Antioxidant, anti-inflammatory and anti-hyperglycemic properties of *Tridax procumbens* in lowering cardio-metabolic risk factors

by

Gauri Shirish Desai

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Approved by

Suresh T. Mathews, Chair, Associate Professor,
Department of Nutrition, Dietetics and Hospitality Management
B. Douglas White, Associate Professor,
Department of Nutrition, Dietetics and Hospitality Management
Kevin W. Huggins, Associate Professor,
Department of Nutrition, Dietetics and Hospitality Management
Barbara W. Kemppainen, Professor of Toxicology,
College of Veterinary Medicine

Abstract

Tridax procumbens L. has traditionally been used in India and Africa for the management of diabetes and inflammation. Phytochemical characterization has revealed presence of carotenoids, saponins, tannins, phenolics and flavonoids. Animal studies have shown that *T. procumbens* exerts antioxidant, anti-hyperglycemic, anti-inflammatory, anti-hypertensive, and anti-atherogenic effects. Therefore, we sought to characterize potential mechanisms mediating anti-hyperglycemic and anti-inflammatory effects and hypothesized that *T. procumbens* supplementation may lower cardiovascular risk factors. An asava (a hydro-alcoholic fermented extract), was prepared according to Ayurveda guidelines, using *Woodfordia fruticosa* L. Kurz flowers. Chemical and microbial analyses indicated the presence of phenolics, flavonoids, and carotenoids, and absence of microbial contamination, aflatoxins, heavy metals, and pesticide residues. *T. procumbens asava* demonstrated strong total antioxidant capacity, H_2O_2 scavenging activity, and inhibition of lipid peroxidation. A 4-week pilot study to evaluate blood glucose lowering effects demonstrated that *T. procumbens asava* supplementation lowered both fasting (decreased by 11% in men and 20% in women) and post-prandial blood glucose (decreased by 26% in men and 29% in women) in individuals with type 2 diabetes. In H4IIE rat hepatoma cells, both *T. procumbens* and *W. fruticosa* activated AMPK (AMP-activated protein kinase), and inhibited dexamethasone-induced gluconeogenic gene expression and hepatic glucose production. The anti-inflammatory effect of *T. procumbens* and *W. fruticosa* was mediated through inhibition of NFκB activation and NFκB transcriptional activity. A randomized, double-

blind, placebo-controlled trial was conducted to evaluate cardio-protective effects of *T. procumbens*. Seventy five individuals with type 2 diabetes were randomized to one of three groups that received either *T. procumbens asava, W. fruiticosa asava*, or placebo for 4 months. *T. procumbens* significantly lowered systolic blood pressure, total cholesterol, and LDL-cholesterol compared to placebo. No significant changes were observed in BMI, waist circumference, diastolic blood pressure, blood glucose, HbA1c, triglycerides, ALT, AST, urea or creatinine. At the end of the study period, a higher prevalence of individuals with HbA1c < 7.5%, was observed in *T. procumbens* group compared to placebo. No adverse effects were reported. Taken together, *T. procumbens* exerts strong antioxidant, anti-hyperglycemic and anti-inflammatory effects in vitro and in intact cells. *T. procumbens asava* supplementation appeared to lower systolic blood pressure, total and LDL-cholesterol, with modest effects on HbA1c.

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Table of Contents

List of Tables

List of Illustrations

List of Abbreviations

ADA	American Diabetes Association
CAM	Complementary and Alternative Medicine
IGT	Impaired glucose tolerance
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
AGEs	Advanced glycation end-products
AMPK	AMP-activated protein kinase
MAPK	Mitogen activated protein kinase
ACC	Acetyl-CoA carboxylase
HbA1c	Glycosylated hemoglobin
NFkB	Nuclear factor kappa B
IkB	Inhibitor of kappa B
PEPCK	Phosphoenolpyruvate carboxykinase
G6Pase	Glucose 6-phosphatase

Introduction

Diabetes is a defined as a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (ADA 2014). According to the National Diabetes Statistic Report, 29.1 million people or 9.3% of the U.S. population have diabetes, with the rate of newly diagnosed cases increasing exponentially (CDC 2014). Type 2 diabetes accounts for 90 to 95% of all diagnosed cases of diabetes, and the risk for developing type 2 diabetes increases with insulin resistance, obesity, impaired glucose metabolism and physical inactivity (CDC 2014). Chronic hyperglycemia associated with type 2 diabetes can lead to long-term damage, metabolic dysfunction, and failure of different organ systems including eyes, nerves, kidneys, heart and blood vessels (ADA 2014). Type 2 diabetes has also been shown to significantly increase the risk of macrovascular complications including coronary heart disease (CHD), stroke, and peripheral vascular disease, resulting in more than 70% of the deaths among type 2 diabetic population (Laakso 2010). Type 2 diabetes has been associated with dyslipidemia, consisting of moderate elevation in triglycerides, low HDL cholesterol, and elevation in small dense LDL particles. Diabetic dyslipidemia is a well-recognized and modifiable risk factor to prevent cardiovascular diseases in individuals with type 2 diabetes (Solano 2006).

It has been estimated that the average medical expenditures for people with diabetes amount to $176 billion with indirect costs including disability, work loss and premature death accounting for $69 billion. Strategies for effective management of diabetes include controlling

blood glucose through drug and insulin therapies, and lifestyle modification including healthy eating, increased physical activity, and weight management. Current algorithm for diabetes management includes oral hypoglycemic agents (metformin, sulfonylureas, biguanides, α-glucosidase inhibitors, glitazones, glucagon-like peptide 1 agonist, dipeptidyl peptidase-IV inhibitors, and sodium/glucose cotransporter 2 (SGLT-2) inhibitors), addition/intensification of insulin, and modification of cardiovascular risk factors (Garber 2013). During the years 2010-2012, approximately 57% of the people age 18 years or older have been reported to use oral medications only, 15% required insulin treatment in addition to oral medications, 14% were treated with insulin alone, and another 14% have been reported to use neither insulin nor oral medications for diabetes management (CDC 2014).

Different classes of oral hypoglycemic agents used in diabetes management have been associated with certain risks. For instance, the glitazones can cause liver toxicity, edema, and increased risk of congestive heart failure; biguanides, DPP-IV inhibitors, and alpha-glucosidase inhibitors have been associated with gastrointestinal discomfort; and hypoglycemia is the major detrimental effect of sulfonylureas (Fowler 2007). Increased cost of conventional anti-diabetic medications and associated side-effects can result in non-adherence and poor blood glucose control, indicating an increased need for the development of cost-effective supplemental therapy (Rubin 2005, Hauber 2009).

According to the National Health Statistics Report, use of Complementary and Alternative Medicine/Therapies (CAM) increased among adults and children in the US, from 2002 to 2007. The most commonly used CAM therapies were non-vitamin, non-mineral, natural products (17.7%), followed by deep breathing (12.7%), and meditation (9.4%) (Barnes, 2008). Plants including *Gymnema sylvestre*, *Panax quinquefolius* (American Ginseng), *Coccinia indica*

(Ivy gourd), *Allium sativum* (Garlic), *Momordica charantia* (Bitter gourd) indicate their use as a drug in diabetes management (Grover 2002, Yeh 2003, Mukherjee 2006, Gupta 2007). So far, higher quality randomized controlled trials (RCTs) for single herb preparations are available for *Coccinia indica*, ginseng species, *Bauhinia forficate*, and *Myrcia uniflora*. Other herbs like *Allium cepa*, *Ocimum sanctum*, *Ficus caric* and *Trigonella foenum* have been studied in poorer-quality RCTs. *Gymnema sylvestre* and *Momordica charantia* have been studied in non-randomized controlled trials (Yeh 2003).

Tridax procumbens Linn. (Family – Asteraceae), commonly known as coat buttons, is a herb native to the tropical Americas, and further introduced to the tropical, subtropical, and mild temperate regions worldwide; is known for several potential therapeutic activities. It has been used traditionally in certain parts of India to alleviate symptoms of inflamed skin, mouth sores, skin infections, bleeding wounds, diarrhea, rheumatism, and epistaxis (Singh 2002, Ayyanar 2005, Upadhyay 2010, Natural Products Alert Database 2012). Ethnomedicinal data suggest that *T. procumbens* may possess properties effective in the management of diabetes. Inhabitants from Mandesh (Maharashtra, India) and Udaipur (Rajasthan, India) have reported the use of *T. procumbens* for diabetes management (Bhagwat 2008, Pareek 2009).

Recent research has shown that *T. procumbens* extracts possess anti-inflammatory, analgesic and anti-diabetic activity in animal models (Anulukanapakorn 1997, Bhagwat 2008, Awasthi 2009, Pareek 2009, Jachak 2011, Prabhu 2011). Pre-clinical studies have shown that acute and sub-acute administration of *T. procumbens* significantly decreased fasting blood glucose levels in alloxan-induced diabetic rats, but not in non-diabetic control rats (Anulukanapakorn 1997, Bhagwat 2008, Pareek 2009). *T. procumbens* has also been shown to reduce plasma concentrations of triglycerides, LDL-, VLDL-, and total cholesterol, as well as

lower atherogenic indices, including cardiac risk ratio, atherogenic coefficient, and atherogenic index of plasma in rats loaded with cholesterol (1g/100g body weight) (Ikewuchi 2009). However, there are no clinical studies examining the efficacy of *T. procumbens* for glucose control and lowering of cardiovascular risk in patients with diabetes. Also, there are limited reports on characterization of the molecular mechanisms underlying the anti-hyperglycemic and anti-inflammatory properties of *T. procumbens*. Therefore, the goal of this study was to evaluate blood glucose lowering properties of *T. procumbens* in individuals with type 2 diabetes, and to examine its impact on body weight, waist circumference, blood pressure and plasma lipid profile. Further, this study aimed at investigating potential anti-hyperglycemic and anti-inflammatory mechanisms using *in vitro* techniques.

Review of Literature

2.1 Diabetes

The American Diabetes Association (ADA) describes diabetes mellitus as a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (ADA 2014). The prevalence of diabetes is increasing worldwide at an alarming rate, with the highest prevalence in China (98.4 million) followed by India (65.1 million) and the United States (24.4 million). The International Diabetes Federation's (IDF) most recent estimates indicate that 8.3% adults (382 million) were reported to be living with diabetes, with 80% of the total number affected living in the low- and middle-income countries. However, these numbers might fail to convey facts as 46% cases of diabetes still remain undiagnosed (IDF 2014).

Diabetes is associated with symptoms including polydipsia (excessive thirst), polyuria (frequent urination), polyphagia (excessive eating), slow healing of wounds, recurrent infections, blurred vision, and unexplained weight loss. Chronic and uncontrolled hyperglycemia can also damage various organs, particularly the eyes, kidneys, nerves, heart and blood vessels (ADA 2014) and increase the risk for macrovascular complications (Cade 2008, Pagano 2013) such as myocardial infarction, ischemic heart disease, peripheral artery disease, stroke, congestive heart failure; and microvascular complications such as retinopathy, nephropathy and neuropathy (Cade 2008).

2.1.1 Diabetes classification and diagnosis

The first worldwide accepted classification for diabetes, based on pharmacologic therapy, categorized diabetes into two major groups namely, insulin-dependent diabetes (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) (National Diabetes Data Group 1979). However, based on recent scientific advancements and valuable contributions from clinicians and researchers from academia, the private sector, ADA, the National Institutes of Health (NIH) and the World Health Organization (WHO), diabetes is currently classified as

- type 1 diabetes (previously known as IDDM or juvenile-onset diabetes)
- type 2 diabetes (previously known as NIDDM or adult-onset diabetes)
- "other specific types" in cases where specific genetic defects, surgery, drugs or other factors have caused hyperglycemia
- Gestational diabetes mellitus (GDM) that develops during pregnancy

Type 1 diabetes accounts for 5-10% of people with diabetes and results from a cellular-mediated autoimmune destruction of the pancreatic β-cells (ADA 2014). Markers for type 1 diabetes include the islet cell cytoplasmic antibodies (ICA) and autoantibodies to insulin, glutamic acid decarboxylase (GAD), tyrosine phosphatase (IA2) and for GM2-1 glycolipid (Panczel 1999). Loss of β-cells often requires treatment with exogenous insulin to maintain normoglycemia. Exogenous insulin is a potent inhibitor of glucagon secretion, thus in addition to the loss of β-cells, type 1 diabetes is also characterized by a defective glucagon response to insulin-induced hypoglycemia (Taborsky 2012). Generally, the first manifestation of type 1 diabetes is ketoacidosis in the presence of infection or stress (ADA 2014). Diabetic ketoacidosis (DKA) results in high levels of ketones in blood and urine from

excessive fat breakdown due to lack of insulin and can be a serious complication of type 1 diabetes.

Type 2 diabetes is the most common type of diabetes accounting for ~ 90-95% of diagnosed cases. It is characterized by insulin resistance and relative insulin deficiency (ADA 2014). Insulin resistance is defined as the inability of a known quantity of exogenous or endogenous insulin to increase glucose uptake and utilization compared to normal population. The mechanisms underlying the development of insulin resistance include genetic abnormalities, fetal malnutrition and increases in visceral adiposity with the risk increasing with age, obesity and lack of physical activity (Lebovitz 2001, ADA 2014).

The "other specific types" can be due to genetically determined abnormalities of insulin action associated with mutations of the insulin receptor; diseases of the exocrine pancreas including pancreatitis, trauma, infection, pancreatectomy and pancreatic carcinoma; excess amounts of hormones such as growth hormone, cortisol, glucagon and epinephrine; and the use of certain drugs such as intravenous pentamidine (ADA 2014).

GDM is defined as any degree of glucose intolerance with onset or first recognition during pregnancy and normally occurs during the 3rd trimester. Approximately 7% of all pregnancies in the U.S are complicated by GDM, resulting in more than 200,000 cases annually. GDM is considered to increase the risk for future development of type 2 diabetes (ADA 2014).

In addition, a condition known as prediabetes is associated with an increased risk of developing diabetes. Prediabetes is defined as a fasting plasma glucose level of 100-125

mg/dl [impaired fasting glucose (IGF)], a 2-hour plasma glucose level after a 75-g oral glucose tolerance test of 140 to 199 mg/dl [impaired glucose tolerance (IGT)] (ADA 2014).

2.2 Oxidative stress

Free radicals including reactive oxygen species (ROS) and reactive nitrogen species (RNS) are chemically reactive molecules that are generated in our body by various endogenous systems following exposure to different physicochemical conditions or pathophysiological states (Devasagayam 2004). In a healthy individual, both ROS and RNS are produced in a well-regulated manner to help maintain homeostasis at the cellular level and they also play an important role in cell signaling (Devasagayam 2004, Valko 2007). The beneficial effects of ROS/RNS occur at low/moderate concentrations and they play a role in number of biological processes including generation of ATP via oxidative phosphorylation, detoxification of xenobiotics, apoptosis of effete and/or defective cells, in defense against infectious agents and in generation of prostaglandins and leukotrienes (Devasagayam 2004). However, higher levels of ROS/RNS are involved in the process of aging and also lead to a number of disease states including ischemia, cancer, compromised immunity and malfunctioning of the endocrine system. The superoxide anion ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) are partially reduced metabolites of O_2 that are generated during various physiological processes. In the presence of transition metal ions, H_2O_2 can get converted to even more reactive hydroxyl radical ($OH^{\bullet}$) (Thannickal 2000).

Enzymatic and non-enzymatic antioxidant defense systems function to keep free radicals in check. Enzymatic antioxidant defense includes superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) while non-enzymatic defense mechanism involves

ascorbic acid (vitamin C), α-tocopherol (α –TOH, vitamin E), glutathione (GSH), β-carotene and vitamin A (Mates 1999). Enzymatic antioxidants catalyze reactions to prevent formation of free radicals, whereas non-enzymatic antioxidants are mainly involved in scavenging free radicals to minimize cellular damage.

The superoxide anion is mostly formed in the mitochondria during oxidative phosphorylation. It is highly unstable in aqueous solutions and undergoes rapid dismutation to H_2O_2 under acidic pH conditions. SOD increases the rate of dismutation reaction by 10^4-fold leading to the rapid conversion of $O_2^{-\bullet}$ to the more stable H_2O_2 (Mates 1999, Thannickal 2000).

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

H_2O_2 is also generated by direct reduction of O_2 in the peroxisomes which is converted to $OH^\bullet$ via the Fenton and Haber-Weiss reactions (Mates 1999).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^\bullet + OH^-$$

$$O_2^{-\bullet} + H_2O_2 \longrightarrow O_2 + OH^\bullet + OH^-$$

In order to prevent the formation of hydroxyl radicals, CAT converts H_2O_2 to water and molecular oxygen. CAT also possesses peroxidase activity and in addition to GPX catalyzes the reduction of hydroperoxides. GPX utilizes the antioxidant GSH to neutralize free radicals and is one of the most important and essential antioxidant defense mechanisms of our body (Mates 1999).

$$2\ H_2O_2 \xrightarrow{CAT} 2\ H_2O + O_2$$

$$ROOH + AH_2 \xrightarrow{CAT} H_2O + ROH + A$$

$$ROOH + 2GSH \xrightarrow{GPX} ROH + GSSG + H_2O$$

Nitric oxide (NO) contains one unpaired electron and is hence considered to be a radical. NO is produced from L-arginine in various biological tissues via the action of nitric oxide synthases (NOSs). NO is an important cell signaling molecule and plays a role in neurotransmission, blood pressure regulation, defense mechanisms, smooth muscle relaxation and immune regulation. Both NO and $O_2^{-\bullet}$ are produced during an immune response. These two molecules can react to produce peroxynitrite anion ($ONOO^-$), which is a potent oxidizing agent that can cause DNA fragmentation and lipid oxidation (Valko 2007).

$$NO + O_2^{-\bullet} \longrightarrow ONOO^-$$

The over-production of ROS leads to oxidative stress and the overproduction of RNS leads to nitrosative stress. This stress is the result of an imbalance between generation of reactive species and the biological system's antioxidant defense mechanism (Valko 2007). The excess reactive species can affect normal functioning of proteins, lipids and DNA and they play an important role in the etiology of various diseases and disorders.

Membrane lipids are the most susceptible to damage by free radicals leading to a chain reaction of lipid peroxidation (LP). Initiation of a peroxidative sequence begins with the attack of a free radical, which abstracts a hydrogen atom from a methylene group (CH_2), leaving behind an unpaired electron on the carbon atom (•CH) (Mylonas 1999, Devasagayam 2004, Niki 2005). The resulting lipid free radical can then undergo oxidation to form peroxyl

radical which can react with other PUFAs producing lipid hydroperoxide and another lipid free radical. Thus a lipid peroxidation chain reaction is propagated and it can cause severe damage to tissues and cell membranes (Mylonas 1999, Grotto 2009).

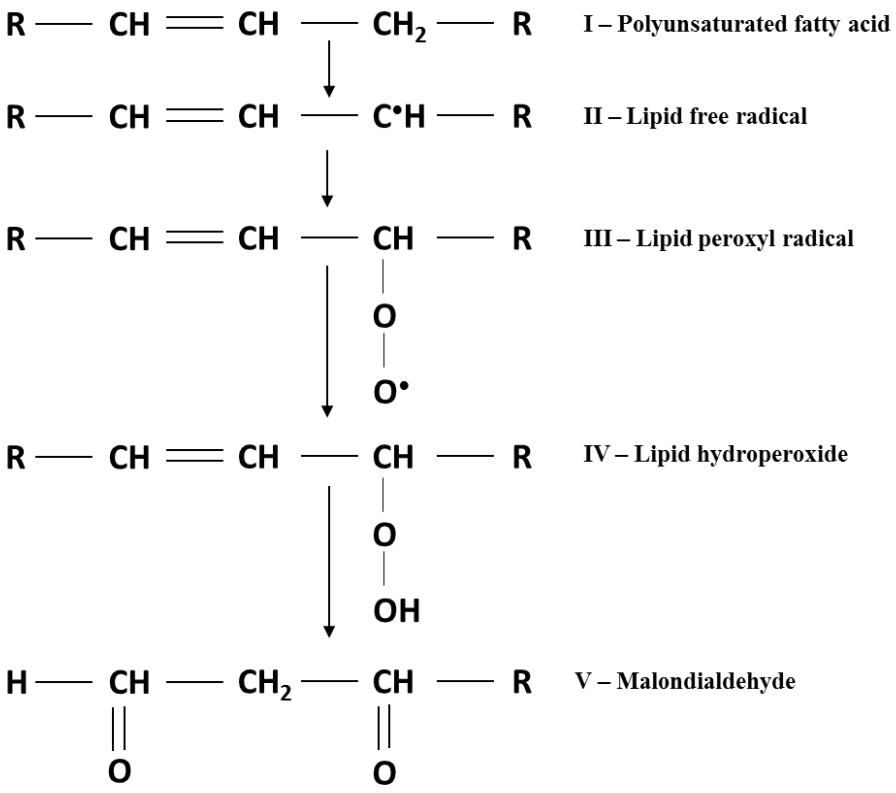

Figure 1. Schematic steps of MDA formation from polyunsaturated fatty acids (Grotto 2009)

The generated lipid hydroperoxide is unstable and is converted to malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), which are toxic and can potentially cause DNA damage (Valko 2007, Grotto 2009).

Lipid peroxidation chain reactions can be terminated by antioxidant such as α-tocopherol which forms a more stable tocopherol phenoxyl radical that is not involved in further chain reactions. This tocopherol phenoxyl radical is further recycled by other antioxidants such as vitamin C and GSH (Devasagayam 2004).

$(LOO^{\bullet}) + LH \longrightarrow L^{\bullet} + LOOH$

$LOO^{\bullet} + \alpha\text{-}TOH \longrightarrow LOOH + \alpha\text{-}TO^{\bullet}$

2.3 Oxidative stress and type 2 diabetes

Oxidative stress is increased in diabetes because of multiple factors including hyperglycemia-induced glucose-autoxidation, cellular oxidation/reduction imbalance, reduction in antioxidant defense, increased levels of proxidants such as ferritin and homocysteine, protein glycation, and formation of advanced glycation end-products (AGEs) (Bonnefont-Rousselot 2000, Rahimi 2005). Various studies have associated diabetes mellitus with increased formation of free radicals and decreased antioxidant potential. Classically used oxidative stress biomarkers involve markers of lipid peroxidation such as thiobarbituric acid reactive substances (TBARS), plasma total antioxidant status including metal chelators, free radical scavengers such as vitamin E and C and antioxidant enzymes (Bonnefont-Rousselot 2000). Determining oxidative age via breath microassays (Phillips 2004) is a potential new diagnostic tool with applications in clinical practice. These microassays measure the levels of methylated alkanes (C4-C20) that are produced as a result of oxidation of polyunsaturated fatty acids by ROS in cell membranes and are excreted in the breath as volatile organic compounds (VOCs).

Type 2 diabetes is associated with increased lipid peroxidation and SOD activity as indicated by high levels of lipid peroxidation end-product, malondialdehyde (MDA) (Song 2007, Bandeira 2012). The increase in total SOD activity in type 2 diabetes suggests an augmented production of reactive species. This increase in activity is probably due to an increased production of $O_2^{-\bullet}$ anion (Bandeira 2012) which in turn causes an increase in the levels of H_2O_2. The higher SOD/CAT ratio in type 2 diabetes (Bandeira 2012) explains, in

part, the increased levels of H_2O_2, which in higher concentrations, has been associated with lesions in the pancreatic beta cells, causing disturbance both in cell signaling and gene expression (Maechler 1999). Mitochondrial oxidative stress leads to oxidative modifications of key enzymes involved in glucose metabolism including aconitase (Beckman 1998) and adenine-nucleotide translocase (Yan 1998). Aconitase participates in the citric acid cycle, hence its inhibition hampers efficient glucose metabolism. Inhibition of aconitase by $O_2^{-\bullet}$ releases free iron from the enzyme which can then react with H_2O_2 and exacerbate the stress by generation of $OH^{\bullet}$ via the Fenton Haber-Weiss reactions (Beckman 1998, Maechler 1999, Mates 1999).

Impaired glucose tolerance (IGT), however, shows decreased erythrocyte SOD activity probably due to non-enzymatic glycation of the enzyme-protein (Song 2007). The observed differences in SOD activity could be due to a lack of correlation between oxidative stress and blood sugar levels or HbA1c levels (Phillips 2004). IGT does not exhibit significant differences in oxidative stress and antioxidant markers compared to normal glucose tolerance. However, IGT is associated with increased levels of MDA (Song 2007) suggesting the beginning of oxidative stress.

The primary causes of diabetes-related oxidative stress include hyperglycemia and glucotoxicity (Bonnefont-Rousselot 2000, Stadler 2012). When glucose is at an abnormally high intracellular concentration, as in type 2 diabetes, it activates the polyol pathway which plays a major role in development of diabetic complications (Bonnefont-Rousselot 2000, Goto 2003, Oyama 2006, Stadler 2012). Glucose metabolism via the polyol pathway involves conversion of excess glucose to sorbitol, by aldose reductase, which is then oxidized to fructose by sorbitol dehydrogenase (Bonnefont-Rousselot 2000, Stadler 2012). Aldose

reductase requires NADPH for its activity, hence activation of the polyol pathway caused by hyperglycemia results in the depletion of cellular NADPH levels. Antioxidant enzymes including glutathione reductase require NADPH as a co-factor. Depletion of intracellular NADPH pool affects the activity of glutathione reductase and hence hampers regeneration of GSH from GSSG contributing to an increase in cellular oxidative stress.

Activation of the polyol pathway is also suggested to augment intimal hyperplasia, which is an increase in the thickness of tunica intima of a blood vessel and can result in the development of diabetic complications including cardiovascular diseases (Goto 2003). Excess of sorbitol and the resulting osmotic stress in retinal cells (Gonzalez 1984) and Schwann cells (Ohtaka 1992, Maekawa 2001) of the peripheral nervous system can lead to the development of retinopathy and peripheral neuropathy in type 2 diabetes. The amount of DNA fragmentation, indicated by levels of 8-hydroxydeoxyguanosine (8-OHdG) is significantly higher under conditions of high glucose and can result in endothelial cell damage (Oyama 2006).

Hyperglycemia can also lead to activation of protein kinase C (PKC) (Geraldes 2010) as a result of an increase in *de novo* diacylglycerol (DAG) synthesis in various tissues and cells including pancreatic islets (Wolf 1990), glomeruli (Craven 1990) and vascular smooth muscle cells (Xia 1994, Hiramatsu 2002). Hyperglycemia-induced increases in DAG levels result from an increase in the glycolytic pathway intermediates including dihydroxyacetone phosphate (DHAP) and glycerol-3-phosphate (G3P) (Wolf 1990, Xia 1994, Czabany 2007). Activation of various PKC isoforms can influence a variety of cellular functions including vascular permeability, angiogenesis, cell growth and apoptosis, changes in vessel dilation, basement membrane thickening, enzymatic activity modifications and alterations in

activation of transcription factors (Geraldes 2010). PKC activation is also known to activate NADPH oxidase (Inoguchi 2000), a membrane-bound enzyme complex that generates $O_2^{-\bullet}$ anion by transferring electrons from NADPH inside the cell across the membrane and coupling it to molecular oxygen, thus contributing to oxidative stress.

AGEs formed as a result of oxidative stress and high intracellular glucose levels can cause severe alterations in cell signaling processes (Thornalley 1998) including increased expression of extracellular matrix proteins, vascular cell adhesion molecules, cytokines and growth factors by binding the receptor of advanced glycation end-products (RAGE) (Rojas 2013). This can further lead to chemotaxis, angiogenesis, increased oxidative stress, cell proliferation and/or programmed cell death (Thornalley 1998). Glucose-derived activation of RAGE increases secretion of pro-inflammatory cytokines including tumor necrosis factor α (TNF-α) (Abordo 1997), NFκB and activates multiple signaling pathways including p21ras, erk1/2 (p44/p42) mitogen-activated protein kinases, p38 and SAPK/JNK mitogen-activated protein kinases, rhoGTPases, phophoinositol-3 kinase (PI3K) and JAK/STAT pathway (Rojas 2013). Accumulation of AGEs in pancreatic β-cells can result in islet dysfunction and is associated with reduced glucokinase levels (Kooptiwut 2005). AGEs can also cause inflammation and activation of macrophages which, if left untreated, can cause tissue lesions and future manifestations of chronic diseases (De Carvalho 2012).

2.4 Oxidative stress and insulin signaling

Insulin is a polypeptide hormone secreted by the beta cells of pancreatic islets of Langerhans, which constitute around 60-80% of all the islet cells (Lomedico 1976). Insulin contains two polypeptide chains (A and B) linked by disulfide bonds, however, it is secreted

as a single inactive polypeptide called preproinsulin (Lomedico 1976, Harper 1981). The mature two-chain structure of insulin involves formation of proinsulin by proteolytic cleavage of the amino-terminal signal sequence involved in insulin secretion from beta cells, followed by selective removal of the connecting or C peptide (Steiner 1974, Harper 1981, Rains 2011). Transcriptional regulation of insulin gene can be modified in response to nutrients or hormonal stimuli via kinase-dependent signaling pathways (Melloul 2002).

Insulin plays a key role in growth and development of tissues and in controlling carbohydrate and fat metabolism (Pirola 2004). Insulin regulates glucose homeostasis by increasing the rate of glucose uptake into skeletal muscle and adipose tissue and by reducing hepatic glucose production (Sesti 2006). Glucose serves as the driving force for insulin secretion with glucose transporter GLUT2 and the enzyme glucokinase playing an important role in glucose sensing. GLUT 2 is mainly expressed in the liver, beta-cells of the pancreas and the basolateral membrane of kidney proximal tubules (Im 2005). Studies also demonstrate the presence of GLUT2 in specific regions of the brain including the arcuate nucleus of the hypothalamus which is in control of energy homeostasis and feeding behavior (Leloup 1998). GLUT2 gene transcription is upregulated in the liver during postprandial hyperglycemia via binding of sterol response element-binding protein (SREBP)-1c to GLUT2 promoter (Im 2005). In addition to GLUT2, the enzyme glucokinase, the sulfonylurea receptor-1 (SUR1), glucagon-like peptide-1 receptor (GLP-1R) and neuropeptide Y (NPY) are involved in central glucose sensing and regulation of food intake (Porzio 1999, Li 2003). Glucokinase facilitates phosphorylation of glucose to glucose-6 phosphate, which is the first step in glucose metabolism. Glucokinase has a high Km, and therefore, low affinity for glucose, thus allowing better glucose-sensing ability. ATP

generated by glucose metabolism serves to regulate insulin secretion from β-cells by closing the ATP-sensitive K^+ channels (Li 2004). Closing of K^+ channels, in turn, opens voltage-gated calcium channels (L-type) on β-cells allowing calcium flux into the cytoplasm. Calcium causes exocytosis of insulin-containing vesicles resulting in insulin secretion (Jung 2009, Cui 2012).

