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A study on the ameliorative effects of goldenseal, goat's rue extracts, and berberine on glucose uptake compared to metformin in a cellular model

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Abstract
This study examines the effects of goat's rue (*Galega officinalis* L.), goldenseal (*Hydrastis canadensis* L.) leaves, root extracts, and berberine on glucose uptake in HepG2 cells. These plant compounds, traditionally used to alleviate diabetes symptoms, were compared to metformin. Glucose uptake was assessed using 2-NBDG, MTT assays for cytotoxicity, Bradford protein assays, and western blotting for GLUT-1 expression. Goldenseal and berberine significantly increased glucose uptake compared to metformin ($p<0.05$). Berberine, goldenseal, and metformin enhanced glucose uptake by 3.4, 2, and 1.8 times respectively ($p<0.001$). GLUT-1 expression increased by 1.7 times with goldenseal and 1.4 times with berberine ($p<0.05$). The results indicate that berberine is more effective than goldenseal in improving glucose uptake, suggesting both may lower blood glucose levels in insulin resistance, and present potential therapeutic alternatives for Type 2 Diabetes.

Keywords: Glucose transporter-1, HepG2 cells, hypoglycaemic herbs, insulin resistance, non-insulin-mediated glucose uptake, Type 2 Diabetes

Introduction
Changing one's diet, maintaining an active lifestyle, and engaging in regular exercise can assist in managing metabolic functions for individuals with borderline Type 2 Diabetes (T2D). However, these interventions tend to be less effective for those in the advanced stages of the condition. Current medicines can have side effects. For example, the biguanide drug metformin may be associated with liver damage, lactose intolerance, B-12 deficiency, nausea, stomach cramps, and diarrhoea [1, 3, 32]. Sulfonylureas can cause hypoglycaemia and weight gain, back pain, low blood sugar levels, and upper respiratory tract infections are among the negative effects of meglitinides. Alpha-glucosidase inhibitors can cause hepatic necrosis while also inhibiting alpha-glucosidase, a digestive enzyme that causes digestive issues and gas formation in the intestines. Thiazolidinediones can cause fluid retention, weight gain, and anaemia, whilst metformin-rosiglitazone combinations can cause irregular menstruation [2, 3]. Currently, an increasing number of individuals are opting for herbal remedies due to their lower cost and potentially reduced side effects compared to conventional anti-diabetic medications [3, 34]. Certain herbs have the efficacy to stimulate pancreatic beta cell regeneration and enhance insulin sensitivity, while others possess antioxidant properties, cholesterol and HbA1c-reducing characteristics [4, 31, 34]. Therefore, using herbal substances could potentially improve insulin resistance and prevent complications. The compounds berberine and metformin, along with extracts from goldenseal and goat's rue, were selected for this research due to their efficacy in treating diabetes symptoms [5-8]. To demonstrate their efficacy, the glucose uptake mechanism and GLUT-1 expression levels were examined. The glucose analogue 2-NBDG was employed to measure non-insulin-mediated glucose uptake in HepG2 cells [30].
HepG2 cells express ubiquitous GLUT-1 and are frequently used as a cellular model of human hepatocytes for biochemical studies because they retain their morphology and most of their function in culture and share a physiological function for lipid or glucose metabolism with normal hepatic cells, were selected for this study after carefully weighing the benefits and drawbacks compared to primary cells [9]. The effect of insulin on HepG2 cell's glucose uptake was not within the scope of this work.
The herbs used in this study have been traditionally used and are noted for their efficacy in providing symptomatic relief for diabetics. However, their application is often limited due to a lack of biochemical evidence and an insufficient understanding of their mechanisms of action

in the existing literature. The aim of this project is to address this research gap by providing data and explaining the mechanisms of action of the selected herbal components. This is the first study to examine glucose uptake in HepG2 cells using goldenseal and goat's rue [33].

Materials and Methods

Compounds and extracts: Berberine chloride hydrate and metformin hydrochloride were sourced from Sigma Aldrich, prepared as 100x stock solutions in DMSO and used at 1000µM (metformin) or 10 µM (berberine), with 1% (v/v) DMSO as control. Goldenseal glycerite extract was provided by Herb Pharm Company, while goldenseal tincture and goat's rue tincture extracts came from "Napiers the Herbalists". Concentrations of 0.312 to 10 µL/mL (v/v) were prepared using DMEM media, with untreated controls and respective solvent controls of 0.2%(v/v) glycerol and 0.6%(v/v) or 0.45%(v/v) ethanol [33].

2-NBDG: The fluorescent glucose analogue 2-NBDG was used to monitor glucose uptake in live cells, prepared as a 10 mM stock solution in methanol and stored at -80 °C [33].

Bradford protein assay: The mean glucose uptake values were divided by the mean protein content to estimate single-cell glucose uptake in short and long-term treated cells. Cells were lysed with 0.1 mL of 1.0 M NaOH per well and rocked for 15 minutes. Frozen-stored plates were thawed, mixed, and aliquots extracted for analysis. A BSA stock solution (10 mg/mL) was prepared in water; its absorbance at 280 nm was expected to be 0.667 for 1.0 mg/mL. Triplicates of 50 µL BSA standard solutions (0, 5, 10, 20, 40, 60, 80, 100 µg/µL) were prepared using distilled water [33].

MTT cytotoxicity assay: The MTT assay uses NAD (P) H-dependent oxidoreductase enzymes to reduce MTT dye to purple formazan, indicating viable cells. These assays were conducted in the dark due to MTT's light sensitivity. Confluent HepG2 cells were tested with optimal phytocompounds after long-term treatment [33].

HepG2 cells: Provided by the Institute of Liver Studies at King's College Hospital, London. They were cultured in DMEM with 10% foetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 2mM L-glutamine (Invitrogen, CA, USA) at 37 °C in a humidified 5% CO_2 atmosphere. Cells were sub-cultured at 75% confluency [33].

Glucose uptake assay by Microplate reader (BMG Labtech)

Procedure and plan for 24-well plate format (short-term treatment): HepG2 cells were seeded in a 24-well plate (Costar) at 5 x 10^4 cells / mL, and 0.5 mL of the cells per well in full medium containing 11 mM glucose. The cells were left to reach the confluency and the media was removed, the cells were then treated with 0.5 mL volumes of varying concentrations of test reagents (0.312 µL/mL to 10 µL/mL) in each well, for each of the different test plates, including goldenseal glycerite, goldenseal tincture extracts. The untreated control was distilled water. Cells were incubated for a further 1-hour treatment, and glucose uptake was measured. The cells in the first three wells of the first column were treated with untreated control, and cells in three wells of columns 2 to 6 were treated with concentrations 10 µL/mL to 0.625 µL/mL. The first three wells of the bottom row were allocated for background fluorescence and the cells in other three wells in the same row were treated with 0.312 µL/mL concentrations [33].

Procedure and plan for 96-well plate format (long-term treatment): The cells were treated for 48 hours with goldenseal tincture, goat's rue tincture, berberine, and metformin was used as positive control, and solvent controls were 0.6% (v/v), 0.45% (v/v),0.24% (v/v) ethanol solutions, and DMSO (1% (v/v)). For the aqueous extracts, distilled water was used as a solvent control. In a 96 well plate the top and bottom rows and the first and last columns were left unused. Columns 2 to 7, in an each column three wells were treated with varying concentrations 10 µL/mL to 0.312 µL/mL of the target extracts, and the three wells in column 8 was allocated for berberine, three wells in column 9 was allocated for metformin, three wells in columns 10 and 11 were allocated for solvent controls 0.6% (v/v), 0.45% (v/v) and the last three wells in column 11 were allocated for solvent control 0.24% (v/v) ethanol solutions. The remaining wells were organised for DMSO (1% (v/v)) and for the untreated control [33].

