Available online at www.ijtpr.com
International Journal of Toxicological and Pharmacological Research 2010; 2(1): 1-5

Research Article ISSN 0975 5160

Antihyperlipidemic Screening of Polyherbal Formulation of *Annona squamosa* and *Nigella sativa*

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ABSTRACT
This study was undertaken to investigate the effect of Polyherbal formulation of *Annona squamosa* and *Nigella sativa* on blood glucose, plasma insulin, tissue lipid profile, and lipidperoxidation in streptozotocin induced diabetic rats. Aqueous extract of Polyherbal formulation of *Annona squamosa* and *Nigella sativa* was administered orally (200 mg/kg body weight) for 30 days. The different doses of Polyherbal formulation on blood glucose and plasma insulin in diabetic rats were studied and the levels of lipid peroxides and tissue lipids were also estimated in streptozotocin induced diabetic rats. The effects were compared with tolbutamide. Treatment with Polyherbal formulation and tolbutamide resulted in a significant reduction of blood glucose and increase in plasma insulin. Polyherbal formulation also resulted in a significant decrease in tissue lipids and lipid peroxide formation. The decreased lipid peroxides and tissue lipids clearly showed the antihyperlipidemic and antiperoxidative effect of Polyherbal formulation apart from its antidiabetic effect.

Keywords: Polyherbal formulation, *Annona squamosa, Nigella sativa,* Streptozotocin, Antihyperlipidemic activity.

INTRODUCTION

Diabetes mellitus is syndrome, initially characterized by a loss of glucose homeostasis resulting from defects in insulin secretion, insulin action both resulting impaired metabolism of glucose and other energy yields fuels such as lipids and protein[1]. Experimental diabetes in animals has provided considerable insight into the physiologic and biochemical derangement of the diabetic state. Many of the derangement have been characterized in hyperglycemic animals. Significant changes in lipid metabolism and structure also occur in diabetes[2]. In these cases the structural changes are clearly oxidative in nature and are associated with development of vascular disease in diabetes[3]. In diabetic rats, increased lipidperoxidation was also associated with hyperlipidemia[4]. Liver, an insulin dependent tissue that plays a pivotal role in glucose and lipid homeostasis and it is severely affected during diabetes[5].

Liver and kidney participates in the uptake, oxidation and metabolic conversion of free fatty acids, synthesis of cholesterol, phospholipids, and triglycerides. During diabetes, a profound alteration in the concentration and composition of lipids occurs. Despite the great strides that have been made in the understanding and management of diabetes, the disease and disease related complications are increasing unabated[6]. In spite of the presence of known antidiabetic medicine in the pharmaceutical market, remedies from medicinal plants are used with success to treat this disease[7]. Many traditional plant treatments for diabetes are used throughout the world. Plant drugs[8] and herbal formulation[9, 10, 11] are frequently considered to be less toxic and freer from side effects than synthetic one.

Based on the WHO recommendations hypoglycemic agents of plant origin used in traditional medicine are important (WHO, 1980). The attributed antihyperglycemic effects of these plants is due to their ability to restore the function of pancreatic tissues by causing an increase in insulin output or inhibit the intestinal absorption of glucose or to the facilitation of metabolites in insulin dependent processes. Hence treatment with herbal drugs has an effect on protecting b-cells and smoothing out fluctuation in glucose Levels [12, 13]. In general, there is very little biological knowledge on the specific modes of action in the treatment of diabetes, but most of the plants have been found to contain substances like glycosides, alkaloids, terpenoids, flavonoids etc., that are frequently implicated as having antidiabetic effects[14].

In the traditional system of Indian medicine, plant formulation and combined extracts of plants are used as drug of choice rather than individual. Various herbal formulations such as diamed[15], coagent db[16], Diasulin[17] and hyponidd[18], are well known for their antidiabetic effects. Polyherbal formulation of *Annona squamosa* and *Nigella sativa* is composed of medicinal plants (Table 1), which are traditionally used for antidiabetic and antihyperlipidemic activity[19, 20]. The present investigation was undertaken to study the effect of the Polyherbal formulation of *Annona squamosa* and *Nigella sativa* on lipidperoxidation and tissue

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lipid profile in streptozotocin induced diabetic rats. The effects produced by this formulation on different parameters were compared with tolbutamide, a reference drug.

MARERIAL AND METHODS

Animals

Male Wistar albino rats (weighing 160–200 g) were procured from Venkateshwara Enterprise, Benglore and they kept in under standard environmental conditions (12 h light/dark cycles at 25–28 ^{0}C, 60–80% relative humidity) in clean and dry cages and maintained in well-ventilated animal house. Animals were divided into 8 groups of five each and were fed with standard diet and water *ad libitum*. The study was approved by the Institutional Animal Ethics Committee.

