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28-HOMOCASTASTERONE ATTENUATED DIABETIC KIDNEY DISEASE BY MODULATING LIVER X RECEPTOR AND TUMOR NECROSIS FACTOR-ALPHA PROTEIN EXPRESSION IN STZ-INDUCED DIABETIC RAT.

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ABSTRACT

28-Homocastasterone (28-HC) exhibited antiglycemic and antilipidemic effects in diabetic rat. In the present study evaluated 28-HC effects on Liver x Receptor (LxR) mRNA expression, antioxidant status and histomorphological changes in diabetic rat kidney. Induction of diabetes was achieved by a single peritoneal injection of STZ (60 mg/kg. bwt) and 100 µg of 28-HC were administrated orally for 15 days. Followed by tissue malondialdehyde (MDA), superoxide dismutase (SOD), catalase, reduced glutathione (GSH), tumor necrosis factor-α (TNF-α), LxR-α, β mRNA and protein expression, tissue histomorphology were analysed along with 28-HC affinity towards inhibitor binding side of COX2 enzyme were evaluated using molecular simulation software. Increased SOD, catalase, reduced GSH, LxR-α and β mRNA levels were noted and reduced levels of MDA and TNF-α protein were registered in 28-HC treated diabetic rat. Kidney histology observed that glomerular damage reduced in 28-HC treated diabetic rat, In Silico analyses detected 28-HC have significant affinity towards inhibitor binding site of COX2 enzyme. 28-HC enhancing antioxidant enzymes catalytic activity thus attenuated lipid peroxidation and glomerular damage through transactivation of LxR and inhibition of COX2 enzyme catalytic activity. It is suggested that 28-HC supplemented diet prevent diabetic renal disease in man.

KEYWORD: 28-Homocastasterone, Diabetic, Kidney, LxR, COX2.

INTRODUCTION

28-Homocastasterone (28-HC) is plant growth regulator comes under brassinosteroid (Bs) family keto steroid are synthesized by CYP72B1 enzyme and regulates a wide range of plant physiological processes such as seed germination, flowering, senescence, photomorphogenesis and stress response.[1] However, human and animals are exposed to 28-HC through consumption of plant materials as food. Assimilation of 28-HC into enterocytes to blood circulation into vital organs were induces metabolic changes in mammalian cells, that observed in bio-physiological changes such as blood and tissues marker alterations.[2] Nevertheless, earlier study in our lab employed with 28-Homobrasslinolide (28-HB) and 28-Homocastasterone (28-HC), an aldo-keto isoform displayed antiglycemic, antilipidemic effects and improvised blood cells indices in diabetic male adult wistar rat.[3,4]

Diabetes mellitus (DM) is a multifactorial metabolic syndrome begins insulin deficiency or dysfunction on target cells resulted to increased blood glucose level called hyperglycemia.[5] Chronic hyperglycemia leads to various complications in the organ that are rich in microvascular including nephropathy. Diabetic nephropathy (DN) reported to develop 20–40 % of patient with diabetes in worldwide.[6] DN characterized by the structural and functional abnormalities such as albuminuria, hyperfiltration, hyperpermiability to macromoleculaes and accumulation of extracellular matrics protein, basement membrane thickening, mesangeal expansion and glomerular epithelial cell loss.[7] Although the underlying mechanisms of diabetic nephropathy remain unclear, even though this multifactorial disease may associate with hyperglycimia, hyperlipidemia, oxidative stress.[8] Kimmelstiel and Wilson reported that, in diabetic the abnormal lipid metabolism causes renal accumulation of lipids drive pathogenesis of diabetic nephropathy.[9] Nevertheless, in the diabetic subject increased renal expression of transcriptional factor, sterol regulatory element binding protein 1c induce 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCoA reductase) transcription resulted to increased synthesis and accumulation of cholesterol. Similarly, carbohydrate response element-binding protein (ChREBP) transcriptional factor induce fatty acid and triglyceride synthesis through transcriptional activation of enzymes responsible for fatty acid and

