AN AYURVEDA BIOLOGY APPROACH FOR UNDERSTANDING THE MULTI-MODULATORY EFFECTS OF AYURVEDA FORMULATIONS FOR MANAGING DIABETES AND OBESITY

A THESIS TO BE SUBMITTED TO

THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY

FOR THE AWARD OF THE DEGREE OF
DOCTOR OF PHILOSOPHY

BY
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UNDER THE GUIDANCE OF

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BENGALURU - 560064

DECLARATION BY THE CANDIDATE

I declare that this thesis entitled "**An Ayurveda Biology approach for understanding the multi-modulatory effects of Ayurveda formulations for managing diabetes and obesity**" submitted for the award of Doctor of Philosophy to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bengaluru, is my original work, conducted under the supervision of my guide Dr CN Vishnuprasad and co-guide, Dr Subrahmanya Kumar. I also wish to inform that no part of the research has been submitted for a degree or examination at any university. References, help and material obtained from other sources have been duly acknowledged

I hereby confirm the originality of the work and that there is no plagiarism in any part of the dissertation.

Place: Bengaluru **Signature of the Candidate**

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CERTIFICATE

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CERTIFICATE

I certify that this thesis entitled **An Ayurveda Biology approach for understanding the multi-modulatory effects of Ayurveda formulations for managing diabetes and obesity** comprises research work carried out by Anjana. T at The University of Trans-Disciplinary Health Sciences and Technology (TDU) under the supervision of Dr. C.N. Vishnuprasad (TDU, Bangalore) and co-supervision of Dr. Subrahmanya Kumar (TDU, Bangalore) during the period 2018-2024 for the degree of Doctor of Philosophy of The University of Trans-Disciplinary Health Sciences and Technology (TDU). The results presented in this thesis have not been submitted previously to TDU or any other University for a Ph.D. or any other degree.

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Chakshurunmilitam Yena Tasmai Sri Gurave Namah'

I offer my humble salutations to Guru, the dispeller of ignorance, and one who opens my eyes to true knowledge

Contents

List of Abbreviation

Abbreviation		Description
5-HT	:	Serotonin
ACHE	:	acetylcholinesterase
ADA	:	adenosine deaminase
ADRA2A	:	adrenoceptor alpha 2A
ADRA2A	:	adrenoceptor alpha
ADRA2B	:	adrenoceptor alpha 2B
ADRA2C	:	adrenoceptor alpha 2C
AGTR2	:	angiotensin II receptor, type 2
AKR1A1	:	aldo-keto reductase family 1 member A1
AKR1B1	:	aldo-keto reductase family 1 member B1
AKR1B10	:	aldo-keto reductase family 1 member 10
AKT1	:	AKT serine/threonine kinase 1
ALB	:	Albumin
ALOX12	:	arachidonate 12-lipoxygenase, 12S type
ALOX15	:	arachidonate 15-lipoxygenase
ALOX5	:	arachidonate 5-lipoxygenase,
AMY2A	:	amylase alpha 2A
APOA1	:	apolipoprotein A1
APP	:	amyloid beta precursor protein
AR	:	Aryl hydrocarbon receptor
AVPR1A	:	arginine vasopressin receptor 1A
AVPR2	:	arginine vasopressin receptor 2
B2M	:	Beta 2 microglobulin
BACE1	:	beta-secretase 1
BCHE	:	butyrylcholinesterase
BCL2	:	BCL2 apoptosis regulator
CA1	:	carbonic anhydrase 1
CA12	:	carbonic anhydrase 12
CA14	:	carbonic anhydrase 14
CA2	:	carbonic anhydrase 2
CA4	:	carbonic anhydrase 4
CA7	:	carbonic anhydrase 7
CACNA1C	:	alcium voltage-gated channel subunit alpha1 C
CACNA1H	:	calcium voltage-gated channel subunit alpha1 H
CASP3	:	Caspase 3
CASP7	:	Caspase 7
CASP8	:	Caspase 8
CASP9	:	Caspase 9
CASR	:	calcium sensing receptor
CaSR	:	calcium-sensing receptor
CCK	:	Cholecystokinin
CCL2	:	C-C motif chemokine ligand 2
CD52	:	cluster of differentiation 52
CES1	:	Carboxyl esterase 1
CHRM4	:	cholinergic receptor muscarinic 4
CHRM5	:	cholinergic receptor muscarinic 5

CHRNA4	:	cholinergic receptor nicotinic alpha 4 subunit
CNDP2	:	carnosine dipeptidase 2
CNR2	:	cannabinoid receptor 2
COMT	:	catechol-O-methyltransferase
CRABP2	:	cellular retinoic acid binding protein 2
CRYAB	:	crystallin alpha B
CSNK2A1	:	casein kinase 2 alpha 1
CTSD	:	cathepsin D
CYP142	:	cytochrome P450 family 142
CYP17A1	:	cytochrome P450 family 17 subfamily A member 1
CYP19A1	:	cytochrome P450 family 19 subfamily A member 1
CYP1A1	:	cytochrome P450 family 1 subfamily A member 1
CYP1A2	:	cytochrome P450 family 1 subfamily A member 2
CYP1B1	:	cytochrome P450 family 1 subfamily B member 1
CYP2B6	:	cytochrome P450 family 2 subfamily B member 6
CYP3A4	:	cytochrome P450 family 3 subfamily A member 4
CYP51A1	:	cytochrome P450 family 51 subfamily A member 1
CYP7A1	:	cytochrome P450 family 7 subfamily A member 1
CYSLTR1	:	cysteinyl leukotriene receptor
DHCR24	:	24-dehydrocholesterol reductase
DHODH	:	dihydroorotate dehydrogenase
DPP4	:	dipeptidyl peptidase 4
DRD2	:	dopamin receptor 2
EBP	:	EBP cholestenol delta-isomerase
EDNRA	:	Endothelin Receptor Type A
EDNRB	:	endothelin receptor type B
EGFR	:	epidermal growth factor receptor
ERAP2	:	endoplasmic reticulum aminopeptidase 2
ESR1	:	estrogen receptor 1
ESR2	:	estrogen receptor 2
F10	:	coagulation factor X
F2	:	coagulation factor 2
F7	:	coagulation factor VII
FAAH	:	fatty acid amide hydrolase
FABP2	:	fatty acid binding protein 2
FABP3	:	fatty acid binding protein 3
FABP4	:	fatty acid binding protein 4
FABP4	:	fatty acid binding protein 4
FABP5	:	fatty acid binding protein 5
FASN	:	fatty acid synthase
FATP4	:	solute carrier family 27-member 4
FFAR1	:	free fatty acid receptor 1
FYN	:	Proto-oncogene tyrosine-protein kinase Fyn
GAA	:	alpha glucosidase
GANC	:	glucosidase alpha, neutral C
GCG	:	Glucagon
GIP	:	gastric inhibitory peptide
GLA	:	galactosidase alpha
GLO1	:	glyoxalase I
GLP-1	:	Glucagon like peptide 1

GLP1R	:	glucagon like peptide 1 receptor
GLP-2	:	Glucagon like peptide 2
GLUT4	:	solute carrier family 2 member 4
GNB1	:	G protein subunit beta 1
GRIN1	:	glutamate ionotropic receptor NMDA type subunit 1
GRIN2A	:	glutamate ionotropic receptor NMDA type subunit 2A
GRIN2B	:	glutamate ionotropic receptor NMDA type subunit 2B
GSK3B	:	glycogen synthase kinase 3 beta
GSTM3	:	glutathione S-transferase mu 3
HCAR3	:	hydroxycarboxylic acid receptor 3
HDAC2	:	histone deacetylase 2
HDAC3	:	histone deacetylase 3
HDAC6	:	histone deacetylase 6
HDAC9	:	histone deacetylase 9
HFD	:	high fat diet
HLA-A	:	human leukocyte antigen
HLAB	:	major histocompatibility complex, class I, B
HLA-C	:	major histocompatibility complex, class I, C
HMGCR	:	3-hydroxy-3-methylglutaryl-CoA reductase
HSD11B1	:	hydroxysteroid 11-beta dehydrogenase 1
HTR1B	:	5-hydroxytryptamine receptor 1B [
HTR2A	:	5-hydroxytryptamine receptor 2A
HTR2C	:	5-hydroxytryptamine receptor 2C
IBMX	:	3-isobutyl-1-methylxanthine
IL6	:	interleukin 6
IMPA1	:	inositol monophosphatase 1
INSL5	:	insulin like 5
ITGAV	:	integrin subunit alpha V
ITGB1	:	integrin beta subunit 1
ITGB3	:	integrin subunit beta 3
IκB kinase-β	:	inhibitor of nuclear factor kappa B kinase subunit beta
JNK1	:	mitogen-activated protein kinase 8
KCL	:	Pottasium hydroxide
KDM1A	:	lysine demethylase 1A
KH_2PO_4	:	pottasium dihydrogen phosphate
LCK	:	LCK proto-oncogene
LGAL3	:	galectin 3
LHCGR	:	luteinizing hormone
LNPEP	:	leucyl and cystinyl aminopeptidase
LOX	:	lysyl oxidase
LPAR2	:	lysophosphatidic acid receptor 2
LPAR3	:	lysophosphatidic acid receptor 3
LSS	:	lanosterol synthase
LTF	:	Lactotransferrin
MAOA	:	monoamine oxidase A
MAPK1	:	mitogen-activated protein kinase 1
MAPK3	:	mitogen-activated protein kinase 3
MAPK8	:	mitogen-activated protein kinase 8
MCC	:	maximum clique centrality
MGAM	:	maltase-glucoamylase

MgCl2	:	magnesium chloride
MGLL	:	monoglyceride lipase
MM-GBSA	:	Molecular Mechanics/Generalized Born Surface Area
MMP2	:	matrix metallopeptidase 2
MMP9	:	matrix metallopeptidase 9
MPO	:	myeloperoxidase
MTRNR2L2	:	MT-RNR2 like 2
NaCL	:	sodium chloride
NaHCO3	:	sodium hydrogen carbonate
$(NH_4)_2CO_3$	:	ammonium carbonate
NaOH	:	sodium hydroxide
NOS2	:	nitric oxide synthase 2
NOS3	:	nitric oxide synthase 3
NOX4	:	NADPH oxidase 4
NPR2	:	natriuretic peptide receptor 2
NR1H2	:	nuclear receptor subfamily 1 group H member 2
NR1H3	:	nuclear receptor subfamily 1 group H member 3
NR1I2	:	nuclear receptor subfamily 1 group I member 2
NR3C2	:	nuclear receptor subfamily 3 group C member 2
OGTT	:	Oral Glucose tolerance Test
OPRM1	:	opiod receptor 1
OSBP2	:	oxysterol binding protein 2
OXM	:	Oxyntomodulin
P2RY4	:	pyrimidinergic receptor P2Y4
PC1	:	prohormone convertase enzyme 1
PC2	:	prohormone convertase enzyme 2
PC3	:	prohormone convertase enzyme 3
PDE5A	:	phosphodiesterase 5A
PEPT1	:	Human peptide transporter 1
PLAT	:	phosphodiesterase 5A
PLAU	:	plasminogen activator, urokinase
PLAUR	:	plasminogen activator, urokinase receptor
PNLIP	:	pancreatic lipase
PNLIP	:	pancreatic lipase
PPAR γ	:	Peroxisome proliferator-activated receptor gamma
PPARA	:	peroxisome proliferator activated receptor alpha
PSMB1	:	proteasome 20S subunit beta 1
PSMB5	:	proteasome 20S subunit beta 5
PSMC6	:	proteasome 26S subunit, ATPase 6
PSMD9	:	PSMD9 proteasome 26S
PTGS2	:	prostaglandin-endoperoxide synthase 2
PTPN1	:	protein tyrosine phosphatase non-receptor type 1
PTPN2	:	protein tyrosine phosphatase non-receptor type 2
PYGL	:	glycogen phosphorylase L
PYY	:	peptide tyrosine tyrosine
RGS4	:	regulator of G protein signaling 4
RORC	:	RAR related orphan receptor C
RPS27A	:	ribosomal protein S27
RPS6KA3	:	ribosomal protein S6 kinase A3
SERPINE1	:	serpin family E member 1

SHBG	:	sex hormone binding globulin
SI	:	sucrase-isomaltase
SLC2A1	:	solute carrier family 2 member 1
SLC3A2	:	solute carrier family 3 member 2
SLC6A4	:	solute carrier family 6 member 4
SLCO1B1	:	solute carrier organic anion transporter member 1B1
SLCO1B3	:	solute carrier organic anion transporter member 1B3
SMILES	:	Simplified Molecular Input Line Entry System
SQLE	:	squalene epoxidase
SRD5A2	:	steroid 5-alpha reductase 2
SREBF2	:	sterol regulatory element binding transcription factor 2
SREBP-1c	:	sterol regulatory element binding transcription factor 1
SULT1E1	:	sulfotransferase family 1E member 1
TAS2R40	:	taste 2 receptor member 40
TAS2R43	:	taste 2 receptor member 43
TF	:	Transferrin
TGBG	:	1,2,4,6 Tetra o Galloyl Beta D Glucose
TGFβ1	:	Transforming growth factor beta 1
TIP4P	:	4-site transferable intermolecular potential
TLR4	:	Toll like receptor 4
TNF	:	tumor necrosis factor
TOP1	:	DNA topoisomerase I
TOP2A	:	DNA topoisomerase II alpha
TP53	:	tumor protein p53
TTR	:	transthyretin
TXNRD1	:	Thioredoxin reductase 1
TYR	:	tyrosinase
UBC	:	ubiquitin C
UGT1	:	UDP glucuronosyltransferase family 1 member A1
VEGFA	:	vascular endothelial growth factor A
XDH	:	xanthine dehydrogenase

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- Thottapillil, A., Kouser, S., Kukkupuni, S. K., & Vishnuprasad, C. N. (2021). An 'Ayurveda-Biology' platform for integrative diabetes management. Journal of ethnopharmacology,268, 113575. https://doi.org/10.1016/j.jep.2020.113575
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- Jaleel, UCA and Chethala N, Vishnuprasad and S, Sathish and Thottapillil, Anjana and K R, Jinu Raj and Kukkupuni, Subrahmanya Kumar and Kulkarni, Prasanna and M, Rakhila and Safeeda, Ayisha and Manuel, Andrew Titus and EPA, Sandesh, Artificial Neural Network Based Self Organizing Maps Analysis for Clinical Trials of Indian Systems of Medicine. Available at SSRN: https://ssrn.com/abstract=4468365 or http://dx.doi.org/10.2139/ssrn.4468365.

Chapter – 1
Introduction

1 Introduction

1.1 Background

The world is seeing an unprecedented rise in metabolic health issues making the global population vulnerable to many risks like complex lifestyle diseases. To confront the global challenge of rising non-communicable diseases (NCD), an integrative healthcare system incorporating the principles of both modern and traditional systems seems to be a plausible strategy. Among the NCDs, there is a rapid rise in the incidence of diabetes and obesity across the world. There are different types of diabetes and all of them are found to be associated with improper lifestyle. Among the different types of diabetes, type 2 diabetes mellitus (T2DM) is the most common and has shown a very profound association with obesity. Due to the predominance, biological complexity and higher co-morbidity association, T2DM is attracting more research attention to understand the disease biology and to come up with novel and affordable management solution. Despite rapid strides in modern healthcare research and innovations, both T2DM and obesity remain a huge challenge in terms of treatment and management strategies (Bansode and Jungari, 2019). The current modern medical practice is more reductionist where the treatment approaches largely break down the individual's biology into cellular and organ systems and the dynamic nature of systemic interactions and biochemical cross-talks are not considered much (Fang and Casadevall, 2011). The fundamental tenets of health stems from this dynamic interaction of biological systems (biomolecules, cells and organs) within the individual (*milieu intérieur* concept developed by the French physiologist Claude Bernard) as well as between the individual and the environment including diet, lifestyle, social interactions and so on and so forth; and these factors are not mutually exclusive (Krahn et al., 2021). The holistic understanding of health and disease as well as their management considers all the physiological and biochemical events as one and attempts to see the entire organism more than the collection of its constituent parts. While modern medicine made rapid advances in prevention and cure of various diseases, primarily infections, it has become increasingly evident that it lacks this holistic perspective that is much needed for the management of the rising 'epidemics' of lifestyle diseases like diabetes, obesity and various associated metabolic diseases associated with them (Paczkowska-Abdulsalam and Kretowski, 2021). The side effects of existing drugs and rising cost of medicines are some of the reasons for the global trend of increasing attention to integrative medicinal approaches

especially for management of complex life style diseases like T2DM and obesity (Padhi et al., 2020). For managing these chronic lifestyle diseases, positing a holistic system focusing on the patient centered disease management and wellness appears to be more advantageous as compared to the current approach which emphasizes a more symptomatic relief treatment.

1.1.1 Global and national scenario of diabetes and obesity: Prevalence and economic burden

Diabetes is one of the most prevalent epidemics globally. Around 537 million adults are currently diagnosed with diabetes mellitus (or T2DM) and this is expected to rise to 643 million by 2030 (Ong et al., 2023). Initially considered as a disease of the affluent, T2DM has now spread to all the developing nations affecting both urban and rural populations (Dunachie and Chamnan, 2019). Although more than 80% of diabetes patients living in low-to-middle income countries, the overall trend is an increase in diabetes prevalence in every country since 1980. Fig-1 shows the global prevalence of T2DM which depicts the incidence of diabetes in low- and middle-income countries. A rapid economic development, urbanization, and transition in nutritional status has spurred exponential diabetic progression in Asia, making it a hub of diabetes (Hu, 2011). India is among the top three countries in in South Asia with the highest number of individuals with diabetes, thereby becoming the epicenter of diabetes (Ramachandran et al., 2008). As per the statistics of International Diabetes Federation (IDF), there are over 74 million people having diabetes in India and around 58% go undiagnosed. Various cross-sectional studies indicate that prevalence of diabetes in India has risen significantly in the last 10-15 years (Balasopoulou et al., 2017). There is also evidence that Asian Indians progress more rapidly through the prediabetes stage as compared to people of other ethnic groups (Yang et al., 2010). The recent Indian Council of Medical Research – India Diabetes (ICMR-INDIAB) study, a cross-sectional population-based survey, reported that the overall weighted prevalence of diabetes is 11.64%, pre-diabetes is 15.3% and generalized and abdominal obesity are 28.6% and 39.5% respectively (Anjana et al., 2023).

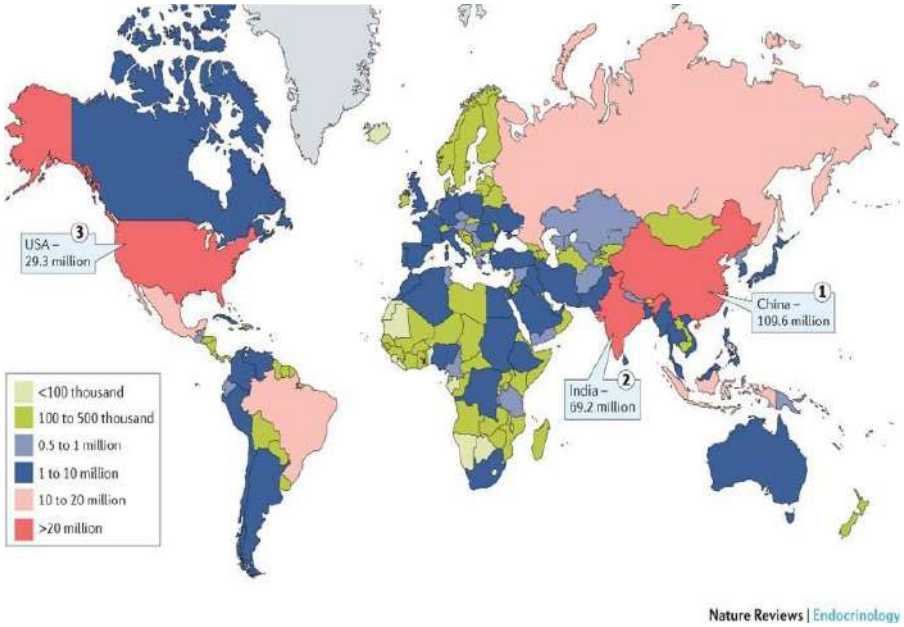

Fig-1. The global prevalence of type 2 diabetes. Figure courtesy (Zheng et al., 2018)

1.1.2 Diabetic complications posing a socio-economic burden

It is well known that diabetes mellitus increases the risk of developing a variety of severe life-threatening health complications (Zheng et al., 2018). Long term hyperglycemia caused by diabetes leads to microvascular complications of diabetes include retinopathy, autonomic neuropathy, peripheral neuropathy and nephropathy and the macrovascular complications broadly including coronary and peripheral arterial diseases. One of the leading diabetic complications in India was found to be diabetic foot ulcers and infection, resulting in more than 30% of the hospitalization related to diabetes (Unnikrishnan et al., 2016). Large population-based studies conducted in India indicate that there is high prevalence and burden of diabetic complication and is also linked to other diseases such as abnormal mental health, cancer, disability, as well as liver disease like Non-Alcoholic Fatty Liver (NAFLD) (Pradeepa and Mohan, 2017;Shaw and Magliano, 2022).

The chronic and progressive nature of diabetes also implicates an economical burden on the patient who suffers significant financial stress for treatment of diabetes and associated complications (Pradeepa and Mohan, 2017). The study concluded that a significant amount from the annual income was spent on diabetes care, more in urban areas than in rural ones (Ramachandran et al., 2007). Global economic projections of diabetes till 2030 also corroborate with this and it predicts that diabetes costs more than 2% of global GDP (Bommer et al., 2018).

This emergent situation requires immediate attention and long-term change in strategies osf diabetes and obesity management.

1.1.3 Etiology and pathophysiology of diabetes mellitus

Diabetes mellitus is a syndrome currently recognized and classified as a group of diseases characterized by signs and symptoms of chronic hyperglycemia which eventually leads to loss of proper insulin response in peripheral tissues, referred as insulin resistance. Epidemiology of T2DM is heterogeneous, and is affected by genetic and environmental factors (Zheng et al., 2018). A strong heritability quotient, proven through various Genome Wide Association Studies (GWAS), is highly associated with the diabetes progression (Grarup et al., 2014). Genetic vulnerability along with the consumption of calorie dense food and sedentary lifestyle habits lead to obesity, a condition characterized with excess adiposity and high body weight and fat content measured as Body Mass index (BMI). It is now well-established that high incidence of obesity is a major determinant leading to T2DM (Pillon et al., 2021). The maintenance of plasma glucose levels within the physiological range is based on a finely controlled autoregulatory system between the pancreatic β- cells, that produce insulin, and the insulin responsive tissues such as liver, adipose and muscle. Abundance of nutrient levels combined with physical inactivity leads to metabolic imbalance leading to higher glucose levels. This in long term leads to impairments in both insulin sensitivity and resistance and impairments in insulin secretion which are crucial factors in the pathophysiology of T2DM (Rachfal et al., 2021). In the early stages of the disease, decreased insulin sensitivity triggers β-cells hyperfunction to achieve a compensatory increase in insulin secretion to maintain normoglycemia. The higher levels of circulating insulin (hyperinsulinemia), thus, prevent hyperglycemia. However, gradually, the increased insulin secretion by β-cells is not able to compensate sufficiently for the decreased in insulin sensitivity. Moreover, β-cell function begins to decline and this β-cell dysfunction eventually leads to insulin deficiency. As a result, normoglycemia can no longer be maintained and hyperglycemia persists.

This insulin resistance leads to decreased glycogen synthesis and protein catabolism in muscles and inhibition of lipoprotein lipase activity in adipocytes, thus increasing free fatty acid (FFA). In the liver, insulin resistance impairs glycogen synthesis, fails to suppress glucose production, enhances lipogenesis, and increases the synthesis proinflammatory factors. In adipose tissue, insulin resistance results in excess lipid accumulation and abnormal production of

proinflammatory adipokines and cytokines which trigger oxidative and ER stress pathways leading to defective insulin response in liver and adipose tissue. Thus, multiple events in these insulin- sensitive tissues triggered by a state of obesity, results in systemic insulin resistance which over time results in β- cell failure and manifestation of T2DM and its complications (Lee et al., 2022).

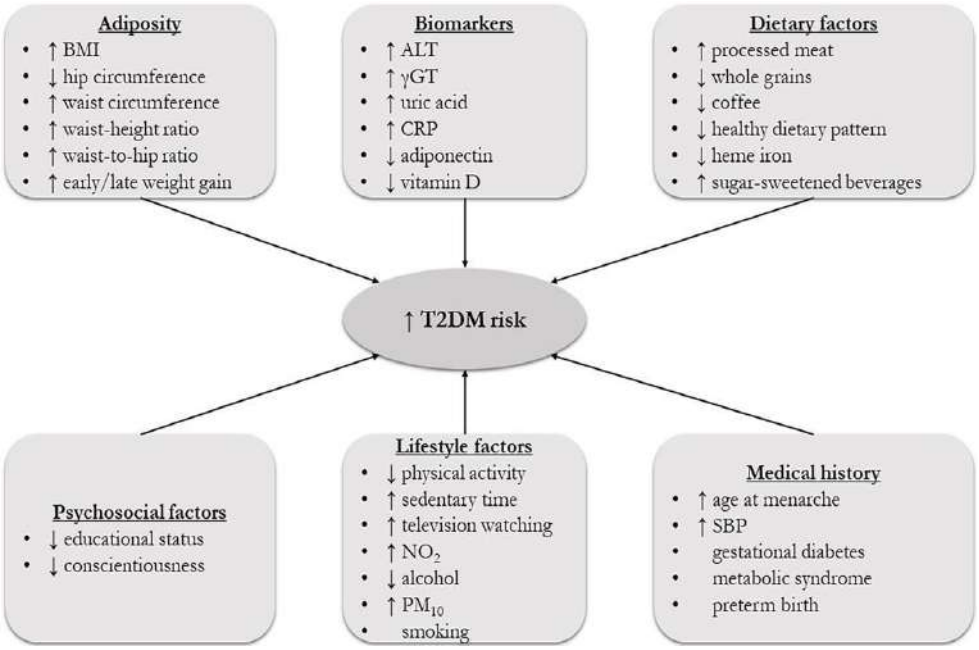

Fig- 2. Illustration depicting the multiple risk factors that can increase the incidence of T2DM. Figure courtesy (Bellou et al., 2018)

Patients of both T2DM and obesity are at risk of developing a spectrum of diseases affecting liver, kidney and macrovascular systems cardiovascular diseases. NAFLD is one such condition where the fat content in liver increases and causes steatosis, finally resulting in liver cell destruction (Godoy-Matos et al., 2020). Similarly, long term hyperglycemia leads to damage of renal cells as well and it leads to chronic diabetic kidney disease (Huang and Khardori, 2017). T2DM patients can also have hypertension which can cause cardiovascular complications (Ohishi, 2018).

There are several risk factors for T2DM and obesity and some of them are modifiable (life style and dietary factors) and some are non-modifiable (genetic) (Fig-2). A nutrient dense diet coupled with lesser physical activity seem to have a marked effect on the onset of obesity and T2DM. Along with the diet, habits such as smoking and alcohol consumption also affect the disease onset. Recent studies have reported additional factors such as exposure to environmental pollutants, depression, short sleep duration, and the built environment (BE) to

be associated with increased diabetes and obesity risks (Kolb and Martin, 2017; Beulens et al., 2022)

1.1.4 Obesity and T2DM

Obesity, is a strong risk factor for T2DM and leads to insulin resistance and metabolic abnormalities. Increased Body Mass Index (BMI) is an index of increasing adiposity and is associated with increased risk of T2DM (Klein et al; Luo and Liu, 2016, 2022). The co-occurrence of obesity and diabetes is referred as 'Diabesity', a term coined way back in 1970 to show the strong association between the two diseases (Colagiuri, 2010; Michaelidou et al., 2023). The co-existence of diabetes and obesity makes individuals more vulnerable to multiple complications like macro and microvascular disorders and diseases like cancer and NAFLD. For obesity and T2DM, the heritability has been estimated to be moderate-to-high, ranging between 30% to 70% (Pillon et al., 2021). With the rapid economic development, our lifestyles have drastically changed, notably change in the food habits and physical activity. Multiple studies have proved that lack of physical activity as a contributing factor for T2DM and obesity (Blüher, 2019). Also, the change in dietary habits from the traditional whole grains to more modern processed foods and sweetened beverages with high glycemic index and glycemic load had increased the risk of developing metabolic disorders (Zheng et al., 2018). Several studies suggest that epigenetic modifications such as DNA methylation might provide new insights into the pathways underlying obesity and T2DM and offer new opportunities for risk stratification and prevention of T2DM (Chambers et al., 2015). Thus, a multi-factorial etiology makes the treatment and management of diabetes and obesity to be a challenging task.

1.1.5 Changing trends in management strategies of T2DM and obesity

Targeted pharmacotherapies, along with lifestyle modifications remain the mainstays in T2DM and obesity management. However, the disease management becomes increasingly difficult due to the chronicity of the systemic complications of diabetes (Mosenzon et al., 2021). Certain drugs such as α-glucosidase inhibitors have been used for pre-diabetic patients which delays the onset of the disease while specific therapies are given for long term glycemic control (Galaviz et al., 2022). Achievement of durable glycemic control to prevent macrovascular and microvascular complications requires more systemic approaches that can manage the diabetic comorbidities. No single drug can reverse the multiple abnormalities in diabetes which results in life time drug dependency and side effects. The socio-economic burden along with the

multifactorial nature of the disease calls for a change in the perspective of treatment and management strategies. Therefore, the need of the hour is a paradigm shift from the targeted to a holistic perspective of disease management with interventions targeting various organ systems involved.

In this context, traditional medical systems following holistic principles of disease management gains relevance. *Ayurveda*, one of the most accepted Indian Systems of Medicines (ISMs), with its person-centered disease management approach can be a worthwhile option to address the heterogeneity and complexity of these diseases. Also, *Ayurveda* is a rich source of multi-component formulations which can target various pathways resulting in a multi-targeted effect. The *Ayurveda* text *Sharangdhara Samhita* emphasizes on the concept of poly-herbalism for a combinatorial effect and greater therapeutic benefits (Mukherjee et al., 2018; Sharangdhar Samhitha, Dr. Bhramanad Tripathi, 2019). This aligns with the wealth of evidence found in various recent studies describing the benefits of combinatorial therapy in T2DM and obesity (Davies et al., 2022; Thottapillil et al., 2021)

In *Ayurveda*, the descriptions of clinical symptoms characteristic of diabetes have a striking similarity with a group of diseases classified as *Prameha*. *Madhumeha*, a type coming under the category of *Prameha* is defined as a disease with excessive excretion of sweet urine. *Madhumeha*, can be corelated to T2DM, is described in *Ayurveda* as a complex multifactorial disease and has hereditary and acquired factors of etiopathogenesis. *Ayurveda* also considers *Sthoulya* (or obesity) to be associated with pathology of *Madhumeha*. The striking similarity between *Madhumeha* and T2DM can be considered as a starting point of expanding the concepts of diabetess to create a trans-disciplinary framework, by integrating the holistic concepts of *Ayurveda* and molecular insights of modern biomedicine, that would enable the designing of novel integrative strategies for diabetes management (Thottapillil et al., 2021).

One of the emerging disciplines in this direction of research is 'Ayurveda Biology' (also referred as Ayurvedic Biology; Ayurbiology), a term introduced by the renowned physician scientist Professor. M. S. Valiathan, which aspires to study *Ayurveda* principles and drugs using molecular biology tools using a transdisciplinary research perspective (Joshi et al., 2022). This new frontier aims to use modern biological techniques to study the Ayurvedic concepts, practices and formulations which can lead to generating novel insights into understanding health and disease progression as well as treatment methods, so as to expand the frontiers of disease biology. This also allows generating the much- sought scientific evidence for this age-

old science and health practice. Thus, an Ayurveda Biology platform maybe an ideal path for developing novel integrative strategies for complex disease management.

1.2 A transdisciplinary approach for management of complex lifestyle diseases like diabetes and obesity

For a complex problem, a solution often calls for collaboration between different disciplines involving a broader community that can integrate cross-cultural concepts logically and thereby emerging as a new discipline leading to innovative solutions. The essence of transdisciplinary research lies in transcending the boundaries of concepts, theories and methods belonging to a singular discipline and form a shared approach to address the complex issues at hand. This requires blending of ideas beyond disciplines, termed as unity of knowledge, leading to the formation of integrated paradigms to understand the societal issues in a holistic way (Archibald et al., 2023). This perspective of transdisciplinary research can lead to impactful and innovative solutions for many of the global health issues, which otherwise would have been impossible.

T2DM and obesity are examples of health conditions which call for an integrative healthcare strategy which effectively merges the concepts from epistemologically and ontologically different health systems, viz; a reductionist approach of modern molecular medicine and a largely holistic approach of traditional medicinal system. Bridging these epistemologically and culturally different health and disease perspectives is an effort in the right direction for creating a transdisciplinary framework of different views. It is notable that one of the components in the recent The National Center for Complementary and Integrative Health's (NCCIH's) whole person research initiative focuses on the impact of multicomponent interventions or therapies and considers the multi-level research as crucial going forward for tackling complex issues (P, Horgusluoglu and Ginexi., 2023). Considering the multi-dimensionality of metabolic syndromes like obesity and diabetes, a united conceptual framework needs to be developed after continuous deliberation and adaptation of different principles. Towards the development of this framework, combining the holistic health perspectives of Indian systems of medicine and modern medicinal technology and tools can be a worthwhile option.

Ayurveda has an advantage in contemporary healthcare domain as its core concepts focuses on a systemic and person-centered approach. This agrees with the current global trend of precision medicine and personalized medicine that is gaining more popularity these days (Van Der Wijst et al., 2018). Making use of the transdisciplinary philosophy, an integration of modern

medicinal system and *Ayurveda* may lead to the development of a holistic and inclusive healthcare, which is the need of the hour.

Ayurvedic treatments are algorithm based which is further fine-tuned or individualized by the *Ayurveda* practitioner based on each patient's body composition, disease condition and treatment requirements. One or more formulations may be prescribed and a number of patients may get the same or slightly varied prescriptions of ayurvedic medicines. Ayurvedic formulations are multi-component therapeutics assumed to work in a systemic way due to the potentially diverse modes of action of the phytochemicals (Patwardhan et al., 2004). Though Ayurvedic treatment modalities have clinical evidence, the mode of action of these formulations are largely unknown and therefore remain a black box for the modern science. An understanding of the *Ayurveda* scheme of diabetes management and correlating it with the molecular understanding of its pathophysiology and management. may be the way forward in formulating an integrative disease management strategy for T2DM and obesity.

1.2.1 *Ayurveda* concepts of health and disease

Ayurveda recognizes health as a condition when the master regulators of physiology viz. *Doshas* (body humors: *Vata*, *Pitta* and *Kapha*), metabolic activities (*Agni*), structural components (*Dhathu*), excretory functions (*Mala-kriya*) as well as the psychological and behavioral contentment of an individual are in complete homeostasis and optimum function. Consequently, a disease is considered as a collective systemic expression of pathological afflictions that disrupt the body's homeostasis and physiology, and exhibited as symptoms. This philosophy has advantages over the molecular viewpoint for designing holistic disease management strategies that are aimed at restoring physiology and pacifying the symptoms. Although they ultimately exert their effects at specific molecular targets, *Ayurveda* perceive their efficacy and success at functional levels like digestion, excretion and various tissue and organ functions. While the algorithms are general guiding principles, the physician can fine-tune and personalize the interventions depending on the severity and chronicity of disease, other comorbidities, patient's physiological strength, behavior as well as socio-cultural background. This makes the disease management more personalized and patient centered, while keeping the pre-defined algorithm general and globally adaptable. Being a science established itself much before modern day biology, the philosophical frameworks used in *Ayurveda* for understanding the life processes are different from the current molecular

framework of biology. Therefore, it is important to note that Ayurvedic epistemology and ontology of health and disease cannot be juxtaposed with the contemporary biomedical languages. This is where a trans-disciplinary knowledge framework is essential which logically integrates the concepts and creates a platform that can be appreciated by both sides (Thottapillil et al., 2021).

1.2.2 Understanding diabetes: An *Ayurveda* perspective

A set of complex clinical disorders with frequent abnormal micturition described in *Ayurveda* are collectively referred to as *Prameha* and is associated with disrupted *Agni* homeostasis in gastro-intestinal tract (GIT) by various etiological factors like genetics, lifestyle issues and un-wholesome diet. *Prameha* has been referred as one of the '*Maharogas*' (intense diseases) and, as per the classical texts, considered to be challenging to treat (Ambikadutta Shastri, 2003; Shastri KN, 2019). The detailed etiology, pathophysiology and management protocols of *Prameha* are mentioned in all major texts of *Ayurveda* viz. *Charaka Samhitha, Sushrutha Samhitha and Ashtangahrudaya*. *Agni* is a physiological phenomenon that optimizes the metabolic transformations in the body by regulating digestion, absorption and bio-assimilation of nutrients from ingested food. The strength and homeostasis of *Agni* functions is critical in defining and determining the overall health of an individual as it represents the central digestive power and governs the whole-body homeostasis. According to *Ayurveda,* disturbed *Agni* is one of the main contributing factors in *Prameha* progression (Thottapillil et al., 2021).

Ayurveda identifies etiology of *Prameha as two types, viz.; Sahaja* (genetically predisposed) and *Apathyanimittaja* (acquired) (Fig-3). *Sahaja* may also indicate the influence of negative intrauterine environment on fetus which can be corelated to a gestational predisposing factor to diabetes. The description of *Sahaja Prameha* can be perhaps better corelated with that of type 1 diabetes which is insulin dependent. *Prameha* classifications based on body types as *Sthula pramehi* and *Krisha pramehi* are also correlatable to the modern day understanding of diabetes. However, the classification based on the *Dosha* dominance are unique to *Ayurveda* and are highly specific in terms of their characteristic clinical manifestations. Twenty different clinical manifestations of *Prameha* have been identified based on the dominance of *Vata, Pitta* or *Kapha-Doshas* (Fig-3).

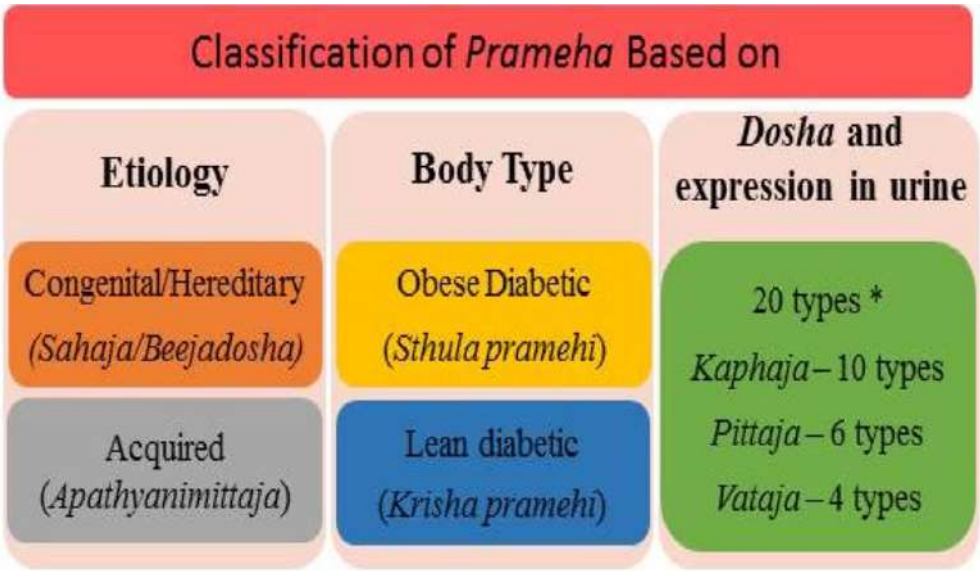

Fig-3. Schematic classification of *Prameha.* Shows the different principles of classification used in *Ayurveda* for classifying the clinical condition of *Prameha*. Figure courtesy (Thottapillil et al., 2021)

The progression of *Prameha* has been described in several stages before it reaches the final disease manifestation (Fig-4). In the initial stage, there is dysregulation of the *Kapha dosha* vitiating *Meda* (fat) and *Kleda* (body fluid), thereby manifesting *Kaphaja Prameha*. Further progression of the disease involves the deregulation of both *Pitta* and *Vata doshas*, ultimately leading to expression of *Prameha* in the form of excretion of excess urine (Sharma and Chandola, 2011;Thottapillil et al., 2021)

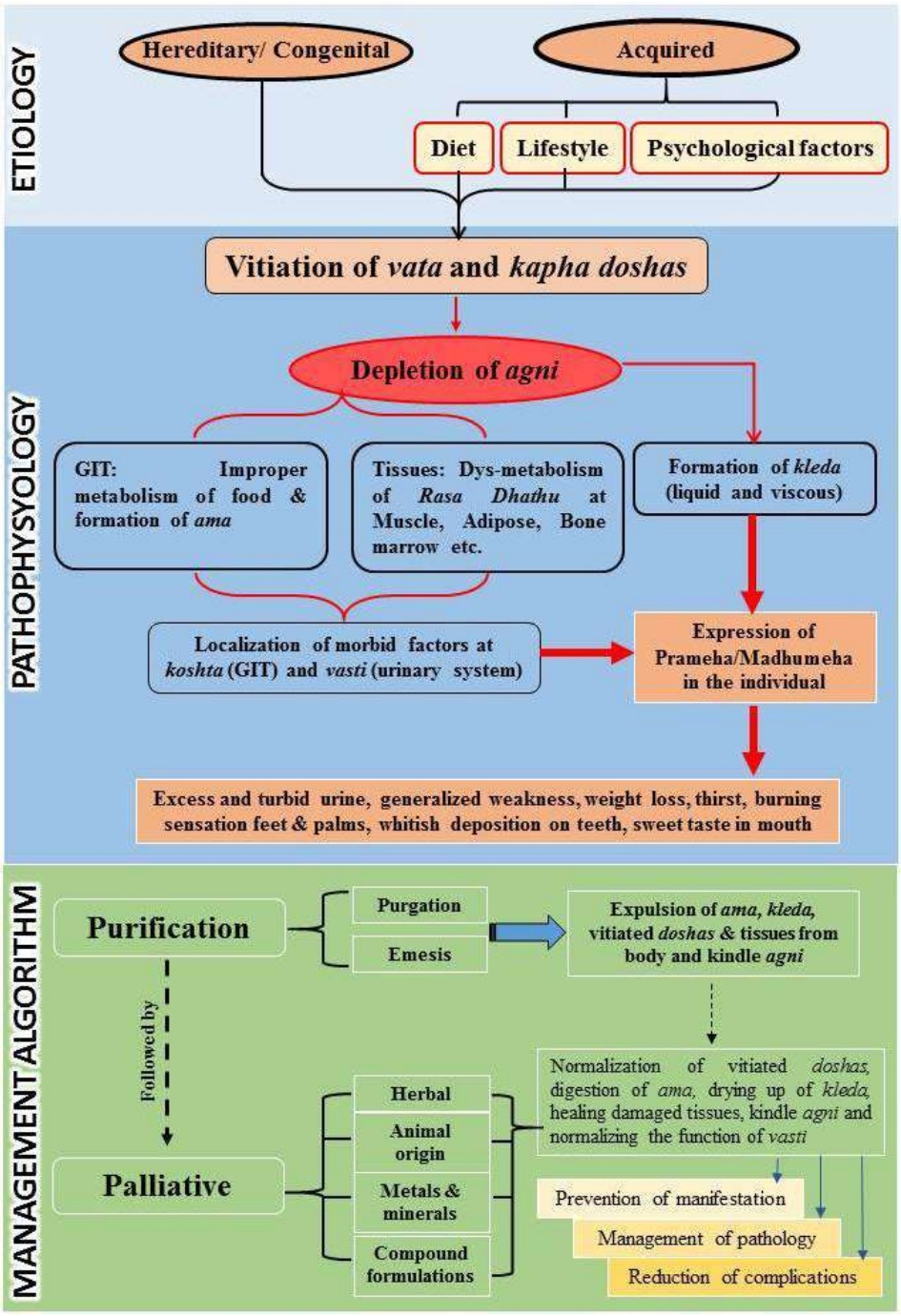

Fig-4. *Ayurveda* perspective of diabetes.. Schematic representation of the etiology, pathophysiology and management algorithm of *Prameha Figure courtesy* (Thottapillil et al., 2021)

1.2.3 *Prameha* and *Sthoulya*

Sthoulya is described as excessive increase in quantity of *Meda* (adipose) and *Mamsa (muscle).* The consumption of foods and substances that are heavy to digest (*Guru*), cold (*Sheeta*), unctuous (*Snigdha*), and sweet or *Kapha*-increasing (*Madhuradi Kaphavardhaka*) as well as lack of exercise and sedentary lifestyle leads to the excessive accumulation of fat (*Meda*). This

results in the excessive nourishment of *Meda* (fat) while other bodily tissues (*Dhatus*) are deprived of proper nourishment. The pathology of *Meda* (fat) and *Mamsa* (muscle) *Dhatus* are involved in the occurrence of *Prameha* as discussed in section 1.2.2. Due to the vitiation in *Meda*, there *is Agni*, imbalance which is again a common factor between *Prameha* and *Sthoulya*, thus establishing a strong relationship exists between *Sthoulya (*obesity*)* and *Prameha*, suggesting that managing one condition can help in controlling the other. One of the key management strategies of *Sthoulya is* life style changes involving dietary restrictions and physical activity which can ameliorate the *Prameha* pathology as well(Hm and Desai, 2022.; Joglekar and Vyas, 2023; Kumari et al., 2013; Zenica D et al., 2019)

Prolonged existence of *Prameha* in an individual can lead to *Madhumeha*, a sub-type hallmarked by sweetness in the urine as mentioned previously in section 1.1.5. Several aspects of *Prameha* overlaps with the modern-day diabetes mellitus, particularly T2DM (Table-1). Classifications of *Prameha* like congenital (*Sahaja*) and acquired (*Apathyanimittaja*) as well as lean (*Krusha*) and obese (*Sthula*) are relatable with similar types of diabetes described in modern biomedicine (Fig-3). Similarities between *Prameha* and T2DM are also seen in their etiology where both *Ayurveda* and biomedicine equally recognize unwholesome diet (excess diet, over nutrition, regular intake of unctuous and heavy food), sedentary lifestyle, lipidemia (~*Medoroga*) and obesity (~*Sthaulya*) as major predisposing factors for *Prameha* and/or T2DM. Consequently, both systems advise on life-style modifications and limiting high glycemic index foods as preventive measures (Sharma, 2000; Mangalasseri et al., 2019).

Prameha	**Diabetes**
Prameha - A disorder associated with urinary system (*Mutravaha srotas*)	Correlates to several metabolic syndrome as well as DM & obesity. Relevant in the context of T2DM
Causes: *Asyasukham and swapna sukham* (comforts and excess of sedentary life), *alasya* (laziness). Excess of ***Dadhini*** (milk products), ***navanna*** (new grains), ***guda-ikshu vikara*** (jaggery and other sugarcane products), ***adyashana*** (repeted eating), ***adyshana*** (frequent eating habits)	Sedentary lifestyle, overnutrition are some of the definitive the causes of T2DM
Psychological factors: *Chinta* (worries)*, Shoka* (grief)*, Bhaya* (fear)	Chronic mental stress is linked with T2DM

Excess and turbid urination, *Madhumeha* (sweet urine)	Polyuria, Glycosuria
Sahaja (hereditary) and *Apathyanimittaja* (acquired).	Hereditary and acquired diabetes
Apachita dhatuvrudhi	Correlated with hyperglycemia
Sthula Pramehi (obese patient), and *Krisha Pramehi* (lean patient)	Correlates with obese and lean diabetes
Prameha is a complication of *Sthoulya* (obesity)	Life style changes like overeating causing a metabolic imbalance leading to **obesity**
Balancing *Agni* is a management strategy for Diabetes	Can be correlated with the role of gut in glucose homeostasis
Dietary managements and physical activities are advised	Management: Adequate exercise and dietary modification with medication

Table-1 – A transdisciplinary corelation of symptoms, etiology and pathophysiology of *Prameha* with that of T2DM.

Madhumeha, which is symptomatically and etiologically more similar to chronic T2DM, belongs to the *Vataja* type (arising due to *Vata* dominance) and considered as the most difficult type of *Prameha* to manage (Sharma and Chandola, 2011; Sharma and Dash, 2007; Shastri, 2003). It is described as an advanced condition characterized by diabetic complications like neuropathy, diabetic ulcers and delayed wound healing and nephropathy which results in vital substances of the body being excreted through the urine resulting in weakness of the body.

1.2.4 Complications of *Prameha* /*Prameha Upadrava*

Prameha patients can develop different types of complications according to their specific *Doshic* subtype dominance. For eg, a *Kaphaja Prameha* patient can have laziness, anorexia, indigestion while *Pittaja Prameha* include hyperacidity, excessive thirst, fever and burning sensation. Complications for *Vataja Prameha* include heaviness in the chest, excessive hunger, insomnia, tremors, pain, constipation, cough, and dyspnea. But it is evident that the complications of *Prameha* are directly or indirectly can be corelated with diabetic complications (Sharma and Chandola, 2011). *Pidaka, Vidradhi (*inflammation and carbuncles in skin), *Trishna* (thirst), *stambhana* (stiffness of body parts), *kampa* (tremor), *Shosha* (Muscles wasting) are other some common notable complications

1.2.5 Prameha management in *Ayurveda*

Various classical *Ayurveda* preparations and *Ayurveda* inspired proprietary formulations have demonstrated diabetes alleviating effects clinically as well as in vitro and in vivo models. Some of the commonly used plants and formulations used in *Ayurveda* are listed in Table-2. Along with the formulations many medicinal herbs have been used extensively for managing diabetes and associated complications.

Prameha management as per *Ayurveda* involves both lifestyle modifications and pharmacological interventions. Ayurvedic drug therapies uses multi-component preparations containing a large number of molecules, derived from natural sources and therefore regarded as generally safe (Parasuraman et al., 2014). Combining herbs of varied strengths enhances the therapeutic results and prevents extreme effects. The combinations of ginger and black pepper, and cumin, black pepper and asafoetida are some of the traditionally used combinations for reducing mucous and enhance digestion respectively. Similarly, *Guduchi (Tinospora cordifolia* (Willd.) Miers) and turmeric (*Curcuma longa* L.) combination is used to improve one's immunity. The various herbal ingredients in the combinations may have a pharmacodynamic synergism which in turn may prove beneficial in complex diseases with various symptoms (Mukherjee et al., 2018).

