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Stevia rebaudiana (Bert.) Bertoni — A Review

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The Sweet Herb of Paraguay, *Stevia rebaudiana* (Bert.) Bertoni is fast becoming a major source of high potency sweetener which produces sweet taste but has no calorific value. Many research activities on its chemical and biological properties have been done in recent past. Several countries including India have started its commercial cultivation. The published literature on this crop is quite scattered. Therefore an effort to compile the literature and review the current status of research and development including cultivation practices has been done in this paper.

Keywords: Natural sweetner, Stevia, *Stevia rebaudiana*, Stevia glycosides, Sweet herb of Paraguay.

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Introduction

Stevia rebaudiana (Bert.) Bertoni (Family — Asteraceae) is one of 154 members of the genus Stevia and one of only two species that produce sweet steviol glycosides. It has been used to sweeten tea for centuries dating back to the Guarani Indians of South America. Glycosides responsible for the plants sweeteners were discovered in 1931. Its extracts are used today as a food additive by the Japanese and Brazilians and as a non-caloric sweetener. In the U.S., however, its use is limited to supplement status only. It is commonly known as Stevia, Sweet leaf, Sweet herb of Paraguay, Honey leaf and Candy leaf. It is a perennial semi-shrub up to 30 cm in height (Plate 1). Leaves are sessile, 3-4 cm long, elongate-lanceolate or spatulate shape with blunt-tipped lamina, serrate margin from the middle to the tip and entire below. The upper surface of the leaf is slightly glandular pubescent. The stem is weak-pubescent at bottom and woody. The rhizome has slightly branching roots. Flowers are composite surrounded by an involucre of epicalyx. The capitula are in loose, irregular, sympodial cymes. The flowers are light purple, pentamerous. The fruit is a five-ribbed spindle-shaped achene[1-5].

Stevia is native to the valley of the Rio Monday in highlands of North-eastern Paraguay in South America where it grows in sandy soils near streams on the edges of marshland, acid infertile sand or muck

Plate 1 — *Stevia rebaudiana*

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soils[1]. Stevia is found growing wild in the highlands of the Amambay at Iguac districts (a border area between Brazil and Paraguay). A large effort aimed at establishing Stevia as a crop in Japan was initiated by Sumida[6].

The first report of commercial cultivation in Paraguay was in 1964 (Refs 1,7,8). Since then it has been introduced as a crop in a number of countries including Brazil, Korea, Mexico, United States, Indonesia, Tanzania and Canada since 1990 (Refs 9-15). Currently its production is centered in China and the major market is in Japan[16].

A lot of work has been done on the ecology, importance of the plant, its production requirements and the agronomic and management aspects of the plant to be grown as a crop, the current status of understanding of the plant and its potential as an alternate source of sweetening to cane sugar[17,18]. Studies showed that Stevia could replace some or all of the sugar (sucrose) in recipes without drastically affecting the visual acceptability or physical characteristics of the food product. Further studies on the safety of Stevia are recommended to determine its potential usefulness as a sugar substitute[19]. The paper presents a comprehensive review on its agrotechniques, bioproduct extraction, phytochemical, biological and toxicological studies done on *S. rebaudiana*.

Cultivation

Stevia is a semi-humid subtropical plant that can be grown easily like any other vegetable crop. India's agro-technologists are actively involved in the cultivation and study of various parameters like mean height, weight of leaves, growth per day, total biomass yield and stevioside content in the plant. The crop could be transplanted in February or March and seed collected in the late summer. Flowering under these conditions should occur between 54-104th day following transplanting, depending on the day length sensitivity of the cultivars used for seed production. Leaf yield increased with increasing density up to 83,000 and 111,000 plants/ha for the first year of production. The concentration of stevioside in the leaves of Stevia increases when the plants are grown under long days[20].

It is harvested just prior to flowering when steviol glycoside content in the leaves is maximum[21]. Weight of one thousand seeds of Stevia usually ranges between 0.15 and 0.30 g and depending on plant density it yields up to 8.1 kg/ha. Seed viability and yield are affected by growing conditions during pollination. Excessive rainfall during pollination can affect both seed yield and germination[22,23].

A variety of plant breeding procedure has been used for better leaf yield and rebaudioside-A concentration in the leaves. Based on the cultivar descriptions from Japan, China and Korea, it appears that sufficient genetic variability exists to make significant genetic gains in leaf yield of rebaudioside A content and the ratio of rebaudioside A to stevioside[9,14,23-25]. Seed is best stored at 0°C, but even under low temperature conditions, germination declines by 50% over three years.

Studies on glycosides revealed that synthesis is reduced at or just before flowering; delaying flowering with long days allows more time for glycoside accumulation. It follows that Stevia herbage production would be best in a long day environment where vegetative period is longer and the resultant steviol glycoside yield will thus be higher. Fertilizer requirements for Stevia grown as an annual crop are moderate. The commercialization of Stevia has forced interest even into *in vitro* propagation[26].

Agrotechnique (Tissue culture) and Bioproduction of stevia glycosides

Wada *et al* reported the induction of somatic embryos from leaf explants on a medium supplement with the cytokinin N-(4-Pyridyl)-N'-phenylurea (4-PU)[27]. Several cell lines were obtained from a predominantly diploid (2n = 22) cell suspension culture of *S. rebaudiana* by colchicine treatment. The cell lines developed were used to initiate predominantly diploid, tetraploid or aneuploid cell suspensions that showed characteristic growth rates and aggregate sizes[28]. Somatic embryogenesis was also obtained from floret explants cultured on MS medium supplemented with 2, 4-D (9.05 and 18.19 µM) and Kinetin (0 to 9.2 µM). On 9.05 µM 2, 4-D supplemented medium maximum without kinetin embryogenic callus formation occurred[29]. A multiple shoot culture was induced from nodal segments on MS medium containing half concentration of macroelements, 1% sucrose and supplemented with NAA (0.01 mg/l)[30].

The composition and content of steviol-glycosides (SGs) in *in vitro* cultures were investigated by Bondarev *et al*[31]. A comparative analysis of production of these compounds in intact plants, *in vitro* plants, dedifferentiated callus and suspension cultures, morphogenic callus and *in vitro* regenerated

shoots was conducted. Qualitative composition of the SGs in *in vitro* plants was found to be identical to that of intact plants, but their content in the former plants appeared to be about five or six times lower. A significant decrease in this value was not observed upon long-term cultivation (for about 5 years) of the plants. Non-differentiated cell cultures, such as callus and cell suspension, were shown to synthesize only minor amounts of the SGs, and their content varied greatly during the growth cycle of the culture. Qualitative composition of the SGs in the cell cultures appeared to be highly scant as compared with that of the donor plants. No correlation between the SG content in organs of the donor plants and that in the cell cultures obtained was found. Factors determining plant cultivation conditions and influencing the accumulation of both fresh and dry cell biomass were not able to completely induce the SG synthesis in non-differentiated cell cultures. This process was restored only after the appearance of morphogenic structures and shoot formation[31].

Its shoots were cultivated in the roller bioreactor to study the production of steviol glycosides (SGs). It was found that, owing to the highly favourable conditions of shoot cultivation created in such an apparatus, the intensity of shoot growth and SG production appeared to be 1.5-2.0 times higher than those of the shoots grown in tubes. The data obtained suggests that the enhanced SG production is due to the differentiation of chlorenchyma cells and formation of specific sub-cellular structures for the glycoside to be accumulated[32].

