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Antidiabetic Activity of *Gnetum Gnemon* Fruits Against Type 2 Diabetes

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ABSTRACT

Type 2 diabetes occurs when the body becomes resistant to insulin or doesn't make enough insulin. The major treatment approach for diabetes is the use of synthetic drugs and insulin. However the side effects associated with the use of synthetic agents attracts the attention of drug discovery professionals to search an effective treatment using natural products. Malaysian plants and herbs have been used as medicinal agents and studies have proved their significance in the treatment of diabetes. As the fruits of *Gnetum gnemon* (family: Gnetacea) locally known as melinjo are traditionally used to control diabetes by Malaysian and Indonesian tribes, the present study was aimed to evaluate the folklore claim about *G. gnemon* fruits to treat diabetes. Hexane extract (GGHE), chloroform extract (GGCE), ethanol extract (GGEE) and water extract (GGWE) were obtained by successive extraction using soxhlet extractor. The preliminary phytochemical analysis was carried out to perceive phytoconstituents in *G. gnemon* extracts. Diabetes was induced in albino rats by single dose of streptozotocin (60 mg/kg b.wt. *i.p.*) followed by nicotinamide (120 mg/kg b.wt. *i.p.*). Streptozotocin-induced diabetic rats were separately treated with GGHE, GGCE, GGEE and GGWE at dose of 400 mg/kg b.wt. and standard drug, glibenclamide (10mg/kg) respectively by oral administration for 14 days and antidiabetic potential of the extracts were assessed. Prelimary phytochemical screening showed the presence of flavonoids, terpenoids, phenolic compounds and tannins. Oral administration of diabetic rats exhibited significant ($P<0.001$) antidiabetic activity. Various serum parameters such as glucose, triglyceride, total cholesterol, HDL, LDL, VLDL, bilirubin, total protein, urea, creatinine and uric acid were significantly ($P<0.001$) restored in the GGEE treated animals compared with diabetic rats and normal control. Histological study supported the protective effect of *G. gnemon* on pancreas in GGEE treated animals. The results illustrate *G. gnemon* to be a promising alternative for diabetes mellitus management, thus supporting its folklore claim.

Keywords: *Gnetum gnemon* fruits, diabetes, extracts, antidiabetic

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INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by absolute (type I) or relative (type II) insulin deficiencies due to the loss of glucose homeostasis that leads to impaired glucose metabolism and other energy-yielding fuels such as lipids and protein [1]. Hyperglycemia resulting from unregulated glucose level generally leads the development of other diabetic complications including nephropathy, neuropathy, retinopathy and hepatopathy [2]. A report revealed that 415 million people were estimated to have diabetes in 2015 worldwide, with this number projected to increase to 642 million in 2040 [3].

In modern medicine, no satisfactory effective therapy is still available to control as well as for the treatment of diabetes mellitus. Scientific studies available on medicinal plants indicate that promising phytochemicals can be developed for many health problems. The common advantages of herbal medicines are effectiveness, safety, affordability and acceptability. For the last few decades, research on herbal medicine has been significantly increased due to its importance and its demand to use for the treatment of diabetes worldwide. Various Malaysian plants and herbs have been used as medicinal agents and studies have proved their significance in the treatment of a variety of diseases. Many medicinal plants have been claimed by folk medicine to reduce the blood sugar level but still it requires the scientific investigation to establish their effectiveness and toxicity to identify a novel alternative herbal drug. One such plant, *Gnetum gnemon*, belonging to the family, Gnetacea is commonly known as melinjo or belinjo in Malaysia. It is native of Assam (North East India) eastward through Malaysia and Fiji [4].

G. gnemon is found throughout Malaysia and all tropical regions. It has been cultivated in South East Asian Countries such as Indonesia, Malaysia, Thailand and Philippines. The fruits of this plant are traditionally used to control the blood sugar level by Malaysians and Indonesians. Also this plant has been used to reduce constipation and inflammation [5] and its leaf sap is used to treat eye complications [6]. The scientific literature review revealed that the ripe fruits are used for stomach cleansing and prevent constipation [7], antimicrobial activity of seed and peel [8] and anti-oxidant property of the plant are also reported [9]. Also the plant seed extracts have been reported for the presence of antioxidant, protein [10] and resveratrol [11] and phenolic compounds from stem bark [12]. The edible parts of *G. gnemon* were reported for their antioxidant and DNA damage prevention activities [13]. There is no available scientific report for the antidiabetic activity of *G. gnemon* fruits. Hence, the antidiabetic activity of *Gnetum gnenom* fruits was undertaken in this present study.

