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EFFECT OF THE AQUEOUS EXTRACT OF *CALEA URTICIFOLIA* (MILL) DC IN ADIPOCYTES 3T3-L1 INSULIN RESISTANCE

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Abstract: Insulin resistance (IR) is a metabolic condition linked to various comorbidities, including type 2 diabetes mellitus (DM-2), fatty liver, atherosclerosis, and hypertension. Several mechanisms contribute to IR, such as insulin receptor insensitivity, dysfunction in glucose transporter (GLUT-4) expression and activity, chronic inflammation marked by elevated proinflammatory cytokines like tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6), and oxidative stress, all of which impede glucose utilization in differentiated adipocytes.

Calea urticifolia (Cu) is traditionally employed for enhancing glucose homeostasis; thus, the biological effects must be evaluated. Mature adipocytes (3T3-L1 line) with induced IR were used to assess the hypoglycemic impact of Cu´s aqueous Cu-Extract (Cu-Extract) Glucose internalization assays in IR adipocytes, exposed to different concentrations of the Cu-Extract (5.5, 11, and 55µg/mL), Metformin (7µg/mL), and a combination of both, were conducted to determine the optimal concentration for GLUT-4 and AKT expression.

The Cu-Extract demonstrated a hypoglycemic effect by facilitating glucose uptake through both insulin-dependent and insulin-independent processes, involving AMPK and AKT phosphorylation.

Keywords: Calea urticifolia, insulin resistance, glucose, low-grade inflammation.

INTRODUCTION

The modern high-caloric diet has led to a global rise in overweight and obesity, causing defects in hormonal regulation, particularly IR, linked to chronic diseases like diabetes mellitus (DM) (Aldhaeefi et al., 2022).

Adipocyte hypertrophy in this context triggers the release of fatty acids, activating immune cells via Toll like receptor 4 (TLR4), initiating a low-grade inflammatory process (IBG) marked by altered adipokines and increased proinflammatory cytokines like TNF-α (Vega-Robledo & Rico-Rosillo, 2019).

TNF-α excess modifies cellular receptor sensitivity to insulin, hindering glucose incorporation into cells through restricted activation of phosphorylation cascades and impaired GLUT-4 translocation (Yaribeygi et al., 2019). Compensatory metabolic pathways in mature adipocytes induced hyperglycemic through gluconeogenesis activation via PKC and AMPK pathways, along with liver glycogenolysis (Göransson et al., 2023; Ho et al., 2019; Hotamisligil, 2006).

Cu, known as negrito, a traditional remedy used for various diseases, including DM (Guzmán-Guzmán, 2010), and is currently under investigation for its hypoglycemic effects.

The aqueous Cu-Extract has been observed to regulate the IBG by inhibition proinflammatory cytokines like TNF-α. In vitro studies have demonstrated that this inhibition occurs through the suppression of the transcription factor NFkB (Segura-Esparragoza, 2017). Additionally, other studies has indicated that the Cu-Extract modulates hypoglycemic activity, possibly by inhibiting the glucose-6-phosphatase enzyme in a model of diabetes induced by streptozotocin (Adolfo Andrade-Cetto et al., 2021).

Chemical analysis reveals germacronolides such as calealactone A, B, C, 2,3-Epoxycalealactone A, Caleine D, Juanislamin, and 2,3-Epoxyjuanislamin in Cu (Yamada et al., 2004); these compounds have inhibited adipocyte differentiation in 3T3-L1 cells (Matsuura et al., 2005). Furthermore, Torres-Rodriguez et al. (2016) identified polyphenolic compounds in the aqueous Cu-Extract of the species with an inhibitory effect on the transcription factor NF-κB. Despite Cu´s recognized hypoglycemic effects, our study aims to delve into the molecular mechanisms

influencing glucose incorporation and GLUT-4 or AKT expression in differentiated insulin-resistant adipocytes.

MATERIALS AND METHODS

COLLECTION OF VEGETAL MATERIAL

The aqueous Cu-Extract was prepared from dry leaves collected during the vegetative phase from plants in the vicinity of el Potrero del Carnero, located in the municipality of Rayón, San Luis Potosí, Mexico (21°52'29.16" North, 99°27'7.92" West, at an altitude of 900 meters above sea level) in September 2017.

