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Stevioside and Rebaudioside A – Predominant *Ent*-Kaurene Diterpene Glycosides of Therapeutic Potential – a Review

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Abstract

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Stevia rebaudiana (Bertoni) is a Paraguayan perennial herb of the family Asteraceae. The leaves contain a great amount of secondary metabolites with a wide range of important biological activities commonly known as steviol glycosides which differ in their molecular configuration, power of sweetness and their taste profile. Out of various steviol glycosides, the main compounds of interest are diterpenoid glycosides of *ent*-kaurene type extracted from the leaves of this plant as non-toxic, thermally stable, low-calorie natural sweeteners stevioside and rebaudioside A. These glycosides are a high-quality sugar substitute or dietary supplement with diverse applications in the medicinal world along with the food and beverage industry. This review article is aimed at the chemistry of stevioside and rebaudioside A, possible biosynthetic pathways, their metabolism and acceptable daily intake along with a broad spectrum of pharmacological and therapeutic applications.

Keywords: *Stevia rebaudiana*; secondary metabolites, therapeutic applications; sweetener

Stevia rebaudiana (Bertoni) (Asteraceae) is a perennial herb native to Paraguay and Brazil. This plant is used all over the world as it possesses medicinal and commercial importance. It contains a significant amount of important nutrients and minerals necessary for regulating and maintaining various metabolic processes in the body (Lemus-Mondaca *et al.* 2012). The leaves of *S. rebaudiana* are extensively studied as a source of high potency sweet tasting. Natural constituents of the plant are *ent*-kaurene diterpene glycosides – stevioside, rebaudiosides A, B, C, D, and E, dulcoside A, and steviolbioside. The foremost active sweet tasting, non-toxic tetracyclic diterpene steviol glycosides of particular interest are stevioside and rebaudiosides A. The structure of these glycosides consists of a diterpene *ent*-kaurene skeleton, linked to a number of glucose units. Out of 230 species in the North and South American genus *S. rebaudiana* has been found to produce these sweet tasting steviol glycosides at high concentration levels. Stevioside (triglucosylated steviol) being the most predominant *ent*-kaurene type diterpene glycoside, approximately 3–8% of dried leaves, was first isolated in the first decade of the twentieth century in impure form (Bertoni 1905, 1918) but its final structure was not elucidated for nearly sixty years (Mosetting *et al.* 1963). Later, (Chaturvedula & Prakash 2011) isolated a new diterpenoid glycoside, 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-(4-*O*-(2-*O*-α-D-glucopyranosyl)-α-D-glucopyranosyl-β-D-glucopyranosyl) ester (1) from the commercial leaf extract of *Stevia rebaudiana* together with the important sweet *ent*-kaurene diterpene glycosides stevioside, rebaudioside A-G, rubusoside, and dulcoside A.

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These non-caloric natural products taste intensely sweet and are estimated to be 150 to 300 times sweeter than sucrose on a weight basis (Richman *et al.* 1999; Potzel & Brouns 2012). Rebaudioside A exhibits more sweetness and palatable taste profile than other associated steviol glycosides. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2007 laid down specifications that steviol glycoside sweeteners should consist of at least 95% of the known steviol glycosides (JECFA 2007).

In Europe, United States, China, Japan, Russia, Korea, and Brazil stevioside and rebaudioside A have been approved for use as a sweetener (Kinghorn & Soejarto 1985). A difference in the structure of stevioside and rebaudioside A is having one less glucose moiety. Recent development in the field of research of these glycosides and their derivatives for extracting, isolating, purifying, and modifying them is an ever growing field. The published literature reported different analytical techniques for determination of steviol glycosides in *S. rebaudiana* which include an electrospray ionisation technique used in mass spectrometry (ESI-MS) (Jackson *et al.* 2009), analysis of infrared light interacting with a molecule via infrared spectroscopy (Dacome *et al.* 2005; Hearn & Subedi 2009), liquid chromatography/mass spectrometry (Wang *et al.* 2004), capillary electrophoresis (Mauri *et al.* 1996; Dacome *et al.* 2005), high-performance thin layer chromatography (HPTLC) (Chester 2012; Londhe & Nanaware 2013) and one of the most reliable and simplest methods recommended by the FAO/WHO Joint Expert Committee on Food Additives (JECFA) at the 73rd Meeting (JECFA 2010) is high-performance liquid chromatography (HPLC) (Woelwer-Rieck *et al.* 2010; Bergs *et al.* 2012; Tada *et al.* 2013). On the other hand, these analytical methods essentially focused on the quantification of either stevioside or rebaudioside A and to a lesser extent on minor glycosides which are also present in the leaf extract contributing to low-calorie sweetness and bioactive properties.

Besides sweetness, various medicinal activities of stevioside and rebaudioside A have been identified which include antihyperglycaemic (Jeppesen *et al.* 2002; Gregersen *et al.* 2004), antineoplastic (Mizushina *et al.* 2005), anticariogenic (Das *et al.* 1992), antihypertensive (Chan *et al.* 2000), anti-inflammatory (Yasukawa 2002), and antioxidant activity (Žlabur *et al.* 2015). Currently, numerous reviews have been published concerning the valuable and abundant potential of steviol glycosides as a sweetener in beverages and variety of food products including soy sauce, sea foods, pickled vegetables, and confectionary or bakery products. The main focus area of this article is to review systematic literature and to summarise the comparative study on the structural features, biosynthetic pathways, safety evaluation, metabolic, pharmacological, and therapeutic applications of the major steviol glycoside sweeteners stevioside and rebaudioside A existing in *S. rebaudiana* leaves.

Chemistry of steviol glycosides – stevioside and rebaudioside A

The effort to explain the chemical structures of *S. rebaudiana* sweeteners started at the beginning of the twentieth century, which was from 1901 to 2000; but the progress was not fast. In different species of *Stevia* plants almost thirty *ent*-kaurene diterpene glycosides have been isolated that are commonly known as steviol glycosides – the aglycone part of such glycosides is steviol (*ent*-13-hydroxy kaur-16-en-19-oic acid) which is involved in constructing a C19-ester linkage between the C19-carboxylic function and a glucose unit, along with the formation of ether linkages

Table 1. The chemical identity of stevioside and rebaudioside A

Steviol glycosides	Common name	Chemical name	Chemical formula	Formula weight	C.A.S. number
Stevioside	stevioside	13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid β-D-glucopyranosyl ester	$C_{38}H_{60}O_{18}$	804.88	57817-89-7
Rebaudioside A	rebaudioside A	13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)-oxy]kaur-16-en-18-oic acid β-D-glucopyranosyl ester	$C_{44}H_{70}O_{23}$	967.03	58543-16-1

