Effect of spray-dried ethanolic extract of *Andrographis paniculata* (Burm. F.) Nees on streptozotocin-induced diabetic female rats

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The objective of this study was to evaluate the effect of commercially prepared spray-dried ethanolic extract of *Andrographis paniculata* (AP) on streptozotocin (STZ)-induced diabetic female rats. **METHODOLOGY:** Rats with regular estrous cycle (EC) prior to STZ induction were randomly divided into five groups. The normal (nondiabetic) and diabetic control groups were given vehicle [0.2 ml of 2% carboxyl methyl cellulose (CMC) by gavaging] daily for 6 weeks. Other diabetic groups were treated with 50, 100 and 200 mg/kg/day of AP extracts respectively. The rats' body weight (BW), fasting blood glucose and insulin level were measured; and daily, EC evaluation was performed throughout the 6-week study period. At the end of the experiment, rats were sacrificed and their pancreases were removed for histological examination. **RESULTS:** The survival rates and estrous cycle of AP-treated diabetic animals were found to be improved compared to nontreated animals. No significant difference in blood glucose level was noted in AP-treated group compared to diabetic control group. Diabetic rats treated with 100 mg/kg of BW of AP extract showed significant reduction in blood insulin level compared to the rats in the diabetic control group. Endocrine cell density was significantly increased in 50 mg/kg AP treated diabetic rats compared to both diabetic and normal control groups. AP spray-dried ethanolic extract at a maximum dose of 200 mg/kg/day was found to have no antihyperglycemic effects in STZ-induced diabetic rats. **CONCLUSION:** The AP extracts used in this study increased the survival rate and endocrine cell density, improved estrous cycle and reduced the 'insulin resistance' phenomenon in STZ-induced diabetic rats.

KEY WORDS: *Andrographis paniculata*, diabetes mellitus, rats

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Diabetes mellitus (DM) is a chronic disease with complex underlying etiologies. It was characterized by hyperglycemia and other metabolic abnormalities due to glucose intolerance.[1,2] The incidence of diabetes mellitus is on the rise worldwide. Based on the World Health Organization (WHO) report, the number of diabetic patients is expected to increase from 171 million in year 2000 to 366 million or more by the year 2030.[3] Herbal medicine has been, and will be, used as antidiabetic therapy alone and along with insulin and other synthetic oral hypoglycemic agents. The use of synthetic agents, on the other hand, has shown several undesirable side effects and has failed to correct the fundamental biochemical lesion and diabetic complications.[4,5] This has increased the use of alternative medicine to treat DM. The consumption of botanicals has been increasing and has continued to be an important area of active research worldwide. The herbal medicines are apparently effective, produce less frequent side effects and are relatively inexpensive as compared to oral synthetic hypoglycemic agents.[6,7] In accordance to the recommendations by the WHO expert committee on diabetes mellitus, investigations on hypoglycemic agents from medicinal plants have become more important.[8]

Andrographis paniculata (Burm. F.) Nees is a traditional medicinal plant belonging to the family of Acanthaceae. This annual herb is indigenous to southeast Asia, China and India. *Andrographis paniculata* (AP) has a broad range of pharmacological effects and high therapeutic value. It is claimed to possess antihepatotoxic, antibacterial, antimalarial, antihepatitis, antitrombogenic, anti-inflammatory, antipyretic properties and has been used for the treatment of snake bites. In Malaysia, this plant has reputable use in treating diabetes and hypertension,[9] and its aqueous extract was effective in reducing the blood glucose level of diabetic animal models.[9,10] The ethanolic extract of AP demonstrated similar glucose-lowering effect in diabetic animal models.[11] The

present study was conducted to evaluate the effect of repeated dose subacute (6 weeks) administration of a commercially prepared spray-dried ethanolic extract of AP on fasting blood glucose, insulin level, estrous cycle and the histology of the islet cells in streptozotocin (STZ)-induced diabetic female Sprague-Dawley (SD) rats. The contents of andrographolide, neoandrographolide and 14-deoxyandrographolide of the test extract were analyzed using high-performance liquid chromatography (HPLC) and compared with the ethanol extract of similar plant prepared in the university lab.

