Mitigation of Aflatoxin metabolites in food and feed using phenolic compounds identified by insilico study

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Abstract

Milk is considered a complete diet that contains nutrients in balance. Apart from different possible concerns, mycotoxins are considered a serious cancer-causing compound from which many are considered extremely toxic. With the industrialization and rapid increase in population, there is a serious concern to be managed with respect to food safety. The permissible limit (0.05µg/l) defined by the EU is being followed in Pakistan. The purposed study was to screen out AFM1 contamination in goat milk and In-silico identification of possible ways to interrupt the pathway of AFM1. The maximum concentration of AFM1 0.0669 µg/l exceeds the permissible limit and minimum 0.0015µg/l below the permissible limit was observed in some Goat milk sample collected in surroundings of Sahiwal Pakistan. 10% milk samples were reported highly contaminated with AFM1 as exceeds permissible limit value. For pathway intruption, an enzyme (O- methylsterigmatocystin oxidoreductase) was identified directly involved in AFB1 synthesis and further conversion. The molecular docking was performed against this enzyme to inhibit the conversion into toxic compound. Essential oil was used to inhibit fungal growth and detoxification of toxic substances. The ligand compounds were extracts of naturally occurring plants such as walnut, black currants, blueberries, raspberries, red currants, cranberries and Adhatoda vasica (Nees). Docking of compounds was performed by AutoDock Vina and after and interaction visualization. A Lead ZINC000030729894 was identified with good docking results and interaction.

1. Introduction

Milk is considered to be a comprehensive diet for all age groups, as for infants milk is only source of diet so they are the most vulnerable to Mycotoxins (Akbar et al., 2019; Arinç et al., 2012). Major problems to which aflatoxins are associated are teratogenicity, carcinogenicity, mutagenicity, genotoxicity and cytotoxicity and are considered to be a serious human health threats globally (Cancer, 1993; COMMUNITIES, 2006; Kazemi Darsanaki & Miri, 2013).

The aflatoxin M1 and M2 secretion in animal's milk of is due to the consumption of contaminated feed with aflatoxin B1 and aflatoxin B2 (Duarte et al., 2013; Fallah, 2010; Food & Administration, 2011). Produced in liver through biotransformation of AFB1 by cytochrome P450 enzymes, aflatoxin M1 is 4-hydroxy metabolite. It exists in biological fluids (i.e., urine, serum, Cerebrospinal fluid, milk etc.). By using different heat treatments for example pasteurization, autoclaving and UHT technique it may be reduced but not completely destroyed (Langat et al., 2016; Montagna et al., 2008). So if raw milk is contaminated then it is obvious that AFM1 will be appeared in processed milk and milk products (Motawee et al., 2004).

Aflatoxins are produced by a complex biosynthesis pathway consisting of at least 27 enzymatic reactions (Yabe & Nakajima, 2004). Two cluster-specific regulators: *aflR* and *aflS* coordinate their expression and the genes coding for these enzymes are grouped in a cluster (Price et al., 2006). In aflatoxin synthesis cytochromes P-450 plays a pivotal role. During biosynthesis these enzymes are to be linked in attaching functional groups (i.e., methyl, acetyl).

Hydroxylation of the toxin also occurs due to microsomal biotransformation of AFB1, resulting in the progression of more polar and relatively less toxic metabolites, mostly AFQ1 and AFM1. CYP1A2 leads to the hydroxylated AFM1 and to both endo and exo-8,9-epoxide, whereas CYP3A4 plays a role for the formation of AFB1-exo-8,9-epoxide and little bit of AFQ1. Formation of AFB1-N7-Gua adduct occurs due to the high affinity of the epoxide intermediate for purine bases of DNA, which promotes mutations in nucleotide sequence. Depurination and thus apurinic site formation is caused by the charged adduct (Marchese et al., 2018; Wild & Turner, 2002). On the site of the original adduct G→T transversion identified which is the predominant mutation caused by AFB1-N7-Gua adduct. Displaying selectivity towards guanine bases with a guanine or a cytosine as 50 base and especially at the third base of codon 249 of the p53 tumor suppressor gene, the mutation has been stated to affect specific base pair locations as presented in Fig. 1. (Macé et al., 1997). In a several number of epidemiological studies on hepatocellular carcinoma (HCC) patients this mutation observed commonly (Marchese et al., 2018).

The studies conducted in Pakistan also show that 25 to 90% of milk samples (S. Z. Iqbal et al., 2011; Shahzad Zafar Iqbal et al., 2014; Muhammad et al., 2010; Raza, 2006) could be contaminated with AFM1. So, it is a matter of global distress over food and feed safety (Polychronaki et al., 2007). However, internationally most commonly adopted permissible limits for AFM1 are 0.5ng/ml (Sartori et al., 2015) and 0.05ppb (Sassahara et al., 2005). Presently, permissible limit (0.5ng/ml) defined by FDA is being followed in Pakistan.

