Extraction of steviol glycosides from Stevia leaves and their biotransformation with the aim to improve its taste and other biological activities

A thesis submitted in partial fulfillment of the requirements for the Degree of **Master of Philosophy in Microbiology**

By

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"IN THE NAME OF ALLAH THE MOST BENEFICENT, THE MOST MERCIFUL"

DEDICATION

My whole struggle and achievement are dedicated to ***ALLAH ALMIGHTY*** *first, then to* ***AL-MASOMEEN S.A.W.W*** *after them, it is dedicated to my* ***parents; Rubina Nazneen*** *and* ***Nadeem Ahmad,*** *and especially* ***Dr. Shaheen Ali, Hafeez Ali, Sajjad Hussain*** *and* ***Ammar Yasir*** *whose love, support, prayers, affection, and belief make me able to reach this point.*

DECLARATION

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Aliya Batool

CERTIFICATE

This thesis submitted by **Aliya Batool** is accepted in its present form by the Department of Microbiology, Quaid-I-Azam University, and Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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LIST OF ABBREVIATIONS

S.no	*Abbreviations*	*Full Form*
1.	PHWE	Pressurized Hot Water Extraction
2.	MAE	Microwave-Assisted Extraction
3.	GRAS	Generally Regarded As Safe
4.	FDA	Food and Drug Administration
5.	EFA	Essential Fatty Acid
6.	ROS	Reactive Oxygen Species
7.	BHA	Butylated Hydroxyanisole
8.	BHT	Butylated Hydroxytoluene BHT
9.	DPPH	2,2-diphenyl-1-picrylhydrazyl
10.	SEB	Stevia Extract in 1-Butanol
11.	SEC	Stevia Extract in Chloroform
12.	SEM	Stevia Extract in Methanol
13.	SEW	Stevia Extract in water
14.	SEH	Stevia Extract in n-Hexane
15.	OH	Hydroxyl
16.	sp^3-CH	Alkane
17.	C-O-C	Ether
18.	C=O	Carbonyl
19.	-C=C	Alkene
20.	MC	Moisture Content
21.	TLC	Thin Layer Chromatography
22.	HPLC	High Performance Liquid Chromatography
23.	FT-IR	Fourier Transform-Infrared Spectroscopy
24.	R_f	Retention Factor
25.	UV	Ultraviolet
26.	IR	Infrared

27.	MS	Microscopy
28.	C	Control
29.	SE	Stevia Extract
30.	E	Experiment
31.	DE	Diluted Extract
32.	rpm	Revolutions per minute
33.	hrs	Hours
34.	mg/L	Milligram per Liter
35.	g/L	Gram per Liter
36.	g	Gram
37.	mg	Milligram
38.	ml	Milli liter
39.	µl	Microliter
40.	cm	Centimeter

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Abstract

Stevia is a natural source of sweetness, that people prefer over other types of sugar and artificial sweeteners as it is a better alternative, composed of various sweetening compounds of diterpene glycosides (steviol glycosides) in which the major constituents are steviosides (along with bitterness) and rebaudioside A. But the main bottleneck in its bulk use is Stevia's bitter aftertaste. The current study was conducted to resolve the taste-related problem of Stevia naturally through biotransformation. The research began by isolating naturally occurring sweet compounds (steviol glycosides) from the dried leaves of Stevia using different solvents i.e., water, methanol, chloroform, and 1-butanol. Furthermore, the extracted sweet compounds were biotransformed by selected bacterial strains in modified M9 media (5% glucose) to reduce the bitterness associated with the steviol glycosides and enhance their biological activities. The present study has shown better transformation of steviol glycosides with *Bacillus stercoris* at 40°C among the selected bacterial strains. The thin-layer chromatographic separation technique was developed to quantify the extracted and biotransformed compounds on the silica plate with different combinations of solvents in a mobile phase. For maximum compounds separation, the optimized mobile phase was acetone/ethyl acetate/water in a specified ratio of 50:40:10 v/v. In addition, the chemical changes in the compounds were determined by FTIR analysis in the range of 400-4000 cm^{-1} indicating bond breaking and compounds formation. These biotransformed compounds had improved biological activities such that the antioxidant activity which was increased to 58% compared to the control extract with 46% activity before biotransformation. This highlighted the value of biotransformed steviol glycosides in the food and pharmaceutical industries due to the prominent health benefits over other sugars. However, further confirmation of steviol glycosides after biotransformation can be obtained by performing high-pressure liquid chromatography (HPLC).

INTRODUCTION

Carbohydrates such as table sugar (sucrose) have been extensively used as a source of sweetener in every field of life, i.e., in the food industry, pharmaceuticals, and beverage industries, etc. but becoming a reason for different health issues including diabetes, high blood pressure, or fatty liver. To reduce the emergence of these problems created by table sugar, the shrub of Stevia is a good naturally occurring alternative (Shivanna *et al.*, 2013). It is an indigenous plant from different regions of the World. Historically, the Paraguayans have utilized this as a sweetener as well as herbal medicine. In the past, Stevia was known to the Spanish around the 16th century, but it remained obscure until M. S. Bertoni once more brought it to the notice of Europeans in 1888 and named Stevia by 1905. This plant has been growing in different regions including China, India, Japan, Thailand, South America, Brazil, Korea, Central America, Paraguay, and Pakistan (Lemus-Mondaca *et al.*, 2012). According to the report published in Mordor Intelligence, the estimated worth of the Stevia market was 638.69 million USD in 2021, and it is anticipated to grow at a CAGR of 8.85 in the next five years till 2028. According to another analysis, A compound annual market growth of Stevia increased to $0.75 billion in 2022 and is estimated to increase to $0.82 billion by 2023 at a CAGR rate of 9.9% which was used to calculate the size of the worldwide Stevia market (*Stevia Market Size, Trends, and Global Forecast to 2032*, 2022).

There are about 154 members of Stevia belonging to the family of Asteraceae including *S. subpubescens, S. salicifolia, S. lucida, S. connata, S. puberula, S. eupatoria,* and *S. serrata*. The perennial herbs of Stevia contain a high concentration of natural, nutritionally important sweeteners in their leaves which make them economically valuable (Kumari *et al.*, 2017). There are various compounds found in the extract of this plant such that tannins, alkaloids, terpenes, lipids, carbohydrates, proteins, polyphenols, and vitamins (Bender, 2018). The most important compounds of Stevia are steviol glycosides that weigh up to 20% weight of dry leaves including, stevioside (5 to 10%), rebaudioside A (2 to 4%), rebaudioside C (1%), rebaudioside D (0.2%), rebaudioside E (0.2%), rebaudioside F (0.2%), steviolbioside (0.1%), dulcoside A (0.4-0.7%), and rebaudioside M (less than 0.1%), etc (Lemus-Mondaca *et al.*, 2012). These compounds have more sweetness as well

as the antioxidant potential to support a healthy lifestyle. These high-intensity glycoside sweeteners have high melting points with low water solubility and 300 times more sugar potential in comparison to that of sugars (Kim *et al.*, 2018; Kaushik *et al.*, 2010). Stevia is also considered a zero calories sweetener as it is not metabolized by the body in highly purified form (Ashwell, 2015). The most important steviol glycoside is stevioside which was discovered in 1931 by French chemists. It is a diterpene glycoside having a chemical formula of $C_{38}H_{60}O_{18}$. The second most important compound is rebaudioside A which is more thermally stable as well as 1.6-1.8% sweeter than stevioside (Urban *et al.*, 2015). On the basis of the intensity of sweetness with respect to structure, the steviol core's glycosyl residues at C-19 (R^1) and C-13 (R^2) are crucial for sweetness. The sweetness is increased by expanding the ester-linked C-19 at glycosyl unit as in rebaudioside A as compared to stevioside (with bitterness) (Ohta *et al.*, 2010).

In unprocessed form, Stevia has 30 times more sweet potential in comparison to refined extract which is 200-300 times extra sweet than normal sugar. As sweetness is related to steviol glycosides, stevioside, and rebaudioside A are more highlighted which are 140 and 240 times sweet respectively. Although other compounds such that rebaudioside M which is about 300 times sweet but is found in very minute quantities (Prakash, Chaturvedula, and Markosyan, 2014). On the basis of the structural stability of steviol glycosides, rebaudioside A appears to be more stable than stevioside and other compounds (Purkayastha *et al.*, 2016).

In addition to sweetness, steviol glycosides possess significant therapeutic or pharmacological properties, including antifungal, antibacterial, antiviral, antioxidant, antitumor, antidiarrheal (gastroprotective) activities, diuretic, as well as immunomodulatory effects and favorable impact on renal function, blood glucose, and blood pressure. Their therapeutical aspect has been making them worthy to be adopted although the research on its compounds is still so vast. They also improve the regeneration of cells, inhibit the formation of tumors, and strengthen or repair the blood vessels (Ruiz-Ruiz *et al.*, 2017).

It is also famous for its industrial use in various food (especially the enhancement of flavors). Stevia leaves are added to coffee, fruit, salads, and different beverages. Stevia is

commonly utilized in a range of different beverage industries including artificial juice and carbonated soft drinks makers i.e., Coca-Cola, Calbee Foods, Unilever, Groupe Danone, PepsiCo, and Nestle (PureCircle, 2019).

Different researchers opt for various ways to extract sweet compounds from Stevia leaves in the past varying from water to reagent extraction such that ethanol or methanol to use in daily life (Myint *et al.*, 2020). In comparison to older extraction techniques like boiling, Soxhlet, and reflux, modern extraction techniques like pressurized liquid extraction, PHWE (pressurized hot water extraction), MAE (microwave-assisted extraction), and supercritical fluid extraction are now more often utilized (Teo *et al.*, 2009). Water can dissolve the majority of compounds more easily than other chemical reagents so it is a good extracting medium for steviol glycosides. Various researchers used this extraction technique to isolate specific substances such as rebaudioside A, steviosides, steviolbiosides, etc., (Chong Saw Peng, Akil, and Bahari, 2020). Another water-dependent ohmic heating extraction method was also adopted in the past to prevent heat-sensitive components from being deteriorated by overheating (Moongngarm *et al.*, 2022). Methanol has better extraction potential in comparison to water even in the temperature range of 110 to 160°C. But had supported a good extraction process when used in combination with water (Kumari *et al.*, 2017). Ethanol is good as methanol for the extraction of compounds from Stevia and most accepted method because it is generally regarded as safe (GRAS) by FDA (Shamima *et al.*, 2019). Another conventional method was reported in the past by using the combination of reagents i.e., water, ethanol, methanol, and their binary combinations for extraction along with microwave and ultrasonic standard extraction methods (Jaitak *et al.*, 2009).

Stevia has a bitter aftertaste due to the presence of stevioside which some people find a little irritating, despite having more sweetness than regular sugar. People have also reported tastes like licorice in it occasionally (Gerwig *et al.*, 2016). Scientists are more concerned about removing the problem of bitterness from Stevia. In the past, different non-biological and biological ways were reported to overcome bitter aftertaste. The non-biological ways involve the chemical processing of the Stevia extract used in the Formigoni *et al.* (2018) study which involved treating the extract with ethanol to lessen the bitterness of the steviol

glycosides found in Stevia (Formigoni *et al.*, 2018). Different food industries use sodium gluconate in different proportions to exclude bitterness to some level but it cannot completely resolve this problem as chemicals have some diverse effects on the body. Sodium gluconate is considered safe by FDA but it is a salt that can raise blood pressure when used in an excessive amount (Grillo *et al.*, 2019). Some sugar acids were also used to reduce the bitterness but that caused different health issues i.e., acidity or can raise blood sugar levels (Hettinga, 2019). This seems to be not suitable for long usage but the biological processing of food is beneficial as it can transform the core compounds in order to improve the taste, and texture, of material, as well as to reduce the toxic effects of the compounds biologically that have no diverse effects on the health (O'Connor, 2020). This kind of biological approach of conversion supports biotransformation processes (changing of organic chemicals from one form to another) that can be either enzymatic or non-enzymatic (Sultana, 2018), accomplished by different microorganisms and their byproducts, including fungus and bacteria (Singh, 2017). Microbial biotransformation is one of the most authentic approaches (Wang *et al.*, 2016). The microbial biotransformation follows the processes of oxidation, hydrolysis, reduction, condensation, isomerization, the introduction of functional groups, regioselective transfer reactions of glycosides, and selective cleavage of tetracyclic terpenoids' side chains to form C19 steroids, and production of new carbon bonds (Sultana and Saify, 2012).

Microbial reduction in biotransformation occurs with a degree of stereochemical and regiochemical consistency that cannot be achieved chemically (Madeira Junior *et al.*, 2013). Another step of biotransformation is hydrolysis which can proceed with enzymes as a substance breaks down during enzymatic hydrolysis after reacting with water in the presence of enzymes. The most favorable hydrolysis is a de-esterification process (Obodovskiy, 2019). While oxidative biotransformation can be done by enzymes including cytochrome P450, monooxygenases, and mixed-function oxidases enzymes supporting the transportation of electrons from one organic compound to another (Meynet, Davenport, and Fenner, 2020). Condensation is the formation of new molecules by combining two pre-existing molecules. This reaction takes place in basic or acidic conditions or in the presence of a catalyst (Enright *et al.*, 2014).

Biotransformation is a more favorable method to change the forms of compounds from one to another as it can involve either natural or synthetic substrates or compounds. It can be supported by more than one enzyme and accomplished in multiple steps even though the endogenous and exogenous processes can easily be accomplished (Ravindran & Basu, 2013).

The sweetness is more related to stevioside and rebaudioside A but people address the bitter aftertaste associated with stevioside which sometimes tastes like licorice. The main focus of the current study was to convert or transform these steviol glycosides compounds through microbial transformation processes to enhance the taste by lowering the bitter aftertaste of specific sweet compound stevioside and to increase its biological activities such that antioxidant potential.

In this study, the extract from Stevia leaves was incubated with pre-isolated microorganisms (*Anoxybacillus, Bacillus stercoris,* and *Bacillus licheniformis)* for biotransformation. The microbial isolates transformed the main steviol glycosides to another form to improve the taste and biological activity such that the antioxidant properties. Hence these biotransformed molecules with improved taste and biological activities can be used in different industries including food and pharmaceuticals after further purification and confirmation through HPLC and other analytical techniques.

Aim and objectives

The aim of this research was the extraction of sweet compounds (steviol glycosides) from the leaves of the Stevia and their biotransformation through microorganisms to improve its taste and biological properties.

Objectives of this research

- Extraction of sweet compounds (steviol glycosides) from Stevia leaves using different solvents.
- Biotransformation of extracted compounds from Stevia by using selected bacterial isolates.
- Examination of targeted compounds through various chromatographic and analytical techniques.
- Comparative analysis for the antioxidant activity of extracted and biotransformed compounds.

LITERATURE REVIEW

2.1. Natural and Artificial Sweeteners

Sugar is consumed as a natural ingredient and has been a part of our diet for a long. It is one of the earliest commodities in history records. It is generally accepted that man originally utilized cane sugar in Polynesia, from where it later traveled to India. The Persian Emperor Darius conquered India in 510 BC. The completed product was being shipped, and the secret of cane sugar was kept under tight lock and key. In the 11th century AD, the Crusades led to the discovery of sugar by western Europeans, in 1069, the first sugar was documented in England (Eggleston, 2019). Later centuries witnessed a significant increase in commerce between East and western Europe, including importing sugar. It was considered to be a significant luxury at the time. In 1747, sugar beet was discovered with sugar-producing properties and was used by Europeans as an alternative to sugar cane in 1880 (Mazumdar, 2020). Although sugar is frequently used by people for flavoring their snacks and food, excessive use can harm health.

There are different artificially available substitutes for sugar being used nowadays. FDA (Food and Drug Administration) of the United State has allowed some substitutes including advantage, acesulfame potassium, neotame, sucralose, and saccharin, etc. although many more substitutes are being used in Europe such that sugar alcohols i.e., xylitol and sorbitol (Pang *et al.*, 2020). Sugar alternatives may aid in short-term weight management for adults and kids who are obese or overweight. This is because sugar replacements frequently have zero or low calories. However, it's unclear if sugar alternatives can support long-term weight management for individuals. According to some studies, long-term everyday use of these sweeteners may increase the risk of heart disease, bladder cancer, brain tumor, stroke, and overall mortality. The elevated risk could be due to other behaviors or a lack of healthy practices (Ebbeling *et al.*, 2020). The researchers analyzed the natural substitute for sugar that have less or no side effects on the human body such as the extract of Stevia leaves which is a plant or herb with great sweetness and biological effects on human health.

2.2. History of Stevia

It is a generic name that describes various kinds of sweeteners including the entire plant of Stevia and its leaves, which contain sweet compounds. It is low calories sweetener (Lemus-Mondaca *et al.*, 2012). Since ancient times, it has been utilized as a flavoring agent and a natural alternative sugar. It is indigenous to South America, where it was initially consumed to sweeten drinks, or its leaves were chewed by locals for sweetness about 200 years ago. Its dried leaves are also known as a sweet herb which was consumed as a pleasant treat or used to sweeten medications as well as beverages. Scientifically, in 1899 it was first introduced as *Eupatorium rebaudianum* by Moises Santiago de Bertoni in Paraguay. It was later identified in 1905 as *Stevia rebaudiana*, belonging to an Asteraceae family member of sunflower (Kumari *et al.*, 2017). In the 1970s, it was originally used commercially as a sweetener in Japan and is still a preferable component there today. Most of the world's Stevia is grown in Kenya, Paraguay, China, United States, as well as in Vietnam, Brazil, Argentina, India, Pakistan, and Colombia (Lemus-Mondaca *et al.*, 2012). It is also referred to as honey leaf, sweet herbs, sweet leaf, sweet weed, and candy leaf.

Globally, 32,000 hectares of Stevia have been grown with 75% of that in China. It is believed that there are 230 species in the genus. Stevia species thrive in mountains that are distinctive to Mexico and South America. Around 120 different species can be found in South America, primarily in the countries of Peru, Northern Argentina, Bolivia, Paraguay, and southern Brazil. There are at least 70 native species in Mexico out of the more than 80 species that are known to exist in North America. According to records, the genus is absent from the Bahamas, Amazonia, and the Antilles.

Stevia rebaudiana Bertoni Asteraceae' specie is also known as Stevia which is the most well-known member of the genus and is famous across the world for producing vast quantities of stevioside, a potent natural sweetener that lacks nutrients. There are numerous regarding plants are there, including *S. salicifolia, S. lucida, S. eupatoria, S. subpubescens, S. connata, S. puberula,* and *S. serrata* (Borgo *et al.*, 2021). The Stevia is classified as follows in the table.

Table 01. Classification of Stevia Plant

KINGDOM	PLANTAE
SUB-KINGDOM	Tracheobionta
DIVISION	Magnoliophyta
SUPER DIVISION	Spermatophyta
CLASS	Manoliopsida
SUBCLASS	Asteridae
ORDER	Asterales
FAMILY	Asteraceae
GENUS	*Stevia*
SPECIES	*Stevia salicifolia, Stevia rebaudiana,* etc.

