Aspalathin, a *C*-glucosyl dihydrochalcone from rooibos improves the hypoglycemic potential of metformin in type 2 diabetic (*db/db*) mice

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Short title: Aspalathin improves the antidiabetic effect of metformin

Summary

Metformin is the first line therapy of type 2 diabetics, but continued reduction of their life expectancy warrants further investigation into alternative treatment strategies. This study reports on the combinational use of metformin with aspalathin, a *C*-glucosyl dihydrochalcone with known glucose lowering and antioxidant properties, as an effective hypoglycemic therapy in a type 2 diabetic (*db/db*) mouse model. When tested as a monotherapy, a low dose of aspalathin (13 mg/kg) showed no effect, while a high dose (130 mg/kg) has already displayed a better potential than metformin in protecting against diabetes associated symptoms in *db/db* mice. Thus, it remains of interest to determine whether this dihydrochalcone can improve the efficacy of metformin. The results showed that this combination therapy was more effective than the use of metformin as a monotherapy in ameliorating diabetes associated symptoms, including abnormal raised fasting plasma glucose levels, impaired glucose tolerance, as well as excessively increased body weights and fat content. The treated mice also had reduced food and water consumption when compared to untreated controls, with a pronounced effect evident in the last week of treatment. Therefore, this study supports further investigations into the ameliorative effect of combination therapy of metformin and aspalathin against diabetes associated symptoms.

Key words

Combination therapy; metformin; aspalathin; diabetes.

Introduction

Metformin is a well-established first-line drug for the treatment of type 2 diabetes mellitus. In addition to lowering blood glucose levels and improving insulin sensitivity, mainly through enhancing skeletal muscle or adipose glucose uptake and suppressing hepatic glucose production, metformin presents some antiinflammatory and oxidative stress ameliorative properties that are important in combating diabetes associated symptoms (Hur and Lee, 2015). However, due to the rapid rise in diabetes related deaths (Interntional Diabetes Federation, 2017), it is hypothesized that the glucose-lowering efficacy of metformin might diminish over time. Furthermore, the efficacy of metformin can be influenced by genetic variation for some individuals, for example those that lack the organic cation transporter 1 (*Oct1*) gene, which is a major thiamine transporter of this biguanide predominantly expressed in the liver (Shu *et al.*, 2007). This suggests that dual therapy approaches which improves the efficacy of metformin in individuals lacking *Oct1* are likely to be beneficial. Indeed, increased exploration of combination drug therapy as an additional mechanism to improve the efficacy of metformin has been evident (Frendo-Cumbo *et al.*, 2016; Wu *et al.*, 2016).

Of interest is the use of metformin in combination with aspalathin, a *C*-glucosyl dihydrochalcone abundantly found in rooibos (*Aspalathus linearis*) with known metabolic benefits. Literature on the beneficial effects of aspalathin or its enriched green rooibos extract has recently been reviewed by our group (Johnson *et al.*, 2018). In addition to its enhanced capacity to reduce elevated fasting blood glucose concentrations in obese and diabetic rodent models, the strong antioxidant and antiinflammatory properties of aspalathin in preventing diabetes associated symptoms are discussed. Furthermore, we have recently demonstrated interesting data showing that an add-on effect of metformin and aspalathin is more effective than the use of each compound alone in preventing shifts in substrate preference and apoptosis in cultured cardiomyocytes exposed high glucose concentrations (Johnson *et al.*, 2016). In a type 2 diabetic (*db/db*) mice, an aspalathin dose of 130 mg/kg performs better than metformin in ameliorating diabetes associated cardiac injury (Dludla *et al*., 2017; Johnson *et al*., 2017). Therefore, it remains of

interest to further assess the combinational use of this biguanide and aspalathin in the modulation of glucose homeostasis and associated complications in a *db/db* mouse model.

Research design and methods

All animal experiments were approved and performed according to the South African Medical Research Council (SAMRC) Ethics Committee for Research on Animals (ECRA no. 07/13), and the Stellenbosch University Ethics Committee (SU-ACUM13-00021). Male C57BLKS/J homozygous (*db/db*) mice and their heterozygous leptin-receptor-deficient nondiabetic lean littermate controls (*db/+*) were obtained from Jackson's Laboratories (Sacramento, USA) and housed, individually in a cage, at the Primate Unit and Delft Animal Centre (PUDAC) of the SAMRC in a controlled environment with a twelve-hour light/dark cycle (lights switched on at 6:00 AM and switched off at 6:00 PM), in a temperature range of 23-25ºC (relative humidity: ~50%). Mice had unlimited access to water and standard mouse chow (Afresh Vention, Cape Town, South Africa).

