Türk Bilimsel Derlemeler Dergisi
E-ISSN: 2146-0132 13(2): 126-138, 2020
Derleme Makalesi

A COMPREHENSIVE OVERVIEW OF *Stevia rebaudiana* AND ITS SECONDARY METABOLITE SWEETENERS

Shuchita Vaghela[a*], Anjali Soni[b]

Department of Biotechnology, Veer Narmad South Gujarat University, Udhana Magdalla Road, Vesu Surat. 395007 (Gujarat) India

Corresponding Author:

E-mail: shuchi.amin@yahoo.in

(Received 12th April 2020; accepted 25th August 2020)

a: ORCID 0000-0002-5565-7566 b: ORCID 0000-0001-7424-9157

ABSTRACT: *Stevia rebaudiana*, member of an Asteraceae family, has a unique property of sweet leaves. The sweetness is because of the secondary metabolite produced, which is a glycoside. Among11 glycosides, primary sweetening agents are Stevioside, rebaudioside A and C. Production of these glycosides are very less in vivo hence there are several attempts performed where these are produced *in vitro*. Various extraction procedures had been applied for maximum steviol glycosides extraction. Further purification and analysis techniques are also used to the samples for having pure steviol glycosides. Previous studies indicated various ill effects of steviol glycosides on human health. Later on, it was proven safe for human consumption in its purest form. Further researches for finding new steviol glycosides are still going on. However, even much work is needed for its biotechnological production. This literature focuses on the work done of *in vitro* production, extraction and health effects of steviol glycosides.

Keywords: Stevioside, Rebaudioside A, Extraction, Purification, Health effects

INTRODUCTION

Stevia rebaudiana, a member of Asteraceae family one of the 154 members, is also known as *Stevia*, Sweetleaf, Honey leaf and Candy leaf. It is also known as the sweet herb of Paraguay as after much dormancy Dr M.S. Bertoni again studied it in 1988 at Paraguay [1]. *Stevia rebaudiana* is a perennial shrub attaining the height of about 1 m and bears 2-3 cm long elliptical, sessile and oppositely arranged leaves. It can be grown in the kitchen garden. The optimum pH requirement is 6.5-7.5, well-drained sandy loam and red soil [2]. It has a weak and woody stem at the bottom and slightly branched roots bearing composite flowers surrounded by an involucre of epicalyx. Flowers are light purple and are pentamerous [3](Madan et al., 2012). Among their 152 co-family members, only two species are found to produce sweet steviol glycosides, which are used as food additives and non-caloric sweeteners in Brazil and Japan [3]. It is proven that steviol glycosides are 300 times sweeter than conventional sugar's 0.4% solution [4]. The chief components of steviol glycosides include Stevioside, steviolbioside, rebaudioside A, B, C, D, E, F and dulcoside A [5, 6](*Medicinal and Aromatic Plants — Industrial Profiles*, 2002). Recently a new glycoside is discovered named rebaudioside M [7].

Stevia is naturally grown in semi-humid sub-tropical regions of 200-400 meters above sea level. Average rainfall for cultivation should be 1500-1800 mm, and temperature conditions are from -60 to 43ºC. *Stevia rebaudiana* is indigenous to Central and South America [8] as well as in Japan [9].

SECONDARY METABOLITES OF *STEVIA REBAUDIANA*

It is found to be proven that the leaves of *Stevia rebaudiana* are the chief source of secondary metabolites. Various qualitative and quantitative analysis of *Stevia* leaves reveals the following data of their composition, macro and micro mineral contents and secondary metabolites. Yadav, S. K., & Guleria, P. (2012) has reported protein to be 20.42%, carbohydrates to be 35.20%, fat to be 4.34% and Ash content to be 13.12% on a dry weight basis., macro elements are Potassium, Calcium, Magnesium, Phosphorus, Sodium and Sulphur and microelements are Copper, Cobalt, Manganese, Zinc, Selenium, and Molybdenum [10].

- The phytochemical analysis reveals the presence of following in the plant:
- Tannins, Alkaloids, Glycosides, Saponins, Sterols and Triterpenes, Anthraquinones [11].
- Different types of diterpenoids, such as non-kaurenoicditerpenoids, labdanesclareol are also present [12].
- Phytosterols are found in the wax of the *Stevia* leaf [13].

STEVIOL GLYCOSIDES AND THEIR BIOSYNTHETIC PATHWAYS

Steviol glycosides render the immense sweetness to the plant leaves. Chief SGs are Stevioside, and rebaudioside A. Their amounts in the leaves from various studies are as Stevioside: 4-14%, Rebaudioside A: 2-4%, Rebaudioside C: 1-2%, Dulcoside A: 0.4-0.7%, Rebaudioside D, E, F; steviolbioside; rubusoside: >0.4%.

SGs are specifically tetracyclic diterpenoids, highly sweet, non-toxic and non-mutagenic [14]. The leaves of *Stevia rebaudiana* have almost nine types of different SGs, and each of them is formed of varying glycosylation and hence possesses distinct organoleptic properties. There is much difference in the sweetness of Stevioside and rebaudioside A [15]. Rebaudioside A is sweeter and less bitter [16]. After extensive functional analysis [17] prove that SGs synthesis is restricted to the green tissue.