2.2.1. Compounds in Stevia

Stevia is a unique combination of steviol glycosides, flavonoids, polyphenols, tannins, terpenes, alkaloids, proteins, carbohydrates, and lipids. The major constituents are steviol glycosides which are responsible for the sugar potential of the Stevia plant. The other compounds are involved in different biological activities.

The compounds in Stevia are as in the figure below;

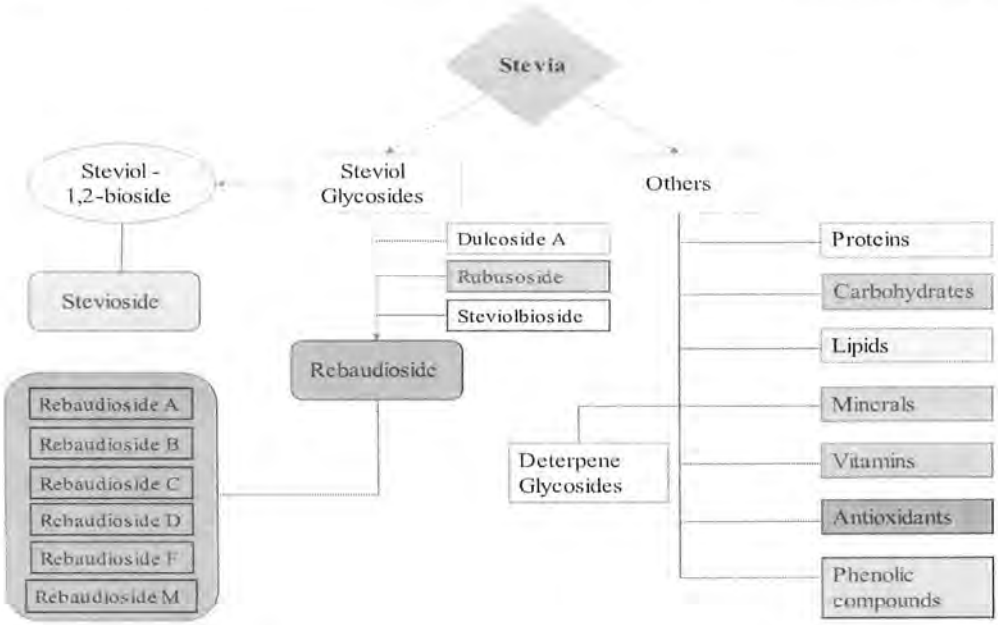


Figure.2.1: *Flowchart of the identified compounds found in the leaves of Stevia.*

2.2.1. Steviol Glycosides

These are the chemical components that give Stevia leaves their sweet flavor and are also the primary constituents of many sweeteners. These are found in different Stevia species such that *Stevia rebaudiana* and *Stevia phlebophylla* and in a plant of *Rubus chingii*. Consumption of these compounds has no glycemic response on the human body as Stevia cannot be metabolized by humans. Their structure is similar to steviol, but the glucose molecule is present instead of carboxyl hydrogen. There are various steviol glycosides in the Stevia extract including steviosides, rubusoside, steviolbioside, and rebaudiana, etc.

2.2.1.1. Stevioside

Stevioside is a sweetening agent and an important steviol glycoside. It was discovered by French chemists in 1931, and they gave it that name. It is one of the essential components of Stevia. According to reports, stevioside (4–13% w/w) is present in the plant leaves in the highest concentration (Lemus-Mondaca *et al.*, 2012). It is a diterpene glycoside with the chemical formula of $C_{38}H_{60}O_{18}$. In aqueous solutions, these compounds are extremely stable over a wide pH and temperature range. It is widely

accepted in Argentina, Brazil, China, Japan, Paraguay, and Korea. According to previous research, stevioside virtually degraded under thermal treatment in the pH range of 1 to 10 for 2 h at 60°C; only minor loss to 5% at pH 2 and 10 were discovered after heating to 80°C. This was driven to decompose in strong acidic circumstances at pH 1.0, which led to total breakdown after incubation at 80°C for two hours. At pH levels between 3-9, the stevioside has excellent heat stability up to 100°C for 1 hour, while at pH levels above 9, fast decomposition takes place. The IUPAC name of stevioside is 3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl (Ceunen and Geuns, 2013). The structure of stevioside is as in Figure (2.2).

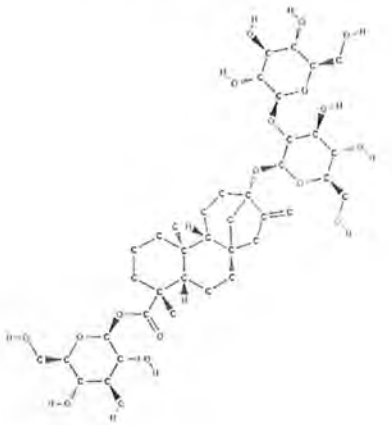


Figure 2.2: Molecular structure of Stevioside

2.2.1.2. *Rebaudioside*

It is the second most important constituent of Stevia. It is diterpene glycoside and is further classified into subclasses varying from A to F, and M. It varies from stevioside in the number of attached glucose units.

2.2.1.2.a. Rebaudioside A

Rubusoside which is a rebaudioside is rebaudioside A in which the beta-D-glucopyranosyloxy group at position 13 alpha has both of its hydroxy groups at positions 3 and 4 transformed to the equivalent beta-D-glucopyranoside. In comparison to stevioside, 1000 mg/L; phosphoric acid rebaudioside A with pH 2.6 displayed somewhat improved thermal stability. The FDA granted use approval for Reb-A in 2008. it is a highly refined

version of Stevia having a chemical formula of $C_{44}H_{70}O_{23}$. The IUPAC name of stevioside is (2S, 3R, 4S, 5S, 6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl. It is present in a ratio of 2 to 4% in the dry matter of Stevia leaves acid (Kakigi *et al.*, 2013). The structure of rebaudioside A is as in figure 2.3 (a);

2.2.1.2.b. Rebaudioside B

It is found in traces in the leaves of Stevia with a chemical formula of $C_{38}H_{60}O_{18}$. It has a melting point of less than 190°C. It is predicted that it has a temperature tolerance of about 1000°C for boiling. The IUPAC name of Rebaudioside B is (1R, 4S, 5R, 9S, 10R, 13S)-13-[[(2S, 3R, 4S, 5S, 6R)-5-hydroxy-6-(hydroxymethyl)-3,4-bis [[(2S, 3R, 4S, 5S, 6R) 3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy]oxan-2yl]oxy]oxan-2-yl]oxy-5,9-dimethyl-14-methylidenetetracyclo[11.2.1.$0^{1,10}$.$0^{4,9}$]hexadecane-5-carboxylic acid (Kakigi *et al.*, 2013). The 2D structure of this compound is as in figure 2.3 (b)

2.2.1.2.c. Rebaudioside C

Rebaudioside C also known as Dulcoside B is a member of the steviol glycosides family. It is a naturally occurring component of the plant Stevia and is used as a natural sweetener by people with diabetes and other dietary restrictions including carbohydrates. It is found in 1.01±0.02% dry by weight in the leaves of Stevia having a chemical formula of $C_{44}H_{70}O_{22}$. It is highly soluble in DMSO and slightly in methanol. It has a melting point of 186-188°C. Its IUPAC name is [(2S, 3R, 4S, 5S, 6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyoxan-2-yl] (1R, 4S, 5R, 9S, 10R, 13S)-13-[[(2S, 3R, 4S, 5S, 6R)-5-hydroxy-6-(hydroxymethyl)-4-[(2S, 3R, 4S, 5S, 6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy]oxan-2-yl]oxy-3-[(2S, 3R, 4S,5S,6R)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxy-5,9-dimethyl-14-methylidenetetracyclo[11.2.1.$0^{1,10}$.$0^{4,9}$]hexadecane-5-carboxylate. The 2D structure of rebaudioside C is in figure 2.3 (c).

2.2.1.2.d. Rebaudioside D

Although Rebaudioside D is a sweetener from Stevia, has better sweetness and organoleptic qualities but its manufacturing is constrained by the plant's extremely low abundance of it in its leaves. It is found in 0.32±0.04% dry by weight in the leaves of Stevia

having a chemical formula of $C_{50}H_{80}O_{28}$ (Prakash, Markosyan, and Bunders, 2014). Its IUPAC name is [(2S, 3R, 4S, 5S, 6R)-4,5-dihydroxy-6-(hydroxymethyl)-3-[[(2S, 3R, 4S, 5S, 6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyoxan-2-yl-] (1R, 4S, 5R, 9S, 10R, 13S)-13-[[(2S, 3R, 4S, 5S, 6R)-5-hydroxy-6-(hydroxymethyl)-3,4-bis [[(2S, 3R, 4S, 5S, 6R) 3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy]oxan-2yl]oxy]oxan-2-yl]oxy-5,9-dimethyl-14-methylidenetetracyclo[11.2.1.$0^{1,10}$.$0^{4,9}$]hexadecane-5-carboxylate. The chemical structure of Rebaudioside D is as in the figure 2.3 (d);

2.2.1.2.e. Rebaudioside F

A line of Stevia that produces a lot of rebaudioside C has leaves that were high in a diterpenoid glycoside production of rebaudioside F with a molecular formula of $C_{43}H_{68}O_{22}$. It has a great temperature tolerance having a boiling point of about 1071°C (Chaturvedula *et al.*, 2011). It has the IUPAC name of [(2S, 3R, 4S, 5S, 6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyoxan-2-yl-] (1R, 4S, 5R, 9S, 10R, 13S)-13-[[(2S, 3R, 4S, 5S, 6R)-5-hydroxy-6-(hydroxymethyl)4- [[(2S, 3R, 4S, 5S, 6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy]oxan-2yl]oxy]oxan-2-yl]oxy-5,9-dimethyl-14-methylidenetetracyclo[11.2.1.$0^{1,10}$.$0^{4,9}$]hexadecane-5-carboxylate. Although it is found in traces (Well *et al.*, 2013). The 2D structure of Rebaudioside F is in figure 2.3 (f)

2.2.1.2.f. Rebaudioside M

A naturally occurring, calorie-free sweetener, high-purity rebaudioside M (sometimes referred to as rebaudioside X). it is a small sweetener found in the South American plant Stevia rebaudiana Bertoni. It is one of at least ten different sweet-tasting steviol glycosides that are present in nature with it. It is a glycoside of the diterpenoid aglycone. The crystalline form of pure rebaudioside M (more than 95 percent) crystallizes out and is only moderately soluble in water and ethanol. At 25 °C, its amorphous substance is 1.1–1.3 percent soluble in water. Its equilibrium solubility is 0.26% in water at 25 °C. Although, at room temperature and with regulated humidity levels, Rebaudioside M dry powder is stable for approximately a year. It is notably less stable in solution below pH 2 and is most stable in the pH range of 4 to 8 (Prakash, Markosyan, and Bunders, 2014). As one might anticipate, stability declines as the temperature rises. It is extremely comparable to rebaudioside A in terms of stability. The chemical formula is $C_{50}H_{90}O_{33}$ with an IUPAC

name of [(2S, 3R, 4S, 5S, 6R)5-hydroxy-6-(hydroxymethyl)-3,4-bis[[(2S, 3R, 4S, 5S, 6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyoxan-2-yl-] (1R, 4S, 5R, 9S, 10R, 13S)-13-[[(2S, 3R, 4S, 5S, 6R)-5-hydroxy-6-(hydroxymethyl)-3,4-bis [[(2S, 3R, 4S, 5S, 6R) 3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy]oxan-2yl]oxy]oxan-2-yl]oxy-5,9-dimethyl-14-methylidenetetracyclo[11.2.1.$0^{1,10}$.$0^{4,9}$]hexadecane-5-carboxylate. It has a chemical structure as shown in the figure 2.3 (f).

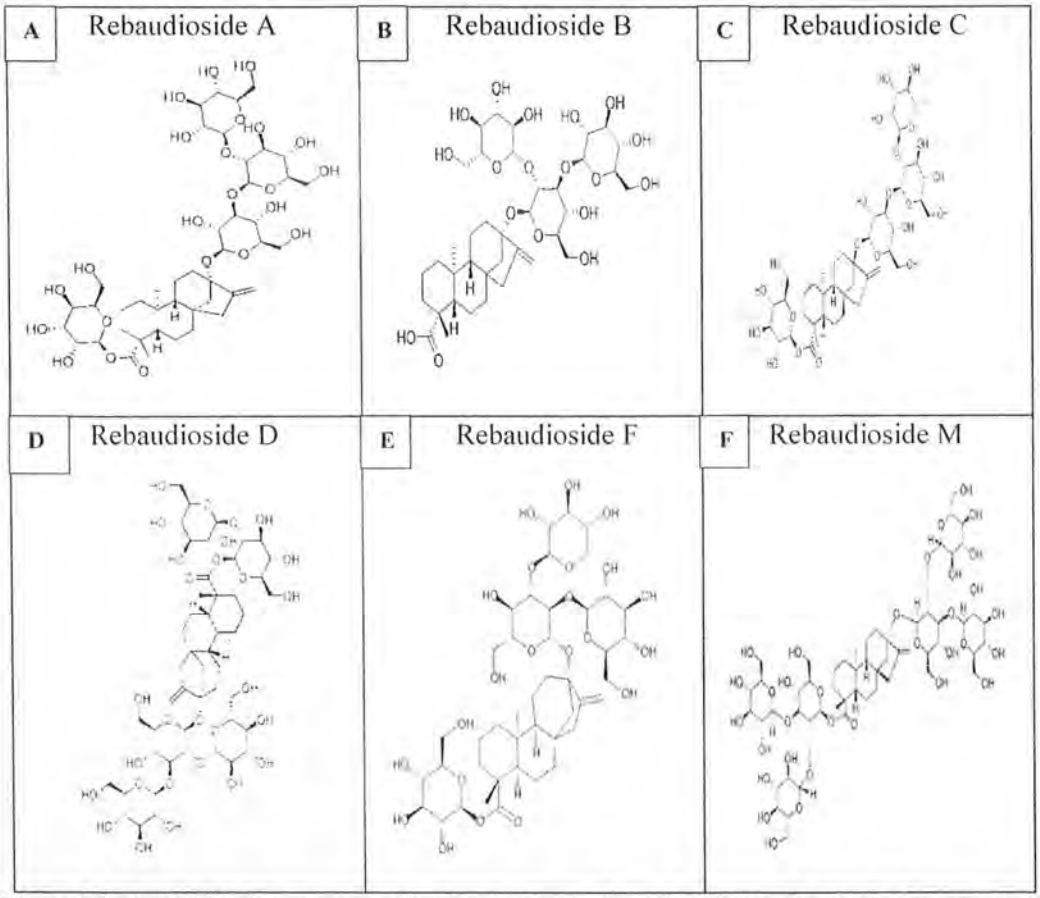


Figure 2.3. *(a) Molecular structure of rebaudioside A, (b) rebaudioside B, (c)rebaudioside C, (d) rebaudioside D, (e) rebaudioside F, (f) rebaudioside M.*

2.2.1.3. Dulcoside A

Dulcoside A is also a member of the group of chemical substances of steviol glycosides. These are prenol lipids that have a steviol moiety and a carbohydrate moiety bonded together glycosidically. Dulcoside A functions as an antioxidant and a sweetener (Barbet-Massin *et al.*, 2015). It has a molecular formula of $C_{38}H_{60}O_{17}$. It is a very weakly basic chemical that is virtually neutral having a structure as shown in figure 2.4.

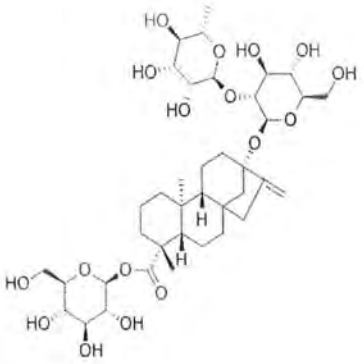


Figure 2.4: Molecular structure of Dulcoside A

2.2.1.4. Rubusoside

It is also a bioactive natural sweetener mostly used in China. It is created by the transformation of the tertiary allylic hydroxy group and carboxy group of steviol to their respective beta-D-glucosides. It also functions as a plant metabolite. It is a beta-D-glucoside bridging tetracyclic diterpenoid molecule and a steviol glycoside (George Thompson *et al.*, 2015). It is similar to a steviol in terms of function. Its molecular formula is $C_{32}H_{50}O_{13}$ with a molecular weight of 642.7 g/mol and possesses a temperature tolerance of up to 86°C. It is also a type of steviol glycoside but is present in low amounts (Yan *et al.*, 2021). The IUPAC name of this compound is [(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl](1R,4S,5R,9S,10R,13S)-5,9-dimethyl-14-methylidene-13-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxytetracyclo[11.2.$0^{1,10}$.$0^{4,9}$]hexadecane-5-carboxylate. Rubusoside's chemical structure is as shown in figure 2.5.

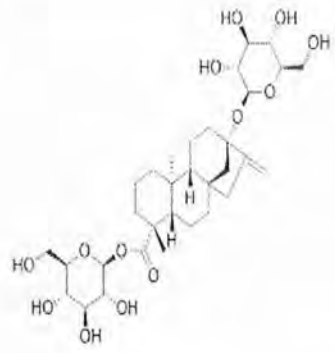


Figure 2.5: Molecular structure of Rubusoside

2.2.1.5. Steviolbioside

This substance is a member of the group of organic substances of steviol glycosides. These are prenol lipids made up of a steviol (a diterpenoid derived from 13-hydroxykaur-16-en-18-oic acid) moiety that is glycosidically connected to a carbohydrate moiety. Its molecular weight is 642.73 g/mol having a molecular formula of that similar to rubusoside $C_{32}H_{50}O_{13}$. According to the study conducted by Khattab *et al.*, steviolbioside was shown to have a sweetness intensity than sucrose with a temperature tolerance of up to 100°C (Khattab *et al.*, 2017)

Figure 2.6. *Molecular Structure of Steviolbioside*

2.2.2. Others

2.2.2.1. Proteins

Proteins are macromolecules made up of amino acids that are essential for the development and maintenance of biological tissues. These are found in a wide range of matrices including vegetables, cereals, fungi, animals, etc. Their significance derives mostly from the fact that protein intake is crucial since they are a necessary component of cells and have to be replaced throughout time (Hajihashemi, 2017). There are nine amino acids present in leaves of Stevia such that tyrosine, alanine, aspartic acid, serine, glutamic acid, lysine, methionine, proline, and isoleucine. There are some other amino acids are also present. Seventeen amino acids are classified into non-essential and essential categories with

arginine regrettably one of the essential amino acids. FAO/UNU/WHO indicated isoleucine, leucine, lysine, valine, threonine, methionine, tryptophan, histidine, and phenylalanine as indispensable (essential) amino acids. Cysteine and tyrosine are among the nearly all-present essential amino acids in Stevia leaves. Tryptophan is the only amino acid that is absent. This indicates that the leftover material after the extraction of stevioside from the leaves may be a significant source of essential amino acids for health products (Koubaa *et al.*, 2015).

