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Original article

Flavonoids from *Enicostema littorale* blume enhances glucose uptake of cells in insulin resistant human liver cancer (HepG2) cell line via IRS-1 /PI3K/Akt pathway

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ABSTRACT

Diabetes mellitus has spread over the world with 347 million people affected. Insulin resistance is a main pathogenic event in Type 2 Diabetes Mellitus (T2DM) leading to a reduction in glucose uptake by peripheral tissue and increased hepatic glucose output. In this study, we have isolated four flavonoid rich fractions fraction A (FA), fraction B (FB), fraction C (FC) and fraction D (FD) from *Enicostema littorale*. All the fractions were preliminary screened for TLC fingerprinting, total flavonoid content. Total eight flavonoids were identified by LC/MS. Insulin resistant HepG2 (IR/HepG2) model was established by inducing insulin resistance in HepG2 cells to investigate the effect of these fractions on IR/HepG2 cell line for their glucose uptake. The results showed the significant dose dependant increase in glucose uptake of cells treated with FD. It showed significant activity at a concentration of 10 μg/ml. The LC/MS results of FD demonstrated the presence of C-glycoside Swertisin which could be responsible for the effect. Further, to investigate the mechanism of action, gene expression for insulin receptor substrate 1 (IRS-1), protein kinase B (Akt-2) and glucose transporter 4 (GLUT-4) genes were evaluated by real time PCR. A significant upregulation of these genes was observed in FD treated samples, thereby indicating the enhancement of glucose uptake rate of cells via IRS-1/PI3K/Akt pathway.

1. Introduction

Diabetes mellitus is a chronic metabolic disorder in which body either produces small amount of insulin or ceases to produce insulin or becomes progressively resistant to its action. Currently, it is estimated that 347 million people worldwide suffer from diabetes mellitus, out of which, 90% of the population suffers from type 2 diabetes mellitus (T2DM) which is characterized by insulin resistance [1]. The limitations with the current therapy have encouraged researchers to discover new antidiabetic drugs which are more safe and potent. The natural products and the botanicals provide a new insight for the development of more efficient and safe drugs. There are many reports which highlight the glucose lowering activity of plants in vitro [2–4]. In T2DM, insulin target tissues are damaged that aggravates the insulin ability to trigger the insulin resistance.

Liver is the major site of glucose metabolism including synthesis, storage and redistribution of carbohydrates and is an important target organ for action of insulin. In this regard, one of the indications of diabetes is the alteration of the molecular regulation of hepatic glucose homeostasis. Therefore, HepG2 cells were used in this study as a model of insulin resistance to mimic in vivo condition. Insulin-resistance in HepG2 cells is mainly associated with deficient glycogenesis, failure to suppress hepatic glucose production and miss-regulation of insulin pathway. This also causes reduction in the amount of total glucose transporters (GLUT) as well as insulin-mediated recruitment of GLUTs from intracellular vesicles to the plasma membrane, is reduced which

Abbreviations: WHO, World Health Organization; T2DM, Type 2 diabetes mellitus; GLUT, Glucose trasporters; IRS-1, Insulin Substrate Receptor-1; AKT-2, Protein Kinase B; FA, Flavonoid Fraction A; FB, Fraction B; FC, Fraction C; FD, Fraction D; AH, Crude alcoholic extract; DPPH1, 1-Diphenyl-2-picrylhydrazyl; FRAP, Ferric ion reducing antioxidant power; ABTS2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid).

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leads to inhibition of glucose uptake in cells [5]. Many reports show the function of all Glut family members. With respect to liver, GLUT-1, GLUT-2, GLUT-9, GLUT-10 are highly expressed whereas GLUT-4 is highly expressed in skeletal, cardiac muscle, brown and white adipose tissue [6–8]. However, few recent reports suggest the function of GLUT-4 in liver [9–11]. Though there are very few reports on it, GLUT-4 has been expressed and can mediate glucose uptake by semicarbazide sensitive amine oxidase mediated effects on insulin receptor signalling in sinusoidal endothelial cells and stellate cells of liver which explains the expression at mRNA level in humans [11]. The insulin stimulates the translocation of GLUT-4 from internal vesicles to the plasma membrane either by PI3K/Akt pathway or insulin receptor mediated phosphorylation of CAP protein. Our study focuses on PI3K/Akt pathway to evaluate whether flavonoids follow the same pathway or not for glucose uptake of IR/HepG2 cells.

Enicostema littorale blume (A. Raynal) a traditional Indian plant belongs to the Gentianaceae family. Its extracts were frequently used as hepatoprotective activity [12], antihelminthic [13], anti-inflammatory [14], and hypoglycemia [15,16]. As one of the major and important bioactive components in *Enicostema littorale,* swertiamarin an secoiridoid glycoside has shown its antidiabetic activity [17,18]. Moreover, there are no reports on flavonoids from *Enicostema littorale* for their antidiabetic activity. Since, flavonoids are polyphenolic compounds which have a positive relationship with disease prevention and can act on various molecular targets and regulate different signaling pathways in pancreatic β-cells (IRS1/2, Glut 4), adipocytes (PPAR-γ), hepatocytes (IRS1/2, Glut 2/4), and skeletal myofibers (IRS1/2, Glut 4) [19]. Hence, the principle approach of this study is identification of flavonoid fractions by LC/MS from extract of *Enicostema littorale*, their antioxidant property, antidiabetic activity of flavonoid fractions by glucose uptake assay on induction of insulin resistance in human liver cancer cell line (HepG2) and pathway involved in the mechanism of action of flavonoids.