Treatment groups

Nine-week old *db/db* mice together with their *db/+* controls (n=six/per group) were randomly divided into 5 groups. Diabetic mice were treated daily for six weeks through oral gavage with metformin (150 mg/kg) monotherapy or a combination of metformin (150 mg/kg) with a low dose (13 mg/kg/day) or high dose (130 mg/kg/day) aspalathin. Untreated nondiabetic (*db/+*) and untreated diabetic (*db/db*) mice served as controls. Aspalathin (batch: SZI-356-54) was synthesized by High Force Research (Durham, UK) as per previously published protocol (Han *et al.*, 2014) while metformin (99% purity) was bought from Sigma-Aldrich (St. Louis, USA). Treatment compounds were dissolved in distilled water before orally administration daily at the same time (08:00–09:00 a.m.), while untreated animals were given water

90 in place of treatment. The doses of metformin and aspalathin were based on previously published studies
91 (Dludla *et al.*, 2017; Johnson *et al.*, 2017).

92

93 **Parameters measured in mice**

94 Body weights, food and water intake as well as fasting plasma glucose concentrations were determined at
95 baseline and every week for six weeks. The cages were changed regularly to avoid dirtiness that may
96 interfere with food measurements. Porcelain containers with stand were used to provide food and the
97 design of the containers prevented them from being tipped over, hence avoiding the spillage of food. The
98 intake for each mouse could be monitored since they were caged individually. Fasting plasma glucose
99 concentrations were determined on a weekly basis on 4 hour fasted mice by tail pricks using a OneTouch
100 Select handheld glucometer (LifeScan, Milpitas, USA). The oral glucose tolerance test was done after the
101 six-week treatment period. Briefly, after a 16-hour fast, mice were given treatments an hour earlier and
102 allowed to settle for an additional hour before a 2 g/kg glucose was orally administered through gastric
103 gavage before plasma glucose concentrations were determined by tail prick at time intervals of 0, 30, 60,
104 and 120 minutes. Homeostatic model assessment-insulin resistance (HOMA-IR) was calculated using
105 fasting plasma glucose and fasting plasma insulin values, as per previously described method (Matthews
106 *et al.*, 1985). Fasting plasma insulin was determined using the radioimmunoassay kit (Linco Research,
107 Inc., St. Charles, MO, USA), as per manufacturer's instructions.

108

109 **Measurement of fat weight in mice**

110 After the six-week treatment period, mice were fasted for 4 hours and body weight measurements
111 documented before being anesthetized with halothane (Safeline Pharmaceuticals; Johannesburg, South
112 Africa). Mice received the anesthetic until no reaction could be recorded by pedal reflex before fats
113 (gonadal and intraperitoneal) were removed and weighed.

Statistical analysis

Results were expressed as the mean ± SEM. Each treatment group contained six mice. Statistical analysis was performed using GraphPad Prism software version 5.0 (Graph- Pad Software, Inc., La Jolla, USA). Comparisons between groups were performed using one-way multivariate ANOVA followed by a Tukey post hoc, while two-way ANOVA was used for multiple comparisons. A p-value < 0.05 was deemed as statistically significant.

Results and discussion

Leptin receptor deficient (*db/db*) mice provide an essential model to study type 2 diabetes associated symptoms. These mice spontaneously become obese and hyperglycemic, and in the process display similar features to type 2 diabetic individuals, for example they develop insulin resistance, which may initiate as early as the sixth week of age (King, 2012). This explains the use of this model to assess the therapeutic potential of various drug compounds, including aspalathin for their antidiabetic activity (Kawano *et al.*, 2009; King, 2012; Johnson *et al.*, 2017).

