Original Article

JHSMR Journal of Health Science and Medical Research

Isosativan from the Nigerian Propolis Activates the mTORC2/AKT Pathway and Suppresses Muscle Atrophy Genes Atrogin-1 and MuRF1 in Diabetic Rats

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Received 14 December 2024 ● Accepted 4 January 2025 ● Published online 30 April 2025

Abstract:

Objective: The study investigated the effects of Isosativan on the mTORC2/AKT pathway and diabetic muscle atrophy in a rat model of the disease.

Material and Methods: Diabetes was induced by a high-fat diet and administration of a low-dose streptozotocin injection. The animals confirmed to be diabetic were subsequently treated with Isosativan (50 mg/kg) and their responses were evaluated based on muscle atrophy and body weight. Key biochemical pathways were assessed, particularly the mTORC2/AKT signaling pathway and the muscle atrophy genes Atrogin-1 and MuRF1, in order to determine the mechanism underlying the effects of this Isosativan.

Results: Administration of Isosativan resulted in significant improvements in diabetic muscle atrophy. The treated rats exhibited increased levels of the mTORC2 and AKT proteins and maintained relatively higher body weights compared to untreated diabetic controls. The muscle atrophy-related genes, Atrogin-1 and MuRF1, were also significantly suppressed in the treated group.

Conclusion: Isosativan shows promise as a therapeutic agent for combating diabetes and diabetic muscle atrophy, operating through the activation of the mTORC2/AKT pathway, thereby leading to better utilization of glucose. This finding suggests that Isosativan could be a viable treatment option for managing diabetes-related metabolic problems, offering a novel approach for therapeutic intervention in diabetic muscle atrophy.

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J Health Sci Med Res
doi: 10.31584/jhsmr.20251195
www.jhsmr.org

Keywords: diabetes mellitus, Isosativan, muscle atrophy, mTORC2/AKT pathway, nigerian propolis

Introduction

Diabetes, a widespread chronic metabolic disease, is marked by sustained hyperglycemia, which can contribute to the development of diverse complications, including skeletal muscle wasting[1]. This condition, known as diabetic muscle atrophy, can significantly impact an individual's quality of life and mobility[2]. In recent years, studies have investigated alternative therapeutic strategies to manage diabetes and mitigate its associated complications, such as diabetic muscle atrophy[2,3]. One promising avenue is the investigation of natural products such as propolis[4] and/or its component compounds[5], such as flavonoids[6], for their potential to modulate the molecular pathways underlying the development, progression, or complications of diabetes[7].

Propolis is a resin material manufactured by honeybees and employed in traditional medicinal applications for a long time[8]. Previous studies have demonstrated the antioxidant and anti-inflammatory properties of propolis and its constituent compounds, including flavonoids such as Isosativan, and their ability to modulate glucose metabolism[7]. Alone, Isosativan has been shown to possess antifungal properties[9], though studies on its therapeutic properties as an isolate-compound from propolis, especially its effect on glucose metabolism, are non-existent. Isosativan has been increasingly isolated from the Nigerian propolis[10,11], and it has been identified as a potent bioactive component, with which the propolis elicits its general effects.

The mTOR complexes (mTORC1 and mTORC2) play a crucial role in regulating skeletal muscle function, growth, and survival[12,13]. mTORC2, in particular, has been shown to activate the Protein kinase B (AKT) pathway, leading to increased protein synthesis and decreased protein degradation[14]. Given the importance of the mTORC2/AKT axis in maintaining muscle integrity, therapies that can activate this pathway may offer a potential strategy to ameliorate diabetic muscle atrophy.

This study aimed to examine the effects of Isosativan, a flavonoid isolated from the Nigerian propolis, on the mTORC2/AKT pathway and its potential to ameliorate diabetic muscle atrophy in a rat model.

Material and Methods

Study design, animal model and approval

Twenty male Wistar rats weighing 280-300 g and maintained under standard laboratory housing with unrestricted access to standard rat chow, were randomly assigned to 4 groups: control, diabetic (untreated), diabetic + metformin, and diabetic + Isosativan. Diabetes was induced by a high-fat diet and administration of a low-dose streptozotocin injection. The diabetic + Isosativan group received daily oral administration of Isosativan (50 mg/kg) for 4 weeks. At the end of the treatment period, the animals were sacrificed humanely, and their gastrocnemius muscles were harvested to prepare tissue lysate for analysis. The study was approved by the Ahmadu Bello University Zaria ethical body for research. International standards for the handling of laboratory animals were maintained throughout the experiment. Pharmacological studies were conducted on the Isosativan to determine the appropriate/chosen dosage.