Preparation of drug

The seeds of *Nigella sativa* obtained from Prgati Ayurvedic Drug store Belgaum and matured fruit of *Annona squamosa* from local market of Belgaum and they were authenticated from Botanical Survey of India, Pune (Maharastra). The extracts of the both antidiabetic plants were mixed and polyherbal formuation was prepared (Table 1). Five hundred grams of each plant (chopped into small pieces) was extracted individually and were soaked overnight in 1 l of water. This suspension was filtered and the filtrates were pooled and the solvents were evaporated in a rotavapor at 40–50 ^{0}C under reduced pressure and lyophilized[21].

Chemicals

Streptozotocin and other biochemicals used in this experiment were purchased from Sigma Chemical Company Inc., St. Louis, Mo, and USA. Enzyme linked immunosorbant assay (ELISA) kit for insulin assay was purchased from Boehringer Mannheim, Germany. Tolbutamide was a generous gift sample from Sun Pharmaceuticals Limited, Baroda, India. All other chemicals used were of analytical grade.

Drug administration

Polyherbal formulation of *Annona squamosa* and *Nigella sativa* was suspended in distilled water and administered orally through intragastric tube at the following doses of 50, 100 and 200 mg/kg body weight.

Streptozotocin-induced diabetes

Rats were made diabetic by single administration of streptozotocin (60 mg/kg/i.p) dissolved in 0.1 M-citrate buffer, pH 4.5. Forty-eight hours later, blood samples were collected and glucose levels were determined to confirm the development of diabetes. Only those animals which showed hyperglycemia (blood glucose levels > 300 mg/dl) were used in the experiment.

Experimental design

In the experiment, a total of 42 rats (30 diabetic surviving rats, 12 normal rats) were used. The rats were divided into seven groups of six rats each after the induction of streptozotocin diabetes.

Group1: Normal treated rats.

Group2: Normal rats given aqueous solution of Polyherbal formulation (200 mg/kg body weight) daily using an intragastric tube for 30 days.

Group 3: Diabetic control rats.

Group 4: Diabetic rats given aqueous solution of Polyherbal formulation (50 mg/kg body weight) daily using an intragastric tube for 30 days.

Group 5: Diabetic rats given aqueous solution of Polyherbal formulation (100 mg/kg body weight) daily using an intragastric tube for 30 days.

Group 6: Diabetic rats given aqueous solution of Polyherbal formulation (200 mg /kg body weight) daily using an intragastric tube for 30 days.

Group 7: Diabetic rats given aqueous solution of Tolbutamide (250 µg/kg bodyweight) daily use an intragastric tube for 30 days.

At the end of 30 days, all the rats were killed by decapitation under pentobarbitone sodium (60 mg/kg) anesthesia. Blood was collected in tubes containing potassium oxalate and sodium fluoride solution for the estimation of blood glucose and plasma was separated for the assay of insulin. Liver and kidney were immediately dissected out, washed in ice-cold saline to remove the blood. The tissues were weighed and 10% tissue homogenate was prepared with 0.025 M Tris–HCl buffer, pH 7.5. After centrifugation at 3000 rpm for 10 min, the clear supernatant was used to measure thiobarbituric acid reactive substances (TBARS) and hydroperoxides. For the determinations of lipids the liver and kidney tissues were weighed and lipids were extracted from tissues[22].

Biochemical analysis

Estimation of blood glucose and plasma insulin

Blood glucose was determined by the O-toluidine method[23]. Plasma insulin was assayed by ELISA, using Boeheringer-Mannheim Kit with a Boeheringer analyzer ES300.

Estimation of lipid peroxidation

Lipid peroxidation in liver and kidney were estimated colorimetrically by thiobarbituric acid reactive substances TBARS[24] and hydroperoxides[25]). In brief, 0.1 ml of tissue homogenate (Tris–HCl buffer, pH 7.5) was treated with 2 ml of (1:1:1 ratio) TBA–TCA–HCl reagent (thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA) and placed in water bath for 15 min, cooled. The absorbance of clear supernatant was measured against reference blank at 535 nm. 0.1 ml of tissue homogenate was treated with 0.9 ml of Fox reagent (88 mg butylated hydroxytoluene (BHT), 7.6 mg xylenol orange and 9.8 mg ammonium ion sulphate were added to 90 ml of methanol and 10 ml 250 mM sulphuric acid) and incubated at 37 ^{0}C for 30 min. The colour developed was read at 560 nm colorimetrically. Hydroperoxides was expressed as mM/100 g tissue.