Methodology

Animals

Adult female SD rats (200-230 g) with regular estrous cycle were obtained from Animal House Unit, Health Campus Universiti Sains Malaysia (USM). The rats were housed in polypropylene cages in an air-conditioned room at a temperature of 22 ± 2°C with 12 h dark and light cycle. The animals were fed with pellet obtained from Gold Coin®, Malaysia, and tap water was given ad *libitum*. The study protocol was approved by the University Animal Ethic Committee (USM/PPSF/050(1) Jld.1).

Preparation of the extract and analysis of the extent of andrographolide decomposition to its 14-deoxy derivative in the extract after spray-drying of the AP leaves at high temperature

Standard and reagents

Andrographolide, neoandrographolide and 14-deoxyandrographolide were isolated in our laboratory and identified by spectroscopic analysis from the 95% ethanolic extract of AP leaves derived from NOVA Pharmaceutical Sdn Bhd, Selangor, Malaysia. The acetonitrile (Licrosov grade) used was purchased from Merck, Germany. Methanol (HPLC grade) for HPLC analysis was purchased from BDH Laboratory Supplies, England. Freshly prepared distilled deionized water was filtered under partial vacuum and degassed prior to HPLC analysis.

HPLC apparatus

The phytochemical analysis of the different samples was performed using a HPLC system consisting of Gilson LC 307 delivery pump connected to a Rheodyne 7125 (USA) containing a 20 µl sample loop, linked in series with a reversed-phase 4.6 x 250 mm Biosphere C_{18} 5 µ column and a Gilson 115 UV/Visible detector monitoring at 230 nm. The signal from the detector was analyzed with a Hitachi D 2500 Chromato-integrator.

Chromatographic conditions

The standards were dissolved in methanol and diluted subsequently with a mobile phase mixture of acetonitrile: water (33:67). The flow rate was maintained at 1 ml/min at room temperature.

Extract preparation

The commercially prepared spray-dried 95% ethanolic extract and the AP leaves for extraction in our lab were supplied by NOVA Pharmaceutical Sdn Bhd, Selangor, Malaysia. About 100 mg of AP powdered leaves were accurately weighed and extracted with 5 × 5 ml 95% ethanol in an ultrasonic bath at 45°C for 20 min per interval. The combined extracts were filtered and evaporated to dryness under nitrogen gas. The dry extract prepared in the lab and the commercially prepared spray-dried extracts were redissolved in HPLC methanol and diluted appropriately with the mobile phase prior to analysis. Three different samples of each extract were analyzed for their andrographolide, neoandrographolide and 14-deoxyandrographolide.

Induction of experimental diabetes rats

Rats were fasted overnight (16-18 h) before being injected with STZ (Sigma-Aldrich Co., USA) at a dose of 50 mg/kg of BW (in sodium citrate buffer, pH 4.5) via the lateral tail vein. The induction of STZ-diabetes was confirmed by determination of high-fasting blood glucose level with polydipsia and polyuria on the third day of STZ administration. Rats with fasting blood glucose level between 12 and 20 mmol/L were selected for experimentation.

Experimental procedure

Sixty rats were randomly divided into five groups. The first group (n = 12), the normal control group, was given vehicle (0.2 ml of 2% CMC solution). Following the confirmation of STZ-induced diabetes, animals of group 2 to 5 were given AP extract at 0, 50, 100 and 200 mg/kg/day respectively. The extract was administered via gavaging between 1000 to 1030 h daily for a period of 6 weeks. Rats' body weight was measured weekly. Estrous cycle was evaluated by performing daily vaginal smear.[12] On the evening of the last day of treatment, rats were fasted overnight (16-18 h) and sacrificed the next morning. Laparotomy was performed under general anesthesia (diethyl ether), blood was collected via posterior vena cava puncture and animals were sacrificed

by an anesthetic overdose. Fasting blood glucose levels were recorded immediately using glucometer (Roche Diagnostics, Germany). Blood was centrifuged at 3000G for 15 min, and serum insulin levels were measured by a rat insulin ELISA kit (DRG Instruments, GmbH, Germany). Pancreases were removed and immediately fixed in 10% formalin, stained with hematoxylin and eosin (H and E) and analyzed using computerized image analyzer.[13,14]

Statistical analysis

Data were analyzed using SPSS version 11. Results were expressed as median (Interquartile range, IR) and analyzed using Kruskal-Wallis (K-W), followed by Mann-Whitney U (MW-U) test to compare between two groups. '*P*' values of <0.05 were considered as statistically significant.