Biochemical and molecular analyses

The study employed commercially available enzyme-linked immunosorbent assay kits from Abcam to quantify the levels of the mTOR complex 2 (mTORC2) protein and phosphorylated AKT. The study also utilized a PIP3 ELISA kit from Echelon Biosciences to quantify the levels of the insulin signaling a second messenger, PIP3. Quantitative gene

expression analysis was performed using PCR reagents procured from Thermo Fisher Scientific. Additionally, Western blotting was utilized to evaluate the protein expression of key constituents within the mTORC2/AKT signaling pathway. Blood glucose levels were monitored using an On-Call Plus glucometer manufactured by Acon Laboratories.

Changes in weight

Measurement of body weight was performed weekly to monitor the effects of Isosativan on weight changes in the diabetic rats. The weight changes were calculated as the percentage change from the initial baseline weight.

% change in weight = (Final weight – Initial weight)/ (Initial weight) x 100.

Quantification of muscle atrophy

The extent of muscle atrophy in the animals was further evaluated by quantifying the mRNA levels of the muscle-specific E3 ubiquitin ligases, Atrogin-1 and MuRF1, through real-time quantitative polymerase chain reaction (rq-PCR). Total RNA was extracted from the muscle tissue samples and reverse-transcribed into cDNA. The gene-specific primer sequences utilized for the quantitative assessment of the mRNA expression levels of the muscle-specific E3 ubiquitin ligases, Atrogin-1 and MuRF1, were as follows:

The forward primer sequence for the Atrogin-1 gene was 5'-ATGCACACTGGTCCTGAGGC-3', and the reverse primer sequence was 5'-GGCTGCTGAACAGATTCTCC-3'. The forward primer sequence for the MuRF1 gene was 5'-ACCTGCTGGTGGAAAACATC-3', and the reverse primer sequence was 5'-CTTCGTGTTCCTTGCACATC-3'. The primer sequences utilized for the GAPDH reference gene in the quantitative gene expression analysis were as follows: GAPDH forward primer, 5'-AGACAGCCGCATCTTCTTGT-3', and GAPDH reverse primer, 5'-CTTGCCGTGGGTAGAGTCAT-3'.

High performance liquid chromatography (HPLC) analysis of Isosativan content in the Nigerian propolis

Ethanolic extract of the Nigerian propolis was evaporated to dryness in a rotary evaporator and the crude extract separated in the HPLC system, equipped with a C18 column, and a mobile phase consisting of acetonitrile and water. In the HPLC chromatogram, a compound exhibiting a retention time of 16.4 minutes was identified as Isosativan (Figure 1 and Table 1). Mass spectrometry also confirmed the molecular weight to be 286.32 g/mol. The Isosativan peak was detected using a UV-Vis detector set at a wavelength of 310 nm.

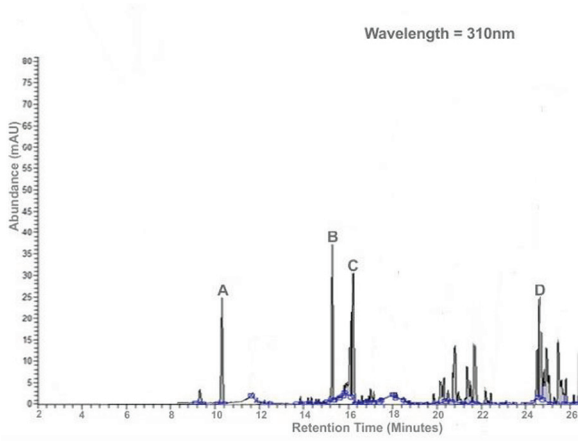


Figure 1 High performance liquid chromatography (HPLC) chromatogram of the Nigerian propolis. Details of labelled elutants are shown in Table 1

Table 1 Details of some compounds isolated from the Nigerian propolis extract with reverse-phase high performance liquid chromatography (HPLC)

