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In-vitro glucose-utilization activity testing of *Medicago sativa* L. (alfalfa) leaf extracts in L6 and HEPG2/C3A cells

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ABSTRACT

Diabetes mellitus (DM) is a metabolic condition defined by chronic and abnormally high blood sugar levels (hyperglycaemia). Globally 537 million adults were affected in 2021 with the possibility of further increase in the future. Current treatment is reported to be inefficient in the long run and has caused undesirable side effects which affect those suffering from it. *In-vitro* glucose-utilization activity of alfalfa leaf extracts was done with the aim of confirming the existing circumstantial evidence of the beneficial anti-diabetic effects that are credited to the consistent drinking of alfalfa extract in the form of herbal tea. The glucose utilization activity testing of *Medicago sativa* L. leaf extracts was performed in L6 muroid skeletal muscle and the C3A/HEPG2 liver cells. The dry, grassy, and pale green leaves were extracted using five solvents: butanol, diethyl-ether, hexane, methanol and water. The resulting activity was compared with the activity of clinical therapeutics: insulin and metformin. The cytoxicity of the test material was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT Assay) in L6 and the C3A/HEPG2 liver cells. The results showed that the butanol and diethyl-ether extracts produced significant increase in glucose utilisation towards the L6 cells, while the diethyl-ether and water extracts revealed sizeable increase in glucose utilisation towards the C3A cells. The potency of glucose use imposed by our extracts at all tested ranges did not induce glucose utilization activity greater than the activity induced by insulin and metformin. The results demonstrated no evidence of significant cytoxicity toward differentiated L6 and C3A cells. Our experiments support the drinking of alfalfa herbal tea for the management of hyperglycaemia with caution because of the increased glucose-utilization activity induced by *Medicago Sativa* L. extracts. The results of this study warrant further examination of the mechanism of action of alfalfa tea on hyperglycaemia and also identification of active compounds for future development of drug or nutritional supplements towards diabetic care.

KEYWORDS: diabetes mellitus (DM), glucose transporter type 4 (GLUT-4), alfalfa (*Medicago sativa* L.), world health organization (WHO), international diabetes federation (IDF).

1. INTRODUCTION

The polypeptide hormone insulin remains the corner stone of bodily blood glucose-level regulation through the varied mechanisms and actions that can include: the promotion of nitric oxide and endothelin production in endothelial cells; glycogenesis in the liver, in the skeletal muscles, and adipocytes; increased glucose uptake through the glucose transporter type 4 (GLUT-4) receptor in the skeletal myocytes and adipocytes; and also, the downregulation of liver gluconeogenic enzymes [1]. The physiologic state of hyperglycaemia will persist as long as the effects of insulin are disabled. This resultant prolonged state of high blood sugar

levels is diagnosed as diabetes mellitus, a chronic and systematic condition that negatively affects the quality of life of the ones affected and effected [2].

The global incidence of diabetes increased sharply from 108 million to 422 million people with DM in the year 2014 [3]. The World Health Organization (WHO) also confirms that diabetes is accused of causing 1.6 million deaths in the year 2016 alone, while high blood glucose level (hyperglycaemia) is said to have caused 2.2 million deaths in the year 2012. The global incidence of diabetes was figured by the International Diabetes Federation (IDF) to be affecting 537 million adults in the year 2021, a figure that is seen to be increasing steadily [4].

The prevalent undesired outcome of diabetic complications includes limb amputations, blindness, or cataracts (retinopathy), heart attacks (vasculopathy), and kidney failure (nephropathy) [4]. The adverse action of advanced glycation end products (AGE's) such as carboxymethyl lysine and pentosidine is also considered to be a major contributor in diabetic complications [5]. There are four mechanisms by which hyperglycaemia has been shown to cause diabetic complications: activation of the polyol pathway; increased formation of advanced glycosylation end products; activation of Protein Kinase C and the hexosamine pathways [6]. The existing literature demonstrates that diabetic complications may arise from reductive and oxidative stress, as a result of excessive and persistent production of ROS [2].

