Chapter 4

4. Inhibition of human salivary aldehyde dehydrogenase by caffeine and citral

4.1. Background

Human salivary aldehyde dehydrogenase (hsALDH) (E.C. 1.2.1.5) is the first line of defence against toxic aldehydes in the oral cavity. HsALDH is primarily a dimeric, class 3 ALDH (ALDH3A1) specific for aromatic and long/medium chain aliphatic aldehydes as the substrates (Bogucka et al., 2009; Dyck, 1995; Giebultowicz et al., 2009). It primarily acts as a phase I drug-metabolizing enzyme in the oral cavity (Li and Bluth, 2011; Vasiliiou et al., 2004). It also acts as a phase II drug-metabolizing enzyme for the detoxification process in other organs and tissues including liver (Muzio et al., 2012). It detoxifies the aldehydes in the oral cavity that are either generated endogenously through metabolism or from the exogenous sources such as foods, pollutants, drugs, etc. (Glatt et al., 2008). These aldehydes are toxic and may cause cancer in the oral cavity if not detoxified (Black et al., 2012; Giebultowicz et al., 2010; Nagler and Dayan, 2006). Thus, activity of hsALDH in the oral cavity is important to maintain the oral health. It has been reported that the lower activity of this enzyme in the oral cavity is a risk factor for oral cancer development (Giebultowicz et al., 2013; Glatt et al., 2008). The activity of hsALDH increases during the initial stage of cancer in response to the cancer development and decreases after the surgical removal of tumor, indicating that carcinogenesis induces the ALDH enzymes (Giebultowicz et al., 2013). The over expression of ALDHs in cancerous tissues results in increased resistance to oxazaphosphorine based chemotherapy (Sladek, 1999).

HsALDH activity and expression are affected by variety of factors such as age, sex, cigarette smoking, alcohol consumption, pollution, diet, drug consumption, etc. (Giebultowicz et al., 2010; Sreerama et al., 1995). It has been found to be induced by various aryl and aromatic hydrocarbons (Korkalainen et al., 1995). HsALDH activity and expression were reported to be induced in the oral cavity upon exposure to cigarette smoke and tobacco products (Bogucka et al., 2009; Giebultowicz et al., 2010; Vasiliiou et al., 1993). The enzyme protects the oral epithelial cells from the cigarette smoke induced cytotoxicity and DNA damage. HsALDH activity was also
observed to be altered by different dietary components, such as vegetables and fruits. Intake of large quantities of coffee and vegetables like broccoli were found to activate and induce the enzyme in the saliva of human subjects (Sreerama et al., 1995). Moderate consumption of coffee was found to decrease the hsALDH activity, whereas consumption of large quantities of coffee increases its activity in the saliva. It has been speculated that the former is due to the inhibition of hsALDH by some coffee component, and the latter is due to induced expression of the enzyme, which is probably a defence mechanism in response to the lowered activity of the enzyme (Giebultowicz et al., 2010; Sreerama et al., 1995). Caffeine, theophylline and related compounds have been reported to inhibit the activity of salivary ALDH (Wierzchowski et al., 2008). The activity of hsALDH in saliva is crucial for maintaining the oral health and prevention of aldehyde mediated toxicity (Dolle and Gao, 2015; Giebultowicz et al., 2013). Therefore, the effect of dietary components frequently encountered by the enzyme, on its activity is important to study for nutrition safety and health (Giebultowicz et al., 2013; Giebultowicz et al., 2010).

Caffeine is an alkaloid present in food items, beverages, coffee, tea, etc. Chemically, it is 1,3,7-trimethylpurine-2,6-dione (Fig. 4.1). It is frequently taken in large quantities on a regular basis around the globe (Barone and Roberts, 1996; Heckman et al., 2010).

![Fig. 4.1. Structure of caffeine (1,3,7-trimethylpurine-2,6-dione).](image)

The caffeine content in various food items ranges from 2-200 mg/150 ml (Barone and Roberts, 1996). A Canadian survey has shown that caffeine content in coffee/tea varies from 30-150 mg/cup (Barone and Roberts, 1996). The quantity of caffeine consumed through beverages among the U.S. population varies from 225-400 mg/day (Mitchell et al., 2014). A small quantity of caffeine intake has been found to be safe and beneficial for health, whereas consumption of large quantity of it causes several adverse health effects (Heckman et al., 2010). Since caffeine affects the activity of
ALDH in the saliva, therefore, a detailed account of its effect on the enzyme kinetics, mechanism of inhibition and interaction with the enzyme is important to examine.

Citral is a monoterpenic aldehyde present in the oils of citrus plants such as lemon, orange, etc. and lemongrass (Ladanyia and Ladaniya, 2008; Shi et al., 2016; Tajidin et al., 2012). Chemically, citral is 3,7-dimethyl-2,6-octadienal with a chemical formula of C₁₀H₁₆O (Fig. 4.2). It is also known as lemonal. It exists as a mixture of two isomeric forms i.e., 40-62% *trans*-isomer and 25-38% *cis*-isomer known as geranial (citral A) and neral (citral B), respectively (da Silva et al., 2008; Manvitha and Bidya, 2014; Onawunmi et al., 1984).

![Fig. 4.2. Structure of citral (3,7-dimethyl-2,6-octadienal). A. *trans*-isomer, geranial (citral A). B. *cis*-isomer, neral (citral B).](image)

Citral is an aroma compound responsible for the characteristic lemon fragrance and is therefore used as a flavouring agent in food items, cosmetics, perfumes, etc. (Ladanyia and Ladaniya, 2008; Shi et al., 2016). It possesses a number of biological activities which includes anti-bacterial, anti-fungal, anti-inflammatory, anti-mutagenic, anti-allergic, anti-cancer, anti-viral, anti-oxidant, and free radical scavenging activity (da Silva et al., 2008; Martins et al., 2017; Mitoshi et al., 2014; Olorunnisola et al., 2014; Onawunmi, 1989; Rabbani et al., 2004; Sheikh et al., 2017; Shi et al., 2016). Citral has been reported to show promising anti-cancer activity. It has anti-proliferative role against a number of cancer cells and induces apoptosis in different cancer cell lines including those of colorectal cancer, breast cancer, hematopoietic cancer, liver cancer, etc. (Chaouki et al., 2009; Dudai et al., 2005; Patel et al., 2015; Sheikh et al., 2017).

Inhibition of hsALDH activity has an important role in oral health and risk of oral carcinogenesis. Further, inhibitors of hsALDH have potential in increasing the sensitivity of certain chemotherapeutic drugs, such as cyclophosphamide. In the present study, we have reported the inhibitory effect of caffeine and citral on the activity (both dehydrogenase and esterase activity) and kinetics of hsALDH. Further, the mechanism
of enzyme inhibition by caffeine or citral, and their binding with hsALDH have also been studied using different biophysical techniques and molecular docking analysis.

4.2. Materials and methods

4.2.1. Materials

6-Methoxy-2-naphthaldehyde, oxidized nicotinamide adenine dinucleotide (NAD$^+$), Citral, Bicinchoninic acid (BCA), 6-methoxy-2-naphthoic acid and Bovine serum albumin (BSA) were purchased from Sigma Chemicals Co., USA. HCl (35%) and NaOH were obtained from Merck Specialities Pvt. Ltd. Caffeine anhydrous pure, $p$-nitrophenyl acetate (pNPA), Dithiothreitol (DTT), Diethylaminoethyl-cellulose (DEAE-cellulose), acetonitrile and 2-morpholinoethane sulphonic acid (MES) were from Sisco Research Lab., India. EDTA, sodium phosphate monobasic anhydrous, acetic acid, NaCl, di-sodium hydrogen phosphate and Tris were obtained from Himedia, India. Sodium acetate was a product of Qualigens Fine Chemicals Pvt. Ltd. All other chemicals used were of analytical grade. HPLC water and glass distilled water were used in all experiments.