The current study showed that untreated nine-week-old *db/db* mice, compared to nondiabetic controls, presented altered glucose homeostasis evident from irregularly elevated fasting plasma glucose concentrations, impaired oral glucose tolerance, as well as raised HOMA-IR, an accomplished measure of insulin resistance (Fig. 1 A, B, C, and D). Some of the additional anomalies displayed by untreated diabetic mice were significantly increased body weight, occurring concurrent to dysregulated food and water intake (Table 1; Fig. 2A, C, and D). This was consistent with an elevated fat to body weight ratio in untreated diabetic mice compared to untreated nondiabetic controls (Fig. 2B). However, the combination therapy presented a better effect than the use of metformin as a monotherapy in ameliorating diabetes associated symptoms assessed in our model (Table 1; Fig. 1 and 2). However, from our results,

138 inconsistencies were observed where treatment did not show a uniform effect for some parameters
139 measured weekly, especially the fasting plasma glucose concentrations (Fig. 1 and 2). Although several
140 factors could explain this consequence, the small sample size of animals used per each group might be
141 responsible, affecting the confidence intervals and *p*-values as previously reported (Du Prel *et al.*, 2009;
142 Dludla et al., 2017). Other factors could relate to the severity of this diabetic model, presenting with high
143 levels of hyperglycemia which could not be properly monitored with the use of One Touch Select
144 glucometers, suggesting that other sensitive methods like ELISA kits should be considered for future
145 studies. Nonetheless, although the effect was moderate and hardly separable between both doses assessed,
146 a low dose (13 mg/kg/day) of aspalathin showed a better effect than its high dose in reducing raised blood
147 glucose concentrations and improving glucose tolerance, while the high dose (130 mg/kg/day) showed a
148 greater effect in reducing fat content and increased body weights than the low dose. Interestingly, the
149 effect of both doses was more pronounced in the last week of treatment (week six), suggesting that long-
150 term treatment with combination therapy might be more effective than short term treatment. However,
151 this hypothesis needs further assessment since it already known that aspalathin demonstrates low
152 bioavailability when assessed using an in vitro intestinal epithelial monolayer (Caco-2) transport model
153 (Bowles et *al.*, 2017).

154 This is the first study to report on the beneficial effect of combining metformin and aspalathin in
155 ameliorating diabetic associated symptoms in a *db/db* mouse model. Furthermore, the results presented
156 here support available data showing the superior effect of metformin when combined with natural
157 products such as resveratrol, a phytoalexin stilbenoid, or salvianolic acid A, a polyphenol derivative
158 isolated from the roots of *Salvia miltiorrhiza*, in combating diabetic symptoms in high fat diet fed or
159 streptozotocin-induced diabetic mice (Frendo-Cumbo *et al.*, 2016; Wu *et al.*, 2016). The beneficial effect
160 of combination therapy from these studies is partially modulated through regulation of
161 phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B (PI3K/AKT), AMP-activated protein
162 kinase (AMPK), as well as nuclear factor (erythroid-derived 2)-like 2 (NRF2), the well-investigated

signaling mechanisms involved in insulin signaling and prevention of hyperglycemia-induced complications such as inflammation and oxidative stress. Interestingly, similar mechanisms have also been identified by studies assessing the antidiabetic potential of aspalathin or in combination with metformin, as recently reviewed by our group (Johnson *et al.*, 2018). Such combination therapy has the potential to provide value to effective management of diabetes mellitus. Aspects that still need investigation are molecular mechanisms associated with the beneficial effect of combination therapy of metformin and aspalathin, the pharmacokinetics profile, and the long-term effect of this treatment.

Acknowledgements

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Conflict of interest

The authors report no conflicts of interest. All authors are responsible for the content and writing of the paper.

