"MOLECULAR MECHANISM AND HERB DRUG INTERACTION OF HYDRO-ALCOHOLIC EXTRACT OF *TERMINALIA ARJUNA* BARK AND ITS AYURVEDIC FORMULATIONS IN METABOLIC SYNDROME"

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For the award of the degree of

Doctor of Philosophy
In the Faculty of Pharmacy

By

Mr. Sushant A. Shengule M.Pharm.

(Registration No: KLEU/Ph.D./14-15/DO1214027)

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Under the Guidance of

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ABSTRACT

Background:

Disorders like obesity, diabetes mellitus (DM), dyslipidemia and hypertension are grouped under metabolic syndrome (MS) with complex etiology, multifactorial pathogenesis and require prophylactic and therapeutic interventions. In our traditional knowledge and Indian civilizations, different herbal medicines have been advocated for DM. It is notable that *Terminalia arjuna* (TA) utilized in dyslipidemia, hyperglycemia, malignancy, high blood pressure and hypercholesterolemia. Arjunarishta (AA) and Arjun Ghritah (AG) are hydroalcoholic and ghee based formulation of TA respectively. These ayurvedic medicines were used to treat a lipid and cardiovascular disorders. Herbs are taken with the belief that they are helpful and safe in reducing side and adverse effects of prescription drugs. This leads to increased incidences of herb-drug interactions (HDIs). As adequate gaps present in the literature about the molecular mechanism, and HDIs, more research study in this direction is required to cover the essential issues.

Objectives:

The objective of the study was to assess the molecular mechanism and HDI of hydro alcoholic TA extract (TAHA) of bark and its ayurvedic formulations in MS

Methodology:

Phytochemical characterisation of arjunetin and arjungenin in TAHA, AA, and AG was performed using RP-HPLC-PDA method. High-fat diet fed intrauterine growth restriction rats were treated with TAHA, AA, metformin (MET), and AG for 3 months. Oral glucose tolerance test and blood biochemistry were performed in all the

groups. Effect of these drugs on the different gene expression was studied in liver tissue using Real-time PCR. The screening of TAHA, AA and AG was performed in adipocyte differentiation and *in vitro* α-amylase and Dipeptidyl Peptidase-4 inhibitory enzyme assays. For HDI study, oral administration of TAHA, AA, and AG was carried out for seven days followed by co-administration of MET till fifteen days. MET plasma PK parameters were measured on the eighth day. PD parameters were measured on the fifteenth day.

Results:

The phytochemically standardised interventions of TAHA, AA and AG could be involved in a partial improvement of body weight, improvement in glucose uptake and lipid compositions in the HFD fed rats. The possible proposed mechanism of action of TAHA, AA, and AG was through inflammatory, obesogenic, insulin-related, energy expenditure related, lipid and carbohydrate metabolism-related genes. TAHA also exhibited its hypoglycemic activity mediated through the α-amylase enzyme inhibition assay. The HDI study evidenced that in diabetic experimental condition, the effect of concomitant administration of TAHA+MET and AG+MET for seven days proved in beneficial PD interaction contributing to better antihyperlipidemic, antihyperglycemic activity and histological changes in tissues without affecting PK parameters of MET.

Conclusion:

This study has shown that the functional aspects and pleiotropic actions of TAHA, AA and AG, especially in MS. The effect of concomitant administration of TAHA and AG with MET proved in beneficial PD interaction without affecting PK parameters of MET in diabetic experimental condition.

Keywords: *Terminalia arjuna*; metabolic syndrome; Arjunarishta; Arjun Ghritah; herb-drug interactions; molecular mechanism; obesity; diabetes mellitus; dyslipidemia; metformin

LIST OF ABBREVIATIONS

AA:	Arjunarishta
ACN:	Acetonitrile
ACO-A oxidase1:	Acyl-Coenzyme A oxidase 1
AdipoqR-2:	Adiponectin receptor 2
AG:	Arjun ghrita
apoB:	apolipoprotein B
APOC III:	Apolipoprotein C-III
aqTAE:	Aqueous extract of TA a
AR:	Aldose reductase
AT2:	Angiotensin II type 2 receptor
AUC:	Area under curve
BMI:	Body mass index
BP:	Blood pressure
BW:	Body weights
C_{max}:	Maximum Concentration
CD-36:	Fatty acid translocase
CHD:	Coronary heart disease
CL:	Clearance rate
CPT-1a:	Carnitine palmitoyltransferase
CRP:	C-reactive protein
CV:	Coefficient of variance
CVD:	Cardiovascular disorders
CYP2D:	Cytochrome P450 2D
CYP3A:	Cytochrome P450 3A

DDI:	Drug-drug interactions
DM:	Diabetes mellitus
DPP-4:	Dipeptidyl Peptidase-4
EA:	Ellagic acid
EMA:	European Medicines Agency
ET-1:	Endothelin 1
FABP:	Fatty acid binding protein
FAS:	Fatty acid synthase
FBS:	Fasting Blood Sugar
FFA:	Free Fatty Acids
GA:	Gallic acid
GAPDH:	Glycerol 3-phosphate dehydrogenase
GC:	Glucocorticoid
GC-MS:	Gas Chromatography - Mass Spectroscopy
GK:	Glucokinase
GLUT4:	Glucose Transporter-4
G6P:	Glucose-6-phosphatase
H:	Hour
HDIs:	Herb-drug interactions
HDL-C:	High Density lipoprotein cholesterol
HFD:	High fat diet
HIV:	Human immunodeficiency viruses
HPLC-MS/MS:	High Performance Liquid Chromatography and Mass Spectroscopy/ Mass Spectroscopy

HPLC-MS-NMR:	High Performance Liquid Chromatography and Mass Spectroscopy and Nuclear Magnetic Resonance
HPLC-PDA:	High Performance Liquid Chromatography and Photo Diode Array
HPTLC:	High-performance thin layer chromatography
IBMX:	3-isobutyl-1-methylxanthine
IC50:	Half maximal inhibitory concentration
IHD:	Ischemic heart disease
IL-1β:	Interleukin-1β
IL-6:	Interleukin-6
IL-18:	Interleukin-18
IR:	Insulin resistance
IRS-1:	Insulin receptor substrate-1
IS:	Internal standard
IUGR:	Intra-uterine growth retarded
K_{el}:	Elimination constant
LDL:	Low-density lipoprotein
LepR:	Leptin Receptor
LOX:	Lectin like oxidized low density lipoproteins
MAPK:	Mitogen-activated protein kinase p38delta
MCP-1:	Monocyte chemoattractant protein-1
MET:	Metformin
mg/dL:	Milligram per decilitre
mg/g:	Milligram per gram
Min:	Minutes

mmHg:	Millimetre of mercury
MNC:	Mononuclear cells
MRF:	Metabolic Risk Factors
MS:	Metabolic syndrome
MWR:	Male Wistar rats
NaF:	Sodium fluoride
NAFLD:	Non-alcoholic fatty liver disease
NF-kB:	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NHANE:	National Health and Nutrition Examination Survey
nm:	Nanometer
OGTT:	Oral glucose tolerance test
OGTT-AUC:	Oral glucose tolerance test – Area under curve
PAI-1:	Plasminogen activator inhibitor -1
PD:	Pharmacodynamic
PEPCK:	Phosphoenolpyruvate carboxykinase
PGC-1α:	PPAR-γ coactivator 1α
PI3K:	Phosphatidylinositol 3 kinase
PK:	Pharmacokinetic
PRKAA-2:	Protein Kinase, AMP-Activated, Alpha 2
R_t:	Retention time
R^2:	Coefficient of regression
RAAS:	Renin Angiotensin aldosterone system
ROS:	Reactive oxygen species
SCD-1:	Stearyl-Coenzyme A desaturase
Sirt-1:	Sirtuline-1

STZ:	Streptozotocin
TA:	*Terminalia arjuna*
TAHA:	TA hydro-alcoholic extract
TBARS:	Thiobarbituric acid reactive substances
TC:	Total cholesterol
TCM:	Traditional Chinese medicines
TG:	Triglycerides
TLR-4	Toll like receptor-4
TM:	Traditional medicines
T_{max}:	Time to reach C_{max}.
TNF-a:	Tumor Necrosis Factor
tPA:	Plasminogen activator
T2DM:	Type 2 Diabetes Mellitus
$T_{1/2}$:	Half life
UHPLC-QToF-MS:	Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry
US:	United States
USFDA:	United States food drug administration
Vd:	Volume of distribution
VLDL-C:	Very low density lipoprotein cholesterol
Vs:	Versus
v/v:	Volume/volume
WHO:	World health organisation

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"MOLECULAR MECHANISM AND HERB DRUG INTERACTION OF HYDRO-ALCOHOLIC EXTRACT OF *TERMINALIA ARJUNA* BARK AND ITS AYURVEDIC FORMULATIONS IN METABOLIC SYNDROME"

1. INTRODUCTION

1.1 Background

Disorders like obesity, diabetes mellitus (DM), dyslipidemia and hypertension are grouped under metabolic syndrome (MS) with complex etiology, multifactorial pathogenesis and require prophylactic and therapeutic interventions. In our traditional knowledge and Indian civilizations, different herbal medicines in various oral formulations have been advocated for DM and claims of cure are on record [1]. It is notable that natural plant-like, Arjuna Bark (*Terminalia arjuna* [TA]) utilized in dyslipidemia, hyperglycemia, malignancy, high blood pressure (BP) and hypercholesterolemia.

As the modern drugs to treat DM have many side effects, the focus has been shifted to herbal drugs due to their safety, efficacy, minimal side effects and low cost [2]. Traditional medicines (TM) came from plants represent a significant part in the treatment of DM [3]. In last decade, Mankil et al. have surveyed various herbal plants utilized in experimental and clinical anti-diabetic studies in TM [4].

Kumar et al. (2009) represented that the TA bark extract significantly reduced the isoprenaline-induced oxidative stress and fibrosis but unable to reduced the increase in the ratio of heart and body weight indicating TA can prevent changes in myocardial induced due to beta-adrenoreceptor activation [5]. According to Sinha et al. 2008, TA ethanolic extract protects NaF-activated oxidative stress in murine hearts, via antioxidant properties [6]. Raghavan and Kumari 2006 represented that the ethanolic

extract of TA represents the antidiabetic and antioxidant effect in diabetic animals [7]. Shailaa et al. 1998 found hypolipidemic and anti-atherogenic activity of TA in rabbits [8]. Based on clinical trial evidence, the drug is considered as cardioprotective and not as cardiotonic. Its antianginal effect and used in different risk factors modification (like obesity, high BP and sugar) open up significant chances in prevention of ischemic heart disease (IHD) [9-14].

There is enhancing the use of complementary and alternative medicines with a prevalent form of herbs in several chronic conditions like cancer, DM and arthritis. Herbs are taken with the belief that they are helpful and safe in reducing side and adverse effects of prescription drugs. This leads to increased incidences of herb-drug (HDIs) interactions [15]. Evidence-based research on integrative use of herbs with prescription drugs lacks detection, assessment and prevention of adverse events and drug interactions associated with herbal drugs. These are due to inherent complex physicochemical nature, lack of quality control & standardization, absence of pharmacokinetic data on active constituents from herbal drugs and unawareness & lack of education among the healthcare systems. Advances in analytical technology have worked it possible to predict clinically relevant pharmacokinetic (PK) drug interactions established on a few numbers of *in vivo* and *in vitro* studies [16]. Recent advances in analytical techniques like HPLC-PDA, GC-MS, HPLC-MS/MS, and HPLC-MS-NMR has shown useful role in quality control and chemical standardization, estimation of concentration of low therapeutic index prescription drugs in biological fluids such as plasma and urine etc. and in PK evaluation studies on constituents from herbal drugs [17-19]. Documentation and reporting of HDI studies are of importance to physician, patients and pharmacists for rationalization of the combination therapy. Several international regulatory agencies such as the WHO,

USFDA, EMA and Australian Regulatory Guidelines for Complementary Medicines [20-22] recommend and advice such studies during the herbal drug development stage. Such studies on traditional western herbal medicines, chinese herbal medicines, Kampo, Cuba and African herbal medicines are available. For examples, Ginger, Garlic, Gingko, St. John wort's, has been studied extensively for their PK as well as pharmacodynamic (PD) interactions [23, 24].

DM is a chronic disease in the metabolism of carbohydrates, proteins, and fats beacause of a relative or absolute lack of insulin secretion with/without a diverging degree of insulin resistance (IR). The countries like India, China and the US are considered as the most significant number of DM patients and will remain the same in the year 2025 [25]. DM is associated with multifactorial pathogenicity, demands a multi-modal therapeutic approach. Among multimodal approaches, the use of herbs alone or along with conventional drug is considered an attractive approach for the treatment of DM [26]. The use of such combination directs to PK/PD interactions with conventional drug and may lead to beneficial, sub-therapeutic and/or toxic effect. Such combination therapies need to be tested for their herb-herb and HDI in early development phase [27]. Traditional Chinese medicines (TCM), western herbal medicines and Japanese Kampo medicines have been studied for their PK and PD interactions with conventionally used oral hypoglycemic [28, 29]. Sho-Saiko-to (Xiao Chai Hu Tang), a primary Chinese traditional polyherbal medicine has been frequently prescribed with other conventional drugs for the treatment of different chronic or acute diseases in Japan. Nishimura, N. et al. reported beneficial PK and PD interaction of Sho-Saiko-to with tolbutamide, oral hypoglycemic when administered concomitantly. Sho-Saiko-to reported to enhance the absorption and potentiate the hypoglycemic effect of tolbutamide [30]. Another study on a hydroalcoholic extract

of *Cassia auriculata*, Ayurvedic herbal medicine by Puranik et al. showed concomitant administration with metformin (MET) does not have significant PK interactions [31]. It has been suggested that *Cassia auriculata* is safe to take with MET during diabetic treatment.

According to the classical references from Ayurveda, TA bark and its traditional formulations have been used in cardiovascular disorders (CVD), hyperlipidemia and DM. It is supposed to promote better cardiac function with hypotensive and cholesterol-lowering effects. Therefore, we have planned this work to study the effects and molecular mechanism of TA hydro-alcoholic extract (TAHA) and its formulation on a cluster of symptoms of metabolic disorders like hypertension, IR (type 2 DM), obesity, dyslipidemia, hypertriglyceridemia and find out its molecular mechanism. These medicines are also likely to be taken along with prescription anti-diabetic drugs like MET and glibenclamide. Therefore, this work has been undertaken to evaluate pharmacological interactions of the above-selected formulations with MET, first line oral hypoglycemic in the normal and diabetic animal model.

MS enhances the possibility of diabetes, coronary heart disease (CHD), and stroke. In CHD condition, a plaque is developed inside the coronary arteries. This plaque blocks the arteries resulted into the decreasing blood supply to the heart muscle and may cause a heart attack, or death [32].

1.1.1. Metabolic Risk Factors (MRF)

The 5 conditions mentioned here are possible MRF. Patient with at least three MRF diagnosed considered with MS [32].

1.1.1.1 Overview

The risk for cardiac disease, DM, and stroke may increase due to several metabolic factors. The MS is connected to overweight, obesity, and a lack of exercise. IR may increase the risk of MS. IR can lead to hyperglycemia and obesity. Other various factors responsible for MS cause are genetics and older age [32].

1.1.1.2 Outlook

MS is becoming more common because of an increase in adiposity in adults. MS may become main MRF for heart disease in the future. MS may be delay or prevent with possible changes in lifestyle [32].

1.1.1.3 Causes

MS has various causes that act together. The patient can control few causes (overweight and obesity, an inactive lifestyle, and IR), cannot control few factors (growing older, genetics). Patients who have MS have other pathological conditions: inflammation and more blood clotting in the body [32].

1.1.1.4 Risk Factors

Patients at most considerable risk for MS have abdominal obesity, an inactive lifestyle and IR. Few patients are at MS risk because medicines prescribed to treat allergies, HIV, and mental illness causes obesity, hypertension, hyperglycemia and hypercholesteremia.

1.1.1.5 Populations affected

Few ethnic and racial population in US are at greater risk for MS than others. Mexican Americans have the higher rate of MS occurance, followed by blacks and whites [32, 33]. Other populations at enhanced risk for MS include a personal history

of DM, a parent or sibling who has DM and women with the polycystic ovarian syndrome.

1.1.1.6 CHD Risk

MS related and other than MS related factors also increases the risk of CHD. Such as, smoking and a low-density lipoprotein (LDL) ("bad") cholesterol level [32].

1.1.1.7 Screening and Prevention

Patient must adopt heart-healthy lifestyle changes and routine health check up to prevent MS [32, 33].

1.1.1.8 Signs and Symptoms

MS is a bundle of MRF that increases the risk for CHD and other complications, like DM and stroke. Many MRF have no indications, while a large waist is a clear sign. Few patients may have indications of T2DM. Symptoms of hyperglycemia include enhanced urination, thirst, especially at fatigue; night; and blurred vision. High BP may have often nosebleeds than usual or mild headaches [33]

1.1.1.9 Diagnosis

1.1.1.9.1 MRF

1.1.1.9.1.1 A Waistline:

A waistline measure of ≥ 40 inches for men or ≥ 35 inches for women is a MRF. A visible waist means patients are at risk for CHD and other complications [33].

1.1.1.9.1.2 A High Triglycerides (TG) Level:

A TG level of ≥ 150 mg/dL is a MRF [34].

1.1.1.9.1.3 A Low HDL-C Level:

An HDL-C concentration of ≤ 40 mg/dL for individual men and ≤ 50 mg/dL for individual women is a MRF [33, 34].

1.1.1.10 High BP:

A BP of > 130/85 mmHg is a MRF. If only 1 of 2 BP numbers are high, a patient still at risk for MS. [34].

1.1.1.11 High Fasting Blood Sugar (FBS)

An FBS level range between 100–125 mg/dL is known as prediabetes. An FBS level of ≥126 mg/dL is considered DM. An FBS level of ≥100 mg/dL is a MRF. About 85 per cent of T2DM patient has MS [35].

1.1.2. Definitions and Epidemiology

1.1.2.1 Definitions

The diabetes WHO consultation group defined MS as the presence of IR (impaired FBS and glucose tolerance, or T2DM) with two other risk factors: obesity, hypertension, hyperlipidemia, or microalbuminuria in 1998 [32].

1.1.2.2 Gender and epidemiology differences in MS

There is an extensive difference in prevalence on the basis of race/ethnicity, gender, age, and the standards used for disease diagnosis. MS impacts a 5^{th} of the US peoples and about a one-fourth of the Europe peoples. South-east Asian population has a lesser MS prevalence but nowadays is increasing like the western world population. [36]. There are also race-and gender-based variations in MS [37]. The MS prevalence in Hispanic women is 26% higher than men and 57% higher in African-American women than men. Among the cluster of diseases of MS, IR is more usual in Hispanics

peoples, high BP in African-Americans peoples, and hyperlipidemia in Whites peoples [38]. An analysis by Miller et al calculated that 10.1% of US adolescents have MS prevalence [39].

1.1.2.3 Pathophysiology

MS is a disease having inflammation, IR, adiposity, dyslipidemia, elevated BP, endothelial dysfunction, and chronic stress (figure 1.1).

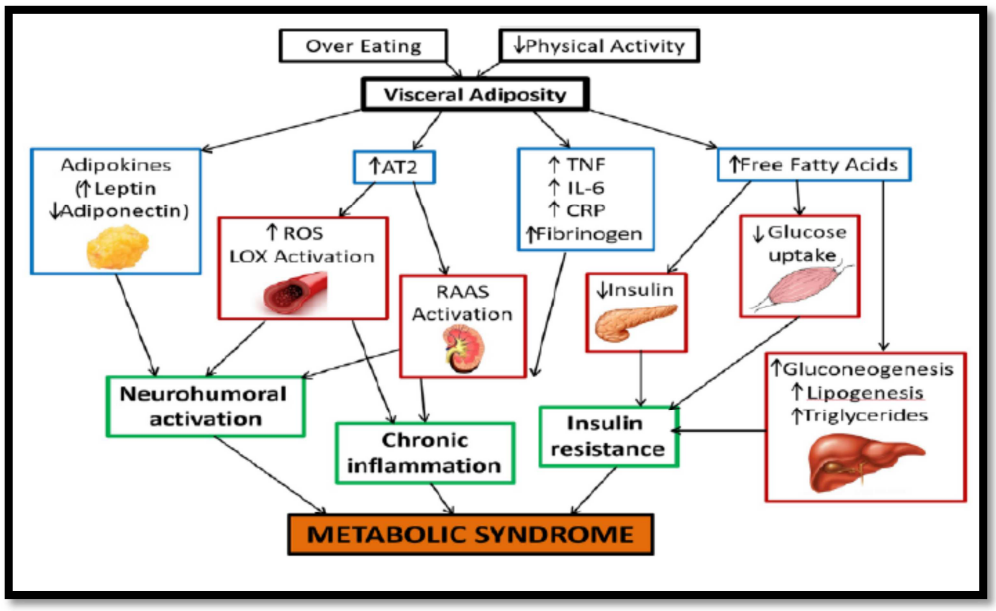

Figure 1.1. Pathophysiological mechanisms in MS. AT2, angiotensin II type 2 receptor; IL-6, interleukin 6; CRP, C-reactive protein; RAAS, renin-angiotensin-aldosterone system; LOX, lectin-like oxidized low-density lipoprotein; ROS, reactive oxygen species; TNF, tumor necrosis factor

1.1.2.4 Abdominal Obesity

Adipose tissue is a group of cells that can changes due to nutrient excess through adipocytes hyperplasia and hypertrophy. Due to increase in adipocytes size and obesity, the blood flow to it may be decreased with consequent hypoxia. It leads to a secretion of adipocytokines. And due to that inflammation in adipose tissue

propagates in systemic inflammation which is responsible for comorbidities in obesity.

1.1.2.5 Free Fatty Acids (FFA)

Adipocytes produce a maximum quantity of FFA, which leads to the fat aggregation in obesity. These FFA induces IR and impairs a pancreatic β-cell function in skeletal muscle and pancreas during acute and chronic exposure respectively [41].

1.1.2.6 TNF-α

It is an intermediator in adipocytes, which decrease the insulin sensitivity in adipocytes. TNF-α inhibits insulin receptor substrate-1 (IRS-1) and adipocytes apoptosis which leads to IR. TNFα is positively related with TGs, and body weight, while, a negatively associated with HDL cholesterol [42].

1.1.2.7 CRP

Elevated CRP levels are related with increased IR, BMI, and hyperglycemia. It is increased in obese IR patients and decreased in obese insulin-sensitive patients. Also, it has been represented that in the MS patient, occurrence of the CVD events mainly predicted on the basis of CRP [43].

1.1.2.8 IL-6

It is released through skeletal muscle and adipose tissue in humans [44]. It is decreased lipoprotein lipase activity. IL-6 is positively related with insulin in fasted condition, BMI, and T2DM development. It is negatively related with HDL [45].

1.1.2.9 PAI-1

PAI-1 exerts its action through the tissue plasminogen activator (tPA) inhibition [46] which is known marker for atherothrombosis and impaired fibrinolysis. PAI-1 is

enhanced in inflammatory states and obese subjects, thus, increasing the risk of adverse cardiovascular outcomes and intravascular thrombus [47].

1.1.2.10 Adiponectin

Adiponectin regulates the increase in the insulin sensitivity, body weight, food intake, lipid and glucose metabolism. It also protects against chronic inflammation. It raises fatty acid oxidation and glucose transport in muscles [48]. It is negatively linked with CVD features like BP, LDL-C, and TGs. Moreover, hypoadiponectinemia is related with IR, hyperinsulinemia that may be responsible for T2DM development without fat mass [49].

1.1.2.11 Leptin

Leptin is required in the energy intake and satiety regulation. Plasma leptin decline in weight loss and increase during the development of obesity. Resistance of leptin is a main pathological condition in obesity [50, 51]. The leptin concentration is associated with hyperleptinemia and adiposity [52].

1.1.2.12 IR

IR is known as a pathological condition in which insulin do not develop a normal insulin response in the peripheral target tissues and the pancreatic beta cell secretes more insulin to overcome the hyperglycemia. This emphasizing of some actions of insulin related to the resistance with other insulin actions results in the clinical MS manifestations [53]. Abdominal subcutaneous fat and visceral fat contributed to IR, an abdominal obesity associated strongly with the IR and the MS than lower body obesity [54].

1.1.2.13 Dyslipidemia

It is defined by lipid abnormalities of atherogenic lipoproteins, which include decreased HDL, increased apolipoprotein B (apoB), TGs and LDL [55].

1.1.2.14 Hypertension

Hypertension is linked with different metabolic disorders, like adiposity, impaired glucose tolerance, and hyperlipidemia. Studies mentioned that both hyperinsulinemia and hyperglycemia stimulate the Renin-angiotensin system (RAS) enhancing, which may responsible to the high BP development with IR in patients [56].

1.1.2.15 Glucocorticoid (GC) Action and Chronic Stress

The cortisol is the stress mediator, which may contribute to the visceral fat accumulation [57]. GCs activates the enzymes required in FA synthesis and gluconeogenic pathway; lipoprotein secretion; differentiation of preadipocytes; and lipolysis or lipid oxidation which associated with IR [58]. These hormonal defects lead to hypersecretion of insulin, obesity, and sarcopenia, leading to hyperlipidemia, high BP, and T2DM [59].

1.1.2.16 Prevalence

MS prevalence is experience a prominent population burden in different part of the world [60]. From few decades, obese U.S. adults have increased adiposity, and ethnic differences in waist circumference and BMI have observed. BMI and waist circumference enhanced linearly, but in women, waistline enhanced at more percentile **[**61]. The age-adjusted MS prevalence in the US is around 40%. The highest MS prevalence in the US was discovered in between Mexican Americans in the NHANE Survey, 1988–1994 [62].

1.1.2.17 Treatment available

Healthy-heart lifestyle modification are the 1st line of MS treatment. If these changes are not enough, then doctor may prescribe drugs. Drugs are used to control and treat diseases, like hypertension, hypertriglyceridemia, hyperlipidemia, and hyperglycemia.

1.1.2.17.1 Treatment Goals

The main goal of MS treatment is to decrease the CHD risk. The first treatment is directed to lower LDL-C, high BP and managing DM. The other goal of treatment is to stop the T2DM onset [62].

1.1.2.17.2 Healthy-Heart Lifestyle Modifications

Healthy-heart lifestyle modifications include eating healthy food, physical activity, quitting smoking, managing stress and weight.

1.1.2.17.3 Medicines

Sometimes lifestyle modification is not enough to restrain the MS risk factors. The doctor may begin statin treatment with patient who has an increased risk of CHD development or have a stroke. The doctor also may prescribe other medications to reduce the chance of heart attack, hypotension, prevent blood clot formation, which may result into a stroke or heart attack and reduce the CHD symptoms.

1.1.2.18 Nutraceuticals in the management of MS

Dietary additives, which provide primary nutritional value with health benefits, are known as nutraceuticals. Several natural compounds derived from plant extracts, herbs, spices, and essential oils have represented usefulness in the MS management.

1.1.2.19 Curcumin

Curcumin is well known for antioxidative and anti-inflammatory effect. It has illustrated to inhibit NF-kB activation by a decreased in the TNF-α and PAI-1 [63].

1.1.2.20 Garlic

Garlic usually used condiment, is known for antithrombotic and antioxidant activities. Reinhart et al, in 29 placebo-controlled trials represented the garlic intake lowers TC and TG levels. Padiya et al, demonstrated that garlic improved insulin sensitivity in diabetic rats. However, Gomez- Arbelaez et al, found the effect of 12 week old garlic extract on adiponectin concentrations in people with MS [64].

1.1.2.21 Neem

Neem known as *Azadirachta indica*. Neem extract enhanced glucose tolerance by decreasing pancreatic and intestinal glucosidase effect [65].

1.1.2.22 Resveratrol

Resveratrol is a polyphenolic compound found in plants like nuts and grapes. It activates the sirtuin molecular pathway, responsible for various cellular functions of oxidation, metabolism, and ageing. It also shows favourable effects on adipogenesis, lipolysis, and cyclooxygenase [66].

1.1.2.23 Sulforaphane

Sulforaphane is extracted from *Brassica* family plants like broccoli, has shown beneficial effects in MS, becuase of its anti-inflammatory and antioxidant effects. Various studies have represented a effect of sulforaphane in several disorders like hyperlipidemia, high BP, and DM, all critical components of MS [67].

1.1.3 Need for new treatment and for that herbal and Ayurveda product exploration

Research is the prime need of contemporary Ayurveda, but modern research on Ayurveda has not been advantageous for Ayurveda itself. Much of it uses Ayurveda to extend modern bioscience. In contrast, Ayurveda needs research designed to test and validate its fundamental concepts as well as its treatments. In this context, if Ayurveda is to be truly explored and validated in all its aspects, scientific inputs should conform to Ayurveda's principles and philosophy. While its evidence base, established since antiquity, may need further verification, research should now focus on the Science of Ayurveda, rather than merely looking for new drugs based on Ayurveda herbals; in-depth research is needed on Ayurveda [68].

1.1.4 The Spectrum of Evidence-Base

One should not believe that Ayurvedic medicine has no evidence-base. Ever since antiquity, All theory and practice of Ayurveda have been evidence-based, in its time frame of course. Like any other branch of traditional or modern knowledge, Ayurveda may need to obtain new evidence from time to time.

The WHO has expressed similar views related to the critical issues in the assessment of therapeutic effects are standardization of diagnostic criteria, clinical epidemiology for clinical research, a systematic review of literature on clinical research, and clinical trials to assess safety and efficacy. Cross-national evaluations using standard protocols have been suggested by individual science activists from a global perspective, while that does not appear to be necessary. We have to prove the evidence using critical evaluation of information from completed studies and

systematic reviews. Research in regular practice settings and the reverse pharmacology approach seem to be correct strategies in ancient medicinal research.

However, then, one cannot help pointing out the conflicting attitudes of different regulatory agencies in various countries. As per the Committee of Herbal Medicinal Products regulation for registration of herbal or traditional medicines in Europe, "The principle behind the actual registration procedure is to enable medicines which have been in extended standing traditional use to be registered because their safety and efficacy can be deduced from their extended standing use [68].

1.1.5 Emerging issues and the sectorial concerns

Ongoing research is progressed in such a way that it is of more appreciate to modern medicine than Ayurveda. Ayurvedic research outcomes have not yet filtered down to professional use. Inadequacies in available regulatory laws are also proving a barrier because various formulations and drugs developed through new research have no drug status, specifically for Ayurvedic physicians. For example, Sarpagandha has known the ayurvedic drug, but reserpine isolated from Sarpagandha is coming under allopathic drugs. Hence, such research is of no use to them. The recent paradigm shift demands research initiatives in the science of Ayurveda including exploration of more recent approaches and methodologies of therapeutic research, in order to enrich Ayurveda as it is and to benefit Ayurvedic practitioners [69].

There must be some procedures and techniques to analyze these drugs (extracts or formulations) for their composition and strength. Thus, there is a need to ensure the standard quality of Ayurvedic products [69].

1.1.5.1 TA

TA has been utilized as a cardioprotective agent and for the treatment of different human ailment to maintain good health. The extract of TA bark has role as an acardiac stimulant for beneficial activity in angina, i.e., "hritshool", and is specified in the treatment of atherosclerosis, hypercholesterolemia and heart failure [68]. Traditionally the bark powder of the stem is prescribed in the form of asava ayurvedic formulation (an alcoholic decoction) or given along with ghrita ayurvedic formulation (clarified butter) or kshirpak ayurvedic formulation (along with boiled milk) [69].

Figure 1.2. TA-bark Flat pieces regular (left). (right: Picture source - http://www.kisalayaherbals.net/terminalia-arjuna-bark-1561916.html)

Figure 1.3. The bark is thin, shiny, smooth, and greenish-grey from outside (right: Picture source - http://www.kisalayaherbals.net/terminalia-arjuna-bark-1561916.html)

TA is described to possess potent hypolipidemic activities beacuase of saponin glycosides that is responsible for inotropic effects, whereas the flavonoids may support vascular strengthening as well as antioxidant activity, therefore showing the multiple effects of this herbal plant [70]. Herbal plants have an important part in health care and are the primary source of raw materials for conventional and traditional medicine manufacturing [71]. They got attention due to unavailability of current medical alternatives, their effectiveness, cultural preferences and rising cost of allopathic medicines [72, 73]. The ethnobotanical fields were utilized to recognise the herbal plants as an food [74], medicines for human health care [75], and medicines for animals [76] and important for economy [77]. The importance of traditional knowledge system has elaborated in view with sustainable growth, protection, and search for novel utilization ways of plant resources [78]. As per WHO, in the world, almost 80% of the people depends on traditional medicine and 60% of the rural Indian population use herbal medicines [78]. During this decade, the utilization of herbal supplements enhanced from 2.5% to 12% [79-81]. In the ancient India, herbal plants were utilised as an excellent source for different drugs and to treat various critical disorders. Ayurveda, Siddha, Unani systems are the part of the Indian traditional medicine. Herbal medicinal drugs are easily available, cheap, safe, effective and have infrequent adverse effects. The assessment of new herbal drugs, has opened a huge area for research and making a changeover from traditional to modern world medicine [82-85].

1.1.6 TAHA and Ayurvedic formulation (Arjunarishta and Arjuna ghrita)

TA and its ayurvedic formulations were used for antidiabetic and cardiotonic activities from ancient times. Various clinical studies have investigated its effect in angina, CHD, hypercholesterolemia and heart failure. The other researchers provided

evidence for the beneficial effect of TAHA to the treatment of DM in diabetic animals [86-88].

In Ayurveda, ancient hydroalcoholic Ayurvedic formulation Arjunarishta (AA) is utilized for the treatment of CVD. It strengthens and nourishes the heart muscle and encourages cardiac functioning by regulating cholesterol and blood pressure [89]. Arjun ghrita (AG) is also mentioned in Ayurveda classics for all types of Hridroga [90].

1.1.7 Need of HPLC characterisation

Characterisation is an essential aspect for the quality of herbal formulations containing multiple ingradients or Ayurvedic formulations. In general, two approached ways were used in the standardization. One approached is fingerprint analysis usiing HPLC and/or HPTLC method and other approach is individual phytochemical markers quantitation. It assures the consistent quality of herbal formulations. A scientific characterisation and validation (quantitation of active constituents, chromatographic fingerprinting) is required for quality control and assurance purposes as there might be variation in the manufacturing process [91].

1.1.8 Animal model for MS

MS consists of various pathological conditions that anticipate the risk for CVD. The risk of MS depends on the environmental and genetic factors in humans [92].

1.1.8.1 Diet-induced MS animal models

There were various dietary approaches reported for MS induction in animals. They have covered the single or a combination of diets, like high-sucrose, high-fructose, high-fat, high-fructose/high-fat, or high-sucrose/high-fat diets. Several studies

investigated MS as diet affects whole-body metabolism. The most usually utilized animal strains in diet-induced MS animal models include Wistar rats, Sprague-Dawley rats, intra-uterine growth retarded rats, Golden Syrian Hamster and C57BL/6 J mice [92].

1.1.8.2 Fat-enriched diet

Fats are most calorically dense and one of the three main macronutrients. The lipotoxicity concept is the standard norms observed in the various diets result in the MS development.

1.1.8.3 MS Genetic animal models

Genetic animal MS models are examine the MS pathogenesis influenced by genetic factors. These MS genetic animal models are timesaving as compared to diet-induced MS animal models. Primitively, leptin- or leptin receptor-deficient rodent animal models are utilized as diabetic and obese experimental genetic models. Various rodent models are developed, like leptin receptor-deficient and leptin-deficient mice; Zucker diabetic fatty, Zucker fatty, Goto-Kakizaki, obese spontaneous hypertensive and DahlS.Z-Lepr fa/Lepr fa rats [92].

1.1.9 HDI

Herbal medicine practice has acquired acceptance around the globe, especially in developing countries because of ease of accessibility, affordability, presumed safety and the notion that it is without adverse effect. This is not necessarily true as herbal medicine practices have its challenges and limitations so Physicians need to know which herb their patients take along with antidiabetic medications as some combinations may be beneficial, harmful or have no effect. In this study, it was summarized the report available on the interaction of herbs and various classes of

antidiabetic drugs whether PD (beneficial, harmful or no effect), PK (whether the herb affects metabolism, distribution, absorption and elimination of the drug) and a brief description of the study. Medicinal herbs and their active ingredients are used globally, and they have become an essential part of clinical medicine [93].

1.1.10 HDI pharmacological factors

Two or more drugs, when coadministered together, can cause pharmacological or chemical interactions. Such interactions may change the effect of either drug, leading to increased or decreased severity or effectiveness of adverse effects. The consequences are dependent on many pharmacological and chemical factors, like how they affect each other PK and PD and the physicochemical nature of the drugs in use [93, 94].

1.1.11 Animal model for HDI study

Appropriate animal models are critical in the drug development process. Although predictions can be made using in vitro data, several key characteristics of drug/xenobiotic disposition can only be determined in vivo, namely the relative contribution of metabolic and excretory routes to total clearance. Moreover, mass balance and the per cent contribution of an enzymatic pathway to overall elimination can only be estimated using in vivo data. Without this data, the appropriateness of PK/PD models cannot be assessed. Information derived from properly designed PK studies can be used to develop or refine PK/PD models. Thus, in addition to helping determine bioavailability and tissue localization of a drug, animal models can provide an estimate of exposure to metabolites after administration of the parent drug. In general, in vitro data are scaled to determine drug interaction liability and whether human in vivo DDI studies are warranted. In some instances, animal models can

provide mechanistic insight into a DDI using an experimental design that is not amenable to humans. A significant disadvantage of animal models is differing metabolic and transport pathways compared with humans because animals can have enzyme and transporter orthologs that differ in tissue expression or substrate specificity [95].

1.2 Literature Review

1.2.1 Test material selection

Disorders like obesity, DM, dyslipidemia and hypertension are often grouped under MS with complex etiology, multifactorial pathogenesis and require prophylactic and therapeutic interventions. In our Indian civilisations and traditional knowledge, many herbal medicines in different oral formulations have been recommended for DM and claims of cure are on record [96]. It is well known that herbal plant-like, TA not only possess hypoglycemic activity but also used in hyperlipidemia, cancer, hypertension and hypercholesterolemia.

As the modern drugs to treat DM have many side effects, the focus has been shifted to herbal drugs due to their safety, efficacy, minimal side effects and low cost [97]. Plant based traditional medicines play a significant role in the DM management [98]. The other author has reviewed many traditional medicinal herbal plants possessing clinical and experimental anti-diabetic activity [99].

1.2.2 Literature regarding TA

The TAHA was screened for α-amylase inhibitory activity and standardisation of the TA for polyphenolic phytochemicals using HPLC-PDA method. The content of arjunetin, arjungenin, gallic acid, ellagic acid, and quercetin was 0.47, 8.22, 2.443, 7.901, and 3.20 mg/g, reported respectively, in a TAHA. The TAHA and acarbose

have shown an inhibitory activity with an IC_{50} value of 145.90 and 62.35 μg/mL, respectively. The TAHA demonstrates α-amylase inhibitory activity because of a synergistic effect of the phytochemical constituents. This study suggests that one of the mechanisms of this plant for anti-diabetic activity is through the inhibition of α-amylase enzyme [100].

TA is an Ayurvedic medicine which has been clinically used in India to treat a variety of ailments like lipid and cardiovascular disorders. TAHA decreased HFD-induced hyperglycemia, hyperlipidemia, and glucose intolerance in the rat. Meanwhile, other author depicted the antihyperglycemic effect of TAHA of leaves in diabetic animals. Also, it decreases total cholesterol (TC) and triglycerides (TG) levels in diabetic animals [101].

TA is a large source of phytochemicals, including triterpenoids, polysaccharides, nucleotides, sterols, steroids, peptides. It has been found that the TAHA activates PPAR-γ coactivator 1α (PGC-1α) which regulates the lipid and glucose metabolism. This work aimed to evaluate the antihyperlipidemic activity of TAHA mediated through lipid and carbohydrate metabolism at the, molecular and biochemical levels in hyperlipidemic rats. The intrauterine growth restriction (IUGR) rats fed with a high fat diet (HFD) can use as a MS model [102].

Evidence from several in vivo, in vitro and clinical trials, reveal the pleiotropic activity of TA like anti-atherogenic, inotropic, anti-inflammatory, hypotensive, antioxidant and anti-thrombotic actions for the treatment of different CVD. It is authenticated that this herbal plant has an excellent safety profile when utilized in co-administration with other allopathic drugs [103].

Potent hypolipidemic activity of an aqueous extract of TA (aqTAE) and regulate the expression of atherogenic proteins highlight the fact that it may be utilized in the management and prevention of atherosclerosis [104].

Microencapsulated herb (TA, 1.8%) added vanilla chocolate dairy drink demonstrated a significant reduction in organ weights (liver and epididymal fats). Furthermore, a significant reduction in lipids like the atherogenic index, TG, TC, VLDL-C, and LDL-C was mentioned in encapsulated TA extract group as compared to control group. Reduction in TBARS and increase in reduced glutathione levels were also observed in both red blood cell and liver lysates with its supplementation. The results illustrated that the phytoconstituents (phytosterols, flavonoids, tannins and saponins) present in the encapsulated TA endure the processing conditions and efficaciously released in the intestine to demonstrate their effects, like antioxidant activities and hypolipidaemic, for better management of CVD [105].

1.2.3 Evidence-based effect regarding TA:

Vascular complications associated with diabetes are a major cause of morbidity and mortality in patients. Traditional herbal medicines are utilized in the treatment of CVD. TA bark extract significantly decreased myocardial injury and cardiac dysfunction in STZ-induced diabetic animals. It also decreased inflammatory cytokine levels, ET-1, and oxidative stress. The reduced heart rate, body weight, BP, and hyperglycemia in diabetic animals did not alter after TA treatment. Results demonstrated that TA bark extract improves the myocardial function in diabetic animals, maybe through decreasing ET-1 levels, cytokine levels, and maintaining antioxidant enzyme actions [106]. The other author demonstrated that the TA bark

extract ameliorates the altered baroreflex sensitivity in diabetic animals, maybe through decreasing cytokine levels and maintaining antioxidant enzyme actions [107].

The Subramaniam et al demonstrated that the three fractions ethyl acetate, diethyl ether and ethanol extract of TA exerted antioxidative and hypolipidemic activities at 175 and 350 mg/kg dose in hyperlipidemic rats. They have mentioned that the ethanolic fraction of TA exhibits the potent hypolipidemic and antioxidant effect than other fractions and has curative potential for CHD prevention [108]. The Biswas et al mentioned that TA leaf demonstrated antihyperglycemic effect due to its antioxidant role in diabetic rats [109].

The other author Raghavan et al demonstrated that the TA bark extract exhibits the antioxidant action mediated via oxidative stress correction in diabetic animals [110]. TA tree bark powder has significant antioxidant action that is comparable to vitamin E [111]. Toppo et al depicted that arjunolic acid an oleanane triterpinoid may be a promising lead to treat NAFLD [112].

The other group of authors independently mentioned that the arjuna fruit extract could be used as a natural antioxidant in reducing protein and lipid oxidation in meat products [113-115].

Chandra shekhar et al reported that TA alcoholic extract of bark depicted protective effect against picrotoxin induced anxiety in mice by alteration of genes linked to neurotransmitters, antioxidant enzymes and synaptic plasticity [116]. The other author demonstrated that the aqueous extract of TA stem bark defends versus IL-18-induced atherosclerosis mediated via NF-κB/PPAR-γ-signalling molecular pathway [117].

