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IDENTIFICATION OF NATURAL COMPOUND FOR ATTENUATION OF DIABETIC NEUROPATHIC PAIN

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CANDIDATE'S DECLARATION

I, Tanya Kalra, Roll no. 2K21/MSCBIO/55, student of M.Sc. (Biotechnology), hereby declare that the project Dissertation titled "**Identification of natural compound for attenuation of Diabetic neuropathic pain**" which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Science, is original and not copied from any source without proper citation. This work has not previously formed the basis for the award of any Degree, Diploma Associateship, Fellowship or other similar title or recognition.

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DATE: 30-05-2023

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CERTIFICATE

I, hereby certify that the Project Dissertation titled "**Identification of natural compound for attenuation of Diabetic neuropathic pain**" which is submitted by Tanya Kalra, Roll no. 2K21/MSCBIO/55, Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Science, is a record of the project work carried out by the student under my supervision. To the best of my knowledge this work has not been submitted in part or full for any Degree or Diploma to this University or elsewhere.

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ABSTRACT

Diabetic neuropathy is a nerve damaging microvascular complication which is a result of long term diabetes contracted by more than 50% of diabetic individuals. It is responsible for pain, infection, numbness, foot ulceration and limb amputations. The cases of diabetes have risen exponentially in all income countries across the globe. As per the current treatment options available, pain management by physical therapy, diet control and certain drugs have been approved. But these drugs come with certain side effects such as blurry vision, headaches, sleep deprivation, nausea, hot flushes etc. along with display of certain mental side effects. To avoid these, certain natural compounds can be used to inhibit multiple pathways involved in diabetic neuropathy progression and do not show excessive side effects. Thus, with the help of in-silico methods, pharmacophore models of FDA approved drugs were generated and a list of natural compounds were curated which was then validated by docking and 2D visualization was done. 2,3-Dimethylbenzoic acid obtained from *Cinnamomum porrectum* was found out to be the best docked phytochemical against calcium channel as compared to FDA approved drugs. *Cinnamomum porrectum* is currently being used in Indian medicinal practices as antirheumatic agent. Thus, it can be used as a substitute for attenuating neuropathic pain.

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LIST OF ABBREVIATIONS

AGE	Advanced glycation endproducts
RAGE	Receptor for advanced glycation endproducts
DKD	Diabetic kidney disease
PARP	Poly (ADP-ribose) polymerase
DRG	Dorsal root ganglia
ROS	Reactive oxygen species
TRPA1	Transient receptor potential cation channel subfamily A member 1
VGCCs	Voltage gated calcium channels
FDA	Food and Drug administration
PDN	Painful diabetic neuropathy
DPN	Diabetic peripheral neuropathy
SNRI	Serotonin and norepinephrine reuptake inhibitor
GABA	Gamma-aminobutyric acid
MMP2	Matrix metallopeptidase 2
IMPPAT	Indian Medicinal Plants, Phytochemistry And Therapeutics
ADME	Absorption, Distribution, Metabolism, Excretion
BBB	Blood brain barrier

CHAPTER 1

INTRODUCTION

Diabetes mellitus is a multifactorial disorder that leads to 1.5 million deaths every year as per WHO [1]. The cases of diabetes have been rising steadily for the past few decades. It is characterized by hyperglycemia or high levels of blood sugar levels. It can be autoimmune (type I) or insulin-independent (type II) diabetes. There are other types of diabetes namely gestational diabetes and type 3 diabetes, which is a progressed form of type 2 diabetes leading to Alzheimer's disease. Diabetes mellitus progresses gradually and leads to several microvascular and macrovascular complications that are responsible for damage to the retina, kidneys, neurons, muscles and heart. Several genetic and lifestyle factors affect the progression of diabetes. Microvascular complications arise due to altered vasculature of blood vessels as a result of leukostasis [2]. Proinflammatory factors, protein glycations and abnormal hemorheological functions alter the activity and structure of the inner lining of blood vessels leading to alteration in blood and insulin supply to organs [3]. Hyperglycemia also induces activation of certain pathways such as PKC, AGE-RAGE, Polyol, Wnt/β catenin and hexosamine pathway upregulation responsible for ROS accumulation and angiogenesis and downregulation of NO synthase responsible for vasoconstriction [4][5][6][7]. These changes further result in hyperpermeability of blood vessels, excess inflammation, ischemia and tissue modifications damaging organs and leading to diabetic complications. Though, there are several pathways that

have been discovered, the exact molecular mechanism for diabetes progression still remains elusive.

1.1 Diabetic nephropathy

Diabetic kidney disease (DKD) or Diabetic nephropathy develops in more than 40% of diabetics and is responsible for end-stage renal disease (ERSD) as a result of excessive oxidative damage and lipotoxicity that cause basement membrane thickness, glomerular structural alterations and podocyte injury affecting the glomerular filtration rate and renal structural integrity[8].

1.2 Diabetic retinopathy

Diabetic retinopathy is a result of polyol pathway activation, accumulation of free radicals and angiogenesis in the retinal region leading to retinal blood vessel damage, hemorrhage, and loss of vision [9]. It is also characterized by fluid retention (edema) in the macula that causes blurry vision, neovascular glaucoma and scarring at the back of the eye. Wnt genes that regulate the development of the retina and are involved in its maintenance are considered a major factor for retinal disease development [10].

1.3 Diabetic myopathy

Due to a shift in metabolic load to muscles in case of insulin resistance and impaired glucose uptake, muscle health gets affected resulting in alteration in the ultrastructure of muscles, fatigue, weakness and defective muscle function. Muscles lose their ability to regenerate due to action on collagen synthetic pathways causing degenerative remodeling of extracellular matrix [11].

1.4 Diabetic neuropathy

As diabetes progresses, up to half the patients experience issues with their autonomic and peripheral nervous systems wherein motor nerve endings get damaged and cause pain, burning, tingling and numbness in limbs mostly towards extremities and sometimes in proximal areas [12]. The neuronal injury activates a cascade of reactions wherein sensitization leads to abrupt nociceptor activity, allodynia and hyperalgesia [13]. Thus, gabapentinoids act as the primary drugs recommended for the attenuation of DPN pain and are used to reduce nociceptive input and neuronal sensitization. These drugs act as calcium channel blockers in the brain [14]. Gabapentinoids namely gabapentin and pregabalin are used to counteract depression as well and have been shown to reduce neuropathic pain when used individually or synergistically with other pain-lowering drugs.

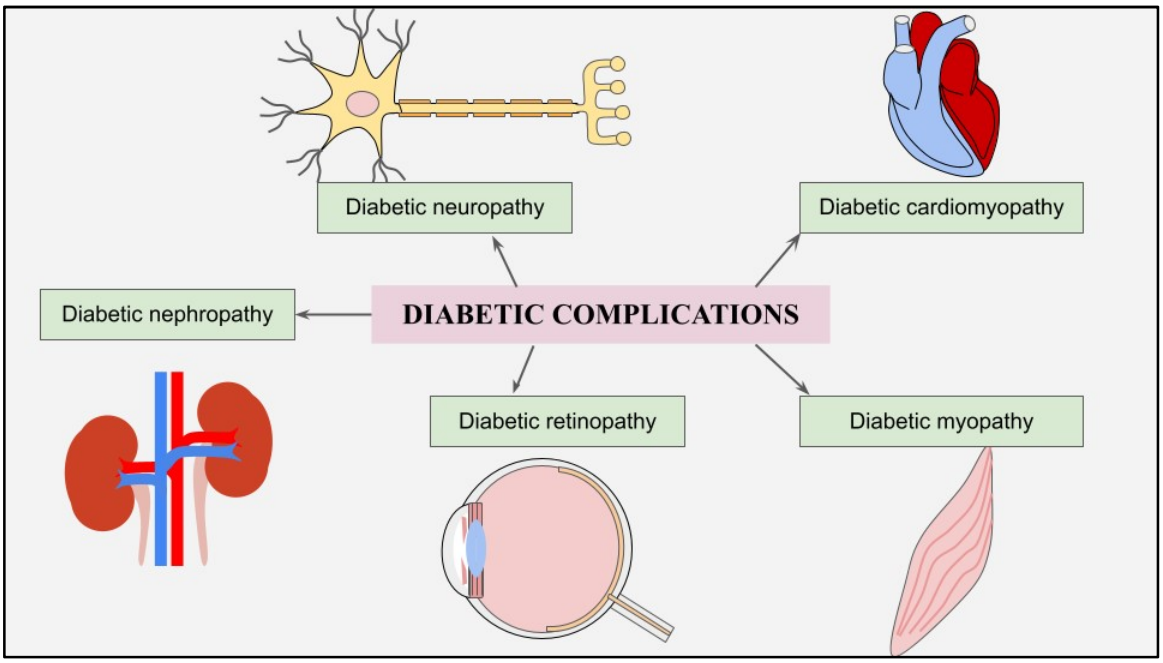


Fig 1.1: Diabetes progression leading to several complications

CHAPTER 2

LITERATURE REVIEW

2.1 Pathogenesis of Diabetic neuropathy

Diabetes targets sensory and autonomic axons in the earlier stages and motor axons in the later stages leading to severe damage to neurons and the foot [15]. The terminal sensory axons start dying out gradually and cause loss of sensation which is also known as the 'stocking and glove' pattern of pain [16]. Long-term diabetes causes alterations in transfer across Schwann cells and axons, changes in translation in the DRG, demyelination and degeneration of axons. Schwann cells play a major role in the regulation of the integrity of axons, nodes of Ranvier protein positioning and axon trafficking, thus damage to these cells affects the mentioned properties of neurons [17].

Experiments have shown that hyperglycemia induces alteration in molecules such as growth-associated protein 43 (GAP43), β-tubulin, HSP and PARP in dorsal root ganglia (DRG) further leading to abnormal protein synthesis, oxidative stress and ultimately loss of peripheral nerve function [18]. Upregulation of certain miRNAs has also been implicated in upregulation of inflammatory and lipid processing pathways in sciatic nerve have also been implicated in sensory neuron alterations. The injury and loss of Schwann cells and DRG neurons occur due to

the accumulation of acylcarnitines under hyperglycemic conditions leading to mitochondrial dysfunction and dysregulated stress response resulting in axonal degeneration [19][20].

Chronic hyperglycemia is responsible for the activation of polyol and hexosamine pathways that enhance the free radical production and inflammatory molecules and result in mitochondrial injury. It can also activate the AGE-RAGE pathway and alter the gene expression to produce more inflammatory molecules and ROS [21]. Similarly, hyperlipidemia due to free fatty acid oxidation generates large amounts of ROS and inflammation occurs that chemotactically infiltrates macrophages into the peripheral nervous system and injures Schwann cells [22].

