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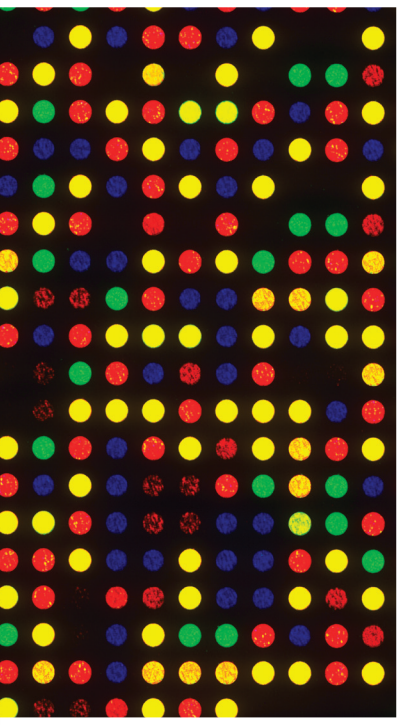
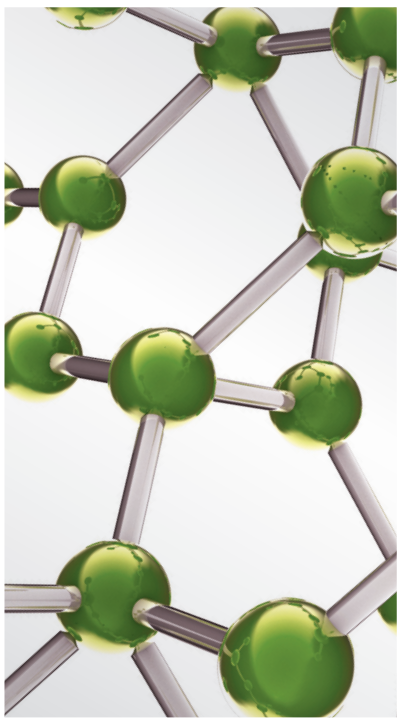
Herbal Medicine as an Alternative Medicine for Treating Diabetes: The Global Burden

Guest Editors: Geeta Watal, Preeti Dhar, Sharad Kr. Srivastava, and Bechan Sharma



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Editorial Board

Contents

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Editorial

Herbal Medicine as an Alternative Medicine for Treating Diabetes: The Global Burden

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Diabetes mellitus (DM) affects 150 million people worldwide including 50 million in India. The incidence rate of the disease is expected to double by 2025. Diabetes emerges due to several factors such as inhibition of glucose absorption, increase in glucose uptake and upregulation of glucose transporters, activation of the nuclear receptor PPARγ, increase in adiponectin release, glycogen metabolism, absent or decreased insulin production and/or impaired function, insulin mimetic and insulinotropic effect, elevation of D-Chloroinositol, incretin mimetics and incretin enhancers, and the role of endogenous opioids on glucose homeostasis and antioxidants. Based on the pancreatic effect, DM is classified as type 1 (insulin dependent diabetes mellitus (IDDM)) and type 2 (noninsulin dependent diabetes mellitus (NIDDM)). DM induced hyperglycemia leads to many clinical complications either at the macrovascular level causing coronary artery and cerebrovascular diseases or at the microvascular level causing renal failure, blindness, limb amputation, neurological complications, and premature death [1, 2]. The symptoms of DM include severe hypoglycemia, lactic acidosis, idiosyncratic liver cell injury, permanent neurological deficit, digestive discomfort, headache, and dizziness.

Existing therapeutics against DM mainly include synthetic hypoglycemic drugs and insulin. These anti-DM drugs usually target a single metabolic pathway to regulate hyperglycemia and are laced with numerous side effects and their efficacies are questionable. Therefore, it is imperative to develop new therapeutic paradigms that can act on more than one key metabolic pathway concerning carbohydrate, fat, or protein metabolism. Herbal preparations from over 200 traditional plants and their bioactive constituents are known to possess antidiabetic property as demonstrated by various screening methods. The extracts or constituents of some plants may act at different levels such as inhibiting glucose absorption from intestines, increasing insulin secretion from the pancreas, enhancing glucose uptake by adipose and muscle tissues, or inhibiting glucose production from hepatocytes [2]. Accordingly, there are ample possibilities to exploit phytochemicals as effective alternative medicines with limited or no side effects.

Given that limited information is available regarding the evidence-based therapeutic usage of many antidiabetic plants, this special issue was proposed. It attracted many submissions; following a peer review, only seven submissions were recommended for publication. These articles include review articles and technical papers that provide comprehensive reports on antidiabetic plants and the mode of action of their active ingredients.

The review article "*Botanical, pharmacological, phytochemical, and toxicological aspects of the antidiabetic plant Bidens pilosa* L." written by W.-C. Yang provides up-to-date information on the pharmacology, phytochemistry, and toxicology of *Bidens pilosa* in regard to type 1 and type 2 diabetes. The author has highlighted medicinal properties of ingredients isolated from the plant, *B. pilosa* L., which is easy-to-grow, widespread, and a palatable perennial. The author has presented the structure and biosynthesis of *B. pilosa* and its polyynes in relation to their antidiabetic action and mechanism. In the article "*Antidiabetic effect of oral borapetol B compound, isolated from the plant Tinospora crispa*, *by*

stimulating insulin release," F. E. Lokman et al. have presented an evaluation of the antidiabetic property of a biologically active small compound borapetol B (C1) isolated from *T. crispa* in normoglycemic control using Wistar (W) and spontaneously type 2 diabetic Goto-Kakizaki (GK) rats. They found that an acute oral administration of the compound significantly improves blood glucose levels in the treated group in comparison to the placebo group. They observed that plasma insulin levels were significantly enhanced by 2-fold in treated W and GK rats compared to the placebo group at 30 minutes. Their study provides evidence that borapetol B (C1)'s antidiabetic property is mainly due to stimulation of insulin release.

N. H. M.-Radzman et al. in the paper "*Stevioside from Stevia rebaudiana Bertoni increases insulin sensitivity in 3T3-L1 adipocytes*" have reported that stevioside from *Stevia rebaudiana* may exert an antihyperglycemic effect on both rat and human subjects. Using methods such as the radioactive glucose uptake assay, they have shown the improvements in insulin sensitivity in 3T3-L1 cells by elevation of glucose uptake as a result of stevioside treatments. They found that stevioside managed to increase uptake activities to a maximum of 2.08 times ($P < 0.001$) under normal conditions and up to 4.40 times ($P < 0.001$) in insulin-resistant states. It was noncytotoxic to the 3T3-L1 cells. The authors have shown that stevioside can have a direct effect on 3T3-L1's insulin sensitivity, by means of the glucose uptake elevation and the increase in expression of proteins in the insulin-signaling pathway. The article "*Potential roles of Stevia rebaudiana* Bertoni *in abrogating insulin resistance and diabetes: A review*" by N. H. M.-Radzman et al. highlights the mechanisms involving free fatty acids, adipocytokines such as TNFα and PPARγ and serine kinases like JNK and IKKβ, asserted to be responsible for the development of insulin resistance. The review article "*Use of laser-induced breakdown spectroscopy for the detection of glycemic elements in Indian medicinal plants*" by P. K. Rai et al. describes how a unique combination of physics, chemistry, and biological techniques can be exploited to evaluate antidiabetic Indian medicinal plants. They show laser-induced breakdown spectroscopy (LIBS) as a sensitive optical technique being widely used for the detection of glycemic elements from medicinal plants.

G. Sartore et al. in the article "*Mediterranean diet and red yeast rice supplementation for the management of hyperlipidemia in statin-intolerant patients with or without type 2 diabetes*" found that the lipid profile can be modified by consuming Mediterranean diet (MD) and red yeast rice (RYR). They indicate that MD counseling alone is effective in reducing low density lipoprotein (LDL) cholesterol levels in moderately hypercholesterolemic statin-intolerant patients with a presumably low cardiovascular risk, and combining MD with the administration of RYR improves patients with LDL cholesterol levels considerably more than when the patient is on MD only.

In a quest to cure many serious diseases using more cost-effective and safer drugs, there has been a paradigm shift in drug discovery that involves using plant-based molecules. More effort, however, is required to explore newer and more promising herbal ingredients that qualify absorption, distribution, metabolism, and excretion (ADME) criteria with a relatively larger therapeutic index.

Geeta Watal
Preeti Dhar
Sharad Kr. Srivastava
Bechan Sharma

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Erratum

Erratum to "Mediterranean Diet and Red Yeast Rice Supplementation for the Management of Hyperlipidemia in Statin-Intolerant Patients with or without Type 2 Diabetes"

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The authors' names were incorrectly listed as Sartore Giovanni, Burlina Silvia, Ragazzi Eugenio, Ferraresso Stefania, Valentini Romina, and Lapolla Annunziata; this error is corrected here.

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Review Article

Botanical, Pharmacological, Phytochemical, and Toxicological Aspects of the Antidiabetic Plant *Bidens pilosa* L.

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Bidens pilosa L. is an easy-to-grow, widespread, and palatable perennial on earth. Hence, it has traditionally been used as foods and medicines without noticeable adverse effects. Despite significant advancement in chemical and biological studies of *B. pilosa* over the past few years, comprehensive and critical reviews on its anti-diabetic properties are missing. The present review is to summarize up-to-date information on the pharmacology, phytochemistry, and toxicology of *B. pilosa*, in regard to type 1 diabetes and type 2 diabetes from the literature. In addition to botanical studies and records of the traditional use of *B. pilosa* in diabetes, scientific studies investigating antidiabetic action of this species and its active phytochemicals are presented and discussed. The structure and biosynthesis of *B. pilosa* and its polyynes in relation to their anti-diabetic action and mechanism are emphasized. Although some progress has been made, rigorous efforts are further required to unlock the molecular basis and structure-activity relationship of the polyynes isolated from *B. pilosa* before their clinical applications. The present review provides preliminary information and gives guidance for further anti-diabetic research and development of this plant.

1. Introduction

Diabetes was coined by a Greek physician, Aretaeus of Cappadocia (30-90 AD), about 2 millennia ago [1]. He first described this devastating disease with symptoms, such as constant thirst (polydipsia), excessive urination (polyuria), and weight loss, which still hold true nowadays [1]. The International Diabetes Federation (IDF) estimated that diabetes afflicted 285 million people, 6.4% of the world population, who were afflicted with diabetes in 2010 and will afflict 439 million people, 7.7% of the world population by 2030 [2]. Over 90% of diabetic patients are diagnosed with type 2 diabetes (T2D) [3, 4] and the rest are diagnosed with type 1 diabetes and others.

Diabetes is a chronic metabolic disease with fatal complications such as cardiovascular diseases, retinopathy, renopathy, and foot ulcers. The cost of health care associated with diabetes continues to grow and is a huge economic burden for afflicted patients and countries. In the states, approximately 17.5 million adults were reported to be receiving treatment for diabetes, where the estimated cost of diabetes was 174 billion dollars in 2007 [5].

Despite much progress made on basic and clinical research into diabetes, this disease has not been cured since antiquity. Main reasons for this mishap are unmet efficacy and significant side effect of the drugs. So far, 1200 plants have been claimed to be remedies for diabetes [6, 7] and one-third of them have been scientifically evaluated for T2D treatment [8]. Among them, *B. pilosa* is commonly used as food and medicine for humans and animals [9, 10]. It is an easy-to-grow herb that is globally distributed. The folkloric use of *B. pilosa* to treat diabetes has been recorded in America, Africa, Asia, and Oceania [11]. Accumulating data have shown the potential of this plant and active compounds to treat

Table 1: Taxonomy of *B. pilosa* [12].

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Asteridae
Order	Asterales
Family	Asteraceae
Genus	*Bidens*
Species	*Bidens pilosa* L.

diabetes. The present review focuses on recent studies on the botany, anti-diabetic action and mechanism, phytochemistry, and toxicology of *B. pilosa*. The information provided here highlights the possible usefulness of *B. pilosa* and its isolated compounds and offers insights into possible future research directions.

2. Botanical Properties

B. pilosa is believed to have originated in South America and subsequently spread everywhere on earth [13]. *Bidens* species and their varieties bear vernacular names based on their sticky seeds or prosperous growth [5]. *B. pilosa* is taxonomically assigned to the *Bidens* genus, up to 240 species, as shown in Table 1 [9, 10]. Different varieties are frequently found in *B. pilosa*. *B. pilosa* is an erect, perennial herb widely distributed from temperate and tropical regions. It has serrate, lobed, or dissected form of green opposite leaves, white or yellow flowers, and long narrow ribbed black seeds (Figure 1) [14]. Apart from morphological traits, chemotaxonomical (Figure 2) and molecular characterization (Table 2) is sometimes helpful in the identification of *B. pilosa* strains [15].

Despite its preference for full sun and semidry soil, *B. pilosa* can grow in arid and barren lands at different altitudes. Food and Agricultural Organization actively promoted the culture of *B. pilosa* in Africa in 1970s due to its fast-growing advantage [16]. *B. pilosa* can be propagated via seeds. After soaking, *B. pilosa* seeds can germinate in 3 to 4 days [17]. Minimal agricultural techniques are required for *B. pilosa* cultivation. *B. pilosa* is recognized as one of the top worst weeds worldwide because of its aggressive invasion [18].

Apart from its use as food ingredient, *B. pilosa* is used as herbal medicines for diabetes and 40 other diseases [5]. All parts of *B. pilosa* plant, the whole plant, the aerial parts (leaves, flowers, seeds, and stems), and/or the roots, fresh or dried, are used as ingredients in folk medicines. Dry powder, decoction, maceration, and tincture are usual formulations for its internal as well as external use [19]. *B. pilosa* can be used alone or together with other medicinal herbs.

3. Antidiabetic Properties

B. pilosa has a variety of pharmacological actions. As far as its anti-diabetic activity is concerned, *B. pilosa* and its anti-diabetic polyynes have been reported to effectively prevent and treat type 1 diabetes and type 2 diabetes, which are etiologically distinct [15, 20–24]. In this section, we will focus our review on the pharmacological action and mechanism of *B. pilosa* extract and its active phytochemicals against both types of diabetes.

Table 2: Percent differences between the internal transcribed spacer 1 (ITS1) regions of the DNA sequences of *B. pilosa* variants (BPR, *B. pilosa* var. *radiata*; BPM, *B. pilosa* var. *minor*; and BPP, *B. pilosa* var. *pilosa*), *B. hintonii*, and *B. biternata*.

ITS1	BPM	BPP	BPR	BH[a]	BB[b]
BPM	0	0.39	3.56	4.74	11.37
BPP	0.39	0	3.16	4.45	11.46
BPR	3.56	3.16	0	5.93	12.55
BH[a]	4.74	4.35	5.93	0	14.45
BB[b]	11.37	11.46	12.55	14.45	0

[a]ITS1 obtained from GeneBank Accession Number AF330101.1.
[b]ITS1 obtained from GeneBank Acession Number EU117248.1.

3.1. Action and Mechanism of B. pilosa for Type 1 Diabetes. T1D is caused by the autoimmune destruction of pancreatic β cells, leading to insulin deficiency, hyperglycemia, and complications. Monotherapy (immune intervention and β-cell replacement/(re)generation) and their combination therapy are common approaches to treat T1D. Despite considerable advances made on these approaches, there has no cure for T1D. Helper T (Th) cell differentiation regulates T1D development. Further, Th1 cell differentiation promotes T1D, whereas Th2 cell differentiation alleviates T1D [25].

To test the immunomodulatory effect of *B. pilosa*, one study showed that *B. pilosa* extract and its butanol fraction could decrease Th1 cells and cytokines and increase Th2 cells and cytokines [23]. This study indicated that IC_{50} value of the butanol fraction was 200 μg/mL. This inhibition was reported to be partially attributed to cytotoxicity, because the butanol fraction at 180 μg/mL could cause 50% death of Th1 cells. Using the bioactivity-directed isolation and identification approach (Figure 3), 3 active polyynes, 3-β-D-glucopyranosyl-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne (**17**), 2-β-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne (**16**), and 2-β-D-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne (cytopiloyne, **19**), as well as 2 index compounds, 4,5-Di-*O*-caffeoylquinic acid, 3,5-Di-*O*-caffeoylquinic acid, and 3,4-Di-*O*-caffeoylquinic acid, were isolated from the butanol extract using high pressure liquid chromatography (HPLC) and, in turns, were structurally identified by nuclear magnetic resonance (NMR) [23, 24]. Only the first three active compounds showed similar effects on Th cell differentiation as the *B. pilosa* butanol fraction. Moreover, compound **19** showed greater activity than compounds **17** and **16** in terms of enhancement (by 277% compared to 34% and 8%) of differentiation of Th0 to Th2 at 10 μg/mL and inhibition (by 60% compared to 17% and 9%) of differentiation to Th1 at the same concentration (Table 3) [23, 24].

Accordingly, the butanol fraction of *B. pilosa* effectively prevented T1D in nonobese diabetic (NOD) mice [23]. Consistently, this prevention involved downregulation of Th1 cells

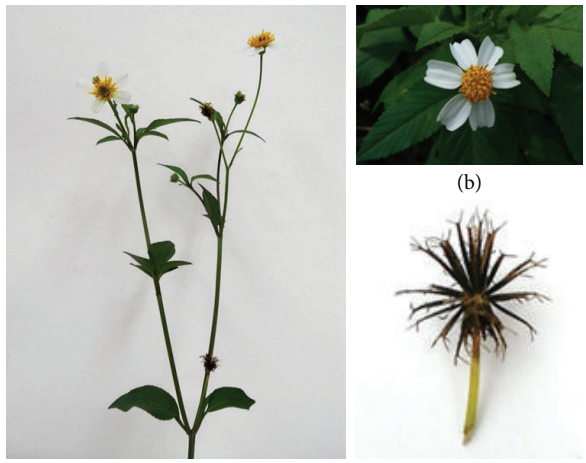


FIGURE 1: Image of *B. pilosa* (a) and its flowers (b) and seeds (c).

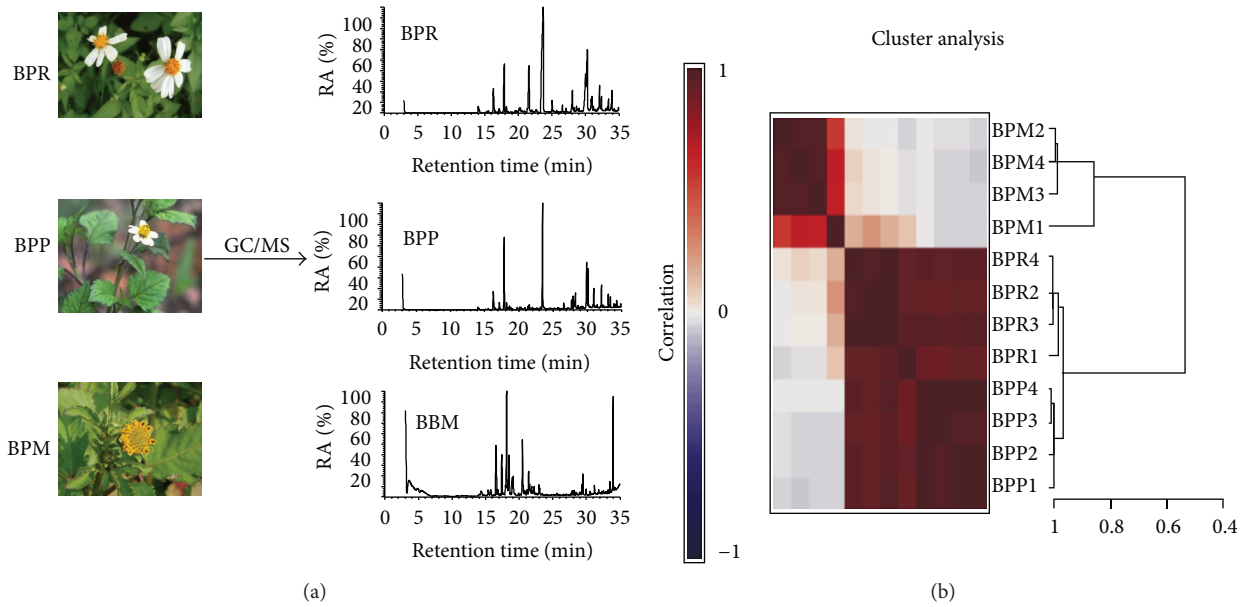


FIGURE 2: Chemotaxonomical comparison of three *B. pilosa* variants (BPR, *B. pilosa* var. *radiata*; BPM, *B. pilosa* var. *Minor,* and BPP, *B. pilosa* var. *pilosa*). Gas chromatography/mass spectrometry (GC/MS) and cluster analysis to assist in determining the taxonomy of 4 samples of the three *Bidens* variants.

or upregulation of Th2 cells. This was proven by intraperitoneal injection of the butanol fraction at a dose of 3 mg/kg body weight (BW), 3 times a week, to NOD mice from 4 to 27 weeks [23]. This dosage resulted in lower incidence of diabetes (33%). At a dose of 10 mg/kg, the butanol fraction of *B. pilosa* totally stopped (0%) the initiation of the disease [23]. Th1 cytokine IFNγ and Th2 cytokine IL-4 favor the production of IgG2a and IgE, respectively. To further confirm whether this butanol *in vivo* regulated Th cell differentiation and Th cytokine profiling, IgG2a and IgE production was measured in the serum of NOD mice. As expected, high levels of IgE and some decline in the levels of IgG2a were observed in the serum [23].

Since cytopiloyne (**19**) had the most potent effect on Th cell differentiation among the aforesaid polyynes [20], another study used cytopiloyne to explore the action and molecular mechanism of cytopiloyne on T1D in NOD mice [20]. NOD mice received intraperitoneal or intramuscular injection of cytopiloyne at 25 μg/kg BW, 3 times per week. Twelve-week-old NOD mice started to develop T1D, and 70% of NOD mice aged 23 weeks and over developed T1D. Remarkably, 12- to 30-week-old NOD mice treated with cytopiloyne showed normal levels of blood glucose (<200 mg/dL) and insulin (1-2 ng/mL). Consistent with T1D incidence, cytopiloyne delayed and reduced the invasion of $CD4^+$ T cells into the pancreatic islets [20]. Albeit

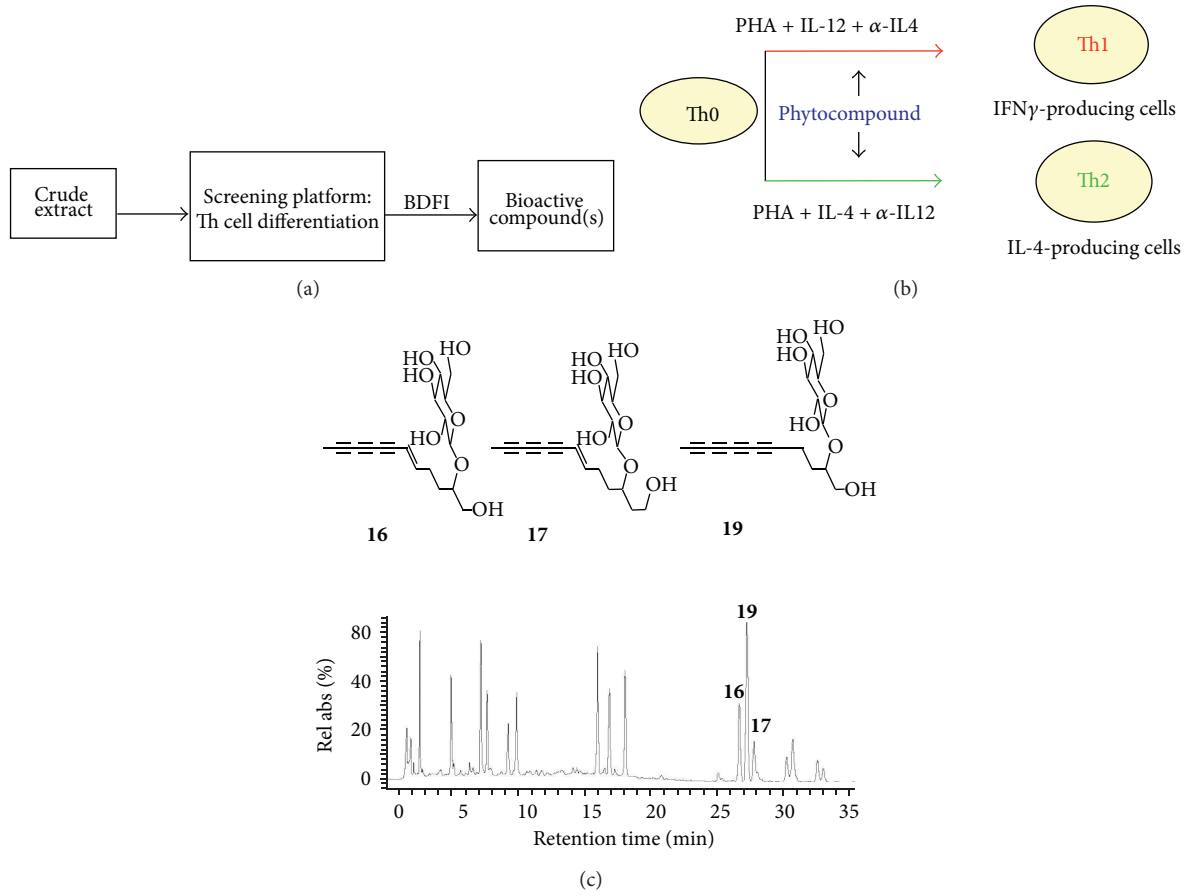


Figure 3: Bioactivity-directed fractionation and isolation approach to identify three active polyynes that regulate Th cell differentiation. A flowchart of the bioactivity-directed fractionation and isolation (BDFI) strategy describes the use of the screening platform and Th cell differentiation assays to determine bioactive compounds from the crude extract and fractions of *B. pilosa* (a). Bioassays are composed of human naïve helper T cells (Th0) which can differentiate into type 1 helper T (Th1) cells and type 2 helper T (Th2) cells in the presence of PHA plus IL-12 and anti-IL-4 antibody and PHA with IL-4 and anti-IL-12 antibody, respectively. The crude extract, fractions, and compounds of *B. pilosa* are added to differentiating cells to test the Th cell differentiation (b). Compounds **16**, **17**, and **19** are active compounds that promote Th2 cell differentiation but inhibit Th1 cell differentiation.

Table 3: Th1 inhibition and Th2 promotion by the extract (150 μg/mL) and polyynes (10 μg/mL) of *B. pilosa*.

	Butanol extract	Compound **19**	Compound **17**	Compound **16**
Reduction of Th1 (%)	32%	75%	17%	9%
Increase of Th2 (%)	103%	277%	31%	6%

less effective than cytopiloyne (**19**), 3-β-D-glucopyranosyl-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne (**17**), and 2-β-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne (**16**) also decreased T1D development in NOD mice.