triglyceride synthesis.[10,11] SREBP1c and ChREBP upregulation in diabetes leads to increase cholesterol and triglyceride synthesis and accumulation in the kidney driving factor for diabetic renal disease.[12] Oxysterol activating nuclear receptor, Liver X Receptors - alpha and beta (LxR-α, β) are master regulator of glucose, cholesterol and triglyceride metabolism by transcriptional down regulation of SREBP1c and ChREBP, HMGCoA reductase and upregulation of ATP-binding cassette transporter-1 (ABCA1) via cholesterol efflux from renal cell.[13, 14] Similarly, LxR induce nuclear factor (erythroid-derived 2)-like 2 (Nrf2) gene expression leads to antioxidant enzymes are upregulated.[15] Therefore, we aimed to investigate 28-homthocastasterone effects on LxR-α, β and antioxidant status in diabetic rat kidney.

MATERIALS AND METHODS

Chemical and experimental rat

All chemicals used in the study are analytical grade and purchased from Sigma-Aldrich, Mo, USA. 28-HC was courtesy of Dr.V.S.Pori. National Chemical Laboratory, Pune, India. Experimental male *albino wistar* rats (3 month old) were purchased from Sri Raghavendra Enterprises, Bengaluru, India. The primers were purchased form European Genomics, Bangalore, India.

Experimental design

Animal use and care were in compliance with that of the CPCSEA regulations and Institutional Animal Ethics Committee (IAEC) guidelines, Pondicherry University, Puducherry, India (IAEC/Approval.No.2013-14/01). Rats were divided into four groups of six rats having an average weight 150-180 gm, Group I: Normal control, Group II: Normal + 28-Homocastasterone (28-HC 100µg), Group III: Diabetic control, Group IV: Diabetic + 28-Homocastasterone (28-HC 100µg). Diabetes was induced by a single intraperitoneal injection of 60 mg/kg bwt streptozotocin (STZ) in citrate buffer (0.1 M, pH 4.5) to 12 hr fasted rats. After 48 hr, blood glucose levels were measured using a glucometer (OneTouch Horizon, Accuva check). Blood glucose level >250 mg/dL were considered to be diabetic. Groups II and IV received 666mg/ kg bwt 28-HC in 50% ethanol by oral gavage daily for 15 consecutive days. Control groups I and IV received 50% ethanol alone.

Tissue Antioxidant status

Malondialdehyde (MDA) was measured by the method of Ohkawa *et al.*[16] The superoxide dismutase (SOD) activity was measurement by the method of Marklund and Marklund.[17] Catalase activity and reduced glutathione level was analysed by method of Beutler *et al.* [18]. Tissue protein content was analysed by method of Lowry *et al.*[19]

Tissue Histology

Following anaesthesia kidney tissues surgically removed from the rat and thoroughly washed with 0.1M phosphate buffer pH 7.4 and fixed in 10% buffered formalin. After an overnight formalin fixation, further tissues process was carried out by dehydration with alcohol, clearing with xylene, paraffin infiltration and paraffin embedding the tissues. The sectioning at 4µm sizes were cutting with the help of a digitalized microtome. The sections were stained with haematoxylin and eosin (H/E) stain.[20] The histomorphological changes of kidney tissues were analysed under camera attached binocular microscopy at 10x and 40x magnifications (Olympus).

Protein Expression Studies

The protein expression was analysed by western blotting technique. Protein samples of rat tissues homogenate were electrophoresed in a 10% SDS-PAGE (Mini Protean II System, Bio-Rad, USA). After the resolved proteins were transferred onto a nitrocellulose membrane (NYTRAN, Keene, NH, USA) confirmed by Ponceau S staining of the blot, then membrane were incubated in a blocking buffer (PBST buffer containing 0.1% Tween and 5% dry milk powder) for 1hr at room temperature with constant shaking. Goat polyclonal primary antibodies against TNF-α protein were used as the probe and the blot were incubated overnight at 4 °C with constant shacking. Following blot were wash with PBST buffer, the blots were further incubated at room temperature for 1hr with horseradish peroxidase conjugated rabbit anti-goat IgG (1:1000 dilution). TMB/ H_2O_2 (Bangalore Genei, Bangalore, India) was used as a substrate to reveal the antibody specific protein. The detected protein bands were taken for picture and quantitated by densitometry.[21]