Effects of sugars, mineral salts and plant growth regulators on the development of stevia shoots cultivated in the roller bioreactor and their production of steviol glycosides (SGs) were investigated. In the medium with fructose or glucose, extension of the shoots and development of their root system was much better than in the medium supplemented with sucrose. Under these conditions, however, accumulation of leaves dry mass decreased, and the content of the SGs in leaves declined. At elevated sucrose concentrations (from 1 to 5%) enhanced development of the root system and an increase in plant dry mass and number of leaf pairs was observed. At the same time 3% sucrose gave optimal SG accumulation. Two fold elevation of the concentration of mineral salts considerably stimulated growth of the shoots, whereas the content of the SGs in their leaves decreased. Addition of 0.1 mg/l 6-benzylaminopurine (BA) together with naphthaleneacetic acid (NAA) resulted in a 1.5-fold increase in the number of shoots. However, the shoots grown on the BA-supplied medium displayed a strong inhibition of the development of their root system. When the medium was supplied with gibberellic acid, lengthening of shoots and roots of stevia were observed. All the plant growth regulators used strongly inhibited production of SGs. The changes in nutrition medium composition had practically no effect on the ratio of individual glycosides in stevia leaves[33].

Biosynthesis of Stevia glycosides

Steviol biosynthesis was first investigated over 40 year's ago[34-36]. The biosynthesis of steviol (II) in *S. rebaudiana* is shown to proceed from mevalonic acid through (−)-kaurene (I) and (−)-kaur-16-en-19-oic acid (III) (Fig. 1). Radiochemical evidence is presented for the formation of (−)-kaurene by *S. rebaudiana*[36]. Acetate-2-C14 and mevalonic acid-2-C14 were applied to the leaves of growing shrubs. While mevalonic acid was not appreciably incorporated into steviol, the radioactive diterpene was isolated in radiochemically pure form after acetate-2-C14 administration[34]. (−)-Kaurene-17-^{14}C was administered to the leaves of a plant and radioactive steviol was isolated by extraction, hydrolysis and chromatography. Its radiochemical purity was demonstrated by dilution with carrier material recrystallization to constant specific activity, conversion to the methyl ester and crystallization. The steviol methyl ester was degraded to establish that essentially the entire radioactivity was located at the 17-carbon atoms[35].

A simple enzymatic method is described for the determination of stevioside in *S. rebaudiana* based on the hydrolysis of stevioside with crude hesperidinase. The reaction is followed by monitoring the production of glucose with a glucose oxidase-peroxidase-2, 2′-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid) system[37]. Since, steviol glycosides are derived from the mevalonic acid pathway (the fundamental metabolic route that provides the two C_5 building block molecules) isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are required for synthesis of all isoprenoid compounds[38, 39].

The diterpenoic compound steviol (ent-kaur-16-en-13-ol-19-oic acid) is the aglycone of sweet glycosides accumulated in Stevia. This compound is the hydroxylated form of ent-kaurenoic acid (ent-kaur-16-en-19-oic acid; ent-KA). The hydroxylation of

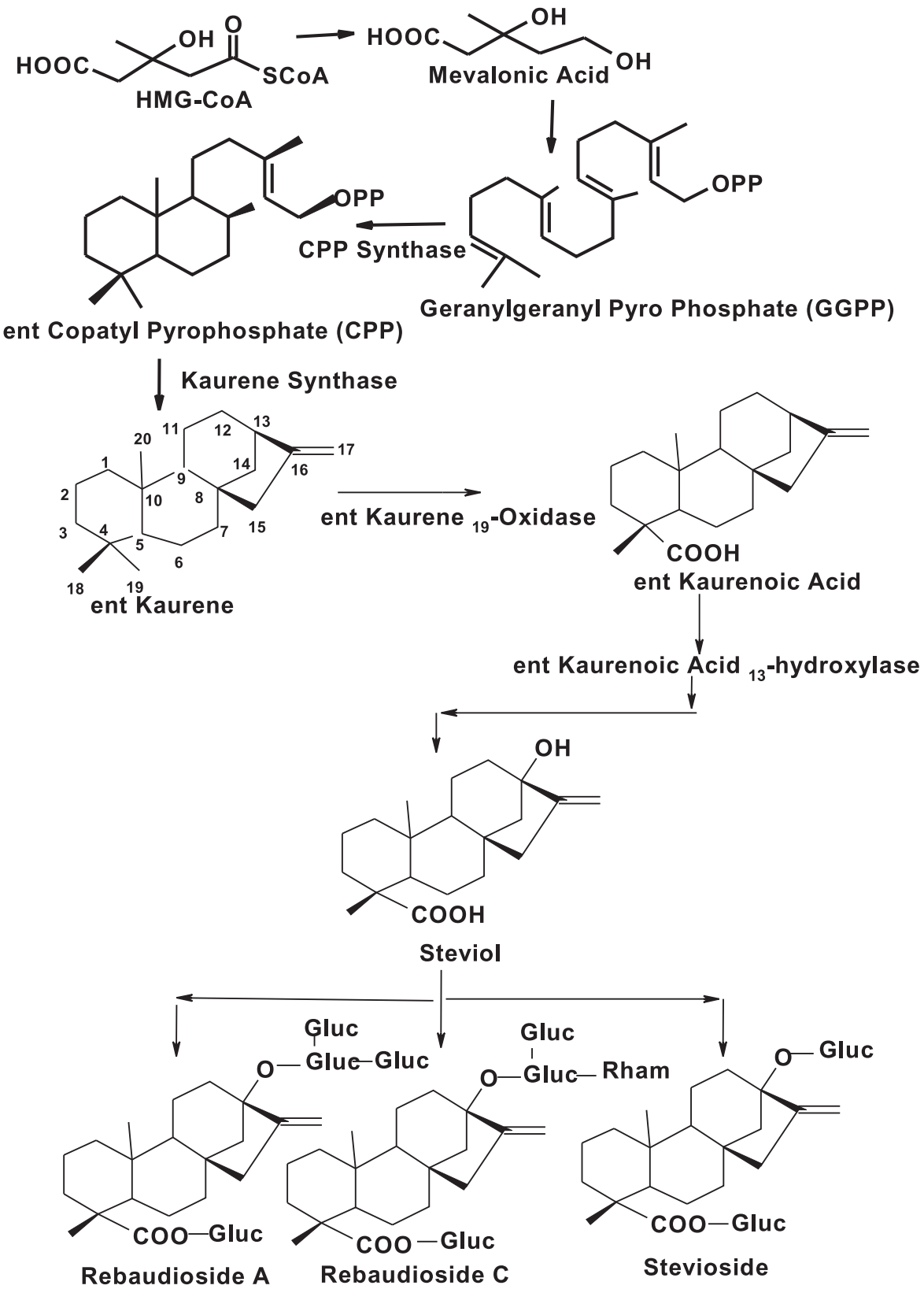

Fig. 1— Biosynthesis of Stevia glycosides

ent-KA to form steviol requiring NADPH and molecular oxygen was detected in stroma prepared from *S. rebaudiana*. The enzyme was purified from leaf extract to apparent homogeneity with a molecular mass of 39 kDa. Taken together with the value of 160 kDa estimated for native enzyme, this suggested that the hydroxylating enzyme is a homotetramer. The N-terminal sequence was determined through 20 residues. The *p*H optimum was 7.5-7.8. Apparent Km values were 11.1 [mu] for ent-KA and 20.6 [mu] for NADPH[40].

It has been established that the initial steps leading to the formation of steviol glycosides from GGPP are identical to those in gibberellin biosynthesis. GGPP is converted to ent-copalyl pyrophosphate (CPP) by CPP synthase (ent-Kaurene synthase A) and ent-Kaurene is produced from CPP by ent-Kaurene synthase (ent-Kaurene synthase B). Subsequent oxidation of this product at the C-19 position to ent-Kaurenoic acid is assumed to occur via the action of one or more P_{450} mono-oxygenases[41]. By incorporation of glucose, steviol and the diterpene aglycone moiety of stevioside, is synthesized in *S. rebaudiana* via the mevalonate-independent methylerythritol phosphate pathway (see Fig. 1)[(Ref. 42)].