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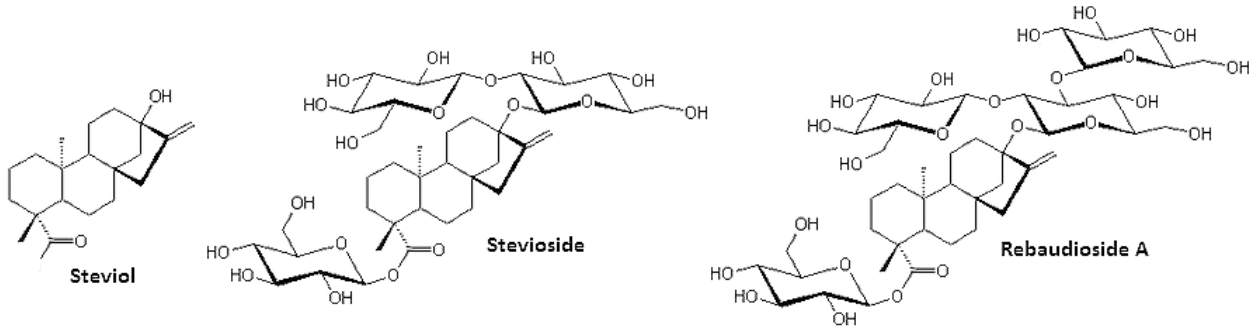

Figure 1. The structures of sweet *ent*-kaurene diterpene glycosides: aglycon steviol, stevioside, and rebaudioside A

using its C13-hydroxy group with combinations of glucose, xylose, and rhamnose moieties. Stevioside and rebaudioside A are two sweet tasting diterpene steviol glycosides found naturally in the leaves of the *Stevia* plant similar in structure to rebaudioside A having one more glucose moiety as compared to stevioside. The chemical identities and key chemical identifiers for the two main sweetener components of the genus *Stevia* are presented in Table 1.

The glucosyl and sophorosyl residue is present in the stevioside attached to the aglycon steviol, finally showing a cyclopentanoperhydrophenanthrene skeleton. Individually the C4 and C13 of steviol are linked to the β-glucosyl and β-sophorosyl group. The rebaudioside A has the same structure as that of stevioside, the only difference is that glucosyl-(1-3)-sophorosyl residue is present in place of sophorosyl residue. The chemical structures of the *Stevia* sweeteners stevioside and rebaudioside A and their similar core and metabolite, steviol are shown in Figure 1.

Biosynthetic pathway

Sweetness in *Stevia* leaves is due to the presence of secondary metabolites steviol glycosides (SGs). Stevioside and rebaudioside A are the vital metabolites among various steviol glycosides. The biosynthetic pathway of steviol glycosides involves 16 steps which are catalysed by numerous enzymes such as four UDP-glycosyltransferases (UGTs) identified as UGT85C2, UGT74G1, and UGT76G1 and kaurenoic acid 13-hydroxylase (KAH) (Yadav & Guleria 2012). It is operated in the leaves and transported to various parts of the *Stevia rebaudiana* plant which synthesises the important sweetening compounds very much related to the gibberellic acid biosynthetic pathway (Humphrey *et al.* 2006). According to different studies steviol glycosides are mostly present in the leaves, a slight amount is in the stem and an untraceable amount in the roots (Pól *et al.* 2007).

The primary seven steps involved in biosynthesis of steviol glycosides are similar to the MEP (2-C-methyl-D-erythritol-4-phosphate) pathway when isopentenyl diphosphate (IPP), dimethylallyl diphosphate (DMAPP), and geranylgeranyl diphosphate (GGDP) are synthesised (Wanke *et al.* 2001). Subsequently, the next four steps are similar to the gibberellic acid (GA) biosynthesis pathway which involves synthesis of kaurenoic acid from geranylgeranyl diphosphate (GGDP). The last five steps include the steviol glycoside (SG) biosynthesis pathway (Figure 2) (Hanson & White 1968).

In the presence of *ent*-copalyl diphosphate (CDP) synthase (CPS) the GGDP is initially converted through protonation initiated cyclisation to (–)-copalyl diphosphate for steviol. Subsequently, kaurene synthase (KS) produces kaurene by ionisation dependent cyclisation of CDP. Further, via the three-step reaction kaurene is oxidised to kaurenoic acid by a novel kaurene oxidase (KO), like in GA biosynthesis (Helliwell *et al.* 1999). Kaurene oxidase (KO) was found to be extremely high in flowers, leaves, succulent stems, and seedling shoots of *S. rebaudiana* (Humphrey *et al.* 2006). Finally, hydroxylation of kaurenoic acid occurs to produce steviol by means of the kaurenoic acid 13-hydroxylase (KAH) enzyme. At this step, steviol glycoside biosynthesis diverges from the gibberellic acid (GA) biosynthesis pathway (Kim *et al.* 1996; Brandle & Telmer 2007).

In the cytoplasm the aglycone steviol is glycosylated by different glucosyltransferases. There are two hydroxyl groups present in steviol – one at C-19 of C-4 carboxyl and the other at C-13. The glycosylation starts at C-13 by UGT85C2 producing steviol which brings about steviolmonoside. The formation of steviolbioside takes place by glycosylation of steviolmonoside. UGT catalysing this step has not been yet identified. As

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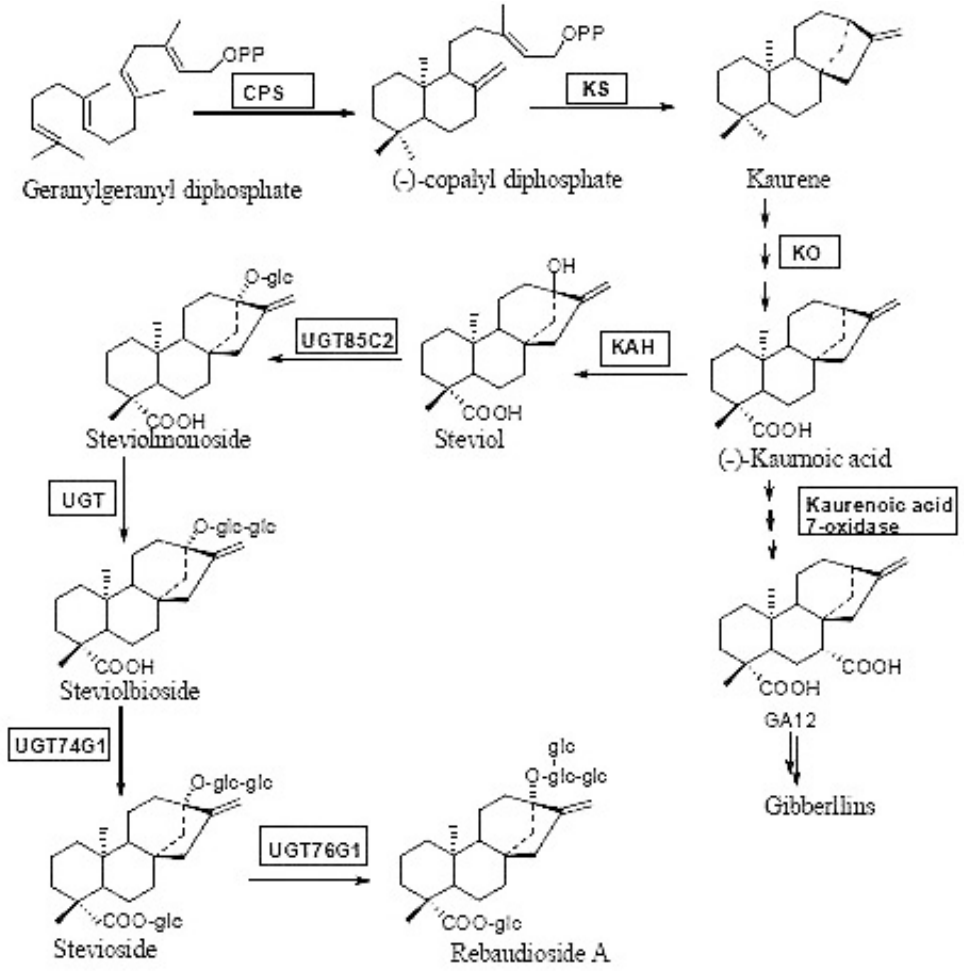

Figure 2. Biosynthetic pathway of steviol glycosides (stevioside and rebaudioside A) (source Brandle & Telmer 2007)
CPS – copalyl diphosphate synthase; KS – kaurene synthase; KO – kaurene oxidase; KAH – kaurenoic acid 13-hydroxylase

a final point, glycosylation of steviolbioside occurs at C-19 position by UGT74G1 to form stevioside (Shibata 1991). Rebaudioside A is synthesised by glucosylation at C-13 of stevioside catalysed by the enzyme UGT76G1 (Brandle & Telmer 2007).

