Effect of bioactive molecules from phytochemical data base as possible therapeutic agents on aortic tissue proteins (ACE2 and MMP-7) in hypertensive rat model using *in silico* and *in vivo* methods

BLDE (DEEMED TO BE UNIVERSITY)
Vijayapura, Karnataka, India.

Doctor of Philosophy
In
Allied Health Sciences
(Biotechnology)

By

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Declaration by the Candidate

I hereby declare that this thesis entitled **"Effect of bioactive molecules from phytochemical data base as possible therapeutic agents on aortic tissue proteins (ACE2 and MMP-7) in hypertensive rat model using *in silico* and *in vivo* methods"** is bonafide and genuine research work carried out by me under the supervision of Professor Kusal K. Das, (Guide), Department of Physiology, Shri B. M. Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura, Karnataka, India and Dr. Sumangala M. Patil, Department of Physiology Shri B. M. Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura, Karnataka, India (Co-guide).

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Faculty of Allied Health Sciences

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Dedicated

To my lovely mother

Ms. Farzana Begum Hattiwale,

Late ***Mr. Khawajaameen Patel,***

my lovely elder sister

Ms. Ameena Patel,

brother and mentors whose constant support, encouragement, affection and love make me able to succeed.

And

A dedicated note to myself

"It takes courage to grow up and became who you really are".

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Abbreviations

NCBI	National Center for Biotechnology Information
NPACT	Naturally Occurring Plant-based anti-cancer compound activity Target
WHO	World Health Organization
FDA	Food and Drug Administration
CT	Cycle Threshold
RT-PCR	Real Time Polymerase Chain Reaction
MMP-7	Matrix metalloproteinase 7
LV	Left ventricle
ANS	Autonomic nervous systems
TIMPs	Tissue inhibitors of metalloproteinases
LVH	Left ventricular hypertrophy
CHF	Chronic heart failure
CKD	Chronic kidney disease
ECM	Extracellular matrix
%	Per cent
µl	Microliter
µM/L	Micro Moles per Liter
ACE	Angiotensin Converting Enzyme
Ang II	Angiotensin II
ANOVA	Analysis of Variance
ANS	Autonomic nervous system
ATP	Adenosine 5'-Triphosphate
AT1	Angiotensin II type 1 receptors
AT2	Angiotensin II type 2 receptors
b. wt	Bodyweight
BP	Blood Pressure
LF	Low frequency
L-NAME	N^G- Nitro L-Arginine methyl Ester
LVA	Low voltage-activated
LVH	Left ventricular hypertrophy
m2	Meter square
MAP	Mean Arterial Pressure

MDA	Malondialdehyde
mg	Milligram
mg/dl	Milligram per decilitre
mg/kg	Milligram per kilogram
mI U/L	Milli International Units per Litre
mm Hg	Millimetre of mercury
mm3	Cubic millimetre
MMPs	Matrix Metalloproteinases
N2	Nitrogen
Na+	Sodium
NaCMC	Sodium Carboxymethyl cellulose
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaNO2	Sodium nitrite
NaOH	Sodium hydroxide
NIBP	Non-invasive blood pressure
nm	Nanometre
nNOS	Neuronal Nitric oxide synthase
NO	Nitric Oxide
NOS	Nitric oxide synthase
NOS2	Inducible NOS
O2	Oxygen
$O2^{-}$	Superoxide anion
OD	Optical density
$ONOO^{-}$	Peroxynitrite
PCR	Polymerase chain reaction
pg/ml	Picogram per millimetre
RAS	Renin Angiotensin System
RAAS	Renin Angiotensin Aldosterone System
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RR	Respiratory rate
RVLM	Rostro ventrolateral medulla
SA	Sinoatrial

SBP	Systolic blood pressure
SD	Standard deviation
Sec	Seconds
SHRs	Spontaneously hypertensive rats
SNS	Sympathetic nervous system
SOD	Superoxide dismutase
SPSS	Statistical Package for the Social Sciences
STAT	Signal transducer and activator of transcription
TBST	Tris-buffered saline with Tween 20
TBS	Tris buffered saline
TMB	3,3’,5,5’-Tetramethylbenzidine
VSMCs	Vascular smooth muscle cells
HRV	Heart rate variability

CHAPTER-1

Introduction

Hypertension is an important worldwide public-health challenge because of its high frequency and high risks of cardiovascular disease. According to WHO reports (2019) 26% of the world's population (972 million people) has hypertension, and the prevalence is expected to increase 25 % by 2025 especially in developing countries (Kearney PM. et al., 2005). Among the multiple risk factors for CVD, high blood pressure (BP) is associated with the strongest evidence for causation, and it has a high prevalence of exposure. Hypertension is indeed the major cause of cardiovascular remodeling, including myocardial ventricular hypertrophy and fibrosis, chronic kidney disease (CKD) and intimal medial thickening, calcification, and fibrosis of vascular wall with increased arterial stiffness. The gradual and chronic target organs remodeling in hypertensive subjects is mediated by different pathogenic molecular mechanisms (i.e., oxidative stress, endothelial and platelet activation, sympathetic nervous system and Renin Angiotensin-Aldosterone system over activity), and included changes in the ECM structure (Bisogni, et al., 2020).

Two specific hypertension regulatory vascular proteins:

ACE2:

The classical regulation of the cardiovascular system relies on the rennin angiotensin system (RAS) pathway, which has evolved over 60 years. Angiotensin II (Ang II) is a key hormone in this pathway, and its metabolism involves a dynamic interplay between angiotensin converting enzyme (ACE) that generates Ang II and ACE2 that removes it by converting it to Ang-(1-7). This process establishes a counterbalance in angiotensin metabolism (Dudoignon E, et al., 2019).

ACE2 plays a crucial role in modulating blood pressure and maintaining homeostasis by negatively regulating the RAS. Both ACE and ACE2 are pivotal

enzymes in synthesizing bioactive compounds within the RAS, and their effects are mediated through three receptors: AT1, AT2, and Mas (Das KK, et al., 2023).

The rennin angiotensin aldosterone system (RAAS) plays a major role in the regulation of blood pressure vascular tone, renal hemodynamic, myocardial contractility via an enzymatic cascade leading to the synthesis of angiotensin 2 (ang-2). Ang-2, synthesized from ang-1 by the angiotensin converting enzyme (ACE), exerts its actions via 2 receptors (AT1 and AT2) present on the surface of many cells (cardiomyocytes, smooth muscle cells, fibroblasts, endothelial cells and immune cells). In chronic condition, the activation of the RAAS has been associated with morbidity and mortality related to cardiovascular damage secondary to structural changes in the myocardium and the development of chronic kidney disease Dudoignon, et al., (2019).

Ang II mediates its biological actions by the selective binding to two different types of G-protein-coupled receptors— AT1 (of which there are two subtypes, AT1A and AT1B) and AT2—that can have opposing effects (Bernstein and Berk 1993, Brede and Hein 2001). The importance of Ang II is supported by the widespread clinical use of ACE inhibitors (and more recently AT1 blockers) in a wide array of cardiovascular and renal diseases. However, the RAS is far more complex than previously envisioned and several new aspects of the RAS have been identified recently. First, tissue Ang II may be generated by non-ACE related enzymes. For example, tissue chymase activity appears to generate Ang II (Wei, et al., 2002). Second, ACE can cleave and inactivate other peptides such as bradykinin and kallidin, which are potent vasodilators that counterbalance the effects of Ang II (Turner and Hooper 2002). Third, another recently discovered enzyme in the RAS, ACE2, also metabolizes various peptides of

the RAS (Donoghue, et al., 2000; Tipnis, et al., 2000; Vickers, et al., 2002). This review examines the properties and *in vivo* role of ACE2 in cardiovascular physiology in the context of the RAS.

MMP-7

MMP-7 is a smallest protein member of MMP family. MMP-7, also called matrilysin, is secreted as a 28 kDa proenzyme and is activated upon the removal of the pro-domain to generate a 19 kDa active enzyme3. Matrix metalloproteinase (MMPs) is a family of proteolytic enzymes that regulate remodeling of the left ventricle (LV). MMPs and their inhibitors (tissue inhibitors of metalloproteinases – TIMPs, which inhibits the proteolytic action of MMPs) play a key role in several physiological processes, including activation of immune cells, cells proliferation, migration, and differentiation, remodeling of extracellular matrix (ECM), and tissue invasion and vascularization (Jablonska-Trypuc, et al., 2016).

Changes in MMPs and TIMPs expression and/or activity have also been described in many inflammatory and cardiovascular diseases (CVD). Macrophages and cardiomyocytes are rich sources of MMP-7, 3, 4 and increased MMP-7 levels are detected in both the remote and infarct regions cardiovascular system. Naturally occurring bioactive compounds are ubiquitous in maximum nutritional better flora for human beings and livestock (Ali, M.A et al., 2012; Egebled. et al., 2012). In systemic hypertension, the bioactive molecules may be explored for their function in modulating MMP-7, thereby regulating systemic hypertension (Cabral-Pacheco and Griselda A, et al., 2020).

MMPs and Hypertension-Mediated Cardiac Remodeling; Persistent high BP values lead to adaptive processes also in the heart, called “cardiac remodeling”, which are characterized by intrinsic cardiomyocyte modifications and interstitium reorganization with increased collagen deposition, fibrosis, and changes in ECM components. MMPs and other proteases over activation has been proposed as a mediator of cardiac remodelling. This latter is characterized by the interruption of connections between cardiomyocytes and blood vessels. Moreover, the excessive production and accumulation of ECM structural proteins results in enhanced stiffness of the myocardium and impedes ventricular contraction and relaxation, leading to distorted architecture and function of the heart (Vilmi-Kerala, et al., 2017).Although these mechanisms are initially compensatory and preserve cardiac contractile performance with development of left ventricular hypertrophy (LVH) and other structural changes, they finally lead to left ventricular dilatation and chronic heart failure (CHF) (Bockelman C, et al., 2018).

To treat in the last three decades, several intensive efforts have been conducted into researching the antihypertensive therapeutic values of local plants and as compared to allopathic treatment, medicinal and bioactive plants have become a vital resource for the treatment of heart problems (Du, G. L. et al., 2017).

Almost 80% of the world population use natural medicines, largely in developing countries, because of their better acceptability with human physiology and lesser side effects. Some reports suggested that plant-based medicine and their bioactive compounds have become used for treatment of cardiovascular disease. FDA approved and clinical candidates, phytomedicine-derived bioactive compounds (or

simply called phytochemicals such as curcumin, quercetin, epigallocatechin gallate EGCG and many others) have also been extensively investigated in search for potential lead molecules/drug candidates (Alazmi M. and Motwalli O. et al., 2021). Therefore, to control blood pressure and heart failure diseases without any side effects is increasing demand for natural products in health care system.

Computational and bioinformatics tools have become very important resource to identify the potential targets for various ligands. Virtual screening is an important consideration. Using these virtual tools, we can analyze to find any phytochemical as a good therapeutic drug (Muhammad S. A, et al., 2015) as *in-silico* screening of phytochemical database has gained tremendous interest in drug discovery research for the identification of new drugs; hence the present study was aimed to assess the effects of screened bioactive molecules on MMP-7 by in silico analysis.

β-sitosterol is a potent micronutrient, and it is present widespread in higher plants. Animals obtain this phytosterol through their diet (Prachi, et a., 2022). Heart Rate Variability (HRV) is a "beat-to-beat" oscillation of the heart rate around its mean value. It represents a physiological phenomenon determined predominantly by the balance between the Autonomic and parasympathetic Nervous systems (ANS) (Patel SK, et al., 2023*)*. Therefore, measuring Heart rate variability is a non-invasive technique that can investigate the dynamic balance between sympathetic and vagal activity (Pereira-Junior PP et al., 2010).

In vivo, vasodilators and vasoconstrictors modulate the endothelial function. It is established that Nitric Oxide (NO) produced in vascular endothelial cells has a potent vasodilator effect. It plays an important role in vascular resistance and growth.

L-arginine analogues such as N-nitro-L-Arginine Methyl Ester (L-NAME) hydrochloride administration inhibit Nitric Oxide Synthase (NOS) activities (Shaikh GB, et al., 2020). Hence, they will reduce NO biosynthesis, which will lead to hypertension. In conditions of NO deficiency, there will be an accumulation of superoxide anion in biological tissues, which can lead to alterations in organ function. NO acts as an endogenous anti-oxidative agent. It reacts with superoxide anions generated in the living tissues and protects from their deleterious effects on many organs, including the heart, kidney, etc. (Diallo MST et al., 2019). There were several adverse reports of therapeutic use of plant-based bioactive compounds as antihypertensive agents; hence, more studies are necessary to understand better their safety and efficacy to prevent possible risks (Aubert AE et al., 1999). As the antihypertensive effects of β-sitosterol have been least studied, the present study assessed its effects on heart rate variability in L-NAME-induced hypertensive rats with particular reference to oxidative stress.

CHAPTER-2

Review of Literature

2.1 Plant based phytochemicals:

Plant species have always been vital to humanity. For medicinal and pharmaceutical purposes, in particular, the formal study of these plants has proven to be a significant tool for comprehending how various indigenous societies interact with natural resources Research on ethnomedicine has been crucial in developing natural and artificial medications (Prachi, et al., 2022).

In recent years, several adequate drug screening studies have employed phytochemical knowledge as a springboard (Heinrich & Bremner, et al., 2006). Traditional medicines remain the primary form of therapy for about 80% of the world's population, mainly the rural populations of developing nations, according to the World Health Organization (WHO). Ethnobotany is the study of this long-standing interaction between humans and plants. Ethnobotany or ethnobiology is the scientific report of plant and human relationships, demonstrating that plants are a fundamental source of need (Prachi, et al., 2022).

The ethnic or tribal population has relied on native flora for therapeutic and other purposes since ancient times. Based on their adventures in maintaining their health, rural and tribal groups' traditional medicine systems represent indigenous knowledge and practices that have been passed down for centuries, though much remains undocumented (Sharma et al., 2012; Mesfin et al., 2013). These systems employ natural remedies to treat illnesses, with herbal pharmaceuticals playing a key role in human health. There is a need to better record the remedies and medicinal knowledge of indigenous and tribal peoples.

As a widespread, invasive species, no special intervention is warranted. Phytochemical analysis shows the plant contains alkaloids, flavonoids, polyphenols, carbohydrates, amino acids, and terpenoids. The ethanolic leaf extract of β-sitosterol demonstrates anti-inflammatory, anti-nociceptive, and hyaluronidase inhibitory activities in mouse models, which may be explained by the presence of secondary metabolites like stigmasterol and lupeol. Existing literature highlights the antitumor potential of phytochemicals like apigenin, luteolin, lupeol, and β-sitosterol found in this plant family (Saha & Paul, 2017; Misra et al., 1999). Specific chemicals isolated from Asteracantha longifolia include 3-methylnocosane, 23-ethylcholesta-11, lupeol, 25-oxo-hentriacontanyl acetate, and methyl 8-n-hexylteracosanoate.

Early phytochemical studies by Doss, et al., (2009) revealed tannins, steroids, cardiac glycosides, saponins, and phenols in Asteracantha longifolia leaf extracts. (Doss and Anand, et al., 2012) examined the folkloric uses of alkaloids, phenolics, tannins, and flavonoids from A. longifolia. Hot water extracts were found to contain excellent sources of essential minerals. A. longifolia may have potential as a nutraceutical and cattle feed supplement due to its antioxidant and macro/micronutrient content (Amit Kumar, et al., 2012). The seed oil contains gamma-linolenic and oleic acids, while the stem has phenolics and flavonoids that can scavenge free radicals and metal ions in hemolytic anemia.

β-sitosterol seed ethanolic extracts demonstrate potent antioxidant and free radical scavenging *in vitro*, including reducing power and iron chelation (Lobo, et al., 2010). This signifies it may help combat oxidative stress, though the specific antioxidative compounds remain unknown. For *in vivo* antioxidant testing in

cadmium-intoxicated mice, the ethyl acetate fraction of β-sitosterol methanolic extracts was selected due to superior in vitro activity (Prachi, et al., 2022).

Petroleum ether, chloroform, alcohol, and aqueous H. spinosa leaf extracts displayed antibacterial activity against E. coli, S. aureus, B. subtilis, and P. aeruginosa using disc diffusion (100 mg/disc), increasing inhibition zones (Patra, et al., 2008). Fractionated H. scricta and P. pellucida extracts showed greater antibacterial effects than crude extracts, especially butanol fractions of P. pellucida and petroleum fractions of H. stricta (18-20 mm inhibition) (Njateng et al., 2002). H. auriculata acetone leaf extracts (10-30 mg/ml) inhibited S. aureus, B. cereus, B. subtilis, and P. aeruginosa growth, with 30 mg/ml showing the largest inhibition zones (Prasanna & Sridhar, et al., 2016). Alcohol extracts exhibited greater antibacterial effects than aqueous (Biswas, et al., 2017).

2.2 Anti-Diabetic activity

Previous research has investigated the anti-diabetic properties of several plant extracts. (Vijaykumar, et al., 2006) examined the hypoglycemic effects of an ethanol extract from Hygrophila auriculata aerial parts in diabetic rats. They found that 250 mg/kg of the extract significantly lowered blood glucose levels. It also reduced oxidative stress by lowering lipid peroxidation and restoring glutathione and antioxidant enzymes in the liver and kidneys of diabetic rats. These results suggest Hygrophila auriculata has anti-diabetic and antioxidant effects. In another study, (Fernando, et al., 1991) looked at hot water extracts of Astracanthus longifolia in normal and diabetic human subjects. At a dose of 20g/kg, the extract improved glucose tolerance in both groups. While it did not directly study insulin secretion, the

results imply Astracanthus longifolia may have hypoglycemic effects by stimulating pancreatic insulin release. Finally, (Solomon, et al., 2011) tested anti-diabetic effects of Hygrophila spinosa root extracts in diabetic rats. Overall, these studies demonstrate specific plant extracts may have therapeutic potential for managing diabetes through various mechanisms such as lowering blood glucose, reducing oxidative stress, and potentially stimulating insulin secretion.

2.3 Antihypertensive activity:

As per literature search on antihypertensive activity, several studies have explored the effectiveness of various compounds in reducing blood pressure. One study focused on newly synthesized xanthone derivatives, showing that compounds 1a, 1b, and 2 exhibited significant antihypertensive activity with minimal toxicity compared to standard drugs like Propranolol and Atenolol Omprakash Goshain et al 2019. Another study evaluated the antihypertensive effect of cleistanthins A and B, demonstrating their ability to reduce blood pressure through alpha-adrenergic receptor blocking properties similar to prazosin (R.P. Priyadharsini, et al., 2014).

Additionally, research has delved into the molecular targets of antihypertensive peptides to understand their mechanisms of action based on hypertension pathophysiology. These peptides target various pathways involved in hypertension, offering insights into potential therapeutic strategies (Kaustav Majumder, et al., 2015). Furthermore, a literature review highlighted new drug targets for hypertension, emphasizing the importance of identifying novel targets for effective management of high blood pressure. These studies collectively contribute to the understanding of anti-hypertensive activity by exploring novel compounds,

peptide targets, and drug development strategies aimed at combating hypertension (Kaustav Majumder, et al., 2015).

2.4: Hypertension

Hypertension is a significant global health issue, with high prevalence worldwide. In India, hypertension is the leading non-communicable disease, affecting an estimated 200 million people. Studies show higher age-adjusted hypertension rates in Indian men (24.5%) versus women (20%), and higher rates among metropolitan, more affluent, and economically developed populations. Unique factors include rising rates among younger adults and in both urban and rural areas (Gupta and Ram, et al., 2019). Despite the high prevalence, awareness, management and control of hypertension in India remain low (Chakraborty et al., 2021). Key risk factors for primary hypertension include older age, obesity, and high dietary sodium intake (Lerman et al., 2019).

2.5: NG-Nitro- L-Arginine Methyl Ester (L-NAME)

Nitric oxide (NO), synthesized by nitric oxide synthases (NOS), plays a key role in hypertension pathophysiology. The three NOS isoforms are: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). eNOS is constitutively expressed in the endothelium, while nNOS is primarily in the nervous system. iNOS is induced by inflammatory stimuli in macrophages and other cell types. eNOS and nNOS are calcium/calmodulin regulated, while iNOS has calmodulin permanently bound. Transient blood pressure increases evoke greater NO release, lowering pressure; drops in pressure reduce NO. Genetic hypertension models have increased NO and NOS activity versus normotensive controls, indicating high blood pressure

increases NO generation via mechanisms that require elucidation. Shear stress and other mechanical factors may impact NO production and eNOS expression (Daff, et el., 2010).

2.6: L- NAME Induced Hypertensive Rat Model

Mice lacking eNOS develop hypertension, supporting its role. Some severe human hypertension cases have reduced NO versus normotensives. L-arginine analogues like L-NAME (NG-nitro-L-arginine methyl ester) acutely reduce renal blood flow, glomerular filtration rate, and increase blood pressure in normal animals. Chronic L-NAME treatment in rats causes sustained increases in blood pressure, glomerular capillary pressure, ultrafiltration, proteinuria, and glomerulosclerosis (Klahr et al., 2001).

L-NAME inhibition of NOS occurs by competitive substrate analogue binding. Some analogues irreversibly inhibit NOS by enzyme binding, unlike L-NAME's reversible inhibition. Chronic in vivo L-NAME treatment is widely used to study NO restriction effects. It reduces NO production and cGMP content in vessels and impairs acetylcholine-induced relaxation. However, lower long-term L-NAME doses may also feedback stimulate NO production by increasing NOS expression, possibly via nuclear factor kB effects on transcription (Kopincova et al., 2012).

L-NAME treatment in normotensive rats is used to model NO-deficient hypertension. Advanced L-NAME doses for 3-6 weeks reduce in vitro vasorelaxation responses and cGMP content. Doses from 10-100 mg/kg/day dramatically increase blood pressure. Increased in vivo hepatic iNOS activity and expression were observed after 7 days of 300 mg/kg/day L-NAME, indicating NOS upregulation. Chronic 40 mg/kg/day L-

NAME impairs vasorelaxation, with consistent NOS activity decreases in cardiovascular tissues and brain, confirming NO reduction (Tucker, et al., 2000).

2.7: L-NAME and NOS expression

L-NAME may increase NOS expression by limiting NO's inhibition of nuclear factor kB activation, a key NOS transcription regulator. Thus, L-NAME reduces NO levels, activating kB to increase NOS (Kopincova, et al., 2011; Hu, et al., 2011).

Oxidative stress, from excessive reactive oxygen species (ROS), contributes to vascular dysfunction, remodeling, renal changes, and sympathetic activation in hypertension. NADPH oxidases are a major cardiovascular ROS source. ROS alter cell signaling and cause oxidative damage via protein modifications. Unchecked ROS generation disturbs redox homeostasis and causes molecular damage (Henrion et al., 2018).

Superoxide (O2-) is membrane-impermeable while hydrogen peroxide (H2O2) is stable, permeable and a useful signaling intermediate. NO reacts with O2- to form peroxynitrite, a potent oxidant. The NO/O2- interaction and O2-/H2O2 dysregulation alter redox status and damage cells and tissues (Incalza, et al., 2018). ROS post-translationally modify proteins, affecting cell function. ROS facilitate vasoactive substance signaling on vascular cells, including angiotensin II, endothelin-1, aldosterone, and catecholamines, and regulate calcium homeostasis for vasoconstriction and cardiac contraction (Ray, et al., 2012).

2.8: Oxidative stress and Vascular Remodeling

In hypertension, increased vascular ROS and oxidative stress cause endothelial dysfunction. Vascular smooth muscle cells from hypertensive vessels have heightened NOX, ROS, Ang II-induced redox signaling, and inflammatory responses versus normotensive cells (Touyz, et al., 2005). Uncoupled NOS also contributes to oxidative stress by generating O2- instead of NO, due to oxidative alterations of NOS cofactors. This mechanism enhances oxidative damage in hypertension (Tejero et al., 2019).

ROS and redox signaling mediate hypertension-related vascular changes like inflammation, remodeling, stiffness, endothelial dysfunction, calcification, and hyperreactivity. Ang II, endothelin-1, aldosterone, catecholamines, growth factors, and other stimuli increase vascular ROS through NADPH oxidase activation, causing vascular pathology. Ang II also transactivates growth factor receptors, amplifying redox-dependent effects (Ushio-Fukai et al., 2009). Thus, oxidative stress plays a key role in hypertension pathophysiology.

2.9: ACE2 protein

ACE2 protein has emerged as a promising target for the treatment of hypertension. In recent years, in silico methods have gained significant attention for their role in analyzing the ACE2 protein and its hypertensive activity. In this literature review, we aim to provide an overview of the current research on *in silico* analysis of ACE2 and its hypertensive activity (Pooja et al., 2015). Methods: A thorough search of scientific databases was conducted to identify relevant studies on *in silico* analysis of ACE2 and hypertensive activity. Results: Several studies have utilized in silico

approaches to investigate the structural and functional properties of ACE2 and its role in hypertension. These studies have employed various computational techniques such as molecular docking, molecular dynamics simulations, and structure-based virtual screening to explore the interactions between ACE2 and potential inhibitors and to predict the impact of ACE2 mutations on hypertensive activity. Some studies have focused on identifying ACE2 inhibitors with potential antihypertensive properties using in silico methods. Conclusion: In silico analysis of ACE2 and its hypertensive activity has provided valuable insights into the structural and functional aspects of this protein. These studies have helped elucidate the interactions between ACE2 and potential inhibitors, as well as the effects of mutations on hypertensive activity. Furthermore, *in silico* methods have facilitated the identification of ACE2 inhibitors with potential antihypertensive effects. This literature review provides a comprehensive overview of the current research on *in silico* analysis of ACE2 and its role in hypertensive activity. Overall, the literature review highlights that in silico analysis of ACE2 and its hypertensive activity has been a subject of considerable research. Researchers have recognized the potential of ACE2 protein as a target for the treatment of hypertension, leading to an increased interest in utilizing in silico methods to explore its structural and functional properties.

2.10: MMP-7 protein

In recent years, *in silico* analysis has emerged as a valuable tool for studying the structural and functional properties of proteins involved in hypertensive activity, including MMPs (matrix metalloproteinases). Several studies have utilized in silico approaches to investigate the role of MMPs, particularly MMP-7, in hypertensive

activity. These studies have employed various computational techniques such as molecular docking, molecular dynamics simulations, and structure-based virtual screening to explore the interactions between MMP-7 and potential inhibitors and to predict the impact of MMP-7 mutations on hypertensive activity. The results of these *in silico* analyses have provided valuable insights into the structural and functional aspects of MMP-7 and its involvement in hypertensive activity. Furthermore, these studies have identified potential MMP-7 inhibitors with antihypertensive properties, offering promising avenues for the development of novel therapeutics. Overall, the literature on in silico analysis of MMPs, particularly MMP-7, in hypertensive activity demonstrates the usefulness and effectiveness of computational methods.

Matrix Metalloprotease-7 (MMP-7) plays a crucial role in various biological processes, particularly in cancer progression and tissue defense mechanisms. In vivo studies have highlighted the multifaceted functions of MMP-7, including its involvement in nucleolar assembly, gene expression regulation, cancer cell proliferation, and tissue repair. Research has shown that MMP-7 functions as an MUC-1 sheddase, activating antimicrobial peptides, generating soluble ligand, and mediating the shedding of syndecan-1. Studies on MMP-7 null mice have demonstrated defects in wound repair but protection against pulmonary damage, indicating its diverse functions in tissue defense, repair, and inflammation (Wei-Hsuan Yu, et al., 2020)

In-silico studies have further complemented *in vivo* findings by analyzing MMP-7 gene expression patterns. These computational analyses have revealed significant correlations between MMP-7 levels and survival outcomes, shedding light

on the molecular mechanisms underlying MMP-7's impact on cancer progression. In silico studies have also contributed to understanding the regulatory networks involving MMP-7 and its potential implications for targeted therapies in cancer treatment (Tibor Szarvas, et al., 2020). The literature on MMP-7 protein in vivo and in silico studies underscores its diverse functions in physiological processes, particularly in cancer biology. The combination of in vivo and *in-silico* approaches provides a comprehensive understanding of MMP-7's role in disease pathogenesis, highlighting its potential as a diagnostic biomarker and therapeutic target in various pathological conditions.

2.11: *In silico* Studies

In silico studies on Matrix Metalloproteinases (MMPs) have been instrumental in understanding the structural and functional aspects of these proteins. Several research articles have delved into the computational analysis of MMPs to elucidate their roles in various physiological and pathological conditions.

A study conducted by (Jaiswal, et al., 2011) analyzed the physicochemical, secondary structural, and functional characteristics of the human MMP family. The research highlighted the importance of MMP-23, MMP-2, and MMP-9 in pathological conditions and proposed further experimental validation to explore their roles.

(Birkedal-Hansen and Moore, et al., 1993) discussed the primary structures, substrate specificities, and activation mechanisms of MMPs in a comprehensive review. This review provided insights into the diverse functions of MMPs and their implications in various biological processes.

A recent study in 2023 presented a strategy for enhancing inhibitor specificity towards individual MMP enzymes through mutagenesis. This approach aimed to modify the activity of N-TIMP2 variants to target specific MMPs effectively (Hezi Hayun, et al., 2023).

An *in-silico* study conducted in 2023 focused on inferring evolutionary relationships between animal models and human MMPs, particularly MMP-1 and MMP-8 (Simone Gomes de Oliveira, et al., 2023). This research provided valuable insights into the conservation of MMP functions across species (Hezi Hayun, et al., 2023).

Another in silico study explored the inhibitory potential of selected terpenoids from Dalbergia sissoo extracts against MMP-1 in 2019. This research shed light on the potential therapeutic applications of natural compounds in modulating MMP activity. *In-silico* studies on MMP proteins have significantly contributed to unraveling the structural characteristics, functional roles, and therapeutic implications of these enzymes. These computational approaches have paved the way for a deeper understanding of MMP biology and the development of novel strategies for targeting MMPs in various diseases (Shagufta Yasmeen, et al., 2019).

CHAPTER-3

Aims and Objectives

3.1: Research Question

Whether selective plant based phytochemical compound(s) can be targeted against aortic tissue remodeling gene to control hypertension?

