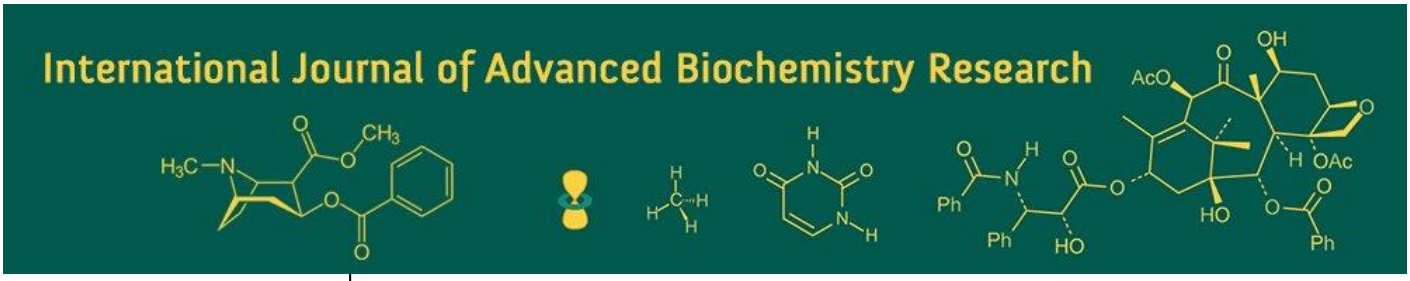

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In silico characterization, homology modelling and structure-based functional annotation of *Labeo rohita* TLR4 protein

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Abstract
Toll-like receptor (TLR4) is one of the pattern recognition receptors and is activated by exogenous molecules from pathogens or endogenous molecules released by injured or narcotic cells. Toll-like receptor 4 (TLR4) activate strong inflammatory response for innate immunity. In the present study structural analysis, homology modelling and molecular docking of *Labeo rohita* TLR4 protein were carried out. TLR4 biochemical analyses revealed the following: Mol. Wt. 94.75 kDa, theoretical isoelectric point (PI) 8.6 and grand average of hydropathicity (GRAVY) values of 0.052. Virtual screening of agonist ligands and molecular docking revealed oxycodone ligand as being highest binding energy value – 8.0 Kcal/mol. Molecular interactions depict conventional hydrogen bonding of Oxycodone was observed with PRO564, LEU563, GLU699, ASN697, PHE604, CYS605, GLY701, VAL702, PRO703 and PRO704.

Keywords: TLR4, homology modelling, molecular docking

Introduction
Innate immune responses are the first line of defence for all host organisms. The pattern recognition receptors (PRRs) are one such important mechanism in the innate immunity spectrum. PRRs are germline-encoded host sensors capable of detecting molecules of pathogenic origin with the ability of disrupting the normal functioning of the host cellular mechanisms (Akira *et al.*, 2006) [1]. Based on the ligand specificity, function, localization and/or evolutionary relationships, PRRs are classified into various types. Among these, the structurally conserved molecules derived from microbes are recognized by single-pass membrane-spanning receptors are known as the Toll-like receptors (TLRs). TLRs are transmembrane proteins which recognize exogenous molecules from pathogens or endogenous molecules released by injured or necrotic cells and launch a strong inflammatory response. TLRs are further divided into two families known as cell surface TLRs and intracellular TLRs (Kawasaki and Kawai, 2014) [15].

Toll-like receptor 4 (TLR4), the protein of our current interest also designated as a Cluster of Differentiation 284 (CD284), is a single-pass membrane-spanning receptor that activates a strong inflammatory response after the recognition of exogenous molecules from pathogens or endogenous molecules released by injured or narcotic cells thereby activating innate immune response (Molteni, Gemma and Rossetti, 2016) [23]. TLR4 displays differential expression across different tissues and is highly expressed in the spleen tissues and moderately expressed in placenta, colon, ovary and peripheral blood leukocytes (Garay-Malpartida *et al.*, 2011) [7]. The signalling pathway responsible for the activation of innate immune system follow one of two pathways the MyD88-dependent and the MyD88-independent pathways. Both pathways induce IRF3-dependent type-I interferon production that ultimately protect the host from microbial infection (Kawasaki and Kawai, 2014) [15]. TLR4 detects lipopolysaccharides (LPS) found in most gram-negative bacteria. Upon detection of LPS, the ligands binding with TLR4 activate either MyD88 dependent or independent pathway which in turn activates innate immune response. Fishes which inherit TLR4 gene can tolerate high level of LPS even with the presence of TLR4 gene as costimulatory molecules essential for its activation are absent from all fish genomes (Iliev *et al.*, 2005; Swain *et al.*, 2008; Pietretti *et al.*, 2013) [13, 24].

In fishes cloning and characterization of TLR4 gene has been successful in zebrafish, rare minnow, common carp, grass carp, channel catfish, rohu and blunt snout bream till date.

(Kongchum *et al.*, 2010; Huang *et al.*, 2012; Quiniou *et al.*, 2013; Tang *et al.*, 2016) [31, 11, 25, 30]. The exact role of TLR4 in fishes, however, is not fully understood. Also, not much is known about the structure and expression profiles of TLR4 in fish including their ligand specificity. Thus, there remains a wide scope for investigating this protein in fishes.

Accumulation of protein sequences have seen an exponential growth, especially in last two decades due to the advances of DNA sequencing technology, which are then deposited on the protein data bank (PDB). But the availability of their functional structures is not common. Determining protein structures is of great significance in biochemical physiology since a very precise arrangement of the atoms in three-dimensional (3D) structures of proteins is required for proper functionality of the proteins. Knowledge of 3D protein structures allows us in better appreciating the working of proteins (Deng *et al.*, 2018) [4]. Homology modelling is a comparative study of protein and amino acid sequences. This computational tool provides understanding about the structural properties of proteins and also has been used to predict the 3D structure of proteins in high resolution and with good accuracy. It is a key component in structural biology and used for the prediction of 3D structures of proteins from their sequences.

Molecular docking is an important tool that mimics the interactions between small ligands and its receptors. The major objective molecular docking is to evaluate the feasible binding geometrics of a putative ligand with respect to a target protein. This method has been used to design a broad range of molecules having potential roles in treating major diseases such as cancers (Sabbah *et al.*, 2015) [27], cardiovascular diseases (Mena-Ulecia *et al.*, 2015; Dong *et al.*, 2016) [22, 5] and anti-infectious agents (De Ruyck *et al.*, 2016) [3].

