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EVALUATION OF ANTIDIABETIC ACTIVITY AND ELUCIDATION OF THE MECHANISM OF ACTIVTY OF THE NOVAL ISOLATED PENTAHYDROXY FLAVANOID OF AVERRHOA BILIMBI L. FRUIT

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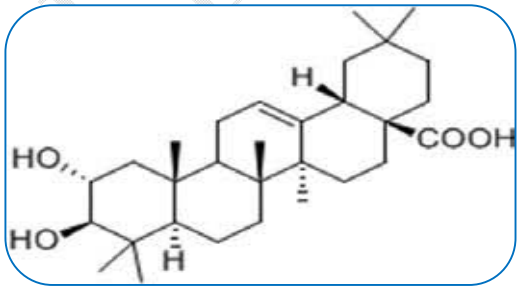
ABSTRACT :

Averrhoa bilimbi is a multipurpose long lived tropical plant. It belongs to the family Oxalidaceae. Known bilimbi or cucmber fruit commonly, the traditional system of treatment uses the different parts of this tree such as leaves and fruits to treat many of the diseases and ailments. In the present work, the pentahydroxyl flavanoid that is isolated for the first time from the fruit is evaluated for its serum glucose lowering and serum lipid lowering effects. Although the hypoglycemic effect of Averrhoa bilimbi fruit extract has been reported, the exact mechanism of this effect has yet to be elucidated. Therefore, we evaluated the effect of hot water extract of Averrhoa bilimbi fruit on glucose uptake through glucose transports in skeletal muscle cells (L-6 cell line)

KEYWORDS : *Averrhoa bilimbi , traditional system , accumulated traditional knowledge.*

1. INTRODUCTION

Plants have been used as drugs for centuries and the accumulated traditional knowledge over the years lead to the discovery of many novel drugs derived from plant based biologically active principles. Even today, vast majority of people in the world rely on indigenous medicinal plants and plant derived products for their every day health care needs. As Cunningham (1988) rightly pointed out a large proportion of the population in developing countries use traditional medicines, either as a result of the high cost of western pharmaceuticals and health care or because the traditional medicines are more acceptable from cultural and spiritual perspective **[2].** According to the reports of WHO, 70% - 95% of citizens in majority of the developing countries rely on traditional medicine as their primary source of medication (Robinson and Zhang, 2011).Nowadays, herbal drugs represent as a major component not only in traditional systems of medicine and health care but also in neutraceuticals and cosmetics industry. As reported by Gurib-Fakim (2006) [3] , one quarter of the medical prescriptions are formulations based on substances derived from plants or plant-derived synthetic analogues. Averrhoa Bilimbi is a tribal medicine belonging to the family Oxalidaceae used for Diabetes, wound healing, hypolipidemic and Anti oxidant agents and plant is botanically identified as dried fruit of Averrhoa bilimbi**.** It is known as pulima in Tamil, vilumpi in Malayalam, gommeraku in Telugu, belambu in Kannada and bilimbi in Hindi. The fruits of thi tree is reported to be used in the treatment of coughs, rectal bleeding, scurvy, diarrhea, hepatitis and inflammatory conditions. It is also considered to be an astringent and stomach refrigerant. Antidaibetic and antihyperlipidemic activity also [4,5,6]. Alkaloids, Saponins,



tanninis, falvaoinds and trterpenes are the main active constituents. Amino acids, citric acid, cyanidin–3–O–h–D–glucoside, phenolics, potassium ion, vitamin A and sugars are found to be chief phytoconstituents.[7] . Due to its diversified uses and ethnopharmacological importance, the present work has been designed to validate the traditional claims on the health beneficial effects of the selected plant with the help of modern scientific tools. The present work has been carried out on a first time isolated pentahydroxy flavanoid[1] for its antidiabeic and antihyperlipidemic activity, assessing its mechanism of activity.

2.EVALUATION OF ANTIDIABETIC ACTIVITY OF A.BILIMBI FRUIT EXTRACT

Diabetes mellitus (DM) is a serious health problem being the third greatest cause of death all over the world, and if not treated, it is responsible for many complications affecting various organs in the body [8]. The chronic hyperglycemia of diabetes is associated with long term damage, dysfunction, and failure of various organs [9]. In diabetic rats, the utilization of impaired carbohydrate leads to accelerate lipolysis, resulted in hyperlipidemia [10]. Despite the presence of known antidiabetic medicine in the pharmaceutical market, diabetes and the related complications continued to be a major problem

3. MATERIALS AND METHODS

Preparation of extracts

Fresh fruits of A. bilimbi were collected from Kottayam, Kerala, India, during the fruiting season and identified. Care was taken that the fruits, were not over-ripe, spoiled, or damaged. Fruits were shade dried and coarsely powdered.

