Review Article

Stevioside and Related Compounds – Molecules of Pharmaceutical Promise: A Critical Overview

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Stevioside, an *ent*-kaurene type of diterpenoid glycoside, is a natural sweetener extracted from leaves of *Stevia rebaudiana* (Bertoni) Bertoni. Stevioside and a few related compounds are regarded as the most common active principles of the plant. Such phytochemicals have not only been established as non-caloric sweeteners, but reported to exhibit some other pharmacological activities also. In this article, natural distribution of stevioside and related compounds, their structural features, plausible biosynthetic pathways along with an insight into the structure–sweetness relationship are presented. Besides, the pharmacokinetics, wide-range of pharmacological potentials, safety evaluation and clinical trials of these *ent*-kaurene glycosides are revisited.

Keywords: biosyntheses / clinical trials / *ent*-Kaurene glycosides / natural distributions / non-caloric sweeteners / pharmacological activities / safety evaluation / stevioside / structure–sweetness relationship

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Introduction

Stevioside is an *ent*-kaurene type diterpenoid glycoside isolated from leaves of *Stevia rebaudiana* (Bertoni) Bertoni, a perennial herb of the asteraceae (compositae) family [1]. Stevioside and related compounds are responsible for the sweet taste of *Stevia* leaves [2]. Due to the high concentration of such sweet principles in leaves of *Stevia* plants, these are known as honey leaf of sweet chrysanthemum or ''sweet herb of Paraguay''. Extracts are being used commercially in many countries for sweetening a variety of products including pickled vegetables, sea foods, soft drinks, soy sauce, and confectionary products. Stevioside is an intense sweetener and the extract of its source (*S. rebaudiana*) finds extensive use in countries like Japan, China, Russia, Korea, Paraguay, Argentina, Indonesia, Malaysia, Australia, New Zealand, South America, and others, to sweeten local teas, medicines, food, and beverages [3]. In addition, *Stevia* leaves are also in use for their medicinal benefits in hypertension, obesity, topical dressing for wounds, and other skin disorders [3, 4]. Although steviol glycosides have not been approved as food ingredient in the United States or the European Union, the leaves of *Stevia* or their extracts are permitted to be sold in the US as dietary supplement, as defined in section [201(ff)(1)] of the Federal Food, Drug, and Cosmetic Act (JECFA) [5]. In 2007, JECFA specified that steviol glycoside sweeteners must be composed of at least 95% of the known steviol glycosides [6].

Extensive reviews on *Stevia* genus and its major constituent stevioside as a low-calorie sweetener and its toxicological aspects have already been published [1, 3, 7]. Hence, the

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Abbreviations: ADI, Acceptable daily intake; **ADME**, Absorption, distribution, metabolism, and excretion; **CHL**, Chinese hamster lung fibroblast cell line; **CPP**, *ent*-Copalyl pyrophosphate; **DMBA**, 7,12-dimethylbenz-[a]-anthracene; **DXP**, 1-Deoxy-D-xylulose 5-phosphate; **DXR**, 1-Deoxy-D-xylulose 5-phosphate reductoisomerase; **DXS**, 1-Deoxy-D-xylulose 5-phosphate synthase; **ESTs**, Expressed sequence tags; **GA**, Gibberellin; **GAP**, Glyceraldehyde 3-phosphate; **GGPP**, Geranylgeranyl pyrophosphate; **HMG-coA**, 3-Hydroxy-3-methylglutaryl co-enzyme A; **ID_{50}**, 50% Inhibitory dose; **IPP**, Isopentenyldiphosphate; **IPP**, Isoprenyl diphosphate; **IVGT**, Intravenous glucose tolerance test; **JECFA**, Joint FAO/WHO Expert Committee on Food Additives; **LD_{50}**, 50% Lethal dose; **MEP**, 2-*C*-Methyl-D-erythritol-4-phosphate; **MEP**, 2-*C*-methyl-D-erythritol-4-phosphate; **MVA**, Mevanolic acid; **NIDDM**, Non-insulin dependent diabetes mellitus; **PAH**, *p*-Aminohippurate; **PEPCK**, Protein levels of phosphoenyl pyruvate carboxy kinase; **PKU**, Phenylketonuria; **SCF**, Scientific Committee on Food; **TPA**, 12-*O*-Tetradecanoyl-phorbol-13-acetate; **UDS**, Unscheduled DNA synthesis; **UGC**, University Grants Commission; **US**, United States.

focusing area of this short review is to retrospect the structural features of *ent*-kaurene glycosides of *Stevia*, structure-sweetness relationship and multidirectional pharmacological potentials of stevioside and related compounds having similar type of skeleton. Despite being a low calorie sweetener and dietary supplement for food [1, 8, 9], stevioside is used for treating hypertension and hyperglycemia [10, 11]. Stevioside and related compounds are also reported to possess anti-tumor activity [12]. Versatile bioactive properties of stevioside provoked scientists to undertake synthesis of several stevioside analogues *i.e.* chemically modified structures (*viz.* sulphopropyl and sodio-sulphopropyl esters) to improve its bioactive properties such as organoleptic activity [13, 14]. Besides a short account of plausible biosynthetic pathways for *ent*-kaurene glycosides, their safety evaluation as well as clinical trials are also discussed in the present resume.

Sources of stevioside and related compounds: Botanical aspects and distribution

The main source of stevioside and many other related glycosides is *Stevia rebaudiana* (Bertoni) Bertoni, a perennial herb belonging to asteraceae (compositae) family (tribe: eupatoriae). It is a small bush native to the valley of the Rio Monday in high lands of Paraguay, between 25 and 26 degrees south latitude. The plant is a small shrubby perennial growing up to 65 cm high in sandy soil near streams, with sessile, oppositely arranged lance (lanceolate to oblanceolate) leaves, serrated above the middle; the flowers are small and white and the seed is an achene with a feathery pappus [15, 16].

The number of species estimated within the genus range from 150 to 300 and the distribution range of this taxon extends from the south-west United States to northern Argentina, through Mexico, the Andes and the Brazilian high lands [17]; *Stevia rebaudiana* species of North and Central American origin are classified according to Grashoff's scheme in three subdivisions – podocephalae, corymbosae and fruticosae, whereas species of South America are classified in breviaristatae and multiaristatae [8, 17, 35]. *S. rebaudiana* and the other 14 *Stevia* species belong to Paraguayan origin also [17]. Stevioside content in leaf was found to vary from 3.17 to 9.94% and that in stem from 1.54 to 3.85%. In terms of weight fraction, the four major steviol glycosides found in *Stevia* plant tissues are 5–10% stevioside, 2–4% rebaudioside A, 1–2% rebaudioside C and 0.5–1% dulcoside A [8, 17, 18, 35].

Rebaudioside B, D, and E may also be present in minute quantities. It is very much interesting to note that only *S. rebaudiana* is the richest source of stevioside and related compounds and none of the other species belonging to this genus has ever been found to produce these compounds at high concentration levels [19]. The yield of stevioside is the highest (2–10%), rebaudioside A (**4**) follows the next (about 1%) and other constituents are the minor components [4, 20, 21]. The two major constituents, stevioside and rebaudioside A, first isolated by two French chemists, Bridel and Lavielle [22], are supposed to be responsible for sweet taste of *Stevia* leaves.