Adipocytokines in obese individuals are also implicated as the cause of oxidative stress and inflammation, where they can even promote insulin resistance and thus induce Type-2 diabetes (non-insulin dependent diabetes) and cardiovascular diseases [7]. Insulin resistance (IR) refers to a metabolic state where the body's ability to utilize glucose is impaired due to a decreased or abnormal response to insulin and also decreased insulin production [8]. The risk factors for IR include obesity; physical inactivity; a diet that is high in carbohydrates; family history, and ethnicity (as it was found to be more prevalent in Africans, Asian-Americans, Hispanics, American Indians, and so on.).

Septic shock is a life threating syndrome that follows untreated sepsis, characterized by hypotension and organ dysfunction [9]. Diabetics are known to be more prone to wounds and sores with poor healing, and this makes diabetics more susceptible to nosocomial infections when they are hospitalized [10].

The non-pharmaceutical approaches towards diabetic control, treatment, management, and diabetic reversal often depends on the severity of the disease, the age of the subject, their lifestyle, and their physical activity levels. The conventional clinical approaches towards diabetic control and management includes the use of sulfonylureas to increase insulin secretion; troglitazone to increase insulin action in fat and muscle cells; metformin to promote insulin mechanisms in the liver; miglitol and acarbose to delay the absorption of carbohydrates from food intake [11]. There are several concoctions that are used to treat COVID-19 patients with DM including dipeptidyl peptidase-4 (DPP-4) inhibitors, glucagon-like peptide receptor agonists, sodium-glucose co-transporter-2 inhibitors, and other analogues of insulin. But there are more novel drugs and compounds that are still required to combat DM that are cheap, and that produces low side effects for the user [12].

The medicinal potential of nature is diverse and has from the beginning of time funded the human and animal health and wellness with sources of beneficial compounds and phytonutrients without causing the negative side effects of synthetic food and drugs. The maxima advantage of plant-based remedies over synthetics is always due to their therapeutic potency versus the side-effects they cause, and the wide diversity of beneficial phytochemicals that plants contain [13, 14, 15].

According to Krakowska and colleagues, one of the most cultivated perennial fodder crop legumes in the world is *Medicago sativa* L. (also called Lucerne or Alfalfa) [16]. *Medicago sativa* L. appears in ancient medicinal herb/plant books such as *the Compendium of Materia Medica* by Li Shizhen (published .1596) and it is described in it as the father of all foods: "alfac-facah" [17].

M. sativa belongs to the legume (Fabaceae) family; it is a perennial plant of 30-100 cm in height. The taproots (invaded by Rhizobium bacteria) are well developed, thick and strong and grow very deep into the soil layers [17]. The leafy stems are erect,

clumped and even supine [18]. The leaflets of *M. sativa* are 5-20 mm long and dentate (toothed) at the apex and sometimes at the base. The papilionaceous flowers of alfalfa are violet to pale lavender and are clustered along the unbranched axis.

The use of any plant or plant part for medicinal purposes indeed is very diverse and in most cases the knowledge is forbidden for the general public. The traditional use of *moriana* or *muthi* (medicine derived from nature) for healing or other uses was and is still guarded by sacred initiations and secretive hierarchy of bodies or organizations that don't formally exist. As such, the scientific exploration of herbal medicines will always be subjected to the scientific method of evidence-based enquiry. But the genus *Medicago* is part of the *Leguminosae* family that contains 83 known species. *Medicago sativa* (L.) has evidently been used for many ages in China, India, Africa, and America for medicinal purposes. The plant has been shown to be rich in saponins, flavonoids, coumarins, terpenoids, vitamins and digestive enzymes.

Our objective was to establish the *in-vitro* glucose utilization activity induced by five *Medicago sativa* L. leaf extracts in L6 and C3A cells as it has never been reported. We also wanted to confirm the cytoxicity of our extracts using the reliable MTT assay. Our aim was to confirm the existing ethnobotanical fact that alfalfa can help reduce hyperglycaemia by increasing glucose utilization in the liver (C3A/HEPG2) and in the skeletal muscles (l6 myoblasts).

