Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 3, 2021, Pages. 2414 - 2421
Received 16 February 2021; Accepted 08 March 2021.

Antidiabetic activity of leaf extracts of *Aegle marmelos linn* in Streptozotocin - Nicotinamide induced diabetic wistar albino rats

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ABSTRACT

The present study was to scientifically investigate the traditional claim of *Aegle marmelos (A. marmelos)* used to subside the symptoms of diabetes mellitus by siddha system of medicine. Hence, the study was carried out to conform the activity. The plant leaves of *A. marmelos* was extracted with chloroform, ethanol and water in Soxhlet apparatus. The extracts obtain were tested for acute toxicity study in wistar albino mice at different dose. The maximum tolerant dose was observed as 2000mg/kg b.wt. No motility and toxic symptoms were observed at a dose of 2000mg/kg, b.wt. So, the 1/5 (400mg) of the maximum tolerant dose was selected for the screening of antidiabetic activity. The 14 days antidiabetic treatment reveals that there is a deviation in the biochemical parameters such as aspartate amino transaminase (AST), alanine amino transaminase (ALT), alkaline phosphatase (ALP), blood glucose (BG), cholesterol (TC), total protein (TP) and total bilirubin (TB). The ethanolic ($P<0.001$) and water ($P<0.01$) extract at a dose of 400mg/kg exhibited significant antidiabetic activity which was compared with the standard drug Gilbenclamide 10mg/kg.

Keywords*: Aegle marmelos*; Anti-diabetic activity; Streptozotocin; Nicotinamide.

INTRODUCTION:

Aegle marmelos belongs to the family Rutaceae is a popular medicinal plant in ayurvedic and siddha systems of medicine. The plant popularly known as vilvam tree is native to Indo, Malayan region and is currently cultivated in India, Pakistan, Burma and Thailand. The leaves are used for diabetic and asthmatic complaint. In pharmacological trials both fruits and root showed antiamyopic and hypoglycaemic activity. As its leaves are offered to god Siva so it is considered as a sacred tree in Hindu temple and worshiped according to Hindu mythology. Diabetes mellitus is possibly the world's largest growing metabolic disorder. The International Diabetes Federation (IDF) estimates the total number of people in India with diabetes to be around 50.8 million in 2010, rising to 87.0 million by 2030. With such incidence, more nutrition and medical therapies cannot be relied upon. Currently a challenge

is to identify such healthy foods there main in the realms of obscurity and to establish them as functional foods to prevent the progression of metabolic complications. *A. marmelos* is one of most widely used medicinal and nutraceutical plant. Leaves, fruits, bark, stem and roots of *A. marmelos* have been used in ethnomedicine to exploit its various medicinal properties including antioxidant, antimicrobial, antidiarrheal, antidiabetic, antiproliferative, hepatoprotective, anti-inflammatory, antihyperlipidemic effect of *A. marmelos* fruit pulp powder on type II diabetic (kirti *et.,al* 2016).

Diabetes mellitus is a metabolic disorder of multiple aetiology, characterized by chronic hyperglycaemia with abnormalities in carbohydrate, fat and protein metabolism due to imbalanced secretion and its action or both. The effects of diabetes include long term damage, dysfunction and failure of various organs. Diabetes may present with characteristics symptoms such as polydipsia, polyuria, polyphagia, blurring of vision, and weight loss (kumar et.,al 2016). In its most severe forms absence of effective treatment leads to ketoacidosis or a non-ketotic hyperosmolar state, may lead to stupor, coma and death(Mohammad and Ahamed, 2016).

The long-term effects of diabetes include progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and or neuropathy with risk of foot ulcers, amputation, Charcot joints and features of autonomic dysfunction including sexual dysfunction. People with diabetes are at increased risk of cardiovascular, peripheral vascular and cerebrovascular disease (Rubino et al., 2016).