MATERIALS AND METHOD

Plant Material

Gnetum gnemon fruits were collected from Kampung Ibok, Kemaman, Terengganu in the month of September 2014. It was authenticated by Dr. J Anbu Jeba Sunilson, Pharmacognosist, School of Pharmacy, KPJ Healthcare University College, Nilai, Malaysia. A voucher specimen was deposited in the departmental herbarium.

Preparation of Extract

Fresh fruit of *G. gnemon* (4kg) was washed thoroughly with tap water and dried under shade. Then the dried fruits were pulverized using ball mill into coarse powder. The coarse powder was successively extracted with organic solvents such as hexane, chloroform, ethanol and water by soxhlet extraction method. The hexane extract (GGHE), chloroform extract (GGCE), ethanol extract (GGEE) and water extract (GGWE) were filtered separately and the excessive solvent was evaporated and concentrated using a rotary vacuum evaporator [14]. The % yield of the extracts was calculated. All the extracts were kept in desiccator until further use.

Preliminary Phytochemical Analysis

All the extracts obtained were subjected to the preliminary phytochemical investigation to identify the phytoconstituents present in *G. gnemon* fruits' extracts by standard methods [14]. The results are shown in Table 1.

Table 1: Preliminary phytochemical analysis of *G. gnenom* fruit extracts

Chemical constituents	Hexane extract	Ethanol extract	Chloroform extract	Water extract
Alkaloids	+	-	+	+
Sterols	+	-	+	-
Carbohydrates	+	+	+	+
Glycosides	-	-	-	-
Fixed oils & fats	+	-	+	-
Tannins & Phenolic compounds	-	+	-	+
Proteins & Amino acids	-	-	-	+
Triterpenoids	+	+	+	+
Saponins	-	-	-	-
Gums & Mucilages	+	-	+	+
Flavonoids	+	+	+	+
Volatile oils	-	-	-	-

+ = Present, - = Absent

Experimental Animals

Healthy adult male swiss albino mice of both sex (20 - 30 g) and male wistar albino rats (200 - 280 g) were used for this investigation and kept at KPJUC vivarium. The animals were housed in

groups of six animals in standard cages and acclimatized for 7 days under standard environmental condition at an ambient temperature (25±2 ^{0}C) with 12 h light-dark cycle. The animals were supplied a standard pellet food & water *ad libitum*. All experiments on animals were conducted according to the ethical norms approved by Animal Ethics Committee, KPJ Healthcare University College, Kota Serimas, Nilai, Negeri Sembilan (KPJUC/CRI/MPT/EC/ 2014/10). All the experiments were performed according to current guidelines for the care of the laboratory animals and the ethical guidelines. The standard orogastric cannula was used for oral drug administration.

Acute Toxicity Studies

Acute toxicity study for the extracts was carried out according to the Organization for Economic Co-operation & Development (OECD) guideline 423. Prior to the acute experimental procedure, the animals were fasted overnight. GGHE, GGCE, GGEE and GGWE were suspended in CMC separately and administered orally with a starting single dose of 5 mg/kg. Any signs of intoxication, lethargy, behavioral modification and morbidity for a period of 2 h, then occasionally for 4 h for severity of any toxic signs and mortality were observed. When no mortality was observed the same dose was additionally administered to one more animal for each group. If no mortality is observed at this dose, the same procedure was repeated for dose levels of 50, 500, 1000 and 2000 mg/kg of extracts on separate newer groups. The LD_{50} was thus determined and 1/10th of LD_{50} value was taken as ED_{50} value for the animal study. The animals were kept under observation up to 14 days after drug administration to find out any delayed mortality [15].