Stevioside and related compounds

ent-Kaurenes are tetracyclic diterpenoids having a perhydrophenanthrene moiety (rings A, B and C) fused with a cyclopentane unit (ring D) formed by a bridge of two carbons between C-8 and C-13; the nomenclature, numbering style and stereochemistry of *ent*-kaurene (Fig. 1a) and *ent*-kaurenoic acid (Fig. 1b) skeleton have already been recommended by the IUPAC [23]. *ent*-Kaurene type of glycosides present in *Stevia* plants are called steviol glycosides – steviol (*ent*-13-hydroxy kaur-16-en-19-oic acid; **1**, Fig. 2), the aglycone part of such glycosides, is involved in constructing a C_{19}-ester linkage between the C_{19}-carboxylic function and a glucose unit, and also in the formation of ether linkages using its C_{13}-hydroxy group with combinations of glucose and rhamnose moieties (see Fig. 2). Stevioside (**2**) is a complex of three glucose molecules and one molecule of steviol aglycone (**1**), whereas rebaudioside A (**3**) bears a total of four glucose units, with the middle glucose of the triplet connected to the central steviol structure [24, 25].

A total of almost twenty *ent*-kaurene diterpene glycosides, isolated from different species of genus *Stevia*, are reported so far (Fig. 2); natural distributions and pharmacological activities of stevioside and related compounds isolated from different species of genus *Stevia* are listed in Table 1.

Biosynthesis

Biosynthesis of steviol glycosides is a subject of much discussion [26–35]. Kim *et al.* [30] observed a high activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in chloroplasts of *Stevia rebaudiana*; hence, the investigators anticipated mevanolic acid (MVA) as an intermediate of steviol biosynthetic route on the basis of the fact that HMG-CoA reductase is a key enzyme of the MVA route to

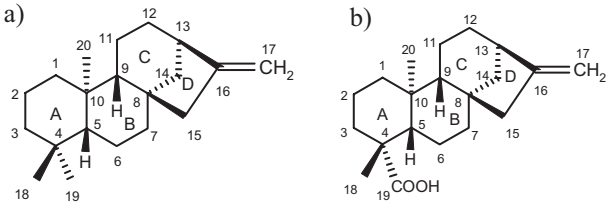


Figure 1. *ent*-Kaurene (a) and *ent*-kaurenoic acid (b).

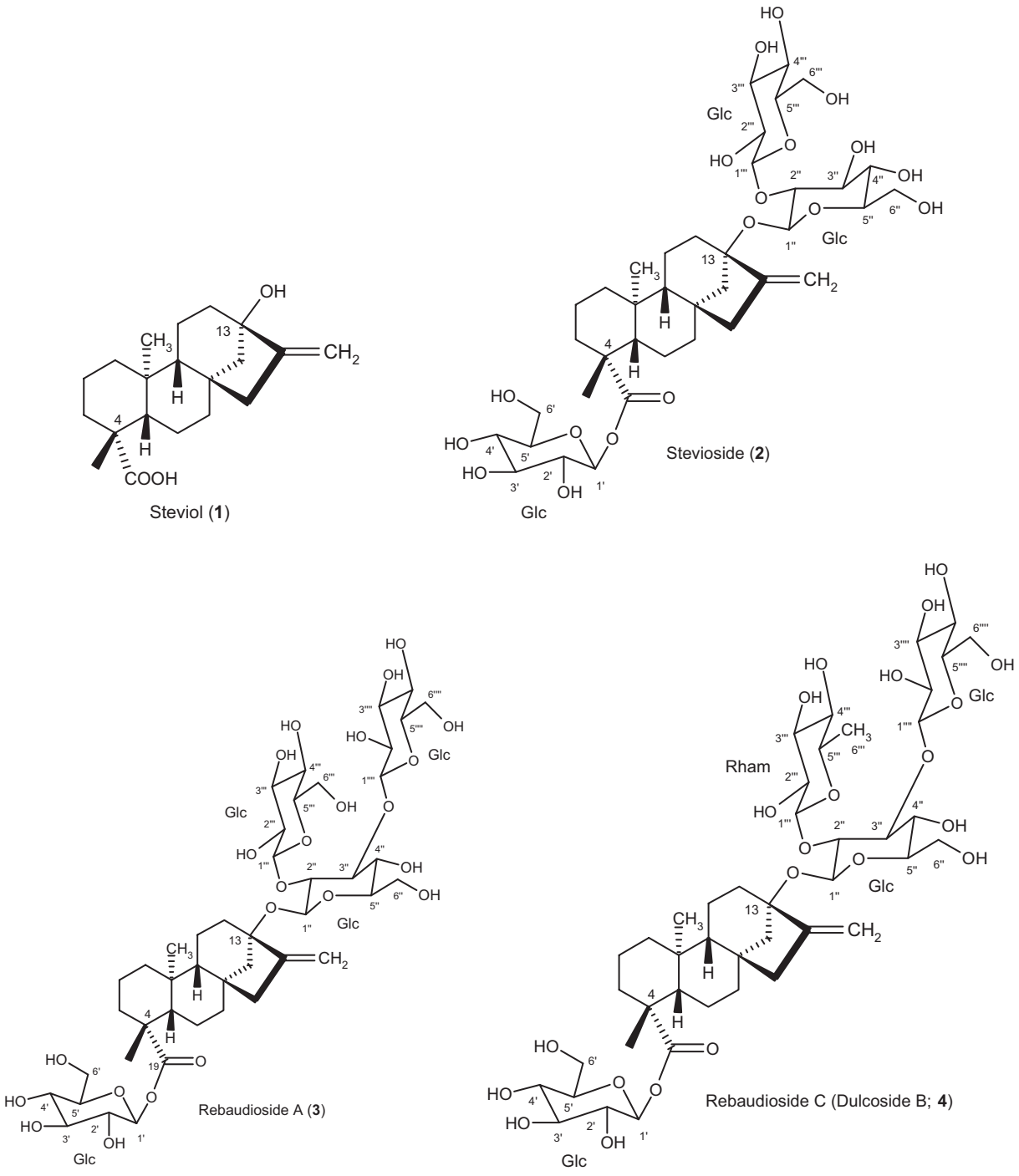


Figure 2. Stevioside and *ent*-kaurene-type diterpenoids isolated from the genus *Stevia*.

isopentenyldiphosphate (IPP), but they could not offer any direct proof to their assumption. Recently, Totté *et al.* [34, 35] demonstrated the involvement of 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway as the biosynthetic route for the *ent*-kaurene skeleton of stevioside and hence also of gibberellins (GAs) – this experimental evidence, thus, discards the hypothesis (*i.e.* involvement of mevalonic acid in the biosynthesis of steviol) of Kim *et al*. [30]. Brandle *et al.* [28] supported the above contention depending upon their gene-discovery experiment in diterpene synthesis involving 5548

