Docking Studies of De Molybednum Rat Xanthineoxidoreductase D428A mutant enzyme with polyphenols

A thesis submitted in the partial fulfillment
of the requirement for the degree of

Master of Science
In
Chemistry

By

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Candidate's Declaration

I hereby declare that the work being presented in this dissertation entitled "Docking Studies of De Molybednum Rat Xanthineoxidoreductase D428A mutant enzyme with polyphenols" in the partial fulfillment of the requirements for the award of the degree of Masters of Science in Chemistry, School of Chemistry and Biochemistry, Thapar Institute of Engineering and Technology, Patiala, is my own work during the period of January 2019 to July 2019, under the supervision of Dr. Manmohan Chhibber (Associate Professor) and Dr. Sanjai Saxena (Professor). I have not submitted the work embodied in this dissertation for the award of my other degree.

Arashdeep Kaur

Place: Patiala
Date: July 15th 2019

Arashdeep Kaur
Reg. No.: 301702008

This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

Dr. Manmohan Chhibber
(Associate Professor)
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Thapar Institute of Engineering and Technology

Patiala

Acknowledgement

In pursuit of this academic endeavour, I feel that I have been singularly fortunate as the inspiration, guidance, direction, co-operation, love along with care have come in my way in abundance and it seems almost an impossible task for me to acknowledge the same in an adequate term. Paramountly, I would like to express my sincere gratitude towards Dr. Amjad Ali, Associate Professor and Head, School of Chemistry & Biochemistry, TIET, Patiala for his generous support. My wholehearted indebtness goes to my erudite guides: Dr. Manmohan Chhibber (Associate Professor), TIET, Patiala and Dr. Sanjai Saxena (Professor), TIET, Patiala for their support and patience. Their invaluable assistance and precious guidance helped me in executing this arduous task from its conception to its completion. I thank Mr. Ashok Rana, Mr. Vagesh Dwibedi & Mr. Rajesh Kondabala, P.h.d scholars at TIET, Patiala for their kind cooperation during the analysis work and most of all for making this experience unforgettable for me throughout my life with their expertised advice. Words fail me to express thanks to my family for their selfless sacrifice, encouragement and heart full blessings that continue to enlighten my life. Above all, I thank almighty God for blessing me with strength and wisdom to complete this project successfully.

Place: Patiala

Date: 15th July; 2019

Arashdeep Kaur

Regards,

Arashdeep Kaur

This thesis is dedicated to my parents

for their endless love, support and encouragement.

Table of Contents

Abstract

Gout is a medical condition characterized by increase of the serum urate levels (sUAs) in extra-cellular fluids that results in its precipitation in the tissues causing joint swelling, uric acid stones and further complications if not cured. Xanthine oxidase (XO) and xanthine oxidoreductase (XOR) are two inter convertible enzymes responsible for uric acid production from hypoxanthine and xanthine. It has been reported that increased expression of XO leads to excessive oxidative stress leading to the formation of ROS and ultimately uric acid. Therefore, inhibitors of XO can act as a drug to treat conditions leading to hyperuricaemia and gout. Allopurinol and febuxostat are two molecules presently in use for the treatment of this medical condition that work by inhibition of XO. Present work involved selection of known XO inhibitors from literature and exploration of their mechanism for inhibition using *in-silico* docking studies. After an extensive literature search nine compounds belonging to flavanone and cinnamic acid derivatives were shortlisted along with Pinobanksin (flavones), ursolic acid and umbelliferone. All the compounds were docked against "de-molybednum rat xanthine oxidoreductase D428A enzyme" that showed pinobanksin as the best inhibitor due to its flexible nature at C-3 carbon having sp^3 hybridization. However, studies on human XO and XOR enzyme need to be done both *in-silico* and in wet experiments for development of these molecules as active drugs.

Chapter 1: Introduction

Gout is a medical condition characterized by increase in the serum urate levels (sUAs) of extra-cellular fluids, resulting in the precipitation of monosodium urate monohydrate crystals in the tissue. [1-8] Monosidium urate is the biological product of uric acid produced as an end product of purine metabolism in *Homo sapiens* and other animals. Hyperuricaemia, the medical term used for such condition can start with symptoms of joint swelling,[9] uric acid stones, [1] but if left untreated can result in kidney failure[10], high blood pressure[11], increased sugar content in blood and heart related diseases.[12-13]

Purines are the constituents of many biomolecules like GTP, ATP, coenzyme A, NADH and many more. Tea, coffee, bear, meat and many dairy products also contain purines. [14] An enzyme Xanthine oxidase (XO) interconvertible from Xanthine oxidoreducatse (XOR) [15] is responsible for Uric acid production from two substrates hypoxanthine and xanthine by formation of reactive oxygen species (ROS). Both xanthine and hypoxanthine are ultimate and penultimate products of purine metabolism respectively. Increased expression of XO leads to excessive oxidative stress leading to formation of ROS and ultimately uric acid. [16] Thus, any inhibitor of XO can act as a drug and can treat conditions leading to hyperuricaemia and gout.

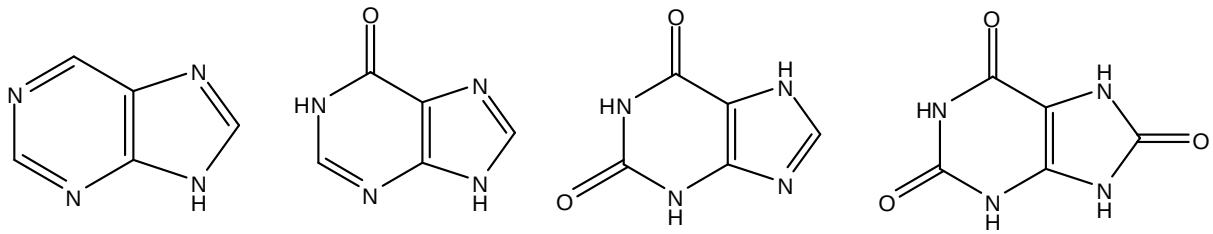


Figure - 1.1: Structures of molecules related to gout (a) General structure of purine (b) Hypoxanthine (c) Xanthine (d) Uric acid.

