

SHORT COMMUNICATION

Aspalathin, a C-glucosyl Dihydrochalcone From Rooibos Improves the Hypoglycemic Potential of Metformin in Type 2 Diabetic (*db/db*) Mice

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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

Aspalathin • Metformin • Combination therapy • Diabetes mellitus

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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 (International 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 metformin efficacy 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 (Dlodla *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.

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, CA, 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 23-25 °C (relative humidity: ~50 %). Mice had unlimited access to water and standard mouse chow (Afresh Vention, Cape Town, South Africa).

Nine-week old *db/db* mice together with their *db/+* controls (n=6/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, MO, USA). Treatment compounds were dissolved in distilled water before orally administration daily at the same time (08:00-09:00 AM), while untreated animals were given water in place of treatment. Metformin and aspalathin doses were based on previously published studies (Dlodla *et al.* 2017, Johnson *et al.* 2017).

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

After the six-week treatment period, mice were fasted for 4 h and body weight measurements documented before being anesthetized with halothane (Safeline Pharmaceuticals; Johannesburg, South Africa). Mice received the anesthetic until no reaction could be

recorded by pedal reflex before fats (gonadal and intraperitoneal) were removed and weighed.

Results were expressed as the mean \pm SEM. Each treatment group contained six mice. Statistical analysis was performed using GraphPad Prism software version 5.0 (GraphPad Software, Inc., La Jolla, CA, 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 $p < 0.05$ was deemed as statistically significant.

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. 1A-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; Figs 1 and 2). However, from our results, inconsistencies were observed where treatment did not show a uniform effect for some parameters measured weekly, especially the fasting plasma glucose concentrations (Figs 1 and 2). Although several factors could explain this consequence, the small sample size of animals used per each group might be responsible, affecting the confidence intervals and *p*-values as previously reported (Du Prel *et al.* 2009, Dłudla *et al.* 2017). Other factors could relate to the severity of this diabetic model, presenting with high levels of hyperglycemia which could not be properly

monitored with the use of One Touch Select glucometers, suggesting that other sensitive methods like ELISA kits should be considered for future studies. Nonetheless, although the effect was moderate and hardly separable between both doses assessed, a low dose (13 mg/kg/day) of aspalathin showed a better effect than its high dose in reducing raised blood glucose concentrations and improving glucose tolerance, while the high dose (130 mg/kg/day) showed a greater effect in reducing fat content and increased body weights than the low dose. Interestingly, the effect of both doses was more pronounced in the last week of treatment (week six), suggesting that long-term treatment with combination therapy might be more effective than short term treatment. However, this hypothesis needs further assessment since it already known that aspalathin demonstrates low bioavailability when assessed using an *in vitro* intestinal epithelial monolayer (Caco-2) transport model (Bowles *et al.* 2017).

