Antidiabetic activity of flavonoid fraction of Manilkara hexandra bark in alloxon-iduced diabetic rats

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Abstract

The present study was undertaken to assess anti-hyperglycemic properties of flavonoid fraction of Manilkara hexandra bark (FFMH), which have been reported in Ayurveda to be useful in diabetes mellitus. Acute oral toxicity studies of FFMH were performed as per OECD-423 guidelines and antidiabetic activity was investigated on alloxon-induced diabetic rats. The levels of plasma insulin, total hemoglobin, glycosylated hemoglobin, glycogen content ad hepatic enzymes (hexokinase and glucokinase) were also estimated in normal and experimental animals. The treatment with FFMH showed significant anti hyperglycemic activity from the first week onwards. The altered levels of plasma insulin, Hemoglobin and glycosylated hemoglobin were returned to near normal range in diabetic rats treated with FFMH at different doses and the results were comparable with that of the standard glibenclamide. In diabetic control hepatic glycogen content decreased significantly by 79.54% as compared to non-diabetic control. Treatment with FFMH led to 60.70% increase in hepatic glycogen content. Treatment with FFMH increased the Glucose -6-phosphate content in the liver, indicating an overall increase in glucose influx. The content of glucokinase and hexokinase was increased by FFMH treated groups. The present study indicates that treatment of FFMH at the doses of 100mg/kg and 200mg/kg brought the parameters altered to near normal level. From this we can conclude that FFMH can be used as a potent anti diabetic agent.

KeyWords: Manilkara hexandra, flavonoid, antidiabetic, alloxan, glibenclamide

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Introduction

In modern medicine no satisfactory effective therapy is still available to control diabetes. Insulin and oral diabetic agents are used as a mono therapy or in combination to achieve better glycogenic control. Management of diabetes without any side effects is a challenge to the medical system. Plants have played a major role in the introduction of new therapeutic agents and the beneficial multiple activities (Tierney et al., 2002; WHO, 1999).

Manilkara hexandra (Mimusopshexandra) is an evergreen tree belongs to family Sapotaceae. Collectively known as Manilkara trees, they occur throughout the tropics. Trees of this genus yield edible fruit, useful wood and latex. The best-known species are M. bidentata (Balata), M. chicle (Chicle) and M. zapota (Sapodilla) (Leland J Cseke et al., 1998). The bark is astringent, sweet refrigerant, aphrodisiac, alexipharmic and anthelmintic, it is useful in otorrhagia, ulitis, odontopathy, fever, colic dyspepsia, helminthiasis, hyper dyspepsia, burning sensation and vitiated conditions of pitta, it retards the fermentation process in toddy (Joshi, 2000).

This kind of scientific study has not been documented so far and most of the literature available on this plant was based on traditional or folklore information. Few reports are available on the use of this plant for its effects on ulcers, antimicrobial activity, antibacterial activity (Shah et al., 2004; Sumitra and Jigna, 2010).

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Hence, in present study, an attempt was made at investigating the anti diabetic of flavonoid fraction of Manilkara hexandra with a view to justifying the use of the plant.

Materials and Methods

Plant material

The bark of Manilkara hexandra was collected from medicinal garden, Kakatiya University, Andhra Pradesh, India and botanically identified by Dr. Raju S. Vastva, Professor, Department of Botany, Kakatiya University, Hanamkonda, Telangana and voucher specimen (PG/10/02) was stored in Department of Pharmacognosy and Phytochemistry, School of Pharmacy, Guru Nanak Institutions Technical Campus, Ibrahimpatnam, Hyderabad, Telangana, India for future reference. The collected bark was shade dried and coarsely powdered. The coarsely powdered bark (750gm) of Manilkara hexandra was extracted successively using petroleum ether (60-80oC), methanol, ethyl acetate and chloroform by soxhlation. The extracts were concentrated in vacuo using rotary evaporator at 40oC. The extracts thus obtained were stored in refrigerator (at 2-4°C) for further use.

Phytochemical screening

All the extracts of Manilkara hexandra bark were analyzed for the presence or absence of various phytochemicals using standard procedures as described by Trease and Evans (1989) and Edeoga et al., 2005. The color intensity or the precipitate formation was used as analytical responses to these tests. The ethyl acetate fraction which shows the presence of flavonoids (designated as FFMH) was used for further studies.

