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Original Article

STUDY OF *IN VITRO* GLUCOSE UPTAKE ACTIVITY OF ISOLATED COMPOUNDS FROM HYDRO ALCOHOLIC LEAF EXTRACT OF CARDIOSPERMUM HALICACABUM LINN

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ABSTRACT

Objective: Herbal medicines have long been used effectively in the treatment of Diabetes Mellitus. *Cardiospermum halicacabum*(*C. halicacabum*) has been used for several centuries for the treatment of various ailments. The present work was undertaken to study the effect of isolated bioactive compounds from leaf of *C. halicacabum* on glucose uptake in L-6 cell lines.

Methods: The hydro alcoholic leaf extract of *C. halicacabum* was subjected to preliminary phytochemical screening and isolated eleven compounds were tested for cytotoxicity by MTT assay. Compound 1 and 8 were selected for further glucose uptake assay based on cytotoxicity concentration CTC_{50} value. Structure of compound 1 and 8 was identified by NMR technique.

Results: Phytochemical analysis showed the presence of alkaloids, flavonoids, tannins and terpenoids. Compound 1 showed moderate cytotoxic activity to L6 cell line when compared to compound 8. Compounds 1 and 8 were identified as Kaempferol-3-o-α-l-rhamnoside and Apigenin-7-o-β-d-glucuronide. In *in vitro* glucose uptake assay Kaempferol-3-o-α-l-rhamnoside showed increase in glucose uptake by 11.38 ± 2.04% and Apigenin-7-o-β-d-glucuronide by 15.97 ± 2.16% over control.

Conclusion: Kaempferol-3-o-α-l-rhamnoside and Apigenin-7-o-β-d-glucuronide isolated from *C. halicacabum* leaf extract enhance the glucose uptake significantly. They exhibit hypoglycemic activity and stimulate glucose uptake in L-6 skeletal muscle cells. This study can bring a promising role for this plant in the management of Diabetes mellitus.

Keywords: Apigenin-7-o-β-d-glucuronide, *Cardiospermum halicacabum*, Diabetes Mellitus, Glucose uptake Assay, Kaempferol-3-o-α-l-rhamnoside, MTT Assay, L-6 skeletal muscle cell.

INTRODUCTION

Diabetes mellitus is a group of metabolic diseases in which a person has high blood sugar, either because the pancreas does not produce enough insulin, or because cells do not respond to the insulin that is produced [1]. Chronic hyperglycemia of diabetes is associated with long term damage, dysfunction and eventually failure of organs, especially the kidney, nerves, heart, eyes and blood vessels [2]. India has the highest number of diabetics of any one country in the entire world. Over 30 million have now been diagnosed with diabetes in India. The Crude prevalence rate in the urban areas of India is thought to be 9 per cent. In rural areas, the prevalence is approximately 3 per cent of the total population. There is more number of drugs used in the treatment of diabetes. They have many side effects with their long term usage [3,4]. Herbal drugs may prefer as alternatives because of their safety and efficacy. In India herbal drugs have long been used in the treatment of diabetes. Many plants have been tested on experimental animals and reported useful for the treatment of diabetes mellitus [5]. Drugs isolated from medicinal plants showed effective antidiabetic activity than oral hypoglyceimic drugs [6]. The active compounds of medicinal plants play an important role in the management of the disease.

Skeletal muscle is a major tissue for blood glucose utilization and a primary target tissue for insulin action and it is responsible for the whole body glucose homeostasis. Postprandial hyperglycemia plays an important role in the etiology of diabetes-related complications and the therapeutic approach for the treatment of diabetes is mainly focused on decreasing postprandial hyperglycemia. Insulin increases glucose uptake in skeletal muscle by increasing functional glucose transport molecules in the plasma membrane [7].

Cardiospermum halicacabum (*C. halicacabum*) is an herbaceous climber distributed in tropical and subtropical area of Asia. It is commonly known as balloon wine belong to family sapindaceae. In India *C. halicacabum* has been used for several centuries in the treatment of rheumatism, stiffness of limbs and snake bite. Roots alone are used for the treatment of nervous diseases. It has analgesic, vasodepressive, anti hyperglycaemic, and also anxiolytic properties [8-10] and its antiparasitic, antimalarial and antifilarial [11-13] activities have been proved. Although the hypoglycaemic effect of *C. halicacabum* has been reported, still there is no report of isolated compounds from *C. halicacabum*on for the same and the exact mechanism also has yet to be elucidated. Hence we under took this study to evaluate the effect of isolated compounds from *C. halicacabum* leaf extract on glucose uptake through glucose transports in skeletal muscle cell (L-6 cell line).

