A Review on the pharmacology and toxicology of steviol glycosides extracted from *Stevia rebaudiana*

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Running title: Steviol glycosides: A review

Abstract

Stevia rebaudiana Bertoni is a sweet and nutrient-rich plant belonging to the Asteraceae family. Stevia leaves contain steviol glycosides including stevioside, rebaudioside (A to F), steviolbioside, and isosteviol, which are responsible for the plant's sweet taste, and have commercial value all over the world as a sugar substitute in foods, beverages and medicines. Among the various steviol glycosides, stevioside, rebaudioside A and rebaudioside C are the major metabolites and these compounds are on average 250-300 times sweeter than sucrose. Steviol is the final product of Stevia metabolism. The metabolized components essentially leave the body and there is no accumulation. Beyond their value as sweeteners, Stevia and its glycosdies possess therapeutic effects against several diseases such as cancer, diabetes mellitus, hypertension, inflammation, cystic fibrosis, obesity and tooth decay. Studies have shown that steviol glycosides found in Stevia are not teratogenic, mutagenic or carcinogenic and cause no acute and subacute toxicity. The present review provides a summary on the biological and pharmacological properties of steviol glycosides that might be relevant for the treatment of human diseases.

Keywords: Stevia; Steviol glycosides; Stevioside; Rebaudiosides A; Cancer; Diabetes; Toxicity

1. Introduction

1.1. Source

Steviol glycosides are natural sweet constituents of *Stevia rebaudiana* Bertoni (Asteraceae), a plant that is widely cultivated in South America particularly in northeast Paraguay, Brazil and Argentina. When this plant is cultivated in fertile soils or grows naturally, its adult form reaches a height of 65-180 centimeters (Fig. 1). Centuries ago, natives of Paraguay, Brazil, Central America, Korea, Thailand, and China were using Stevia leaves as a natural sweetener [1, 2]. In 1888, Dr. Moises Santiago Bertoni discovered Stevia in Paraguay; but it was a few years later in 1905 when a chemist named Dr. Rebaudi named this plant *S. Rebaudiana* [3]. The sweet taste of Stevia leaf is due to the presence of steviol glycosides in its leaves [4]. Plant leaves also contain some essential fats, tannins, and flavonoids that are responsible for the bitter aftertaste [5]. Steviol glycosides are extracted from the leaves of *S. rebaudiana* with hot water, followed by solvent purification of the water-soluble extract. Additionally, ion-exchange resins can be used during the purification process. Steviol glycosides are determined and identified with different methods that involve liquid chromatography. Obtained extracts contain high percentage of stevioside and rebaudioside A, and smaller amounts of other steviol glycosides [6]. These natural sweet constituents are stronger sweetener than sucrose while they have lower absorption rate and contribute to the control of blood glucose levels in patients with type 2 of diabetes, and also blood pressure [7].

1.2. Applications and Properties

Stevia is widely used as sweetener and sugar substitute in many countries and is produced as a commercial product in several countries. The reaping time of Stevia is immediately before its

blossoming period since the amount of sugar is at the highest level, and after reaping, the leaves are cut and processed. Due to the very low concentration of glycosides in the stems and roots, these parts of plant are not subjected to industrial processing [8]. Aside from its industrial applications, several lines of evidence have shown that *S. Rebaudiana* possesses different medicinal properties including anti-diabetic [7-9], anti-microbial [10], anti-viral [11], anti-fungal [12], anti-tumor [10], anti-hypertensive [13-15], anti-inflammatory [16-19], hepatoprotective [20, 21], and immuno-stimulating [16, 22-24] properties. Moreover, toxicological studies have shown that steviol glycosides of Stevia leaves are not teratogenic, nor mutagenic and carcinogenic, and no allergic responses have been reported after its use as a sweetener [1, 6].

1.3. Safety

Steviol glycosides meet the safety standard of health authorities such as Expert Joint Committee of the Food and Agriculture Organization/World Health Organization (FAO/WHO) on food additives (JECFA). Additionally, Acceptable Daily Intake (ADI) of 0–2 mg/kg body weight/day has been accepted for steviol glycosides by the Joint FAO/WHO Expert Committee on Food Additives [6].