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

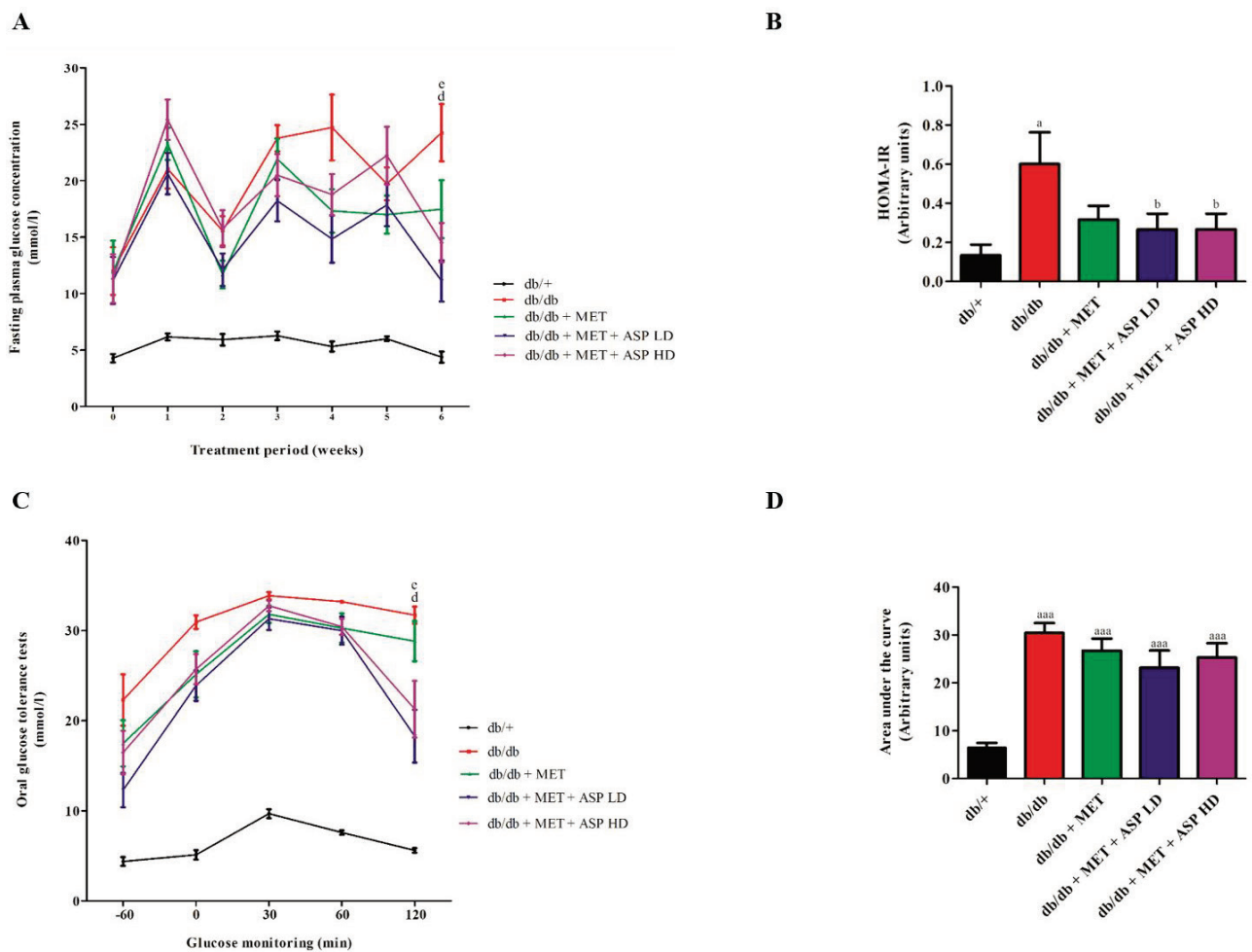


Fig. 1. The combination effect of metformin and aspalathin on fasting plasma glucose levels (**A**), homeostasis model assessment: insulin resistance (HOMA-IR; **B**), impaired glucose tolerance (**C**), and area under the curve (**D**) in *db/db* mice. Each value represents the mean \pm SEM of six mice. 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 $p < 0.05$ 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).

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 \pm 1	35 \pm 2 ^{aa}	36 \pm 2 ^{aa}	37 \pm 2 ^{aa}	36 \pm 2 ^{aa}
CFI (g)	148 \pm 7	279 \pm 10 ^{aaa}	260 \pm 5 ^{aaa, b}	247 \pm 5 ^{aaa, bb}	265 \pm 7 ^{aaa, bb}
CWI (ml)	494 \pm 7	1,075 \pm 47 ^{aaa}	1,015 \pm 32 ^{aaa}	951 \pm 36 ^{aaa, bb}	916 \pm 33 ^{aaa, bbb, c}
INS (ng/ml)	0.4 \pm 0.1	1.9 \pm 1	1.1 \pm 0.2	0.7 \pm 0.1 ^b	0.6 \pm 0.3 ^b

Results are represented as the Mean \pm 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.