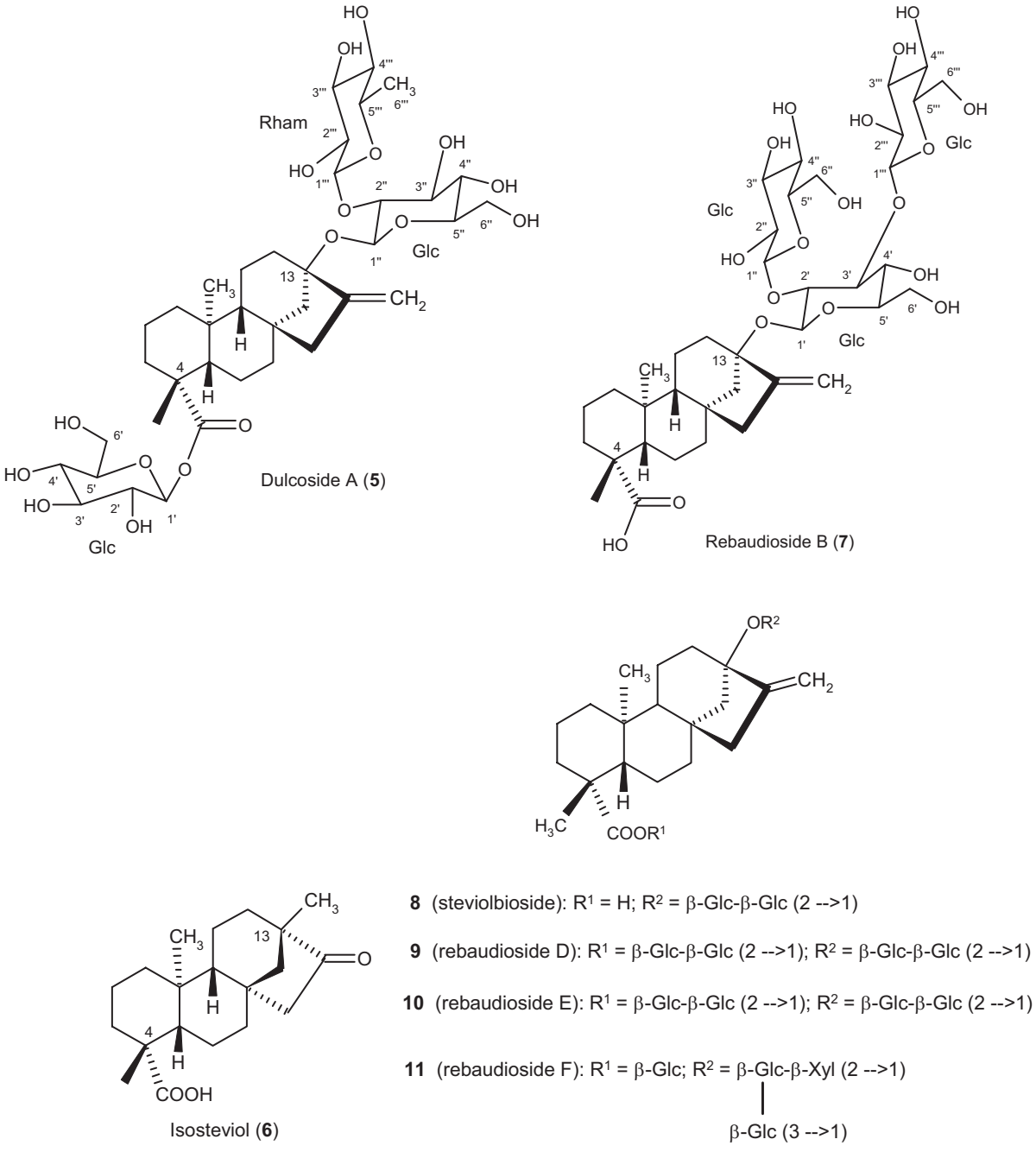
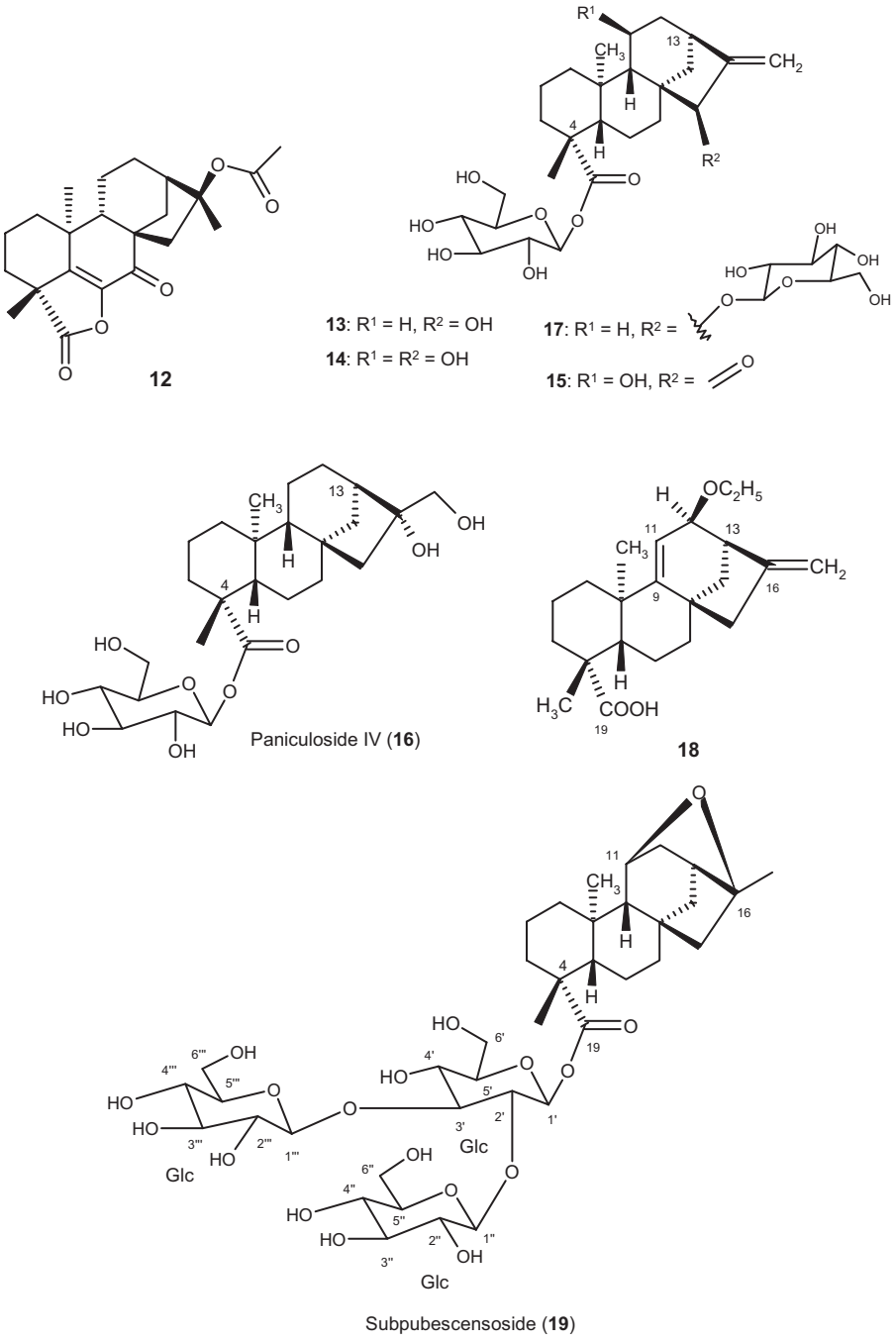


Figure 2. *Continued*.

expressed sequence tags (ESTs) from a *Stevia* leaf cDNA library – many candidates genes specific to the MEP pathway and no members of the MVA pathway were identified, thereby, indicating the involvement of MEP pathway as the prime route to yield isopentenyldiphosphate (IPP) for diterpene biosynthesis [36–40]. IPP is subsequently converted to geranylgeranyl pyrophosphate (GGPP) followed by its conversion to *ent*-copalyl pyrophosphate (CPP) by CPP synthase (also called *ent*-kaurene synthase A), and thereafter *ent*-kaurene is produced from CPP by *ent*-kaurene synthase (also called *ent*-kaurene synthase B) [36, 37]. Subsequent oxidation of this product at the C-19 position to *ent*-kaurenoic acid (Fig. 1b) is assumed to occur *via* the action of one or more P450 monooxygenases; at this point the pathways to the