The exact role of TLR4 in fishes is not fully understood. Also, the expression profiles of TLR4 in fish are poorly understood along with its ligand specificity in fishes. Given the significance of TLR4 proteins in the innate immune response in general and the lack of information on the protein in particular, the present study was proposed. The most top produced freshwater aquaculture species from India and an Indian major carp, rohu (*Labeo rohita*) was used as a model organism for the study.

Materials and Methods

Retrieval of protein sequence from NCBI

Labeo rohita TLR4 gene sequence with accession number KX21824.1 was retrieved from the NCBI (www.ncbi.nlm.nih.gov) and was used for further analysis.

Computing of physiochemical properties

Physicochemical properties like molecular weight, isoelectric point, instability index, aliphatic index, etc. were estimated using ProtParam tool (https://web.expasy.org/protparam) for the analysis of TLR4 protein.

Secondary structure analysis and verification

SOPMA server was used for secondary structure prediction and PSIPRED server was used for the graphical presentation of TLR4 protein.

Homology modelling

Homology modelling was done by the Modeller 10.1 software. The template sequence was selected using the BLAST search on the NCBI database and four most closely related ligands were selected based on the query cover, identity percentage and the e-value of the alignment. The 3D crystalline structure of the four targeted proteins were retrieved from the protein data bank (http://www.rcsb.org/). Retrieved proteins obtained from PDB were 3FXI, 4G8A, 3VQ1, 2Z64. Visualization of the proteins model was done by Discovery Studio program. The model with the lowest value of DOPE (Discrete Optimized Protein Energy) scores, or with the highest GA341 assessment scores was selected for further assessment. ModRefiner was used to improve the quality of the generated structure.

Three-dimensional structure analysis

A stereo chemical quality of the predicted models and accuracy of the protein model was evaluated after the refinement process using Ramachandran plot computed with the PROCHECK program.

Docking studies

Eight ligands were selected and retrieved from PubChem database (https://pubchem.ncbi.nih.gov). The drug-likeness properties of compounds were obtained using the OSIRWAS property explorer. The VSDK (virtual screening through docking), a program based on auto dock was used for docking studies.

Phylogenetic analysis and sequence alignment

Multiple sequence alignment between TLR4 protein sequences from different fish species was performed using the MEGAX software. Phylogenetic and molecular evolutionary studies were done on the basis of the deduced amino acid sequences of other species available on the public databases by using a neighbour-joining method with 1000 bootstrap replicates using Molecular Evolutionary Genetics Analysis software (MEGA version X).

Results

In silico representation and protein analysis of TLR4

Labeo rohita TLR4 gene is composed of 2729 nucleotides, with a coding region (CDS) of 2468 bp (Fig. 1a). This gene sequence of TLR4 encodes an 822 amino acid (aa) protein in *L. rohita* (AOM81178.1) (Fig. 1b).

The total amino acid sequence length of TLR4 proteins is 822, and the molecular weight is 94.75 kDa. The compound pI value of TLR4 protein was 8.6, more than 7 (pI>7) which indicates that TLR4 protein was considered as basic. The total number of negatively charged residues (Asp + Glu) and total number of positively charged residues (Arg + Lys) of TLR4 protein was 72 and 81. The instability index of TLR4 protein is 42.82. Aliphatic index of protein is 105.11, the aliphatic index is high for TLR4 protein sequence of *L. rohita*. Grand average of hydropathicity (GRAVY) indices of TLR4 protein was 0.052. This low range of value indicates the possibility of better interaction with water. All other physiochemical characterization of protein sequence of TLR4 analysis are given in Table 1.

The Secondary protein structure prediction and verification revealed that the alpha helix (Hh) (46.35%) was most dominant in the TLR4 protein of *L. rohita*. This was followed by random coil (Cc) 34.31% and extended strand (Ee) 16.79% (Fig. 2). Graphical representation of secondary structures (The extended strand, alpha helix, random coil and disorder,

etc.) by using PSIPRED is displayed in Fig. 3.

Three-dimensional structure analysis and validation

Four different templates were selected to generate the 3D structure of TLR4 protein of *L. rohita* (Table 2). 3D structure of proteins was determined by homology modelling, using Modeller 10.1. Visualization of the proteins model was done by Discovery Studio program. α-helix, β-sheets, loop, N-terminus and C-terminus region of TLR4 protein are shown in the (Fig. 4). A stereo chemical quality of the predicted models and accuracy of the protein model was evaluated after the refinement process using Ramachandran plot computed with the PROCHECK program. Ramachandran plot analysis revealed that amino acid residues 728 (96.84%) are in the highly preferred region, 24 residues (2.10%) lie in preferred observations region and 9 residues (1.05%) lie in the Questionable region (Table 3 and Fig. 5).

Docking studies

The drug-likeness properties of eight best compounds are showing in the table 4 which were obtained using the OSIRWAS property explorer. The binding energy of eight selected ligands ranges from – 8.0 to – 2.7 kcal/mol. Oxycodone ligand shared the highest binding energy of – 8.0 kcal/mol with TLR4 protein of L. rohita (Table 5). The binding site of Oxycodone ligand interaction with amino acid residues was at residues PRO564, LEU563, GLU699, ASN697, PHE604, CYS605, GLY701, VAL702, PRO703 and PRO704. Two-dimensional interaction of TLR4 with Oxycodone ligand is represented in (Fig. 6). The hydrogen bonds interaction of Oxycodone is represented in (Fig. 7).

Phylogenetic analysis and sequence alignment

Phylogenetic and molecular evolutionary analysis of *L. rohita* TLR4 protein revealed close resemblance to *T. putitora*, *C. auratus*, *S. anshuiensis*, *S rhinocerous*. The Phylogenetic analysis of TLR4 protein is represented in the Fig. 8, disclosed that all fish formed an independent clade while birds and mammals of TLR4 for another separate clade.

Discussions

In the present study we constructed a three-dimensional structure of the *L. rohita* TLR4 protein using homology modelling and then optimized the three-dimensional structure for docking studies by molecular modelling techniques. The optimized model was then docked with different drug targets and their ligand-receptor interaction sites and binding affinities were predicted. The TLR4 properties and results of docking studies are discussed.