A dichloromethane fraction and an ultrafiltered water extract of leaves of a new cultivar of *S. rebaudiana* ('UEM-320') were submitted to separate downstreaming fractionation in silica gel columns. An unusual sweetener ratio on behalf of rebaudioside A, a glycoside almost devoid of bitter after-taste, was found in the new cultivar as compared to other native cultivars, where the bitter stevioside was the major diterpenic glycoside[43].

The leaves accumulate a mixture of at least eight different steviol glycosides. The pattern of glycosylation heavily influences the taste perception of these intensely sweet compounds. The majority of the glycosides are formed by four glucosylation reactions that start with steviol and end with rebaudioside A. The steps involve the addition of glucose to the C-13 hydroxyl of steviol, the transfer of glucose to the C-2' and C-3' of the 13-O-glucose and the addition of glucose to the hydroxyl of the C-4 carboxyl group. By using a collection of expressed sequence tags (ESTs), an UDP-glucosyltransferase (UGT)-specific electronic probe and keyword searches to identify candidate genes resident in the collection. Fifty-four ESTs belonging to 17 clusters were found using this procedure. Full-length cDNAs for 12 of the UGTs were isolated, cloned into an expression vector and produced recombinant enzymes in *Escherichia coli*. An *in vitro* glucosyltransferase activity enzyme assay was conducted using quercetin, kaempferol, steviol, steviolmonoside, steviolbioside, and stevioside as sugar acceptors, and ^{14}C-UDP-glucose as the donor. Thin layer chromatography was used to separate the products and three of the recombinant enzymes produced labeled products that co-migrated with known standards. HPLC and LC-ES/MS were then used to further define those reaction products. It was determined that steviol UGTs behave in a regioselective manner. A modified pathway for the synthesis of rebaudioside A from steviol was proposed[44].

As the sweet steviol glycosides are derived from the diterpene steviol which is produced from a branch of the gibberellic acid (GA) biosynthetic pathway, an understanding of the spatial organisation of the two pathways including subcellular compartmentation, provided important insight into the metabolic engineering of steviol glycosides as well as other secondary metabolites in plants. The final step of GA biosynthesis, before the branch point to steviol production, is the formation of (−)-kaurenoic acid from (−)-kaurene, catalysed by kaurene oxidase (KO). Downstream of this, the first committed step in steviol glycoside synthesis is the hydroxylation of kaurenoic acid to form steviol, which is then sequentially glucosylated by a series of UDP-glucosyltransferases (UGTs) to produce the variety of steviol glycosides. The subcellular location of KO and three of the UGTs involved in steviol glycoside biosynthesis was investigated by expression of GFP fusions and cell fractionation which revealed KO to be associated with the endoplasmic reticulum and the UGTs in the cytoplasm. It has also been shown by expressing the Stevia UGTs in *Arabidopsis* that the pathway can be partially reconstituted by recruitment of a native *Arabidopsis* glucosyltransferase[45].

Cyclodextrin glucanotransferases (CGTases) produced by mesophilic, thermophilic, alkaliphilic and halophilic bacilli were used for transglycosylating stevioside and rebaudioside A with the use of starch as a donor. CGTases produced by *Bacillus stearothermophilus* B-5076 and *B. macerans* BIO-4m were the most effective biocatalysts. The proposed method can be successfully used for direct transglycosylation of Stevia extract without purification of its individual components[46].

Comprehensive two-dimensional liquid chromatography (LC × LC) connected on-line to electrospray ionisation time-of-flight mass spectrometry (ESI-TOF-MS) was employed for analysis of aqueous extract of *S. rebaudiana*. Different combinations of strong cation-exchange (SCX), amino (NH_2) and octadecyl siloxane (C18) stationary phases were tested in the separation of all nine known sweet Stevia glycosides. A combination of C18 as the first-dimension column and NH_2 as the second-dimension column fully separated all the glycosides from the matrix. The method proved to be quantitative and repeatable. The limit of detection (S/N = 3) for stevioside was 43.4 ng/g in dry leaves. The RSD for retention time was <0.1% and that of peak areas 4.5%[(Ref. 47)].

Commercial and purified stevioside was modified by hydrolytic enzymes from *Gibberella fujikuroi*. The screening was carried out on six strains of the fungus in order to select the most active. The production of the enzymes by the fungus was induced by its culture in a medium containing stevioside as the sole carbon source and the enzymatic extract was then used in the experiments. The products obtained were analyzed by HPLC-UV and HPLC-MS/MS. Results showed a significant increase in the concentration of rebaudioside A in the final product, which has better organoleptic properties than stevioside[48].

Phytochemical properties

Eight of Stevia glucosides were discovered, viz. dulcosides A, rebaudiosides A-E, steviobioside and stevioside[49-51]. In addition, the triterpenes amyrin acetate and 3 esters of lupeol and the sterols like stigmasterol, sitosterol and campesterol were also isolated from leaves[52,53].

Yamasaki *et al*[54] established the structures of some diterpene glucosides using ^{13}C NMR. Later, the diterpene glycosides, dulcosides A and B were isolated from *S. rebaudiana* and their structures were established[55]. Sholichin *et al* isolated some labdane type diterpenes from the plant[56]. In addition to the sweet diterpenoid glycosides, several other diterpenes have been isolated from Stevia. The first to be characterized were jhanol and austroinulin, previously obtained from other plants, and 6-O-acetylaustroinulin[56]. Jhanol, austroinulin, 6-O-acetylaustroinulin and 7-O-acetylaustroinulin as well as stevioside and rebaudioside A have also been obtained from Stevia flowers[57]. Six flavonoid glycosides have been isolated from aqueous methanol extract of leaves, viz apigenin-4'-O-glucoside, luteolin-7-O-glucoside, kaempferol-3-O-rhamnoside, quercetin, quercetin-3-O-glucoside and quercetin-3-O-arabinoside and 5,7,3'-trihydroxy-3, 6,4'-trimethoxy flavone (centauredin)[58].

The major components identified in the essential oil were the sesquiterepenes: δ-caryophyllene, trans-δ-farnesene, humulene, candinene, caryophyllene oxide and nerolidol and the monoterpenes: linalool, terpinen-4-ol and terpineol[59,60]. Some scientists have used hydrophilic columns for determination of few components of Stevia using HPLC.

S. rebaudiana, the sweetest species contains in its leaves all of the eight ent-kaurene glycosides[51] with stevioside being the major constituent (3-8% by wt of dried leaves)[61]. Additional labdane-type diterpenoids, sterebins E, F, G and H, were isolated from leaves and their structures were elucidated on the basis of fresh weight, spectral and chemical studies[62]. Stevioside (6-18% fresh weight in the leaves) is the sweetest glycoside and was tested and found to be 300 times sweeter than sucrose in a report[63]. From the extracts of *S. rebaudiana* I, two glucosyltransferases (GTases I and IIB) acting on steviol and steviol-glycosides were isolated. Another, distinct transferase (GTase IIA) acting on steviol was detected. Purified GTase I (subunit Mr 24,600) catalyzed glucose transfer from UDP-glucose to steviol and steviolmonoside (steviol-13-O-glucopyranoside), but not to other steviol-glycosides. GTase IIB (subunit Mr 30,700) showed a broad substrate specificity, acting on steviol, steviolmonoside, steviolbioside (13-O-β-sophorosyl-steviol) and stevioside. The two enzymes had a similar optimum *p*H at 6.5. They also acted effectively on ubiquitous flavonol aglycones, quercetin, and kaempferol and utilized kaempferol at a higher rate than steviol and steviol-glycosides. These glycosides are mainly compounds of the diterpene derivative steviol[64].