2.2.2.2. *Carbohydrates*

Carbohydrates are sugars or carbs. They perform various functions inside living organisms. They are categorized into a variety of types on the number of sugar units from simpler (monosaccharides and disaccharides) to complex (polysaccharides) (Slavin and Carlson, 2014). Monosaccharides serve as a primary source of energy for the metabolic process of the human body whereas polysaccharides function as structural elements and are used as energy storage (Lemus-Mondaca *et al.*, 2012). These substances have also been related to additional advantageous health effects. These have a prebiotic impact in addition to other uncommon anti-inflammatory or antioxidant effects. The health advantages of Stevia leaves are mostly attributable to their nutritional component, which includes a good amount of crude fiber and carbohydrates that boost well-being and lower the chances of various diseases. The leaves and roots of Stevia contain fructooligosaccharides which are a type of inulin, a naturally occurring plant polysaccharide with significant functional features related to dietary fiber and prebiotics, including lipid metabolism, and diabetes management as mentioned by Lemus-Mondaca *et al.*, Purified fructooligosaccharides yielded from the plant's leaves and roots were 0.46% and 4.6% respectively by which extract of this plant may serve as dietary supplements (Lemus-Mondaca *et al.*, 2012).

2.2.2.3. Minerals

Minerals are chemical elements that an organism needs as important nutrients in order to carry out life-sustaining processes. Some minerals are referred to as essential trace elements because they are necessary for metabolism but only in extremely small concentrations in the human diet. They are categorized by the requirement of the body as well as their relative proportion. The primary components include macronutrients such as

magnesium, sodium, sulfur, chlorine, phosphorus, calcium, and potassium, and micronutrients which are minor elements, including manganese, chromium, cobalt, iron, copper, molybdenum, zinc, iodine, and selenium (Lall, 2022). The growth and maintenance of essential bodily functions depend on the micro- and macronutrient content of food that's why these substances are deemed necessary for the body normally. They take part in all processes related to reproduction, development, and wellness, as well as the growth of cells, tissues, and organs. Stevia is a mineral-rich substance. It contains these vital nutrients in significant proportions required to protect the body, as well as to maintain the numerous metabolic processes. Elements such that calcium, potassium, sodium, and magnesium are found in the leaves of Stevia. These minerals' high concentration would be very advantageous to health (Cantabella *et al.*, 2017). The average amount of these micro and macro components is found in dried Stevia leaves. Despite appearing to be quite low, research has found that potassium has a remarkably high level. In addition to being present in both plant- and animal-based meals; iron and zinc are also found in Stevia leaves. Mineral zinc functions as an anti-oxidant, in such a manner its consumption can aid in reducing oxidative damage to the cells. The primary biological role of iron is oxygen transportation to various parts of the body. Hence, the deficiency of mineral iron can cause anemia. The high iron content of Stevia leaves may once more be beneficial in maintaining a healthy level of hemoglobin in the body. Additionally, its leaves can be used to make a variety of sweet dishes to treat anemia, a serious nutritional illness that is prevalent in developing nations (Lemus-Mondaca *et al.*, 2012).

2.2.2.4. Vitamins

Vitamins are chemical compounds that are found in food in extremely minute amounts yet are essential for metabolism. They are essential components of the diet. Due to their chemical similarity and shared physiological roles, they are classed together. There are about 13 main vitamins including vitamins A, B, C, D, E, and K, in which the vitamin B further contains thiamine, biotin, folate, niacin, riboflavin, pantothenic acid, B6, and B12 (Peteliuk *et al.*, 2021). A deficiency in these vitamins can lead to the emergence of different disorders. These vitamins are divided into two groups; four fat-soluble vitamins such that A, D, E, and K and nine water-soluble vitamins i.e., Vitamin C and Vitamin B with sub-classified vitamins. These substances play a variety of biological roles. Some regulate the

metabolism of minerals in ways that are similar to hormones, such as vitamin D, and different forms of vitamin A for the regulation of cells and also for the development and differentiation of tissues. Others i.e., vitamins C, E, and rarely B serve as antioxidants. The majority of vitamins, including the B complex function as cofactor precursors for enzymes (Bernal *et al.*, 2011). The vitamins are present in the leaves and calluses of the Stevia plant. The leaf extracts have noticeably higher levels of vitamin C, vitamin B2, and folic acid, in comparison to that extract of callus of Stevia. Folic acid and vitamin C are shown to be the two main compounds in the leaf extract. While Vitamin C is the main ingredient followed by vitamin B in the extract of callus (Peteliuk *et al.*, 2021).

2.2.2.5. Lipids

Lipids are a wide category of natural substances. They are responsible for primary biological functions such as the storage site of energy, crucial signaling molecules, and cell membranes' structural components. Although lipids are broken down and synthesized by a variety of metabolic routes in mammals, including humans. Some necessary lipids are taken from food as they cannot be produced by the body. The three primary categories of lipids are sterols, phospholipids, and triglycerides. Esters (triglycerides) are a common type of lipid that is produced via a chemical process of alcohol and carboxylic acid. Carboxylic acids include fatty acids having variable unsaturated or saturated aliphatic tails (Ahmed, Ahmed, and Shah, 2018). They are crucial to living things as dietary components. For human metabolism, long-chain polyunsaturated fatty acids, notably those of the n-3 class, like a-linolenic acid, are crucial. They have a wide range of positive effects, such as the prevention of various ailments, including inflammation, coronary heart disease, autoimmune disorders, hypotriglyceridemia, and hypertension. An EFA (essential fatty acid) for optimal health is linolenic acid, which is just as beneficial as linoleic acid. EFAs play an essential role in the creation of numerous cellular structures and other chemicals with biological significance. Other polyunsaturated fatty acids, which carry out a variety of functions for the body including maintaining cell membranes and producing prostaglandins, which act as regulators of numerous bodily processes like blood clotting and inflammation, are also crucial. Additionally, fats are required in the diet as a source of the fat-soluble vitamins A, D, E, and K as well as because they can be absorbed to control the metabolism of cholesterol. Lipids are found in Stevia leaf oil. The leaf oil contained

palmitoleic, palmitic, oleic, stearic, linolenic, and linoleic acids. Palmitic acid has the highest concentration of fatty acids, whereas stearic acid had the lowest concentration. Linolenic acid is abundantly found in the leaf oil of Stevia. This high level of this linolenic acid helps to keep the appropriate ratio of fatty acids in the human diet (Ahmad *et al.*, 2018).

2.2.2.6. Diterpene Glycosides

Glycosides are substances made up of a sugar molecule linked to a non-carbohydrate component. These substances are primarily found in plants. They can be transformed into an aglycone (sugar component) and a non-sugar component through hydrolytic cleavage (Basharat *et al.*, 2021). They are introduced based on the sugar they contain in their structure such that pentosides (pentose), fructosides (fructose), or glucosides (glucose), etc. Stevia leaves naturally contain diterpenes called steviol glycosides, which have been extracted including dulcoside, steviolbioside, stevioside, and rebaudioside A to F (Perera and McChesney, 2021). These are Stevia's phytochemical components. These compounds are discussed earlier.

2.2.2.7. Non-glycosidic Diterpenes

In 2007, Ibrahim was able to confirm the existence of the previously characterized substances iso-austroinulin, austroinulin, sterebin E acetate, and sterebin E, as well as the newly discovered substance sterebin A acetate. These compounds were identified by MS, IR, and UV spectroscopy, together with 1H- and 13C NMR. The discovered structure of sterebin A, I to N are as in Figure (6).

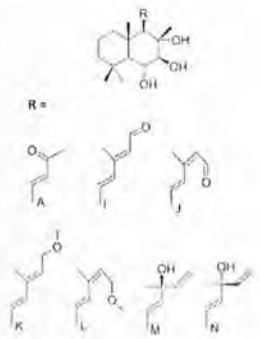


Figure 2.7. *Structure of sterebin A, I to N*

New Stevia lines are important in order to increase the levels of sweet-tasting diterpene glycosides while reducing the concentration of low-polarity sterebins (Ibrahim *et al.*, 2006; Wölwer-Rieck, 2012).

2.2.2.8. Antioxidants

Antioxidants are molecules that can stop or delay the harm that free radicals cause to cells. The body produces these unstable chemicals (free radicals) in response to stresses from the environment and other factors. These free radicals along with their reactive metabolites are key intermediates for cell signaling, death processes, and infection control (Ware, 2018). Antioxidants are electron donors that can neutralize free radicals and their reactive metabolites, making them capable of promoting good health. Antioxidants work by regulating the antioxidant enzyme system or directly transferring electrons from free radicals to other free radicals to scavenge reactive oxygen species (ROS) and free radicals. A number of antioxidant substances, including quercetin, apigenin, luteolin, isoquercitrin, Miocene, chlorogenic acid, caffeic acid, and kaempferol, have been identified from Stevia (Bender, 2018).

2.2.2.9. Phenolic compounds

Polyphenols are vital to plant metabolites as they have the capacity to capture free radicals. They are thought to serve a preventive function in a number of degenerative disorders linked to oxidative stress because they operate as antioxidants and may aid in antioxidative action. The main phenolic compounds found in Stevia include chlorogenic acid, pyrogallol, 4-methylcatechol, 4-methoxybenzoic acid, cinnamic acid, p-coumaric acid, sinapic acid, flavonoids (apigenin, luteolin, quercetin, quercetin-3-O-arbinoside, kaempferol-3-O-rhamnoside, apigenin-4-O-glucoside), caffeic acid, ferulic acid and its derivatives, rosmarinic acid, and its derivatives. Flavonoids are one of the main classes of phenolic chemicals that are extensively present in plants. In vitro, flavonoids have a considerable antioxidant impact; they are good at chelating metals, reducing/ oxidizing free radicals, and regenerating antioxidants (Bender, 2018).

2.3. Physiochemical Properties

2.3.1. pH

The concentration of hydrogen ions in a specific solution is measured as pH, which reflects both the acidity and alkalinity of the solution. At the room temperature of 25 °C, aqueous solutions with a pH of less than 7 are neutral, while those with a pH of more than 7 are alkaline. However, pH 7.0 is neutral (Schmiermund, 2022). The Stevia powder dissolved in deionized water possesses a pH of 6.1, which is slightly acidic which is close to neutral indicating that the concentration of hydrogen ions is less than that of hydronium (H_3O^+) ions (YALÇIN *et al.*, 2022). According to climate settings, the cultivated Stevia's pH was previously reported to be 5.95 (Goyal *et al.*, 2009). However, other research indicated that the pH of Stevia ranges from 5.95-6.24 (Gasmalla *et al.*, 2014).

2.3.2. Bulk Density

It is the weight-to-volume ratio of particles. Stevia leaf powder seems to be deficient in this quality. The analysis of Segura-Campos in 2014 discovered that the bulk density of Stevia powder was 0.443 gm/ml rather than the manufacturers' claimed value of 0.547 or 0.061 gm/ml (Segura-Campos *et al.*, 2014).

2.3.3. Water Holding Capacity

Protein- or fiber-rich meals are crucial in the production of food products because of their varied qualities that regulate how much water they can hold. WHC (Water holding capacity) is connected to a variety of olfactory, dietary, physiological, and practical aspects. The WHC of native Stevia was determined to be 3.9330.049 ml/gm. Stevia's WHC is advantageous for managing recipes and is taken into account because of its high protein content. Proteins increase the body's ability to hold onto water, and Stevia has a sizable amount of high-quality protein demonstrated by Segura-Campos that Stevia's capacity to store water ranges from 2.87 to 4.07 ml/g, while Savita discovered 4.7 ml/g (Savita *et al.*, 2004; Segura-Campos *et al.*, 2014).

2.3.4. Swelling Capacity

During processing, the product's composition and the amount of stress depend on swelling power. Therefore, the native Stevia's swelling ability or power, which is 5.003+/-

0.029gm/gm, was determined to be consistent with the findings of (Segura-Campos *et al.*, 2014), which stated that it was 5.01 gm/gm.

2.3.5. Fat Absorption

The amount of oil trapped during meal preparation depends on the fat absorption ability. According to reports, Stevia powder has a sufficient capacity for absorbing fat, which aids in the processing of food by preserving tastes and improving mouthfeel. Native Stevia exhibits good fat absorption, with an apparent capacity of 5.963+/- 0.069 ml/gm, making it desirable for use as a standard sweetener in the baking and beverage industries (GUPTA *et al.*, 2016).

2.3.6. Climate and Temperature

The delicate perennial Stevia plant is cultivated as an annual through vegetative propagation. Parent plants kept indoors over the winter produce stems that can be rooted in the spring. Stevia is indigenous to subtropical climes, semi-humid, where average daily temperatures fall between -6°C to 43°C. Hard frosts will damage the plant's roots even though it is tolerant of light frost. Although the steviol glycosides have high-temperature tolerance such that stevioside up to 198°C and rebaudioside A up to 200°C. But stevioside must be kept in a cool and dry environment to retain its quality. The breakdown of stevioside is one of the chemical reactions that can be accelerated by temperature more than optimium range. It should therefore be kept away from heat sources in a temperature-controlled setting. (Amien *et al.*, 2020).

2.3.7. Biological Activities

The majority of the pharmacological properties of Stevia extracts are connected to their anti-oxidant, antiproliferative, anti-viral, anti-parasitic, and anti-inflammatory properties.

2.3.7.1. Antitumor

Cancer is a condition that affects the cells in the body. Damage to the cells' DNA occurs during its development and builds up over time. For years, people have used Stevia and its metabolites as a natural, nutrient-free substitute for sucrose as well as other nutritive sweeteners throughout the world. Stevia leaf extract contains a substance called labdane sclareol that possesses cytotoxic and anti-tumor effects (Wang *et al.*, 2018). Studies have

shown that leaf extracts of Stevia and the polyphenolic components inside them suppress the development and spread of tumors. According to studies, stevioside, aglycones, and their metabolites prevent tumor promotion by preventing the induction of the Epstein-Barr virus early antigen. Isosteviol, a byproduct of stevioside hydrolysis has a strong inhibitory effect on DNA replication and the development of human cancer cells (Słoczyńska *et al.*, 2014). Stevioside reduced the tumor promotion caused by a TPA (tumor-promoting agent) in skin cancer development. The toxicological effects of low stevioside concentrations on serum-induced apoptosis utilizing the cell system. Stevioside increased the amount of cytochrome c and Bax released into the cytoplasm, which indicated that stevioside impacts normal apoptotic regulation. This enhancement was brought about by serum deprivation-induced apoptosis.

Martinez-Rojo *et al.* (2020) investigated the anticancer efficacy of methanolic root extracts from other Stevia species such that *S. eupatoria* and *S. pilosa* on prostate cancer cells including human fibroblast cell line, as well as androgen-independent (PC-3) androgen-dependent (LNCaP) prostate cancer cell lines. Trypan Blue exclusion testing for 48 hours determined the viability of the cells and a wound-healing experiment for the analysis of their migration. In every concentration tested, both extracts drastically decreased the vitality and migration of prostate cancer cells. The Stevia extracts had a stronger antiproliferative effect on cancer cells than on healthy cells (Martínez-Rojo *et al.*, 2020).

2.3.7.2. Antioxidant

Antioxidants guard against the oxidative harm caused by free radicals. They can stop the oxidation process by reacting with free radicals, chelating catalytic metals, and acting as scavengers of oxygen. Numerous physiologically active components of plants, such as flavonoids and phenolic compounds (also known as phenolics), are known to have potent antioxidant effects (Borgo *et al.*, 2021). The concentration of each antioxidant that enters a composition determines the antioxidant capacity of medicinal herbs. The medicinal potentials of plants as antioxidants in preventing tissue damage caused by free radicals have recently attracted more attention. Although there are many commercially available synthetic antioxidants, including butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). They are highly dangerous, and there are issues with their toxicity.

As a result, their use has been subject to rigorous limitations, and antioxidants that are found in nature are increasingly being used in their place (Shukla *et al.*, 2012). Natural plant-based antioxidants, particularly flavonoids, and phenols have been used commercially as nutritional supplements or antioxidant additives.

The discovery of plants having antioxidant capacity received a lot of interest in recent years. Plants contain a wide variety of antioxidants, therefore it is exceedingly challenging to quantify each antioxidant independently. As a result, various techniques have been created to assess the antioxidant activity such that ORAC (oxygen radical absorption capacity), FRAP (the ferric reducing ability of plasma), TRAP (total radical absorption potentials), and TEAC (Trolox equivalent antioxidant capacity) are regularly utilized and serve as representative techniques in scientific research (Martínez-Rojo *et al.*, 2020). Another technique that can be used for many samples in a short amount of time and is sensitive enough to find natural substances at low concentrations is the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, in which the percentage of DPPH free radical inhibition determined the antioxidant activity. This assay is the most accepted technique for testing antioxidant potential. A stronger DPPH radical scavenging activity is associated with a lower IC50 value which was measured in terms of the inhibition percentage of DPPH radicals as well as IC50 (required concentration to inhibit DPPH radicals by 50%). Stevia was shown to have the highest antioxidant capacity when extracted with five various solvents, including distilled water, acetone, methanol, ethanol, and acetic acid (Chong Saw Peng *et al.*, 2020).

2.3.7.3. Antiprotozoal

In past years, researchers evaluated the antiprotozoal in vitro activity of quercetin against *Leishmania donovani, Trypanosoma cruzi*, and *Trypanosoma brucei rhodesiense*. A remarkable leishmanicidal potential with an IC50 of 1.0 g/mL was found. Additionally, Boniface and Ferreira reported quercetin's antimalarial, antileishmanial, and efficacy against Dengue. With an IC50 value of 5.5 g/mL, quercetin demonstrated antimalarial efficacy against *P. falciparum* (Boniface and Ferreira, 2019). It had inhibitory effects on the *L. mexicana* rCPB2.8 proteinase, with an IC50 value of 18.03 M. Additionally, quercetin demonstrated in vitro antileishmanial action against the promastigote and

intracellular amastigote forms of *L. amazonensis* (IC50 = 0.7 M and 4.3 M, respectively). In an in vivo test, quercetin (30 mg/kg) decreased the size of the lesion in mice infected with Leishmania amazonensis from 1.8 mm (vehicle) to 0.2 mm (treatment). Additionally, *L. amazonensis* exposed to quercetin demonstrated arginase inhibitory effects with IC50 values of 3.8 M. Additionally, quercetin inhibited *L. amazonensis* (66 percent inhibition at 96 M; IC50 = 31.4 M) (Verma *et al.*, 2019).

2.3.7.4. Antimutagenic

Mutagenicity or antimutagenic activity is the induction of long-term modifications in an organism's DNA sequence, which may lead to a heritable alteration in the properties of living systems. Mutagens can be neutralized by antimutagenic substances which can be both inorganic or organic. In the past researchers assessed the antimutagenic action of methanolic extracts made from the roots and leaves of *Stevia eupatoria* and *Stevia pilosa*. The scientists discovered that 2-amino anthracene-induced mutagenicity in strain TA98 was inhibited by both species. The leaves of both species and the blooms of *S. eupatoria* produced the best results (Borgo *et al.*, 2021). In the strain TA100, the N-ethyl-N'-nitro-N-nitrosoguanidine-induced mutations were similarly diminished. Extracts from *Stevia eupatoria* and *Stevia pilosa*'s roots and flowers, respectively, demonstrated inhibition of about 93 percent. A decrease of 87 percent was achieved with the extract of *S. eupatoria's* leaves using mitomycin-C on the strain TA102. Additionally, proven more than 90% is the extracts' antioxidant capacity (Al-Taweel *et al.*, 2021).