Biotransformation

Researches on the biotransformation of major *ent*-kaurene diterpene glycosides showed that stevioside and rebaudioside A are safely metabolised by the body and do not differ in elimination though similar pathways followed in both humans and animals (Roberts & Renwick 2008). Stevioside owing to its high molecular weight is not readily absorbed from the upper small intestine of the human body and no digestive enzymes from the gastro-intestinal tract can breakdown stevioside to steviol. Alternatively, stevioside is degraded by the bacterial flora of the caecum or colon producing free steviol which is further converted into its glucuronide derivative in the liver and excreted from the body through urine (Geuns *et al.* 2007). An *in vitro* method of digesting steviosides by various digestive enzymes was studied by Hutapea *et al.* (1997) and it was found that none of the enzymes digested the stevioside but the microflora present in the intestinal tract hydrolysed the stevioside to further compounds steviol and steviol-16,17alpha-epoxide. Eventually, steviol-16,17alpha-epoxide was then completely transformed back into steviol and excreted as steviol glucuronide from the body in urine (Chatsudthipong & Muanprasat 2009).

Rebaudioside A is metabolised by microbes in the colon of the digestive tract to stevioside, which is further transformed to produce the end product glucose molecule and steviol. According to Renwick and Tarika (2008) the bacterium or *Bacteroides* sp. in the

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colon use the released glucose molecule which is not absorbed into the blood stream and the metabolised components essentially leave the body. Koyama *et al.* (2003) studied the human digestive tract and observed through human faeces that steviol is the end product of *Stevia* metabolism and it is not altered at high or low concentrations. The major steviol glycosides (stevioside and rebaudioside A) are absorbed and glucuronidated in the liver and a small part of glucuronidate that stays behind the colon is excreted through faecal matter and the newly bonded glucuronide is released in the blood and filtered via the kidneys into urine. Wingard *et al.* (1980) also reported the conversion rate of stevioside and rebaudioside A in animals (rats) and humans; it was recognised that in individual species the conversion from stevioside to steviol is more rapid than that of rebaudioside A to stevioside. Furthermore, quantitative and qualitative similarities have been found in the gut (microflora) of both the rat organism and the human body.

Acceptable daily intake (ADI)

Numerous renowned food safety and regulatory agencies have made their apprehension accurately with *Stevia* based ingredients (SCF 1985; JECFA 1999; FDA 2007). *S. rebaudiana* has been reported as the first natural non-caloric sweetener with medicinal properties which is safe for people of all ages (Gupta *et al.* 2013). Extracted forms of *Stevia* usually have a high percentage of the principal sweetening glycosides stevioside and rebaudioside A which can be marketed as sweeteners after meeting the regulatory purity criteria laid down in Commission Regulation (EU) No 231/2012 of 9th March 2012.

The US FDA has approved the most important steviol glycosides as a safe dietary supplement and considered rating in the USA (GRAS Notification 287 for steviol glycosides with stevioside and rebaudioside A as main components) as appears to have an adequate daily intake (ADI) of approximately 7.9 mg/kg BW (body weight) in humans and 25 mg/kg BW in rats (Xili *et al.* 1992; Geuns *et al.* 2003). Although the authors did not test the concentrations of stevioside higher than 793 mg/kg BW, this ADI is supposed to be considered as a minimum value. In 1994 the United States passed the Dietary Supplement Health and Education Act (DSHEA), which permitted the use of steviol glycosides (SGs) as an ingredient in dietary supplements (Williams & Burdock 2009). On 20th September 2012, at the 10th Meeting of Food Authority held at FDA Bhavan, New Delhi, the use of steviol glycoside was also approved as an artificial sweetener in a variety of foods.

Worldwide, for any marketed food ingredient correct and reliable specifications are important for commercial, regulatory, and safety reasons. For the comparison of intake and safety limits, the entire steviol glycosides are converted to their steviol equivalents (JECFA 2007). On the basis of relative molecular weights, 0.33 is multiplied by rebaudioside A quantities and 0.40 is multiplied by stevioside quantities so that both can be converted to steviol equivalents.

The current Joint FAO/WHO Expert Committee on Food Additives (JECFA) conducted a systematic scientific review on steviol glycosides at the 58th, 63rd, and 68th Meetings and established both temporary specifications and ADI for steviol glycosides of 0–2 mg/kg BW/day on a steviol equivalent basis and it corresponds to 0–6 mg of rebaudioside A/kg BW/day using this molecular weight conversion. The predictable permanent ADI for rebaudioside A is 0–12 mg/kg BW/day based on an expected permanent JECFA and ADI for steviol equivalents of 0–4 mg/kg BW/day. After getting sufficient information, the European Commission's Scientific Committee on Food (SCF) reviewed and recommended that it would decrease the safety factor to 100 and make the ADI permanent (SCF 1985, 1999a, b). JECFA also concluded that natural sweeteners made from *Stevia rebaudiana* are not dangerous and are safe for use in foods and beverages.

In 2010 The European Food Safety Authority (EFSA) established an ADI of steviol glycosides from *Stevia* and assessed their safe use. The steviol equivalents are expressed as ADI of 4 mg/kg BW (Carakostas *et al.* 2008). On 11th November 2011, the European Commission permitted the wide-scale usage of steviol glycosides as a food additive in Europe (Stoyanova *et al.* 2011).

Therapeutic applications

Stevioside and rebaudioside A are the most common active principles of the plant *S. rebaudiana*. These compounds have achieved universal attention due to their potent sweetness and exhibit diverse activities which play an important part in the medicinal world as traditional medicine and have been recommended as a treatment against different chronic and non-chronic diseases like diabetes, cancer, cardiovascular

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disease, high blood pressure, renal disease, fertility, and teratogenicity.

Glucoregulation

The isolation of active principles from medicinal plants is a traditional practice for the treatment of diabetes mellitus (Kujur *et al.* 2010). A number of clinical studies have suggested that stevioside and rebaudioside A are now recognised as glucogonostatic, insulinotropic, and antihyperglycaemic. It may offer therapeutic benefits for subjects with type 2 diabetes mellitus and have a direct effect on the β-cells of the islets of Langerhans of pancreas to produce insulin. Holvoet *et al.* (2015) published a research article which supports the *Stevia* products that help in the reduction of insulin resistance by improving the glucose metabolism and enhancing the insulin secretion by the breakdown of fat and bile acids, which aids in the control of weight.