The data of microplate reader-based experiments was fluorescence at excitation/emission maxima of λ_{ex} 485 nm and λ_{em} 530 nm. These values were triplicates of samples. Solvent control expresses intrinsic fluorescence of the samples, whereas 2-NBDG treated samples express fluorescence of 2-NBDG uptake. To obtain average adjusted fluorescence, average intrinsic fluorescence values were subtracted from average fluorescence values of the 2- NBDG treated medium [33].

Glucose uptake assay by flow cytometry (Guava easycyte)

HepG2 cells were cultured in six separate flasks (Corning, surface area 25 cm^2) until they reached confluency, then treated for 48 hours with goldenseal ethanolic extracts (10 µL/mL), berberine (10 µM), and positive control metformin (1000 µM), solvent controls DMSO (1% (v/v)) and ethanol (0.6% (v/v)) and an untreated non-fluorescent negative control. At the end of the treatment, medium was removed, and the cells were washed once with glucose free medium. The cells were trypsinised and collected, followed by quenching with ice cold glucose free medium in six, 15ml tubes. Cells were treated with 400µL of 0.1 mM 2-NBDG and incubated for 15 minutes. The cells were collected in 18 Eppendorf tubes (1.5 mL) and spun down at 800 g for 5 minutes for settling down at the bottom of the tube. Medium was removed from the tube and 1.0 mL ice cold PBS was added. The cells were spun down at 5000 rpm for 20 seconds. PBS was removed from the tube and 1.0 mL ice cold PBS was added and the cells were suspended. The cells were analysed with a Guava flow cytometer controlled using Guava Express Plus software, version 4.1 (Merck Millipore). The HepG2 cells which took up 2-NBDG displayed fluorescence with excitation and emission at the wavelength of the fluorescence λ_{ex}= 485 nm and λ_{em}= 535 nm respectively, and the effect was measured in the channel used to detect fluorescence [10, 33].

Bradford assay procedure: 50 µL of each cell lysate was taken per well in a 96 well plate, and each lysate assayed in triplicates, and applied 50 µL of BSA protein standard per well and each standard assayed in triplicates. Added 150 µL of Pierce Bradford reagent by using 8-channel pipette, and mixed Bradford reagent and sample in a plate reader by shaking for

10 seconds, and left at room temperature for 15 minutes, and then read absorbance at 595 nm in Omega Fluostar plate reader. In protein estimation experiments standard curve preparation is essential; thus, the known properties of the standard solution were measured, and graph was plotted, this typical standard curve allows the similar or same properties to be estimated for the unknown or test samples by interpolation of the standard curve [33].

MTT assay procedure for 24 well plates (Nunc cell culture plates): Thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich, Poole, UK), was dissolved in PBS (5 mG/mL). 100 µL of MTT solution was added to each well of cells in a 24 well plate. After 2 hours incubation at 37^0C the medium was removed and 500 µL of DMSO was added and the cells were incubated further 30 minutes. And the absorbance was read at 570 nm and 670 nm in a plate reader (Fluostar omega microplate reader) [33].

Statistical analysis: The experimental data are presented as mean ± standard deviation (S.D) of the mean of n independent measurements. Statistical significance was determined as the difference from the vehicle control to target treatments by employing one-way ANOVA with Bonferroni's multiple comparison post hoc tests, $p>0.05$(not significant), $*p<0.05$, $**p< 0.01$, $***p<0.001$, and $****p<0.0001$.

Western blotting analysis for detection of GLUT-1: HepG2 cells were seeded in four 75 cm^2 flasks (Costar); 12 mL of cells with a density of 2.5 x 10^5 cells/mL were seeded in each flask and incubated until they reach confluence, and then treated with berberine (10 µM), goldenseal (10 µL), DMSO (1% (v/v), and EtOH (0.6% (v/v)), and incubated at 37°C for 48 h. The total protein was then extracted from cells using RIPA lysis buffer and supplemented with a protease inhibitor cocktail. The total protein concentration was determined with the bicinchoninic acid reaction. And 20 µg/lane of protein samples were separated using 10% SDS-PAGE, then transferred onto PVDF membranes that were blocked for 90 minutes at room temperature using 6% non-fat milk. Membranes were subsequently incubated with the following primary antibodies at 4°C overnight: The first antibody tried was from Santa Cruz (sc-7903, Bioscience UK) and was a polyclonal rabbit raised against amino acids 218-260 of human GLUT-1. It was expected to detect GLUT-1 as a 55 kDA protein in human cell lysates. A variety of conditions were tried at first, including 3% or 5% blocking buffer and dilutions 1:500, 1000, 2000. To minimise background staining a dilution of 1:1000 in 5% blocking buffer was used. The first antibody did not give interpretable results, and a second antibody was tried, Abcam 15309 (Abcam, UK). This was also a rabbit polyclonal antibody, raised against an undisclosed peptide of the human sequence from the C-terminus of the protein. It was used at a dilution of 1:500 in 4% blocking buffer. The membrane was incubated with primary antibody solution with constant rotation at room temperature for at least 2 hours. Alternatively, the membrane in primary antibody was left overnight at 4 °C then incubated for one hour with constant rotation at room temperature. Following the primary antibody incubation, the membrane incubated with the secondary antibody, donkey anti-rabbit IgG conjugated with horse radish peroxidase (Santa Cruz sc-2313, Bioscience, UK) at a 1:2000 dilution in blocking buffer. The membrane was incubated on a rotating platform at room temperature for at least 1 hour. Protein bands were observed on a gel imaging system (GE Healthcare) using a SuperSignalTM West Pico PLUS Chemiluminescent substrate (Thermo Fisher Scientific, Inc.), and the band density was measured using densitometric analysis with ImageJ V1.8.0 software [33].

Results

Effect of goldenseal glycerite and goldenseal ethanolic extracts on 2-NBDG uptake in HepG2 cells

To test the efficacy of the extracts, studies were first conducted with glycerite extracts in the short-term treatment. However, because ethanolic extracts have a higher bioavailability, they were employed in the long-term treatment [11]. Berberine's effects were also examined in comparison to those of the common medication metformin. To demonstrate the average effect, the mean value of the net fluorescence was divided by the mean value of the protein content of the treated cells, and then net fluorescence was normalised to the net fluorescence of 2-NBDG uptake in the untreated control. The results of the protein assays were used to normalise the fluorescence values of the 2-NBDG and are not presented individually in this paper. In confluent cells, a short-term treatment with goldenseal glycerites and ethanolic extracts stimulated glucose uptake at the optimal concentration (10 µL/mL), by 6.5 times and 5.4 times respectively, compared with untreated cells. And in the long-term treatment ethanolic extracts, at the optimal concentration stimulated glucose uptake 13.5 times compared with the untreated control, positive control metformin stimulated 9 times, berberine stimulated 14.2 times more glucose uptake, compared with the untreated control [33].