At present, Allopurinol and Febuxostat are two molecules used for the treatment of this condition by inhibition of XO.[1,17,18] Besides this a number of molecules have been reported in literature known to inhibit XO. Present work involves selection of known XO inhibitors and in silico study of their interactions with the enzyme to get an insight of the mechanism involved.

Chapter 2: Review of Literature

Xanthine oxidoreductase (XOR) consists of two interconvertible enzymes namely Xanthine dehydrogenase (XDH) and Xanthine oxidase (XO). Both XDH and XO are important enzymes in purine metabolism where they catalyze conversion of hypoxanthine to xanthine and ultimately to uric acid as shown in **Figure - 2.1.** [1,5,19-23]

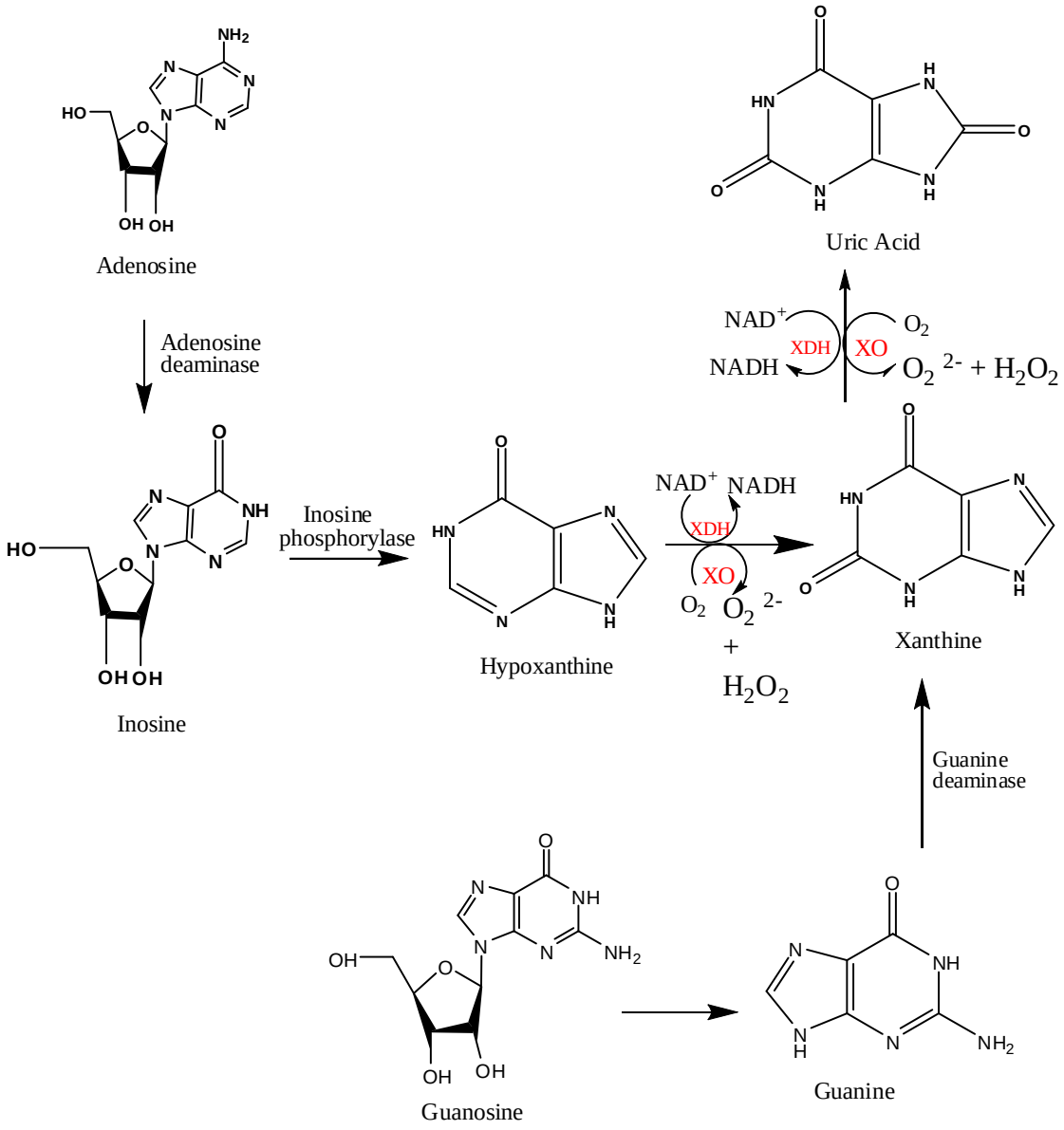


Figure - 2.1: Mechanism of formation of Uric acid

It has been documented that in mammals XOR normally exists as dehydrogenase (XDH), but under certain conditions this enzyme post- translationally converts into oxidase (XO) either reversibly or irreversibly.[15] This results in increased production of uric acid due to its exceptional binding ability with oxygen in comparison to NAD^+.[24]Also, the reactive oxygen species (ROS) produced in the process gives rise to super oxide radicals resulting in further complications. Thus, inhibition of Xanthine oxidase is one of the best solutions to deal with the issue. **Figure - 2.2** shows the structure of some of the known inhibitors of XO used for the treatment of such a medical condition. [1,17,18,25]

