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Krishna K R Kumar
krishnakumar_kr@yahoo.co.in

Jayaprakash A P

Srinivasan K K

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Antidiabetic evaluation of *Hemionitis arifolia* leaves by *in vitro* methods

Krishna Kumar K R^{1*}, Jayaprakash A P³ and Srinivasan K K³

1) University College of Pharmacy, M G University, Cheruvandoor, Ettumanoor-686 631, Kottayam, Kerala, India.

2) University College of Pharmacy, M G University, RIMSR, Kottayam

3) Shri Madhwa Vadiraja Institute of Technology, Vishwothama Nagar, Bantakal, Udipi, Karnataka, India.

ABSTRACT

In the present article, antidiabetic activity of the extracts of *H. arifolia* leaves prepared with 95% ethanol (HALE) and 50% ethanol (HALH) were evaluated by various *in vitro* methods. Initially, preliminary phytochemical studies were performed and the presence of phenolic, flavonoids and their glycosides were identified. In the antidiabetic activity evaluation enzyme inhibition and glucose uptake studies on muscle cell lines were performed. In the enzyme inhibition studies, models such as alpha amylase inhibition and DPP-IV inhibition assays were performed. In the alpha amylase inhibition studies IC₅₀ values of HALE and HALH were found to be above 1000 µg/ml. In the DPP-IV inhibition studies, the IC₅₀ value was found to be above 500 µg/ml. HALH exhibited only 15.83% inhibition of DPP-IV at a concentration of 500 µg/ml. Glucose uptake studies and the mechanism of action identification were performed on L-6 muscle cell lines of rats. HALH demonstrated better glucose uptake (26.0±0.22% over control) compared to HALE glucose uptake values (14.0±4.17% over control) in the experiment. In the evaluation of mechanism of action of glucose transport by HALH on L-6 cell lines, Glut-4 upregulation and PPARγ upregulation were observed. Glut-4 gene upregulation of 0.15 and 0.09 fold over normal control were demonstrated by HALH at a concentration of 500 and 250 µg/ml, respectively. In the other mechanism of action identified of glucose transport by HALH on L-6 cell lines, 0.20 and 0.15 fold upregulation of PPARγ genes over normal at a concentration of 500 and 250 µg/ml concentration were also observed. In the MTT assay studies, CTC₅₀ values of both the extracts were found to be above 1000 µg/ml. The above all data are indicative of the antidiabetic potential of *H. arifolia* leaves.

Key words: *Hemionitis arifolia*, leaf extract preparation; Phytochemistry; alpha amylase inhibition studies; DPP-IV inhibition studies; glucose uptake studies on L-6 muscle cell lines; Glut-4 translocation; PPARγ upregulation; MTT assay; antidiabetic evaluation

INTRODUCTION

Antidiabetic activity is one among the acclaimed properties of *H. arifolia* in folk medicine of Kerala. Later studies on various other plants revealed the relation between the antioxidant property and the presence of phytoconstituents such as phenolics, flavonoids and phytosterols¹. In the phytochemical analysis of *H. arifolia* leaf extract in ethanol, presence of phenolics and flavonoids were detected and quantified^{2,3}. The reported components are apigenin, kaempferol, quercetin and some of its glycosides such as rutin.

In *diabetes mellitus*, NOS (iNOS (inducible NOS) over expression causes the destruction of β-cells of pancreas

* For Correspondence:

E-mail: krishnakumar_kr@yahoo.co.in

by inhibition of mitochondrial enzymes and via different cytokines released from activated lymphocytes⁴. Peroxynitrite (ONOO⁻) which is another powerful antioxidant causes tyrosine nitration, thiol oxidation, lipid peroxidation, DNA strand break, guanosine nitration/oxidation and ultimately cell death. When, the overall generation of ROS and RNS exceeds the total antioxidant activity in the body, oxidative stress will develop. Antioxidants prevent free radical induced tissue damage by preventing the formation of radicals, scavenging them, or by promoting their decomposition⁵. Antioxidant studies of both the extracts were performed by ABTS, DPPH, nitric oxide scavenging and lipid peroxidation inhibition methods and the results were promising and were reported⁶. In this case, it is important to observe the nitric oxide scavenging activity demonstrated by the extracts.