In the current study we observed that the full-length of *L. rohita* TLR4 gene is 2729 bp with an open reading frame of 2468 bp. The molecular weight of TLR4 gene is 97.75 kDa and total length of the TLR4 protein is 822 aa. Lai *et al.*, (2016) observed *Megalobrama amblycephala* TLR4 gene comprised of 2862 bp, including a 146 bp 5′-untranslated region (UTR), an open reading frame (ORF) of 2364 bp encoding a protein of 787 amino acid residues, and a 352 bp 3′ - UTR. In the 3′ - UTR, an mRNA instability signal (ATTTA) and a typical polyadenylation signal (ATTAAA) were found. Han, *et al.*, (2018) [9] observed *Acipenser dabryanus* TLR4 cDNA with 2902 bp in length, including 83 bp of 5’ - UTR, 2493 bp of ORF encoding 830 a.a, and 326 bp of 3’ - UTR. *Acipenser dabryanus* TLR4 contained an in-frame stop codon upstream of the start codon (ATG) and a poly adenylation signal (AATAA motif) before the poly A tail, suggesting that the complete ORF of both genes had been obtained.

Length of TLR4 gene in humans is 11,467 bp, with the molecular weight of 95.68 kDa and total protein length of 839 AA. However, TLR4 gene in mouse is of 15,337 bp, with the total length of TLR4 protein is 835 AA (Smirnova *et al.*, 2000) [28].

The molecular weight of *L. rohita* TLR4 protein is 94.75 kDa. The calculated isoelectric point (pI) of 8.6 being more than 7.0 (pI > 7) indicate these proteins are considered alkaline. The number of negatively charged residues (Asp + Glu) and positively charged residues (Arg + Lys) of the TLR4 protein is 72 and 81. The predicted instability index of 42.82, indicates that it’s an unstable protein. The average value of hydrophilicity (GRAVY) was 0.052. This value range suggests bad interaction with water. The aliphatic index score was 105.11 which indicates that these proteins are stable over a wide temperature range.

Hamta *et al.*, (2020) [8], computed physical and chemical parameters of human TLR4 protein (839 amino acids) and found that its molecular weight is 95.68 kDa, instability index of 43.05, aliphatic index of 101.86, GRAVY of 0.033 and theoretical pI of 5.88. Lai *et al.*, (2016), studied physicochemical properties of *Megalobrama amblycephala* TLR4 and stated that the molecular mass is 90.22 kDa and the predicted isoelectric point is 8.50. Han *et al.*, (2018) observed Dabry’s sturgeon (*Acipenser dabryanus*) TLR4 protein has an isoelectric point (pI): 6.65, extinction coefficient: 75075, instability index: 40.78 (indicating being unstable), aliphatic index: 106.42 (indicating being thermostable proteins) and GRAVY: 0.021 (indicating to have high affinity to water).

Majority of our current findings align with the previous observations of Hamta *et al.*, (2020) [8] and Han *et al.*, (2018). However, the current isoelectric point (pI) of 8.6 varied with respect to the findings of Hamta *et al.*, (2020) [8], (5.88) and (6.65), This indicates that the Human TLR4 and Dabry’s sturgeon TLR4 holds pI value of less than 7, while *L. rohita* TLR4 of 8.6, (pI value ranges from 2-14), meaning the human and Dabry’s sturgeon contains more positive charge (H+) and less negative charge (OH–) than L. rohita TLR4.

In present study, homology modelling approach was used to predict the three-dimensional structure of TLR4 protein. This approach uses predetermined template structures to predict the 3D structure of the protein. The three-dimensional structure of TLR4 was validated using the Ramachandran plot (Procheck server), which showed that 728 amino acid residues (96.84%) were in the highly preferred region, 24 residues (2.10%) were in preferred observations region and 9 residues (1.05%) were in questionable region.

Protein model containing more than 90% of the residues in the highly preferred regions suggesting that they may be high quality models (Laskowski *et al.*, 1993) [21]. However, Kubarenko *et al.*, (2010) [19], validated the three-dimensional structure of human TLR4 with Ramachandran plot and found 78.8% were in most favoured region, 20.4% in additionally allowed region, 0.6% in generously allowed region and 0.2% in disallowed region. Kharor *et al.*, (2018) [16] validated three-dimensional structure of human TLR4 and mouse TLR4 with Ramachandran plot and found that, in human TLR4, 81.2% residues were in the most favoured regions, 18.1% residues were in additional allowed regions, 0.5% in generously

allowed region and 0.2% in disallowed regions and in mouse TLR4 77.8% residue in the most favoured region, 20.9% residue in the allowed region, 0.7% residue in the generally allowed region and 0.5% residue in the disallowed region.

The quality of generated structure and the alignment of the outcomes with the previous observation's states that the generated 3D structure of *L. rohita* TLR4 is of good quality and fit for further docking studies.

In this study, different opioids-based agonist ligands were docked against the *L. rohita* TLR4 protein. TLR4 protein of *L. rohita* showed best binding affinity (– 8.0) with oxycodone ligand. Oxycodone is an opioid drug mainly used as controlled-release tablets for chronic pain. The immediate-release solution and tablets are used for acute pain or for breakthrough pain (Kalso *et al.*, 2005) [14]. Opioid-based based agonist ligand binds and activates the human TLR4, this then increases levels of proinflammatory cytokines and chemokines to modulate innate immunity through TNF-α kinase pathway resulting in pain (Kodukula *et al.*, 2018). House mouse and rat TLR4 show similar activity when bound with opioid based agonist ligands (Eisenstein, 2019[6]; Hutchinson, 2012) [12]. The fishes which inherit TLR4 have no well-defined role as costimulatory molecules essential for its activation are absent from all fish genomes (Iliev *et al.*, 2005 [13]; Swain *et al.*, 2008; Pietretti *et al.*, 2013) [24].

Although, the role of TLR4 in fish species is not clear but the interaction of *L. rohita* TLR4 with opioid drugs, aligns with the findings of the Eisenstein (2019) [6] and Hutchinson (2012[12] who stated that the opioid drug binds and activates the TLR4 gene of humans, mouse and rat. Thus, an in vivo research using oxycodone can help in understanding the role of *L. rohita* TLR4 and similarity of its effects with human, house mouse and rat TLR4 (Kodukula *et al.*, 2018; Eisenstein, 2019 [6]; Hutchinson, 2012) [12], this will also help to define the role of TLR4 in fish in journal.