Total of 16 enzymes is identified for SGs biosynthesis. These enzymes are classified into three groups according to the similar pathway they follow in other plants. SGs biosynthesis occurs via three distinct pathways. They are MEP pathway, GA biosynthesis pathway and remaining steps are specific for SG biosynthesis [1]. From the initial seven steps, isopentenyldiphosphate (IPP) and dimethylallyldiphosphate (DMAPP) are synthesized. Pyruvate and Glyceraldehyde 3-phosphate is the precursors for these reactions. These steps are common within the MEP pathway [18]. These IPP and DMAPP are converted into Geranylgeranyl Diphosphate (GGDP). GGDP is converted to kaurenoic acid in the next four steps. These steps are common within the GA pathway [19]. Kaurenoic acid underwent hydroxylation and converted to steviol. From steviol, the biosynthetic pathway diverges, and SG synthesis specific steps are involved [17]. The remaining four steps are catalyzed by UDP-glucosyltransferases (UGTs). Out of these four UGTs, three are identified which converts steviol to steviolmonoside, steviolbioside to Stevioside and Stevioside to rebaudioside A. one UGT which converts steviolmonoside to steviolbioside is not yet identified [17].

SGs biosynthesis also takes place in different locations of the leaf tissue because the associated enzymes are related to the various parts. Formation of kaurene occurs in the chloroplast. The enzymes for the formation of steviol from kaurene are associated with Endoplasmic reticulum. The four UGTs from which 3 UGTs named UGT85C2, UGT74G1, and UGT76G1 are present in the cytoplasm [20].

IN VITRO PRODUCTION OF SGs

Different tissue culture methods had been incorporated for the *in vitro* propagation of *Stevia rebaudiana* which includes somatic embryogenesis [21] cell line development from colchicine-treated diploid cell suspension culture [22] and multiple shoot generation from nodal segments[23]. Totté, N. et al., 2003 has reported that *in vitro* production of Stevioside depends on the biomass yield and cultivation, medium components and osmotic stress [4]. Plant tissue culture is advantageous technique over conventional farming for secondary metabolite production as a minimal amount of plant material is used for quite a high cell growth and biogenesis and desired product may be produced in a notably short period. Efforts have been made to develop suitable media for the production of biomass, Stevioside and rebaudioside A in callus as well as in cell suspension culture. In a study of media standardization by changing its components and its strength wherein various media formulation such as Nitsch media, Murashige and Skoog media, Linsenmayer and Skoog media and Gamborg's B5 media with multiple concentrations of auxins and 2,4-D was examined for sustainable growth [24]. Media composition for different carbon sources was also discussed. Different sugar viz. glucose, sucrose, galactose, and fructose were examined for carbon utilization in Stevioside production [25]. Babu P. et al., 2011 has done compiling work on above and concluded that explants cultured on the half-strength MS medium containing 3% sucrose accumulated higher biomass and produced maximum Stevioside followed by glucose [26].

Researchers have also examined the effects of different stress conditions. Effect of salts particularly NaCl and Na_2CO_3 on callus and suspension culture for SGs production experiment reveals that the quantity of SGs increased by 1.43 and 1.57% with 0.10% NaCl and 0.25% Na_2CO_3 respectively compared to 0.27% in control in callus. Whereas 2.61% and 5.14% in cell suspension culture compare to 1.36% in control [27]. Effect of drought stress has been examined by using proline and polyethylene glycol. Proline plays an important role in the osmotic adjustment in osmotically stressed plant tissue and the protection of plasma membrane integrity. At the same time, PEG is a non-ionic water polymer and used *in vitro* to induce water stress in plants. The highest amount of fresh and dry weight in callus and suspension culture was obtained with 5mM proline, and 5% PEG and SGs content increased from 0.27 (control) to 1.09% and 1.83% with 7.5mM proline and 5% PEG in callus and from 1.36% (control) to 5.03% and 6.38% in suspension culture [28]. It was also proven that SGs are synthesized in green tissues; hence, it is imperative to have green callus for the initiation of cell suspension culture for the biosynthesis of SGs. For obtaining such green callus, several experiments have suggested various combinations of plant hormones. The study suggests that MS medium fortified with combinations of BAP, NAA and 2,4-D produce green callii [29].

Newly emerged method of tissue culture, namely BIT®, had also been employed for the production of biomass as well as total Stevioside. From the study, it was concluded that morphological quality was best and fresh and dry weight was seen seven times in BIT® compare to the semi-solid or liquid medium. The total Stevioside quantity was also higher in a bioreactor [30].

An overall brief review of the *in vitro* culture studies by far of *Stevia rebaudiana* for various purposes are listed below in chronological order. (Table 1)