Molecular Biology Studies

RNA was isolated from kidney tissues using the TRIzol reagent and protocol were followed as for manufacturer guidelines. The cDNA synthesised from isolated RNA employing reverse transcriptase polymerase chain reaction (RT-PCR) technique by using AMV reverse transcriptase and a total RNA (100 ng) from tissue as a template. The cDNA used template to amplify the gene of interest using specific primers, (LxR-α) 5'-GCGTCCATTCAGAGCAAGTGT-3' , 3'-TCACTCGTGGACATCCCAGAT-5', (LxR-β) 5'-CTCTGCCTACATCGTGGTCATCT-3', 3'-ATGAAGGCATCCATCTGGCAGGT-5'. Amplified gene product were then separated by electrophoresis on a 2% (w/v) agarose gel and stained with ethidium bromide. The polynucleotide band intensities were measured by densitometry.[22]

Bioinformatics – In Silico

The 3D crystal structure of COX2 (PDB ID: 2cox) were retrieved from RCSB protein databank (http://www.rcsb.org). Retrieved protein prepared before molecular modeling using PyMol software (Schrodinger LLC, Cambridge, USA). The protein crystal structure water molecules were removed and polar hydrogens, Koliman charge were added to the protein using AutoDock software 4.0 and saved in .pdb script for further molecular docking.

28-HC (CID: 5487654) and aspirin (CID: 2244) were obtained from PubChem database (http://pubchem.ncbi.nlm.nih.gov/) in .SDF file format, converted to .mol2 format using Open Babel software version 2.4.1. Followed by the inhibitors aspirin and 28-HC were prepared and optimized by means of ligand preparation script in AutoDock 4.0 program. The inhibitors (28-HC and aspirin) were prepared for docking as detecting root, torsion tree were set and saved .pdbqt file format.

Grid generation were carried out using the prepared crystallographic structures of COX2 protein. The inhibitor molecules 28-HC and aspirin in the complexes of protein structure were picked to form grid. Therefore, the centroid of inhibitor molecule in complex structures were chosen to generate grid points X = 60, Y = 60, and Z = 60 axis set for further docking analysis. The grid file generated by means of "grid generation panel" in AutoDock software version 4.0.

Molecular docking carried out AutoDock 4.0 suite docking simulation. Different docked conformations were obtained and conformations with strongest binding affinity towards inhibitor binding cavity of protein were selected as the possible binding conformation and taken into for further interaction analysis. The final evaluations of protein–ligand binding were done with Glide score.

RESULTS

MDA level in kidney was reduced to 66.31% and 36.70% in 28-HC treated control and diabetic rat, whereas MDA level was increased 23.71% in diabetic rat. Catalase activity was increased to 23.52% and 28.57% in 28-HC treated rat, although 23.52% catalase activity was reduced in diabetic rat. SOD activity noted to be increased 22.22% and 42.85% in normal and diabetic 28-HC treated rat, on other hand 22.22% reduction was observed in diabetic rat. GSH content noted to be increased to 19.10% and 73% in 28-HC treated control and diabetic rat respectively and 43% of GSH reduction was registered in diabetic rat compared to control (Table 1).

Table 1: MDA, Catlase, SOD and reduced GSH levels

Group	MDA	Catalase	SOD	GSH
Control	0.249±0.02	19± 2	18±1.84	22.77 ± 1.2
C + 100µg 28-HC	0.125±0.03*	24± 3	22±1.52	27.58 ± 1.8*
Diabetic	0.316±0.05	15±2	14±1.73	14.64 ± 1.52
D + 100µg 28-HC	0.218±0.06*	20±2*	20±1.84*	31.52 ± 1.6*

MDA levels expressed nmol MDA /min/mg protein. Catalase activity expressed nmol H_2O_2 produced/min/mg protein. SOD activity nmol pyrogallol oxidized/mim/mg protein. GSH level expressed mg/gm protein. Each group contain six rat (n=6) and p<0.01* considered to be significant.

