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Acacia gerrardii Leaf Extracts Inhibit Genetic Diversity Induced by Streptozotocin in Male Rats

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This study investigated the prevention effect of *Acacia gerrardii ý*(AG) leaf extracts on genetic variation in male rats exposed to streptozotocin (STZ)-induced diabetes. Male rats (n=70) were treated with *A. gerrardii* leaf extract using concentrations ranging from 100 to 500 mg/kg body weight/day for four weeks against STZ-induced possibly genetic variation. Blood samples and pancreatic tissues were collected at the end of the experiment. Ten Inter-Simple Sequence Repeat(ISSR) primers and tenStart Codon Targeted Polymorphism(SCoT) primers were used to detect genetic variations between the different groups. The biochemical results indicated a reduction inserum glucose levels, especially at doses of 400 and 500 mg/kgbody weight. Molecular analysis showed that ISSR primers and SCoT detected 119 and 97 amplicons, with 0.43% and 0.55% polymorphic respectively. Based on the previous markers analyzed, cluster analysis revealed that genetic variation was high in the pancreatic tissues of STZ-treatedrats in the groups treated with 0 (C+) > 100 (T1) > 200 (T2) > 300 (T3) mg/kg body weight/day. However, low genetic variation was observed in the pancreatic tissues of STZ-treatedrats in the groups treated with 400 (T4) > 500 (T5) mg/kg body weight/day. These results indicate that the A. *gerrardii* leaf extracts are rich in antioxidants preventing genetic variation in the pancreatic tissues. Therefore, A. *gerrardii* leaf extracts could be recommended as good source of dietary antioxidant.

Keywords: *Acacia gerrardii ý*, Genetic diversity, ISSR and SCoT marker, Streptozotocin, Diabetes, Rats.

400 million people had diabetes worldwide in 2013 and this number will increase to almost 600 million by 2035[1]. Most people with diabetes live inlow or medium-income countries, where rapid changes in lifestyle have increased the prevalence of cancer, diabetes and heart diseases. These countries are expected to experience the greatest increase in diabetes in the next 20 years[2].

Development of diabetes can be attributed to the combined effect of genetic and environmental factors[3]. The most effective method to investigate genetic variations in diabetes uses molecular markers, which in biotechnology and molecular biology contexts, identify specific sequences within a wider pool of unknown DNA. These markers can be used to detect sequence changes, such as insertion, duplication, inversion, deletion or nucleotide changes[4], using either long or short sequences[5].

Such markers include:Single-Nucleotide Polymorphism (SNP), Inter-Simple Sequence Repeat (ISSR) and Start Codon Targeted

Polymorphism (SCoT)[6].Of these, ISSRs are cheap and comparatively easy to use, so are well-suited to beginners or for use with organisms where genetic information is lacking[7]. ISSRs refer to genomic regions that are bounded by microsatellite sequences. A single-primer PCR amplification of these regions yields multiple amplification products, enabling a dominant multi-locus marker system to identify genome variations in a range of organisms. Similarly, SCoT is relatively simple and new to the market. Dependent on the short-conserved genetic region, SCoT marker primers focus on the translation initiation codon,ATG, and its surrounding conserved regions[8-9]. In this procedure, single primers are formed from the above-mentioned region without knowing any genomic sequence information[10]. SCoT markers are deemed to have more precision than either Random Amplified Polymorphic DNA (RAPD) or ISSR. Furthermore,Gorji *et al*[11]have proposed that the reproducibility of SCoT markers are not solely dependent on annealing temperature or primer length.Theprimer has also demonstrated its value in the analysis of genetic variation among a group of plant species[12].

The relationship between genetic variation, detected using different molecular marker assays, and genes responsible for morphological and physiological traits,has been identifiedusing advanced bioinformatics analysis techniques[13].

Different medicinal plants have clearly shown the presence of antidiabetic compounds[14]. Herbal drugs and medicinal plants may provide the best substitution to synthetic drugs as they are more affordable and have a long history of safe and effective use in both conventional and folk medicine[14].Herbs are used as a drug source to treat diseases and ailments such as diarrhea, colds, flu, dysentery, malaria, wounds, sexually transmitted infections (STIs), as well as being used as an ethno-veterinary and colic medicine[15].