MATERIALS AND METHODS

Extraction of plant material

Fresh leaves of *C. halicacabum* were collected from the tropical areas of MallurTaluk, Salem district, Tamil Nadu, India. The leaves were thoroughly washed in water, dried at room temperature, and powdered. The dry fine powder was suspended in different solvents of increasing polarity for cold maceration for 4 days. Petroleum ether, chloroform, acetone and Hydroalcoholic solvent (80% methanol + 20% distilled water). The extracts were filtered by using a muslin cloth and concentrated at 40 ± 5°C.

The sole purpose of such maceration procedures for crude drugs is to obtain the therapeutically desirable portion and eliminate the inert material by treatment with a selective solvent.

Chemicals

3-(4,5–dimethyl thiazol–2–yl)–5–diphenyltetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and

antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from E. Merck Ltd., Mumbai, India.

Qualitative phytochemical evaluation

All the leaf-extracts of *C. halicacabum* were subjected to phytochemical tests for its active constituents according to the Harborne method [14]. Hydro alcoholic extract of *C. halicacabum* leaf showed maximum number of phytochemicals in phytochemical screening assay. So further isolation of active principles was carried out with the same extract.

Bioactive compounds from *C. halicacabum* Leaf

The hydro alcoholic leaf extract of *C. halicacabum* then subjected to column chromatography to isolate the active compound using hexane as a solvent. Preparative Thin Layer chromatography [15-19] was performed in order to select the mobile phase. Different solvent systems were tried as mobile phase, and finally tried with chloroform: methanol (9:1). Six major fractions (A1-F1) were collected. The TLC plates were used to observe the separation of individual compounds as a single spot. Totally eleven compounds were obtained.

Cell Lines and Culture Medium

L-6 cell cultures were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in Dulbecco's modified Eagle's medium (DMEM). Medium was supplemented with 10% inactivated Fetal Bovine Serum (FBS). Penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of Test Solutions

For cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serially two fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of Cell Viability by MTT Assays

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 105 cells/ml using medium containing 10% FBS and was used for the determination of cell viability by MTT assays as described by Francis and Rita [20] respectively. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC50) values is generated from the dose-response curves for each cell line.

% Growth inhibition =

$$100 - \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

Based on CTC_{50} Value Compound 1 and 8 were selected for structural elucidation. Compound 1 was identified as Kaempferol-3-o-α-l-rhamnoside and compound 8 as Apigenin-7-o-β-d-glucuronide. Three independent experimental values in duplicates were taken to determine CTC50 value.

***In vitro* Glucose Uptake Activity [21] of Kaempferol-3-o-α-l-rhamnoside and Apigenin-7-o-β-d-glucuronide**

Cells were cultured on 6 well plates and incubated for 48 hrs at 37°C in CO_2 incubator. When semi confluent monolayer was formed the culture were renewed with serum free DMEM containing 0.2 % BSA and incubated for 18 hrs at 37°C in CO_2 incubator. After 18 hrs the media was discarded and cells were washed with KRP buffer once. The cells are treated with Insulin. Standard drug and plant extract and added glucose (1M) and incubated for half an hour. The supernatant was collected for glucose estimation and glucose uptake was terminated by washing the cells three times with 1 ml ice-cold KRP buffer. Cells were subsequently lysed by freezing and thawing three times. Cell lysate was collected for glucose estimation. Glucose uptake was calculated as the difference between the initial and final glucose content in the incubated medium by GOD-POD method [22]. Three independent experimental values in duplicates were taken to determine the percentage enhancement of glucose uptake over controls.

CALCULATION

Glucose concentration in the sample can be calculated using the following formula: Glucose = Absorbance of Sample/ Absorbance of Standard X Conc. of Std.

RESULTS

Table 1 shows the qualitative phytochemical analysis of petroleum ether, chloroform, acetone, ethanol and hydroalcoholic extract. Hydro alcoholic leaf extract of *C. halicacabum* showed the presence majority of bioactive compounds including alkaloids, tannins, flavonoids and terpenoids.

Based on CTC_{50} value Compound 1 and 8 were selected for further *in vitro* glucose uptake assay. Figure 1 and 2 shows the structure of compounds, Kaempferol-3-o-α-l-rhamnoside (compound 1) and Apigenin-7-o-β-d-glucuronide (compound 8).

