In vitro and *in silico* analysis proving DPP4 inhibition and diabetes associated gene network modulation by a polyherbal formulation – *Nisakathakadi Kashaya*.

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Abstract

Frontiers of disease biology started recognizing the importance of systems and network medicine approach for managing chronic disease like diabetes. Polyherbal preparations like *Ayurveda* formulations are known to exert a multicomponent-multitargeted mode of action that makes them an ideal tool for delineating new biological insights into this systemic mechanism of disease manifestation and management. Additionally, these formulations are rich repository for identifying novel ligands that interact with drug targets having systemic effects. The current study aims at identifying DPP4 inhibitory potential and modulation of diabetes associated gene network by a clinically established Ayurvedic anti-diabetic formulation *Nisakathakadi Kashaya* (NK) using *in vitro* and *in silico* methods. DPP4 inhibitory potential of NK was evaluated by standard enzyme inhibition assay. Bioinformatics and computational biology tools were used to identify the potential bioactives responsible for the DPP4 inhibitory activity of NK. Molecular docking and dynamics studies of the identified compounds provided insights about the molecular interactions involved in DPP4 inhibition. Target mapping of phytochemicals using network pharmacology tools *viz.* STITCH, CHEMBL and BindingDB databases was used to depict the multi-targeted interaction of the formulation and a sub network for diabetes related genes and their

relationship with other diabetes associated comorbidities were depicted with the help of EnrichR. NK demonstrated a dose dependent DPP4 inhibition with an IC_{50} of 2.06 μg GAE/mL. Further, the *in silico* studies identified three compounds namely Terchebin, Locaracemoside B and 1,2,4,6 Tetra o Galloyl Beta D Glucose showing stable interactions with DPP4 when compared to the standard drug Vildagliptin. Network pharmacology studies with the phytochemicals identified from NK revealed a number of genes like TNF, TGFβ, SOD1, SOD2, AKT1, DPP4 and GLP1R in its protein-protein interaction network which are vital to diabetic progression and complications. This suggests that a polyherbal formulation like NK can exert its action through various phytomolecules present. The present work demonstrated that the polyherbal formulation NK has DPP4 inhibition potential and modulates a large number of diabetes related genes and pathways. The approach adopted in the current study by combining *in vitro* and *in silico* methods allowed us to understand the mechanism of DPP4 inhibition by the formulation and also the possible pharmacological networking through which the formulation modulate diverse disease related targets.

1. Introduction

Dipeptidyl-peptidase IV (DPP4) inhibitors have emerged as an important class of anti-diabetic medication that provide better glycemic control with less adverse effects owing to their role in modulating incretin physiology. One of the major substrates of DPP4 is Glucagon Like Peptide - 1 (GLP1), an important incretin hormone secreted by the enteroendocrine cells, known for its multi-organ interactions to modulate the body's glucose homeostasis (Brubaker and Drucker, 2004). The GLP1 mediated incretin effects are highly compromised in type 2 diabetes primarily due to its degradation by the serine protease action of DPP4 and therefore DPP4 inhibitors, that can prolong GLP1 action in the body, are shown to significantly improve glucose homeostasis without posing any risk of hypoglycemia or weight gain (Holst et al., 2011; Thornberry and Gallwitz, 2009; Balkan et al., 1999). Five such DPP4 inhibitors namely Sitagliptin (Januvia, Merck), Vildagliptin (Glavus, Novartis), Saxagliptin (Onglyza, Bristol-Myers Squibb), Alogliptin (Nesina, Takeda), and Linagliptin (Tradjenta, Boehringer-Ingelheim) are already in active clinical use and more including Gemigliptin, Teneligliptin, etc. are in the clinical trial stage as well (Scheen, 2012; Andukuri et al., 2009; Sharma et al., 2016). However, the marketed DPP4 inhibitors are reported to have several adverse effects and are relatively expensive. This spurs scientists to look for more affordable and safer alternatives from various alternative sources. Additionally, the incretin hormone biology and DPP4 actions are crucial players of the gut centred systemic

glucose metabolism of the body (broadly referred as gastrointestinal mediated glucose disposal - GIGD) and therefore targeting DPP4 and incretins become an important strategy for developing more holistic and multi-targeted drug interventions (Thottapillil et al., 2021). Such a systemic disease management approach is imperative for long term management of chronic lifestyle diseases like diabetes and diabetes-associated comorbidities.

Ayurveda formulations, by virtue of their multicomponent herbal ingredients, form a rich repository of bioactive molecules and serves as a source for screening potential drug candidates (Patwardhan et al., 2004). Embracing the *Ayurveda* concept of '*Agni*' (unique concept that describes the biology of metabolism) as well as its gut centric perspective of managing metabolic diseases further enhances the scope of screening *Ayurveda* formulations for identifying potential drug candidates that inhibit DPP4 and thereby modulate incretin biology. In this background, the present study attempts to investigate the DPP4 inhibitory effect of an Ayurvedic polyherbal formulation *Nisakathakadi Kashaya* (NK), prescribed for the clinical management of diabetes and its comorbidities, using *in-vitro* assay. The study also illustrates the molecular interactions of key bioactives from NK with DPP4 protein using docking and molecular dynamic (MD) simulation tools.

At the frontiers of disease biology, systems and network medicine concepts have begun to redefine the 'one disease-one target-one drug' dogma prevalent in current biology (Nogales et al., 2022). Emerging insights into the mechanism of action of polyherbal formulations also suggests the possibility of herbal formulations (including Ayurvedic formulations) exerting their overall biological effects through a multitarget and multi-pathway crosstalk (Zhang et al., 2021). The Ayurvedic formulation, NK, selected in this study is a combination of eight herbal ingredients with known hypoglycemic effects (Table-1). A variety of phytochemicals identified from these ingredient plants are known for their multitargeted mode of action. Although in our study the formulation is shown to inhibit DPP4, we hypothesized that the diverse number of bioactives present in the formulation can interact with their putative gene targets to exert a network pharmacology mode of action in the human body (Patwardhan and Chandran, 2015). This prompted us to further investigate the spectrum of diabetes related genes that are modulated by the formulation. Network pharmacology and bioinformatics tools allowed us to curate and capture the physiologically relevant signalling interactome of multi-component formulation and the pharmacological networking of phytochemicals was deduced via the openly available software tools. A disease-gene overlap analysis performed in the study further provided information on the possible molecular basis for the

polypharmacological action of NK in diabetes, diabetic complications and diabetes associated comorbidities.

2. Materials and Methods

2.1.Reagents and NK quantification.

Nisakathakadi kashaya (NK) was procured from a leading *Ayurveda* drug manufacturer and the details about the product were recorded in the Sample Data Sheet (SDS) book maintained in the University. The DPP4 enzyme assay kit was procured from Enzo Life Sciences (Cat. No: BML-AK499-0001). In order to quantitatively use the formulation for DPP4 inhibition assay, total tannins present in the NK formulation was estimated using standard Folin - Ciocalteu method using gallic acid as standard. Briefly, 10 µL of the formulation mixed with 40 µL of water, 50 µL Folin's reagent and 100 µL of 3.5% Na_2CO_3 was incubated at room temperature for 30 min. A set of gallic acid standards (50, 25, 12.5, 6.25, 3.125 µg/mL) were prepared in the same manner. The absorbance was measured at 700 nm using a multi-well plate reader (xMark Microplate Spectrophotometer, BioRad, USA). The experimental concentrations of test samples were expressed as 'µg of gallic acid equivalent tannin (GAE)/mL of sample'

DPP4 inhibition assay

The DPP4 inhibition assay was carried out as per the kit instructions. Briefly, various concentrations of NK were prepared with the assay buffer. The test samples, positive control and the standard inhibitor (given in the kit) were incubated with DPP4 enzyme for 20 minutes at room temperature. The fluorogenic substrate provided with the kit was added and the plate was read for 30 mins using a fluorometer (Biotek Synergy H1 microplate reader) at Ex:380/EM:460 nm**.**

The percentage remaining activity in the presence of inhibitor was calculated using the formula below

% activity remaining (with inhibitor)= (slope of inhibitor sample/control slope) x 100

2.3.Phytochemical data collection

The phytochemicals present in the eight medicinal plants were extracted from two publicly available databases *viz.* Dr Duke's Phytochemical and Ethnobotanical Database (https://phytochem.nal.usda.gov) and IMPPAT - Indian Medicinal Plants, Phytochemistry And Therapeutics (https://cb.imsc.res.in/imppat/home) (Mohanraj et al., 2018).

Phytochemicals reported from the respective parts used in the formulation were shortlisted and considered for further studies. The detailed chemical information about the shortlisted phytochemicals were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and used for molecular docking and other bioinformatics studies.

2.4.Preparation of DPP4 structure for docking

The three-dimensional (3D) structure of DPP4 interacted with Vildagliptin (PDB ID: 6B1E) was retrieved from Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB; http://www.rcsb.org). The protein was then prepared using the *protein preparation wizard* module available in the Schrodinger suite (Schrödinger, LLC, Newyork, United States, 2020). For acceptable ionisation states, h-bonds corresponding to pH-7 were provided to both basic and acidic amino acid residues followed by energy minimization of the protein structure using the OPLS3e force field. The ligand-binding site of the DPP4 protein was identified from a detailed study of literature. CASTp server was used to validate the binding sites that were found in the literature. The prepared protein generated from the Protein preparation wizard was considered to generate the grid box for the binding of the ligand to the protein molecule. This step was carried out using *the Receptor Grid Generation* module available in Schrodinger maestro Suite. Active site residues of the DPP4 protein (consisting of residues Glu205, Glu206, Asp708, His740, Ser630, Arg125, Ser209, Phe357, Tyr547, Tyr631, Ile651, Trp659, Tyr662, Tyr666, Arg669 and Val711) was selected to define the size of the Grid box. All catalytic active site residues (such as Glu205, Glu206, Asp708, His740 and Ser630) and substrate binding site residues were personally verified and confirmed to be correctly restricted within the rectangular grid box.