1.4. Study Aim

The purpose of this review is to provide a summary on the biological and pharmacological properties of steviol glycosides that might be relevant for the treatment of human diseases.

2. Active Ingredients of Stevia Leaf

Previous studies have shown that Stevia leaf contains nutritive ingredients such as nine essential amino acids (glutamic acid, aspartic acid, lysine, serine, isoleucine, alanine, proline, tyrosine

and methionine) [25], six fatty acids (palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acids) [26], water-soluble vitamins (folic acid, vitamin C and vitamin B2) [27], phytochemicals (austroinullin, β-carotene, dulcoside, nilacin, rebaudi oxides, riboflavin, steviol, stevioside and thiamine) [10], secondary metabolites (tannins, alkaloids, cardiac glycosides, saponins, sterols and triterpenes, reducing compounds and anthraquinones) [26], minerals (calcium, phosphorous, sodium, potassium, iron, magnesium and zinc) [25, 26, 28-31].

Stevia produces sweet glycosides which all have the steviol backbone (Fig. 2); commonly known as steviol glycosides [32]. Steviol glycosides are structurally four-ring diterpene. The presence of a hydroxyl group in the C-13 position and a carboxyl group in the C-19 position (Fig.2) is necessary for the sweet taste of these compounds [33]. Main steviol glycosides that are present in Stevia leaf are stevioside, rebaudioside (A to F), steviolbioside, and isosteviol (Fig. 3) [34]. Among these, stevioside (4–13% w/w), rebaudioside A (2–4% w/w) and rebaudioside C (1–2% w/w) are the most abundant steviol glycosides present in the plant leaves [35]. These complexes are concentrated in Stevia's leaves and their amount depends on the plant genotype and environmental conditions [5, 32, 36-38]. Bondarev et al. studies showed that steviol glycosides only exist in tissues with chloroplast [39]. It has been reported that rebaudioside A is 250-450 times sweeter than sucrose, while stevioside and rebaudioside C are 250-300 times and 50-120 times sweeter, respectively [40-42]. Under alkaline hydrolysis, rebaudioside A and rebaudioside D can be converted into rebaudioside B [43]. These complexes are highly soluble in water and are metabolized in the body without any side effects. They are also pH-stable as well as heat-stable up to 200^{o}C, and are not fermentable which makes them a potentially effective instrument in improving health [44].

3. Metabolism of Steviol glycosides

The main ingredients of Stevia such as steviol glycosides are eliminated through similar metabolic reactions in humans and animals [34, 45]. Rebaudioside A is first metabolized to stevioside by human colon microbiome. Stevioside will then decompose to glucose and steviol. The resulting glucose is consumed by colon bacteria and is not absorbed into systemic circulation [46]. Studies in both human and mouse have shown that the conversion of stevioside to steviol is faster compared with the conversion of Rebaudioside A to stevioside. A study on human's alimentary canal showed that steviol is the final product of stevia metabolism [46, 47]. Moreover, this research also showed that most steviol glycosides are absorbed and undergo glucuronidation in the liver. Glucoronidated metabolites are filtered by the kidneys, and will eventually excreted in urine [47]. Stevioside complexes are not subject to decomposition or structural rearrangement by the stomach fluid and digestive enzymes. Digestive ability of stevioside through various digestive enzymes was evaluated *in vitro* by Hutapea et al., and it was reported that no human enzyme can digest steviol [48]. Although human microbiome of the alimentary canal hydrolyzes steviol to steviol 16, 17 alpha-epoxide, the latter will be reconverted to the parent compound and excreted in urine in the glucoronidated form [41, 48].