3.2: Aims

The aim of the study is to assess effects of bioactive molecules on vascular pathophysiology of aortic tissue in experimentally induced hypertension in rat models.

3.3: Objectives

1. Screening and identification of potential bioactive molecules against aortic tissue proteins (ACE2 and MMP-7) using ***in silico*** analysis and to further find out the potent bioactive molecules with the better effector protein from either ACE2 or MMP-7 or both.
2. To find out the effect of L-NAME induced experimental hypertension in rats on cardiovascular electrophysiology with or without supplementation of bioactive compound(s) from phytochemical database ***in silico***.
3. Validation of potent bioactive molecule on target gene expression and cardiovascular pathophysiology by *electrophysiology, oxidative stress, target gene expression* and *histopathology* in rats by using hypertensive rat model ***in vivo.***

3.4: Hypothesis

Aortic tissue specific target gene (ACE2 and MMP-7) involve in hypertension pathophysiology may be modulated by certain plant based phytochemical compound(s).

CHAPTER- 4

Materials and Methods

4.1 Sources of data collection for *in-silico* analysis:

4.1.1 List of databases and tools/software used in *in silico* analysis:

The present investigation was performed in Intel® Core™ i3-7th gen *hp* processor laptop. The software's and databases used for current study:

1. Pubmed (Harjacek M, et al 2000)
2. Pubchem (Kim S, et al., 2019)
3. NCBI (Sayers E.W, et al., 2020)
4. Swiss model (Waterhouse A, et al 2018)
5. Uniport (UniProt Consortium. 2019)
6. PDB sum (Laskowski R.A, et al., 2018)
7. NOPACT (Shapovalov M.V, et al 2011)
8. Auto dock 4.2 (Morris G.M, et al., 2009)
9. Discovery studio visualizer (BIOVIA, Dassault Systèmes. 2017)
10. Protein data bank (Burley S. K, et al 2017)

4.2: Methodology (*In silico*)

4.2.1: Retrieving Target Proteins

The target proteins were retrieved by RCSB PDB (https:// www.rcsb.org/) with their MMP-7 (PDB ID: 2DDY) and ACE2 protein (PDB ID: 1R4L). The target proteins were retrieved from PDB and thus cannot be directly employed for molecular docking as they may be bound with heavy atoms, co-crystallized ligands, water molecules, ions, and co-factors that bring up the need for a preprocessing by adding hydrogen atoms, removing the surrounding water molecules, removing co-bound ligand(s)/ ions, and adding polar charges. Once the preprocessing was done, these were converted from .pdb to pdbqt.

4.2.2: Active site of protein prediction

This was executed using PDBsum to find the active sites/binding pockets in the selected target proteins where the drugs are most likely to bind with stable free energy.

4.2.3: Retrieval of ligands

The best bioactive compounds from the phytochemical database were screened thoroughly to prepare an exhaustive list of bioactive compounds that are known to encapsulate to treat high blood pressure and improve properties, specifically in cardiac vascular pathophysiology, which may exhibit antihypertensive activities. Subsequently, 130 phytochemicals of these bioactive compounds were retrieved from NOPACT, and the chemical structure was downloaded from PubChem and saved in ".sdf" files and visualized in Discovery Studio Visualizer.

4.2.4: Pharmacokinetic Parameters

In order to select the best, soluble, druggable, lead-like, and non-violating drug compounds for the progression of this study, the bioactive compounds were subjected to clusters for ADME (Absorption, Distribution, Metabolism, Excretion, and Toxicity Analysis) analysis using Swiss ADME online tool. This tool evaluates and analyses essential pharmacokinetic properties. Evaluate structural analogues, predict physical descriptors and pharmaceutically relevant properties, and calculate critical parameters such as logP (Octanol/Water), log S, molecular weight, and adhere to Lipinski's rule of 5 for rational drug design.

4.2.5: Molecular Docking Studies

Generating a grid around the defined active site of the target protein is an important prerequisite for docking studies. A 10 x 10 x 10 Å cubic grid box was centered on the ACE2 and MMP-7 active sites using Glide v5.9 (Prachi P Parvatikar, et al., 2023). To soften the potential for nonpolar parts of the receptor, the scaling factor for protein van der Waals radii was set to 1.0 during receptor grid generation, along with a partial atomic charge of 0.25. Docking was performed twice, once for each ligand-protein pair, following the same initial preprocessing steps. The Glide XP docking method was used, which helps remove false positives and improve correlations between good binding poses and scores (Friesner et al., 2006). In XP docking, the ligand center was positioned at various 1 Å grid points for rotating the ligand around three Euler angles, computing crude score values and eliminating false binding modes. Binding solutions were evaluated and refined using OPLS-2005, including torsional and rigid-body motions of each ligand via a Monte Carlo post-docking minimization. The best

docking pose was selected using the Glide scoring function Prachi P Parvatikar, et al., (2023) an empirically-based model Eldridge, et al., (1997) where the Glide Emodel combines the Glide score, Coulombic and van der Waals energies, and strain energy Friesner, et al., (2006).

XP Gscore = *E*coul + *E*vdW + *E*bind + *E*penalty

Where

*E*coul and *E*vdW are coulombic and van der Waal's contribution for ligand binding. *E*bind is principal terms that favors binding and *E*penalty is principal terms that hinder binding.

*E*bind = *E*hyd_enclosure + *E*hb_nn_motif + *E*hb_cc_motif + *E*PI +*E*hb_pair + *E*phobic_pair

Where

*E*hyd_enclosure = hydrophobic enclosure reward; *E*hb_nn_motif = neutral-neutral

hydrogen-bonding motifs; *E*hb_cc_motif = special charged-charged hydrogen-bond motifs;

EPI = rewarding pi stacking and pi-cation interactions; *E*hb_pair = hydrogen-bonded pair

reward; and *E*phobic_pair = hydrophobic pair term

*E*penalty = *E*desolv + *E*ligand_strain

Where

*E*desolv =desolvation penalties; and *E*ligand_strain = contact penalties

The best docking pose per ligand was chosen using a Glide Scoring function (XP G-Score). Comparison of obtained hits with published inhibitors and co-crystal ligands wascarried out, top ranked hits obtained through XP docking and MM-GBSA binding free energy analysis was selected for further studies.

4.3: Sources of data collection for *In vivo*

The rats used in this study were healthy adult male Wistar Albino rats aged 60-70 days and weighing 180-250g. They were obtained from the animal facility at Shri B.M. Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University) in Vijayapura, Karnataka, India. The rats were housed in the lab for 7 days prior to the start of the experiment to allow them to acclimate. During this acclimation period, the rats were kept at 22-24°C with a 12-hour light/dark cycle and given unlimited access to food and water.

Study Design: Experimental animal study.

Study Duration: Four years (October 2020-November 2023)

Sample Size: n=6 per group

Calculation of sample size: The sample size was calculated using the resource equation approach (Bagali S, et al., 2019)

Sample Size= DF/k +1

DF=Degree of Freedom

k=No. of groups (4 in the present study)

Minimum n/group= 10/k+1=10/4+1=3.5

Maximum n/group=20/k+1=20/4+1=6

Inclusion criteria:

Apparently healthy Male Wister rats.

Body: 180-250g

Age: 8-10 weeks

Exclusion criteria:

Female Wister rats

Pretreated rats

Diseased Rats

Body weight >250 g

Table 4.3: Experimental groups of rats

Groups	Number of rats	Intervention
1. Control	n=6	Normal water and food.
2. L-NAME	n=6	L-NAME (L-NG-Nitroarginine methyl ester), 40 mg/day/kg body weight, orally in distilled water for 28 days[17].
3. β-sitosterol	n=6	Dosage: β-sitosterol 10 mg/day/kg body weight, orally in distilled water for 28 days[18]
4. L-NAME+β-sitosterol	n=6	L-NAME (L-NG-Nitroarginine methyl ester), 40 mg/day/kg body weight, orally in distilled water for 28 days. β-sitosterol: 10 mg/day/kg body weight, administrated orally for 28 days.

Values with different superscripts are significantly different from each other (p <0.05)*

Note: Group 2 and 4, rats were divided into two sets (6+6) where one set of aortas (n=6) collected for molecular biology analysis and another set of aortas (n=6) collected for histopathology.

Details;

Group 1= 6

Group 2 = 6 (For Molecular biology for aorta) + 6 (histopathology study of aorta)

Group 3 = 6

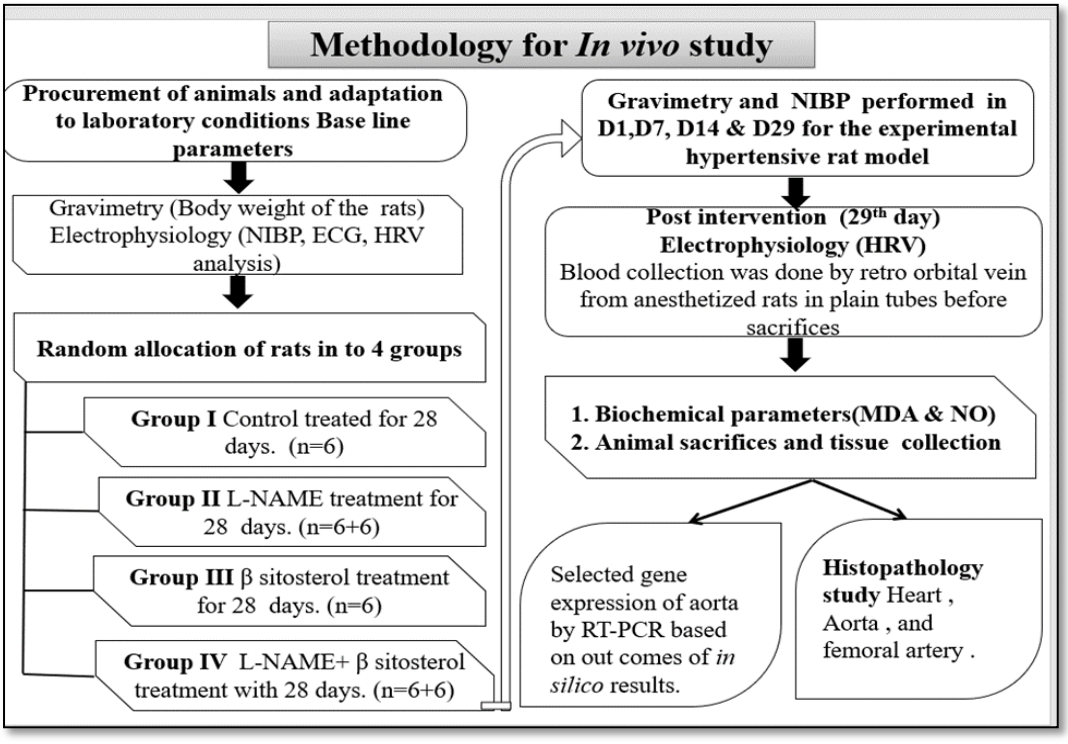
Group 4 = 6 (for Molecular biology for aorta) + 6 (histopathology study of aorta)

The reason is one aorta weigh is not sufficient to carry out both molecular biology and histopathology. Moreover, aorta histopathology needs intact aorta.

Further IAEC permitted only limited number of animal use. Hence, we tried to do it in more carefully and economy way.

The Total sample size is 36.

Protocol



4.4: Methodology (*In vivo*)

4.4.1: Gravimetry

The gravimetry (body weight) of all rats was recorded on day 1 of the experiment (initial body weight) and after 28 days of intervention (29th day, final body weight) using an electronic balance (Practicum 1102-10IN, Sartorius Lab Instruments, Germany). The percentage change in body weight was calculated using the following formula: The rats of all groups were matched for weight at the onset of the experimental protocol.

$$Change\ in\ body\ weight\ (\%) = \frac{Final\ body\ weight - Initial\ body\ weight}{Initial\ body\ weight} \times 100$$

4.4.2: Evaluation of cardiovascular electrophysiology

a. Recording of Blood Pressure

b. Recording of ECG

c. Heart rate variability (HRV) analysis

a. Recording of Blood Pressure

Blood pressure (systolic and diastolic) was measured weekly for 4 weeks in conscious rats. Prior to each recording, the rats were kept in restrainers for 10-20 minutes per day to acclimate them. Their tails were warmed for 30 minutes beforehand to improve detection of tail artery pulsations. Blood pressure was recorded non-invasively using a tail cuff sensor (NIBP) attached to a Bio Pac MP 100 instrument (a PC Windows-based animal electrophysiology system). The Bio Pac Student Lab 4.1 software analysed all parameters. Both systolic blood pressure (SBP)

and diastolic blood pressure (DBP) were measured. Three measurements were taken for each rat and averaged to obtain the final value.

Mean arterial pressure (MAP) was calculated by using formula

MAP =Diastolic blood pressure+1/3Pulse pressure.

b. Recording of ECG

ECG was recorded using needle electrodes (Biopac MP 45: PC windows-based animal electrophysiological system). All the recordings were performed in the morning following overnight fasting in anaesthetized animals (Ketamine, 60mg/kg and Xylazine, 6mg/kg)14. From the recorded ECG, heart rate was obtained.

c. Heart rate variability analysis

RR intervals obtained from the recorded ECG (using Biopac Student Lab 4.1 software) were exported to Kubios software for heart rate measurement and HRV analysis. HRV analysis was done using the frequency domain method to assess the level of sympathetic activity, parasympathetic activity, and sympathovagal balance.

4.4.3: Collection of Blood

All the animals were fasted overnight and blood sample were collected after 28 days of intervention on day 29^{th} by retro orbital vein from anaesthetized rats in plain tubes for further analysis of biochemical and gene expression respectively.

4.4: Biochemical Parameters

a. Oxidative stress parameters- Serum Malondialdehyde (MDA) level.

b. Nitrosative stress parameters- Serum Nitric oxide (NO) level

a. **Oxidative stress parameters- Serum Malondialdehyde (MDA) level:**

Introduction

Malondialdehyde (MDA) is a marker of oxidative stress in biological systems. It is a byproduct of the lipid peroxidation process. It is produced when a free radical chain reaction breaks down polyunsaturated fatty acids (PUFA). It is one of the numerous reactive electrophile species that cause oxidative stress and is a reactive aldehyde.

Principle

MDA, which is produced when PUFA break down, functions as an easy way to gauge how much lipid peroxidation has occurred. It reacts with thiobarbituric acid (TBA) to produce a pink colour that may be detected at 535 nm.

Sample: Serum

Chemicals required:

1. Trichloroacetic acid (TCA)

2. 2-Thiobarbituric acid (TBA)

3. Hydrochloric acid (HCl)

4. Malonaldehyde (dimethyl acetal)

Preparation:

1. TCA-TBA-HCl reagent

21 l of concentrated HCl was diluted to 100 ml with 0.25 N HCl (DW). 100ml of 0.25N HCl were used to dissolve 15% TCA and 0.375% TBA. The reaction mixture was heated and maintained at 40°C in order to dissolve the components.

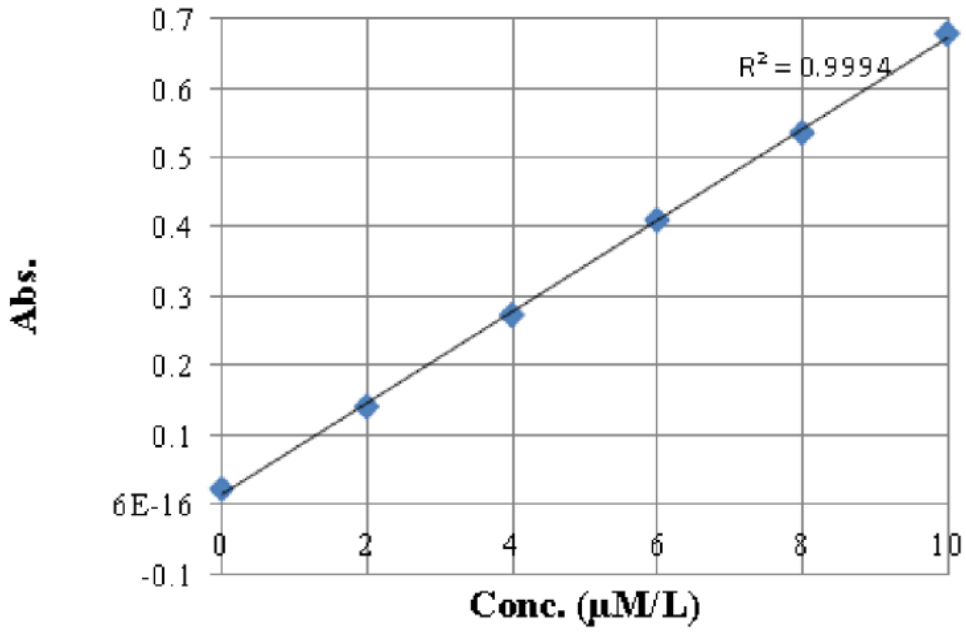
2. MDA standard (stock-164µg/ml): To make 100 ml, 4µl of the standard MDA solution were diluted with distilled water

3. MDA working standard (working- 1.64µg/ml) The stock was made 10ml from 100µl l using distilled water.

The procedure for standardization: Standardization (Range 2-10µM/L) All of the reagents were added in accordance with the values listed in the table and standardization was performed using the table.

Sl. No.	Vol of MDA (ml)	Vol of DW (ml)	Conc. of MDA(µM/L)	TBA-TCA-HCl (ml)	
B	0.0	1	0.0	1	Keep in boiling water bath for 15 min
1	0.2	0.8	2.0	1	
2	0.4	0.6	4.0	1	
3	0.6	0.4	6.0	1	
4	0.8	0.2	8.0	1	
5	1	0.0	10.0	1	

Standard curve:



Sample Preparation:

500 µl of serum was obtained by diluting 100 µl of serum with distilled water.

Procedure:

a. 1 ml of TCA-TBA-HCl reagent was added to the diluted serum sample.

b. The samples were maintained in a boiling water bath for 15 minutes.

c. After cooling, the reaction mixture was centrifuged.

d. The optical density of the pink color generated in the supernatant was measured at 535 nm using a UV-visible spectrophotometer (Shimadzu, Model: UV 1800).

e. By comparing the obtained absorbance to a reference graph, the MDA concentration in the sample was determined. The optical density of the resulting pink color was directly proportional to the amount of MDA present.

Calculations:

The optical densities of the test samples obtained by plotting against the reference graph and multiplying by the respective dilution factors yielded values that were directly correlated to the MDA concentrations. The final MDA concentration was measured in µM/L.

b. Estimation of Serum Nitric Oxide Level by Griess Reaction Method:

Principle:

The stable nitric oxide byproduct, nitrate, was coupled to N-naphthyl ethylene diamine and then reduced to nitrite using cadmium. The colored complex produced was measured by a spectrophotometer at 540 nm.

Reagents:

Cadmium granules stored in 0.1M H_2SO_4

Glycine-NaOH buffer (pH 9.7): 7.5 g glycine dissolved in 200 ml water, pH adjusted to 9.7 with 2M NaOH, diluted to 500 ml

Sulfanilamide: 2.5 g dissolved in 250 ml 3M HCl, allowed to cool

N-Naphthyl ethylene diamine: 50 mg dissolved in water, diluted to 250 ml

Sodium nitrite standard solution:

a. Stock (0.1M): 690 mg sodium nitrite dissolved in 100 ml 10 mM sodium borate
b. Working (10μM): 10 μl stock diluted to 100 ml with 10 mM sodium borate

$ZnSO_4$ solution (75 mM)
NaOH solution (55 mM)
H_2SO_4 solution (0.1 M)
$CuSO_4$ solution (5 mM): 125 mg $CuSO_4$ dissolved in 100 ml glycine-NaOH buffer

Procedure:

a. Deproteinization: 0.5 ml serum added to 2 ml $ZnSO_4$ (75 mM), mixed. 2.5 ml NaOH (55 mM) added and centrifuged 10 mins. Supernatant filtered to remove proteins.
b. Activation of cadmium granules: Granules washed in water and agitated 1-2 mins in 5 mM $CuSO_4$. Drained and rinsed in glycine-NaOH buffer. Used within 10 mins of activation. Rinsed in 0.1 M H_2SO_4 after use.

Nitrite Assay:

a. 3 flasks labeled Blank (B), Test (T) and Standard (S).
b. 1 ml glycine-NaOH buffer added to each. Then 1 ml water (B),
c. 1 ml sample (T) or 1 ml standard (S).
d. 2.5-3 g activated cadmium granules added and swirled thoroughly.
e. After 90 mins, diluted to 4 ml with water.
f. 2 ml from each flask pipetted into tubes B, S and T.
g. 1 ml sulfanilamide and 1 ml NED added to each tube.
h. Tubes shaken and OD of S and T read at 540 nm against blank after 20 mins.

Serum nitric oxide calculated: OD Sample/OD Standard x Standard Conc. x DF

4.5 : The sacrifice of animals and collection of tissues:

After 28 days of intervention, the rats were sacrificed by an overdose of ketamine (150 mg/kg, ip) following blood collection. The rats were carefully dissected to separate the heart, aorta, and femoral artery. The individual weights of the aorta, heart, and femoral artery were immediately measured. The organ-somatic index was calculated for the aorta and heart by taking the ratio of the organ weight (mg) to the body weight (g). Typically, 250-300 g rats have aortic weights of 0.08-0.15 mg, giving a normal ratio of 0.05-0.15. Portions of the tissue samples were preserved in 10% neutral buffered formalin for subsequent histopathological examination, while the remaining specimens were stored at -20°C to prepare tissue homogenates for RT-PCR analysis of gene expression.

4.6: Preparation of aortic tissue homogenate and mRNA extraction for RT-PCR:

1. Weigh the tissue (5-15mg).
2. Clean and remove excess debris with ethanol, finely chop it, and homogenize thoroughly using a handy homogenate.
3. Add 200 microliters of lysis buffer to the tissue homogenate, and centrifuge at 1300 rpm for 1 minute.
4. After centrifugation, perform the first wash with 700 microliters of washing solution 1 and centrifuge at 1300 rpm for 2 minutes.
5. Subsequently, conduct three washes with 500 microliters of washing solution 2, allowing for a 0-second interval between each wash.
6. Elevate RNA with 40-60 micro liter preheated Elusion solution

a. Put the filter cartridge into fresh collection tubes.

b. Pipette out 40 micro liter of elution solution add into fresh tubes and centrifuge for 1 min.

c. Divide into three parts and again centrifuge for 1 mint.

d. After centrifugation aliquoted sample 20, 40, 60 micro liter in PCR tubes and stored in -20^0C. Sample preparation composition for aortic tissue homogenate given in table 4.2.

Table 4.6: Sample preparation for aortic tissue homogenate

Components	Volume in µL
RNA	1
Control RNA	-
Oligo (dt)	8
10mM dNTPs mix	20
DEPC treated water	200
10X RT buffer	40
25 mM $Mgcl_2$	80
0.1 mM DTT	40
RNaseOUT	8

Values with different superscripts are significantly different from each other (p <0.05)*

4.7: RT-PCR Kit Protocol

4.7.1: First-Strand Synthesis Using Oligo(dT) or GSP (Ponchel F, et al., 2023)

Starting material: 1 ng–5 µg total RNA or 50–500 ng poly(A)+ RNA

Control reactions: Use 1 µl of Control RNA (50 ng/µl)

a. Mix and briefly centrifuge each component before use.

b. For each reaction, combine the following in a sterile 0.2- or 0.5-ml tube:

c. Component Amount: RNA n µl, 10 mM dNTP mix 1 µl, Primer (0.5 µg/µl oligo(dT)12–18, or 1 µl 2 µM gene- specific primer), DEPC-treated water to 10 µl 3. Incubate the RNA/primer mixture at 65°C for 5 minutes, then place on ice for at least 1 minute.

d. In a separate tube, prepare the following 2X reaction mix, adding each component in the indicated order.

e. Component:

    a) 1 Rxn; 10X RT buffer 2 µl, 25 mM MgCl2 4 µl, 0.1 M DTT 2 µl, RNaseOUT™ (40 U/µl) 1 µl.

    b) 10 Rxns; 10X RT buffer 20 µl, 25 mM MgCl2 40 µl, 0.1 M DTT 20 µl, RNaseOUT™ (40 U/µl) 10 µl.

f. Add 9 µl of the 2X reaction mix to each RNA/primer mixture from step 3, mix gently, and collect by brief centrifugation.

g. Incubate at 42°C for 2 minutes. Add 1 µl of SuperScript™ II RT to each tube. Minus RT Control: Add 1 µl DEPC-treated water instead of the RT.

h. Incubate at 42°C for 50 minutes.

i. Terminate the reaction at 70°C for 15 minutes. Chill on ice.

j. Collect the reaction by brief centrifugation. Add 1 µl of RNase H to each tube and incubate for 20 minutes at 37°C. The reaction can be stored at -20°C or used for PCR immediately.

4.8: Ct value conformation by using High-Capacity cDNA Reverse Transcription Kit:

Prepare the 2X RT master mix (20-µL reaction)

a. Allow the kit components to thaw on ice.

b. Calculate the volume of components needed to prepare the required number of reactions.

c. Place the 2X RT master mix on ice and mix gently.

Table 4.8(a): Master mix composition of selected gene(s)

Components	Volume in µL
Cyber green	90
Forward primer	14.4
Reverse primer	14.4
Molecular gradient water	43.2

Table 4.8(b): Master mix composition for housekeeping gene (ß-actin)

Components	Volume in µL
Cyber green	90
Forward primer	14.4
Reverse primer	14.4
Molecular gradient water	43.2

Prepare the reverse transcription reactions

1. Pipette 10 µL of 2X RT master mix into each well of a 96-well reaction plate or individual tube.
2. Pipette 10 µL of RNA sample into each well, pipetting up and down two times to mix.
3. Seal the plates or tubes.
4. Pipette centrifuge the plate or tubes to spin down the contents and to eliminate any air bubbles.
5. Place the plate or tubes on ice until you are ready to load the thermal cycler.

Perform reverse transcription:

1. Program the thermal cycler using the conditions below.

2. Set the reaction volume to 20 µL.

3. Load the reaction plates or tubes into the thermal cycler.

4. Start the thermal cycler run**.**

Settings	Step 1	Step 2	Step 3	Step 4
Temp.	25°C	37°C	85°C	4°C
Time	10 minutes	120 minutes	5 minutes	Hold

4.9: Histopathological examination

The heart, aorta and femoral artery were fixed in 10% neutral buffered formalin were embedded in paraffin blocks, sectioned with a microtome (0.7 µ thickness) & finally stained by Hematoxylin & Eosin and were subjected to histopathological examination.

4.10: Statistical Analysis:

Statistical analysis was done using SPSS19.0 version software using Student's t-test, and a p-value <0.05 is considered statistically significant. A one-way ANOVA was used to analyze various groups, following tests such as Tukey's post hoc test to ascertain significant intergroup differences. The p< 0.05 value is considered statistically significant.

4.11: Ethical Clearance:

Institutional Ethics Committee clearance was approved by the Institutional Animal Ethics Committee (IAEC), BLDE (Deemed to be University, Vijayapura (Ref. NO.09/BLDE(DU)/2021 dated 10.07.2021).

CHAPTER-5

Results and Discussion

Bioinformatics, an interdisciplinary field, has evolved through the incorporation of diverse disciplines such as biology, mathematics, computer science, and statistics. It revolves around the analysis of biological data and finds applications in various domains, including medicine, microbial genomics, internet-based database integration, genome comparisons, and the identification of gene product functions. One of its invaluable aspects involves the exploration of three-dimensional structures, providing crucial insights into protein shape, domains, functions, enzyme mechanisms, post-translational modifications, and experimental evidence for transmembrane domains and interactions with small ligands, substrates, and inhibitors. The active conformation of enzymes in three-dimensional structures serves as a foundational platform for the development of new drug molecules using molecular modeling techniques, particularly molecular docking.

5.1: Retrieving Target Proteins

The crystal structure of MMP-7 (PDB ID: 2ddy) and ACE2 (PDB:1R4L) protein was retrieved from PDB. The MMP-7 protein is composed of Modelled Residue Count: 173 Deposited Residue Count: 173 residues with molecular weight of 19 kDa and single motif. The ACE2 protein is composed of Modelled Residue Count: 655 and Deposited Residue Count: 673 residues with molecular weight 76.98 kDa as shown in figure 5.1(a) and 5.1(b).

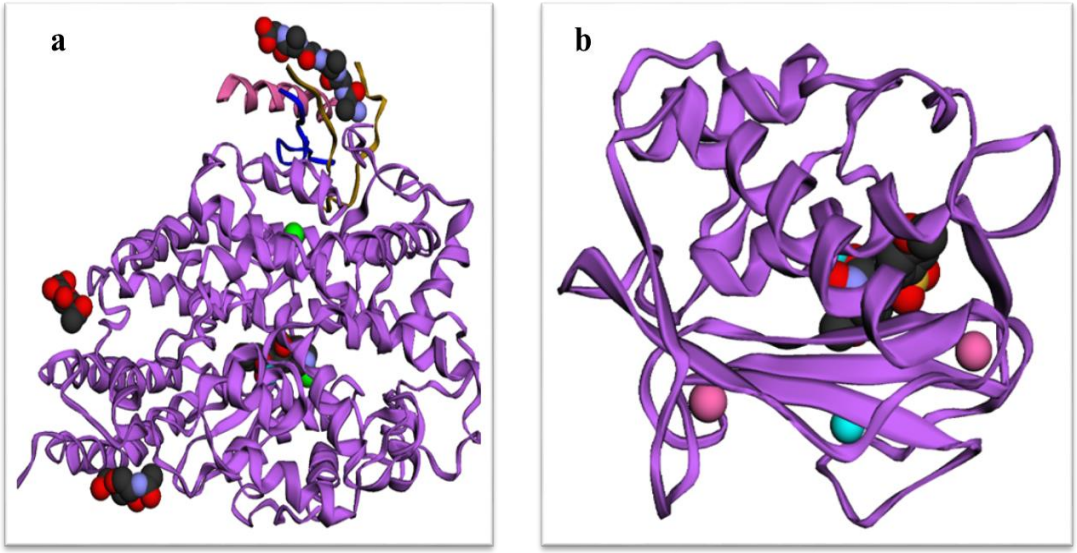


Figure 5.1 (a): 3D Structure of Protein (ACE2) PDB:1R4L
Figure 5.1 (b): 3D Structure of Protein (MMP-7) PDB:2DDY

5.2: Active site of protein prediction

As per literature survey binding site information of target protein was predicted by performing PDBsum. The ligand plot obtained from PDBsum showed binding site region of MMP-7 receptor containing 15 amino acid residues of chain A, His 120, His 124, Glu 121, His 130, Leu 82, Ala 83, Ala 117, Thr 81, Pro 140, Tyr 116, Tyr 142, Thr 141, Ile 112 and MDW 178. These residues possess higher ligand efficiency and interaction with higher number of amino acids which are used for setting the grid of molecular docking. The ligand plot obtained from PDBsum showed binding site region of ACE2 receptor containing 12 amino acid residues of chain A, Asp 615(A), Ala 614(A), Unk 910(C), Unk 901(B), Unk 902(B), Unk 903(B), Unk 908(C), Unk 907(C), Unk 904(B), Unk 905(B) and Unk 906(B). These residues possess higher ligand efficiency and interaction with higher number of amino acids which are used for setting the grid of molecular docking with their chemical structures as shown in figures 5.2(a), 5.2(b) and 5.2(c).