Multiple classes of phytochemicals such as phenols, flavonoids, phytosterols, tannin, proanthocyanidin and terpenes, as well as many minerals have been recognized in the roots and leaves ofAcaciaspecies[16].

Acacia gerrardii ý is a small tree belonging to the Fabaceae family. It is found in tropical and subtropical areas[17]and is an essential component of hilly desert and wadi areas, due to its nitrogen fixation ability. It could therefore be considered a keystone species[18]. Because it is a good source of tannins and gums, A. *gerrardii* is considered one of the most essential tree species in Saudi Arabia[19].

There is now published data about the relationship between medicinal herbs and decreased genetic variation in induced diabetic animal models. The present study was therefore conducted to determine genetic variation among diabetic rats treated with different concentrations of A. *gerrardii* leaf extracts using different marker systems.

MATERIALS AND METHODS

Plant materials

A. *gerrardii* leaveswerecollected from theTaif region, south-east of Jeddah and Makkah, in the Kingdom of Saudi Arabia.

Chemicals

Gentamicin grade, used for induction of chronic kidney disease (CKD) among rats was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Kits for determining the parameters were purchased from Sigma-Aldrich Corp., MO, USA. Streptozotocin-induced diabetic rats were purchased from Upjohn Company, USA.The DNeasy Mini Kit, ISSR and SCoT primers were purchased from QIAGEN, Germany.

Methanol extraction from *Acacia gerrardii* leaves

The mill machine was used to obtain A. *gerrardii* leaf powder extracts, which was stored in amber bottles to prevent degradation. About 50ml methanol water (4:1 v/v) was used to dissolve 5g of the extract powder at room temperature, using an orbital shakerovernight. The methanol extract solution was filtered using centrifugation (5000rpm for 10min) and the supernatant was subjected to rotary evaporation at 40ºC for concentration. After the solvent evaporated, the remaining extract was dissolved in distilled water.

Animals

Male albino rats (n=70), weighing 155-185g each, were acquired from the King Saud University College of Pharmacy and transported to the King Fahd Medical Research Center in Jeddah. Their basal diet which consisted of corn starch (65%), casein(15%, containing 12.6% protein),

vitamin mixture (1%), salt mixture (4%), and cellulose (5%) was prepared according to AOAC[20]. Rats were kept separately in wired cages at 25 ± 2°C and under good healthy environmental conditions.

Experimental diabetes induction

Diabetes was induced in rats that had been fasted for 12 hours via a single intraperitoneal injection of STZ (50 mg/kg) dissolved in citrate buffer (0.01 M, pH 4.5), while a control group was injected with citrate buffer only. The threshold to be considered diabetic wasa blood glucose level exceeding 250 mg/dl[21]and these rats were added to the study 72h post-injection.

Experimental setup

All rats were fed on the basal diet for one-week at the start of the study for environmental acclimatization. They were then divided into two groups: Group 1 (n=10) was fed on the basal diet and labelled control-ve (C-). Group 2 (n=60) were induced with diabetes by injecting streptozotocin dissolved in citric acid (0.1M, pH 4.5) IP into the leg muscle (5mg /100g body weight) after fasting overnight[22]. The second main group was divided into six sub-groups (n=10 rats each) 48h after the injection and fed on different diets for four weeks as follows: Group (1): Negative control (C-), fed on basal diet. Group (2): Control positive (C+), fed on basal diet. Groups (3-7): Fed on basal diet, with 100, 200, 300, 400 and 500 mg/kg body weight/day ofA. *gerrardii* leaf extract, taken orally. Blood samples of fasting rats from retro-orbital venous plexus were obtained under diethylether anesthesia. These blood samples were drawn at several intervals, collected in dry, clean centrifuge tubes and left to clot. For the purpose of glucose estimation, the blood serum was then separated from samples[23]. The clear sera was separated though centrifugation (3000 rpm for 15 min at 4°C), then stored at –20ºC. After scarifyingether-anesthetized rats, the pancreatic tissues were gathered for molecular biological assays.

DNA Extraction

Genomic DNA was obtained from the pancreasusing the DNeasy Mini Kit (QIAGEN). DNA quality and quantity was determined through comparison with standard lambda DNA on 1%(w/v) agarose gel[24].