Chemicals

Streptozotocin (STZ) and glibenclamide were purchased from Alfa Aesar, Great Britain. Glucose standard strip/kits and ACCU-CHEK Active glucometer was purchased from Roche, Germany, Petroleum ether, ethyl acetate, chloroform and methanol were purchased from Labort fine Chem Pvt. Ltd, India. All other chemicals and reagents used were of analytical grade.

Animals

Albino rats (180-220gm) of both sexes and male Swiss albino mice (20-25gm) were used for the present investigation. They were obtained from the experimental facility of the School of Pharmacy, Guru Nanak Institutions Technical Campus, Ibrahimpatnam, Hyderabad, Telangana. They were housed in colony cages (four per cage) under conditions of standard lighting, temperature (22±3° C) and humidity, and were fed with standard pellet diet (Hindustan Lever, Kolkata, India) and water ad libitum. The animals were acclimatized to laboratory condition for one week before start of experiment. All these procedures described were reviewed and approved by the Institutional Animal Ethical Committee (IAEC), School of Pharmacy, Guru Nanak Institutions Technical Campus, Ibrahimpatnam, Hyderabad, Telangana (Approval number: 1374/PO/Re/S/10/CPCSEA).

Acute toxicity studies

For the limit test to determine the acute toxicity of the plant extract five female, Swiss albino mice (non-pregnant, nulliparous, 20-30g, age of 8-12 weeks) were used as recommended by OECD (Organization for Economic Co-operation and Development) (2008). Initially, 3-4 h fasted one mouse was orally given FFMH (1000 mg/kg) dissolved in distilled water and observed strictly for any sign of toxicity for first 4h and then for 24 h. After survival of the first mouse, the other four mice (fasted for 3-4 h) were also given FFMH at the same dose and were observed for physical and behavioral sign of toxicity up to 14 days.

Induction of diabetes

Albino Wistar rats were made diabetic by a single i.p injection of alloxan monohydrate (150 mg/kg) in saline (Sunil Mistry et al., 2008). Alloxan is commonly used to produce diabetic mellitus in experimental animals due to its ability to destroy the beta cells of pancreas possibly by generating excess reactive oxygen species such as H2O2, O2 and HO (Shah et al., 2004). The development of hyperglycemia in rats was confirmed by plasma glucose estimation 72 h post alloxan injection. The rats with fasting plasma glucose level of >175mg/dl were included in the study. In the experiment a total of 30 rats (24 diabetic surviving rats, six normal rats) were used. The rats were divided in to 5 groups and followed the treatment protocol as mentioned below.

Treatment protocol

Animals were divided into five groups, Group I was

normal control and received normal saline only. Group II was diabetic control and received alloxan (150mg/kg) and normal saline. Group 3 was standard and received alloxan (150mg/kg) and Glibenclamide (600 µg/kg) in aqueous solution daily using intragastric tube for 21 days (Maikai et al., 2008), Group 4 received alloxan (150mg/kg) + FFMH 100mg/kg in normal saline daily using intra gastric tube for 21 days, Group 5 received alloxan (150mg/kg) + FFMH 200mg/kg in normal saline daily using intra gastric tube for 21 days. After 21 days of treatment, blood was collected retro orbitally from the inner canthus of the eye under light ether anesthesia using capillary tubes. Blood was collected in fresh plasma tubes coated with EDTA and Plasma was separated in a T8 electric centrifuge (Remi, New Delhi) at 2000 rev/min for 2 min. Then animals were sacrificed by decapitation, liver tissue samples were collected for the assessment of plasma insulin, total hemoglobin and glycosylated hemoglobin hepatic glucokinase and hexokinase, glucose -6- phosphate and along with glycogen content in liver tissues.

Estimation of biochemical parameters

Estimation of blood glucose

Blood glucose was estimated by commercially available glucose kit (One Touch Ultra) Johnson Johnson based on glucose oxidase method (Trinder, 1969).