1.2.4 HPLC method literature review:

Dhanani et al first identified and quantified five tannin-related markers gallic acid, chebulagic acid, corilagin, ellagic acid and chebulinic acid in the bark and fruit extracts of T. arjuna using HPLC–PDA **[118].** Saha et al reported that the aqueous extract of TA bark contains flavon‐3‐ols such as (+)‐catechin, (+)‐gallocatechin and (−)‐epigallocatechin. Phenolic acids such as GA, EA and its derivatives were also found in TA extract. **[119].**

The other author represented the HPLC-PDA method developed for the standardisation of Arjunarishta by quantitative estimation of major antioxidant compounds, EA, GA, quercetin, kaempferol, and ethyl gallate as markers. Arjunic acid and arjunolic acid were not detected in the formulation **[120].** Some other authors described a reversed phase HPLC-PDA method for the simultaneous quantification of arjunolic acid, arjunic acid, arjunetin and arjungenin in TA extract **[121].** The GC-MS analysis of TA extract identified 21 compounds, respectively [122]. A wide range of constituents of TA was characterised and broadly grouped as 27 GA and 52 EA derivatives [123]. Avula et al identified 37 compounds using nontargeted UHPLC-QToF-MS analysis of contributed to the differences between Terminalia species [124, 125].

1.2.5 Animal model literature:

1.2.5.1 Fetal nutrient restriction and programming of metabolic diseases in offspring

There is an extensive body of evidence, that relates compromised fetal growth to later metabolic diseases. This has been aptly demonstrated by different models in which

IUGR can be obtained through dietary, pharmacological, and surgical manipulation of maternal animal [126-129].

IUGR is resulting from the malnutrition programs the fetus to adapt low energy diet permanently inducing following changes in the offspring. First, the combination of a post-weaning HFD and a prenatal low nutrient diet has a far more significant effect on adiposity than does a postnatal HFD alone [130]. Second, the studies indicate multiple components of the adipose phenotype are altered. These include central effects like food preference and appetite control of the offspring impaired pregnancies that have a preference for higher fat foods and are hyperphagic. Provocatively, IUGR rats have neuroendocrine alterations in the hypothalamus [131].

Interestingly, the phenotype is similar irrespective of its mode of induction, proposing common fundamental genetic and epigenetic mechanisms such as DNA methylation (Figure 2.2). This modification involves the add-on of a methyl group, by a methyl donor, to the five positions of the cytosine pyrimidine ring or the number 6 nitrogen of the adenine purine ring. This can alter the expression of the respective gene. Methyl donors come from one-carbon metabolism. One such mechanism is that low-protein diets have a high concentration of methionine. High concentrations of methionine may, therefore lead to hyperhomocysteinemia [132, 133]. We have used a similar kind of model in this study. This model has been bread for several generations to induce permanent genetic and epigenetic changes. The multi-generational inheritance of this model makes it a unique model to study interventions with certain drugs.

1.2.5.2 Effect of Adverse intrauterine milieu on peripheral organs

IUGR affects insulin dependent organs like pancreas, liver, adipose tissue and skeletal muscle. Various groups have covered IUGR and related metabolic defects in insulin signalling at length [134].

1.2.5.2.1 Pancreas

Insulin is a vital hormone for fetal growth, and thus, maternal undernutrition results in reduced body weight, smaller organ mass, hyperinsulinism at the time of birth. Fetal malnutrition in the last trimester of pregnancy in rats decreases β-cell mass by half and increases fetal insulin content. The adult offsprings of the same show a decrease in insulin content and β-cell mass predisposing these rats to IGT. Second and third generation offspring of STZ induced severely and mildly hyperglycemic mothers display the similar disorders such as macrosomic, hyperinsulinemic fetus with islet hyperplasia, IGT associated with defects in insulin secretion in adults [134].

1.2.5.2.2 Liver

Rats with fetal malnutrition are resistant to the actions of insulin, as evidenced by the decreased infusion rates of glucose to maintain euglycemia. This resistance to insulin was found to be the result of a decreased responsiveness of the liver, i.e., a dampened suppression of glucose production during hyperinsulinemia, whereas insulin action at the peripheral tissues remained normal [135, 136].

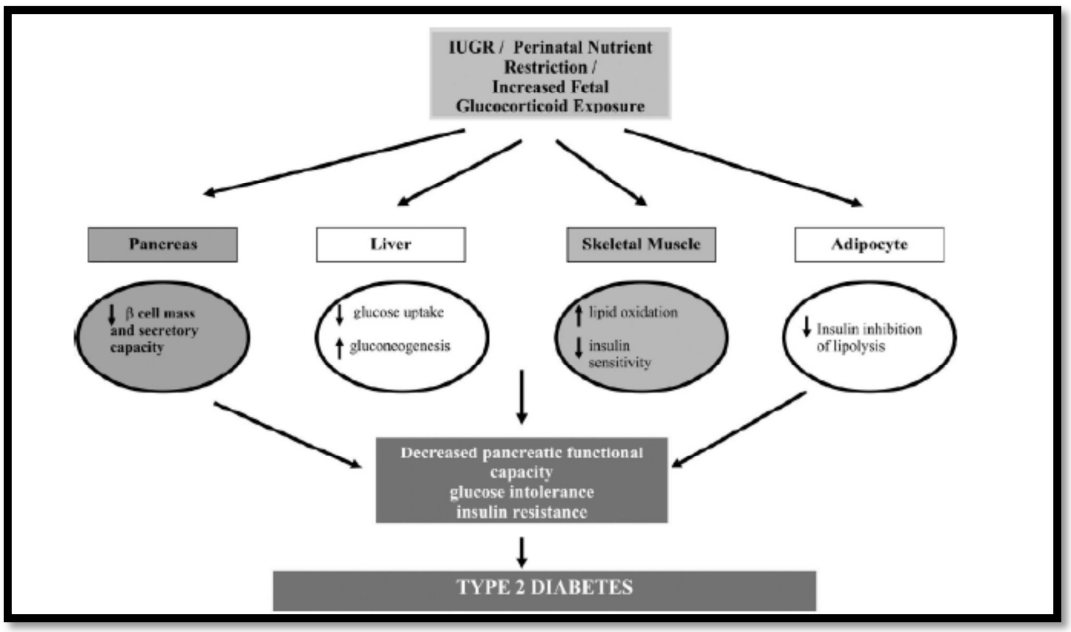

Figure 1.4: Different pathways underlying the perinatal programming of T2DM [134]

1.2.5.2.3 Adipose tissue

Basal glucose uptake was increased in adipocytes from subcutaneous, epididymal, and intra-abdominal fat depots; however, insulin- induced adipocyte glucose consumption is lesser than low-protein offspring as compared to control group. The GLUT4 protein expression showed no difference from the low-protein offspring in the adipocytes. At 15 month of lifespan, expression of insulin receptor was similar as compare to low-protein offspring and controls in adipocytes, indicating that the molecular change that results into IR must be at the post-receptor stage. At 3 and 15 months of lifespan, insulin- induced phosphatidylinositol 3-kinase effect was decreased, and this is linked with a decrease in the p110 beta subunit level from low-protein offspring in adipocytes. At 15 month, insulin- induced protein kinase B was also decreased from low-protein offspring in the adipocytes, which may justify the IR noticed in the adipocyte at this age.

From this data, it is clear that all the insulin-sensitive tissues are adversely affected by adverse intrauterine milieu. Therefore we have used a similar model to study the mechanistic aspects of TAHA and its ayurvedic formulations [137].

1.2.6 Herb-drug interaction related literature:

TA possesses different therapeutic activities. The author Varghese et al, indicated that the active phytochemicals of TA extract can suppress the in vitro CYP3A and CYP2D isoenzymes, which may, in turn, lead to PK HDI [138]. The same author investigated the PK-PD interaction of TA aqueous extract with metoprolol succinate in rats.

The alcoholic, aqueous and ethyl acetate extracts of TA bark indicated strong reversible non-competitive suppression of CYP2D enzyme in microsomes of the rat liver. Arjunic acid, arjunetin and arjungenin did not depict significant suppression of CYP2D enzyme in microsomes of rat liver. PK studies indicated that aqueous bark extract of TA co-administered with metoprolol succinate resulted to a significant reduction in $AUC_{0\text{-}24h}$ and C_{max} of metoprolol succinate in rats. PD studies show a significant decrease in the curative action of metoprolol succinate on co-administration with aqueous extract of TA bark. On the basis of in vivo and in vitro results, until further drug interaction clinical experiments are executed, the co-administration of drugs, cleared via CYP2D catalysed metabolism, with TA extracts should be done with precaution [139].

The author Bhamra et al mentioned that majority of patients who were on prescription medicines did not inform the doctor regarding any herbal medicines consumed simulataneously; this increases a concern about people's information of HDI, cooperation and effect on prescribed allopathic medication program [140].

AIM AND OBJECTIVES

Aim

The aim of the study was to assess the molecular mechanism and HDI of TAHA bark and its ayurvedic formulations in metabolic syndrome

Study Objectives

1. To develop method of TAHA and its ayurvedic formulations using HPLC analysis method
2. To investigate the effect and molecular mechanism of TAHA and its ayurvedic formulations in high fat fed rat model
3. To evaluate the activity of TAHA and its ayurvedic formulations in enzyme inhibition assays and their effects on adipocyte differentiation
4. To determine the effect of TAHA and their ayurvedic formulations on levels of MET and glucose uptake in normal and diabetic rats

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 List of chemicals and materials used for experiments is given below:

Table 2.1. List of chemicals and materials

Sr. No.	Particulars	Make/Source
1	TA bark	procured from the local market of Pune, and authenticated by Agharkar Research Institute (ARI), Pune, India
2	HPLC grade methanol	Merck, India
3	Water	Milli-Q (Millipore)
4	HPLC grade acetonitrile	(Merck)
5	Arjunetin and arjungenin	HPLC phytochemical markers from the Natural Remedies Pvt. Ltd. Bangalore, India.
6	Kits	Crest Biosystem (India)
7	Normal and HFD diets	VRK Nutritional Solutions, Pune, India.
8	Marketed formulations, i.e., Arjunarishta (AA)	(Batch No. GA-06, Sandu Pharmaceuticals Ltd. Goa, India)
9	Arjun Ghritam (AG)	(Batch No. 116, Nagarjun Pharmaceuticals P. Ltd. Ahmedabad, India)

2.1.2 List of equipment used for experiments is given below.

Table 2.2. List of equipment

Sr. No.	Particulars	Make/Source
1	Soxhlet extraction	Borosil
2	Rotary evaporator	Buchi Rotavapor
3	HPLC system	Dionex P680

2.2 Experimental Design

The plan of work was as follows:

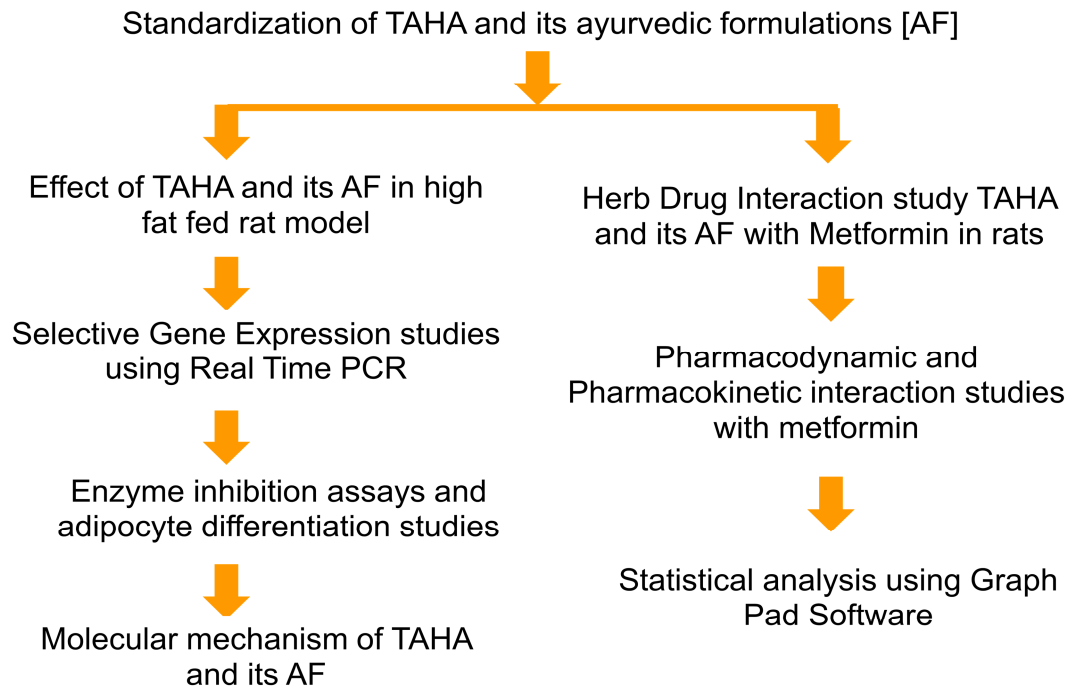

Figure 2.1: Experimental Design

2.2.1. Preparation of Standard

Both phytochemical markers stock solution was prepared in methanol. Please (for detailed methodology) refer Annexure II.

2.2.2 Preparation of Sample

Please (for detailed methodology) refer Annexure II.

2.2.3. HPLC System

Please (for detailed methodology) refer Annexure II [141, 142].

2.2.4 Estimation of arjunetin and arjungenin in a TAHA bark extract, AA, AG

Please (for detailed methodology) refer Annexure II [141-143].

2.2.5. Data Analysis for HPLC analysis

Please (for detailed methodology) refer Annexure II.

2.3 Method validation

Please (for detailed methodology) refer Annexure II [144].

2.4 Animal study

2.4.1 Animals and experimental design:

Male Wistar (MWR) and IUGR rats of 8-10 weeks were purchased from the APT Testing and Research Pvt. Ltd. (Previously known as National Toxicology Centre), Pune, India. The Institutional Animal Ethics Committee approved the preclinical experiment.

Please (for detailed methodology) refer Annexure II.

Table 2.3. HFD-composition

Ingredient	Grams
Casein, Lactic, 30 Mesh	200.00 g
Cystine, L	3.00 g
Lodex 10	125.00 g
Sucrose, Fine Granulated	72.80 g
Solka Floc, FCC200	50.00 g
Lard	245.00 g
Soybean Oil, USP	25.00 g
Mineral	50.00 g
Choline Bitartrate	2.00 g
Vitamins	1.00 g
Dye, Blue FD&C #1, Alum. Lake 35-42%	0.05 g

2.4.2 Plasma lipid profiles

TGs, TC, and HDL were measured using the commercial kit (Crest Biosystem, India) [88].

2.4.3 OGTT

The last oral dose of vehicle, TAHA or MET was administered 24 hr before the OGTT. Please (for detailed methodology) refer Annexure II [88].

2.4.4 Gene expression related results

2.4.4.1 RT-PCR analysis

Please (for detailed methodology) refer Annexure II.

Table 2.4. Primer details

Gene Name		5'-3' FP	5'-3'(RP)	Product size
Apolipoprotein C-III	APOC III	GGCCACCACAGCTATATCAGACTC	GATCCTTGCTGCTGGGCTCTA	103
Acyl-Coenzyme A oxidase 1	ACO-A oxidase1	AGGTGCGGTCGGGGAAGTT	GCTGTGGCTGGATCCGCTG	77
Stearyl-Coenzyme A desaturase	SCD-1	AGATCCTCCCTACCTCCACCCCTA	CCCTCTCGTTCAGTGGTTGCCTC	164
Phosphoenolpyruvate carboxykinase	PEPCK	GGAGTGGATGTTCGGACGCA	TCCGAACAGCTCCTCCACGT	126
Fatty acid binding protein	FABP	ATGTGTGATGCCTTTGTGGGGA	TACTCTCTGACCGGATGACGACC	169
Carnitine palmitoyltransferase	CPT-1a	CCTGTGAATACTTGGGCGTCTG	TCCCCAGGCCTAACCATTCC	142
Fatty acid translocase	CD-36	GCTGCACGAGGAGGAGAATGG	CCAAACACAGCCAGGACAGCAC	84
Glycerol 3-phosphate dehydrogenase	GAPDH	CATCGTAGGCTCCGGCAACT	AGTTAACTTTCTGCCCCCGATG	132
Glucose-6-phosphatase	G6P	CAAGAGCTGCAAAGGAGAACTG	TCAGCGAGTCAAAGAGATGCAG	94
Aldose reductase	AR	TCCCCAGACAGGCCTAGTGC	CGGCCACGTTCCTCTCTATATG	135

Glucokinase	GK	ATGAGGCGATGGGACACGAA	CGGTAGTTGGGTCGACAGCA	131
Mitogen-activated protein kinase p38delta	MAPK	CGGAGATGACTGGCTATGTGGTG	TGATGCAGCCAACAGACCAGATA	107
Phosphatidylinositol 3 kinase	PI3K	CCAGGAGCGGTACAGCAAAG	AGTCTCCTCCTGCTGTCGATGA	134
C-reactive protein	CRP	AGTCTGCAGAAGGGCTACATTGTG	TCCCACCAAAGACTGATTCGC	104
Insulin growth factor - binding protein	IGF-BP	GCTGCTCTTGCTGCTGCTGT	CTCTCGCACCAAGCTCGGC	147
Fatty acid synthase	FAS	CAACCTGATAGTGAGCGGGAAAG	AGACAGACTCGGACTCGGCG	100
sn-glycerol-3-phosphate acyltransferase	GPAT	AATGCTGCGGAAAAACTACGG	TGAAGGAAGGATTGCTGGTAACA	144
SREBF chaperone	SREBF	GGGAAGTACAGTGGGGTCAGCC	TCGGAGGCTGCAGTTGGGG	149
Stearoyl-coenzyme A desaturase 1	SCD-1	CCACAACTACCATCACGCCTTCC	CAGTCGATGAAGAACGTGGTGAAG	86
Squalene synthetase	SS	GAGAGCAAGGAGAAGCACCGA	CCCACCAGTCCAGCAACATAGT	202
Monocyte chemoattractant protein-1	MCP-1	TGCTGTCTCAGCCAGATGCAGTTA	TACAGCTTCTTTGGGACACCTGCT	100
Interleukin-1β	IL-1β	AGCAGCTTTCGACAGTGAGGAGAA	TCTCCACAGCCACAATGAGTGACA	144
Tumor Necrosis factor-α	TNF-α	AGAACAGCAACTCCAGAACACCCT	TGCCAGTTCCACATCTCGGATCAT	149

Protein Kinase, AMP-Activated, Alpha 2	PRKAA-2	AAGCATCGATGATGAGGTGGTGGA	AGACAGTGAATGGTTCTCGGCTGT	107
Sirtuline-1	Sirt-1	ACAACCTCCTGTTGGCTGATGAGA	TCACTAGAGCTTGCGTGTGATGCT	134
Interleukin-6	IL-6	AACTCCATCTGCCCTTCAGGAACA	AAGGCAGTGGCTGTCAACAACATC	131

2.4.4.2 Herb-drug interaction study

Please (for detailed methodology) refer Annexure II.

2.4.4.3 Animal study protocol

Please (for detailed methodology) refer Annexure II [88, 146].

2.4.4.4. Induction of Diabetes

Please (for detailed methodology) refer Annexure II.

2.4.4.4.1. OGTT

Please (for detailed methodology) refer Annexure II [146].

2.4.4.4.2. Bodyweight

All animals were monitored for body weight during the treatment period.

2.4.4.4.3. Biochemical Analysis

Please (for detailed methodology) refer Annexure II.

2.4.4.4.4. Histopathology:

Please (for detailed methodology) refer Annexure II.

2.4.4.4.5. HPLC-PK method

Please (for detailed methodology) refer Annexure II.

2.5 Enzyme Inhibition Assay

2.5.1 Assay of alpha-amylase inhibition

Please (for detailed methodology) refer Annexure II [147, 148].

2.5.2 DPP-IV inhibition assay

Please (for detailed methodology) refer Annexure II [149].

2.6. Adipocyte differentiation assay

In brief, 3T3-L1 preadipocyte was seeded as 5 X 10^3 cells/well in 24 well plates. After reaching the confluence (48 h), the preadipocytes were induced by IBMX + insulin (10 mg/ml) medium to differentiate to adipocytes for another 48 h. The TAHA, AA, AG and Cerulinin (a known anti-differentiating agent) were added at different concentration. Please (for detailed methodology) refer Annexure II [143].

3. STATISTICAL ANALYSIS

Please (for detailed statistical analysis) refer Annexure II [29].

4. RESULTS

The retention time for arjungenin and arjunetin were found to be 7.68 and 4.95 minutes, respectively. The spectral overlays were matched with the UV spectra of the reference standard at 220 nm. Quantitative estimation of these phytochemical compounds was carried out using an external standard calibration curve method. The calibration curve of concentration vs peak area was constructed in the range of 31.25-1000 µg/ml.

4.1 Validation data of arjunetin and arjungenin HPLC-PDA method

4.1 Specificity

The specificity of the developed HPLC analysis method was checked by changing the solvent B (from methanol to ACN). Retention time and lower limits of quantification were determined and were shown in Table 4.1, together with regression equations and coefficients of regression (R^2). The value of R^2 affirmed the linearity of the HPLC method. The robustness of the HPLC method was evaluated by changing the mobile phase (solvent B from 80:20 to 90:10); minor variation in mobile phase illustrated no effect on the resolution of the peak. The linearity of arjunetin and arjungenin solutions were obtained as 31.25-1000.0 µg mL^{-1}, as shown in Table 4.1. The calibration curve was constructed using linear regression of the theoretical concentration of an analyte vs the corrected peak area. The mean regression equations and R2 were evaluated to be $Y = 0.046 X + 0.628$ and $R^2 = 0.992$ for arjungenin and $Y = 0.069 X + 0.660$ and $R^2 = 0.991$ for arjunetin,.

As shown in Figure 4.1. Phytochemical evaluation of arjunetin and arjungenin HPLC chromatograms at 220 nm

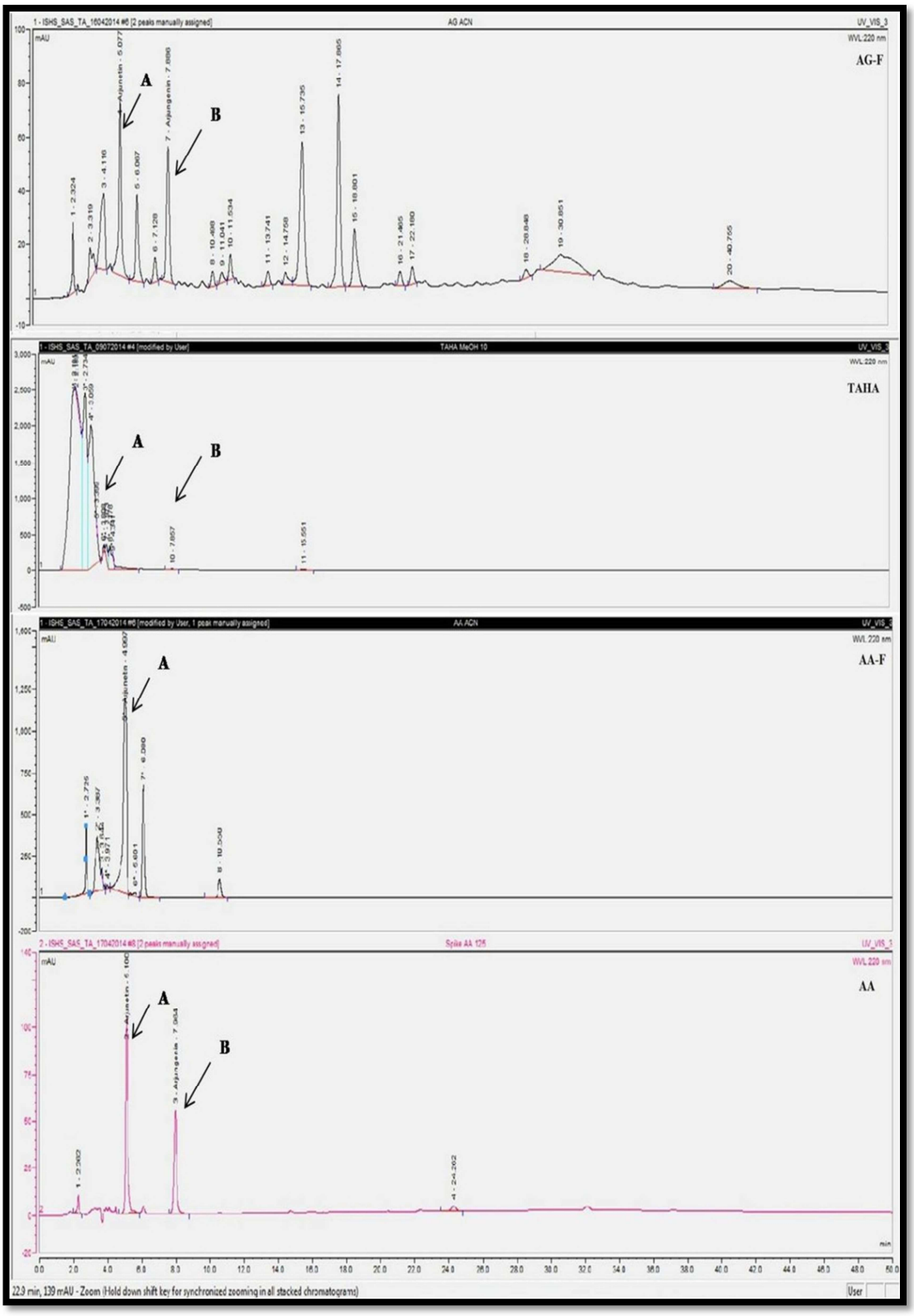

Figure 4.1: Phytochemical profiling HPLC chromatograms of arjunetin and arjungenin

HPLC chromatograms at 220 nm. (I) Arjuna ghritah formulation (AG-F); (II) extract of T. arjuna (TAHA); (III) Arjunarishta formulation (AA-F); (IV) chromatogram of Spiked Arjunetin (A) and Arjungenin (B) markers.

Table 4.1. Linearity, linear regression equation, and LLOQ of arjunetin and arjungenin HPLC method

Phytochemical Marker	RetentionTime	R^2	Conc. Range µg/ml	LLOQ µg/ml	Linear Regression Equation
Arjunetin	4.945 ± 0.002	0.991	31.25-1000	31.25	Y=0.069X + 0.660
Arjungenin	7.685 ± 0.003	0.992	31.25-1000	31.25	Y=0.046X + 0.628

Results were expressed as mean ± SD.

4.1.2 Precision and accuracy

The repeatability of the sample was evaluated as an intra-day variation, whereas the intermediate precision was determined by measuring the inter-day variation for the evaluation of arjunetin and arjungenin at different concentrations of 1000-31.25 µg/ml. The standard solutions, at four different concentrations, were determined at least six times within the same day, and % CV values obtained were between 5.19 - 16.86 % and 5.57 - 10.90% for arjunetin and arjungenin, respectively. Similarly, to determine the inter-day variability, the same concentration of the two standards was analyzed at least six following days, and the values were between 6.86 - 9.29% and 5.74 - 10.47 % for arjunetin and arjungenin. The accuracy (% bias) values ranged within -8.98 to 6.94 and -9.79 to 8.08 for arjunetin and arjungenin, respectively. The results of the intermediate and repeatability precision were expressed in CV (%) and shown in Table 4.2; the low values of the CV (%) indicated the repeatability of the proposed method.

Table 4.2. Intra and inter-day precision and accuracy of arjunetin and arjungenin HPLC method

C_{nom} (µg/ml)	Precision Intra-day (n=6)		Accuracy	Precision Inter-day (n=6)		Accuracy
	C_{obs} (µg/ml)	Precision (% CV)	% bias	C_{obs} (µg/ml)	Precision (% CV)	% bias
Arjunetin						
1000	1047 ± 54.28	5.19	-4.7	947.0 ± 65.02	6.86	5.3
250	245.5 ± 21.73	8.85	1.8	246.9 ± 22.93	9.29	1.24
62.5	68.11 ± 4.9	7.19	-8.98	60.70 ± 4.853	8.00	2.88
31.25	29.08 ± 4.91	16.86	6.94	29.70 ± 2.586	8.71	4.96
Arjungenin						
1000	1084 ± 60.39	5.57	-8.4	1037.0 ± 59.48	5.74	-3.7
250	229.8 ± 19.82	8.62	8.08	256.7 ± 16.36	7.15	-2.68
62.5	68.62 ± 6.25	9.11	-9.79	62.58 ± 5.68	9.07	-0.128
31.25	32.14 ± 3.502	10.90	-2.85	33.88 ± 3.55	10.47	-8.42

Results were expressed as mean ± SD.

4.2. Phytochemical characterizations of TAHA, AA and AG

Phytochemical characterization of AA, AG, and TAHA was determined using the selective phytochemical approach. Arjungenin and arjunetin were used as phytochemical markers for characterization purpose. The marker contents were estimated using earlier reported HPLC-PDA method. The HPLC-PDA analysis showed proper resolution of all the peaks. The presence of marker contents in the TAHA, AA, and AG was identified using UV spectra matching and retention time with corresponding reference standards (Figure 4.1). The content of arjunetin and arjungenin was 0.59 ± 0.04 and 0.85 ± 0.01 mg/g in TAHA; 254.6 ± 23.39 and 0.00 µg/ml in AA; 166.6 ± 2.69 and 228.7 ± 9.15 µg/ml in AG (Table 4.3).

Table 4.3. Quantitation of arjunetin and arjungenin in TAHA, AA and AG

Compound Name	Rt (min)				(µg/ml of AG)	(µg/ml of AA)	(mg/g of TAHA)
	Reference Standard	AG	AA	TAHA			
Arjunetin	4.95 ± 0.0	5.06 ± 0.01	5.10 ± 0.02	5.09 ± 0.02	166.6 ± 2.69	254.6 ± 23.39	0.59 ± 0.04
Arjungenin	7.69 ± 0.0	7.87 ± 0.02	ND	7.88 ± 0.04	228.7 ± 9.15	ND	0.85 ± 0.01

Results were expressed as mean ± SD.

Table 4.4. Effect of TAHA, AA and AG on body weight of HFD fed rats

	0 Month	**1 Month**	**2 Month**	**3 Month**
Normal	235.33 ± 22.33	259.67 ± 16.50**	310.17 ± 21.88**	347.00 ± 20.36***
HFD Control	284.83 ± 36.72	339.67 ± 42.50	394.50 ± 38.11	443.17 ± 33.70
Metformin	296.17 ± 32.50	331.17 ± 32.98	332.20 ± 13.97*	366.20 ± 25.85**
TAHA 250	298.17 ± 42.48	354.50 ± 57.82	364.67 ± 45.72	391.67 ± 49.74
TAHA 500	296.67 ± 57.73	337.50 ± 57.86	360.00 ± 54.01	395.83 ± 48.16
AA 0.9	275.2 ± 24.1	311.0 ± 53.6	346.8 ± 59.7	387.8 ± 55.8
AA 1.8	281.0 ± 37.3	326.0 ± 74.9	353.6 ± 59.6	387.8 ± 55.8
Ghee	282.5 ± 34.4	305.6 ± 68.3	348.1 ± 48.9	392.0 ± 49.8
AG 450	283.2 ± 15.3	303.8 ± 24.7	315.0 ± 29.3**	340.0 ± 44.8***
AG 900	276.2 ± 29.2	292.0 ± 33.1	318.6 ± 30.1*	342.0 ± 48.3***

Results are expressed as mean ± SD (n=6, Two-way ANOVA Bonferroni post-tests to compare replicate means by row). Values are statistically significant at *p<0.05 Vs. The high-fat diet fed rats (control groups).

4.3 Effect of TAHA, AA and AG intervention on body weights (BW)

At the end of the study, the BW dissented in the order of HFD control, TAHA 500, Ghee, TAHA 250, AA 0.9, AA 1.8, MET, AG 900, AG 450 and normal group animals (Table 4.4). The average BW of rats in the HFD control group (443 ± 34 g)

was significantly higher than the normal group (347 ± 20 g; Table 4.4). Among these treatments, only AG 450, AG 900, MET group represented a significant BW reduction as compared to HFD control group (340 ± 44; 342 ± 48; 366 ± 26 vs 443 ± 34 g). TAHA 250, TAHA500, AA 0.9, AA 1.8, group rats (387 ± 56; 387 ± 55; 392 ± 50 and 396 ± 48 vs 443 ± 34 g) showed a tendency, although not at a significant level, toward reduction of BW (Table 4.4).

4.4 Effect of TAHA, AA and AG intervention on OGTT

Fasting glucose, before an OGTT, represented no significant differences in HFD control or the MET; TAHA 500, TAHA 250, AA 0.9, AA 1.8, AG 900, AG 450 treated rats, compared to vehicle treatment (Figure 4.2). However, level of glucose was higher for the HFD control group (102.7 ± 23.7 vs. 90.8 ± 3.0 mg/dl) and the MET, TAHA 250, TAHA 500 AA 0.9, AA 1.8, Ghee, AG 450, AG 900 treated rats (87.6 ± 10.4; 96.7 ± 18.3; 89.8 ± 12.1; 93.6 ± 3.2; 96.0 ± 7.6; 100.2 ± 4.5; 88.6 ± 4.1; 100.2 ± 3.7 vs. 102.7 ± 23.7 mg/dl) at the 60 minutes, as shown in Figure 4.2. These glucose levels remained significantly elevated until the 120 minutes only for the HFD control group. MET, TAHA 500, AA 0.9, AA 1.8, and AG 450 treated rats showed lower glucose values at the 120 minutes compared to HFD control group.

Table 4.5. Effect of TAHA, AA and AG intervention on Oral Glucose Tolerance Test (OGTT)

	Normal	Control	Met	TAHA 250	TAHA 500	AA 0.9	AA 1.8	Ghee	AG 450	AG 900
0 min	61.0 ± 4.5	60.3 ± 5.8	60.4 ± 7.2	66.3 ± 7.9	60.8 ± 9.8	59.8 ± 4.8	63.4 ± 12.5	64.0 ± 2.6	61.4 ± 5.1	63.6 ± 3.8
60 min	90.8 ± 3.0	102.7 ± 23.7	87.6 ± 10.4	96.7 ± 18.3	89.8 ± 12.1	93.6 ± 3.2	96.0 ± 7.6	100.2 ± 4.5	88.6 ± 4.1	100.2 ± 3.7
120 min	87.7 ± 9.0	131.7 ± 25.9	109.6 ± 6.8	119.2 ± 22.6	101.0 ± 11.4	110.8 ± 4.2	114.6 ± 8.9	124.5 ± 3.8	102.2 ± 4.9	122.2 ± 9.5
180 min	75.5 ± 7.3	97.5 ± 19.4	82.4 ± 13.2	88.3 ± 16.0	80.7 ± 6.4	72.4 ± 2.3	79.4 ± 7.6	83.3 ± 6.1	69.2 ± 3.2	72.8 ± 5.8
240 min	61.3 ± 5.1	82.8 ± 14.4	68.8 ± 9.2	75.0 ± 12.2	66.0 ± 5.1	67.8 ± 1.9	68.2 ± 7.6	74.3 ± 3.2	64.4 ± 2.3	62.2 ± 3.4
OGTT-AUC	18235 ± 1224**	24055 ± 4387	20652 ± 1893	22540 ± 3845	19945 ± 1157	20436 ± 1355	21348 ± 1674	22630 ± 1027	19374 ± 3221	21486 ± 2672

The AUC histogram results were showed the significant change in the glucose for HFD control group rat (31% increase vs normal group, $p < 0.05$) and MET, TAHA 250, TAHA 500 AA 0.9, AA 1.8, and AG 450 treated rats showed reduction, but results were non-significant compared to HFD control group rats (Figure 4.2), demonstrating an impaired glucose excursion during an OGTT (Table 4.5).

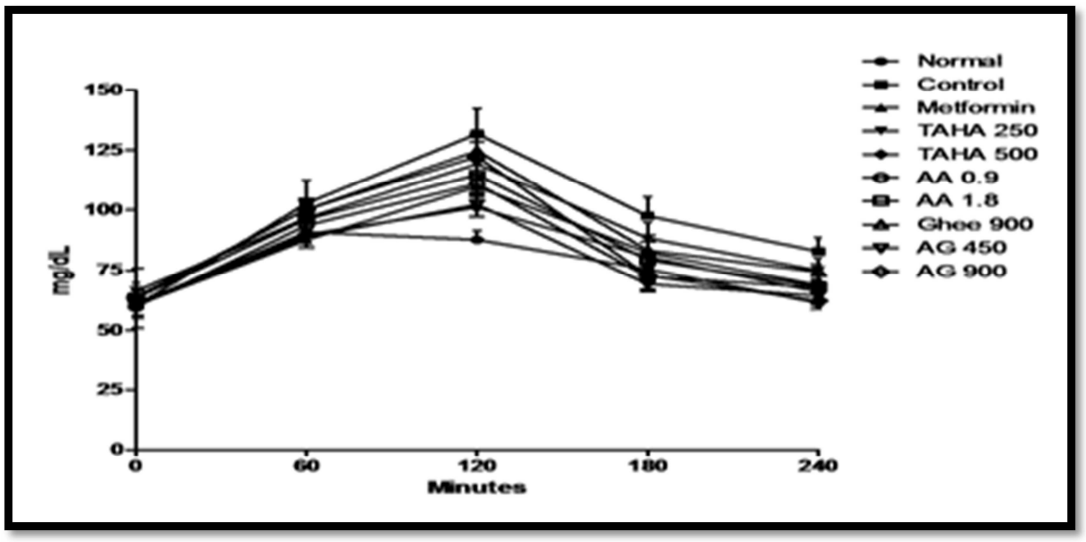

Figure 4.2: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) intervention on Oral Glucose Tolerance Test (OGTT)

Results were expressed as mean ± SD. *p<0.05, ** p<0.01, *** p<0.001 when compared with control group.

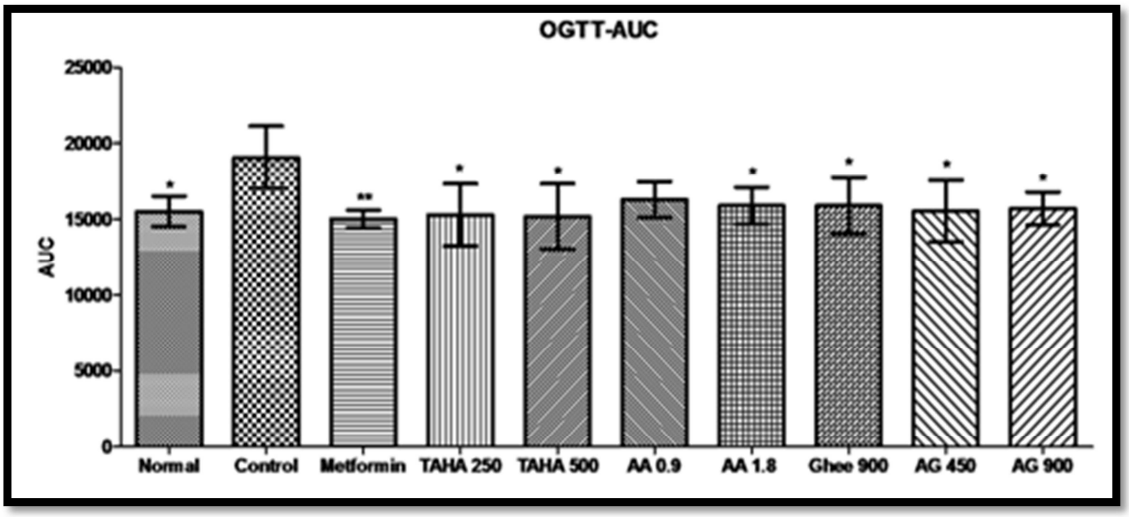

Figure 4.3: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on intervention on Oral Glucose Tolerance Test (OGTT)-Area under curve (AUC).

Results were expressed as mean ± SD. *p<0.05, **p<0.01, ***p<0.001 when compared with control group.

As shown in Figure 4.3, the results were also represented as the OGTT-AUC histogram showed the significant decrease in the normal, MET; TAHA 500, TAHA 250, AA 1.8, AG 900, AG 450 treated rats compared to control group rats.

4.5. Lipid Profile (HFD)

Table 4.6: Effect of TAHA AA and AG on plasma TG, TC, and HDL-C in HFD fed rats

	TG	HDL	TC
Normal	88.00 ± 3.74***	33.50 ± 1.29**	142.40 ± 18.55***
HFD Control	118.00 ± 12.36	23.45 ± 2.55	193.20 ± 21.91
Metformin	72.00 ± 7.07***	31.85 ± 1.58*	149.15 ± 6.26**
TAHA 250	51.75 ± 5.56***	29.53 ± 4.56	88.60 ± 10.26***
TAHA 500	53.00 ± 9.93***	38.40 ± 5.83***	79.17 ± 21.93***
AA 0.9	76.2 ± 7.29***	36.52 ± 2.56***	182.0 ± 10.94
AA 1.8	72.0 ± 5.29***	32.15 ± 1.11*	165.76 ± 11.06
Ghee	88.5 ± 9.29***	28.18 ± 2.42	88.17 ± 29.59***
AG 450	83.75 ± 19.76***	27.0 ±1.93	85.2 ± 40.97***
AG 900	88.75 ± 9.88 ***	33.5 ± 1.27*	109.0 ± 51.98***

Results are expressed as mean ± SD (n=6, one-way ANOVA). Results were statistically significant at *p<0.05 vs. the high-fat diet fed rats (HFD control groups).

As shown in Table 4.6, HFD fed rats in the control group had a significant higher in plasma TG (118.0 ± 12.4 vs. 88.0 ± 3.7 mg/dl, $p < 0.001$) and TC (193.2 ± 21.9 vs. 142.5 ± 18.5 mg/dl, $p < 0.001$) compared to the normal rats. MET, TAHA250,

TAHA500, AA 0.9, AA 1.8, Ghee, AG 450 and AA 900 treated rats showed significant reduction in the plasma TG levels as compared to the HFD control group (72.0 ± 7.0; 51.7 ± 5.5; 53.0 ± 9.9; 76.2 ± 7.29; 72.0 ± 5.29; 88.5 ± 9.29; 83.75 ± 19.76; 88.75 ± 9.88 vs. 118.0 ± 12.3 mg/dl, $p < 0.001$) by 39.0, 56.1, 55.1, 35.4, 39.0, 25.0, 29.0, 24.8 % respectively. MET, TAHA250, TAHA500, Ghee, AG 450 and AA 900 treated rats showed significant reduction in the plasma TC levels as compared to the HFD control group (149.1 ± 6.2; 88.6 ± 10.2; 79.2 ± 21.9; 88.17 ± 29.59; 85.2 ± 40.97; 109.0 ± 51.98 vs. 193.2 ± 21.9 mg/dl, $p < 0.01$) by 22.8; 54.1, 59.6, 54.3, 55.9, 43.6 % respectively. In addition, HFD fed rats in the control group had a significant lower the plasma HDL (33.5 ± 1.2 vs. 23.5 ± 2.5 mg/dL, $p < 0.01$) by 30 %. The MET, TAHA500, AA 0.9, AA 1.8 and AG 900 intervention significantly enhanced the level of HDL compared to the HFD control (31.8 ± 1.5; 38.4 ± 5.8; 36.52 ± 2.56; 32.15 ± 1.11; 33.5 ± 1.27 vs. 23.5 ± 2.5, $p < 0.05$ mg/dl, $p < 0.05$) by 35.8, 63.8, 55.4, 36.8, 42.6 % respectively.