Rebaudioside A and steviobioside have been isolated by HPTLC methods[65]. Stevia also contains steviol, a product formed by enzymatic hydroxylation within the plant. Isolation of the principal sugars of Stevia has also been studied[40,66]. The sweet diterpenoid glycoside, rebaudioside F, was isolated from leaves of a high rebaudioside C producing line of the species, and its structure was established by chemical and spectral studies[67]. *S. rebaudiana* produces in remarkably high yield several high-potency low-calorie sweeteners in its leaf tissues. The

major compound stevioside, rebaudiosides A and C and dulcoside A, glycosides of the diterpene steviol (ent-13-hydroxykaur-16-en-19-oic acid), exhibit characteristic organoleptic properties[67].

The structure, stereochemistry and absolute configuration of steviol and isosteviol were established, through a series of chemical reactions and correlations over 20 years after the pioneering work of Bridel and Lavielle[68-71].

Both rebaudioside A and rebaudioside D could be converted to rebaudioside B by alkaline hydrolysis showing that only their ester functionality differed[49,72]. On the basis of IR, MS, 1H and 13C NMR as well as chemical evidences, the structure of rebaudioside B was assigned as 13-O-[β-glucosyl (1-2)-β-glucosyl (1-3)]-β-glucosyl-steviol. Kobayashi *et al* [55] reported the dulcosides A and B, the later was reported as having the same structure as rebaudioside C. Fractionation of leaf extracts led to the isolation and identification of the sweet glycosides, rebaudioside C, D and E[72,73]. Simple enzymatic method for the extraction of stevioside is described based on the hydrolysis of stevioside with crude hesperidinase. The reaction is followed by production of glucose with a glucose oxidase-peroxidase-2, 2'-azino-di-(3-ethylbenzothiazoline-system) and the stevioside content in the leaves was found to show large variation[37].

A variety of known sesquiterpene lactones as well as one new guaianolide, two known longipinene derivatives, two ent-labdanes and several other common compounds have been isolated from Stevia species[74]. Minor products from the acid-catalysed hydrolysis of the diterpenoid glycoside, stevioside have been identified and the location of the deuterium has been established when the hydrolysis is carried out in the presence of deuterium bromide[75]. The percent relative abundance of steviol glycosides was determined by evaporative light scattering detector (ELSD)[76, 77]. Natural longipinene isolated from Stevia species undergo molecular rearrangements to generate compounds with novel hydrocarbon skeletons[78].

Polymeric adsorbents with both functions of adsorption and decolorization were synthesized by introducing quaternary ammonium groups into conventional resinic adsorbent used to adsorb Stevia glycosides in production. The relation between the adsorption capacity of the bifunctional adsorbents for Stevia glycosides and the structures of adsorbents, and that between the decolorization efficiency and the structure were investigated, which revealed that the adsorption capacity for Stevia glycosides decreased somewhat as the increase of the content of quaternary ammonium groups in the adsorbent, while the decolorization efficiency increased. Mechanism of adsorption and decolorization was also studied, and revealed that adsorption of Stevia glycosides was based on hydrophobic interactions, but decolorization was based on both ion exchange and hydrophobic interactions. Introduction of $-N^{+}(CH_3)_3$ group into polymeric adsorbent also changed the adsorption selectivity for various Stevia glycosides and it was demonstrated to use bifunctional adsorbent to prepare Stevia glycoside containing high amount of rebaudioside A [(Ref.79)].

Six new labdane-type, non-glycosidic diterpenes, sterebins I-N, were isolated from the leaves of *S. rebaudiana* and their structures, analogous to those of the previously described sterebins A-H were elucidated on the basis of spectroscopic and chemical studies[80].

Extraction of Stevia glycosides

Besides the known extraction methods, new methods for glycoside-based extraction from Stevia were developed and it was found that water was very effective for extracting glycosides at selected *p*H and temperatures. It was also reported that a multi-stage membrane process was successfully able to concentrate the glycoside sweeteners. The bitter-tasting components were washed out from the sweetener concentrate in the nanofiltration process. It has been demonstrated that a membrane-based separation process for refining glycoside-based sweeteners could be viable and needs to be investigated further[81].

Analytical methods

Several analytical techniques have been employed to assess the distribution and level of sweet diterpenoid glycosides in *S. rebaudiana.* These include thin layer chromatography[17,51,82] over pressured layer chromatography[83], droplet counter-current chromatography[84] and capillary electrophoresis[65,85].

Stevioside and rebaudioside have also been analyzed by HPLC after conversion to the *p*-bromophenacyl esters of steviolbioside and rebaudioside[86]. Stevioside levels have also been determined enzymatically[73] and by near infrared reflectance spectroscopy[87] in plant strains producing mainly stevioside.

Amino columns have also been used to measure stevioside and related glycosides in foods and beverages[88-90]. The most common analytical method, however, has been high performance liquid chromatography (HPLC). Although separations have also been achieved using silica gel[83], hydrophilic[59], hydroxyapatite and size exclusion[91,92] columns, amino bonded columns have been used most frequently for the analysis of the sweet glycosides[51,85,93,94].

An improved analytical method was developed which may be applied to quality control of stevioside and rebaudioside A contents in dried leaves of *S. rebaudiana.* The procedure developed involves two steps: solvent extraction followed by an isocratic HPLC analysis. After the extract is cooled, it is filtered and analyzed by HPLC using an NH (2) column (250 × 4.6 mm) and mixture of acetonitrile: water (80:20, v/v) as mobile phase and *p*H 5 was adjusted with acetic acid. The detection was in the UV range at 210 nm. Quantitation was performed by means of an external standard calibration curve obtained from standard solutions of pure stevioside and rebaudioside A[(Ref. 95)]. These sweet constituents of *S. rebaudiana* were purified by droplet counter-current chromatography[96].

A simple method for stevioside analysis based on the water extraction, hydrophobic chromatography (Sep-Pak C18 cartridges) and HPLC using linear gradient of acetonitrile in water is also described. The feasibility of procedure was tested analyzing the stevioside level in Stevia leaves as well as in tea ("Fruit tea with Stevia"). Stevioside content did not show statistically significant difference when a set of 20 tea bags randomly chosen from five boxes was used. The method proved to be fast and friendly to the environment due to minimization of organic solvent consumption[97]. The HPLC separation and quantitation of stevioside, rebaudioside A and rebaudioside C [(Ref. 92)], rebaudiosides B, D, and E, dulcoside A, and steviolbioside was achieved[93] by an improved HPLC separation of the sweet diterpene glycosides using linear gradient elution[94].

Another simple and highly sensitive reversed-phase HPLC method has been developed for the determination of steviol from *S. rebaudiana* using dihydroisosteviol (DHISV) as an internal standard. Steviol and DHISV were derivatized by reaction of the acids[98].

A HPTLC method was developed and validated as per the ICH (International Conference on Harmonization) guidelines for simultaneous quantification of three steviol glycosides, i.e. steviolbioside, stevioside and rebaudioside A from *S. rebaudiana* leaves. For achieving good separation, mobile phase of ethyl acetate: ethanol: water (80:20:12, v/v/v) on pre-coated silica gel 60 F254 HPTLC plate was used. The densitometric quantification of steviol glycosides was carried out at λ = 510 nm in reflection–absorption mode after spraying with acetic anhydride: sulphuric acid: ethanol reagent. The calibration curves were linear in the range of 160-960 ng/spot for steviolbioside, 1-6 µg/spot for stevioside and 0.5-3 µg/spot for rebaudioside A with good correlation coefficients (0.998-0.999). The method was found to be reproducible for quantitative analysis of steviol glycosides in *S. rebaudiana* leaves collected from ten different locations and will serve as a quality control indicator to monitor the commercial production of stevioside and its allied molecules during different stages of processing[99].