Here, it can be correlated with the above cited reports of Young I S and Wood side J V⁵. In the studies performed on *Wistar albino* rats using alcoholic extract, antidiabetic activity was reported.⁷ During the collection of plants, first author personally observed the interest of village goats/sheeps to eat *H. arifolia*. The above all points motivated us to investigate further to find and evaluate the methods by which the acclaimed antidiabetic activity works out in both the extracts and the components identified and reported.

MATERIALS AND METHODS

Extracts of *H. arifolia* leaf was prepared according to reported procedure² and, pure quercetin and rutin were used for the studies. Ethanol 95% extract was named as HALE and ethanol 50% extract was named HALH. The plant was identified by Dr K V George, Professor of Botany, CMS College, Kottayam and the voucher specimen number is KRK/UCP/CMS/506.

Alpha amylase inhibition studies

Chemicals and reagents

Alpha amylase: 2 mg/ml in sterile water prepared using alpha amylase enzyme (Himedia Rm 638), E.C.No. 3.2.1.1, 1:2000 IU, Phosphate buffer (pH-6.9), Starch solution 1% w/v, Dinitrosalicylic acid reagent: Dinitrosalicylic acid (1gm) and sodium hydroxide (12 gm) dissolved in distilled water (100 ml), DMSO.

Plates used:

Micro-well plate: Tarson 96 well polystyrene, non-treated (Cat. no. T941196, Tarsons, India)

Preparation of the test solutions

Extracts HALE, HALH, quercetin, rutin and acarbose (10 mg each) were weighed accurately and separately dissolved in distilled DMSO (1 ml) and respective 10 mg/ml stock solutions were prepared. Serial dilutions of the above stock solutions were done with DMSO to get the required concentrations for enzyme inhibition studies.

Procedure

Bernfeld method with modifications was used in the *in vitro* alpha amylase inhibition studies⁸. The test solutions of extract and individual components (100 μ l in DMSO) were allowed to react with 200 μ l of α -amylase enzyme according to the standard procedure. After completion of the reaction 200 μ l each of the solutions were taken in the wells of a micro-titer plate and the absorbance was measured at 540

nm using a micro-plate reader. The percentage inhibition of alpha amylase enzyme was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{\text{control} - \text{test}}{\text{control}} \times 100$$

Respective reagent blank and controls were also prepared simultaneously and used in the study. All the experiments were performed thrice. Results are as shown in Fig.1, Fig.2 and Fig. 3.

DPP-IV inhibition studies

Chemicals and Reagents

Enzyme: Dipeptidyl aminopeptidase (E.C. 3.4.14.5) IV from porcine kidney [D7052, Sigma, USA], Substrate: Gly-Pro-p-nitroanilide hydrochloride [G0513, Sigma, USA], Positive control: Ile-Pro-Ile [I9759, Sigma, USA], Trizma base [T6066, Sigma, USA], Tris-hydrochloride [RM613, Himedia, India], Tris HCl buffer – pH 8.2, Sodium acetate buffer 1M (pH-4.0), DPP-IV enzyme solution 0.01 u/ml in Tris HCl buffer – pH 8.2, Gly-Pro-p-nitroanilide hydrochloride 1.59 mM in Tris HCl buffer – pH 8.2.

Micro-titer plates:

Costar 384 well polystyrene, non-treated. (Cat. no. 3702, Corning)

Preparation of test solutions

The standard and sample extract were dissolved in DMSO (62.5 μ l) and the volume was then made up to 2 ml with 50 mM Tris buffer (pH 7.5) to get 1mg/ml solutions. The required dilutions were prepared from the stock solution by diluting with DMSO.