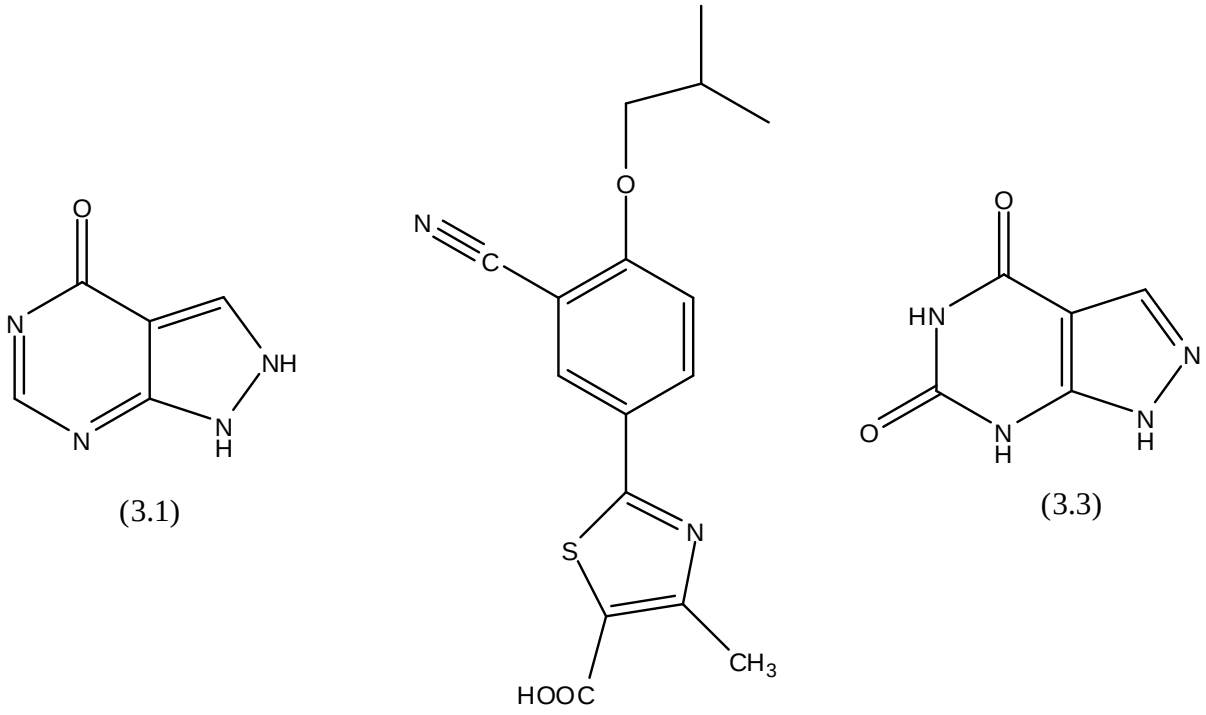


Figure - 2.2: Known inhibitors of XO namely Allopurinol (3.1), Febuxostat (3.2) and Oxypurinol (3.3)

Despite presence of these drugs in market there is need to discover more inhibitors of XO which are safe and have fewer side effects unlike these drug molecules.[20,26,27,28-38] Following paragraphs discuss some molecules from literature that have been reported over the years.

A new class of Xanthone derivatives shown in **Figure - 2.3** was synthesized as XO inhibitor out of which 8a, 8c, 8i, 8g and 8r had spectular inhibition results. Their SAR and docking studies were done showing insertion of 8i and 8r into narrow tunnels of the enzyme towards molybdenum cofactor region along with hydrogen bonding enabling interactions with Gln 1040, Ser 1082, Gln 1261, Gly 797, Gln 797 as well as Cys 150. The 5 potent inhibitors showed IC_{50} values lower than 10 µM which was way far better than allopurinol. [39]

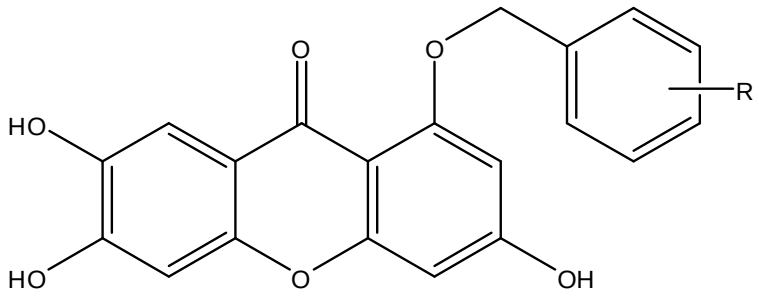


Figure - 2.3: General structure of Xanthone derivative where R= Alkyl group that can be replaced with 2- CH_3 (8a), 4- CH_3 (8c), 4- Cl (8i), 2- Cl (8g), 4- CN (8r)

Recently, 4-aryl/hetroaryl-4H-fused pyrans were synthesized depicting 5n as a remarkable inhibitor among them with an IC_{50} value of 0.59 µM as shown in **Figure - 2.4.** Due to presence of a chiral centre in 5n both of its R and S enantiomer were analysed via computational docking studies showing better results in case of S-enantiomer which is able to fit in a better way in the binding site. [20]

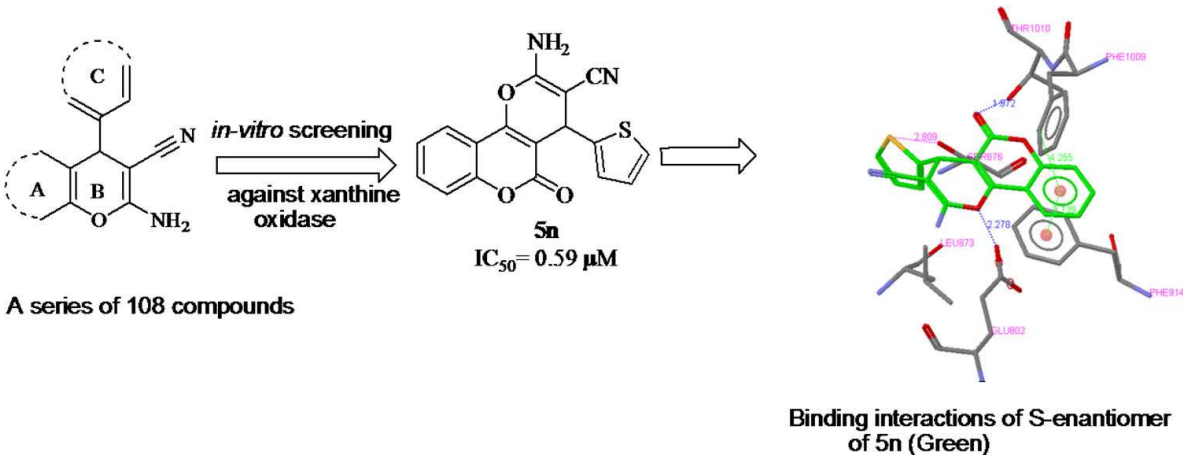
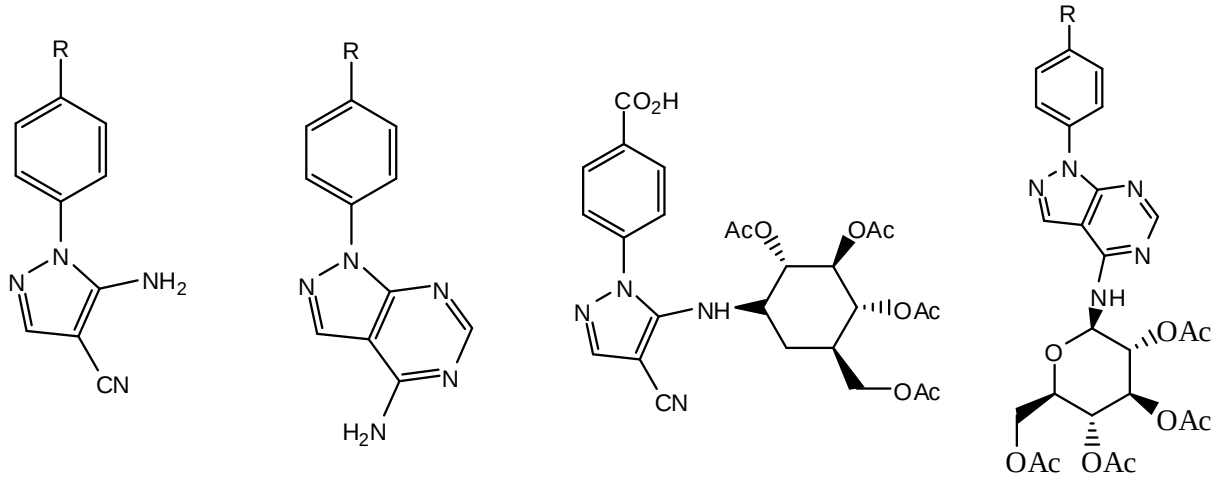


