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A Review on Chemical Composition, Biosynthesis of Steviol Glycosides, Application, Cultivation, and Phytochemical Screening of *Stevia rebaudiana* (Bert.) Bertoni

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Authors' contributions

This work is carried out in collaboration between all three authors. Author MDKMG designed the study and prepared the manuscript. Author RMUSS prepared the part of the manuscript and author WTPSKS proof read the manuscript. All authors read and approved the final manuscript.

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Review Article

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ABSTRACT

Aims: *Stevia rebaudiana* which is commonly known as sweet leaf herb is used to extract non caloric sweet steviol glycosides. Thus based on available reports about its chemical composition, biosynthesis of steviol glycosides, application, cultivation, and phytochemical screening an attempt has been made to review to *Stevia rebaudiana* in context of its medicinal and pharmaceutical importance.
Methodology: Literature search have been done in the web using google scholar, PubMed as search platform. More than two-thirds of the references are within 15 years.
Results: According to the literature search *S. rebaudiana* leaves contain non caloric steviol

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glycosides and pharmaceutically important other phytochemicals. Micropropagation is the best propagation method to overcome difficulties in conventional propagation methods.
Conclusion: It is concluded that *in vitro* cultures has potential to extract important phytochemicals used in various pharmaceutical, food (flavoring agents, food additives) and perfume industries.

Keywords: Stevia rebaudiana; steviol glycosides; biosynthesis; non caloric sweeteners; miccropropagation; phytochemicals.

1. INTRODUCTION

Stevia rebaudiana (Bert.) Bertoni is a herbaceous plant species in the genus Stevia of Family Asteraceae. It is commonly known as honey leaf, sweet leaf or candy leaf herb due to the sweetness in its leaves. The chemical compounds which produce its sweetness are known as Steviol glycosides and are non-caloric sweeteners [1]. The green powder of Stevia leaf has sweetness of 20 to 25 times greater than cane sugar while the pure extract that is, Stevioside is 300 times sweeter than that [2]. Due to the presence of non caloric sweetness this plant has given an extra value especially for hyperglycemic patients for their consumption as an alternative for sugar.

2. ORIGIN AND DISTRIBUTION OF *S. rebadiana*

S. rebaudiana originated in the highland regions of Northeastern Paraguay (on the Brazilian border) [3]. It is native to the Rio Monday Valley of the Amambay mountain region and distributed from the Southwestern United States, southward through Mexico and Central America. It also occurs from non Amazonian South America, southward to Central Argentina. In Brazil, thirty six species have been recorded and distributed mainly in southern and central regions. In the native state it grows naturally on the edges of marshes or in grassland on soils with shallow water tables. The suitable climate condition for optimum growth can be considered as semi-humid subtropics, with temperatures ranging from 6 to 43 □C, with an average of 23 □C, and rainfall ranging from 1500 to 1800 mm per annum [4].

Commercial cultivation of *S. rebaudiana* was first reported in Paraguay in 1964. It was exported to Japan in 1968, and from there awareness and cultivation of this species spread throughout the world [5]. From then, Stevia has been introduced to many other countries, including United States, Brazil, Canada, Korea, Mexico, Indonesia, Tanzania and India and recently introduced to Sri Lanka. Its production is centered in China and the major market is in Japan [4].

3. TAXONOMY OF *S. rebaudiana*

S. rebaudiana is an annual and perennial herb which grows as a shrubs or sub shrubs. However under cultivation conditions plants grow more vigorously.

Kingdom : Plantae
Subkingdom : Viridiplantae
Infrakingdom : Streptophyta
Superdivision : Embryophyta
Division : Tracheophyta
Subdivision : Spermatophytina
Class : Magnoliopsida
Superorder : Asteranae
Order : Asterales
Family : Asteraceae
Genus : Stevia
Species : *Stevia rebaudiana*

(Source:https://www.itis.gov/servlet/SingleRpt/SingleRpt) [6].
]
Taxonomic hierarchy of *S. rebaudiana* was obtained from Integrated Taxonomic Information System (IT IS) which is based on the latest scientific consensus available and is provided as a general reference source since it is the most reliable source with up-to-date information.

The plant was first named as *Eupatorium rebaudianum* Bert. in honour of Rebaudi, the first chemist to study the chemical characteristics of the substances extracted. Its name was later changed to *S. rebaudiana [7*]. According to Yadav et al. [4], the genus Stevia consists of approximately 150-200 species of herbaceous plants. Although all those species contain important phytochemicals, only two species along with *S. rebaudiana* contain Steviol glycosides which give the sweetness to its leaves [8].

S. eupatoria, S.triflora, S.micrantha, S.ovata, S.plummerae, S.rhombifolia, S.serrata, S.viscid,S.myriadenia,S.commixta, S.satureiaefilia, S.leptophylla, S.phlebophylla and *S.oligophylla* are few other species belongs to genus Stevia [4]. Out of these, *S. phlebophylla* is the other species which produce Steviol glycosides [9]. However, recent experiments done by Ceunen et al. [10] reported a re-evaluation of this claim and concluded that *S. phlebophylla* is unlikely to contain significant amounts of steviol glycosides, if any.

4. MORPHOLOGY OF *S. rebaudiana*

S. rebaudiana is a herbaceous plant grows up to 30 cm height. It is a perennial plant in tropical and sub-tropical climates, however usually grown as an annual plant in cooler climates. It has small, sessile leaves with an alternate leaf arrangement and the leaf shape is lanceolate [11]. They are slightly glandular pubescent and trichomes on the leaf surface are of two distinct sizes. Leaf margin is serate and tip is blunt. Stevia leaves vary widely due to many environmental factors, including soil conditions, irrigation methods, sunlight, air purity, farming practices. Leaves have a pleasantly sweet, refreshing taste that can linger in the mouth for hours. The material contains the sweet components, surrounded by the bitter components in the veins [12].

The stem is weak-pubescent at bottom and woody. The rhizome has slightly branching roots. It has loosely paniculated inflorescence and white, small flowers with pale purple throat corollas [13]. The tiny white florets are dioecious [14]. Seeds are very small and contained in slender achenes about 3 mm in length. They have a very small endosperm and dispersed in the wind via hairy pappus. Fertile seeds can be usually observed in dark in color, whereas infertile seeds are usually pale in color [4] (Figs 1 and 2). Seed germination and seedling development are slow may be due to small size of seeds [15]. Seed viability is also recorded as very low and the viability decreases with storage. Mostly in an open environment seed viability lost within a week or two [16].

