Journal of Applied and Natural Science 9 (4): 2114 -2126 (2017)

JANS

Chemistry and analytical techniques for ent-kaurene-glycosides of *Stevia rebaudiana* Bertoni - A review

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Received: December 2, 2016; Revised received: April 28, 2017; Accepted: October 2, 2017

Abstract: The *Stevia* genus encompasses about 200 herbs and shrubs species. *Stevia rebaudiana*, one of the members has gained commercial importance as a natural low-calorie sweetener, due to the presence of high concentration of stevioside and rebaudioside - A (25% to 45% of stevioside content) in the leaves. The major processes involved in the production and quantification of steviol glycosides are extraction, purification and estimation. Various extraction methods have been used for extraction of steviol glycosides in the world. The extraction methods of steviol glycosides mostly differed at the stage of clarification of extracts. The present study is an attempt to summarize the scattered literature and reports on a single podium. Moreover, it also depicts upto date literature regarding numerous extraction, purification and quantitative estimation methods for steviol glycosides

Keywords: Extraction, Purification, Quantitative Analysis, Rebaudioside-A, *Stevia,* Stevioside

INTRODUCTION

Stevia rebaudiana is a perennial plant of tribe Eupatorieae and family Asteraceae. *Stevia* genus comprises of about 150-200 species of herbs and shrubs (Gentry, 1996) and native to Brazil and Paraguay regions of South America (Soejarto, 2002; Ramesh *et al.,* 2006). *S. rebaudiana* is also known as sweet leaf, sweet herb, sweet weed and honey leaf (Carakostas *et al.,* 2008; Inamake *et al.,* 2010).In the native state it occurs on the edges of marshes or in grassland communities on soils with shallow water table (Shock,1982) with semi-humid subtropical climate, temperatures ranging from -6 to 43.8°C, with an average of 23.8°C and rainfall ranging from 1500 to 1800 mm per annum (Yadav *et al.,* 2011). *S. rebaudiana* is a short day plant that grows up to height of 1m (Mishra *et al.,* 2010). It has 2 to 3cm long and elliptical leaves having alternate arrangement and bears a brittle stem and an extensive root system. Flowers are white in colour with a pale purple throat.They are small in size and arranged in the form of small corymbs (Madan *et al.,* 2010; Yadav and Guleria 2012). The fruit is a five-ribbed spindle shaped achene (Katayama *et al.,* 1976; Blumenthal, 1996).

The plant has gained commercial importance as a natural low calorie sweetener, due to the presence of high concentration of stevioside and rebaudioside-A in leaves (Kinghorn, 2002; Ramesh *et al.,* 2006). Natural low caloric sweeteners not only create a calorie deficit but are also an appropriate tool against the health problems (Surana *et al.,* 2006). Stevioside passes through the digestive processes without chemical break down, thus making stevia safe for the diabetic peoples (Yadav *et al.,* 2011). Historically, plant has been used for various purposes throughout the world (Goyal *et al.,* 2010). Leaves of *S. rebaudiana* has therapeutic properties like anticariogenic (Yabu *et al.,*1977; Gardana *et al.,* 2010), antimicrobial (Satishkumar *et al.* 2008), antiviral (Kedik *et al.,* 2009), antifungal (Silva *et al.,* 2008), anti-hypertensive (Chan *et al.,* 1998; Lee *et al.,* 2001;Hsieh *et al.,*2003), anti-hyperglycaemic (Jeppesen *et al.,* 2002; Benford *et al.,* 2006), anti-tumour (Satishkumar *et al.,* 2008; Kaushik *et al.,* 2010), anti-inflammatory (Ghanta *et al.,* 2007; Arya *et al.,* 2012), hepatoprotective (Mohan and Robert, 2009), diuretic, anti-diarrhoeal, anti-human rotavirus activities (Das *et al.,* 1992; Takahashi *et al.,* 2001), anti-HIV (Takahashi *et al.,* 1998) and immunomodulatory (Chatsudthipong and Muanprasat, 2009). In recent times, plant has gained significance in the pharmaceutical, food and cosmetic industries (Kienle, 2007; Hansen, 2010; Kienle, 2010; Kroyer, 2010; Herranz *et al.,* 2010). Several studies have shown steviol glycosides as a substitute for sugar (Crammer and Ikan, 1986; Anton *et al.,* 2010; Gasmalla *et al.,* 2014).

There are numerous extraction, purification and estimation methods of steviol glycosides (Vanek *et al.,* 2001; Choi *et al.,* 2002; Yoda *et al.,* 2003; Erkucuk *et al.,* 2009) developed in the world. So in this regard,

this paper is an attempt to summarize the scattered literature and reports on a single podium.