2. METHODOLOGY

2.1. Plant material and extraction

The test material (*M. sativa* L. leaves) was purchased from a nursery and a medicinal plant farm North of Pretoria (South Africa) with the Batch Number MH 71(10kg), sold as herbal tea indicated for diabetes, cancer treatment side effects and others. Tea is to be prepared form the herbs. The plant material was cultivated by Zizameleni Farming based in Mamogalieskraal, Northwest Province of South Africa. The test material was cultivated using regenerative natural farming principles and the fertilizers used were natural and certified organics. The material was air-dried and stored in a cool dry area away from light and heat. The dry, grassy, and pale green leaves were extracted using five solvents: butanol, diethyl-ether, hexane, methanol and water. Sixty (60) grams of powdered plant material was extracted in 1000 mL of each solvent, and the extracts were then filtered using a Buchner funnel and Whatman No.1 filter paper. The extracts were frozen at -40 ^{o}C and freeze-dried for 48 hours that resulted in a yield of 9 g of dried extracts. The dried extracts were stored at -4 ^{o}C until analysis. Dimethyl sulfoxide (DMSO) was used to reconstitute the extracts prior to analysis.

2.2. Cell lines, media, reagents and assay kits

The cells HepG2/C3A and L6 myoblasts were sourced from Highveld Biological, South Africa. Eagle's minimum essential medium (EMEM), 2-(4, 5-dimethylthiazol-2yl) (MTT)-2, and 5 diphenyl tetrazolium bromide were supplied by Sigma Aldrich, South Africa. Fetal calf serum (FCS) and phosphate-buffered saline (PBS) were supplied by Lonza Biologics. The other materials and reagents used for the analysis were sourced either from Merck Chemicals, or Sigma Aldrich.

2.3. Maintenance of cell cultures

The L6 cells were routinely grown in low glucose Dulbecco's modified eagle medium (DMEM); they were then supplemented with 10% Fetal calf serum (FCS) and sub-cultured by trypsinisation. HepG2/C3A cells were grown in EMEM supplemented with 10% FCS and sub-cultured by trypsinisation.

2.4. Cytotoxicity evaluation assay

At the end of the glucose utilisation assay, all the remaining medium was removed. 100 µl of fix solution (0.4% formaldehyde in PBS) was added to each well and the plate was incubated at room temperature for 1 hour. After the removal of the fix solution, 100 µl of crystal violet (0.1%) was added and the plate was incubated at room temperature again for 20 minutes. The plates were washed with water to remove excess unbound dye and plates were then dried overnight at 37 °C. 100 ul of acetic acid was then added to each well and the absorbance was read at 570 nm using the BioTek® SYNERGY Mx fluorometer (Winooski, VT, USA). The cytotoxicity is expressed as a percentage of the untreated control.

2.5. The glucose utilization activity testing of *Medicago sativa* L (alfalfa) in L6 and HepG2/C3A cells

2.5.1. Glucose utilization in L6 cell assay procedure

The L6 cells were seeded into 96-well plates at a density of 5000 cells/well. At least one row was kept devoid of cells to represent the no cell control. At approximately 80% confluence, the medium was replaced with differentiation medium (DMEM containing 2% horse serum). The cells were then incubated for an additional 5 days to allow sufficient time for full differentiation.

The treatment was continued for 48 h. The spent culture medium was then removed, and the cells were washed once with phosphate-buffered saline (PBS). Fifty micro-litres of 8 mM glucose solution (Roswell Park Memorial Institute medium (RPMI) diluted with PBS and supplemented with bovine serum albumin (BSA) to a final concentration of 0.1%) was added. For the positive control, 1000 ng/ml insulin was used. The plates were returned to the incubator. After 2 hours 5 µl culture medium was transferred to a new 96-well plate and 200 ul glucose assay reagent (glucose oxidase/peroxidase colorimetric reagent) was added and the plate was incubated again at 37 °C for 10-20 minutes. The absorbance was measured at 510 nm using the BioTek® SYNERGY Mx fluorometer (Winooski, VT, USA). Glucose utilisation was calculated as the difference between the mean of the no cell wells and the test sample and expressed as a percentage of the untreated control.