Fig. 1. (a) Habit, (b) leaves, (c) flowers and (d) seeds of *S. rebaudiana* [17]

5. CHEMICAL COMPOSITION

Stevia leaves are the source of sweet Steviol glycosides [19]. There are also other related compounds including flavonoid glycosides, coumarins, cinnamic acids, phenylpropanoids and some essential oils [20]. In addition, the triterpenes amyrin acetate and three esters of lupeol and the sterols like stigmasterol, sitosterol and campesterol are also extracted from the leaves [21]. Even more, Stevia contains a high percentage of phenols, flavonoids and antioxidants.

5.1 Steviol Glycosides

All Steviol glycosides have similar structures and Steviol is the aglycone of all of the principle and secondary sweetener compounds [22] (Fig. 3). This aglycone is connected at C-4 and C-13 to mono-, di-, or tri saccharides consisting of glucose and/or rhamnose residues [23-24] (Table 1).

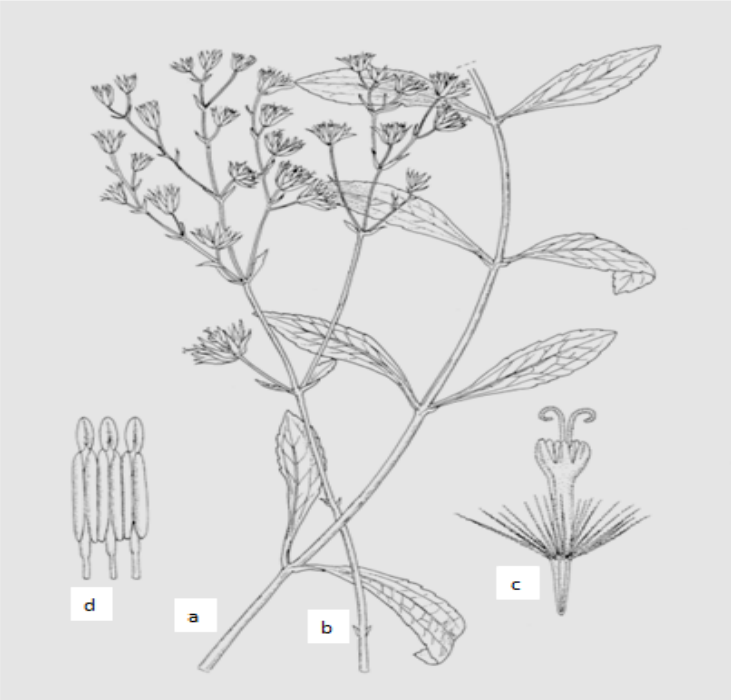

Fig. 2. Descriptive morphological structures of *S. rebaudiana*; a) leafy twig, b) flowering branch, c) flower and d) anthers
(Source:https://uses.plantnetproject.org/en/Stevia_rebaudiana_%28PROSEA%29) [18]

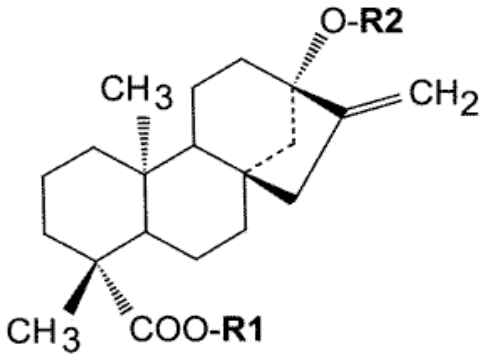

Fig. 3. Steviol- the aglycone of sweetener compounds [22]

Table 1. Chemical structures of main steviol glycosides

H : Hydrogen
Glc : Glucose
Rha : Rhamnose
Xyl : Xylose

(Wallin, 2004; Shah et al., 2012) [22,24]

Compound name	R1	R2
Steviol	H	H
Stevioside	β-Glc	β-Glc-β-Glc(2→1)
Rebaudioside A	β-Glc	β-Glc-β-Glc(2→1)| β-Glc(3→1)
Rebaudioside B	H	β-Glc-β-Glc(2→1)| β-Glc(3→1)
Rebaudioside C	β-Glc	β-Glc-α-Rha(2→1)| β-Glc(3→1)
Rebaudioside D	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1)| β-Glc(3→1)
Rebaudioside E	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1)
Rebaudiosie F	β-Glc	β-Glc-β-Xyl(2→1)| Glc(3→1)
Dulcoside A	β-Glc	β-Glc-α-Rha(2→1)
Steviolbioside	H	β-Glc-β-Glc(2→1)

There are around ten Steviol glycosides present in the plant namely Stevioside, Rebaudioside A, B, C, D, E, F, Dulcoside A and Steviolbioside [1]. Four major sweeteners are Stevioside, Rebaudioside A, Rebaudioside C and Dulcoside A Fig. 4 with the sweetness of 210, 242, 30 and 30 times respectively in relation to sucrose [25]. The percent content of steviol glycosides present in the total dry weight of Stevia leaves are; 5-10% of Stevioside, 2-5% of Rebaudioside A, 1% of Rebaudioside C, 0.5% of Dulcoside A, 0.2% of Rebaudioside D,E,F and 0.1% of Steviolbioside [4,26].

Among two main Steviol glycosides, majority of the sweetener content (about 65% of the total glycosides) is made up by Stevioside. Other than sweetness, sometimes it has a licorice taste responsible for the bitter aftertaste. Due to this lingering effect Stevioside is not accepted by some people [4]. However there is high interest for Rebaudioside A, because it has the sweetest taste and no bitter aftertaste. Presence of Rebaudioside A content is about 30% of total glycosides [27].

5.2 Other Phytochemical Compounds

Over 100 phytochemicals have been discovered in *S. rebaudiana* and rich in terpenes and flavonoids. The main phytochemicals present in Stevia include: apigenin, austroinulin, avicularin, beta-sitosterol, caffeic acid, campesterol, caryophyllene, centaureidin, chlorogenic acid, cosmosiin, cynaroside, daucosterol, foeniculin, formic acid, gibberellin, indole-3-acetonitrile, isoquercitrin, jhanol, kaempferol, kaurene, lupeol, luteolin, polystachoside, quercetin, quercitrin, scopoletin, sterebin A-H, stigmasterol, umbelliferone, and xanthophylls [30,31] (Fig. 5).