2.5.Preparation of Ligands

A total of 206 compounds from NK have been shortlisted for the virtual screening of ligands interacting with DPP4. The 3D structures of all compounds in Spatial Data File (SDF) format were downloaded from the PubChem database. *The LigPrep* module of Schrodinger (LigPrep, Schrödinger, LLC, New York, 2021) was used to prepare all the 206 ligands at a pH of 7.0 ± 0.1, and partial atomic charges were applied and ionisation states were developed. All compounds were minimised using OPLS 2005 force field and each ligand was

subjected to energy minimization until it passed an RMSD (root mean square deviation) limit of 0.001.

2.6.Molecular Docking

The molecular docking of the 206 compounds of NK with DPP4 was done using the XP (extra precision) docking mode of the GLIDE program with default parameters (Glide, Schrödinger, LLC, New York, 2021) (Friesner et al., 2006). In this process, ligand molecules were treated as flexible and the receptor is considered as rigid entity to attain the most significant interaction with binding site residues of DPP4. Then, using the Prime-MM-GBSA module of the Schrodinger Maestro suite, the binding free energy calculations were performed. Based on the XP-docking score, GLIDE score, binding free energy and interaction with the active site residues, top 3 ranked complexes and one protein-known inhibitor (Vildagliptin) complex were considered for further assessment.

2.7.Molecular Dynamics (MD) Simulation:

The top three bioactive molecules and one known inhibitor (Vildagliptin) complexed with DPP4 protein were subjected to 50ns simulation production run using Desmond (v5.6) package (Bowers et al., 2006). For each protein-ligand complex, the TIP4P water model was used as a solvation medium and the periodic boundary conditions were set to orthorhombic with the size of 10 × 10 × 10 Å. The necessary number of ions was adjusted to neutralise the system and the salt concentration was maintained at 0.01M. To equally distribute the ions and solvent around the protein-ligand complex, each system was equilibrated using the NPT (respectively number of the particle, system pressure and temperature) with a constant temperature of 300K. Using the relaxation model, the simulation production run was carried out for 50ns for all four complexes. Using Simulation Interaction Diagram and Simulation Event Analysis modules of Schrodinger suite, RMSD of the protein backbone, RMSF of individual amino acids and atoms of ligand, RoG of the ligand, SASA of the ligand, intra H-bond calculation of the ligand, etc. were calculated from MD simulation trajectory. The simulation interaction diagram was used to study the interaction details of protein and ligands during the simulation. The thermal_mmgbsa.py script was used to calculate the average binding free energy from the simulation trajectory based on MM-GBSA using a step size of 10.

2.8.Target mapping of phytochemicals using network pharmacology tools.

The potential target genes of the phytochemicals were obtained from three database sources *viz*. ChEMBL (https://www.ebi.ac.uk/chembl), STITCH (http://stitch.embl.de/) and BindingDB (https://www.bindingdb.org) (Gaulton et al., 2012; Kuhn et al., 2008; Liu et al., 2007). The putative targets with active interaction sources from Experiments and Databases and with the minimum confidence score of 0.400 was used during the STITCH search. The SMILES notation of the compounds were used in BindingDB and the conversion from CID to SMILES was done using the PubChem identifier exchanger (https://pubchem.ncbi.nlm.nih.gov/idexchange/idexchange.cgi). A similarity cut-off of 0.9 was used during the BindingDB search.

2.9.Network construction and disease associated gene identification

A phytochemical target gene network was obtained using Cytoscape_v3.9.0 (Paul Shannon et al., 1971). Every chemical with its representative targets is arranged and files are loaded in Cytoscape. The hub genes and hub compounds were identified from the network using the Cytoscape plugin Cytohubba (Chin et al., 2014). It provides 11 topological analysis methods, out of which MCC is used which captures more essential proteins in the top-ranked list in both high degrees as well as low degrees. MCC indicates that every node is connected to every other node in that subgraph.

2.10. EnrichR analysis for identifying gene-disease overlap.

The reported phytochemical targets from the databases were corrected for duplicate entries and processed for gene disease overlap using the publicly available tool DigiNet (http://www.disgenet.org/web/DisGeNET/), OMIM, and ClinVAR databases containing information about relationships between human/animal genes and diseases that was accumulated from various sources mainly through text-mining approaches.

The KEGG pathway database and Cluego Plugin in cytoscape was used for understanding the biological pathways regulated by the potential network constructed for the formulation(Bindea et al., 2009;Kanehisa et al., 2017). A Venn diagram analysis was done for common targets between diabetic complications and associated metabolic diseases.

3. Results

3.1. NK inhibits DPP4 dose dependently in vitro

NK formulation showed a strong dose dependent inhibition of DPP4 with a maximum of 97% inhibition at 25µg of GAE/mL and a minimum of 64% at the lowest concentration of 3.125µg of GAE/mL concentration tested in the study with an IC50 of 2.09 µg GAE/mL (Fig-1).

3.2. Molecular Docking studies showed key phytochemicals identified in NK directly interacting and inhibiting DPP4 activity.

There are 206 phytochemicals identified from eight medicinal plants present in NK. In order to identify the phytoconstituents of NK responsible for DPP4 inhibition, a virtual screening of all the 206 compounds were performed by docking them to DPP4 protein. Well known DPP4 inhibitor Vildagliptin was used as a positive control. The top-ranked 35 compounds, based on their docking score, GLIDE score, and MM-GBSA are listed in Table-2. The docking and MM/GBSA scores for Vildagliptin were -3.899 kcal/mol and -26.7 kcal/mol, respectively. The compounds listed in Table 2 showed a better Glide score compared to that of Vildagliptin. The interaction details of the top 3 compounds from the table *viz.* Terchebin, Locoracemoside B and 1,2,4,6 Tetra o Galloyl Beta D Glucose (TGBG) having G binding energy -47.12 kcal/mol, -45.45kcal/mol, and -60.73 kcal/mol, respectively, are reported in the study along with Vildagliptin as a control. DPP4 amino acid residues Glu206, Tyr666 and Asn710 that are involved in Vildagliptin-DPP4 interaction are found to interact with the top 3 compounds identified from the virtual screening (Fig-2).

3.3. MD simulation analysis of DPP4 ligand complex

The top three compounds selected from the docking study, Terchebin, Locoracemoside B, and TGBG as well as Vildaglyptin were further subjected to a detailed interaction analysis using MD simulation. MD is a powerful computer simulation technique that is now being investigated in the field of computer-aided drug discovery research to better understand how the protein-ligand complex behaves in a dynamic environment at the atomic level over a user-specified time period. A 50ns MD simulation analysis of the DPP4-ligand docked complex was performed and the RMSD profiles of the protein and ligands during the 50ns simulations are analysed (Fig-3). The average RMSD of DPP4 and Vildagliptin was found to be 1.75Å and 2.0Å, respectively (Fig-3A). Whereas, the average RMSD of DPP4 and Terchebin was 2.8Å and 3.5Å; DPP4 and Locoracemoside B was 2.1Å and 1.5Å and DPP4 and 1,2,4,6 Tetra o Galloyl Beta D Glucose was 2.1Å and 1.8Å, respectively (Fig-3B-D). All ligands were found to have stable interactions in the active site.

3.4. Stability analysis of phytochemicals interacting with DPP4

The protein-ligand interactions are depicted for 4 interactions (hydrogen bond, hydrophobic bond, water bridge and ionic bond) using the clubbed bar graph. The interactions with the specific amino acids throughout the run are depicted using normalised values. The stability is majorly defined by the hydrogen bonds being formed between the ligand and the protein. The DPP4-Vildagliptin interactions showed 4 hydrogen bonds being formed throughout the run at positions Glu205, Glu206 and His740. Strong hydrophobic interactions were seen at positions Val656 and Tyr666 (Fig-4A). For DPP4-Terchebin interaction, 8 hydrogen bonds at positions Ser206, Ser242, Tyr545, Lys554, Arg560, Trp627, Asn710 and Asp739 as well as hydrophobic interactions at positions Lys122, Arg125, Tyr547, Trp627 and Trp629 were identified (Fig-4B). Similarly, a total of 10 hydrogen bonds at positions Glu206, Val207, Ser209, Arg358, Tyr547, Ser552, Gln553, Tyr385 and Ser630 and hydrophobic bonds at Phe357, Tyr547, Tyr585, Tyr631, Tyr662, Tyr666 and Val711 were found in the DPP4-Locoracemosids B interaction (Fig-4C). The DPP4-TGBG interaction showed hydrogen bonds at Glu205, Glu206, Val207, Ser209, Tyr547, Pro550, Tyr662, Arg669, Tyr670 and Asn710 positions and hydrophobic bonds at positions Phe357, Tyr547, and Tyr666 (Fig-4D). The trajectory was subjected to detailed binding energy analysis using thermal_mmgsa script. Free energy of binding for Vildagliptin, Terchebin, Locoracemoside B and TGBG found to be -28.6 kcal/mol, -66.5kcal/mol, -58kcal/mol and -90kcal/mol, respectively.

