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Production and Utilization of Insulin from Stevia Leave (*Rebaudiana Bertoni*) for the Treatment of Diabetes Mellitus

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Abstract

Stevia (*Rebaudiana bertoni*) leave, is an ancient perennial shrub mostly found in South America in countries like Paraguay and Brazil. It contains a low-calorie sweetener, which is about 300 times sweeter than sucrose. Diabetes mellitus is among the common metabolic disorders affecting about 2.8% of the world's population and is reported to reach 5.4% by the year 2025. In this work, insulin was successfully extracted from *Rebaudiana bertoni* to serve as an alternative to bovine insulin extracted from animals which is devoid of the risk of transferring infectious diseases from the animals to the diabetic patient as is the case with convectional animal-based insulin. Gas Chromatography-Mass Spectroscopy (GC-MS) and Fourier Transform InfraRed (FTIR) analysis were used to analyze and determine the functional groups available in the isolated compound and the results shows that it has retention time of 14.11 min; abundance/peak area (% PA) of 86.613 %; mass number (m/z) of 413 g/mol; molecular peak ion of 149; fragmentation patterns of 7; and $C_{20}H_{29}BrO_4$ as molecular formula and phthalic acid, 8-bromoctyl butyl ester as IUPAC name. The insulin was tested on three groups of rats (*Rattus norvegicus*) whereby the glucose level was monitored, and the result shows that the extracted insulin was found to be effective in addition to having antidiabetic effect.

Keywords: Insulin, Diabetes mellitus, *Rebaudiana bertoni*, Stevia leave, *Rattus norvegicus*, Phthalic acid

Production and Utilization of Insulin from Stevia Leave (Rebaudiana Bertoni) for the Treatment of Diabetes Mellitus

Introduction

Stevia (*Rebaudiana bertoni)*, is an ancient perennial shrub generally found in South America in countries including Paraguay and Brazil (Chang *et al.*, 2005). It contains glycosides considered as low calorie sweeteners, which is about 300 times sweeter than sucrose (Ilca *et al.*, 2017; Lemus-mondaca *et al.*, 2012; Witono and Chandra, 2020). The leaf extract of Stevia possesses many phytochemicals, which include austroinullin, β-carotene, dulcoside, nilacin, rebaudioxides, riboflavin, steviol, stevioside, and tiamin with known antimicrobial properties against many pathogens (Theophilus *et al.*, 2015). Stevia extracts have therapeutic properties, antioxidant effect, antimicrobial and antifungal activity. (Lemus-mondaca *et al.*, 2012) reported different techniques for the extraction of stevioside from stevia leaves which include solvent extraction, chromatographic adsorption, ion exchange, selective precipitation, membrane processes and supercritical fluids. Diabetes mellitus is among the common metabolic disorders affecting about 2.8% of the world's population and is reported to reach 5.4% by the year 2025 (Eddouks *et al*., 2014; Patel *et al*., 2012). Insulin is a peptide hormone that is isolated from animal pancreas and is the main hormone for glucose regulation, fats and proteins (Koona *et al.,* 2010). The existence of the protein hormone insulin in plants is not widely researched in literature, however, reviews show the presence of insulin in plants, which has continued to receive attention among researchers (Xavier-filho *et al.*, 2003).

Insulin is isolated from the pancreas of animals which might carry along infectious diseases to the patient. The use of insulin extracted from Stevia leaves for the treatment of diabetes could be safe and non-carcinogenic compared to the use of bovine insulin and synthetic drugs which results in many complications. Insulin extracted from stevia leave will serve as a substitute or alternative to insulin extracted from pancreas of animals, with minimal risk of carrying along infectious diseases from the pancreas of animals to diabetic patients. Insulin is from Latin word "insula", meaning "island". It is a peptide hormone that is produced by beta cells of the pancreatic cell, considered to be the main anabolic hormone of the body (Sangeetha and Vasanthi, 2009). It promotes the absorption of glucose from the blood into liver, fat and skeletal muscle cells. In these tissues, the absorbed glucose is converted into either glycogen via glycogenesis or fats (triglycerides) via lipogenesis, or, in the case of the liver, into both (Ahmed, 2020). Diabetic patient cannot produce or properly use insulin, as they have high blood glucose (Anwer *et al.*, 2012). Traditionally, insulin is isolated from animal pancreas, but recent researches reveal that insulin are present in plant, bacteria and even fungi. Some drawbacks of isolating insulin from animal pancreas includes the following and as such, a substitute is paramount: (a) only 1-pound pure insulin is gotten from animal source, by killing 10,000 animals, (b) if the pancreas of the animal is infected by some diseases like cancers etc., there is always a probability of it being carried along with the insulin and (c) plant cultivation offers higher percentage of insulin than animal extracted insulin which is costly.