Screening of Antidiabetic Activity on streptozotocin-nicotinamide induced diabetic rats

The antidiabetic activity of the various extracts of *G. gnemon* fruits were assessed on streptozotocin-nicotinamide induced diabetic rats by the method developed by Masiello *et al*., (1998) [16] with some modifications. Forty two (42) male wistar albino rats (200 - 280gram) were divided into six (6) groups of six rats each (n = 6). Prior to the experimental procedure, the animals were fasted overnight. The weight of each rat was recorded before administering Streptozotocin. Streptozotocin (60 mg/kg) was prepared in fresh cold 0.1M citrate buffer (pH 4.5). Diabetes was induced by single intraperitonial injection of streptozotocin (60 mg/kg) in all rats except group I which received 1% w/v Sodium CMC suspension and served as normal group. 2% glucose solution (20ml/kg b.wt) was given to the streptozotocin injected animals to overcome the drug-induced hypoglycemic shock. After 15 minutes the animals were given the single dose of nicotinamide 120 mg/kg body weight dissolved in distilled water. On the 3rd day, a drop of blood from the rats' tail vein was withdrawn by the tail tipping method and the blood glucose level was checked using digital blood glucometer. The rats injected with streptozotocin showed elevated

levels of blood glucose. Rats which showed the blood glucose level of 185 to 460 mg/dl were taken for the experiment. The diabetic rats in group II also received 1% Sodium CMC suspension and served as diabetic control. The diabetic rats in group III served as standard and received Glibenclamide (5 mg/kg, *p.o.*). The diabetic rats in group IV – VII were treated with a single dose of GGHE, GGCE, GGEE and GGWE (400 mg/kg, *p.o.*) respectively for consecutive 14 days. The blood glucose level was estimated on day 1, 7 and 14 for all groups of animals.

Biochemical analysis

At the end of the treatment period (14 days), the animals in the active extract treated group were fasted for at least 16 hours. Blood samples were collected by retro-orbital sinus puncture using a capillary tube under diethyl ether anesthesia in Eppendroff's tubes (1 ml) containing 50 µl of anticoagulant (10% trisodium citrate). The serum was separated by centrifuging the blood samples at 4000 rpm for 15 min for biochemical analysis. Triglycerides, Total cholesterol (TC), High density lipoprotein (HDL), low density lipoprotein (LDL), very low density lipoprotein (VLDL), total protein (TP), urea, uric acid and total bilirubin (TB) were measured for the normal control group, glibenclamide treated group and the active extract treated group [17].

Histopathological Study

After collection of blood sample, all the animals were sacrificed by cervical dislocation and pancreas was removed and immersed in 10% buffered formalin solution. The tissue were embedded in paraffin wax and cut into 5 µm thickness, then stained with haematoxylin-eosin staining for histopathological examination and pathological changes were observed [18].

Statistical analysis

Data were reported as mean ± standard error of the mean (S.E.M.) and was compared using one-way analysis of variance (ANOVA) for determining the significant difference. The inter group significance was analyzed using Dunnet's post hoc test using SPSS software version 18.0. p values <0.01, <0.001 were considered as significant [19].

RESULTS AND DISCUSSION

The percentage yield of *G. gnemon* fruits root extracts was calculated. Among the four extracts, GGEE had the highest percentage yield (18.72%) followed by GGWE (14.26%) and CCGG (8.58%) whereas GGHE had the lowest percentage yield (5.68%). Phytochemical tests revealed the presence of carbohydrates, triterpenoids and flavonoids where as volatile oils, saponins and glycosides were absent in all the extracts. Phenolic compounds and tannins were found in ethanol and water extracts only (Table 1). The oral administration of the extracts of *G. gnemon* fruits did

not exhibit any mortality in mice up to a dose level of 2000 mg/kg in acute toxicity studies. This indicates the non-toxic therapeutic index of *G. gnemon* fruits. So the dose of the extracts was fixed at 200 mg/kg, i.e. 1/10th of the maximum tolerated dose.

In diabetic animals, marked elevations (P<0.001) in blood glucose level was noted and these levels were observed to continuously increase until day 14. Daily administration of the various extracts of *G. gnemon* fruits (400 mg/kg p.o. for 14 days) to the respective groups of diabetic rats antagonized this action and showed a remarkable reduction in blood glucose level (Table 2). GGEE of *G. gnemon* fruits showed significant (P<0.001) reduction in blood glucose level followed by GGWE (P<0.01). An increased activity of serum AST, ALP, ALT, BG, and TC and decreased activity of serum TP were observed in streptozotocin induced diabetic rats. Oral treatment with the standard drug, glibenclamide (5 mg/kg) and GGEE of *G. gnemon* fruits significantly (P<0.001) restored these enzyme levels to near normal range followed by water extract (P<0.01) at a dose of 400 mg/kg (Table 3). On the other hand, these extracts had no effect on blood glucose concentrations of normal control animals. Histopathological studies support the antidiabetic effect of the ethanol extract of *G. gnemon* fruits (Figure 1a-d.)