Figure - 2.4: Assay of 4-aryl/hetroaryl-4H-fused pyrans[20]

In the same way 16 compounds (10 of them new) of pyrazole or pyrazolopyrimidine type named as pyrazolo [3,4-d] pyrimidines were synthesized shown in **Figure - 2.5** out of which the ones with cyano, nitro, trifluromethyl group or glycine methyl ester derivatives showed maximum potency of inhibition against XO along with a few of them revealing IC_{50} values below 1mM. [20]



(w) (x) (y) (z)

Figure - 2.5: General structure of pyrazolo [3,4-d] pyrimidines derivative where R= Alkyl group that can be replaced with R= CH_3; OCH_3; COOH; CN; NO_2; CF_3; Cl; $CONHCH_2COOCH_3$.

Certain derivatives of 18b-glycyrrhetic acid (18b- GA) as shown in **Figure - 2.6**were also synthesized marking towards highest XO inhibition in case of moieties having lactone in them after measuring xanthine oxidase activity with allopurinol as standard and xanthine as substrate. [21, 40].

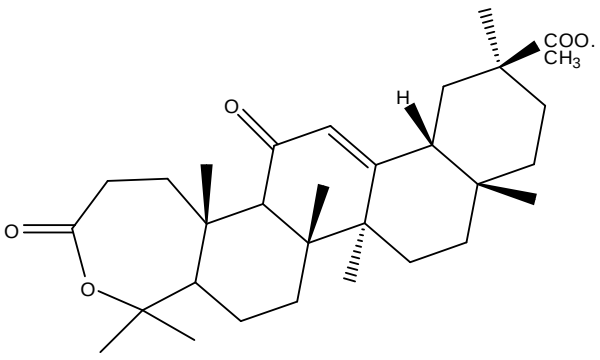


Figure - 2.6: 18b- GA compound with remarkable XO inhibition.

Besides above mentioned synthesis based molecules, many natural sources have also been used for the XO inhibition. Later on, active ingredients of all such natural sources were also identified.

In 1994, Chiang et.al isolated six compounds from the (HOOK) of leaves of *Alsophila spinulosa* Tryon. Among these caffeic acid ($2.X_1$) was the most potent constituent having IC_{50} = 39.21 µM for uncompetitive inhibition against XO using substrate xanthine as control. Nagae et.al studied inhibition of xanthine oxidase by both non- planar and planar flavones and flavonols having 7-hydroxyl group, planar ones like chrysin, quercitin, luteolin, kaempferol, myricetin and isorhamnetin showed IC_{50} values from 0.40 to 5.02 µM. On the other hand, non planar flavonoids, anthocyanidins and isoflavones had lesser inhiboitory effect suggesting application of former as anti- hyperuricemic agents. [41]

A massive study that included 26 species from 18 families of plants showed excellent inhibitory activities. Almost 20% of the plants had greater than 50% inhibition for XO. [42]. Among these Quercitin and kaempferol had 90% and 85% XO inhibitory activity respectively. It was hypothecated that phenolic groups impart hydrophillicity that impart inhibition to XO. The work concluded that 5 potent inhibitors from *Achillea millefolium, Larix laricina, Ledum groenlandicum., Populus balsamifera* and *Veronica officinale* inhibited XO more than 50% due to presence of phenolic and tannin content.[54] Butanol extract of *Spartium junceum L.* flowers was used to isolate 5 flavonoid glycosides out of which antioxidant activity was maximum for luteolin 4'*β*-glucoside and azaleatin 3*β*-glucoside (quercetin 5-methylether 3*β*-glucoside) having value of 22.59 and 19.08 U/ mL respectively. [43]

In 2018, certain triterpenes were isolated from ethanolic extracts of *Tribulus arabicus* to access XO inhibitory activity. A special compound- Ursolic acid shown in **Figure - 4.1** isolated from the plant showed IC_{50} value of about 10.3 µg/mL leading towards reduction of uric acid level by 79.9% in in-vivo trials.[44]

As currently there are no clinical measures focusing on inhibiting generation and enhancing uric acid excretion, a novel xanthone possessing natural biological activity named norathyriol (1,3,6,7-tetrahydroxy-9H-xanthen-9-one) shown in **Figure - 2.7** was investigated. It is mainly found in medicinal plants like *Mangifera indica, Hypericum elegans, Gentianaceae and Tripterospermum lanceolatum*. Reported results signify decreased serum urate levels in mice

upto 27%, 34%and 37% at intake of norathyriol doses in` quantities of 0.92, 1.85, 3.7 mg/kg respectively. Moreover, the potent inhibition activities tested in- vitro proved that the novel candidate is similar to allopurinol, but inferior to febuxostat. [13]

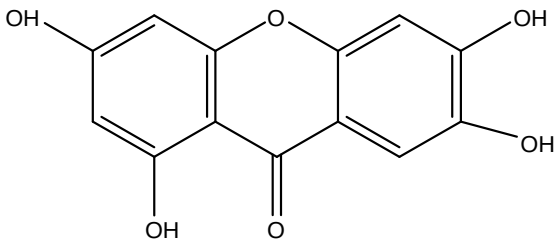


Figure - 2.7: Chemical Structure of Norathyriol.