The genus Stevia, consists of about 200 species of herbaceous, shrub and sub-shrub plants (Shivanna *et al.*, 2013), and is one of the most distinctive genera within the tribe eupatorieae, because of the morphological uniformity of its flowers and capitula, which consist of five tubular flowers and five involucral bracts. It is distributed from the southwestern United States southward through Mexico and Central America. It also occurs from non-Amazonian South America, southward to Central Argentina. In Brazil, more than 30 species have been found, distributed mainly in southern and central regions. *Rebaudiana bertoni* originated in the highland regions of northeastern Paraguay (on the Brazilian border), between latitudes 23^{o} and 24^{o}, where the unique sweetening power of its leaves and its medicinal properties have been

known by the local Guarani Indians many centuries. The first seeds were exported to the United Kingdom where it could not be brought under cultivation. In 1968, it was exported to Japan, and from there awareness of and cultivation of the plant spread throughout the world. Subsequently, the crop has been introduced to many countries, including Brazil, Korea, Mexico, the United States of America, Indonesia, Tanzania, Canada and India *(Claudio Gardana et al.*, 2003; Ferrazzano *et al.*, 2015; Jamil *et al*., 2020; Yadav *et al*., 2011). *Rebaudiana bertoni* is commonly known as candyleaf, sweetleaf or sugarleaf (Jamil et al., 2020). In other terms, it is also called sweet lead flowering plant in the aster family (asteraceae), normally grown for its sweet taste (Ahmed, 2020). Stevia is a small perennial shrub that grow up to 65-80 cm in height, with directly, oppositely arranged leaves. It may be found on the banks of swamps, on infertile, acidic sands or muck soils. Stevia is a semi-humid subtropical plant that can be grown easily even in kitchen garden just like any other vegetable crop. The shrub grows in a well-drained red sandy loam soil with pH range of 6.5-7.5 as well as on dark, damp, sandy soils (highly-permeable soils). However, (Goyal et al., 2010) suggest that saline soils be avoided when cultivating the plant. In addition and according to (Kobus-Moryson and Gramza-Michałowska, 2015; Yadav et al., 2011), *Rebaudiana bertoni* grows naturally on subtropical meadows at an altitude ranging from 200–500m above sea level, with temperatures ranging from –6-43°C, and an average temperature of 23°C. Rainfall requirement of stevia is between 1500–1800 mm. The objectives of the research are to recover insulin from the stevia leave extract, characterize the insulin using GC-MC and FTIR analysis and test the insulin effectiveness on rats during diabetic treatment.

Material and Equipments

Materials and equipment that were used in the experiment are shown in Table 1.

Table 1: Materials and Equipment Used

Material/Equipment	Model	Company
Stevia (*Rebaudiana bertoni)*	-	-
Rat	-	-
Cages	-	-
Saw dust	-	-
Beaker	-	Tianjin Glass Instrument Factory
Measuring Cylinder	-	Tianjin Glass Instrument Factory
Separating Funnel	-	Tianjin Glass Instrument Factory
Conical Flask	-	Tianjin Glass Instrument Factory
Glass Rod	-	Tianjin Glass Instrument Factory
Syringe	-	-
Mortar	-	-
Pestle	-	-
Spatula	-	-
Water bath	XMTD-4000	Zhengzhou Keda Machinery Co., Ltd
Furnace	SX 25 10	PEC Medical USA
Digital weighing Balance	MT-5000D	Metlar, India
Capillary tube	75mm Length	Smart Diagnosis, China
Glucometer	XEG349-P2B7C	Abbott Diabetics care Ltd, UK
TLC plate	60 F254	E. Merck, Darmstadt Germany

GC-MS	QP-2010 PLUS	Shimadzu Europa GmbH, Germany
FTIR	8400S	Shimadzu Europa GmbH, Germany

Reagent

The reagents that were used in the experiment is shown in Table 2.

Table 2: Reagents and Manufacturer Information

Reagent	Purity	Manufacturer
Ethyl acetate	A.R	JHD Chemical Reagent Co. ltd.
N-Hexane	99%	BDH, VWR International Limited.
Silica Gel 60-120 mesh	-	Qualikems Laboratory Reagent
Alloxan monohydrate	98%	Sigma Aldrich
Distilled Water	-	-
Tween 20	-	Sigma Aldrich

Sample Collection and Seedlings Transplanting

Seedlings of stevia were obtained from Raw Materials Research and Development Council (RMRDC) Abuja. Plate 1 shows the seedlings of stevia obtained from the Council and its transplanting in a sandy loam soil 35×120cm in size.

Plate 1: (a) Seedlings of Stevia Plant and (b) Transplanted Stevia Plant in a Sandy Loam Soil

IRRIGATION

Irrigation was done immediately after transplanting. Water was applied to the plant frequently three times a day, in the early morning, late morning and night. Plate 2 demonstrates how the stevia plant were irrigated in the night and a depiction of the farm after its irrigation.

Plate 2: (a) Irrigation of the Plant at Night and (b) Afterwards of the Plant Irrigation

Weeding and Insect Management

Weeding was done at interval of 2 weeks. Normally, the control of weed is important as the growth of weed limits the plant growth. Insecticide was applied on the plants to stop the spray of disease.

Manure and Fertilizer Application

Farm yard manure was applied moderately to the plant after 21 days of transplanting. Plate 3 shows the manure that was used on the stevia plant.