2.3.7.5. Antiviral and antiparasitic activity

Numerous flavonoids have demonstrated antiviral and antiparasitic activity, making them potent for the creation of medications to treat leishmaniasis, dengue, malaria, and Chagas diseases (Boniface & Ferreira, 2019). The flavonoids quercetin-3-O-D-Gucopyranoside, isolated from *Stevia nepetifolia* and *Stevia rebaudiana*, and quercetin-3-O-D-Galactopyranoside isolated from S. soratensis, *S. serrata, and S. nepetifolia*, have demonstrated antiplasmodial and leishmanicidal action respectively.

2.4. Sweet potential/Tolerance of Stevia

In its raw form, Stevia is 30 times sweeter than sugar while after being refined, it is 250 to 300 times sweeter. Many nations employ stevioside, a substance found in large quantities

in Stevia rebaudiana leaves, as a non-caloric sweetener due to its high sweetness of approximately 140 times sweeter than sucrose while rebaudioside A possesses 240 times more sweetness and is said to be more sweetness exhibiting compound in Stevia. Although rebaudioside M is present in a minute quantity having 300 times more sweetness (Prakash, Chaturvedula, and Markosyan, 2014). Other compounds are found in limited quantity but have great sweet potential. The sweet tolerance of different steviol glycosides as mentioned in the table 02;

Table 02. *Sweet potential of various steviol glycosides.*

Compounds	Molecular formula	Sweetness potential
Rubusoside	$C_{32}H_{50}O_{13}$	114
Stevioside	$C_{38}H_{60}O_{18}$	210
Dulcoside A	$C_{38}H_{60}O_{17}$	30
Rebaudioside A	$C_{44}H_{70}O_{23}$	200-400
Rebaudioside B	$C_{38}H_{60}O_{18}$	150
Rebaudioside C	$C_{44}H_{70}O_{22}$	30
Rebaudioside D	$C_{50}H_{80}O_{28}$	221
Rebaudioside E	$C_{44}H_{70}O_{23}$	174
Rebaudioside F	$C_{43}H_{68}O_{22}$	200
Rebaudioside M	$C_{56}H_{90}O_{33}$	250
Steviolbioside	$C_{32}H_{50}O_{13}$	90

2.5. Uses of Stevia

2.5.1. Industrial Use

They taste best when consumed directly or in any kind of herbal tea. Although the leaves can be used to make different sauces. Powdered or ground leaves are also being used in bulk forms or tea bags. The leaves are added to coffee, fruit, salads, and more to add sweetness, flavor, and color. In the food and beverage sector, Stevia is primarily utilized as a sweetener and flavor booster. The second most important use is in the health sector. By-products or the material left over from the plant after the best leaves have been plucked for tea or extraction are also comprised of market significance (PureCircle, 2019). The by-

products are being used as animal feeds and bio-fertilizers such as the remaining components of plants including seeds, stems, blossoms, as well as leaves. These are also involved in the production of natural sweeteners. The plant is also significantly selected as a source of phytosterols, chlorophyll, and other nutrients. Fresh leaves of Stevia in their purest and most basic form, taste faintly of licorice. The plant's remaining that weren't chosen for industrial use, are gathered and turned into fertilizer. It is a perennial shrub that is widely used in medicines too.

2.5.2. Medicinal Use

WHO (World Health Organization) research shows that Stevia controls blood pressure, prevents cavities, stimulates the pancreas to make more insulin, and functions as a bactericide (Ahmad *et al.*, 2020). No harmful clinical findings have surfaced in any of the nations where Stevia is easily accessible. Worldwide, the use of medicinal plants to treat various diseases is growing in popularity. People with diabetes and obesity should use Stevia. Later, it might be helpful in preventing type 2 diabetes. Additionally, it exhibited hypotensive, cardiotonic, antibacterial, anti-fertility, antiseptic, diuretic, anti-inflammatory, and other properties. Dermatitis, wrinkles, acne outbreaks, eczema, skin imperfections, rashes, scars, and itching have all been successfully treated with it. Steviol acts as a digestive tonic and controls blood sugar levels in insulin-deficient people by improving both insulin secretion and utilization. It is anticipated to give hope to diabetics who crave sweets (Mahmud *et al.*, 2015). Since Stevia has no effect on blood sugar levels, people with diabetes or those trying to control their blood sugar levels can use it. Stevia's allure as a better substitute for sugar molecules, which may be connected to inflammation and other detrimental health impacts, is also aided by its natural origin and lack of artificial additives (Ahmad *et al.*, 2020). Steviosides, which are incredibly sweet chemicals that are especially beneficial to diabetics, make up about 10 percent of its leaves. Stevia's raw material is a natural synthesizer of rheumatoid arthritis treatment, oral contraceptives, a cholesterol-lowering medication, and anticancer activity against prostate tumors. It has therapeutic benefits for controlling blood sugar, reducing hypertension, treating skin conditions, and preventing tooth decay. It nourishes the pancreas, aiding in the organ's return to normal operation. It also has a significant antioxidant activity due to the large percentage of phenols and flavonoids in it. The secondary metabolites are known as

phenols lower the incidence of cancer and cardiac disease. The herb may have activities known as cardiac tonics that balance blood pressure and control heart rate. People with hypertension and normotension exhibit vasodilator effects from the plant. It has improved diuretic and natriuretic effects in people and has also assisted in the situations of lowering blood pressure (Borgo *et al.*, 2021).

2.5.4.3. Food and Beverages

Since 2018, Stevia become more and more popular as a flavoring agent for beverages and foods. Stevia is presently the market leader in a variety of areas where high-intensity sweeteners are utilized. These categories include plant-based drinks, frozen yogurt, dairy-based ice cream, ready-to-drink iced tea, food dressings, and vinegar. Stevia is widely used in a variety of beverages, including carbonated soft drinks from Coca-Cola, Calbee Foods, Unilever, Groupe Danone, PepsiCo, and Nestle, some meal replacements, and others (PureCircle, 2019).

2.6. Pros and cons

Stevia has several health advantages over other materials, and there aren't as many concerns involved with utilizing it. Stevia is regarded as one of the safest sweeteners as it comes from a genuine plant and isn't manufactured artificially. The American Heart Association warns that some artificial sweeteners may have harmful side effects i.e., elevating your risk of stroke.

2.6.1. Pros of Stevia

Stevia is widely accepted by people because of its maximum positive effects on lives. It is more popular as of the following beneficial aspects;

- **Low Calories**

  Being calorie-free is one of the primary advantages of Stevia. Stevia can be used to sweeten a variety of meals without adding sugar or extra calories to them. According to a meta-analysis of September 2015 published in the International Journal of Obesity, artificial sweeteners like Stevia may aid in weight loss when taken as a substitute for sugar. This makes sense because switching from sugar to a low-calorie alternative reduces the overall calorie content of the dish (Rogers *et al.*, 2015).

- **Combination with blood sugar**

The blood sugar levels may be regulated by Stevia as well. Researchers in June 2015 discovered that taking Stevia alleviated their symptoms when 114 persons with diabetes underwent tests as mentioned in the Journal of Medicinal Plant and Herbal Therapy Research that the patients who received Stevia showed lower blood pressure and blood sugar level than the control group (Pallarés *et al.*, 2015).

- **Role in oral care**

Stevia is good for dental health. According to a July 2012 research published in the International Journal of Advances in Pharmacy, Biology, and Chemistry, it inhibits the growth of Streptococcus mutants which is a kind of bacterium that is a significant contributor to tooth decay (Shukla *et al.*, 2012).

- **Disease Prevention**

According to a November 2012 publication in Experimental and Toxicologic Pathology, Stevia has been demonstrated to have antioxidant benefits. These results appear to be explained by the phenolic components in Stevia (Shukla *et al.*, 2012).

A May 2014 research published in BMC Medicine found a relationship between consuming phenolic chemicals and a reduced risk of heart disease. This group looked at 7,172 persons who had a high risk of developing heart disease. Patients' mortality rates were lowered by 37% when they consumed more phenolic substances (Tresserra-Rimbau *et al.*, 2014).

- **Against Cancer**

Additionally, comprehensive short- and long-term investigations have been conducted to determine the toxicity of Stevia. It is also safe for human ingestion because no significant toxic, genotoxic, regulation, or carcinogenic effects were found in mammalian species (Ray *et al.*, 2020).

2.6.2. Cons

Stevia is not known to have any negative side effects when it is taken moderately. Numerous Stevia products contain other chemicals, such as sugar alcohols, which may have negative consequences. Here are a few potential drawbacks of Stevia.

- **Interference with Gut**

Some adverse reactions such as nausea and bloating have been frequently mentioned after consuming Stevia. Although Stevia has not been proven to cause gastrointestinal problems, according to research published in the International Journal of Dentistry in October 2016, many Stevia products have sugar alcohols added to them creating some health problems (Mäkinen, 2016). According to a January 2019 study in Advances in Nutrition, researchers indicates that Stevia might disrupt good flora in your stomach. However, further study is required in this area (Ruiz-Ojeda *et al.*, 2019).

- **Reaction to medications**

Before using Stevia, people who are taking medicine to treat their diabetes or to lower their blood pressure should see their doctors. According to a May 2020 study published in the International Journal of Clinical Research Trials, Stevia may drastically drop blood pressure and blood sugar, which raises the possibility that taking it with these medications could result in hypotension or hypoglycemia (Ray *et al.*, 2020).

- **Bitter aftertaste**

Although Stevia comprises more sweetness as compared to normal sugar but has some bitter aftertaste that slightly annoys people. Sometimes people feel that it tastes like licorice.

2.7. Extraction methods of Stevia compounds

As with any other plant extraction, the efficiency of compound extraction varies for the same resource depending on factors including the solvents, extraction process, extraction duration, temperature, pressure, pH, and particle size of dried leaves, even the way in which fresh leaves are dried. Most acceptable extraction methods involved solvents such that water, ethanol, methanol, etc (Myint *et al.*, 2020).

Modern extraction methods like pressurized liquid extraction PHWE (pressurized hot water extraction), MAE (microwave-assisted extraction), and supercritical fluid extraction are now more widely used to extract bioactive compounds from plants than traditional methods like boiling, Soxhlet, and reflux. Pressurized liquid extraction and supercritical fluid extraction using carbon dioxide as well as aqueous methanolic extraction have been discovered yet (Teo *et al.*, 2009).

2.7.1. Water extraction

1. Hot water Extraction

The majority of chemicals and substances are dissolved by water easily, which is a universal solvent. In addition, because water is a polar solvent, its molecules are drawn to other polar ones, including those of glycosides. This indicates a high-water solubility of Stevia (Chong Saw Peng, Akil, and Bahari, 2020). According to the study of Anvari and Khayati (2016), the hot water extraction was carried out using a modified version of the method using a heating mantle. For which they had considered 40 g of Stevia leaves, which were powdered to a mesh size of 20-30, were cooked for one hour in 4 L of soft water. After cooling, the crude extract was collected, and the leftover Stevia powder was then cooked for one hour in an additional 4L of soft water. Three times the boiling process was carried out while adding 4 L of soft water each time (Anvari and Khayati, 2016). The Stevia also possesses heat stability that supports hot water extraction. This extraction method was adopted by different researchers to isolate particular compounds such as rebaudioside A, steviosides, steviolbiosides, etc (Anvari and Khayati, 2016).

Another water-dependent extraction adopted in the past was assisted by ohmic heating. According to the research of Moongngarm *et al.* (2022), ohmic heating uses the food's natural electrical resistance to produce heat. Ionic components like conductive acids and salts are present in the majority of dietary ingredients. These substances produce heat inside the food material under OH by converting electrical energy into heat energy, resulting in a rapid rise in temperature. This prevents heat-sensitive components from being damaged by overheating. OH has been used widely in the evaporation, pasteurization, extraction as well as blanching of food (Moongngarm *et al.*, 2022). High yields of important chemicals were produced as a result of the breakdown of plant material cell walls caused by electric energy

used under the influence of OH during the extraction process. To determine the number of phytochemicals and steviol glycosides in Stevia leaves, OH-AWE (ohmic heating-assisted water extraction) was used. It helped in measuring the extract's total flavonoids, total phenolic components, phenolic acids, and antioxidant activity. According to the technique described by Loypimai *et al.* (2009), 20 percent, 30 percent, and 40 percent deionized water was applied to the ground leaves to increase the moisture content (MC). The soaked Stevia leaves were locked in a zip-lock plastic bag and the MC was then balanced for 12 hours in the refrigerator (Moongngarm *et al.*, 2022). After that leaves were treated with OH. During this, temperature, current, and voltage were continually recorded with a data logger controller. For rebaudioside A and stevioside, the treated Stevia extract was undergone through orbital shaking at 100rpm for 1 hour at 55°C (Chong Saw Peng, Akil and Bahari, 2020).

2.7.2. Methanolic extraction

Methanol is a good extracting solvent used to isolate the compounds of Stevia such that steviosides. It has better extraction power than water even in the temperature range of 110 to 160°C (Pól *et al.*, 2007). Different researchers have used various methanolic extracts as one of the accepted methods was done by Howlader, (2016) according to which the methanolic green Stevia extract was prepared by using a mortar and pestle to crush the fresh Stevia leaves vigorously and flux them with 80% methanol followed by the 30 minutes centrifugation of the mixture at 6000 rpm. The supernatant was collected and dried at 45°C in a hot air oven to analyze the compounds further (Howlader *et al.*, 2016). Another methanol-based extraction was done by Kumari *et al.* (2017) in which Stevia was refluxed with methanol in a water bath several times which was filtered and underwent chloroform treatment to exclude compounds other than steviol glycosides. the remaining solution was rotary evaporated and vacuum dried to get the final crystals of steviol glycosides after refluxing with n-butyl alcohol (Kumari *et al.*, 2017). This combined water and methanol extraction supported a good extraction process.

2.7.3. Ethanolic extraction

Ethanol is good as methanol for the extraction of compounds from Stevia. According to the study of Shamima *et al.* (2019) Stevia leaves powder was soaked in a precise proportion

(1:15) to a 70% ethanol solution for two hours at room temperature. After then, this solution was purified. The process was repeated on the solid portion. Three times reflux of the procedure was done to extract all of the sweetness from the Stevia powder. This is one of the most accepted methods in all processes (Shamima *et al.*, 2019).

2.7.4. Conventional Extraction

Another extraction was conducted by Jaitak *et al.* (2009) in which Stevia leaf material was air dried, ground into a mesh powder in a mortar, and then kept at room temperature. The leaf powder was extracted using microwave and ultrasonic standard extraction methods with the help of water, ethanol, methanol, and their binary combinations. Following extraction, the supernatant was filtered and dried at 50°C in a rotary evaporator under reduced pressure. Hexane was used to defat dried extracts, and the resultant supernatants were thrown away. The leftover material was evaporated and then dissolved in a 20:80 ratio mixture of water and acetonitrile to get a final extract of sweet compounds (Jaitak *et al.*, 2009).

2.8. Control of bitterness of Stevia

The compound of Stevia such that stevioside which is added to food and beverages have some unfavorable sensory qualities of bitter taste in addition to sweetness. The main challenge for researchers is the exclusion of this bitterness which is annoying the people. Different researchers had used various methods to overcome this issue including both non-biological and biological methods.

2.8.1. Non-biological methods

It involved the chemical treatment of Stevia extract as studied by Formigoni *et al.* (2018) who treated the extract with ethanol to reduce the bitterness of steviol glycosides present in Stevia. In this study, Stevia leaves were subjected to an ethanolic process using a column. The leaves were weighed and packed with 100 percent ethanol. The same solvent was then gravity-eluted, yielding approx. 14 ethanolic fractions. The leaves and fractions were then dried and kept in preparation for later extraction of the sweeteners and characterization of the fractions (Formigoni *et al.*, 2018). The food industries use some chemicals for reducing the bitter taste such that sodium gluconate which should be added in amounts ranging from

0.11% - 0.18% by weight of the sweetened product, ideally 0.13% to 0.16% by weight of the sweetened food.

2.8.2. Biological methods

2.8.2.1. Biotransformation

It is a process that changes organic chemicals from one form to another in order to lessen their persistence and toxicity. A wide variety of microorganisms and their byproducts, including enzymes, fungi, and bacteria aid in this process. Chemicals or materials can also be synthesized through this process when synthetic methods are impossible to be done. The process of natural transformation is slow, unfocused, and less effective (Sultana, 2018). Microbial biotransformation is becoming more and more important and widely used to produce metabolites in greater quantities and with greater selectivity. The process of biotransformation is classified into two main types; enzymatic and non-enzymatic processes.

2.8.2.1.1. Types of biotransformation

- **Non- Enzymatic**

  It is a random biotransformation process that involves physiological factors such that pH. It is mostly related to unstable, highly active compounds i.e., hexamine (Singh, 2017).

- **Enzymatic**

  The enzymatic biotransformation is caused by numerous enzymes found in the body or comes from microorganisms. As Wang *et al.,* indicated by combining the functions of *Arabidopsis thaliana* AtSUS1 sucrose synthase and *S. rebaudiana's* recombinant UDP-glucosyltransferase UGT76G1. The conversion was accomplished by AtSUS1 regenerating UDP-glucose. For UDP-glucose recycling, UDP might be used as the starting material instead of UDP-glucose (Wang *et al.,* 2016). It is further conducted with the help of *E. coli* (Host Strain). The plasmids pEUGT (formed by the cloning of the UGT76G11 gene in strain) or pEUGT-SUS (by the cloning of AtSUS1) were used

to transform E. coli cells into individual colonies, which were then cultured in Luria Bertani media (Wang *et al.*, 2016). The enzymatic activity was analyzed by glucosyltransferase activity assay and HPLC. The microbial enzymatic biotransformation is frequently used to change a range of chemicals, pharmaceuticals, contaminants, hydrocarbons, and metals (Singh, 2017). It follows the processes of oxidation, hydrolysis, reduction, condensation, isomerization, the introduction of functional groups, regioselective transfer reactions of glycosides, and selective cleavage of tetracyclic terpenoids' side chains to form C19 steroids, and production of new carbon bonds. It has long been recognized as a vital technique for reducing the production of several chemicals used in the agrochemical, pharmaceutical, food, and other sectors. For microbial transformation, the most common materials employed are spores, vegetative cells, resting cells, immobilized enzymes/ cells, and enzymes (Sultana and Saify, 2012). When developing cultures, the strain is grown in an appropriate medium before a concentrated substrate solution is added after the culture has grown as intended.

2.8.2.1.2. Process of biotransformation

The four primary processes involved in biotransformation are reduction, oxidation, conjugation, and hydrolysis which support the two main phases of biotransformation (Phang-Lyn and Llerena, 2020).