3.5. Target mapping of phytochemicals identified in NK formulation for network analysis.

The constituent bioactives in a herbal formulation can interact with diverse bioactives to bring out their systemic biological effects and this can be studied using network pharmacology analysis methods, enabling us to delineate the possible mode of action of the formulation and hypothesise new biological pathways and interactomes involved in its pharmacological interactions (Patwardhan and Chandran, 2015).

The targets of 206 phytochemicals were curated from three databases as mentioned in materials and methods viz, STITCH, ChEMBL and BindingDB. Out of 206 bioactives studied, only 139 bioactives were found to have reported protein targets in these three databases and a total of 1555 proteins were identified for these 139 compounds. The remaining 67 compounds were found to have no interaction reports in the top databases widely used for target identification.

Using the Cystoscape, the phytochemicals and their identified targets were then converted to a compound-target network to represent the possible biological crosstalk modulated by NK. The network has 1694 nodes and 3264 edges identified (Fig-5). The compound-target network is a bipartite one, representing the interaction between phytochemicals and their putative targets. From this network, we observed that curcumin has the maximum interactions with 138 targets. Several hub compounds like chrysin and rutin which showed large interactions with proteins in the network were also reported to inhibit DPP4 *in* vitro (Kalhotra et al., 2018; Lee et al., 2021). The complete list of hub genes and hub compounds are given in Table 3 and 4. The network pharmacology studies also revealed compounds such as rutin, typhaneoside, myricitrin, narcissin, kaempferol 3-O-beta-D-galactoside and quercitrin having DPP4 as their targets aligning with our docking results which showed strong DPP4 inhibition by these compounds.

In order to understand the significant pathways associated with these proteins, a KEGG pathway analysis was done. The analysis revealed a number of significant (p-value 0.05) diabetes associated pathways like PI3K-Akt signalling pathway, MAPK signalling pathway, lipid and atherosclerosis, Ras signalling pathway, chemokine signalling pathway, diabetic cardiomyopathy, insulin signalling pathway, cellular senescence, HIF-1 signalling pathway, AGE-RAGE signalling pathway in diabetic complications and insulin resistance are associated with these proteins.

Also, a Clue GO analysis performed to identify the potential biological processes that are regulated by the formulation showed several metabolic processes are associated with these identified target proteins (Fig-6). The complete list of pathways and processes is given in Supplementary data 1 and 2. These observations are important in this study as it is supporting the Ayurvedic rationale of using NK for the management of diabetic associated symptoms and complications.

3.8 Disease overlap analysis of the gene targets mapped for phytochemicals in NK formulation

To get a better idea on the disease associated implications of NK, we used the openly accessible tool EnrichR, a comprehensive gene enrichment analysis tool containing diverse gene set libraries available for analysis (Kuleshov et al., 2016). The EnrichR tool was used for disease analysis of the 1555 targets mapped, and data was extracted from three sources viz, DigiNet, ClinVar and OMIM expanded (Supplementary data 5). A total of 9000 disease

indications emerged from disease analysis, from which a p-value significance of 0.05 was applied to short list the indications. Further, using the key words “Diabetes mellitus”, “Insulin resistance”, “Hyperglycemia”, “Hypoglycemia”, we categorised 614 diabetes associated genes, from the entire list of disease conditions obtained (Supplementary Data 3). A diabetes genes associated sub network can be seen in figure 8 containing 743 nodes with 1612 edges. This showed the putative diabetic interactome of NK including a number of critical genes in diabetes and related complications and comorbidities, further validating the anti-diabetic actions of the formulation.

For investigating the diabetic complications and associated risk conditions, the 614 genes were again analysed in the EnrichR tool. The resulting disease analysis showed various diabetic complications like nephropathy, neuropathy, cardiomyopathy and retinopathy. and also revealed the presence of risk factors/related conditions such as fatty liver, obesity and general metabolic disturbances along with inflammation which is a characteristic of all these diseases (Luo and Lin, 2021). The complete list of indications and key categories is given in supplementary data (Supplementary Data 3 and 4).

From the Venn diagram analysis in Fig-7A, we found that 37 genes emerged as common among all the diabetes associated comorbidities and diabetic complications. The associated co morbidities used for analysis were inflammation, fatty liver, obesity and metabolic dysregulation which are strongly diabetes associated. Proinflammatory markers like NFKB1 and TNF, characteristic of the insulin resistance were present in the cluster along with lipid regulatory and obesity-linked genes like PPARα and PPARγ (Chen et al., 2015;Yang et al., 2009 ;Stienstra et al., 2007). The other critical markers were insulin signalling molecules like AKT1 and PI3K, demonstrating that these core pathways involved in diabetic pathogenesis can be regulated by multitargeted action of NK (Huang et al., 2018).

To analyse the common genes among the diabetic complications, we also performed a specific gene overlap analysis between the categories of diabetic complications, namely, diabetic retinopathy, cardiomyopathy, nephropathy and neuropathy as shown in Fig-7B.

A cluster of 23 genes were identified as common among all diabetic complications and included markers such as TNF, TLR4, CCL2, SOD1, TGFβ and SOD2, all of which are deregulated and involved in various stages of diabetic pathogenesis (Huang et al., 2018; Yehualashet, 2020; Zhao et al., 2020; Tesch, 2008; Pickering et al., 2018).

The presence of DPP4 and GLP1R in the gene overlap of obesity, fatty liver and diabetic complications aligns with the recent evidence from various studies establishing DPP4 as a crucial gene in the pathogenesis of these disorders (Trzaskalski et al., 2020).

From our *in silico* studies, we observed that various compounds in NK formulation can target multiple genes and pathways which are therapeutically relevant for diabetes and related complications and related metabolic disorders. Specifically, from our invitro and in silico network pharmacology analyses, we can infer that one of the disease regulatory mechanisms of the formulation NK can be possibly through DPP4 inhibition, thereby modulating GLP1 levels.

4 Discussion

DPP4 has gained importance as a key therapeutic target over the last few years due to its primary role in increasing endogenous GLP-1 levels, which is found to be important for improved glycemic control and systemic glucose homeostasis. Though there are several approved DPP4 inhibitors, they are relatively expensive and are associated with some side effects such as pancreatitis, joint pain and a possible increased risk of cancer (Mohanty et al., 2019; Kalhotra et al., 2018; Hermansyah et al., 2021;Shirakawa and Terauchi, 2020). Hence there is an increasing interest in identifying new compounds that are cheap, safe and accessible.

Ayurveda formulations, by virtue of their long established clinical efficacy and multi-component-multi-target mode of action, have always been a source for identifying novel bioactives with better efficacy and less side effects. Also, the concepts of Ayurveda have been a treasure for science-curious people to unearth new biological underpinnings of health and disease manifestations. Recent insights into the Ayurveda concepts and polyherbal preparations have gained global interest and are being scientifically explored through evidence based research for newer transdisciplinary understandings of biological activities. The present study is an attempt to provide transdisciplinary understanding for the anti-diabetic action of a clinically established formulation called NK with a special focus on DPP4 inhibition activity. NK, as per Ayurveda, is prescribed for reducing diabetes symptoms as well as diabetes associated complications (Nishteshwar K, 2007). The herbal components of this formulation are described to have the potential to support the management of diabetes (*prameha*), and alleviate diabetic complications like wound healing (*vrana*) and eye disorders *(netra roga*) like retinopathy and cataract (Government of India, Ministry of Health and

Family Welfare, 2005). Various in vitro, in vivo and clinical research evidences also support the hypoglycemic potential of the component plants of NK *viz. Curcuma longa*, *Emblica officinalis*, *Salacia reticulata* , *Symplocos racemosa*, *Vetiveria zizanioides*, *Strychnos potatorum*, and *Aerva lanata* (Marton et al., 2021;Karłowicz-Bodalska et al., 2017;D'Souza et al., 2014;Medagama, 2015;Acharya et al., 2016;Acharya et al., 2016;Yadav et al., 2014;Riya et al., 2015).The background information from ayurveda and modern biology make NK one of the suitable candidates for *Ayurveda Biology* research.

In our study, the *in vitro* DPP4 inhibition study demonstrated a strong inhibition of the enzyme with an IC_{50} of 2.06 μg GAE/mL suggesting a beneficial role in improving the incretin biology. Parallel studies conducted in our laboratory have also shown that NK increases GLP1 secretion from enteroendocrine cells (unpublished data). The *in silico* data mining and virtual screening of phytochemicals present in NK showed three compounds Terchebin, Locoracemoside B and TGBG having strong affinity for DPP4 with stable interactions comparable to the standard inhibitor Vildagliptin. Terchebin and TGBG are reported from *Emblica officinalis*, which is considered as a 'wonder plant' in Ayurveda and it is widely reported for its antidiabetic and other biological actions (Sharma et al., 2020;Baliga and Dsouza, 2011). Locoracemoside B is a glycoside isolated from *Symplocos racemosa*, which is another important plant grouped under the anti-obesity (*medo-hara*) groups of plants in Ayurveda (Kumari et al., 2013). The known inhibitor Vildagliptin and the NK phytochemicals are found to have common residues such as Glu205, Glu206, Tyr666 and Asn710 on DPP4 interaction. The binding site of the DPP4 protein consists of Glu205, Glu206, Asp708, His740 and Ser630 as their essential active site residues(Berger et al., 2018). In addition to these essential active site residues, other binding site residues such as Arg125, Ser209, Phe357, Tyr547, Tyr631, Ile651, Trp659, Tyr662, Tyr666, Arg669, and Val711 are also found to play important role in protein-ligand binding (Ojeda-Montes et al., 2018;Patel and Ghate, 2014).