2.5.2. Glucose utilization in HEPG2/C3A cell assay procedure

The HEPG2/C3A cells were seeded into 96-well plates at a density of 5000 cells/well. At least one row was kept devoid of cells to represent the no cell control. Cells were incubated for an additional 2-3 days to become confluent. The spent culture medium from differentiated cells was then removed and replaced with fresh medium containing test compounds at the concentrations of 200 ul/well. Metformin was used as positive control. The treatment was continued for 48 h. Spent culture medium was then removed, and the cells were washed once with PBS. Fifty micro-litres of 8 mM glucose solution (RPMI medium diluted with PBS and supplemented with BSA to a final concentration of 0.1%) was added. The plates were then returned to the incubator. After 3 hours 5 µl was transferred into a new plate and 200 ul glucose assay reagent (glucose oxidase/peroxidase colorimetric reagent) was added and incubated at 37 °C for 10-20 min. Absorbance was measured at 510 nm using the BioTek® SYNERGY Mx fluorometer (Winooski, VT, USA). Glucose utilisation is calculated as the difference between the mean of the no cell control and the test sample and expressed as a percentage of the untreated control.

3. RESULTS

The results of studies measuring glucose absorption in HEPG2/C3A cells using five leaf extracts of Medicago sativa L. (butanol, diethyl-ether, hexane, methanol, and water) as well as metformin as the control are shown in Figure 1. The glucose utilization observed in HEPG2/C3A cells was considerably uniform but the water extract at 50 µg/mL produced glucose use almost similar to our control metformin (Figure 1). The cytotoxicity potential of the plant materials that could be used for drug development is important to ensure the consumer's safety, and our experiments demonstrated no evidence of significant cytoxicity towards differentiated HEPG2/C3A cells.

Figure 2 displays the outcomes of cytotoxicity testing and glucose uptake of five leaf extracts of Medicago sativa L. (butanol, diethyl-ether, hexane, methanol, and water) using l6 myoblast cells. Metformin was employed as the control. The glucose utilization observed in L6 myoblast cells was considerably uniform but butanol and diethyl-ether produced glucose use almost similar to our control insulin (Figure 2). The cytotoxicity potential of the plant materials that could be used for drug development is important to ensure the consumer's safety, and our experiments demonstrated no evidence of significant cytoxicity towards differentiated L6 cells.

4. DISCUSSION

From our experiments we observed the Butanol and Diethyl-ether extracts producing a sizable increase in glucose utilisation in skeletal L6 cell line (Figure 2), whereas the diethyl-ether and water extracts revealed significant increase in glucose utilisation towards the hepatic C3A cell line (Figure 1).