CU-EXTRACT PREPARATION

Aqueous Cu-Extract was prepared with 1 kg of dry leaves pulverized with 1 liter of distilled water; the mixture was brought to a boil for 10 minutes on a calefactory plate. The decoction at room temperature was filtered using a Whatman number 2 filter. The filtered solution was frozen at -20°C and subsequently lyophilized using a Freeze Dryer TFD5505 Ilshin. The lyophilized powder was stored at 4°C until use and reconstituted in distilled water for the experiments.

CELL CULTURE OF 3T3-L1

A preadipocyte 3T3-L1 cell line derived from Mus musculus, obtained from ATCC®, was used in this study. Three types of media were used for cell culture: The base media (MB) consisted of DMEM-supplemented media with 1g/L of glucose, 10% fetal bovine serum (FBS), 4mM glutamine, 1mM sodium pyruvate, and 10nM HEPES, all from Gibco™. The differentiation media (DM) was prepared from MB and adding insulin (1µg/mL), 3-Isobutyl-1-metylxantine (IBMX) (0.5nM), Dexamethasone, and Rosiglitazone (1µM), all from Sigma-Aldrich, and finally, the maintenance media (MM) was formed by adding insulin (1µg/mL) to the MB.

The cells were slowly thawed and counted in a Neubauer cell using 5mL of MB for viability and cell counting. The cells were seeded in a 24-well plate at a density of 10,000 cells per plate in 0.5 mL of DM. The plates were incubated at 27°C with 5% CO_2 for 48 hours to induce differentiation. After incubation, the cells were washed with PBS buffer and maintained in MM. This washing and media substitution process was repeated every 48 hours until complete differentiation (15-20 days). Differentiation was confirmed by observing distinctive morphological characteristics of the cells and their differential staining.

DIFFERENTIAL STAINING

Mature adipocyte cells were fixed with 10% formaldehyde and washed with PBS at pH 7.2. Subsequently, the cells were stained with 0.3% red oil from Sigma-Aldrich in an isopropanol-water solution (1:3) for 60 minutes. The cells were kept in soft shaking during the staining process to prevent colorant precipitation.

MITOCHONDRIAL METABOLIC ACTIVITY

The cellular viability was measured through mitochondrial activity by the conversion oxide-reduction of resazurin to resofurin. Differentiated adipocytes were starved for 24 hours in a medium without FBS to reduce interference in the effects of the treatments (Urzì et al., 2022). After this period, the cells were treated with different concentrations of Cu-Extract (5.5µg/mL, 11µg/mL, and 55µg/mL) to determine the optimal concentration that allows a biological effect without causing damage to the cells.

A 75µM of H_2O_2 was used to control cytotoxicity and cell death. The cultures were incubated with 5% CO_2 at 27°C for 4 hours. The red-violet coloration was quantified

using a plate reader model Synergy HT from BioTek at 600nm with 530nm excitation. The experiment was performed in triplicates and repeated in three independent experiments.

INTERNALIZATION OF GLUCOSE ASSAY

The capacity of glucose incorporation in adipocytes was determined independently (MB), and depended on the insulin pathway, known as a classical pathway adding 1µg/mL of insulin (MBI), under two conditions of glucose concentration (5.55 and 22.20mmol/L independently) in the culture media.

The cells were incubated with the different treatments: lyophilized Cu-Extract (11µg/mL), metformin (7µg/mL), and a combination of both by 60 minutes. The glucose concentration incorporating was quantified at 15, 30 and 60 minutes by glucose oxidase test kit from SpinReact following the insert instructions. The experiment was performed in triplicates and repeated in at least three independent experiments.

INDUCTION OF IR IN THE CELL LINE 3T3-L1

IR was induced in mature 3T3-L1 cells using the Mohallem method with modifications (Mohallem & Aryal, 2020). Cells were cultured in a medium without FBS for 24 hours at 27°C with 5% CO_2. After the incubation period, the cells were cultured in two types of culture media: a medium without insulin; a medium with 1µg/mL insulin, both with TNF-α (2ng/mL) to induce IR.

Three groups of treatments were established: cells without stimuli (Control), cells treated with 11µg/mL Cu-Extract, and cells treated with 7µg/mL Metformin. Glucose levels were measured using a glucose oxidase test kit from SpinReact. All treatments were performed in triplicates, and at least three independent experiments were conducted.