4.2.2. Preparation of crude saliva and purification of hsALDH

Preparation of crude saliva and the purification of hsALDH by DEAE-cellulose column chromatography were carried out according to the standard procedure described in section 2.2.2 and section 2.2.3, respectively. The purified stock solution of hsALDH (350 µg/ml) used was in 50 mM Tris-HCl buffer, pH 8.0 containing 0.5 mM EDTA and 0.5 mM DTT. It was stored at 4°C for immediate use and at -80°C for long-time storage. The collection of human saliva samples was approved by the institutional ethical committee of Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh, India.

4.2.3. Preparation of caffeine and citral stock solutions

Caffeine (5 mg) was dissolved in 1 ml of 50 mM sodium phosphate buffer (pH 7.5), containing 0.5 mM EDTA and 0.5 mM DTT. 1 mM stock solution of
caffeine was then prepared by diluting the above solution in the same buffer for activity studies. For all the spectroscopic studies, caffeine solution was prepared in 50 mM sodium phosphate buffer (pH 7.5), without EDTA and DTT. Citral (1.8 µl) from the 5.6 M stock solution (Sigma Chemicals Co.) was dissolved in a total volume of 10 ml in 50 mM sodium phosphate buffer (pH 7.5), to make its 1 mM solution. Further dilutions were made in the same buffer to obtain the desired concentrations. Solutions were always prepared fresh prior to use and stored at 4°C.

4.2.4. HsALDH dehydrogenase activity assay

The dehydrogenase activity of hsALDH was assayed by using 6-methoxy-2-naphthaldehyde as substrate and NAD$^+$ as the co-enzyme as described in section 2.2.4.

4.2.5. Effect of caffeine or citral on the dehydrogenase activity of hsALDH

The effect of caffeine or citral on the dehydrogenase activity of crude and purified hsALDH was studied by incubating the enzyme for 1 min in the presence of varying concentration of caffeine (0-1000 µM) or citral (0-200 µM). Activity was then determined under standard assay conditions as described in section 2.2.4. The amount of the crude and the pure enzyme used was 40 and 15 µg/ml, respectively in the case of caffeine, and 40 and 20 µg/ml, respectively in the case of citral. Data were fitted to non-linear regression analysis curve of log[caffeine] (or [citral]) versus activity under ‘Dose-response-Inhibition’ function in “GraphPad PRISM” (Version 5.03, 2009) to determine the IC$_{50}$ value.

4.2.6. Determination of kinetic parameters in the absence/presence of caffeine or citral

The dehydrogenase activity of hsALDH at varying concentration of the substrate (0-50 µM) was determined in the absence and presence of caffeine (150 µM) or citral (85 µM) (at IC$_{50}$ value), using the standard assay method described above. Enzyme kinetic parameters ($K_m$ and $V_{max}$) were then determined by fitting the data in non-linear regression analysis curve of Michaelis-Menten equation (i) and Lineweaver-Burk equation (ii) under ‘Enzyme-kinetics’ function in “GraphPad PRISM” (Version 5.03, 2009) (Lineweaver and Burk, 1934; Michaelis et al., 2011):
\begin{align*}
V &= \frac{v_{\text{max}}[S]}{K_m + [S]} \quad \text{(i)} \\
\frac{1}{V} &= \frac{K_m}{v_{\text{max}}} \times \frac{1}{[S]} + \frac{1}{v_{\text{max}}} \quad \text{(ii)}
\end{align*}

Where, \( V \) and \( v_{\text{max}} \) are the initial and maximum velocities, respectively, \( K_m \) is the Michaelis-Menten constant and \([S]\) is the substrate concentration.

4.2.7. Effect of caffeine or citral on the esterase activity of hsALDH

The esterase activity of hsALDH was assayed by using pNPA as the substrate and the rate of formation of \( p \)-nitrophenol at 400 nm was measured. Absorbance at 400 nm was recorded on a double-beam Perkin Elmer Spectrophotometer (Lambda 25). The standard reaction mixture of 1 ml contained 50 mM sodium phosphate buffer (pH 7.5), 1 mM pNPA and 15 µg the enzyme. The reaction was started by the addition of pNPA at 25°C and the absorbance was recorded continuously for 5 min. A fresh stock solution of pNPA was prepared in 100% acetonitrile to minimize its spontaneous hydrolysis. A negative control without the enzyme was always taken to record the spontaneous hydrolysis of pNPA during the reaction time (5 min), and was subtracted from the test reading. Enzyme activity was reported in terms of change in absorbance (ΔA) at 400 nm per min per microgram of the enzyme.

The effect of caffeine or citral on the esterase activity of hsALDH was determined by incubating the enzyme (15 µg) for 1 min in the presence of varying concentration of caffeine (0-500 µM) or citral (0-200 µM). The activity was then measured under standard assay conditions as described above.

4.2.8. Effect of caffeine or citral on the nucleophilicity of the catalytic cysteine (Cys) residue of hsALDH

The effect of caffeine or citral on the nucleophilicity of the catalytic Cys residue of the enzyme was determined by measuring the pH dependence of esterase activity in the absence and presence of caffeine (150 µM) or citral (85 µM). Variation in the pKₐ value of the catalytic Cys was then determined in the absence and in presence of caffeine or citral. The esterase activity of hsALDH was measured over a pH range of 5.0-9.5. The buffer systems used were: 15 mM sodium acetic acid buffer
(pH 5.0), 15 mM MES buffer (pH 6.0-7.0) and 60 mM Tris buffer (pH 7.5-9.5). The pH was adjusted to the desired value using either HCl or NaOH (Perez-Miller et al., 2010). The pKₐ values were then calculated from the pH-activity curve both in the absence and in the presence of caffeine or citral by fitting the data in non-linear regression analysis curve of pH versus activity under ‘EC50 Shift’ function in “GraphPad PRISM” (Version 5.03, 2009).

4.2.9. UV-Vis Spectroscopy measurements

Absorbance spectra of hsALDH (100 µg or 75 µg for caffeine and citral, respectively) in presence of varying concentration of caffeine (0-500 µM) or citral (0-250 µM) were recorded in the wavelength range of 200-400 nm on a double beam Perkin Elmer spectrophotometer (Lambda 25) at 25°C. Absorption spectra of caffeine or citral alone were also recorded as a control for the correction of background noise if any. Spectra were then plotted and analyzed using ‘Microsoft Office Excel 2007’.

4.2.10. CD Spectroscopy measurements

Far-UV CD spectroscopy was employed to determine the secondary structural changes that occur in hsALDH upon binding to caffeine or citral. HsALDH (2 µM) was incubated with various concentrations of caffeine (0-500 µM) or citral (0-250 µM) for 20 min at 25°C. Far-UV CD measurements were made on a Jasco Spectropolarimeter (J-815) equipped with a Jasco Paltier-type temperature controller (PTC-424S/15) under constant nitrogen flush. The spectra were recorded in the wavelength range of 190-250 nm with a spectral bandwidth of 1.0 nm. The spectrum of caffeine or citral alone served as a control for the correction of background noise. The observed CD values (mdeg) were converted into mean residue ellipticity (MRE) in deg cm² dmol⁻¹ by using the following relation:

$$\text{MRE} = \frac{\theta_{\text{obs}}(\text{mdeg})}{10 \times n \times C \times l}$$

Where, $\theta_{\text{obs}}$ is the observed CD value in mdeg, n is the total number of peptide bonds in the protein, l is the path length of cell in cm and C is the molar concentration of protein.
The secondary structural contents (α-helix and β-sheet) were then estimated in the absence and in presence of caffeine or citral using the freely available software ‘K2D2’ (Perez-Iratxeta and Andrade-Navarro, 2008).