Insulin signaling occurs by activation of insulin receptor, which belongs to the family of receptor tyrosine kinases. Binding of insulin to the α-subunit of insulin receptor phosphorylates and activates insulin receptor substrate-1 (IRS-1), which in turn mediates the signal by binding various signaling molecules containing the SRC homology 2 (SH-2) domain, such as PI3K (Wilden 1996, Ogawa 1998). Studies have coupled insulin receptor signaling with cellular oxidant generating mechanisms. Upon stimulation of insulin receptor with insulin, there is an increase in cellular H_2O_2 mediated by NADPH oxidase. H_2O_2 generation enhances tyrosine phosphorylation of insulin receptor and IRS-1 along with oxidative inhibition of cellular protein-tyrosine phosphatases (Mahadev 2004). However, prolonged exposure to H_2O_2 results in impaired insulin-mediated glucose uptake via reduction in insulin-stimulated GLUT4 translocation (Rudich 1998). It also disrupts IRS-1 and PI3K binding (Rudich 1998) and cellular distribution between the cytosol and an internal membrane pool resulting in a 90% reduction in Akt serine 473 phosphorylation (Tirosh 1999). Increased ROS production in mitochondria also interferes with insulin action by increasing serine phosphorylation of IRS-1 and decreasing insulin-stimulated tyrosine phosphorylation of IRS-1 and serine phosphorylation of Akt (Imoto 2006). Serine phosphorylation of insulin receptor or IRS-1 impairs recruitment of downstream signaling molecules and disrupts the ability of IRS-1 to interact with insulin receptor (Rains 2011).

Inhibition of the insulin signaling pathway due to oxidative stress and elevated levels of inflammatory cytokines leads to the development of an insulin resistant state (Koyama 1997, Sesti 2006, Rains 2011). In order to maintain normal glucose homeostasis under conditions of insulin-resistance, there is an increase in the secretion of insulin, relative to glucose, leading to hyperinsulinemia (Koyama 1997). Hyperinsulinemia is paralleled by oxidative stress, alterations in mitochondrial physiology, changes in mitochondrial biogenesis-related gene expression (Abhijit 2013) ultimately resulting in mitochondrial dysfunction (Yang 2012). Mitochondrial dysfunction can then create a feedback loop, adding to overall oxidative stress environment and exacerbating the condition of insulin-resistance (Rains 2011).

2.5 Antioxidants and type 2 diabetes

Considering the involvement of oxidative stress in diabetes, supplementation with antioxidants can help in lowering the levels of free radicals thus allowing a delay in development of type 2 diabetes and its complications (Bonnefont-Rousselot 2000, Rahimi 2005, Pazdro 2010). Free radical scavengers such as vitamin E and vitamin C have been widely studied for their protective role against tissue damage. Vitamin E and C are likely to act synergistically where vitamin E is primarily involved in the termination of lipid peroxidation chain reaction, forming the tocopheroxyl radical in the process, while vitamin C plays an important role in regeneration of tocopherol and protection of the protein thiol group from oxidation (Naziroglu 2005, Rahimi 2005). Supplementation with vitamin E, vitamin C and lipoic acid can improve insulin signaling and insulin-sensitivity by inhibiting IRS-1 serine phosphorylation and JNK pathway while preserving the free radical defense system (Vinayagamoorthi 2008). Vitamin C, the strongest physiological antioxidant in aqueous

environment, can be used as a marker of fruit intake and has been observed to reduce oxidative stress in type 2 diabetes (Rahimi 2005, Hegde 2013). Daily consumption of fruits and green leafy vegetables has been shown to be inversely associated with development of chronic diseases (Joshipura 1999, Bazzano 2002, Hung 2004), including type 2 diabetes, as opposed to the positive association of fruit juice consumption and the incidence of type 2 diabetes ((Bazzano 2008) reference?). These associations were found to be independent of known risk factors for type 2 diabetes, including age, BMI, family history, smoking, physical activity, total energy intake, and consumption of whole grains, nuts, processed meats, coffee and potatoes (Bazzano 2008). The beneficial effects of consuming whole fruits in place of fruits juices can be attributed to the presence of high fiber and micronutrients in whole fruits compared to the lack of fiber and other phytochemicals, and a high sugar load in fruit juices.

Phytochemicals are defined as bioactive non-nutrient plant compounds in fruits, vegetables, grains, and other plant foods, classified as carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur compounds (Figure 2) (Liu 2004).

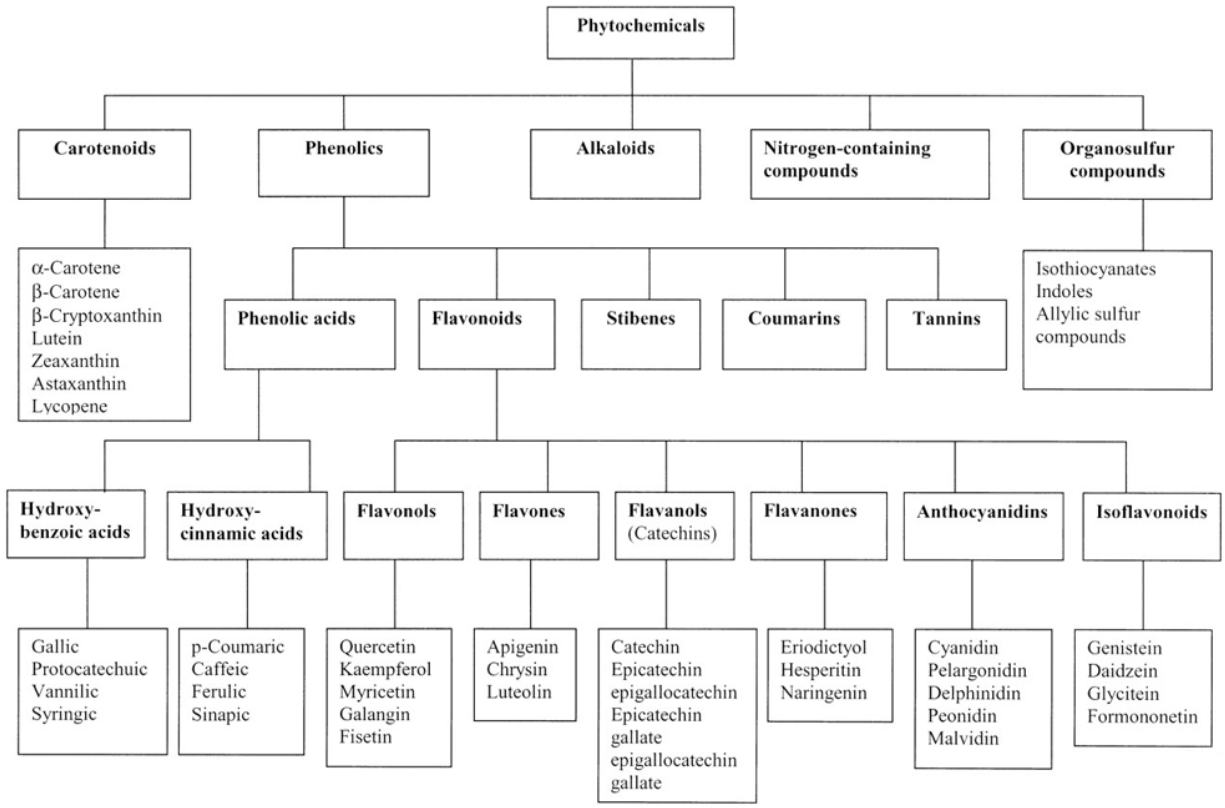

Figure 2. Classification of dietary phytochemicals (Liu 2004)

The protective effects of fruits and vegetables are ascribed to the presence of phenolic phytochemicals, which include flavonoids such as anthocyanins, dihyrochalcones, flavan-3-ols, flavanones, flavons, flavonols, isoflavones; and non-flavonoids such as gallic acid, ellagic acid, tannins, phenolic acids, hydroxycinnamates (coumaric acid, caffeic acid) and stilbenes (resveratrol, piceatannol) (Crozier 2009). The most widely consumed dietary sources of phenolics and polyphenolics include green tea, black tea, red wine, coffee, and cocoa/chocolate, followed by fruits, vegetables, herbs, spices, nuts, algae and olive oil. In the U.S., estimated total flavonoid intake (189.7 mg/d) was mainly from flavan-3-ols (83.5%), followed by flavanones (7.6%), flavonols (6.8%), anthocyanidins (1.6%), flavones (0.8%), and isoflavones (0.6%) with the highest flavonoids obtained from tea (157 mg), then from citrus fruit juices (8 mg), wine (4 mg) and citrus fruits (3 mg) (Chun 2007).

Absorption of phenolics depends significantly on the modulation of the flavonoid aglycone and the type of sugar attached. Most of the phenolics and polyphenolics are absorbed into the circulatory system in the small intestine, with formation of glucuronide, methyl and sulfate metabolites. Some pass over to the large intestine where they are subject to cleavage by colonic microflora, forming phenolic acid and non-phenolic catabolites. These can then be absorbed and are ultimately excreted in the urine (Borges 2007, Crozier 2009, Daniele 2010).

Dietary phenolics are known to benefit human health due to their high antioxidant potential. The most important structural feature of a flavonol, for example, quercetin is the catechol or dihydroxylated B-ring (R_1, R_2 – OH) (Figure 3) followed by the presence of unsaturation in the C-ring and the presence of a 4-oxo function (black ring) in the C-ring (Williams 2004, Crozier 2009).

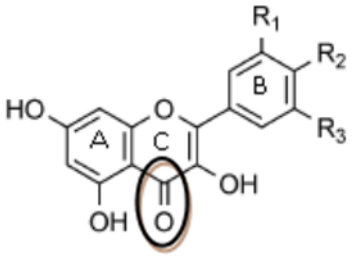

Figure 3. Representative chemical structure of flavonol, quercetin (Williams 2004)

In addition to their role as hydrogen donors, flavonoids may exert protective effects by influencing intracellular signaling cascades (Kong 2000, Williams 2004). Flavonoids have been shown to protect neurons against apoptosis under stress-activated conditions by strongly inhibiting Ca^{2+}-dependent activation of extracellular signal-regulated kinases 1/2 (ERK1/2) and c-Jun-N-terminal kinase (JNK). They also inhibit cleavage of procaspase-3 which can form the active caspase-3 (cysteine-aspartic acid protease-3) enzyme involved in cell apoptosis (Kobuchi 1999, Schroeter 2001). Quercetin exhibits antidiabetic properties via its

action β-cells. It stimulates insulin secretion by increasing Ca^{2+} influx through interaction with L-type Ca^{2+} channels (Bardy 2013).

Cell surface expression and mRNA levels of intercellular adhesion molecule 1 (ICAM-1) in phorbol 12-myristate 13-acetate (PMA)- and TNFα-treated human endothelial cells is down-regulated by quercetin treatment. Quercetin has not been observed to alter PMA or TNFα- induced NFκB activation, however a dose-dependent down-regulation of activator protein-1 (AP-1) was observed. ICAM-1 is associated with increased production of proinflammatory cytokines while AP-1, a redox-sensitive transcriptional factor, and linked to growth regulation, cell transformation, inflammation, and innate immune response (Kobuchi 1999).

Another possible mechanism by which phenolics exhibit health benefits, especially in type 2 diabetes, is inhibition of active sodium-dependent glucose transporter SGLT1 in the small intestine, thus reducing glucose absorption (Kottra 2007). Studies also demonstrate that some phytochemicals can exert modulatory effects in cells through selective actions on multiple intracellular signaling cascades, which are vital for cellular functions such as growth, proliferation and apoptosis (Crozier 2009).

2.6 Anti-diabetic drugs and their mechanism of action

2.6.1 Alpha-glucosidase inhibitors

Alpha-glucosidase (EC 3.2.1.20) is an enzyme that selectively catalyzes the hydrolysis of α-1,4 glucosidic linkages and is involved in carbohydrate metabolism (Wardrop 2010). Since this enzyme converts carbohydrates into simple sugars to facilitate intestinal absorption, inhibitors of alpha-glucosidase have been used as oral anti-diabetic agents.

Alpha-glucosidase inhibitors such as acarbose, miglitol and voglibose are saccharides that act as competitive inhibitors of alpha-glucosidase in the brush border of small intestine and reduce the rate of carbohydrate absorption. Treatment with alpha-glucosidase inhibitors has been shown to improve glycemia and post-prandial glucose levels in individuals with type 2 diabetes. Diarrhea (soft stools) and flatulence are two of the most common side-effects of this treatment, however, it offers the advantage of not causing weight gain and/or hyperinsulinemia and poses lower risk of incidences of clinical and subclinical hypoglycemia (Johnston 1998, Johnston 1998).

2.6.2 Dipeptidyl peptidase (DPP) – IV inhibitors

Inhibitors of the enzyme DPP-IV provide a novel strategy for treatment of type 2 diabetes (Ahren 2007). DPP-IV is an enzyme that rapidly inactivates the incretin hormones glucagon-like peptide (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) also known as gastric inhibitory polypeptide (GIP) (Green 2006). GLP-1 and GIP are the two primary incretin hormones secreted from the intestine upon ingestion of glucose or nutrients to stimulate insulin secretion from pancreatic β-cells. In addition to this, they have also been shown to enhance β-cell proliferation and inhibit apoptosis (Yabe 2011). The primary route to degradation of incretins is via cleavage by DPP-IV. Hence, DPP-IV inhibitors, such as sitagliptin, saxagliptin, vildagliptin, linagliptin, and alogliptin have been used to prolong and enhance the activity of endogenous GLP-1 and GIP (Green 2006, Dicker 2011). Prolonged incretin activity demonstrates enhanced insulin secretion, delayed gastric emptying, reduced glucagon secretion, and inhibition of β-cell apoptosis (Salvatore 2009). Clinical studies with DPP-IV inhibitors have shown efficacy and tolerability without

weight gain or hyperglycemia. They can be used as a monotherapy or in combination with other classes of drugs (Green 2006, Salvatore 2009).

2.6.3 5'-AMP-activated protein kinase (AMPK) activators

AMPK is a phylogenetically conserved serine/threonine kinase that plays an important role in energy homeostasis by co-ordinating anabolic (synthesis and storage of glucose and fatty acids) and catabolic (oxidation of glucose and fatty acids) processes (Viollet 2009). AMPK is a heterotrimeric enzyme composed of a catalytic subunit (α1/2) and two regulatory subunits (β1/2 and γ1/2/3) encoded by separate genes (Hardie 2008, Viollet 2009). AMPK is activated in response to binding of AMP to the γ-subunit as a consequence of increased AMP/ATP ratio. AMP acts as an allostertic activator of AMPK and promotes phosphorylation of the α-subunit at Thr-172 by upstream kinase LKB1. AMPK is also activated in response to elevated cytosolic Ca^{2+} via phosphorylation of Thr-172 by calmodulin-dependent kinase kinase β (CaMKK β). Thr-172 is dephoshorylated by protein phosphatase PP2C, switching active AMPK to the inactive form (Hardie 2008, Viollet 2009, Zhang 2009).

Activation of AMPK drives metabolic events in tissues including liver, skeletal muscle adipose and hypothalamus. In liver, AMPK activation has been shown to decrease hepatic glucose output by downregulation of PEPCK and G6Pase gluconeogenic gene expression (Shih 2013). AMPK also decreases hepatic lipogenesis by phosphorylation and inactivation of acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) thus inhibiting fatty acid biosynthesis and cholesterol synthesis

respectively (Zhang 2009). In skeletal muscle, AMPK activation increases glucose uptake and glycogen synthesis (Friedrichsen 2013).

AMPK activator, AICAR (5-aminoimidazole-4-carboamide ribonucleoside), has been shown to increase glucose uptake in muscle by inducing selective recruitment of GLUT4 to the plasma membrane and activating p38MAPK α and β, which may be involved in the activation of GLUT4 (Lemieux 2003). AICAR gets metabolized to its monophosphorylated derivative ZMP (5-aminoimidazole-4-carboxamide ribonucleoside). ZMP mimics AMP and leads to direct allosteric activation of AMPK. ZMP also promotes phosphorylation of AMPK by upstream kinases (Corton 1995). In accordance with this finding, it has been shown that AICAR treatment phosphorylates AMPK in an LKB1-independent manner via activation of ATM (ataxia telangiectasia mutated). ATM is a serine/theronine protein kinase involved in repairing DNA damage and may function as a potential AMPK kinase in response to AICAR treatment (Sun 2007).

The anti-hyperglycemic agent metformin activates AMPK (Hawley 2002). However, the mechanism by which metformin activates AMPK is still under speculation. It has been shown that metformin does not affect regulation of AMPK by upstream kinases and phosphatases, and activates AMPK without affecting the ADP-to-ATP ratio (Hawley 2002). It has also been shown that activation of AMPK by metformin suppresses expression of a key lipogenic transcription factor, SREBP-1. Metformin-treated rats have reduced SREBP-1 mRNAs and protein (Zhou 2001).

Physiological activators of AMPK include adiponectin, leptin and ghrelin. Adiponectin has been shown to induce phosphorylation and activation of AMPK in skeletal muscle and

liver. Leptin was reported to activate AMPK by acting directly on muscle as well as by influencing the hypothalamic-sympathetic nervous system axis. Ghrelin has also been indicated to increase AMPK activity in the hypothalamus leading to inhibition of fatty acid biosynthesis via ACC inhibition (Zhang 2009).

2.7 Plants with anti-diabetic potential

Various plants and plant-derived compounds are considered to have medicinal properties and many investigators have studied the effects of various phytochemicals on diabetes (Grover 2002, Rahimi 2005, Mukherjee 2006). Plants that demonstrate hypoglycemic potential mainly belong to the family Leguminosae, Liliaceae, Cucurbitaceae, Asteraceae, Moraceae, Rosaceae and Araliaceae (Patel 2012). Phytochemicals that mainly contribute to the therapeutic capacity of a plant include vitamin C, vitamin E, carotenoids, phenolics, alkaloids, glycosides, galactomannan gum, polysaccharides, peptidoglycans, hypoglycans, guanidine, steroids, carbohydrates, terpenoids, amino acids and inorganic ions (Marles 1995, Patel 2012).

More than 400 plants have been reported to possess glucose-lowering potential based on evidence from animal experiments or traditional use; however, most of these findings are not supported by rigorous clinical trials (Ernst 1997). A systematic review of herbs and dietary supplements studied for glycemic control in diabetes illustrated 58 controlled clinical trials (42 randomized and 16 nonrandomized) examining 36 herbs (single or in combination) involving 4,565 patients with diabetes or impaired glucose tolerance. Of these 58 trials, the direction of the evidence for improved glucose control was positive in 76% (44 of 58) with very few adverse effects reported (Yeh 2003).

The best evidence for efficacy from adequately designed randomized controlled trials is available for *Coccinia indica* (ivy gourd, tindora) followed by ginseng species. A double blind control trial for *C. indica* reported marked improvement in glucose tolerance of individuals with type 2 diabetes following 6 weeks of treatment with crushed, dried *C. indica* leaves (Azad 1979). There are other open-label prospective trials offering supporting evidence of a hypoglycemic effect and no adverse events have been reported in these trials (Yeh 2003). The mechanism of action of *C. indica* is not well understood, but pectin isolated from this plant has been shown to significantly lower blood glucose and increase hepatic glycogen in normal rats (Kumar 1993).

Among ginseng species, American ginseng (*Panax ginseng*) has been shown to lower post-prandial hyperglycemia in both diabetic and non-diabetic subjects when taken prior to a 25-g oral glucose challenge (Vuksan 2000, Vuksan 2000). In a double-blind placebo-controlled study involving 36 patients with type 2 diabetes, ginseng therapy for 8 weeks was shown to elevate mood, improve psychophysical performance, and reduce fasting blood glucose and body weight (Sotaniemi 1995).

Other herbs with possible hypoglycemic potential, including *Allium sativum* (garlic), *Gymnema sylvestre* (gurmar) , *Citrullus colocynthis* (bitter cucumber), *Trigonella foenu-greacum* (fenugreek), *Momordica chanratia* (bitter melon) and *Ficus bengalensis* (banyan) have been either been studied in non-randomized controlled trials or in poorer-quality RCTs as indicated by their Jaded score (Yeh 2003, Patel 2012). *Gymnema inodorum*, has been reported to exhibit high antioxidant index (14.8), without the suppression of sweetness, and lacking the bitter taste of *G. sylvestre* and was shown to lower blood glucose by reducing intestinal glucose absorption (Shimizu 1997, Chanwitheesuk 2005). *Allium cepa* L. (onion)

administration demonstrated alleviation of hyperglycemia, increased fasting serum high-density lipoprotein (HDL) cholesterol, and decreased superoxide dismutase activity, with no increase in lipid hydroperoxides and lipoperoxides, in STZ-induced diabetes in rats (Campos 2003). *Allium cepa* and *Allium sativum* L. (garlic) juices have also shown a decrease in plasma levels of glucose, urea, creatinine and bilirubin; and reduced activities of aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH); and alkaline and acid phosphatases in the plasma of alloxan-induced diabetic rats (El-Demerdash 2005). One of the most widely prescribed anti-diabetic drug, metformin, is a derivative of the alkaloid galegine (isoamylene guanidine), which was the active ingredient identified with the hypoglycemic potential from *Galega officinalis* (Witters 2001).

2.8 Ayurveda

Ayurveda is a Sanskrit word that, translated, means knowledge (*veda*) of life (*ayur*). It refers to the traditional medicine developed and practiced in India for roughly three thousand years. There are three important treatises in Ayurveda written from around 800 B.C. to A.D. 1000 and are collectively referred as the Senior Triad which include the *Charak Samhita*, the *Susruta Samhita* and the *Ashtanga Hridaya Samhita*. The Junior Triad comprises of *Madhava Nidana*, the *Sarangdhara Samhita* and the *Bhava Prakasha*.

The *Charak Samhita*, complied by physician *Charak*, contains the basic philosophy of disease, based on the imbalance of three basic humors known as *vata*, *pitta* and *kapha*. It catalogs fundamental aspects of medical philosophy and contains detailed sections on aphorism (*sutra*), diagnosis (*nidana*), methodology (*vimana*), body (*sharira*), sense organs (*indriya*), treatment (*chikitsa*), preparation (*kalpa*) and purification (*siddha*). Treatment in

Ayurveda attempts to balance the body humors of *vata*, *pitta* and *kapha*, and differs based on a person's body constitution (Sharma 1998, Hardy 2001).

One of the strongholds of Ayurvedic medicine is its sophisticated system of drug preparation and use (Hardy 2001, Sekar 2008). The *Charak Samhita* has classified herbal medicines into roughly 50 categories based on their pharmaceutical action such as diuretic, analgesic, emetic, promoting longevity, and digestion. Ayurvedic preparations include herbal teas, infusions, decoctions, tinctures, capsules, powders, infused oils, ointments, creams, lotions and the unique forms of *asavas* (fermented infusions) and *arishtas* (fermented decoctions) (Sekar 2008, Mishra 2010). *Asavas* contain self-generated alcohol (in the range of 7 to 12% by volume) and 1 to 3% w/w of sugar content (Nandre 2013). They are prepared by allowing herbal juices to undergo fermentation with the addition of sugars. According to Ayurveda, fermentation is initiated with addition of *dhataki* (*Woodfordia fruticosa*) flowers, which contain wild species of yeast in the dry nectariferous region and therefore serve as an inoculum (Vohra 2001, Sekar 2008). Presence of alcohol in *asava* preparation confers long term stability and enhances therapeutic properties probably due to improved efficiency of extraction from the herb and improved bioavailability (Sekar 2008, Gupta 2011, Nandre 2013). A study comparing differences in volatile compounds between tincture (alcoholic extract of plant or animal material with 40-60% alcohol, 25% being the most common for herbal tinctures) and *asava* made from ginger rhizomes or jujube fruits suggests that alcoholic fermentation during *asava* preparation hydrolyzes glycosides which might be helpful in absorption of effective aglycones (Okutsu 2007).

The most common conditions for which Ayurvedic therapies have been published include diabetes mellitus, infectious diseases, hepatitis, hypercholesterolemia, CNS disorders such as

dementia and depression, and cardiovascular diseases. There are studies reporting beneficial effects of *yoga asanas*, *pranayam* (breathing exercises) and meditation as a means to reduce stress, restore and maintain physiological balance, and in turn contribute to effective blood glucose control. However, the most widely cited literature for diabetes management with Ayurveda is herbal therapy (Elder 2004, Aljasir 2010). At present, there is great heterogeneity in the available literature on Ayurvedic interventions for diabetes management, with few randomized controlled and/or controlled trials and small number of subjects, but there is sufficient data to warrant further research (Sridharan 2011).

2.9 *Tridax procumbens* L.

Tridax procumbens Linn. commonly known as "coat buttons" or "tridax daisy", is a perennial herb belonging to the family Asteraceae (Figure 4). Native to Central America, the plant has been naturalized in many tropical regions of Asia, Africa and Australia. In the United States, *T. procumbens* is given a native status in the lower 48 states, Hawaii, U.S. Virgin Islands and Puerto Rico, and is presently found in the states of Florida and Hawaii. The U.S. federal government has classified this plant as a noxious weed and the state of Alabama has listed *T. procumbens* as a Class A noxious weed (USDA).

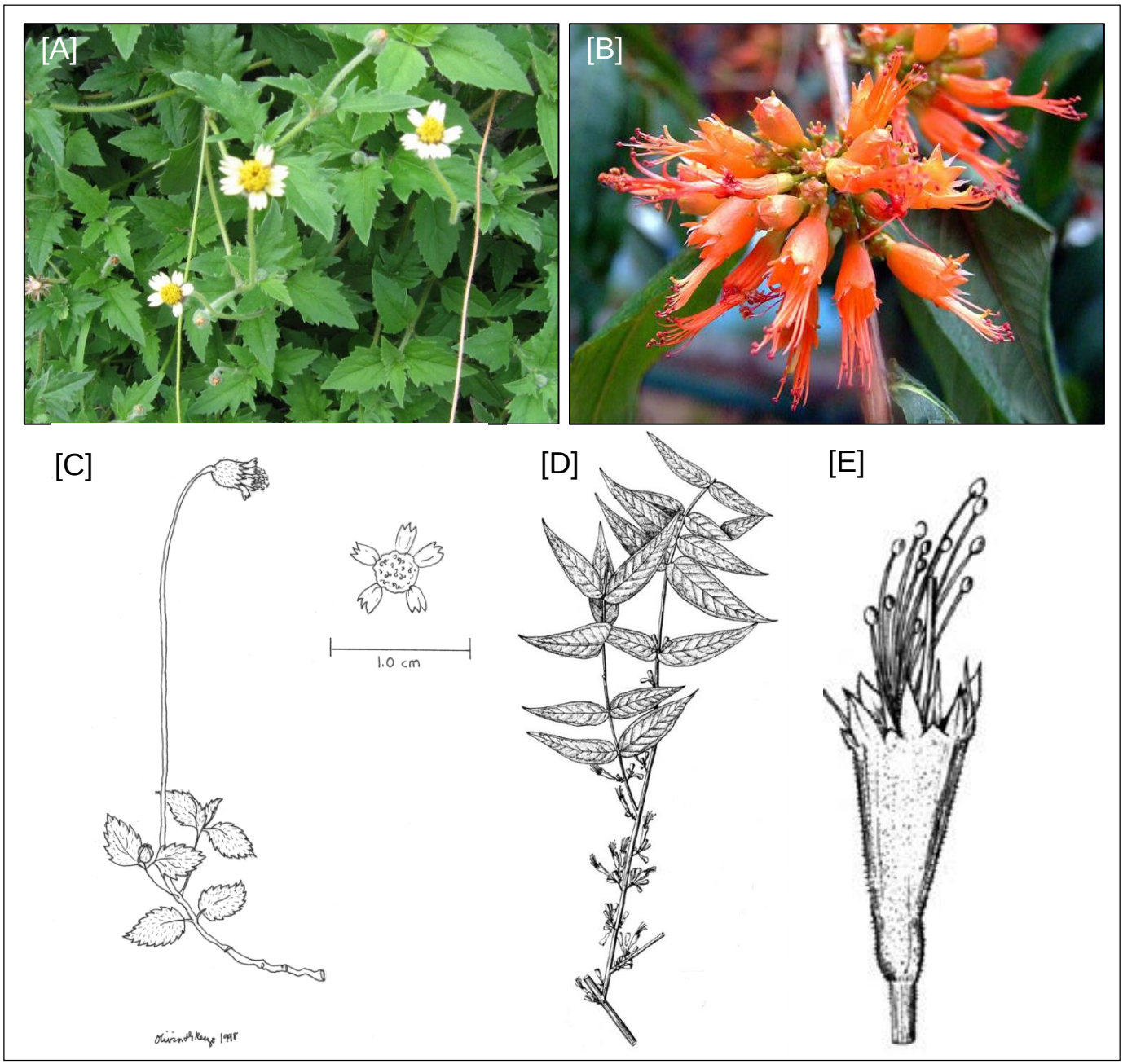

Figure 4. Tridax procumbens L. [A,C], *also known as coat buttons, grows in the tropical, sub-tropical and temperate regions of the world. W. fruticosa L. Kurz* [B,D], *also known as fire flame bush, grows in western and northern regions of India. W. fruticosa flower* [E] *is widely used in Ayurveda as a source of wild yeast for fermentation.*

In India, *T. procumbens* is widespread and abundant especially in fallow fields, agricultural fields and disturbed wastelands. It is reported to grow throughout the state of Maharashtra, Madhya Pradesh, Rajasthan, Kerala and Tamil Nadu, and is predominant in districts of Coorg, Dharwar, Hassan, Mysore, North Kanara and Shimoga of Karnataka State, in India. *T. procumbens* is also widely distributed in the north-eastern *Terai* grasslands of Uttar Pradesh (Portal, 2009). The leaves of *T. procumbens* are cooked and eaten as a vegetable and the plant is widely reported in folkloric medicine for its antimicrobial activity and potential to treat wounds, inflamed skin, mouth sores, skin infections, rheumatism, body heat, epistaxis, bronchial catarrh, dysentery and diarrhea (Samy, 1999, Ali 2001, Subramaniam 2013).

2.9.1 *T. procumbens*: Ethnomedicine

There is particularly high know-how about medicinal properties and uses of *T. procumbens* among local inhabitants belonging to diverse ethnic groups. The Kani tribes of Kouthalai region in Tamil Nadu, India reported external application of *T. procumbens* leaf paste for relief from swellings (Ayyanar 2005). Extensive field excursions and collection of ethnobotanical information from local inhabitants of Gond and Kharwar communites from Sonabhardra district, Uttar Pradesh, India also revealed the use of *T. procumbens* plant paste for bleeding wounds, cuts and stings (Singh 2002). Ethnomedicinal information collected from 6 districts, viz., Alwar, Bharatpur, Dausa, Dholpur, Karouli and Sawai Madhopur within the eastern parts of Rajasthan, India verified that *T. procumbens* was commonly used as ethnomedicine (21.63% informants reported use of *T. procumbens*), third in line following *Azadirachta indica* (neem) and *Ocimum sanctum* (holy basil) (Upadhyay 2010). *T. procumbens* juice is widely used to arrest bleeding from cuts and bruises and is known to

expedite epithelial cell growth and collagen synthesis, while retarding scar formation and granulation (Diwan 1983). The effects of various extracts including whole plant extract, aqueous extract, butanol extract and ether fraction were compared, and it was found that whole plant extract had the greatest pro-healing activity (Raina 2008).