The main steviol glycoside stevioside is an effective antihyperglycaemic agent. It directly acts on the β-cells and enhances the insulin secretion without altering the K^+-ATP channel activity and cAMP level in the islets (Jeppesen *et al.* 2000). Stevioside possesses a hypoglycaemic effect by suppressing the secretion of glucagon from the α-cells of the pancreas (Shibata *et al.* 1995). The direct effect of stevioside on glucose transport activity in skeletal muscle was studied by Lailerd *et al.* (2004). It was reported that the low concentration of stevioside has an immense action in glucose transport in skeletal muscle. Chen *et al.* (2005) suggested that stevioside enhances the insulin secretion and also helps in insulin utilisation by regulating blood glucose levels in insulin-deficient rats. It was due to the stevioside action of slowing down gluconeogenesis which decreases the phosphoenolpyruvate carboxykinase (PEPCK) gene expression in rats' liver. The long-term exposure of fatty acids in pancreas halts the activity of *α*- and β-cells causing diabetes. In this view the function of stevioside has also been studied by Hong *et al.* (2006) and they concluded that stevioside is a strong antidiabetic agent as it can upregulate the activity of genes responsible for fatty acid metabolism, namely oxidation, conversion, and gene disposal by counterbalancing the hypersecretion of *α*-cells caused by fatty acids.

Gregersen *et al.* (2004) conducted an experiment in diabetic humans for insulin sensitivity, where 1 mg of stevioside reduced the postprandial blood glucose levels by 18% relative to the control (1 mg of maize starch) and promoted the insulin to glucose ratio in serum by 40%.

The role of rebaudioside A in the stimulation of pancreas for the secretion of insulin in mouse islets in a dose, glucose, and Ca^{2+}-dependent manner was studied by Abudula *et al.* (2008); it was noticed that the presence of 16.7 mM glucose and a high concentration of 10^{-9} M of rebaudioside A significantly increased the ATP/ADP ratio without changing the intracellular cAMP level and by reducing the ATP-sensitive potassium channel [K(ATP)] conductance in a glucose-dependent manner. Additionally, rebaudioside A stimulates the secretion of insulin from MIN6 cells in a dose and glucose dependent manner. Thus, from the experimental findings it was specified that rebaudioside A may reduce the risk of hypoglycaemia and can be recommended over sulphonylureas. The purified forms of rebaudioside A were granted the GRAS (Generally Recognised as Safe) status by the US FDA.

Consequently, for supporting the positive energy balance, healthy glucoregulation and for pancreatic gland rejuvenation the table sugars can be substituted with low-calorie sweet tasting, excellent natural sugar alternatives stevioside and rebaudioside A of *Stevia*.

Saravanan and Ramachandran (2013) studied the modulating efficacy and protective effects of a diterpenoid rebaudioside A on the antioxidant status and lipid profile in experimental streptozotocin (STZ)-induced diabetic rats. Wistar rats were induced diabetes by a single intraperitoneal administration of STZ (40 mg/kg BW). The diabetic Wistar rats showed decreased levels of insulin and elevated levels of plasma glucose, thiobarbituric acid reactive substances, and hydroperoxides. There was a decreased activity of enzymatic antioxidants (superoxide dismutase, catalase, and glutathione peroxidase) and the levels of non-enzymatic antioxidants (vitamin C, vitamin E, and reduced glutathione) in diabetic rats. The lipid profile levels were significantly increased in plasma such as triglycerides, total cholesterol, phospholipids, free fatty acids, low density lipoproteins (LDL-cholesterol), and very low-density lipoproteins while plasma high-density lipoproteins were significantly decreased in diabetic rats. The administration of rebaudioside A (200 mg/kg BW) orally reversed the lipid peroxidation products, insulin, plasma glucose, enzymatic, non-enzymatic antioxidants, and lipid profile levels close to normal. The outcome of this study suggests that the natural low-calorie sweet-

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ener rebaudioside A exhibits antilipid peroxidative, antihyperlipidaemic, and antioxidant properties.

Genotoxicity

Intense *in vitro* and *in vivo* studies have been done on genotoxicity of steviol glycosides (stevioside and rebaudioside A). Twenty expert panels along with international food safety agencies have concluded that the extensively used sweet steviol glycosides, stevioside, and rebaudioside A, are not genotoxic. In the latest publications a major concern has been expressed on the basis of selected studies and overall database suggests that steviol glycosides may be mutagenic, but it has been recommended that additional *in vivo* genotoxicity studies are required to complete their safety profiles (Urban *et al.* 2013). A statement from the Joint Expert Committee for Food Additives in 2005 concluded that stevioside and rebaudioside A did not show any evidence of genotoxicity either *in vitro* or *in vivo* (JECFA 2005).

Brusick (2008) reviewed many literature sources on the genotoxicity of stevioside, two out of 16 studies confirmed genotoxic activity for stevioside. In contrast, Awney *et al.* (2011) presented the data which is well-designed and well-conducted on subchronic toxicity studies showing that no adverse effects are noticed after the consumption of rebaudioside A.

The steviol glycoside, chiefly stevioside, is subjected to both *in vitro* and *in vivo* assays so as to detect damage to DNA (Brusick 2008). The tests consist of measuring chromosome alterations, mutations, and simple breakage of DNA. The exception is with a single positive trial in strain TA98 of the Ames test (stevioside at 50 mg/plate which exceeded the recommended upper concentration limits for that assay), for steviol glycosides including rebaudioside A, all *in vitro* test results, do not produce any evidence that DNA damage is induced by steviol glycosides. The *in vivo* study is based on assessing the capability of stevioside to induce the breakage of DNA strand in rats and mice and a micronucleus test for chromosome damage in the mouse. No genotoxicity was seen by *in vivo* assays conducted in rats and mice when given a dose up to 2000 mg/kg BW. In another study on crude crystals of stevioside it was specified that the compound is not mutagenic. This has been shown again in numerous bacterial assays, specifically the forward mutation test in *B. subtilis*, spore rec assay, Ames test in *S. typhimurium* and *in vitro* tests for chromosomal aberrations in mammalian cells in Classical Hodgkin Lymphoma *(*CHL) and human lymphocytes (Joint FAO/WHO Expert Committee on Food Additives 1999).

Stevioside of 99% purity showed positive results in *Salmonella typhimurium* (*S. typhimurium)* strain TA98 at 50 mg/plate (Suttajit *et al.* 1993). The results of data analysis showed a 4-fold increase in revertants without S9 extract and a 2-fold increase with bacterial strain S9. The study used pre-incubated stevioside with and without β-glucosidase. The same mutagenic results are shown by both treated and untreated samples which reveal that at 50 mg/plate, stevioside (without β-glucosidase or S9), stevioside metabolite(s) (stevioside + S9), steviol (stevioside + β-glucosidase), and steviol metabolite (s) (stevioside + β-glucosidase + S9) are all mutagenic in TA98. Suttajit *et al.* (1993) also stated that human lymphocytes incubated with 1, 5, and 10 mg/ml stevioside for 24 h did not cause any chromosomal aberrations.

However, Klongpanichpak *et al.* (1997) concluded on the basis of results that stevioside is not mutagenic in *S. typhimurium* strain TA98 at a concentration of 50 mg/plate though S9 extract is used from mice, hamsters, rats, and guinea pigs where Suttajit *et al.* (1993) showed the strongest outcome or results without S9 extract.