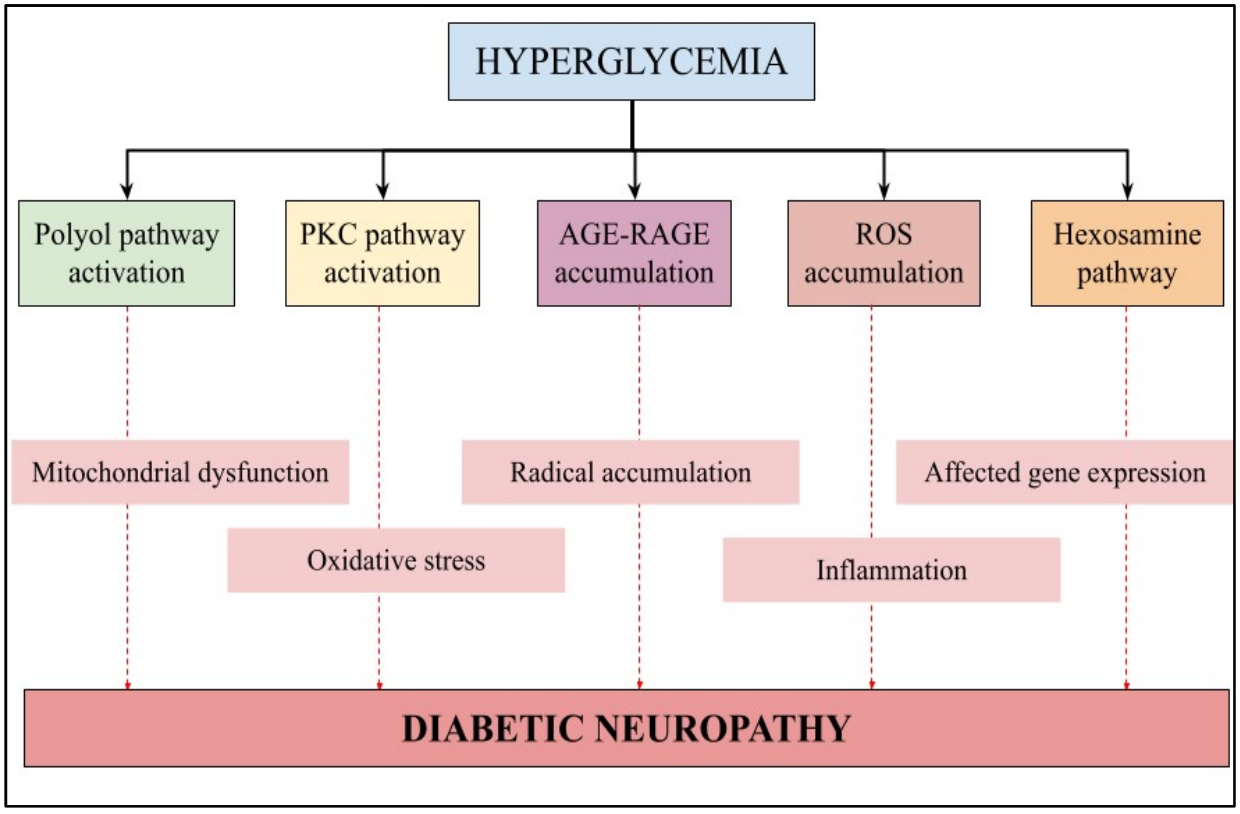


Fig 2.1: Several pathways are activated due to hyperglycemia that lead to mitochondrial dysfunction, oxidative stress, inflammation, ROS accumulation and alter gene expression that lead to diabetic neuropathy

2.2 Mechanism of pain

Around 30-50% of diabetic neuropathic patients complain of burning pain. The pain develops as a consequence of hyperexcitability and depolarization of neurons even in the absence of required pain stimulus causing changes in stimulus-response of neurons [23]. These alterations vary depending on the location and type of proteins or channels involved.

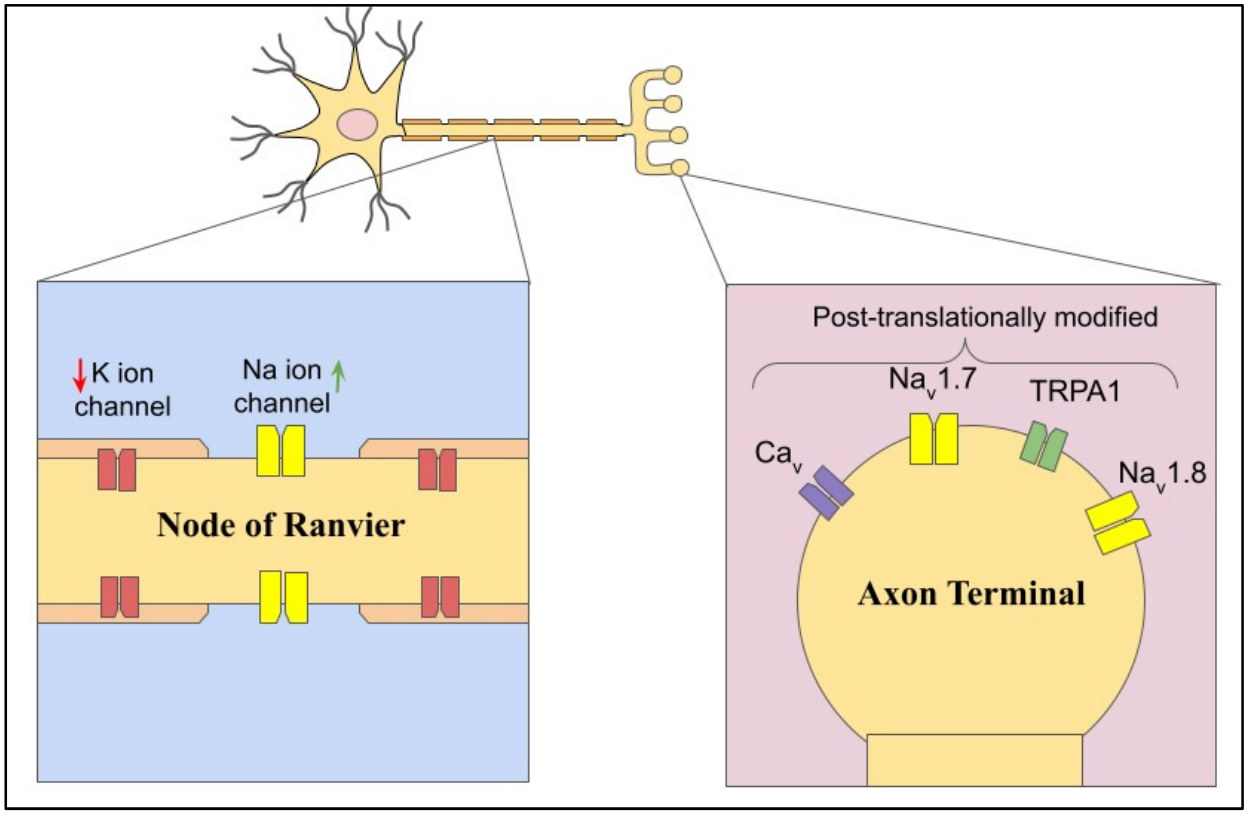


Fig 2.2: Alteration in ion channel levels in neurons in diabetic neuropathy patients

One of the mechanisms for the pathophysiology of pain includes glycation of ion channels that lead to enhanced function of these channels. The threshold of nociceptors (nerve cell endings that initiate pain) reduces in case of nerve damage or inflammation which can then get activated

by minimal stimulus leading to hyperalgesia (heightened sensitivity and response to pain). The signals travel to DRG and activate ion channels that then trigger the release of neurotransmitters and neuromodulators which are responsible for central sensitization and secondary hyperalgesia[23].

In the perikaryon region, the expression of voltage-gated sodium channels, $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ increases are also responsible for hyperexcitability and enhanced stimulus leading to excessive nociceptor activity in the spinal cord where they activate microglial cells and further lead to hyperexcitability [17].

Other factors include genetic variation in ion channels that alter the level of expression, trafficking and post-translational modifications. For instance, the upregulated expression of $Na_v1.8$ in sensory neurons of diabetic mice models reduces the conduction failure in nociceptors leading to enhanced signal conduction and hence increased neuropathic pain [24]. AGE accumulation during diabetes can also post-translationally modify $Na_v1.8$ and a cation channel TRPA1, leading to its enhanced function and hyperexcitability contributing to pain [25]. The variation in gene encoding $Na_v1.7$, which is *SCN9A* has been implicated as a major contributor to neuropathic pain in chronic diabetics, due to impaired inactivation only after the onset of diabetes [26].

2.3 Role of voltage-gated calcium channels

Voltage gated calcium channels are channel proteins that allow influx or efflux of calcium ions which is an important ion for electrical activity or excitation of neurons. Influx of calcium ions via these channels triggers gene transcription, release of neurotransmitters such as glutamate, neurite outgrowth and activation of certain enzymes. Theα_1, β, γ and $\alpha_2\delta$ subunits constitute this

channel. The $\alpha_2\delta$ and β subunits have isoforms that play a role in increasing the function of high voltage-activated channels. It is the $\alpha_2\delta$-1 isoform that is present in the brain and is the primary target for lowering neuropathic pain via certain antidepressant drugs [14]. They are also present in all other types of muscle tissues and their elevated levels are responsible for leading to increased pain stimulus. Damage to neurons and increased expression of $\alpha_2\delta$-1 takes months to come down to normal and resolve the evoked behavior. A lack of injury to neurons and a genetic increase of $\alpha_2\delta$-1 subunits can also initiate neuropathic pain. Also, increased levels of $\alpha_2\delta$-1 can cause spinal sensitization due to increased glutamate release that further contributes to the sensitization of postsynaptic neurons.

