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Synergistic antihyperglycaemic effect of combination therapy with gallic acid and andrographolide in streptozotocin-induced diabetic rats

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ABSTRACT

Previous studies had reported on the antidiabetic effect of gallic acid and andrographolide, as monotherapy, in diabetic rats. However, studies on the combination effect of gallic acid and andrographolide in treating diabetes are lacking. Hence, the objective of this study was to investigate the antihyperglycaemic activity of combined gallic acid and andrographolide in streptozotocin-induced diabetic rats compared to single compound treatment. Diabetes was induced with streptozotocin (40 mg/kg, intraperitoneally) in male Sprague Dawley rats. After 7 days, all rats were divided into 8 groups as follows: normal control and diabetic control groups given distilled water, diabetic groups treated with gallic acid (10 or 20 mg/kg), andrographolide (5 or 10 mg/kg), or both in 2:1 combination ratio of gallic acid:andrographolide (10:5 or 20:10 mg/kg). All treatments were given orally, once daily for 15 consecutive days. Non-fasting blood glucose, food and water intake were measured weekly. On day 14, an oral glucose tolerance test was performed. Our results showed that the combination treatment of gallic acid and andrographolide synergistically reduced blood glucose compared to single compound treatment, with higher synergistic effect and favourable dose reduction at higher combination dose. Furthermore, the combination treatment significantly improved glucose tolerance, with significantly reduced area under the curve in diabetic rats. The combination treated diabetic group also demonstrated normalization of their daily food and water intake. In conclusion, our results indicated that the combination therapy at 2:1 combination ratio of gallic acid:andrographolide demonstrated synergistic hypoglycaemic activity with favourable dose reduction in experimental diabetic rats.

1. Introduction

According to the World Health Organization (WHO), diabetes is currently ranked sixth in the cause of death globally, having killed 1.6 million people in 2015 compared with less than 1 million in 2000 (World Health Organization, 2017). Current oral antidiabetic drugs for the management of diabetes have limitations due to side effects such as weight gain, hypoglycaemia (Aquilante, 2010), secondary failure (Ekstrom et al., 2015) and diarrhoea (Bouchoucha et al., 2011). Hence, there is a need to discover new antidiabetic agents with better therapeutic efficacy and fewer side effects.

Previous studies had reported on the antidiabetic effect of gallic acid (GA) (Gandhi et al., 2014) and andrographolide (AGP) (Subramanian et al., 2008), as monotherapy, in diabetic rats. However, studies on the combination effect of GA and AGP in treating diabetes are lacking. Hence, the objective of this preliminary study is to investigate the combination effect of GA and AGP on the blood glucose of streptozotocin-induced diabetic rats.

As previous animal studies have shown the beneficial effect of GA and AGP as monotherapy on diabetes, we hypothesize that the combination of GA and AGP at certain combination ratio may produce synergistic effect on improving diabetes condition as compared to single compound treatment.

2. Materials and methods

2.1. Animal selection and diabetes induction

A total of 48 male Sprague-Dawley rats (5–7 weeks old) obtained from Chenur Supplier, Selangor, Malaysia were used in this study. The rats were housed in standard polypropylene cages (three rats/cage) and maintained under controlled room temperature (24 ± 1 °C) and humidity (55 ± 5%), with a 12 h light-dark cycle (light on at 8:00 a.m. and off at 8:00 p.m.). They were given *ad libitum* access to commercial

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standard rodent pellet (702 P, Gold Coin, Malaysia) and water. The care and use of the animals were according to accepted international and national guidelines. This study has received ethical approval from the Universiti Kebangsaan Malaysia Animal Ethical Committee (Approval code: UTM/2016/FADZILAH/28-JAN./723-APR.-2016-APR.-2019).

After 7 days of acclimatization, 42 rats were given a single intraperitoneal (IP) injection of streptozotocin (STZ) (Sigma, USA) (40 mg/kg BW, dissolved in 0.1 M cold citrate buffer, pH 4.5) to induce diabetes (Moodley et al., 2015). 6 normal control rats were given single IP injection of vehicle citrate buffer (2 mL/kg BW).

After 3–7 days of STZ injection, rats with non-fasting blood glucose (BG) of ≥16.7 mM tested on glucometer (Accu-chek Active, Roche Diagnostics, Germany) were considered diabetic and were selected for further studies. Rats with BG < 16.7 mM were given a 2nd dose of STZ at 40 mg/kg BW IP and observed for BG again after 7 days.