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List of tables

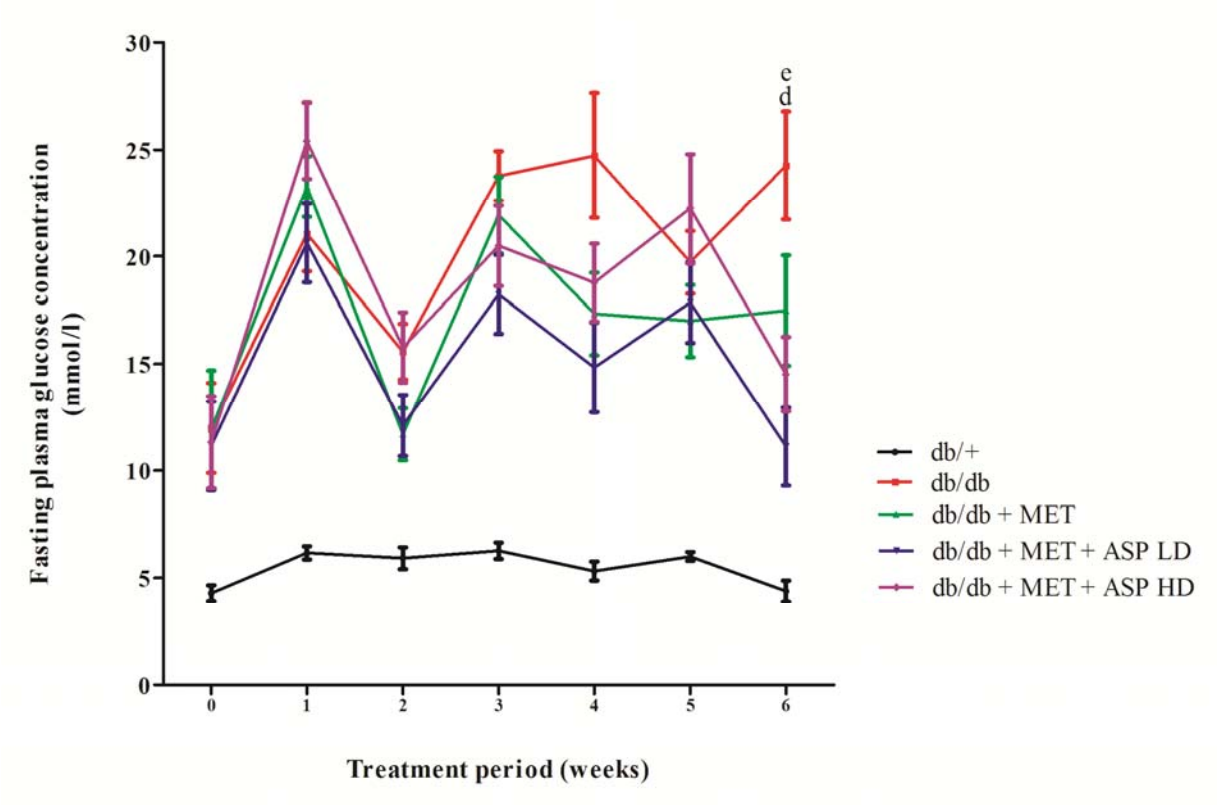
Table 1. Body weights, cumulative food and water intakes, and insulin concentrations of rats treated for 6 weeks with metformin or a combination of metformin and aspalathin.

	db/+	db/db	db/db+MET	db/db+MET+ASP LD	db/db+MET+ASP HD
BW (g)	27 ± 1	35 ± 2 ^{aa}	36 ± 2 ^{aa}	37 ± 2 ^{aa}	36 ± 2 ^{aa}
CFI (g)	148 ± 7	279 ± 10 ^{aaa}	260 ± 5 $^{aaa\ b}$	247 ± 5 $^{aaa\ bb}$	265 ± 7 $^{aaa\ bb}$
CWI (ml)	494 ± 7	1075 ± 47 ^{aaa}	1015 ± 32 ^{aaa}	951 ± 36 $^{aaa\ bb}$	916 ± 33 $^{aaa\ bbb\ c}$
INS (ng/ml)	0.4 ± 0.1	1.9 ± 1	1.1 ± 0.2	0.7 ± 0.1 ^{b}	0.6 ± 0.3 ^{b}