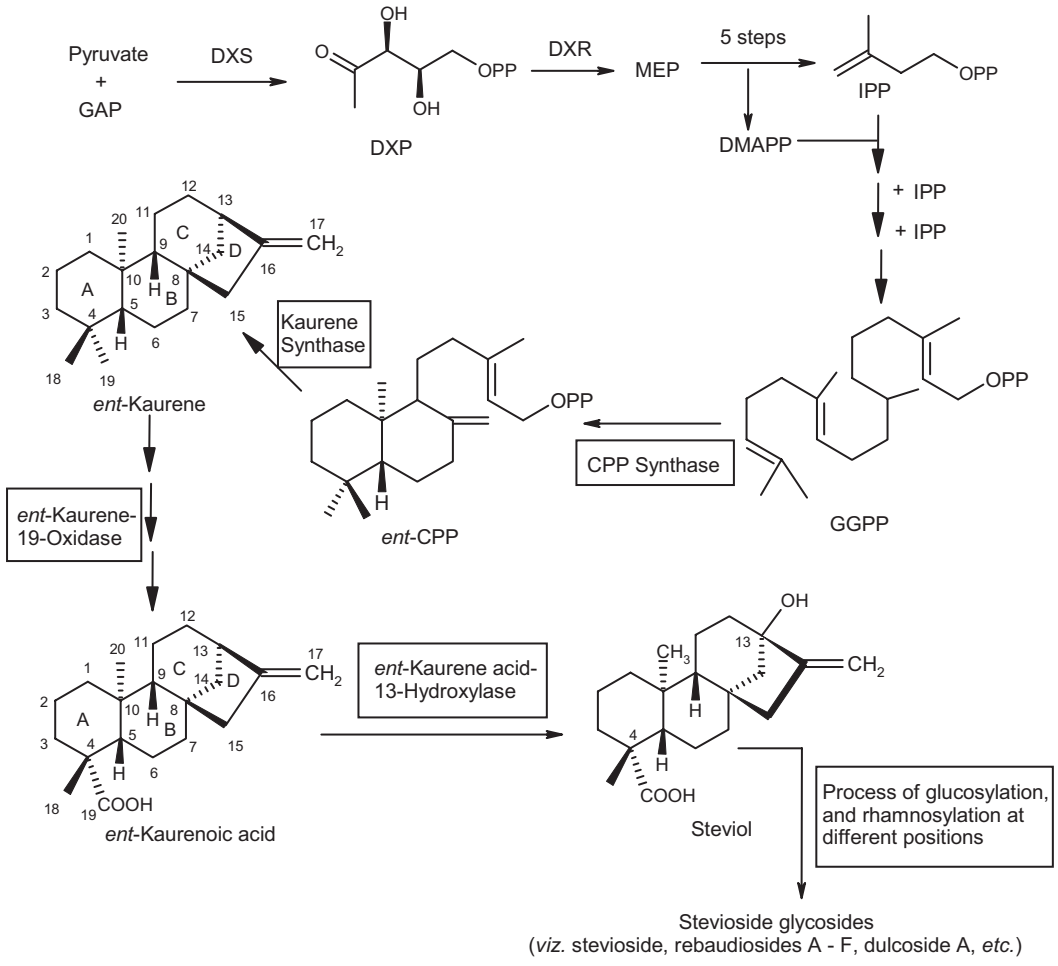


(Glc: β-D-glucopyranosyl: Rham: α-L-rhamnopyranosyl; Xyl: β-D-xylopyranosyl)

Figure 2. *Continued.*

steviol glycosides and the gibberellins diverge [36, 41]. Steviol (**1**) is produced by further hydroxylation of *ent* kaurenoic acid at the C-13 position by the action of *ent*-kaurenoic acid 13-hydroxylase [42–45]; steviol glycosides are then produced through glycosylations at different positions [36, 45] (see Scheme 1).

Alves and Ruddat [26] estimated that gibberellins such as GA_{20} are present in *Stevia* leaves at concentrations of



(CPP: *ent*-copalyl pyrophosphate; DXP: 1-deoxy-D-xylulose 5-phosphate; DXR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS: 1-deoxy-D-xylulose 5-phosphate synthase; GAP: glyceraldehydes 3-phosphate; GGPP: geranylgeranyl pyrophosphate; IPP: isoprenyl diphosphate; MEP: 2-*C*-methyl-D-erythritol-4-phosphate)

Figure 3. Schematic representation of possible MEP biosynthesis pathway leading to steviol, stevioside, and related *ent*-kaurene glycosides [34–36, 41, 42, 45].

1.2 μg/kg fresh weight – the amount is over 10 000 times lower than steviol glycosides. This vast difference in concentration between such structurally related compounds has spurred curiosity among the scientists, leading to various investigations [26, 27, 29, 32, 33, 46]. It was found that the expression levels are highest in mature tissues in comparison to young growing tissues, thereby, suggesting the possibility of temporal and spatial separation that prevents an overlap of steviol and GA biosynthesis. On the basis of their experimental findings, Richman *et al.* [32] remarked that considerable changes in the regulation of copalylphosphate synthase and kaurene synthase expression in *Stevia* leaves enables the synthesis and accumulation of such sweeteners in high concentrations.

Stevioside and related compounds: zero-calorie sweeteners

With the increased incidence of diabetes and obesity and also due to growing concern over the safety of some chemical sweeteners such as alitame, aspartame, cyclamate, saccharin, sucralose, *etc.*, the need for natural non-calorie sweeteners

Table 1. *ent*-Kaurene diterpenoids from *Stevia* plants.

Compound	Source	Parts	Bioactivity	Reference
Steviol (**1**)	*S. rebaudiana*		antihyperglycemic; mutagenic; anticancerous	[71, 79, 85, 100]
Stevioside (Steviol-13-*O*-β-sophoroside-19-*O*-β-D-glycopyranosyl ester) (**2**)	*S. rebaudiana*; *S. phelbophylla*	leaves	organoleptic; anti-inflammatory; antihypertensive	[11, 111–113]
Rebaudioside A (**3**)	*S. rebaudiana*	leaves	organoleptic; anti-inflammatory	[111, 113]
Rebaudioside C (dulcoside B) (**4**)	*S. rebaudiana*	leaves	organoleptic; anti-inflammatory	[111, 113]
Dulcoside A (**5**)	*S. rebaudiana*	leaves	organoleptic; anti-inflammatory	[111, 113]
Rebaudioside B (**7**)	*S. rebaudiana*	leaves	–	[114]
Steviolbioside (**8**)	*S. rebaudiana*	leaves	–	[84]
Rebaudioside D (**9**)	*S. rebaudiana*	leaves	–	[114]
Rebaudioside E (**10**)	*S. rebaudiana*	leaves	–	[114]
Rebaudioside F (**11**)	*S. rebaudiana*	leaves	–	[114]
ent-Kaurenoic acid (Fig. 1b)	*S. lucida*	aerial parts, roots		[115]
Stevionolide (**12**)	*S. lucida*	aerial parts	–	[115]
Paniculoside (I-V) (**13–17**)	*S. ovate*	leaves	–	[68]
12β-Ethoxy-*ent*-kaur-9(11), 16-dien-19-oic acid (**18**)	*S. eupatoria*	aerial parts	–	[116]
11β,16-Oxo-*ent*-kauran-19-oic acid 19-*O*-[β-D-glucopyranosyl (1 → 2)]-[β-D-glucopyranosyl (1 → 3)]β-D-glucopyranosyl (subpubescensoside; **19**)	*S. subpubescens*	aerial parts, roots	–	[117]

with acceptable taste and relatively safe is demanding. With increasing health consciousness, uses of low-calorie/non-calorie sweeteners as food additives are now encouraged so as to slow down the world-wide sugar consumption. Chemical sweeteners in place of sugar are being used now-a-days in various food preparations including cordials, juices, jams and sweets, and hence, *Stevia* products may find potential market [3, 19, 47]. Stevioside (**2**) and related compounds are responsible for the sweet taste of the leaves of *Stevia* plants and these compounds range in sweetness from 40 to 300 times sweeter than sucrose [48]. Moreover, the sweetness is superior in quality to that of sugar in terms of mildness and refreshment [49]. These glycosidic constituents of *Stevia* tend to produce a sweet taste less instantly than sucrose, but the taste lasts for a longer period [49]. Rebaudioside A (**3**) is reported not only to exhibit sweetness more pronouncedly than the other steviol glycosides, but also to show a palatable taste profile, having less of the metallic/liquorice taste, often associated with steviol glycosides [50].