Inter-Simple Sequence Repeat (ISSR) Technique

Ten ISSR primers, detailed in Table 1, were used in this study. Our PCR reaction and amplification method replicated that used by Attallah et al[24]. 10µl of the ISSR-PCR products were resolved using 1.5% agarose gel electrophoresis with ethidium bromide. A standard for DNA molecular weight was determined using a 100bp DNA marker (Fermentas), and the Gel Doc™ System (Bio-Rad) was used to visualize and photographically record the results.

SCoT (Start Codon Targeted) Technique

The study used ten SCoT primers, detailed in Table 1. The PCR reaction and amplification program was performed as described byIbrahim *et al*[25]. The amplification products were separated, visualized and photographed as mentioned in the ISSR assay.

Statistical analysis

The statistical procedure of analysis of variance was applied. Means were compared using Duncan's Multiple Range test (DMRT)at (*P d"* 0.05). This analysis was carried out using SAS[26].A present (1) and absent (0) reading system was used in recording PCR fragments and the Dice's similarity matrix coefficient was used to measure the genetic variation between control and treatment through the unweighted pair group method (UPGMA). The obtained data matrix was used to construct phylogentic trees using Dendro UPGMA(genomes.urv.es/UPGMA).

RESULTS AND DISCUSSION

Effect of A. *gerrardii* leaf extract on serum glucose levels

The effect of different concentrations of A. *gerrardii* leaf extracts on serum glucose levels in STZ-treated rats is summarized in Table 2. Rats treated with STZ showed significantly increased ($P<0.01$) levels of serum glucose at the onset and the end of the experiment. Supplementation of STZ-treated rats with 100 mg/kg body weight/day with A. *gerrardii* leaf extract did notsignificantly decrease the glucose levels compared with rats treated with STZ. The serum glucose levels started to decrease significantly in STZ treated-rats with the dose of 100 mg /kg body weight/day from *A. gerrardii* leaf extract. The glucose levels decreased to levels similar to the negative control at the highest dose (500 mg/kgbody weight/day) of*A. gerrardii* leaf extract. Blood glucose levels in STZ-induced diabetic experimental rats increased

significantlycompared with the control group. Similar results have been reported by others[27-29]. STZ is diabetogenic agent with alkylating properties and is cytotoxic to mammalian pancreatic beta cells (*â*-cells)[30].The consumption of STZ molecules results in the formation of superoxide radicals in *â*-cells and also*â*-cell destruction by necrosis by the of NO moiety from STZ[31].Moreover, Asad et al[29]reported that a significant decrease in blood glucose was observed in diabetic rats treated with *Acacia nilotica* leaf extract in comparison to diabetes controlled rats.It is evident from this investigation that the *Acacia* extract was effective in maintaining the blood glucose levels in STZ-induced diabetic rats.

ISSR- and SCoT PCR Analysis

This study successfully used ISSR and SCoTto differentiate between five treated pancreatic tissues and its control by components

Table 1. The primer name (PN) and primer sequence of ISSR and SCoT PCR primers

PN	PS
ISSR- 7	5'-GACGATAGATAGATAGATA-3'
ISSR- 8	5'-AGACAGACAGACAGACGC-3'
ISSR-10	5'-GACAGACAGACAGACAAT-3'
ISSR-11	5'-ACACACACACACACACY*A-3'
ISSR-12	5'-ACACACACACACACACYC-3'
ISSR-13	5'-AGAGAGAGAGAGAGAGYT-3'
ISSR-17	5'-TCTCTCTCTCTCTCTCA-3'
ISSR-18	5'-HVHCACACACACACACAT-3'
ISSR-19	5'-HVHTCCTCCTCCTCCTCC-3'
ISSR-20	5'-HVHTGTGTGTGTGTGTGT-3'
SCoT-1	5'-ACGACATGGCGACCACGC-3'
SCoT-2	5'-ACCATGGCTACCACCGGC-3'
SCoT-3	5'-ACGACATGGCGACCCACA-3'
SCoT-4	5'-ACCATGGCTACCACCGCA-3'
SCoT-5	5'-CAATGGCTACCACTAGCG-3'
SCoT-6	5'-CAATGGCTACCACTACAG-3'
SCoT-7	5'-ACAATGGCTACCACTGAC-3'
SCoT-8	5'-CATGGCTACCACCGGCCC-3'
SCoT-9	5'-ACAATGGCTACCACTGCC-3'
SCoT-10	5'-ACAATGGCTACCACCAGC-3'