CHEMICAL CONSTITUENTS

Stevia rebaudiana is essentially well-known for a mixture of diterpenoid glycosides (steviol glycosides) in leaves, which are based on aglycone steviol (Prakash *et al.,* 2008, Tavarini and Angelini, 2013). Goyal *et al.*(2010) reported eight sweet diterpenoid glycosides (steviol, stevioside, rebaudiosides (A, B, C, D), steviolbioside and dulcoside A) in leaves of the plant. However in the same year, Joint FAO/WHO Expert Committee on Food Additives (JECFA) listed nine different diterpenoid glycosides viz. Stevioside, rebaudioside-A, rebaudioside-B,rebaudioside-C, rebaudioside-D, rebaudioside-F, dulcoside-A, steviolbioside and rubusoside (JECFA, 2010). Out of various steviol glycosides reported to date, major glycosides are stevioside (6–10%) and rebaudioside-A (2 to 4%). While percentage of other minor glycosides in the leaves are 1–2 per cent only (Geuns, 2003). Some minor diterpene glycosides, differs in the substitution on R_1, R_2 and/or R_3 of the ent –kaurene body (Table 1; Fig. 1).

Stevioside has chemical formula of a diterpene glycoside ($C_{38}H_{60}O_{18}$) and is accountable for the sweetening properties. It is about 300 times sweeter than sucrose (Debnath, 2008; Giuffre *et al.,* 2013) (Table 2), but has an unpleasant bitter aftertaste (Schiffman *et al.,* 2000; Abelyan *et al.,* 2004; Mitchell, 2006; Carakostas *et al.,* 2008). Rebaudioside-A, normally present in lower amount (25% to 45% of stevioside) in leaves, possess no bitter aftertaste and has a sweetening power of 1.2 to 1.6 times higher than stevioside (Kinghorn and Soejarto, 1985). Reason behind the bitterness of the stevioside is the presence of essential oils, tannins and flavonoids (Phillips, 1987). Stevioside is stable at high temperature (100°C) and over a range of pH values (3 to 9) (Chang and Cook, 1983; Kinghorn and Soejarto, 1985), non-fermentable and does not darken upon cooking (Kroyer, 2010; Abdullateef and Osman, 2012; Reshu *et al.,* 2014). However, Serio (2010) reported the compound stability at 200°C.

The minor diterpenoid glycosides are 30-80 times sweeter than sugar (Brandle, 1999; Oddone, 1999). Three new minor diterpenoid glycosides were isolated in addition to eight known steviol glycosides including stevioside, rebaudiosides A–F and dulcoside A. These compounds were 13-[(2 – O – b – D – glucopyranosyl – b – D - glucopyranosyl) oxy] 17hydroxy – kaur – 15 – en -18-oic acid b – D - glucopyranosyl ester, 13-[(2 - O - b- D -glucopyranosyl -b- Dglucopyranosyl) oxy]-kaur – 16 – en – 18 – oicacid -(6 – O - bxylopyranosyl b – D - glucopyranosyl) ester and 13-[(2 – O – b - D-glucopyranosyl – b – D - glucopyranosyl)oxy] -17 – oxo – kaur – 15 – en – 18 - oic acid b-D-glucopyranosyl ester (Chaturvedula *et al.,* 2011). Chaturvedula *et al.* (2013a, b) isolated two new diterpenoid glycosides viz. rebaudioside-N and rebaudioside-O. Similarly, Rebaudioside-R and S have been isolated by Ibrahim *et al.* (2016). Chaturvedula (2014) recognised a minor penta β-D glucopyranosyl diterpene from *S. rebaudiana* leaves. Markovic *et al.* (2008) identified the compounds; nerol, safranal, aromadendrene, α- amorphene and T-muurolol and β-cyclocitral in leaves of *S. rebaudiana*. Prakash *et al.* (2012) reported three reduced derivatives of rebaudioside- B, C and D by using palladium hydroxide as catalyst. Prakash *et al.* (2011) evaluated steviol glycosides in mock beverage solutions by simulating formulations used in root beer soft drinks (pH 4.2), lemon-lime soft drinks (pH 3.8) and commercial cola soft drinks (pH 2.8 and pH 3.2) but lacking the flavour components. Results indicated that steviol glycoside yielded two minor compounds (13-[(2-O-β-D-glucopyranosyl-3-O-β-D-gluopyranosyl-β-D-glucopyranosyl)oxy] ent-kaur-15-en-19-oic acid β-D-glucopyranosyl ester and 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-16β-hydroxy-ent-kauran-19-oic acid β-D-glucopyranosyl ester) on the basis of MS and hydrolysis studies.