Plate 3: Farm Yard Manure

Fertilizer (N.P.K) were applied after 30 days of transplanting because nutrient management was very vital in stevia and it yields higher leaf.

Identification of Plant and Pretreatment of Sample

The plant collected was identified at the herbarium of Faculty of Pharmacy, University of Maiduguri, Nigeria with the Voucher No. of UMM/FPH/ASR/005. The leaves were harvested after growth and cut into pieces manually, air dried in the laboratory and grinded using pestle and mortar. The ground powder was stored in an air tight container.

Extraction

Cold maceration technique was adopted; where 25 g of the powered sample was put into separating funnel, mixed with 250 mL of ethyl acetate as shown in Plate 4a and left for 48 hours.

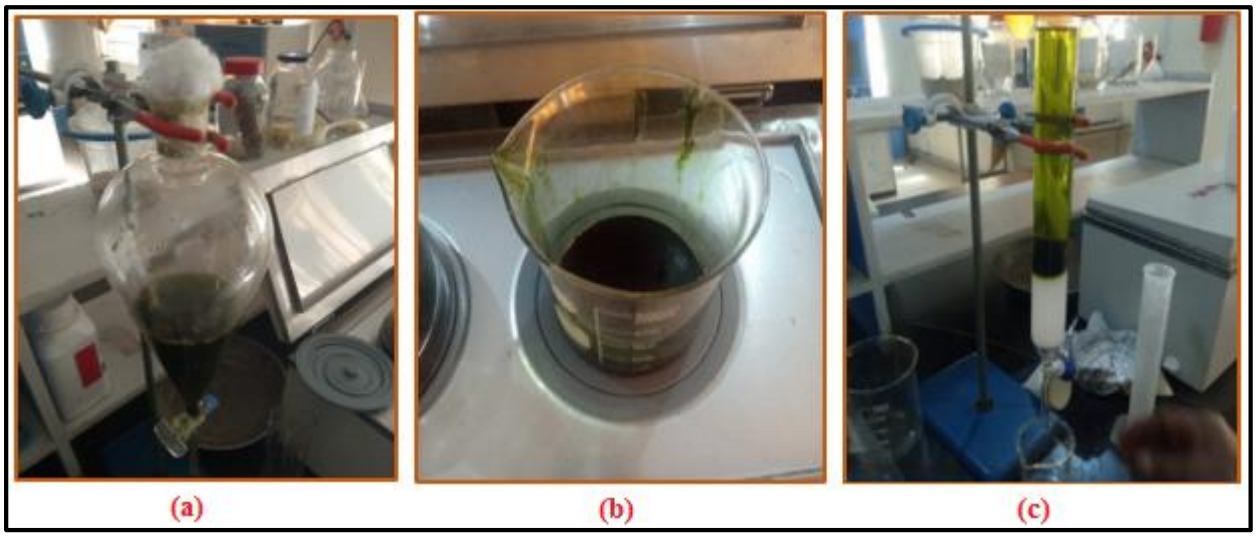

Plate 4: (a) Cold Maceration of Stevia Powder and Ethyl acetate, (b) Evaporation of Solvent from Extract using Water bath and (c) Isolation of Compounds by Column Chromatography

The extract was collected in a 1000 mL beaker and subjected to heat at 64.7℃ to evaporate the solvent as shown in Plate 4b. A batch of 4.2g of the extract was mixed with 10g of silica gel 60-120 mesh and dropped into glass column as shown in Plate 4c. Solvent system of 7:3 of n-hexane and ethyl acetate was used. TLC was then used to isolate the insulin from the extract. The samples were spotted in the form of a band (3.0 mm) with disposable capillary tube on TLC aluminum plate precoated with silica gel 60-120 mesh as in Plate 5. The development was carried out in linear ascending manner through glass chamber (20 × 10 cm) saturated with n-hexane:ethyl acetate (7:3). The optimized chamber saturation time for mobile phase was 20 min at room temperature and the chromatogram was developed up to the length of 72 mm. Subsequent to the development, the TLC plate was air dried. The plate was kept in furnace at 100°C for 5 min followed by air drying to visualize the chromatogram.