Apart from being a source of structurally diverse group of molecules, polyherbal preparations are reported to have multi-targeted effect possibly due to synergistic action of phytocompounds(Hopkins, 2008;Patwardhan and Chandran, 2015). To gain an insight into the polypharmacological action of NK, we did a network pharmacological analysis of the phyto actives which revealed a network of 1555 target proteins. The hub proteins with maximal interaction include carbonic anhydrases like CA9, CA7 and CA12 which have been shown to have a possible role of these proteins in diabetic complications such as

cardiomyopathy and retinopathy (Torella et al., 2014). Some other important hub proteins, like aldose reductase (AKR1B1) and Aryl hydrocarbon receptor (AR) are implicated in the pathogenesis of diabetic retinopathy and activated in hyperglycemic conditions respectively (Kaur and Vanita, 2016;Zapadka et al., 2021;Wang et al., 2011). DPP4, also a hub protein, showed multi protein interactions. This demonstrates that the PPI network of NK involves many genes which are central to diabetic complications.

A detailed disease analysis of NK targets in diabetes gave us 619 diabetes specific genes pertaining to diabetes mellitus, insulin resistance and hyperglycemia. A comparison of genes between the diabetic complications such as retinopathy, neuropathy, nephropathy and myopathy, wound healing and micro and macrovascular complications revealed a cluster of 23 markers. Among the 23 markers, inflammatory mediators like TNFα and pro fibrotic TGFβ are present, which is associated with the development of insulin resistance and is crucial in diabetic nephropathy progression respectively. AKR1B1, a hub gene, also present in the overlap of diabetic complications is an emerging target for diabetic complications(Ramasamy and Goldberg, 2010). Oxidative stress induced by chronic hyperglycemia is one of the main pathways for development of macrovascular and microvascular complications (Rehman and Akash, 2017);(Pickering et al., 2018). One of the main mechanisms mediated by polyherbal formulations in alleviating diabetes is by reducing oxidative stress by multiple mechanisms (Unuofin and Lebelo, 2020). The genes present in the central cluster in the overlap analysis between the diabetic complications show the presence of oxidative stress markers like GSTM1, SOD1 and SOD2, indicating the possibility of NK acting through oxidative stress signalling also (Bid et al., 2010). These observations indicate that NK through its various phytoactives may modulate these markers or related pathways to exert its actions in diabetes and associated complications

A comparison of gene categories between diabetic complications and the associated conditions/risk factors showed a set of 37 markers, including important signalling kinases like AKT1, obesity associated PPARα and PPARγ and pro-inflammatory transcription factor NFKB1. This shows the complex interplay between insulin signalling pathways, and lipid synthesis which are deregulated in lifestyle diseases like diabetes, obesity and NAFLD. Occurence of DPP4 and GLP1R in the gene overlap of fatty liver, obesity and diabetic comorbidities stresses the importance of the gut and its incretin axis in the possible polypharmacological drug network of NK

Leveraging the power of computational tools like docking and network pharmacology enables us to determine the workings of multicomponent therapeutics like Ayurvedic formulations. Through our *in vitro* and *in silico* experiments, we observed that DPP4 is one of the therapeutically important targets of NK. Our network analysis revealed the possible involvement of critical diabetes regulatory markers in the multi-level mechanisms of NK. Further experimental studies are needed to gain more clarity on the specific molecules and pathways involved.

5. Conclusion

The present work as evidenced through in vitro and in silico observations suggested that the anti-diabetic formulation NK has a strong DPP4 inhibitory potential. Further, our network pharmacology studies of the constituent phytochemicals revealed a complex molecular interaction network consisting of crucial genes and significant pathways in diabetes, diabetes-related complications and associated comorbidities. This integrated approach of combining in vitro and in silico methods offers the much needed scientific rationale for traditionally prescribed polyherbal Ayurvedic formulations in the treatment of complex diseases like type 2 diabetes. The pathways and molecular targets obtained from our work merits detailed investigation which will enable us to have more insight into the action of this Ayurvedic formulation.

Acknowledgement

The authors acknowledge the financial support received by Anjana from Tata Education and Development Trust and Rural India Support Trust. Authors also acknowledge the financial support for a Research Fellow from TDU, Bangalore. The authors also acknowledge the DS Endowment support received from TDU for the project.

Conflict of interest

Authors declare no conflict of interest.

Tables

S.No	Scientific Name	Common Name	PART USED
1	*Curcuma Longa*	Turmeric	Rhizome
2	*Strychnos potatorum L.*	Kataka	Seed
3	*Ixora coccinea L.*	Paranti	Root/Stem
4	*Symplocos racemosa Roxb.*	Lodhra	Stem Bark
5	*Emblica officinalis Gaertn.*	Amla	Fruit
6	*Aerva lanata (L.) Juss. ex Schult*	Gorakshaganja	Entire Plant
7	*Vetiveria zizanioides (L.) Nash*	Ushira , Khas Vetiver	Root
8	*Salacia reticulata Wight*	Salacia	Stem/Root

Table 1: List of herbs present in *Nisakathakadi Kashaya* (NK)

Table 2: Top scored 35 compounds from the Virtual Screening of NK phytochemicals with DPP4. The docking score, Glide score and binding free energy from MM-GBSA analysis are reported

Sl. No.	Compound Name	Docking Score	GLIDE Score	MM-GBSA
1	Vildagliptin	-3.899	-3.899	-26.7
2	Terchebin	-11.766	-12.441	-47.12
3	Locoracemosides B	-10.145	-10.145	-45.45
4	1,2,4,6 Tetra o Galloyl Beta D Glucose	-9.556	-9.752	-60.73
5	Tercatain	-9.252	-9.757	-28.37
6	Rutin	-9.244	-9.273	-41.90
7	Typhaneoside	-8.782	-8.811	-45.47
8	1,6-Bis-O-Galloyl-Beta-D-Glucose	-8.45	-8.551	-44.24
9	Kotalanol	-8.374	-8.374	

10	Chebulinic Acid	-8.314	-8.506	-19.48
11	Kaempferol-7-O-α-rhamnoside	-8.309	-8309	-41.87
12	Quercitrin	-8.012	-8.04	-36.48
13	1,6-Bis-O-Galloyl-Beta-D-Glucose	-8.003	-10.106	-15.06
14	Glucogalin	-7.993	-8.043	-38.18
15	Epicatechin	-7.992	-7.992	-39.34
16	catechin	-7.992	-7.992	-39.34
17	Sucrose	-7.643	-7.643	-28.54
18	Quercetin	-7.498	-7.53	-23.39
19	Benzoylsalireposide	-7.403	-7.403	-33.55
20	Procyanidin	-7.382	-7.382	-22.21
21	Salirepin	-7.253	-7.254	-36.51
22	Myricitrin	-7.142	-7.176	-27.44
23	Symplocomoside	-6.989	-6.898	-31.26
24	Luteolin	-6.985	-7.024	-29.11
25	Narcissin	-6.613	-6.641	-42.08
26	Corilagin	-6.541	-7.085	
27	Bisacurone	-6.345	-6.345	-10.71
28	Kaempferol 3-O-beta-D-galactoside	-6.272	-6.381	-39.39
29	Proanthocyanidin	-6.114	-6.312	4.71
30	Salacinol	-6.099	-6.099	-40.66
31	Kaempferol-7-oglucoside	-6.076	-6.076	-28.39

32	Punicafolin	-6.065	-8.352	-5.8
33	Symplososide	-6.021	-6.022	-32.88
34	Symploveroside	-6.009	-6.009	-36.55
35	Epicatechin-4β-8	-6.008	-6.206	-30.48
36	Symponoside	-5.317	-7.458	-23.85