The current study has been planned for *insilico* identification and mitigation of biosynthetic pathway analysis of AFB1 mediated by cytochromeP450 enzymes system present in human's liver using phenolic compounds.

2. Methodology

AFM1 is a secondary metabolite of aflatoxin B1 synthesize in human liver by CYP1A2. After identify the enzyme (O-methylsterigmatocystin oxidoreductase) that directly involved in biosynthesis of AFB1 regulated by CYP450 enzyme system present in the liver, the molecular docking was performed against O-methylsterigmatocystin oxidoreductase and a lead compound for that enzymatic reaction was identified.

2.1. Sample Collection

40 goat's milk samples were collected from the surrounding villages of Sahiwal City, Punjab, Pakistan. Milk samples were collected in food grade bottles and were kept in thermos during sampling at a temperature of 4°C. After collection, the samples were bought to Laboratory at COMSATS University Islamabad, Sahiwal Campus and were held in refrigerator at -4°C.

2.2. Retrieval of sequence and 3D model prediction

Amino acid sequences of O-methylsterigmatocystin oxidoreductase enzyme was retrieved from UniProt (Universal Protein Resource) database (http://www.uniprot.org/) in FASTA format for computational analysis (Bairoch et al., 2005). Tertiary structure is overall three-dimensional arrangement of all atoms in a

protein. For 3D model prediction online server, I-TASSER was used (https://zhanglab.ccmb.med.umich.edu/I-TASSER/).

2.3. Structure refinement and selection

The ProCheck tool generated the Ramachandran plot of the given protein model and results depicted the quality of 3D protein structure as the more than 90% residues lied within favorable region so the model was selected without refinement on the bases of Ramachandran results (SuKo et al., 2012).

2.4. Preparation of protein

The three- dimensional structure of the protein was purified by removing water molecules, adding polar hydrogen, Gasteiger charge and Kollman's charge by using AutoDock Vina.

2.5. Ligands Retrieval and preparation

Different natural compounds reported in plants were selected from literature survey. Three dimensional (3D) structures of selected compounds were downloaded from PubChem. The ligand molecules were prepared for docking by using AutoDock Vina. The prepared molecules were saved in pdbqt format in Table 1.

Table 1
Natural compounds identified from literature

Source	Name	PubChem ID	Source	Name	PubChem ID
Walnut	Alpha tocopherol	14985	Blueberries	delphinidin-3-O-galactoside	13915667
	Beta tocopherol	6857447		delphinidin-3-O-glucoside	13915667
	Gemma tocopherol	92729		petunidin-3-O-galactoside	136664458
	Cholesterol	5997		cyanidin-3-O-arabinoside	12137509
	Brassicasterol	5281327		malvidin-3-O-galactoside	5484292
	24-Methylene Cholesterol	24779661		peonidin-3-O-arabinoside	12137510
	Campesterol	173183		malvidin-3-O-arabinoside	12137511
	Campestanol	119394		myricetin-3-O-galactoside	12311099
	Stigmasterol	5280794	Raspberries	cyanidin-3-O-sophoroside	11169452
	7-Campesterol	91746241		pelargonidin-3-O-sophoroside	23724704
	5.23-Stigmastadienol	6427344		cyanidin-3-O-rutinoside	29232
	Clerosterol	5283638		ellagic acid	5281855
	beta-Sitosterol acetate	521199		quercetin-O-galactosylrhamnoside	44259236
	Sitostanol	241572		Quercetin 3-O-glucosyl-rutinoside	102332276
	5-Avenasterol	5281326		quercetin-3-O-galactoside	5281643
	5.24-Stigmastadienol	6432810		quercetin-3-O-glucoside	5280804
	7-Stigmastenol	129726103		Ellagic acid-4-acetylxyloside	101757027
	7- Avenasterol	12795733			

Source	Name	PubChem ID	Source	Name	PubChem ID
	Butirospermol	12302182	Black Currants	delphinidin-3-O glucoside	25201902
	Cycloartenol	92110		delphinidin-3-O galactoside	13915667
	2.4-Methylcycloartenol	94204		delphinidin-3-O rutinoside	74319441
	Docosanol	12620		cyanidin-3-O glucoside	441667
	Tetracosanol	10472		cyanidin-3-O rutinoside	441674
	Hexacosanol	68171		peonidin-3-O rutinoside	138319215
	Octacosanol	68406		myricetin-3-O rutinoside	44259428
	Chlorophyll a	12085802		caffeic acid-O glucoside	5281759
	Chlorophyll b	11593175			
	β-Carotene	5280489	Cranberries	procyanidin dimer	131752343
	β-Cryptoxanthin	5281235		cyanidin-3-O-galactoside	441699
	Lutein	5281243		trans-p-Coumaric acid 4-glucoside	13783633
	Zeaxanthin	5280899		cyanidin-3-O-arabinoside	91810602
	Violaxanthin	448438		peonidin-3-O-galactoside	11454027
	Neoxanthin	5281247		peonidin-3-O-glucoside	14311151
				peonidin-3-O-arabinoside	91810651
Red Currants	4-Hydroxybenzoic acid	135		malvidin-3-O-arabinoside	91810654
	caffeic acid-O-glucoside	5281759		Myricetin 3-arabinoside	21672568
	Cyanidine 3-sambubioside	78302543		Flavone	10680