The underlying mechanism by which cytopiloyne and its derivatives inhibited T1D covered inactivation of T cells, polarization of Th cell differentiation, and Th cell depletion, leading to islet protection [20] and is illustrated in Figure 4. First, [^{3}H] thymidine incorporation assay showed that cytopiloyne inhibited ConA/IL-2- and CD3 antibody-mediated T cell proliferation, implying that cytopiloyne could inhibit T cell activation. Second, *in vitro* study showed that cytopiloyne (**19**) inhibited the differentiation of naïve Th (Th0) cells (i.e., CD4$^+$ T cells) into Th1 cells and promoted differentiation of Th0 cells into Th2 cells [24]. The *in vitro* data are consistent with the *in vivo* results, indicating that cytopiloyne reduced Th1 differentiation and increased Th2 differentiation as shown by intracellular cytokine staining and FACS analysis [20]. Cytopiloyne also enhanced the expression of GATA-3, a master gene for Th2 cell differentiation, but not the expression of T-bet, a master gene for Th1 cell differentiation, further supporting its role in skewing Th differentiation [20]. In line with the skewing of Th differentiation, the level of serum IFN-γ and IgG2c decreased, while that of serum IL-4 and serum IgE increased compared to the negative controls (PBS-treated mice). Third,

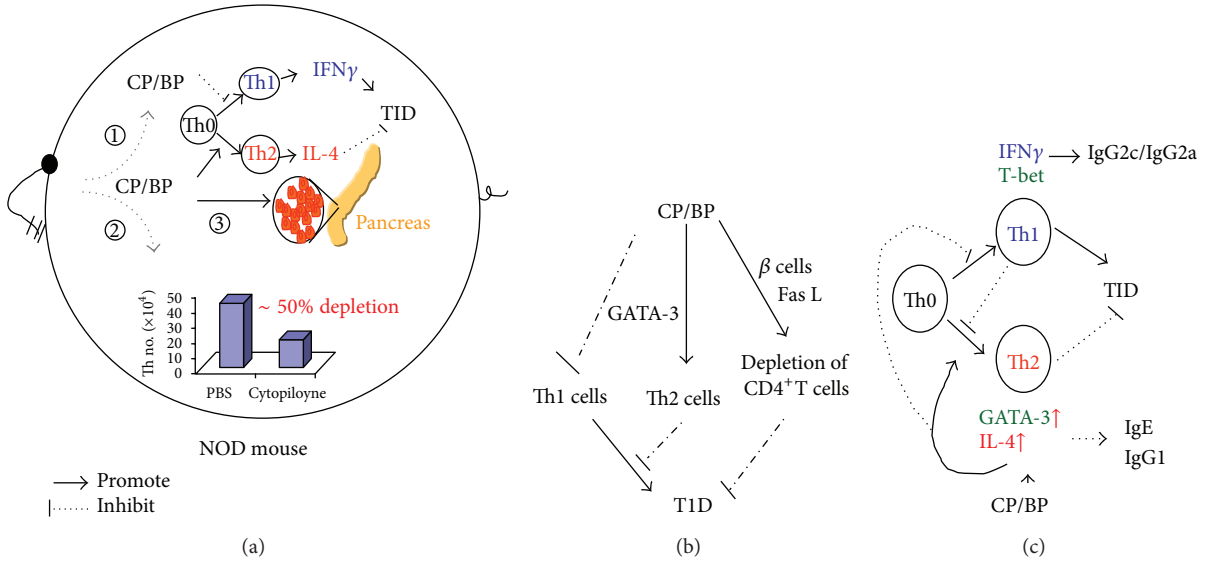


FIGURE 4: The underlying mechanism of the crude extract of *B. pilosa* (BP) and its active compound, compound **19** (CP), in T1D. BP and/or CP can suppress T1D development via regulation of T cells (① and ②) and β cells (③) in NOD mice (a). Their regulation of T cells involves Th cell activation and differentiation (①) and partial depletion of Th0 cells (②) as depicted (b). CP and/or BP augment the expression of GATA-3 gene and, in turn, promote the expression of IL-4 and Th2 cell differentiation. In contrast, CP and/or BP do not affect the expression of T-bet (c).

cytopiloyne partially depleted $CD4^+$ rather than $CD8^+$ T cells in NOD mice [20]. Coculture assays showed that the depletion of $CD4^+$ T cells was mediated through the induction of Fas ligand expression on pancreatic islet cells by cytopiloyne, leading to apoptosis of infiltrating $CD4^+$ T cells in the pancreas via the Fas and Fas ligand pathway. However, cytopiloyne did not induce the expression of TNF-α in pancreatic islet cells and, thus, had no effect on $CD8^+$ T cells [20].

Due to the antidiabetic mechanisms of action, it was hypothesized that cytopiloyne protects NOD mice from diabetes by a generalized suppression of adaptive immunity. To evaluate this hypothesis, ovalbumin (Ova) was used as a T-cell dependent antigen to prime NOD mice, which had already received cytopiloyne or PBS vehicle. Ova priming enhanced similar anti-Ova titers in cytopiloyne-treated mice and PBS-treated mice, but a different profile of immunoglobulin isotype was observed in the two groups. Therefore, it was concluded that cytopiloyne is an immunomodulatory compound rather than an immunosuppressive compound [20, 24].

Collectively speaking, the mechanism of action of cytopiloyne and, probably, its polyyne derivatives in T1D includes inhibition of T-cell proliferation, skewing of Th cell differentiation and partial depletion of Th cells, and protection of β pancreatic islets.

3.2. Action and Mechanism of B. pilosa for Type 2 Diabetes. Mounting evidence from epidemiological studies proposes environmental and genetic factors as the primary causes of T2D. Both factors contribute to insulin resistance and loss of β-cell function, leading to impairment in insulin action, insulin production, or both. As a result, this impairment accompanies the development of hyperglycemia, a major pathological feature of T2D [26]. Hyperglycemia can cause damage to β cells and other peripheral tissues, named glucotoxicity. As a consequence, cardiovascular disease, nephropathy, retinal blindness, neuropathy, and peripheral gangrene develop and contribute to mortality [27]. Therefore, maintenance of glycemic homeostasis has been a golden standard for T2D therapy. Moreover, aberrant lipid metabolism in adipose and other tissues can cause lipotoxicity, which can further worsen diabetic complications. The β cells in the pancreas are the key players in glycemic homeostasis [28].

Plants are an extraordinary source for anti-diabetic agents. Over 1200 plant species have be claimed to treat diabetes [6, 7]. One of them, *B. pilosa*, has traditionally been used as an anti-diabetic herb in America, Africa, and Asia [7, 29, 30]. More recently, *B. pilosa* has scientifically been investigated for anti-diabetic activity. One seminal study by Ubillas et al. showed that the aqueous ethanol extract of the aerial part of *B. pilosa* at 1 g/kg BW lowered blood glucose in db/db mice, a T2D mouse model [30]. They also used a bioactivity-guided identification strategy to identify two polyynes, compounds **17** and **16**. Moreover, the mixture of the compounds (**17** : **16**) in a 2 : 3 ratio significantly reduced blood glucose level and food intake on the second day when administered at doses of 0.25 g/kg twice a day to C5BL/Ks-db/db mice. When evaluated at 0.5 g/kg, a more substantial decrease in blood glucose level as well as the stronger anorexia (food intake reduced from 5.8 g/mouse/day to 2.5 g/mouse/day) was noticed [30]. This study suggested that compounds **17** and **16** were active ingredients of *B. pilosa* for diabetes [30]. The anti-diabetic effect of both polyynes was partially caused by the hunger suppressing effect. However, the anoxic effect of the ethanol extract of *B. pilosa* was not observed in the studies described below. In another

study [31], water extracts of *B. pilosa* (BPWE) were tested in diabetic db/db mice, aged 6–8 weeks, with postmeal blood glucose levels of 350 to 400 mg/dL. Like oral anti-diabetic glimepiride, which stimulates insulin release, one-single dose of BPWE reduced blood glucose levels from 374 to 144 mg/dL. The antihyperglycemic effect of BPWE was relevant to an increase in serum insulin levels, implying that BPWE drops blood glucose concentration through an upregulation of insulin production. However, BPWE showed different insulin secretion kinetics from glimepiride [31]. One drawback in current anti-diabetic agents is their decreasing efficacy over time. Therefore, the authors investigated the long-term anti-diabetic effect of BPWE in db/db mice. BPWE lowered blood glucose, boosted blood insulin, improved glucose tolerance, and reduced the percentage of glycosylated hemoglobulin (HbA1c). Both one-time and long-term experiments strongly support the superior action of BPWE on diabetes [31]. Unlike glimepiride, which failed to preserve pancreatic islets, BPWE was significantly protected against islet atrophy in mouse pancreas. The group also evaluated anti-diabetic effect of 3 *B. pilosa* varieties, *B. pilosa* var. *radiate* (BPR), *B. pilosa* var. *pilosa* (BPP), and *B. pilosa* var. *minor* (BPM) in db/db mice [15]. A single oral dose (10, 50, and 250 mg/kg BW) of BPR, BPP, or BPM water extracts decreased postprandial blood glucose levels in db/db mice for up to four hours and this reduction was dose-dependent. Of note, BPR extract resulted in a higher reduction in blood glucose levels when administered at the same dose as the other two varieties. Further, the BPR extract increased serum insulin levels in db/db mice to a greater extent than the other varieties at the same dose, 50 mg/kg. Three polyynes, compounds **16**, **17**, and **19**, were identified from the three *Bidens* strains though their varied contents. Compound **19** at 0.5 mg/kg exerted a better stimulation for insulin production in db/db mice than compounds **17** and **16**. On the contrary, 28-day treatment with the *Bidens* extracts and three polyynes were then performed using diabetic mice with postprandial glucose levels from 370 to 420 mg/dL and glimepiride was used as positive control. The applied dosages ranged from 10 mg/kg to 250 mg/kg BW. Results showed that the positive control as well as the crude extracts of the three varieties lowered the blood glucose levels in db/db mice. However, only BPR extract, containing a higher content of cytopiloyne (**19**), reduced blood glucose levels and augmented blood insulin levels more than BPP and BPM. The percentage of glycosylated hemoglobin A1c (HbA1c), a long-term indicator of blood homeostasis, was also monitored. HbA1c in the blood of 10- to 12-week-old diabetic mice was 7.9 ± 0.5%. However, treatment with BPR crude extract (50 mg/kg), glimepiride (1 mg/kg), and compound **19** (0.5 mg/kg) led to the HbA1c of 6.6 ± 0.2%, 6.1 ± 0.3%, and 6.2 ± 0.3% in the blood of age-matched mice, respectively [15]. Since cytopiloyne (**19**) was the most effective polyyne found in *B. pilosa*, against T2D, it was used for further study on anti-diabetic action and mechanism in another study [22]. The data confirmed that cytopiloyne reduced postmeal blood glucose levels, increased blood insulin, improved glucose tolerance, suppressed HbA1c level, and protected pancreatic islets in db/db mice. Nevertheless, cytopiloyne never managed to decrease blood glucose in streptozocin- (STZ-) treated mice whose β cells were already destroyed. In addition, cytopiloyne dose-dependently promoted insulin secretion and expression in β cells as well as calcium influx, diacylglycerol, and activation of protein kinase Cα. Taken together, the mechanistic studies suggest that cytopiloyne acts to treat T2D via regulation of β cell functions (insulin production and β cell preservation) involving the calcium/diacylglycerol/PKCα cascade (Figure 5).

The above studies point to the conclusion that cytopiloyne (**19**) and related polyynes (compounds **16** and **17**) are anti-diabetic agents in animal models. The data unfold a new biological action of polyynes. However, like all drugs developed for diabetes, cytopiloyne could neither prevent nor stop diabetes completely but reduced diabetic complications [22]. Intriguingly, 36 polyynes have been found in *B. pilosa* so far. It remains to be investigated whether all the polyynes present in this plant have anti-diabetic activities.

4. Phytochemistry

Broad application of *B. pilosa* all over the world has led to over 120 publications on its exploitation and use in medicines, foods, and drinks. *B. pilosa* is an extraordinary source of phytochemicals and 201 compounds have so far been identified from this plant, including 70 aliphatics (36 polyynes), 60 flavonoids, 25 terpenoids, 19 phenylpropanoids, 13 aromatics, 8 porphyrins, and 6 other compounds [32]. Mounting evidence suggests that phytochemical complexity of *B. pilosa* can account for its diverse bioactivities. The structures and likely bioactivities of these compounds were recently reviewed in the previous publications [5, 32], which are out of our scope. In this section, we focus on the chemical structures of 36 polyynes found in *B. pilosa* (Table 4) and their bioactivities (Table 5). We also discuss the likely biosynthesis of the polyynes in *B. pilosa*. Although their biosynthesis is not well defined, those polyynes are thought to derive from desaturation and acetylenation of fatty acids (Figure 6) in *B. pilosa* and other plants.

5. Toxicology

Food and Agricultural Organization of the United Nations has reported *B. pilosa* as a staple food and promoted its cultivation in Africa since 1975. Taiwanese Department of Health also allows its use as an ingredient in food for human consumption. Despite lack of systemic toxicological study of *B. pilosa* in humans, some information about acute and/or subchronic toxicities was revealed in rodents. Frida and colleagues reported that one-single oral dose of the water extract of *B. pilosa* leaves at 10 g/kg BW showed no obvious mortality or changes in the appearance of rats [61]. The same extract at 0.8 g/kg BW/day, once a day, showed no obvious sub-chronic toxicity in rats over 28 days, as shown by survival rate, body weight, and gross examination of organs [61]. They also evaluated the acute toxicity of hydroethanol extracts of *B. pilosa* in mice [61]. Five- to six-week-old mice, weighing between 28 and 35 g, received a peritoneal injection of both extracts at the different doses. The LD_{50}, the dose

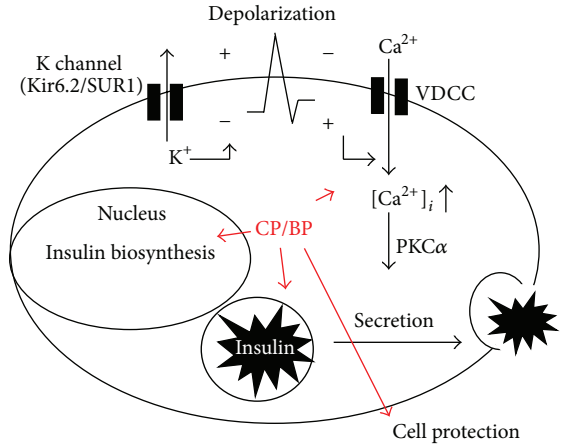


FIGURE 5: The underlying mechanism of the crude extract of *B. pilosa* (BP) and its active compound, compound **19** (CP), in T2D. BP and/or CP can treat T2D development via control of β cell function in db/db mice. Their anti-diabetic actions are through upregulation of insulin expression/secretion and protection of β cells involving secondary messengers (calcium and diacylglycerol) and their downstream PKCα pathway.

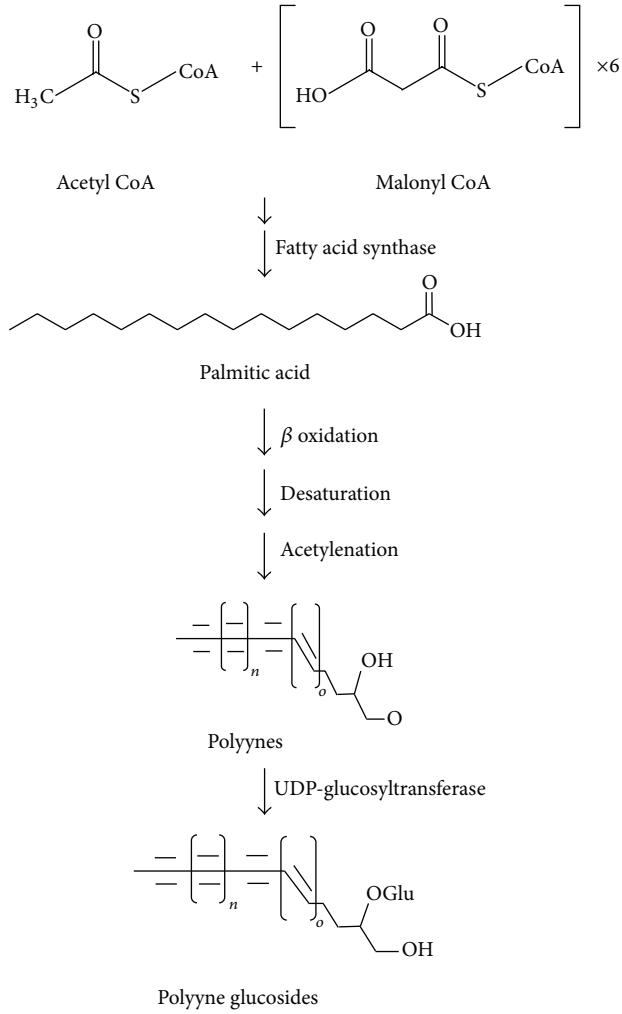


FIGURE 6: A scheme describing the biosynthesis of polyynes and its glucosides. Saturated fatty acids (e.g., palmitic acid, etc.) are synthesized from their common precursors, acetyl CoA and malonyl CoA. To generate acetylenic fatty acids (i.e., polyynes), the saturated fatty acids are proposed to undergo β oxidation, desaturation, and acetylenation. UDP-glucosyltransferase can attach a glucose moiety or more to polyynes to produce polyyne glucosides.

Table 4: Polyynes isolated from *B. pilosa* [32].

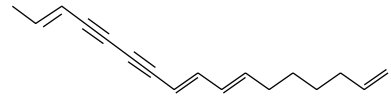
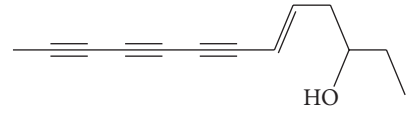
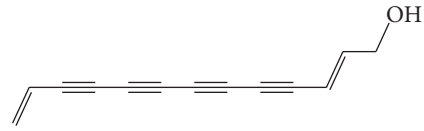
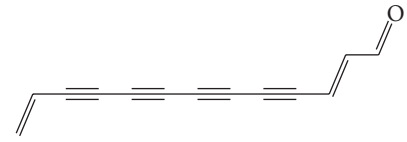
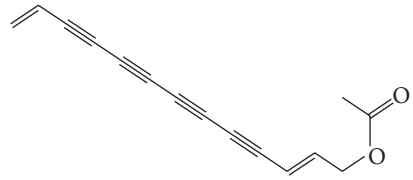
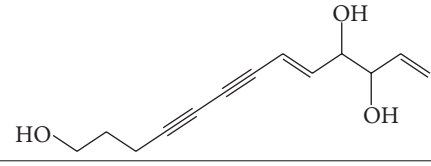
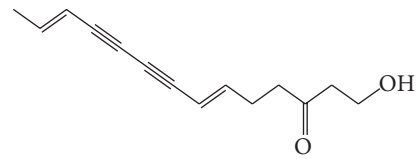
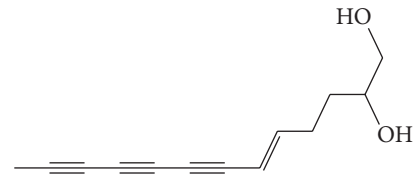
S. number	IUPAC names	Common names	Structure	Plant part (country)	References
1	1,7*E*,9*E*,15*E*-Heptadecatetraene-11,13-diyne	Heptadeca-2*E*,8*E*,10*E*,16-tetraen-4,6-diyne		Not found (China)	[33]
2	1,11-tridecadiene-3,5,7,9-tetrayne			Roots (not stated)	[34]
3	1-Tridecene-3,5,7,9,11-pentayne	Pentadiene		Leaves (not stated) and not found (Egypt)	[34, 35]
4	5-Tridecene-7,9,11-triyne-3-ol			Not found (Egypt)	[35]
5	2,10,12-Tridecatriene-4,6,8-triyn-1-ol			Part not specified (not stated)	[36]
6	2,12-Tridecadiene-4,6,8,10-tetrayn-1-ol	1,11-Tridecadiene-3,5,7,9-tetrayn-13-ol		Roots (not stated) and not found (Egypt)	[34, 35]
7	2,12-Tridecadiene-4,6,8,10-tetraynal	1,11-Tridecadiene-3,5,7,9-tetrayn-13-al		Roots (Germany)	[37]
8	2,12-Tridecadiene-4,6,8,10-tetrayn-1-ol,1-acetate	1,11-Tridecadiene-3,5,7,9-tetrayn-13-acetate		Roots (not stated)	[34]
9	(5*E*)-1,5-Tridecadiene-7,9-diyn-3,4,13-triol			Aerial (China)	[38]
10	(6*E*,12*E*)-3-oxo-tetradeca-6,12-dien-8,10-diyn-1-ol			Aerial (China)	[38]
11	(*E*)-5-Tridecene-7,9,11-triyne-1,2-diol	1,2-Dihydroxy-5(*E*)-tridecene-7,9,11-triyne		Whole (Taiwan)	[39]

Table 4: Continued.

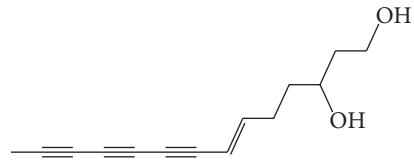
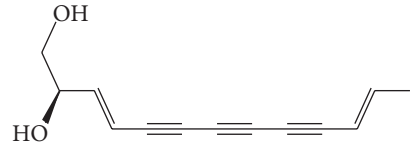
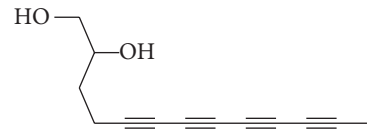
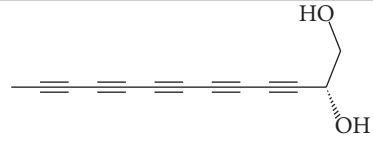
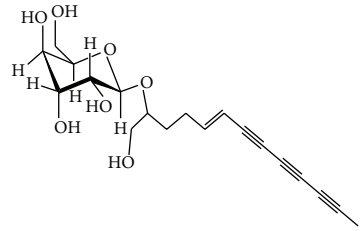
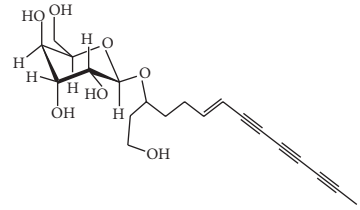
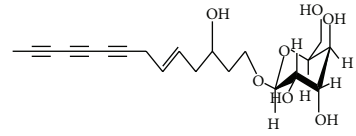
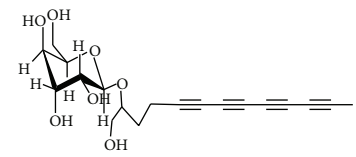
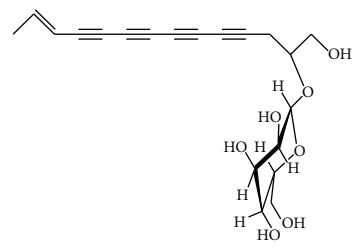
S. number	IUPAC names	Common names	Structure	Plant part (country)	References
12	(*E*)-6-Tetradecene-8,10,12-triyne-1,3-diol	1,3-Dihydroxy-6(*E*)-tetradecene-8,10,12-triyne		Whole (Taiwan)	[39–41]
13	(2*R*,3*E*,11*E*)-3,11-Tridecadiene-5,7,9-triyne-1,2-diol	Safynol		Not found (Egypt and China)	[33, 35]
14	5,7,9,11-Tridecatetrayne-1,2-diol	1,2-Dihydroxytrideca-5,7,9,11-tetrayne		Whole (Taiwan)	[39, 40, 42]
15	(*R*)-3,5,7,9,11-Tridecapentayne-1,2-diol	(*R*)-1,2-Dihydroxytrideca-3,5,7,9,11-pentayne		Aerial (Japan)	[43]
16	(4*E*)-1-(Hydroxymethyl)-4-dodecene-6,8,10-triyn-1-yl-*β*-*D*-glucopyranoside	2-*β*-*D*-Glucopyranose-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne		Aerial (USA); whole (Taiwan); leaves (Taiwan)	[15, 23, 24, 30, 41]
17	(4*E*)-1-(2-Hydroxylethyl)-4-dodecene-6,8,10-triyn-1-yl-*β*-*D*-glucopyranoside	3-*β*-*D*-Glucopyranose-1-hydroxy-6(*E*)-tridecene-8,10,12-triyne		Aerial (USA and China); whole (Taiwan); leaves (Taiwan)	[15, 23, 24, 30, 41, 44]
18	3-Hydroxy-6-tetradecene-8,10,12-triynyl-*β*-*D*-glucopyranoside	*β*-*D*-Glucopyranoyloxy-3-hydroxy-6*E*-tetradecene-8,10,12-triyne		Whole (Mexico)	[45]
19	1-(Hydroxymethyl)-4,6,8,10-dodecatetrayne-1-yl-*β*-*D*-glucopyranoside	Cytopiloyne, 2-*β*-*D*-Glucopyranose-1-hydroxytrideca-5,7,9,11-tetrayne		Whole (Taiwan); leaves (Taiwan)	[15, 20, 24]
20	2-*O*-*D*-Glucosyltrideca-11*E*-en-3,5,7,9-tetrayn-1,2-diol			Leaves (Brazil)	[46]

Table 4: Continued.

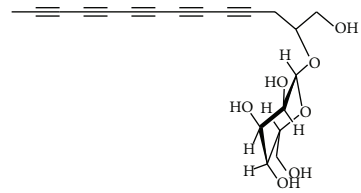
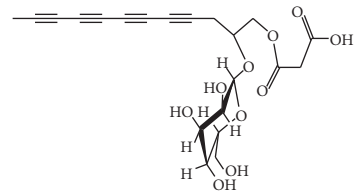
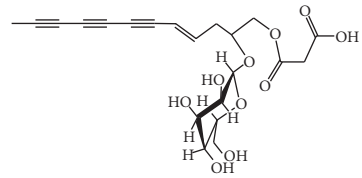
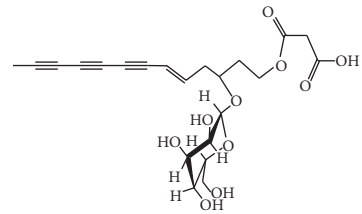
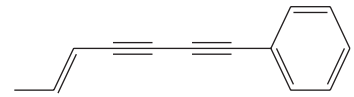
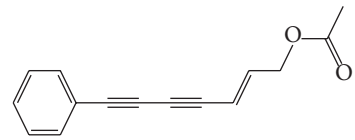
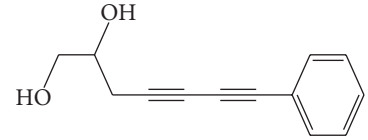
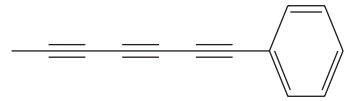
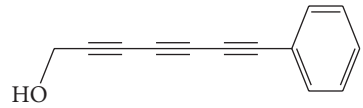
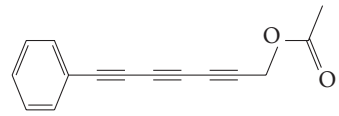
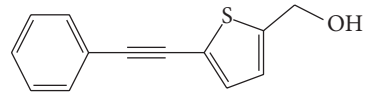
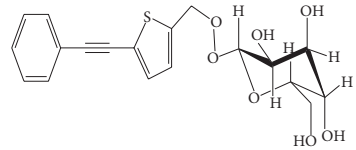
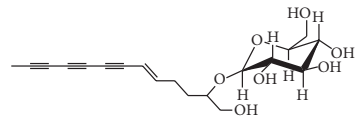
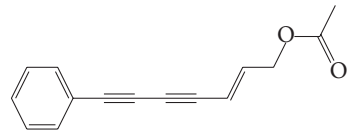
S. number	IUPAC names	Common names	Structure	Plant part (country)	References
21	(*R*)-1-(Hydroxymethyl)-2,4,6,8,10-dodecapentayn-1-yl-*â*-*D*-glucopyranoside	2-*β*-*D*-Glucopyranose-1-hydroxytrideca-3,5,7,9,11-pentayne		Aerial (China and Japan)	[43, 44]
22	1-[[(Carboxyacetyl)oxy]methyl]4,6,8,10-dodecatetraynyl-*â*-*D*-glucopyranoside			Aerial (Japan)	[47]
23	(4*E*)-1-[[(Carboxyacetyl)oxy]-methyl]-4-dodecene-6,8,10-triynyl-*â*-*D*-glucopyranoside			Aerial (Japan)	[47]
24	(4*E*)-1-[[(Carboxyacetyl)oxy]-ethyl]-4-dodecene-6,8,10-triynyl-*â*-*D*-glucopyranoside			Aerial (Japan)	[47]
25	(5*E*)-5-Heptene-1,3-diyn-1-yl-benzene	1-Phenylhepta-1,3-diyn-5en		Whole (Taiwan)	[48]
26	7-Phenyl-2(*E*)-heptene-4,6-diyn-1-ol			Roots (not stated); aerial (China)	[34, 38]
27	7-Phenyl-2(*E*)-heptene-4,6-diyn-1-ol-acetate			Roots not stated and brazil	[34, 49, 50]
28	7-Phenyl-4,6-heptadiyn-2-ol	Pilosol A		Whole (Taiwan); aerial (China)	[38, 48]
29	7-Phenylhepta-4,6-diyn-1,2-diol			Aerial (China)	[38]

Table 4: Continued.