An inedible and non-toxic fungus, *Tyromyces fissilis* was used to develop methanolic extract from its fruiting bodies to isolate 8 compounds shown in **Figure - 2.8** which showed excellent inhibitory activity due to involvement of salicylic acid and hydrophobic alkyl side chain. Maximum non- competitive `inhibition was remarked by 2-hydroxy-6-pentadecylbenzoic acid (IC_{50} value 58.9 ± 2.2% at 25 μM) [45]

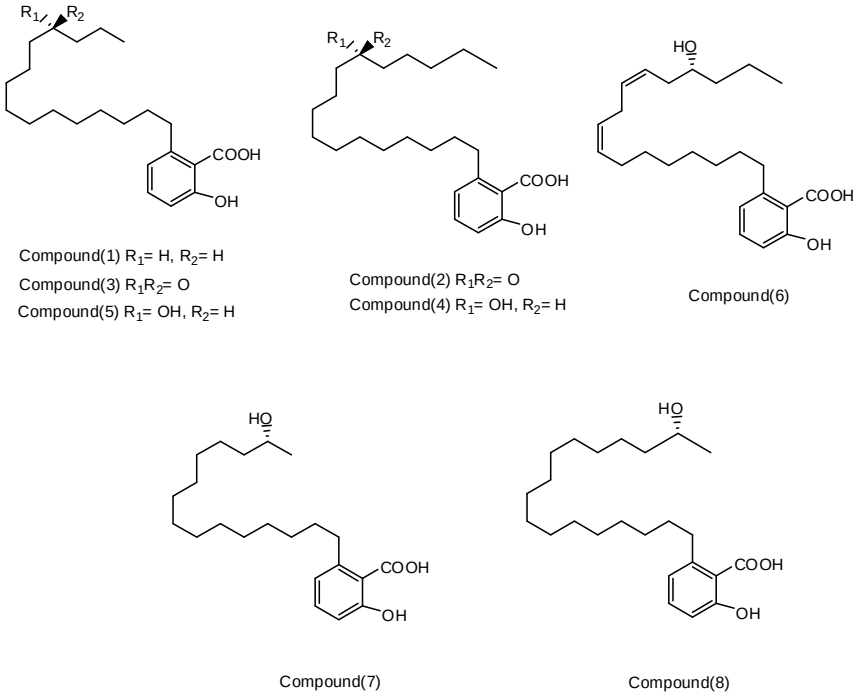


Figure - 2.8: Chemical Structures of 8 compounds isolated from *Tyromyces fissilis.* [45]

Present work involved screening of some compounds obtained from biological resources in literature and understand their mechanism of inhibition for XO by in- silico docking studies.

Chapter 3: Materials and Methodology

General: For docking studies, Autodoc (AutoDockTools- 1.5.6rc3) available at internet was downloaded on the laptop along with chemdraw software (Chemdraw Ultra 10.0). From the literature forty different compounds were shortlisted that have shown activity against Xanthine Oxidase (XO) enzyme of different organisms. All the compounds were categorized on the basis of their structure. A total of twelve compounds were shortlisted on the basis of their IC_{50} values and non availability of literature for interactions with amino acids of the enzyme. **Figure - 4.1** lists the compounds that were chosen for the study.

3.1: Human XOR Enzyme: Crystal structure of the only human xanthine oxidoreductase available on open database was the structure of mutant xanthine oxidoreductase with PDB ID: 2E1Q.[46] The amino acid sequence of 2E1Q was downloaded and homology molecular modeling was performed using SWISS Model due to missing crystal structure for 24 amino acid residues. Three new crystal structure models were obtained having PDB ID: 3AN1, 2E3T and 2CKJ with 90.31, 90.23, 99.92% similarity along with 0.99, 0.98, 0.98 A° resolution respectively. Thus, 3AN1 was chosen as the best model due to maximum resolution which is demolybdo-form of the D428A mutant of rat xanthine oxidoreductase henceforth named **as deMo-rXOR D428A** Enzyme.

3.2: Docking Studies: All the compounds chosen for analysing the inhibitory activity via docking studies were drawn in Chemdraw Ultra 10.0 and later subjected to energy minimization techniques with the help of MM_2 and saved as pdb format.

- Preparation of Receptor (**deMo-rXOR D428A**)

Water molecules were removed and polar hydrogens along with kollman charges were added using autodoc and the atoms were assigned AD_4 type before saving the file in PDBQT format.

- Preparation of ligand

With the same tool mentioned above ligand file can also be saved in PDBQT format after adding gasteizer and setting number of rotatable bonds below 6 i.e. the number of torsions set lesser than 6.

- Preparation of gridbox

After fulfilling the above- mentioned steps docking was performed in three sets via choice of 3 sets of grid box highlighted in **Table 3.1**.

Table 3.1: Grid paramaters of docking studies done.

	Set: 1 Blind Docking for Allopurinol	Set: 2 Docking site of Allopuranol	Set: 3 Active site as per Literature
Points in 3D	126 each	41 each	40 each
Centre (X) (Y) (Z)	 -3.468 11.632 -17.33	 25.912 10.369 -30.876	 -8.314 18.908 -26.198
Spacing	0.819A°	0.375A°	0.986A°

3.3. Procedure

Firstly, the standard drug allopurinol was left in the vicinity of macromolecule in Autodock to dock the protein blindly at the most potent active site. The grid parameters covered the whole macromolecule and the energy results were noted down. The second set included grid parameters covering a smaller area surrounding the site where allopurinol was bound with the protein in first set and docking experiments were performed on standard as well as 12 other ligands chosen from literature. While in the third set the only difference lied in choosing grid box according to the common binding sites in the active pocket site mentioned in review of literature. Finally, the docking results were compared to that of set: 1 and as a whole with standard allopurinol through programming of config file with command prompt using vina file stored in Local disk: C. The best energy scoring molecules were chosen to represent their most stable conformation for direct relation with binding affinity of the chosen ligands.