6. BIOSYNTHESIS OF STEVIOL GLYCOSIDES

S. rebaudiana produce sweet steviol glycosides in its leaves and its aglycone is steviol. Many studies reported that steviol was synthesized from kaurene via MEP pathway [33] and there are two main stages in biosynthesis. In the first stage, Pyruvate and Glyceraldehyde 3-Phosphate (G-3-P) which are molecules of primary metabolism synthesis isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) through 2-C-methyl-D-erythritol-4 phosphate pathway (MEP pathway). Then these IPP and DMAPP synthesis Geranylgeranyl diphosphate (GGDP) (Fig. 6).

In the second stage, GGDP is converted into steviol glycosides in several steps. First GGDP produce (-) Copalyl disphosphate by cyclation and Kaurene is produced from it by ionization dependent cyclation. Kaurene is then oxidized to

kaurenoic acid. From here, the Steviol glycoside biosynthetic pathway takes a diversion from gibberellin biosynthesis pathway. The next step is hydroxylation of kaurenoic acid to form Steviol (the aglycone) and this is the first committed step for steviol glycoside production [33,34] (Fig. 6).

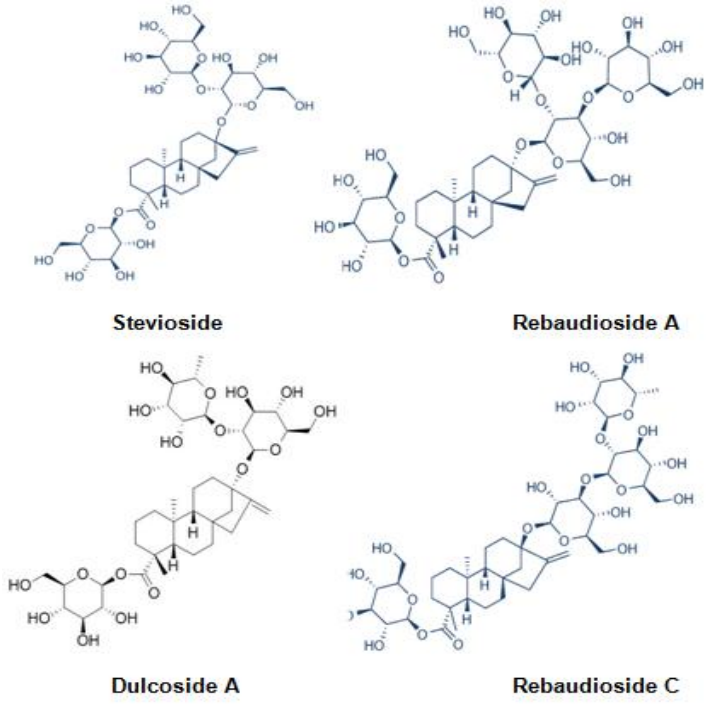

Fig. 4. Structures of four main steviol glycosides
(Source: https://www.selleckchem.com/ https://www.sigmaaldrich.com/catalog/product/sial/30987lang=e&region=LK)[28,29]

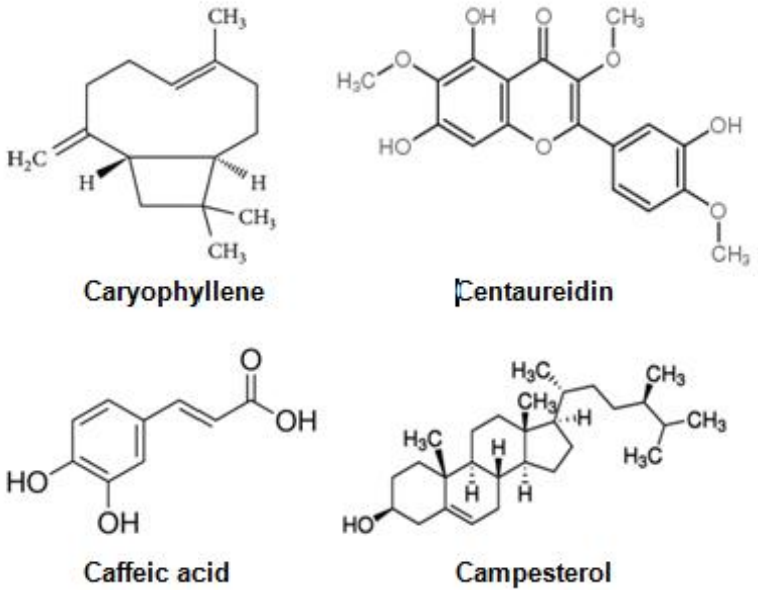

Fig. 5. Chemical structures of few other phytochemicals present in *S. rebaudiana*
(Source: https://www.sigmaaldrich.com/catalog/product/sigma) [32]