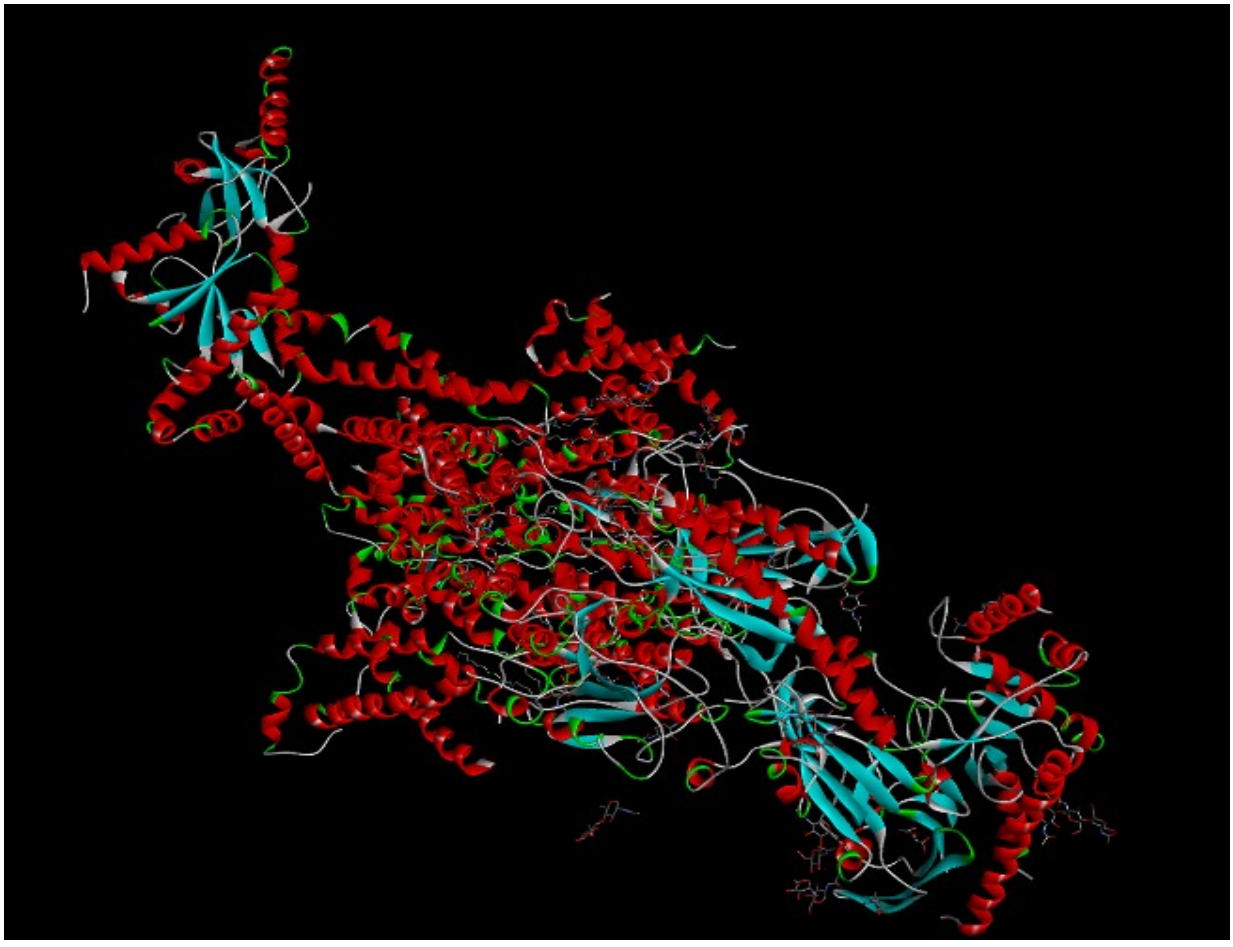


Fig 2.3: The 3D structure of voltage gated calcium ion channel protein present in neurons

Nerve injury or inflammation reduces the nociceptor threshold. The signals are transmitted to the spinal dorsal root that causes changes in membrane potential and hence voltage-gated calcium channels (VGCCs) are activated. This triggers a flow of calcium ions into the neuron and the release of neurotransmitters and neuromodulators such as glutamate, substance P, BDNF (brain-derived neurotrophic factor) etc. the increased release of glutamate in DRG leads to enhanced activation of postsynaptic nociceptor neurons along with neuroplastic changes further responsible for persistent central sensitization and hyperalgesia [14].

2.4 Current treatment strategies for painful diabetic neuropathy

Current cures for the management of PDN include the use of antidepressants, anticonvulsants and opioids other than topical analgesics and pain management exercises. Some FDA-approved anticonvulsants are Pregabalin, Gabapentin, Valproic acid etc. but these drugs come with certain side effects such as confusion, dizziness, drowsiness, digestive issues and tachyphylaxis [27]. Amongst the antidepressants, duloxetine which is a serotonin and norepinephrine reuptake inhibitor prescribed for depression is used as first line of treatment for PDN [27][28]. Its side effects include somnolence and hyperhidrosis. Other examples include Venlafaxine and TCAs such as amitriptyline. Opioid such as tapentadol is an FDA-approved opioid but is used as third-line therapy due to the risk of misuse and abuse. Thus they are used at the lowest effective dose for treatment if being used [29].

Alpha-Lipoic acid and Actovegin are some novel agents that are also available currently for the pharmacotherapy of DPN. α-Lipoic acid is an antioxidant that lowers the oxidative stress, and improves blood flow to tissues and nerve conduction velocity [30]. But due to its availability

without prescription in countries such as the USA, its purity and safety are the reasons for concern for its usage in pain management in PDN.

TABLE 2.1: FDA approved drugs for treatment of diabetic neuropathy along with their category and mechanism of action

Drug category	Drugs	Mechanism of action	Reference
Anticonvulsants	Pregabalin, Gabapentin	Inhibit voltage gated calcium channel	[31]
Antidepressants	Duloxetine, Venlafaxine, TCAs	SNRI or SSRI or inhibition of voltage gated sodium channels, NDMA receptors	[32]
Opioids	Tapentadol	μ-opioid receptor agonist and NRI	[33]
Topical agents	Capsaicin	Vanilloid receptor agonist	[34]
	Lidocaine	Inhibit voltage gated sodium channel	[35]
Novel agents	α-Lipoic acid	Antioxidant	[36]
	Actovegin	Anti-hypoxia agent	[37]

Intravenous therapies include the administration of lidocaine and ketamine which can significantly reduce pain, and allodynia and provide relief. But they either become ineffective after a month of regular administration or become an addiction similar to opioids so they are not used that commonly. Further studies are needed to approve them for consistent use in PDN [27].

Certain studies have also indicated the positive effect of 4 hours of brisk walking per week to reduce the frequency of neuropathy in diabetics. Training the muscles to bear weight can also improve metabolic dysregulation and may even promote nerve regeneration [38]. Thus dietary changes and exercise bring about lifestyle intervention in diabetics and have shown good results for neuropathy management.

2.5 Gabapentinoids for analgesia

Gabapentinoids are structural analogs of γ-aminobutyric acid (GABA) that can bind to calcium channels and block them. They don't directly act on GABA receptors. They tend to bind to $\alpha_2\delta$ subunit of the calcium channel thus affecting the calcium influx in neurons. They are categorized under anticonvulsant drugs, for example, gabapentin and pregabalin. They are absorbed by facilitated transport and reach peak concentration within 1-3 hrs. But looking at their pharmacokinetic parameters, they are not readily metabolized by the liver and hence are eliminated from the body with an estimated half-life of 6 hrs [14]. They also exhibit anti-allodynic effects by preferentially affecting medium-sized neurons that are associated with nociceptive transmission. They act on high-voltage calcium channels to inhibit the forward trafficking of $\alpha_2\delta$ and calcium channels to the cell membrane of axons thus altering the excitability of dorsal neuron terminals. Gabapentinoids can also attenuate synaptic transmission by improving neurotransmitter release, modulating the descending serotonergic pathway which is associated with pain and increasing the activity of excitatory amino acid transporters (EAAT)-3 that endocytose excess glutamate [14][39].

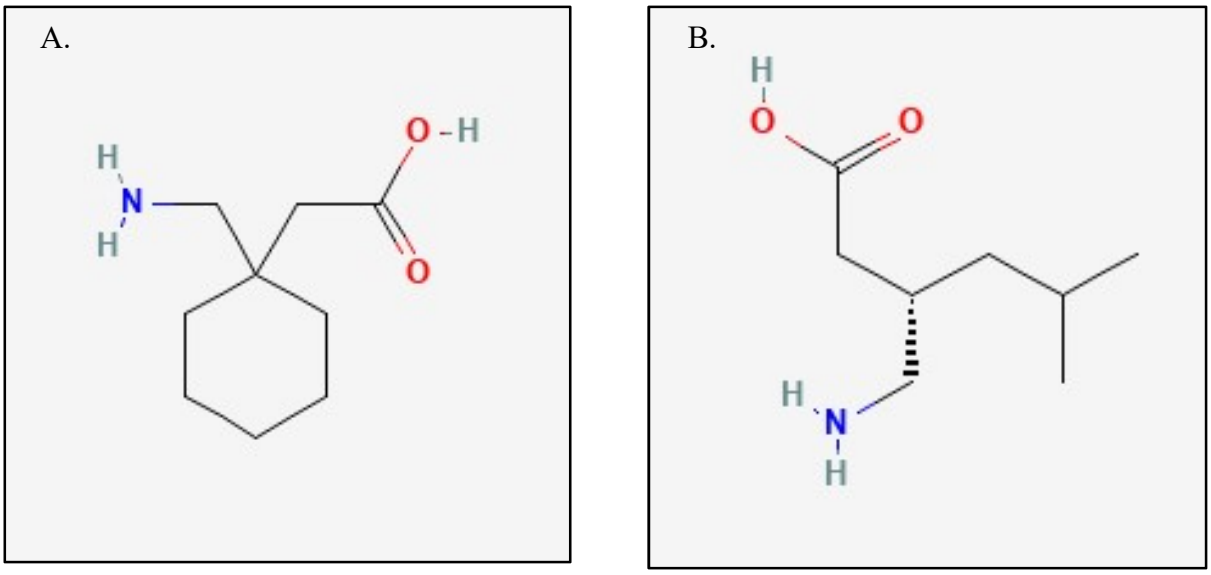


Fig 2.4 FDA approved gabapentinoids for neuropathy treatment. A. Gabapentin, B. Pregabalin[40][41]

2.6 Flavonoids and other natural compounds to rescue

Flavonoids are phenolic compounds that include six classes of compounds namely flavonols, flavones, flavanones, isoflavones, flavanols, and anthocyanidins which are the secondary metabolites of plants and fungi and have various potential roles as therapeutic agents for certain diseases such as diabetes, obesity etc [42]. These flavonoids, when ingested, are metabolized by the liver and converted to a more usable form that is more than the parent compound. They mainly show anti-inflammatory and antioxidant effects by reducing the inflammatory cytokine and acting against the free radicals in diabetes patients. They have also been shown to regulate glucose metabolism, liver enzymes, the proliferation of islet beta cells and their apoptosis, insulin signaling cascade, lipid accumulation in adipose tissues and act as inhibitors of certain nuclear factors and proteins involved in glucose signaling [43].

Some of the flavonoids that have been used in the amelioration of diabetic neuropathy in animal models are Isoquercitrin (ISQ), Formononetin (FMT), Naringenin, Hesperidin, Epigallocatechin-3-gallate (EGCG), Resveratrol, Catechin etc. ISQ is a quercetin derivative that inhibits the Wnt/β-catenin pathway to block the upregulation of β-catenin and MMP2 in spinal nerves which generally show an increased expression of Wnt pathway proteins and MMP2, and hence reduce neuropathic pain [44]. FMT on the other hand can control inflammation, limit the AGE production and accumulation, and improve nerve growth factors to allow the regeneration of axons. It can also lower blood sugar levels, improve nerve conduction velocity and reduce apoptosis in sciatic nerves. It can also control damage caused due to hyperglycemia by activating a histone deacetylase SIRT1 in sciatic nerve tissue [45]. The levels of nerve growth factor (NGF) during diabetes go down which affects axon regeneration, synaptogenesis and remyelination thus resulting in apoptosis of neurons and the development of neuropathy. But this can be attenuated by administration of FMT, which can increase NGF levels. Naringenin on the other hand, can show an analgesic effect by inhibiting voltage-gated sodium channels and reducing the influx of calcium in DRG neurons along with the reduction in serum glucose levels, improved insulin levels, reduced hyperalgesia and reduced levels of inflammatory cytokines [46]. Thus it can be seen that natural compounds, mainly flavonoids, act at multiple sites and show synergistic action to ameliorate disease. They possess least to no side effects and hence can be used for the treatment of certain diseases.