2.9.2 Chemical and phytochemical profile of *T. procumbens*

Proximate analysis indicates high moisture content for *T. procumbens* leaves (90.05 ± 0.00 % wet weight) and stem (88.30 ± 0.02 % dry weight) suggesting short shelf-life and susceptibility to microbial contamination. Hence, dehydration of the plant has been suggested to increase the relative concentrations of food nutrients and improve plant shelf-life. Dehydrated *T. procumbens* plant can be a good source of carbohydrates, proteins, minerals including calcium, potassium, sodium, and vitamins, including vitamin A (Raina 2008, Ikewuchi 2009, Ikewuchi 2009, Jude 2009). Methanolic and acetone extracts of *T. procumbens* leaves indicate the presence of saponins, phlobatannins, resins, lipids, steroids, tannins, glycosides, reducing sugars, phenols, carbohydrates, anthraquinone, catachol, sterols and flavonoids (Manjamalai 2010). However, phytochemical screening of an aqueous extract showed absence of steroids, phlobatanins and glycosides, suggesting that a combination of solvent systems may be beneficial in improving extraction efficiency (Dhandapani 2008). *T. procumbens* flowers had the highest sterol content (18.6 mg/g dw) followed by leaves (15 mg/g dw), pedicle (9.5 mg/g dw), bud (9.2 mg/g dw), root (7 mg/g dw) and stem (4.7 mg/g dw) (Jindal 2013).

T. procumbens leaves have high saponin content (10.30 mg/ 100 g wet weight) and drying further increases the relative concentration of saponins to 103.52 mg/100 g dry weight

(Jude 2009). Saponins constitute a very heterogenous group of substances preferentially found in plants. They are glycosides consisting of a sterol or triterpene ring with attached sugar, and are known to prevent cholesterol absorption by forming insoluble complexes with cholesterol in the intestine (Price 1987, Petit 1995). Saponin treatment has been shown to induce a significant decrease in plasma total cholesterol ($p < 0.01$), HDL free cholesterol ($p < 0.05$), and VLDL-LDL total cholesterol ($p < 0.05$) in normal and diabetic rats without affecting plasma triglyceride, plasma insulin and blood glucose (Petit 1995). GC-MS analysis of essential oil obtained from *T. procumbens* identified peaks for alpha-pinene [$C_{10}H_{16}$], beta-pinene [$C_{10}H_{16}$], 1-phellandrene [$C_{10}H_{16}$] and sabinene [$C_{10}H_{16}$] (Manjamalai 2010). Alpha-pinene is an organic compound of the terpene class and is known for its anti-inflammatory properties. Intraperitonial administration of alpha-pinene has been shown to reduce the production of pancreatic TNF-α, IL-1β and IL-6 during cerulean-induced acute pancreatitis in mice (Bae 2012). It also reduces inflammation by inhibition of NF-κB activation and iNOS enzyme activity (Gonçalves 2008).

Structural elucidation of polysaccharide components in *T. procumbens* leaves identified the presence of a novel water-soluble high molecular weight arabinogalactan and a linear low molecular weight galactan. Arabinogalactan is a biopolymer consisting of arabinose and galactose monosaccharides. Often found attached to proteins, the resulting arabinogalactan protein (AGP) functions as a cellular signaling molecule. Arabinogalactan can activate the immune cells involved in body's first line of defense and also promote cytokine production. Arabinogalactan activity is may be enhanced in the presence of antioxidants such as vitamin C (Raju 1994, Currier 2003, Nosalova 2011).

Lipid constituent characterization of *T. procumbens* revealed the presence of β-sitosterol along with isolation of eight novel lipid compounds (Verma 1988, Gadre 1992, Saxena 2005, Shamshul 2010). β- and γ-sitosterols have been studied for their anti-diabetic, antioxidant, immunomodulatory and hypocholesterolemic properties. Oral administration of γ-sitosterol isolated from a creeping perennial herb, *Lippia nodiflora* L., has been shown to significantly reduce blood glucose, glycated hemoglobin, serum total cholesterol, triglycerides and VLDL-cholesterol, coupled with a corresponding increase in plasma insulin, body weight, food intake and HDL-cholesterol in streptozotocin (STZ)-induced diabetic rats (Balamurugan 2011). β-sitosterol exerts beneficial effects on glucose and lipid metabolism by phosphorylating AMPK and ACC to stimulate glucose uptake and it has been reported to work synergistically with stigmasterol in promoting hypoglycemia (Jamaluddin 1994, Hwang 2008). β-sitosterol has also been shown to promote insulin sensitivity in rats fed a high-fat diet. β-sitosterol significantly reduced hyperglycemia, hyperinsulinemia, insulin resistance, oxidative damage, nitrosative stress, hepatic lipid accumulation and elevated aminotransferases (Radika 2013). In addition to being a cholesterol analog, β-sitosterol is also considered to be a phytoestrogen and has been demonstrated to possess antioxidant properties that prevent oxidative stress and membrane lipid peroxidation (Shi 2013).

2.9.3 Pre-clinical studies on *T. procumbens*

T. procumbens has been investigated for anti-diabetic, anti-inflammatory, analgesic, hepatoprotective, hypotensive and wound healing properties in animal models (Table 1).

Table 1: Pre-clinical studies demonstrating anti-diabetic, anti-inflammatory, anti-hypertensive and hepatoprotective effects of *Tridax procumbens*

T. procumbens	Extract	Animal model	Activity	Reference
Leaves	Aqueous, alcoholic, petroleum ether	Alloxan-treated Wister rats	Anti-diabetic	Bhagawat D.A. et al. Int J Green Pharm, 2:126, 2008
Whole plant	95% methanol	Alloxan-treated Wister rats	Anti-diabetic	Pareek H. et al. BMC Complement Altern Med, 9:48, 2009
Aerial	95% ethanol	Alloxan-treated Sprague Dawley	Anti-diabetic	Anulukanapakom, K. et al. Thai J. Pharm. Sci., 21:211, 1997
Aerial	Ethyl acetate, methanol, 70% ethanol	Carrageenan-treated Sprague Dawley rats	Anti-inflammatory	Jachak S.M. et al. Fitoterapia, 82:173, 2011
Aerial	95% ethanol	DGalN/LPS-treated Wister rats	Hepatoprotective	Ravikumar V. et al. J Ethnopharmacol, 101:55, 2005
Leaves	Aqueous	Salt-loaded albino rats	Anti-hypertensive	Ikewuchi J.C. et al. PJST, 1:381, 2012

Oral administration of 50% methanolic extract of *T. procumbens* whole plant has showed a significant reduction ($p < 0.05$) in fasting blood glucose levels in diabetic rats. An acute dose of 250 and 500 mg/kg body weight showed more effective lowering of blood glucose (68.26% and 71.03% resp.) compared to 10 mg/kg glibenclamide (57.29%), with maximum reduction observed at 6 h. Significant anti-hyperglycemic effect was also evident when *T. procumbens* extract was administered sub-chronically (30 days) (Pareek 2009). *T.*

procumbens has been shown to lower blood glucose in both normal and alloxan-induced diabetic rats in a dose-dependent manner with 26.6% reduction at 100 mg/kg, 32.1% at 250 mg/kg and 48.2% at 500 mg/kg bw (Kumar 2009). Aqueous and alcoholic extracts of *T. procumbens* leaves (200 mg/kg) orally administered for 7 days have also resulted in a significant decrease in blood glucose levels of alloxan-induced diabetic rats compared to a very weak anti-diabetic activity of a petroleum ether extract (Bhagwat 2008). Aqueous extract of *T. procumbens* leaves is rich in sitosterol and tannic acid, which are known for their hypoglycemic activity. Tannic acid has been shown to stimulate insulin-induced glucose uptake via phosphorylation of insulin receptor and translocation of GLUT4 in 3T3-L1 adipocytes (Liu 2005).

Jachak et al. examined the anti-inflammatory activity of *T. procumbens* using ethyl acetate, methanol and 70% ethanolic extracts of *T. procumbens* aerial parts and demonstrated significant inhibition of carrageenan-induced rat paw edema (Jachak 2011). Further, these authors identified two flavonoids viz., centaureidin and centaurein, and a glycoside, bergenin that might contribute to the anti-inflammatory and immunomodulatory activities of *T. procumbens* (Chang 2007, Nazir 2007, Jachak 2011). Fresh leaf juice and ethyl acetate extract of *T. procumbens* exhibited significant anti-inflammatory activity compared to a dose of 50-100 mg/kg b.w of ibuprofen and the effect was enhanced when *T. procumbens* and ibuprofen were administered together (Awasthi 2009, Das 2009, Jachak 2011). In addition to the anti-inflammatory effect, *T. procumbens* also possesses analgesic potential. Oral administration of *T. procumbens* extract has been shown to significantly inhibit late phase moderate pain caused by formalin injection, acetic-acid induced abdominal constriction and CFA- (Complete Freund's adjuvant) induced hyper-analgesia in male C57 BL6/J mice and

Sprague-Dawley rats. The anti-nociceptive property of *T. procumbens* has been attributed to the presence of flavonoids such as procumbentin and quercetin, and sterols such as β-sitosterol (Prabhu 2011).

T. procumbens also displays high antioxidant activity demonstrated by significant free radical scavenging potential, which might play a protective role to reduce inflammation-induced oxidative stress (Nia 2005, Agrawal 2009, Jachak 2011). The antioxidant activity of *T. procumbens* also bestows a hepatoprotective role. Pretreatment of rats with *T. procumbens* extract exerted a protective effect against D-galactosamine/lipopolysaccharide (D-GalN/LPS)-induced hepatitis. Induction of rats with D-GalN/LPS causes a marked increase in lipid peroxidation with a subsequent decline in activities of enzymatic antioxidants including superoxide dismutase, catalase, glutathione peroxidase and glutathione S-transferase, and non-enzymatic antioxidants including reduced glutathione, vitamin C and vitamin E. These levels are normalized in *T. procumbens*-treated rats (Ravikumar 2005). The hepatoprotective effect of *T. procumbens* was also evident by reversal of elevated activites of AST, ALT, alkaline phosphatase, lactate dehydrogenase and gamma glutamyl transferase. It also improves histological appearance of liver sections (Ravikumar 2005, Joshi 2011). *T. procumbens* extract was also effective in protecting the liver from drug-induced damage. Paracetamol overdose can lead to intracellular stress, accompanied by structural and functional changes of liver cell membranes, which affects DNA integrity, membrane-bound ATPase and inorganic cations homeostasis. The oxidative stress in paracetamol-induced hepatic damage in rats has been shown to be alleviated with *T. procumbens* treatment (Hemalatha 2008).

An altered lipid profile is often associated with diabetes and is an independent risk factor for cardiovascular diseases. Aqueous extract of *T. procumbens* leaves (300 mg/kg) significantly lowered plasma triglyceride and VLDL cholesterol levels in alloxan-induced diabetic rats and lowered total-, HDL-, LDL- and non-HDL-cholesterol levels. It was reported to significantly increase plasma calcium, ocular ascorbic acid content, hemoglobin concentration, and neutrophil count while positively affecting the hematopoietic system, and integrity and function of liver and kidney (Ikewuchi 2012). *T. procumbens* treatment was also shown to improve the lipid profile and atherogenic indices of cholesterol-loaded rats, suggesting possible implications in the management of obesity and cardiovascular diseases (Ikewuchi 2009, Bharathi 2011).

To determine the effect of *T. procumbens* on hypertension, plasma lipid profile and atherogenic indices were studied in sub-chronic, salt-loaded Wister rats, receiving a diet consisting of 8% salt. Administration of *T. procumbens* aqueous extract (150 and 200 mg/kg bw) significantly lowered mean daily weight gain, plasma LDL and non-HDL cholesterol, while reductions in plasma triglyceride, atherogenic indices, total and VLDL cholesterol were not significant. There was an increase in plasma HDL cholesterol, without significant alterations in organ weights and sizes (Ikewuchi 2011).

At a dose of 200 mg/kg bw, *T. procumbens* immediately lowered the systolic and diastolic blood pressure in sub-chronic salt-loaded rats, which was maintained for 72 hours. The extract also prevented salt-induced upsurge in pulse pressure and lowered pulse rates compared to control (Ikewuchi 2011). Heart rate was lowered by 36% at 6 mg/kg dose and by 56.7% at 9 mg/kg dose of *T. procumbens* in male albino rats. Injecting *T. procumbens* immediately before treatment with adrenalin (2 µg/kg) did not produce any significant effect

on arterial blood pressure and heart rate compared to adrenaline alone. However, injecting acetylcholine (2 µg/kg) following *T. procumbens* pretreatment showed higher reduction in mean arterial blood pressure compared to acetylcholine alone. Also, pretreatment of rats with muscarinic receptor blocker, atropine (6 mg/kg) significantly prevented the hypotensive effect of *T. procumbens* extract suggesting that the blood pressure lowering effect is probably mediated through muscarinic cholinergic receptors (Salahdeen 2004). The anti-hypertensive effect of *T. procumbens* might also be mediated by alteration of plasma electrolyte profile. Chronically salt-loaded rats have shown significant reduction ($p < 0.05$) in plasma sodium and chloride levels with no effect on potassium and calcium levels, suggesting a possible mechanism for the hypotensive action. Also, *T. procumbens* treatment had positive effects on the hematopoietic system indicated by significantly higher percent packed cell volume in test animals compared to control (Jude 2010). The vasorelaxant property of *T. procumbens* was evident by direct action of aqueous leaf extract on mechanical responses of smooth muscles in aortic rings isolated from normotensive rats. *T. procumbens* (0.15-1.05 mg/ml) induces a concentration-dependent relaxation of smooth muscles pre-contracted with either noradrenalin (10^{-7} M) or KCl (60 mM) in a concentration-dependent manner with EC_{50} 0.45 ± 0.01 mg/ml in noradrenalin pre-contracted aortic rings and 0.75 ± 0.01 mg/ml in KCl pre-contracted rings. The contractile response was attenuated and the concentration-response curve was shifted to right for noradrenalin, KCl and serotonin in the presence of *T. procumbens* suggesting interference with availability of Ca^{2+} for the contractile process (Salahdeen 2012).

Acute toxicity study for ethyl acetate extract of *T. procumbens* aerial parts, based on intraperitoneal administration, showed no visible signs of toxicity at 10 mg/kg (Abubakar

2012). Visual signs of potential toxicity including salivation, rubbing at the site of application, rubbing of nose and mouth on the floor of cage, and restlessness were observed at 100 mg/kg and 1000 mg/kg dosages. There was significant gain in body weight, with the highest increase at 50 mg/kg and lowest at 1600 mg/kg. The median lethal dose (LD_{50}) was calculated to be 2100 mg/kg. Short-term toxicity study showed hypoglycemic effects (less than 4.0 mmol/L glucose) at doses of 400 mg/kg and above, and a significant increase in ALT activity at 800 mg/kg. Histopathological studies showed sporadic infiltration of inflammatory cells in liver and absence of hemosiderin at 50 mg/kg, while there was significant hemosiderin deposition at 400 and 800 mg/kg (Abubakar 2012). Chronic toxicity study with oral administration of ethanolic extract (300 mg/kg bw) has shown significant reduction in protein content of liver, kidney, uterus and ovary of female albino rats compared to control groups suggesting a possible toxic effect at higher dose (Ahirwar 2010).

3.0 Study Objectives

Tridax procumbens Linn, has been recognized for several potential therapeutic activities including antioxidant, anti-inflammatory and anti-diabetic effects. Recent research using pre-clinical animal models have demonstrated that *T. procumbens* has the potential to modify cardiovascular risk factors. However, there are no clinical studies evaluating antidiabetic effects of *T. procumbens*. Further, our understanding of the molecular mechanisms that mediate these effects is limited.

The **overall goal** of this proposal was to evaluate the impact of antioxidant, anti-inflammatory and anti-hyperglycemic potential of *T.procumbens* on cardiovascular health.

We proposed to test the **hypothesis** that *T. procumbens* asava (a hydro-alcoholic extract prepared according to Ayurveda guidelines) can minimize cardiovascular risk factors due to

its antioxidant, anti-inflammatory and blood glucose lowering potential. The following were the study objectives:

Objective 1:

a. Characterization of *T. procumbens* asava and evaluation of antioxidant potential
b. Evaluate blood glucose lowering properties of *T. procumbens* in individuals with type 2 diabetes: a pilot clinical study

Objective 2:

Characterization of potential mechanisms mediating anti-diabetic and anti-inflammatory effect of *T. procumbens* asava and *W. fruticosa* using *in vitro* and cell culture techniques

Objective 3:

a. Conduct a randomized, double-blind, placebo-controlled clinical trial to assess antihyperglycemic and cardioprotective effects of *T. procumbens*

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Anti-hyperglycemic effects of *Tridax procumbens* L. in individuals with type 2 diabetes

Gauri S. Desai[1,2,#], Shirish V. Desai[2,##], Rajendra S. Gavaskar[3,§] and Suresh T. Mathews[1]*

[1] Department of Nutrition and Dietetics, Boshell Diabetes and Metabolic Diseases Research Program, Auburn University, Auburn, AL 36830

[2] Bhagawati Industrial and Organic Products, Kolhapur, Maharashtra, India

[3] GTL Foundation, Sindhudurg, Maharashtra, India

[#]lead graduate student

[##]contributed to recruitment of subjects, sample collection

[§]physician for the study

*principal investigator

*Corresponding author:

Suresh T. Mathews, Ph.D, FACN
Associate Professor & Graduate Program Director
Department of Nutrition and Dietetics
260 Lem Morrison Dr., 101 PSB
Auburn University, AL 36849
Phone: 334-844-7418
Fax: 334-844-3268
Email: mathest@auburn.edu

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Abstract

Traditional knowledge, in vitro studies, and studies using animal models suggest that *Tridax procumbens* L. exhibits blood glucose lowering properties and anti-inflammatory effects. In this study we evaluated blood glucose lowering effect of *T. procumbens* supplementation in individuals with type 2 diabetes. An extract *(asava)* of *T. procumbens* L. was prepared following Ayurveda guidelines. Chemical and microbial analyses indicated presence of phenolics, flavonoids, and carotenoids, and absence of microbial contamination, aflatoxins, heavy metals, and pesticide residues. *T.procumbens asava* demonstrated strong total antioxidant capacity, Fe^{3+} reducing potential, Fe^{2+} chelation, H_2O_2 scavenging activity, and inhibition of lipid peroxidation. We recruited twenty type 2 diabetic individuals from Kolhapur, India. Participants received 15 ml of *T. procumbens* asava, twice daily, for 4 weeks, while continuing their prescribed antidiabetic medications. Fasting blood glucose decreased by 11% in men ($p < 0.01$) and 20% in women ($p < 0.05$), and, post-prandial blood glucose concentrations were lowered by 26% in men ($p < 0.001$) and 29% in women ($p < 0.001$) following 4-weeks of *asava* supplementation. No adverse events or side effects were reported. This is the first clinical study demonstrating a significant blood glucose lowering effect of *T. procumbens asava* in type 2 diabetes.

Introduction

Tridax procumbens Linn., (Family-Asteraceae), a common herb, native to the tropical Americas is known for several potential therapeutic activities. Traditionally, it has been used, in certain parts of India, to alleviate symptoms of inflamed skin, mouth sores, skin infections, bleeding wounds, diarrhea, rheumatism, and epistaxis (Ayyanar 2005, Upadhyay 2010). Ethnomedicinal data suggest that *T. procumbens* may possess properties effective in the management of diabetes. Inhabitants from regions including Mandesh (Maharashtra, India) and Udaipur (Rajasthan, India) have reported the use of *T. procumbens* for diabetes management (Bhagwat 2008, Pareek 2009).

Diabetes is one of the major causes of premature illness and death worldwide. According to the International Diabetes Federation, 382 million people globally have diabetes and this is projected to rise to 592 million by 2035 (International Diabetes Federation 2013). Conventional drug therapy uses oral hypoglycemic agents as the first line of therapy in the management of diabetes. However, increasing costs of conventional antidiabetic therapy and associated side-effects can result in non-adherence and poor blood glucose control (Hauber 2009).

According to the National Health Statistics Report, the use of Complementary and Alternative Medicine/Therapies (CAM) increased among adults and children in the US, from 2002 to 2007, with natural products accounting for 17.7% of the most commonly used CAM therapies (Barnes 2008). Plants including *Gymnema sylvestre*, *Panax quinquefolius* (American Ginseng), *Coccinia indica* (Ivy gourd), *Allium sativum* (Garlic), *Momordica charantia* (Bitter gourd) have been used for studies on blood glucose control (Yeh 2003).

Recent research has shown that *T. procumbens* extracts possess anti-inflammatory, analgesic and anti-diabetic activity in relevant animal models (Pareek 2009, Jachak 2011). Acute and sub-acute administration of *T. procumbens* significantly decreased fasting blood glucose levels in alloxan-induced diabetic rats, but not in non-diabetic control rats (Anulukanapakorn 1997, Pareek 2009). However, there are no clinical studies examining the efficacy of *T. procumbens* for glucose control in patients with diabetes. In this study we evaluated blood glucose lowering properties of *T. procumbens* in individuals with type 2 diabetes and examined its antioxidant potential.

Materials and Methods

Chemicals

Total antioxidant capacity (TAC) assay kit (Cat# K274-100) was purchased from BioVision, Milpitas, CA. Ammonium thiocyanate, ferrous chloride, ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine), and hydrogen peroxide were obtained from VWR International, Radnor, PA. Trichloroacetic acid (TCA), linoleic acid, potassium ferricyanide, ferric chloride, butylated hydroxyanisole (BHA), butylated hydroxyltoluene (BHT), α-tocopherol and trolox were obtained from Sigma (Sigma-Aldrich GmbH, St. Louis, MO). Curcumin was purchased from Cayman Chemicals, Ann Arbor, MI. All other chemicals used were of analytical grade and were obtained from either Sigma-Aldrich or VWR International.

Plant Material

Tridax procumbens Linn. plants (whole plants) were collected from the town of Athani, Belgavi district, Karnataka State, India following Good Agricultural Practices for medicinal plants (World Health Organization 2003). *Tridax procumbens* Linn. was authenticated by Aditi Organic Certifications, Bangalore, India Whole, mature flowers of *Woodfordia fruticosa* (L.) Kurz. *(*known as *Dhataki* in Sanskrit*)*, a common ingredient of fermented Ayurvedic preparations, were obtained commercially from local vendors (Chaudhary 2011).

Preparation of *Tridax procumbens asava*

An extract of *T. procumbens* was prepared following Ayurveda (a system of traditional Indian Medicine) guidelines, hitherto referred to as *T. procumbens asava* (the word *asava* in Ayurveda means ‘fermented infusion’). *T. procumbens* L. whole plants were initially rinsed in

running water, shade dried and then coarsely powdered. The powdered *T. procumbens* plant material (10 kg) and dried whole flowers of *Woodfordia fruticosa* (L.). Kurz. (4 kg) were added to a stainless steel process vessel containing 100 L of potable water and dissolved pharmaceutical-grade sugar (35 kg/100L). *W. fruticosa* flowers harbor wild species of yeast in the dry nectariferous region, which served as the inoculum (Sekar 2008). Contents in the closed process vessel were kept undisturbed for 45 days to allow completion of the natural fermentation process. The temperature of the process vessel was maintained between 30°-35° C using a water jacket. Following the fermentation period, the *asava* was filtered using cheese-cloth, bottled, and sealed following Good Manufacturing Practices. The *asava* was commercially analyzed for chemical characteristics, microbial load, and pesticide residues. Total phenolics, flavonoids, and carotenoids were analyzed as reported previously (Scott 2001, Waterhouse 2001, Marinova 2005).

Antioxidant activity

For all antioxidant activity assays, *T. procumbens asava* dilutions 1:100, 1:50, 1:35, 1:25, and 1:10, which correspond to 1, 2, 3, 4, and 10 mg of dried *T. procumbens*/ml were prepared in distilled water and compared with 45 µg/ml BHA, BHT, trolox, α-tocopherol and curcumin. Total antioxidant activity of *T. procumbens asava*, BHA, BHT, trolox, α-tocopherol and curcumin were measured using TAC assay kit from BioVision following manufacturer's recommended protocol. Ferric ion reducing antioxidant power (FRAP) assay was used to measure the antioxidant reducing potential as described earlier (Ak 2008). Inhibition of lipid peroxidation was measured using the ferric thiocyanate assay (Ak 2008).

Ferrous ion chelating activity of *T. procumbens* was evaluated by the method of Dinis et al (Dinis 1994). Metal ion chelating activity was calculated using the following equation: Chelating effect (%) = (1 – As/Ac) x 100 (where *Ac*: absorbance of control, *As*: absorbance in presence of *T. procumbens* or other antioxidants).

Hydrogen peroxide scavenging activity was determined according to Ak and Gulcin (Ak 2008). Percent H_2O_2 scavenging activity was calculated using the following equation: H_2O_2 scavenging activity (%) = (1 – As/Ac) x 100 (where *Ac*: absorbance of blank solution, *As*: absorbance in presence of *T. procumbens* or other scavengers).

Clinical study: Experimental design

This study was approved by the Kolhapur Independent Ethics Committee (KIEC), Kolhapur, India. Participants were Asians from the Indian subcontinent, between the ages of 40 and 90 years. Eligibility requirements included having established type 2 diabetes for at least 5 years before enrollment in the study. Twenty subjects with established type 2 diabetes were recruited from Kolhapur area in Maharashtra, India, through the physician's office. Children and pregnant women were excluded because the trial was a pilot study of small sample size. Written informed consent was received from all participants before the start of the study. A single group, open-label experimental design was adopted for this study. Participants received an oral dose of 15 ml *T. procumbens asava*, twice a day, for 4 weeks. *T. procumbens asava* was administered supplementary to their prescribed antidiabetic medications, which included monotherapy with sulfonylurea or α-glucosidase inhibitors, combination therapy with sulfonylurea and α-glucosidase inhibitors; and combination therapy with sulfonylurea, thiazolidinedione and biguanide. Three out of twenty subjects were on insulin therapy combined with an oral dose of α-

glucosidase inhibitors. Participants were asked to continue their normal diet and physical activity patterns. Fasting and 2-hour post-prandial blood samples were obtained at the beginning of the study and at the end of the 4-week period. Blood glucose concentrations were analyzed in a clinical laboratory. To assess intervention compliance and dose validation, participants returned any unused *T. procumbens asava* at the end of the study period.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 (La Jolla, CA). A Students two-tailed paired t-test was used to analyze differences between pre- and post-treatment. One-way ANOVA followed by Tukey or Dunnett's test was used to compare results of antioxidant assays. A two-way ANOVA was performed to analyze lipid peroxidation status in the presence on antioxidants over time. Statistical significance was accepted if $p < 0.05$. Data are expressed as mean ± SD.

Results

Chemical, microbial, and pesticide analyses

Gas-chromatography analysis of *T. procumbens asava* showed no traces of methanol. The ethanol content estimated by distillation method was 8.2% (v/v). Microbial analyses of *T. procumbens asava* revealed absence of *E.coli, Salmonella spp., Staphylococcus aureus, Pseudomonas aeruginosa,* and aflatoxins A and B. Heavy metals, including cadmium, arsenic and mercury were not detected; lead was present at 0.06 ppm, which was below the maximum permissible limits set by WHO and AYUSH for assessing quality of herbal preparations (World Health Organization 2007, Department of Commerce - Government of India 2008) (Table 1). Pesticide residues were undetectable (*see Supplemental Table 1*). Phytochemical analysis of *T. procumbens asava* showed the presence of phenolics, carotenoids, and flavonoids content (Table 1).

Antioxidant activity

T. procumbens asava showed a strong dose-dependent antioxidant reducing potential as indicated by significantly high trolox equivalents (Fig.1A) and ferric ion reducing potential (Fig.1B). Our data demonstrate that *T. procumbens asava* exert comparable antioxidant reducing potential as BHA, BHT, Trolox, α-T and Cur, at concentrations ranging from 1 - 2 mg/ml. *Tridax procumbens* extract showed the highest antioxidant reducing potential at a concentration of 10 mg/ml.

Next, we examined the ferrous ion chelating effect and hydrogen peroxide scavenging activity of *T. procumbens asava*. Forty five micrograms per milliliter of BHA, BHT, Trolox, α-T, and Cur exhibited 10, 8, 8, 9, and 10% ferrous ion chelating effect, respectively (Fig.1C), and

scavenged 31, 30, 26, 0, and 45% of H_2O_2 (Fig.1D). *T. procumbens asava* at 10, 4, 3, 2, and 1 mg/ml concentrations exhibited 74, 62, 51, 42, and 23% ferrous ion-chelating effect, and scavenged 96, 91, 89, 81, and 56% of H_2O_2. This suggests that *T. procumbens* extract (as low as 1 mg/ml) has high ferrous-ion chelating and H_2O_2 scavenging effect.

The ferric thiocyanate assay was used for quantitation of lipid peroxidation, as this was shown to be sensitive, and responsive to hydroperoxides of mono- and di-unsaturated fatty acids (Mihaljevic 1996). Peroxidation of linoleic acid, indicated as an increase in the ferric thiocyanate complex, increased significantly with time, from 5 -30 hours ($p < 0.001$). This increase in lipid peroxidation was significantly inhibited in the presence of antioxidant standards and *T. procumbens asava*, starting from 5 h through 30 h (Fig.1E) ($p < 0.001$). At the 30 h time point, *T. procumbens asava* demonstrated significantly higher inhibition ($p < 0.001$) of lipid peroxidation compared to Trolox and α-tocopherol (Fig.1F).

Clinical study

We recruited 20 individuals (10 men and 10 women) into the study. The average age was 56 ± 8 years for men and 61 ± 15 years for women (Table 2). Recruited participants had established diabetes for an average period of 16 ± 8 years. In men, blood glucose concentrations averaged 137 ± 17 mg/dl in the fasted state, and 227 ± 36 mg/dl, post-prandially. In women, blood glucose concentrations averaged 168 ± 40 mg/dl in the fasted state, and 298 ± 89 mg/dl, post-prandially (Table 2). While the majority of participants included animal products in their diet, three participants reported they were lactovegetarians. No adverse events or side effects were reported and none of the subjects withdrew from the study.

After obtaining fasting and 2-h post-prandial blood samples at the start of the study, participants were given a 4-week supply of *T. procumbens asava*. Participants were instructed by the physician to take 15 ml of the *asava* twice a day, using a plastic measuring cup, which provided a dose of 3 g dried *T. procumbens* per day.

At the end of the 4-week supplementation period, fasting blood glucose (Fig.2A, B) was significantly reduced in both men and women. Fasting blood glucose concentrations decreased by 11% from 137 ± 17 mg/dl to 122 ± 17 ($p < 0.01$) (Fig.2E). In women, fasting blood glucose decreased from 168 ± 40 mg/dl to 135 ± 15 ($p < 0.05$), demonstrating a 20% reduction (Fig.2E).