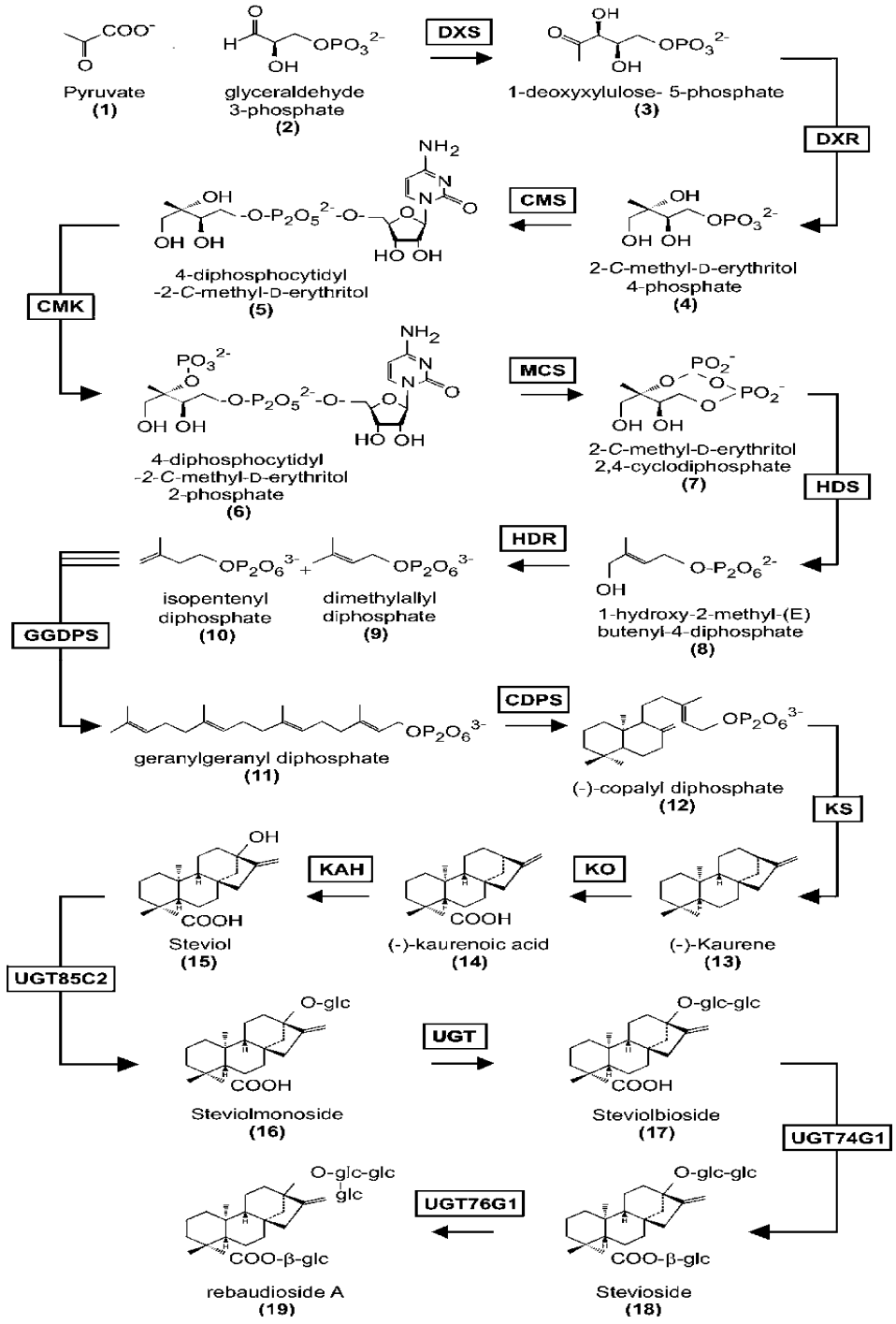

Fig. 6. Steviol glycoside biosynthesis via MEP pathway [33]

Biosynthesis of Steviol glycosides occurred in leaves and the products are transported to various parts of the plant. Hedden and Phillips, [35] suggested that early steps of steviol glycosides biosynthesis pathway occur only in green tissues and it confirmed by Bondarev et al. [36] stating steviol glycosides are only present in chloroplast bearing tissues. Steviol glycoside biosynthesis pathway occurs in different organelles. Initial steps up to Kaurene (the

precursor of diterpenoids) synthesis are occurred in chloroplasts. Kaurene is then transferred into endoplasmic reticulum (ER) and converted to steviol by the activity of enzymes [37] present on ER membrane. Finally steviol is transferred to cytosol and steviol glycosides are synthesized. They are ultimately accumulated in the vacuole [34] (Fig. 7).

Chloroplasts in leaves are playing an important role in steviol glycosides biosynthesis by acting as a precursor. Therefore, Stevia leaves contain more glycosides than other parts of the plant [11]. Due to the fact that chloroplasts play an important role in glycoside biosynthesis, plant parts with low or no chloroplasts contain very low or no steviol glycosides. Different studies confirmed that steviol glycosides mostly present in leaves, small amount in stem and undetectable in roots [38] supporting the above fact. More sweetener content is contained in older leaves than younger leaves, because Steviol glycosides tent to accumulate in tissues as they grow mature. Glycosides content in leaves are decrease with its flowering [4] as it's been used as an energy source for the process.

7. APPLICATION OF *S. rebaudiana*

Stevia is mainly used as a sweetener and flavor enhancer in the food and beverage industry [39]. The health market is second in order of importance. The third most important market is by-products, which consists of the remainder of the plant after the best leaves have been harvested for chemical extraction including steviol glycosides. The remaining parts of the plant, including stems, seeds, flowers and even leaves that were not selected for industrialization, are collected and processed into animal feed or fertilizers [39].

7.1 As a Sweetener and Flavor Enhancer

Stevia has been used for centuries as a bio-sweetener globally in selected countries. White crystalline compound (Steviol glycosides) is the natural herbal sweetener with no calories and is over 100-300 times sweeter than table sugar. The leaves have been traditionally used for hundreds of years in Paraguay and Brazil to sweeten local teas as a 'sweet treat' [40]. People in many parts of Central and South America, where this species is indigenous use Stevia as a sweetener [39,41].

With its extracts having up to 300 times the sweetness of cane sugar, Stevia has garnered attention with the rise in demand for sugar and calorie free food alternatives. As Stevia has a negligible effect on blood glucose, it is attractive as a natural sweetener to people on carbohydrate-controlled diets and people suffering from hyperglycemia [4].

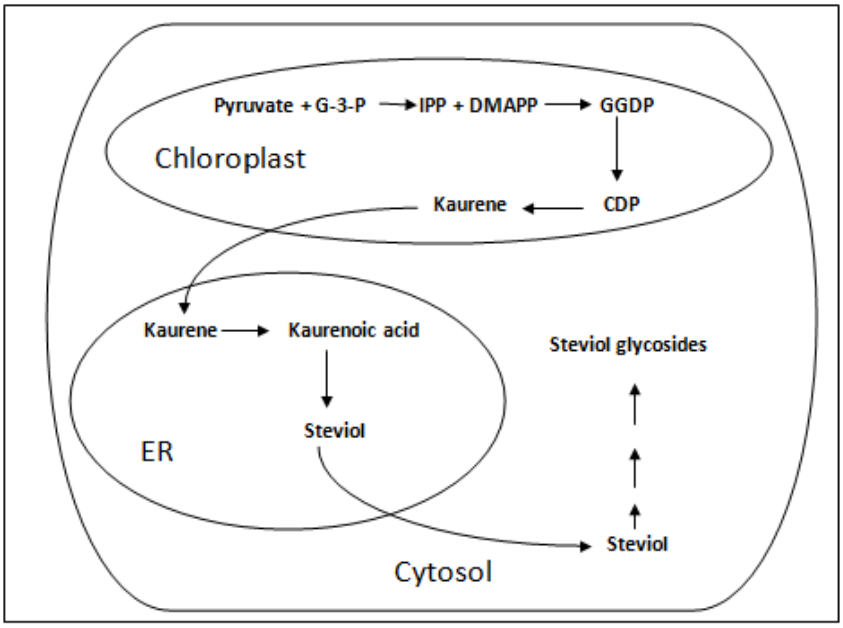

Fig. 7. Steviol glycosides biosynthesis occur in different organelles [17]