Table 2. Effect of A. gerrardii leaf extract concentrations on serum glucose levels in STZ-treated rats

Treatment	Glucose level (mg/dl)	
	Day 0*	Week 4
Control (C-)	86.5 ± 10.3	84.2 ± 11.2^{d}
Control (C+)	349.2 ± 18.5	341.6 ± 22.3^{a}
T1	332.8 ± 22.1	318.3 ± 10.5^{a}
T2	337.6 ± 20.8	254.6 ± 11.6^{b}
T3	331.4± 17.5	158.2 ± 9.4^{c}
T4	336.5± 21.3	127.2 ± 8.2^{cd}
T5	330.9 ± 24.7	98.6 ± 7.6^{d}

Day 0*: blood sugar reached >250 mg/dl (2-3 days post-STZ treatment); Control (C-): Negative control (C-) fed on basal diet. Control (C+): Positive control treated with STZ and fed on basal diet. T1-T5: Male rats treated with STZ and fed on basal diet and orally injected with 100, 200, 300, 400 and 500 mg /kg/ day body weight from A. gerrardii leaves extract, respectively.

Table 3. Primer Name (PN); primer sequence (PS); number of scored bands (NSB); number of polymorphic bands(NPB);mean band frequency (MBF) for the ISSR primers used in this study

PN	NSB	NPB	%P	MBF
ISSR- 7	12	6	50.0%	0.5
ISSR- 8	9	5	55.6%	0.8
ISSR- 10	11	6	54.5%	0.7
ISSR- 11	9	0	0.0%	1
ISSR- 12	9	2	22.2%	0.8
ISSR- 13	14	5	35.7%	0.9
ISSR- 17	15	7	46.7%	0.7
ISSR- 18	16	8	50.0%	0.8
ISSR- 19	11	3	27.3%	0.8
ISSR- 20	13	4	30.8%	0.9
Total	119	46	38.7%	
Average	11.9	4.6	38.7%	0.79

Table 4. Statistical analysis of SCoT primers used in this study and the amplification results

PN	NSB	NPB	%P	MBF
SCoT -1	8	4	50.00%	0.7
SCoT -2	16	4	25.00%	0.5
SCoT -3	10	1	10.00%	0.4
SCoT -4	12	0	0.00%	0.7
SCoT -5	8	5	62.50%	0.8
SCoT -6	6	3	50.00%	0.8
SCoT -7	8	6	75.00%	0.9
SCoT -8	9	5	55.56%	0.8
SCoT -9	13	6	46.15%	0.6
SCoT -10	7	5	71.43%	0.9
Total	97	39		7.1
Average	9.7	3.9	40.21%	0.71

of *A. gerradii*. The ability to detect polymorphic loci among the control and the treatments was compared between ISSR and SCoT assays. ISSR analysis found a total of 119 polymorphic and mono-morphic bands, using ten primers in n=7 groups, each with a distinct scorable fragment. The PCR products' molecular weight ranged from 144bp to 2,307bp, where the number of observed and scorable fragments ranged from 9 to 16, with a mean of 11.9 bands per PCR primer (Fig.1). Out of 119 bands, 46 were polymorphic with an average of polymorphism of 38.7% across all treatments.