4. Pharmacological effects

4.1. Anti-Diabetic Effect

Type 2 diabetes is a global health concern that is associated with insulin resistance and hyperinsulinemia [49]. Maintaining a low-calorie diet and control of carbohydrate intake is integral to the successful management of type 2 diabetes. Medicinal plants have been used for a long time in several traditional systems of medicine to control diabetic complications, and

hitherto over 1200 types of medicinal plants have been identified with anti-diabetic properties [50]. Stevia is one of such plants which has been proven to be very effective in diabetes treatment. Sweets produced in stevia, despite their taste, would not be absorbed by body and therefore will not increase blood glucose [7]. Clinical trial studies have shown that leaf extract of *Stevia rebaudiana* lowers blood glucose in patients with type 2 diabetes [7].

Jeppesen et al. reported that stevioside and steviol treatment have a direct effect on β cells in the presence of glucose and would prompt the release of insulin in mice and rat [51, 52] . In other studies, it was reported that rebaudioside A and stevioside stimulate secretion of insulin from pancreatic β cells and also interfere with glucose metabolism in adipocytes, leading to insulin resistance in adipocytes but not pancreatic cells [38, 53, 54]. Also, it was found that stevioside stimulates secretion of insulin in pancreatic islets isolated from Naval Medical Research Institute (NMRI) mice without desensitization of pancreatic β cells induced by the anti-diabetic drug glyburide [54]. Mechanistically, it was suggested that stevioside protects the function of pancreatic β cells when exposed to high levels of glucose through regulating acetyl-coenzyme A carboxylase [55].

In summary, steviol glycosides, especially stevioside, rebaudioside A and steviol, have noticeable antihyperglycemic effects and can be helpful in the treatment of type 2 diabetes.

4.2. Effects on Blood Pressure

Studies have shown that stevia glycosides can help lowering blood pressure. Jeppesen et al. reported that stevioside has a dual anti-hyperglycemic and hypotensive effect [52]. Chan et al. showed that in normotensive mice, intravenously administration of stevioside lowers blood pressure but has no effect on heart performance and plasma levels of catecholamines [14]. In

another study, it was observed that intraperitoneal administration of stevioside could relax the vasopressin-induced vasoconstriction through inhibition of calcium influx into rat blood vessel [13]. In a similar study, it was revealed that isosteviol reduces vasoconstriction in rats through opening ATP-sensitive potassium channel and Ca^{2+}-sensitive small conductance potassium channel [56]. To summarize, steviol glycosides could reduce blood pressure through modulation of calcium and potassium channels without any side effect. Hence, these natural compounds could serve as promising therapeutic agents to lower blood pressure

4.3. Renal Effects

Melis evaluated the effect of stevioside on the performance of healthy kidney and high blood pressure. Stevioside was found to improve vasodilation of and reduce blood pressure, while increasing urination in two groups of normal and hypertensive mice. Moreover, multiple administration of stevioside in both normal and hypertensive groups led to increased glomerular filtration rate and renal plasma flow [57]. Yuajit et al. studied the effects of steviol and its derivatives on cyst growth in polycystic kidney disease using a cyst model of Madin-Darby canine kidney (MDCK) cells [58]. Results showed that steviol delays the development of MDCK cyst through direct controlling of cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel, and reduction of CFTR expression through CFTR proteasome decomposition [58]. Based on the findings of the above-mentioned study, steviol and its analogues are potential drug candidates for the treatment of renal polycystic disease [58].