Estimation of lipids

Lipids were extracted from tissues by the method of using chloroform–methanol mixture[22] (CHCl3:MeOH) (2:1 v/v).

For total cholesterol estimation[26] 0.1 ml of the lipid extract, 9.9 ml of ferric chloride–acetic acid reagent was added and allowed to stand for 15 min and then centrifuged. To 5 ml of the supernatant, add 3 ml of Conc. H_2SO_4. The colour developed was read after 20 min at 560 nm against a reagent blank. Values were expressed as mg/100 g tissue.

For Triglycerides estimation[27] an aliquot of lipid extract, evaporated to dryness. 0.1 ml of methanol was added followed by 4 ml of isopropanol. 0.4 g of alumina was added to all the tubes and shaken well for 15 min. Centrifuged and then 2 ml of the supernatant was transferred to labeled tubes. The tubes were placed in a water bath at 65 ^{0}C for 15 min for saponification after adding 0.6 ml of the saponification reagent followed by 0.5 ml of acetyl acetone reagent. After mixing, the tubes were kept in a water bath at 65 ^{0}C for 1 h, the contents were cooled and absorbance was read at 420 nm. The triglyceride content was expressed as mg/100 g tissue.

Phospholipid content was determined [28] by 0.1 ml of lipid extract, added 1 ml of 5 N H_2SO_4 and 1 ml of Conc. HNO_3 and digested to a colourless solution. The phosphorus content

in the extract was determined[29]. The values were expressed as g/100 tissue.

Free Fatty acids were estimated[30] by 0.1 ml of lipid extract was evaporated to dryness. One milliliter of phosphate buffer, 6 ml of extraction solvent and 2.5 ml of copper reagent were added. All the tubes were shaken vigorously. Two hundred milligrams of activated silicic acid was added and left aside for 30 min. The tubes were centrifuged and 3 ml of the copper layer was transferred to another tube containing 0.5 ml of diphenyl carbazide and mixed carefully. The absorbance was read at 550 nm immediately. The amount of free fatty acids was expressed as mg/100 g tissue.

Statistical analysis

The data for various biochemical parameters were analyzed using analysis of variance (ANOVA) and the group means were compared by Duncan's Multiple Range Test (DMRT). Values were considered statistically significant when at $p < 0.05$[31].

Table 1: Polyherbal Formulation of *Annona Squamosa* and *Nigella Sativa* (Composition and Concentration).

S. No.	Botanical Name	Common Name	Family	Part used	Conc. (%)
1	Annona squamosa	Sharifa	Annonnaceae	Matured fruits	50
2	Nigella sativa	Kalonji	Ranunculaceae	Seeds	50

Table 2: Changes in blood glucose and plasma insulin levels of control and experimental animals.

Group	Fasting blood glucose (mg/dl)	Plasma insulin (IU/ml)
Normal	81.04 ± 2.29	11.26 ± 0.96
Diabetic control	262.24 ± 22.23	3.48 ± 0.69
Diabetic + Polyherbal formulation (50 mg/kg)	209.58 ± 12.46	5.59 ± 0.34
Diabetic + Polyherbal formulation (100 mg/kg)	155.58 ± 11.69	6.03 ± 0.45
Diabetic + Polyherbal formulation (200 mg/kg)	104.16 ± 6.56	7.15 ± 0.45
Diabetic + Tolbutamide (250 mg/kg)	110.65 ± 9.35	6.32 ± 0.48

Values are given as mean ± SD for six rats in each group.
Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT).

RESULTS

Table 2 shows the level of blood glucose and plasma insulin in control and experimental diabetic animals. There was a significant elevation in blood glucose level with significant decrease in plasma insulin levels in streptozotocin diabetic rats, compared with normal rats. Administration of Polyherbal formulation and Tolbutamide tended to bring blood glucose and plasma insulin towards near normal levels. The effect of Polyherbal formulation at 200 mg/kg was significantly better than 50 and 100 mg/kg; therefore the higher dose was used for further biochemical studies. The administration of Polyherbal formulation and Tolbutamide to normal rats showed a significant effect in lowering blood glucose and increasing plasma insulin.

Table 3 represents the concentration of TBARS and Hydroperoxides in tissues of control and experimental animals. There was asignificant elevation in tissue TBARS and Hydroperoxides during diabetes, when compared to the corresponding control group. Administration of Polyherbal formulation and Tolbuatamide tends to bring the values to near normal.

Table 3: Changes in levels of TBARS and Hydroperoxides in liver and kidney of control and experimental animals (mm/100 g tissue).