4.2.11. Fluorescence quenching measurements

Quenching of intrinsic fluorescence of hsALDH by caffeine or citral was monitored at 25°C on a Shimadzu Spectrofluorophotometer (RF-5301PC) equipped with temperature control. HsALDH was titrated against varying concentration of caffeine (0-500 µM) or citral (0-500 µM) and the emission spectra were then recorded in the wavelength range of 300-500 nm with excitation of either tryptophan residues at 295 nm. Slit width was 5 nm for both excitation and emission. The inner filter effect was corrected as mentioned in section 2.2.4. The data were plotted using Stern-Volmer equation (iii) and modified Stern-Volmer equation (iv) as shown below:

\[
\frac{F_0}{F} = K_{sv}[Q] + 1 = K_q\tau_o[Q] + 1 \quad \text{(iii)}
\]

\[
\log\left[\frac{F_0}{F} - 1\right] = n\log[Q] + \log[K_b] \quad \text{(iv)}
\]

Where, \(F_0\) and \(F\) are the fluorescence intensities in the absence and presence of quencher (caffeine or citral), respectively, \(K_{sv}\) is the Stern-Volmer quenching constant, \(Q\) is the concentration of quencher in molar, \(K_q\) is the quenching rate constant, \(\tau_o\) is the average lifetime of the protein in absence of quencher, \(n\) is the stoichiometric binding ratio and \(K_b\) is the binding constant.

4.2.12. van’t Hoff plot and the effect of temperature on the binding of caffeine or citral with hsALDH

The effect of temperature on the binding of caffeine or citral with hsALDH was studied by determining the binding constant \((K_b)\) using fluorescence quenching method at 15, 25 and 37°C. The \(K_b\) value was determined at each temperature using Stern-Volmer and modified Stern-Volmer plot. The van’t Hoff equation (v) and the thermodynamic equation (vi) were used to determine the thermodynamic parameters i.e., \(\Delta H\), \(\Delta S\) and \(\Delta G\).
\[
\log(K_b) = \frac{\Delta S}{R} - \frac{\Delta H}{RT} \quad (v)
\]
\[
\Delta G = \Delta H - T\Delta S \quad (vi)
\]

Where, \(K_b\) is the binding constant, \(\Delta S\) is the entropy change, \(\Delta H\) is the enthalpy change, \(R\) is the gas constant, \(T\) is the temperature in Kelvin and \(\Delta G\) is the free energy change.

4.2.13. Fluorescence resonance energy transfer (FRET) analysis

FRET analysis for caffeine was not possible because caffeine does not absorb significantly at the emission wavelength range of the enzyme. For citral, FRET analysis was done by recording the emission spectrum of hsALDH (50 µg) and absorption spectrum of citral (100 µM) in fluorescence spectrophotometer and UV-Vis spectrophotometer, respectively, as mentioned above. Absorption spectrum of the compound was then plotted with the emission spectrum of hsALDH to obtain the overlapping region of energy transfer. Data were then fitted into the following equation to obtain the efficiency of energy transfer (\(E\)).

\[
E = \frac{R^6}{R^6 + r^6} = 1 - \frac{F}{F_0}
\]

Where, \(r\) is the distance between donor and acceptor, \(F\) and \(F_0\) are the fluorescence intensities in the presence and absence of citral, respectively, and \(R_0\) is the distance at which transfer efficiency become 50%, which is calculated from the following relation:

\[
R_0^6 = 8.79 \times 10^{-25}K^2n^{-4}\varnothing J
\]

Where, \(K^2\) is the factor related to the geometry of the donor acceptor dipoles, \(n\) is the refractive index of the medium, \(\varnothing\) is the fluorescence quantum yield of the donor in the absence of acceptor, and \(J\) is the overlap integral of donor fluorescence emission and the acceptor absorption spectra, which is calculated as:

\[
J = \frac{\int_0^\infty F_\lambda \varepsilon_\lambda \lambda^4 d\lambda}{\int_0^\infty F_\lambda d\lambda}
\]

Where, \(F_\lambda\) is the fluorescence intensity of the donor at wavelength \(\lambda\), \(\varepsilon_\lambda\) is the molar extinction coefficient of acceptor at wavelength \(\lambda\). For hsALDH, \(K^2\), \(\varnothing\) and \(n\) were taken as 2/3, 0.118 and 1.33, respectively.
4.2.14. Molecular docking analysis

Molecular docking analysis was employed to determine the binding site, the amino acid residues and the type of interactions involved in the ALDH3A1-caffeine or ALDH3A1-citral complex formation. ALDH3A1 pdb file was obtained from the RCSB protein data bank (http://www.rcsb.org/pdb/home/home.do) (PDB ID: 3SZA). The structure of caffeine and citral (both geranial and neral) were obtained from PubChem data base (https://pubchem.ncbi.nlm.nih.gov) (PubChem CID: 2519, 638011, 643779 for caffeine, geranial and neral, respectively). Docking was performed using AutoDock Vina (http://vina.scripps.edu) and confirmed by using commercial docking software ‘GOLD’. Docking analysis was performed by taking grid size of 60 each along x, y and z axes with a grid spacing of 0.375 Angstrom. Ten best results out of the docking scores were further analyzed. ‘Discovery Studio 4.0’ was used for the visualization and identification of the amino acid residues involved in the binding.

4.2.15. Measurement of ligand efficiency (LE), percentage/potency efficiency index (PEI) and binding efficiency index (BEI)

The ligand efficiency of caffeine and citral has been measured and compared based on LE, PEI and BEI parameters. LE is the measurement of binding energy per heavy atom of a ligand molecule interacting with a receptor such as an enzyme (Kuntz et al., 1999). It indicates how efficiently the non-hydrogen atoms of a ligand are used in the binding of the ligand molecule to the target (receptor/protein/enzyme). LE is an important parameter utilized in drug discovery for screening, selection and optimization of small ligand molecules to narrow down to lead compounds (Abad-Zapatero, 2007; Hopkins et al., 2004). Mathematically, LE is the ratio of Gibbs free energy ($\Delta G$) to the number of non-hydrogen atoms (heavy atoms) (HA) of a ligand molecule:

\[
LE = -\frac{\Delta G}{HA} = -2.303RT \cdot \log\left(\frac{K_b}{C^o}\right)
\]

Where, $R$ is the ideal gas constant ($1.99 \times 10^{-3}$ kcal/K/mol) and $T$ is the temperature in Kelvin (K), $C^o$ is the standard concentration, and $K_b$ is the binding constant.

LE can also be defined in terms of IC$_{50}$ value where, $p$(IC$_{50}$) is $-\log$(IC$_{50}$):

\[
LE = \frac{p$(IC_{50})}{HA} = -1.4\left[\frac{\log$(IC_{50})}{HA}\right]
\]
PEI is the percent inhibition of a ligand molecule divided by the molecular weight (MW) in kDa. It measures the potency of a compound per kDa molecular weight and is calculated as:
\[
\text{PEI} = \frac{\% \text{ inhibition in fraction } (0 - 1)}{\text{MW(kDa)}}
\]
Percent (\%) inhibition is taken as fraction (0-1) at a given compound concentration and is measured in a single-point assay under the same conditions to compare the potencies of different ligand molecules \textit{in vitro} (Abad-Zapatero, 2007; Abad-Zapatero and Metz, 2005).

BEI is the measurement of the binding efficiency of a ligand molecule in relation to its molecular weight (MW). It relates the potency or affinity of a ligand molecule to its molecular size expressed in kDa (Abad-Zapatero and Metz, 2005). It is also an important parameter utilized in drug discovery for screening, selection and optimization of lead compounds (Abad-Zapatero, 2007). Mathematically, BEI is expressed as:
\[
\text{BEI} = \frac{p(\text{IC}_{50})}{\text{MW(kDa)}} = -1.4\left[\log(\text{IC}_{50})\right]_{\text{MW(kDa)}}
\]

### 4.2.16. Statistical analysis

All the experiments were carried out twice in triplicates independently and the data are presented as mean ± S.D. Non-linear regression analysis was employed for fitting the data into the equations mentioned above i.e., Michaelis-Menten equation and Lineweaver-Burk equation. ‘GraphPad PRISM’ (Version 5.03, 2009) was used to calculate the kinetic parameters \((K_m \text{ and } V_{max})\), \(\text{IC}_{50}\) and the \(pK_a\) values. ‘Microsoft Office Excel 2007’ was used for drawing and analyzing the spectral graphs.