Table 3: List of hub genes ranked as per their degree values

S. No	Hub Gene	Degree	S. No	Hub Gene	Degree	S.No.	Hub Gene	Degree	S.No.	Hub Gene	Degree
1	CA2	26	38	DHCR24	8	75	CNR2	6	112	GANC	5
2	CYP19A1	22	39	AKR1B10	8	76	CHRM4	6	113	ALOX12	5
3	CA7	18	40	TOP2A	8	77	AGTR2	6	114	PLAT	5
4	ESR1	17	41	ALB	8	78	PNLIP	6	115	TP53	5
5	F2	16	42	ADRA2B	8	79	CASP8	6	116	PYGL	5
6	AKR1B1	16	43	CYP1A2	8	80	BCL2	6	117	SULT1E1	5
7	CA12	16	44	ALOX5	8	81	UGT2B15	6	118	CSNK2A1	5
8	AR	15	45	PSMB5	8	82	TYR	6	119	GSK3B	5
9	CASP3	15	46	NR1I2	8	83	XDH	6	120	SI	5
10	ACHE	15	47	CA1	8	84	GLO1	6	121	TTR	5
11	PTPN1	13	48	GAA	8	85	NR3C2	6	122	AKR1A1	5
12	RORC	13	49	CHRM5	8	86	SLC2A1	6	123	FAAH	5
13	SHBG	13	50	HMGCR	7	87	HDAC6	6	124	GSTM3	5
14	MMP9	13	51	GRIN2B	7	88	SERPINE1	6	125	LOX	5
15	ESR2	12	52	ITGB3	7	89	PTPN2	6	126	DHODH	5
16	NR1H3	12	53	MAPK3	7	90	PLAU	6	127	CYP3A4	5
17	UGT1A7	12	54	CASP7	7	91	CYP51A1	6	128	VEGFA	5
18	UGT1A8	12	55	F7	7	92	MGLL	6	129	PSMB1	5
19	UGT1A10	12	56	RGS4	7	93	AMY2A	6	130	CACNA1H	5
20	CA4	12	57	ADRA2C	7	94	RPS6KA3	6	133	COMT	5
21	CYP17A1	11	58	CASR	7	95	CA14	6	134	MGAM	5
22	CYP1A1	11	59	GLA	7	96	BACE1	6	135	PLAUR	5
23	CRYAB	10	60	PDE5A	7	97	CTSD	5	136	IMPA1	5
24	EGFR	10	61	CASP9	7	98	CYP142	5	137	EBP	5
25	TOP1	10	62	UGT1A1	7	99	ITGAV	5	138	LSS	5
26	F10	10	63	CYP1B1	7	100	GRIN1	5	139	TNF	5
27	HSD11B1	10	64	NOX4	7	101	CYP7A1	5	140	AVPR1A	5
28	PTGS2	9	65	LCK	7	102	MPO	5	141	SLCO1B3	5
29	NR1H2	9	66	KDM1A	7	103	CRABP2	5	142	APP	5
30	MAPK1	9	67	SQLE	7	104	GNB1	5	143	CYSLTR1	5

31	AKT1	9	68	GRIN2A	6	105	HTR1B	5	144	EDNRB	5
32	ADRA2A	9	69	OSBP2	6	106	LPAR3	5	145	FABP4	5
33	UGT1A9	9	70	SREBF2	6	107	MTRNR2L2	5	146	FABP5	5
34	MAOA	9	71	CYP2B6	6	108	HCAR3	5	147	FFAR1	5
35	DPP4	9	72	MAPK8	6	109	TAS2R40	5	148	NOS2	5
36	UGT1A3	9	73	BCHE	6	110	TAS2R43	5			
37	MMP2	9	74	LPAR2	6	111	P2RY4	5			

Table 4: List of hub compounds ranked as per their degree values

S.No	Hub Compound	Degree
1	Curcumin	138
2	Chrysin	121
3	Kaempferol	106
4	Oleic Acid	87
5	Quercetin	82
6	Stearic_Acid	80
7	Caffeic Acid	78
8	Camphor	78
9	Ursolic_Acid	75
10	Ascorbic Acid	75
11	Sucrose	74
12	Oleanolic Acid	67
13	Palmitic_Acid	67
14	Ellagic Acid	61
15	Quercitrin	60
16	Kaempferol 3-O-Beta-D-Galactoside	58
17	Strychnine	56
18	Cinnamic Acid	55

Figure Legends

Figure-1: Inhibition of DPP4 by NK formulation. Graph shows a concentration dependent inhibition of DPP4 enzyme action upon treatment with various concentrations of NK

Figure-2: Interaction of DPP4 with various ligands. Interactions of Vildagliptin (A), Terchebin (B), Locoracemosids B (C) and 1,2,4,6 Tetra o Galloyl Beta D Glucose (D) with DPP4.

Figure-3: RMSD plot. RMSD plot of Protein and Vildagliptin (A); Protein and Terchebin (B); Protein and Locoracemosids B (C); Protein and 1,2,4,6 Tetra o Galloyl Beta D Glucose (D).

Figure-4: 2D interaction diagram of ligands with protein during the simulation. (A)-Vildagliptin, (B)-Terchebin, (C)-Locoracemosids B and (D)-1,2,4,6 Tetra o Galloyl Beta D Glucose with DPP4.

Figure-5: The compound-target network of Nishakathakadi. (A) - The yellow nodes represent the compounds from plants used in the Nishakathakadi formulation and the pink nodes represent the target genes. (B) - The network of phytochemicals of NK and diabetes-related genes. The blue color nodes represent the compounds and the orange nodes represent the diabetes associated target genes.

Figure-6: Clue-go analysis of the 1500 target proteins. (A) analysis of the 1500 target proteins involved in different biological processes and (B) analysis of the 1500 target proteins involved in different pathway analysis

Figure-7: Venn diagram analysis. (A) represents the analysis of diabetes and associated gene overlap and (B) shows the overlap analysis of genes between diabetic complications. Abbreviations: INFLAM – Inflammation; FAT LIV - Fatty liver; MET DYS - Metabolic disturbances; DIA COMP - Diabetic complications; CARDIO – Cardiomyopathy; NEPHRO – Nephropathy; NEURO – Neuropathy; RETINO – Retinopathy; OTHER - Other diabetic associated complications like diabetic foot disorder, delayed wound healing, microvascular and macrovascular complications

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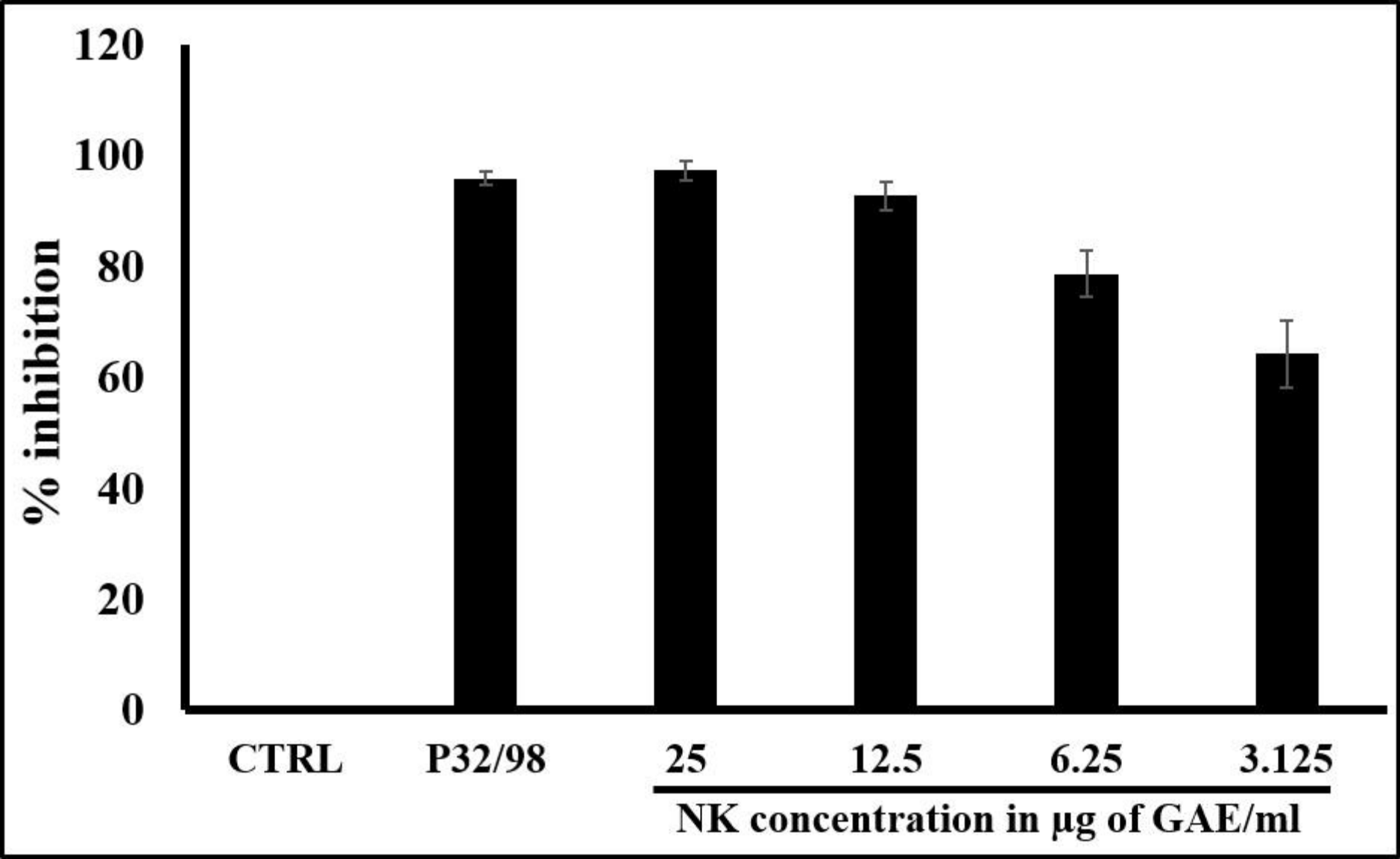
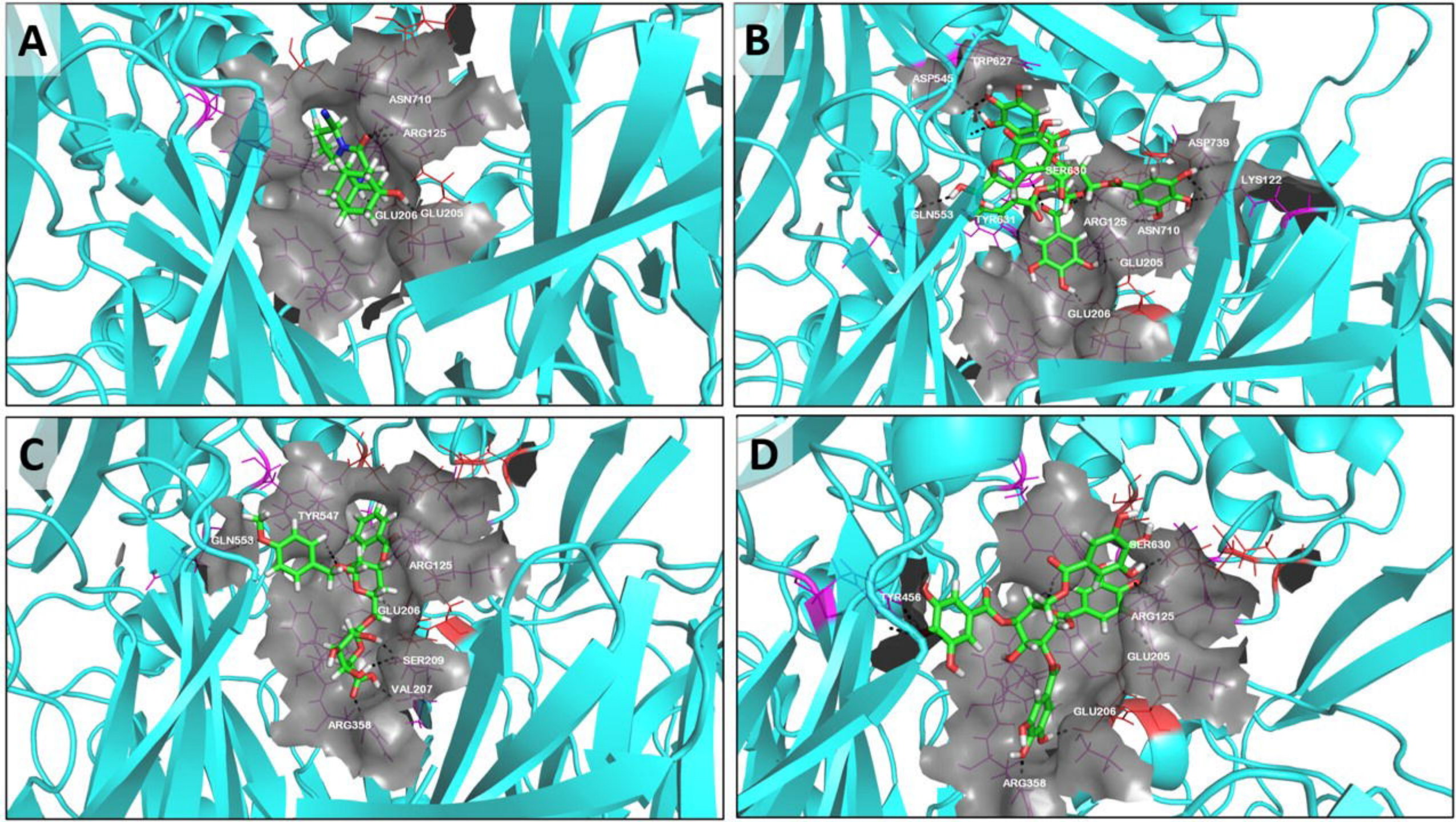