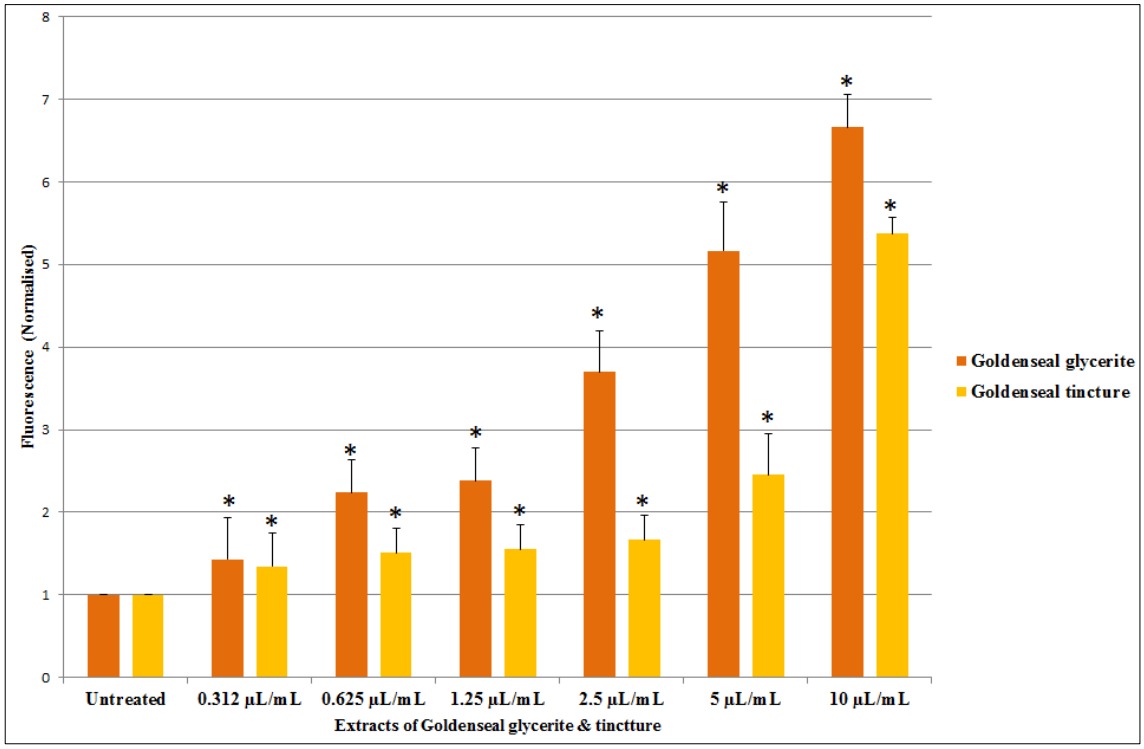


Fig 1: The effect of a 1-hour treatment with goldenseal glycerite and ethanolic extracts on 2-NBDG uptake in confluent HepG2 cells.

Data are changes in fluorescence normalised for protein content, and normalised to untreated control, and are the mean + SD from three experiments with goldenseal glycerite extract and five experiments with goldenseal ethanolic extracts. The treatments in each experiment were conducted in triplicate wells. Significant difference from untreated control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, *$p<0.05$ [33].

Effect of goldenseal ethanolic extracts on 2-NBDG uptake in HepG2 cells after long- term treatment.

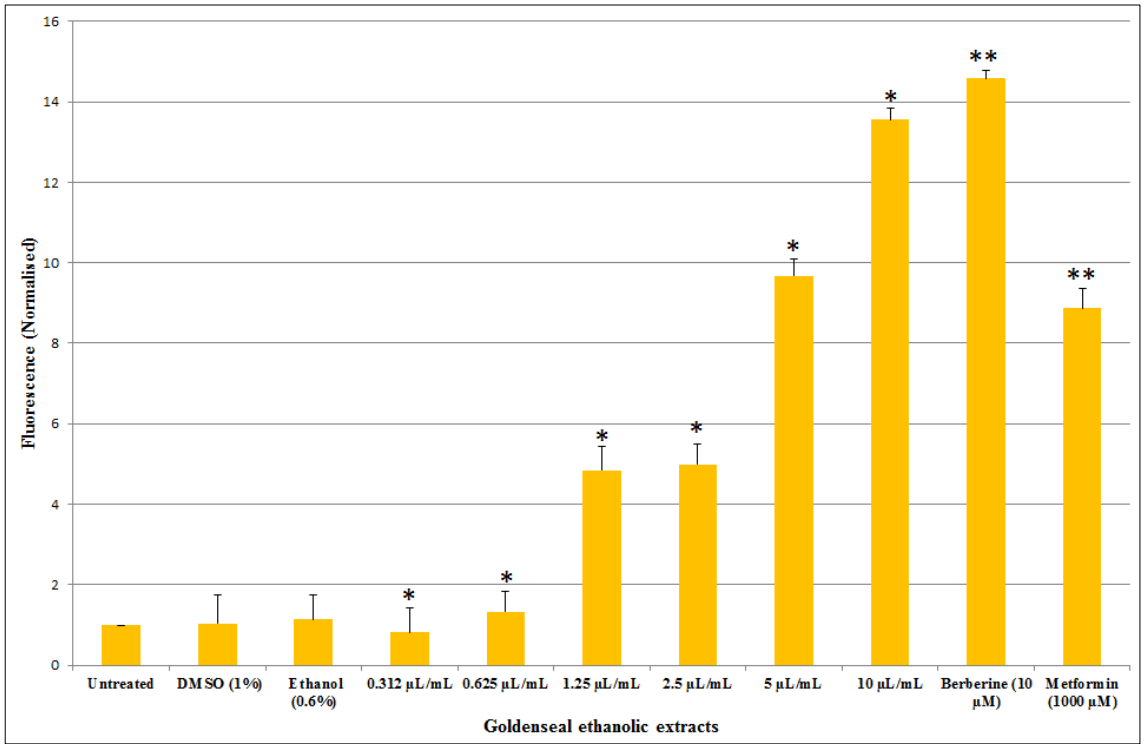


Fig 2: Effect of long-term (48 hours) treatment of goldenseal ethanolic extracts on uptake of 2-NBDG by confluent HepG2 cells.

Data are changes in fluorescence normalised for protein content, and normalised to untreated cells, and are the mean + SD from five experiments with goldenseal ethanolic extracts, and the treatments in each experiment were conducted in triplicate wells. Significant difference from untreated control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, *$p<0.05$ and **$p<0.01$[33].

Effect of goat's rue ethanolic extracts on 2-NBDG uptake in HepG2 cells after long-term treatment

In confluent cells, the long-term treatment with goat's rue ethanolic extracts, the optimal concentration (10 µL/mL) upregulated glucose uptake 10 times, and the positive control metformin stimulated 9 times more augmentation, whereas berberine augmented 14.2 times more glucose uptake, compared with the untreated control [33].