The powder was successfully extracted with boiling water using soxhlet extractor cooled and filtered using Whatman No.1 filter paper. The filtrate was centrifuged and the sediment was discarded. The supernatant was concentrated upto 100 mL on rotary vapour evaporating apparatus under reduced pressure. The concentrated crude extract was lyophilized into powder (5 g) and used for the study.

Experimental animals

Male Wistar albino rats (180- 200 g) were used were fed with a standard diet (Sai Foods, Bangalore) and water ad libitum. Male rats were induced diabetes by injecting a single injection of 120 mg/kg body weight of alloxan monohydrate [11] intraperitonially.

Serum glucose level was checked after 72 h. Animals with serum glucose levels > 300 mg/100 ml were considered diabetic and were used for the study[12].

The diabetic rats were divided into five groups of six rats per group were compared with normal animals as tabulated below:

TABLE 1

Sl No:	Group	No. of Animals	Treatment
1	Group 1 (control)	6	Normal saline
2	Group 3 (standard Drug)	6	Alloxan(120mg/kg ip)+ Metformin (100mg/kg io)
3	Group 4	6	Alloxan + aqueous extract of A.bilimbi 100mg/kg po)
4	Group 5	6	Alloxan + aqueous extract of A.bilimbi 200mg/kg po)
5	Group 6	6	Alloxan + aqueous extract of A.bilimbi 300mg/kg po)

In single dose study, after administration of a single dose of extracts, blood samples were collected from the tail vein just prior to and 1, 2, 4 and 6 h intervals. Serum was separated and glucose levels were estimated by enzymatic glucose oxidase-method [13] .

For multi dose study, the same groups of diabetic animals were used with the same dose level once daily, up to 14 days. At intervals of 3rd, 5th, 7th, 9th and 14th day, the blood glucose level was monitored.

DATA AND STATISTICAL ANALYSIS

Effect of plant extracts on serum glucose level in fasted normal rats (single dose).

TABLE 2

Treatment	Serum glucose Serum glucose level (mg/100ml)100ml)				
	Fasting	1h	2h	4h	6h
Control	75.7±2.8	76.1±2.8^{A}	75.1 ±2.3^{A}	73.3±3.2^{A}	73.2±3.1^{A}
A.Bilimbi L.Fruit ext 100mg/kg b.w	81.4±5.6	47.0±4.6^{B*}	39.9±3.3^{B**}	43.2±3.2^{B*}	63.1±4.5^{AB*}
A.Bilimbi L.Fruit ext 200mg/kg b.w	80.5±6.1	49.1±7.3^{B*}	38.1±6.5^{B**}	41.3±5.2^{B*}	52.5± 4.4^{B*}
A.Bilimbi L.Fruit ext 300mg/kg b.w	81.3±7.3	60.1±7.1^{AB*}	57.3±6.2^{AB**}	62.2± 5.2^{A}	64.1 ± 5.1^{A}
Standard Drug Metformin 100 mg/ kg b.w	74.3±2.1	42.7±2.6^{A}	35.1 ±2.1^{A}	45.3 ±3.2^{A}	53.2 ± 3.1^{A}

Value are Mean ± SD ; n = 6 **P <0.001 are significant as compared to control : capital letters indicate between group comparison.

TABLE 3

Effect of plant extracts on serum glucose level in fasted normal rats (continuous study).

Treatment	Serum glucose level (mg/100ml)				
	3rd day	5th day	7th day	9th day	14th day
Control	75.6± 2.9	76.2 ±3.2	75.7± 3.3^{A}	75.0± 2.9^{A}	76.3± 2.1^{A}
A.Bilimbi L.Fruit ext 100mg/kg b.w	55.9 ±5.4	57.7± 6.9	55.4 ±5.7^{B*}	55.8± 5.9^{C*}	57.7± 5.3^{BC*}
A.Bilimbi L.Fruit ext 200mg/kg b.w	62.9 ±8.8	59.1 ±8.1	55.7 ± 6.4^{B*}	56.6 ± 5.5^{C*}	54.1 ± 4.8^{C*}
A.Bilimbi L.Fruit ext 300mg/kg b.w	72.8 ±6.5	69.6 ± 5.8	68./8 ±5.3^{A}	66.6± 4.2^{ABC*}	62.5 ± 4.6^{ABC*}
Standard Drug Metformin 100 mg/kg b.w	45.6± 2.9	46.2 ±3.7	45.7± 3.1^{A}	45.0± 2.9^{A}	46.3± 2.1^{A}

Value are Mean ± SD ; n = 6

*P <0.05 are significant as compared to control : capital letters indicate between group comparison

Effect of plant extracts on serum glucose level in Diabetic rats (single dose).