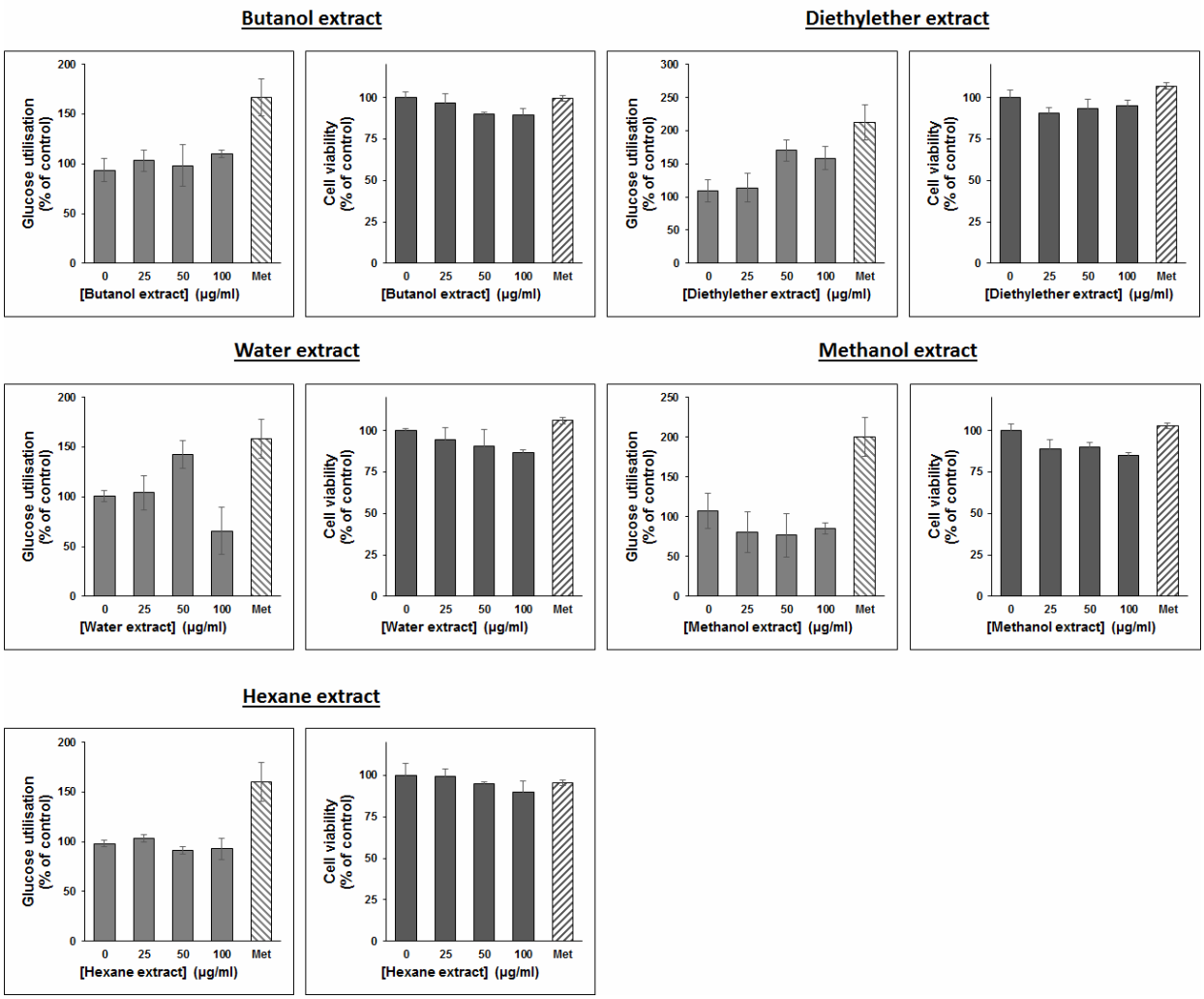

Figure 1. Glucose utilisation and corresponding cytotoxicity towards C3A/HEPG2 cells. Data is expressed as a percentage of the untreated control and metformin included as a positive control.

The overall cytotoxicity test was weak and is unlikely to have any meaningful physiological impact (Figures 1 and 2). The extracts did not induce potent activity (glucose utilization) compared to the control's insulin and metformin, which are well used in clinical setting to treat cystic fibrosis, DM, and others.

To measure the glucose utilization bioactivity induced by our five *Medicago sativa* L. extracts using liver cell line C3A/HEPG2 we used metformin as a positive control (Figure 1). The cell line C3A is a clonal derivative of HEPG2 cell line that was selected due to its strong contact inhibition of growth, high albumin production, high production of alpha fetoprotein (APF) and the ability to grow in glucose deficient medium. The corresponding cytotoxicity towards C3A cells is shown in Figure 1, where data is expressed as a percentage of the untreated control. And according to the results only the diethyl ether and water extracts demonstrated significant increase in glucose utilisation relative to metformin; however we are not saying other extracts did not induce increased glucose use. In this context, increased glucose utilization by *Medicago sativa* could subsequently lead to the decrease of glucose in the blood; that is to say it alleviates hyperglycemia but only in laboratory conditions.

Glucose is a six-carbon structure (C6H1206) that is bio-processed for the generation of energy that