Source	Name	PubChem ID	Source	Name	PubChem ID
	myricetin-3-O-rutinoside	44259428		Quercetin	5280343
	Myricetin 3-rhamnoside	5352000			
	quercetin-3-O-rutinoside	102332276	Adhatoda Vasica(Nees)	Vasicinolone	13970119
	quercetin-3-O-galactoside	5359430		Vasicine	442929
	quercetin-3-O-glucoside	5280804		Vasicine acetate	vasicine acetate
	Quercetin 3-O-malonylglucoside	5282159		2-acetyl benzylamine	22379528
	Kaempferol-3-rutinoside	122173234		Vasicinone	442935
				Epitaraxerol	12443227
				Glutinol	312242816

2.6. Molecular Docking Analysis

Molecular docking of protein was done with selected natural phenolic compounds. The .pdbqt files of protein and ligands were used in this process. The results were generated in the form out.pdbqt file. By using Pymol software protein ligand complexes were generated. The complex files were saved in pdb format and visualized by LigPlot to study protein-ligand interactions. The top ten Protein-ligand complexes showing lowest binding affinities and reasonable hydrogen bond interactions were selected for further study.

2.7. Pharmacophore Generation

A pharmacophore is an abstract description of molecular features that are necessary for molecular recognition of a ligand by biological macromolecules. Pharmacophores for all the three targets were generated by using the Ligand Scout 4.1.10. by inserting or open the sdf files of lead Compounds making them active and selecting the option of create pharmacophore by keeping all other parameter set as default and save the file by selecting option of save as file in .pmz format.

2.8. Database Creation

After the pharmacophore creation the library of different size was retrieved from ZINC specialized database in the .smi format for database creation in LigandScout. There were different libraries for Natural and Synthetic compounds in ZINC specialized database corresponding to selected compounds. In LigandScout

by selecting the sign of database creation and giving the .smi file as input and giving the path for saving .ldb file in respective folder in output option. Natural database was created for screening purpose.

2.9. Virtual Screening

Ligand based virtual Screening was performed by using the LigandScout for hit compounds identification. After screening calculates standard properties option was selected to add the different features i.e. RO5 and other matching features etc. First top 10 hits were selected for further analysis for lead identification.

2.10. Molecular Docking

After that hit compounds were filtered on basis of different parameter for drug likeness properties etc. The structure of these Hits was retrieved from ZINC database in the .sdf format. AutoDock was used to prepare the pdbqt files of these hit compounds. Then docking was performed ten times for each ligand with same pdbqt file of protein and configure file to predict the different binding mode for different binding energy. Then low binding energy compounds were selected and binding interactions were also studied by using the LigPlot.

2.11 Lead Identification

After the detailed analysis of ligand–receptor interaction and docking score, the most active inhibitors were identified against the target. The compounds or ligands having the best interactions among all has been identified and selected as Lead which will be extracted from herbs using HPLC.

3. Results

3.1. Retrieval of sequence

40 goat milk sample were collected from four directions in surrounding areas of Sahiwal indicated as east, west, north and south as 10 samples from each area. The samples were screened to identify the Aflatoxin M1 contamination present in goat milk. High performance liquid chromatography (HPLC) results exhibited different part per billion (ppb) values of milk samples that showed presence of AFM1 contamination in milk samples.

There were total four samples# 1, 10, 14 and 24 which contained ppb values greater than European union recommended permissible value 0.05 μgL^{-1} Only sample 19 didn't show any ppb value which means AFM1 was not detected in sample#19. All remaining samples of four regions contains AFM1 contamination. Samples collected from region 2 (West) have relatively high contamination of AFM1 as all the 10 samples exhibit higher values as compared to remaining three regions (East, North and south) presented in Table 2

Table 2
Aflatoxin M1 concentration(ppb) in Sahiwal goat milk samples

Area	No of Samples	Min conc.	Max ± conc.	Mean ± SD	No of samples > MRL
East	10	0.0016	0.0556	0.02738 ± 0.01305	1
West	10	0	0.0363	0.031056 ± 0.00329	0
North	10	0.0016	0.0669	0.02789 ± 0.017955	1
South	10	0.0015	0.0541	0.0274 ± 0.016561	2
Total	40	0.0015	0.0669	0.028364 ± 0.013647	10%

Retrieval of sequence

Amino acid sequences of O-methylsterigmatocystin oxidoreductase enzyme was retrieved from UniProt (Universal Protein Resource) database (http://www.uniprot.org/) in FASTA format for computational analysis.