Results

Amount of andrographolide, neoandrographolide and 14-deoxyandrographolide in extract of leaves samples and commercial extract of AP.

The amount of andrographolide in the leaf extract (8.11% ± 1.3) prior to large-scale extraction and spray drying at high temperature was 3.56 times higher than its content (2.28% ± 0.16) after large-scale extraction and spray drying at high temperature [Table 1]. Its lower content was due to high-temperature decomposition of andrographolide, thus resulting in the corresponding 3.30 times increase in concentration of 14-deoxyandrographolide, its decomposed product, from 2.78% ± 0.8 [Table 1] to 9.17% ± 0.7 [Table 2]. The content of neoandrographolide remained almost similar.

Survival rate and body weight

The effect of AP extract on survival rate of rats is shown in Table 3. Only three rats (25%) survived in the diabetic control group. The survival rates of AP-treated diabetic rats were found to be increasing in a dose-dependent manner (50, 58.33 and 66.67% in animals that received 50, 100 and 200 mg/kg/day extract respectively). The body weight of diabetic rats was found to be reducing throughout of the study [Figure 1]; however, the percentage changes in body weight of the AP-treated diabetic rats were not significantly different compared to the diabetic control rats [Table 3].

Fasting blood glucose and serum insulin levels

Fasting blood glucose levels were persistently high in diabetic control and AP-treated diabetic rats throughout the study and they were not significantly different [Table 3]. There was a significant difference in the fasting insulin levels ($P < 0.05$, KW). Rats that received AP extract at a dose of 100 mg/kg/day showed a significant reduction in fasting insulin level compared to normal and diabetic control rats ($P < 0.5$, MW-U). Diabetic control rats showed the highest insulin level; however, it was not significantly different as compared to normal control rats.

Estrous cycle

The diabetic rats showed a prolonged anovulatory estrous cycle. Treatments with AP extract showed significant improvement in the estrous cycle of diabetic rats. There was a significant increase in the number of ovulatory cycles in animals that received 100 mg/kg/day extract compared to the nontreated diabetic rats [Table 3].

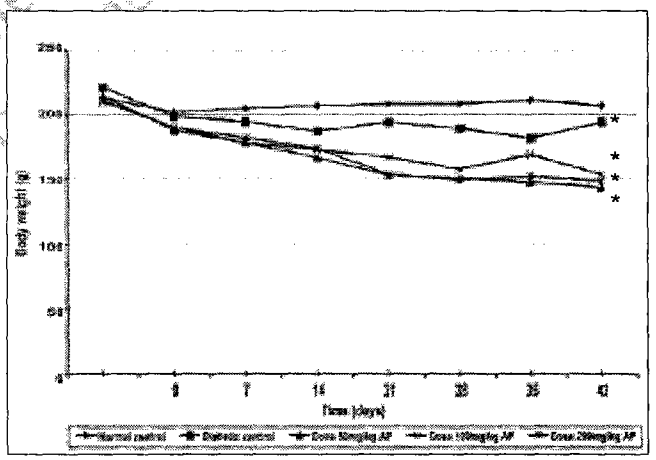

Figure 1: Effects of different doses of spray-dried 95% ethanol extract of *Andrographis paniculata* on body weight over a 6-week period in STZ-induced diabetic rats. *'P' < 0.05 when compared with normal control group (n = 12 for each group at the start of experiments)

Table 1: Amount of andrographolide, neoandrographolide and 14-deoxyandrographolide in the 95% ethanol *Andrographis paniculata* extract of leaf samples supplied by NOVA Pharmaceutical Sdn Bhd for analysis prior to large-scale extraction and spray drying at high temperature

HB leaf extract	Andrographolide			Neoandrographolide			14-deoxyandrographolide		
	1	2	Mean ± SD	1	2	Mean ± SD	1	2	Mean ± SD
Sample 1	9.73%	11.06%	10.40%	0.29%	0.33%	0.31%	3.41%	3.87%	3.64%
Sample 2	7.20%	7.10%	7.15%	0.20%	0.21%	0.21%	2.40%	2.44%	2.42%
Sample 3	6.77%	6.81%	6.79%	0.20%	0.21%	0.21%	2.26%	2.30%	2.28%
			8.11% ± 1.30			0.24% ± 0.60			2.78% ± 0.80

Table 2: Amount of andrographolide, neoandrographolide and 14-deoxyandrographolide in the 95% ethanol *Andrographis paniculata* extract of leaf samples supplied by NOVA Pharmaceutical Sdn Bhd for analysis after large-scale extraction and spray drying at high temperature

