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Hypoglycemic Effect of Mucilage and Methanol Extract of *Aegle marmelos* (L.) Correa fruits in Stereptozotocin induced Diabetic Rats

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ABSTRACT

Purpose: *The present study aims to evaluate the mucilage and methanol extract of Aegle marmelos (L.) Correa fruit as antidiabetic agents in STZ -induced diabetic rats.*

Methods: *Mucilage of Aegle marmelos (L.) Correa fruit was isolated to yield (15% w/w) and analyzed by using GLC technique revealed the presence of glucose as a major sugar (36.61%), in addition to galactose, arabinose, xylose, mannitol, rhaminose and galacturonic acid. the mucilage and methanol extract of fruit investigated for their possible hepatoprotective effects against liver disorders induced by reactive oxygen species associated with diabetic complications in diabetic rat. The investigated parameters included blood glucose, insulin (to investigate diabetic condition), total cholesterol, triglycerides, total lipid content as indices of liver steatosis, Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline Phosphatase (ALP), total protein content and total albumin as markers of liver functions, as well as hepatic Nitric Oxide (NO) Andmalondialdehyde (MDA) (marker of lipid peroxidation) in addition to, hepatic gluconeogenic phosphoenol pyruvate carboxy kinase (PECK), Glycolytic enzymes lactate Dehydrogenase (LDH), Pyruvate Kinase (PK) and Hexokinase (HK). Also histopathological studies were investigated. Results: Significant beneficial glycemic control, scavenging free radical, normalized liver function, inhibited lipid synthesis associated with diabetic complication.*

Conclusion: *Mucilage and extract of Aegle marmelos (L.) Correa fruit have principle role in treatment and amelioration of liver damage associated with diabetic status at the biochemical and cellular levels. Further clinical studies must be done in trial to investigate there efficacy in human as new candidate drug.*

Keywords: *Aegle marmelos*, Mucilage, Hepatoprotective, Histopathological examination

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INTRODUCTION

Aegle marmelos (L.) Correa commonly known as Beal belonging to the family Rutaceae, has beenwidely used in indigenous systems of Indian medicine due to its various medicinal properties [1]. Sunita et al. [1], reported that fruit used in chronic diarrhea, tonic for heart and brain and had anti-viral activity, hypoglycemic activity, antibacterial activity and antiproliferative activity. The anti hyperlipidaemic activity of aqueous extract of *A. marmelos* (L.) Correa fruits was demonstrated by Marinzene et al. [2], using the streptozotocin-induced diabetic rats [3]. Also Singh et al. [2], demonstrated that aqueous extract of bael fruit pulp and seeds are effective in the treatment and prevention of CCl_4 induced hepatic toxicity. In addition, the aqueous extract of *A. marmelos* (L.) Correa leaves, was evaluated for hypoglycemic and antioxidant effect by Upadhya et al. [4], in male albino rats. In addition, it was reported that the extract of *A. marmelos* (L.) Correa leaves is able to regenerate damaged pancreatic cells in diabetic rats [5]. Seema et al. [6], declared that the *A. marmelos* (L.) Correa leaves is effective as insulin in the restoration of blood glucose and body weight to normal levels.

Periannan et al. [7] evaluated the antibacterial activities of different parts of plant. Various chemical constituents were isolated from different parts of *A. marmelos* (L.) Correa as coumarins, alkaloids carotenoids, mucilage and tannins according to Pandian et al. [8].

Based on the broad range of activities of fruit pulp of *A. marmelo*s (L.) Correa, the present research is extended to evaluate the efficiency of the methanol and the mucilage isolated from fruit on stereptozotocin induced diabetic rats.

MATERIALS AND METHODS

Plant material

The fresh plant *Aegle marmelos* (L.) Correa fruit were collected from El-Zohrya botanical garden, Giza, Egypt in April 2008. The plant was identified by Mrs. ThreaseLabib consultant of plant taxonomy at the Ministry of Agriculture and former director of El-Orman botanical garden. A voucher specimen (No. 00017 1Ac 04-02-05-17) was kept at the Herbarium of El-Orman Botanical Garden The collected fruits were air dried, separately powdered and kept in tightly-closed containers. Successively extracted using Soxhlet apparatus with solvents of increasing polarities. Then the methanol extract was evaporated to dryness under vacuum at 40°C, weighted and were ready for biological and chemical examination.

Investigation of the methanol extracts of *Aegle marmelos* (L.) Correa fruit by LC/MS using the following technique

The high-resolution mass spectra were obtained with an LTQ Orbit rap Spectrometer (Thermo Fisher, USA) equipped with a HESI-II source. The spectrometer was operated in positive mode (1 spectrum s-1;mass range: 200-1000) with nominal mass resolving power of 60,000 atm/z 400 with a scan rate of 1 Hz with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation using an internal standard; Bis(2-ethylhexyl)phthalate: m/z=391.284286. The spectrometer was attached with an Agilent 1200 HPLC system (Santa Clara, USA) consisting of LC-pump, PDA detector (λ=205 nm), auto sampler (injection volume 10 µL) and column oven (30ºC). MS/MS experiments were performed by CID (Collision Induced Decay, 35 eV) mode. Following parameters were used for experiments: spray voltage 5 kV, capillary temperature 260ºC, tube lens 70 V. Nitrogen was used as sheath gas (50 arbitrary units) and auxiliary gas (five arbitrary units). Helium served as the collision gas. The separations and purifications were performed by using a Nucleodur Gravity column (50 × 2 mm, 1.8 µm particle size) from Macherey–Nagel (Düren, Germany) with a H_2O (+0.1% HCOOH, +10 mM NH4Ac) (A)/acetonitrile (+0.1% HCOOH) (B) gradient (flow rate 300 µL min^{-1}). Samples were analyzed by using a gradient program as follows: 90% A isocratic for 2 min, linear gradient to 100% B over 13 min, after 100% B isocratic for 5 min, the system returned to its initial condition (90% A) within 0.5 min, and was equilibrated for 4.5 min.

Preparation of mucilage using cold extraction method

The powdered plant fruit (100 g) was mixed with one litre of distilled water, acidified with HCl to pH 3.5, stirred at 28ºC for 12 hrs and the liquid was filtered [9,10]. The process was repeated for three times, the mucilage was precipitated from the extract by adding, slowly, while stirring four volumes of ethanol. The precipitate was washed several times with absolute ethanol till free from chloride ions. The mucilage was then vigorously stirred in absolute ethanol, filtered and dried in vacuum desiccators over anhydrous calcium chloride. The mucilage was purified by re-dissolved in water and re-precipitated by ethanol as mentioned before. Then washed successively with ethanol and ether and dried in vacuum desiccators over anhydrous $CaCl_2$. The mucilage was de-proteinated by shaking its aqueous solution with chloroform and centrifuged. This technique was repeated several times to get rid of most of the proteins.

Hydrolysis of mucilage

The mucilage obtained (0.1 g) was hydrolyzed by adding 10 mL 1 HCL for 5 hrs in boiling water bath. Add amount of $Ba(OH)_2$ then centrifuge and wash the precipitate twice by water then filtrate and evaporate until the volume reach to 2 ml [11].

GLC analysis of mucilage hydrolysate deravatization of sugarhydrolysate

Evaporate 0.5 ml of mucilage hydrolysate in small screw-topped septum vials to dryness under stream of nitrogen at 40ºC. When almost dry add 0.5 ml isopropanol and complete the drying under stream of nitrogen until dry solid residue remains was dissolved in 2.5% hydroxylamine hydrochloride in pyridine. Mix and heat for 30 min at 80ºC then allow to cool. Inject 1 ml silylating reagent (trimethyl cholorosilane; N, 0-bis-(trimethylsilyl) acetamide, 1:5 by volume). Mix and heat for 30 min at 80ºC and allow cooling. 1 µ is injected in GLC for analysis using the following condition [12].

Condition for GLC analysis of mucilage of *A. marmelos* (L.) Correa

The column which used was (ZB-170, 30 mx 0.25×025 µm, 14% cyanopropyl phenyl methyl)**.** Analyses were carried out using helium as carrier gas at a flow rate 1.2 ml/min under pressure 10.6 psi with velocity and the following temperature program Initial temperature: 150ºC and Initial time: 2 min with Rate : 7 c/min Final temperature: 200ºC Final time: 20 min.