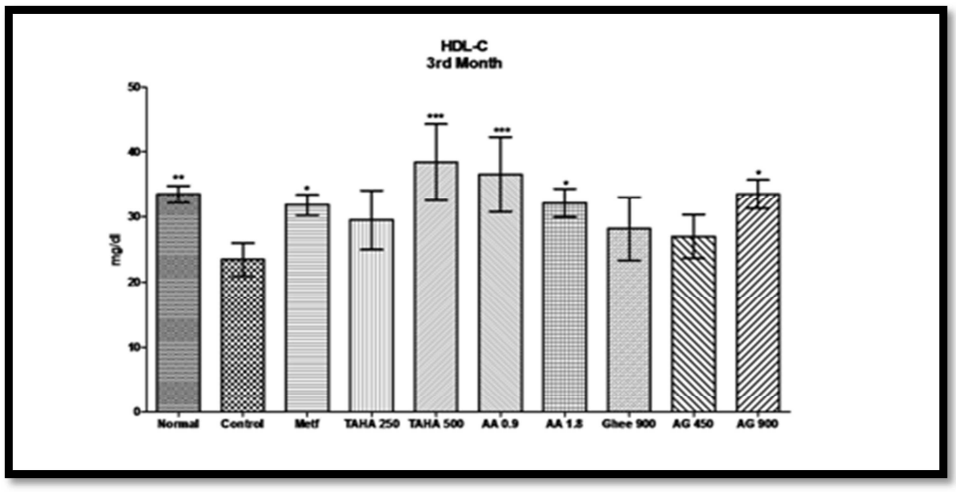

Figure 4.4: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on High density lipoprotein (HDL) cholesterol levels. Results were expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

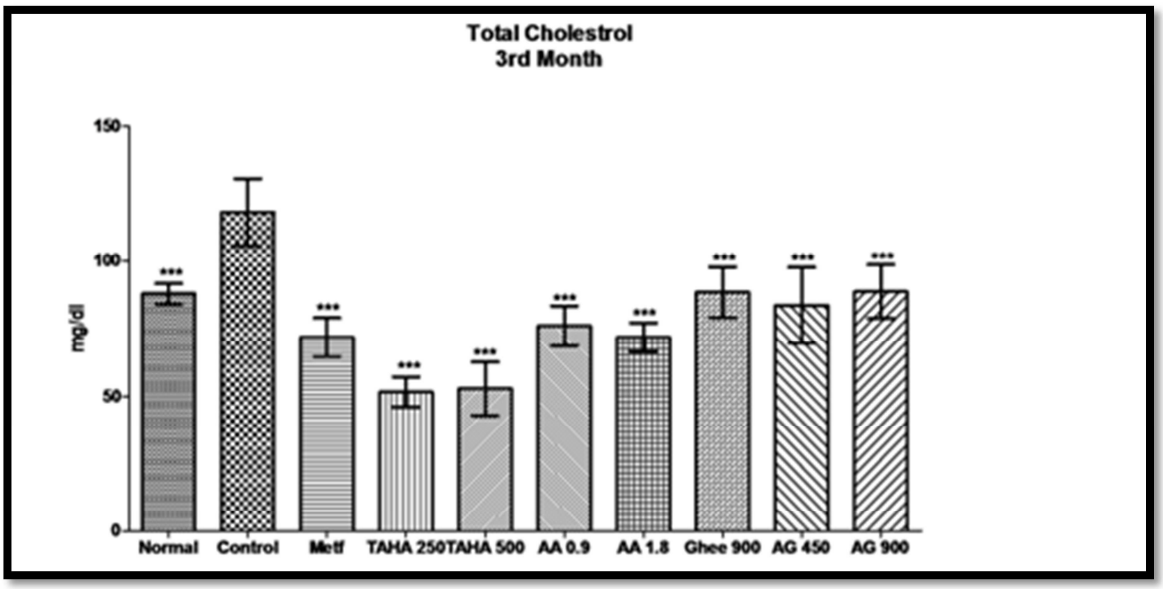

Figure 4.5: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on total cholesterol levels. Results were expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

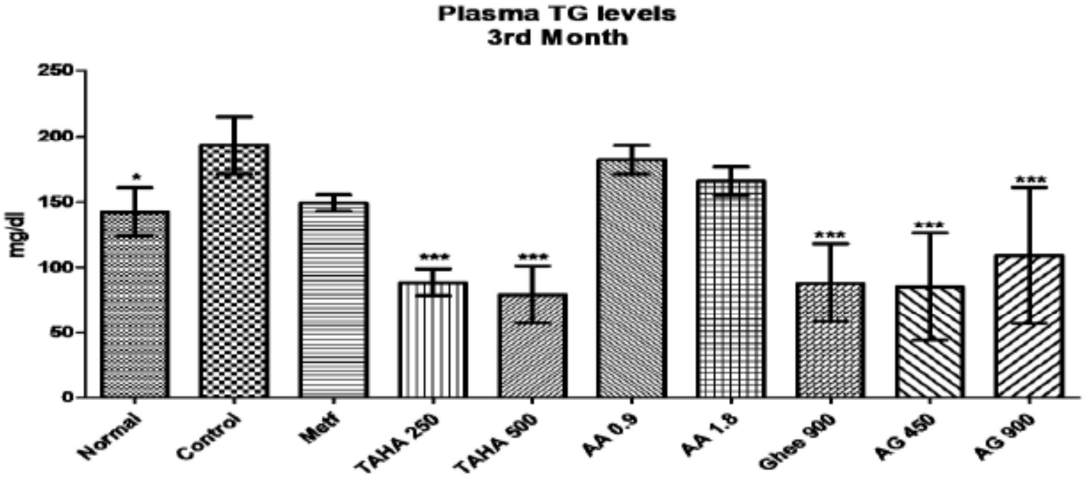

Figure 4.6: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on TG levels. Results were expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

4.6 Effect of TAHA, AA, AG on gene expression involved in metabolic syndrome

4.6.1 Obesity-related genes

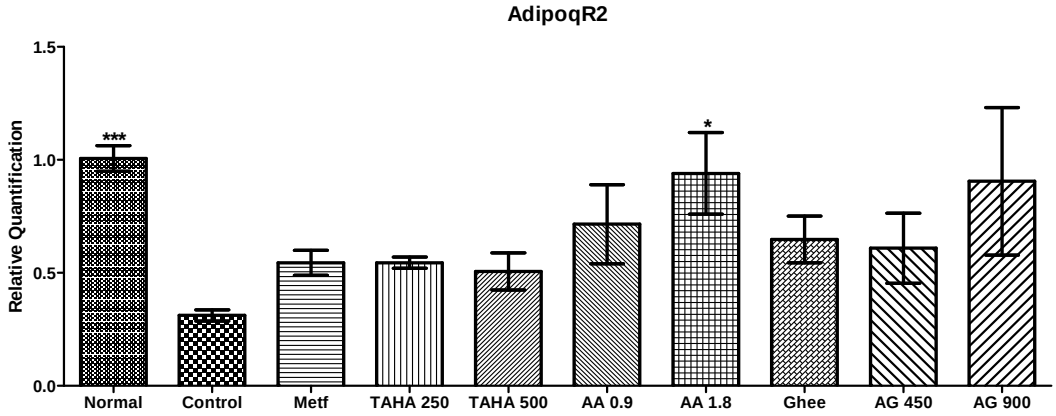

Figure 4.7: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on AdipoqR2 gene expression. Results were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

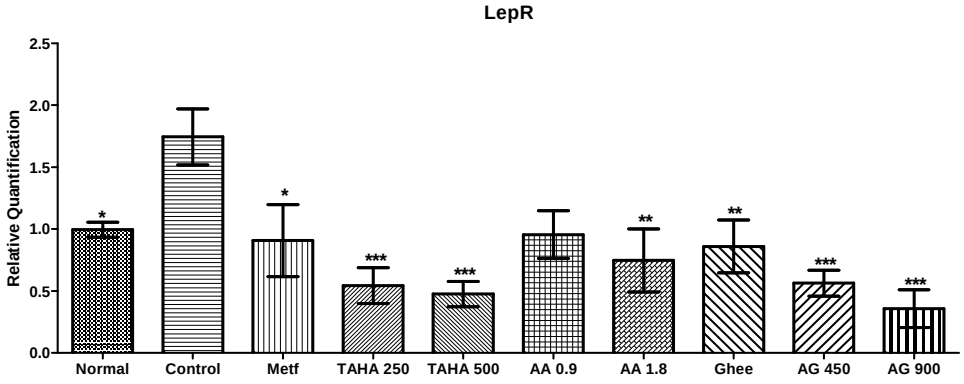

Figure 4.8: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on LepR gene expression. Values were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group

The expression of AdipoqR-2 was significantly downregulated in HFD fed rats and increased in AA1.8 treated rats than the HFD control group by 2.0 fold, respectively

[Figure 4.7]. The expression of LepR was significantly upregulated in HFD fed rats and decreased in MET, TAHA250, TAHA500, AA1.8, AG450, and AG900 treated rats than the HFD control group [Figure 4.8].

4.6.2 Inflammation-related genes

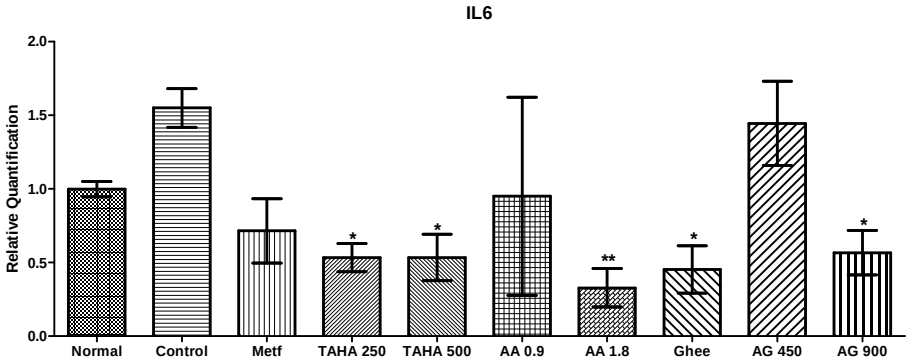

Figure 4.9: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on IL-6 gene expression. Results were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

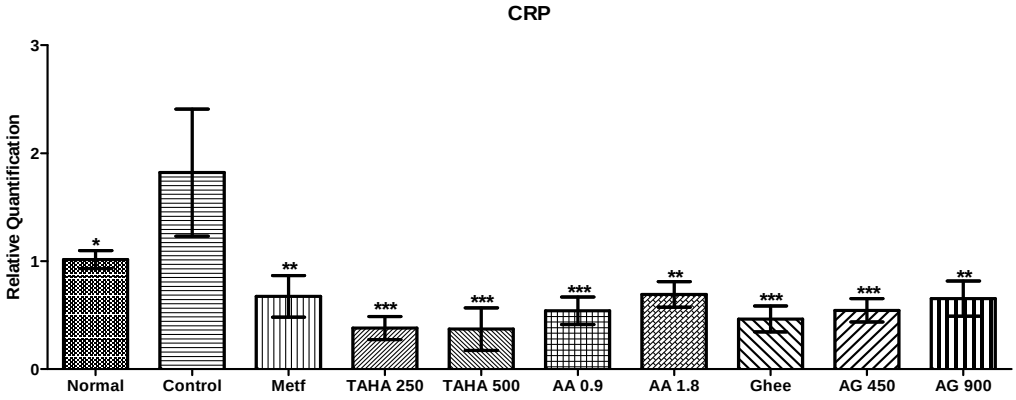

Figure 4.10: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on CRP gene expression. Results were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

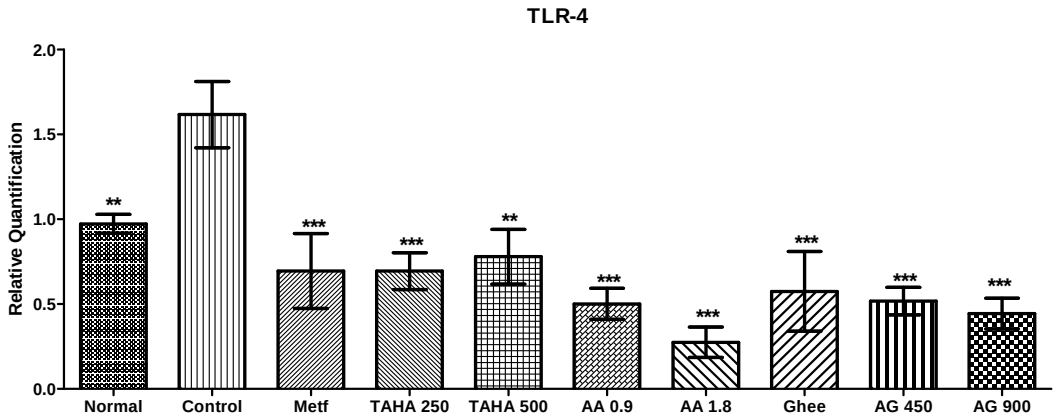

Figure 4.11: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on TLR-4 gene expression. Results were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

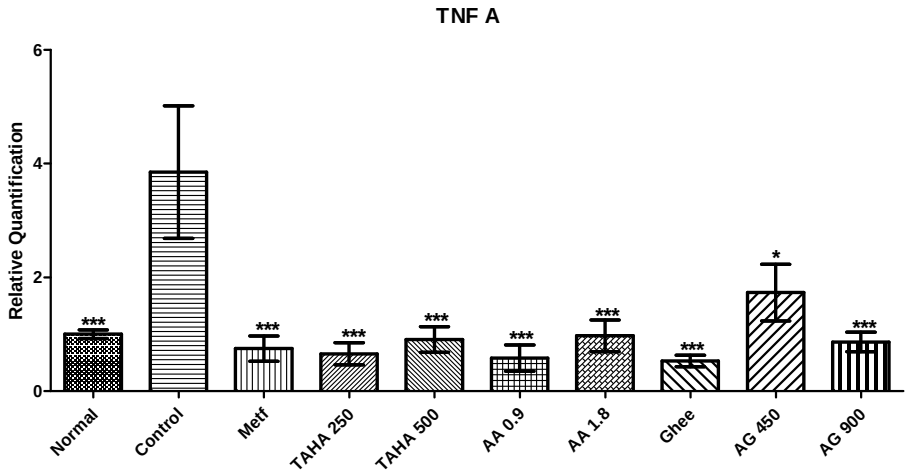

Figure 4.12: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on TNF-α gene expression. Results were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

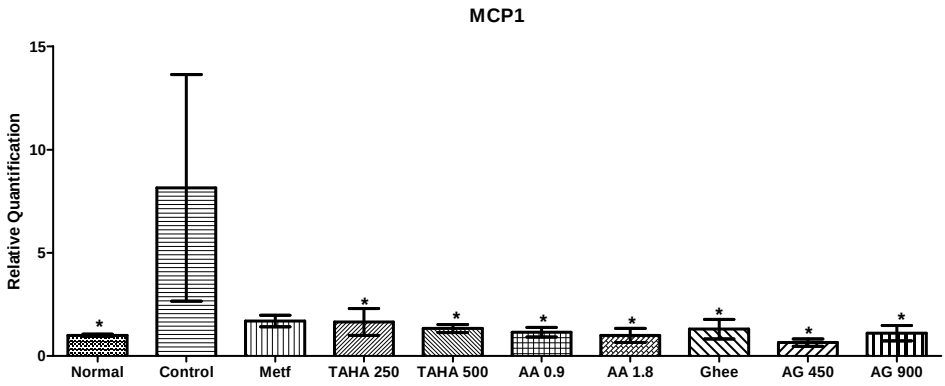

Figure 4.13: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on MCP1 gene expression. Results were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

As shown in (Figure 4.9 and Figure 4.10) CRP and IL-6 levels in liver tissue was significantly increased ($p<0.001$) in the HFD control rats as compared to the normal rats while these levels were significantly reduced in diabetic rats treated with MET, TAHA250, TAHA500, AA0.9, AA1.8, AG450 and AG900 treatment.

The expression of TLR-4, which converts immature adipocytes to mature adipocytes, was also higher in HFD fed rats and less in MET, TAHA250, TAHA500, AA0.9, AA1.8, AG450, and AG900 treated rats than the HFD control group (Figure 4.11). The expression of TNF-α was significantly upregulated in HFD fed rats and decreased in MET, TAHA250, TAHA500, AA0.9, AA1.8, AG450 and AG900 treated rats than the HFD control group by 7.7, 3.0, 3.1, 3.5, 1.8 and 2.4 fold respectively (Figure 4.12). The expression of MCP-1 was significantly upregulated in HFD fed rats and decreased in MET, TAHA250, TAHA500, AA0.9, AA1.8, AG450 and AG900 treated rats than the HFD control group by 4.0, 2.7, 2.9, 4.1, 3.0, 4.9, and 2.6 fold respectively (Figure 4.13).

4.6.3 Insulin resistance-related gene

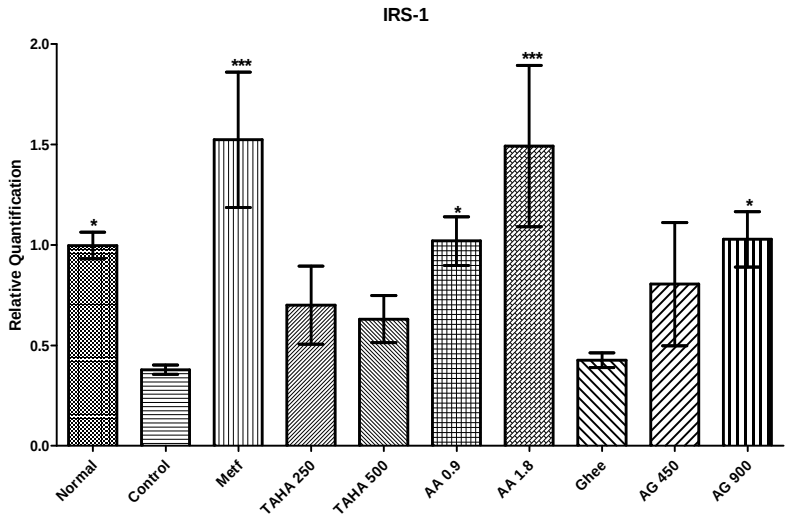

Figure 4.14: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on IRS-1 gene expression. Results were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

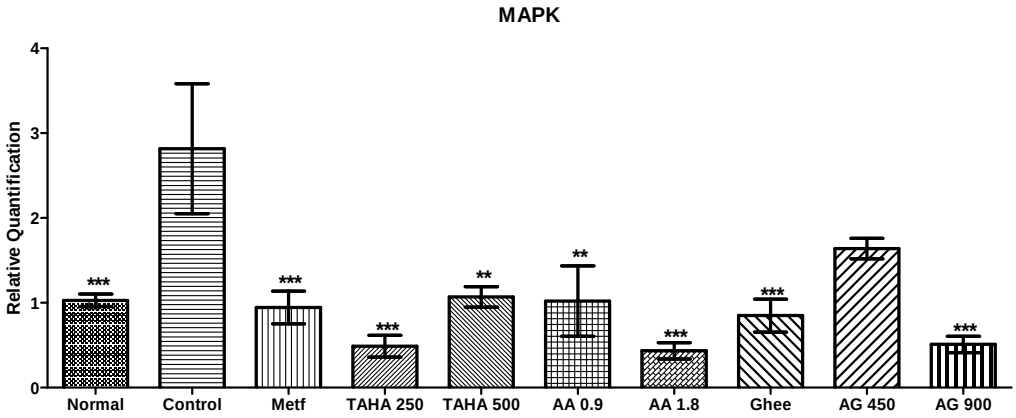

Figure 4.15: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on MAPK gene expression. Results were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

The expression of IRS-1 was significantly downregulated in HFD fed rats and increased in MET, AA0.9, AA1.8, and AG900 treated rats than the HFD control group by 3.0, 1.7, 3.1, and 1.6 fold respectively (Figure 4.14). The expression of MAPK was significantly upregulated in HFD fed rats and decreased in MET, TAHA250, TAHA500, AA0.9, AA1.8, and AG900 treated rats than the HFD control group by 2.0, 4.6, 1.6, 1.8, 5.4, and 4.5 fold respectively (Figure 4.15).

4.6.4 Energy expenditure related genes

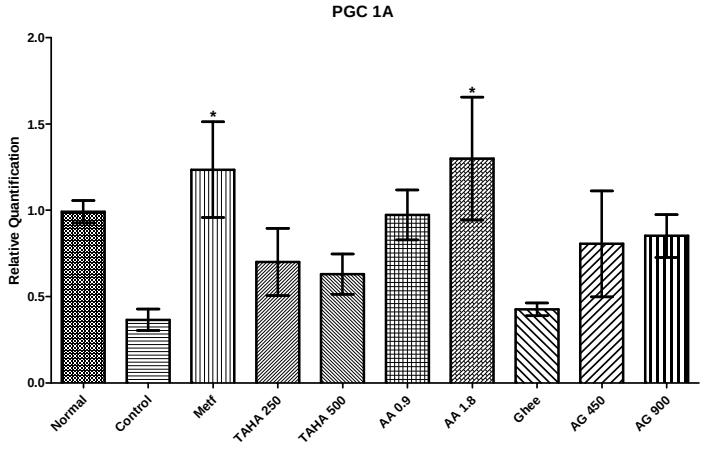

Figure 4.16: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on PGC-1α gene expression. Results were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

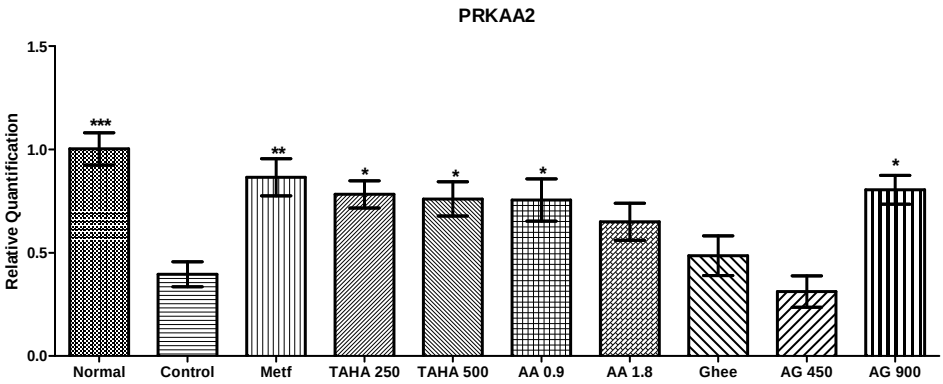

Figure 4.17: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on PRKAA2 gene expression. Results were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

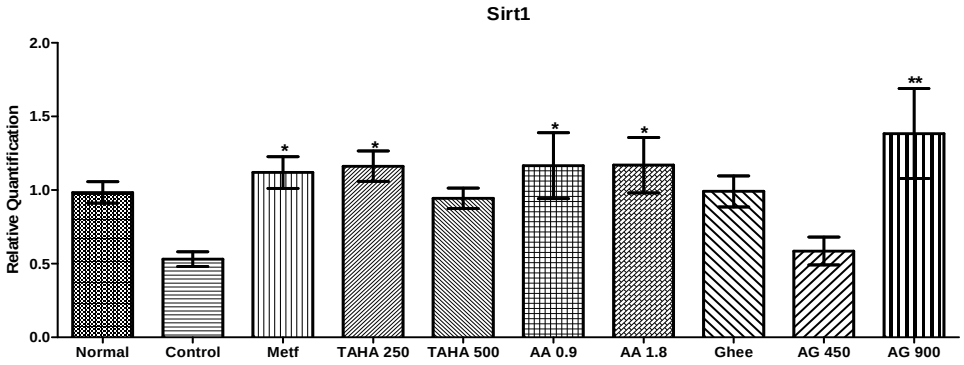

Figure 4.18: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on Sirt1 gene expression. Results were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

The expression of PGC-1α was significantly downregulated in HFD fed rats and increased in MET, and AA1.8 treated rats than the HFD control group by 2.4, and 2.5 fold, respectively (Figure 4.16). The expression of PRKAA-2 was significantly downregulated in HFD fed rats and increased in MET, TAHA250, TAHA500, AA0.9, and AG900 treated rats than the HFD control group (Figure 4.17). The expression of

Sirt-1 was significantly downregulated in HFD fed rats and increased in MET, TAHA250, AA0.9, AA1.8, and AG900 treated rats than the HFD control group (Figure 4.18).

4.6.5 Carbohydrate metabolism-related genes

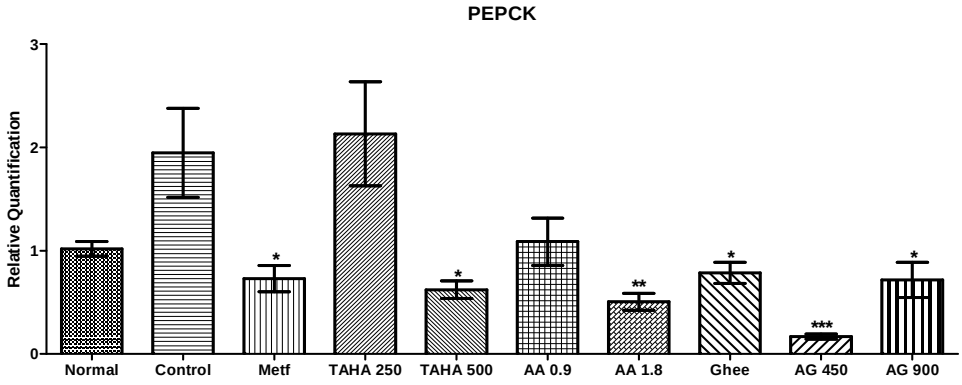

Figure 4.19: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on PEPCK gene expression. Results were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

To figure out the molecular mechanism involved in the reduction of blood glucose level following TAHA intervention, the expression of some genes involved in glucose metabolism in the liver was analyzed, and the results were presented in Figure 4.19. There was no significant difference in the level of the gene expression related to gluconeogenesis, i.e., PEPCK between HFD control and normal groups. In contrast, MET and TAHA500 down-regulated PEPCK that was upregulated in hyperlipidemic rats (Figure 4.19).

4.6.6 Lipid metabolism related genes:

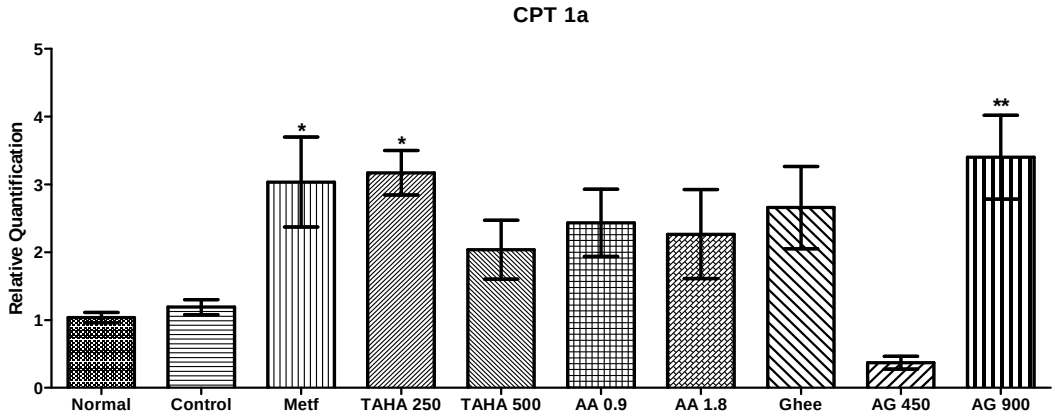

Figure 4.20: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on CPT-1a gene expression. Results were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

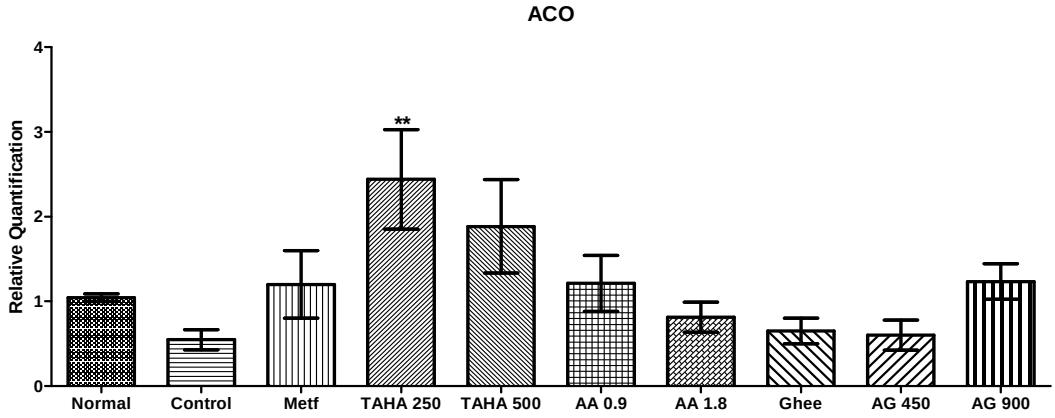

Figure 4.21: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on ACO gene expression. Results were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

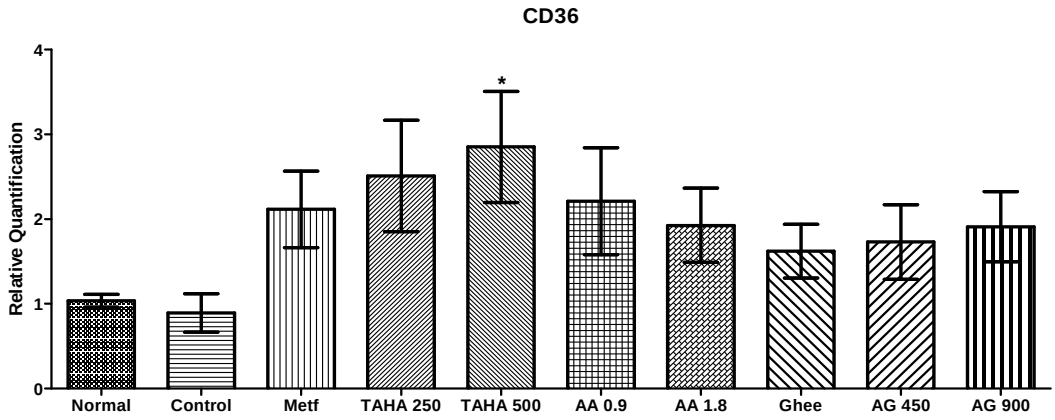

Figure 4.22: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on CD36 gene expression. Results were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

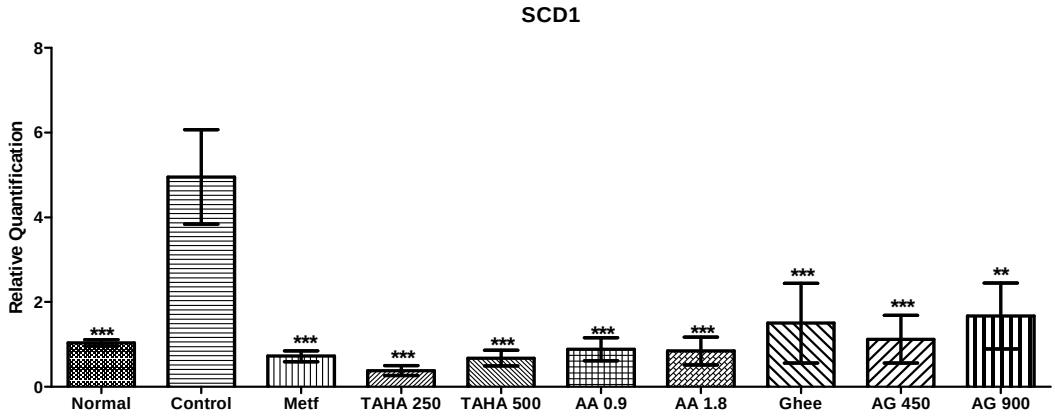

Figure 4.23: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on SCD1 gene expression. Results were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

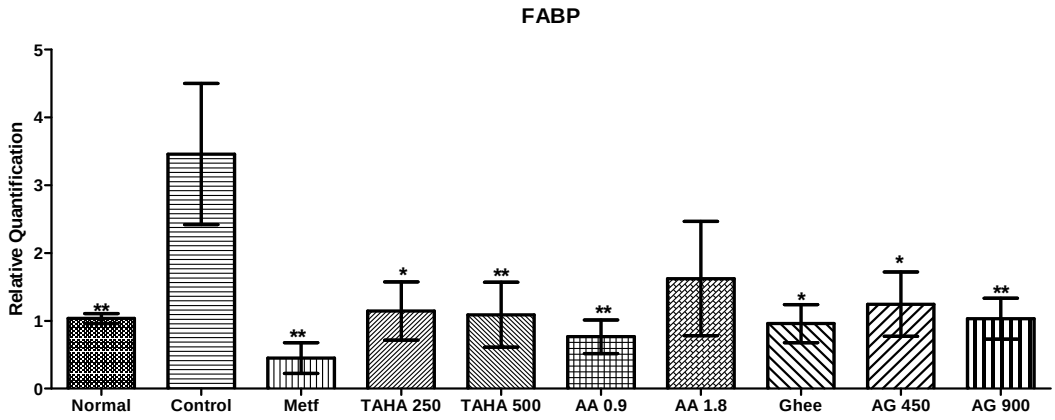

Figure 4.24: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on FABP gene expression. Results were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

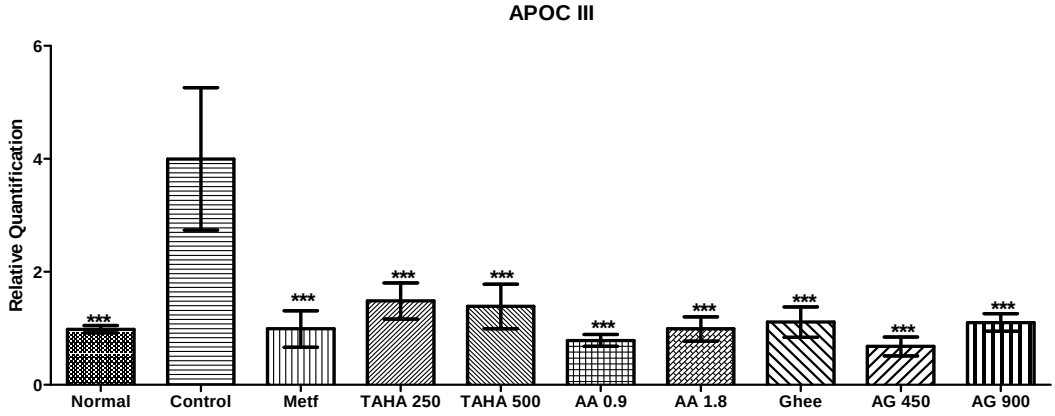

Figure 4.25: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) on APOC III gene expression. Results were expressed as mean ± SD. N = 8; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with control group.

TAHA250 and AG900 significantly increased mRNA expression of CPT-1 as compared to HFD control rats. When MET concomitant administered with HFD, it ameliorated this downregulation significantly. However, Aco1 (responsible for lipid β-oxidation) have the enhanced expression in hyperlipidemic rats following the TAHA250 administration. The suppression was more apparent in the HFD control rats than TAHA treated rats. TAHA treatment increased the mRNA levels of Aco1 (Figure 4.21), the rate-limiting enzyme in peroxisomal β-oxidation, in the liver. The levels were 4.22-fold higher in the TAHA250-fed rats, respectively.

The more significant FAT/CD36 (Figure 4.22) expression in HFD fed rats than the normal rat indicated that HFD could enhance liver lipid uptake. In the TAHA500-fed rats, the expressions of FAT/CD36 were upregulated by 3.19 fold, respectively. These results show that TAHA mediated its action through the expression of PPAR-α target genes in the liver.

The expression of SCD-1 changes SFA into monounsaturated FA, was also more prominent in HFD fed rats and less expressed in MET, TAHA250, and TAHA500 treated rats than the HFD control group (Figure 4.23). The expression of FABP was significantly upregulated in HFD fed rats and decreased in MET, TAHA250 and TAHA500 treated rats than the HFD control group by 7.7, 3.0 and 3.1 fold, respectively (Figure 4.24). The expression of APOC-III was significantly upregulated in HFD fed rats and decreased in MET, TAHA250 and TAHA500 treated rats than the HFD control group by 4.0, 2.7 and 2.9 fold respectively (Figure 4.25). These results indicate that TAHA treatment suppresses the HFD-induced hyperlipidemia and hepatic TG accumulation.

4.7. Effect of TAHA, AA, AG on pharmacokinetic and pharmacodynamic parameters of MET

4.7.1. HPLC Chromatogram

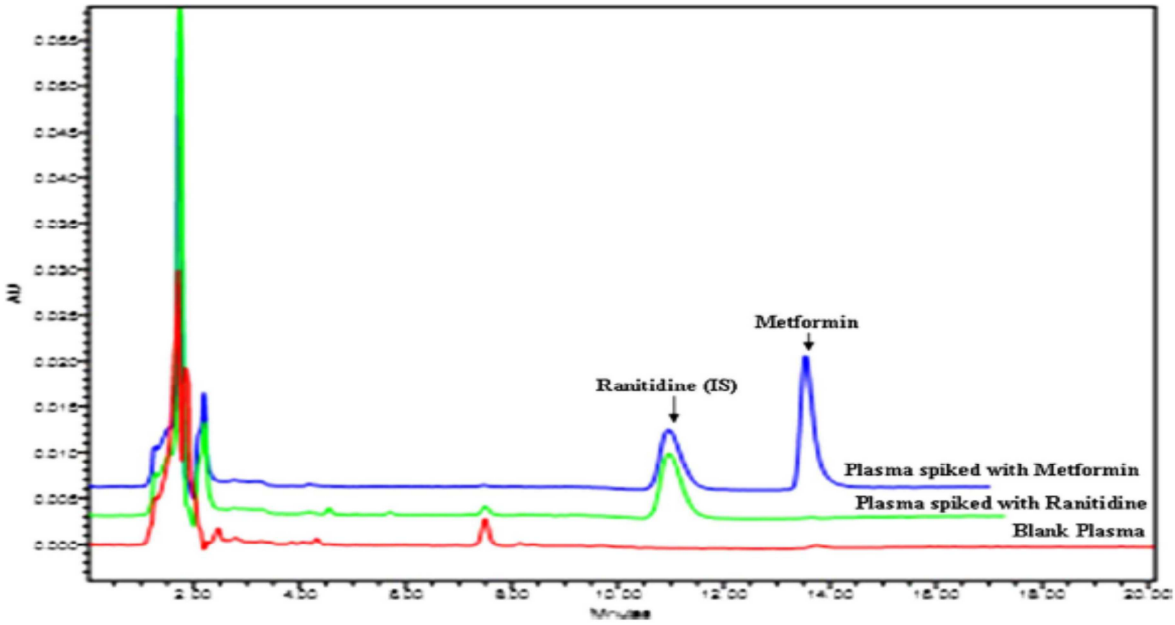

Figure 4.26: HPLC-DAD chromatograms of blank plasma, plasma spiked with ranitidine (Internal Standard - IS), plasma spiked with a mixture of ranitidine, and metformin (MET).

Quantitative estimation of MET in rat plasma samples was done using previously reported HPLC method. Optimized chromatographic conditions showed a good separation of IS and MET standards. The retention times for MET and IS were found to be 11.02 min and 13.65 min, respectively.

Plasma sample preparation is an important step, employed to remove interferences from biological samples using simple procedure having a suitable recovery. In the present study, liquid-liquid extraction with sodium hydroxide and 1-butanol/n-hexane (50:50, v/v) resulted in consistent good recoveries of analytes. Ranitidine was used as IS for MET because of similar chromatographic and extraction characteristics.

As seen in the above figure, no interfering peaks from endogenous compounds were observed at the retention times of analytes and IS in the matrix suggesting specificity of the method.

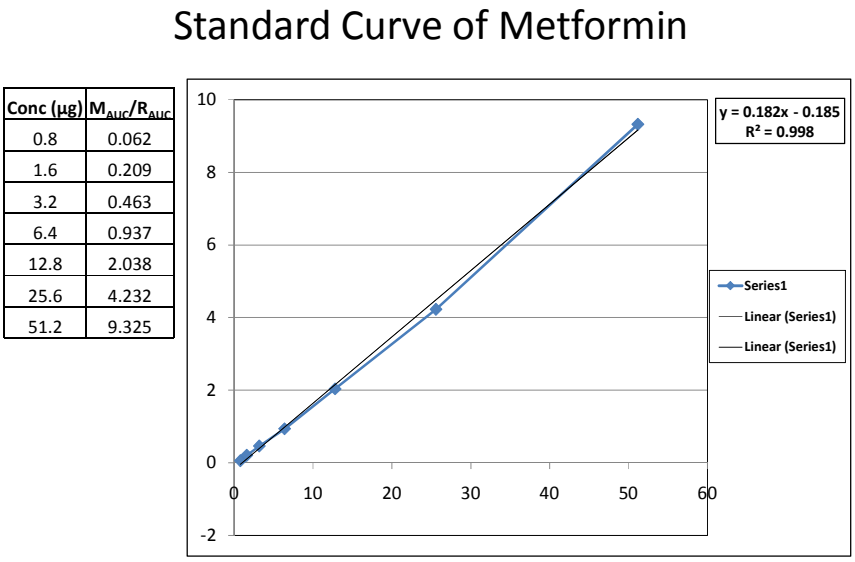

Figure 4.27: Standard Curve of Metformin

Table 4.7. HPLC validation data (Metformin)

Concentration added (ug/ml)	**Concentration measured (ug/ml)**	**Recovery[a] (%)**	**CV (%)**
0.8	1.388 ± 0.076	173.5 ± 9.46	5.46
6.4	6.382 ± 0.386	99.71 ± 6.04	6.06
51.2	52.46 ± 3.816	102.5 ± 3.04	7.27

Concentration of Metformin (0.8, 6.4, and 51.2 ug/ml)) added and showed CV 5.46, 6.06, and 7.27 respectively.