120
100
80
60
40
20
0
% inhibition
CTRL
P32/98
25
12.5
6.25
3.125
NK concentration in µg of GAE/ml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ASN710
ARG125
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GLU205
B
TRP627
ASP545
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LYS122
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ARG125
ASN710
GLU205
GLU206
C
TYR547
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GLU206
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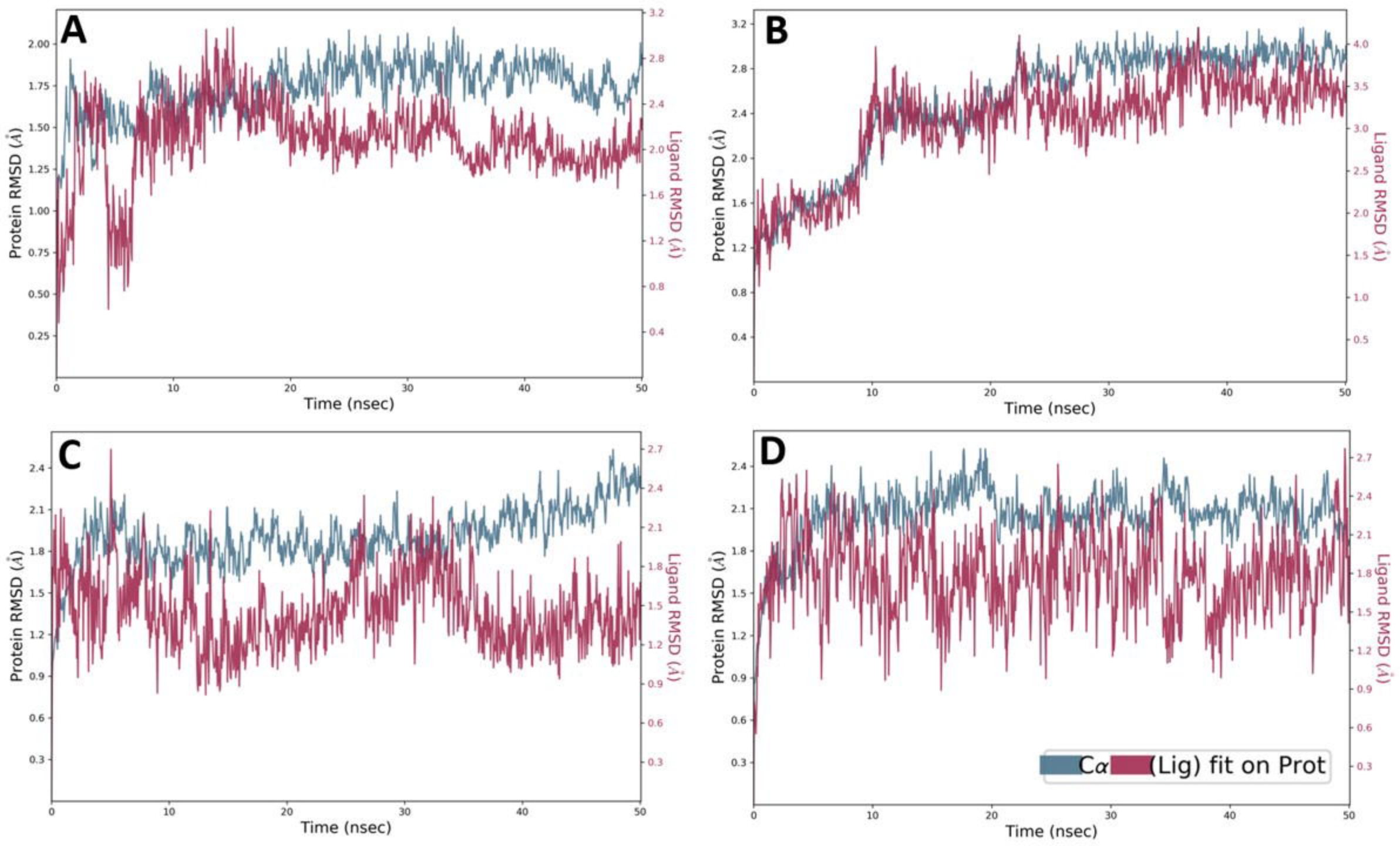
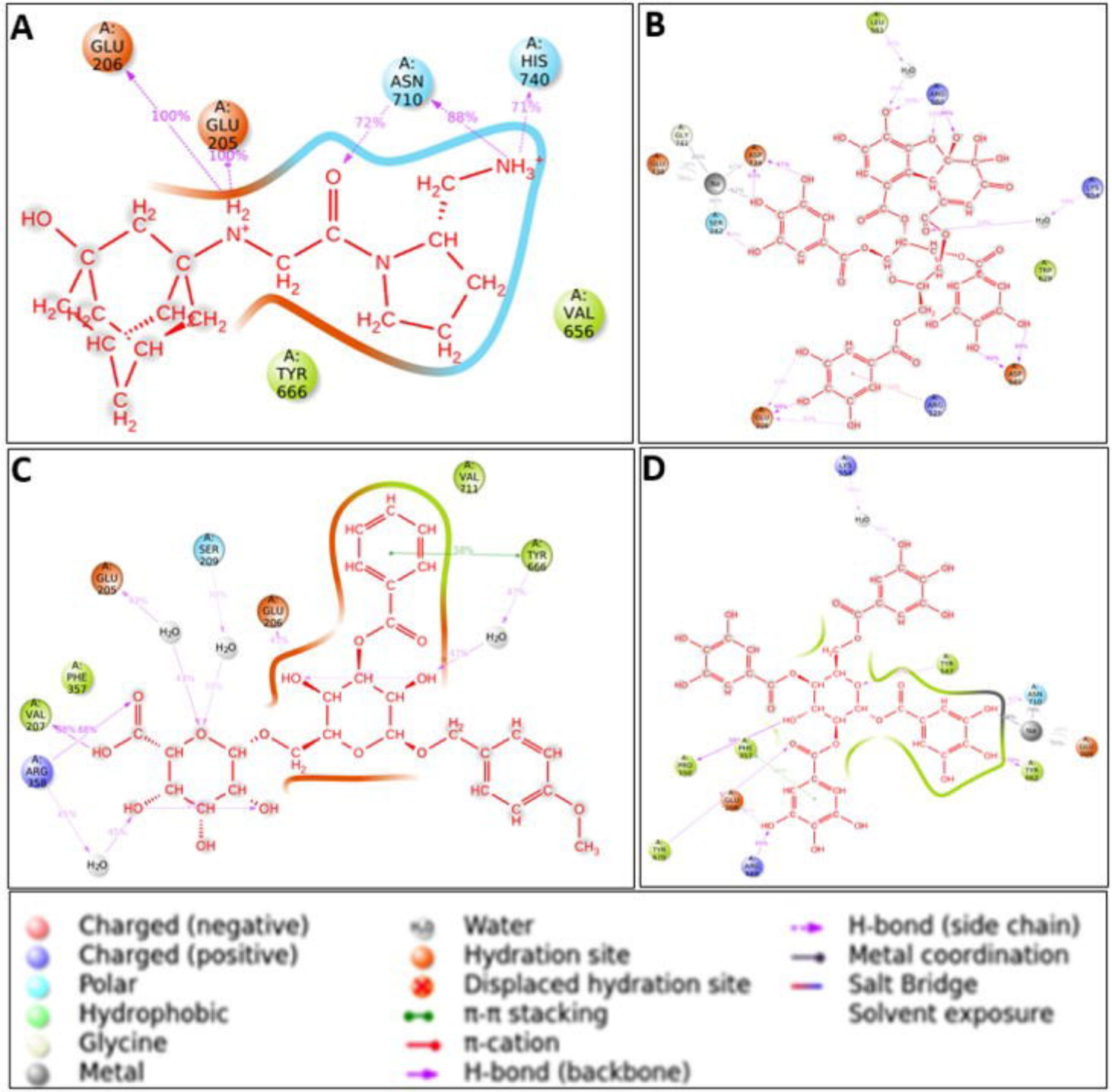
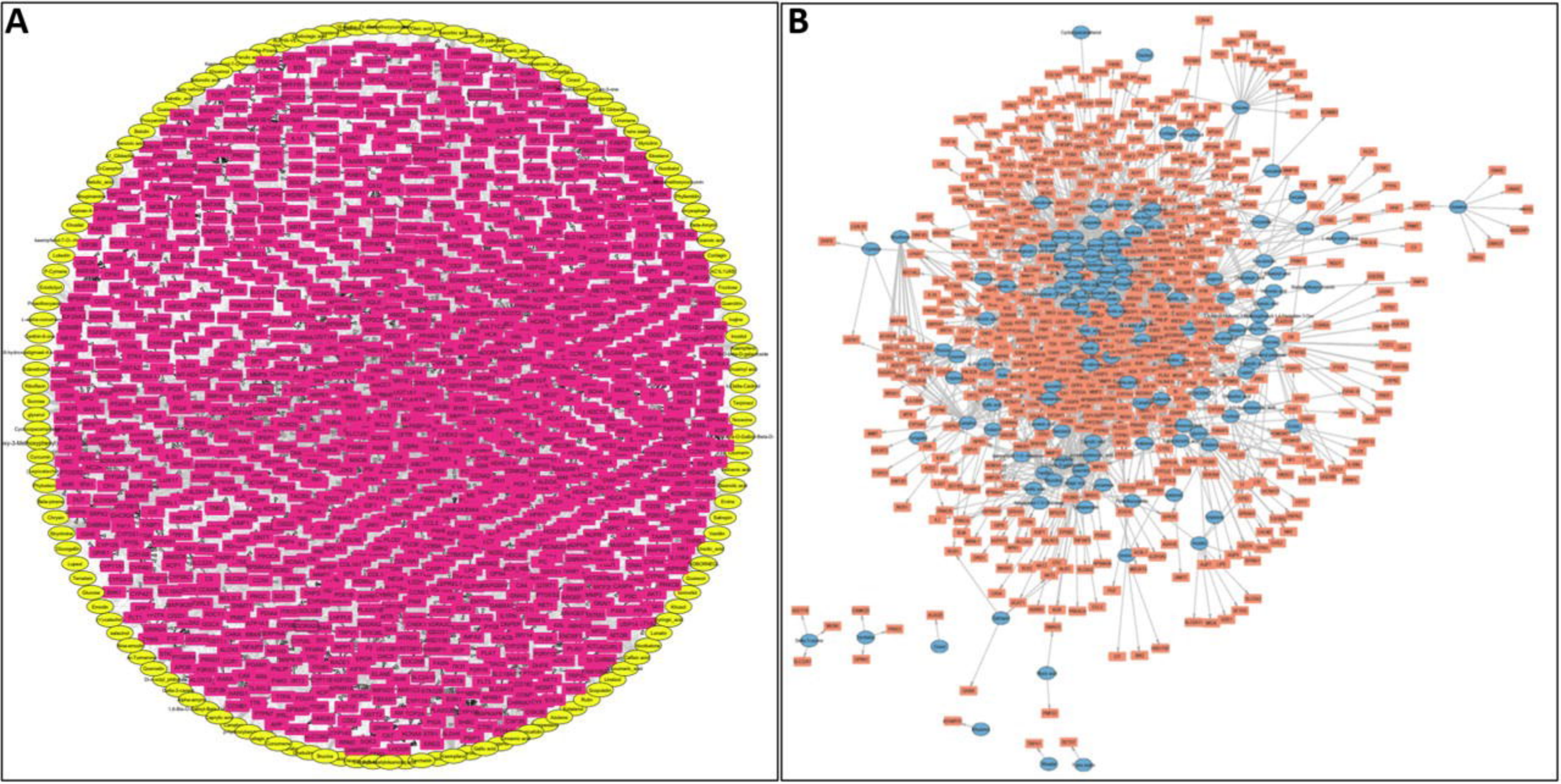
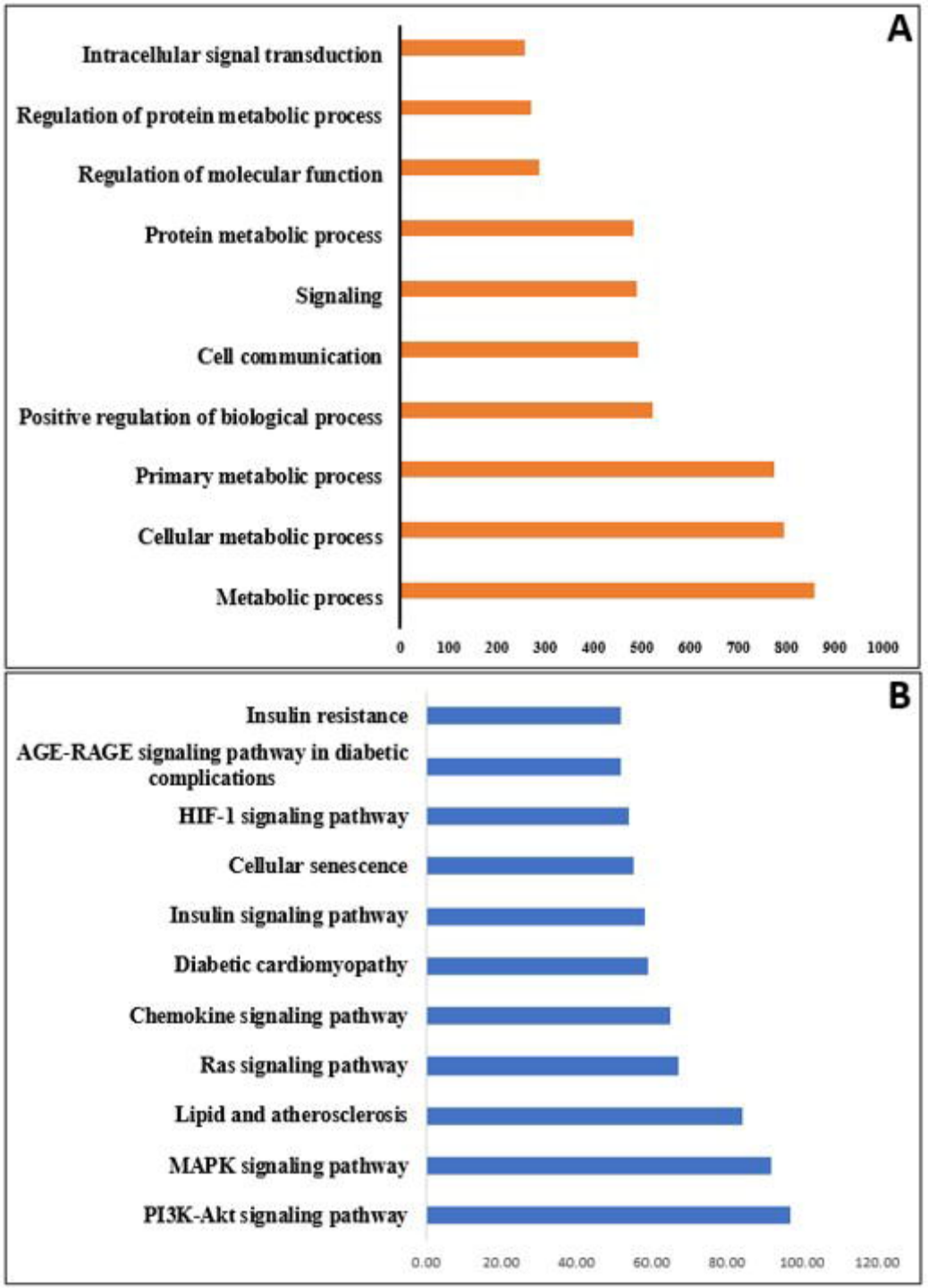