3.2. Protein 3D model prediction

I-TASSER server generated the refined 3D model of protein with iterative threading assembly refinement (I-TASSER) first generates three-dimensional (3D) atomic models from multiple threading alignments and iterative structural assembly simulations. Figure 2

3.3. Structure refinement and selection

The ProCheck tool generated the Ramachandran plot of the given protein model and results depicted the quality of 3D protein structure as the more than 90% residues lied within favourable region so the model was selected without refinement on the bases of Ramachandran results in Fig. 3

3.4. Docking of Novel Compounds

Molecular docking of protein with selected natural compounds was conducted using AutoDock Vina. Top ten ligands with the least binding energies were selected for further analysis as a result of docking. These docking complexes were visualized by LIGPLOT and protein ligands interactions (Hydrogenbonding and Hydrophobic interaction) were analyzed by using LIGPLOT computer program as presented in Table 3. Interactions studies having distance range of 4 or less were keep into consideration (Wallace et al., 1995) see in Table 3

Table 3
Protein Ligand interaction studies using LigPlot

Compound name	B. E.	Hydrogen Bonds	Distance	Total HB	Hydrophobic Residues	Hydrophobic Bonds	Total Bonds
101757027	-9.6	Glu297:OE2-O7	3.11	1	Val432(1), Ser366(1), Phe433(3), Val479(2), Pro371(2), Ala104(1), Asn219(1), Ile210(6), Phe106(2), Trp112(2), Phe207(2), Glu297(2), Met300(1), Cys440(4), Thr305(2), Gly301(2)	34	35
12311099	-9.2	Val432:O-O9 Val432:O-O10 Thr305:OG1-O11 Arg438:O-O8 Arg438:NE-O5 Arg438:NH2-C11	3.00 2.93 2.84 3.04 3.23 2.81	6	Val432(4), Ser366(7), Ala370(2), Ile439(1), Arg438(5), Pro441(1), Cys440(2), Glu297(1), Ala117(2), Pro371(2), Val479(3), Leu368(2), Gly434(1), Phe433(2)	35	41
12795733	-9.2	Ile210:O-O	3.13	1	Cys440(1), Arg438(1), Gly369(2), Pro371(1), Leu368(3), Ala370(4), Ile210(5), Trp216(3), Val479(3), Thr305(1), Ile480(1), Glu297(2), Gly301(1)	28	29

Compound name	B. E.	Hydrogen Bonds	Distance	Total HB	Hydrophobic Residues	Hydrophobic Bonds	Total Bonds
12137511	-9.1	Val432:O-O8 Arg129:NH1-O5 Arg438:O-O6 Arg438:NE-O4 Arg438:NH2-O4 Arg438:NH2-O3 Ala117:O-O10	3.02 3.03 3.05 2.88 3.09 2.94 2.80	7	Phe433(2), Val432(2), Gly434(2), Cys440(3), Arg129(3), Ile439(1), Pro441(1), Arg438(6), Glu297(1), Ile101(1), Ala117(8), Ala370(3), Ile210(1), Gly301(1), Val479(1), Gly369(1), Leu368(1), Ser366(2), Thr305(1)	41	48
312242816	-8.7	-	-	0	Ala370(3), Arg438(1), Asn219(1), Ala104(1), Thr118(1), Ile210(1), Phe106(1), Trp112(2), Ala117(3), Val479(1), Glu297(1), Cys440(1)	17	17
173183	-8.6	Glu297:O-O	3.18	1	Leu390(2), Asn219(2), Trp216(3), Phe220(1), Ala104(3), Phe106(1), Phe207(1), Ile210(3), Met300(2), Ala117(1), Gly301(1), Glu297(4), Val479(1), Trp112(4), Pro102(3), Pro371(2)	34	35

Compound name	B. E.	Hydrogen Bonds	Distance	Total HB	Hydrophobic Residues	Hydrophobic Bonds	Total Bonds
14985	-8.4	0			Met300(2), Phe207(4), Trp112(3), Ile210(4), Thr118(2), Ala107(2), Phe106(1), Ile101(1), Pro102(1), Pro371(4), Trp216(2), Phe211(1), Leu368(3), Val479(5), Gly369(2), Ala370(1), Gly301(1), Glu297(1)	40	40
138319215	-8.1	Arg443:NH2-O52 Arg443:NE2-O8 Asp447:OD2-O49 Glu448:OE2-O37 Glu448:OE2-O38 Lys357:NZ-O19	2.94 3.08 2.70 3.02 3.06 3.27	6	Ile88(1), Phe431(2), Phe435(2), Arg443(6), Asp447(4), Phe444(1), Glu448(3), Arg346(4), Pro423(2), Asp353(3), Ser347(2), Lys357(3), Asp427(1), Lys430(3), Ile439(1), Met429(2), Pro428(2)	42	48
12443227	-7.9	-	-	0	Ile439(2), Arg443(1), Lys430(3), Phe435(3), Ile88(4), Pro428(2), Met429(3), Asp427(1)	19	19