Matsui *et al.* (1996) reported that doses of stevioside up to 5 mg/plate were not mutagenic in strains of *S. typhimurium* TA97, TA98, TA100, TA102, and TA104 with or without S9 or in *S. typhimurium* strains TA1535 and TA1537, and *E. coli* WP2 *uvrA*/pKM101 with S9. This compound was not mutagenic in *S. typhimurium* strain TM677 with or without S9 at 10 mg/ml, either. The negative results were also given by stevioside in the *umu test* with or without S9 and were negative in the spore and streak *rec-assays* with or without S9 at 10 mg/disk of stevioside.

Williams and Burdock (2009) investigated the potential of rebaudioside A in inducing genotoxicity in three *in vitro* and two *in vivo* assays [conducted according to the Organisation for Economic Cooperation and Development (OECD) guidelines]. It was found that this compound at concentrations up to 5000 μg/ml was not mutagenic in a chromosomal aberration test using Chinese Hamster V79 cells, in the Ames test using *Escherichia coli* and *S. typhimurium*, and in a mouse lymphoma assay using L5178Y+/– cells, with and without metabolic activation. Moreover, rebaudioside A at doses up 750 mg/kg BW was non-genotoxic in a bone marrow micronucleus test in mice and in

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rats at 2000 mg/kg BW in an unscheduled DNA synthesis test. Thus, this study supports the generally recognised as safe determination of rebaudioside A and provides enough evidence that at tested doses rebaudioside A is not genotoxic.

Rebaudioside A does not cause any chromosome damage, mutations, or DNA strand breakage in quite a few *in vitro* and *in vivo* studies (Pezzuto *et al.* 1985; Nakajima 2000a, b; Sekihashi *et al.* 2002).

Carcinogenicity

Stevioside and rebaudioside A are the most important secondary metabolites in leaves of *Stevia rebaudiana*, which is approved in non-toxic category whereas toxicity was studied under acute oral conditions due to its structural behaviours and historical uses (Medon *et al.* 1982). Studies suggest that crude crystals of the compounds stevioside and rebaudioside A are not mutagenic. The anti-inflammatory and cancer protecting property of *S. rebaudiana* is due to the presence of water soluble and bioactive compounds like chlorophylls, xanthophylls, stevioside, and rebaudioside A (Koubaa *et al.* 2015) which help in the phagocytic function of cells by completely engulfing and processing the particles, and protecting the body from potential threats by means of boosting the immune system (Salvador 2014).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1999 clearly declared that stevioside does not acquire a carcinogenic potential.

The carcinogenic effects of stevioside were studied on urinary bladder initiation and promotion. Findings showed that no development of neoplastic or preneoplastic lesions was observed in the urinary bladder by stevioside (Hagiwara *et al.* 1984). The combined outcome of an oral 24-month carcinogenicity and chronic toxicity study of stevioside with 85% purity in Wistar rats showed that no preneoplastic or neoplastic lesions were seen in any rat tissue. On the other hand, the lack of toxicity was also observed in subchronic studies after giving the maximum dose of 600 mg/kg BW/day (Xili *et al.* 1992). The stevioside hydrolysed product isosteviol inhibits the human cancer cell growth *in vitro* (with LD_{50} values of 84 to 167 μmol) and DNA replication (Mizushina *et al.* 2005).

In a two-stage model of mouse skin carcinogenesis with subsequent sequential exposure to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 7,12-dimethylbenz[a] anthracene (DMBA) have been reported to inhibit and reduce the tumour formation and tumour promotion by blocking the Epstein-Barr virus early antigen (EBV-EA) induction (Konoshima & Takasaki 2002; Yasukawa *et al.* 2002; Takasaki *et al.* 2009).

The mutagenicity of stevioside was tested for chromosomal effects on cultured human lymphocytes and in *S. typhimurium* strains TA98 and TA100; results revealed that stevioside does not show any significant chromosomal effect in healthy donors' cultured blood lymphocytes although it was not mutagenic at concentrations up to 25 mg/plate but showed the direct mutagenicity to only TA98 at 50 mg/plate (Suttajit *et al.* 1993). The stevioside effect against tumour was studied and results showed that stevioside slowed the tumour promoting agent (TPA) induced tumour promotion in a mice skin carcinogenesis (Nakamura *et al*. 1995).

The highlighting novel study by Paul *et al.* (2012) reported that the consumption of stevioside reduces a risk of breast cancer. It was observed that stevioside decreases certain stress pathways in the body that contribute to the cancer cell growth and enhances cancer apoptosis (cell death). The low concentration of stevioside was studied for toxicological effects on apoptosis induced by serum deprivation using the PC12 cell system by means of DNA electrophoresis and TUNEL signal assays. On the basis of analysed data it was established that stevioside enhanced apoptosis induced by serum deprivation and this is due to increased expression of Bax and of cytochrome c released into the cytosol, suggesting that stevioside affects the regulation of the normal apoptotic condition (Takahashi *et al.* 2012). The sweet glycoside stevioside extracted by methanol and ethanol solvent showed anticancer activity against Caco cell line with IC_{50} value 10 and 12 μg/ml and cytotoxic property against CaSki cell line with IC_{50} value of 20 and 5 μg/ml, respectively (Deshmukh & Kedari 2014).

Administration of rebaudioside A in a single dose of 2000 mg/kg BW to male Wistar rats does not show any signs of toxicity after observation for 16 h post dosing (Williams & Burdock 2009).

The bacterial reverse mutation test (Ames test) was used to study the toxicity of rebaudioside A using standard *S. typhimurium* and *Escherichia coli*; results showed no significant increase in the number of relevant colonies exposed to rebaudioside A at concentrations up to 5000 μg/plate. Rebaudioside A in the Ames test was found to be non-mutagenic in these two bacterial strains (Williams & Burdock 2009).

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The mutagenic potential of rebaudioside A was evaluated in cultured human lymphocytes; the analysed data showed no significant increase in the incidence of chromosomal aberrations or polyploidy in cultured Chinese Hamster V79 cells following 4 and 20 h treatments at any of the doses tested with or without S9 metabolic activation (Williams & Burdock 2009). Rebaudioside A was tested for the mutagenic potential on mouse bone marrow cells by means of the mammalian erythrocyte micronucleus. The results of the test showed that the maximum dose of 5000 µg/ml did not produce any statistically significant increase in the incidence of polychromatic immature erythrocytes (Williams & Burdock 2009). Additionally, rebaudioside A was shown not to cause any signs of toxicity when administered to the mice at doses of 150, 275, or 750 mg/kg of BW compared to the plain saline control (Williams & Burdock 2009).

