Isolation, Characterization and Biological Activity of 
Annona squamosa Bark and Cuscuta reflexa Areal Part

A Synopsis
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Introduction:

Diabetes mellitus (DM) is the name given to a group of disorders characterized by chronic hyperglycaemia, polyurea, polydipsia, polyphagia, emaciation and weakness due to disturbance in carbohydrate, fat and protein metabolism associated with absolute or relative deficiency in insulin secretion and / or insulin action (Elgawish et al., 1999). It is a global disease that is a major cause of morbidity in the world. The worldwide prevalence of diabetes mellitus is expected to be more than 240 million by the year 2010 (http://plants.usda.gov/java/profile?symbol=CURE).

Diabetes is a chronic disorder of carbohydrate, fat and protein metabolism characterized by increased fasting and post prandial blood sugar levels. The global prevalence of diabetes is estimated to increase, from 4% in 1995 to 5.4% by the year 2025. WHO has predicted that the major burden will occur in developing countries. Studies conducted in India in the last decade have highlighted that not only is the prevalence of diabetes high but also that it is increasing rapidly in the urban population (Edwards et al., 1988) It is estimated that there are approximately 33 million adults with diabetes in India. This number is likely to increase to 57.2 million by the year 2025.

Commonly practiced pharmacologic treatment of diabetes mellitus includes oral hypoglycaemic agents and insulin. In the last few years there has been an exponential growth in the field of herbal medicine and these drugs are gaining popularity both in developing and developed countries because of their natural origin and less side effects. Many traditional medicines in use are derived from medicinal plants, minerals and organic matter. There is an increasing demand by patients for the use of natural products and other dietary modulators with anti diabetic activity. This tendency is because insulin, to date, cannot be used orally and its repeated injections have many undesirable adverse effects. In addition, certain oral hypoglycaemic agents are not effective in lowering the blood sugar in chronic diabetic patients. The global information on ethno-botanicals includes about 800 medicinal plants are used for controlling diabetes mellitus. A number of plants, including vegetables, are commonly consumed in India and other parts of the world; and many of these are purported to possess anti diabetic potential (Elgawish et al., 1999) More than 100 medicinal plants are mentioned in the Indian system of medicines including folk medicines for the management of diabetes.
Though pathophysiology of diabetes remains to be fully understood, experimental evidences suggest the involvement of free radicals in the pathogenesis of diabetes (Shanmugasundaram et al., 1990) and more importantly in the development of diabetic complications (Yang et al., 1990; Murakami et al., 1996; Baskaran et al., 1990). Free radicals are capable of damaging cellular molecules, DNA, proteins and lipids leading to altered cellular functions. Many recent studies reveal that antioxidants capable of neutralizing free radicals are effective in preventing experimentally induced diabetes in animal models (Ji et al., 2002; Oberlay, 1988) as well as reducing the severity of diabetic complications. For the development of diabetic complications, the abnormalities produced in lipids and proteins are the major etiologic factors. In diabetic patients, extra-cellular and long lived proteins, such as elastin, laminin, collagen are the major targets of free radicals. These proteins are modified to form glycoproteins due to hyperglycaemia. The modification of these proteins present in tissues such as lens, vascular wall and basement membranes are associated with the development of complications of diabetes such as cataracts, micro-angiopathy, atherosclerosis and nephropathy (Shabana et al., 1990). During diabetes, lipoproteins are oxidized by free radicals. There are also multiple abnormalities of lipoprotein metabolism in very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) in diabetes. Lipid peroxidation is enhanced due to increased oxidative stress in diabetic condition. Apart from this, advanced glycation end products (AGEs) are formed by non-enzymatic glycosylation of proteins. AGEs tend to accumulate on long-lived molecules in tissues and generate abnormalities in cell and tissue functions (Brownlee, 1996; Akhtar & Iqbal, 1991). In addition, AGEs also contribute to increased vascular permeability in both micro and macro vascular structures by binding to specific macrophage receptors. This results in formation of free radicals and endothelial dysfunction. AGEs are also formed on nucleic acids and histones and may cause mutations and altered gene expression.

**Review of Literature:**

Diabetes mellitus is a widespread disorder, which has long been in the history of medicine. Before the advent of insulin and oral hypoglycaemic drugs the major form of treatment involved the use of the plants. But now from the last two decades there has been a new trend in the preparation and marketing of herbal drugs.

In traditional systems, a number of plant extracts have been used for their hypoglycaemic activity like Karela (*Momordica charantia*), Jambul (*Syzigium cumini*), fenugreek (*Trigonella foenum-graaecum*).
foenum-gracecum), Gudmar (Gymnema sylvestre). Gymnemic acids 1 – 4, guarmarin in Gymnema sylvestre shows antidiabetic activity.


