RESEARCH ARTICLE

Activation of Human Salivary Aldehyde Dehydrogenase by Sulforaphane: Mechanism and Significance

Md. Fazle Alam, Amaj Ahmed Laskar, Lubna Maryam, Hina Younus*

Enzymology Laboratory, Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh, India

* hyounus.cb@amu.ac.in

Abstract

Cruciferous vegetables contain the bio-active compound sulforaphane (SF) which has been reported to protect individuals against various diseases by a number of mechanisms, including activation of the phase II detoxification enzymes. In this study, we show that the extracts of five cruciferous vegetables that we commonly consume and SF activate human salivary aldehyde dehydrogenase (hsALDH), which is a very important detoxifying enzyme in the mouth. Maximum activation was observed at 1 μg/ml of cabbage extract with 2.6 fold increase in the activity. There was a ~1.9 fold increase in the activity of hsALDH at SF concentration of $\geq$ 100 nM. The concentration of SF at half the maximum response (EC_{50} value) was determined to be 52 ± 2 nM. There was an increase in the V_{max} and a decrease in the K_m of the enzyme in the presence of SF. Hence, SF interacts with the enzyme and increases its affinity for the substrate. UV absorbance, fluorescence and CD studies revealed that SF binds to hsALDH and does not disrupt its native structure. SF binds with the enzyme with a binding constant of 1.23×10^7 M^{-1}. There is one binding site on hsALDH for SF, and the thermodynamic parameters indicate the formation of a spontaneous strong complex between the two. Molecular docking analysis depicted that SF fits into the active site of ALDH3A1, and facilitates the catalytic mechanism of the enzyme. SF being an antioxidant, is very likely to protect the catalytic Cys 243 residue from oxidation, which leads to the increase in the catalytic efficiency and hence the activation of the enzyme. Further, hsALDH which is virtually inactive towards acetaldehyde exhibited significant activity towards it in the presence of SF. It is therefore very likely that consumption of large quantities of cruciferous vegetables or SF supplements, through their activating effect on hsALDH can protect individuals who are alcohol intolerant against acetaldehyde toxicity and also lower the risk of oral cancer development.

Introduction

The aldehyde dehydrogenase (ALDH) superfamily consists of 19 isozymes, which catalyze the oxidation of endogenous and exogenous toxic aldehydes (short, long aliphatic and aromatic)

into non-toxic corresponding acids [1,2]. Modest consumption of ethanol is believed to be beneficial for health, whereas large consumption can cause several complications [3,4]. Ethanol is first oxidized by alcohol dehydrogenase into the primary metabolite acetaldehyde, which is mutagenic and placed in group 1 carcinogens [5]. ALDH than detoxifies acetaldehyde by oxidizing it to acetic acid. More than 40% East Asian population (~ 560 million or ~ 8% of the world population) cannot oxidize the toxic acetaldehyde into the non-toxic acid because of having a point mutation in the ALDH2 gene which results in almost no enzyme activity, leading to the accumulation of the toxic acetaldehyde [6–8]. ALDH2 deficient people are very sensitive to alcohol, a small dose of it can cause severe consequences like nausea, facial flushing, tachycardia and long-lasting headache [9]. There are many reports indicating the chances of occurrence of malignancy and other serious health problems due to ALDH polymorphism [10]. Particularly, the occurrence of squamous cell carcinoma in the upper aerodigestive track (UADT) among the middle aged East Asian populations is associated with the ALDH2 polymorphism [6,11]. There is more than 80 fold increased risk for squamous cell carcinomas in the UADT of heavy drinker heterozygotic individual (ALDH*1/*2) compared with only about 4 fold increase in the wild-type (ALDH*1/*1) heavy drinkers [6,12–14].

Human salivary aldehyde dehydrogenase (hsALDH) found in the saliva is basically ALDH3 isoform of the ALDH enzyme. ALDH3 is highly expressed in the epithelial lining of the UADT, kidney, mammary gland, liver and stomach [15,16]. HsALDH catalyzes the oxidation of the aromatic aldehydes having bulky side chains, medium/long chain aliphatic aldehydes and α,β-hydroxyalkenal aldehydes, but not acetaldehyde under basal condition [17]. Moderate consumption of ethanol by ALDH2*1/*2 heterozygote individuals leads to a significant increase in the acetaldehyde concentration in the saliva which forms an adduct interacting with DNA, resulting in severe health issues [18]. There are reports on strategies to increase the rate of acetaldehyde elimination especially in ALDH2*1/*2 heterozygotes through the modulation of the activity of ALDH2 by chemical chaperones, and by inducing and recruiting other ALDH isoforms [4,19,20].

The cruciferous vegetables which include broccoli, cauliflower, radish, kale, cabbage and brussels sprouts are a rich source of health beneficial secondary metabolites [21]. Much of the health benefits have been attributed to the physiological effects of the isothiocyanates, especially sulforaphane (SF) which has been shown to protect against various types of cancers, diabetes, atherosclerosis, respiratory diseases, cardiovascular diseases, neurodegenerative disorders and ocular disorders [22,23]. A number of mechanisms by which SF protects cells have been reported which includes induction of apoptosis, cell cycle arrest, anti-inflammatory effects, inhibition of phase 1 (cytochrome P450) enzymes and induction of phase 2 detoxification enzymes [23–25]. Therefore, modulation of important enzymes like quinone reductase, glutathione S-transferase, ALDHs and ribonucleoside diphosphate reductase by natural bioactive compounds like SF or synthetic chemical compounds may be a significant component of their anticarcinogenic action [26,27]. Recently it has been shown that SF accelerates acetaldehyde metabolism by inducing ALDHs and hence may protect individuals who are alcohol intolerant against acetaldehyde toxicity [20].

The present study was undertaken after getting very encouraging results from one of our previous studies where a chemical activator or chaperone (Alda 1) was designed which activated ALDH2 and restored near wild type activity of ALDH2*2 [4]. In this study, we aimed to investigate the effect of different types of cruciferous vegetables extracts and their bio-active compound SF on the activity of hsALDH. Since they activated this very useful detoxifying enzyme, we than evaluated the mechanism of activation of the enzyme by SF through kinetic measurements and different biophysical techniques. Molecular docking analysis was done to

determine the binding site and the amino acid residues of the enzyme involved in the interaction with SF.

Materials and Methods

Materials

SF, 6-methoxy-2-naphthaldehyde, NAD^+, NADH, DTT and acetaldehyde were purchased from Sigma. Acetone, ethanol, acetonitrile, n-hexane and chloroform were obtained from SRL, India. Fresh cruciferous vegetables like cabbage, cauliflower, radish, turnip and broccoli were purchased from the local market of Medical Road, Aligarh, India in the month of November, 2015. These vegetables are grown in the agricultural field of the locality and made available to us through local vendors. EDTA was the product of Himedia chemicals, India. All other chemicals and reagents used were of analytical grade.

Preparation of crude extract of cruciferous vegetables

Fresh broccoli, cabbage, cauliflower, turnip and radish were obtained. The edible parts were collected in each case, washed and air dried in portions. The dried portions were combined and homogenized in 50 ml methylene chloride for 5 min. The crushed homogenate was left to autolyze at room temperature for 5 min. After autolysis, the homogenate was filtered and again extracted with 50 ml methylene chloride. The extracted fractions were combined and salted with anhydrous 5 mM sodium sulphate. The methylene chloride fractions were dried at 30°C under vacuum on a rotatory evaporator. The residue was dissolved in acetonitrile and then filtered through a 0.22 μm filter and stored for further use.