TABLE 2.2: List of some flavonoids and their mechanism of action against neuropathy amelioration

Flavonoid	Category of flavonoid	Mechanism of action	Reference
Isoquercitrin (ISQ)	Flavone	Inhibition of Wnt/β-catenin pathway thus reducing c-myc, β-catenin and MMP2 levels	[44]
Formononetin (FMT)	Isoflavone	Activates PI3K/Akt pathway in neuron, suppresses inflammation of cortical neurons, ADAM10-sAPPα pathway and AGE generation pathways, increases SIRT1 expression	[45]
Naringenin	Flavanone	Inhibit voltage gated Na-channels, reduce Ca influx in DRG, decrease serum glucose, increase insulin levels, lower inflammatory cytokine levels	[46]
Hesperidin	Flavanone	Anti-inflammatory as it lowers proinflammatory cytokine levels, analgesic by decreasing central sensitization of neurons and decreased expression of P_2X_3 mRNA levels in DRG, anti-hyperglycemic	[46]
Epigallocatechin-3-gallate (EGCG)	Flavan-3-ol	Reduces inflammation, oxidative stress and modulates tumorigenesis, lowers hyperalgesia, decreases pro-inflammatory cytokines in DRG, inhibits fatty acid synthase activity, downregulates CX_3CL_1 protein levels thus lowering hyperalgesia	[46]

Catechin	Flavan-3-ols	Upregulated function of antioxidant enzymes SOD and CAT, decreases lymphocyte infiltration and MDA (Malondialdehyde) levels thus exhibiting neuroprotective and antioxidant function	[47]

CHAPTER 3

METHODOLOGY

3.1 Identification of FDA-approved drugs

The FDA-approved drugs that are used as calcium channel blockers were identified and obtained from PubChem in 3D SDF format. These downloaded structures were then converted to .mol2 format using BIOVIA Discovery Studio 2021.

3.2 Pharmacophore model generation

The .mol2 structures of the FDA-approved drugs were loaded on ZINCPharmer [48] using the load features option. It scans the complete ZINC database [49] for structural similarity to the loaded compound. After submitting the query, hits for the drug were calculated by the software and results appeared in the right-hand column. It was done for all the drugs and the highest Zinc score was taken. The results gave the highest hit compound as **ZINC67172138,** which is basically 6-boc-hydrazino pyridine-3-carboxylic acid.

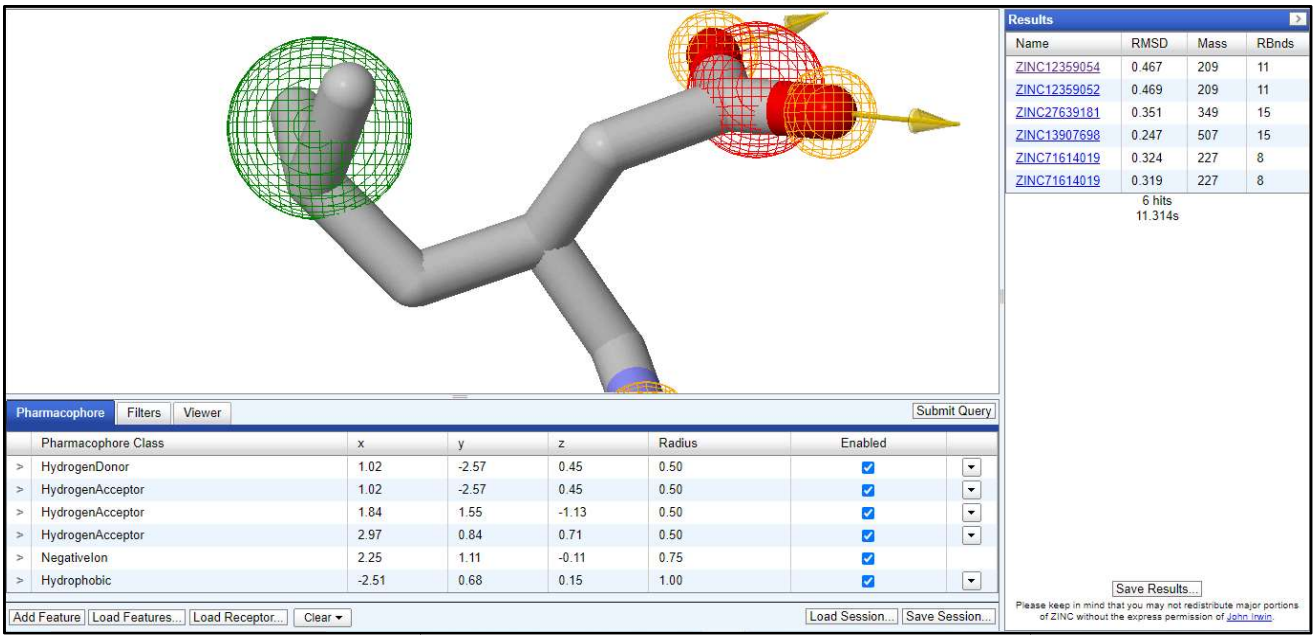


Fig 3.1: Pharmacophore model generation using ZINCPharmer. Right pane shows the ZINC IDs of best hits

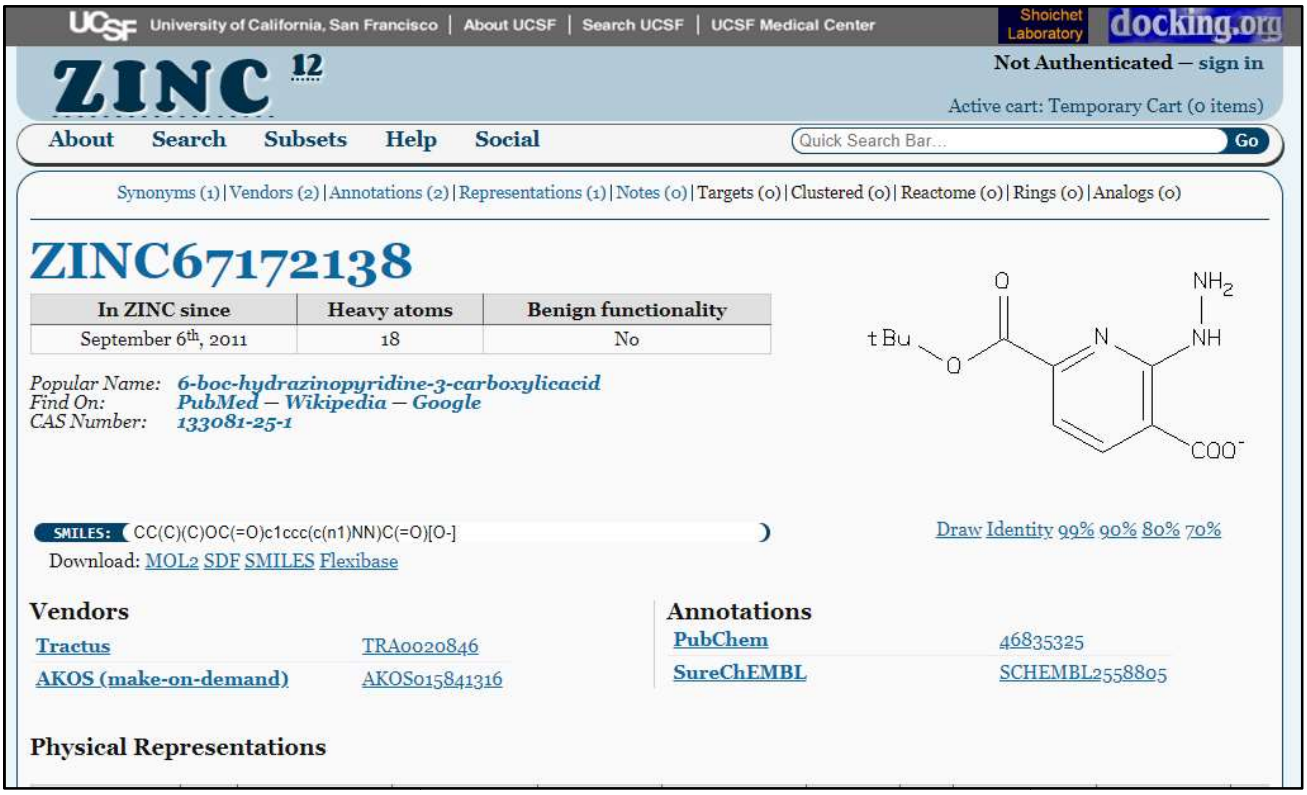


Fig 3.2: The compound obtained using ZINCPharmer that gave the highest hit (ZINC67172138)

3.3 Natural compound selection

After obtaining the highest zinc score compound, the SMILES of the compound were obtained and entered in the chemical similarity filter of IMPPAT which is a curated database for more than 4000 Indian medical plants containing more than 17000 phytochemicals [50][51].

3.4 ADME Analysis

The SMILES of these compounds were obtained and using SwissADME [52], the ADME profiling for all compounds was done. The compounds that were blood barrier permeant and followed the Lipinski rule of 5 were further downloaded in .pdb format for docking.

3.5 Protein and ligand preparation for docking

Protein preparation:

The protein or calcium channel was downloaded in .pdb format after which the file was opened in BIOVIA discovery studio to delete hetero atoms and bound ligands if any [53]. AutoDock tools (MGLTools) were used to delete water, and add hydrogen and Kollman charges to the structure. It was then saved in .pdbqt format. Kollman charges play a role in mimicking molecular electrostatic potential around the molecule.

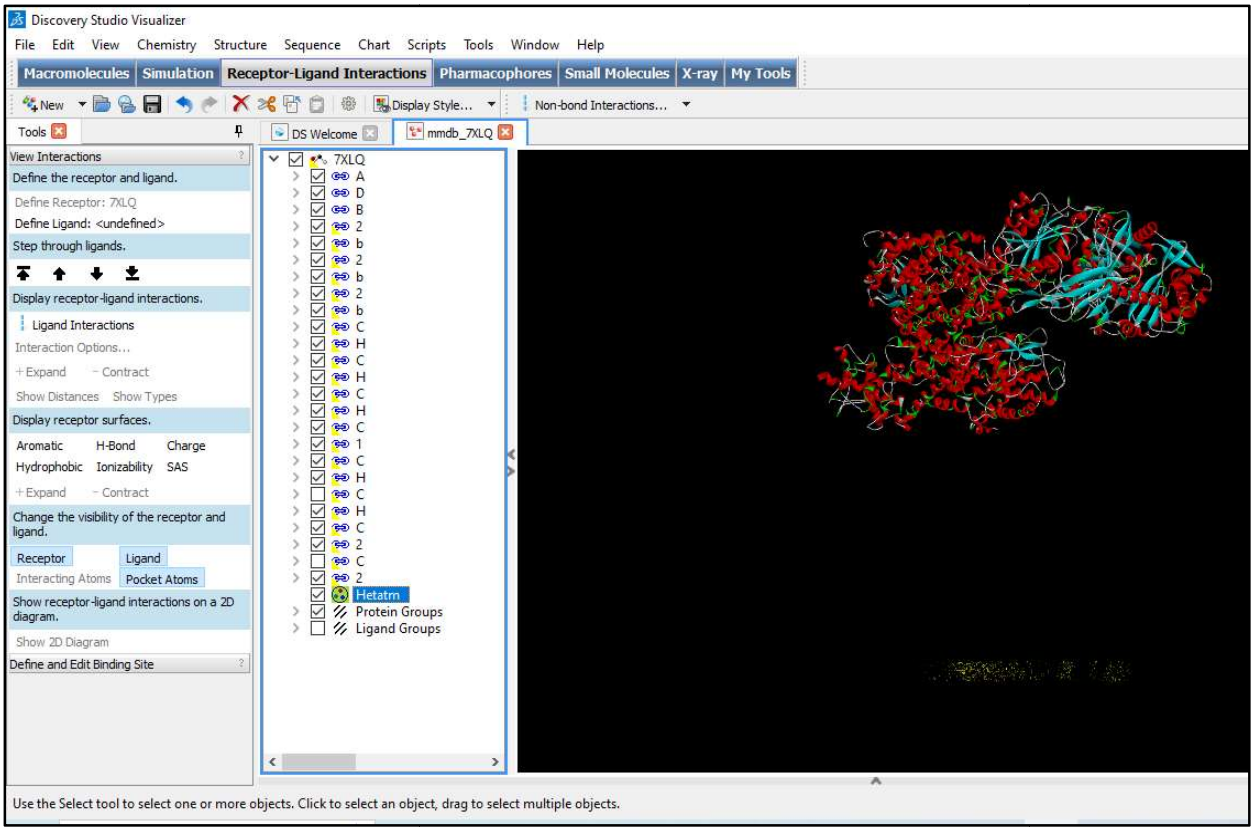


Fig 3.3: Protein preparation by removal of Het atoms and bound ligands in BIOVIA Discovery Studio

Ligand preparation:

The ligands were the natural compounds retrieved from IMPPAT that gave the best ADME scores and were BBB permeant. PubChem was used to obtain their SDF structures and BIOVIA Discovery Studio was used for format conversion to .pdb. These ligands were then individually prepared in MGLTools of Autodock tools and all hydrogens were added. The non-polar ones were merged and the Gasteiger charges were computed. This was followed by the detection of the torsion tree root and the files were saved in .pdbqt format.