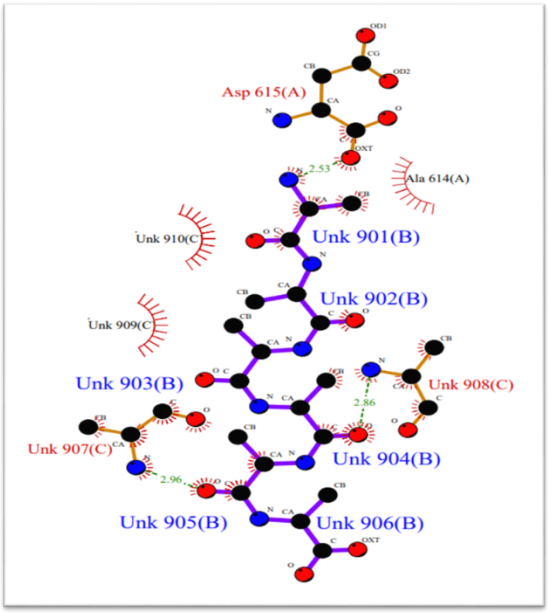


Figure 5.2 (a): Binding sites of Protein ACE2

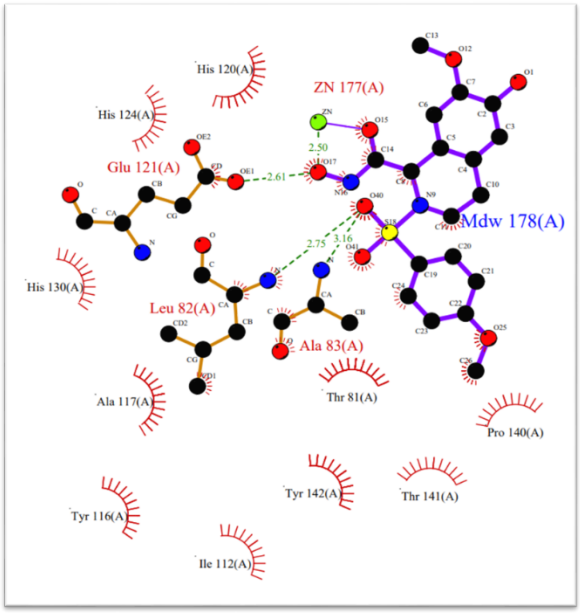


Figure 5.2(b): Binding sites of Protein MMP-7

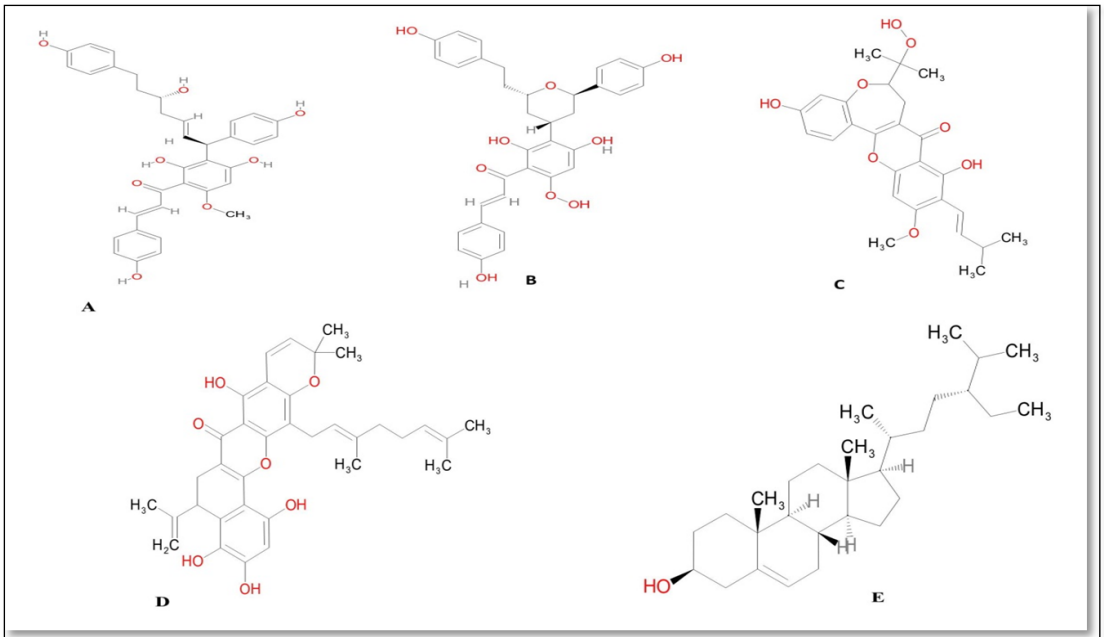


Figure 5.2(c): 2D structure of 5 docked bioactive compounds ; (A). Calyxins B, (B). Calyxins F, (C). Artoindonesianin B, (D). Artoindonesianin V,

(E). β- Sitosterol

5.3: Prediction of pharmacokinetic properties

The prepared 130 bioactive compounds were subjected to pharmacokinetic (PK) absorption, distribution, metabolism, excretion, and toxicity (ADMET) analysis, pharmacodynamic, and physicochemical analysis. Bioactive compounds that had a higher absorption, solubility, blood–brain barrier permeability, good logP values (partition coefficient), and those which did not violate any of the rules namely-Lipinski's RO5, Finally, bioactive compounds that had a good bioavailability, lead-likeness score with better synthetic accessibility were selected. After ADME analysis, bioactive compounds were subjected to toxicity screening to select those bioactive compounds that exhibited no susceptible toxicity or binding to any toxic receptor. Table 5.1 showcase the results of ADME/T properties. The 5 bioactive compounds followed the Lipinski's rule of five without any violation with respect to molecular weight (≤ 500KDa), number of H-bond acceptors (≤ 8) and number of H-bond donors (≤ 6). The Lipinski's screening is an essential filter that determines if a compound is suitable for drug designing and their chemical structures. Table 5.3 represents the Pharmacokinetic properties of selected 130 bioactive molecules.

Table 5.3: Pharmacokinetic properties of selected 130 bioactive molecules.

SL. No	Compound Name	Molecular Weight (< 500 g / mole)	Rotatable Bonds	Hydrogen Bond Acceptor	Hydrogen Bond Donor	Druglikenes Lipinski Rule	Violation	Pharmacokinetics Log Kp cm/s
1	Calyxins B	582.6	5	8	3	Yes	0	-5.04 cm/s
2	Artoindonesianin B	468.5	6	7	4	Yes	1	-5.74 cm/s
3	Calyxins F	582.6	12	8	6	No	2	-5.07 cm/s
4	Artoindonesianins V	570.7	6	7	4	Yes	1	-3.71 cm/s
5	β- Sitosetrol	414.7	6	1	1	Yes	1	-2.20 cm/s
6	Butein	272.2	3	5	4	yes	0	-5.96 cm/s
7	Calyxins A	582.6	12	8	6	no	2	-4.99 cm/s
8	Calyxins C	582.6	10	8	5	yes	1	-5.74 cm/s
9	Calyxins D	582.6	10	8	5	yes	1	-5.74 cm/s
10	Calyxins E	582.6	10	8	5	yes	1	-5.69 cm/s
11	Calyxins G	582.6	7	8	4	yes	1	-5.76 cm/s
12	Calyxins H	582.6	12	7	5	yes	1	-4.69 cm/s
13	Calyxins J	582.6	7	9	4	yes	1	-5.57 cm/s
14	Artoindonesianin P	368.3	0	7	4	yes	0	-6.58 cm/s
15	Artoindonesianins A	570.7	5	7	3	yes	1	-4.22 cm/s
16	Artoindonesianins G	570.7	6	6	3	yes	0	-3.66 cm/s

17	Artoindonesianins H	368.3	6	6	3	yes	0	-3.66 cm/s
18	Artoindonesianins I	368.3	6	7	4	yes	1	-4.75 cm/s
19	Artoindonesianins U	570.7	8	7	4	yes	1	-3.44 cm/s
20	Baicalein	270.24	6	11	6	no	2	-8.23 cm/s
21	Cajanol	316.30	3	6	2	yes	0	-6.20 cm/s
22	Biochanin A	284.26	2	5	2	yes	0	-5.91 cm/s
23	Blepharocalyxins A	879.0	17	18	2	no	3	-4.39 cm/s
24	Blepharocalyxins B	879.0	17	11	8	no	3	-4.39 cm/s
25	Blepharocalyxins C	879.0	12	7	6	no	2	-5.21 cm/s
26	Blepharocalyxins D	592.7	12	7	6	no	2	-5.21 cm/s
27	Blepharocalyxins E	879.0	17	11	8	no	3	-4.34 cm/s
28	Burttinone	438.5	6	6	3	yes	0	-5.34 cm/s
29	Subtrifloralactone A	454.5	1	6	1	yes	0	-7.07 cm/s
30	Subtrifloralactone B	270.2	1	6	1	yes	0	-7.12 cm/s
31	6-(2-Hydroxy-3-methyl-3-butenyl)-8-prenyl-eriodictyol	582.6	6	7	5	yes	0	-5.66 cm/s
32	6,8-Diprenyleriodictyol	440.4	6	7	5	yes	0	-5.66 cm/s
33	6-hydroxycalyxin F	582.6	9	9	6	no	2	-5.86 cm/s

34	7,7"-dimethyllanaraflavone	570.7	6	10	3	yes	1	-5.53 cm/s
35	7"-methylagathisflavone	414.7	4	10	5	yes	1	-5.86 cm/s
36	7-OH-flavanone	240.2	1	3	1	yes	0	-5.79 cm/s
37	candenatenin B	228.2	3	2	1	yes	0	-6.12 cm/s
38	acacetin	582.6	2	5	2	yes	0	-5.66 cm/s
39	Agathisflavone	582.6	3	10	6	no	2	-6.01 cm/s
40	Amentoflavone	538.4	3	10	6	no	2	-6.01 cm/s
41	Anthocyanin 1	634.2	9	16	9	no	3	-11.80 cm/s
42	Subtrifloralactone K	502.5	1	9	2	yes	1	-9.02 cm/s
43	Apigenin	582.6	1	5	3	yes	0	-5.80 cm/s
44	apigenin-7-O-beta-D-glucopyranoside	368.3	4	10	6	yes	4	-7.65 cm/s
45	Artelasticin	490.5	7	6	4	yes	0	-3.54 cm/s
46	Artobiloxanthone	570.7	1	7	4	yes	0	-5.57 cm/s
47	Artoindonesianin B	368.3	5	8	3	yes	0	-5.74 cm/s
48	Artoindonesianin P	368.3	0	7	4	yes	0	-6.58 cm/s
49	Artoindonesianins A	570.7	5	7	3	yes	1	-4.22 cm/s

50	Artoindonesianins G	270.24	6	6	3	yes	0	-3.66 cm/s
51	Artoindonesianins H	316.30	6	6	3	yes	0	-3.66 cm/s
52	Artoindonesianins I	284.26	6	7	4	yes	1	-4.75 cm/s
53	Artoindonesianins U	879.0	8	7	4	yes	1	-3.44 cm/s
54	Artoindonesianins V	879.0	6	7	4	yes	1	-3.71 cm/s
55	Baicalein	879.0	1	5	3	yes	0	-5.70 cm/s
56	Baicalin	592.7	4	11	6	no	2	-8.23 cm/s
57	Cajanol	879.0	3	6	2	yes	0	-6.20 cm/s
58	Biochanin A	438.5	2	5	2	yes	0	-5.91 cm/s
59	Blepharocalyxins A	570.7	17	11	8	No	0	-4.39 cm/s
60	Blepharocalyxins B	270.2	17	11	8	No	0	-4.39 cm/s
61	(-)-Epicatechin	290.2	1	6	5	yes	0	-7.82 cm/s
62	(+)-Gallocatechin	468.5	1	7	6	yes	1	-8.17 cm/s
63	(3S)-3',7-dihydroxy-2',4',5',8-tetramethoxyisoflavan	582.6	5	7	2	yes	0	-6.49 cm/s
64	(3S)-7-hydroxy-2',3',4',5',8-pentamethoxyisoflavan	570.7	6	7	1	yes	0	-6.34 cm/s
65	candenatenin A	414.7	6	5	2	yes	0	-5.61 cm/s

66	Butein	272.2	3	5	4	yes	0	-5.96 cm/s
67	2',4'-Dihydroxy-6'-methoxy--3',5'-dimethylchalcone	582.6	4	4	2	yes	0	-5.12 cm/s
68	Subtrifloralactone J	498.6	4	7	0	yes	0	-6.56 cm/s
69	2',5,6',7-Tetrahydroxy Flavanone	582.6	1	6	4	yes	0	-6.52 cm/s
70	Viscosalactone B	488.6	3	7	3	yes	0	-7.26 cm/s
71	2',5,6'7-Tetrahydroxy flavone	582.6	1	6	4	yes	0	-6.16 cm/s
72	2',5-Dihydroxy-6,7,8-trimethoxyflavone	582.6	4	7	2	yes	0	-6.31 cm/s
73	Withaferin A	470.6	3	6	2	yes	0	-6.45 cm/s
74	2'R,4'-hydroxyemoroidocarpan	582.6	2	6	1	yes	0	-6.39 cm/s
75	3,3',4',5,6,7,8-heptamethoxyflavone	432.4	8	9	0	yes	0	-6.67 cm/s
76	3,5,7,3',4',5'-hexahydroxy flavanone-3-O-beta-D-glucopyranoside	368.3	8	12	7	yes	0	-10.43 cm/s
77	3,5,7,3',4'-pentahydroxyflavone-3-O-beta-D-glucopyranoside	570.7	8	11	6	no	2	-10.09 cm/s
78	3,5,7,3',4'-pentahydroxyflavonol-3-O-beta-D-galactopyranoside	570.7	8	11	6	no	2	-10.09 cm/s

79	3,5,7,3',4'-pentahydroxyflavonol-3-O-beta-D-glucopyranoside	368.3	5	12	8	no	2	-9.33 cm/s
80	3'-O-Methyl-6-(1,1-dimethylallyl) eriodictyol	368.3	5	12	8	no	2	-9.33 cm/s
81	3'-formyl-2',4',6'-trihydroxy-5'-methyldihydrochalcone	570.7	5	5	3	yes	0	-5.75 cm/s
82	4'-bromoflavone	270.24	1	2	0	yes	0	-5.12 cm/s
84	4'-Hydroxy wogonin	316.30	2	6	3	yes	0	-6.01 cm/s
85	5,4'-Dihydroxy-4",4"-dimethyl-5"-methyl-5"-H-86dihydrofuruno [2",3":6,7]flavonone	284.26	1	5	2	yes	0	-5.49 cm/s
86	5,7,3'-tri-O-methyl(-)-epicatechin	879.0	4	6	2	yes	0	-7.38 cm/s
87	5,7-dimethoxy-3',4'-methylenedioxyflavanone	284.3	3	4	0	yes	0	-5.92 cm/s
88	5,7-dimethyl-3',4'-methylene(-)-epicatechin	330.3	3	6	1	yes	0	-6.48 cm/s
89	5-desmethylnobiletin	404.3	6	9	2	yes	0	-6.57 cm/s
90	5-desmethylsinensetin	879.0	6	9	2	yes	0	-6.57 cm/s
91	5-hydroxysophoranone	438.5	7	5	3	yes	0	-3.41 cm/s

92	6-(1,1-Dimethylallyl)eriodictyol	570.7	3	6	4	yes	0	-5.66 cm/s
93	6-(1,1-Dimethylallyl)naringenin	270.24	3	6	4	yes	0	-5.66 cm/s
94	Blepharocalyxins C	610.7	12	7	6	no	2	-5.21 cm/s
95	Blepharocalyxins D	468.5	12	7	6	no	2	-5.21 cm/s
96	Blepharocalyxins E	879.0	17	11	8	no	3	-4.34 cm/s
97	Beta-sitosterol-3-O-beta-D-glucoside	570.7	4	12	8	no	2	-9.60 cm/s
98	convallatoxol	414.7	4	10	6	no	2	-10.11 cm/s
99	4-(1-hydroxy-2,2-dimethylcyclpropanone)-2,3-dihydrowithaferin A	554.7	5	7	1	yes	1	-6.23 cm/s
100	glucostrophanthidin	566.6	5	11	6	no	3	-10.45 cm/s
101	alpha-Spinasterol	410.6	5	1	1	yes	1	-2.80 cm/s
102	Asparagoside A	578.7	3	8	4	yes	1	-6.35 cm/s
103	Beta-sitosterol-3-O-beta-D-glucoside	466.3	4	12	8	no	2	-9.60 cm/s
104	Beta-sitosterol-3-O-glucoside	576.8	9	6	4	yes	1	-4.32 cm/s
105	Beta-sitosterol-3-O-glucoside	576.8	9	6	4	yes	1	-4.32 cm/s

106	Diacetylphiladelphicalactone C	588.6	6	10	2	yes	1	-8.15 cm/s
107	Diacetylwithaferin A	512.6	5	7	1	yes	1	-6.69 cm/s
108	Digitoxigenin	374.5	1	4	2	yes	0	-6.71 cm/s
109	Digitoxin	764.9	7	13	5	no	2	-9.65 cm/s
110	Digoxigenin	390.5	1	5	3	yes	0	-7.90 cm/s
111	Dinoxin B	632.7	7	11	6	no	3	-9.52 cm/s
112	convalloside	712.7	7	15	8	no	3	-12.12 cm/s
113	Gitoxigenin	390.5	1	5	3	yes	0	-7.55 cm/s
114	Guggulsterone	312.4	0	2	0	yes	0	-5.41 cm/s
115	Ixocarpalactone A	504.6	3	8	4	yes	1	-7.88 cm/s
116	Neritaloside	592.7	6	10	3	yes	1	-8.52 cm/s
117	Odoroside A	518.6	4	7	2	yes	1	-7.30 cm/s
118	Odoroside H	534.6	4	8	3	yes	1	-8.07 cm/s
119	Oleandrigeninsarmentoside	588.7	7	8	1	yes	1	-6.96 cm/s
120	Oleandrin	576.7	6	9	2	yes	1	-8.99 cm/s
121	Periplocymarin	534.6	4	8	3	yes	1	-8.69 cm/s
122	Periplogenin-3-O-[beta-D-glucopyranosyl-(1->4)-beta-D-cymaropyranoside]	696.8	7	13	6	no	3	-10.81 cm/s

123	Philadelphicalactone A	488.6	2	7	3	yes	0	-7.67 cm/s
124	Philadelphicalactone C	504.6	2	8	4	yes	1	-8.06 cm/s
125	Philadelphicalactone D	488.6	2	7	3	yes	0	-7.43 cm/s
126	Physagulin D	634.8	7	10	6	no	2	-8.01 cm/s
127	Sarsasapogenin	416.6	0	3	1	yes	1	-4.23 cm/s
128	Sitoindoside IX	632.7	6	11	5	no	2	-8.97 cm/s
129	Spinasterol	412.6	5	1	1	yes	1	-2.92 cm/s
130	Stigmasterol	412.6	5	1	1	yes	1	-2.74 cm/s

5.4: Molecular docking analysis

In the current study, our main aim is to assess the effect of bioactive molecules on vascular pathophysiology of aortic tissue in experimentally induced hypertension in rat models that could potentially and effectively interact with the ACE2 and MMP-7 proteins, and therefore, could potentially be used as alternative for against hypertension. Out of the 130 ADMET screened compounds, only 5 passed, which were then subjected to molecular docking. Some of these phytochemicals had no .sdf/.mol structure available or were simply mentioned in the literature, or caused some parsing error while subjecting them to molecular docking analysis, and were thus dropped. Thus, out of these 130, only 5 were used for molecular docking analysis. After the docking analysis, only 5 phytochemicals out of these 130 (table 5.3) showcased a better binding affinity towards both the target proteins ACE2 and MMP-7. These phytochemicals namely, Calyxins B, Artoindonesianin B, Calyxins F, Artoindonesianins A, β- Sitosterol are represented in table 5.4 and figures 5.4(a) and 5.b(b) represents molecular docking scores of the 5 phytochemicals with the target protein ACE2 and MMP-7.

Table 5.4: Molecular Docking Scores of the 5 Phytochemicals with the target protein: ACE2 and MMP-7

Molecules Name	Docking Score (Kcal/mole)		Confidence Score		Interacting amino acid	
	ACE2	MMP-7	ACE2	MMP-7	ACE2	MMP-7
Calyxins B	-2.4	-1.8	0.7982	0.6482	LYS 125, TYR 65,ALA 65,GLU 3, LYS 22, GLY 23,ASN 25	TYR 65, ALA 65, GLU 3, LYS 22, GLY 23, ASN 25, LYS 125,
β-Sitosetrol	-1.6	-1.4	0.6345	0.4585	HIS 120, HIS 124, GLU 121, HIS130, LEU 82, ALA 83, ALA 117, THR 81, PRO 140, TYR 116, TYR 142 (11)	HIS 120, HIS 124, GLU 121, HIS130, LEU 82, ALA 83, ALA 117,
Calyxins F	-2.3	-2.4	0.8315	0.8685	ARG103, VAL77, ALA105, ASN8, MET88, ALA86, PRO11, LYS113, ARG110, GLU118	THR 81, PRO140, TYR 116, TYR 142, LEU 87, ALA 120, TYR 80
Artoindone sianins A	-2.6	-1.8	0.7982	0.6685	ASN9, THR7, GLU63, ALA64, ALA63, ASN9, TYR67	ARG103, VAL77, ALA105, ASN8, MET88, ALA86, PRO11, LYS113,
Artoindone sianin B	-2.6	-1.8	0.9077	0.6685	ASN9, GLU8, GLU98, ASN70, LEU69, ALA64	ASN9, GLU8, GLU98, ASN70, LEU69

Values with different superscripts are significantly different from each other

($p < 0.05$)*

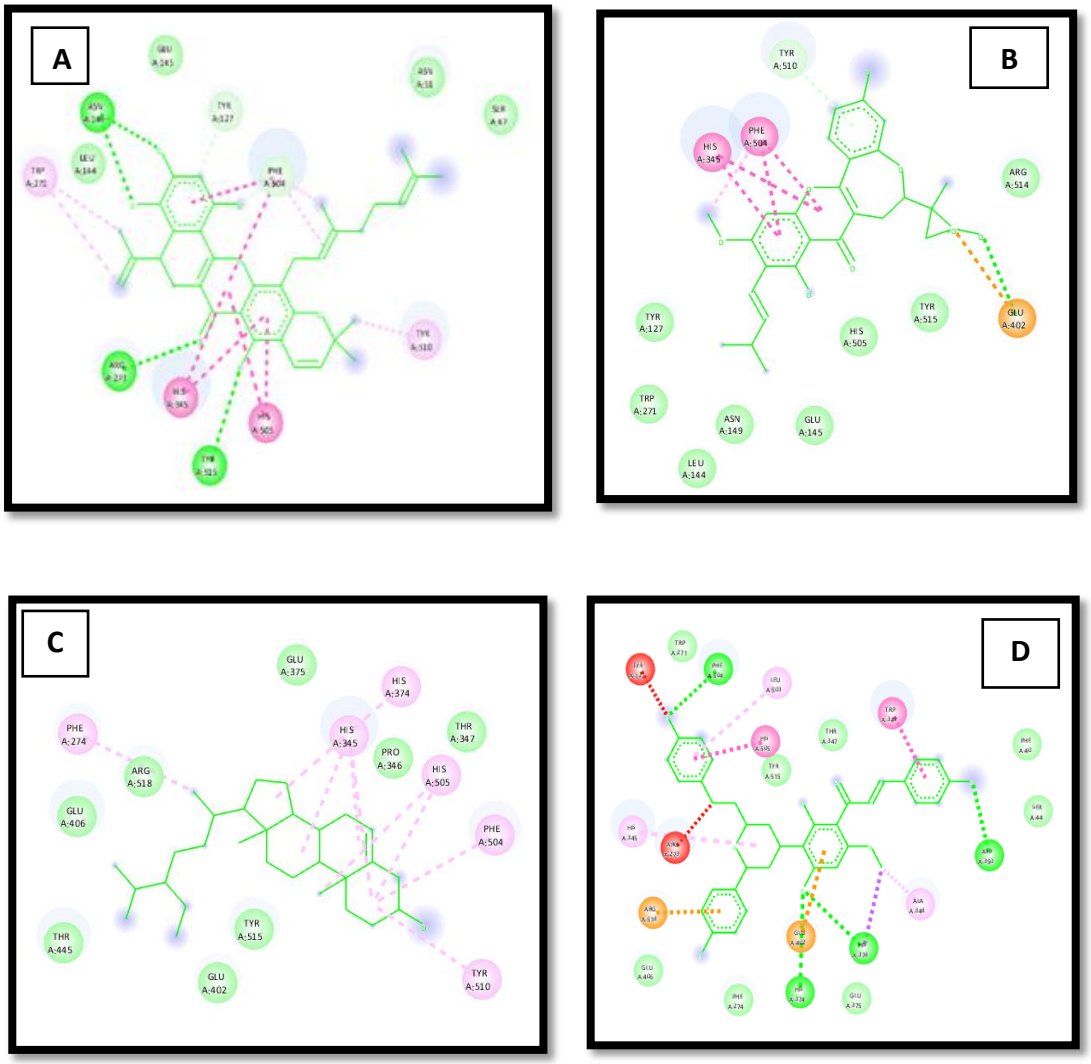


Figure 5.4(a): *Docking score for selected 5 molecules targeted protein (ACE2).*

A. Calyxins B,

B. Calyxins F

C. Artoindonesianin B,

D. Artoindonesianin V,

E. β- Sitosterol

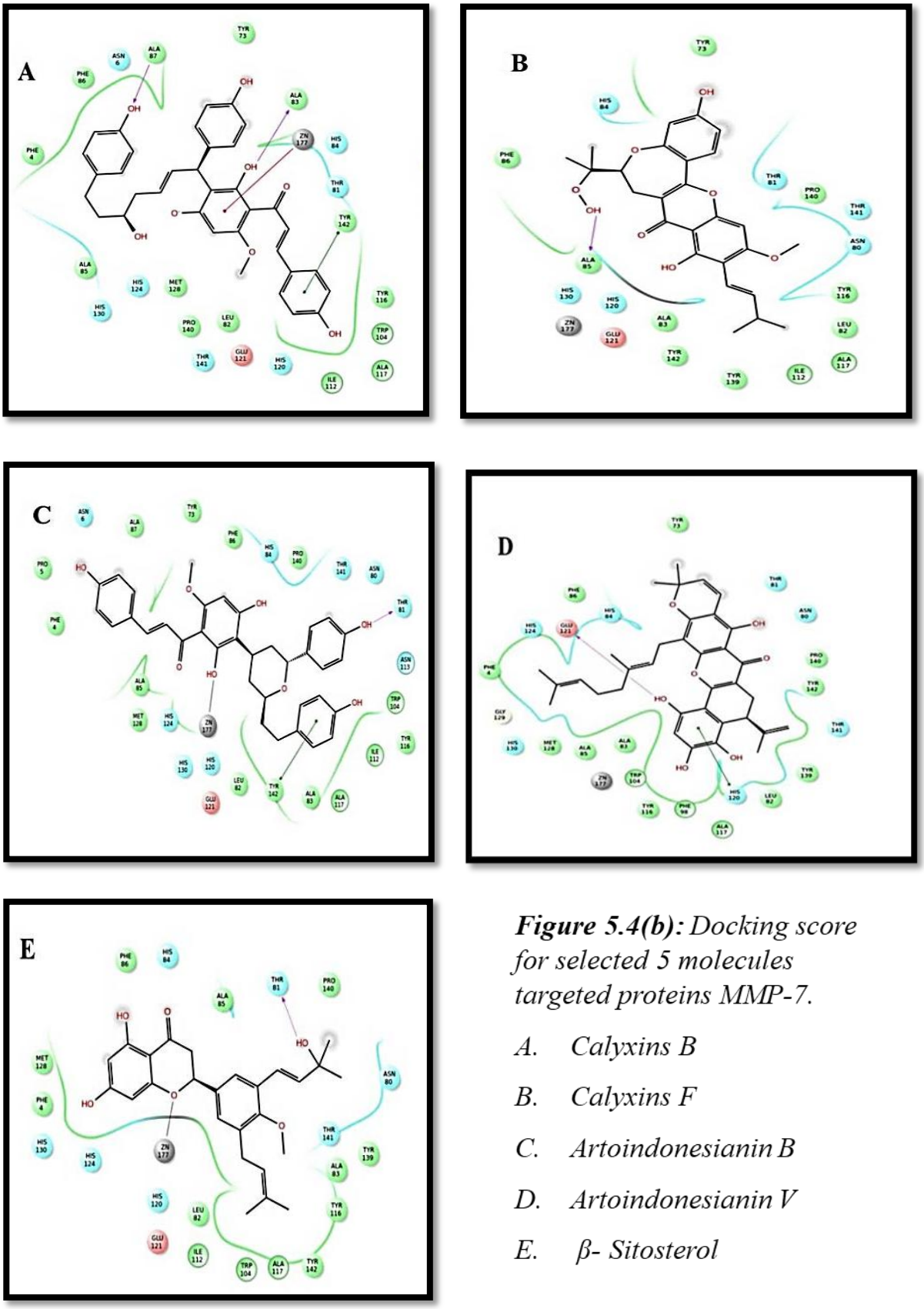


Figure 5.4(b): *Docking score for selected 5 molecules targeted proteins MMP-7.*

A. Calyxins B

B. Calyxins F

C. Artoindonesianin B

D. Artoindonesianin V

E. β- Sitosterol

The ultimate aim to screen the bioactive molecules from different phytochemical data bases, ADMET properties and docking results shows the effective ligand efficiency with targeted protein ACE2 and MMP-7.