HsALDH activity measurements

Preparation of crude saliva and purification of hsALDH by DEAE-Cellulose column was carried out according to our published procedure [28]. The collection of human saliva samples was approved by the institutional ethical committee of Interdisciplinary Biotechnology Unit (IBU), Aligarh Muslim University, Aligarh, India. The participants were the research students of IBU (MFA, AAL, LM) who gave a verbal consent to their research supervisor (HY) and the members of the ethical committee, which was approved. The activity assay of crude and pure hsALDH (ALDH3A1) was done using fluorogenic naphthaldehyde substrate. In particular, 6-methoxy-2-naphthaldehyde is used to measure selectively the activity of the ALDH3A1 isoform [29]. All the activity assays were performed in 50 mM sodium phosphate buffer, pH 7.5 at 25°C, in the presence of 0.5 mM DTT and 0.5 mM EDTA. Substrate, 6-methoxy-2-naphthaldehyde (5 μM) and coenzyme NAD^+ (100 μM) were used to measure the activity of hsALDH [30,31]. The reaction was started by the addition of the enzyme at 25°C and monitoring continuously for 5 min. Fluorescence background noise, if any, was measured prior to enzyme addition and subtracted from the final slope. Fluorimetric assays were run on a Shimadzu RF-5301PC instrument with excitation and emission wavelengths as 315 and 360 nm, respectively. The activity of hsALDH was expressed in terms of nanomolar (nM) concentration of flourogenic product or (NADH) formation per min per μg of the enzyme under the standard assay conditions using a standard plot of the respective product.

Effect of SF on the activity of hsALDH

The effect of the different vegetable extracts and SF on the activity of purified hsALDH was studied by incubating the enzyme for 1 min in presence of different concentration of the vegetable extracts (0–2.5 μg/ml) or SF (0–500 nM). Activity was then determined under standard

assay condition as described above. Data were fitted in the case of SF into non-linear regression analysis curve of log (SF) vs activity to determine the EC_{50} value under "Dose response Stimulation" using "Graphpad Prism 6.0".

Substrate dependent activity assay in absence/presence of SF

The activity of hsALDH was determined by varying the concentration of the substrate (0–20 µM) in absence and presence of 50 nM SF at standard reaction conditions. All the apparent enzyme kinetic parameters (V_{max} and K_m) were determined by fitting the data in non-linear regression analysis curve of Michaelis-Menten plot and Line-weaver Burk plot under 'Enzyme kinetic' function in "Graphpad Prism 6.0".

UV absorption analysis

Absorption measurements were performed using a Perkin-Elmer (Lambda 25) double beam UV-VIS Spectrophotometer. Fixed concentration of hsALDH (0.2 mg/ml) and varying concentration of SF (0–300 nM) were taken and spectra were recorded from 250–350 nm at 25˚C.

Fluorescence quenching measurements

Fluorescence quenching measurements were done on a Shimadzu 5301 PC fluorescence spectrophotometer equipped with a constant temperature holder and the temperatures were maintained by a constant temperature water circulator (Julabo Eyela). The excitation and emission slit widths were set at 3 nm. Titration of SF (0–60 nM) with hsALDH solutions (0.2 mg/ml) was carried out in a dual-path length fluorescence cuvette (10 x 3.5 mm). Intrinsic fluorescence was measured by exciting the sample at 280 nm. The emission spectra were recorded in the range of 300–450 nm and the data were plotted by taking the emission intensity at 339 nm. The decrease in fluorescence intensity at 339 nm was analyzed using the following Stern-Volmer Eq (1):

$$F_o/F = K_{sv}[Q] + 1 = Kq\tau_o[Q] + 1 \qquad (1)$$

where, F_0 and F are fluorescence intensities in absence and presence of SF, K_{sv} is the Stern-Volmer quenching constant, K_q is the bimolecular rate constant of the quenching reaction and τ_0 is the average integral fluorescence life time of tryptophan, which is $\sim 10^{-9}$ s. For the correction of inner filter effect of protein and ligand we used following Eq (2) [32,33]:

$$F_{cor} = F_{obs}10^{(A_{ex}+A_{em})/2} \qquad (2)$$

Where F_{cor} and F_{obs} are corrected and observed fluorescence intensity, Aex and Aem are the absorption of the system at excitation (280 nm) and emission (339 nm) wavelength, respectively. Binding constant and number of binding sites were obtained from the following modified Stern-Volmer Eq (3):

$$\log(F_o/F - 1) = \log K_b + n \log[Q] \qquad (3)$$

Where, K_b is binding constant and n is number of binding sites.

Far UV-Circular Dichroism (CD) measurements

The CD studies of hsALDH in the absence and presence of SF were carried out with a JASCO-J815 Spectropolarimeter equipped with a Peltier-type temperature controller. The instrument was calibrated with D-10-camphorsulfonic acid. All the CD measurements were performed at 25˚C. Spectra were collected with a 50 nm min^{-1} scan speed, 0.1 nm data pitch

and a response time of 2 s. Each spectrum was the average of 2 scans. The far UV-CD spectra were recorded in the wavelength range of 190–250 nm. All the spectra were smoothed by the Savitzky-Golay method with 25 convolution width. 0.2 mg/ml hsALDH concentration was used for the experiment.

Effect of SF on the activity of hsALDH towards acetaldehyde

The activity of hsALDH towards acetaldehyde in the absence and presence of SF was determined using the above standard activity assay method using acetaldehyde (50 μM) as the substrate. NADH fluorescence was measured to determine the activity in terms of NADH formation. The instrumental settings for NADH were: Excitation at 340 nm and emission at 460 nm, with spectral bandwidths of 10 nm for both excitation and emission beams. The amount of NADH formed was determined from the standard curve of NADH at 460 nm.

Molecular docking studies

To determine the binding site and the amino acid residues of ALDH3A1 interacting with SF, *in silico* docking studies were performed by AutoDock Vina (http://vina.scripps.edu/) and further confirmed by commercially available docking software GOLD. The crystal structure of apoform of ALDH3A1 was obtained from Protein Data Bank (PDB ID: 3SZA), and sdf file of SF (CID: 5350) was obtained from PubChem database. Docking analysis was carried out with the grid size set as 60, 60 and 60 along the X, Y and Z axes with 0.375 Angstrom grid spacing. The 10 best solutions based on docking score were retained for further analysis. Discovery studio 3.5 was used for visualization and for the identification of residues involved in binding.

Results and Discussion

SF derived from its glucosinolate precursor contained in cruciferous vegetables has been reported to increase the phase II antioxidant enzymes in the human upper airway and has recently been shown to induce ALDHs [20,34]. The level of hsALDH was found to be high in the saliva of subjects who continuously ingest large quantities of broccoli [16]. Therefore, in the present study, the direct effect of different cruciferous vegetable extracts and SF on the activity of pure hsALDH has been determined.