TABLE 4

Groups	Treatment	Serum glucose level (mg/100ml) (mg/100ml)				
		Fasting	1h	2h	4h	6h
1	Control	342.5± 22.4	347.3± 16.1	359.3 ±15.0	366.5 ±13.6	375.7 ± 6.9^{A}
2	A.Bilimbi L.Fruit ext 100mg/kg b.w	397.8± 39.9	363.0 ± 34.5	349.1 ±31.0	347 .1 ±45.1	438.5 ±71.1^{A**}
3	A.Bilimbi L.Fruit ext 200mg/kg b.w	381.8± 54.9	367.3 ±51.3	343.9 ±50.2	331.9± 49.2	334.7± 53.1^{B}
4	A.Bilimbi L.Fruit ext 200mg/kg b.w	398.8 ±22.3	389.1± 21.2	365.9 ±21.5	345.1± 20.5	354.9 ±19.3^{B}
5	Standard Drug Metformin 100 mg /kg b.w	242.5± 21.4	247.3± 16.4	259.3 ±15.7	266.5 ±13.1	275.7 ± 6.1^{A}

Value are Mean ± SD ; n = 6

**P <0.001 are significant as compared to control : capital letters indicate between group comparison.

Effect of plant extracts on serum glucose level in Diabetic rats (continuous dose).

TABLE 5

Groups	Treatment	Serum glucose level (mg/100ml)				
		3rd day	5th day	7th day	9th day	14th day
1	Control	410.2± 10.4^{A}	428.8 ±13.5^{A}	459.6 ±22.1^{A}	381.3± 23.6^{A}	321.3 ±18.3^{A}
2	A.Bilimbi L.Fruit ext 100mg/kg b.w	4033.0 ±41.7^{A}	337.2± 28.3^{B**}	290.7 ±29.9^{BC**}	252.6 ±26.4^{BC**}	239.3 ±22.9^{B**}
3	A.Bilimbi L.Fruit ext 200mg/kg b.w	302.5± 63.2^{C**}	271.9 ±53.5^{C**}	262.4 ±55.4^{C**}	253.8 ±51.9^{BC**}	240.6 ±50.0^{B**}
4	A.Bilimbi L.Fruit ext 300mg/kg b.w	368.4± 21.4^{AB*}	349.4 ±21.7^{B**}	312.5 ±15.2^{B**}	280.3 ±13.3^{B**}	228.3 ±14.0^{B**}
5	Standard Drug Metformin 100 mg/kg b.w	285.2± 10.1^{A}	278.8 ±13.7^{A}	259.6 ±21.1^{A}	281.3± 23.1^{A}	221.3 ±16.1^{A}

Value are Mean ± SD ; n = 6

**P <0.001 are significant as compared to control : capital letters indicate between group comparison.

In vitro glucose uptake studies in skeletal muscle cell line

Skeletal muscle is the main tissue involved in insulin-induced stimulation of glucose uptake. Insulin increases glucose uptake in skeletal muscle by increasing functional glucose transport molecules in the plasma membrane. Glucose transport in skeletal muscle can also be stimulated by contractile activity. The maximal effects of insulin and contractile activity on glucose transport are additive.

In skeletal muscle, both insulin and contractile activity stimulate translocation of glucose transporter GLUT-4 protein from an intracellular membrane pool to the plasma membrane. Resistance to this stimulatory effect of insulin is a major pathological feature of diabetes.

A relatively little-studied response to adrenoceptor activation is facilitation of glucose uptake. Adrenoceptors are classified into three main subtypes: α1-, α2-, and β-adrenoceptors. β-Adrenoceptor stimulation increases glucose uptake in rodent skeletal muscle and brown adipose tissue. This effect is mediated primarily by the β3-adrenoceptor in brown adipose tissue, it is also mediated by β2-adrenoceptors in skeletal muscle cells.

L6 cells represent a good model for glucose uptake because they have been used extensively to elucidate the mechanisms of glucose uptake in muscle, have an intact insulin signaling pathway, and express the insulin-sensitive GLUT4.

Although the hypoglycemic effect of Averrhoa bilimbi fruit extract has been reported, the exact mechanism of this effect has yet to be elucidated. Therefore, we evaluated the effect of hot water extract of Averrhoa bilimbi fruit on glucose uptake through glucose transports in skeletal muscle cells (L-6 cell line).

Plant extract

The collected fruits were shade dried, coarsely powdered and extracted with hot water by maceration process. The extract was filtered and concentrated in vacuum and kept in a vacuum desiccators for complete removal of solvent. Hot water extract of fruits of *A. bilmbi* was obtained with an yield of 1.24%.

Chemicals Fetal bovine serum was purchased from Invitrogen (Carlsbad, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and other culture products were purchased from GIBCO BRL (San Diego, CA, USA). TPVG solution, Bovine serum albumin (BSA),

Insulin, Metformin, Glucose kit was obtained from Randox, Dibasic sodium hydrogen phosphate, sodium bicarbonate, magnesium chloride, calcium chloride, potassium chloride, sodium chloride were from Ranbaxy Laboratories Ltd., Mohali and SD Fine Chem., Mumbai, India. All chemicals and solvents used were of analytical grade.