Defatted HALH extract dilutions were prepared in the same manner as mentioned above.

Procedure

This study was performed as per the method of Kojima et al., with some modifications⁹. Serially diluted test extract and individual components (25 μ l) in Tris HCl buffer (pH-8.2), DPP-IV enzyme-0.01 U/ml (50 μ l), prepared in Tris HCl buffer, were taken in the wells of a 96 well micro-titer plate, mixed and performed the assay as per the standard procedure. After completion of the reaction, the absorbance was measured at 405 nm using a micro-plate reader (PHERAstar, BMG LABTECH, Germany). Controls were run without test samples. Respective sample blank and control blank were done with all reagents except the enzyme. The experiment was done in triplicate.

The % inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (test)}}{\text{Absorbance (control)}} \times 100$$

Log- probit analysis was used to calculate IC_{50} values. The results are depicted in table 1.

Materials and methods for cell line studies

Phosphate Buffered Saline (PBS), 3-(4, 5-dimethyl thiazol-2-yl)-5-diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Bovine Serum Albumin (BSA), D- glucose, Dulbecco's Modified Eagle's Medium (DMEM) and Trypsin were obtained from Sigma Aldrich Co., St Louis, USA.

EDTA, antibiotics and other chemicals were procured from Hi-Media Laboratories Ltd, Mumbai. Insulin from (Torrent Pharmaceuticals, 40 IU/ml) Dimethyl Sulfoxide (DMSO) and Propanol from E.Merck Ltd, Mumbai, India. Membrane filter (Millipore, 0.22 μ m, 047 mm (dia.) and filtration assembly.

(Respective reagents were used from the above prepared reagents for the various cell line studies performed as per the requirement.)

Culturing of L-6 cells (Myoblast)

Muscle cell lines, L-6 (Rat, skeletal muscle) were 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), as per the standard procedure of NCCS in 25 cm² culture flasks. All experiments were carried out in 96 well micro-titer plates (Tarsons India Pvt Ltd, Kolkata, India).

Preparation of test solutions

The test extracts were separately dissolved in distilled DMSO and the 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. Serial two fold dilutions of the test extracts were prepared from their respective stock solutions for carrying out cytotoxic and glucose uptake studies. Similarly, the standard was also prepared.

MTT assay- Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.5 x 10⁴ cells/ml using DMEM containing 10% FBS. The diluted cell suspension (0.1ml) containing approximately 1500 cells were added to each well of the

96 well micro titer plates. After 24 hours, when a partial monolayer of 70% confluency is formed, the supernatant was flicked off, and the monolayer was washed with medium. The serially diluted different dilutions (100 μ l) of both the test and standard drugs were added on to the partial monolayer formed in designated wells of the micro-titer plates and performed the experiment as per the procedure of Mosmann T et al., with necessary modifications¹⁰. A micro-plate reader was used to measure the absorbance at 540 nm. The percentage growth inhibition was calculated using the following formula and concentrations of test drug needed to inhibit cell growth by 50%, (CTC_{50} values) were determined from the dose-response curves for the respective extract.