High blood pressure and cardiovascular effects

A lot of reports have broadly shown that various classes of plant-derived substances like diterpenoids exert significant cardiovascular and antihypertensive effects (Melis 1991; Tirapelli 2008). The diterpenoids stevioside and rebaudioside A show a great potential source of the latest prototypes for the discovery and development of new cardiovascular therapeutic agents. Leaves of *S. rebaudiana* possess valuable biological properties due to the presence of the important glycosides stevioside and rebaudioside A. An alternative therapy was offered by these isolated glycosides, non-caloric sweeteners whose consumption may possibly exert valuable effects on human health by functioning as heart tonic for lowering the elevated blood pressure, regulating the heartbeat, relaxing the arteries, inhibiting vascular contractility and diuretic action (Gardana *et al.* 2010). Usual consumption of these compounds decreases the content of blood cholesterol by maintaining the lipid profile levels (Atteh *et al.* 2008). It also improves blood coagulation and cell regeneration, suppresses neoplastic growth and strengthens blood vessels (Wingard *et al.* 1980; Jeppesen *et al.* 2003; Barriocanal *et al.* 2008; Maki *et al.* 2008).

The role of stevioside in inhibiting the atherosclerosis by improving insulin signalling and antioxidant defence in obese insulin-resistant mice was studied by Geeraert *et al.* (2010). Results showed that stevioside action in obese insulin-resistant mice enhanced adipose tissue maturation and increased glucose transport, insulin signalling, and antioxidant defence in white visceral adipose tissues. Additionally, stevioside also helps in reduction and inhibition of the plaque volume in the aortic arch by decreasing the number of macrophages, lipid, and oxidised low-density lipoprotein (ox-LDL) content of the plaque.

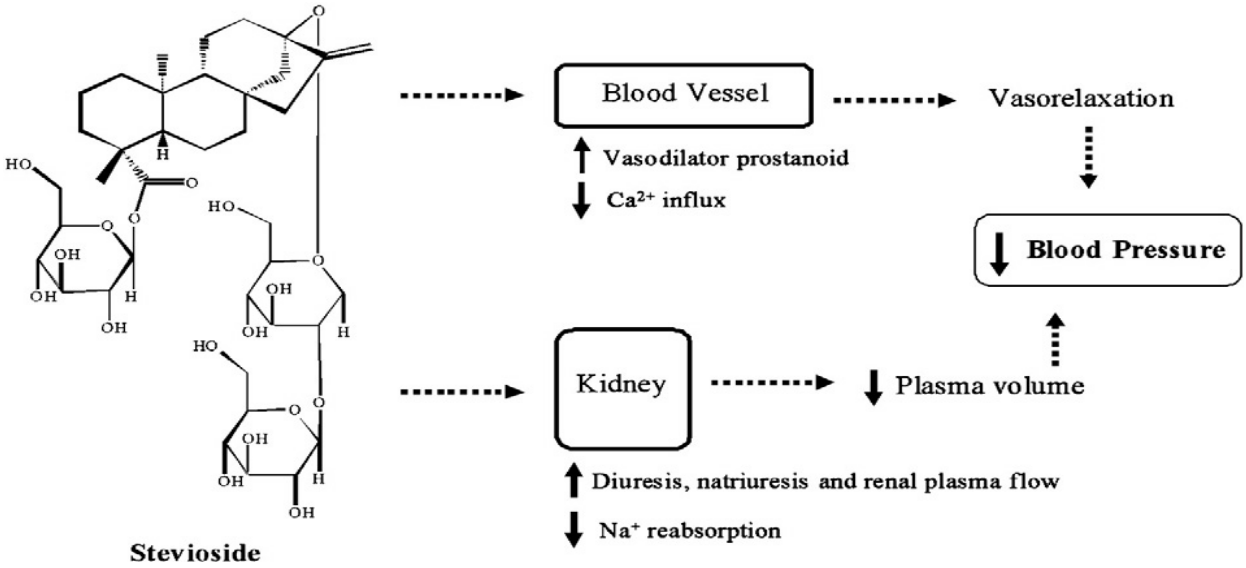

Figure 3. Mechanism of stevioside action on the cardiovascular system. Stevioside decreases the vascular resistance which promotes the blood pressure reduction by means of the inhibition of extracellular Ca^{2+} influx which stimulates the release of a vasodilator prostaglandin. Stevioside promotes diuresis, natriuresis, and reduction of Na^{+} reabsorption ensuing in a reduction of the extracellular fluid volume (Tirapelli *et al.* 2010)

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A group of patients treated with stevioside was well-tolerated with no side-effects and reported to have significantly superior quality of life scores than the placebo group. However, more patients in the placebo group developed left ventricular hypertrophy (Hsieh 2003), high blood pressure may often cause the abnormal thickening of the heart muscle. A placebo-controlled double-blind study established that stevioside taken orally at doses of 250 mg three times a day in patients with mild to moderate hypertension for one year resulted in a significant, long-term decrease in both the systolic and diastolic blood pressure (Chan *et al.* 2000). Following the initial study by a similar research team by means of an increased dose of stevioside (1.5 mg) stimulated the decrease in blood pressure (Hsieh *et al.* 2003). Studies revealed that purified stevioside produces changes in prostaglandin activity and induces diuresis, natriuresis, and hypotension in rats (Melis *et al.* 1985).

The first study on stevioside was conducted in 1977 to investigate the cardiovascular effects of stevioside in rats (Humboldt & Boech 1977). It was established that stevioside induces diuresis and a marked decrease in the mean arterial pressure and heart rate. The mechanism behind the role of stevioside in cardiovascular effects was investigated initially and it was found that an intravenous infusion of stevioside (8 and 16 mg/kg/h) in normotensive rats produced a noticeable dose-dependent hypotensive effect with diuresis and natriuresis (Melis & Sainati 1991).

More recently, studies on stevioside suggested that it induces a reduction in the mean arterial pressure and lower renal vascular resistance by promoting renal vasodilatation. This vasodilator effect is due to the blockage of Ca^{2+} channels because verapamil, a Ca^{2+} channel blocker, improved the systemic effect of stevioside, while $CaCl_2$ infusion reduced the vasodilator response of stevioside (Melis & Sainati 1991).

The stevioside in a dose-dependent manner relaxed the endothelium-intact and endothelium-denuded arteries in rat isolated aortic rings, contracted with vasopressin (Lee *et al.* 2001). The stevioside-induced relaxation was not influenced by methylene blue, a guanylate cyclase inhibitor, presenting that the relaxation was not mediated by the cyclic guanosine monophosphate (cGMP)-NO pathway. The authors used the cultured aortic smooth muscle cells (A7r5) and found that the Ca^{2+} influx was blocked by stevioside but it was ineffective in inhibiting the intracellular Ca^{2+} release. Therefore, the resulting data indicate that stevioside induces vasorelaxation primarily by inhibiting the extracellular Ca^{2+} influx. Additionally, *in vitro* effects of stevioside showed that stevioside induces hypotension in conscious hypertensive rats (Lee *et al.* 2001).

The precise fundamental mechanism of stevioside underlying the cardiovascular actions is summarised in Figure 2. Stevioside affects vascular resistance by inhibiting the extracellular Ca^{2+} influx and the release of vasodilator prostaglandin which in general helps in a reduction of blood pressure. This compound also results in a reduction of the extracellular fluid volume and produces diuresis and natriuresis. Toxicity of stevioside in rodents was investigated and results showed that the intake of stevioside as high as 7.9 mg/kg BW produces no acute toxicity (Xili *et al.* 1992). These major findings might provide significant information about the possible use of stevioside in the treatment of arterial hypertension. A randomised, double-blind 4-week study by Maki *et al.* (2008) evaluated hemodynamic effects of the consumption of 1000 mg/day rebaudioside A against placebo in 100 individuals with normal and low-normal systolic blood pressure (SBP) and diastolic blood pressure (DBP). The study groups were mostly female (76% rebaudioside A and 82% placebo) with an average age of around 41 (range 18–73) years. The mean resting seated systolic blood pressure/diastolic blood pressure was 110.0/70.3 and 110.7/71.2 mm Hg for the rebaudioside A and placebo groups, respectively. On comparing with the placebo, rebaudioside A does not significantly change the resting seated systolic blood pressure/diastolic blood pressure, mean arterial pressure, heart rate, or 24-h ambulatory blood pressure responses in patients with low-normal to normal blood pressure. The results of the present study suggest that 1000 mg/day consumption of rebaudioside A does not produce any clinically vital changes in the blood pressure in healthy adults with normal and low-normal blood pressure.