Groups	TBARS		Hydro peroxide	
	Liver	Kidney	Liver	Kidney
Normal	0.58 ± 0.066	0.68 ± 0.05	70.38 ± 4.54	55.34 ± 4.55
Normal + Polyherbal formulation (200 mg/kg)	0.56 ± 0.079	0.78 ± 0.06	68.59 ± 5.14	52.48 ± 4.75
Diabetic control	1.54 ± 0.06	1.65 ± 0.12	99.98 ± 5.98	78.29 ± 4.58
Diabetic + Polyherbal formulation (200 mg/kg)	0.64 ± 0.053	0.97 ± 0.07	80.55 ± 5.69	60.99 ± 4.78
Diabetic + Tolbutamide (250 mg/kg)	0.67 ± 0.088	1.02 ± 0.08	84.35 ± 5.45	62.45 ± 5.56

Values are given as mean ± SD for six rats in each group.
Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT).

Table 4: Changes in levels of cholesterol, free fatty acids, triglycerides and phospholipids in liver of control and experimental animals (mg/100 g wet tissue).

Groups	Cholesterol	Free fatty acids	Triglycerides	Phospholipids
Normal	335.44 ± 18.90	606.10 ± 1.76	344.50 ± 23.20	1598.00 ± 19.30
Normal + Polyherbal formulation (200 mg/kg)	328.38 ± 5.74	601.93 ± 9.00	341.10 ± 21.00	1593.10 ± 24.10
Diabetic control	496.56 ± 13.10	921.60 ±44.60	622.50 ± 18.80	1858.60 ± 18.70
Diabetic + Polyherbal formulation (200 mg/kg)	398.65 ± 17.54	769.16 ± 3.30	456.25 ± 17.30	1718.80 ± 14.40
Diabetic + Tolbutamide (250 mg/kg)	405.21 ± 9.79	802.80 ± 3.77	530.80 ± 35.70	1769.30 ± 17.60

Values are given as mean ± SD for six rats in each group.
Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT).

Table 5: Changes in levels of cholesterol, free fatty acids, triglycerides and phospholipids in kidney of control and experimental animals (mg/100 g wet tissue).

Groups	Cholesterol	Free fatty acids	Triglycerides	Phospholipids
Normal + Polyherbal formulation (200 mg/kg)	371.08 ± 8.28	432.51 ± 1.60	278.75 ± 14.60	1442.50 ± 43.30
Diabetic control	546.90 ± 23.80	743.00 ± 5.70	501.10 ± 34.10	2041.50 ± 33.60
Diabetic + Polyherbal formulation (200 mg/kg)	435.20 ± 12.18	556.80 ± 8.50	382.90 ± 9.28	1684.00 ± 28.80
Diabetic + Tolbutamide (250 mg/kg)	449.90 ± 13.49	600.30 ± 3.40	438.66 ± 39.30	1819.30 ± 34.70

Values are given as mean ± SD for six rats in each group.
Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT).

Table 4 and 5 show the levels of cholesterol, triglycerides, free fatty acids and phospholipids in liver and kidney of control and experimental rats, respectively. Liver and kidney of diabetic rats showed significantly increased levels of cholesterol, triglycerides, free fatty acids and phospholipids, when compared with normal rats. In rats treated with Polyherbal formulation and Tolbutamide there was a

significant decrease in the content of cholesterol, triglycerides, free fatty acids and phospholipids in both the tissues, when compared with diabetic control rats.

DISCUSSION

Diabetes mellitus is one of the most common chronic disease and is associated with hyperlipidemia and co-morbidities such as obesity, hypertension. Hyperlipidemia is a metabolic complication of both clinical and experimental diabetes[32]. Streptozotocin induces a wide variety of animal species by damaging the insulin secreting pancreatic b-cell, resulting in a decrease in endogenous insulin release, which paves the ways for the decreased utilization of glucose by the tissues[33].

In our study, we have observed that Polyherbal formulation decreases blood glucose in streptozotocin diabetic rats. The possible mechanism of action of extract could be correlated with the reminiscent effect of the hypoglycemic sulphonylureas that promote insulin secretion by closure of K^+–ATP channels, membrane depolarization and stimulation of Ca^{2+} influx, an initial key step in insulin secretion. In this context, number of other plants has also been reported to have antihyperglycemic and insulin stimulatory effects[34, 35]. Like the plant extract, tolbutamide also produced significant reduction in blood glucose levels of streptozotocin diabetic rats.