Previously analysed the binding affinities of TLR4/MD2 ligands in humans. The docking scores for VS1 and 4-methoxybenzyl were –15.1 kcal/mol and –12.5 kcal/mol.

In current work, multiple sequence alignments between TLR4 protein sequences from different species showed high amino acid sequence similarities between the TLR4 proteins of 13 species in all based on the best match scores. Comparisons revealed the *L. rohita* TLR4 protein to be more closely related with that of *Tor putitora* followed by *Carassius auratus*, *Sinocyclocheilus spp* and *Megalobrama amblycephala* (Fig. 8). One more thing that became evident from the figure was that the outgroup comprising of humans (*Homo sapiens*), the House mouse (*Mus musculus*), and the Red junglefowl (*Gallus gallus*) segregated themselves into a separate cluster and that the cluster was situated farthest away from the remainder 10 fish species revealed multiple sequence alignments based on amino acid sequences of Dabry's sturgeon TLR4 had a single copy of TLR4 in sturgeon and spotted gar, forming a clade supported by a bootstrap value of 100 in the phylogenetic tree. The TLR4 appeared to have duplicated independently in Cyprinidae and Ictaluridae as the two catfish TLR4s sat tightly together in the tree.

Fig 1a: Nucleotide sequence of TLR4 gene (KX21824.1) of *Labeo rohita*

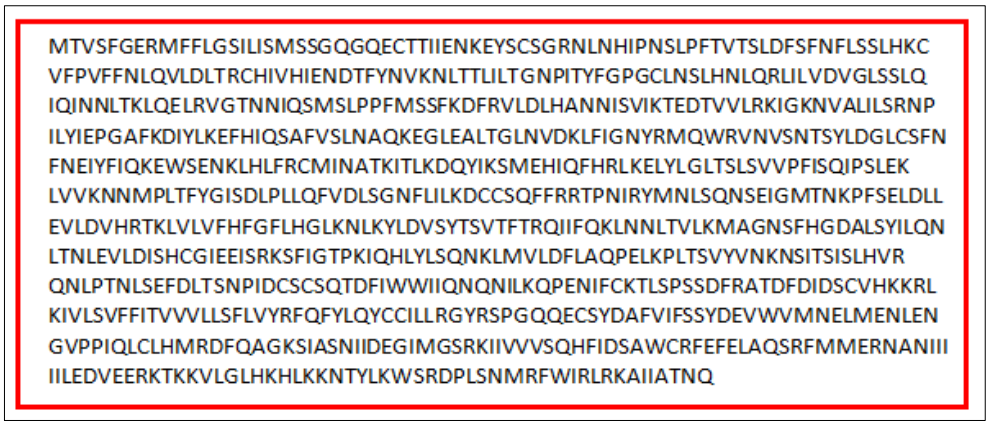

Fig 1b: Protein sequence of *Labeo rohita* TLR4 (AOM81178.1)