EXPRESSION OF GLUT-4 AND AKT EVALUATION

Differentiated cells were exposed to the same treatments as in the induction of IR in the 3T3-L1 cell line for 60 minutes. After the exposure, the cells were detached from the plate using a scraper and 0.125% trypsin to improve cell separation and suspended in a fresh medium. The cell concentration was adjusted to $2x10^5$ cells/mL, ensuring a viability of over 95%. The expression of GLUT-4 was determined using flow cytometry with the addition of a primary antibody of Santa Cruz biotechnology sc-53566 (200µg/ml) at a 1:200 dilutions. For the expression of AKT, the cells were permeabilized and incubated in the dark at 4°C with the primary antibody sc-5298 (200 µg/ml) at a 1:200 dilutions. Subsequently, a secondary antibody of Merck SAB4600389-50UL (200µg/ml) labeled with Alexa 488 was added and maintained in complete darkness. Finally, the cells were analyzed using a Becton Dickinson flow cytometer and Flow Jo v10 software. All treatments were performed in triplicates, and at least three independent experiments were conducted.

STATISTICAL ANALYSIS

The data obtained from the experiments were analyzed using IBM SPSS Statistics software. Descriptive statistics were used for non-parametric data. One and two-way analyses of variance (ANOVA) were performed with post-test analysis using the Kruskal-Wallis test and Bonferroni multiple comparisons. The significance level for determining differences was set at $p < 0.05$.

RESULTS

CU-EXTRACT PERFORMANCE

The aqueous lyophilized Cu-Extract yields 19.88g/100 g of dry plant material.

MORPHOLOGIC CHARACTERISTICS OF ADIPOCYTES 3T3-L1

Differential morphological characteristics were observed in the differentiated adipocytes. These cells exhibited large lipid vacuoles within the cytoplasm varying in shape between spherical or ellipsoid forms. The existence of these lipid vacuoles was confirmed through red oil staining.

MITOCHONDRIAL METABOLIC ACTIVITY

Various concentrations of Cu-Extract (5.5µg/mL, 11µg/mL, and 55µg/mL) did not demonstrate a concentration-dependent impact on mitochondrial metabolic activity (Figure 2). On the other hand, treatment with H_2O_2 lead to a noteworthy 46% reduction ($p<0.05$) in mitochondrial activity. This decreases was attributed to the induction of reactive oxygen species (ROS) causing disruptions in redox balance and damage to mitochondrial enzyme (Zorov et al., 2006)

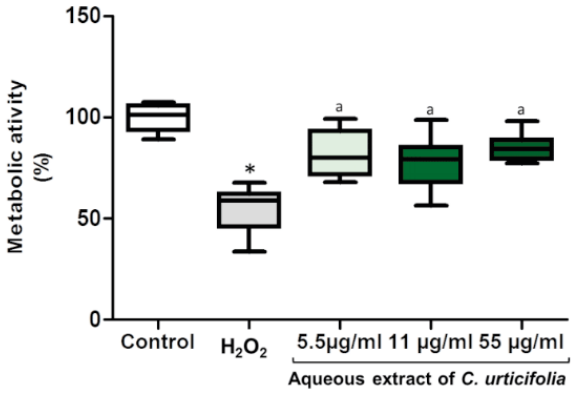


Figure 2. Effect of the metabolic activity mitochondrial of the lyophilized Cu-Extract. One-way ANOVA of Kruskal Wallis test and post hoc Bonferroni were performed for this experiment. *Control vs H_2O_2, [a]Extract vs H_2O_2 ($p< 0.05$).

INTERNALIZATION OF GLUCOSE ASSAY

Figure 3 illustrates the dynamic changes in glucose incorporation within 3T3-L1 adipocytes under an insulin dependent and independent conditions in the presence of low and high glucose media.