Effect of different cruciferous vegetable extracts on the activity of hsALDH

The effect of five common cruciferous vegetables on the activity of hsALDH has been studied. The relative activity of the enzyme in the presence of varying concentration of each vegetable extract is shown in Fig 1. Each of the vegetable extract in the concentration used (till 2.5 μg/ ml) activated the enzyme. Maximum activation was observed at 1 μg/ml of cabbage, broccoli, cauliflower and turnip extract with 2.6, 2.0, 1.7 and 1.6 fold increases in activity, respectively. For these vegetable extracts, when the concentration of the extract was increased above 1 μg/ ml, the activating effect decreased gradually. However, the enzyme activity still remained higher than that in the absence of the vegetable extract even at 2.5 μg/ml of the extract. For the radish extract in the concentration range used, the relative activity kept increasing with increasing concentration of the extract. There was 1.3 fold increase in the activity at 1 μg/ml of the extract. Therefore, it is evident from the data that out of all the five cruciferous vegetables used in this study, cabbage exhibited the maximum activating effect on the activity of hsALDH. The level of glucoraphanin from which SF is derived is highly variable in different cruciferous vegetables and broccoli is a rich source of glucoraphanin [35]. However, still
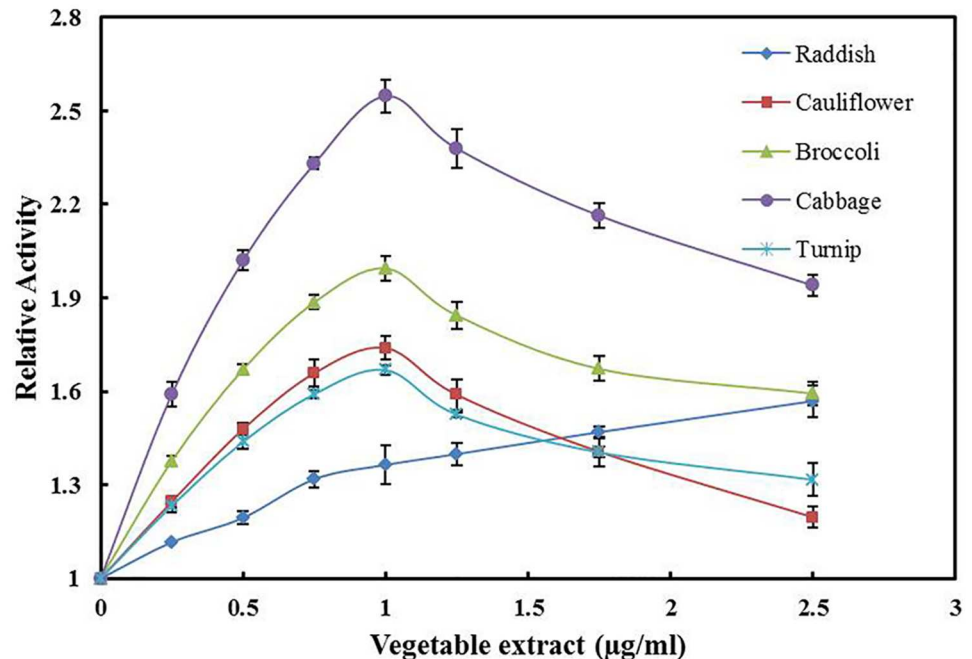
Fig 1. Effect of cruciferous vegetable extracts on the activity of hsALDH. The amount of hsALDH used in the reaction was 20 μg, concentration of the substrate (6-methoxy-2-naphthaldehyde) was 5 μM, concentration of NAD was 100 μM and the reaction time was 5 min. Each point represents the mean of two experiments carried out in triplicates.

doi:10.1371/journal.pone.0168463.g001

higher amounts are present in some cultivars of cabbage [36]. Therefore, the higher level of SF in cabbage extract may be the reason why this extract exhibited the maximum activating effect on hsALDH activity.

Effect of SF on the activity of hsALDH

The effect of SF on the activity of hsALDH was investigated. It was found that the activity of hsALDH remained the same till 20 nM concentration of SF (Fig 2). However, with further increase in the concentration of SF, the activity of the enzyme increased gradually upto 100 nM concentration of SF, after which the activity remained constant till 500 nM concentration of SF (Fig 2). With further increase in the concentration of SF (above 500 nM), there was a slight decrease in the enzyme activity which may be due to molecular crowding or non-specific binding of SF to the enzyme (data not shown). However, even at these high concentrations of SF examined, the activity was still higher than that in the absence of SF. Therefore, SF exerted an overall activating effect on the enzyme. The activity of hsALDH was increased by about 1.9 fold in presence of 100 nM SF. The concentration of SF at half maximum response (EC$_{50}$ value) was determined to be 52 ± 2 nM. Therefore, SF increases the activity of hsALDH to a good extent. However, crude cruciferous vegetables exhibited a greater stimulating effect on the activity of the enzyme than SF alone. This might be because of the presence of other beneficial compounds other than SF that shows cumulative effect to activate the enzyme. Ushida and Talalay (2013) similarly observed that out of the 20 compounds they examined, 15 of them including isothiocyanates such as SF, flavonoids, terpenoids, etc. increased the total ALDH specific activity in Hepa1c1c7 cells [20]. Some studies have shown that the ingestion of cruciferous vegetables lead to an increase in the activity of ALDH in human saliva and these
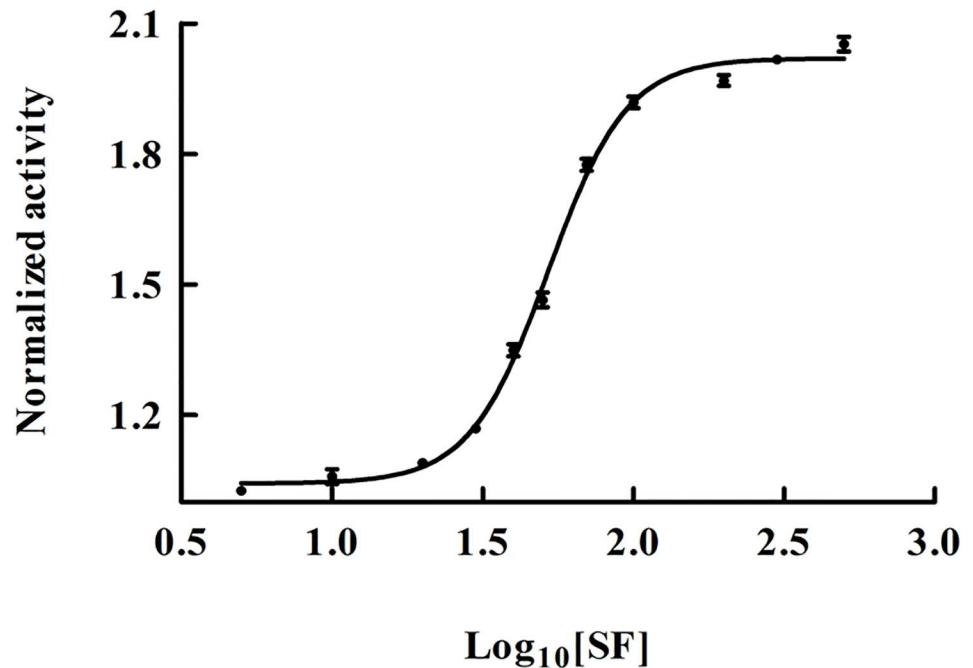
Fig 2. **Dose response curve of SF.** HsALDH activity was determined in presence of 0–500 nM concentration of SF using 15 µg of the enzyme in 1 ml of total reaction mixture. Each point represents the mean of two experiments carried out in triplicates.

vegetables were found to be inducers of the enzyme [16,20]. This *in vitro* study reveals that the binding of SF to hsALDH is strong because the binding constant (K_b) is high i.e 1.23 x 10^7 M^{-1} (see fluorescence quenching studies). Therefore, it is very likely that SF will bind with the enzyme *in vivo* and lead to its activation.

Substrate dependent activity assay in the absence/presence of SF

The activity of hsALDH with varying concentration of substrate (0–20 µM) was determined in the absence and presence of 50 nM SF. The apparent V_{max} and K_m were calculated by using the Michaelis-Menten plot (Fig 3A) and the Lineweaver-Burk plot (Fig 3B). It was found that the K_m of hsALDH towards the substrate (MONAL-62) decreased and the V_{max} increased in the presence of SF (Table 1). Therefore, SF increases the affinity of hsALDH for the substrate. SF interacts with the enzyme and favours the binding with the substrate. It is proposed that SF due to its antioxidant property, protects the catalytic Cys 243 amino acid residue from oxidation, which leads to the increase in the catalytic efficiency of the enzyme.