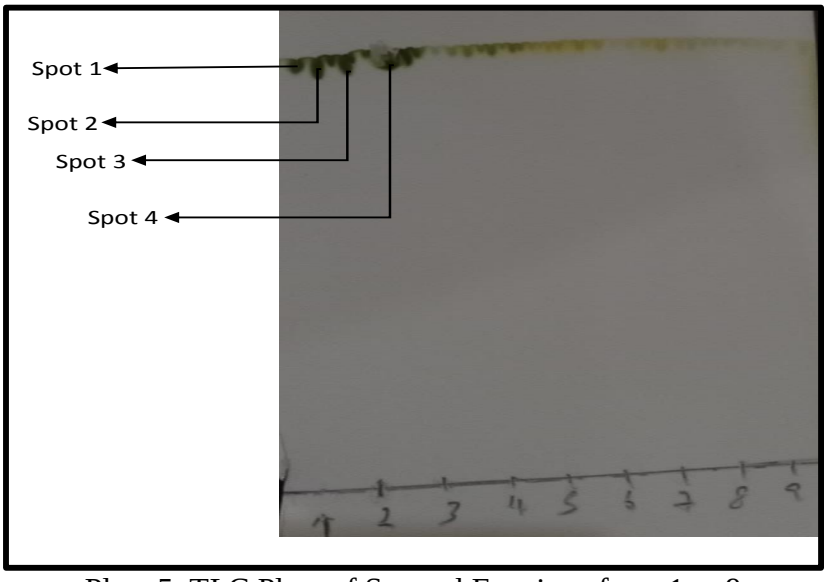

Plate 5: TLC Plate of Spotted Fractions from 1 to 9

Retention factor was used for analyzing and comparing of different substances in the isolation process. It provided collaborative evidence as to the identity of a compound. Spot 1-4 has the same compound based on the profile on TLC as seen in Plate 5 and it has R_f of 0.88 as calculated in Appendix I. It was named Compound A. No other spot was observed, thus

indicating that no other compounds were found. Compound A was further purified to give pure isolate.

Purification

The compounds isolated were further purified using thin layer chromatography to obtain a pure isolate as demonstrated in Plate 6a. The development was carried out in linear ascending manner through glass chamber (20 × 10 cm) saturated with n-hexane**:**ethyl acetate (8:2) which was observed and purified based on the TLC profile shown in Plate 6b.

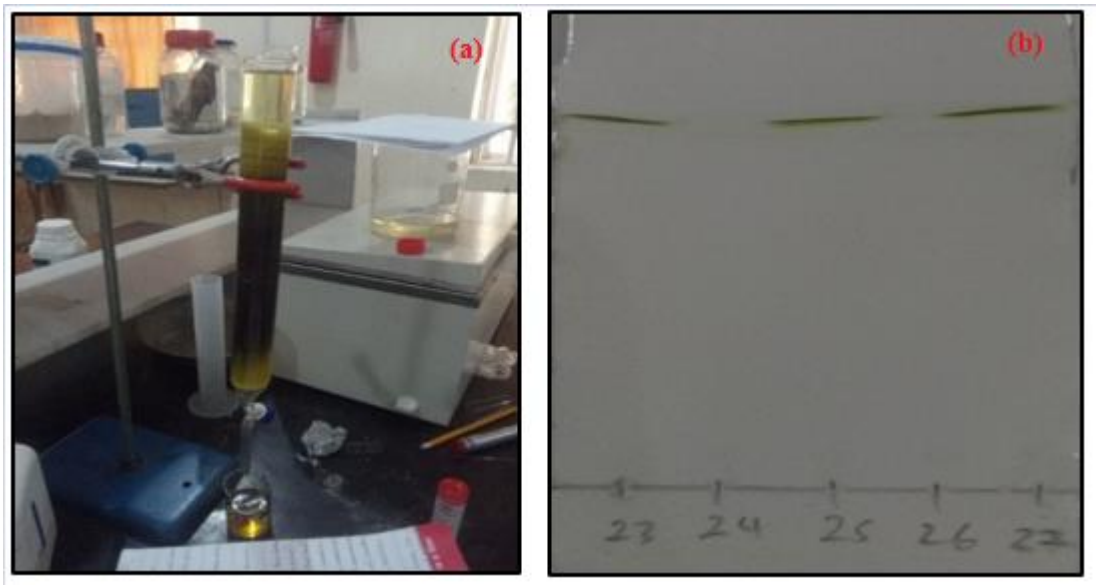

Plate 6: (a) Purification of Isolates and (b) Spotted TLC Plate of Pure Isolated Compound A

Characterization

FTIR analysis was conducted on the extracted insulin using FTIR 8400S Spectrophotometer, GC-MS analysis was conducted on the extracted insulin using GC-MS-QP2010 PLUS.

Test for Effectivity

Four rats (*Rattus norvegicus)* were grouped into; Group 1, Group 2, Group 3 and Group 4 as shown in Plate 7a. The weight of each group was taken as shown in appendix I and the glucose base level of each group was determined using glucometer. Distilled water was given to Group 1 as normal control group and alloxan monohydrate was induced to Group 2, Group 3 and Group 4 as described in Plate 7b, based on their weight to rise the glucose level to diabetic. Thereby the insulin extracted was administered to diabetic Groups as shown in Plate 7c and the glucose level was monitored after 1 hr, 3 hrs, 6 hrs, 8 hrs, 24 hrs and 48 hrs.