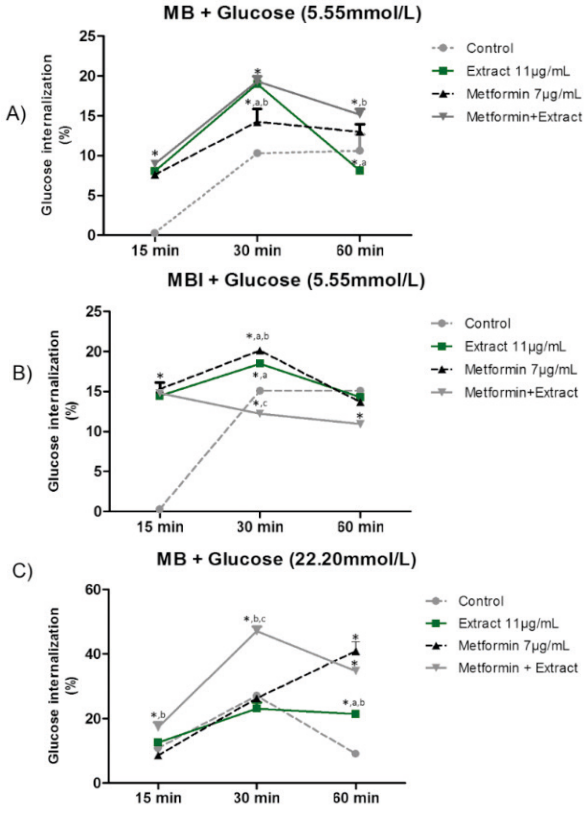
For the insulin-independent scenario (Figure. 3-A MB+Glucose (5.55mmol/L), the group control exhibited a low glucose internalization percentage at 15 minutes (0.3%), in contrast to treated groups displaying entries of 8-9% (Cu-Extract 8.1%, Metformin 7.6%, Mixture 9.0%, p=0.017). By 30 minutes, the maximum glucose internalization was observed, with percentages of 19% Cu-Extract, 14.8% Metformin and 19.3% for the Mixture (p=0.022). However, this effect reverted to baseline over time. The control group sustained glucose incorporation from 30 to 60 minutes at 10.3% to 10.6 % respectively (p=0.023).

Respect to the insulin-dependent condition (Figure 3-B MBI+Glucose (5.55mmol/L)), glucose internalization doubled compared to the insulin-independent condition, with 14.5% for Cu-Extract, 15.3% for Metformin, and 14.8% for the Mixture (p=0.069). At 30 minutes, treatments reached their maximum glucose internalization under these conditions (Cu-Extract 18.5%, Metformin 20.1% and Mixture 12.2%, p=0.015). The control group responded later but reached a percentage like the treatments at 15 minutes (15.1%) at 30 minutes, which returned to initial concentrations by 60 minutes, with the Mixture showing a notable 10.9% (p=0.017).

Figure 3-C MB+Glucose (22.20mmol/L)) depicts the impact of treatments under high glucose without insulin stimulation. Control, Cu-Extract, and Metformin groups showed low internalization percentages at 15 (10.8%, 12.6% and 8.6% respectively) and 30 minutes (Control 27%, Cu-Extract 23.1%, Metformin

26.3%). However, the mixture significantly enhanced glucose internalization at 30 minutes (47.0%), sustained up to 60 minutes (34.7%). Compared to cells treated only with Metformin, the mixture demonstrated sustained, time-dependent glucose internalization (26.3% and 40.9%, p=0.019).

In Figure 3-D (MBI+Glucose 22.20mmol/L). Under the influence of 1 µg/mL insulin, Control and Metformin-treated cells exhibited similar effects to those observed in MBI+glucose (5.55mmol/L) throughout the exposure time. Control (2.5%), Metformin (10.9%), Cu-Extract (19.5%), and the Mixture (20.7%) displayed a parallel effect (p=0.024), increasing intracellular glucose concentrations by 33.3% at 48.5%. However, the group treated with the Mixture demonstrated an enhanced effect at 30 minutes.



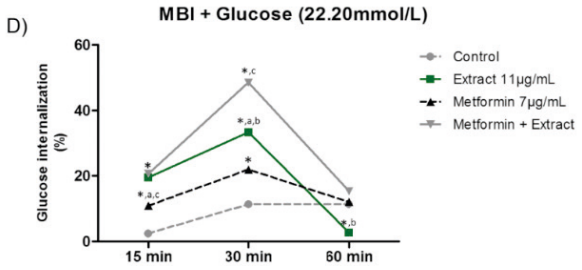


Figure 3. Assessment glucose internalization concentrations and the temporal impact of treatments. Statistical analyses involved One-way ANOVA, Kruskal Wallis test and post hoc Bonferroni for non-parametrical data. *Control vs treatments; [a]Extract vs Metformin, [b]Extract vs Mixture, [c]Metformin vs Mixture at each exposure time ($p<0.05$).

INDUCTION OF INSULIN RESISTANCE IN THE CELL LINE 3T3-L1

The figure 4 illustrate the impact of treatments on insulin-resistance cells induced by TNF-α. Controls cells exhibit elevated extracellular glucose levels, a phenomenon effectively reserved by the aqueous Cu-Extract of and metformin. This suggests that both may operate through mechanisms counteracting IR. In the absence of insulin, the Cu-Extract demonstrate a 46.5% glucose internalization, while Metformin shows 46.34%. In the presence of insulin (1µg/mL), these percentage increase to 55.1% and 67.6%, respectively.