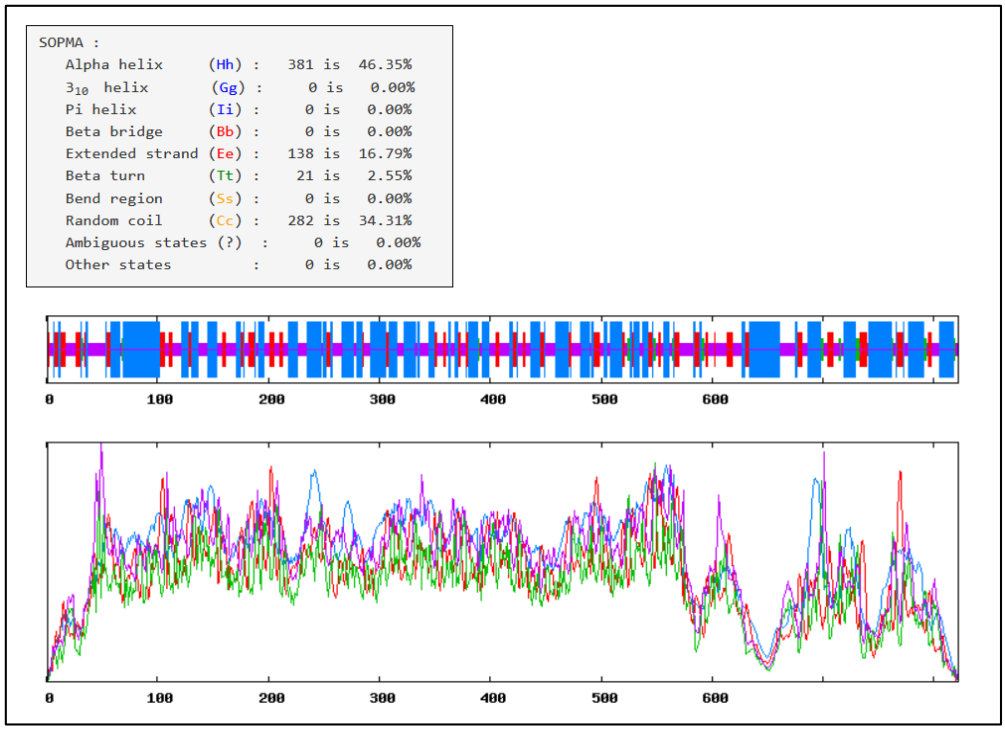

Fig 2: Secondary structure of TLR4 protein

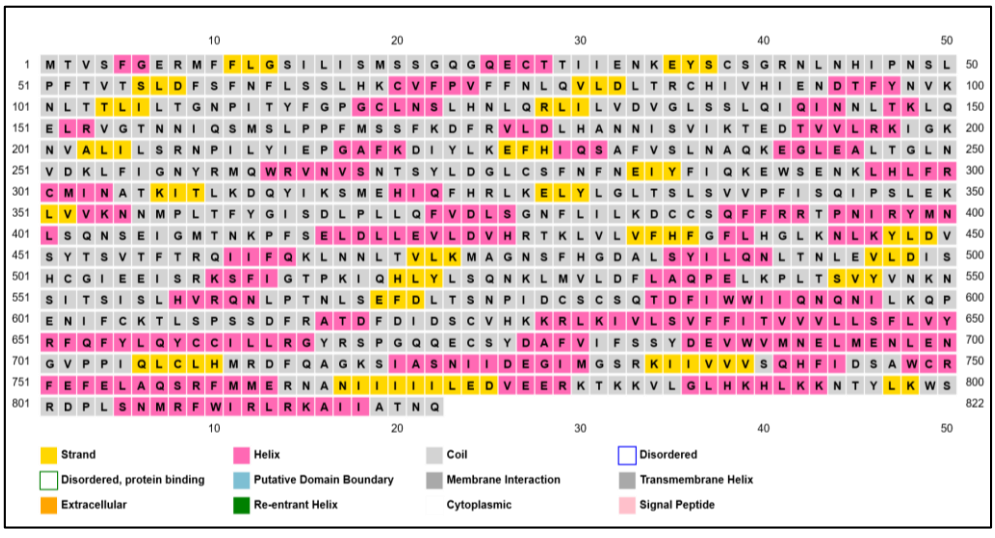

Fig 3: Amino acids which comprise strand, helix, random coil and disorder, etc. of TLR4

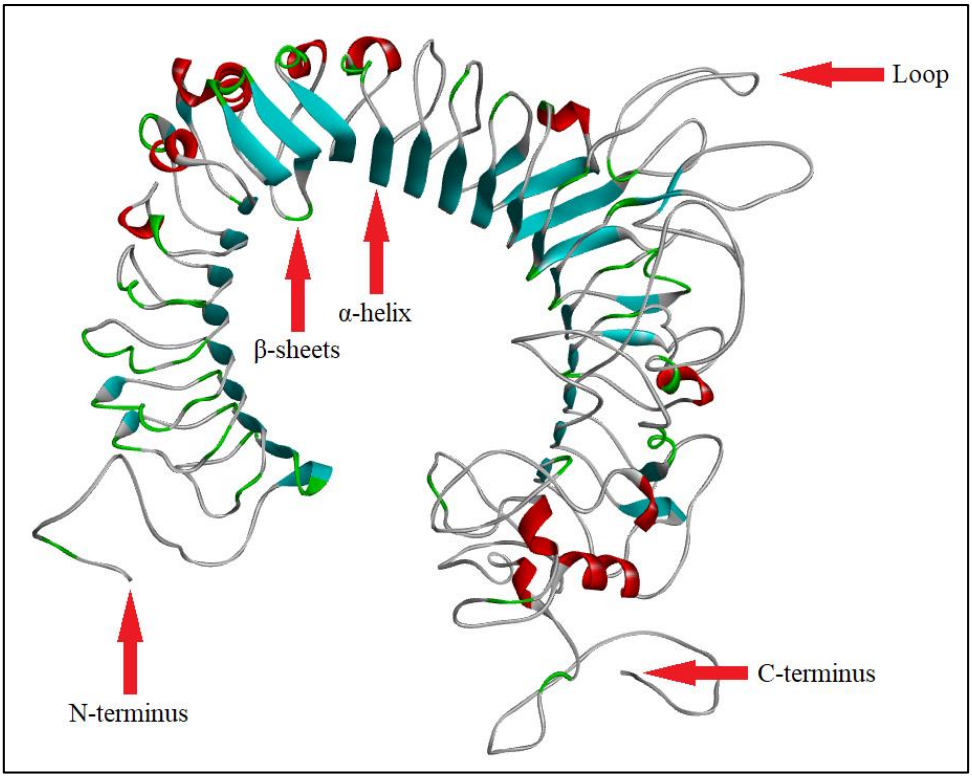

Fig 4: 3D structure of TLR4 protein of *Labeo rohita*

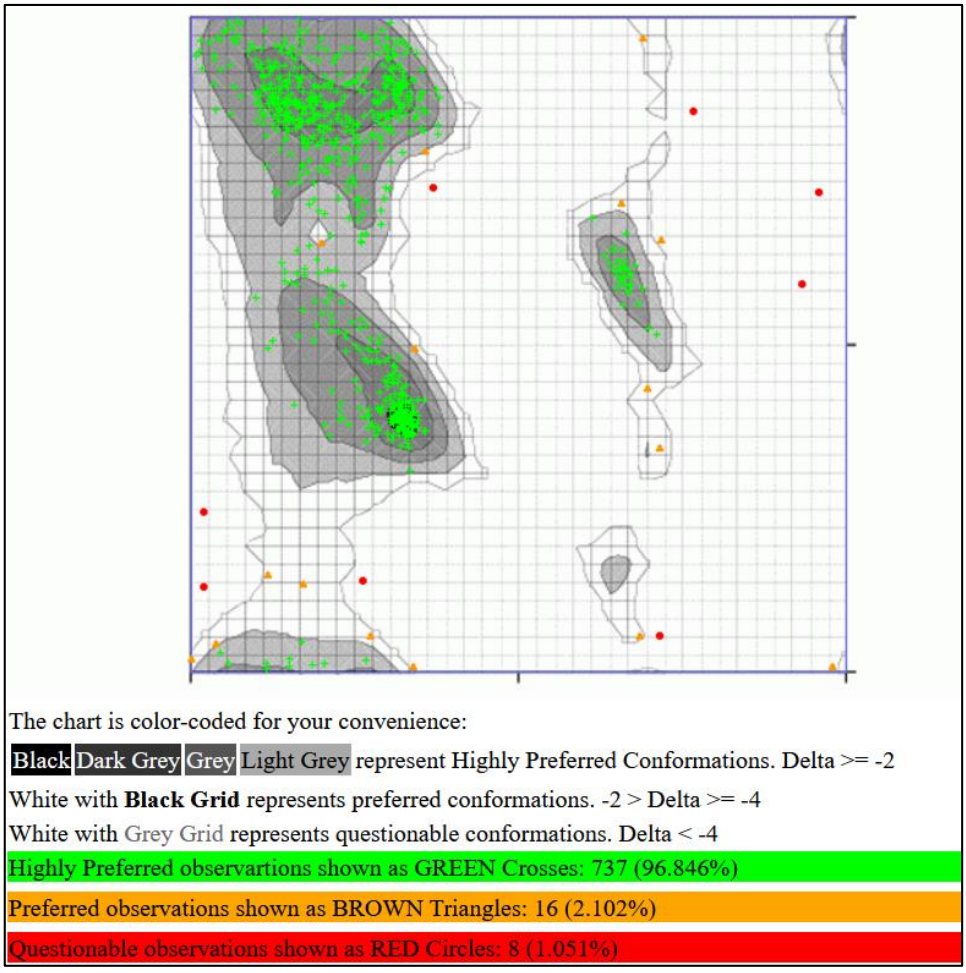

Fig 5: Ramachandran Plot of TLR4 amino acid residues showing residues in favoured region and allowed region