Chapter 4: Results and Discussion

After an extensive literature search, two major categories of compounds along with Umbelliferone and Ursolic acid were shortlisted for docking studies with Xanthine Oxidoreductase (XOR) enzyme **(Figure - 4.1)**. Five compounds each from the two categories consisting of flavones, flavanone and cinnamic acid derivatives were taken up due extensive literature reports of their antioxidant and anti-hyperuricaemic activity as reported in the previous section.

For docking studies, a mutant human xanthine oxidoreductase (PDB ID: 2E1Q) protein was optimized by homology modeling using SWISS Model. Three sequences with structure similarity of 90.31, 90.23 and 99.92 percent having PDB IDs 3AN1, 3E3T and 2CKJ were identified having a resolution of 0.99, 0.98 and 0.98 A°. Finally, 3AN1 with highest resolution was taken for further docking studies with above mentioned compounds.

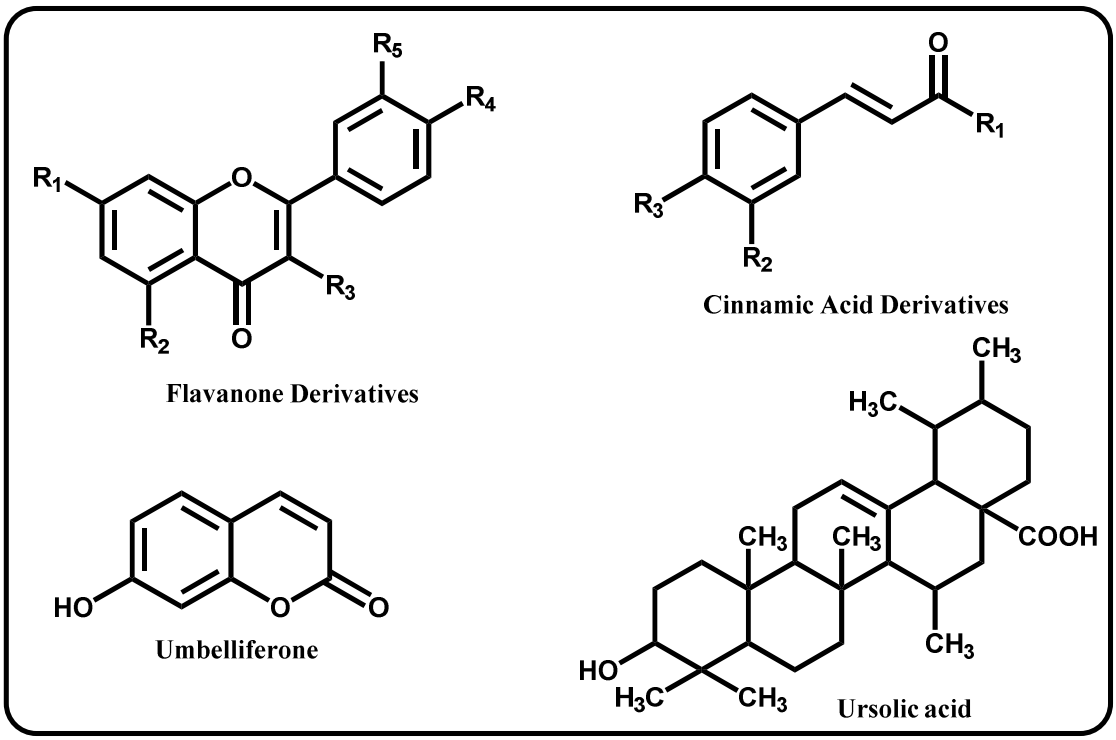


Figure - 4.1: Flavanone and Cinnamic acid derivatives along with Umbelliferone and Ursolicacid shortlisted for docking studies with XOR enzyme.

Docking studies with Autodock software were performed using both blind docking and active site docking. Active site of the protein was identified using literature reports for the binding of

various inhibitors including allopurinol. Allopurinol was also used as a reference compound for the studies. **Table- 4.1** shows the energy scores for all the shortlisted flavanone and flavone compounds after interaction with demolybdo-form of the D428A mutant of rat xanthine oxidoreductase[R1]. It can be seen that Pinobanksin, a flavanone, containing sp^3 hybridized carbon stabilizes the active site to maximum extent having energy score of -9.6 kcal/mol followed by flavones. Isorhamnetin with energy value of -9.4 kcal/mol stabilizes the XOR enzyme maximum at blind docking site. Stabilization by Azaleatin though much higer than standard Allopurinol was lowest among all the flavanone compounds. **Figure-4.2**shows that the binding site of standard drug used in the study, Allopurinol, with blind docking was entirely different from the active site reported in literature. Although the values obtained in both cases were approximately same.

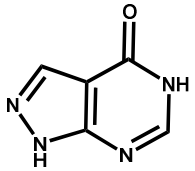
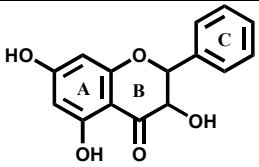
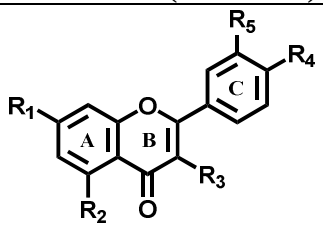
Compound Name and Structures						**Blind Docking**	**Active Site**
						Energy Score (kcal/mol)	
 Allopurinol (Standard)						-6.7	-6.4
 Pinobanksin (Flavanone)						-8.7	**-9.6**
 General Structure (Flavone)							
Name	**R_1**	**R_2**	**R_3**	**R_4**	**R_5**		
Galangin	OH	OH	OH	H	H	**-9.2**	**-9.2**
Azaleatin	OH	OCH_3	OH	OH	OH	**-9.6**	**-9.1**
Rhamnetin	OCH_3	OH	OH	OH	OH	**-9.8**	**-9.3**
Isorhamnetin	OH	OH	OH	OH	OCH_3	**-9.8**	**-9.4**

Table 4.1: Minimum energy results of flavanone derivatives after docking with demolybdo-form of the D428A mutant of rat xanthine oxidoreductase.