EXPRESSION OF GLUT-4 AND AKT EVALUATION

Table 1 shows the variations in the percentage expression of GLUT-4 and AKT in the presence of Cu-Extract and Metformin. While the results did not demonstrate statistical differences, there is a noticeable trend that may be associated with the classical and insulin independent activation of glucose metabolism (GLUT4 and AKT) in the presence of both the Cu-Extract and Metformin.

DISCUSSION

The process of glucose internalization is essential for establishing glucose homeostasis and ensuring proper metabolic and energetic activities. Adipocytes without IR were treated with the Cu-Extract, Metformin, and a combination of both. Regardless of insulin stimulation, all treatments significantly enhanced glucose homeostasis by the cells, moreover, glucose concentration was identified as factor influencing the internalization process, with longer times required at low concentrations and shorter times at high concentrations. Notably, the Mixture yielded a more pronounced suggesting a synergistic mechanism enhancing glucose utilization in mature adipocytes.

Recent studies show that glucose uptake into cells relies on activation of exocytosis of existing intracellular vesicles containing GLUT-4. These transports aiding glucose incorporation in response to various stimuli, including cellular stress, metabolic changes, and hormonal and oxidative cues. These mechanisms occur with or without insulin stimulation (LaMoia & Shulman, 2021; Mueckler & Thorens, 2013)

In adipocytes, adiponectin activates AMP-activated protein kinase (AMPK), triggering cascades that promote GLUT-4 translocation. This enables glucose internalization even without insulin or under IR conditions (Abel et al., 2001; Chen & Hess, 2008; Coelho et al., 2013; LaMoia & Shulman, 2021; Miranda et al., n.d.). Another insulin-independent mechanism involves AKT phosphorylation pathways external to the activation, leading to GLUT-4 vesicles exocytosis and subsequent glucose internalization (Foster & Klip, 2000; Hou & Pessin, 2007)

The Cu-Extract enhances glucose internalization, even in cells without insulin stimulation, like Metformin, which activates AMPK, booting glucose internalization through diverse pathways. Additionally, Metformin inhibits the Acetyl CoA Carboxylase enzyme, reducing malonyl CoA formation, critical for synthesis of novo lipogenesis (Miranda et al., n.d.; Saltiel & Kahn, 2001).

Hyperglycemia, can mimic IR effects, creating an emergent state in adipocytes to balance glucose levels. Studies indicate that under IR conditions, adipocytes trigger lipolysis, releasing fatty acids and glycerol for energy production.(Hou & Pessin, 2007).

The study on 3T3-L1 adipocytes using the IR model revealed that TNF-α negatively impacts the insulin receptor signaling pathway, hindering glucose uptake by inhibiting GLUT-4 translocation (Saltiel & Kahn, 2001). Additionally, TNF-α suppresses adipocyte AMPK activation, disrupting glucose homeostasis (Daval et al., 2006), leading to signs of IR in the cells.

Regarding GLUT-4 expression, the Cu-Extract increased its expression under standard culture conditions. In contrast, the control group exhibited enhanced expression in the presence of insulin due to the insulin receptor activation. Under IR conditions, GLUT-4 expressions decreased across all treatments, reflecting a steady state of glucose reuptake. Despite the lack of increase GLUT-4 expression, observable glucose entry into the cell interior suggests potential involvement of other transporters or mechanisms stimulated by both Cu-Extract and Metformin, such as GLUT-1 and GLUT-3 or facilitated diffusion, among others. Further evaluation is necessary to understand the precise mechanism employed by the Cu-Extract to generate this effect (Chadt & Al-Hasani, 2020).

On the other hand, in adipocytes, Metformin enhances AKT expression in IR, supporting its function. Additionally, under IR conditions with insulin stimulation (IR + I), the Cu-Extract and Metformin subtly increase the expression of AKT. It is crucial

Expression	GLUT-4			AKT		
Treatments	Control %	Cu-Extract %	Metformin %	Control %	Cu-Extract %	Metformin %
MB	21.23	23.10	19.33	25.50	24.30	24.35
MBI	22.97	19.82	20.58	24.90	25.35	24.30
MB+TNF-α	19.33	20.55	20.45	24.05	26.10	26.15
MBI+TNF-α	21.37	20.03	21.70	24.95	24.80	27.00

Table 1. Percentage of expression measured by flow cytometer of GLUT-4 and AKT in 3T3-L1 cells (Cu-Extract 11µg/mL and Metformin 7µg/mL).