4.7.2. Effect of TAHA, AA and AG on the PK parameters of MET

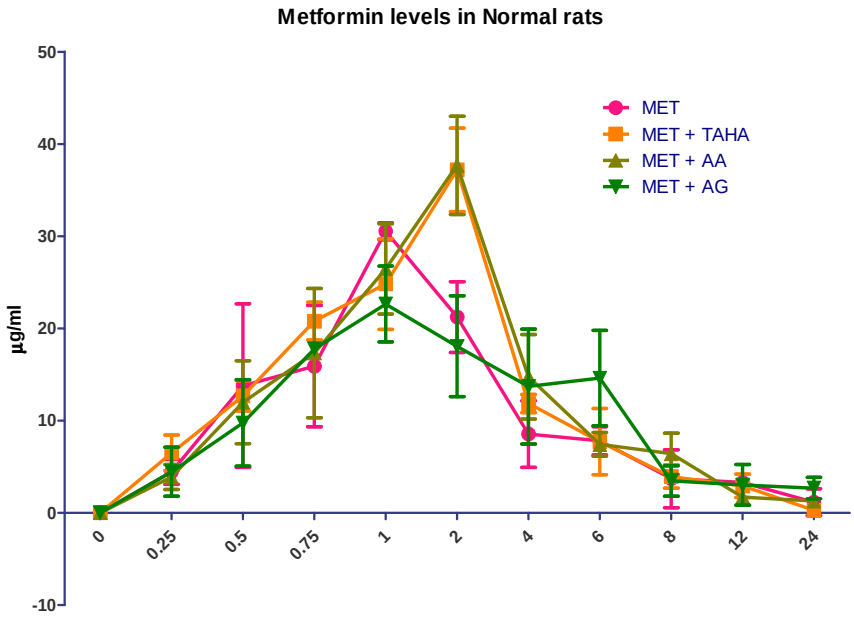

Figure 4.28: Effect of TAHA, AA and AG on Metformin levels in Normal Rats

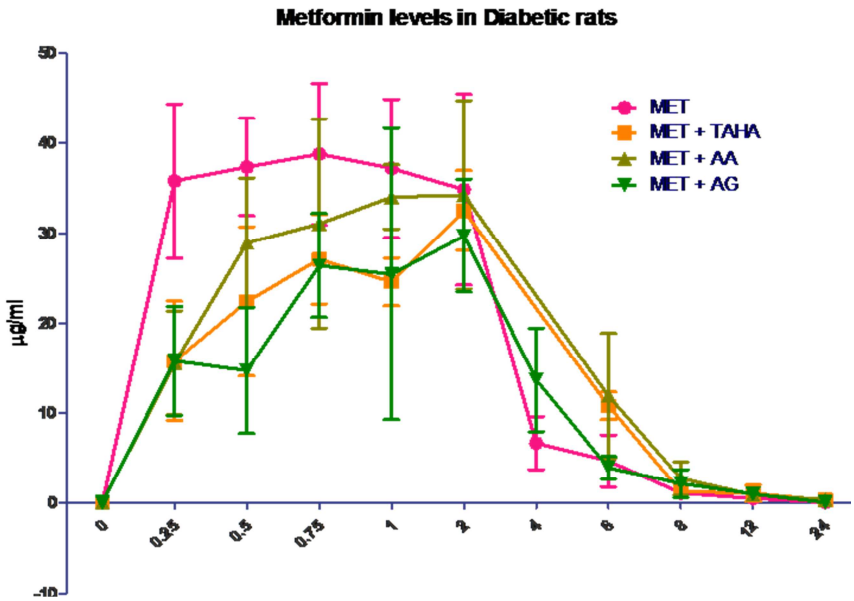

Figure 4.29: Effect of TAHA, AA and AG on Metformin levels in Diabetic Rats

Table 4.8. Effect of TAHA, AA and AG on the Pharmacokinetic (PK) parameters of Metformin (MET) in normal and diabetic rats

Normal	**MET**	**MET +TAHA**	**MET + AA**	**MET + AG**
C_{max} (µg/mL)	33.23 ± 5.57	37.2 ± 4.54	34.88 ± 7.07	34.69 ± 12.76
T_{max} (min)	80.00 ± 34.64	120.0 ± 0	105.0 ± 30	90.00 ± 34.64
AUC_t (µg.h mL)$^{-1}$	131.3 ± 18.39	128.5 ± 1.388	130.8 ± 6.72	122.8 ± 21.15
AUC_∞	119.3 ± 37.1	129.4 ± 1.9	124.4± 11.9	122.4 ± 21.4
CL	4.59 ± 1.73	3.86 ± 0.06	3.88 ± 0.47	4.18 ± 0.71
$T_{1/2}$	2.37 ± 0.89	2.54 ± 0.61	3.38 ± 0.68	2.97 ± 2.1
Vd	15.05 ± 5.7	14.17 ± 3.4	19.09 ± 5.8	19.15 ± 15.14
Kel	0.326 ± 0.11	0.286 ± 0.07	0.211 ± 0.04	0.346 ± 0.22
Diabetes	**MET**	**MET +TAHA**	**MET + AA**	**MET + AG**
C_{max} (µg/mL)	45.88 ± 2.00	37.7 ± 10.87	39.44 ± 3.54	34.88 ± 2.21
T_{max} (min)	35.00 ±22.91	86.25 ± 39.45	50.0 ± 8.66	55.00 ± 8.66
AUC_t (µg.h mL)$^{-1}$	193.0 ±21.24	140.6 ± 30.18	170.7 ±15.05	132.2 ± 16.21
AUC_∞	194.7 ± 20.2	140.7 ± 30.16*	176.9 ± 12.2	135.9 ± 17.7*
CL	2.59 ± 0.27	3.67 ± 0.7*	2.83 ± 0.19	3.72 ± 0.49*
$T_{1/2}$	1.65 ± 0.48	4.16 ± 2.44	1.55 ± 0.20	1.79 ± 1.15
Vd	6.13 ± 1.8	22.71 ± 14.4	11.94 ±10.63	8.99 ± 4.85
Kel	0.45 ± 0.13	0.22 ± 0.12	0.45 ± 0.06	0.50 ± 0.24

Results were expressed as mean ± SD.

AUC, area under the plasma MET concentration-time curve; AUC_t, up to the last concentration measured; C_{max}, maximum concentration; T_{max}, time to maximum concentration

4.7.2.1 Effect of TAHA, AA and AG on the PK of MET in normal animals

The MET concentration vs time curve was plotted for the PK parameters calculation (as shown in Figure. 4.28, Table 4.8). The PK parameters such as T_{max}, C_{max}, AUC_t, CL, and Vd were calculated (Table 4.8). In normal animals, the MET reached a C_{max} of 33.23 ± 5.57μg/mL at 80.00 ± 34.64 min, and the AUCt was 131.3 ± 18.39 μg h/mL. Co-administered TAHA (500 mg/kg) has a non-significant change in PK parameters like C_{max}, T_{max}, AUC_t, Vd and CL. Similarly, co-administered AA and AG has a non-significant change in PK parameters such as T_{max}, C_{max}, AUC_t, Vd and CL of MET.

4.7.2.2. Effect of TAHA, AA, and AG on the PK of MET in diabetic animals

In diabetic animals, the mean plasma concentration of MET reached a C_{max} of 45.88 ± 2.00 μg/mL at 35.00 ± 22.91 min, and the AUC_t was 193.0 ± 21.24μg h/mL. Co-administered TAHA at a dose of 500 mg/kg has a non-significant change in PK parameters like C_{max}, T_{max}, AUC_t, Vd and CL. Similarly, co-administered AA and AG have a non-significant change in PK parameters like C_{max}, T_{max}, AUC_t, Vd and CL of MET. (Fig. 4.29, Table 4.8)

4.7.3 Effect of TAHA, AA and AG on pharmacodynamic parameters of MET

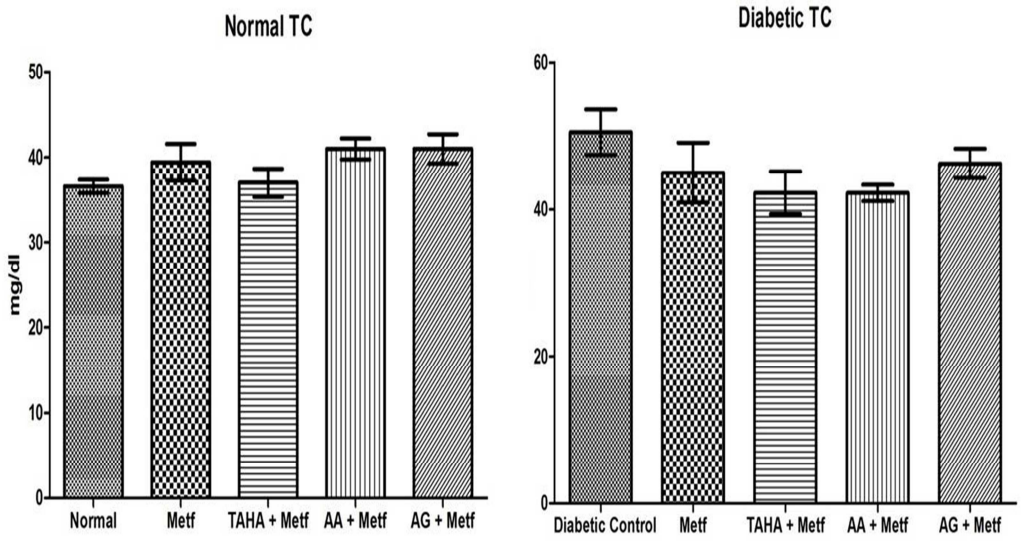

Figure 4.30: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) co-administered with MET on TC levels in Normal and Diabetic animals. Values were expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with metformin alone group.

4.7.3.1 Total cholesterol (TC)

MET alone and co-administered with TAHA, AA and AG decreased the TC levels in diabetic animals as compared to the diabetic control group. (Table 4.9)

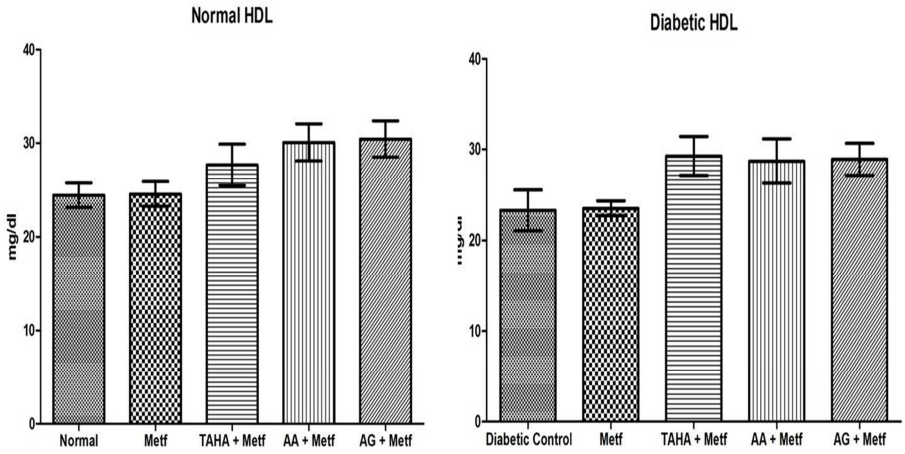

Figure 4.31: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) co-administered with MET on HDL levels in Normal and Diabetic animals. Values were expressed as mean ± SD.

4.7.3.2 HDL-cholesterol

In diabetic animals, MET co-administered with TAHA, AA and AG increased the HDL cholesterol levels as compared to the diabetic control group (Table 4.9).

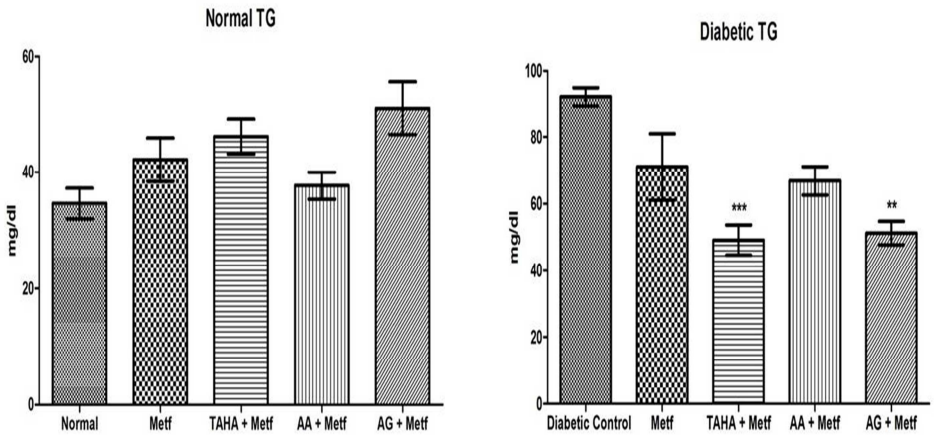

Figure 4.32: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) co-administered with MET on TG levels in Normal and Diabetic animals. Values were expressed as mean ± SD. ** $p < 0.01$, *** $p < 0.001$ when compared with the diabetic control group.

4.7.3.3 Triglyceride levels (TG)

In diabetic animals, MET co-administered with TAHA and AG decreased the TG levels significantly while MET alone and co-administered with AA showed non-significant results (Table 4.9).

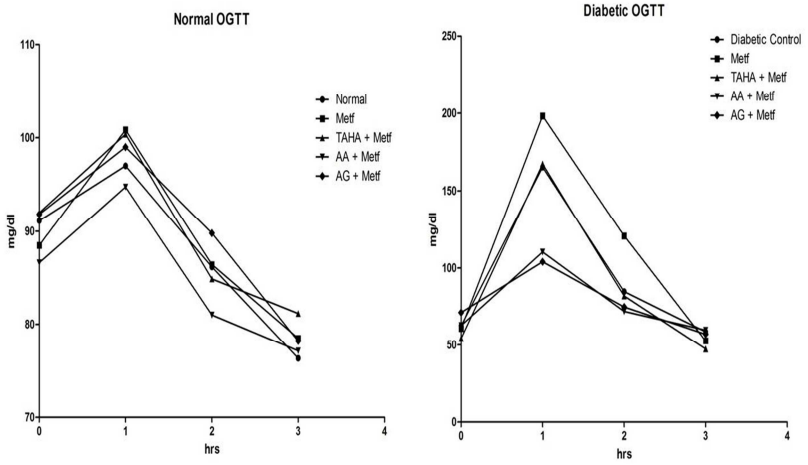

Figure 4.33: Effect of Arjun ghritah (AG), Arjunarishta (AA) formulations and hydro-alcoholic extract of *Terminalia arjuna* (TAHA) co-administered with MET on OGTT in Normal and Diabetic animals. Values were expressed as mean.

4.7.3.4. OGTT

Glucose homeostasis was assessed in normal and diabetic animals treated with MET alone or co-administered with TAHA, AA and AG by OGTT before and at the end of the experiment. MET co-administered with TAHA, AA and AG reduced blood glucose levels as compared to the diabetic control group (Fig. 4.33).

Table 4.9. Effect of TAHA, AA and AG on pharmacodynamic parameters of MET (TG, HDL, TC)

Group Non-diabetic	**HDL** (mg/dL)	**TC** (mg/dL)	**TG** (mg/dL)
Normal	24.39 ± 1.30	36.57 ± 0.78	34.63 ± 2.60
MET	24.51 ± 1.32	39.43 ± 2.16	42.17 ± 3.73
TAHA + MET	27.64 ± 2.24	37.00 ± 1.68	46.14 ± 3.00
AA + MET	30.06 ± 1.97	41.00 ± 1.23	37.67 ± 2.32
AG + MET	30.42 ± 1.92	41.00 ± 1.70	51.00 ± 4.56
Group Diabetic			
Control	23.25 ± 2.21	50.5 ± 3.07	92.00 ± 2.68
MET	23.48 ± 0.80	45.00 ± 4.08	71.00 ± 9.95
TAHA + MET	29.25 ± 2.14	42.25 ± 2.93	49.14 ± 4.49
AA + MET	28.68 ± 2.46	42.20 ± 1.11	66.80 ± 4.27
AG + MET	28.88 ± 1.75	46.25 ± 2.02	51.20 ± 3.49

Results are expressed as mean ± SEM. *$p<0.05$ and ***$p<0.001$

4.7.3.5. Effect of TAHA, AA and AG on histopathology of kidney and liver tissue

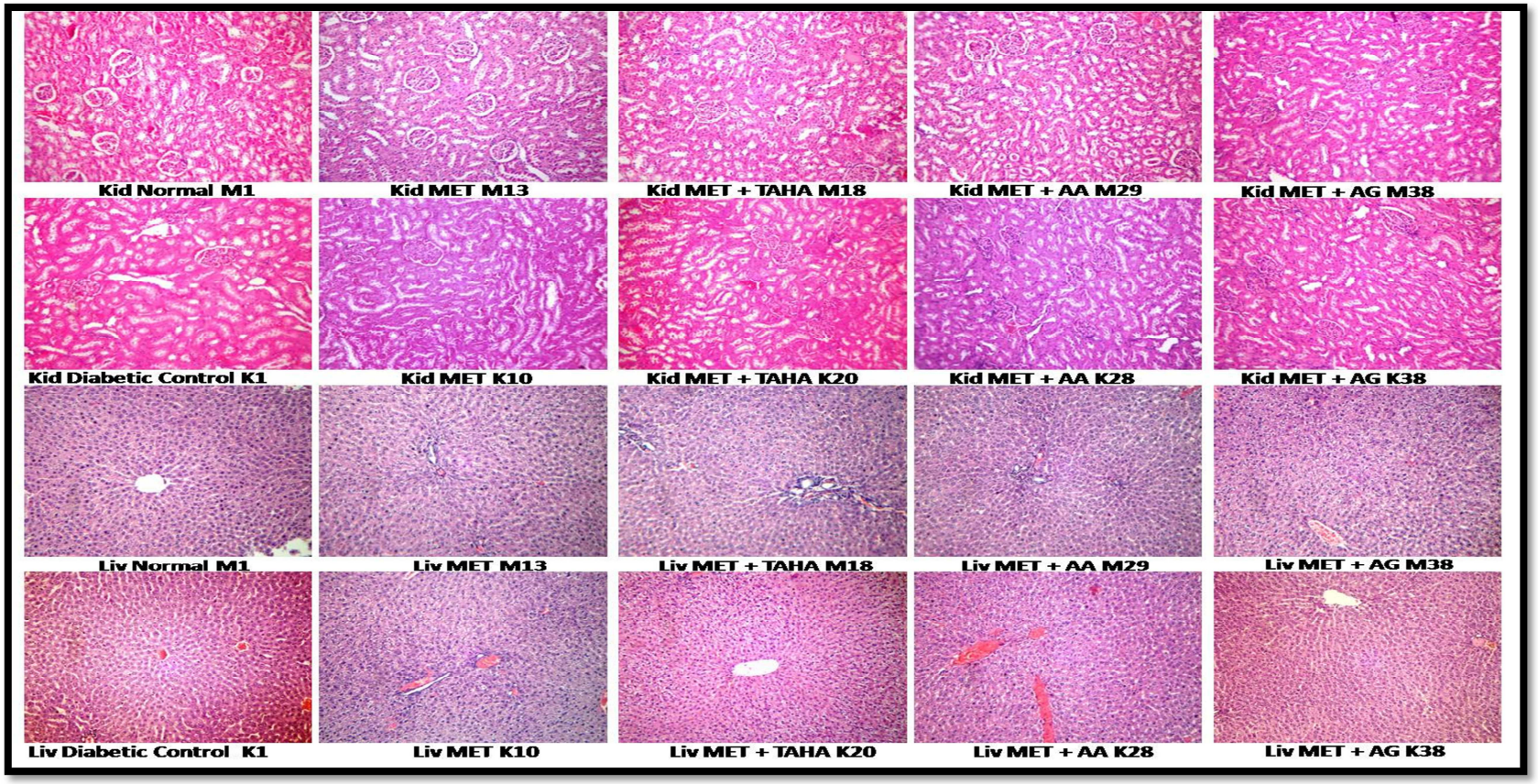

Figure 4.34 Histopathological changes in kidney and Liver tissues in normal and STZ diabetic animals treated with metformin (MET) alone or co-administered with T. arjuna hydroalcoholic extract (TAHA), Arjunarishta (AA), Arjun Ghritah (AG)

Morphological features of kidney remained normal in the normal group (Fig. 4.34 Kid Normal M1). The STZ-induced diabetic control group (Fig.4.34 Kid Diabetic Control K1) showed congestion and haemorrhages; focal tubular swelling, granular and vacuolar changes in the cytoplasm; infiltration of mononuclear cells. The STZ-induced damage to the kidney cells was not reversed in MET alone group (Fig. 4.34 Kid MET K10) only along with tubular degeneration, sloughing of the tubular epithelium; necrotic changes of tubular epithelium and glomerular changes. MET co-administered with TAHA, AA and AG (Fig. 4.34 Kid MET+TAHA K20, Kid MET+AA K28, Kid MET+AG K38) showed reversal changes MET alone group in diabetic treated animals. The liver cells of the normal group (Fig. 4.34 Liv Normal M1) showed eminent hepatocytes with central vein along with portal triad. The damage to the liver cells in the form of sinusoidal congestion and haemorrhages; focal cellular swelling, granular and vacuolar changes in the cytoplasm of hepatocytes; degenerative changes in hepatocytes; infiltration of mononuclear cells (MNC), granuloma/lymphoid aggregation could be seen in STZ received group (Fig. 4.34 Liv Diabetic Control K1). The damage to the liver cells was not reversed in MET alone group (Fig. 4.34 Liv MET K10) only. MET co-administered with TAHA, AA and AG (Fig. 4.34 Liv MET+TAHA K20, Liv MET+AA K28, Liv MET+AG K38) showed reversal changes MET alone group in diabetic treated animals except for sinusoidal congestion and haemorrhages; focal cellular swelling, granular and vacuolar changes in the cytoplasm of hepatocytes.

4.8 Effect of TAHA, AA, AG on enzyme inhibition assay

4.8.1 Effect of TAHA, AA, AG on α-amylase inhibition assay

Table 4.10. Effect of TAHA, AA and AG on α-amylase inhibition assay

Conc	**% inhibition**			
	Acarbose	**TAHA**	**AA**	**AG**
Blank	0	0	0	0
5 µg/mL	7.57 ± 1.40	**NA**	**NA**	**NA**
10 µg/mL	15.43 ± 1.27	8.43 ± 1.51	2.11 ± 0.17	1.44 ± 0.32
20 µg/mL	23.71 ± 1.60	13.71 ± 1.60	4.80 ± 1.93	5.11 ± 1.86
40 µg/mL	39.00 ± 2.65	20.86 ± 2.34	7.13 ± 1.27	7.19 ± 2.12
80 µg/mL	52.86 ± 1.77	33.43 ± 5.06	9.80 ± 1.82	10.34 ± 2.16
160 µg/mL	76.86 ± 3.72	50.86 ± 2.67	12.80 ± 3.12	11.78 ± 2.59
320 µg/mL	**NA**	71.00 ± 4.66	15.92 ± 3.64	17.43 ± 4.53

TAHA - Hydroalcoholic extract of *Terminalia arjuna;* AA - Arjunarisht; AG - Arjun Ghritah

In this assay, acarbose is utilised as a standard for α-amylase inhibition assay. Acarbose (concentrations 5–160 µg/mL) demonstrated α-amylase inhibition from 7.57±1.40 to 76.86±3.72% with an IC_{50} value 62.35±5.39 µg/mL (Table 4.10).

The percentage inhibition of the TAHA, AA and AG on α-amylase enzyme was studied in a range of 10–320 µg/mL. The TAHA extract has shown inhibition of from 8.43±1.51 to 71.00±4.66% with an IC_{50} value 145. 90±16.34 µg/mL. The IC_{50} value for a TAHA extract was 145.90±16.34 µg/mL, which is lesser to standard anti-diabetic drug acarbose, which was 62.35±5.39 µg/ml (Table 4.10). The AA and AG did not show α-amylase inhibition activity.

4.8.2 Effect of TAHA, AA, AG on DPP-4 enzyme inhibition assay

Table 4.11. Effect of TAHA, AA, AG on DPP-4 enzyme inhibition assay

Concentration	**% inhibition**
	Sitagliptin
Blank	0
1 nM	11.33 ± 4.44
5 nM	22.67 ± 3.57
10 nM	38.44 ± 5.53
20 nM	58.11 ± 7.11
40 nM	70.22 ± 5.72
80 nM	81.44 ± 5.08
100 nM	87.00 ± 3.04

Conc	**% inhibition**		
	TAHA	**AA**	**AG**
Blank	0	0	0
10 µg/ml	1.88 ±0.92	3.11 ± 1.17	5.44 ± 1.59
50 µg/ml	3.77 ±1.71	3.89 ± 0.93	7.11 ± 1.76
100 µg/ml	6.33 ±1.94	5.13 ± 1.27	9.89 ± 2.52
200 µg/ml	10.33 ±2.35	7.89 ± 1.62	11.44 ± 3.36
400 µg/ml	14.67 ±2.69	9.80 ± 1.62	13.78 ± 2.99
800 µg/ml	18.56 ±2.79	12.87 ± 2.21	15.22 ± 2.82
1000 µg/ml	24.67 ±2.40	16.89 ± 2.09	18.67 ± 3.04

TAHA - Hydroalcoholic extract of *Terminalia arjuna*, AA – Arjunarisht, AG - Arjun Ghritah

Sitagliptin, which had an IC50 (half of the maximal inhibitory concentration) of 18 nM, was used as control (Table 4.11). The TAHA, AA and AG did not show DPP-4 inhibition activity. Table... shows that the DPP-4 inhibitory activity of TAHA, AA and AG against DPPIV were not decreased in a dose-dependent manner.

4.9 Effect of TAHA, AA, AG on adipocyte differentiation assay

Table 4.12. Effect of TAHA, AA, AG on Adipocyte Differentiation Assay

Group	Concentration (µg/mL)	% induction	% reduction
Control		100	0
TAHA	50	100	0
	100	97	3
AA	50	100	0
	100	94.1	5.9
AG	50	96.5	3.5
	100	93.3	6.7
Cerulenin	5	66.0 ± 9.6	34.0 ± 9.6
	10	56.5 ± 19.4	43.5 ± 19.4
Rosiglitazone	5	109.7 ± 16.0	-9.7 ± 16.0
	10	113.1 ± 20.7	-13.1 ± 20.7

The TAHA, AA and AG did not show a reduction in oil droplet formation. Positive control, i.e. Rosiglitazone, showed a concentration-dependent increase in the oil drop formation and adipocyte differentiation. Whereas Cerulenin control showed a concentration-dependent reduction in and adipocyte differentiation and oil drop formation.

5. DISCUSSION

Characterization is an essential prospect of showing the calibre of any traditional herbal formulations. There are numerous studies on cardioprotective actions and phytochemical analysis of AA and TAHA; yet, no reports on the characterization of AG [150-152]. This work attempted to characterize the TA related ayurvedic formulations for its specific phytochemicals such as arjunetin and arjungenin. The chromatograms of standards (arjunetin and arjungenin), TA bark extract and TA related ayurvedic formulations were compared.

Medicinal herbal plants were essential to the individuals and communities health [153-158]. TA is a general medicinal herbal plant utilized in the pharmacological system to care for different degenerative disorders. In previously reported work, the phytochemical analysis mentioned a huge quantity of phytosterol, lactones, flavonoids, phenolics compounds and glycosides and tannins present in methanol extract of TA bark. Natural antioxidants found in the plant sources, like tocopherols, phenolic acids, flavonoids, etc [159]. The flavonoid's antioxidative property is because of several mechanisms, like chelation of metal ions, inhibition of enzymes responsible for a free radical generation and scavenging of free radicals, [160, 161]. More than 2000 flavonoids have been reported among woody and non-woody plants [162].

HFD-fed model is a suited model for the investigation of drugs influencing the pathogenetic mechanisms of MS. HFD feeding effectively induced obesity in animals, as evidenced by a significant body weight gain. At the end of the work, the average body weight of animals in the HFD control group was significantly increased by 27.7

% than the normal group. Among all these treatments, only MET, AG450 and AG900 group showed a significant body weight reduction by 17.4, 23.2 and 22.8 % as compared to the HFD control group respectively. TAHA250, TAHA500, AA0.9 and AA1.8 group rats showed a tendency, though not at a significant level, toward reduction of body weight gain by 11.6, 10.7, 12.5 and 12.5 % caused by HFD respectively.

To evaluate the antihyperlipidemic effect of the TAHA, AA, and AG in dyslipidemia, we have investigated the plasma TG, TC and HDL concentration in HFD-fed rats treated for 12 weeks. As per previous studies, HFD fed animals showed enhance levels of both plasma TGs and cholesterol in the circulation, compared to vehicle. During an OGTT, HFD fed rats were represented enhanced glucose levels at 120 minutes followed by a four hr return to normal glucose levels. Additionally, we noticed that after 12 weeks of HFD administration rats depict glucose tolerance, as presented during the OGTT by the impaired glucose excursion. We consider that this effect might be because of insulin resistance development [163, 164].

TAHA, AA and AG intervention could be involved in the partial improvement of body weight, improvement in glucose uptake and lipid compositions in the HFD fed rats. Furthermore, the expression of different genes involved in lipogenesis, lipid storage, and gluconeogenesis was evaluated to understand the molecular mechanism of TAHA, AA, and AG intervention. The PEPCK enzyme associated with gluconeogenesis is a rate-limiting. Due to HFD, PEPCK expression was up-regulated in rats [164].

In this study, PEPCK expression was not increased significantly in the HFD control group. However, administration of TAHA, AA and AG altered expression of the

PEPCK gene. Therefore, TAHA, AA and AG induced antihyperglycemic effect through hepatic glucose production suppression due to reduction in the expression of PEPCK in HFD fed rats. Reconstitution of a fat store in the liver of TAHA treated rats may be complex, involving a reduction in FA oxidation and an enhanced in de novo FA synthesis and FA uptake from peripheral fat stores. However, in these circumstances, the effort of de novo lipogenesis is playing an essential role as TAHA, AA and AG treatment did induce APOC-III, FABP, and SCD-1 expression in MS rats. The HFD enhanced the FABP expression in the liver due to increased fatty acid flux derived from dietary fat [165]. Evidence suggests that proteins related to lipoprotein transport, like, APOC-III may significantly alter the procedure of body fat accumulation, which increased adiposity in response to an HFD in rat [166]. To determine the changes related with the lipogenesis, of the ACC gene expression was assessed, and among all groups, there was no significant difference in the expression levels. The Acox-1 plays an essential role in lipid constitutions. FA degradation takes place via β-oxidation [167]. In this study, the Acox-1 gene expression was increased by over 5-folds in the MS rats following TAHA treatment, which may provide clear evidence that TAHA could decrease TG concentration via leading lipid β-oxidation.

TAHA showed a precise effect on lipid metabolism via regulation of the lipogenesis and lipolysis. Moreover, PPAR related genes, like Acox1 and CPT1α, were up-regulated. These TAHA, AA and AG-regulated changes in gene expression were related to the improvement in the hyperlipidemia, and TG content, which probably simulated the alteration in the metabolism. Throughout lipogenesis, the malonyl-CoA developed from the ACC reaction enhances and inhibited CPT-1α, leading in the β-oxidation pathway inhibition [168]. In this study, we detected a significant rise in mRNA levels of the CPT1α, responsible for the fatty acid transport into mitochondria.

In the liver, FA may either undergo the mitochondrial β-oxidation for energy production.

The decreased expression of the CPT-1α gene in HFD fed rats may explain the minimization of FA oxidation, which responsible for elevating TG synthesis. This study results also suggest that acquisition of the TG levels in HFD fed rats livers was due to an increase in the lipid flux. In HFD fed rats, the antihypertriglyceridemic effect of TAHA and AG was expressed, with a decrease in TG and TC levels through faster lipoprotein clearance. This discussion is confirmed by the enhanced FAT/CD36 and reduced APOC-III expression in TAHA treated rats. Enhanced FAT/CD36 gene expression could express the enhancement in FA usage in the liver as formerly reported [169]. Therefore, based on these results, the effect of TAHA, AA and AG may mediate through the glucose and lipid metabolism-related genes in the HFD fed rats.

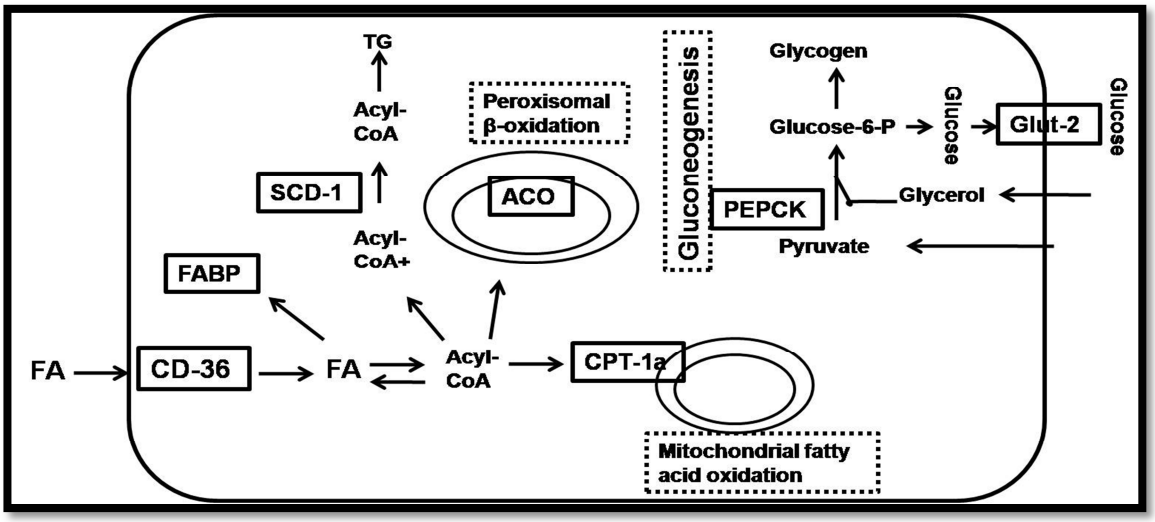

Figure 5.1. A schematic representation summarising hepatic glucose and fatty acid metabolism in the liver

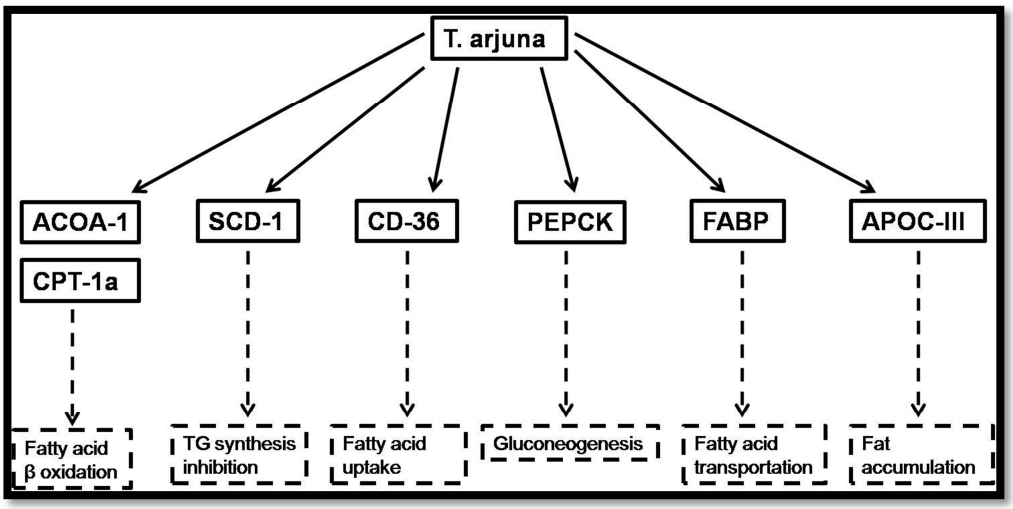

Figure 5.2. Effect of T. arjuna on carbohydrate and lipid metabolism-related genes

Systemic inflammation in IUGR rats

Chronic hyperglycemia also triggers a rise in various secondary mediators, which can be of inflammatory and non-inflammatory origins. In brief, chronic multi-generational caloric restriction triggers an increase in visceral adiposity, which in turn converts macrophages to its active form. Macrophages activation elicits a systemic inflammatory response indicated by the rise in TNF-α and IL-6 [170]. TLR-4 plays an important role in the conversion of activated M2 phenotype to M1 phenotype [171]. Rise systemic inflammation also activates cytoplasmic mediators like IKKB and Jun to enhance transcription of NF-kB, a master regulator that then activates the inflammatory cascade. Each of the above-mentioned secondary mediators has been implicated in the pathogenesis of T2DM [172]. For example, it is well appreciated that TNF-α and IL-6 can impair insulin signal transduction by impairing the tyrosine phosphorylation of the insulin receptor, thus accelerating insulin resistance. NF-kB also inhibits the triad of AMPK-SIRT1-PGC-1α that is vital in mitochondrial energetics and maintaining insulin sensitivity [173]. Sirt1 has been implicated in the ageing, cell proliferation and involved in protection from metabolic syndrome at a

molecular level. Both, SIRT1 and AMPK are up-regulated with caloric restriction and exercise which in turn can trigger GLUT and IRS-1 to stimulate glucose uptake in the cell [174]. However, postnatal protein undernutrition is known to down-regulate both IRS and SIRT1 [175].

In normal physiology, NF-kB and the AMPK-SIRT1-PGC-1α triad are well regulated. However, in the case of chronic inflammation, NF-kB expression exceeds and down-regulates the triad. Therefore, attempts are on to find small molecules that stimulate this triad or independent elements in a triad. Vachharajani et al. now show that sirtuin can dampen NF-κB-dependent gene expression, thus showing a possible connection between ageing, metabolism, and inflammation [176]. Resveratrol, a polyphenol is known to do so [177]. Metformin also stimulates AMPK from this triad [178]. Antihyperglycemic activity of metformin is attributed to its AMPK modulating activity. Adiponectin is also a potent modulator of PGC-1α and PPAR-γ [179]. Adiponectin also up-regulates GLUTs to bring insulin sensitization [180]. Thus targeting the inflammatory mediators in congruence with the AMPK-SIRT1-PGC-1α triad can achieve modulation of multiple downstream events that can bring insulin sensitivity.

TAHA, AA and AG decrease systemic inflammation to bring insulin sensitivity in insulin resistance rats. Gene expression studies demonstrate the primary activity of TAHA, AA and AG as an anti-inflammatory. The deletion of TLR4 decreases the impact of HFD diet-induced obesity on insulin resistance supports a direct connection to the conversion of activated M2 to an activated M1 phenotype [181]. This suggests the TAHA, AA and AG might be bringing anti-hyperglycemic activity and insulin sensitization by its anti-inflammatory activity. TA is rich in polyphenols. Polyphenols

are known to inhibit NF-kB [182]. Therefore, TAHA, AA and AG bring about insulin sensitization by its anti-inflammatory effect.

Effect of TAHA, AA and AG on Adiponectin, AMPK-SIRT1-PPAR-γ

Polyphenols act as a positive modulator of AMPK and SIRT-1 [183]. Activated AMPK increases the production of ATP by regulating fatty acid and glucose metabolism. TAHA, AA and AG represented increased in the expression of AMPK (PRKAA-2). Thus TAHA, AA and AG demonstrate cardioprotective activity. Following figure 4.30 suggests possible mechanisms of action of TAHA, AA and AG.

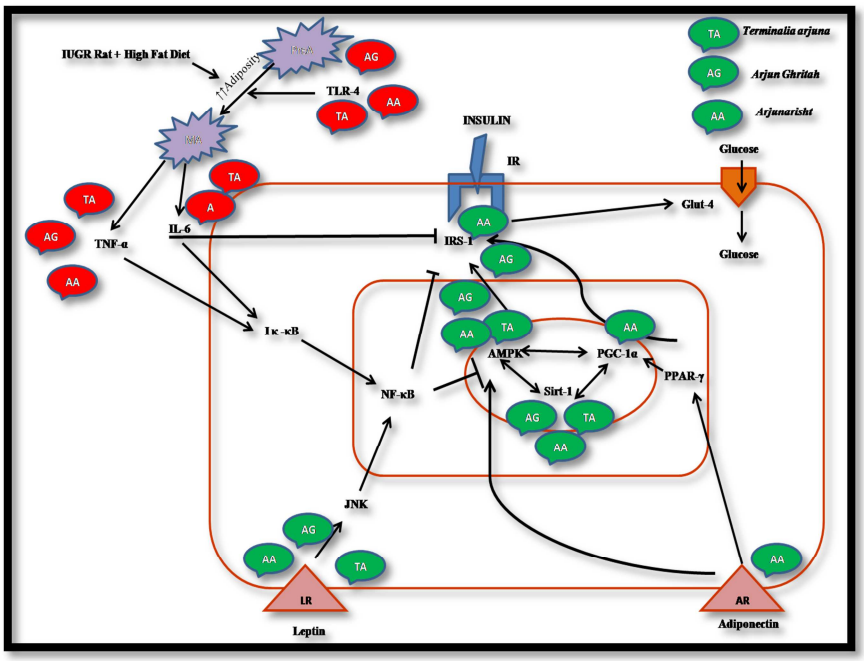

Figure 5.3. Possible mechanisms of TAHA, AA and AG

Treatment of AA for three months increased adiponectin receptor two significantly. Adiponectin has a direct correlation with insulin sensitivity and CVDs. High molecular weight adiponectin is a predictor of insulin resistance, T2DM and MS [184]. Adiponectin exerts its effect through modulation of AMPK [185]. This suggests that the effect of TAHA, AA and AG on AMPK might be regulated through

the increase of adiponectin. However, we have not looked specificity at the ratio of adiponectin to its high molecular weight fraction.

One of the mechanisms by which TAHA exhibited its hypoglycemic potential in vitro assay is through the inhibition of α-amylase. AA and AG did not show α-amylase inhibition activity [186]. However, the methanolic, aqueous and 50% methanolic extract of TA was reported for α-amylase inhibition activity [187, 188].

The diabetic patient is utilizing complementary alternative medicine (CAM) from 17% to 73% of herbal forms [189]. TA is a crucial traditional medicine use for dyslipidemia and CVD from various years. Patients are ingesting TA with allopathic hypoglycemic drugs. Therefore, there is a need for risk versus benefits assessment when TA is co-administered with allopathic hypoglycemic drugs.

This investigation aimed to assess the PK and PD interactions of TAHA, AA, AG with metformin (MET), in diabetic rats.

The effect of TAHA, AA and AG on the PK parameters of MET in normal and diabetic animals has been studied. Co-administration of TAHA, AA and AG did not affect MET pharmacokinetic parameters. TAHA, AA and AG did not represent any change in C_{max}, T_{max}, AUC_{∞}, V_d and CL of MET in both normal and diabetic animals except co-administration of AG significantly decreased AUC_{∞} and significantly increased CL parameters in diabetic animals.

PD interactions of TAHA, AA, and AG with MET were studied in an STZ-induced diabetic rat model. Effect of TAHA+MET, AA+MET and AG+MET was studied on their antihyperglycemic and antihyperlipidemic potential. Co-administration of TAHA, AA, and AG with MET did not show any change in HDL and TC levels in

normal and diabetic animals. Similarly, Co-administration of TAHA, AA, and AG with MET did not show any change in glucose homeostasis in normal animals, but co-administration of TAHA, AA, and AG with MET showed a significant decrease in blood glucose levels as compared to a diabetic control group. Co-administration of TAHA, AA, and AG with MET did not show any change in TG levels in normal animals, but co-administration of TAHA and AG with MET showed a significant reduction in TG levels as compared to a diabetic control group. The diabetic animals indicated elevated levels of plasma TC and TG and decreased levels of HDL as compared to a normal group ($p < 0.05$). In histology of kidney and liver, STZ-induced damage to the kidney and liver cells was not reversed in MET alone group but MET co-administered with TAHA, AA and AG showed reversal changes as compare to MET alone group in diabetic treated animals. Rats have been utilized for better effect of sub-chronic concomitant administration of TAHA+MET and AG+MET for seven days resulted in beneficial PD interaction leading to better antihyperlipidemic, antihyperglycemic action and histological changes in tissues without affecting PK parameters of metformin. However, these results warranted further studies after chronic co-administration as case studies or controlled trials in humans.