Compound name	B. E.	Hydrogen Bonds	Distance	Total HB	Hydrophobic Residues	Hydrophobic Bonds	Total Bonds
91746241	-7.8	-	-	0	Val404(1), Pro428(3), Asp427(3), Met429(2), Asp447(6), Arg346(4), Phe444(3), Arg443(4), Lys430(4), Phe435(3), Phe431(2), Ile88(1), Thr426(2)	38	38

3.5. Pharmacophore Model

On the basis of binding energies, top 10 selected least binding energy inhibitors were subjected to generate a pharmacophore model. This merge pharmacophore model with matching features such as Hydrogen Bond Donors (green), Hydrogen Bond Acceptors (red), and hydrophobic (yellow) is shown in Fig. 4

3.6. Virtual Screening

Zbc Leads library of biogenic lead-like compounds of ZINC database was used for Virtual Screening. Virtual screening was performed by LigandScout to find the novel compounds that have features similar to those of pharmacophore model. 7626 hits were identified out of 30736 compounds. On the basis of Pharmacophore-fit score, top 10 compounds from Zinc library were used for molecular docking with O-methylsterigmatocystin oxidoreductase. 2D chemical structures of novel compounds were retrieved via Zinc Database.

3.7. Docking of Novel Compounds

Molecular docking of O-methylsterigmatocystin oxidoreductase with selected novel compounds was conducted using AutoDock Vina. As a result of docking ligands with the least binding energies were identified. LIGPLOT was used to visualize these docking complexes to study protein ligands interactions. ZINC000030729894 was found with the least binding energies of -7.8 in Fig. 5

3.8. Lead Identification

Among all the compounds from library, some showing good binding energies, so ZINC000030729894 (4β-methylzymosterol-4α-carboxylic acid from Saccharomyces cerevisiae) with least binding energies and good interactions score may be considered as potential drug and will be extracted using HPLC.

4. Discussion

Milk has a significant potential of establishing aflatoxin deposits from tissues of animal to human diet. Furthermore, milk is the vital nutrient for growing children and young babies, who are more delicate and possess higher vulnerability than the adults, the manifestation of aflatoxin M1 in processed milk, lactating mothers and milk foodstuffs is of major concern and a major issue of food asepticism. Aflatoxins are produced by filamentous species of fungi and are heterocyclic compounds. The main fungal species that produce aflatoxins are Asp. flavus and Aspergillus parasiticus, they may cause contamination in a vast range of agricultural and food artefacts e.g. grain, cereals, silage, seeds (Mahmoudi, 2014). Hence, because of common incidence and injurious health effects aflatoxins contamination, it is of great importance and need of the hour to AFM1 detection and quantification in milk in Pakistan.

The study has been planned to observe AFM1 contamination in goat milk samples. The current study reveals the AFM1 occurrence in milk samples. There were 40 samples of goat milk collected from four directions in surrounding areas of Sahiwal city namely as east, west, north and south as 10 samples per each area. The samples were analyzed for screening of AflatoxinM1 contamination. Over all 10% milk samples were reported highly contaminated because their ppb values of AFM1 concentration were found above the permissible limit value0.050µg/L

AFM1 is the potential cause of liver cancer and secondary metabolite of aflatoxin B1 so after analyzing the biosynthetic pathway of AFB1, an enzyme (O-methylsterigmatocystin oxidoreductase) was identified directly involved in AFB1 synthesis and further conversion. The molecular docking was performed against this enzyme to inhibit the conversion into toxic compound. Essential oil are used to inhibit fungal growth and detoxification of toxic substances (Marchese et al., 2018). The ligand compounds were extracts of naturally occurring plants such as walnut, black currants, blueberries, raspberries, red currants, cranberries and Adhatoda vasica (Nees). Docking of compounds was performed by AutoDock Vina in order to get ligands with high binding affinity and good interactions with target proteins. Lead was identified after screening, docking and interaction visualization. Compound with good interaction, best fit score after virtual and high binding affinity was selected as lead compound.

Conclusion

Over all 10% samples lied above the permissible limit range 0.050µg/L while 90% showed the ppb values below permissible limit range 0.050 µg/L indicate there were few milk samples have higher concentration of AFM1. AFM1 is the potential cause of liver cancer and secondary metabolite of aflatoxin B1 so after analyzing the biosynthetic pathway of AFB1 the molecular docking was performed to inhibit the conversion into toxic compound. Among all the compounds from library, some showing good binding energies, so ZINC000030729894 (4β-methylzymosterol-4α-carboxylic acid from Saccharomyces cerevisiae) with least binding energies and good interactions score may be considered as potential lead for the mitigation of aflatoxin metabolites

Declarations

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Data Availability

The Data provided in manuscript is available with author and will be produced on demand.