Table 1

Year of research	The technique used or treatment given	Result of the study	Reference
1981	Callus induction from leaf explants using the different concentration of IAA, BA, Kinetin, Zeatin, 2- ip and 4-PU	Medium supplemented with 0.1 mg/l of NAA and 10.0 mg/l of 2- iP, the highest callus formation and 4- PU induced root formation.	(Wada et al., 1981)
1990	Establishment of multiple shoot culture from nodal segments with NAA and BAP and subculturing them in a bioreactor	Seeds were cultured on ½ MS+0.01mg/L NAA then multiple shoots were obtained in MS + 2.1 mg/L BAP and then 2.0 g of above culture was added to liquid hormone-free MS medium in bioreactor and the hardened on Sand: vermiculite (1:1)	[23](Abeyaratne & Bandara, 1990)
1994	Mass propagation of shoots of *Stevia rebaudiana* using a large scale bioreactor	Shoot primordia were cultured and multiplied in liquid shake cultures in MS medium containing 0.1 rng/1 NAA, 1 mg/l BA from which 10 g/L primordia was inoculated in a bioreactor which resulted in 64.6 kg fresh weight shoots, and then 90 % of them were acclimatized in soil.	[25]
2001	Comparative study of the production of steviol glycosides in in vivo plants, *in vitro* callus culture of stem and leaf and suspension culture, was done.	The highest amount of total steviol glycosides were found in leaves of *in vitro* plants viz., 5900 µg/g dry weight and the lowest amount was in dedifferentiated callus viz., NIL.	[65]
2003	Effects of different concentrations of different cytokinin and Auxin was examined for *in vitro* culture of *Stevia rebaudiana*	Maximum shoot generation was achieved with 8.87 µM BA, and 5.71 µM IAA and the maximum rooting response was shown in half-strength MS medium supplemented with 4.90 µM IBA giving 12-13 thick roots per shoot and cocopit was found to be most effective for acclimatization.	[66]
2007	Development of a method for clonal propagation of *Stevia rebaudiana*	Highest multiple shoots were induced in the MS medium supplemented with 1.5 mg/L BA and 0.5 mg/L Kinetin whereas highest rooting response (97.66%) achieved on MS medium with 0.1 mg/L IAA.	[75]

2008	Use of cyanobacterial medium for *in vitro* callusing of *Stevia rebuadiana*	Leaf explants of *Stevia rebaudiana* Bertoni induced callusing when putting on a mixture of different cyanobacterial cultures as a medium for regeneration. Maximum proliferation was obtained on media containing cyanobacterial media (5ml/l and 10ml/l) for initiation and maturation medium respectively + Sucrose (3% w/v)+ $CaCl_2$ (0.44mg).	[67]
2009	Indirect organogenesis studies on leaf derived callus of *Stevia rebaudiana*	Juvenile leaf produced the highest amount of callus on MS medium supplemented with 11.31 µM 2,4- D and 2.22 µM BAP. Highest shooting response from this callus was achieved with MS medium supplemented with 4.44 µM BAP and 1.34 µM NAA in 28 days to which highest rooting response was achieved with half-strength MS medium supplemented with 2.46 µM IBA	[68]
2011	Incorporation of a roller bottle system for the establishment of adventitious root culture of *Stevia rebaudiana* Bertoni	Adventitious roots were induced on Murashige and Skoog (MS 1962) media supplemented with 10.7 µM of NAA. These cultures were successfully maintained in the same medium for six months with regular subcultures after four weeks. After that, the 1.0 to 1.5 cm long segments of the roots were transferred to the roller bottle system containing a fresh root inducing liquid MS medium supplemented with 10.7 µM NAA. In this study, the best conditions for cultivation were investigated, considering culture volume (25 ml), culture period (4 weeks), salt concentrations in the nutrient medium (33%) and optimal initial inoculum (0.2 g) of S. rebaudiana roots.	[69]
2011	Optimization of media composition and strength for maximum biomass and stevioside contents	MS medium supplemented with 2,4-D was found to best for callusing. Stevioside content was not found to be dependent on the strength of the medium, but the highest biomass was achieved on half strength.	[26]
2012	Development of an efficient method for micropropagation of *Stevia*	Liquid culture MS hormone-free medium with 1% sugar exhibited better multiplication of nodes and as well as increased shoot length as	[70]

	rebaudiana	compare to multiplication on solid medium. The maximum numbers of roots per shoot (21.2) were observed in medium containing half strength of MS salt and 100 ppm charcoal with an average root length of 4.22 cm.	
2014	Study of effects of different concentrations salts (NaCl and Na_2CO_3) on callus and suspension culture of *Stevia rebaudiana* for the steviol glycoside production	Incorporation of sodium salt in growth medium resulted in the inhibition of growth, and at lower concentrations viz., 0.1% of NaCl and 0.025% of Na_2CO_3 resulted in higher level accumulation of stevioside and rebaudioside A both in callus and suspension culture.	[27]
2015	Effects of drought on the production of stevioside and rebaudioside A in callus and suspension culture of *Stevia rebaudiana* by incorporation of proline and polyethylene glycol in growth medium	Highest biomass produced in the callus as well as suspension culture with five mM proline and 5 % PEG. In comparison, maximum production of steviol glycoside in callus and suspension culture was achieved with 7.5mM proline and 5% PEG. The overall amount of steviol glycoside was higher in case of suspension culture then callus culture.	[28]
2016	Effect of salt and drought stress on the production of SGs in *in vitro* generated shoots	NaCl and proline found to be better for shoot induction then Na_2CO_3 and PEG. Highest (2.60%) amount of total SGs (Stevioside and Rebaudioside A) were found with shoots treated with 0.025% Na_2CO_3followed by 5.0 mMProline -1.65%, 0.10% NaCl-1.25% and 5% PEG-1.15%, which were 3.3, 2, 1.6 and 1.5 times higher than control (0.79%) respectively.	[71]
2016	Study on the establishment of callus and suspension culture for production of SGs.	Callus produced with the combination of BAP, NAA and 2,4-D was good to initiate cell suspension culture and gave the highest yield of SGs compared to other treatments used in that study. Callus was generated on MS medium supplemented with 1 mg/L BAP, 1 mg/L NAA and 2.5 mg/L 2,4-D. Suspension culture was also established on the same medium. This treatment gave 33.87 mg per gram of tissue SGs content.	[29]

