Detection of insulin-like protein and some active compounds in *Bauhinia variegata* Linn. leaf ethanolic extracts and the effect in reducing blood glucose levels in mice

كشف البروتين-شبيه الانسولين وبعض المركبات الفعالة في الخلاصة الايثانولية لأوراق نبات خف الجمل *Bauhinia variegate* Linn. والتأثير في خفض مستويات سكر الدم في الفئران

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Abstract

The study aims to detect the insulin-like protein and determine the active compounds in *Bauhinia variegata* L. leaves ethanolic extracts that help in reducing the blood glucose levels for white albino mice. The chemical detection of leaves ethanolic extract revealed the presence of tannins, terpenes, steroids, and flavonoids as active compounds. High performance liquid chromatography (HPLC) analysis method, using C18 column resulted in presence of insulin-like protein in the leaves extracts. Results showed significant reduction of blood glucose levels after 3 days of the treatment, and high reduction obtained after 6, 9, 12 days as compared with the human swine insulin used as a control. It was concluded that the crude ethanolic extracts and the partial purified insulin of *B. variegata* was used to examine the existence of insulin-like protein in this plant. In this study, an attempt has been made to report plant insulin that may be useful to the health professionals and scholars working in the field of pharmacology and therapeutics to develop alternative medicine to cure diabetes in animals and man.

Key words: *Bauhinia*, Active compounds, HPLC analysis

المستخلص

تهدف الدراسة للكشف عن البروتين-شبيه الانسولين وتحديد المركبات الفعالة في المستخلصات الايثانولية لأوراق نبات خف الجمل B*auhinia variegata* Linn. التي تساعد في خفض مستويات سكر الدم للفئران البيضاء . اظهر الكشف الكيميائي للمستخلص الايثانولي للأوراق وجود التانينات tannins، التربينات terpenes، الستيرويدات steroids، والفلافينويدات flavonoids كمركبات فعالة . استعمال العمود C18 بتحليل HPLC نتج عنه وجود البروتين-شبيه الانسولين في مستخلصات خف الجمل *B. variegate* . اظهرت النتائج انخفاض معنوي في مستويات السكر بعد 3 ايام من المعاملة وانخفاض عالي بعد 6، 9، 12 يوم بالمقارنة مع الانسولين البشري المستعمل كونترول. وقد استنتج ان الخلاصات الايثانولية الخام والانسولين المنقى جزئياً لنبات خف الجمل *B. variegata* قد استعمل لفحص وجود البروتين – شبيه الانسولين في هذا النبات . في هذه الدراسة اجريت محاولة لتوثيق الانسولين النباتي الذي ربما يكون مفيد للمحترفين في الصحة وفي حقل العلوم الصيدلانية والعلاجية لتطوير طب بديل لعلاج داء السكر في الحيوانات والانسان .

الكلمات المفتاحية : المركبات الفعالة ، نبات خف الجمل ، HPLC

Introduction

Demand for insulin will continue to grow as the incidence of diabetes increases worldwide and new delivery technologies, these new delivery technologies require a greater supply of insulin due to the increased dosing requirement of inhaled products. This reduce capital costs by 70% and cost-of-goods by more than 40% compared with current production methods, which is to achieve commercial levels of insulin expression in commercial plant system [1].

Diabetes mellitus is a systemic metabolic disease characterized by hyperglycemia, hyperlipedemia, hyperaminoacidemia, and hypoinsulinaemia it leads to decrease in both insulin secretion and insulin action. The worldwide prevalence of diabetes for all age groups was estimated to be 2.8% in 2000 and it is projected to be 5.4% in 2025. Currently available therapies for diabetes include insulin and various oral antidiabetic agents such as sulfonylureas, biguanides, α-glucosidase inhibitors and glinides [2]. Presently, there is growing interest in herbal remedies due to the side effects associated with the oral hypoglycemic agents (therapeutic agent) for the treatment of diabetes mellitus. So the traditional herbal medicines are mainly used which are obtained from plants, it plays important role in the management of diabetes mellitus [3].