1. Reduction

An ion, atom, or molecule's oxidation status is decreased by the process of reduction. It might also just mean that one electron is being gained. Enzymatic reduction occurs with a degree of stereochemical and regiochemical uniformity that is challenging to attain chemically. These enzymatic techniques have enantioselectivity that is frequently predictable using empirical guidelines. One of the most common biotransformations is the reduction of a carbonyl, especially to produce a new chiral center (Madeira Junior *et al.*, 2013). A hydroxyl group may be introduced into a molecule during microbial incubation at a location far from any other functions. Microorganisms' capacity to hydroxylate

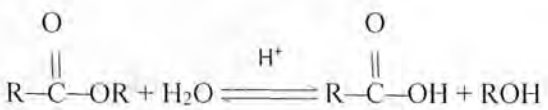
substances at chemically inert locations is a useful synthetic tool. The reduction process is always followed by an oxidation process to support the redox reaction.

***Table 03**: Chemical Reactions involved in the reduction process.*

Processes	Reactions
Dehydroxylation	R-OH → R-H
Hydrogenation	R-C=O → R-C-OH
Decarboxylation	R-COOH → R-C=O
Amination	R-C=O → R-C-NH_2
Methylation	R-C-H → R-CH_3

2. Hydrolysis

In the hydrolysis process, water is chemically incorporated together with the breakdown of a substance into simpler components. It can proceed with enzymes as a substance breaks down during enzymatic hydrolysis after reacting with water in the presence of enzymes. It has been widely employed in the food industry and is mostly performed for the continuous manufacture of numerous important products. It is opposite to the condensation process. In this process, one fragment of the target molecule acquires a hydrogen ion. It disrupts a chemical link in the molecule (Obodovskiy, 2019). Plants frequently undergo hydrolysis reactions, which involve nitrile, amide, or ester bonds. The process of converting an ester into a carboxylic acid with the formation of alcohol by using water is de-esterification or ester hydrolysis. The reaction of hydrolysis is as follows;



De-esterification might be thought of as a type of bioactivation since the ester is turned into acid during the process and the gradient become steeper for the entry of new ester, it will also heighten or sustain the concentration gradient (Zang *et al.*, 2020). Another hydrolysis process involves the conversion of amides (especially for the acylanilide group) and nitriles etc.

3. Oxidation

It is a chemical process that occurs when a substance interacts with oxygen or another oxidizer. It is a biological process in which an electron with a negative charge is transferred from one organic substance to another. Cytochrome P450, monooxygenases, and mixed-function oxidases enzymes are some of the oxidizing enzymes that help in the biotransformation process (Meynet, Davenport, and Fenner, 2020). The involved reactions can be;

Table 04. *Types of chemical reactions in the oxidation process*

Processes	Reactions
Hydroxylation	R-H $\rightarrow$ R-OH
Dehydrogenation	R-C-OH $\rightarrow$ R-C=O
Carboxylation	R-C=O $\rightarrow$ R-COOH
Deamination	R-C-NH_2 $\rightarrow$ R-C=O
Demethylation	R-CH_3 $\rightarrow$ R-C-H

4. Isomerization

The process of changing a substance into one of its isomeric forms, which have the same chemical makeup but a different configuration or structure and, consequently, often have distinct physical and chemical characteristics of isomerization. Isomerases facilitate alterations inside a single molecule. The end product of the reaction has the same chemical formula but a different physical structure because they transform one isomer into another. Although there are many different types of isomers, they may typically be divided into stereoisomers and structural isomers (Vanzan *et al.*, 2017).

5. Condensation

In a condensation reaction, two molecules join to form one new molecule, typically with the loss of water. The reaction is a dehydration process when water is lost during the formation of a molecule. Normally, the addition of the two molecules happens step-by-step to the additional product which is generally in equilibrium (Jo *et al.*, 2022). It is a diverse

family of reactions that can take place in basic or acidic circumstances or the presence of a catalyst, and it may also include the functional groups of the molecule. The production of fatty acids and the creation of peptide bonds between amino acids which is a necessary component of life depend on this class of processes (Enright *et al.*, 2014).

2.8.2.2. Significance of biotransformation

It is a significant biological method for bioconversion as;

- The substrate for biotransformation can be either artificial or natural.
- Cell cultures that express several enzyme activities can carry out multiple reactions.
- The biotransformation process can be straightforward when it is mediated by one or more enzymes and involves a few steps.
- Even non-producing cell cultures can occasionally be used to synthesize the intended end product by utilizing the right precursor.
- Since the yield declined as the number of processes increased, one-step biotransformation is fairly efficient (Ravindran and Basu, 2013).

3. MATERIALS AND METHODS

3.1 Study Area

The study was conducted in the AEG (Applied, Environmental, and Geomicrobiology) Laboratory located in the department of Biological Sciences Quaid I Azam University Islamabad under complete observation.

3.2 Materials

The required chemical reagents including methanol, acetone, and ethyl acetate were brought from SIGMA-ALDRICH, ethanol from ACI LABSCAN, and others such that chloroform (EMPARTA), n-Butyl alcohol or 1-Butanol (EMPROVE), n-Hexane (EMPLURA), acetonitrile (Li Chrosolv), DPPH, ascorbic acid, M9 salt media having Na_2HPO_4 (12.8 g/L), KH_2PO_4 (3g/L), NaCl (0.5 g/L), $MgSO_4$ Stock Solution (1ml/L), $CaCl_2$ Stock Solution (100µl/L), NH_4Cl(1g/L), and 20%glucose (20ml/L), Nutrient Agar.

3.3 Collection and Preparation of Plant Material

3.3.1 Collection of Stevia Plants

Stevia plants came from the nurseries located in the capital territory of Islamabad, Pakistan. They were baby plants and collected in the spring season. They were looked after for a month to grow up to the required length which was suitable for further experimentation.

3.3.2 Transportation of Stevia Plants

Once the plants were grown to an appropriate length and their leaves were transported in proper packaging to the AEG (Applied, Environmental, and Geomicrobiology) Lab of Quaid I Azam University for further processing.

3.3.3 Preparation of Plant Material

The process started with the proper washing of the collected leaves of each plant three times with water to remove all the dust from them. The leaves were spread over a clean paper and placed in a dry and cool place for drying them for about 3-4 days at room temperature. The dried leaves were weighed (6g) and ground to a fine powder by using a

grinder machine after which the final 5g of Stevia leaves powder was stored in a clean and dry bottle.

3.4 Processing of Plant Material

3.4.1 Extraction of compounds

The process of extraction was initiated with the separation of all the compounds found in Stevia leaves after which the particular compounds were separated for further processing. The extraction was conducted in two different ways; one by using methanol (methanolic extraction) and the other by water (water extraction).

3.4.1.1 Extraction Processes

The extraction of compounds was conducted by using two reagents; methanol and water in multiple steps;

i. For methanolic extraction of sweet compounds, the method of Kumari *et al.* (2017) was followed for the leaves powder of one plant, by which 75ml of 70% methanol was added to 5g of powder in a beaker, covered with aluminum foil, and placed in a water bath for 2 hours at 64.7°C. On the other hand, for other powdered leaves, water was used to extract the sweet compounds according to the method by Chong Saw Peng *et al.*, (2020). For this, 15g of powdered leaves were taken and dissolved with distilled water of 150ml (1:10 w/v) in a beaker, covered with aluminum foil, and placed in a hot water bath at a temperature of 97°C for one hour.

ii. The solution from the methanolic solution and water solution were filtered with Whatman filter papers and the filtrates were collected in separate round bottom flasks respectively. The residual residues of the methanolic extract were resoaked in 75ml (70%) methanol for two hours while the residues of the water solution were treated with distilled water (150ml) for 1 hour before being filtered once more. The filtrates were collected again in separate flasks. The residues from selected mediums were processed with three refluxes of methanol and water respectively to get maximum filtrate.

Note: All filtrates from methanolic and water refluxes were combined in separate flasks for further processing.

iii. The whole combined filtrate from methanolic refluxes was evaporated by a rotary evaporator to get compounds residues which were dried in a vacuum or oven. While the water extracted compounds were treated with 60ml of n-Hexane and heated at 50°C for 30 minutes. The layers that appeared were collected separately in two separate flasks.

iv. Both methanol and water mixtures were refluxed with chloroform but in different proportions i.e., methanolic compounds were refluxed with 10ml of chloroform while the decant layer after n-Hexane processing was treated with 200ml chloroform in separating funnels for 1 hour in a hot water bath at a temperature of 61.2°C and 55°C mixed well respectively. Chloroform layers from both solutions were collected in different flasks separately. The reflux was repeated with the chloroform until both solutions became colorless.

v. The decant layers of chloroform from methanol-based extract were combined, dissolved in 5ml of methanol, and stored in the refrigerator for the whole night.

vi. While there was a distinct layer formed in the separated chloroform layer of water-based extract which was rotatory evaporated, dissolved in methanol, and stored. The water-based remaining solution other than the chloroform solution was stored in the refrigerator.

vii. Methanolic washing and filtering were performed on the settled bulk of both methanol and water-based stored solutions and methanol was distilled off from both by using a vacuum or oven.

viii. For both dried extracts, 8 ml of distilled water was used to dissolve the residual leftovers. The methanol-based extract solution was repeatedly refluxed with n-butyl alcohol in the concentration of 4ml, 4ml, 5ml, 5ml, and 6ml while water-based compounds were refluxed in ratios of 100ml, 60ml, and 40ml respectively for extracting sweet compounds (steviol glycosides).

ix. For the final compounds, the n-butyl alcoholic extracts underwent rotational evaporation. After being separated, the mixtures of both extracts were blended with methanol separately, dried by air, and kept in the fridge overnight.

x. Both extracts were mixed with chilled ethanol for the crystallization of sweet compounds.

xi. The mixtures were spread over the paper and dried to get the final powder of sweet compounds.

3.4.2. Experimentation of Biotransformation

The experimentation was conducted with selected bacterial strains of *Anoxybacillus, Bacillus licheniformis,* and *Bacillus stercoris* to observe the changes in extracted compounds of Stevia after biotransformation. The process was conducted in the following steps;

3.4.2.1. Strains Growth

The bacterial strains were provided by my research fellow in the laboratory that were pre-isolated from soil and I had refreshed them for my experimentation. Strains were streaked over plates of autoclaved nutrient agar and placed at ambient temperatures (*Anoxybacillus* 50°C, *Bacillus licheniformis,* and *Bacillus stercoris* at 40°C) for 24 hours.

3.4.2.2. Broth Inoculation

The nutrient broth was used as a growth medium for bacterial cultures which were *Anoxybacillus, Bacillus licheniformis,* and *Bacillus stercoris*. Initially, three separate flasks with 20ml (each) of nutritional broth were autoclaved for 90 minutes at 121°C. After cooling a full loop of each bacterial strain; *Anoxybacillus, Bacillus licheniformis,* and *Bacillus stercoris* were added to each flask by using a sterile inoculating loop and covered properly with aluminum foil respectively in a laminar flow hood. The flasks were placed in Shaking incubators at their optimum growth temperature i.e., *Anoxybacillus* at 50°C, *Bacillus lichenifirmis,* and *Bacillus stercoris* at 40°C for 24 hours respectively.

3.4.2.3. Preparation of M9 Media

The M9 salt media is minimal salt media which only consists of nitrogen and salts. It is a well-known microbiological growth medium for the culture of microorganisms. This buffered minimal microbial medium is typically supplemented as needed with vitamins, amino acids, and glucose.

i. For the process of biotransformation, specified M9 media was prepared in one-fold (1x) which contains autoclaved $CaCl_2$, $MgSO_4$, Na_2HPO_4, KH_2PO_4, NaCl, NH_4Cl, distilled water, and filtered 5% glucose.

ii. Three glass flasks were taken. In each empty clean flask 18.36ml of distilled water was added in which Na_2HPO_4, KH_2PO_4, NaCl, and NH_4Cl were mixed in various concentrations mentioned in the table 3.1and covered properly with plugs and aluminum foil. In separate flasks, the stock solutions of additionals (10ml stock solution of $CaCl_2$ and $MgSO_4$ respectively) were prepared. The concentrations were as in Table 3.1.

iii. All the solutions were autoclaved properly.

iv. 5% glucose solution was prepared with autoclaved distilled water separately.

Table 3.1. The Chemical Composition of Selected M9 Salt Media.

Chemicals	Concentration
Na_2HPO_4	0.256g
KH_2PO_4	0.06g
NaCl	0.01g
NH_4Cl	0.02g
$MgSO_4$ (Stock Solution)	0.12g/10ml for stock solution 0.4ml for medium
$CaCl_2$	0.11g/10ml for stock solution 200µl for medium
Glucose (5%)	6ml/20ml

3.4.2.3. Experimentation

Separate experiments were done with prepared extracts of Stevia from methanol and water.

1. For Methanolic Extract

i. First, the M9 media was prepared by combining the autoclaved salts medium with additionals of glucose, $MgSO_4$, and $CaCl_2$.

ii. In all three flasks of 18.36ml of salt solution, 0.4ml of $MgSO_4$ and 200µl of $CaCl_2$ were mixed respectively by using a 200µl pipette in a biosafety cabinet.

iii. The 6ml of prepared glucose solution was filtered by a syringe filter and 1.6ml of it was added to each flask of prepared media by using a 1000µl pipette in a biosafety cabinet and the flasks were labeled as an experiment.

iv. Three separate autoclaved empty flasks were taken and labeled as control.

v. The 10ml of media from each flask were added in labeled control flask (three controls for three experiments)

vi. The inoculum and extract were added in the same proportion (1:1) in the experiment flask and only the extract was added in the control flask.

vii. The extract was measured to 1.87g diluted in 5 ml of methanol under serial dilution in methanol to reach 29.86mg/ml for each separate experiment.

viii. For the first experiment, 500µl of *Anoxybacillus* inoculum and 500µl of extract (14.93mg) were added to the experiment flask and covered while 500µl(14.93mg) of the extract was added to the control flask and covered properly with aluminum foil.

ix. For the second experiment, 500µl of *Bacillus licheniformis* inoculum and 500µl of extract (14.93mg) were added to the experiment flask and covered while 500µl of the extract was added to the control flask and covered properly with aluminum foil.

x. For the third experiment, 500µl of *Bacillus stercoris* inoculum and 500µl of extract (14.93mg) were added to the experiment flask and covered while 500µl(14.93mg) of the extract was added to the control flask and covered properly with aluminum foil.

xi. Both experimental and control flasks were placed in a shaking incubator at their respective temperatures (50°C for *Anoxybacillus*, 40°C for *Bacillus licheniformis*, and *Bacillus stercoris*).

xii. The experiment was conducted for 4 days with the measurement of changes after every 24 hours at the optimum temperature tolerance of microbial strains

xiii. Every 24 hours, 1ml of the experiment and 1ml of the control were collected from every flask and were centrifuged at 4°C for 10 minutes at a speed of 10000 rpm to remove the palate, and the supernatants were transferred to clean Eppendorf tubes, respectively.

xiv. The pH changes were measured and the Eppendorf tubes were stored inside the refrigerator

xv. Thin layer chromatography was performed for the chemicals' alterations to be confirmed after the experiment was run for 4 days.

2. For Water Extract

i. The 20ml of M9 media was prepared and distributed equally into two parts (10ml for experiment and 10ml for control).

ii. The extract was measured at 1.67g which was dissolved in 6ml of methanol initially and serially diluted by taking 1ml from it and dissolving it in 5ml methanol then 1ml was taken from it to reach the amount of 55.66mg/ml.

iii. The experiment was conducted with *Bacillus stercoris*. 500µl of *Bacillus stercoris* inoculum and 500µl of extract (27.83mg) were added to the experiment flask and covered while 500µl (27.83mg) of the extract was added to the control flask and covered properly with aluminum foil.

iv. Both experimental and control flasks were placed in a shaking incubator at their respective temperatures of 40°C for *Bacillus stercoris*.

v. The experiment was conducted for 3 days with the measurement of changes after every 24 hours at the optimum temperature tolerance of microbial strain.

vi. Every 24 hours, 1ml of the experiment and 1ml of the control were collected from both flasks and were centrifuged at 4°C for 10 minutes at a speed of 10000 rpm to

remove the palate, and the supernatants were transferred to clean Eppendorf tubes, respectively.

vii. The pH changes were measured and the Eppendorf tubes were stored inside the refrigerator

3.5 Analysis Techniques

3.5.1 Thin Layer Chromatography (TLC)

The analysis technique of TLC is adsorption-based separation. The separation depends on the affinity of the testing sample (stationary phase) with different chemical regents acting as mobile phase.

Here, it was conducted to identify the compounds and changes in them after the biotransformation process. The identification through chromatography was conducted as follows;

3.5.1.1. Optimization of Thin Layer Chromatography (TLC) for extract

Thin Layer Chromatography (TLC) was performed to separate the compounds present in the final extract. The process was followed by using three different mobile phases for the process optimization.

i. Three separate beakers were taken.
ii. The mobile phase of Acetone: Ethyl Acetate: Water, Chloroform: Methanol: Water, and Water: Ethyl acetate: Methanol were prepared in ratios of 50:40:10, 32.5:15:5, and 12:80:20 in beakers respectively.
iii. The beakers were covered with aluminum foil and placed aside.
iv. Silica TLC plates were taken and cut to a length of 12cm. Two lines were drawn at a distance of 1.5 cm from both the bottom (stationary phase) and top (mobile front) of each TLC plate. 2µl of experimental Stevia extracts (S.E1, S.E2, S.E3) and commercial Stevia (CS) were dropped at a distance of 0.5cm from each other over the line of stationary phase and left to stand for 10 minutes.
v. The plates were placed vertically in each beaker of different mobile phases with the care that it didn't move.

vi. Observe the plates till the mobile phase reached the line of the mobile front.
vii. Remove the plates from all beakers and air dried
viii. Observe the TLC plates under the UV lamp at 270nm and 360nm to check the separation of compounds over the plate.
ix. The Rf (retention factor) values of compounds were calculated.

3.5.1.2. Thin Layer Chromatography (TLC) of extract

The optimized mobile phase of the Stevia extract was used to analyze the compounds in biotransformed extract of Stevia.

1. For Methanolic Extract Biotransformation by *Anoxybacillus*, *Strain10*, and *Bacillus stercoris*

i. The mobile phase of Acetone: Ethyl Acetate: Water was prepared in the ratio of 50:40:10 in a beaker, covered with aluminum foil, and placed aside.
ii. The four silica TLC plates were taken and cut to a length of 11cm. Two lines were drawn at a distance of 1.5 cm from both the bottom (stationary phase) and top (mobile front) of both TLC plates.
iii. 2µl of main Stevia extracts (S.E), 24hr control (24C), 24hr experiment (24E), 48hr control (48C), and 48hr experiment (48E), 72hr control (72C), and 72hr experiment (72E) of *Anoxybacillus*, *Bacillus licheniformis*, *Bacillus stercoris* were dropped on plate 1 to 4 respectively, by using a pipette (0.5-20µl) and left to stand for 10 minutes.
iv. The plates were placed vertically in beakers of mobile phases (two of *Anoxybacillus* in one beaker while the plates of *Strain10* and *Bacillus stercoris* in the second beaker) with the care that they didn't move.
v. Observe the plates till the mobile phase reached the line of the mobile front.
vi. Remove the plates from the beaker and air dried
vii. Observe the TLC plates under the UV lamp at 270nm and 360nm to check the separation of compounds over the plate.
viii. The Rf (retention factor) values of compounds were calculated.