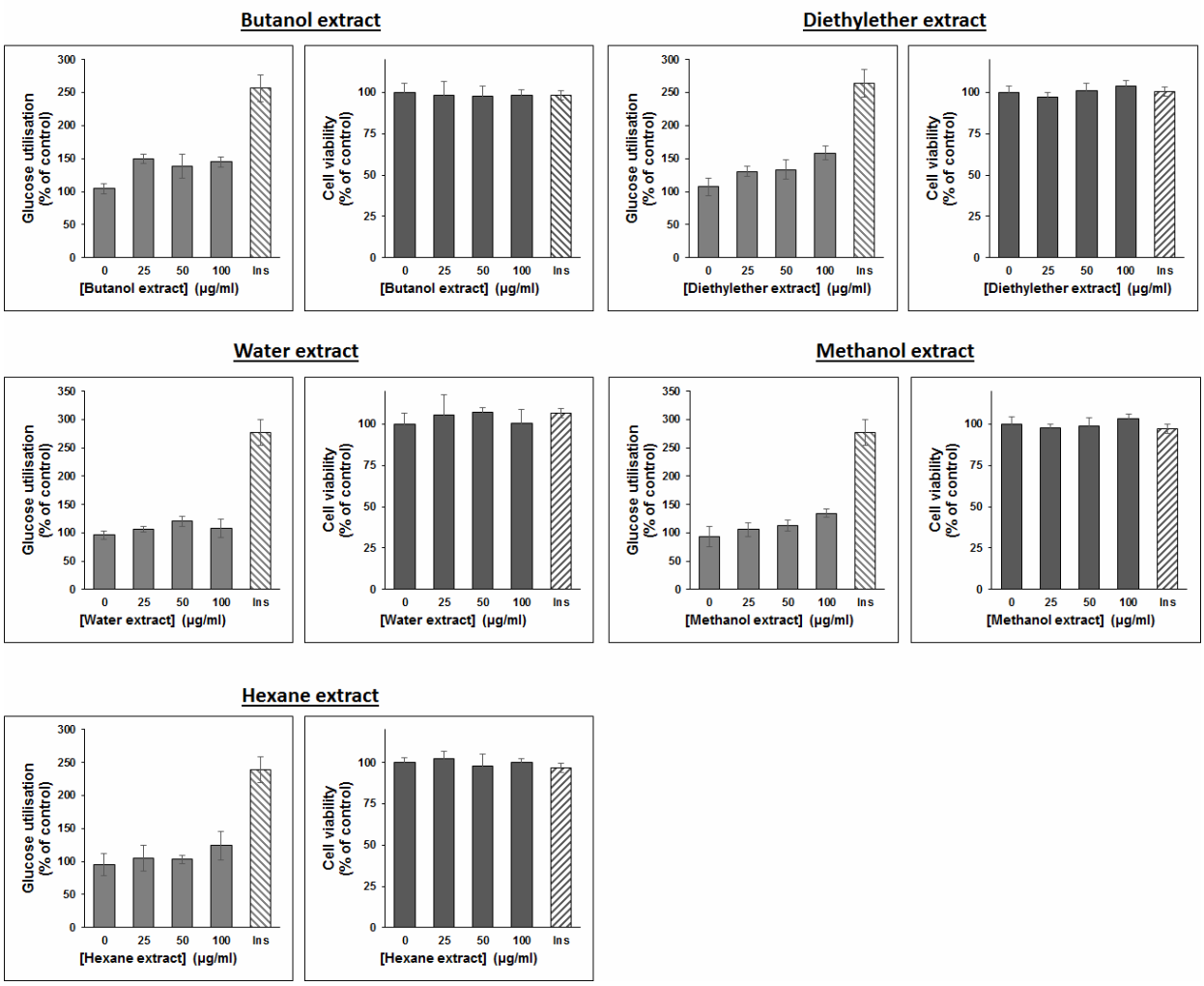

Figure 2. Glucose utilisation and corresponding cytotoxicity towards L6 skeletal muscle cell line. Data is expressed as a percentage of the untreated control and insulin employed as a positive control.

is required by organisms to function. Glucose is consumed *via* several pathways and processes that ultimately convert it to energy in the form of adenosine triphosphate (ATP). Glucose homeostasis is therefore an essential action of maintaining the energy levels of the body in the form of glucose that is circulating in the blood or that is stored in the liver in the form glycogen. The feedback mechanism that regulates blood glucose levels depends on the effective and efficient functioning of the hormone insulin.