Several species of herbal drugs have been described in the scientific and popular literature as having antidiabetic activity. Due to their perceived effectiveness, fewer side effects in clinical experience and relatively low costs, herbal drugs are prescribed [4, 5]. *Bauhinia variegata* named orchid tree, belongs to the family leguminosae, grows 10-12 meter tall with a spreading crown of briefly deciduous leaves which are 10-20 cm across and rounded with lobed ends and heart shaped bases. The flowers are purplish blue or even white. It is a very popular ornamental tree in subtropical and tropical climates; it blooms for several months. The genus *Bauhinia* were used in traditional medicine for their interesting biological activities such as, anti-diabetic, anti-inflammatory, antimicrobial, analgesic, astringent and diuretic effects [6]. The hypoglycemic activity of Bauhinia species has been most extensively researched in *Bauhinia forficata*, this species leaves are currently used in Brazil, in the form of aqueous extract. *Bauhinia variegata* has been used in dyspepsia, bronchitis, leprosy, ulcer, to prevent obesity, as an astringent, tonic and anthelmintic. Bauhinia contained many kinds of chemical constituents, primarily including flavonoids, steroids, terpenoids, tannins, lactones, glycolipids, glycosyl steroids and quinines. Five flavonoids isolated from the different parts of *Bauhinia* has been identified as quercetin, rutin, apigenin and apigenin 7-O-glucoside [7, 8].

The first report on the presence of insulin-like antigens in plants was published by Khanna et al. who isolated and patented an active principle called p-insulin from the fruits of *Momordica charantia*. In addition, materials resembling insulin were described in spinach, rye and *Lemna gibba*, which were recognized by broad spectrum anti-pork and anti-chicken insulin antibodies and had molecular weights, chromatographic properties and biological activities similar to those of vertebrate insulin [9].

It was reported the detection of insulin-like antigens in a large range of species utilizing a modified ELISA plate assay and Western blotting. [10]. The presence of insulin-like molecules was recently demonstrated in the leaves of *B. variegata* where a protein was found that has a partial amino acid sequence identical to that of bovine insulin. This protein may be responsible for the lowering of blood glucose concentrations when it is injected in diabetic mice [11]. It is frequently associated with the development of micro and macro vascular diseases which include neuropathy, nephropathy, cardiovascular and cerebrovascular diseases [12, 13]. Acute toxicity studies revealed the non-toxic nature of the ethanolic extracts of *B. variegata* L. LD_{50} studies indicate that ethanolic of *B. variegata* L. is safer to use in animals even at a dose of 200 mg/kg of body weight. This study aims to detect the insulin-like protein and the active compounds in *Bauhinia variegata* leaves that help in reducing the blood glucose levels, and to achieve a source of insulin to meet the future growth of insulin demand.

Materials and Methods

This study was carried out in March, 2012 in the Cllege of medicine laboratories- Baghdad University and the Center of Biotechnology-Al-Nahrain University.

Plant material and extraction method

The leaves of *Bauhinia variegata* Linn. with white flowers were collected from Al-Nahrian University gardens in March/2012. The leaves of Bauhinia plant were washed and dried at room temperature and then reduced to coarse powder using grinding machine. In order to prepare the extracts, 50 g of the powder was separately extracted with 250 ml of ethanol, stirring for 24 hours, and then the solvent was evaporated in vacuum pressure at 40 C°.[14].

Buffer preparation steps and chloroplast purification

Chloroplasts were isolated by the procedure described by Marcos and Flores [17]. Five grams of *B. variegata* leaves was gently homogenized at 4ºC in a mortar with two volumes (w/v) of a grinding buffer containing 0.35 M sucrose, 3 mM EDTA, 0.1% (w/v) BSA, 50 mM Tris-HCl, pH 7.2, and 10 mM mercaptoethanol. The homogenate was filtered through four layers of cheesecloth and the filtrate was centrifuged at 250 *g* for 10 min at 4ºC. The supernatant was then centrifuged at 3000 *g* for 10 min at the same temperature. and the pellet was resuspended in 2 mL of grinding buffer [15].

Extraction and purification of insulin–like protein from electrophoresis gel

Chloroplast proteins were extracted from 2-mm thick 15% SDS-PAGE gels by the syringe maceration extraction method [16]. After electrophoresis the band, with a mass similar to that of bovine insulin, was sliced horizontally into 0.5-cm sections. The gel slices (~1 g of gel material) were placed in a 3-mL syringe and forced through the opening into a second syringe. This procedure was repeated five times.