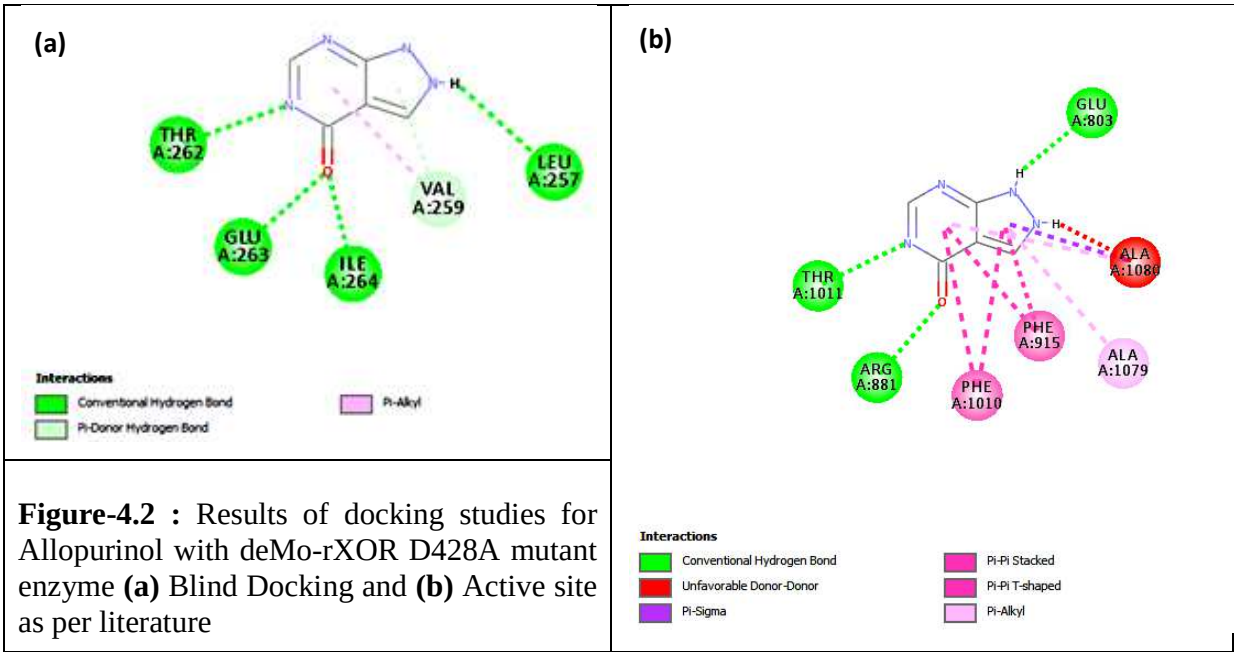


Figure-4.2 : Results of docking studies for Allopurinol with deMo-rXOR D428A mutant enzyme **(a)** Blind Docking and **(b)** Active site as per literature

As shown in **Table 4.1**, both in case of flavanones and flavones the rings were named A, B and C for convenience to discuss the interactions between the functional groups of the molecules and the amino acids of the enzyme. A comparison of **Figure 4.2 (b)** and **Figure - 4.3** shows that binding site of Allopurinol, flavanones and flavones, except for Azaleatin, were closely placed but different amino acids interacted in each case. Azaleatin was exceptional because its binding site was markedly different from rest of the compounds of flavanone category.

It can be seen that Ala1084 was common in case of Pinobanksin, Isorhamnetin and Galangin involved in σ to π interaction between A, A and C aromatic rings respectively. In case of Isorhamnetin the same AA residue was also involved in stronger hydrogen bonding interaction between OH of ring A and carbonyl of the chain. No visible interactions were seen between this residue and Rhamnetin.

It is interesting to note that Rhamnetin and Isorhamnetin, both isomers of each other, flipped their positions by 180° to accommodate only OCH_3 group present in their structure for hydrogen bonding interactions with Ala 1080. This is also one of the reasons for Ala 1084 not interacting with the former. Ala1080 residue, although visible in case of Pinobanksin, is actually positioned away from the flavanones for any interaction.

Arg 913 is common amino acid residue involved in σ to π interaction in all four Pinobanksin, Isorhamnetin, Galangin and Rhamnetin. The ring in case of Pinobanksin, Isorhamnetin, Galangin

(C ring) is different than in case of Rhamnetin (A ring). This once again proves that the Rhamnetin and Isorhamnetin are flipped by 180°.