Two hour post-prandial blood glucose concentrations were also significantly reduced in both men and women (Fig.2C,D). Post-prandial blood glucose concentrations decreased by 26% (169 ± 20 mg/dl) compared to pre-treatment values (227 ± 36 mg/dl) in men ($p < 0.001$) (Fig.2F). In women, a 29% reduction in 2-h post-prandial blood glucose concentration was observed after 4-weeks of *T. procumbens* supplementation (Post: 210 ± 49 versus Pre: 298 ± 89 mg/dl, $p < 0.001$) (Fig.2F).

Discussion

In this study, we demonstrate, for the first time, that *T. procumbens asava*, taken in a complementary manner with other medications, has significant blood glucose lowering properties in patients with established type 2 diabetes. Administration of the *asava* lowered fasting blood glucose by 13% and 2-h post-prandial blood glucose concentrations by 26% in all participants. These findings are consistent with the studies of Pareek et al (Pareek 2009) and Bhagwat et al (Bhagwat 2008) in alloxan-induced diabetic rats who demonstrated that oral administration of a methanolic extract of *T. procumbens* L. whole plant (250 mg/kg body weight) for a period of 30 days, lowered fasting blood glucose by 62% in diabetic rats (Pareek 2009). These authors also showed that acute administration of *T. procumbens* extract (250 mg/kg body weight) lowered blood glucose by 67% in 8 hours only in alloxan-induced diabetic rats, but not in normal rats (Pareek 2009). While our studies have not examined mechanisms underlying blood glucose control, it may be surmised that *T. procumbens* may exert its effects through improved peripheral glucose utilization and/or suppression of hepatic glucose production.

In our studies, an *asava* of *T. procumbens,* a liquid preparation containing self-generated alcohol, was prepared as defined by the Ayurvedic Formulary of India (Department of Ayurveda 2000). Ayurveda, derived from "*ayur*" (meaning life) and "veda" (meaning knowledge), is a traditional system of medicine in India practiced since 5000 B.C. *Asava* is a fermented infusion described in Ayurveda, in addition to other dosage forms including herbal teas, decoctions, tinctures, capsules, powders, infused oils, ointments, creams, and lotions (Sekar 2008). The Ayurvedic Formulary of India is based on traditional Sanskrit literature including '*Charak Samhita*', '*Sushruta Samhita'*, and '*Astanga Hridaya'*, that describe Ayurvedic preparations and use of medicinal plants (Sekar 2008). In our study, an *asava* of the whole plant was prepared.

Asava formulation has the advantage of extracting heat-sensitive, aqueous and ethanol-extractable bioactives and extend shelf life (Ross 2002). Further, microbial biotransformation during asava preparation could potentially improve bioavailability of phytochemicals and its therapeutic potential (Ross 2002). In our study, *T. procumbens asava* preparation demonstrated an 8.2% (v/v) ethanol content, which was within the acceptable limit for *asava* preparations (Kalaiselvan 2010).

Phenolic content of *T. procumbens asava* (1.0 g GAE/L) was comparable to that of red wine (1.8 g GAE/L), and the flavonoid content was similar to that present in citrus fruits (Waterhouse 2001, Chun 2007). Phenolic compounds, shown to be strong antioxidants, and inversely associated with incidence of diabetes and cardiovascular diseases, exert modulatory effects on intracellular signaling cascades vital for growth, proliferation and apoptosis (Crozier 2009). In our study, *T. procumbens asava* demonstrated Trolox equivalent antioxidant capacity (TEAC) of 1.39 mM at a concentration of 10 mg/ml, which was comparable to TEAC of lettuce (1.33 mmol Trolox/kg fresh weight) and pineapple juice (1.50 mmol Trolox/L) (Pellegrini 2003). *T. procumbens asava* prepared using *W. fruticosa* flowers showed a calculated TEAC value of 3 mM/kg dry-weight of *T. procumbens*. In other foods, spinach demonstrated highest TEAC (8.49 mmol Trolox/kg fresh weight) among vegetables; blackberry (20.24 mmol Trolox/kg fresh weight) among fruits; and coffee (36.54 mmol Trolox/L) among beverages followed by citrus juices (Pellegrini 2003).

Few commercially available fermented Ayurvedic infusions, including *Kumaryasava*, *Dashamoolarishta*, and *Ashwagandharishtha* have been studied for their antioxidant potential compared to ascorbic acid (Manmode 2012, Manwar 2013, Pawar 2013). *Kumaryasava* prepared by fermentation using three different inoculums, viz. *Woodfordia fruticosa* flowes, *Madhuca*

indica flowers and *Saccharomyces cerevisiae* SC1011 showed significant dose-dependent reduction ($p < 0.05$) in DPPH free radical scavenging activity, H_2O_2 scavenging activity and total reducing power indicate by ferric ion reducing potential, within the concentration range 100-1000 µl/ml. *Kumaryasava* prepared using *W. fruticosa* flowers showed strong antioxidant potential, with an IC_{50} value of 49.6 µl/ml in ferric-ion reducing potential assay and IC_{50} of 50.13 µl/ml in H_2O_2 scavenging activity assay (Manmode 2012). *Ashwagandharishtha* prepared by fermentation using *W. fruticosa* flowers demonstrated 78.75% DPPH free radical scavenging activity at 1 mg/ml and 69.62% H_2O_2 scavenging activity at 100 µg/ml (Manwar 2013). In our study, *T. procumbens asava* prepared using *W. fruticosa* flowers demonstrated 55.9% H_2O_2 scavenging activity at a concentration of 1 mg/ml. The half maximal effective concentration (EC_{50}) of *T. procumbens asava* was 1.62, 4.79 and 1.2 mg/ml respectively in total antioxidant capacity assay, ferric-ion reducing antioxidant potential assay and ferrous-ion chelating activity assay. *T. procumbens asava* also showed significant inhibition of lipid peroxidation. Further, *T. procumbens asava* inhibited pro-oxidant reactions by chelating ferrous ions and scavenging hydrogen peroxide (Mates 1999), suggesting that the antioxidant properties may contribute to lower elevated oxidative stress associated with diabetes.

A few studies have examined the toxicity of *T. procumbens* in Wistar rats. In acute studies, no fatal consequences or visible symptoms of toxicity were observed with administration of a methanolic extract of *T. procumbens* up to a dose of 5 g/kg body weight (Pareek 2009). Further, short-term toxicity studies using ethyl actetate extract of *T. procumbens* (50, 100, 200, 400 and 800 mg/kg) for 14 days have demonstrated increased body weight and percent organ/body weight ratio for liver and spleen in a dose-dependent manner (Abubakar 2012). In DGal-N/LPS-induced hepatotoxicity and liver damage, administration of *T. procumbens* restored

elevated serum alanine aminotransferase and aspartate transaminase, bilirubin and lipids to normal levels (Ravikumar 2005). However, Abubakar et al. showed increased ALT activity, but not AST activity, in Wistar rats administered 800 mg/kg ethyl acetate extract of *T. procumbens* (Abubakar 2012). These authors showed that the LD_{50} of the ethyl acetate extract of *T. procumbens* was 2100 mg/kg body weight. Further, hemosiderin deposition was highest at 800 mg/kg body weight, and lowest at 50 mg/kg body weight. In our study, 15 ml of *T. procumbens asava* was administered twice daily, which gave a calculated dose of approximately 50 mg *T. procumbens*/kg body weight.

To our knowledge, this is the first clinical report on the anti-hyperglycemic potential of *T. procumbens*. A major limitation of this pilot, clinical study was that only blood glucose concentrations were assayed. Body weights of participants were not obtained, either pre- or post-supplementation. However, this 4-week study provides encouraging preliminary data for further evaluation of blood glucose lowering potential of *T. procumbens* in a randomized, double-blind, placebo-controlled trial.

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Conflict of Interest

G.S. Desai, S.V. Desai: serving on Board of Directors; R.S. Gavaskar, S.T. Mathews: No conflicts of interest.

Figure Legends

Fig.1. Antioxidant reducing potential of *T. procumbens asava*. Total Antioxidant Capacity (TAC), expressed as Trolox equivalents (mM) [A], ferric ion reducing potential [B], ferrous-ion chelating effect [C], H_2O_2 scavenging activity [D], and lipid peroxidation [E,F] were determined. *** $p < 0.001$, compared to butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), trolox (Tro), α-tocopherol (□-T) and curcumin (Cur); ### $p < 0.001$, compared to *T.procumbens asava* and antioxidant standards; ‡‡‡ $p < 0.001$, compared to Tro and α-T.

Fig.2. Blood glucose lowering effect of *T. procumbens asava*. Twenty type 2 diabetic patients (10 men, 10 women) were administered 15 ml *T. procumbens asava*, twice daily, for 4 weeks. Fasting and 2-h post-prandial blood glucose concentrations in men [A, C] and women [B, D] were analyzed before and after *T. procumbens* supplementation, and are shown as before-after graph. Percent reductions for fasting [E] and post-prandial [F] blood glucose concentrations following 4-week supplementation are depicted. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, compared to pre-supplementation values.

Table 1: Total phenolics, flavonoids, carotenoids, chemical and microbial characteristics of *T. procumbens asava*

T. procumbens asava	**Characteristics**
pH	4.5
Specific gravity	1.024
Total solids	2.9% w/w
Ethanol	8.2% v/v
Methanol	None
Reducing sugar	1.4% w/w
Non-reducing sugar	1.0% w/w
Total acidity	0.713
Total Phenolics	1.0 ± 0.3 g GAE / L
Flavonoids	2.5 ± 0.03 mg QE/mL
Carotenoids	20 ± 0.4 µg/mL
Lead	0.06 ppm
Cadmium	None
Mercury	None
Arsenic	None
Total bacterial count	$< 10^5$cfu/g
Total fungal count	$< 10^3$cfu/g
E.coli	Absent
Salmonella spp.	Absent
S. aureus	Absent
P. aeruginosa	Absent

Table 2: Participant characteristics, duration of diabetes, and blood glucose concentrations prior to *T. procumbens* supplementation.

Parameters	**Men (n=10)**	**Women (n=10)**
Age (years)	56 ± 8	61 ± 15
Duration of diabetes (years)	14 ± 6	18 ± 10
Fasting blood glucose (mg/dl)	137 ± 17	168 ± 40
2-h post prandial blood glucose (mg/dl)	227 ± 36	298 ± 89

Figure 1

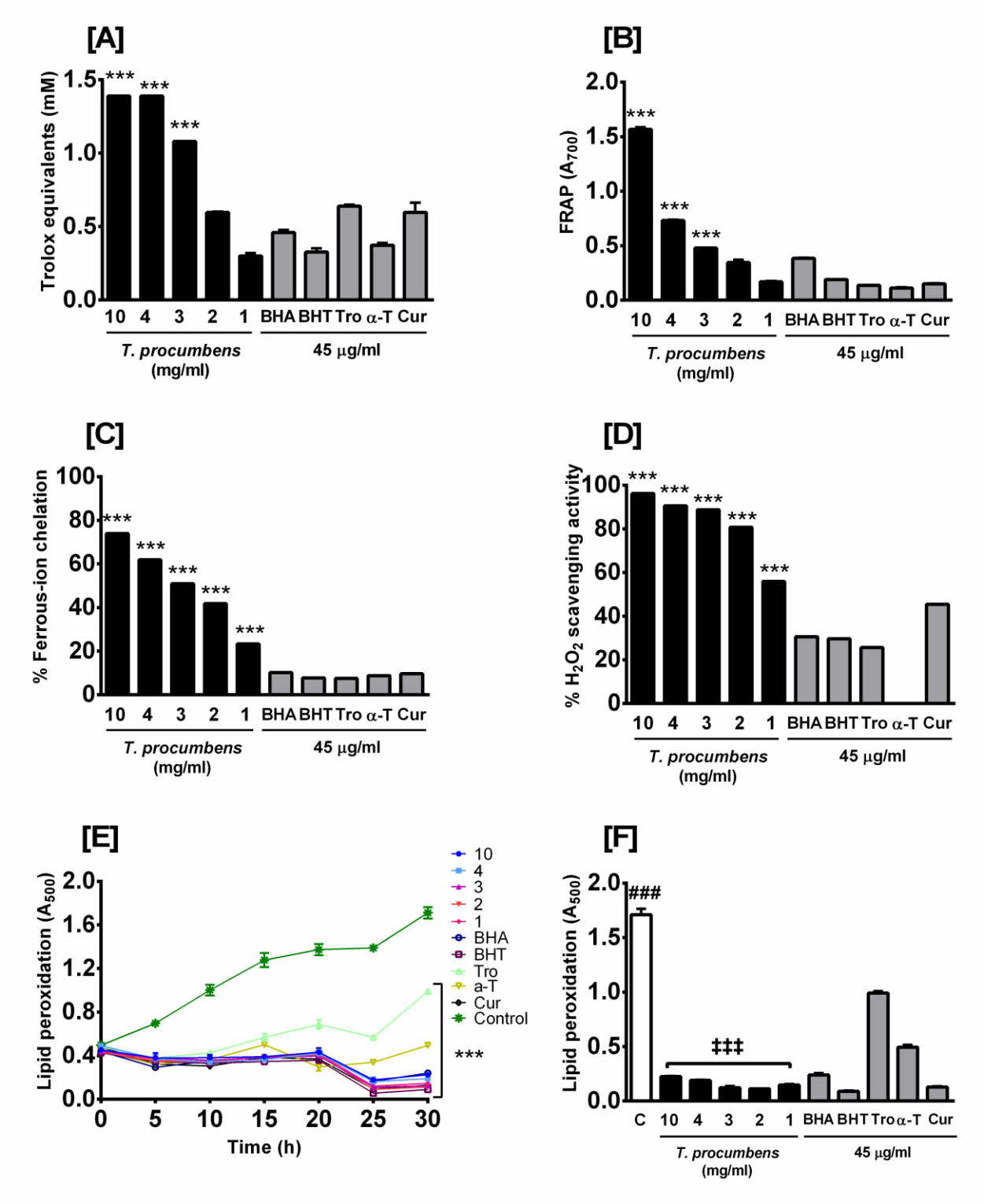
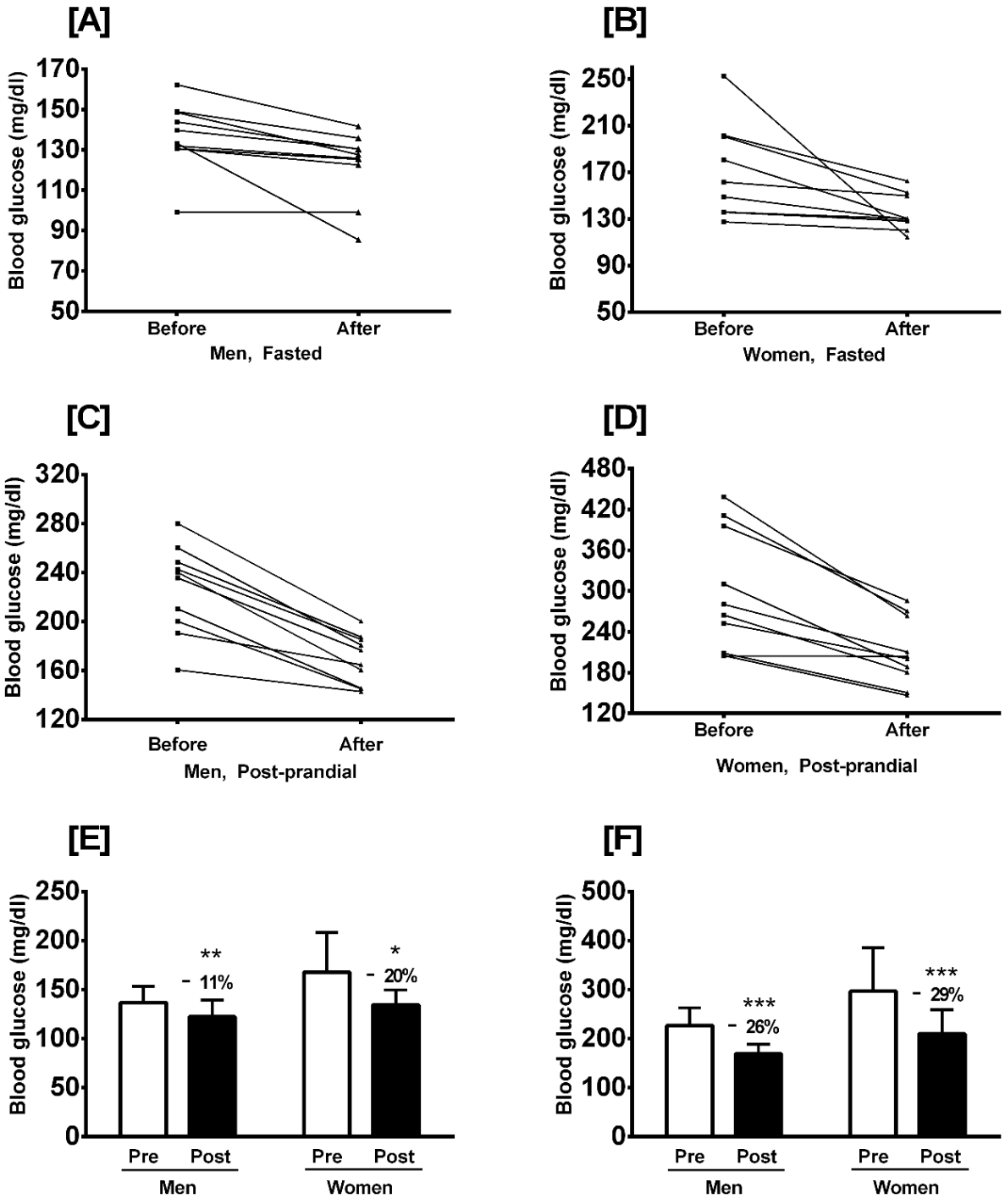

[A]
Trolox equivalents (mM)
1.5
1.0
0.5
0.0
***
***
***
10 4 3 2 1 BHA BHT Tro α-T Cur
T. procumbens (mg/ml)
45 μg/ml
[B]
FRAP (A700)
2.0
1.5
1.0
0.5
0.0
***
***
***
10 4 3 2 1 BHA BHT Tro α-T Cur
T. procumbens (mg/ml)
45 μg/ml
[C]
% Ferrous-ion chelation
100
80
60
40
20
0
***
***
***
***
***
10 4 3 2 1 BHA BHT Tro α-T Cur
T. procumbens (mg/ml)
45 μg/ml
[D]
% H2O2 scavenging activity
100
80
60
40
20
0
***
***
***
***
***
10 4 3 2 1 BHA BHT Tro α-T Cur
T. procumbens (mg/ml)
45 μg/ml
[E]
Lipid peroxidation (A500)
2.0
1.6
1.2
0.8
0.4
0.0
0 5 10 15 20 25 30
Time (h)
10
4
3
2
1
BHA
BHT
Tro
a-T
Cur
Control
***
[F]
Lipid peroxidation (A500)
2.0
1.5
1.0
0.5
0.0
###
‡‡‡
C 10 4 3 2 1 BHA BHT Tro α-T Cur
T. procumbens (mg/ml)
45 μg/ml

Figure 2


[A]
Blood glucose (mg/dl)
170
150
130
110
90
70
50
Before
After
Men, Fasted
[B]
Blood glucose (mg/dl)
250
210
170
130
90
50
Before
After
Women, Fasted
[C]
Blood glucose (mg/dl)
320
280
240
200
160
120
Before
After
Men, Post-prandial
[D]
Blood glucose (mg/dl)
480
420
360
300
240
180
120
Before
After
Women, Post-prandial
[E]
Blood glucose (mg/dl)
250
200
150
100
50
0
**
- 11%
*
- 20%
Pre
Post
Pre
Post
Men
Women
[F]
Blood glucose (mg/dl)
500
400
300
200
100
0
***
- 26%
***
- 29%
Pre
Post
Pre
Post
Men
Women

Supplemental Table 1: Quantitation of pesticide residues in *T. procumbens asava* using LCMS / GCMS

No.	Pesticide	Quantitation Limit	Quantitation Method	Residue content (mg/kg)
1	Atrazine	0.01	LCMS/MS	BQL
2	Acephate	0.01	LCMS/MS	BQL
3	Diazinon	0.01	LCMS/MS	BQL
4	Dimethoate (Sum of diomethoate & Omethoate expressed as Dimethoate)	0.01	LCMS/MS	BQL
5	Ethion	0.01	LCMS/MS	BQL
6	Malathion	0.01	LCMS/MS	BQL
7	Methamidophos	0.01	LCMS/MS	BQL
8	Monocrotophos	0.01	LCMS/MS	BQL
9	Omethoate (Sum of Dimethoate & omethoate expressed as Omethoate)	0.01	LCMS/MS	BQL
10	Phosalone	0.01	LCMS/MS	BQL
11	Phosphamidon	0.01	LCMS/MS	BQL
12	Profenophos	0.01	LCMS/MS	BQL
13	Quinalphos	0.01	LCMS/MS	BQL
14	Triazophos	0.01	LCMS/MS	BQL
15	Simazine	0.01	LCMS/MS	BQL
16	Metalaxyl	0.01	LCMS/MS	BQL
17	Carbaryl	0.01	LCMS/MS	BQL
18	Carbofuran	0.01	LCMS/MS	BQL
19	Carbosulfan	0.01	LCMS/MS	BQL
20	Indoxycarb	0.01	LCMS/MS	BQL
21	Mithomyl	0.01	LCMS/MS	BQL
22	Thiodicarb	0.01	LCMS/MS	BQL
23	Fenarimol	0.01	LCMS/MS	BQL
24	Bitertanol	0.02	LCMS/MS	BQL
25	Flusilazole	0.01	LCMS/MS	BQL
26	Hexaconazol	0.01	LCMS/MS	BQL
27	Myclobutanil	0.01	LCMS/MS	BQL
28	Penconazole	0.01	LCMS/MS	BQL
29	Propiconazole	0.01	LCMS/MS	BQL
30	Tebuconazole	0.01	LCMS/MS	BQL
31	Triademifon	0.01	LCMS/MS	BQL
32	Difenoconazole	0.01	LCMS/MS	BQL
33	Etrimphos	0.01	LCMS/MS	BQL
34	Carbendazim (Sum of Benomyl & Carbendazim expressed as Carbendazim)	0.01	LCMS/MS	BQL
35	Iprobenphos	0.01	LCMS/MS	BQL
36	Thiophanate-methyl	0.01	LCMS/MS	BQL

No.	Pesticide	Quantitation Limit	Quantitation Method	Residue content (mg/kg)
37	Acetamiprid	0.01	LCMS/MS	BQL
38	Clothinidin	0.01	LCMS/MS	BQL
39	Imidacloprid	0.01	LCMS/MS	BQL
40	Thiacloprid	0.01	LCMS/MS	BQL
41	Thiamethoxam	0.01	LCMS/MS	BQL
42	Cymoxanil	0.025	LCMS/MS	BQL
43	Dimethomorph	0.01	LCMS/MS	BQL
44	Oxydemeton methyl	0.025	LCMS/MS	BQL
45	Buprofezin	0.01	LCMS/MS	BQL
46	Cartap hydrochloride	0.01	LCMS/MS	BQL
47	Azoxystrobin	0.01	LCMS/MS	BQL
48	Flufenoxuron	0.025	LCMS/MS	BQL
49	Propargite	0.01	LCMS/MS	BQL
50	Fenpyroximate	0.01	LCMS/MS	BQL
51	Famoxadone	0.01	LCMS/MS	BQL
52	Fenamidone	0.01	LCMS/MS	BQL
53	Diflubenzuron	0.01	LCMS/MS	BQL
54	Trifloxystrobin	0.01	LCMS/MS	BQL
55	Pyraclostrobin	0.01	LCMS/MS	BQL
56	Triadimenol	0.01	LCMS/MS	BQL
57	Emamectin Benzoate	0.05	LCMS/MS	BQL
58	Spinosad	0.01	LCMS/MS	BQL
59	Abamectin	0.01	LCMS/MS	BQL
60	Kresoxym methyl	0.01	LCMS/MS	BQL
61	Iprovalicarb	0.01	LCMS/MS	BQL
62	Difenthiuron	0.01	LCMS/MS	BQL
63	Aldrin	0.01	GCMS/MS	BQL
64	Cis Chlordan	0.01	GCMS/MS	BQL
65	Chlorothalonil	0.01	GCMS/MS	BQL
66	DDT (sum of p,p'-DDT, o,p'-DDT, p-p'-DDE expressed as DDT)	0.01	GCMS/MS	BQL
67	Endosulphan	0.01	GCMS/MS	BQL
68	Endrin	0.01	GCMS/MS	BQL
69	BHC	0.01	GCMS/MS	BQL
70	Heptachlor (Sum of heptachlor & Heptachlor epoxide expressed as Heptachlor)	0.01	GCMS/MS	BQL
71	Lindane	0.01	GCMS/MS	BQL
72	4-bromo-2-chloro phenol	0.01	GCMS/MS	BQL
73	Chlorofevinphos	0.01	GCMS/MS	BQL
74	Chloropyriphos Ethyl	0.01	GCMS/MS	BQL
75	Chloropyriphos methyl	0.01	GCMS/MS	BQL
76	Dichlorvos	0.01	GCMS/MS	BQL

No.	Pesticide	Quantitation Limit	Quantitation Method	Residue content (mg/kg)
77	Fenitrothion	0.01	GCMS/MS	BQL
78	Parathion Ethyl	0.01	GCMS/MS	BQL
79	Parathion methyl	0.01	GCMS/MS	BQL
80	Trans chlordane	0.01	GCMS/MS	BQL
81	Dicofol	0.01	GCMS/MS	BQL
82	Cyfluthrin (Sum of Isomer)	0.01	GCMS/MS	BQL
83	Cypermethrin (Sum of Isomer)	0.01	GCMS/MS	BQL
84	Deltamethrin (cis-deltamethrin)	0.01	GCMS/MS	BQL
85	Ethofenprox	0.01	GCMS/MS	BQL
86	Fenvalerate & Esfenvaleiate (Sum of RR & SS isomers)	0.01	GCMS/MS	BQL
87	Fenvalerate & Esfenvaleiate (Sum of RS & SR isomers)	0.01	GCMS/MS	BQL
88	Lambda Cyhalothrin	0.01	GCMS/MS	BQL
89	Permethrin	0.01	GCMS/MS	BQL
90	Iprodione	0.01	GCMS/MS	BQL
91	Oxyfluorfen	0.025	GCMS/MS	BQL
92	2,4 DDE	0.01	GCMS/MS	BQL
93	4,4 DDE	0.01	GCMS/MS	BQL
94	Endosulphan Sulphate	0.01	GCMS/MS	BQL

LCMS: Liquid Chromatography with Mass Spectrometry
GCMS: Gas Chromatography with Mass Spectrometry
BQL: Below Quantitation Limit

Tridax procumbens L. activates AMPK in rat hepatoma cells and inhibits NFκB activation in RAW 264.7 murine macrophages

Gauri S. Desai[1] and Suresh T. Mathews[1]*

[1] Department of Nutrition and Dietetics, Boshell Diabetes and Metabolic Diseases Research Program, Auburn University, Auburn, AL 36830

*Corresponding author:

Suresh T. Mathews, Ph.D, FACN
Associate Professor & Graduate Program Director
Department of Nutrition and Dietetics
260 Lem Morrison Dr., 101 PSB
Auburn University, AL 36849
Phone: 334-844-7418
Fax: 334-844-3268
Email: mathest@auburn.edu

Abstract

Traditional knowledge and anecdotal evidence suggest that *Tridax procumbens* Linn may have anti-inflammatory and blood glucose lowering properties. Recent studies have shown that *T. procumbens* extract was effective in lowering blood glucose in alloxan- and streptozotocin-induced diabetic rats. *T. procumbens* extract exerts strong antioxidant and anti-inflammatory properties evidenced through Trolox equivalent antioxidant capacity, ferric-ion reducing potential, hydrogen peroxide scavenging activity, metal ion chelating effect, and inhibition of COX-1 and COX-2 activities. Previously, we demonstrated that, oral supplemenation of *T. procumbens* extract (prepared following Ayurveda guidelines), for a period of 4 weeks, significantly lowers fasting and post-prandial blood glucose in individuals with type 2 diabetes. The goal of this study was to characterize molecular mechanisms underlying blood glucose lowering properties and anti-inflammatory effects of *T. procumbens* extract using cell culture techniques. *T. procumbens* extract significantly increased AMPK phosphorylation (Thr 172), compared to basal, in H4IIE rat hepatoma cells. Also, *T. procumbens* extract suppressed dexamethasone-induced phosphoenol pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) gene expression with a concomitant decrease in hepatic glucose production in H4IIE cells. However, *T. procumbens* extract did not activate insulin signaling via phosphorylation of Akt (Ser 473) or MAPK (p44/42) in HIRcB cells (rat-1 fibroblasts overexpressing human insulin receptors).The anti-inflammatory potential of *T. procumbens* extract was demonstrated by inhibition of NFκB and IκBα activation in LPS-stimulated RAW 264.7 murine macrophages. *T. procumbens* extract also suppressed LPS-stimulated NFκB transcriptional activityin NFκB luciferase reporter HeLa cell line. Our studies indicate that *T. procumbens* extract exhibits strong anti-inflammatory properties through NFκB

inhibition. Activation of AMPK, together with the suppression of hepatic glucose production, may be a potential mechanism that contributes to the glucose-lowering property of *T. procumbens*.

Introduction

Tridax procumbens Linn., (Family-Asteraceae), commonly known as coat-buttons, is a perennial weed and flowering plant native to the tropical Americas. It grows throughout the tropical and sub-tropical regions of the world, and is abundant especially in fallow fields, agricultural fields and wastelands. In the United States, *T. procumbens* has been introduced in the Lower 48 States (L48), Hawaii (HI), Puerto Rico (PR) and the U.S. Virgin Islands (VI), and is listed as a noxious weed by the United States Department of Agriculture (USDA 2014). *T. procumbens* has been a part of indigenous medicine in India and Africa, and used in the management of diabetes and inflammation.

Studies in relevant animal models have also demonstrated the anti-inflammatory and blood glucose lowering effects of *T. procumbens* (Pareek 2009, Jachak 2011). *T. procumbens* whole plants extract (250 mg/kg) lowered fasting blood glucose by 68% at the end of 6 hours in alloxan-induced diabetic rats. At a higher dose of 500 mg/kg, fasting blood glucose was lowered by 71% in diabetic animals. In the acute toxicity study, *T. procumbens* (2500-5000 mg/kg) did not show any visible signs of toxicity including hyperactivity, sedation, loss of reflex or convolutions. Further, no mortality was reported within 48 hours of *T. procumbens* administration (Pareek 2009). The anti-inflammatory activity of *T. procumbens* has been studied using carrageenan-induced rat paw edema assay in female Sprague-Dawley rats. Ethyl acetate, methanol and 70% ethanol extract of *T. procumbens* (200 mg/kg) demonstrated significant reduction ($p < 0.05$) in paw volume compared to normal control group. Further, ethanol extract of *T. procumbens* inhibited inflammation by approximately 35%, which was significantly lower compared to the non-steroidal anti-inflammatory drug, Ibuprofen (~52% inhibition) (Jachak 2011).