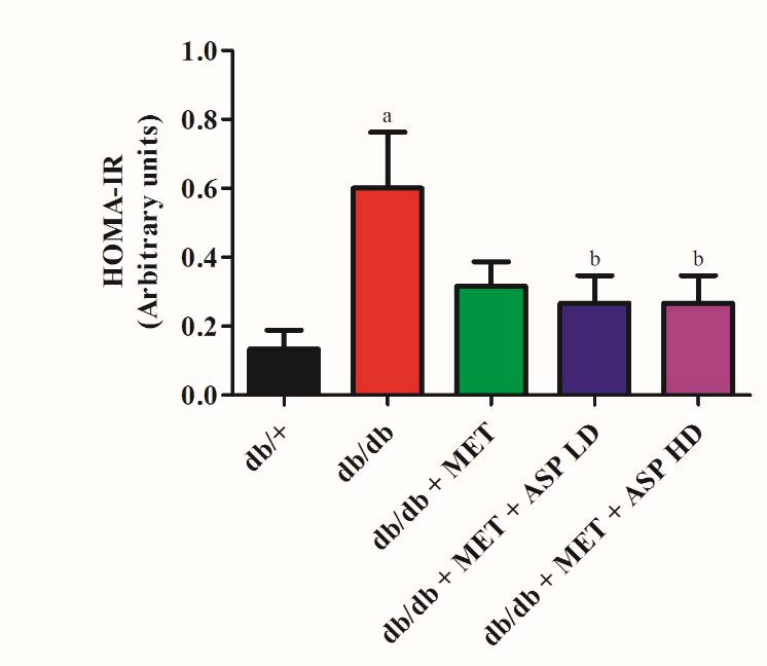
Results are represented as the Mean ± SEM. ^{aaa} P<0.001, ^{aa} P<0.01 vs untreated nondiabetic control (db/+); ^{bbb} p<0.001, ^{bb} p<0.01, ^{b} p<0.05 vs untreated diabetic control (db/db); and ^{c} p<0.05 vs diabetic group treated with metformin only (db/db+MET). Abbreviations: ASP LD, aspalathin low dose; ASP HD, aspalathin high dose; BW, body weight; CFI, cumulative food intake; CWI, cumulative water intake; INS, insulin; MET, metformin.

List of figures

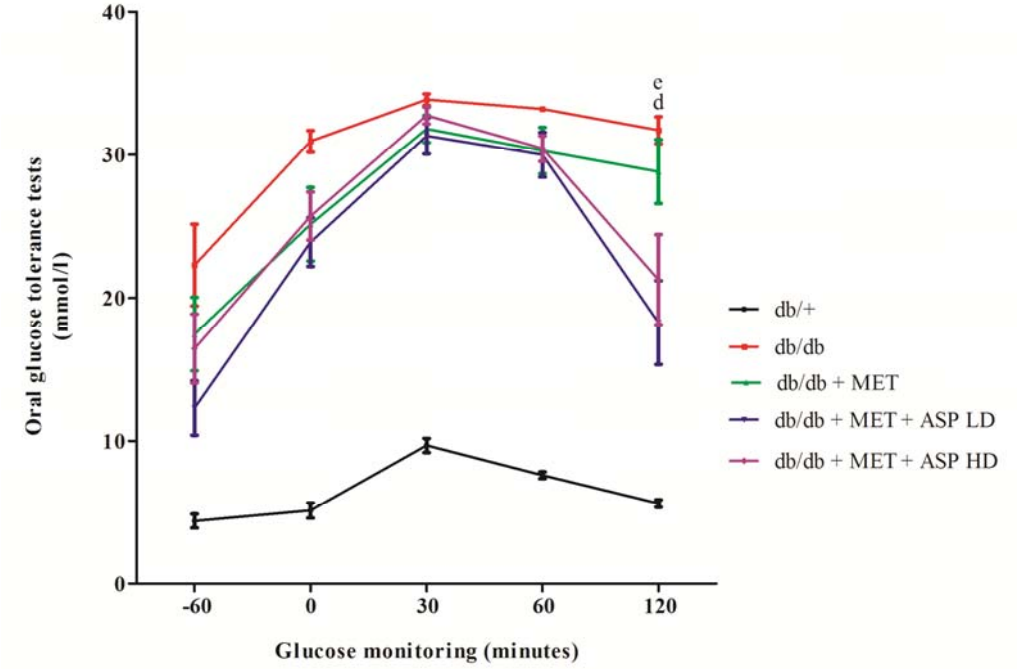
A



B



C


Oral glucose tolerance tests (mmol/l)
40
30
20
10
0
-60
0
30
60
120
Glucose monitoring (minutes)
e
d
db/+
db/db
db/db + MET
db/db + MET + ASP LD
db/db + MET + ASP HD