6. SUMMARY

The present thesis **"Molecular Mechanism and Herb-Drug Interaction of TAHA and its Ayurvedic Formulations in Metabolic Syndrome"** aimed to develop a method of TAHA and its ayurvedic formulations using HPLC analysis method. To study the effect and molecular mechanism of TAHA and its ayurvedic formulations in a HFD-fed rat model. To assess the activity of TAHA and its ayurvedic formulations in enzyme inhibition assays and adipocyte differentiation. To determine the effect of TAHA and its ayurvedic formulations on MET levels and glucose uptake in normal and diabetic animals.

Based on the literature, TA can be judged as a cardioprotective herbal drug with huge therapeutic prospect. Since CVD is complex, the pleiotropic actions of TA in modifying severe cardiovascular risk factors place this herbal medicine on a unique stage for its development in future. As adequate gaps present in the literature about the molecular mechanism, and HDIs, more research study in this direction using a rigorous systems biology concept is required to cover the essential issues mentioned above.

TAHA, AA and AG intervention could be involved in a partial improvement of body weight, improvement in blood glucose uptake and lipid compositions in the HFD fed rats. The proposed mechanism of action of TAHA, AA, and AG was through inflammatory, obesogenic, insulin-related, energy expenditure related, lipid and carbohydrate metabolism-related genes.

The results of this study suggest the standardization of arjungenin and arjunetin phytochemical specific markers in AA, and AG. This work can be used for quality

assurance and control process of manufacturing of AA and AG formulations. TAHA exhibited its hypoglycemic activity mediated through the α-amylase enzyme inhibition assay.

The HDI study demonstrated that the effect of sub-chronic concomitant administration of TAHA+MET and AG+MET for seven days proved in beneficial PD interaction contributing to better antihyperlipidemic, antihyperglycemic activity and histological changes in tissues without affecting PK parameters of MET in diabetic condition.

This study has shown that the functional aspects and pleiotropic actions of TA and its ayurvedic formulations, especially in metabolic syndrome. Though several pharmacological previous studies affirm its benefit in the hyperglycemia, CVD, hyperlipidemia as per traditional application, using more in-depth state of the art technology is warranted to evaluate the efficacy of TA and its ayurvedic formulations.

7. CONCLUSION

Phytochemically standardised TAHA, AA and AG intervention could be involved in a partial improvement of body weight, improvement in glucose uptake and lipid compositions in the HFD fed rats. The possible proposed mechanism of action of TAHA, AA, and AG was through inflammatory, obesogenic, insulin-related, energy expenditure related, lipid and carbohydrate metabolism-related genes. TAHA exhibited its hypoglycemic activity mediated through the α-amylase enzyme inhibition assay.

The HDI study evidenced that in diabetic experimental condition, the effect of sub-chronic concomitant administration of TAHA+MET and AG+MET for seven days proved in beneficial PD interaction contributing to better antihyperlipidemic, antihyperglycemic activity and histological changes in tissues without affecting PK parameters of MET. This study has shown that the functional aspects and pleiotropic actions of TA and its Ayurvedic formulations, especially in metabolic syndrome.

8. REFERENCES

1. Balasaraswati R, Sadasivam S et al. An antiviral protein from *Bougainvillea spectabilis* roots; purification and characterisation. Phytochemistry. 1998;47(8):1561-5.

2. Surana SJ, Gokhale RB et al. Antihyperglycemic Activity of Various Fractions of *Cassia auriculata* Linn. in Alloxan Diabetic Rats. Ind J Pharm Sci. 2008; 70:227-9.

3. Shokeen P, Anand P et al. Antidiabetic activity of 50% ethanolic extract of *Ricinus communis* and its purified fractions. Food Chem. Toxicol. 2008; 46:3458-66.

4. Mankil J, Moonsoo P et al. Antidiabetic Agents from Medicinal Plants. Curr. Med. Chem. 2006;13(10):1203-18.

5. Kumar S, Enjamoori R. A Catecholamine-induced myocardial fibrosis and oxidative stress is attenuated by *Terminalia arjuna* (Roxb.). J. Pharm. Pharmacol. 2009;61:1529-36.

6. Sinha M, Manna P et al. *Terminalia arjuna* protects mouse hearts against sodium fluoride-induced oxidative stress. J Med Food. 2008;11(4):733-40.

7. Raghavan B, Kumari SK. Effect of *Terminalia arjuna* stem bark on antioxidant status in liver and kidney of alloxan diabetic rats. Indian J. Physiol. Pharmacol. 2006;50:133-42.

8. Shailaa HP, Udupa SL et al. Hypolipidemic activity of three indigenous drugs in experimentally induced atherosclerosis. Int. J. Cardiol.1998;67:119-24.

9. Sharma PC, Yelne MB et al. Database on Medicinal Plants used in Ayurveda, CCRAS 2005;7.

10. Arora RC, Agarwal N et al. Evaluation of CTI (cardioprotective drug) in subjects of coronary artery disease, hypertension and diabetes mellitus. Flora Fauna. 1995;1: 203-5.

11. Dwivedi S, Agarwal A et al. Role of *Terminalia arjuna* in ischaemic mitral regurgitation. Int. J. Cardiol. 2005;100:507-8.

12. Gupta R, Singhal S et al. Antioxidant and hypocholesterolaemic effects of *Terminalia arjuna* tree-bark powder: a randomised placebo-controlled trial. J. Assoc. Physicians India. 2001;49:231-5.

13. Dwivedi IS, Jauhari R. Beneficial effects of *Terminalia arjuna* in coronary artery disease. Indian Heart J. 1997;49:507-10.

14. Dwivedi S, Agarwal MP. Antianginal and cardioprotective effects of *Terminalia arjuna*, an indigenous drug, in coronary artery disease. J. Assoc. Physicians India. 1994;42:287-9.

15. Izzo AA, Ernst E. Interactions between herbal medicines and prescribed drugs: a systematic review. Drugs. 2001;61:2163-75.

16. Huang SM, Strong JM, et al. New era in drug interaction evaluation: US Food and Drug Administration update on CYP enzymes, transporters, and the guidance process. J. Clin. Pharmacol. 2008;48:662-70.

17. Patil D, Gautam M, et al. 2010. Physicochemical stability and biological activity of *Withania somnifera* extract under real-time and accelerated storage conditions. Planta. Med. 2010;76:481-8.

18. Montoro P, Maldini M, et al. Metabolite fingerprinting of *Camptotheca acuminata* and the HPLC-ESI-MS/MS analysis of camptothecin and related alkaloids. J. Pharm. Biomed. Anal. 2010;51:405-15.

19. Chakradhar L, Kallem R, et al. A rapid and highly sensitive method for the determination of glimepiride in human plasma by liquid chromatography-electrospray ionization tandem mass spectrometry: application to a pre-clinical pharmacokinetic study. Biomed. Chromatogr. 2008;22:58-63.

20. World Health Organization Geneva. WHO guidelines on safety monitoring of herbal medicines in pharmacovigilance systems.2004;1-18.

21. European Medicine Agency. Committee for Human Medicinal Products (CHMP) 2010 Guideline on the Investigation of Drug Interactions CPMP/EWP/560/95/Rev. 1-38.

22. Guidance for Industry Botanical Drug Products U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) June 2004;1-47.

23. Hu Z, Yang X, et al. Herb-drug interactions: a literature review. Drugs. 2005;65:1239-82.

24. Iwata H, Tezukay Y, et al. Inhibition of human liver microsomal CYP3A4 and CYP 2D6 by extracts from 78 herbal medicines J. Trad. Med. 2004;21:41-50.

25. King H, Aubert RE, et al. Global burden of diabetes, 1995- 2025- Prevalence, numerical estimates, and projections. Diabetes Care. 1998;21:1414-31.

26. Tiwari AK, Rao JM. Diabetes mellitus and multiple therapeutic approaches of phytochemicals: Present status and future prospects. Current Science. 2002;83:30-8.

27. Zhou S, Zhou W, et al. Identification of drugs that interacts with herbs in drug development. DDT. 2007;12:664-73.

28. Gardiner P, Phillips R, et al. Herbal and dietary supplement-drug interactions in patients with chronic illnesses. Am. Fam. Physician. 2008;77:73-8.

29. Xu H, Williams KM, et al. Effects of St John's wort and CYP2C9 genotype on the pharmacokinetics and pharmacodynamics of gliclazide. Br. J. Pharmacol. 2008;153:1579-86.

30. Nishimura N, Naora K, et al. Effects of Sho-saiko-to on the pharmacokinetics and pharmacodynamics of tolbutamide in rats. J. Pharm. Pharmacol. 1998;50:231-6.

31. Puranik A, Halade G, et al. Cassia auriculata: Aspects of safety pharmacology and drug interaction. eCAM. 2010.1-10.

32. Grundy SM, Hansen B, et al. American Heart Association, National Heart, Lung, and Blood Institute, American Diabetes Association. Clinical management of metabolic syndrome: report of the American Heart Association/National Heart, Lung, and Blood Institute/American Diabetes Association conference on scientific issues related to management. Arterioscler. Thromb. Vasc. Biol. 2004;24(2):e19–4.

33. Shaw JE, Chisholm DJ. Epidemiology and prevention of type 2 diabetes and the metabolic syndrome. Medical Journal of Australia. 2003;179(7):379-83.

34. Kylin E. Studien ueber das Hypertonie-Hyperglyca “mie- Hyperurika” miesyndrom. Zentralblatt fuer Innere Medizin. 1923;44:105-27.

35. Vague J. Sexual differentiation. A factor affecting the forms of obesity. Presse Medicale. 1947;30:S39-S40.

36. Beltrán-Sánchez H, Harhay MO, et al. Prevalence and trends of metabolic syndrome in the adult U.S. population, 1999-2010. J Am Coll Cardiol 2013;62(8):697-703.

37. Ford ES, Giles WH, et al. Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. JAMA. 2002;287(3):356-9.

38. Grundy SM. Metabolic syndrome pandemic. Arterioscler. Thromb. Vasc. Biol. 2008;28(4):629-36.

39. Miller JM, Kaylor MB, et al. Prevalence of metabolic syndrome and individual criterion in US adolescents: 2001-2010 National Health and Nutrition Examination Survey. Metab Syndr Relat Disord. 2014;12(10):527-32.

40. Rochlani Y, Pothineni NV et al. Metabolic syndrome: pathophysiology, management, and modulation by natural compounds. Ther Adv Cardiovasc Dis. 2017;11(8):215-25.

41. Kahn SE, Prigeon RL, et al. Obesity, body fat distribution, insulin sensitivity and islet □-cell function as explanations for metabolic diversity. Journal of Nutrition. 2001;131(2):354S-60S.

42. Xydakis AM, Case CC, et al. Adiponectin, inflammation, and the expression of the metabolic syndrome in obese individuals: the impact of rapid weight lose through caloric restriction. J Clin Endocrin Metab. 2004;89(6):2697–2703.

43. Clearfield MB. C-reactive protein: a new risk assessment tool for cardiovascular disease. Journal of the American Osteopathic Association. 2005;105(9):409-16.

44. Diamant M, Lamb HJ, et al. The association between abdominal visceral fat and carotid stiffness is mediated by circulating inflammatory markers in uncomplicated type 2 diabetes. J Clin Endocrin Metab. 2005;90(3):1495-1501.

45. Zuliani G, Volpato S, et al. High interleukin-6 plasma levels are associated with low HDL-C levels in communitydwelling older adults: the InChianti study. Atherosclerosis. 2007;192(2):384-90.

46. Alessi MC and Juhan-Vague I. PAI-1 and the metabolic syndrome: links, causes, and consequences. Arteriosclerosis, Thrombosis, and Vascular Biology. 2006;26(10):2200-07.

47. Kohler HP and Grant PJ. Plasminogen-activator inhibitor type 1 and coronary artery disease. The New England Journal of Medicine. 2000;342(24):1792-1801.

48. Matsuzawa Y, Funahashi T, et al. Adiponectin and metabolic syndrome. Arteriosclerosis, Thrombosis, and Vascular Biology. 2004;24(1):29-33.

49. Fumeron F, Aubert R, et al. Adiponectin gene polymorphisms and adiponectin levels are independently associated with the development of hyperglycemia during a 3-year period: the epidemiologic data on the insulin resistance syndrome prospective study. Diabetes. 2004;53(4):1150-7.

50. Hutley L and Prins JB. Fat as an endocrine organ: relationship to the metabolic syndrome.The American Journal of the Medical Sciences. 2005;330(6):280-9.

51. Shirasaka T, Takasaki M, et al. Cardiovascular effects of leptin and orexins. The American Journal of Physiology—Regulatory Integrative and Comparative Physiology. 2003;284(3):R639-51.

52. Considine RV, Sinha MK, et al. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. The New England Journal of Medicine. 1996;334(5):292-5.

53. Gill H,Mugo M, et al. The key role of insulin resistance in the cardiometabolic syndrome. The American Journal of the Medical Sciences. 2005;330(6):290-294.

54. Jensen MD, Haymond MW, et al. Influence of body fat distribution on free fatty acid metabolism in obesity. Journal of Clinical Investigation. 1989;83(4):1168-73.

55. Lewis GF, and Steiner G. Acute effects of insulin in the control of VLDL production in humans: implications for the insulinresistant state," Diabetes Care, 1996;19(4):390-3.

56. Briones AM, Cat AND, et al. Adipocytes produce aldosterone through calcineurin-dependent signaling pathways: implications in diabetes mellitus-associated obesity and vascular dysfunction. Hypertension. 2012;59(5):1069-78.

57. Charmandari E, Tsigos C, et al. Endocrinology of the stress response. Annual Review of Physiology. 2005;67:259-84.

58. Guillaume-Gentil C, Assimacopoulos-Jeannet F, et al. Involvement of non-esterified fatty acid oxidation in glucocorticoid-induced peripheral insulin resistance in vivo in rats. Diabetologia. 1993;36(10):899-906.

59. Chrousos GP. Stress and disorders of the stress system. Nature Reviews Endocrinology. 2009;5(7):374-81.

60. Hossain P, Kawar B, et al. Obesity and diabetes in the developing worldda growing challenge. N Engl J Med. 2007;356:213-5.

61. Olshansky SJ, Passaro DJ, et al. A potential decline in life expectancy in the United States in the 21st century. N Engl J Med 2005;352:1138-45.

62. Ford ES, Giles WH, et al. Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. JAMA 2002;287:356-9.

63. Pendurthi UR and Rao LV. Suppression of transcription factor Egr-1 by curcumin. Thromb Res. 2000; 97(4):179–189.

64. Gomez-Arbelaez D, Lahera V, et al. Aged garlic extract improves adiponectin levels in subjects with metabolic syndrome: a doubleblind, placebo-controlled, randomized, crossover study. Mediators Inflamm. 2013;2013:285795.

65. Bhat M, Kothiwale SK, et al. Antidiabetic properties of Azardiracta indica and Bougainvillea spectabilis: in vivo studies in murine diabetes model. Evid Based Complement Alternat Med. 2009;2011:561625.

66. Mendez-del Villar M, Gonzalez-Ortiz M, et al. Effect of resveratrol administration on metabolic syndrome, insulin sensitivity, and insulin secretion. Metab Syndr Relat Disord. 2014;12(10):497–501.

67. Song M-Y, Kim E-K, et al. Sulforaphane protects against cytokine- and streptozotocin-induced β-cell damage by suppressing the NF-κB pathway. Toxicol Appl Pharmacol. 2009;235(1):57–67.

68. Yi-Wei W, Si-Jia H, et al. Metformin: a review of its potential indications. Drug Des Devel Ther. 2017;11:2421-9.

69. Singh RM. Exploring issues in the development of Ayurvedic research methodology. J Ayurveda Integr Med. 2010 Apr-Jun;1(2):91-5.

70. Dwivedi, S. *Terminalia arjuna* Wight &Arn.—a useful drug for cardiovascular disorders. Journal of Ethnopharmacology. 2007;114:114-29.

71. WHO. World Health Organization, Traditional Medicine Strategy Report. 2002 Document WHO/EDM/TRH/2002.1.

72. Heinrich M. Ethnobotany and its role in drug development. Phytother Res. 2000;14:479–488. [PubMed: 11054835]

73. Tabuti JRS, Lye KA, et al. Traditional herbal drugs of Bulamogi, Uganda: plants, use and administration. J Ethnopharmacol. 2003;88:19-44. [PubMed: 12902048]

74. Pieroni A. Evaluation of the cultural significance of wild food botanicals traditionally consumed in Northwestern Tuscany, Italy. J Ethnobiol. 2001;21:89– 104.

75. Kim H, Song MJ. Ethnomedicinal practices for treating liver disorder of local communities in the southern regions of Korea. J Evid Based Complement Altern Med. 2013.

76. Upadhyay B, Singh KP, et al. Ethnoveterinary uses and informants consensus factor of medicinal plants of Sariska region, Rajasthan, India. J Ethnopharmacol. 2011;133:14–25. [PubMed: 20817085]

77. Reyes-Garcia V, Huanca T, et al. Cultural, practical, and economic value of wild plants: a quantitative study in the Bolivian, Amazon. Econ Bot. 2006;60:62–74.

78. Gosh A. Herbal folk remedies of Bantura & Medinipur districts, West Bengal (India) Indian J Tradit Knowl. 2003;2:393–6.

79. Stickel F, Schuppan D. Herbal medicine in the treatment of liver diseases. Dig Liver Dis. 2007;39:293–304. [PubMed: 17331820]

80. Gopinath K, Venkatesh KS, et al. Green synthesis of gold nanoparticles from leaf extract of *Terminalia arjuna*, for the enhanced mitotic cell division and pollen germination activity. Ind Crop Prod. 2013;50:737–42.

81. Yallappa S, Manjanna J, et al. Microwave assisted rapid synthesis and biological evaluation of stable copper nanoparticles using T. arjuna bark extract. Spectrochim Acta A. 2013;110:108–15.

82. Edison TJI, Sethuraman MG. Instant green synthesis of silver nanoparticles using Terminalia chebula fruit extract and evaluation of their catalytic activity on reduction of methylene blue. Process Biochem. 2012;47:1351–7.

83. Sharma J, Gairola S, et al. The treatment of jaundice with medicinal plants in indigenous communities of the Sub-Himalayan region of Uttarakhand, India. J Ethnopharmacol. 2012;143:262–91. [PubMed: 22759701]

84. Ram A, Lauria P, et al. Hypocholesterolaemic effects of *Terminalia arjuna* tree bark. J Ethnopharmacol. 1997;55:165–169. [PubMed: 9080336]

85. Bachaya HA, Iqbal Z, et al. In vitro and in vivo anthelmintic activity of *Terminalia arjuna* bark. Int J Agric Biol. 2009;11:273–8.

86. Phani Kumar G, Navya K, et al. DNA damage protecting and free radical scavenging properties of *Terminalia arjuna* bark in PC-12 cells and plasmid DNA. Free Radic Antioxid. 2013;3:35–9.

87. Dwivedi S. *Terminalia arjuna* Wight &Arn.- a useful drug for cardiovascular disorders. J Ethnopharmacol. 2007;114:114–29.

88. Shengule SA, Mishra S, et al. Anti-hyperglycemic and anti-hyperlipidaemic effect of Arjunarishta in high-fat fed animals. J Ayurveda Integr Med. 2018 Jan-Mar; 9(1):45–52.

89. Mandal S, Patra A, et al. Analysis of phytochemical profile of *Terminalia arjuna* bark extract with antioxidative and antimicrobial properties. Asian Pac J Trop Biomed. 2013 Dec;3(12):960–6.

90. Nikode K, Kuchewar V. Comparative study of Goghrita and Arjun- Ghrita on biological parameters in healthy human volunteers, J of Ayurveda and Hol Med (JAHM). 2018;6(1):13-29.

91. Lal UR, Tripathi SM, et al. HPLC Analysis and Standardization of Arjunarishta An Ayurvedic Cardioprotective Formulation. Sci Pharm. 2009;77:605–16.

92. Wong SK, Kok-Yong C, et al. Animal models of metabolic syndrome: a review. Nutr Metab (Lond). 2016;13:65.

93. Aluefua OF, Chika A, et al. Interactions Between Herbs and Antidiabetic Drugs: A Systematic Review. Res. J. Pharmacol. 2017;11(5-6):6-17.

94. Dhande SR, Bhutkar SP et al. Wadk. Study of pharmacokinetic interaction of arjuna with haritaki and aloe vera. World Journal of Pharmacy and Pharmaceutical Sciences. 2016;5(6).

95. Brantley SJ, Argikar AA, et al. Herb–Drug Interactions: Challenges and Opportunities for Improved Predictions. Drug Metab Dispos. 2014 Mar; 42(3): 301–17.

96. Balasaraswati R, Sadasivam S et al. An antiviral protein from Bougainvillea spectabilis roots; purification and characterisation. Phytochemistry. 1998;47(8):1561-5.

97. Surana SJ, Gokhale RB et al. Antihyperglycemic Activity of Various Fractions of Cassia auriculata Linn. in Alloxan Diabetic Rats. Ind J Pharm Sci. 2008;70:227-9.

98. Shokeen P, Anand P et al. Antidiabetic activity of 50% ethanolic extract of Ricinus communis and its purified fractions. Food Chem. Toxicol. 2008;46:3458-66.

99. Mankil J, Moonsoo P et al. Antidiabetic Agents from Medicinal Plants. Curr. Med. Chem. 2006;13(10):1203–18.

100. Shengule SA, Mishra S, et al. Inhibitory Effect of A Standardized Hydroethanolic Extract of *Terminalia arjuna* Bark On Alpha- Amylase Enzyme. Asian J Pharm Clin Res. 2018;11(4):366-9.

101. Biswas M, Kar B, et al. Antihyperglycemic activity and antioxidant role of *Terminalia arjuna* leaf in streptozotocin-induced diabetic rats. Pharm. Biol. 2011;49:335-40.

102. Chen LH, Liang L, et al. Effects of intrauterine growth restriction and high-fat diet on serum lipid and transcriptional levels of related hepatic genes in rats. Zhongguo Dang Dai Er Ke Za Zhi. 2015;17:1124-30.

103. Kapoor D, Vijayvergiya R, et al. *Terminalia arjuna* in coronaryartery disease: Ethnopharmacology, pre-clinical, clinical & safety evaluation. Journal of Ethnopharmacology. 2014;155:1029-45.

104. Rather RA, Malik VS, et al. Aqueous *Terminalia arjuna* extract modulates expression of key atherosclerosis-related proteins in a hypercholesterolemic rabbit: A proteomic-based study. Proteomics Clin Appl. 2016;10(7):750-9.

105. Sawale PD, Pothuraju R, et al. Hypolipidaemic and anti-oxidative potential of encapsulated herb (*Terminalia arjuna*) added vanilla chocolate milk in high cholesterol fed rats. J Sci Food Agric. 2016;96(4):1380-5.

106. Khaliq F, Parveen A, et al. Improvement in myocardial function by *Terminalia arjuna* in streptozotocin-induced diabetic rats: possible mechanisms. J Cardiovasc Pharmacol Ther. 2013;18(5):481-9.

107. Khaliq F, Parveen A, et al. *Terminalia arjuna* improves cardiovascular autonomic neuropathy in streptozotocin-induced diabetic rats. Cardiovasc Toxicol. 2013;13(1):68-76.

108. Subramaniam S, Ramachandran S, et al. Anti-hyperlipidemic and antioxidant potential of different fractions of *Terminalia arjuna* Roxb. bark against PX-407 induced hyperlipidemia. Indian J Exp Biol. 2011;49(4):282-8.

109. Biswas M, Kar B, et al. Antihyperglycemic activity and antioxidant role of *Terminalia arjuna* leaf in streptozotocin-induced diabetic rats. Pharm Biol. 2011;49(4):335-40.

110. Raghavan B, Kumari SK. Effect of *Terminalia arjuna* stem bark on antioxidant status in liver and kidney of alloxan diabetic rats. Indian J Physiol Pharmacol. 2006;50(2):133-42.

111. Amalraj A and Gopi S. Medicinal properties of *Terminalia arjuna* (Roxb.) Wight & Arn.: A review. J Tradit Complement Med. 2017;7(1):65–78.

112. Toppo E, Sylvester Darvin S, et al. Curative effect of arjunolic acid from *Terminalia arjuna* in non-alcoholic fatty liver disease models. Biomed Pharmacother. 2018;107:979-88.

113. Chauhan P, Pradhan S, et al. Inhibition of lipid and protein oxidation in raw ground pork by *Terminalia arjuna* fruit extract during refrigerated storage. Asian-Australas J Anim Sci. 2018.

114. Kalem IK, Bhat ZF, et al. The effects of bioactive edible film containing *Terminalia arjuna* on the stability of some quality attributes of chevon sausages. Meat Sci. 2018;140:38-43.

115. Swain KK, Mishra PM, et al. Biosorption of praseodymium (III) using *Terminalia arjuna* bark powder in batch systems: isotherm and kinetic studies. Water Sci Technol. 2018;77(3-4):727-38.

116. Chandra Sekhar Y, Phani Kumar G, et al. *Terminalia arjuna* bark extract attenuates picrotoxin-induced behavioral changes by activation of serotonergic, dopaminergic, GABAergic and antioxidant systems. Chin J Nat Med. 2017;15(8):584-96.

117. Bhat OM, Kumar PU, et al. *Terminalia arjuna* prevents Interleukin-18-induced atherosclerosis via modulation of NF-κB/PPAR-γ-mediated pathway in Apo E-/- mice. 2018;26(2):583-98.

118. Dhanani T, Shah S et al. A Validated High- Performance Liquid Chromatography Method for Determination of Tannin- Related Marker Constituents Gallic Acid, Corilagin, Chebulagic Acid, Ellagic Acid and Chebulinic Acid in Four Terminalia Species from India. Journal of Chromatographic Science. 2014;1–8.

119. Saha A, Pawar VM et al. Characterisation of Polyphenols in *Terminalia arjuna* Bark Extract. Indian J. Pharm. Sci. 2012;74(4):339-47.

120. Lal UR, Tripathi SM, et al. HPLC Analysis and Standardization of Arjunarishta-An Ayurvedic Cardioprotective Formulation. Sci Pharm. 2009;77;605–16.

121. Singh DV, Verma RK, et al. RP-LC determination of oleane derivatives in *Terminalia arjuna*. Journal of Pharmaceutical and Biomedical Analysis. 2002;28:447–52.

122. Gupta D, Kumar M. Evaluation of in vitro antimicrobial potential and GC-MS analysis of Camellia sinensis and *Terminalia arjuna*. Biotechnol Rep (Amst). 2016;13:19-25.

123. Singh A, Bajpai V, et al. Profiling of Gallic and Ellagic Acid Derivatives in Different Plant Parts of *Terminalia arjuna* by HPLC- ESI-QTOF-MS/MS. Nat Prod Commun. 2016 Feb;11(2):239-44.

124. Avula B, Wang YH, et al. Metabolic Profiling of Hoodia, Chamomile, Terminalia Species and Evaluation of Commercial Preparations Using Ultrahigh-Performance Liquid Chromatography Quadrupole-Time-of-Flight Mass Spectrometry. Planta Med. 2017;83(16):1297-1308.

125. Zou D, Chen T, et al. An Efficient Protocol for Preparation of Gallic Acid from Terminalia bellirica (Gaertn.) Roxb by Combination of Macroporous Resin and Preparative High-Performance Liquid Chromatography. J Chromatogr Sci. 2016;54(7):1220-4.

126. McMillen IC, Robinson JS. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. Physiol Rev. 2005;85:571-633.

127. McMillen IC, Adam CL, et al. Early origins of obesity: programming the appetite regulatory system. J Physiol. 2005;565:9-17.

128. Ozanne SE, Martensz ND, et al. Maternal low protein diet in rats programmes fatty acid desaturase activities in the offspring. Diabetologia. 1998;41:1337-42.

129. Merezak S, Hardikar AA, et al. Intrauterine low protein diet increases fetal beta-cell sensitivity to NO and IL-1 beta: the protective role of taurine. J Endocrinol. 2001;171:299-308.

130. Gallagher EA, Newman JP, et al. The effect of low protein diet in pregnancy on the development of brain metabolism in rat offspring. J Physiol 2005;568:553-8.

131. Vickers MH, Breier BH, et al. Fetal origins of hyperphagia, obesity, and hypertension and postnatal amplification by hypercaloric nutrition. Am J Physiol Endocrinol Metab. 2000;279:E83-7.

132. Ikenasio-Thorpe BA, Breier BH, et al. Prenatal influences on susceptibility to diet-induced obesity are mediated by altered neuroendocrine gene expression. J Endocrinol. 2007;193:31-7.

133. Steegers-Theunissen RP, Steegers EA. Nutrient-gene interactions in early pregnancy: a vascular hypothesis. Eur J Obstet Gynecol Reprod Biol. 2003;106:115-7.

134. Ganguly P, Alam SF. Role of homocysteine in the development of cardiovascular disease. Nutr J. 2015;14:6.

135. Holemans K, Aerts L, et al. Evidence for an insulin resistance in the adult offspring of pregnant streptozotocin-diabetic rats. Diabetologia. 1991;34:81-5.

136. Aerts L, Van Assche FA. Animal evidence for the transgenerational development of diabetes mellitus. Int J Biochem Cell Biol. 2006;38:894-903.

137. Nasu R, Seki K, et al. Effect of a high-fat diet on diabetic mother rats and their offspring through three generations. Endocr J. 2007;54:563-9.

138. Varghese A, Saboo P, et al. Bioactivity guided fractionation of methanolic extract of *Terminalia arjuna* for its CYP3A and CYP2D inhibition in rat liver microsomes. Biopharm Drug Dispos. 2018;39(3):143-51.

139. Varghese A, Savai J, et al. In Vitro CYP2D Inhibitory Effect and Influence on Pharmacokinetics and Pharmacodynamic Parameters of Metoprolol Succinate by *Terminalia arjuna* in Rats. Drug Metab Lett. 2016;10(2):124-35.

140. Bhamra SK, Slater A, et al. The Use of Traditional Herbal Medicines Amongst South Asian Diasporic Communities in the UK. Phytother Res. 2017;31(11):1786-94.

141. Lal UR, Tripathi SM, et al. HPLC analysis and standardization of arjunarishta – An ayurvedic cardioprotective formulation. Sci Pharm. 2009;77:605-16.

142. Singh DV, Verma RK, et al. RP-LC determination of oleane derivatives in *Terminalia arjuna*. J Pharma Biomed Anal. 2002;28:447-52.

143. Deshpande MS, Tondare PR, et al. Evaluation of antioxidant, anti-inflammatory and adipocyte differentiation inhibitory potential of Ziziphus mauritiana bark extract. Pharmacognosy Journal. 2013;5:205-10.

144. SA Shengule, S Mishra, et al. Phytochemical characterization of ayurvedic formulations of *Terminalia arjuna*: A potential tool for quality assurance. Indian Journal of Traditional Knowledge. 2019;18(1):127-32.

145. Jaiswal S, Mishra S, et al. 2018. Neuroprotective effect of epalrestat mediated through oxidative stress markers, cytokines and TAU protein levels in diabetic rats. Life Sci. 2018;207:364-71.

146. Shengule S, Kumbhare K, et al. Herb-drug interaction of Nisha Amalaki and Curcuminoids with metformin in normal and diabetic condition: A disease system approach. Biomedicine and Pharmacotherapy. 2018;101:591-8.

147. Apostolidisa E, Lib L, et al. In vitro evaluation of phenolic- enriched maple syrup extracts for inhibition of carbohydrate hydrolyzing enzymes relevant to Type 2 diabetes management. J Funct Foods. 2011;3:100.

148. Dada FA, Oyeleye SI, et al. Phenolic constituents and modulatory effects of Raffia palm leaf (Raphia hookeri) extract on carbohydrate hydrolyzing enzymes linked to Type-2 diabetes. J Trad Complem Med. 2017;7:494-500.

149. Al-masri IM, Mohammad MK, et al. Inhibition of dipeptidyl peptidase IV (DPP IV) is one of the mechanisms explaining the hypoglycemic effect of berberine. J Enzyme Inhib Med Chem. 2009;24(5):1061-6.

150. Chitlange SS, Kulkarni Ps, et al. High Performance Liquid Chromatographic Fingerprint For Quality Control Of *Terminalia arjuna* Containing Ayurvedic Churna Formulation. J Aoac Inter. 2009;92:4.

151. Lal UR, Tripathi SM, et al. Chemical Changes During Fermentation Of Abhayarishta And Its Standardisation By Hplc-Dad. Nat Prod Commun. 2010;5(4):575-9.

152. Lal UR, Triptathi SM, et al. HPLC analysis and standardisation of Arjunarishtaa – An Ayurvedic cardioprotective formulation. Scient Pharma. 2009;77:606-16.

153. Dhanani T, Shah S, et al. A Validated High-Performance Liquid Chromatography Method for Determination of Tannin-Related Marker Constituents Gallic Acid, Corilagin, Chebulagic Acid, Ellagic Acid and Chebulinic Acid in Four Terminalia Species from India. J Chromatograph Sci. 2014;1–8.

154. Pascaline J, Charles M, et al. Phytochemical constituents of some medicinal plants used by the Nandis of South Nandi district, Kenya. J Anim Plant Sci. 2011;9:1201-10.

155. Sofowora A. Medicinal plants and traditional medicine in Africa. New York: John Wiley and Sons;1993:191-289.

156. Brown JE, Rice-Evans CA. Luteolin rich artichoke extract protects low density lipoprotein from oxidation in vitro. Free Radic Res.1998;29:247-55.

157. Krings U, Berger RG. Antioxidant activity of roasted foods. Food Chem. 2001;72:223-9.

158. Nema R, Jain P, et al. Antibacterial and antifungal activity of *Terminalia arjuna* leaves extract with special reference to flavanoids. Basic Res J Med Clin Sci. 2012;1(5):63-5.

159. Ali SS, Kasoju N, et al. Indian medicinal herbs as source of antioxidants. Food Res Int. 2008;41:1-15.

160. Benavente-Garcia O, Castillo J, et al. Uses and properties of Citrus flavonoids. J Agric Food Chem. 1997;45(12):4505-15.

161. Marjorie C. Plant products as antimicrobial agents. Clin Microbiol Rev. 1999;12:564-82.

162. Harborne JB. Plant phenolics. In: Bell EA, Charlewood BV, editors. Secondary plant products. Berlin: Verlag Springer;1980:320.

163. Samuel VT, Shulman, GI. The pathogenesis of insulin resistance: integrating signaling pathways and substrate flux. J Clin Invest. 2016;126:12–22.

164. Song S, Andrikopoulos S, et al. Mechanism of fat-induced hepatic gluconeogenesis: effect of metformin. Am J Physiol Endocrinol Metab. 2001;281:E275-82.

165. Bass, N. Fatty acid-binding protein expression in the liver: its regulation and relationship to the zonation of fatty acid metabolism. Molecular and Cellular Biochemistry. 1990;98:167-76.

166. Raposo HF, Paiva AA, et al. Apolipoprotein CIII overexpression exacerbates diet-induced obesity due to adipose tissue higher exogenous lipid uptake and retention and lower lipolysis rates. Nutr Metab (Lond). 2015;12:61.

167. Hiltunen JK, Qin Y. Beta-oxidation-strategies for the metabolism of a wide variety of acyl-CoA esters. Biochim Biophys Acta. 2000;1484:117–28.

168. Roden M, Price TB, et al. Mechanism of free fatty acid-induced insulin resistance in humans. J Clin Invest. 1996;97:2859–65.

169. Koonen DP, Jacobs RL, et al. Increased hepatic CD36 expression contributes to dyslipidemia associated with diet-induced obesity. Diabetes. 2007;56:2863-71.

170. GA Duque and A Descoteaux. Macrophage Cytokines: Involvement in Immunity and Infectious Diseases. Front Immunol. 2014;5:491.

171. Bi Y, Chen J, et al. M2 Macrophages as a Potential Target for Antiatherosclerosis Treatment. Neural Plasticity. 2019;Article ID 6724903:21.

172. Baker RG, Hayden MS, et al. NF-κB, inflammation and metabolic disease. Cell Metab. 2011;13(1):11–22.

173. Cantó C, Auwerx J. PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. Curr Opin Lipidol. 2009;20(2):98-105.

174. Sergi D, Naumovski N, et al. Mitochondrial (Dys)function and Insulin Resistance: From Pathophysiological Molecular Mechanisms to the Impact of Diet. Front. Physiol. 2019.

175. Chen J-H, Martin-Gronert MS, et al. Maternal Protein Restriction Affects Postnatal Growth and the Expression of Key Proteins Involved in Lifespan Regulation in Mice. PLoS ONE 2009;4(3):e4950.

176. Vachharajani VT, Liu T, et al. Sirtuins Link Inflammation and Metabolism. Journal of Immunology Research.2016;Article ID 8167273:10.

177. Zang M, Xu S, et al. Polyphenols stimulate AMP-activated protein kinase, lower lipids, and inhibit accelerated atherosclerosis in diabetic LDL receptor-deficient mice. Diabetes. 2006;55(8):2180-91.

178. Aatsinki S, Buler M, et al. Metformin induces PGC-1α expression and selectively affects hepatic PGC-1α functions. Br J Pharmacol. 2014;171(9):2351–63.

179. Ma X, Wang D, et al. Deciphering the Roles of PPARγ in Adipocytes via Dynamic Change of Transcription Complex. Front Endocrinol (Lausanne). 2018;9:473.

180. Achari AE and Jain SK.Adiponectin, a Therapeutic Target for Obesity, Diabetes, and Endothelial Dysfunction. Int J Mol Sci. 2017;18(6):1321.

181. Castoldi A, de Souza CN, et al. The Macrophage Switch in Obesity Development. Front Immunol. 2015;6:637.

182. Yahfoufi N, Alsadi N, et al. The Immunomodulatory and Anti- Inflammatory Role of Polyphenols. Nutrients. 2018;10:1618.

183. Rahnasto-Rilla M, Tyni J, et al. Natural polyphenols as sirtuin 6 modulators. Sci Rep. 2018;8:4163.

184. Horáková D, Azeem K, et al.Total and High Molecular Weight Adiponectin Levels and Prediction of Cardiovascular Risk in Diabetic Patients. International Journal of Endocrinology. 2015;Article ID 545068:6.

185. Ehsan M, Singh KK, et al. Adiponectin limits monocytic microparticle-induced endothelial activation by modulation of the AMPK, Akt and NFκBsignaling pathways. Atherosclerosis. 2016;245:1-11.

186. Shengule SA, Mishra S, et al. Inhibitory Effect of A Standardized Hydroethanolic Extract of *Terminalia arjuna* Bark On Alpha- Amylase Enzyme. Asian J Pharm Clin Res, 2018;11(4):366-9.

187. Jaiswal P, Kumar P. Alpha amylase inhibitory activity of different extract of *Terminalia arjuna* bark. Curr Trends Biotech Pharm. 2017;11:253-8.

188. Saha S, Verma R. Inhibitory potential of traditional herbs on Î±-amylase activity.

Pharm Bio. 2012;50:326-31.