2.2. Animal treatment

A total of 48 rats were randomly divided into 8 groups of 6 rats each as follows:

Group 1: Normal control rats received vehicle alone (10 mL/kg distilled water).

Group 2: Diabetic control rats received vehicle alone (10 mL/kg distilled water).

Group 3–4 were diabetic rats given commercially obtained gallic acid (GA) (Sigma, USA) at 10 and 20 mg/kg BW, respectively.

Group 5–6 were diabetic rats given commercially obtained andrographolide (AGP) (Sigma, USA) at 5 and 10 mg/kg BW, respectively.

Group 7–8 were diabetic rats given combination of GA + AGP (2:1) at 10 mg/kg:5 mg/kg and 20 mg/kg:10 mg/kg, respectively.

All treatment drugs were dissolved in distilled water and given once daily by oral gavage in the afternoon (1–3 p.m.) for 15 days. BG, food and water intake were determined weekly.

2.3. Oral glucose tolerance test

Oral glucose tolerance test (OGTT) was determined on day 14 of the treatment. Animals were fasted for 6-h prior to beginning OGTT. Glucose in solution (20% w/v) was given by oral gavage (2 g/10 mL/kg BW) after 30-min post-administration of drugs or vehicle. Blood glucose was assayed at time 0 (pre-glucose administration) and 30-, 60- and 120-min post-glucose administration.

On day 15, after 1-h post-administration of drugs or vehicle, the rats were anaesthetized with ketamine/xylaxine/zoletil-50 mixture (3.33 mg/kg ketamine:3.33 mg/kg xylazine:3.33 mg/kg tiletamine-zolazapam; 0.1 mL/100 g BW) via tail IV injection and killed by exsanguination via cardiac puncture and incision of the heart. Blood samples from the cardiac puncture and various tissue samples were collected and stored for future studies.

2.4. Combination analysis

The dose-effect curve was plotted using the CompuSyn software (ComboSyn, Inc.). The dose-effect curve was also transformed into the linear form by the median-effect plot based on the general median-effect equation developed by Chou (2006):

$$\frac{f_a}{f_u} = \left(\frac{D}{D_m}\right)^m \tag{1}$$

where D is the dose of a drug, D_m is the median-effect dose (ED_{50}) that reduces the blood glucose by 50%, f_a is the fraction affected by D (i.e. percentage effect/100), f_u is the fraction unaffected ($f_u = 1 - f_a$), m is the slope of the median plot signifying the shape of the dose-effect curve. A rearrangement of Equation (1) gives:

$$\log\left(\frac{f_a}{f_u}\right) = m\log(D) - m\log(D_m) \tag{2}$$

where $y = \log(f_a/f_u)$ versus $x = \log(D)$ in the median-effect plot.

The nature of the interaction, i.e. synergy, additivity, or antagonism, of the GA-AGP combinations as a function of their doses and blood glucose reduction were assessed by using the combination index method (CI) using CompuSyn software (ComboSyn, Inc.). The CI method is based on the Loewe additivity model for mutually exclusive agents and calculated using the following equation:

$$\mathrm{CI} = \frac{D_{\mathrm{A},x}}{\mathrm{ED}_{x,\mathrm{A}}} + \frac{D_{\mathrm{B},x}}{\mathrm{ED}_{x,\mathrm{B}}} \tag{3}$$

where ED_x is the dose of each compound alone required to produce x effect level of blood glucose reduction, and $D_{A,x}$ and $D_{B,x}$ are the doses of the two compounds in combination that produce the same effect. A CI of < 1 implies synergy, = 1 is additivity, and > 1 is antagonism.

The dose reduction index (DRI) of the two-compound combination and two-crude was measured using the formula:

$$\mathrm{DRI} = \frac{\mathrm{ED}_{50}\ of\ compound\ alone}{\mathrm{ED}_{50}\ of\ compound\ in\ combination\ with\ combination\ partner} \tag{4}$$

DRI measures how many-fold the combination dose may be reduced as compared to the doses of each compound or extract alone to produce the same blood glucose reduction effect. DRI > 1 is beneficial as it indicates reduction of doses of combination compounds while retaining the same therapeutic efficacy (Chou, 2006).