HB leaf extract	Andrographolide			Neoandrographolide			14-deoxyandrographolide		
	1	2	Mean ± SD	1	2	Mean ± SD	1	2	Mean ± SD
Sample 1	1.99%	2.38%	2.19%	0.30%	0.34%	0.32%	8.05%	9.20%	8.63%
Sample 2	2.34%	2.38%	2.36%	0.36%	0.38%	0.37%	9.65%	9.81%	9.73%
Sample 3	2.17%	2.39%	2.28%	0.33%	0.37%	0.35%	8.61%	9.69%	9.15%
			2.28% ± 0.16			0.35% ± 0.03			9.17% ± 0.70

Table 3: Effect of different doses of spray-dried *Andrographis paniculata* 95% ethanol extract on the survival rate, percentage change in body weight (BW), fasting blood glucose (FBG) and fasting serum insulin (FSI) levels, number of ovulatory estrous cycles and endocrine cells density in STZ-induced diabetic rats

Parameter	Normal control (I)	Diabetic control (II)	Diabetic 50 mg/kg AP (III)	Diabetic 100 mg/kg AP (IV)	Diabetic 200 mg/kg AP (V)	*P* values
Survival rates (%)	100.00	25.00	50.00	58.33	66.67	-
Changed in BW (%)	3.49 (14.85)	-3.96 (0.00)	-21.29 (21.71)	-18.32 (17.78)	-21.99 (7.71)	0.703
FBG at day 0 (mmol/L)	4.45 (0.45)	12.60 (0.00)	16.55 (2.4)	16.50 (3.6)	16.50 (4.63)	0.877
FBG at day 42 (mmol/L)	5.95 (1.93)	24.69 (0.00)	27.65 (7.03)	24.50 (11.50)	22.65 (13.98)	0.491
FSI at day 42 (μg/L)	0.16 (0.32)	0.24 (0.00)	0.05 (0.21)	0.04 (0.09)*	0.12 (0.17)	0.029
Number of ovulatory estrous cycle	8.00 (0.00)	1.00 (0.00)	2.00 (0.00)	5.00 (0.00)°	1.00 (0.00)	0.042
Endocrine cells density (cell/[μm]^2)	0.06 (0.11)	0.07 (0.13)	0.15 (0.15)*	0.06 (0.15)	0.07 (0.09)	0.119

Each value represents median (IR), except survival rate. Comparisons were made between group II, III, IV and V (Kruskal-Wallis test). '*P*' < 0.05 was considered significant difference. *'*P*' < 0.05, compared with group I and II, °'*P*' < 0.05, compared with group II (Mann-Whitney U test), AP - *Andrographis paniculata*.

The histology of Islet cells of Langerhans

The effect of AP extract on the endocrine cell density is shown in Table 3. The endocrine cell density of diabetic animals that received the AP extract at a dose of 50 mg/kg/day was increased significantly when compared to both the normal and diabetic control groups. The Islet cell size however was not significantly different. Islet of Langerhans of control diabetic rats showed hydropic degeneration with accumulation of intracellular fluid, and cells have cytoplasm with distortion of nucleus. The islet of Langerhans of diabetic rats that received AP extract at a dose of 100 mg/kg/day showed reduction in hydropic degeneration compared to the control diabetic rats.

Discussion

Large-scale production of plant extract is necessary for long-term research and development program. Production of spray-dried AP extract at high temperature has led to increased decomposition of andrographolide to 14-deoxyandrographolide. Therefore, the amount of andrographolide was significantly reduced and that of 14-deoxyandrographolide was significantly increased in the spray-dried extract compared to the extract that was not exposed to high-temperature drying.

The present study demonstrated that the spray-dried ethanol extract of AP had no antihyperglycemic effect in STZ-induced diabetic rats throughout the 6 weeks of study. Many animals showed severe hyperglycemia and died. However, this extract seems to increase the survival rate of diabetic rats in a dose-dependent manner. AP extract was reported to have antioxidant effect in STZ-induced diabetic rats.[11] These findings may suggest that the increase in survival rate could be due to the effect of antioxidative protective properties of AP extract on prolonged hyperglycemic state or due to other unknown mechanism.