In case of control rat, normal kidney architecture was observed. 28-HC treated control rat kidney showed unremarkable changes in glomerulus. Diabetic control rat kidney tissue observed that most of the glomeruli distorted and enlarged, some of the tubules showing signs of necrosis and loss of brush border. In 28-HC treated diabetic kidney noted most of the glomeruli unremarkable with few tubules showing loss of brush border and mild interstitial lymhocytic infiltration (Figure 1).

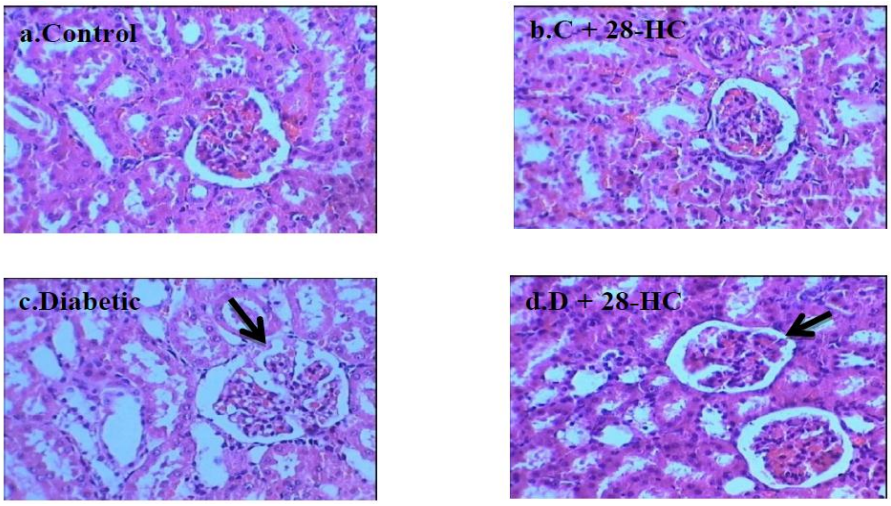


Figure. 1: Histological sections of kidney tissue (a) Control, (b) Control+28-HC, (c) Diabetic, (d) Diabetic+28-HC following 15 d oral administration of 100 µg 28-HC Hematoxylin and Eosin Staining at X 40 magnification.

Figure. 1: Effect of 28-HC on normal and diabetic rat kidney histology.

Densitometry analysis of TNF-α protein expression was carried out following western blotting after SDS-PAGE analysis under reducing conditions. TNF-α expression in the kidney reduced 30% in 28-HC treated control and 35% in 28-HC treated diabetic rat. The diabetic control tissue yielded 143% increase in TNF-α band intensity compared to control rat kidney tissue (Figure 2).

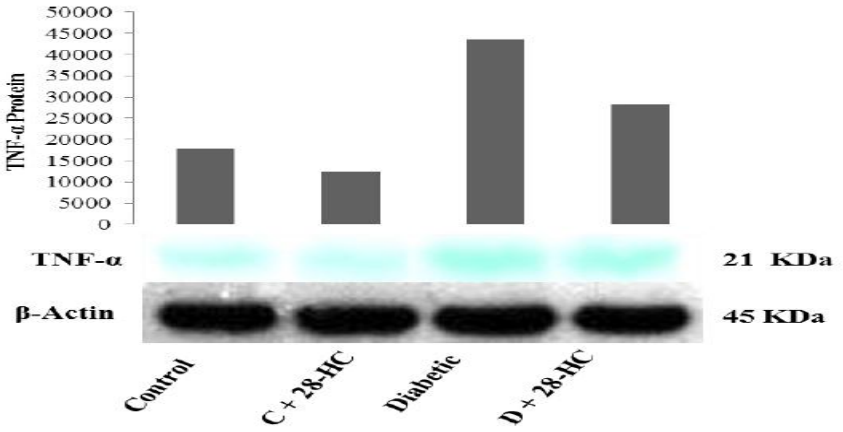


Figure. 2. TNF-α; Tumor necrosis factor-alpha, protein expressions profile in rat kidney tissue following 15 day oral administration of 28-HC in normal and diabetic rat.