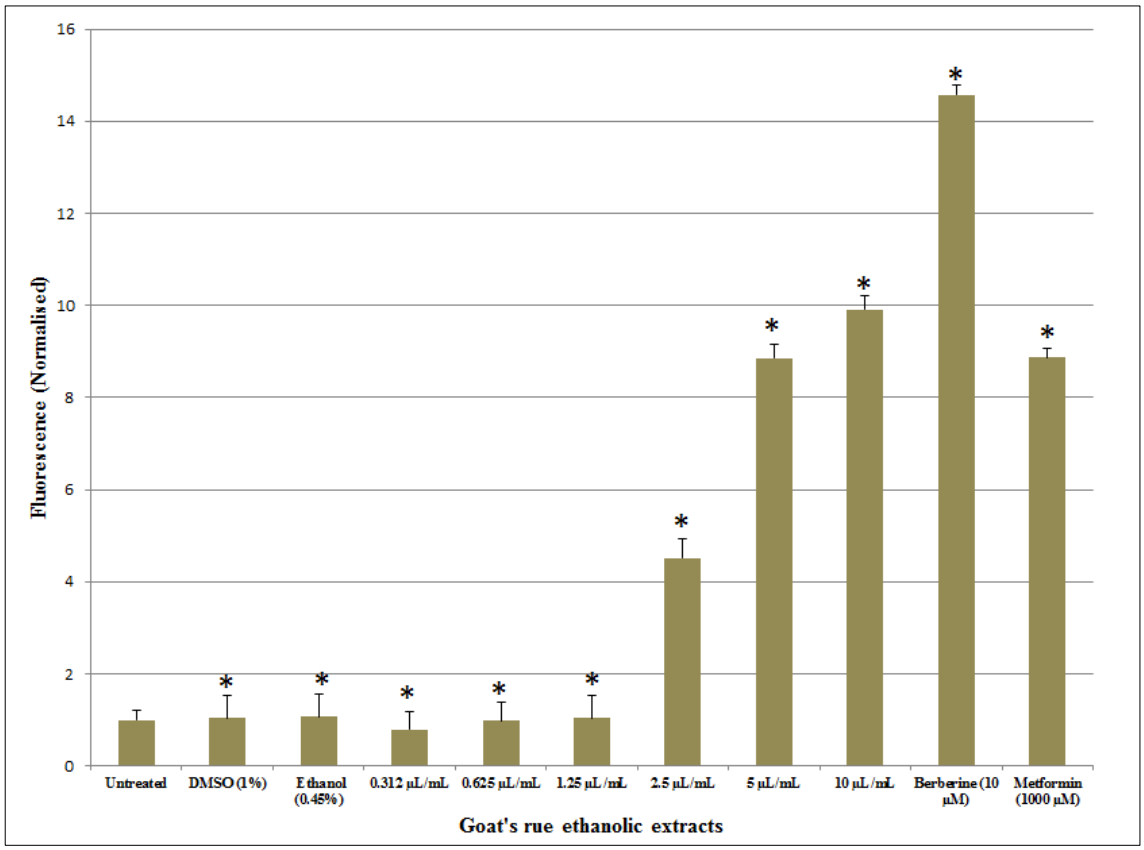


Fig 3: Effect of long-term (48 hours) treatment of goat's rue ethanolic extracts on 2-NBDG uptake by confluent HepG2 cells.

Data are changes in fluorescence normalised for protein content, and normalised to untreated cells, and are the mean +SD from five experiments with goat's rue ethanolic extracts, and the treatments in each experiment were conducted in triplicate wells. Significant difference from untreated control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, $*p<0.05$ [33].

As berberine and goldenseal (10 µL/mL) consistently demonstrated significant glucose uptake when compared to the standard drug metformin throughout the trial, these were considered to focus and investigate the cytotoxicity assays, flow cytometry-based glucose uptake assays, and GLUT-1 expression assays [33].

Cytotoxicity assays (MTT assays)

Relative live cells were initially tested to ensure that the chosen treatments had no negative effects under the conditions that would be employed in the subsequent tests, normally cells grown to 70-80% confluency before long-term treatment. Goldenseal (10 µL/mL), berberine (10 µM), and the respective solvent controls ethanol (0.6% (v/v)), and DMSO (1% (v/v)), and a positive control metformin (1000 µM), and an untreated control were assessed. The reduction of tetrazolium salts reflects relative live cells in a cell population, the yellow tetrazolium MTT was reduced by live and active cells by dehydrogenase enzymes and generate reducing compounds NADH and NADPH, and the resultant purple formazan was quantified using a plate reader [33].

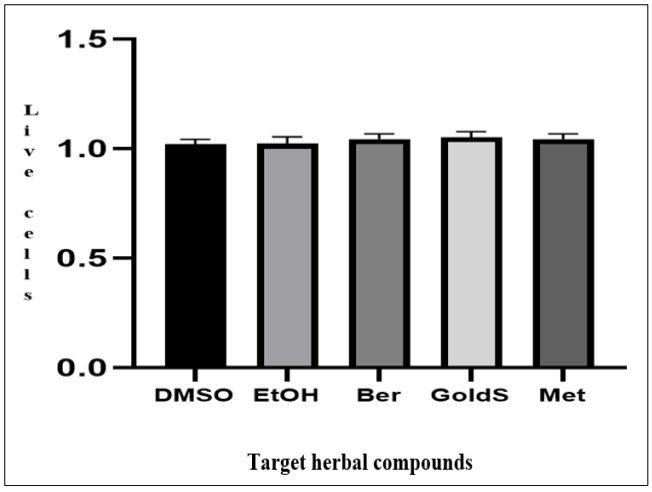


Fig 4: Effect of the target herbal compounds on cell viability after long-term treatment of the confluent HepG2 cells.

Data are changes in absorbance and are the mean +SD from three experiments, and the treatments in each experiment were conducted in triplicate wells. Significant differences between the control and target treatments were assessed by employing Graph Pad Prism 9, with a student t-test, and between the treatments and the control $p<0.05$ for Ber and Met and for Gold S $p<0.01$ [33].

As shown in the above Fig 4, in confluent HepG2 cells after 48 hours treatment, there were no signs of toxicity. Following this viability assay, it was confirmed the identified key compounds were nontoxic at the concentrations used, and the research was

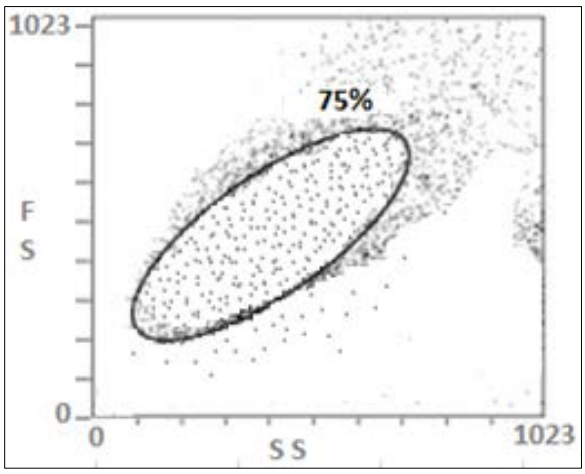
further explored by assay of glucose uptake with the selected key compounds, and by flow-cytometry to complement the glucose uptake assays performed by the plate reader method [33].

Flow cytometric detection of 2-NBDG uptake in HepG2 cells stimulated by berberine and goldenseal

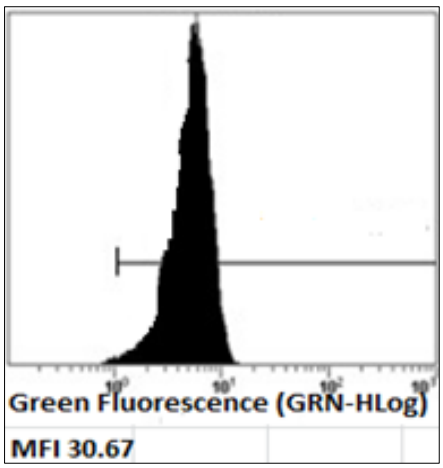
This technique allows for a direct measurement of each cell's fluorescence and allows for a quantitative comparison of cells treated with test compounds, controls, and untreated non-fluorescent negative control cells. The 2-NBDG uptake assay is limited because it is sensitive to light and is used by cells. So, it's important to limit the number of samples so that the last one can be evaluated within 30 minutes of the first one. For these investigations, confluent HepG2 cells were treated for 48 hours with goldenseal ethanolic extracts (10µL/mL), berberine (10 µM), and positive control metformin (1000 µM), solvent controls DMSO (1% (v/v)) and 0.6% (v/v) ethanol. Additionally, to allow the setup for analysis to be accurately understood, untreated non-fluorescent negative control cells were present, and a gating method was used to separate the populations of fluorescent cells [33]. An active gate was drawn based on the cell size and granularity, to avoid the heavily granulated cells which are generally dead cells, and often found at the bottom of the plot, and this gate excludes debris. Also, the auto-fluorescence of the unstained gated cells in the dot plot (Plot-1A) was plotted in the histograms (Plot-1B). The gated cells' fluorescence was plotted separately in the histograms (Plot-B), which shows the green-fluorescent profile of the gated population. On the X-axis of Plot-B, cells with green fluorescence greater than 100 were looked at, and the mean intensity of the green fluorescence was used to measure how much 2-NBDG was taken up [33].