Chemicals

All chemicals used were of high analytical grade, products of Merck, Germany and Sigma, USA. Kits used for the quantitative determination of different parameters were purchased from Biogamma, Stanbio, West Germany. Streptozotacine was used for induction of diabetes in rats. Glibenclamide (Daonil) (Sanofi Aventis) was used as standard antidiabetic drug.

Animals

100 male albino rats (150-200 g) were obtained from animal house of National Research Center, Dokki, and Giza, Egypt. Rats were fed a standard diet and free access to tap water. They were kept for two weeks to acclimatize to the environment condition.

Animal rights

Appropriate anesthetic and sacrifice procedures were followed ensuring that animals didn't suffer at any stage of the experiments. Anesthetic procedures complied with the legal ethical guidelines approved by the Ethical Committee of the Federal Legislation and National Institutes of Health Guidelines in USA and were approved by the ethical committee of the National Research Centre in Egypt with registration No. 13-017 for hypoglycemic potency study. Body and liver weight of all groups were recorded in grams, and then the ratio of liver weight to body weight (relative liver weight) was calculated.

Induction of diabetes

Diabetes was induced by streptozotocin, each rats will injected intraperitoneally with a single dose of streptozotocin (65 mg/kg body weight dissolved immediately in 0.01 M citrate buffer before use [13].

EXPERIMENTAL DESIGN

The rats were divided into two main groups

The first main group, consists of three healthy normal groups (groups 1-3, each of ten rats):

Group 1: Normal healthy control group (not received any medication).

Group 2: mucilage extract–treated normal rats.

Group 3: Methanol extract–treated normal rats.

Mucilage and methanolic extract of *A. marmelos* (L.) given orally in a dose 120 and 250 mg/kg body weight/day for 30 consecutive days respectively [14,15].

The second main group, consists of six diabetic groups (groups 4-9 each of ten rats), diabetes was induced by streptozotocin, each rats will injected intraperitoneally with a single dose of streptozotocin (65 mg/ kg body weight dissolved immediately in 0.01 M citrate buffer before use [13]. After injection, they had free access to food and water and given 5% glucose solution to drink overnight to counter hypoglycemic shock [16]. After 2 days, fasting blood samples were obtained and fasting blood glucose was measured (>300 mg/dl). Hyperglycemic rats will be used for the experiments.

Group 4: Diabetic +ve control group sacrificed after 2 days.

Group 5: Diabetic +ve control group sacrificed after 10 days.

Group 6: Diabetic +ve control group sacrificed after 40 days and considered as recovery group.

Group 7: Diabetic animal (after 10 days) treated with polysaccharides extracts of *Aegle marmelose* fruit for 30 successive days.

Group 8: Diabetic animal (after 10 days) treated with methanol extract of *Aegle marmelose* fruit for 30 successive days.

Group 9: Diabetic animal (after 10 days) treated with 300 mg/kg body weight tolbutamide (glucosulfa) as reference drug for 30 days. All treatments will be given as the same manner of healthy groups.

Preparations of serum samples

After 30 days of drugs treatment, the animals were fasted overnight (12-14 hrs), the blood samples were collected from each animals by scratching sub-tongual vein into sterilized tubes. Serum were separated by centrifugation at 3000 rpm for 10 min and used for biochemical analysis.

Preparation of liver homogenates

After blood collection, rats of each group sacrificed under anaesthesia, the livers were removed immediately, weighed and homogenized in 5-10 volumes of appropriate medium using electrical homogenizer, centrifuged at 4000 rpm for 15 min, and the supernatant was collected and liquated in epindorff tubes and stored at 20ºC. The supernatant were used for different biochemical tissue analysis. The homogenization was carried out as described [17].

Determination of blood glucose

Glucose was determined in serum by colorimetric assay according to Trinder et al. [18].

Human insulin enzyme immunoassay

Insulin was determined by Insulin quantitative test kit according the method of Sacks [19].

Determination of total cholesterol in rat serum

Cholesterol was determined by the method of Richmond [20] (using a diagnostic kit Bio systems Spain).

Determination of triglycerides in rat serum

Triglyceride was measured by the method of Fossati and Prencipe [21] using a diagnostic kit (Biosystems).

Determination of total lipids in rat serum

Total lipids was measured by the method of Zollner and Kirsch [22] using a diagnostic kit (Bio diagnostic, Egypt).

Determination of alkaline phosphatase in rat serum

Alkaline phosphatase was measured by the method of Belfield and Goldberg [23] using a diagnostic kit (Biosystems).

Determination of Alanine aminotransferase (ALT)

ALT was determined in blood according to Bergmeye et al. [24].

Determination of Aspartate aminotransferase (AST)

AST was determined in blood serum samples according to the method of Reitman and Frankel [25] using QCA Diagnostic kits (Spain).

Determination of Albumin

Albumin was measured in blood serum according to Rodkey [26].

Liver tissue biochemical analysis

Determination of Hexokinase (HK)

Hexokinase was assayed in liver homogenate tissue according to Abrahao–Neto et al. [27].

Determination of lactate dehydrogenase (LDH) activity

Lactate dehydrogenase was determined in tissue liver homogenate according to Bergmeyer et al. [24].

Determination of pyruvate kinase (PK) activity

Pyruvate kinase activity was determined in tissue liver homogenate according to Bucher and pfleiderer [28].

Determination of phosphoenolpyruvate carboxy kinase (PEPCK) activity

Phosphoenol pyruvate carboxy kinase was determined in tissue liver homogenate according to Suarez et al. [29].

Determination of nitric oxide

Nitric oxide was determined in tissue liver homogenate according Moshage et al. [30]

Determination of lipid peroxidatrion products (thiobarbituric acid reactive substances TBARs)

Lipid peroxidation was determined in tissue liver homogenate according to Ruizlarre et al. [31].

Determination of total proteins in tissue homogenate

Total proteins was assayed in tissue liver homogenate by colorimetric assay according to Bradford [32].

Calculation

Percentages of change and improvement were calculated according to the following equations:

% Change=Mean of control–Mean of treated × 100/Mean of control

% Improvement=Mean of treated–Mean of disease × 100/Mean of control

Statistical analysis

Statistical analysis was carried out by one way analysis of variance (ANOVA), SPSS computer program combined with post–Hoc (LSD; Least Significance Difference where significance is considered at $P \leq 0.005$.

RESULTS

LC/MS analysis of the methanol extract of fruits of *A. marmelos (L.)Correa* using computer programing X-Caliber® resulted in the identification of two alkaloid compounds, marmesiline and shahidine. The identification of the compounds was based on the comparison of their mass spectral fragmentation data including molecular ion peak and their CID fragmentations pattern with the previously reported data. Mucilage of *Aegle marmelos* (L.) *Correa* fruits are prepared as mentioned by the cold method and gave 15% (g/100 g). GLC analysis of mucilage hydrolysatesrevealed the identification of seven sugars represented (73.14%) of the total sugar hydrolysate. Glucose represented the major (36.61%), followed by galactose (24.55%), arabinose (5.54%), rhaminose (2.68%), mannitol (1.88%), xylose (1.55%) and galacturonic acid (0.33%) (Table 1).