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

Results are depicted in Fig.4

Glucose uptake studies (*In vitro*) on L-6 muscle cell lines-Procedure

Among the cell lines used in glucose uptake studies L-6 skeletal muscle cell lines derived from rat skeletal muscle is most frequently utilized as a cellular model system for investigating the insulin-stimulated glucose transport system¹¹. The 24 hour cell cultures with 70-80% confluency in 40mm petri plates were allowed to differentiate by maintaining in DMEM with 2% FBS for 4-6 days. The differentiated cells were serum starved over-night at the time of experiment. Cells were washed with HEPES buffered KRP buffer once and incubated with KRP buffer with 0.1% BSA for 30 minutes at 37°C. Cells were treated with serially diluted concentrations of test extract and standard drug (metformin), for 30 minutes along with normal controls at 37 °C. D-glucose solution (20 μ l) was added simultaneously to each well and incubated at 37°C for another 30 minutes. After incubation, the uptake of the glucose was stopped by aspiration of solutions from wells and washed three times with ice-cold KRP buffer solution. Then, the cells were lysed with 0.1M NaOH solution and the cell-associated glucose was measured using the respective aliquots of the lysed cells. Glucose assay kit (Biovision Inc, USA) was used to measure the glucose level in cell lysates. The experiments were performed in triplicate and the values were used to determine the glucose uptake percentage enhancement over controls.

Results are depicted in Table 2.

Materials and methods for the evaluation of mechanism of action

GLUT-4 gene expression studies

RT-PCR Procedure

The mRNA expression levels of Glut-4 was carried out using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The L-6 cells were cultured in 60 mm petridish and maintained in DMEM medium for 48 hours. The DMEM medium was supplemented with FBS and amphotericin. The serially diluted concentration of test samples and standard were added to the petridish and incubated for 20 minutes. Isolation of the total cellular RNA from the untreated (control) and treated cells were performed according to manufacturer's protocol using Trizol reagent. The cDNA was synthesized from the isolated total RNA by a reverse transcriptase kit according to the manufacturer's instructions (Thermo scientific). The mRNA expression levels of Glut-4 were determined by a PCR reaction using the specific primers and constructed cDNA. The primers used in this study were chosen as per the earlier literature¹² and the specifically designed primers procured from Eurofins India. The expression levels of mRNA of a house keeping gene (GAPDH) were used as an internal control.

Amplification conditions for Glut-4 gene

After the initial DNA synthesis for 30 minutes, denaturation was done at 95 °C for 1 minute followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 66 °C for 30 seconds and an extension at 72 °C for 45 seconds was done. This was followed by a final extension at 72°C for 10 minutes.

Primer used:

For I strand synthesis: Random primer

For II strand synthesis: 5'CGG GAC GTG GAG CTG
GCC GAG GAG 3'-Forward
5' CCC CCT CAG CAG CGA
GTG A 3'- Reverse

Product size: 318 bp.

Analysis of amplified sequences

After PCR, the amplified samples were analysed through electrophoresis in 1.8% agarose gels stained with ethidium bromide (0.5µg/ml). The gel was scanned with ultraviolet illumination using digital imaging (UV Tech, UK) and

relative sample expression levels were calculated using Alpha View, version 3.3.1.0 Cell Biosciences, Inc and were expressed relative to GAPDH and corrected for, between run variability. Results are depicted in Fig. 5, Fig. 6 and Table 3.

Gamma PPAR gene expression studies

RT-PCR Procedure

The mRNA expression levels of PPAR γ was carried out using semi-quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) as explained with GLUT-4 gene amplification studies.

The mRNA expression levels of PPAR γ were determined by a PCR reaction using the specific primers and constructed cDNA. The primers used in this study were chosen as per the earlier literature¹² and the specifically designed primers procured from Eurofins India.

Amplification conditions for PPAR γ gene

After the initial DNA synthesis for 30 minutes, denaturation was done at 95 °C for 1 minute followed by 35 cycles of denaturation at 95 °C for 15 seconds, annealing at 64 °C for 30 seconds and extension at 72 °C for 45 seconds was done. This was followed by a final extension at 72°C for 10 minutes.

Primer used:

For I strand synthesis: Random primer

For II strand synthesis: 5' GGA TTC ATG ACC AGG
GAG TTC CTC 3'-Forward
5' GCG GTC TCC ACT GAG
AAT AAT GAC 3'- Reverse

Product size: 155 bp.

Analysis of amplified sequences

After PCR, the amplified samples were analyzed in the same procedure followed for Glut-4 analysis. Results are depicted in Fig. 7, Fig. 8 and Table 4.