The results of individual test compounds and their respective solvent control and an untreated negative control were obtained based on the mean fluorescence intensity of the green fluorescence values of the Plot-B. Due to the large amount of data obtained in these four experiments, a single set of data is presented the below, and to distinguish the population of fluorescent cells from the negative control cells for goldenseal and berberine only presented below to understand the results of the experiments and the similar pattern observed for the remaining samples [33].

5.1. Untreated non-fluorescent negative control cells

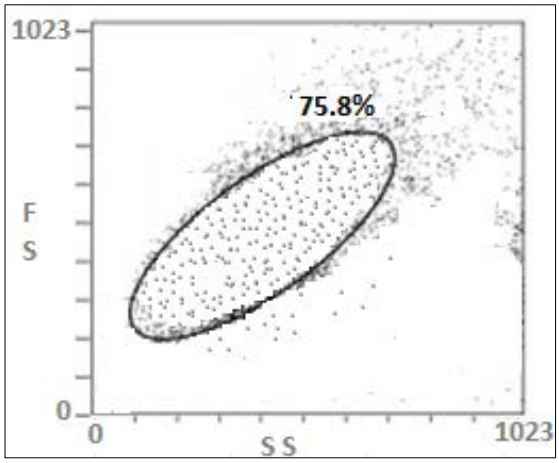


Plot-1A (Dot plot)

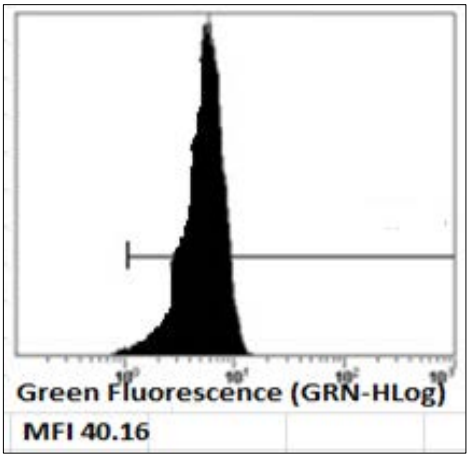


Plot-1B (Auto-fluorescent profile)

5.2. DMSO (1% (v/v)) treated cells

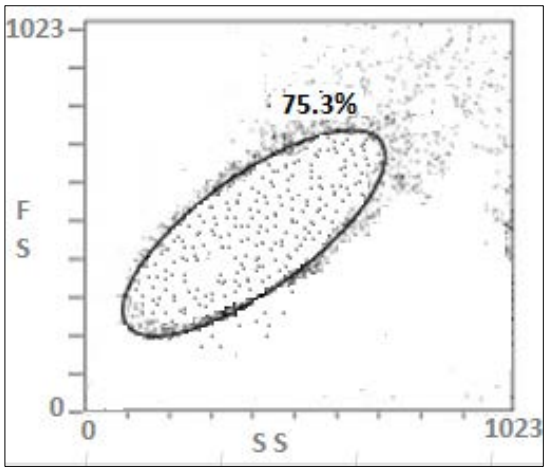


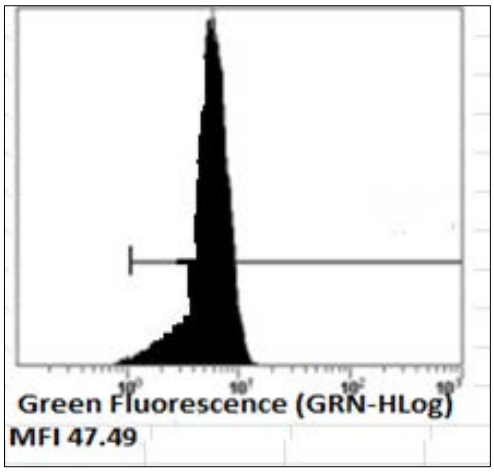
Plot-2A (Dot plot)



Plot-2B (Fluorescent profile)

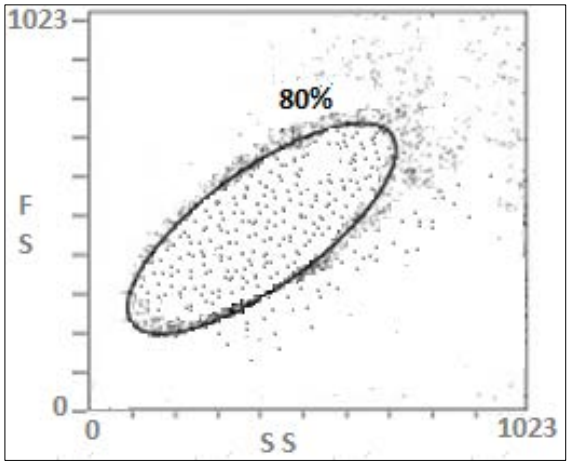
5.3. Ethanol (0.6% (v/v))

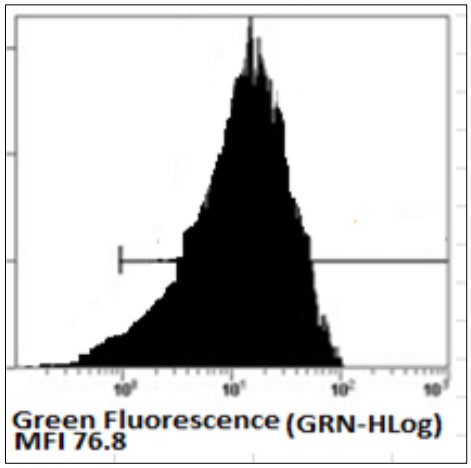




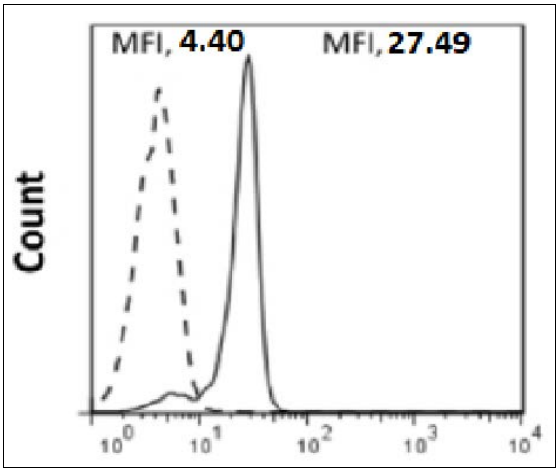
Plot-3A (Dot plot) Plot-3B (Fluorescent profile)