Competing Interest

No, I Muhammad Wasim Sajid declare that the authors have no competing interests as defined by Springer, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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Figures

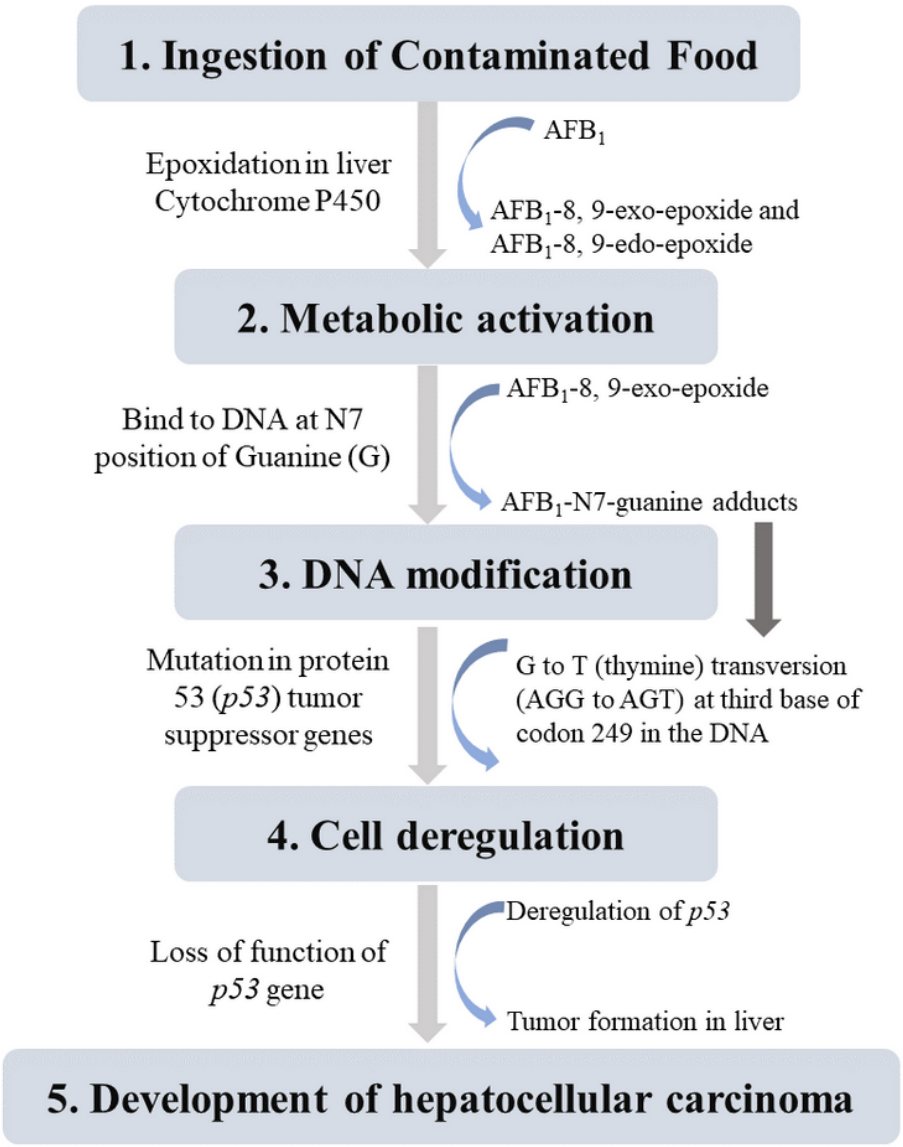

Figure 1

Pathway of development of hepatocellular carcinoma (HCC) by conversion of AFBI in liver