Oral stevioside (**2**) is not taken up by the human body (or the uptake is extremely low) and none of the digestive enzymes from the gastro-intestinal tract of different animals and human body are able to degrade stevioside into steviol. Nonetheless, bacterial flora of the caecum or colon (caecum of mice, rats, hamsters and chickens and colon of pigs and man) were found to degrade stevioside into steviol [2, 51–53]. In one experiment, the bacteria from the human colon were also found to form steviol epoxide *in vitro*, which was again metabolized to steviol [2]. Renwick *et al.* [54] reviewed the literature on the metabolism of stevioside and rebaudioside A by intestinal microbiota; steviol was reported to be the only metabolite in feces which is not further metabolized, thereby, playing the role of low calorie sweetener.

Several studies revealed that the ratio of the number of glucose units at the 13-hydroxyl group to that at 19-carboxyl group seems to have a significant relationship with the sweetness as well as the quality of taste for glucosides of this type [21, 31, 55–58]. Detailed studies with stevioside revealed that elongation of 13-*O*-glucosyl moiety up to a total number of four glucosyl units, accompanied by reduction of glucosylation at 19-*O*-glucosyl moiety, is associated with the enhancement of intensity of sweetness of the compound [59].

Uptake and metabolism of *ent*-kaurene glycosides

Studies on the absorption and metabolism of *ent*-kaurene glycosides in rats showed that stevioside is not readily absorbed from the upper small intestine owing to its high molecular weight. However, stevioside is degraded by bacteria of the colon, resulting in free steviol, part of which is absorbed by the colon and transported to the liver and part is excreted in feces. Steviol is then converted into its glucuronide derivative in the liver and excreted from the body through urine [60]. It was also reported that stevioside itself had a clearance rate less than that of *p*-amino hippuric acid

but greater than that of insulin, which suggests that steviol glycosides are actively secreted by renal tubular epithelium [61]. Absorption, distribution, metabolism, and excretion (ADME) of *ent*-kaurene glycosides have been extensively reviewed earlier [1, 61–63].

Pharmacological aspects

Other than being zero-calorie sweeteners and food additives, stevioside and related compounds have also been found to exhibit diverse kind of pharmacological activities; some of these significant efficacies are presented herein.

Antitumor and anticancer activity

Toyoda *et al*. [64] established that oral administration of 96.6% pure stevioside at a dose of 2.5% and 5% prevents carcinogenicity in Fischer 344 rats. The anti-carcinogenicity of stevioside was supported by various studies [65–67]. The stevioside was found to be similar in activity to many triterpenoids like heliantriol C, pachymic acid, 3-*O*-acetyl-16α-hydroxytrametenolic acid and poricoic acid B on tumor promotion by 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) [68, 69].

Yasukawa *et al.* [12] also reported the inhibitory effect of stevioside on tumor promotion by TPA in two-stage carcinogenesis in mouse skin; four steviol glycosides such as stevioside (**2**), rebaudiosides A (**3**) and C (**4**), and dulcoside A (**5**) isolated from *Stevia rebaudiana* were found to exhibit strong inhibitory activity against TPA-induced inflammation in mice. The ID_{50} (50% inhibitory dose) values of these compounds for TPA-induced inflammation were determined as 54.1, 92.2, 92.5, and 291.6 μg/ear, respectively for the test compounds **4**, **3**, **5**, and **2** [12]. The same investigators [12] reported also that application of a sample (1.0, 0.2, 0.04, or 0.008 mg/ear) inhibited the TPA-induced inflammation in a dose-dependent manner. The inhibitory effects of these compounds were compared with antitumor–promoting agent quercetin and anti-inflammatory drugs indomethacin and hydrocortisone; rebaudiosides A and C and dulcoside A were found to be similar in activity to hydrocortisone, and stevioside was found to be more effective than indomethacin. Rebaudioside C (**4**) was reported to exhibit 50% inhibition of the swelling at doses 10 and 100 times lower than those of indomethacin and quercetin [12]. Furthermore, a mixture of the steviol glycosides at a dose of 1.0 and 0.1 mg/mouse (administered 30 min before each TPA treatment) was found to inhibit inflammation as well as promoting effects of TPA (from 1 week after initiation with the single topical application of DMBA, it was applied at a dose of 1.0 μg/mouse twice weekly) on skin tumor formation initiated with 7,12-dimethylbenz [a] anthracene (DMBA; a single topical application at a dose of 50 μg/mouse) [12]. Yasukawa and his coworkers also studied the time course of skin tumor formation as well as average number of tumors/mouse treated with DMBA plus TPA, with or without stevioside mixture – the group treated with DMBA plus TPA produced 8.1 tumors/mouse at 20 weeks, whereas the groups treated with DMBA plus TPA and a stevioside mixture (at doses of 0.1 mg and 1.0 mg) had 2.2 and 0.3 tumors/mouse, respectively [12]. Hence, the treatment with stevioside mixture of 0.1 mg and 1.0 mg caused 73% and 96% reductions, respectively, in the average number of tumors/mouse at the stipulated time [12].

Chaiwat *et al*. [70] examined the effects of stevioside and its metabolite, steviol, on human colon carcinoma cell lines. High concentrations of stevioside (2–5 mM) and steviol (0.2–0.8 mM) were observed to decrease cell viability in T84, Caco-2, and HT29 cells (assayed by MTT method). Stevioside (**2**; at a dose of 2 mM) potentiated TNF-α mediated IL-8 release in T84 cells. However, steviol (0.01–0.2 mM) was found to suppress TNF-α-induced IL-8 release significantly in all the three cell lines. In T84 cells, steviol attenuated TNF-α stimulated IκB → NF-κB signaling. Chloride transport was stimulated by steviol (0.1 mM) more potently than stevioside (1 mM) observed at 30 min on treatment with the drugs. Thus, steviol was evaluated for its significant efficacies in stimulating Cl^- secretion and attenuating TNF-α-stimulated IL-8 production in colon; the immunomodulatory effects of steviol appear to involve NF-κB signaling, while at nontoxic concentrations stevioside affects only Cl^- secretion [70].

The anticancer efficacy of stevioside (**2**) and six related compounds including the aglycones steviol (**1**) and isosteviol were also evaluated by Takasaki *et al*. [71] in an *in-vitro* assay for inhibitory effects on Epstein-Barr virus early antigen activation; the compounds **1**, **2**, and isosteviol (**6**) were found to show significant activity in this assay and also exhibited strong inhibitory effects in a two-stage carcinogenesis test using mouse skin induced by 7,12-dimethylbenz[a]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The inhibitory effects of these three compounds were found to be greater than that of glycyrrhizin. Furthermore, these three compounds significantly inhibited mouse skin carcinogenesis initiated by peroxynitrite and promoted by TPA. Their activities were found to be comparable to that of curcumin [71].