Phytochemical screening has shown presence of flavonoids, carotenoids, saponins, tannins and sterols including campesterol, stigmasterol and β-sitosterol in leaves, stem and flowers of *T. procumbens* (Gadre 1993, Saxena 2005, Jude 2009). *T. procumbens* has been shown to inhibit carrageenan-induced rat paw edema. It has been suggested that the anti-inflammatory potential of *T. procumbens* can be attributed to the presence of phytosterols (Kale 2013). Further, in a pilot clinical study in individuals with type 2 diabetes, *T. procumbens* extract supplementation significantly lowered fasting blood glucose by 11% in men and 20% in women. Also, 2-h post-prandial blood glucose concentrations were significantly lowered by 26% in men and 29% in women, following 4 weeks of *T. procumbens* supplementation (Desai 2014).

However, there are no studies characterizing the molecular mechanisms mediating anti-hyperglycemic effects of *T. procumbens*. It has been previously suggested that the anti-inflammatory activity of *T. procumbens* could be due to inhibition of cyclooxygenase enzymes COX-1 and COX-2, and the free radical scavenging potential of plant polyphenols (Jachak 2011). In this study, we have examined the effects of *T. procumbens* on AMPK activation, hepatic gluconeogenesis, insulin signaling and the NFκB pathway.

Materials and Methods

Plant Material

Tridax procumbens Linn. plants (whole plants) were collected from Athani (Belgavi district) in Karnataka, India following Good Agricultural Practices for medicinal plants (WHO 2003). *Tridax procumbens* Linn. was authenticated by Aditi Organic Certifications, Bangalore, India Whole, mature flowers of *Woodfordia fruticosa* (L.) Kurz., a common ingredient of fermented Ayurvedic preparations, were obtained from local vendors (Chaudhary 2011).

Preparation of *Tridax procumbens asava*

T. procumbens asava, a fermented infusion, was prepared following Ayurveda (a system of traditional Indian Medicine) guidelines. *T. procumbens* L. whole plants were initially rinsed in running water, shade dried and then coarsely powdered. The powdered *T. procumbens* plant material (10 kg) and dried, whole flowers of *Woodfordia fruticosa* (L.). Kurz. (4 kg) were added to a stainless steel process vessel containing pharmaceutical-grade sugar (35 kg/100L) in 100 L of potable water. *W. fruticosa* flowers harbor wild species of yeast in the dry nectariferous region, which served as the inoculum (Sekar 2008). Fermentation was carried out for 45 days in the closed process vessel, maintained between 30-35° C. Following fermentation, the *asava* was filtered using cheese-cloth, bottled, sealed, and tested for microbial, heavy metal, or pesticidal contamination following Good Manufacturing Practices. Total phenolics, flavonoids, and carotenoids were analyzed as reported previously (Scott 2001, Waterhouse 2001, Marinova 2005).

Reagents and antibodies

Recombinant human insulin was purchased from Roche Diagnostics (Indianapolis, IN); dexamethasone from Sigma-Aldrich (St. Louis, MO); antibodies against AMPK,

pAMPKα(Thr172), ACC, pACC(Ser79), Akt, pAkt(Ser473), ERK1/2, pMAPKp44/42(Thr202/Tyr204), NFκB, pNFκB(Ser536), IκBα, and pIκBα(Ser32) from Cell Signaling Technology (Beverly, MA). Antibody against GAPDH was purchased from Abcam, Cambridge, MA.

Cell culture

H4IIE rat hepatoma cells and RAW 264.7 murine macrophages were purchased from the American Type Culture Collection (Manassas, VA, USA). HIRcB cells (Rat-1 fibroblasts stably transfected with wild-type human insulin receptor cDNA, overexpressing human insulin receptor at ~ 1.25 $x10^6$ receptors/cell) was a gift from Dr. Jerrold Olefsky, USCD/VAMC, San Diego, CA. NFκB Luciferase Reporter HeLa Stable Cell Line was purchased from Signosis (Santa Clara, CA). H4IIE cells were cultured in MEM-α, HIRcB and RAW 264.7 cells were grown in DMEM, high glucose supplemented with 10% FBS, and 1% Penicillin-Streptomycin-Neomycin. All cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Western blot

Cells were washed with ice-cold PBS three times and lysed in 300 µl of cell lysis buffer [50 mM HEPES pH7.4, 100 mM Sodium pyrophosphate, 100 mM sodium fluride, 10 mM EDTA, 10 mM sodium orthovanadate, 2 mM PMSF, 1% Triton X-100 and protease inhibitor cocktail tablet (Roche diagnostics, Indianapolis)]. Complete cell lysis was ensured by sonication for 20 sec (pulse 05 sec, stop 03 sec) at 50% amp, followed by mechanical disruption on a rotating mixer at 4°C for 45 min. Cell pellet was separated by centrifugation at 4°C, 14000 rpm for 20 min. Pierce 660 nm protein assay reagent (Thermo Fischer Scientific Inc., Rockford, IL) was used to quantitate total protein. Equal quantities of cell lysate proteins were separated by 4-20% SDS-PAGE precast

gels (Nu-Sep Inc., Austell, GA) and transferred to 0.45 µm nitrocellulose membrane (Bio-Rad Laboratories Inc., Berkeley, CA) at 12 V for 1 h. The membrane was blocked using 5% milk blocking buffer followed by incubation with primary antibodies against pAMPKα(Thr172), AMPK, pAkt(Ser473), Akt, pMAPK(p44/42), ERK1/2, pNFκB(Ser536), NFκB, pIκBα(Ser32), IκBα and GAPDH. The blots were then probed with respective horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies. Reactive bands were visualized using enhanced chemiluminescence detection system (Thermo Fischer Scientific Inc., Rockford, IL) and image analysis were performed with UVP-Biochimie Bioimager and LabWorks Software (UVP, Upland, CA).

Quantitative real-time PCR

H4IIE cells were grown to confluence in 6-well plates and starved for 6 hours using serum-free MEM-α supplemented with 0.1% bovine serum albumin and 1% penicillin/ streptomycin/ neomycin. Cells were then treated with combinations of dexamethasone (5 µM), insulin (100 nM) and *T. procumbens* extract for 15 hours. Following incubation, cells were washed thrice with ice-cold PBS and collected using trypsin. RNA was extracted using RNeasy Mini RNA isolation kit (QIAGEN, Valencia, CA). cDNA was synthesized using iScript cDNA synthesis kit (BIO-RAD, Hercules, CA) and real-time PCR was performed using iQSYBR Green Supermix (BIO-RAD) following manufacturer's instructions. Primer sequences for real-time PCR were as follows: phosphoenol pyruvate carboxy kinase (PEPCK) forward 5'-GGG TGC TAG ACT GGA TCT GC-3', PEPCK reverse 5'-GAG GGA GAA CAG CTG AGT GG-3'; glucose-6 phosphatase (G6Pase) forward 5'-GGG TGT AGA CCT CCT GTG GA-3', G6Pase reverse 5'- GAG CCA CTT GCT GAG TTT CC-3'; fructose 1,6 bisphosphatase (Fru1,6bPase) forward 5'-TCA TCG CAC TCT GGT CTA CG-3', Fru1,6bPase reverse 5'-GCC CTC TGG TGA ATG TCT GT-3'; β-actin

forward 5'-CCT CTA TGC CAA CAC AGT GC-3', β-actin reverse 5'-CAT CGT ACT CCT GCT TGC TG-3'. Reaction conditions were as follows: step 1: 95 °C, 3 min; step 2: 95 °C, 30s; 55 °C, 30s; 72 °C, 30s (step 2 is repeated 35x); step 3: 72 °C, 7 min; step 4: 4 °C, hold. Expression levels were normalized to β-actin and gene expression was calculated as $2^{-\Delta\Delta CT}$. All assays were carried out in triplicate.

Glucose production assay

Confluent 6-well plates of H4IIE cells were starved in serum-free MEM-α for 5 hours, including 1 hour pre-treatment with *T. procumbens* extract, and incubated overnight with 5 μM dexamethasone. Insulin (100 nM) was used a positive control to demonstrate suppression of glucose production. Following incubation, cells were washed thrice with PBS and treated with glucose production media (glucose-free DMEM supplemented with 2 mM pyruvate and 20 mM sodium lactate), dexamethasone, *T. procumbens* and insulin for 3 hours. Glucose production was measured using a Glucose UV Reagent (Cliniqa Corp., San Marcos, CA) and normalized to total protein content of whole-cell lysates.

Luciferase Reporter assay

HeLa cell line stably transfected with pTA-NFκB-luciferase reporter vector was purchased from Signosis, Inc. (Santa Clara, CA). Cells were seeded in a 96-well plate and incubated with freshly prepared LPS (2 μg/ml) for 1 hour at 37°C in the presence or absence of *T. procumbens* extract. Following incubation, cells were lysed and luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI).

Statistical analyses

Statistical analysis was performed using GraphPad Prism 6 (La Jolla, CA). A Student's two-tailed unpaired *t* test was used to determine statistical significance at $p < 0.05$. Data are expressed as mean ± SD.

Results

T. procumbens does not alter insulin signaling

To investigate the mechanisms underlying its blood glucose lowering potential, we examined the effect of *T. procumbens* extract on phosphorylation and activation of two key nodes of insulin signaling, viz., MAPK (ERK1/2) and Akt (Ser473). In our study, *T. procumbens* extract did not increase phosphorylation of MAPK (ERK1/2) or Akt (Ser473) in HIRcB rat-1 fibroblasts (Fig.1A), suggesting that *T. procumbens* may not directly exert an effect on insulin signaling.

T. procumbens activates AMPK signaling

Several phytochemical compounds have been previously identified in *T. procumbens* extract that could potentially activate the energy sensing protein complex AMPK. H4IIE cells were treated with *T. procumbens* concentrations (10, 2, 1 mg/ml) in serum-free MEM-α for 30 min (Fig. 1B). Synthetic AMP-analog, AICAR (0.5 mM), was used as a positive control. Our results show that *T. procumbens* extract increased AMPK (Thr172) phosphorylation by 1.9- and 2.2-fold at 10 and 2 mg/ml respectively, which was significantly higher compared to basal AMPK phosphorylation. Also, as expected, AICAR demonstrated 3-fold increase in AMPK phosphorylation at 0.5 mM concentration. Since activation of AMPK has been shown to phosphorylate and inactivate its downstream enzyme acetyl-CoA carboxylase (ACC) (Hardie 1997), we, therefore, examined the effect of *T. procumbens* on phosphorylation of ACC (Fig.1C). Our results indicate that *T. procumbens* significantly increased phosphorylation of ACC by 1.8, 2.5 and 1.3-fold at 10, 2 and 1 mg/ml respectively. AICAR induced 1.8-fold increase in phospho-ACC at 0.5 mM concentration, which was comparable to *T. procumbens* at 10 mg/ml.

T. procumbens inhibits hepatic glucose production

Earlier studies have shown that insulin exerts direct effects in inhibiting hepatic glucose production (HGP) with gluconeogenesis being the predominant mechanism responsible for liver glucose output (Beck-Nielsen 2002, Edgerton 2006). In our study, insulin significantly suppressed dexamethasone-induced HGP ($p < 0.05$). Glucose production in the presence of 100 nM insulin was 21%. *T. procumbens* exhibited mean HGP of 34% at 10 mg/ml, which was significantly lower ($p < 0.05$) compared to cells treated with dexamethasone alone (Fig.1D).

T. procumbens suppresses dexamethasone-induced gluconeogenic gene expression

Since activation of AMPK has been shown to suppress expression of G6Pase and PEPCK genes; and lower endogenous glucose production, we wanted to study the effects of *T. procumbens* on gluconeogenic gene expression (Horike 2008, Mues 2009). Consistent with our findings on *T. procumbens* effects of AMPK activation and HGP, our studies showed that dexamethasone increased gene expression of PEPCK and G6Pase by 5.2- and 2.7-fold, respectively. *T. procumbens* decreased gene expression dose-dependently, with significant inhibition ($p < 0.001$) at the highest concentration of 10 mg/ml. (Fig.1E,F).

T. procumbens inhibits NFκB activation

Since NFκB activation is a key event in the pathogenesis of type 2 diabetes and phosphorylation NFκB p65 subunit on Ser536 at the C-terminal transactivation domain is critical to induce transcription of target genes (Buss 2004, Patel 2009), we investigated the role of *T. procumbens* on LPS-stimulated NFκB p65 (Ser536) phosphorylation in RAW 264.7 murine macrophages (Fig.2A). LPS induced an ~1.3-fold increase in phosphorylation of NFκB p65. This increase in phopho-NFκB p65 was significantly higher ($p < 0.01$) compared to basal levels in

unstimulated cells. *T. procumbens* treatment significantly inhibited ($p < 0.05$) LPS-induced NFκB p65 (Ser536) phosphorylation. Interestingly, NFκB p65(Ser536) phosphorylation in cells pretreated with *T. procumbens* was comparable to basal values. This suggests that *T. procumbens* can potentially suppress translocation of NFκB p65 from the cytoplasm to the nucleus, thus impairing a vital step in activation of NFκB-regulated genes.

T. procumbens inhibits IκBα phosphorylation

To better understand *T. procumbens* inhibitory effects on NFκB activation, we examined its effect on LPS-induced phosphorylation of IκBα on Ser32 in RAW 264.7 murine macrophages (Traenckner 1995). LPS increased phosphorylation of IκBα by 1.5-fold, which was significantly higher than basal. Our results indicate that *T. procumbens* significantly inhibited LPS-induced IκBα(Ser32) phosphorylation compared to cells treated with LPS alone (Fig.2B). The inhibition was dose-dependent with highest inhibition at 10 mg/ml. Inhibition of IκBα phosphorylation protects it from proteasomal degradation, allowing IκBα to continue to exert its inhibitory effect on NFκB activation.

T. procumbens inhibits NFκB transcriptional activity

Next, we evaluated the transcriptional activity of NFκB using the NFκB luciferase reporter HeLa cell line stably transfected with a vector containing 4 repeats of NFκB binding sites followed by the firefly luciferase coding region. In our study, LPS significantly increased ($p < 0.01$) NFκB transcriptional activity compared to basal. In the presence of *T. procumbens*, there was significant reduction ($p < 0.01$) in LPS-induced NFκB transcriptional activity. In cells treated with 10 mg/ml of *T. procumbens* extract, LPS-induced NFκB transcriptional activity was comparable to NFκB transcriptional activity at basal level (Fig.2C).

Discussion

Tridax procumbens has been evaluated for antioxidant, anti-inflammatory, anti-diabetic, anti-hypertension, and anti-atherogenic properties with promising findings (Salahdeen 2004, Bhagwat 2008, Ikewuchi 2009, Pareek 2009, Jachak 2011). In this study, we report for the first time potential mechanisms mediating anti-hyperglycemic and anti-inflammatory properties of *T. procumbens.* Our studies have identified that *T. procumbens* activates AMPK and inhibits hepatic gluconeogenic gene expression of PEPCK and G6Pase with a concomitant reduction of hepatic glucose production in H4IIE rat hepatoma cells. Further, these studies have shown that *T. procumbens* extract inhibited LPS-stimulated NFκB and IκBα activation in RAW 264.7 murine macrophages. *T. procumbens* extract also suppressed LPS-stimulated NFκB transcriptional activity in an NFκB luciferase reporter HeLa cell line.

Investigations on phytochemical evaluation have revealed the presence of carotenoids, saponins, tannins, β-sitosterol, phenolics and flavonoids, including catechins, luteolin, glucoluteolin, flavones, glycoside, and quercetin (Yadava 1998, Ali 2001, Ali 2002, Edeoga 2005, Jude 2009, Muthusamy 2013, Desai 2014). Several plant derived polyphenols, including epigallocatechin-3-gallate (EGCG), resveratrol, quercetin and ferulic acid exert effects on liver function and glucose homeostasis (Hanhineva 2010, Roghani 2010). One of the mechanisms by which polyphenols mediate their hypoglycemic effects is by activation of the energy sensing protein complex, AMP-activated protein kinase (AMPK) (Collins 2007, Pandey 2009). Under normal physiological conditions, AMPK is activated in response to increase in AMP:ATP ratio during hypoxia, starvation, glucose deprivation or muscle contraction (Kahn 2005, Hardie 2012). *T. procumbens* extract demonstrated approximately 2-fold increase in AMPK (Thr172) phosphorylation, and increased phosphorylation of ACC (Ser 79), its downstream substrate.

Phosphorylation of ACC leads to its inactivation, and decreases malonyl CoA production and inhibits lipogenesis (Harada 2007).

Individuals with type 2 diabetes often exhibit fasting hyperglycemia associated with increased rate of hepatic gluconeogenesis. It has been shown that activation of AMPK represses the rate of expression of two key gluconeogenic genes, PEPCK and G6Pase in an insulin-independent manner (Lochhead 2000). Dexamethasone, a corticosteroid, induces gluconeogenesis in liver cells possibly by increasing phosphoenolpyruvate formation (Sistare 1985, Jones 1993). *T. procumbens* significantly suppressed dexamethasone-induced expression of PEPCK and G6Pase genes compared to cells treated with dexamethasone alone. Dexamethasone treatment has been shown to stimulate glucocorticoid receptor and Forkhead transcription factor FOXO1 binding to glucocorticoid response element (GRE) on proximal G6Pase promoter (Vander Kooi 2005). Induction of PEPCK and G6Pase genes is regulated by binding of cyclic-AMP response element (CRE) activation through binding of CRE-binding protein (CREB) (Herzig 2001, Vander Kooi 2005). Also, AMPK activation has been shown to promote TORC2 phosphorylation and inhibition of nuclear translocation thus contributing towards suppression of gluconeogenesis (Koo 2005, Lee 2010). Recent evidence suggests a role of GSK3β (Ser9) phosphorylation in suppression of CRE in the PEPCK promoter (Horike 2008). Further, using transcriptional profiling of AICAR-treated hepatocyte cell lines, the dual specificity phosphatase Dusp4 and the immediate early transcription factor Egr1 have been identified as transcriptional targets of AMPK that are necessary for its ability to fully repress glucose production (Berasi 2006). Since insulin plays a crucial role in regulating hepatic glucose production (Michael 2000), we evaluated *T. procumbens* effects on hepatic glucose production induced by dexamethasone. While insulin showed 79 ± 1.1% inhibition of dexamethasone-induced endogenous glucose production, *T. procumbens* extract (10 mg/ml)

inhibited HGP by 66 ± 12.5%. While the specific mechanisms need further elucidation, our data suggest that AMPK-mediated inhibition of gluconeogenic gene expression and a reduction of HGP may contribute to the anti-hyperglycemic effects of *T. procumbens* extract.

T. procumbens possesses anti-inflammatory potential as demonstrated by significant inhibition of carrageenan-induced rat paw edema (Awasthi 2009, Jachak 2011). Compounds isolated from *T. procumbens*, centaureidin and centaurein, are reported to exhibit anti-inflammatory properties through inhibition of COX-1 and COX-2; and bergenin is known to inhibit production of pro-inflammatory cytokines (IL-2, IFNγ, TNFα) (Jachak 2011). In our study, we observed that *T. procumbens* significantly inhibits the canonical NFκB signaling pathway by reducing LPS-induced phosphorylation of NFκB-p65 and IκBα. The ability of *T. procumbens* extract to suppress inflammation is also demonstrated by significant inhibition of NFκB transcriptional activity in the presence of LPS. NFκB plays a critical role in activation of pro-inflammatory cytokines, chemokines and cell adhesion molecules responsible for initiating inflammatory processes. Several studies in cell and animal models suggest that NFκB activation is a key event in the pathogenesis of type 2 diabetes (Patel 2009). Phosphorylation of Ser536 at the C-terminal transactivation domain (TAD) of NFκB p65 subunit has been shown to initiate its interaction with components of the basal transcription factor machinery to induce transcription of target genes (Buss 2004). It has been shown that systemic inflammation is often associated with impaired glucose metabolism in T2D. Compared to healthy subjects, patients with type 2 diabetes show increased NFκB binding activity and JNK phosphorylation following LPS treatment. Also, AMPK phosphorylation is compromised in inflammation-associated hyperglycemia (Andreasen 2011). Celastrol, a triterpene extracted from the medicinal plant *Triptergium wilfordii*, has been shown to inhbit NFκB resulting in an improvement of insulin resistance and lipid abnormalities in

db/db mice (Kim 2013). The inability of NFκB to initiate transcription of target genes would contribute to down-regulation of the inflammatory response. Increasing evidence suggest a role of AMPK in dampening the inflammatory response to stimuli such as LPS, TNFα and IL-6. In T2D, which is often marked by chronic low-grade inflammation, there is increased tissue macrophage infiltration contributing towards inflammation especially in the liver and adipose tissue (Johnson 2013). Macrophages activated with LPS acquire an inflammatory M1 phenotype, demonstrating enhanced production of pro-inflammatory cytokines and ROS (O'Neill 2013). It has been hypothesized that activation of AMPK switches macrophage polarization to the anti-inflammatory M2 phenotype, depressing the energy-demanding inflammatory response. Further, activation of AMPK has been shown to inhibit NFκB signaling by suppressing LPS-induced IκBα degradation (Salminen 2011, O'Neill 2013). The association between pathways regulating glucose disposal and NFκB pathway involved in inflammation suggests that there might be a synergistic mechanism operating behind the anti-hyperglycemic effect of *T. procumbens*.

In conclusion, we have shown that *T. procumbens* extract activates AMPK and suppresses hepatic gluconeogenic gene expression and endogenous glucose production, without direct effect on phosphorylation and activation of insulin signaling pathways mediated through Akt or MAPK (ERK1/2). The anti-inflammatory effects of *T. procumbens* are mediated, at least in part, via inhibition of canonical NFκB signaling pathway. Taken together, our current studies, while offering a mechanistic basis, lends support to the anti-hyperglycemic and anti-inflammatory properties of *T. procumbens* and suggest that *T. procumbens* may have a potential therapeutic application in the management of diabetes and other inflammatory disorders.

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Figure Legends

Fig.1. Molecular mechanisms mediating blood glucose lowering effect of *T. procumbens asava.* *T. procumbens asava* did not increase phosphorylation of Akt [A] and p44/42 MAPK [B]. Treatment with *T. procumbens asava* increased activation of AMPK [C] and ACC [D], and suppressed dexamethasone-induced gene expression of PEPCK [E] and G6Pase [F], and hepatic glucose production [G] in H4IIE rat hepatoma cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to basal; ## $p < 0.01$, ### $p < 0.001$, compared to dexamethasone.

Fig.2. Molecular mechanisms mediating anti-inflammatory effect of *T. procumbens asava*. *T. procumbens asava* inhibited LPS-induced phosphorylation of NFκB p65 [A] and IκBα [B] in murine macrophages. *T. procumbens* also inhibited LPS-induced transcriptional activity of NFκB in HeLa cells [C]. * $p < 0.05$, ** $p < 0.01$, compared to LPS; ## $p < 0.01$, compared to basal.

Figure 1

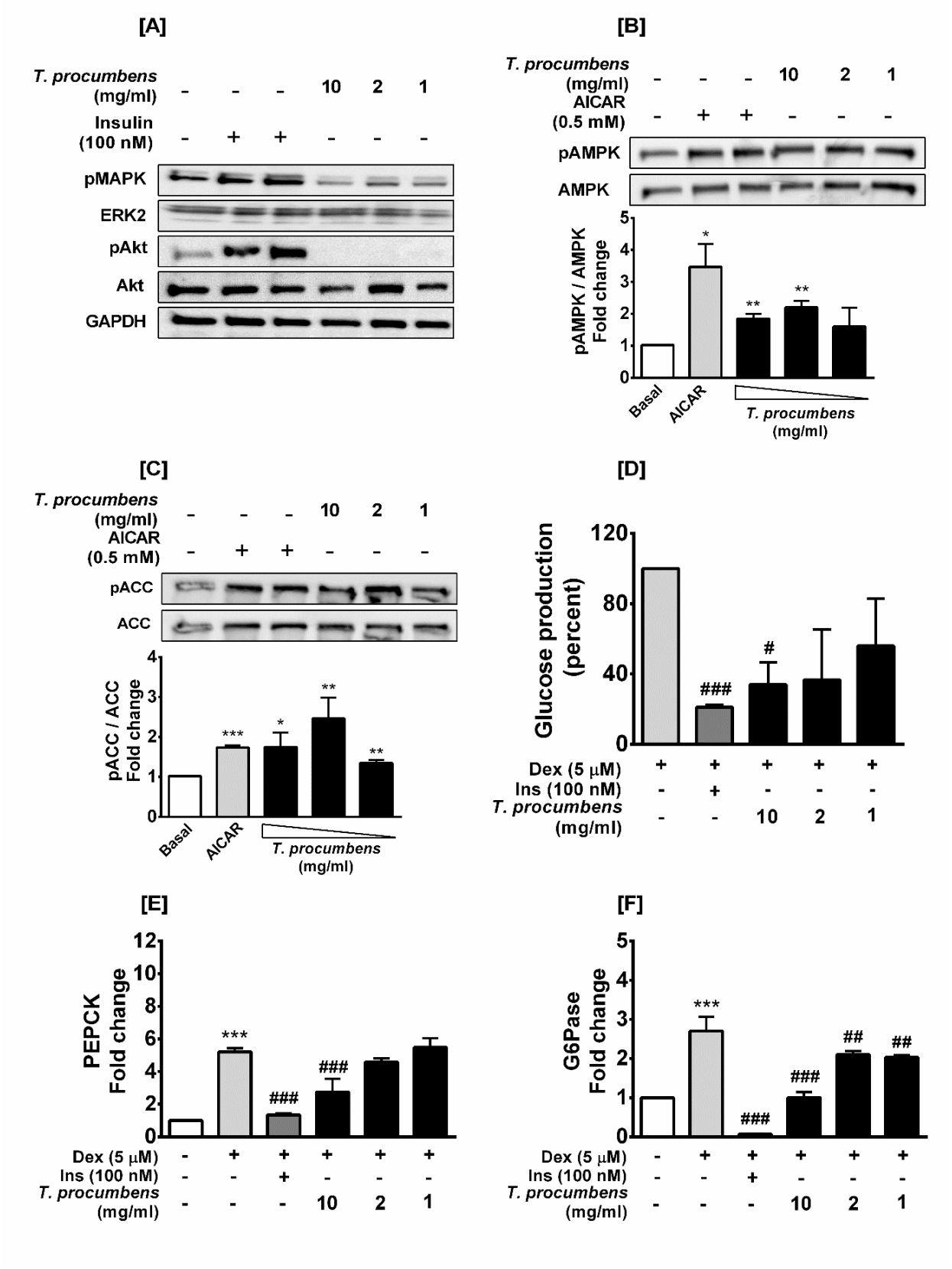
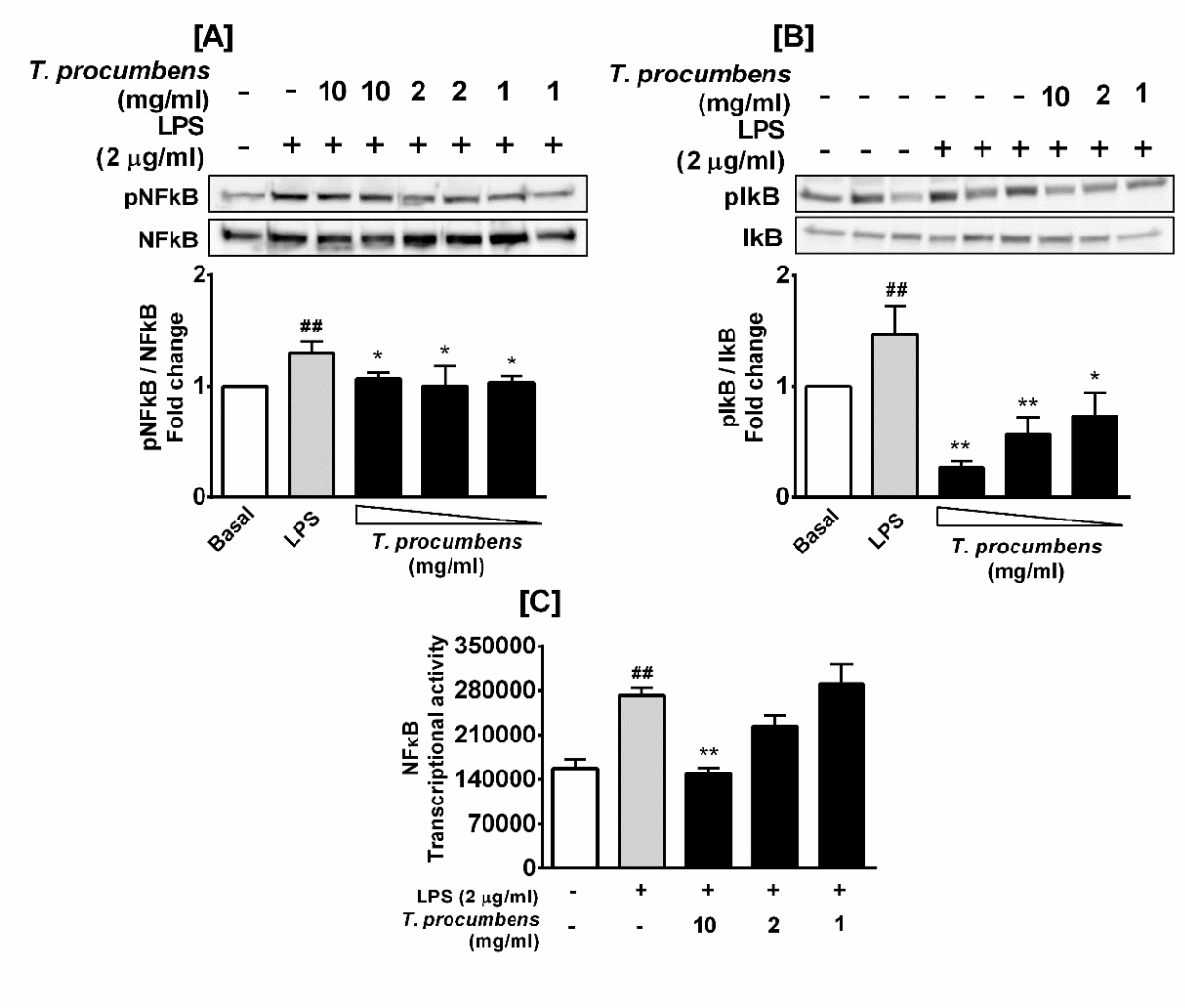

[A]
T. procumbens (mg/ml) - - - 10 2 1
Insulin (100 nM) - + + - - -
pMAPK
ERK2
pAkt
Akt
GAPDH
[B]
T. procumbens (mg/ml) - - - 10 2 1
AICAR (0.5 mM) - + + - - -
pAMPK
AMPK
pAMPK / AMPK Fold change
Basal
AICAR
T. procumbens (mg/ml)
[C]
T. procumbens (mg/ml) - - - 10 2 1
AICAR (0.5 mM) - + + - - -
pACC
ACC
pACC / ACC Fold change
Basal
AICAR
T. procumbens (mg/ml)
[D]
Glucose production (percent)
Dex (5 μM) + + + + +
Ins (100 nM) - + - - -
T. procumbens (mg/ml) - - 10 2 1
[E]
PEPCK Fold change
Dex (5 μM) - + + + + +
Ins (100 nM) - - + - - -
T. procumbens (mg/ml) - - - 10 2 1
[F]
G6Pase Fold change
Dex (5 μM) - + + + + +
Ins (100 nM) - - + - - -
T. procumbens (mg/ml) - - - 10 2 1

Figure 2


[A]
T. procumbens (mg/ml) - - 10 10 2 2 1 1
LPS (2 μg/ml) - + + + + + + +
pNFkB
NFkB
pNFkB / NFkB Fold change
Basal
LPS
T. procumbens (mg/ml)
[B]
T. procumbens (mg/ml) - - - - - - 10 2 1
LPS (2 μg/ml) - - - + + + + + +
pIkB
IkB
pIkB / IkB Fold change
Basal
LPS
T. procumbens (mg/ml)
[C]
NFκB Transcriptional activity
350000
280000
210000
140000
70000
0
LPS (2 μg/ml) - + + + +
T. procumbens (mg/ml) - - 10 2 1

Characterization of molecular mechanisms mediating anti-inflammatory and blood glucose lowering properties of *Woodfordia fruticosa* L. Kurz

Gauri S. Desai[1] and Suresh T. Mathews[1]*

[1] Department of Nutrition and Dietetics, Boshell Diabetes and Metabolic Diseases Research Program, Auburn University, Auburn, AL 36830

*Corresponding author:

Suresh T. Mathews, Ph.D, FACN
Associate Professor & Graduate Program Director
Department of Nutrition and Dietetics
260 Lem Morrison Dr., 101 PSB
Auburn University, AL 36849
Phone: 334-844-7418
Fax: 334-844-3268
Email: mathest@auburn.edu

Abstract

Woodfordia fruticosa L. Kurz has been a part of indigenous medicine in the Indian subcontinent and South East Asia. Pre-clinical studies have shown that *W. fruticosa* lowers blood glucose in alloxan- and streptozotocin-induced diabetes in rats and mice. *W. fruticosa* has also demonstrated effective anti-inflammatory potential in Wistar rats. The goal of this study was to characterize molecular mechanisms underlying the anti-inflammatory and blood glucose lowering effects of *W. fruticosa*. *W. fruticosa* demonstrated strong antioxidant reducing potential and ferrous-ion chelating effect. *W. fruticosa* significantly increased phosphorylation of AMPK on Thr172 ($p < 0.05$); and Akt (Ser473), but had no effect on p44/42 MAPK phosphorylation. W. fruiticosa suppressed dexamethasone-induced expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) genes, and significantly inhibited hepatic glucose production in H4IIE rat hepatoma cells. The anti-inflammatory potential of *W. fruticosa* was demonstrated by inhibition of NFκB-p65 and IκBα activation in LPS-stimulated RAW 264.7 murine macrophages. *W. fruticosa* also suppressed LPS-stimulated NFκB transcriptional activity in NFκB Luciferase Reporter HeLa cell line. Our studies indicate that *W. fruticosa* extract exhibits strong antioxidant potential and anti-inflammatory effect mediated by inhibition of NFκB pathway.Activation of Akt, and AMPK, together with suppression of gluconeogenic gene expression and hepatic glucose production, may be potential mechanisms that contribute to the glucose-lowering activity of *W. fruticosa*.