S. No	Plant and the parts used	Activity	Reference
1	*Syzygium cumini (L.) Skeels* (seeds)	Seed extract exhibits significant insulin-sensitizing, anti-dyslipidemic, antioxidant, anti-inflammatory and beta-cell salvaging activity in HFD-STZ-induced type 2 diabetic rats. Reduced the blood glucose level significantly and also regulated the insulin levels in hyperglycemic rats. Beneficial effect of SC seed extract on insulin resistance and β-cell dysfunction in HFD-STZ-induced type 2 diabetic rats.	(Sharma et al., 2012) (Raza et al., 2017)
2	*Pterocarpus marsupium* Roxb. (bark and wood)	Extracts reduce oxidative stress and inflammation in hyperglycaemic rats The extracts may have potent DPP-4 inhibitory action, and their hypoglycemic action attributed through	(Pari et al., 2018) (Kosaraju et al., 2014)

		an increase in plasma active GLP-1 levels.	
3	*Aegle marmelos* (L.) Correa (root, wood and leaves)	*Aegle marmelos* used as a complementary therapy reduced the biochemical parameters in T2DM	(Nigam and Nambiar, 2019)
4	*Cedrus deodara* (Lamb.) G. Don (bark and wood)	Lowers blood glucose levels in STZ induced diabetic rats.	(Singh et al., 2013)
5	*Momordica charantia* L.(fruit)	GLP-1 secretion in diabetic rats increases upon administration of aqueous extracts.	(Bhat et al., 2018)
6	*Trigonella foenum graecum* L. (seed)	Consumption of seeds reduced fasting glucose levels and HbA1c levels in T2DM patients. A network pharmacology approach to prove the anti-diabetic and hypolipidemic effect of Trigonella plant	(Ranade and Mudgalkar, 2017) (Banerjee et al., 2019)
7	*Berberis aristate* DC. (wood and root)	Antioxidant and anti-hyperglycaemic activity in diabetic rats.	(Singh and Kakkar, 2009)
8	*Curcuma longa* L. (rhizome)	Extract reduced blood glucose levels in STZ-induced diabetic rats. Increase in GLP-1 secretion in GLUTag cells via the Ca2+/calmodulin dependent kinase II pathway.	(Essa et al., 2019) (Alli-Oluwafuyi et al., 2019)
9	*Emblica officinalis* L. (fruit)	Inhibits α-amylase, α-glucosidase, and DPP-4. Decreased blood glucose levels in STZ induced rats	(Majeed et al., 2020) (Ansari et al., 2014)
10	*Gymnema sylvestre (*Retz.) Schult. (entire plant)	Hypoglycaemic activity observed when diabetic rats were administered by leaf extracts. Inclusion of herb in diet reduced glucose levels and HbA1c levels in T2DM patients.	(El Shafey et al., 2013) (Kumar et al., 2010)

11	*Acacia catechu* L.f.)Willd. (heart wood)	Extracts showed significant hypoglycaemic activity in glucose-induced diabetic mice.	(Rahmatullah et al., 2013)
12	*Azadirachta indica* A.Juss (leaves, bark)	Leaf extract improves glucose tolerance and insulin signaling impairment levels in STZ- induced diabetic rats.	(Satyanarayana et al., 2015)
13	*Albizzia lebbeck Albizia lebbeck* (L.) Benth. (bark, fruits)	Extract of the bark exhibited anti-hyperglycaemic activity in T2DM rats. .	(Patel et al., 2015)
14	*Ficus glomerata* L. (bark, root, leaves)	Leaf extract exhibited hypoglycaemic activity in STZ-induced early diabetic rats.	(Shaikh et al., 2020).
15	*Salacia reticulata* Wight (stem, roots)	*Salacia reticulata* Wight stem extracts down-regulate gluconeogenic pathway thereby reducing fasting blood glucose levels in mice.	(Medagama , 2015) (Stohs and Ray, 2015)
16	*Semecarpus anacardium* L.f.	*Semecarpus anacardium* improves cardiac dysfunction through modulating energy metabolism	(Panchanadham et al., 2011)
17	*Asparagus racemose* Willd	*Asapargus racemosa* inhibits digestive enzyme inhibition	(Vadivelan et al., 2019)
18	*Terminalia chebula* Retz	Aqueous extract of *Terminalia chebula* reduced inflammation and lipids clinically	(Pingali et al., 2020)
19	*Aerva lanata* (L.) Juss. ex Schult.	*Aerva lanata* reduced the blood glucose levels in alloxan induced type 2 diabetic rats	(Riya et al., 2015)
20	*Cyperus rotundus* L. (rhizomes)	*Cyperus rotundus* demonstrates anti-diabetic and anti-oxidant activity in high sugar induced model of Drosophila model of diabetes	(Dechakhamphu et al., 2023)
Ayurvedic compound formulations			
S. No	**Formulation**	**Activity**	**Reference**

1	*Nishamalaki*	Controls hyperglycaemia and reduces lipid levels.	(Dawane et al., 2016)
		Reduces blood glucose level in pre diabetic patients	(Munshi et al., 2023)
2	*Nyagrodhadi Ghanavati* (combined with *Virechana Karma* – purgation therapy)	Combined therapy of *Virechana* and *Nyagrodhadi Ghanavati* provided better relief in T2DM patients.	(Dave et al., 2010)
3	*Triphala Churna*	Improves lipid profile in diabetic patients	(Phimarn et al., 2021)
4	*Ayaskriti*	Decrease in blood glucose level was observed in patients with T2DM.	(Ram et al., 2016)
5	*Lodhrasavam*	*Lodhrasavam* digest inhibits α-amylase and α-glucosidase.	(Butala et al., 2017a)
6	*Chandraprabha Vati*	Reduced blood glucose levels, cholesterol and triglycerides in alloxan induced diabetic rats.	(Wanjari et al., 2016a)
7	*Nisakathakadi kashayam*	*Nisakathakadi Kashayam* inhibits DPP4 and exhibits multi-modulatory potential	(Thottappillil et al., 2023)
8	*Chaturmukha Rasa*	Inhibits α-amylase, α-glucosidase and sucrase in STZ induced rats.	(Sharma et al., 2019)
9	*Arjunarishta*	*Arjunarishta* improves anti-lipidemia and anti-hypertension in high fat diet rats	(Shengule et al., 2018)

Table-2 depicting some of the commonly used medicinal herbs and compound ayurvedic formulations in mitigating diabetes, obesity and associated comorbidities

According to modern biomedicine, T2DM is characterized by chronic hyperglycemia associated with metabolic alterations in all types of diabetes and hallmarked by insulin resistance in the case of T2DM and obesity (Kahn et al., 2014). In response to the elevated glucose levels, insulin is secreted more from the pancreas. But over a period of time, the resultant hyperinsulinemia causes a burnout of pancreatic cells. The pathogenesis of diabetes is complex and heterogeneous resulting from a complex interplay of genetic, environmental, lifestyle and psychosocial factors. Altered insulin signaling and deregulated metabolism affects many peripheral organs like adipose, liver and muscles (Shoelson et al., 2006; Zheng et al., 2018)

Glucose homeostasis is maintained by balancing the production of glucose in liver and disposal and metabolism into peripheral tissues like muscles and adipose. In response to rising glucose

levels in the blood, GLUT2 present in the pancreatic beta cells take in the glucose which results in changed ATP/ADP ratio and subsequent membrane depolarization and exocytosis of insulin granules (Röder et al., 2016). Secreted insulin travels through blood and interact with insulin receptors present in the peripheral tissues, primarily muscle and adipose, where insulin hormone suppresses endogenous glucose production and stimulates glucose uptake in these tissues. Insulin signaling increases glucose transport through the regulated trafficking of the glucose transporter, GLUT4, from intracellular stores to the cell surface in muscle and adipose cells through various signaling mechanisms leading to increased glucose uptake. Insulin signaling also increases lipid storage in adipose tissue (Röder et al., 2016). Studies have shown that proximal signaling issues like decreased IR expression, tyrosine phosphorylation, and kinase activity contribute to insulin resistance (Petersen and Shulman, 2018). It is quite interesting to note that recent studies have shown that insulin mediated GLUT4 translocation and uptake can also take place in other tissues like bone (osteoblasts) and it suggest a broader systemic role of insulin and glucose in body's metabolic and energy homeostasis, than it was understood earlier (Li et al., 2016).

1.3.1 Insulin resistance: the road to metabolic maladies

As mentioned in section 1.3, one of the main features of metabolic diseases such as T2DM and obesity is insulin resistance, a phenomenon defined as the decreased ability of peripheral target tissues viz, muscles, adipose and liver, to respond properly to normal circulating concentrations of insulin. The most common risk factor of insulin resistance is obesity, characterized by the accumulation of excessive body fat, hyperglycemia and dyslipidemia. Elevated glucose levels, circulating free fatty acids and low-grade inflammation associated with obesity act as triggers for pancreatic cells to secrete more insulin eventually leading to loss of β-cell mass. Multiple events such as Endoplasmic reticular (ER) stress, unfolded protein response (UPR) and reactive oxygen species (ROS) production ensue which drive the T2DM progression (Fig-5) (Petersen and Shulman, 2018; Halban et al., 2014).

1.3.2 Peripheral tissues in insulin resistance: Adipose, Liver and skeletal muscle

Insulin resistance manifests in adipose, liver and muscle and the deregulated cross talk between these vital organs play a crucial role in T2DM pathogenesis as shown in Fig-5. The main function of adipose tissue is storage and release of lipids/fatty acids (FAs) as needed depending on the fuel/food availability. Adipose tissue not only functions as a storage organ, but also as an endocrine organ that secretes many hormones (termed as 'adipokines'), inflammatory

mediators and free fatty acids (FFAs) which impair glucose metabolism and muscle ATP synthesis, promote the synthesis of toxic lipid metabolites, and alter insulin signaling. Insulin acts on adipose tissue leading to two major biological processes viz. 1) stimulating glucose uptake and triglyceride synthesis and *2*) suppression of triglyceride hydrolysis and release of FFA and glycerol into the circulation (Luo and Liu, 2016).

Obesity is characterized by dysfunctional adipose tissue functions, in which adipocytes initially become hypertrophic during periods of caloric excess and secrete adipokines that result in the recruitment of additional pre-adipocytes, which differentiate into mature adipocytes as compensatory protection against some of the adverse metabolic consequences of obesity (Longo et al., 2019). When the capacity for adipocyte recruitment and hypertrophy is overwhelmed, fat accumulates in ectopic sites such as visceral depots, the liver, skeletal muscle, and pancreatic beta cells. These changes are accompanied by inflammation, insulin resistance and other features of the metabolic syndrome and T2DM. Some of the inflammatory adipokines are TNFα, IL1, IL6 and chemokines like CCL2. TNFα downregulates lipolysis and causes accumulation of lipids in other tissues like muscles and also causes decrease in expression of genes like PGC1α required for mitochondrial functions. It also inhibits GLUT4 mediated glucose uptake through various mechanisms (Rosen and Spiegelman, 2006).

Skeletal muscles are the main site for insulin induced glucose uptake. Under normoglycemic conditions around 80% of the glucose uptake happens in the muscle (DeFronzo and Tripathy, 2009). In early stages of T2DM, impaired glycogen synthesis in muscle is the primary defect in insulin resistance. Defects in muscle glycogen synthesis play a significant role in insulin resistance, and three potentially rate-controlling steps in muscle glucose metabolism have been implicated in its pathogenesis: action of glycogen synthase and hexokinase in glycogen and glucose respectively and the translocation of insulin mediated glucose transporter, GLUT4 (Petersen and Shulman, 2018).

Liver is a vital organ regulating glucose and lipid metabolism, in which higher circulating insulin levels are necessary to adequately control blood glucose levels. In healthy individuals, postprandially, contrasting actions of insulin and glucagon signal the liver to increase glucose consumption, stop glucose production, and store excess nutrients in the form of glycogen and lipids (Meshkani and Adeli, 2009). In pathologic states, such as obesity and T2DM, due to the insulin resistance hepatic metabolism is dysregulated, leading to excess production of glucose despite accelerated rates of lipid synthesis, a condition now commonly referred to as selective hepatic insulin resistance (Santoleri and Titchenell, 2019). Consequently, insulin-resistant

disorders such as obesity and T2DM are closely associated with nonalcoholic fatty liver disease (NAFLD), a disorder that can lead to liver dysfunction and progress to deadly nonalcoholic steatohepatitis (Utzschneider and Kahn, 2006).

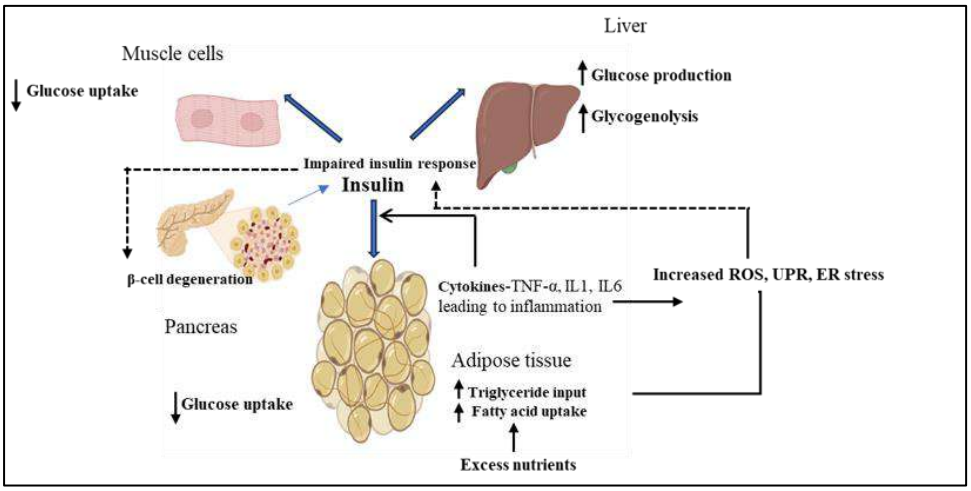

Fig-5. Mechanisms of insulin resistance. The mechanisms of IR where an excess of nutrients causes dysregulation of insulin responsive tissues that ultimately result in
1. pancreatic beta cell degeneration are depicted

Systemic inflammation is a well-documented contributor to insulin resistance. Increased levels of pro-inflammatory cytokines, such as IL-6 and TNFα and increased numbers of macrophages and other inflammatory cells are observed in adipose tissue, liver and sera of patients and animals in insulin-resistant states (Chen et al., 2015; Nieto-Vazquez et al., 2008;Moller, 2000). In obesity, the enlarged adipocytes secrete a number of pro-inflammatory cytokines which exacerbate insulin resistance by activating downstream kinases, including IκB kinase-β (IKKβ), JUN amino-terminal kinase 1 (JNK1; also known as MAPK8) and p38 MAPK, which can contribute to the phosphorylation of serine residues in IRS proteins leading to their reduced action (Shoelson et al., 2006). Macrophage infiltration, common in hypertrophic adipocytes, can also drive inflammation by activating Toll-like receptors (TLRs) which are an important component of the innate immune response and is activated by fatty acids (Benomar and Taouis, 2019).

1.3.4 The spectrum of diabetic complications

Diabetes is a disease strongly associated with both macrovascular complications (including ischemic heart disease, cerebrovascular disease, and peripheral vascular disease) and microvascular complications (including nephropathy, retinopathy, and neuropathy) (Fig-6) (Harding et al., 2019). Hyperglycemia promotes the development of microvascular

complications through the activation of six major pathways, including i) enhanced polyol pathway flux, ii) iii) increased formation of advanced glycation end products (AGEs), iv) increased AGE receptor expression, v) activation of PKC isoforms, enhanced hexosamine flux and increased intracellular reactive oxygen species (Shaw and Magliano, 2022a). Reactive oxygen species impair angiogenesis, activate several proinflammatory pathways and cause epigenetic changes that result in long-lasting expression of proinflammatory genes that persists after glycaemia is normalized. These same biochemical and molecular mechanisms that contribute to the microvascular complications also contribute to the macrovascular complications. Apart from the well-known macro and microvascular complications, there is growing evidence that diabetic population is also vulnerable to a number of other conditions like cancer and declining cognitive function (Harding et al., 2019).

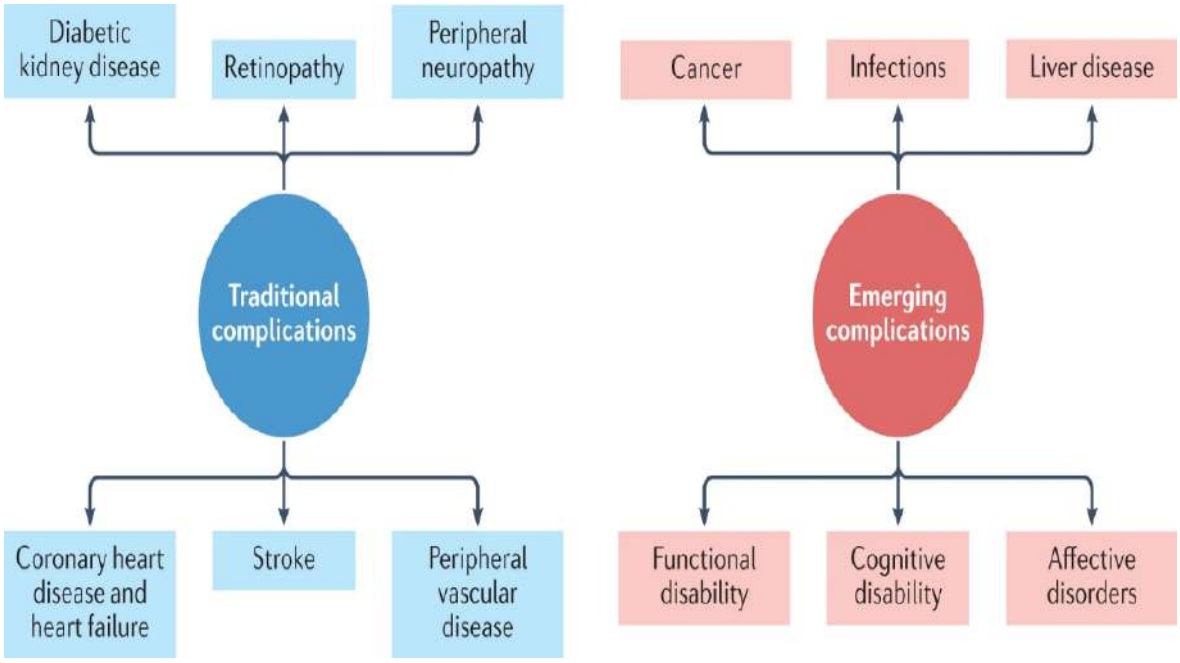

Fig-6. T2DM complications. Schematic depiction of well-known complications such as microvascular and macrovascular disorders and other conditions uncovered more recently. Figure courtesy (Shaw and Magliano, 2022b)

1.3.5 Contemporary management strategies for T2DM

For managing T2DM, several targeted drug classes like biguanides, sulfonylureas, meglitinide, thiazolidinedione (TZD) are given along with insulin and suggestions for lifestyle modifications. Metformin remains the first-line therapy of choice for patients with T2DM and

it reduces hepatic glucose output, enhances peripheral tissue sensitivity, and stimulates GLP-1 secretion. Furthermore, metformin effectively lowers HbA1c concentration by about 1–2%, is weight neutral, does not cause hypoglycemia, and can have modest beneficial effects on blood pressure and lipid profile. Sulfonylureas, such as gliclazide and glimepiride, act on β cells to stimulate insulin secretion while Thiazolidinediones (TZD), also known as PPAR γ agonists, (rosiglitazone, pioglitazone) improve insulin sensitivity in target organs. TZDs have been associated with adverse cardiovascular outcomes. Other medications that may lower blood sugar include alpha-glucosidase inhibitors like voglibose and acarbose (Padhi et al., 2020).

The newer classes of anti-diabetic drugs include incretin-based therapies such as GLP-1 receptor agonists and oral DPP-IV inhibitors (both regulated the incretin effect) as well as SGLT-2 inhibitors which increase urinary glucose excretion by inhibiting glucose reabsorption in the renal proximal tubule. GLP1 agonists bring about GLP1-like effects while DPP4 inhibitors prevent GLP1 from enzymatic degradation, resulting in higher amount of circulating GLP1 that helps in increased insulin secretion, reduced glucagon secretion, reduced hepatic glucose output, delayed gastric emptying, and increased satiety. A lesser number of therapies are currently approved for obesity and they include Orlistat, a lipase inhibitor, a combination of Naltrexone/bupropion and the recently FDA-approved GLP1 agonist (Müller et al., 2022). Though non pharmacological approaches like bariatric surgery have shown success in weight loss, maintaining long term reduction in body weight remains challenging.

Despite the presence of several treatments for diabetes and its co-morbidities, the pipeline for novel drug therapies is still very active in its pursuit for better and efficacious ones. The involvement of multiple vital organs in the pathogenesis of T2DM necessitates a combinatorial approach to alleviate various pathological aspects of the disease. Melanie J. Davies et al in the American Diabetes consortium report stated the many advantages of combinatorial therapies such as 1) increased durability of the glycemic effect, 2) simultaneous targeting of the multiple pathophysiological processes characterized by T2DM, 3) impacts on medication burden, medication-taking behavior, and treatment persistence, and 4) complementary clinical benefits (e.g., on glycemic control, weight and cardiovascular risk profiles) (Davies et al., 2022).

Out of the emerging therapies, the most effective ones are gut based therapeutics which includes GLP1 receptor agonists and DPP4 inhibitors owing to their proven weight loss and beneficial cardiac and renal outcomes. Another recently approved gut-based drug for obesity and T2DM is tirzepatide, a dual agonist for GLP1 and GIP which has also shown positive

outcomes in terms of weight loss and HbA1c reduction (Lynch and Llano, 2023). Several other gut hormones are also being explored for their anti-obesity and anti-diabetic activities in combination with GLP1 and GIP. The gastrointestinal tract plays a multifaceted role with its diverse ecosystem of gut peptides and gut microbes, and harnessing the potential of gut in designing novel and effective therapies for T2DM and obesity management holds great promise (Holst, 2007; Alexiadou et al., 2019).The central role of gut in glucose homeostasis

The gut or gastrointestinal tract (GIT) is the first anatomic site that interfaces the ingested food (primary source of glucose and energy) and body's metabolic homeostasis. It is the largest endocrine organ that regulates nutrient absorption and energy homeostasis of the body. The gut harbors a variety of cells especially in the small intestine, with receptors responding to various nutritional cues, and secretes hormones and nutrient metabolizing enzymes to crosstalk with major physiological systems like the liver, kidney, brain, pancreas and heart (Gribble and Reimann, 2019). Enteroendocrine (EE) cells are specialized hormone secreting cells that are dispersed throughout the mucosal epithelial layer of the gastrointestinal (GI) tract. EE cells consist of a spectrum of cell types, synthesizing and secreting a combination of more than 20 hormones in response to a variety of luminal and basolateral stimuli. Gut-derived hormones influence a range of physiological processes, including metabolic pathways. They perform these regulatory roles in glucose homeostasis, centrally-mediated appetite control and adiposity. The regulation of whole-body metabolism involves the integrated action of multiple metabolically active tissues, including the GIT, pancreas, adipose tissue, liver and the central nervous system (CNS). The release of one or a combination of gut hormones either postprandially (GLP-1, GIP, PYY, 5-HT, CCK, OXM) or during periods of fasting (ghrelin, 5-HT) significantly influences both glucose homeostasis and overall energy status. Each of these hormones can exert biological effects independently or can act in a synergistic manner. (Gribble and Reimann, 2019; Psichas et al., 2015; Sanchez et al., 2022)

1.3.6 Nutrient sensing and gut hormone secretion

The primary site of nutrient absorption is the apical side of the polarized epithelial cell layer of the upper small intestine, while nutrients activate metabolic and sensory signaling pathways in the mucosal layer to exert whole-body biological responses before being absorbed into the circulation. Stem cells within the GI tract differentiate into multiple cell types, including secretory cells such as a hormone secreting subtype enteroendocrine cells (EECs). They are heterogeneous and secrete a number of hormones like GLP-1, GLP-2, CCK, GIP and PYY

among others. Luminal glucose is taken up mainly by SGLT1 and leads the release of gut peptides. Luminal small oligopeptides and amino acids are taken up by PepT1 transporter and amino acid transporters, respectively, into the enterocyte and enteroendocrine cells. Small intestinal protein sensing stimulates the release of CCK and GLP-1 and regulates feeding and glucose homeostasis through a number of amino- and oligopeptide-sensing GPCR receptors, mainly CaSR, a Gαq-coupled receptor. Small intestinal long-chain fatty acids are taken up (via CD36/FATP4 and/or simple diffusion) by enterocytes to form triglycerides and eventually packaged into chylomicrons released on the basolateral side. LCFA are also taken up by enteroendocrine cells to undergo ACSL3-dependent metabolism and activate PKCs to potentially stimulate CCK and/or GLP-1 release. Luminal LCFA may activate GPR40/120 to stimulate peptide release (Psichas et al., 2015; Sanchez et al., 2022).

Enteroendocrine cells spread throughout the GI tract also express a variety of neurotransmitter receptors, which form the gut brain network. The gut hormones can act as paracrine factors, neurotransmitters, and circulating hormones and acts as a relay center for triggering local and distant physiological responses in response to the nutrient availability (Barton et al., 2023).

1.3.7 Incretin effect and its role in whole body glucose homeostasis

Among the various enteric hormones secreted from enteroendocrine cells, incretin hormones identified in the 1980s garnered considerable interest in diabetes management because of their pleiotropic roles in enhancing insulin secretion, gastro-intestinal motility and modulation of gastro- intestinal crosstalk with other organs (Holst et al., 1987; Moody et al., 1984). The "Incretin effect" is defined as a biological phenomenon of 2–3 fold higher stimulation of insulin secretion in response to an oral glucose administration than an intravenous glucose infusion, even with the same plasma glucose profile (Andersen et al., 2018). Glucagon-like peptide-1 (GLP-1) and Gastric inhibitory peptide (GIP) are the two primary incretin hormones secreted from L cells present in ileum and colon and K cells located in the duodenum (Müller et al., 2019; Holst, 2019). Dietary components like fat and glucose are the primary stimulants for incretin secretion. Their biology, mechanism of action and pleiotropic role in glucose homeostasis are extensively studied and their potential in diabetes management is well established (Holst, 2006; Tolhurst et al., 2009). GLP-1 and GIP are synthesized as pro-peptide, cleaved into hormones by pro-convertases, and are inactivated by the enzyme dipeptidyl peptidase-4 (DPP-4). GLP-1 is encoded by the preproglucagon (*Gcg*) gene, which is processed into different peptides such as GLP-1 or glucagon under the action of tissue-specific

proconvertases such as PC2 and PC1/3. GLP-1 is produced as a 37 amino acid peptide after post-translational maturation, GLP-1(1-37). Around 25% of the active GLP-1 reaches the liver where it is also degraded by hepatic DPP-4 (40–50%) and 10–15% of active GLP-1 reaches the bloodstream. Beside GLP-1 incretin's effect on insulin secretion, GLP-1 exerts myriad metabolic effects, such as the control of food intake, gastric emptying and motility, increased muscle insulin sensitivity, and glucose uptake (Fig-7). The enteric nervous system (ENS) which is present throughout the gut relays the information to and from the brain forming the gut brain network for the integration of complex physiological processes driving the energy homeostasis (Holst, 2007).

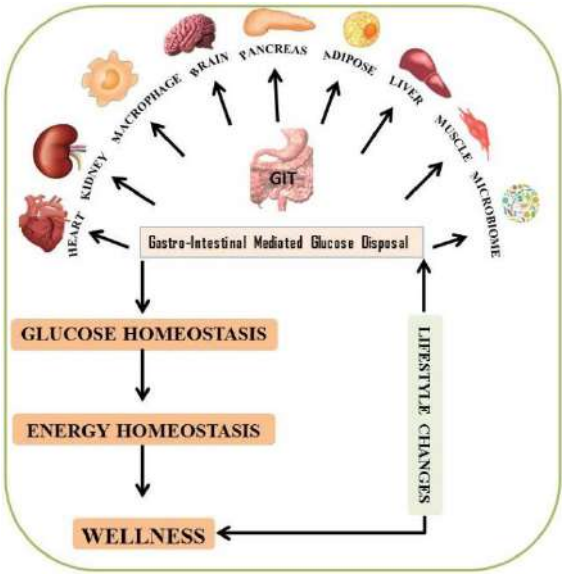

Fig-7. The pleotropic role of GIT in glucose homeostasis Figure courtesy (Thottapillil et al., 2021)

1.3.8 Loss of incretin effect in T2DM

One of the characteristics of T2DM is a more or less complete loss of the incretin effect (Holst et al., 2011). Studies of dose response profiles of incretin hormones in response to oral glucose proved that incretin effect is one of the major gastrointestinal mechanisms by which glycemic control is secured. It has been observed that secretion of GLP1 itself is decreased in T2DM and obesity and the mechanisms underlying the same are not very clear and still under investigation. Some studies showed that while GIP responsiveness is lost in T2DM, GLP1 still retains the ability to restore insulin secretion levels to values similar to those observed in healthy controls. This led to more focus being given for developing GLP1 based therapies (Nauck and Meier, 2016).

1.3.9 Extra-pancreatic effect of GLP1

Several studies have proved that GLP1 exerts its effects on other physiological functions also through downstream signaling via GLP1R (Fig-8). One such effect is the protective effect on cardiovascular system. This is through its receptor GLP1R signaling, which is expressed in cardiomyocytes and also an increase of cardiomyocyte survival via inhibition of apoptosis, and improving endothelial dysfunction. Numerous clinical and animal studies have demonstrated the ability of GLP1 to reduce body weight through regulation of satiety and appetite. Studies showed that the mechanism of food intake inhibition is mediated by complex mechanisms mainly dependent on GLP1R signaling in hindbrain by circulating GLP1. GLP1 receptors are also expressed in the kidney and studies in humans and rodents have demonstrated that GLP1R agonists improved renal functions. This has been attributed to its induction of sodium excretion (natriuresis) in healthy and diseased subjects. The beneficial renal outcomes are also as a result of its actions on conventional risk factors like glycemic control, weight control and BP. (Drucker, 2022, 2018; Holst, 2007; Holst et al., 2009; Smith et al., 2019)

Apart from this, GLP1 also influences functions of adipose tissue and liver which are centrally involved in lipid and glucose metabolism by increasing glucose uptake and inhibiting hepatic gluconeogenesis. GLP1 agonists have been shown to enhance hepatic and adipose insulin sensitivity. Animal studies revealed a novel role for vagal afferent neuron (VAN) GLP-1R signaling in the regulation of EE (energy expenditure) and brown adipose tissue (BAT) thermogenesis (Krieger et al., 2018)

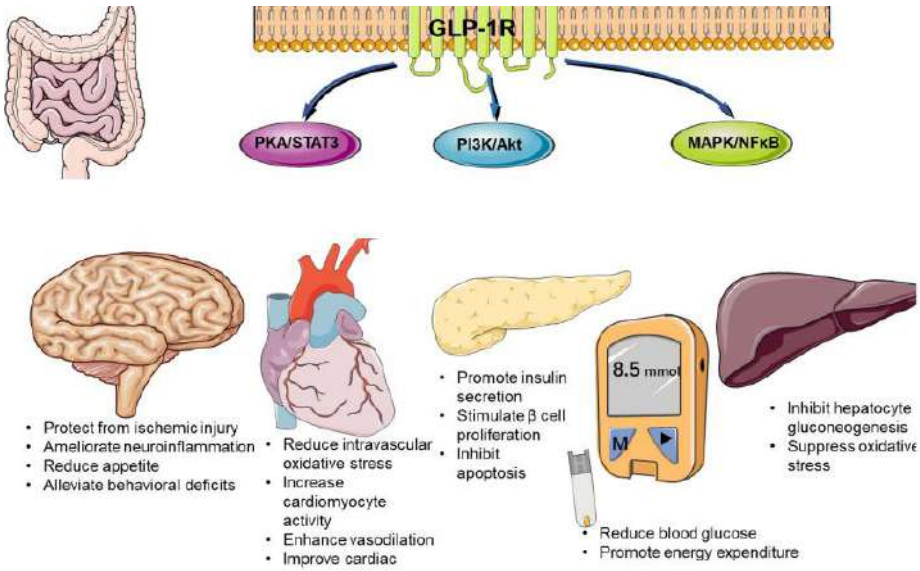

Fig-8. GLP1 signaling. Illustration depicting GLP1 and actions through major signaling pathways such as AKT1, NFKB1 and PKA. Figure courtesy (Chen et al., 2022)

1.3.10 Role of GIP

GIP is another enteroendocrine hormone secreted from K cells lining the more distal segments of the intestine. Both GLP-1 and GIP have functional similarities in their insulinotropic effects through G-protein coupled receptor (GPCR) signaling in pancreatic beta cells, and both have their insulinotropic effects inactivated by the enzyme dipeptidyl peptidases–4 (DPP4). They are also shown to enhance pancreatic beta cell proliferation and increase pancreatic beta cell mass by inhibiting apoptosis, whereas they suppress glucagon secretion from pancreatic alpha cells (Perfetti et al., 2000; Ramracheya et al., 2018). While they are similar in their insulinotropic activity, GLP-1 is shown to inhibit glucagon secretion from pancreatic alpha cells, whereas GIP stimulates glucagon secretion during hypoglycemia. Therefore, GIP can serve as a bifunctional hormone to work against both extreme excursions of glucose. Recent studies have shown the role of GIP in influencing body weight by regulating fat deposition and adipocyte biology, where Tirzeptide, a dual agonist of GIP and GLP-1 found to reduce body weight (Müller et al., 2019; Frias et al., 2018). Similarly, GLP-2, which is also secreted by L cells of the intestine, is another potential target for whole body glucose homeostasis owing to their important role in energy homeostasis and intestinal integrity as evidenced by several studies (Amato et al., 2016).

1.3.11 Other gut peptides

Apart from the incretin effect, the human gut has several key functions during postprandial glucose metabolism. Although incretins play an important role in all these gut-centric events, there are a host of other peptide hormones secreted by gut epithelium that regulate many of these metabolic functions in the body. They also regulate the important process of gastric emptying which is an important determinant of postprandial blood glucose levels. They are broadly grouped under two classes, orexigenic (appetite stimulant) and anorexigenic (appetite reducers). While the majority are anorexigenic, only ghrelin and INSL5 are orexigenic in action. Some of other gut peptides such as oxyntomodulin, PYY, neurotensin, nesfatin, secretin, cholecystokinin and apelin are now considered as potential gut hormone polyagonists for therapeutic applications for diabetes and obesity owing to their role in food intake and gastric functions (Knauf et al., 2020; Michell et al., 2008).

The cross talk of gut with other systems like immune cells, liver and kidney plays a key role in maintenance of normal glycemic levels. The gut modulates hepatic glucose metabolism as well

as renal function through regulating electrolyte and fluid homeostasis (Michell et al., 2008; Muskiet et al., 2014; Radziuk and Pye, 2001).

1.3.12 Gut microbiome

Gut microbiome is a unique entity residing in the gastrointestinal tract and plays a crucial role in host digestion, nutrition and interactions with other tissue and organ systems in the body (Shreiner et al., 2015). Diabetes being a majorly influenced by diet, gut microbiome is shown to be a critical player in pathophysiology and management of T2DM and obesity. Metagenomics and metabolomics-based studies show that diabetes causes imbalances in gut microbiome homeostasis (Allin et al., 2018; Karlsson et al., 2013; Vangipurapu et al., 2020). Various mechanisms such as metabolic endotoxemia, modulation of incretin secretion and butyrate production have been proposed to explain the influence of the microbiota on insulin resistance and T2DM (Jia et al., 2017; Larraufie et al., 2018; Madsen et al., 2019). Substantial body of knowledge points out the relevance of gut microbiome for mitigating T2DM and several studies are underway to explore this (Gurung et al., 2020). Due to the multi-system involvement of the gastrointestinal tract, gut is an important therapeutic target for T2DM and associated metabolic diseases.

1.3.13 Gut - a converging node for *Ayurveda* and Biomedicine

As per the *Ayurveda* perspective also, gut plays a significant role in *Prameha* or T2DM pathophysiology and management. Three unique concepts of *Ayurveda* viz. *Agni*, *Ama* and *Rasa-dhathu* are central in understanding the pathophysiology of *Prameha*. *Agni* is a physiological phenomenon that optimizes the metabolic transformations in the body by regulating digestion, absorption and bio-assimilation of nutrients from ingested food. The strength and homeostasis of *Agni* is critical in determining the overall health of an individual. *Ama* is antithesis to *Agni* and has a vicious negative feedback effect on *Agni*. It is defined as the detrimental products derived from improperly digested and assimilated food due to malfunctioning of *Agni*. The third concept, *Rasa-dhathu*, is the circulatory liquid form of the bio-assimilable part of the digested food, which is the primary structural component for the formation of all other tissue systems in the body. According to *Ayurveda*, urine production starts in the GIT and therefore the pathological cascades of *Prameha* also begins at the gut, but clinically manifest in the urinary system (*Vasti*) as shown in Fig-4 in section 1.2.2. Various etiological factors like genetics, lifestyle issues, unwholesome diet etc., disrupt the *Agni* homeostasis in the GIT and cause *Kapha-dosha* domination in the body. Due to improper *Agni*,

accumulation of metabolic wastes (*Ama*) happens in the gut and other tissues that hamper the *Rasa-dhathu* metabolism and its supportive functions to tissues like muscle, fat and bone marrow ultimately resulting in manifestation of signs and symptoms of *Prameha* (Murthy and Singh, 1989). Hence the diabetes management (both prophylactic and therapeutic) in *Ayurveda* begins with restoration of *Agni* homeostasis and reduction of *Ama* accumulation, and thereby improving absorption, bio-assimilation and utilization of nutrients for physiological functions. The prescribes *Ayurveda* formulations with their multifunctional natural molecules utilize gut as the most suitable site that naturally disseminates bioactivity all over the body. *Ayurveda* being a wellness science, it emphasizes more on prophylaxis than treatment. Nevertheless, the same algorithm or logic is followed for both prevention and cure. The *Prameha* management algorithm of *Ayurveda* is broadly categorized into purification process (*Shodhana-chikitsa*) and palliative therapy (*Shamana-chikitsa*). The purification process is aimed at restoration of '*Agni*' to augment digestion and metabolism by expelling '*Ama*', the metabolic wastes, accumulated over a period of time. Palliative therapy is the medication approach used to restore the physiological balance using various herbal and herbo-mineral formulations (Fig-4). In real-life clinical practice, *Ayurveda* physicians perform a process of elimination of metabolic wastes by *Shodhana-chikitsa* to normalize the *Agni* functions in the body; followed by *Shamana-chikitsa* to restore the functions of '*Agni*' to augment digestion and metabolism by expelling '*Ama'*, the metabolic wastes, accumulated over a period of time. Palliative therapy is the medication approach used to restore the physiological balance using various herbal and herbo-mineral formulations (Fig-4). To sum up, gut and gut derived factors play an important role in the systemic management of *Prameha* (Thottapillil et al., 2021).

Ayurveda's gut centered view to explain the manifestations and management of metabolic diseases overlaps with modern medicine's view of gut being crucial in glucose homeostasis. Emerging as a point of confluence for modern medicine and *Ayurveda,* gut-based therapies have become integral in designing innovative therapies for T2DM and obesity management

1.3.14 Gastro-intestinal mediated glucose disposal (GIGD) - a gut parameter evolving as a concept for holistic glucose metabolism

The whole-body glucose homeostasis is centrally regulated by gut mediated factors; the entire gut centered events of postprandial glucose metabolism can be collectively referred as gastro-intestinal mediated glucose disposal (GIGD). It is primarily measured as a parameter to estimate the glucose clearance by incretin effect, which is the most effective gut determinant of glucose metabolism to date. Higher the ingested glucose, greater is the incretin effect. Therefore, as a parameter GIGD describes how effectively the body alleviates the postprandial glucose excursions driven primarily by the incretin effect. Besides incretin hormones, there are several other components like gut microbiota, first-pass hepatic glucose uptake, glucosidase inhibitors, immunomodulation, inhibition of intestinal glucose absorption as well as hitherto unknown factors which collectively contribute to the postprandial glucose disposal as well as maintenance of the body's glucose homeostasis (Fig-9) (Holst, 2019). Glucose being the primary energy source for all the tissues in the body, perhaps, GIGD components may have a broader role in optimizing the physiology and metabolism of all the tissue systems in the body by regulating the glucose metabolism. Therefore, it is imperative to consider gut as a whole and GIGD as one of the important nodal concepts that converge the principles of diabetes management in Ayurveda and biomedicine.

With ever increasing prevalence, it is vital to find better integrated therapies for chronic multi-factorial diseases like T2DM and obesity. Combining the holistic healing principles of Ayurveda along with the scientific rigor of modern medicine can usher novel therapy ideas for complex lifestyle disorders. Although the Ayurveda formulations contain many medicinal plants which are well studied, the formulations as such, lack scientific evidence for their pharmacological actions. To decipher the mode of action of these multi-component therapies, it is important to devise innovative methods along with corelating Ayurveda principles rationally. A trans-disciplinary 'Ayurveda-Biology' research framework focused on developing unique in vitro and in vivo methods for studying the systemic action of the multi-component therapies holds great promise for developing holistic diabetes care protocols.

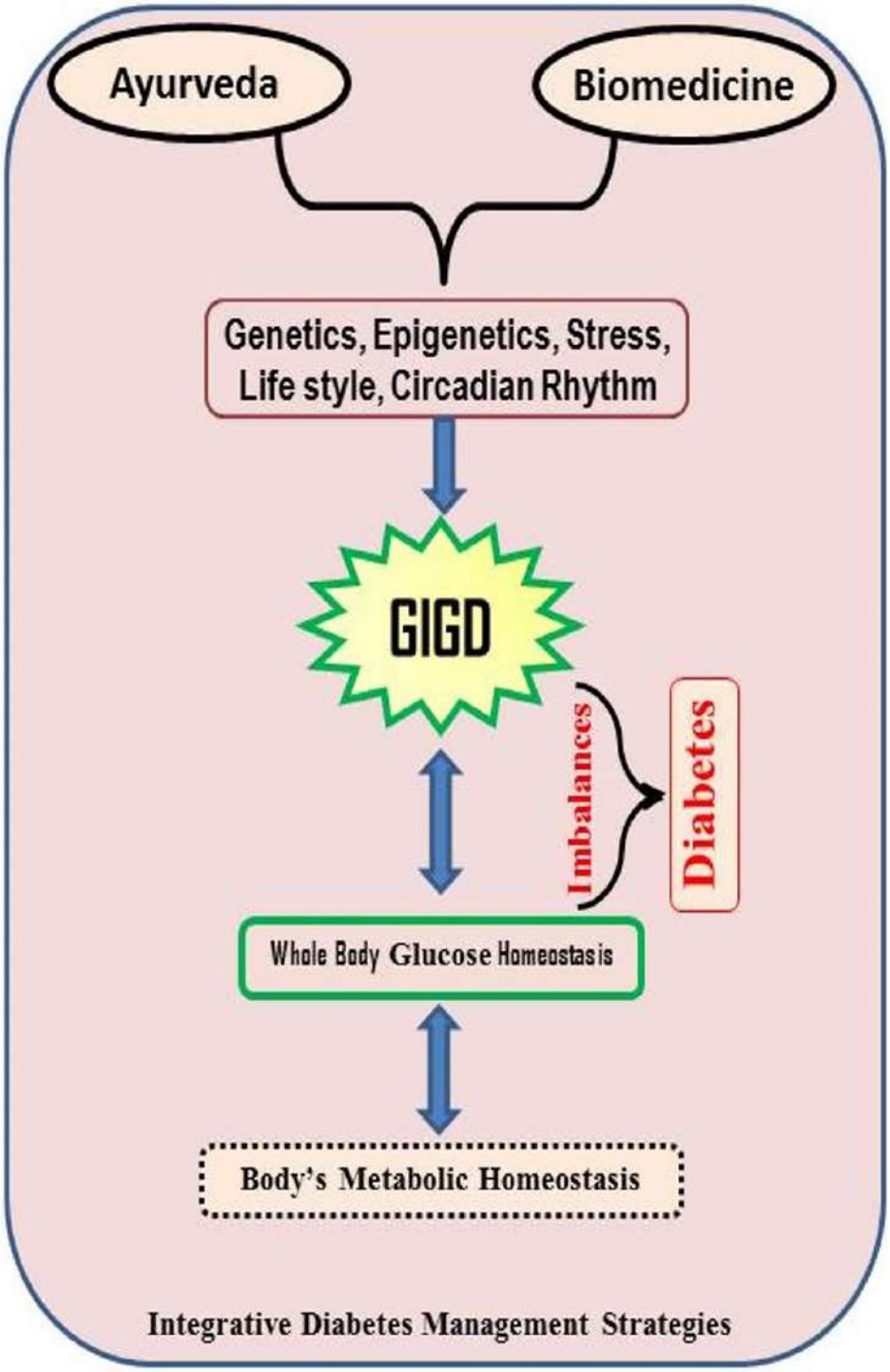

Fig-9. A converging role for gut in *Ayurveda* and Biomedicine. Figure courtesy (Thottapillil et al., 2021)

With this premise, the thesis puts forth a study of four classical *Ayurveda* formulations which are clinically prescribed for T2DM or *Prameha*. This study has been carried out with four objectives; 1**)** To create a transdisciplinary knowledge platform and establish in vitro and in vivo assays for mode of action of Ayurveda formulations; 2) To study the differential action of multicomponent Ayurveda formulations used in diabetes and obesity management; 3) To study the modulation of gut /GIGD components by Ayurveda formulation; 4) To delineate the mechanism of action, at molecular and systemic levels, of Ayurveda formulations in modulating body's glucose homeostasis.

For achieving these objectives, the thesis hypothesized that Ayurvedic concept of holistic diabetes management through *Agni* correction can be correlated with GIGD because of its multifunctional effect. To delineate this multi modulatory effects of the formulations, various *in vitro*, *in silico* and *in vivo* approaches were adopted. The *in vitro* methods included modulation of key digestive enzymes (α-glucosidase and α-amylase), DPP4, GLP1 secretion and adipogenesis. *In silico* methods included network pharmacology and molecular docking to understand the multi-targeted effect of the complex formulations and finally in vivo studies were conducted in a diabetic rat model to understand the effect and mode of action.

Chapter – 2
Materials and Methods

2 Materials

2.1.1 Shortlisting of *Ayurveda* formulations

Ayurveda clinical management uses several formulations for the management of diabetes depending on the patient physiology, disease stage, severity and the co-morbidities associated with it. Among the several formulations used, this thesis work selected four '**classical *Ayurveda* formulations**' (descriptions given in results Chapter – 3.1) namely *Nisakatakadi Kashaya* (NK), *Varanadi Kashaya* (VA), *Vasantha Kusumakara Rasa* (VK), and *Chandraprabha vati* (CV) based on the discussions had with the clinicians at the Institute of *Ayurveda* and Integrative Medicine (IAIM), Bangalore and other *Ayurveda* experts at the University of Transdisciplinary Health Sciences and Technology, Bangalore. These are among the top prescribed formulations by the physicians in their clinical practice for managing diabetes and associated co-morbidities. The names and the sources of formulations is described in the table below (Table - 3).

S. No	Name of the formulation	Source	Physical form
1	*Nishakatakadi Kashaya*	Vaidyaratnam Oushadhashala, Kerala. Batch No: 16A3404 (For *in vitro* studies); Batch: 22A4680, 23A1800 (for *in vivo* studies)	Liquid
2	*Varanadi Kashaya*	The Arya Vaidya Pharmacy, Tamil Nadu. Batch No: AEA156 (for *in vitro* studies); Batch No: AII-092 (for *in vivo* studies)	Liquid
3	*Chandraprabha vati*	Vaidyaratnam Oushadhashala, Kerala. Batch no: 18C0034	Tablet form
4	*Vasantakusumakara rasa*	Amrita Drugs, Telengana Batch no:30	Tablet form

Table-3. Name and source of the compound formulations used in this study

2.1.2 Cell lines

Murine GLUTag cell line was obtained as a generous gift from Dr. Tohru Hira, Hokkaido University, Japan with the kind permission of Dr. Daniel Drucker, University of Toronto, Canada. 3T3-L1 fibroblasts used in the study was procured from National Centre for Cell Sciences, Pune, India. All cells were maintained and used for various assays as described under section 2.3.2.5 in this chapter.

2.1.3 Chemicals and Reagents

The enzymes α-amylase (Origin: *Aspergillus oryzae*; Cat. No. 10065) and α-glucosidase (Origin: *Saccharomyces cerevisiae*; Cat. No. G5003); Thiazolyl Blue Tetrazolium Bromide (Cat.No: M2128); Benzylamine (Cat. No. 100-46-9); Dimethyl Sulfoxide (Cat. No. 67-68-5) as well as other fine chemicals were purchased from Sigma-Aldrich (St. Louis, MI). Dulbecco's Modified Eagle's Medium (Cat. No. 11965084) and Fetal Bovine Serum (Cat. No. 10270106) were purchased from Thermo Fisher Scientific Inc. The DPP-4 drug discovery kit was procured from Enzo Life Sciences (Farmingdale, New York, NY, USA). GLP1 kit was purchased from Ray biotech (CAT NO-EIAR-GLP1-1, RAT GLP-1 EIA,). Triglycerides Cholesterol were purchased from Biosystems Catalog Number:11258 and 11505 respectively. For Metformin, Glycomet (Metformin 500 mg Tablet), and Metformin hydrochloride (Honeychem pharma) for the pure compound.

2.2 Rationale for various assays performed

The thesis used various *in vitro*, *in silico* and *in vivo* experiments as well as retrospective analysis of clinical data from the hospital to ascertain the Ayurveda Biology framework and to understand the mechanism of action of the selected formulation. This section summarizes the rationale of various experiments used in the study.

2.2.1 α-amylase and α-glucosidase inhibition

The enzyme α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) is a key therapeutic target that has been targeted for developing several synthetic drugs such as acarbose, voglibose, and miglitol. The α-amylase, mainly seen in the saliva and pancreas, helps to start the chemical breakdown of carbohydrates by hydrolyzing the glycosidic bonds in starch and related substrate polysaccharides, transforming them into oligosaccharides and simple absorbable sugars (Stiefel and Keller, 1973). Upon entry into the small intestine, partially hydrolyzed starch is further converted by the pancreatic amylases which targets the α-1,4 bonds of the carbohydrate releasing dextrins (des Gachons and Breslin 2016). While the salivary amylase is produced by the salivary glands and initiates carbohydrate digestion in the mouth, a large amount of pancreatic amylase is secreted by the pancreas into the duodenum to continue the digestion of incoming starch.

The final step in carbohydrates metabolism is mediated by α-glucosidases (EC 3.2.1.20) in the brush border of the enterocytes. The enzymes contain duplicated glycoside hydrolases (GH31) domains and they catalyze the hydrolysis of α-glucosidic linkages of disaccharides and

oligosaccharides (Jongkees and Withers 2014; Lombard et al. 2014). Polysaccharides and monosaccharides resulting from the action of α-amylase and α-glucosidase are absorbed at different rates by the body with monosaccharide units being absorbed more quickly. The inhibition of α-amylase and α-glucosidase activity can therefore retard glucose liberation from complex carbohydrates modulating the onset of postprandial hyperglycemia, thereby rendering it an ideal target for the management of T2DM.

2.2.2 Anti-adipogenic assay

Obesity, one of the important pre disposing factors of T2DM, is a complex, chronic disorder caused by the interaction of different contributing parameters, including dietary, lifestyle, genetic, and environmental factors. Obesity is associated with an increase in adipose tissue, which is caused not only by increased adipocyte size (hypertrophy) but also by increased adipocyte number (hyperplasia). Hyperplasia is regulated by the de novo differentiation of preadipocytes, which are in the stromal-vascular fraction of adipose tissue. The regulation of adipocyte differentiation might be of pivotal importance for developing strategies for the prevention and treatment of obesity, diabetes and other associated co-morbid conditions (Baker et al., 2022).

2.2.3 DPP4 inhibition and GLP1 secretion

The incretin hormone glucagon-like peptide-1 (GLP-1), regulates appetite and satiety, inhibits glucagon secretion and promotes insulin production by stimulating pancreatic cells for more insulin secretion and also exerts beneficial effects on physiology by exerting effects on other tissues beyond the pancreas. They are produced by enteroendocrine cells in the gastrointestinal tract and are rapidly degraded by dipeptidyl peptidase-4 (DPP-4). Analogues and receptor agonists of GLP-1 as well as DPP-4 inhibitors are newer classes of antihyperglycemic agents used for the management of T2DM (Scheen, 2012)

2.2.4 *In silico* Studies

In silico studies for herbal formulations provides a holistic understanding of the complex interactions between multiple compounds in Ayurveda formulations and their biological targets. Ayurveda formulations containing multiple active ingredients and a systems approach through network analysis aids in identifying how these compounds exert their influence on various biological pathways and targets simultaneously. For this, the phytochemicals extracted from the herbs in formulations were mapped to putative target genes from various databases

and further studied for disease associations, pathways and biological processes. Additionally, as a part of the in-silico studies, virtual screening of phytochemicals extracted from the formulations was done to identify the potential DPP4 inhibitors followed by molecular docking and simulation to evaluate the stability between compounds and the target DPP4 (Patwardhan and Chandran, 2015a; Uma Chandran, Neelay Mehendale, Saniya Patil, 2002).

2.2.5 *In Vivo* studies

To explore the mechanism of action of the anti-diabetic *Ayurveda* formulations *in vivo* and to corroborate the in vitro findings, a type 2 diabetic model was chosen. For the *in vivo* experiments, high-fat diet-fed, streptozotocin (HFD/STZ) induced rat model was chosen. This model utilizes a high-fat diet, to induce hyperinsulinemia, insulin resistance, and glucose intolerance. Subsequently, the administration of the β-cell toxin STZ significantly reduces functional β-cell mass. This model captures the metabolic profile of T2D to a large extent making it a suitable model. After the T2D was induced in the obese rats, various experiments such as glucose tolerance test, GLP1 secretion and blood glucose, serum lipids were assayed followed by histopathological examinations of liver, pancreas, intestine and kidney (Skovsø, 2014; Srinivasan et al., 2005a). In addition to understand the mode of action, the in vivo studies also is means to assess the safety and toxicity of the *Ayurveda* polyherbal formulations.

2.3 Methods.

The current thesis used *in vitro*, *in silico* and *in vivo* methods to delineate the mode of action of four formulations selected for the study. Additionally, a preliminary retrospective analysis of the clinical data of patients treated with these formulations at the IAIM was also performed to understand the relevance of using multi-drug interventions, as per the *Ayurveda* disease management algorithms, and its relevance in achieving a personalized or ‘precision-medicine’ therapy for accomplishing the desired biological effects. The figure below (Fig-10) gives an overview of the methods used in the study, which is followed by a detailed description of every method.