RESULTS

Both the extracts HALE and HALH were found to inhibit alpha amylase activity at a concentration of 1000 µg/ml and above. The pure component quercetin showed alpha amylase inhibition and the IC₅₀ value was 931±9.28 µg/ml and its glycoside rutin showed IC₅₀ value above 2000 µg/ml concentration only. Therapeutically using antidiabetic

acarbose showed alpha amylase inhibition and the IC₅₀ value was 82.0±0.85 µg/ml.

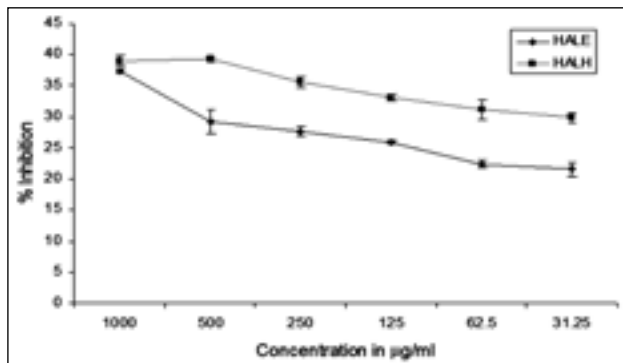


Fig.1 Alpha amylase inhibition activity of HALE and HALH

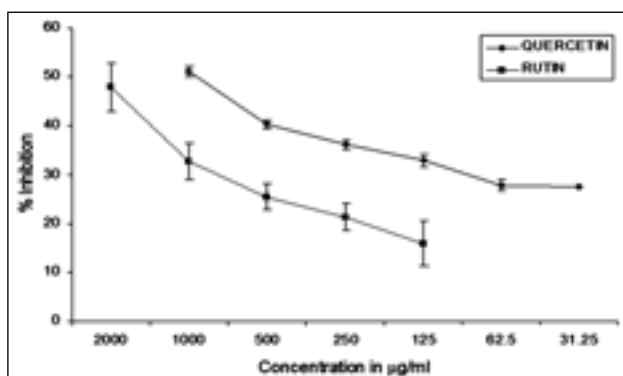


Fig.2 Alpha amylase inhibition activity of Quercetin and Rutin

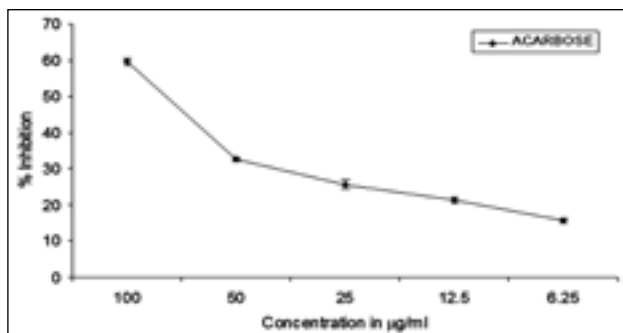


Fig.3 Alpha amylase inhibition activity of Acarbose

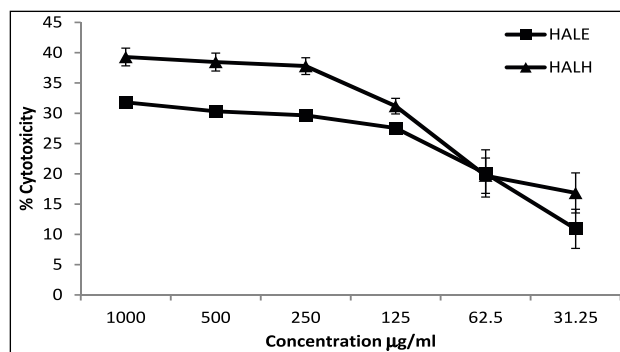


Fig. 4 MTT assay of HALE and HALH

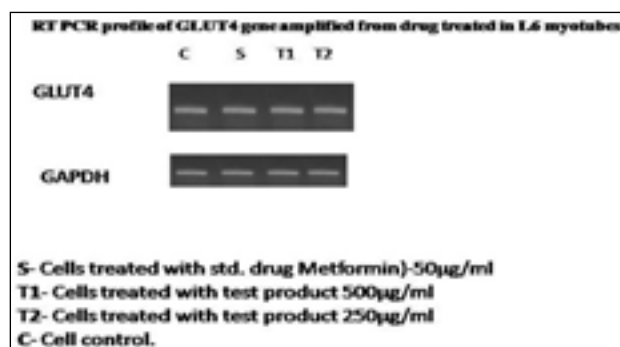


Fig. 5 RT-PCR profile of Glut-4 gene amplified from drug treated L-6 cells (defatted HALH).

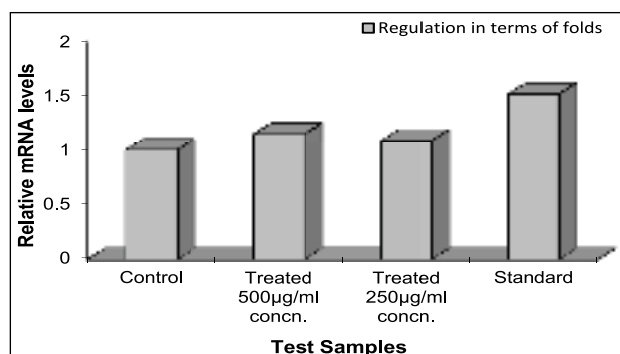


Fig.6 Densitometric analysis of gene transcripts. The relative level of Glut-4 gene expression is normalized to GAPDH (defatted HALH). Values shown depicted in arbitrary units.

Table 1. Results of DPP-IV inhibition studies of HALE and HALH

Sl. No.	Name of drug	Test concentration. in µg/ml	% Inhibition ± SD	IC ₅₀ in µg/ml
1	Ile-Pro-Ile	50.00	86.83±0.50	6.51
		25.00	75.29±2.25	
		12.50	71.90±2.40	
		6.25	48.69±4.58	