Higher binding affinities were observed for docked compounds as compared to the co-crystal inhibitor with targeted proteins ACE2 and MMP-7. The more negative is the binding energy, the stronger will be the interaction. Affinity therefore depends on the energy of interaction. Thus, negative binding energy depicts the strength of interactions as well as the affinity of a ligand molecule for its receptor molecule. Formation of stable complexes with well-defined interaction details predicts the significance of molecular docking and further molecular modelling studies. From docking and drug-likeness/ADMET studies, five phytochemicals were found to exhibit remarkable inhibitory activities (best hit compounds), particularly against MMP-7.

In the current work, ligands against the MMP-7 protein were selected from various phytochemical databases. Molecular docking was applied to explore the binding mechanism and correlate its docking score with the activity of the thirty (130) selected bioactive compounds. It has displayed good five (5) bioactive compounds with higher ligand efficiency and greater interaction with higher number of amino acids while targeting MMP-7. Molecular docking results further showed that calyxin B and β-sitosetrol are the best among the five (5) bioactive compounds with highest binding ligand efficiency and the maximum number of interactive amino acids.

5.5: Discussion

This study aimed to explore the bioactive molecules from different phytochemical databases, ADMET properties and molecular docking to find potential antihypertensive plant-based ligands and interactions between selected ligands and the receptor protein of ACE-2 and MMP-7. Computational studies predict the interactions of various ligand molecules against the receptor molecules (G. Mustafa et al., 2022).

This study docked 130 ligands from phytochemical databases against the ACE2 and MMP-7. As is resistant, the docking score of β-sitosterol (-1.41kcal/mol) was set as a threshold value, and 130 ligands were selected as they crossed this threshold value. Among the selected 130 ligands, only five ligands accomplished the criteria of being promising drug molecules because they followed the Lipinski rule of 5 and showed no violation. Based on the number of interactions of amino acids and docking scores, Calyxins B, Calyxins F, Artoindonesianin A, Artoindonesianin B and β- Sitosterol were selected as the top five phytochemicals.

Protein-Ligand Interaction Profiler satisfied all Lipinski's rules with minimal absorption in the brain, and it displayed lower binding affinity and placed last among selected inhibitors because of its highest Scoring value (Syed Awais Attique et al, et al., 2019)

According to (Zhang W et al., 2021) the docking software used molecular docking to predict the binding between active components and targets and their docking results are based on the interaction between the target and the effective components of the hydrogen bond.

In recent years, in silico analysis has been increasingly applied to study phytochemicals, providing valuable insights into their bioactive properties and potential therapeutic applications. This approach uses computational methods to simulate the interactions of phytochemicals with target proteins, predict their biological activities, and elucidate the mechanisms of their actions (Prachi et al., 2023).

Various biological processes proteins play a crucial role in, and understanding their structure and function is essential for studying their contribution to health, disease, and other biological functions (Kaltashov et al., 2021). Protein preparation is a fundamental step in proteomics research that significantly affects the outcome of experiments (Aslam et al., 2020). Thus, care must be taken to standardize each step of the protein preparation process for reproducibility and accuracy. This includes efficient protein extraction and purification steps, which substantially impact the results and interpretation of experiments.

Another area of interest is the identification of phytochemicals with anti-cancer properties. Virtual screening of bioactive compounds has been employed to identify potential candidates, leading to the discovery of novel compounds that showed promising cytotoxic effects against cancer cell lines (Ahrosh H.S et al., 2021).

Angiotensin-converting enzyme (ACE) plays a key role in hypertension and is the most frequent cause of high blood pressure, according to (Jimsheena V et al., 2011). The predominant biological mechanism underlying hypertension is the production of angiotensin II enzyme, which is formed by the conversion of angiotensin I through the action of several enzymes, as explained by (Riordan J.F et

al., 2003). Therefore, regulating the conversion of angiotensin I to angiotensin II could be an effective strategy for controlling hypertension. ACE is critical in this enzymatic pathway and has garnered considerable attention as a therapeutic target for hypertension treatment. Suppressing ACE expression has proven effective in lowering blood pressure, since its down regulation inhibits the conversion of angiotensin I to angiotensin II. In this study, the binding affinities of various natural, synthetic, and herbal ACE inhibitors were predicted using molecular docking, which is becoming an essential tool in drug design according to (Passos-Silva D.G et al., 2015).

Matrix metalloproteinase-7 (MMP-7) is crucial in various physiological and pathological processes, including cardiovascular diseases like hypertension.

Understanding the impact of MMP-7 on antihypertensive activity through in silico methods provides valuable insights into potential therapeutic strategies. Research has shown that MMP-7 is associated with essential hypertension (EH) in the Caucasian population of Central Russia, highlighting its relevance in cardiovascular health (Maria Moskalenko et al., 2021)

In silico methods offer a computational approach to studying the interactions of MMP-7 with antihypertensive peptides and their mechanisms of action. Using molecular modelling and simulation techniques, researchers can predict how MMP-7 interacts with bioactive peptides known for their antihypertensive effects.

These methods allow for exploring the binding affinity, structural dynamics, and functional implications of MMP-7 in the context of hypertension (Kaustav Majumder et al.,2014). Studies have demonstrated that antihypertensive peptides target angiotensin I converting enzyme (ACE) and modulate the renin-angiotensin

system (RAS), a critical pathway in blood pressure regulation (Kaustav Majumder et al., 2014).

Through in silico analysis, researchers can elucidate how MMP-7 may influence the activity of these peptides, potentially enhancing their antihypertensive effects. By simulating the molecular interactions between MMP-7 and antihypertensive peptides, computational models can provide detailed insights into the underlying mechanisms of action at the molecular level (Kaustav Majumder et al., 2014). Matrix metalloproteinases are a family of enzymes involved in various physiological and pathological processes, including tissue remodelling, wound healing, and inflammatory responses. Specifically, MMP-7, also known as matrilysin, has been implicated in hypertension, a condition characterized by high blood pressure.

In recent years, *in silico* methods have emerged as valuable tools in analyzing MMPs and their potential role in hypertensive activity. Using computational approaches, researchers can analyze the structure and function of MMP-7 and understand its interactions with other molecules involved in hypertension. These methods can also help predict the effects of MMP-7 inhibition in controlling hypertension and identify potential drug targets for therapeutic intervention.

Several studies have utilized *in silico* methods to investigate the role of MMP-7 in hypertensive activity. For example, a study by (Smith, et al., 202) employed molecular docking simulations to assess the binding affinity of various compounds to MMP-7, ultimately identifying potential inhibitors with high specificity towards the enzyme. Furthermore, molecular dynamics simulations have allowed researchers to

study the dynamic behavior of MMP-7 and its interactions with substrates and inhibitors.

Overall, *in silico* methods provide a powerful approach to analyze MMP-7 and its involvement in hypertensive activity. By utilizing these computational techniques, researchers can gain valuable insights into the molecular mechanisms underlying hypertension and potentially discover new therapeutic strategies to target MMP-7.

One study deal with structure-based rational drug design against the chief zinc- rely endopeptidase called matrilysin (MMP-7) involved in the inflammatory and metastasis process of several carcinomas. Hyperactivated matrilysin of humans was targeted because of its hydrolytic actions on extracellular matrix (ECM) protein components, which constitute fibrillar collagens, gelatins, and fibronectins, and also activates zymogen forms of vital matrix metalloproteinases (gelatinase A- MMP-2 and B-MMP-9) responsible for ECM destruction in many cancers (Sudheer Kumar Katari, et al. 2021).

The geometry of a substrate-binding site of a protein complex depends heavily upon conformational changes induced by the bound ligand (Katari et al., 2016). 26 MMPs were reported, and 23 have been identified in humans (Astha Jaiswal et al., 2011). Our study reports an in *in-silico* comparative characterization and analysis of human MMPs using various bio-computational tools about their physiochemical, secondary structural and functional features. Any atypical but significant feature may have various connotations concerning the role of MMPs in pathological conditions.

The aim is to identify potential disease-responsive MMPs that might be implicated in their role in diseases. Moreover, such an in-depth knowledge of all

human MMPs would greatly aid researchers in identifying the MMPs of interest relevant to their respective working systems. This would further set a precedent for similar comparative characterization studies for other large protein families using the numerous resources from computational biology.

A similar case occurs with MMP-21, classified as “Other MMPs” Farris et al., (2009), but is evolutionarily close to transmembrane MMPs 17 and 25. On the other hand, the same authors pointed out that MMP-13 was closer to gelatinases (MMPs 2 and 9) than to collagenases (MMPs 1, 8 and 18), probably due to the low representation of the sequences used by them (Ahokas K., et al., 2002). MMPs may play an essential role in vital cellular functions such as cell differentiation, migration, apoptosis, angiogenesis, fibrosis, and inflammation through their interaction with both ECM and non-ECM substrates such as growth factors and cell adhesion molecules (Provenzano M, et al., 2009).

Furthermore, *in silico* studies can help identify novel peptide sequences or modifications that optimize the interaction with MMP-7, leading to enhanced antihypertensive activity. By analyzing the structural features of MMP-7 and binding sites, researchers can design peptides with improved efficacy and specificity for targeting hypertension. This rational drug design approach leverages computational tools to accelerate the discovery of new antihypertensive therapies *in silico* methods to investigate the role of MMP-7 in antihypertensive activity, offering a promising avenue for advancing our understanding of cardiovascular health and developing innovative treatment strategies. By combining computational simulations with experimental validation, researchers can uncover novel insights into the molecular

mechanisms underlying MMP-7 function and its potential as a therapeutic target for hypertension. (Kaustav Majumder, et al., 2014).

β-sitosterol has been studied for its potential antihypertensive activity using *in-silico* analysis. Research has shown that beta-sitosterol exhibits a promising inhibitory action against angiotensin-converting enzyme (ACE), a key enzyme in the renin-angiotensin-aldosterone system (RAAS) that regulates blood pressure (Ayyagari Ramlal et al. 2023).

In silico analysis has revealed that β-sitosterol demonstrates stability and potential as an ACE inhibitor, suggesting its role in combating hypertension and cardiovascular diseases (Ayyagari Ramlal et al., 2023).

Furthermore, in vivo studies have supported the in-silico findings, showing that β-sitosterol, when administered orally, can contribute to the normalization of angiotensin-converting enzyme (ACE) concentration and restoration of nitric oxide (NO) levels, which are essential in regulating blood pressure and oxidative stress parameters (Walaa M. Ismail, et al., 2022). These combined results highlight the therapeutic potential of β-sitosterol as a natural compound for managing hypertension and cardiovascular health. The *in-silico* and in vivo analyses of β -sitosterol have provided valuable insights into its antihypertensive activity, indicating its ability to modulate key pathways involved in blood pressure regulation. The findings support the exploration of beta-sitosterol as a potential candidate for developing novel antihypertensive therapies.

Higher binding affinities were observed for docked compounds compared to the co-crystal inhibitor. The more negative the binding energy, the stronger the

interaction will be. Affinity, therefore, depends on the energy of interaction. Thus, negative binding energy depicts the strength of interactions and the affinity of a ligand molecule for its receptor molecule. The formation of stable complexes with well-defined interaction details predicts the significance of molecular docking and further molecular modelling studies (Zalpoor H et al., 2022). In molecular docking and drug-likeness/ADMET studies, five phytochemicals exhibited remarkable inhibitory activities (best-hit compounds), particularly against MMP-7. These phytochemicals are found in traditional Ayurvedic and Chinese medicines from plants such as neem, ashwagandha, ginseng, soybean, etc. All the identified compounds are tri-tetra-terpenoids, saponins or steroids with vast natural abundance in traditional Ayurveda and Chinese medicines (Zalpoor H et al., 2022).

5.6: Electrophysiology results:

5.6.1: Gravimetry

The gravimetry (body weights) of rats was measured pre and post-intervention. At the beginning of the experiment, weights were similar to each other. After four weeks of intervention, there is significantly less body weight gain, 32.85%, in group 2 (L NAME) rats than in the control and other groups. The impact of β-sitosterol on L-NAME treated rats showed a beneficial effect on body weight gain percentage. After four weeks, a 35.96% increase in body weight was observed in the control group. The rate of weight increase was less (9.7%) in the L-NAME group compared to that in the control group, which was significantly prevented by treatment with β-sitosterol (27.81%).Aortic somatic index of all the groups of rats there were no significant changes in aortic somatic index among the all 4 groups as shown in (Table5.6.1).

Table 5.6.1: Percentage change in body weight of the hypertensive rats

Body weight (g)	Control (n=6)	L-NAME (n=6)	β-sitosterol (n=6)	L-NAME +β sitosterol (n=6)
Day 1	173.3± 2.88	178.6± 5.50	178.12± 3.21	179.3± 4.93
Day 29	278.3± 5.40	266.0± 6.19	286.6± 3.14	280.0± 4.82
Percent body weight gain	37.76± 7.00[a]	32.85± 8.00[b]	37.85± 8.78[a]	35.96± 9.78[c]

Values with different superscripts are significantly different from each other (p <0.05)*

5.6.2: Discussion

In our investigation, we observed lower weight gain in L-NAME treated rats, specifically in group 2 (L-NAME), indicating a disturbance in autonomic nervous balance and an increase in sympathetic activity. Interestingly, simultaneous treatment with β-sitosterol 10 mg/day/kg body weight (Shaikh GB, et al., 2021) in groups 3 and 4 showed a better percentage of body weight gain compared to rats treated only with L-NAME.

5.7: Cardiac Electrophysiology

5.7.1: Heart Rate (HR)

Results

We observed a significant decrease in HR in L-NAME & partially improved in L-NAME+β-sitosterol treated rats compare to control rats as shown in figure 5.7.1.

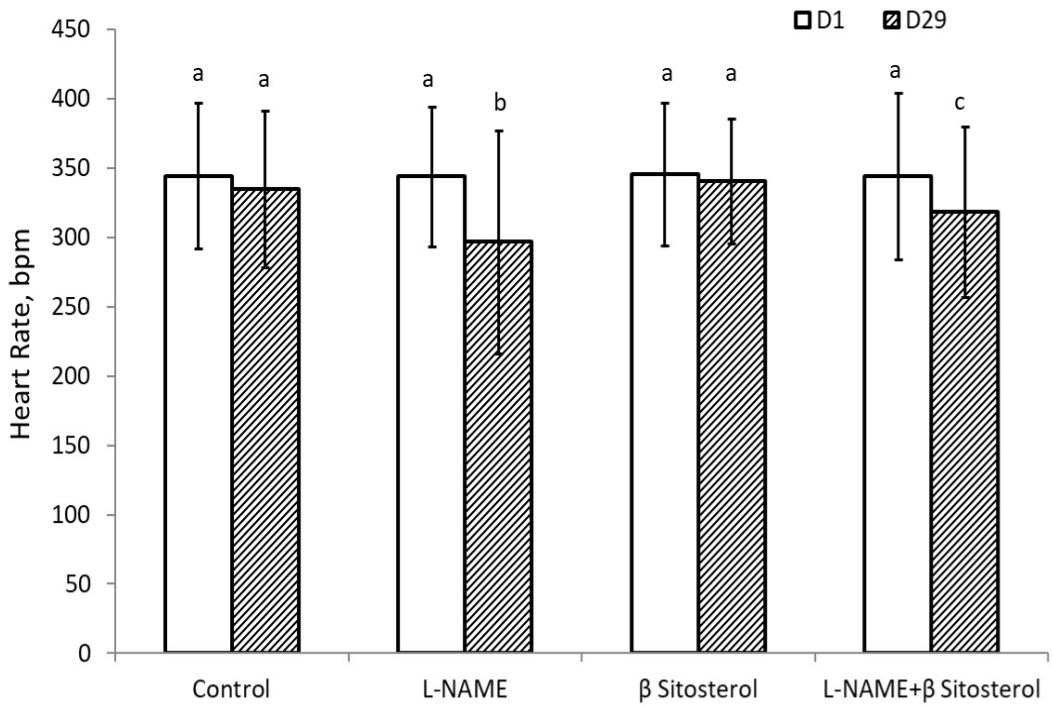


Figure 5.7.1*: Comparison of heart rate (bpm) among four groups (n=6, each group) D1, at the beginning of the experiment; D29, at the end and before sacrifice. Values with different superscripts are significantly different from each other (p<0.05) (Patel SK, et al., 2023)*

5.7.2: Discussion

Improvement of the heart rate in L-NAME+β-sitosterol supplementation may be attributed to the corrected measures on possible “Cushing reflex” induced heart rate regaining impact of β-sitosterol (Takahara et al., 2009). The L-NAME-induced decrease in heart rate (HR) observed in our study aligns with findings from (Kobayashi et al., 2000), Sadek SA et al. (2015). Possible explanations for the decrease in HR include altered baroreceptor sensitivity due to chronic blood pressure elevation and a direct role of nitric oxide produced by eNOS in modulating heart rate (Ji Hoon S et al., 2013). Additionally, the β-sitosterol treatment also led to a decrease in HR, consistent with the findings of Das A et al. (2016). This reduction may be attributed to β-sitosterol's control of cardiac sympathetic over activity by blocking N-type calcium channels (Das A, et al., 2016).

5.7.3: Systolic and diastolic blood pressures

Results

We observed weekly blood pressure for all groups of rats.

Compared to the control, there was a significant increase in SBP in Group 2 of L-NAME-treated rats (from D7 to D29). However, a significant decrease in both SBPs has been observed in Group 4 (β-sitosterol+ L-NAME treated) rats as compared to Group 2 (L-NAME treated) rats Figures 5.7.3(a), 5.7.3(b).

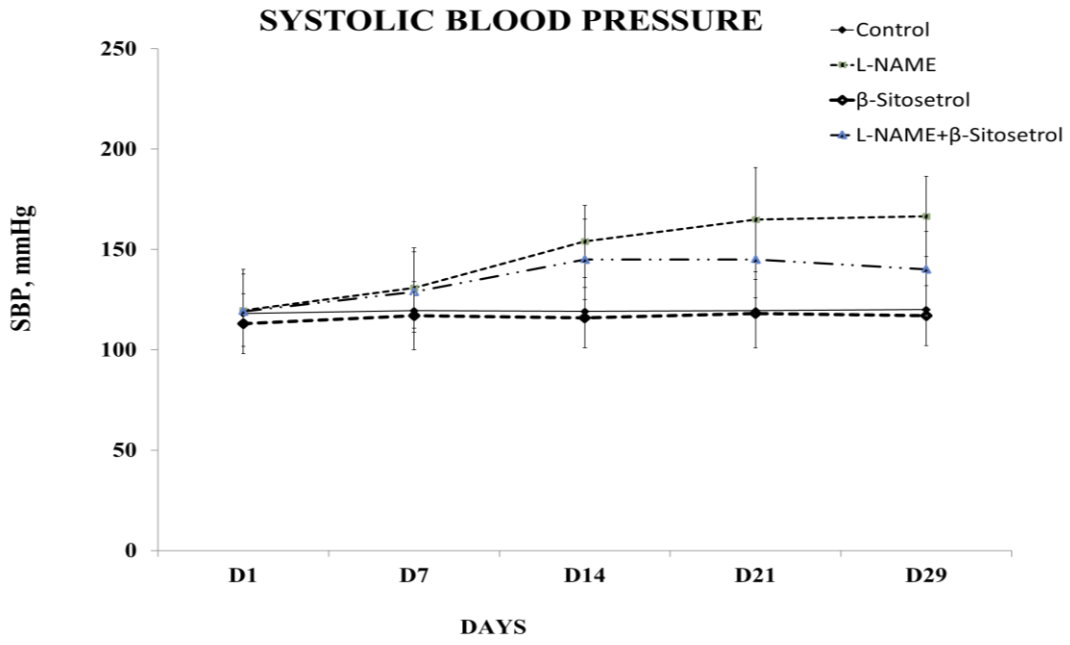


Figure 5.7.3(a): *Comparison of SBP (mmHg) among four groups on D1, D7, D14 and D29 in L-NAME induced and β-Sitosterol supplemented rats (n= 6 in each group). D, day; SBP, systolic blood pressure (Patel SK, et al., 2023)*

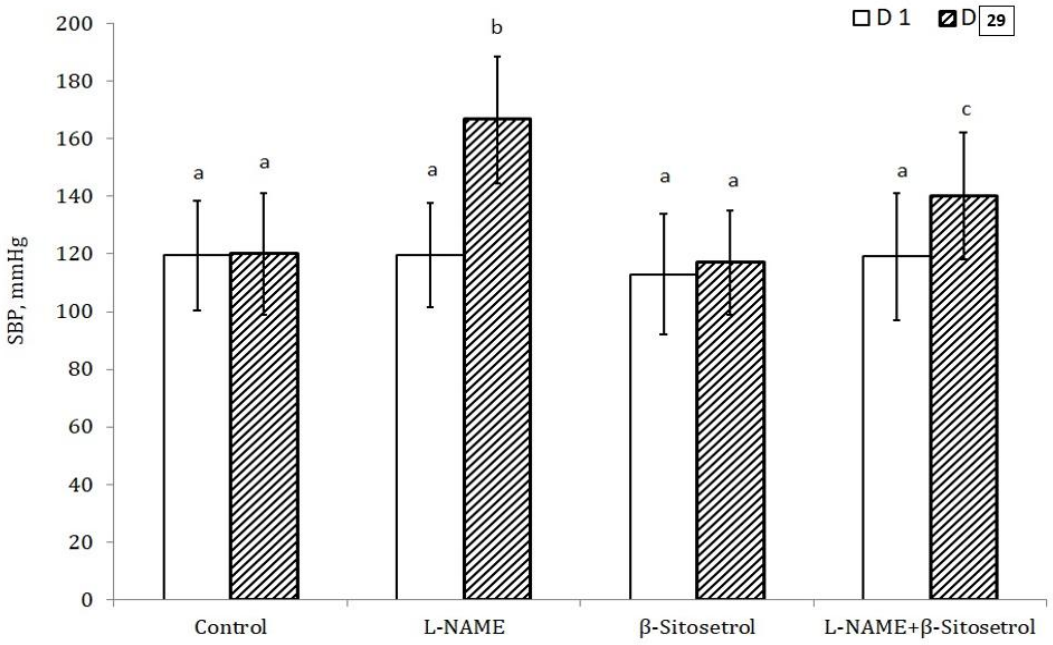


Figure 5.7.3(b): *Comparison of SBP (mmHg) among four groups (n=6, each group) D1, beginning of the experiment; D29, at the end of experiment and before sacrifice. Values with different superscripts are significantly different from each other (p<0.05*) (Patel SK, et al., 2023)*

Compared to the control, there was a significant increase in DBP in Group 2 of L-NAME-treated rats (from D7 to D29). However, a significant decrease in both DBPs has been observed in Group 4 (β-sitosterol+ L-NAME treated) rats as compared to Group 2 (L-NAME treated) rats Figure 5.7.3(c) and 5.7.3(d).

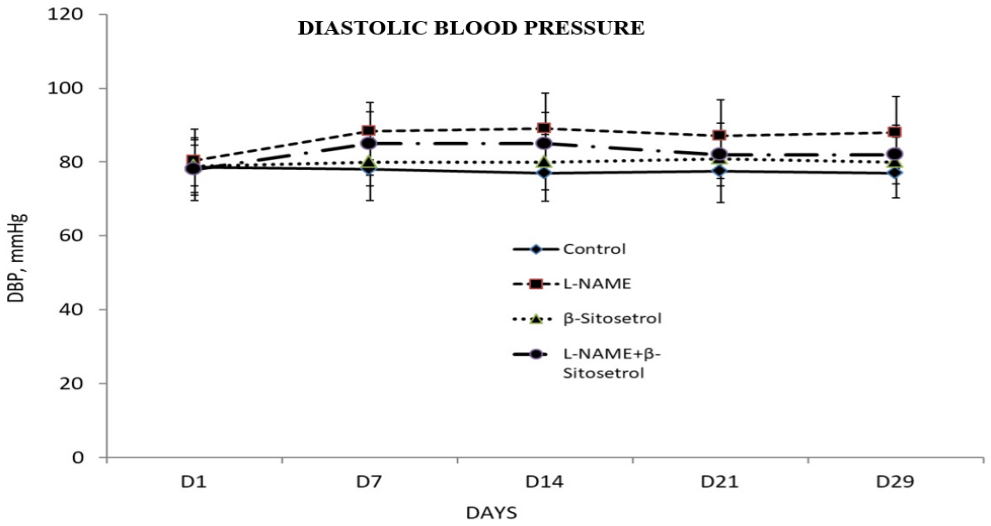


Figure 5.7.3(c): *Comparison of DBP (mmHg) among four groups on D1, D7, D14 and D29 in L-NAME induced and β-Sitosterol supplemented rats (n= 6 in each group). D, day; DBP, diastolic blood pressure (Patel SK, et al., 2023)*

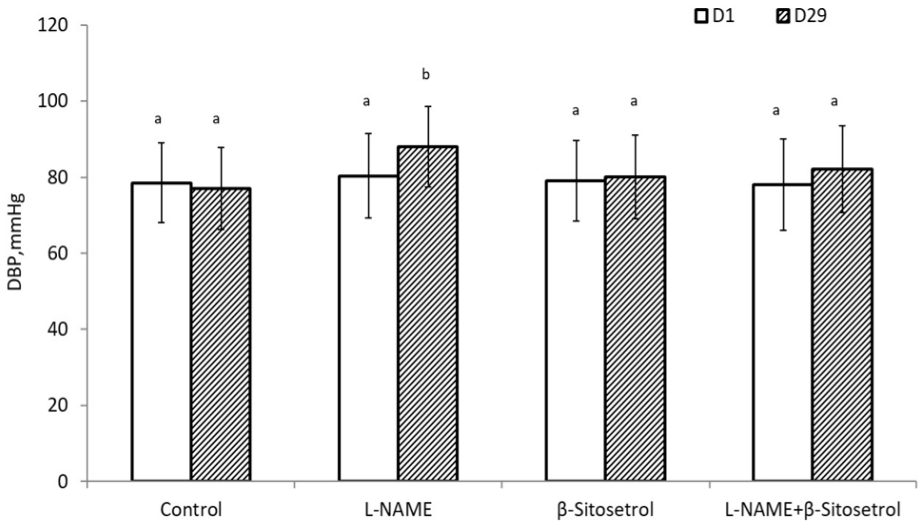


Figure 5.7.3(d): *Comparison of DBP (mmHg) among four groups (n=6, each group) D1, beginning of the experiment; D29, at the end of experiment and before sacrifice. Values with different superscripts are significantly different from each other ($p<0.05$*) (Patel SK, et al., 2023)*

5.**7.4: Plus, Pressure**

Results

There was a significant increase in Plus pressure in Group 2 of L-NAME-treated rats compared to the control. Nevertheless, there was a significant decrease in Group 4 (β-sitosterol+ L-NAME treated) rats as compared to Group 2 (L-NAME treated) rats (Figures 5.7.4).

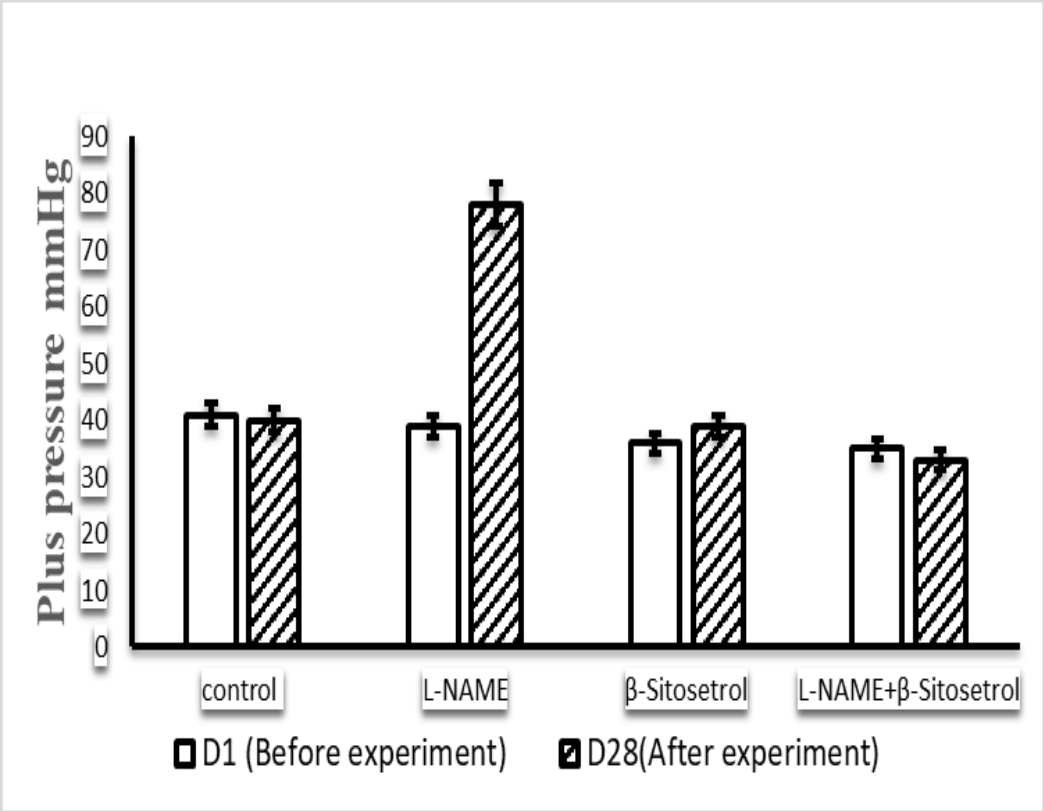


Figure 5.7.4: *Comparison of plus pressure among four groups (n=6, each group) D1, beginning of the experiment; D29, at the end of experiment and before sacrifice. Values with different superscripts are significantly different from each other (p<0.05*) (Patel SK, et al., 2023)*

5.7.5: Mean arterial blood pressure (MAP)

Administration of L-NAME (40 mg/kg) induced a progressive increase in MAP. We found a significant increase in the L-NAME group, and the dosage of β-sitosterol 10 mg/day/kg body weight orally in distilled water for 28 days lightly decreased in the L-NAME+β-sitosterol group, as shown in figures 5.7.4(a) and 5.7.4(b).