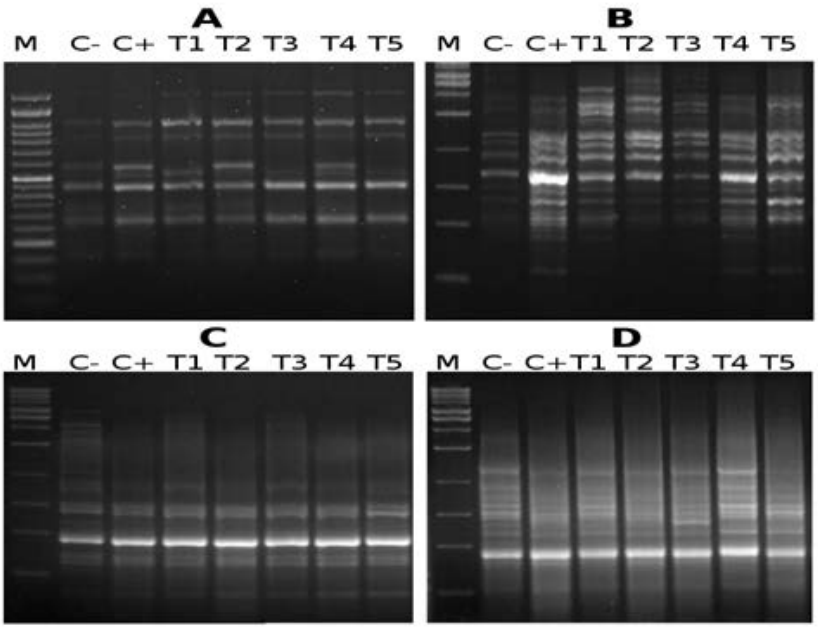

Fig. 1. PCR products with ISSR marker profiles. M: Marker 1kb DNA ladder, C-; control negative, C+; control positive, T1 to T5; treatments A:Primer ISSR8 , B: Primer ISSR18, C: Primer ISSR19 and D: primer ISSR20

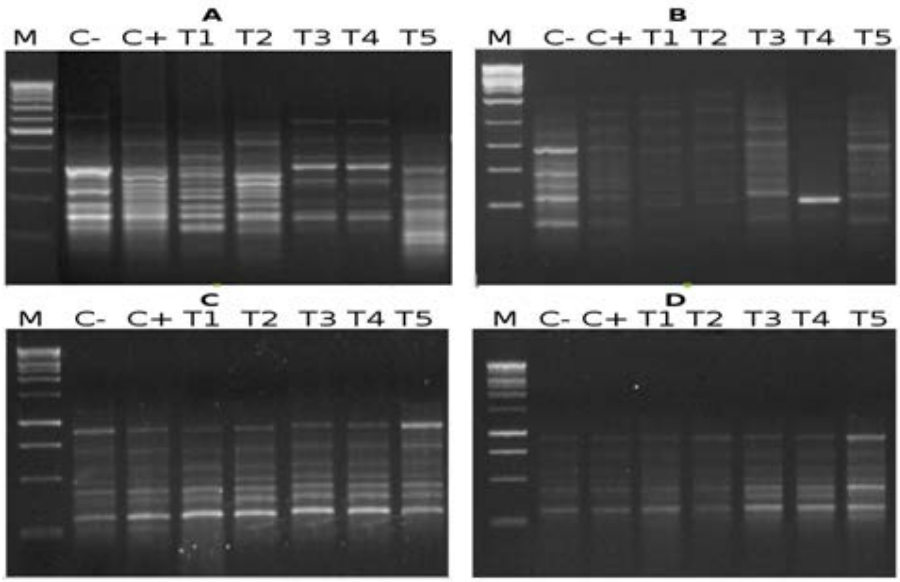

Fig. 2. PCR products with SCoT marker profiles. M: Marker 1kb DNA ladder,C-; control negative, C+; control positive, T1 to T5; treatment A:Primer SCoT9 , B: Primer SCoT2, C: Primer SCoT4 and D: primer SCoT1

The maximum number of polymorphic bands was 8 (ISSR-18) (Table 3).

ISSR-18 was the most polymorphic, with 8 bands. This was followed by ISSR-17 (7 bands), ISSR-7 (6 bands) and ISSR-10 (6 bands). Polymorphism resulting from ISSR primers spanned from 0% (ISSR-11, with no monomorphic band) to 55% (ISSR-8). The band frequency mean ranged from 0.5% (ISSR-7, which yielded high polymorphic bands) to 1% (ISSR-11, with 0% polymorphism efficiency). Full results are given in Table 3. Using the same primers,Hatti et al[32]achieved the same results as our study. Following Ali[33], we calculated polymorphism as the percentage of polymorphic bands from the total number of bands produced by each primer.

Variable factors influence the number of bands amplified by different primers as the annealing sites in the genome and primer structure[34-35].