### 4.3. Results and discussion

#### 4.3.1. Caffeine

#### 4.3.1.1. Effect of caffeine on the dehydrogenase activity of hsALDH

The effect of caffeine on the activity of crude and purified hsALDH was studied. It was observed that the activity remained unchanged till 30 and 10 \(\mu\text{M}\)}
concentration of caffeine for the crude (Fig. 4.3 A) and purified (Fig. 4.3 B) enzyme, respectively, at 5 µM substrate concentration. For both the enzyme preparations, with further increase in the caffeine concentration, the activity gradually decreased till 1000 µM concentration of caffeine, after which the activity almost remained constant at the minimum value of about 20-25% of the initial activity. The half maximal inhibitory concentration (IC$_{50}$ value) of caffeine was determined to be 287 ± 5 and 151 ± 3 µM for the crude and pure hsALDH, respectively. Therefore, caffeine inhibits hsALDH to a good extent. The IC$_{50}$ values obtained here for the crude and pure hsALDH are somewhat different from those reported by Wierzchowski et al., (2008), as these values are partially dependent on the enzyme preparation (crude vs pure) as well as the substrate concentration used as mentioned by Wierzchowski et al., (2008).

Fig. 4.3. Effect of caffeine on the activity of crude (A) and purified (B) hsALDH. The activity was determined in the presence of 0-1000 µM caffeine with 40 or 15 µg of crude or purified enzyme, respectively, in a total volume of 1 ml of the reaction mixture at 25°C.

4.3.1.2. Determination of kinetic parameters in the absence/presence of caffeine

The activity of hsALDH with varying concentration of the substrate (0-50 µM) was determined in the absence and presence of 150 µM caffeine. Variation of kinetic parameters ($K_m$ and $V_{max}$) brought about by caffeine were determined using Michaelis-Menten plot (Fig. 4.4 A) and Lineweaver-Burk plot (Fig. 4.4 B). Caffeine increased the $K_m$ value of hsALDH from 0.25 ± 0.06 µM to 0.60 ± 0.07 µM and reduced the $V_{max}$ value from 1.49 ± 0.15 to 0.70 ± 0.08 EU/µg. The increase in the $K_m$ value implies that caffeine decreases the affinity of the enzyme for the substrate. This may be because of the binding of caffeine to the enzyme, which partially competes with the binding of the
substrate to the enzyme. The decrease in the apparent $V_{\text{max}}$ value also shows that hsALDH is inhibited by caffeine. Therefore, when caffeine binds to the enzyme, both the $K_m$ and $V_{\text{max}}$ values are altered, which indicates a mixed type of inhibition. It was also observed from the Lineweaver-Burk plot (Fig. 4.4 B) that the pattern of the lines is in between those of competitive and non-competitive inhibition. Therefore, caffeine is a partial competitive and non-competitive inhibitor of the dehydrogenase activity of hsALDH. Similar results were also reported by Wierzchowski et al., (2008), where it was observed that caffeine partially competes with the substrate at higher substrate concentrations. We have observed in molecular docking studies (section 4.3.1.9) that caffeine binds to the active site of the enzyme which further confirms the possible competition between caffeine and the substrate to bind to the active site, as also indicated by increased apparent $K_m$ value in presence of caffeine.

![Fig. 4.4. Michaelis-Menten plot (A) and Lineweaver-Burk plot (B) showing the variation in the kinetic parameters ($K_m$ and $V_{\text{max}}$). The activity of hsALDH was measured in absence and in the presence of 150 µM caffeine with the substrate varied from 0-50 µM.](image)

**4.3.1.3. Effect of caffeine on the esterase activity**

The effect of varying concentration of caffeine (0-500 µM) on the esterase activity of purified hsALDH was studied. The esterase activity of the enzyme remained almost constant till 50 µM concentration of caffeine, after which it decreased gradually with increasing concentration of caffeine up to 300 µM (Fig. 4.5). With further increase in caffeine concentration, the activity remained constant at the minimum value of about 25% of the initial activity. Therefore, caffeine also inhibits the esterase activity of the enzyme. Since the same catalytic Cys residue is involved in the esterase as well as the dehydrogenase activity of the enzyme (Koppaka et al.,
we speculated that perhaps caffeine binds to the active site near the Cys residue and influences its nucleophilicity. To further confirm this, we have studied the effect of caffeine on the nucleophilicity of catalytic Cys residue by measuring its $pK_a$ value in the absence and presence of caffeine as described in section 4.3.1.4.

Fig. 4.5. Effect of caffeine on the esterase activity of hsALDH. The effect of caffeine on the esterase activity of purified hsALDH (15 µg) was measured by incubating the enzyme for 1 min in the presence of varying concentration of caffeine (0-500 µM) at 25°C.

**4.3.1.4. Effect of caffeine on the nucleophilicity of catalytic Cys residue**

The pH dependence of the esterase activity of hsALDH was studied in the absence and in presence of 150 µM caffeine over a pH range of 5.0-9.5 to see the effect of caffeine on the nucleophilicity of the catalytic Cys residue (Fig. 4.6).

Fig. 4.6. Determination of $pK_a$ values in the absence and in the presence of 150 µM caffeine. The esterase activity of hsALDH was measured over a pH range of 5.0-9.5. The buffer systems used were: 15 mM sodium acetic acid buffer, 15 mM MES buffer and 60 mM Tris-HCl buffer.
The activity of the enzyme increased gradually with an increase in the pH from 7.0 to 9.5, both in the absence and presence of caffeine. However, the activity was lower in the presence of caffeine than in its absence at each pH value. The $pK_a$ values were determined to be $7.91 \pm 0.02$ and $8.23 \pm 0.03$ in the absence and presence of caffeine, respectively. There is a significant increase in the $pK_a$ value of the enzyme in the presence of caffeine. Hence, caffeine decreases the nucleophilicity of the catalytic Cys residue by increasing its $pK_a$ value. Therefore, caffeine reduces the catalytic efficiency of the enzyme. This is also evident from the decrease in the apparent $V_{max}$ value of the enzyme in the presence of caffeine for the dehydrogenase activity.

4.3.1.5. UV-Vis Spectroscopy

UV absorption spectrum of hsALDH was recorded in the absence and in presence of increasing caffeine concentration. The characteristic spectrum of the enzyme was observed to be altered in the presence of caffeine (Fig. 4.7).

![Fig. 4.7. UV-Vis absorption spectra of hsALDH. Spectra of the enzyme were recorded in absence and in presence of caffeine in the wavelength range of 200-400 nm at 25°C. The curves denote spectra of hsALDH at 0 μM (E only), 70 μM (E+ 70 μM), 150 μM (E+ 150 μM) caffeine and the spectrum of 150 μM caffeine alone as control (C only).]

There was a shift in the absorption peaks (218 and 280 nm) of the enzyme in the presence of increasing caffeine concentration. The decrease in absorbance at around 218 nm shows that the peptide backbone structure is altered in presence of caffeine.
This probably suggests that there is a change in the secondary and tertiary structure of the enzyme. Absorbance at 280 nm was found to increase in presence of caffeine showing hyperchromic effect which may be due to alteration of the secondary and tertiary structure of the enzyme and hence exposure of chromogenic groups. Caffeine itself gives a significant amount of absorbance at around 280 nm which was nullified using the control solution of caffeine alone. Changes in the absorption properties of the enzyme indicate that caffeine binds and forms a complex with the hsALDH.