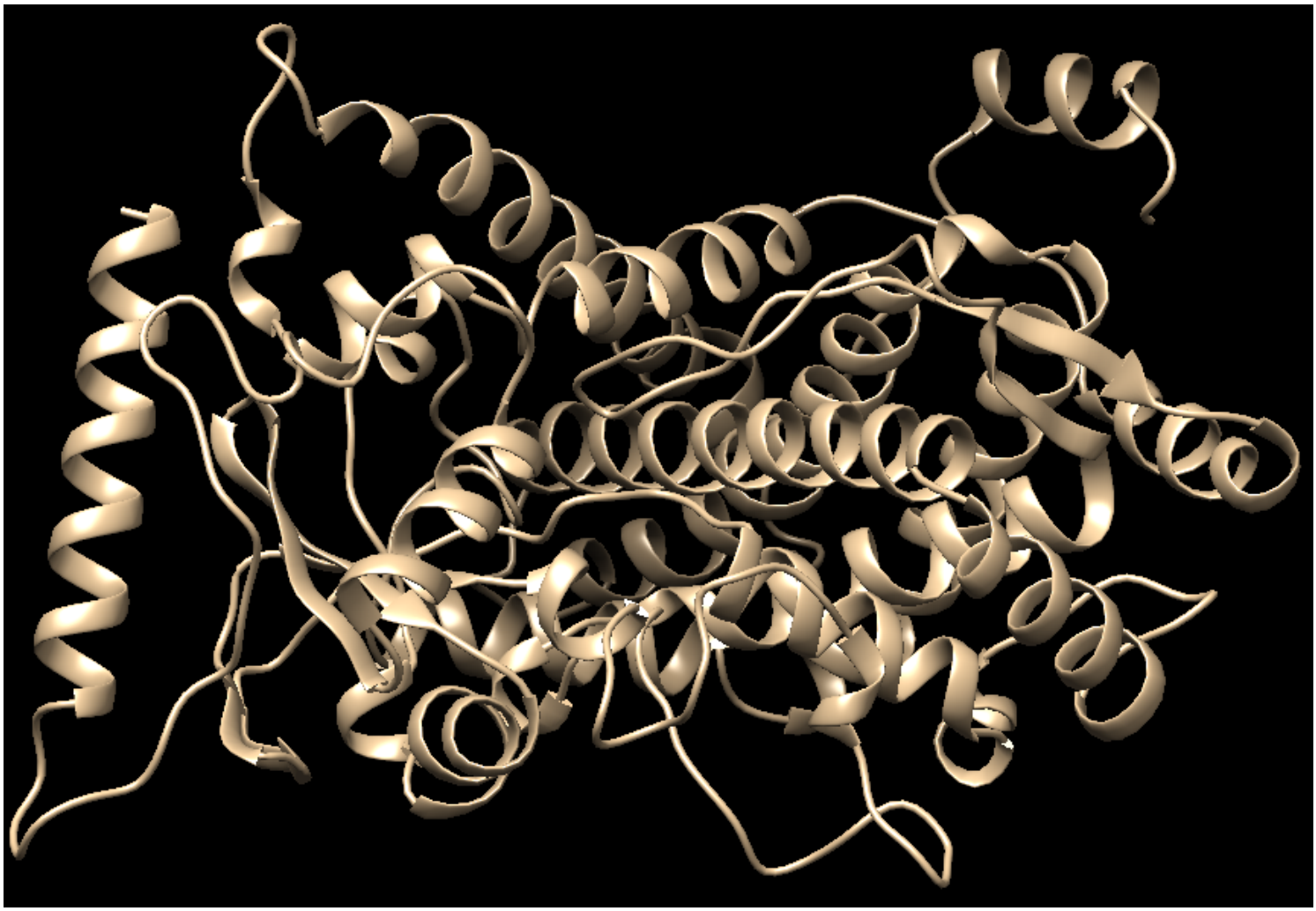

Figure 2

Three-dimensional(3D) model of O-methylsterigmatocystin oxidoreductase

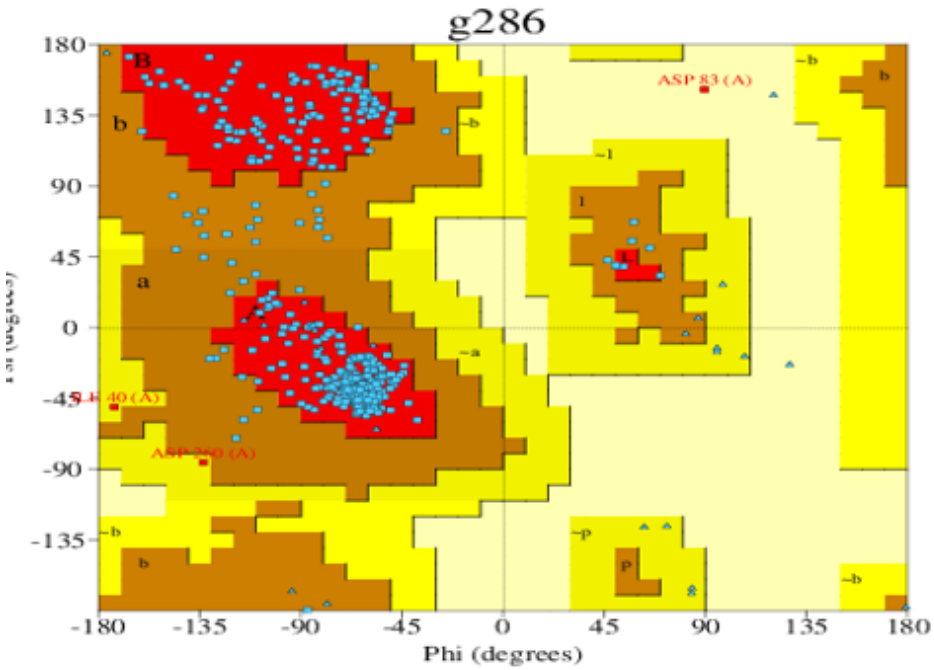

Figure 3

Graphical representation of Ramachandran plot of 3Dmodel of O-methylsterigmatocystin oxidoreductase