This study found significant variation between differently-treatedrat pancreatic tissues, in concordance with Raghunathachari *et al*[36], Saker *et al*[37]and Abdulateef *et al*[38].The present study obtained results using ISSR assay, indicating that $(AC)^8$ repeats are more frequent in the*A. gerrardii* genome than in the TG repeats.

SCoT Polymorphism

39 PCR markers exhibited the total number of scored bands (97) and polymorphism. SCoT-7 and 9 primers had the highest number of bands (6 each) and the SCoT-4 primer did not reveal any polymorphic bands. Figure 2 illustrates some SCoT primer PCR patterns. Polymorphism averaged at 40.2%.SCoT-2 produced 0% polymorphism, while SCoT-7 produced the highest percentage (75%). The mean band frequency average across all primers was 0.71. Scot-7 and Scot-10 produced the highest value (0.90), while SCoT-3 produced the lowest value (0.50), illustrated in Table 4.

Of the 97 scored bands of PCR products, 39 were polymorphic, while 17 were unique, reflecting the findings of Gorji *et al*[11]and Ibrahim *et al*[25], in their SCoT assays in potato and grape respectively.

Cluster analysis using PCR markers

The study used cluster analysis to estimate the differences between control and treatment polymorphism. This resulted from the banding patterns of ISSR and SCoT markers (Tables 3 and 4, and Figs. 3A and 3B).

A dendrogram, constructed using a distance matrix, using the UPGMA method, depending on genetic distances from ISSR marker analysis, showed that three major groups were observed. T1 was separated into a single group (group I), whereas T2 and T3 were placed in a second group (group II), with a similarity ranging from 0.82 to 0.91. T5 and T4 were separated into athird group, with a similarity range from 0.82 to 0.88.

The control (C and C+) and treatments

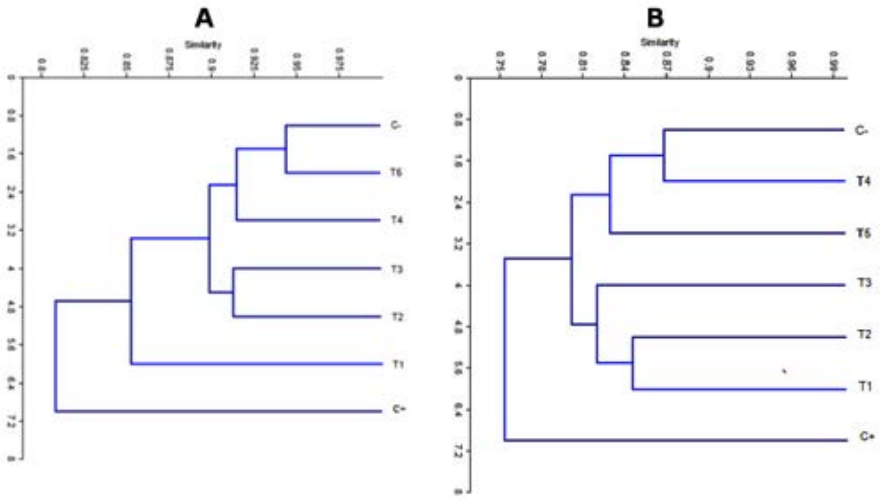

Fig. 3. Dendrogram [(A) ISSR; (B) SCoT] illustrated genetic fingerprint and relationships between control and treatments

could be divided into three groups. Group I consisted of C-, T4 and T5; Group II of T3 (which wassimilar to T2 and T1); and Group III contained only C+. These findings agreed with those ofXiong *et al*[39], who concluded that the highly polymorphic nature of SCoT markersmake them useful for genetic analysis of functional genetic variation and examining the relationships between different genotypes.

CONCLUSION

The molecular biological analysis revealed that genetic diversity was high in the pancreatic tissues of STZ-treatedrats collected from the groups treated with 0 (C+) > 100 (T1) > 200 (T2) > 300 (T3) mg/kg body weight/day. However, low genetic variation was observed in the pancreatic tissues of STZ-treatedrats collected from the groups treated with 400 (T4) > 500 (T5) mg/kg body weight/ day. From this, we concluded that the *A. gerrardii* leaf extracts are rich in antioxidants, preventing genetic variation in pancreatic tissues. Therefore, *A. gerrardii* leaf extracts could be recommended as good source of dietary antioxidant.

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