Figure 2: Effect of 28-HC on TNF-α protein expression.

Gene expression studies were carried out on a set of specific primer, generating corresponding cDNA using AMV reverse transcriptase and amplified each cDNA population by the polymerase chain reaction standardized for use of the pair of the specific primers that ranged between 19-24 mers. Following PCR amplification the ethidium bromide band intensity of the amplicons was assessed using densitometric software in a gel documentation system. LxR mRNA expression in control and treated kidney tissue yielded difference in the band intensity for LxR-α band were only 8% and 40% higher than the normal and diabetic control. In the LxR-β bands were only 70% and 10% higher than the normal and diabetic control (Figure 3a, 3b).

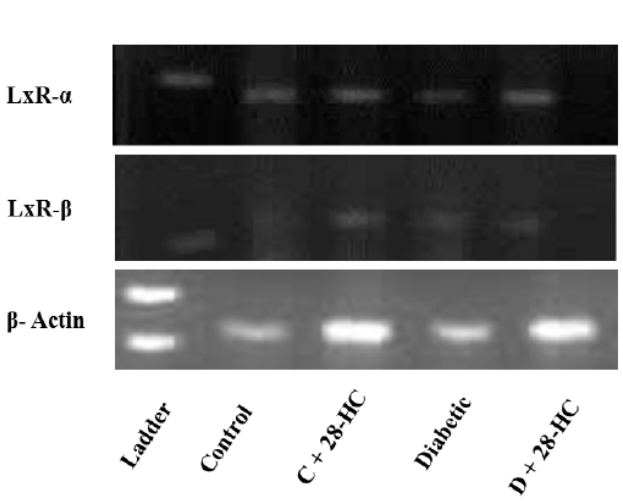


Figure 2 a. Kidney gene electrophoretic bands

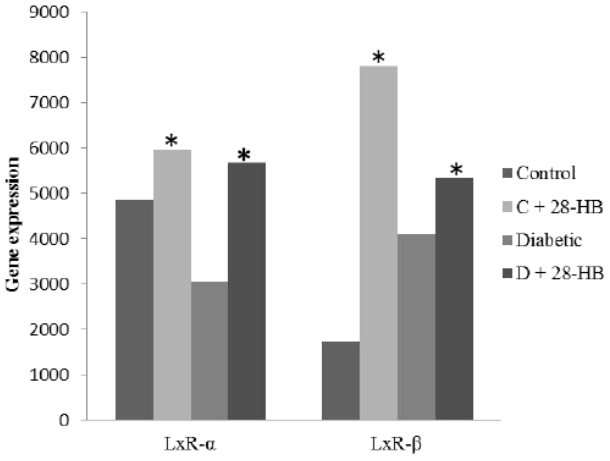


Figure 2b. Kidney gene expression quantification

Figure 3 a & b. 28-HC, 28-homocastasterone; LxR-α and β, Liver x Receptor-alpha & beta, *P<0.005 considered to be significant.

Figure 3: Liver X Receptor and hexokinase-I mRNA expression in normal and diabetic rat.