Besides having sweet tasting diterpenoid glycosides and some minor glycosides, *S. rebaudiana* leaves accumulate several other important chemical constituents, such as flavonoids, alkaloids, hydroxycinnamic acid, vitamins, phytosterols, jhanol, austroinulin, 6-0-acetyl austroinulin, β-amyrin acetate and lupeol esters in leaves and flowers (Darise *et al.,* 1983; Kinghorn and Soejarto, 1985). Labdane type diterpenoids, called sterbins, I-N, A---H and Q_1 –Q_5 (Oshima *et al,*. 1986 and 1988; McGarvey *et al.,* 2003; Kamauchi *et al.,* 2015); bactericide agents (Koshiro, 1980) and gums as well as organic acids (Cheng and Chang, 1983) have also been reported in leaves.

Cioni *et al.,* (2006) identified forty components like spathulenol (13.4-40.9%), caryophyllene oxide (1.3-18.7%), beta-caryophyllene (2.1-16.0%) and beta pinene (5.5-21.5%) in the essential oil of the aerial parts of *S. rebaudiana* genotypes cultivated on the Tuscan coast (Italy) through using crystal chromatography and GC/MS (Gas Chromatography/ Mass Spectroscopy). However, some new essential oil compounds, such as ledene oxide-(II), beta-guaiene, geranyl vinyl ether, tricyclo (5.2.2.0 (1, 6) undecan-3-ol, indole, aristolene epoxide, 1, 2, 3, 5, 6, 7, 8, 8a-octahydro-1, 4 -dione and 2, 6, 6-trimethyl-2-cyclohexene-1, 4-dionewere were identified for the first time by Hossain *et al.,* (2010). Additionally, twenty four compounds were also identified viz. betulin, α-amyrin, cyclopropyl ursane-type triterpene, 13, 27-cycloursan-3-ol and acetate (3β,13β,14β) etc. (Lasekan and Naidu, 2013).

Table 1. Diterpenoid glycosides of *S. rebaudiana.*

Name of compound	R_1	R_2	Reference(s)
Steviol monoside	H	Glcβ1−	Kaur *et al.,* 2014
Stevioside	Glcβ1−	Glcβ1− 2Glcβ1−	Kaur *et al.,* 2014
Steviolbioside	H	Glcβ1−2Glcβ1−	
Rebaudioside A	Glcβ1−	Glcβ1 −2(Glcβ1−3)Glcβ1−	Carakostas *et al.*, 2008
Rebaudioside B	H	Glcβ1−2(Glcβ1−3)Glcβ1−	Kaur *et al.*, 2014
Rebaudioside C	Glcβ1−	Rhaα1−2(Glcβ1−3)Glcβ1−	JECFA, 2010
Rebaudioside D	Glcβ1−2Glcβ1	Glcβ1−2(Glcβ1−3)Glcβ1−	Prakash *et al.*, 2014a
Rebaudioside E	Glcβ1−2Glcβ1−	Glcβ1−2Glcβ1−	
Rebaudioside F	Glcβ1−	Xylβ1−2(Glcβ1−3)Glcβ1−	JECFA , 2010
Rebaudioside G	Glcβ1−	Glcβ1−3Glcβ1−−	Wolwer, 2012
Rebaudioside H	Glcβ1−	Glcβ1−3Rhaα1−2(Glcβ1−3) Glcβ1−	Wolwer, 2012
Rebaudioside I	Glcβ1−3Glcβ1−	Glcβ1−2(Glcβ1−3)Glcβ1−	Wolwer, 2012
Rebaudioside J	Rhaα1−2Glcβ1−	Glcβ1−2(Glcβ1−3)Glcβ1−	Wolwer, 2012
Rebaudioside K	Glcβ1−2Glcβ1−	Rhaα1−2(Glcβ1−3)Glcβ1−	Wolwer, 2012
Rebaudioside L	Glcβ1−	Glcβ1−6Glcβ1−2(Glcβ1−3) Glcβ1−	Wolwer, 2012
Rebaudioside M	Glcβ1−2(Glcβ1−3)Glcβ1−−	Glcβ1−2(Glcβ1−3)Glcβ1−	Prakash *et al.*, 2014b
Rebaudioside N	Rhaα1−2(Glcβ1−3)Glcβ1−	Glcβ1−2(Glcβ1−3)Glcβ1−	Chaturvedula *et al.,* 2013b
Rebaudioside O	Glcβ1−3Rhaα1−2(Glcβ1−3)Glcβ1−	Glcβ1−2(Glcβ1−3)Glcβ1−	Chaturvedula *et al.,* 2013a
Rebaudioside R	-	-	Ibrahim *et al.,* 2016
Rebaudioside S	-	-	Ibrahim *et al.,* 2016