Plasma insulin:

Plasma insulin was determined by ELISA method using a Boehringer–Mannheim kit (Anderson et al., 1993) with an ES300 Boehringer analyzer (Mannheim, Germany).

Estimation of total haemoglobin and glycosylated hemoglobin

Total hemoglobin was determined by the method of Drabkin and Austin (1932) and glycosylated hemoglobin was determined by the method of Sudhakar Nayak and Pattabiraman (1981).

Estimation of Hepatic Glucokinase and Hexokinase activity

The liver was per fused with ice cold 0.15M KCL and 1mM EDTA solution and homogenized with twice its weight of ice cold buffer (0.01 cysteine and 1mM EDTA in 0.1 ml Tris –HCl, PH 7.40) and glucose -6-phospahte dependent spectrophotometric method (Crane and Sols, 1955). The liver was homogenized with 40 times its weight of ice cold buffer (0.1citrate –KOH, pH 6.5) and filtered through cheese cloth. Glucose -6-phosphatase activity was measured by phosphate release by the method of Marjorie, 1950. The calorimetric method for determination of phosphoric acid concentration in the supernatant of the assay mixture was employed (Fiske and Subbaraw, 1925).

Estimation of Glycogen content

The tissue sample was digested by hot concentrated 30% Potassium Hydroxide and treated with anthrone reagent. Glycogen content was determined as glucose colorimetrically (Morales et al., 1973).

Statistical analysis

The data for various biochemical parameters were analyzed using analysis of variance (ANOVA), and the group means were compared by Newman-Keuls multiple range test (NKMRT). Values were considered statistically significant at $p<0.001$.

Results and Discussion

Currently available drug regimens for the management of diabetes mellitus have certain draw backs (Marjorie, 1950) and therefore, there is a need for safer and more effective anti-diabetic drugs. Thus the present study was therefore undertaken to assess anti-hyperglycemic properties of flavonoid fraction of Manilkara hexandra, which have been reported in Ayurveda to be useful in diabetes mellitus.

Extraction

Various extracts like petroleum ether, methanol, ethyl acetate and chloroform of Manilkara hexandra bark were prepared by successive solvent extraction. The colour, nature and percentage yields of all obtained extracts were presented in the table 1.

Table 1: Colour, nature and percentage yields of all extracts

S.No	Extract	Weight (% W/W)	Color	Consistency
1	Petroleum ether	2.8	Yellow	Solid
2	Methanol	11.5	Brown	Solid
3	Ethyl acetate	4.7	Yellow	Solid
4	Chloroform	0.72	Yellow	Solid

Phytochemical screening

In present investigation, four different solvent extracts of Manilkara hexandra bark are subjected to phytochemical evaluation. Standard tests and reagents were employed to detect various phytochemical studies. The tests revealed the presence of polyphenols, steroids, flavonoids, tannins, terpenoids, phlobatanins, and saponins. The results were given in table 2.

Table 2: Phytochemical analysis of different type of extracts

S.No	Test for	Petroleum ether extract	Methanol extract	Ethyl acetate extract	Chloroform extract
1	Alkaloids	-	-	+	-
2	Flavonoids	-	+	+++	-
3	Glycosides	-	+	+	+
4	Saponins	-	+	+	++
5	Steroids	++	-	+	-
6	Carbohydrates	-	-	-	-
7	Proteins	-	-	-	-
8	Aminoacids	-	-	-	-
9	Phenolics/tannins	-	++	-	++

Acute Toxicity study

Acute toxicity is a toxicity produced by a pharmaceutical when administered in one or more doses within a period not exceeding 24 h. Changes in general behaviors, variations in body weight and mortality are critical for the evaluation of the effect of a compound on test animals, since such changes are often the first signs of toxicity (Deressa et al., 2010).

Accordingly, the acute toxicity study of the present investigation indicated that no mortality in mice was caused within two weeks of follow up period after oral administration of the ethyl acetate fraction of Manilkara hexandra at a dose of 1000 mg/kg. Gross physical and behavioural observations of the experimental mice also revealed that there were no visible signs of toxicity like lacrimation, hair erection, diarrhoea, comma and feeding activities. It can be concluded that the dose of 1000 mg/kg body weight of FFMH is safe.