S. number	IUPAC names	Common names	Structure	Plant part (country)	References
30	1,3,5-Heptatriyn-1-yl-benzene	1-Phenylhepta-1,3,5-triyne		Leaves (not stated); leaves of tissue culture (not stated); aerial (Tanzania and China); whole (Taiwan); roots (Brazil)	[34, 38, 48, 50–53]
31	7-Phenyl-2,4,6-heptatriyn-1-ol			Leaves (not stated) and aerial (China)	[34, 38]
32	7-Phenyl-2,4,6-heptatriyn-1-ol-acetate			Leaves (not stated)	[34]
33	5-(2-Phenylethynyl)-2-thiophene methanol			Aerial (China)	[38]
34	5-(2-Phenylethynyl)-2β-glucosylmethyl-thiophene			Aerial (China)	[38]
35	3-β-*D*-Glucopyranosyl-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne			Leaves (Cameroon)	[54]
36	1-Phenyl-1,3-diyn-5-en-7-ol-acetate			Leaves (Brazil)	[46]

that causes 50% lethality, of the hydroethanol extracts in mice was 12.3 g/kg BW and 6.2 g/kg BW, respectively [61]. Ezeonwumelu et al. showed that oral delivery of the water extract of the *B. pilosa* whole plant at 1 g/kg BW/day, once a day, seems nontoxic in rats over 28 days in Wistar rats [62], which is in line with our observations in rat [5]. Overall, these data suggest that consumption of *B. pilosa* aqueous extract at up to at 1 g/kg BW/day, once a day, is highly safe in rats. A complete toxicology and drug-drug interaction of *B. pilosa* with other drugs in humans are required prior to its further medical use.

6. Conclusions

B. pilosa is a worldwide plant and widely used as folk remedies and foods. It has long used to treat diabetes in different continents. However, a comprehensive up-to-date review of research on *B. pilosa* for diabetes has hitherto been not available. In this paper, scientific studies on the use of *B. pilosa* as an anti-diabetic remedy have been summarized and critically discussed from botanical, phytochemical, pharmacological, and toxicological aspects. Thirty-six polyynes identified from this plant were identified and three of which were showed to treat both T1D and T2D. The anti-diabetic utility of *B. pilosa* and its modes of action in relation to its known polyynes were discussed herein. Cautions should be taken in the anti-diabetic use of *B. pilosa* alone and in combination with other medicines since its overdose may cause dramatic hypoglycemia.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

Table 5: Polyynes of *B. pilosa* and their biological activities.

S. number	Name	Classification	Molecular formula	Biological activities
11	1,2-Dihydroxy-5(*E*)-tridecene-7,9,11-triyne [55]	Polyyne	$C_{13}H_{14}O_2$	Antiangiogenetic [55] Antiproliferative [55]
12	1,3-Dihydroxy-6(*E*)-tetradecene-8,10,12-triyne [56]	Polyyne	$C_{14}H_{16}O_2$	Antiangiogenetic [56]
14	1,2-Dihydroxytrideca-5,7,9,11-tetrayne [56]	Polyyne	$C_{13}H_{12}O_2$	Antiangiogenetic [56]
15	(*R*)-1,2-dihydroxytrideca-3,5,7,9,11-pentayne [43]	Polyyne	$C_{13}H_8O_2$	Antimalarial [43] Antibacterial [43]
6	2-*β*-*D*-Glucopyranose-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne [54]	Polyyne	$C_{19}H_{24}O_7$	Antidiabetic [54] Anti-inflammatory [57] Antimalarial [43] Antibacterial [43]
30	1-Phenylhepta-1,3,5-triyne [58]	Polyyne	$C_{13}H_8$	Antimicrobial [59] Antimalarial [9] Cytotoxic [9] Antifungal [60]
35	3-*β*-*D*-Glucopyranosyl-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne [54]	Polyyne	$C_{20}H_{26}O_7$	Antidiabetic [54] Anti-inflammatory [57]

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Research Article

Mediterranean Diet and Red Yeast Rice Supplementation for the Management of Hyperlipidemia in Statin-Intolerant Patients with or without Type 2 Diabetes

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Lipid profile could be modified by Mediterranean diet (MD) and by red yeast rice (RYR). We assessed the lipid-lowering effects of MD alone or in combination with RYR on dyslipidemic statin-intolerant subjects, with or without type 2 diabetes, for 24 weeks. We evaluated the low-density lipoprotein (LDL) cholesterol level, total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, triglyceride, liver enzyme, and creatinine phosphokinase (CPK) levels. We studied 171 patients: 46 type 2 diabetic patients treated with MD alone (Group 1), 44 type 2 diabetic patients treated with MD associated with RYR (Group 2), 38 dyslipidemic patients treated with MD alone (Group 3), and 43 dyslipidemic patients treated with MD plus RYR (Group 4). The mean percentage changes in LDL cholesterol from the baseline were $-7.34 \pm 3.14\%$ ($P < 0.05$) for Group 1; $-21.02 \pm 1.63\%$ ($P < 0.001$) for Group 2; $-12.47 \pm 1.75\%$ ($P < 0.001$) for Group 3; and $-22 \pm 2.19\%$ ($P < 0.001$) for Group 4 with significant intergroup difference (Group 1 versus Group 2, $P < 0.001$; Group 3 versus Group 4, $P > 0.05$). No significant increase in AST, ALT, and CPK levels was observed in all groups. Our results indicate that MD alone is effective in reducing LDL cholesterol levels in statin-intolerant patients with a presumably low cardiovascular risk, but associating MD with the administration of RYR improves patients' LDL cholesterol levels more, and in patients with type 2 diabetes.

1. Introduction

The prevalence of metabolic syndromes (MS) and the associated cardiovascular diseases (CVDs) is increasing rapidly around the world. Lifestyle measures, including dietary changes and physical activity, play a crucial role in preventing these conditions, and the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) has already suggested dietary intervention to contain this epidemic [1].

Cardiovascular risk factors in MS could be modified by dietary interventions. The Mediterranean diet (MD) is characterized by a high consumption of monounsaturated fatty acids (primarily from olive oil) and a daily consumption of fruit, vegetables, whole-grain cereals, and low-fat dairy products; weekly consumption of fish, poultry, tree nuts, and legumes; a relatively low consumption of red meats (approximately twice a month) [2]. The beneficial effect of the MD on all-cause mortality, CVD, and cancer, as well as on obesity and type 2 diabetes, has already been reported, based on the results of many epidemiological studies and clinical trials [3–5]. The results of a meta-analysis revealed the protective role of the MD on components of MS such as waist circumference, high-density lipoprotein cholesterol, triglycerides, systolic and diastolic blood pressure, and blood glucose levels [6]. However, acceptable total cholesterol (TC) or LDL-C levels are difficult to be achieved with lifestyle changes alone in order to significantly reduce the cardiovascular risk.

Some studies have shown that red yeast rice (RYR) reduces low-density lipoprotein (LDL) cholesterol levels

in hypercholesterolemic patients [7, 8]. Becker et al. also demonstrated that RYR and therapeutic lifestyle changes reduced LDL cholesterol levels in dyslipidemic patients unable to tolerate statin therapy [9].

A combination of RYR extract, policosanol, berberine, folic acid, and Q_{10} coenzyme reportedly induced a significant metabolic improvement in elderly patients with dyslipidemia [10].

In nondiabetic patients with dyslipidemia, a combination of RYR, policosanol, berberine, folic acid, and Q_{10} coenzyme in addition to dietary counseling was found to amplify the effect of diet on central obesity, improve lipid profiles and blood pressure, and reduce the incidence of MS [11].

To our knowledge, little is known about the efficacy of RYR extract in patients with type 2 diabetes. In particular, no data are available on the effect of RYR supplementation combined with a Mediterranean diet on the lipid profiles of such patients. Hence, this randomized, parallel-group controlled study lasting six months to investigate whether adding a combination of RYR extract, artichoke extract, resveratrol, chrome, folic acid, and coenzyme Q_{10} (Redulip, For Farma srl) to the Mediterranean diet could improve the lipid profile of dyslipidemic patients with and without type 2 diabetes.

2. Methods

This study was designed as a controlled, randomized, parallel-group study and complied with the content of the Helsinki Declaration. The Local Institutional Review Board approved the study protocol and all participants provided written informed consent.

We studied consecutive patients attending our outpatient clinic from January to October 2010. Patients were included in the study if they had total cholesterol levels higher than 200 mg/dL and/or low-density lipoprotein (LDL) cholesterol levels higher than 130 mg/dL and a cardiovascular risk (as assessed according to the Progetto Cuore) of ≤10% for dyslipidemic patients and ≤15% for diabetic patients [12] and if they had discontinued at least one statin because of myalgias with a resolution of muscle pain when the medication was discontinued. The exclusion criteria were as follows: the presence of intima media thickness or carotid plaques on echo-color Doppler B-mode images obtained with a high-resolution imaging system (always by the same operator); use of statins up to 6 months before the study; a history of secondary dyslipidemia, except for diabetes; abnormal baseline laboratory values (a CPK level >500 U/L; AST or ALT levels >1.5 times the normal upper limit). Current and former smokers and pregnant or breast-feeding women were also ruled out.

The sample of 171 eligible participants included 90 type 2 diabetic patients with dyslipidemia and 81 dyslipidemic patients without type 2 diabetes.

At the baseline visit, we used 24-hour recall, a self-reporting method for collecting data on eating behavior and measuring energy intake by means of structured interviews, as described elsewhere [13]. A Mediterranean style dietary pattern, as previously reported [2], was recommended to all patients to improve their lipid profiles. In particular, the main goals of these recommendations were a calorie intake (between 1500 and 1800 calories) tailored to reach and/or maintain a desirable body weight, a fat intake amounting to less than 30% of the total calories (15–20% of monounsaturated fatty acids, primarily from olive oil), and a carbohydrate intake of 55–60% in dyslipidemic patients and 45–55% in diabetic patients. Patients were randomly assigned to four groups. Group 1 consisted of 46 type 2 diabetic patients treated with MD alone; Group 2 consisted of 44 type 2 diabetic patients treated with the MD plus a nutraceutical-combined pill (NCP) containing RYR extract 200 mg (equivalent to 3 mg monacolin K), artichoke extract 400 mg, resveratrol 15 mg, chromium 200 mcg, folic acid 200 mcg, and coenzyme Q_{10} 10 mg (Redulip, For Farma srl); Group 3 consisted of 38 dyslipidemic patients treated with the MD alone; Group 4 consisted of 43 dyslipidemic patients treated with the MD plus the NCP. Adherence to the medication was ascertained by means of pill counts on the study medication returned at follow-up visit.

The primary outcome was the low-density lipoprotein (LDL) cholesterol level measured at the baseline and after 24 weeks. Secondary outcomes included total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, triglyceride, liver enzyme, and creatinine phosphokinase (CPK) levels.

Patients attended follow-up visits after 24 weeks of treatment. None of the patients dropped out of the study.

At each visit, all patients were assessed in terms of body mass index (BMI), diastolic and systolic blood pressure (measured with patients being seated, using a standard sphygmomanometer), and waist circumference (midway between the lowest rib and the iliac crest).

A fasting blood sample was obtained at the baseline and at week 24 to measure LDL cholesterol, TC, HDL cholesterol, triglyceride, CPK, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels. All analyses were performed at the laboratory of the University Hospital of Padua, Italy.

At weeks 24, patients' dietary compliance was assessed using the 24-hour recall method [12] and an analysis of the food diaries completed weekly by the patients.

2.1. Statistical Analysis. Values are expressed as mean ± SD or SEM. Data were normalized according to each patient's baseline situation by calculating the difference (after-before) at the two follow-up times considered (24 weeks after starting the study) and expressing the value as a percentage of the parameters at the baseline. Negative values therefore indicate the effective percentage decrease in the parameter following the treatment. ANOVA followed by the *post hoc* test was used to identify statistical differences between the groups at the two different follow-up times. The statistical significance of the differences (after-before) induced by the treatment within each group of patients was tested with Student's *t*-test for paired data. Differences were considered statistically significant when $P < 0.05$ (two-tailed test).

3. Results

3.1. Baseline Characteristics. Table 1 shows the clinical characteristics of the four groups of patients at the baseline. Groups 1 and 2 differed significantly only in terms of BMI and systolic and diastolic blood pressure; Groups 3 and 4 differed significantly in terms of BMI, body weight waist circumference, and ALT levels. As regards the lipid profile, groups 1 and 2 did not differ significantly, but Groups 3 and 4 differed significantly in terms of TC, HDL cholesterol, LDL cholesterol, and triglycerides. In particular, Group 4 had higher levels of TC ($P < 0.001$), higher levels of LDL cholesterol ($P < 0.001$), higher levels of HDL cholesterol ($P < 0.001$), and lower levels of triglycerides ($P < 0.05$).

3.2. Changes in Clinical Characteristics. Table 2 shows the normalized changes in the parameters after 24 weeks of treatment. Only the dyslipidemic patients treated with MD alone (Groups 3) experienced a significant reduction in body weight, BMI, and waist circumference from the baseline to week 24; the mean percentage change in these patients' BMI was $-3.89 \pm 0.92\%$ ($P < 0.001$), and as regards waist circumference the reduction was -2.42 ± 0.82 cm ($P < 0.01$). All the patients kept to a Mediterranean-style diet for all 24 weeks.

3.3. Primary Outcome Measures. Table 2 shows the differences in the parameters in the four groups after 24 weeks of treatment. It is worth noting that the changes observed with the different treatments were significant and comparable between the groups, independently from the within-group differences at the baseline, since all the results were expressed as normalized values, taking the baseline condition of each patient for reference in the calculation. This normalization was also considered to evaluate and compare the effectiveness of the treatments appropriately.

A significant drop in TC was seen in all groups of patients. In particular, the average reduction in TC 24 weeks after the baseline was $-4.65 \pm 1.92\%$ in Group 1 ($P < 0.05$), $-15.45 \pm 1.26\%$ in Group 2 ($P < 0.001$), $-11.96 \pm 1.43\%$ in Group 3 ($P < 0.001$), and $-16.94 \pm 1.51\%$ in Group 4 ($P < 0.001$), with significant between-group differences (Group 1 versus Group 2, $P < 0.001$; Group 3 versus Group 4, $P > 0.05$).

The mean percentage changes in LDL cholesterol levels from the baseline were -7.34 ± 3.14 ($P < 0.05$) for Group 1, they were $-21.02 \pm 1.63\%$ ($P < 0.001$) for Group 2, they were $-12.47 \pm 1.75\%$ ($P < 0.001$) for Group 3, and they were $-22 \pm 2.19\%$ ($P < 0.001$) for Group 4. The mean percentage change in LDL levels differed significantly between Groups 1 and 2 ($P < 0.001$), and between Groups 3 and 4 ($P < 0.01$).

We found no significant differences in terms of HDL cholesterol and triglycerides levels in any of the groups (Table 2).

3.4. Safety Data. Regarding the safety of treatment with NCP, there was no significant increase in CPK or liver-associated enzyme levels in any of the patients after 24 weeks of treatment. None of the patients discontinued the treatment with NCP and no side effects were observed. As regards liver function, we observed after 24 weeks of treatment a significant drop in AST and ALT levels in type 2 diabetic patients (Groups 1 and 2) from the baseline. In particular, we observed in Group 1 a decline in AST levels of $-6.48 \pm 2.83\%$ ($P < 0.05$) and in ALT levels of $-6.60 \pm 2.58\%$ ($P < 0.05$); in Group 2 we observed a decline in AST levels of $-7.52 \pm 3.31\%$ ($P < 0.05$) and in ALT levels of $-7.12 \pm 2.96\%$ ($P < 0.05$).

4. Discussion

This controlled, randomized, parallel-group study demonstrated that, in statin-intolerant patients, associating a MD with a combination of nutraceuticals (NUTs) based on red yeast rice extract can significantly improve dyslipidemic patients' lipid profiles by comparison with diet alone.

The effects of MD on lipid profile and in protecting against cardiovascular risks are well known.

In a recent meta-analysis, MD was also found associated with a lower risk of MS; in particular, several studies showed the beneficial role of MD on HDL cholesterol and triglyceride levels [6]. This meta-analysis considered studies on the general population, type 2 diabetic patients, overweight subjects, and hypercholesterolemic patients. Several randomized, controlled trials have also demonstrated that MD reduces total cholesterol and LDL cholesterol levels by 5–15% and increases HDL cholesterol levels by 3–15% [14, 15].

In agreement with previous studies, our findings showed that adherence to MD alone significantly reduced BMI, waist circumference, TC, and LDL cholesterol levels in overweight dyslipidemic patients without type 2 diabetes. The beneficial effects of MD on BMI and waist circumference were not seen in type 2 diabetic patients. These findings are consistent with other reports of weight loss programs proving less effective in overweight and obese diabetic patients [16–18]. On the other hand, we observed a significant decline in liver enzyme concentrations in type 2 diabetic patients treated with MD alone or in combination with NCP. These data are in agreement with a previous study that demonstrated that the adherence to a diet similar to MD can improve the liver function in MS subjects [19]. The treatment with RYR did not affect the beneficial effect of MD.

Our results also indicate that associating a combination of NUTs with MD can add to the lipid-lowering effect of MD, in particular on LDL cholesterol, in terms of a 10% improvement in dyslipidemic patients.

A previous study demonstrated the effect of a combination of red yeast rice extract and berberine (an isoquinoline alkaloid found in plants of the genus *Berberis* and *Coptis,* with neuroprotective and antiatherosclerotic actions [20]) associated with dietary restrictions on lipid profiles after 16 weeks of treatment, globally achieving a 23.5% reduction in LDL cholesterol levels [11]. Our present investigation is the first to be conducted on a combination of NUTs associated with MD and administered for 24 weeks. The combination of NUTs used in our study contained no berberine, which has a lipid-lowering effect in its own right, by upregulating LDL cholesterol receptors on the liver cell surface [21].

Table 1: Baseline anthropometric and haematochemical parameters in the four groups of patients. Data are the mean ± standard deviation (SD).

	Group 1 Diabetic patients/ diet ($n = 46$)	Group 2 Diabetic patients/ diet + red rice ($n = 44$)	Group 3 Dyslipidemic patients/ diet ($n = 38$)	Group 4 Dyslipidemic patients/ diet + red rice ($n = 43$)	P Group 1 versus 2	P Group 3 versus 4
Gender (M/F)	19/27	18/26	20/18	14/29	n.s.	n.s.
Age (yrs)	53.28 ± 10.41	54.73 ± 11.40	52.37 ± 9.83	51.28 ± 9.68	n.s	n.s.
Height (m)	1.64 ± 0.09	1.66 ± 0.11	1.67 ± 0.08	1.66 ± 0.09	n.s.	n.s.
Body weight (kg)	84.68 ± 10.25	82.10 ± 17.26	77.95 ± 14.15	70.25 ± 12.01	n.s.	•
BMI	31.57 ± 3.73	29.70 ± 5.02	27.77 ± 4.26	25.36 ± 3.16	•	••
Waist (cm)	98.64 ± 11.47	96.75 ± 12.69	94.61 ± 12.07	86.70 ± 10.43	n.s	••
TC (mg/dL)	231.13 ± 22.01	234.80 ± 19.09	251.37 ± 32.76	275.81 ± 32.20	n.s.	• • •
LDL (mg/dL)	149.07 ± 21.95	148.72 ± 20.38	168.23 ± 31.70	188.98 ± 28.79	n.s.	• • •
HDL (mg/dL)	54.15 ± 13.17	60.00 ± 15.46	49.66 ± 12.50	61.26 ± 16.94	n.s.	• • •
TG (mg/dL)	139.52 ± 53.69	130.36 ± 58.32	167.42 ± 100.75	127.91 ± 65.82	n.s.	•
CPK (ng/mL)	112.47 ± 40.68	110.59 ± 35.74	114.03 ± 40.95	114.23 ± 46.57	n.s	n.s.
AST (mU/mL)	27.37 ± 17.34	29.18 ± 19.92	23.03 ± 7.83	22.44 ± 6.14	n.s	n.s.
ALT (mU/mL)	30.74 ± 18.58	32.41 ± 17.62	32.95 ± 23.89	22.09 ± 7.37	n.s	••
GGT (mU/mL)	26.53 ± 19.45	28.43 ± 20.17	29.55 ± 28.17	22.05 ± 10.89	n.s	n.s.
Systolic BP (mmHg)	142.80 ± 15.00	134.05 ± 14.39	130.53 ± 17.00	124.77 ± 15.08	••	n.s.
Diastolic BP (mmHg)	86.07 ± 10.74	81.52 ± 8.15	81.18 ± 10.36	77.56 ± 8.89	•	n.s.

To assess statistical differences between groups, ANOVA followed by *post hoc* test was used for continuous data; for frequency data, χ^2 test was used.

• • •: $P < 0.001$; ••: $P < 0.01$; •: $P < 0.05$; n.s.: not significant.

Abbreviations: BMI: body mass index; TC: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; TG: triglyceride; CPK: creatinine phosphokinase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma glutamyl transferase; BP: blood pressure.

Table 2: Differences (Δ) (after – before), expressed as percentage of basal value, in parameters observed in the four groups of patients, following 24 weeks of treatment.

Difference (after – before) % of basal at 6 months	Group 1 Diabetic patients diet (n = 46)		Group 2 Diabetic patients diet + red rice (n = 44)		Group 3 Dyslipidemic patients diet (n = 38)		Group 4 Dyslipidemic patients diet + red rice (n = 43)		P	
									Group 1 versus 2	Group 3 versus 4
Δ-Body weight % (kg)	0.89	±0.83	−0.86	±0.55	−3.89	±0.92†††	−0.87	±0.51	n.s.	••
Δ-BMI %	0.89	±0.83	−0.86	±0.55	−3.89	±0.92†††	−0.87	±0.51	n.s.	••
Δ-Waist % (cm)	−0.89	±0.32	−0.93	±0.44	−2.42	±0.82††	−0.98	±0.55	n.s.	n.s.
Δ-TC % (mg/dL)	−4.65	±1.92†	−15.45	±1.26†††	−11.96	±1.43†††	−16.94	±1.51†††	•••	•
Δ-LDL % (mg/dL)	−7.34	±3.14†	−21.02	±1.63†††	−12.47	±1.75†††	−22.00	±2.19†††	•••	••
Δ-HDL % (mg/dL)	2.87	±2.38	−2.93	±1.72	−0.11	±2.67	−1.94	±1.53	•	n.s.
Δ-TG % (mg/dL)	2.30	±4.32	−3.77	±4.33	−7.24	±7.53	−7.52	±3.83	n.s.	n.s.
Δ-CPK % (ng/mL)	−2.84	±1.32	−3.51	±1.84	−1.15	±1.36	2.14	±5.62	n.s.	n.s.
Δ-AST % (mU/mL)	−6.48	±2.83†	−7.52	±3.31†	0.52	±4.52	−0.78	±2.40	n.s.	n.s.
Δ-ALT % (mU/mL)	−6.60	±2.58†	−7.12	±2.96†	−12.37	±6.02†	1.60	±3.30	n.s.	•
Δ-GGT % (mU/mL)	8.47	±15.74	10.30	±18.64	−6.89	±4.39	−1.48	±4.67	n.s	n.s.
Δ-Systolic BP % (mmHg)	3.42	±2.13	−2.06	±1.45	−0.47	±1.83	1.51	±1.75	•	n.s.
Δ-Diastolic BP % (mmHg)	3.03	±2.80	−0.41	±1.33	−0.23	±1.75	0.49	±1.56	n.s.	n.s.

Statistical significance for difference (after – before); treatment within each group of patients was checked with Student's t-test for paired data. $^{\dagger\dagger\dagger}P < 0.001$; $^{\dagger\dagger}P < 0.01$; $^{\dagger}P < 0.05$.
To assess statistical differences between groups, ANOVA followed by *post hoc* test was used. • • •: $P < 0.001$; ••: $P < 0.01$; •: $P < 0.05$; n.s.: not significant.
Abbreviations: BMI: body mass index; TC: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; TG: triglyceride; CPK: creatinine phosphokinase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma glutamyl transferase; BP: blood pressure.
Data are the mean ± standard error of the mean (SEM). Negative values indicate a decrease of the parameter after respective treatment.

It is worth emphasizing that our results relate exclusively to the hypolipidemic effect of RYR that contains monacolin K, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase [22], and sterols, compounds that are known to block cholesterol absorption in the gut [23, 24], with a synergic effect on lipoproteins.

Associating a combination of NUTs with a Mediterranean-style diet could prove a valuable therapeutic option for dyslipidemic statin-intolerant patients at low cardiovascular risk without excessively high LDL cholesterol levels.

The composition of the NUTs used in some previous studies varied considerably, particularly in terms of the concentration of monacolin K, which ranged from 9.6 mg to 3 mg. The dose of monacolin K in the NUTs used in our study was 3 mg. Becker et al. [9] obtained the same reduction in LDL cholesterol levels as us, but with twice as much monacolin K (6 mg). Our findings therefore suggest that a lower dose of monacolin K (about 3 mg/day) may suffice to achieve an evident therapeutic effect.

We recorded none of the adverse effects described elsewhere in patients treated with red yeast rice [25–27], probably because of the lower dose of monacolin K used in our study. Further studies are needed, however, to investigate this issue over a longer period of treatment.