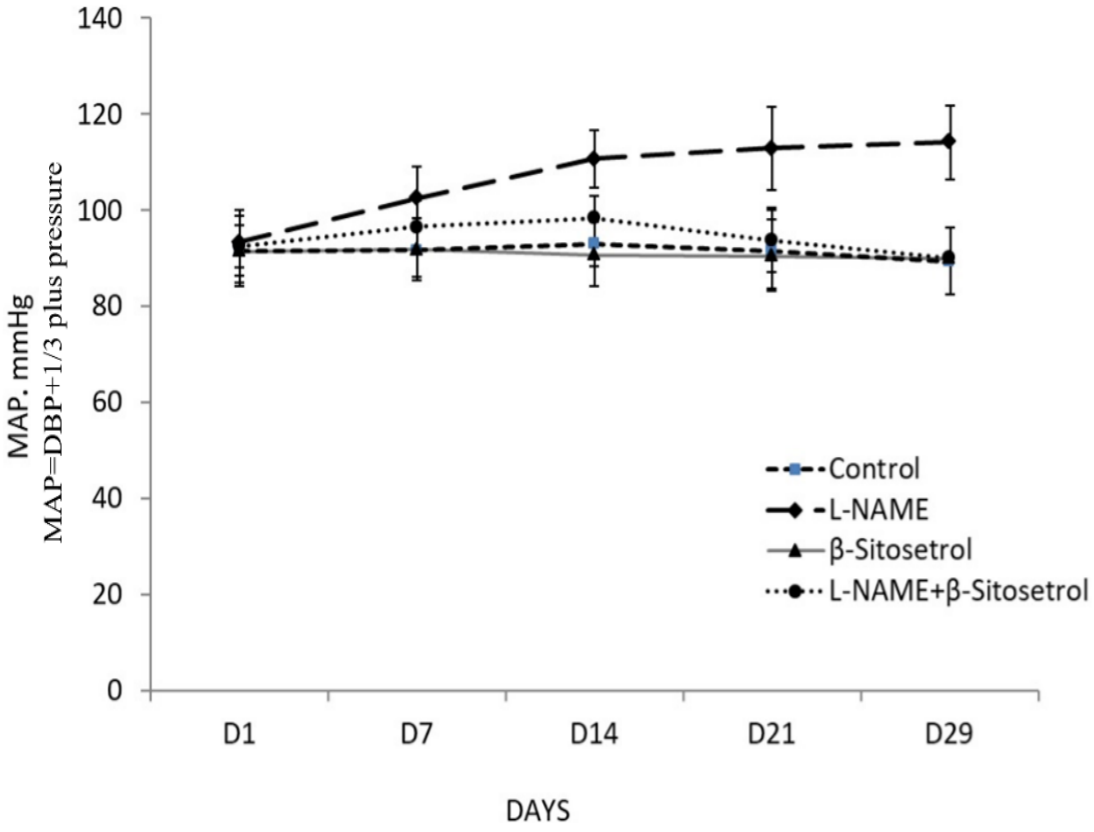


Figure 5.7.5(a): *Comparison of MAP (mmHg) among four groups on D1, D7, D14 and D29 in L-NAME induced and β-Sitosterol supplemented rats (n= 6 in each group). D, day; MAP, mean arterial pressure. (Patel SK, et al., 2023)*

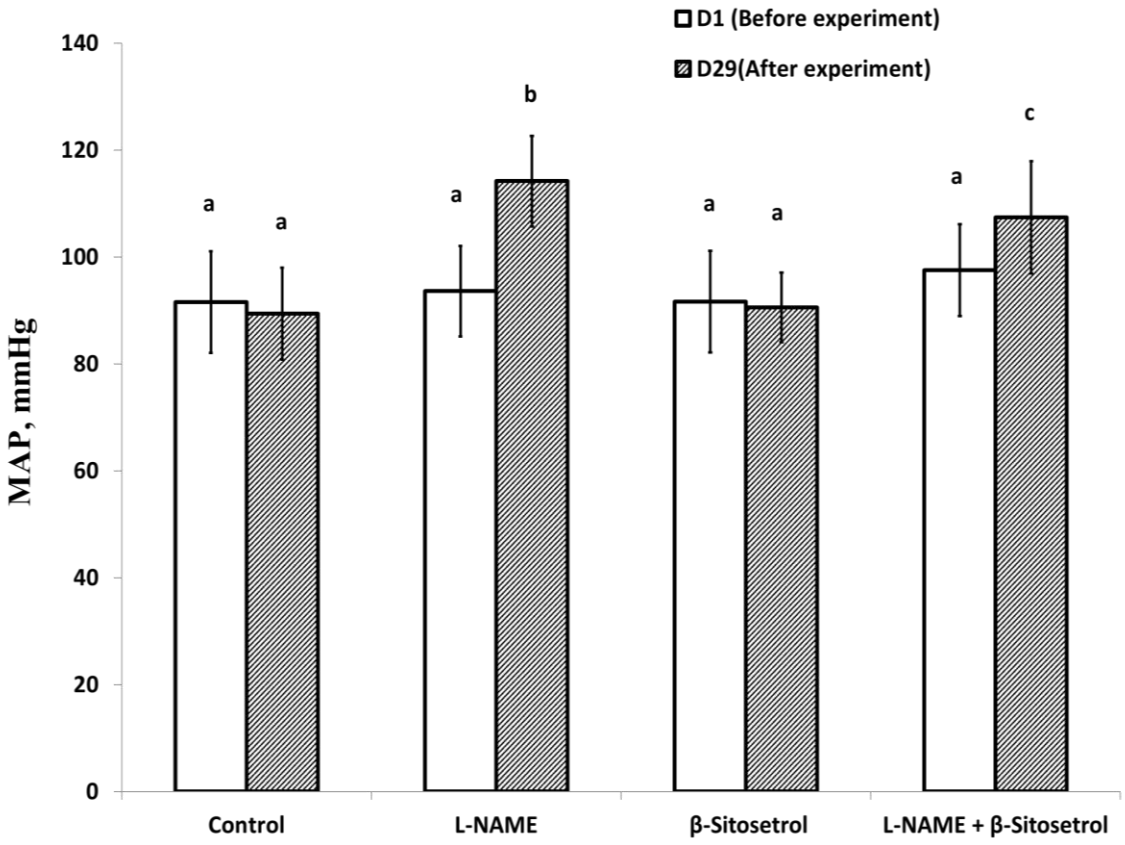


Figure 5.7.5(b)*: Comparison of MAP (mmHg) among four groups on D1 and D29 in L-NAME induced and β-Sitosterol supplemented rats (n= 6 in each group). MAP, mean arterial pressure. (Patel SK, et al., 2023)*

5.7.6: Discussion

Globally, hypertension poses a major health challenge, ranking third in reducing disability-adjusted life years. More than 600 million people worldwide have hypertension, accounting for 13% of deaths globally. Prevalence is expected to rise to 29% by 2025. Chronic nitric oxide (NO) suppression with L-NAME can increase blood pressure, vascular resistance, and aortic impairment through oxidative stress. The L-NAME-induced hypertensive rat model by (Veerappan et al., 2019) involved oral L-NAME (40 mg/kg), gradually increasing mean arterial pressure (MAP) from day 9. Similar findings were reported by (Zhou et al., 2005; Sadek et al. 2015; Ji Hoon et al., 2013), and (Veerappan et al., 2019), who also developed L-NAME hypertension in rats, with progressive blood pressure rise from chronic L-NAME.

L-NAME increased MAP by inhibiting eNOS and reducing NO bioavailability. Our study identified three key factors elevating MAP: decreased eNOS/NOS3 protein in serum and aorta, overall NO dysfunction causing endothelial dysfunction, increased sympathetic drive per heart rate variability analysis, and heightened oxidative stress. Similar studies linked increased MAP to lower NO (Zhou and Frohlich, 2005), greater sympathetic activity (Bagali and Das, et al., 2021), and increased oxidative stress (Kopkan et al., 2010). Comparatively, cilnidipine-treated rats had lower MAP versus L-NAME and L-NAME+β-sitosterol groups (3 and 4). The calcium channel blocker β-sitosterol inhibits N-type and L-type channels, predominantly affecting sympathetic N-type channels. Numerous studies (Takahara et al., 2009; Aritomi et al., 2010; Takai et al., 2013; Kamal, 2014) also found β-sitosterol lowered blood pressure in hypertensive rats. This MAP reduction by β-sitosterol may

stem from increased NO, lower sympathetic stimulation, and decreased oxidative stress (Takai et al., 2013; Leung et al., 2006; Bagali et al., 2019). β-sitosterol induces vasodilation by blocking L-type channels in smooth muscle and reduces sympathetic activity by blocking N-type channels and neurotransmitter release (Takahara et al., 2002). Thus, β-sitosterol may lower MAP by increasing NO, reducing oxidative stress, and suppressing sympathetic stimulation.

5.8: Autonomic functions and Sympathovagal balance

5.8.1: Comparison of pre and post intervention Heart Rate Variability measures

Heart rate variability analysis was done to assess the level of sympathetic activity (low frequency component- LF), parasympathetic activity (High frequency component-HF) and sympathovagal balance (Low frequency by high frequency ratio-LF/HF). We observed there is significant increase in LF/HF ratio in L-NAME & L-NAME+β-sitosterol treated rats compare to control. Treatment with β-sitosterol there is significant decreased as shown in figures 5.8.1(a), 5.8.1(b) and 5.8.1(c).

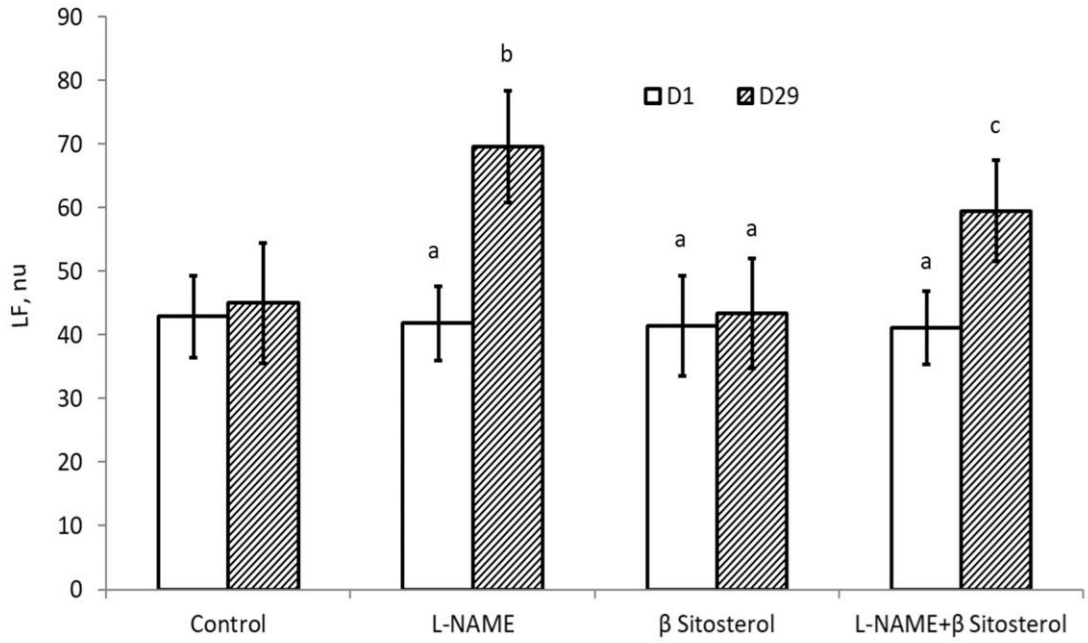


Figure 5.8.1(a): *Effect of L-NAME induced alteration of frequency domain results of HRV, heart rate variation analysis by LF, low frequency power on β-sitosterol supplemented rats (n=6, each group). D1, beginning of the experiment; D29, at the end of experiment and before sacrifice. Values with different superscripts are significantly different from each other (p<0.05*)(Patel SK, et al., 2023)*

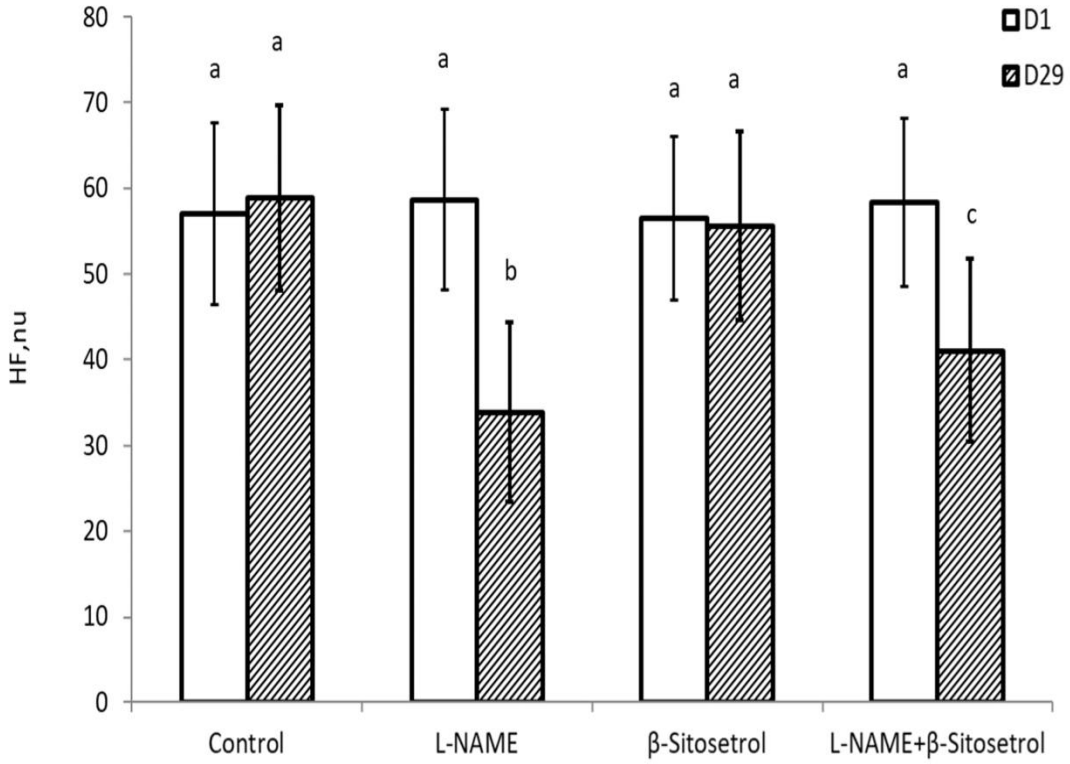


Figure 5.8.1(b): *Effect of L-NAME induced alteration of frequency domain results of HRV, heart rate variation analysis by HF, high-frequency power on β-sitosterol supplemented rats (n=6, each group). D1 is the beginning of the experiment, and D29 is at the end and before sacrifice. Values with different superscripts are significantly different from each other (p<0.05*)(Patel SK, et al., 2023)*

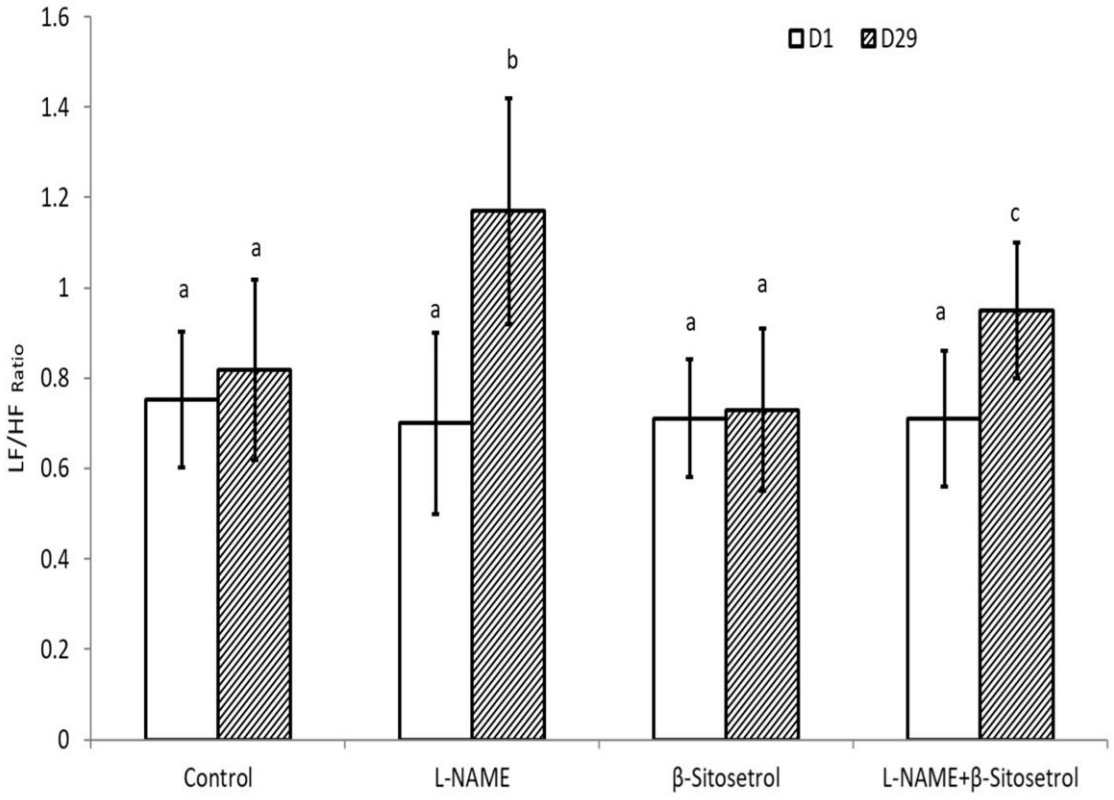


Figure 5.8.1(c): *Effect of L-NAME induced alteration of frequency domain results of HRV, heart rate variation analysis by LF/HF ratio on β-sitosterol supplemented rats (n=6, each group). D1, beginning of the experiment; D29, at the end of experiment and before sacrifice. Values with different superscripts are significantly different from each other (p<0.05*)(Patel SK, et al., 2023)*

5.8.2: Discussion

Heart rate variability (HRV), or variations in the time between heartbeats, is commonly analyzed to non-invasively assess cardiovascular autonomic function. This involves examining HRV indices in the time and frequency domains. The two main frequency bands are low frequency (LF) (0.04-0.15 Hz), reflecting sympathetic activity, and high frequency (HF) (0.15-0.4 Hz), reflecting parasympathetic activity. The LF/HF ratio serves as a measure of the heart's sympathovagal balance (Shaffer, et al., 2017).

In our study, L-NAME and L-NAME + β-sitosterol treated rats showed increased LF, decreased HF, and a notable rise in the LF/HF ratio. However, β-sitosterol resulted in a significant decrease in the LF/HF ratio. Elevated sympathetic vasomotor tone, linked to nitric oxide (NO) inhibition-induced hypertension, was observed, aligning with previous findings (Biancardi et al., 2007; Esler et al., 2010; Zambrano et al., 2019). Interestingly, (dos Santos et al., 2010) reported no renal sympathetic nerve activity increase after L-NAME treatment in rats, highlighting potential variable responses.

The interplay of glutaminergic neurotransmission and NO concentrations in the nucleus tractus solitarius (NTS) and rostral ventrolateral medulla (RVLM) was discussed. Low NO promoted sympathoexcitation, while high NO induced sympatho-inhibition. This suggests increased NO concentration in the brain may be critical for sympathoexcitation and hypertension in L-NAME-treated rats. Stimulation of the rennin angiotensin system (RAS) in response to NO inhibition and the role of

NADPH oxidase in oxidative stress were also considered as potential contributors to sympathoexcitation (Takahara, 2009; Takahara, 2002; Konda et al., 2006).

In both in-vitro and in-silico models, relationships exist between β-sitosterol treatment and sympathetic adrenergic activities (Takahara, 2009; Takahara, 2002; Konda et al., 2006).

Our study found significant changes with β-sitosterol treatment (Group 4) and a significant decrease in MAP compared to L-NAME-treated rats. We showed decreased LF/HF ratio and MAP in Group 4 (β-sitosterol + L-NAME) rats, suggesting β-sitosterol supplementation regulates sympathetic overdrive in L-NAME-induced hypertension. The observed reduction in oxidative stress and improved NO levels further confirmed the efficacy of β-sitosterol supplementation on cardiovascular abnormalities during L-NAME-induced hypertension in rats (Zhou et al., 2002; Aritomi et al., 2011).

4.9: Oxidative Stress Parameters

4.9.1: Serum Malondialdehyde (MDA) and Nitric oxide levels

There is significant increase in oxidative stress was noted in hypertensive rats. MDA levels in µmol/L are increased significantly in L-NAME (2.610±5.4) & L-NAME+β-sitosterol (1.650±0.3) treated rats compare to control rats (1.620±0.2). Malondialdehyde (MDA) is product of lipid peroxidation and has been used as an indirect biomarker of oxidative stress**.** We also observed there is significant decrease in nitric oxide levels in L-NAME (0.440±0.3) & L-NAME +β-sitosterol (0.500±0.3) treated rats compare to control.

Table 5.9.1: Effect of β-Sitosterol on oxidative stress parameter in L-NAME treated hypertensive rats

Parameters	Control (n=6)	L-NAME (n=6)	B-sitosterol (n=6)	L-NAME+β-sitosterol (n=6)
MDA in serum µmoles/L	1.620±0.2[a]	2.610±5.4[b]	1.640±0.4[a]	1.650±0.3[a]
Nitric oxide in serum µmoles/L	0.520±0.2[a]	0.440±0.3[b]	0.550±0.6[a]	0.500±0.3[a]

Values with different superscripts are significantly different from each other (p <0.05)*

4.9.2: Discussion

Excessive reactive oxygen species (ROS) generation during oxidative stress disrupts the oxidation-reduction (redox) state, leading to significant impacts on various molecular processes. In hypertension, these effects contribute to impaired vascular function, cardiovascular remodeling, renal dysfunction, immune cell activation, and sympathetic nervous system excitation, involving inflammation, proliferation, apoptosis, migration, and fibrosis (Oparil S et al., 2018; Stanley CP et al., 2019). These molecular events also give rise to protein oxidation and dysregulated cell signaling.

Oxidative stress, characterized by the overproduction of ROS, can result in the oxidative degradation of lipid, protein, carbohydrate, and nucleic acid

macromolecules, potentially leading to cell death in extreme cases (Hanna Ali H., 2012). Lipid peroxidation, particularly targeting polyunsaturated fatty acids (PUFA), produces lipid peroxyl radicals and hydroperoxides, generating various oxidation products. Lipid hydroperoxides, along with aldehydes like malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal (4-HNE), are prominent byproducts of this process (Ayala et al., 2014).

MDA, a byproduct of omega-3 and omega-6 fatty acid peroxidation, is commonly used as an indirect biomarker of oxidative stress (Buege and Aust, 1978), and in our study, tissue oxidative stress was assessed by measuring MDA levels in the aorta, heart, and femoral artery, while systemic oxidative stress was evaluated through MDA estimation in serum.

Our observations revealed that animals treated with L-NAME and L-NAME + β-sitosterol exhibited significantly higher blood and serum MDA levels compared to control rats. However, MDA levels significantly decreased following β-sitosterol administration. Consistent with our findings, other studies (Ji Hoon S., 2013; Veerappan R, Malarvili T., 2019) have also demonstrated that L-NAME induces oxidative stress by inhibiting NO. Various forms of hypertension pathophysiology have been associated with oxidative stress, where the suppression of nitric oxide synthesis by L-NAME can lead to increased ROS production by vascular NADPH oxidase, resulting in endothelial dysfunction (Zalba G., 2001).

5.10: Molecular Biology

5.10.1: MMP-7 gene expression

Results:

We utilized QuantStudio5 appliedbiosystem based qPCR to profile the RNA levels of aortic tissue in all four groups of rats in experimental hypertensive model. To aid in the visual presentation of these data, results are shown as the CT value, which provides an accurate approximation of the level of expression of MMP-7 gene. So as a result, we found over expression of MMP-7 figure 5.10 in L-NAME (2.29 log2 fold change**)** treated group whereas improved in L-NAME+β-sitosterol (1.49 log2 fold change) treated group as compare to control (0.00log2 fold change) as shown in the figure 5.10.1, Table 5.10.1(a) and table 5.10.1(b) shows the study results.

Table 5.10.1(a): Primer pair used for quantitative RT-PCR for mRNA expression

Genes Name		**Sequence 5' to 3'**	**Length (bp)**
β -actin	Forward Primer	TACAACCTCCTTGCAGCTCC	20
β -actin	Reverse Primer	ACAATGCCGTGTTCAATGG	19
MMP-7	Forward Primer	CTTCAGGCAGAACATCCA	18
MMP-7	Reverse Primer	ATTATTGATCATCTACCAC	20

Table 5.10.1(b): MMP-7 gene expression of aortic tissue

Groups	**MMP-7 CT**	**β actin CT**	**2^-ddCt** ΔΔCT = ΔCT (a target ample)−ΔCT (a reference sample)	**log2 fold change**
Control (n=6)	36.17	34.42	1.00	0.00
L-NAME (n=6)	33.87	34.41	4.89	2.29
β – sitosterol (n=6)	35.04	36.73	2.22	0.37
β – sitosterol + L-NAME(n=6)	35.81	35.54	2.80	1.49

Values with different superscripts are significantly different from each other (p <0.05)*

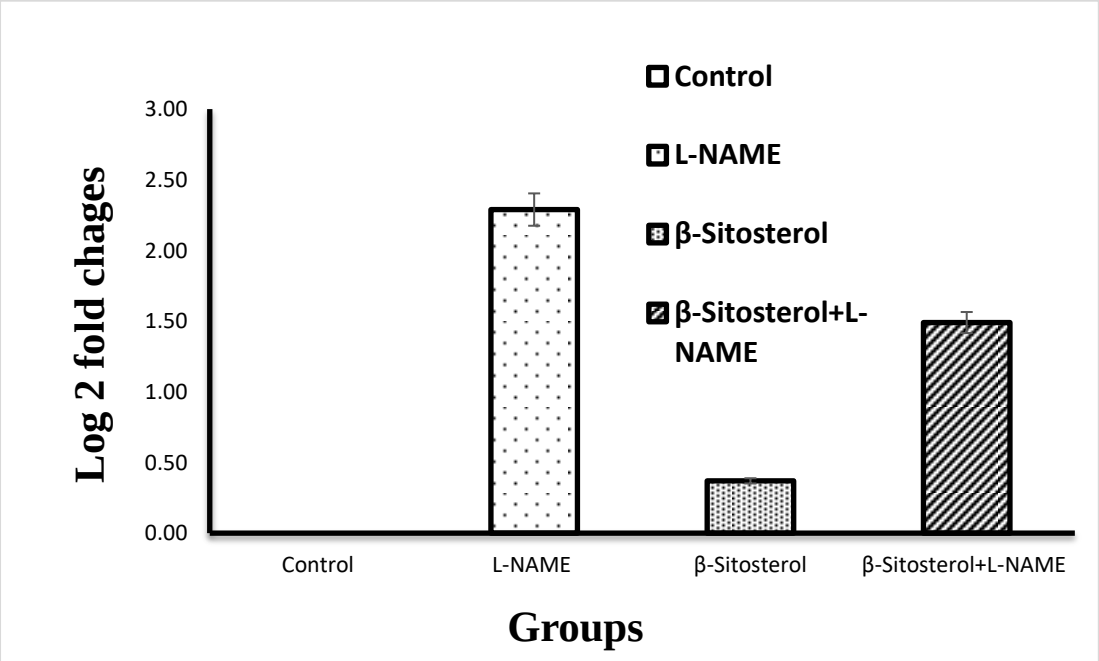


Figure 5.10.1: *MMP7 gene expression is 2.29-fold upregulated in L-NAME Group compare to control. MMP7 gene is 1.49-fold down regulated in β-sitosterol+L-NAME Group compare to control.*

5.10.2: Discussion

In our study, Real-time PCR was conducted to evaluate the precision and consistency of results between SSII one-step and two-step real-time RT-PCR methodologies. In previous studies, absolute transcript numbers for each gene were determined by synthesizing and quantifying RNA transcripts, followed by creating serial dilutions of their cDNA within the range of 10^8 to 10^1 transcripts (Robert K Nuttall, et al., 2004). However, we observed that when analyzing equal numbers of transcripts for different genes, the variations in CT values were consistently less than five. Consequently, we consider the CT value a reliable overall indicator of relative RNA abundance.

Additionally, our findings reveal that MMP-7 mRNA is highly abundant in the aortic tissue is expressed at lower levels in L-NAME treated groups, but its RNA is present in all tissues. Many transmembrane MMPs exhibit high expression across various tissues.

Myocyte growth is partly regulated by collagen presence, and it is noteworthy that around the time of parturition, there is an upregulation in the expression of several MMPs, including MMP-7. This suggests a breakdown of ECM extra cellular matrix degradation components, leading to the termination of myocyte growth stimuli.