4.3.1.6. CD Spectroscopy

The changes in the secondary structure of hsALDH in the absence and presence of caffeine were determined by far-UV CD Spectroscopy. There was an increase in the two negative peaks corresponding to 208 and 222 nm in the presence of increasing caffeine concentration (Fig. 4.8). The α-helix and β-sheet contents in the absence and in presence of caffeine were found to be 34.93% α-helix, 26.14% β-sheet and 30.15% α-helix, 29.09% β-sheet, respectively. This implies that caffeine upon binding with the enzyme decreases the α-helix content by about 5% and increases the β-sheet content by almost 4%. Therefore, the binding of caffeine to hsALDH slightly changes the secondary structural elements of the enzyme. Changes in the secondary structural elements may further contribute to the inhibition process by altering the substrate and/or co-enzyme binding ability of the enzyme.

![CD Spectra](image.png)

Fig. 4.8. Far-UV CD spectra of hsALDH in absence and in presence of caffeine. HsALDH (2 μM) was incubated with various concentrations of caffeine (0-500 μM) for 20 min at 25°C. Far-UV CD spectra were then recorded in the wavelength range of 190-250 nm with a spectral bandwidth of 1.0 nm. Spectra corresponding to hsALDH at 0 μM, 100 μM and 150 μM caffeine are presented in the figure.
4.3.1.7. Fluorescence quenching measurements

Titration of hsALDH with varying concentration of caffeine has shown the quenching of intrinsic fluorescence of tryptophan as evident from the gradual decrease in emission intensity of hsALDH with the increase in caffeine concentration (Fig. 4.9).

Fig. 4.9. Fluorescence emission spectra of hsALDH. Spectra of hsALDH were recorded in the wavelength range of 300-500 nm with increasing concentration of caffeine (0-500 µM) at 25°C. Samples were excited at 295 nm to record specifically the tryptophan fluorescence emission.

Fig. 4.10. Stern-Volmer plot (A) and Modified Stern-Volmer plot (B) of hsALDH-caffeine binding at 25°C. The concentration of hsALDH used was 100 µg. The Stern-Volmer and Modified Stern-Volmer graphs were plotted using equation (iii) and (iv), respectively, as mentioned in the methods.
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The Stern-Volmer plot (Fig. 4.10 A) was used to determine the Stern-Volmer quenching constant \( (K_{sv}) \) and it was found to be \( 2.74 \pm 0.08 \times 10^4 \) M\(^{-1} \). Modified Stern-Volmer plot (Fig. 4.10 B) revealed the binding constant \( (K_b) \) of \( 2.5 \pm 0.03 \times 10^5 \) M\(^{-1} \) and a stoichiometric binding ratio of 0.98 (Table 4.1). \( K_{sv} \) and \( K_b \) value indicates that the binding of caffeine to hsALDH is very strong and of the order of static binding (complex formation) with a 1:1 binding ratio.

### Table 4.1. Binding parameters of hsALDH-caffeine interaction at 25°C.

<table>
<thead>
<tr>
<th>Temperature (K)</th>
<th>( K_{sv} ) (10(^4) M(^{-1}))</th>
<th>( K_b ) (10(^5) M(^{-1}))</th>
<th>n</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>298</td>
<td>2.74±0.08</td>
<td>2.5±0.03</td>
<td>0.98±0.04</td>
<td>0.997</td>
</tr>
</tbody>
</table>

4.3.1.8. van’t Hoff plot and the effect of temperature on the binding of caffeine to hsALDH

The effect of temperature on the binding of caffeine to hsALDH was determined and it was found that the binding constant decreased from \( 3.2 \times 10^5 \) M\(^{-1} \) to \( 1.8 \times 10^5 \) M\(^{-1} \) with the increase in temperature from 15°C (288 K) to 37°C (310 K), respectively. This decrease in binding constant with increase in temperature indicates that the quenching of fluorescence by caffeine may be static in nature and there might be the formation of a complex between caffeine and hsALDH. With the increase in temperature, the caffeine-hsALDH complex probably destabilizes and thereby decreases the binding constant.

![Fig. 4.11. van’t Hoff plot of hsALDH-caffeine binding showing the effect of temperature on the binding constant. The graph was plotted using equation (v) as mentioned in the methods.](image-url)
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### Table 4.2. Thermodynamic parameters of hsALDH-caffeine interaction at 25°C.

<table>
<thead>
<tr>
<th>T</th>
<th>ΔH</th>
<th>ΔS</th>
<th>TΔS</th>
<th>ΔG</th>
</tr>
</thead>
<tbody>
<tr>
<td>298 K</td>
<td>-8.56 kJ mol⁻¹</td>
<td>32.72 J K⁻¹ mol⁻¹</td>
<td>9.75 kJ mol⁻¹</td>
<td>-18.31 kJ mol⁻¹</td>
</tr>
</tbody>
</table>

van’t Hoff plot was used to calculate the thermodynamic parameters i.e., ΔH, ΔS and ΔG. Fig. 4.11 shows the dependence of binding constant ($K_b$) with temperature (1/T) with a slope $-ΔH/R$ and intercept of $ΔS/R$. Protein-ligand interaction primarily involves the non-covalent interactions such as hydrogen bonding, electrostatic interactions, hydrophobic interactions and van der Waals forces. Caffeine contains three methyl groups on its surface along with two ring –C=O groups (Fig. 4.1). The methyl groups along with the aromatic ring might provide the hydrophobic and van der Waals forces for binding to the enzyme. Also, the partial negative charge on the carbonyl oxygen and ring nitrogen may contribute to the binding through hydrogen bond formation and ionic interactions. This was further confirmed by molecular docking analysis. A negative ΔH and a positive ΔS value (Table 4.2) shows that the electrostatic interactions may also be involved in the hsALDH-caffeine complex formation. Negative ΔG and ΔH shows that the binding of caffeine to hsALDH is a spontaneous process.

4.3.1.9. Molecular docking analysis

Molecular docking analysis revealed that caffeine fits into the active site of ALDH3A1 close to the catalytic Cys 243 and other highly conserved amino acid residues, through π-π interactions, hydrophobic interactions and hydrogen bonding. The amino acid residues involved in the interaction are Thr 112, His 289, Phe 335 and Trp 113 (Fig. 4.12). Trp 113 forms hydrogen bond with the ring carbonyl oxygen of caffeine and multiple hydrophobic interactions with its methyl groups. Thr 112 forms hydrogen bond with ring carbonyl oxygen whereas His 289 forms hydrophobic interaction with the side chain methyl group of caffeine. Phe 335 binds through multiple π-π interactions with the main rings of caffeine and forms additional hydrophobic interaction with the side chain methyl group of caffeine. Therefore, caffeine forms a strong complex with ALDH3A1 in its active site through multiple non-covalent interactions with some of the highly conserved amino acid residues.
4.3.1.10. Measurement and analysis of ligand efficiency metrics

The efficiency, selectivity and potency of caffeine were measured and compared based on its calculated LE, PEI and BEI values. The LE, PEI and BEI were determined to be 0.38, 1.80 and 27.60, respectively. The mean LE of most of the commercial oral drug molecules is 0.51 which is also the LE of an ideal ligand molecule under standard conditions (Abad-Zapatero, 2007; Hopkins et al., 2014). Additionally, the recommended drug discovery benchmark value of LE is ≥0.30 (Hopkins et al., 2004; Schultes et al., 2010). Based on a large sample of marketed oral drugs, the reference value of PEI and BEI for an ideal ligand molecule have been determined as 1.5/2.7 and 27, respectively (Abad-Zapatero, 2007; Abad-Zapatero and Metz, 2005). The values of these three parameters determined for caffeine are in good agreement with the recommended reference values, indicating that it may be a possible drug candidate with optimized physicochemical and pharmacokinetic properties. Therefore, caffeine may be a good candidate for its better ligand efficiency, potency and druggability for the enzyme and hence may be used as an efficient ligand molecule for the inhibition of hsALDH.
4.3.2. Citral

4.3.2.1. Effect of citral on the dehydrogenase activity of hsALDH

The effect of citral on the activity of crude and purified hsALDH was studied. It was observed that the activity remained almost unchanged till 50 and 20 µM concentration of citral for the crude (Fig. 4.13 A) and purified (Fig. 4.13 B) enzyme, respectively at 5 µM substrate concentrations.