To our knowledge, ours is the first study to show that associating MD with NUTs can improve, in terms of 21%, LDL cholesterol levels of type 2 diabetic statin-intolerant patients. It is often difficult to obtain a normalization of TC, or even of LDL cholesterol, with dietary restrictions alone in diabetic patients, so adding a NUT based on red yeast rice might be a good therapeutic option for type 2 diabetic patients, at low cardiovascular risk with no evidence of vascular damage or other complications, who have previous statin intolerance. The reduction in LDL cholesterol levels achieved with MD plus NUTs was similar to the reduction obtained using statins, as already reported in dyslipidemic patients with statin intolerance [7].

The limitation of this study is the small size of the sample studied; further studies on larger samples will be needed to confirm the validity of this patient management approach, particularly in cases of type 2 diabetes.

5. Conclusions

Despite its limitations, this study provides useful new insight into the nutraceutical/dietary treatment of lipid profiles, even in patients with type 2 diabetes. Our results indicate that MD counseling alone is effective in reducing LDL cholesterol levels in moderately hypercholesterolemic patients with a presumably low cardiovascular risk, but associating MD with the administration of RYR improved patients' lipid profiles considerably more, also in patients with type 2 diabetes with statin intolerance.

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Research Article

Stevioside from *Stevia rebaudiana* Bertoni Increases Insulin Sensitivity in 3T3-L1 Adipocytes

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Stevioside from *Stevia rebaudiana* has been reported to exert antihyperglycemic effects in both rat and human subjects. There have been few studies on these effects *in vitro*. In this paper, radioactive glucose uptake assay was implemented in order to assess improvements in insulin sensitivity in 3T3-L1 cells by elevation of glucose uptake following treatment with stevioside. Oil Red-O staining and MTT assay were utilized to confirm adipocyte differentiation and cell viability, respectively. Findings from this research showed a significant increase in absorbance values in mature adipocytes following Oil Red-O staining, confirming the differentiation process. Stevioside was noncytotoxic to 3T3-L1 cells as cell viability was reduced by a maximum of 17%, making it impossible to determine its IC_{50}. Stevioside increased glucose uptake activities by 2.1 times ($p < 0.001$) in normal conditions and up to 4.4 times ($p < 0.001$) in insulin-resistant states. At times, this increase was higher than that seen in positive control group treated with rosiglitazone maleate, an antidiabetic agent. Expressions of pY20 and p-IRS1 which were measured via Western blot were improved by stevioside treatment. In conclusion, stevioside has direct effects on 3T3-L1 insulin sensitivity via increase in glucose uptake and enhanced expression of proteins involved in insulin-signalling pathway.

1. Introduction

Epidemic-level emergence of many noncommunicable diseases as a result of modern lifestyle and dietary habits is worrying. Insulin resistance has commonly been linked to metabolic syndromes resulting in type 2 diabetes mellitus, as well as obesity. In fact, it is purported to underlie the progression of type 2 diabetes [1] and in cases of burn trauma, hyperlipidaemia and cancer cachexia [2]. It is important to note that insulin resistance occurs in an impaired insulin-signalling pathway, indicative of patients with type 2 diabetes. In normal individuals with fully functioning insulin-signalling activities, insulin will be secreted once blood glucose levels are increased following intake of food, and will subsequently bind to its receptor. This binding action will lead to several stages of signalling and phosphorylation cascades, resulting in migration of glucose transporter 4 (GLUT4) from cytoplasm to cellular membrane to take up extracellular glucose. However, in a state of insulin resistance, these signalling activities and cascades are interrupted, blocking said migration of GLUT4, if not disrupting the protein's expression altogether [3]. Hence, a better understanding of these mechanisms will possibly lead to breakthroughs in unravelling the secrets of both insulin resistance and diabetes.

As is often the case, traditional communities use local herbs in their folk and traditional medicines for treating hyperglycaemia and diabetes. Among these is *Stevia rebaudiana* Bertoni, a perennial herb commonly grown in tropical and subtropical regions, specifically in South America and Asia. In recent years, Malaysians too have taken a particular interest in this herb as it has been promoted as a sweetening alternative to sucrose, beneficial specifically for those with obesity and diabetes. *Stevia rebaudiana* has little to no caloric value despite its sweetening abilities, thus will not jeopardise patients' blood glucose levels, while fulfilling their cravings for sweet food and drinks [4]. *Stevia rebaudiana* is sweet

due to its constituents of steviol glycosides including stevioside, rebaudioside A and rebaudioside C [5]. Furthermore, previous reports showed this plant has antioxidant [6] and antihyperglycaemic [7] properties, increasing its potential for use in adjuvant management of diabetes mellitus and associated conditions. There has been little investigation into such assertions which has prompted this study to evaluate how stevioside can affect insulin sensitivity, particularly through observation of glucose uptake, and expression of proteins involved in insulin-signalling pathway at a cellular level through use of 3T3-L1 adipocytes.

2. Methods

2.1. Materials. 3T3-L1 preadipocytes were commercially acquired from ATCC (American Type Culture Collections, USA). Chemicals, including stevioside, cell supplements, and media, were mostly purchased from Sigma-Aldrich Co. (Germany) and Lonza (USA). Ultima Gold LLT scintillation cocktail and 2-deoxy-[1-3H]-glucose were commercially obtained from Perkin Elmer (USA). The antidiabetic drug rosiglitazone maleate (AVANDIA) was bought from a local drugstore. β-Actin, p-IRS1, and pY20 primary antibodies, and donkey anti-goat and goat anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibodies were purchased from Santa Cruz (USA).

2.2. Cell Culture and Differentiation. 3T3-L1 preadipocytes were cultured in Dulbecco's Modified Eagle's Media (DMEM). Cells were later differentiated with supplements of insulin, dexamethasone (DMX), and 3-isobutyl-1-methylxanthine (IBMX) following the procedures described by Ahn et al. [8] and Ismail et al. [9]. In order to induce insulin resistance, cells were treated with tumour necrosis factor-α (TNF-α) at 1.0 ng/mL for 4 days prior to treatment.

2.3. Oil Red-O Staining. 3T3-L1 cells were washed with phosphate-buffered saline (PBS) before being fixed with a solution of 10% formaldehyde in PBS. After overnight incubation, the solution was discarded and Oil Red-O dye was introduced to cells for 10 minutes at room temperature. Subsequently, excess dye was washed with de-ionised water. The stain was later eluted out with 100% isopropanol and measured spectrophotomerically at 520 nm.

2.4. Cell Viability Test. 3T3-L1 adipocytes were cultured in 96-well plates and were pretreated with stevioside (25–300 μM) for 14, 24, 48, and 72 hours. After pretreatment, the cells received 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and were further incubated for 4 hours. The purple formazan crystals were then dissolved with dimethyl sulfoxide (DMSO) before their absorbance values were read on a microplate reader at 590 nm.

2.5. Glucose Uptake Assay. Glucose uptake levels by differentiated 3T3-L1 adipocytes were analysed using a method previously described by Adam et al. [10], with minor modifications. Briefly, 3T3-L1 adipocytes were differentiated on 12-well plates. For insulin-resistance studies, cells were initially induced to insulin resistance with TNF-α treatment, as mentioned earlier. Cells were serum-starved for 2 hours. Next, they were washed with Krebs-Ringer bicarbonate (KRB) buffer and preincubated with a range of stevioside concentrations (30–150 μM and 30–120 μM) for half an hour, with or without insulin addition, with rosiglitazone as a positive control. 2-Deoxy-[1-3H]-glucose (1 μCi/mL) was then added to each well to initiate glucose uptake, and incubated for a further hour. Cells were then washed with ice-cold KRB buffer and solubilised by 0.1% sodium dodecyl sulphate (SDS). Finally, samples were collected and mixed with 15 mL of Ultima Gold LLT scintillation cocktail before being measured in a liquid scintillation counter.

2.6. Western Blotting. 3T3-L1 cells were cultured and differentiated in 6-well plates. Cells were given TNF-α to induce insulin resistance, as described earlier, prior to treatment with stevioside. Treatments of stevioside and rosiglitazone maleate were given, in 60 and 90 μM concentrations, for 24 hours. Cells were stimulated with insulin for 5 minutes prior to harvesting. From the preparation of cell lysates and loading of samples to Western blot analysis, procedures described by Ismail et al. [9] were followed, with minor modifications.

2.7. Statistical Analysis. Data were presented as mean ± standard error mean (SEM) for a given number of tests. The results were processed statistically by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc tests, using the Sigma Plot version 12 software. Statistically different means were recognised at $p < 0.05$.

3. Results

3.1. Oil Red-O Staining. Effects of the supplements (insulin, DMX, and IBMX) on adipocyte differentiation are presented in Figure 1. In order to proceed with further experiments, adipocyte differentiation was confirmed by conducting the Oil Red-O staining procedure. Lipid stain from fully differentiated adipocytes was eluted out and measured quantitatively in a spectrophotometer, where the readings were found to be significantly increased when compared to control group.

3.2. MTT Cytotoxicity Test. Cells were previously differentiated to mature adipocytes before treatment with stevioside (25–300 μM) for 14, 24, 48, and 72 hours. Cell viability was determined from absorbance readings at 590 nm, corresponding to formazan crystals formed by living cells (Figure 2). There were no significant differences in cell viabilities in any of the stevioside treatment groups, at any treatment period, with the exception of those treated for a period of 72 hours. Viability of cells treated with 250 μM of stevioside for 72 hours was reduced by 17% at most. No half-maximal inhibitory concentration (IC_{50}) was found, as cell viability did not fall lower than the 80% benchmark.

3.3. Glucose Uptake Assay

3.3.1. Optimum Insulin Concentration. Prior to investigating effects of stevioside on glucose uptake in 3T3-L1 adipocytes,

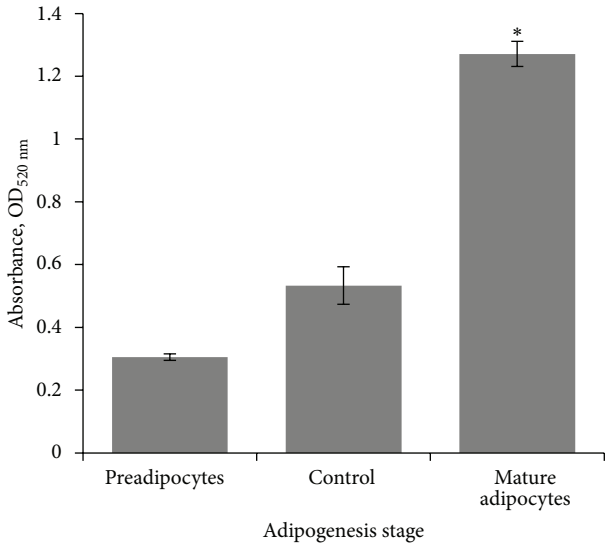

Figure 1: Effect of induction of differentiation on lipid accumulation in 3T3-L1 cells, presented by Oil Red-O staining. To quantify lipid accumulation in cells as a result of differentiation, the stain was eluted with 100% isopropanol and measured spectrophotometrically at 520 nm. Mean ± SEM ($n = 3$). * Significantly different from control ($p < 0.05$, ANOVA & Dunnett's test).

separate assays were conducted to find the optimum concentrations of insulin and TNF-α to be used. From Figure 3(a), it can be observed that starting from 100 nM of insulin, there was a highly significant increase in glucose uptake, with maximum uptake at 125 nM. There was no significant difference between these two groups. Therefore, 100 nM insulin was selected as the optimum concentration to be used for further experiments.

A separate glucose uptake assay was conducted to find the optimum TNF-α concentration for inducing an insulin-resistant state in 3T3-L1 adipocytes. TNF-α reduced glucose uptake in the cells up to a concentration of 1.0 ng/mL, beyond which there was an increase in glucose uptake at 2.5 ng/mL (Figure 3(b)). Since glucose uptake went down furthest at 1.0 ng/mL, this concentration was selected as the optimum TNF-α concentration to induce insulin resistance in cells.

3.3.2. Glucose Uptake in Normal 3T3-L1 Adipocytes. Glucose uptake assay was next conducted on normal, non-insulin-resistant adipocytes to study effects of stevioside. Glucose uptake by adipocytes was increased by treatment with stevioside or with rosiglitazone (Figure 4). Stevioside (30 μM) elicited significant increase in glucose uptake, in both insulin-stimulated and non-insulin-stimulated groups. However, glucose uptake was higher in the insulin-stimulated group, by 2.1 times ($p < 0.001$) compared to control. This was better than what was observed in cells that were treated with rosiglitazone maleate, where glucose uptake in presence of insulin was increased by 1.7 times ($p < 0.001$) at a far higher concentration of 120 μM.

3.3.3. Glucose Uptake in TNF-α Induced Adipocytes. 3T3-L1 cells were induced to insulin resistance using TNF-α, to test whether stevioside could exert similar effects on these cells as on normal adipocytes in terms of enhancing glucose uptake. Cells were pretreated with TNF-α prior to stevioside treatment and their glucose uptake was measured. In insulin-resistant adipocytes, stevioside was still able to stimulate glucose uptake although at a higher concentration of 90 μM compared to the previous 30 μM observed in normal adipocytes (Figure 5). Maximum increase in glucose uptake was observed in rosiglitazone-treated group (90 μM) without insulin stimulation, with an elevation of up to 4.6 times ($p < 0.001$) when compared to control. This was followed by treatment with stevioside (90 μM) with insulin stimulation, which elevated glucose uptake by 4.4-times ($p < 0.001$).

3.3.4. Western Blotting. In this study, expressions of two proteins (p-IRS1 and pY20) that are essential to the insulin-signalling pathway were measured by Western blotting technique. Both proteins were poorly expressed or not expressed at all in the insulin-resistant control group (Figure 6). Treatment with stevioside led to expression of both proteins while treatment with rosiglitazone elicited an increase in expression of pY20, though the effects of both treatments were hardly distinguishable from those observed in normal, non-insulin-resistant cells.

4. Discussion

Aim of this study was to investigate effects of stevioside on insulin sensitivity of 3T3-L1 cells. Oil Red-O is a common lipid stain used to colour lipid droplets *in vitro*, for quantification and for microscopic and imaging purposes. This specific staining method was used here to determine the degree to which adipocyte differentiation process (also called adipogenesis) had progressed since droplets secreted by adipocytes upon full differentiation to mature adipocytes will be stained (also referred to as lipid positive) [11]. Microscopic observation also revealed that lipid droplets were present in cytoplasm of fully differentiated adipocytes in abundance, compared to their absence in preadipocytes before the differentiation process.

As confirmation, there was a significant increase in absorbance values of eluted Oil Red-O stains from mature differentiated adipocytes (Figure 1). The stains from mature adipocytes were increased by 2.4 times ($p < 0.05$) when compared to control, which was the stain from empty wells; and were 4.2 times ($p < 0.001$) higher when compared to preadipocytes. This showed that the insulin-DMX-IBMX supplements combination was successful at hormonally triggering adipocyte differentiation in cells, most possibly via activation of peroxisome proliferator activator receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα), as reported by Jin et al. [12].

Fully differentiated 3T3-L1 adipocytes were then subjected to MTT assay to determine effect of stevioside on cell morphology and viability. 3T3-L1 Cells were treated with stevioside at concentrations ranging from 25 to 300 μM, for several different treatment periods: 14, 24, 48, and 72 hours.

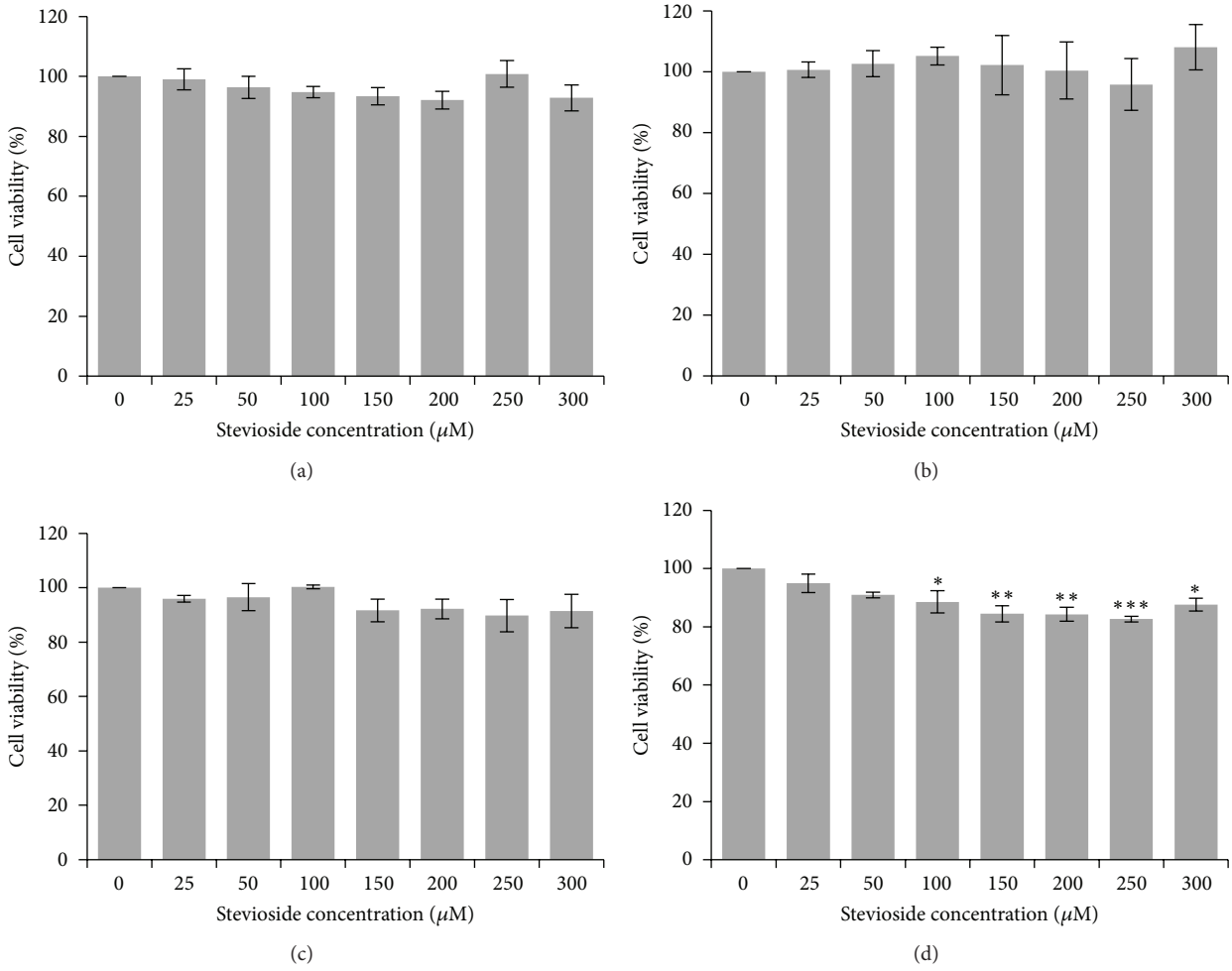

Figure 2: Viability of 3T3-L1 adipocytes treated with stevioside for (a) 14 hours, (b) 24 hours, (c) 48 hours, and (d) 72 hours. Mean ± SEM ($n = 4$). Statistically significant compared to control ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, ANOVA & Dunnett's test).

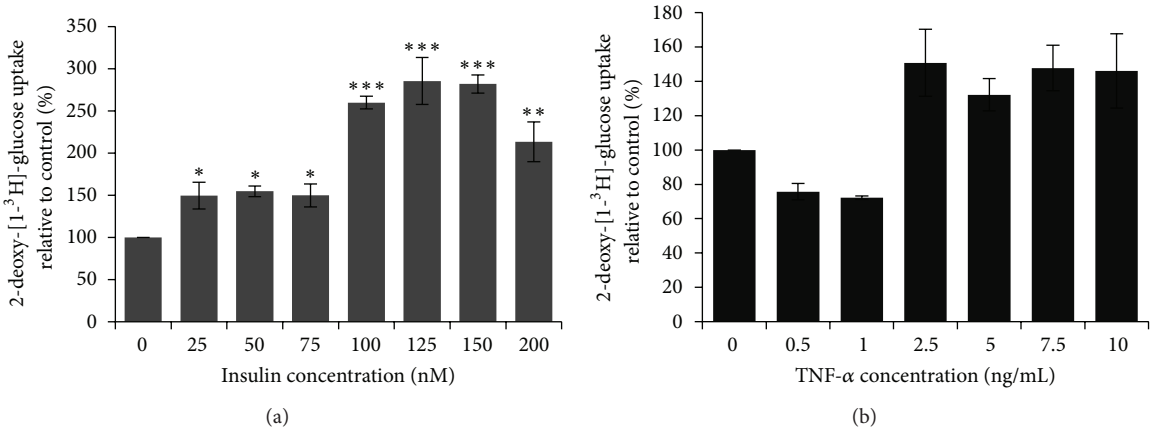

Figure 3: Glucose uptake in 3T3-L1 adipocytes exposed to (a) insulin and (b) TNF-α. Optimum insulin concentration to stimulate glucose uptake was 100 nM, while optimum TNF-α concentration to reduce glucose uptake was 1.0 ng/mL. Mean ± SEM ($n = 4$). Statistically significant compared to control ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ANOVA & Dunnett's test).

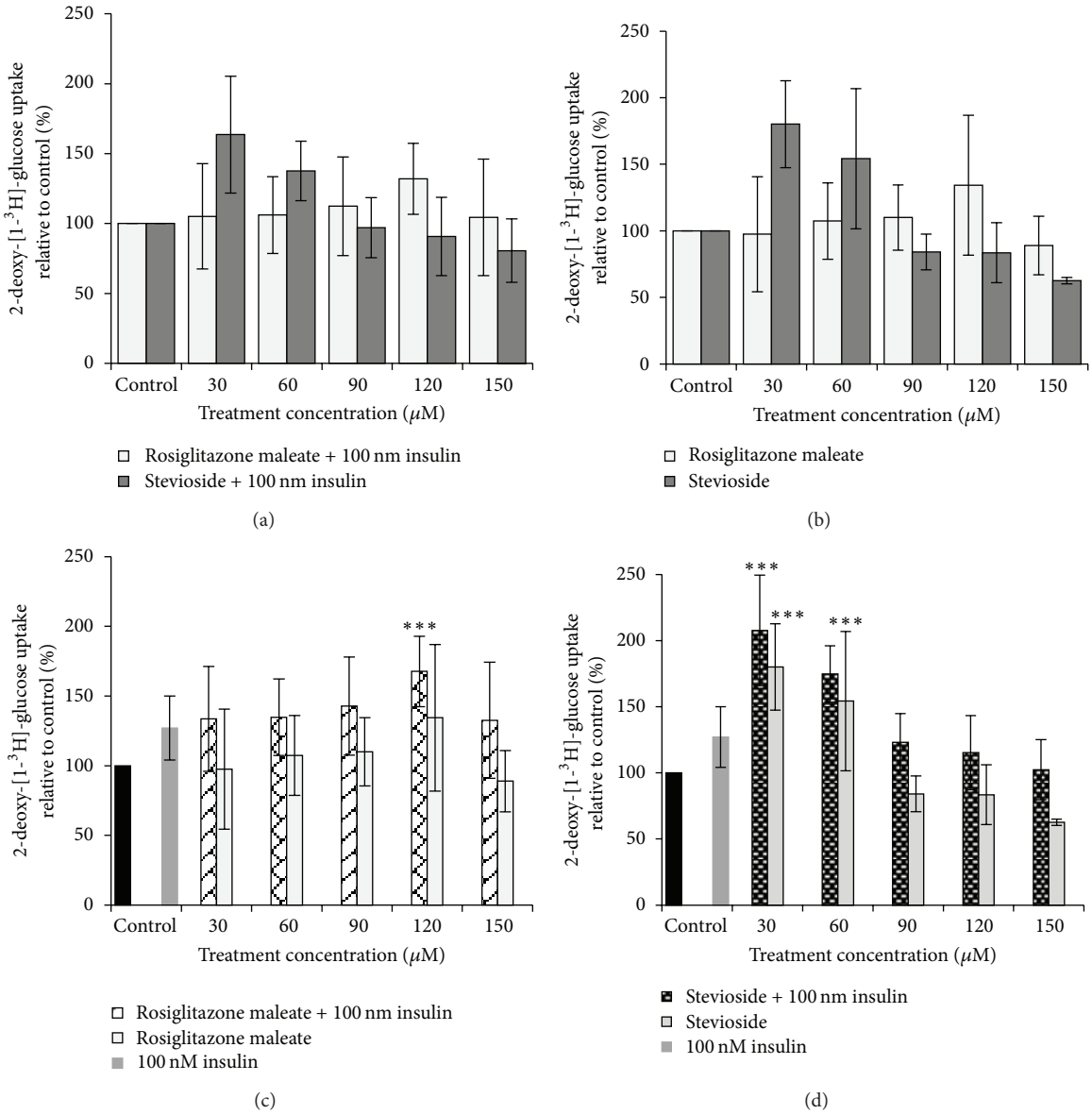

Figure 4: Effects of stevioside and rosiglitazone maleate on glucose uptake in normal 3T3-L1 adipocytes. (a) Treatment with stevioside or rosiglitazone maleate with insulin stimulation. (b) Treatment with stevioside or rosiglitazone maleate without insulin stimulation, (c) insulin-stimulated and non-insulin-stimulated groups treated with rosiglitazone maleate, and (d) insulin-stimulated and non-insulin-stimulated groups treated with stevioside. Mean ± SEM ($n = 3$). Statistically significant compared to control ($^{***}p < 0.001$, ANOVA & Dunnett's test).

Treatment for 14 hours was done as it was the stipulated doubling time for 3T3-L1 adipocytes [13]. Viability of cells was not markedly altered by exposure to stevioside except at concentrations greater than 100 μM following incubation for 72 hours. Even then cell viability was still higher than 80% with a maximum decrease of only 17%. Thus, median inhibitory concentration (IC_{50}) could not be estimated from this study. Some slight, nonsignificant increase in cell viability of 3T3-L1 adipocytes was observed as has been previously reported [11, 14, 15]. It can be surmised that stevioside did not exert cytotoxicity on 3T3-L1 adipocytes.

To test effects of stevioside on insulin-sensitivity of 3T3-L1 adipocytes, its effects on glucose uptake by the adipocytes was determined. Radioactively labelled glucose uptake assays were conducted with use of antidiabetic agent, rosiglitazone as a positive control, as previously reported [10].

Prior to subjecting 3T3-L1 adipocytes to stevioside, response of the cells to insulin and to TNF-α were determined in separate assays to estimate optimum concentrations. These studies showed that glucose uptake was elevated with increase in insulin concentration in concentration-dependent manner reaching a plateau at 100 nM of insulin. This insulin concentration was taken as the optimum concentration for use in further glucose uptake assays using 3T3-L1 adipocytes as previously reported [16].