The ancient Egyptian '*Chief of Dentists and Physicians*', Hesy-Ra was the first in 1552 B.C. to describe the well-known symptom of diabetes which is frequent urination in the ancient book called *Ebers papyrus.* The earlier 'diagnostic tests' for DM involved the observations of abnormally frequent urination, the tasting of urine for sweetness and what I call the ant-urine-attraction test/assay (where the positive diabetes test = ants being attracted to urine). The combination of these observations helped to create the name of the condition DM where mellitus means honey (sweetness), and diabetes refers to a process of siphoning (frequent urination).

Human insulin (or insulin human) is a drug synthesized through DNA recombinant techniques, to control and regulate the rate of glucose and amino acid cellular uptake. Insulin is injected subcutaneously to affect the blood glucose levels

in type 1 and type 2 DM. The common side effects of insulin therapy include excessive hypoglycaemia, weight gain, gut discomfort, nausea, and the usual organ failure (liver and heart).

There are many other therapeutic tools used to manage and control DM, such as the use of microcapsules that carry pig-derived islets of Langerhans, which are then implanted beneath the skin of a diabetic patient to restore glucose homeostasis. Metabolic syndrome is a complex mix of abnormalities that can include obesity, insulin resistance (IR), hypertension, glucose intolerance and dyslipidaemia [19].

Obesity often disrupts the Krebs's Cycle and as a result can cause mitochondrial dysfunction which has been established to promote excessive production of ROS. Adipocytokines in obese individuals are also implicated as the cause of oxidative stress and inflammation, where they can promote insulin resistance and thus induce type-2 diabetes (non-insulin dependent diabetes) and cardiovascular diseases [7]. Insulin resistance (IR) refers to a metabolic state where the body's ability to utilize glucose is impaired due to a decreased or abnormal response to insulin and decreased insulin production [8].

The oral treatment with water extracts of *Terminalia paniculata* bark, demonstrated a decreased blood glucose and glycosylated haemoglobin levels in streptozotocin-nicotinamide-induced diabetic rats [20]. The experiment further established the *in-vivo* inhibition of the enzymes pancreatic alpha-amylase and alpha-glucosidase. The chemical profiling of *Terminalia paniculata* using high performance liquid chromatography (HPLC) indicated the presence of bioactive biomarkers gallic acid, ellagic acid, catechin and epicatechin [20].