4.4. Anti-Tumor Effects

Chemical carcinogenesis is a multistage process leading to malignancy, and inhibition of promotion stage has an important role in cancer chemoprevention. Konoshima and Takasaki investigated *in vivo* cancer chemopreventive effect of stevioside A, steviol and isosteviol in comparison with glycyrrhizin, a compound with known antitumor-promoting activity in chemical carcinogenesis. The results demonstrated that these three sweet compounds exerted stronger inhibitory effect compared with glycyrrhizin in the two-stage mouse skin carcinogenesis model induced by 7, 12 dimethylbenz [44]anthracene and 12-*O*-tetradecanoylphorbol-13-acetate [59, 60]. In agreement with this finding, it has been reported that stevioside, steviol and isosteviol, and their metabolites can retard tumor development through repressing the activation of the Epstein-Barr virus early antigen [60, 61]. In an *in vitro* study conducted by Mizushina et al., isosteviol was shown to suppress a panel of human cancer cells (human T cell acute lymphoblastic leukemia cell line, MOLT-4, human B cell acute lymphoblastoid leukemia cell line, BALL-1, and human gastric cancer cell line, NUGC-3) through inhibiting DNA replication [62]. Additionally, Jayaraman et al. evaluated anti-tumor effects of the acetonic extract of Stevia leaf using MTT assay and reported that this extract is toxic against human laryngeal epithiloma cells (HEp-2) while having not toxicity on the Vero normal cell line [10]. Hence, the extract could exert selective cytotoxicity but still considerable data is required from *in vivo* studies and studies on other cell lines to confirm any cytotoxic effect for Stevia leaf extract. In summary, Steviol glycosides are potential chemopreventive agents in carcinogenesis, and good natural compounds for cancer therapy.

4.5. Anti-inflammatory and immunomodulatory effects

Pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 are over-expressed in cancer cells and cause inflammatory disease. Increased production of pro-inflammatory cytokines is mediated by the MAPK pathway, TLR2 and NF-κB, and is inhibited by IκBα. Wang et al. revealed that stevioside attenuates inflammation through decreasing gene expression of IL-6, TNF-α and IL-1β cytokines in mice mammary glands infected by *S. aureus*. It was found that stevioside inhibits gene expression of the cytokines via inactivating MAPK pathway, TLR2 and NF-κB [18]. Several lines of evidence support these results, as it has been reported that stevioside inhibits pro-inflammatory cytokines through similar mechanisms in RAW 264.7 (mouse macrophage) cell line, acute lung injury, and epididymal, epithelial, and intestinal cells [19, 24, 63, 64]. Boonkaewwan and Burodom reported that steviol and stevioside decrease the expression of IL-6, TNF-α and IL-1β via inactivation of NF-κB and activation of IκBα in human colon carcinoma and THP-1, *in vitro*. It was concluded that steviol and stevioside inhibit pro-inflammatory cytokines through increasing IκBα level [65, 66]. In another study, Ahirwal et al. showed that Stevia leaf extract could efficiently modulate immune response and inhibit immunological disorders [67]. Sehar et al. also confirmed the same effect of stevioside by modulating B and T cell responses, and increasing phagocytic function [23].

In conclusion, stevioside can reduce inflammation and mediate immunomodulation through inhibition of pro-inflammatory cytokines at the level of gene expression.

4.6. Obesity Treatment

Increased consumption of carbohydrates has a significant impact on health and leads to various nutritional diseases such as obesity. Continuous consumption of sugar-based food and drinks also cause various metabolic diseases. Therefore, an approach for weight control could be

substitution of sugar and sweets with low calorie sweeteners [68]. Stevioside and rebaudioside of Stevia leaf have zero calorie and do not produce energy, while they are 250-300 times sweeter than sucrose [69, 70]. Clinical studies have shown that sweetness of 1 gram of raw stevia leaf extract solved in water is between 100 to 150 times the sweetness of equal amount of sucrose [71]. Thus, stevia sweeteners could replace sugar in low-calorie foods and drinks as they can restrict or control calorie intake and, eventually, control or reduce weight. In this context, Curry and Roberts have also shown that consuming a high dose of rebaudioside A (50,000 ppm) for 13 weeks reduces the weight of male and females rats which may be attributed to initial distaste and lower caloric density [72].

4.7. Effect on Tooth Decay

Continuous use of food sweeteners as well as high-calorie carbohydrates could lead to the growth of harmful bacteria in mouth leading to the formation of dental plaques and gingivitis. Therefore, substitution of sucrose with other natural and less harmful carbohydrates is vital [73]. Stevia is an edible sweetener with zero calorie that can act as a bacteriostatic and bactericidal substance and prevent tooth decay and gingivitis. The leaf extract of stevia and most of its secondary metabolites, including steviol, isosteviol, stevioside, and rebaudiosides A, B, C,D E, and F do not cause tooth decay and will restrain the accumulation of glucan by microorganisms that are responsible for tooth decay [74]. Addition of stevioside, but not sucrose, glucose, and fructose, in culture media has been reported to limit the growth and acid-producing capacity of *streptococcus mutans* which is the main microorganism causing tooth cavity [75].