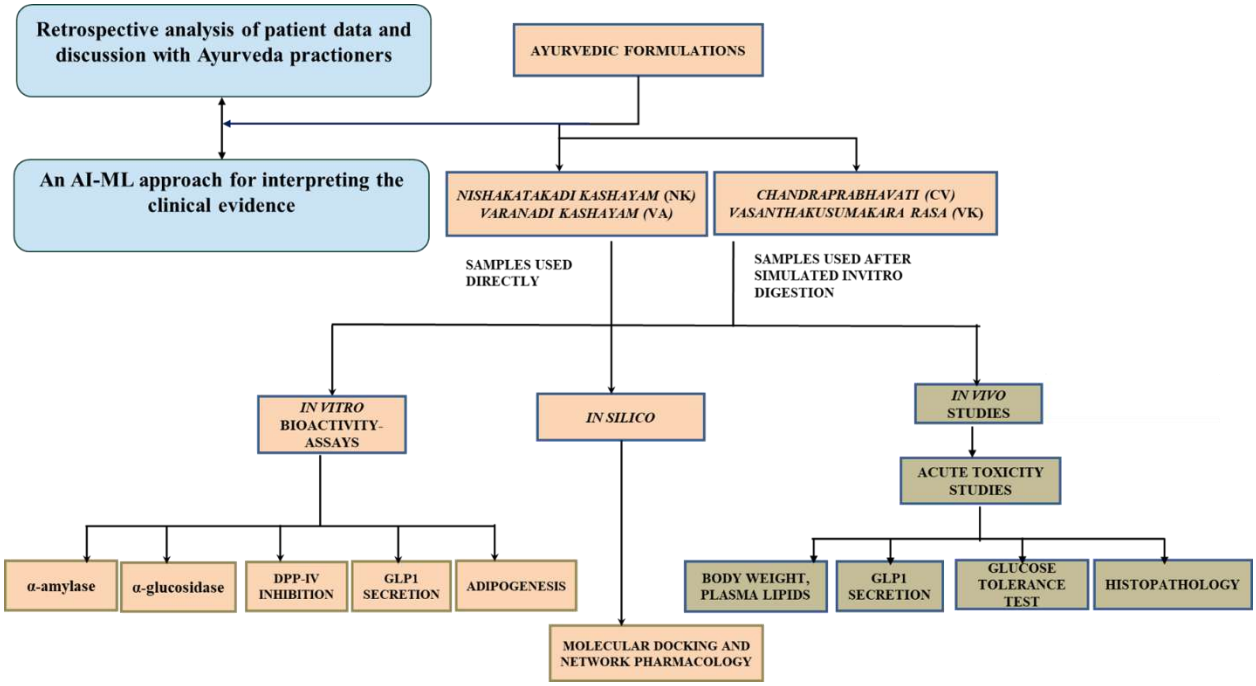

Fig 10: Overview of experimental methodology

2.3.1 Retrospective clinical data analysis of the shortlisted formulation

The current study used the clinical outcome data of 230 patients who visited the diabetic clinic (*Swasthavritta* department) of the Institute of Ayurveda and Integrative Medicine (IAIM), The University of Transdisciplinary Health Sciences and Technology (TDU), Bengaluru, Karnataka, India between 2011 and 2017 and got treated for diabetes were retrospectively collected and documented. The protocol was approved by the institutional ethics committee for human research (Protocol number as **TDU/IEC/10E/2018-19/PR02**; **Version:2**; dated **22nd March 2019**). Personal identification details of the patients were kept confidential. The primary inclusion criteria were that the patient received one or a combination of the five classical *Ayurveda* formulations viz. *Nishamalaki* (NA), *Nisakathakadi* (NK), *Varanadi Kashaya* (VA), *Chandraprabha Vati* (CV), *Vasantha kusumakara Rasa* (VK). However, the data shows some of the patients among the study population received one or many of 27 other drugs (classical Ayurvedic preparations and *Ayurveda* inspired proprietary herbal preparations), in addition to the above mentioned five formulations. This prescription was as per the discretion of the physician following the principles of personalization and management framework emphasized by the Ayurvedic system. In the present study all these 'other drug interventions' were grouped into a single separate set for the convenience of data handling. In

the current study, the Self-Organizing Maps (SOM) was implemented using the XLSTAT software and validated using the R language in the posit.cloud framework. The use of XLSTAT provided an efficient tool for SOM implementation, while the R language allowed for the customization of the SOM visualization and analysis. The validation of SOM using R language in the posit.cloud framework ensured the accuracy and reproducibility of the results.

2.3.2 *In Vitro* Studies

2.3.2.1 Sample preparation – simulated *in vitro* digestion of *Ayurveda* formulations

The present study used two types of poly-herbal formulations. Liquid (*Kashaya*) preparations viz. NK and VA, and solid (tablets) preparations viz. VK and CV. Both NK and VA are directly used for the assay after estimating total tannins. Whereas both VK *(*1.25g), and CV (2.5 mg) were subjected to a simulated *in-vitro* digestion protocol (standardized in the lab for *Ayurveda* formulations) to convert the samples into assay compatible liquid forms (Minekus et al., 2014a). Briefly, the electrolyte solutions of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as per the details given in Table – 4 (Butala et al., 2018). For simulated gastric digestion, human equivalent doses of VK (1.25g) and CV (2.5g) were mixed with 250 mL of SGF containing 2500 U/mL pepsin and 0.16 mM $CaCl_2 \cdot 2H_2O$ (pH was adjusted to 2.0 by adding 6N HCl) was incubated at 37 °C for 2 h in a shaking water bath. Following digestion, the gastric chyme was mixed with 250 mL SIF containing pancreatin-bile solution (final concentration of 500 µg/mL pancreatin and 3 mg/mL bile) and 0.6 mM $CaCl_2 \cdot 2H_2O$ (pH adjusted to 7.0 by adding 5N NaOH) and incubated at 37 °C in a shaking water bath for another 2 h to complete the digestion. The digest was heated at 65°C for 30 mins to inactivate it from any residual enzyme actions. The digests were filtered through cotton plug and the filtrate was collected and stored at −80 °C for further use. All samples were estimated for total tannins and the samples were quantitatively used as 'µg of GAE/mL' (as per the protocol mentioned in 2.3.2.2).

S. No	Component	Final Concentration (mM)	
		SGF	SIF
1	KCl	6.9	6.8
2	KH_2PO_4	0.9	0.8
3	$NaHCO_3$	25	85
4	NaCl	47.2	38.4
5	$MgCl_2$	0.1	0.330
6	$(NH_4)_2CO_3$	0.5	-
7	HCl	Adjust to pH 2.0	

8	NaOH		Adjust to pH 7.0

Table – 4. The composition of SGF and SIF electrolytes.

2.3.2.2 Estimation of total gallic acid equivalent using Folin - Ciocalteu method.

Both NK and VA as well as digests of VK and CV were estimated for total tannins following standard Folin - Ciocalteu method with modifications to suit to samples, using gallic acid as standard (Ainsworth and Gillespie, 2007). Briefly, 10µL of the formulation or 10µL of the digest is mixed with 40 µL of water, 50 µL Folin's reagent and 100 µL of 3.5% Na_2CO_3 as 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 (Mark Microplate Spectrophotometer, BioRad, USA). The experimental concentrations of test samples were expressed as 'µg of gallic acid equivalent tannin (GAE)/mL of sample'

2.3.2.3 α-amylase and α-glucosidase inhibition assay

Both α-amylase and α-glucosidase inhibition assays were carried out following standard protocol with modifications to suit to 96-well plate format. For α-amylase, all incubations were done at room temperature. Increasing concentrations of the samples were taken and the volume was made up to 50 µL using 0.02M sodium phosphate buffer (pH 6.9). To this, 50 µL of α-amylase (0.5 mg/mL) was added and incubated for 30 min. Followed by this, 50 µL of 1% starch (soluble potato starch) was added as substrate and incubated for another 10 min. The reaction was stopped by adding 50 mL of 1% dinitrosalicylic acid (DNS) and the plate was heated at boiling water bath for 5 min till the development of an orange red color. The absorbance was read at 550 nm, using plate reader (Butala et al., 2017b). For α-glucosidase, similar to α-amylase, different concentrations of the samples were taken and the volume was made up to 50 µL with 0.02 M sodium phosphate buffer (pH 6.9). To this, 50 µL of α-glucosidase (0.5 U/mL) was added and incubated for 10 min at room temperature, followed by the addition of 50 µL of 3.0 mM p-nitrophenyl glucopyranoside (pNPG) as substrate and incubation for 20 min at 37 °C. The reaction was stopped by adding 50 µL of 0.1 M Na_2CO_3.

For α-amylase and α-glucosidase the absorbance was read at 550 and 405 nm, respectively, using plate reader. Test samples with 50 µL of buffer was used as control for enzyme activity for both α-amylase and α-glucosidase and a set of test samples without enzyme was used to measure the basal level of reducing sugars present in the test samples. The absorbance was

subtracted from the corresponding test readings. The percentage inhibition of enzyme activity for α-amylase was calculated as follows.

$$\% \text{ Inhibition} = [(Abs_{(control)} - Abs_{(test)})/Abs_{(control)}] \times 100$$

2.3.2.4 DPP4 inhibition assay

The DPP4 inhibition assay was carried out as per the kit instructions. Briefly, various concentrations of NK and VA 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 minutes 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,

$$\text{Percentage (\%) activity remaining (with inhibitor)} = [Slope_{(inhibitor)} / Slope_{(control)}] \times 100$$

2.3.2.5 GLUTag cell maintenance and GLP-1 secretion assay

GLUTag cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (PS) solution at 37°C in a 5% CO_2 humidified atmosphere.

For GLP-1 secretion assay, cells seeded in poly-L-lysine coated 24-well plates (85000 cells/well) were grown for 48 h and then serum starved for an hour. Following which, the wells were treated with glucose free media (DMEM) with or without different concentrations of NK (12.5, 25, 50 μg of GAE/mL) and incubated for 2 h in a 5% CO2 atmosphere at 37°C. The media was collected and briefly centrifuged to remove any debris and secreted GLP-1 levels were assayed using GLP-1 ELISA kit (Ray biotech) following the manufacturer's instruction

2.3.2.6 Maintenance and differentiation of 3T3-L1 fibroblasts

3T3-L1 fibroblasts procured from NCCS were cultured in DMEM containing 10% FBS and 1% PS in 5% CO_2 incubator at 37°C. Differentiation of 3T3-L1 fibroblasts was induced by incubating two days post-confluent plates (day-0) in differentiation induction medium (MDI) containing 500μM IBMX, 250 nM dexamethasone and 100 nM insulin. On day-3, the medium was replaced with fresh culture medium and was replaced every two days till the cells attained complete adipocyte morphology

2.3.2.7 MTT assay

The cytotoxicity of the digests (VK and CV) and formulations (NK and VA) were evaluated using the MTT assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase in viable cells. The respective cell lines (GLUTag or 3T3-L1) were seeded in 96-well plates and grown till confluency. They were treated with different concentrations of digests and formulations and incubated for 24 h at 37˚C. After incubation, 500µg/mL MTT solution was added to each well and incubated for 4 h at 37˚C. The violet formazan crystal in each well was dissolved in DMSO and the absorbance of each well was measured at 570 nm using an ELISA microplate reader (xMark Microplate Spectrophotometer, BioRad, USA). The cell viability was calculated and least toxic concentration was used for further cell-based assays.

2.3.2.8 Anti-adipogenesis assay and Oil-Red O staining

Anti-adipogenesis assay and Oil-Red O staining were done following published protocols with minor modifications (Butala et al., 2017b ; Nidhina et al., 2011). Briefly, two days post-confluent cells, grown in 48 well plates, were co-treated with MDI and different concentrations of VK for three days and followed the standard adipocyte differentiation protocol as mentioned in cell culture and differentiation. Cells treated with only MDI or vehicle were used as positive and negative controls respectively. After differentiation, Oil Red O staining was performed to qualitatively and quantitatively evaluate the anti-adipogenic effect of the sample. Cells were washed twice with phosphate buffer, and fixed with 4% formaldehyde for 30 mins. Cells were then stained with freshly diluted Oil Red O solution (0.5% Oil Red O in isopropanol diluted to 3:2 with H2O, and filtered) for 20 mins at 37 °C. Images of cells stained with Oil Red O were captured using phase contrast microscope (Olympus-IX-71, Olympus America Inc, USA). For quantitative measurement, the stain retained was eluted by 100% isopropanol and absorbance was read at 540 nm.

2.3.2.9 Triglyceride quantification assay

The cellular triglyceride accumulation was measured using standard assay kits. Briefly, the cells were washed with PBS, scraped into 250 µl 0.1% PBST and pulse sonicated with 45% amplitude for 45 seconds. The lysates were assayed for their total triglyceride content using assay kit and cellular protein was estimated using the Bradford method. The triglyceride

content was normalized to the total protein and the results were expressed as μg of triglyceride per mg of total protein.

2.3.2.10 Gene expression studies in 3T3-L1 and GLUTag cells.

Total RNA was extracted from differentiated 3T3-L1 adipocytes (treated with and without VK/CV) and GLUTag (treated with VA and NK) using Trizol reagent (Invitrogen, CA, USA). One microgram of total RNA was subjected to first strand cDNA synthesis with oligo (deoxythymidine) primers (Table-4) and Superscript II reverse transcriptase (Invitrogen, CA, USA). The target cDNA was amplified using the following sense and antisense primers

Total RNA was extracted from GLUTag cells, (treated with and without NK) using Trizol reagent (Invitrogen, CA, USA). One microgram of total RNA was subjected to first strand cDNA synthesis with oligo (deoxythymidine) primers (Table-5) and Superscript II reverse transcriptase (Invitrogen, CA, USA). The target cDNA was amplified using the following sense and antisense primers

Gene name	Primer	Sequence	mRNA accession number
PPAR γ	Forward primer	TTTTCAAGGGTGCCAGTTTC	NM_011146
	Reverse primer	AATCCTTGGCCCTCTGAGAT	
SREBP-1c	Forward primer	GTGAGCCTGACAAGCAATCA	NM_011480
	Reverse primer	GGTGCCTACAGAGCAAGAGG	
Glut-4	Forward primer	ACATACCTGACAGGGCAAGG	NM_009204.2
	Reverse primer	CGCCCTTAGTTGGTCAGAAG	
FASN	Forward primer	TGGGTTCTAGCCAGCAGAGT	NM_007988
	Reverse primer	ACCACCAGAGACCGTTATGC	
FABP4	Forward primer	AAGGTGAAGAGCATCATAACCCT	NM_024406.3
	Reverse primer	TCACGCCTTTCATAACACATTCC	
Leptin	Forward primer	GCTCATGTCCCTGTGGTTAG	NM_008493.3
	Reverse primer	GCCCTGAAATGCGGTATGTA	
DPP4	Forward primer	TCAACAGTCATGAGCAGAGC	NM_010074.3
	Reverse primer	GGTCTTCATCCGTGTACCAC	

Table-5. List of primers. The primers were used for qPCR experiments for adipocyte differentiation markers and DPP4 in GLUTag cells

2.3.3 *In-Vivo* studies

Experiments were conducted in an *in vivo* diabetic model of rats to corroborate the *in vitro* and *in silico* observations for both VA and NK. For this, a combined high fat and streptozotocin (HFD-STZ) was selected and various parameters such as effect on body weight and other plasma parameters were assessed.

2.3.3.1 Study animals and ethics committee approval.

All *in-vivo* experiments were carried out in Acharya BM Reddy college, Bengaluru. Male Sprague Dawley rats (SD rats) weighing 160±180 g were procured and maintained in Acharya BM Reddy college animal housing facility. Animals were maintained under laboratory conditions (temperature 21-25°C and normal humidity conditions). The animals were fed with commercial High Fat Diet (HFD) diet procured from VRK nutritional solutions, Pune. The composition of HFD and the normal diet are given in the Table-6. The study protocol was approved by institutional animal ethics committee (IAEC/ABMRCP/2023-2024/5).

S. No	Composition of HFD diet	Value	Composition of normal diet	Value
1	Protein	18.15%	Crude protein	7.47%
2	Fat	35.10%	Fat	3.25%
3	Fiber	2.5%	Fiber	2.81%
4	Carbohydrates	20%	Carbohydrates	63%
5	Calcium	1.25%	Calcium	1.25%
6	Total ash	3.5%	Total ash	4.7%
7	Phosphorous	0.67%	Phosphorous	0.66%
8	Energy through fat	3120 KCAL/KG (60%)	Moisture	7.47%
9	Energy through protein	1050 KCAL/KG (20%)	Energy	3060 KCAL/KG
10	Energy through carbohydrates	1030 KCAL/KG (20%)		
11	Energy from palm oil	2700 KCAL/KG (52%)		
12	Moisture	2.5%		
13	Cholesterol	2%		
14	Cholic acid	0.5%		
13	Energy	5200 KCAL/KG		

Table-6 listing the composition of High Fat Diet (HFD) and normal diet

2.3.3.2 Acute toxicity studies

Based on the limit test standard of the Organization for Economic Cooperation and Development (OECD) No 425 Guideline described in the reference for acute toxicity testing of drugs in rodents, two groups of 2 female SD rats were administered orally with 2mL/Kg of both NK and VA (Kifle and Belayneh, 2020). After 24 hrs of observation for physical or behavioral change, the animals were further administered with a higher dose of 5mL/Kg of each formulation and observed for 14 days for any sign of toxicity. Formulations were well tolerated and showed no fatality or no signs of any abnormal behavior.

For administration of formulations in diabetic condition, the doses were calculated according to the conversion table based on surface area as rat equivalent volume of adult human dose, derived by multiplying the adult human dose by 0.018 per 200 g of body weight of rat. (Ghosh MN, 2015). The adult human dose of formulations was considered to be 30 ml as high dose and 15 ml as low dose after discussion with the *Ayurveda* physicians. Formulations were administered by oral gavage.

2.3.3.3 Feeding of high fat diet and induction of diabetes in experimental animals

Male SD rats weighing 160±180 g were randomly divided into two nutritional groups: a standard diet (control group) and a high-fat diet (HFD - 60% energy from fat, 20% from carbohydrate, and 20% from protein; Total energy 5200 kcal/kg). Before the HFD feeding, animals were weighed and their initial blood glucose level were recorded. The animals were fed with high fat diet and daily food intake was recorded. Animals were weighed weekly to evaluate the weight gain. After 45 days of HFD diet, animals were fasted for 3 hrs (with free access to water) and injected streptozotocin at a dose of 30 mg/kg (STZ) prepared in citrate buffer.; pH: 4.4 (Ghasemi and Jeddi, 2023). The control animals received an IP injection of citrate buffer solution (vehicle). After 72 hrs of STZ injection, the HFD rats injected with STZ with the fasting blood glucose level ≥ 200 mg/dL were grouped into 7 groups as shown in Table-7

Groups	Treatment	No of animals
I	Control + Water	6
II	HFD+STZ (HFDS)	6
III	HFDS + Metformin	6
IV	HFDS and NK high dose 540mg/200g body weight	6
V	HFDS and NK low dose 270mg/200g body weight	6
VI	HFDS and VA high dose 540mg/200g body weight	6
VII	HFDS and VA low dose 270mg/200g body weight	6

Table -7. The different treatment groups of animals

The experimental rats were administered with the respective drugs with an oral gavage for a period of 30 days after STZ induction. The high fat diet was continued throughout the

formulations' treatment for the respective groups. 50 mg/1000g of metformin was given to the animals for the first 2 weeks and later was increased to 100 mg/1000g.

2.3.3.4 Oral glucose tolerance test (OGTT)

The OGTT was done after 45 days of HFD, and repeated after STZ induction on 48th day and finally on 26th day after drug intervention. Briefly, on the day of OGTT, animals were fasted for 3 hrs, following that 2 gm/Kg of glucose was administered orally. Blood samples were collected from the caudal vein, by means of a small incision at the end of the tail, at 0, 30, 60 and 120 min after glucose administration. Blood Glucose Level (BGL) was estimated by the enzymatic glucose oxidase method using a commercial glucometer (Accucheck Active glucometer and glucose strips). The results were expressed as the integrated area under the curve for glucose (AUC glucose), which was calculated using GraphPrism 10.1.1 Version.

2.3.3.5 Evaluation of various biochemical parameters upon treatment with formulations.

At the end of the experimental day, blood was collected from the retro-orbital sinus and centrifuged at 3500 rpm for 10 min at 4 °C. The supernatant was obtained for GLP1 measurement. The plasma samples collected from the animals were assayed for GLP1 secretion using Raybiotech GLP1 kit, catalogue no: EIA-GLP1. Plasma was separated and analyzed spectrophotometrically for triglyceride and cholesterol levels using the estimation kits mentioned in section 2.1.3. The liver, kidney, and pancreas tissues were surgically removed from each rat for H&E staining.; control group and 6 drug intervention groups described in the table

2.3.3.6 Histopathological examination of Liver, Kidney, Pancreas and Intestine in the animal groups

At the end of the experiments, the liver, kidney, pancreas and intestine in each animal group were immediately removed and fixed in 10% formalin. The organs from a single animal representing each group were given for histological studies. The specimens were submitted to Dr Vamshi's Biological Sciences and Research Center, Rajajinagar, Bangalore for detailed examination of the tissues.

2.3.4 *In-silico* studies

2.3.4.1 Phytochemical data collection for formulations VA and NK

The total phytochemicals reported from the ingredient plants of NK (8 ingredients) and VA (16 ingredients) were retrieved 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 of the ingredient plants 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.

2.3.4.2 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.

2.3.4.3 Preparation of Ligands

A total of 206 compounds from NK and 248 compounds from VA 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 minimized 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.

2.3.4.4 Molecular Docking

The molecular docking of all the compounds NK and VA 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, binding free energy and interaction with the active site residues, Top 3 ranked complexes and one protein-vildagliptin (known inhibitor) complex were considered for further assessments.

2.3.4.5 Molecular Dynamics (MD) Simulation

The top three compounds with maximal docking and MMGBSA scores for NK and VA and one known inhibitor (Vildagliptin) complexed with DPP4 protein were subjected to 100ns simulation production run using Desmond (v5.6) package (Bowers et al., 2006). For each protein-ligand complex, the TIP4P water model was used as 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.

For VA, the Prime MM-GBSA (v3.0) module of the Schrodinger Maestro suite was used for binding free energy calculations of the protein-ligand complexes. Molecular dynamics simulations were performed using the Desmond (v7.3) package with an OPLS4 force field for energy calculation. The protein-ligand complexes were solvated with the TIP4P water models

and simulated in an orthorhombic box with the periodic boundary conditions of size 10 x 10 x 10 Å. The system is neutralised by adjusting the number of ions and the salt concentration was maintained at 0.15M.

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 100ns for all four complexes. Using Simulation Interaction Diagram and Simulation Event Analysis modules of Schrodinger suite, RMSD of the protein backbone, RMSF (Root mean square fluctuation) 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.

2.3.4.6 Target mapping of phytochemicals using network pharmacology tools

The potential target proteins 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 was 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 0.85 was used during the BindingDB search.

2.3.4.7 Network construction and disease associated gene identification

A phytochemical target protein 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 proteins 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.

2.3.4.8 EnrichR analysis for identifying target-disease overlap

The reported phytochemical targets from the databases were corrected for duplicate entries and processed for protein 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 proteins 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 to obtain the common targets between diabetic complications and associated metabolic diseases.

Chapter – 3
Results and Discussion

3 Results and Discussion

3.1 Selection and processing of the formulations for study.

Therapeutic procedures in *Ayurveda* adopts a multi-formulation intervention that can address the systemic biological changes underlying the disease manifestation. This is aimed at delivering a personalized intervention, comparable to precision medicine concept in modern biomedicine, that can address the patient physiology and resilience (*Rogi bala*), stage and severity of disease progression (*Roga bala*) as well as severity and the co-morbidities associated with a particular disease. *Rogi bala* and *Roga bala* can be understood as 'strength of the patient' and 'strength of the disease', respectively. In general, the drugs of choice in *Ayurveda* clinics can be broadly divided into two groups *viz*. classical *Ayurveda* formulations and proprietary *Ayurveda* formulations. Classical *Ayurveda* formulations can be defined as those formulations described in identified *Ayurveda* texts recognized by the regulatory authorities of India. These formulations are expected to follow the principles of *Ayurveda* pharmacology (*Dravyaguna Vijnana*) derived from the unique epistemology and ontology of health and diseases in *Ayurveda*. Whereas the proprietary formulations are the group of formulations that are prepared from *Ayurveda*-inspired herbal extracts and these drugs are expected to follow the principles of modern biomedical pharmacology. The current thesis work selected four 'classical *Ayurveda* formulations' (details given in section 2.1.1 under chapter - Materials) namely *Nishakatakadi Kashaya, Varanadi Kashaya, Vasantha Kusumakara Rasa and Chandraprabha vati*. These are the top most frequently prescribed formulations by physicians for diabetes in the clinical practice. Although patient centered management of diabetes in *Ayurveda* involves both pharmacological interventions as well as life style changes, multi-component formulations are prescribed based on a persons' constitution as per their treatment algorithm. This personalized treatment regimen is unique to *Ayurveda.* Though there were a number of proprietary formulations prescribed, classical formulations were chosen owing to their concordance to the pharmacology principles mentioned in *Ayurveda* texts. Among the formulations selected for this study, NK and VA are in decoction form prepared from various herbs whereas VK and CV are in tablet form prepared from herbo-mineral ingredients.

While studies generally make organic solvent extracts of formulations to perform various bioassays, this study hypothesized that making extracts of a formulations using conventional

methods may result in a product that may not represent the formulation's phytochemistry qualitatively and quantitatively. Therefore, this work used whole formulations for all the studies. The decoctions or *kashaya* are used directly for the assays because they are in assay compatible liquid form. Whereas the tablets where subjected to a simulated *in-vitro* digestion and the digests were used for different assays. The formulations and digests were estimated for total tannins and the samples were quantitatively used as 'µg of GAE/mL' as described in Methods section 2.3.2.2. The subsequent sections of this chapter, from 3.2 to 3.5, gives a detailed narration of the results of each formulation studied using different model systems.

While this thesis selected four formulations for detailed analysis for their multi-targeted mode of action, it was quite interesting to note that in the real clinical practice a large set of classical and proprietary preparations are given to the patients for the management of diabetes and its co-morbidities. This practice is essential for achieving the personalization effects. However, this heterogeneity of interventions makes *Ayurveda* clinical data analysis a difficult task. Randomized controlled trials (RCTs), the gold standard of clinical trials, are largely used for comparing the efficacy of interventions with a placebo control in a carefully selected homogeneous population. Their clinical outcomes are evaluated through appropriate biochemical markers as readouts, and significance is measured by applying linear regression statistics between control and test groups. This is successfully adopted in molecular medicine, wherein the drugs precisely interact with their targets and modulate specific biochemical pathways to exert their biological effects. But the personalized treatment framework of *Ayurveda* interventions is often influenced by several factors and therefore a simple control versus test group comparison using descriptive and inferential statistical analysis tools may not be sufficient to address the heterogeneity of the data. The need of the hour is a methodology that can encompass and analyze multiple variables by appropriately clustering them to subgroups that justify the personalization principles which influence the clinical efficacy (Jaleel et al., 2023).

To accommodate the *Ayurveda* method of a heterogenous yet personalized treatment, clinical design and execution need to be re-thought incorporating various features. Based on this idea, using a dataset of multivariable clinical management data for diabetes (known as "*Prameha*" or "*Madhumeha*" in *Ayurveda*) treated with Ayurvedic formulations, an exercise was done to test the viability of AI-approach for future research in *Ayurveda* clinical design and interpretation. The data showed multiple features influencing the decision-making process on efficacy and safety of clinical interventions. Therefore, a binary classification of patients and

regression statistical methods would be insufficient, rather inappropriate, to map the patient groups to the drug combination giving a successful result. In this background, the aim of the study is to see the possibility of using higher dimensional statistical analysis to cluster the patients into comparable subgroups with matching features that contribute or influence the diagnostic and clinical outcome parameters, safety and efficacy of interventions as well as the personalization concepts. The study utilized Self Organizing Maps to explore the natural grouping of clinical features and project multidimensional data onto a two-dimensional display in a non-linear manner. This was carried out as a parallel study of this thesis and the results of the study is described in the section 3.6 in this thesis.

3.2 *Nisakatakadi kashayam* (NK) exerted antidiabetic effects through a multi-targeted mode of action involving digestive enzyme inhibition and modulation of incretin effect.

Nisakatakadi kashyam (NK) is an effective *Ayurveda* preparation for diabetes as mentioned in one of the *Ayurveda* texts '*Sahasrayogam* (Nishteshwar K, 2007). This formulation is a combination of eight herbal ingredients with known hypoglycemic effects (Table-8). *Ayurveda* physicians prescribe NK for patients showing manifestations of diabetes and associated complications. Various *in vitro*, *in vivo* and clinical research evidences 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* (Acharya et al., 2016; Goyal et al., 2011a; Gul et al., 2022; Karan et al., 2013; Pivari et al., 2019; Sharma et al., 2020; Yadav et al., 2014).

Scientific name	Sanskrit name	Part used	
***Curcuma Longa* L.**	*Haridra*	rhizome	3.75 g
***Strychnos potatorum* L.f.**	*Kataka*	seed	3.75 g
***Ixora coccinea* L.**	*Paranti*	root/stem	3.75 g
***Symplocos racemosa* Roxb.**	*Lodhra*	stem bark	3.75 g
***Emblica officinalis* Gaertn.**	*Amla*	fruit	3.75 g
***Aerva lanata* (L.) Juss. ex Schult.**	*Gorakshaganj*	entire plant	3.75 g

***Vetiveria zizanioides* (L.) Nash**	*Nash Ushira Khas Vetiver*	root	3.75 g
***Salacia reticulata* Wight**	*Saptachakra*	stem/root	3.75 g

Table-8. The list of plants in NK

In the present work, NK was studied using *in vitro, in silico and in vivo* model systems. *In vitro* model systems were used to assess the effect of NK on digestive enzyme inhibition, DPP4 inhibition and DPP4 secretion and *in silico* and network pharmacology analysis were carried out to delineate the possible multi-targeted actions of the formulation. *In vivo* studies were performed to support the in-vitro data as well as the *Ayurveda* claims of the formulation.

3.2.1 NK inhibited α-glucosidase dose dependently but did not inhibit α-amylase enzyme

NK was subjected to α-amylase and α-glucosidase inhibition assay, both of which are cell free *in vitro* enzyme assays. Both enzymes play a crucial role in hydrolyzing carbohydrates into glucose. Their inhibition will delay the sudden rise in blood glucose levels, thereby aiding in maintenance of euglycemia. The results showed a a significant dose dependent inhibition of α-glucosidase by NK (Fig-11A). But it did not show any inhibition of α-alpha amylase enzyme (data not presented). NK exhibited the highest inhibition of α-glucosidase activity at 10 µg of GAE/ml with 74 % inhibition and lowest inhibition of 28% at 2.5 µg of GAE/ml.

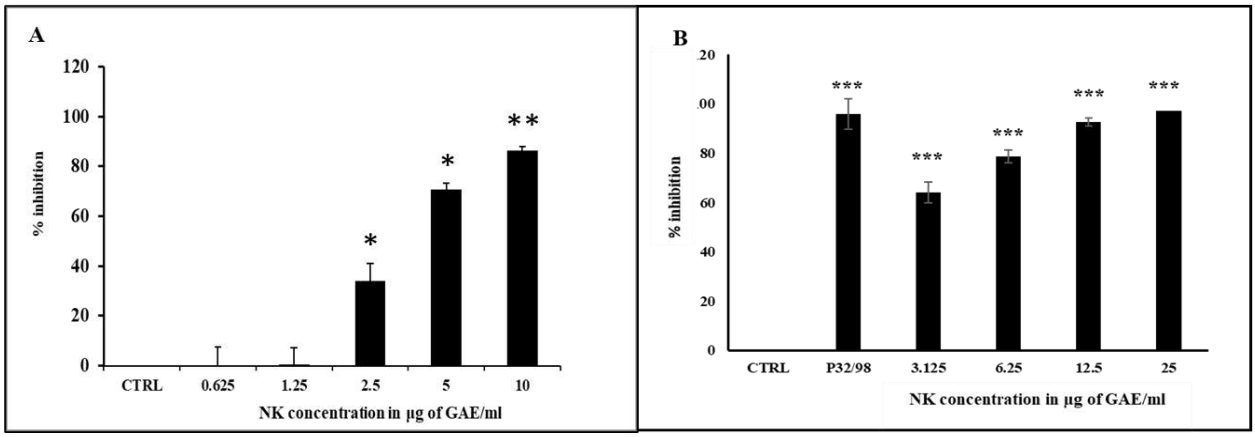

Fig-11: **α-glucosidase and DPP4 inhibition effect of NK.** (A) Graph shows a concentration-dependent inhibition of α-glucosidase enzyme action upon treatment with various concentrations of NK (p value ≤ *** 0.001, p value ≤ ** 0.01, p value ≤ * 0.05). (B) Graph shows a concentration-dependent inhibition of DPP4 enzyme action upon treatment with various concentrations of NK (p value ≤ *** 0.001)

3.2.2 NK inhibited DPP4 dose dependently *in vitro* as well as enhanced GLP1 secretion from an intestinal cell line GLUTag

One of the important therapeutic targets in emerging therapies for type 2 diabetes is DPP4, the inhibition of which results in prolonged half-life of GLP1, a pleotropic gut hormone with multiple physiological actions which improves diabetes symptoms (Trzaskalski *et al*., 2020).

Evaluation of NK for DPP4 inhibition showed that NK inhibited DPP4 enzyme dose dependently with an IC_{50} of 2.09 µg of GAE/mL. NK exhibited a maximum inhibition of 97% at 25µg of GAE/mL and a minimum inhibition of 64% at the lowest concentration of 3.125µg of GAE/mL ($p<0.001$) (Fig-11B). The strong inhibition observed by NK is very relevant in its potential to regulate incretin effect and thereby modulating the whole body glucose homeostasis.

It has been reported that, in addition to the inhibition of DPP4 enzyme action, enteric inhibition of DPP4 expression can also enhance incretin action (Mulvihill et al., 2017 Waget et al., 2011). Hence in addition to the *in vitro* enzyme inhibiton assay, the effect of NK treatment on DPP4 expression in intestinal cell line GLUTag was checked. But NK did not show any inhibition of DPP4 mRNA expression in GLUTag cells in formulation treated cells.

3.2.3 NK enhanced GLP1 secretion from GLUTag cells

In addition to DPP4 inhibition, the formulation was also studied for its effect on GLP1 secretion, one of the well-studied incretin hormones, using an enteroendocrine cell model GLUTag. Cytotoxicity studies were performed to check and find out the non-toxic concentration range of the formulation for GLP1 secretion studies (Fig-12A). The cells treated with 12.5, 25 and 50 µg of GAE/ml of the formulation for 2 hrs showed a concentration dependent increase in GLP1 secretion when compared to untreated control (Fig-12B). It was observed that NK enhanced GLP1 secretion significantly by 18%, 26% and 40% at concentrations 12.5, 25 and 50 µg GAE/ml respectively.

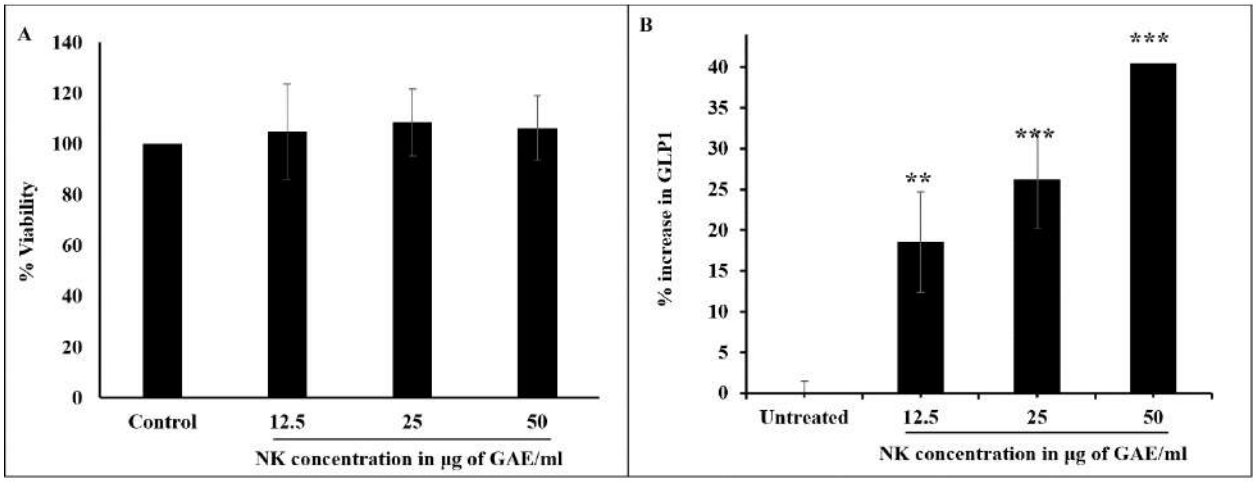

Fig-12. Effect of NK on GLP1 secretion in GLUTag cells. A) Graph shows the viability of GLUTag cells when treated with NK at various concentration of NK for 2 hours followed by MTT assay. B) Graph showing concentration dependent increase in GLP1 secretion from GLUTag cells, when treated with various concentrations of NK. (p value ≤ 0.001, *** p value ≤ 0.01**)

These initial enzyme inhibition results indicate a possible multi-targeted mode of anti-diabetic action for NK and also it corroborates with the clinical usage of this formulation for diabetes management.

3.2.4 Molecular Docking studies showed key phytochemicals identified in NK directly interacting and inhibiting DPP4 activity Phytochemical data mining

NK, containing 8 medicinal plants, is the source of a large number of phytochemicals potentially involved in for its pharmacological actions. Since the NK as a formulation exhibited DPP4 inhibition, identifying phytoactives that are involved in DPP4 inhibition can help in providing a detailed understanding of the pharmacodynamics and pharmacokinetics of the formulation as well as in discovering new druggable candidates for diabetes management. To take this forward, *in silico* approach was adopted wherein phytochemical data was extracted from different databases mentioned in methodology section 2.3.4.1, identified from 8 medicinal herbs mentioned in Table 8. From the data mining, 206 phytochemicals identified which were further screened for DPP4 inhibition using virtual screening procedures.

A virtual screening of all 206 compounds were performed by docking them to DPP4 protein retrieved from Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB; http://www.rcsb.org). Well known DPP4 inhibitor Vildagliptin was used as a positive control. The top-ranked 35 compounds, based on their docking score, and MM-GBSA are listed in Table-9. The docking and MM/GBSA scores for Vildagliptin were -3.899 kcal/mol and -26.7 kcal/mol, respectively. The compounds listed in Table 9 showed a better docking 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-13A-D). Among the 35 compounds, MM/GBSA scores were not obtained for Kotalanol and Corilagin.

Sl. No.	Compound Name	Docking Score kcal/mol	MM-GBSA kcal/mol
1	Vildagliptin	-3.899	-26.7
2	Terchebin	-11.766	-47.12
3	Locoracemosides B	-10.145	-45.45
4	1,2,4,6 Tetra o Galloyl Beta D Glucose	-9.556	-60.73
5	Tercatain	-9.252	-28.37
6	Rutin	-9.244	-41.90
7	Typhaneoside	-8.782	-45.47

8	1,6-Bis-O-Galloyl-Beta-D-Glucose	-8.45	-44.24
9	Kotalanol	-8.374	*Not obtained
10	Chebulinic Acid	-8.314	-19.48
11	Kaempferol-7-O-α-rhamnoside	-8.309	-41.87
12	Quercitrin	-8.012	-36.48
13	1,6-Bis-O-Galloyl-Beta-D-Glucose	-8.003	-15.06
14	Glucogalin	-7.993	-38.18
15	Epicatechin	-7.992	-39.34
16	Catechin	-7.992	-39.34
17	Sucrose	-7.643	-28.54
18	Quercetin	-7.498	-23.39
19	Benzoylsalireposide	-7.403	-33.55
20	Procyanidin	-7.382	-22.21
21	Salirepin	-7.253	-36.51
22	Myricitrin	-7.142	-27.44
23	Symplocomoside	-6.989	-31.26
24	Luteolin	-6.985	-29.11
25	Narcissin	-6.613	-42.08
26	Corilagin	-6.541	*Not obtained
27	Bisacurone	-6.345	-10.71
28	Kaempferol 3-O-beta-D-galactoside	-6.272	-39.39
29	Proanthocyanidin	-6.114	4.71
30	Salacinol	-6.099	-40.66
31	Kaempferol-7-oglucoside	-6.076	-28.39
32	Punicafolin	-6.065	-5.8
33	Symplososide	-6.021	-32.88
34	Symploveroside	-6.009	-36.55
35	Epicatechin-4β-8	-6.008	-30.48
36	Symponoside	-5.317	-23.85

Table 9. The top scored 35 compounds from the virtual screening of docking of NK phytochemicals with DPP4. The docking score and binding free energy from MM-GBSA analysis are given.

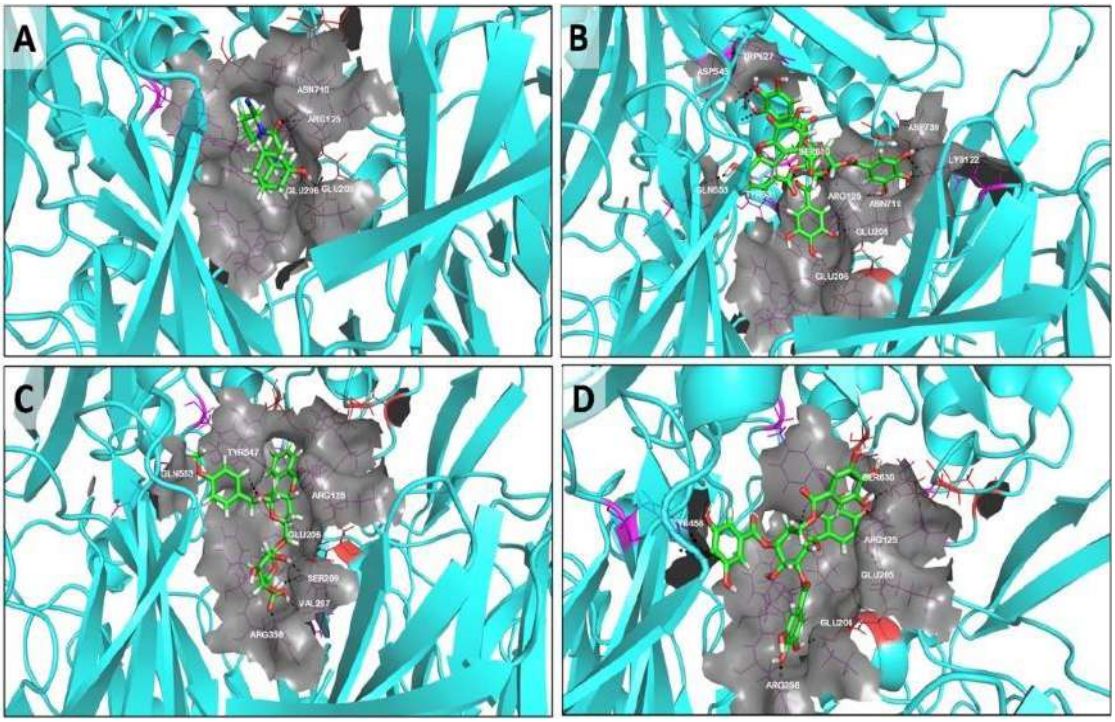

Fig-13. Interaction of DPP4 with various ligands. Interactions of Vildagliptin (A), Terchebin (B), Locoracemoside B (C) and 1,2,4,6 Tetra o Galloyl Beta D Glucose (D) with DPP4.

3.2.5 MD simulation analysis of DPP4 ligand complex

The top three compounds selected from the docking study, Terchebin, Locoracemoside B, and TGBG as well as the standard Vildagliptin were further subjected to a detailed interaction analysis using MD simulation. MD is a powerful computer simulation technique that is now being used in the field of computer-aided drug discovery research to deepen the understanding of how the protein-ligand complex behaves in a dynamic environment at the atomic level over a user-specified time period. A 100ns MD simulation analysis of the DPP4-ligand docked complex was performed and the RMSD profiles of the protein and ligands during the 100ns simulations are analyzed (Fig-14). The average RMSD of DPP4 and Vildagliptin was found to be 1.75Å and 2.0Å, respectively (Fig-14A). 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-14B-D).

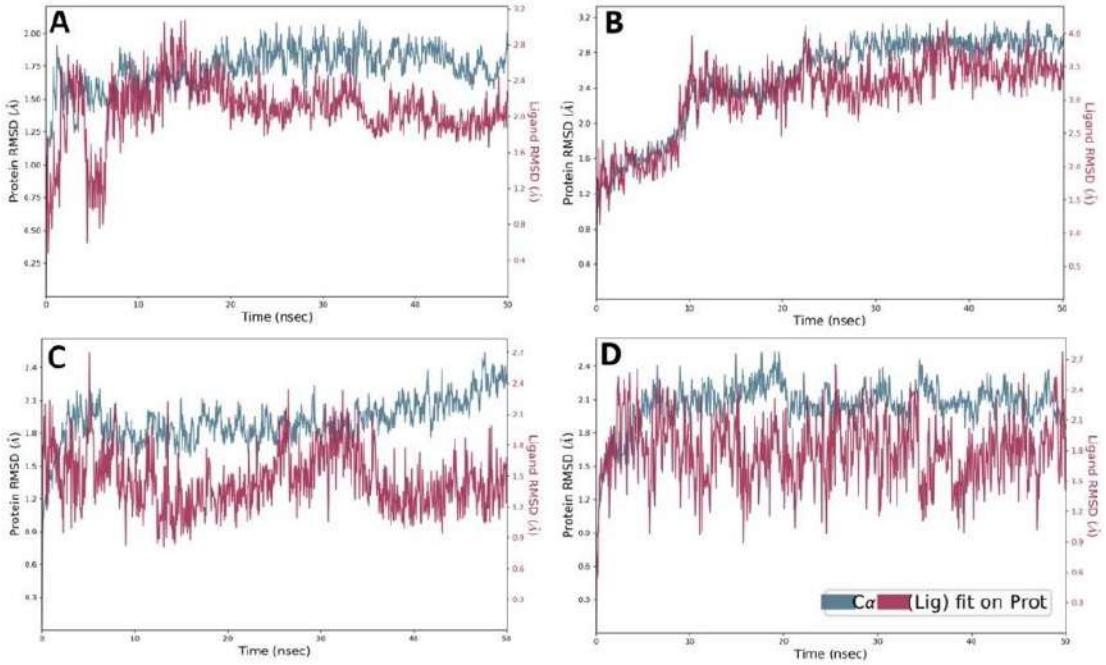

Fig-14. RMSD plot of top ranked ligands in NK. RMSD plot of Protein and Vildagliptin (A); Protein and Terchebin (B); Protein and Locoracemoside B (C); Protein and 1,2,4,6 Tetra o Galloyl Beta D Glucose (D).

All ligands were found to have stable interactions in the active site.

3.2.6 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) in Fig-15.

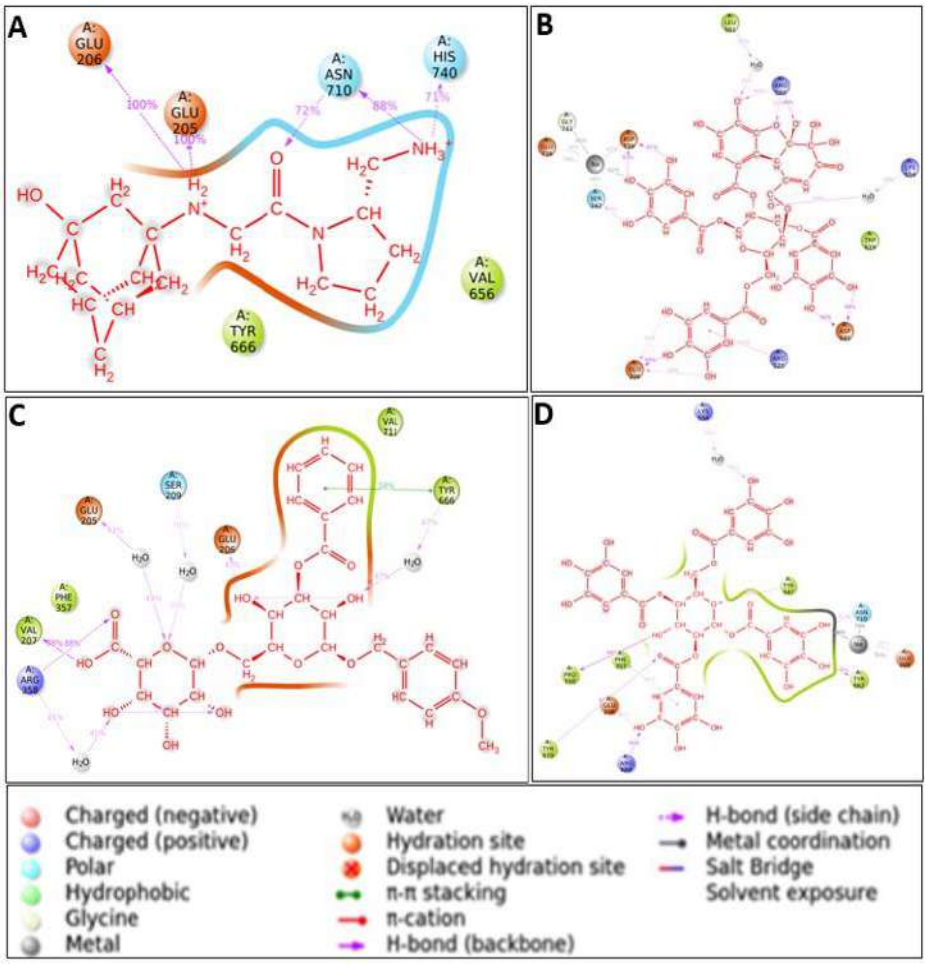

Fig-15. 2D interaction diagram of ligands with protein during the simulation. (A)-Vildagliptin, (B)-Terchebin, (C)-Locoracemoside B and (D)-1,2,4,6 Tetra o Gallovl Beta D Glucose with DPP4.

The interactions with the specific amino acids throughout the run are depicted using normalized 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-15A). For DPP4-Terchebin interaction, 8 hydrogen bonds at positions Ser206, Ser242, Tyr545, Lys554, Arg560, Trp627, Asn710 and Asp739 as well as hydrophobic nteractions at positions Lys122, Arg125, Tyr547, Trp627 and Trp629 were identified (Fig-15B). 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-15C). The DPP4-TGBG interaction showed 10 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-15D). 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. The interaction details of the amino acids for Vildagliptin and compounds from NK with DPP4 are shown in the clubbed graph Fig-16.

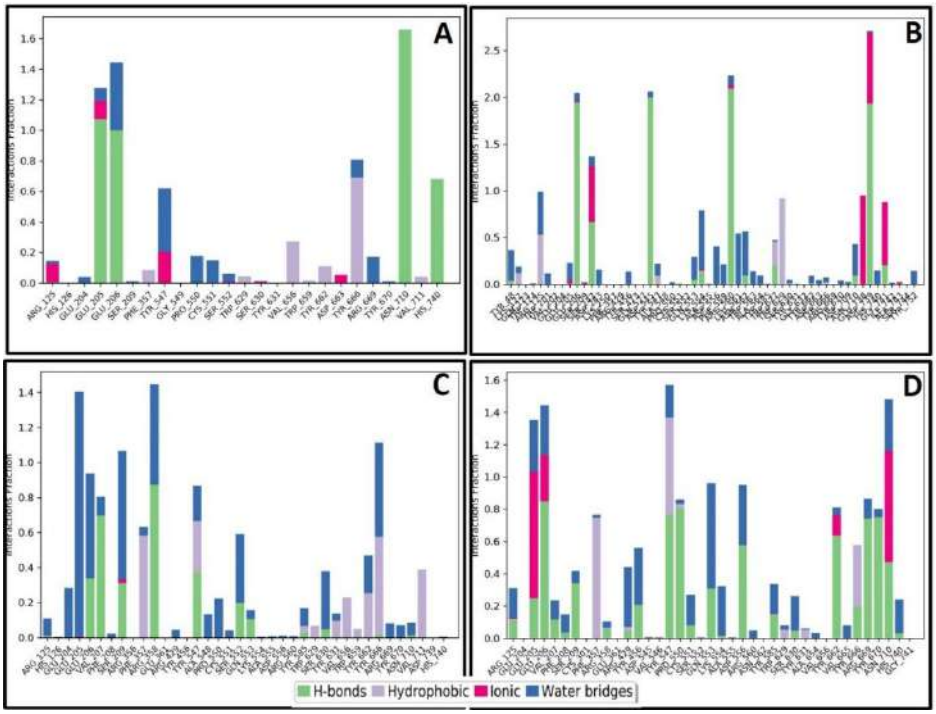

Fig-16. Protein interactions of the interacting amino acids in NK compounds. Graph shows Hydrogen Bonds, Hydrophobic, Ionic and Water Bridges involved in protein and compound interaction A) Protein and Vildagliptin C) Protein and Terchebin, C) Protein and Locaracemoside B, Protein and TGBG

3.2.7 Target mapping of phytochemicals identified in NK formulation for network analysis

The constituent bio-actives in a herbal formulation can interact with multiple target genes to exert 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 hypothesize 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 software, 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-17A). 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* (Kanehisa *et al*., 2017; Lee *et al*., 2021). The list of all the phytochemicals and their targets can be accessed from supplementary Data in (Thottappillil et al., 2023) . The complete list of top ranked hub proteins having high degree values is given in Table 10. 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, which aligned with the docking results, showing strong DPP4 inhibition by these compounds.