2. **For Water Extract Biotransformation by *Bacillus stercoris***

i. Two mobile phases of Acetone: Ethyl Acetate: Water was prepared in the ratio of 50:40:10 and 60:45:5 in two separate beakers respectively, covered with aluminum foil, and placed aside.

ii. The two silica TLC plates were taken and cut to a length of 12cm. Two lines were drawn at a distance of 1.5 cm from both the bottom (stationary phase) and top (mobile front) of both TLC plates.

iii. 3µl of main Stevia extracts (S.E), 24hr control (24C), 24hr experiment (24E), 48hr control (48C), 48hr experiment (48E), 72hr control (72C), and 72hr experiment (72E) were dropped at a distance of 0.5cm from each other over the line of the stationary phase of both plates and left to stand for 5 minutes.

iv. One plate was placed vertically in a beaker with one mobile phase and a second in the beaker of the other mobile phase with the care that they didn't move.

v. Observe the plates till the mobile phase reached the line of the mobile front.

vi. The plates were removed from the beakers and air-dried.

vii. Observe the TLC plates under the UV lamp at 270nm and 360nm to check the separation of compounds over the plate.

viii. Calculate the retention factor (Rf) values of separated compounds.

3.5.2. FT-IR (Fourier Transform-Infrared Spectroscopy)

i. The liquid samples of extracted Stevia compounds, control, and final biotransformed products of Stevia extract (steviol glycosides) of *Bacillus stercoris* were characterized by using an FT-IR spectroscopy.

ii. The samples were placed in an equivalent amount over the Horizontal Attenuated Total reflectance and were scanned through an FTIR spectroscope in the region of 400 to 4000cm^{-1} with 4 cm^{-1} resolution.

iii. Every spectrum was calibrated using the air spectrum's background as a reference. For each scan of the sample, a new air spectrum background reference was acquired.

iv. The spectra's values were noted as values of absorbance.

3.5.3. Antioxidant Activity Assay

This assay is based on the free radicals scavenging ability of DPPH on reacting with the selected solutions (samples). It depends on the electron transfer that produces a crystal violet color when dissolved in methanol or ethanol. At room temperature, free radicals are stable enough that are reduced when the antioxidant molecules are present in the methanolic/ethanolic solution. The procedure was as follows;

i. Sample of a main Stevia extract was dissolved in methanol.
ii. 3mg of ascorbic acid (as control) was weighed and dissolved in 1 ml of methanol concerning the estimated extract compounds present in the biotransformed sample.
iii. The assay was performed in three different quantities of extract solution, 24, 48, 72 hours control and 24, 48, and 72 hours biotransformed sample of *Bacillus stercoris* varying from 5µl followed by 10µl and 20µl in duplicate.
iv. 95µl of DPPH was added 5µl, 90µl to 10µl sample, and 80µl to 20µl of sample.
v. The plate was incubated at room temperature for 30 minutes in the dark.
vi. After incubation, the absorbance was measured at 517nm.

4. RESULTS

4.1. Preparation of Plant Material

Fresh leaves directly from the Stevia plant were plucked, washed, dried, and converted into fine powder at room temperature

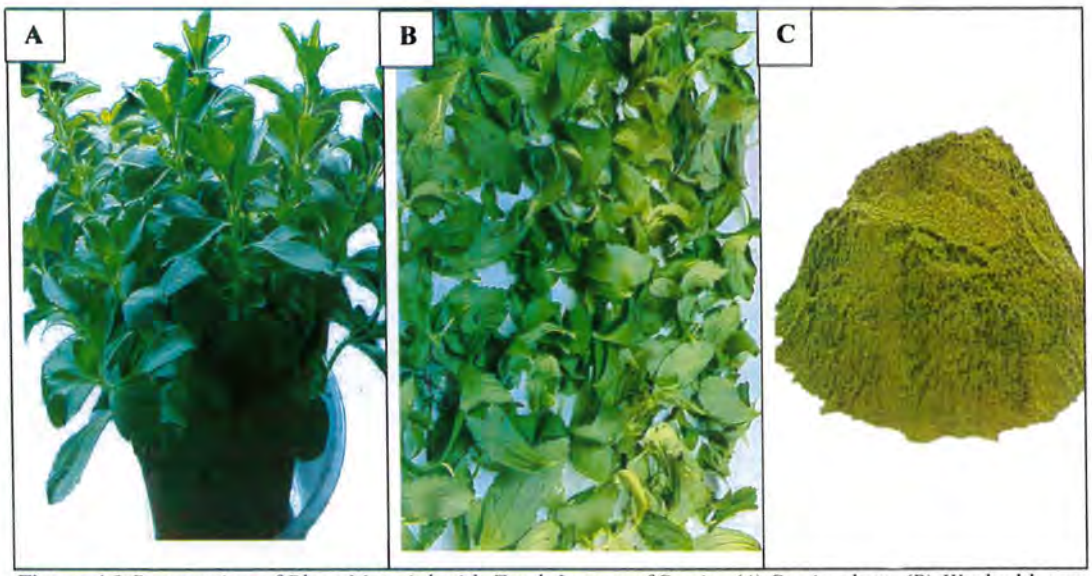


Figure 4.1 *Preparation of Plant Material with Fresh Leaves of Stevia. (A) Stevia plant, (B) Washed leaves of Stevia for drying, and (C) Green powder of dried Stevia leaves.*

4.2. Processing of Plant Material

4.2.1. Methanolic Extraction of compounds

Figure (4.2.1.) showed the extraction of sweet compounds from Stevia plant by using methanol as main extracting reagent. The powder of Stevia leaves underwent methanolic reflux and filtration to get an initial liquid extract of plant material (filtrate) which was further treated with chloroform to exclude the non-polar compounds along with oils. The polar mixture went through n-butyl (1-Butanol) reflux to get respective sugar compounds which were dissolved in ethanol to give the final white powder of sugars.

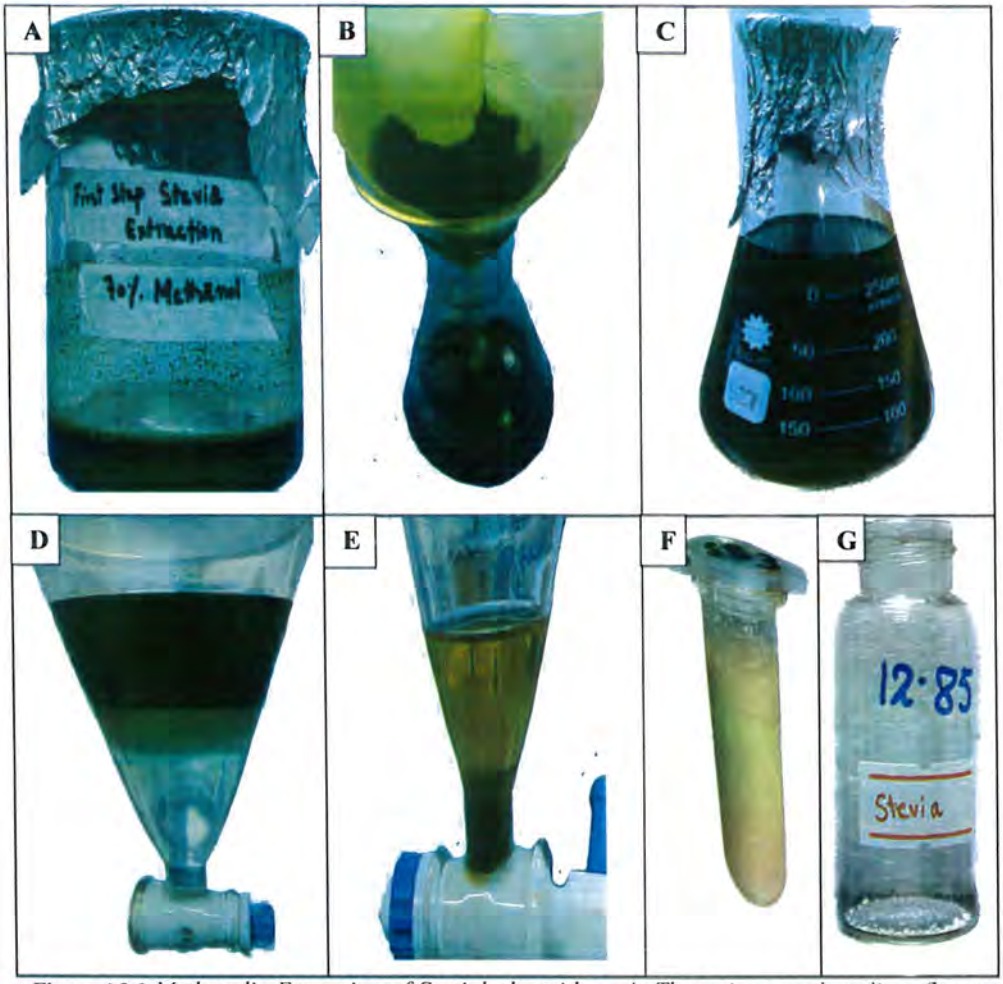


Figure 4.2.1 *Methanolic Extraction of Steviol glycosides. A: Three-times methanolic reflux at 64.7°C, B: Filtration of methanolic plant material by Whatman filter paper after every reflux, C: Final liquid Stevia compounds after separating it from residues, D: the refluxed process of an extract with chloroform to separate polar, non-polar compounds and oils, E: Extraction of final sweet compounds with n-butyl alcohol for scavenging sugars from water dissolved solution, F shows the rotary evaporated compounds dissolved in chilled ethanol, and G: the final white powder of compounds.*

4.2.2. Water Extraction

Figure (4.2.2.) exhibited the process of water-based extraction of sweet compounds from Stevia leaves. The powder of Stevia leaves was treated with distilled water at 97°C three times, and filtered to get the filtrate, after which it was treated with n-hexane one and underwent rotary evaporation. The remaining extract solution was refluxed with chloroform till no color remained in the chloroform. The colored material was mixed with

methanol and treated with n-butyl alcohol. The final compound went through rotary and mixed with chilled ethanol to get a white powder of compounds.

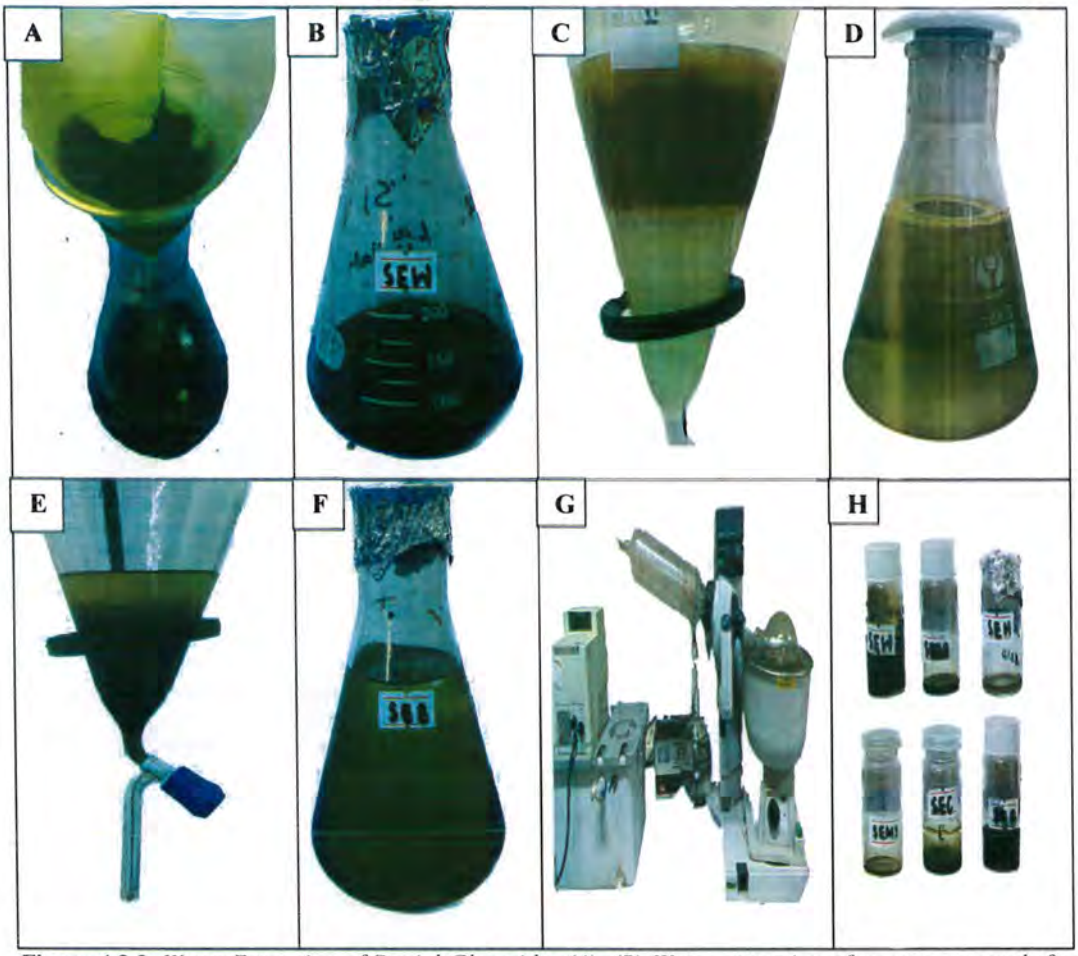


Figure 4.2.2. *Water Extraction of Steviol Glycosides (A), (B) Water extraction of sweet compounds from powdered leaves via filtration process, (C) showing the reflux process of an extract with chloroform to separate polar and non-polar compounds, (D) showing separate layers in chloroform layer after six refluxes, (E), (F) showing the reflux of remaining extract with 1-butanol, while (G) show the final separation of steviol glycosides by rotary evaporation and (H) shows the separated compounds in different reagents (SEB: n-butanol extract, SEC: chloroform extract, SEM: methanolic extract and SEH: n-Hexane extract).*

4.3. Experiment of Biotransformation

4.3.1. Inoculum preparation

The cultures of *Anoxybacillus, Bacillus licheniformis,* and *Bacillus stercoris* were streaked over the separate nutrient agar plates and incubated at 50°C and 40°C respectively. The growth was observed after 24 hours. A full loop of bacterial cultures was mixed with

separate nutrient broth and incubated for further 24 hours at optimum temperatures of 50°C and 40°C for inoculum preparation.

4.3.2. Biotransformation of a methanolic extract with *Anoxybacillus, Bacillus licheniformis,* and *Bacillus stercoris*

The experiments were conducted by mixing the inoculums of *Anoxybacillus* (Figure 4.3.2), *Bacillus licheniformis* (Figure 4.3.4), and *Bacillus stercoris* (Figure 4.3.6), with methanolic Stevia extract in 1:1 respectively along with the control for three to four days. After every 24 hours, the sample was collected from every flask, centrifuged at 4°C, and stored. Here, the samples were observed after 24 hours, then 48 hours, 72 hours, and a maximum of 120 hours. The color of the extract changed in biotransformed flask due to the microbial breakdown of compounds.

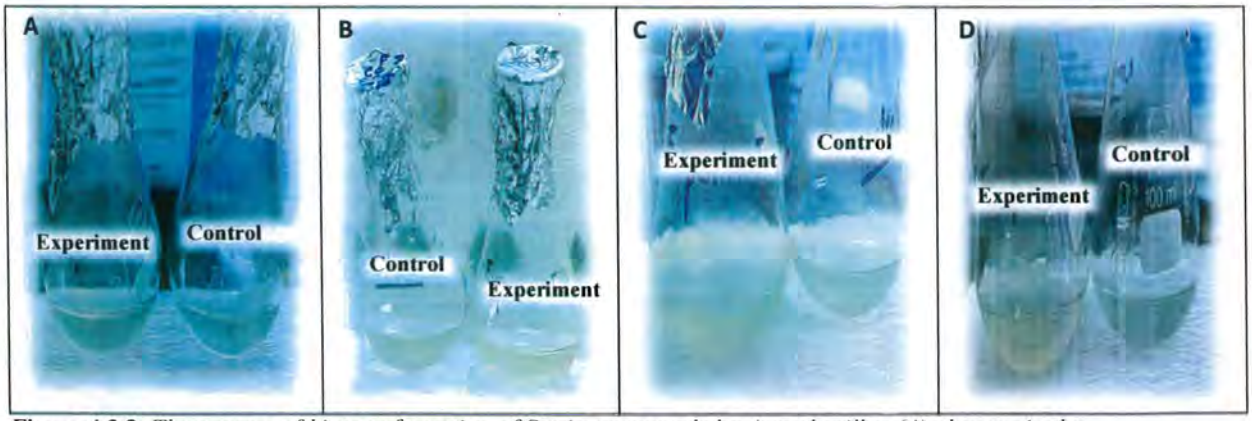


Figure 4.3.2. *The process of biotransformation of Stevia compounds by Anoxybacillus (A) changes in the experiment and control after 24hrs, (B) changes after 48hrs, (C) changes after 72hrs, and after 120hrs of experimentation*

pH change

After centrifugation of every 24 hours sample, the pH was checked, and observed the changes with time (Table 4.1). The Figure (4.3.3.) showed the graphical comparison of different pH.

Table 4.1: pH change in control and experiment of biotransformation by Anoxybacillus on different time intervals

Time (hrs)	pH change	
	Control	Experiment
0	6.653	6.653
24	6.653	7.297
48	6.631	6.272
72	6.62	6.596

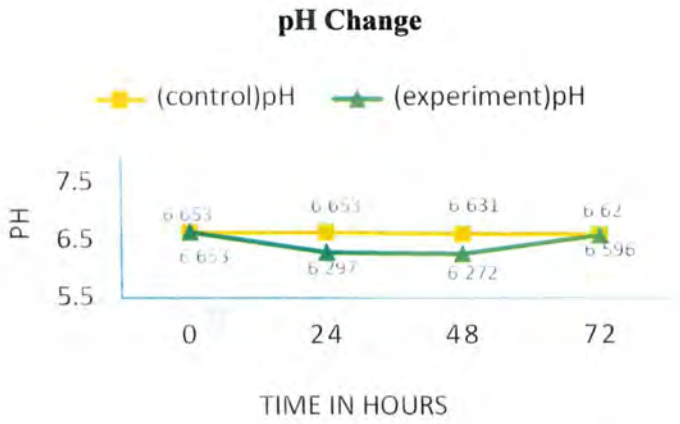


Figure 4.3.3. *pH changes during the process of biotransformation.by Anoxybacillus*

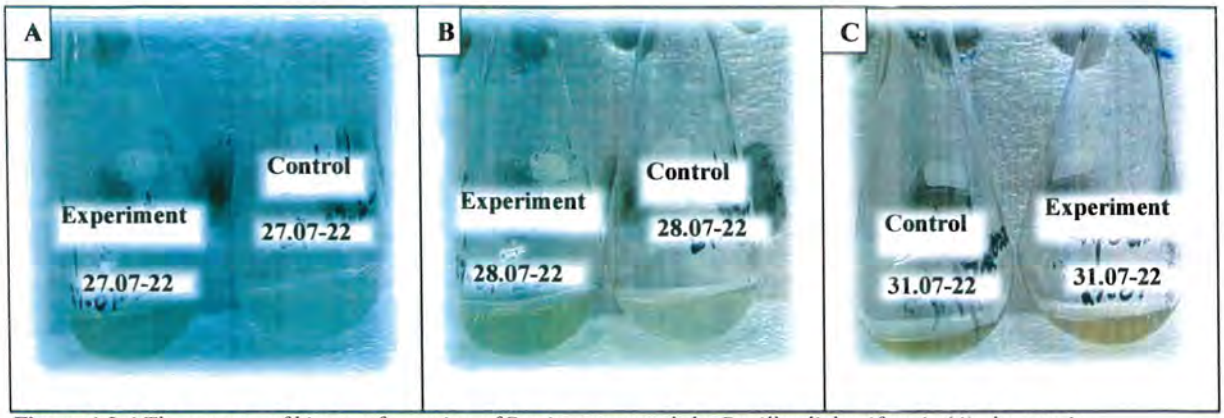


Figure 4.3.4 *The process of biotransformation of Stevia compounds by Bacillus licheniformis (A) changes in the experiment and control after 24hrs, (B) changes after 48hrs, and (C) changes after 120hrs of experimentation*

pH change

After centrifugation of every 24 hours sample, the pH was checked and observed the changes with time (Table 4.2) and differences were compared as shown in Figure (4.3.5).