D

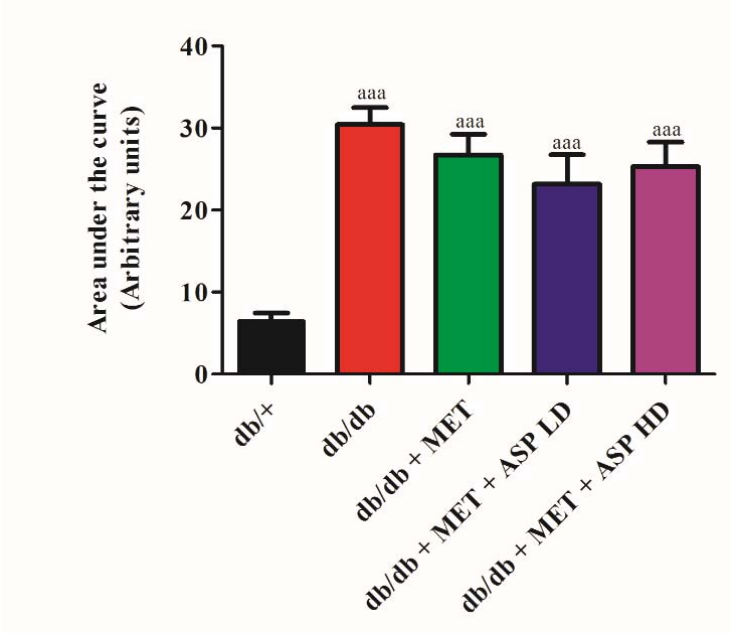
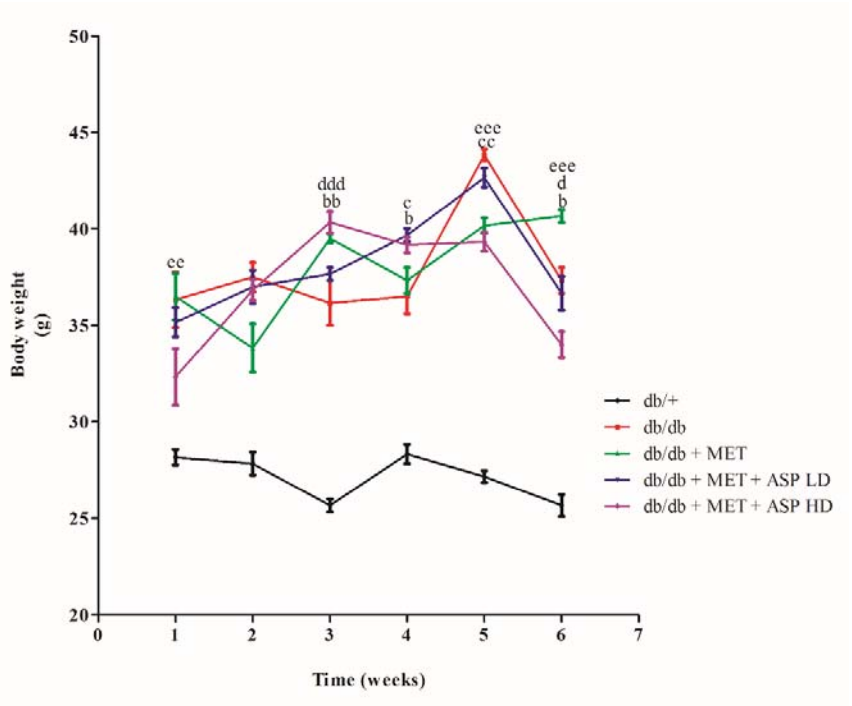
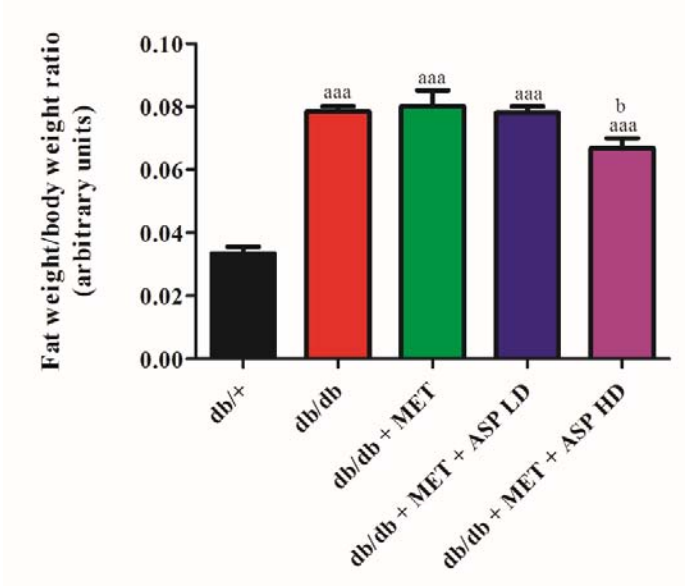