The blood glucose and insulin levels in serum of normal control, control- treated, diabetic and diabetic treated groups (Table 2). It is obvious that, there is no significant change between normal control and different control- treated groups either in blood glucose or in insulin levels. Concerning diabetic groups, significant increase is noticed in blood glucose levels which is concomitant with significant reduction in insulin levels at day 2, 10 and 40 post injection of streptozotacin recorded 311.40 ± 8.59, 323.52 ± 6.65 and 320.75 ± 9.03 mg/dl with percentage increase +177.05, +187.83 and +185.36% for glucose, respectively and 1.18 ± 0.25, 1.16 ± 0.21

Table 1: Carbohydrates identified by GLC analysis of the mucilage of *Aegle marmelos* (L.) Correa fruits

Peak No	R_t	Relative area%	Sugar
1	8.09	5.54	Arabinose
2	8.15	1.55	Xylose
3	9.22	2.68	Rhaminose
4	11	1.88	Mannitol
5	11.75	2.34	Unkown
6	12.1	24.55	Galactose
7	12.33	36.61	Glucose*
8	12.69	24.52	Unkown
9	13.57	0.33	Galacturonic acid
Total identified sugar represented			**(73.14%)**

Table 2: Efficiency of the mucilage and the methanol extract of *Aegle marmelos* (L.) Correa fruits on blood glucose and insulin levels in control and different therapeutic groups

Groups / Parameter	Normal Control (1)	Normal-treated mucilag (2)	Normal-treated Methanol extract (3)	Diabetes 2 days (4)	Diabetes 10 days (5)	Diabetes 40 days (recovery group) (6)	Diabetes (after10 days) treated mucilage (7)	Diabetes (after10 days)treated methanol extract (8)	Diabetes treated glibenclamide (9)
Glucose	112.40 ± 1.58	113.15 ± 2.88	113.5 ± 1.67	311.40 ± 8.59	323.52 ± 6.65	320.75 ± 9.03	133.85 ± 4.93	129.25 ± 7.1	119.00 ± 5.35
LSD	(4-8)	(4-8)	(4-8)	(1-3,5-9)	(1-4,7-9)	(1-4,7-9)	(1-6,9)	(1-6,9)	(4-8)
Insulin	5.750 ± 0.51	5.74 ± 0.33	5.81 ± 0.51	1.18 ± 0.250	1.16 ± 0.214	1.17 ± 0.28	3.65 ± 0.33	3.97 ± 0.10	4.56 ± 0.32
LSD	(4-9)	(4 – 9)	(4 – 9)	(1-3,7-9)	(1-3,7-9)	(1-3,9)	(1-6,9)	(1-6,9)	(1 – 8)

*Glucose was expressed in mg/dl and insulin in IU/ml
*Data are means ± SD of 10 rats in each group
*Statistical analysis is carried out using one-way analysis of variance (ANOVA) combined with post-hoc, where (SPSS Program) least significance difference between groups (LSD) at $P \leq 0.05$

and 1.17 ± 0.28 µIU/ml with percentage reduction reached to -79.48%, -79.83% and -79.65%, for insulin, respectively. Significant amelioration is noticed in blood glucose and insulin levels in all diabetic–treated groups recorded the most pronounced effect for methanol extract (+14.99% for glucose and -30.96% for insulin) followed by mucilage (+19.08 and -36.52.8%, respectively) comparing to glibenclamide as reference drug (+5.87 and -20.69%, respectively).

Table 3 shows the levels of lipid profile, total cholesterol, triglycerides and total lipid in normal control, normal-treated, diabetic and diabetic–treated rats. It can be easily noticed that, there is no significant change in total cholesterol, triglycerides and total lipid between different normal-treated rats as compared to untreated control one except total cholesterol shows significant reduction in methanol extract (-7.56%) compared to normal control group. With regard to diabetic rats, significant elevation in lipid profile is observed 2 days post streptozotacin injection as compared to normal control group, recorded 201.00 ± 10.36, 212.00 ± 10.29 and 1686.50 ± 10.75 mg/dl with percent of elevation +48.33, +100.00 and 66.96% for total cholesterol, triglycerides and total lipid

Table 3: Efficiency of the mucilage and the methanol fruits extract of *Aegle marmelos* (L.) Correa fruits on serum lipid profile in control, and different therapeutic groups

Groups / Parameter	Normal Control (1)	Normal -treated mucilag (2)	Normal-treated Methanol extract (3)	Diabetes 2 days (4)	Diabetes 10 days (5)	Diabetes 40 days(recovery group) (6)	Diabetes (after10 days) treated mucilage (7)	Diabetes (after10 days) treated methanol extract (8)	Diabetes treated glibenclamide (9)
Total Cholesterol	135.50 ± 5.32	129.00 ± 2.71	125.25 ± 2.87	201.00 ± 10.36	227.25 ± 6.85	230.75 ± 2.87	148.50 ± 2.38	141.50 ± 2.38	131.00 ± 5.83
LSD	(3 – 7)	(4 – 8)	(1,4-8)	(1-3, 5-9)	(1 – 4, 7 – 9)	(1-4, 7-9)	(1 – 6 , 9)	(2 – 6 , 9)	(4 – 8)
Triglycerides	106.00 ± 2.18	102.00 ± 2.16	100.25 ± 1.26	212.00 ± 10.29	239.75 ± 7.09	263.75± 5.19	145.00 ± 4.55	131.00 ± 4.08	117.00 ± 6.78
LSD	(4 – 9)	(4 – 9)	(4 – 9)	(1-3 , 5-9)	(1-4 , 6-9)	(1-5 , 7)	(1-6 , 8-9)	(1 – 7,9)	(1 – 8)
T-Lipid	1010.13 ± 26.92	986.35 ± 7.57	978.33 ± 7.66	1686.50 ± 10.75	1820.00 ± 21.60	1888.75±6.29	1414.25±45.23	1373.00±19.89	1144.75 ± 36.84
LSD	(4 – 9)	(4 – 9)	(4 – 9)	(1-3 , 5-9)	(1-4 , 7-9)	(1-4 , 6-9)	(1-6 , 8-9)	(1-7 , 9)	(1-8)

*Lipid profile was expressed in mg/dl
*Data are means ± SD of 10 rats in each group
*Statistical analysis is carried out using one-way analysis of variance (ANOVA) combined with post-hoc, where (SPSS Program) least significance difference between groups (LSD) at $P \leq 0.05$

respectively. On the other hand, nearly simultaneously elevated levels in lipid profile is recorded 10 and 40 days post streptozotacin injection with percentage of increase +67.71, 126.18 and 80.17% for total cholesterol, triglycerides and total lipid after 10 days and +70.29, +148.82 and +86.98% after 40 days, respectively. All diabetic-treated groups (G7-G9) show significant enhancement in lipid profile level as compared to normal control group, where in mucilage –treated diabetic group, total cholesterol recorded 148.50 ± 2.38 mg/dl with percentage increase +9. 59%. While in methanol extract, total cholesterol reaches to 141.50 ± 2.83 mg/dl with percent +4.43%. With respects to glibenclamide as reference drug, total cholesterol shows significant reduction amounted 131.00 ± 5.83 mg/dl with percent -3.32% as compared to normal control group. Remarkable, significant enhancement is noticed in triglycerides level post different types of treatments, recorded 145.00 ± 4.55, 131.00 ± 4.08 and 117.00 ± 6.78 mg/dl for mucilage, methanol extracts and glibenclamide drug, respectively as compared to normal control rats with elevated percent amounted +36.79, +23.58 and 10.38%, respectively. Total lipid reveals an enhanced significant mean value of 1414.25 ± 45.23, 1373.00 ± 19.89 and 1144.75 ± 36.84 mg/dl for mucilage, methanol extracts and glibenclamide drug as compared to normal control(1010.13 ± 26.92) with percent +40.00, +35.92 and +13.33%, respectively.