Label	Peak	Area	Height (mAU)	Retention time	Class	Molecular formula
A	Epicatechin	33154	26.12	10.2	Flavonoid	$C_{15}H_{14}O_6$
B	Chrysin	24500	37.94	15.3	Flavonoid	$C_{15}H_{10}O_4$
C	Isosativan	60007	32.08	16.4	Flavonoid	$C_{17}H_{18}O_4$
D	Oleanolic acid	39012	26.23	24.5	Triterpene	$C_{30}H_{48}O_3$

mTORC2 pathway analysis

The activation of the mTORC2/AKT pathway was assessed by measuring the levels of mTOR complex 2 and phosphorylated AKT using commercially available ELISA kits by Abcam. This analysis also included examination of the expression of pAKT (phosphorylated AKT) and its downstream targets through Western blotting.

PIP3 analysis

To prove that the activation of the mTORC2/AKT pathway by Isosativan was somewhat independent of insulin sensitization, the levels of PIP3 (phosphatidylinositol-3,4,5-trisphosphate), a key second messenger in the insulin signaling cascade, were assayed. PIP3 levels were measured in the muscle tissue samples using a specific ELISA kit.

Blood glucose measurement

The study also assessed the effect of Isosativan on blood glucose levels in the diabetic rats. Fasting blood glucose was measured weekly using glucometer (Acon Laboratories).

Statistical analysis

All data were presented as mean ± standard error of mean (SEM). One-way ANOVA followed by Tukey's post-hoc test was used to compare the differences between groups. A p-value<0.05 was considered statistically significant.

Results

Amelioration of weight loss

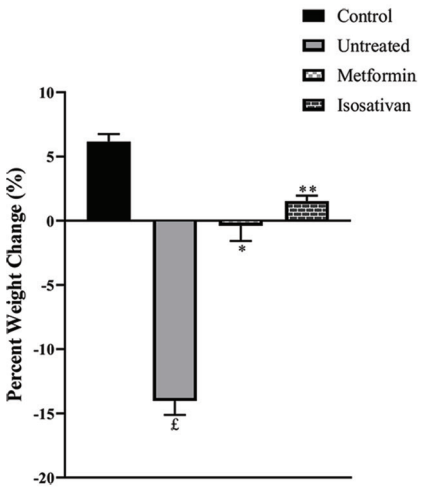
The diabetic animals treated with Isosativan exhibited a significant improvement in weight maintenance compared to the untreated diabetic group. The percentage change in body weight from the initial baseline was substantially lower in the Isosativan-treated diabetic rats, with the animals even gaining about 1.5% in weight (Figure 2), indicating that Isosativan was able to ameliorate the weight loss typically observed in the diabetic condition.

Isosativan ameliorates diabetic muscle atrophy

In Figure 3, quantitative gene expression analysis using the 2-ΔΔCt method, with GAPDH as the reference gene and the control group as the baseline, revealed significantly elevated mRNA levels of the muscle atrophy-related genes Atrogin-1 and MuRF1 in the diabetic group compared to controls. In contrast, diabetic animals treated with Isosativan displayed substantially lower mRNA expression of Atrogin-1 and MuRF1.

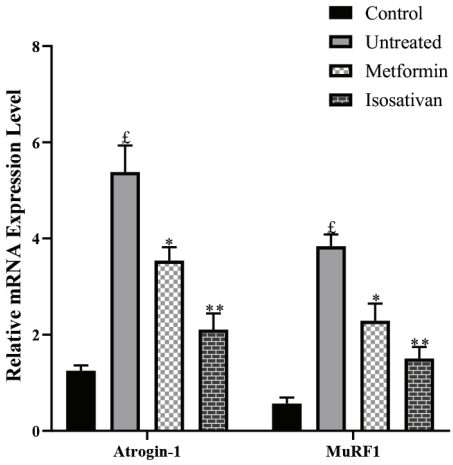
Isosativan activates the mTORC2/AKT pathway

The ELISA-based analysis showed that the levels of the mTORC2 complex were significantly lower in the diabetic group compared to the control group. Conversely, administration of Isosativan to the diabetic animals led to a substantial increase in mTORC2 levels, which approached those observed in the non-diabetic control group (Figure 4).