A few other plants with hypoglycaemic activity: Panax ginseng, Dioscorea dumatorum, Cuminum nigrum, Ocimum sanctum, Curcuma longa, Phyllanthus embelica.

Oral administration of the extract of Asteracantha longifolia Nees. (20 g/kg of starting material) can significantly improve glucose tolerance in healthy human subjects and diabetic patients (Aderibigbe & Emudianughe, 1999)

Oral administration of 2, 3, and 4 g/kg of Achyranthes aspera L produced a significant dose-related hypoglycaemic effect in normoglycaemic and alloxan-induced diabetic rabbits. In these animals, water and methanol extracts also decreased blood sugar levels. The plant may act by providing certain necessary elements like calcium, zinc, magnesium, manganese and copper to the beta-cells (Yoshikawa et al., 1996)

The antidiabetic activity of Mangifera indica L (Mango) was seen when an extract of the leaves of M indica was given to rats 60 min before the glucose. The hypoglycaemic effect of the aqueous extract was compared with that of an oral dose of chlorpropamide (200 mg/kg). The hypoglycaemic action of this plant may be due to a reduction in the intestinal absorption of glucose (Chandra et al., 2007)

Male Swiss mice were orally loaded with glucose after the extracts of Daucus carota L had been given by oral loading. The extract of Daucus carota L. was prepared by boiling the dried material with water or macerating it with 80 % ethanol. It was shown that the extract improved the glucose tolerance (Swatson-Flatt et al., 1990)

Seeds of Coriandrum sativum L (Coriander), when supplied in the diet (6.25 % by weight) and infusion (1 g/400 ml) in place of drinking, reduced the hyperglycaemia during the development of streptozotocin-induced diabetes in mice (Born et al., 1990)

The anti hyperglycaemic effect Cuminum cyminum L. was studied in healthy rabbits subjected to weekly subcutaneous glucose tolerance tests after gastric administration of water, tolbutamide or a traditional preparation of the plant. The results showed that the C. Cyminum significantly decreased the area under glucose tolerance curve and the hyperglycaemic peak (Ohnishi et al., 1996)

Oral administration of the flavonoids content (8%) of the seeds of Cuminum nigrum caused a significant blood glucose lowering at a dose range of 0.5 to 1.5 g/kg, both in normoglycaemic
and alloxan-induced diabetic rabbits. The maximum of decrease in glycaemia was obtained within 4-8 h; the normal level of glycaemia was reached within 24 h of drug administration. In contrast, the alkaloids isolated from *C. nigrum* (0.01%) had no significant hypoglycaemic effect in either normoglycaemic or diabetic rabbits. A high dose of 5g/kg did not produce any adverse effects in a 7-day acute toxicity study in rabbits (Sitasawad et al., 2000).

Oral administration of the aqueous fraction of an alcoholic extract of leaves of *Vinca rosea* L. *Catharanthus roseus* Don leads to marked lowering of glycaemia in normal and streptozotocin-induced diabetic rats. This effect was comparable with that of tolbutamide (http://plants.usda.gov/java/profile?symbol=CURE). Three suspension cultures of *C. rosea* were obtained from three different cell lines (CWS, CW-A and CWS-G). In the production medium, the first cell line produced 0.1 % ajmalicine and the cell extract caused a 71 % decrease in glycaemia in diabetic rats. In contrast, in the growth medium, CWS produced trace amounts of alkaloids and the extract did not show any anti-diabetic activity. The CWA cell line synthesized 0.036 % ajmalicine. The extract had no hypoglycaemic effect while in the growth medium the cells produced trace amounts of alkaloids and the extract induced an 86 % decrease in blood sugar. The CWS-G cell line did not produce significant levels of alkaloids and had no hypoglycaemic effect (Sindurani & Rajamohan, 2000).

The extract of *Rhazya stricta* Decne at oral doses of 0.5, 2.0 and 4.0 g/kg reduced glycaemia 1 h (2 and 4 g/kg) and 2 h (4 g/kg) after administration to streptozotocin-diabetic rats. The insulin concentration increased 1, 2 and 4 h after administration of the extract at 2 and 4 g/kg. Treatment of control animals with the extract did not affect glycaemia insulin or glucagon levels for up to 4 h after the administration of the extract. Simultaneous treatment of healthy and diabetic rats with the extract (0.5, 2.0 and 5.0 g/kg) and glibenclamide (5.0 mg/kg) exacerbated the effects on glucose, insulin and glucagon. At doses of 0.5, 2.0 and 4.0 g/kg/day for 6 consecutive days the glycaemia was reduced by approximately 6, 8 and 30 %, respectively (Vats et al., 2002).