5.4. Goldenseal (10 µL/mL)





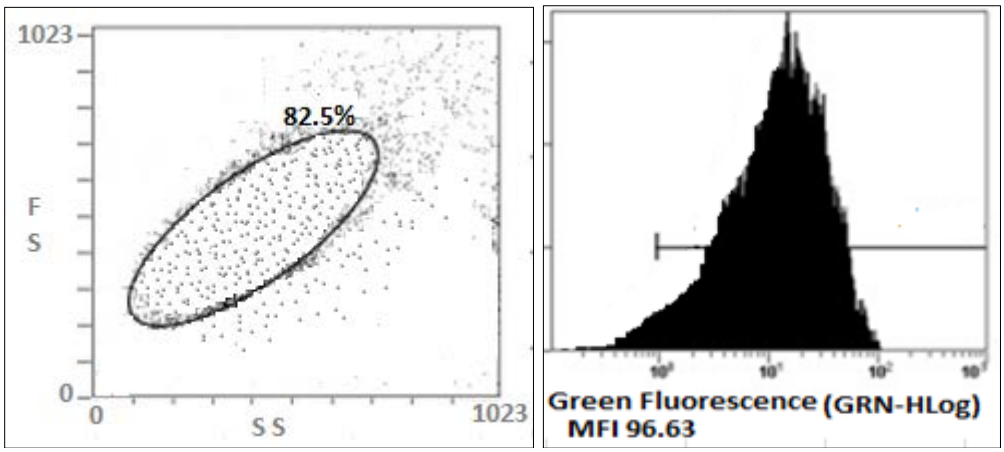
Plot-4A (Dot plot) Plot-4B (Fluorescent profile)



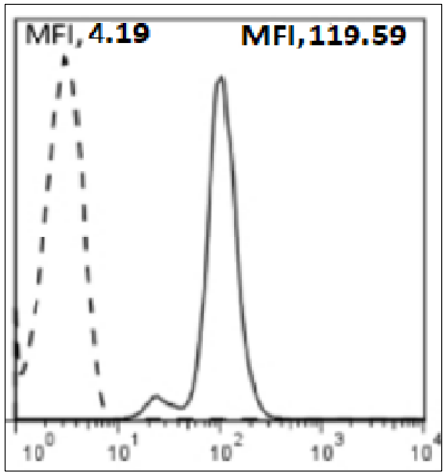
Plot-4C (Negative control and Goldenseal sample)

Plot-4C shows the negative control (- - - -), and the goldenseal with 2- NBDG treated sample (—). The negative controls MFI is 4.40, whereas the goldenseal sample's MFI is 27.49.

5.5. Berberine (10 µM)



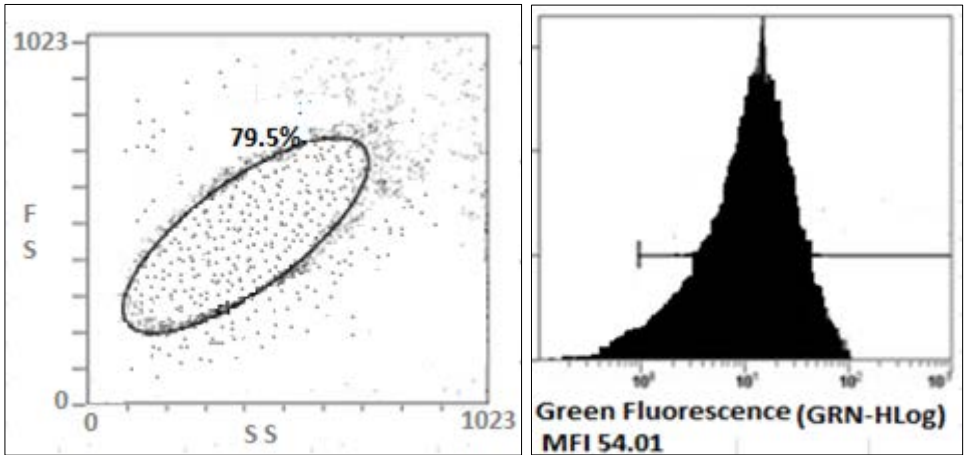
Plot-5A (Dot plot) Plot-5B (Fluorescent profile)



Plot-5C (Negative control and Berberine sample)

Plot-5C shows the negative control (- - - -), and the berberine with 2-NBDG treated sample (—). The negative control's MFI is 4.19, whereas the berberine sample's MFI is 119.59 [33].

5.6. Metformin (1000 µM)



Plot-6A (Dot plot) Plot-6B (Fluorescent profile)

Fig 5: Flow cytometric analysis of 2-NBDG uptake by HepG2 cells.

Plots A show dot plots of forward vs side-scatter. The gated cell population chosen for analysis of 2-NBDG uptake, as assessed by green fluorescence, is shown by the oval in each plot. The Plots B display the green-fluorescent profile of the gated population of the corresponding Plot-A. The cells that have green fluorescence greater than 10^0 on the X-axis of the Plot B, and the mean fluorescence intensity of the green fluorescence was the criteria to quantify 2-NBDG uptake [33].

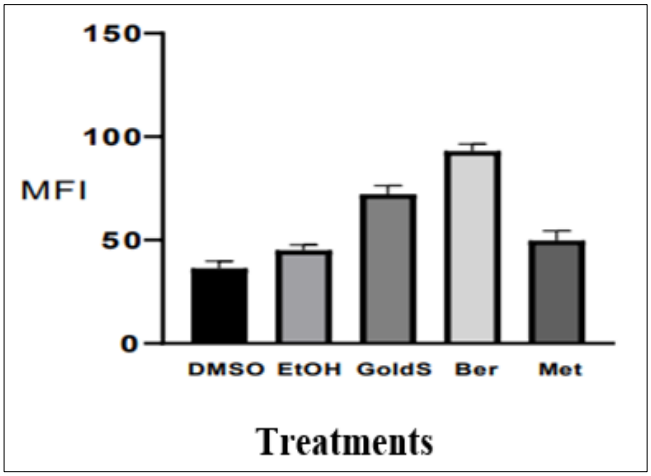


Fig 6: Uptake of 2-NBDG by HepG2 cells assessed by flow cytometry.

The treatments in each experiment were carried out in triplicate wells, and the data represent changes in the mean fluorescence intensity of the green fluorescence and are the mean + SD from four trials. Graph Pad Prism 9, a student t-test was used to determine whether there was a significant difference between the target treatments and the solvent control (DMSO), and $p<0.001$ in all groups [33].

Table 1: A single set of the data of the mean values of the green fluorescence in plots B of a negative control, DMSO (1%), and ethanol (0.6%), and goldenseal (10 µL/mL), berberine (10 µM), and metformin (1000 µM).

MFI (Mean fluorescence intensity of the Green fluorescence)						
	Negative Control	**Dmso**	**EtOh**	**GoIS**	**Ber**	**Met**
	30.67	40.16	47.49	76.8	96.63	54.01
	33.29	40.5	42.33	77.96	95.01	55.05
	30.29	41	45.4	77.7	96.5	54.97
Average	31.41	40.55	45.07	76.82	96.04	54.67
S.D	1.63	0.42	2.59	0.87	0.9	0.57

The outcome of the experiments shows that berberine stimulates 2.5 times more glucose uptake ($p<0.001$), than the solvent control (DMSO), and goldenseal is the second most active compound with 1.98 times more uptake ($p<0.001$), and metformin stimulates 1.37 times uptake ($p<0.001$), compared to the DMSO. Following the glucose uptake experiments, the research has explored the effect of goldenseal and berberine on stimulation of GLUT-1 in the long-term treated HepG2 cells. These data confirm qualitatively the stimulation of 2-NDBG uptake by berberine, goldenseal and metformin measured using a plate reader (Fig 2) [33].