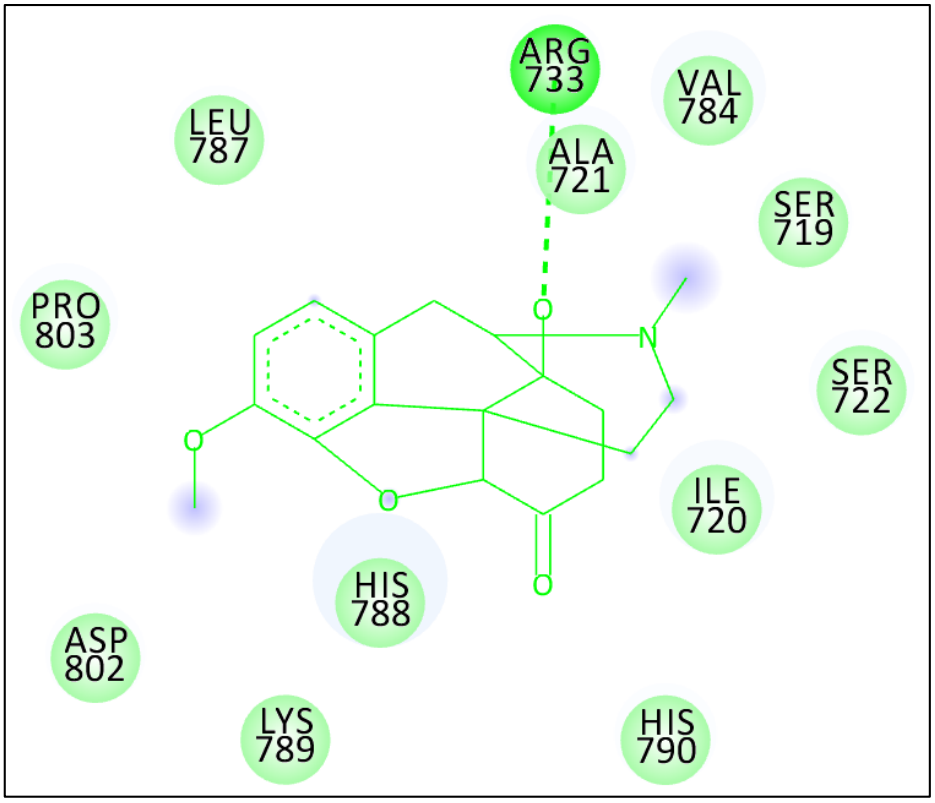

Fig 6: Two-dimensional structure of TLR4 protein and Oxycodone ligand interaction with active site