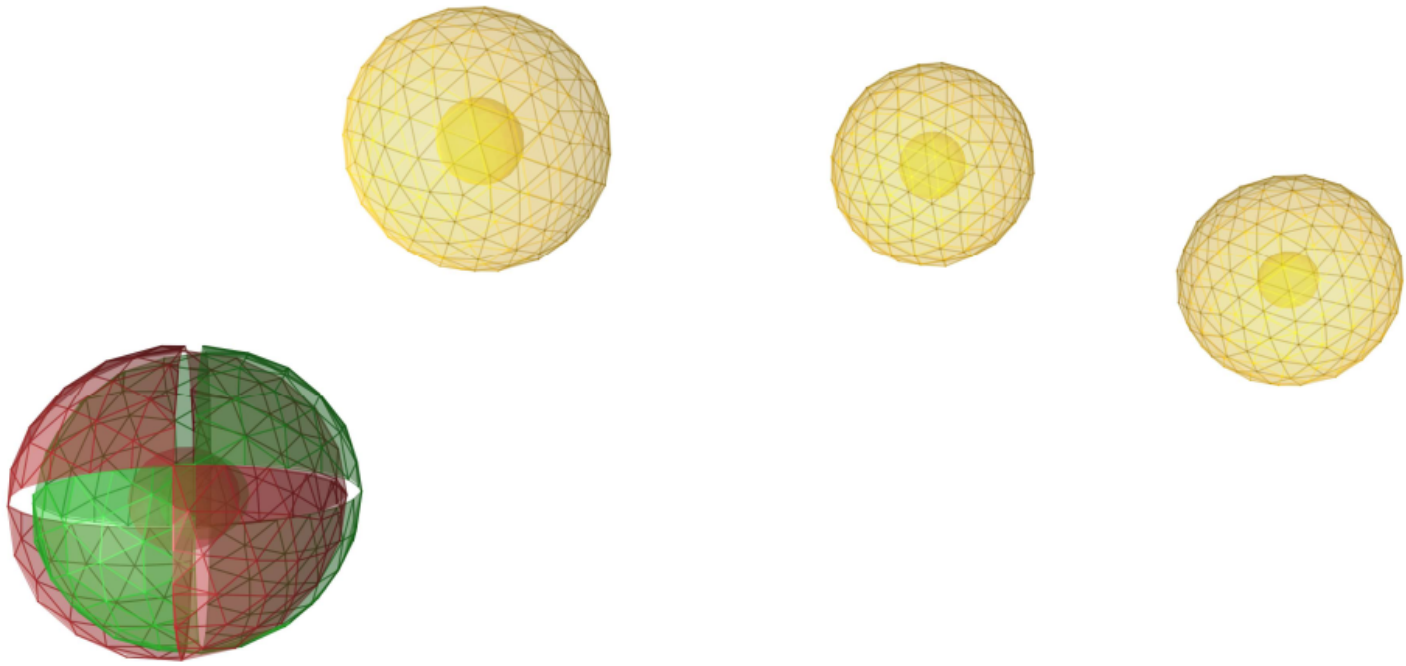

Figure 4

Merged pharmacophore of herbal compounds identified from literature

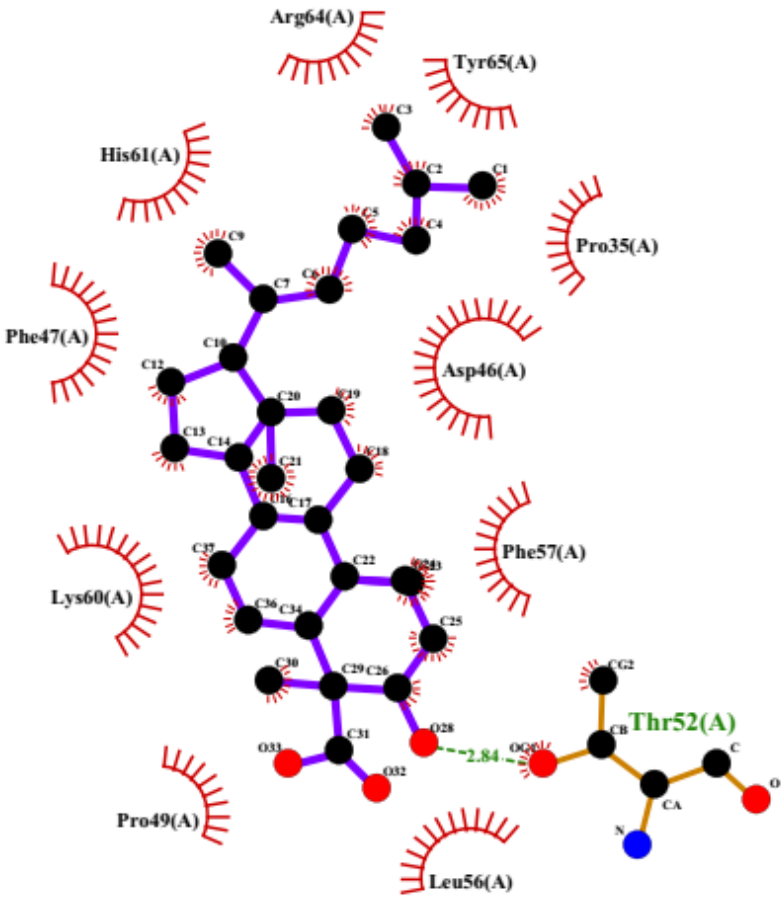

Figure 5

Protein Ligand interaction of ZINC000030729894 with O-methylsterigmatocystin oxidoreductase