TNF-α has been implicated in the progression of insulin resistance; it disrupts phosphorylation of several proteins involved in insulin-signalling pathway, including IRS1,

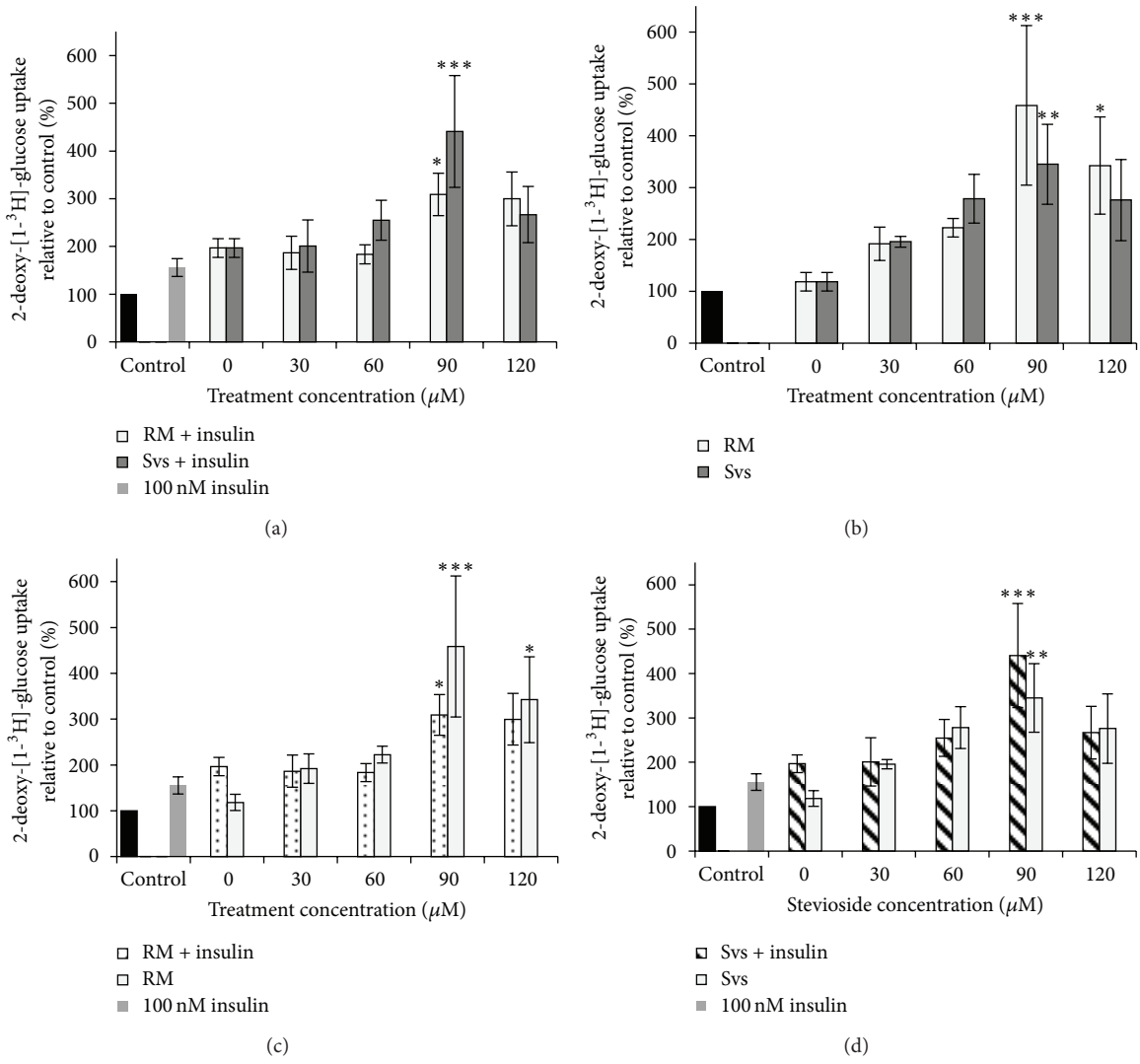

Figure 5: Effect of stevioside and rosiglitazone maleate on glucose uptake in TNF-α induced insulin-resistant 3T3-L1 adipocytes. (a) Treatment with stevioside or rosiglitazone maleate with insulin stimulation. (b) Treatment with stevioside or rosiglitazone maleate without insulin stimulation, (c) insulin-stimulated and non-insulin-stimulated groups treated with rosiglitazone maleate, and (d) insulin-stimulated and non-insulin-stimulated groups treated with stevioside. Mean ± SEM ($n = 3$). Statistically significant compared to control ($^* p < 0.05$, $^{**} p < 0.01$, $^{***} p < 0.001$, ANOVA & Dunnett's test). RM: rosiglitazone maleate, Svs: stevioside.

tyrosine, and most importantly, the downregulation of glucose transporter 4 (GLUT4) [3, 17]. TNF-α is also implicated in a number of catabolic states linked to insulin resistance such as sepsis and cancer even though its exact mechanism of actions remains unclear [18].

TNF-α caused a maximal decrease in glucose uptake at 1 ng/mL, beyond which glucose uptake was increased when compared to control. This observation contradicted initial expectation of a concentration-dependent decrease in glucose uptake by TNF-α, as was previously reported [19].

Normally differentiated, mature 3T3-L1 adipocytes which were not exposed to TNF-α were used to evaluate effect of stevioside on insulin sensitivity in normal, non-insulin-resistant state to ascertain effects of its usage in health. Our data showed that stevioside was better than the positive control, rosiglitazone, at increasing glucose uptake in normal adipocytes. Glucose uptake was maximally increased by 30 μM of stevioside, both in presence or in absence of insulin stimulation, surpassing that of rosiglitazone at 120 μM.

In TNF-α induced insulin resistance, glucose uptake was maximally increased by rosiglitazone or insulin at 90 μM either in presence or absence of insulin. Since effect of rosiglitazone on glucose uptake was not different from that of stevioside, it may be concluded that both treaments were equally effective at enhancing glucose uptake in insulin-resistant cells.

There appeared to be interaction between rosiglitazone and insulin in the insulin-resistant state with regards to glucose-uptake, which was lower in presence of 100 nM insulin compared to in its absence. This observation was not seen

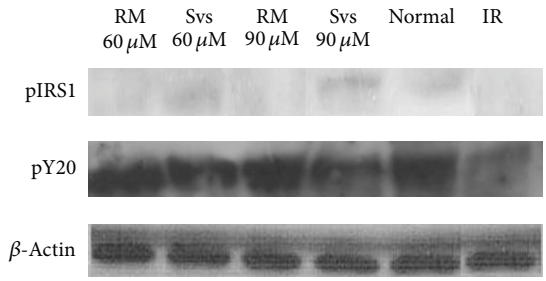

Figure 6: Band intensities observed via Western blotting, showing the different expression levels of phosphorylated insulin receptor substrate 1 (p-IRS1) and phosphorylated tyrosine (pY20), in groups treated with stevioside (Svs) and rosiglitazone maleate (RM) in comparison to the normal and insulin-resistant (IR) groups. β-actin was used as a loading control. The experiment was repeated thrice.

with stevioside as presence of insulin further elevated glucose uptake compared to its absence. A similar trend of enhanced glucose uptake by stevioside and rosiglitazone was observed in normal adipocytes. It may be assumed that stevioside's interaction with insulin was unaffected by insulin resistance unlike that of rosiglitazone except higher concentration of stevioside was needed to elicit significant increase in glucose uptake during insulin-resistant state compared to normal state. The different behaviour of insulin-stimulated versus non-insulin stimulated cells to rosiglitazone has important bearing in hyperinsulinaemic states which often occur in insulin resistance, though not always. It is possible that stevioside is suitable for use in hyperinsulinaemic states, which, besides diabetes, often occur in certain cancers [20].

This study investigated two proteins—the phosphorylated insulin receptor substrate 1 (p-IRS1), and phosphorylated tyrosine (pY20) that act upstream of the insulin-signalling pathway. Our data showed both proteins were poorly expressed or not expressed at all in insulin-resistant control group, which imply disruptions in the insulin-signalling pathway as previously reported by Solomon et al. [17]. Such disruptions were successfully counteracted by stevioside which restored expression of both proteins, while rosiglitazone was also seen to increased expression of pY20 in insulin-resistant cells. Expression of pY20 was elevated by treatment with rosiglitazone or stevioside (60 μM & 90 μM) which provided support for potential ability of stevioside at sensitising cells to insulin signalling. p-IRS1 expression was observed in normal cells and in those treated with stevioside, although the bands were of low resolutions and intensities. No p-IRS1 bands were observed in insulin-resistant cells, including those treated with rosiglitazone. This showed that stevioside was able to restore p-IRS1 expressions, similar to those observed in normal group. Stevioside possibly have an effect upstream of the insulin-signalling pathway, by elevating levels of pY20 and probably p-IRS1 as well, which will result in GLUT4 translocation to increase uptake of extracellular glucose. Antihyperglycemic effects of stevioside on cell metabolism as a whole has also been previously reported [21].

5. Conclusion

Stevioside proved to be as effective as the antidiabetic agent, rosiglitazone, at enhancing glucose uptake in normal 3T3-L1 adipocytes or in cells induced to insulin resistance via exposure to TNF-α. In normal adipocytes, stevioside at a concentration that was a quarter that of rosiglitazone produced greater increase in glucose uptake than the antidiabetic agent, both in absence or presence of insulin-stimulation. In insulin-resistant cells, stevioside was as effective as rosiglitazone as similar concentrations of both produced comparable stimulation of glucose uptake in absence or presence of insulin. Enhancement of glucose uptake by stevioside was accompanied by increased expression of p-IRS1 and pY20, denoting involvement of GLUT-4 translocation. Further studies are needed to unravel mechanisms underlying use of stevioside in adjuvant management of diabetes mellitus and its associated complications.

Acknowledgments

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Review Article

Potential Roles of *Stevia rebaudiana* Bertoni in Abrogating Insulin Resistance and Diabetes: A Review

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Insulin resistance is a key factor in metabolic disorders like hyperglycemia and hyperinsulinemia, which are promoted by obesity and may later lead to Type II diabetes mellitus. In recent years, researchers have identified links between insulin resistance and many noncommunicable illnesses other than diabetes. Hence, studying insulin resistance is of particular importance in unravelling the pathways employed by such diseases. In this review, mechanisms involving free fatty acids, adipocytokines such as TNFα and PPARγ and serine kinases like JNK and IKKβ, asserted to be responsible in the development of insulin resistance, will be discussed. Suggested mechanisms for actions in normal and disrupted states were also visualised in several manually constructed diagrams to capture an overall view of the insulin-signalling pathway and its related components. The underlying constituents of medicinal significance found in the *Stevia rebaudiana* Bertoni plant (among other plants that potentiate antihyperglycemic activities) were explored in further depth. Understanding these factors and their mechanisms may be essential for comprehending the progression of insulin resistance towards the development of diabetes mellitus.

1. Introduction

The emergence of many non-communicable diseases has been prominent over the last century. One of the global epidemics is diabetes, that has progressively affected the human populations for 20 centuries.

Diabetes mellitus is a metabolic disorder signified by high levels of glucose in the blood and can be categorised into two main groups. The first group (Type I) is often used to describe the onset of diabetes, which is triggered by the inability of the pancreas to produce sufficient amounts of insulin for glucose uptake and metabolism. Leney and Tavaré [1] report that the insufficiency of insulin in Type I diabetes results from the destruction of the autoimmune response, which disrupts the pancreatic β-cells.

The second group is noninsulin-dependent diabetes mellitus (NIDDM), also referred to as Type II diabetes, which is primarily related to insulin resistance. Many researchers agree that Type II diabetes is predominantly caused by impairment of the insulin-signalling pathway, even though the exact disease pathogenesis is yet to be understood. Even so, insulin resistance has been closely related to reduced metabolic responsiveness to normal insulin circulation [2]. Additionally, insulin resistance involves an abnormal biological response of the body systems with regard to physiological levels of insulin, and this pathological feature of the disease is the key to the metabolic syndrome.

There have also been reported cases in the American population of increased susceptibility to Type II diabetes due to family history and lack of cardiorespiratory fitness [3]. Although Type II diabetes has been asserted to have a genetic linkage [4], the key here is insulin resistance, which can be exacerbated by lifestyle changes and unhealthy dietary intake [3]. DeFronzo [5] states that insulin resistance and Type II diabetes have been linked to clusters of cardiovascular and metabolic disorders including hypertension, obesity, glucose

intolerance, dyslipidemia, and endothelial dysfunction. Apart from that, Type II diabetes has also been referred to as obesity-associated insulin resistance and implicated in the development of hypertension and atherosclerosis.

Theoretically, insulin resistance is defined as a state in a cell, tissue, system, or body for which levels of insulin needed to produce a quantitatively normal response are greater than normal. It is claimed that insulin has diverse effects and actions, depending on the different types of cells and tissues it is reacting to in the body. Insulin resistance is also closely related to hyperinsulinemia, though high blood glucose is observed in the former while high insulin is observed in the latter. Insulin resistance also occurs in clinical settings such as pregnancy, cancer cachexia, obesity, starvation, burn trauma, sepsis, and as an outcome of several experimental treatments, both *in vivo* and *in vitro* [6]. Insulin-mediated glucose disposal is essentially impaired in most identified cases, as glucose levels are the main feedback signal for compensatory hyperinsulinemia [7, 8]. The past 20 years have seen various schemes put forward to categorise the different mechanisms of insulin resistance with respect to the diverse molecular pathways involved.

Aside from that, the prevalence of metabolic syndromes like insulin resistance has triggered a quest for developing alternative treatments, if not new drugs, for these diseases. Communities around the world, particularly in rural areas, have been practising folk medicines using their own local resources. In the case of diabetes, many herbs and fruits with antihyperglycemic effects have been studied, prompted by their use in folk medicines, particularly those from the tropical and Asian regions. For example, in Bangladesh, leaves from several species of fruit tree have been tested for their ability to reduce serum glucose levels in mice; these include *Averrhoa carambola*, *Ficus hispida*, and *Syzygium samarangense* [9]. Fruit peels have also proved to have anti-hyperglycemic properties, as shown by the evaluation of blood glucose levels in Wistar rats fed with raw *Psidium guajava* (guava) fruit peels [10]. *Cynodon dactylon* is another example; a weed, known and popularised by the name "Doob" in India, has been found to be highly potent in its anti-hyperglycemic activities, as observed in Streptozotocin-induced diabetic rats [11].

Indirectly, these studies support the development of natural products from plant extracts and fruit products as sources of hypoglycemic agents and potential alternatives to off-the-shelf antidiabetic drugs. Among the many herbs prevalent in ancient and traditional folk medicine practices, *Stevia rebaudiana* Bertoni, a perennial shrub from South America, is a prime example [12]. The sweetness and unique properties of this plant provide an interesting platform for revealing its potential medicinal effects, specifically with regard to insulin resistance.

2. Insulin-Signalling Pathway

In order to tackle insulin resistance, it is important to understand the major insulin-signalling pathways involved and their impact on the regulation of blood glucose levels. Since the discovery of insulin, its correlation with fat, carbohydrate, and protein metabolism has been well established, but the molecular mechanisms of its actions remain difficult to define. It is well understood that insulin action begins with the binding of insulin to its receptor. The insulin receptor (IR) protein has been described as a heterotetramer of two identical extracellular α-subunits and two transmembrane β-sub-units which span the cell membrane [13]. The α-chain lies in the extracellular portion of the cell membrane, while the β-chain spans the cell membrane in a single transmembrane segment, with parts of it lying in the intracellular compartment of the membrane, and these are all held together by disulphide bonds [14]. This insulin receptor is a tyrosine kinase that has two different ligand binding regions: a high-affinity site and a low-affinity site [15].

Physiologically, insulin triggers the insulin receptor into exerting its effects, leading to the phosphorylation of insulin receptor substrate (IRS) proteins [16]. On detection of high levels of glucose released from the ingestion and uptake of food into the body, β-cells in the pancreas will first release insulin in response, which is prior to the binding of insulin to specific cell surface receptors. These specific cell receptors are embedded in the cell membrane of a fat, brain, or muscle cell. The binding will then lead to the activation of "second messengers" acting as intracellular mediators, initiating and stimulating a cascade of phosphorylation and dephosphorylation activities, which are responsible for a series of pathways and metabolic mechanisms, including glucose transport.

The first step in the cascade involves the activation of the IR tyrosine kinase by the autophosphorylation of the β-sub-unit, via the self-addition of phosphate groups in the intracellular domain of the receptor. This causes a conformational change in the receptor, which will also help in other adenosine triphosphate (ATP) binding and facilitate the gathering of other substrates for the ensuing phosphorylation activities. Phosphorylation of insulin receptor substrate 1 (IRS1) together with other intracellular substrates will ensue later, via the action of the activated IR tyrosine kinase.

Generally, these phosphorylated substrates will each provide unique docking sites for particular effector proteins with Src homology 2 (SH2) domains, which will recognise those residues with high specificity—and in this case, the SH2 domain of the phosphoinositide 3-kinase (PI3-K) will specifically identify the phosphotyrosine residue of IRS1 and will subsequently bind with it, passing down the signal for the next step in the signalling pathway.

Kinases are important components of signalling pathways and phosphorylation, in terms of transmitting the signal from one compartment to the other. In this mechanism, the signal corresponds to the level of blood glucose and is transmitted from the extracellular environment to the intracellular cavity. Following the binding process, the enzyme is activated, thus triggering the PI3-K pathway later leading to the phosphorylation of phosphatidylinositol-(4,5)-bisphosphate (PIP_2) into phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3). The generation of PIP_3 activates sets of specific proteins, enzymes, substrates, and molecules; and this includes phosphoinositide-dependent kinase 1, which initiates a number of downstream proteins including protein kinase B (PKB) or Akt. The activation of Akt through its

translocation to the membrane is directly assisted by PIP_3 via the pleckstrin homology domain. Akt has an important and central role in insulin-stimulated glucose uptake, as it is a major target in PI3-K activities, directly associating upstream insulin signalling with Glucose Transporter 4 (GLUT4) translocation [17]. Metabolic enzymes like glycogen synthase kinase 3 and 6-phosphofructo-2-kinase are regulated by Akt activation—apart from it stimulating the translocation of GLUT4 to the plasma membrane from the intracellular storage compartment, in order to take up the ingested extracellular glucose (Figure 1) [17].

3. Molecular Mechanisms of Insulin Resistance

The involvement of insulin resistance in diabetes was initially proposed in 1939 by Sir Harold Percival Himsworth, a British scientist [18]. Previously, diabetes was believed to be caused only by the deficiency of insulin. Since this breakthrough, the research on insulin resistance (particularly its molecular mechanism) is still progressing, mainly with regard to fatty acids, adipocytokines like tumour necrosis factor (TNFα), peroxisome proliferator activator receptor γ (PPARγ), and serine kinases like c-Jun NH_2-terminal kinase (JNK) and the inhibitor of nuclear factor κB kinase β (IKKβ).

3.1. Free Fatty Acids. The mediation of insulin resistance in tissue remains complex and difficult to define, but researchers have found that it can be facilitated by factors such as free fatty acids (FFAs). Fat accumulation has been strongly linked to elevated glucose production and insulin resistance and hence to increased susceptibility to Type II diabetes [19]. Based on the observations of Savage et al. [2], there is a strong correlation between circulating FFAs and obesity and insulin resistance, which supports this hypothesis. In addition, the elevation of FFA levels observed *in vitro* among 3T3-L1 adipocytes caused mitochondrial dysfunction in the cells, apart from causing decreased insulin-stimulated glucose uptake [20]. The same occurrences were observed *in vivo* in Zucker *fa/fa* rats, where FFA levels were proportional to the levels of reduced insulin-mediated glucose uptake [21]. Adipose cells and tissues are of particular importance in this case, as they administrate fat confiscation in whole-body metabolism processes, which also links high concentrations of FFAs and triglycerides circulation with the deficiency in adipose tissues [22].

Moreover, FFAs were also revealed to have induced insulin resistance by initially disrupting the phosphorylation process in the insulin-signalling pathway and consequently reducing glucose oxidation and glycogen synthesis (Figure 2) [23]. Reduced glucose oxidation and glycogen synthesis increase FFA oxidation, which causes an increase in and accumulation of glucose-6-phosphate, inhibiting the action of hexokinase II in the glycogen synthesis pathway [23]. Such inhibitory effects cause the glucose level in the cells to increase, prompting glucose uptake to halt; thus, the glucose levels in the bloodstream will also rise. This will eventually lead to insulin resistance and diabetes, as a long-term impact.

Similar observations have been recorded where increased FFA oxidation has led to increased reactive oxygen species (ROS) levels, which may lead to increased fat accumulation [24]. This strongly supports the theory that a pro-oxidant environment corresponding to metabolic disorders like insulin resistance may have been hugely influenced by the inability of the cells to combat oxidative stress. Furthermore, in a study implementing the TNFα cytokine, it was suggested that JNK might be the mediator to ROS-induced insulin resistance [6]. Both JNK and TNFα play major roles in the progression of insulin resistance and are discussed later in this review.

These observations also suggest that ideal glucose homeostasis and insulin sensitivity go hand in hand with sufficient adipose tissue with respect to a person's body size. The importance of adipose tissue in a body's metabolism activities and in homeostasis is emphasised by the fact that adipose tissues play a major role in adipokines secretion.

3.2. Tumour Necrosis Factor α (TNFα). The functionality of an adipocyte's role as an endocrine cell to secrete biologically active enzymes and proteins such as adipokines increases as a result of an elevation in adiposity. An example of these adipokines is TNFα, which has a major role in inflammatory responses in the cell. TNFα is recognised as a multifunctional proinflammatory cytokine, which is expressed as a 26-kDa transmembrane prohormone and produces a 17-kDa soluble form of the TNFα molecule on proteolytic cleavage. It also performs countless biological functions in the cell. Recently, TNFα has become the main focus of this particular field of research, the aim being to unravel its physiological and pathophysiological functions, which include transcriptional regulation, fatty-acid metabolism, hormone-receptor signalling, glucose metabolism, and adipocyte differentiation. Many current studies have concluded that TNFα actions and contributions to the system are implicated in metabolic disturbances like obesity and insulin resistance. This provides a platform for the implementation and use of TNFα in insulin-resistance studies, as a factor to induce insulin resistance in the cells of interest.

TNFα is often found in adipose tissue as well as in human fat, and the levels of its mRNA have been closely linked to the prevalence of hyperinsulinemia and obesity. In *in vivo* testing, a decrease in TNFα level is correlated to a loss in body weight [25]. The basis of this hypothesis is that TNFα was found to contribute to the induction of insulin resistance, although its exact mechanism of action is yet to be established. As a proinflammatory cytokine, TNFα is responsible for the development of metabolic syndromes and the maintenance of metabolic homeostasis, exerting its actions through the immune and inflammatory pathways [26]. States indicative of Type II diabetes were indicated by insulin resistance induced by TNFα, through the inhibition of tyrosine (tyr) phosphorylation of IRS1 [27] (Figure 2). Sethi and Hotamisligil [28] report that TNFα is highly responsible in lipid metabolism, where its increased levels are directly proportional to the increased levels of basal lipolysis, a major biochemical site in this process (Figure 2). In both *in vitro* and *in vivo* states, lipolysis can be initiated—together with an elevation of circulating free-fatty-acid concentrations—through the administration of exogenous TNFα. In addition

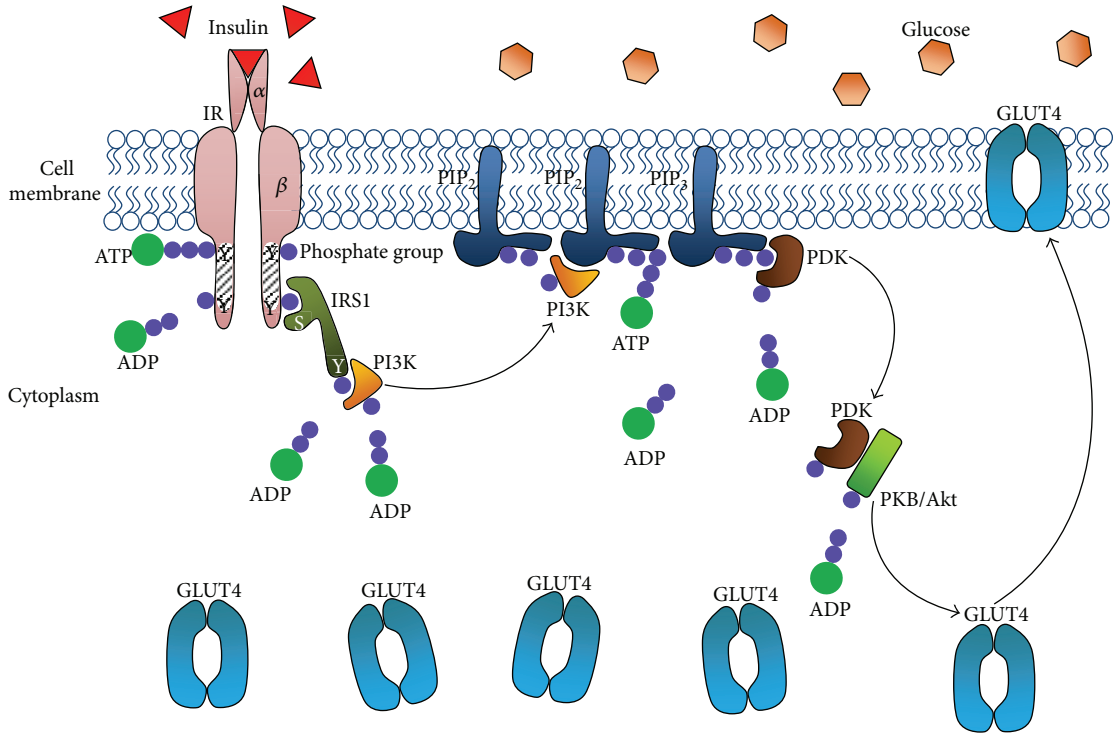

Figure 1: Manually derived and structured mechanisms of the insulin-signalling pathway in a normal state triggered by high glucose levels in the blood, prompting insulin binding and cascades of phosphorylation by ATP bindings, finally leading to the migration of GLUT4 from the cytoplasm to the cell membrane for extracellular glucose uptake. IR: insulin receptor; Y: tyrosine; S: serine; ATP: adenosine triphosphate; ADP: adenosine diphosphate; IRS1: insulin receptor substrate 1; PI3K: phosphoinositide kinase 3; PIP_2: phosphatidylinositol 4,5-bisphosphate; PIP_3: phosphatidylinositol 3,4,5-trisphosphate; PDK: PIP_3-dependent kinase; PKB/Akt: protein kinase B; GLUT4: glucose transporter 4.

to that, TNFα has the ability to inhibit lipoprotein lipase (LPL) activities that occur in fatty-acid uptake derived from lipolysis [29].

Expressions of free-fatty-acid transporters were also claimed to be reduced, resulting in reduced FFA uptake, which leads to hyperlipidemia—all in the course of TNFα's actions. This implies that TNFα also controls the escalations in lipolysis that lead to hyperlipidemia. There were also reduced expressions of key enzymes like acetyl-CoA carboxylase, fatty-acid synthase, and acyl-CoA synthetase, all of which affect insulin-mediated glucose uptake. Moller [21] mentions similar states, in which TNFα suppressed the expression of gene-encoding proteins with the likes of acetyl-CoA carboxylase and LPL, in charge of lipogenesis. Moller [21] also suggests that downregulation of GLUT4 (among other metabolic components) may lead to better understanding of the mediation of TNFα in inducing insulin resistance.