189. Kamble B, Gupta A, et al. Effects of *Gymnemasylvestre* extract on the pharmacokinetics and pharmacodynamics of Glimepiride in streptozotocin-induced diabetic rats. *Chemico-Biological Interactions.* 2016;245:30-8

ANNEXURE I - PUBLICATIONS

Publications related to Ph.D work

1. Shengule SA, Mishra S, Joshi K, Apte K, Patil D, Kale P, Shah T, Deshpande M, Puranik A. Anti-hyperglycemic and anti-hyperlipidaemic effect of Arjunarisht in high-fat fed animals. J Ayurveda Integr Med. 2018; 9: 45-52.
2. Shengule SA, Mishra S, Patil D, Joshi KS, Patwardhan B. Phytochemical characterization of Ayurvedic formulations of T arjuna: A potential tool for quality assurance. Indian J of Traditional Knowled 2019;18[1]:127-32. [IF 1.061]
3. Shengule S, Mishra S, Bodhale S. Inhibitory effect of a standardized hydroethanolic extract of *Terminalia arjuna* bark on alpha-amylase enzyme. Asian J Pharm Clin Res. 2018; 11(4):366-9.
4. Shengule SA, Mishra S, Joshi K, Apte K, Patwardhan B. Possible mechanism of action of T arjuna bark extract mediated through lipid metabolism-related genes in experimental hyperlipidemia. Fitoterapia [Under Preparation]
5. Shengule SA, Mishra S, Joshi K, Apte K, Patwardhan B. Effect of *Terminalia arjuna* bark extract on hepatic genes in a diabetic rat model: biochemical and molecular mechanism study. [Under Preparation]
6. Shengule SA, Kumbhare K, Patil D, Mishra S, Apte K, Patwardhan B. Effects of *Terminalia arjuna* extract and its formulations on the PK and pharmacodynamics of Metformin in healthy and diabetic rats. [Under Preparation]

Fulltext of published papers were included in the last of the thesis for reference

Oral / Poster presentations:

02/2019 Poster presentation at 23rd National Convention of Society of Pharmacognosy & and International Conference on "New Age Opportunities and Challenges for Quality, Safety and GMP's in Herbal Drug Development". CSIR-NBRI, Lucknow, India

02/2015 Oral presentation at 2nd International Congress of Society for Ethnopharmacology, "validation of medicinal plants and traditional medicine - global perspectives" Nagpur, India

Publications other than PhD work

1. Shengule S, Kumbhare K, Patil D, Mishra S, Apte K, Patwardhan B. Herb-drug interaction of Nisha Amalaki and Curcuminoids with metformin in normal and diabetic condition: A disease system approach. Biomedicine and Pharmacotherapy. 2018; 101:591-8. [IF 3.457]
2. Jaiswal S, TS, Shengule SA. Neuroprotective effect of epalrestat mediated through oxidative stress markers, cytokines and TAU proteins levels in diabetic rats. Life Sci. 2018;207:364-71. [IF 3.234]
3. Cota D, Mishra S, Shengule SA. Beneficial role of *Terminalia arjuna* hydro-alcoholic extract in colitis and its possible mechanism. J Ethnopharmacol. 2019;230:117-125. [IF 3.115]
4. Cota D, Rasal V, MS, Shengule SA. Cardioprotective effect of Oregano oil against doxorubicininduced myocardial infarction in rats. Pharmacognosy Magzine. 2018;14[57]:363-368.[IF 1.525]
5. Shelke S, Shahi S, Jalalpure S, Dhamecha D, Shengule S. Formulation and evaluation of thermoreversible mucoadhesive in-situ gel for intranasal delivery of naratriptan hydrochloride. J Drug Deliv Sci Technol. 2015;29:238-244 [IF 2.606]
6. Cota D, Mishra S, Shengule SA, et al. Assessment of in vitro biological activities of *Terminalia arjuna* and Arjunarishta in IBD and colorectal cancer. Indian J of Expt Biology. [Under Revision]

ANNEXURE II: MATERIALS AND METHODS

2.1. Materials

2.1.1. List of chemicals and materials used for experiments is given below:

Table 2.1. List of chemicals and materials

Sr. No.	Particulars	Make/Source
1	TA bark	procured from the local market of Pune, and authenticated by Agharkar Research Institute (ARI), Pune, India
2	HPLC grade methanol	Merck, India
3	Water	Milli-Q (Millipore)
4	HPLC grade acetonitrile	(Merck)
5	Arjunetin and arjungenin	HPLC phytochemical markers from the Natural Remedies Pvt. Ltd. Bangalore, India.
6	Kits	Crest Biosystem (India)
7	Normal and HFD diets	VRK Nutritional Solutions, Pune, India.
8	Marketed formulations, i.e., Arjunarishta (AA)	(Batch No. GA-06, Sandu Pharmaceuticals Ltd. Goa, India)
9	Arjun Ghritam (AG)	(Batch No. 116, Nagarjun Pharmaceuticals P. Ltd. Ahmedabad, India)

2.1.2. List of equipment used for experiments is given below:

Table 2.2. List of equipment

Sr. No.	Particulars	Make/Source
1	Soxhlet extraction	Borosil
2	Rotary evaporator	Buchi Rotavapor
3	HPLC system	Dionex P680

2.2. Experimental Design

The plan of work was as follows:

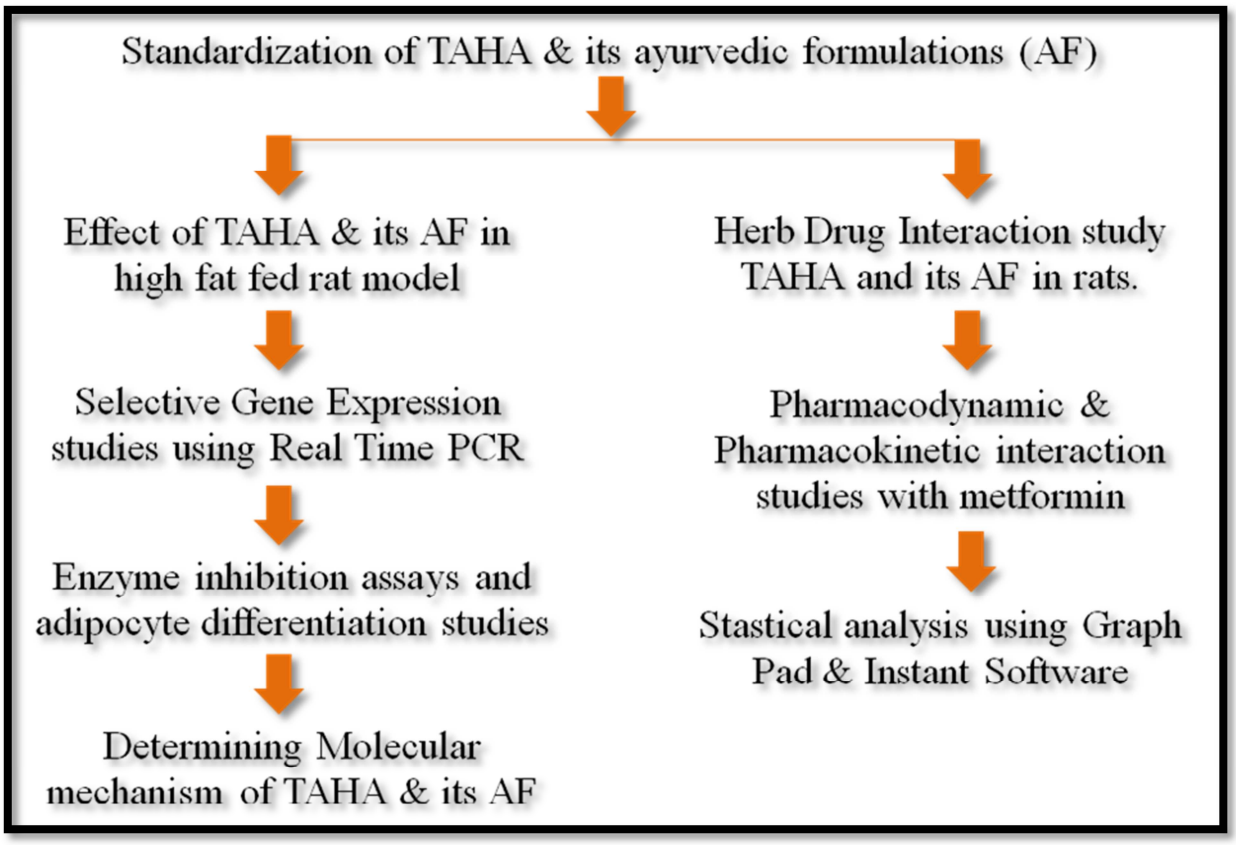

Figure 2.1. Experimental Design

2.2.1. Preparation of Standard

Stock solutions of both phytochemical markers prepared separately in methanol. Final used concentration (1000, 500, 250, 125, 62.5, 31.25 µg/ml) was made up by further diluting with methanol.

2.2.2. Preparation of Sample

AA 5 mL sample was added to a centrifuge tube, and then 10 mL of methanol included and vigorously stirred for 5 minutes then stand for 10 minutes to settle down the precipitated sugars. After 10 minutes, the methanol extract was separated. Then, the separated solution was filtered through a 0.45 µm (Millipore) filter, and 20 µL was used for HPLC analysis. AG 10 mL sample was poured on 10 g silica gel powder, and 20 mL n-hexane was added and vigorously shakes for 5 minutes then stand for 10 minutes to the precipitate the ghritah component and silica gel powder in 50 mL centrifuge tube. After that, n-hexane was pipette out and removed without upsetting silica gel powder. This procedure was repeated thrice. Then add 20 mL acetonitrile (ACN) solvent and vigorously shake for 5 minutes then stand for 30 minutes to settle down the precipitated silica gel powder. After that, ACN was pipetted out. The supernatant (1 mL) was filtered through a 0.45 µm (Millipore) filter, and 20 µL was injected for HPLC analysis.

2.2.3. HPLC System

Conditions

A Dionex P680 HPLC system including autosampler, a Dionex UVD 170U/340U photodiode array detector and thermostatted column compartment (Dionex Corp., Germering, Germany) was used for analysis. The RP C18 BDS Hypersil column (250

× 4.6 mm, 5 µm particle size) from Thermo Corp. (PA). Dionex Chromeleon 6.70 software was used for analysis [141, 142].

2.2.4. Estimation of arjunetin and arjungenin in a TAHA bark extract, AA, AG

The analysis was achieved with an RP C18 BDS Hypersil column (250 × 4.6 mm, 5 µm particle size) at a column temperature 26°C. The separation was performed with the gradient program for pump A (ACN: water, 30:70) and pumped B (ACN: water, 70:30) as follows: initially 30% B, flow rate 0.8 mL/minutes; increased gradually to 50-70% B up to 30 minutes, flow rate 1.2 ml/minutes. Then washed the column for 20 minutes, 30% B, a flow rate of 0.8 ml/minutes. The detection wavelength was used 220 nm, and the 20µL injection volume was used for standard and sample [141-143].

2.2.5. Data Analysis for HPLC analysis

Data analysis was performed by similarity and variations observed in peak areas, spectral patterns, and retention time values of the peaks obtained in the chromatograms of ayurvedic formulations and TA bark extract.

2.3 Method validation

The HPLC analysis method validation was performed according to the industrial guideline for analytical method validation.

2.3.1 Specificity

The specificity of the HPLC analysis method was investigated by analysing samples from at least six different batches to evaluate the potential interferences at the HPLC peak region for analytes. The acceptance criterion for the HPLC analysis method was that at least four out of six batches should have response less than five times of the LLOQ level response in the same solvent.

2.3.2 Linearity

The linearity of the HPLC analysis method was generated by evaluation of five calibration curves containing six different concentrations. The calibration curve for arjunetin and arjungenin (1000, 500, 250, 125, 62.5, 31.25 µg/mL) was demonstrated by plotting the peak area each analyte against the nominal concentration of calibration standards in methanol. This action was repeated on six consecutive days with freshly prepared standards. Each calibration curves were evaluated individually by fitting the area response for analyte as a function of standard concentration, using least square weighted or no weighted ($1/x^2$ or $1/x$) linear regression and excluding the point of origin [144]. The acceptance criteria for each back-calculated standard point were ± 15 % deviation from the nominal value except at LLOQ, which was set at ± 20 %.

2.3.3 Precision and Accuracy of the HPLC method

Arjungenin and Arjunetin samples (at a concentration of 1000, 500, 250, 125, 62.5, 31.25 µg/ml) were prepared. Inter- and intra-day assays were repetitively performed on the samples at six different days and same day for three different times, respectively. The precision was evaluated by calculating the % CV of variances intra- and inter-day while accuracy was calculated from the nominal concentration (C_{nom}) and the mean value of observed concentration (C_{obs}) as follows: accuracy (bias, %) = $[(C_{nom} - C_{obs}) / C_{nom}] \times 100$.

2.4 Animal study

2.4.1 Animals and experimental design:

Male Wistar (MWR) and IUGR rats of 8-10 weeks were purchased from the APT Testing and Research Pvt. Ltd. (Previously known as National Toxicology Centre), Pune, India. The Institutional Animal Ethics Committee approved the preclinical

experiment. Rats were acclimatized at a temperature of 25 °C with 40-65% humidity and a photoperiod of 12 hr day/night cycle. Food and water were supplied ad libitum.

General procedures

After acclimatization for a week, the rats were randomly divided into ten groups, as follows:

Normal Group: MWR + Normal Diet + Water (n=6)

HFD Control Group: IUGR + HFD Diet + Water (n=6)

MET Group: IUGR + HFD Diet + MET (n=6)

TAHA250 Group: IUGR + HFD Diet + TAHA 250 $mg.Kg^{-1}$ (n=6)

TAHA500 Group: IUGR + HFD Diet + TAHA 500 $mg.Kg^{-1}$ (n=6)

AA0.9 Group: IUGR + HFD Diet + AA 0.9 $ml.Kg^{-1}$ (n=6)

AA1.8 Group: IUGR + HFD Diet + AA 1.8 $ml.Kg^{-1}$ (n=6)

Ghee Group: IUGR + HFD Diet + Ghee 900 $mg.Kg^{-1}$ (n=6)

AG450 Group: IUGR + HFD Diet + AG 900 $mg.Kg^{-1}$ (n=6)

AG900 Group: IUGR + HFD Diet + AG 450 $mg.Kg^{-1}$ (n=6)

The drugs were administered once a day. The duration of the animal experiment was 90 days. Oral glucose tolerance test (OGTT) was performed on day 88. Rats were fed a regular diet and HFD containing 13.8% of protein, 4.4% of fibre, 5.1% of minerals and vitamins, 48.7% of carbohydrates and 28% of fat. The weight of the rats was

measured at the beginning and every week until the end of the experiment. Blood samples were obtained through cardiac puncture under general anaesthesia before rat sacrifice. Plasma samples were obtained by centrifugation at 2,000 x g for 15 min and used for laboratory analysis. Liver samples were stored at -80 °C for isolation of total RNA.

Table 2.3. HFD-composition

Ingredient	Grams
Casein, Lactic, 30 Mesh	200.00 g
Cystine, L	3.00 g
Lodex 10	125.00 g
Sucrose, Fine Granulated	72.80 g
Solka Floc, FCC200	50.00 g
Lard	245.00 g
Soybean Oil, USP	25.00 g
Mineral	50.00 g
Choline Bitartrate	2.00 g
Vitamins	1.00 g
Dye, Blue FD&C #1, Alum. Lake 35-42%	0.05 g

2.4.2. Plasma lipid profiles

TGs, TC, and HDL were measured using the commercial kit (Crest Biosystem, India) [88].

2.4.3. Oral glucose tolerance test (OGTT)

The last oral dose of vehicle, TAHA or MET was administered 24 hr before the OGTT. Rats were food-deprived for 12 hr, and a glucose load of 2 g/kg was administered orally. The glucose levels were measured using a glucometer (AccuChek Active, Roche Diagnostics USA). The blood was collected through the tail vein, before and at 60 minutes, 120 minutes, 180 minutes, and 240 minutes after glucose dose and the area under the curve (AUC) of blood glucose were used to compare differences in the glucose excursion curves among groups. The trapezoidal method was used to calculate the total area under the blood glucose curve [88].

2.4.4 Gene expression related results

2.4.4.1 RT-PCR analysis

After sacrifice, rat livers were removed and stored at −80 °C for total RNA extraction using the TRI reagent (Sigma-Aldrich). Total RNA was extracted from liver samples, as stated before [88]. Total RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, USA). For RT-PCR analysis, First strand cDNA was synthesized using High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, USA). RT-PCR was carried out in a 20 μL volume reaction containing each primer, 50 ng of cDNA and ten μL of Power SYBR Green Master Mix (Applied Biosystems, USA). Gene expression was normalized using the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Each sample was analyzed in duplicates, and the relative transcript quantity was calculated according to the method of $2^{-\Delta\Delta CT}$. All primers were obtained from IDT-Integrated DNA Technologies, Inc (Coralville, IA, USA).

Table 2.4. Primer details

Gene Name		Forward Primer 5'-3'	Reverse Primer 5'-3'	Product size
Apolipoprotein C-III	APOC III	GGCCACCACAGCTATATC AGACTC	GATCCTTGCTGCTGGGCTC TA	103
Acyl-Coenzyme A oxidase 1	ACO-A oxidase1	AGGTGCGGTCGGGGAAGT T	GCTGTGGCTGGATCCGCT G	77
Stearyl-Coenzyme A desaturase	SCD-1	AGATCCTCCCTACCTCCAC CCCTA	CCCTCTCGTTCAGTGGTTG CCTC	164
Phosphoenolpyruvate carboxykinase	PEPC K	GGAGTGGATGTTCGGACG CA	TCCGAACAGCTCCTCCAC GT	126
Fatty acid binding protein	FABP	ATGTGTGATGCCTTTGTGG GGA	TACTCTCTGACCGGATGAC GACC	169
Carnitine palmitoyltransferase	CPT-1a	CCTGTGAATACTTGGGCG TCTG	TCCCCAGGCCTAACCATTC C	142
Fatty acid translocase	CD-36	GCTGCACGAGGAGGAGAA TGG	CCAAACACAGCCAGGACA GCAC	84
Glycerol 3-phosphate dehydrogenase	GAPD H	CATCGTAGGCTCCGGCAA CT	AGTTAACTTTCTGCCCCCG ATG	132
Glucose-6-phosphatase	G6P	CAAGAGCTGCAAAGGAGA ACTG	TCAGCGAGTCAAAGAGAT GCAG	94
Aldose reductase	AR	TCCCCAGACAGGCCTAGT GC	CGGCCACGTTCCTCTCTAT ATG	135
Glucokinase	GK	ATGAGGCGATGGGACACG AA	CGGTAGTTGGGTCGACAG CA	131

Mitogen-activated protein kinase p38delta	MAPK	CGGAGATGACTGGCTATGTGGTG	TGATGCAGCCAACAGACCAGATA	107
Phosphatidylinositol 3 kinase	PI3K	CCAGGAGCGGTACAGCAAAG	AGTCTCCTCCTGCTGTCGATGA	134
C-reactive protein	CRP	AGTCTGCAGAAGGGCTACATTGTG	TCCCACCAAAGACTGATTCGC	104
Insulin growth factor - binding protein	IGF-BP	GCTGCTCTTGCTGCTGCTGT	CTCTCGCACCAAGCTCGGC	147
Fatty acid synthase	FAS	CAACCTGATAGTGAGCGGGAAAG	AGACAGACTCGGACTCGGCG	100
sn-glycerol-3-phosphate acyltransferase	GPAT	AATGCTGCGGAAAAACTACGG	TGAAGGAAGGATTGCTGGTAACA	144
SREBF chaperone	SREBF	GGGAAGTACAGTGGGGTCAGCC	TCGGAGGCTGCAGTTGGGG	149
Stearoyl-coenzyme A desaturase 1	SCD-1	CCACAACTACCATCACGCCTTCC	CAGTCGATGAAGAACGTGGTGAAG	86
Squalene synthetase	SS	GAGAGCAAGGAGAAGCACCGA	CCCACCAGTCCAGCAACATAGT	202
Monocyte chemoattractant protein-1	MCP-1	TGCTGTCTCAGCCAGATGCAGTTA	TACAGCTTCTTTGGGACACCTGCT	100
Interleukin 1 beta	IL-1β	AGCAGCTTTCGACAGTGAGGAGAA	TCTCCACAGCCACAATGAGTGACA	144
Tumor Necrosis Factor	TNF-α	AGAACAGCAACTCCAGAACACCCT	TGCCAGTTCCACATCTCGGATCAT	149
Protein Kinase AMP-Activated, Alpha-2	PRKAA-2	AAGCATCGATGATGAGGTGGTGGA	AGACAGTGAATGGTTCTCGGCTGT	107

Sirtuline-1	Sirt-1	ACAACCTCCTGTTGGCTG ATGAGA	TCACTAGAGCTTGCGTGTG ATGCT	134
Interleukin-6	IL-6	AACTCCATCTGCCCTTCAG GAACA	AAGGCAGTGGCTGTCAAC AACATC	131

2.4.4.2 Herb-drug interaction study

Male Wistar rats (160 and 200 g) were supplied by National Toxicology Centre, Pune, India. The animals were housed in standard conditions of temperature (22 ± 5°C), humidity (55 ± 15 %), in 12 h light-dark cycles and fed with standard laboratory animal pellet diet as well as tap water, *ad libitum*. The Institutional Animal Ethics Committee approved the protocol for the study of National Toxicology Centre, Pune, India.

2.4.4.3 Animal study protocol

A study was performed to evaluate the effects of TAHA extract, AA and AG formulations on pharmacokinetic of Metformin in healthy and diabetic Wistar rats. The study was performed into two parts, i.e., healthy and diabetic animals. Forty healthy rats were randomly divided into their respective treatment groups (Eight rats in each group). The detailed study groups were as follows:Group I: MWR+Vehicle (distilled water); Group II: MWR+MET (200 mg/kg; p.o.); Group III: MWR+TAHA (500 mg/kg; p.o.); Group IV:MWR+AA (1.8 ml/kg; p.o.); Group V:MWR+AG (0.9 g/kg; p.o.). The above animals in group I to V were treated with their respective treatments from day 1–7. From day 8–15, all animals except vehicle were co-administered with MET tablet (200 mg/kg; p.o.). To avoid any physicochemical influence on GLM absorption, GLM was given to all the animals by gavage an hour later.

Antidiabetic and hypolipidemic activities of AA were evaluated at the dose of 1.8 ml/kg once daily, which was extrapolated from the adult dose commonly used in humans [88]. According to the dose translation from animal to human studies, we used the body surface area (BSA) normalization method to convert the dose from human to rat. Rats were fasted for 12 h before the test parameter evaluation and taking final blood samples, but they were allowed free access to tap water. The duration of the experiment was 16 days. Oral glucose tolerance test was performed on day 15. On day 16 of the experiment, heparinised blood samples were drawn by exsanguinations from barbiturate-anaesthetized animals. Plasma was separated immediately by centrifugation.

The time intervals were selected with commonly used strategies for pharmacokinetic studies knowing the total blood consumption should not be over 20 % of the body blood volume.

Animals were anaesthetized with ketamine-xylazine anaesthesia, and blood was collected from the retro-orbital route (0.3 mL) in EDTA vacutainer (3 mL, BD Biosciences). After drug administration, eight animals were further subdivided into two groups with four animals each. The sample size was calculated as per the resource equation method [146]. Sampling intervals were collected at 0, 0.25, 1, 4, 8 and 24 h interval in the first subgroup and 0, 0.5, 2, 6 and 12 h in the second subgroup. Animal blood samples were centrifuged at 8000 g for 15 min at 4°C to obtain the plasma which was stored at -20 °C until analysis using HPLC-PDA method. Body weight was monitored on day 0, 7, 14, and 16. Other parameters such as FBGL, TG, HDL and TC were measured on the 16^{th} day.

2.4.4.4 Induction of Diabetes

The overnight fasted rats were induced diabetes by single intraperitoneal injection of freshly prepared STZ (45 mg/kg BW) in 0.1M citrate buffer (pH 4.5) in a volume of 1 mL/kg BW and control normal rats (NC group, n=8) received vehicle (citrate buffer). The fasting glucose levels were assessed by tail vein puncture method using one-touch glucometer (Accu-Chek Active). The inception value of fasting glucose level to diagnose diabetes was considered to be more than 250 mg/dL after 72 h and used for HDI study. Forty diabetic rats were randomly divided into their respective treatment groups (Eight rats in each group) as per healthy rat study.

2.4.4.4.1. Oral glucose tolerance test (OGTT)

On completion of 16 days of dosing, all rats were fasted for 14 h before being subjected to OGTT by administering glucose solution 2 g/kg by the oral route. Blood samples were obtained through a cut-tail method at 0, 60, 120, and 180 minutes for glucose determination [146]. The trapezoidal method was used for the calculation of the total area under the blood glucose curve. Blood glucose content was measured using a commercial glucometer (Counter, Bayers).

2.4.4.4.2. Bodyweight

All animals were monitored for body weight during the treatment period.

2.4.4.4.3. Biochemical Analysis

At the end of the treatment period, after overnight fasting and under anaesthesia with ketamine: xylazine (65:10 mg/kg), the blood was collected in EDTA tubes from the animals' heart. The Plasma was separated by centrifugation at 8,000 rpm for 10 min from the collected blood samples and analyzed for total cholesterol (TC), HDL-C and TG levels by using diagnostic kits (Crest Biosystem, India).

2.4.4.4.4. Histopathology:

After blood collection, animals were sacrificed by cervical dislocation under anaesthesia at the end of the experimental period followed by fixation of liver and kidney in 10% neutral buffered formalin for routine histopathological examination. The formalin-fixed tissues were dehydrated in a graded series of ethanol and embedded in paraffin wax before sectioning. The block of tissues was sectioned, stained with haematoxylin and eosin (H&E) and examined under a light microscope.

2.4.4.4.5. HPLC-PK method

Previously reported HPLC-PDA method was modified and used for the analysis of metformin from rat plasma, using ranitidine as internal standard (IS). The suitability of the method was justified by validating the bioanalytical method in terms of selectivity, linearity, sensitivity, precision, accuracy and recovery following USFDA Guidance for Industry for bioanalytical method validation. Blank rat plasma was spiked with working solutions of metformin to prepare calibration standards at concentrations 0.8, 1.6, 3.2, 6.4, 12.8, 25.6 and 51.2 µg/mL. Similarly, quality control samples were prepared in six to replicate each at 0.8 (LQC), 6.4 (MQC) and 51.2 (HQC) µg/mL.

100 µL plasma sample was vortex mixed with 20 µL of internal standard solution (30µg / mL ranitidine in methanol) followed by addition of 100 µL of 8M NaOH and 1.3 mL of 1-butanol/n-hexane (50:50, v/v) to the tube followed by 2 min shaking. After centrifugation at 10,000 g for two min., the organic layer was separated. Then, 100 µL of 1% acetic acid was added, and the mixture was a vortex, followed by centrifugation for 2 min. The organic phase was removed, and 50 µL volume of the

aqueous phase was injected into the HPLC system (Dionex) with a gradient pump having a photodiode array detector.

Metformin and internal standard were eluted on a Hypersil Gold phenyl column (250mm×4.6 mm, 5µm: Thermo Fisher Scientific, Mumbai, India) by using acetonitrile and phosphate buffer (0.020 M; pH 7.0 with 35: 65 v/v) as mobile phase in an isocratic elution program of 10 minutes duration. The flow rate was 1.5 mL/minutes. The column and autosampler were maintained at 40 °C and 4 °C, respectively. The selectivity of the HPLC analysis method was evaluated by analyzing the plasma sample of 7 different untreated animals along with LOQ sample in six replicate. The precision of the HPLC analysis method was evaluated by analyzing the quality control samples in six replicates in two different days along with freshly spiked calibration standards. Recovery of the method was evaluated at LQC level by comparing the instrument response of analyte and IS in extracted plasma sample with the instrument response of same in unextracted (Neat) sample. Data acquisition was performed with Chromeleon software.

2.5 Enzyme Inhibition Assay

2.5.1 Assay of alpha-amylase inhibition

A mixture of 50 µL of extract or acarbose and 50 µL 0.02 mol sodium phosphate solution (pH 6.9 with six mmol sodium chloride) containing alpha-amylase solutions (13 U/mL) were incubated for 10 minutes at 25°C. After pre-incubation, 50 µL 1% soluble starch solution in 0.02 mol sodium phosphate solution (pH 6.9 with six mmol NaCl) was added to each well at timed intervals, then incubated for 10 minutes at 25°C, followed by addition of 1 ml dinitrosalicylic acid colour reagent. Then, the test tubes were placed in hot water (at 80°C for 10 min) to stop the reaction. The reaction

mixture was diluted with 1 ml distilled water and absorbance was read at 540 nm [147, 148].

Each experiment was repeated three times, and the data averaged for reporting. All values were expressed as mean ± standard deviation (SD). The inhibition percentage was estimated using the following formula:

% Inhibition = [(Abs control - Abs sample)/Abs control] *100

The IC50 values were determined through nonlinear regression by fitting to a sigmoid dose-response equation with variable slope using GraphPad Prism5 software (GraphPad Software, Inc. La Jolla, CA, USA).

2.5.2 DPP-IV inhibition assay

DPP-IV assay was performed in triplicate following the modified method of Al masri et al. [149]. In a 96-well titer plate reader, the chromogenic substrate is cleaved by the serine protease DPPIV resulting in the release of para-nitroaniline (pNA), a yellow coloured product (measured at 405 nm). Sitagliptin was diluted to various concentrations (1, 5, 10, 20, 40, 80, 100 nM) using Tris–HCl buffer (50 mM, pH 7.5) and the final volume was made to 35 µL. Absorbance was measured at 405 nm in a multiplate reader (Bio-TEK, USA). 15 µL of the DPP-IV enzyme (0.05 U/mL) was added to the above mixture. One unit enzyme activity was mentioned as the amount of enzyme that catalyzes the release of 1 µmol pNA from the substrate/min under assay conditions.

After adding the enzyme, the mixture was pre-incubated for 10 min at 37 ◦C to enhance the binding capacity of the inhibitor. This was followed by addition of 50 µL of Gly-pro-p-nitroanilide (GPPN 0.2 mM in Tris–HCl) as a substrate and incubated at

37 ◦C for 30 min. The reaction was terminated using 25 µL of 25% glacial acetic acid, and the absorbance was measured at 405 nm using a microtiter plate reader. Experiments were performed in triplicates.

The results obtained were compared with the negative control (no inhibitor). The TAHA extract, AA and AG (100 mg) was dissolved in dimethyl sulphoxide (DMSO) (0.5%) 10 mL of distilled water to make a stock concentration of 10 mg/mL. From the stock, the following working concentrations (0.1, 0.5, 1, 2, 4, 8 and 10 mg/mL) were prepared. 20 µL of each of above stock concentrations was made to 35 µL using Tris–HCl buffer (50 mM, pH 7.5) to obtain final inhibitory concentrations of 10, 50, 100, 200, 400, 800 and 1000 µg/mL, respectively, in a total well volume of 100 µL. The assay was performed in triplicates according to the standardized procedure of sitagliptin. The hydrolysis of the substrate was monitored at 405 nm wavelength using a Microplate reader from Electronics Corporation of India Limited. The activity was expressed as DA 405 nm/min. The experiment was performed in triplicate and compared with negative control (enzymatic solution without inhibition), while standard DPP-IV inhibitor drug sitagliptin employed as a positive control.

2.6.3 Adipocyte differentiation assay

The ABTS, IBMX, Insulin, Dexamethasone, Cerulenin, Glucose and Carrageenan were obtained from Sigma-Aldrich Chemical Co., USA. All chemicals used were of Analytical grade and purchased from a local supplier. Cultures of 3T3-L1 preadipocytes (ATCC no. CL-173) were obtained from National Centre for Cell Science, Pune, India.

In brief, 3T3-L1 preadipocyte was seeded as 5 X 10^3 cells/well in 24 well plates. After reaching the confluence (48 h), the preadipocytes were induced by IBMX + insulin (10 mg/mL) medium to differentiate to adipocytes for another 48 h. The TAHA, AA, AG and Cerulinin (a known anti-differentiating agent) were added at different concentration and incubated at 37 °C under humidified 5% CO_2 atmosphere. The medium was then changed every two days with DMEM containing 10% FBS and five µg/ml insulin. On day 6, the medium was removed entirely for triglyceride estimations. Formaldehyde was added slowly to each well and kept for 30 min at room temperature. Formaldehyde was then aspirated, and Oil Red O solution (0.5 g/100 µL isopropanol) was added in each well and incubated for 1 h at room temperature. The stain was removed entirely and washed with distilled water twice. After drying the plate, photographs were taken. The Oil Red O stained oil droplets were extracted in Isopropyl alcohol and absorbance was determined spectrophotometrically at 520 nm [143].

3. STATISTICAL ANALYSIS

The experimental results were represented as the mean ± standard deviation (SD) for six rats in the group. One-way analysis of variance (ANOVA) with Dunnett's multiple comparison tests were used to evaluate experimental data. Differences were believed significant at $p<0.05$. Statistical analyses were performed using Prism, GraphPad 5 Software (San Diego, USA).

Results were expressed as mean±SEM. The PK parameters such as the area under the plasma concentration-time curve (AUC), terminal elimination half-life ($t_{1/2}$) and oral clearance (CL) were estimated. The maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were obtained directly from the plasma concentration-time

curve. The area under the plasma MET concentration-time curve (AUC) up to the last concentration measured (AUC_t) was determined using the linear trapezoidal rule. The AUC was extrapolated to infinity AUC_∞ using C_t/K_{el}, where C_t is the last measured MET concentration, and K_{el} is the elimination rate constant determined from the terminal slope of the log concentration-time plot. Half-life ($t_{1/2}$) was calculated as $0.693/K_{el}$. The CL was calculated as dose/AUC_∞ [28]. All values were expressed as mean ± SD. Results were evaluated using one-way ANOVA, followed by Dunnett's post-hoc test to determine significant differences among the means. Differences were believed significant at $p<0.05$. GraphPad Prism version 5.0, GraphPad Software Inc., was used for statistical analysis.

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Original Research Article (Experimental)

Anti-hyperglycemic and anti-hyperlipidaemic effect of *Arjunarishta* in high-fat fed animals

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ABSTRACT

Background: *Arjunarishta* (AA), a formulation used as cardiotonic is a hydroalcoholic formulation of *Terminalia arjuna* (Roxb.) Wight and Arn. (TA) belonging to family Combretaceae.
Objective: To evaluate the anti-hyperglycemic and anti-hyperlipidemic effect of *Arjunarishta* on high-fat diet fed animals.
Materials and methods: High-fat diet fed (HFD) Wistar rats were randomly divided into three groups and treated with phytochemically standardized *Arjunarishta* (1.8 ml/kg), and hydroalcoholic extract of *T. arjuna* (TAHA) (250 mg/kg) and rosuvastatin (10 mg/kg), for 3 months. Intraperitoneal glucose tolerance test, blood biochemistry, liver triglyceride and systolic blood pressure were performed in all the groups. Effect of these drugs on the expression of tumor necrosis factor-α (TNF-α) and insulin receptor substrate-1 (IRS-1) and peroxisome proliferators activated receptor γ coactivator 1-α (PGC-1α) were studied in liver tissue using Quantitative Real-time PCR.
Results: HFD increased fasting blood glucose, liver triglyceride, systolic blood pressure and gene expression of TNF-α, IRS-1 and PGC-1α. Treatment of AA and TAHA significantly reduced fasting blood glucose, systolic blood pressure, total cholesterol and triglyceride levels. These treatments significantly decreased gene expression of TNF-α (2.4, 2.2 and 2.6 fold change); increased IRS-1 (2.8, 2.9 and 2.8 fold change) and PGC-1α (2.9, 3.7 and 3.3 fold change) as compared to untreated HFD.
Conclusion: Anti-hyperglycemic, anti-hyperlipidemic effect of *Arjunarishta* may be mediated by decreased TNF-α and increased PGC-1α and IRS-1.

1. Introduction

Natural products have been considered the leading source of treatment for several human diseases due to their wide chemical diversity and biological actions. Diabetes remains the most common metabolic disorder, generally differentiated by hyperglycemia with disturbance of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, action or both [1,2].

Type 2 diabetes (T2DM) is associated with abnormal glucose utilization, insulin resistance, and hyperlipidemia. Lipoprotein abnormalities are the major cause of cardiovascular (CV) morbidity and mortality in diabetes patients who require additional lipid-lowering agents along with their antidiabetic medications while, available antidiabetic agents do not exert a favourable effect on lipid abnormalities [3]. The commonly used anti-hyperlipidemic agents such as statins adversely manipulate glycemic control. Due to this reason, the need for novel treatment strategies which offer more benefits to patients suffering from T2DM was created, focusing on treatments that produce better glycemic control, appetite regulation, blood pressure, lipid reduction and weight loss. Also, there has been a growing emphasis on therapy of

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hyperlipidemia associated with diabetes to develop newer antidiabetic agents with potential anti-hyperlipidemic effects [4].

Traditional Indian Medicinal System (TIMS) utilizes a large number of plants and derived preparations, which are often difficult to characterize through phytochemical, pharmacological, and toxicological investigations. *Terminalia arjuna* (Roxb.) Wight and Arn. (TA) and its traditional preparations were used in Ayurveda for their cardiotonic and antidiabetic effects for many years [5,6]. Several clinical investigations have suggested its usefulness in relieving angina pain and in the treatment of coronary artery disease, heart failure and possibly hypercholesterolemia [7–9]. Raghavan and Krishnakumari have provided evidence for the beneficial effect of hydroalcoholic bark extract of TA (TAHA) in relation to the treatment of diabetes, reporting a reduction in serum glucose levels as well as protection against the destruction of pancreatic beta cells and kidney damage in animals with alloxan-induced diabetes [10].

Few studies have been undertaken to understand the mechanism of this plant in diabetes and its related complications [11]. Earlier studies indicated that the anti-inflammatory and antioxidant activities of TA could be the one of the mechanisms behind its antidiabetic effects [12]. Till date, the exact molecular mechanism of TA bark in diabetes is unknown. Chemically, TA contains polyphenols such gallic acid, ellagic acid, and triterpenoids like arjunolic acid, arjunic acid, arjunetin, arjungenin, arjunglucoside I and II [13].

In Ayurveda, *Arjunarishta* (AA) is an ancient hydroalcoholic Ayurvedic formulation having the highest percentage of TA and used for the treatment of CVD. It nourishes and strengthens the heart muscle and promotes cardiac functioning by regulating blood pressure and cholesterol [14].

Therefore, the present study was undertaken to evaluate the anti-hyperglycemic and anti-hyperlipidemic effects of phytochemically standardized AA in high-fat diet fed (HFD) animals.

2. Materials and methods

2.1. Test materials, extraction, and formulation

The dried bark of TA was purchased from Trimurti Traders, Pune, India. A botanist authenticated the plant material from Agharkar Research Institute, Pune, India. The sample was deposited at Agharkar Research Institute, Pune, India with voucher specimen no. S/B-109. The dried bark was extracted with ethanol : water (70:30 v/v) using Soxhlet extractor for 3 consecutive days at 65 °C. The extract was dried under vacuum using rotary evaporator at 45 °C. AA; traditional formulation containing TA was procured from the local market (Batch no. 25; Sandu Pharmaceuticals Ltd., Mumbai, India).

2.2. Chemical characterization of selected test materials using HPLC-PDA analysis

Chemical characterization was carried using polyphenolics such as gallic acid, ellagic acid, and quercetin by HPLC method [15]. The method was modified as per laboratory conditions [15,16]. Prominence HPLC system (Schimatzu, Japan) equipped with the binary pump, autosampler, a column oven and a photodiode array detector was used. Chromatographic separations were carried out using C-18 analytical column (150 × 4.6 mm, 5 μm particle size; Syncronis, Thermo Scientific, USA). Gradient elution with water containing 0.5% acetic acid as component **A** and acetonitrile : water containing 0.5% of acetic acid (80:20 v/v) as component **B** were used.

The non-linear gradient elution program: 0–10 min 10% of B; 10–20 min 20% of B; 20–30 min 40% of B; 30–40 min 60% of B; 40–45 min 70% of B; 45–55 min 10% of B and equilibrated with initial conditions for another 5 min. The flow rate and oven temperature were used at 1 ml/min and 25 °C respectively. All chromatograms were monitored at 270 nm. The method was validated for linearity, accuracy, and precision.

2.2.1. Reference compound preparation

Each reference compound (10 mg) was dissolved in 10 ml of methanol. Serial dilutions were carried out from the working stock solution in methanol (600 μg/ml). Calibration curves were plotted from concentration range of 3.125–100 μg/ml in triplicate.

2.2.2. Sample preparation

Sample preparation was done as per previously reported method [15]. Briefly, 10 mg of TAHA extract was dissolved in 10 ml methanol, and the solution was filtered through 0.45 μm membrane filter. AA (1 ml) was dried on a rotary evaporator for 0.5 h, and 5 ml of methanol was added. It was sonicated for 10 min and then centrifuged at 3000 rpm for 10 min. The supernatant (1 ml) was passed through 0.45 μm membrane filter. Sample solutions of 20 μl were used for HPLC analysis. The peaks of ellagic acid, gallic acid, and quercetin were identified by comparing their retention time values and UV spectra with those of standards. The marker contents for TAHA and AA were expressed as mg/g and μg/ml respectively.

2.3. Study animals and ethics committee approval

Male Wistar rats, weighing from 200 to 250 g body weight were used in this study. Animals were procured and maintained at National Toxicology Centre, APT Research Foundation, Pune, India. They were housed in makrolon cages under standard laboratory conditions (light and dark cycle of 12 h at temperature 21 ± 2 °C with a relative humidity of 55 ± 10%). The animals were fed with commercial HFD (45%) procured from VRK Nutritional Solutions, Pune, India. The composition of HFD is mentioned in Supp. Table no. 1. All animals had free access to water during the experimental period. The study protocol was approved by Institutional Animal Ethics Committee (Certificate was attached as Supp. Figure. No. 1). All institutional and national guidelines for the care and use of laboratory animals were followed.

2.4. Experimental design

Forty animals were randomly divided into five groups containing eight animals in each group. The detailed study groups were as follow: (Group I) Normal Diet + Vehicle (distilled water), (Group II) HFD + Vehicle (distilled water), (Group III) HFD + Rosuvastatin (10 mg/kg), (Group IV): HFD + AA (1.8 ml/kg), (Group V) HFD + TAHA (250 mg/kg).

According to the conversion table based on surface area, the adult human dose multiplied by 0.018 gave the dose for rat weighing 200 g [17]. The dose was selected based on adult human dose (20 ml per day). It was converted using USFDA guidance considering the weight of rat as 200 g. Animals were studied for glucose tolerance using intraperitoneal administration of glucose (2 g/kg). Blood was collected for evaluation of serum biochemical parameters such as total cholesterol (TC), triglyceride (TG) and high density lipoproteins (HDL). The animals were then sacrificed, and liver tissue was collected and stored at −70 °C for RNA isolation. RNA was isolated for gene expression studies.

2.5. Collection of blood samples and lipid profile test

The blood samples were collected on the 90th day from the retro-orbital venous plexus of rats without any coagulant for the

separation of serum. Serum samples were analyzed for biochemical parameters with the help of commercially available kits produced by Crest Biosystem, Pune, India (Crest Biosystems a division of Coral Clinical Systems is a part of the innovative Tulip Group of Companies). Low density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C) were calculated by using Friedewald's equation [18].

2.6. Intraperitoneal glucose tolerance test (IPGTT)

Animals were fasted overnight before IPGTT. An IPGTT was performed on male Wistar rats 90 days after the onset of treatment. Rats were injected with D-glucose at 2 g/kg body weight. Blood samples were drawn from the tail vein before glucose administration at 60, 120 and 180 min thereafter. Blood glucose content was measured using a commercial Glucometer (Contour, Bayers). The results were expressed as area under the curve ($AUC_{0-180\ min}$) calculated by using GraphPad Prism 5.00 Software [San Diego, USA] [19,20].

2.7. Systolic blood pressure determination

Systolic blood pressure was measured in consciously restrained animals with a tail-cuff sphygmomanometer, using a non-invasive blood pressure instrument (AD Instrument, Australia). Thirty minutes before the measurements, the animals were placed into restrainers, with the tail exposed. The tail cuff was fitted to the base of the tail, and the pulse sensor was place just behind the tail cuff. The pressure in the occlusion cuff and the pulse signal were recorded in a Power Lab/400 system. The initiation of the pulse signals, after the inflation peaks, was correlated with the pressures in the occlusion cuff to obtain the mean systolic blood pressure readings for each animal.

2.8. TG levels in liver, peritoneal and epididymal tissue

TG was extracted from liver, peritoneal and epididymal tissue overnight in 500 μl heptane-isopropanol (3:2) at 4 °C. TG content was measured using a colorimetric kit as described above. The organic layer was collected and dried. The residue was dissolved in isopropanol for measuring triglyceride content with a colorimetric kit. All values of tissue triglyceride content were corrected for respective tissue weight and intra-assay co-efficient variation was 7.8% [21].

2.9. Hematology

To evaluate the toxicological effect of chronic supplementation of TAHA and AA, hematological parameters such as white blood cells (WBC), red blood cells (RBC), platelet count, hemoglobin (Hb) level, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV) were determined by the use of automated hematological analyzer.

2.10. RNA isolation and real-time PCR analysis

Total RNA was isolated using the TRI reagent (Sigma–Aldrich), according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed into first-strand cDNA (ABI) following the

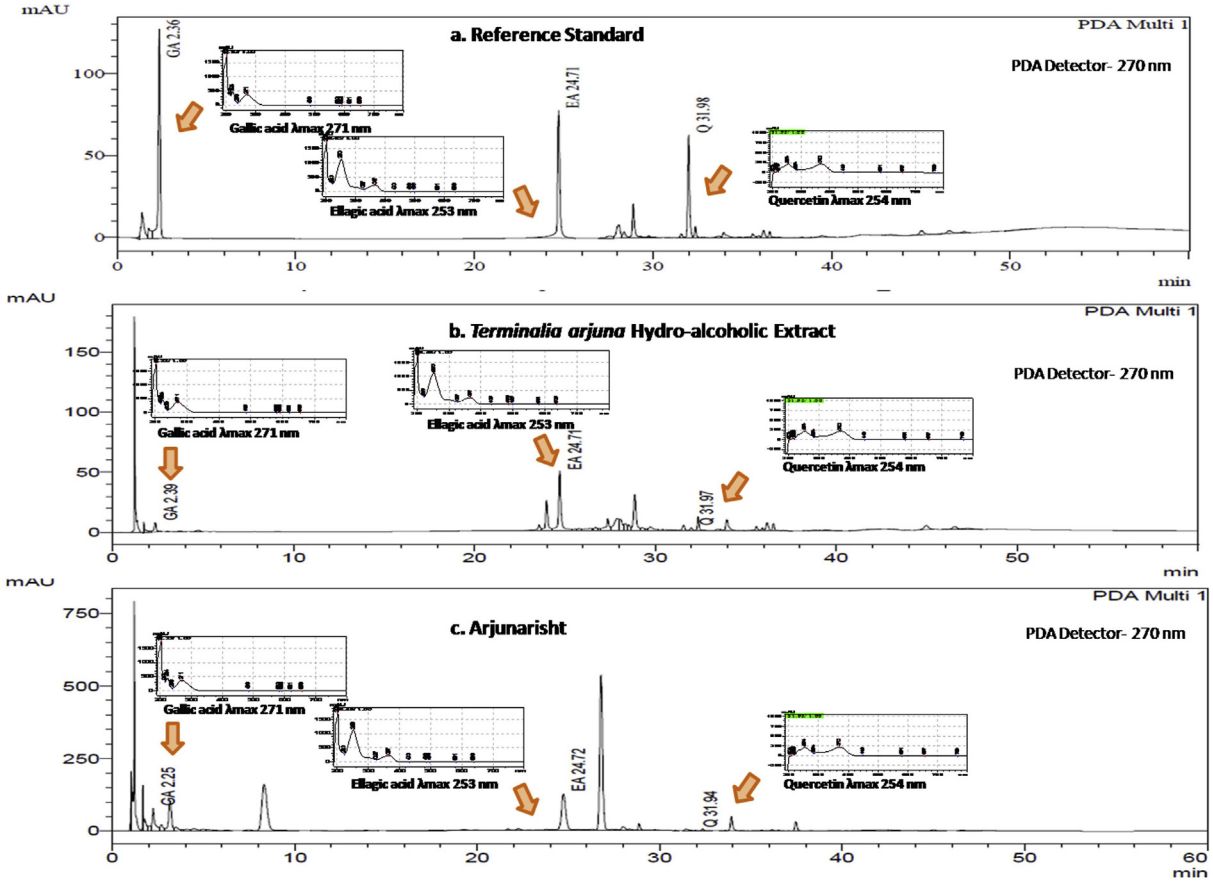

Fig. 1. HPLC chromatogram of a. Reference standard spike polyphenols gallic acid, ellagic acid, and quercetin; b. Hydroalcoholic extract of *T. arjuna* (TAHA); c. *Arjunarishta* (AA).