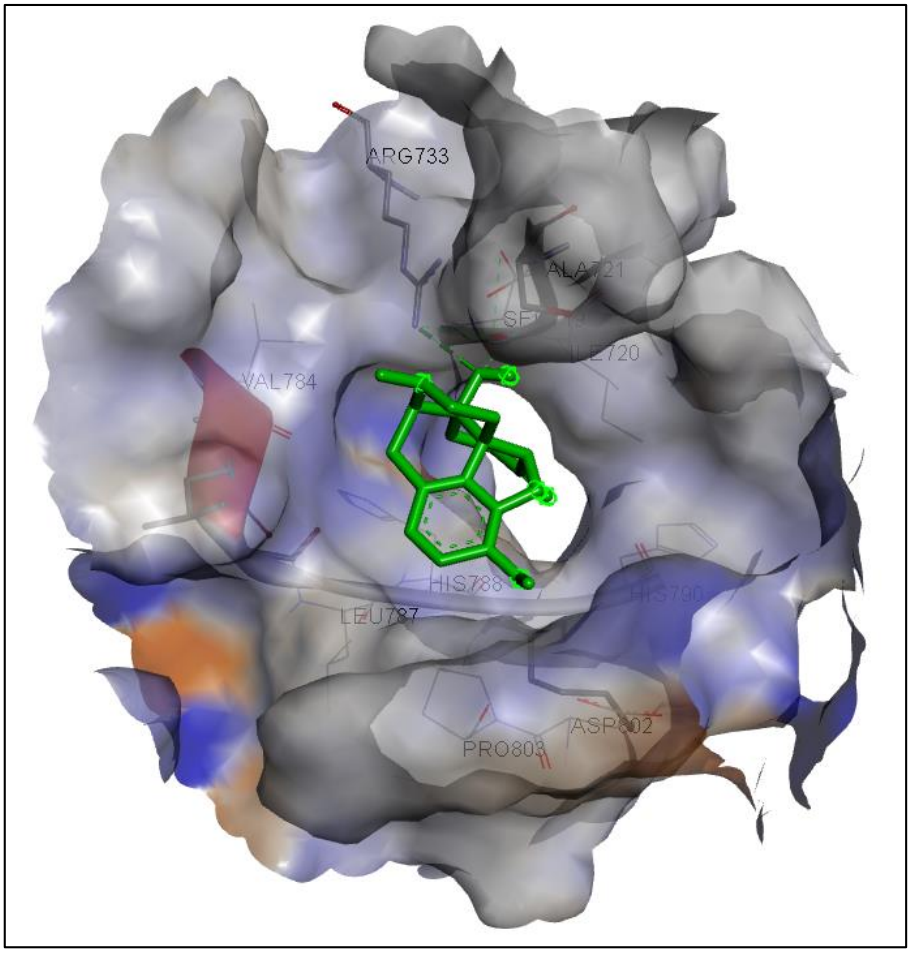

Fig 7: Hydrogen bond interaction of TLR4 and Oxycodone ligand

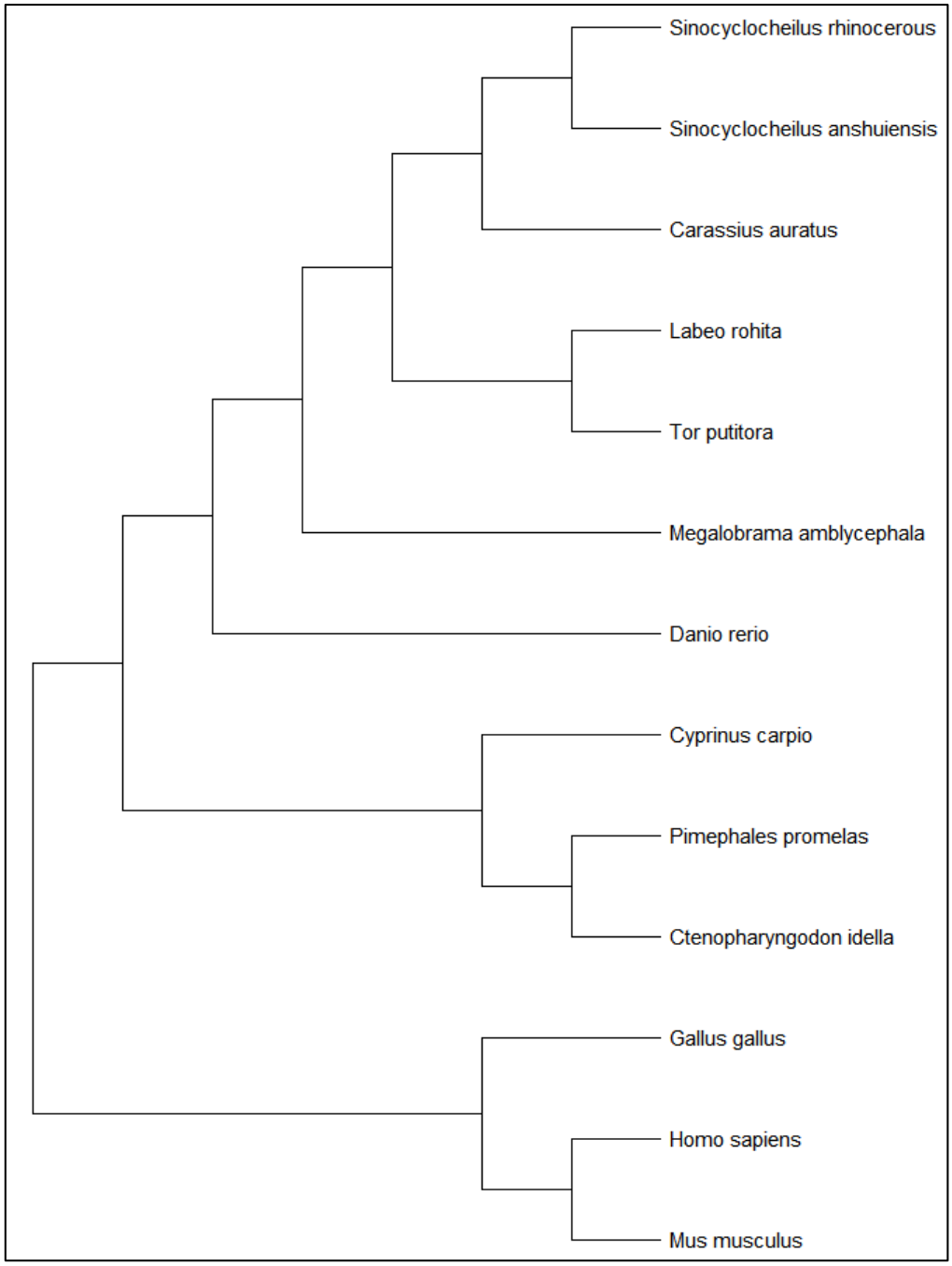

Fig 8: Phylogenetic tree of *L. rohita* TLR4 protein in comparison with other vertebrates

Table 1: Physicochemical properties of TLR4 protein of *Labeo rohita* (https://web.expasy.org/cgi-bin/protparam/protparam)

Sr. No.	Parameter	Value
1	Number of amino acids	822
2	Molecular weight	94753.21
3	Theoretical pI	8.6
4	Total Number of negatively charged residues (Asp + Glu)	72
5	Total Number of positively charged residues (Arg + Lys)	81
6	Total number of atoms	13432
7	Estimated half-life	30 hours (mammalian reticulocytes, in vitro)
8	Instability index (II)	42.82
9	Aliphatic index	105.11
10	Grand average of hydropathicity (GRAVY)	0.052

Table 2: Selected TLR4 protein templates used in developing 3D structure of *Labeo rohita* TLR4

Sr. No.	Protein ID	Organism	Query Cover (%)	Percent Identity (%)
1	3FXI	*Homo sapiens*	71	33.96
2	4G8A	*Homo sapiens*	71	33.96
3	3VQ1	*Mus musculus*	73	32.51
4	2Z64	*Mus musculus*	72	32.61

Table 3: Structure validation of developed TLR4 using Ramachandran Plot Server

Regions	Amino Acid Residues	Amino Acid Residues (%)
Highly Preferred	728	96.84
Preferred	24	2.10
Questionable	9	1.05

Conclusion

Labeo rohita is one of the top produced freshwater aquaculture species found natively in India. TLR4 is found present in the *Labeo rohita* genome. But its functions are not completely studied so far. Protein structure analysis and docking studies would help in understanding the proper functioning of the TLR4 in *L. rohita*.

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Disclosure statement

No potential conflict of interest was reported by the author (s).