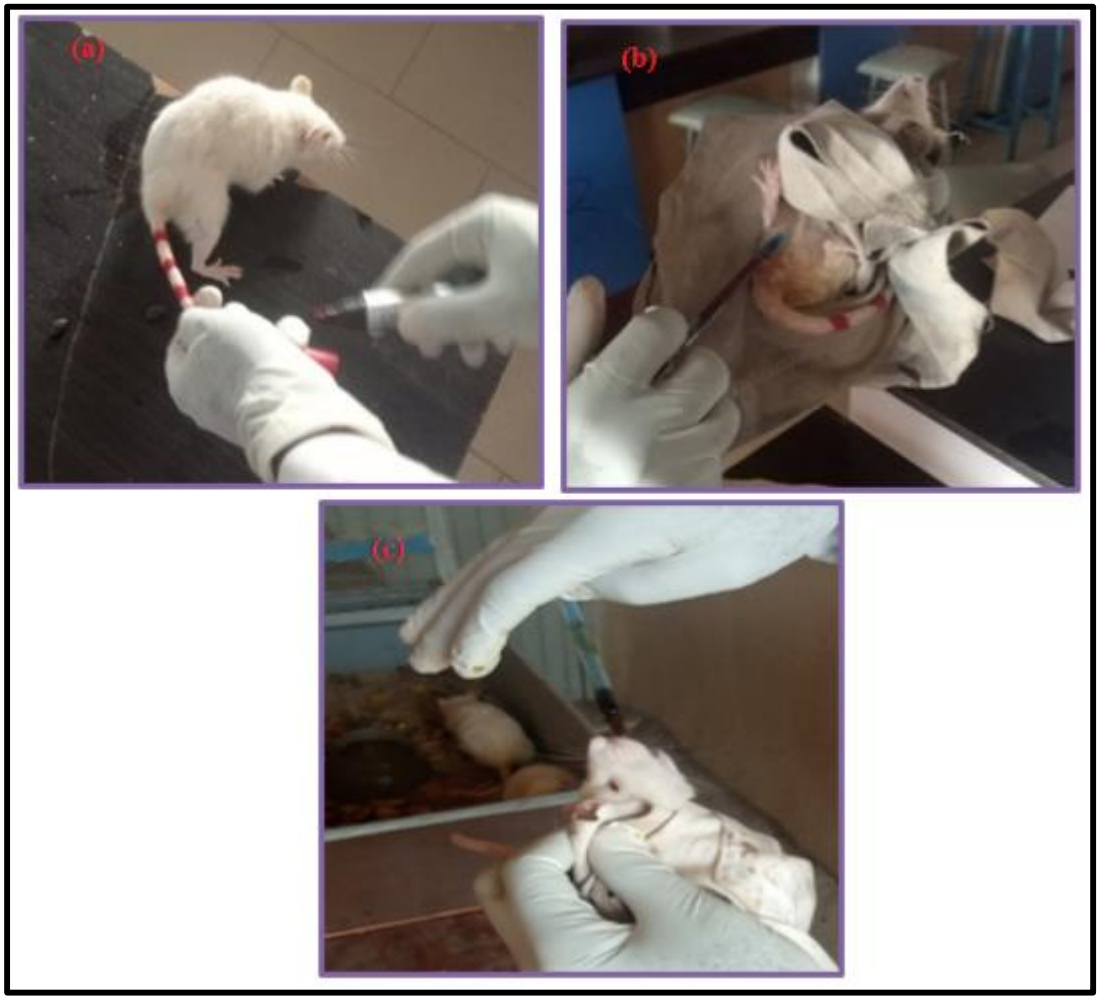

Plate 7: (a) Labelling/Grouping of Rats, (b) Inducing Alloxan monohydrate to Rat in Group 2 and (c) Administration of Insulin to Rat of Group 3

Results and Discussion

Functional Group Analysis

Fourier Transform InfraRed (FTIR) spectroscopy was used to analyze and determine the functional groups available in the compound isolated. FTIR analysis was conducted on pure isolated stevia compound and the result of the analysis is depicted in Figure 1.