3. Results and discussion

3.1. Blood glucose analysis

Non-fasting blood glucose (BG) data was used to determine the antihyperglycaemic effect of the compounds in this study instead of fasting blood glucose (FBG) data. This was due to the high variability of FBG values after 7 days of STZ 40 mg/kg i. p. injection. Some rats showed non-fasting BG in the range between 16.7 and 22 mM which indicates diabetes, but their FBG was between 4.5 and 10 mM after 6-h of fasting (data not shown). Although their non-fasting BG values indicated that the animals had diabetes, their FBG values were in the non-diabetic range. This could be due to the moderate beta cells damage from the low dose of STZ given, whereby the remaining surviving beta cells are able to produce insulin to reduce blood glucose level during the 6-h fasting period. It has been reported that FBG is not an authentic indicator for the diagnosis of diabetes and non-fasting BG measurements are required in some situations for the diagnosis or confirmation of diabetes (Islam, 2011).

Our study demonstrated that the combination of GA:AGP at 2:1 ratio reduced BG much more compared to single compound treatment in STZ-induced diabetic rats after 15 days of treatment (Fig. 1 and Table 1).

The BG data from the GA and AGP treatment group in our study is in

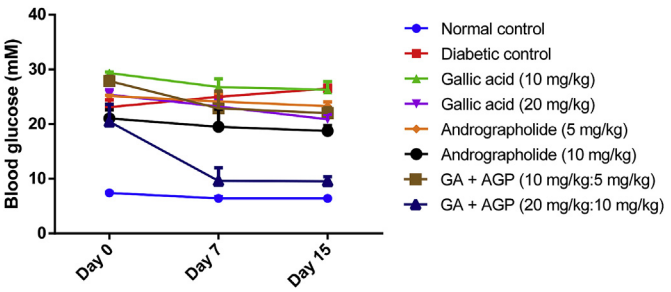

Fig. 1. Non-fasting blood glucose during 15 days of treatment.

Table 1
Antihyperglycaemic activity in diabetic rats.

Group	n	Blood glucose (mmol/L)			Reduction/(Gain) (%)
		Before treatment	After treatment		
			Day 7	Day 15	
Normal control	6	7.40 ± 0.17	6.4 ± 0.31	6.43 ± 0.03	13.06
Diabetic control	5	23.10 ± 1.35	24.97 ± 0.55	26.53 ± 0.58	(22.84)
GA (10 mg/kg)	6	29.35 ± 0.15	26.80 ± 1.50	26.30 ± 1.50	10.39
GA (20 mg/kg)	6	25.37 ± 0.95	23.23 ± 1.13	20.90 ± 0.85	17.61
AGP (5 mg/kg)	6	25.15 ± 0.15	24.15 ± 1.45	23.30 ± 0.80	7.91
AGP (10 mg/kg)	6	21.05 ± 1.55	19.50 ± 3.20	18.75 ± 0.95	10.93
GA + AGP (10 mg/kg:5 mg/kg)	6	27.90 ± 0.90	22.90 ± 3.10	22.00 ± 1.00	21.15
GA + AGP (20 mg/kg:10 mg/kg)	6	20.45 ± 3.15	9.60 ± 1.96	9.55 ± 0.85	53.30

Values were expressed as mean ± S.E.M. Reduction/(Gain) (%) = [(Before treatment – After treatment Day 15)/Before treatment] × 100.

contrast to previously reported studies which showed greater improvement to the weekly BG using the same dosage of GA (Gandhi et al., 2014; Latha and Daisy, 2011) or AGP (Subramanian et al., 2008).

3.2. Combination analysis

Based on Chou's theory on the mass-action law (Chou, 2006), a specific curve can be drawn from the median-effect plot with a minimum of two data points using the CompuSyn software (Fig. 2). This allow for the conservation of animal resources in *in vivo* drug combination studies.

