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Effect of Alpha-Glucosidase Inhibitory Property and Glucose Uptake Assay In L6 Cell Line By *Griffithsia Pacifica Kylin*

R. Lalitha[1 & 2 *] S. Palani[3]
1 *Kamban College of Arts and Science For Women, Tiruvannamalai, Tamilnadu, India.*
2 Research Scholar, Bharathiar University, Coimbatore, Tamilnadu, India.
3.*Department of Biotechnology, Anna Bio Research Foundation, Arunai Engineering college, Tiruvannamalai, Tamilnadu, India.*

Abstract: *The whole red algae Griffithsia Pacifica Kylin (GPK) has not yet been known for its in vitro anti-diabetic property. This research was performed to investigate the ability of GPK fractions as an anti-diabetic agent. On the first stage extraction of GPK was performed using ethanol. On the second stage in vitro anti-diabetic activity was performed using α-glucosidase enzyme and determination of cytotoxic properties of GPK in selected L6 cell line of rat skeletal muscles.In brief, the 24 hr. cell cultures with 70-80% confluences in 40mm petri plates were allowed to differentiate by maintaining in DMEM with 2% FBS for 4-6 days. The extent of differentiation was established by observing multinucleation of cells. For the inhibition activity toward α-glucosidase enzyme, ethanol extract of red algae showed higher activity at 1000µg/ml. Dose comparable with standard RosiGlutozone. This had the highest potential as a type 2 anti-diabetic agent. It was concluded that ethanol extract of GPK showed potential anti diabetic property.*

Key Words:*Griffithsia Pacifica Kylin, Red algae, α-glucosidase, Type 2 anti-diabetic agent, hyperglycemia.*

1.Introduction

Diabetes is a metabolic disorder with a disturbed carbohydrate metabolism due to any defect in insulinsecretion, action or both (Report of a WHO consultation). About 2-3% of the world's population is affected by diabetes (Abid M, et al 2010). Diabetes is more common in the developed countries. It ranks among the top 5 significant diseases in the world (Bhagwat Det al2008). Factors such as age, obesity, physical inactivity, excess eating may prevent the use of insulin by the body. This stops the metabolism of carbohydrates. Sometimes the body's immune system may demolish thebeta cells of pancreas, thus leading to diabetes. The factorsinvolved are genetics, family history and environmental factors(Riaz Set al.2009). The major symptoms are polydipsia, polyuria and polyphagia(Bastaki S.et al 2009).

One therapeutic approach for treating diabetes is to decrease postprandial hyperglycemia. This can be obtained by delaying the absorption of glucose through the inhibition of carbohydrate hydrolyzing enzymes, alpha glucosidase in the digestive tract. The alpha glucosidase inhibitors can retard the liberation of glucose from dietary complex carbohydrates and delay glucose absorption, resulting in reduced postprandial plasma glucose levels and suppress postprandial hyperglycemia. (Martim et al 2003).

Alpha-Glucosidase Inhibitors (AGI) reversibly inhibit a number of alpha-glucosidase enzymes (eg, maltase), consequently delaying the absorption of sugars from the gut. In a recent study among healthy subjects it was suggested that the therapeutic effects of AGIs are not only based on a delayed digestion of complex carbohydrates, but also on metabolic effects of colonic starch fermentation (Campbell et al 1996). AGIs might be a reasonable option as first-line drug in the treatment of patients with DM2 as it specifically targets postprandial hyperglycemia, a possible independent risk factor for cardiovascular complications (Ceriello et al 2005). Although rare cases of hepatic injury were described, AGIs are expected to cause no hypoglycemic events or other life-threatening events, even at overdoses, and cause no weight gain (Chiasson et al 2003).Therapeutic strategies for T2DM lead to serious adverse effects (Ramachandran S, et al 2013). It causes decreased bone mineral density in the femoral neck and hip in diabetic patients during therapy (Abid M, et al 2010).Acarbose and miglitol, the conventionally used glucosidase inhibitors for the management of PPH in diabetic patients are known to be associated with several side effects (Fujisawa et al. 2005); therefore, search for glucosidase inhibitors from natural sources with lesser side effects attains more interest in scientific community (Chiasson JL, et al 2003).

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(Gopalakrishnan et al 1976).

Griffithsia Pacifica Kylin is a well-known ceramaceous red algal genus, which has characteristic large vegetative cells visible to the unaided eye and thousands of nuclei in a single cell at maturity and has served as a useful tool for many developmental studies(WaalandJ.R. 1982).