In vitro glucose uptake activity

Preparation of cell culture

Monolayer of L-6 cells was maintained at sub confluent conditions in growth media containing DMEM with 4.5 g/l glucose, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum. Cells were maintained in a humidified 37°C incubator with ambient oxygen and 5% CO_2. Cells were maintained in continuous passage by trypsinization of sub confluent cultures using TPVG solution.

Glucose uptake assay

Cells were cultured on 6 well plates and incubated for 48 h at 37°C in a CO_2incubator. When semi confluent monolayer was formed, the culture was renewed with serum free DMEM containing 0.2% BSA and incubated for 18 h at 37°C in the CO_2 incubator. After 18 h, the media was discarded and cells were washed with KRP buffer once. The cells were 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 thrice with 1 ml ice-cold KRP buffer. Cells were subsequently lysed by freezing and thawing thrice. 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 as follows:

Mix 10 µl of sample and 1 ml of reagent, incubate for 25 min at 15-25°C or 10 min at 37°C. Measure the absorbance of the standard ($A_{standard)}$ and the sample (A_{sample}) against the reagent blank within 60 min, the time interval from sample addition to read time must be exactly the same for standard/control and sample.

$$\text{Glucose concentration mmol / 1} = \frac{\text{Asample}}{\text{Astandard}} \times 55.5$$

$$\text{mg/dl} = \frac{\text{Asample}}{\text{Astandard}} \times 100$$

Six groups containing five wells of plate (n = 5) each were taken
Incubation medium used for glucose uptake assay in L-6 cell line
Groups are tabulated in table no:6

TABLE 6

Sl. No:	Incubation medium
Group 1	900 µl of KRP buffer and 100 µl of glucose solution (1M) (control group)
Group 2	800 µl of KRP buffer, 100 µl of Insulin (10 IU/ml) and 100 µl of glucose solution (1M).
Group 3	800 µl of KRP buffer, 100 µl of metformin (1mg/ml) and 100 µl of glucose solution (1M).
Group 4	700 µl of KRP buffer, 100 µl of Insulin (10 IU/ml),100 µl of metformin (1mg/ml) and 100 µl of glucose solution (1M).
Group 5	800 µl of KRP buffer, 100 µl of plant extract (2mg/ml) and 100 µl of glucose solution (1M).
Group 6	700 µl of KRP buffer, 100 µl of Insulin (10 IU/ml), 100 µl of Averrhoa bilimbi fruit extract (2 mg/ml) and 100 µl of glucose solution (1M).

Results

Glucose utilization in L-6 cell lines was studied in vitro. The results are given in Table. The given results show that hot water extract of fruit of Averrhoa bilimbi enhance the glucose uptake by 28.99 % over control at 200 µg/ml dose. Results were compared with insulin and metformin , which were used as the standard antidiabetic drugs.

Insulin (1IU/ml) and metformin (100 µg/ml) enhance the glucose uptake by 148.79 % and 71.50% over control. Extract was also tested with insulin to confirm any synergistic effect, but results indicate that extract does not have any synergistic effect with insulin. Extract and insulin enhance the glucose uptake in L-6 cells by 149.28% over control when used in combination.

Effect of hot water extract of fruits of A bilimbi on glucose uptake in L-6 cell line.

TABLE 7

Sl. No:	**Incubation Medium**	**% Glucose uptake over control**
Group 1	Insulin(1 IU/ml)	148.79
Group 2	Metformin (100 µg/ml)	71.5
Group 3	Insulin (1 IU/ml) + metformin (100 µg/ml)	151.69
Group 4	Averrhoa bilimbi fruit extract (200 µg/ml)	28.99
Group 5	Averrhoa bilimbi fruit extract (200 µg/ml) + insulin (1 IU/ml)	149.28

Skeletal muscle is the primary site responsible for postprandial glucose use. Furthermore, it 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 (NIDDM). GLUT4 is the major glucose transporter expressed in insulin responsive tissue such as skeletal muscle and adipose tissue, where

they respond to an acute insulin challenge by translocating GLUT4 rapidly from an intracellular membrane storage site to the plasma membrane.

The results obtained in the present study clearly demonstrate that the Averrhoa bilimbi hot water extract 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.

CONCLUSION

The results obtained from the pharmacological screening have led to the conclusions that, aqueous extract of Averrhoa bilimbi fruit have marked antidiabetic activity. Hence it can be used as such or in combination with the existing therapeutic agents for the treatment of diabetes. The mechanism of hypoglycemic activity of the noval isolated pentahydroxy flvaoid is by enhancin 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.

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