Next, the gel material was collected into a 2-mL test tube and 1 mL of water was added. The mixture was vortexed for 30 s and left at room temperature for 5 min. The gel material was pelleted by centrifugation at 12,000 *g* for 1 min and the supernatant was collected. Partial purification of protein-like protein was conducted due to the procedure by [12], The supernatant (400 µL) containing the chloroplast protein extracted from gels was fractionated with a 2 x 24 cm Sephadex G-50 column. The column was previously equilibrated with 0.1 M ammonium bicarbonate, pH 7.6, and eluted at a flow rate of 0.5 mL/min with the same buffer. Fractions (2 mL) were collected and absorbance at 280 nm was measured. Fractions were analyzed for the presence of insulin-like by HPLC through a C18 hydrophobic column [16].

Detection of some active compounds

Detection of tannins

The extract of *B. variegata* was boiled in a boiling water bath for 10 minutes, then filtered and the filtrate was treated with 5 drops of 1% lead acetate solution. The development of greenish-blue precipitate is an indicator for the presence of tannins [17].

Detection of terpenes and steroids

One milliliter of ethanol extract was participated in a few drops of chloroform, then 1 drop of acetate anhydride and 1 drop of concentrated sulfuric acid were added, brown precipitate appeared which representing the presence of terpene, and the appearance of dark blue color after 4-5 minutes would ensure the present of steroids [18].

Detection of flavonoids

Ethanol extract was partitioned with petroleum ether using Buckner funnel; the aqueous layer was mixed with the ammonia solution. The appearance of dark color is an evidence for the presence of flavonoids [18].

High performance liquid chromatography (HPLC) analysis

HPLC analysis was performed using the same procedure of [19]. Ethanolic and buffer extracts of *B. variegata* leaves were subjected for HPLC analysis to detect the insulin-like protein. Symmetry C18 column was used, HP-100 system (Agilent-USA), consisting of a pump with degasser, multi-wave detector (MWD). A mobile phase consisting of methanol and water (70:30), pH was adjusted to 2.5 using formic acid. The flow rate was 0.5 ml/min. and injection volume was 15 µl. HPLC identification was confirmed by comparison of retention indices with that of authentic standard human insulin [19].

Experimental animals and diabetes induction

Healthy 25 adult albino (male) mice of Swiss albino strain were obtained from the animal house of Biotechnology Research Center, Al-Nahrain University. The age of the mice was 8 weeks, and the weight was 25 gram. The animals were housed in a clean plastic cages, sterilized weekly with 70% ethanol. Five mice kept in each cage with natural 14 hours light, 10 hours dark, and a controlled temperature at 24-28C°. The animals were fed chow and water. The protocol was proved by [20]. The animals were fasted for 24 hours, then diabetes was induced by a single intraperitoneal (IP) injection of alloxan monohydrated dissolved in distilled water at a dose of 150 mg/kg of mice body weight in volume of 0.1 ml. The diabetic state was confirmed 72 hours after alloxan injection. Blood glucose value was reached 320 mg/dl which indicate hyperglycemia (while it was 120-140 mg/dl as standard before treatment), and there was 1% mortality in animals treated with alloxan [21].

Experimental groups

The animals were divided into five groups (five mice per each group); the first group, control, normal mice administrated with 0.1 ml distilled water. Second group, diabetic mice administrated with 0.1 ml of alloxan. Third group, diabetic mice administrated with 500 ug/kg b.w. of glibenclamide. Fourth group, diabetic mice administrated with 0.1 ml of *B. variegata* leaf ethanolic extract 200 mg/kg.

Blood sample collection

For 15 days after the experiment, blood samples were collected every three days (3, 6, 9, 12, 15) days, from the tail vein of the mice under the experiment, and glucose was assayed immediately using glucometer apparatus. Statistical analysis was carried out using ANOVA followed by F-test for comparison between groups. $P<0.001$ was considered as a significant.

Results and discussion

Active compounds

Table (1) revealed the presence of flavonoids, tannins, terpenes and steroids in the ethanolic and buffer extracts of *B. variegata*. The phytoconstituents present in the extracts may be responsible for antioxidant and antidiabetic activity in lowering of blood glucose levels. Tannins are commonly found in medicinal plants, and are non-toxic. They are soluble in water and alcohol. Tannins have pronounced physiological actions both internally and externally. When ingested they produce localized tissue reactions, such as reduce inflammation, relieve diarrhea, reduce swelling. A major flavonoid compound of the n-butanol fraction from *Bauhinia forficata* leaves leads to a significant hypoglycemic effect in normal and in alloxan-induced diabetic rats [17].