Table 4.2: pH change in control and experiment of biotransformation by Bacillus licheniformis on different time intervals

Time (hrs)	pH change	
	Control	**Experiment**
0	6.653	6.653
24	6.65	6.330
48	6.649	6.505
120	6.6489	6.534

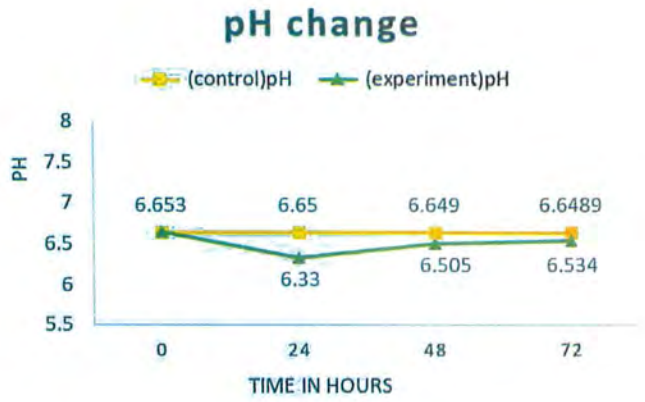


Figure 4.3.5. *The graphical representation of pH changes during the process of biotransformation.by Bacillus licheniformis*

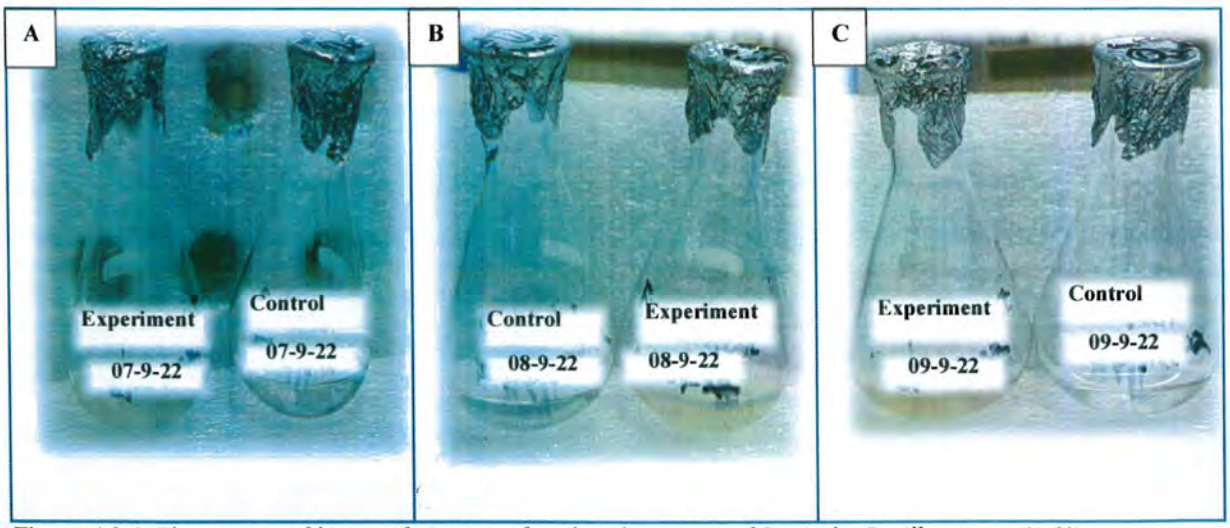


Figure 4.3.6. *The process of biotransformation of methanolic extract of Stevia by Bacillus stercoris (A) changes in the experiment and control after 24hrs, (B) changes after 48hrs, (C) changes after 72hrs of experimentation*

pH change

The figure below showed that after centrifugation, the pH was checked and the changes were observed in the selected time laps time (Table 4.3). the alteration in pH level were graphically represented as in Figure (4.3.7).

Table 4.3: pH change in control and experiment of biotransformation by Bacillus stercoris on different time intervals

Time (hrs)	pH change	
	Control	Experiment
0	6.697	6.697
24	6.653	6.269
48	6.638	6.470
72	6.643	6.515

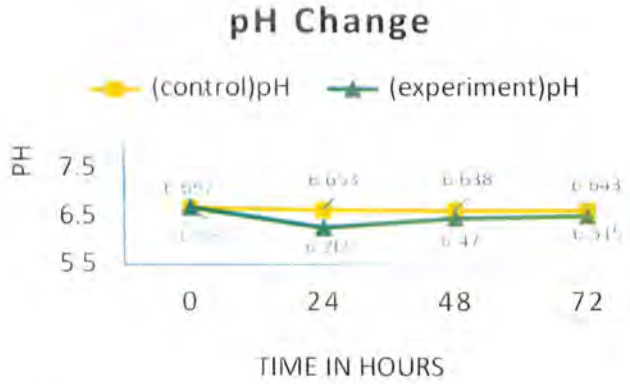


Figure 4.3.7. *The graphical representation of pH changes during the process of biotransformation by Bacillus stercoris*

4.3.3. Biotransformation of a water extract with *Bacillus stercoris*

Figure (4.3.8.) showed the biotransformational experiment that was conducted by mixing inoculum of *Bacillus stercoris* and Stevia extract in 1:1 along with the control for four days. After every 24 hours, the sample was collected, centrifuged, and stored. Here, the sample was observed after 24 hours, then 48 hours, and 72 hours.

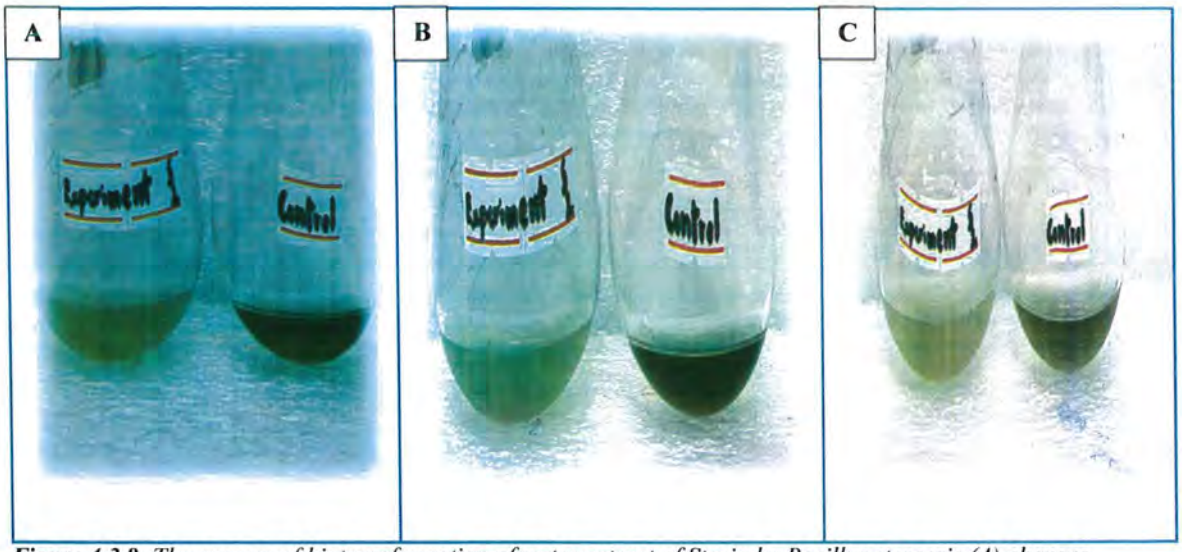


Figure 4.3.8. *The process of biotransformation of water extract of Stevia by Bacillus stercoris (A) changes in the experiment and control after 24hrs, (B) changes after 48hrs, (C) changes after 72hrs of experimentation*

pH change

The figure below showed that after centrifugation, the pH was checked and the changes were observed in the selected time laps time (Table 4.3) and differences were graphically compared in Figure (4.3.9).

Table 4.4: pH change in control and experiment of biotransformation by Bacillus stercoris on different time intervals

Time (hrs)	pH change	
	Control	Experiment
0	7.026	7.026
24	7.026	6.348
48	7.026	6.429
72	7.020	6.567

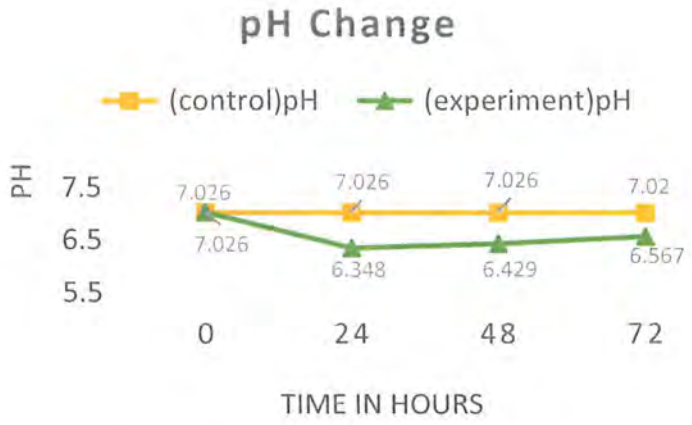


Figure 4.3.9. *The graphical representation of pH changes during the process of biotransformation of water extract by Bacillus stercoris.*

4.4. Analysis of compounds

4.4.1. TLC (Thin Layer Chromatography) of extracted compounds

The separation of methanol-extracted compounds of Stevia was done by running the stationary phase of extract in various mobile phases in the Thin Layer Chromatography. As shown in figure (4.4.1), the compounds were separated by using the mixture of ethyl acetate, water and acetone in a specified ratio of 4:1:5 which was observed under the UV lamp.

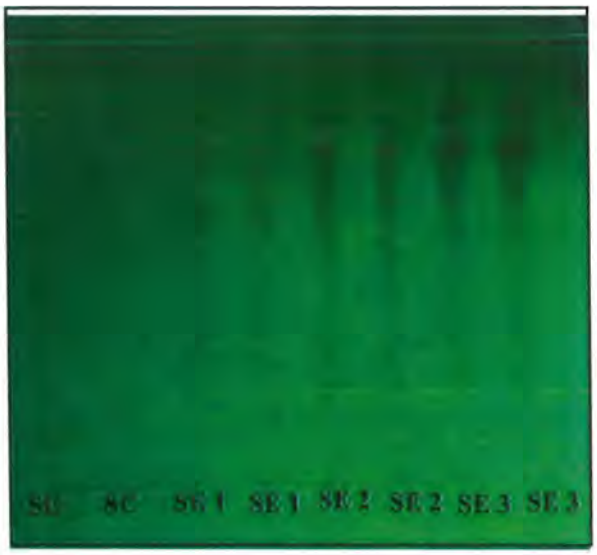


Figure 4.4.1. The visualization of separated compounds of Stevia by TLC in the mobile phase of acetone, ethyl acetate, and water under the UV radiations.

Figure 4.4.2. showed the chromatographic analysis of methanol-based extracted compounds in the mobile phase of chloroform, methanol, and water in which the compounds were not separated properly.

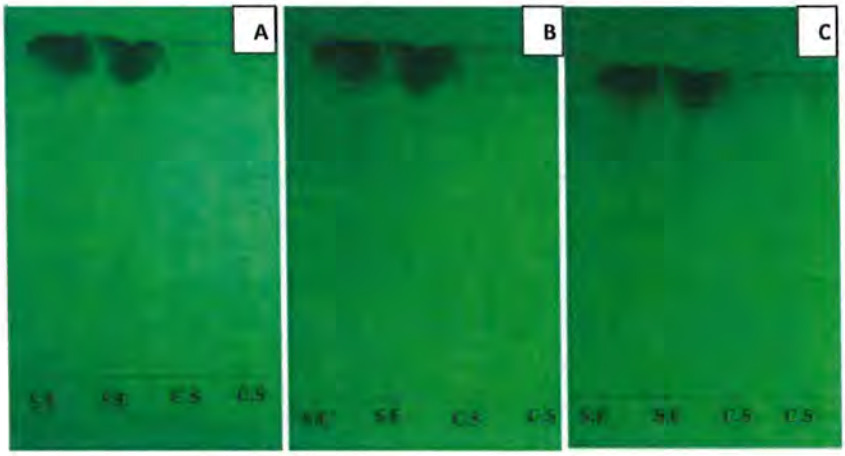


Figure 4.4.2 *(A), (B), and (C) show the separation of the compounds on the TLC plate in the chloroform, methanol, and water mobile phase*

The separation of water-extracted compounds of Stevia was done by running the stationary phase of extract in various mobile phases in the Thin Layer Chromatography. Figure (4.3.3). depicted the compound separation in a mobile phase mixture of acetic acid: chloroform: water, acetonitrile: water, and acetone: water: ethyl acetate in two different ratios respectively. There was no proper separation in the mediums of chloroform/water/acetic acid and acetonitrile/water as shown in the figure.

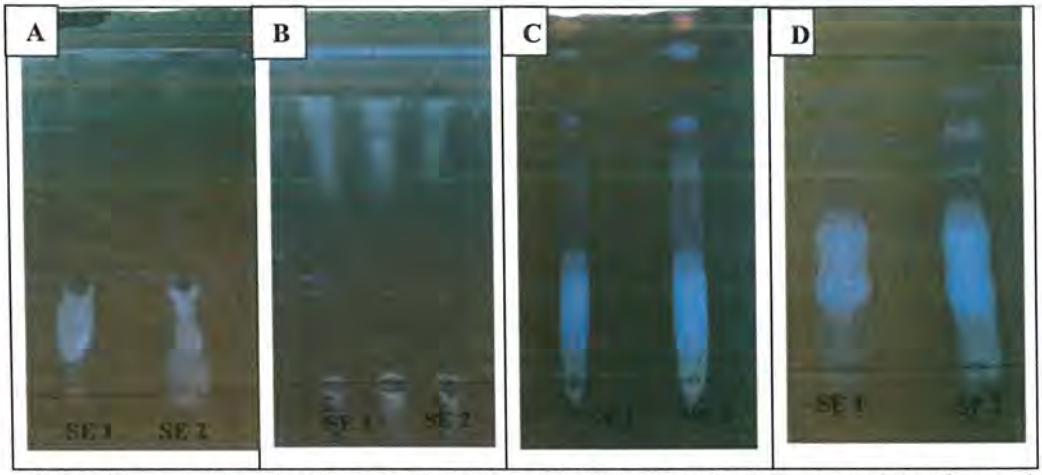


Figure 4.4.3. *The running of stationary phase (Steviol Glycosides) in different mobile phases. (A) The running of stationary phase (Steviol Glycosides) in different mobile phases. in the mobile phase of Chloroform: Acetic Acid: Water, (B) The running of stationary phase (Steviol Glycosides) in the mobile phase of Acetonitrile: Water, 3 C The running of stationary phase (Steviol Glycosides) in the mobile phase of Acetone: Ethyl acetate: Water in a ratio of 50:40;10, and (D) The running of stationary phase (Steviol Glycosides) in the mobile phase of Acetone: Ethyl Acetate: Water in the ratio of 60:35:5*

4.4.1.2. Rf values of extracted compounds

(i) Methanolic Extract

Various values of the retention factor (R_f)of extracted compounds after TLC were as in Table 4.5.

Table 4.5: The calculated R_f of separated compounds after TLC

Samples	Compound 1 (R_f)	Compound 2 (R_f)	Compound 3 (R_f)	Compound 4 (R_f)	Compound 5 (R_f)	Compound 6 (R_f)	Compound 7 (R_f)
(SC)	0.742	0.639	-	-	-	-	-
(S.E1)	0.258	0.329	0.567	0.742	0.762	-	-
(S.E2)	0.216	0.298	0.567	0.742	0.762	-	-
(S.E3)	0.258	0.329	0.567	0.742	0.762	0.855	0.896

4.4.2. TLC (Thin Layer Chromatography) of biotransformed compounds by *Anoxybacillus*

Figure (4.4.4) exhibited the separated bio-transformed compounds by *Anoxybacillus* by performing TLC in the optimized mobile phase of acetone: ethyl acetate: water in a ratio

of 50:40:10 and observed under the UV lamp of 260nm which clearly differentiate the transformation by observing the thickness of the band increase compared to the control.

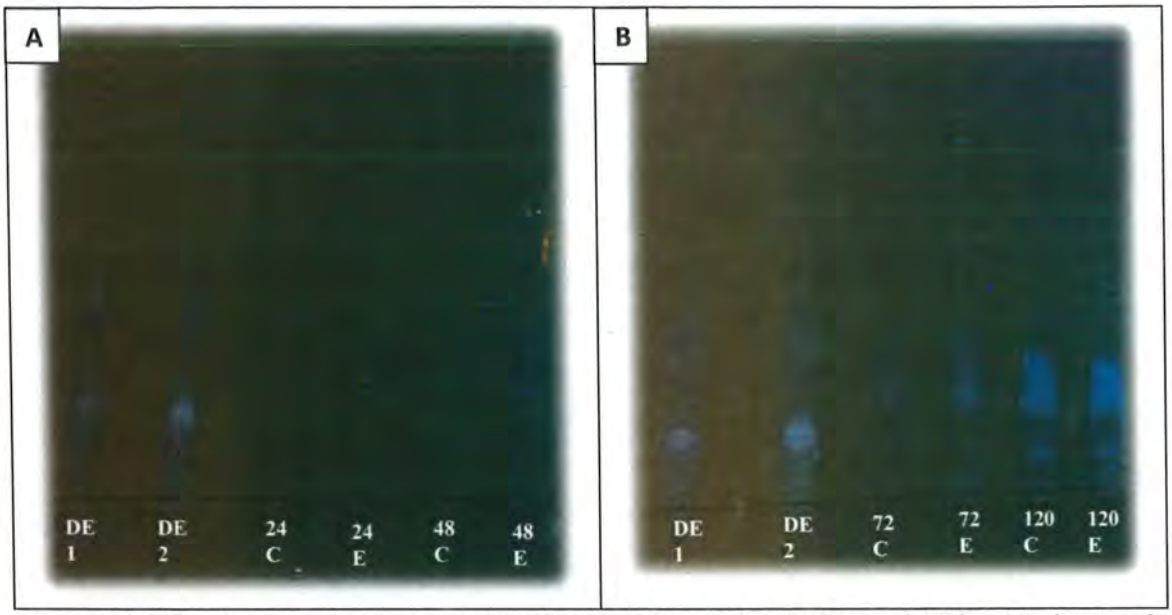


Figure 4.4.4 *The change in the Stevia compounds after 24hrs, 48 hrs, 72 hrs, and 120hrs (A) showing the separated compounds in diluted Stevia extracts (DE) and biotransformed extract in 24 and 48 hours controls (24 C), (48C), and experiments (24E),(48E).*

The calculated R_f values of separated compounds in the tested samples are as in Table 4.6.