Therefore, the current study was designed to investigate the *in vitro* anti-diabetic effects of GPK extract on alpha glucosidase and L6 cell line of rat muscle.

II Materials And Methods

Red Algae Collection

Algal materials were collected from the Rameswaram, Tamilnadu, India. The algae were obtained by catching method. The collected red algae were washed with tap water to remove salts and other adhering particles. The whole red algae was dried under shade and then powdered with a mechanical grinder to obtain a coarse powder. Equal quantity of powder was passed through 40 mesh sieve and extracted with ethanol in sox let's apparatus to 60'c. The solvent was completely removed by rotary vacuum evaporator. The extract was freeze dried and stored in vacuum desiccators. The red algae were further processed to test for its inhibition of α-glucosidase activity.

Chemicals:

A-Glucosidase enzyme, Phosphate buffer (pH-6.8) 0.2M Sodium carbonate and 5mM p-Nitrophenyl α-D glucopyranosidase (pNPG).

Preparation Of Test Soulution:

2mg each of the test substance was dissolved in 750 µl of phosphate buffer separately to obtain solutions of 1000µg/ml of concentrations. Each of these solutions was serially diluted separately to obtain lower concentrations.

A-Glucosidase Inhibition Assay:

In vitro α-glucosidase inhibition was studied by Kim et al method. In brief, 50µl of the α-glucosidase was pre incubated with 60µl of different concentration of test substance for 20 minutes. Then the reaction is initiated by adding 50µl of p-Nitro phenyl α-D glucopyranosidase (pNPG) as a substrate. The reaction mixture was incubated at 37° C for 20 minutes and stopped by adding 160µl of sodium carbonate. The α-glucosidase activity was determined by measuring the yellow-colouredparanitrophenol released from pNPG at 405nm and the percentage inhibition of α-glucosidase enzyme was calculated using the formula,

$$\text{Inhibition } (\%) = 100\left(\frac{\text{control} - \text{test}}{\text{control}}\right).$$

Suitable reagent blank and inhibitor controls were simultaneously carried out.

***In Vitro* Antidiabetic Activity Of Test Drugs In L6 Cell Line:**

Cell Lines And Culture Medium:

L-6 (Rat, Skeletal muscle) cell culture was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells of L-6 were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), Penicillin (100 IU/ml) and amphotericin B (5µg/ml) in a humidified atmosphere of 5%CO_2 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 25cm^2 culture flasks and all experiments were carried out in 96µl plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation Of Test Solution:

For in vitro studies, test substance dissolved in DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination Of Cell Viability Of MTT Assay (Francis And Rita, 1986):

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hrs, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test

drugs were added on to the partial monolayer in microtitre plates atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a micro plate 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% (CTC_{50}) values is generated from the dose-response curves for each cell line.

$$\% \text{ Growth Inhibition} = 100 - \left(\frac{\text{Mean OD of individual test group}}{\text{OD of control group}} \right) \times 100$$

***IN VITRO* GLUCOSE UPTAKE ASSAY (**HISAKO ET AL., 2003; ANGELINE ET AL., 2007**)**

Glucose uptake activity of test substance was determined indifferentiated L6 cells. In brief, the 24 hr cell cultures with 70-80% confluence in 40mm petri plates were allowed to differentiate by maintaining in DMEM with 2% FBS for 4-6 days. The extent of differentiation was established by observing multi nucleation of cells. The differentiated cells were serum starved overnight and at the time of experiment cells were washed with HEPES buffered Krebs Ringer Phosphate solution (KRP buffer) once and incubated with KRP buffer with 0.1% BSA for 30min at 37^0C. Cells were treated with different non-toxic concentrations of test and standard drugs for 30 min along with negative controls at 37^0C. 20µl of D-glucose solution was added simultaneously to each well and incubated at 37^0C for 30 min. After incubation, the uptake of the glucose was terminated by aspiration of solutions from wells and washing thrice with ice-cold KRP buffer solution. Cells were lysed with 0.1M NaOH solution and an aliquot of cell lysates were used to measure the cell-associated glucose. The glucose levels in cell lysates were measured using glucose assay kit (ERBA). Two independent experimental values in duplicates were taken to determine the percentage enhancement of glucose uptake over control.