In another experiment, the endothelial function in hypertensive rats given L-NAME alone was found to result mainly from impaired NOS function in vascular endothelial cells, indicated by a significant decrease in eNOS mRNA expression in aortic tissue. In that study further observed that addition of β-sitosterol partially improved systolic blood pressure and endothelium-dependent relaxation in the aorta

and heart (Oh, J. *et al*., 2001). It was further noticed a full recovery of eNOS mRNA expression in aortic tissues. The dysfunction of the endothelial vasodilator mechanism in L-NAME hypertensive rats aligns with previous reports, and treatment with angiotensin-converting enzyme inhibitors showed no significant difference in restoring endothelial vasodilator function and eNOS mRNA expression (Wells., et al., 2003).

Contrary to its beneficial role in regulating blood pressure, an upsurge in inducible nitric oxide synthase (iNOS) expression can have adverse effects on hypertension. This heightened expression leads to an excess production of nitric oxide (NO), which, when reacting with superoxide anions (O2 −), forms peroxynitrite (ONOO−), consequently inducing oxidative damage within the system. Furthermore, iNOS is implicated in the modulation of arginase activity, resulting in reduced bioavailability of NO by uncoupling endothelial nitric oxide synthase (eNOS). This uncoupling amplifies the generation of O2 − instead of NO, exacerbating oxidative stress within the vasculature (Forstermann et al., 2012).

Studies conducted on both animal models and human subjects have consistently demonstrated elevated iNOS expression in aortic and cardiac tissues obtained from hypertensive rats and heart failure patients. This increased iNOS activity is accompanied by heightened levels of nitrotyrosine, a marker of oxidative stress, further corroborating the deleterious impact of iNOS overexpression in hypertensive conditions (Stathopulos et al., 2001).

Notably, experiments utilizing iNOS knockout mice subjected to myocardial infarction have unveiled significantly lower mortality rates and reduced oxidative

stress and cardiac remodeling compared to their wild-type counterparts These findings underscore the pivotal role of iNOS in exacerbating cardiovascular complications associated with hypertension (Liu, et al., 2005). The mechanisms driving these detrimental effects involve augmented matrix metalloproteinase (MMP) activity and elevated concentrations of reactive oxygen species (ROS) within the tissues. These processes, coupled with impaired NO activity due to iNOS upregulation, converge to disrupt various cellular signaling pathways, culminating in functional and proliferative aberrations within the vasculature of hypertensive individuals (Prado et al., 2021).

Our result of 2.29 -fold change indicates higher gene expression of MMP-7 in (L-NAME treated group 2) aortic tissue of experimental hypertensive rats. Therefore, it may be speculated that, in addition to MMP-7 and other MMP families could also be involved in the pathogenesis of experimental hypertensive rats. Although other MMP families might have been involved in protecting regulation of hypertension development (Johnson J, et al., (2003).

5.11: Cardiovascular Histopathology

Results

Histological examination of aorta, heart and femoral artery tissue:

Histopathology of heart, aorta and femoral artery tissue of hypertensive rats are as following :-

a. Heart

Control group: Section studied under H&E stain shows large sized artery consisting of inner tunica intima, middle tunica media and outer tunica adventitia. The tunica intima is lined by endothelial cells. The tunica media is consisting of smooth muscle cells with elastic fibers. The tunica adventitia is composed of loose connective tissue.

No evidence of arteriosclerosis, atherosclerosis, arteritis, calcification, aneurysm, dysplasia. Microscopic Impression: Normal aorta, No significant pathology.

L-NAME group: Section studied under H&E stain shows myocardium composed of branching and anastomosing striated muscle fibres arranged in parallel fashion, separated by capillaries. The myocardial fibres are connected to each other by *intercalated* discs. There is evidence of focal myocardial hypertrophy and degeneration and capillary congestion. There is no evidence of atherosclerosis, calcification, aneurysm. Microscopic Impression: Focal myocardial hypertrophy and capillary congestion. Moderate arteriosclerosis and congestion of coronary arteries.

β sitosterol: Section studied under H&E stain shows large sized artery consisting of inner tunica intima, middle tunica media and outer tunica adventitia. The tunica intima is lined by endothelial cells. The tunica media is consisting of smooth muscle cells with elastic fibers. The tunica adventitia is composed of loose connective tissue.

No evidence of arteriosclerosis, atherosclerosis, arteritis, calcification, aneurysm, dysplasia. Microscopic Impression: Normal aorta. No significant pathology**.**

L-NAME+ β sitosterol: Section studied under H&E stain shows myocardium composed of branching and anastomosing striated muscle fibers arranged in parallel fashion, separated by capillaries. The myocardial fibers are connected to each other by intercalated discs. Coronary arteries appear normal. No evidence of myocardial hypertrophy, arteriosclerosis, atherosclerosis, arteritis, calcification, aneurysm, infarction. Microscopic Impression: Normal myocardium.

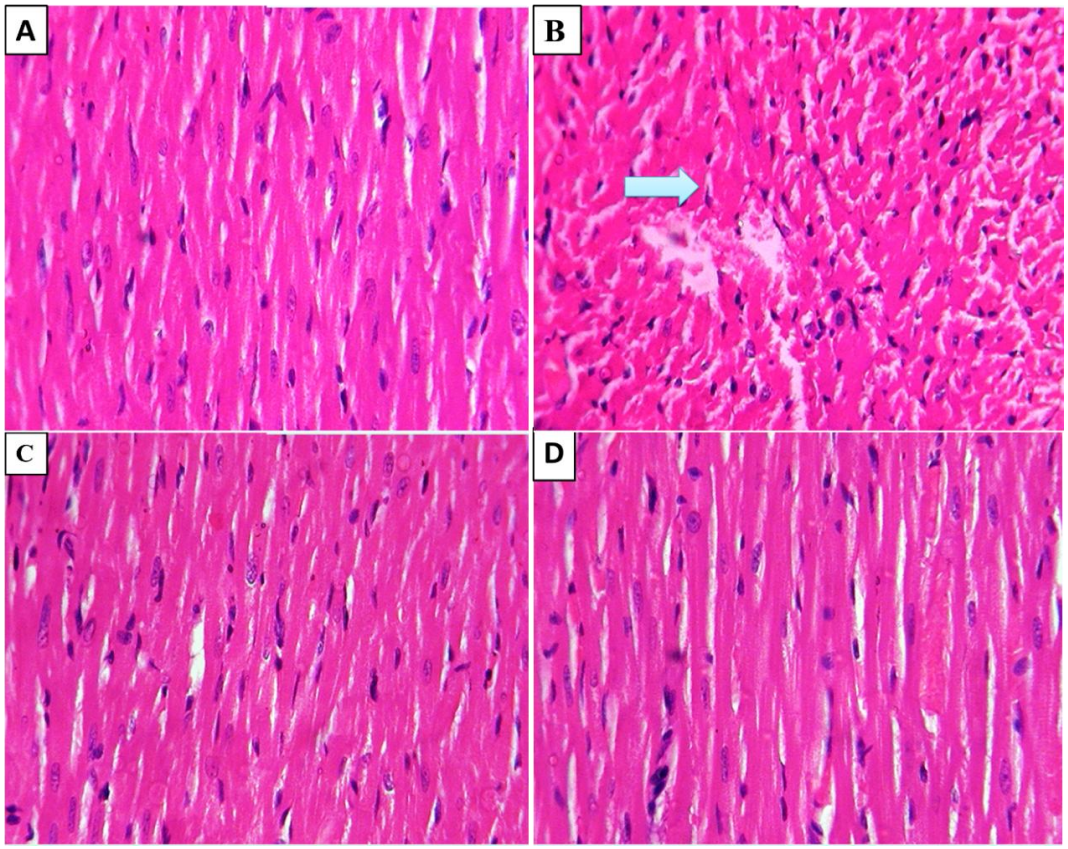


Figure 5.11(a): Histopathology of rats heart tissue in β-sitosterol supplemented L-NAME induced hypertensive rats. A; control, B; L-NAME, C; β-sitosterol, D; L-NAME+ β-sitosterol Focal myocardial hypertrophy and capillary congestion. Moderate arteriosclerosis and congestion of coronary arteries B marked as arrow sign.

Aorta

Control group: Section studied under H&E stain shows myocardium composed of branching and anastomosing striated muscle fibers arranged in parallel fashion, separated by capillaries. The myocardial fibers are connected to each other by intercalated discs. Coronary arteries appear normal. No evidence of myocardial hypertrophy, arteriosclerosis, atherosclerosis, arteritis, calcification, aneurysm, infarction. Microscopic Impression: Normal myocardium.

L-NAME group: Section studied under H&E stain shows large sized artery consisting of inner tunica intima, middle tunica media and outer tunica adventitia. The tunica intima is lined by endothelial cells and shows mild thickening. The tunica media is consisting of hyperplastic smooth muscle cells with elastic fibers. The tunica adventitia is composed of loose connective tissue. No evidence of arteriosclerosis, aneurysm, arthritis, calcification, dysplasia. Microscopic Impression: Mild thickening of tunica intima. Medial sclerosis with hyperplastic smooth muscle cells.

B-sitosterol: Section studied under H&E stain shows myocardium composed of branching and anastomosing striated muscle fibers arranged in parallel fashion, separated by capillaries. The myocardial fibers are connected to each other by intercalated discs. Coronary arteries appear normal. No evidence of myocardial hypertrophy, arteriosclerosis, atherosclerosis, arteritis, calcification, aneurysm, infarction. Microscopic Impression: Normal myocardium.

L-NAME+ β sitosterol: Section studied under H&E stain shows large sized artery consisting of inner tunica intima, middle tunica media and outer tunica adventitia. The tunica intima is lined by endothelial cells and shows mild thickening. The tunica

media is consisting of normal smooth muscle cells with elastic fibers. The tunica adventitia is composed of loose connective tissue. No evidence of medial sclerosis, arteriosclerosis, aneurysm, arteritis, calcification, dysplasia. Microscopic Impression: Mild thickening of tunica intima. No significant pathology noticed.

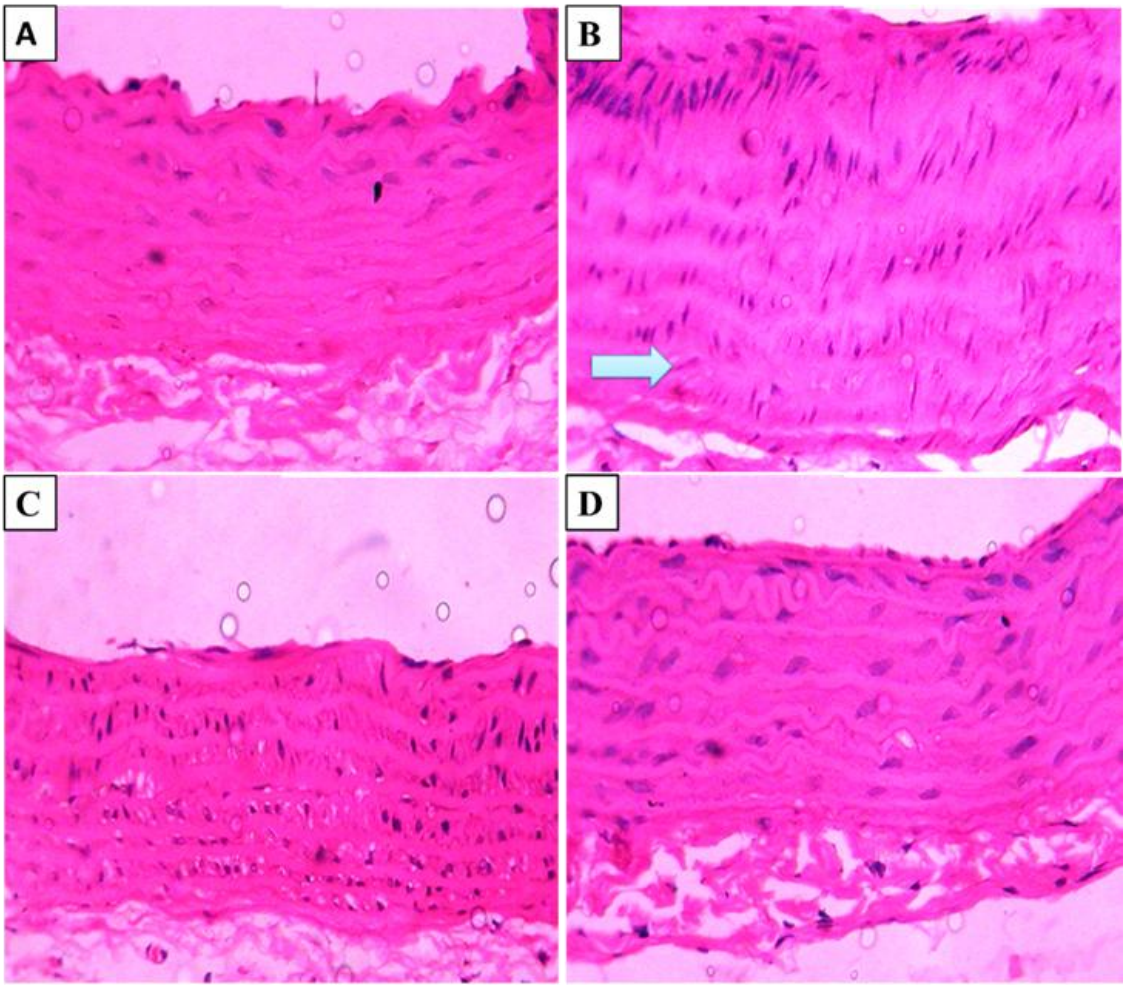


Figure 5.11(b): Histopathology of rats aortic tissue in β-sitosterol supplemented L-NAME induced hypertensive rats. A; control, B; L-NAME, C; β-sitosterol, D; L-NAME+ β-sitosterol Mild thickening of tunica intima and medial sclerosis with hyperplastic smooth muscle cells in B marked as arrow sign.

b. Femoral artery

Control group: Section studied under H&E stain shows medium sized artery consisting of inner tunica intima, middle tunica media and outer tunica adventitia. The tunica intima is lined by endothelial cells. The tunica media is consisting of smooth muscle cells with elastic fibers. The tunica adventitia is composed of loose connective tissue. No evidence of arteriosclerosis, atherosclerosis, arteritis, calcification, aneurysm, dysplasia. Microscopic Impression: Normal femoral artery

L-NAME group: Section studied under H&E stain shows medium sized artery consisting of inner tunica intima, middle tunica media and outer tunica adventitia. The tunica intima is lined by endothelial cells and shows mild thickening. The tunica media is consisting of hyperplastic smooth muscle cells with elastic fibers. The tunica adventitia is composed of loose connective tissue. No evidence of arteriosclerosis, aneurysm, arteritis, calcification, dysplasia. Microscopic Impression; Mild thickening of tunica intima. Medial sclerosis with hyperplastic smooth muscle cells**.**

β sitosterol: Section studied under H&E stain shows medium sized artery consisting of inner tunica intima, middle tunica media and outer tunica adventitia. The tunica intima is lined by endothelial cells. The tunica media is consisting of smooth muscle cells with elastic fibers. The tunica adventitia is composed of loose connective tissue. No evidence of arteriosclerosis, atherosclerosis, arteritis, calcification, aneurysm, dysplasia.

Microscopic Impression: Normal femoral artery

L-NAME+ β sitosterol: Section studied under H&E stain shows medium sized artery consisting of inner tunica intima, middle tunica media and outer tunica adventitia. The tunica intima is lined by endothelial cells and shows mild thickening. The tunica media is consisting of normal smooth muscle cells with elastic fibers. The tunica adventitia is composed of loose connective tissue. No evidence of medial sclerosis, arteriosclerosis, aneurysm, arteritis, calcification, dysplasia. Microscopic Impression; Mild thickening of tunica intima. No significant pathology noticed.

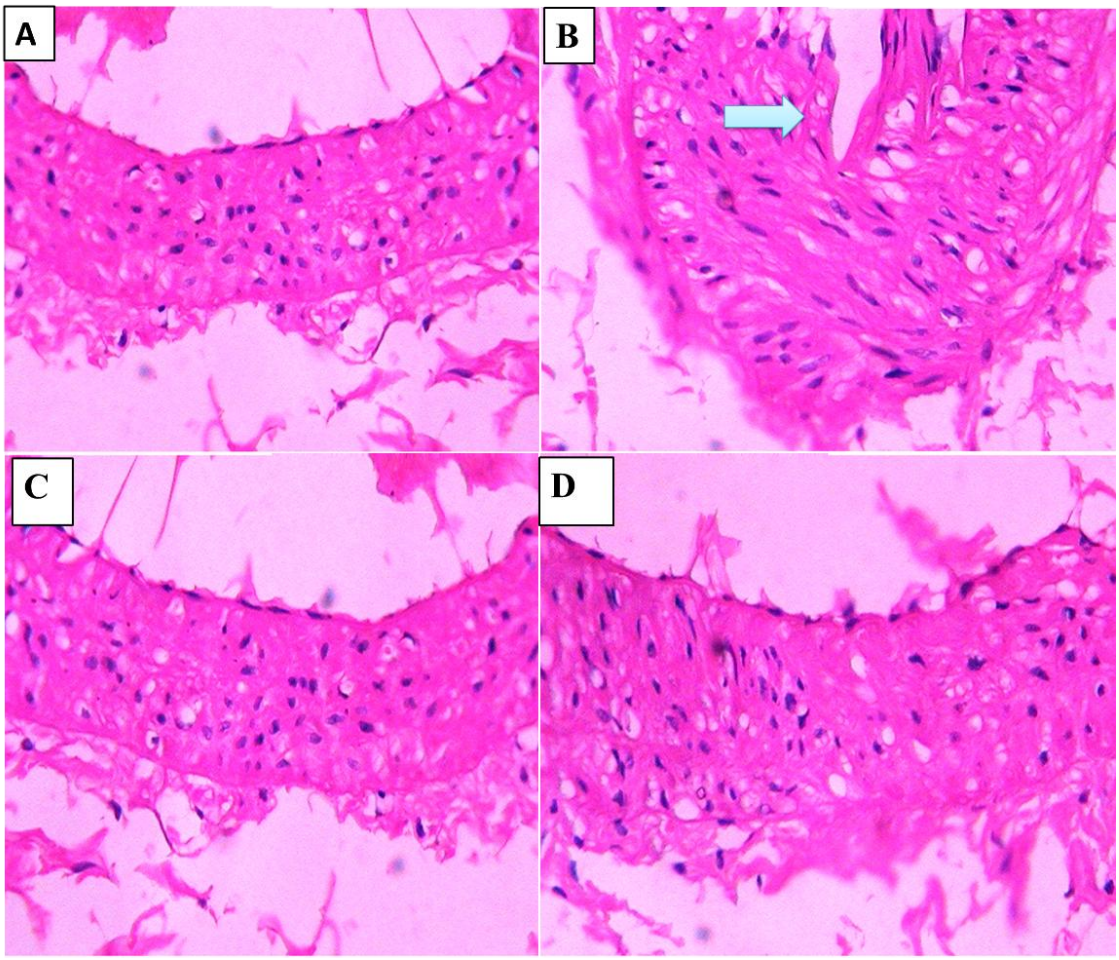


Figure 5.11(c): Histopathology of rats femoral artery tissue in β-sitosterol supplemented L-NAME induced hypertensive rats. A; control, B; L-NAME, C; β sitosterol, D; L-NAME+ β sitosterol Mild thickening of tunica intima. Medial sclerosis with hyperplastic smooth muscle cells B marked as arrow sign.

5.11.1: Discussion

In the present study, histopathological examination of the aorta, heart, and femoral artery revealed a large-sized artery structure comprising the inner tunica intima, middle tunica media, and outer tunica adventitia under Haematoxylin and Eosin stain. The tunica intima was lined with endothelial cells, while the tunica media was composed of elastic fiber-covered smooth muscle cells, and the tunica adventitia consisted of loose connective tissue.

Control rats exhibited no evidence of arteriosclerosis, atherosclerosis, arteritis, calcification, aneurysm, or dysplasia. However, in L-NAME-treated rats, mild thickening of the tunica intima was observed, with no significant changes in the tunica media or adventitia. In rats treated with L-NAME + β-sitosterol, the tunica intima showed mild thickening, the tunica media exhibited hyperplastic smooth muscle cells with elastic fibers, and the tunica adventitia displayed no significant abnormalities. β-sitosterol treatment resulted in near-normal histology of the aorta.

The chronic inhibition of nitric oxide (NO) and increased oxidative stress in the present study may have contributed to mild intimal thickening and smooth muscle cell hyperplasia. Similar observations were reported by (Rossi MA et al., 2001), noting prominent intima thickness in L-NAME-treated rat aortas. (Girardi et al., 2011) suggested that prolonged NO inhibition by L-NAME administration causes various vascular complications, including endothelial dysfunction, vascular hypertrophy, cardiac fibrosis, atherosclerosis, perivascular inflammation, renal failure, and heightened vascular responses to adrenergic stimuli.

The vascular damage observed in our study may result from NO inhibition, increased blood pressure, mechanical stress, oxidative stress, and elevated Ang II levels. β-sitosterol treatment

appeared to mitigate these effects by increasing NO bioavailability, reducing oxidative stress, and decreasing mean arterial pressure. (Takai S. et al., 2013) found that NADPH oxidases in vascular smooth muscle and endothelial cells are activated by mechanical stress, while cilnidipine attenuated oxidative stress in hypertensive rats by reducing the gene expression of a NADPH oxidase subunit.

The renin-angiotensin-aldosterone system (RAAS) peptide, Angiotensin-II, plays a crucial role in regulating vascular tissue structure and function, aligning with the findings of our study (Veerappan R, Malarvili T., 2019).

CHAPTER-7

Summery and Conclusion

Summary:

Hypertension is the most important noncommunicable disease in India, estimated burden of 200 million persons. Hypertension is more common in developed countries, urban populations, and individuals with better socioeconomic status. The prevalence of hypertension is continuously increasing across the globe. Despite being a high-prevalence disease, the awareness, management, and control of hypertension in the community are deficient. In previous studies, MMP-7 has been implicated in human pathologies such as cardiac diseases.

Plant-based bioactive compounds have extreme potential to protect vascular pathophysiology due to hypertension. Presently, bioactive molecules like β-sitosterol, found in mainly plants like *Mucuna Purines, Phyllanthus Emblica,* etc., are in the limelight and researchers' attention for medical uses.

The present study was undertaken to evaluate the effect of L-NAME (L-NG-Nitroarginine methyl ester), a nitric oxide synthase inhibitor-induced experimental hypertension in rats with β-sitosterol, a plant-derived compound supplementation on the pathophysiology of experimental hypertension by *in silico* and *in vivo* experimental rat models.

In silico results show that after screening bioactive molecules from different phytochemical databases, ADMET properties and molecular docking results show effective ligand efficiency in hypertensive targeted proteins like ACE2 and MMP-7. Further, it has been observed that out of 130 screened cardio-protective ligands, only 05 ligands have effective binding with MMP-7 and ACE2 protein molecules. However, it has also been found that the MMP-7 protein molecule is more efficiently bound with β-sitosterol as MMP-7 has more interacting amino acids as ligands while

docking with β-sitosterol compared to ACE2. This *in silico* study indicates a higher potentiality of MMP-7 than ACE2 as cardiovascular protection.

For *the in vivo* study, we included 36 adults male Wistar strain albino rats in the study (Rattus Norvegicus), divided into four groups at random. Group 1 is the control group, Group 2 is treated with L-NAME (40mg/kg/day orally in distilled water) for 28 days, Group 3 is treated with (β-sitosterol 10 mg/day/kg body weight, orally in distilled water) for 28 days and Group 4 is treated with L-NAME (40 mg/day/kg body weight, orally in distilled water) for 28 days and β-sitosterol: (10 mg/day/kg body weight, administrated orally) for 28 days simultaneously.

The gravimetric analysis based on percentage body weight change between the first and last day of experimental protocol on the experimental model revealed that rats treated with L-NAME significantly reduced body weight gain compared to the control group, an effect mitigated by β-sitosterol supplementation. Additionally, heart rate decreased significantly in L-NAME-treated rats, while an improvement has been observed in the case of β-sitosterol supplementation. Mean arterial blood pressure (MAP) significantly increased in L-NAME-treated rats. In contrast, β-sitosterol supplemented rats showed an improvement of MAP compared to only L-NAME treated rats.

Heart rate variability analysis demonstrated an alteration in sympathetic and parasympathetic activities, with an increased LF/HF ratio in L-NAME-treated rats, indicating sympathovagal imbalance reflecting cardiac autonomous malfunction. This treatment mitigated the β-sitosterol effect, suggesting a potential role in modulating autonomic balance.

Furthermore, serum biomarker analysis revealed increased malondialdehyde (MDA) levels, indicative of higher oxidative stress, in both L-NAME-treated and L-NAME+β-sitosterol-treated rats compared to controls. Conversely, nitric oxide levels were significantly decreased in the L-NAME treated group, suggesting endothelial dysfunction.

In a study on MMP-7 gene expression by RT-PCR, it has been found that overexpression of the MMP-7 gene in the aortic tissue of L-NAME-treated rats indicates potential cardiovascular remodelling associated with hypertension-induced alteration of the pathophysiology of the aorta. The simultaneous supplementation of β-sitosterol in L-NAME-treated hypertensive rats suggests the protective role of β-sitosterol against only L-NAME-treated rats on vascular pathophysiology remodelling.

Histological examination of the aorta, heart, and femoral artery tissues revealed architectural changes in all three cardiac and vascular components in L-NMAE-treated hypertensive rats. The architectural changes included the thickening of tunica intima and medial sclerosis. However, β-sitosterol supplementation possibly ameliorates the adverse impact of L-NAME on cardiovascular histopathology.

The study demonstrates the potential possible therapeutic effects of β-sitosterol to modify hypertension-induced alterations in cardiovascular electrophysiology, oxidative stress, and MMP-7 gene expression to regain vascular remodelling in near-normal pathophysiology of the cardiovascular system in rats. Hence, these findings suggest a promising plant-based bioactive compound as a possible antihypertensive therapeutic agent to regulate hypertension and its related changes in vascular pathophysiology.

Conclusion:

In conclusion, β-sitosterol supplementation demonstrates promising therapeutic possibilities in modifying cardiovascular complications associated with L-NAME-induced hypertension in rats. Its beneficial effects encompass regulating body weight, heart rate, blood pressure, autonomic functions, oxidative stress, and MMP-7 gene expression in cardiovascular remodelling. This study provides valuable insights into the various mechanisms underlying the cardioprotective effects of β-sitosterol. It highlights its significance as a potential adjunctive therapy for hypertension and associated cardiovascular disorders. Further research is needed to elucidate its precise mechanisms of action and therapeutic efficacy in clinical settings.

Clinical implications:

This study clearly shows a possibility of selected plant origin bioactive compounds like β-Sitosterol may be considered along with antihypertensive medication.

Limitations:

The *in vivo* study only targeted aortic MMP-7 gene for β-sitosterol supplemented hypertensive rats as per the outcome of i*n silico* findings. Hence β-sitosterol may not be considered absolutely a single cardio protective and antihypertensive agent until multiple other cardiovascular regulatory genes (except ACE2) are thoroughly investigated.

Future scopes:

Role of entire family of MMP on cardiovascular diseases and the possible therapeutic uses of plant based bioactive compounds like β- sitosterol may be explored.

CHAPTER-8

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Annexure

BLDE (DEEMED TO BE UNIVERSITY)

Annexure -I

PLAGIARISM VERIFICATION CERTIFICATE

1. Name of the Student: **Ms Sanakousar Patel** Reg No: **19PHD001**
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2. Patel SK, Aithala M, Patil S, Das KK. β-sitosterol on heart rate variability in L-NAME induced hypertensive rats. J Krishna Inst Med Sci Univ 2023, 12(1): 97-106 Scopus

3. Patel SK, Shutter AK, Patil R, Desangi A, Malali V, Patil J, Patil1 S, Das KK, Parvatikar PP. In-Vitro Antioxidant, Anti-Inflammatory and Cytotoxic effects of different Solvent Extraction Terminalia chebula, Terminalia Billerica Phyllanthus emblica. Research J. Pharm. and Tech2022;.15(7). Scopus

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RESEARCH ARTICLE

In-Vitro Antioxidant, Anti-Inflammatory and Cytotoxic effects of different Solvent Extraction *Terminalia chebula, Terminalia billerica, Phyllanthus emblica*

Sanakousar K. Patel[1], Arun K Shutter[2], Ria Patil[3], Archana Desangi[3], Vishalakshi Malali[3], Jyoti Patil[3], Sumangala Patil[1], Kusal K. Das[1], Prachi P. Parvatikar[1]*

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ABSTRACT:

Objective: The phytochemical analysis of *Terminalia chebula, Terminalia bellirica, Phyllanthus emblica* fruit extract as well as to evaluate the potential synergistic activity of screened plant extracts for anti-oxidant, anti-inflammatory and anti-cancer activity on MCF-7 breast cancer cell line. **Methodology:** In present study phytochemical constituent from selected plant material was extracted in different solvents. Antioxidant activity was evaluated by DPPH and FRAP assay against standard antioxidant. MTT assay was performed to find out cytotoxic and anti-inflammatory effect on MCF- breast cancer cell line. **Result:** Phytochemical analysis of the performed which showed that alkaloids, phenols, flavonoids and saponins. In context of antioxidant assays almost selected extracts showed moderate activity but ethanol extract of *Terminalia chebula*, proven to be having significant antioxidant activity over all extracts. Anti-inflammatory activity of MCF-7 breast cancer cell line exhibited that good activity with IC_{50} value 228.82µg/ml**.** Whereas Methanol extract of *Terminalia bellirica* and Aqueous extract of *Phyllanthus emblica* shown 432.45µg and 259.02µg respectively. **Conclusion:** The present study concludes that in among three tested extracts, Ethanol extract of *Terminalia chebula* exhibited prominent anticancer activity against Breast cancer (MCF-7) with significant IC_{50} value 228.82 µg. However further studies are needed to find out the structural of bioactive compounds and investigate mechanisms of antioxidant and anticancer activities of the bioactive compounds in *in-vivo* animal study.

KEYWORDS: *Terminalia chebula*, Phytochemical constitutes, Antioxidant, Breast cancer, MTT assay.