Table 1
Retention time and relative concentration of polyphenols in *Arjunarishta* (AA) and the hydroalcoholic extract of *T. arjuna* (TAHA).

Sr. no.	Compound name	R_t (min)			(μg/ml of AA)	(mg/g of TAHA)
		Reference standard	AA	TAHA		
1	Gallic acid	2.362	2.248	2.358	92.18 ± 1.184	2.443 ± 0.090
2	Ellagic acid	24.711	24.720	24.709	244.6 ± 8.676	7.901 ± 0.786
3	Quercetin	31.987	31.944	31.974	15.82 ± 1.832	3.20 ± 0.351

Results were expressed as mean ± SEM.

manufacturer's procedure. The synthesized cDNA (50 ng/μl) was used as a template for polymerase chain reaction (PCR) amplification. Real-time PCR was performed using step one Real-time PCR system (ABI).

PCR was carried out for 45 cycles using the following conditions: denaturation at 95 °C for 45 s, annealing at 62.7 °C for 30 s, and elongation at 72 °C for 15 s. The relative expression levels of the target genes were calculated as a ratio to the housekeeping gene GAPDH. All the samples were in triplicate and each time no template control was done during plate run. Melting curve analysis was performed to assess the specificity of the amplified PCR products. A dissociation curve analysis of all primers showed a single peak. The primers used for SYBR Green Real-time PCR are mentioned in the Supp. Table 2. All relative quantification analysis was represented in the form of relative expression to the normal group (delta delta Ct) [22].

2.11. Statistical analysis

The experimental data were expressed as mean ± SEM. The significance of difference among the various treated groups and control group were analyzed by mean of one-way ANOVA followed by Dunnett's multiple comparison tests using GraphPad Prism 5.00 Software [San Diego, USA]. $p < 0.01$ and $p < 0.05$ were considered as statistically significant.

3. Results

3.1. HPLC-PDA analysis

Phytochemical standardization of AA and TAHA was carried out using selected marker-based approach. Polyphenolic compounds such as gallic acid, ellagic acid, and quercetin were used as chemical markers for standardization purpose. The marker contents were estimated using earlier reported HPLC-PDA method modified with column and mobile phase gradient. The optimized chromatographic conditions showed good resolution of all the peaks. The presence of marker contents in the AA and TAHA was identified using retention time (t_R) and UV spectra matching with corresponding reference standards (Fig. 1). The t_R for gallic acid, ellagic acid, and quercetin was found to be 2.36, 24.71 and 31.98 min respectively. The spectral overlays showed the presence of UV spectra at 270 nm and were matched with the reference standard (see inset of Fig. 1). Quantitative estimation of these marker compounds was carried out using external standard calibration method. The calibration plots of concentration versus peak area were constructed in the range of 3.125–100 μg/ml.

The content of gallic acid, ellagic acid, and quercetin was 2.443 ± 0.090, 7.901 ± 0.786 and 3.20 ± 0.351 mg/g respectively in TAHA whereas 92.18 ± 1.184, 244.6 ± 8.676 and 15.82 ± 1.832 μg/ml respectively in AA (Table 1 and Fig. 1). These quantitative estimations were consistent with earlier reports on AA and TAHA.

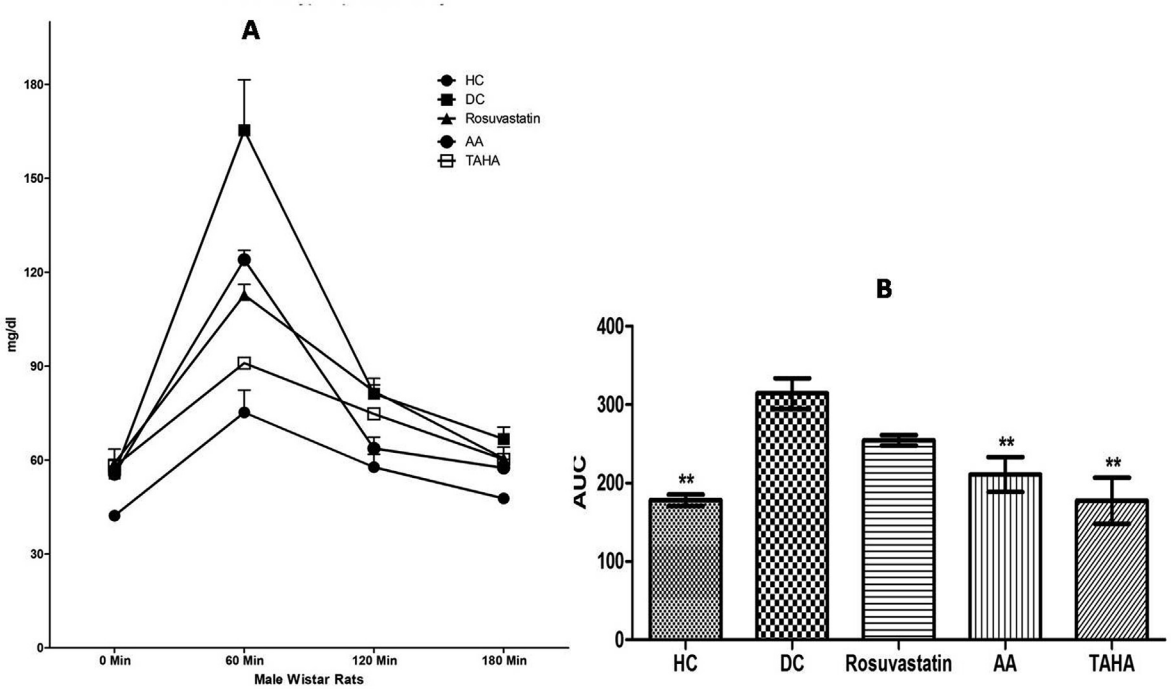

Fig. 2. Effect of *Arjunarishta* (AA) and the hydroalcoholic extract of *T. arjuna* (TAHA) on IPGTT in HFD fed rats (A) blood glucose levels and (B) $AUC_{0-180\ min}$. Results were expressed as mean ± SEM [N = 8]. $^{*}p < 0.05$ and $^{***}p < 0.001$ as compared to diabetes control.

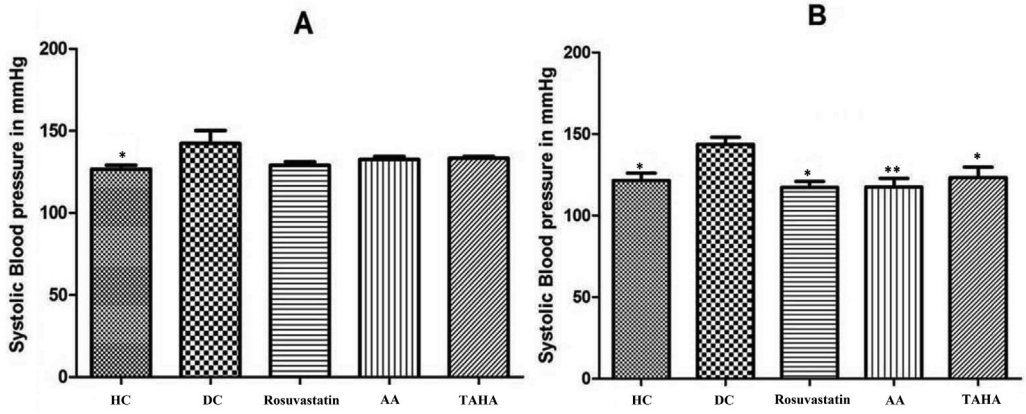

Fig. 3. Effect of *Arjunarishta* (AA) and the hydroalcoholic extract of *T. arjuna* (TAHA) on systolic blood pressure in HFD fed rats (A) 1 month and (B) 3 month. Results were expressed as mean ± SEM [N = 8]. $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared to diabetes control.

3.2. Hematology

Treatment with rosuvastatin, AA, and TAHA for 90 days did not alter the hematological investigations. No significant changes were observed on WBC, RBC, platelet, hemoglobin, percent hematocrit, MCV, MCH and MCHC in treated animals.

3.3. Intraperitoneal glucose tolerance test (IPGTT)

Glucose homeostasis was assessed in HFD fed animals treated with test materials by glucose tolerance test before and at the end of the experiment. Oral treatment of AA and TAHA significantly reduced AUC of blood glucose levels by 33.1% ($p < 0.01$) and 43.6% ($p < 0.01$; $AUC_{0–180\ min}$) as compared to diabetes control (DC) animals respectively (Fig. 2).

3.4. Systolic blood pressure

Hypertension effect was tested on experimental animals after 30 and 90 days of initiating the study. Elevated systolic blood pressure was observed in the DC animals by 12% in comparison to healthy control (HC) animals. As shown in Fig. 3, a significant reduction of systolic blood pressure was demonstrated in animals treated with AA ($p < 0.01$), TAHA ($p < 0.05$), and rosuvastatin ($p < 0.05$) as compared to DC animals.

3.5. Metabolic parameters

HFD-fed animals showed a significant increase in TC, TG, LDL-C, VLDL-C ($p < 0.001$) and a significantly decreased level of HDL-C ($p < 0.001$) compared with the HC animals. HFD-fed animals with TAHA, AA, and rosuvastatin showed a significant fall in the level of TC, TG, LDL-C, VLDL-C ($p < 0.05$) and a significantly increased level of HDL-C compared with the DC animals ($p < 0.05$) (Table 2).

3.6. TG levels in liver, peritoneal, and epididymal tissues

HFD-fed animals showed a significant increase in TG levels in liver, peritoneal and epididymal tissue after 30 and 90 days ($p < 0.05$) compared with the HC animals. HFD-fed animals with TAHA, AA, and rosuvastatin showed a significant fall in the level of TG in liver, peritoneal, and epididymal tissue after 30 and 90 days compared with the DC animals ($p < 0.05$) (Table 3).

3.7. Molecular mechanism study

AA and TAHA were found to be modulating expression of insulin sensitizing genes. Significant reduction in pro-inflammatory cytokine TNF-α gene expression was observed in animals treated with AA and TAHA.

3.7.1. Inflammatory gene expression

The expression of the inflammatory gene in the liver is given in Fig. 4. The inflammatory gene, TNF-α was markedly upregulated in DC animals compared to HC animals, indicating the increase in cytokine levels which results into inflammation. Whereas, treatment with AA, TAHA, and rosuvastatin markedly downregulated ($p < 0.05$) mRNA expression of TNF-α (2.4, 2.2 and 2.6 fold change respectively) as compared to DC animals.

3.7.2. Insulin sensitizer gene expression

The expression of insulin sensitizer gene in the liver is given in Fig. 5. The insulin sensitizer genes including IRS-1 and PGC-1α were downregulated in DC animals as compared to HC animals ($p < 0.05$), indicating the insulin resistance through the inflammatory pathway. AA, TAHA, and rosuvastatin treatment significantly enhanced mRNA expression of IRS-1 (2.8, 2.9 and 2.8 fold change) and PGC-1α (2.9, 3.7 and 3.3 fold change) as compared to DC animals.

Table 2
Effect of *Arjunarishta* (AA) and the hydroalcoholic extract of *T. arjuna* (TAHA) on metabolic parameters in HFD fed rats.

	Metabolic parameters				
	TG (mg/ml)	TC (mg/ml)	HDL (mg/ml)	LDL (mg/ml)	VLDL (mg/ml)
Healthy control	110.25 ± 3.33***	157.25 ± 4.77***	41.0 ± 2.65***	93.75 ± 4.56***	22.05 ± 0.66***
Diabetic control	264.5 ± 6.99	303.0 ± 9.11	23.75 ± 1.25	226.0 ± 8.09	52.9 ± 1.40
Rosuvastatin	150.75 ± 5.76***	243.75 ± 14.65*	31.75 ± 2.25*	181.5 ± 13.88*	30.15 ± 1.15***
AA	182.75 ± 5.93***	239.25 ± 7.28***	33.0 ± 2.19*	169.5 ± 9.53**	36.55 ± 1.18***
TAHA	200.5 ± 5.38***	235.75 ± 6.68***	32.25 ± 1.70*	163.0 ± 8.40***	40.1 ± 1.08***

Results were expressed as mean ± SEM [N = 8]. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when compared with diabetic control group. TG, Triglyceride; TC, Total Cholesterol; HDL, High-density lipoprotein; LDL, Low-density lipoprotein; VLDL, Very low-density lipoprotein.

Table 3
Effect of *Arjunarishta* (AA) and the hydroalcoholic extract of *T. arjuna* (TAHA) on triglyceride levels in liver, peritoneal, and epididymal tissues after 30 and 90 days treatment.

Group		Liver (mg/dl)	Peritoneal (mg/dl)	Epididymal (mg/dl)
Healthy control	After 30 days	266.4 ± 16.15*	332.8 ± 22.25*	318.9 ± 36.35*
	After 90 days	464.2 ± 23**	296.3 ± 58.75***	236.9 ± 27.71***
Diabetic control	After 30 days	381.1 ± 25.24	530.9 ± 36.05	600.9 ± 61.75
	After 90 days	753.5 ± 50.51	533.6 ± 20.99	421.5 ± 10.15
Rosuvastatin	After 30 days	280.5 ± 14.15*	272.1 ± 17.5**	314.6 ± 23.5**
	After 90 days	553.6 ± 41.1*	373.4 ± 40.64*	260 ± 22.5**
AA	After 30 days	237.9 ± 42.51**	285.6 ± 31.51**	190 ± 37.24***
	After 90 days	327.3 ± 20.4***	304.7 ± 43.65**	263 ± 4.676***
TAHA	After 30 days	238.3 ± 19.11**	327.2 ± 25.09**	503.7 ± 6.35
	After 90 days	251.0 ± 6.35***	249.3 ± 28.32***	240.1 ± 8***

Values were expressed as mean ± SEM [N = 8]. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when compared with diabetic control group.

4. Discussion

The hyperglycemic state developed in HFD fed animal was due to the association of insulin resistance and glucose intolerance. The treatment effect on glucose tolerance and tissue utilization of glucose was studied using IPGTT. The IPGTT is advantageous as it was performed under physiological conditions and simulated a post-absorptive state in which the production and release of insulin and its responsiveness are necessary [23]. The IPGTT and AUC measure the rate of tissue uptake and glucose utilization. Oral treatment of AA, TAHA and rosuvastatin significantly reduced in blood glucose excursion as compared to DC animals (Fig. 2). A similar effect was produced by rosuvastatin and TAHA in diabetic animals [24]. The IPGTT results were in accordance with previous reports on the anti-glycemic potential of TA [25].

Regulation of dyslipidemia remains important in the diabetes etiology and complications. The study results indicate that prophylactic treatment with AA, TAHA, and rosuvastatin improves altered biochemical parameters in the HFD-fed animals, suggesting its beneficial effects in hyperlipidemia by significantly lower levels of TG, LDL-C, VLDL-C, TC and enhanced level of HDL-C. The study results as shown in Table 2 are supported with previous reports on the anti-hyperlipidemic potential of TAHA and rosuvastatin [26,27]. DC animals demonstrated the significant increase in TC, TG, LDL-C, VLDL-C, whereas significantly decreased a level of HDL-C was observed in comparison to HC animals. HFD-fed animals with TAHA, AA, and rosuvastatin showed a significant fall in the level of total cholesterol, TG, LDL-C, VLDL-C and a significantly increased level of HDL-C compared with the DC animals.

Hyperglycemia and hyperlipidemia play an important role in the development of hypertension. Therefore, the anti-hypertensive effect was tested on experimental animals after 30 and 90 days of initiating the study. Elevated systolic blood pressure was observed in DC animals as compared to HC animals. As shown in Fig. 3, a significant reduction upto normal level was demonstrated in animals treated with AA, TAHA, and rosuvastatin as compared to DC animals. Similar findings on TAHA study has been previously reported [28–30]. Also, hematological results were similar to earlier reports for TAHA [26].

An abnormal accumulation of TG in liver, peritoneal, and epididymal tissues plays an important role in the pathophysiology of insulin resistance, atherosclerosis, hypertriglyceridemia, and T2DM [31,32]. As shown in Table 3, study results demonstrated that tissue TG levels in DC animals were increased significantly as compared to HC animals. However, significant fall in the TG level was observed in AA, TAHA and rosuvastatin treated animals. Therefore, study findings on TG levels suggest improved condition of hypertriglyceridemia leading to T2DM. Even if the diabetes animals revealed a hypertriglyceridemic condition, it should be stressed that AA and TAHA displayed a higher capacity than that showed by rosuvastatin treatment.

The liver is an important principal organ for metabolism of carbohydrate, lipids and proteins leads to energy storage in the form of TG. HFD feeding conditions enhance cholesterol and TG in

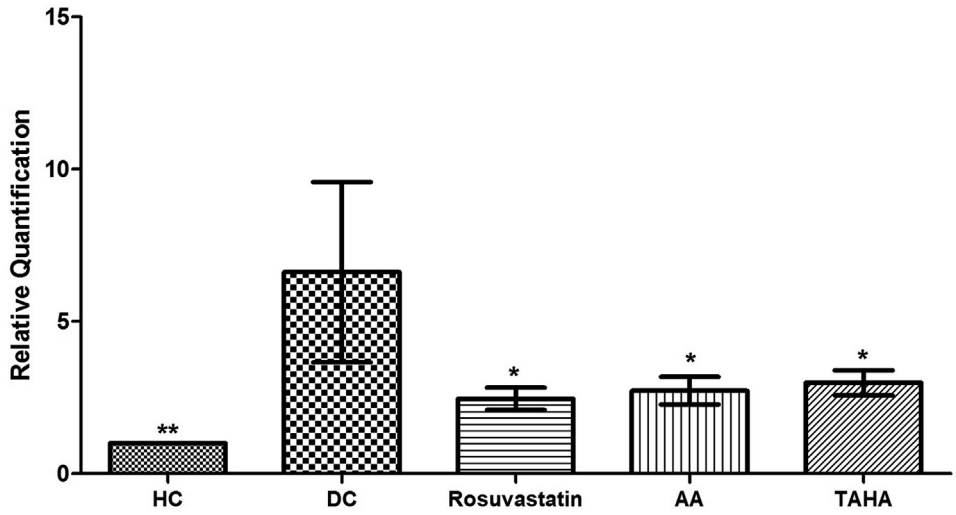

Fig. 4. Effect of *Arjunarishta* [AA] and the hydroalcoholic extract of *T. arjuna* [TAHA] on the inflammatory gene in liver tissues after 90 days treatment. Values were expressed as mean ± SEM [N = 8]. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when compared with diabetes control group.

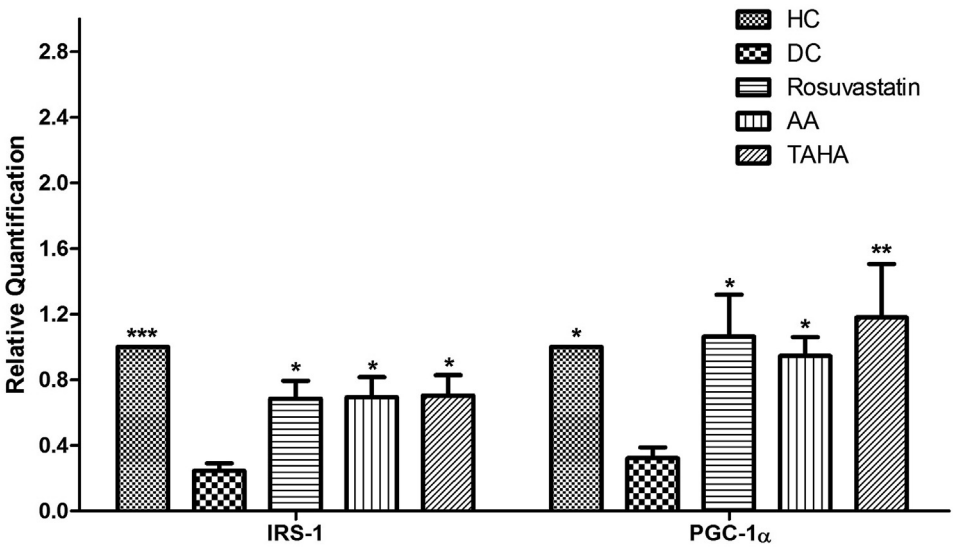

Fig. 5. Effect of *Arjunarishta* [AA] and the hydroalcoholic extract of *T. arjuna* [TAHA] on insulin sensitizer genes in liver tissues after 90 days treatment. Values were expressed as mean ± SEM [N = 8]. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when compared with diabetes control group.

the liver exhibits hypertriglyceridemia and hyperlipidemia eventually in T2DM. To study the possible mechanism behind the anti-hyperglycemic, anti-hyperlipidemic and anti-hypertriglyceridemic activity, a gene expression study was investigated on liver-targeted genes involved in inflammatory and insulin signaling pathways.

Previous reports on TAHA demonstrated that cardiotonic and anti-hyperglycemic activity is associated with their anti-inflammatory effects [12,33]. TNF-α has acted as a potential therapeutic target for new onset diabetes and mechanistic correlations between TNF-α and BMI, HOMA-IR, HOMA-B and insulin have been established in T2DM [34,35]. To explore possible mechanism of AA and TAHA on animals, we have investigated the expression level of TNF-α related to anti-inflammation. As shown in Fig. 4, TNF-α was markedly upregulated in DC animals in comparison to HC animals, indicating HFD-induced enhancement of cytokine secretion, which results in inflammation. These findings were consistent with an earlier report published on TNF-α [36]. Whereas treatment with AA, TAHA, and rosuvastatin markedly downregulated ($p < 0.05$) mRNA expression of TNF-α as compared to diabetes control. Gene expression studies suggested the anti-inflammatory effect could be one of the mechanisms behind the anti-hyperglycemic potential of AA and TAHA.

Our study suggested that anti-glycemic potential of AA could be due to increase in facilitated glucose transport into the cell mediated through IRS-1. However, confirmatory studies on other intermediate genes involved in insulin signaling pathway need to be analyzed to understand the precise mechanism. Another targeted insulin sensitizer gene: PGC-1α is transcription co-activator to modulate glucose/fatty acid metabolism through enhanced gluconeogenesis and promotion of fatty acid oxidation suggesting key drug target for anti-hyperglycemic and insulin resistance-related disorders [37–40]. Study results as illustrated in Fig. 5, IRS-1 and PGC-1α were downregulated in DC animals as compared to HC animals indicating HFD-induced cytokine secretion, which results in insulin resistance through the inflammatory mechanism. AA, TAHA, and rosuvastatin treatment significantly enhanced mRNA expression of IRS-1 and PGC-1α. Gene expression finding suggests that treatment with AA and TAHA might be restoring insulin sensitization along with anti-hyperglycemic effect by its anti-inflammatory activity. Further, upregulation of PGC-1α expression is well documented to induce gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase and glucose 6-phosphate, thereby enhancing glucose uptake. This supports our study finding in a similar manner.

Treatment with AA and TAHA showed considerable modulation of targeted genes involved in inflammation and insulin signaling pathway as compared to rosuvastatin. Rosuvastatin is a competitive HMG-CoA reductase inhibitor indicated for the treatment of dyslipidemia, hypercholesterolemia, and hypertriglyceridemia [41]. The gene expression results of rosuvastatin are found to be consistent with earlier expression reports of statins [42].

Overall, the study demonstrated that AA and TAHA have multiple therapeutic effects mediated by insulin sensitization through its anti-inflammatory mechanism; decrease in the systolic blood pressure, TC, glucose and TG levels, which were elevated in the disease condition. Additionally, these results might be correlated with previous evidence of arjunolic acid, which acts as an anti-diabetes against hyperglycemia [43].

5. Conclusion

The present study represents the action of AA and TAHA in the treatment of hyperglycemia, hyperlipidemia, and hypertriglyceridemia. Activation of PGC-1α and IRS-1 due to a decrease in the gene expression of TNF-α is the key mechanism of activity. Further studies are needed to confirm signaling pathways for Ayurvedic formulation AA with multiple doses.

Conflict of interest

None

Sources of funding

None.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaim.2017.07.004.

References

[1] Maniyar Y, Bhixavatimath P. Antihyperglycemic and hypolipidemic activities of aqueous extract of Carica papaya Linn. leaves in alloxan-induced diabetes rats. J Ayurveda Integr Med 2012;3:70–4.

[2] Gaballu FA, Gaballu YA, Khyavy OM, Mardomi A, Ghahremanzadeh K, Shokouhi B, et al. Effects of a triplex mixture of Peganum harmala, Rhuscoriaria, and Urticadioica aqueous extracts on metabolic and histological parameters in diabetes rats. Pharm Biol 2015;53:1104–9.

[3] Tang WH, Maroo A. Ischemic heart disease and congestive heart failure in diabetes patients. Med Clin North Am 2004;88:1037–61.

[4] Datta A, Bagchi C, Das S, Mitra A, Patil AD, Tripathi SK. Antidiabetes and antihyperlipidemic activity of hydroalcoholic extract of Withania coagulans Dunal dried fruit in experimental rat models. J Ayurveda Integr Med 2013;4:99–106.

[5] Singh G, Singh AT, Abraham A, Bhat B, Mukherjee A, Varma R, et al. Protective effects of *Terminalia arjuna* against Doxorubicin-induced cardiotoxicity. J Ethnopharmacol 2008;117:123–9.

[6] Amalraj A, Gopi S. Medicinal properties of *Terminalia arjuna* (Roxb.) Wight &Arn.: a review. J Tradit Complement Med 2017;7:65–78.

[7] Subramaniam S, Subramaniam R, Rajapandian S, Uthrapathi S, Gnanamanickam VR, Dubey GP. Anti-Atherogenic activity of ethanolic fraction of *Terminalia arjuna* bark on hypercholesterolemic rabbits. Evid Based Complement Altern Med 2011, 487916.

[8] Bharani A, Ganguly A, Bhargava KD. Salutary effect of *Terminalia Arjuna* in patients with severe refractory heart failure. Int J Cardiol 1995;49:191–9.

[9] Gauthaman K, Banerjee SK, Dinda AK, Ghosh CC, Maulik SK. *Terminalia arjuna* (Roxb.) protects rabbit heart against ischemic-reperfusion injury: role of antioxidant enzymes and heat shock protein. J Ethnopharmacol 2005;96:403–9.

[10] Raghavan B, Kumari SK. Effect of *Terminalia arjuna* stem bark on antioxidant status in liver and kidney of alloxan diabetes rats. Indian J physiol Pharmacol 2006;50:133–42.

[11] Lele RD. Beyond reverse pharmacology: mechanism-based screening of Ayurvedic drugs. J Ayurveda Integr Med 2010;1:257–65.

[12] Halder S, Bharal N, Mediratta PK, Kaur I, Sharma K. Anti-inflammatory, immunomodulatory and antinociceptive activity of *Terminalia arjuna* Roxb bark powder in mice and rats. Indian J Exp Biol 2009;47:577–83.

[13] Jain S, Yadav PP, Gill V, Vasudeva N, Singla N. *Terminalia arjuna* a sacred medicinal plant: phytochemical and pharmacological profile. Phytochem Rev 2009;8:491–502.

[14] Ragini H, Amita P, Jain AK. An approach to standardize Arjunarishta: a well known ayurvedic formulation using UV and Colorimetric method. J Med Pharm Allied Sci 2012;01:77–84.

[15] Lal UR, Tripathi SM, Jachak SM, Bhutani K, Singh I. HPLC analysis and standardization of Arjunarishta – an Ayurvedic cardioprotective formulation. Sci Pharm 2009;77:605–16.

[16] Chitlange SS, Kulkarni PS, Patil D, Patwardhan B, Nanda RK. High- fingerprint for quality control of *Terminalia arjuna* containing Ayurvedic churna formulation. J AOAC Int 2009;92:1016–20.

[17] Ghosh MN. Fundamentals of experimental pharmacology. 6th ed. Hilton & Company; 2015.

[18] Wu Z, Shen S, Jiang J, Tan D, Jiang D, Bai B, et al. Protective effects of grape seed extract fractions with different degrees of polymerisation on blood glucose, lipids and hepatic oxidative stress in diabetes rats. Nat Prod Res 2015;29:988–92.

[19] Sattar EA, Harraz FM, Ghareib SA, Elberry AA, Gabr S, Suliaman MI. Antihyperglycaemic and hypolipidaemic effects of the methanolic extract of Caralluma tuberculata in streptozotocin-induced diabetes rats. Nat Prod Res 2011;25:1171–9.

[20] Lee B, Shi L, Kassel DB, Asakawa T, Takeuchi K, Christopher RJ. Pharmacokinetic, pharmacodynamic, and efficacy profiles of alogliptin, a novel inhibitor of dipeptidyl peptidase-4, in rats, dogs, and monkeys. Eur J Pharmacol 2008;589:306–14.

[21] Deshpande MS, Shengule S, Apte KG, Wani M, Piprode V, Parab P. Anti-obesity activity of Ziziphus mauritiana: a potent pancreatic lipase inhibitor. Asian J Pharm Clin Res 2013;6:168–73.

[22] Hardikar AA, Satoor S, Karandikar M, Joglekar M, Puranik A, Wong W, et al. Multigenerational undernutrition increases susceptibility to obesity and diabetes that is not reversed after dietary recuperation. Cell Metab 2015;22:312–9.

[23] Mopuri R, Ganjayi M, Banavathy KS, Parim BN, Meriga B. Evaluation of anti-obesity activities of ethanolic extract of Terminalia paniculata bark on high fat diet-induced obese rats. BMC Complement Altern Med 2015;15:76.

[24] Simsek S, Schalkwijk CG, Wolffenbuttel BH. Effects of rosuvastatin and atorvastatin on glycaemic control in Type 2 diabetes—the CORALL study. Diabet Med 2012;29:628–31.

[25] Parveen K, Khan R, Siddiqui WA. Antidiabetes effects afforded by *Terminalia arjuna* in high fat-fed and streptozotocin-induced type 2 diabetes rats. Int J Diabetes Metab 2011;19:23–33.

[26] Ram A, Lauria P, Gupta R, Kumar P, Sharma VN. Hypocholesterolaemic effects of *Terminalia arjuna* tree bark. J Ethnopharmacol 1997;55:165–9.

[27] Nigam GK, Ansari MN, Bhandari U. Effect of rosuvastatin on methionine-induced hyperhomocysteinaemia and haematological changes in rats. Basic Clin Pharmacol Toxicol 2008;103:287–92.

[28] Dwivedi S, Agarwal MP. Antianginal and cardioprotective effects of *Terminalia arjuna* an indigenous drug in coronary artery disease. J Assoc Physicians India 1994;42:287–9.

[29] Sandhu JS, Shah B, Shenoy S, Chauhan S, Lavekar GS, Padhi MM. Effects of Withania somnifera (Ashwagandha) and *Terminalia arjuna* (Arjuna) on physical performance and cardiorespiratory endurance in healthy young adults. Int J Ayurveda Res 2010;1:144–9.

[30] Khaliq F, Parveen A, Singh S, Gondal R, Hussain ME, Fahim M. Improvement in myocardial function by *Terminalia arjuna* in streptozotocin-induced diabetes rats: possible mechanisms. J Cardiovasc Pharmacol Ther 2013;18:481–9.

[31] Shimabukuro M, Zhou YT, Levi M, Unger RH. Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes. Proc Natl Acad Sci U S A 1998;95:2498–502.

[32] McGarry J. Banting lecture: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. Diabetes 2002;51:7–18.

[33] Parveen A, Babbar R, Agarwal S, Kotwani A, Fahim M. Mechanistic clues in the cardioprotective effect of *Terminalia arjuna* bark extract in isoproterenol-induced chronic heart failure in rats. Cardiovasc Toxicol 2011;11:48–57.

[34] Swaroop JJ, Rajarajeswari D, Naidu JN. TNF-α with insulin resistance in type 2 diabetes mellitus. Stud IJMR Assoc 2012:127–30.

[35] Joshi K, Awte S, Bhatnagar P, Walunj S, Gupta R, Joshi S, et al. Cinnamomum zeylanicum extract inhibits proinflammatory cytokine TNF∝: in vitro and in vivo studies. Res Pharm Biotechnol 2010;2:14–21.

[36] Borst SE, Conover CF. High-fat diet induces increased tissue expression of TNF-alpha. Life Sci 2005;77:2156–65.

[37] Liang H, Ward WF. PGC-1alpha: a key regulator of energy metabolism. Adv Physiol Educ 2006;30:145–51.

[38] Handschin C, Spiegelman BM. Peroxisome proliferator-activated receptor gamma coactivator 1 coactivators, energy homeostasis and metabolism. Endocr Rev 2006;27:728–35.

[39] Sumantran VN, Tillu G. Cancer, inflammation, and insights from ayurveda. Evid Based Complement Altern Med 2012, 306346.

[40] Gupta R, Walunj S, Awate S, Kulkarni R, Joshi S, Sabharwal S, et al. Ethanolic extract of cinnamon potentiates in vitro pparγ reporter activity in presence of pgc1α and src1 and improves glucose tolerance in mice. Int Res J Biotechnol 2011;2:47–57.

[41] Olsson AG, McTaggart F, Raza A. Rosuvastatin: a highly effective new HMG-CoA reductase inhibitor. Cardiovasc Drug Rev 2002;20:303–28.

[42] Wang W, Wong CW. Statins enhance peroxisome proliferator-activated receptor gamma coactivator-1alpha activity to regulate energy metabolism. J Mol Med (Berl) 2010;88:309–17.

[43] Manna P, Ghosh J, Das J, Sil PC. Streptozotocin induced activation of oxidative stress responsive splenic cell signaling pathways: protective role of arjunolic acid. Toxicol Appl Pharmacol 2010;244:114–29.

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Phytochemical characterization of *ayurvedic* formulations of *Terminalia arjuna*: A potential tool for quality assurance

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Ayurveda has gained worldwide attention due to its efficacy. With the growing need for safer drugs, attention has been drawn to their quality and standards of the Ayurvedic formulations. Ayurveda describes the formulation of *Terminalia arjuna* (*T. arjuna*) as a potent drug for dyslipidemia, cardiac disorders, and diabetes. It is administered as arishta, ghritah (medicated ghee) or as a powder. Thus, the main objective of the present work was to characterize Arjunarishta (AA) and Arjuna ghritah (AG) - ayurvedic formulations using arjunetin and arjungenin by High-Performance Liquid Chromatography-Photodiode Array Detector (HPLC-PDA) method. The presence of marker contents in the AA, AG, and *T. arjuna* hydroalcoholic extract (TAHA) was identified using retention time (Rt) and UV spectra matching with corresponding reference standards. The content of arjunetin and arjungenin was 0.47 and 8.22 mg/g in TAHA; 206.38 and 0.00 µg/mL in AA; 190.58 and 285.48 µg/mL in AG. These quantitative estimations were consistent with earlier reports on TAHA. The results of the present study indicate the charecterization of Arjunetin and Arjungenin phytochemical markers in AG, and AA formulations, which have not been, reported so far. These finding will certainly help in the quality assurance during manufacturing of AG and AA formulations.

Keywords: Arishta, Arjunetin, Arjungenin, *Ayurveda*, Ghritah, HPLC, Standardization.

IPC Code: Int. Cl.[18] A61K 36/185, A61K 31/56, A61K 36/185, A61K 36/00, A61K 36/00, C07K 1/16, A61K 36/00

Ayurveda is a traditional system of medicine in India. Ayurvedic preparations were used to prevent diseases for centuries[1,2]. Formulations were prepared using well-documented methods consisting of several herbs in different forms like decoction, asavas, fresh juices, tablets, oils, ghee, and arishtas[3,4]. Ayurveda has gained worldwide attention due to its efficacy. With rising requirement for better drugs, attention has been focussed on the quality and standards of the Ayurvedic formulations[5]. However, different constituents with diverse physical and chemical properties made the quality control of formulations difficult[6]. Ayurvedic pharmacy advocates the use of quality control tests to make sure that the prepared medicines adhere to the standards mentioned in Ayurveda. Those were based on observation parameters. Hence, characterization of Ayurvedic formulations using modern techniques of analysis is extremely important[5].

The bark of *Terminalia arjuna* (*T. arjuna*) is used in traditional Indian medicines either alone or as a constituent in lots of herbal medicines for treating various ailments. Ayurveda describes the formulation of *T. arjuna* as a potent drug for dyslipidemia, cardiac disorders, and diabetes. It is administered as arishta, ghritam (medicated ghee) or as a powder[3]. The usefulness of this drug in cardiovascular disorders is well documented in the literature[7-10]. The plant was reported to contain triterpenoids, flavonoids, glycosides, and tannins, etc. *T. arjuna* contains polyphenols such gallic acid, ellagic acid and triterpenoids like arjunolic acid, arjunic acid, arjunetin, arjungenin, arjunglucoside I and II. However characterized and validated methods for quantification were not available for Ayurvedic formulations such as Arjunarishta (AA) and Arjun

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ghritam (AG)[3,11-13]. Pharmacopoeial standards for Ayurvedic formulations published by the Central Council for Research in Ayurveda and Siddha provide physicochemical parameters (pH, total solids, sugar content), thin layer chromatography profiling and gallic acid content of the AA formulation. Therefore, it is essential that there are definite and accurate analytical tools to ascertain consistency and quality of AA and AG[14-16]. Thus, the objective of this work was to characterize AA and AG using specific phytochemical analytical markers.

Methodology

Instrumentation

A Dionex P680 HPLC system including autosampler, thermostatted column compartment and a Dionex UVD 170U/340U photodiode array detector (Dionex Corp., Germering, Germany) was used to acquire chromatograms. The column used was RP C18 BDS Hypersil column (250 × 4.6 mm, 5 µm particle size) from Thermo Electron Corp. (Bellefonte, PA). Chromeleon 6.70 software from Dionex was used to acquire data for fingerprint analysis.

Chemicals

HPLC grade methanol (Merck, Mumbai, India) was used to prepare samples; water was purified by using the Milli-Q (Millipore, USA) system; and HPLC grade acetonitrile (Merck) was used to prepare the mobile phase. Arjunetin and Arjungenin were purchased as HPLC markers from the Natural Remedies Pvt. Ltd. Bangalore, India.

Plant materials and extraction

The *T. arjuna* bark sample was collected from a local market in Pune, India and authenticated by Agharkar Research Institute, Pune, India. The sample was deposited as voucher specimens no. S/B-109. The dried bark was extracted with ethanol: water (70:30 v/v) using Soxhlet extraction for 8 hours for consecutive three days at 65°C. The extract was dried under vacuum using rotary evaporator at 45°C and stored at 2-8°C until use.

Formulations

Marketed formulations, i.e., Arjunarishta (Batch No. GA-06, Sandu Pharmaceuticals Ltd. Goa, India) and Arjun Ghritam (Batch No. 116, Nagarjun Pharmaceuticals P. Ltd. Ahmedabad, India) manufactured by Indian-based Ayurvedic drug manufacturing companies were used for the analysis. These formulations were procured from the market.

Preparation of standard solution

Stock solutions of both markers prepared separately in methanol. Final concentration (31.25, 62.5, 125, 250, 500, 1000 µg/mL) was made up by further diluting with methanol.

Preparation of sample

AA 5 mL sample was added to 50 mL centrifuge tube, and then 10mL of methanol added and vigorously shook for 5 min then stand for 10 min to settle down the precipitated sugars. After 10 min, methanol extract was pipetted out. Then, 1 mL of supernatant was passed through 0.45 µm filter (millipore) and 20µL was injected for quantification. AG 10 mL sample was soaked on 10 g silica gel powder in 50 mL centrifuge tube, and 20 mL n-hexane was added and vigorously shaken for 5 min then allowed to stand for 10 min to settle down the precipitated ghritah component and silica gel powder. After that, n-hexane was pipetted out and discarded without disturbing silica gel powder. This procedure (wash with n-hexane) was repeated thrice. Then added 20 mL acetonitrile solvent and vigorously shaken for 5 min then allowed to stand for 30 min to settle down the precipitated silica gel powder. After that, acetonitrile was pipetted out. 1 mL of supernatant was passed through 0.45 µm filter (millipore) and 20 µL was injected for quantification.

HPLC system parameters

The analysis was performed with an RP C18 BDS Hypersil column (250 × 4.6 mm, 5 µm particle size) at a column temperature 26°C. Separation was achieved with two pumps. The gradient program for pump A (acetonitrile: water, 30:70) and pump B (acetonitrile: water, 70:30) as follows: initially 30% B, flow rate 0.8 mL/min; increased gradually to 50-70% B up to 30 min, flow rate 1.2 mL/min. Then washed the column for 20 min, 30% B, flow rate 0.8 mL/min. The detection wavelength was 220 nm, the absorption close to both the compounds. Injection volume for standard and sample was 20 µL[17].

Data analysis of chromatogram

Data analysis was performed by variations and similarity observed in retention time values, peak areas and spectral patterns of the peaks obtained in the

chromatograms of ayurvedic formulations and *T. arjuna* hydroalcoholic bark extract (TAHA).

Method validation

A complete method validation was carried out according to the industrial guideline for analytical method validation[18].

Specificity and selectivity

The specificity of the method was evaluated by analyzing methanol samples from at least six different lots to investigate the potential interferences at the LC peak region for analytes. The acceptance criterion for the experiment was that at least four out of six lots should have response less than five times of the LLOQ level response in the same solvent.

Linearity

The linearity of the method was generated by analysis of five calibration curves containing six non-zero concentrations. The six-point calibration curve for arjunetin and arjungenin (31.25, 62.5, 125, 250, 500, 1000 µg/mL) was constructed by plotting the peak area each analyte against the nominal concentration of calibration standards in methanol. This operation was repeated on six consecutive days with freshly prepared calibration standards to select the most appropriate regression model. Each calibration curves were analyzed individually by fitting the area response for analyte as a function of standard concentration, using no weighted or least square weighted ($1/x$ or $1/x^2$) linear regression and excluding the point of origin[18]. The acceptance criteria for each back-calculated standard concentration were ± 15% deviation from the nominal value except at LLOQ, which was set at ± 20%.