Antidiabetic activity

Antidiabetic activity was evaluated using alloxan induced diabetic animals. In all groups prior to alloxan administration, the basal levels of plasma glucose of the rats were not significantly different. However, 72 hrs after alloxan administration, plasma glucose levels were significantly higher in the rats selected for the study. In contrast Non-diabetic control remained persistently glycemic throughout the study. Table 3 shows the effect of treatment with FFMH on plasma glucose levels.

Table 3: The effect of 3 weeks treatment with extract of FFMH on glucose levels (mg %) in alloxan

induced diabetic rats.

Groups	0 Day	3 Day	14th day	21 day
Group I Normal control	58.10 ± 1.10	59.4 ± 1.31	61.07 ± 2.2	60.4 ± 1.9
Group II Diabetic control	60.4 ± 2.01* a	199.7 ± 4.8*a	180.6 ± 5.1*a	182.4 ± 3.2*a
Group III Standard control (Glibenclamide)	59.3 ± 1.26	205.5 ± 6.4	120.6 ± 3.6	103.4 ± 1.10
Group IV FFMH 100 mg/kg	63.1 ± 2.85*b	186.5 ± 5.2*b	148.1 ± 4.9*b	138.3 ± 2.9*b
Group V FFMH 200 mg/kg	61.2 ± 6.74*b	179.4 ± 5.5*b	145.6 ± 3.4*b	131.7 ± 4.1*b

*values were expressed as mean ± SEM
*a-- Values were significantly different from normal control at P<0.001
*b-- Values were significantly different from diabetic control at P<0.001

In the present study treatment with FFMH showed significant anti hyperglycemic activity. In mild diabetes, the maximum percent reduction in glucose levels were seen in groups Group 4 and Group 5 receiving 100mg/kg and 200 mg/kg of FFMH. In the treated groups (Group 4 & Group 5) significant anti-hyperglycemic (P<0.01) effect was evident from the first week onwards, the decrease in plasma sugar was maximum on completion of the third week (P<0.001) in the group receiving 100mg/kg and 200mg/kg of FFMH respectively.
This is probably indicative of efficacy of the plants only in mild to moderate degree of diabetes. Moreover, it indirectly indicates that part of the anti hyperglycemic activity of these plants is through release of insulin from the pancreas.
Table 4 illustrates the levels of plasma insulin, total hemoglobin and glycosylated hemoglobin in normal and experimental animals in each group. In Group 2 treated animals plasma insulin, and total hemoglobin levels were decreased to 11.6±0.20, 6.10±0.69 respectively and glycosylated hemoglobin level was increased in diabetic animals as compared to normal control animals. However the levels of plasma insulin, Hemoglobin and glycosylated hemoglobin returned to near normal range in diabetic rats treated with FFMH at 100mg/kg and 200mg/kg and diabetic rats treated with glibenclamide.

Glycogen content

Glycogen content of liver tissues was estimated in control, diabetic control, positive control and treatment control groups. On the 21 day, hepatic enzymes (hexokinase, glucokinase) and substrate (Glucose-6-phosphate) were also estimated in all groups treated animals. The results have been complied in Table 5 and compared with non diabetic control values. Mean levels of enzymes hexokinase, glucokinase and substrate (Glucose -6-phosphate) values decreased in the diabetic controls.

Table 4: Effect of FFMH on plasma insulin, Hemoglobin and glycosylated hemoglobin in normal and treated animals

Group	Plasma insulin µ/ml	Haemoglobin gm/100ml	Glycosylated hemoglobin HbA1%
Group I Normal control	25.3 ± 0.60	12.10 ± 0.98	0.25 ± 0.04
Group II Diabetic control	11.6 ± 0.20* a	6.10 ± 0.69* a	0.92 ± 0.09* a
Group III Standard control (Glibenclamide)	20.4 ± 0.23	10.7 ± 1.02	0.36 ± 0.06

Group IV FFMH 100 mg/kg	18.6 ± 0.41*b	9.24 ± 0.53*b	0.45 ± 0.04*b
Group V FFMH 200 mg/kg	18.9 ± 0.53*b	9.54 ± 0.34*b	0.41 ± 0.06*b

*values were expressed as mean ± SEM
*a-- Values were significantly different from normal control at P<0.001
*b-- Values were significantly different from diabetic control at P<0.001

Hepatic glycogen content was reduced significantly in diabetic control as compared to non-diabetic controls. In diabetic controls hepatic glycogen content decreased significantly by 79.54% as compared to non-diabetic controls. Treatment with FFMH prevented this alteration in glycogen content. It led to 60.70% increase in hepatic glycogen content. This prevention of depletion of glycogen in the liver is possibly due to either stimulation of insulin release from Beta cells.