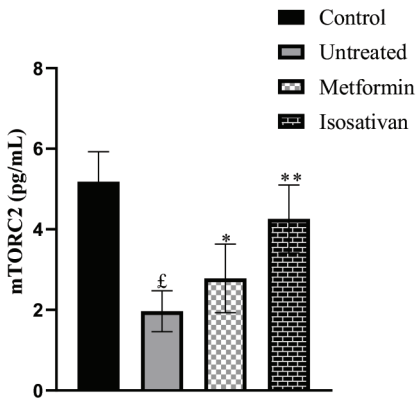
A p-value<0.05 was accepted as statistically significant. (£) p-value<0.001 compared with the control. (*) p-value<0.001 compared with the untreated. (**) p-value<0.0001 compared with the untreated.

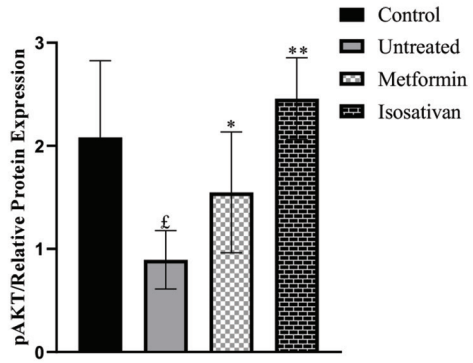
Figure 2 This shows the amelioration of weight loss by Isosativan. Negative graph implies loss of weight. Results are presented as mean±SEM with differences between groups compared using one-way ANOVA followed by Tukey's post-hoc test



A p-value<0.05 was accepted as statistically significant. (£) p-value<0.001 compared with the control. (*) p-value<0.001 compared with the untreated. (**) p-value<0.0001 compared with the untreated.

Figure 3 Isosativan ameliorates diabetic muscle atrophy. Results are presented as mean±SEM with differences between groups compared using two-way ANOVA followed by Tukey's post-hoc test





A p-value<0.05 was accepted as statistically significant. (£) p-value<0.001 compared with the control. (*) p-value<0.05 compared with the untreated. (**) p-value<0.001 compared with the untreated.

Figure 4 Isosativan activates the mTORC2/AKT pathway. Results are presented as mean±SEM with differences between groups compared using one-way ANOVA followed by Tukey's post-hoc test

Furthermore, in Figure 4, the phosphorylated AKT (pAKT) levels, a readout of mTORC2 activity, were significantly lower in the diabetic group compared to the control group. Notably, the diabetic rats treated with Isosativan exhibited a substantial increase in phosphorylated AKT levels.

In Figure 5, the analysis of Western blot data shows that the phosphorylation levels of the AKT pathway's downstream targets, including 4E-BP1 and S6K1, were substantially diminished in the diabetic group.

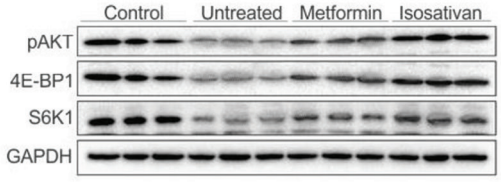
In contrast, the diabetic rats administered Isosativan exhibited elevated phosphorylation of 4E-BP1 and S6K1.

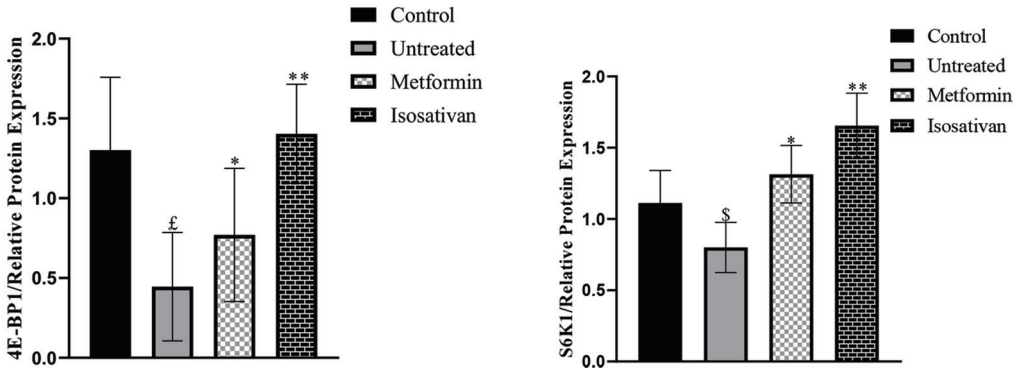
Insulin-independent action of Isosativan