Glc=Glucose; Rha=Rhamnose; Xyl=Xylose

Table 2. Relative sweetening power of diterpene glycosides of *S. rebaudiana.*

Diterpene glycoside	Relative sweetening power			
	Sharma *et al.*, 2009	Puri *et al.*, 2011	Carkostas *et al.*, 2012	Prakash *et al.*, 2014
Stevioside	100-270	250-300	150-250	210
Steviol	nd	Nd	nd	nd
Steviolbioside	10-15	Nd	90	90
Rebaudioside-A	150-320	350-450	200-300	200
Rebaudioside-B	10-14	300-350	150	150
Rebaudioside-C	40-60	50-120	30	30
Rebaudioside-D	200-250	200-300	221	221
Rebaudioside-E	150-200	250-300	nd	174
Rebaudioside-F	nd	Nd	nd	200
Rebaudioside-M	nd	Nd	nd	250
Rubusoside	nd	Nd	nd	114
Dulcoside-A	40-60	20-120	30	30
Dulcoside-B	40-60	Nd	nd	nd

nd=not determined

EXTRACTION OF MAJOR SWEET COMPOUNDS

Worldwide, there are many patents for steviol glycosides extraction (Giavanetto, 1990; Payzant *et al*., 1999; Jonnala *et al*. 2006). The main processing steps involved in the production of steviol glycosides comprise of extraction, pretreatment, separation and refining. Water is most popular solvent used for extraction; however, methanol/ethanol alone or in combination with water is also used for extracting the sweet diterpenoid glycosides. The extract obtained

after solvent extraction contains dark brown colour and possess leaf pigments, soluble polysaccharides, proteins, pectins, flavonoids and other impurities. Less polar, other non-polar compounds and chlorophyll are removed with solvents such as chloroform/hexane (Masuyama 1980, Kinghorn *et al.,* 1982).

Plentiful extraction methods have been used for extraction of sweet steviol glycosides (Table 3) but, conventional solvent extraction is the most commonly used method (Puri *et al.,* 2012). Extraction of steviol glycosides from the leaves is often done by water (Inamake *et al.,* 2010;Rao *et al.,* 2012; Afandi *et al.,* 2013). The crude extract so obtained, is dark brown, foul-smelling and bitter-tasting. Consequently, further purification is essential for preparation of quality products (≥90% purity). Hot water is used for extraction of the same, as rebaudioside-A is more soluble than stevioside in water (Mondaca *et al.,* 2012). More solubility of rebaudioside-A in water than stevioside is preferably due to the presence of additional glucose unit in its molecule (Kohda *et al.,* 1976). On the other hand, Abou *et al.* (2010) found that stevia sweeteners were highly soluble in methanol and less soluble in water. On this basis, Liu *et al.* (1997) extracted stevioside from *S. rebaudiana* leaves with hot methanol. Pol *et al.* (2007) used pressurized fluid extraction method for the extraction of stevioside from *S. rebaudiana* leaves using methanol and water.

Conventional extraction methods have few demerits over the non-conventional extraction methods like their lower yields, lack of selectivity and use of large volume of organic solvents. Sorecently, modern extraction methods such as pressurized hot water extraction, microwave assisted extraction (MAE), ultrasound assisted extraction (UAE), enzyme assisted extraction, and supercritical fluid extraction have been developed (Puri *et al.,* 2012; Khoddami *et al.,* 2013; Piasecka *et al.,* 2014; Tiwari *et al.,* 2014). Conventional extraction processes are time consuming and involve use of large amounts of solvents. However, improved methods such as UAE and MAE techniques have some advantages over conventional extraction methods viz. reduction in extraction time, higher extract yield and less solvent consumption. MAE and UAE techniques are much easier and effective techniques as they produce extracts with similar qualitative characteristics, however with high quantitative differences (Khaled *et al.,* 2015).MAE yielded maximum amount of stevioside (0.7658 mg/g of dry leaf powder) with lesser extraction time (120 seconds) and a little solvent amount used (10 ml/g) as compared to conventional method (Javad *et al.,* 2014). On the other hand, Zlabur *et al.* (2015) compared UAE with conventional solvent extraction and found maximum yield of steviol glycosides (stevioside: 96.48 mg g^{-1} extract; and rebaudioside-A content: 36.92 mg g^{-1} extract), total phenolic compounds and flavonoids with UAE. However, on comparison with UAE, Jaitak *et al.* (2009) reported MAE method as a fast and capable extraction method for stevioside estimation. They suspended powdered leaf samples of 100mg in 10ml of different solvents (methanol, ethanol, water) and mixture of solvents (methanol: water: 80:20, ethanol: water: 80:20), filtered, concentrated and dried. Extraction was done at different power levels ranging from 20 to 160 W with extraction time ranging from 30 seconds to5 minutes and temperature from 10–90° C. MAE yielded 8.64 and 2.34 percent of stevioside and rebaudioside-A, respectively, while UAE yielded 4.20 and 1.98 percent of stevioside and rebaudioside-A, respectively.