Introduction

Woodfordia fruticosa (L.) Kurz (syn. *Woodfordia fluoribunda* Salisb.), commonly known as Fire Flame bush, belongs to the family Lythraceae. It grows abundantly in the Western Ghats of Maharashtra State (India) and northern regions of Indian Himalayas ascending up to an altitude of 1500 m. *W. fruticosa* has also been found in regions of South East and Far East Asia, Sri Lanka, China, Japan, Pakistan and Africa (Das 2007, Baravalia 2011).

W. fruticosa is popularly known as *Dhataki* in Sanskrit, and the flowers are abundantly used as a common ingredient in fermented Ayurvedic preparations (Kroes 1993, Chaudhary 2011). Extracts of *W. fruticosa* flowers possess beneficial pharmacological properties and they have been used traditionally in parts of India to alleviate symptoms of skin infections, leprosy, dysentery, and blood disorders (Das 2007). (Baravalia 2012, Verma 2012, Bhatia 2013).

Phytochemical screening has shown presence of phenolics, flavonoids, sterols, and fatty alcohols in the stem, leaves and flowers of *W. fruticosa* (Chauhan 1979, Yoshida 1990). The non-phenolic constituents identified from *W. fruticosa* include sapogenin, hecogenin, and meso-inositol from flowers, and triterpenoids, lupeol, botulin, betulinic acid, oleanolic acid and ursolic acid from leaves (Das 2007).

The ethanolic extract of *W. fruticosa* flowers (250 and 500 mg/kg) has been shown to significantly reduce fasting blood glucose (40 and 69%, resp.) and improve insulin levels ($p < 0.001$) in streptozotocin-induced diabetic rats. The extract has also been shown to significantly increase glycolytic enzyme activity and decrease gluconeogenic enzyme activity in diabetic rats (Verma 2012). Methanolic extract of *W. fruticosa* flowers significantly lowered ($p < 0.001$) blood glucose in alloxan-induced diabetic rats, with at 100, 400, and 800 mg/kg body weight. The maximum effect was observed at 6 hour. Acute toxicity study showed that *W. fruticosa* did not

exert any visible symptoms of toxicity up to 1000 mg/kg (Bhatia 2013). Pre-clinical studies have also shown that methanolic extract of *W. fruticosa* flowers (400 mg/kg) significantly inhibited carrageenan-induced rat paw edema, with maximum inhibition (69%, $p < 0.01$) at the late phase. Further, the extract also inhibited formation of granuloma and reduced the frequency of formaldehyde-induced paw licking in Wistar rats. *W. fruticosa* has also been shown to inhibit inflammation induced by pro-inflammatory mediators including histamine, dextran, and serotonin (Baravalia 2012).

While *W. fruticosa* has been shown to be effective in lowering blood glucose and suppressing inflammatory processes in animal models, there are no studies characterizing the molecular mechanisms mediating anti-hyperglycemic and anti-inflammatory effects of *W. fruticosa*. In this study, we demonstrate that *W. fruticosa* activates AMPK, and inhibits hepatic gluconeogenic gene expression, and NFκB activation.

Materials and Methods

Reagents and antibodies

Ferrous chloride, and ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine), were obtained from VWR International, Radnor, PA. Potassium ferricyanide, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), α-tocopherol and trolox were obtained from Sigma (Sigma-Aldrich GmbH, St. Louis, MO). Recombinant human insulin was purchased from Roche Diagnostics (Indianapolis, IN); dexamethasone from Sigma-Aldrich (St. Louis, MO); antibodies against AMPK, pAMPKα(Thr172), Akt, pAkt(Ser473), ERK1/2, pMAPKp44/42 (Thr202/Tyr204), NFκB, pNFκB(Ser536), IκBα, and pIκBα(Ser32) from Cell Signaling Technology (Beverly, MA). Antibody against GAPDH was purchased from Abcam, Cambridge, MA. All other chemicals used were of analytical grade and were obtained from either Sigma-Aldrich or VWR International.

Preparation of *Woodfordia fruticosa* extract

Whole, mature flowers of *Woodfordia fruticosa* (L.). Kurz. *(*known as *Dhataki* in Sanskrit*)*, were obtained commercially from vendors in India. An extract of *W. fruticosa* was prepared following Ayurveda (a system of traditional Indian Medicine) guidelines. Whole flowers of *Woodfordia fruticosa* (L.). Kurz. (4 kg) were added to a stainless steel process vessel containing 100L of water and dissolved pharmaceutical-grade sugar (35 kg/100L). Contents in the closed process vessel were kept undisturbed for 45 days to allow completion of the natural fermentation process. The temperature of the process vessel was maintained between 30°-35°C using a water jacket. *W. fruticosa* flowers harbor a consortium of yeasts including *Saccharomyces cerevisiae* and *Rhodutorula mucilaginosa,* which served as the inoculum (Bhondave 2013). Following the

fermentation period, the *asava* was filtered using cheese-cloth, bottled, and sealed following Good Manufacturing Practices.

Cell culture

H4IIE rat hepatoma cells, HIRcB rat-1 fibroblasts overexpressing human insulin receptor and RAW 264.7 murine macrophages were purchased from the American Type Culture Collection (Manassas, VA, USA). NFκB Luciferase Reporter HeLa Stable Cell Line was purchased from Signosis (Santa Clara, CA). H4IIE cells were cultured in MEM-α, HIRcB and RAW 264.7 cells were grown in DMEM, high glucose supplemented with 10% FBS, 1% PSN and 1.25 µg/ml fungizone. All cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Determination of total phenolics, flavonoids and carotenoids

Total phenolics in *W. fruticosa* extract were measured by the Folin-Ciocalteau (FC) colorimetric assay using gallic acid standards and results were expressed as mean ± SD in mg/ml gallic acid equivalent (GAE) (Waterhouse 2001). Total flavonoid content was measured with the aluminium chloride colorimetric assay using quercetin standards (Marinova 2005). Total flavonoid content of *W. fruticosa* was expressed as mean ± SD in mg/l quercetin equivalent (QE).

Carotenoid content of *W. fruticosa* was determined by measuring absorbance of the extract at 450 nm (Scott 2001). Concentration of carotenoid, expressed as µg/ml, was calculated using the following equation: Carotenoid conc. = $[(A \times V_1)/A^{1\%}] \times C^{1\%}$ (where *A*: absorbance of sample, V_1: dilution factor, $A^{1\%}$: absorbance of 1% carotenoid solution (Extinction coefficient: 2500), $C^{1\%}$: concentration of a 1% carotenoid solution, 10 mg/ml).

Antioxidant activity

Sample preparation

For antioxidant activity assays, *W. fruticosa* extract dilutions 1:50, and 1:10, which correspond to 0.8 and 4 mg of dried *W. fruticosa*/ml were prepared in distilled water and compared with 45 µg/ml BHA, BHT, trolox and α-tocopherol. Results were expressed as mean ± SD.

Ferric ion reducing antioxidant power (FRAP)

FRAP assay was used to measure the antioxidant reducing potential of *W. fruticosa*, BHA, BHT, trolox and α-tocopherol (Akinmoladun 2007, Ak 2008, Habila 2010). Briefly, *W. fruticosa,* BHA, BHT, trolox and α-tocopherol were mixed with sodium 0.2 M phosphate buffer, pH 6.6 and 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min, and the absorbance was measured at 700 nm.

Ferrous ion chelating activity

Ferrous ion chelating activity of *W. fruticosa* was evaluated by the method of Dinis et al (Dinis 1994). Briefly, *W. fruticosa*, BHA, BHT, trolox and α-tocopherol were diluted with 70% ethanol, mixed with 2 mM $FeCl_2$ and 5 mM ferrozine and kept in the dark for 5 min. Absorbance was measured at 562 nm in a spectrophotometer (Ak 2008). Metal ion chelating activity was calculated using the following equation: Chelating effect (%) = (1 – As/Ac) x 100 (where *Ac*: absorbance of control, *As*: absorbance in presence of *W. fruticosa* or other antioxidants).

Western blot

Cells were washed with ice-cold PBS three times and lysed in 300 µl of cell lysis buffer [50 mM HEPES pH7.4, 100 mM Sodium pyrophosphate, 100 mM sodium fluride, 10 mM EDTA,

10 mM sodium orthovanadate, 2 mM PMSF, 1% Triton X-100 and protease inhibitor cocktail tablet (Roche diagnostics, Indianapolis)]. Complete cell lysis was ensured by sonication for 20 sec (pulse 05 sec, stop 03 sec) at 50% amp, followed by mechanical disruption on a rotating mixer at 4°C for 45 min. Cell pellet was separated by centrifugation at 4°C, 14000 rpm for 20 min. Pierce 660 nm protein assay reagent (Thermo Fischer Scientific Inc., Rockford, IL) was used to quantitate total protein. Equal quantities of cell lysate proteins were separated by 4-20% SDS-PAGE precast gels (Nu-Sep Inc., Austell, GA) and transferred to 0.45 µm nitrocellulose membrane (Bio-Rad Laboratories Inc., Berkeley, CA) at 12 V for 1 h. The membrane was blocked using 5% milk blocking buffer followed by incubation with primary antibodies against pAMPKα(Thr172), AMPK, pAkt(Ser473), Akt, pMAPK(p44/42), ERK1/2, pNFκB(Ser536), NFκB, pIκBα(Ser32), IκBα and GAPDH. The blots were then probed with respective horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies. Reactive bands were visualized using enhanced chemiluminescence detection system (Thermo Fischer Scientific Inc., Rockford, IL) and image analysis were performed with UVP-Biochimie Bioimager and LabWorks Software (UVP, Upland, CA).

Quantitative real-time PCR

H4IIE cells were grown to confluence in 6-well plates and starved for 6 hours using serum-free MEM-α supplemented with 0.1% bovine serum albumin and 1% penicillin/ streptomycin/ neomycin. Cells were then treated with combinations of dexamethasone (5 µM), insulin (100 nM) and *W. fruticosa* extract for 15 hours. Following incubation, cells were washed thrice with ice-cold PBS and collected using trypsin. RNA was extracted using RNeasy Mini RNA isolation kit (QIAGEN, Valencia, CA). cDNA was synthesized using iScript cDNA synthesis kit (BIO-RAD, Hercules, CA) and real-time PCR was performed using iQSYBR Green Supermix (BIO-RAD)

following manufacturer's instructions. Primer sequences for real-time PCR were as follows: phosphoenol pyruvate carboxy kinase (PEPCK) forward 5'-GGG TGC TAG ACT GGA TCT GC-3', PEPCK reverse 5'-GAG GGA GAA CAG CTG AGT GG-3'; glucose-6 phosphatase (G6Pase) forward 5'-GGG TGT AGA CCT CCT GTG GA-3', G6Pase reverse 5'- GAG CCA CTT GCT GAG TTT CC-3'; β-actin forward 5'-CCT CTA TGC CAA CAC AGT GC-3', β-actin reverse 5'-CAT CGT ACT CCT GCT TGC TG-3'. Reaction conditions were as follows: step 1: 95 °C, 3 min; step 2: 95 °C, 30s; 55 °C, 30s; 72 °C, 30s (step 2 is repeated 35x); step 3: 72 °C, 7 min; step 4: 4 °C, hold. Expression levels were normalized to β-actin and gene expression was calculated as $2^{-\Delta\Delta CT}$. All assays were carried out in triplicate.

Glucose production assay

Confluent 6-well plates of H4IIE cells were starved in serum-free MEM-α for 5 hours, including 1 hour pre-treatment with *W. fruticosa* extract, and incubated overnight with 5 μM dexamethasone. Insulin (100 nM) was used a positive control to demonstrate suppression of glucose production. Following incubation, cells were washed thrice with PBS and treated with glucose production media (glucose-free DMEM supplemented with 2 mM pyruvate and 20 mM sodium lactate), dexamethasone, *W. fruticosa* and insulin for 3 hours. Glucose production was measured using a Glucose UV Reagent (Cliniqa Corp., San Marcos, CA) and normalized to total protein content of whole-cell lysates.

Luciferase Reporter assay

HeLa cell line stably transfected using pTA-NFκB-luciferase reporter vector was purchased from Signosis, Inc. (Santa Clara, CA). Cells were seeded in a 96-well plate and incubated with LPS (2 μg/ml) for 1 hour at 37°C in the presence or absence of *W. fruticosa*.

Following incubation, cells were lysed and luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI).

Statistical analyses

Statistical analysis was performed using GraphPad Prism 6 (La Jolla, CA). A Student's two-tailed unpaired *t* test was used to determine statistical significance at $p < 0.05$. Data are expressed as mean ± SD.

Results

Antioxidant activity

Phytochemical analysis of *W. fruticosa dried flower* extract showed presence of phenolics, flavonoids and carotenoids. Total phenolic content of *W. fruticosa* dried flower extract was 16 ± 0.5 g GAE/L. The stem extract of *W. fruticosa* has been reported to have total phenolic content within the range of 0.12 – 0.4 g GAE/g (Shahwar 2012). The flavonoid and carotenoid content of *W. fruticosa* dried flower extract was 3.0 ± 0.09 mg QE/ ml and 6.8 ± 0.31 µg/ml respectively.

W. fruticosa dried flower extract showed a strong dose-dependent antioxidant reducing potential indicated by significantly high ferric ion reducing potential (Fig.1A). Our data demonstrate that *W. fruticosa* exerts significantly higher antioxidant reducing potential at concentrations ranging from 4 – 0.8 mg/ml, compared to 45 µg/ml BHA, BHT, Trolox, and α-T. *W. fruticosa* dried flower extract showed the highest antioxidant reducing potential at a concentration of 4 mg/ml. Next, we examined the ferrous ion chelating effect of *W. fruticosa*. Forty five micrograms per milliliter of BHA, BHT, Trolox, and α -T exhibited 10, 8, 8, and 9% ferrous ion chelating effect, respectively (Fig.1B). Dried flower extract of *W. fruticosa* at 4 and 0.8 mg/ml concentrations exhibited 64 and 75% ferrous ion-chelating effect. *W. fruticosa* showed the highest ferrous-ion chelating effect at the lowest concentration of 0.8 mg/ml. This indicates that 0.8 mg/ml *W. fruticosa* has significantly higher ferrous-ion chelating effect, compared to other antioxidant standards.

W. fruticosa activates Akt but has no effect on p44/42 MAPK phosphorylation

To evaluate the molecular mechanisms underlying the anti-hyperglycemic effect of *W. fruticosa*, we examined the effect of *W. fruticosa* extract on phosphorylation and activation of two

fundamental markers of insulin action, viz., Akt (Ser473) and p44/42 MAPK. In our study, *W. fruticosa* increased phosphorylation of Akt(Ser473) by 4-fold in HIRcB rat-1 fibroblasts, compared to basal. There was no effect on phosphorylation of p44/42 MAPK (Fig.2A).

W. fruticosa activates AMPK and suppresses dexamethasone-induced gluconeogenic gene expression

We evaluated the effect of *W. fruticosa* dried flower extract on AMPK activation and hepatic glucose production in H4IIE rat hepatoma cells. *W. fruticosa* significantly increased phosphorylation of AMPK (Thr172) at a concentration of 4 mg/ml, compared to basal (Fig.2B). AICAR (5-aminoimidazole-4-carboxamide riboside), 0.5 mM was used a positive control. AICAR demonstrated approximately 3.5-fold increase in AMPK phosphorylation, which was higher compared to *W. fruticosa*.

Further, we examined the effect of *W. fruticosa* dried flower extract on gluconeogenic gene expression and hepatic glucose production. Our studies showed that dexamethasone (5 µM) increased gene expression of PEPCK and G6Pase ~ 6.8- and 3.6-fold, respectively. *W. fruticosa* dried flower extract decreased gene expression dose-dependently, with significant inhibition ($p < 0.001$) compared to dexamethasone (Fig.2C,D). This finding corresponds with *W. fruticosa*-mediated increase in AMPK phosphorylation and the reported inhibition of gluconeogenesis by activation of AMPK (Berasi 2006).

Hepatic glucose production was also suppressed significantly with *W. fruticosa* treatment compared to dexamethasone (Fig.2E). Insulin, which was used as a positive control, inhibited dexamethasone-induced glucose production by approximately 80%. *W. fruticosa* inhibited dexamethasone-induced HGP by approximately 50%.

W. fruticosa inhibits NFκB activation

NFκB has been widely known for its role in initiating inflammatory processes. We investigated the effect of *W. fruticosa* on LPS-stimulated NFκB p65 (Ser536) and IκBα (Ser32) phosphorylation in RAW 264.7 murine macrophages (Fig.2A,B). In our study, LPS induced approximately 1.4-fold increase ($p < 0.01$) in phosphorylation of NFκB p65 at 2 µg/ml, compared to basal. *W. fruticosa* dried flower extract treatment significantly inhibited LPS-induced NFκB p65(Ser536) phosphorylation compared to cells treated with LPS alone. Interestingly, NFκB p65(Ser536) phosphorylation in cells pretreated with *W. fruticosa* concentrations 4, and 0.8 mg/ml was significantly lower ($p < 0.01$ and $p < 0.05$ respectively) compared to basal values. To further evaluate *W. fruticosa*-mediated NFκB inhibition, we examined the effect of *W. fruticosa* on NFκB upstream inhibitor IκBα. LPS increased phosphorylation of IκBα by 1.6-fold and *W. fruticosa* significantly inhibited LPS-induced IκBα(Ser32) phosphorylation compared to cells treated with LPS alone (Fig. 2B). The inhibition was dose-dependent with the highest inhibition at 4 mg/ml. Further, *W. fruticosa* treatment at 4, and 0.8 mg/ml significantly reduced IκBα phosphorylation ($p < 0.001$, $p < 0.01$ respectively) compared to basal levels.

W. fruticosa suppresses NFκB transcriptional activity

We evaluated the transcriptional activity of NFκB using the NFκB luciferase reporter HeLa cell line. This cell line is transfected with a vector containing 4 repeats of NFκB binding sites followed by the firefly luciferase coding region. In our study, LPS (2 µg/ml) significantly increased ($p < 0.01$) NFκB transcriptional activity compared to basal (Fig.2C). In the presence of *W. fruticosa*, there was a significant reduction in LPS-induced NFκB transcriptional activity at concentrations of 4, 0.8 and 0.4 mg/ml. Further, *W. fruticosa*-treated cells demonstrated significant reduction in LPS-induced NFκB transcriptional activity compared to basal levels.

Discussion

W. fruticosa is a major herb used for fermentation in Ayurvedic medicine, the traditional Indian System of Medicine; in particular, the dried flowers of *W. fruticosa* has been used in the preparation of asavas by slow fermentation process (Sekar and Mariappan 2008). Recently, several chemical compounds including tannins (especially those of macrocyclic hydrolysable class), flavonoids, anthraquinone glycosides, and polyphenols have been isolated from this species (Das 2007). Studies in animal models have demonstrated that *W. fruticosa* Kurz exerts anti-inflammatory and blood glucose lowering effects (Baravalia 2012, Verma 2012, Verma 2012, Bhatia 2013). Thus, it was of significant interest to characterize molecular mechanisms mediating blood glucose lowering and anti-inflammatory properties of *W. fruticosa*.

Phenolic compounds exhibit strong antioxidant potential and have been shown to be inversely associated with incidence of diabetes and cardiovascular diseases (Crozier 2009). Antioxidants have been studied for their protective effects against hyperglycemia-induced oxidative stress in diabetes (Rahimi 2005). *W. fruticosa* has been previously shown to exert antioxidant effects through increased antioxidant enzyme activity in thioacetamide-induced oxidative stress in rats (Nitha 2012). In our study *W. fruticosa* exhibited strong antioxidant activity, as indicated by high ferric-ion reducing potential and ferrous-ion chelating effect. It has been suggested that phenolic compounds exert modulatory effects on intracellular signaling cascades vital for growth, proliferation and apoptosis (Pandey and Rizvi 2009). Anti-diabetic effects of polyphenols may be mediated through different mechanisms, including inhibition of glucose absorption in the gastrointestinal tract, reduced glucose uptake by peripheral tissues, reduced carbohydrate absorption through inhibition of α-glucosidase, suppressed GLUT-1 mediated glucose transport, and enhanced glucose homeostasis (Pandey and Rizvi 2009). Polyphenols such

as resveratrol, apigenin, EGCG, and curcumin have been reported to activate AMPK through phosphorylation at Thr172 (Zang 2006, Collins 2007, Kim 2009). Several studies have shown that AMPK activation represses the rate of expression of two key gluconeogenic genes, PEPCK and G6Pase (Lochhead 2000). Both PEPCK and G6Pase gene expression are critical for gluconeogenesis, and it is tightly regulated in the presence of glucocorticoid stimulation. Dexamethasone, a corticosteroid, induces gluconeogenesis in liver cells possibly by increasing phosphoenolpyruvate formation (Sistare and Haynes 1985, Jones 1993). In our study, *W. fruticosa* demonstrated an ~1.3-fold increase in AMPK (Thr172) phosphorylation, and suppressed dexamethasone-induced gene expression of PEPCK and G6Pase. Further, *W. fruticosa* also suppressed hepatic glucose production in H4IIE rat hepatoma cells. We evaluated the effect of *W. fruticosa* on two key elements of the insulin signaling pathway, viz. Akt and p44/42 MAP kinase (MAPK). In this study *W. fruticosa* demonstrated a significant increase in Akt (Ser473) phosphorylation, without affecting p44/42 MAPK phosphorylation status, indicating that the effects on *W. fruticosa* on insulin signal transduction might be specific to the PI3-kinase-Akt pathway. These findings suggest that activation of Akt and AMPK, together with suppression of gluconeogenesis and hepatic glucose production may contribute towards the blood glucose lowering effects of *W. fruticosa*.

Type 2 diabetes is also marked by chronic low-grade inflammation, with increased tissue macrophage infiltration (Johnson and Olefsky 2013). The anti-inflammatory activity of several plant-derived compounds has been shown to be mediated by suppression of nuclear factor κB (NFκB) (Chen 2000, Singh 2002, Nakamura 2003). Luteolin, a plant flavonoid, has been shown to inhibit NFκB transcriptional activity in rat-1 fibroblasts, without inhibition of IκBα degradation, and NFκB nuclear translocation, suggesting a competitive inhibition for coactivator binding (Kim

2003). *W. fruticosa* leaf extract has previously been shown to contain flavonoids, including luteolin (20%), myricetin (9%), isoquercetin (3%), orientin (2%), and catechin (1.6%) (Khan 2012). In our study, *W. fruticosa* significantly inhibited LPS-induced canonical NFκB signaling pathway by suppressing IκBα degradation and NFκB p65 nuclear translocation. Further, *W. fruticosa* showed significant inhibition of LPS-induced NFκB transcriptional activity, contributing towards down-regulation of inflammatory processes.

In conclusion, this is the first study that offers a mechanistic support for the anti-inflammatory and blood glucose lowering effects of *W. fruticosa*. While we acknowledge that additional studies would be needed to further characterize the effects of *W. fruticosa* on insulin signaling, AMPK activation, and inhibition of NFkB signaling pathway, this study suggests that *W. fruticosa* may be useful in the management of diabetes and inflammatory disorders.

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Figure Legends

Fig.1. Antioxidant reducing potential of *W. fruticosa*. Ferric ion reducing potential [A], and ferrous-ion chelating effect [B], were determined. *** $p < 0.001$, compared to butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), trolox (Tro), and α-tocopherol (α -T).

Fig.2. Molecular mechanisms mediating blood glucose lowering effect of *W. fruticosa. W. fruticosa* increased phosphorylation of Akt and had no significant effect on MAPK phosphorylation [A]. *W. fruticosa* increased activation of AMPK [B], suppressed dexamethasone-induced expression of PEPCK [C] and G6Pase [D] genes, and inhibited hepatic glucose production [E] in H4IIE rat hepatoma cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to basal; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, compared to dexamethasone.

Fig.3. Molecular mechanisms mediating anti-inflammatory effect of *W. fruticosa*. *W. fruticosa* inhibited LPS-induced phosphorylation of NFκB p65 [A] and IκBα [B] in murine macrophages (Raw 264.7). *W. fruticosa* inhibited LPS-induced transcriptional activity of NFκB in HeLa cells [C]. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to LPS; # $p < 0.05$, ## $p < 0.01$, compared to basal.