In past few years, the use of Stevia as a sweetener and food additive become increases in South-east Asia (Fig. 8). Stevia is commercially grown in Paraguay, Brazil, Central America, Korea, China and Thailand [43]. Currently, the largest consumer of steviol glycosides extracted from Stevia leaves is Japan [44] and according to Goyal et al. [39] Japanese people use Stevia as a sweetener in several products such as seafood, candies and soft drinks. However, in Unites States use of Stevia is limited to supplement status only [1].

7.2 Medicinal Value of Stevia

Medicinal plants are becoming very popular for the treatment of different diseases all over the world. However, Stevia has been used in ancient medical systems for antimicrobial, antifungal, anti-oxidative, hepatoprotective, hypoglycemic, antitumor, anti-hypertension and antiviral activity [19,45]. Goyal et al. [39] stated that, in traditional medicine dried leaves have been used in herbal drinks or chew the leaves as medicine by Paraguayan Gurani Indians as a remedy for certain ailments. Although native Paraguayan has been used Stevia without health problems for many years, there are concerns over health and safety issues of use of Stevia in past two decades. Yet no negative clinical outcomes are reported in the Pacific Rim countries like China, Korea and Japan where Stevia is regularly used in preparation of food and pharmaceutical products without [46].

In addition to steviol glycosides, other metabolites such as β-carotene, thiamin, various terpenes, austroinullin, riboflavin and flavanoids play an important role in medicinal purpose [34,47]. According to World Health Organization (WHO), Stevia has been recommended for treatments against diabetes, blood pressure, stomach infections, obesity and also to avoid dental caries [48].

Type 2 diabetes usually occurs due to the body becoming resistant to insulin or when the pancreas is unable to produce sufficient amount of insulin. Steviol glycosides reactivate the glycogen synthase system to improve insulin secretion in abnormality of liver glycogen synthesis (glycogenolysis) in diabetes [40,49,50]. Stimulation of insulin secretion via a direct action on beta cells occurred by Stevioside and Steviol because of its long lasting reversible insulinotropic effect in the presence of glucose [40]. Studies on animal models revealed that steviol glycosides not only enhance insulin secretion but also regulate utilization of insulin in insulin deficient animals [51]. Therefore, it is used to control blood glucose level and is also used as a digestive tonic. Stevia leaves contain approximately 10% of Steviosides which are intensely sweet compounds however, due to the anti-hyperglycemic glucogonostatic and insulinotropic effects it brings hope to diabetic people who have craving for sweets [52-54].

Stevia reported to decrease in blood pressure through vasodilatory actions by relaxing the muscular walls of arteries [55] and also dose dependently relaxes the vasopressin- induced vasoconstriction [56]. Apart from the direct effect on the cardiovascular system it causes diuresis and natriuresis per milliliter of glomerular filtration rate [57]. Steviol glycosides have hypolipidaemic effect and used to reduce cardiovascular diseases by inhibiting angiotensin- II-induced cell proliferation and endothelin -1 secretion via attenuation of reactive oxygen species [58]. According to Sharma and Mogre, [59], cholesterol level in human is reduced by consuming Stevia extracts. It was observed the level of cholesterol, triglyseride and low-density lipoprotein cholesterol were reduced while increasing desirable high-density lipoprotein cholesterol in the presence of Steviol glycosides [60].

Several studies concluded that steviol glycosides play an important role in preventing dental cavities. When compare the amount of sugar and Stevioside as a sweetener, required level of Stevioside is rather low. Matsukubo and Takazoe, [61] stated that, both Stevioside and Rebaudioside A are non-cariogenic sweeteners. Thus they can be used as a substitute for cariogenic compounds present in sucrose [62]. Also steviol glycosides have antibacterial effect and it helps to reduce the dental cavity forming bacterial growth [34]. So by reducing the level of consumption of sugar and also antibacterial activity, Stevia reduces the dental cavity formation. Thus this could possibly has a potential to use in tooth paste manufacturing industry.

Stevia extracts are potent anti-rotavirus inhibitors which inhibits the replication of all four serotypes of Anti-human rotavirus (HRV) by blockade of the virus binding reducing the progression of the diarrhea [63,64]. As Steviol glycosides have antibacterial and antiviral effects [65] it acts as an anti-diarrheal agent by helping to prevent

Fig. 8. Application of Stevia as flavor enhancer in different products
(Source: https://sweetleaf.com/sweet-products/) [42]

intestinal damage and malfunctioning caused by diarrhea [66]. According to Chatsudthipong and Muanprasat, [67], steviol glycosides also decrease the loss of intestinal fluid by inhibiting the factors targeting chloride channels activate by bacterial enterotoxins. Singh and Rao, [11] stated that Stevia tea is used to manage weight, proper digestion and as an appetite stimulant.

Steviol glycosides are calorie free sweetener and act as a sugar substitute. Therefore it is used to treat people with obesity which caused by consumption of excessive amount of sugar [68]. Steviol glycosides are not metabolized in body to produce energy and they are used for weight control and weight loss by controlling calorie intake [69]. Jain et al. [70] stated that cravings for sweet foods can be reduce by consumption of Stevia extracts.