Precision and accuracy of the analytical method

Arjunetin and Arjungenin samples (at a concentration of 31.25, 62.5, 125, 250, 500 and 1000 µg/mL) were prepared. Intra- and inter-day assays were repetitively carried out on the samples at three different times of the same day and six different days, respectively. The standard spiked solutions were examined six times within one day to determine the intra-day precision. The inter-day precision was established by analyzing each sample on six consecutive days; each sample was injected three times on each day. Analytical precision was evaluated by calculating the % CV of variances Intra- and inter-day while accuracy was calculated from the nominal concentration (C_{nom}) and the mean value of observed concentration (C_{obs}) as follows: accuracy (bias, %) = $[(C_{nom} - C_{obs}) / C_{nom}] \times 100$[19].

Statistical analysis

The experimental data were expressed as Mean ± SD. Precision was expressed as % coefficient of variation using Graph Pad Prism 5.00 Software (San Diego, USA).

Results and discussion

Standardization is an important aspect for establishing the quality of the any traditional formulations. Reports on cardioprotective activity and phytochemical investigation of TAHA and AA are numerous[13-15, 20-22] however there are no reports on standardization of AG. We have attempted to standardize the formulation with respect to its specific phytochemical constituents like arjungenin and arjunetin that may be responsible for the therapeutic action. The chromatograms of standards, TAHA, AA, and AG, were compared. Arjunetin and Arjungenin were separated under the optimum conditions. No interference peaks from the endogenous constituents of the TAHA, AA, and AG were found in the region of the investigated compounds.

Validation parameters

The retention time for arjunetin and arjungenin were found to be 4.95 and 7.68 min respectively. The spectral overlays showed the presence of UV spectra at 220 nm and were matched with the UV spectra of the reference standard. Quantitative estimation of these marker compounds was carried out using external standard calibration method. The calibration plots of concentration versus peak area were constructed in the range of 31.25-1000 µg/mL.

Specificity

Specificity of the developed method was checked by changing the solvent B (from methanol to acetonitrile). Retention time, lower limits of quantification were determined and are shown in Table 1, together with regression equations and their coefficients of regression (R^2). The value of R^2 confirmed the linearity of the method. The robustness of the method was studied by changing the mobile phase (solvent B from 80:20 to 90:10); minor changes in mobile phase showed no effect on peak resolution. The linearity range of arjunetin and arjungenin solutions were obtained as 31.25-1000.0 µg/mL as shown in Table 1 The calibration curve was

constructed using a linear regression of the theoretical concentration of an analyte versus the corrected peak area. The mean regression equations and their correlation coefficients were calculated to be $Y = 0.069 X + 0.660$ and $R^2 = 0.991$ for arjunetin, and $Y = 0.046 X + 0.628$ and $R^2 = 0.992$ for arjungenin.

Precision and accuracy

The precision was considered at two levels, the repeatability, and the intermediate precision. The repeatability of the sample application was determined as an intra-day variation, whereas the intermediate precision was determined by carrying out the inter-day variation for the determination of arjunetin and arjungenin at different concentration levels of 31.25, 62.5, 125, 250, 500 and 1000 µg/mL. The standard solutions, at four different concentration levels, were analyzed at least six times within the same day, and % CV values obtained were in the range between 5.19 - 16.86% and 5.57 - 10.90% for arjunetin and arjungenin. Similarly, to measure the inter-day variability, the same concentration of the two standards was run over at least six consecutive days, and the values were in the range between 6.86 and 9.29% and 5.74 and 10.47% for arjunetin and arjungenin. The accuracy (% bias) values ranged 8.98 to 6.94 and -9.79 to 8.08 for arjunetin and arjungenin respectively. The results of the repeatability and intermediate precision were expressed in CV (%) and shown in Table 2; the low values of the CV (%) indicated the repeatability of the proposed method.

Phytochemical characterization of AA and AG

Phytochemical characterization of AA, AG, and TAHA was carried out using selected marker-based approach. Arjunetin and arjungenin were used as phytochemical markers for characterization purpose. The marker contents were estimated using earlier reported HPLC-PDA method suitably modified on column and mobile phase gradient. The optimized chromatographic conditions showed good resolution of the all the peaks. The presence of marker contents in the AA, AG, and TAHA was identified using retention time and UV spectra matching with corresponding reference standards (Fig. 1). The content of arjunetin and arjungenin was 0.47 and 8.22mg/g in TAHA; 206.38 and 0.00 µg/mL in AA; 190.58 and 285.48 µg/mL in AG (Table 3). These quantitative estimations were consistent with earlier reports on TAHA[3]. Previously, standardisation of AA reported using HPLC method with gallic acid, ellagic acid, and quercetin markers. Lal *et al*. mentioned that AA formulation did not contain any specific *T. arjuna* phytochemical markers[3]. This may be due to less amount of arjunetin present in the formulation or non-specific sample processing and analysis method.

Table 1 — Linearity, LLOQ, linear regression equation of arjunetin and arjungenin HPLC method

Phytochemical Marker	Retention Time	R^2	Conc.Range µg/mL	LLOQ µg/mL	Linear Regression Equation
Arjunetin	4.945 ± 0.002	0.991	31.25-1000	31.25	Y=0.069X + 0.660
Arjungenin	7.685 ± 0.003	0.992	31.25-1000	31.25	Y=0.046X + 0.628

Results are expressed as mean ± SD.

Table 2 — Intra and inter-day precision and accuracy of arjunetin and arjungenin HPLC method

C_{nom} (µg/mL)	Precision Intra-day (n=6)		Accuracy	Precision Inter-day (n=6)		Accuracy
	C_{obs} (µg/mL)	Precision (% CV)	% bias	C_{obs} (µg/mL)	Precision (% CV)	% bias
			Arjunetin			
1000	1047 ± 54.28	5.19	-4.7	947.0 ± 65.02	6.86	5.3
250	245.5 ± 21.73	8.85	1.8	246.9 ± 22.93	9.29	1.24
62.5	68.11 ± 4.9	7.19	-8.98	60.70 ± 4.853	8.00	2.88
31.25	29.08 ± 4.91	16.86	6.94	29.70 ± 2.586	8.71	4.96
			Arjungenin			
1000	1084 ± 60.39	5.57	-8.4	1037.0 ± 59.48	5.74	-3.7
250	229.8 ± 19.82	8.62	8.08	256.7 ± 16.36	7.15	-2.68
62.5	68.62 ± 6.25	9.11	-9.79	62.58 ± 5.68	9.07	-0.128
31.25	32.14 ± 3.502	10.90	-2.85	33.88 ± 3.55	10.47	-8.42

Results are expressed as mean ± SD.

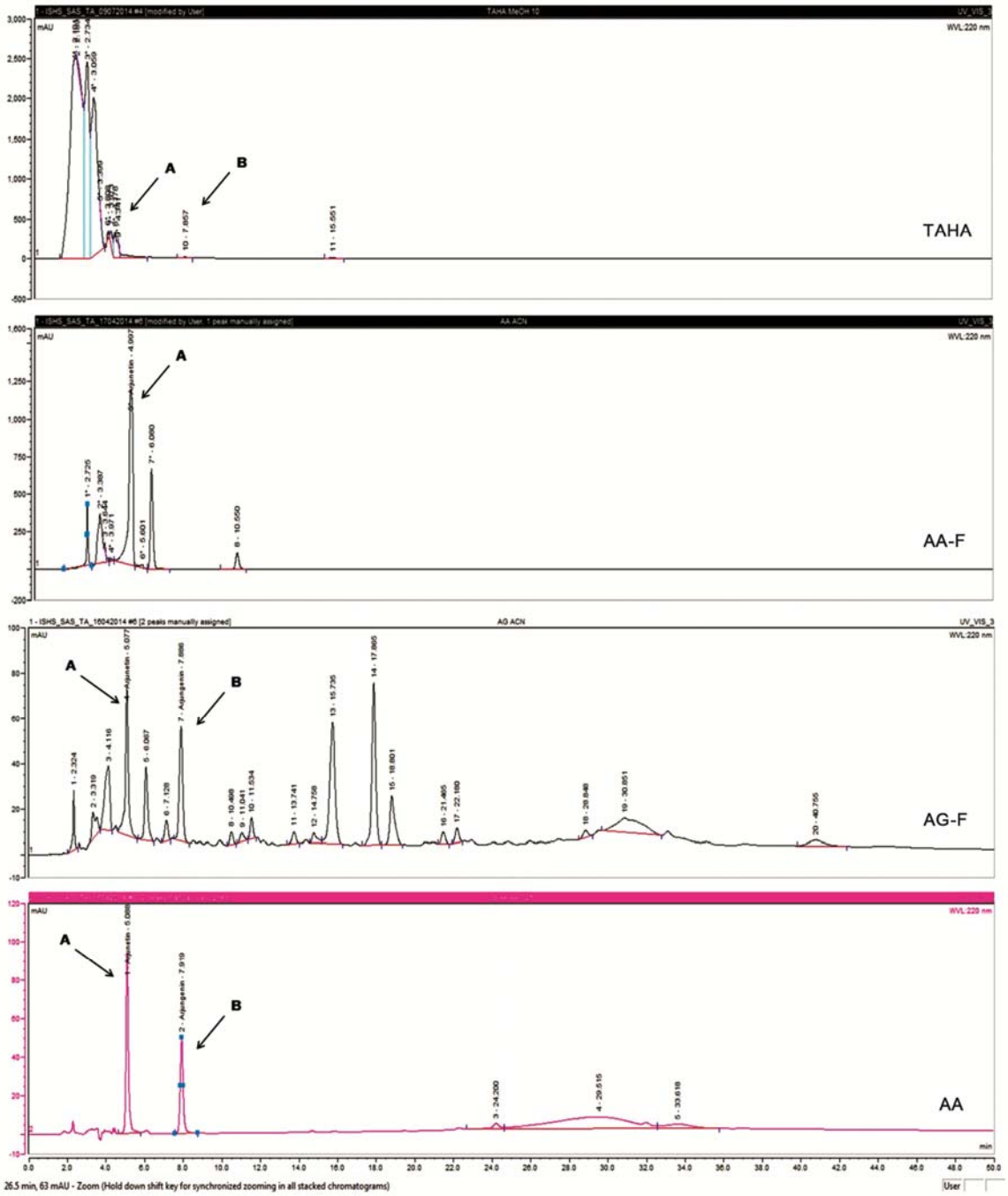

Fig. 1 — Phytochemical profiling HPLC chromatograms of arjunetin and arjungenin HPLC chromatograms at 220 nm. (I) extract of *T. arjuna* (TAHA); (II) Arjunarishta formulation (AA-F); (III) Arjuna ghritah formulation (AG-F); (IV) chromatogram of Spiked Arjunetin (A) and Arjungenin markers (B).

Table 3 — Quantitation of arjunetin and arjungenin in formulations and extract of *T. arjuna* (TAHA)

Compound Name	Rt (min)				(µg/mL of AG)	(µg/mL of AA)	(mg/g of TAHA)
	Reference Standard	AG	AA	TAHA			
Arjunetin	4.95 ± 0.0	5.06 ± 0.01	5.10 ± 0.02	5.09 ± 0.02	166.6 ± 2.69	254.6 ± 23.39	0.59 ± 0.04
Arjungenin	7.69 ± 0.0	7.87 ± 0.02	ND	7.88 ± 0.04	228.7 ± 9.15	ND	0.85 ± 0.01

Results are expressed as mean ± SD.

Conclusion

The results of the present study indicate the characterization of Arjunetin and Arjungenin phytochemical markers in AG, and AA formulations, which have not been, reported so far. This work can be utilized as baseline data of characterization and quality assurance of *T. arjuna* formulations with specific phytochemical markers. These finding will certainly help in the quality control process of manufacturing of AG and AA formulations.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1 Joshi KS, Nesari TM, Dedge AP, Dhumal VR & Shengule SA, *et al*, Dosha phenotype specific Ayurveda intervention ameliorates asthma symptoms through cytokine modulations: Results of whole system clinical trial, *J Ethnopharmacol*, 197 (2017) 110-7.

2 Shengule S, Kumbhare K, Patil D, Mishra S & Apte K, *et al*, Herb-drug interaction of Nisha Amalaki and Curcuminoids with metformin in normal and diabetic condition: A disease system approach, *Biomed Pharmacother*, 101 (2018) 591-8.

3 Lal UR, Triptathi SM, Jachak SM, Bhutani KK & Singh IP, HPLC analysis and standardisation of Arjunarishtaa – An Ayurvedic cardioprotective formulation, *Sci Pharm*, 77 (2009) 606–16.

4 Cota DL, Rasal VP, Mishra S & Shengule S, Cardioprotective effect of oregano oil against doxorubicin-induced myocardial infarction in rats, *Phcog Mag,*14 (2018) 363-8.

5 Elamthuruthy AT, Shah CR, Khan TA, Tatke PA & Gabhe SY, Standardisation of marketed Kumariasava—an Ayurvedic Aloe vera product, *J Pharm Biomed Anal*, 37 (2005) 937–41.

6 Garg S & Bhutani K, Chromatographic Analysis of Kutajarista—an Ayurvedic Polyherbal Formulation, *Phytochem Anal*, 2008, 19 (2008) 323–8.

7 Bharani A, Ganguly A & Bhargava KD, Salutary effect of *Terminalia arjuna* in patients with severe refractory heart failure, *Int J Cardiol*, 49 (1995) 191-9.

8 Dwivedi S & Jauhari R, Beneficial effects of *Terminalia arjuna* in coronary artery disease, *IHJ,* 49 (5) (1997) 507-10.

9 Singh N, Kapur KK, Singh SP, Shanker K & Sinha JN, *et al*, Mechanism of Cardiovascular Action of *Terminalia arjuna*, *Planta Med*, 45 (1982) 102-10.

10 Nammi S, Gudavalli R, Behara S, Lodagala DS & Boini KM, Possible mechanisms of hypotension produced 70% alcoholic extract of *Terminalia arjuna* (L.) in anaesthetized dogs, *BMC Complement Altern Med*, 3 (5) (2003) 1-4.

11 Aravind SG, Arimboor R, Rangan M, Madhavan SN & Arumughan C, Semi-preparative HPLC preparation and HPTLC quantification of tetrahydroamentoflavone as marker in Semecarpus anacardium and its polyherbal formulations, *J Pharm Biomed Anal*, 48 (2008) 808–13.

12 Saha A, Pawar VM & Jayaraman S, Characterisation of Polyphenols in *Terminalia arjuna* bark extract, *Ind J Pharm Sci*, (2012) 339-47.

13 Kalola J & Rajani M, Extraction and TLC Densitometric Determination of Triterpenoid Acids (Arjungenin, Arjunolic Acid) from *Terminalia arjuna* Stem Bark Without Interference of Tannins, *Chromatographia*, 63 (2006) 475–81.

14 Chitlange SS, Kulkarni PS, Patil D, Patwardhan B & Nanda RK, High performance liquid chromatographic fingerprint for quality control of *Terminalia arjuna* containing ayurvedic churna formulation, *J AOAC INT*, 92 (2009) 4.

15 Lal UR, Tripathi SM, Jachak SM, Bhutani KK & Singh IP, Chemical Changes during Fermentation of Abhayarishta and its Standardisation by HPLC-DAD, *Nat Prod Commun*, 5 (4) (2010) 575-9.

16 Dhanani T, Shah S & Kumar S, A Validated High-Performance Liquid Chromatography Method for Determination of Tannin-Related Marker Constituents Gallic Acid, Corilagin, Chebulagic Acid, Ellagic Acid and Chebulinic Acid in Four Terminalia Species from India, *J Chromatogr Sci*, (2014) 1–8.

17 Singh DV, Verma RK, Singh SC & Gupta MM, RP-LC determination of oleane derivatives in *Terminalia arjuna*, *J Pharm Biomed Anal*, 28 (2002) 447–52.

18 Anonymous, FDA, Analytical procedures and methods validation fpr drugs and biologics: Guidance for industry. (2015) 7-8.

19 Patil D, Gautam M, Mishra S, Karupothula S & Gairola S, *et al*, Determination of withaferin A and withanolide A in mice plasma using high-performance liquid chromatography-tandem mass spectrometry: Application to pharmacokinetics after oral administration of *Withania somnifera* aqueous extract, *J Pharm Biomed Anal*, 80 (2013) 203-12.

20 Shengule SA, Mishra S, Joshi K, Apte K & Patil D, *et al*, Anti-hyperglycemic and anti-hyperlipidaemic effect of Arjunarisht in high-fat fed animals, *J Ayurveda Integr Med*, 9 (2018) 45-52.

21 Shengule S, Mishra S & Bodhale S, Inhibitory effect of a standardized hydroethanolic extract of *Terminalia arjuna* bark on alpha-amylase enzyme, *AJPCR*, 11 (2018) 366-9.

22 Cota D, Mishra S & Shengule S. *Terminalia arjuna* hydroalcoholic extract ameliorates trinitrobenzenes ulphonic acid induced colitis mediated through inhibition of inflammation, oxidative stress and improvement in structure of gut microbiota, *J Ethnopharmacol,* 230 (2019) 117-125.

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Research Article

INHIBITORY EFFECT OF A STANDARDIZED HYDROETHANOLIC EXTRACT OF *TERMINALIA ARJUNA* BARK ON ALPHA-AMYLASE ENZYME

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ABSTRACT

Objective: The present study was initiated to screen the hydroethanolic bark extract for α-amylase inhibitory activity and standardization of the *Terminalia arjuna* for polyphenolic phytochemicals using high-performance liquid chromatography-photo diode array (HPLC-PDA) method.

Methods: The *T. arjuna* bark sample was extracted with ethanol: water (70:30 v/v) using Soxhlet extraction. A Dionex P680 HPLC system was used to acquire chromatograms. The screening of extract of *T. arjuna* bark has performed for *in vitro* α-amylase inhibitory assay. Each experiment was repeated 3 times. All values were expressed mean ± standard deviation.

Results: The content of arjunetin, arjungenin, gallic acid, ellagic acid, and quercetin was 0.47, 8.22, 2.443, 7.901, and 3.20 mg/g, respectively, in a hydroethanolic extract of *T. arjuna*. The hydroethanolic extract of *T. arjuna* bark and acarbose has shown an inhibitory activity with an IC_{50} value 145.90 and 62.35 µg/mL, respectively.

Conclusion: The hydroethanolic extract *T. arjuna* bark demonstrates α-amylase inhibitory activity due to a synergistic effect of the phytochemical constituents present in it. This study suggests that one of the mechanisms of this plant for antidiabetic activity is through the inhibition of α-amylase enzyme.

Keywords: *Terminalia arjuna*, Acarbose, Diabetes, Enzyme inhibition, Arjunetin, Arjungenin.

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INTRODUCTION

Diabetes mellitus (DM) is a complex disease developed due to unbalanced carbohydrate, protein, and fat metabolism. A progressive metabolic disorder finally leads to micro- and macro-vascular changes causing secondary complications [1]. According to the World Health Organization, 346 million people worldwide have diabetes and among 90% of them suffer from type 2 diabetes (T2DM) [2,3]. Type 1 diabetes results from the deficient synthesis of insulin by β-cells of the pancreas, while T2DM is developed due to insulin resistance condition or β-cell dysfunction [1].

The treatment of T2DM is based on oral antidiabetic drugs include sulfonylureas, biguanides, and thiazolidinediones. However, they have demonstrated some side effects which suggest the need for other alternative therapy [4-6]. One of the treatment approaches is to decrease the after-meal blood glucose concentrations [5]. Inhibition of the α-amylase enzyme in the intestine delays the degradation of starch before absorption that reduces postprandial blood glucose concentrations [4,7].

Voglibose, acarbose, and Miglitol are the α-amylase inhibitory drugs available in the market. These are prescribed as combinatorial therapy with sulfonylurea and metformin for the treatment of T2DM to reduce glycated hemoglobin (HbA1c) levels. However, the previous studies have reported few side effects such as abdominal discomfort, flatulence, and diarrhea for these drugs [8]. The side effects caused and drug resistance is two major concerns in the usage of these drugs after prolonged treatment [2].

Traditional medicinal plants have used to treat various diseases. Plant extracts or phytochemical constituents have been reported scientifically for biological activities [5]. *Terminalia arjuna* Roxb. (Combretaceae) commonly known as Arjuna, has been traditionally used for several medicinal purposes such as cardiac diseases, dyslipidemia, and lowering blood glucose [9-11]. The hydroethanolic extracts of the *T. arjuna* (TAHA) leaves and stem bark have been reported to possess anti-diabetic activities in diabetic rats [9,12]. The previous study by Saha *et al*. have reported the α-amylase inhibitory activity of methanolic, aqueous and 50% methanolic extract of *T. arjuna* [13,14]. While there is no evidence in the literature that has determined TAHA bark extract containing arjunetin, arjungenin, ellagic acid, gallic acid, and quercetin compounds for α-amylase inhibition activity. This study will be useful to determine one of the possible mechanisms of effect of TAHA as antidiabetic activity. Therefore, the objective of the present study was to screen the inhibitory activity of TAHA bark extract for α-amylase enzyme and standardization for polyphenolic phytochemicals using high-performance liquid chromatography-photo diode array (HPLC-PDA) method.

MATERIALS AND METHODS

Materials/chemicals

Solvents such as HPLC grade methanol and acetonitrile (Merck, Mumbai, and India) were used to prepare samples and mobile phase, respectively. Water was purified using the Milli-Q (Millipore, Billerica, MA) system. Arjunetin, arjungenin, gallic acid, ellagic acid, and quercetin were purchased as HPLC markers from the Natural Remedies Pvt. Ltd. Bangalore, India. Enzyme α-Amylase was purchased from Sigma-Aldrich, Bangalore, India.

Plant material and extraction

The *T. arjuna* bark sample was collected from a local market in Pune, India, and authenticated by Agharkar Research Institute, Pune, India. The sample was deposited as voucher specimens no. S/B-109. The dried bark was extracted with ethanol: water (70:30 v/v) using Soxhlet

extraction for 8 h for consecutive 3 days at 65°C. The extract was dried under vacuum using rotary evaporator at 45°C and stored at 2–8°C until use.

Estimation of arjunetin and arjungenin in a TAHA bark extract

A Dionex P680 HPLC system including autosampler and thermostatted column compartment and a Dionex UVD 170U/340U photodiode array detector (Dionex Corp., Germering, Germany) was used to acquire chromatograms. The column used was RP C18 BDS Hypersil column (250 × 4.6 mm, 5 µm particle size) from Thermo Electron Corp. (Bellefonte, PA). Chromeleon 6.70 software from Dionex was used to acquire data for fingerprint analysis.

The analysis was performed with an RP C18 BDS Hypersil column (250 × 4.6 mm, 5 µm particle size) at a column temperature 26°C. The gradient program for pump A (acetonitrile: water, 30:70) and pump B (acetonitrile: water, 70:30) as follows: Initially 30% B, flow rate 0.8 ml/min; increased gradually to 50–70% B up to 30 min, flow rate 1.2 ml/min, then 20 min, 30% B, flow rate 0.8 ml/min. The detection wavelength was 220 nm. Injection volume for standard and sample was 20 µl [15]. Data analysis was performed by variations and similarity observed in retention time values, peak areas and spectral patterns of the peaks obtained in the chromatograms of *T. arjuna* bark extract.

Estimation of ellagic acid, gallic acid, and quercetin in a TAHA bark extract

Phytochemical characterization was carried out using gallic acid, ellagic acid, and quercetin by HPLC method [16-18]. Briefly, prominence HPLC system (Shimadzu, Japan) was used. Chromatographic separations were carried out using C-18 column (150 × 4.6 mm, 5 µm particle size; Syncronis, Thermo Scientific, USA). Following gradient elution with water containing 0.5% acetic acid (component A) and acetonitrile: water containing 0.5% of acetic acid (80:20 v/v) as component B was used.

The nonlinear gradient elution program: 0–10 min 10% of B; 10–20 min 20% of B; 20–30 min 40% of B; 30–40 min 60% of B; 40–45 min 70% of B; and 45–55 min 10 % of B and equilibrated with initial conditions for another 5 min. The flow rate and oven temperature were used at 1.0 ml/min and 25°C, respectively. All chromatograms were monitored at 270 nm.

Assay of alpha-amylase inhibition

A mixture of 50 µl of extract or acarbose and 50 µl 0.02 mol sodium phosphate solution (pH 6.9 with 6 mmol sodium chloride) containing alpha-amylase solutions (13 U/ml) were incubated at 25°C for 10 min. After pre-incubation, 50 µl 1% soluble starch solution in 0.02 mol sodium phosphate solution (pH 6.9 with 6 mmol NaCl) was added to each well at timed intervals. The reaction mixtures were then incubated at 25°C for 10 min followed by addition of 1 ml dinitrosalicylic acid color reagent. Then, the test tubes were placed in hot water (at 80°C for 10 min) to stop the reaction. The reaction mixture was diluted with 1 ml distilled water and absorbance was read at 540 nm [19,20].

Statistical analysis

Each experiment was repeated 3 times, and the data averaged for reporting. All values were expressed mean ± standard deviation (SD). Percentage of inhibition was calculated using the formula:

% Inhibition = [(Abs control - Abs sample)/Abs control] *100

The IC_{50} values were determined through nonlinear regression by fitting to a sigmoid dose-response equation with variable slope using GraphPad Prism5 software (GraphPad Software, Inc. La Jolla, CA, USA).

RESULTS

Phytochemical standardization of TAHA bark extract

Phytochemical standardization of TAHA extract was carried out using selected marker-based approach. Arjunetin, arjungenin, gallic acid, ellagic acid, and quercetin were used as phytochemical markers for standardization purpose. The marker contents were estimated using earlier reported HPLC-PDA method suitably modified on column and mobile phase gradient. The optimized chromatographic conditions showed good resolution of the all the peaks. The presence of marker contents in the TAHA extract was identified using retention time and ultraviolet (UV) spectra matching with corresponding reference standards. The retention time for arjunetin and arjungenin was found to be 4.95 and 7.68 min, respectively. The t_R for gallic acid, ellagic acid, and quercetin was found to be 2.36, 24.71, and 31.98 min, respectively. The spectral overlays for arjunetin and arjungenin showed the presence of UV spectra at 220 nm. The spectral overlays for gallic acid, ellagic acid, and quercetin showed the presence of UV spectra at 270 nm. The calibration plots of arjunetin and arjungenin versus peak area were constructed in the range of 31.25–1000 µg/ml. The calibration plots of gallic acid, ellagic acid, and quercetin versus peak area were constructed in the range of 3.125–100 µg/ml. The content of arjunetin and arjungenin was 0.47 and 8.22 mg/g in the TAHA extract (Table 1). These quantitative estimations were consistent with earlier reports on a TAHA extract. The content of gallic acid, ellagic acid, and quercetin was 2.443±0.090, 7.901±0.786, and 3.20±0.351 mg/g, respectively, in the TAHA extract (Table 1). These quantitative estimations were consistent with earlier reports of a TAHA extract.

Effect of TAHA extract and acarbose on α-amylase activity

In this study, acarbose is used as a standard drug for α-amylase inhibitor assay. Acarbose (at a concentrations 5–160 µg/ml) showed α-amylase inhibitory activity from 7.57±1.40 to 76.86±3.72% with an IC_{50} value 62.35±5.39 µg/ml (Table 2).

The percentage inhibition of the TAHA extract on α-amylase enzyme was studied in a concentration range of 10–320 µg/ml. The TAHA extract has shown inhibition of from 8.43±1.51 to 71.00±4.66% with an IC_{50} value 145. 90±16.34 µg/ml. The IC_{50} value for a TAHA extract was 145.90±16.34 µg/ml which is lesser to standard anti-diabetic drug acarbose which was 62.35±5.39 µg/ml (Table 2).

DISCUSSION

Many herbal extracts have reported and used in Ayurveda for the treatment of diabetes. However, medicinal plants have not gained much importance due to lack of proper standardization of herbal medicines and scientific support [5].

Several reports on the phytochemical analysis of *T. arjuna* have been recently published. The major isolated compounds include the five oleane derivatives, namely, arjunic acid, arjunolic acid, arjungenin, arjunetin, and arjunglucoside I from stem bark extract [21]. Also, flavon-3-ols such as (+)-catechin, (+)-gallocatechin, and (-)-epigallocatechin; phenolic acids such as gallic acid, ellagic acid and its derivatives such as 3-O-methyl-ellagic acid 4-O-β-D-xylopyranoside, and 3-O-methyl ellagic acid 3-O-rhamnoside were reported [22]. However, not much data are available related to α-amylase inhibition.

Effective synthetic α-amylase inhibitors (acarbose and voglibose) are available but cause various negative gastrointestinal symptoms

Table 1: Quantitation of arjunetin and arjungenin in the TAHA extract

Compound name	Rt (min)		(mg/g of TAHA)
	Reference standard	TAHA	
Arjunetin	4.95±0.0	5.09±0.02	0.59±0.04
Arjungenin	7.69±0.0	7.88±0.04	0.85±0.01
Gallic acid	2.36±0.0	2.36±0.05	2.44±0.09
Ellagic acid	24.71±0.0	24.71±0.06	7.90±0.79
Quercetin	31.99±0.0	31.97±0.1	3.20±0.35

Results were expressed as mean±SD; n=6, SD: Standard deviation

Table 2: Effect of acarbose and TAHA extract for α-amylase inhibitory activity

Drug/extract	Concentration of acarbose (µg/ml)	% of inhibition	IC_{50} value (µg/ml)
Acarbose	0	0	62.35±5.39
	5	7.57±1.40	
	10	15.43±1.27	
	20	23.71±1.60	
	40	39.00±2.65	
	80	52.86±1.77	
	160	76.86±3.72	
TAHA	0	0	145.90±16.34
	10	8.43±1.51	
	20	13.71±1.60	
	40	20.86±2.34	
	80	33.43±5.06	
	160	50.86±2.67	
	320	71.00±4.66	

Results were expressed as mean±SD; experiments were performed 3 times in triplicate; n=9, SD: Standard deviation

and hepatic disorders [23,24]. Phenolic α-amylase inhibitors from herbal extracts are potentially safer, and therefore, may be a suitable alternative for inhibition of carbohydrate breakdown and control of glycemic index of food products.

While there is no published report in the literature to date that has tested TAHA extract containing arjunetin, arjungenin, ellagic acid, gallic acid, and quercetin compounds for α-amylase inhibition activity. However, the methanolic, aqueous and 50% methanolic extract of *T. arjuna* was reported for α-amylase inhibition activity [13,14]. Table 2 represents the inhibitory effect of different concentration of the TAHA extract and acarbose. A TAHA extract and acarbose exhibited α-amylase inhibitory effects with an IC_{50} value of 145.90±16.34 and 62.35±5.39 µg/ml. In this study, acarbose (10–160 µg/ml) extract has shown an inhibition ranging from 15% to 76% and *T. arjuna* (10–160 µg/ml) though exhibited a minimum inhibitory potential ranging from 8% to 50% against α-amylase, it was less and not statistically significant. These investigations suggest that this may be due to the presence of potential α-amylase inhibitors.

In a recent study, methanol and free flavonoids extract of *T. arjuna* has been reported for α-amylase inhibitory effects with an IC_{50} valve 5.16 and 38.28 mg/ml [13]. Previously, Saha *et al*., reported that methanolic, aqueous and 50% methanolic extract of *T. arjuna* showed α-amylase inhibitory activity. IC_{50} value of methanolic, aqueous and 50% methanolic extract of *T. arjuna* was 1503±0.71 µg/ml, 592±0.34 µg/ml, and 302±0.55 µg/ml [14]. Further, our results suggest that the health promoting benefits of *T. arjuna* herbs used in traditional Indian medicine for treatment of DM may involve the α-amylase inhibiting activity of polyphenolic compounds, and that α-amylase inhibiting activity may be promoted by mechanistic synergies among the present phenolic substances.

Previous studies on the *in vivo* antidiabetic potential of *T. arjuna* bark in Wistar rats concluded that the hydroethanolic extract of this plant possessed strong glucose-lowering property in both alloxan and streptozotocin-induced diabetic rats [12,25], but the mechanism of action remained elusive. The present study suggests that one of the mechanisms by which *T. arjuna* exhibited its hypoglycemic potential in the reported animal study is through the inhibition of α-amylase. This inhibitory activity of the TAHA extract might be due to the presence of several polyphenols such as arjungenin, arjunetin, arjunolic acid, arjunic acid, quercetin, gallic acid, and ellagic acid in it.

The results of this study direct further research to evaluate the therapeutic effect of *T. arjuna* in the management of postprandial hyperglycemia in T2DM either alone or a combinatorial therapy. Further research is needed to investigate the potential α-amylase inhibitory activity of polyphenolic compounds present in *T. arjuna* and at elucidating putative phenolic synergies that may promote inhibition of α-amylase activity.

CONCLUSION

The TAHA bark extract demonstrates good α-amylase inhibitory activity due to a synergistic effect of the phytochemicals present in it. This study suggests that one of the mechanisms by which this plant displayed its antidiabetic potential is by the inhibition of α-amylase.

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AUTHORS CONTRIBUTION

Shushant A Shengule, has majorly performed the experiments in the laboratory. Sanjay Mishra, has provided the experimental concept, data analysis, and sincerely authored the article. Shweta Bodhale, has role for experimental protocol and conducting the experiment along with mentorship.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

REFERENCES

1. Kazeem MI, Adamson JO, Ogunwande IA. Modes of inhibition of α-amylase and α-glucosidase by aqueous extract of *Morinda lucida* Benth Leaf. BioMed Res Inter 2013;527570:6.
2. Jyothi KS, Hemalatha P, Avanthi A, Challa S. A comparative analysis on the alpha amylase inhibitory potential of six ornamental medicinal plants. J Nat Prod Plant Resour 2013;3:1-6.
3. Prabu M, Kumuthakalavalli R. Antidiabetic potential of the oyster mushroom *Pleurotus florida* (mont.) singer. Int J Curr Pharm Res 2017;9:51-4.
4. Shareef MI, Reddy PJ, Gopinath SM, Dayananda KS, Mandal A, Purushotham KM. *In vitro* α-amylase inhibitory activity of the leaves of *Tinospora cordifolia*. Inter J Innovat Res in Sci Eng Tech 2014;3:10091-6.
5. Dineshkumar B, Mitra A, Manjunatha M. A comparative study of alpha amylase inhibitory activities of common anti-diabetic plants at kharagpur 1 block. Inter J of Green Pharm 2010;4:115-21.
6. Rupeshkumar M, Kunchu K, Pallab H. Role of herbal plants in the diabetes mellitus therapy: An overview. Int J App Pharm 2014;6:1-3.
7. Reka P, Thahira B, Seethalakshmi M. Alpha amylase and alpha glucosidase inhibition activity of selected edible seaweeds from south coast area of India. Int J Pharm Pharm Sci 2017;9:64-8.
8. Chakrabarti R, Singh B, Prakrith VN, Vanchhawng L, Thirumurugan K. Screening of nine herbal plants for *in vitro* a-amylase inhibition. Asian J Pharm Clin Res 2014;7:84-9.
9. Biswas M, Kar B, Bhattacharya S, Kumar RB, Ghosh AK, Haldar PK. Antihyperglycemic activity and antioxidant role of *Terminalia arjuna* leaf in streptozotocin-induced diabetic rats. Pharm Biol 2011;49:335-40.

10. Dube N, Nimgulkar C, Bharatraj DK. Validation of therapeutic anti-inflammatory potential of arjuna ksheera paka: A traditional ayurvedic formulation of *Terminalia arjuna*. J Trad Complem Med 2017;7:414-20.
11. Desai S, Patil B, Kanthe P, Potekar R. Effect of ethanolic extract of *Terminalia arjuna* on liver functions and histopathology of liver in albino rats fed with hyperlipidemic diet. Int J Pharm Pharm Sci 2015;7:302-6.
12. Ragavan B, Krishnakumari S. Effect of *Terminalia arjuna* stem bark extract on the activities of marker enzymes in alloxan induced diabetic rats. Anc Sci Life 2005;25:8-15.
13. Jaiswal P, Kumar P. Alpha amylase inhibitory activity of different extract of *Terminalia arjuna* bark. Curr Trends Biotech Pharm 2017;11:253-8.
14. Saha S, Verma R. Inhibitory potential of traditional herbs on α-amylase activity. Pharm Bio 2012;50:326-31.
15. Singh DV, Verma RK, Singh SC, Gupta MM. RP-LC determination of oleane derivatives in *Terminalia arjuna*. J Pharma Biomed Anal 2002;28:447-52.
16. Lal UR, Tripathi SM, Jachak SM, Bhutani K, Singh I. HPLC analysis and standardization of arjunarishta – An ayurvedic cardioprotective formulation. Sci Pharm 2009;77:605-16.
17. Shengule SA, Mishra S, Joshi K, Apte K, Patil D, Kale P, *et al*. Anti-hyperglycemic and anti-hyperlipidaemic effect of Arjunarisht in high-fat fed animals. J Ayurveda Integr Med 2017; DOI.org/10.1016/j.jaim.2017.07.004.
18. Das P, Panneerselvam T, Prabhu P, Reddy J. Method development and validation of gallic acid and ellagic acid in Argwadharistam. Int J Curr Res 2017;9:61417-20.
19. Apostolidisa E, Lib L, Leea C, Seeramb NP. *In vitro* evaluation of phenolic-enriched maple syrup extracts for inhibition of carbohydrate hydrolyzing enzymes relevant to Type 2 diabetes management. J Funct Foods 2011;3:100-6.
20. Dada FA, Oyeleye SI, Ogunsuyi OB, Olasehinde TA, Adefegha SA, Oboh G, *et al*. Phenolic constituents and modulatory effects of Raffia palm leaf (*Raphia hookeri*) extract on carbohydrate hydrolyzing enzymes linked to Type-2 diabetes. J Trad Complem Med 2017;7:494-500.
21. Singh DV, Verma RK, Gupta MM, Kumar S. Quantitative determination of oleane derivatives in *Terminalia arjuna* by high performance thin layer chromatography. Phytochem Anal 2002;13:207-10.
22. Saha A, Pawar VM, Jayaraman S. Characterisation of polyphenols in *Terminalia arjuna* bark extract. Indian J Pharm Sci 2012;74:339-47.
23. Sudha P, Zinjarde SS, Bhargava SY, Kumar AR. Potent α-amylase inhibitory activity of Indian ayurvedic medicinal plants. BMC Compl Altern Med 2011;11:5.
24. Chipiti T, Ibrahim MA, Singh M, Islam MS. In vitro α-amylase and α-glucosidase inhibitory effects and cytotoxic activity of *Albizia antunesiana* extracts. Pharmacogn Mag 2015;11:S231-6.
25. Khaliq F, Parveen A, Singh S, Gondal R, Hussain ME, Fahim M. Improvement in myocardial function by *Terminalia arjuna* in streptozotocin-induced diabetic rats: Possible mechanisms. J Cardiovasc Pharmacol Ther 2013;18:481-9.

"MOLECULAR MECHANISM AND HERB DRUG INTERACTION OF HYDRO-ALCOHOLIC EXTRACT OF *TERMINALIA ARJUNA* BARK AND ITS AYURVEDIC FORMULATIONS IN METABOLIC SYNDROME"

An Errata submitted to

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI

Accredited **'A' Grade by NAAC (2nd Cycle)** Placed in Category 'A' by MHRD (GoI)

[Deemed-to-be-University u/s 3 of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]

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In the Faculty of Pharmacy

By

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March 2020

Errata submission of the PhD thesis:

Observations	Clarifications / Corrected as	Corrected thesis copy P. N.
1. With respect to publication from the current work, out of 6 publications shown, only one paper candidate is senior author and the same is not relevant work. Publication No. 5 is not relevant and published in 2015. Except one paper all others have no direct relevance to work	The publications in the thesis are from the PhD thesis work only. The full text of the articles has been included in thesis in addition to list – - The first published article [Journal of Ayurveda and Integrative Medicine] is centred on preliminary study hypothesis to endorse the Arjunarishta treatment effect in the direction of possible antihyperglycemic and antihyperlipidemic potential in High Fat Diet Fed Rat Model since it is well documented for cardiotonic effect only. This finding is discussed in the introduction section. - Second article is published in Indian Journal of Traditional Knowledge [IJTK] and was based on the phytochemical characterization of Ayurvedic formulations i. e. Arjunarishta and Arjun - Ghritah along with T. arjuna extract. [formulations standardization] - Third article is published in Asian Journal of Pharmaceutical and Clinical Research and was based on the alpha-amylase enzyme inhibition activity of T. arjuna. - 4th research articles has been already submitted to 'Fitoterpia Journal' and is based on lipid metabolism related genes mechanism study. The study findings are mentioned at page nos. 59 – 63 in results section whereas discussed at page nos. 80-83 in discussion section. - 5th research article is under manuscript preparation. It is based on molecular mechanism of T. arjuna and study findings are discussed in result [P. Nos.: 52-59] and discussion section [P. Nos.: 83-87]. - 6th research articles is under manuscript preparation and is based on herb drug interaction findings. **Pointed publication No. 5 which was published in 2015 is related with other than PhD work publications and is clearly mentioned on top of the page No. 119.**	The full text of the published research articles has been incorporated at the end of the thesis. [Separate Page Nos. are not assigned to avoid confusion in page nos.]

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2. Formatting of different sections is very poor	We apologise for such mistake and as per kind suggestion, respective changes were made uniformly in the different section of result and discussion.	Page Nos.: 47- 48, 50, 66-67, 69-70, 72, 74, 76-78, 91-112.
3. Title for tables are not properly written as per standard format, please see Table title 4.5, 4.8, 4.10, 4.11 and 4.12	We accept the raised point. The table titles in detail were not written to avoid the plagiarism percentage [self publications and others]. However, the necessary details were written at first citation at appropriate places. As per suggestion, respective changes are made in the title of 4.5, 4.8, 4.10, 4.11 and 4.12 tables along with necessary details.	Page Nos.: 47, 67, 76, 77, and 78
4. No title for Table in Page 68	The respective table was shifted from particular page to next page while printing as well as two tables were there together on that page and a common title was mentioned. Now, both the tables has been merged together and accordingly table title has been incorporated	Page No.: 67
5. Figure legends are incomplete Ref Fig 4.2, 4.3 and 4.4	As previously justified for avoidance of plagiarism the figure legends were mentioned in short. The suggested figures legends details have been incorporated. In addition other figure legends were also corrected in similar manner.	Page Nos.: 48, 48, 50, 51, and 66.
6. Figures are not of uniformly represented	Since, colour printer was used for printing of coloured as well as black and white pages, therefore some figure borders were red in colour and some were black. Such figures have been rearranged with uniformity at respective places in the thesis.	Page No.: 66, 69, 70, and 72.

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7. Figure 4.34 Histopathology feature are not clearly visible	The histopathological figure was presented in vertical page layout as well as was small in size. Therefore, was not clearly visible. The figure is presented in landscape page layout with clear visibility along with proper descriptions.	Page No. 74
8. References are not in uniform style or format	We apologise for such mistake. The cited references are now arranged in uniform style and **due to this the page no. of the thesis has been reduced from 138 to 134.** Accordingly, the page nos. after Reference section has been changed	Page No.: XXIII [Table of Content] Page Nos.: 91 - 112

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