Renal function

Abundance of information was offered by traditional herbalism regarding the treatment of the kidney disease. The presence of diterpene glycosides in the herb *S. rebaudiana* exhibits a high degree of natural antioxidant activity and is used as high potency sweeteners.

Toskulkao *et al.* (1994) studied the interaction between urinary enzyme levels and changes in

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plasma creatinine, and blood urea nitrogen levels in rats treated with stevioside by means of concurrent changes of the kidney. There is an increase in blood urea nitrogen after subcutaneous injection with stevioside (1.5 g/kg BW) at 3 h onward. The blood urea nitrogen and creatinine level increases to a maximum after stevioside injection with approximately 180 and 132% at 9 hours. At this point in time stevioside causes a significant increase in alkaline phosphatase (AP), glucosuria and glutamyl transpeptidase (GTP), however, no significant changes in proteinuria, *N*-acetyl-D-glucuronidase (NAG), or glutathione-*S*-transferase (GSH-S-TF). Degeneration of the proximal convoluted tubule cells was detected after histopathological examination of the kidney induced by stevioside but no lipid peroxidation was found. Results revealed that stevioside causes nephrotoxicity at the proximal convoluted tubules rather than at the other tubules or glomeruli. It was most probably by a defect of the cell volume regulation due to depletion of intracellular ATP and disruption of microvilli, and nuclear dysfunction.

In another study by Melis *et al.* (1992) the effect of stevioside from *S. rebaudiana* leaves on the renal function of normal and hypertensive rats was studied. The examined stevioside functions as a systemic vasodilator which aggravates hypotension, diuresis, and natriuresis in both the normal and hypertensive rats. The administration of stevioside continuously to both normal and hypertensive rats increased the renal plasma flow and glomerular filtration rate, which was due to the vasodilation of both the afferent and efferent arterioles. A similar study shows that long-term oral intake or acute intravenous administration of stevioside lead to a decreased plasma volume producing diuresis and natriuresis. On the other hand, the infusion of stevioside directly into rats' artery induces diuresis. This reaction was due to decreased proximal tubular reabsorption as indicated by lithium clearance (Chatsudthipong & Thongouppakarn 1995), signifying that stevioside targets at the proximal tubule of the kidneys.

This research was designed to investigate the effect of stevioside on the transepithelial transport of *p*-aminohippurate in isolated S2 segments of rabbit proximal renal tubules using *in vitro* micro-perfusion. The result shows that stevioside, at a concentration of 0.70 mM, inhibits the transepithelial transport of *p*-aminohippurate by interfering with the basolateral entry step, the rate-limiting step for transepithelial transport. The absence of the effect of stevioside on the transepithelial transport of *p*-aminohippurate on the luminal side and its reversible inhibitory effect on the basolateral side indicate that stevioside does not permanently change the *p*-aminohippurate transport and does not harm the renal tubular function at normal human intake levels (Jutabha *et al.* 2000).

In a recent study, Hashemi *et al.* (2014) investigated the feasible protective effects of rebaudioside A on acetaminophen (APAP)-induced oxidative stress in the kidney of mice. The oxidative stress was induced in the kidney of BALB/c mice by the intraperitoneal (*i.p.*) administration of a single dose of 300 mg/kg acetaminophen. Thirty minutes after acetaminophen injection a number of these mice were treated with rebaudioside A (700 mg/kg) (*i.p.*). Later, after two and six hours of acetaminophen injection all BALB/c mice were sacrificed and glutathione (GSH), malondialdehyde (MDA), free acetaminophen, and glutathione conjugated acetaminophen (APAP-GSH) were determined in the kidney tissues of sacrificed mice. Thus, findings suggest that though rebaudioside A was not successful in preventing the initiation of acetaminophen-induced oxidative stress, as indicated by GSH depletion and lipid peroxidation in kidneys of mice, but afterwards it attenuated lipid peroxidation by reducing acetaminophen conversion to its activated metabolite, specifically *N*-acetyl-*p*-benzoquinone imine (NAPQI), which produced APAP-GSH conjugate in kidneys of mice. Thus, rebaudioside A acts as a principal compound in the alleviation of acetaminophen induced oxidative stress in kidneys of mice after acetaminophen overdoses.

Fertility and teratogenicity

It is controversial whether the plant *S. rebaudiana* or their extracts possess some contraceptive effect (Planas & Kuc 1968). Paraguayan Matto Grosso Indian tribes used the leaves of this herb *S. rebaudiana* in tea or beverages as a male or female oral contraceptive.

The legislative body mentioned in the application that accessible results do not explain that stevioside induces teratogenic or embryotoxic effects and this report is scientifically supported by three studies, one in the hamster (Yodyingyuad & Bunyawong 1991) and two in the rat (Mori *et al.* 1981; Usami *et al.* 1995). The effect of stevioside on growth and reproduction was studied. Stevioside at different doses (500, 1000, and 2500 mg/kg BW/day, 90% purity)

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was fed to hamsters during the mating time and they were permitted to bear three litters. In the course of treatment mating performance, fertility, pregnancy, number of foetuses, in addition growth and fertility of the offspring were not affected. On the other hand, no teratogenic effects were studied (Yodyingyuad & Bunyawong 1991). In another study stevioside was applied in a diet of Wistar rats (0.15, 0.75, and 3%, equivalent to 150, 750, and 3000 mg/kg BW/day, 96% purity). Before and during the mating period males were treated for 60 days while females for 14 days before the mating period and for 7 days during gestation. The analysed result shows no treatment related effects on fertility or mating performance, and no developmental malformations were seen in the foetuses (Mori *et al.* 1981).

Stevioside of 95.6% purity, dissolved in distilled water, was given to four groups of 26 pregnant Wistar rats once a day at doses of 0, 250, 500, and 1000 mg/kg BW/day from 6th day to 15th day of pregnancy, on 20th day of gestation the rats were sacrificed, and the foetuses of pregnant Wistar rats were examined for malformations. At the final stage the points examined were number of live foetuses, number of resorptions or dead foetuses, maternal and foetal body weight, sex distribution, and incidence of malformations. It was concluded that no teratogenic effects were induced in pregnant rats upon the administration of stevioside orally (Takanaka *et al.* 1991; Usami *et al.* 1995). Perhaps, in view of the numerous studies, it can be concluded that stevioside does not cause any developmental toxicity at high doses. Although some data suggest that stevioside can affect the male reproductive organ system and reproductive performance, for example decreased seminal vesicle weight, reduced spermatogenesis, and interstitial cell proliferation in the testes (Yamada *et al.* 1985).