Biological properties

Effect as a sweetening agent

Stevia is used in many parts of the world as a non-caloric sweetener[100]. As stevia leaf powder with no processing is highly safe to use, calorie free and moreover around 20-30 times sweeter than cane sugar, it can replace cane sugar easily. Use of stevia in bakery, confectionery, soft drink and beverage industry and in household products is recommended by various researchers. It was found to have similar potency with regard to sweetness as a 10% sucrose solution at either *p*H 3.0 or 7.0. Stevia has been evaluated for sweetness in animal response testing[101]. Matsukubo and Takazone reported the detailed characteristics of sucrose substitutes currently in use and their role in caries prevention and promotion of oral health[102].

Antioxidant activity

Stevioside, along with steviolbioside, isosteviol and steviol caused inhibition of oxidative phosphorylation in rat liver mitochondria[103]. The effect of several natural products extracted from the leaves of *S. rebaudiana* on rat liver mitochondria was investigated. They inhibited oxidative phosphorylation including on ATPase, NADH-oxidase, succinate-oxidase, succinate dehydrogenase and glutamate dehydrogenase activity. The ADP/O ratio

decreased and substrate respiration increased at low concentrations and at higher concentrations there was complete inhibition. It was concluded that, in addition to the inhibitory effects, *S. rebaudiana* natural products may also act as uncouplers of oxidative phosphorylation[104].

Four steviol glycosides, stevioside, rebaudiosides A and C, and dulcoside A, showed strong inhibitory activity against 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice. The 50 per cent inhibitory dose of these compounds markedly inhibited the promoting effect of TPA (1µg/mouse) on skin tumour formation initiated with 7, 12-dimethylbenz {a} anthracene[105].

Leaf extract of *S. rebaudiana* promotes effects on certain physiological systems such as the cardiovascular and renal systems and influences hypertension and hyperglycaemia. Since, these activities may be correlated with the presence of antioxidant compounds, leaf and callus extracts of *S. rebaudiana* were evaluated for their total phenols, flavonoids content and total antioxidant capacity. Total phenols and flavonoids were analyzed according to the Folin-Ciocalteu method and total antioxidant activity of water and methanolic extracts of Stevia leaves and callus was assessed by ferric reducing/antioxidant power (FRAP) assay as well as 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The total phenolic compounds were found to be 25.18 mg/g for Stevia leaves and 35.86 mg/g for callus on dry weight basis. The flavonoids content was found to be 21.73 and 31.99 mg/g in the leaf and callus, respectively. The total antioxidant activity was expressed as mg equivalent of gallic acid, ascorbic acid, BHA and trolox per gram on dry weight basis. Total antioxidant activity was reported in the range 9.66 to 38.24 mg and 11.03 to 36.40 mg equivalent to different standards in water and methanolic extracts of Stevia leaves, respectively. In case of Stevia callus, it was found to be 9.44 to 37.36 mg for water extract and 10.14 to 34.37 mg equivalent to standards for methanolic extract. The concentrations required for 50% inhibition (IC_{50}) of DPPH radicals were 11.04, 41.04 and 57.14 µg/ml for gallic acid, trolox and butylated hydroxyanisole (BHA), respectively. The per cent inhibition of DPPH radical of various extracts of Stevia leaves and callus ranged from 33.17 to 56.82%. The highest per cent inhibition was observed in methanolic extract of callus[106].

Anti-inflammatory and Immunomodulatory activity

Studies were carried out to elucidate the anti-inflammatory and immunomodulatory activities of stevioside and its metabolite, steviol. Stevioside at 1 mM significantly suppressed lipopolysaccharide (LPS)-induced release of TNF-α and IL-1β and slightly suppressed nitric oxide release in THP-1 cells without exerting any direct toxic effect, whereas steviol did not do so even at 100 µM. Activation of IKK β and transcription factor NF-kappa B were suppressed by stevioside, as demonstrated by Western blotting. Furthermore, only stevioside induced TNF-α, IL-1β, and nitric oxide release in unstimulated THP-1 cells. Release of TNF-α could be partially neutralized by anti-TLR4 antibody. The study suggested that stevioside attenuates synthesis of inflammatory mediators in LPS-stimulated THP-1 cells by interfering with the IKK beta and NF-kappa B signaling pathway, and stevioside-induced TNF-α secretion is partially mediated through TLR4 (Ref. 107).

Stevioside was tested for its immunomodulatory activity on different parameters of the immune system at three different doses (6.25, 12.5 and 25 mg/kg p.o.) on normal as well as cyclophosphamide treated mice. Stevioside was found effective in increasing phagocytic activity, haemagglutination antibody titre and delayed type hypersensitivity. In parallel, stevioside substantially increase proliferation in the LPS and Con A stimulated B and T cells, respectively. Thus, the drug holds promise as immunomodulating agent, which acts by stimulating both humoral as well as cellular immunity and phagocytic function[108].

Effect on reproductive system

The effect of the active principles of *S. rebaudiana* (SR) on endocrine parameters of male rats was studied upon chronic administrations (60 days) of a concentrated, crude extract of its leaves, starting at prepubertal age (25-30 days old). The SR-treated group did not significantly differ from the control group, with the exception of the seminal vesicle weight, which fell by about 60%. Thus SR extract does not have potential to decrease male rat fertility[109]. In addition, the fructose content of the accessory sex glands and the epididymal sperm concentration also decreased. Stevia treatment tended to decrease the plasma testosterone level, probably by a putative affinity of glycosides of extract for a certain androgen receptor, but no alteration occurred in luteinizing hormone level[109,110].

The safety of Thai medicinal plant, *Aegle marmelos* and *S. rebaudiana* on the reproduction of female rats was evaluated by Saenphet *et al*. Female rats were treated orally with aqueous extract of *A. marmelos* (6%) and *S. rebaudiana* at various concentrations (0, 0.2, 1 or 10%) for 60 days (1 ml/day) before mating. The control rats received only distilled water. At the end of the treatment period, treated females were mated with untreated males and the effects on reproduction were examined at day 14 of pregnancy. No notable abnormalities were observed in any of the pregnant rats. The number of corpus lutea, implanted and dead fetuses, as well as the sizes of the fetuses in the treated rats were not significantly different from those of the controls. Thus the aqueous extracts of *A. marmelos* and *S. rebaudiana* do not alter the reproduction of female rats[111].

Mutagenic and Bactericidal activity

Stevioside and steviol have been subjected to extensive genetic testing. The majority of the findings show no evidence of genotoxic activity. Neither stevioside nor its aglycone steviol have been shown to react directly with DNA or demonstrate genotoxic damage in assays relevant to human risk. The mutagenic activity of steviol and some of its derivatives, exhibited in *S. typhimurium* strain TM677, was not reproduced in the same bacteria having normal DNA repair processes. The single positive *in vivo* study measuring single-strand DNA breaks in Wistar rat tissues by stevioside was not confirmed in experiments in mice and appears to be measuring processes other than direct DNA damage. Neither stevioside nor steviol-induced clastogenic effects was observed at extremely high dose levels *in vivo*. Application of a Weight-of-Evidence approach to assess the genetic toxicology database concludes that these substances do not pose a risk of genetic damage following human consumption[112].