Table 4 demonstrates liver function enzymes AST, ALT, ALP enzyme activities, total protein and total albumin levels in serum of different control, diabetic and diabetic-treated groups. It is obvious that insignificant change is recorded in AST and ALT levels in serum of normal treated rats either with mucilage or methanol extract. On the contrary significant inhibition is noticed in ALP activity in normal treated rats (-8.56% and -8.02% for mucilage and methanol extract, respectively). With respect to total protein and albumin contents, insignificant change is observed in their levels in normal treated groups as compared to untreated control one except total albumin exhibits significant increase in normal treated methanol extract rats. With regard to diabetic condition, significant increase in all enzyme activities is noticed at day 2,10 and 40 post streptozotacin injection reached to 70.04 ± 0.43, 76.15 ± 2.91 and 78.53 ± 1.31 mole/mg protein/min for AST with percentage increase +350.99, +390.41 and +405.67%, respectively. While, ALT recorded 60.43 ± 1.27, 64.44 ± 1.58 and 66.33 ± 0.90 mol/mg protein/min with percentage increase +199.31%, +219.17% and +228.53%, respectively. ALP shows a value of 6.25 ± 0.20, 6.63 ± 0.04 and 6.74 ± 0.17 mole/mg protein/min with percentage of elevation +67.11, +77.27 and +80.21%, respectively. Total protein content shows significant reduction at different durations post streptozotacin injection (-14.49, -17.77 and -26.49 at 2, 10 and 40 days, respectively). Albumin exhibited the same significant reduction manner as total protein 3.09 ± 0.12, 2.75 ± 0.13 and 2.68 ± 0.17 mg/dl as compared to normal control rats with reduction percent -18.89, -27.82 and -29.66% at 2,10 and 40 days, respectively. The curative effect of mucilage and methanol extracts and glibenclamide drug on diabetic rats can easily be noticed through the normalization of all enzymes tested returned more or less to the level of normal control, with elevated percent amounted +47.52, +17.84, +25.21, +14.07, +21.93 and +8.56% for AST, ALT and ALP and for mucilage and methanol extract, respectively. Insignificant change in all enzymes tested in glibenclamide treated diabetic rats as compared to normal control. Total protein content still recorded significant reduction post both extracts treatment (in spite of its normalization with glibenclamide) amounted 94.40 ± 3.77 and 101.65.25 ± 2.69 mg/ml with percentage decrease -14.244 and -7.66%, respectively. In contrast total albumin shows insignificant change in different diabetic treated groups as compared to normal control one.

Table 4: Efficiency of mucilage and methanol extract of *Aegle marmelos* (L.) Correa fruits on liver function enzyme activities, total protein content and albumin level in control and different therapeutic groups

Groups / Parameter	Normal Control (1)	Normal-treated mucilage (2)	Normal-treated methanol extract (3)	Diabetes 2 days (4)	Diabetes 10 days (5)	Diabetes 40 days(recovery group) (6)	Diabetes (after10 days) treated mucilage (7)	Diabetes (after10 days) treated methanol extract (8)	Diabetes treated Glibenclamide (9)
Total Protein	110.08 ± 1.46	109.15 ± 2.89	112.83 ± 1.82	94.13 ± 4.77	90.52 ± 0.032	80.91 ± 1.02	94.40 ± 3.77	101.65 ± 2.69	112.88 ± 2.04
LSD	(4 – 8)	(4 – 8)	(4 – 8)	(1-3,6,8,9)	(1-3, 6-9)	(1-5, 7-9)	(1-6,8,9)	(1-7,9)	(4-8)
AST	15.53 ± 0.49	15.25 ± 0.13	15.20 ± 0.18	70.04 ± 0.43	76.15 ± 2.91	78.53 ± 1.31	22.91 ± 2.12	18.30 ± 0.55	16.35 ± 0.99
LSD	(4 – 8)	(4 – 8)	(4 – 8)	(1-3 , 5-9)	(1-4) , 6-9)	(1-5 , 7-9)	(1-6 , 8,9)	(1-7)	(4-7)
ALT	20.19 ± 0.83	20.29 ± 0.48	20.25 ± 0.30	60.43 ± 1.27	64.44 ± 1.58	66.33 ± 0.90	25.28 ± 1.57	23.03 ± 0.75	21.00 ± 0.82
LSD	(4 – 8)	(4 – 8)	(4 – 8)	(1-3, 5-9)	(1-4 , 6-9)	(1-5 , 7-9)	(1-6 , 8-9)	(1-7 , 9)	(4 – 8)
ALP	3.74 ± 0.19	3.42 ± 0.13	3.44 ± 0.07	6.25 ± 0.20	6.630 ± 0.040	6.74 ± 0.17	4.56 ± 0.78	4.06 ± 0.15	3.56 ± 0.27
LSD	(2 – 8)	(1, 4 – 8)	(1, 4-8)	(1-3 , 5-9)	(1-4 , 7-9)	(1-4 , 7-9)	(1- 6 , 8-9)	(1-7 , 9)	(4 – 8)
Albumin	3.81 ± 2.39	4.98 ± 0.11	5.03 ± 0.11	3.09 ± 2.39	2.75 ± 0.13	2.68 ± 0.17	3.75 ± 0.33	4.24 ± 0.19	4.72 ± 0.20
LSD	(3)	(4 – 7)	(1 , 4 – 7)	(2, 3, 9)	(2, 3, 8 , 9)	(2, 3, 8, 9)	(2, 3)	(5, 6)	(4 – 6)

*AST, ALT and ALP are expressed in umole/mg protein/min. *Total protein content is expressed in mg/ml, while total albumin is expressed in mg/dl

*Data are means ± SD of 10 rats in each group

*Statistical analysis is carried out using one-way analysis of variance (ANOVA) combined with post-hoc, where (SPSS Program) least significance difference between groups (LSD)

Table 5 shows the manipulation of nitric oxide (NO) and lipid peroxidation (MDA) in liver tissue homogenates of different control, diabetic and diabetic–treated groups. It can be easily noticed that MDA levels is insignificantly affected post various extracts-treated normal rats as compared to untreated control one. In response to diabetic state, MDA shows significant increase in its level of a value 145.33 ± 4.30, 150.18 ± 4.26 and 156.100 ± 2.27 µg/g tissue with percentage increase +698.96, +725.62 and +758.16% at day 2, 10 and 40 post streptozotacin injection, respectively. The level of MDA is significantly improved as a result of different treatments, shows the best pronounced effect for methanol extract as compared to normal control rats amounted 22.70 ± 2.12 ug/g tissue with percentage change amounted +24.79 (+57.39 for mucilage treatment). While insignificant change is observed in MDA

Table 5: Efficiency of mucilage and methanol extract of *Aegle marmelos* (L.) Correa fruits on nitric oxide (NO) and lipid peroxidation (MDA) in liver of control and different therapeutic groups

Groups / Parameter	Normal Control (1)	Normal-treated mucilag (2)	Normal-treated Methanol extract (3)	Diabetes 2 days (4)	Diabetes 10 days (5)	Diabetes 40 days(recovery group) (6)	Diabetes (after10 days) treated mucilage (7)	Diabetes (after10 days) treated methanol extract (8)	Diabetes treated glibenclamide (9)
MDA	18.19 ± 0.66	17.73 ± 1.3	16.59 ± 0.42	145.33 ± 4.30	150.18 ± 4.26	156.100 ± 2.27	28.63 ± 7.33	22.70 ± 2.12	18.75 ± 1.24
LSD	(4 – 8)	(4 – 8)	(4 – 8)	(1-3 , 5-9)	(1 – 4, 6– 9)	(1-5, 7-9)	(1–6 , 8- 9)	(1–7 , 9)	(4 – 8)
NO	43.22 ± 2.22	39.60 ± 0.58	38.69 ± 1.34	81.88 ± 2.76	84.20 ± 0.80	85.28 ± 2.60	47.50 ± 2.61	43.40 ± 2.77	41.38 ± 2.18
LSD	(2 – 7)	(1.4 –8)	(1,4 – 8)	(1-3 , 6-9)	(1-3 , 7-9)	(1-4 , 7-9)	(1-6 , 8-9)	(2-7)	(4-7)

*NO and MDA are expressed in ug/g tissue.
*Data are means ± SD of 10 rats in each group.
*Statistical analysis is carried out using one-way analysis of variance (ANOVA) combined with post-hoc, where (SPSS Program) least significance difference between groups (LSD) at $P \leq 0.0$.