UV-visible absorption studies

UV-visible absorption spectroscopy is an important tool for steady-state studies of protein-ligand and DNA-ligand interaction as well as other biomolecules [37–41]. Changes in the far and near UV regions correspond to the secondary and tertiary structural changes, respectively. In proteins, we can discriminate the various internal chromophoric groups that give rise to electronic absorption bands. The aromatic amino acids contribute to bands in the range of 255–330 nm. Fig 4 shows that the absorption peak of hsALDH centers at 280 nm mainly due to the tryptophan residues. The formation of hsALDH-SF complexes is evident from the
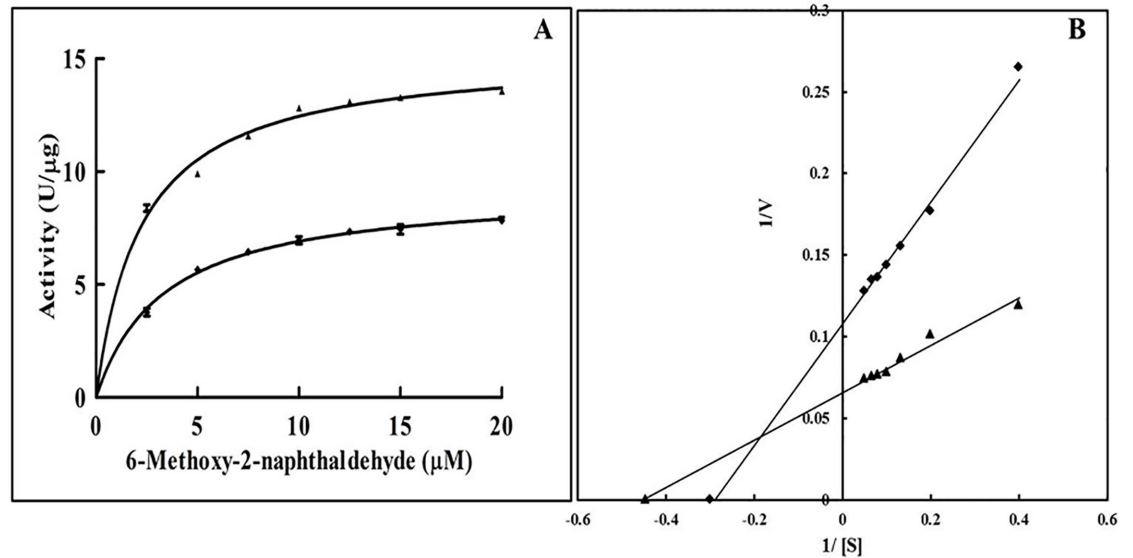
Fig 3. Substrate dependent activity of hsALDH in the absence/presence of SF (A) Michealis Menten plot (B) Line weaver Burk plot. Absence (♦) and presence (▲) of 50 nM SF. Each point represents the mean of two experiments carried out in triplicates.

doi:10.1371/journal.pone.0168463.g003

spectral data since absorbance decreases with the increase in SF concentration. The shift at 280 nm is not prominent. From these observations we can conclude that SF forms a complex with hsALDH and increases the compactness of the enzyme.

Fluorescence quenching studies

Fluorescence measurements give information about the molecular environment in the vicinity of the fluorophore molecules [42–45]. The fluorophores in protein are tryptophan, tyrosine and phenylalanine, however, tryptophan contributes maximally to the fluorescence [46]. Therefore, conformational changes of hsALDH were evaluated by the intrinsic fluorescence intensity before and after the addition of SF. The fluorescence intensity of hsALDH decreased gradually with increasing concentration of SF (Fig 5), which indicated that SF interacts with hsALDH. The decrease in fluorescence intensity upon addition of SF was analyzed according to the Stern-Volmer equation (Fig 6A). There is a linear dependence between F_0/F and the molar concentration of SF in the Stern-Volmer plot.

There are two types of quenching mechanisms, static and dynamic. In the dynamic mode of quenching, there is collision between ligand and protein which generally increases with increasing temperature, while in the case of static quenching, there is complex formation between ligand and protein which decreases with increasing temperature. When the value of K_q is greater than the maximum scatter collision quenching constant (2.0×10^{10} mol^{-1} sec^{-1}), it shows that quenching is not initiated by dynamic diffusion but occurs by formation of a strong complex between ligand and protein [33,47]. Here, it was observed that the value of K_q is

Table 1. Kinetic parameters of hsALDH in absence/presence of SF (50 nM).

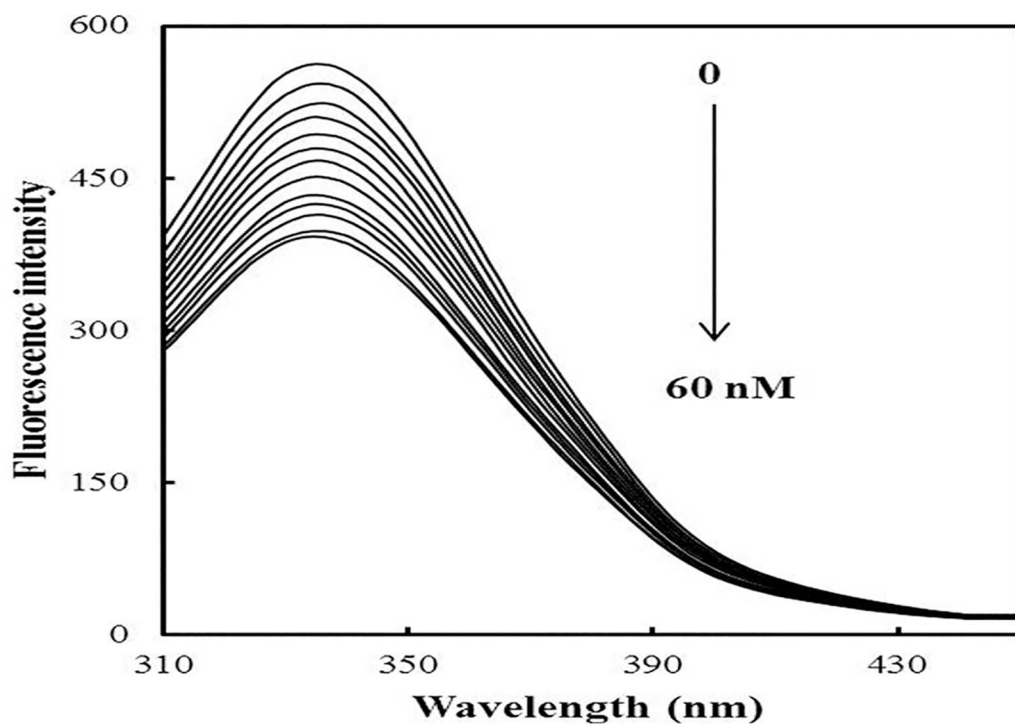| Kinetic parameters	Without SF	With SF
V_{max} (U/μg)	9.22 ± 0.18	15.24 ± 0.22
K_m (μg)	3.34 ± 0.25	2.41 ± 0.15

doi:10.1371/journal.pone.0168463.t001
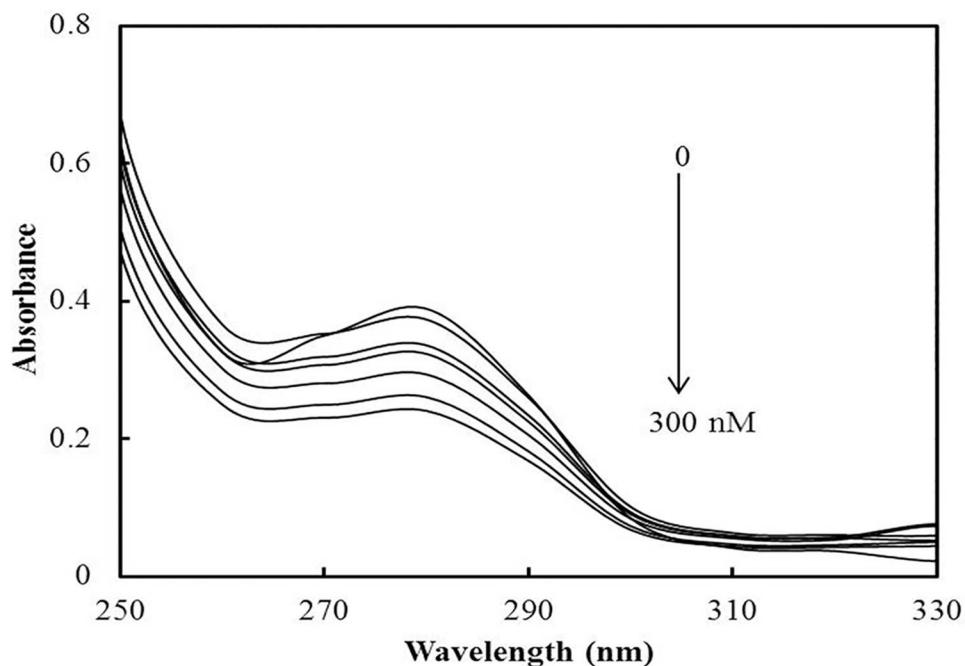
Fig 4. UV absorption spectra of hsALDH in the absence/presence of SF. UV spectra of hsALDH (0.2 mg/ml) in increasing concentration of SF (0–300 nM) were recorded.