Earlier reports suggest that the flavonoids, steroidal compounds and tannins are responsible for anti-diabetic activity. However, preliminary phyto-chemical study reveals the presence of, steroids, flavonoids and tannins in the alcoholic extract of *Bauhinia variegata*. Thus the anti-diabetic effect produced by the extract of *Bauhinia variegata* may be due to presence of any of these active ingredients. Flavonoids are potent antioxidants that occur naturally in foods and they can inhibit carcinogenesis in rodents [22]. Many studies are in harmony with this study results which were indicated that phenolic compounds; flavonoids protect against many types of diseases. Therefore these specific phytochemicals provide a promising area of research for future human studies and potential nutraceutical for disease prevention and treatment [23].

Table (1): Detection of some active compounds in ethanol and buffer leaves extracts of *Bauhinia vareigata* Linn

Phytochemical compound	Ethanol extract	Buffer extract
Flavonoids	+	+
Tannins	+	+
Terpenes	+	+
Steroids	+	+
(+) present		

HPLC analysis

The HPLC analysis method resulted first in a chromatogram with one clear peak for standard human insulin at retention time of 15.698 and peak area of 95.935 Figure (1), also it was resulted in a chromatogram with three distinct peaks (Figure 2), one being the insulin-like protein peak at retention time of 15.240 and peak area of 76.552, and the other being some compounds found in *B. variegata* ethanolic extract. Figure (3) illustrates calibration curves for the insulin-like protein from *B. variegata* buffer extract at retention time of 15.172 and peak area of 67.658.

These results showed that insulin like protein was found in ethanolic and buffer extracts that prepared from the leaves of *B. variegata* plant. There were no significant differences $p< 0.01$ between the curves as determined by ANOVA with repeated measures. A new method for the economical manufacture of biopharmaceuticals from oilseeds. Plant-derived insulin accumulates to significant levels in transgenic seed and can be enzymatically treated *in vitro* to generate a product, the biological activity of this product *in vivo* and *in vitro* was demonstrated using an insulin tolerance test in mice and phosphorylation assay performed in a mammalian cell culture system, respectively [24].

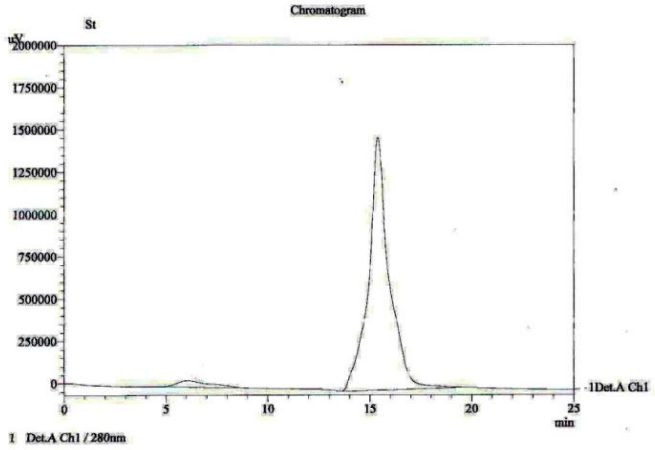

Fig. (1): Sample chromatogram for insulin by HPLC techniques. Single peak represent standard human insulin

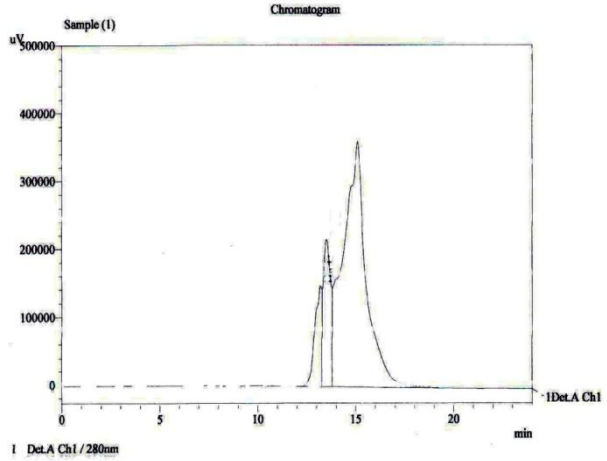

Fig. (2): Sample chromatogram for insulin by HPLC techniques. Peak represents insulin in ethanolic extract of *Bauhinia variegata* leaves