level in glibenclamide treated diabetic rats. On the other hands, NO level demonstrated significant reduction in normal mucilage and methanol extract leaf amounted 39.60 ± 0.58 and 38.69 ± 1.34 ug/g tissue (-8.38 and -10.48%, respectively) as compared to normal control (43.22 ± 2.22 ug/g tissue). However significant increase is noticed in NO level streptozotacin injection amounted 81.88 ± 2.76, 84.20 ± 0.80 and 85.28 ± 2.60 ug/g tissue at 2, 10 and 40 days with percent of elevation amounted +89.44, +94.82 and +97.32%, respectively. Significant amelioration is observed in NO level post mucilage treatment of diabetic rats amounted 47.50 ± 2.61 ug/g tissue (+ 9.90%) as compared to normal control level. Although, NO level returned to its normal level post both total methanol extract and glibenclamide drug, where insignificant change is recorded. Table 6 recorded the level of gluconeogenic and glycolytic enzymes in liver tissue homogenates of the different studied groups. Careful inspection of the data would reveal that gluconeogenic and glycolytic enzymes show insignificant change in different normal-treated groups as compared to the normal untreated control one. It can be deduced that all enzymes either gluconeogenic or glycolytic are strongly affected with diabetic condition, where PEPCK shows significant increase amounted 14.75 ± 0.72, 15.55 ± 0.46 and 15.95 ± 0.13 umole/mg protein/min. with percentage increase amounted +90.94, +101.29 and +106.47% at 2, 10 and 40 days, respectively post streptozotacin injection as compared to control. However HK, PK and LDH glycolytic enzymes show significant inhibition reached to 29.55 ± 0.64, 25.100 ± 0.22 and 19.39 ± 0.13 mole/mg protein/min. for HK with percentage of inhibition -73.08, -77.13 and -82.33% and amounted 16.60 ± 1.16, 15.00 ± 0.14 and 14.54 ± 0.43 mole/mg protein/min, for PK (with percent -74.22, -76.71 and -77.42%), while LDH

Table 6: Efficiency of mucilage and methanol extract of *Aeglemarmelos* (L.) Correa fruits on some glycolytic and gluconeogenic enzymes in liver of control and different therapeutic groups.

Groups / Parameter	Normal Control (1)	Normal -treated mucilage (2)	Normal -treated methanol (3)	Diabetes 2 days (4)	Diabetes 10 days (5)	Diabetes 40 days(recovery group) (6)	Diabetes (after10 days) treated mucilage (7)	Diabetes (after10 days) treated methanol extract (8)	Diabetes treated Glibenclamide (9)
PEPCK LSD	7.7250 ± 0.31 (4 – 8)	7.93 ± 3.86 (4 – 8)	8.09 ± 2.18 (4 – 8)	14.75 ± 0.72 (1-3 , 5-9)	15.55 ± 0.46 (1-4 , 7-9)	15.95 ± 0.13 (1-4 , 7-9)	9.39 ± 0.42 (1-6 , 9)	8.90 ± 0.08 (1-6 , 9)	8.00 ± 0.08 (4-8)
HK LSD	109.75 ± 4.11 (4 – 8)	109.00 ± 0.82 (4 – 9)	110.75 ± 1.71 (4 – 8)	29.55 ± 0.64 (1-3 , 5-9)	25.100 ± 0.22 (1-4 , 6-9)	19.39 ± 0.13 (1-5 , 7-9)	86.40 ± 2.70 (1-6 , 8,9)	98.85 ± 2.63 (1-7 , 9)	112.83 ± 4.00 (2,4-8)
PK LSD	64.40 ± 4.05 (4 – 8)	66.17 ± 0.82 (4 – 8)	66.15 ± 2.41 (4 – 8)	16.60 ± 1.16 (1-3, 7-9)	15.00 ± 0.14 (1-3 , 7-9)	14.54 ± 0.43 (1-3 , 7-9)	48.63 ± 1.84 (1-6 , 8-9)	54.38 ± 1.98 (1-7 , 9)	65.54 ± 2.30 (4 – 8)
LDH LSD	36.90 ± 2.05 (4 – 8)	37.95 ± 1.30 (4 – 8)	37.57 ± 1.82 (4-8)	9.950 ± 0.06 (1-3 , 7-9)	9.22 ± 0.22 (1-3 , 7-9)	8.78 ± 0.46 (1-3 , 7-9)	30.53 ± 1.26 (1- 6 , 8-9)	34.15 ± 0.64 (1-7 , 9)	36.25 ± 1.26 (4 – 8)

* All enzymes are expressed in umole/mg protein/min. (Unit /min).
* Data are means ± SD of 10 rats in each group.
* Statistical analysis is carried out using one-way analysis of variance (ANOVA) combined with post-hoc, where (SPSS Program) least significance difference between groups (LSD) at $P \leq 0.05$

recorded activity of 9.95 ± 0.06, 9.22 ± 0.22 and 8.78 ± 0.46 mole/mg protein/min. with percent of inhibition -73.03, -72.57 and -76.21% at 2,10 and 40 days, respectively post STZ injection and as compared to normal control. Treatment of the diabetic rats with extracts produced obvious improvement in all gluconeogenic and glycolytic enzymes tested as compared to both normal control and glibenclamide treated –diabetic groups. Since PEPCK demonstrated significant increase with percent +21.55, +15.21 as a result of treatment with mucilage and methanol extract respectively. While normalization in PEPCK value is noticed after glibenclamide treatment, where insignificant change is recorded as compared to normal control. HK, PK and LDH glycolytic enzymes show also significant amelioration amounted 86.40 ± 2.70, 98.85 ± 2.63 umole/mg protein/min, for HK with percent of inhibition -21.28 and -9.93% post both extracts treatment, respectively. While for PK, the curative effect of extracts reached to 48.63 ± 1.84 and 54.38 ± 1.98 umole/mg protein/min with percent -24.49 and -15.56%, respectively. Significant normalization can be also seen in the level of LDH post treatment of diabetic rats with mucilage and methanol extracts recorded 30.53 ± 1.26 and 34.15 ± 0.64 umole/mg protein/min. with inhibition percent of -17.26 and -7.45%, respectively as compared to normal control rats. In addition, normalization in all diabetic glycolytic enzymes is noticed after treatment with glibenclamide drug, where insignificant change is recorded as compared to their normal control levels.

Histopathological examination of pancreas tissue

The histological structure of the control pancreas consisted of closely packed lobules of pancreatic acini. Islets of Langerhans were embedded within the exocrine portions and cells located on the periphery (Figure 1). Control pancreas of rats treated with mucilage and methanol extract showing nearly normal structure of Islet's of Langerhans showing well preserved pancreatic islets with no change in the exocrine region (Figures 2 and 3). The present light microscopic study of diabetic rats revealed pathological changes of both exocrine and endocrine part of the pancreas represented by vacuolation and marked decrease of β-cells. Some exocrine acini

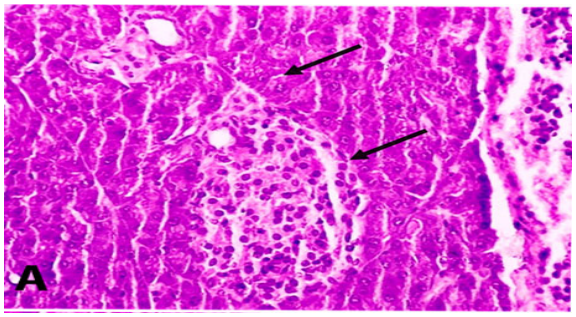

Figure 1: Photomicrograph section (A) of rat pancreatic tissue of the control group showing Islet's cells forming cords separated by a network of blood capillaries stained with (H&E x200)

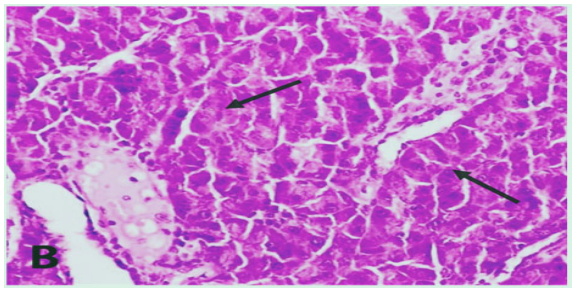

Figure 2: Photomicrograph (B) of a pancreatic section from healthy group treated with methanol extract showing well preserved pancreatic islets with no change in the exocrine region (↖) (H&E x200)

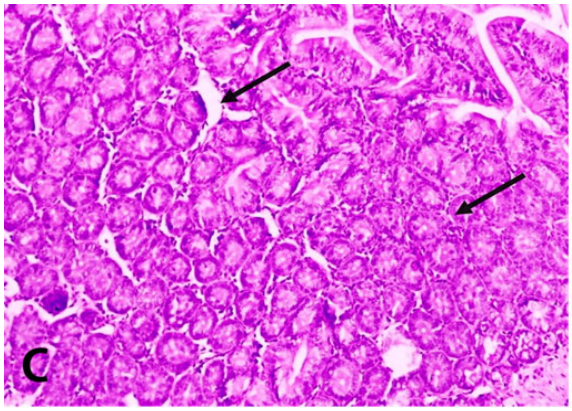

Figure 3: Photomicrograph (C) of a pancreatic section from healthy group treated with mucilage showing no change in pancreatic cells.