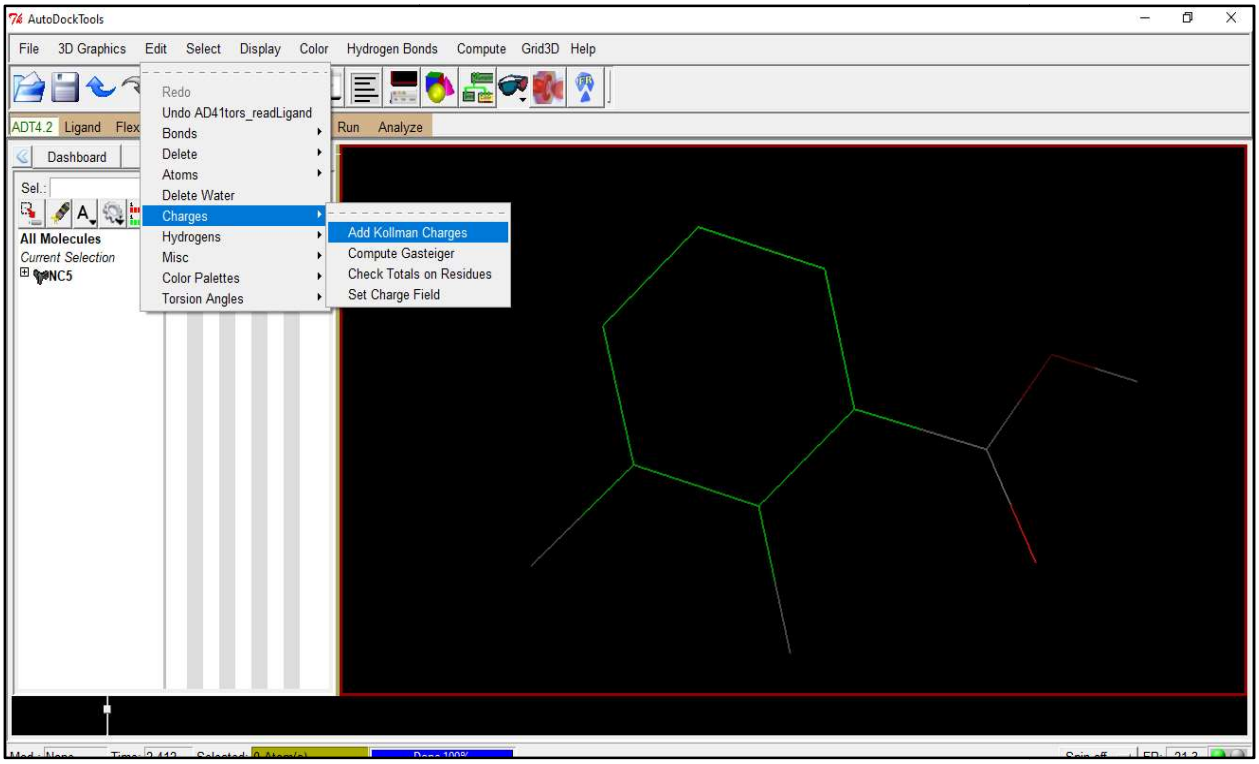


Fig 3.4: Ligand preparation by addition of hydrogen, kollman chargers and Gasteiger charges in MGLTools

3.6 Grid box formation and docking

- The protein or calcium channel in .pdbqt format was opened in MGLTools. After the selection of the molecule, a grid box was generated with points on X, Y and Z dimensions set to maximum (126).
- To cover the protein completely with the box, spacing was increased using a slider.
- The dimensions of the grid box were then exported in .txt format.
- AutoDock was initiated to analyze the binding of protein with different ligands. The results of binding energy were exported in .txt format and the ligand that gave the minimum binding energy (more negative) was further used for visualization using Chimera and the 2D image was downloaded using BIOVIA Discovery Studio.

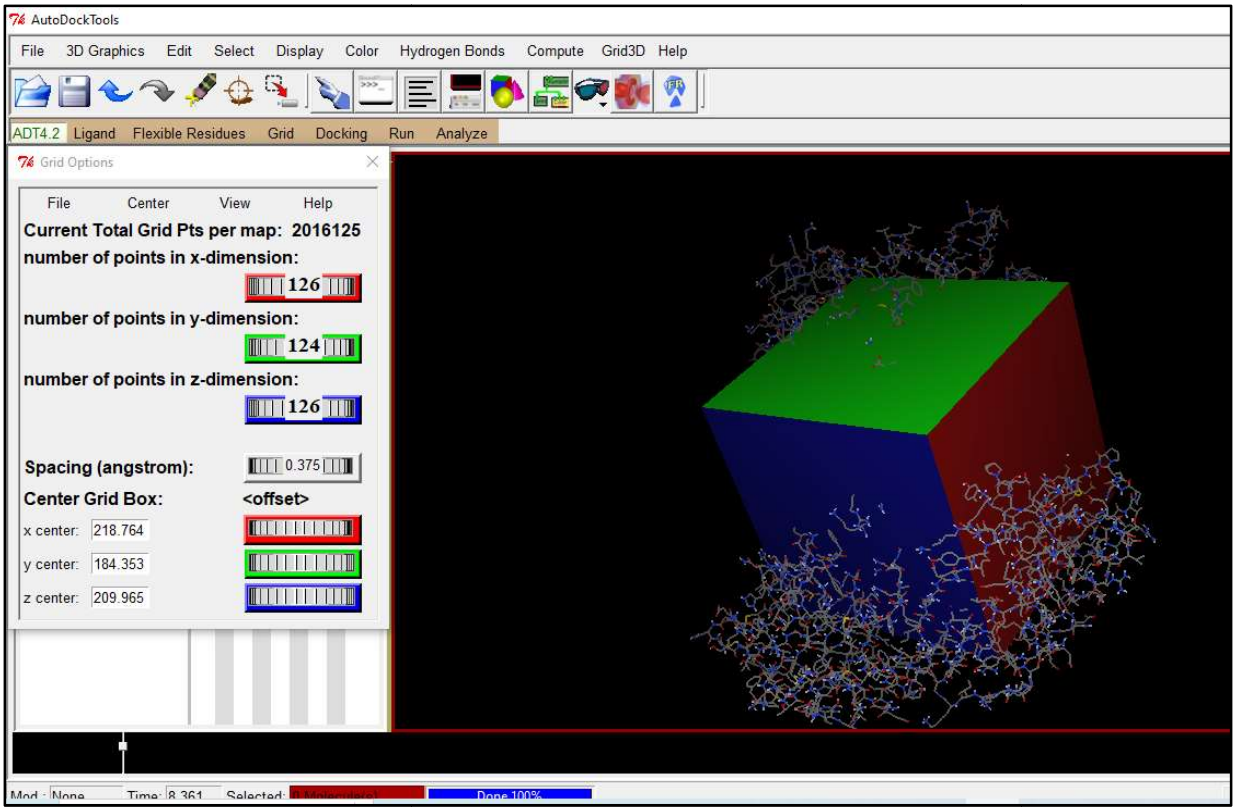


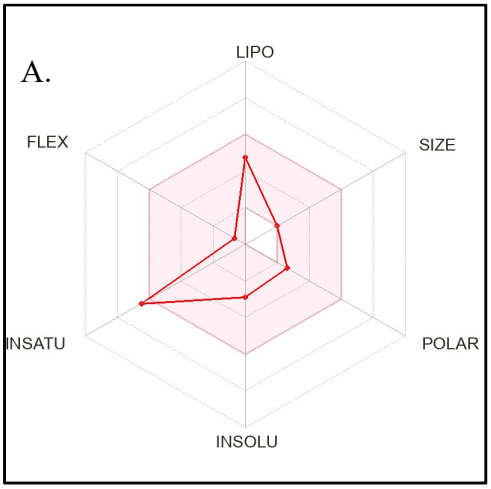
Fig 3.5: Grid box generation around protein in AutoDock

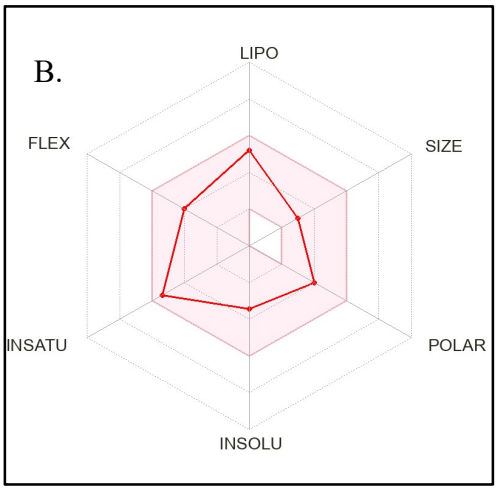
CHAPTER 4

RESULTS

4.1 ADME analysis

The phytochemicals procured after SMILES input into the IMPPAT were subjected to ADME analysis. Of the 10 best hits (Appendix 1, Table S1), 7 were BBB permanent. They also showed good ADME aspects and passed the Lipinski rule of 5. These compounds were- 2,4-Dimethylbenzoic acid, 2-((Isobutoxycarbonyl)amino)benzoic acid, 2,3-Dimethylbenzoic acid, 2,5-Dimethylbenzoic acid, 2,5-Dimethoxybenzoic acid, tert-Butyl 1H-imidazole-1-carboxylate and Methyl N-acetylanthranilate. These were then docked to assess their binding energies.