2016	Study of Plant Growth Regulator Effects on *In vitro* Propagation and Stevioside Production in *Stevia rebaudiana* Bertoni	Researchers suggest plant growth regulator free medium for getting maximum production of stevioside and rebaudioside A. Biomass yield was higher in woody plant medium supplemented with BAP and NAA.	[74]
2017	Study on the effect of zinc oxide (ZnO) nanoparticles on physiology and steviol glycosides production in micropropagated shoots of *Stevia rebaudiana* Bertoni	Maximum shooting response and highest accumulation of SGs was achieved in the MS medium supplemented with 1 mg/L ZnO nanoparticles compared to control and other concentration used.	[72]
2017	Comparative study of different *in vitro* micropropagation methods of *Stevia rebaudiana* B. including temporary immersion bioreactor (BIT®)	Morphological quality of BIT®-derived shoots was best and coupled with shoot fresh and dry weight that was more than seven times higher in BIT® compared with micropropagation in liquid or semi-solid media. In turn, the total content of steviol glycosides produced was also higher in bioreactors.	[30]
2018	Study of the effect of nitrogen and phosphate on *in vitro* growth and metabolite profiles of *Stevia rebaudiana* Bertoni (Asteraceae)	Reduction of nitrogen and phosphate levels to half resulted in tallest shoots. An increased concentration of nitrogen significantly lowered the amount of rebaudioside A accumulation compared to control. The highest amount of stevioside was found in the micropropagated plants on half phosphate MS medium.	[73]

EXTRACTION, ISOLATION, AND ANALYSIS OF SGS

Extraction of SGs from ex vivo and *in vitro* material has always been the subject of interest and extensively studied using different chemical methods to get the purest form of Stevioside and rebaudioside A.

Persinos, G. J., 1980 was the very first attempt recorded for the extraction of SGs in 1973 [31]. The patented invention noted the extraction of SGs from the grounded, defatted and extracted with organic solvents. Afterwards, various patents are given in this field based on the choice of different solvents [32], ion exchange [33], adsorption chromatography [34] and use of solvent plus decolorizing agent [35]. Conventional methods include steps like aqueous or solvent extraction, ion exchange, precipitation or coagulation with filtration followed by crystallization and drying. Reports are there for the pretreatment of the extracts with lime [36]. The advanced techniques applied for extraction of SGs involves supercritical fluid extraction

with CO_2 and co-solvents such as methanol, ethanol and acetone, selective adsorption on zeolites X and A [38], supercritical fluid extraction by two-step process [39], pressurized fluid extraction using water or methanol [40]. More recent techniques developed include advanced techniques like sonication [41], ultrasound-assisted extraction [42], microwave-assisted extraction [43] and pressurized hot water extraction [44]. Use of sonication for extraction gave the highest value of 3561 mg/g of rebaudioside A when power is kept 360 W with time 18 min.

In comparison, ultrasound-assisted extraction gave highest 36.92 mg/g content of rebaudioside A with probe diameter 22 mm and 10 min time. MAE resulted in a total of 2.34%content of rebaudioside A in 1 min time with power 80 W at 50ºC. Above all, the most suitable technique seems to be PHWE as it provides effective green extraction method. From studies, it had been concluded that water changes its polarity at high temperatures and thus can dissolve many nonpolar compounds also. Rebaudioside A can be extracted using PHWE at 100℃, 11-13 bar pressure with 1.5 ml/min flow rate in 15 min.

For determination of distribution and content of SGs in in vivo as well as extracted samples, various analytical methods have been employed. Majority of them are thin layer chromatography [45, 9, 46], overpressure layer chromatography [47], droplet countercurrent chromatography [48] and capillary electrophoresis [49] & [50]. Stevioside and rebaudioside were specifically analyzed using HPLC by converting above to their derivative [51], enzymatically [52], by near-infrared reflectance spectroscopy [53] and HPTLC [43]. The most successful method for analysis of Stevioside and rebaudioside among all is HPLC coupled with mass spectroscopy.

HEALTH EFFECTS OF SGS

As *Stevia* is used in confectionaries and beverages as the natural sweetener with no caloric value in many parts of the world, it has always been the matter of concern that whether it is safe for human consumption. Multiple researchers have examined Its effects on various physiological processes at different time zones.

The significant health associated risk was thought to be associated with carcinogenic properties of *Stevia*. Mutagenic properties of *Stevia* had been examined. Two studies previously have detected mutagenic effects of steviol extracts [54, 55] while in the same study steviosides are found to have no such effects. Later in next year study, both the compounds were found to have no mutagenic effects [56]. However, the final research proves the SGs as non-mutagenic substance [57], and also various studies showed that stevioside has an antiproliferative effect on human colon cancer HT-29 cells [58], CPUK 02 an Ent-kaurenoid derivative of stevioside exhibited demethylating properties and hence exhibited potent anticancer activity in vivo and *in vitro* [59]. *Stevia* was also found safe for consumption by longitudinal tracking of pancreatic acinar carcinoma development, growth, and lethality in a sensitized mouse model [60].

As steviosides are used as artificial sweeteners, one question arises that if they have any effect on pancreatic activity and hence on blood sugar. It was found that stevioside has a blood-glucose-lowering effect. The study on normal mouse islets and the β-cell line INST for stevioside and steviol resulted in enhance insulin secretion in the presence of glucose in both [61]. A study on rebaudioside A proves that in the presence of Ca^{2+}, it induces insulin stimulation at high glucose [62]. Recent studies on TRPM 5, a Ca^{2+} activated cation channel show induced insulin secretion and can prevent type 2 diabetes [63]. In one such research, radioactive glucose uptake assay was implemented to assess improvement in insulin sensitivity in 3T3-L1 cells by elevation of glucose uptake following treatment with stevioside, and the result suggested that stevioside has direct effects on 3T3-L1 insulin sensitivity via an increase in glucose uptake and enhanced expression of a protein involved in the insulin-signalling

pathway [64].