Other efficient methods of extracting steviol glycosides are PHWE (Pressurized Hot Water Extraction) and SCFE (Supercritical Fluid Extraction). Teo *et al.* (2009) reported higher extraction efficiency of PHWE and MAE as compared to heating under reflux. Extraction of *S. rebaudiana* plant samples were carried out with water at different extraction temperature for different periods. Maximum stevioside (2137.8 mg/100gm) and rebaudioside-A yield (2080.7 mg/100gm) was found with MAE for extraction period of 20 minutes. In SCFE method, glycosides from stevia leaves were obtained in two steps; extraction with CO_2 at 200 bar pressure and 30°C temperature and extraction with CO_2 and water (Pasquel *et al.,* 2000; Choi *et al.,* 2002; Yoda *et al.,* 2003; Erkucuk *et al.,* 2009). While, patent has claimed that CO_2 could be used as a solvent and methanol, ethanol and acetone as a cosolvant for steviol glycosides extraction (Kienle, 1990).

Extractions of sweet glycosides using various enzymes have been also used successfully (Puri *et al.,* 2012; Rao *et al.,* 2015). Puri *et al*. (2012) revealed a novel enzyme-mediated extraction (EME) method for stevioside extraction from leaves of *S. rebaudiana*. The study investigated the effects of cellulase, hemicellulase and pectinase enzymes on stevioside yield. Hemicellulase gave the highest stevioside yield (369.23 ± 0.11 µg) in 1 hour with comparison to cellulase (359 ± 0.30 µg) and pectinases (333 ± 0.55 µg). Enzymes used for stevioside extraction in both studies were cellulase, hemicellulase and pectinase. Rao *et al*. (2015) extracted stevioside through treatment of dry leaves of stevia with the help of hydrolytic enzymes aided by transition metal salts ($FeCl_3$). The metal salt assisted enzyme extraction of stevioside resulted in increased stevioside yield to 72 per cent with 98 per cent purity.

PURIFICATION OF MAJOR SWEET COMPOUNDS

After extraction, extract purification is needed. Various methods for extraction of steviol glycosides mostly differ at the stage of clarification of extracts. Extract

Table 3. Extraction methods for diterpene glycosides of *S. rebaudiana.*

Extraction methods	Reference(s)
Water extraction	Abou *et al.,* 2010; Inamake *et al.,* 2010; Rao *et al.,* 2012; Afandi *et al.,* 2013; Deshmukh and Kedari, 2014; Gonzalez *et al.,* 2014
Methanol extraction	Liu *et al.,* 1997; Pol *et al.,* 2007; Deshmukh and Kedari, 2014
Ethanol extraction	Erkucuk *et al.,* 2009; Deshmukh and Kedari, 2014
Pressurized fluid extraction (PFE)	Pol *et al.,* 2007
Microwave-assisted extraction (MAE)	Jataik *et al.,* 2009; Teo *et al.,* 2009; Javad *et al.,* 2014
Ultrasonic-assisted Extraction (UAE)	Zlabur *et al.,* 2015
Pressurized hot water extraction(PHWE)	Teo *et al.,* 2009; Tiwari *et al.,* 2014
Supercritical fluid extraction (SFE)	Pasquel *et al.,* 2000; Choi *et al.,* 2002; Yoda *et al.,* 2003; Pol *et al.,* 2007; Erkucuk *et al.,* 2009
Enzymatic extraction	Puri *et al.,* 2012; Rao *et al.,* 2015