Since streptozotocin is known to destroy pancreatic b-cells, the present findings appear to be in consonance with the earlier suggestion[36] that sulphonylureas have extrapancreatic antihyperglycemic mechanism of action secondary to their insulin secreting effect and the attendant glucose uptake into, and utilization by, the tissues.

Apart from the regulation of carbohydrate metabolism, insulin also plays an important role in the metabolism of lipids. Insulin is potent inhibitor of lipolysis. Since it inhibits the activity of the hormone sensitive lipases in adipose tissue and suppresses the release of free fatty acids[37]. During diabetes, enhanced activity of this enzyme increases lipolysis and releases more free fatty acids into the circulation[38]. Increased fatty acids concentration also increases the b-oxidation of fatty acids, producing more acetyl CoA and cholesterol during diabetes. In normal condition, insulin increases the receptor-mediated removal of LDL-cholesterol and decreased activity of insulin during diabetes causes hypercholesterolemia. Hypercholesterolemia and hypertriglyceridemia have been reported to occur in diabetic rats[39]. The increased concentration of cholesterol could result in a relative molecular ordering of the residual phospholipids resulting in a decrease in membrane fluidity[40].

The increased concentration of free fatty acids in liver and kidney may be due to lipid breakdown and this may cause increased generation of NADPH, which results in the activation of NADPH dependent microsomal lipid peroxidation. Liver and kidney phospholipids were increased in diabetic control rats. Phospholipids is present in cell membrane and make up vast majority of the surface lipoprotein forming a lipid bilayer that acts as an interface with both polar plasma environment and non-polar lipoprotein of lipoprotein core[41].

Phospholipids are vital part of biomembrane rich in PUFA, which are susceptible substrate for free radicals such as O^{2-} and OH. Radicals[42].Increased phospholipids levels in tissues were reported[43,44] in streptozotocin diabetic rats. Administration of Polyherbal formulation decreased the levels of tissue free fatty acids and phospholipids. Accumulation of triglycerides is one of the risk factors in Coronary Heart Disease (CHD). The significant increase in the level of triglycerides in liver and kidney of diabetic control rats may be due to the lack of insulin. Since under normal condition, insulin activates the enzyme lipoprotein lipase and hydrolysis triglycerides[45]. Polyherbal formulation reduces triglycerides in tissues of streptozotocin-induced diabetic rats and may prevent the progression of CHD. The results show increased lipid peroxidation in the tissues (liver and kidney) of diabetic control group. Previous studies have reported that there was an increased lipid peroxidation in liver, heart, kidney and brain of diabetic rats[46, 47]. This may be because the tissues contain relatively high concentration of easily peroxidizable fatty acids.

Liver during diabetes, showed a relatively severe impairment in antioxidant capacity than kidney. The kidney exhibits a characteristic pattern of changes during diabetes[48]. The increase in oxygen free radicals in diabetes could be primarily due to increase in blood glucose levels, which upon autoxidation generate free radicals and secondarily due to the effects of diabetogenic agent streptozotocin[49]. In diabetes, hypoinsulinaemia increases the activity of the enzyme, fatty acyl coenzyme, coenzyme A oxidase, which initiates b-oxidation of fatty acids resulting in lipid peroxidation[50, 51].

Increased lipid peroxidation impairs membrane functions by decreasing membrane fluidity, and changing the activity of membrane- bound enzymes[51]. Its products (lipid radicals and lipid peroxide) are harmful to the cells in the body and are associated with atherosclerosis and brain damage[51]. Administration of Polyherbal formulation and Tolbutamide reduced the lipid peroxidative markers in liver and kidney tissues of diabetic rats. This indicates that Polyherbal formulation inhibit oxidative damage due to the effect of ingredients present in Polyherbal formulation. This could be correlated with previous study that reported that *Annona squamosa* and *Nigella sativa* antihyperlipidemic effect of diabetic animals. Antidiabetic and antihyperlipidemic effect of Polyherbal formulation of *Annona squamosa* and *Nigella sativa* may be due to the effect of active constituents of both plants alkaloid, phytosterols, which may be responsible for scavenging free radicals liberated by streptozotocin in diabetic rats. On the basis of above results, it could be concluded that Polyherbal formulation of *Annona squamosa* and *Nigella sativa*, exert a significant antihyperlipidemic. This could be due to combined effect of *Annona squamosa* and *Nigella sativa*. Hence the antihyperlipidemic effect of Polyherbal formulation of *Annona squamosa* and *Nigella sativa* in particular could be considered as of possible therapeutic value.

ACKNOWLEDGMENTS

Authors are thankful to KLESs College of Pharmacy, Belgaum for providing necessary facilities to conduct the experimental work.

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