A
B
C
D
Protein RMSD (Å)
Ligand RMSD (Å)
Time (nsec)
Cα
(Lig) fit on Prot


A
A: GLU 206
A: GLU 205
A: ASN 710
A: HIS 740
A: VAL 656
A: TYR 666
100%
100%
72%
88%
71%
B
C
A: VAL 211
A: SER 209
A: GLU 205
A: GLU 206
A: TYR 666
A: PHE 357
A: VAL 207
A: ARG 358
D
Charged (negative)
Charged (positive)
Polar
Hydrophobic
Glycine
Metal
Water
Hydration site
Displaced hydration site
π-π stacking
π-cation
H-bond (backbone)
H-bond (side chain)
Metal coordination
Salt Bridge
Solvent exposure


A
B


A
Intracellular signal transduction
Regulation of protein metabolic process
Regulation of molecular function
Protein metabolic process
Signaling
Cell communication
Positive regulation of biological process
Primary metabolic process
Cellular metabolic process
Metabolic process
0
100
200
300
400
500
600
700
800
900
1000
B
Insulin resistance
AGE-RAGE signaling pathway in diabetic complications
HIF-1 signaling pathway
Cellular senescence
Insulin signaling pathway
Diabetic cardiomyopathy
Chemokine signaling pathway
Ras signaling pathway
Lipid and atherosclerosis
MAPK signaling pathway
PI3K-Akt signaling pathway
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40.00
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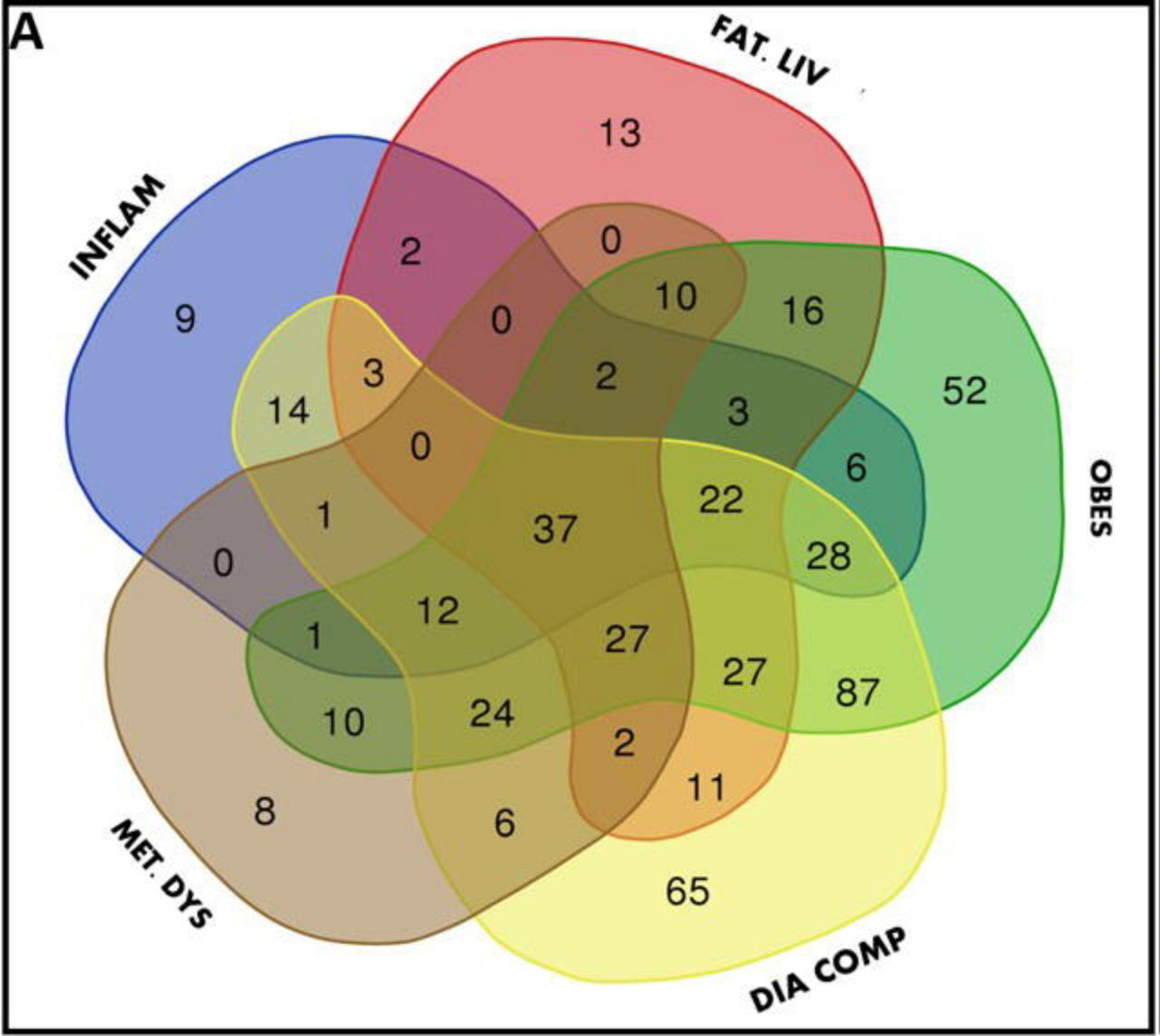
A
FAT. LIV
INFLAM
OBES
MET. DYS
DIA COMP
13
2
0
9
0
10
16
3
2
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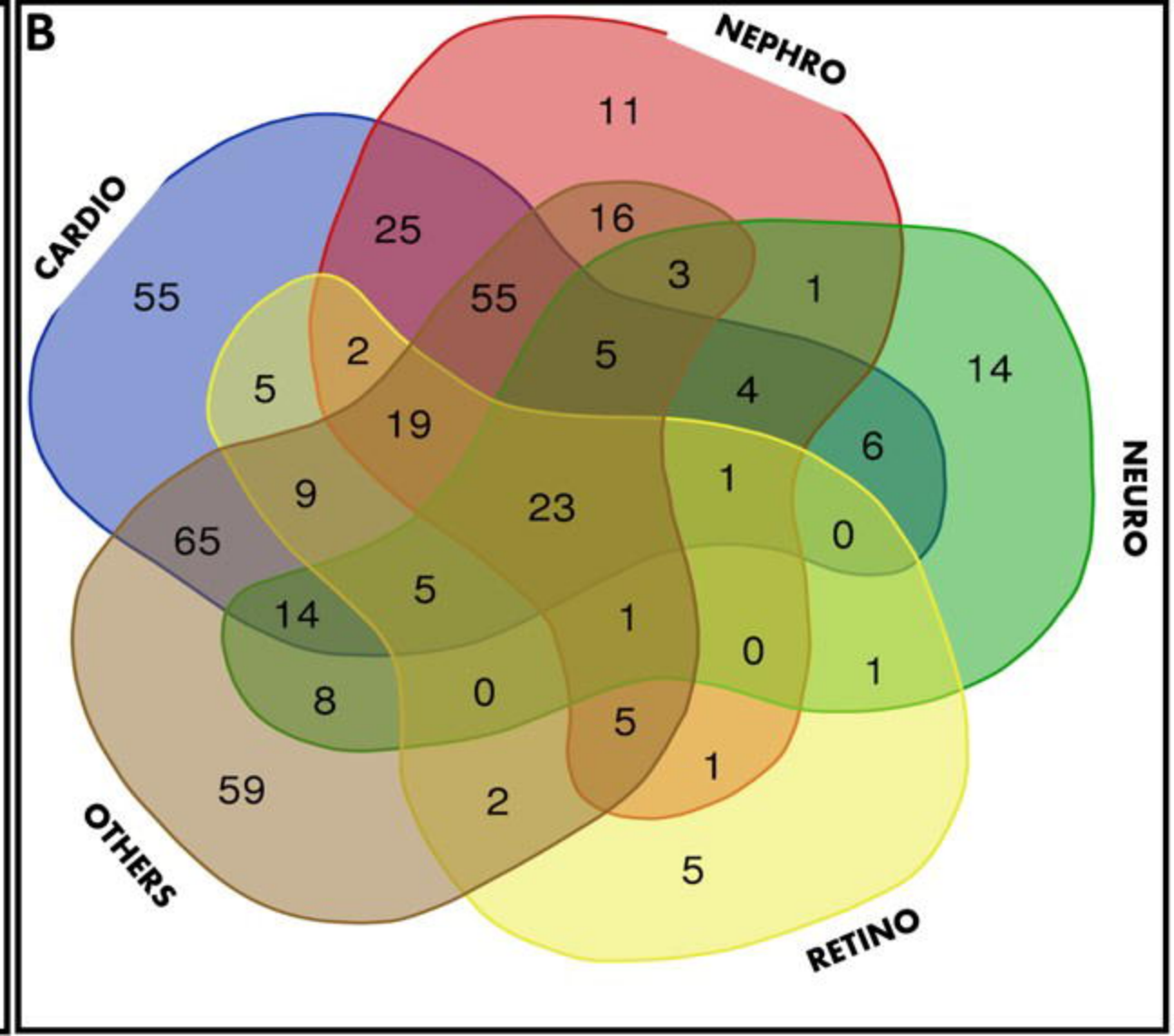
B
NEPHRO
CARDIO
NEURO
OTHERS
RETINO
11
25
16
55
55
3
1
2
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14
5
4
19
6
9
23
1
65
0
14
5
1
0
8
0
1
5
59
1
2
5