Table 4. HPLC methods for estimation of steviol glycosides of *S. rebaudiana.*

Analytical method	Columns	References
HPLC	Amino	Kolb *et al.,* 2001; Woelwer *et al.,* 2010; Hoekstra *et al.,* 2009; Kumari *et al.,* 2016
HPLC	C18	Vanek *et al.,* 2001; Abou *et al.,* 2010; Afandi *et al.,* 2013; Gonzalez *et al.,* 2015; Gonzalez *et al.,* 2014; Kubica *et al.,* 2015; Samah *et al.,* 2013; Javad *et al.,* 2014
HPLC	HILIC	Wolwer *et al.,* 2010; Zimmermann *et al.,* 2011
HPLC	HILIC and Develosil ODS HG	Lorenzo *et al.,* 2014
RPLC	C18 and amide	Fu *et al.,* 2012
RP-HPLC	Reverse phase C18	Chaturvedula and Zamora, 2014; Rodenburg *et al*., 2016

purification is an important step as it reduces the chances of occurrence of problems afterwards. On the other hand, purification processes have some demerits viz. organic solvents and metallic ions leave residue and thus harming human health whereas application of ultra filtration membranes and other advanced technologies increases final cost of the products.

Rebaudioside-A is commercially more desirable compound due to its more pleasant sweet taste than stevioside. Besides, separation of rebaudioside-A from other steviol glycosides is a tricky job, as all compounds are having almost similar chemical structures. Several patents have mentioned the purification of steviol glycosides (Abelyan *et al.,* 2010; Magomet *et al.,* 2011; Purkayastha *et al.,* 2012). In these patents, leaves were dried and powdered, extracted with water followed by filtration and the filtrate so obtained was treated with calcium hydroxide and iron chloride. The filtrate was deionized using amberlite beds e.g., amberlite FPC23H, amberlite FPA51 and amberlite FPA98Cl. The filtrate was vacuum dried, concentrated and spray dried. The dried material was extracted with methanol at 20 to 25°C for 0.5 to 1.0 hour with agitation. Consequently, precipitation occurred and solution was filtered to obtain stevioside precipitate. The filtered precipitate was dried and analyzed to obtain about 90% stevioside. The remaining solution was evaporated to remove methanol and vacuum dried. Extract obtained is dissolved in ethanol and filtered to get 90% rebaudioside-A.

Membranes have been utilised to obtain each fractional component of a solution on the basis of molecular weight differences and have been applied to remove impurities from fermentation broth (Kuo and Chiang, 1987) and prefiltered fruit juices (Yu and Chiang, 1986). Membrane processes have some advantages over other separation processes like occurrence at room temperature without phase change and with no heating or solvent involved. Fuh and Chiang (1990)

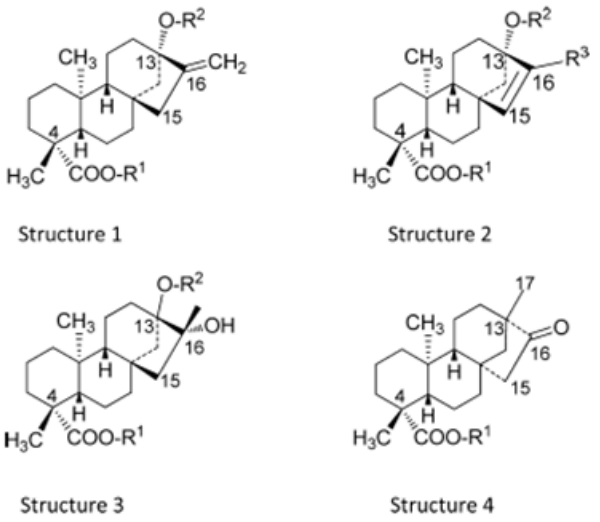

Fig. 1. *Ent-kaurene body structures of steviol glycosides* (Woelwer, 2012).

applied membranes for carrying out ultrafiltration, diafiltration and reverse osmosis to remove majority of the pigments in *S. rebaudiana* leaf extract (>90%). However, nano filtration practice has also been applied to purify the stevia extract (Zhang *et al.,* 2000). Chaya *et al.* (2011) purified stevia extract through ultrafiltration membranes using four different ultrafiltration membranes of 5, 10, 30 and 100kDa molecular weights. 30kDa membrane was found to be most appropriate.Vanneste *et al.* (2011) evaluated the performance of tailor-made polyethersulphone (PES) membranes for purification of steviol glycosides from *S. rebaudiana*. Similarly, Roy and De (2014) attempted to purify the steviol glycosides by using polymer blend of cellulose acetate phthalate (CAP) and polyacrylonitrile (PAN). Optimum flux, recovery and purity were 11 L/m^2 h, 68% and 34%, respectively.