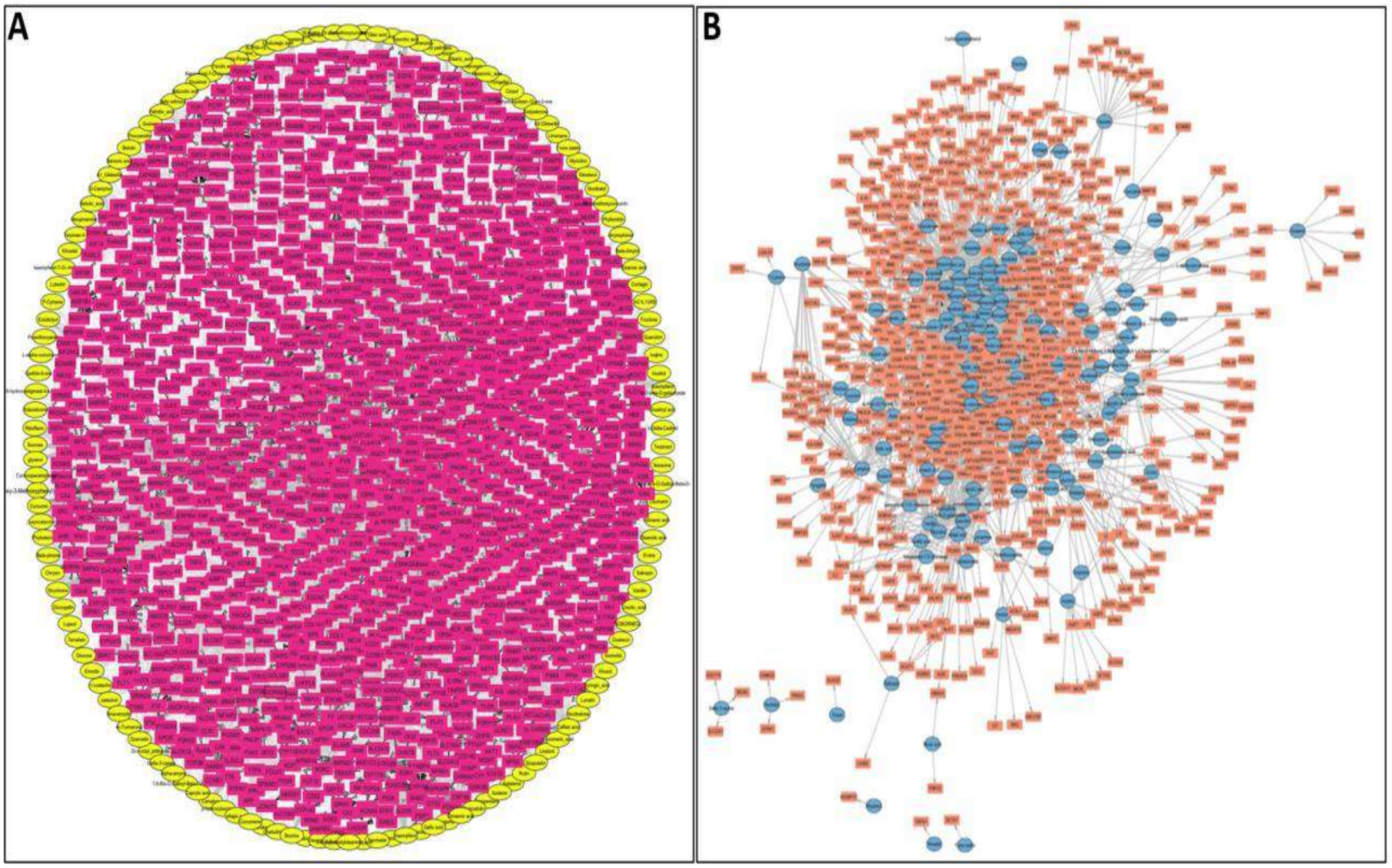

Fig-17. The compound-target network of NK (A) - The yellow nodes represent the compounds from plants used in the NK formulation and the pink nodes represent the target proteins. (B) - The network of phytochemicals of NK and diabetes-related proteins. The blue color nodes represent the compounds and the orange nodes represent the diabetes associated target proteins

S. No.	Hub Gene	Degree	Rank	S. No.	Hub Gene	Degree	SL no	Hub protein	Degree	Rank
1	CA2	26	1	38	DHCR24	8	19	UGT1A10	12	8
2	CYP19A1	22	2	39	AKR1B10	8	20	CA4	12	8
3	CA7	18	3	40	TOP2A	8	21	CYP17A1	12	9
4	ESR1	17	4	41	ALB	8	22	CYP1A1	11	9
5	F2	16	5	42	ADRA2B	8	23	CRYAB	11	10
6	AKR1B1	16	5	43	CYP1A2	8	24	EGFR	10	10
7	CA12	16	5	44	ALOX5	8	25	TOP1	10	10
8	AR	15	6	45	PSMB5	8	26	F10	10	10
9	CASP3	15	6	46	NR1I2	8	27	HSD11B1	10	10
10	ACHE	15	6	47	CA1	8	28	PTGS2	10	11
11	PTPN1	13	7	48	GAA	8	29	NR1H2	9	11
12	RORC	13	7	49	CHRM5	8	30	MAPK1	9	11
13	SHBG	13	7	50	HMGCR	7	31	AKT1	9	11
14	MMP9	13	7	51	GRIN2B	7	32	ADRA2A	9	11
15	ESR2	12	8	52	ITGB3	7	33	UGT1A9	9	11
16	NR1H3	12	8	53	MAPK3	7	34	MAOA	9	11
17	UGT1A7	12	8	54	CASP7	7	35	DPP4	9	11
18	UGT1A8	12	8	55	F7	7	36	UGT1A3	9	11
							37	MMP2	9	11

Table-10. The list of top ranked hub proteins in the compound target network of NK

A ClueGO analysis was 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-18A). To understand the important pathways associated with these proteins, a KEGG pathway analysis was performed. The analysis revealed a number of diabetes associated pathways significantly associated with NK protein targets (p-value < 0.05) like PI3K-AKT, MAPK signaling, lipid and atherosclerosis, Ras signaling, chemokine signaling, diabetic cardiomyopathy, insulin resistance and insulin signaling, cellular senescence, HIF-1 signaling and AGE-RAGE signaling pathways in diabetic complications associated with the proteins in NK network (Fig-18B). These observations support the

Ayurveda rationale of using NK for the management of diabetic associated symptoms and complications

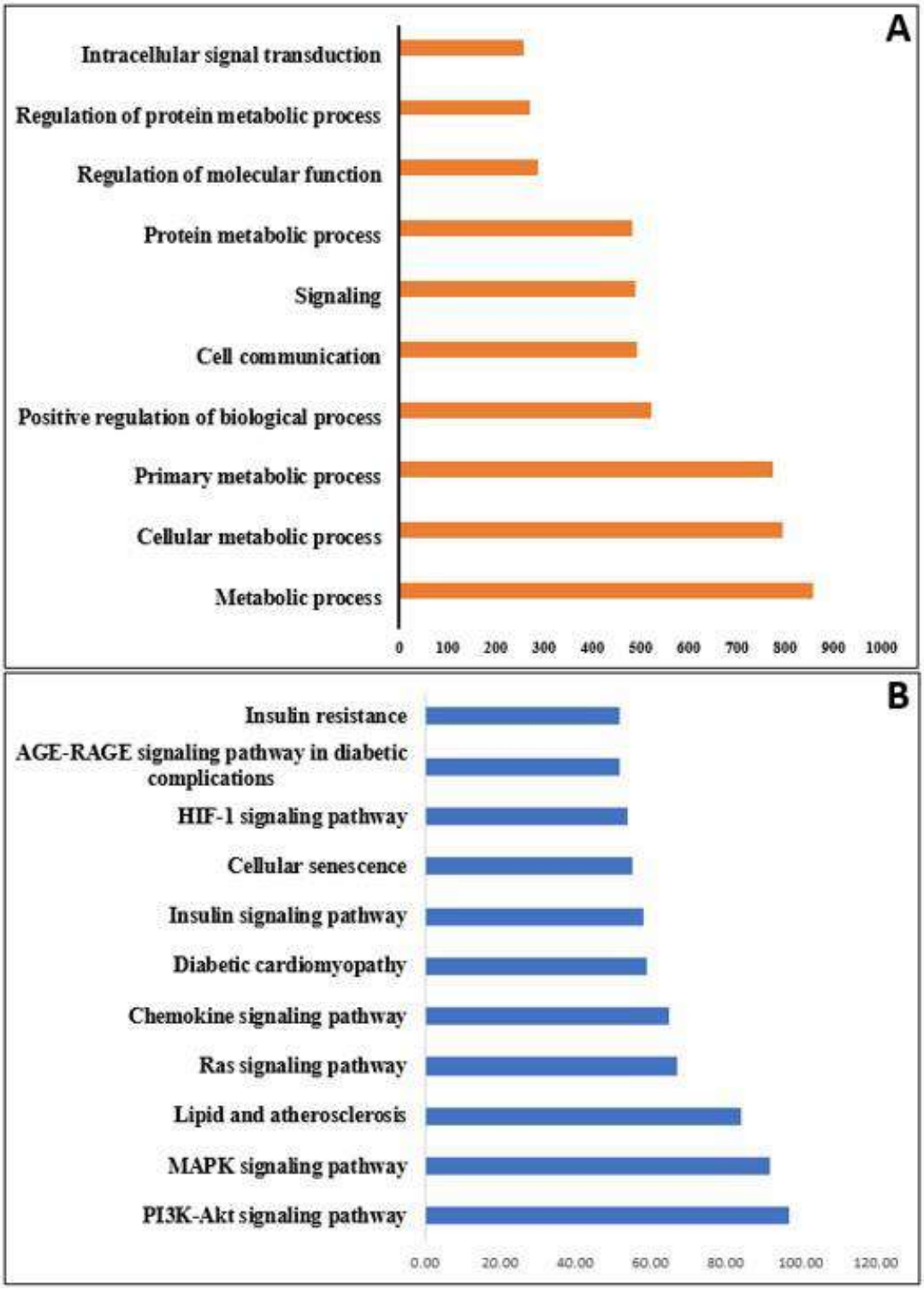

Fig-18. ClueGO and KEGG analysis of the 1555 target proteins. (A) analysis of the 1555 target proteins involved in different biological processes and (B) analysis of the 1555 target proteins involved in different pathways

3.2.8 Disease overlap analysis of the protein targets mapped for phytochemicals in NK formulation

To gain more insight into the diseases associated with the proteins of NK network, a comprehensive gene enrichment analysis tool (EnrichR) containing diverse gene set libraries was utilized (Kuleshov *et al*., 2016). The tool was used for disease analysis of the 1555 targets mapped and data was extracted from three sources viz, DigiNet, ClinVar and OMIM expanded. A total of 9000 disease indications emerged from EnrichR analysis, for which a p-value of ≤ 0.05 was applied to short list those diseases. Further, from the entire list of disease conditions, we used the key words "Diabetes mellitus", "Insulin resistance", "Hyperglycemia", "Hypoglycemia", to shortlist 614 diabetes associated proteins. From this, a sub-network of diabetes specific proteins was created which contained 743 nodes and 1612 edges (Fig-17B). This showed the putative diabetic interactome of NK comprising a number of critical regulators in diabetes related complications and comorbidities (Thottappillil et al., 2023)

For investigating the association of NK targets in diabetic complications and diabetes associated risk conditions, the 614 proteins were again subjected to EnrichR analysis. The resulting disease analysis showed various diabetic complications like nephropathy, neuropathy, cardiomyopathy and retinopathy. and also revealed the presence of 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). These conditions have been grouped into 5 specific categories viz; fatty liver, obesity, general metabolic disturbances, inflammation and diabetic complications. The details of these categories and the complete list of indications can be accessed from supplemental data in (Thottappillil et al., 2023).

To gain an insight into the gene association of diabetes with these 5 categories, a Venn diagram analysis was performed. The results of Venn analysis as observed in Fig-19A showed that 37 proteins were common among all the diabetes associated diseases and diabetic complications. Proinflammatory markers like NFKB1 and TNFα, characteristic of the insulin resistance, were present in the cluster along with lipid regulatory and obesity-linked PPARα and PPARγ (Diehl, 2004; Stienstra *et al*., 2007). The other critical markers were insulin signaling 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). One of the overlap categories in the Venn analysis is obesity, fatty liver and diabetic complications which have 27 shared proteins. The presence of both DPP4 and GLP1R aligned with the recent evidence from various

studies establishing showing DPP4 as a crucial gene in the regulation of fatty liver, obesity and diabetic complications (Trzaskalski *et al*., 2020). The experimental results showing DPP4 inhibition and increased GLP1 secretion indicate that NK can possibly act via gut regulatory mechanisms involving GLP1 pathway. It further corroborated the *Ayurveda* indications of NK with our experimental data of DPP4 inhibition and GLP1 secretion.

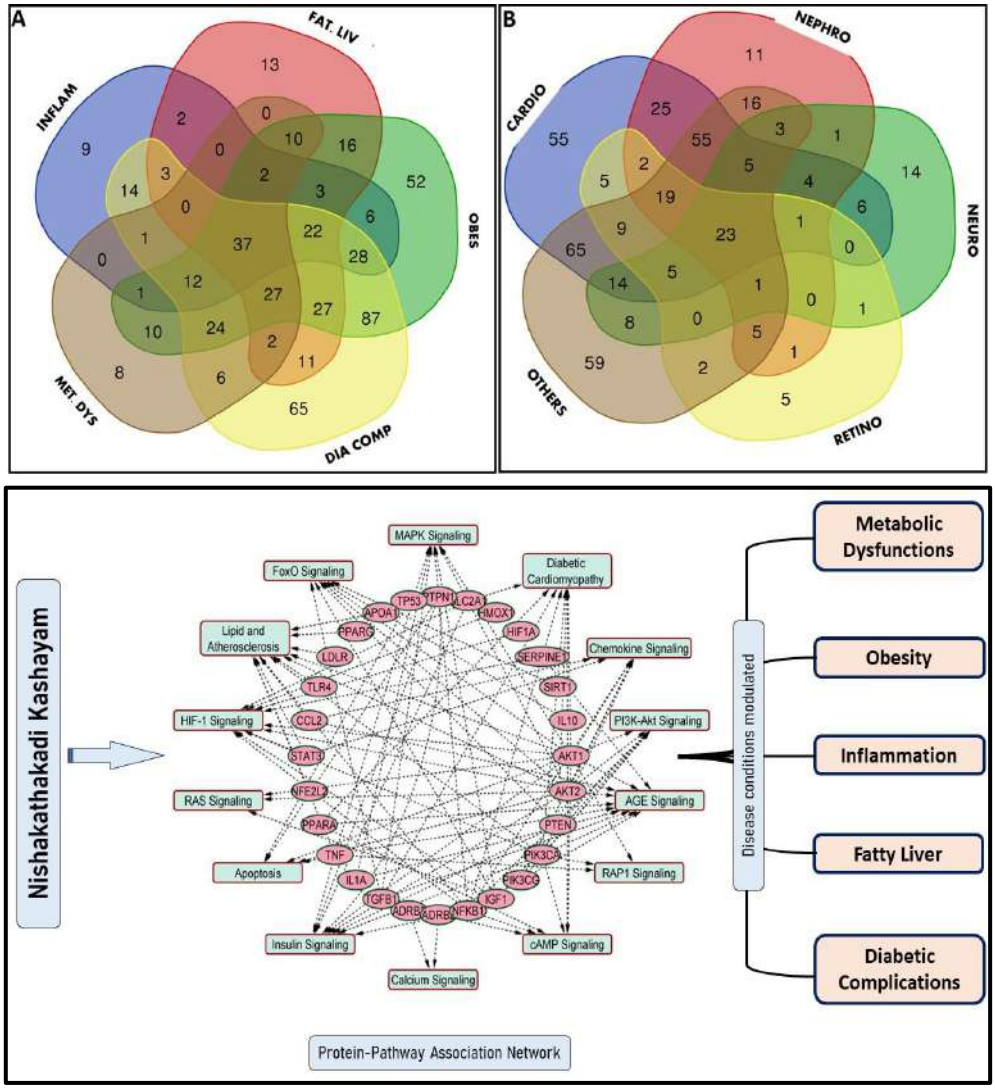

Fig-19. Venn diagram analysis of NK protein targets. (A) represents the protein association between diabetic complications and associated diseases; (B) shows the proteins within diabetic complications; (C) indicates the possible modulation of 14 key pathways associated with 5 metabolic dysfunctions related to glucose metabolism by one formulation. 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

For a specific analysis of the possible overlap within the 'diabetic complications' category, we further grouped the 'diabetic complications' into 5 groups viz. diabetic retinopathy, cardiomyopathy, nephropathy and neuropathy and 'other diabetic complications. From the Venn diagram analysis of diabetic complications, a cluster of 23 proteins were emerged as common (Fig-19B) and included several important markers such as TNFα, TLR4, CCL2, SOD1, TGFβ1 and SOD2, all of which are deregulated and involved in various stages of diabetic pathogenesis (Stienstra *et al*., 2007; Diehl, 2004; Yehualashet, 2020; Forbes and Cooper, 2013). The proteins present in each category and Venn diagram overlaps can be accessed from Supplementary Data in (Thottappillil et al., 2023). From *in silico* studies, it was found that the bioactives in NK can target multiple proteins and pathways which are therapeutically relevant for diabetes and associated complications. Fig-19C summarizes the possible modulation of 14 key pathways associated with 5 metabolic dysfunctions related to glucose metabolism by one formulation.

3.2.9 NK reduced the increased blood glucose levels and improved the oral glucose tolerance in HFD-STZ diabetic rats

Further to validate the *Ayurveda* claims of the anti-diabetic potential of NK as well as to corroborate with the observations from the *in vitro* experiments, an animal study was conducted using animals fed with diet rich in fat as mentioned in materials and methods. The animal study protocol was followed as mentioned in the section 2.3.3. Though the animal group had six animals to begin with, due to the observed mortality with STZ induction and the resultant hyperglycemia, the results of all further experiments are expressed for animal group containing 4 animals. (n=4)

The high fat animals gained considerably more body weight than the normal diet control (Table-12). Following the high fat feeding, the animals were induced with a low dose of STZ (25 mg/1000g) to induce diabetes, while the control animals were given citrate buffer.

As given in Table-11, the blood glucose levels increased with STZ induction of T2D compared to the initial blood glucose levels in all groups except for control. The untreated diabetic group (HFD-STZ) showed higher blood glucose levels at the end of the study, whereas the animals treated with both low and high doses of NK showed a significant reduction of glucose levels compared to the untreated HFD-STZ group, on the 26^{th} day post treatment, thereby demonstrating an alleviation of diabetes.

Blood glucose levels in mg/dL			
Total days of study	**Day 0**	**Day 49**	**Day 75**
Group name	**Initial**	**3rd day after STZ induction**	**26 days after Drug**
Control	**98.75±3.75**	**77±5.93**	**94.25±5.93**
HFD-STZ	**112±6.48**	**330.75±63.94**	**503.75±35.63**
Metformin	**104.25±4.51**	**357±32.11**	**322.25±69.18**
NK LD	**109.25±3.88**	**354.75±40.11**	**203±42.07****
NK HD	**107.75±5.43**	**454.25±28.63**	**305.25±56.29****

Table-11- The effect of NK on blood glucose levels. Table showing blood glucose levels in NK groups when compared to the untreated diabetic control HFD-STZ (High fat diet combined with STZ) group. Data given as mean ±SEM n=4 for all groups. (p value $\leq$ ** 0.01). Standard error mean (SEM). The results were statistically significant in NK HD Vs HFD-STZ on Day 75.

After 72 hrs of STZ induction, the OGTT was carried out to determine the glucose tolerance efficiency of the animals. It was observed that while the control animals retained their ability for glucose clearance and had normal glycemic levels, the HFD-STZ animals had >200mg/dL glucose and exhibited significant impairment of glucose tolerance. The area of under curve (AUC) of control animals were in the range of 10000 mg/dL while the HFD-STZ animals showed AUC around 40000-60000 mg/dL. The HFD-STZ animals with impaired oral glucose tolerance and glucose levels >200mg/dL were randomly grouped into different treatment groups.

To check if the high fat feeding had an impact on glucose clearance in the body, OGTT was performed by giving glucose gavage of 2 g/Kg and estimating the glucose levels at different time points (0, 30, 60, 90 and 120 minutes) were checked using a glucometer. The area under the curve for the glucose for the high fat fed animals were higher (ranging from 13900-14901 mg/dL) whereas for the control, it was just ranging in 12000 mg/dL. This showed a slightly impaired glucose tolerance level in the high fat fed animal groups.

OGTT was performed to evaluate the ability of the animals to dispose a glucose challenge after STZ induction. In the OGTT experiment, HFD-STZ diabetic rats showed statistically elevated blood glucose levels compared to normal control rats after glucose load and this indicated severe glucose intolerance in that group as evidenced by increase in AUC (##p < 0.01, Fig-20). Diabetic rats treated with NK high and low doses exhibited a reduced AUC (Fig-18) where low

dose had a significant improvement in glucose tolerance. (**p value < 0.01). The results suggested that NK at both doses improved the impaired glucose tolerance and the low dose (NK LD) group appears to have better effect.

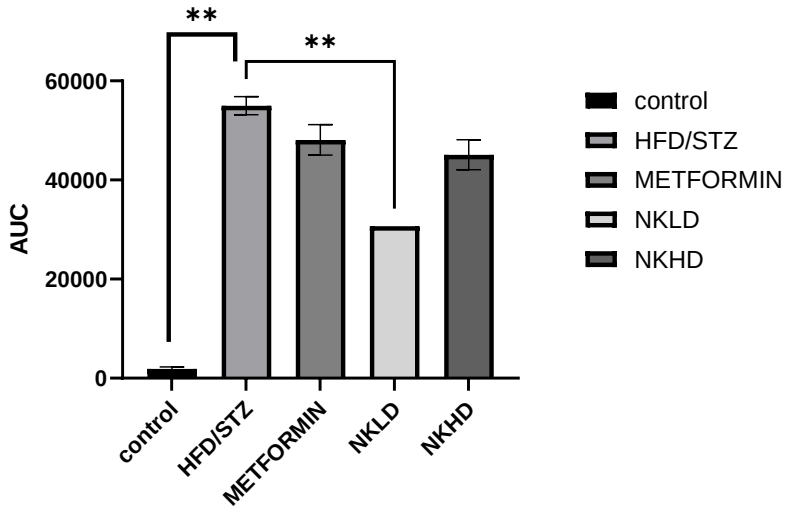

Fig-20. Effect of NK on AUC for glucose in OGTT. The graph showing the effect of NK (NKLD and NKHD) low and high doses on area under curve (AUC) when compared to the untreated diabetic control HFD/STZ group along with the standard Metformin. Data is given as mean with Standard error mean (SEM) (p value $\leq$ ** 0.01), n=4

3.2.10 Treatment of HFD-STZ rats with NK reduced body weight

The designated groups of animals, administered with low and high doses of NK as shown in the table, exhibited prominent body weight alterations. After STZ administration, the body weight reduced significantly indicating the onset of a chronic type 2 diabetes which is a well-known effect of STZ induction due to pancreatic cell loss and subsequent induction of chronic T2DM (Akinlade et al., 2021). The effect of formulation on the body weight of rats can be observed (Table-12) from the Day 49 to Day 79. The alterations in animals' body weight showed that it has reduced, though not statistically significant, in the higher dosage of NK treatment (NK HD) whereas the low dose group (NK LD) showed not much difference in weight on 15 days after administering the formulation when compared to the un treated HFD-STZ group. In Metformin treated group, after the initial loss of weight after STZ induction, there is no further loss, It is well known that chronic T2DM diabetes results in weight loss and generalized weakness (Furman, 2021). A gain in weight in the low dose treatment group of the formulation may indicate alleviation of chronic disease symptoms and improvement of metabolic dysregulation.

3.2.11 NK treatment lowered the plasma lipids in diabetic rats

Parameters of blood lipids such as Triglycerides and Cholesterol were examined . An increase was seen in un-treated diabetic rats as seen in Table 13. A reduction in the parameters like triglycerides and cholesterol was observed in NK treated groups, but it was not statistically significant. In the present study, Metformin also did not show any significant change on the

Group Name	Body weight measurements				
	Day 0	Day 45 (after HFD)	Day 49 (3rd day after STZ)	Day 64 (15th day after drug)	Day 79 (28th day after drug)
Control	170.25±7.972	201.25±6.920	212.25±10.52	218±9.721	217.5±12.155
HFD-STZ	159.5±12.861	237±19.761	227±16.477	210.75±7.951	195±11.098
Metformin	176.±.768	259.5±9.742	247.75±6.587	215.25±10.734	214±14.736
NK LD	165±18.0046	249.25±15.315	242.75±16.469	241.75±16.484	265.5±20.449
NK HD	162.5±13.88	235.75±18.629	230.5±17.437	202.75±8.984	184.25±12.071

Table-12. The effect of NK treatment on body weight. The table shows the changes in body weight at different time points, initial, after STZ induction, 15th day and 28th day after formulation treatment

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Group name	Control	HFD-STZ	Metformin	NKLD	NKHD
Triglyceride in mg/dL	107.70 ±27.7	215.74±52.4	261.76±44.9	94.07±16.8	107.96± 24.15
Cholesterol mg/dL	27.75±5.3777	286.88±74.371	488.82±149.21	117.91±23.31	286.20±69.67

Table-13. The effect of NK treatment on plasma lipid parameters, Triglyceride and Cholesterol. The table shows the reduction in plasma lipids 30 days post treatment. Data given as mean with SEM, n=4

3.2.12 Treatment of diabetic animals with low and high doses of NK improved plasma GLP1 levels.

In the T2DM rats, the plasma levels of GLP1 reduced markedly Fig-21. T2DM individuals are associated with reported to have impaired incretin effect which is evaluated by impaired OGTT response. In HFD-STZ diabetic control, the reduced glucose tolerance (Fig-20) can be attributed to a loss of incretin effect.

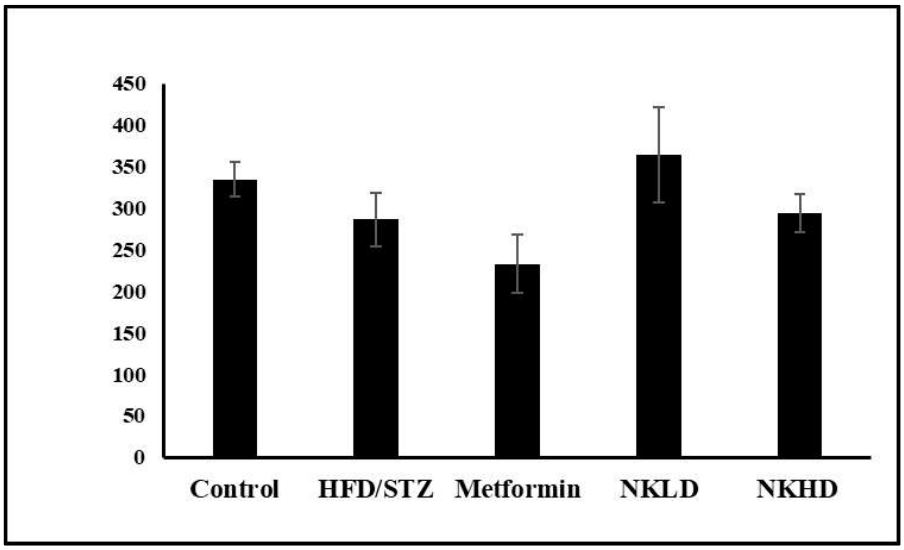


Fig-21. The effect of NK on plasma GLP1 levels. Graph showing GLP1 levels in NK treated groups when compared to the untreated diabetic control HFD/STZ group. The results were not statistically significant. Data given as mean with standard error of mean (SEM) n=4

In Fig-21, it can be observed that with low dose of NK, plasma GLP1 levels increased when compared to the untreated diabetic control but the higher dose did not have any effect. The increase in plasma GLP1 can be one of the factors for the improved OGTT response in NK LD group. This also validated the *in vitro* results of NK enhancing the GLP1 levels from intestinal cells.

3.2.13 Histopathology of key organs like pancreas, liver, kidney and intestine showed that NK treated animals showed reduced inflammation and improved morphology of cells when compared to HFD-STZ

The histopathology of liver showed that HFD-STZ rats had moderate inflammation as indicated by the arrows and steatosis which indicates the fat accumulation and the resulting inflammatory response (Fig-22B). The liver cells of NK treated animal group showed an improved morphology and lesser steatosis and inflammation as observed in (Fig-22D-E). This indicated that at both low and high doses, NK had a positive outcome on liver, which plays a vital role in T2DM progression and pathogenesis. Metformin, the standard drug treated rat liver showed milder inflammation and steatosis when compared to HFD-STZ group (Fig-22C). Histopathological analysis of kidney in the HFD-STZ group, the inflammation is shown to be increased in interstitium with degenerated tubules while the NK treated groups showed lesser

inflammation in interstitium as shown in Fig-23D-E. Metformin showed a slight improvement in the morphology of kidney cells while there was no apparent effect on other cellular aspects like interstitial inflammation (Fig-23C). Histopathological analysis of pancreas showed that in HFD-STZ group, there were a smaller number of islets with degenerative beta cells (Fig-24B). In NK treated group, an increase in islets is observed. Metformin also appeared to increase the beta islets which aligned with its known protective effect on pancreas (Fig-24C-E). The analysis of intestine treated with HFD-STZ showed inflammation and edema in the intestinal lining with presence of lymphocytes (Fig-25B). The intestinal layer also showed extensive loss of crypts and goblet cells. The intestine in the NK treated groups showed milder inflammation and loss of goblet untreated group. In the Metformin treated group also, the intestinal cells showed lesser inflammation when compared to the un treated diabetic group (Fig-25C-E).

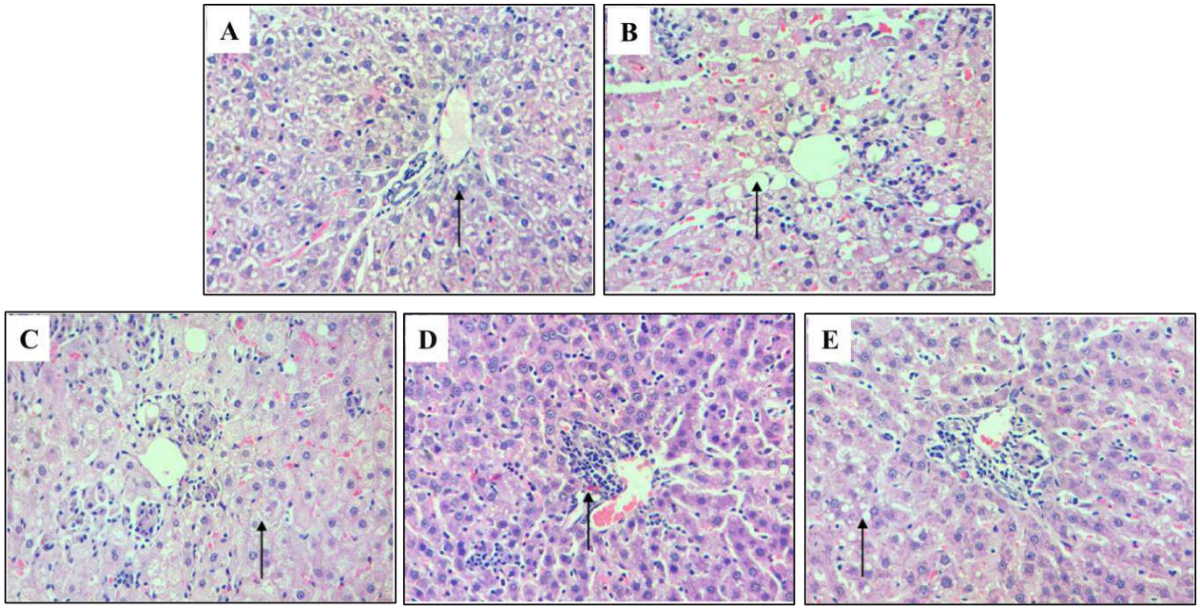


Fig-22. A-E showing the histopathological analysis of liver in NK treated animal groups . A) Control B) HFD-STZ, C) Metformin, D) NK LD E) NK HD

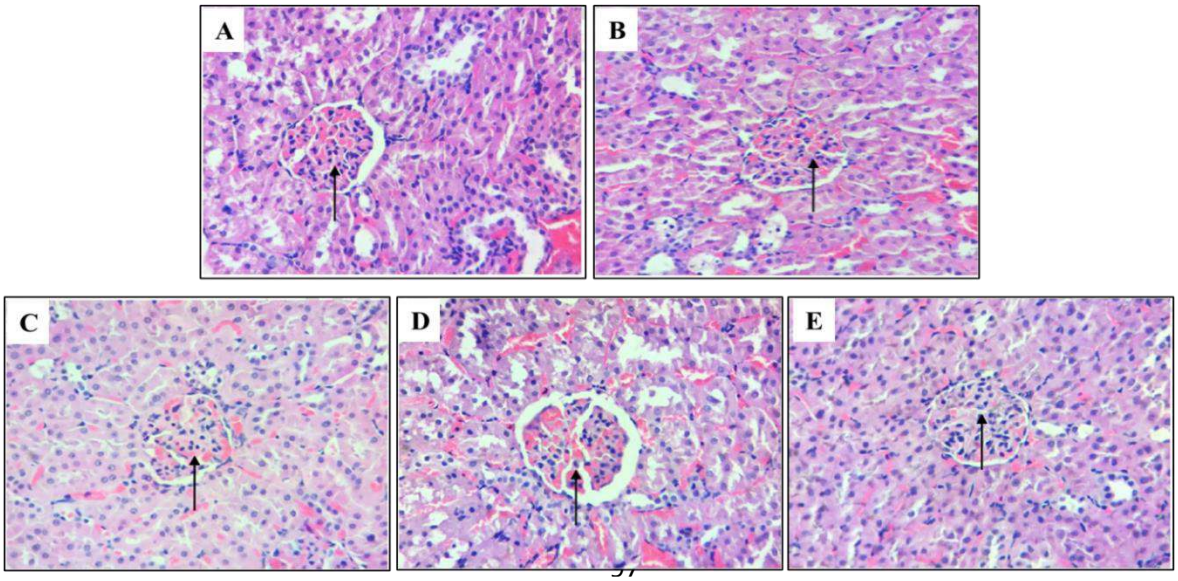


Fig-23. A-E showing the histopathological analysis of kidney in NK treated animal groups. A) Control B) HFD-STZ, C) Metformin, D) NK LD E) NK HD

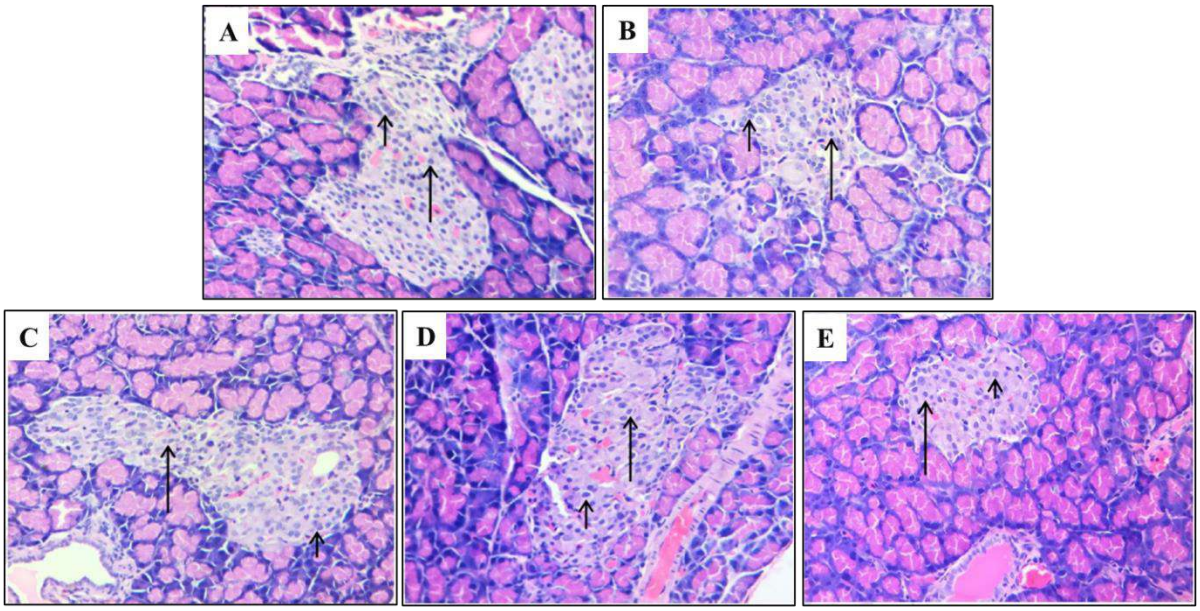
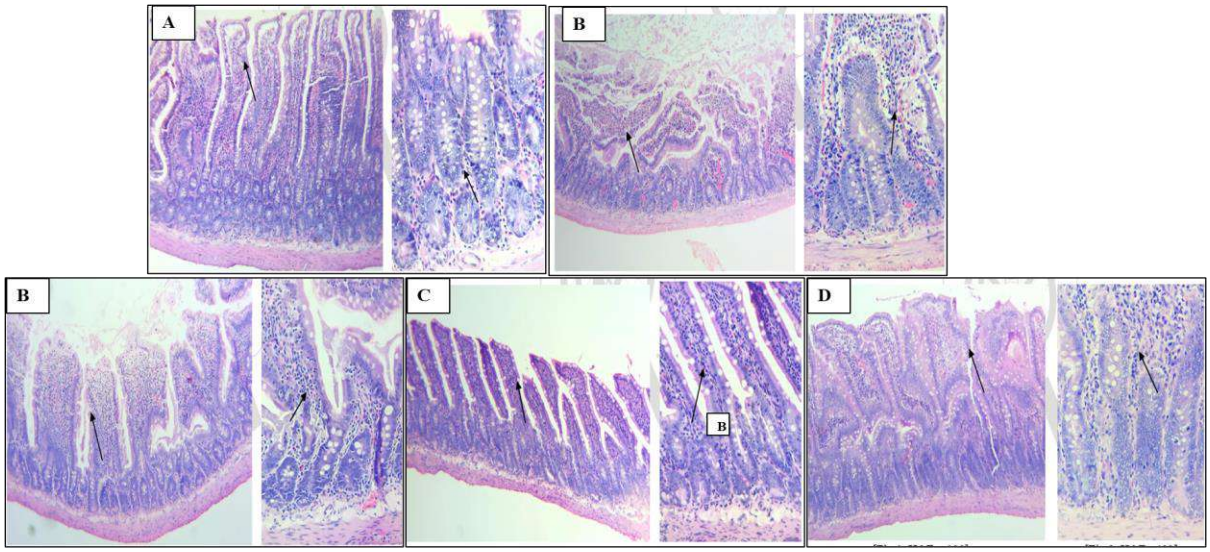


Fig-24. A-E showing the histopathological analysis of pancreas in NK treated animal groups. A) Control B) HFD-STZ, C) Metformin, D) NK LD E) NK HD



A-E showing the histopathological analysis of intestine in NK treated animal groups. A) Control B) HFD-STZ, C) Metformin, D) NK LD E) NK HD

From these observations, NK treatment appeared to have a positive effect on the vital organs such as kidney, liver, pancreas and intestine.

3.2.14 Summary

NK demonstrated digestive enzyme inhibition and strongly reduced DPP4 activity *in vitro*. It also enhanced GLP1 secretion from enteroendocrine cells. In the *in vivo* HFD-STZ rat model of diabetes, NK treatment reduced the T2DM fasting blood glucose levels, and improved oral glucose tolerance levels. *In silico* studies for NK revealed a multi-protein, multi-pathway biological network, and helped understand the potential therapeutical mode of action of NK.

3.3 *Varanadi kashayam* (VA) demonstrated anti-diabetic effect through DPP4 enzyme inhibition, GLP1 secretion and inhibition of digestive enzyme α-glucosidase.

Varanadi Kashayam (VA), as per the classical *Ayurveda* text *Ashtanga hridaya*, is indicated for correction of slow metabolism and reducing *meda* or fat (Singh, R.H., 2013). The formulation consists of 16 medicinal herbs, many of which have been reported to have anti diabetic and hypoglycemic effects as listed in Table-14. Many plants like *Terminalia chebula, Semecarpus anacardium, Aerva lanata* and *Moringa oleifera* are some of the well-studied herbs for their medicinal properties in diabetes other disease like cancer as well. (Goyal et al., 2011b; Bag et al., 2013; Semalty et al., 2010)

Scientific name	**Sanskrit name**	**Part used**	**Quantity**
***Crateva magna* (Lour.) DC**	*Varuna*	stem bark	0.625 g
***Aerva lanata* (L.) Juss. ex Schult.**	*Bhadra*	entire plant	0.625 g
***Asparagus racemosus* Willd.**	*Shatavari*	tuberous root	0.625 g
***Plumbago zeylanica* L.**	*Chitraka*	root	0.625 g
***Marsdenia tenacissima* (Roxb.) Moon.**	*Murva*	stem and root	0.625 g
***Aegle marmelos* (L.) Corrêa**	*Bilwa*	root/ root bark	0.625 g
***Aristolochia bracteolata* Lam**	*Kitamari*	root	0.625 g
***Solanum indicum* L.**	*Brihati*	root	0.625 g
***Barleria strigosa* Willd.**	*Sahachara*	entire plant	0.625 g
***Pongamia pinnata* (L.) Pierre**	*Karanja*	stem bark/seeds	0.625 g
***Moringa oleifera* Lam.**	*Shigru*	stem bark	0.625 g
***Premna corymbosa* (Burm.f.) Rottl. & Willd.**	*Agnimantha*	Root	0.625 g
***Terminalia chebula* Retz.**	*Haritaki*	Fruit	0.625 g
***Holoptelea integrifolia* (Roxb.) Planch**	*Chiribilwa*	Bark	0.625 g
***Desmostachya bipinnata (*L.) Stapf**	*Darbha*	entire plant	0.625 g
***Semecarpus anacardium* L**	*Bhallataka*	Seed	

Table-14 The list of plants in VA

There has been a limited number of studies attempted to understand the mode of action of *Varanadi* in the context of diabetes and related conditions. Chinchu et al., 2020 reported the anti-obesity and anti-inflammatory action of *Varanadi kashayam in vitro* and *in vivo*, where they have shown that the action is mainly through downregulation of lipogenic regulators such as PPARγ. Since the formulation is a combination of several potent hypoglycemic herbs, investigating the modes of action of the whole preparation using different approaches would aid greatly in a better understanding of its biological effects.

VA was subjected to multiple assays such as α-amylase and α-glucosidase inhibition, DPP4 inhibition, GLP1 secretion, and anti-adipogenic effects to explore the multi-modular mechanism of action (section 2.3.2). VA was found to be toxic to fibroblasts at different concentration ranges used in the study and therefore the antiadipogenic experiments were not taken forward. The formulation was also investigated for its biological action *in vivo* based on the results of the *in vitro* and *in silico* experiments for its anti-diabetic actions.

3.3.1 *Varanadi kashayam* (VA) exhibited a dose dependent inhibition of α-glucosidase but did not inhibit α-amylase

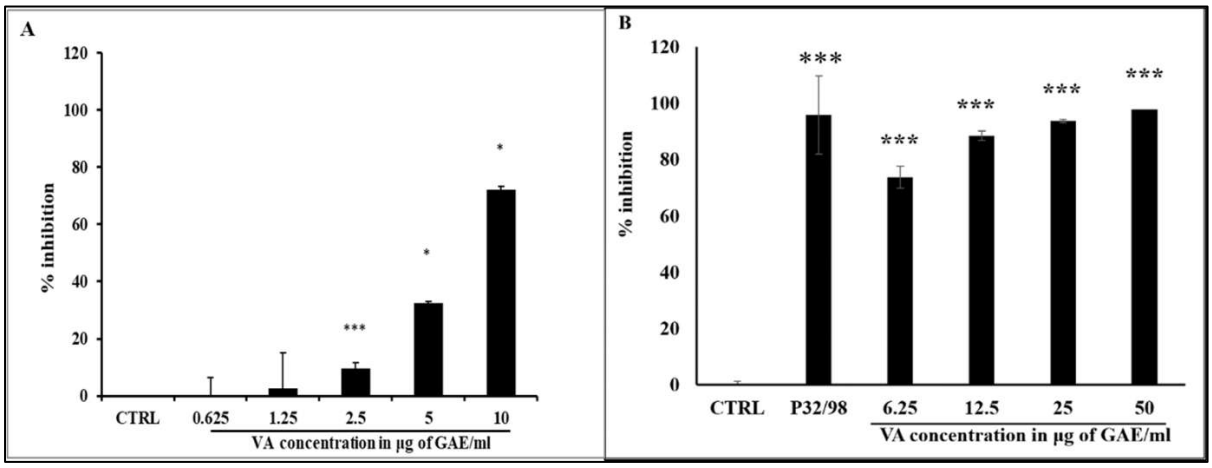


Fig-26. α-glucosidase and DPP4 inhibition effect of VA. (A) Graph shows a concentration-dependent inhibition of α-glucosidase enzyme action upon treatment with various concentrations of VA (p value ≤ *** 0.001, p value ≤ ** 0.01, p value ≤ * 0.05). (B) Graph shows a concentration-dependent inhibition of DPP4 enzyme action upon treatment with various concentrations of VA (p value < *** 0.001)

As mentioned in methodology 2.3., the polyherbal formulation was evaluated for their ability to inhibit digestive enzymes. Similar to NK, VA als showed a dose dependent inhibition of the

digestive enzyme α- glucosidase and the IC_{50} of the inhibition was found to be 7.738 μg of GAE/ml (Fig-26A). VA showed 72 % inhibition at the highest concentration of 10 μg of GAE/ml tested and at 2.5 μg of GAE/ml showed a 9 % inhibition. Further again, similar to NK, VA too did not exhibit any inhibitory effect on α-amylase activity in the concentration range 0.625 to 10 μg of GAE/ml used in the study. This observation requires more studies to understand the chemistry and biology of these formulations with respect to digestive enzyme inhibition.

3.3.2 VA inhibited DPP4 enzyme action as well as its expression in enteroendocrine cell model GLUTag

Following the digestive enzyme inhibition, VA was studied for its effect on incretin hormone regulating enzyme DPP4. VA was found to inhibit DPP4 enzyme action in a dose dependent manner with an IC_{50} of 1.22 μg of GAE/ml (Fig-26B). At a concentration of 50 μg of GAE/ml, VA inhibited DPP4 by 97% and at lowest concentration of 6.25 μg of GAE/ml showed an inhibition of 73 %. This indicates a strong DPP4 inhibitory potential of VA that further suggests a possible incretin modulatory effect for VA in the management of diabetes and associated disease conditions.

As explained in section 3.2.2, studies have shown that inhibition of DPP4 expression at mRNA level can reduce the circulating concentration of DPP4 and this is also considered as a good strategy for positively modulating incretin effect. With this in mind, the effect of VA on DPP4 expression in GLUTag cells was evaluated. The toxicity of the formulation was checked in cells by MTT assay (Fig-27A). The highest non-toxic concentration was selected and the qPCR studies showed that VA downregulated the expression of DPP4 by 1.5 folds when compared to untreated control which suggested a transcriptional downregulation of DPP4 in addition to its *in vitro* enzyme inhibition (Fig-27B). Both these activities suggest the ability of VA to positively modulate incretin effect. It is also important to note that among the two formulations studied, only VA showed inhibition of DPP4 expression and enzyme action.

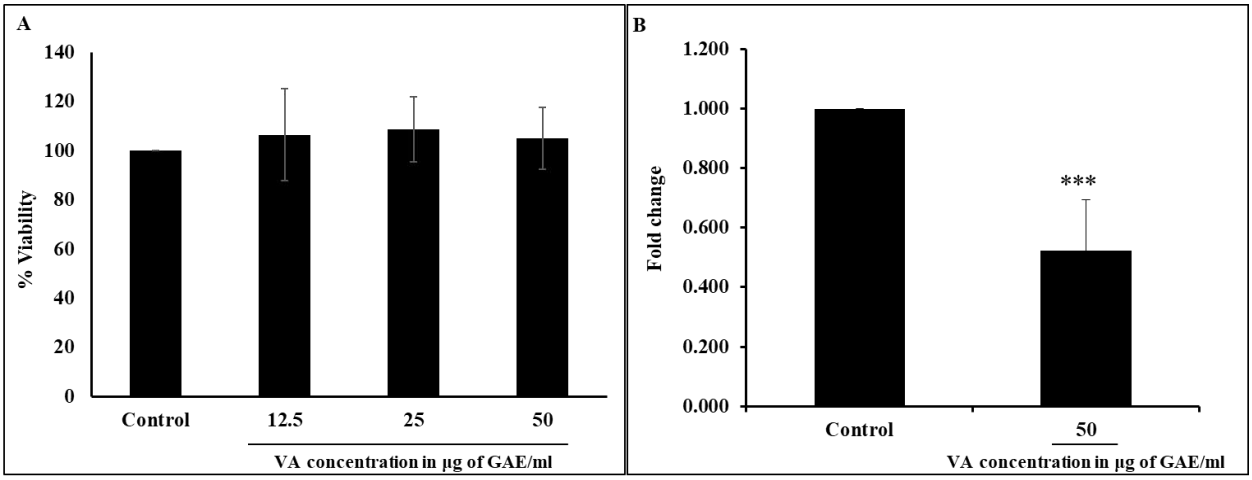


Fig-27. Effect of VA on DPP4 expression in GLUTag cells. (A) Graph shows a concentration-dependent effect on cell viability upon treatment with various concentrations of VA. (B) Graph shows a downregulation of DPP4 expression at 50 µg of GAE/ml (p value ≤ *** 0.001)

3.3.3 VA enhanced GLP1 secretion from enteroendocrine cells

Similar to the formulation NK, VA was also studied for its ability to stimulate GLP1 secretion using GLUTag cells. GLUTag cells treated with different concentrations of VA showed a modest but significant increase in GLP1 levels (Fig-28). At concentrations 25 and 50 µg of GAE/ml, there was an increase of 11% and 17% increase in GLP1 secretion. This showed that VA can potentially modulate incretin effect through DPP4 inhibition, downregulation of DPP4 expression and increased secretion of GLP1.

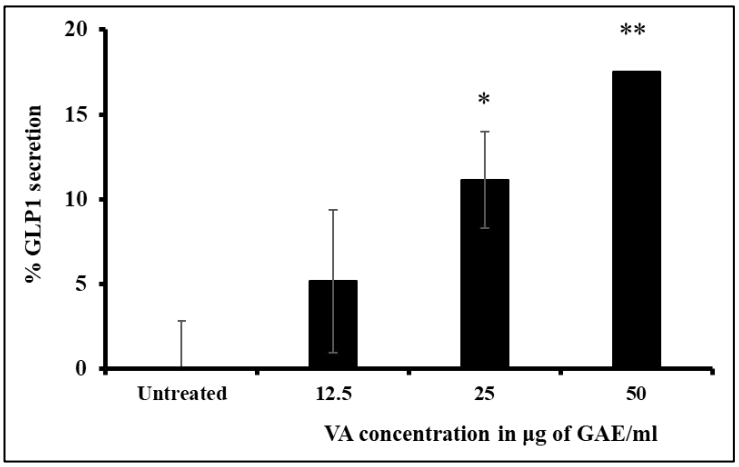


Fig-28. The effect of VA on GLP1 secretion in GLUTag cells. Graph shows a concentration dependent increase in GLP1 release from GLUTag cells upon treatment with various concentrations of VA (p value ≤ 0.01**, ≤ 0.05 *)

3.3.4 Phytochemical Data mining and Molecular Docking of compounds from

From the initial *in vitro* results, it was observed that VA inhibited DPP4 enzyme action and its expression in enteroendocrine cells as well as increased GLP1 secretion from the endocrine cells. Similar to the methodology carried out for NK, an *in silico* approach was adopted to identify the phytochemicals that could be potentially DPP4 inhibitors. As mentioned in section 2.3.4.1, phytochemical data mining was carried out 248 compounds were retrieved from the 16 constituent herbs present in the formulation.

This was followed by a virtual screening of phytochemicals for DPP4 interaction with Vildagliptin as a standard DPP4 inhibitor. Molecular docking and MMGBSA studies were carried out for all the compounds and listed in Table-15 and the top 3 compounds, Terchebin, Chebulinic acid and Chebulagic acid, were selected and taken for molecular dynamics and simulation studies since some of the other candidate compounds with higher docking scores like Terflavin A, 1,2,3,4,6-Penta-O-galloyl-a-D-glucopyranose and Pentagalloyl Glucose were not found to be suitable as they did not exhibit stable interactions with the DPP4 in molecular dynamics and simulation studies. Among the compounds, Terchebin came as a top candidate in NK also. The MMGBSA scores of Terchebin, Chebulinic acid and Chebulagic acid were found to be and -47.12, -64.61, and -46.51 kcal/mol respectively whereas the docking scores were -11.766, -10.631 and-9.322 kcal/mol respectively. The 2D interaction diagram of the compounds with DPP4 are represented in Fig-29. The protein-Vildagliptin complex can be referred to Fig-15. DPP4 amino acid residues Glu206, Tyr666 and Asn710 that are involved in Vildagliptin-DPP4 interaction are also found to interact with the top 3 compounds identified from the virtual screening. The compounds chebulinic acid and chebulagic acid interacted with amino acid residues Glu205 and Glu206 respectively which are critical for protein-ligand stable interaction. Chebulagic acid also interacted with other important residues in DPP4 binding such as a Tyr666, His740 and Gly 741. These interactions are stabilized by prominent hydrogen bonds and water bridges which significantly contribute for a stable ligand protein interaction (Fig-29 and Fig-30).

S. No	Compound name	MMGBSA dG (kcal/mol)	Docking score (kcal/mol)
	Vildagliptin	-26.7	-3.899
1	Chebulinic Acid	-64.61	-10.631
2	Terchebin	−47.12	-11.766
3	1,2,3,4,6-Penta-O-galloyl-a-D-glucopyranose	-61.17	-13.724
4	Pentagalloyl Glucose	-48.67	-11.443
5	Terflavin A	-60.68	-12.644
6	Chebulagic Acid	-46.51	-9.332
7	Moupinamide	-41.62	-6.873
8	Anacardoside	-41.28	-10.33
9	Sennoside A	-38.09	-10.489
10	Maltose	-32.81	-9.504
11	Typhaneoside	-32.53	-11.747
12	Galluflavanone	-32.38	-7.194
13	Beta-Glucogallin	-31.38	-7.884
14	chebulic acid	-29.91	-7.504
15	Rutin	-28.44	-9.113
16	Amentoflavone	-26.85	-6.839
17	Asclepobiose	-26.08	6.666
18	Quercetin	-24.64	-5.968
19	beta-Marsdenin	-24.49	-6.575
20	Sorbitol	-13.63	-7.188

Table-15. Top scored 20 compounds from the virtual screening of VA phytochemicals with DPP4. The docking score and binding free energy from MM-GBSA analysis are reported.

Chebulinic acid and DPP4 interaction had two hydrogen bonds involved in the residues Arg560 and Asp545 stabilizing the complex while chebulagic acid had five hydrogen bonds. The protein–ligand interactions are depicted for four interactions (hydrogen bond, hydrophobic bond, water bridge, and ionic bond) using the clubbed bar graph through normalized values as seen in Fig-30.