Effect of goldenseal extract and berberine on expression of GLUT-1 in HepG2 cells

To further explore the mechanisms, an analysis of the expression of the GLUT-1 was carried out by western blotting. Recently the practice of using housekeeping controls, such as β-actin, to normalise expression data from western blots has come under criticism, and instead total staining of blotted proteins is recommended practice by some journals, including the Journal of Biological Chemistry [12, 33]. Accordingly, equivalent amounts of protein per sample were loaded, according to Bradford assay of sample lysates, and after blotting, all blotted proteins were transiently stained and recorded as described in methods. Confluent HepG2 cells were treated with goldenseal extract (10 µL/mL), and berberine (10µM), and the respective solvent controls ethanol (0.6% (v/v)), and DMSO (1% (v/v)) for 48 hours. GLUT-1 protein was detected by using a rabbit primary anti-GLUT-1 antibody, and a goat anti-rabbit IgG labelled with horse-radish peroxidase secondary antibody. GLUT-1 was identified as a band of about 55 kDa, in accordance with published values [13].

Treatments of HepG2 cells with goldenseal (10 µL/mL), and berberine (10 µM) induced a noticeable increase in expression of GLUT-1 compared with the solvent controls' ethanol (0.6% (v/v)) and DMSO (1% (v/v)) [33].

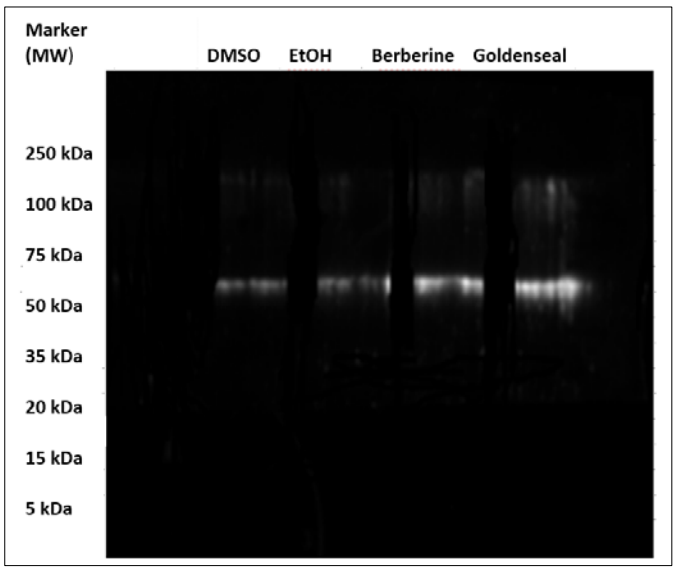


Fig 7: GLUT-1 expression in treated HepG2 cell lysates.

A representative western blot showing changes in expression of GLUT-1 in HepG2 lysates induced by goldenseal (10µL/mL), and berberine (10 µM), compared with control treatments ethanol (0.6% (v/v)), and DMSO (1% (v/v)) [33].

The band intensities on gels were quantified using Image J software to compare the expression of GLUT-1 in each sample. A rectangular region was chosen around each band, and the

intensity of each sample was quantified. Intensities were normalised to intensities of protein blots of the same sample. The data in the Fig 7 suggest that extracts from goldenseal (10 µL/mL), and berberine (10 µM), could stimulate an increased expression of GLUT-1 protein in HepG2 cells [33].

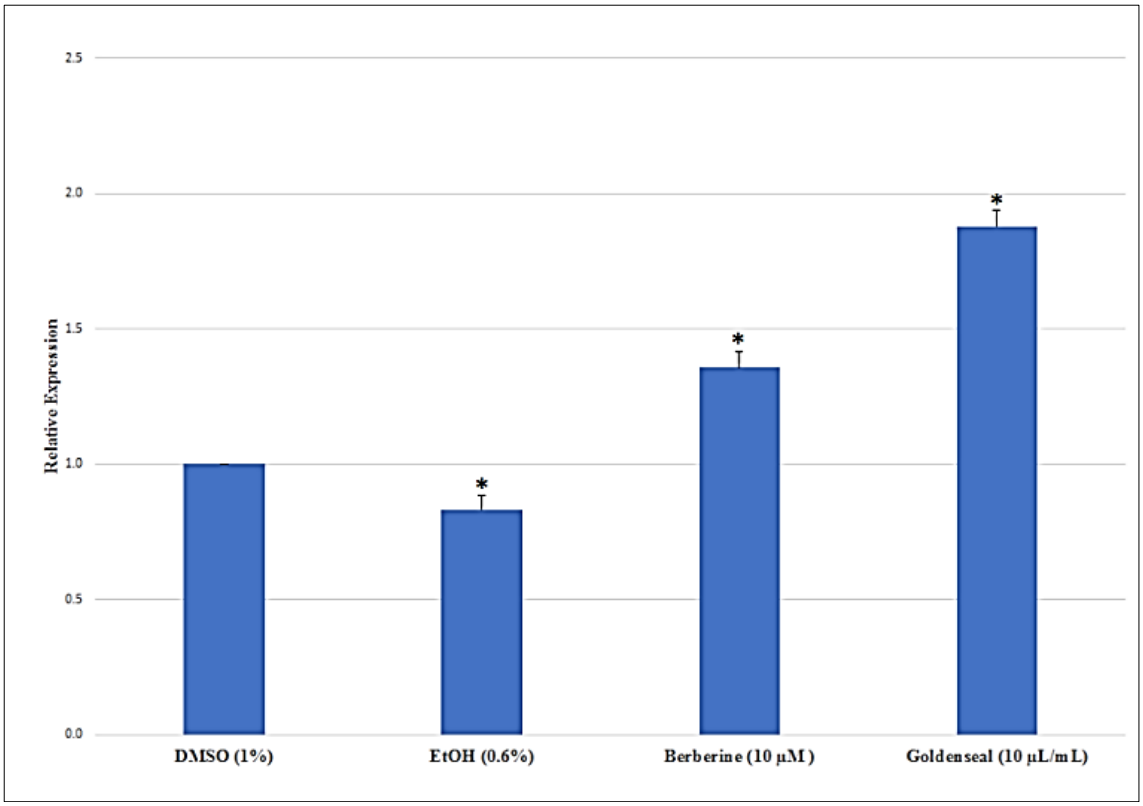


Fig 8: Quantification of the expression of GLUT-1 protein detected by western blotting in HepG2 cells.

A plot of the average relative intensities of the GLUT-1 bands from the image of four blots, quantified using ImageJ. Data are changes in the intensity normalised to the DMSO control and are the mean +SD from four experiments. Significant difference from the control to target treatments were assessed by employing one-way ANOVA with Bonferroni's multiple comparison post hoc test, $*p<0.05$ [33].

As shown in the Fig 8, goldenseal stimulates the expression of GLUT-1, 1.7 times ($p<0.05$), and berberine stimulates 1.4 times ($p<0.05$), compared to the control. These data provide an explanation for the enhanced uptake of 2-NBDG induced by goldenseal and berberine [33].

Discussion and Conclusion

The goal of the study was to evaluate and contrast the effects of pure compounds and phyto extracts on the glucose uptake in HepG2 cells with that of the drug metformin, which belongs to the most used biguanide class today. This study focussed on the basal glucose uptake through GLUT-1, which facilitates basal glucose uptake due to its lower Km value (3-7 mM) [14]. Generally, a lower Km value indicates a high affinity to glucose uptake. In contrast, GLUT-2 has a higher Km value (17 mM), which leads a lower affinity to glucose uptake. The higher Km value enables glucose sensing, and the rate of glucose uptake is proportional to blood glucose levels [14]. The major glucose transporter in HepG2 cells is GLUT-1 [15], reflecting the original cloning of GLUT-1 as the first glucose transporter to be cloned, from HepG2 cells [13, 33].