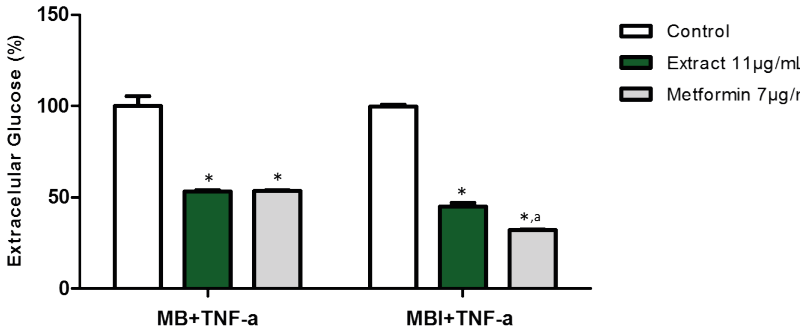


Figure 4. Glucose concentrations were quantified in the supernatants of the cultures after 60 minutes of stimulation. Results are shown in means ± SEM analyzed by IBM SPSS Statistics, *Control vs Treatments, [a]Extract vs Metformin ($p<0.05$). One-way ANOVA of Kruskal Wallis test and post hoc Bonferroni analysis were performed for these experiments.

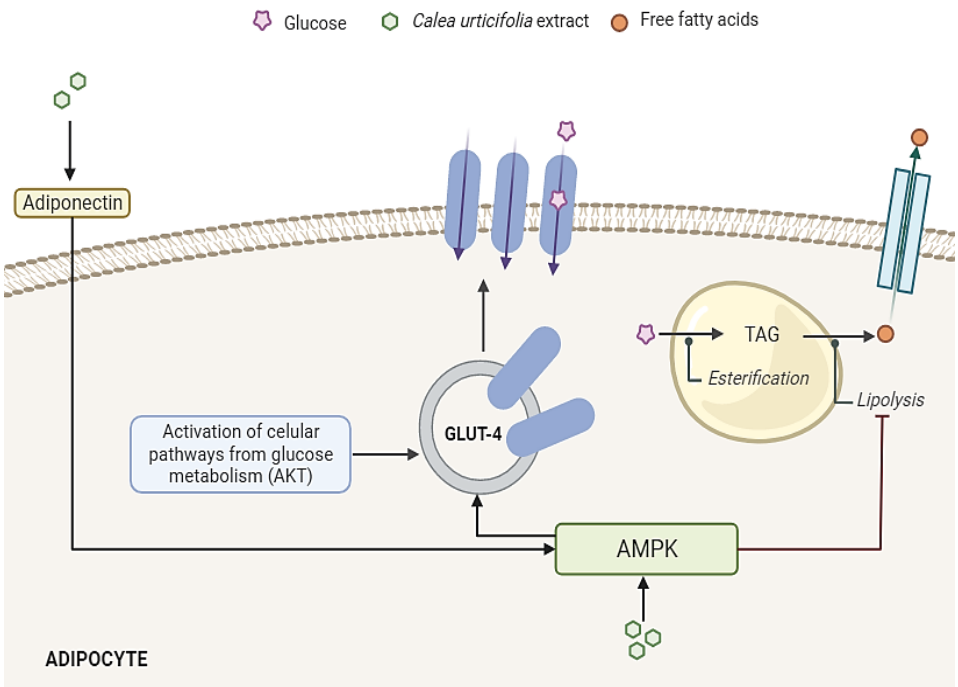


Figure 6. Proposed Cu-Extract mechanism: Activates AMPK, inhibiting lipolysis, restoration of glucose homeostasis. AMPK acts as an energy sensor in adipocytes, activated directly or by stimulating adiponectin secretion.

to note that the AKT protein is constitutive, meaning its expression remains unmodified. However, detailed studies on AKT phosphorylation and GLUT-4 expression will provide a more accurate understanding of the dual mechanism employed by adipocytes to internalize available glucose.

Figure 6. depicts a potential mechanism of action aqueous extract of *Calea urticifolia*.

CONCLUSIONS

The Cu-Extract exhibits a hypoglycemic effect in an IR model in 3T3-L1 adipocytes, enhancing glucose reuptake. This implies potential insulin-dependent and insulin-independent processes, including AMPK and AKT phosphorylation. Further research is needed to delve into the Cu-Extract's mechanisms and clinical implications for diabetes and related metabolic disorders.

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