METHODOLOGY:

Collection of plant materials:

Healthy and uninfected 5kg of leaves of *A. marmelos* were collected from Bragadhishwara Temple Thanjavur district, Tamil Nadu, India. The plant authenticated by Prof. Dr. John Britto. S. J, Rapinat herbarium, St. Joseph's college in Tiruchirappalli, Tamil Nadu, India. And specimen was deposited in the herbarium [voucher specimen NO:GR001].

Preparation of extracts:

The leaves of *A. marmelos* were dried in shade and then coarsely powdered in a blender. The powdered drug was extracted in a Soxhlet apparatus successively with chloroform, ethanol and water. Then it was concentrated in a rotary vacuum evaporator which resulted in chloroform extract and ethanol extract, water extract. These extracts were used for the pharmacological studies.

Animals:

The use of rats and all the experimental procedures were approved by the Animal Ethics Committee, KPJUC, School of Pharmacy, Kota Seriemas, Nilai (REF NO: KPJUC/ IAEC/ 2017/ 4) and were performed in accordance with the Code of Practice for the Care and Use of Animals for Scientific Purposes. A total of thirty-six (36) Wistar albino rats (150±30g) were obtained from the animal house. They were housed in polycarbonyl cages and separated into five groups of six in each. The animals were acclimatized to the environmental condition of vivarium for at least a week (25 ± 2ºC with 12-hour light/12-hour dark cycles and 35-60% relative humidity) prior to the experiment. All animals were supplied with commercial pellet food and water *ad libitum* (chehraghi *et al.*, 2013).

Acute toxicity studies:

Acute toxicity study of the extracts was carried out according to the Organization for Economic Co-operation & Development (OECD) guideline 423, (2001). Male wistar albino rats (150-180gm) were used in the study (Dharmalingam et at.,2017). The rats were fasted overnight and the weight of each rats were recorded before use(dha. Chloroform, Ethanol and Water extracts of *A. marmelos* were given orally at the starting dose level of 2000mg./kg bodyweight as per guidelines of OECD 423. The rats were observed continuously for 2 hours for behavioural, neurological and autonomic profiles and after 24 hours and 72 hours for any mortality (OECD,2001).

Preparation of dose:

Chloroform extracts of *A. marmelos* (AMCE), Ethanol extract of *A. marmelos* (AMEE), and Aqueous extract of *A. marmelos* (AMAE) were chosen for *in vivo* experiment because of insignificant percentage yield. 400mg/kg of the respective extracts were dissolved in distilled water to make the respective stock solutions and glibenclamide (10mg./kg) (Zibula *et al.*, 2000) were prepared from the respective stock solution by diluting with distilled water prior *in vivo* experimentation for antidiabetic activity. The doses were freshly prepared in each day.

Anti-diabetic activity in STZ and NAM induced diabetic rats:

The anti-diabetic activity of the various extracts of *A. marmelos* was assessed on Streptozotocin-nicotinamide induced diabetic rats by the method developed by (Masiello *et al.*,1998). with some modifications. Thirty-six (36) Male wistar albino rats (150-180gram) 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 (60mg./kg) was prepared in fresh cold 0.1M citrate buffer (pH 4.5). Diabetes was induced by single intraperitonial injection of Streptozotocin (60mg./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 hypoglycaemic shock. After 15 minutes the animals were given a single dose of Nicotinamide 120mg./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 (10 mg./kg, p.o.). The diabetic rats in group IV–VI were treated with a single dose of AMCE, AMEE and AMAE (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 anaesthesia in Eppendorf's tubes (1ml.) containing 50µl of anticoagulant (10% trisodium citrate). The serum was separated by centrifuging the blood samples at 4000 rpm for 15 min for bio chemical 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. The serum collected was used to analyse biochemical parameters such as aspartate amino transaminase (AST), alanine amino transaminase (ALT), alkaline phosphatase (ALP), blood glucose (BG), cholesterol (TC), total protein (TP), and total bilirubin (TB) (Said et., al 2017). Biochemical assay kits were used to estimate the biochemical parameters (Alemnji *et al.,* 2010).