5. Toxicity of Steviol Glycosides

Safety of steviol glycosides has been confirmed in numerous toxicological studies including acute and subacute toxicity, reproductive toxicity, genotoxicity and carcinogenicity investigations [72]. Consequently, steviol glycosides meet the safety standard of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [6].

5.1. Acute Toxicity

Acute toxicity tests determine the dose at which half of the animals die, so-called LD_{50} or median lethal dose. It was found that Stevioside has a large LD_{50} value in mouse, rat and hamster, thereby a low acute oral toxicity [76-78]. Purified stevioside (purity of 93-95%) at a dose as high as 15g/kg body weight was not lethal to either mice, rats or hamsters by oral intake [76, 78]. In dogs, rats and mice, an LD_{50} of 0.5 g/kg body weight was found for the isosteviol by oral Intake [77]. It was concluded that as stevioside is 300 times sweeter than sugar, a LD_{50} of 8.2 g/kg corresponds to an exceedingly high value of about 2.5 kg sugar/kg body weight [79].

5.2. Subacute Toxicity

In subacute toxicity studies, the maximum concentration at which tested compound do not show any adverse effect, so called- NOEL (No Effect Level), is determined. Finally, acceptable daily intake (ADI) is determined through dividing the NOEL by 100. Subacute toxicity studies were performed on male and female rats, F344 rats and hamsters fed with up to 2.5g/kg/day of purified stevioside during 3 months. Stevioside showed no adverse effect at dosage of 2.5g/kg/day in the tested animals. Consequently, it was concluded that the NOEL for stevioside is higher than 2.5g/kg/day and the ADI can be at least 25 mg/kg body weight. In another study, male and female rats were fed diets containing up to stevioside 1.2 % for 24 months. Results

showed no adverse effect on rats, so the NOEL was obtained 1.2 %. Therefore, it was suggested that ADI of stevioside in human is 7.938 mg/kg body weight/day [80]. Subacute toxicity of rebaudioside A was studied at dietary concentrations up to 50,000 ppm in rats during a 13-week study. The NOEL was considered to be approximately 4161 and 4645 mg/kg body weight/day in male and female rats, respectively, that are about 1000 folds higher than likely human exposures to rebaudioside A through its use as a natural sweetener [72]. In a similar 13-week toxicity study of rebaudioside A in rats at a dosage of up to 20000 mg/kg body weight/day, it was concluded that the NOEL is 2000 mg/kg body weight/day [81]. However, the JECFA cut-off level of ADI of 0–2 mg/kg body weight/day is 2000 folds less than the value obtained in the above-mentioned study [6]. It was emphasized that the temporary ADI established by JECFA is based on data for other steviol glycosides and Stevia extracts, not rebaudioside A. Hence, an ADI higher than the current temporary ADI of 0–2 mg/kg body weight/day is supported by the NOAL in the 13-week toxicity study [72].

5.3. *Reproductive toxicity*

Yodyingyuad and Bunyawong studied toxicity of stevioside on the growth and reproduction performance of male and female hamsters. Results revealed that growth and fertility in both sexes (parents, F1 and F2 generations) fed by stevioside were normal. It was found that pregnancy length, number of fetuses, parturition frequency, as well as histological features of reproductive tissues were not affected. Finally the authors concluded that growth and reproduction performance in hamsters fed by stevioside at a dose as high as 2.5 g/kg body weight/day does not show any abnormality [82]. In a similar study, two-generation reproductive toxicity study on male and female rats fed by rebaudioside A showed no adverse effects on