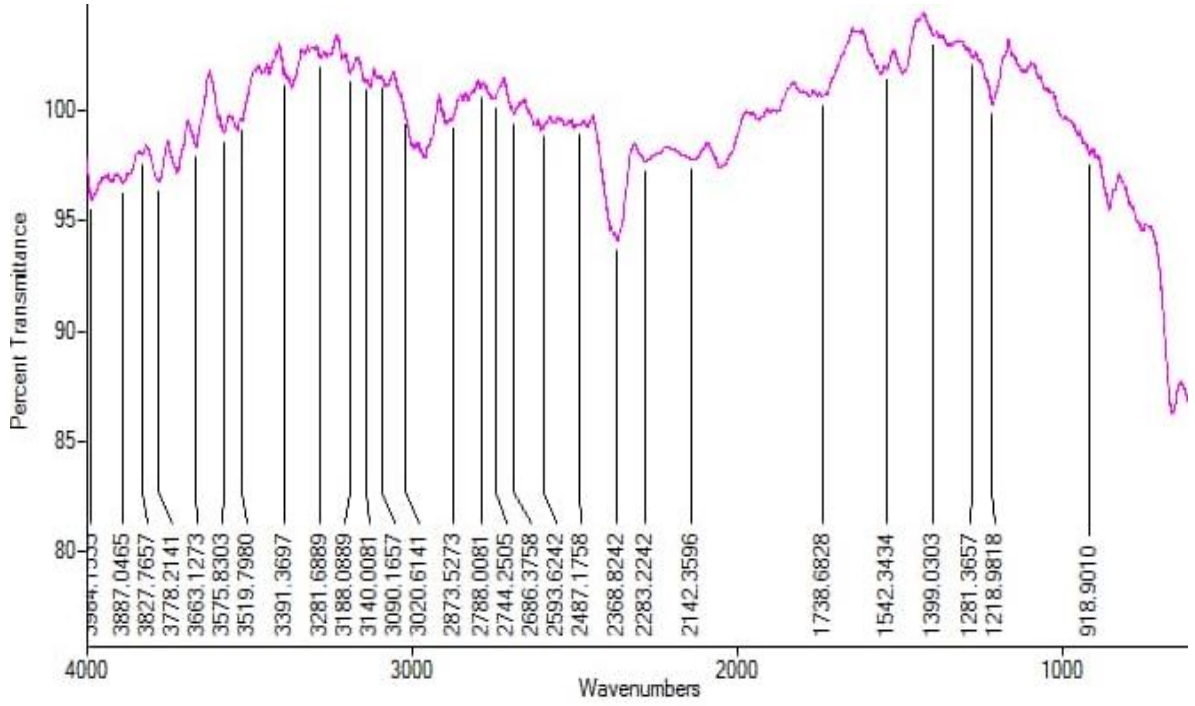

Figure 1: FTIR Analysis of Pure Isolated Compound

In Figure 1, the peaks in the region of 4000 cm^{-1} to 3000 cm^{-1} wavelengths represent aromatics and double bonds carboxylic acids which are characteristics of most insulin-like compounds from plants. Region between 2000 cm^{-1} to 1000 cm^{-1} wavelength represent the functional groups present in the compound for aromatics, carboxyls and bromide. The analysis was carried out in accordance with (Berthomieu and Hienerwadel, 2009; Franca and Oliveira, 2011) .

Gas Chromatography Mass Spectrometry (GC-MS)

GC-MS was used to identify different substances or components in a sample. GC-MS analysis was conducted on the pure isolated stevia compound where the result of the analysis was depicted in Figures 2 and 3.