INTRODUCTION:

Breast cancer globally reported that second rank amongst all prevalent cancer[1]. Among the Indian population, breast cancer is the highest, with a death ratio of 2:1[2]. Despite the effective therapies available for the treatment of breast cancer, their side effects and the chances of re-occurrence of the malignancy or metastatic cancer still projects a considerable risk[3]. Therefore, there has been a hunt for the novel anticancer agent from natural products, which could endure as an alternative medicine for cancer treatment[4,5].

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Since ancient time plants used in traditional medicine, medicinal plants are essential for the well-being and continued survival of man. The traditional knowledge and use of medicinal plants are widespread from various perspectives, like pharmaceuticals.[6]

Terminalia chebula, a medicinal plant belonging to the family *Combretaceae* and found in different Indian regions. Some species of family have been traditionally used as folk medicines in the treatment of various diseases[7]. *Terminalia bellirica* is such a medicinal plant that has extensive usage as pharmaceutical and nutraceuticals[8]. *Phyllanthus emblica* is a tree native to Southeast Asia's tropical climates. The fruit of the tree is known as Indian Gooseberry or Alma. For millennia, the extract from these fruits has been utilized in traditional

medicine to cure ailments ranging from constipation to tumor treatment[9].

The current study is focused on phytochemical extraction of selected plant material through successive solvent extraction and evaluate the antioxidant, anti-inflammatory and cytotoxic properties against human breast cancer cell line MCF-7[10].

MATERIALS AND METHODS:

Collection of plant material:

Fresh, dried fruits of *Terminalia chebula*, *Terminalia bellirica* and *Phyllanthus emblica* were collected from the Department of Pharmacology, BLDE AVS Ayurveda Mahavidyalaya, Vijayapura-586108, Karnataka, India. The powder was stored in airtight containers at -20°C for further use for crude solvent extraction.

Preparation of plant extract:

About 25g of powdered material was extracted with 250 ml of Chloroform, Ethyl acetate, Methanol, Ethanol and Distilled water in series extraction method using Erlenmeyer flask and kept at room temperature for 24hours under dark condition. The extract was filtered using what-man filter paper and stored in the airtight bottle at 40C until use.

Phytochemical analysis:

The crude solvent extracts of *Terminalia chebula*, *Terminalia bellirica* and *Phyllanthus emblica* was qualitatively tested for the presence of different phytochemical constituents.[11]

Determination of antioxidant activity by using *in-vitro* methods:

Ferric ion reducing antioxidant power assay (FRAP):

Ferric ions reducing power assay was measured according to standard method with slight modification[12]. Different solvent extracts of *Terminalia chebula*, *Terminalia bellirica* and *Phyllanthus emblica* taken in different concentrations ranging from 100µl to 500µl. The absorbance was measured at 700nm using a UV-VIS Spectrophotometer. Ascorbic acid was used as the reference standard. All samples were assayed in triplicates.

Hydrogen peroxide scavenging assay (H2O2):

The antioxidant activity of selected plant material was carried with ascorbic acid as a standard was assessed based on their ability to scavenge the hydrogen peroxide [13]. 0.6ml of 4mM H2O2 solution (pH-7.4) was added to 0.5ml of known concentration of standard ascorbic acid and tubes containing different concentrations ranging from 100µl to 500µl of plant extracts in phosphate buffer (pH-7.4). The absorbance of the solution was measured at 230nm after 10min against the blank solution containing phosphate buffer without hydrogen peroxide. Control was prepared by replacing the sample or standard with phosphate buffer.

DPPH free radical-scavenging ability assay

According to the standard method, radical scavenging activities of solvent extracts of plant extract were determined using the DPPH radical as a reagent, according to the standard method [14]. 100µL of a DPPH radical solution in ethanol (60 µM) were mixed with 100µL of sample solution. The mixture was incubated for 30 min in the dark at room temperature, and then absorbance was measured at 517nm using a UV-VIS Spectrophotometer. Ascorbic acid was used as a reference standard. The DPPH scavenging activity of each sample was calculated using the following equation:

% inhibition= Ac-At/Ac x 100

Where Ac- the absorbance of the control; At- the absorbance of the test. The IC50 value was calculated for all the samples used.

Evaluation of *In-vitro* anti-inflammatory activity:

Anti-inflammatory activity of Different solvent extracts of selected plant was evaluated by protein denaturation method[15]. The reaction mixture consisting of 2ml of known concentration of Selected plant extracts (100 µg/ml) with standard Diclofenac sodium (100µg/ml) and 2.8ml of phosphate buffered saline (pH 6.4) was mixed with 2ml of egg albumin (from fresh hens egg) and incubated at (27±1)°C for 15 min. Denaturation was induced by keeping the reaction mixture at 70°C in a water bath for 10 min. After cooling, the absorbance was measured at 660nm by using double distilled water as blank. The percentage inhibition of protein denaturation was calculated.

Determination of cell viability by MTT Assay:

The effect of plant extract on the viability of breast cancer (MCF-7) cells was determined using the standard colorimetric MTT assay using the 3-(4,5-dimethylthiazol- 2-yl)-2,5-dimethyl tetrazolium bromide dye (Sigma, St. Louis, MO, USA)[16]. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC50) values is generated from the dose-response curves for each cell line[17].

Inhibition Percentage = OD of Test sample ÷ OD of control × 100

Statistical analysis:

All the tests were carried out in triplets (n = 3) and statistically analyzed and is presented as mean±S.E. using ANOVA statistical programme.

RESULTS AND DISCUSSION:

Phytochemical analysis:

The qualitative analysis of the various extract of selected plant material was found to possess more phytoconstituents which was revealed by qualitative analysis indicating alkaloids, flavonoids, glycosides, phenols, saponins, tannins, terpenoids, steroids and carbohydrates**.**

Antioxidant assay:

FRAP Assay

In the present study, different concentrations of plant extract were subjected to FRAP assay. Ethanol extract of *T.chebula* ethanol extract of known concentration (500µl) showed good antioxidant activity with absorbance values (1.232). In the case of *T.bellirica*, methanol extract exhibited higher activity among other sections (1.262), an aqueous extract of *P.emblica* showed higher activity. (Table.1)

Hydrogen peroxide scavenging assay:

H_2O_2 assay is one of the common methods used to investigate the antioxidant capacity of the extracts. The results revealed that aqueous extract of *P emblica* showed highest scavenging activity (80.8667) than standard and among all other plant extracts (Table 2).

Table.1. FRAP Assay of selected of plant extracts. (C-chloroform, E-ethyl acetate, M-methanol, Et-ethanol, A-aqueous)

Name of the plants			*Terminalia chebula*			*Terminalia bellirica*			*Phyllanthus emblica*		
Sl. No	**Concentration**	**Std Ascorbic acid**	**E**	**M**	**A**	**M**	**E**	**A**	**M**	**E**	**A**
1	100µl	0.4650 ± 0.00300	0.5527± 0.00503	0.4367± 0.00416	0.2220± 0.01000	0.3490± 0.00265	0.2687± 0.00503	0.2917± 0.00351	0.2653± 0.00321	0.3327 ± 0.00702	0.3890± 0.00300
2	200 µl	0.7530 ± 0.00458	0.7177± 0.00351	0.6133± 0.00802	0.3303± 0.01550	0.4310± 0.00361	0.3947± 0.00306	0.4433± 0.00416	0.5543± 0.00321	0.4513 ± 0.00833	0.5783± 0.00252
3	300 µl	0.9943 ± 0.00416	0.8433± 0.00611	0.8167± 0.00611	0.4907± 0.00611	0.6737± 0.01457	0.5120± 0.00200	0.5797± 0.00153	0.7463± 0.00379	0.5287 ± 0.00902	0.6870± 0.00458
4	400 µl	1.1353 ± 0.00416	0.9467± 0.00416	0.8743± 0.00950	0.5793± 0.00416	0.8617± 0.00751	0.6433± 0.00416	0.7710± 0.00964	0.8767± 0.00451	0.6680 ± 0.00872	1.0417± 0.00252
5	500 µl	1.4647 ± 0.00306	1.2320± 0.00755	1.0247± 0.00503	0.6427± 0.00503	1.2620± 0.00917	0.7160± 0.00529	0.8867± 0.00379	1.0113± 0.00404	0.7820 ± 0.00400	1.1533± 0.00416

Table.2. DPPH activity of selected plant extract of plant extracts. (C-chloroform, E-ethyl acetate, M-methanol, Et-ethanol, A-aqueous)

Name of the plants			*Terminalia chebula*			*Terminalia bellirica*			*Phyllanthus emblica*		
Sl. No	**Concentration**	**Std Ascorbic acid**	**M**	**E**	**A**	**M**	**E**	**A**	**M**	**E**	**A**
1	10µg	67.7867±0.17898	51.6300 ±0.35000	57.0633± 0.52596	42.7350 ±0.81862	73.8867 ±0.46501	58.2300 ±0.29206	68.7200 ±0.41146	57.8800 ± 0.35679	46.0761± 0.58662	68.2167± 0.35572
2	20 µg	73.6533±0.23502	57.4500 ±0.35000	58.9300± 0.41146	55.6332 ±0.48524	77.3067 ±0.35572	65.6500 ±0.35679	72.8533 ±0.32347	67.2033 ±0.35572	59.4794± 0.59808	70.1167± 0.37434
3	30 µg	78.2000±0.30806	69.8900 ±0.29206	69.4233± 0.44287	62.9370 ±0.23310	80.7100 ±0.19975	68.2700 ±0.15100	74.8967 ±0.48840	72.5700 ±0.29206	60.5112± 0.48524	75.9067± 0.27135
4	40 µg	80.8000±0.35679	73.1100 ±0.35679	75.3667± 0.24420	66.9386 ±0.52558	84.0200 ±0.73980	71.6367 ±0.24420	77.4233 ±0.17898	78.3567 ±0.40624	72.4941± 0.46620	80.3333± 0.24420
5	50 µg	83.9100±0.35000	77.2700 ±0.35000	78.9000± 0.42297	74.8251 ±0.46620	86.0100 ±0.30806	73.6267 ±0.56889	78.7833 ±0.23502	81.0733 ±0.17786	78.0108± 0.48524	83.0200± 0.24021

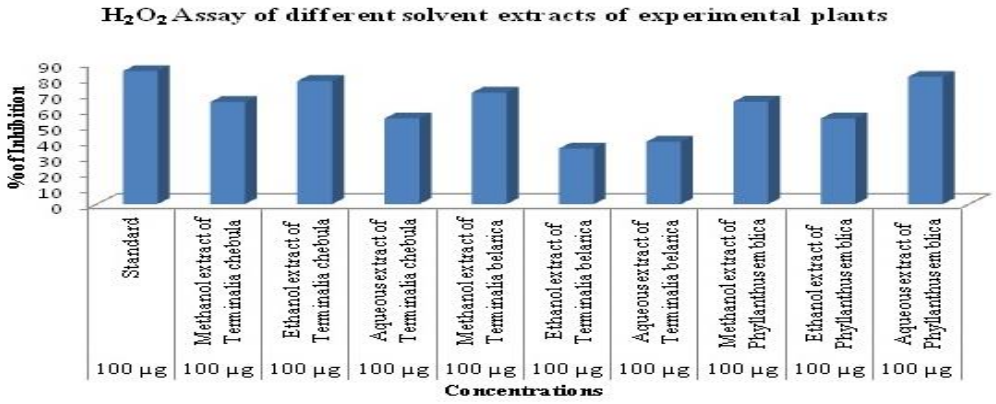

Figure 1: Percentage of inhibition of different solvent extracts of selected plant extracts by hydrogen peroxide assay at 100µg/ml

DPPH free radical-scavenging ability assay:

The antioxidant capacity of the extracts was compared with ascorbic acid as a standard antioxidant[17]. In the case of *T.chebula*, all solvent extract shown good scavenging activity but inhibition percentage (83.9100). The DPPH assay of *T Billerica* shows that methanol extract exhibited more significance than others (86.0100). In case of *P.emblica* among all three sections showed promising antioxidant activity with the percentage of inhibition (Table 3).

***In-vitro* anti-inflammatory assay:**

Anti-inflammatory study of known concentrations (100µg) of plant extracts was subjected for anti-inflammatory activity through protein denaturation assay. The results revealed that methanol extract of *T.bellirica* exhibited significant anti-inflammatory activity with the percentage of inhibition 86.9133% than standard drug and all the quotes. In comparison, the standard drug Diclofenac sodium showed 94.2467% of inhibition of protein.

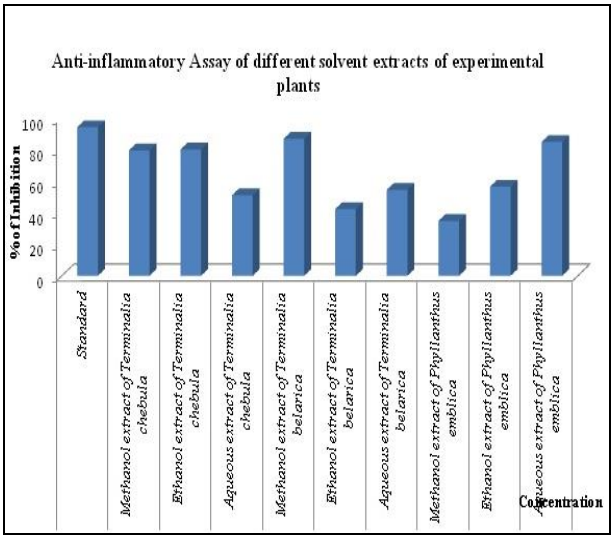

Figure 2: Ani-inflammatory activity of different solvent extracts of selected plant extracts at 100µg/ml

Cell Viability and Morphological observation

The cell viability assay was performed on a triple-negative MCF-7 cell line taken as the control group. In the present study, different concentrations (100µg - 500 µg) of standard drug and plant extract were taken to study morphological changes and cell growth inhibition in a cell line.

An increase in the cell viability was observed at minimum concentration only. As the concentration increases, the viability of cells was decreased. Among three tested extracts, the Ethanol extract of *Terminalia chebula* exhibited prominent activity with a significant IC50 value of 228.82µg. Whereas Methanol extract of *Terminalia bellirica* and Aqueous extract of *Phyllanthus emblica* shown 432.45µg and 259.02µg, respectively.

Table.3. Comparison of Effect of different solvent extracts of experimental plants on MCF-7 (Breast Cancer) Cell viability

Treatment	Cell Line	Concentration in µg	Cell Viability in Percentage (%)	IC_{50} in µg
Ethanol extract of *Terminalia chebula*	MCF-7	100	68.7142±0.015	228.82
		200	56.0645±0.0165	
		300	32.4310±0.0215	
		400	13.4825±0.0185	
		500	6.9234±0.0015	
Methanol extract of *Terminalia bellirica*	MCF-7	100	94.3779±0.005	432.45
		200	85.4763±0.0545	
		300	70.0676±0.0650	
		400	53.9781±0.0380	
		500	39.6147±0.0305	
Aqueous extract of *Phyllanthus emblica*	MCF-7	100	87.4544±0.0035	259.02
		200	67.0900±0.0105	
		300	34.0551±0.0420	
		400	17.7782±0.0125	
		500	5.4138±0.0090	
Standard Drug Cisplatin	MCF-7	20	6.9755±0.0050	≥15

CONCLUSION:

In the present study, the phytochemical analysis helped detect the secondary metabolites in the selected extracts of the plant. Phytoconstituents were evaluated by different quantitative biochemical tests. Phytochemical

analysis of plant extrac*t* contains a broad spectrum of bioactive compounds that included alkaloids, glycosides, saponins, tannins, phenols and flavonoids. In the context of antioxidant assays, almost all selected extracts showed moderate activity, but ethanol extract of *T. chebula*, methanol extract of *T. bellirica* and aqueous extract of *P.emblica* proven to be having significant antioxidant activity overall sections. The *in-vitro* anti-inflammatory activity of the extracts was comparable to the reference drug. The results revealed that methanol extract of *T.bellirica* exhibited effectiveness with the percentage of inhibition 86.9133%.In anticancer activity, comprehensive studies showed that ethanol extract of *T. chebula* has solid anticancer activity against Breast cancer cells MCF-7. However, further studies are needed to determine the structure of bioactive compounds and investigate the antioxidant and anticancer activities of the bioactive compounds in *the in-vivo* animal study.

CONFLICT OF INTEREST:

Authors have no conflict of interest to declare.

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ORIGINAL ARTICLE

β-sitosterol on heart rate variability in L-NAME induced hypertensive rats

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Abstract

Background: β-sitosterol is a bioactive compound extracted from *Mucuna pruriens* and found to be effective in protecting against cerebrovascular diseases. *Aim and Objectives:* The present study is aimed to assess the effect of β-sitosterol on heart rate variability in L-NAME induced hypertensive rats. *Material and Methods:* The study involved laboratory-bred 24 adult male Wistar rats (*Rattus norvegicus*) randomly allocated into four groups. Group 1: Control (n=6), Group 2: L-NAME (n=6), Group 3: β-sitosterol (n=6) and Group 4: L-NAME+β-sitosterol (n=6) for 28 days. All the experimental animals were subjected to Heart Rate Variability (HRV) analysis at the beginning as well as at the end of 28 days of before and after L-NAME treatment and simultaneous supplementation of β-sitosterol. Animals were subjected for assessment of other hemodynamic parameters such as heart rate and blood pressure. Oxidative stress parameters like serum Malondialdehyde (MDA) and Nitric Oxide (NO) were also assessed. *Results:* The present study demonstrated alteration in HRV with increased LF (sympathetic function) and HF (parasympathetic function) ratio in Group 2 L-NAME treated hypertensive rats. In Group 4 (β-sitosterol supplemented), hypertensive rats showed remarkable reduction in the values of LF and HF ratio and other vascular parameters as compared to Group 2. Increased level of serum MDA and decrease level of serum NO in Group 2 with concomitant decreased level of MDA and increased level of NO were also observed in Group 4 rats. *Conclusion:* This study clearly indicates an alteration in cardiovascular physiology with altered sympathovagal balance in L-NAME treated hypertensive rats. Administration of β-sitosterol in hypertensive rats was found to be beneficial against L-NAME induced hypertension.

Keywords: Heart Rate Variability analysis, oxidative stress, L-NAME induced hypertensive rat model, β-sitosterol.

Introduction

β-sitosterol is a bioactive phytosterol which is naturally present in plant cell membranes with chemical structure similar to the mammalian cell-derived cholesterol. They are abundantly present in lipid-rich plant foods such as nuts, seed, legumes and olive oil. β-sitosterol is a potent micronutrient and it is present widespread in higher plants. Animals obtain these phytosterol through their diet [1].

Heart Rate Variability (HRV) is "beat-to-beat" oscillation of the heart rate around its mean value and represents physiological phenomenon determined predominantly by the balance between Autonomic sympathetic and parasympathetic Nervous System (ANS). Therefore, the measurement of the HRV is a non-invasive technique that can be used to investigate the dynamic balance between sympathetic and vagal activity [2].

In vivo, vasodilators and vasoconstrictors modulate the endothelial function. It is established that Nitric Oxide (NO) produced in vascular endothelial cells has a potent vasodilator effect. It plays an important role in vascular resistance and growth. L-arginine analogues such as N-nitro-L-Arginine

Methyl Ester (L-NAME) hydrochloride administration inhibit Nitric Oxide Synthase (NOS) activities [3]. Hence, they will reduce NO biosynthesis leading to hypertension. In conditions of NO deficiency, there will be accumulation of superoxide anion in biological tissues which can lead to alterations in organ function. NO acts as an endogenous anti-oxidative agent. It reacts with superoxide anions generated in the living tissues and provides protection from their deleterious effects on many organs including heart, kidney etc. [4]. There were several adverse reports of therapeutic use of plant based bioactive compounds as antihypertensive agents hence more studies are necessary for greater understanding of their safety and efficacy to prevent possible risks [5]. As antihypertensive effects of β-sitosterol have been least studied, the present study was undertaken to assess it's effects on heart rate variability in L-NAME induced hypertensive rats with special reference to oxidative stress.

Material and Methods

Experimental animals

The study involved laboratory-bred adult male Wistar rats (*Rattus norvegicus*), weighing about 180-250g (age 8-10 weeks) obtained from the animal house of Shri B. M. Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura. The experimental animals were kept at 22-24^{0}C and exposed to 12 hours light/dark cycle with food and water being made available *ad libitum*. Utmost care was taken to avoid any laboratory induced stress in animals. The procedure was approved by Institutional Animal Ethics Committee (IAEC) and the animals were acclimatized to the laboratory conditions for one week before initiating the experimental protocol. All the experimental procedures were done in accordance with national guidelines (Committee for the Purpose and Control and Supervision of Experiments on Animals, Government of India).

Experimental groups

The acclimatised animals were randomly allocated to the following four groups: (Table 1)

Table 1: Experimental groups and dosages

Groups	Number of rats	Intervention
Control	n=6	Normal water and food.
L-NAME	n=6	L-NAME, 40 mg/kg/day orally in distilled water for 28 days
β-sitosterol	n=6	HPCM emulsion, 0.05 mg added in 5ml distilled water 1 to 2 unit/kg/day as per body weight administrated orally for 28 days.
L-NAME+β-sitosterol	n=6	L-NAME, 40 mg/kg/day orally in distilled water for 28 days.HPCM emulsion, 0.05 mg added in 5ml distilled water and β-sitosterol 0.14 to 0.26 mg/kg/day 1 to 2 unit/kg/day as per body weight administrated orally for 28 days.

Gravimetry

The body weights of all rats were recorded on day 0 of the experiment (initial body weight) and after 28 days of intervention (day 29, final body weight) (as the period of intervention was 28 days i.e. from day1-day 28) with an electronic balance (Practum 1102-10IN, Sartorius Lab Instruments, Germany). Percentage change in body weight was calculated using the formula shown in Table 2.

Change in body weight = Final body weight-Initial body weight/Initial body weight ×100

Administration of drugs

L-NAME procured from Sumedha Research World, India. L-NAME was refrigerated at -20^0 C for further use. L-NAME daily dose (40 mg/kg/day) was calculated and given in the morning by oral gavage at once in distilled water to Group 2 and Group 4 rats for 28 days [6]. β-sitosterol was extracted from *Mucuna prueins* plant in our Laboratory of Vascular Physiology and Medicine following all the laboratory protocol [7]. Extracted β-sitosterol was stored in the refrigerator (-20^{0}C) until further use. The daily dose of β-sitosterol was prepared as follows –HPCM emulsion 0.05 mg added in 5ml distilled water and β-sitosterol 0.14 to 0.26 mg/kg/day 1 to 2 unit/kg/day as per body weight was prepared freshly every day and was administered by oral gavage once in the morning to Group 3 and Group 4 rats for 28 days [7].

Evaluation of cardiovascular parameters

Blood pressure: Blood pressure was recorded non-invasively (NIBP) using a tail-cuff sensor (BioPac 200A) after placing the animal in a re-strainer. Systolic Blood Pressure (SBP) and Diastolic Blood Pressure (DBP) were recorded. Three values were obtained and the average of the three readings was considered for computation. Mean Arterial Pressure (MAP) was calculated using the formula (MAP = Diastolic Blood Pressure+ 1/3(Systolic blood pressure – diastolic blood pressure).

Electrocardiogram (ECG): ECG was recorded using needle subcutaneous electrodes using MP45 Biopac instrument with a PC based BSL 4.1 (Biopac Student Lab 4.1) software. All the recordings were performed in the morning hours following overnight fasting. Ten minute ECG was recorded in anaesthetized rats (Ketamine, 60 mg/kg b. wt.i.p and Xylazine, 6 mg/kg b. wt.i.p) in dorsal recumbency by inserting the needle electrodes into right (negative) and left (positive) front legs of the animal. From the recorded ECG, heart rate was calculated.

Heart Rate Variability (HRV) analysis: HRV components of low (LF, sympathetic index), high (LH, parasympathetic index), very low (VLF, injury index) frequencies and the LF/HF ratio were calculated. The recorded ECG was inspected offline for artefacts and ectopic beats that were manually deleted from the recording. RR intervals obtained from the recorded ECG were exported to Kubios software version 2.0 (developed by Department of Physics, University of Kuopio, Finland) for HRV analysis. Short term HRV analysis using 5 min ECG RR interval data was done to assess cardiac autonomic balance. Frequency-domain method of HRV analysis was used to assess the level of sympathetic activity, parasympathetic activity and sympathovagal balance.

Assessment of oxidative stress

Malondialdehyde (MDA): It is a product of lipid peroxidation. Concentration of MDA is frequently used as a marker for oxidative stress. MDA concentration was estimated in the serum by the method of Buege and Aust (1978). MDA reacts with thiobarbituric acid to give a pink colour and absorbance was read at 535 nm using spectrophotometer (Schimadzu UV 800, Schimadzu Corporation, Japan)[8].

Serum nitric oxide (NO): It is a stable by product of nitric oxide; nitrate was coupled to N-naphthyl ethylene diamine and then reduced to nitrate using cadmium reduction process. A spectrophotometer was used to measure the coloured complex produced at 540 nm by modified Griess reaction method [9-10].

Statistical analysis

SPSS software (version 20.0) was used for statistical analysis. Data were presented as Mean ± SD. To analyse multiple groups, one way ANOVA was used followed by Tukey's *post hoc* test to ascertain significant intergroup differences. Value of $p < 0.05$ was considered statistically significant.

Results

Gravimetry

Table 2 shows percentage change of body weight at the onset and before sacrifice of the rats. There was significant less body weight gain in group 2 (L-NAME) rats as compared to the control as well as other groups.

Effect of β-sitosterol on systolic and diastolic blood pressures

There were significant increase in SBP and DBP in the Group 2 of L-NAME treated rats (from D7 to D29) as compared to control. But a significant decrease in both SBP and DBP have been observed in Group 4 (β-sitosterol+ L-NAME treated) rats as compared to Group 2 (L-NAME treated) rats (Table 3, Figures 1 and 2). Table 3 also shows percentage change difference of SBP and DBP before and after treatment in different groups of rat.

Table 2: Percentage change in body weight of the rats

Body weight (g)	Control (n=6)	L-NAME (n=6)	β-sitosterol (n=6)	L-NAME+ β-sitosterol (n=6)
Day 1	173.3 ± 2.88	178.6 ± 5.50	178.12 ± 3.21	179.3 ± 4.93
Day 29	278.3 ± 5.40	266.0 ± 6.19	286.6 ± 3.14	280.0 ± 4.82
Percent body weight gain	37.76 ± 7.00^{a}	32.85 ± 8.00^{b}	37.85 ± 8.78^{a}	35.96 ± 9.78^{a}

Values with different superscripts are significantly different from each other ($p < 0.05$)*

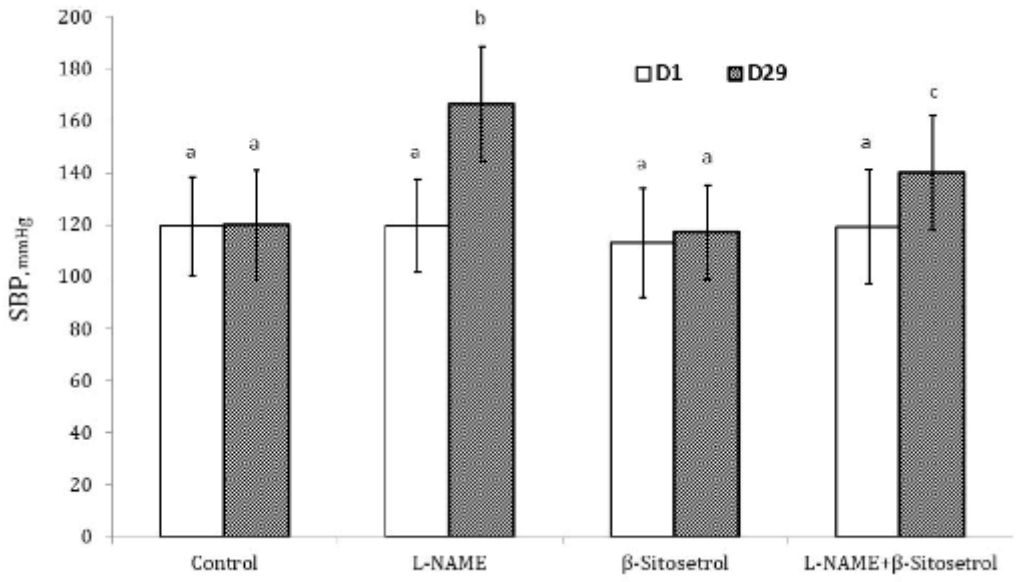

Figure 1: Effect of L-NAME induced alteration of SBP (mmHg) on β-sitosterol supplemented rats (n=6, each group) on D1, beginning of the experiment; D29, at the end of experiment and before sacrifice. Values with different superscripts are significantly different from each other ($p<0.05$*).

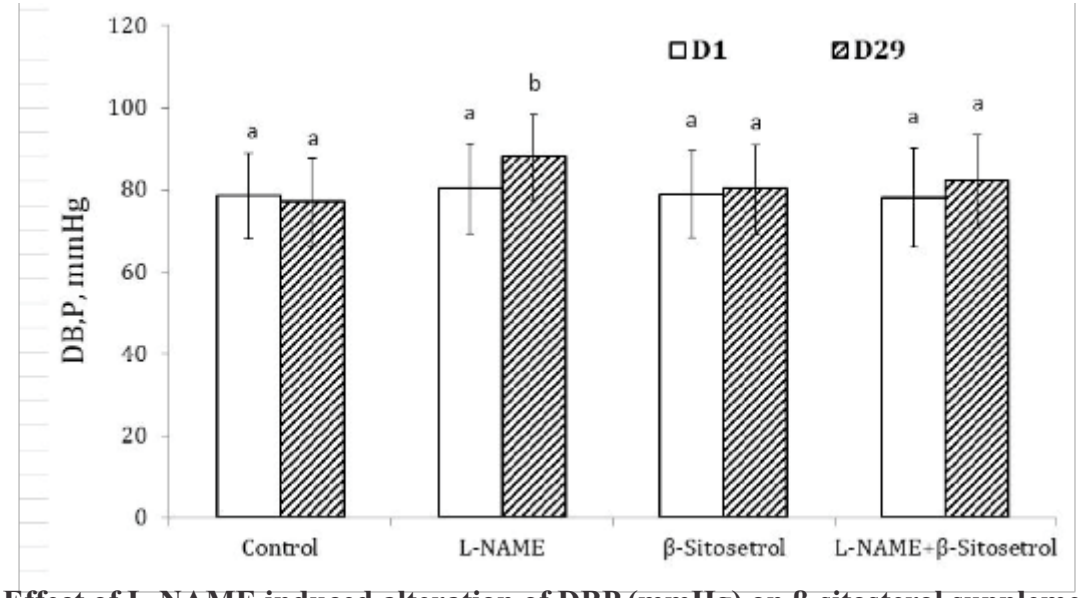

Figure 2: Effect of L-NAME induced alteration of DBP (mmHg) on β-sitosterol supplemented rats (n=6, each group) on D1, beginning of the experiment; D29, at the end of experiment and before sacrifice. Values with different superscripts are significantly different from each other ($p<0.05$*).