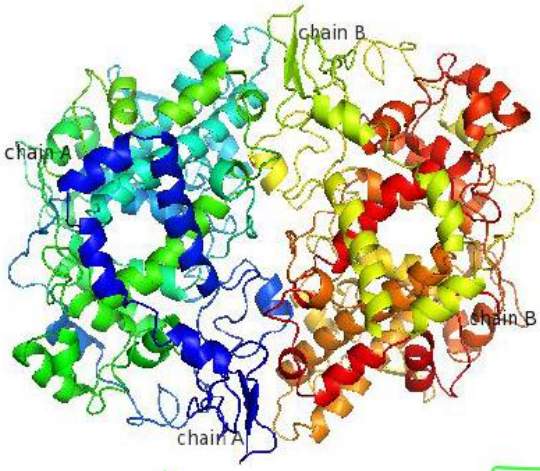


Figure 4a: COX2 protein

Figure 4b: 28-homocastasterone

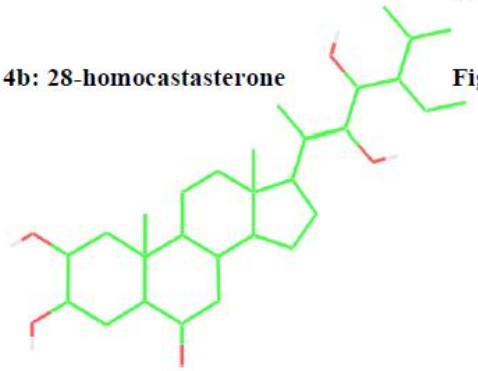


Figure 4c: Aspirin

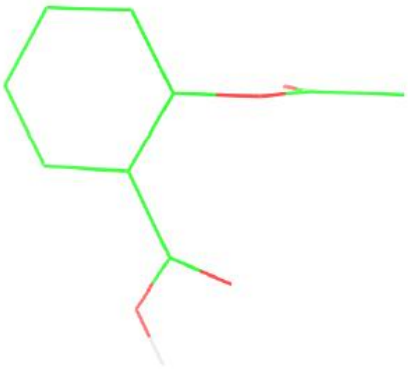


Figure. 4: Shows COX2 enzyme and ligands structure.

Figure 5a: COX2 and ligand

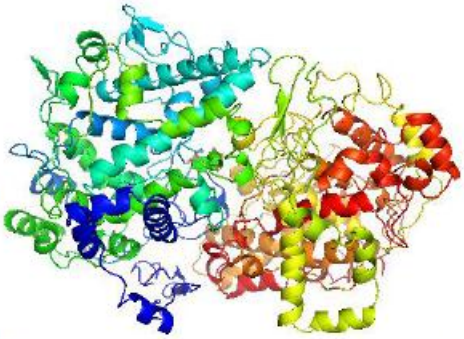
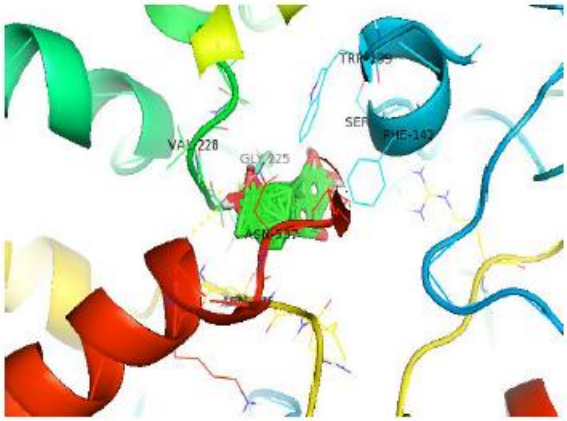


Figure 5b: COX2 and 28-HC

Figure 5c: COX2 and Aspirin



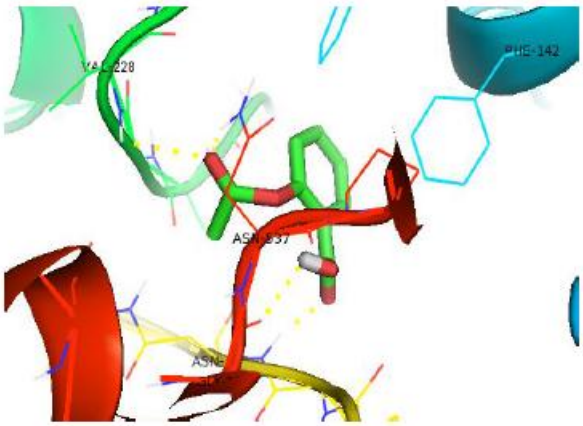
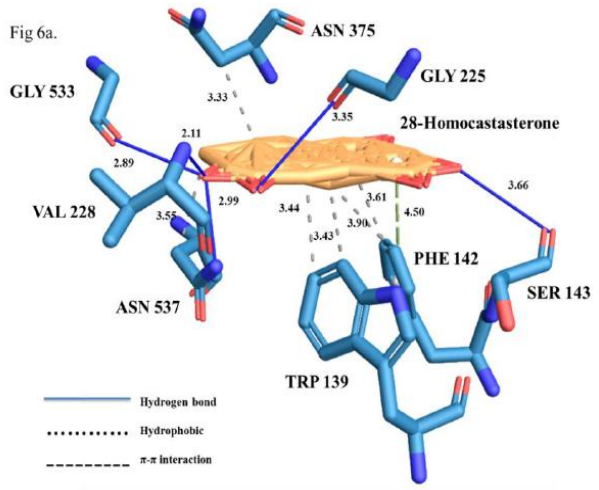