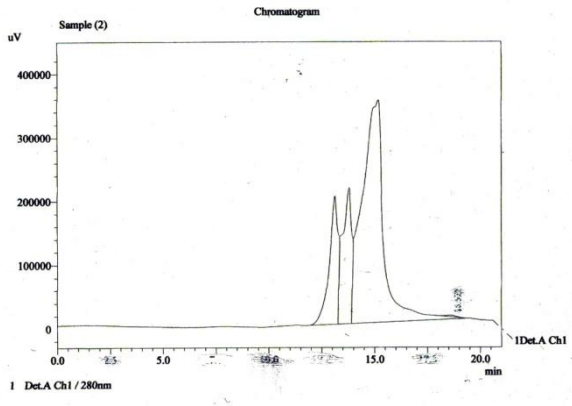

Fig. (3): Sample chromatogram for insulin by HPLC techniques. Peak represents insulin in ethanolic extract (Buffer preparation) of *Bauhinia variegata* leaves

Table (2) shows that the daily treatment with *B. variegata* ethanolic extract of 200 mg/kg b.w. led to a significant reduction in the blood glucose levels after 3, 6, 9 days of the treatment which recorded 194.3, 141.3, 121.6 mg/dl respectively. The effect seems to reach maximum on 12th day of the treatment period 119.6 mg/dl with ethanolic extract and became stable after 15 days of treatment which recorded

121.0 mg/dl. Significant reduction in the blood glucose level was observed as compared to the normal group 117.6 mg/dl and diabetic group 339.0 mg/dl after 15 days and glibenclamide treated group with gradually reduction till reached 119.3 mg/dl at the end of the test period 15 days.

These results showed that *B. variegata* leaves ethanolic extract have hyperglycemic activity as compared to the drug glibenclamide, and there were no significant differences appeared when ethanolic extract used in this experiment. The effect of the insulin-like protein and the active compounds such as flavonoids that found in *B. variegata* leaves on glucose levels in the serum of diabetic mice caused a significant decrease in blood glucose levels in diabetic mice similar to that reported on the hypoglycemic activity of *Bauhinia variegate* in diabetic patients by [25]. Several published study showed that streptozotocin-diabetic rats treated with a decoction of *Bauhinia forficata* leaves presented a significant reduction in serum and urinary glucose as compared to streptozotocindiabetic controls [26]. Another study, utilizing the oral administration of an n-butanol fraction of *B. forficata* leaves showed a significant decrease in blood glucose levels in normal and diabetic rats [27].

Table (2): Effect of *Bauhinia variegata* leaves ethanolic extract on reducing blood glucose levels of white albino mice after different treatments

Group/treat. (n=5)	Dose	Blood glucose level (mg/dl) average					
		0 Day	3 days	6 days	9 days	12 days	15 days
Normal mice (control)	0.1 ml distilled water	118.6	120.0	116.6	119.3	118.6	117.6
Induced diabetic mice with alloxan (control)	0.1 ml (150 mg/kg)	330.3	335.3	329.0	336.3	339.0	338.3
Diabetic mice (glibenclamide)	0.1 ml (500 µg/kg)	326.0	202.0	132.3	121.6	121.3	119.3
Diabetic mice (ethanolic extract)	0.1 ml (200 mg/kg)	320.0	194.3	141.3	121.6	119.6	121.0
F-test		3159.5	1325.7	1498.9	8526.3	10288.8	14390.5
P-value		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

N= number of mice for each treatment,

In view of the results reported here and of others previously published, it is tempting to speculate that insulin in chloroplasts may be involved in carbohydrate biosynthesis and transport [28]. Insulin in plant exhibit metabolic functions as those of animal insulin by promoting several metabolic activities through glucose transportation into the cell and by phosphorylating proteins regulating carbohydrate metabolism as evidenced by many studies. However, isolated Insulin-like protein is found to have synergistic effect in reducing blood glucose level in experimental animals and most of the insulin-like prptein of different plant species shows peptide sequence homology with insulin. Plant insulin ingested together with protease inhibitors is protected from hydrolysis in the digestive tract, crosses the intestinal barrier and promotes lowering of blood glucose levels [29].