Ginseng polypeptides (GPP) isolated from the root of *Panax ginseng* Mey. (*Asiatic ginseng*) decreased the level of blood sugar and liver glycogen when injected i.v. to rats at doses of 50-200 mg/kg without affecting total blood lipid concentrations. When mice were injected subcutaneously with daily doses of 50 and 100 mg/kg for 7 successive days, GPP was also found to decrease blood glucose and liver glycogen and stimulated the release of insulin. In addition, GPP was found to decrease hyperglycaemia induced experimentally by injection of adrenaline, glucose and alloxan (Li et al., 1990; Morita et al., 2002). The oral administration of the water extract of *Ginseng Radix* (GR) to normal and adrenaline-induced
hyperglycaemic mice caused a significant decrease in blood glucose level 4 h after its administration. The hepatic content of the facilitative glucose transporter isoform 2, liver type glucose transporter (GLUT 2) protein significantly increased in the orally GR-treated healthy and adrenaline-induced hyperglycaemic mice compared to that in the controls (Benjamin et al., 1994). Recently, ginseng, which is among five crude drugs included in the traditional Chinese prescription, Byakkoka- ninjin-to, was investigated using genetically obese diabetic KK-CA(y) mice and alloxan-diabetic mice. The water extract of ginseng, when individually tested, markedly lowered blood glucose level in diabetic animals (Saikat et al., 2009).

On 4 separate occasions, 10 non-diabetic subjects and 9 subjects with type 2 diabetes mellitus received 3 Panax quinquefolius L. (American ginseng) or placebo capsule, either 40 minutes before or together with a 25 g oral glucose challenge (gc). Significant reduction in glycaemia was observed only when ginseng was taken 40 min before gc in non-diabetic subjects and the same result was seen in diabetic subjects (Roman-Ramos et al., 1995).

Saponin isolated from the leaves of Acanthopanax senticosus injected to mice (100, 200 mg/kg, i.p.) decreased experimental hyperglycaemia induced by injection of adrenaline, glucose and alloxan, without affecting the levels of blood sugar in untreated mice (Baynes & Thorpe, 1997).

Elatosides E was isolated from the root cortex of Aralia elata Seem. (Japanese Angelica) Seem. It was shown to affect the elevation of plasma glucose levels in an oral sugar tolerance test in rats. The structures of elatosides E and F have been elucidated. Moreover, the hypoglycaemic activity of oleanolic acid and nine oleanolic acid glycosides isolated from the root cortex of this plant were tested (Li et al., 1990). Five new saponins named elatosides G, H, I, J, and K were isolated from a garnish foodstuff “Taranome” which is the young root shoot of A. elata Seem. Elatosides G, H, and I were found to exhibit potent hypoglycaemic activity in the oral glucose tolerance test in rats (Benjamin et al., 1994). Nine oleanolic acid oligoglycosides were isolated from the cortex of A. elata (Trinder, 1969).

In order to identify the antidiabetic agent from the stem bark of Kalopanax pictus Nakai, seven kinds of chemical constituents including hederagenin glycosides and phenolic glycosides were isolated. The antidiabetic evaluation of these isolates in streptozotocin-induced diabetic rats showed that kalopanax saponin A has a potent antidiabetic activity in contrast to a mild activity of hederagenin (Araya et al., 2002).

To investigate the relationship between the intestinal bacterial metabolism of kalopanaxsaponin B and H from K. pictus, and their antidiabetic effect, kalopanaxsaponin B and H were metabolized by human intestinal microflora and the antidiabetic activity of their
metabolites was measured. The main metabolites of kalopanaxsaponin B were kalopanaxsaponin A and hederagenin. The main metabolites of kalopanax H were kalopanaxsaponin I and hederagenin. Among kalopanaxsaponin B, H and their metabolites, kalopanaxsaponin A showed the most potent antidiabetic activity, followed by hederagenin (Raza et al., 1996).

The hypoglycaemic effect of neutral detergent fiber from *Cocos nucifera* L. (*coconut*) was tested in rats fed 5%, 15% and 30% glucose. Increase in fiber intake caused a significant lowering in glycaemia and serum insulin. Moreover, it increases the fecal excretion of Cu, Cr, Mn, Mg, Zn and Ca. The results suggest the beneficial effect of inclusion of coconut fiber in the diet (Pal et al., 2003).

Oral administration of an extract from *Pergularia tomentosa* Span.to normoglycaemic rats produced a hypoglycaemic effect comparable to Daonil (Seth & Sharma, 2004).