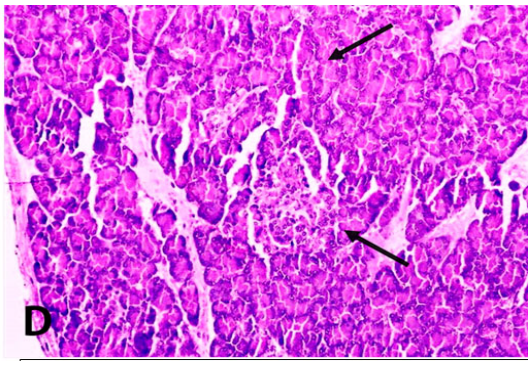

Figure 4: photomicrograph section (D) of rat pancreatic tissue of the diabetic group 48 hr. showing degenerated of islet's cells with nuclear pyknosis and nuclear fragmentation Inset (H&E x200) (↖)

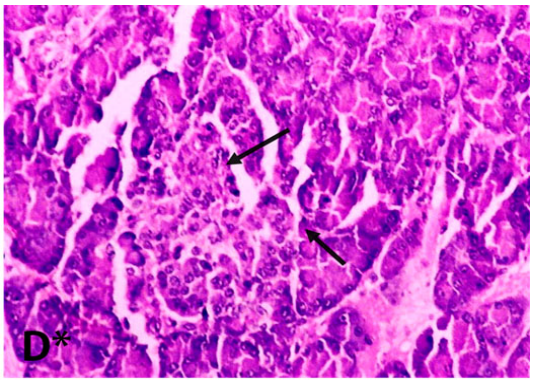

Figure 5: Other Magnification of section (D*) showing the empty spaces leaved after cell degeneration filled with amyloid-like material (H&E x400)

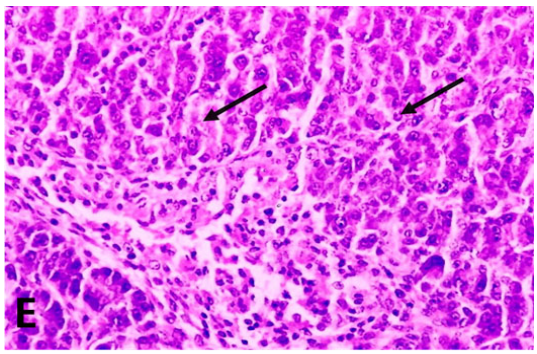

Figure 6: Photomicrograph section (E) of rat pancreatic tissue of the diabetic group after 10 days showing the inflammatory cells infiltration inside the islet (↖) (H&E x200)

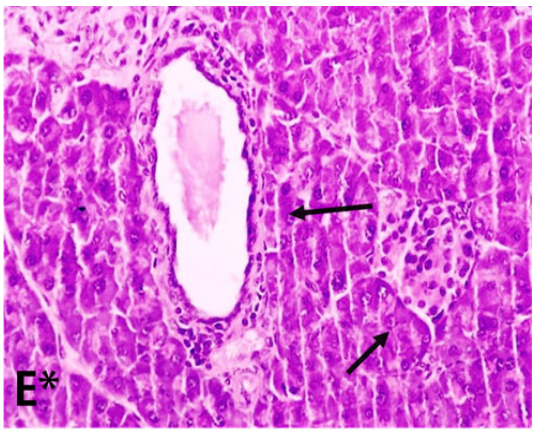

Figure 7: Other Magnification of (E*) noticed cellular residues within the cytoplasm of phagocytic cells, the phagocytic cells are surrounded by empty spaces (H&E x400)

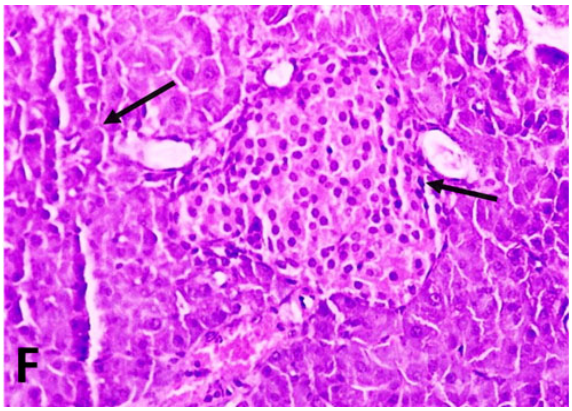

Figure 8: Photomicrograph section (F) of rat pancreatic tissue of the methanol extract treated group showing increase islet cell density and reduction in the inflammatory cells infiltration (↖) (H&E x400)

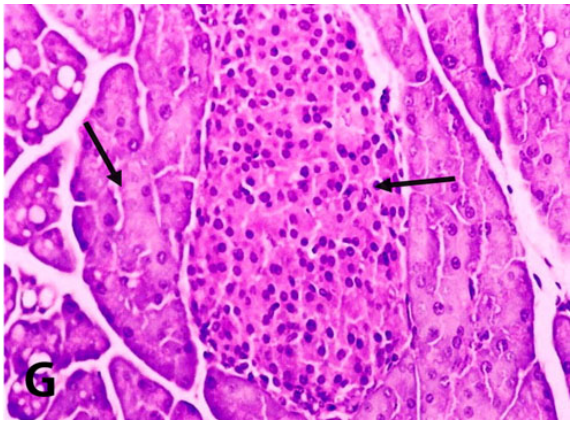

Figure 9: Photomicrograph section (G) of rat pancreatic tissue of the glibenclamide treated group showing islet's of Langerhans with distinct border surrounding exocrine part (↖) (H&E x400)

revealed focal acinar damage represented by cytoplasmic vacuolation and pyknotic nuclei of some acinar cells (Figures 4-7). After supplementation with methanol extract and mucilage the pancreas section showing increase islet's cell density and reduction in the inflammatory cells infiltrationappeared similar to the control and most of the Islet's of Langerhans were intact with no alteration except few vacuoles (Figures 8 and 9).

Histopathological study of liver tissue

The investigation examined the histology of healthy control and treated rats with methanol and mucilage extract of fruits showing

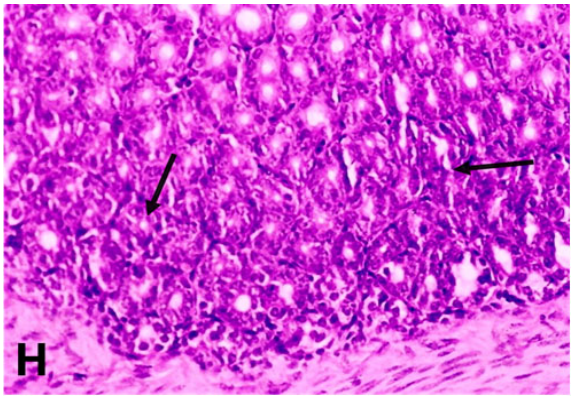

Figure 10: Photomicrograph section (H) of rat pancreatic tissue of the mucilage treated group showing nearly control pancreatic islet's with no change in the exocrine region (H&E x400)

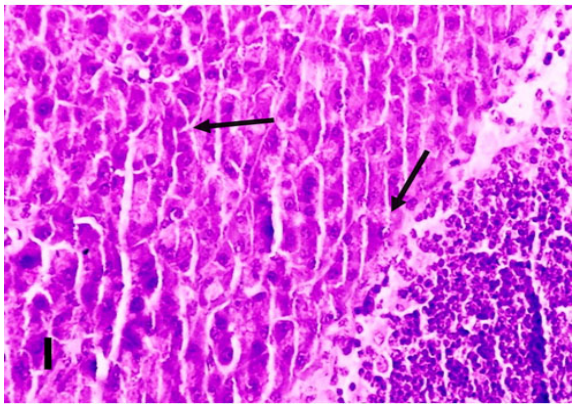

Figure 11: photomicrograph section (I) of rat pancreatic tissue of the diabetic group left without therapeutic treatment for 40 days showing abnormal pancreatic cells, whereas nuclear fragmentation Inset still present (H&E x200). (↖)