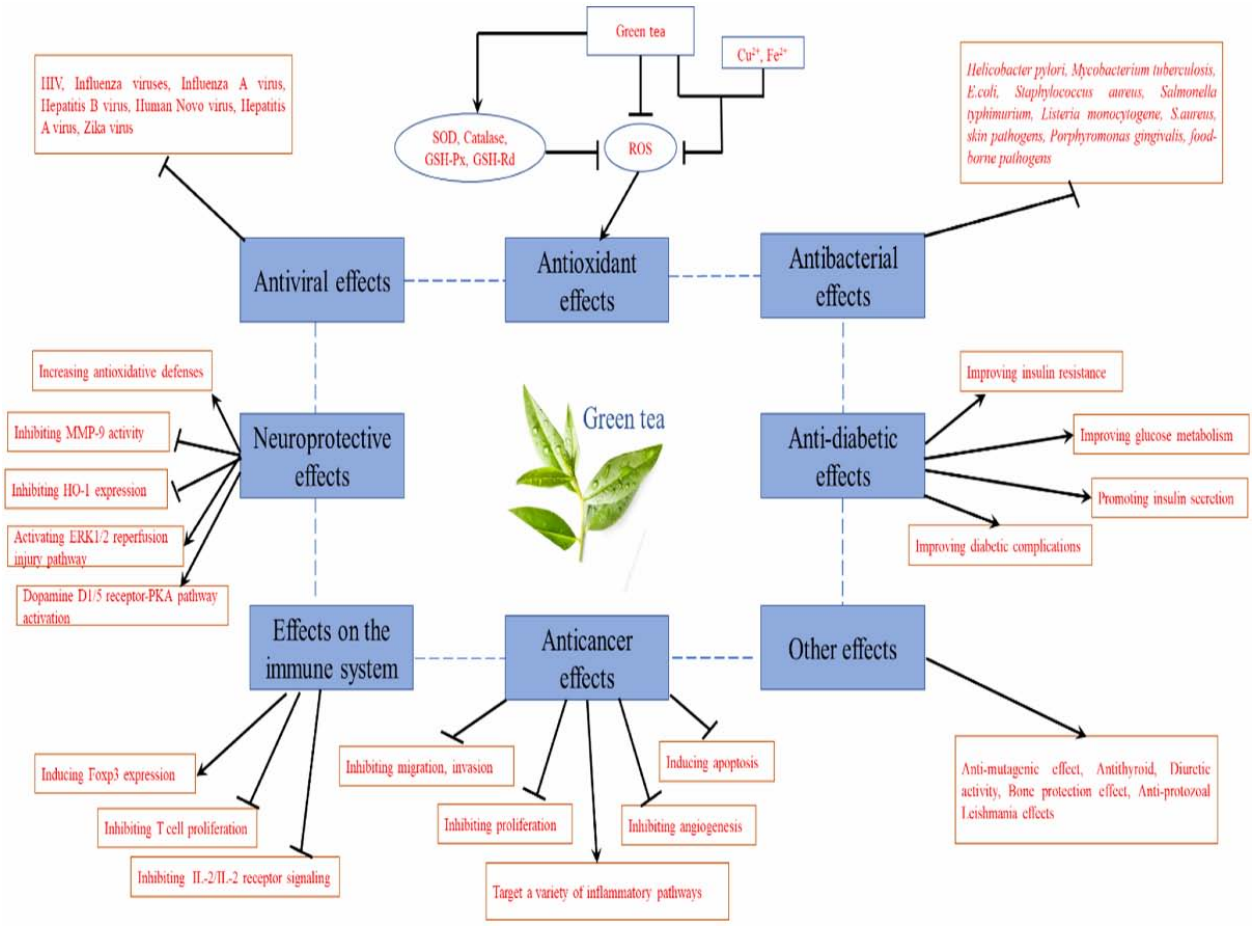

Figure 3. Pharmacological effects of green tea [21] (https://www.mdpi.com/1420-3049/27/12/3909/htm).

The active catechins (epicatechin gallate, epigallocatechin gallate) contained in green tea were demonstrated to inhibit the activity of glucose transporter 1 (GLUT-1) which is excessively expressed in cancer cells. This observation indicated the mechanism by which tea polyphenols can act as competitive inhibitors of glucose uptake through binding to GLUT-1 (Figure 3) and as thus induce anti-diabetic effects.

Our objective with the experiment was to determine and report the *in-vitro* glucose utilization activity of *Medicago sativa* L. leaf extracts towards L6 and C3A cell lines using five different solvents. The aim was to use the results to validate the existing evidence of anti-diabetic effects of drinking alfalfa tea.

5. CONCLUSION

Diabetes is a global concern that threatens the competence of modern medical care. To curb the emergent exponential rise of DM prevalence, affordable solutions are needed urgently. The sensitivity of contextualizing experimental results is explained by David Klatzow in his book '*Justice denied*', where he argues that the detection of opiates in a test sample for example, can lead to erroneous conclusions if we ignore that the test subject ate a roll of bread with poppy seeds sprinkled on top before the test. Thus, the spiritual aspects of traditional and African healing are also difficult to measure and to record using empirical methods.

Our experiments demonstrated clearly that every one of our five extracts we tested did impose a considerable glucose utilization enhancement both at the liver (C3A cell line) and skeletal muscle level (L6 cell line). We admit that our test extracts did not produce the bioactivity that is as strong as the activity induced by the clinical standards that we used (metformin and insulin). Our experimental results can corroborate the religious drinking of alfalfa tea for health and wellness. The result of our experiments hereby confirms the ethnobotanic and traditional uses of alfalfa towards the control of hyperglycaemia. *Medicago sativa* L. appears in ancient medicinal herb/plant books such as the *Compendium of Materia Medica* by Li Shizhen and it is described in it as the father of all foods: "*alfac-facah"* [17] and our experiments can be used to scientifically to validate that opinion. Our recommendation is further analysis at molecular level to establish the possible mechanisms of action exhibited by alfalfa and further isolation and testing of the active compound(s) of interest.

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CONFLICT OF INTEREST STATEMENT

We confirm no conflict of interests.

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