Fig 5. Fluorescence emission spectra of hsALDH in the absence/presence of SF. Spectra of hsALDH (0.2 mg/ml) were recorded in the wavelength range of 300–450 nm in the presence of increasing concentration of SF (0–60 nM).
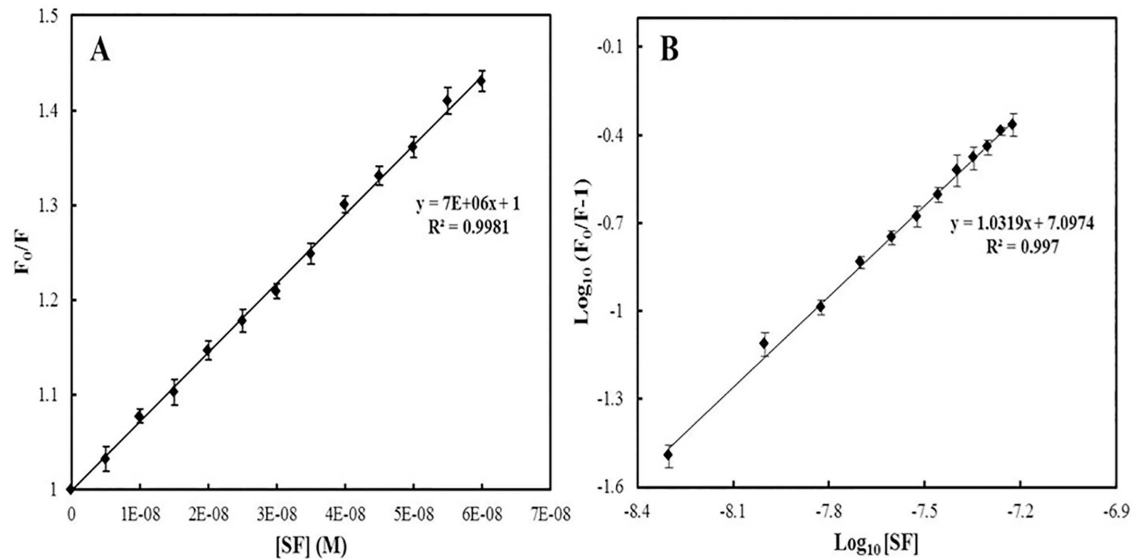
Fig 6. (A) Stern-Volmer plot (B) Modified Stern-Volmer plot for the binding of SF with hsALDH.

doi:10.1371/journal.pone.0168463.g006

greater than that of maximum scatter collision quenching constant (Table 2). Therefore, quenching is not initiated by collision but by complex formation between hsALDH and SF.

To determine the binding constant and the number of binding sites, log (Fo/F-1) vs. log [SF] was plotted (Fig 6B). From the slope and intercept of modified Stern-Volmer plots (Eq 3), the number of binding sites and the value of binding constant were calculated. The observed values of K_{sv}, K_q, K_b and n are listed in Table 2. Therefore, there is one binding site on hsALDH for SF, and the binding between the two is quite strong.

Far UV-CD studies

CD is an important technique by which the secondary and tertiary structure of biomolecules is studied [48,49]. It also helps in the elucidation of intermediate states like molten globule (MG) during conformational alteration of the proteins [50]. In order to know the conformational changes in hsALDH after interaction with SF, far UV-CD spectra were recorded in absence and presence of SF (Fig 7). The CD spectrum of hsALDH exhibited two negative minima in the UV region at 208 and 222 nm, which is characteristic of the α-helix structure of the protein [51,52]. The binding of SF to hsALDH leads to a small increase in both the negative minima peaks, clearly indicating that the α-helical structure in the enzyme increases to a small extent upon interaction with SF. Further, the CD spectra of hsALDH in the absence and in presence of SF were found to be similar in shape, revealing that the structure of hsALDH is predominantly α-helical even after the addition of SF. Therefore, it appears that the binding of SF with hsALDH stabilizes the native structure of the enzyme without any significant conformational changes. The enzyme appears to become slightly more compact.

Table 2. Binding parameters of hsALDH and SF complex.

Complex	K_{sv} (M^{-1})	K_q (M^{-1}sec^{-1})	K_b (M^{-1})	N	R^2
HsALDH-SF	7 x 10^6	1.22 x 10^{15}	1.23 x 10^7	1.03	0.997

doi:10.1371/journal.pone.0168463.t002
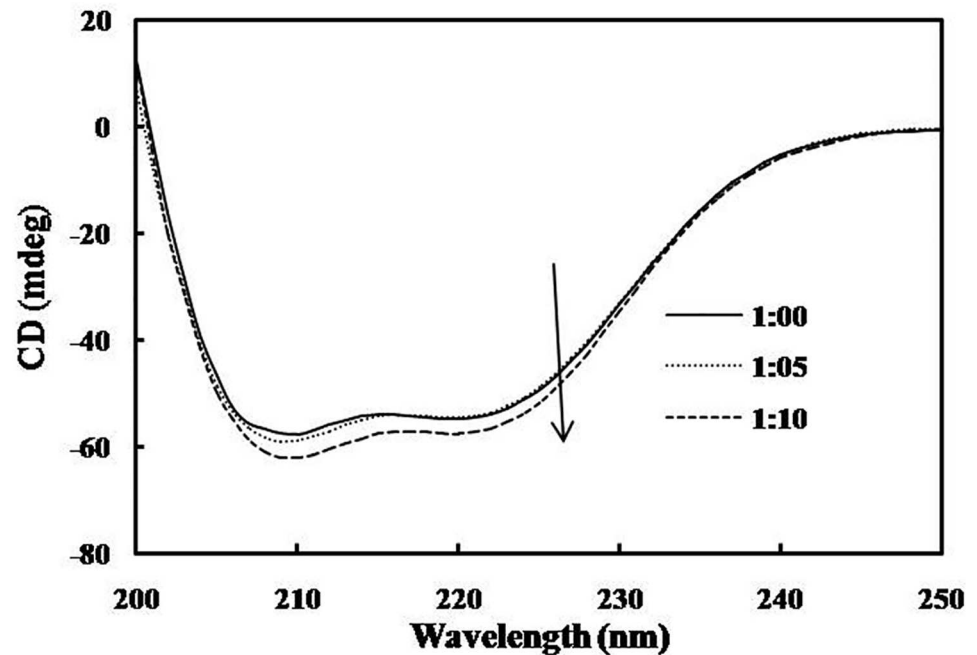
Fig 7. CD spectra of hsALDH in the absence/presence of SF. Far UV-CD spectra of hsALDH (0.2 mg/ml) were recorded with different molar ratios of hsALDH: SF (1: 00, 1: 05 and 1: 10).