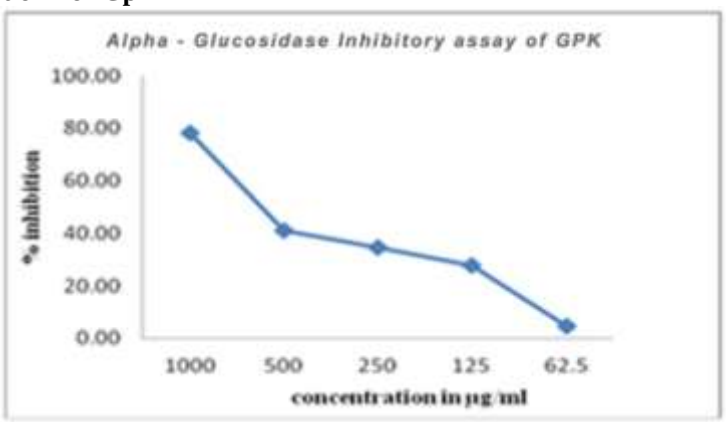
III. Results and Discussion

A- Glucosidase Enzyme Inhibition Assay:-Glucosidase inhibitor effectiveness of ethanol extract of GPK was studied on the basis of its IC_{50} value inhibited the activity of α-Glucosidase with an 80µg/ml.

Table1 1: Alpha-Glucosidase Inhibitory Activity Of Gpk

Sl No.	Name of test substance	Test Conc. (µg/ml)	% Inhibition	IC 50 (µg/ml)
1	Ethanolic extract of GPK	1000	80.99 ± 2.15	646.75 ± 10.08
		500	41.21 ± 1.91	
		250	34.48 ± 0.17	
		125	27.71 ± 0.18	
		62.5	4.68 ± 0.21	

A-Glucosidase Inhibition For Gpk



MTT Assay:

The MTT assay is used to assess the viability and the proliferation of cells (Freshney RL, Culture of Animal Cells: A Manual of Basic Techniques, Wiley-Liss, New York, 2000). It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials, since those agents would stimulate or inhibit cell viability and growth. The absorbance of the GPK extract with the L6 muscle cell line at the different concentrations (62.5-1000µg/ml) compared with control(untreated L6 cells). The results confirm that there was "nill or low" toxicity effect of GPK red algae extract at higher concentrations.

Table 2: Cytotoxic properties of ethanolic extract of GPK drugs against L6 cell line.

Sl. No	Name of Test sample	Test Conc. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	Ethanolic extract of GPK	1000 500 250 125 62.5	24.68±1.7 18.78±0.9 17.44±1.8 16.17±2.1 14.33±1.1	>1000

Glucose uptake results:

Table 3: In vitro glucose uptake studies for ethanolic extract of GPK substances in L-6 cell line

Sl No.	Name of the Test substances	Test Conc. In mcg/ml	Glucose uptake percentage (%)
1	**Control**	-	1.44±1.30
2	**Std Rosiglutozone**	100	116.67±0.66
3	ethanolicectract of GPK	500	89.64±1.37
	ethanolicectract of GPK	250	40.57±3.46

Discussion

Management of the blood glucose level is a critical strategy in the control of diabetes complications. Inhibitors of saccharide hydrolyzing enzyme alpha glucosidase have been useful as oral hypoglycemic drugs for the control of hyperglycemia especially in patients with type-2 diabetes mellitus. The ability of GPK extracts to inhibit alpha glucosidase activity *in vitro* was investigated and the result is presented in Table 1 and graph. The results revealed that GPK extract inhibited alpha glucosidase in a dose dependent manner (62.5-1000µg/ml). The enzyme inhibition assays are given in Table 1 and Graph. Table 2 shows cytotoxic property of extract against L6 cell line.

Glucose utilization in L6 cell line was studied *in vitro*. The results revealed in Table 3. The results show that the ethanol extract of GPK enhances the glucose uptake by 89.64% over control of 500µ/ml. The results were compared with control and rosiglutozone, used as standard antidiabetic agent. It enhances the glucose uptake by 116.61% over control of 100µg/ml. The results indicated that GPK extract has no synergic effect than standard drug.

Skeletal muscle is the primary site responsible for postprandial glucose use. Furthermore, it is the most abundant tissue in whole body, and thus proper function of skeletal tissue is most important to maintain normal blood glucose level (Defronzo R.A 1981). Defects in insulin stimulated skeletal muscle glucose uptake are common pathological states in noninsulin dependent diabetes mellitus (Baron AD 1981).

The results obtained in the present study clearly demonstrate that GPK extract enhances glucose uptake under in vitro conditions. This may be due to its effect on the number of receptors located in skeletal muscle cell line.

IV Conclusion

We conclude from the above results and discussion on the ethanol extract of *Griffithsia Pacifica Kylin* has significant effect on inhibition of alpha glucosidase, cytotoxicity and glucose uptake in L6 cell line.

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