In a study, berberine and metformin were found to be the most active phytochemicals in augmenting glucose uptake, these experiments were carried out by Kandunuri K and White K [29] before the publication of Xu *et al*, 2014 [16], which supported these findings HepG2 cells treated with berberine or metformin, at the concentrations used in the present study, 10 µM and 1.0 mM respectively, showed enhanced uptake of glucose. The focus of the study of Xu *et al*, 2014, was on the biochemical pathway involved and the effects were found to be independent of the key metabolic regulatory pathway involving AMP-activated protein kinase (AMPK) [16, 33].

It was of interest to assess the activity of an herbal extract with a well-established history of diabetes treatment, and so an ethanolic extract of goat's rue (*Galega officianalis* L.) was tested for its effect on the glucose uptake. In confluent cells, a 48-hour application of goat's rue ethanolic extracts at the top concentration (10 µL/mL) augmented glucose uptake 10 times, compared with nine times by metformin and 14 times by berberine, all compared with the untreated cells ($p<0.05$) (Fig 3). Goat's rue has been used in traditional medicine to treat diabetes since the early 1900's [17, 18, 33]. The active constituent in Goat's rue extracts is known to be Galegine, a bisguanidine, and it was originally sold under the trade name synthalin. 2, 3, 4, 5. It was galegine from which the most sought after and cost-effective drug for the treatment of T2D, metformin was derived [18, 33]. Galegine, promotes glucose lowering by lowering the surplus basal rates of hepatic gluconeogenesis [19-22, 33]. Also, it is likely, that galegine hinders the glucose 6-phosphate produced from glycogen breakdown to be transformed to glucose through an inhibition of glucose 6-phosphatase and phosphoenolpyruvate carboxykinase, as seen metformin as studied [20-24, 33]. According to a thorough search of reliable academic literature search engines like PubMed and Medline, no *in vitro* studies have been done on the glucose uptake properties of goat's rue using HepG2 cells and the glucose analogue 2NBDG. This is likely the first study done so far [33].

In confluent cells, an hour application with goldenseal glycerites and ethanolic extracts stimulated glucose uptake at

the concentration 10 µL/mL, by 6.5 times and 5.4 times correspondingly, compared with untreated control ($p<0.05$) (Fig 1). Ethanolic extracts, at the highest concentration stimulated glucose uptake 13.5 times, metformin 9 times, berberine 14.2 times more glucose uptake, compared to the untreated control ($p<0.05$) (Fig 2). There are no previous *in vitro* reports available on the glucose uptake property of the goldenseal using GLUT-1 in HepG2 cells, perhaps this is the first report generated so far; therefore, it could be the novelty of this study [33].

In the above the experiments, goldenseal and berberine were showed the marked effects on the glucose uptake in HepG2 cells compared to metformin. Therefore, goldenseal ethanolic extracts (10µL/mL), and berberine (10µM) were identified as the key elements and their properties were explored further with cytotoxicity assays, flow cytometry-based glucose uptake assays, western blotting GLUT-1 analysis [33].

The MTT assay was performed in confluent cells, after long-term application, the cells were well tolerated with the applied concentrations, but the cells treated with goldenseal (10 µL) were displayed 20% increase in the proliferation, in this case the treated cells could be more metabolic active and trying to deal with the multiple compounds in the extract, or the wholesome effect of the multiple compounds could be potentiating the cells. A relative low concentration of berberine (10 µM) didn't affect the cell viability, and the positive control metformin had no toxic effects on cell viability. Therefore, it was evident that, the chosen compounds with respective concentrations were safe to HepG2 cells (Fig 4) [33].

Flow cytometry-based glucose uptake assays were used to supplement the plate reader-based tests. Berberine came out on top in these experiments, beating out goldenseal and metformin. It stimulates 2.5 times more glucose uptake than goldenseal, which stimulates 1.98 times more uptake, and metformin stimulates 1.37 times more uptake than DMSO (Fig 5&6). Therefore, it is evident that the pure compound berberine stimulates more uptake than its founder crude extracts of goldenseal. These data confirm qualitatively the stimulation of 2-NDBG uptake by berberine, goldenseal and metformin measured using the plate reader [33].

Likely the other components in the crude extract that reduced the effect of alkaloids berberine and hydrastine, or the pure compound generates a more intensified action than the crude extracts, for instance, in HepG2 hepatocytes and C2C12 myotubes, berberine stimulates not only GLUT-1, but also GLUT-4 and retinol-binding protein-4 in favour of glucose uptake, activates glycolysis by AMPK pathway, and also influences the peroxisome proliferator-activated receptor γ molecular targets of the phosphorylation of insulin receptor substrate-1, and eventually reduces insulin resistance [16]. Perhaps the crude extracts with the right dose could be beneficial in other ways also, such as the nutrients, alkaloids and flavonoids in the extracts could act as buffering agents and antioxidants protect from the free-radicals and autophagy, thereby promoting cell viability [25, 33]. Also, goldenseal seems to have antibacterial activity compared with berberine, probably because of the efflux pump inhibitory activity of bacteria [26, 33].

Insulin-mediated glucose uptake and non-insulin-mediated glucose uptake are the two primary mechanisms by which glucose is absorbed by living cells. Only insulin-sensitive tissues, such as skeletal muscle, adipose tissue, and liver tissue, can absorb glucose via an insulin-mediated process. However, even though both insulin-sensitive and non-insulin-sensitive tissues can absorb glucose without being insulin-mediated, the non-insulin mediated glucose uptake pathway accounted for most of the glucose absorption in insulin resistance situations. Furthermore, while T2D is distinguished by a significant impairment in insulin-stimulated glucose uptake, the status of non-insulin-mediated glucose uptake mechanisms in T2D is unknown. This study directly measured non-insulin-mediated glucose uptake in HepG2 cells using the glucose analogue 2-NBDG and the targeted herbal compounds. Dialysed foetal calf serum was used in the cell culture media to ensure it was insulin-free. In addition, there have been few studies on the acute effects of the targeted herbal components on the function of the insulin-insensitive GLUT-1. Because of this, we investigated the acute effects that the targeted herbal substances have on the non-insulin-mediated glucose uptake in HepG2 cells, which express the ubiquitous GLUT-1 [33].

In the GLUT-1 expression assays, goldenseal and berberine stimulated expressions 1.7 times and 1.4 times respectively in comparison to the control. The alkaloids in the goldenseal extracts, specifically berberine and hydrastine [27, 19, 33], could contribute to a slight increase in GLUT-1 expression relative to pure berberine (Fig 7 & 8) [33]. According to the European Pharmacopoeia, it is mandatory to market goldenseal as a food supplement only if its alkaloid concentrations are at least 3% berberine and 2.5% hydrastine [28]. Consequently, the goldenseal extract utilised in this research contained a minimum of 3% berberine (890 µM), with a final berberine concentration of 8.9 µM. It suggests that the combined presence of berberine and hydrastine in goldenseal extract may enhance GLUT-1 expression more effectively than pure berberine alone [33].

In summary, berberine and the extract of goldenseal exhibit similar activities in improving glucose uptake in HepG2 cells and demonstrate greater efficacy than metformin. It is evident that berberine shows better efficacy than the extract of goldenseal in enhancing glucose uptake. Therefore, goldenseal extracts and berberine are promising candidates for developing new oral anti-diabetic agents for T2D treatment.

Conflict of Interest Statement: We hereby declare that there are no conflicts of interest to disclose.

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