Increase proteolysis of protein in skeletal muscle and lipolysis of adipose tissue lead to reduced body weight in diabetic state.[15] The diabetic rats' body weight was reduced throughout the treatment period with AP extract. At the same time, the fasting blood glucose levels were not significantly different in 'AP extract'-treated diabetic rats compared to the diabetic control rats. These findings are in contradiction to that of previous reports that AP decreased blood glucose level in diabetic animal models.[9-11] However, the composition of the chemical constituent of the extracts studied by those researchers was not documented. Reduction of glucose transporter expression to prevent cytotoxic effect of STZ[16,17] and

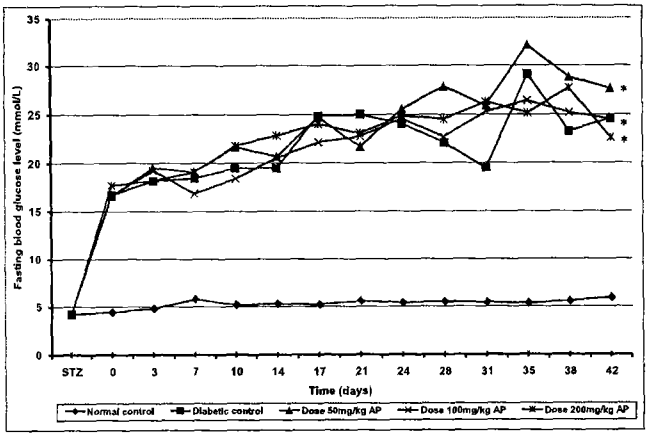

Figure 2: Effects of different doses of spray-dried 95% ethanolic extract of Andrographis paniculata on fasting blood glucose levels during the 6-week period in STZ-induced diabetic rats. *'*P*' < 0.05 when compared with normal control group (n = 12 for each group at the start of experiments)

decrease of β-cell sensitivity to glucose due to long-term hyperglycemia[18,19] are believed to be the possible causes of high glucose level in the bloodstream of STZ-induced diabetic rats. The difference in chemical composition of the extract or differences in the dose of extract studied could be the factors that may contribute to the different antidiabetic properties of the studied AP extract.

Our data showed decreased level of serum insulin in STZ-induced diabetic rats following 6 weeks' treatment with AP extract. This finding is similar to our finding in type 2 diabetes patients.[20] Renu also reported that AP significantly reduced HbA_{1c} level of type 2 diabetes. However, the reduction in insulin level is in opposition to the findings of a previous report that AP extract did not change the serum insulin level in STZ-induced diabetic rats.[11] Insulin resistance is a common phenomenon observed in STZ-induced diabetic animals.[21-23] The postulation could be that the extract may improve insulin sensitivity in the peripheral tissue via a nitric oxide (NO)-related glucose uptake.[23]

Studies on the effect of AP extract treatment on the estrous cycle of diabetes-induced rats have not been reported previously. In the STZ-induced diabetic rat model, STZ seems to disrupt the estrous cycle, with the rats showing prolonged anestrous cycle. The number of ovulatory estrous cycles was increased in diabetic rats treated with the AP extract, especially those that received 100 mg/kg/day. This finding suggests that the AP extract has the ability to normalize the anovulatory estrous cycle of the diabetic rats. However, the number of growing follicles (histology) of the ovary was not evaluated in this study.

The endocrine cells density of the Islet of Langerhans in STZ-induced diabetic rats was increased significantly with 50 mg/kg/day AP extract compared to the nontreated animals. At the same time, the diabetic rats treated with the AP extract at 100 mg/kg/day showed a reduction of hydropic degeneration. These findings suggest that the AP extract may accelerate the recovery of Islet cell of Langerhans or may have a rejuvenating effect on these cells.

Conclusion

The present study shows that the 95% spray-dried commercially prepared ethanol extract of AP has higher 14-deoxyandrographolide and lower andrographolide content compared to the extract that was prepared in the university laboratory. The commercial extract given at a maximum dose of 200 mg/kg/day had no antihyperglycemic effect in STZ-induced diabetic rats. However, the extract seems to increase the survival rate, reduce serum fasting insulin levels and improve the estrous cycle of STZ-induced diabetic rats. The recovery effect of AP extract on the Islet cells and the ability of the extract to reduce insulin resistance require more detailed examination. The reduction in body weight of the extract-treated animals suggests that there could be another effect of the herb on carbohydrate metabolism.

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