mating performance, fertility, gestation length, oestrous cycles, and sperm mobility, concentration, or morphology in either the F_0 or F_1 generations. Also, it was found that rebaudioside A does not have any side effects on pre-weaning reflex development, overall body weight gain, and the timing of sexual maturation of the F_1 and F_2 offsprings. Consequently, it was found that rebaudioside A at a dosage of 2048–2273 mg/kg body weight/day is safe for the survival, development, and general condition of the parents and offspring rats [83]. Usami et al. evaluated teratogenicity of stevioside at doses of 0, 250, 500 and 1000 mg/kg/day in pregnant rats. Result showed that stevioside was not teratogen and no detectable adverse effect in the pregnant rats and their fetuses was observed. So it was concluded that over 1000 mg/kg/day of stevioside could not cause any fetal malformation and is safe for both pregnant rats and rat fetus [84].

5.3. Genotoxicity

Based on the *in vitro* and *in vivo* studies including Ames, mouse lymphoma and micronucleus assays , stevioside and rebaudioside A are not mutagenic and do not cause any chromosomal damage nor any break in DNA structure [85-93]. There has been only one evidence by Nunes et al. for showing DNA damage with stevioside according to the results of comet assay [94]. The validity of this assay has been questioned [95, 96], and JECFA has rejected this result and concluded that stevioside and rebaudioside A do not possess any genotoxic activity. In addition, an objective weight-of-evidence assessment of the complete genotoxicity profile for steviol glycosides has shown that consuming stevioside and rebaudioside A do not introduce a risk of genetic aberration in human [97].

5.4. Carcinogenicity

Animal studies have not shown any evidence for the carcinogenicity of stevioside and rebaudioside [98]. Hagiwara et al. have studied 344 laboratory mice in a diet enriched with 5% stevioside for 36 weeks, and did not report a new neoplasm in their cysts [99]. Consistently, Xili et al. did not report any new neoplasm after a 24-month study on laboratory mice following feeding with stevioside (dose range: 0.2-1.2%) [80]. Finally, Toyoda and colleagues in their research on 344 laboratory mice receiving 2.5%, 5%, and 65.6%-rich stevioside diet, did not report any neoplasm [100].

6. *Conclusion*

Stevia has been used as a medicinal plant in the traditional medicine for a long time. Carbohydrates such as steviol, isosteviol, stevioside, and rebaudiosides A, B, C, and E are among the ingredient of this plant. The main characteristics of Stevia carbohydrates are their low gastrointestinal absorption, zero calorie, and considerable greater sweetness compared with sucrose which make them ideal sweeteners for healthy low-calorie drinks. Besides, several benefits against cardiovascular disease, cancer, diabetes, and obesity have been attributed to the carbohydrates present in Stevia. However, there is still need for confirmatory evidence from randomized controlled trials to ascertain the preventive and/or therapeutic value of supplementation with Stevia and its glycoside ingredients against the above-mentioned diseases. Furthermore, future studies are recommended to explore the mechanisms underlying the health benefits of Stevia, and the main active ingredients.

Conflict of interests

None.

Reference

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Figure 1. *Stevia rebaudiana*; Left, plant body and right, leaves.

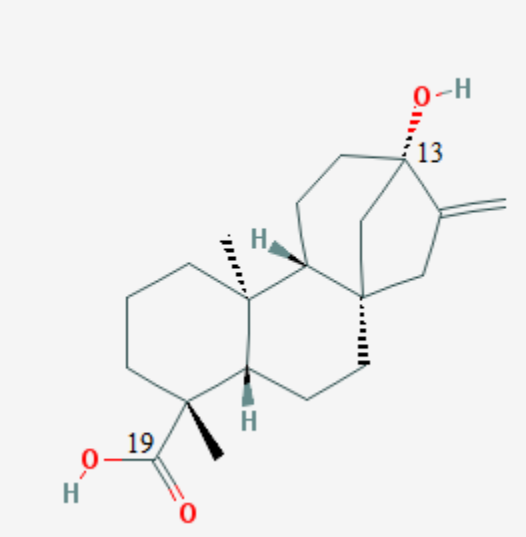

Figure 2. Chemical structure of steviol; the basic building block of steviol glycosides.