Figure 1

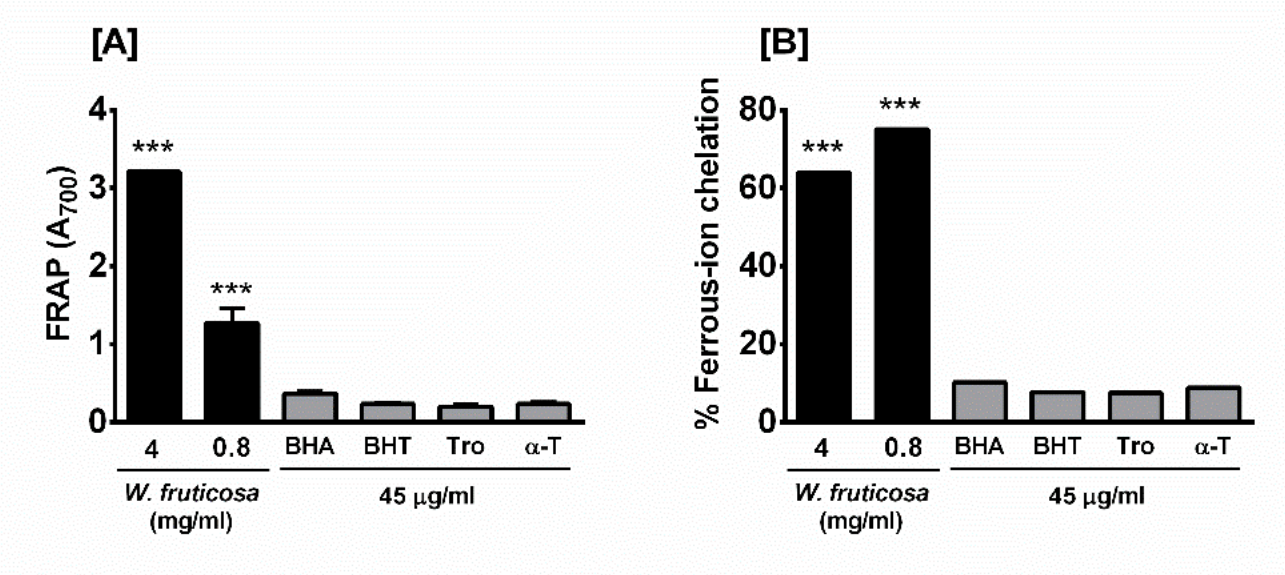

[A]
FRAP (A_{700})
4
3
2
1
0
***
***
4
0.8
BHA
BHT
Tro
α-T
W. fruticosa (mg/ml)
45 μg/ml
[B]
% Ferrous-ion chelation
80
60
40
20
0
***
***
4
0.8
BHA
BHT
Tro
α-T
W. fruticosa (mg/ml)
45 μg/ml

Figure 2

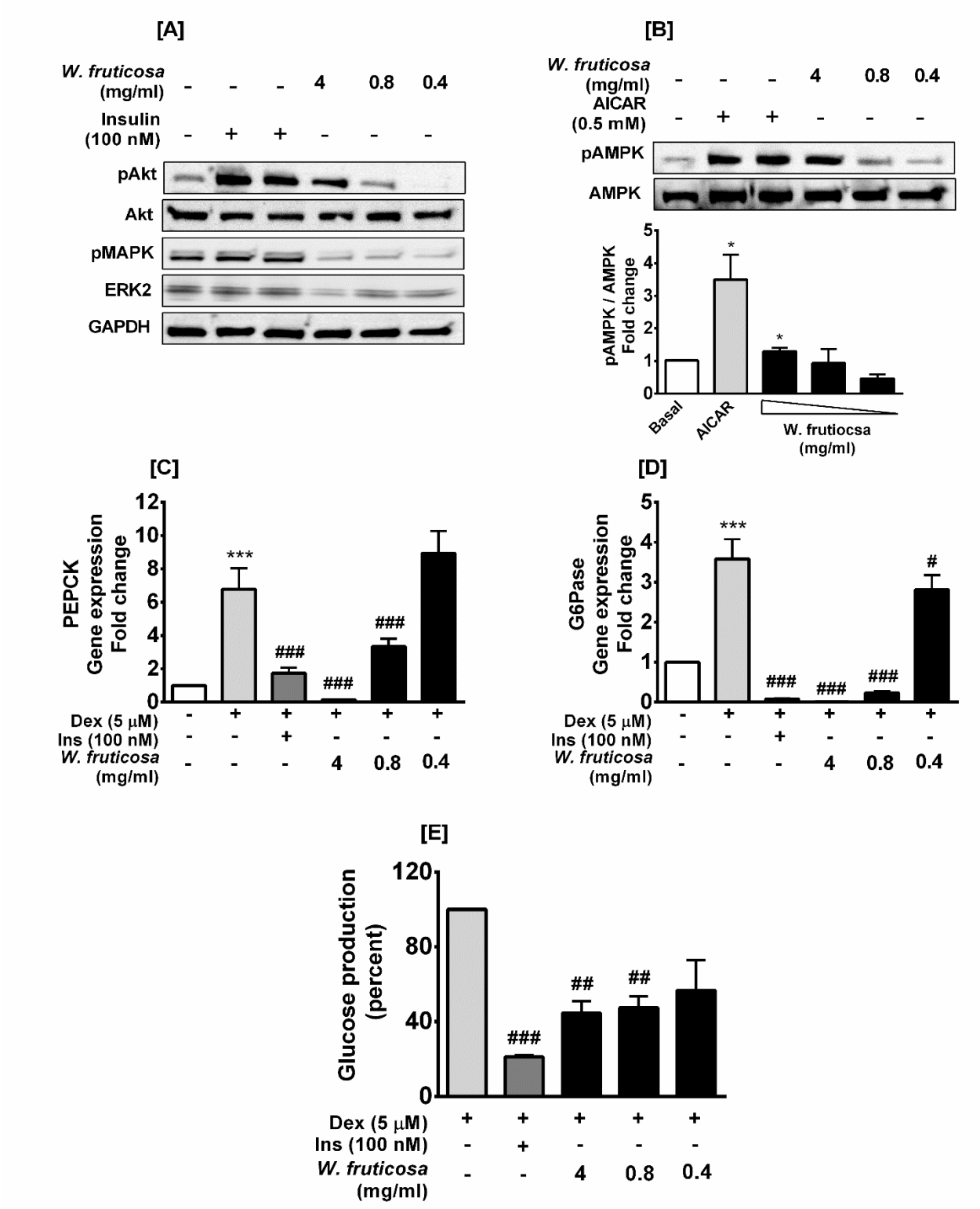
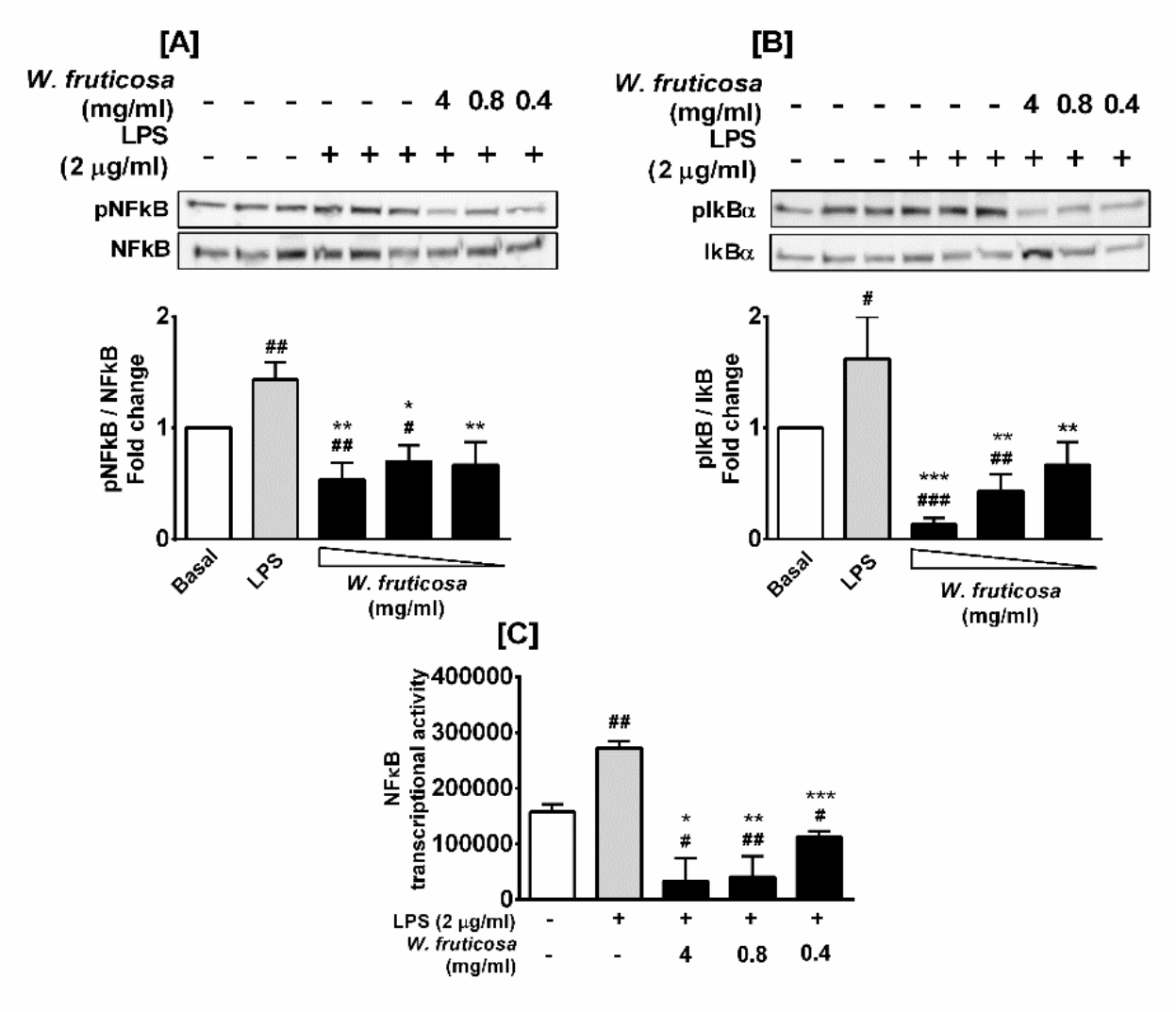

[A]
W. fruticosa (mg/ml) - - - 4 0.8 0.4
Insulin (100 nM) - + + - - -
pAkt
Akt
pMAPK
ERK2
GAPDH
[B]
W. fruticosa (mg/ml) - - - 4 0.8 0.4
AICAR (0.5 mM) - + + - - -
pAMPK
AMPK
pAMPK / AMPK Fold change
Basal
AICAR
W. frutiocsa (mg/ml)
[C]
PEPCK Gene expression Fold change
Dex (5 μM) - + + + + +
Ins (100 nM) - - + - - -
W. fruticosa (mg/ml) - - - 4 0.8 0.4
[D]
G6Pase Gene expression Fold change
Dex (5 μM) - + + + + +
Ins (100 nM) - - + - - -
W. fruticosa (mg/ml) - - - 4 0.8 0.4
[E]
Glucose production (percent)
Dex (5 μM) + + + + +
Ins (100 nM) - + - - -
W. fruticosa (mg/ml) - - 4 0.8 0.4

Figure 3


[A]
W. fruticosa (mg/ml) - - - - - - 4 0.8 0.4
LPS (2 μg/ml) - - - + + + + + +
pNFkB
NFkB
pNFkB / NFkB Fold change
Basal
LPS
W. fruticosa (mg/ml)
[B]
W. fruticosa (mg/ml) - - - - - - 4 0.8 0.4
LPS (2 μg/ml) - - - + + + + + +
pIkBα
IkBα
pIkB / IkB Fold change
Basal
LPS
W. fruticosa (mg/ml)
[C]
NFκB transcriptional activity
LPS (2 μg/ml) - + + + +
W. fruticosa (mg/ml) - - 4 0.8 0.4

Anti-hyperglycemic and cardio-protective effects of *T. procumbens* L.: A randomized, double-blind, placebo-controlled trial in individuals with type 2 diabetes

Gauri S. Desai[1,2,#], Shirish V. Desai[2,##], Vasundhara R. Borakhade[3§], Nedret Billor[4§§], Bin Wu[4§§], Ramchandra G. Kulkarni[2,##], Faiyaz S. Satvilkar[2,##] and Suresh T. Mathews[1]*

[1] Department of Nutrition and Dietetics, Boshell Diabetes and Metabolic Diseases Research Program, Auburn University, Auburn, AL 36830

[2] Bhagawati Industrial and Organic Products, Kolhapur, Maharashtra, India

[3]Yashwant Ayurvedic College, Department of Kayachikitsa, Kolhapur, Maharashtra, India

[4]Department of Mathematics and Statistics, Auburn University, Auburn, AL

[#]lead graduate student

[##]contributed to recruitment of subjects, sample collection

[§]physician for the study

[§§]contributed to statistical analyses

*principal investigator

*Corresponding author:

Suresh T. Mathews, Ph.D, FACN
Associate Professor & Graduate Program Director
Department of Nutrition and Dietetics
260 Lem Morrison Dr., 101 PSB
Auburn University, AL 36849
Phone: 334-844-7418
Fax: 334-844-3268
Email: mathest@auburn.edu

Abstract

Tridax procumbens L. has been shown to lower blood glucose, and exert hypotensive and cholesterol lowering effects in several animal studies. Previously, we have shown that a 4-week oral supplemenation of *T. procumbens asava* (hydro-alcoholic fermented infusion prepared following Ayurveda guidelines) significantly lowered fasting and post-prandial blood glucose in individuals with type 2 diabetes. In this study we evaluated blood glucose lowering and cardio-protective effects of *T. procumbens* in a randomized, double-blind, placebo-controlled clinical trial in individuals with established type 2 diabetes. Seventy five type 2 diabetic individuals were recruited and randomized into 3 groups, to receive 15 ml of *T. procumbens extract, W. fruiticosa extract* or a placebo, twice daily, for a period of 4 months, as supplement along with their prescribed anti-diabetic medications. Systolic blood pressure, total cholesterol, and LDL-cholesterol were significantly lowered with *T. procumbens supplementation* compared to placebo. No significant changes were observed in BMI, waist circumference, blood glucose, HbA1c, triglycerides, or HDL-cholesterol at the end of the study period. At the end of the study period, a higher prevalence of individuals with HbA1c < 7.5%, was observed in *T. procumbens* group compared to placebo. *T. procumbens* did not significantly alter biochemical markers of liver and kidney function including alanine aminotransferase (ALT), aspartate aminotransferase (ALT), urea, blood urea nitrogen, and serum creatinine. No adverse events or side effects were reported. *T. procumbens* supplementation may be beneficial in lowering cardio-metabolic risk in individuals with type 2 diabetes.

Introduction

Tridax procumbens Linn. (Family – Asteraceae), is a perennial weed and flowering plant, native to the tropical Americas. It is widespread throughout the tropical and sub-tropical regions of the world, and commonly inhabits uncultivated fields and wastelands. In the United States, *T. procumbens* has been introduced in the lower 48 States, Hawaii, Puerto Rico and the U.S. Virgin Islands, and is listed as a noxious weed by the United States Department of Agriculture (USDA NRCS 2014). *T. procumbens* is known in India and Africa, as part of indigenous medicine, for its hypotensive effects and has been used in the management of diabetes (Salahdeen 2004, Bhagwat 2008, Pareek 2009, Jain 2012).

Diabetes is one of the major causes of illness and premature deaths, with type 2 diabetes accounting for 90-95% of all diagnosed cases (CDC 2014). Type 2 diabetes has been associated with hypertension, and dyslipidemia, consisting of moderate elevation in triglycerides, low HDL cholesterol, and elevation in small dense LDL particles. The American Heart Association has identified diabetes to be a major risk factor for cardiovascular complications including coronary heart disease, stroke, peripheral arterial disease, nephropathy, retinopathy, and cardiomyopathy (Grundy 1999). Cardiovascular diseases contribute to more than 65% deaths in individuals with diabetes, with atherogenic/diabetic dyslipidemia and hypertension being well-established risk factors. Diabetic dyslipidemia is a well-recognized and modifiable risk factor to prevent cardiovascular diseases in individuals with type 2 diabetes (Solano MP 2006).

High blood pressure currently affects more than 72 million Americans, which is 1 in every 3 adults. High blood pressure, if not diagnosed or treated, can lead to life-threatening conditions, including heart attack, heart failure, stroke, and kidney failure. Normal blood pressure is defined by the American Heart Association as systolic < 120 mmHg and diastolic <

80 mmHg, with 140/90 considered as high blood pressure. Blood pressure between 120-139 systolic and 80-89 diastolic is considered "pre-hypertension" range, with increased risk of developing high blood pressure (Chobanian 2003).

Studies in animal models have demonstrated blood glucose lowering, anti-hypertensive and anti-dyslipidemic effects of *T. procumbens* (Salahdeen 2004, Bhagwat 2008, Ikewuchi and Ikewuchi 2009, Pareek 2009). *T. procumbens* whole plant extract (250 mg/kg) lowered fasting blood glucose by 68% at the end of 6 hours in alloxan-induced diabetic rats. At a higher dose of 500 mg/kg, fasting blood glucose was lowered by 71% in diabetic animals (Pareek 2009). The hypotensive effect of *T. procumbens* was demonstrated in normal Sprague-Dawley rats. *T. procumbens* reduced mean arterial blood pressure (MABP) in a dose-dependent mannerwith highest reduction in MABP (44.5 ± 5.7 mm Hg) observed at 9 mg/kg. Aqueous extract of *T. procumbens* also reduced heart rates of rats at higher dose of 6 mg/kg and 9 mg/kg (Salahdeen 2004). *T. procumbens* leaf extract (20 mg/kg) has been shown to significantly improve plasma lipid profile in cholesterol-loaded rats. *T. procumbens* significantly lowered plasma triglycerides, total cholesterol, LDL-cholesterol, and VLDL-cholesterol, while HDL-cholesterol values were significantly higher (2.49 mmol/L) in the experimental group compared to control (Ikewuchi 2009).

A pilot clinical study in individuals with type 2 diabetes demonstrated that *T. procumbens* supplementation significantly reducted fasting (11% in men, 20% women) and 2-hour post-prandial blood glucose (26% in men, 29% in women) (Desai 2014). To the best of our knowledge, there are no randomized clinical trials evaluating the effect of *T. procumbens* on glycemic control and cardio-metabolic risk factors. In this study, we evaluated the blood glucose

lowering and cardio-protective effects of *T. procumbens* in a randomized, double-blind, placebo-controlled trial.

Subjects and Methods

Subjects

This study was approved by the Kolhapur Independent Ethics Committee (KIEC), Kolhapur, India. Participants were Asians from the Indian subcontinent, Maharashtra State, Kolhapur district in India, between the ages of 30 and 80 years. Eligibility requirements included willingness and ability of the participants to provide written informed consent, and having established type 2 diabetes for at least 3 years before enrollment in the study. Eligible participants could be using conventional oral anti-hyperglycemic agents. Pregnant women, lactating mothers, and patients on insulin therapy for diabetes management were excluded from the study. Study participants were recruited through free medical check-up camps organized jointly by the Kayachikitsa Department, Yashwant Ayurvedic College and Bhagawati Industrial & Organic Products at Mangale, Savarde, Tandulwadi, Kodoli, Minche and Ghunki villages in Kolhapur district. Written informed consent was received from all participants before the start of the study.

Plant Material

Tridax procumbens Linn. plants (whole plants) were collected from the town of Athani, Belgavi district, Karnataka State, India following Good Agricultural Practices for medicinal plants (World Health Organization 2003). *Tridax procumbens* Linn. was authenticated by Aditi Organic Certifications, Bangalore, India Whole, mature flowers of *Woodfordia fruticosa* (L.) Kurz. *(*known as *Dhataki* in Sanskrit*)*, a common ingredient of fermented Ayurvedic preparations, were obtained commercially from local vendors (Chaudhary 2011).

Preparation of *asava* and placebo

An extract of *T. procumbens* was prepared following Ayurveda (a system of traditional Indian Medicine) guidelines, hitherto referred to as *T. procumbens asava* (the word *asava* in Ayurveda means 'fermented infusion'). *T. procumbens* L. whole plants were initially rinsed in running water, shade dried and then coarsely powdered. The powdered *T. procumbens* plant material (10 kg) and dried, whole flowers of *Woodfordia fruticosa* (L.). Kurz. (4 kg) were added to a stainless steel process vessel containing 100 L of potable water and dissolved pharmaceutical-grade sugar (35 kg/100L). *W. fruticosa* flowers harbor wild species of yeast in the dry nectariferous region, which served as the inoculum (Sekar 2008). Contents in the closed process vessel were kept undisturbed for 45 days to allow completion of the natural fermentation process. The temperature of the process vessel was maintained between 30°-35° C using a water jacket. Following the fermentation period, the *asava* was filtered using cheese-cloth, bottled, and sealed following Good Manufacturing Practices. Similarly, an asava of *W. fruticosa* was prepared as above, without *T. procumbens* plant material to serve as control. A placebo was prepared without *T. procumbens* and *W. fruticosa*, using water and natural caramel color to resemble an *asava* in appearance.

Study design and intervention

This was a 4-month, randomized, double-blind, placebo-controlled intervention trial comparing the effects of *T. procumbens asava, W. fruticosa asava,* or placebo on blood glucose and cardio-metabolic risk factors in individuals with type 2 diabetes. Participants (n = 75) were randomly assigned to either *T. procumbens, W. fruiticosa*, or placebo group for the duration of the study. A Randomized Block Design was used to create a randomization sequence to ensure equal number of participants (n = 25) in each of the 3 study groups.

Participants received an oral dose of 15 ml *T. procumbens asava* or *W. fruticosa asava* or placebo, twice a day, for 4 months as a supplement to their prescribed anti-diabetic medications. Participants were asked to continue their normal diet and physical activity patterns. Fasting and 2-hour post-prandial blood samples were obtained at the beginning, at the 2-month time point and at the end of the 4-month study period. BMI, waist circumference, blood pressure and a 3-day diet log was recorded at each visit. Diet analysis was conducted using Food Processor 10.13 software, ESHA Research Inc, Salem, OR. Glucose, HbA1c, total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, ALT, AST, urea, and creatinine, were assayed in a clinical laboratory. To assess intervention compliance and dose validation, participants returned any unused *asava*/placebo at the end of the study period.

Statistical Analysis

Data were analyzed using a two-way repeated-measures analysis of variance, followed by Tukey's test for pairwise comparisons within and between experimental groups. Mauchly's Sphericity test was used to evaluate variances of differences between the 3 study groups. Violation of sphericity was considered at $p < 0.05$, and the Greenhouse-Geisser Epsilon correction was used. Baseline characteristics between groups were analyzed using one-way analysis of variance, followed by Tukey's multiple comparison test. Statistical analyses were performed using GraphPad Prism 6 (La Jolla, CA). Data are expressed as mean ± SD.

Results

In this study, we recruited 75 individuals with established type 2 diabetes. The average age of the study population was 61 ± 11 years. Participants were randomized into one of the three study groups at the beginning of the study such that each study group had 25 participants. Of the 75 enrolled patients, 22 patients from the *T. procumbens* group, 21 patients from the *W. fruticosa* group and 22 patients from the placebo group completed the study. Ten individuals withdrew from the study because of unrelated injury/illness (n = 3), reluctance to provide blood samples according to the study protocol (n = 3), or relocation (n = 4). No adverse events were reported. Five individuals (*W. fruticosa:* n = 2, placebo: n = 3,) did not receive any conventional treatment for diabetes from the start of the study, and 6 individuals (*T. procumbens:* n = 1, *W. fruticosa:* n = 2, and placebo: n = 3,) discontinued use of their anti-diabetic medications during the 4 month study period. Baseline characteristics were not significantly different between the groups (Table 1). At the end of the study, mean total calorie intake was not significantly different in *T. procumbens* (Pre: 1621 ± 332 cal vs. 4-month: 1701 ± 414 cal), *W. fruticosa* (Pre: 1779 ± 427 cal vs. 4-month: 1789 ± 359 cal) and placebo (Pre: 1647 ± 463 cal vs. 4-month: 1759 ± 394 cal) groups. Similarly, no significant differences were observed between groups.

Glycemic control

At the end of 4-month, there were no significant differences in both fasting and 2-hour post-prandial blood glucose concentrations between the 3 groups (Table 2). Fasting blood glucose increased significantly from 138 ± 45 mg/dl at baseline to 157 ± 43 mg/dl ($p < 0.01$) at 4-month, in *T. procumbens* group. Similar increase in fasting glucose was also observed in placebo (Pre: 144 ± 48 mg/dl vs. 4-month: 177 ± 69 mg/dl) and *W. fruticosa* (Pre: 121 ± 37

mg/dl vs 4-month: 152 ± 41 mg/dl) groups. No significant changes were observed in 2-hour post-prandial blood glucose in all 3 groups.

In *T. procumbens* group, HbA1c tended to increase from 7.1 ± 0.8% at baseline to 7.5 ± 0.9% at 2-month ($p = 0.06$). At 4-month, HbA1c tended to decrease to 7.1 ± 1.2% ($p = 0.07$) compared to 2-month. There was a significant increase ($p < 0.05$) in HbA1c in *W. fruticosa* group at 4-month compared to baseline. No changes in HbA1c were observed in placebo group. At the end of the study period, prevalence of individuals with HbA1c < 7.5% was highest in *T. procumbens* group (Pre: 77% vs Post: 73%), followed by *W. fruticosa* (Pre: 76% vs Post: 67%) and placebo (Pre: 64% vs Post: 41%) (Figure 2).

There were no significant changes in body mass index (BMI) within the study groups. Waist circumference was reduced by 5% in placebo group ($p < 0.001$), and 3% in *T. procumbens* group ($p < 0.01$). Waist circumference in *W. fruticosa* group tended to decrease ($p = 0.059$) by 2%. The percent change in waist circumference was not statistically significant between groups (data not shown).

Cardio-metabolic risk factors

Mean systolic blood pressure at baseline was in the prehypertension range in all 3 groups (Table 2). Supplementation of *T. procumbens* asava significantly lowered SBP at the 2-month and 4-month time points (6.7% reduction at 4-month time point) (Figure 2). Supplementation with *W. fruticosa* asava demonstrated a 5.2% reduction in SBP ($p < 0.01$) at the 2-month time-point, but this effect was reversed at the end of the 4-month time-point. No changes were observed in diastolic blood pressure in any of the 3 groups.

Serum total cholesterol decreased significantly in all the study groups compared to their respective pre-treatment values. *T. procumbens* lowered total cholesterol by 20.0%, which was significantly higher ($p < 0.001$) compared to 9.8% reduction in placebo group and 12.1% reduction in *W. fruticosa* group (Figure 2). Serum triglycerides were also significantly lowered in both placebo (14.5%) and *T. procumbens* (18.5%) groups (Table 2).

LDL-cholesterol was significantly lowered ($p < 0.001$) in *T. procumbens* group (25.1% reduction in LDL-cholesterol at 4-month compared to pre-treatment). LDL-cholesterol tended to decrease ($p = 0.0667$) in the placebo group (8.7% reduction in LDL-cholesterol at 4-month compared to pre-treatment). The 25.1% change in LDL-cholesterol in *T. procumbens* group was significantly different ($p < 0.05$) compared to the 8.7% change in placebo group (Figure 2). The total/HDL cholesterol ratio (TC:HDL) was significantly lower in both *T. procumbens* ($p < 0.001$) and *W. fruticosa* ($p < 0.05$) groups compared to baseline. TC:HDL was reduced by 20.2% in *T. procumbens* group and by 8.6% in *W. fruticosa* group ($p = 0.07$). The LDL/HDL cholesterol (LDL:HDL) ratio was also lowered significantly in *T. procumbens* group (25%). No changes were observed in HDL-cholesterol at the end of the study period. None of the groups lowered HDL-c and the mean HDL-cholesterol in all 3 groups was maintained at HDL-c > 40 mg/dl (Table 2).

Markers of liver and kidney function

There were no significant differences in ALT and AST levels post-treatment compared to pre-treatment values in all 3 groups (Table 3). At 4-month, ALT was within the normal range of 10-40 IU/L for both placebo (37 ± 19 IU/L) and *T. procumbens* (36 ± 16 IU/L). *W. fruticosa* group had a mean ALT of 43 ± 16 IU/L.

Blood urea and blood urea nitrogen (BUN) were within their normal ranges of 20-40 mg/dl and 6-20 mg/ml, respectively, at both pre- and post-treatment in placebo, *W. fruticosa* and *T. procumbens* groups. No significant changes were observed in serum creatinine values, which were within the normal range of 1.0-2.0 mg/dl for all 3 groups. The creatinine clearance rate did not increase significantly with time and was within the normal range of 90-120 ml/min in all study groups.

Discussion

This study is the first report of a randomized, double-blind, placebo-controlled clinical trial to evaluate blood glucose lowering and cardio-protective effects of *T. procumbens*. In our study, there were no significant reductions in either fasting or 2-hour post-prandial blood glucose, or HbA1c in any of the study groups. The prevalence of HbA1c ≤ 7.5% at the end of 4 months was highest in *T. procumbens* group, compared to both placebo and *W. fruticosa*, suggesting modest effects on HbA1c control. HbA1c levels, both pre- and post-treatment, correlated significantly with fasting and 2-hour post-prandial blood glucose. However, the percent change in HbA1c at the end of 4 months did not correlate with percent change in blood glucose. Further, HbA1c was not correlated with BMI, waist circumference or age in any of the study groups. There were significant reductions in waist circumference at the end of 4 months, with no significant changes in body weight or diet of study participants. While the reduction in waist circumference was significant between pre- and post-treatment for *T. procumbens* and placebo, the percent change in waist circumference between these two groups was not statistically significant.

T. procumbens has been shown to reduce blood pressure and heart rate in both normotensive and sub-chronic salt-loaded rats (Salahdeen 2004, Ikewuchi 2011). In our study, supplementation with *T. procumbens* lowered systolic blood pressure by 7% at the 4-month time point. The diastolic blood pressure was close to normal, and was not further reduced by *T. procumbens supplementation*. There were no significant changes in either SBP or DBP in both placebo and *W. fruticosa* groups, suggesting that the blood pressure lowering effect may be attributed to *T. procumbens*. It has been shown that *T. procumbens* mediates vasorelaxant effects on rat vascular smooth muscle through attenuation of contractile responses to KCl (Salahdeen

and Murtala 2012). It has also been demonstrated that *T. procumbens* may have calcium antagonistic potential, suggesting that *T. procumbens* may facilitate non-specific, non-competitive inhibition of Ca^{2+} influx and inhibition of Ca^{2+} mobilization from intracellular stores to exert relaxant effects on smooth muscles (Salahdeen 2014).

Diabetic dyslipidemia is one of the major risk factors for cardiovascular diseases (Mooradian 2009). The National Cholesterol Education Program has identified elevated serum LDL-cholesterol (> 100 mg/dl) as a major cause of coronary heart disease, with significant increase in atherogenesis even at borderline high (130-159 mg/dl) levels (NCEP 2002). *T. procumbens* has been shown to significantly lower plasma concentrations of triglycerides, LDL-, VLDL- and total cholesterol in cholesterol-loaded normal rats, and alloxan-induced diabetic rats (Ikewuchi and Ikewuchi 2009, Ikewuchi 2012). In our study, *T. procumbens* significantly lowered total cholesterol, LDL-cholesterol, and TC:HDL ratio or atherogenic index, compared to placebo and *W. fruticosa*. Triglycerides were lowered by 19% in *T. procumbens* group, which was significant compared to the pre-treatment values. However, we observed 15% reduction in the placebo group, and the percent reduction was not significant between placebo and *T. procumbens*, suggesting a potential placebo-effect.

A few studies have examined the toxicity of *T. procumbens* in Wistar rats. In acute studies, no fatal consequences or visible symptoms of toxicity were observed with administration of a methanolic extract of *T. procumbens* up to a dose of 5 g/kg body weight (Pareek 2009). Further, short-term toxicity studies using ethyl actetate extract of *T. procumbens* (50, 100, 200, 400 and 800 mg/kg) for 14 days have demonstrated increased body weight and percent organ/body weight ratio for liver and spleen in a dose-dependent manner (Abubakar 2012). In DGal-N/LPS-induced hepatotoxicity and liver damage, administration of *T. procumbens* restored

elevated serum alanine aminotransferase and aspartate transaminase, bilirubin and lipids to normal levels (Ravikumar 2005). Abubakar et al. showed that the LD_{50} of the ethyl acetate extract of *T. procumbens* was 2100 mg/kg body weight. In our study, 15 ml of *T. procumbens asava* was administered twice daily, which gave a calculated dose of approximately 50 mg *T. procumbens*/kg body weight. At the end of 4 months, there were no changes in ALT and AST activity, compared to pre-treatment in *T. procumbens* group ALT was within the normal range of 10-40 IU/L for both placebo and *T. procumbens* groups, with close to normal values in *W. fruticosa* group. AST values were also within the normal range of 10 – 34 IU/L for all 3 groups. Blood urea nitrogen and serum creatinine values are commonly used in clinical practice to determine renal function (Lyman 1986). In our study, BUN and serum creatinine were within their normal range for all study participants. The results of biochemical assays for markers of liver and kidney function indicate that *T. procumbens* did not exert toxic effects on liver and kidney function during the 4-month study period.

The results of our previous pilot clinical study had demonstrated significant reduction in both fasting and 2-hour post-prandial blood glucose following 4-week *T. procumbens asava* supplementation in individuals with type 2 diabetes (Desai 2014). In this study, we did not observe a significant blood glucose lowering effect for fasting or 2-hour post-prandial blood glucose following 4 months of *T. procumbens* supplementation. This may have been due to the extended study duration, differences in *T.procumbens* source and batch, and/or sample size.

The current study had important strengths. To our knowledge, this is the first randomized, double-blind, placebo-controlled clinical report evaluating blood glucose lowering and cardio-protective effects of *T. procumbens asava*. The inclusion criteria were fairly broad to include most type 2 diabetic patients on oral medications. Our study had several limitations including

small sample size, homogenous sample with study participants predominantly adults of Indian origin, and study population recruited from a small geographic area. Further, the small sample size limited our ability to analyze gender-based differences. In this study, blood samples were not obtained at the 1-month time point. Therefore, the findings of this study could not be compared with the results of our previous pilot clinical study. In this study, we measured the effect of a single dose of *T. procumbens asava*. Future research with *T. procumbens* can be directed towards optimization of the effective dose for glycemic control. A major finding of this study was that *T. procumbens asava* significantly reduced systolic blood pressure, total cholesterol, LDL-cholesterol, and atherogenic index, while maintaining HDL-cholesterol within normal range. These parameters are considered critical in diabetes management and are identified risk factors for cardiovascular diseases.