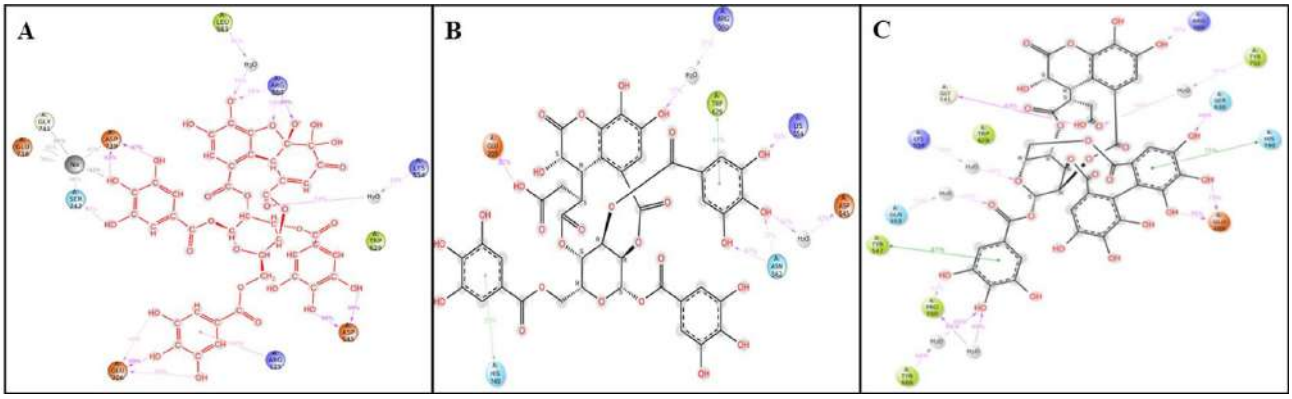


Figure 29. 2D interaction diagram of ligands with protein during the simulation. (A) Terchebin, (B) Chebulinic acid and (D) Chebulagic acid with DPP4

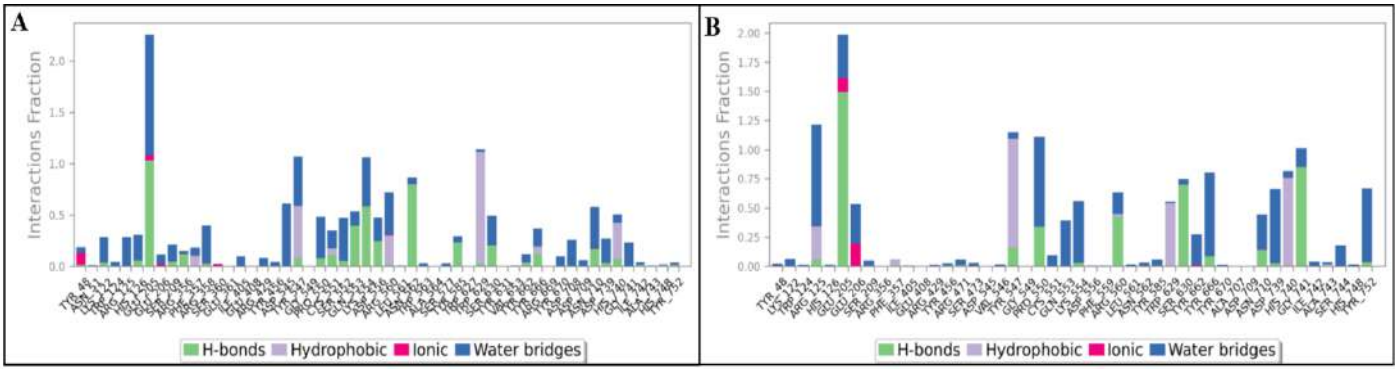


Figure 30. Protein interactions of the interacting amino acids in VA compounds. Graph shows bonds categorized into Hydrogen Bonds, Hydrophobic, Ionic and Water Bridges. Graph shows A) Protein and Chebulinic acid and B) Protein and Chebulagic acid

3.3.5 Molecular Dynamics, stability and simulation studies

A 100ns MD simulation analysis of the DPP4-ligand docked complex was performed and the RMSD profiles of the protein and ligands during the 100ns simulations are analyzed (Fig-31). The average RMSD of DPP4 and Vildagliptin was found to be 1.75Å and 2.0Å, respectively (Fig-31A). On the basis of simulation and molecular dynamic studies, 3 compounds were selected as most suitable as DPP4 inhibitors namely, Terchebin, Chebulinic acid and Chebulagic acid. The RMSD studies were done for all the three compounds and all exhibited stable binding with DPP4. The average RMSD of DPP4-Chebulinic acid for the last 50ns (Cα) was 1.8 Å and DPP4-Chebulagic acid was 1.6Å. (Fig-31C-D). The RMSD of DPP4-Terchebin interaction has already been mentioned in section 3.2.5. The fluctuations between ligand and protein were studied through Root Mean Square Fluctuation (RMSF) analysis and minimal fluctuations were observed.

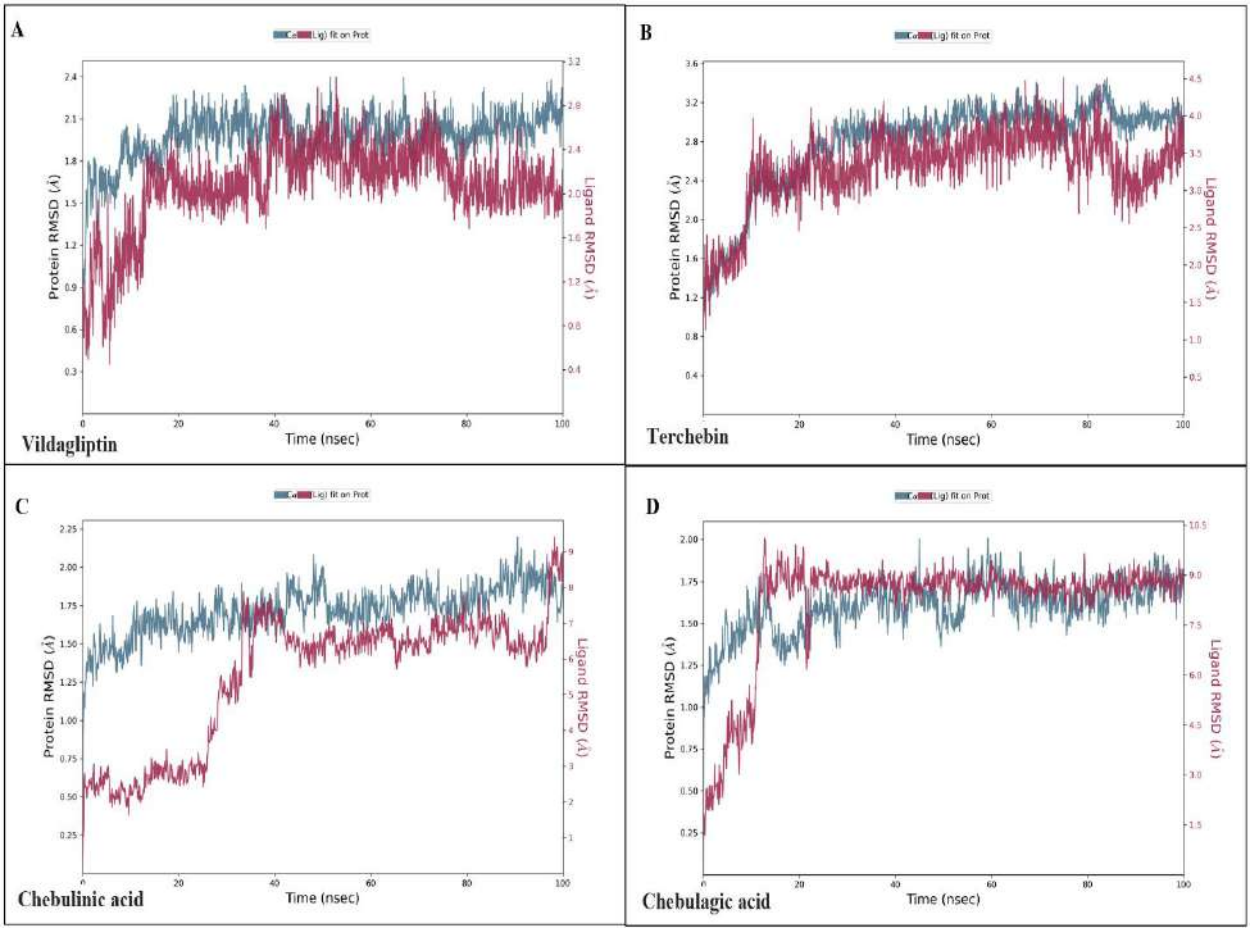


Fig-31. RMSD plot of top ligands in VA. RMSD of Protein and Vildagliptin (A); Protein and Terchebin (B); Protein and Chebulinic acid B (C); Protein and chebulagic acid (D)

3.3.6 Target mapping of phytochemicals identified in VA formulation for network analysis

Further to delineate the pharmacological networking of VA, analysis was carried out where each phytochemical was mapped to its potential targets sourced from the databases described in methods. Using the Cytoscape tool, the phytochemical-target ingredient, a bipartite network was constructed, in which 172 phytochemicals were mapped to 4581 genes. To create a diabetes specific subnetwork, key words “Diabetes mellitus”, “Insulin resistance”, “Hyperglycemia”, “Hypoglycemia” were used.

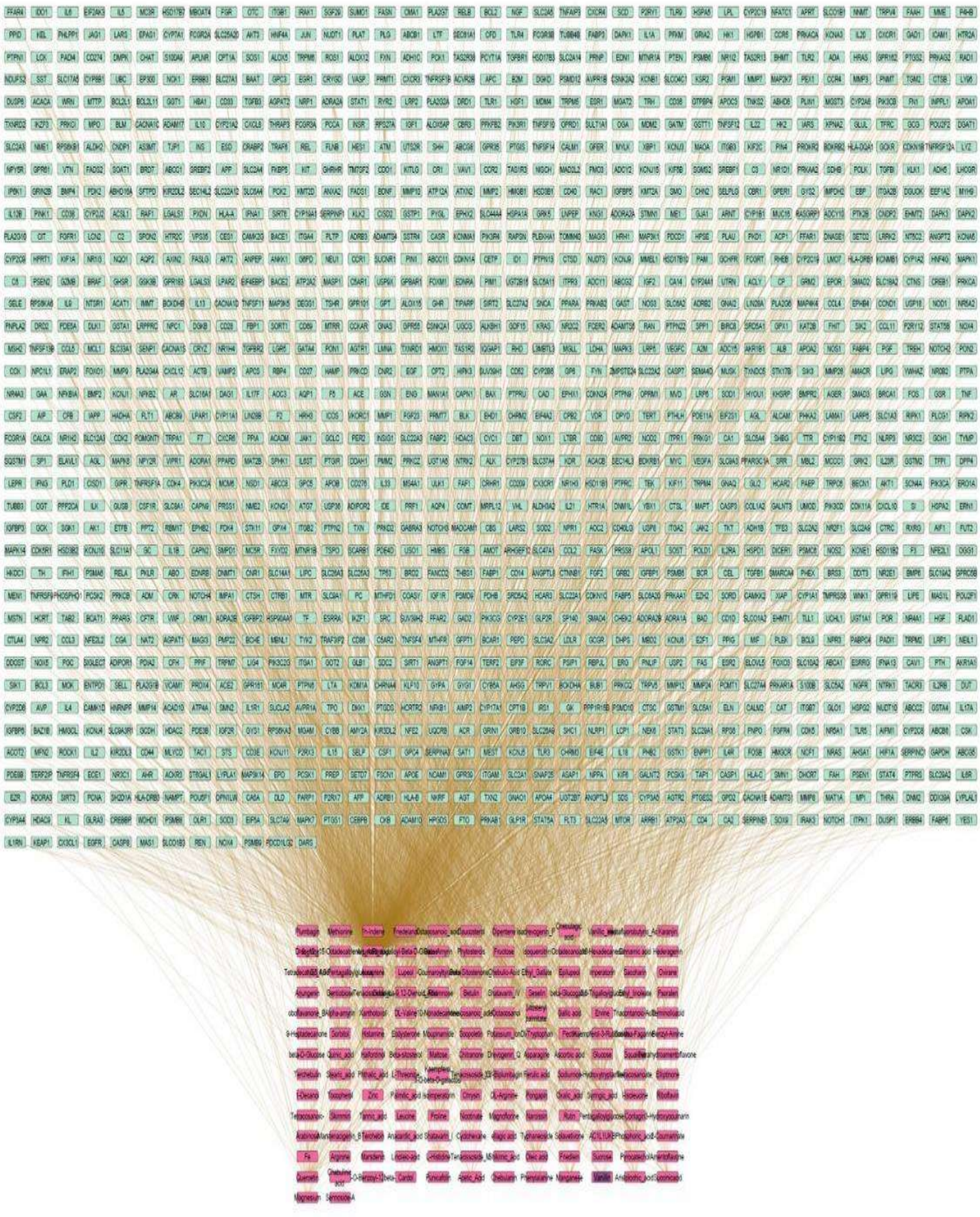


Fig-32. The compound-target network of VA The network of phytochemicals of VA and diabetes-related proteins. The pink color nodes represent the compounds and the green nodes represent the diabetes associated target proteins

The sub network comprised of 1379 proteins constitutng the potential diabetic network of VA formulation and had a large number of genes playing relevant roles in pathogenesis of diabetes and associated metabolic conditions (Fig-32). From the compound-target network, using the plug-in Cyto hubba, the genes having maximum interactions were identified and are listed in Table-16. Among these, DPP4 and GLP1R are in the top 10 hub proteins having maximal interactions. The 1379 targets were further subjected to a disease association analysis to understand the potential regulation of diabetes and associated co morbidities.

SL no	Hub protein	Degree	Rank	Sl no	Hub protein	Degree	Rank	Sl no	Gene Hub protein	Degree	Rank
1	CA2	23	1	36	MMP2	9	7	71	BCL2	7	9
2	AKR1B1	15	2	37	ALB	9	7	72	CD52	7	9
3	MMP9	13	3	38	VEGFA	9	7	73	PYGL	7	9
4	CASP3	13	3	39	HTR2A	9	7	74	AVPR1A	6	10
5	FABP4	13	3	40	HLA-C	9	7	75	EDNRA	6	10
6	FFAR1	13	3	41	EGFR	9	7	76	ADA	6	10
7	CYP19A1	12	4	42	SLC3A2	9	7	77	SLCO1B1	6	10
8	UBC	12	4	43	PNLIP	9	7	78	GRIN1	6	10
9	ADRA2A	12	4	44	RPS6KA3	8	8	79	BCHE	6	10
10	AR	12	4	45	KDM1A	8	8	80	NOS3	6	10
11	FABP5	12	4	46	PSMB5	8	8	81	GAA	6	10
12	F2	12	4	47	CYP17A1	8	8	82	CACNA1C	6	10
13	ESR1	11	5	48	MAPK1	8	8	83	COMT	6	10
14	RPS27A	11	5	49	OPRM1	8	8	84	TF	6	10
15	ERAP2	11	5	50	PSMC6	8	8	85	SOD2	6	10
16	PTGS2	11	5	51	NOX4	8	8	86	TXNRD1	6	10
17	PDE5A	11	5	52	CES1	8	8	87	CYP1A2	6	10
18	GRIN2B	10	6	53	ESR2	8	8	88	SRD5A2	6	10
19	HLA-A	10	6	54	DPP4	8	8	89	NPR2	6	10
20	CA1	10	6	55	LNPEP	7	9	90	GLP1R	6	10
21	CYP1B1	10	6	56	HDAC2	7	9	91	TNF	6	10
22	NR1I2	10	6	57	MAPK8	7	9	92	LHCGR	6	10
23	ADRA2B	10	6	58	AVPR2	7	9	93	ITGB1	6	10
24	EDNRB	10	6	59	GCG	7	9	94	FABP2	6	10
25	PPARG	10	6	60	CHRNA4	7	9	95	LPAR2	6	10
26	HLA-B	10	6	61	HDAC9	7	9	96	SLC6A4	6	10
27	B2M	10	6	62	RORC	7	9	97	DRD2	6	10
28	ALOX15	9	7	63	HSD11B1	7	9	98	HTR2C	6	10
29	AKT1	9	7	64	SHBG	7	9	99	MPO	6	10
30	CYP1A1	9	7	65	LTF	7	9	100	PPARA	6	10
31	NR1H3	9	7	66	CA14	7	9	101	CNDP2	6	10
32	HDAC3	9	7	67	CASR	7	9	102	PLAT	6	10
33	LGALS3	9	7	68	IMPA1	7	9	103	NOS2	6	10
34	AMY2A	9	7	69	FYN	7	9	104	FABP3	6	10
35	PTPN1	9	7	70	PSMD9	7	9				

Table-16. The list of hub proteins in VA target network ranked as per their degree values.

3.3.7 Disease overlap analysis of the protein targets mapped for phytochemicals in VA formulation

To create a diabetes specific subnetwork, key words “Diabetes mellitus”, “Insulin resistance”, “Hyperglycemia”, “Hypoglycemia” were used. The sub network comprised of 1379 proteins constituting the potential diabetic network of VA formulation and had a large number of genes playing relevant roles in pathogenesis of diabetes and associated metabolic conditions. A Cluego and KEGG pathway analysis of the mapped proteins was done to understand their role in biological processes and pathways (Fig-33) and (Fig-34). From the Cluego analysis, several key processes such as Nitric Oxide Synthase process, lipoprotein process, negative regulation of Cholesterol transport were found VA can modulate key processes involved in metabolism. Similarly, the KEGG pathway analysis showed top pathways like AGE-signaling, Lipid and atherosclerosis, insulin resistance and regulation of lipolysis in adipocytes and these are highly important in the pathogenesis of T2DM and obesity.

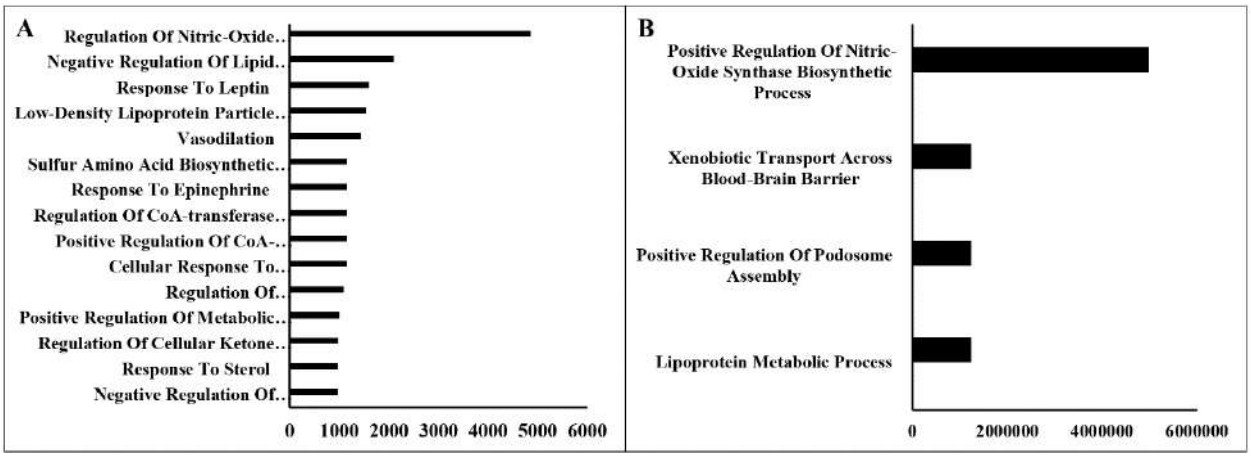


Fig-33. ClueGO analysis of biological processes. Biological processes from the Cluego analysis of **1379** target proteins in the diabetic network of VA. The proteins are found to be involved in different biological processes

Using EnrichR algorithm, from various databases like Clinvar, Diginet and OMIM expanded databases, the 1379 diabetes proteins were analyzed for diabetic complications and also associated comorbidities. These include nephropathy, neuropathy, cardiomyopathy and retinopathy and also risk factors/related conditions such as fatty liver, obesity and general metabolic disturbances along with inflammation which is a characteristic of all these diseases. From the Venn diagram analysis, it was observed that 16 genes were overlapping among the

specific diabetic complications such as neuropathy, nephropathy, cardiomyopathy and retinopathy and others like diabetic foot and ulcers (Fig-35-A-B). Between the diabetic pre-disposing conditions like obesity and fatty liver and general metabolic disturbances and inflammation which are associated symptoms in diabetes, an overlap of 59 genes was observed. This suggested that a common cluster of genes are playing a predominant role in the pathogenesis and complications of diabetes. Among the 16 genes found to be common in diabetic complications, inflammatory markers like TNFα, TLR4 and TGFβ1 and growth factors like HGF and IGF1, were present. In the Venn diagram analysis of diabetic complications with co morbidities, many critical genes like TNFα, PPARγ, AKT1, CCL2, TGFβ1 and APOA1 were found among others. DPP4 and GLP1R were observed in the overlap between the diabetic complications, obesity and fatty liver. From the network pharmacology analysis, it could be inferred that VA potentially modulated a vast number of proteins involved in diabetes and associated co morbidities and detailed studies need to be carried out understand the precise mode of actions and mechanisms in the pharmacological action of VA.

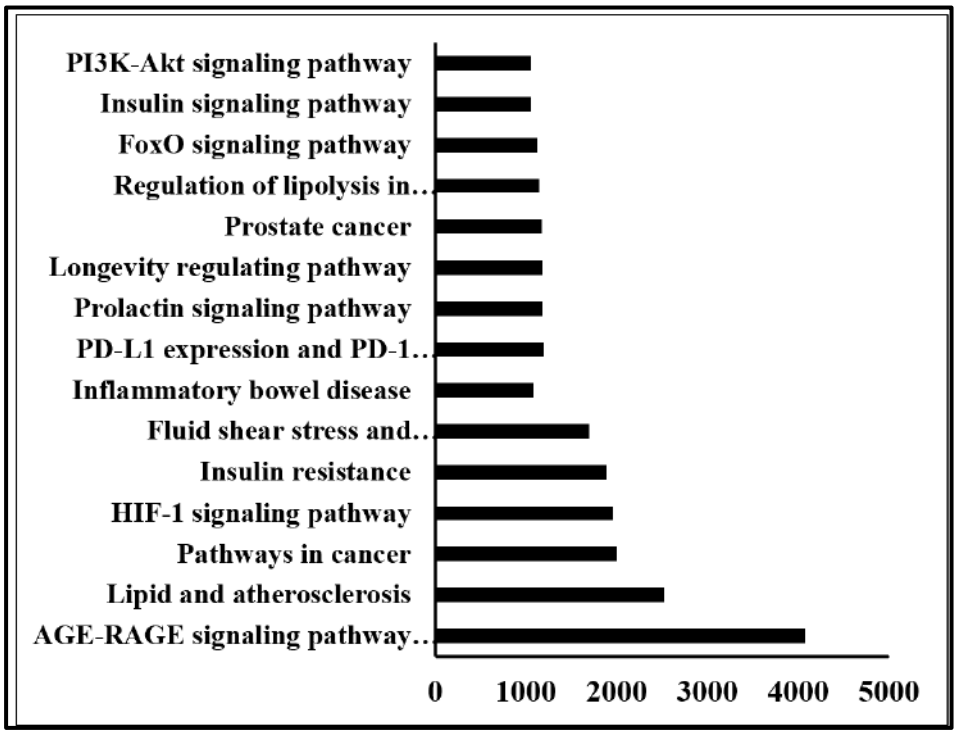


Fig-34. KEGG pathway analysis of VA target proteins. Pathways from the KEGG analysis of 1379 target proteins in the diabetic network of VA

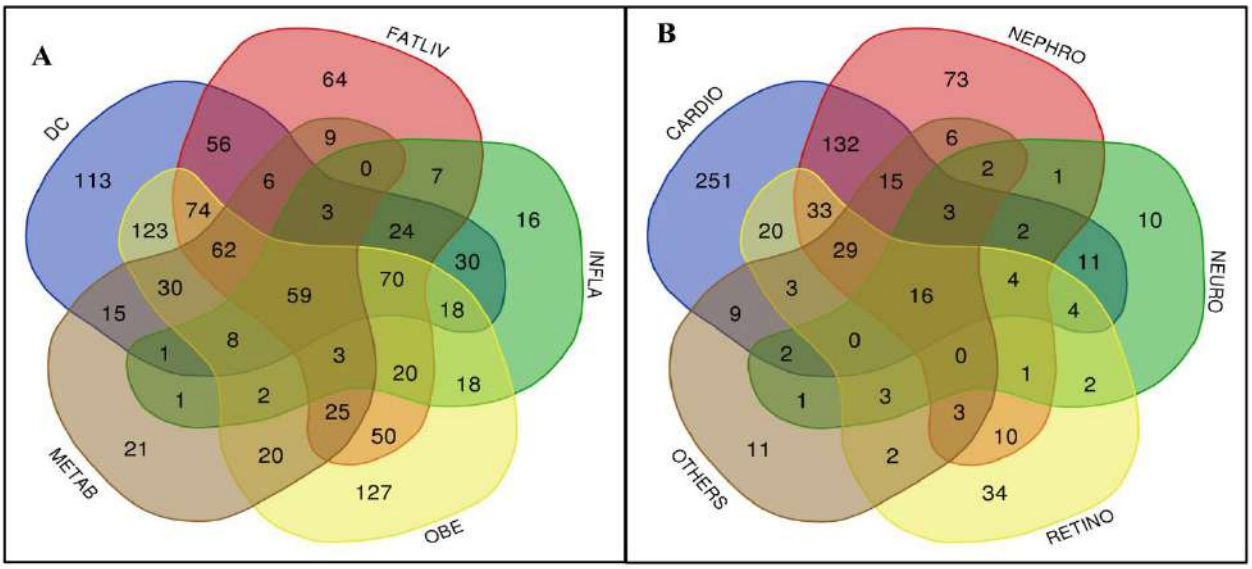


Fig-35. Venn diagram analysis of VA target proteins. (A) represents the analysis of protein association between diabetic complications and associated diseases (B) shows the Venn analysis of proteins within diabetic complications. Abbreviations: INFLAM – Inflammation; FAT LIV - Fatty liver; MET DYS - 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

3.3.8 VA treated type 2 diabetic rats exhibited lower fasting blood glucose concentrations and improved glucose tolerance.

To corroborate the *in vitro* findings of anti-diabetic effects of VA, *in vivo* experiments were carried out in diabetic model of high fat diet combined with induction of STZ and treated with low and high doses of VA. Similar to NK, the results of experiments are given for each group containing 4 animals (n=4). A significant reduction in the fasting blood glucose levels was observed in the VA formulation treated rats when compared to the untreated diabetic control HFD-STZ as seen in Table-17. Further, an improvement of glucose tolerance was also was observed in the oral glucose tolerance test carried out. The Fig-36 showed a decrease in area under curve (AUC) of blood glucose in VA treated groups of animals. The improvement in oral glucose tolerance induced by VA administration was not significant statistically in the present study.

Blood glucose levels (mg/dL)			
Total days of study	**Day 0**	**Day 49**	**Day 76**
Group Name	**Initial**	**3rd day after induction STZ**	**26 days after Drug**
Control	**98.75±3.75**	**77±5.93**	**94.25±5.93**
HFD-STZ	**112±6.48**	**330.75±63.94**	**503.75±35.63**
Metformin	**104.25±4.51**	**357±32.11**	**322.25±69.18**
VA LD	**117.75±3.81**	**420.75±51.21**	**283.75*±74.09**
VA HD	**113.75±1.54**	**454.25±9.05**	**335.5*±45.77**

Table-17- The effect of VA on blood glucose levels. Table showing blood glucose levels in VA treated groups when compared to the untreated diabetic control HFD-STZ group. Data are given as mean with Standard Error Deviation (SEM) (p value ≤ 0.05 *)

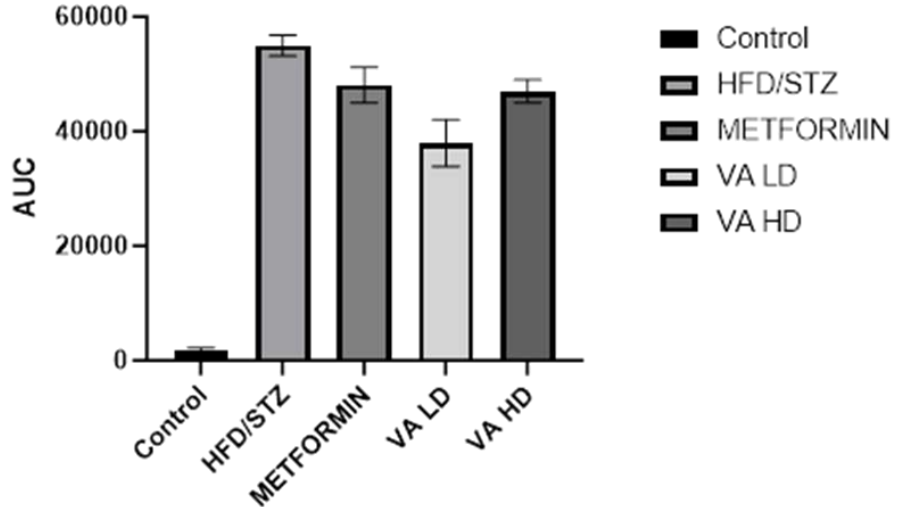


Fig-36. AUC for glucose for VA treated groups. The effect of VA low and high doses (VA LD and VA HD) on AUC when compared to the untreated diabetic control HFD-STZ group. Data given as mean with Standard Error Mean (SEM). n=4.

3.3.9 Treatment of HFD-STZ rats with VA reduced body weight

Treatment of HFD-STZ rats with VA high and low doses (VA LD and VA HD) also showed body weight alterations as seen in the Table-18. After induction of STZ there was a decrease in body sweight across groups as a result of effects of STZ induction as explained in section 3.2.10. 15 days after the formulation treatment, there is a significant weight loss when compared to the HFD-STZ group in the high dose group of VA while in the lower dose no

change was observed. At the end of treatment, weight reduction is not significant which may indicate a protective effect of formulation. It may be worthwhile to study the effects of lower dose of VA to understand fully the mode of action since the lower dose seemed to maintain the body weight till the end of study.

Body weight measurements					
Total days of study	**Day 0**	**Day 45**	**Day 49**	**Day 64**	**Day 79**
Group Name	**Initial**	**After HFD 45 days**	**Day 49 (3rd day after STZ)**	**Day 64 (15th day after drug)**	**Day 79 (28th day after drug)**
Control	**170.25±7.97**	**201.25±6.920**	**212.25±10.52**	**218±9.721**	**217.5±12.155**
HFD-STZ	**159.5±12.86**	**237±19.761**	**227±16.477**	**210.75±7.951**	**195±11.098**
Metformin	**176.±.76**	**259.5±9.742**	**247.75±6.587**	**215.25±10.734**	**214±14.736**
VA LD	**164.75±5.67**	**250±7.0710**	**236.5±10.531**	**232±22.524**	**232.75±28.726**
VA HD	**154.75±9.586**	**233±20.569**	**218±17.141**	**174.25±16.428**	**175±15.942**

Table-18. The effect of VA treatment on body weight post 30 days. The table shows the changes in body weight at different time points, initial, after STZ induction, 15th day and 28th day after formulation treatment. Data given as mean with (SEM), n=4

3.3.10 VA treated HFD-STZ induced diabetic rats exhibited lower plasma lipid parameters

VA as mentioned in section 3.3, is prescribed by *Ayurveda* physicians for obesity management and for improving the deregulated metabolic balance. Treatment of VA in HFD-STZ rats led to a reduction in concentrations of plasma triglycerides and cholesterol in both low and high dose groups as seen in Table-19. These results suggested anti-hyperlipidemic action providing a basis for the clinical usage as an anti-obesity treatment.

Group name	**Control**	**HFD/STZ**	**Metformin**	**VALD**	**VAHD**
Triglyceride in mg/dL	107.7±27.7	215.74±52.4	261.76±44.9	171.17±20.75	121.85±29.55
Cholesterol in mg/dL	27.75 ±5.37	286.88±74.37	488.82±149.21	269.51±127.55	194.12±65.23

Table-19. The effect of VA on plasma lipid parameters. The table showing reduction in triglycerides and Cholesterol in VA treated animals when compared to the untreated diabetic control HFD-STZ group. Data given as mean with Standard Error Deviation (SEM), n=4

3.3.11 Treatment of diabetic animals with low and high doses of VA improved plasma GLP1 levels.

In HFD-STZ group, there was a reduction in plasma GLP1 levels indicative a defective incretin response in the chronic diabetic condition (Bhat et al., 2018; Lalitha et al., 2020). After 30 days of administration of VA at both low and high doses, there is a non-significant increase in GLP1 levels (Fig-37). This also aligned with the *in vitro* observations wherein VA treated enteroendocrine cells had higher GLP1 levels. The slight improvement in OGTT response seen in Fig-37, though not statistically important, could be due to improved incretin response.

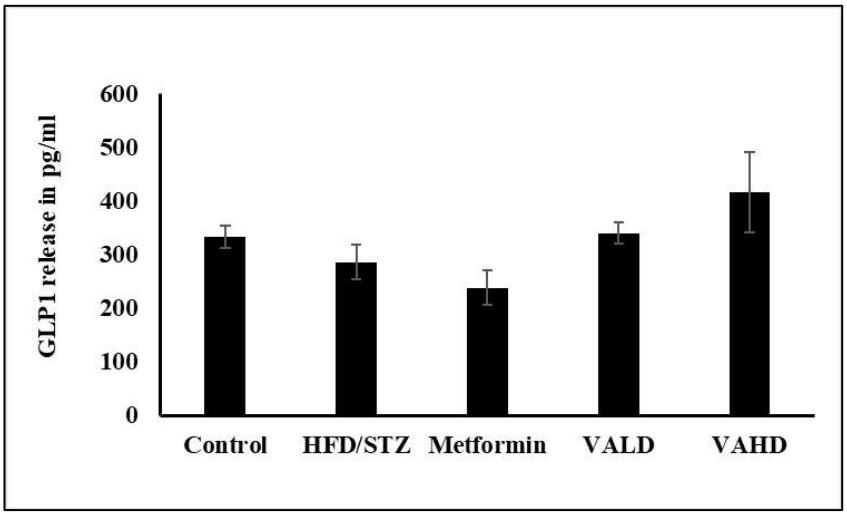


Fig-37. The effect of VA treatment on plasma GLP1 levels. Graph showing increased GLP1 levels in VA treated groups when compared to the untreated diabetic control HFD-STZ group. Data given as mean with Standard Error Deviation (SEM), n=4

3.3.12 Histopathology of key organs like pancreas, liver, kidney and intestine showed that VA treated animals showed reduced inflammation and improved morphology of cells when compared to HFD-STZ

The histopathology of liver showed that HFD-STZ rats had moderate inflammation as indicated by the arrows and steatosis which indicates the fat accumulation and the resulting inflammatory response (Fig-38B). The formulation treated liver cells showed an improved morphology and lesser steatosis and inflammation when compared to the un treated group (Fig-38D-E). VA also appeared to decrease the liver inflammation and steatosis. The formulation treated kidney cells showed lesser inflammation when compared to the un treated group and showed normal glomerulus and tubules. (Fig-39D-E). The formulation treated pancreatic islets cells showed lesser inflammation when compared to the un treated diabetic group and an increased in beta islets were observed when compared to the diabetic group which indicated the protective effect of VA on pancreas (Fig-40D-E). The histological analysis of VA treated intestine showed

comparatively lesser damage to intestinal lining and milder inflammation and no edema was observed while in HFD-STZ group, there was extensive damage and loss of goblet cells accompanied by inflammation (Fig-41B-E). Overall, VA showed an improvement in the intestinal morphology.

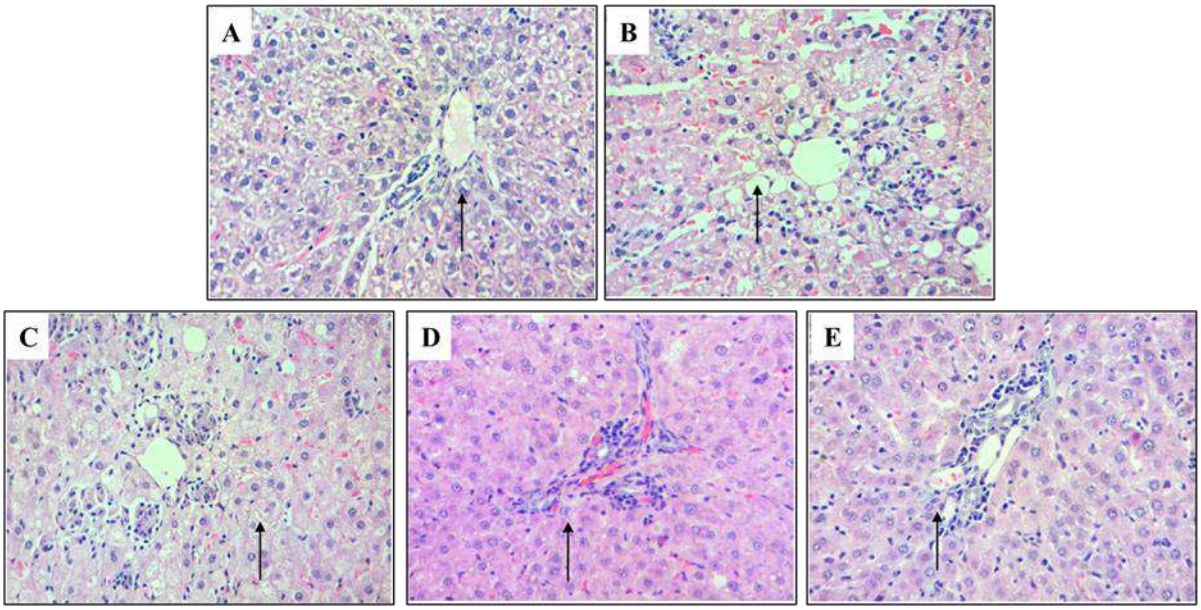


Fig-38- A-E showing the histopathological analysis of liver in animal groups. Images showing A) Control B) HFD-STZ, C) Metformin, D) VA LD E) VA HD

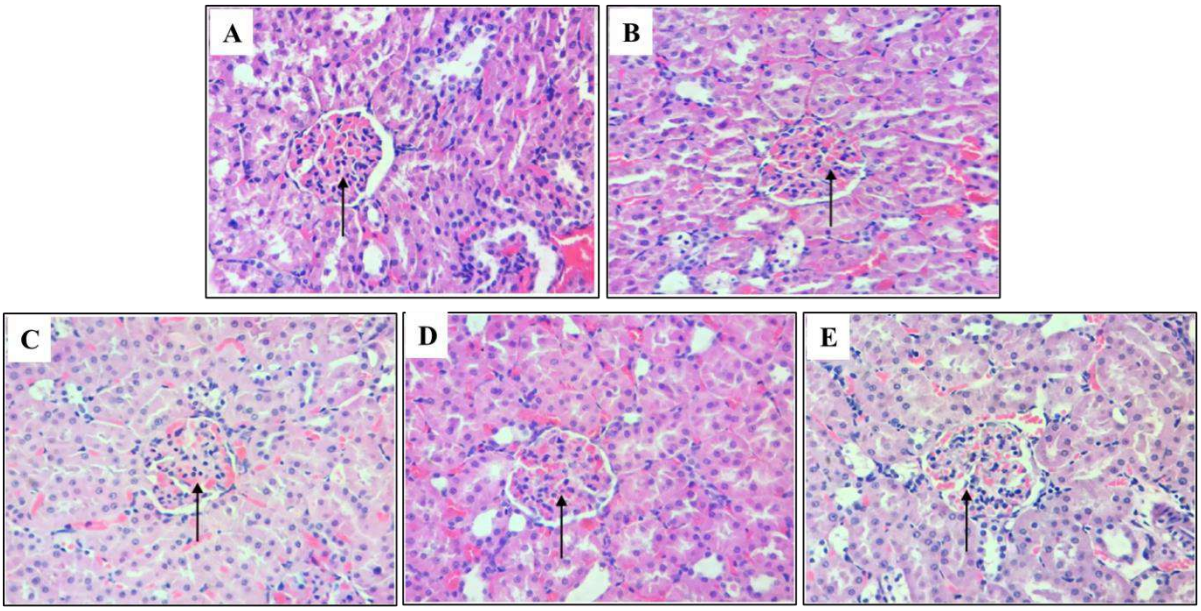


Fig-39A-E. The histopathological analysis of kidney in animal groups. Images showing A) Control B) HFD-STZ, C) Metformin, D) VA LD E) VA HD

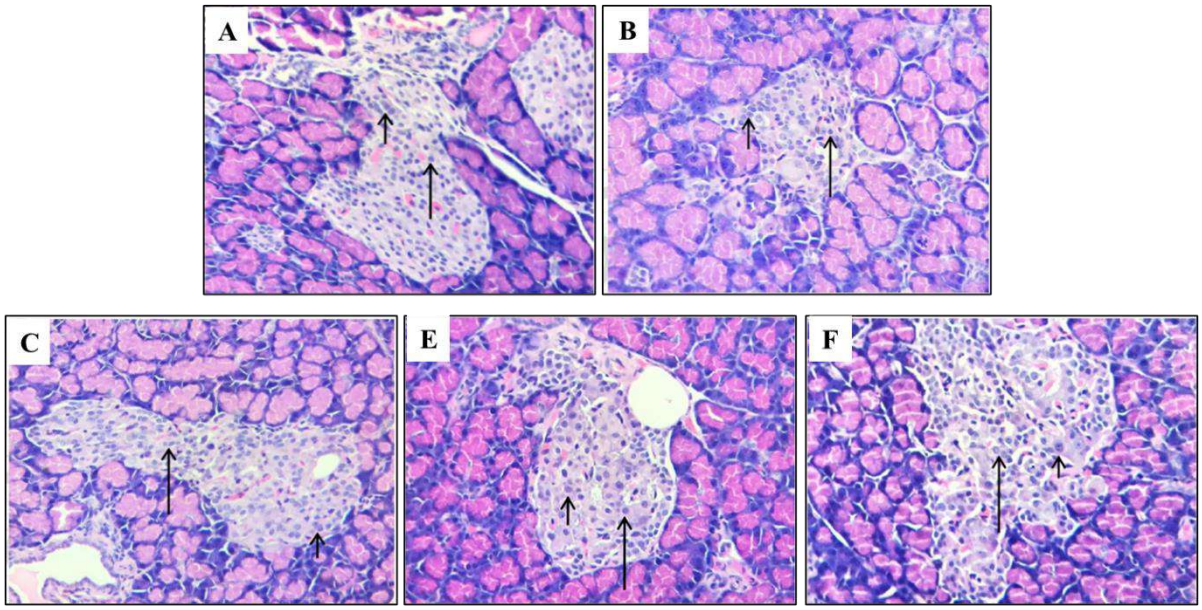


Fig- 40. A-E. The histopathological analysis of pancreas in animal groups. Images showing A) Control B) HFD-STZ, C) Metformin, D) VA LD E) VA HD

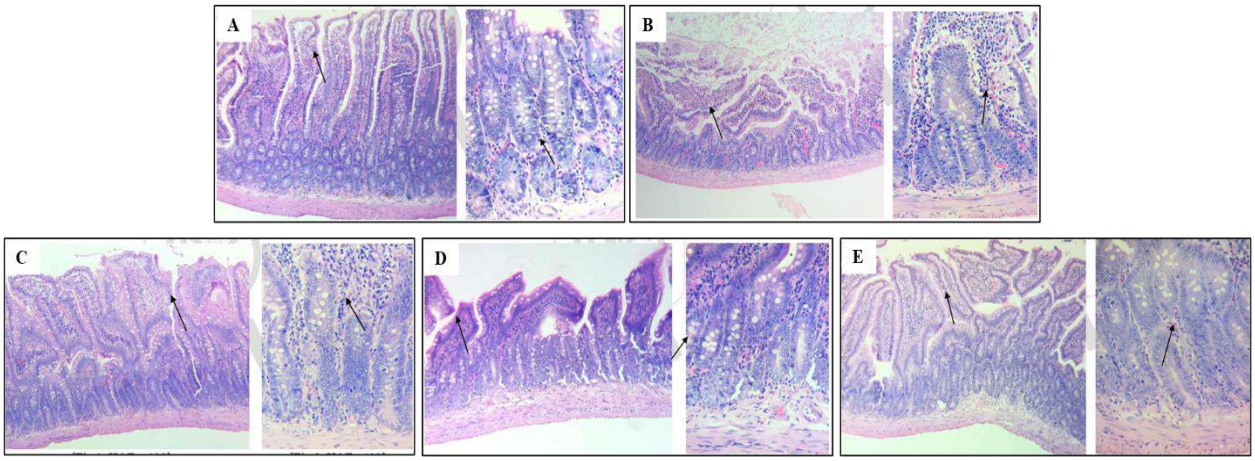


Fig-41. A-E The histopathological analysis of intestine in animal groups. Images showing A) Control B) HFD-STZ, C) Metformin, D) VA LD E) VA HD

3.3.13 Summary

VA, a classical *Ayurveda* anti-obesity formulation is demonstrated to inhibit digestive enzyme, DPP4 enzyme activity and modulate GLP1 secretion from intestinal cells. The hypoglycemic potential of the formulation is further observed in an *in vivo* rat model where VA treatment resulted in a reduction of blood glucose levels. With *in silico* methods like phytochemical data mining, virtual screening and molecular docking, potential DPP4 inhibitors and their multi-targeted effect is explored. This revealed potential targets, pathways and processes which can be further studied further to delineate the mode of action in detail.

3.4 *Vasanthakusumakara Rasa* (VK) demonstrated anti-diabetic and anti-obesogenic effects through inhibition of α-amylase and down-regulation of key adipocyte differentiation genes.

Vasanthakusumakara rasa (VK) is one of the unique herbo-mineral preparations in *Ayurveda* prescribed for restoration of metabolic homeostasis in diabetes and obesity. The constituents in VK are listed in Table-20 According to *Ayurveda*, VK is said to have *Rasayana* properties (preventive, curative and rejuvenative action) which helps in balancing the metabolic functions of all the tissues (Dr. G. Prabhakara Rao, 2014) . Despite its wide clinical use, not many studies exist to understand the pharmacological actions of VK and delineate its mechanism of action. As per *Ayurveda* texts it is known to exhibit many properties like *Pramehagna, Rasayana,* and rejuvenative (Subodh, 2018).

Vasantakusumakara rasa	**Sanskrit name**	**Parts used**	**Quantity**
Calcined Argentinum	*Rajata Bhasma*	NA	2 parts
Calcined Ferrum	*Kanta lauha bhasma*	NA	3 parts
Calcined Stannum	*Vanga Bhasma*	NA	3 parts
Calcined Plumbum	*Naga Bhasma*	NA	3 parts
Calcined Mica	*Abhraka Bhasma*	NA	4 parts
Calcined Coral CaCO3	*Pravala Bhasma*	NA	4 parts
Calcined Pearl CaCO3	*Mukta Bhasma*	NA	4 parts
Musk	*Kasturi*	Musk deer	
Milk	*Godugdha*	Cow	
Saccharum officinarum L.	*Ikshu*	Stem & roots	
Justicia adhatoda L.	*Vasa rasa*	leaves	
Laccifer Lacca	*Laksha rasa*	oleo resin	
Valeriana wallichii DC	*Sugansh bala*	root and stem	
Musa paradisiaca L.	*Kadali kanda rasa*	inner part of stem and tuberous part	
Nelumbo nucifera Gaertn.	*Kamala*	flower	

Jasminum officinale L.	*Malati*	flower	
Curcuma longa L.	*Turmeric*	rhizome	

Table-20. The plant and mineral ingredients in the formulation VK

Notably two recent studies reported the protective effect of VK on alleviating diabetic retinopathy in rat models as well as reducing neuropathic complications in diabetic patients (Tamoli et al., 2020). In addition, the mineral and herbal ingredients of VK like *Nagabhasma,* and other plant constituents such as *Laccifer lacca, Valeriana wallichii*, *Musa paradisiaca*, *Nelumbo nucifera, Jasminum officinale*, and *Curcuma longa* are well documented for their anti-diabetic, anti-hyperglycemic and immunomodulatory effects in both *in vivo* and *in vitro* model systems (Ji *et al*., 2019;sDeshmukh et al., 2013;Gulfraz et al., 2011; Dubey et al., 2018; Poorassar *et al*., 2020; Shodehinde et al., 2015; Ono et al., 2006; Lekshmi et al., 2014).

Although studies on individual plants and mineral ingredients are supporting the beneficial effects of VK, they need not necessarily represent the biological effects of VK as a whole formulation. Also, *Ayurveda* formulations when administered orally, undergo complex post-digestive modifications that potentially influence their overall pharmacodynamics and kinetics. Therefore, it would be more appropriate to study them in their whole form rather than looking at individual component plants or selected bio-actives present in them. The present study used the whole formulation which was subjected to simulated *in vitro* digestion following the protocol in Methods section 2.3.2.1, to mimic the transformations happening to the formulation during physiological digestion, using standardized protocol (Minekus et al., 2014b). The digest (referred as VK) was estimated for total tannins and the digest was quantitatively used as "µg of GAE/mL".

3.4.1 VK inhibits α-amylase, but not α- glucosidase activity

VK being an oral formulation, its digestive enzyme inhibition potential was evaluated *in vitro* using α-amylase and α-glucosidase inhibition assays. The digested form of formulation showed a concentration dependent inhibition of α-amylase ranging from 8% with the lowest concentration (0.625µg of GAE/mL) to 80% with the highest concentration (10µg GAE/mL) used in the study (Fig-42). However, in the present study, the VK showed only α-amylase inhibition and not α-glucosidase in the concentration range

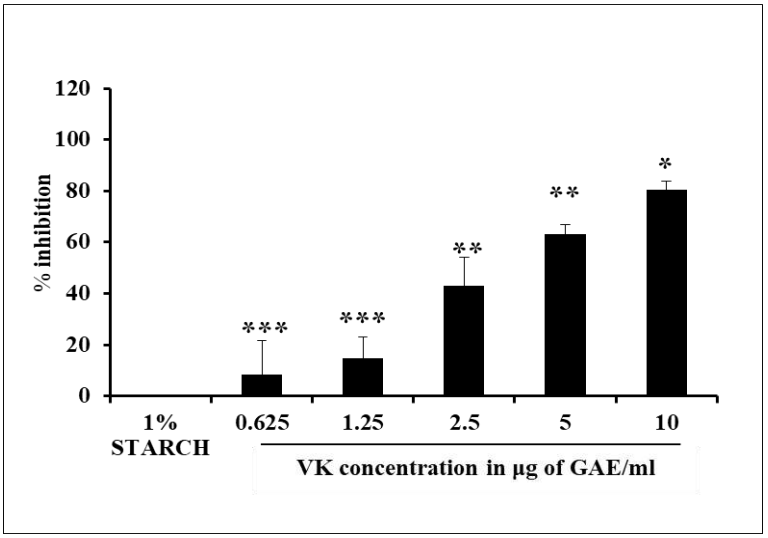


Fig-42. The effect of VK on α-amylase activity. The graph showed a dose dependent inhibition of α-amylase by various concentrations of VK

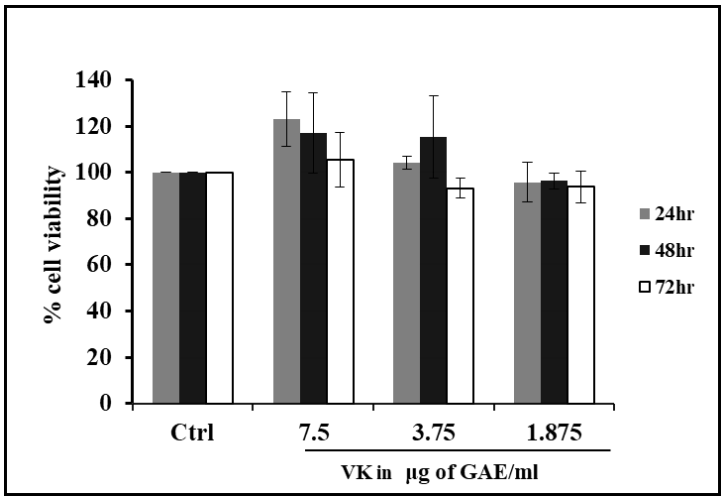
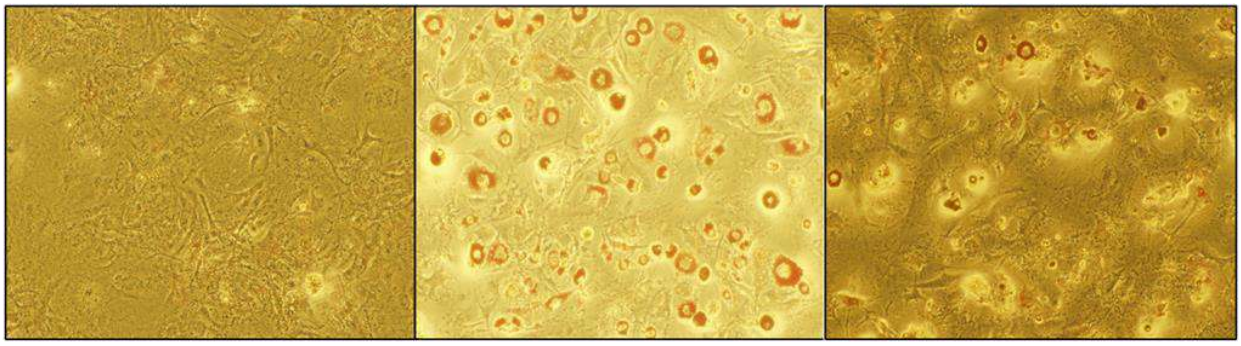


Fig-43A. MTT assay of 3T3L1 adipocytes. Cells were treated with varying concentrations of VK and cell viability was measured using MTT.