Decreased enzymatic activity of glucokinase, hexokinase has been reported in diabetic animals resulting in depletion of liver and muscle glycogen. To establish diabetes, plasma glucose was determined 72 hrs after alloxan administration, only those mice with over 180 mg/ kg body weight.

Table 5: Effect of FFMH on enzymes in carbohydrate metabolism andglycogen content in liver tissues

Group	Glycogen µg/g(tissue)	Hexokinase µg/mg	G-6-P µg/mg	Glucokinase µg/mg
Group I Normal control	43.50 ± 1.6	0.192 ± 0.010	0.380 ± 0.062	24.10 ± 1.50
Group II Diabetic control	8.90 ± 1.26* a	0.086 ± 0.006* a	0.162 ± 0.007* a	5.15 ± 0.86* a
Group III Standard control (Glibenclamide)	26.20 ± 4.6	0.142 ± 0.009	0.286 ± 0.20	16.42 ± 2.01
Group IV FFMH 100 mg/kg	22.65 ± 3.8* b	0.116 ± 0.004* b	0.228 ± 0.011*b	12.06 ± 1.60*b
Group V FFMH 200 mg/kg	20.16 ± 2.9* b	0.112 ± 0.006*b	0.236 ± 0.016*b	10.48 ± 1.36* b

*values were expressed as mean ± SEM
*a-- Values were significantly different from normal control at P<0.001
*b-- Values were significantly different from diabetic control at P<0.001

Treatment with FFMH increased the Glucose -6-phosphate content in the liver, indicating an overall increase in glucose influx. The content of glucokinase and hexokinase was increased by FFMH treated groups. This seems to have an overall effect in increasing glucose utilization. The respective percentage decrease was 54.97, 78.63 and57.36% in diabetic controls. Treatment with FFMH led to a rise in % of these parameters by 34.88, 57.29 and 40.74% respectively (P<0.001).

Flavonoids are known to have different pharmacological effect on various mammalian cell systems (Kikuchi et al., 2019). In a related study, flavonoids were found to lower blood glucose level in animal studies (Tundis et al., 2010; Vareda et al., 2014). Phenolic compounds have been reported as effective antihyperglycemic agents (Gebreyohannis et al., 2013). In present study, phytochemical screening indicated the presence of flavonids in ethylacetate fraction of Manilkara hexandra bark. Flavonoids such as quercetin, myricetin (Liu et al., 2005; Vessal et al., 2003), kaempferol glycosides and kaempferol-containing plant extracts (Arunachalam and Parimelazhagan, 2014) have exhibited that the antidiabetic activities in various animal models. Flavonoids exhibit antidiabetic activity due to their ability to regenerate pancreatic β-cells (Gebreyohannis et al., 2013; Al-Ishaq et al., 2019).

In accordance with these mechanisms, the potential

effect of fraction of Manilkara hexandra bark could be due to stimulation of insulin secretion from undamaged β-cells of islets or regenerated β-cells, enhanced transport of glucose to the peripheral tissues, and reduction of hepatic gluconeogenesis and glycogenolysis.

Conclusion

The chemical analysis revealed presence of different classes of flavoinds in ethylacetate fraction of Manilkara hexandra bark which together with other constituents could be responsible for the antidiabetic activity. The possible mechanisms of antidiabetic properties of the bark extract could be due to stimulation of insulin secretion from undamaged β-cells of islets or regenerated β-cells and inhibition of glucose absorption. Taken together, these results justify its traditional use for the treatment of diabetic-related disorders. Further studies on the crude and active fraction are recommended to confirm the above potential mechanism and the constituents responsible for the effect.

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