The activation of the mTORC2/AKT pathway by Isosativan was not necessarily due to an increase in insulin sensitivity and signaling, as evidenced by the lack of significant difference in PIP3 levels, a key second messenger in the insulin cascade, between the diabetic and diabetic + Isosativan groups. See Figure 6.

Blood glucose control

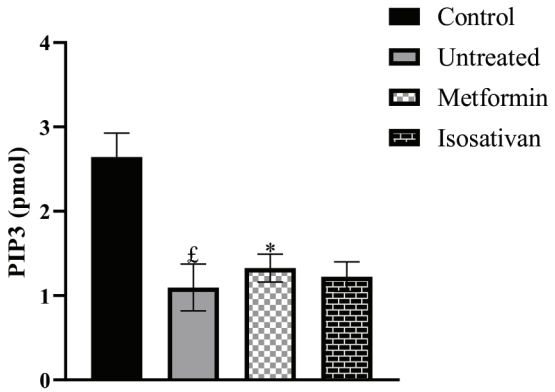
The diabetic control (Untreated) group exhibited significantly elevated blood glucose levels compared to the non-diabetic control group throughout the study period. Interestingly, the diabetic rats treated with Isosativan showed a gradual reduction in blood glucose over the 4-week treatment duration, Figure 7.





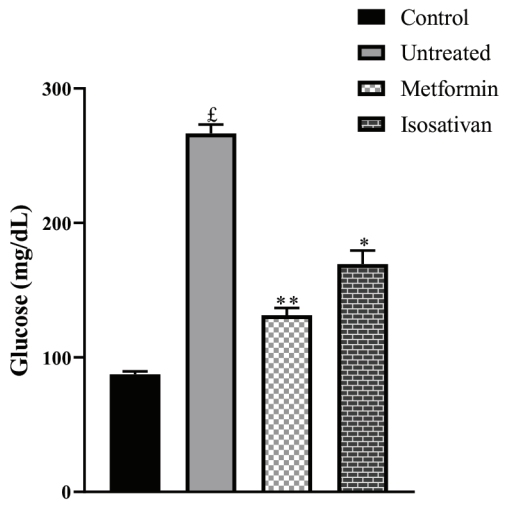
A p-value<0.05 was accepted as statistically significant. (£) p-value<0.001 compared with control. ($) p-value<0.05 compared with the control. (*) p-value<0.05 compared with the untreated. (**) p-value<0.001 compared with the untreated.

Figure 5 Isosativan-treated rats exhibit elevated levels of AKT pathway downstream targets including 4E-BP1 and S6K1. Results are presented as mean±SEM with differences between groups compared using one-way ANOVA followed by Tukey's post-hoc test



A p-value<0.05 was accepted as statistically significant. (£) p-value<0.001 compared with the control. (*) p-value<0.05 compared with the untreated.

Figure 6 Insulin-independent action of Isosativan. Results are presented as mean±SEM with differences between groups compared using one-way ANOVA followed by Tukey's post-hoc test



A p-value<0.05 was accepted as statistically significant. (£) p-value<0.001 compared with the control. (*) p-value<0.05 compared with the untreated. (**) p-value<0.01 compared with the untreated.

Figure 7 Blood glucose control. Results are presented as mean±SEM with differences between groups compared using one-way ANOVA followed by Tukey's post-hoc test

Discussion

The results of this study demonstrate that Isosativan, a seemingly novel flavonoid compound isolated from Nigerian propolis, effectively ameliorates diabetic muscle atrophy in a rat model. Remarkably, the study seems to have also uncovered a mechanism by which Isosativan activates the mTORC2/AKT pathway in an insulin-independent manner.

Studies have shown the ability of propolis to influence mTOR and AMPK pathways[15]. The present study demonstrates that Isosativan from the Nigerian propolis can stimulate the activation of the mTORC2 complex, which in turn leads to the phosphorylation and activation of AKT.