3.4.2 VK inhibited MDI mediated adipogenesis and reduced triglyceride accumulation in 3T3-L1 cells

The anti-adipogenic action of VK was evaluated in 3T3L1 adipocytes, following the standard adipogenesis protocol mentioned in section 2.2.2. The cytotoxicity of the digest was assessed first using MTT assay to find the non-toxic concentration (Fig-43A). Cells treated with different concentrations of VK (7.5, 3.75, 1.875 µg GAE/mL) were evaluated for viability for three time points (24, 48 and 72 hrs). All subsequent experiments were carried out at the highest non-toxic concentration 7.5 µg of GAE/mL.



MDI	-	+	+
VK	-	-	+

Fig-43B. Anti-adipogenic action of VK by inhibition of oil droplet formation. Microscopic images of Basal, MDI and VK treated adipocytes shows a reduction in oil droplet formation in VK treated adipocytes as indicated by (+ and – signs)

Intracellular accumulation of lipids is an indication of adipogenesis and this can be assessed by Oil-Red O staining and triglyceride assays. VK treated adipocytes showed a reduction of oil droplet formation in 3T3-L1 cells (Fig-43B). The VK treated adipocytes showed a 20% reduction in oil droplet formation as quantified by Oil-Red O assay (Fig-44A)

In order to confirm the qualitative and quantitative results obtained from Oil Red O staining of lipid droplets, triglyceride assay was carried out parallelly. Similar to lipid droplet reduction, VK reduced triglyceride formation in 3T3-L1 cells supporting the potential anti-adipogenic effect of VK. The VK treated adipocytes showed ≈40% ($p < 0.05$) reduction in triglyceride accumulation and it corroborates with the reduction in oil droplet formation (Fig-44B). Together, these data support the anti-obesity effect of VK

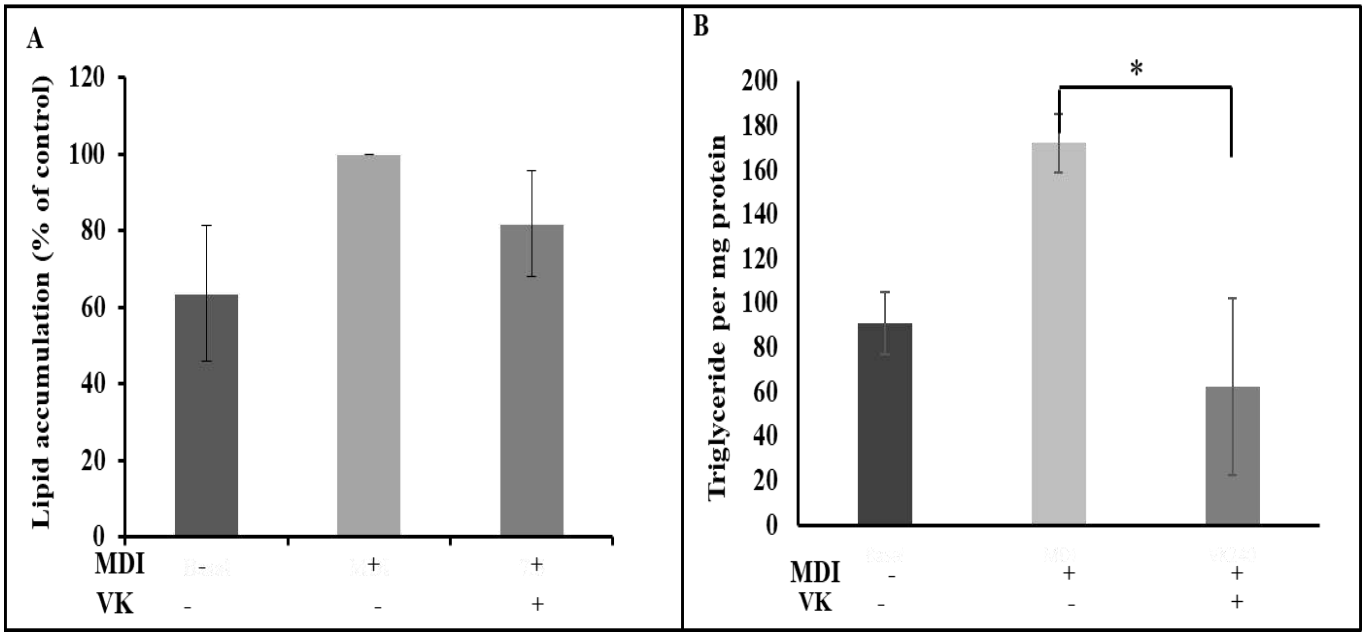


Fig-44 A-B. The anti-adipogenic action of VK in differentiated 3T3L1 adipocytes. Graph showing A) quantification of lipids in adipocytes. B) Quantification of Triglycerides in adipocytes. (p value $\leq$ * 0.05)

in adipocytes. (p value $\leq$ * 0.05)

3.4.3 VK suppressed adipogenesis by downregulation of adipogenic transcriptional factors.

To understand the molecular markers involved in anti-adipogenic action of VK, expression levels of key transcriptional regulators and adipocyte specific genes in VK treated 3T3-L1 cells

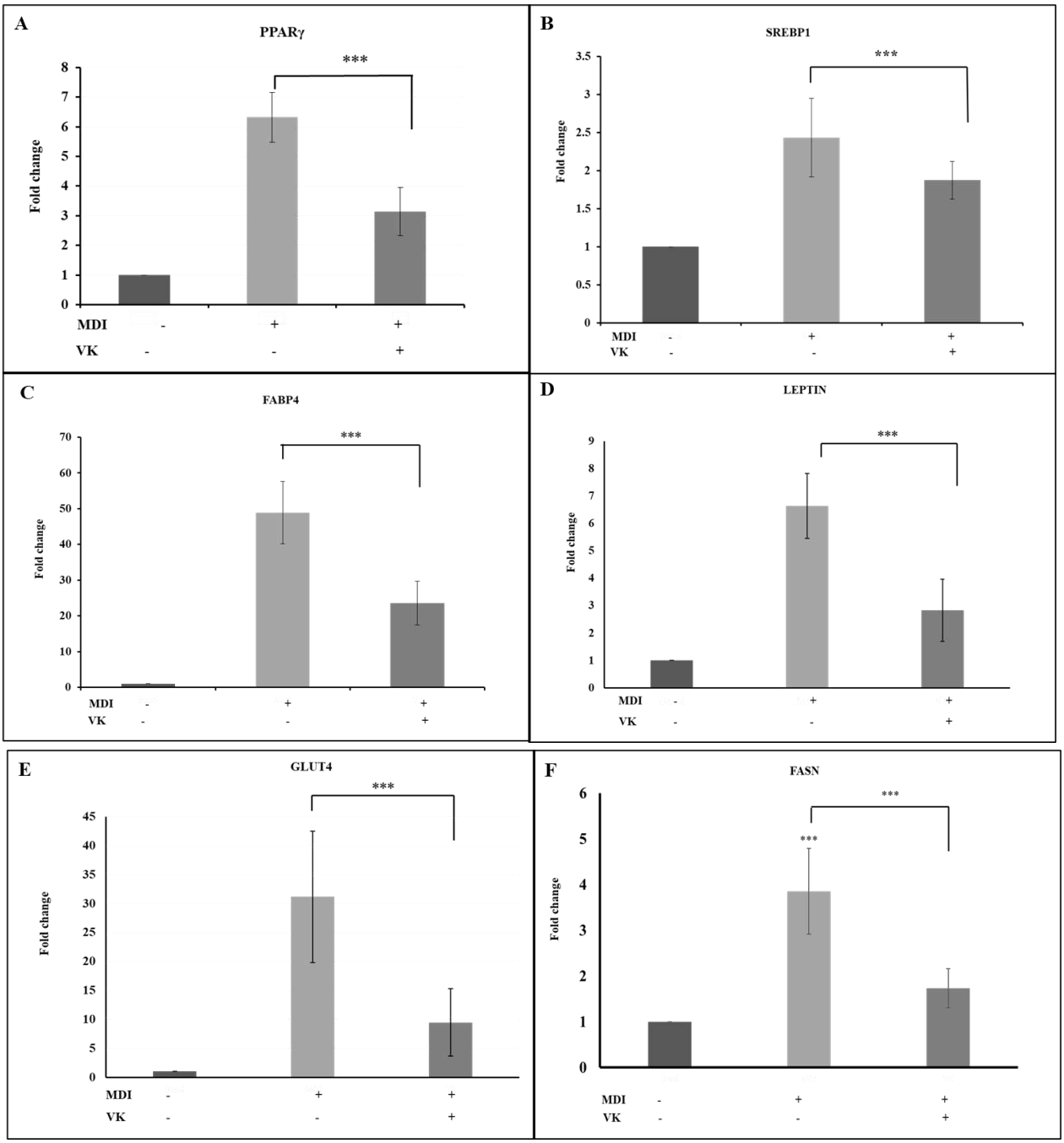


Fig 45A-F. qPCR analysis of VK treated adipocytes. Graph showing 3T3L1 adipocytes treated with VK showed reduced expression of A) PPARγ B) SREBP c) FABP4 D) Leptin, E) GLUT4, F) FASN (p value < *** 0.001).

were analyzed using qRT-PCR. Results demonstrated a significant reduction in expression of early markers of adipogenesis like PPARγ by 3-fold (p <0.001) and SREBP1c by 0.6-fold (p

0.001) by VK treatment (Fig-45A-B). It was also observed that with VK treatment, there is a strong reduction in the expression of FABP4, an adipogenic marker, affirming that downstream targets of PPARγ are also getting inhibited (Fig-45C) (Berndt et al., 2007; Furuhashi et al., 2014). In addition, VK also downregulated the expression of Leptin ($p < 0.001$), an important adipokine secreted by adipocytes (Fig-45D) (Palhinha et al., 2019a). Expression of FASN, a central enzyme in lipogenesis is inhibited by 2.1 folds and GLUT4, a key insulin regulated glucose transporter, is also strongly reduced with VK treatment (Fig-43E-F) (Favaretto *et al*., 2014). Thus, these observations suggested that VK suppressed the adipogenic program through downregulation of key adipocyte regulators and downstream genes.

3.4.4 Summary

The results demonstrated *in vitro* experimental evidence for the anti-diabetic and anti-obesity effects of VK, an Ayurvedic herbo-mineral formulation. VK exerted its anti-diabetic and anti-obesity effects through digestive enzyme inhibition and suppression of key adipogenic markers. Further studies are warranted to understand more about the other possible modes of action of this formulation, which can give insights into the action of VK.

3.5 *Chandraprabhavati* (CV) inhibited α-amylase and differentiation of 3T3L1 adipocytes

Chandraprabha vati (CV) is a classical *Ayurveda* formulation prescribed for a wide number indications including urinary disorders. It is used in Ayurvedic system of medicine for various indications such as, *Pandu* (Anaemia), *Netraroga* (Eye disorder), *Aruchi* (Tastelessness), *Mandagni* (Impaired digestive fire), *Daurbalya* (Weakness) and *Prameha* (Wanjari et al., 2016). *Chandraprabha vati* is prescribed in mitigation of all 20 types of *Prameha* described by *Ayurveda* and can be corelated with obesity, metabolic syndrome and diabetes mellitus. It contains 37 herbomineral ingredients (Table 21). Most of these ingredients exhibited both glucose and lipid lowering activities in experimental studies. The ingredients like *Acorus calamus ,Cyperus rotundus Phyllanthus niruri , Tinospora cordiofolia Curcuma longa, Berberis aristata, Piper longum, Coriandrum sativum,Terminalia chebula Terminalia belerica, Embelica officinalis , Embelia ribes , Zingiber officinale, Piper nigrum, Hordeum vulgare, Ipomoea turpethum, Cinnamomum zeylanicum,* and *Asphaltum punjabianum* and *Commiphora wightii* showed remarkable antidiabetic and hypolipidemic effects in several studies (Gray and Flatt, 1999; Liu et al., 2015; Nabi et al., 2013; Nugroho et al., 2012; Raut and Gaikwad, 2006; Singh et al., 2013; Sotoudeh et al., 2019;Madhvi et al., 2014).

Chandraprabhavati	**Sanskrit name**	**Part used**	**Quantity**
Cinnamomum camphora (L.) J. Presl	*Chandraprabha*	exudate	8.18 mg
Acorus calamus L.	*Vacha*	rhizome	8.18 mg
Cyperus rotundus L.	*Musta*	rhizome	8.18 mg
Andrographis paniculata (Burm. fil.) Nees	*Bhunimba*	entire plant	8.18 mg
Tinospora cordifolia (Willd.) Miers	*Guduchi*	stem	8.18 mg
*Cedrus deodara (*Lamb.) G. Don	*Daruka*	heartwood	8.18 mg
Curcuma longa L.	*Haridra*	rhizome	8.18 mg
Aconitum heterophyllum	*Ativisha*	tuberous root	8.18 mg
Piper longum L.	*Pippalimula*	root	8.18 mg
Plumbago zeylanica L.	*Chitraka*	root	8.18 mg
Berberis aristata. DC.	*Daruharidra*	stem and root	8.18 mg
Coriandrum sativum L.	*Dhanyaka*	fruit	8.18 mg

Terminalia chebula Retz.	*Haritaki*	fruit	8.18 mg
Terminalia bellirica (Gaertn.) Roxb.	*Bibhitaka*	fruit	8.18 mg
Piper nigrum L	*Maricha*	fruit	8.18 mg
Piper longum L	*Pippali*	Fruit	8.18 mg
Hordeum vulgare L	*Yava*	Whole plant	8.18 mg
Operculina turpethum (L.) S. Manso	*Trivrit*	root	8.18 mg
Baliospermum montanum (Willd.) Müll.Arg.	*Danti*	seed	32.72 mg
Cinnamomum verum J. S. Presl	*Patrak*	Leaves and bark	32.72 mg
Elettaria cardamomum (L.) Maton	*Sukamaila*		32.72 mg
Commiphora wighti (Hook.) Engl.	*Guggulu*	Resin	261. 76 mg
Bambusa bamboo (L.) Voss	*Vamshalochana*	exudate	32.72 mg
Calcined iron			65.44 mg
Calcined Copper pyrite	*Makshika dhatu bhasma*	NA	8.18 mg
Carbonate of Soda	*Sarji Kshara*	NA	8.18 mg
Asphalt	*Shilajit*	NA	261.76 mg
Souchal sal	*Sauvarchala lavana*	NA	8.18 mg
Black salt	*Vida lavana*	NA	8.18 mg
Rock salt	*Saindhava lavana*	NA	8.18 mg

Table-21- The plants and mineral ingredients in *Chandraprabha vati*

3.5.1 CV inhibited α-amylase dose dependently and did not significantly inhibit α-glucosidase

It was observed that CV inhibited α-amylase enzyme dose dependently, with maximal inhibition of 76% at 10 µg of GAE/ml and lowest inhibition of 21% at 0.625 µg of GAE/ml as seen in Fig-46. But CV did not show any inhibition on α- glucosidase enzyme activity in this

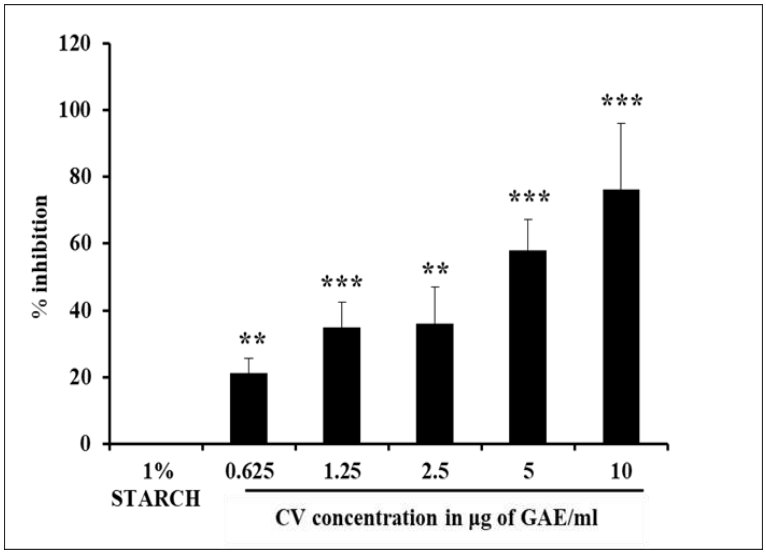


Fig-46. Effect of CV on α-amylase inhibition. The graph showed a dose dependent inhibition of α-amylase by various concentrations of CV

3.5.2 CV inhibited adipogenesis in 3T3L1 adipocytes

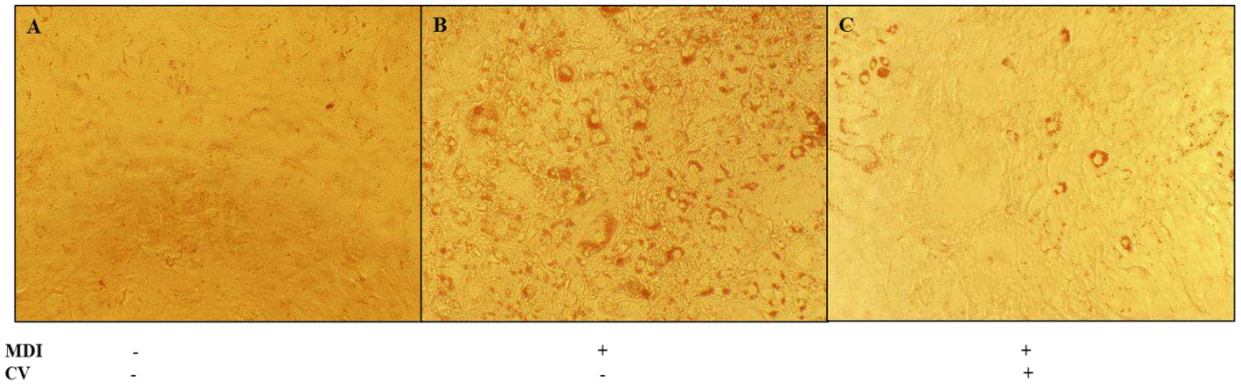


Fig-47. Anti-adipogenic action of CV on 3T3L1 adipocytes. Microscopic images showing reduced oil droplets in CV treated adipocyte when compared to MDI. A) Basal, B) MDI C) CV treated adipocytes.

CV was also assayed for anti-adipogenic action and the results from the experiment showed that CV at a concentration of 2.5 µg GAE/ml inhibited the oil droplet formations in mature adipocytes when compared to untreated MDI cells (Fig-47). This demonstrated the anti-adipogenic mode of action of CV and this was similar to VK

3.5.3 CV inhibited adipocyte differentiation markers in 3T3L1 adipocytes

To further delve into the mechanism of anti-adipogenic action of CV, the adipogenic gene expression was checked in CV treated adipocytes with qPCR Fig-48 (A-D). PPARγ is a major adipogenic transcription factor which regulates activation of factors necessary for lipid accumulation and fat metabolism. SREBP1 is another transcription factor that stimulates the

transcription of lipogenesis genes. FABP4 is an adipocyte differentiation marker and leptin is an adipokine secreted from adipocytes and regulates fat metabolism. CV downregulated both PPARγ and SREBP1 by 2 folds and 0.5 folds respectively (Fig-48A-B). The expression of FABP4 and Leptin were also observed to be significantly reduced, more than 80- and 2-folds reduction respectively for FABP4 and Leptin in treated adipocytes, when compared to the untreated fully mature adipocytes (Fig-47C-D). This proved that the adipogenic program is downregulated by CV and one of the major mechanisms is the transcriptional inhibition of key transcription factors like PPARγ and SREBP1 which initiate the adipogenesis.

CV was evaluated for DPP4 enzyme inhibition and GLP1 secretion but did not show any effect on the same.

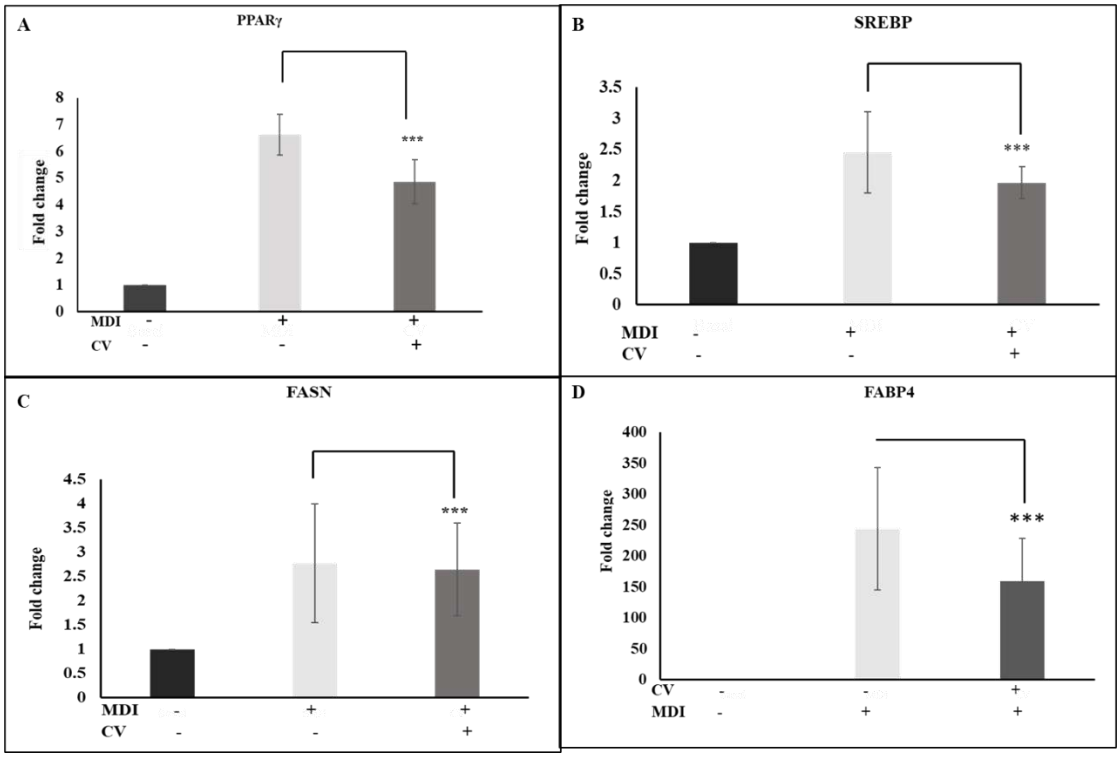


Fig-48. qPCR analysis of CV treated adipocytes. Graph showing 3T3L1 adipocytes treated with CV showed reduced expression A) PPARγ B) SREBP1, C) FABP4 and D) LEPTIN in 3T3L1 cell model.

3.6 An AI-ML analysis for Clinical Trials of Indian Systems of Medicine-A retrospective clinical study

Clinical studies and their data analysis are important for evaluating the efficacy and safety of therapeutic interventions. *Ayurveda*'s personalized framework makes clinical data analysis challenging due to the multi formulation-based treatment regimen. Both the efficacy and safety of these interventions are often influenced by several factors, so a simple control versus test group comparison using descriptive and inferential statistical analysis tools may not be sufficient to address the heterogeneity of the data. The need of the hour is a methodology that can encompass and analyze multiple variables by appropriately clustering them to subgroups that justify the personalization principles which influence the clinical efficacy. The proposed study emphasizes the demonstration of a model using a dataset of multivariable clinical management data for diabetes (known as "*Prameha*" or "*Madhumeha*" in *Ayurveda*) treated with Ayurvedic formulations. The study attempted to find novel approaches to analyze the multivariable Ayurveda clinical data by incorporating multiple features, as shown in Fig-49.

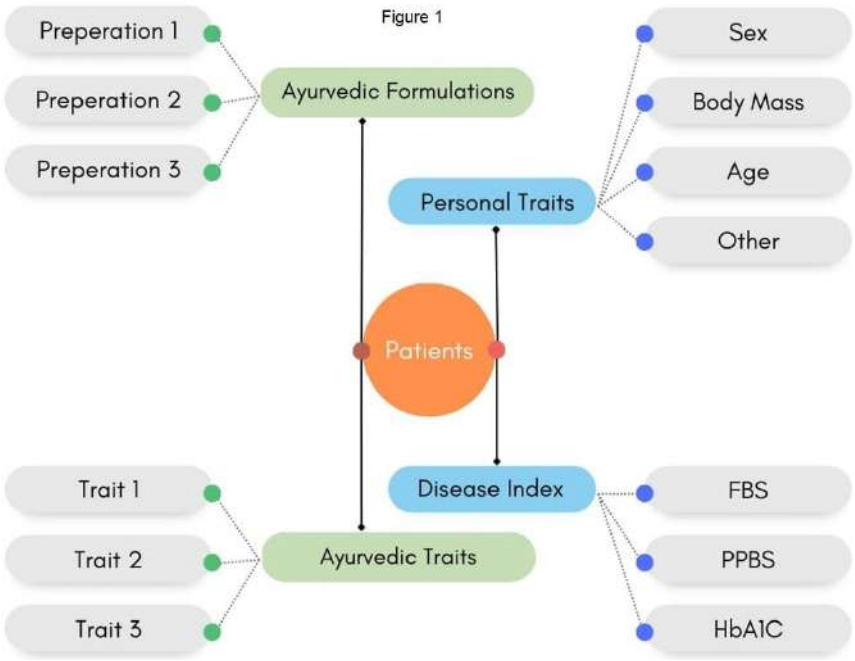


Fig-49. Illustration of the concept of analyzing the multivariable Ayurveda clinical data by incorporating multiple features.

The aim of the study is to see the possibility of using higher dimensional statistical analysis to cluster the patients into comparable subgroups with matching features that contribute or influence the diagnostic and clinical outcome parameters, safety and efficacy of interventions as well as the personalization concepts. While the patients received a variety of drug interventions during the course of treatment, the present study focused on analyzing the efficacy of five classical *Ayurveda* formulations viz. *Nishamalaki* (NA), *Nisakathakadi* (NK), *Chandraprabha Vati* (CV), *Vasantha kusumakara Rasa* (VK), *Varanadi Kashaya* (VK), on improving the diabetic indices viz. HbA1C, Fasting Blood Sugar (FBS) and Postprandial Blood Sugar (PPBS). The study utilized Self Organizing Maps to explore the natural grouping of clinical features and project multidimensional data onto a two-dimensional display in a non-linear manner. The data showed multiple features influencing the decision-making process on efficacy and safety of clinical interventions. Therefore, a binary classification of patients and regression statistical methods would be insufficient to map the patient groups to the drug combination giving a successful result. In this background, the aim of the study is to see the possibility of using higher dimensional statistical analysis to cluster the patients into comparable subgroups with matching features that contribute or influence the diagnostic and clinical outcome parameters, safety and efficacy of interventions as well as the personalization concepts.

Manually curating and analyzing these humongous data for knowledge generation is challenging and therefore automated analysis and visualization of massive multidimensional datasets have been the focus of research to recognize regularities and relationships in the data, thereby gaining access to hidden and potentially useful knowledge (Liu et al., 2017.; Tatu et al., 2009; Thanos et al., 2023). Artificial Neural Networks (ANNs), a class of algorithms that are inspired from and aimed to simulate the neural structures of the brain, are a promising tool in this direction. They can be applied in complex problems that involve a higher number of parameters and are robust classifiers and superior pattern recognizers as well (Sutariya et al., 2013). A fairly well-known neural network and one of the most popular unsupervised learning algorithms introduced by Finnish Professor Teuvo Kohonen in the early 1980's is the Self-Organizing Maps (SOM) (Kohonen, 2013; The Self-organizing Map, 1990) SOMs comprehensively visualize natural groupings and relationships in the data and have been successfully applied in a broad spectrum of research areas ranging from speech recognition to financial analysis (Goddard et al., 2019). The non-linear projection of multidimensional data onto a two-dimensional display by SOM is topology-preserving, which means the more alike

two data samples in the input space, the closer they will appear together in the final map. This allows the user to identify ‘clusters’, i.e. groupings of a certain type of input pattern (Penn, 2005). Further examination may then reveal what features the members of a cluster have in common (Silva and Marques, 2015; Tatu et al., 2009). Despite the popular use of the algorithm for clustering and information visualization, a system that combines the fast execution of the algorithm with powerful visualization of the maps and effective tools for their interactive analysis is absent. The current study therefore used SOM to explore the natural grouping of clinical features and project multidimensional data onto a two-dimensional display in a non-linear manner. While the regression statistical models of RCTs map the biological effects of molecular drug interventions directly to its specific biochemical readouts, this model based on the concepts of AI and ML caters to a patient centered approach. It is inclusive of an individual's physiological constitution, lifestyle, dietary patterns, daily regimens, comorbidities as well as multiple pharmacological interventions to map with the clinical outcome. The Self Organizing Map (SOM) is a well-known neural network which can visualize the natural relationships in a multi-dimensional data and create clusters having common features. Despite the popular use of the algorithm for clustering and information visualization, a system that combines the fast execution of the algorithm with powerful visualization of the maps and effective tools for their interactive analysis is absent. This study analyzes several algorithms that are highly impactful in the analysis of self-organizing maps. Self-organizing maps applied in different areas of studies were validated with the SOM module available in XLSTAT version 21.4.63677 of XLSTAT 2019. Implementation and development of various algorithms related to SOM used in the study were obtained from (Wehrens and Maintainer, 2023).

The current study performed SOM based analysis of clinical data where the results are represented as the medicines given on a particular visit and the results of them in the following visit. Here the biological readouts considered for this study are FBS, PPBS and HbA1C and the medicines are *Nishamalaki* (NA), *Nisakathakadi* (NKK), *Chandraprabha Vati* (CP), *Vasanta Kusumakara Rasa* (VKR), *Varanadi Kashayam* (VK) and other medicines and are grouped respectively from 1 – 6. Fig-50 is a representative figure of the results in which (a), the code plot, reveals the correlation between various medicines and diabetes indices; (b), the count plot, provides an understanding of the population distribution for a specific result; and (c), variable influence plot, quantifies the contribution of each component, offering a comprehensive view of their influence and the color code. The results are quantified in such a

way that red color indicates high and blue color indicates low value in variables influence plot. (Jaleel et al., 2023).

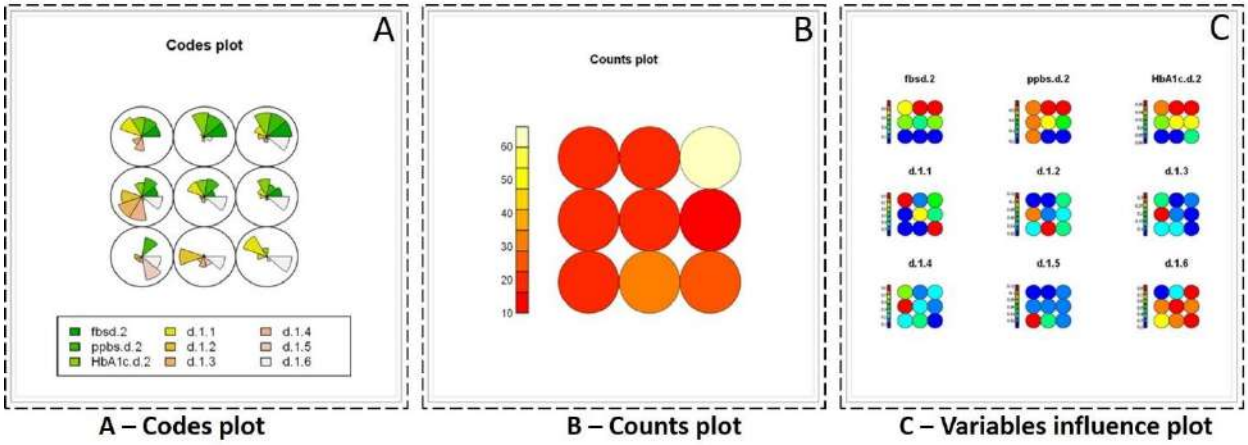


Fig-50. SOM analysis. The figure showing the three types of plots given by SOM as results after analysis of multi-variable patient data.

The results from the current study showed that *Nishamalaki, Nishakathakadi and Vasanthakusumakara Rasa* were found to be highly active in the highly populated area of the clinical study. Studies from the lab also showed supporting results for these formulations. Apart from the *Ayurveda* formulations, the analysis also revealed a number of proprietary Ayurvedic medicines and it is required to perform a detailed study on all these drugs.

The most important outcome of this study is that a protocol was set for sorting and analyzing the multi-component, multi-variable based clinical trials used in CAM like *Ayurveda*. The study used retrospective clinical data of *Ayurveda* management of diabetes and the protocol further needs to be validated using prospective observational studies. Since the SOM system can accommodate multi-level data including placebo settings, the present study provides a better data analysis method for *Ayurveda*-like diseases management systems. The management strategies of complex non-communicable diseases in CAMs are yet to be unraveled. The methods involving AI and ML may show a new light on the same, which may even help to deepen the overall understanding about the pathology and help to design much more efficient (integrative) management strategies as well (Jaleel et al., 2023).

However, the major limitation of this study was the nature of the data that was collected. Since the clinical data was collected retrospectively, the format used for documenting the data was unstructured and the population size was relatively small. Another limitation of the data

collection process may stem from the employment of mean imputation, which harbors the potential to introduce bias. This concern is particularly pronounced in datasets characterized by non-random missingness or in instances where the data distribution exhibits skewness. This can be overcome by increasing the number of patients and collecting their data in a structured format. Such a prospective observational data analysis will further corroborate this study and establish new methods for clinical data analysis in multivariable diseases management systems like Ayurveda and other CAMs.

3.7 Discussion

The exponential rise in prevalence for non-communicable diseases like T2DM and obesity have resulted in substantial health and socio-economic burden globally. The recent pan-Indian study reported the high prevalence of T2DM across the different states of India. This calls out for an urgent need for a well-defined long-term strategy for disease management. Of the recent pharmacological interventions, gut-based incretin therapies have emerged with several promising outcomes. There are several ongoing clinical trials which attempt to develop gut-peptides with multiple targets resulting in diversifying the beneficial effects. The pleotropic nature of the gut, from being a source of various gut peptides and the gut microbiome, can be leveraged in varied ways thus making GI tract, a key area for metabolic research (Brandt et al., 2018).

Ayurveda, one of the Indian traditional medical sciences, has laid emphasis on the role of gut in disease management. Despite the epistemological and ontological differences, an integrative disease management protocol combining the principles and practices of both modern medicine and *Ayurveda*, can significantly help in overcoming the challenges in managing lifestyle diseases like T2DM and obesity.

Ayurveda formulations are a rich in various classes of phytochemicals like polyphenols, tannins, and flavonoids, and can interact and modulate many receptors and pathways in the body. Several studies referred in the introduction and result sections in this thesis substantiate this statement. The formulations' multi-pronged effect can be an advantage in mitigating the complex pathophysiology and associated complications of diabetes. However, scientific studies demonstrating their molecular targets and mechanism of action are still lacking, and that is limiting our understanding into the pharmacological actions. Therefore, exploring how *Ayurveda* formulations exert their biological effects, using various approaches like *in vitro* and *in vivo* models as well as *in silico* approaches, can illuminate our understanding about *Ayurveda* concepts of disease pathophysiology as well as helps in discovering novel molecules and targets for disease managements.

The present study aimed at investigating the various modes of action exerted by four clinically prescribed classical anti-diabetic formulations NK, VA, VK and CV. The four formulations contain a number of medicinal herbs and VK and CV are particular because they have metals and minerals as part of their composition.

To explore the multi-targeted functionality of these formulations, they were assessed for various effects on several therapeutically relevant targets and pathways related glucose and lipid metabolism. Various biological assays including digestive enzyme (α-amylase and α-glucosidase) inhibition, anti adipogenic assay, DPP4 inhibition, GLP1 secretion and up- and down-regulation of key marker genes were studied using in-vitro model systems. Computational biology approaches like virtual screening, molecular docking, molecular simulation and network pharmacology studies were carried out for better understanding of the systemic action of these herbal formulations. Based on the observations from *in vitro* and *in silico* studies, two formulations, NK and VA, were further studied using in-vivo models of diabetic rats.

One of the promising strategies for reducing post prandial glucose levels is through inhibition of the carbohydrate-hydrolyzing enzymes, α-amylase and α-glucosidase, that are present in the brush border of small intestine. They are playing a partial role in gluco-regulatory function of the gut. Therefore, targeting digestive enzymes to curb postprandial hyperglycemia has been one of the successful strategies for diabetes management (Joshi et al., 2015; P et al., 2011). Digestive enzyme inhibitors like acarbose and their derivatives are widely used as blood glucose regulators by delaying postprandial glucose rise and this strategy is found to be effective in high carbohydrate consuming populations like Indians (Rosak and Mertes, 2012,; Choudhury et al., 2016). Retarding the amount of carbohydrate absorption through the inhibition of these enzymes can prevent the steep rise in postprandial blood glucose levels. It is well reported that many of the anti-diabetic herbs exhibit digestive enzyme inhibition and therefore the four formulations were evaluated for the digestive enzyme inhibition.

NK as mentioned in section 3.2, consists of 8 herbs and various *in vitro*, *in vivo* and clinical research evidence support the hypoglycemic potential of herbal ingredients (Marton *et al*., 2021; D'Souza *et al*., 2014; Medagama, 2015; Acharya *et al*., 2016; Butala *et al*., 2017; Karan *et al*., 2013; Biswas *et al*., 2012; Riya *et al*., 2015). VA like NK, is also a polyherbal formulation but having a broader set of indications prescribed by *Ayurveda* practitioners for correction of *Agni* imbalance and alleviating the *Doshic* dysregulation widely present in disease conditions like, obesity, chronic liver diseases including ascitis and other abdominal bloating conditions and chronic arthritis (K.R. Srikantha Murthy, 2022; Singh, R.H., 2013). The classical texts *Ashtanga Hridaya* and *Susrutha samhitha* describes a category of medicinal herbs used for purifying and detoxifying the vitiated *Doshas* and *Dhathus* known as *Varanadi gana.* VA consists of 16 herbs, many of which are extensively studied for their biological

activities. Some of the well-known herbs like *Aerva lanata, Asparagus racemosus, Aegle marmelos, Moringa oleifera, and Terminalia chebula* among others, are extensively studied for their anti-diabetic and anti-oxidant activities (Murali et al., 2007; Rao and Nammi, 2006). VK, a herbo-mineral preparation is known to alleviate *Prameha* symptoms and contain several constituents which are known to exert anti-lipidemic and anti-diabetic effects (Tamoli et al., 2020; Dr. G. Prabhakara Rao, 2014). CV is another herbo mineral formulation which has a wide spectrum of disease indications including *Prameha* and associated complications. Given their extensive usage for disease management in the clinical practice of *Ayurveda* and a dearth of scientific evidence for their mode of actions, the four formulations are attractive candidates for Ayurveda Biology research

The results from the evaluation of the all four formulations for the preliminary digestive enzyme inhibition demonstrated a differential dose dependent modulation of the enzymatic activity. NK and VA inhibited α-glucosidase dose dependently while VK and CV did not exhibit significant inhibition for this enzyme. On the contrary, VK and CV inhibited α-amylase dose dependently whereas NK and VA did not significantly inhibit α-amylase. These differential effects of formulations could be due to the presence of different herbs and constituents or could be because of the chemical modifications these formulations have undergone during preparation. Further studies on the phytochemistry of these preparations may reveal the detailed mechanism involved in the enzymatic inhibition.

The dose dependent inhibition of α-glucosidase by NK demonstrated that one of the modes of actions of the formulation is by digestive enzyme inhibition. Some of the herbs present in the NK formulation are reported to inhibit digestive enzymes viz. *Curcuma long*a and *Emblica officinalis Salacia reticulata* and *Symplocos racemosa*. Medicinal herbs in VA such as *Crateva magna, Premna Corymbosa, Terminalia chebula, Moringa oleifera* also have been reported to show α-glucosidase and α-amylase inhibiting properties (Faheem et al., 2021; Bag et al., 2013b; D'Souza et al., 2014. Similarly, *Nelumbo nucifera Gaertn, Jasminum officinale* and *Curcuma longa* which are the constituent herbs in VK have showed anti-diabetic, anti-hyperglycemic effects through inhibition of digestive enzymes (Ono et al., 2006; Dubey et al., 2018; Lekshmi et al., 2014). CV also shares some well-known herbs with the other three formulations such as *Curcuma longa, Embilica officinalis,* and *Terminalia chebula* and is widely indicated for improving digestion (Wanjari et al., 2016b) and the phytochemicals present in these may be involved in the digestive enzyme inhibition.

As mentioned in section 1.2.1 and 1.4.8, one of the concurrent modern medicinal findings on the relevance of gut in glucose homeostasis can be corelated with *Agni* concept of *Ayurveda* and its correction which is central to balancing the disturbed metabolism of obesity and diabetes. One of the recent widely accepted and most promising therapies are gut peptide based, which enhances enteric secretion of GLP1, an incretin hormone, or by inhibiting DPP4 enzyme to prolong the physiological levels of GLP1. The actions of GLP1 are pleotropic having a direct role in many mechanisms regulating glucose metabolism and energy homeostasis. The role of gut in glucose homeostasis or GIGD is a phenomenon which largely is governed by incretin effect and can be considered as a focal point for gut centric perspective of *Ayurveda* and modern medicine.(Gimeno et al., 2020)

The most of the effects seen upon DPP4-inhibitor treatment are ascribed to an increase in GLP-1 levels. Dipeptidyl-peptidase 4 inhibitors exert glucose regulatory actions by prolonging the effects of GLP-1 and GIP, ultimately increasing glucose-mediated insulin secretion and suppressing glucagon secretion. The importance of DPP4 for the scientific and medical community raised substantially since the approval of DPP4 inhibitors for the treatment of type 2 diabetes mellitus. Beside the glucose-lowering properties of DPP4 inhibitors, emerging evidence suggests that incretin-based therapies may also have a positive impact on inflammation, cardiovascular and hepatic health, sleep, and the central nervous system.

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 economical, 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. The diverse phyto-actives present can be novel inhibitors of DPP4 and may act as one of the modes of action of anti-diabetic formulations.

So, to test the potential of formulations to inhibit DPP4, *in vitro* DPP4 assay was carried out. Out of the four formulations tested, VA and NK showed a significant dose dependent inhibition of DPP4, while CV and VK did not demonstrate DPP4 inhibition activity. The dose dependent inhibition of DPP4 by VA and NK hinted that the formulations are incretin modulatory in nature and several phytochemicals present in the formulation may be potentially contributing

to this DPP4 inhibitory in nature. Both the formulations were also assessed for their ability to enhance GLP1 secretion from the enteroendocrine cells, which also showed significant GLP1 release. In addition to the inhibition of DPP4 enzyme inhibition *in vitro*, the formulations were assessed for their effect on intestinal DPP4 expression. VA at a higher concentration showed reduced DPP4 expression in intestinal cells when compared to the untreated control cells while NK did not have any significant effect on DPP4 expression

Overall, these results suggested a potentiation of incretin pathway through inhibition of enzyme activity, transcriptional downregulation of DPP4, as well as at the level of modulating GLP1 secretion for VA, whereas for NK, the incretin modulatory effect may come through DPP4 inhibition and enhancing the secretion machinery of GLP1, which needs to be studied further.

An *in vivo* HFD-STZ rat model was employed to further corroborate the *in vitro* findings of the formulations VA and NK. A high fat diet combined with low dose of streptozotocin (STZ) was chosen as a suitable model which recapitulates many of the typical characteristics of type 2 diabetes such as a consistent hyperglycemia and insulin resistance (Srinivasan et al., 2005b). Further since a low dose of STZ is associated with lesser animal mortality as well as this model was able to closely mimic the physiological progression of type 2 diabetes.

As seen in results section of 3.2.9, the animals on HFD diet had impaired glucose tolerance with a higher AUC, although the glucose levels of all the high fat diet fed groups returned to normal levels at the end of 120 min. But after STZ induction to induce chronic hyperglycemia, the AUC was much higher in all the HFD groups suggestive of defective insulin action due pancreatic cell destruction by the STZ, and is similar to a chronic type 2 diabetic condition.

A 30-day treatment of the diabetic groups with low and high doses of VA and NK led to a significant amelioration of the increased fasting blood glucose levels and also improved the OGTT in the animals when compared to untreated diabetic control. The low doses of both the formulations had a more pronounced effect in reducing the glucose excursion as see in AUC in Fig-20 and Fig-36.

Accumulation of triglycerides is one of the conditions found as a result of metabolic imbalance in T2DM (David et al., 2017). In VA treated groups, the higher dose had a greater impact on the plasma triglyceride levels whereas both doses of NK treatment had a good effect in triglyceride reduction which could be observed in the Tables-13 and 19 respectively. Both doses of formulations VA and NK also reduced plasma cholesterol levels, demonstrating a hypocholesterolemia effect. These results match with the *Ayurveda* indications of these

'*kashayas*' or decoctions as substances that are correcting the irregular metabolism and improve digestive functions to reduce the lipids or '*meda*', in plasma levels.

It is well established that incretin effect is severely compromised in T2DM (Holst et al., 2009). This could be observed by the decreased OGTT response in the diabetic control animals which reflected partially the affected incretin response in HFD-STZ animal groups before the start of drug administration. After the drug administration, this glucose response was seen to be improved markedly in formulation treated groups especially in lower dose of NK treated group when compared to the untreated HFD-STZ control. For the formulation VA also, the low dose seemed to have a better effect on glucose tolerance level, though not significant. The beneficial effect in the low dose has been reported for many herbal formulations and is referred to as a phenomenon called 'hormesis' where phytochemicals exhibit different effects in various doses. Juhasz et al reported a cardioprotective effective of resveratrol at the lowest dose and attribute the effect to a positive influence on the immune system (Juhasz et al., 2010). Similarly, the compounds like rutin and naringenin have shown increased longevity in *Drosophila melanogaster* model at lower doses. Many flavonoids including these two are present in both VA and NK formulations, and they may exert their positive effect on various signalling pathways in diabetes and obesity at lower doses resulting in a response in the lower treatment groups. (Jodynis-liebert and Kujawska, 2020). Though there are several reports of these differential dose responses for herbal formulations, the exact mechanisms involved needs to be deciphered.

The plasma GLP1 levels in HFD-STZ group was lowered in the diabetic control rats whereas the treatment groups, the levels showed an increased trend compared to disease group. VA low and high doses had a modest effect *in vitro* and *in vivo* enhancing the GLP1 levels. NK treated groups also had a slight increase in GLP1 levels though statistically insignificant when compared to the diabetic control.

From the *in vitro* and *in vivo* experiments, the present work could generate evidence for one of the core principles of action of formulations, ie, modulation of *Agni* through regulating metabolism. NK and VA being polyherbal *Ayurveda* formulations it is expected that they will have multiple bioactive compounds that can act on targets individually or in a synergistic manner. Given the complex composition of *Ayurveda* concoctions and their multiple mechanisms of biological effects, it would be desirable to use *in silico* approaches to get insight into their multi targeted effects. While many study the ingredient plants or fractions of whole

formulation to better their understanding on their mode of action, it would not correctly present the action of the formulation as a whole.

Computational approaches have become key component in drug discovery especially in traditional medicinal research. The advancement of computer in biology and medicine has garnered significant attention for their applications in deciphering the mode of action of complex medicinal preparations (Patwardhan and Chandran, 2015b). *In silico* technology is used to predict and study these mechanisms of poly-herbal formulations to circumvent the challenges associated with classical drug discovery (Yi *et al*., 2018). With this focus, *in silico* data mining and virtual screening of phytochemicals present in NK and VA were carried out followed by molecular docking and computational biology analyses.

In silico data mining of eight medicinal plants in NK identified 206 phytochemicals identified from eight medicinal plants and was followed by virtual screening followed by and molecular docking studies were done for DPP4. Virtual screening of phytochemicals present in NK gave 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).

Similar to NK, through *in silico* data mining, 248 ingredient compounds in VA were identified, followed by a virtual screening protocol to search for potential DPP4 inhibitors. Though there were no common plants between these two formulations, compounds such as Terchebin, Chebulinic acid, Rutin and Chebulic acid, were present in the predictive compound lists of NK and VA. Based on molecular docking and MMGBSA scores, the top three candidates were

terchebin, chebulinic acid and chebulagic acid demonstrated stable interaction with DPP4 and the interactions were comparable to the standard drug Vildagliptin. Terchebin and Chebulinic acid as mentioned before, were obtained in the virtual screening protocol of NK also. The ligand-protein interaction and stability studies of these ligands with the protein revealed several key residues such as Glu206, Tyr547. His740 and Tyr666 demonstrating that the compounds haves a stable binding profile with the protein. In VA, these compounds were reported from *Terminalia chebula*. *Terminalia chebula* is a very important plant referred to as 'King of medicine' and is a constituent of *Triphala*, a famed traditional preparation used as a medicine in many conditions (Ratha and Joshi, 2013). *Terminalia* plants are known to exert their antihyperlipidemic and immune-modulatory effect in multiple *in vitro* and *in vivo* studies (Pingali et al., 2020; Subramani et al., 2020).

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 and VA, a network pharmacological analysis of the phyto-actives was carried out using various databases mentioned in section 2.3.4. The target mapping of the formulations revealed a large networks complex networks comprising of 1555 and 4581 proteins respectively. The hub proteins with maximal interaction for NK 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 target network of NK involves many genes which are central to diabetic complications. Since the putative network of VA was quite complex and vast, for ease of analysis, a subnetwork of diabetes containing 1379 proteins was created by shortlisting diabetes associated proteins. The hub protein analysis of diabetic network of VA also showed the presence of carbonic anhydrases like CA2, CA1, CA14, DPP4 and GLP1R along with key diabetic regulators such as PPARγ, AKT1, FABP5 and TNFα among others. The presence of many common proteins in the target network of both formulations suggested that these shared several pathways in their mode of actions. The *in vitro and in vivo* results aligned with the predictive analysis findings that DPP4 is an important target in the mode of action of NK and VA, thereby hinting at incretin enhancing potential for both.

The results of the KEGG and Cluego analysis of mapped targets of both NK and VA showed an enrichment of diabetes associated pathways and processes. The commonly enriched pathways in NK and VA included insulin resistance, Lipid and atheroscelorosis, PI3K-AKT signaling, MAPK signaling, and AGE- related signaling, HIF1 signaling and FOXO signaling pathways. Certain pathways like cellular senescence and chemokine signaling in NK pathway enrichment results may suggest additional regulatory mechanisms through which the formulation brings the molecular effects. The predictive results of biological processes of VA presented several key processes such as lipoprotein metabolic process, Nitric oxide synthase regulation, negative regulation of lipid localization, low density lipoprotein, regulation of metabolic process along with neuroinflammatory response, response to leptin and vasodilation, all of which play a major role in T2DM progression. One of the major *Ayurveda* described action of VA is *Agni* correction which can be corelated to the multiple metabolic roles played by gastrointestinal tract. Also, the lipo regulatory effect and anti-inflammatory effect of VA has been reported by (Chinchu et al., 2020,;J.U et al., 2020) *in vitro* and *in vivo*. The experimental results matched the predictive analysis, thus giving a rationale for its clinical usage for obesity or *medohara* effect as well as in correction of dysregulated metabolic processes.

To get a deeper understanding into the broader disease associated effects of NK and VA, a Venn analysis of diabetic networks of both VA and NK was carried out. The NK and VA diabetic interactome containing 1379 and 619 proteins were categorized into diabetes complications such as retinopathy, neuropathy, nephropathy and myopathy and macrovascular complications. A comparison was also made between related metabolic diseases like NAFLD, pre-disposing factor like obesity and conditions like inflammation as well as metabolic dysregulation. The Venn analysis of diabetic complications of NK revealed a cluster of 23 markers. Among the 23 markers, inflammatory mediators like TNFα and pro fibrotic TGFβ1 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). For formulation VA, the results from Venn analysis of the four main classes of complications of diabetes such as nephropathy, neuropathy, cardiomyopathy and retinopathy showed 16 proteins common among the diabetic

complications. In this also, cytokines like TNFα and TGFβ1, and inflammatory regulators like RELA and MMP2, MMP9 were present, indicating various molecules playing a role in progress of diabetes and associated complications. The Venn analysis of diabetes and associated disease conditions revealed protein clusters consisting of 37 and 59 markers for NK and VA respectively. The set of 37 markers in NK analysis, include 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. Similarly for VA, the 59 common proteins included TNFα, AKT1, CCL2, TGFβ1, all of which are involved in the progression of type 2 diabetes and its complications. Lipid regulatory molecules like PPARγ, PPARα, LDL, APOA1, APOE1, ADIPO1 emerged in the cluster of both NK and VA validating the lipid *or 'meda'* regulatory effect that the formulations. Apart from the anti-obesity action, one of the main mechanisms mediated by polyherbal formulations in alleviating diabetes is by reducing oxidative stress by different mechanisms. (Unuofin and Lebelo, 2020). The presence of oxidative stress markers like GSTM1, SOD1, CAT and SOD2 in the central cluster in the overlap analysis between the diabetic complications for NK and VA show, indicate that the formulations acting through oxidative stress signaling also (Bid *et al*., 2010). Since the downregulation of DPP4 expression was observed in VA treated intestinal cells, in the mapped VA network the presence of upstream regulators of DPP4 was examined. One of the possibilities found was NFKB1, an important transcription factor, which is reported to increase the genomic expression of DPP4 by binding to DPP4 promoter (Tang et al., 2021).

Occurrence of DPP4 and GLP1R in the overlap of fatty liver, obesity and diabetic comorbidities for both VA and NK demonstrated the potential role of gut and its incretin axis in their poly pharmacological drug network. Leveraging the power of computational tools like docking and network pharmacology enable deciphering the actions of multicomponent therapeutics like *Ayurveda* formulations. Through the *in vitro* and *in silico* experiments, it was observed that DPP4 is one of the important targets of NK and VA. The network analysis revealed the possible involvement of critical diabetes regulatory markers in the multi-level mechanisms of NK and VA.