LIMITATIONS AND FUTURE SCOPE OF THE STUDIES

As *Stevia* has been known to the scientific community since long, almost all type of necessary studies related to its physiology, composition, genetic makeup, cultivation and extraction of steviol glycosides have been completed. However, there is scope of transcriptome analysis with the advancement in molecular biology. Transcriptome level studies can add new insights about the change in the concentrations of these important glycosides during biotic and abiotic stress. At present no or less information is available regarding the role of different transcriptional factors, enzymes and hormones in response to different stresses, age of the plant and growth conditions for *Stevia*. Higher Rebaudioside A production at the mass level is still a difficult task due to paucity of standard protocol and analytical method. Despite all these achievements, a fundamental need of biochemical method to check the concentration of steviol glycosides in the extracts is still there.

CONCLUSION

The review presented here focused on the magic herb, its contents and their biosynthetic pathway and biotechnological approach for their production. Stevioside and rebaudioside A has been proved to be safe for human consumption; still, *Stevia* leaf and crude *Stevia* extracts are not included in GRAS (Generally Recognized As Safe) and do not have FDA approval for use in food. Hence there is a great need of having the purest form of safe steviol glycosides- stevioside and rebaudioside A. Moreover conventional extraction using alcohol from in vivo explant do not give desirable purity, and amount of above said SGs as well as not safe for consumption. From the present review, it is concluded that further work is to be done for rebaudioside A as far as its biotechnological production, green extraction and consumption studies are concerned.

REFERENCES

[1] Yadav, S. K., & Guleria, P. (2012): Steviol Glycosides from *Stevia*: Biosynthesis Pathway Review and their Application in Foods and Medicine. Critical Reviews in Food Science and Nutrition, 52(11): 988–998.

[2] Goyal, S. K., Samsher, & Goyal, R. K. (2010): *Stevia* (*Stevia rebaudiana*) a bio-sweetener: A review. International Journal of Food Sciences and Nutrition, 61(1): 1–10.

[3] Madan, S., Ahmad, S., Sing, G. N., Kohli, K., Kumar, Y., S, R., & M, G. (2012): *Stevia rebaudiana* Bertoni : A review. Indian Journal of Natural Products and Resources, 1(September): 267–287.

[4] Totté, N., Van Den Ende, W., Van Damme, E. J. M., Compernolle, F., Baboeuf, I., & Geuns, J. M. C. (2003): Cloning and heterologous expression of early genes in gibberellin and steviol biosynthesis via the methylerythritol phosphate pathway in *Stevia rebaudiana*. Canadian Journal of Botany, 81(5): 517–522.

[5] Medicinal and Aromatic Plants — Industrial Profiles. (2002).

[6] Starratt, A. N., Kirby, C. W., Pocs, R., & Brandle, J. E. (2002): Rebaudioside F, a diterpene glycoside from *Stevia rebaudiana*. Phytochemistry, 59(4): 367–370.

[7] Prakash, I., Bunders, C., Devkota, K. P., Charan, R. D., Ramirez, C., Priedemann, C., & Markosyan, A. (2014): Isolation and characterization of a novel rebaudioside M isomer from a bioconversion reaction of rebaudioside A and NMR comparison studies of rebaudioside M isolated from *Stevia rebaudiana* Bertoni and *Stevia rebaudiana* Morita. Biomolecules, 4(2): 374–389.

[8] Melis, M. S. (1992): Renal excretion of stevioside in rats. Journal of Natural Products, 55(5): 688–690.

[9] Kinghorn, A. D., Soejarto, D. D., Compadre, C. M., & Makapugay, H. C. (1984): A phytochemical