The leaves of plants of many *Bauhinia* species are used in antidiabetic treatments by many populations of the world. The presence of insulin-like molecules was recently demonstrated in the leaves of *B. variegata* where a protein was found that has a partial amino acid sequence identical to that of bovine insulin. This protein may be responsible for the lowering of blood glucose concentrations when it is used in treatment of diabetes [30]. Insulin-like protein from the leaves of *B. variegata* was detected in the chloroplasts by immunocytochemical techniques. Its presence in these organelles was also confirmed by its detection in chloroplasts isolated by centrifugation, gel filtration chromatography and RP-HPLC [31].

It was concluded that the crude ethanolic extract and the partial purified insulin-like protein of *B. variegate* was used to examine the existence of insulin-like protein in this plant using HPLC techniques. For this, therapies developed along the principles of western medicine (allopathic) are often limited in efficacy, carry the risk of adverse effects, and are often too costly, especially for the developing world. Therefore, treating diabetes mellitus with plant derived compounds which are accessible and do not require laborious pharmaceutical synthesis seems highly attractive. In this research, an attempt has been made to report that plant insulin-like protein and the active compound in this plant may be useful to the

health professionals, scientists and scholars working in the field of pharmacology and therapeutics to develop evidence-based alternative medicine to cure diabetes in man and animals.

References

1. Pereira, R. C.,Oliveira, M. TR. and Lemos, G. CS. (2004). Plantas utilizadas como medicinais no municipio de Campos de Goylacazes – RJ. Rev. Brsa. Farmacogn. 14(1): 37-40.
2. Thiruvenka, R. and Jayakar, B. (2010). Anti-Hyperglycemic and Anti-Hyperlipidaemic activities of *Bauhinia*. Der Pharmacia Lettre. 2(5): 330-334.
3. Sharma, R. K., Rajani, G. P. and Komala, V. S. (2011). Effect of Ethanolic and Aqueous Extracts of *Bauhinia Variegata* Linn. On Gentamicin-Induced Nephrotoxicity in Rats. Ind J. Pharm. Edu. Res. Vol 45/ Issue 2.
4. Rajani, G. P. and Purnima, A. (2009). *In vitro* antioxidant and antihyperlipidemic activity of *Bauhinia variegata* Linn. Indian J. Pharmacol. 41(5)227-32.
5. Ghaisas, M. M., Shaikh, S. A. and Deshpande, A. D. (2009). Evaluation of the immunomodulatory activity of ethanolic extract of the stem bark of *Bauhinia variegata* Linn. Int. J. Green Pharm. 3:70-4.
6. Chu, W. L. (2005). SemBioSys Reports Positive Preclinical Results of Plant Produced Insulin. In the Plant Biotechnology Journal.
7. Silva, K. L. and Cechine-Filho, V. (2002). Plantas do genero Bauhinia: Composicao quimica epotencial farmacologico. Quim. Nova. 25: 121-125.
8. Rajani G. P., Purnima, A. (2009). *In vitro* antioxidant and antihyperlipidemic activity of *Bauhinia variegata* Linn. Indian J Pharmacol. 41(5)227-32.
9. Garcia Flores, C., Aguilar, R., Reyes, de la Cruz H., Albores, M., Sanchez de Jimenez, E. A. (2001). Maize insulin-like growth factor signals to a transduction pathway that regulates protein synthesis in maize. Biochem J. 358: 95-100.
10. Barbosa-Filho, JM., Vasconcelos, THC., Alencar, AA., Batista, LM., Oliveira, RAG., Guedes, DN., Falcao, HS., Moura, MD., Diniz, MFFM. and Modesto-Filho, J. (2005). plants and their active constituents from south, Central, and North America with hypoglycemic activity. Rev. Bras. Farmacogn. 15: 392-413.
11. Azevedo, C. R. (2003). Caracterização parcial de insulina de folhas de *Bauhinia variegata*. [Master's thesis]. Campos dos Goytacazes: Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense.
12. Feldman, J. M. (1988). In Diabetes Mellitus, 9th ed. Indianapolis, Eli Lilly & co. pp: 28-42.
13. Strojek, K. (2003). Features of macrovascular complications in type 2 diabetic patients. Acta Diabetologica. 40, 334–337.
14. Akueshi, C. O., Kadiri, C. O., Akueshi, E. U., Agina, S. E. and Gurukwem, B. (2001). Antimicrobial potential of *Hyptis sauvedens* Poit (Lamiaceae), Nigeria. J. Bot. 15: 37-41.
15. Marcos, J. F. and Flores, R. (1990). Subcellular location of avocado sunblotch viroid in avocado leaves. Plant Sci. 67: 237-244.
16. Scheer, J. M., Ryan C. A. (2001). A method for the quantitative recovery of proteins from polyacrylamide gels. Anal Biochem. 298: 130-132.
17. Evans, W. C. (1989). Pharmacognosy 13th (Eds) Balliere Tindal, London. 419-420.
18. Harborne, J. B. (1984). Phytochemical methods. A guide to Modern Technique of Plant Analysis, Chapman Hall, London.
19. Almela, L., Sanches-Muno, B., Frenandez-Lopez, J., RRoca, M. and Rabe, V. (2006). Liquid Chromatographic-Mass Spectrometric analysis of phenolic and free radical scavenging activity of rosemary extract from different raw materials. J. Chromatog. 1120(1-2):22-229.
20. Institutional Animal Ethical Committee JKKMMRF/CP/PhD (2008).
21. Mukhtar, H. M., Ansari, S. H., Ali, M., Naved, T. and Bhat, Z. A. (2004). J. Nat. Remed. 4 (2): 150-154.
22. Dhanabal, S. P., Kokate, C. K., Ramanathan, M., Elango, K., Kumar, E. P. and Subburaj, T. (2004). Indian Drugs. 41(11): 690-695.
23. National Center for Complementary and Alternative Medicine. (2012). Antioxidant Supplements for Health: An Introduction. Web. March 18.
24. Cory, L. N., Joseph, G.. B., Elizabeth, W. M., Richard, G. K., Goren, H. J., Nancy, A. M. and Maurice M. M. (2005). Transgenic expression and recovery of biologically active recombinant human insulin from *Arabidopsis thaliana* seeds. Plant Biotechnology Journal, Blackwell Publishing Ltd.3.