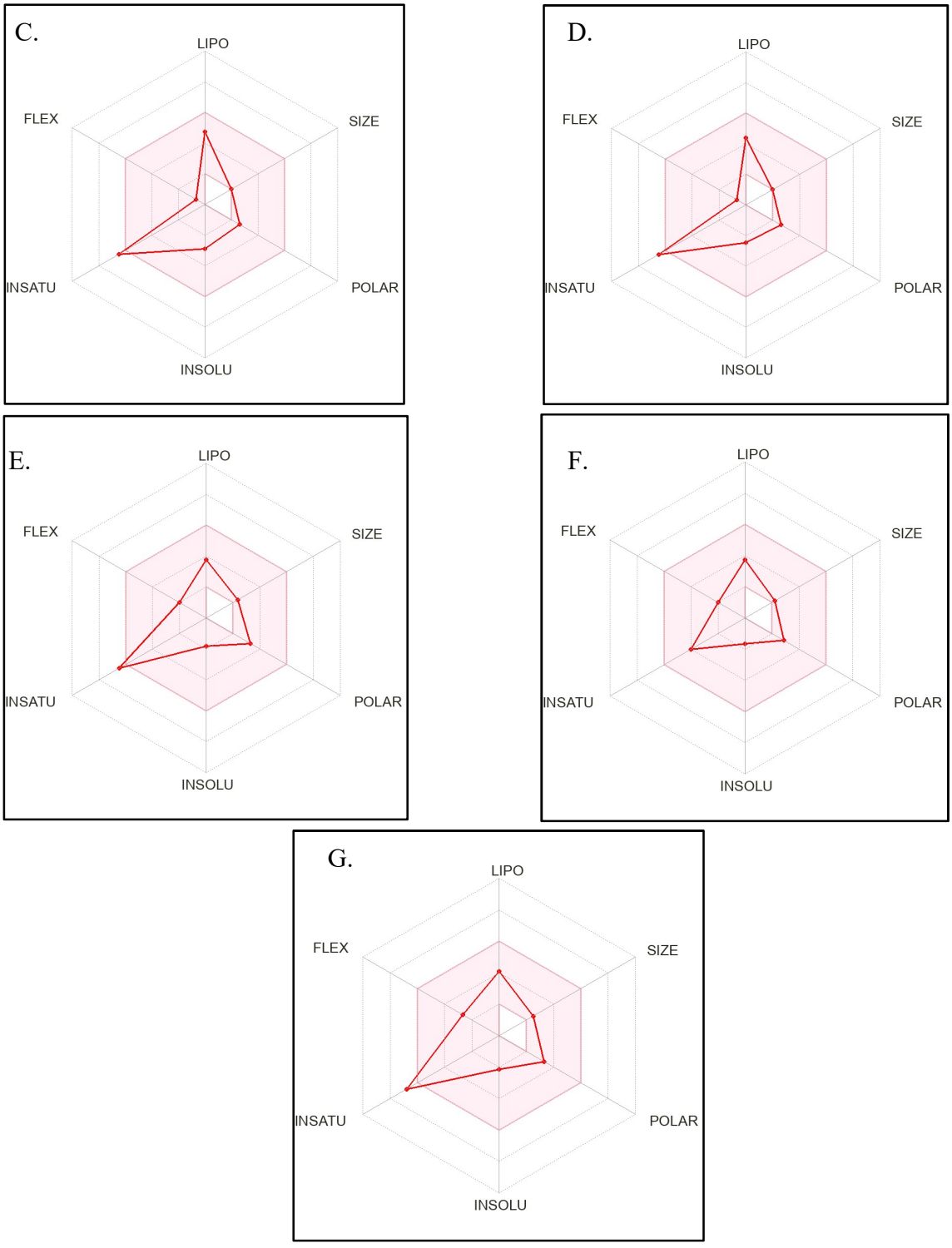


Fig 4.1: Phytochemicals with best ADME scores- A. 2,4-Dimethylbenzoic acid, B. 2-((Isobutoxycarbonyl)amino)benzoic acid, C. 2,3-Dimethylbenzoic acid, D. 2,5-Dimethylbenzoic acid, E. 2,5-Dimethoxybenzoic acid, F. tert-Butyl 1H-imidazole-1-carboxylate and G. Methyl N-acetylanthranilate

4.2 Docking results

The binding energies of both control drugs and natural compounds obtained from IMPPAT were obtained after docking with the protein.

(The 3D structure of 2-((Isobutoxycarbonyl)amino)benzoic acid was not available thus, docking could not be performed for this compound)

TABLE 4.1: List of docked phytochemicals and their binding energies

Phytochemical name	Binding energy(kcal/mol)	Distance from rmsdl.b.	Distance from rmsdu.b.
2,4-Dimethylbenzoic acid	-6.3	0	0
2,3-Dimethylbenzoic acid	-6.4	0	0
2,5-Dimethylbenzoic acid	-5.9	0	0
2,5-Dimethoxybenzoic acid	-5.8	0	0
tert-Butyl 1H-imidazole-1-carboxylate	-5.2	0	0
Methyl N-acetylanthranilate	-6.3	0	0
Gabapentin (Control)	**-5.7**	**0**	**0**

2,3-Dimethylbenzoic acid showed the least binding energy (-6.4 kcal/mol) as compared to other natural compounds thus the best binding affinity towards calcium channel in contrast to the control drug Gabapentin (-5.7 kcal/mol).

4.3 Visualize 2D structure

The 2D image of the best docked compound (2,3-Dimethylbenzoic acid) and FDA approved drug Gabapentin (control) was obtained using BIOVIA Discovery Studio 2021 to analyze the interactions.

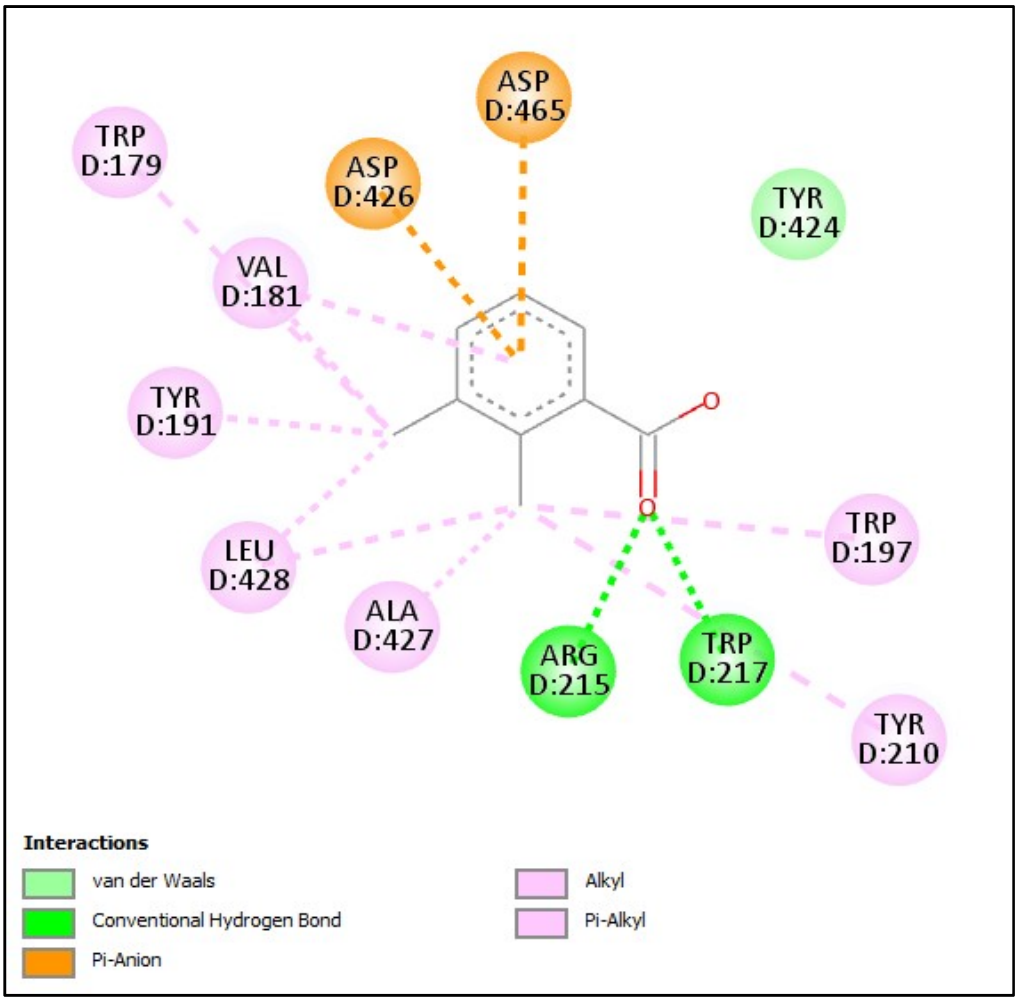


Fig 4.2: 2D image of 2,3-Dimethylbenzoic acid after docking

The compound 2,3-Dimethylbenzoic acid forms 7 alkyl bonds with amino acids Trp 179, Trp 191, Trp 197, Trp 210, Leu 428, Ala 427 and Val 181 and two conventional hydrogen bonds with Arg 215 and Trp 217. It also forms 2 pi-anion bonds with amino acids Asp 426 and Asp 465.

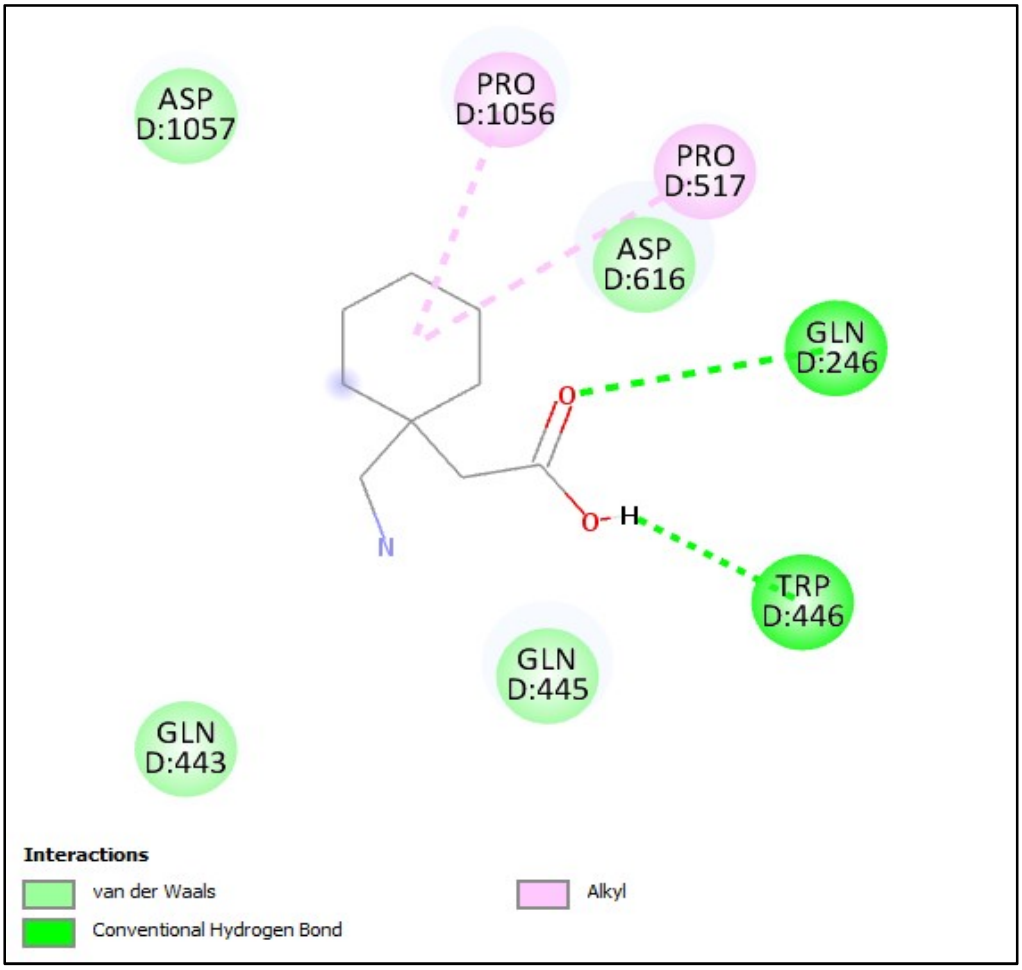


Fig 4.3: 2D image of Gabapentin after docking

The FDA-approved Gabapentin forms 2 alkyl bonds with amino acids Pro 1056 and Pro 157. Additionally there are formation of two conventional hydrogen bonds with amino acids Gln 246 and Trp 446 indicating that the natural compound forms better and more bonds with the channel thus giving better results as compared to the control.