screening procedure for sweet ent-kaurene glycosides in the genus *Stevia*. Journal of Natural Products, 47(3): 439-444.
[10] Yadav, S. K., & Guleria, P. (2012): Steviol glycosides from *Stevia*: biosynthesis pathway review and their application in foods and medicine. Critical reviews in food science and nutrition, 52(11): 988-998.
[11] Manish, T., & Subhash, R. (2006): Preliminary studies on Stevia Rebaudiana leaves: proximal composition, mineral analysis and Phytochemical Screening. J. Med. Sci, 6: 321-326.
[12] Kaushik, R., Narayanan, P., Vasudevan, V., Muthukumaran, G., & Antony, U. (2010): Nutrient composition of cultivated *Stevia* leaves and the influence of polyphenols and plant pigments on sensory and antioxidant properties of leaf extracts. Journal of Food Science and Technology, 47(1): 27–33.
[13] Markovi, I. S., Darmati, Z. A., & Abramovi, B. F. (2008): Chemical composition of leaf extracts of *Stevia rebaudiana* Bertoni grown experimentally in Vojvodina. Journal of the Serbian Chemical Society, 73(3): 283–297.
[14] Bondarev, N. I., Sukhanova, M. A., Reshetnyak, O. V., & Nosov, A. M. (2003): Steviol glycoside content in different organs of Stevia rebaudiana and its dynamics during ontogeny. Biologia Plantarum, 47(2): 261-264.
[15] Kohda, H., Kasai, R., Yamasaki, K., Murakami, K., & Tanaka, O. (1976): New sweet diterpene glucosides from *Stevia rebaudiana*. Phytochemistry, 15(6): 981-983.
[16] DuBois, G. E., Bunes, L. A., Dietrich, P. S., & Stephenson, R. A. (1984): Diterpenoid Sweeteners. Synthesis and Sensory Evaluation of Biologically Stable Analogues of Stevioside. Journal of Agricultural and Food Chemistry, 32(6): 1321–1325.
[17] Brandle, J. E., & Telmer, P. G. (2007): Steviol glycoside biosynthesis. Phytochemistry, 68(14): 1855–1863.
[18] Wanke M, Skorupinska-Tudek K, Swiezewska E. (2001): Isoprenoid biosynthesis via 1-deoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (DOXP/MEP) pathway. Acta Biochim Pol., 48(3): 663-672.
[19] Kim, K. K., Sawa, Y., & Shibata, H. (1996): Hydroxylation of ent-kaurenoic acid to steviol in *Stevia rebaudiana* Bertoni--purification and partial characterization of the enzyme. Archives of Biochemistry and Biophysics, 332(2): 223–230.
[20] Humphrey, T. V., Richman, A. S., Menassa, R., & Brandle, J. E. (2006): Spatial organization of four enzymes from *Stevia rebaudiana* that are involved in steviol glycoside synthesis. Plant Molecular Biology, 61(1–2): 47–62.
[21] WADA, Y., TAMURA, T., KODAMA, T., YAMAKI, T., & UCHIDA, Y. (1981): Callus cultures and morphogenesis of *Stevia* rebaudiana Bertoni. Journal of Japan Oil Chemists' Society, 30(4): 215-219.
[22] Handro, W., Ferreira, C. M., & Floh, E. I. S. (1993): Chromosomal variability and growth rate in cell suspension cultures of *Stevia rebaudiana* (Bert.) Bertoni. Plant Science, 93(1–2): 169–176.
[23] Abeyaratne, W. M., Bandara, D. C., & Senanayake, Y. D. A. (1990): In Vitro Propagation of Nadun (Pericopsis mooniana). Tropical Agricultural Research, 2: 775–776.
[24] Lee, J. I., Kang, K. H., & Park, H. W. (1982): New high rebaudioside-A *Stevia* variety" Suweon 11". The Research Reports of the Office of Rural Development (Korea R.).
[25] Motomu Akita, Takeo Shigeoka, Yoko Koizumi, and M. K. (1994): Mass propagation of shoots of *Stevia rebaudiana* using a large scale bioreactor. Plant Cell Reports, 13: 180–183.
[26] Babu, P., Chikkasubbanna, V., & Kp, G. R. (2011): Optimization of media composition for higher biomass and stevioside production. Biosci. Biotech. Res. Comm., 4(1): 1–5.
[27] Gupta, P., Sharma, S., & Saxena, S. (2014): Effect of salts (NaCl and Na_2CO_3) on callus and suspension culture of *Stevia rebaudiana* for steviol glycoside production. Applied Biochemistry and Biotechnology, 172(6): 2894–2906.
[28] Gupta, P., Sharma, S., & Saxena, S. (2015): Biomass Yield and Steviol Glycoside Production in Callus and Suspension Culture of *Stevia rebaudiana* Treated with Proline and Polyethylene Glycol. Applied Biochemistry and Biotechnology, 176(3): 863–874.
[29] Javad, S., Naz, S., Ilyas, S., & Aftab, A. (2016): Production of stevioside from callus and cell suspension cultures of *Stevia rebaudiana* (Bert.). Journal of Animal and Plant Sciences, 26(5): 1374–1382.