Previously, it has been theorised that TNFα is responsible for a number of catabolic states, such as sepsis, burn trauma, and cancer. TNFα produces its effects through several actions targeting insulin sensitivity: insulin receptor signalling, glucose transport, improved lipid metabolism, and leptin production [28]. The main mechanisms of TNFα actions are yet to be defined, but it appears to be able to downregulate GLUT4 directly (based on significantly reduced GLUT4 mRNA levels, documented after TNFα treatment) [30]. Phosphorylation of IRS1 at serine residues instead of tyrosine (which blunts insulin signalling) was observed to have increased via the induction of TNFα in cultured adipocytes. These incidences caused a conformational change in the multifunctional docking protein, forcing it to inhibit the insulin receptor (IR) tyrosine kinase on its binding site. The correlation between IRS1 and PI3-K in the downstream events of insulin signalling is also reduced due to this IRS1 modification. Further evidence includes an increase in insulin-signalling activity and efficiency by the effective genetic and pharmacological blockade of TNFα actions, observed *in vivo* on rat specimens [31].

3.3. Peroxisome Proliferator-Activator Receptor γ (PPARγ). Another factor worthy of mention here would be PPARγ, which serves as an important function in adipocyte functionality and is a major transcriptional regulator in adipogenesis. PPARγ works together with the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors in the regulation of adipogenesis. PPARγ can also react with insulin sensitisers, which serve as its agonists and ligands [32]. Therefore, many researchers have been targeting PPARγ pharmacologically for drug developments, especially concerning diabetes. PPARγ agonists have been studied in the past; they were given as treatments both *in vitro* and *in vivo*, resulting in normalised serum insulin and glucose concentrations in insulin-resistance models [33]. PPARγ showed great potential

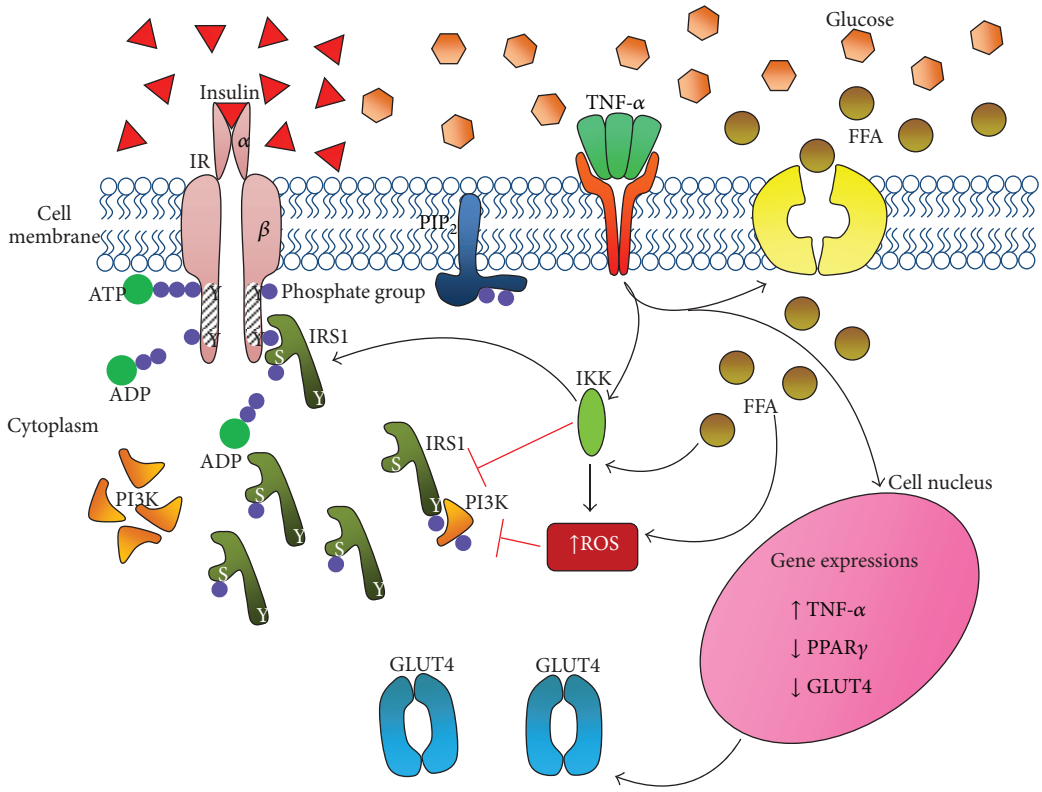

Figure 2: The disruptions in the insulin-signalling pathway in an insulin-resistant state caused by elevated actions of TNF-α and FFA. IRS1 is no longer phosphorylated on its tyrosine residues but on serine residues, resulting in nonfunctional, inhibitory proteins. TNF-α also influences increased gene expressions of TNF-α but decreases PPARγ and GLUT4 expressions, resulting in lower levels of GLUT4 proteins. Glucose uptake is reduced, leading to hyperglycemia and hyperinsulinemia. IR: insulin receptor; Y: tyrosine; S: serine; ATP: adenosine triphosphate; ADP: adenosine diphosphate; IRS1: insulin receptor substrate 1; PI3K: phosphoinositide kinase 3; PIP_2: phosphatidylinositol 4,5-bisphosphate; TNF-α: tumour necrosis factor α; FFA: free fatty acid; IKK: a type of serine kinase; ROS: reactive oxygen species; PPARγ: peroxisome proliferator activator-receptor γ; GLUT4: glucose transporter 4.

for sensitising the insulin-signalling pathway in the observed insulin-resistant states.

Apart from its insulin-sensitising activities, PPARγ also plays a role in adipocyte differentiation. There have been numerous studies demonstrating relationships between the posttranscriptional covalent modifications of PPARγ (through phosphorylation and sumoylation of said protein) to the progression of metabolic deteriorations, including diabetes. Researchers have established that one of the ways to combat insulin resistance and diabetes therapeutically is to tackle the covalent modifications of PPARγ, though more extensive studies need to be done [34]. Nonetheless, pharmacological functions of PPARγ in promoting glucose uptake were seen to have been restored, with the prevention of sumoylation in 3T3-L1 adipocytes [35].

Another potential line of enquiry is that PPARγ is closely linked to TNFα, as TNFα can downregulate the expression of PPARγ in observed 3T3-L1 adipocytes [36]. The fact that PPARγ facilitates the maintenance of normal insulin sensitivity leads to the conclusion that its inhibition by TNFα could possibly account for TNFα-induced insulin resistance. In addition, this finding is backed up by a study showing PPARγ agonists averting lipolysis and preventing an increased elevation of FFAs in 3T3-L1 cells that were initially subjected to the actions of TNFα [37]. PPARγ transcriptional activities are also affected by treatments with antidiabetic drugs such as thiazolidinediones and pioglitazones [38], and all of these indirectly support its involvement and importance in maintaining normal glucose homeostasis.

3.4. Serine Kinases. Several studies on TNFα plausibly connect TNFα with influencing ROS levels in cells [6]. Both ROS and TNFα are reported to be able to cause insulin resistance and to be potent activators of JNK—also conceivably a factor contributing to the metabolic syndrome. Guo et al. [16] explain that the inhibitory effects of both ROS and TNFα on insulin sensitivity might involve several serine/threonine kinase cascades, which suggest the implication of JNK and IKKβ as major candidates. The significance of and possibilities for JNK mediating the role of TNFα in insulin resistance were also proposed in this study. Guo et al. [16] report that levels of JNK activation and the phosphorylation of IRS1 on its serine residues were significantly increased with *in vitro* TNFα treatment in 3T3-L1 cells. The authors elaborate on the role of JNK; they observed a prevention of insulin resistance with the gene knock-down of JNK1 protein expression. Apart

from that, insulin sensitivity of the 3T3-L1 adipocytes treated with TNFα was also seen to have improved, merely by inhibiting JNK activation.

Houstis et al. [6] provide a similar take on this; in their studies, decreased phospho-JNK levels in the 3T3-L1 adipocytes led to an elevation of insulin-mediated glucose uptake. JNK normally functions as a sensing juncture for inflammatory status and cellular stress, but it also targets the serine (ser-307) site of IRS1, as it also vigorously phosphorylates IRS1 on that particular site [27]. As phosphorylation on this site produces blunt insulin signalling, the presence and action of JNK in facilitating this process further decrease insulin sensitivity and lead to insulin resistance.

Because of current findings linking insulin resistance to diabetes, many researchers are now focusing on the diverse molecular mechanisms in the progressions of both metabolic conditions, which underlines the importance of understanding the metabolic syndrome. Additionally, adipocytokines like TNF-α, serine kinases and free fatty acids are among the many factors and channels that may contribute to insulin resistance, type II diabetes mellitus, and other diseases (Figure 3 and Table 1).

4. Stevia as an Antidiabetic Agent

To date, out of the 150 known species of Stevia, *Stevia rebaudiana* Bertoni is the only one of its kind found to have particular attributes: firstly, it is unique in the potency of its sweetness [39]. Furthermore, this particular plant has been used by the Guarani Indians of Paraguay and Brazil to treat diabetes, due to its therapeutic qualities [12]. Even though the plant's leaves give out a distinctly sweet taste, they contain no calories [40], though they are rich in metabolites such as β-carotene, thiamine, austroinulin, riboflavin, diverse terpenes, and flavonoids, which give the plant its medicinal advantages [41]. This zero-calorie property can also be beneficial to patients suffering from obesity and diabetes, as it will not elevate their blood-glucose levels. Contrast this with the effects of sucrose (normally extracted from sugar beets and sugar cane), which may cause stomach infections and dental caries [39].

On the whole, researchers worldwide agree on the antidiabetic effects of Stevia; but they differ on *how* the effects contribute towards combating this metabolic disease. It is important to note that there are many steviol glycosides, which are compounds with multiple carbohydrate molecules, bound to a noncarbohydrate, aglycone moiety (steviol) that can be extracted from the *Stevia rebaudiana* Bertoni plant, most commonly are stevioside, rebaudioside A, rebaudioside C and dulcoside, among many other available glycosides [42, 43]. Some assert that Stevia's utility is due to its antioxidant properties; this is supported by analysis of the phenols that may be extracted from the plant. Stevia has a large overall proportion of phenols, up to 91 mg/g; it is proposed that these constituents extracted from the leaves are the major agents contributing towards the antihyperglycemic activities exerted by the plant [44]. This is further supported by the fact that the leaves have a greater ability to scavenge free radicals and prevent lipid peroxidation than controls such as butylated hydroxytoluene, butylated hydroxyanisole, and tertiary butyl hydroxyquinone [44].

Such findings concur with the results of other studies of Type 1 diabetes, modelled by streptozotocin-induced diabetic rats, in which phenolic compounds prevented several diabetic complications [45]. In addition, Shivanna et al. [44] observed a significant decrease (about 30%) in peroxidation in the livers of Stevia-pre-fed rats, compared to those of their control groups. This is a good indicator of reduction in the progression of diabetic complications, as diabetic tissue damage is commonly linked to the peroxidation of lipids, likewise the condition of hyperglycemia, which increases the production of reactive oxygen species (ROS) in the tissues due to high blood-glucose levels, which makes the tissues susceptible to oxidation [46].

4.1. Maintenance of Blood-Glucose Levels. As previously discussed, it is highly likely that Stevia's antioxidants are the source of its most medicinally beneficial effects. One such effect is the maintenance of blood-glucose levels, which is the most common measure used by researchers to evaluate the effectiveness of an anti-hyperglycemic agent. Susuki et al. [47] observed a significant decrease in blood-glucose levels over four weeks in rats fed with Stevia (combined with high-carbohydrate and high-fat diets). Similarly, NMRI-Haan laboratory mice induced to hyperglycemia using glucose experienced a significant reduction in glycemia after a week's treatment with stevioside, a major component of the leaf extract [47]. The same trend was seen in those assigned to the adrenaline load test after the same treatment periods.

It was further reported that there was a significant reduction (an average of 18%) in postprandial glucose levels in Type II diabetic patients given test meals supplemented with stevioside [48]. A study by Anton et al. [49] confirmed this; postprandial glucose levels were significantly lowered in patients supplemented with Stevia, compared to those given aspartame (a type of synthetic sweetener) or sucrose (normal table sugar). Interestingly, patient satiety as an after-effect of the different sweeteners was also tested; it was found that subjects given lower-calorie sweeteners (Stevia or aspartame) did not compensate by eating more than those given sucrose.

4.2. Anti-Inflammatory Response. A study on the C57BL6J insulin-resistant mice model shows that stevioside is also able to downregulate the nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) pathway, as well as enhancing whole-body insulin sensitivity, glucose infusion rate, and the level of the glucose-lowering effect of insulin [40]. Additionally, and interestingly, the expression of TNFα (the previously discussed proinflammatory cytokine contributing to the reduction of insulin sensitivity) was significantly downregulated, together with the expressions of interleukin 6 (IL6), interleukin 1β (IL1β), and interleukin 10 (IL10), among other chemotactic and pro-inflammatory cytokines [40]. Therefore, stevioside was seen to be able to potentiate in the reduction of insulin resistance through reducing the inflammation in adipose tissues by regulating TNFα.

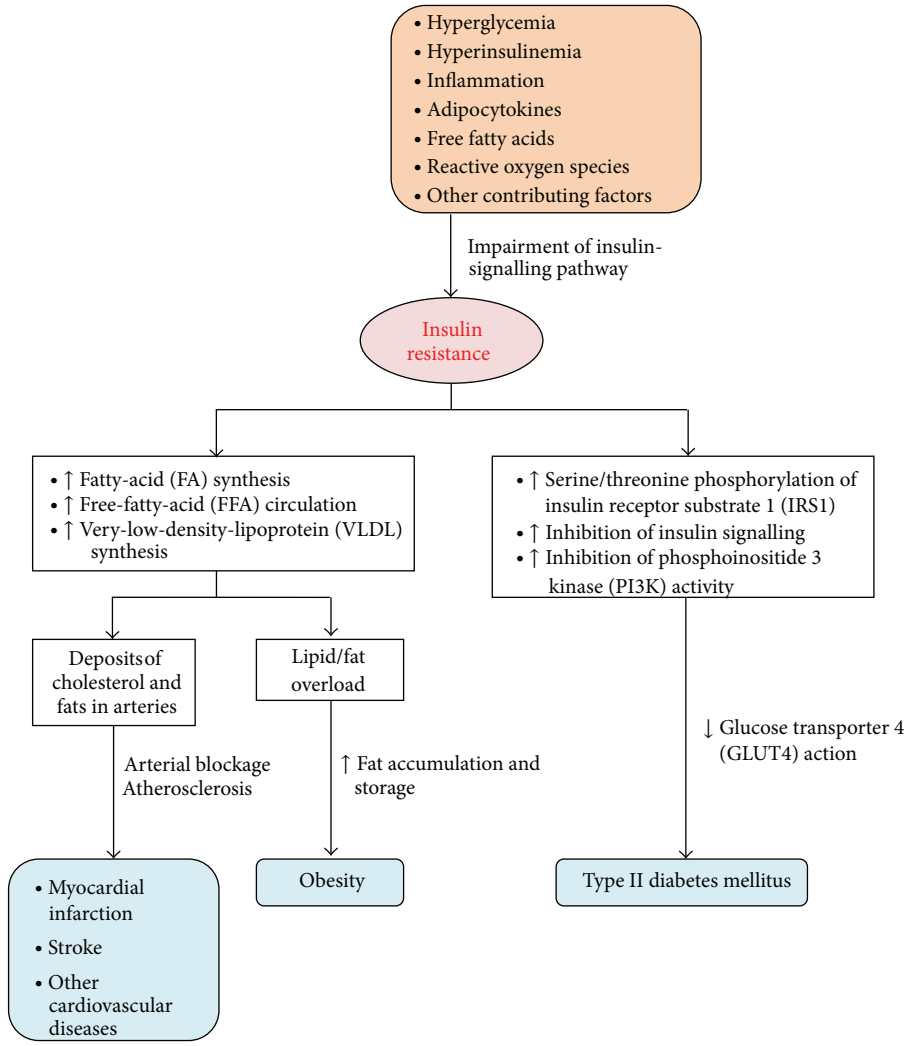

Figure 3: Manually constructed flowchart summarising the factors leading to insulin resistance that will eventually result in many related diseases.

4.3. Influence on Insulin Secretion. Various hypotheses have been constructed as to how stevioside causes such significant reductions in blood-glucose levels; these include theories of glucose disposal [50], modulation of glucose transport [51], and improvement in insulin sensitivity and secretion [52]. Jeppesen et al. [53] first reported that insulin release can be directly influenced by both stevioside and steviol solely, as they observed an increase in insulin secretion in both the INS-1 pancreatic β-cell line and in normal mouse islets. They also hypothesised that stevioside may only exert its glucose-depleting effects in specific high-blood-glucose conditions (as in a diabetic setting), as they failed to prove otherwise in non-hyperglycemic conditions [48]. This is a very encouraging finding; it signifies that stevioside can be target-specific, lowering glucose levels at specific settings without jeopardising the patient's health by risking severe hypoglycemia.

4.4. Insulinotropic, Glucagonostatic, and Nutrient-Sensing Effects. With the regulation of hormones with the likes of insulin comes nutrient sensing, which is literally what the term suggests: an organism's ability to sense and target available nutrients in order to control and regulate the related metabolic pathways and fluxes. Unlike prokaryotes that can manage their own nutrient sensing, eukaryotes are more complex, in terms of the influences of nutrient availability on the metabolic processes (particularly by both neuronal and hormonal signal transductions, such as glucagon and insulin). In recent years, it has been shown that nutrient sensing can operate both autonomously and in coordination with other endocrine pathways as a response to macronutrient fuel substrates such as glucose, amino acids, and lipids [54]. These pathways are essential to the regulation of cellular homeostasis, full utilisation of available nutrients, and for survival during starvation [55].

In a more recent publication by Jeppesen et al. [56], the authors state that stevioside contributes to insulinotropic and glucagonostatic effects by increasing insulin secretions while suppressing glucagon, apart from being anti-hyperglycemic to Goto-Kakizaki (GK) rats, as non-obese Type II diabetic

Table 1: Summarised effects based on several factors involved in the mechanisms of insulin resistance and insulin signalling, including previous figures.

Factor	Effects	References
FFA	↑FFA, ↑FFA oxidation, ↑ROS, ↓glucose uptake, ↑IR	[14, 15, 18]
TNF-α	↑TNF-α, ↓tyr phosphorylation of IRS1, ↓glucose uptake, ↑IR	[22]
PPARγ	↑PPARγ, ↓FFA, ↑glucose uptake, ↓IR	[30]
JNK and IKKβ	↑TNF-α, ↑JNK, ↑IKKβ, ↑ser phosphorylation of IRS1, ↓tyr phosphorylation of IRS1, ↓glucose uptake, ↑IR	[12, 21]

FFA: free fatty acid; ROS: reactive oxygen species; IR: insulin resistance; TNF-α: tumour necrosis factor α; tyr: tyrosine; IRS1: insulin receptor substrate 1; PPARγ: peroxisome proliferator-activator receptor γ; JNK: c-Jun NH_2-terminal kinase; IKKβ: inhibitor of nuclear factor κB kinase β; ser: serine.

animal models. Insulin depletion and elevation in glucagon levels in a Type II diabetes condition have been closely linked with dysfunction in the α-pancreatic cells, contributing (along with the more commonly implicated culprit, insulin resistance) to the development of the disease [57]. This supports the theory of Jeppesen et al. [58] that stevioside's glucagonostatic effects might be brought about by an indirect insulin-induced inhibitory response to glucagon, the increase in effectiveness of glucose recognition, or a straightforward inhibition of glucagon production by the α-pancreatic cells.

Furthermore, elevated levels were observed of the genes responsible for glycolysis, which may have contributed to the elevated insulin secretions. This is also thought to improve nutrient sensing in the specimen, as is the downregulation of proteins such as phosphodiesterase 1 (PDE1), responsible for the cyclic adenosine monophosphate (cAMP) degradation concomitant with stevioside treatments. In cases where PDE1 is downregulated, cAMP (essential in amplifying insulin secretions physiologically induced by glucose) will be increased, suggesting stevioside's ability to holistically amplify the expressions of glucose-responsive genes and improve nutrient sensing [58].

5. Conclusion

Research in this field has established that the metabolic syndrome encompassing diabetes, obesity, and insulin resistance is highly correlated to various aspects, from the selection of a cell culture model through the understanding of each and every step in the mechanisms involved, with proper comprehension of the function of each component on the pathways. In order to counter the metabolic syndrome as a whole, it is essential to go through all the tiny details of each metabolic process. Even so, it is essential for researchers to look into the potential healing ability (bestowed on us by nature, but often well hidden) of diverse herbs and plants.

It is postulated that the *Stevia rebaudiana* Bertoni plant could benefit the community medicinally through several different pathways, all eventually leading to its antihyperglycemic qualities. Although there are many unknowns and anomalies in our knowledge of insulin-signalling pathways, the mechanisms of glucose uptake, and the metabolic processes involved in insulin resistance, these loopholes could be addressed if researchers were to focus more on key factors such as IRS1, its phosphorylation, the translocation of GLUT4, and the roles of cytokines such as TNFα, not forgetting how PPARγ, JNK, and IKKβ contribute to insulin resistance.

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Research Article

Antidiabetic Effect of Oral Borapetol B Compound, Isolated from the Plant *Tinospora crispa*, by Stimulating Insulin Release

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Aims. To evaluate the antidiabetic properties of borapetol B known as compound 1 (C1) isolated from *Tinospora crispa* in normoglycemic control Wistar (W) and spontaneously type 2 diabetic Goto-Kakizaki (GK) rats. *Methods*. The effect of C1 on blood glucose and plasma insulin was assessed by an oral glucose tolerance test. The effect of C1 on insulin secretion was assessed by batch incubation and perifusion experiments using isolated pancreatic islets. *Results*. An acute oral administration of C1 improved blood glucose levels in treated versus placebo groups with areas under glucose curves 0–120 min being 72 ± 17 versus 344 ± 10 mmol/L ($P < 0.001$) and 492 ± 63 versus 862 ± 55 mmol/L ($P < 0.01$) in W and GK rats, respectively. Plasma insulin levels were increased by 2-fold in treated W and GK rats versus placebo group at 30 min ($P < 0.05$). C1 dose-dependently increased insulin secretion from W and GK isolated islets at 3.3 mM and 16.7 mM glucose. The perifusions of isolated islets indicated that C1 did not cause leakage of insulin by damaging islet beta cells ($P < 0.001$). *Conclusion*. This study provides evidence that borapetol B (C1) has antidiabetic properties mainly due to its stimulation of insulin release.

1. Introduction

Type 2 diabetes mellitus is a heterogeneous disorder associated with impaired insulin secretion from pancreatic β-cells and decreased insulin sensitivity which leads to hyperglycemia [1, 2].

The drugs that are currently available in the treatment of diabetes are mainly targeted either to improve insulin sensitivity or improve insulin secretion or both. Even after the discovery and use of insulin and availability of existing modern antidiabetic agents such as sulphonylureas, biguanides, and incretins, the search of more effective drugs of plant origin for the treatment of diabetes continues as an alternative [3–6]. Several medicinal plant parts have demonstrated promising results in terms of achieving normoglycemia by improving insulin secretion from pancreatic beta cells, while some have shown to increase peripheral utilization of glucose [7, 8] or improve hepatic insulin sensitivity [9–11].

Tinospora crispa (*T. crispa*) belongs to the *Menispermaceae* plant family and is known by various vernacular names such as "*Akar patawali*" or "*Akar seruntum*" (Malays). It comprises a climbing vine found throughout the southwestern part of China to southeast Asia including Malaysia. The aqueous extract of *T. crispa* is used in traditional medicine for treatment of type 2 diabetes [12, 13]. The antidiabetic effects of *T. crispa* extract have been previously demonstrated both *in vivo* and *in vitro* [12–16]. In this study, we have investigated the antidiabetic effect of borapetol B (C1), a compound isolated from *T. crispa* by evaluating the blood glucose levels and stimulation of insulin secretion in normoglycemic control Wistar (W) and diabetic Goto-Kakizaki (GK) rats, an animal model of type 2 diabetes [17].

2. Materials and Methods

2.1. Animals. Male normoglycemic control Wistar (W) and spontaneously type 2 diabetic Goto-Kakizaki (GK) rats (200–350 g) were used in this study. GK rats, originating from W rats, were bred in our department [17]. W rats were purchased from a commercial breeder (Charles River). The animals were kept at 22°C with an alternating 12-hour light-dark cycle (6 am–6 pm) and were allowed access to food and water before being anesthetized for isolation of pancreatic islets. The study was approved by the Laboratory Animal Ethics Committee of the Karolinska Institutet.

2.2. Plant Material. *T. crispa* vines were collected in Kota Belud (Sabah, Malaysia) in May 2005, identified by Berhaman Ahmad (Universiti Malaysia Sabah) and voucher specimen (FRI54832) deposited at the Forest Research Institute Malaysia. The stems were cleaned, air dried (3 days), and ground into coarse powder. Stem powder was sealed and stored at 4°C in a dry cabinet.

2.3. Bioassay-Guided Isolation of Borapetol B. Isolation and purification of borapetol B (C1) from *T. crispa* were modified from a previous study [18]. During the isolation procedure, fractions stimulating insulin secretion in a bioassay with isolated pancreatic islets from W rats were selected for subsequent purification [17].

The stem powder (5 kg) was extracted by sonicating with solvents at room temperature (25°C) for 15 minutes. It was first defatted with hexane (20 L) followed by methanol-water (4 : 1 by volume, 20 L) solvent extraction. Each extraction was repeated 3 times. Extracts were consolidated and reduced to one-third volume by vacuum evaporation yielding a brown syrup. The syrup was acidified to pH 2 with sulphuric acid (50% v/v) and partitioned four times with chloroform. The chloroform layer was evaporated to dryness to obtain brownish mass which showed prominent insulin stimulatory effect.

The brown mass was chromatographed over normal phase silica gel eluted with 100% chloroform followed by chloroform methanol (9.5 : 0.5) and subsequently increasing eluent polarity with methanol. Chromatographic fractions were monitored by thin layer chromatography (TLC) visualized at 365 nm. Fractions containing spots possessing R_f value within the range of 0.20–0.75 (chloroform methanol 9.5 : 0.5) were examined further as these fractions were also inducing insulin secretion.

These fractions were consolidated and re-chromatographed to yield 10 subfractions. When cooled (4°C), subfractions seven and eight yielded colourless, monoclinic crystals. Upon recrystallization with chloroform-methanol, crystals (450 mg) were recovered by vacuum filtration and washed with cold chloroform. TLC revealed a single compound known as C1 (Figure 1) which stimulated insulin secretion. The identity of C1 as borapetol B was further confirmed using ^{1}H-NMR [18, 19].