doi:10.1371/journal.pone.0168463.g007

Effect of SF on the activity of hsALDH towards acetaldehyde

ALDH2 deficient alcohol consumers are exposed to high concentrations of salivary acetaldehyde and have been found to have an increased risk of upper digestive tract cancers [53]. Also, rinsing with ethanol-containing mouthwashes causes an increase in the acetaldehyde level in the saliva [54]. However, hsALDH is reported to be virtually inactive towards acetaldehyde [29]. Therefore, we examined whether SF can also activate hsALDH to oxidize acetaldehyde. The activity of hsALDH towards acetaldehyde as the substrate in the absence and presence of SF was studied (Fig 8). In the absence of SF, hsALDH showed virtually no activity towards acetaldehyde. However, in the presence of 50 nM SF, the enzyme exhibited significant activity towards acetaldehyde. Therefore, it is expected that SF should protect individuals from salivary acetaldehyde induced toxicity by activating hsALDH to oxidize acetaldehyde.
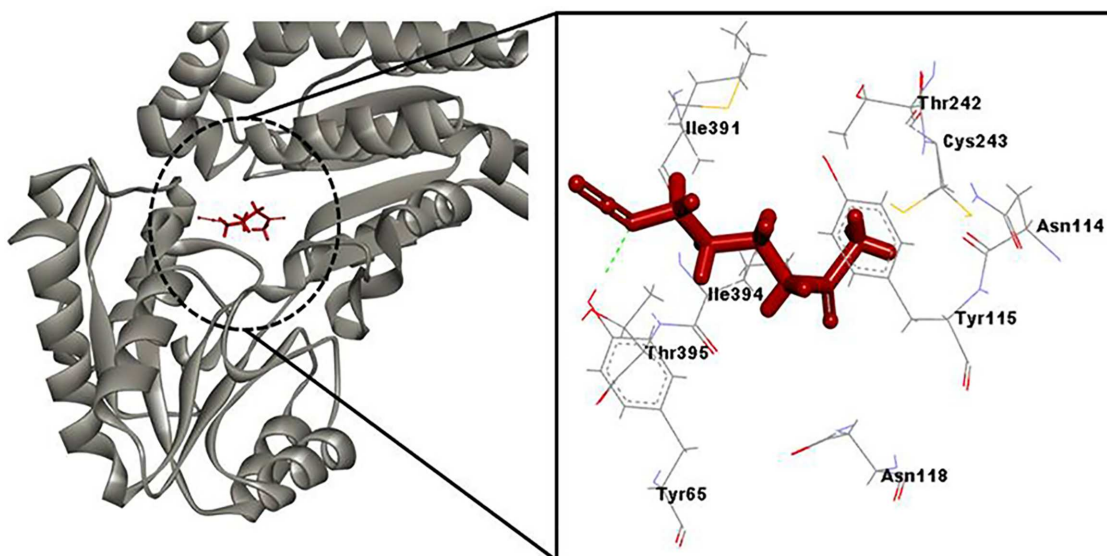
Molecular docking analysis

The docking results clearly showed that SF binds to the active site cavity of the ALDH3A1 and predominantly interacts with Ile 391, Thr 242, Tyr 115, Asn 114, Cys 243 and Tyr 65 (Fig 9A and 9B). The amino acid residues of the enzyme interact with SF mainly through hydrophobic interactions and hydrogen bonding. Tyr 65 is hydrogen bonded with SF while Ile 391, Thr 242 and Tyr 115 interact with SF through hydrophobic interactions. SF binds near the Cys 243 residue which acts as a nucleophile and plays a major role in the reaction catalyzed by the enzyme. It is very likely that SF due to its antioxidant property, protects the catalytic Cys 243 residue from oxidation, which leads to the increase in the catalytic efficiency and hence the activation of the enzyme. Also, SF interacts with Asn 114 which is a highly conserved residue in the catalytic domain of ALDHs and is having a role in the stabilization of oxyanion form of the thiohemiacetal during catalysis [55]. Therefore, SF fits inside the catalytic center and occupies some
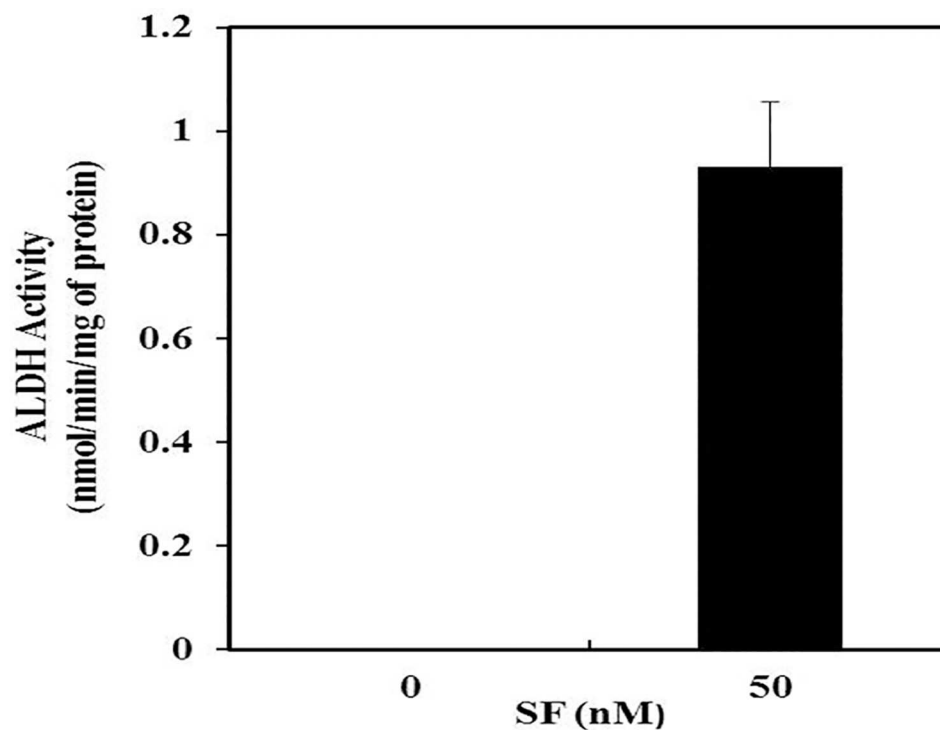
Fig 8. Activity of hsALDH towards acetaldehyde in the absence/presence of SF. The amount of enzyme used in the reaction assay was 20 µg. The concentration of acetaldehyde, NADH and SF used were 50 µM, 100 µM and 50 nM, respectively, and the reaction time was 5 min. The experiment was performed thrice in triplicates.

doi:10.1371/journal.pone.0168463.g008

Fig 9. Docking structure. (A) Molecular docking structure of hsALDH and SF (B) Amino acid residues interacting with SF.

doi:10.1371/journal.pone.0168463.g009

portion of the active site and thus decreases the overall size of active site cavity. We speculate that the reduced cavity now becomes capable of fitting the small acetaldehyde molecule, and therefore hsALDH starts catalyzing it.

Conclusions

The present study clearly shows that the cruciferous vegetables and their key bio-active compound SF activate hsALDH which is a very important detoxifying enzyme in the mouth. UV absorbance, fluorescence and CD studies revealed that SF binds to hsALDH and does not disrupt its native structure. The thermodynamic parameters indicate the formation of a spontaneous strong complex between hsALDH and SF. SF interacts with the enzyme and increases its affinity for the substrate. Molecular docking analysis revealed that SF occupies some portion of the active site of the enzyme and interacts with the important amino acid residues present in it, including the catalytic Cys 243 which acts as a nucleophile during catalysis. We propose that SF due to its antioxidant property, protects the catalytic Cys 243 residue from oxidation, which leads to the increase in the catalytic efficiency and hence the activation of the enzyme. In addition, the enzyme showed significant activity towards acetaldehyde in the presence of SF and hence can protect individuals who are alcohol intolerant against acetaldehyde toxicity. It is therefore very likely that consumption of large quantities of cruciferous vegetables or SF supplements can lower the risk of acetaldehyde mediated toxicity and oral cancer development.

Acknowledgments

Author Contributions

Conceptualization: HY.

Data curation: MFA AAL HY.

Formal analysis: MFA AAL HY.

Investigation: MFA AAL LM.

Methodology: MFA AAL HY.

Project administration: HY.

Resources: MFA AAL HY.

Software: MFA AAL HY.

Supervision: HY.

Validation: MFA AAL HY.