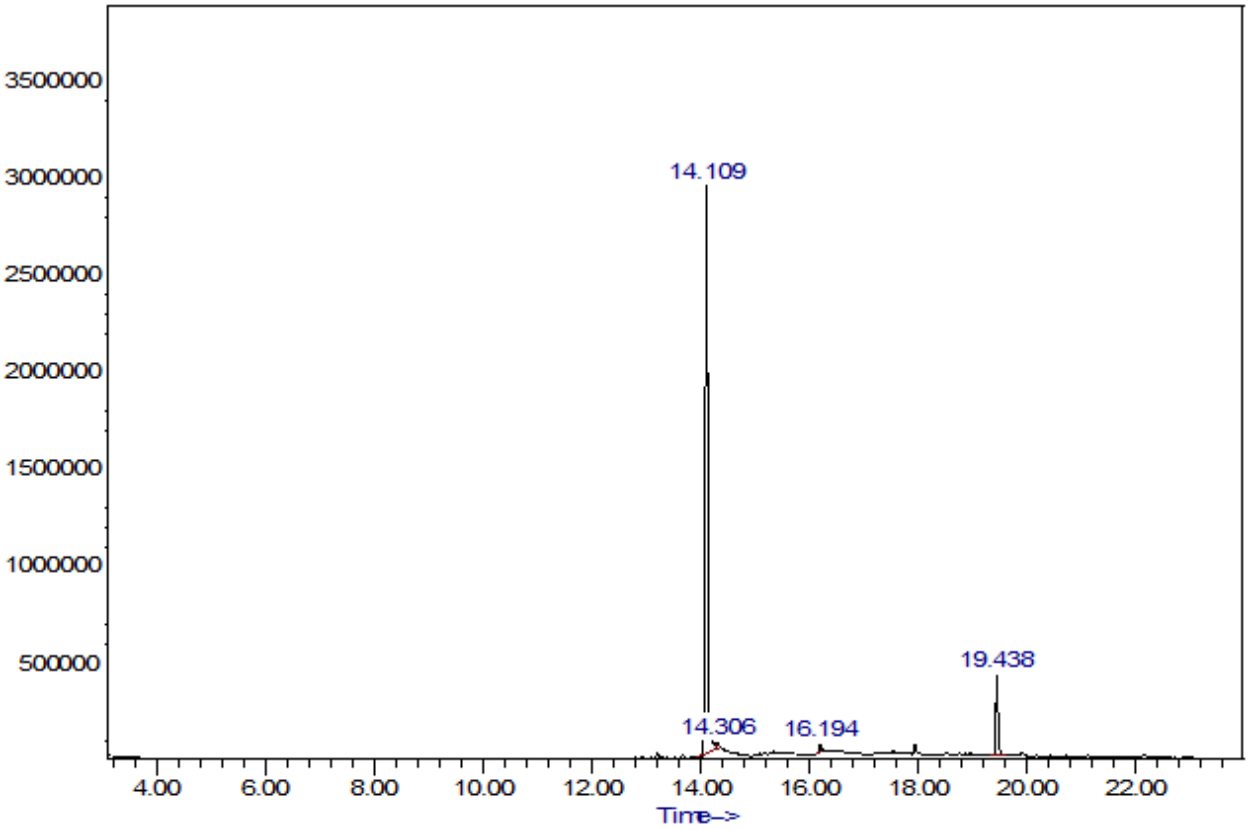

Figure 2: Chromatogram for the Isolated Compound

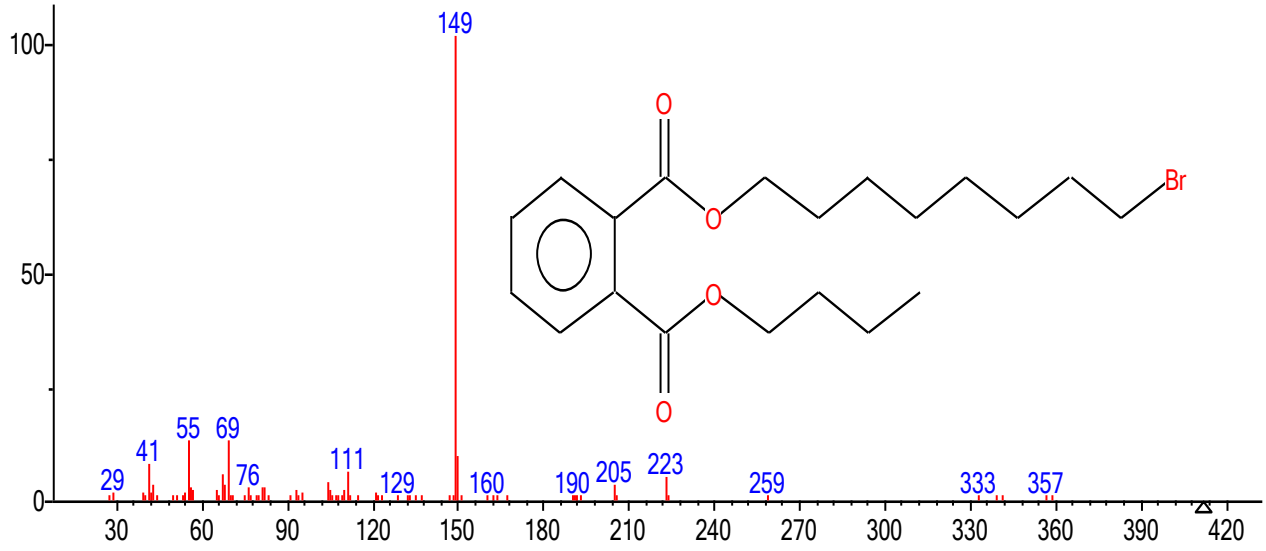

Figure 3: Mass Spectra of Isolated Compound

Figures 2 and 3 shows the characteristics of the isolated compound (i.e., phthalic acid, 8-bromoctyl butyl ester – $C_{20}H_{29}BrO_4$), starting from retention time = 14.11 min, abundance/peak area (PA) = 86.613%, mass number, -m/z = 413 g/mol, molecular peak ion = 149, and fragmentation patterns = 7, molecular formula. The isolated compound or $C_{20}H_{29}BrO_4$, is an insulin-like protein compound in plants.

Effectiveness of the Insulin

The results of the test for antidiabetic effect of the insulin is presented in Figure 4.