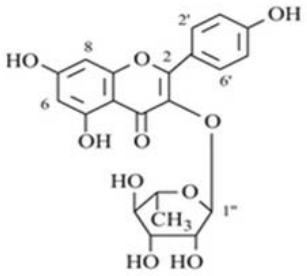


Fig. 1: Compound-1 Kaempferol-3-o-α-1-Rhamnoside

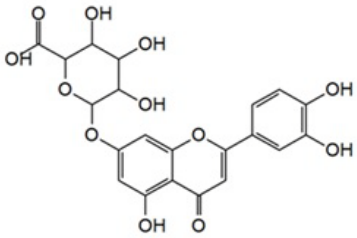
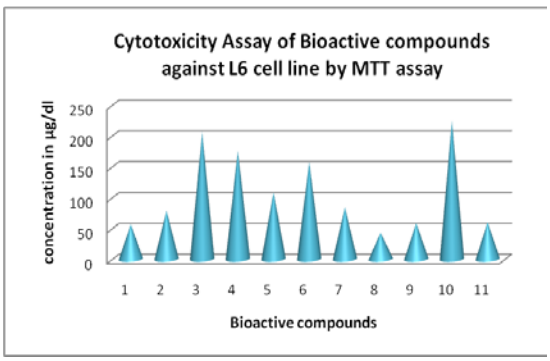
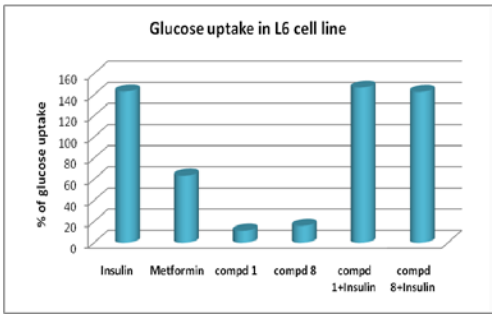


Fig. 2: Compound-8 Apigenin-7—o-β-d-glucuronide



Graph 1: Graphical representation of CTC_{50} value of isolated compounds

Graph 2 and Table 3 shows the *in vitro* glucose uptake assay. The results were compared with insulin and metformin, which were used as the standard antidiabetic drugs. Kaempferol-3-o-α-l-rhamnoside and Apigenin-7-o-β-d-glucuronide were also tested with insulin.



Graph 2: Graphical representation of *in vitro* glucose uptake assay

DISCUSSION

Insulin is the major regulator of blood glucose levels in the fed state when skeletal muscle becomes the primary consumer of glucose [23]. The determinant of glucose utilization by muscle is its uptake mediated by glucose transporters [24]. Insulin stimulates glucose uptake in skeletal muscle cells and fat cells by promoting the rapid translocation of GLUT4 glucose transporters to the plasma membrane. It regulates the release of GLUT4 from sequestered intracellular storage pools, and also has effects on docking and fusion of GLUT4 vesicles with plasma membrane [25]. Regulation of GLUT4 activity by insulin will enhance the muscle cell glucose uptake. Medicinal plants enhance the glucose uptake by GLUT4 translocation and were proven by *in vitro* glucose uptake model [26-29]. The L-6 cell line is the best characterized cellular model origin to study glucose uptake and GLUT4 translocation [30,31]. Hence in this study we have used L-6 muscle cells to determine the glucose uptake activity of *C. halicacabum* leaf extract. Eleven compounds were isolated from the hydro alcoholic leaf extract of *C. halicacabum*.

The isolated compounds were evaluated for its cytotoxic activity by MTT assay and the results were shown in Table 2. The ability of the cells to survive a toxic insult has been the basis of most assays. The assay depends both on the number of cells present and on the mitochondrial activity per cell. As the compound 1 and 8 showed minimum values, it was selected for antidiabetic work.

From the NMR spectrum the compounds were identified as Kaempferol-3-o-α-l-rhamnoside (compound 1) and Apigenin-7-o-β-d-glucuronide (compound 8). The *in vitro* glucose uptake assay of the compounds showed the enhancement in glucose uptake by 11.38 ± 2.04% and 15.97 ± 2.16% over control. Insulin (1IU/ml) and metformin (100 µg/ml) enhance the glucose uptake by 143.07±2.17 % and 63.45±2.69% over control. Kaempferol-3-o-α-l-rhamnoside+ insulin enhance the glucose uptake in L-6 cells by 146.83 ± 1.30% and Apigenin-7-o-β-d-glucuronide+insulin enhance by 142.70 ± 1.86% over control when used in combination. In the above results compound 8 was found to be prominent over compound 1. Skeletal muscle is the most abundant tissue in the whole body, and thus, proper function of skeletal tissue is important to maintain normal blood glucose level. Defects in insulin stimulated skeletal muscle glucose uptake are common pathological states in non-insulin-dependent diabetes mellitus [32]. Present study showed that the isolated bioactive compounds enhance the glucose uptake which may be due to its effect on the receptors on the cell membrane in L-6cell lines.