Area under the curve (Arbitrary units)
40
30
20
10
0
aaa
aaa
aaa
aaa
db/+
db/db
db/db + MET
db/db + MET + ASP LD
db/db + MET + ASP HD

Figure 1. The combination effect of metformin and aspalathin on fasting plasma glucose levels (A), homeostasis model assessment: insulin resistance (HOMA-IR; B), impaired glucose tolerance (C), and area under the curve (D) in *db/db* mice. Each value represents the mean ± SEM of six mice. Comparisons between groups were performed using one-way multivariate ANOVA followed by a Tukey post hoc, while two-way ANOVA was used for multiple comparisons. A p value of <0.05 was deemed as statistically significant. Although not represented on graphs A and B, all diabetic animals (*db/db*) showed significant difference ($p<0.001$) when compared to nondiabetic control (*db/+*). $^{a}p < 0.05$, $^{aaa}p < 0.001$ versus *db/+*; $^{b}p < 0.05$ versus *db/db*; $^{d}p < 0.05$ versus diabetic mice treated with metformin and a low dose aspalathin (db/db + MET + ASP LD); and $^{e}p < 0.05$ versus diabetic mice treated with metformin and a high dose aspalathin (db/db + MET + ASP LD).

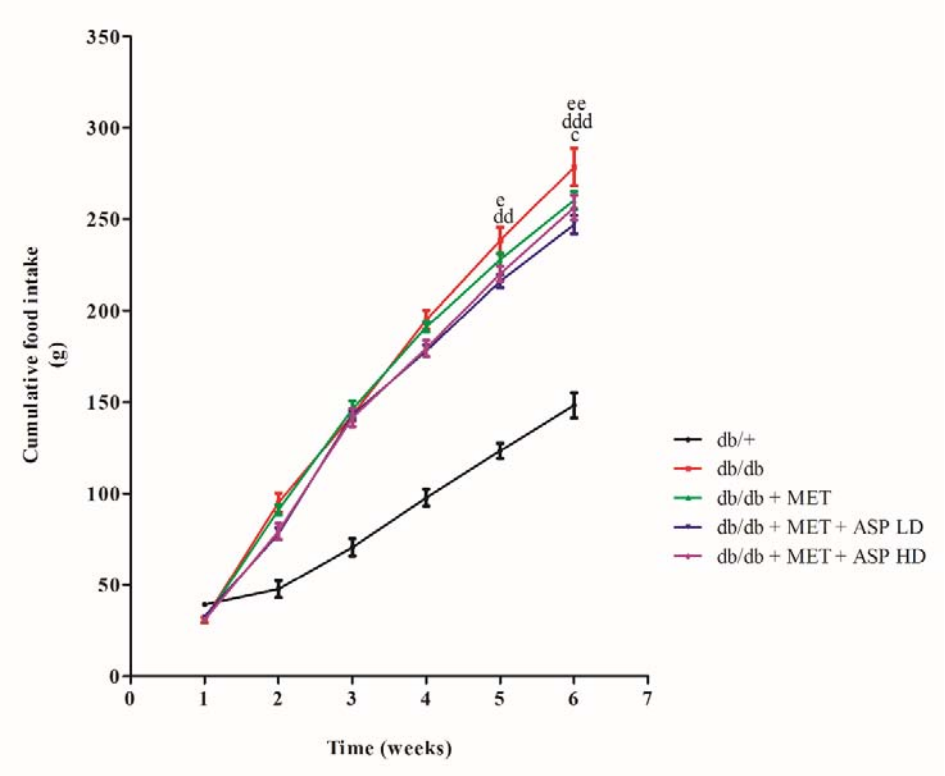
A


Body weight (g)
Time (weeks)
db/+
db/db
db/db + MET
db/db + MET + ASP LD
db/db + MET + ASP HD