Tackling obesity, one of the strongest pre-disposing factors for metabolic syndrome and complications, such as, diabetes and cardiovascular diseases can be one of the most effective approaches for reducing incidence of T2DM. Medicinal plants have attracted extensive attention for anti-obesity associations and many of the components like plant polyphenols,

flavonoids and terpenoids have been studied for the same (Karri et al., 2019). To explore the modulatory action of four *Ayurveda* formulations on adipogenesis and downstream markers, anti-adipogenic assay was carried out using the well-studied 3T3L1 adipocytes.

Lipid droplets are important organelles contribute adipocyte intracellular signaling and adipokine regulation. Ectopic lipid accumulation has been one of the most important pro-obesogenic factors associated with obesity. Hence reducing accumulation of oil droplets can be a counter measure to reduce the detrimental effects of lipid droplets (Sanjabi et al., 2015; Zadoorian et al., 2023). There have been several reports of phytochemicals leading to reduction of lipid accumulation *in vitro* (Wong et al., 2014). From the anti-adipogenesis assays, it was observed that treatment of adipocytes with formulations VK and CV led to reduced formation of lipid droplets. Further, elution of lipids showed that VK treatment demonstrated that there is a significant decrease in triglycerides. VA and NK formulations appeared to be toxic to the adipocytes.

Many of the ingredients present in these formulations are reported to show anti-diabetic and anti-obesogenic effects in *in vivo* animal studies. However, the mechanism by which they exert their pharmacological action is not clear. The observations from the present study suggest that one of the mechanisms of actions of both these formulations involve modulation of the adipogenic process and thereby fat metabolism. Adipogenesis, characterized by conversion of fibroblast into mature adipocytes is a complex process accompanied by sequential activation and enhanced expression of several transcriptional factors. Triglyceride storage in mature adipocytes is induced by various transcription factors including like PPARγ and SREBP1 and adipocyte specific genes (Rosen et al., 2000). CV and VK downregulated pro-adipogenic factors to reduce the accumulation of lipids, which gave a scientific basis for their hypolipidemic action in the clinical reports of obese patients. VK and CV inhibited PPARγ and SREBP1 significantly demonstrating the anti-adipogenic action. PPARγ is a nuclear receptor transcription factor which initiates adipogenesis and is a master regulator of the adipocyte program (Ma et al., 2018). Another transcription factor, SREBP1 expressed in the adipocytes regulates de novo lipid synthesis genes like FASN in adipose tissue. FABP4, which is highly expressed in differentiated adipocytes, is a downstream gene of PPARγ and is involved in lipid transport and lipolysis. GLUT4 is involved in glucose uptake and is a marker for increased adipocyte differentiation (Furuhashi, 2019). Leptin is a secreted adipokine and is reported to accelerate the differentiation of preadipocytes (Palhinha et al., 2019b). In this study, VK

inhibited the expression of transcriptional regulators of adipogenesis like PPARγ and SREBP1 along with crucial effectors like FABP4, GLUT4 and adipokines like Leptin. Both CV and VK formulations also downregulated the expression of FABP4, which is highly expressed in differentiated adipocytes and has been identified as an adipokine in maintaining glucose homeostasis. VK in addition to these markers, also downregulated GLUT4 and FASN, but CV did not show significant effect on them. Further studies need to be done to understand better the differential downregulation of markers.

Through the various approaches, it is demonstrated that *Ayurveda* formulations target relevant therapeutic targets and can have a multi-modal effect. Through the various approaches, it is demonstrated that *Ayurveda* formulations target multiple glucose and lipid metabolism relevant therapeutic targets and can have a multi-targeted effect. In the thesis, the concept of *Agni*, representing the metabolic and digestive processes in the body, is corelated with the concept of GIGD, which describes the role of gut in glucose homeostasis (Thottapillil et al., 2021; Tripathi Js, 2013). For T2D/*Prameha*, the *Agni*-modulatory effect of NK and VA are explored through bioassays such as digestive enzyme inhibition and GLP1 hormone secretion. The stimulation of GLP1 secretion, DPP4 inhibition and digestive enzyme inhibitory potential of these formulations clearly indicated their *Agni*-GIGD modulatory properties. Further in a diabetic rat model, it was observed that the formulations improved the oral glucose tolerance, thus demonstrating the *Agni* enhancing potential. One of the other cardinal principles of *Ayurveda* for *Prameha* management is focused on reducing *meda* or adiposity. The *in vitro* results for all four formulations, along with in vivo observations for NK and VA, supported their *medohara* (anti-obesity) effect. Additionally, in-silico analysis of both formulations identified several genes and pathways associated with *Prameha* (T2D) and *Sthoulya* (obesity), further affirming the rationale for prescribing these formulations. Thus, using a molecular investigation, the mode of action of these formulations was explored, and novel mechanisms were uncovered, thereby underscoring the relevance for *Ayurveda* biology.

A summary of the biological actions of the formulations is listed in table 22. This systemic effect is often lacking in modern medicines which are targeted and may produce un wanted side effects over long term usage. The perturbed metabolism in obesity and diabetes manifested in vital organs like pancreas, liver, adipose and muscle tissue result in many complications. There has been an increase in the studies which reports the holistic effect of *Ayurveda* in

managing T2DM (Chattopadhyay et al., 2023; Thomas, 2023).While the modern medicine boasts of targeted therapy regimen and evolved into prescribing combinatorial regimen and lifestyle interventions, it still fails to account for differences from patient to patient to a large extent. *Ayurveda* has the potential to address this challenge since a personalized approach is integral to its epistemology from its inception. *Ayurveda* formulations, if supported by scientifically sound know-hows, it can contribute in managing the chronic diseases in a better way.

This thesis attempted to explore the mode of actions of four different formulations using different cell models and *in silico* methods and demonstrates the differential effects. It also tries to corelate certain concepts like *Agni* and lays a molecular context to them. In an age, where metabolic disease management pose a high public health burden, the thesis has great potential to serve as a framework for further deep dive studies into the actions of formulations which can fully uncover their therapeutic potential.

Formulation name			*Nisakatakadi*	*Varanadi Kashaya*	*Vasantakusumara rasa*	*Chandraprabhavati*
Form			Liquid	Liquid	Tablet	Tablet
Number of ingredients			8	16	15	30
Major therapeutic claim of *Ayurveda*			Effective against *Prameha* and associated complications	Effective against obesity (*Medohara*) and enhancing metabolic activity (*Agni*)	Effective against all types of *Prameha*	Effective against all types of *Prameha*
Experiments done	*In vitro* cell free	α-glucosidase	++	++	No activity	No activity
		α-amylase	No activity	No activity	++	++
		DPP4 inhibition	++	++	No activity	No activity
	In vitro cell based	adipogenesis	No activity	No activity	+	+
		GLP1 secretion	++	+	-	-
	In silico	Phytochemical data mining	206 compounds	248 compounds		
		Molecular docking	Top 3 compounds - Terchebin, TGBG and Locoracemoside B	Top 3 compounds - Terchebin, Chebulinic acid and Chebulagic acid	NA*	NA*
		Network pharmacology	1555 total proteins and 614 diabetic associated targets	4581 total proteins and 1379 diabetes associated targets		
			Venn analysis – 37 overlapping genes for diabetes associated conditions. 23 overlapping genes for diabetic complications.	Venn analysis – 59 overlapping genes for diabetes associated conditions. 16 overlapping genes for diabetic complications.		
			Modulation of 14 pathways connected to 5 glucose metabolism associated diseases conditions	Modulation of 15 pathways connected to 5 glucose metabolism associated diseases conditions		
	In vivo (HFD-STZ rat model)	Blood Glucose	++	+	NA*	NA*
		OGTT	+	+		
		Triglycerides	+	+		
		Cholesterol	+	+		
		GLP1 secretion	+	+		
		Histopathology	Improvement in the morphology of kidney; Reduction of steatosis and inflammation in liver; Decreased inflammation in pancreas; Increased number of islet cells; Lesser inflammation in intestine compared to diabetic control	Improved morphology in kidney; Reduced liver steatosis and inflammation; Reduced damage to pancreatic islet cells; Lesser inflammation and damage of intestine.		
		Strong Activity	NA* - Not applicable			
		Moderate Activity				

Table -22: A summary of the various bioactivity assays. This demonstrates the multitargeted effect of *Ayurveda* formulations through different methods

3.8 Limitations of the thesis

While the study presents promising results to explain the Ayurveda Biology framework and multi-targeted mode of action of Ayurveda formulations, there are some limitations as well for this study. Though four formulations have been taken for the experimental studies, the form in which they have been used (kashaya, tablet etc) are not well suited for the model systems used, particularly cell lines. Most of the model systems available for biological studies are designed to suit the analysis of single molecule – single target mode of action. The presence of multiple bioactives in the test material may result in biological effects that may not be read properly. Also, the toxicity posed by certain formulations like NK and VA in cell-based system, due to the presence of multiple bioactives, could not be addressed properly using the methods utilized. The computational methods by which the phytochemical composition was obtained is limited by the available information from the databases, leaving a gap in the actual composition of these preparations. Though biological effects were evaluated a detailed molecular and signaling pathway analysis is not included in the present study. While the predictive analysis of proteins and biological pathways mapped were performed, it need to be further validated using experimental approaches. Advanced omics studies using RNA-seq and metabolomics should be utilized for learning the deeper molecular signatures exerted by these multi-component therapies.

4 Conclusions and Future directions

The present thesis can be looked as one of its kind which attempts to study the formulations in their whole form by combined various approaches like *in silico*, *in vivo* and *in vitro* thereby generating a scientific rationale for the clinical usage of classical *Ayurveda* formulations. By trying to integrate and corelate *Ayurveda* concepts using modern biology techniques, the study engages in a transdisciplinary thought process. The findings from the thesis are relevant especially today when there is an urgent need to manage this twin burden of diabesity. The thesis through its Ayurveda Biology framework endeavors to herald an era of forming integrative approach for complex disease management.

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Appendix

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This is to certify that the research proposal entitled **"Exploring the anti-diabetic and incretin modulatory effect of two Ayurveda formulations Nishakathakadi Kashaya and Varanadi Kashaya on rat model"** submitted by **Mrs. Anjana T,** research scholar of The University of Trans-Disciplinary Health Sciences and Technology has been approved by IAEC.

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Review

An '*Ayurveda-Biology*' platform for integrative diabetes management

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ABSTRACT

Ethnopharmacological relevance: Diabetes is a multifactorial disease with complex multi-organ-multi-target crosstalk in the body. Currently, the theoretical assumptions framing the diabetes management strategies are reductionist and largely focus on reducing hyperglycemia through targeted molecular drugs. While they effectively reduce hyperglycemia, they are inadequate to address the multifactorial etiopathology, chronicity and systemic complications of diabetes. Therefore, a holistic and systemic approach is essential for its successful management. We hypothesize an integrative diabetes management strategy, combining holistic principles of diabetes management with its molecular understandings, would be more appropriate to fill this gap. The holistic disease management principles of Ayurveda, the Indian system of medicine, can play a pivotal role in this context. This narrative review discusses the scope of a trans-disciplinary ' Ayurveda-Biology ' approach for deepening the holistic understanding of the pathophysiology of diabetes as well as designing novel integrative strategies for managing diabetes and restoring whole body glucose homeostasis.

Methodology: The article analyses the Ayurveda scheme of diabetes management and correlates it with the molecular understanding of its pathophysiology and management. The sources of information used in this article include classical texts of Ayurveda , medical books, published research articles and scientific databases like PubMed, Google Scholar, Science-Direct, etc.

Results: While Ayurveda and modern biomedicine uses different epistemology and ontology for describing diabetes, both the systems recognize the central role of gut and gut derived factors in postprandial glucose disposal and whole body glucose homeostasis. Essentially, the principles of both Ayurveda and modern biomedicine overlap at a gut centred view of diabetes management; and Gastro-intestinal mediated glucose disposal , a holistic concept of glucose metabolism, is emerging as a converging node for designing innovative integrative diabetes management strategies.

Conclusions: An integrative disease management strategy, combining holistic and reductionist perspectives of traditional medicine and biology respectively, would be the prerogative for successful management of diabetes. Creating an ' Ayurveda-Biology' knowledge framework integrating the patient centred holistic management principles of Ayurveda and the molecular approaches of modern biology can give better insights into the biology of whole body glucose homeostasis and offer novel strategies for cost effective, holistic and multi-targeted management of diabetes.

1. Introduction

Health is a harmonious state of physiological, biochemical and psychological functions of the body, and disturbances to this harmony result in various diseases (Piko and Brassai, 2016). 'Healing' is a process of restoration of this body homeostasis by synchronising its functions, both at systemic and molecular levels. Philosophy of science has two predominant schools of thought *viz.* holism and reductionism. Holistic philosophy of science recognizes health or disease as a collective expression of several biochemical and physiological events in the body, whereas reductionism deconstructs them into more tractable cellular and molecular components (Fang and Casadevall, 2011). However, both these perspectives are equally important for comprehensively understanding the biological phenomena of health and disease in an individual; and that is always more than the sum of independent molecular and cellular events happening in the body. Therefore, an appropriate

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and effective merger of both holistic and reductionist views is imperative for successful health and disease management. This is the context where integrative medicine or medical pluralism becomes relevant. Its aim is to recognise and logically bridge epistemologically and culturally different perspectives of health and disease management. It neither rejects nor accepts one system; but mutually integrates positives from systems. Although pluralism in medicine is criticised for multiple reasons, the notion of integrative medicine is becoming more popular in the contemporary healthcare sector, particularly in the management of chronic lifestyle diseases.

Diabetes, a complex multifactorial disease resulting from pathophysiological insults that disrupts the whole body glucose homeostasis, is a classic example that entails an integrative medicine approach for its holistic management. The pathophysiological network of diabetes along with obesity, inflammation and insulin resistance makes it an extremely difficult disease to manage. The body's glucose homoeostasis is a tightly regulated physiological process involving multi-organ networking and cellular cross-talks and therefore, diabetes affects virtually all systems in the body. However, the current diabetes management strategies largely follow a reductionist approach focusing on reducing hyperglycemia through targeted molecular drugs (Chatterjee and Davies, 2015). This reductionist approach is inadequate to address the chronicity and systemic complications of diabetes and fails to provide long-term effectiveness, ultimately resulting in lifetime dependency on drugs with a gradual, but constant increase in dose.

The need of the hour is a paradigm shift in the perspective of diabetes management, from reductionist to holistic, which can target multiple organ systems involved in glucose homeostasis and pathophysiology of diabetes (Shroff, 2011). Holistic perspective and principles on wellness and disease management practiced in *Ayurveda* may be a worthwhile option in this context. We hypothesize, a trans-disciplinary framework of medical pluralism built on pragmatic acculturation of holistic concepts of *Ayurveda* and molecular insights of modern biomedicine would be the prerogative for designing novel integrative strategies for diabetes management. Contemplating on a trans-disciplinary medicine approach, focusing on holistic concepts of lifestyle, wellness, diet and energy homeostasis would also generate novel insights into the multifaceted physiology and biochemistry of whole-body glucose homeostasis. This narrative review discusses the scope of an '***Ayurveda-Biology***' platform for designing integrative management strategies for diabetes. Forging bridges between disciplines is the path-breaking strategy for innovation in science. Therefore, creating a trans-disciplinary knowledge framework between *Ayurveda* and modern biomedicine could be the paradigm shift in the global healthcare sector to fulfil the contemporary healthcare demands (Vishnuprasad, 2018).

2. Patient centred disease management algorithm of *Ayurveda*

Ayurveda recognizes health as a condition when the master regulators of physiology *viz. Doshas* (body humors*: Vata, Pitta and Kapha*), metabolic activities (*Agni*), structural components (*Dhathu*), excretory functions (*Mala-kriya*) as well as the psychological and behavioural contentment of an individual are in complete homeostasis and optimum function. Consequently, disease is considered as a collective systemic expression of pathological afflictions that disrupt the body's homeostasis and physiology, and exhibited as symptoms. This philosophy has advantages over the molecular viewpoint for designing holistic disease management strategies that are aimed at restoring physiology and pacifying the symptoms. Although they ultimately exert their effects at specific molecular targets, *Ayurveda* perceive their efficacy and success at functional levels like digestion, excretion and various tissue and organ functions. For example, if a disease is diagnosed as a result of *Agni* malfunction, *Ayurveda* algorithms prescribe strategies to normalize *Agni* functions, i.e., correction of digestion and metabolism, through physician guided interventions like lifestyle modifications, diet and medications. While the algorithms are general guiding principles, the physician can fine-tune and personalize the interventions depending on the severity and chronicity of disease, other co-morbidities, patient's physiological strength, behaviour as well as socio-cultural background. This makes the disease management more personalized and patient centred, while keeping the pre-defined algorithm general and globally adaptable. Being a science established itself much before modern day biology, the philosophical frameworks used in *Ayurveda* for understanding the life processes are different from the current molecular framework of biology. Therefore, it is important to note that Ayurvedic epistemology and ontology of health and disease cannot be juxtaposed with the contemporary biomedical languages. This is where a trans-disciplinary knowledge framework is essential which logically integrates the concepts and creates a platform that can be appreciated by both sides.

3. Understanding diabetes - the *Ayurveda* perspective

Ayurveda identifies diabetes as a urinary system disorder exhibited when the *Agni* homeostasis in gastro-intestinal tract (GIT) gets perturbed by various etiological factors like genetics, lifestyle issues and unwholesome diet. *Prameha* and *Madhumeha* are the two diabetes-equivalent clinical manifestations described in *Ayurveda*. *Prameha* is a broad group of disorders characterised by excess and turbid urination, with symptomatic similarities to the pre-diabetes stage. Prolonged existence of *Prameha* in an individual can lead to *Madhumeha*, a sub-type hallmarked by sweetness in the urine. Several aspects of *Prameha* overlap with the modern-day diabetes mellitus (Table-1). Classifications of *Prameha* like congenital (*Sahaja*) and acquired (*Apathyanimittaja*) as well as lean (*Krusha*) and obese (*Sthula*) are relatable with similar types of diabetes described in modern biomedicine (Fig-1). However, *Prameha* classifications based on the *Dosha* dominance are unique to *Ayurveda* and are highly specific in terms of their characteristic clinical manifestations. Twenty different clinical manifestations of *Prameha* have been identified based on the dominance of *Vata, Pitta* or *Kapha-Doshas* (Fig-1). *Madhumeha*, which is symptomatically and etiologically more similar to chronic type-2 diabetes mellitus, belongs to the *Vataja* type (arising due to *Vata* dominance) and considered as the most difficult type of *Prameha* to manage (Sharma and Chandola, 2011; Sharma and Dash, 2007; Shastri, 2003). Similarities between *Prameha* and diabetes are also seen in their etiology where both *Ayurveda* and biomedicine equally recognise unwholesome diet (excess diet, over nutrition, regular intake of unctuous and heavy food), sedentary lifestyle, lipidemia (∼*Medoroga*) and obesity (∼*Sthaulya*) as major predisposing factors for *Prameha* and/or diabetes. Consequently, both systems advise on life-style modifications and limiting high glycemic index foods as preventive measures (Sharma, 2000; Mangalasseri et al., 2019).

4. Gut-centric view of pathophysiology and management of diabetes in *Ayurveda*

Ayurveda has a gut-centric and systemic view of describing the pathophysiology and management of diabetes. Three unique concepts of *Ayurveda* viz. *Agni*, *Ama* and *Rasa-dhathu* are central in understanding the pathophysiology of *Prameha*. *Agni* is a physiological phenomenon that optimises the metabolic transformations in the body by regulating digestion, absorption and bio-assimilation of nutrients from ingested food. The strength and homeostasis of *Agni* is critical in determining overall health of an individual. *Ama* is antithesis to *Agni* and has a vicious negative feedback effect on *Agni*. It is defined as the detrimental products derived from improperly digested and assimilated food due to malfunctioning of *Agni*. The third concept, *Rasa-dhathu,* is the circulatory liquid form of the bio-assimilable part of the digested food, which is the primary structural component for the formation of all other tissue systems in the body.

According to *Ayurveda*, urine production starts in the GIT and therefore the pathological cascades of *Prameha* also begins at the gut,

Table 1
Transdisciplinary correlation of symptoms, etiology and pathophysiology of *Prameha* with Diabetes: Table provides an *'Ayurveda-Biology'* comparison of symptoms, etiology and pathophysiology of *Prameha* with that of diabetes. Pathophysiological events like *Agnimandya* and *Apachitdhathuvrudhi* can be correlated to various events in Gastrointestinal mediated glucose disposal (GIGD).

PRAMEHA	DIABETES	REFERENCES
Symptomatic and Etiologic Correlations		
Prameha: Disease affecting *Mutravaha srotas* (urinary system) manifested as excess and turbid urination	Diabetes or Polyuria	Defronzo (2009); Forbes and Cooper (2013); Sharma and Dash (2007).
Madhumeha (sweetness in urine)	Hyperglycaemia and Glycosuria	
Sahaja Prameha (*Prameha* by birth)	Diabetes caused by genetic predisposition (Correlated to Type-1 diabetes mellitus)	Sharma and Dash (2007); Temelkova-Kurktschiev and Stefanov, 2012.
Apathyanimittaja (acquired through lifestyle changes).	Diabetes acquired through an unwholesome diet and lifestyle (correlated to Type-2 diabetes mellitus)	
Sthula Pramehi (obese diabetes patient)	Diabetes associated with obesity.	
Krusha Pramehi (lean diabetes patient)	Diabetes without obesity	George et al. (2015); Sharma and Dash (2007).
Pathophysiological Correlations		
Agnimandya [reduced *Agni* (digestion and metabolism) functions]	Improper digestion and metabolism of nutrients leading to altered whole body glucose homeostasis.	Gribble and Reimann (2019); Sharma and Dash (2007).
Apachitdhatuvrudhi [Accumulation of improperly metabolized *dhathu* (tissues)]	Improper bio-assimilation and metabolism of digested food components leading to hyperglycemia and central adiposity.	Defronzo (2009); Holst et al. (2016); Sharma and Dash (2007).
Sthoulya (obesity)	Chronic over-nutrition leading to obesity.	Sharma and Dash (2007); Temelkova-Kurktschiev and Stefanov, 2012.
Karshya (drastic weight loss due to diseases)	Pathological weight loss	Lien et al. (2018); Sharma and Dash (2007).
Shosha and *Balahani* (muscle wasting and general debility)	Muscular atrophy or pathological wasting of muscle due to prolonged insulin resistance and inflammation	Perry et al. (2016); Sharma and Dash (2007).

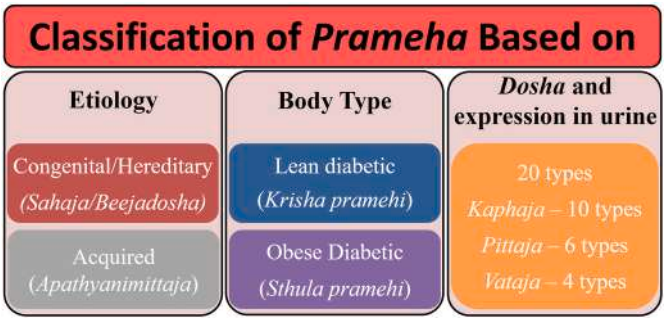

Fig. 1. Scheme of classification of *Prameha*: Shows the different principles used in *Ayurveda* for classifying the clinical condition *Prameha*.

but clinically manifest in the urinary system (*Vasti*). Various etiological factors like genetics, lifestyle issues, unwholesome diet etc., disrupt the *Agni* homeostasis in the GIT and cause *Kapha-dosha* domination in the body. Due to improper *Agni*, accumulation of metabolic wastes (*Ama*) happens in the gut and other tissues that hamper the *Rasa-dhathu* metabolism and its supportive functions to tissues like muscle, fat and bone marrow ultimately resulting in manifestation of *Prameha* symptoms (Fig-2) (Murthy and Singh, 1989). Accordingly, the diabetes management (both prophylactic and therapeutic) in *Ayurveda* begins with restoration of *Agni* homeostasis and reduction of *Ama* accumulation, and thereby improving absorption, bio-assimilation and utilisation of nutrients for physiological functions. *Ayurveda* being a wellness science, it emphasizes more on prophylaxis than treatment. Nevertheless, the same algorithm or logic is followed for both prevention and cure. The *Prameha* management algorithm of *Ayurveda* is broadly categorised into purification process (*Shodhana-chikitsa)* and palliative therapy (*Shamana-chikitsa)*. The purification process is aimed at restoration of *'Agni'* to augment digestion and metabolism by expelling '*Ama*', the metabolic wastes, accumulated over a period of time. Palliative therapy is the medication approach used to restore the physiological balance using various herbal and herbo-mineral formulations (Fig-2). In the real-life clinical practice, *Ayurveda* physicians perform a one-time elimination of metabolic wastes by *Shodhana-chikitsa* to normalize the *Agni* functions in the body; followed by *Shamana-chikitsa* to restore the physiological balance for the management of diabetes. To sum up, the systemic management of *Prameha* has a significant role played by the gut and various gut-derived factors.

5. Role of gut in holistic management of diabetes

The gut or GIT is the first anatomic site that interfaces the ingested food (primary source of glucose and energy) and body's metabolic homeostasis. It is the largest endocrine organ that regulates nutrient absorption and energy homeostasis of the body. The gut harbours a variety of cells especially in the small intestine, with receptors responding to various nutritional cues, and secretes hormones and nutrient metabolizing enzymes to crosstalk with major physiological systems like the liver, kidney, brain, pancreas and heart (Gribble and Reimann, 2019). Similarly, the resident microorganisms in the GIT, the gut microbiome, also play an important role in physiology by improving both post-prandial glucose disposal and fasting blood glucose levels (Gérard and Vidal, 2019). These versatile roles of gut make it an important target for designing innovative holistic strategies for management of metabolic diseases like diabetes and obesity. Granting gut plays diverse roles in digestion, absorption, metabolism and assimilation of food and nutrients, gut-centred glucose metabolism events can be broadly grouped into two, incretin mediated and other non-incretin events.

6. Incretin effect and its role in whole body glucose homeostasis

In response to the ingested food, enteroendocrine cells (EECs) present in the gut epithelium secrete several peptide hormones which modulate insulin release and food satiety signals. Of these, incretin hormones, identified in the 1980s, garnered considerable interest in diabetes management because of their pleiotropic roles in enhancing insulin secretion, gastro-intestinal motility and modulation of gastro-intestinal crosstalk with other organs (Holst et al., 1987; Moody et al., 1984). The "*Incretin effect*" is defined as a biological phenomenon of 2–3fold higher stimulation of insulin secretion in response to an oral glucose administration than an intravenous glucose infusion, even with the same plasma glucose profile (Andersen et al., 2018). Glucagon-like peptide-1 (GLP-1) and Gastric inhibitory peptide (GIP) are the two primary incretin hormones secreted from L cells present in ileum and colon and K cells located in the duodenum (Müller et al., 2019; Holst, 2019b). Dietary components like fat and glucose are the primary stimulants for incretin secretion. Their biology, mechanism of action and pleiotropic

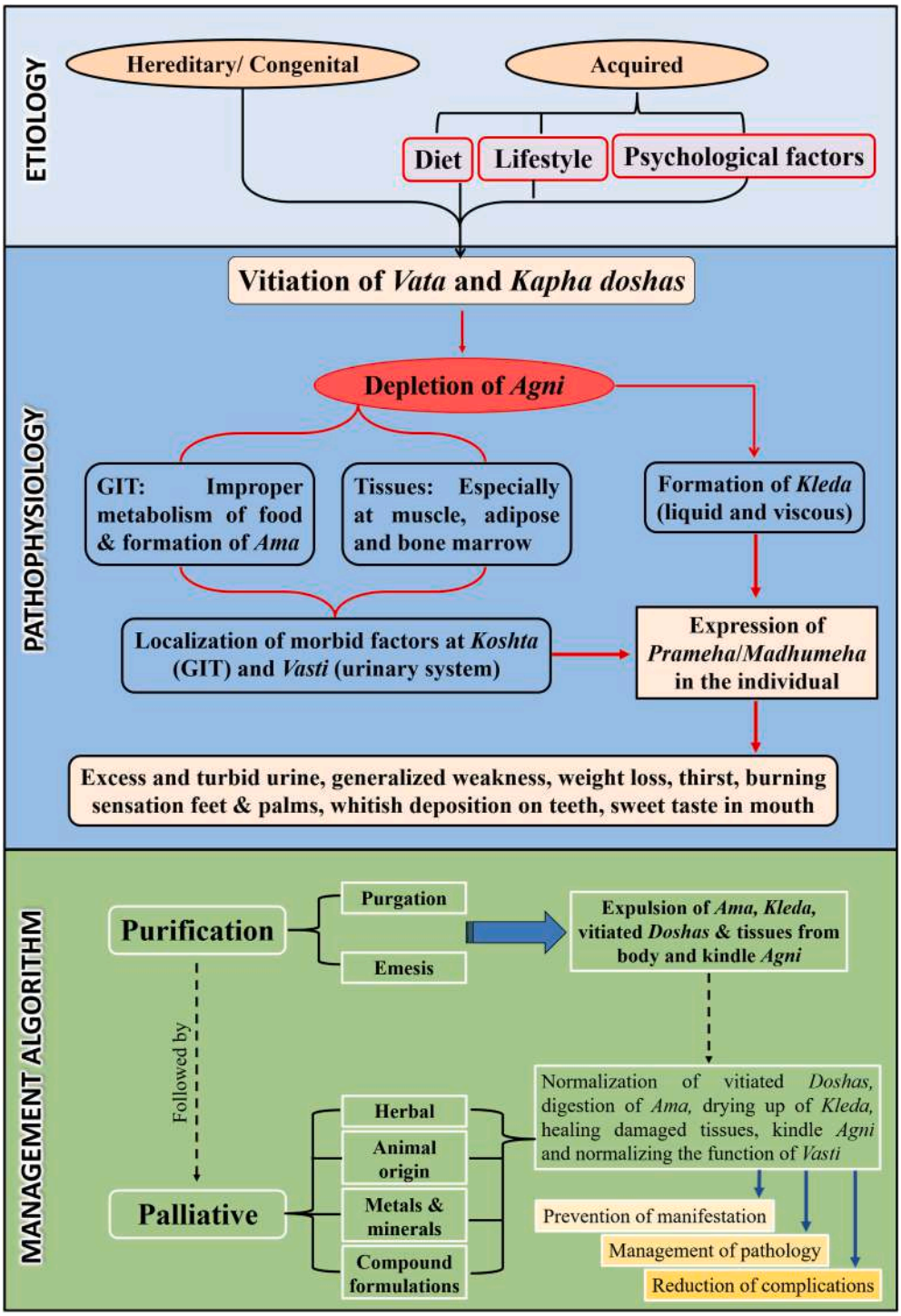

Fig. 2. *Ayurveda* perspective of diabetes: Schematic representation of the etiology, pathophysiology and management algorithm of *Prameha*.

role in glucose homeostasis are extensively studied and their potential in diabetes management is well established (Holst, 2006; Tolhurst et al., 2009). Both GLP-1 and GIP have functional similarities in their insulinotropic effects through G-protein coupled receptor (GPCR) signalling in pancreatic beta cells, and both have their insulinotropic effects inactivated by the enzyme dipeptidyl peptidases–4 (DPP4). They are also shown to enhance pancreatic beta cell proliferation and increase pancreatic beta cell mass by inhibiting apoptosis, whereas they suppress glucagon secretion from pancreatic alpha cells (Perfetti et al., 2000; Ramracheya et al., 2018). While they are similar in their insulinotropic activity, GLP-1 is shown to inhibit glucagon secretion from pancreatic alpha cells, whereas GIP stimulates glucagon secretion during hypoglycemia. Therefore, GIP can serve as a bifunctional hormone to work against both extreme excursions of glucose.

Although both GLP-1 and GIP are shown to have extra-pancreatic interactions with brain, liver, kidneys, intestine and gastric parietal cells to regulate various biological functions, the effects of GLP-1 are well-established than GIP (Seino et al., 2010). Extra-pancreatic actions of GLP-1 include: inhibition of gastric emptying and food intake; inhibition of hepatic gluconeogenesis; inhibition of adipogenesis; regulation of food satiety and appetite; regulation of peripheral glucose disposal; insulin mimetic action in muscles and adipocytes as well as cardio-,

neuro- and reno-protective effects makes it a potential holistic target for managing diabetes and its co-morbidities (Capozzi et al., 2018; Nauck et al., 1997; Wettergren et al., 1993). Studies have shown that GIP is an obesogenic hormone, where it positively correlates with weight gain and typically elevated in both genetically and diet induced obese mice and obese human. Nevertheless, growing scientific evidence suggests that, similar to GLP-1, GIP agonism also has metabolic benefits and improves glucose metabolism. Recent studies have shown the role of GIP in influencing body weight by regulating fat deposition and adipocyte biology, where a dual agonist of GIP and GLP-1 found to reduce body weight (Müller et al., 2019; Frias et al., 2018). Similarly, GLP-2, which is also secreted by L cells of the intestine, is another potential target for whole body glucose homeostasis owing to their important role in energy homeostasis and intestinal integrity as evidenced by several studies (Amato et al., 2016).

The key role of incretins in postprandial glucose homeostasis and appetite control as well as their severe reduction in type 2 diabetes has made them an actionable target for diabetes management (Holst et al., 2011). Incretin modulators, GLP-1 analogues and DPP4 inhibitors have become a novel class of therapeutics for diabetes (Deacon, 2019). Additionally, their pleiotropic effects like anti-inflammatory effects and cardiovascular improvement make it a better target for diabetes complications as well (Guo et al., 2016; Lu et al., 2019; Madsbad, 2019; Virtue and Vidal-Puig, 2010; Zhao et al., 2019).

7. Other gut-centric actions in glucose metabolism

Apart from the incretin effect, the human gut has several key functions during postprandial glucose metabolism. For example, the gut-brain axis constituting the enteric nervous system has a crucial role in regulating various gastrointestinal functions like intestinal motility, blood flow, secretion, barrier function, and interactions with immune and gut endocrine system and it is often referred as the 'second brain' (Knauf et al., 2020). A growing body of evidence confirms the gut's interactions with immune cells, liver and kidney all of which have key roles in maintenance of normal glycemic levels. The gut modulates hepatic glucose metabolism as well as renal function through regulating electrolyte and fluid homeostasis (Michell et al., 2008; Muskiet et al., 2014; Radziuk and Pye, 2001). Although incretins play an important role in all these gut-centric events, there are a host of other peptide hormones secreted by gut epithelium that regulate many of these metabolic functions in the body. They are broadly grouped under two classes, orexigenic (appetite stimulant) and anorexigenic (appetite reducers). While the majority are anorexigenic, only ghrelin and INSL5 are orexigenic in action. Some of other gut peptides such as oxyntomodulin, PYY, neurotensin, nesfatin, secretin, cholecystokinin and apelin are now considered as potential gut hormone polyagonists for therapeutic applications for diabetes and obesity owing to their role in food intake and gastric functions (Knauf et al., 2020; Michell et al., 2008). The growing interest in developing polyagonist therapies for type-2 diabetes and obesity proves that gut peptides have a lot of scope in novel therapeutics.

Gut microbiome is another exciting area of interest for holistic health and disease management owing to their roles in host digestion, nutrition and interactions with other tissue and organ systems in the body (Shreiner et al., 2015). Diabetes being a major disease associated with food and nutrition, gut microbiome plays an important role in its pathophysiology and management. Metagenomics and metabolomics-based studies show that diabetes causes imbalances in gut microbiome homeostasis (Allin et al., 2018; Karlsson et al., 2013; Vangipurapu et al., 2020). Various mechanisms have been proposed to explain the influence of the microbiota on insulin resistance and type-2 diabetes such as metabolic endotoxemia, modulation of incretin secretion and butyrate production (Jia et al., 2017; Larraufie et al., 2018; Madsen et al., 2019).

8. Gut as a converging node for '*Ayurveda-Biology*' understanding of diabetes management

Now the pertinent question is how can these holistic and reductionist (conventional biomedicine) principles of diabetes management be logically integrated? Do these different epistemologies intersect? The answer is, yes. It is quite interesting to note that the Complementary and Alternative Medicines (CAMs) in general and *Ayurveda* in particular, emphasize the role of gut and gut-derived factors in the body's metabolic homeostasis, wherein diet is considered to be one of the primary pillars of health. What you eat, how much you eat and when you eat are important in determining the metabolic balance. Dys-regulations in GIT and the defective digestive capacity are linked to metabolic diseases. Essentially, *Ayurveda* follows a gut-centred view to explain the manifestations and management of metabolic diseases like diabetes and this is perfectly overlapping with the concepts in modern biomedicine (Udupa, 2004). Furthermore, the multicomponent therapeutics (e.g., herbal formulations) prescribed for diabetes management utilizes gut as the most suitable site that naturally disseminates bioactivity all over the body. Corroborating this, some of the multicomponent anti-diabetic medicaments of *Ayurveda* uses combinations of different herbs that are reported to have anti-hyperglycemic, hepato-protective, anti-hyperlipidemic effects, along with components for enhanced digestion and calming the mental stress (Butala et al., 2017). While multicomponent therapeutics are necessary to achieve a desired network pharmacological effect, it is very important to highlight that such therapeutic interventions are not random combinations but rationally designed based on unique pharmacological algorithms pertaining to a particular CAM, which are time tested and reproducible (Parasuraman et al., 2014; Hopkins, 2008). A deeper understanding of gut-mediated glucose homeostasis from the parallels drawn between the *Ayurveda* and biomedicine can provide valuable holistic strategies ultimately resulting in better long-term containment of diabetic morbidities.

9. Gastro-intestinal mediated glucose disposal (GIGD) – a gut parameter evolving as a concept for holistic glucose metabolism

Gut being a central player in the whole body glucose homeostasis and several factors govern the gut mediated glucose metabolism; the entire gut centred events of postprandial glucose metabolism can be collectively referred as gastro-intestinal mediated glucose disposal (GIGD). It is primarily measured as a parameter to estimate the glucose clearance by incretin effect, which is the most effective gut determinant of glucose metabolism to date. Higher the ingested glucose, greater is the incretin effect. Therefore, as a parameter GIGD describes how effectively the body alleviates the postprandial glucose excursions driven primarily by the incretin effect. Besides incretin hormones, there are several other components like gut microbiota, first-pass hepatic glucose uptake, glucosidase inhibitors, immunomodulation, inhibition of intestinal glucose absorption as well as hitherto unknown factors which collectively contribute to the postprandial glucose disposal as well as maintenance of the body's glucose homeostasis (Fig-3) (Holst, 2019a). Glucose being the primary energy source for all the tissues in the body, perhaps, GIGD components may have a broader role in optimising the physiology and metabolism of all the tissue systems in the body by regulating the glucose metabolism. Therefore, it is imperative to consider gut as a whole and GIGD as one of the important nodal concepts that converge the principles of diabetes management in *Ayurveda* and biomedicine (Fig. 4).

10. Conclusions and future perspectives of '*Ayurveda-Biology*' framework for diabetes management

The holistic concepts of diabetes management and the rich repository of safe and food-equivalent herbal materials attracted scientists

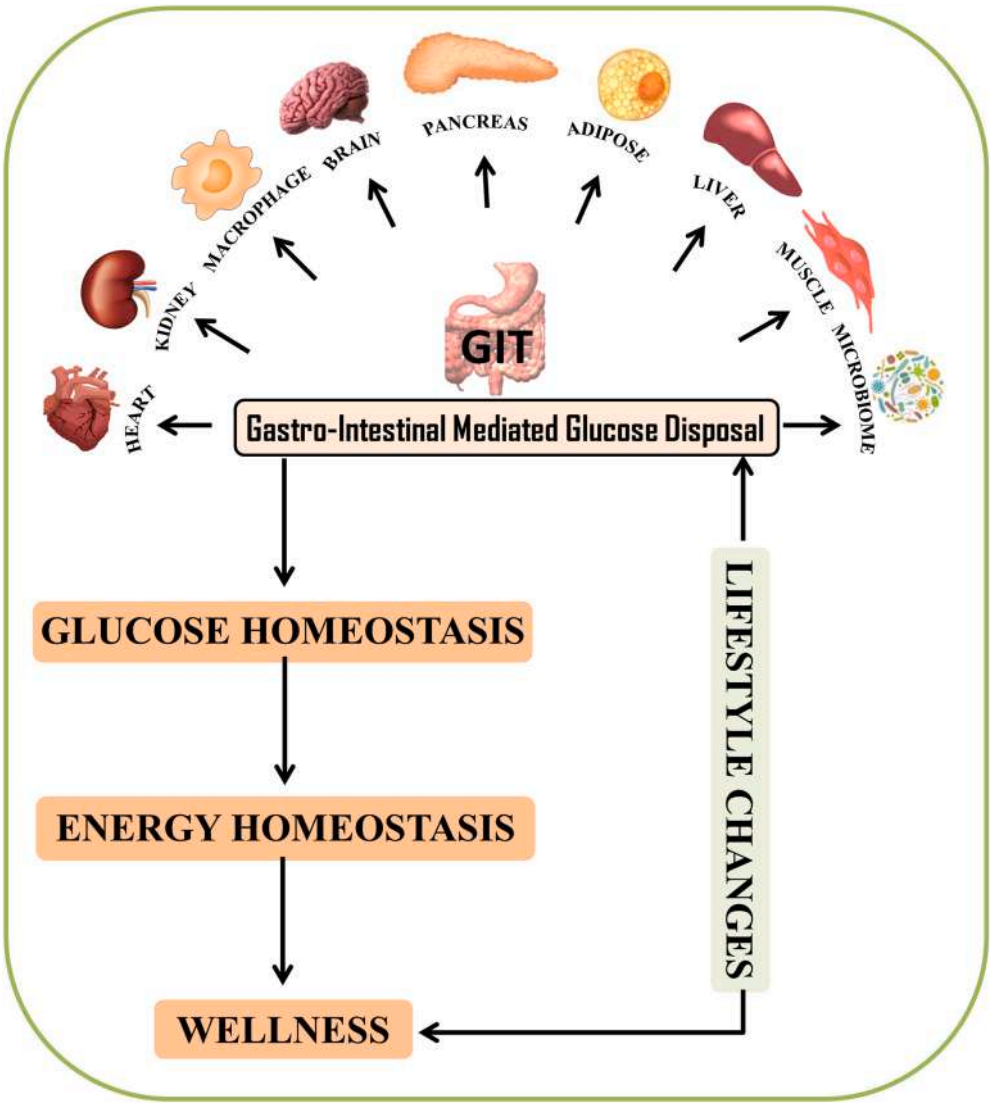

Fig. 3. Gastrointestinal mediated glucose disposal: Figure conceptualizes multi-system interaction of gut in the whole body glucose homeostasis and wellness.

and physicians to explore *Ayurveda* for developing novel drug candidates and holistic management strategies for diabetes and associated diseases. Review of research literature indicates that every plant mentioned in *Ayurveda* for *Prameha* management as well as some of the anti-diabetic herbal formulations were studied for their chemistry, pharmacodynamics, pharmacokinetics, molecular mechanisms of action as well as repurposing potentials using *in silico, in vitro, in vivo* and clinical model systems (Supplementary data, Table- S1 and S2). However, the majority of these studies are rooted in the reductionist frameworks of experimental and clinical pharmacology, which are primarily designed for studying molecular drugs. While these strategies had remarkable advancements in deepening the understandings of chemistry, pharmacodynamics, pharmacokinetics and mechanisms of action of plants and their bioactives, they had limited success in understanding or hypothesising the complex multifactorial biological effects of these plants. The robustness and multi-system modulation of glucose homeostasis is the result of network interactions of several independent molecular signalling pathways. In order to unravel the complexity and to get more insight into the biology of diabetes, a trans-disciplinary '*Ayurveda-Biology*' research framework is necessary that can logically bridge these epistemologically different knowledge systems.

So, how do we envision a new road map in diabetes management? With the number of diabetes and pre-diabetes populations on the rise, it is vital to develop innovative and effective management solutions. To overcome the shortcomings of the existing single system-based diabetes care, it is important to formulate a platform synergizing the healing oriented diabetic management ways of *Ayurveda* with modern biomedicine resulting in an optimized integrated therapy regimen. However, it is necessary that more efforts be invested in active trans-disciplinary research for: 1) understanding and correlating the gut-centred principles of pathophysiology and management of diabetes in different systems; 2) developing unique *in vitro* and *in vivo* assay systems for studying the systemic action of multi-component therapeutics; 3) Developing and implementing globally acceptable trans-disciplinary assessment standards for safety and efficacy of multi-component therapeutics and, last but not the least; 4) develop globally acceptable, cost effective, integrative diabetes management protocols, which essentially includes non-pharmacological approaches as well. In short, gut and gut-derived factors can serve as the bridging concept for developing an efficacious and holistic framework of integrative diabetes management.

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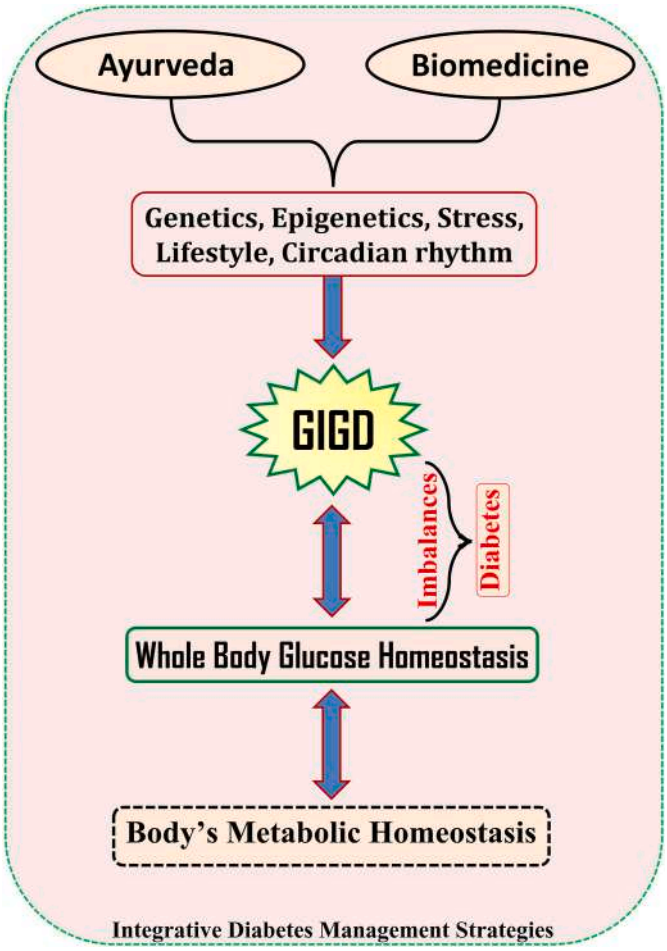

Fig. 4. Integrative diabetes management: Conceptualize GIGD mediated the integrative diabetes management framework.

Declaration of competing interest

Authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2020.113575.

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

Anjana Thottappillil[a], Sthitaprajna Sahoo[b,c], Abhijnan Chakraborty[b,d], Sania Kouser[a], Vidhya Ravi[b], Soumya Garawadmath[a], Pranav Banvi[a], Subrahmanya Kumar Kukkupuni[a], S Suma Mohan[b] and Chethala N. Vishnuprasad[a]

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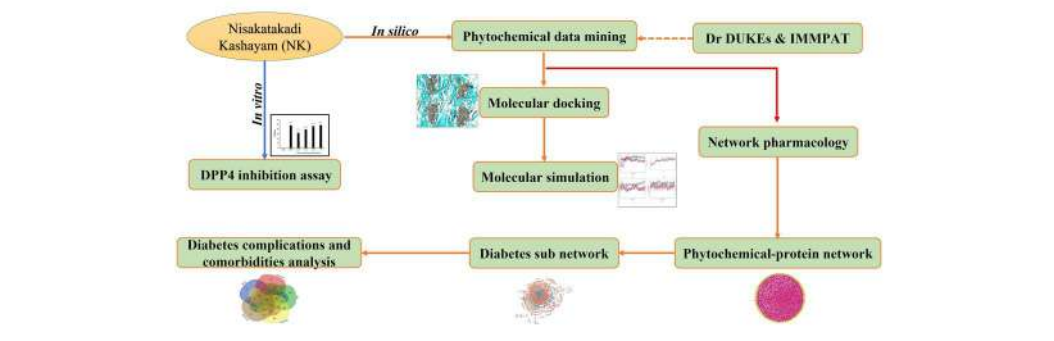
ABSTRACT

Dipeptidyl-peptidase IV (DPP4) inhibitors are an important class of anti-diabetic drugs recognised for their systemic biological actions. Polyherbal preparations like *Ayurveda* formulations are considered to be ideal sources for discovering novel DPP4 inhibitors owing to their rich phytochemical composition. The current study reports the DPP4 inhibitory potential of a clinically established Ayurvedic anti-diabetic formulation *Nisakathakadi Kashaya* (NK) using *in vitro* assay and substantiates it by identifying potential bioactives responsible for DPP4 inhibition using computational biology tools. NK showed a dose-dependent DPP4 inhibition with an IC_{50} of 2.06 μg GAE/mL, and the molecular docking and simulation studies showed three compounds, namely Terchebin, Locaracemoside B and 1,2,4,6 Tetra o Galloyl Beta D Glucose having stable interactions with DPP4 similar to the standard drug Vildagliptin. Further, for the reason that polyherbal formulations exert a network pharmacology mode of action, *in silico* analysis was carried out to identify the other putative phytochemical-protein networks modulated by NK. The complex pharmacological network of the formulation was explored further using a subnetwork of diabetes proteins and their relationship with diabetes-associated comorbidities. A number of key targets like TNFα, TGFβ1, SOD1, SOD2, AKT1, DPP4 and GLP1R were identified in the protein-protein interaction network that is vital to diabetic progression and complications. A combination of *in vitro* and *in silico* methods allowed us to prove the DPP4 inhibition potential of NK as well as provided insights into the possible pharmacological networking through which NK potentially exerts its systemic effect in diabetes management.



Abbreviations: AKR1B1: Aldo-keto reductase family 1 member B; AKT1: AKT serine/threonine kinase 1; AKT2: AKT serine/threonine kinase 2; CA2: Carbonic anhydrase 1; CA7: Carbonic anhydrase 2; CA4: Carbonic anhydrase 4; CA12: Carbonic anhydrase 12;CCL2,C-C motif chemokine ligand 2; DPP4: Dipeptidyl peptidase 4; EGFR: Epidermal growth factor receptor; GLP1R: Glucagon like peptide 1 receptor;GSTM1,glutathione S-transferase mu 1; IL1α: Interleukin 1 alpha; IL10: Interleukin 10; MMP9: Matrix metallopeptidase 9; MMP2: Matrix metallopeptidase 2; PPARα: Peroxisome proliferator activated receptor alpha; PPARβ: Peroxisome proliferator activated receptor beta; NFKβ1: Nuclear factor kappa B subunit 1; PIK3Cα: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PIK3Cγ: Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit gamma isoform; PTEN: Phosphatase and tensin homolog; SOD1: Superoxide dismutase 1; SOD2: Superoxide dismutase 2; TGFβ1: Transforming growth factor beta 1; TLR4: Toll like receptor 4.

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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 in regulating body's glucose homeostasis (Brubaker & Drucker, 2004). The GLP1-mediated incretin effects are highly compromised in type 2 diabetes primarily due to the serine protease action of DPP4 resulting in GLP1 degradation. Consequently, DPP4 inhibitors, that can prolong GLP1 action in the body, are considered to be beneficial for improving glucose homeostasis without posing any risk of hypoglycaemia or weight gain (Scheen, 2012; Sharma et al., 2016). 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; Thornberry & Gallwitz, 2009). As incretin biology and DPP4 actions are crucial players of body's gut-centred systemic glucose metabolism (broadly referred as gastrointestinal mediated glucose disposal - GIGD) targeting them for developing more holistic, multi-targeted and systemic drug interventions for the management of diabetes and diabetes-associated comorbidities become an important strategy (Thottapillil et al., 2021).

Ayurveda formulations, by virtue of their multicomponent herbal ingredients, form a rich repository of bioactive molecules 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. Despite the important role DPP4 inhibitors playing in whole-body glucose homeostasis, the marketed DPP4 inhibitors are reported to have several adverse effects and are relatively expensive (Mohanty et al., 2019). This also spurs scientists to look for more affordable and safer substitutes of DPP4 inhibitors from alternative sources. In this background, the present study investigates the DPP4 inhibitory effect of an *Ayurveda* polyherbal formulation *Nisakathakadi Kashaya* (NK), prescribed for the clinical management of diabetes and associated comorbidities. The study also identifies the phytochemicals responsible for DPP4 inhibition and their mode of action using *in silico* methods of docking and molecular dynamic (MD) simulation.

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 suggest the possibility of herbal formulations (including *Ayurveda* formulations) exerting their overall biological effects through a multitarget and multi-pathway crosstalk (Zhang et al., 2021). The *Ayurveda* formulation NK selected in this study is a combination of eight herbal ingredients with known hypoglycaemic effects (Table 1). A variety of phytochemicals identified from these ingredient plants are known for their multitargeted mode of action. Therefore, our study hypothesised that the formulation, besides inhibiting DPP4 enzyme, could also exert a network pharmacology mode of action by interacting with putative genes in the human body (Patwardhan & Chandran, 2015). This prompted us to further investigate the spectrum of diabetes-related genes that are modulated by the formulation. Network pharmacology and structural bioinformatics tools allowed us to capture and collate the pharmacological networking of phytochemicals in NK and deduce the physiologically relevant signalling interactome of this multi-component formulation. A disease-gene overlap analysis performed in this 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 (Vaidyaratnam Oushadhashala, Batch No:16A3404) 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). The GLP1 ELISA kit was procured from Raybiotech (Cat. No: EIAM-GLP1-1, Mouse GLP-1 EIA). All cell culture reagents used in the study were purchased from Gibco-BRL. All other routine laboratory chemicals used were of analytical grade and purchased from SD-Fine chemicals.