2. Materials and methods

2.1. Chemicals

Sodium carbonate (Na_2CO_3), Potassium dihydrogen phosphate (KH_2PO_4), di-Potassium hydrogen phosphate (K_2HPO_4), concentrated Sulphuric acid (H_2SO_4) 98%, Potassium ferricynide [$K_3Fe(CN)_6$], Ethyl acetate (purity 99%), Petroleum ether (40°−60°), Ferric chloride ($FeCl_3$), Sodium chloride (NaCl), Potassium chloride (KCl), Ammonium molybdate were bought from Fisher Scientific, India. Methanol of AR grade was purchased from Merck specialities PVT. Ltd, India whereas Quercetin (purity > 99.0%) from SD Fine Chemicals. Ascorbic acid, Trichloroacetic acid and Folin Ciocalteau reagent were procured from Lobachemie. Sodium phosphate AR grade was purchased from SRL PVT Ltd. Ethanol (purity 99.9%) were from Changshu hongsheng fine chemicals co Ltd. 1, 1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) tablet and TRIzol reagent were purchased from Sigma Aldrich. Standard Apigenin (purity > 95.0%) was procured from Natural remedies private limited, Bangalore, India. Swertiamarin (purity > 95.0%) was a gift sample from Baroda university, Gujarat, India. For cell culture Dulbecco's modified eagle's medium low (1 g/L) and high glucose (4.5 g/L), Fetal bovine serum, Dulbecco's phosphate buffered saline (1X) were obtained from Cell clone Genetix Biotech, Asia PVT. Ltd, India and Insulin (10X) was procured from BD Biosciences, India. API Metformin was a gift sample from USV Ltd, India. First strand cDNA synthesis kit was purchased from Takara Bio Company, USA. SYBR select master mix was purchased from Applied Biosytems by Life Technologies, USA whereas primers of IRS-1, Akt-2 and GLUT-4 were purchased from Eurofins Genomic India Pvt Ltd.

2.2. Plant material

Five kg of the whole plant powder of *Enicostema littorale* blume (A. Raynal) was purchased from recognised ayurvedic supplier from Mumbai, Maharashtra, India on 10th Sept 2013. It was authenticated by Agharkar Research Institute, Plant Science Division, Pune, Maharashtra, India. The Voucher no WP-114 was allotted and has been deposited.

The plant material was defatted with pet ether. The phytoconstituents were extracted by soxhlation at 60 °C for 72 to 120 h to obtain Alcoholic Hot extract (AH) as a crude extract using ethanol as a solvent in 1:6 ratios (w/v). The extract was concentrated on rotary evaporator to obtain alcoholic extract which was weighed and percent yield was calculated as 10.4%. The extract was then kept in capped glass vials, properly labeled and stored in air tight desiccators at 27 °C.

2.3. Isolation of flavonoid fractions from E. littorale and their preliminary screening

The isolation of flavonoids was done by liquid-liquid solvent extraction procedure which was adapted with few modifications by Ghosal and Jaiswal [20]. Briefly, the crude AH was extracted with distilled water in 1:5 (w/v) ratio. This mixture was then centrifuged to obtain aqueous filtrate (AF) and yellowish brown residue. The brown residue was dissolved in hot ethyl acetate and sonicated till maximum residue gets dissolved. The undissolved residue was labelled as Fractions A (FA) and the dissolved ethyl acetate fraction was labeled as Fractions B (FB). The AF was extracted with ethyl acetate thrice in separating funnel, out of which ethyl acetate fraction is Fractions C (FC) and aqueous fraction was incubated at 0° C which was labeled as Fractions D (FD). The incubation of FD at 0° C was optimized for one month for better yield. Total four flavonoid fractions obtained from alcoholic crude extract AH were FA, FB, FC and FD and the percent yield of all fractions were calculated as 10.72, 8.72, 6.88 and 2% respectively. Solutions of 1 mg/ml powdered fractions were dissolved in methanol and were taken ahead for screening.

2.4. TLC

1 mg/ml concentration of each extracts was made in Methanol and 10 μl loaded onto the TLC plate. The solvent system was standardized as Toluene: Acetone: Methanol: Formic acid (6.9: 2.0: 2.5: 0.15). The plate was observed under UV at 254 nm and 366 nmThe TLC plate was sprayed with natural product reagent which is specific derivatizing agent for flavonoids.

2.5. Total flavonoid content

The total flavonoid content was determined using $AlCl_3$ method and was calculated by using calibration curve of quercetin. The content was expressed as milligram of Quercetin Equivalents/g (mg QE/g extract) total flavonoid content [21].

2.6. Identification of isolated flavonoid fractions by LC/MS

The four flavonoid fractions Fractions A (FA), Fractions B (FB), Fractions C (FC), Fractions D (FD) were obtained from alcoholic crude extract (AH) containing mixture of flavonoids which were identified by LC/MS. Solutions of 1 mg/ml powdered fractions were dissolved in methanol and were taken ahead for screening. Jasco RP-HPLC-PDA was used for the standardization of HPLC method.