The aglycone of stevioside, steviol, is mutagenic towards *Salmonella typhimurium* strain TM677 in the presence of a metabolic activating system derived from the liver of Aroclor 1254-pretreated rats. The required activating component is localized in the microsomal fraction of rat liver, suggestive of a cytochrome P-450-mediated reaction. Partially purified epoxide hydrolase does not inhibit steviol-induced mutagenicity, indicating that an active metabolite is not an epoxide that serves as a substrate for this enzyme preparation. The 13-hydroxy group of steviol is required for the expression of mutagenicity since ent-kaurenoic acid is nonmutagenic and acetylation of steviol at this position negates mutagenicity. Similarly, diterpenes bearing a strong structural resemblance to steviol, cafestol and kahweol, were found to demonstrate no mutagenic activity toward *Salmonella typhimurium* TM677, as did their respective acetates and palmitic acid esters. 19-O-[β]-glucopyranosyl steviol, a potential hydrolysis product of stevioside, is mutagenic and bactericidal in the presence of a metabolic activating system. Structural differences among these naturally occurring and semi-synthetic diterpenes appear to impart major differences in biological activity that may relate to human health upon dietary ingestion[113].

Stevia extract has exhibited strong bactericidal activity against a wide range of pathogenic bacteria, including certain *E.coli* strains. The chronic administration of stevioside in hamsters showed no adverse effects, including histological evidence, on the reproductive systems in both male and female animals[114].

Effect on Renal function

Study conducted on stevioside for its possibility in acting as calcium antagonist in rats using classical clearance techniques and arterial pressure measurements showed that stevioside produced a fall in systemic blood pressure as well as diuresis and natriuresis per milliliter of glomerular filtration rate. Verapamil tended to increase the renal and systemic effects of stevioside, whereas an infusion of $CaCl_2$ in rats prepared with stevioside induced a marked attenuation of the vasodilating responses of stevioside[115].

The effects of administration of *S. rebaudiana* extract for 20, 40 and 60 days on renal function and mean arterial pressure in normal Wistar rats were evaluated by various workers. Results showed that the *S. rebaudiana* treated rats group for 20 days did not significantly differ from the control group. Chronic administration of a crude extract for 40 and 60 days induced hypotension, diuresis and natriuresis with constant glomerular filtration rate. Increased renal plasma flow was exclusively observed for the group treated for 60 days. The results suggested that oral administration to rats of an aqueous extract of Stevia dried leaves induced systemic and renal vasodilation, causing hypotension, diuresis and natriuresis[115-117].

The stevioside and its aglycone metabolite, steviol, have been shown to inhibit transepithelial transport of

para-aminohippurate (PAH) in isolated rabbit renal proximal tubules by interfering with basolateral entry. The study was carried out to determine which of the cloned basolateral organic anion transporters were involved in the renal transport of stevioside and steviol. The question was addressed in *Xenopus laevis* oocytes expressing human organic anion transporter 1 (hOAT1), 3 (hOAT3), and winter flounder OAT (fOat1). The parent compound, stevioside, had no inhibitory effect on either PAH (hOAT1) or ES (estrone sulfate; hOAT3) uptake. In contrast, steviol showed significant, dose-dependent inhibition of PAH and ES uptake in hOAT1- or hOAT3-expressing oocytes, respectively. The IC (50) of steviol for hOAT1-mediated PAH transport was 11.1 μm compared with 62.6 μm for hOAT3-mediated ES uptake. The Michaelis-Menten inhibition constants [K (i)] for steviol transport mediated by hOAT1 and hOAT3 were 2.0 +/- 0.3 and 5.4 +/- 2.0 μm, respectively. Trans-stimulation of PAH efflux by steviol was assessed to determine whether steviol itself was transported by hOAT1 or hOAT3. A low concentration of 1 μm steviol increased the efflux of [(3) H] PAH (trans-stimulated) via both hOAT1 and hOAT3. In addition, it was shown by electrophysiology that steviol entry induced inward current in fOat1-expressing oocytes. Thus stevioside has no interaction with either hOAT1 or hOAT3, whereas hOAT1, hOAT3 and fOat1 are capable of steviol transport and can play a role in its renal transport and excretion[118].

Effect on blood pressure

The plant was found to possess vasodilatory actions in both normo and hypertensive animals[120]. Stevia has previously been shown to reduce blood pressure in studies in animals. Stevia produces decrease in blood pressure and increase in diuretic and natriuretic effects in rats[117,119].

The effect of intravenously introduced stevioside on the blood pressure was studied by Chan *et al* in spontaneously hypertensive rats (SHR). In the conscious SHR, hypotensive effect on both systolic and diastolic blood pressure was dose-dependent for intravenous doses of 50, 100 and 200 mg/kg. Serum dopamine, nor-epinephrine and epinephrine levels did not change significantly 60 min after intravenous injection of stevioside at 100 mg/kg in anesthetized SHR. The study showed that stevioside given intravenously to conscious SHR was effective in blood pressure reduction and there was no change in serum catecholamines in anaesthetized animals with this natural compound[120].

Intraperitoneal injection of stevioside 25 mg/kg had antihypertensive effect. In isolated aortic rings from normal rats, stevioside dose-dependently relaxed the vasopressin-induced vasoconstriction in both the presence and absence of endothelium. However, stevioside had no effect on phenylephrine and KCl-induced phasic vasoconstriction. In addition, stevioside lose its influence on vasopressin-induced vasoconstriction in Ca^{+2} free medium. Stevioside caused vasorelaxation via an inhibition of Ca^{+2} influx into the blood vessel, which was confirmed in cultured aortic smooth muscle cells (A7r5). Using 10^{-5} M methylene blue for 15 min, stevioside could still relax10^{-8} M vasopressin-induced vasoconstriction in isolated rat aortic rings, showing that this vasorelaxation effect was not related to nitric oxide[116].

Feri *et al* studied the antihypertensive effect of crude stevioside obtained from the leaves of *S. rebaudiana* on previously untreated mild hypertensive patients. Patients with essential hypertension were submitted to a placebo phase for 4 weeks. During the investigation, blood pressure (BP) was measured biweekly and the remaining data was collected at the end of each stevioside dose step. Systolic and diastolic BP decreased ($p < 0.05$) during the treatment with crude stevioside, but a similar effect was observed in the placebo group also. Oral crude stevioside is reported to be safe and supports the view taken on well-established tolerability during long-term use as a sweetener in Brazil[121].

Studies with isosteviol, a derivative of stevioside were conducted by Wong *et al* to examine whether it alters angiotensin-II-induced cell proliferation in rat aortic smooth muscle cells. Cultured rat aortic smooth muscle cells were preincubated with isosteviol, then stimulated with angiotensin II, after which [(3) H] thymidine incorporation and endothelin-1 secretion were examined. It was speculated that isosteviol inhibits angiotensin-II-induced cell proliferation and endothelin-1 secretion via attenuation of reactive oxygen species generation. The study provides important insights that may contribute to the effects of isosteviol on the cardiovascular system[122].

Effect on blood glucose

The mechanism for the blood glucose-lowering effect of stevioside was elucidated and the impact of stevioside and its aglycon steviol on insulin release from normal mouse islets and the β-cell line INS-1

were studied. Both stevioside and steviol enhanced insulin secretion from incubated mouse islets in the presence of glucose. Stevioside and steviol had a long-lasting and apparently reversible insulinotropic effect in the presence of glucose and stimulate insulin secretion via a direct action on β-cells[123].

Extract of leaves has been used to unreveal the antihyperglycaemic effect of stevioside *in vivo* in non-obese animal model of type 2 diabetes. An i.v. glucose tolerance test (IVGT) was carried out with and without stevioside in the type 2 diabetic Goto-Kakizaki (GK) rats, as well as in the normal Wistar rats. Stevioside and D-glucose were administered as i.v. bolus injections in anaesthetized rats. Stevioside significantly suppressed the glucose response in GK rats. The glucagon level was suppressed by stevioside during the IVGT. In the normal Wistar rats, stevioside enhanced insulin levels above basal during the IVGT, however, without altering the blood glucose response or the glucagon levels. Stevioside exerts anti-hyperglycaemic, insulinotropic and glucagonostatic actions in the type 2 diabetic GK rats and may have the potential of becoming a new antidiabetic drug for use in type 2 diabetes[124].