Table 1: Phytochemical analysis of different extracts of *C. halicacabum* leaf

Phytochemicals	Petroleum Ether	Chloroform	Acetone	Ethanol	Hydro alcohol (80% methanol + 20% distilled water)
Alkaloids	-	-	+	+	+
Phytosterols	+	+	+	+	+
Carbohydrates	-	-	+	+	+
Phenols	-	+	+	+	+
Protein	-	-	+	+	+
Diterpenoids	-	-	+	+	+
Glycosides	-	-	-	-	+
Cardiac glycosides	-	-	-	+	+
Saponins	-	-	+	+	+
Free amino acids	-	-	-	-	+

In bioassay guided isolation eleven compounds were isolated and the cytotoxicity of all the compounds was evaluated by MTT assay (graph 1). Table 2 shows the CTC_{50} values of all the eleven compounds in L-6 cell lines. Compound 8 showed higher cytotoxicity against L6 cell line, whereas compound 1 showed moderate cytotoxic activity to L6 cell line when compared to compound 8.

Table 2: The result of *in vitro* cytotoxic concentration of *C. halicacabum* leaf

Compound	Concentration in µg/ml	% cytotoxicity (µg/ml)	CTC_{50} (µg/ml)
Compound 1	500	90.14±8.02	56.4±4.98µg/ml
	250	72.58±6.05	
	125	68.73±5.28	
	62.5	51.49±5.12	
Compound 2			78.6±6.24 µg/ml
	500	93.07±7.06	
	250	84.19±6.04	
	125	67.09±5.22	
	62.5	43.6±3.31	
Compound 3			206±19.35 µg/ml
	500	84.19±7.15	
	250	63.08±6.01	
	125	32.72±2.11	
	62.5	5.49±4.30	
Compound 4			176±15.58 µg/ml
	500	84.03±7.22	

	250	63.97±5.01	
	125	21.42±2.36	
	62.5	8.12±8.04	
Compound 5			108.4±9.12 µg/ml
	500	92.13±8.89	
	250	74.05±7.21	
	125	52.16±4.74	
	62.5	32.56±3.00	
Compound 6			157.5±14.02 µg/ml
	500	90.16±8.12	
	250	70.48±6.12	
	125	43.95±3.03	
	62.5	24.17±2.02	
Compound 7			83.9±6.98 µg/ml
	500	85.47±7.01	
	250	63.18±6.03	
	125	58.29±4.03	
	62.5	43.15±4.24	
Compound 8			43±3.33 µg/ml
	500	89.06±7.98	
	250	74.18±6.01	
	125	64.38±5.33	
	62.5	57.90±4.11	
Compound 9			58.6±5.54 µg/ml
	500	91.42±8.16	
	250	88.19±7.99	
	125	76.26±6.03	
	62.5	52.14±4.85	
Compound 10			224.1±20.58 µg/ml
	500	69.87±6.08	
	250	55.49±5.96	
	125	43.92±3.67	
	62.5	12.40±9.36	
Compound 11			59.8±5.68 µg/ml
	500	87.43±7.21	
	250	79.93±7.03	
	125	61.84±6.16	
	62.5	52.47±5.22	

Table 3: The results of *In vitro* glucose uptake activity of hydro alcoholic extract of *C. halicacabum* leaf

S. No.	Incubation medium	Test Conc.	% Glucose uptake over control
1	Insulin	1 IU/ ml	143.07 ± 2.17
2	Metformin	100 µg/ml	63.45 ± 2.69
3	Comp 1	56.4 µg/ml	11.38 ± 2.04
4	Comp 8	43 µg/ml	15.97 ± 2.16
5	Comp 1+ Insulin	56.4 µg/ml + 1 IU/ ml	146.83 ± 1.30
6	Comp 8+ Insulin	43 µg/ml + 1 IU/ ml	142.70 ± 1.86

CONCLUSION

The results obtained in the present study clearly demonstrate that the isolated compounds Kaempferol-3-o-α-l-rhamnoside and Apigenin-7-o-β-d-glucuronidefrom hydro alcoholic extract of *C. halicacabum* enhances glucose uptake under *in vitro* conditions. This may be due to its effect on the number of receptors located in the skeletal muscle cell line. Since herbal drugs are used for the treatment of diabetes for a long time because of its high potency and negligible side effects, this study will definitely help in the field of herbal medicine. Further *in vivo* anti diabetic effects of compound 1 and 8 is in progress which will support the present study.

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CONFLICT OF INTERESTS

Declared None

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