Furthermore, inorganic salt treatment, ion exchange, adsorption column chromatography, adsorption by activated charcoal and use of macroporous resins have been widely used for clarification of stevia sweeteners (Akashi *et al,.* 1975; Okane and Kamata, 1977; Takamura *et al.,* 1977; Ishizone, 1979; Kuroda and Kamiyama, 1979; Cheng *et al.,* 1985; Fuh and Chiang, 1990; He *et al.,* 1994; Rajab *et al.,* 2009; Li *et al.,* 2012; Deshmukhand Kedari, 2014; Kaur *et al.,* 2014; Hubert *et al.,* 2015; Anvari and Khayat, 2016). Selective adsorption using zeolites was also found suitable for stevia extract purification (Mantovaneli *et al.,* 2004; Silva *et al.,* 2007).

QUANTITATIVE ESTIMATION OF STEVIOL GLYCOSIDES

Various quantitative methods have been used to evaluate the distribution and per cent of sweetent-kaurene glycosides in *S. rebaudiana*. Thin layer chromatography (Nikolova *et al.,* 1994),High Performance Liquid Chromatography (HPLC) (Kolb *et al.,* 2001; Woelwer *et al.,* 2010; Pieri *et al.,* 2011; Samah *et al.,* 2013; Lorenzo *et al.,* 2014; Gonzalez *et al.,* 2015; Kumari and Chandra, 2015; Kumari *et al.,* 2016a), High Pressure Thin Layer Chromatography (HPTLC) (Chester *et al.,* 2012, Saifi *et al.,* 2014), Reverse Phase High Pressure Liquid Chromatography (RP-HPLC) (Jadhao *et al.,* 2011, Chaturvedula and Zamora, 2014; Meneni and Chaturvedula, 2015), Ultra High Pressure Liquid Chromatography (UHPLC) (Gardana *et al,.* 2010; Cacciola *et al.,* 2011), HPLC-UV (Gonzalez *et al.,* 2014), Liquid chromatography (LC), counter current chromatography (Huang *et al.,* 2010; Englert *et al.,* 2016), capillary zone electrophoresis, micellar kinetic capillary electrophoresis (Mauri *et al.,* 1996; Dacome *et al.,* 2005) and near infrared reflectance spectroscopy (Hearn and Subedi, 2009)have been used to quantify steviol glycosides from *S. rebaudiana*. However, HPLC is the most commonly used method (Table 4). Saifi *et al*. (2014) developed a validated HPTLC method for simultaneous identification and quantification of stevioside and rebaudioside-A from *S. rebaudiana* leaf extract. Stevioside and rebaudioside-A recovery was 98.97 and 97.68%, respectively. Stevioside per cent ranged from 3.63 to 7.80% in *S. rebaudiana* leaves collected from Haryana and Kashmir, respectively. However, rebaudioside-A per cent varied from 1.74 to 4.40 % in leaves collected from Haryana and Madhya Pradesh, respectively. Gonzalez *et al*. (2014) determined the per cent of dulcoside-A, steviolbioside, rebaudioside-C and rebaudioside-B in Morita II and Criolla varieties of *S. rebaudiana* through using isocratic HPLC-UV method. Average recovery varied between 92.29 to 104.49% for the minor glycosides (dulcoside A, steviolbioside, rebaudioside-C and rebaudioside-B). Dulcoside-Aand rebaudioside-C content (%)varied between 0.4– 0.7 and 1–2%, respectively, in both varieties. Kumari *et al*. (2016) reported the effects of different growing conditions (open field and polyhouse) on stevioside and rebaudioside-A per centin *S. rebaudiana* leaves using HPLC method. Maximum stevioside (9.19%) and rebaudioside-A content (7.00%) was found in leaves under polyhouse and open field conditions, respectively. However, minimum stevioside and rebaudioside-A content (<1.00%) was found in green and woody stems of the plants.