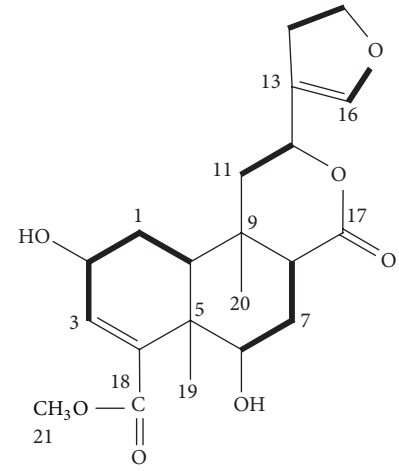

Figure 1: Chemical structure of C1 or borapetol B isolated from *T. crispa*.

2.4. Study Design. The protocol of this study is presented in Figure 2. The effect of C1 on the blood glucose and plasma insulin was assessed by an oral glucose tolerance test in normoglycemic control W and GK rats. For *in vitro* studies, the stimulation of insulin secretion was assessed by performing batch incubation and perifusion experiments using isolated W and GK pancreatic islets.

2.4.1. Oral Glucose Tolerance Test (OGTT). An OGTT was performed to identify the effect of C1 on the blood glucose levels in W and GK rats. The same rats ($n = 5$) were used for both control (placebo) and treatment groups with 7 days between each type of treatment. The rats were fasted overnight (14-15 hours), allowing access only to plain drinking water. For the treatment group, 10 μg/100 g body weight of C1 was administrated orally by gavage 30 min prior to an oral glucose challenge (0.2 g/100 g body weight). Control rats were given water only. Blood for glucose determination was measured by tail-prick method at different time points: −30 min (before the administration of the compound), 0 min (before glucose load), then at 30, 60, and 120 min after glucose administration. Blood glucose level was measured using a glucometer, Accu-check Aviva (Roche Diagnostic GmbH, USA). Blood samples were also collected for the measurement of plasma insulin levels (about 20 μL/serum sample) at 0 and 30 min.

2.4.2. Isolation of Pancreatic Islets. Islets from W and GK rats were used for *in vitro* experiments. The isolation of islets was performed using collagenase digestion method. Hank's balanced salt solution (HBBS) (Statensveterinäranstalt, Sweden) containing collagenase (Sigma-Aldrich, USA) was injected through the bile duct. For W and GK rats, 9 mg and 24 mg of collagenase were added to 10 mL of HBBS, respectively. The pancreas was then collected, incubated in 37°C water bath without shaking for 24 min, followed by several washing and centrifugation steps with HBBS, Histopaque 1119 (Sigma-Aldrich, USA), and Histopaque 1077 (Sigma-Aldrich, USA).

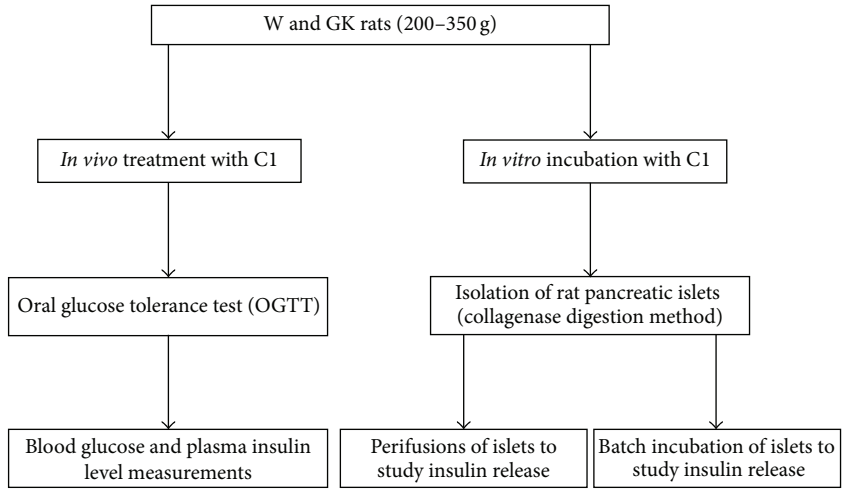

Figure 2: Study design. The *in vivo* and *in vitro* studies were carried out to identify the effects of *T. crispa* C1 in W and GK rats.

The islets were hand-picked under a stereomicroscope and then cultured for 24 hours at 37°C, with an atmosphere of 5% CO_2-95% air in RPMI 1640 culture medium (SVA, Sweden) supplemented with 30 mg L-glutamine (Sigma-Aldrich, USA), 11 mM glucose (Sigma-Aldrich, USA), and antibiotics (100 IU/mL penicillin and 0.1 mg/mL streptomycin) (Invitrogen, USA). Heat-inactivated fetal calf serum (10%) was added to RPMI 1640 medium before the incubation of islets [17].

2.4.3. Batch Incubations for Insulin Secretion. The medium used was Krebs-Ringer bicarbonate (KRB) buffer solution [20] containing 10 mmol/L *HEPES* (Sigma-Aldrich, USA) and 0.2% bovine serum albumin. Following overnight incubation, the islets were preincubated at 3.3 mM glucose for 30–45 min at 37°C with an atmosphere of 5% CO_2-95% air. After washing islets twice with the incubation medium, batches of 3 islets of similar size were incubated at 3.3 mM or 16.7 mM glucose with or without C1 compound at different concentrations (0.1, 1, and 10 μg/mL). The tubes containing islets and solutions were then incubated for 60 min in 37°C waterbath, slowly shaking. After incubation, 200 μL of the solutions was transferred to new tubes for RIA and kept in freezer −20°C until being assayed for insulin by RIA.

2.4.4. Perifusions of Islets. Perifusions of islets were done to investigate how C1 affects the kinetics of insulin release [20]. Batches of 30 or 50 isolated W and GK rat islets each were layered between polystyrene beads (Bio-Rad Laboratories, Inc., USA) in a perifusion chamber and perifused by use of peristaltic pump (Ismatec SA, Zurich, Switzerland) as previously described [21]. Perifusion medium was collected in fractions every 2 min to establish the basal insulin secretion rate at 3.3 mM glucose for 20 min (−20 to min 0). At min 0 to 15, the glucose concentration was maintained at 3.3 mM glucose and increased to 16.7 mM glucose at 15 to 30 min. Finally, the glucose concentration was then switched back to 3.3 mM glucose. C1 (10 μg/mL) was added at min 0–15 in 3.3 mM glucose and at min 15–30 in 16.7 mM glucose. The fractions were collected and stored in −20°C for insulin radioimmunoassay (RIA).

2.5. Insulin RIA. Aliquots obtained from batch incubations and perifusions experiments were analyzed for insulin content using RIA [22].

2.6. Statistical Analysis. The results are presented as mean ± SEM. Difference between experimental groups was analyzed using paired t-test for OGTT and insulin secretion experiments whereas 2-way ANOVA was used for perifusions of islet experiment. P value of less than 0.05 was considered to be significant. All data were analyzed using Prism Graph Pad Software (CA, USA).

3. Results

3.1. Oral Glucose Tolerance Test (OGTT) in W and GK Rats. After the oral administration of glucose, the blood glucose levels reached a peak at 30 minutes and then gradually decreased in both W and GK rats. In W rats, the blood glucose levels at 30, 60, and 120 min were significantly decreased in the treated group as compared to the placebo group (at 120 min 4.7 ± 0.1 versus 6.1 ± 0.4 to mmol/L; $P < 0.01$) (Figure 3(a)) and with areas under the glucose curves (AUCs) (0 min to 120 min) being 72 ± 17 versus 344 ± 10 mmol/L ($P < 0.001$) (Figure 3(b)). In GK rats, the blood glucose levels at 60 and 120 min were significantly decreased in the treated group as compared to the placebo group (at 120 min 12.5 ± 0.8 versus 15.3 ± 0.9 mmol/L; $P < 0.05$) (Figure 4(a)) with AUCs (0–120 min) being 492 ± 63 versus 862 ± 55 mmol/L ($P < 0.01$) (Figure 4(b)). In W and GK rats, plasma insulin levels were increased from 0 min to 30 min in both placebo and treated groups (Figures 5 and 6) and there was a significant difference observed between the placebo and treated groups at 30 min ($P < 0.05$). In W rat, the mean values for plasma insulin in the treated and placebo groups at 0 min were 18 ± 4 μU/mL versus 10 ± 1 μU/mL respectively and at 30 min,

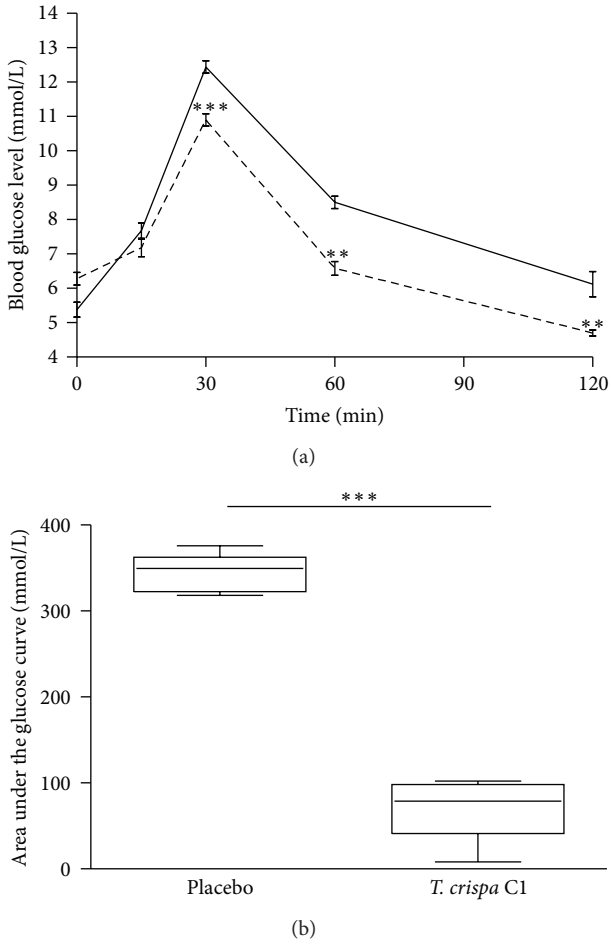

Figure 3: (a) Blood glucose level in the oral glucose tolerance test in W rats. 10 μg/100 g of b.w. of *T. crispa* C1 (- - -) or placebo (—) was given orally 30 minutes prior to the glucose challenge (0.2 g/100 g of b.w.). Data are presented as means ± SEM ($n = 5$). $^{**}P < 0.01$ versus placebo; $^{***}P < 0.001$ versus placebo. (b) Area under the glucose curve in the oral glucose tolerance test in W rats. Data are presented as means ± SEM ($n = 5$). $^{***}P < 0.001$ versus placebo

61 ± 9 μU/mL versus 27 ± 4 μU/mL ($P < 0.05$), respectively (Figure 5). In GK, the mean values for plasma insulin in the treated and placebo groups at 0 min were 18 ± 4 μU/mL versus 11 ± 1 μU/mL and at 30 min, 64 ± 8 μU/mL versus 30 ± 4 μU/mL ($P < 0.05$), respectively (Figure 6).

3.2. Effects of C1 on Insulin Secretion of W Rat Islets and GK Rat Islets. In W rat islets, the incubation of islets with C1 at 0.1, 1, and 10 μg/mL in 3.3 mM glucose significantly increased the insulin secretion 6.3-fold ($P < 0.01$), 8.1-fold ($P < 0.05$) and 9.1-fold ($P < 0.001$), respectively, compared to the control group (Table 1). At 16.7 mM glucose concentration, C1 (0.1, 1, and 10 μg/mL) stimulated insulin secretion 1.5-fold ($P < 0.05$), 1.9-fold ($P < 0.05$), and 5.0-fold ($P < 0.001$), respectively, compared to the control group. The incubation of GK rat islets with C1 at 0.1, 1, and 10 μg/mL in 3.3 mM glucose significantly increased the insulin secretion 3.9-fold, 6.3-fold and 8.8-fold (all $P < 0.05$), respectively, compared

Table 1: The effect of different concentrations of *T. crispa* C1 at low (3.3 mM) and high (16.7 mM) glucose on insulin secretion from W ($n = 5$) and GK ($n = 3$) rat islets.

Addition to incubation medium		Insulin release (μU/islet/h)	
Glucose (mM)	*T. crispa* C1 (μg/mL)	W islets	GK islets
3.3	None	2.4 ± 0.1	1.2 ± 0.2
	0.1	15.1 ± 2.2**	4.7 ± 0.3*
	1	19.5 ± 4*	7.5 ± 0.9*
	10	21.9 ± 2***	10.5 ± 1.8*
16.7	None	32.5 ± 1.8	13.6 ± 0.6
	0.1	48.8 ± 6$^{\#}$	20.6 ± 1.1$^{\#}$
	1	63.1 ± 8.9$^{\#}$	30.9 ± 3.8$^{\#}$
	10	164.5 ± 11.3$^{\#\#\#}$	57.3 ± 6.3$^{\#}$

Data are presented as means ± SEM. W: $^{*}P < 0.05$ versus 3.3 mM glucose; $^{**}P < 0.01$ versus 3.3 mM glucose; $^{***}P < 0.001$ versus 3.3 mM glucose; $^{\#}P < 0.05$ versus 16.7 mM glucose; $^{\#\#\#}P < 0.001$ versus 16.7 mM glucose GK: $^{*}P < 0.05$ versus 3.3 mM glucose; $^{\#}P < 0.05$ versus 16.7 mM glucose.

to the control group. At 16.7 mM glucose, C1 at 0.1, 1, and 10 μg/mL stimulated insulin release 1.5-fold, 2.3-fold, and 4.2-fold (all $P < 0.05$), respectively, compared to the control group.

3.3. Kinetics of Insulin Secretion of Isolated Islets. Insulin secretion was increased as 10 μg/mL of C1 was added to perifusate containing 3.3 mM (0–16 min) and 16.7 mM (16–30 min) glucose in both W (Figure 7) and GK (Figure 8) rat islets. In W, the addition of C1 stimulated insulin secretion by 5.5-fold from 0.2 ± 0.01 μU/islet/min (0 min) to 1.1 ± 0.16 μU/islet/min (12 min) and further increased to 7.5-fold (1.5 ± 0.16 μU/islet/min) (22 min). There was a significant difference observed from 2 min to 32 min ($P < 0.001$) in the treated group compared to control (Figure 7). In GK rat, C1 stimulated insulin secretion by 2.5-fold from 0.17 ± 0.03 μU/islet/min (0 min) to 0.43 ± 0.05 μU/islet/min (2 min) ($P < 0.001$). The insulin secretion was increased to 0.8 ± 0.03 μU/islet/min (20 min) ($P < 0.001$) in 16.7 mM glucose. There was a significant difference observed between the treated and control group at 2 to 4 min ($P < 0.001$) and between 20 to 30 min ($P < 0.001$) (Figure 8). The insulin secretion returned to basal level in both W and GK when C1 was omitted from the perifusate.

4. Discussion

We show that oral administration of *T. crispa* C1 30 minute before an oral glucose challenge significantly decreased blood glucose levels in W rats. This was most likely mediated through enhanced insulin secretion, since the plasma insulin level in treated W rats increased by 2-fold compared to the placebo group. This is further supported by our findings that C1 stimulates insulin secretion from isolated pancreatic islets, both in batch incubations and in perifusions.

A major defect behind type 2 diabetes is inadequate insulin secretion, that would be needed to compensate

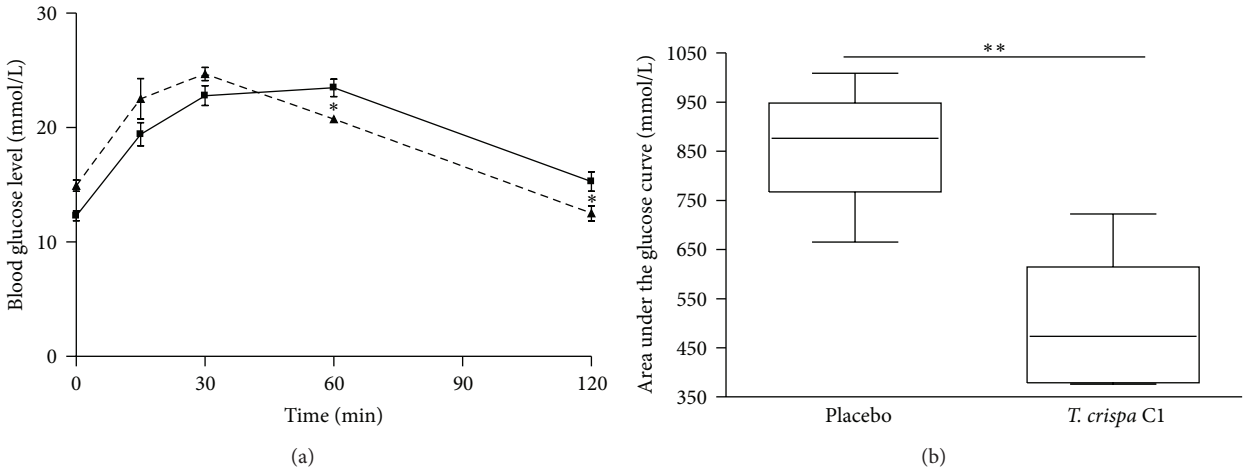

Figure 4: (a) Blood glucose level in the oral glucose tolerance test in GK rats. 10 μg/100 g of b.w of *T. crispa* C1 (- - -) or placebo (—) was given orally 30 minutes prior to the glucose challenge (0.2 g/100 g of b.w.). Data are presented as means ± SEM ($n = 5$). $^{*}P < 0.05$ versus placebo. (b) Area under the glucose curve in the oral glucose tolerance test in GK rats. Data are presented as means ± SEM ($n = 5$). $^{**}P < 0.01$ versus placebo.

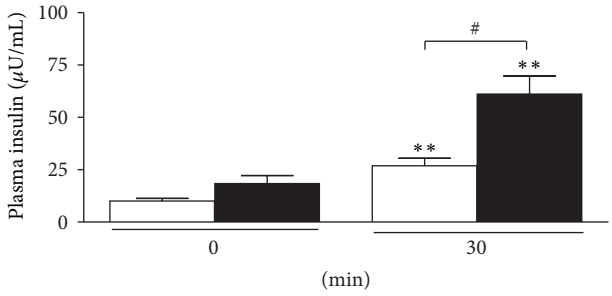

Figure 5: Plasma insulin level at 0 and 30 min in the oral glucose tolerance test from W rats. Data are presented as means ± SEM ($n = 5$). *T. crispa* C1 (■) or placebo (□). $^{**}P < 0.01$ versus 0 min (placebo and *T. crispa* C1, resp.); $^{\#}P < 0.05$ versus 30 min (placebo).

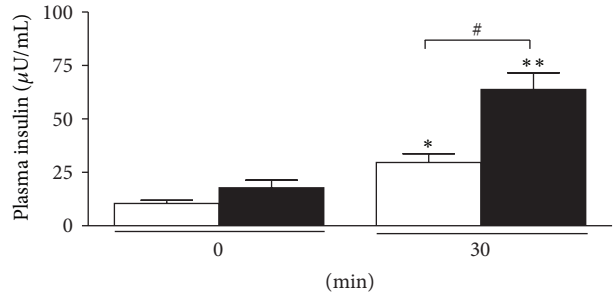

Figure 6: Plasma insulin level at 0 and 30 min in the oral glucose tolerance test from GK rats. Data are presented as means ± SEM ($n = 5$). *T. crispa* C1 (■) or placebo (□). $^{*}P < 0.05$ versus 0 min (placebo); $^{**}P < 0.01$ versus 0 min (*T. crispa* C1); $^{\#}P < 0.05$ versus 30 min (placebo).

for decreased insulin sensitivity [1, 2]. This in turn leads to development of hyperglycemia. To further assess the insulinotropic properties of C1 in type 2 diabetes, studies were performed in spontaneously diabetic Goto-Kakizaki (GK) rats. The GK rat strain was established from normoglycemic W rats by repeated inbreeding in each successive generation of the siblings with the highest blood glucose levels during an OGTT [23]. GK rats are lean and develop mild hyperglycemia early in life due to impaired insulin secretion, in particular a consistently low insulin response to glucose stimulation [17, 24]. Impaired glucose-stimulated insulin secretion has been demonstrated *in vivo*, in the perifused isolated pancreas and in isolated pancreatic islets of GK rats [24].

We now demonstrated that oral treatment with C1 decreased blood glucose levels in parallel with an increase in plasma insulin levels during the OGTT, not only in W rats but also in GK rats. In addition, C1 at different concentrations increased insulin release from GK rat islets in low and high glucose and the stimulatory effect was observed in a dose-dependent manner. In the perifusions of islets experiment, C1 stimulated insulin secretion in both W and GK rat islets. The insulin secretion gradually returned to basal level on the removal of C1, supporting that C1 did not cause nonspecific insulin leakage by damaging islets beta cells.

Previous findings have shown the effectiveness of extract and isolated compounds from *T. crispa* in the stimulation of insulin release and insulin sensitivity in normal and diabetic animal models [12, 14]. A two-week treatment with *T. crispa* extract significantly reduced the blood glucose level and caused a significant increase in plasma insulin in moderately diabetic rats with some functional β-cells. No effect was observed in severely diabetic animals suggesting that the hypoglycemic effect of *T. crispa* extract was not due to extra pancreatic action but through the stimulation of insulin secretion [13].

An *in vitro* study showed that *T. crispa* extract induced a dosage-dependent stimulation and also potentiated basal and glucose-stimulated secretion of insulin in rat islets and HIT-T15 cells. The insulin secretion in perifused isolated human

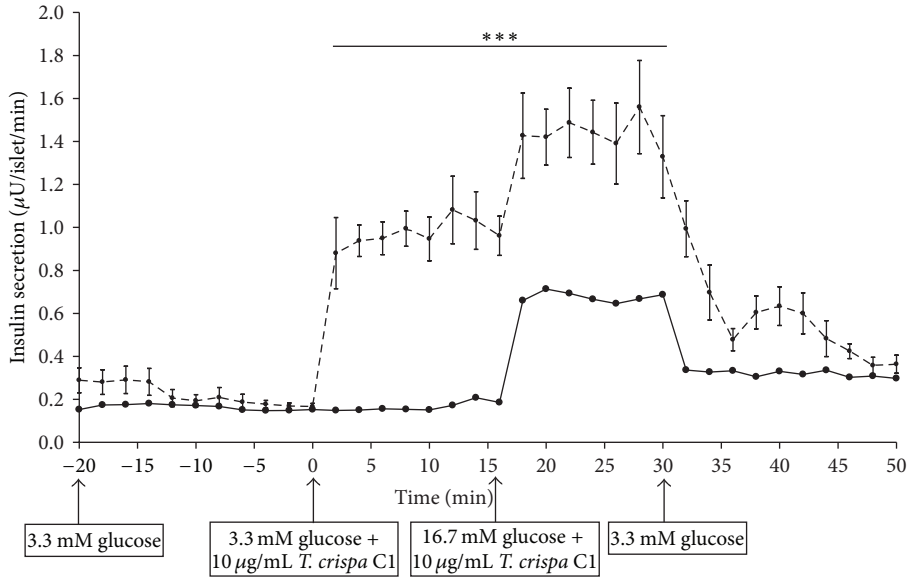

Figure 7: Effect of *T. crispa* C1 on kinetics of insulin secretion of W rat islets. Data are presented as means ± SEM from six separate experiments. Aliquots of the medium were collected and then determined by RIA. *T. crispa* C1 (- - -) or control (—). $^{***}P < 0.001$ versus control.

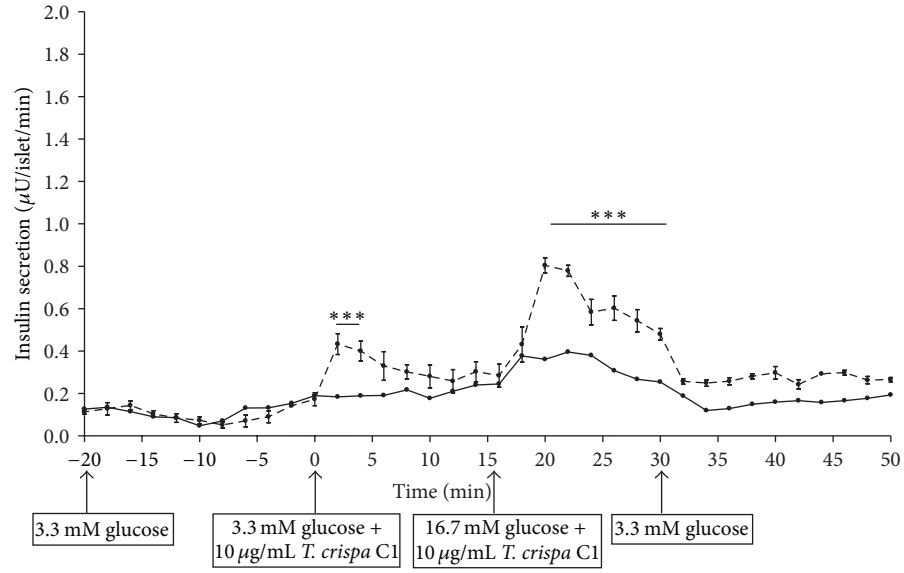

Figure 8: Effect of *T. crispa* C1 on kinetics of insulin secretion of GK rat islets. Data are presented as means ± SEM from three separate experiments. Aliquots of the medium were collected and then determined by RIA. *T. crispa* C1 (- - -) or control (—). $^{***}P < 0.001$ versus control.

and rat islets as well as HIT-T15 β cells returned to basal levels as *T. crispa* extract was omitted from perifusate indicating that the insulintropic activity was not due to toxicity effect [14].

Another compound isolated from *T. crispa* borapetoside C reduced plasma glucose levels in normal and type 2 diabetic mice and streptozotocin induced type 1 diabetic mice but increased plasma insulin levels in normal and type 2 diabetic mice only. The hypoglycemic effect was associated with increase of glucose utilization in peripheral tissues and the reduction of hepatic gluconeogenesis [15]. In another study, borapetoside C increased glucose utilization, delayed the development of insulin resistance, and enhanced insulin sensitivity in diabetic mice [16].

It would have been interesting to explore the anti-diabetic effects of borapetoside C with borapetol B (C1) and to compare with the effects of C1 in W and GK rats. Since GK rats in addition to defective insulin secretion display decreased insulin sensitivity, it is possible that a stronger antidiabetic effect would be obtained by treatment with

a combination of C1 and borapetoside C. However, due to limitations and difficulties to obtain borapetoside B, we have focused on the effects of C1.

5. Conclusion

We demonstrate that *T. crispa* C1 improves the diabetic condition in GK rats by stimulating insulin secretion. Further studies are needed to understand the mechanisms involved by which *T. crispa* C1 induces insulin release from pancreatic islets.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Review Article

Use of Laser-Induced Breakdown Spectroscopy for the Detection of Glycemic Elements in Indian Medicinal Plants

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The demand for interdisciplinary research is increasing in the new millennium to help us understand complex problems and find solutions by integrating the knowledge from different disciplines. The present review is an excellent example of this and shows how unique combination of physics, chemistry, and biological techniques can be used for the evaluation of Indian medicinal herbs used for treating diabetes mellitus. Laser-induced breakdown spectroscopy (LIBS) is a sensitive optical technique that is widely used for its simplicity and versatility. This review presents the most recent application of LIBS for detection of glycemic elements in medicinal plants. The characteristics of matrices, object analysis, use of laser system, and analytical performances with respect to Indian herbs are discussed.