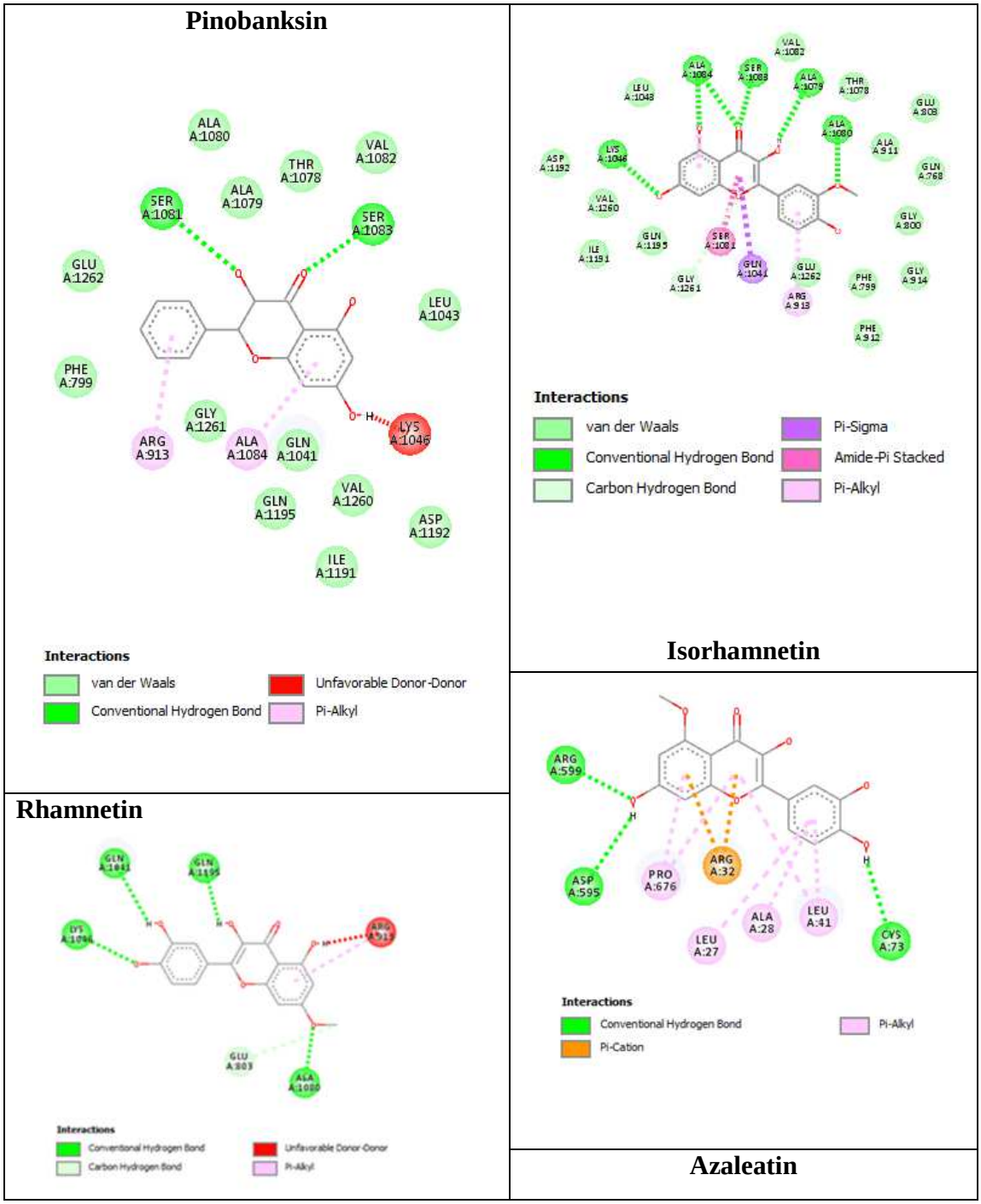


Figure-4.3 : Docking studies results of flavanone and flavones with deMo-rXOR D428A mutant enzyme

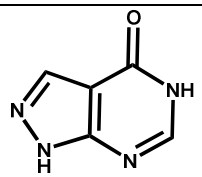
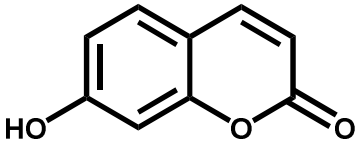
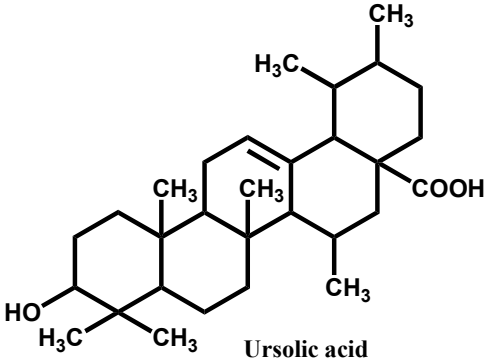
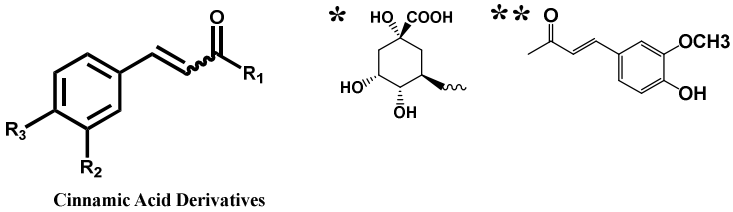
Compound Name and Structures				Blind Docking	Active Site
				Energy Score (kcal/mol)	
 Allopurinol (Standard)				-6.7	-6.4
 **Umbelliferone**				-7.0	-7.4
 **Ursolic acid**				-9.3	-6.9
 **Cinnamic Acid Derivatives**					
Name	**R_1**	**R_2**	**R_3**		
Cis- Cinnamaldehyde	H	H	H	-6.1	-5.9
Trans- Cinnamaldehyde	H	H	H	-6.2	-6.4
Trans- Ferulic Acid	OH	OH	OH	-7.0	-7.3
Chlorogenic Acid	*	OH	OH	-8.6	-8.1
Curcumin	**	OH	OCH_3	-9.6	-8.5

Table 4.2: Minimum energy results of cinnamaldehyde derivatives and other compounds after docking with deMo-rXOR D428A mutant enzyme.

Finally, high stability of deMo-rXOR D428A mutant enzyme complexed with Pinobanksin and Isorhamnetin (E = -9.6 kcal/mol and -9.4 kcal/mol respectively) can be attributed to stabilization of B ring carbonyl and hydroxyl groups by the serine residues. In case of Pinobanksin **(Figure-4.3 a)** both these groups are stabilized via two different serine residues at position 1081 and 1083 while in case of Isorhamnetin Ser1083 stabilizes carbonyl and the OH is stabilized by Ala1084 as discussed above **(Figure-4.3 b)**. The highest stability of Pinobanksin can be attributed to hydroxyl (OH) group positioned on sp^3 hybridized carbon that leads to stronger hydrogen bonding interactions than in case of Isorhamnetin.

Comparing Isorhamnetin, Rhamnetin and Azaleatin **(Figure 4.3 b, c and d)** it can be reasoned that the methoxy group at 5- position of A ring drifts the molecule out of active site cavity by making C-4 carbonyl inaccessable due to steric hinderance. This is the reason that Rhamnetin did not position itself in the active site pocket.

Table 4.2 shows minimum energy value for other compounds shortlisted for this study. As compared to above discussed category of compounds not very impressive values were obtained for active site docking results. Curcumin the conjugated yellow colored compound among these was the most active with minimum energy value of -8.5kcal/mol. However the binding site pocket of this and the ursolic acid was different from the active site pocket of the enzyme.

Chapter 5: Conclusion

Thus, it can be concluded that flavanone are possible potential inhibitors of the deMo-rXOR D428A mutant enzyme enzyme due to their flexible nature. However, studies on human XO and XOR enzyme need to be done both in silico and in experiments to confirm the results for use of these molecules as active drugs. [47]

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