Table 2: Effect of *G. gnenom* fruit extracts on blood glucose level (mg/dl)

Treatment	**Serum Glucose (mg/dl)**			
	Time (days) After Treatment			
	Normal	**Day 1**	**Day 7**	**Day 14**
Normal Control	74.7 ± 3.2	75.6 ± 2.6	73.6 ± 4.2	75.5 ± 5.2
Diabetic Rats	75.6 ± 2.8	479.2 ± 6.5	486.4 ± 7.1	508.6 ± 5.3
Diabetic rats treated with Glibenclamide (10 mg/kg)	74.7 ± 4.2	356.7 ± 8.2	132.4 ± 2.1★★	82.9±8.5★★
Diabetic rats treated GGHE (400 mg/kg)	75.7 ± 3.2	479.2 ± 9.5	361.2 ± 4.6	417.2 ± 11.4
Diabetic rats treated with GGCE (400 mg/kg)	75.2 ± 2.62	475.4 ± 8.3	358.4 ± 6.6	414.8 ± 10.6
Diabetic rats treated with GGEE (400 mg/kg)	76.2 ± 3.2	463.1 ± 9.2	199.6 ± 2.3*	86.5 ± 3.2**
Diabetic rats treated with GGWE (400 mg/kg)	75.5 ± 2.9	496.2 ± 5.1	267.5 ± 6.6*	223.5 ± 3.6*

Values are mean ± S.E.M., n=6, [a]P<0.01, [b]P<0.001, statistically significant between diabetic control group and normal group, *P<0.01, **P<0.001, statistically significant between extract treated groups and Diabetic control group

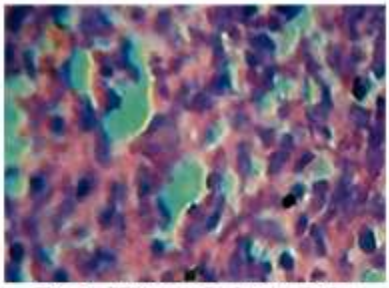

Fig.a. Histopathology of the pancreas of control rat showing normal cell Architecture (45X)

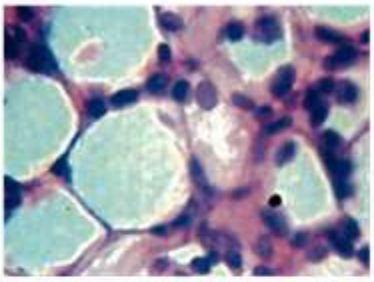

Fig. b. Histopathology of the pancreas of STZ induced diabetic rat showing massive cells Necrosis (10X)

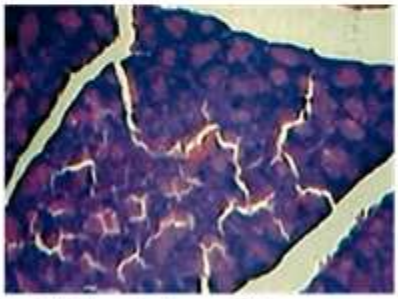

Fig. c. Histopathology of the pancreas of STZ induced diabetic rat administered with Glibenclamide showing regeneration of cells (10X)

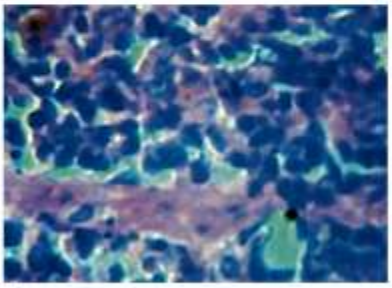

Fig. d. Histopathology of the pancreas of STZ induced diabetic rat administered with ethanolic extract of *Gnetum gnemon* (400mg/kg) showing regeneration with patchy necrosis (10X)