CHAPTER 5

DISCUSSION

It is evident from the part in sections 2.2 and 2.3 that an alteration in expression levels of voltage-gated ion channels, gain of function of ion channels due to their glycation, trafficking and post-translational modification of ion channels occurs in neurons due to hyperglycemia. Thus there is a change in the stimulus-response of neurons.

Both Na^{+} and Ca^{+2} channels act as major contributors to the development and progression of neuropathic pain. An increased influx of calcium into neurons results in their hyperexcitability and hence leads to hyperalgesia and allodynia [14][17]. Certain drugs such as antidepressants and anticonvulsants are employed for attenuating this pain. Antidepressants such as gabapentinoids (GABA analogs) that include Gabapentin and Pregabalin act by binding to the $\alpha_2\delta$ subunit of the calcium channel and inhibit trafficking of the calcium channel subunit to the cell membrane, improve neurotransmitter release and increase glutamate endocytosis [14]. But these drugs come with certain side effects such as hyperhidrosis, and fatigue and people may become addicted to these drugs. It is thus important to introduce alternatives that are less toxic and help ameliorate neuropathic pain.

Thus, certain flavonoids and other natural compounds are being tested and experimented for their potential to treat diabetic neuropathy. These compounds not only act on multiple targets but also are stable and less or non-toxic for the human body [54]. These plant-derived compounds have been in practice in Ayurveda and Yunani medicine since ages without exhibiting toxicity or

unwanted consequences. Thus discovery and validation of such compounds can be used to replace FDA-approved drugs with high toxicity or side effects.

The IMPPAT database provides such a platform to curate a list of such compounds and detect their potential use by high throughput screening methods. The compounds can be checked for their binding affinity with desired protein or receptor using docking that allows the binding of ligand and protein in an in-silico setup.

Docking analysis gave 2,3-Dimethylbenzoic acid which is a benzoid, as the phytochemical with the least binding energy as compared to the control Gabapentin. It is obtained from *Cinnamomum porrectum* root and wood and is used as an antirheumatic agent [50][51].

CHAPTER 6

CONCLUSION

The present work involved the in-silico determination of a natural compound that can be used as a calcium channel blocker to attenuate neuropathic pain. Initially, the FDA-approved drugs for neuropathic pain were curated and a pharmacophore model for the same was generated using ZINCPharmer. The natural compounds with structural similarity to the best-hit ZINC compound were extracted after which their bioavailability testing was done to determine their blood-brain barrier permeability. After docking, 2,3-Dimethylbenzoic acid obtained from *Cinnamomum porrectum* root and wood gave the best docking results. Though FDA-approved drugs are more specific to target and show quicker relief, their toxicity in the blood, brain and other organs of the body is a major concern that calls for the need to switch to natural compounds for therapeutic purposes. Thus, 2,3-Dimethylbenzoic acid can act as a potential replacement of gabapentinoids for attenuation of painful symptoms of diabetic neuropathy.

APPENDIX

APPENDIX 1: SUPPLEMENTARY INFORMATION

S1. Lead compounds obtained from IMPPAT

10 lead compounds were obtained after input of pharmacophore SMILES structure into IMPPAT search engine. These compounds are calculated on the basis of structural similarity and Tanimoto coefficient.

TABLE S1. Phytochemical ID, Tanimoto coefficient and name of photochemical that best match with the best pharmacophore structure

Similar Phytochemical	Tanimoto coefficient	Phytochemical name
IMPHY013518	0.32	Cerberic acid
IMPHY016998	0.31	2,4-Dimethylbenzoic acid
IMPHY006175	0.3	4-Methoxysalicylic acid
IMPHY017255	0.29	2-((Isobutoxycarbonyl)amino)benzoic acid
IMPHY016995	0.29	2,3-Dimethylbenzoic acid
IMPHY016997	0.29	2,5-Dimethylbenzoic acid
IMPHY006151	0.28	2,5-Dimethoxybenzoic acid
IMPHY013974	0.27	tert-Butyl 1H-imidazole-1-carboxylate
IMPHY003115	0.27	4,5-Dimethoxyphthalic acid
IMPHY017171	0.27	Methyl N-acetylanthranilate

APPENDIX 2: LIST OF PUBLICATIONS

1. Kalra Tanya, and Kumar Pravir (2023). Identification of novel therapeutic compounds against Diabetic nephropathy: A drug repurposing approach. IEEE Bangalore Humanitarian Technology Conference (IEEE B-HTC)- Accepted

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Chair - IEEE Mysore Subsection
Professor, Dept. of ECE,
SJCE (JSSSTU), Mysuru

Dr. Prashant M Vishwanath
Dean (Research)
JSS AHER, Mysuru -15

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Identification of novel therapeutic compounds against Diabetic nephropathy: A drug repurposing approach

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***Abstract*- Chronic diabetes often leads to complications such as diabetic nephropathy which develops into end stage-renal disease over time marked by glomerular fibrosis, endothelial dysfunction, proteinuria, and ultimately kidney failure. One of the major causes of such deteriorating situations is buildup of reactive oxygen species or ROS that leads to oxidative stress in renal tissue. eNOS or endothelial nitric oxide synthase is an enzyme that takes care of ROS buildup. But in hyperglycemic patients there is a reduced amount of eNOS. We thus curated a list of 100 drugs that were assessed via molecular docking for their potential to enhance eNOS expression or activity and/or improve NO bioavailability to overcome oxidative stress. The molecular docking conducted gave the results that pemafibrate, a drug used in treatment of dyslipidemia is a potential drug that can increase eNOS and help overcome oxidative damage.**

KEYWORDS: Diabetic nephropathy, eNOS(endothelial nitric oxide synthase), drug repurposing, molecular docking, pemafibrate

I. INTRODUCTION

A. Diabetic nephropathy

Diabetes has affected a total of 422 million people worldwide and its prevalence is rising every year leading to around 1.5 million deaths every year(https://www.who.int/health-topics/diabetes#tab=tab_1). Its progression leads to damage to organs such as pancreas, retina, kidneys, heart and nervous system becoming a cause of retinopathy, nephropathy, myopathy and neuropathy. Amongst the various genetic factors that are responsible for pathogenesis and progression of diabetic complications, mutations in *MTHFR, APOE, ADRA2B, PPAR, ACE* and *eNOS* genes play a major role and are majorly studied [1].

Diabetic nephropathy (DN) is the major cause of development of end stage renal disease (ESRD). Diabetic nephropathy may or may not develop as a direct cause of hyperglycemia or glucotoxicity; the pathogenic pathways are diverse; major pathways being RAAS, AGE accumulation, PKC and MAPK activation and ROS generation [2].

eNOS or endothelial nitric oxide synthase enzymes plays a role in production of nitric oxide which regulates the vascular diameter, keeps superoxide radicals under control and prevents platelet aggregation that leads to initiation of clot formation cascade**.** Thus, eNOS genetic anomalies specifically at rs2070744, rs1799983 and rs869109213 (4a/b) locations can cause alteration in its activity leading to diabetic complications of nephropathy [3]. A mutation in this gene is also related to cardiac autonomic neuropathy, uremic neuropathy along with hypertension and atherosclerosis.

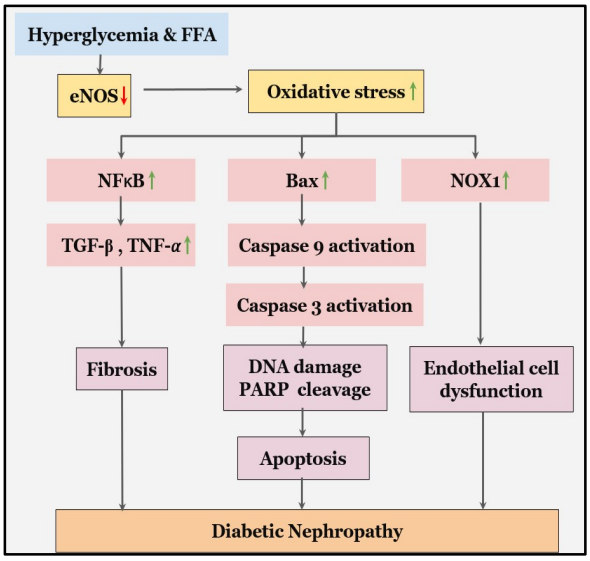

Fig. 1. Role of eNOS and oxidative stress in progression of hyperglycemia to diabetic nephropathy. eNOS downregulation results in increased oxidative stress that upregulates certain signaling pathways in the renal cells leading to fibrosis, apoptosis and endothelial cell dysfunction ultimately progressing to diabetic nephropathy.

As depicted in Fig. 1, oxidative stress caused due to accumulation of reactive oxygen species is evident in diabetic patients, which leads to increased proinflammatory cytokines by infiltrating macrophages causing local inflammation that damages podocytes, mesangial cells and endothelial cells resulting in fibrosis [2]. Excess blood sugar causes uncoupling of eNOS, cofactor dissociation, decrease in phosphorylation of eNOS at its important Ser and Thr residues that leads to its decreased activity and promote its acetylation [4]. Decreased eNOS also increases VEGF levels, lead toinflammation and aggravates DN by altering vascular morphology [5]. Thus, targeting the acetylation and phosphorylation of eNOS, recoupling of eNOS and improving bioavailability of NO in kidney can help alleviate renal damage due to oxidative stress and treat DN. Improving eNOS availability can also regulate angiogenesis and improve glomerular injury repair.

B. *SwissDock*

Swissdock is an online platform that allows prediction of molecular interactions between target protein and small molecules such as drug, inhibitor, ligand, etc and gives output in the form of CHARMM energies after local or blind docking (http://www.swissdock.ch/). It is based on Ajax/HTML interface with a built-in S3DB database that has manually curated complexes, whose working is similar to AutoDock Vina, AutoDock and Molegro Virtual Docker, based on Vital-IT clustering and is used for two-step SBVS(Structure-based virtual screening) docking[6] [7] [8].