The mTORC2 complex is a critical regulator of skeletal muscle homeostasis[16], as it stimulates the AKT signaling cascade[17], which in turn promotes protein synthesis and suppresses protein degradation. Dysregulation of the mTORC2/AKT axis has been implicated in the pathogenesis of diabetic muscle atrophy[14]. Our findings indicate that Isosativan is able to directly activate mTORC2, leading to increased phosphorylation of AKT and its downstream targets, thereby ameliorating the muscle wasting observed in diabetic animals. This is proven by the increased mTORC2 levels, elevated phospho-AKT, and enhanced phosphorylation of 4E-BP1 and S6K1, and, by the way, indicating the possible activation of mTORC1 also in the Isosativan-treated diabetic group.

Some prior studies have also demonstrated that metformin, a widely used antidiabetic drug, can indirectly activate the AMPK pathway, which in turn regulates the mTOR complexes and positively impacts muscle homeostasis[18]. The current study extends these findings by demonstrating that the natural compound Isosativan can directly target the mTORC2/AKT axis leading to enhanced AKT phosphorylation and the subsequent downstream signaling, and do it, possibly without the requirement of insulin, thereby mitigating diabetic muscle atrophy.

In addition to overall weight measurements to assess loss of mass due to diabetes, the study also investigated the effects of Isosativan on the muscle-specific markers of atrophy. The up-regulated expression of the ubiquitin ligases Atrogin-1 and MuRF1 is a hallmark of muscle atrophy processes[19]. Quantitative gene expression analysis revealed that Isosativan was able to suppress the increased mRNA levels of these 2 muscle atrophy-related genes in the diabetic animals, suggesting that this may have contributed to the preservation of muscle mass and the amelioration of the diabetic muscle wasting that was observed in this study.

Phosphatidylinositol 3,4,5-trisphosphate, PIP3, intracellular levels increase with the binding of insulin to its receptor[20]. To further ascertain that the observed effects of Isosativan were not limited to enhanced insulin sensitivity and signaling, the study examined the levels of the key insulin signaling intermediate PIP3. The lack of significant difference in PIP3 levels between the diabetic and diabetic + Isosativan groups indicated that a possible insulin-independent activation of the mTORC2/AKT pathway by Isosativan was responsible for the protective effects on muscle integrity in the diabetic state. Although Isosativan was able to independently activate mTORC2 in the Isosativan-treated group, the presence of some key insulin signaling intermediate PIP3 was still likely necessary for complete AKT activation and downstream signaling in the muscle. Since this study did not block the insulin receptor and, therefore, the generation of PIP3[21], the insulin-independent activation of the mTORC2/AKT pathway by Isosativan likely worked in conjunction with the insulin signaling pathway to produce the observed effects.

Finally, it is worthy of note that the observed blood glucose control activity of Isosativan is also likely due to its ability to activate the mTORC2/AKT pathway since this leads to the translocation of more glucose transporter (GLUT 4) proteins to the cell membrane[22], after phosphorylation of the AKT substrate AS160[23], inhibiting it[24], and activating Rab protein[25], thereby enhancing glucose uptake and storage. This finding indicates that in addition to its effects on muscle atrophy, Isosativan also had a significantly beneficial impact on glycaemic control in the diabetic animal model. The ability of Isosativan to ameliorate both muscle wasting and hyperglycemia suggests its potential as a multi-targeted therapeutic approach for managing diabetes.

Conclusion

This study demonstrates that the flavonoid compound Isosativan, from the Nigerian propolis, is an effective treatment for diabetic muscle atrophy. The activation of the mTORC2/AKT pathway by Isosativan plays a key role in preserving muscle mass and function in diabetes. By suppressing the increased expression of muscle atrophy genes Atrogin-1 and MuRF1, and restoring the phosphorylation of mTORC2 downstream targets, Isosativan was able to counteract the detrimental effects of diabetes on skeletal muscles. These findings highlight the potential of Isosativan as a natural approach to manage diabetes and its attendant muscle wasting. Further research is however needed in order to ascertain the molecular mechanisms underlying its observed effects, and subsequently, explore its therapeutic applications in the clinical management of diabetes and diabetic muscle atrophy.

Conflict of interest

No conflict of interest declared.

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