Leaf extracts of Stevia has shown anti-tumor property. Polyphenolic compounds present in leaf extracts have inhibitory effects on tumor promotion and initiation due to the activity of labdane sclareol present in Stevia leaf extract [71]. Steviol glycosides and its aglycone steviol have the ability to inhibit tumor promotion and formation [72] and inhibit DNA replication in cancer cells grown in vitro [73]. The leaves of Stevia inhibit oxidative phosphorylation inducing ATPase, NADH-oxiade, succinate- oxidase, succinate dehydrogenase and glutamate dehydrogenase activity in the mitochondria causing an antioxidant effect. The ADP/ oxygen ration decreases and substrate respiration increases at low concentrations and at higher concentrations causing complete inhibition [74].

8. ASSOCIATED RISKS OF THE USE OF STEVIOL GLYCOSIDES

S. rebaudiana sweeteners are used sparingly and there seems to be no threat to public health, although caution should be taken at daily intake levels. Despite their widespread use in several parts of the world, no evidence of adverse reactions due to the ingestion of *S. rebaudiana* extracts by humans has appeared in the biomedical literature [75]. Mutagenicity and chromosomal effects on cultured human lymphocytes with Stevioside and steviol has been tested and no mutagenic or clastogenic effects were observed [76]. Food and Agriculture Organization (FAO) of the United Nations, the WHO and the Food and Drug Administration (FDA) claimed that high purity Stevia extract is safe for consumption by the general public when consumed within the recommended levels. The Dietary Supplement Health and Education Act (DSHEA) passed in the US in 1994 also approved steviol glycosides to be used as a functional ingredient in dietary supplements [77]. Use of steviol glycoside as an artificial sweetener in various foods has been approved by FDA by the minutes of the Tenth Meeting of Food Authority held on 20th September, 2012 [55]. According to European Food Safety Authority (EFSA) Stevioside and Rebaudioside A do not show evidence of genotoxicity *in vitro* or *in vivo*. They also stated that the results of toxological testing indicated that steviol glycosides are not genotoxic, carcinogenic, nor associated with any reproductive or developmental toxicity. However, some Stevia products contain added sugar alcohols that may cause unpleasant symptoms in

individuals that are very sensitive to the chemicals. Although hypersensitivity to sugar alcohol is rare, its symptoms can include: nausea, vomiting, cramping and indigestion. Thus it is recommended to use purified forms of steviol glycosides in foods and beverages.

According to US FDA Stevioside and Rebaudioside A; main components in steviol glycosides, have an ADI of 25 mg/kg in rats which is 7.9 mg/kg in humans [78]. According to Carakostas et al. [79] consumption of 5-6 mg/kg of Stevia extracts daily as a dietary sweetener is reported to be safe. Joint FAO/WHO Expert Committee on Food Additives (JECFA) [80] conducted a thorough scientific review of all the available scientific data and concluded Stevia sweeteners are safe for use in foods and beverages and an acceptable daily intake of steviol glycoside is up to 4 mg/kg of body weight was recommended [55].

9. RELATIONSHIP BETWEEN STEVIOL GLYCOSIDES AND CARDIAC GLYCOSIDES

Glycosides are molecules in which a sugar is bound to another non sugar functional group via a glycosidic bond. The sugar group is known as the glycone and the non-sugar group as the aglycone or genin part of the glycoside [81]. Cardiac glycosides have steroidal nucleus as the aglycone part [82] while steviol glycosides have steviol as the aglycone part. Cardiac glycosides are compounds that are derived from the foxglove plants such *as Digitalis purpurea* and *Digitalis lanata* [83,84]. Their therapeutic value was first described by William Withering in 1785. Initially, they were used to treat edema and experiments found that they were most useful for edema that was caused by a weakened heart (i.e., heart failure) [83]. Therefore Cardiac glycosides are used in the treatment of heart diseases such as congestive heart failure and cardiac arrhythmias. However, their relative toxicity prevents them from being widely used [85].

10. CULTIVATION OF STEVIA

Stevia is a semi humid sub tropical plant. The suitable climatic condition for optimum growth can be considered as semi humid, with temperatures ranging from 6 to 43 □C, with an average of 23 □C, and rainfall ranging from 1500 to 1800 mm per annum [78]. The best time for crop transplantation is February to March and seeds can be collected in late summer. Under these conditions flowering occur between 50-100th day. It can be varying with day length and cultivars used [1]. Leaves are harvested prior to flowering stage where steviol glycoside content is maximum. Long day environment is best for high steviol glycoside yield; because it longer the vegetative period by delaying flowering and it allows more time for glycoside accumulation [86]. Stevia plant production can be done by seed germination, vegetative propagation with limited success and using tissue culture techniques [34].

10.1 Seed Germination for Mass Propagation

Stevia has two types of seeds, brown and black in color [14]. Seeds produced without fertilization are brown in color and they are non-viable while viable seeds with more germination potential are black in color [34]. Reproduction in wild is mainly occurred by seeds, however seed germination is often poor and unsuccessful. Propagation through seeds is not a common method owing to the problem of low seed production, low viability and poor germination capacity [87].

10.2 Vegetative Propagation

Some plant varieties produce virtually no viable seed therefore vegetative propagation is the only possibility of multiplication. Stem cuttings are usually used for the propagation of Stevia, but require high labour inputs [4]. Although rooted plants were established, survival percentage was low. The direct planting of stem cuttings in the field was found to have a limited success due to poor rooting [88]. Stem cuttings are dipped in fungicides and rooting media prior to planting in soil keeping two or three nodes above the soil [12]. Several factors affect the sprouting percentage and shoot growth; location of the plant in which cuttings are taken, length of the cutting, number of internodes in cuttings and also season [10]. It is recommended that cuttings 15 cm long with four internodes taken from top part of the main shoot on February are suitable for vegetative propagation [89]. According to Gantait et al. [90] and Philippe et al. [91] the main limitation of vegetative propagation is obtaining quality planting materials due to less number of individuals multiply from a single cutting.

10.3 Tissue Culture of *S. rebaudiana* for Mass Propagation

Due to the limitations in Stevia propagation through seeds and stem cuttings, suitable alternative method for large scale Stevia production within a short period is the use of *in vitro* culture technology. Several studies have been conducted on miccropropagation of *S. rebaudiana* over the past several years. Protocols have been developed for regeneration of *S. rebaudiana* plantlets in vitro through organogenesis (direct organogenesis or indirect organogenesis from callus derived from leaf segments and internodes) using leaves, nodal segments, internodes and shoot apex as explants [92-94].