References

1. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124:783-801.
2. Amin K, Anwar M, Kamel M, Kassem E, Syam Y, Elseginy S. Synthesis, cytotoxic evaluation and molecular docking study of novel quinazoline derivatives as PARP-1 inhibitors. Acta Pol Pharm. 2013;70(5):833-849.
3. De Ruyck J, Brysbaert G, Blossey R, Lensink M. Molecular docking as a popular tool in drug design, an *In silico* travel. Adv. Appl. Bioinform. Chem. 2016;9:1-11.
4. Deng H, Jai Y, Zhang Y. Protein structure prediction. Int. J Mod Phys B. 2018;32(18).
5. Dong M, Chen H, Ren Y, Shao F. Molecular modeling studies, synthesis and biological evaluation of dabigatran analogues as thrombin inhibitors. Bioorg Med Chem. 2016;24:73-84.
6. Eisenstein T. The role of opioid receptors in immune system function. Front Immunol. 2019;10:2904.
7. Garay-Malpartida H, Mourao R, Mantovani M, Santos I, Sogayar M, Goldberg A. Toll-like receptor 4 (TLR4) expression in human and murine pancreatic beta-cells affects cell viability and insulin homeostasis. BMC Immunol. 2011;12:18.
8. Hamta A, Komijani M, Hezaveh S. Prevalence of human herpes virus 8 infection and pattern of TLR4 rs4986791 polymorphism in women with breast cancer. Jentashapir J Cell Mol Biol. 2020;11(4):e109192.
9. Han P, Wang S, Zhang Q, Zhang S, Shao R, Xu W, *et al.* Molecular characterization and expression analysis of TLR1 and TLR4 from the endangered fish Dabry's sturgeon (*Acipenser dabryanus*). Dev Comp Immunol. 2018;86:180-188.
10. Honegr J, Malinak D, Dolezal R, Soukup O, Benkova M, Hroch L, *et al.* Rational design of novel TLR4 ligands by *In silico* screening and their functional and structural characterization in vitro. Eur J Med Chem. 2017;146:38-46.
11. Huang R, Dong F, Jang S, Liao L, Zhu Z, Wang Y. Isolation and analysis of a novel grass carp toll-like receptor 4 (tlr4) gene cluster involved in the response to grass carp reovirus. Dev Comp Immunol. 2012;38:383-388.
12. Hutchinson M, Northcutt A, Hiranita T, Wang X, Lewis S, Thomson J, *et al.* Opioid activation of toll-like receptor 4 contributes to drug reinforcement. J Neurosci. 2012.
13. Iliev D, Roach J, Mackenzie S, Planas J, Goetz F. Endotoxin recognition: In fish or not in fish? FEBS Lett. 2005;579:6519-6528.
14. Kalso E. Oxycodone. J Pain Symptom Manage. 2005;29:S47-S56.
15. Kawasaki T, Kawai T. Toll-like receptor signaling pathways. Front Immunol. 2014;5:461.
16. Kharor N, Bansal P, Kumar R, Singh J. *In silico* structure analysis of type 2 diabetes associated toll-like receptor-4 (TLR4). J Adv Scholarly Res Allied Educ. 2018;15(7):130-137.
17. Kodukula S, Zeng S. Signal crosstalk between TLR4 and opioid receptor pathways. Transl Perioper Pain Med. 2018;5(1):27-32.
18. Javed Khan, Fawad Ali. Effect of pituitary gland extract (PGE) and ovaprim on induced breeding in Rohu (*Labeo rohita*) and grass carp (*Ctenopharyngodon idella*). Int. J Biol. Sci. 2021;3(2):12-16. DOI: 10.33545/26649926.2021.v3.i2a.30
19. Kubarenko A, Ranjan S, Colak E, George J, Frank M, Weber A. Comprehensive modelling and functional analysis of Toll-like receptor ligand-recognition domains. Protein Sci. 2010;19:558-569.
20. Lai R, Liu H, Jakovlic I, Zhan F, Wei J, Yang P, *et al.* Molecular cloning and expression of toll-like receptor 4 (tlr4) in the blunt snout bream (*Megalobrama amblycephala*). Dev Comp Immunol. 2016;59:63-76.
21. Laskowski R, MacArthur M, Moss D, Thorton J. PROCHECK: A program to check the stereochemical quality of protein structures. J Appl. Crystallogr.

1993;26:283-291.
22. Mena-Ulecia K, Tiznado W, Caballero J. Study of the differential activity of thrombin inhibitors using docking, QSAR, molecular dynamics, and MM-GBSA. PLoS ONE. 2015;10(11):e0142774.
23. Molteni M, Gemma S, Rossetti C. The role of toll-like receptor 4 in infectious and non-infectious inflammation. Mediators Inflamm. 2016;2016:6978936.
24. Pietretti D, Forlenza M, Fink I, Wiegertjes G. A closer look at Toll-like receptor 4 (TLR4) and toll-like receptor 20 (TLR20) of common carp (*Cyprinus carpio*). Fish Shellfish Immunol. 2013;34:1635-1691.
25. Quiniou S, Boudinot P, Bengten E. Comprehensive survey and genomic characterization of Toll-like receptors (TLRs) in channel catfish, *Ictalurus punctatus*: identification of novel fish TLRs. Immunogenetics. 2013;65:511-530.
26. Samanta M, Basu M, Swain B, Paichha M, Lenka S, Das S, *et al.* Molecular cloning and characterization of LrTLR4, analysis of its inductive expression and associated down-stream signalling molecules following lipopolysaccharide stimulation and Gram-negative bacterial infection. Fish Shellfish Immunol. 2016;60:164-176.
27. Sabbah D, Saada M, Khalaf R, Bardaweel S, Sweidan K, Al-Qirim D, *et al*. Molecular modeling based approach, synthesis, and cytotoxic activity of novel benzoin derivatives targeting phosphoinostide 3-kinase (PI3Kα). Bioorg Med Chem Lett. 2015;25:3120-3124.
28. Smirnova I, Poltorak A, Chan E, McBride C, Beutler B. Phylogenetic variation and polymorphism at the Toll-like receptor 4 locus (TLR4). Genome Biol. 2000;1(1):research002.1-002.10.
29. Swain P, Nayak S, Nanda P, Dash S. Biological effects of bacterial lipopolysaccharide (Endotoxin) in fish: A review. Fish Shellfish Immunol. 2008;25:191-201.
30. Tang L, Xiang X, Jiang Y, Lv Y, Zhou Y, Zhong H, *et al.* Identification and characterization of a novel Toll-like receptor 4 homologue in blunt snout bream, *Megalobrama amblycephala*. Fish Shellfish Immunol. 2016;57:25-34.
31. Kongchum P, Palti Y, Hallerman E, Hulata G, David L. SNP discovery and development of genetic markers for mapping innate immune response genes in common carp (*Cyprinus carpio*). Fish Shellfish Immunol. 2010;29:356-361.