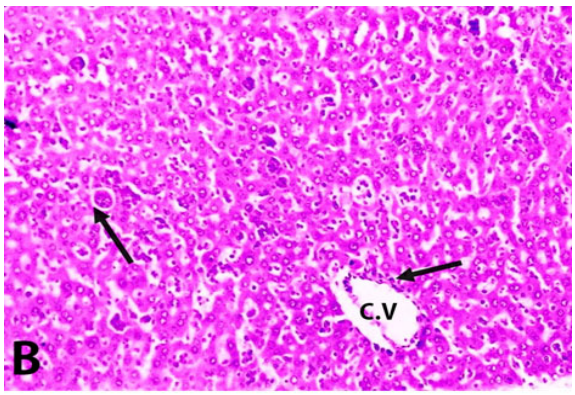

Figure 12: Photomicrograph (B) of liver section of healthy group treated with methanol extract showing no change in centrilobular region and periportal region with no change (H&E x100)

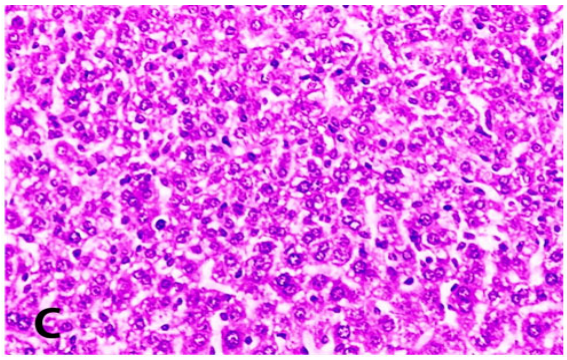

Figure 13: Photomicrograph (C) of liver section of healthy group treated with mucilage showing nearly normal hepatic cells (H&E x200)

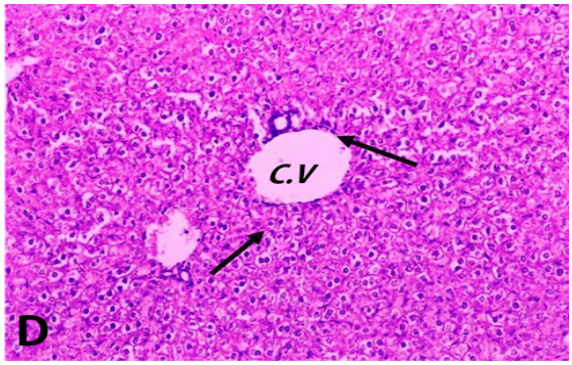

Figure 14: Photomicrograph (D) of toxicity liver section, of rat stained with (H&E ×100 showed centrilobular region; Periportal region showed severe morphological changes as a result of giving streptozotacin (48 hr)

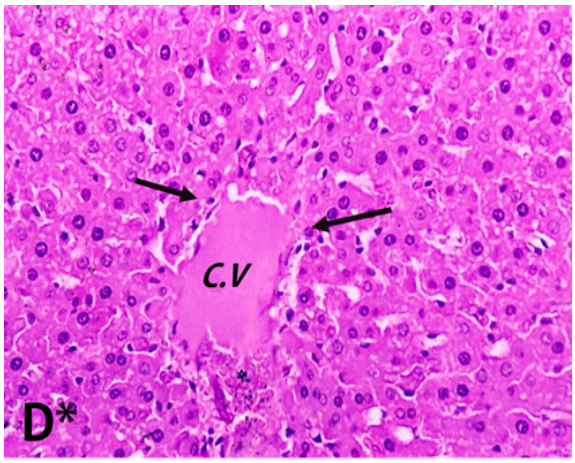

Figure 15: Other section of higher Magnification of (D*) Noticed cellular residues within the cytoplasm of phagocytic cells, the phagocytic cells are surrounded by empty spaces (↖) (H&E x400)

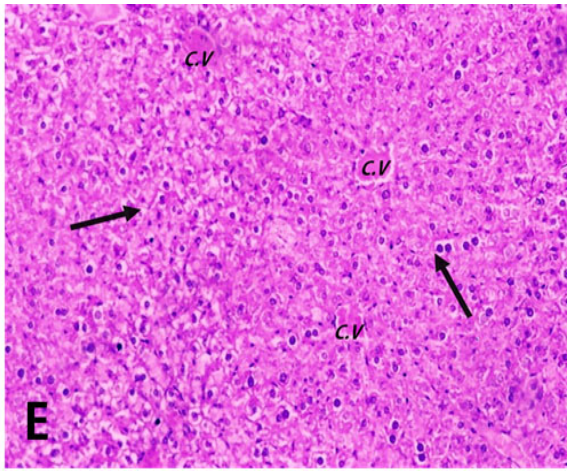

Figure 16: Photomicrograph (E) of toxicity liver section of rat stained with (H&E ×100) showed hydropic degeneration, lymphocyte infiltration, and congestion of central vein as a result of giving streptozotacin (10 days)

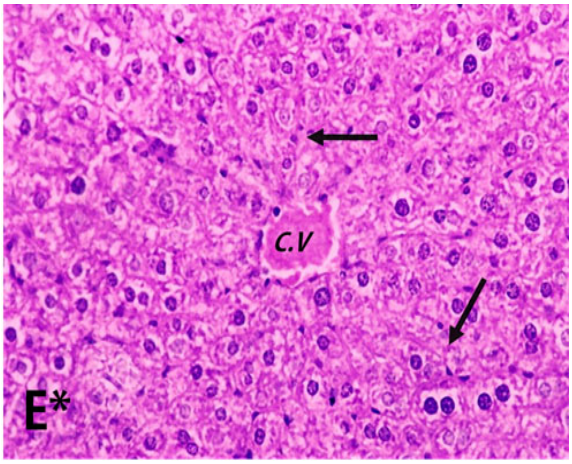

Figure 17: Other liver section with higher magnification(E*) stained with (H&E x200)

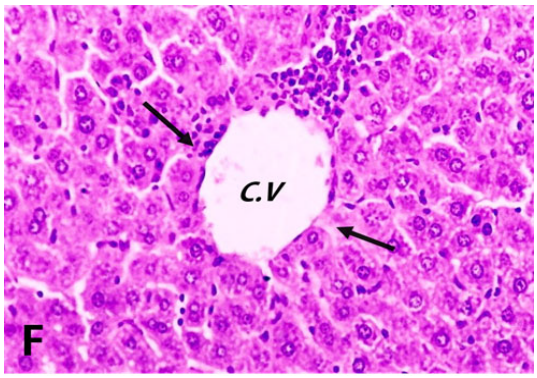

Figure 18: Photomicrograph (F) of intoxicated rat liver section, treated with methanol extract showing improvement in the liver cells stained with (H&E x200) showed the normal structure of liver control

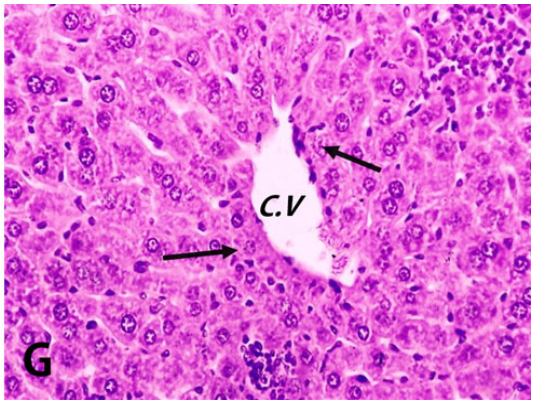

Figure 19: Photomicrograph (G) of intoxicated rat liver section, treated with mucilage showing improvement in the liver tissue stained with (H&E x200)