For both the enzyme preparations, with further increase in the citral concentration, the activity gradually decreased till 125-130 µM concentration of citral, after which the activity almost remained constant at the minimum value of about 52-55% and 29-33% of the initial activity for the crude and pure enzyme, respectively. The half maximal inhibitory concentration (IC₅₀ value) of citral was determined to be 83.7 ± 1.8 and 86.8 ± 1.3 µM for the crude and pure hsALDH, respectively. Therefore, citral inhibits hsALDH to a good extent. Boyer and Petersen (1991) studied the inhibitory effect of citral against rat liver ALDH isozymes and found that citral at 100 µM concentration, reduced the initial velocity of high-affinity mitochondrial ALDH by almost 85%. Kikonyogo et al., (1999) also studied the inhibitory effect of citral against three different ALDH isozymes (ALDH1, ALDH2 and ALDH9) and reported the inhibitory constants within sub-micromolar to micromolar range. Here also, it has been observed that citral inhibits the activity of hsALDH (ALDH3) with IC₅₀ value within the micromolar range (86.8 ± 1.3 µM for the purified enzyme).
4.3.2.2. Determination of kinetic parameters in the absence/presence of citral

The activity of hsALDH with varying concentration of the substrate (0-50 µM) was determined in the absence and presence of 85 µM citral. Variation of kinetic parameters \( (K_m \text{ and } V_{\text{max}}) \) due to inhibition by citral was determined using Michaelis-Menten plot (Fig. 4.14 A) and Lineweaver-Burk plot (Fig. 4.14 B). Citral increased the \( K_m \) value of hsALDH from \( 0.32 \pm 0.03 \) µM to \( 1.81 \pm 0.19 \) µM and reduced the \( V_{\text{max}} \) value from \( 3.16 \pm 0.09 \) to \( 2.78 \pm 0.04 \) EU/µg. The increase in the \( K_m \) value implies that citral decreases the affinity of the enzyme for the substrate. This may be either because of competition between citral and the substrate to bind to the active site or any structural changes which disrupts the binding of the substrate to the enzyme, or a combined effect of both. It was observed that the extent of inhibition is reduced at higher concentration of the substrate (Fig. 4.14 A), indicating a possible competition between citral and the substrate. The decrease in the apparent \( V_{\text{max}} \) value also shows that there is partial non-competitive inhibition by citral. Citral alters both the \( K_m \) and \( V_{\text{max}} \) value, which indicates linear mixed-type inhibition with more of competitive behavior and a little but significant non-competitive inhibition.

![Fig. 4.14. Michaelis-Menten plot (A) and Lineweaver-Burk plot (B) showing the variation in the kinetic parameters \( (K_m \text{ and } V_{\text{max}}) \). The activity of hsALDH was measured in absence and in the presence of 85 µM citral with the substrate varied from 0-15 µM.](image)

It was also observed from the Lineweaver-Burk plot (Fig. 4.14 B) that the pattern of the lines is in between those of competitive and non-competitive inhibition with more of competitive in nature as evident from the large decrease in the \( K_m \) value and small decrease in the \( V_{\text{max}} \) value. Therefore, citral is a partial competitive and partial non-competitive inhibitor of the dehydrogenase activity of hsALDH. Similar results
showing a linear mixed-type of inhibition were also reported by Boyer and Petersen (1991) for the inhibition of high-affinity mitochondrial ALDH by citral. Kikonyogo et al. (1999) reported that citral is a non-competitive inhibitor of E2 (ALDH2) and E3 (ALDH9) isozyme and is a substrate for the E1, E2 and E3 isozymes. Therefore, it is very likely that citral binds to the active site of hsALDH (ALDH3) and competes with the substrate for its binding to the active site. Molecular docking study (section 4.3.2.11) further confirmed that citral binds to the active of the enzyme.

4.3.2.3. Effect of citral on the kinetics of co-substrate (NAD\(^+\))

Effect of citral on the kinetics of co-substrate (NAD\(^+\)) catalysis by hsALDH was studied by varying the concentration of NAD\(^+\) (0-50 μM) in the standard reaction mixture and determining the activity in absence and presence of citral (85 μM). Enzyme kinetic parameters (\(K_m\) and \(V_{max}\)) were then determined by fitting the data in non-linear regression analysis curve of Michaelis-Menten equation (i) and Lineweaver-Burk equation (ii) under ‘Enzyme-kinetics’ function in “GraphPad PRISM” as mentioned in the section 4.2.6. It was found that citral inhibited the catalysis of NAD\(^+\) by hsALDH and reduced the \(K_m\) and \(V_{max}\) value (Fig. 4.15 A). The \(K_m\) and \(V_{max}\) value decreased from 2.73 ± 0.37 to 1.41 ± 0.28 μM and 3.38 ± 0.10 to 2.51 ± 0.09 EU/μg, respectively.

Fig. 4.15. Michaelis-Menten plot (A) and Lineweaver-Burk plot (B) showing the variation in the kinetic parameters (\(K_m\) and \(V_{max}\)). The activity of hsALDH was measured in absence and in the presence of 85 μM citral with the NAD\(^+\) varied from 0-50 μM.
The Lineweaver-Burk plot (Fig. 4.15 B) shows that citral is an uncompetitive inhibitor of NAD$^+$ catalysis by hsALDH. Boyer and Petersen (1991) reported that citral inhibits the high-affinity mitochondrial ALDH isozyme and the inhibition does not involve binding of citral to the NAD$^+$-binding site. ALDH isozymes share a common NAD$^+$-binding Rossmann fold domain which is conserved among almost all the ALDH isozymes (Lindahl and Hempel, 1991). Therefore, it is very likely that citral binds to a site other than the NAD$^+$-binding site, and alters the NAD$^+$-binding affinity of hsALDH.

4.3.2.4. Effect of citral on the esterase activity

The effect of varying concentration of citral (0-200 µM) on the esterase activity of purified hsALDH was studied. No significant change in the esterase activity was observed in presence of citral (Fig. 4.16).

![Fig. 4.16. Effect of citral on the esterase activity of hsALDH. The effect of citral on the esterase activity of purified hsALDH (20 µg/ml) was measured by incubating the enzyme for 1 min in the presence of varying concentration of citral (0-200 µM) at 25°C. Reaction progress over a time of 5 min, in the absence and presence of 85 µM citral is shown in the figure.]

Therefore, citral does not inhibit the esterase activity of the enzyme. Since the same catalytic Cys residue is involved in the esterase as well as the dehydrogenase activity of the enzyme (Koppaka et al., 2012), we speculated that perhaps citral binds to the active site near the Cys residue and influences its nucleophilicity. To further confirm this, we have studied the effect of citral on the nucleophilicity of catalytic Cys residue by measuring its pK$_a$ value in the absence and presence of caffeine as described in section 4.3.2.5.
4.3.2.5. Effect of citral on the nucleophilicity of catalytic Cys residue

The pH dependence of the esterase activity of hsALDH was studied in the absence and in presence of 85 µM citral over a pH range of 5.0-9.5 to see the effect of citral on the nucleophilicity of the catalytic Cys residue (Fig. 4.17). The activity of the enzyme increased gradually with an increase in the pH from 7.0 to 9.5, both in the absence and presence of citral. However, the activity was slightly lower in the presence of citral than in its absence at higher pH value. The pK\textsubscript{a} values were determined to be 8.19 ± 0.02 and 8.26 ± 0.05 in the absence and presence of citral, respectively. There was no significant change in the pK\textsubscript{a} value of the enzyme in the presence of citral. Hence, citral does not alter the nucleophilicity of the catalytic Cys residue, indicating that it is not a significant contributing factor in the decrease of apparent \( V_{max} \) value of the enzyme in presence of citral.