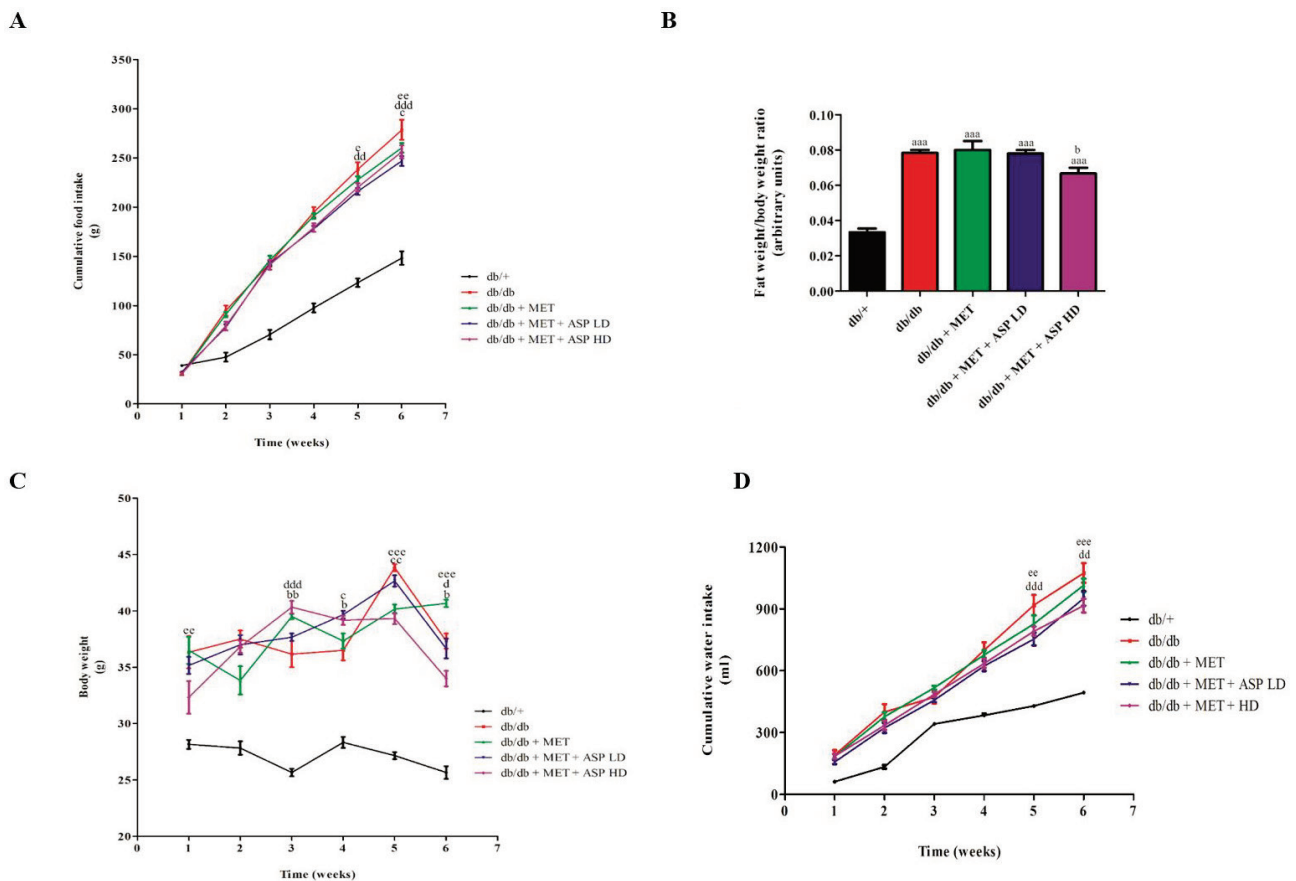


Fig. 2. The combination effect of metformin and aspalathin on body weights (**A**), fat weight to body weight (**B**), cumulative food intake (**C**), and cumulative water intake (**D**) in *db/db* mice. Each value represents the mean \pm SEM of six mice. Although not represented on the graph A, C and 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).

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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