Stevioside has also shown dual positive effect by acting as an antihyperglycaemic and a blood pressure lowering substance[125].

The influence of rebaudioside A on the insulin release from mouse islets using static incubations, as well as perifusion experiments was studied. Rebaudioside A dose-dependently stimulated the insulin secretion in the presence of glucose. The effect of rebaudioside A is critically dependent on the presence of extracellular Ca^{2+}, i.e., rebaudioside A-induced insulin stimulation at high glucose disappears in the absence of extracellular Ca^{2+}. Thus, rebaudioside A also possesses insulinotropic property and may serve a potential role as treatment in type 2 diabetes mellitus[126].

The effect of stevioside (SVS) treatment on skeletal muscle glucose transport activity in both insulin-sensitive lean and insulin-resistant obese Zucker rats was studied. SVS was administered (500 mg/kg body weight) 2 hours before an oral glucose tolerance test (OGTT). Acute oral SVS increased whole-body insulin sensitivity, and low concentrations of SVS (0.01 to 0.1mmol/l) modestly improved *in vitro* insulin action on skeletal muscle glucose transport in both lean and obese Zucker rats[127].

The positive effects of stevioside in type 2 diabetic patients led to the hypothesization that supplementation of test meal with stevioside causes a reduction in postprandial blood glucose levels. Twelve type 2 diabetic patients were included in an acute, paired crossover study. A standard test meal was supplemented with either 1 g of stevioside or 1 g of maize starch. Blood samples were drawn at 30 min before and at 240 min after ingestion of the test meal. Stevioside was found to reduce postprandial blood glucose levels in type 2 diabetic patients, indicating beneficial effects on the glucose metabolism[128].

Effect of stevioside was examined on the glucose and insulin metabolism in 2 models of diabetes in rats, viz. STZ-induced diabetic rats and NIDDM induced diabetic rats (by feeding with fructose). Stevioside (0.5 mg/kg) lowered the blood glucose levels in STZ-induced rats. Stevioside administered twice-daily also demonstrated dose dependent hypoglycaemic activity in both diabetic rat models. Stevioside reduced the rise in glucose during glucose tolerance testing in normal rats. It showed dose-dependent decreased protein levels of phosphoenol pyruvate carboxykinase (PEPCK) and PEPCK mRNA after 15 days of treatment. Stevioside also reduced insulin resistance in the diabetic animals as shown by the glucose lowering effects of tolbutamide and able to regulate blood glucose levels by enhancing not only insulin secretion, but also insulin utilization in insulin-deficient rats; the later was due to decreased PEPCK gene expression in rat liver by stevioside's action of slowing down gluconeogenesis[129].

The combination of stevioside and a dietary supplement of soy protein possesses beneficial qualities in the treatment of type 2 diabetes and the metabolic syndrome. Male Zucker diabetic fatty rats were randomized into 4 groups and fed with different test diets for 10 weeks and their plasma glucose, blood pressure, weight and food intake were measured once weekly. The animals were equipped with an intra-arterial catheter, and at week 10, the conscious rats underwent an intra-arterial glucose tolerance test. Stevioside exerted beneficial effects in type 2 diabetic Zucker diabetic fatty rats; it lowered blood glucose and reduced free fatty acids. The combination of stevioside and soy supplementation appears to have the potential of an effective treatment of a number of the characteristic features of the metabolic syndrome, viz hyperglycaemia, hyper-tension and dyslipidemia[130].

Repeated oral administration of stevioside delayed the development of insulin resistance in rats on a high-fructose diet. Increased insulin sensitivity by stevioside administration was further identified using the plasma glucose-lowering action of exogenous insulin in streptozotocin-induced diabetic rats. Oral administration of stevioside at 0.2 mg/kg three times daily into STZ-diabetic rats for ten days increased the response to exogenous insulin. It was demonstrated that oral administration of stevioside improves insulin sensitivity and seems suitable as an adjuvant for diabetic patients and/or those that consume large amounts of fructose[131].

Stevioside in combination with glyburide did not reverse glyburide-induced increase in basal insulin secretion, whereas both stevioside and glucagon-like peptide-1 counteracted glyburide-induced desensitization of glucose-stimulated insulin secretion. Stevioside was found to counteract the desensitizing effects of glyburide and may be a putative new drug candidate for the treatment of type 2 diabetes mellitus[132].

The reduction of glycaemia promoted by the treatment with *S. rebaudiana* leaves was mediated, at least in part, by an inhibition of hepatic gluconeogenesis of 15-h fasted rats. However, this effect did not involve stevioside and the activation of PPAR gamma receptors[133].

Anti-viral activity

S. rebaudiana extracts are potent anti-rotavirus inhibitors *in vitro* and *in vivo*[134]. Anti-human rotavirus (HRV) activity of hot water extract from *S. rebaudiana* (SE) showed inhibition of replication of all four serotypes of HRV *in vitro*. Binding assay with radiolabeled purified viruses indicated that the inhibitory mechanism of SE is due to the blockade of virus binding. The SE inhibited the binding of anti-VP7 monoclonal antibody to HRV-infected MA104 cells. The inhibitory components of SE were found to be heterogeneous anionic polysaccharides with different ion charges. The component analysis suggested that the purified fraction named as stevian with the highest inhibitory activity consists of an anionic polysaccharide with molecular weight of 9800 containing the amino acids Ser and Ala. Analyses of sugar residues suggest uronic acid as sugar components. It did not contain amino and neutral sugars and sulfate residues. Thus SE may bind to 37 kD VP7 and interfere with the binding of VP7 to the cellular receptors by steric hindrance, which results in the blockade of the virus attachment to cells[135].

Intestinal metabolism

Stevia mixture, extracted from the leaves consisting mainly of stevioside and rebaudioside A and its α-glucose derivative was investigated for its human intestinal metabolism by LC/MS/ESI analysis. Degradation was examined by incubating Stevia mixture, enzymatically modified Stevia, stevioside, rebaudioside A, α-monoglucosyl-stevioside, α-monoglucosyl-rebaudioside A and the aglycone, steviol with pooled human faecal homogenates for 0, 8 and 24 h under anaerobic conditions. Stevioside and rebaudioside A were completely eliminated within 24 h, whereas no degradation of steviol appeared to be found during the incubation period. Stevia mixture, stevioside and rebaudioside A appeared to be hydrolyzed to steviol by human intestinal microflora. This observation is consistent with previous rat metabolism studies. Similarly, enzymatically modified stevia appeared to be metabolized via Stevia components and finally to steviol[136].

Gastroprotective activity

The effect of a hot water extract of the stem of *S. rebaudiana* on the smooth muscle of isolated guinea pig ileum was investigated. The butyl alcohol layer of the extract antagonized the contractions of the isolated guinea pig ileum induced by histamine (1×10^{-5} M) and acetylcholine (1×10^{-5} M) in a concentration-dependent manner. The butyl alcohol layer of the extract also showed inhibition of $CaCl_2$ (1×10^{-3}-3.8×10^{-1} M)-induced contractions. The antagonism of the extract was considered to be non-specific, but this action might be related to an influx of extracellular Ca^{2+}. With column chromatography preparation, the active component was found to be stevioside. The antagonistic effect exerted by the stem extract of *S. rebaudiana* contributed to the gastroprotective activity of the extract in animals fed dietary histamine[137].

Kinetic study

It has been demonstrated that the stevioside is converted to steviol by bacteria in colon of pig. However, no stevioside or steviol could be detected in the blood of the animals, not even after converting steviol into the (7-methoxycoumarin-4-yl) methyl ester of steviol, a very sensitive fluorescent derivative with a detection limit of about 50 pg. The intestinal transport characteristics of stevioside, rebaudioside A and steviol were also studied in the Caco-2 system.