![Fig. 4.17. Determination of pK\textsubscript{a} values in the absence and presence of citral (85 µM). The esterase activity of hsALDH was measured over a pH range of 5.0-9.5. The buffer systems used were: 15 mM sodium acetic acid buffer, 15 mM MES buffer and 60 mM Tris-HCl buffer.](image)

4.3.2.6. UV-Vis Spectroscopy

UV absorption spectrum of hsALDH was recorded in the absence and in presence of increasing citral concentration (0-250 µM). The characteristic spectrum of the enzyme was observed to be altered in the presence of citral (Fig. 4.18). There was an increase in the absorption peak corresponding to 218 and 280 nm of the enzyme in the presence of increasing citral concentration (Fig. 4.18 and the inset). The increase in absorbance at around 218 nm shows that the peptide backbone structure is altered.
in presence of citral. This probably suggests that there is a change in the secondary and tertiary structure of the enzyme. Further, the absorbance at 280 nm was found to increase in presence of increasing citral concentration (Fig. 4.18 inset), showing hyperchromic effect which may be due to alteration of the secondary and tertiary structure of the enzyme and hence the exposure of chromogenic groups. Changes in the absorption properties of the enzyme indicate that citral binds and forms a complex with the hsALDH and causes structural changes in the enzyme.

![UV-Vis absorption spectra of hsALDH](image)

**Fig. 4.18.** UV-Vis absorption spectra of hsALDH. Spectra of the enzyme were recorded in the absence and in presence of increasing citral concentration (0-250 µM) in the wavelength range of 200-400 nm at 25°C. Increase in the absorbance at 280 nm with increase in citral concentration is shown in the inset.

### 4.3.2.7. CD Spectroscopy

The changes in the secondary structure of hsALDH in the absence and in presence of varying concentration of citral were determined by far-UV CD Spectroscopy. There was a significant decrease in the two negative peaks corresponding to 208 and 222 nm in the presence of increasing concentration of citral (Fig. 4.19). The α-helix and
β-sheet contents in the absence and in presence of citral were found to be 37.45% α-helix, 22.16% β-sheet and 31.43% α-helix, 29.25% β-sheet, respectively. Citral upon binding with the enzyme decreases the α-helix content by about 6% and increases the β-sheet content by almost 7%. This shows that binding of citral to hsALDH changes the secondary structure, leading to destabilization of the native structure, which may contribute to the inhibition process by subsequent alteration of the substrate and/or co-enzyme binding ability of the enzyme.

![Far-UV CD spectra of hsALDH in the presence of varying concentration of citral. HsALDH (2 µM) was incubated with various concentrations of citral (0-250 µM) for 30 min at 25°C. Far-UV CD spectra were then recorded in the wavelength range of 200-240 nm with a spectral bandwidth of 1.0 nm. Spectra corresponding to hsALDH at 0 µM (0), 10 µM (1) 50 µM (2) 100 µM (3), 150 µM (4) and 250 µM (5) citral are presented in the figure.](image)

4.3.2.8. Fluorescence quenching measurements

Titration of hsALDH with varying concentration of citral (0-500 µM) showed the quenching of intrinsic fluorescence of tryptophan as evident from the gradual decrease in emission intensity of hsALDH with the increase in citral concentration (Fig. 4.20). The Stern-Volmer plot (Fig. 4.21 A) was used to determine the Stern-Volmer quenching constant ($K_{sv}$) and it was found to be $2.71 \pm 0.05 \times 10^3$ M$^{-1}$. Modified Stern-Volmer plot (Fig. 4.21 B) revealed the binding constant ($K_b$) of $6.06 \pm 0.02 \times 10^3$ M$^{-1}$ and a stoichiometric binding ratio (n) of 1.09 (Table 4.3). $K_{sv}$ and $K_b$ value indicates that the binding of citral to hsALDH is very strong and of the order of static binding (complex formation) with a 1:1 binding ratio.
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Fig. 4.20. Fluorescence emission spectra of hsALDH. Spectra of hsALDH were recorded in the wavelength range of 290-450 nm with increasing concentration of citral (0-500 µM) at 25°C. Samples were excited at 295 nm to record specifically the tryptophan fluorescence emission.

Fig. 4.21. Stern-Volmer plot (A) and Modified Stern-Volmer plot (B) of hsALDH-citral binding at 25°C. The concentration of hsALDH used was 100 µg. The Stern-Volmer and Modified Stern-Volmer graphs were plotted using equation (iii) and (iv), respectively, as mentioned in the section 4.2.11.

Table 4.3. Binding parameters of hsALDH-citral interaction at 25°C.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>$K_{sv}$ $(10^3 \text{ M}^{-1})$</th>
<th>$K_b$ $(10^7 \text{ M}^{-1})$</th>
<th>n</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>298 K</td>
<td>2.71±0.05</td>
<td>6.06±0.02</td>
<td>1.09±0.04</td>
<td>0.98</td>
</tr>
</tbody>
</table>
4.3.2.9. van’t Hoff plot and the effect of temperature on the binding of citral to hsALDH

The effect of temperature on the binding of citral to hsALDH was determined and it was found that the binding constant decreased from $6.74 \times 10^3$ M$^{-1}$ to $5.19 \times 10^3$ M$^{-1}$ with the increase in temperature from $15^\circ C$ (288 K) to $37^\circ C$ (310 K), respectively. This decrease in binding constant with increase in temperature indicates that the quenching of fluorescence by citral may be static in nature and there might be the formation of a complex between citral and hsALDH. With the increase in temperature, the citral-hsALDH complex probably destabilizes and thereby decreases the binding constant.

![Fig. 4.22. van’t Hoff plot of hsALDH-citral binding showing the effect of temperature on the binding constant. The graph was plotted using equation (v) as mentioned in section 4.2.12.](image)

**Table 4.4.** Thermodynamic parameters of hsALDH-citral interaction at 25°C.

<table>
<thead>
<tr>
<th>T</th>
<th>ΔH</th>
<th>ΔS</th>
<th>TΔS</th>
<th>ΔG</th>
</tr>
</thead>
<tbody>
<tr>
<td>298 K</td>
<td>-3.41 kJ mol$^{-1}$</td>
<td>19.87 J K$^{-1}$ mol$^{-1}$</td>
<td>5.92 kJ mol$^{-1}$</td>
<td>-9.33 kJ mol$^{-1}$</td>
</tr>
</tbody>
</table>

The van’t Hoff plot was used to calculate the thermodynamic parameters i.e., ΔH, ΔS and ΔG (Table 4.4). Fig. 4.22 shows the dependence of binding constant ($K_b$) with temperature (1/T) with a slope $-\Delta H/R$ and intercept of $\Delta S/R$. Protein-ligand interaction primarily involves the non-covalent interactions such as hydrogen bonding, electrostatic interactions, hydrophobic interactions and van der Waals forces. Citral contains three methyl groups on its surface along with one carbonyl (–C=O)
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group and two double bonds (Fig. 4.2). The methyl groups might provide the hydrophobic forces for binding to the enzyme. The partial negative charge on the carbonyl oxygen may contribute to the binding through hydrogen bond formation and ionic interactions. Also, the double bonds with degenerated π electrons may interact with enzyme through π-π interactions. This was further confirmed by molecular docking analysis. A negative ΔH (-3.41 kJ mol⁻¹) and a positive ΔS (19.87 J K⁻¹ mol⁻¹) value (Table 4.4) shows that the electrostatic interactions may also be involved in the hsALDH-citral complex formation. Negative ΔG (-9.33 kJ mol⁻¹) and ΔH (-3.41 kJ mol⁻¹) shows that the binding of citral to hsALDH is a spontaneous process.