2	HALH (defatted)	500.00	15.83±8.20	>500
		250.00	06.05±3.31	
		125.00	0	
		62.50	0	
		31.25	0	

n=3

IC₅₀ values were above 500 µg/ml for defatted HALH samples. Defatted HALH exhibited 15.83% inhibition of DPP-IV activity at a concentration of 500µg/ml.

Table 2. *In vitro* glucose uptake studies of HALE and HALH on L-6 cell lines

Sl No	Name of the extract	Test concentration ($\mu\text{g/ml}$)	% glucose uptake over control \pm SD
1	HALE	1000	14.0 \pm 4.17
2	HALH	1000	26.0 \pm 5.22
3	Insulin	1 IU/ml	131.50 \pm 17.62

n=3

Table 4. RT PCR Glut-4 gene amplification data of defatted HALH

Test sample	Control	Treated concentration (500 $\mu\text{g/ml}$)	Treated concentration (250 $\mu\text{g/ml}$)	Standard
Regulation in terms of folds	1	1.15	1.09	1.52

Table 5 PPAR γ upregulation details of HALH

Test sample	Control	Treated concentration (500 $\mu\text{g/ml}$)	Treated concentration (250 $\mu\text{g/ml}$)	Standard
Regulation in terms of folds	1	1.20	1.15	1.34

Inference

In this study the results show upregulation of Glut-4 gene in test samples (treated) and standard when compared with the control. The test sample of 500 and 250 $\mu\text{g/ml}$ concentrations were upregulated 0.15 folds and 0.09 folds, respectively compared to control. The standard was upregulated 0.37 and 0.43 folds from treated concentration of 500 and 250 $\mu\text{g/ml}$, respectively and 0.52 folds from control.