Figure. 5: COX2 enzyme and inhibitors interactions.

Table. 2: COX2 protein with 28-HC and Aspirin interactions.

Protein COX 2 (PDB: 2cox)	G-Score	H-band	Hydrophobic	π-π
28-HC	**-12.2**	**SER143B, GLY225B, VAL228A, GLY533A, ASN537A**	**TRP139B, PHE142B, ASN375A, ASN537A**	**PHE142B**
Aspirin	**-4.66**	**VAL228A, ASN375A, GLY536A, ASN537A**	**TRP1398, ASN375A**	**PHE142B**

Molecular docking was carried out to examine the binding affinity and favorable binding orientation of COX2 protein with 28-HC and aspirin are selected for docking molecular simulation. Different docked conformations generated and docking conformation which having lowest glide score were selected as a most probable affinity towards inhibitor binding site of COX2 enzyme. The carefully chosen docking conformations of 28-HC and aspirin with COX2 enzyme were taken further analysis for binding mode of interaction. Three-dimensional crystal structures of the COX2 enzyme and ligand molecules are shown in Figure 4a and 4b. The binding modes of 28-HC in the inhibitor binding cavity of COX2 enzyme are shown in Figures 5a and 5b.



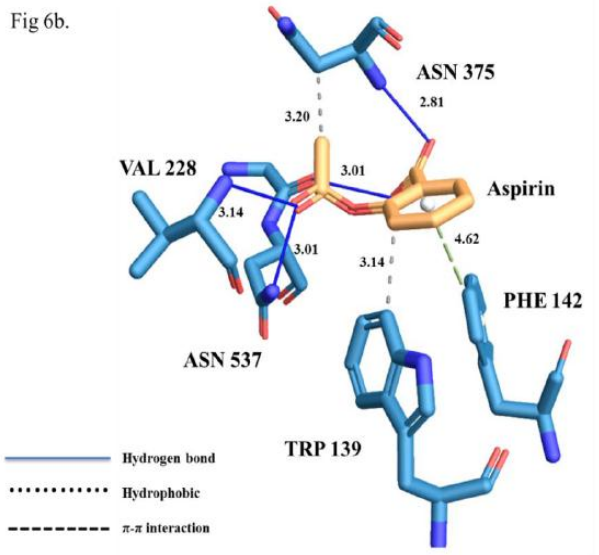


Figure. 6a & 6b: 28-HC and COX2 enzyme amino acid residues interactions and Aspirin and COX2 amino acid residues interactions.

Figure 6: COX2 enzyme amino acids and inhibitors interactions.

Docked complexes of COX2 enzyme and 28-HC exhibited glide score -12.2 kcal/mol indicating more favourable binding affinity towards inhibitor binding cavity of COX2 enzyme compared to known inhibitor aspirin glide score were registered - 4.66 kcal/mol. The complex of COX2 enzyme and 28-HC shows strong binding interaction with inhibitor binding cavity of the enzyme formed H-bond with residues SER143B, GLY225B, VAL228A, GLY533A, ASN537A and residues TRP139B, PHE142B, ASN375A, ASN537A forming hydrophobic interaction, the residues PHE142B formed π-π stacking interaction with 28-HC (Figure 6a and Table 2). However, complex of COX2 enzyme and aspirin shows binding interaction with binding cavity of the protein formed H-bond with residues VAL228A, ASN375, GLY536A, ASN537A and residues TRP1398, ASN375A forming hydrophobic interaction, the residues PHE142B formed π-π stacking interaction with 28-HC (Figure 6b and Table 2). The docking complex of COX2 enzyme with reported inhibitor aspirin and 28-HC revealed that in structural component a chain A and chain B involved in hydrogen bond, hydrophobic and π-π stacking interactions with 28-HC and aspirin.