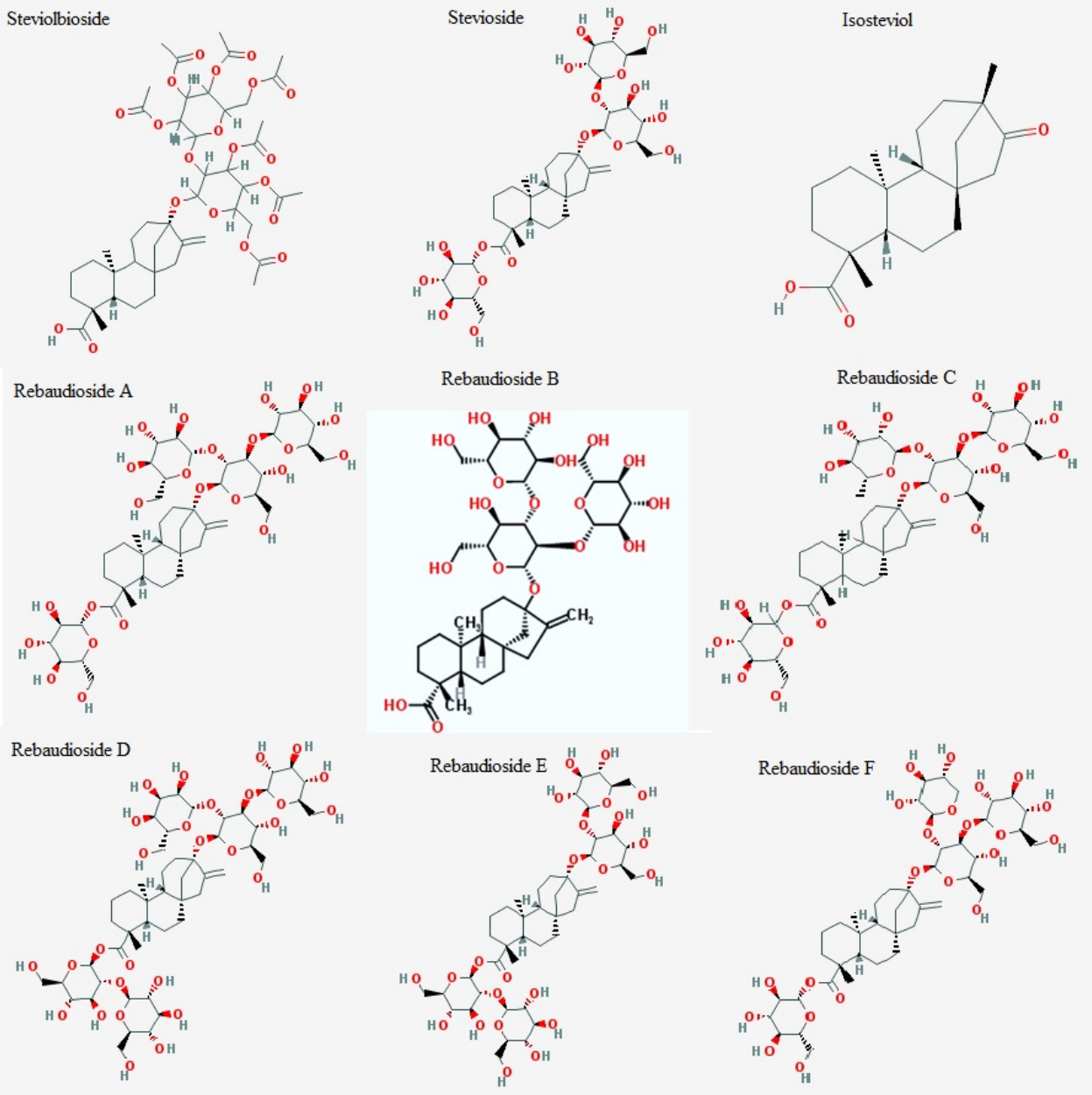

Figure 3. Chemical structures of the main steviol glycosides of Stevia leaves.