Several studies have been carried out on indirect organogenesis where calli derived from different explants including leaves, nodal segments, internodes, flowers and anthers [46,95-97]. Uddin et al. [46] reported the use of *S. rebaudiana* nodal segments, internodes and leaf discs for establishment of callus cultures. Callus initiation from internodes was earlier than nodal segments and leaf discs. Also the best medium for callus initiation was MS medium with 3.0 mg/L 2, 4-D and callus initiation decrease with higher concentrations [46].

However, leaf discs cultured on MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA found to be the best for callus induction of Stevia [98]. Sharma et al. [99] reported an efficient callus regeneration protocol through leaf disc explants in MS medium supplemented with various concentrations and combinations of 2,4-D, BAP, Kin and NAA. The best combination was MS medium supplemented with 1.0 mg/L NAA and 1.0 mg/L 2,4-D. In another study callus was induced from leaf disc explants on MS medium supplemented with BAP, 2,4-D and NAA in different concentrations. From them leaf discs cultured on MS medium supplemented with 1.0 mg/L BAP, 1.0 mg/L NAA and 2.5 mg/L 2,4-D found to be the best medium and they produced friable green calli with the highest biomass [100]. According to Gupta et al. [95], leaf discs were the best explants for callus initiation and the best growth regulator combination was NAA and 2,4-D. The best growth regulators combination for callus initiation was 2,4-D with Kin and BAP with NAA was best for callus maintenance [96].

Direct organogenesis is another method for mass propagation of Stevia using different explants. There are number of reports on direct organogenesis of *S. rebaudiana*. In direct organogenesis shoot buds were directly induced from explants without an intermediate callus. Number of reports are available on the use of nodal explant for direct organogenesis of Stevia [48,97,101,102,103]. Singh et al. [7] stated that the most important factor affecting shoot multiplication is the type of cytokinin used in the medium. According to Sivram and Mukundan, [92] higher shoot multiplication on shoot apex and leaf explants was observed at high BAP concentration. However, medium supplemented with Kin resulted in elongated shoots.

Effect of different concentrations of BAP in combinations with various auxins (NAA, IAA, IBA) on direct multiple shoot bud regeneration was studied [102]. BAP with IAA found to be the best combination for shoot bud regeneration at the level of 1.0 mg/L BAP and 0.5 mg/L IAA. Another study suggested that the size of the shoot tip and number of leaf primordia present can also affect the multiple shoot regeneration [92].

According to Sreedhar et al. [104] shoot buds were directly induced from the midrib of Stevia leaf explants when they were cultured on medium supplemented with BAP and Kin. Also shoot buds can be regenerated from the margins of Stevia leaves when they were cultured on media with high concentrations of Kin [7]. However, direct shoot induction from leaf discs and internodes found to be less compared to the nodal segments [93,94,105]. Gunasena and Senarath, [16] stated that high number of shoots were directly induced from nodal segments when they were cultured on MS medium supplemented with 2.0 mg/L BAP Fig. 9.

Rooting and hardening of tissue-culture-raised *S. rebaudiana* plantlets can be achieved by using full or half strength of MS medium supplemented with different concentrations of IBA and NAA [7]. IBA showed significant and more effective root induction than NAA in all tested concentrations. According to Ahmed et al. [97] maximum root induction was observed on medium supplemented with 0.1 mg/L IAA and was observed that root induction gradually decreased with increasing concentrations [101].

When well developed and well rooted in vitro raised plantlets acclimatized, after one month growth lesser amount of total chlorophyll and total carotenoid contents were observed compare to naturally grown plants. However after

three months growth both total chlorophyll and carotenoid contents were comparatively higher than those of naturally grown plants [17]. When compared the Stevioside content of naturally grown plants in different growth stages; before flowering (one month old), ontime flowering (three months old) and after flowering (four months old), highest Stevioside content was observed in three months old ontime flowering plants. Therefore it could be suggested that ontime flowering is the best growth stage to harvest Stevia leaves to obtain high content of Steviol glycosides. Leaf extracts of three months old naturally grown and tissue cultured plants were analyzed using HPLC to compare the Steviol glycosides content (Rebaudioside A, Stevioside, Rebaudioside C and Dulcoside A) and according to results highest contents were observed in tissue cultured plants. These results supported the fact that there is a positive correlation between chlorophyll content and the secondary metabolites synthesis especially Steviol glycosides [17].

11. CELL SUSPENSION CULTURES OF *S. rebaudiana* FOR SECONDARY METABOLITE SYNTHESIS

The callus and cell cultures have a higher rate of metabolism than intact plants due to the fast proliferation of cell mass that results in a condensed biosynthetic cycle. The ability of these cells seems to be important for the formation of the secondary metabolites. There are several records on Stevia cell suspension cultures.

Sharma et al. [99] reported that cell suspension cultures were initiated with callus induced on MS medium supplemented with 1.0 mg/L NAA and 1.0 mg/L 2,4-Dtransferring into a liquid MS medium supplemented with the 2,4-D, BAP and ascorbic acid. The media supplemented with ammonium nitrate in addition of 4.5% sucrose showed the highest cell growth response on twentieth day.

According to Bondarev et al. [106] cell suspension cultures synthesized only minor amounts of the steviol glycosides, and their content varied greatly during the growth cycle of the culture. The amount of steviol glycosides in the cell suspension cultures (15 µg/g Stevioside, trace amount of Rebaudioside A and 0 µg/g Rebaudioside C) were very low compared to the mother plants (3300 µg/g Stevioside, 1900 µg/g Rebaudioside A and 700 µg/g Rebaudioside C). However in contrast to that, Javad et al, (2014) [100] concluded that callus cultures can grow into cell suspension cultures and can be exploited for commercial scale production of Stevioside. To establish cell suspension cultures, callus induced from leaf disc explants on MS medium supplemented with 1.0 mg/L BAP, 1.0 mg/L NAA and 2.5 mg/L 2,4-D were used. Cell suspension cultures of S. rebaudiana were also established on same media with optimum conditions of pH 5.5, and subculturing interval of 30 days [100].