Visualization: MFA AAL HY.

Writing – original draft: MFA AAL HY.

Writing – review & editing: MFA AAL HY.

References

1. Vasiliou V, Pappa A (2000) Polymorphisms of human aldehyde dehydrogenases. Pharmacology 61: 192–198. PMID: 10971205

2. Chen CH, Ferreira JCB, Gross ER, Mochly-Rosen D (2014) Targeting aldehyde dehydrogenase 2: new therapeutic opportunities. Physiological reviews 94: 1–34. doi: 10.1152/physrev.00017.2013 PMID: 24382882

3. Crabb DW, Matsumoto M, Chang D, You M (2004) Overview of the role of alcohol dehydrogenase and aldehyde dehydrogenase and their variants in the genesis of alcohol-related pathology. Proceedings of the Nutrition Society 63: 49–63. PMID: 15099407

4. Perez-Miller S, Younus H, Vanam R, Chen CH, Mochly-Rosen D, Hurley TD (2010) Alda-1 is an agonist and chemical chaperone for the common human aldehyde dehydrogenase 2 variant. Nature structural & molecular biology 17: 159–164.

5. Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, Bouvard V, et al. (2007) Carcinogenicity of alcoholic beverages. The lancet oncology 8: 292–293. PMID: 17431955

6. Brooks PJ, Enoch MA, Goldman D, Li TK, Yokoyama A (2009) The alcohol flushing response: an unrecognized risk factor for esophageal cancer from alcohol consumption. PLoS Med 6: e1000050.

7. Eng MY, Luczak SE, Wall TL (2007) ALDH2, ADH1B, and ADH1C genotypes in Asians: a literature review. Alcohol Research and Health 30: 22. PMID: 17718397

8. Impraim C, Wang G, Yoshida A (1982) Structural mutation in a major human aldehyde dehydrogenase gene results in loss of enzyme activity. American journal of human genetics 34: 837. PMID: 7180842

9. Li D, Zhao H, Gelernter J (2012) Strong protective effect of the aldehyde dehydrogenase gene (ALDH2) 504lys (*2) allele against alcoholism and alcohol-induced medical diseases in Asians. Human Genetics 131: 725–737. doi: 10.1007/s00439-011-1116-4 PMID: 22102315

10. Druesne-Pecollo N, Tehard B, Mallet Y, Gerber M, Norat T, Hercberg S, et al. (2009) Alcohol and genetic polymorphisms: effect on risk of alcohol-related cancer. Lancet Oncology 10: 173–180. doi: 10.1016/S1470-2045(09)70019-1 PMID: 19185835

11. Oze I, Matsuo K, Wakai K, Nagata C, Mizoue T, Tanaka K, et al. (2011) Alcohol drinking and esophageal cancer risk: an evaluation based on a systematic review of epidemiologic evidence among the Japanese population. Japanese journal of clinical oncology 41: 677–692. doi: 10.1093/jjco/hyr026 PMID: 21430021

12. Yokoyama A, Kato H, Yokoyama T, Tsujinaka T, Muto M, Omari T, et al. (2002) Genetic polymorphisms of alcohol and aldehyde dehydrogenases and glutathione S-transferase M1 and drinking, smoking, and diet in Japanese men with esophageal squamous cell carcinoma. Carcinogenesis 23: 1851–1859. PMID: 12419833

13. Yokoyama A, Muramatsu T, Ohmori T, Yokoyama T, Okuyama K, Takahashi H, et al. (1998) Alcohol-related cancers and aldehyde dehydrogenase-2 in Japanese alcoholics. Carcinogenesis 19: 1383–1387. PMID: 9744533

14. Pelucchi C, Gallus S, Garavello W, Bosetti C, La Vecchia C (2008) Alcohol and tobacco use, and cancer risk for upper aerodigestive tract and liver. European journal of cancer prevention 17: 340–344. doi: 10.1097/CEJ.0b013e3282f75e91 PMID: 18562959

15. Yoshida A, Rzhetsky A, Hsu LC, Chang C (1998) Human aldehyde dehydrogenase gene family. European Journal of Biochemistry 251: 549–557. PMID: 9490025

16. Sreerama L, Hedge MW, Sladek NE (1995) Identification of a class 3 aldehyde dehydrogenase in human saliva and increased levels of this enzyme, glutathione S-transferases, and DT-diaphorase in the saliva of subjects who continually ingest large quantities of coffee or broccoli. Clinical cancer research 1: 1153–1163. PMID: 9815907

17. Sladek NE (2003) Human aldehyde dehydrogenases: potential pathological, pharmacological, and toxicological impact. Journal of biochemical and molecular toxicology 17: 7–23. doi: 10.1002/jbt.10057 PMID: 12616643

18. Balbo S, Meng L, Bliss RL, Jensen JA, Hatsukami DK, Hecht SS (2012) Time course of DNA adduct formation in peripheral blood granulocytes and lymphocytes after drinking alcohol. Mutagenesis 27: 485–490. doi: 10.1093/mutage/ges008 PMID: 22406526

19. Chen CH, Cruz LA, Mochly-Rosen D (2015) Pharmacological recruitment of aldehyde dehydrogenase 3A1 (ALDH3A1) to assist ALDH2 in acetaldehyde and ethanol metabolism in vivo. Proceedings of the National Academy of Sciences 112: 3074–3079.

20. Ushida Y, Talalay P (2013) Sulforaphane accelerates acetaldehyde metabolism by inducing aldehyde dehydrogenases: relevance to ethanol intolerance. Alcohol and alcoholism 48: 526–534. doi: 10.1093/alcalc/agt063 PMID: 23825090

21. Manchali S, Murthy KNC, Patil BS (2012) Crucial facts about health benefits of popular cruciferous vegetables. Journal of Functional Foods 4: 94–106.

22. Fahey JW, Talalay P, Kensler TW (2012) Notes from the field:"green" chemoprevention as frugal medicine. Cancer Prevention Research 5: 179–188. doi: 10.1158/1940-6207.CAPR-11-0572 PMID: 22307565

23. Elbarbry F, Elrody N (2011) Potential health benefits of sulforaphane: a review of the experimental, clinical and epidemiological evidences and underlying mechanisms. Journal of Medicinal Plants Research 5: 473–484.

24. Talalay P, Fahey JW, Holtzclaw WD, Prestera T, Zhang Y (1995) Chemoprotection against cancer by phase 2 enzyme induction. Toxicology letters 82: 173–179. PMID: 8597048

25. Mein JR, James DR, Lakkanna S (2012) Induction of phase 2 antioxidant enzymes by broccoli sulforaphane: perspectives in maintaining the antioxidant activity of vitamins A, C, and E. Frontiers in genetics 3: 7. doi: 10.3389/fgene.2012.00007 PMID: 22303412

26. Zhang Y, Talalay P, Cho C-G, Posner GH (1992) A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proceedings of the national academy of sciences 89: 2399–2403.

27. Moosavi-Movahedi AA, Hakimelahi S, Chamani J, Khodarahmi GA, Hassanzadeh F, Luo FT, et al. (2003) Design, synthesis, and anticancer activity of phosphonic acid diphosphate derivative of adenine-containing butenolide and its water-soluble derivatives of paclitaxel with high antitumor activity. Bioorganic & medicinal chemistry 11: 4303–4313.