In conclusion, the present study suggests that *T. procumbens asava* is effective in improving cardio-metabolic health by lowering cardio-metabolic risk factors including dyslipidemia, total cholesterol/HDL cholesterol ratio, LDL/HDL cholesterol ratio and hypertension, when used in a complementary approach. *T. procumbens* has demonstrated significant anti-hyperglycemic effect in animal models of diabetes, and in an open-label pilot clinical study. However, additional research is needed to evaluate blood glucose lowering effects of *T. procumbens* in human subjects.

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Figure Legends

Fig 1. Flow diagram of the study. We recruited 75 individuals with established type 2 diabetes and randomly assigned them to one of the three study groups. Out of 75 participants, 65 completed the study. Ten participants dropped out for reasons mentioned in the figure.

Fig 2. Glycemic control and cardio-protective effects of *T. procumbens*. Seventy five type 2 diabetic patients were randomly assigned to placebo, *W. fruticosa* or *T. procumbens* groups. Participants were supplemented with 15 ml asava/placebo, twice daily, for 4 months. Prevalence of HbA1c $\leq$ 7.5% following 4 month supplementation is depicted [A]. Percent change in systolic blood pressure [B], total cholesterol [C] and LDL-cholesterol [D] was significant in *T. procumbens* group compared to placebo. # $p < 0.05$ compared to placebo. *T. pro: T. procumbens*.

Table 1: Baseline characteristics of study population

Characteristic	**Placebo (n=22)**	***W. fruticosa* (n=21)**	***T. procumbens* (n=22)**	**P value**
Age (years)	61 ± 12	61 ± 12	59 ± 11	0.808
Men (n)	12	17	12	
Women (n)	10	4	10	
Body weight (kg)	60 ± 14	68 ± 12	67 ± 14	0.108
BMI (kg/m^2)	24.1 ± 4.1	25.6 ± 4.7	27.4 ± 5.2	0.072
Waist circumference (cm)	89 ± 11	93 ± 11	92 ± 12	0.486
SBP (mmHg)	133 ± 25	134 ± 23	138 ± 23	0.761
DBP (mmHg)	81 ± 12	83 ± 12	83 ± 10	0.798
FPG (mg/dl)	144 ± 48	121 ± 37	138 ± 45	0.211
2hPG (mg/dl)	251 ± 102	193 ± 80	211 ± 80	0.093
HbA1c (%)	7.3 ± 1.3	6.7 ± 1.0	7.1 ± 0.8	0.174
Cholesterol (mg/dl)	211 ± 48	220 ± 44	236 ± 45	0.193
Triglycerides (mg/dl)	182 ± 44	171 ± 49	190 ± 45	0.403
LDL (mg/dl)	141 ± 51	135 ± 51	155 ± 42	0.251
HDL (mg/dl)	44 ± 3	43 ± 4	43 ± 4	0.589
Urea (mg/dl)	30 ± 10	29 ± 9	29 ± 10	0.925
BUN	14.2 ± 4.6	13.4 ± 4.4	13.4 ± 4.8	0.803
Creatinine (mg/dl)	1.1 ± 0.3	1.1 ± 0.2	1.1 ± 0.2	0.373
CCr (ml/min)	51 ± 16**	52 ± 11**	68 ± 20	0.001
AST (IU/L)	31 ± 8	32 ± 8	37 ± 18	0.233
ALT (IU/L)	39 ± 10	40 ± 9	42 ± 12	0.626

Data are expressed as Mean ± SD. ** $p < 0.01$, compared to *T. procumbens*.

Table 2: Blood glucose and cardiovascular health factors

Characteristic	Placebo			*W. fruticosa*			*T. procumbens*		
	Pre	Post		Pre	Post	Post	Pre	Post	Post
		2-mo	4-mo		2-mo	4-mo		2-mo	4-mo
Waist cir (cm)	89 ± 11	87 ± 12*	84 ±11***	93 ± 12	93 ± 10	91 ± 11	92 ± 12	90 ± 12	89 ± 11**
BMI (kg/m^2)	24.1 ± 4.1	23.9 ± 4.3	24.0 ± 4.1	25.6 ± 4.7	25.2 ± 4.9	26.0 ± 4.6	27.4 ± 5.2	27.3 ± 5.3	27.5 ± 5.2
SBP (mmHg)	133 ± 25	131 ± 19	129 ± 27	134 ± 23	127 ± 25**	136 ± 29	138 ± 23	131 ± 22*	127 ± 18*
DBP (mmHg)	81 ± 12	79 ± 11	80 ± 11	83 ± 12	83 ± 13	85 ± 14	83 ± 10	80 ± 11	81 ± 10
FPG (mg/dl)	144 ± 48	187 ± 93*	177 ± 69**	121 ± 37	146 ± 57*	152 ± 41	138 ± 45	160 ± 64*	157 ± 43**
2hPG (mg/dl)	251 ± 102	296 ± 129*	286 ± 124	193 ± 80	242 ± 118**	221 ± 82	211 ± 80	270 ± 101**	237 ± 96
HbA1c (%)	7.3 ± 1.3	7.5 ± 1.2	7.6 ± 1.4	6.7 ± 1.0	7.1 ± 0.7	7.2 ± 1.0*	7.1 ± 0.8	7.5 ± 0.9	7.1 ± 1.2
Cholesterol (mg/dl)	211 ± 48	225 ± 56	187 ± 38*	220 ± 44	220 ± 44	187 ± 37**	236 ± 45	223 ± 31	186 ± 32***
Triglycerides (mg/dl)	182 ± 44	157 ± 26**	152 ± 33**	171 ± 49	154 ± 15	160 ± 49	190 ± 45	182 ± 67	148 ± 32**
LDL (mg/dl)	132 ± 44	151 ± 54	114 ± 35	141 ± 51	144 ± 42	114 ± 34	155 ± 42	143 ± 33	113 ± 26***
HDL (mg/dl)	44 ± 3	43 ± 5	43 ± 6	43 ± 4	45 ± 4	42 ± 5	43 ± 4	43 ± 4	44 ± 6
TC:HDL	4.9 ± 1.3	5.3 ± 1.7	4.4 ± 0.9	5.1 ± 1.3	5.0 ± 1.2	4.5 ± 0.9*	5.6 ± 1.5	5.2 ± 1.0	4.3 ± 0.7***
LDL:HDL	3.1 ± 1.2	3.6 ± 1.5	2.7 ± 0.8	3.2 ± 1.4	3.3 ± 1.2	2.7 ± 0.8	3.7 ± 1.3	3.4 ± 0.9	2.6 ± 0.6***

Data are expressed as Mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to pre-treatment value.

Table 3: Biochemical markers of liver and kidney function

Characteristic	Placebo			*W. fruticosa*			*T. procumbens*		
	Pre	Post		Pre	Post		Pre	Post	
		2-mo	4-mo		2-mo	4-mo		2-mo	4-mo
ALT (IU/L)	39 ± 10	40 ± 16	37 ± 19	40 ± 9	46 ± 19	43 ± 16	42 ± 12	53 ± 28	36 ± 16
AST (IU/L)	31 ± 8	28 ± 8	33 ± 12	32 ± 8	31 ± 15	34 ± 9	37 ± 18	35 ± 20	33 ± 14
Urea (mg/dl)	30 ± 10	31 ± 8	32 ± 7	31 ± 9	31 ± 11	32 ± 9	29 ± 10	32 ± 12	35 ± 10
BUN	14 ± 5	14 ± 4	15 ± 3	13 ± 4	15 ± 5	15 ± 4	13 ± 5	15 ± 5	16 ± 5
Creatinine (mg/dl)	1.1 ± 0.3	1.0 ± 0.2	1.2 ± 0.2	1.1 ± 0.2	1.0 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.4	1.2 ± 0.3
CCr (ml/min)	51 ± 16	55 ± 20	49 ± 16	52 ± 11	57 ± 19	54 ± 13	68 ± 20	72 ± 27	62 ± 16

Data are expressed as Mean ± SD.

Figure 1

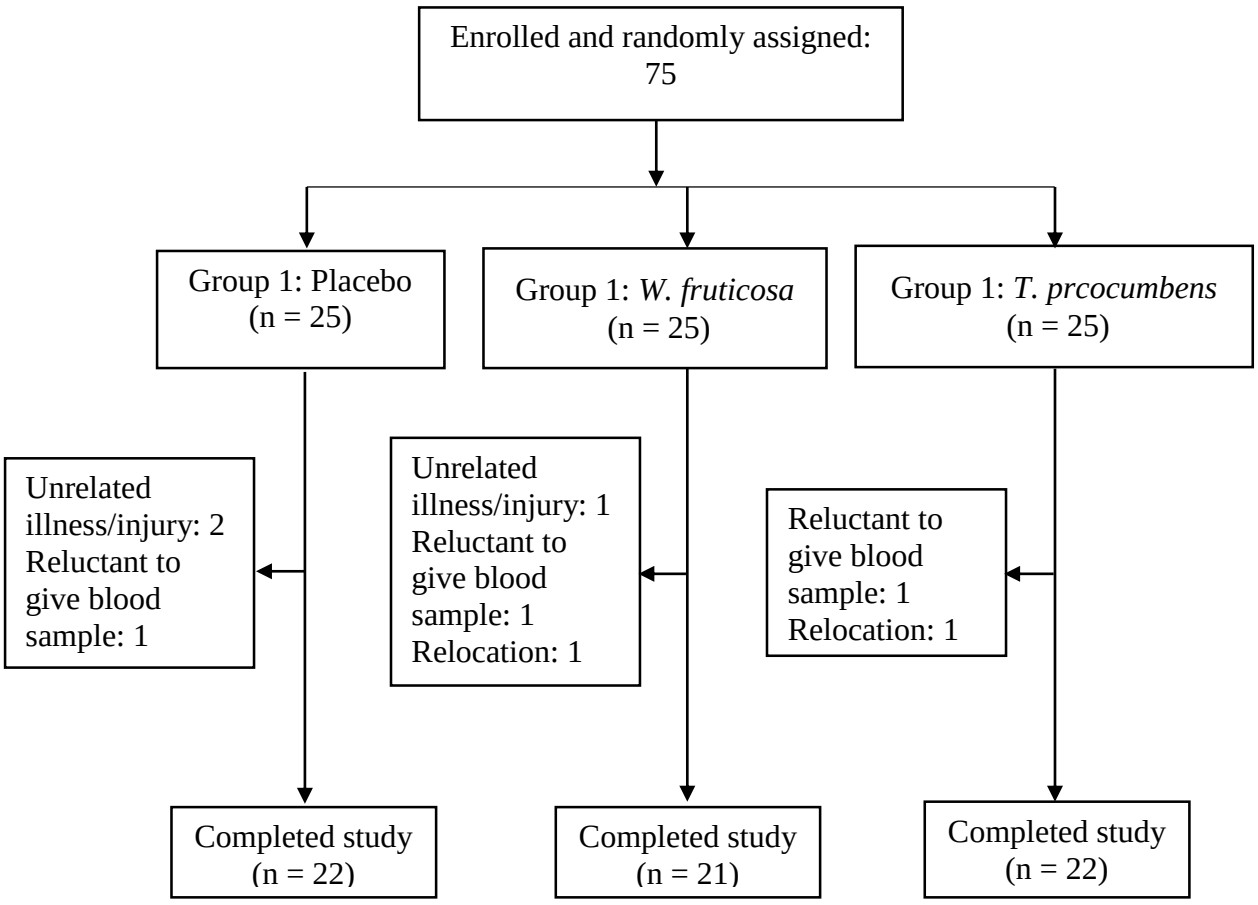
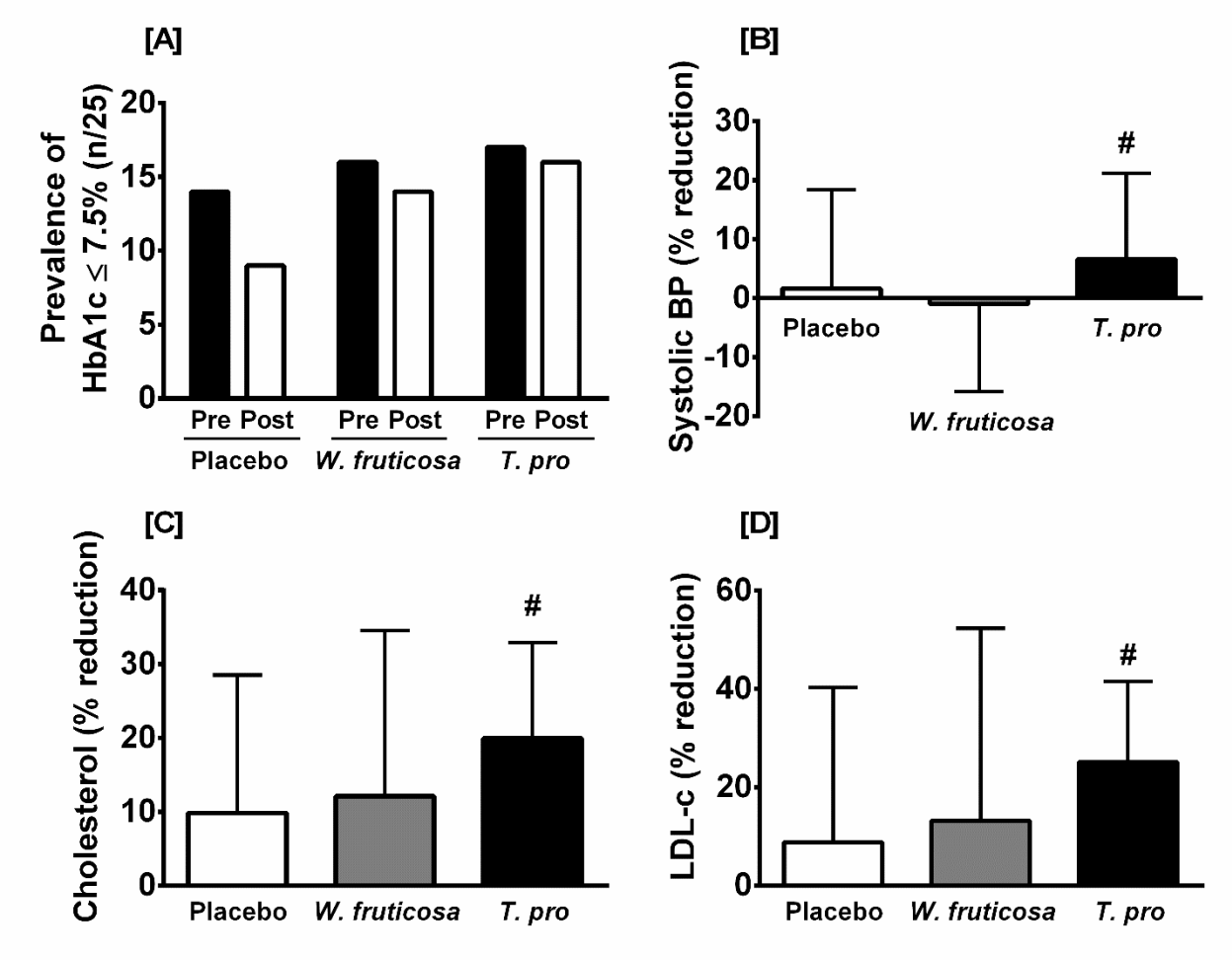

Enrolled and randomly assigned: 75
Group 1: Placebo (n = 25)
Group 1: *W. fruticosa* (n = 25)
Group 1: *T. prcocumbens* (n = 25)
Unrelated illness/injury: 2
Reluctant to give blood sample: 1
Unrelated illness/injury: 1
Reluctant to give blood sample: 1
Relocation: 1
Reluctant to give blood sample: 1
Relocation: 1
Completed study (n = 22)
Completed study (n = 21)
Completed study (n = 22)

Figure 2


[A]
Prevalence of HbA1c ≤ 7.5% (n/25)
20
15
10
5
0
Pre Post
Pre Post
Pre Post
Placebo
W. fruticosa
T. pro
[B]
Systolic BP (% reduction)
30
20
10
0
-10
-20
#
Placebo
W. fruticosa
T. pro
[C]
Cholesterol (% reduction)
40
30
20
10
0
#
Placebo
W. fruticosa
T. pro
[D]
LDL-c (% reduction)
60
40
20
0
#
Placebo
W. fruticosa
T. pro

Summary and Conclusion

The global prevalence of type 2 diabetes for all age groups is estimated to rise from 2.8% in 2000 to 4.4% in 2030, with 366 million people worldwide expected to be diagnosed with diabetes (Wild 2004). Poor diabetes control, including chronic hyperglycemia and diabetic dyslipidemia are known to increase risk of cardiovascular complications (Mitka 2007). Current data suggests that increasing number of individuals in the US use one or more Complementary and Alternative Medicine (CAM) therapies for chronic medical conditions, with diabetic individuals being 1.6 times more likely to use CAM compared to non-diabetic individuals. (Egede 2002). *Tridax procumbens* L. has been a part of indigenous medicine in India and Africa and has been known for its anti-hyperglycemic, anti-inflammatory, and anti-hypertensive effects. Anecdotal evidence and pre-clinical studies in animal models led to our interest in *T. procumbens.* Due to lack of information on the safety and efficacy of CAM therapies, especially botanicals, the National Center for Complementary and Alternative Medicine (NCCAM) has focused research efforts towards strengthening scientific evidence-based studies in evaluating herbal supplements for management of diabetes and metabolic syndrome. Therefore, our studies focused on characterization of molecular mechanisms mediating anti-diabetic and anti-inflammatory effects of *T. procumbens*, along with evaluating the effectiveness of *T. procumbens* in improving blood glucose control and cardio-metabolic health in a randomized, double-blind, placebo-controlled clinical trial in individuals with type 2 diabetes.

Our studies demonstrate that *T. procumbens*, supplemented as a fermented infusion, in a complementary manner along with conventional anti-diabetic medications, was effective in

maintaining a high prevalence of HbA1c $\leq$ 7.5%. The open-label pilot clinical study in individuals with type 2 diabetes demonstrated significant lowering of both fasting and 2-hour post-prandial blood glucose. However, the reductions in blood glucose were not reflected in the randomized study design, suggesting the need for further research targeted towards *T. procumbens* dose optimization combined with lifestyle modifications. *T. procumbens* significantly improved cardio-metabolic health by lowering systolic blood pressure, total cholesterol and LDL-cholesterol. To our knowledge, this is the first clinical report on the anti-diabetic and cardio-protective effects of *T. procumbens*. Future research is required to confirm the traditional claim of *T. procumbens* in lowering blood glucose. However, our findings validate the anti-hypertensive and cholesterol lowering effects of *T. procumbens* in human subjects suggesting its potential use in reducing cardiovascular complications associated with type 2 diabetes.

In our studies we have used a fermented hydro-alcoholic extract of *T. procumbens*, termed *asava*, which was formulated following Ayurveda guidelines. Our *asava* preparation used *W. fruticosa* flowers, as inoculum. *W. fruticosa* is a medicinal plant and the flowers are known in traditional medicine for its anti-diabetic and anti-inflammatory effects. Therefore, we have characterized the molecular mechanisms, for both *T. procumbens asava* and *W. fruticosa asava*.

T. procumbens asava demonstrated presence of phenolics, flavonoids, and carotenoids, and strong antioxidant reducing potential, ferrous-ion chelating activity, hydrogen peroxide scavenging effect, and inhibition of lipid peroxidation. Our studies have shown that the blood glucose lowering effect of *T. procumbens* may be mediated through activation of AMPK, and suppression of gluconeogenic gene expression and hepatic glucose production. Our studies have

also shown that the anti-inflammatory activity of *T. procumbens* was mediated through inhibition of NFκB pathway.

W. fruticosa asava also demonstrated significant antioxidant reducing potential, activation of AMPK, and suppression of hepatic glucose production, and expression of PEPCK and G6Pase. Interestingly, *W. fruticosa* phosphorylated Akt at Ser473, with no effect on p44/42 MAPK, suggesting a potential effect on insulin signal transduction specific to the PI3-kinase-Akt pathway. *W. fruticosa* also exerted its anti-inflammatory effects through inhibition of NFκB pathway, and NFκB transcriptional activity in murine macrophages suggesting a potential synergistic action between *T. procumbens* and *W. fruticosa*.

These studies suggest that *T. procumbens asava* offers a novel and promising complementary approach in the management of diabetes, with potential beneficial effects on diabetic dyslipidemia and hypertension. Future studies can be directed to include assessment of different doses of *T. procumbens asava* for optimum blood glucose control in human subjects. Conducting cell culture based mechanistic studies using an extract of *T. procumbens* alone can further validate present findings, and can be used to perform phytochemical screening and fractionation for bioactivity guided assays. Additional studies are required to examine the effect of *T. procumbens* on glucose uptake in skeletal muscle cells, independent of the insulin signaling pathway. In this study, we show that *W. fruticosa* phosphorylates and activates Akt, which provides an interesting lead to further explore the effects of *W. fruticosa* on insulin signaling.

In conclusion, our study is the first clinical report demonstrating potential benefits of *T. procumbens* in management of diabetes through improved cardio-metabolic health, while offering a mechanistic basis for the traditionally claimed anti-diabetic and anti-inflammatory properties.

References

Egede LE, Ye X, Zheng D and Silverstein MD (2002). The Prevalence and Pattern of Complementary and Alternative Medicine Use in Individuals With Diabetes. Diabetes Care 25(2): 324-329

Mitka M (2007). Report quantifies diabetes complications. Jama 297(21): 2335-2338

Wild S, Roglic G, Green A, Sicree R and King H (2004). Global Prevalence of Diabetes: Estimates for the year 2000 and projections for 2030. Diabetes Care 27(5): 1047-1053

Appendix I

Ethics Committee Approval

Pilot Clinical Study

KOLHAPUR INDEPENDENT ETHICS COMMITTEE

Regd. Off. 1182/98, Mali Colony, Takala, Kolhapur, Maharashtra – 416 008 Phone: 0231- 2532034

Ref. No. 01/13/1/11 Date: 13/JAN/11

To
Gauri Shirish Desai
Mrs. Gauri Aniket Shirgaokar
Institute:Implentation Research Division,
Bhagawati Industrial & Organic Products Pvt. Ltd.,
C/O Standard Plates & Vessels Pvt. Ltd.,
Plot 9, M.I.D.C. Shiroli, Kolhapur- 416 122
Maharashtra,India.

Dear Mrs. Gauri Aniket Shirgaokar,

The Institutional Ethics Committee / Independent Ethics Committee (Kolhapur Independent Ethics Committee, as appropriate) reviewed and discussed your application to conduct the clinical trial **Protocol: BIO/TRIDAXEN/01 titled: "Diabetes Management Potential Of TRIDAXEN, An Ayurvedic Asava Of *Tridax procumbens*- Pilot Study" dated 13 Jan 2010.**

The following documents submitted for the above-mentioned clinical project were reviewed

Sr. No.	Document
1.	Approval Of The Head Of The Division
2.	Protocol
3.	Ethical Issues in Study & Plans to Address These Issues
4.	Proformae, questionnaires, follow up card,etc
5.	Patient Information Sheet & Informed Consent in Local Language
6.	For Any Drug/ Device Trial, All Relevant Pre-Clinical Animal Data & Clinical Trial Data From Other Countries, If Available

KOLHAPUR INDEPENDENT ETHICS COMMITTEE

Regd. Off. 1182/98, Mali Colony, Takala, Kolhapur, Maharashtra – 416 008 Phone: 0231- 2532034

7.	Statement Describing Compensation For Study Subjects fro Participation and/ or Study related Injuries
8.	CV of All Investigator With Relevant Publications In Last Five Years
9.	Source of Funding and Financial Requirements for The Project
10.	An Agreement To Rreport Any Serious Side Effects Or Adverse Drug Reactions To IEC
11.	Statement Of Conflicts Of Interest, If Any
12.	Research Article
13.	Scientific design And Conduct Of Study

The following members of the Ethics Committee were present at the meeting held on (13/ JAN/ 2011, **10.00 am & Hotel Vrushali)**

Name	Qualification	Designation/ Title	Affiliations as to the Institution Yes/No
Dr. Udayprakash Sant	M.S. (Gen. Surgeon)	Chairperson	No
Mr.B.S. Mohite	M. Phil., Ph d.	Member Secretary	No
Mr.Pravin Metrani	B.E. (IT)	Lay Person	No
Mrs.Sadhna Zadbuke	M.A. Sociology, M. Phil Social Work, Diploma in Higher Education	Social Worker/ Female Candidate	No
Dr.Vasant Deshmukh	M.S.		No

KOLHAPUR INDEPENDENT ETHICS COMMITTEE

Regd. Off. 1182/98, Mali Colony, Takala, Kolhapur, Maharashtra – 416 008 Phone: 0231- 2532034

As mentioned in the SOP for 'Kolhapur Independent Ethics Committee'- KCCIEC/PW/SOP/03 in section 27- Annexure A the following members who were not present have conveyed in writing to the committee their favorable opinions.

Name	Edu. Qualification	Designation/Title	Affiliations as to the Institution Yes/No
Adv. Ramesh Hirve	L.L.B.	Legal Representative	No
Mr. M.B. Shaikh	B.E. Civil	Engineer	No
Dr.Ajit Patil	M.D.,DNB (Gynaec)	Clinicians	No

We approve the trial to be conducted in its presented form

Kolhapur Independent Ethics Committee expects to be informed about the progress of the study, any SAE occurring in the course of the study, any changes in the protocol and patient information/informed consent and asks to be provided a copy of the final report.

We hereby confirm that the *(Kolhapur Independent Ethics Committee)* is organized and operates as per GCP and applicable regulations.

Yours Sincerely,

Signature and Date: 13 JAN 2011

Name : Mr. B.S. Mohite
Member Secretary
Kolhapur Independent Ethics Committee,
1182/98, E-Ward, Mali Colony,
Takala, Kolhapur,
Maharashtra-416 008, India.

KOLHAPUR INDEPENDENT ETHICS COMMITTEE

Appendix II

Ethics Committee Approval

Pilot Clinical Study

KOLHAPUR INDEPENDENT ETHICS COMMITTEE

Regd. Off. 1182/98, Mali Colony, Takala, Kolhapur, Maharashtra - 416 008 **Phone :** 0231-2532034

Date: 15 JAN 2013

To,
Mrs. Gauri S. Desai,
Bhagwati Industrial &
Organic Products PVt. Ltd.,
Implementation Research Division,
C/O Standard Plates & Vessels Pvt. Ltd.,
Plot No. 9, M.I.D.C. Shiroli, Kolhapur 416122,
Maharashtra, India.

Sub: Protocol: **BIO/TRIDAXEN/01** titled "Diabetes Management Potential of TRIDAXEN, An Ayurvedic ASAVA Of *Tridax procumbens*- Pilot Study"

Dear Mrs. Gauri,

The Kolhapur Independent Ethics Committee reviewed and discussed your application to conduct the clinical study entitled "**BIO/TRIDAXEN/01** titled "Diabetes Management Potential of TRIDAXEN, An Ayurvedic ASAVA Of *Tridax procumbens*- Pilot Study" on 15 Jan 2013, at Kolhapur Independent Ethics Committee, 1182/98, E-Ward, Mali Colony, Takala, Kolhapur 416 008, Maharashtra, India.

The following amendments were reviewed:

Sr. No	Description	Provisions as per Approved Research Proposal	Amendments Requested
1.	Title	Diabetes management potential of 'Tridaxen' an Ayurvedic asava of Tridax procumbens-Pilot Study	Role of antioxidant, anti-inflammatory properties of hyperglycemic properties of Tridax procumbens in lowering cardiovascular risk factors
2.	Name of the Institute/Hospital/Field Area/where	Name of the Institut-Implementation Research Division, Bhagawati Industrial & Organic Products Pvt. Ltd., C/O Standard Plates & Vessels Pvt.	Name of the Institute-Kayachikitsa Department, Yashwant Ayurvedic College, Post Graduate

KOLHAPUR INDEPENDENT ETHICS COMMITTEE

Regd. Off. 1182/98, Mali Colony, Takala, Kolhapur, Maharashtra - 416 008 **Phone :** 0231-2532034

	research will be conducted	Ltd., Plot No. 9, M.I.D.C. Shiroli, Kolhapur 416122, Maharashtra, India.	Training and Research Centre, Kodoli, Tal. Panhala, Dist. Kolhapur, Maharashtra, India.
3.	Approval of the Head of the Division	BIO/IRD/1210/01 Dtd. 2nd Dec 2010	**YACK/PG/58/2013 Dtd.1st Jan 2013 enclosed**
4.	Protocol of the proposed research	As per annexure-II of Approved Research Proposal. Protocol:BIO/TRIDAXEN/01 Titled: Diabetes management potential if 'Tridaxen' an Ayurvedic asva of Tridax procumbens- Pilot Study Dated:13th Jan 2010	**As per Annexure-II (Revised) enclosed** Protocol: BIO/TRIDAXEN/01 Titled: Role of antioxidant, anti-inflammatory and anti-hyperglycemic properties of Tridax procumbens in lowering cardiovascular risk factors. Dated:14th SEP 2012
5.	Proformae, Questionnaires, follow up card, etc.	As per Annexure-IV Approved Research Proposal	**As per Annexure-IV (Revised) Proposal**
6.	Patient Information Sheet and informed consent form	As per Annexure-V Approved Research Proposal	**As per Annexure-V (Revised) Proposal**
7.	Curriculum vitae of all the investigators	As per Annexure-VIII Approved Research Proposal	**As per Annexure-VIII (Revised) Proposal**
8.	Sources of funding requirements for the project	As per Annexure- X of Approval Research Proposal	**As per Annexure- X (Revised) enclosed**

KOLHAPUR INDEPENDENT ETHICS COMMITTEE

Regd. Off. 1182/98, Mali Colony, Takala, Kolhapur, Maharashtra - 416 008 **Phone :** 0231-2532034

The following members of the Ethics Committee were present at the meeting held on 15 JAN 2013, at Kolhapur Independent Ethics Committee, 1182/98, E-Ward, Mali Colony, Takala, Kolhapur 416 008, Maharashtra, India.

1. Dr. Udayprakash Sant - Chairperson
2. Dr. B.S. Mohite - Member Secretary
3. Adv. Ramesh Hirave - Member
4. Dr. Reshma Pawar - Member
5. Smt. Anuradha Bhosale - Member
6. Dr. Ajit Patil - Member
7. Mr. Pravin Metrani - Member
8. Dr. Vasant Deshmukh - Member

We approve the trial to be conducted in its presented form.

The Kolhapur Independent Ethics Committee expects to be informed about the progress of the study, any SAE occurring in the course of the study, any changes in the protocol and patient information / informed consent and asks to be provided a copy of the final report.

Yours Sincerely,

15 JAN 2013

The Member Secretary,
Dr. B. S. Mohite,
Kolhapur Independent Ethics Committee.

KOLHAPUR INDEPENDENT ETHICS COMMITTEE