Table 3: Percentage change difference of SBP and DBP before and after treatment in different groups of rats

Parameters	Groups (n=6 /group)	Before treatment (D1)	After treatment (D29)	Percentage change (D1 Vs D29)
SBP (mmHg)	**Controls**	119.50 ± 18.50 [a]	120.00 ± 21.00 [a]	(+) 0.42
	L-NAME	119.66 ± 20.00 [a]	166.50 ± 24.25 [b]	(+) 39.14
	β-sitosterol	113.00 ± 16.75 [a]	117.00 ± 14.20 [a]	(+) 3.53
	L-NAME + β-sitosterol	119.16 ± 18.00 [a]	140.00 ± 15.25 [c]	(+) 17.48
DBP (mmHg)	**Controls**	78.50 ± 9.75 [a]	77.00 ± 10.00 [a]	(-) 1.91
	L-NAME	80.33 ± 9.56 [a]	88.00 ± 9.56 [b]	(+) 9.55
	β-sitosterol	79.00 ± 8.75 [a]	80.00 ± 10.25 [a]	(+) 1.26
	L-NAME + β-sitosterol	78.00 ± 9.00 [a]	82.00 ± 9.75 [a]	(+) 5.13

SBP-systolic blood pressure; DBP-diastolic blood pressure; D1,day one (before treatment), D29, day 29 (after treatment). Values with different superscripts are significantly different from each other ($p<0.05$).*

Effect of β-sitosterol on Mean Arterial Pressure (MAP)

Administration of L-NAME (40 mg/kg/day) induced a progressive increase in MAP. We found progressive increase in MAP with L-NAME treated groups as compared to Control Group (from D7 to D29). We also observed a slow increase till D14 and subsequently decrease in MAP in Group 4 (β-sitosterol + L-NAME treated) rats as compared to L-NAME treated Group 2 rats (Figure 3). Results also reflect the impact of β-sitosterol alone on MAP during pre-established hypertension from D1 to D7,D14,D21 and D29.

Effect of β-sitosterol on HRV analysis

The alteration in HRV was characterized by increased LF (sympathetic function) and HF (parasympathetic function) ratio in Group 2 (L-NAME treated) hypertensive rats. In Group 4 (β-sitosterol + L-NAME) rats showed remarkable reduction in LF and HF ratio as compared to Group 2 rats (Figure 4).

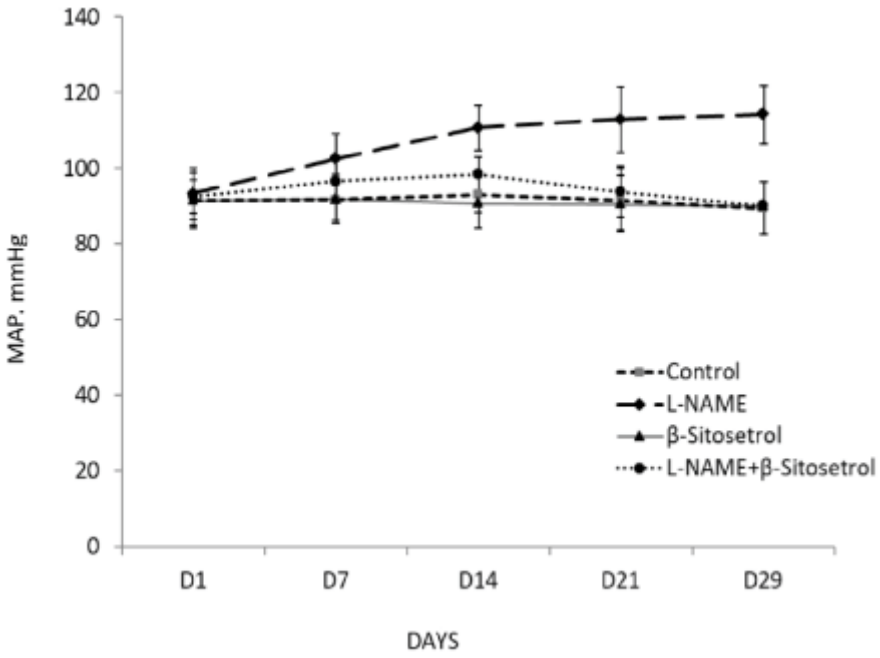

Figure 3: Comparison of MAP (mmHg) among four groups on D1, D7, D14 and D29 in L-NAME Induced and β-sitosterol supplemented rats (n= 6 in each group)

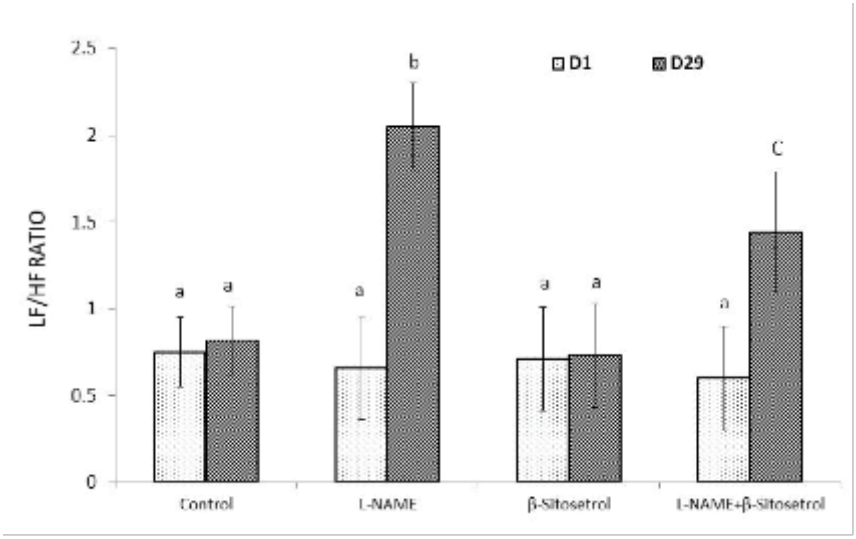

Figure 4: Effect of L-NAME induced alteration of frequency domain results of HRV analysis by LF/HF ratio on β-sitosterol supplemented rats (n=6, each group). D1, beginning of the experiment; D29, at the end of experiment and before sacrifice. Values with different superscripts are significantly different from each other ($p<0.05$*).

Table 4: Oxidative stress parameters

Parameters	Control (n=6)	L-NAME (n=6)	β-sitosterol (n=6)	L-NAME+ β-sitosterol(n=6)
MDA in serum µmoles/L	1.620.02[a]	2.610.54[b]	1.640.04[a]	1.650.03[a]
Nitric oxide in serum µmoles/L	0.520.02[a]	0.440.03[b]	0.550.06[a]	0.500.03[a]

Values with different superscripts are significantly different from each other (p<0.05).*

Oxidative stress

We observed significant increase in MDA levels in serum of Group 2 (L-NAME treated) rats when compared to respective control group 1. We also observed significant reduction in MDA levels in serum of Group 4 (β-sitosterol + L-NAME) rats (Table 3). In case of serum nitric oxide (NO) there was a significant decrease in NO levels in Group 2 (L-NAME treated) rats as compared to control. With the supplementation of β-sitosterol in Group 4 rats, the level of NO elevated significantly to near control level (Table 4).

Discussion

Decreased body weight in case of L-NAME treated rats after the end of treatment reflects rise of blood pressure induced stress phenomenon [3]. Present studies revealed that β-sitosterol- a bioactive compound of *Mucuna prueins* possesses cardio protective effects on L-NAME induced hypertensive rats. Chronic blockage of NO synthesis by L-NAME is well established method of inducing experimental hypertension in small animals [11]. Although this model cannot be extrapolated to human being, but it provides a possibility of understanding the mechanism of elevated blood pressure to a certain extent in relation to NO bioavailability. Sufficient magnitude of NO is needed for maintaining normal blood pressure. Another mechanism of endothelial dysfunction might be NO synthase inhibition by L-NAME with elevation of the Reactive Oxygen Species (ROS) via vascular NADPH oxidase [12]. When treated with β-sitosterol (Group 4), there was a significant decrease in MAP as compared to L-NAME treated rats. Our results showed decreased LF/HF ratio along with MAP in case of Group 4 (β-sitosterol+ L-NAME) rat, which might be due to regulating sympathetic overdrive during L-NAME induced hypertension by β-sitosterol supplementation. The observation of reduction of oxidative stress parameter and improvement in NO levels in our study further confirmed efficacy of β-sitosterol supplementation on cardiovascular abnormalities during L-NAME induced hypertension in rats [13-15].

β-sitosterol, by nature, is a phytosterol. Accumulating evidence indicates that cardiovascular protective effects of bioactive plant extracts are mainly via their cholesterol lowering ability, modulation of endothelial function and antioxidant capacity. More importantly, the cardiovascular protective effect of β-sitosterol, a key component of cholesterol controlling functional foods, has been related to its antioxidant and hypocholesterolemic capacity [16-17]. Further β-sitosterol possibly increases eNOS activity and decreases

NADPH oxidase in the vascular system which leads to increased level of NO production and ameliorate L-NAME induced elevated blood .pressure in experimental animals [18]. As β-sitosterol, a plant derived bioactive compound may protect against hypertension in animals, further studies are required to consider it as a possible therapeutic agent against hypertension in human.

Conclusion

The present study indicates altered cardiovascular physiology in L-NAME treated hypertensive rats but simultaneous supplementation of bioactive phytocompound β-sitosterol was found to be cardio-protective against L-NAME induced hypertension. These findings may be further extrapolated in clinical research as possible therapeutic measure against hypertension. This finding highlights the tremendous scope for using medicinal plants as possible therapeutic measure against cardiovascular diseases.

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Repurposing of potential bioactive compounds from various database to study their effects on MMP-7 by virtual screening.

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Abstract

Matrix metalloproteinase-7 (MMP7), a member of the matrix metalloproteinase (MMP) family, is involved in the mediation of both agonist-induced vascular tone and cardiac remodelling. We aimed to study the effect of a few bioactive molecules on (MMP-7) by in silico analysis. Data of bioactive molecules were collected from Pubchem and NPACT databases. PDB database was used for the generation of the 3D structure of protein MMP-7.

ADME/T properties showed 5 bioactive molecules obeying Lipkin's rule. Based on molecular docking, β-Sitosetrol and calyxin B are the top two compounds possessing higher ligand efficiency and interactive with higher number of amino acids while targeting MMP-7. The findings of this in silico study indicate 5 bioactive molecules obeying Lipkin's rule and out of these, two molecules may be considered as possible inhibitors of MMP-7.

Keywords: Bioactive molecules, MMP-7, ADME, Molecular Docking.

Introduction

Using bioactive compounds approved for one clinical use in another disease or syndrome is referred to as 'repurposing'. Most of the drive for repurposing is the high cost of developing a drug and the very long time it takes to determine the safety and specificity of a completely new drug[1].

Drug repositioning (DR) utilizes computational and experimental approaches to explore new clinical indications of existing drugs on a rational basis. Repurposing has investigated the clinical usefulness of many existing drugs as depicted above including some of the natural products such as ivermectin, colchicine etc. as prophylactic agents. FDA approved and clinical candidates, phytomedicine-derived bioactive compounds (or simply called phytochemicals such as curcumin, quercetin, epigallocatechin gallate EGCG and many others) have also been extensively investigated in search for potential lead molecules/drug candidates[2]. MMP-7 is a smallest protein member of MMP family. Matrix metalloproteinase (MMPs) are a family of proteolytic enzymes that regulate remodelling of the left ventricle (LV). MMP-7, also called matrilysin, is secreted as a 28 kDa proenzyme and is activated upon the removal of the pro-domain to generate a 19 kDa active enzyme[3]. Macrophages and cardiomyocytes are rich sources of MMP-7, 3, 4 and increased MMP-7 levels are detected in both the remote and infarct regions cardiovascular system. Naturally occurring bioactive compounds are ubiquitous in maximum nutritional better flora for human beings and livestock[4,5]. In systemic hypertension, the bioactive molecules may be explored for their function in modulating MMP-7, thereby regulating systemic hypertension[6, 7].

As *in sillico* screening of phytochemical database has gained tremendous interest in drug discovery research for the identification of new drugs, hence the present study was aimed to assess the effects of screened bioactive molecules on MMP-7 by *in silico* analysis.

Martial and Methods

Protein preparation: The crystal structure of human MMP-7 protein (PDB ID -2DDY) was obtained from the Protein Data Bank[8,9]. The protein structure was processed using Accelrys Discovery Studio by removing all non-receptor atoms including water, ion and various compounds. The refined and processed structure was saved as a "pdb" file format and viewed in Discovery studio[10]. The binding site for the inhibitor was searched based on a structural association of template with experimental evidence by using PDB-sum supported by a literature survey[11].

Ligand Preparation: A total of 130 biologically active plant-derived compounds (phytochemicals) with a wide range of structural diversity belonging to different phytochemical classes were selected based on their potential medicinal/biological interests as reported in traditional as well as modern phytomedicines. The 3D structures of compounds were downloaded from the PubChem database and saved in "sdf" files. Ligands were energetically minimized using the CHARMm-based minimizer on Biovia Discovery Studio (DS 2020)[12].

Pharmacokinetic Parameters: ADMET study is an essential step of drug screening for pharmacokinetic properties. The SWISS ADME tool analysed the properties including structural analogues; it predicts significant physical descriptors and pharmaceutically relevant properties. It consists of principle descriptors and physicochemical properties with a detailed analysis of the

logP (Octanol/Water), log S, molecular weight etc. It also calculates the analogues depending on Lipinski's rule of 5, an essential parameter for rational drug design[13].

Molecular Docking Studies: Maestro Schrödinger and molecular docking[14] 4.2 were used for selected 30 compounds (Table 1). Using genetic algorithm, extra precision docking was performed with the prepared protein and the ligands. Structures of ligands were kept flexible to generate different conformations. Receptor grid generation work flow was used to define a grid (box) around the ligand and to keep all the functional residues in the grid. Docking was performed on Intel® Core™ i3-7th gen laptop with 8 GB RAMS, Windows 10 system. All the results were visualized in Discovery studio.

Results

Structure of protein: The crystal structure of MMP-7 protein (PDB ID: 2ddy) was retrieved from PDB[15]. The MMP-7 protein is composed of 173 residues with molecular weight of 19 kDa and single motif. It is made of single A chain, contains 1 beta alpha beta unit, 1 beta hairpin, 1 psi loop, 7 strands, 3 helices, 22 beta turns and 2 gamma turns (Figure 1).

Binding site prediction: As per literature survey binding site information of target protein was predicted by performing PDBsum[16]. The ligand plot obtained from PDBsum showed binding site region of MMP-7 receptor containing 15 amino acid residues of chain A (Figure 2), viz. His 120, His 124, Glu 121, His 130, Leu 82, Ala 83, Ala 117, Thr 81, Pro 140, Tyr 116, Tyr 142, Thr 141, Ile 112 and MDW 178. These residues possess higher ligand efficiency and interaction with higher number of amino acids which are used for setting the grid of molecular docking.

Prediction of pharmacokinetic properties: *In silico* predictions of pharmacokinetic based on criteria via absorption, distribution metabolism and excretion (ADME)[17] properties have become important in drug selection and to determine their success for human therapeutic use.

Table 1
30 bioactive compounds selected for docking based on pharmacokinetics parameters

S.N.	Compound Name	Family	Molecular Weight
1	Calyxins B	Flavonoid	582.6
2	Artoindonesianin B	Flavonoid	468.5
3	Calyxins F	Flavonoid	582.6
4	Artoindonesianins V	Flavonoid	570.7
5	β- Sitosetrol	Flavonoid	414.7
6	Butein	Flavonoid	272.25
7	Calyxins A	Flavonoid	582.6
8	Calyxins C	Flavonoid	582.6
9	Calyxins D	Flavonoid	582.6
10	Calyxins E	Flavonoid	582.6
11	Calyxins G	Flavonoid	582.6
12	Calyxins H	Flavonoid	582.6
13	Calyxins J	Flavonoid	582.6
14	Artoindonesianin P	Flavonoid	368.3
15	Artoindonesianins A	Flavonoid	570.7
16	Artoindonesianins G	Flavonoid	570.7
17	Artoindonesianins H	Flavonoid	368.3
18	Artoindonesianins I	Flavonoid	368.3
19	Artoindonesianins U	Flavonoid	570.7
20	Baicalein	Flavonoid	270.24
21	Cajanol	Flavonoid	316.30
22	Biochanin A	Flavonoid	284.26
23	Blepharocalyxins A	Flavonoid	879.0
24	Blepharocalyxins B	Flavonoid	879.0
25	Blepharocalyxins C	Flavonoid	879.0
26	Blepharocalyxins D	Flavonoid	592.7
27	Blepharocalyxins E	Flavonoid	879.0
28	Burttinone	Flavonoid	438.5
29	Artoindonesianins V	Flavonoid	570.7
30	Apigenin	Flavonoid	270.24

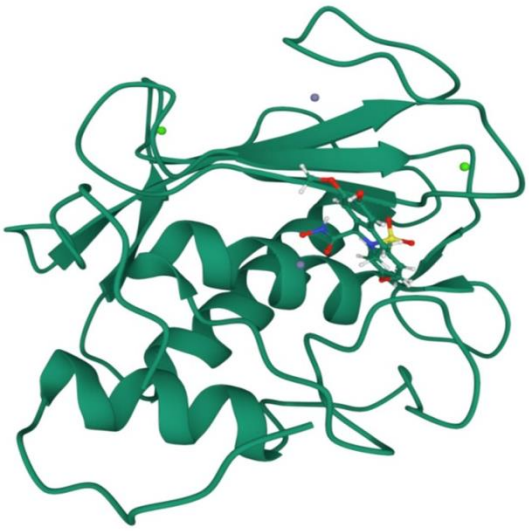

Figure 1: 2D structures of MMP-7

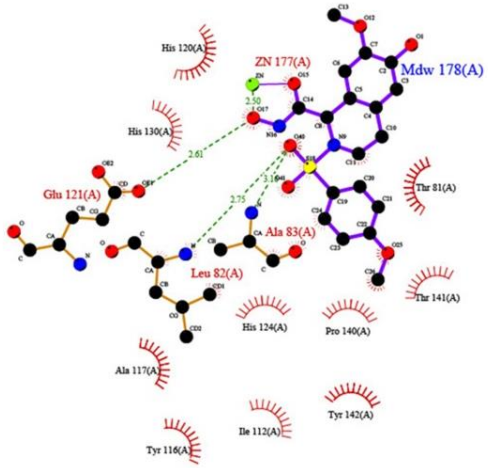

Figure 2: Amino acid residues lining higher ligand efficiency site of MMP-7.

Table 2
Top five compounds of ADME/T properties

S.N.	Compound Name	Molecular Weight (g / mole)	Rotatable Bonds	Hydrogen Bond Acceptor	Hydrogen Bond Donor	Lipinski Rule	Violation
01	Calyxins B	468.5	5	8	3	Yes	0
02	Artoindonesianin B	570.67	6	7	4	Yes	1
03	Calyxins F	582.64	12	8	6	No	2
04	Artoindonesianins V	582.64	9	8	5	Yes	1
05	β- Sitosetrol	414.7	6	1	1	Yes	1

So, these physiochemical properties were calculated to determine the ADME properties of the drugs. Bioactive molecules selected for present study were based on Lipinski's rule of five. All five ligands (Calyxins B, Artoindonesianin B, Calyxins F, Artoindonesianins V, β-Sitosetrol) have shown strong higher binding energy efficiency with target protein MMP-7 (-3.04 to -2.69 Kcal/mol). The said compounds followed the Lipinski's rule in table 2 of five without any violation with respect to molecular weight (≤ 600KDa), number of H-bond acceptors (≤ 8) and number of H-bond donors (≤ 6). The Lipinski's screening is an essential filter that determines if a compound is suitable for drug designing and their chemical structures had shown (Figure 3).

Molecular Docking Study: The human MMP-7 showed higher ligand efficiency (-3.04, -5.17, -5.89, -3.7 and -2.69) and interaction with amino acids as shown in table 3 and

figure 4. Finally, comparing the higher ligand efficiency (-3.04 to -2.69) and interaction with amino acids scores of all five known inhibitors of human MMP-7. β-sitosterol and calyxins B were proposed in the study as possible inhibitors for the human MMP-7[18].

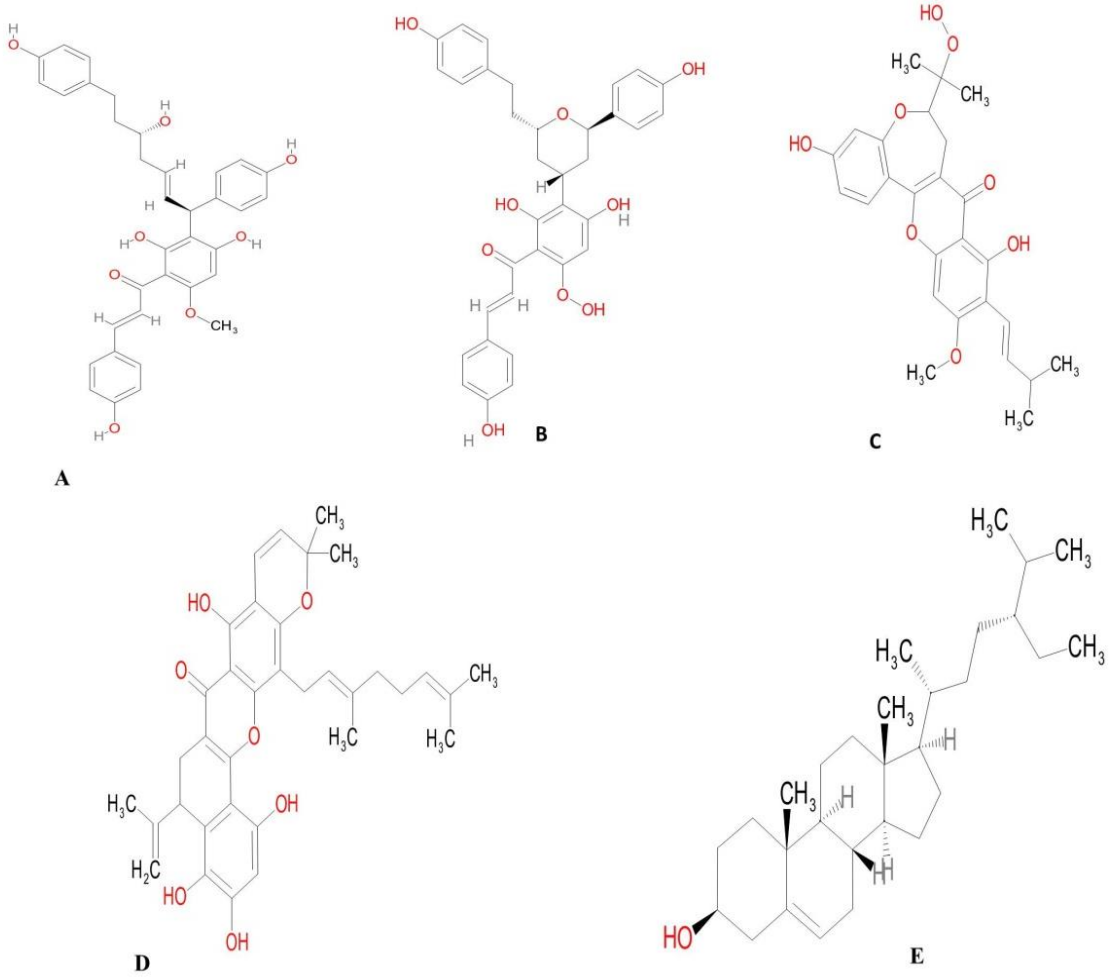

Figure 3: (A) Calyxins B (B) Calyxins F (C) Artoindonesianin B (D) Artoindonesianins V and (E) β- Sitosetrol

Table 3
Molecular Docking score

Molecules Name	Binding Energy (Kcal/mole)	Ligand Efficiency	Inhibition Constant	Interacting amino acid
Calyxins B	-3.04	-0.14	5.92	LYS 125, TYR 65,ALA 65, GLU 3, LYS 22, GLY 23,ASN 25
β- Sitosetrol	-2.69	-0.15	6.34	His 120, His 124, Glu 121, His130, Leu 82, Ala 83, Ala 117, Thr 81, Pro 140, Tyr 116, Tyr 142
Calyxins F	-5.89	-0.93	8.54	ARG103, VAL77, ALA105, ASN8, MET88, ALA86, PRO11, LYS113, ARG110, GLU118
Artoindonesianins V	-3.7	-0.18	1.94	ASN9, THR7, GLU63, ALA64, ALA63, ASN9, TYR67
Artoindonesianin B	-5.17	-0.25	10.29	ASN9, GLU8, GLU98, ASN70, LEU69,

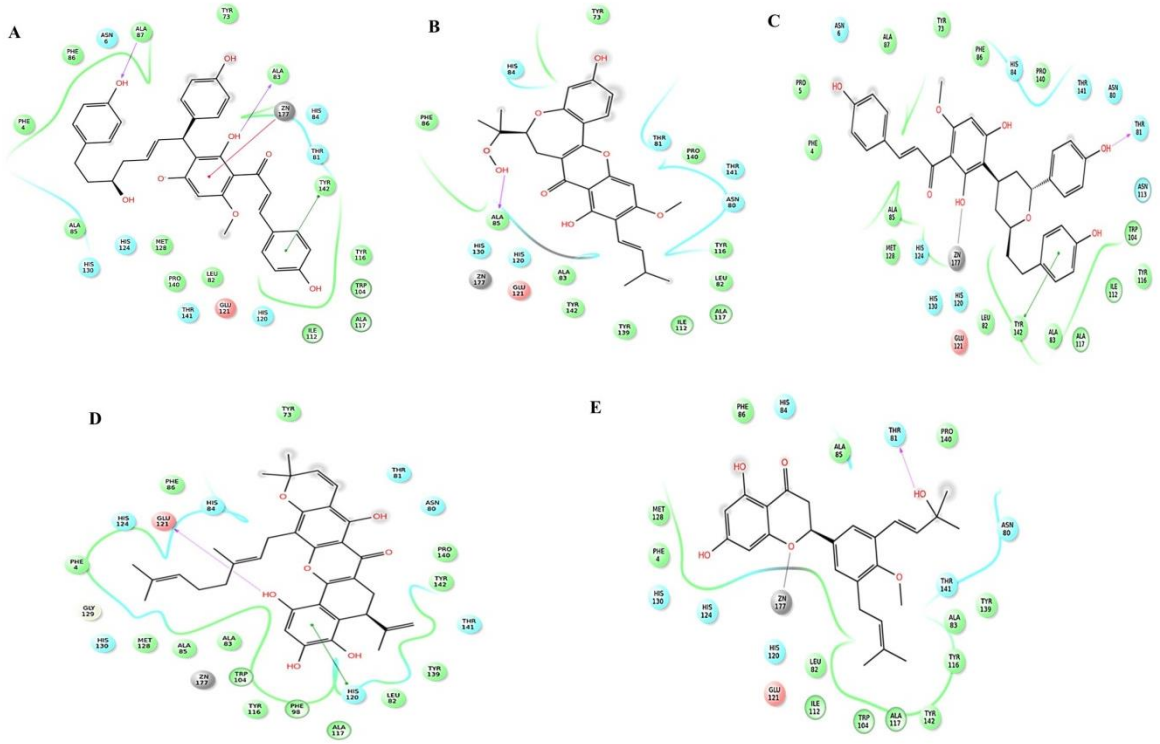

Figure 4: (A) Calyxins B (B) Calyxins F (C) Artoindonesianin B (D) Artoindonesianins V and (E) β- Sitosetrol

Discussion

Higher binding affinities were observed for docked compounds as compared to the co-crystal inhibitor. The more negative is the binding energy, the stronger will be the interaction. Affinity therefore depends on the energy of interaction. Thus negative binding energy depicts the strength of interactions as well as the affinity of a ligand molecule for its receptor molecule. Formation of stable complexes with well-defined interaction details predicts the significance of molecular docking and further molecular modelling studies[19].

From docking and drug-likeness/ADMET studies, five phytochemicals were found to exhibit remarkable inhibitory activities (best hit compounds), particularly against MMP-7. These phytochemicals are found in traditional Ayurvedic and Chinese medicines from plant sources such as neem, ashwagandha, ginseng soybean etc. All the identified compounds are basically tri-tetra-terpenoids, saponins or steroids with their wide natural abundance in traditional Ayurveda and Chinese medicines[19].

In the current work, ligands against the MMP-7 protein were selected from various phytochemical databases. Molecular docking was applied to explore the binding mechanism and correlate its docking score with the activity of the thirty (30) selected bioactive compounds. It has displayed good five (5) bioactive compounds with higher ligand efficiency and greater interaction with higher number of amino acids while targeting MMP-7. Molecular docking results further showed that calyxin B and β-sitosetrol are the best among the five (5) bioactive compounds with highest binding ligand efficiency and the maximum number of interactive amino acids. This present study can be useful for the design and development of novel compounds having better inhibitory activity against several diseases.

Conclusion

The results of the study indicate out of 30 selected bioactive compounds, 5 compounds were having higher ligand efficiency and interactivity with higher number of amino acids targeting MMP-7. Further out of 5 bioactive compounds, calyxin B and β-sitosetrol possesses the maximum ligand efficiency and interactivity with higher number of amino acids.

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