28. Alam MF, Laskar AA, Choudhary HH, Younus H (2016) Human Salivary Aldehyde Dehydrogenase: Purification, Kinetic Characterization and Effect of Ethanol, Hydrogen Peroxide and Sodium Dodecyl Sulfate on the Activity of the Enzyme. Cell Biochemistry and Biophysics 74: 307–315. doi: 10.1007/s12013-016-0742-9 PMID: 27324040

29. Giebultowicz J, Wolinowska R, Sztybor A, Pietrzak M, Wroczynski P, Wierzchowski J (2009) Salivary aldehyde dehydrogenase: activity towards aromatic aldehydes and comparison with recombinant ALDH3A1. Molecules 14: 2363–2372. doi: 10.3390/molecules14072363 PMID: 19633610

30. Giebultowicz J, Dziadek M, Wroczynski P, Woznicka K, Wojno B, Pietrzak M, et al. (2010) Salivary aldehyde dehydrogenase—temporal and population variability, correlations with drinking and smoking habits and activity towards aldehydes contained in food. Acta Biochimica Polonica 57: 361–368. PMID: 20931090

31. Wierzchowski J, Wroczynski P, Laszuk K, Interewicz E (1997) Fluorimetric detection of aldehyde dehydrogenase activity in human blood, saliva, and organ biopsies and kinetic differentiation between class I and class III isozymes. Analytical Biochemistry 245: 69–78. doi: 10.1006/abio.1996.9921 PMID: 9025970

32. Vahedian-Movahed H, Saberi M, Chamani J (2011) Comparison of binding interactions of lomefloxacin to serum albumin and serum transferrin by resonance light scattering and fluorescence quenching methods. Journal of Biomolecular Structure and Dynamics 28: 483–502. doi: 10.1080/07391102.2011.10508590 PMID: 21142219

33. Lakowicz JR, Weber G (1973) Quenching of protein fluorescence by oxygen. Detection of structural fluctuations in proteins on the nanosecond time scale. Biochemistry 12: 4171–4179. PMID: 4200894

34. Riedl MA, Saxon A, Diaz-Sanchez D (2009) Oral sulforaphane increases Phase II antioxidant enzymes in the human upper airway. Clinical immunology 130: 244–251. doi: 10.1016/j.clim.2008.10.007 PMID: 19028145

35. Sasaki K, Neyazaki M, Shindo K, Ogawa T, Momose M (2012) Quantitative profiling of glucosinolates by LC—MS analysis reveals several cultivars of cabbage and kale as promising sources of sulforaphane. Journal of Chromatography B 903: 171–176.

36. Herr I, Lozanovski V, Houben P, Schemmer P, Büchler MW (2013) Sulforaphane and related mustard oils in focus of cancer prevention and therapy. Wiener Medizinische Wochenschrift 163: 80–88. doi: 10.1007/s10354-012-0163-3 PMID: 23224634

37. Anwar S, Khan MA, Sadaf A, Younus H (2014) A structural study on the protection of glycation of superoxide dismutase by thymoquinone. International Journal of Biological Macromolecules 69: 476–481. doi: 10.1016/j.ijbiomac.2014.06.003 PMID: 24933520

38. Nienhaus K, Nienhaus GU (2005) Probing heme protein-ligand interactions by UV/visible absorption spectroscopy. Protein-Ligand Interactions: methods and applications: 215–241.

39. Chamani J, Tafrishi N, Momen-Heravi M (2010) Characterization of the interaction between human lactoferrin and lomefloxacin at physiological condition: Multi-spectroscopic and modeling description. Journal of Luminescence 130: 1160–1168.

40. Laskar AA, Khan MA, Rahmani AH, Fatima S, Younus H (2016) Thymoquinone, an active constituent of Nigella sativa seeds, binds with bilirubin and protects mice from hyperbilirubinemia and cyclophosphamide-induced hepatotoxicity. Biochimie 127: 205–213. doi: 10.1016/j.biochi.2016.05.020 PMID: 27265787

41. Shakir M, Hanif S, Alam MF, Younus H (2016) Molecular hybridization approach of bio-potent CuII/ZnII complexes derived from N, O donor bidentate imine scaffolds: Synthesis, spectral, human serum albumin binding, antioxidant and antibacterial studies. Journal of Photochemistry and Photobiology B: Biology 165: 96–114.

42. Tang B, Huang Y, Ma X, Liao X, Wang Q, Xiong X, et al. (2016) Multispectroscopic and docking studies on the binding of chlorogenic acid isomers to human serum albumin: Effects of esteryl position on affinity. Food Chemistry 212: 434–442. doi: 10.1016/j.foodchem.2016.06.007 PMID: 27374553

43. Chaturvedi SK, Ahmad E, Khan JM, Alam P, Ishtikhar M, Khan RH (2015) Elucidating the interaction of limonene with bovine serum albumin: a multi-technique approach. Molecular BioSystems 11: 307–316. doi: 10.1039/c4mb00548a PMID: 25382435

44. Chamani J, Vahedian-Movahed H, Saberi MR (2011) Lomefloxacin promotes the interaction between human serum albumin and transferrin: A mechanistic insight into the emergence of antibiotic's side effects. Journal of pharmaceutical and biomedical analysis 55: 114–124. doi: 10.1016/j.jpba.2010.12.029 PMID: 21273024

45. Baig U, Gondal M, Alam MF, Wani WA, Younus H (2016) Pharmacological evaluation of poly (3-methylthiophene) and its titanium (IV) phosphate nanocomposite: DNA interaction, molecular docking, and cytotoxic activity. Journal of Photochemistry and Photobiology B: Biology 164: 244–255.

46. Ajmal MR, Zaidi N, Alam P, Nusrat S, Siddiqi MK, Badr G, et al. (2015) Insight into the Interaction of anti-tubercular and anticancer compound Clofazimine with Human Serum Albumin: spectroscopy and molecular modelling. Journal of Biomolecular Structure and Dynamics: 1–42.

47. Pasban Ziyarat F, Asoodeh A, Sharif Barfeh Z, Pirouzi M, Chamani J (2014) Probing the interaction of lysozyme with ciprofloxacin in the presence of different-sized Ag nano-particles by multispectroscopic techniques and isothermal titration calorimetry. Journal of Biomolecular Structure and Dynamics 32: 613–629. doi: 10.1080/07391102.2013.785919 PMID: 23659247

48. Greenfield NJ (2006) Using circular dichroism spectra to estimate protein secondary structure. Nature protocols 1: 2876–2890. doi: 10.1038/nprot.2006.202 PMID: 17406547

49. Baig U, Gondal M, Alam MF, Alam M, Wani WA, Younus H (2016) Design, Facile Synthesis, Molecular Docking, DNA Binding and Cytotoxic Activity of Polythiophene and Polythiophene-Titanium (IV) phosphate Nanocomposite. International Journal of Polymeric Materials and Polymeric Biomaterials.

50. Moosavi-Movahedi A, Chamani J, Ghourchian H, Shafiey H, Sorenson CM, Sheibani N (2003) Electrochemical evidence for the molten globule states of cytochrome c induced by N-alkyl sulfates at low concentrations. Journal of protein chemistry 22: 23–30. PMID: 12739895

51. Whitmore L, Wallace BA (2008) Protein secondary structure analyses from circular dichroism spectroscopy: methods and reference databases. Biopolymers 89: 392–400. doi: 10.1002/bip.20853 PMID: 17896349

52. Kabiri M, Amiri-Tehranizadeh Z, Baratian A, Saberi MR, Chamani J (2012) Use of spectroscopic, zeta potential and molecular dynamic techniques to study the interaction between human holo-transferrin and two antagonist drugs: comparison of binary and ternary systems. Molecules 17: 3114–3147. doi: 10.3390/molecules17033114 PMID: 22410420

53. Helminen A, Väkeväinen S, Salaspuro M (2013) ALDH2 genotype has no effect on salivary acetaldehyde without the presence of ethanol in the systemic circulation. PloS one 8: e74418. doi: 10.1371/journal.pone.0074418 PMID: 24058561

54. Moazzez R, Thompson H, Palmer RM, Wilson RF, Proctor GB, Wade WG (2011) Effect of rinsing with ethanol-containing mouthrinses on the production of salivary acetaldehyde. European Journal of Oral Sciences 119: 441–446. doi: 10.1111/j.1600-0722.2011.00886.x PMID: 22112029

55. Hempel J, Kuo I, Perozich J, Wang BC, Lindahl R, Nicholas H (2001) Aldehyde dehydrogenase. European Journal of Biochemistry 268: 722–726. PMID: 11168411