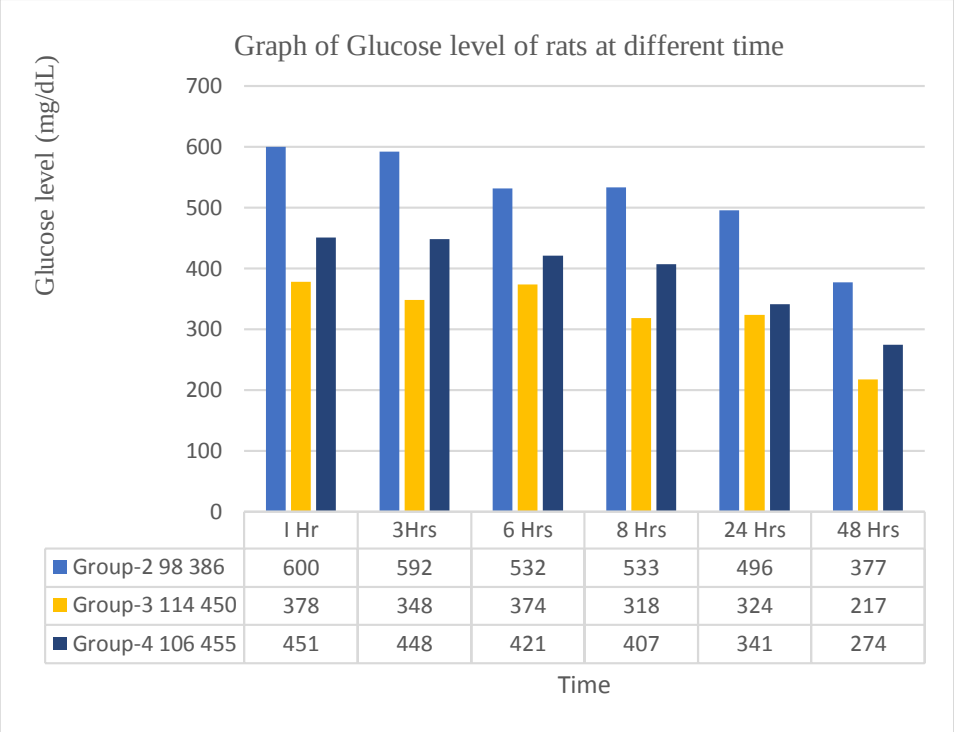

Figure 4: Glucose Level of Group 2, Group 3 and Group 4 Rats at Different Time

From Figure 4, it can be seen that glucose level of the rat in Group 2 increased from 98 mg/dl to 386 mg/dl after 48 hrs because alloxan monohydrate was induced to the rat. Precisely, 0.3 mL of the insulin was orally administered to rat of Group 2. After an hour, the glucose level rise to 600 mg/dl, which shows that the insulin did not start working until after 3 hrs. Successive drop in glucose level are as follows: the glucose level first drop to 592 mg/dl, after 6 hours it drop to 532 mg/dl, after 8 hours it continue to 533 mg/dl, after 24 hours it drop to 496 mg/dl and after 48hours it finally drops to 377 mg/dl – which is still diabetic, as low dose of insulin was administered showing low activity. Rat from Group 3 has glucose level rise from 114 mg/dl to 450 mg/dl after 48 hrs as alloxan monohydrate was induced to the rat. After 0.6 mL of the insulin was orally administered to Group 3 rat, after an hour the glucose level drop to 378 mg/dl. After 3 hours, it then drops to 348 mg/dl, rises to 374 mg/dl after 6 hrs, drops again to 318 mg/dl after 8 hrs, continue to drop up to 324 mg/dl after 24 hrs and finally after 48hours, it drops to 217 mg/dl. Group 3 rat was cured from diabetics as the glucose level is less (300 mg/dl). Rat from Group 4 has glucose level rise from 106 mg/dl to 455 mg/dl after 48 hours as alloxan monohydrate was induced to the rat. After 1.5 mL of the insulin was orally administered to Group 4 rat, after an hour the glucose level drop to 451 mg/dl, which further drops to 448 mg/dl after 3 hrs. After 6 hrs, it drops to 421 mg/dl, 407 mg/dl after 8 hrs, 341 mg/dl after 24 hrs and after 48hours it drops to 274 mg/dl. Group 4 rat was cured from diabetics as the glucose level is less (300 mg/dl).

Conclusion

Insulin was successfully extracted from stevia leave to serve as an alternative to bovine insulin extracted from pancreas of animals. Results of the insulin characterized by GC-MS and FTIR spectroscopy to analyze and determine the functional groups available in the compound isolated shows that, compound isolated was an insulin like protein with the molecular formula, $C_{20}H_{29}BrO_4$ and IUPAC name: Phthalic acid, 8-bromoctyl butyl ester. The insulin extracted was tested on *Rattus norvegicus* where it proves effective. Group 3 and Group 4 rats were cured

from diabetics while Group 2 ray was found to be still diabetic because a low dose of insulin was administered. From the foregone, it is recommended that insulin should be extracted from local biomass such as mango tree leave etc. Furthermore, government should collaborate with agencies, industries and companies to commercialize the production of *Rebaudiana bertoni* leave which has many medicinal benefits. Other methods of extraction should be compared with this research for further improvement.

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Appendix

$$Retention\ Factor\ (R.f)\ = \frac{Distance\ travel\ by\ spot}{Distance\ travel\ by\ solvent}$$

$$Retention\ Factor\ (R.f)\ of\ spot\ 1-4\ = \frac{7.2}{8.2} = 0.88$$

Weight of Rats

Group 1=215 g

Group 2= 175 g

Group 3= 214 g

Group 4= 253 g

Dose of Alloxan monohydrate used= 150 mg/Kg

$$\text{Weight of Alloxan monohydrate (mg)} = \frac{Weight\ of\ Rat\ (g) \times 150\ mg/Kg}{1000}$$

$$\text{Volume of Alloxan monohydrate to induced (ml)} = \frac{Weight\ of\ Alloxan\ monohydrate\ (mg)}{50\ mg/ml}$$

Dose of Insulin used

Low dose= 300 mg/Kg

Medium dose= 600 mg/Kg

High dose= 1200 mg/Kg

$$Weight\ of\ Insulin\ (mg) = \frac{Weight\ of\ Rat\ (g) \times Dose\ of\ Insulin\ (mg/kg)}{1000}$$

$$\text{Volume of Insulin to administer} = \frac{Weight\ of\ Insulin\ (mg)}{200\ mg/ml}$$