Histology of tissue samples

After collection of blood sample, all the animals were sacrificed by cervical dislocation and dissected. The pancreas was removed, weighed and rapidly transferred in 10% formalin no longer 48hours[7]. Pancreas specimen were further processed for histological studies. The collected tissues were fixed in 10% neutral buffered formalin, dehydrated in a graded ethanol series, (50-100%), cleared in xylene and embedded in paraffin wax. The tissues embedded in paraffin wax were sectioned for 5µm thick sample and were stained using haematoxylin and eosin (H& E) method. Light microscopy was used for tissue samples observations.

Statistical Analysis:

The result is expressed as mean ± S.E.M. and subjected to one way. ANOVA followed by Dunnett's t-test. Values of $p<0.01$ and $p<0.001$ were considered statistically significant.

RESULTS:

In acute toxicity studies the oral administration of the extract of *A. marmelos* leaves did not produce any mortality in mice up to dose level of 2000 mg/kg This maybe to non-toxic therapeutic index of this plant. So, the dose of the extracts was fixed at 400mg/kg i.e., $1/5^{th}$ a maximum tolerate dose. In diabetic animal marked evaluations ($p<0.001$) in the blood glucose level was noted and these levels were observed to continuously increase until day 7. Daily administration of various extracts *of A. marmelos* (400mg/kg p.o. for 14 days). To the respective groups of diabetic rats antagonized is action and showed a remarkable reduction in blood glucose level. (Table1&2) Ethanol extract of *A. marmelos* showed significant ($p<0.001$) reduction of blood glucose level. Increased activity of serum AST, ALP, ALT, BG and TC and decreased activity of serum TP were observed in streptozotocin and nicotinamide induced diabetic rats. Oral treatment with the standard drug Gilbenclamide (10mg/kg) and ethanol extract 400mg/kg of *A. marmelos* significantly ($P<0.001$) restored the enzyme activities and the body weight to near normal range followed by water extract ($P<0.01$) at a dose of 400mg/kg.

Table 1: Acute toxicity studies *of A. marmelos* extracts

Group (n=6)	**Dose**	**Signs of toxicity**	**Onset of toxicity**	**Duration of observation**
Chloroform extract	2000 mg/kg	No toxicity	Nil	7 days
Ethanol extract	2000 mg/kg	No toxicity	Nil	7 days
Aqueous extract	2000 mg/kg	No toxicity	Nil	7 days

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Table2. Anti-diabetic effect of different extracts of *A. marmelos* in STZ and NAM induced diabetic rats

Treatment	**Serum glucose(mg/dl)**			
	Times (days) after treatment			
	normal	1st	7th	14th
Normal control	72.8±2.8	74.7±3.1	74.8±4.8	75.1±3.1
Diabetic rats	76.2±2.3 510	462.3±20.1	491.1±18.3	510.7±6.9
Diabetic rats treated with chloroform extract (400 mg/kg)	75.8±2.9	468.5±18.5	403.6±9.6	418.3±12.
Diabetic rats treated with ethanol extract (400 mg/kg)	75.2±3.9	451.0±23.6	203.7±3.5**	89.6±2.5*
Diabetic rats treated with aqueous extract (400 mg/kg)	76.5±2.6	478.5±31.1	284.5±7.8*	199.6 ± 18.6*
Diabetic rats treated with Gilbenclamide extract (400 mg/kg)	73.4±4.8	401.3±11.3	156.5±5.6**	83.7±2.1**

*P<0.01, **P<0.001 (compared with control), n=6

Histopathological studies

Streptozotocin and nicotinamide intoxicated rats (Fig.1) showed perihepatitis characterised by degeneration and coagulative necrosis of majority of the Pancreas cells. The cells at the periphery showed slight granular swelling. The Pancreas showed congested central veins, sinusoid and multi focal area of necrosis and fatty changes when compared with that of the control group (Fig.1.a) which showed normal histological features.

Extracts treated groups

While comparing the histopathological photographs of all the extract treated groups (Fig.1c, d, e) with Gilbenclamide (Fig.1.b) treated group (10mg/kg b. wt); the Ethanolic extract (Fig.1.f) of *A. marmelos* treated group showed only mild degenerative changes of Pancreatic cells, which can be comparable with control group. In addition, there was less degree of perihepatitis in few pancreatic lobules. All the above histopathological findings were well comparable with the biochemical estimations. When compared with the various extracts *of A. marmelos* and Gilbenclamide, the ethanolic extract *of A. marmelos* and Gilbenclamide showed a significant activity at 400mg, 10mg/kg b/wt. dose levels respectively.

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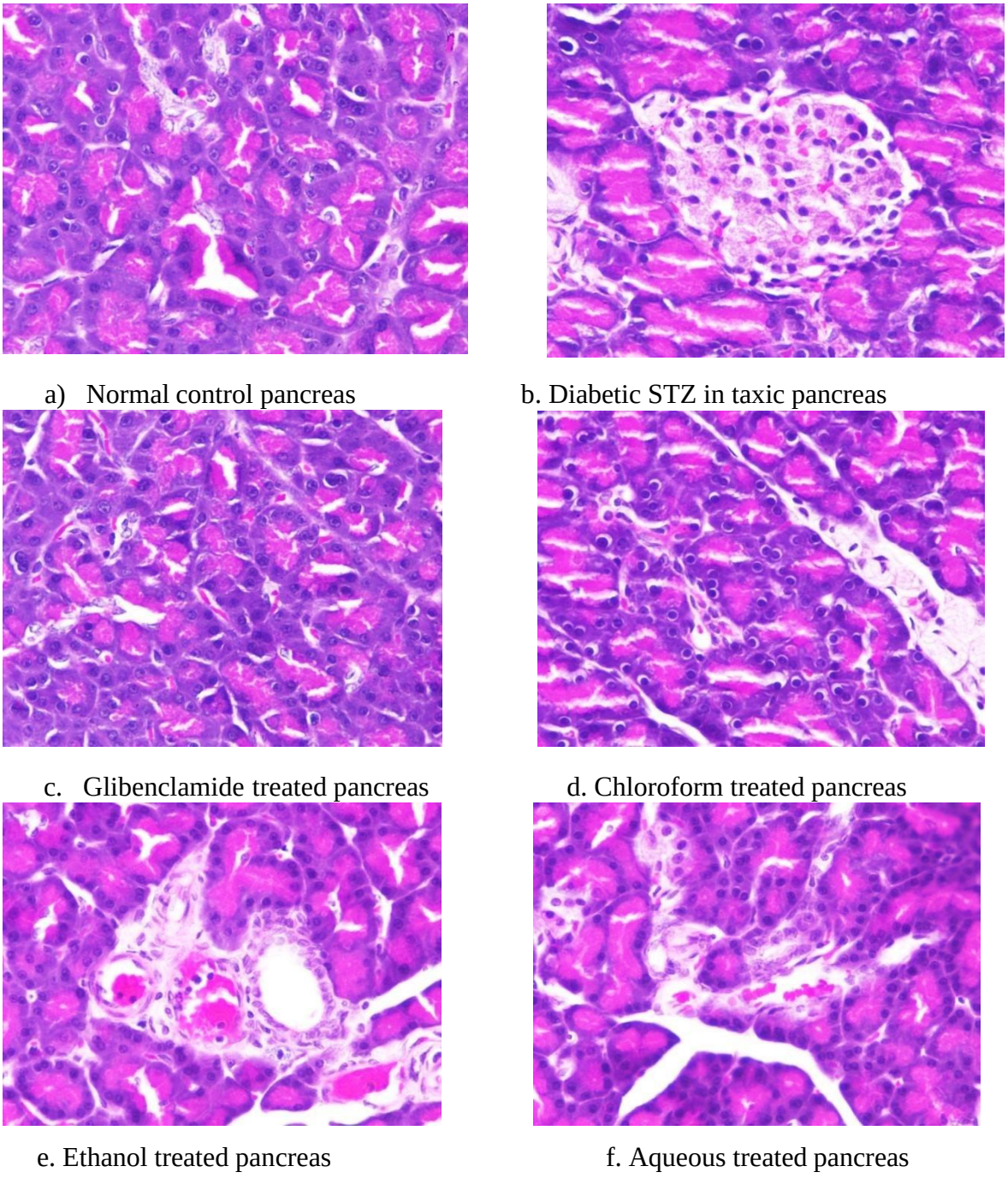

a) Normal control pancreas

b. Diabetic STZ in taxic pancreas

c. Glibenclamide treated pancreas

d. Chloroform treated pancreas

e. Ethanol treated pancreas

f. Aqueous treated pancreas

Figure1. Anti-diabetic effect of different extracts *of A. marmelos* in STZ and nitotineamide induced diabetic rats.

DISCUSSION

Streptozotocin Nicotinamide induced diabetes has been identified as a useful experimental model to examine the action of hypoglycaemic agent. Streptozotocin is a diabetogenic agent which destroys pancreatic beta cells by the generation of excessive free radicals and reduces insulin biosynthesis and secretion. After induction of diabetes with a low dose of STZ, regeneration is also possible due to the presence of many surviving β-cells. The treatment

with certain medicinal plants has an effect on protecting β-cells and smoothening out fluctuation in glucose levels which may be due to their ability to restore its function by different mechanisms. In the present study, reduction in body weight was observed in diabetic rats which might be due to the degradation of structural proteins and the deficiency of carbohydrate. An increased activity of serum AST, ALP, ALT, BG, and TC and decreased activity of serum TP were also observed which might be due to the damage of the podocytes. Oral treatment with ethanol and water extracts of *A. marmelos* leaves at a dose of 400mg/kg exhibited good anti-hyperglycaemic activity against the streptozotocin-induced diabetes. The anti-diabetic activity of these extracts was evident by the significant restoration of the body weight changes in the diabetic rats. Also, a significant ($P<0.001$) decrease in the concentration of blood glucose was observed in diabetic rats on the 14th day of treatment. The antidiabetic activity of the ethanol and water extracts of *A. marmelos* leaves was supported by significant restoration ($P<0.001$), ($P<0.01$) of the marker enzymes which were able to condition the hepatocytes as to protect the membrane integrity against Streptozotocin induced leakage of marker enzymes into blood circulation. The anti-diabetic effect of *A. marmelos* leaves might be due to the Presence of secondary metabolites. Extensive research on medicinal plants is focused on the isolation of several classes of secondary metabolites which are due to the responsible for anti-diabetic property. These results demonstrated that the significant anti-diabetic activity of ethanol in the wistar albino rats. The findings revealed that no significant antidiabetic activity was found in diabetic rats treated with chloroform extracts. The earlier studies on plants the blood glucose lowering activity is because of enhanced peripheral glucose utilization by skeletal muscle which may be due to the stimulation of β-cells to release more insulin (Ramulu *et al.*,2013).

CONCLUSION:

The findings of the present study suggest that *A. marmelos* leaves possess promising anti-diabetic activity which was evident by the restoration of biochemical parameters to near normal range in diabetic animals treated with ethanol extracts of *A. marmelos* leaves. Further studies are necessary to determine the mechanism of action of *A. marmelos* leaves for its anti-diabetic effect. Currently, fractionation of the active extract of *A. marmelos* leaves is in progress to isolate and characterize the bio active compounds responsible for anti-diabetic activity.

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