Only a minor fraction of stevioside and rebaudioside A was transported through the Caco-2 cell layer giving a Papp value of 0.16×10^{-6} and 0.11×10^{-6} cm/s, respectively. The Papp value for the absorptive transport of steviol was about 38.6×10^{-6} cm/s while the Papp value for the secretory transport of steviol was only about 5.32×10^{-6} cm/s suggesting carrier-mediated transport. The relatively high absorptive transport of steviol and the lack of steviol in the blood may be explained by the fact that in the Caco-2 study, steviol is applied as a solution facilitating the uptake, whereas in the colon, steviol probably is adsorbed to the compounds present in the colon and of which the content is getting concentrated by withdrawal of water[138].

Effect on K^+ ion channel

Role of potassium channels has been elucidated in the action of isosteviol on intracellular calcium concentrations in cultured vascular smooth muscle cells using the calcium ion sensitive dye Fura-2 as an indicator. The increase of calcium ion in cells produced by vasopressin or phenylephrine was attenuated by isosteviol. This attenuation was inhibited by glibenclamide, apamin and 4-aminopyidine but not by charybdotoxin. The inhibitory action of isosteviol on calcium ions was blocked when cells co-treated with glibenclamide and apamim in conjunction with 4-aminopyridine were present. Therefore, not only did the ATP-sensitive potassium channel affect the action of isosteviol in cells, but also on the small conductance calcium-activated potassium channels and voltage-gated channels[139]. Isosteviol also tended to relax isolated aortic strips of Wistar rat[140].

Anticancer activity

Isosteviol (one of the hydrolysis product of stevioside) and related compounds were reported to be produced from stevioside by microbial transformation and chemical conversion; they were assayed for their inhibitory activity towards DNA metabolic enzymes and human cancer cell growth. Among twelve compounds obtained, only isosteviol potently inhibited both mammalian DNA polymerases and human DNA topoisomerase II. This compound had no inhibitory effect on higher plant (cauliflower) pols, prokaryotic pols, human topo I, and DNA metabolic enzymes such as human telomerase, T7 RNA polymerase and bovine deoxyribonuclease-I. Isosteviol acted non-competitively with the DNA template-primer and nucleotide substrate. It prevented the growth of human cancer cells. The compound also caused a marked reduction in TPA (12-O-tetradecanoylphorbol-13-acetate)-induced inflammation. The relationships between the structure of stevioside-based compounds and these activities have been discussed[141].

Hemodynamic effects

Stevioside and rebaudioside were evaluated for their hemodynamic effects in a randomized double blind trial following consumption of 1000 mg/day of rebaudioside A versus placebo in 100 indiviuals with normal and low normal systolic blood pressure (SBP) and diastolic blood pressure. Subjects chosen were predominantly female in the mean age of 41 year (range is 18-73 years). At the base line, mean resting seated SBP and DBP was found to be 110/70.3 mm and 110.72/71.2 mm of Hg for the rebaudioside A and placebo group, respectively. Compared with placebo, rebaudioside A did not significantly alter resting seated SBP, DBP, MAP (mean arterial pressure), heart rate or 24 hours ambulatory blood pressure responses. These results indicated that consumption of as much as 1000 mg/day of rebaudioside A did not produce any important clinical changes in healthy adults with normal and low normal blood pressure[142].

Toxicology

Stevioside does not promote bladder carcinogenesis[141]. Short-term studies of 13 weeks at a concentration of 5% in diet were found suitable to be maximum tolerable dose of stevioside for a 2-year study in rats[143]. Stevioside at dose up to 2500 mg/kg body weight per day was found to affect neither growth nor reproduction in hamsters[110]. Acute toxicity studies of steviosides given as single doses to rodents showed no lethality within 14 days after administration and no clinical signs of toxicity or morphological or histopathological changes were found[144].

The genotoxic potential of stevioside in eukaryotic cells was studied using Wistar rats treated with stevioside solution (4 mg/ml) through oral administration (*ad libitum*) and the DNA-induced damage was evaluated using the single cell gel electrophoresis (comet assay). The results showed that treatment with stevioside generates lesions in peripheral blood, liver, brain and spleen cells in different levels; the largest effect being in liver. The undesired effects need to be better understood, once the data presented point to possible stevioside mutagenic properties[145].

Clinical trials

Stevia extract induced significant changes in glucose, insulin and electrolytes in a double-blind study of 60 healthy volunteers. Patients were tested in both catabolic and anabolic phases. Significant reductions in blood glucose were found in both the anabolic and catabolic phases with the 200 mg dose but not with the 50 mg dose[146].

The study was undertaken to investigate the long-term (2-year) efficacy and tolerability of stevioside in patients with mild essential hypertension and to determine the effects of stevioside on left ventricular mass index (LVMI) and quality of life (QOL). This was a multicenter, randomized, double blind and placebo-controlled trial on Chinese men and women aged between 20 and 75 years with mild essential hypertension [systolic blood pressure (SBP) 140-159 mm Hg and diastolic blood pressure (DBP) 90-99 mm Hg]. Patients were given capsules containing 500 mg stevioside powder or placebo 3 times daily for 2 years. Blood pressure was measured at monthly clinic visits; patients were also encouraged to monitor blood pressure at home using an automated device. LVMI was determined by 2-dimensional echocardiography at baseline and after 1 and 2 years of treatment. QOL was assessed using the Medical Outcomes Study 36-Item Short-Form Health Survey. Electrocardiographic, laboratory and QOL parameters were assessed at the beginning of treatment, and at 6 months, 1 year and 2 years. One hundred seventy-four patients (87 men, 87 women) were enrolled in the study and 168 completed it; 82 (42 men, 40 women; mean age, 52 years) in the stevioside group and 86 (44 women, 42 men; mean age, 53 years) in the placebo group. After 2 years, the stevioside group had significantly decreased mean SBP and DBP compared with baseline [SBP, from 150 (7.3) to 140 (6.8) mm Hg; DBP, from 95 (4.2) to 89 (3.2) mm Hg]. In this 2-year study on Chinese patients with mild hypertension, oral stevioside significantly decreased SBP and DBP compared with placebo. QOL was improved, and no significant adverse effects were noted[147].

Usage and Precautions

Stevia uses in folk medicine include against hypertension, diabetes and as a contraceptive. It is a new promising renewable raw material for the food market. Acute and subacute toxicity studies revealed a very low toxicity of Stevia and stevioside[148]. It is suited for both diabetics and PKU (Phenylketonuria) patients, as well as for obese persons intending to lose weight by avoiding sugar supplements in the diet. No allergic reactions to it seem to exist[149].

Adverse cardiovascular and kidney/genitor-urinary effects have been documented with Stevia. A slight decrease in mean arterial pressure (approximately 9.5%) and bradycardia was reported in healthy subjects (age 24 to 40 years) after ingestion of a tea made from stevia leaves for 30 days. Intravenous administration of stevioside to rats has resulted in natriuresis. This effect is partially dependent on prostaglandins[115]; uresis has also been reported in animal studies[43]. Stevia extracts were found to decrease the fertility of male rats while steviosides induced diuresis and natriuresis and a fall in renal tubular reabsorption of glucose[150].

Conclusion

From the present review, it can be concluded that very exhaustive work has been done on the plant but there is still need for research work on the pharmacological aspects of rebaudioside, adverse effects and ADME (Absorption, distibution, metabolism and excretion) studies of stevioside on human beings. Attempt should also be made for the isolation of alkaloids from Stevia plant as well as to improve the after taste of stevioside. Because the safety of Stevia for human consumption remains controversial, there is a clear need for further experimentation with respect to the metabolic fate of steviol glycosides and to clarify its risk towards genotoxicity.

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