B


Fat weight/body weight ratio (arbitrary units)
db/+
db/db
db/db + MET
db/db + MET + ASP LD
db/db + MET + ASP HD

C


Cumulative food intake (g)
Time (weeks)
db/+
db/db
db/db + MET
db/db + MET + ASP LD
db/db + MET + ASP HD
e
dd
ee
ddd
c

D

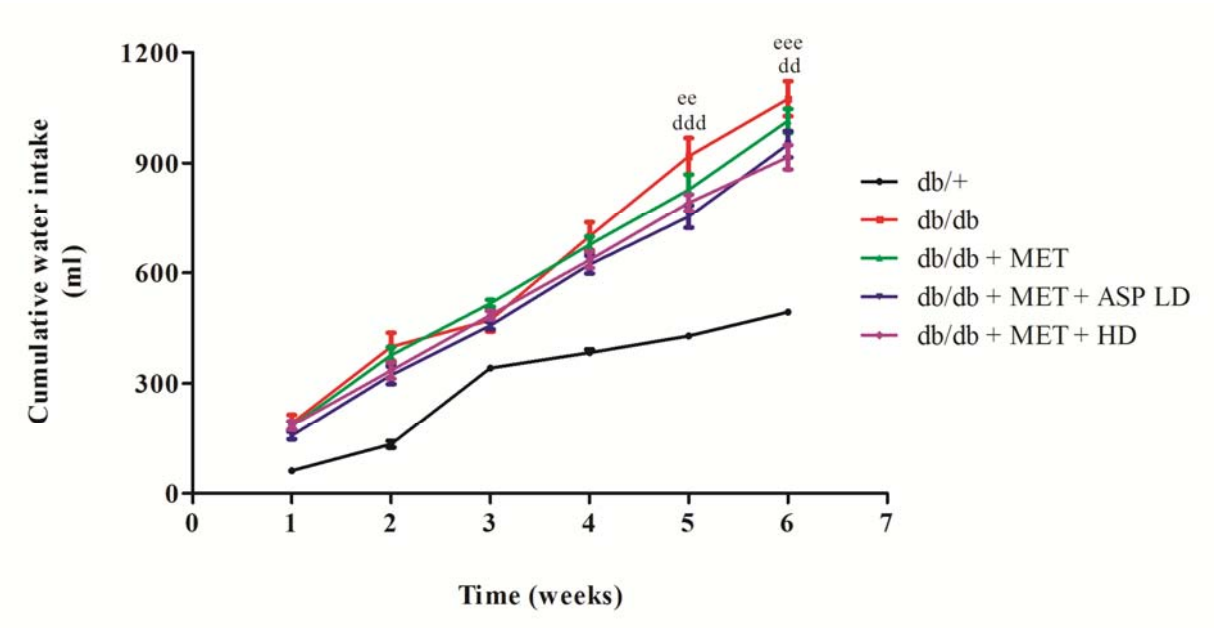

Cumulative water intake (ml)
Time (weeks)
db/+
db/db
db/db + MET
db/db + MET + ASP LD
db/db + MET + HD
ee
ddd
eee
dd

Figure 2. The combination effect of metformin and aspalathin on body weights (A), fat weight to body weight (B), cumulative food intake (C), and cumulative water intake (D) in *db/db* mice. Each value represents the mean ± SEM of six mice. Although not represented on the graph A, C and D, all diabetic animals (*db/db*) showed significant difference ($p<0.001$) when compared to nondiabetic control (*db/+*). $^{aaa}p < 0.001$ versus *db/+*; $^{b}p < 0.05$, $^{bb}p < 0.001$ versus *db/db*; $^{c}p < 0.05$, $^{cc}p < 0.01$ versus diabetic mice treated with metformin only (db/db + MET); $^{d}p < 0.05$, $^{dd}p < 0.01$, $^{ddd}p < 0.001$ versus diabetic mice treated with metformin and a low dose aspalathin (db/db + MET + ASP LD); $^{e}p < 0.05$, $^{ee}p < 0.01$, $^{eee}p < 0.001$ versus diabetic mice treated with metformin and a high dose aspalathin (db/db + MET + ASP LD).