In humans: GS4 (400 mg/day) extracted from leaves of *Gymnema sylvestre* R. Br., was administered to type II diabetic patients for 18-20 months as a supplement to the conventional oral drugs. During GS4 supplementation, the patients showed a significant reduction in blood glucose, glycosylated haemoglobin and glycosylated plasma proteins, and conventional drug dosage could be decreased. Five of the 22 diabetic patients were able to discontinue their conventional drugs and maintain their blood glucose homeostasis with GS4 alone. These data suggested that pancreatic beta cells may be regenerated and/or repaired in type II diabetic patients on GS4 supplementation. This is supported by the appearance of raised insulin levels in the serum of patients after GS4 supplementation (Naziroglu & Cay, 2001).

Furthermore, GS4 was administered (400 mg/day) to 27 patients with insulin-dependent diabetes mellitus (type I). GS4 therapy appears to enhance endogenous insulin release, possibly by regeneration/revitalisation of the residual beta cells (Chattopadhyay et al., 1991).

Investigation of the hypoglycaemic activity of saponin constituents from gymnemic acid, a crude saponin fraction of *G. sylvestre*, identified not only two new saponins, gymnemosides a and b, but also gymnemic acid V as the active principle (Bierer et al., 1998).

Recently, effects of the water soluble fraction of an alcoholic extract of *G. sylvestre* leaves on glycogen content of isolated rat hemidiaphragm was studied in normal and glucose fed hyperglycaemic rats. In glucose fed rats, the leaf extract lowered the glycogen content of the tissue and this effect was amplified by insulin (Ghosh, 2005).

Cryptolepine is a natural product isolated from *Cryptolepis sanguinolenta*. A series of substituted and heterosubstituted cryptolepine analogues have been synthesised. Antihyperglycaemic activity was measured *in vitro* and in a NIDDM mouse model to
generate the first structure bioactivity study of the cryptolepine nucleus (Barham & Trinder, 1972).

**Objectives:**
1. To collect the selected crude drugs and authenticate it.
2. To extract the authenticated crude drugs.
3. To perform phytochemical screening of extracts.
4. To evaluate anti-diabetic & antimicrobial activity of extract.

**Need of Work:**
The plants *Annona squamosa* and *Cuscuta reflexa* are claimed to possess various medicinal properties. As the chemical investigation of the bark of Annona and Cuscuta is not dealt in deep, detailed phytochemical and pharmacological investigation can be done. This will give us an insight mechanism into the therapeutic action which can be useful for research and therapeutic activities.

Oxidative stress is one of major causative factors in induction of many chronic and degenerative diseases including atherosclerosis, diabetes mellitus, cancer, Parkinson's disease, Alzheimer's disease, chronic renal failure, immune dysfunction and is involved in aging. The anti-diabetic potential of certain indigenous plants is still largely unexplored. In the recent past; there has been growing interest in exploiting the anti-diabetic activities of different Ayurvedic medicinal herbs, owing to their natural origin, cost effectiveness and lesser side effects.

**Methodology:**

**Source of data**

Data obtained from journals and publications of different literature and related articles, which will be searched from science direct, pubmed, med line, google-scholar, blog search ISPUB & Delnet.
Collection of Different Medicinal Plants:

Crude Drug A: Annona squamosa bark

Annona squamosa is also called as sugar-apple, belongs to the family Annonaceae, species of Annona native to the tropical America (Sitasawad, 2000). The sugar apple tree ranges from 10 to 20 ft (3-6 m) in height with open crown of irregular branches, and some-what zigzag twigs. Deciduous leaves, alternately arranged on short, hairy petioles, are lanceolate or oblong, blunt tipped, 2 to 6 in (5-15 cm) long and 3/4 to 2 in (2-5 cm) wide; dull-green on the upperside, pale, with a bloom, below; slightly hairy when young; aromatic when crushed (Kubish et al., 1997). 10-hydroxy-16-hentriacetanone (Matteucci & Giampietro, 2000), Squamocenin a new Acetogenin (Ali, 1997), Annotemoyin-2, Reticulatain-2, Samaquasine A, benzoquinazeline alkaloid ( Chattopadhyay et al., 1991), Annonacin, Annonacin-A, Annonastatin (http://ayurvedicmedicinalplants.com/plants/3133.html), Annotemoyin-1, Annotemoyin-2 (Akhtar & Iqbal, 1991), Squamocin (Mukhlesur et al., 2005), cholesteryl glycopyranoside (Park et al., 1998), Anonin I, Anonin VI (Nahar, 1993), Squamocin-O(1) and Squamocin-O(2) (Morton, 1987) have been Isolated from Annona squamosa.