Steviol glycosides have been separated using various columns viz. hydroxyapatite (Kasai *et al.,* 1987), silica gel (Nikolova *et al.,* 1994), hydrophilic (Hashimoto *et al.,* 1978) and size exclusion (Ahmed and Dobberstein, 1982) columns. Amino bonded columns have been commonly employed for the analysis of steviol glycosides (Kolb *et al.,* 2001; Ahmed and Smith, 2002; Hoekstra *et al.,* 2009; Musa *et al.,* 2014). Amino columns have also been used to determine stevioside and related glycosides content in foods and beverages (Fujinuma *et al.,* 1986; Kitada *et al.,* 1989). Ahmed *et al.* (1980) used HPLC technique for determination of stevioside and rebaudioside- B per cent after conversion to p-promophenacyl esters. Application of amino columns for steviol glycosides analysis is burden some particularly for aglycone steviol, as steviol glycosides retains poorly on the column and decreases its efficiency. Therefore, RP-HPLC has been employed for the quantification of steviol glycosides (Minne *et al.,* 2004; Martono *et al*., 2016). Two-dimensional reversed-phase liquid chromatography/hydrophilic interaction liquid chromatography (2D-RPLC/HILIC) have been used for characterization of diterpene glycosides from *S. rebaudiana* by using C18 and amide columns (Fu *et al.*, 2012). As a result, the methods uccessfully purified the low-abundance compounds from natural products. Liquid Chromatography with Ultraviolet detection method was used for simultaneous analysis of steviol and other glycosides using a

mixed mode reverse phase weak anion exchange chromatography column (Jaworska *et al.,* 2012). Similarly, Ayyappa *et al.* (2015) developed electro kinetic chromatographic method for the simultaneous separation and determination of steviol glycosides in stevia samples by capillary electrophoresis. The method recommended that using a separating agent (TM-b-CD) greatly improves the separation efficiency of steviol glycosides. Wang *et al.* (2015) developed UHPLC -UV method for detection of adulterants (glucose) in stevia products.

Several spectroscopy techniques have been commonly used for quantification of diterpene glycosides in stevia. Mass spectroscopy (MS) is the precise detection method for steviol glycosides (Rajasekaran *et al.,* 2008; Jackson *et al.,* 2009). Shah *et al.* (2012) developed a LC-MS/MS method for the characterization of steviol glycosides in foods. Kakigi *et al.* (2013) developed an analytical method for characterizing stevia sweeteners in soft drinks by using LC and MS methods. Kubica *et al.* (2015) reported a method (high-performance liquid chromatography and tandem mass spectroscopy with electrospray ionisation (HPLC-ESI-MS/MS) for the determination of synthetic sweeteners (acesulfame-K, aspartame, alitame, cyclamate, saccharine, sucralose, neohesperidin dihydrochalcone, neotame) and steviol glycosides (steviol, steviolbioside, rebaudioside A, rebaudioside C and stevioside) in beverages.

Another method for detection of steviol glycosides is NMR spectroscopy. Stevioside and rebaudioside A−C have been detected by 1H NMR spectroscopy (Inamake *et al.,* 2010). Furthermore, reference compounds are not used for identification of desired compounds in NMR spectroscopy as compared to HPLC and LC-UV method (Kakigi *et al.,* 2013).

FUTURE PROSPECTS

Stevia extracts are increasingly used in ice creams, soft drinks and juices and various other products. Apart from this, stevia sweeteners have found its use in bakery and confectionery items too. According to future market insight report, stevia market is expected to reach about 15 percent of the total sweetener market by 2020 worldwide. Additionally, stevia market is affected by the requirement of efficient alternatives for artificial sweetener products due to changing consumer lifestyle and increasing product visibility in urban areas. At the same time, many health problems resulted by using intense sweeteners. Consequently, consumers shifted their preference to natural low calorie sweeteners which is a main factor for driving growth of stevia market. *Stevia rebaudiana* has high sweetening potential due to stevioside and rebaudioside-A, but the conventional methods of isolation of these glycosides involve long extraction and purification process. So, to make best use of product yield is a challenging problem. Therefore, main emphasis must be on developing new methods for isolation of these glycosides. Simultaneously, development of new varieties having more rebaudioside-A content than stevioside and higher leaf stem ratio and quality are desirable.

Conclusion

Stevia rebaudiana is a sweet diterpenoid glycosides containing plant of Asteraceae family. Stevioside and rebaudioside-A are two major sweet diterpene glycosides present mostly in the leaves. Stevioside is 300 times sweeter than sugar but with bitter after taste however, rebaudioside-A is having more sweeteness than stevioside. There are thousands of patents, literature available for extraction, purification and quantitative estimation of steviol glycosides. In this review, we have tried to provide the all available information regarding different extraction, purification and estimation processes of steviol glycosides. Extraction methods are generally categorised into conventional and non-conventional methods. Modern extraction or non-conventional extraction methods are the most popular methods for the same because of its merits like reduction in extraction time, higher extract yield and less solvent consumption. But, still conventional methods are being used for steviol glycosides extraction in small scale industries due to its lower cost.

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