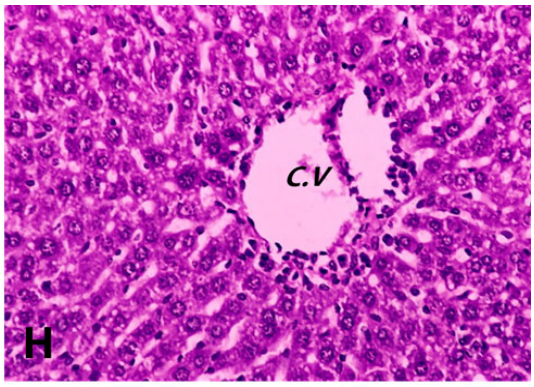

Figure 20: Photomicrograph (H) of intoxicated rat liver section, treated with glibenclamide showing improvement in the liver tissue stained with (H&E x200)

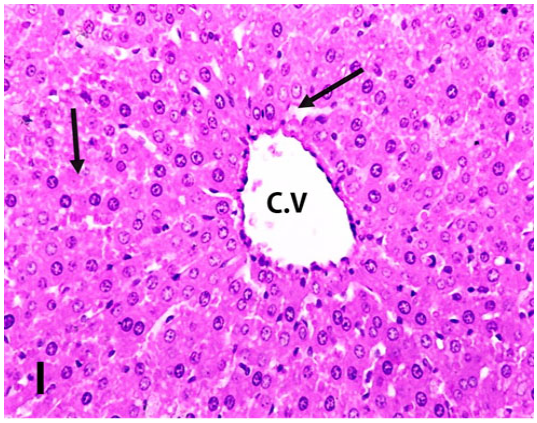

Figure 21: Photomicrograph(I) of liver section of intoxicated rat after 40 days, left free of treatment with therapeutic substance, showing that lymphocyte infiltration still present (H&E x200)

normal hepatic cells with no change in centrilobular region and periportal region with no change (Figures 10-12). Diabetic rats showed severe morphological changes in centrilobular region; Periportal region as a result of giving streptozotacin 48 hrs (Figures 13-15) and hydropic degeneration, lymphocyte infiltration, Congestion of central vein after (10 days) as shown in (Figures 16-18). Administration of methanol, mucilage and glibenclamide show that most of hepatic lobules are almost similar to that of the control group with remarkable improvement compared to the streptozotacin-treated group (Figures 19-21).

DISCUSSION

The current work declared improvement in blood glucose and insulin levels in diabetic rats supplemented with mucilage and methanol extract of *A. marmelos* (L.) Correa fruits with improvement percentage 168.74 and 172.83%, for glucose, 43.3% and 48.87%, for insulin, respectively. Also, diabetic rats treated with standard drug, glibenclamide attenuated the blood glucose and insulin to normal values (181.96% and 59.13%, respectively). Kamalakkannan and Prince [33] agreed with our results as aqueous extract of *A. marmelos* (L.) Correa fruits had hypoglycaemic activity. Sharma et al. [34] postulated that, the aqueous extract of *Aegle marmelos* (L.) Correa plant may have bioactive substances that are able to induce β-cells to enhance insulin secretion improving enzymes responsible for carbohydrate metabolism resulting in normalization of blood glucose value. In addition, Narendhirakannan et al. [35] demonstrated certain inorganic trace elements in the leaves of *A. marmelos* (L.) Correa that may have the potential role in preserve normal glycaemia by stimulating pancreas β-cells. With respect to lipid profile in this study, marked improvement in lipid profile was detected in diabetic rats treated with the mucilage and methanol extract with percentages 58.11 and 63.28% respectively for total cholesterol as compared to 71.03% in glibenclamide-treated diabetic rats. Triglycerides recorded enhanced level of 89.39 and 102.59%, respectively comparing to glibenclamide (115.8%). Also, total lipid was ameliorate by 40.17 and 44.25%, respectively (66.92% for glibenclamide).

In concomitant, with the present results Kamalakkannan et al. [36] found the elevated serum and tissue lipids in diabetic rats were reduced after aqueous *A. marmelos* (L.) Correa fruits extract supplementation. The antihyperlipidemic effect exerted by using 250 mg/kg fruits extract was found to be higher than 125 mg/kg glibenclamide. This hypolipidemic effect may be related to the active constituents in *A. marmelos* (L.) Correa as, the dry pulp of fruits contains chiefly mucilage pectin like substance.

The present results demonstrate increment in liver enzyme activities AST, ALT, ALP at different durations after injection of STZ linked with inflammatory reaction leading to damage of liver cells and apoptosis. In a parallel work, Ahmed et al. [37] declared elevation in liver enzymes activities in diabetes comparing to normal value.

Marked improvement in liver function enzyme markers as well as total protein content was noticed upon treatment of diabetic rats with mucilage and methanol extract with percent of improvement 342.8%, 372.5%, respectively for AST relative to 385.06%, for glibenclamide. ALT recorded 193.95% and 205.1%, respectively (215.15% for glibenclamide). ALP showed ameliorated level amounted to 55.34% and 68.72% respectively as well as 82.08% for glibenclamide. Our results are consistent with previous studies that, the enzyme activities of serum AST, ALT and ALP, were enhanced after treatment of diabetic as well as carbon tetrachloride intoxicated rats with ethanol and aqueous leaf extracts of *A. marmelos* (L.) Correa as compared to control [38]. Total protein content exhibited ameliorated level by 3.56 and 10.11%, for mucilage and methanol extract compared with 20.31% for glibenclamide, in accordance to the present results. The aqueous and alcoholic extracts of *A. marmelose* (L.) Correa leaves treated animals for 14 days at 250 mg/kg body weight significantly decreased of protein catabolism as compared to untreated diabetic animals. However, the rate of protein catabolism was still higher than controls [34].

Significant increase was achieved in oxidative stress markers MDA and NO in streptozotacin induced diabetic rats. However significant amelioration was noticed after treatment of diabetic rats with mucilage and methanol extracts of A. *marmelose* (L.) Correa recorded percent of improvement 668.22% and 700.82%, for lipid peroxidation as compared to glibenclamide which exhibited 722.54% respectively. NO revealed percent of amelioration of 84.91 and 94.4% respectively (99.07% for glibenclamide). From previous results [39], aqueous extract of *Aegle marmelos* (L.) Correa fruits exhibited antidiabetic and anti-oxidative activity in streptozotacin induced diabetic rats. In addition, Sharma et al. [34] declared *A. marmelos* (L.) Correa leaf extract treatment to diabetic rats induced by alloxan decreases MDA as the product of lipid peroxidation. Hence, the beneficial effects of *A. marmelose* (L.) Correa extracts is well investigated at cellular level either in hepatocytes or pancreas cells by normalization and neutralizing oxidative stress.

Considering enzymes responsible for the metabolism of carbohydrate, LDH, PK, HK and PEPCK. Treatment of diabetic rats with mucilage and methanol extract showed high percentages of improvement in both pathways of enzymes as they declared 79.74% and 86.08% respectively for (PEPCK) comparing to 97.67%, for glibenclamide. While glycolytic enzymes recorded percent of improvement reached to 55.85%, 67.19%, 52.22%, 61.14%, 57.75% and 67.56% respectively for HK, PK and LDH (79.93%, 78.47% and 73.25% for glibenclamide respectively). In connection with the obtained data Aly et al. [40] found that in diabetes condition, glycogen was degraded so gluconeogenesis are enhanced, although the utilization of glucose is decreased. This leads to increment in liver glucose-6-phosphatase, initiating the release of glucose into the circulation. While, hexokinase which is responsible glucose phosphorylation is an insulin and glucokinase-independent and inhibited in diabetes. As a consequence glucose output by liver was go on, constant. In this circumstance the ordinary liver must stop and precipitate glycogen. In concomitant with the present results Ismail [41] suggested that the *A. marmelose* (L.) Correa leaves produced antihyperglycemic effect probably by enhancing the peripheral utilization of glucose, correcting the impaired hepatic glycolysis and limiting its gluconeogenic formation similar to insulin.

CONCLUSION

It could be concluded that, mucilage and methanol extracts of *A. marmelose* (L.) Correa fruit demonstrate anti-hyperglycemic, antioxidant, hypolipidemic effects. These may be attributed to the identified constituents in extracts which have free radical scavenging activity, ameliorating liver enzyme activities, improved lipid profile levels as well as they have important role in preserved liver, pancreas architectures. So, these extracts can be effective in improving liver damage as complication of diabetes which needs further clinical investigation for future using as new candidate natural antidiabetic supplements.

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