To quantitatively use the formulation for DPP4 inhibition assay, total tannins present in the NK formulation were estimated using the standard Folin - Ciocalteu method using

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

S.No	Scientific Name	Common Name	PART USED
1	*Curcuma longa* L.	*Haridra*, Turmeric	Rhizome
2	*Strychnos potatorum* L.	*Kataka*, clearing nut	Seed
3	*Ixora coccinea* L.	*Paranti*	Root/stem
4	*Symplocos racemosa* Roxb.	*Lodhra*	Stem bark
5	*Emblica officinalis* Gaertn.	*Amla*, *Amalaki*, Indian Gooseberry	Fruit
6	*Aerva lanata* (L.) Juss. ex Schult	*Gorakshaganja*	Entire plant
7	*Vetiveria zizanioides* (L.) Nash	*Ushira*, Khas Vetiver	Root
8	*Salacia reticulata* Wight	*Saptachakra*, Salacia	Stem/root

gallic acid as standard (Ainsworth & Gillespie, 2007). 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'.

2.2. 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 min at room temperature. The fluorogenic substrate provided with the kit was added and the plate was read for 30 min 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,

$$\text{Percentage } (\%) \text{ activity remaining } (\text{with inhibitor}) = (\text{slope of inhibitor sample/control slope}) \times 100$$

2.3. Cell culture and GLP1 secretion assay

Murine GLUTag cell line was obtained as a generous gift from Dr. Tohru Hira, Hokkaido University, Japan with the kind permission of Dr. Daniel Drucker, University of Toronto, Canada. Cells were maintained in 18% Foetal Bovine Serum (FBS) containing Dulbecco's Modified Eagle's Medium (DMEM) at 37 °C in a 5% CO_2-humidified atmosphere. Cells seeded in poly-L-lysine coated 24-well plates (85000 cells/well) were grown for 48 h, and then serum starved for an hour. Following which, the wells were treated with glucose-free media (DMEM) with or without different concentrations of NK (12.5, 25, 50 µg of GAE/mL) and incubated for 2 h in a 5% CO2 atmosphere at 37 °C. The media was collected and briefly centrifuged to remove any debris, and secreted GLP-1 levels were assayed using GLP-1 ELISA kit (Ray biotech) following the manufacturer's instruction. The OD was read at 450 nm as per kit protocol.

2.4. 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. Detailed chemical information about the shortlisted phytochemicals was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and used for molecular docking and other bioinformatics studies.

2.5. Preparation of DPP4 structure for docking

The three-dimensional (3D) structure of DPP4 interacting 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 minimisation of the protein structure using the OPLS3e force field. The ligand-binding site of the DPP4 protein was identified from a detailed study of the 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.

2.6. 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, 2020) 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 minimisation until it passed an RMSD (root mean square deviation) limit of 0.001.

2.7. 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, 2020) (Friesner et al., 2006). In this process, ligand molecules were treated as flexible, and the receptor is considered as a 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, 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.

2.8. Molecular dynamics (MD) simulation

The top three bioactive molecules and one known inhibitor (vildagliptin) complexed with DPP4 protein were subjected to 100 ns simulation production run using Desmond (v5.6) package (Bowers et al., 2006) using OPLS_2005 force field. For each protein–ligand complex, the TIP4P water model was used as solvation medium, and the periodic boundary conditions were set to orthorhombic with the size of $10 \times 10 \times 10$ Å. The necessary number of ions was adjusted to neutralise the system and the salt concentration was maintained at 0.01 M. 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 100 ns for all four complexes. Using Simulation Interaction Diagram and Simulation Event Analysis modules of Schrodinger suite, RMSD of the protein backbone, RMSF (Root mean square fluctuation) of individual amino acids and atoms of ligand, RoG of the ligand, SASA of the ligand, PSA (Polar Surface Area), 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 100.

2.9. Target mapping of phytochemicals using network pharmacology tools

The potential target proteins 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 a minimum confidence score of 0.400 were used during the STITCH search. The SMILES notation of the compounds was 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 0.85 was used during the BindingDB search.

2.10. Network construction and disease-associated gene identification

A phytochemical target protein network was obtained using Cytoscape_v3.9.0 (Shannon et al., 1971). Every chemical with its representative targets is arranged, and files are loaded in Cytoscape. The hub proteins 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.

2.11. EnrichR analysis for identifying target-disease overlap

The reported phytochemical targets from the databases were corrected for duplicate entries and processed for protein 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 proteins 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 to obtain the common targets between diabetic complications and associated metabolic diseases.

3. Results

3.1. NK inhibits DPP4 dose-dependently in vitro as well as enhances GLP1 secretion from an intestinal cell line GLUTag

NK showed a strong dose-dependent inhibition of DPP4 enzyme with an IC_{50} of 2.09 μg GAE/mL and a maximum inhibition of 97% at 25 μg of GAE/mL and a minimum inhibition of 64% at the lowest concentration of 3.125 μg of GAE/mL tested in the study ($p < 0.001$) (Figure 1A). GLP1 being one of the most therapeutically relevant and glucose metabolism-related targets of DPP4, a parallel study conducted in our laboratory focusing on the effect of NK on incretin hormone modulation showed NK enhances GLP1 secretion from murine enteroendocrine GLUTag cell line in a dose-dependent manner (Figure 1B). Both DPP4 inhibition and GLP1 secretion results substantiate the possibility of incretin modulation by NK to exert its anti-diabetic activity. However, more studies need to be conducted to deepen the understanding of NK-mediated GLP1 secretion and its biology in metabolic health.

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. The list of herbs present in NK is listed in Table 1. To identify the phytoconstituents of NK responsible for DPP4 inhibition, a virtual screening of all 206 compounds was performed by docking them to the DPP4

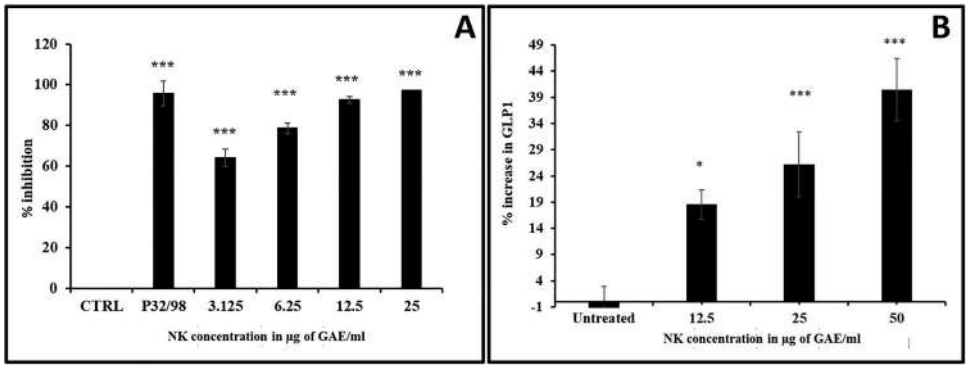

Figure 1. DPP4 inhibition and GLP1 secretion effect of NK. (A) Graph shows a concentration-dependent inhibition of DPP4 enzyme action upon treatment with various concentrations of NK (p value $\leq$ 0.001, ***). (B) The graph shows a concentration-dependent increase in GLP1 release from GLUTAG cells upon treatment with various concentrations of NK (p value $\leq$ 0.001, *** p value $\leq$ 0.01**, $\leq$ 0.05 *).

Table 2. Top scored 35 compounds from the virtual screening of NK phytochemicals with DPP4. The docking score and binding free energy from MM-GBSA analysis are reported.

Sl. No.	Compound Name	Docking Score (kcal/mol)	MM-GBSA (kcal/mol)
1	Vildagliptin	−3.899	−26.7
2	Terchebin	−11.766	−47.12
3	Locoracemosides B	−10.145	−45.45
4	1,2,4,6 Tetra o Galloyl Beta D Glucose	−9.556	−60.73
5	Tercatain	−9.252	−28.37
6	Rutin	−9.244	−41.90
7	Typhaneoside	−8.782	−45.47
8	1,6-Bis-O-Galloyl-Beta-D-Glucose	−8.45	−44.24
9	Kotalanol	−8.374	*Not obtained
10	Chebulinic Acid	−8.314	−19.48
11	Kaempferol-7-O-α-rhamnoside	−8.309	−41.87
12	Quercitrin	−8.012	−36.48
13	1,6-Bis-O-Galloyl-Beta-D-Glucose	−8.003	−15.06
14	Glucogalin	−7.993	−38.18
15	Epicatechin	−7.992	−39.34
16	catechin	−7.992	−39.34
17	Sucrose	−7.643	−28.54
18	Quercetin	−7.498	−23.39
19	Benzoylsalireposide	−7.403	−33.55
20	Procyanidin	−7.382	−22.21
21	Salirepin	−7.253	−36.51
22	Myricitrin	−7.142	−27.44
23	Symplocomoside	−6.989	−31.26
24	Luteolin	−6.985	−29.11
25	Narcissin	−6.613	−42.08
26	Corilagin	−6.541	*Not obtained
27	Bisacurone	−6.345	−10.71
28	Kaempferol 3-O-beta-D-galactoside	−6.272	−39.39
29	Proanthocyanidin	−6.114	4.71
30	Salacinol	−6.099	−40.66
31	Kaempferol-7-oglucoside	−6.076	−28.39
32	Punicafolin	−6.065	−5.8
33	Symplososide	−6.021	−32.88
34	Symploveroside	−6.009	−36.55
35	Epicatechin-4β-8	−6.008	−30.48
36	Symponoside	−5.317	−23.85

*MM-GBSA score not obtained.

protein. Well-known DPP4 inhibitor vildagliptin was used as a positive control. The top-ranked 35 compounds, based on their docking 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. A comparison of the docked complex of DPP4-Vildagliptin with the cocrystalized complex was done to validate the docking protocols and reported a minimal RMSD of 0.156 Å (Supplementary Figure 1). The compounds listed in Table 2 showed a better docking 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 binding free energy from MM-GBSA analysis, −47.12 kcal/mol, −45.45 kcal/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 are involved in Vildagliptin-DPP4 interaction and are found to interact with the top 3 compounds identified from the virtual screening (Figure 2A–D).

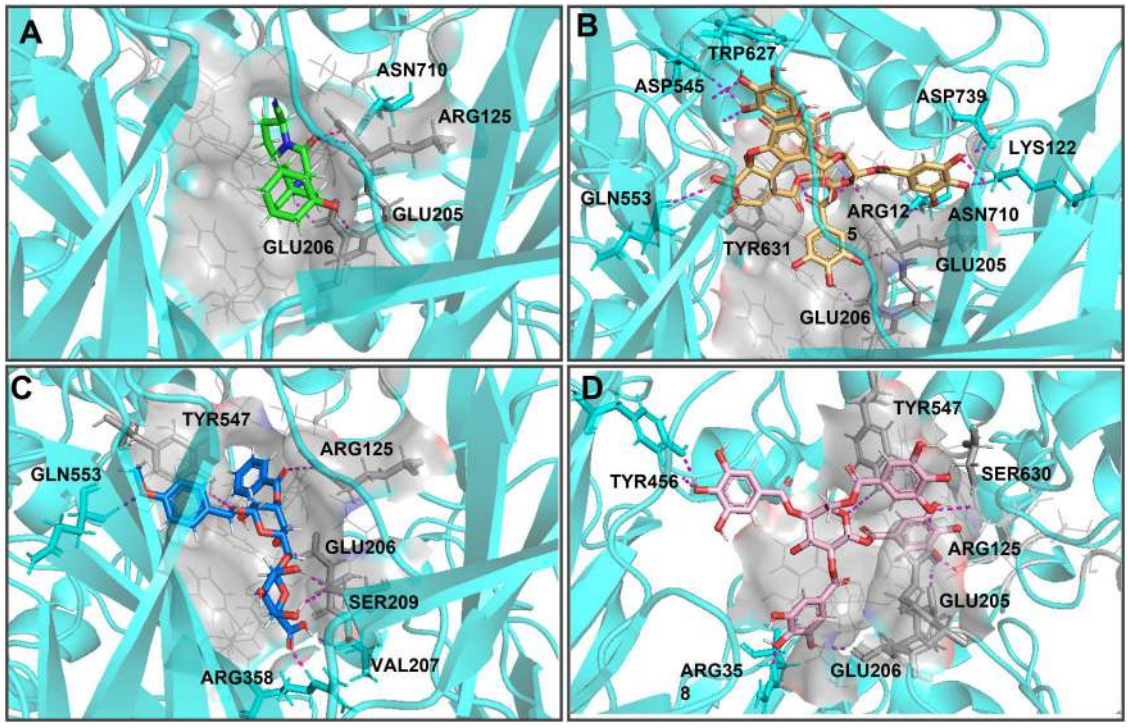

Figure 2. Interaction of DPP4 with various ligands. Interactions of (A) Vildagliptin, (B) Terchebin, (C) Locoracemoside B and (D) 1,2,4,6 Tetra o Galloyl Beta D Glucose with DPP4.

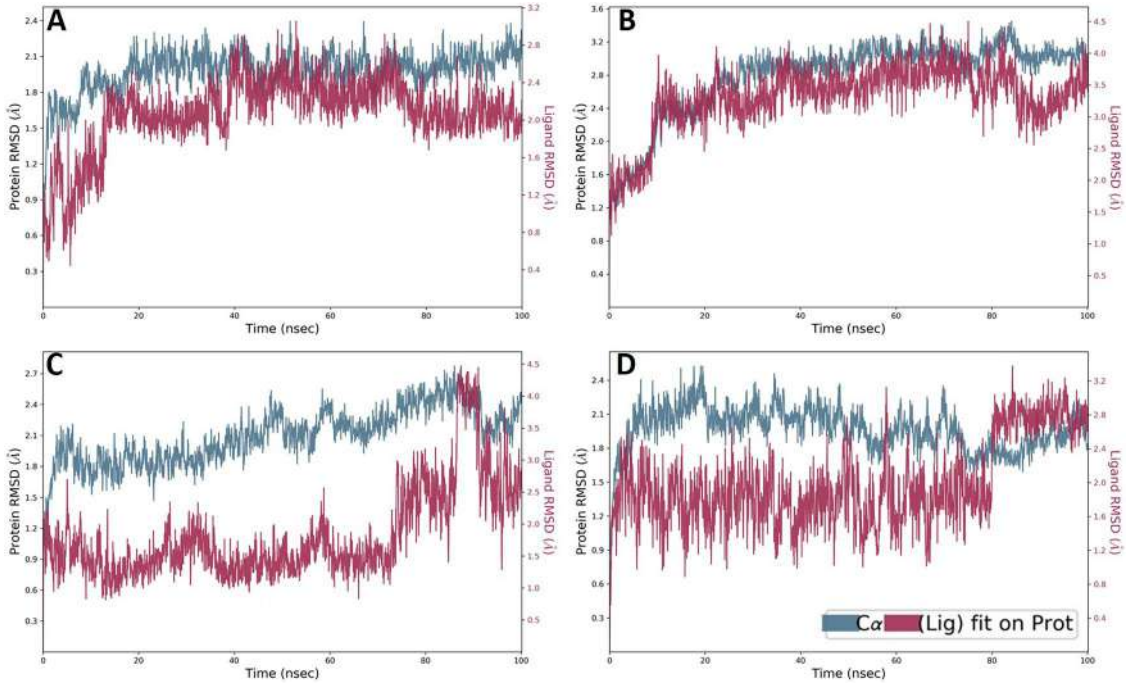

Figure 3. RMSD plot. RMSD plot of (A) Protein and Vildagliptin; (B) protein and Terchebin; (C) Protein and Locoracemoside B; (D) protein and 1,2,4,6 Tetra o Galloyl Beta D Glucose.

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 the standard vildagliptin were further subjected to a detailed interaction analysis using MD simulation. MD is a powerful computer simulation technique that is being used in the field of computer-aided drug discovery research to deepen the understanding of how the protein–ligand complex behaves in a dynamic environment at the atomic level over a user-specified time period. A 100 ns MD simulation analysis of the DPP4-ligand docked complex was performed, and the RMSD profiles of the protein and ligands during the 100 ns simulations are analysed (Figure 3). The average RMSD of the last 50 ns simulations of DPP4 (Cα atoms) and Vildagliptin with respect to protein was found to be 2.03 and 2.20 Å, respectively (Figure–3A). Whereas the average RMSD of DPP4

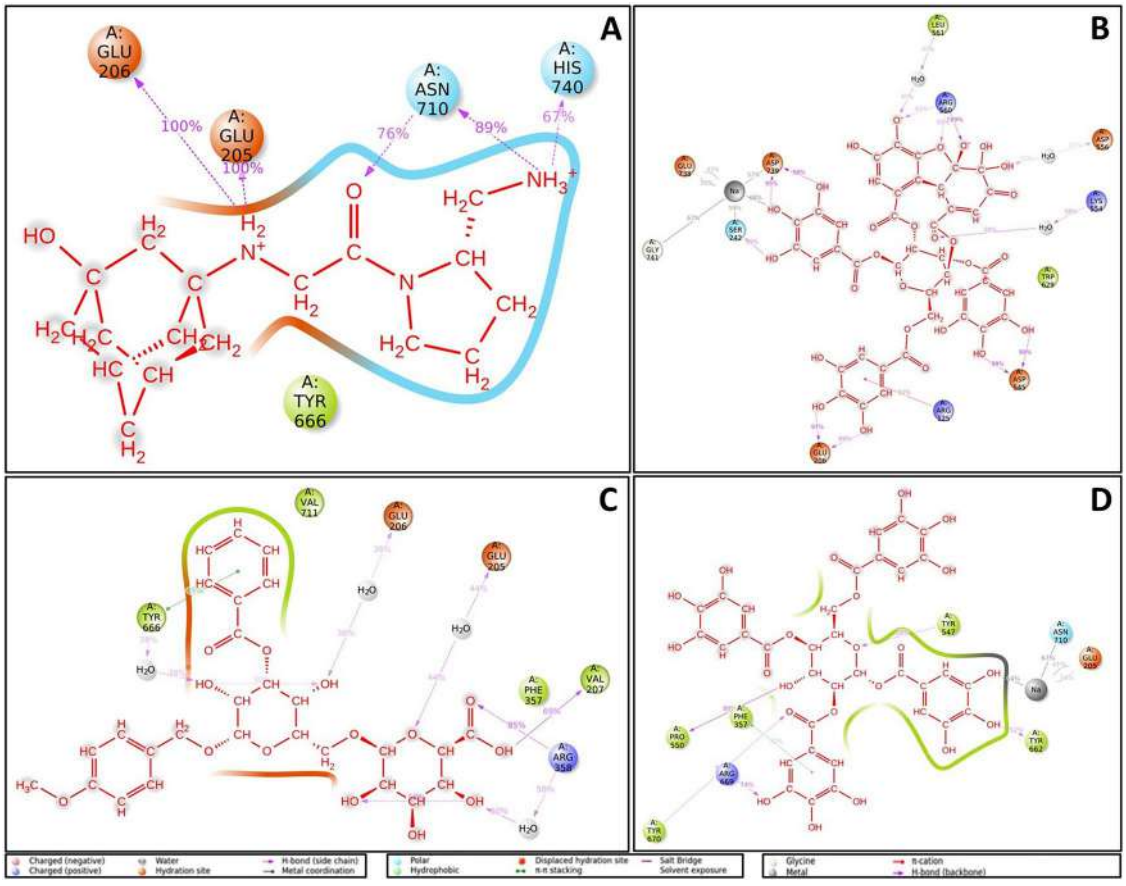

Figure 4. 2D interaction diagram of ligands with protein during the simulation. (A) Vildagliptin, (B) Terchebin, (C) Locoracemoside B and (D) 1,2,4,6 Tetra o Galloyl Beta D Glucose with DPP4.

and Terchebin was 3.07 and 3.54 Å; DPP4 and Locoracemoside B were 2.29 and 2.18 Å and DPP4 and 1,2,4,6 Tetra o Galloyl Beta D Glucose was 1.88 and 2.19 Å, respectively (Figure 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 four interactions (hydrogen bond, hydrophobic bond, water bridge, and ionic bond) using the clubbed bar graph (Supplementary Figure 2). 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 four hydrogen bonds being formed throughout the run at positions Glu205, Glu206, Asn710 and His740. Hydrophobic interactions were seen at positions Val656 and Tyr666 (Figure 4A, Supplementary Figure 2A). For DPP4-Terchebin interaction, five prominent hydrogen bonds at positions Glu206, Ser242, Tyr545 Arg560, and Asp739 as well as hydrophobic interactions at positions Lys122, Arg125, Tyr547, Trp627 and Trp629 were identified (Figure 4B, Supplementary Fig-2B). Similarly, seven prominent hydrogen bonds at positions Glu206, Val207, Ser209, Arg358, Tyr547, Ser552, Gln553, Tyr585 and Ser630 and hydrophobic bonds at Phe357, Tyr547, Tyr585, Tyr631, Val656, Trp659, Tyr662, Tyr666 and Val711 were found in the DPP4-Locoracemosids B interaction (Figure 4C, Supplementary Figure 2C). The DPP4-TGBG interaction showed prominent hydrogen bonds at Glu206, Ser209, Tyr547, Pro550, Asp556, Tyr662, Arg669, Tyr670 and Asn710 positions and hydrophobic bonds at positions Phe357, Tyr547, and Tyr666 (Figureure 4D, Supplementary Figure 2D).

We performed the analysis of the fluctuations of the protein and ligand during the simulations. The Root Mean Square Fluctuations (RMSF) of the protein during these simulations, involving four distinct ligands, are depicted in Supplementary Figure 3. Minor variations in fluctuation tendency were observed in specific local regions of the protein when different ligands were introduced. Notably, the active site residues exhibited minimal fluctuations. The fluctuations of ligand atoms are presented in Supplementary Fig-4 (A-D). As observed in the ligand interaction diagram in Figure 4, the atoms of ligands involved in interactions showed the least fluctuations. The properties of the ligands such as Surface Area (MolSA), Solvent Accessible Surface Area (SASA) and Polar Surface Area (PSA) are also analysed and is reported in Supplementary Figure 5 (A–D). The MolSA, quantifying the van der Waals surface area for all compounds, demonstrated remarkable stability, with minor fluctuations. The SASA during the simulations was most stable for Terchebin. The polar surface area contributed by oxygen and nitrogen atoms remained stable for Vildagliptin, Terchebin,

and Locoracemoside B. However, fluctuations were observed in the case of TGBG.

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 −64.29 kcal/mol, −68.99 kcal/mol, −59.51 kcal/mol and −88.51 kcal/mol, respectively. Compared to vildagliptin, Terchebin and TGBG demonstrated stronger binding energies.

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

The constituent bio-actives in herbal formulations can interact with diverse targets 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 & 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 software, 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 (Figure 5A). 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* (Kanehisa et al., 2017; Lee et al., 2021). The list of all the phytochemicals and their targets can be found in Supplementary Data 1. The complete list of hub proteins and hub compounds are given in Tables 3 and 4 (abbreviations can be accessed from Supplementary Data 6). 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, which aligned with our docking results showing strong DPP4 inhibition by these compounds.

To understand the important pathways associated with these proteins, a KEGG pathway analysis was performed. The analysis revealed a number of significant (p value $\leq$ 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 ClueGO 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 (Figure 6). The complete list of pathways and processes is given in Supplementary Data 2. These observations support the *Ayurveda* rationale of using NK for the management of diabetic-associated symptoms and complications.

3.6. Disease overlap analysis of the protein targets mapped for phytochemicals in NK formulation

To get a better idea of 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 (Supplementary data 5), and data was

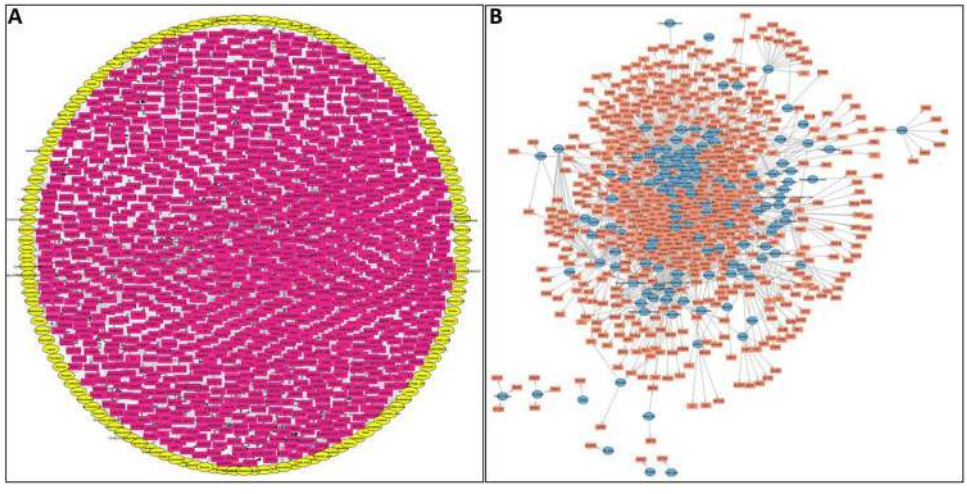

Figure 5. Network analysis of NK. (A) The compound-target network of NK - the yellow nodes represent the compounds from plants used in the NK formulation and the pink nodes represent the target proteins. (B) The network of phytochemicals of NK and diabetes-related proteins. The blue colour nodes represent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

Table 3. List of hub proteins ranked as per their degree values.

S. No.	Hub Gene	Degree	Rank	S. No.	Hub Gene	Degree	Rank	S. No.	Hub Gene	Degree		S. No.	Hub Gene	Degree	Rank
1	CA2	26	1	38	DHCR24	8	12	75	CNR2	6	15	112	GANC	5	16
2	CYP19A1	22	2	39	AKR1B10	8	12	76	CHRM4	6	15	113	ALOX12	5	16
3	CA7	18	3	40	TOP2A	8	12	77	AGTR2	6	15	114	PLAT	5	16
4	ESR1	17	4	41	ALB	8	12	78	PNLIP	6	15	115	TP53	5	16
5	F2	16	5	42	ADRA2B	8	12	79	CASP8	6	15	116	PYGL	5	16
6	AKR1B1	16	5	43	CYP1A2	8	12	80	BCL2	6	15	117	SULT1E1	5	16
7	CA12	16	5	44	ALOX5	8	12	81	UGT2B15	6	15	118	CSNK2A1	5	16
8	AR	15	6	45	PSMB5	8	12	82	TYR	6	15	119	GSK3B	5	16
9	CASP3	15	6	46	NR1I2	8	12	83	XDH	6	15	120	SI	5	16
10	ACHE	15	6	47	CA1	8	12	84	GLO1	6	15	121	TTR	5	16
11	PTPN1	13	7	48	GAA	8	12	85	NR3C2	6	15	122	AKR1A1	5	16
12	RORC	13	7	49	CHRM5	8	12	86	SLC2A1	6	15	123	FAAH	5	16
13	SHBG	13	7	50	HMGCR	7	13	87	HDAC6	6	15	124	GSTM3	5	16
14	MMP9	13	7	51	GRIN2B	7	13	88	SERPINE1	6	15	125	LOX	5	16
15	ESR2	12	8	52	ITGB3	7	13	89	PTPN2	6	15	126	DHODH	5	16
16	NR1H3	12	8	53	MAPK3	7	13	90	PLAU	6	15	127	CYP3A4	5	16
17	UGT1A7	12	8	54	CASP7	7	13	91	CYP51A1	6	15	128	VEGFA	5	16
18	UGT1A8	12	8	55	F7	7	13	92	MGLL	6	15	129	PSMB1	5	16
19	UGT1A10	12	8	56	RGS4	7	13	93	AMY2A	6	15	130	CACNA1H	5	16
20	CA4	12	8	57	ADRA2C	7	13	94	RPS6KA3	6	15	133	COMT	5	16
21	CYP17A1	11	9	58	CASR	7	13	95	CA14	6	15	134	MGAM	5	16
22	CYP1A1	11	9	59	GLA	7	13	96	BACE1	6	15	135	PLAUR	5	16
23	CRYAB	10	10	60	PDE5A	7	13	97	CTSD	5	16	136	IMPA1	5	16
24	EGFR	10	10	61	CASP9	7	13	98	CYP142	5	16	137	EBP	5	16
25	TOP1	10	10	62	UGT1A1	7	13	99	ITGAV	5	16	138	LSS	5	16
26	F10	10	10	63	CYP1B1	7	13	100	GRIN1	5	16	139	TNF	5	16
27	HSD11B1	10	10	64	NOX4	7	13	101	CYP7A1	5	16	140	AVPR1A	5	16
28	PTGS2	9	11	65	LCK	7	13	102	MPO	5	16	141	SLCO1B3	5	16
29	NR1H2	9	11	66	KDM1A	7	13	103	CRABP2	5	16	142	APP	5	16
30	MAPK1	9	11	67	SQLE	7	13	104	GNB1	5	16	143	CYSLTR1	5	16
31	AKT1	9	11	68	GRIN2A	6	14	105	HTR1B	5	16	144	EDNRB	5	16
32	ADRA2A	9	11	69	OSBP2	6	14	106	LPAR3	5	16	145	FABP4	5	16
33	UGT1A9	9	11	70	SREBF2	6	14	107	MTRNR2L2	5	16	146	FABP5	5	16
34	MAOA	9	11	71	CYP2B6	6	14	108	HCAR3	5	16	147	FFAR1	5	16
35	DPP4	9	11	72	MAPK8	6	14	109	TAS2R40	5	16	148	NOS2	5	16
36	UGT1A3	9	11	73	BCHE	6	14	110	TAS2R43	5	16				
37	MMP2	9	11	74	LPAR2	6	14	111	P2RY4	5	16				

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

extracted from three sources viz, DigiNet, ClinVar and OMIM expanded (Supplementary data 5). A total of 9000 disease indications emerged from EnrichR analysis, for which a p value of $\leq$ 0.05 was applied to short list the diseases. Further, from the entire list of disease conditions, we used the key words "Diabetes mellitus", "Insulin resistance", "Hyperglycemia", "Hypoglycemia", to shortlist 614 diabetes-associated proteins (Supplementary Data 3). From this, a subnetwork of diabetes-specific proteins was created, which contained 743 nodes and 1612 edges (Figure–5B). This showed the putative diabetic interactome of NK comprising a number of critical regulators in diabetes-related complications and comorbidities.

For investigating the association of NK targets in diabetic complications and diabetes-associated risk conditions, the 614 proteins were again subjected to EnrichR analysis. The resulting disease analysis showed various diabetic complications like nephropathy, neuropathy, cardiomyopathy and retinopathy. and also revealed the presence of related conditions such as fatty liver, obesity and general metabolic disturbances along with inflammation which is a characteristic of all these diseases (Luo & Lin, 2021). We have grouped these conditions into five specific categories viz; fatty liver, obesity, general metabolic disturbances, inflammation and diabetic complications. The details of these categories and the complete list of indications are given in Supplementary Data 3 and 4.

In order to gain insight into the gene association of diabetes with these five categories, we performed a Venn diagram analysis. The results of Venn analysis as observed (Figure 7A) showed that 37 proteins were common among all the diabetes-associated diseases and diabetic complications. Proinflammatory markers like NFKB1 and TNFα, characteristic of the insulin resistance, were present in the cluster along with lipid regulatory and obesity-linked PPARα and PPARγ (Diehl, 2004; Stienstra et al., 2007). The other critical markers were insulin signalling molecules like AKT1 and PI3K,

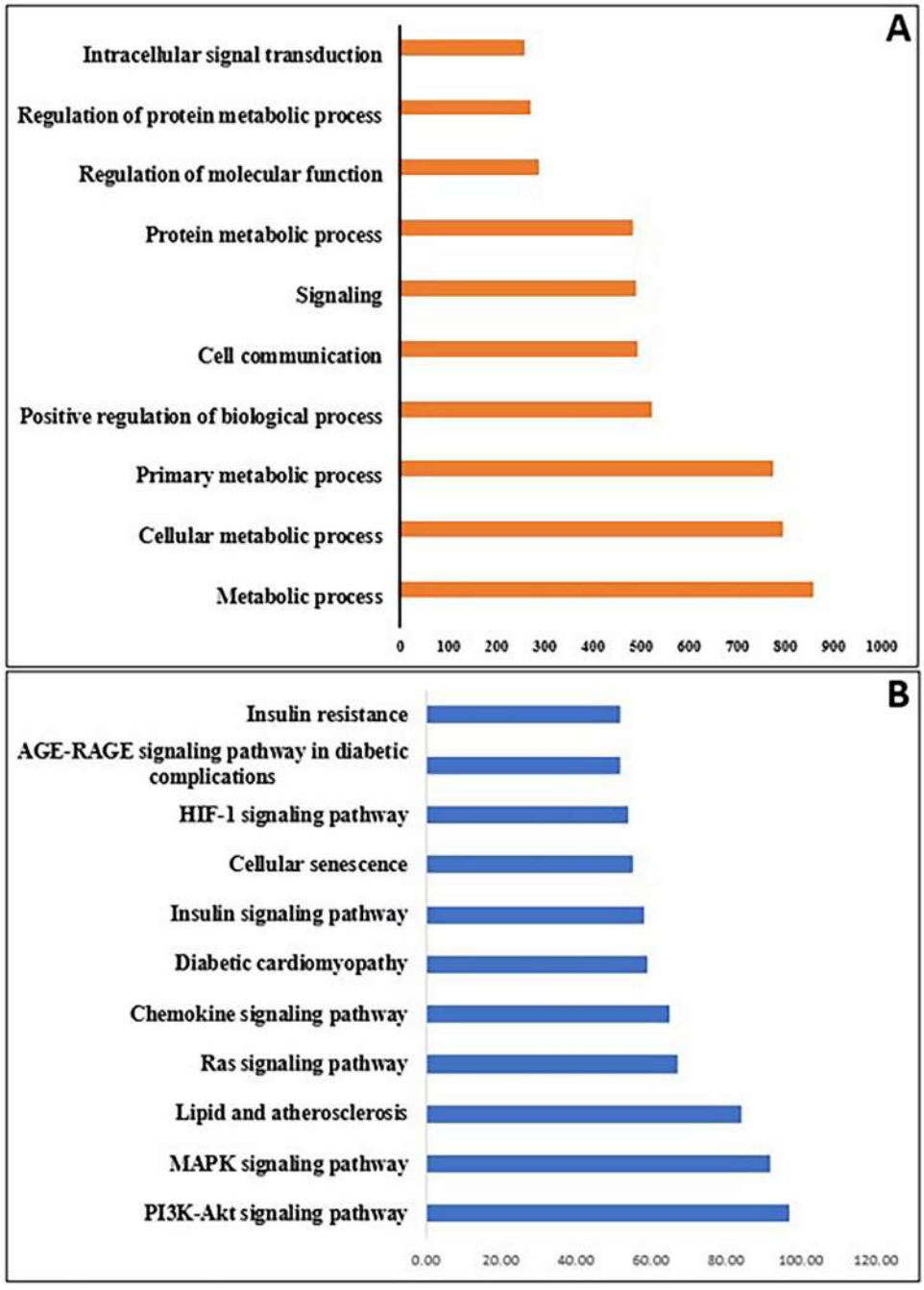

Figure 6. ClueGO analysis of the 1555 target proteins. (A) analysis of the 1555 target proteins involved in different biological processes and (B) analysis of the 1555 target proteins involved in different pathway analysis.

demonstrating that these core pathways involved in diabetic pathogenesis can be regulated by the multitargeted action of NK (Huang et al., 2018). One of the overlap categories in the Venn analysis is obesity, fatty liver and diabetic complications which have 27 shared proteins. We observed that both DPP4 and GLP1R were present in this cluster that supports the observations from recent studies showing DPP4 as a crucial gene in the regulation of fatty liver, obesity and diabetic complications (Trzaskalski et al., 2020). It further corroborated the Ayurvedic indications of NK with our experimental data on DPP4 inhibition and GLP1 secretion and Ayurvedic indications of NK.

For specific analysis of the possible overlap within the 'diabetic complications' category, we further divided it into five groups viz. diabetic retinopathy, cardiomyopathy, nephropathy and neuropathy and 'other diabetic complications. A Venn analysis of this group showed a cluster of 23 proteins identified as common (Figure 7B) and included several important markers such as TNFα, TLR4, CCL2, SOD1, TGFβ1 and SOD2, all of which are deregulated and involved in various stages of diabetic pathogenesis (Diehl, 2004; Forbes & Cooper, 2013; Stienstra et al., 2007; Yehualashet, 2020).The proteins present in each category and Venn diagram overlaps are listed in Supplementary Data 4. From our *in silico* studies, we observed that the bioactives in NK can target multiple proteins and pathways which are therapeutically relevant for diabetes and associated complications.

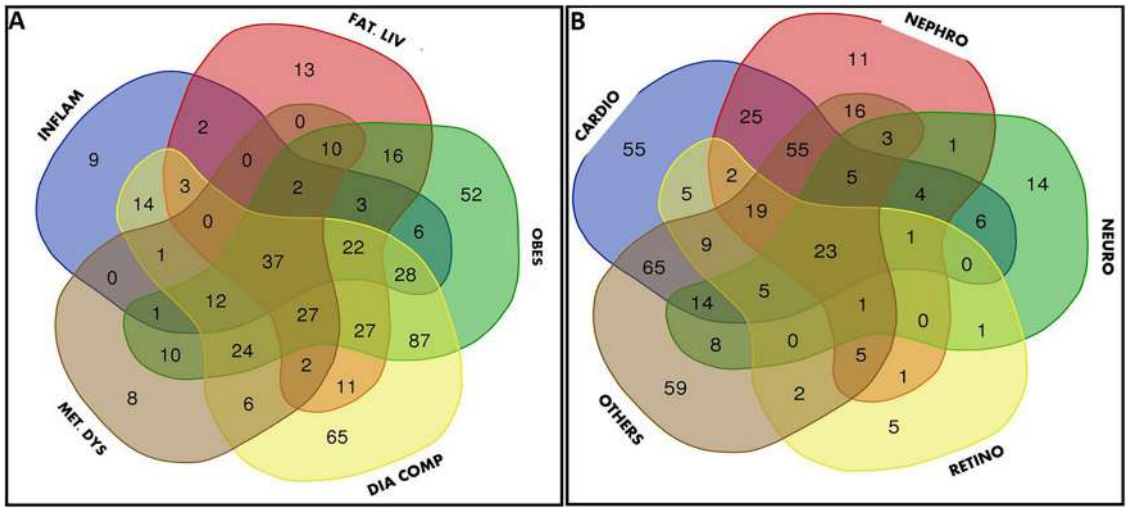

Figure 7. Venn diagram analysis. (A) represents the analysis of protein association between diabetic complications and associated diseases (B) shows the Venn analysis of proteins within diabetic complications. Abbreviations: INFLAM – inflammation; FAT LIV - Fatty liver; MET DYS - Metabolic disturbances; 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.

4. Discussion

DPP4 inhibitors have gained importance as a key therapeutic agent over the last few years due to their primary role in increasing endogenous GLP-1 levels, which is found to be important for improving 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 increased risk of cancer (Mohanty et al., 2019; Shirakawa & Terauchi, 2020). Hence, there is an increasing interest in identifying new compounds that are cheap, safe and accessible.

Ayurveda formulations and their component plants, by virtue of their long-established clinical efficacy and multi-component-multi-target mode of action, have been a source for identifying novel bioactives with better efficacy and potentially fewer side effects (Patwardhan et al., 2004). Furthermore, the concepts of *Ayurveda* have been a treasure for science-curious people to unearth new biological underpinnings of health and disease manifestations. The *Ayurveda* strategy of holistic healing and the use of poly-herbal preparations have gained global interest and are being scientifically explored through evidence-based research for better understanding of their biological actions (Patwardhan & Mashelkar, 2009). The present study evaluates the polypharmacological action of a clinically established formulation NK with a focus on its ability to inhibit DPP4, one of the relevant targets for diabetes and a potential target for many other complex metabolic diseases. As per the Ayurveda pharmacology, the herbal ingredients of this formulation have the potential to manage *prameha* (≈ diabetes) as well as alleviate disease conditions like *vrana* (wounds) and *netra roga* (eye disorders like retinopathy and cataract), which are clinically considered as diabetic complications (Nishteshwar, 2007). Therefore, Ayurveda physicians prescribe NK for patients showing manifestations of diabetes and diabetes-associated complications. Various *in vitro*, *in vivo* and clinical research evidence support the hypoglycaemic potential of these herbal ingredients of NK *viz. Curcuma longa*, *Emblica officinalis*, *Salacia reticulata*, *Symplocos racemosa*, *Vetiveria zizanioides*, *Strychnos potatorum* and *Aerva lanata* (Acharya et al., 2016; Biswas et al., 2012; Butala et al., 2017; D'souza et al., 2014; Karan et al., 2013; Marton et al., 2021; Medagama, 2015; Riya et al., 2015). *Curcuma longa* (Essa et al., 2019) and *Emblica officinalis* have been extensively studied for their anti-diabetic effects *in vitro* and *in vivo* models (Majeed et al., 2020). The ayurveda indications of NK and biomedical research evidence of its constituent plants make it an attractive candidate for Ayurveda biology research.

In our study, *in vitro* DPP4 inhibition analysis demonstrated strong inhibition of the enzyme with an IC_{50} of 2.06 μg GAE/mL, suggesting a beneficial role of NK in improving the incretin biology. Further, we also observed an increase in GLP1 secretion from the intestinal cells (GLUTag) hinting at a dual modulatory action of NK, through both DPP4 inhibition and enhancing GLP1 secretion. Although the formulation as a whole is shown to inhibit DPP4, it is important to know the nature and mode of action of bioactive(s) responsible for the inhibition. It is well-known that Ayurveda formulations will have multiple bioactive compounds that can act on targets individually or in a synergistic manner. Computational methods are used for predicting and studying these mechanisms of poly-herbal formulations to circumvent the challenges associated with classical drug discovery (Yi et al., 2018). With this focus, an *in silico* data mining and virtual screening of phytochemicals present in NK was carried out, followed by molecular docking and computational biology analyses. The results showed three compounds Terchebin, Locoracemoside B and TGBG having strong affinity and stable interactions with DPP4 comparable to the standard inhibitor Vildagliptin used in our study. Among the screened compounds, TGBG showed a high affinity with the binding energy of −88.51 kcal/mol followed by Terchebin (−68.99 kcal/mol). The DPP4 protein consists of Glu205, Glu206, Asp708, His740 and Ser630 residues that are

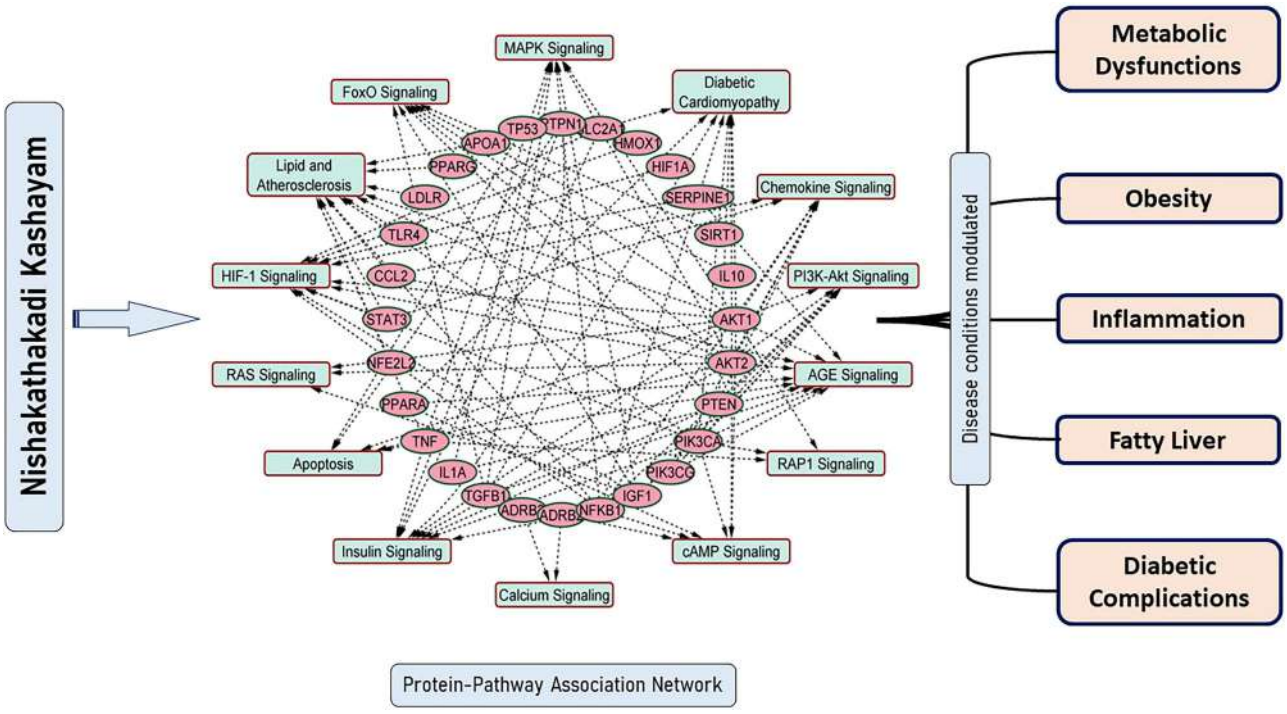

Figure 8. A Pictorial depiction of the common protein cluster that emerged from the Venn analysis between the five disease categories potentially modulated by NK and their associated signalling pathways.

essential for Vildagliptin binding and inhibition. In addition to these, other residues such as Arg125, Ser209, Phe357, Tyr547, Tyr631, Ile651, Trp659, Tyr662, Tyr666, Arg669, and Val711 are also found to play important roles in protein-ligand binding. Both Vildagliptin (standard inhibitor) and the phytochemicals data mined from NK virtual screening are found to interact with the same residues such as Glu205, Glu206, Tyr666 and Asn710 that are involved in DPP4 inhibition mechanism. This validates our *in vitro* DPP4 inhibition experiment and provides a possible mechanism of action through which the formulation inhibits DPP4. Terchebin and TGBG are reported from *Emblica officinalis*, considered a 'wonder plant' in *Ayurveda* that is widely reported for its antidiabetic and other biological actions (Baliga & Dsouza, 2011; Sharma et al., 2020). 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).

Being a repository of structurally and functionally diverse groups of molecules, polyherbal preparations are expected to interact with multiple targets in the human body resulting in a pharmacological networking. In one of the landmark papers on network pharmacology, Hopkins *et al.* emphasised the imperative and relevance of this poly-pharmacology in overcoming the challenges of complex disease management. He suggested that complex diseases may require a multi-targeted therapeutic strategy for getting the desired results (Hopkins, 2008). This concept of pharmacological networking is much relevant in the context of Ayurveda formulations, due to their multi-component nature and systemic actions. Studies conducted on *Triphala* and *Nishamalaki* have shown the importance of network pharmacology approaches for studying *Ayurveda* formulations (Chandran et al., 2015). Taking this hypothesis forward, we analysed the phytoactives identified from NK using *in silico* methods and unveiled 1555 proteins as the possible targets. The hub proteins with maximal interaction included carbonic anhydrases (like CA2, CA7, CA4, CA12 and CA12) as well as some of the important proteins like epidermal growth factor receptor (EGFR), aldose reductase (AKR1B1), Matrix metalloproteinases (MMPs) and Aryl hydrocarbon receptor (AR) that are significantly involved in the pathophysiology of diabetes and diabetic complications. The carbonic anhydrases (CAs) are a group of ubiquitously expressed metalloenzymes that are involved in numerous physiological processes like gluconeogenesis, lipogenesis, ureagenesis and tumorigenicity (Imtaiyaz Hassan et al., 2013; Torella et al., 2014). Some of the recent studies indicate their potential as novel targets in obesity and diabetes management (Supuran, 2008). In addition to the above proteins, DPP4 is also identified as a hub protein with multiple protein interactions. This demonstrated that the NK interactome may possibly involve many targets, other than DPP4 and GLP1, which are central to diabetic complications resulting in the multi-modal actions of the formulation.

To delve deep into the diabetic-specific PPI network of NK we used the EnrichR tool for disease protein association analysis. The 1555 protein targets of NK were submitted in the EnrichR tool, from which 614 diabetes-specific proteins were extracted using the key words 'diabetes mellitus', 'insulin resistance' and 'hyperglycemia'. Since NK is a formulation used for diabetes and related complications, we wanted to see the overlap of NK diabetic network with related conditions like obesity, inflammation, fatty liver, metabolic dysfunction and other diabetic complications. The Venn diagram analysis of the data showed a cluster of 37 common proteins including important signalling kinases (AKT1, PIK3Cα and

PIK3Cγ), key obesity-associated transcription factors (PPARα and PPARβ) and the pro-inflammatory mediators (IL1α, IL10, TNFα, CCL2, TGFβ1, and NFKβ1). This demonstrated the complex interplay between insulin signalling pathways and lipid synthesis which are deregulated in lifestyle diseases like diabetes, obesity, and NAFLD. Apart from this, 87 proteins were found to be overlapping between diabetic complications and obesity, which affirms the well-established role of obesity as a predisposing factor for diabetes. The presence of DPP4 and GLP1R as common proteins between fatty liver, obesity, and diabetic comorbidities stresses the importance of the gut and its incretin axis in the possible polypharmacological drug network of NK.

A Venn analysis conducted between the diabetic complications such as retinopathy, neuropathy, nephropathy and myopathy, wound healing and micro and macrovascular complications showed 23 proteins common among all these complications. Among these, inflammatory mediators like TNFα, IL10, CCL2, TLR4 and TGFβ1, MMP9 and MMP2 were found to be associated with the development of insulin resistance and diabetic nephropathy progression (Forbes & Cooper, 2013; Kowluru et al., 2012). AKR1B1, present in the common cluster of proteins, is involved in the conversion of excess glucose into sorbitol. Recent studies prove that overexpression of AKR1B1 is involved in many diabetic complications like cardiovascular diseases and retinopathy (Ramasamy & Goldberg, 2010). Oxidative stress induced by chronic hyperglycemia is one of the main pathways for development of macrovascular and microvascular complications (Rehman & Akash, 2017). One of the main mechanisms mediated by polyherbal formulations in alleviating diabetes is by reducing oxidative stress through multiple mechanisms (Unuofin & Lebelo, 2020). The Venn diagram between the diabetic complications showed the presence of oxidative stress markers like GSTM1, SOD1, and SOD2 in the central cluster, indicating the possibility of NK acting through oxidative stress signalling.

Out of 37 common proteins identified from the Venn analysis, 28 proteins are found to be associated with five well-studied and critical biochemical pathways in the pathogenesis of diabetes, its comorbidities and complications (Figure 8). Among those, the PI3K/AKT signalling pathway regulates cell proliferation, differentiation, metabolism, and cytoskeletal reorganisation. Insulin is one of the main ligands for activation of PI3K signalling through which it governs a variety of physiological processes in adipose, muscle, brain, liver and pancreas. Multiple proteins like AKT1, AKT2, PIK3Cγ, PIK3Cα and PTEN are associated with these two pathways. They are also involved in MAPK, HIF1α and AGE signalling and all of which play important roles in the progression of diabetic complications like fatty liver disease, obesity and macrovascular and microvascular complications. Similarly, the lipid and atherosclerosis pathway also includes many proteins like TLR4, IL1α, NFKβ, CCL2 and TNFα, all of which are studied for their inflammatory action in diabetes associated disorders. TLR4 and CCL2 are also emerging targets as they play a key role in many diabetic complications like nephropathy and retinopathy. Other important pathways associated with the disease overlap of proteins include HIF1α, cAMP, RAP1 and Calcium signalling as well as the apoptotic pathway.

5. Conclusion

The present work, as evidenced through *in vitro* and *in silico* observations, suggests that the anti-diabetic formulation NK has a strong DPP4 inhibitory potential. Further, our network pharmacology studies of the phytochemicals identified from NK revealed a complex molecular interactome consisting of crucial proteins and significant pathways in diabetes, diabetes-related complications and associated metabolic diseases. This integrated approach of combining *in vitro* and *in silico* methods provides the much-needed scientific rationale for the multi-targeted mode of action of traditionally prescribed polyherbal *Ayurveda* formulations in the treatment of complex diseases like type 2 diabetes. The pathways and molecular targets delineated from our work merit detailed investigation, which will provide more insight into the biological action of this *Ayurveda* formulation.

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Disclosure statement

Authors declare no conflict of interest.

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Competing interests

The authors declare no financial or non-financial interests that are directly or indirectly related to the work submitted for publication.

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Anti-diabetic and anti-adipogenic effects of polyherbal formulation *Varanadi kashayam* with a focus on incretin modulation.

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Abstract

Managing complex disease like diabetes requires a systems and network medicine approach. Polyherbal formulations used in Indian Systems of Medicines (ISMs) like *Ayurveda* and their multi-targeted mode of action could be an effective way to bring out this systems biology pharmacology effect required for chronic diseases. These multi-component formulations are also served as sources for identifying novel ligands that interact with drug targets having systemic effects. The present study uses *Varanadi Kashayam* (VK), a polyherbal preparation used in *Ayurveda* clinical management of diabetes and its comorbidities, to demonstrate the systemic pharmacological action exerted by a formulation with a special focus on incretin modulation. *In-vitro* assays demonstrated the inhibitory potential of VA on α-glucosidase and DPP4 enzymes and adipogenesis. It also showed an increase in GLP-1 secretion from GLUTag cells. These observations were corroborated in the *in-vivo* high-fat fed model of SD

Artificial Neural Network based Self Organizing Maps analysis for Clinical Trials of Indian Systems of Medicine

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Abstract:

Clinical studies are imperative to confirm the efficacy and safety of pharmacological interventions. Contemporary clinical study protocols are largely rooted in molecular concepts of drug interactions wherein specific molecular markers are used as clinical readouts. Standard regression analysis models are used to compare between various control and test groups in the