Antihypertensive activity

Chan *et al*. [72] reported that intravenous (*i.v.*) administration of 95% pure stevioside (at a dose of 50, 100 or 200 mg/kg b.w.) demonstrated a significant hypotensive effect in spontaneously hypertensive rats without any adverse effect on the heart rate or serum catecholamine levels. In another study by the same group with humans, stevioside was administered at a dose of 250 mg thrice a day for 1 year to 60

hypertensive volunteers [73]; the study revealed that after 3 months the systolic and diastolic blood pressure decreased significantly and the effect was also found to be persisting. Besides, no significant adverse effects in any blood-biochemistry parameters were observed as after effect [73] – hence, the investigators concluded that stevioside is a well tolerated and an effective compound that may be considered as an alternative or supplementary therapy for patients with hypertension. From their study with anesthetized dogs to highlight the underlying mechanism of the hypotensive effect of stevioside, Liu *et al*. [74] reported that the antihypertensive effect of stevioside (at a dose of 200 mg/kg b.w.) was due to inhibition of Ca^{2+} influx from extra-cellular fluid.

Lee *et al*. [11] also studied the inhibitory effect of stevioside on Ca^{2+} influx to produce antihypertention. The study revealed that intraperitoneal injection of stevioside (25 mg/kg) caused antihypertensive effect, while it showed no effect on phenylephrine and KCl-induced phasic vasoconstriction. In addition, stevioside was also found to lose its influence on vasopressin-induced vasoconstriction in Ca^{2+} free medium. Thus, the results indicated that stevioside caused vasorelaxation *via* an inhibition of Ca^{2+} reflux into the blood vessel. In another study with rats, Melis and Sainati established that the cardiovascular action of stevioside is mediated *via* a prostaglandin-dependent mechanism – since the drug was found to reduce heart rate as well as mean arterial blood pressure in the animals, the effects that are blocked by indomethacin [75].

The role of potassium channels in the vasodilator effect of isosteviol (**6**) was investigated by Wong *et al*. [76] using potassium channel blockers on isosteviol-induced relaxation of isolated aortic rings prepared from Wistar rats. Isosteviol (**6**) was found to relax dose-dependently the vasopressin (10^{-8} M)-induced vasoconstriction in isolated aortic rings with or without endothelium. However, in the presence of potassium chloride (3×10^{-2} M), the vasodilator effect of isosteviol on arterial strips disappeared. Only the inhibitors specific for the ATP-sensitive potassium (K_{ATP}) channel or small conductance calcium-activated potassium (SK_{Ca}) channel inhibited the vasodilator effect of isosteviol in isolated aortic rings contracted with 10^{-8} M vasopressin. From their detailed studies, the present investigators [76] demonstrated that vasodilatation induced by isosteviol (**6**) is related to the opening of SK_{Ca} and K_{ATP} channels.

Antihyperglycaemic activity

Steviol glycosides do not induce a glycemic response when ingested, making them attractive as natural zero-calorie or low calorie sweeteners to diabetics and others on carbohydrate-controlled diets. Due to the presence of high concentration of stevioside and other steviol glycosides, the leaves extract of *S. rebaudiana* has been used traditionally in the treatment of diabetes. Jeppensen *et al*. [77] reported the antihyperglycaemic, insulinotropic, and glucagonostatic effects of stevioside in type 2 diabetic Gotokakizaki (GK) rats as well as in normal Wister rats. Stevioside was found to suppress significantly the glucose response and concomitantly increase the insulin response during the *i.v.* glucose tolerance test (IVGT) with GK rats; but in normal Wister rats, stevioside was found to enhance insulin levels above basal during the same test, without altering the blood glucose response. Thus, the investigators concluded that stevioside being antihyperglycaemic, insulinotropic, and glucagonostatic, may have the potential of becoming a new antidiabetic drug for use in type 2 diabetes [77].

Chen *et al*. [78] also studied the effect of stevioside (**2**) on the glucose and insulin metabolism in two models of diabetes in rats, *i.e.* STZ-induced diabetic rats and NIDDM diabetic rats induced by feeding with fructose. Stevioside at a dose of 0.5 mg/kg was reported to lower the blood glucose levels in STZ-induced rats, peaking at 90 min, while stevioside administered twice daily also demonstrated dose-dependent hypoglycemic activity in both diabetic rat models. Besides, stevioside was also found to reduce insulin resistance in the diabetic animals as like as the glucose lowering effects of tolbutamide. Thus, the investigators concluded that stevioside was able to regulate blood glucose levels by enhancing not only insulin secretion, but also insulin utilization in insulin-deficient rats, which was due to the decreased PEPCK (Protein levels of phosphoenyl pyruvate carboxy kinase) gene expression in rat liver, caused by stevioside's action of slowing down gluconeogenesis [78].

Chatsudthipong and Jutabha [79] studied the effect of steviol (**1**) on transepithelial transport of *p*-aminohippurate (J_{PAH}) in isolated S_2 segments of rabbit renal proximal tubule using *in-vitro* microperfusion, and clearly showed that steviol can have a direct inhibitory effect on renal tubular transport by competitive binding with organic anion transporter. This prevents the entry of PAH into the cell, leading to the depression of transepithelial transport of PAH [79, 80]. The inhibitory effect was found to be dose-dependent and reported to be maximum at a concentration of 0.05 mM after 20 min of steviol treatment [79].

Recently, Abudula *et al.* [81] demonstrated that rebaudioside A (**3**) potently stimulates the insulin secretion from isolated mouse islets in a dose-, glucose-, and Ca^{2+}-dependent manner; it was found that in the presence of 16.7 mM glucose, addition of rebaudioside A at the maximally effective concentration of 10^{-9} M increases the ATP/ADP ratio significantly, while it does not change the intracellular cAMP level. The investigators also showed that rebaudioside A (**3**) and stevioside (**2**) at respective doses of 10^{-9} and 10^{-6} M reduced the ATP-sensitive potassium channel [K(ATP)] conductance in a glucose-dependent manner. Moreover, rebaudioside A also stimulated the

insulin secretion from MIN6 cells in a dose- and glucose-dependent manner; the insulinotropic effect of the test compound (**3**) was supposed to be mediated *via* inhibition of ATP-sensitive K(+)-channels, which requires the presence of high glucose level [81]. The inhibition of ATP-sensitive K(+)-channels is probably induced by changes in the ATP/ADP ratio; the experimental findings, thus, indicate that rebaudioside A may offer a distinct therapeutic advantage over sulphonylureas because of less risk of causing hypoglycemia.

Geeraert *et al.* [82] reported that stevioside (**2**) treatment is associated with improved insulin signaling and antioxidant defense in both the adipose tissue and the vascular wall, leading to inhibition of atherosclerotic plaque development and inducing plaque stabilization in obese insulin-resistant mice (twelve-week-old) when they were treated with the drug (10 mg/kg, $n = 14$) or placebo ($n = 20$) for 12 weeks. Treatment with the test compound (**2**) was also found to be associated with a two-fold increase of adiponectin responsible for improved insulin signaling and antioxidant defense in both the adipose tissue and the aorta of stevioside-treated mice [82]. In addition, stevioside also reduced plaque volume in the aortic arch by decreasing the macrophase, lipid and oxidized low-density lipoprotein (ox-LDL) content of the plaque; the decrease in ox-LDL in the plaque was likely due to an increase in the antioxidant defense in the vascular wall, as evidenced by increased Sod1, Sod2, and Sod3 [82].