[30] Vives, K., Andújar, I., Lorenzo, J. C., Concepción, O., Hernández, M., & Escalona, M. (2017): Comparison of different *in vitro* micropropagation methods of *Stevia rebaudiana* B. including temporary immersion bioreactor (BIT®). Plant Cell, Tissue and Organ Culture, 131(1): 195–199.
[31] Persinos, G. J. (1980): Method of producing Stevioside. United states patent 3,723,410.
[32] Haga, T., Ise, R. and Kobayashi, T. (1976): A method for purifying stevioside (English abstr.). Jap. Patent 51-131900.
[33] Unesh, H., Ise, R., & Kobayashi, T. (1977): Purification of a *Stevia* sweetening agent. Japanese Patent, 54, 030199.
[34] Itagaki, K., & Ito, T. (1979): Purification of stevioside. Japanese Patent, 54-041898.
[35] Ogawa, T., Nozaki, M., & Matsui, M. (1980): Total synthesis of stevioside. Tetrahedron, 36(18): 2641–2648.
[36] Giovanetto, R. H. (1990): US Patent No. 4,892,938. Washington, DC: US Patent and Trademark Office.
[37] Moraes, É. D. P., Regina, N., & Fernandes, C. (2001): Clarification of *Stevia rebaudiana* (Bert .) Bertoni extract by adsorption in modified zeolites. Acta Scientarium, 23: 1375–1380.
[38] Yoda, S. K., Marques, M. O. ., Petenate, A. J., & Meireles, M. A. A. (2003): Supercritical fluid extraction from *Stevia rebaudiana* Bertoni using CO_2 and CO_2+water: extraction kinetics and identification of extracted components. Journal of Food Engineering, 57(2): 125–134.
[39] Pól, J., Varaďová Ostrá, E., Karásek, P., Roth, M., Benešová, K., Kotlaříková, P., & Čáslavský, J. (2007): Comparison of two different solvents employed for pressurized fluid extraction of stevioside from *Stevia rebaudiana*: Methanol versus water. Analytical and Bioanalytical Chemistry, 388(8): 1847–1857.
[40] Gasmalla, M. A. A., Yang, R., & Hua, X. (2015): Extraction of Rebaudioside-A by sonication from *Stevia rebaudiana* Bertoni leaf and decolourization of the extract by polymers. Journal of Food Science and Technology, 52(9): 5946–5953.
[41] Žlabur, J. Š., Voća, S., Dobričević, N., Brnčić, M., Dujmić, F., & Brnčić, S. R. (2015): Optimization of ultrasound-assisted extraction of functional ingredients from *Stevia rebaudiana* Bertoni leaves. International Agrophysics, 29(2): 231–237.
[42] Jaitak, V., Bandna, Singh, B., & Kaul, V. K. (2009): An efficient microwave-assisted extraction process of stevioside and Rebaudioside-A from *Stevia rebaudiana* (Bertoni). Phytochemical Analysis, 20(3): 240–245.
[43] Teo, C. C., Tan, S. N., Yong, J. W. H., Hew, C. S., & Ong, E. S. (2010): Pressurized hot water extraction (PHWE). Journal of Chromatography A, 1217(16): 2484–2494.
[44] Ramesh, K., Singh, V., & Megeji, N. W. (2006): Cultivation of *Stevia* [*Stevia rebaudiana* (Bert.) Bertoni]: A Comprehensive Review. Advances in Agronomy, 89(05): 137–177.
[45] Nikolova-damyanova, B., Bankova, V., & Popov, S. (1994): Separation and Quantitation of Stevioside and Rebaudioside A in Plant Extracts by Normal-Phase High Performance Liquid Chromatography and Thin-Layer Chromatography: A Comparison. Phytochemical Analysis, 5: 81–85.
[46] Kinghorn, A. D., Compadre, C. M., & Pezzuto, J. M. (1989): U.S. Patent No. 4,808,409. Washington, DC: U.S. Patent and Trademark Office.
[47] Kinghorn, A. D., Nanayakkara, N. P. D., Soejarto, D. D., Medon, P. J., & Kamath, S. (1982): Potential sweetening agents of plant origin. I. Purification of *Stevia rebaudiana* sweet constituents by droplet counter-current chromatography. Journal of Chromatography A, 237(3): 478–483.
[48] Mauri, P., Catalano, G., Gardana, C., & Pietta, P. (1996): Analysis of *Stevia* glycosides by capillary electrophoresis. Electrophoresis, 17(2): 367–371.
[49] Liu, J., & Li, S. F. Y. (1995): Separation and Determination of *Stevia* Sweeteners by Capillary Electrophoresis and High Performance Liquid Chromatography. Journal of Liquid Chromatography, 18(9): 1703–1719.
[50] Ahmed, M. S., Dobberstein, R. H., & Farnsworth, N. R. (1980): Use of p-bromophenacyl bromide to enhance ultraviolet detection of water-soluble organic acids (steviolbioside and rebaudioside B) in high-performance liquid chromatographic analysis. Journal of Chromatography A, 192(2): 387–393.
[51] Sakamoto, I., Yamasaki, K., & Tanaka, O. (1977): Application of 13C NMR spectroscopy to chemistry of plant glycosides: Rebaudioside-D and -E, new sweet diterpene-glucosides of *Stevia*