Crude Drug B: Cuscuta reflexa

Cuscuta reflexa is also called as Amar bel (meaning, immortal vine) is an unusual parasitic vine related to the Morning glory family (Convolvulaceae). It grows in a prolific manner over host plants (or other support) with inter-twined stems, giving it a common name of Devils Hair and Giant Dodder ( Chattopadhyay, 1998; Lipinski, 2001). The Amar bel extract will be prepared from arial parts.

Selection of Animals for Antidiabetic study:
The Wistar albino rats of either sex weighing between 100 – 150 gm would be procured to study acute toxicity and antidiabetic activity studies. The animals would be stabilized for 1 week; in standard condition at room temp; 60 ± 5% relative humidity and 12 light dark cycles. The Animal house facilities are approved by Institutional Animal Ethics Committee.

Pharmacological studies:

Acute Toxicity Studies:
The acute toxicity study would be carry out in adult female albino rats by “fix dose” method of OECD (Organization for Economic Co-operation and Development) Guideline No. 420.
Fixed dose method as in Annex 2d: Test procedure with a starting dose of 2000 mg/kg body weight would be administered orally to overnight fasted animals. Then the animals have to observe continuously for three hour for general behavioural, neurological, autonomic profiles and then every 30 min for next three hour and finally for mortality after 24 hour till 14 days (Kim et al., 1998).

**Anti Diabetic Activity:**
For the assessment of anti diabetic activity, two dose levels have to choose in such a way that, one dose was approximately one tenth of the maximum dose during acute toxicity studies, and a high dose, which was twice that of one tenth dose (200mg/kg, 400mg/kg).

**Induction of Diabetes:**
Overnight fasted albino rats have to make diabetic by injecting alloxan monohydrate (in the ice cold normal saline) intraperitoneally at a dose of 120 mg/kg body weight. After that the animals have to left aside for 4 hrs and then 10% glucose solution was placed in the cages for 24 hrs. Rats with blood glucose level above 250 mg/dl would consider as diabetic (Saikat et al., 2009).

**In vivo Experimental Design:**
The animals were randomly divided into 7 groups of six animals each after the induction of diabetes.
Group 1: Non diabetic control rats received 1-2% vehicle, 2ml/kg body weight per oral, once daily for 5 weeks.
Group 2: Diabetic control rats received 1-2% vehicle, 2ml/kg body weight per oral, once daily for 5 weeks.
Group 3: Diabetic rats received standard drug Glibenclamide, 10 mg/kg body weight, in 1% vehicle, orally, once daily for 5 weeks.
Group 4: Diabetic rats given ethanolic extract, 200 mg/kg body weight of *Annona squamosal* bark, made a fine suspension with 1-2% vehicle, orally for 5 weeks.
Group 5: Diabetic rats given ethanolic extract, 400 mg/kg body weight of *Annona squamosal* bark, made a fine suspension with 1-2% vehicle, orally for 5 weeks.
Group 6: Diabetic rats received aqueous extract, 200 mg/kg body weight of *Annona squamosal* bark, made a fine suspension with 1-2% of vehicle, given orally for 5 weeks.
Group 7: Diabetic rats received aqueous extract, 400 mg/kg body weight of *Annona squamosal* bark, made a fine suspension with 1-2% vehicle, given orally for 5 weeks.

Group 8: Diabetic rats received P-Ether extract, 200 mg/kg body weight of *Annona squamosal* bark, made a fine suspension with 1-2% of vehicle, given orally for 5 weeks.

Group 9: Diabetic rats received P-Ether extract, 400 mg/kg body weight of *Annona squamosal* bark made a fine suspension with 1-2% vehicle, given orally for 5 weeks. Same Procedure Have to be follow for screening of antidiabetic activity of *Cuscuta reflexa* areal part.

The blood samples were collected from the retro orbital plexus at 1, 3, 5, 7, 24 hrs (Acute study) and at the end of 1, 3 and 5 weeks (Chronic study) (Lieb et al., 1990; Vuksan et al., 2000; Wang et al., 1990). Blood glucose levels were determined by the glucose oxidase method (Kim et al., 1998; Kimura et al., 1999).

**In-vitro Experimental Design:**

A simple model system has touse to evaluate effects of clove bud extracts on glucose Movement *in vitro*. This model was adapted from a method described by Edwards et al. (1988) which involved the use of a sealed dialysis tube into which 15 ml of a solution of glucose and NaCl (0.15 M) was introduced and the appearance of glucose in the external solution was measured. Glucose concentrations measure by using the glucose oxidase method of Analysis.
References:


