

***Aegle marmelos* Leaf Extract is an Effective Herbal Remedy in Reducing Hyperglycemic Condition: A Pre-clinical Study**

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Abstract

Diabetes is one of the alarming health problem in Pakistan. According to international diabetes federation (IDF), about 7 million Pakistanis are victims of diabetes accounting a total of 3% population. Most of Pakistanis still prefer to use herbal medicines for almost all of their health issues. *Aegle marmelos* (commonly known as the bael tree) is highly reputed ayurvedic medicinal tree as it has been used in traditional medicine for centuries. In this pre-clinical work, 32 mice were induced for diabetes via injecting alloxan intraperitoneally at a dose of 100 mg/kg body weight and mice having 104-170 mg/dl sugar level were considered diabetic. All mice were divided into two groups, each group with three sub-groups containing normal, control and diabetic mice. One group was given normal feed whereas other group was given a food containing *Aegle marmelos* leaf extract at 300 mg/kg body weight as an effective dose against alloxan induced hyperglycemia. The experiments lasted for 60 days and blood samples (0.5 ml to 1.0 ml) were collected from coccygeal vein of rats on 1st day, 30th day and 60th day of the experiment in heparinized tubes and blood glucose level was measured using spectrophotometer by enzymatic kit at a wavelength of 540nm. It was observed that the *Aegle marmelos* leaf extract is an effective herbal remedy in reducing and maintaining the glucose level in normal and hyperglycemic mice.

Keywords: Diabetes mellitus, *Aegle marmelos* leaf, Hyperglycemia, Pre-clinical study

Introduction

Diabetes mellitus (DM) is a major endocrine disorder of carbohydrate disturbed metabolism and growing health problem in the world. It has been suggested that formation of free radicals is involved in the pathogenesis of diabetes and in the development of diabetic complications because a prolonged exposure of the antioxidant defense system to hyperglycemic condition (Sander *et al* 2001). It is also a syndrome problem resulting from variable interactions of hereditary and environmental factors and characterized by the depleted insulin secretion, hyperglycemia and altered metabolism of lipids, carbohydrates and proteins, in addition to damaged β -cells of pancreas and an increased risk of complications of vascular diseases. Hence, it is characterized by high level of glucose in the blood resulting from defects in insulin production (insulin deficiency), insulin action (insulin resistance) or both (Davis and Granner, 1996, Ruth *et al.*, 1999, Robert *et al.*, 1999). Insulin (Latin insula, "island") is a polypeptide hormone primarily playing a pivotal role in the regulation of

carbohydrate metabolism as well in metabolism of fats and proteins. Alloxan is a toxic glucose analogue, which selectively destroys insulin-producing cells in the pancreas when administered to rodents and many other animal species causing an insulin-dependent diabetes mellitus called alloxan diabetes in these animals that is similar to type 1 diabetes in humans (Berg *et al* 2001, Fuller *et al.*, 1990, Ruddon and Gilman, 2000).

According to the latest statistics given by jointly Pakistan diabetes federation (PDF) and international diabetes federation (IDF), Pakistan has 7 million diagnosed diabetes patients on an average of 3% of national population which is ranked 14 in case of diabetes prevalence in the world (<http://www.idf.org/membership/mena/pakistan>) as shown in figure 1. Presently control of diabetes mellitus relies on the injection of IV insulin and the usage of many chemicals and plant extracts in subcontinent. A number of plants have been studied for this purposes and their extracts were prepared and are available in market as remedy for diabetes

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(Vats *et al.*, 2002). Some medicinal plants have been found playing extra pancreatic effect and acting directly on liver, muscle tissues altering the activities of the regulatory enzymes of glycolysis, gluconeogenesis and other pathways. Since the plant products have fewer side effects, so these have the potential as good hypoglycemic drugs and can provide us clues for the development of new and better oral drugs (Shukia *et al.*, 2000, Mukherjee *et al.*, 2006).

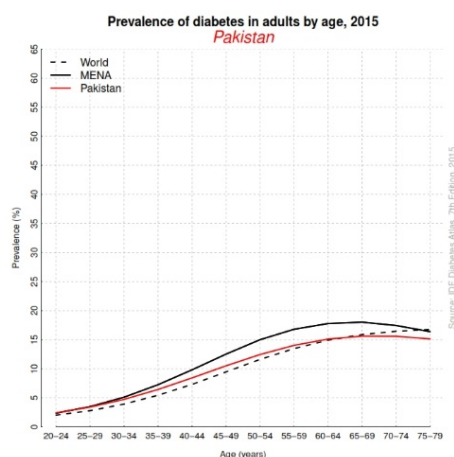


Figure 1. Prevalence of diabetes and its comparison with world and Middle East region (MENA). (<http://www.idf.org/membership/mena/pakistan>)

The medicinal values of plants have been tested by trial and error method for a long time by different workers. Even today great opportunities are still open for scientific investigations of herbal medicines for the cure of diabetes and its complications. *Aegle marmelos* (AM) commonly known as Bael is a spiny tree belonging to class Rutaceae. It is an indigenous tree found in India, Myanmar, Pakistan and Bangladesh. The leaves, roots, bark, seeds and fruits are edible and have medicinal values as described in the Ayurveda (Khair, 2004) and can act as a hypoglycemic agent (Alam *et al.*, 1990, Grover *et al.*, 2002, Mukherjee *et al.*, 2006, Kamalakkannan and prince, 2003, Kesari *et al.*, 2006).

Preliminary reports indicate blood glucose lowering agents in green leaves of *Aegle Marmelos* plants. However, limited scientific evidence exists to validate these claims since there are only few available reports on the exact mode of action of these extracts and pharmacological actions of this plant (Ponnachan *et al.*, 1993. Das *et al.* 1996; Schdewa *et al.* 2001; Nema *et al.* (1991) Riyanto *et al.* (2001) Upadhya *et al.* 2004, Kesari *et al.* 2006). This work was aimed to study the glucose lowering effect of AM leaves in different groups of diabetic animals.

Materials and Methods

The experimental work was conducted in animal house of the Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore, Pakistan.

Aqueous leaves extract of *Aegles Marmelos* (Bael)

Plant material and Preparation of Extract

Aegle marmelos leaves were collected from the Botanical garden of Punjab University and thoroughly washed with water and dried in shade. Then these leaves were grinded to make powder and were kept in airtight containers for the experimental purpose. About 500 g of the shade dried powdered leaves were ground into fine powder and boiled in 5 liters of distilled water for 7-8 hours and stirred occasionally. After this, the mixture was filtered using Whatmann No. 1 filter paper and 50 g of the paste was obtained. Then this paste was dissolved in 0.5 ml water just prior to the administration.

Induction of diabetes in mice

Alloxan induced hyperglycemia has been described as a useful experimental model to evaluate the activity of hypoglycemic agents (Junod *et al.* 1996). Diabetes was induced by a single intraperitoneal injection of alloxan prepared in 0.1 mol/L citrate buffers at a dose of 100 mg/kg body weight. Diabetes was confirmed in the alloxan treated rats by measuring the fasting blood glucose concentration 48 h of post injection as described by other researchers (Karunanayke *et al.* 1984 and Grover *et al.* 2002).

Enzymatic Kits

Commercial kits of the company Randox, UK were used to determine serum glucose in mice by spectrophotometer.

Animals

Total 32 mice of both sexes weighing between 35-36 g were selected for the experiment. These animals were housed in steel cages under controlled laboratory conditions. The mice were fed standard diet with free access to fresh water (Reeves *et al.*, 1993)

Experimental Design

32 rats were divided into four groups (A, B, C and D) with eight mice in each group. Each group was having both normal and diabetic mice as described in table 1.

Table 1. Animal grouping, their diet and treatment research plan

| Groups | | Animal Conditions | Treatment |
|--------|---|--------------------------------------|--|
| 1 | A | Normal (control) | Normal feed (3% b.w.) |
| 2 | B | Normal + (<i>Aegle Marmelos</i>) | Normal feed + aqueous Leaves extract of <i>Aegle Marmelos</i> (300 mg/kg). |
| 3 | C | Diabetic (control) | Normal feed (3 % b.w.) |
| 4 | D | Diabetic + (<i>Aegle Marmelos</i>) | Normal feed (3% b.w.) + aqueous Leaves extract of <i>Aegle Marmelos</i> (300 mg/kg). |

Blood Collection

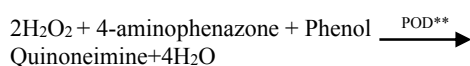
1 ml blood was collected after 12 h of fasting from coccygeal vein of mice at 1st day, 21st day and 42nd day of the experiment from.

Blood Analysis

Collected blood was allowed to clot and then centrifuged at 3000 r.p.m. for 10 minutes and serum was separated. The specific enzymatic kits were used to assess serum glucose levels of mice using spectrophotometer.

Estimation of Serum Glucose**Principle**

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a Reddish-pink quinoneimine dye as an indicator.

Reaction Principle

*GOD Glucose oxidase

**POD Peroxidase

Sampling

Glucose is stable for 24 hours at +2 to +8°C if the serum was prepared within 30 min after collection.

Reagents and Parameters**Reagents**

1. Enzyme Reagent
2. Standard (100 mg/dl)

| Standard | Blank | Standard | Tests |
|----------------|-------|------------|------------|
| (100 mg/dl) | ---- | 10 μ l | ---- |
| Tests | ---- | ---- | 10 μ l |
| Enzyme reagent | 1 ml | 1 ml | 1 ml |

Parameters of Study

Wavelength λ : 500 nm, Hg 546 nm
 Cuvette: 2 cm path length
 Temperature: 15-25°C or 37°C
 Measurement: against reagent blank

10 test tubes were taken. Two tubes out of 10 test tubes were labeled as blank and standard. Remaining 8 tubes were labeled as 1, 2, 3...8 for each sample of mice serum from each group. 1ml reagent was taken in all the tubes by pipette. 10 μ l of standard solution from the kit was added to the tube labeled as standard and 10 μ l of each serum sample was taken in tubes labeled as 1, 2, 3,...8.

All the tubes were shaken well and then incubated at 37°C for 10 minutes. After incubation, the absorbance of standard (AbsStd) and the sample (AbsS) was measured at wavelength of 546 nm against the blank (AbsSRB).

Normal values

Serum, plasma (fasting): 75-115 mg/dl

Linearity

This method is linear upto 400 mg/dl. Samples having higher glucose concentration were diluted at 1:2 with saline solution and the measured calculation was multiplied by 2.

Calculations

The concentration of glucose in serum was calculated by the following formula;
 Glucose conc. (mg/dl) = (Δ test / Δ standard) \times 100

Statistical Analysis

The data thus obtained was subjected to statistical analysis represented by mean \pm S.D for 8 mice in each group of experiments. Comparison among the different groups was determined by ANOVA test

and differences were considered significance when $p < 0.05$. The level of significance was set at 5% according to the method of Steel and Torri, 1982.

Results

The present work was carried out to investigate the effect of feeding *Aegle marmelos* leaves on serum glucose level in diabetic mice.

Serum Glucose level (mg/dl)

The change in blood glucose level of mice blood serum as a function of the *Aegles marmelos* leaves extract for a feeding period of 8 weeks is presented in figure 2.

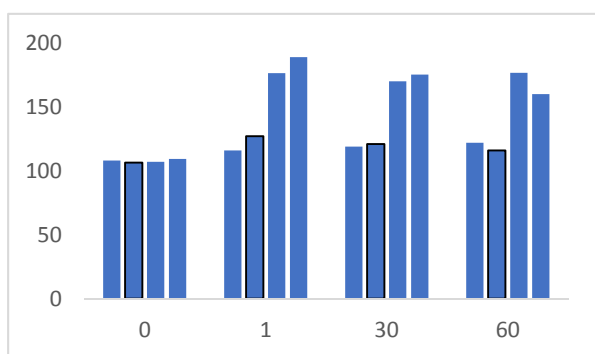


Figure 2. Change in blood glucose level of mice blood serum

In control group A, the mean glucose level was 108.2 ± 7.28 before alloxan injection, which increased to 116.1 ± 8.53 at 1st day after treatment and then further raised to 119 ± 8.26 at 30th day and 122 ± 8.42 at 60th day.

In normal group B, the mean glucose level was 106.5 ± 9.81 before alloxan injection, which increased to 127.14 ± 8.23 at 1st day after alloxan injection and then further raised to 121 ± 8.26 at 30th day and 116.1 ± 8.53 at 60th day.

In diabetic control group C, the mean glucose level was 107.2 ± 6.99 before alloxan injection, which increased to 176.5 ± 3.64 at 1st day and then further raised to 170.2 ± 6.10 at 30th day and 176.8 ± 10.97 at 60th day.

In diabetic group D, the mean glucose level was 109.4 ± 6.55 before alloxan, which increased to 189.1 ± 8.02 at 1st day and then decreased to 175.4 ± 12.62 at 30th day, and further decreased to 160.1 ± 7.33 at 60th day.

According to the analysis of variance, at before given alloxan, Non-significant difference was observed in Group A (control + normal feed) as $P > 0.05$, while at day 1, 30 and 60 of the experiment there was significance difference among the mean value of glucose level in the groups i.e. Group B (Normal + *Aegle marmelos* leaf extract), Group C

(Diabetic + normal feed) and Group D (Diabetic + *Aegle marmelos* leaf extract) as $P < 0.05$ (Figure 3).

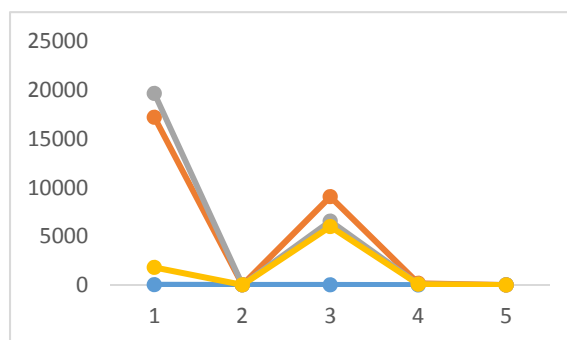


Figure 3. Analysis of Variance of Glucose level

At day zero before alloxan injection, non-significant difference was observed in groups i.e. Group A (control + normal feed, group), Group B (normal + aq. leaves extract of AML) and Group C (diabetic + normal feed) with Group B (normal + aq. leaves extract of AML), Group C (diabetic + normal feed) and Group D (diabetic + aq. leaves extract of AML) as $P > 0.05$ (Table S1).

At 1st day of experiment, significant difference was observed in groups i.e. Group A (control + normal feed), Group B (normal + aq. leaves extract of AML) and Group C (diabetic + normal feed) with Group B (normal + aq. leaves extract of AML), Group C (diabetic + normal feed) and Group D (diabetic + aq. leaves extract of AML) as $P < 0.05$. At 30th day of experiment, non-significant difference was observed in Group A (control + normal Feed), Group C (diabetic + normal feed) with Group B (normal + aq. leaves extract of AML) and Group D (diabetic + aq. leaves extract of AML) respectively as $P > 0.05$, while significant difference was observed in Group A (control + normal feed) with groups i.e. Group C (diabetic + normal feed) and Group D (diabetic + aq. leaves extract of AML) and Group B (normal + aq. leaves extract of AML) with groups i.e. Group B (diabetic + normal Feed) and Group C (diabetic + aq. leaves extract of AML) as $P < 0.05$.

At 60th day of experiment, Non-significant difference was observed in Group A (control + normal feed) with Group B (normal + aq. leaves extract of AML) as $P > 0.05$, While significant difference was observed in Group A (control + normal feed) with Group C (diabetic + normal feed) and Group D (diabetic + aq. leaves extract of AML) and also significance difference observed in groups i.e. Group B (normal + aq. leaves extract of AML) and Group C (diabetic + normal feed) with groups i.e. Group C (diabetic + normal feed) and Group D (diabetic + aq. leaves extract of AML) as $P < 0.05$.

Discussion

Diabetes arises from destruction of the β -islet cells of the pancreas, due to degradation or reduction of insulin secretion (Ramkumar *et al* 2004). The elevation in plasma insulin in the *Aegle marmelos* leaf extract treated Alloxan-diabetic mice could be due to the insulin tropic substances present in the fractions, which induce the intact functional β -cells of the Langerhans islet to produce insulin, or the protection of the functional β -cells from further deterioration so that they remain active and produce insulin (Bell and Hye, 1983).

Diabetic mice induced by alloxan show an increased sensitivity to oxygen free radicals and hydrogen peroxide, the breakdown products of the liver, which impose oxidative stress in diabetes and would damage inner endothelial tissue; this would eventually be directly responsible for high blood glucose (Reddi and Bollineni, 2001). Alloxan and STZ are widely used to induce experimental diabetes in animals. The mechanism of their action in β -cells of pancreas has been intensively investigated and now is quite well understood. Experimental evidences suggest that the free radicals and reactive oxygen species are involved in high number of diseases. Free radicals (Ca^{2+} , Fe^{2+} , Fe^{3+} , H_2O_2 , Hydroxyl Oxide and Nitric Oxide inhibit Aconitase activity and participate in DNA damage of β -cell (Szkudelski, 2001).

The present research work was aimed to study the effect of *Aegle marmelos* leaf extract on serum glucose level in mice. The outcomes of present study showed that *Aegle marmelos* leaf lowers the glucose level in hyperglycemic mice. The experimentally induced diabetes significantly ($P < 0.05$) increased the fasting blood glucose level of the control level. However, the treatment of Alloxan-induced diabetic mice with the AML reduced their blood glucose levels, in comparison to the diabetic group.

This study showed that AML-extract supplementation improved glucose tolerance in diabetic mice. It seems that the hypoglycemic effect of AML is due to the increased level of serum insulin and the enhancement of peripheral metabolism of glucose (Skim *et al* 1999). The present experiment

reports the anti-diabetogenic and hypoglycemic effects of aqueous extract of *Aegle marmelos* leaves on alloxan or streptozotocin-induced diabetic mice. Loss of body weight had also been observed very distinctly in alloxan induced diabetic mice of the present study. After treatment with *Aegle marmelos* plant leaf extract, the mice regained their weight, which is close to the control (non-diabetic mice)

level which was also reported previously in 2004 (Sathishsekar and Subramanian, 2005).

This was also confirmed by the alteration in the fasting blood glucose levels in diabetic mice followed by its regeneration after the plant extract treatment, then there is no significance alteration in fasting blood glucose level in the control mice. Further the same extract causes the significance reduction of sugar within 2 hours and this fact strengthens the antidiabetogenic potentiality of this plant extract (Karunanayake *et al* 1984, Grover *et al* 2002). The aqueous extract of *Aegle marmelos* contains some biomolecules that sensitize the insulin receptors to insulin or stimulate the β -cells of islets of Langerhans to release insulin which may finally lead to improvement of carbohydrate metabolizing enzymes towards the re-establishment of normal blood glucose level (Gupta *et al* 2005)

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Table s1. Multiple Comparisons of glucose level among different groups

| Dependent Variables | Groups | Mean Difference | Std. Error | Sig. | |
|----------------------------|--|--|------------|---------|--------|
| Before Alloxan | Group A (Control) + Normal Feed | (Group B) (Normal) + aq. Leaves extract of AML | 1.7143 | 4.22054 | .688** |
| | | (Group C) Diabetic + Normal Feed | 1.0000 | 4.22054 | .815** |
| | | (Group D) Diabetic + aq. Leaves extract of AML | -1.1429 | 4.22054 | .789** |
| | Group B (Normal) + aq. Leaves extract of AML | (Group C) Diabetic + Normal Feed | -.7143 | 4.22054 | .867** |
| | | (Group D) Diabetic + aq. Leaves extract of AML | -2.8571 | 4.22054 | .505** |
| | Group C Diabetic + Normal Feed | (Group D) Diabetic + aq. Leaves extract of AML | -2.1429 | 4.22054 | .616** |
| Day 1st | Group A (Control) + Normal Feed | (Group B) (Normal) + aq. Leaves extract of AML | -11.0000 | 3.94951 | .010* |
| | | (Group C) Diabetic + Normal Feed | -60.4286 | 3.94951 | .000* |
| | | (Group D) Diabetic + aq. Leaves extract of AML | -73.0000 | 3.94951 | .000* |
| | Group B (Normal) + aq. Leaves extract of AML | (Group C) Diabetic + Normal Feed | -49.4286 | 3.94951 | .000* |
| | | (Group D) Diabetic + aq. Leaves extract of AML | -62.0000 | 3.94951 | .000* |
| | Group C Diabetic + Normal Feed | (Group D) Diabetic + aq. Leaves extract of AML | 12.5714 | 3.94951 | .004* |
| Day 30th | Group A (Control) Normal Feed | (Group B) (Normal) + aq. Leaves extract of AML | -2.0000 | 4.87845 | .685** |
| | | (Group C) Diabetic + Normal Feed | -51.2857 | 4.87845 | .000* |

| | | | | | |
|----------------------------|--|--|----------|---------|--------|
| | | (Group D) Diabetic + aq. Leaves extract of AML | -56.4286 | 4.87845 | .000* |
| | Group B (Normal) + aq. Leaves extract of AML | (Group C) Diabetic + Normal Feed | -49.2857 | 4.87845 | .000* |
| | | (Group D) Diabetic + aq. Leaves extract of AML | -54.4286 | 4.87845 | .000* |
| | Group C Diabetic + Normal Feed | (Group D) Diabetic + aq. Leaves extract of AML | -5.1429 | 4.87845 | .302** |
| Day 60th | Group A (Control) Normal Feed | (Group B) (Normal) + aq. Leaves extract of AML | 6.5714 | 4.76595 | .181** |
| | | (Group C) Diabetic + Normal Feed | -54.1429 | 4.76595 | .000* |
| | | (Group D) Diabetic + aq. Leaves extract of AML | -37.4286 | 4.76595 | .000* |
| | Group B (Normal) + aq. Leaves extract of AML | (Group C) Diabetic + Normal Feed | -60.7143 | 4.76595 | .000* |
| | | (Group D) Diabetic + aq. Leaves extract of AML | 44.0000 | 4.76595 | .000* |
| | Group C Diabetic + Normal Feed | (Group D) Diabetic + aq. Leaves extract of AML | 16.7143 | 4.76595 | .002* |

** = Non-significant value as $P > 0.05$

* = Significant value as $P < 0.05$