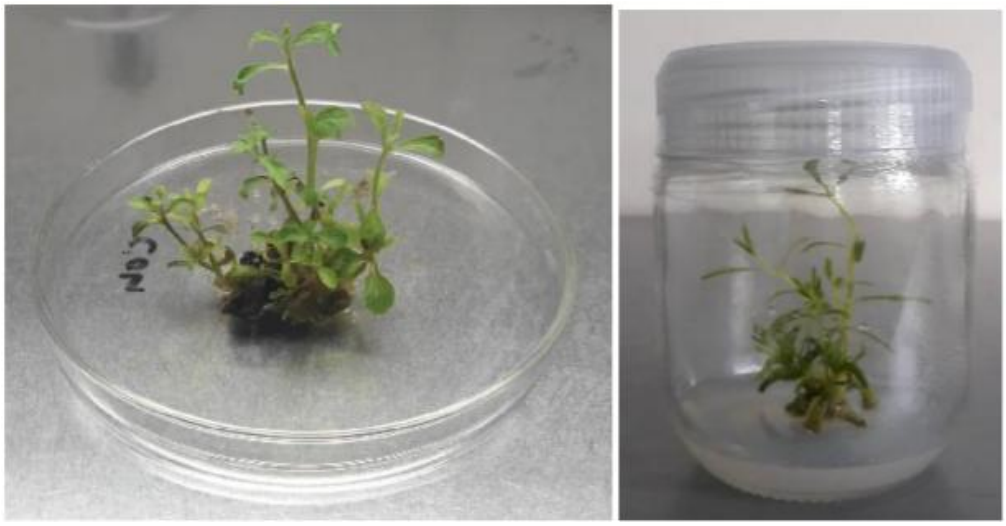

Fig. 9. Multiple shoots obtained from nodal segments cultured on MS medium supplemented with 2.0 mg/L BAP [16]

12. PHYTOCHEMICAL ANALYSIS OF *S. rebaudiana*

12.1 Phytochemical Extraction of *S. rebaudiana*

Extraction and purification method affect the distribution of steviol glycosides in extracts [39]. Some Stevia extracts have bitter aftertaste and out of major glycosides least bitterness is reported in Rebaudioside A [107]. Although leaves are the main choice of plant parts used to extract steviol glycosides, they contain high amount of moisture [108] and have to dry before extraction. Gonzalez et al. [109] applied four different drying methods (radiation, convection, shade and sun drying) on *S. rebaudiana* leaves and the aqueous extracts were analyzed to quantify the steviol glycosides. According to their results highest steviol glycosides content was in sun dried samples (17.29 g/100 g) and lowest was in radiation dried samples (14.34 g/100 g).

There are several methods for extraction and purification of Steviol glycosides namely; solvent extraction, microwave assisted extraction, high pressure liquid extraction (PFE), supercritical fluid extraction and enzyme assisted extraction. In solvent extraction method, glycosides are initially extracted into hot water followed by extracted into a water immiscible organic solvent such as chloroform or hexane. The organic phase is separated, concentrated to obtain the solid mass and it is dissolved in hot methanol. During cooling, Steviol glycosides crystals are formed and washed with cold methanol. Finally they are recrystallized from methanol or water to obtain high purity steviol glycosides. It contains 97-98% of steviol glycosides and about 4% water [22].

12.2 Phytochemical Screening of *S. rebaudiana*

For phytochemical screening mainly two techniques are used in the previous studies. High Performance Liquid Chromatography (HPLC) technique was used for identification of Steviol glycosides and the Gas chromatography-mass spectrometry (GC-MS) has been used for identification of other phytochemicals. Other than that, enzyme hydrolysis, capillary electrophoresis, Thin Layer Chromatography (TLC), Liquid Chromatography (LC) coupled with fluorescence or ultraviolet are also been used. Recommended method for determination of Steviol glycosides is HPLC and therefore it is the most commonly used analytical method [1,110].

Typically, Polar amine or C18 column is used to determine the steviol glycosides by HPLC. According to the monograph published by JECFA [80], steviol glycosides can be determined by UV detection at 210 nm using 70:30 deionized water: acetonitrile using as the mobile phase. Gonzalez et al. [111] used aqueous leaf extracts for determination of minor glycosides in *S. rebaudiana*. HPLC method was performed using C18 column (250 × 4.6 mm), UV detector set at 210 nm and mobile phase consisted of 32: 68 (v/v) mixture of acetonitrile and sodium phosphate buffer. Retention time for Rebaudioside C and Dulcoside A were observed as 9.52 min, 10.30 min and 2.24 ± 0.04 g/100g Rebaudioside C and 0.61 ± 0.04 g/100g Dulcoside A was obtained. Jadhao et al. [112] also used aqueous extracts of dried Stevia leaves to determine the Stevioside content and obtained 7.87 ± 0.08 % and 7.50 ± 0.04 % w/w.

Other than steviol glycosides which gives the sweetness and determined by HPLC, *S. rebaudiana* extracts consists of sesquiterpenes, alcohols, labdanic diterpenes, aliphatic hydro carbons, sterol, essential oils andtriterpenes [113]. Separation and analysis of these compounds can be performed by GC-MS. The chemical composition of stevia leaf extracts analyzed using GC-MS. Markovic et al. [114] reported the presence of fatty acids, alkanes, aldehydes, ketones, terpenes, sterols etc. in ethyl acetate extracts of leaves. According to Maheshwari et al. [115] different phytochemicals have been identified in stevia by GCMS including saturated fatty acid, ester and hydrocarbon, which are used in various industries and ailments such as solid detergent, flavouring agent, lubricants, transformer oils, anti-corrosion agents, plasticizers, ingredient in resins, perfumes.

13. CONCLUSIONS

Popularity of *S. rebaudiana* has developed in many countries in past few years due to the production of non caloric sweet steviol glycosides. It has many therapeutic values such as anti-cancer, anti-hypersensitive, anti-hyperglycemic and anti-microbial activity due to the presence of Steviol glycosides and other important phytochemicals. Propagation through seeds is not a common method owing to the restrictions in seed production, low viability and

poor germination capacity etc. Vegetative propagation is usually done by stem cuttings, but required high labour inputs and the survival percentage is very low. Therefore micropropagation, or *in vitro* culture appears to be the best method to overcome those problems. HPLC is the common most analytical method used to screen steviol glycosides in Stevia extracts and other phytochemicals are screened by GC-MS.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable

ETHICAL APPROVAL

It is not applicable

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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