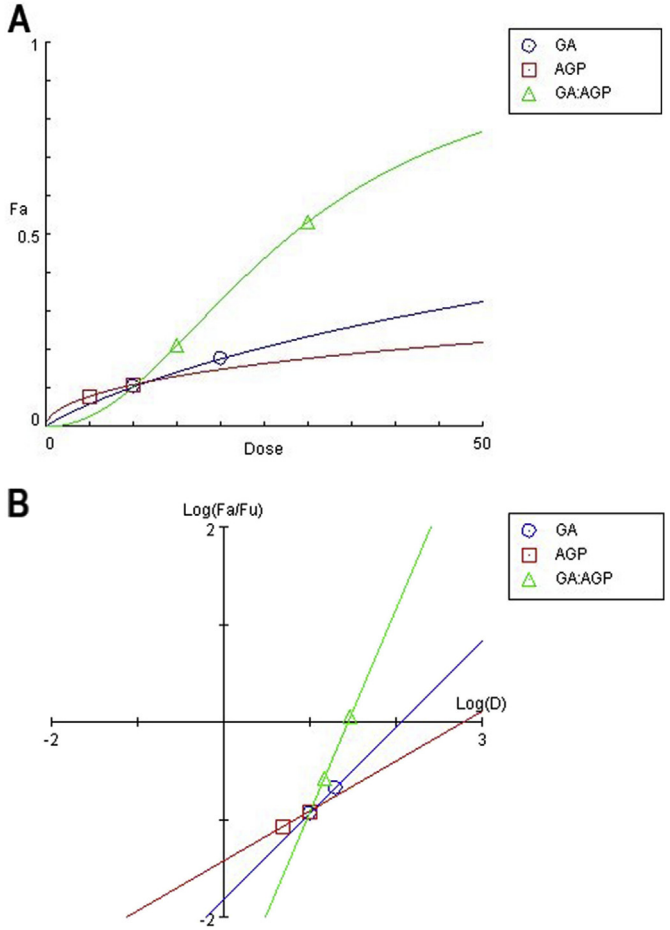

Fig. 2. Combination analysis. (A) Dose-effect curve and its linearization with the (B) median-effect plot for a single and combination treatment of GA and AGP.

The median effect plot allows the estimation of ED_{50}, which is the median-effect dose required for 50% blood glucose reduction. The antilog of the x-intercept gives the Dm value (ED_{50}) of the median-effect equation in Equation (2), which can be computed from the CompuSyn software. The ED_{50} values from the single and combination treatment of GA and AGP were then used to generate the combination index (CI) plot (Fig. 3). CI < 1, = 1, and > 1 indicates synergism, additive effect and antagonism, respectively. Our analysis demonstrated that the two combination data points are on the synergy side (CI < 1).

The simulation at low Fa (Fa < 0.15) showed substantial antagonism. Low Fa is less relevant to therapy than high Fa (i.e. reducing blood glucose in small fraction is not useful in diabetes therapy). The simulation from the experimental data showed that higher synergism could be seen from higher dose of GA:AGP combination treatment at 2:1 combination ratio, effecting higher blood glucose reduction.

Our dose reduction index (DRI) analysis (Table 2) indicated that reduction of BG by 53.3%, requires 131.75 mg/kg GA or 761.92 mg/kg AGP. However, combination of GA:AGP at 2:1 combination ratio requires only 6.59-fold less GA plus 76.19-fold less AGP (i.e. 20 mg/kg GA + 10 mg/kg AGP) to achieve the same 53.3% BG reduction. Hence, the combination of GA:AGP at 2:1 ratio has the potential to provide better therapeutic effect with potentially lower side effects due to the lower dose needed from each compound in the combination treatment.

3.3. Oral glucose tolerance test

After 14 days of treatment, combination treatment group showed significantly greater postprandial glucose response compared to single compound treatment (Fig. 4). Previous studies have reported that both

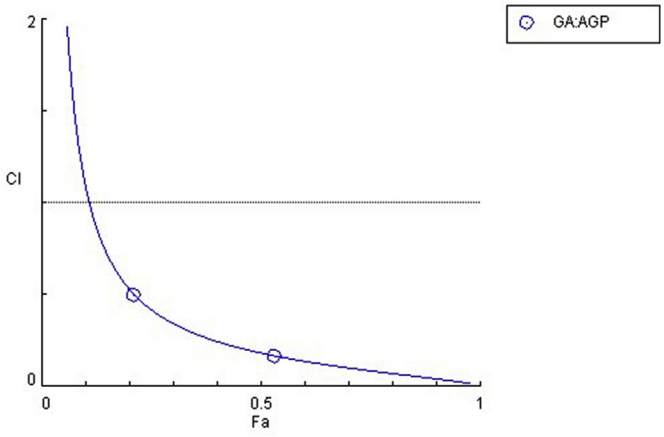

Fig. 3. Combination index plot. Combination index (CI) of 1, < 1, and > 1 indicates additive effect, synergism and antagonism, respectively. As the fraction affected (Fa) or treatment dose increases, the CI decreases.