Anti-diarrheal activity

Stevioside and its major metabolite, steviol (**1**), were reported to affect ion transport in many types of tissues, such as the kidney, pancreas, and intestine [83]; such effect of stevioside (**2**), steviol (**1**), and its analogs on intestinal Cl^--secretion was investigated in detail using human T84 epithelial cell line by Pariwat *et al*. [83]. Short-circuit current measurements showed that steviol and its analogs isosteviol, dihydroisosteviol and isosteviol 16-oxime inhibit forskolin-induced Cl^--secretion in a dose-dependent manner with IC_{50} values of 101, 100, 9.6, and 50 μM, respectively, whereas the parent compound stevioside had no such effect. Apical current measurement indicated that dihydroisosteviol targeted the cystic fibrosis transmembrane regulator (CFTR); the inhibitory action of this compound was found reversible and was not associated with changes in the intracellular Camp level. In addition, it did not affect calcium-activated chloride secretion and T84 cell viability. *In vivo* studies using a mouse closed-loop model of cholera toxin-induced intestinal fluid secretion showed that intraluminal injection of 50 μM dihydroisosteviol reduced intestinal fluid secretion by 88.2% without altering fluid absorption, thereby indicating that dihydroisosteviol and similar compounds could be a new class of CFTR inhibitors that may be useful for further development as anti-diarrheal agents.

Enzyme inhibitory activity

Stevioside, at concentration level of ~1.5 mM, was found to have no effect on activity of glutamate dehydrogenase of rat or bovine liver [84]; however, the drug exhibited inhibitory effects or various enzymatic activities like ATP dependent swelling, NADH oxidase activity, DNP-stimulated ATPase succinate dehydrogenase and succinate oxidase activity at quite high concentration as compared to other *ent*-kaurane analogs of *Stevia* [84].

Mutagenicity of *ent*-kaurene glycosides

Pezzuto *et al*. [85] reported that stevioside (**2**) is not mutagenic as judged by utilization of *Salmonella typhimurium* strain TM677, either in the presence or in the absence of a metabolic activating system; while the steviol, the aglycone of stevioside, was found to be highly mutagenic when evaluated in the presence of supernatant fraction (S-9) derived from the livers of aroclor 1254-pretreated rats. The investigators [85] also indicated that unmetabolized steviol and structurally related species, isosteviol was not active regardless of metabolic activation. Similarly, chemical reduction of the unsaturated bond linking the carbon atoms 16 and 17 positions of steviol resulted in the generation of two isomeric products, dihydrosteviol A and B, that were not mutagenic. In addition, *ent*-kaurenoic acid (Fig. 1b) was also found to be inactive. The study revealed that a metabolite of an integral component of stevioside is mutagenic; structural features of requisite importance for the expression of mutagenic activity include a hydroxyl group at position 13 and an unsaturated bond joining the carbon atoms at position 16 and 17 [85]. The aglycone, steviol (**1**), was also found to produce dose-related positive responses in some mutagenicity tests, *i.e.* the forward mutation assay using *Salmonella typhimurium* TM677, the chromosomal aberration test using Chinese hamster lung fibroblast cell line (CHL) and the gene mutation assay using CHL [86]. Metabolic activation systems containing supernatant fraction (S-9) of liver homogenates prepared from polychlorinated biphenyl or phenobarbital plus 5,6-benzoflavone-pretreated rats were required for mutagenesis and clastogenesis [86]. Steviol was weakly positive in the umu test using *S. typhimurium* TA1535/pSK1002 either with or without the metabolic activation system. Steviol, even in the presence of the S-9 activation system, was negative in other assays, *i.e.* the reverse mutation assays using *S. typhimurium* TA97, TA98, TA100, TA102, TA104, TA1535, TA1537 and *Escherichia coli* WP2 uvrA/pKMlOl and the rec-assay using *Bacillus subtilis*. Thus, steviol was found negative in the mouse micronucleus test but the mutagenic in a forward mutation assay, and caused chromosome aberrations and gene mutations in mammalian cells [86] and plasmid mutagenesis [87]. Stevioside and steviol were not mutagenic toward *S. typhimurium* TA97, TA98,

TA100, TA102, and TA104 either with or without S-9 mix at doses up to 5 mg per plate. They were not toxic to *S. typhimurium* even at the highest dose. Neither stevioside nor steviol were mutagenic in *S. typhimurium* TA1535, TA1537 and *E. coli* WP2 wvM/pKM101 in the presence of S-9 mix. These results suggested that neither stevioside nor steviol is mutagenic in *S. typhimurium* TA strains and *E. coli* WP2 wvM/pKMlOl either with or without metabolic activation.

Stevioside (**2**) was found to induce no significant increase of the mutation frequency of *S. typhimurium* TM677, even at the highest dose of 10 mg/mL, either with or without S-9 mix. However, steviol induced a significant dose-related increase in the mutation frequency when S-9 mix was present. Steviol increased not only the mutation frequency but also the raw number of 8-AZ resistant colonies (mutants) per plate, ruling out the possibility that the mutagenicity of steviol was an artefact due to the analysis of the data [88]. In the absence of S-9 mix, steviol did not give rise to an increase in the mutation frequency. To determine the genetic requirements for the mutagenicity of steviol, the authors compared the sensitivities of three isogenic tester strains in the presence of S-9 mix. Of the three strains examined, *S. typhimurium* TM677 (*uvrB*, *rfa*, pKMlOl) exhibited much higher sensitivity toward steviol than did *S. typhimurium* TM35 (*uvrB*, *rfa*) or KH75 (*rfa*, pKMlOl). These results suggest that steviol is mutagenic to *S. typhimurium* TM677 in the presence of S-9 mix and also that *rfa* mutation, deficiency of excision repair and presence of plasmid pKMlOl are all required for the maximum mutagenesis. Suttajit *et al*. [89] reported positive results for reverse mutations in the *S. typhimurium* strain TA98 with and without S-9 extract at a 50 mg/plate for 99% pure stevioside with and without S-9 extract. However in another study, Klongpanichpak *et al*. [90] did not find stevioside to be mutagenic in TA98 at similar concentration. However, they used S9 extract from rats, mice, hamsters and guinea-pigs, while Suttajit *et al*. [89] showed the strongest result without S-9 extract. The ability of stevioside and rebaudioside A to cause reverse mutations as indicated by TA98 needs to be further investigated, because such mutations suggest the possibility of carcinogenesis.

Besides, stevioside was also found to induce no significant increase in the specific *p*-galactosidase activity of *S. typhimurium* TA1535/pSK1002 either with or without S-9 mix as observed upon treatment with the compound at various doses ranging from 1250–5000 μg/mL (incubation period, 2 days) [86]. However, steviol was reported to induce an increase (∼2-fold) in the specific activity of β-galactosidase at concentrations of 313–1250 μg/mL (specific enzymatic activity of 53.6 U/A_{600} at 1250 μg/mL) in the absence of S-9 mix and 625–2500 μg/mL in the presence of S-9 mix (specific enzymatic activity = 99.9 U/A_{600} at 2500 μg/mL; incubation period, 2 days). Under the same conditions, the positive controls furylfuramide (specific enzymetic activity = 1759 U/A_{600} at 0.03 μg/mL without S-9 mix) and 2-aminoanthracene (specific enzymetic activity = 1848 U/A_{600} at 3.3 μg/mL with S-9 mix) substantially increased the specific activity of β-galactosidase of *S. typhimurium* TA1535/pSK1002; these results suggested that steviol is weakly positive in the umu test either with or without metabolic activation. It is also interesting to note that mutation frequency of steviol (0.60×10^4 at the maximal dose of 10.0 mg/mL) in the absence of S-9 mix is ten times lower than that (66.0×10^4 at the same dose) in the presence of S-9 mix [86]. Stevioside was also found to cause DNA breakage in blood, spleen, liver, and brain cells in rats [91]. Thus it was concluded that metabolically-activated steviol was found to cause dose-related positive responses in several mutagenicity tests, thereby, indicating that a steviol derivative is likely responsible for its mutagenic activity, but the metabolite has not been identified [92].