1. Introduction

The World Health Organization estimates that about 80% of the world's population relies on herbal medicines for primary healthcare [1, 2]. Knowledge of herbal medicines evolved by trial and error and was passed orally from generation to generation [3, 4]. It is only during the last five decades that ethnobotanical research has documented numerous medicinal plants that were earlier not known to the scientific world [5–9]. This body of research has been possible in part due to the availability of several analytical techniques and isolation processes for studying the phytoconstituents of plants.

One important analytical technique that has led to advances in natural product research is laser induced breakdown spectroscopy (LIBS). In this paper, we review the applications of this technique and then review its use for the evaluation of Indian medicinal plants used for treating diabetes mellitus. Specifically, we review what these glycemic elements are and how LIBS has been used to detect these elements in plants known to have hypoglycemic property.

A number of plant species have hypoglycemic property [10, 11]. Despite the availability of many antidiabetic medicines, screening for antidiabetic drugs from natural sources is an attractive proposition. First, natural medicines are widely used in many developing and underdeveloped countries. Second, natural medicines are believed to have minimal side effects and are therefore preferred over synthetic medicines.

2. Laser-Induced Breakdown Spectroscopy (LIBS)

The LIBS technique is used for qualitative and quantitative analysis of trace elements. It was used for the first time in 2008

to study the presence of certain trace elements in medicinal plants with hypoglycemic effects [12]. This technique is used to analyze the spectral emission from laser-induced plasmas, the plasma emission intensity being proportional to the abundance of an element in the sample. The relative simplicity and capability of fast multielemental analyses of solid, liquid, or gaseous samples makes LIBS an ideal tool to study a wide range of samples. These include metallurgical and solid samples, colloidal and liquid samples, particles, and gases. While the qualitative analysis of a sample is straightforward, the quantitative results of elemental compositions from LIBS measurements require much more effort. Although the use of LIBS has been most popular in metallurgical samples, in recent years, it has been used to study environmental and biological samples, advanced materials such as semiconductors, for online sample analysis, for remote analysis of nuclear power stations, and for depth profiling of a field.

2.1. LIBS Applications

2.1.1. Liquids. Initially, liquid analysis with LIBS was not popular because of problems such as sloshing, splashing, and focal length changes with a high repetition rate laser. Apart from this, high local density within the liquid caused intrinsic complexity with LIBS analysis; the spectral transition in comparison to that for a rarefied gas, considerably broadens when the high collision rate within the plasma is confined in a liquid. If attributes like minimal sample preparation and high detection sensitivity are resolved, then LIBS offers great potential for detection of elements in liquids. Consequently, various attempts were made by several researchers to overcome the problems encountered for liquid samples [13–15].

2.1.2. Aerosols and Gases. In today's world, *in situ* and real time techniques have a range of applications in the analysis of small particles that range from submicrometer to several micrometers in diameter. Such techniques are useful in atmospheric sciences, process monitoring and control, and effluent waste stream monitoring. The LIBS technique was used to detect chromium in aerosols using a constant-output aerosol generator called atomizer [16]. In multiphoton ionization, the dominant mechanism for plasma formation is at 266 nm and avalanche ionization at 1064 nm, while both are almost equal contributors at 532 nm. The results show that, to have a maximum efficiency of ionization, an increase of laser energy/pulse is needed. Once the maximum efficiency for ionization is reached, no further increase in incident energy is necessary. For calcium and magnesium based aerosols, LIBS was used for quantitative analysis of size, mass, and composition of individual micron- to submicron-sized aerosol particles over a range of characterized experimental conditions [17].

2.1.3. Metallurgical Samples. LIBS has been used for direct and rapid determination of various types of trace metals. For analyzing the various trace elements, such as Mg, Cu, Cr, Si, and Ca, in rock samples, a time-resolved LIBS (TRELIBS) technique was used by Song et al. [18]. The analytical signal of trace elements was integrated within 20 n sec after an optimal gate delay time of 200 n sec. The detection limit (S/N ratio = 3) was in the order of 5–100 parts per million (ppm). Precision was typically 5–10% relative standard deviation (RSD). This methodology was used to determine several elements (Al, Cu, Fe, Pb, and Sn) in solid zinc alloys [19].

2.1.4. Environmental Samples. With the increased research awareness, LIBS has also been used for environmental monitoring, specifically for online and remote analysis of potential hazards. The United States Environmental Protection Agency (EPA) encourages facilities to evaluate the use of real-time emissions monitoring technology in industry. Theriault et al. used LIBS for screening heavy-metal-contaminated soils using an *in situ* probe [20]. The detection limits can meet the EPAs site screening levels (SSLs) for several key metal contaminants in sand, although the probe response is affected by the soil matrix conditions like rain amount and water content. Knight et al. used LIBS for stand-off analysis of soil at reduced air pressure and in a simulated Martian atmosphere (5–7 Torr pressure of CO_2) showing the feasibility and scope of the use of LIBS in space exploration [21]. However, the extent to which the method can provide quantitative information in space remains to be seen and must be thoroughly studied before the technique is deployed.

2.1.5. Nonmetallic Solids. LIBS was adapted in mining and coal industries for both exploration as well as ore body imaging; it is employed by using an optical fiber bundle with wide acceptance angle, placed at a distance to find out the elemental content of the mineral core drill sample. LIBS was also used in the study of archaeological objects and for conservation and restoration of cultural heritage, though the main focus has been on laser artwork cleaning. Mineral assaying applications were performed using TRELIBS as reported by Bolger [22]. They used a Q-switched Nd:YAG laser to test lengths of drill core, with remote LIBS signal acquisition via a bare optical fiber bundle coupled to a spectrometer. High linear correlations ($R^2 > 0.92$) were obtained for Cr, Cu, Mn, Ni, and Fe, which appeared in concentration range of 200 ppm but were actually 10% of 200 ppm, as compared to the normalized atomic emission intensities of these elements in the laboratory result. The detection limits for these and other elements were extrapolated to be around 300 ppm. Fabre et al. used LIBS in geological materials for the determination of lithium in melt inclusions, quartz, and associated fluid inclusions and then compared the results (obtained with LIBS) with the result of electron microprobe technique. The results obtained with LIBS were in good agreement with bulk and microprobe data obtained for the same minerals [23].

2.1.6. Plant Materials. In recent years, LIBS has received much attention with applications to solid and liquid analysis of plants materials [24–30]. Due to shock wave generation and splashing phenomenon, repetition rate becomes an important parameter when analyzing a sample in the liquid phase. It has been observed that the average spectra is

preferred instead of single-shot spectrum for enhancement of the signal-to-background and signal-to-noise ratio and to get reproducibility [15]. The essential challenge in LIBS spectroscopy is the calibration. Classical approach is the use of an internal standard of known or constant concentration [31–33].

2.1.7. Advanced Materials. In the field of surface analysis, including composition mapping and in-depth profile analysis of advanced materials, the most advanced LIBS techniques have been used. Major progress at the nanometric range has been achieved for depth-resolved measurement. Detailed spatial information by imaging mode of LIBS has been achieved by point-to-point mapping using a spherical lens focusing system, when a multidimensional detector is used. Romero and Laserna used multichannel LIBS to generate selective chemical images for Ag, Ti, and C from silicon photovoltaic cells [34]. Both surface and depth distributions were amenable with the help of this approach. Lateral resolution of 80 nm and depth resolution of better than 13 nm for TiO_2 coatings were achieved. The surface analyses of photonic-grade silicon were also tested using LIBS technique [35]. A total area of 3–2.1 mm^2 was analyzed with a lateral resolution of 70 mm and depth resolution of about 0.16 mm. Two-dimensional (2D) and three-dimensional (3D) distribution maps of carbon contamination on silicon were presented. The use of LIBS for quantitatively mapping the multielement distribution on polish rock and for copper in printed circuits was proposed by Yoon et al. and Kim et al. [36, 37].

2.1.8. Miscellaneous Applications. LIBS application for the online multielement analyses of glass melts in a vitrification process of high-level liquid waste (HLLW) was investigated by Yun et al. [38]. Twelve different HLLW glass melts with a complex composition of about 27 chemical elements were simulated on a laboratory scale, varying the HLLW component concentration. The analytical method was calibrated by real-time analyses of the reference glass at 1200°C. The LIBS results were also well presented, and comparisons were done with those determined by XRF and ICP-AES. The influence of the matrix on the LIBS of magnesium was presented by Gornushkin et al. and Rai et al. [39, 40]. The surface density normalization method works well for the reduction of the matrix effect in the determination of Mg in powdered samples of different bulk compositions. According to Kurniawan et al. [41], the relative error of 10% and a precision of 10–20% were obtained for the determination of Mg in several certified samples.

3. Diabetes, Natural Products, and LIBS

3.1. Diabetes Mellitus and Its Incidence. Diabetes mellitus, hereafter referred to as diabetes, is a heterogeneous disorder characterized by excess blood glucose due to improper metabolism of proteins, fats, and carbohydrates. Diabetes is characterized by elevated fasting blood glucose (FBG) and postprandial glucose (PPG) levels. The body is unable to utilize available blood sugar (glucose), either due to unavailability of insulin caused by inactivation of β-cells of pancreas or due to improper utilization of insulin (also known as insulin resistance) due to insensitiveness of cell receptors to insulin [42].

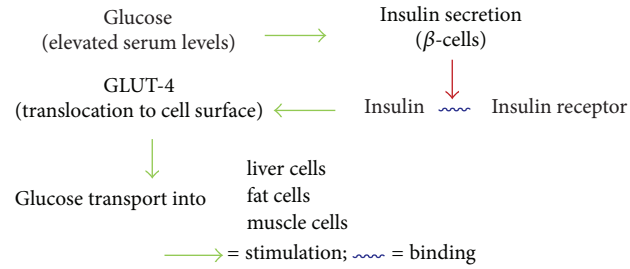


Figure 1: Mechanism of glucose delivery.

The World Health Organization (WHO) has listed diabetes as one of the major killers of our time. About 225 million people worldwide are estimated to be suffering from diabetes. This number may probably double by the year 2030. It is the 7th leading cause of death even in a developed country like the US [43]. India has been labeled as "Diabetes capital of the world" due to highest incidence of diabetes. Each day, more than 2,200 people are diagnosed with diabetes. Unfortunately, more than 50% of diabetics are not aware of their condition.

3.2. Pathogenesis of Diabetes. Insulin is secreted, in response to elevated serum glucose levels, by β-cells of the islets of Langerhans in the pancreas. The α-cells of islets of Langerhans secrete glucagon, a hormone with action nearly opposite of insulin. The role of insulin is to stimulate the GLUT-4 glucose transporter. GLUT-4 is the most important of the glucose transporter molecules, and its insertion into the muscles and adipose cell membranes serves to facilitate glucose delivery into these cells. This is the only mechanism by which the glucose can be delivered to fat, muscle, and also liver cells (Figure 1). Failure at any step of the above-mentioned mechanism of glucose delivery triggers diabetes.

3.3. Types of Diabetes

3.3.1. Type 1. Formerly known as insulin-dependent diabetes mellitus (IDDM), it occurs when the β-cells of the pancreatic islets of Langerhans are destroyed, such that insulin production is grossly impaired. Thus, type 1 diabetes is invariably treated with insulin. It is also referred to as juvenile diabetes.

3.3.2. Type 2. Formerly known as noninsulin-dependent diabetes mellitus (NIDDM), typical type 2 diabetes is rarely found as an isolated abnormality. Obesity, hypertension, dyslipidemia, and hyperurinemia appear to cluster in the same individuals. Insulin resistance may be the underlying cause of this type of metabolic syndrome. Typical dyslipidemia associated with type 2 diabetes is hypertriglyceridemia and hypocholesterolemia, [44–47]. The mechanism by which hyperinsulinemia can lead to hypertension and hypertriglyceridemia has already been proposed [48–53].

Table 1: Intensity ratio of different elements of some antidiabetic plants with respect to C III (229.6 nm).

Intensity ratio (element/ref)	*Cynodon dactylon*	*Emblica officinalis*	*Ficus benghalensis*	*Moringa oleifera*	*Momordica charantia*	*Psidium guajava unripe*	*Trichosanthes dioica*	*Withania coagulans*
$C\ III_{229.6\,nm}/C\ III_{229.6\,nm}$	1	1	1	1	1	1	1	1
$C_{247.8\,nm}/C\ III_{229.6\,nm}$	0.58326	0.87216	0.72842	0.77376	0.99845	0.63213	0.65925	4.43244
$Mg\ II_{279.5\,nm}/C\ III_{229.6\,nm}$	1.53617	3.06977	3.19302	1.31796	2.74925	4.26175	1.48591	4.33553
$Mg\ II_{280.2\,nm}/C\ III_{229.6\,nm}$	0.70947	2.04378	1.82947	0.84332	2.95627	2.39152	1.13031	3.39804
$Ca_{393.3\,nm}/C\ III_{229.6\,nm}$	2.58442	39.2093	2.72951	3.37054	1.93864	0.72139	3.38716	1.98661
$Ca_{396.8\,nm}/C\ III_{229.6\,nm}$	1.95627	18.31737	2.94815	4.18424	1.67391	0.51283	2.02546	0.76354
$Ca_{422.7\,nm}/C\ III_{229.6\,nm}$	0.98174	5.41176	1.87245	2.74294	0.98351	0.39271	0.55465	

3.3.3. Type 3. The term "type 3 diabetes" reflects the fact that Alzheimer's disease (AD) represents a form of diabetes that selectively involves the brain and has molecular and biochemical features that overlap with both type 1 and type 2 diabetes mellitus. The characteristic molecular and biochemical abnormalities associated with AD include cell loss, increased activation of prodeath genes and signaling pathways, impaired carbohydrate metabolism, chronic oxidative stress, DNA damage, and so forth. Currently, there is a rapid growth in the literature pointing toward insulin deficiency and insulin resistance as mediators of AD-type neurodegeneration, but this surge of new information is riddled with conflicting and unresolved concepts regarding the potential contributions of type 2 diabetes mellitus and obesity to AD pathogenesis [54]. However, extensive disturbances in brain insulin signaling could account for the majority of molecular and biochemical lesions in AD. It has been reported that type 2 diabetes mellitus causes brain insulin resistance and oxidative stress. Experimental brain diabetes produced by intracerebral administration of streptozotocin (STZ) shares many features with AD and is treatable with insulin sensitizer agents, that is, drugs currently used to treat type 2 diabetes mellitus (T2DM) [54].

4. Indian Plants and Their Glycemic Elements

Several Indian plants and their glycemic elements have been discussed in the literature.

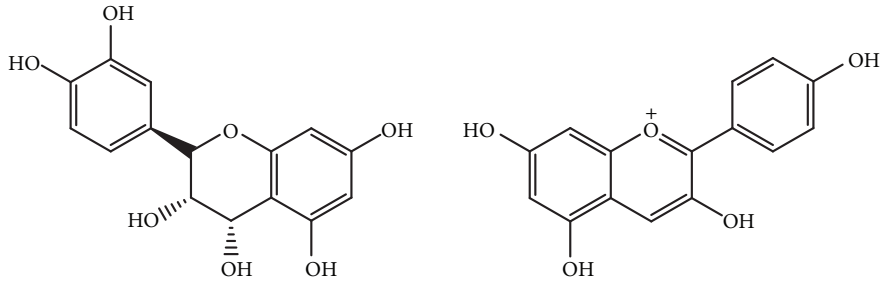
4.1. Cynodon dactylon (Family: Poaceae). Commonly known as "Dhoob" in India, is a weed, and is regarded to possess antidiabetic properties. Aqueous and ethanolic extracts of whole plant were found to have hypoglycemic and hypolipidemic effects in STZ-induced diabetic rats at a dose of 500 mg kg^{-1} [55, 56]. Recently, antioxidant activity and glycemic elemental study have been reported on the aqueous extract of the leaves of this plant [24, 25]. Results of the LIBS analysis of *C. dactylon* indicate that its extracts consist of elements like Mg, C, and Ca in the spectral range 200–500 nm.

Because the intensity of a spectral line of a particular element present is directly proportional to its concentration, the proportion of the concentration of these elements could be evaluated by taking the ratio of intensity of these elements and the intensity of a reference line. In the aforesaid study, C III (229.4 nm) is the reference as carbon (C) being an essential constituent of trace elements present in plants, is abundant. C III (229.4 nm) line is interference-free line and, hence, fulfills the requirement to be chosen as a reference line. The intensity ratio of Mg/C and Ca/C has been calculated and tabulated in Table 1.

4.2. Emblica officinalis (Family: Euphorbiaceae). Commonly known as "Amla" in Hindi, grows in India as well as tropical and subtropical regions of the world. The fruit of *E. officinalis* has been reported to have potent antimicrobial, antioxidant, hepatoprotective, antitumor, and hypolipidemic properties [57, 58]. The aqueous extract of the *E. officinalis* seeds has antidiabetic and antioxidant activities [59, 60]. The LIBS spectrum of *E. officinalis* fruit extract was taken in different spectral ranges at optimized experimental conditions. It revealed the presence of Mg, Na, Cl, Ca, H, O, C, and N elements in the spectral range of λ 200–900 nm. According to the Boltzmann distribution law, intensity is directly related to concentration [61], therefore, the intensity of observed spectral lines corresponding to the major and minor elements present in the extract indicates their concentrations and helps define their role in STZ-induced diabetes and its stress management [12, 24, 25, 55–60].

4.3. Ficus bengalensis (Family: Moraceae). Commonly known as "Indian Banyan Tree or Bur," is distributed throughout India. A glycoside called bengalenoside was isolated from the bark and showed more potent hypoglycemic action compared to the crude ethanolic bark extract, and the activity being was half that of the synthetic drug tolbutamide [62]. Oral administration of bark extract showed significant antihyperglycemic effect in STZ diabetic rats by raising serum insulin levels. Leucocyanidin and pelargonidin compounds (Scheme 1) isolated from the bark have also shown hypoglycemic activity [63–65]. Most recently, the hypoglycemic as well as antidiabetic properties have been reported in aerial roots of this tree [66]. The LIBS results showed a higher concentration of Mg and Ca in aqueous extract of *Ficus bengalensis* as compared to other elements present.

4.4. Moringa oleifera (Family Moringaceae). Commonly known as Drumstick tree, is indigenous to Northwest India.



I II

Scheme 1: Leucocyanidin (I) and pelargonidin (II) isolated from the bark of *Ficus bengalensis* show hypoglycemic activity.

Most parts of this tree possess antimicrobial activity [67]. The tree is well known for its pharmacological actions and is used for the traditional treatment of diabetes mellitus, hepatotoxicity [68], rheumatism, venomous bites, and cardiac stimulation. The leaves of *M. oleifera* are lopped for fodder [69] and have been used as antiulcer, diuretic, antiinflammatory and for wound healing [67, 70]. Ethanolic extract of leaves has shown antifungal activity against a number of dermatophytes [71, 72]. The aqueous extract of the leaves has been found to possess antidiabetic activity [73]. The LIBS spectrum of *M. oleifera* fruit extract was taken at optimized experimental conditions. It revealed the presence of Mg, Ca, H, O, C, and N elements in the spectral range of 200 nm to 400 nm. The proportion of the concentration of these elements could be evaluated by taking the ratio of intensity of these elements with intensity of reference lines (C and O, respectively, as they are essential elements of plant materials and hence are in abundance).

4.5. Momordica charantia (Family: Cucurbitaceae). Commonly known as bitter gourd (melon) or Karela, is widely planted in tropical areas and is usually consumed as a vegetable. Bitter gourd has also been frequently used as a medicinal herb in Asia, Africa, and South America because of its antidiabetic, anthelmintic, abortifacient, antibacterial, antiviral, and chemopreventive functions [74, 75]. In addition, freeze-dried *M. charantia* powder was found to have antidiabetic activity [26]. LIBS spectrum of the freeze-dried *M. charantia* fruit powder was recorded to identify its glycemic trace elements that may be responsible for diabetes management in biological systems. The LIBS results point out that dried fruit pellet of *M. charantia* consists of elements like Na, K, Mg, Ca, Fe and Al in the spectral range of 200 nm–500 nm. The concentrations of these elements were determined by calibration-free (CF)-LIBS method. This approach requires extensive data processing and, hence, a data processing algorithm was developed [76] to quantify the effect of different CF parameters using a code in MATLAB [26].

4.6. Psidium guajava (Ripe Fruit Peel): (Family: Myrtaceae). Is a semideciduous tropical tree commonly known as "Amrood" in Hindi and is widely grown throughout India for its fruit. A high percentage of vitamin C, carotene, vitamins B_1, B_2, and B_6, and free sugars (glucose, fructose, and sucrose) have been reported in this fruit [77]. The *P. guajava* fruit has been discovered as a new source of antioxidants and is reported to have hypoglycemic effect [78] on blood glucose level (BGL) of normal and STZ-induced mild as well as severely diabetic rats during glucose tolerance test (GTT) and PPG studies, respectively. Surprisingly, aqueous extract of the ripe fruit peel was discovered to be hyperglycemic [79], and the observed hyperglycemic effect has been correlated to its low Mg concentration [27].

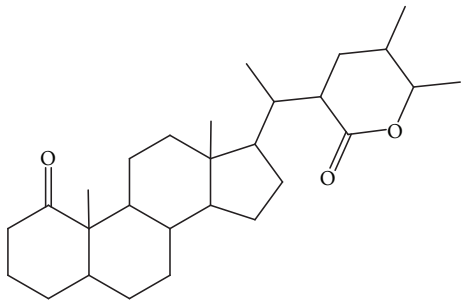
4.7. Trichosanthes dioica Roxb. (Family: Cucurbitaceae). It is a dioecious perennial herbaceous vegetable. It is commonly known as "Parval" in Hindi and is widely grown throughout India. Its fruits are a rich source of vitamin C and minerals (Mg, Na, K, Cu, and S). Normal animals fed with a diet consisting of shade dried fruits of *Trichosanthes dioica*, have shown hypocholesterolemic, hypotriglyceridemic, and hypophospholipedemic effects [80]. Normal and diabetic human volunteers given the direct intake of fruit and pulp also have shown hypocholesterolemic and hypotriglyceridemic effects [81]. Direct feeding of the seeds of this fruit was also found to be effective in lowering the serum lipid profile of normal and mild diabetic human subjects [82] and albino rabbits [83]. Effect of fruit powder has been studied on blood sugar and lipid profile of normal albino rabbits [84]. Aqueous extract of seeds and leaves have glycemic elemental profile and have shown antihyperglycemic, hepatoprotective properties [28–30, 85–88].

4.8. Withania coagulans (Family: Solanaceae). Is commonly known as Indian cheese maker. A steroidal lactone, withanolide (Scheme 2), isolated from the aqueous extract of fruits of *W. coagulans*, has cardiovascular effect [89]. Alcoholic extract has shown antibacterial and antihelminthic activities [90]. The hot aqueous extract of *W. coagulans* fruits has shown to exert hepatoprotective, anti-inflammatory and antidiabetic effects [91–93]. The hot aqueous extract of *W. coagulans* fruits increases the glucose utilization in isolated rat hemidiaphragm cells [94].

4.9. Cajanus cajan (Family: Leguminosae). It is an annual or perennial herb or shrub. It is commonly known as pigeon pea

Table 2: Intensity ratio of different elements of some hypoglycemic plants with respect to C III (229.6 nm).

Intensity ratio (element/ref)	*C. cajan*	Ripe fruit peel of *P. guajava*	*M. paradisiaca*
$C\ III_{229.6\ nm}/C\ III_{229.6\ nm}$	1	1	1
$C_{247.8\ nm}/C\ III_{229.6\ nm}$	0.64383	0.75183	0.78036
$Mg\ II_{279.5\ nm}/C\ III_{229.6\ nm}$	1.77763	1.51083	0.51094
$Mg\ II_{280.2\ nm}/C\ III_{229.6\ nm}$	2.10679	0.86273	0.36734
$Ca_{393.3\ nm}/C\ III_{229.6\ nm}$	2.18003	2.98471	3.39804
$Ca_{396.8\ nm}/C\ III_{229.6\ nm}$	1.30575	1.57927	1.98661
$Ca_{422.7\ nm}/C\ III_{229.6\ nm}$	0.57595	0.62948	0.76354



Scheme 2: General carbon skeleton of withanolides is shown above and these are known to exert cardiovascular effects.

or red gram in English and Arhar in Hindi. It is one of the most important pulse crops cultivated in India [95]. Arhar is consumed in the form of split pulse or dal or, when tender, as a vegetable. The green leaves and tops of the plant are used as fodder and as green manure [96]. *C. cajan* leaves have hyperglycemic activity. LIBS spectra of *C. cajan* clearly reveal that the extract consists of elements like Mg, C, and Ca in the spectral range 200–500 nm. The intensity ratios of Mg/C and Ca/C have been calculated and found that these ratios for *C. cajan* are lower in comparison to the above-mentioned plant extracts known to have antidiabetic activity (Table 2).

4.10. Musa paradisiaca (Family: Musaceae). Is a perennial tree-like herb. It is commonly known as banana and is widely found in Northern India. Ayurvedic physicians of Karnataka and Kerala recommended *Musa paradisiaca* for the treatment of urinary stones [97]. The stem juices of *Musa paradisiaca* have been reported for dissolving preformed stones and in preventing the formation of stones in the urinary bladder of rats [98, 99]. The juice of the stem is also used in nervous affectations like epilepsy, hysteria, and also in dysentery and diarrhea. Several sugars comprising fructose, xylose, galactose, glucose, and mannose occur naturally in banana, making it an excellent prebiotic for the selective growth of beneficial bacteria in the intestine [100]. The stem juice of *M. paradisiaca* has hyperglycemic activity [94].

Since all the three hyperglycemic extracts are sharing Mg/C as well as Ca/C, their opposite effects on BGL lead us to believe that the ratio of concentration of magnesium and calcium over carbon plays a vital role in determining whether a plant is hyperglycemic or hypoglycemic. Hence, to analyze the role of these elements in diabetes management, understanding of the specific proportion of these elements in all the extracts is essential. Additionally, other essential constituents like H, O, N, and C are found in equal proportion in both the extracts and show no significant role in diabetes management.

5. Conclusion

The present review describes some advanced applications of LIBS. This technique is gaining popularity for qualitative and quantitative analysis of trace elements present in any material. The use of LIBS does not involve complicated sample preparation, is portable and easy to use, with high reproducibility. The technique is based on the principle of the spectral analysis of radiation emanating from microplasma generated by focusing a high power pulsed laser beam on the surface of the sample. The characteristic emission from plasma is recorded as spectrum, which provides a fingerprint of elements present in the target material. Almost all metals and some nonmetals in different matrices have been target elements in trace determinations by LIBS and can be explored further by the pharmaceutical industry. Further research in this direction (detection of glycemic elements from medicinal plants) will be on developing on-site and doing remote analysis of elements, improving precision in measurement, developing accurate calibration procedures and detection limits.

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