Figure 1: Histopathology of Pancreas (Haematoxylin and Eosin stained)

Table 3: Effect of *G. gnenom* fruit extracts on serum biochemical constituents

Treatment	Triglycerides (mg/dl)	Total Cholesterol (mg/dl)	High density lipoprotein (mg/dl)	Low density lipoprotein (mg/dl)	Very low density lipoprotein (mg/dl)	Total protein (gm/dl)	Blood urea (mg/dl)	Serum creatinine (mg/dl)	Uric Acid (mg/dl)
Normal	77.0 ± 0.57	57.1 ± 0.47	78.5 ± 0.76	47.83 ± 0.67	16.17 ± 0.60	09.00 ± 0.57	23.50 ± 0.76	0.48 ± 0.042	2.52 ± 0.075
Diabetic control	150.02 ± 0.6	120.2 ± 0.60	30.67 ± 0.42	160.2 ± 0.60	27.00 ± 0.58	06.00 ± 0.51	43.67 ± 0.71	1.22 ± 0.04	6.36 ± 0.049
Glibenclamide	84.50 ± 0.42**	60.17 ± 0.60 **	70.50 ± 0.42**	61.50 ± 0.42**	18.83 ± 0.30**	07.61 ± 0.66**	26.33 ± 0.88**	0.58 ± 0.06**	3.86 ± 0.06**
Ethanol extract	78.33 ± 0.76**	55.50 ± 0.76**	75.50 ± 0.42**	54.33 ± 0.47**	16.71 ± 0.62**	08.83 ± 0.54**	23.67 ± 0.66**	0.51 ± 0.06**	2.63 ± 0.061**

Values are mean ± S.E.M., n=6, [a]P<0.01, [b]P<0.001, statistically significant between diabetic control group and normal group, *P<0.01, **P<0.001, statistically significant between extract treated groups and Diabetic control group

DISCUSSION

The present study was undertaken to scientifically validate the traditional claim about *G. gnemon* fruits. The various extracts of *G. gnemon* fruits were obtained by successive solvent extraction method. The antidiabetic activity was assessed for all the extracts obtained and the active antidiabetic ethanol extract was subjected to biochemical analysis and histopathological study. The observed antidiabetic activity of the *G. gnemon* fruits extracts on type 2 diabetic induced rats scientifically proves its folkloric use in the management/treatment of diabetes. The findings suggest the phytoconstituents such as phenolic compounds and tannins, flavonoids, terpenoids present in this fruit might be responsible to exert the antidiabetic effect.

Prashant *et al.* (2013)[18] have reported that phenolic compounds, flavonoids, terpenoids potentiate the antidiabetic activity in streptozotoci*n*-induced diabetic rats. An *in-vivo* study in streptozotocin-nicotinamide induced diabetic rats and normal rats reveals that the flavonoid compounds such as boswellic acid, ellagic acid, quercetin, and rutin, exhibited significant antidiabetic activity [20]. A review also supported that flavanoids-rich food play positive roles in maintaining blood glucose levels, glucose uptake and insulin secretion and modulating immune function to prevent specific diabetes mellitus [21]. Previous studies have indicated the antidiabetic activity of terpenoids [22]. The antidiabetic potential of the terpenoids is further supported by a study done by Shehla *et al*., (2013) [23]. Recently, polyphenolic compounds have become subjects of interest because of their beneficial effects on human health [24]. An earlier report by Asmawati *et al*., (2014) [25] revealed that the phenolic compounds have been found to be beneficial in controlling diabetes. The antidiabetic activity of the phenolic compounds is supported by the study reported by Deepak *et al*., (2014) [26]. Therefore, the findings suggest that the anti-diabetic property of the *G. gnemon* fruit could be linked with the presence of phytoconstituents flavonoids, terpenoids, phenolic compounds and tannins.

CONCLUSION

From the findings, it was found that ethanol extract has significant anti-diabetic activity at a dose of 400mg/kg bwt and the activity was significantly comparable with the standard drug. This study scientifically proves the folklore claim of antidiabetic effects of *Gnetum gnemon*. However the active constituent(s) responsible for the anti-diabetic effect is to be isolated that will be part of the future research which may help to discover novel herbal anti-diabetic agent.

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