Inference

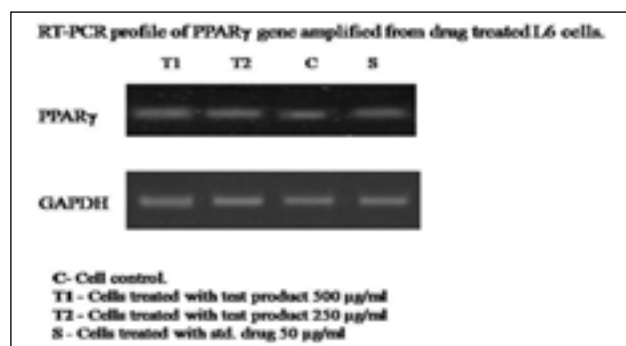
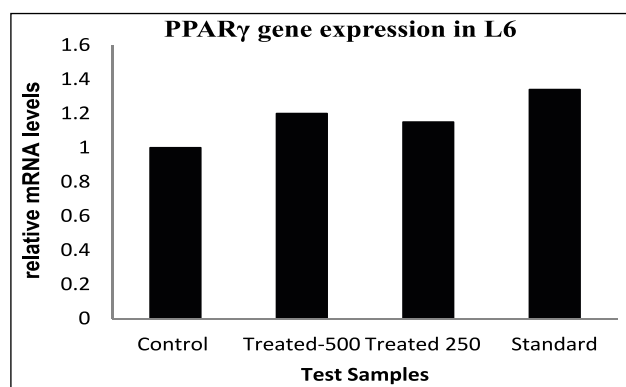
In this study the results show upregulation of PPAR γ gene in test samples (Treated) and Standard, when compared with the Control. The test sample of 500 conc. and 250 conc. is 0.20 folds and 0.15 folds upregulated respectively, compared to control. The Standard is 0.14 and 0.19 folds upregulated from treated of 500 and 250 conc. respectively and 0.34 folds from control.

DISCUSSION

In the alpha amylase inhibition studies performed, both the extracts HALE and HALH were demonstrating IC₅₀ values above 1000 $\mu\text{g/ml}$ concentration. This is indicative of the minimal antidiabetic activity effected by alpha amylase inhibition by both the extracts. IC₅₀ values of the pure components quercetin and rutin were 931 \pm 9.28 $\mu\text{g/ml}$ (3.03 mM) and above 2000 $\mu\text{g/ml}$ (3.2 mM) respectively. Early reports^{2,3} of the presence of flavonoids apigenin, kaempferol, quercetin and their glycosides can be correlated here. In the year 2006, Tadeja *et al.*,¹³ reported the alpha amylase inhibitory action of quercetin, kaempferol and apigenin and was reported again in the year 2013 by Orhan *et al.*,¹⁴. Thus, we can conclude that, the demonstrated alpha amylase inhibition can be due to the above reported phytoconstituents present in the extract.

If we consider the μM ratio, it can be seen that the efficiency is almost equal for quercetin and rutin. The IC₅₀ of quercetin was 3.08 mM and that of rutin (Q-3-O-rutinoside) was 3.2 mM, an almost same value of molar mass. Thus, it can be concluded that the effect were produced by quercetin, the aglycone of rutin.

On comparing to the alpha amylase inhibition action of acarbose, the inhibition produced by both the extracts, quercetin and rutin are less.


Fig. 7 RT-PCR profile of PPAR γ gene amplified by HALH treated L6 cells.

Fig. 8 Densitometric analysis of gene transcripts. The relative level of PPAR γ gene expression is normalized to GAPDH. Values shown depict arbitrary units.