C. *Therapeutic intervention*

eNOS activation and increasing NO bioavailability in the renal tissues can help reduce oxidative stress and overcome renal fibrosis and endothelial damage. eNOS can be activated directly by increasing its transcription or via phosphorylation or by enhancing its coupling to allow efficient production of NO. Indirect ways included mainly via activation of PI3K-Akt-eNOS signaling or MAPK-Erk signaling pathways or via drugs that metabolize to release NO.

Using eNOS activators can help slow the progression to ESRD and promote recovery of damaged endothelium at a faster pace [4]. For this, the conventional approaches for drug discovery and formulation tend to be really laborious and time intensive, thus it becomes necessary to opt for newer and better methods such as drug repurposing to target the molecule and check its efficiency using molecular docking software.

II. METHODOLOGY

A. *Data collection*

A list of 100 FDA approved drugs and natural molecules was curated through literature and drugbank which had the potential to activate eNOS and/or improve NO bioavailability. These compounds were then screened for their ability to directly or indirectly activate eNOS or improve NO levels at the target site via literature review. Out of 100 compounds, 28 compounds were found to directly activate eNOS. The SDF structures for all these compounds were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/). The nitric oxide synthase enzyme structure was also downloaded from the PDB database.

B. *Preparation of target and ligand*

For carrying out docking of enzyme and shortlisted compounds, the enzyme eNOS (target) was prepared using BIOVIA Discovery Studio. It was done by

removing Hetatm (hetero atoms), water molecules and ligand groups from the PDB structure of target. The structure was then saved in PDB format. The SDF structures of ligands (drugs) were converted to MOL2 format using BIOVIA Discovery Studio, which is a visualization tool and saved.

C. Molecular docking using SwissDock

The eNOS PDB file was uploaded in the target section of SwissDock (http://www.swissdock.ch/)- 'submit docking' section. The structure and aspect ratio of the uploaded structure were checked by Swiss Dock and accepted within a few minutes. The MOL2 file of each ligand was uploaded in the ligand section of SwissDock- 'submit docking' section, one after the other and SwissDock checked the topology of structures uploaded. Docking was then started. The results for free energy estimates of each docking were obtained via email which were then retrieved via Chimera for future analysis. Compounds with free binding energy of -8.27 kcal/mol or less were considered and ranked.

III. RESULT

Only 8 compounds met the criteria of binding energy less than -8.27 kcal/mol out of 28 compounds, whose 2D structure has been depicted in Fig. 3, that could directly activate eNOS. Pemafibrate with binding energy of -9.18 kcal/mol gave the best output and hence was considered as a winner in this case. Table 1. compares the binding energy of top 8 compounds.

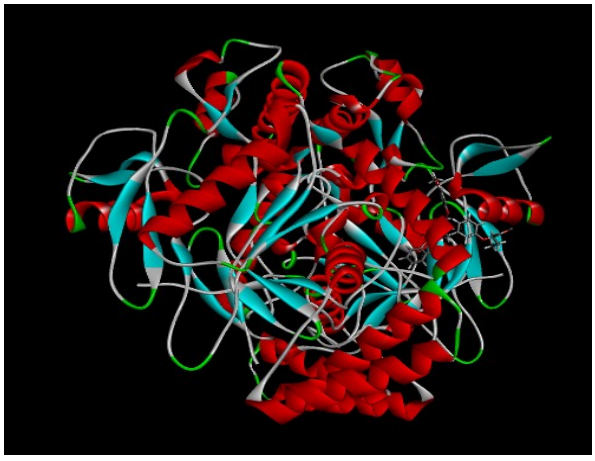

Fig. 2. Pemafibrate 3D-docked structure inSwissDock

K-877 Pemafibrate (EC_{50}= 1 nM) (https://drugs.ncats.io/drug/17VGG92R23) sold as Parmodia® is a selective peroxisome proliferator-activated receptor(PPAR)-α agonist, developed by Kowa Company Ltd., which is used for treating dyslipidemic patients, got its approval in the year 2017 [9]. It is also currently under Phase III of clinical trials for treatment of cardiovascular abnormalities by reduction in triglyceride levels in diabetics (https://clinicaltrials.gov/ct2/show/NCT03071692).

TABLE 1. ESTIMATED BINDING ENERGY OF ENOS ACTIVATING DRUGS.

Drugs	Estimated ΔG (kcal/mol)
Pemafibrate	-9.18
Pravastatin	-8.66
Icariin	-8.58
Berberine	-8.53
BM-573	-8.5
Nebivolol	-8.4
Chlorogenic acid	-8.32
Pioglitazone	-8.31
Astragaloside IV	-8.27

As per recent studies, pemafibrate's role in enhancing eNOS activity and expression had been discovered. Thus it can help in overcoming oxidative stress and improve DN by decreasing Bax activation in mitochondria which signals for caspase activation that lead to PARP cleavage and DNA damage which can subsequently lead to apoptosis of cells. Fibrosis of renal tubules is also prevented as there is decreased activation of TGF-β, TNF-α and NFκB which activate the signaling cascade for arteriolar constriction and glomerular sclerosis.

It can also prevent detachment of podocytes, ECM accumulation, proteinuria and tubular atrophy that are otherwise responsible for DN.

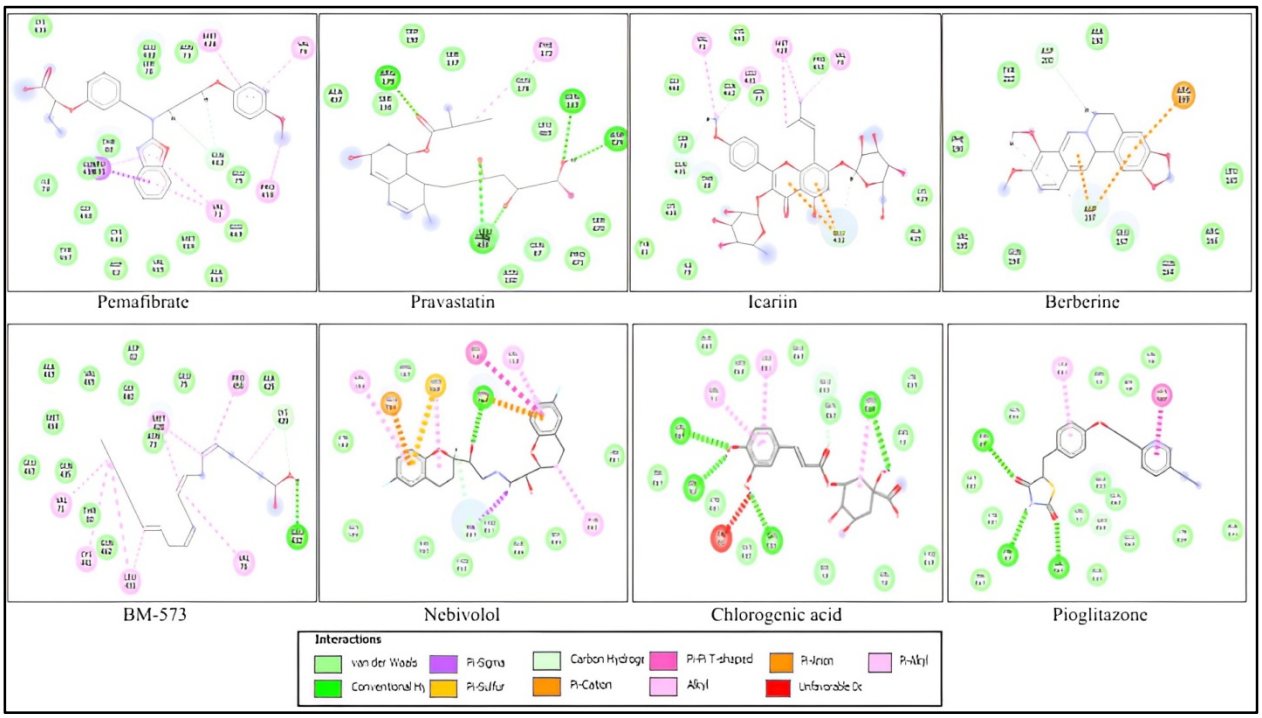

Fig. 3. 2D- structures of docked drugs with binding energy less than -8.27 kcal/mol

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Therefore, pemafibrate can be repurposed as a potential drug for treatment of DN other than its current use in treatment of dyslipidemia.

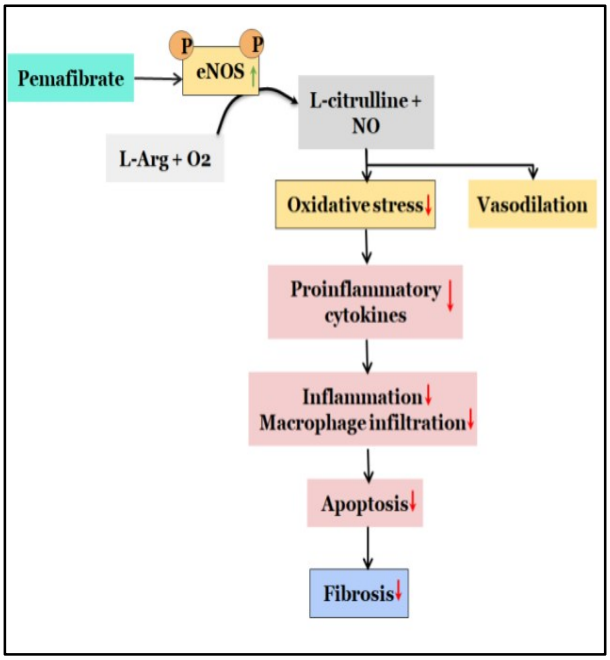

Fig. 4. Pemafibrate action on eNOS and alleviation of DN

IV. DISCUSSION

The therapeutic strategies for diabetic complications mainly involve blood pressure control and glucosecontrol via diet, exercise and drugs to which people develop resistance over time. Other drugs that are used in case of DN are ACE-inhibitors and endothelin receptor antagonists but these have severe side effects such as risk of hyperkalemia and congestive heart failure [2]. Thus, therapies targeting oxidative stress and medicinal interventions via use of bioinformatic tools for molecular docking can help skip the long durations of drug development process. Via this approach and technology, repurposing already available drugs can help save money and time and screen potential drugs for therapeutic purposes.

Using this approach, we tried to repurpose Pemafibrate as a potent eNOS activator for treatment of diabetic nephropathy. It is a promising drug in overcoming oxidative stress with EC50 of 1nM and better docked than other drugs such as pravastatin, nebivolol, etc. It thus results in overcoming renal damage caused due to oxidative stress. As per the

studies done so far, a dosage of 0.1-0.3 mg/kg/day Pemafibrate had been administered in mice models for lowering triglyceride levels while in human trials 0.4 mg/kg/day was administered to see positive effects on patients. Though, establishing a fixed dose requirement for diabetic nephropathy would require undertaking further studies on animal models and human clinical trials[10][11].

V. CONCLUSION

The results of this study helped in identifying a drug that can be used for treatment of diabetic nephropathy with considerable low free energy of binding as compared to other drugs. Thus, this drug other than its use in dyslipidemia treatment can be used for preventing DN. Further studies need to be carried out on Pemafibrate to validate the proposed results.

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