Furthermore, few researchers reported that no developmental toxicity was evidenced from published chronic toxicity (Xili *et al.* 1992; Toyoda *et al.* 1997), subchronic toxicity (Aze *et al.* 1991), and developmental and reproductive toxicity (Mori *et al.* 1981; Yodyingyuad & Bunyawong 1991; Usami *et al.* 1995) studies that purified stevioside or rebaudioside A have an undesirable influence on the male or female reproductive systems.

On the basis of past database and continuous debate over the reproductive effects of stevia extracts additional investigations were conducted to prove the reproductive safety of high-purity rebaudioside A. The initial phase of the evaluation included a histopathological examination of the testes in high-dose males from both 28 and 90 day feeding studies (Curry *et al.* 2008). The results were unexceptional after macroscopic and microscopic examinations of the testes from the 28-day study in 100 000 ppm group and from the 90-day study of all the male reproductive organs in the 50 000 ppm group. It was concluded from both studies that in both male and female reproductive systems no treatment-related adverse effects were observed.

Sloter (2008) tested the rebaudioside A via gavage in an embryo/foetal developmental toxicity study in pregnant rats. Intrauterine growth and survival were not affected by the administration of rebaudioside A and there were no developmental variations or foetal malformations at any dosage level. A dose level ≥ 2000 mg/kg BW/day (highest dose administered) was considered to be the NOAEL for maternal and embryo/foetal developmental toxicity.

Biotechnological approach

Sixty thousand years back in the middle Palaeolithic age traditional medicinal systems developed by incorporating plants or herbs as a mode of therapy, which can be tracked from fossil studies (Solecki 1975).

In the past few years a number of major advances have occurred in molecular techniques mainly focusing on their benefits and limitations; moreover, the latest technologies improve existing techniques or build up new approaches so as to produce information more quickly, accurately, easily, or in a more straightforward repeatable manner than the existing methods. Accordingly, for *Stevia* a number of advanced molecular techniques are applied, for instance nuclear magnetic resonance (NMR) studies were approved for determining the physical and chemical properties and confirming the structure of natural sweeteners stevioside and rebaudioside A (Steinmetz & Lin 2009).

Biotechnology plays an important role in accelerated development of consistent plant based drugs. For multiplication and genetic enhancement of the medicinal plant like *S. rebaudiana* (Bertoni) biotechnological tools are important which help in adopting new techniques, for example *in vitro* regeneration, genetic transformations, and plant tissue culture. The biotechnologists show enormous interest in the production of valuable secondary products by using the cell culture technique. In the current situation plant tissue culture is a noble, innovative, effective,

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and efficient method for obtaining their substances on a large scale and for converting less medicinally vital plant metabolites to a valuable product.

An extremely low germination percentage was shown by seeds of *S. rebaudiana* (Felippe & Lucas 1971; Monteiro 1980; Toffler & Orio 1981) and a practice of cutting the plant through vegetative propagation is restricted to a small number of individuals (Sakaguchi & Kan 1982). The simple and quick process for the mass propagation of *S. rebaudiana* is through tissue culture techniques which include many areas such as cell nutrition, cell division, differentiation, and cell preservation. But at present, bulk cells are cultivated *in vitro* or as clone from single cells to produce whole plants from the isolated meristem, subsequently the callus is induced and complete plantlets are developed by organogenesis or by embryogenesis. Plant propagation by means of *in vitro* method holds a remarkable potential for the production of high-quality plant-based medicines (Murch *et al.* 2000). The micropropagation of *S. rebaudiana* through shoot tip culture was reported by Das *et al*. (2011). In support of multiple shoot proliferation within 35 days of culture, Murashige and Skoog (MS) medium (Murashige & Skoog 1962) supplemented with 2 mg/l kinetin was found to be the most excellent option producing more than 11 shoots from a single shoot tip explant. MS media without growth regulators worked vigorously for root induction but when supplemented with indole-3-acetic acid (IAA) and benzyl adenine (BA), they had an adverse effect on root induction. The peroxidase assay was performed along with inter-simple sequence repeat (ISSR) fingerprinting to authenticate the genetic clonality of *in vitro* generated propagules. Thus, an overall study shows that the shoot tip culture in agar medium containing a high concentration of kinetin helps in the micropropagation of *S. rebaudiana*.

The practice of metabolic engineering experiments may disclose undiscovered branches to already known metabolic pathways. The achievements in metabolic engineering of diterpenoids help in determination of biological activities of transgenic plants engineered for a diterpenoid pathway. The presence of diterpene steviol glycosides (stevioside and rebaudioside A) in leaves of *S. rebaudiana* provides significant applications in industrial products for instance flavouring agents, antimicrobial agents, commercial sweeteners, and pharmaceuticals. The essential role of these glycosides is plant-plant communication, plant-environment interaction, plant-insect and plant-animal interactions (Pichersky & Gershezon 2002).

Few scientists performed a small number of molecular level investigations from the *Stevia* leaf cDNA library. Brandle *et al.* (2002) one-time sequenced 5548 expressed sequence tags (ESTs) and studied the association of the MEP pathway for steviol biosynthesis without involving the mevalonic acid (MVA) pathway.

S. rebaudiana has many applications in nanotechnology and includes a potential to recommend positive applications for food and health. The dried stevia leaf extract was used as a shadow in $AgNO_3$ solution to synthesise silver nanoparticles (Yilmaz *et al.* 2011). X-ray diffraction and transmission electron microscopy (TEM) specify that nanoparticles are polydispersed and spherical with diameter ranging from 2 nm to 50 nm.

The "nutraceutical" application of the *Stevia* extract comprises foods and beverages as pharmaceutical compositions such as protective hydrocolloids (like proteins, modified starches), secondary metabolites, bioactive compounds, antioxidants, and antibacterial agents that may possibly be added to soft drinks, nutritious bars or candies, with the aim that an adult can consume a large amount of preferably 100–500 mg of *Stevia* extract.

The diverse applications of *Stevia* have brought the interest of researchers to start research in a variety of fields for understanding the chemistry, biochemistry, structure, behaviour, and role of these steviol glycosides. Researchers have been working for a long time in the field of immunology to clarify the biological nature and safety aspects of steviol glycosides (Ahmed *et al.* 2011).

CONCLUSION

The leading food scientists of the world consider bioactive constituents of *S. rebaudiana* as the "sweeteners of the future or millennium". *Stevia* has diverse applications in various fields such as in global food industry and medicinal world. Traditionally, the leaves of *S. rebaudiana* are acknowledged as producer of diterpenoid steviol glycosides which are low in calories, non-hazardous, anti-microbial, and effective sugar substitute of commercial value in a number of countries. The chemistry, biochemistry, and biotechnology have played a vital role in understanding the structure, behaviour, and role of these steviol glycosides. The main active principal metabolites of

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these glycosides are stevioside and rebaudioside A, which have a huge impact on human physiology and are reported to possess a potential therapeutic mode of applications in diabetes, cardiovascular, cancer, renal, and reproductive system. There is a requirement for much skill for the toxicological evaluation of these glycosides to settle issues related to safety concerns. Therefore, some biotechnological techniques must be implemented to cover a broad spectrum of scientific applications for understanding the potential benefits offered by the remarkable plant *S. rebaudiana*.

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