In the DPP-IV activity inhibition evaluation, HALH exhibited 15.83% inhibition only at a concentration of 500 µg/ml. This data is also indicative of minimum contribution to the antidiabetic activity produced by HALH. The mechanism of DPP-IV inhibitors is to increase incretin levels (GLP-1 and GIP), which inhibit glucagon release, which in turn increases insulin secretion and decreases blood glucose levels¹⁵. Early report of DPP-IV inhibitory action of twenty one phenolics can be correlated here¹⁶. Out of them, luteolin and apigenin are the most potent. The minimum DPP-IV inhibitory action demonstrated here can be correlated with the early report of flavonoids and especially apigenin². Thus, further enrichment of flavonoid/phenolic content by purification of HALH may improve the activity.

In the MTT assay, both extracts were showing CTC₅₀ values at a concentration above 1000 µg/ml. This can be correlated to the observation of the first author about the interest of the goats/sheep in the villages to eat this plant. The above result indicates the nontoxic nature of the plant. The results of cell line studies indicate the glucose uptake facilitation by both the extracts and HALH treated cells demonstrated a better activity among the two. HALH demonstrated 26.0±5.22% of glucose uptake compared to the normal control.

In the evaluation of mechanism of action, upregulation of Glut-4 gene was demonstrated by HALH and the standard drug metformin treated cells. HALH at 500 and 250 µg/ml concentrations upregulated Glut-4 genes 0.15 folds and 0.09 folds, respectively compared to the house keeping gene (GAPDH) as the internal control. The standard upregulated Glut-4 gene 0.52 fold is compared to normal control at a concentration of 500 µg/ml. Glut-4 translocation facilitation by apigenin¹⁷ and Glut-4 activation by quercetin¹⁸ are already reported. As the presence of apigenin, kaempferol, quercetin and their glycosides such as rutin are reported, the above gene translocation can be considered as an effect of the above components.

Determination of expression of the receptor and possible involvement in insulin action/resistance in skeletal muscle, PPARγ mRNA abundance and its regulation by insulin were quantified in muscle tissue and cultures using competitive reverse transcription-polymerase chain reaction (RT-PCR), Park K S¹⁹. PPARγ agonists, regulating the expression of several genes involved in the regulation of glucose, lipid and protein metabolism and enhances the action of insulin in insulin-sensitive tissue by increasing glucose uptake in

skeletal muscle and adipose tissue and decreasing hepatic glucose production²⁰.

In the year 2013, Annapurna et al, reported the effect of apigenin on Glut-4 and PPARγ up regulation²¹. In addition to that, Murase et al., reported the upregulation of PPARγ by quercetin and gallic acid esters²².

The upregulation of PPARγ demonstrated in this experiment can be correlated with the above all cited activity contributions by the flavonoids quercetin and apigenin and their glycosides.

CONCLUSION

In this study the acclaimed antidiabetic property of *H. arifolia* was evaluated on its leaf extracts HALE and HALH. Even though the alpha amylase inhibitory activity and DPP-IV inhibitory activity results alone cannot explain the antidiabetic action demonstrated by the alcoholic extract in the animal studies reported by Nair et al⁷, we can find that the above two enzyme inhibition methods are also contributing towards the antidiabetic action. In the glucose uptake studies performed on L-6 muscle cell lines, glucose uptake facilitation by both the extracts was also demonstrated. In the evaluation of mechanism for glucose uptake, upregulation of Glut-4 genes is clearly demonstrated. MTT assay result is indicative of the non-toxic nature of the extract also. Thus, it can be concluded that, the acclaimed antidiabetic property of *H. arifolia* is true and the effect is produced by a combined effect of enzyme inhibitions such as alpha amylase inhibition, DPP-IV inhibition and also by glucose uptake facilitation in L-6 muscle cell lines. The glucose uptake by L-6 muscle cells is the most important property required for the normal activities of human beings/animals. It is facilitated by this extract and the extract is nontoxic as well. Hence, this plant can be considered as antidiabetic. Further fractionation of the extract, enrichment of the components in each fraction and more specific studies will help in the identification of a nontoxic, renewable, better and economically viable antidiabetic agent.

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