rebaudiana Bertoni. Chemical & Pharmaceutical Bulletin, 25(12): 3437–3439.
[52] Paula Nishiyama, M. A. and L. G. E. V. (1992): Quantitative Analysis of Stevioside in the Leaves of *Stevia rebaudiana* by Near-Infrared Reflectance Spectroscopy. J Sci Food Agric, 59: 277–281.
[53] Matsui, M., Matsui, K., Kawasaki, Y., Oda, Y., Noguchi, T., Kitagawa, Y., Sofuni, T. (1996): Evaluation of the genotoxicity of stevioside and steviol using six *in vitro* and one in vivo mutagenicity assays. Mutagenesis, 11(6): 573–579.
[54] Pezzuto, J. M., Nanayakkara, N. D., Compadre, C. M., Swanson, S. M., Kinghorn, A. D., Guenthner, T. M., & Lam, L. K. (1986): Characterization of bacterial mutagenicity mediated by 13-hydroxy-ent-kaurenoic acid (steviol) and several structurally-related derivatives and evaluation of potential to induce glutathione S-transferase in mice. Mutation Research/Genetic Toxicology, 169(3): 93-103.
[55] Klongpanichpak, S., Temcharoen, P., Toskulkao, C., Apibal, S., & Glinsukon, T. (1997): Lack of mutagenicity of stevioside and steviol in Salmonella typhimurium TA 98 and TA 100. Journal of the Medical Association of Thailand= Chotmaihet Thangphaet, 80: S121-8.
[56] Carakostas, M. C., Curry, L. L., Boileau, A. C., & Brusick, D. J. (2008): Overview: the history, technical function and safety of rebaudioside A, a naturally occurring steviol glycoside, for use in food and beverages. Food and Chemical Toxicology, 46(7): S1-S10.
[57] Ren, H.-P., Yin, X.-Y., Yu, H.-Y., & Xiao, H.-F. (2017): Stevioside induced cytotoxicity in colon cancer cells via reactive oxygen species and mitogen-activated protein kinase signalling pathways-mediated apoptosis. Oncology Letters, 13(4): 2337–2343.
[58] Mokarram, P., Mohammadi, Z., Khazayel, S., & Dayong, Z. (2017): Induction of epigenetic alteration by CPUK02, an ent- kaurenoid derivative of stevioside. Avicenna Journal of Medical Biotechnology, 9(1): 13–18.
[59] Dooley, J., Lagou, V., Dresselaers, T., van Dongen, K. A., Himmelreich, U., & Liston, A. (2017): No Effect of Dietary Aspartame or *Stevia* on Pancreatic Acinar Carcinoma Development, Growth, or Induced Mortality in a Murine Model. Frontiers in Oncology, 7(February): 1–7.
[60] Jeppesen, P. B., Gregersen, S., Poulsen, C. R., & Hermansen, K. (2000): Stevioside acts directly on pancreatic β cells to secrete insulin: Actions independent of cyclic adenosine monophosphate and adenosine triphosphate-sensitive K+-channel activity. Metabolism: Clinical and Experimental, 49(2): 208–214.
[61] Abudula, R., Jeppesen, P. B., Rolfsen, S. E. D., Xiao, J., & Hermansen, K. (2004): Rebaudioside A potently stimulates insulin secretion from isolated mouse islets: Studies on the dose-, glucose-, and calcium-dependency. Metabolism: Clinical and Experimental, 53(10): 1378–1381.
[62] Philippaert, K., Pironet, A., Mesuere, M., Sones, W., Vermeiren, L., Kerselaers, S., Vennekens, R. (2017): Steviol glycosides enhance pancreatic beta-cell function and taste sensation by potentiation of TRPM5 channel activity. Nature Communications: 8.
[63] Mohd-Radzman, N. H., Ismail, W. I. W., Jaapar, S. S., Adam, Z., & Adam, A. (2013): Stevioside from *Stevia rebaudiana* Bertoni Increases Insulin Sensitivity in 3T3-L1 Adipocytes. Evidence-Based Complementary and Alternative Medicine : ECAM, 938081.
[64] Bondarev, N., Reshetnyak, O., & Nosov, A. (2001): Peculiarities of diterpenoid steviol glycoside production in *in vitro* cultures of *Stevia rebaudiana* Bertoni. Plant Science, 161(1): 155–163.
[65] Sivaram, L., & Mukundan, U. (2003): *In vitro* culture studies on *Stevia rebaudiana*. In vitro Cellular & Developmental Biology - Plant, 39(5): 520–523.
[66] Banerjee, Meenakshi; Sarkar, P. (2008): *In vitro* callusing in *Stevia rebaudiana* Bertoni using cyanobacterial media- a novel approach to tissue culture Algal culture. International Journal of Integrative Bio, 3(3): 163–168.
[67] Janarthanam, B., Gopalakrishnan, M., Lakshmi Sai, G., & Sekar, T. (2009): Plant regeneration from leaf derived callus of *Stevia rebaudiana* Bertoni. Plant Tissue Culture and Biotechnology, 19(2): 133–141.
[68] Reis, R. V., Borges, A. P. P. L., Chierrito, T. P. C., de Souto, E. R., de Souza, L. M., Iacomini, M., ... & Gonçalves, R. A. C. (2011): Establishment of adventitious root culture of *Stevia* rebaudiana Bertoni in a roller bottle system. Plant Cell, Tissue and Organ Culture (PCTOC), 106(2): 329-335.
[69] Modi, A. R., Patil, G., Kumar, N., Singh, A. S., & Subhash, N. (2012): A Simple and Efficient *In vitro* Mass Multiplication Procedure for *Stevia rebaudiana* Bertoni and Analysis of Genetic Fidelity of *In vitro* Raised Plants Through RAPD. Sugar Tech, 14(4): 391–397.

[70] Gupta, P., Sharma, S., & Saxena, S. (2016): Effect of abiotic stress on growth parameters and steviol glycoside content in *Stevia rebaudiana* (Bertoni) raised *in vitro*. Journal of Applied Research on Medicinal and Aromatic Plants, 3(4): 160-167
[71] Javed, R., Usman, M., Yücesan, B., Zia, M., & Gürel, E. (2017): Effect of zinc oxide (ZnO) nanoparticles on physiology and steviol glycosides production in micropropagated shoots of *Stevia rebaudiana* Bertoni. Plant Physiology and Biochemistry, 110: 94-99.
[72] Magangana, T. P., Stander, M. A., & Makunga, N. P. (2018): Effect of nitrogen and phosphate on in vitro growth and metabolite profiles of *Stevia* rebaudiana Bertoni (Asteraceae). Plant Cell, Tissue and Organ Culture (PCTOC), 134(1): 141-151.
[73] Röck-Okuyucu, B., Bayraktar, M., Akgun, I. H., & Gurel, A. (2016); Plant Growth Regulator Effects on *In vitro* Propagation and Stevioside Production in *Stevia rebaudiana* Bertoni. HortScience, 51(12): 1573–1580.
[74] Ahmed, M. B., Salahin, M., Karim, R., Razvy, M. A., Hannan, M. M., Sultana, R., & Islam, R. (2007): An efficient method for *in vitro* clonal propagation of a newly introduced sweetener plant (*Stevia rebaudiana* Bertoni.) in Bangladesh. American-Eurasian Journal of Scientific Research, 2(2): 121-125.