Although steviol (**1**) was mutagenic and clastogenic in bacteria and cultured mammalian cells, it did not exhibit any positive response in the mouse micronucleus test. This *in-vivo* test result does not necessarily mean that neither mutagenic nor clastogenic metabolites are generated from steviol *in vivo.* It could be possible that steviol produced adverse metabolites *in vivo* but they did not reach the bone marrow, the target organ for the micronucleus test. In fact, dimethylnitrosamine and diethylnitrosamine, potent hepatocarcinogens, do not give rise to a substantial increase in the number of icronucleated cells in mouse micronucleus test, probably because the short-lived active metabolites generated in the liver cannot reach the bone marrow [93, 94]. It might also be possible that the genotoxic metabolites of steviol could reach bone marrow but that they predominantly induced point mutations, such as base change or frameshifts, rather than chromosome aberrations, so that no micronucleated blood cells were found in the steviol-treated mice. Thus, further work is necessary to predict the genotoxic risk of steviol to human beings. Since steviol requires S-9 activation for mutagenesis and clastogenesis *in vitro*, the genotoxic damage in the liver of rats or mice should be examined and for this purpose the liver unscheduled DNA synthesis (UDS) assay or a transgenic mutagenicity assay were suggested to be appropriate for the further assessment of the genotoxic potential of steviol *in vivo* [86].

Safety evaluation

The toxicology and safety of stevioside used as a sweetener were studied by different investigators [19, 29, 50, 60, 91]. Stevioside (**2**) does not appear to be carcinogenic [84]. Recent studies have demonstrated that a portion of stevioside is absorbed and degraded to steviol, which appear to undergo further metabolism [60]. Other studies indicate that none of

the digestive enzymes from gastro-intestinal tract of different animals and man are able to degrade stevioside into steviol [2, 51–53]. Nevertheless, in feeding experiments with rats and hamsters stevioside was metabolized to steviol by the bacterial flora of the caecum [1]. Although animal studies did not show any adverse effects or toxicity associated with stevioside consumption [64, 66], the limited data is available on its metabolism and safety in humans to approve its use as a non nutritive sweetener; only the herbal form of the *Stevia* plants is allowed for use in foods as a flavor enhancer and also as a tea [96]. Stevioside was found to bear a very low acute oral toxicity (LD_{50} between 8.2 and 17g/kg) in the mouse, rat, and hamster [97, 98]. The safety of oral stevioside in relation to carcinogenic activity is evidenced by the works of different groups with rats [65, 67, 99, 100]. Stevioside was evaluated for safety by the 51st meeting of the JECFA (Joint FAO/WHO Expert Committee on Food Additives) in 1998 (WHO TRS no. 891). The JECFA considered the toxicity data of steviol glycosides in 1999 but was unable to recommend on ADI (acceptable daily intake) due to insufficient data, including a lack of human metabolism studies, a lack of information on the purities of the product, and lack of adequate *in-vivo* mutagenicity studies; later on, by 2004, JECFA set a temporary ADI of 2 mg/kg b.w./day for *Stevia* at that time and requested extensive additional information to be submitted by 2007 on the effects of steviol glycosides in humans, including special populations such as people with diabeties or hypertension [101]. Melis *et al*. [102] evaluated the renal excretion of steviol, and also clarified the actual participation of this compound on the renal excretion of glucose in rats, which has been previously suggested as the preferential action of steviol on the Na-glucose renal tubular transport system; on the basis of their detailed experimental observations the investigators concluded that steviol is secreted by renal tubular epithelium, causing diuresis, natriuresis, kaliuresis, and a fall in renal tubular reabsorption of glucose [102]. Recently, Maki *et al*. [103] demonstrated that consumption of as much as 1000 mg/day of rebaudioside A produced no clinically important changes in blood pressure in healthy adults with normal and low-normal blood pressure.

Yamada *et al*. [100] demonstrated that no significant effect was observed on spermatogenesis, nor on the interstitial cell proliferation as well as tumor formation in the testes of F344 rats when fed a ration containing up to 1% stevioside (95.2% purity) for 22 months. This observation was supported by various studies on fertility or reproduction in mice, rats or hamsters [67, 104–107]. However, steviol, the metabolite of stevioside, was found to be toxic to pregnant hamsters and their fetuses when administered on day 6 through 10 of gestation at doses of 0.5–1.0 g/kg body weight/day [25]. It was observed that the drug produces decreased maternal weight gain and high maternal mortality; the number of live births per litter and mean fetal weight decreased. Moreover, the maternal kidneys showed a dose-dependent increase in severity of convoluted tubules in the kidneys [25].

In 2006, the World Health Organization (WHO) performed a thorough evaluation of recent experimental studies of *Stevia* extracts conducted on animals and humans, and concluded that ''stevioside and rebaudioside A are not genotoxic *in vitro* or *in vivo* and that the genotoxicity of steviol and some of its oxidative derivatives *in vitro* is not expressed *in vivo*''. They also found no evidence of carcinogenic activity of *Stevia* extracts and suggested the possibility of its health benefits; but at the same time the organization recommended for a further study to determine its proper dosage [108]. Extensive scientific research by Scientific Committee on Food (SCF) has reported the safety of the common sweeteners like acesulfame K, aspartame, cyclamate, saccharin, sucralose, and also stevioside by several *in-vitro* and *in-vivo* animal studies, tests in human and in some cases of epidemiological studies and also recommended their ADI (acceptable daily intake) – the SCF observed that the consumption of sweeteners in the quantities within the ADI does not constitute a health hazard to consumers [5]. Among these synthetic or semi-synthetic sweeteners, stevioside is a naturally occurring molecule of interest because of its low toxicity level.

Clinical trials

Several clinical studies [72–78] reveal that no significant effects on blood pressure and blood sugar occur with high doses of steviol glycoside applied for several months [63]. However, two clinical trials on hypertensive patients reported reduction of blood pressure after long-term treatment with stevioside (**2**) [63]. Another clinical trial using stevioside proved a beneficial effect on post-prandial glucose homeostasis in type 2 diabetic patients. Clinical studies were also conducted to examine the effects of rebaudioside A (**3**) on the blood pressure of healthy subjects and on glucose homeostatis in type 2 diabetics, and it was concluded that 1000 mg/day of rebaudioside A had no clinically significant effects on blood pressure or on glucose homeostats or blood lipids in type 2 diabetic patients. However, no adverse effects were observed in these studies [96, 109].

Comments and conclusion

Stevioside and related *ent*-kaurene glycosides have been established as natural zero-calorie/low-calorie sweeteners, and many of them in the form of crude plant products are being used commercially in many countries as food additives for sweetening a variety of products; potent sweetness intensities of these glycosides in comparison to sucrose have projected

them as cost-effective substitutes of sugar. However, these *ent*-kaurene glycosides have still not been approved as food ingredient in the United States or the European Union; but *Stevia* in the leaf or extracted form is permitted to be sold in the US only as dietary supplement. Such phytochemicals have not only been established as non-caloric sweeteners, but reported also to exhibit some other significant pharmacological activities like antitumor and anticancer, antihypertensive, antihyperglycemic, anti-diarrheal, and enzyme inhibitory activities. Besides the pharmacological activities, the present article also deals with the natural distribution of such compounds, their structural features, plausible biosynthetic pathways, pharmacokinetics along with an insight into the structure–sweetness relationship, safety evaluation and clinical trials of these *ent*-kaurene glycosides. It has been demonstrated that steviol, a metabolite of stevioside, produced in the human intestinal microflora is genotoxic and induces developmental toxicity. Hence, rigorous researches not only on their prospective uses as pharmaceutical agents including zero-calorie sweeteners, but also on their toxicological evaluation are demanding to resolve issues pertaining to safety concerns.

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