4.3.2.10. FRET analysis

Fig. 4.23. FRET analysis showing the overlapping region. Emission spectrum of hsALDH and absorption spectrum of citral were recorded at 25°C.

FRET is used to determine the possibility of energy transfer and the distance between a donor protein and a ligand molecule. When the emission spectrum of donor and the absorption spectrum of acceptor overlap with an intermolecular distance (r) of less than 7 nm, energy is effectively transferred. Here, it was found that both the spectrum overlaps (Fig. 4.23) with an r value of 2.33 nm and therefore, energy transfer took place between hsALDH and citral. The value of E, Ro and J were determined to be 0.155, 1.75 nm, 1.3x10⁻¹⁵ M⁻¹ cm⁻¹, respectively. Also, it was found that 0.5Ro < r < 1.5Ro, which indicates that the quenching is static due to complex formation between citral and hsALDH.
4.3.2.11. Molecular docking analysis

Molecular docking analysis revealed that citral (both geranial and neral) fits into the active site of ALDH3A1 close to the catalytic Cys 243 and other highly conserved amino acid residues, through \( \pi-\pi \) interactions, hydrophobic interactions, ionic interactions and hydrogen bonding. The amino acid residues involved in the interaction of geranial with ALDH3A1 are Asn 114, Thr 112, His 289, Phe 335 and Trp 113 (Fig. 4.24 A). Trp 113 forms hydrogen bond with the carbonyl oxygen of geranial and \( \pi-\pi \) interactions with its double bond \( \pi \) electrons. Thr 112 forms hydrogen bond with the carbonyl oxygen, whereas His 289 forms hydrophobic interaction with the methyl group of geranial. Asn 114 forms hydrogen bond with the carbonyl oxygen of geranial. Phe 335 binds through multiple hydrophobic interactions with the methyl groups of geranial. The amino acid residues involved in the interaction of neral with ALDH3A1 are Trp 113, Gly 187, His 289 and Phe 335 (Fig. 4.24 B). Trp 113 forms multiple hydrophobic interactions with the methyl group of neral. Gly 187 forms ionic interaction with the carbonyl oxygen of neral. Phe 335 and His 289 bind through multiple hydrophobic interactions with the methyl groups of neral. Therefore, both the isomers of citral form strong complex with ALDH3A1 in its active site through multiple non-covalent interactions with some of the highly conserved amino acid residues.

4.3.2.12. Measurement and analysis of ligand efficiency metrics

The efficiency, selectivity and potency of citral were measured and compared based on its calculated LE, PEI and BEI values. The LE, PEI and BEI were determined to be 0.52, 3.62 and 37.49, respectively. The mean LE of most of the commercial oral drug molecules is 0.51, which is also the LE of an ideal ligand molecule under standard conditions (Abad-Zapatero, 2007; Hopkins et al., 2014). Additionally, the recommended drug discovery benchmark value of LE is \( \geq 0.30 \) (Hopkins et al., 2004; Schultes et al., 2010). Based on a large sample of marketed oral drugs, the reference value of PEI and BEI for an ideal ligand molecule have been determined as 1.5/2.7 and 27, respectively (Abad-Zapatero, 2007; Abad-Zapatero and Metz, 2005). The values of these three parameters determined for citral are found to be in good agreement with the recommended reference values, indicating that it may be a possible drug candidate with
Fig. 4.24. Molecular docking analysis of hsALDH-citral binding. Both geranial (A) and neral (B) were docked into the active site of “Apo structure of human ALDH3A1 (PDB ID: 3SZA).” Each figure highlights the complex of ALDH3A1 with geranial or neral (a), interactions of residues with the molecule (b), and the key residues involved in the interaction (c).
improved physicochemical and pharmacokinetic properties. Therefore, citral may be a better drug candidate for its good ligand efficiency, potency and druggability for the enzyme and hence may be used as an efficient ligand molecule for the inhibition of hsALDH.

4.4. Conclusions

Caffeine was found to inhibit both the dehydrogenase and esterase activity of hsALDH. It reduced the substrate binding affinity and the catalytic efficiency of the enzyme. The mode of inhibition is mixed type with partial competitive and non-competitive behavior. Caffeine increased the pK\textsubscript{a} value of the enzyme and hence, the nucleophilicity of the catalytic Cys residue is reduced. It partially altered the secondary structure of the enzyme. Biophysical investigation revealed that caffeine strongly binds with hsALDH and forms a complex in a static manner. Molecular docking analysis revealed that caffeine binds to the active site of the enzyme and interacts with some of the highly conserved amino acid residues through non-covalent interactions. Ligand efficiency metrics values indicate that it is an efficient ligand for the enzyme in terms of its physicochemical and pharmacokinetic properties. Therefore, it is very likely that caffeine binds and inhibits the activity of hsALDH by decreasing the substrate binding affinity and partially competing with the substrate to bind to the active site. Also, it reduces the catalytic activity of the enzyme by decreasing the nucleophilicity of catalytic Cys residue and partially altering the secondary structure of the enzyme.

Citral was found to inhibit the dehydrogenase activity of hsALDH. There was no significant change in the esterase activity of the enzyme in presence of citral. It reduced the substrate binding affinity and to a lesser extent, the catalytic efficiency of the enzyme. Citral altered both the $K_m$ and $V_{max}$ values which indicated linear mixed-type inhibition with more of competitive behavior and a little but significant non-competitive inhibition. Binding of citral does not influence the pK\textsubscript{a} value of the enzyme and hence, the nucleophilicity of the catalytic Cys residue is not a significant contributing factor in the inhibition process. Citral shows uncompetitive inhibition towards the co-enzyme (NAD\textsuperscript{+}), which may be a contributing factor in the decrease of $V_{max}$ value and hence the reduction in catalytic efficiency of the enzyme. It partially altered the secondary structure, leading to destabilization of the enzyme. Biophysical
Inhibition of hsALDH by caffeine and citral

studies revealed that citral forms a complex with hsALDH in a static manner. Molecular docking analysis revealed that both the isomers of citral bind to the active site of the enzyme and interact with some of the highly conserved amino acid residues through multiple non-covalent interactions. Ligand efficiency metrics values indicate that citral is an efficient ligand for the enzyme in terms of its physicochemical and pharmacokinetic properties. Therefore, it can be inferred that citral binds and inhibits the dehydrogenase activity of hsALDH by decreasing the substrate binding affinity and competing with the substrate to bind to the active site. Further, it reduces the catalytic activity of the enzyme by uncompetitively inhibiting the co-enzyme catalysis, and partially destabilizing the enzyme by altering its secondary structure.

The present work shows that both the natural compounds inhibit hsALDH with IC50 value of 85 µM and 150 µM for citral and caffeine, respectively. Comparisons of ligand efficiency metrics values show that both caffeine and citral are efficient ligands for the enzyme, with citral as better ligand than caffeine in terms of physicochemical and pharmacokinetic properties. Therefore, citral is a stronger inhibitor of hsALDH than caffeine. This indicates that oral intake of caffeine or citral through different means may have a harmful effect on the oral health and may increase the risk of aldehyde related pathogenesis including oral carcinogenesis. ALDH3A1 enzyme is occasionally over expressed in neoplastic tissues (Giebultowicz et al., 2008), resulting in increased resistance to oxazaphosphorine chemotherapy (Sladek, 1999). Therefore, it can be speculated that a cocktail of chemotherapeutic drug with ALDH3A1 inhibitors, such as caffeine or citral may act as an adjuvant and is expected to increase the sensitivity of these drugs through their inhibitory effect on the enzyme.