Table 2
DRI value of GA and AGP in combination dose.

% BG reduction	Dose GA (mg/kg)	Dose AGP (mg/kg)	DRI GA[a]	DRI AGP[a]
21.15	25.82	45.70	2.58	9.14
53.30	131.75	761.92	6.59	76.19

[a] DRI > 1 indicates favourable dose reduction for the combination.

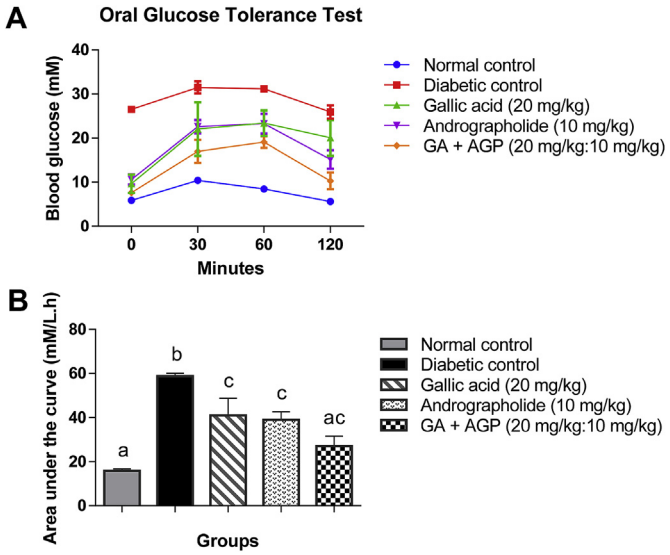

Fig. 4. (A) Oral glucose tolerance test (OGTT) and the respective (B) area under the curve estimated from the OGTT plot. Different letters indicate significant differences analysed by one-way ANOVA followed by Tukey post hoc test ($p < 0.05$).

Table 3
Daily food and water intake (n = 5–6) after 14 days of treatment.

Group	Average daily food intake (g/rat)	Average daily water intake (mL/rat)
Normal control	23.3	43.3
Diabetic control	41.7	151.0
GA (10 mg/kg)	30.5	114.0
GA (20 mg/kg)	32.3	92.7
AGP (5 mg/kg)	32.0	94.0
AGP (10 mg/kg)	30.7	91.7
GA + AGP (10 mg/kg:5 mg/kg)	24.7	82.3
GA + AGP (20 mg/kg:10 mg/kg)	29.0	47.0

GA and AGP have the ability to regenerate beta cells and enhance glucose uptake via GLUT4 (Gandhi et al., 2014; Latha and Daisy, 2011; Nugroho et al., 2011; Yu et al., 2008; Zhang et al., 2009). Hence, the greater tolerance to glucose following an OGTT could be a function of either greater recovery of the pancreatic beta cells, hence greater insulin production, or enhanced glucose uptake of peripheral tissues.

3.4. Food and water intake

Following 14 days of treatment, combination treatment of 20 mg/kg GA and 10 mg/kg AGP normalizes the food and water intake of diabetic rats (Table 3). Among the characteristics of diabetes are excessive food intake (polyphagia) due to insufficient glucose uptake by peripheral tissues, increased thirst (polydipsia) due to high blood glucose that raises the osmolarity of blood, and increased frequency of urination (polyuria) due to excessive water intake. As the GA:AGP combination-treated diabetic rats improved their ability to utilize glucose in the blood as shown in OGTT, food and water intake eventually normalize.

4. Conclusion

This preliminary study demonstrated synergistic blood glucose reduction activity of the combination treatment of GA and AGP on the diabetic rats at 2:1 GA:AGP combination ratio. This study also demonstrated favourable dose reduction of individual compounds from the combination treatment. Further studies to understand the effect of the combination treatment on the blood biochemistry profile, as well as at the histological and molecular level are currently ongoing.

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Author contributions

T.S.W., Z.H. and F.A.A.M. conceived and planned the experiment; T.S.W. and H.F.I. carried out the experiment; T.S.W. analysed the data; T.S.W. wrote the manuscript and all authors have approved the final manuscript.

Declarations of interest

None.

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