DISCUSSION

Protein and gene expression studies through western blot and PCR analysis based on band intensity assessment suggested increased expression of LxR-α and β in 28-HC treated diabetic rat kidney. In contrast, TNF-α expression reduced in 28-HC treated diabetic rat kidney.[6] Suggestive of specific gene expressions caused by this ketosteroid conforming the biopotency at the genetic level in the mammalian. It was established from earlier studies [22, 23] that rat testicular testosterone induction by the isoform 28-homobrasslinolide and 28-homocastasterone was mediated through the transcriptional regulation by the both LxR-α and β nuclear receptors. The increase in the LxR-α and β gene expression in the kidney thought that a similar signaling cascade is in process in rat kidney as a response to 28-HC administration to the experimental animal.[23]

Chronic hyperglycemia causes excessive generation of reactive oxygen species (ROS) and reduction of antioxidants SOD, catalase, GSH level resulted to oxidative stress induced tissue damages in diabetic subject.[24] Although present study observed increased lipid peroxidation marker, MDA level and decreased GSH level, SOD, catalase activity in the STZ-induced diabetic rat kidney tissue. However, orally administration of 28-HC to the diabetic rat for 15 days, reduces MDA level and increases SOD, catalase activity in kidney tissue. The phyto steroid 28-HC capable to induce antioxidant enzymes in mammalian system, moreover, the antioxidative effect of 28-HC may result of Nrf-2 gene transcriptional induction by LxRs, in the present study not analysed Nrf-2 gene expression, even though, LxRs similar signaling cascade of transcriptional regulation of Nrf-2 gene expression resulted to increases SOD, catalase gene expression in rat kidney tissue.[25]

Although, chronic hyperglycemia affect renal structure and function as a consequence of enhanced ROS formation, abnormal lipid metabolism and lipid deposition in tissue.[10] The degenerative changes in the kidney histology, distorted and enlarged glomerular structure observed in STZ-induced diabetes.[26, 27] Although orally administration of 28-HC to the diabetic rat for 15 days, diabetic kidney histology observed that most of the glomeruli unremarkable, thus suggestive of 28-HC may prevent hyperglycimic induced renal histomorphological changes.

Earlier studies with diabetes rat renal tissue suggested upregulation of renal COX-2 enzyme and increases prostaglandin production that precedes to increases TNF-α expression, structural and functional alteration in diabetic renal tissue.[28-30] Inhibition of COX-2 enzyme catalytic activity resulted to reduction of prostaglandin and TNF-α level, attenuate the development of glomerulosclerosis. The inhibition of COX-2 enzyme preventing prostaglandin accelerated renal damage in diabetic rat, thus reflected decreased TNF-α level and structural and functional improvement in diabetic rat renal tissue. However, in the present study molecular docking interaction with 28-HC observed that 28-HC showed high affinity towards inhibitor binding side of the enzyme and secured -12.2 kcal/mol glide score compared to known inhibitor aspirin registered -4.66 kcal/mol (Table 2). Thus suggestive of 28-HC inhibits COX2 downstream events such as prostaglandin and TNF-α formation and attenuate progression of diabetic renal disease.

CONCLUSION

Phyto steroid attracted attention as possible exogenous modulators of mammalian gene and protein expression. The plant steroid 28-HC is now recognized exogenous ligand for LxR-α, β. The experimentally observed increased two antioxidant enzyme activity and reduced lipid peroxidation, glomeruli damage is to be considered a cellular response to 28-HC action mediated through liver X receptor in rat kidney. The remarkable ability to decrease TNF-α level, significant affinity toward COX2 enzyme inhibitor binding side by 28-HC is a lead observation in the study and reflects the significant biological potency associated with 28-HC. We suggest that attempts at drug development for renal disease control include LxRs transcriptional regulation along with COX2 enzyme inhibition as a potent target.

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