25. Azevedo, C. R., Maciel1, F. M., Silval, L. B., Ferreira1, A. T. S., Cunha, M. and Machado, O. L. T. (2006). Isolation and intracellular localization of insulin-like proteins from leaves of Bauhinia variegata. Braz J Med Biol Res. 39(11):1435-1444.

26. Silva, F. R., Szpoganicz, B., Pizzolatti, M. G., Willrich, M. A. and de Sousa, E. (2002). Acute effect of *Bauhinia forficata* on serum glucose levels in normal and alloxan-induced diabetic rats. *J* Ethnopharmacol. 83: 33-37.

27. Carpi, A., Di Maira, G., Vedovato, M., Rossi, V., Naccari, T., Floriduz, M. (2002). Comparative proteome bioinformatics: identification of a whole complement of putative protein tyrosine kinases in the model flowering plant Arabidopsis thaliana. Proteomics. 2: 1494-1503.

28. Sanchéz-de Jiménez, E., Beltrán-Penã, E., Ortíz-López, A. (1999). Insulin stimulated ribosomal protein synthesis in maize embryonic axes during germination. Physiol Plant. 105: 148-154.

29. Chiou, T. J. and Bush, D. R. (1996). Molecular cloning, immunochemical localization to the vacuole, and expression in transgenic yeast and tobacco of a putative sugar transporter from sugar beet. Plant Physiol. 110: 511-520.

30. Silva, K. L. and Cechinel, V. (2002). Plantas do gênero *Bauhinia*: composiçãoquímica e potencial farmacológico. Quim Nova. 25: 449-454.

31. Pepato, M. T., Keller, E. H., Baviera, A. M., Kettelhut, I. C., Vendramini, R. C. and Brunetti, I. L. (2002). Anti-diabetic activity of *Bauhinia forficata* decoction in streptozotocin-diabetic rats. J. Ethnopharmacol. 81: 191-197.