Table 4.6: The calculated R_f of observed compounds.

Samples	C1 (R_f)	C2 (R_f)	C3 (R_f)	C4 (R_f)	C5 (R_f)
DE 1	0.091	0.321	0.505	0.701	0.782
DE2	0.080	0.298	0.505	0.689	0.747
(24 C)	0.114	0.252	-	-	-
(24 E)	0.126	0.333	-	-	-
(48 C)	0.068	0.126	0.275	-	-
(48 E)	0.068	0.126	-	-	-
(120.C)	0.068	0.126	0.218	0.301	0.517
(120.E)	0.057	0.126	0.229	0.528	-

(120 C)	0.057	0.126	0.183	0.252	0.528
(48 E)	0.068	0.126	0.183	0.241	0.528

4.4.3. TLC (Thin Layer Chromatography) of biotransformed compounds by *Bacillus licheniformis*

Figure (4.4.5) showed the bio-transformed compounds in water extract by *Bacillus licheniformis* that were separated by running TLC and observed under the UV lamp of 260nm. The image showed clear bands of different compounds, formation after 48hrs and 72 hrs of the biotransformation in comparison to 24 hrs. The R_f values of compounds were as in Table 4.7 illustrating the changes in the compounds in comparison to the main extracted Stevia compounds.

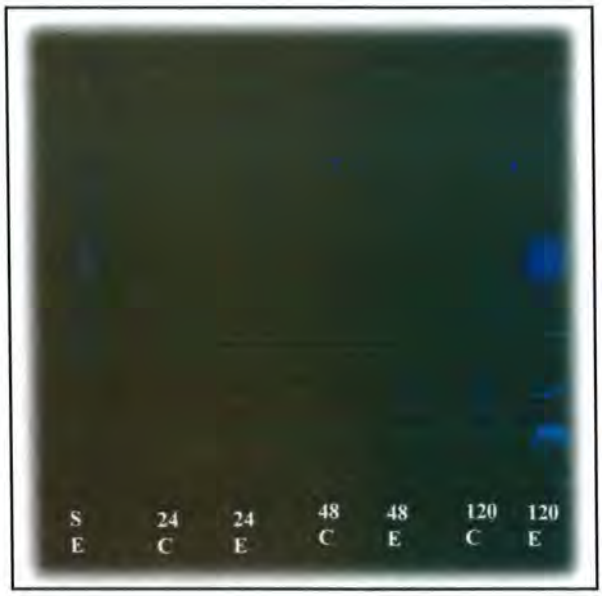


Figure 4.4.5. *The change in the Stevia compounds after 24hrs, 48 hrs, and 120 hrs by Bacillus licheniformis.*

The R_f values of compounds in the selected samples were as in Table 4.7.

Table 4.7: The calculated R_f of observed compounds.

Samples	C.1 (R_f)	C.2 (R_f)	C.3 (R_f)	C. 4 (R_f)	C.5 (R_f)
(SE)	0.2267	0.386	0.533	0.667	0.76
(24 C)	0.133	0.173	0.266	0.40	-
(24 E)	0.066	0.173	0.266	0.333	0.40
(48 C)	0.066	0.133	0.213	0.333	-
(48 E)	0.053	0.133	0.213	0.333	-
(120. C)	0.066	0.12	0.266	0.333	0.40
(120. E)	0.666	0.173	0.126	0.333	0.426

4.4.4. TLC (Thin Layer Chromatography) of bio-transformed compounds by *Bacillus stercoris*

1. Methanolic Extract

Figure (4.4.6,) showed the separated bio-transformed compounds in the methanolic extract carried out by *Bacillus stercoris*, by running TLC in a selected mobile phase of acetone/ethyl acetate/water in a ratio of 50:40:10 and observed under the UV lamp of 260nm. The image showed clear bands of different compounds, formation after 48hrs and 72 hrs of the biotransformation in comparison to 24 hrs. The R_f values of compounds were as in Table 4.8 illustrating the changes in the compounds in comparison to the main extracted Stevia compounds.

Figure 4.4.6 The change in the Stevia compounds after 24hrs, 48 hrs, and 72 hrs by Bacillus stercoris.

Table 4.8: The calculated R_f of observed compounds after biotransformation with *Bacillus stercoris*.

Samples	C1 (R_f)	C2 (R_f)	C3 (R_f)	C4 (R_f)	C5 (R_f)	C6 (R_f)	C7 (R_f)
S.E	0.056	0.157	0.325	0.393	0.629	0.730	0.831
24 hrs. C	0.101	0.202	0.494	-	-	-	-
24 hrs E	0.056	0.112	0.236	0.652	-	-	-
48hrs. C	0.101	0.202	-	-	-	-	-
48hrs. E	0.101	0.213	0.506	0.629	0.741	-	-
72 hrs. C	0.1011	0.179	0.831	-	-	-	-
72 hrs. E	0.078	0.011	0.225	0.517	0.652	0.798	-

2. Water Extract

The (Figure 4.4.7) demonstrated that bio-transformed compounds in water extract by *Bacillus stercoris* and control extract without transformation were separated by running on TLC plate and observed under the UV lamp of 260nm. The R_f values of compounds were as in Table 4.9. The Rf values and images of TLC clearly shows a difference in bands of

biotransformed extract in comparison with control. Further, it shows a clear distinction of bands in biotransformed extract after 48 hours and 72.

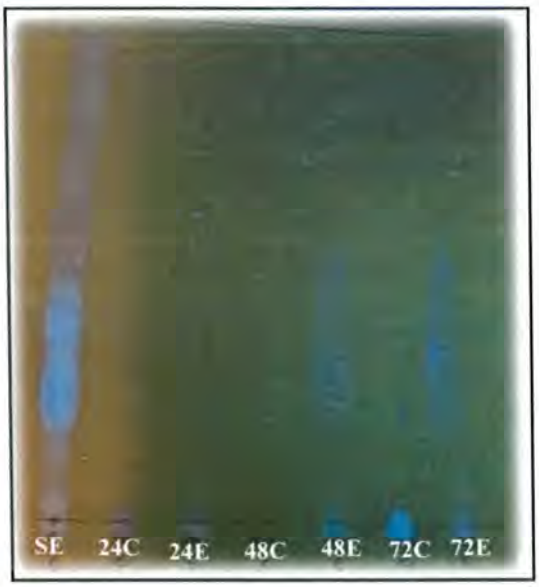


Figure 4.4.7 The change in the Stevia compounds of water extract after 24hrs, 48 hrs, and 72 hrs by Bacillus stercoris.

Table 4.9: The calculated R_f of observed compounds after biotransformation with *Bacillus stercoris*.

Samples	C.1 (R_f)	C.2 (R_f)	C.3 (R_f)	C.4 (R_f)	C.5 (R_f)	C.6 (R_f)	C.7 (R_f)	C.8 (R_f)	C.9 (R_f)
S.E	0.08	0.121	0.164	0.263	0.340	0.384	0.483	0.714	0.879
24 hrs. C	0.164	0.121	0.340	0.384	0.714	0.879	-	-	-
24 hrs E	0.164	0.121	0.340	0.652	0.714	0.835	0.869	-	-
48hrs. C	0.164	0.121	0.340	0.714	0.879	-	-	-	-
48hrs.E	0.054	0.121	0.186	0.219	0.4505	0.516	0.780	0.868	-
72 hrs.C	0.164	0.121	0.340	0.714	0.879	-	-		
72 hrs.E	0.054	0.121	0.186	0.219	0.4505	0.516	0.692	0.780	0.868

4.4.6. FT-IR

The figure (4.4.8) showed the FTIR analysis of the selected samples of 48-hour and 72-hour control and biotransformed Stevia extract done by *Bacillus stercoris* which indicated changes in the wideness and appearance of different peaks after the biotransformation. The peaks between 1000-1500 cm^{-1} and 2000-2500 cm^{-1} disappeared illustrating the bond-breaking and altered structures of functional groups.

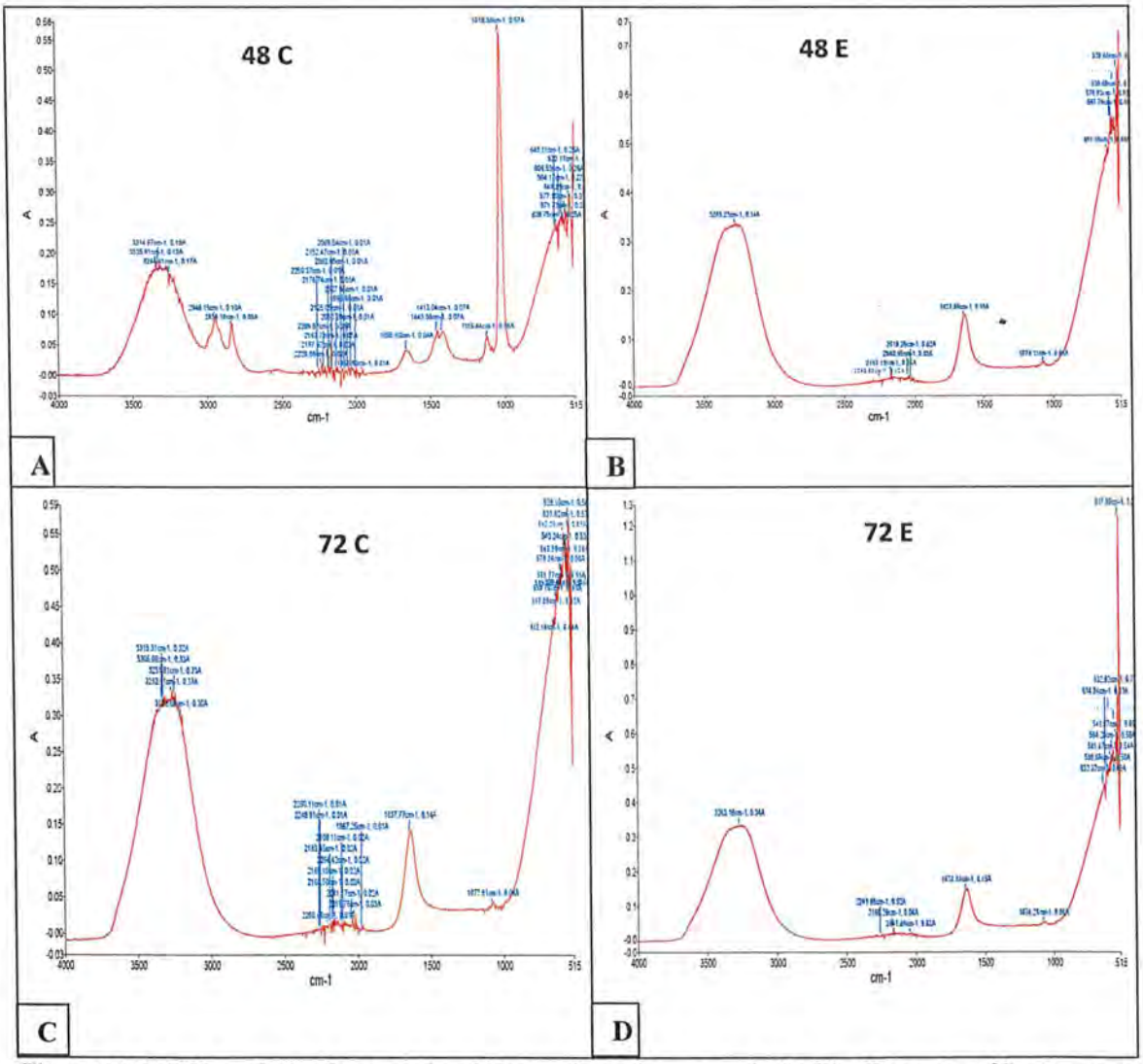


Figure 4.4.8 *FTIR analysis of extracted and biotransformed compounds (A) The IR spectra of Stevia extract control after the incubation of 48 hours while (B) indicating the biotransformed compounds of Stevia present in methanolic extract. (C) and (F) indicated the IR spectra data about the water extract control of 72 hours and its biotransformed compounds by Bacillus stercoris respectively.*

4.4.7. Antioxidant Activity Assay

Table 4.10 showed the variations in antioxidant activity of selected samples of best-biotransformed compounds carried out by *Bacillus stercoris* and the control of every 24 hours sample with respect to the positive control of Ascorbic acid for antioxidant assay. The DPPH scavenges the free radicals that changed the color from purple/violet to yellow or colorless as in figure (4.4.9). The comparison of changes in the antioxidant potential was as in figure (4.4.10).

Table 4.10: Antioxidant Potentials of samples.

Samples	Percentage % inhibition at various concentration		
	5µl	**10µl**	**20µl**
Ascorbic Acid	89	91.04	91.35
24-hour Control	26.47	46.5	46
24-hour Experiment	56	57	65.3
48-hour Control	29	39.93	39.93
48-hour Experiment	51.3	55	55.7
72-hour Control	16.60	18.2	21
72-hour Experiment	41	55	58

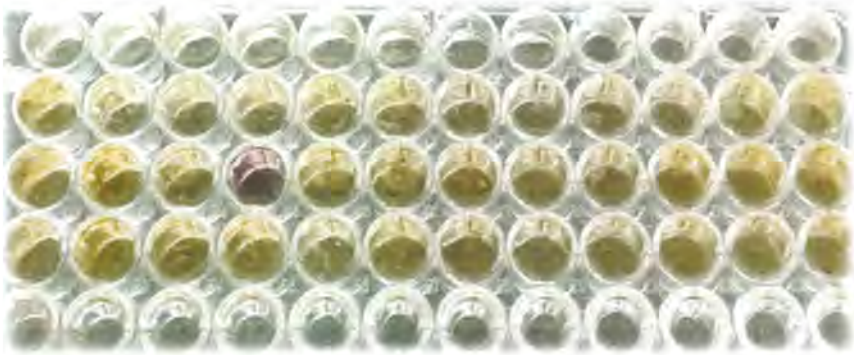


Figure 4.4.9. The Color of DPPH changed from purple to yellow indicating the antioxidant potential of samples.

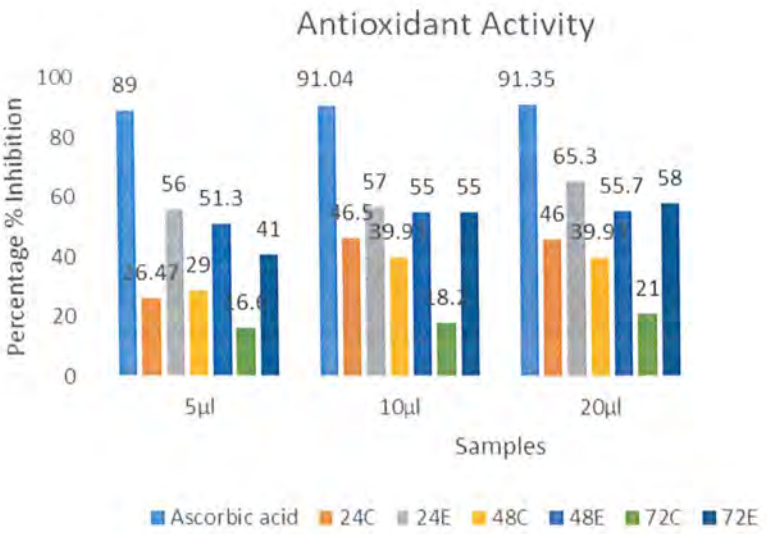


***Figure 4.4.8**: Antioxidant potential of extracted and biotransformed compounds. The Percentage inhibition of samples including Ascorbic Acid, 24-hour control (24C), experiment (24E), 48-hour control (48C), experiment (48E), 72-hour control (72C), and experiment (72E) during DPPH scavenging assay.*

DISCUSSION

This study was conducted for the extraction of commercially important steviol compounds found in *Stevia* and biotransforming them to improve its taste and other biological activities. The process was initiated with the extraction of sweet compounds. The solvent extraction technique was used to separate the Stevia extract from blended Stevia leaves by using methanol, an important solvent by comparing with previously done extractions in the past. It was a preferable solvent for the maximum rate of extraction as it has high polarity and extraction yield as compared to water which was used for being a green alternative. Previously, extraction with methanol was conducted by Howlader *et al.*, (2016) by using 80% methanol (Howlader *et al.*, 2016). In our study, 70% methanol was used for the extraction of sweet compounds (steviol glycosides) at the optimum temperature of 62.8°C (boiling point of methanol). Heating at that temperature enhances the extraction power of solvent to reduce the repetition of a process that depends on at least ten to thirteen times extraction. Although the temperature tolerance of Stevia compounds such that steviol, Stevioside, and Rebaudiana A is more than 200°C. So that a high-temperature range can support the optimum isolation of compounds.

Although it was believed that methanol cannot extract whole sweetness from the leaves extract. For the complete extraction of sweet compounds, powder of leaves was refluxed with methanol for three times with filtration as shown in figure 4.2.1 (A), (B), and (C). There were different compounds found in Stevia including materials like oils. The greasy material and bulk of less polar and colored chemicals were extracted from methanolic extract after the solvent had completely evaporated. This was done by several times refluxes using chloroform as shown in figure 4.2.1 (D). It has more density than water making it suitable to separate esters and other colored compounds (Fatima, 2017). The refluxes continued till the green color in the chloroform layer completely vanished. The chloroform layer had fewer sweet compounds as compared to another methanolic layer with maximum sweet potential. The extremely polar, water-soluble steviol compounds i.e., sweet steviol glycosides and oligosaccharides were isolated from these other compounds, by repeatedly extracting the aqueous solution of the resulting mass with n-butyl alcohol as shown in figure 4.2.1 (E). It is supportive in gradient elution as being more polar. Before

extracting the desired chemicals from the plant material or after they have been extracted, the undesirable compounds must be eliminated. The immiscible solvents; water and butanol were utilized together to eliminate contaminants from the residue, leaving the butanol layer with polar compounds. The diterpenoid class of sugary chemicals found in Stevia are glycosidic in origin and were retained by n-butyl alcohol (1-butanol) (Huang *et al.*, 2010). The final extract was in light brown color that turned to a lighter color with the appearance of crystals after adding methanol to it. That crystallized extract was treated with chilled ethanol to reduce the phenolic compounds as well as the bitterness of Stevia. It further enhanced the appearance of particular Stevia compounds to boost the white to off-white colored powder of Stevia as shown in figure 4.2.1 (F) and (G).

On the other side, water extraction was performed in an almost similar pattern to that of methanolic extraction. The extraction by water was considered a green alternative. In this method, the initial refluxes were performed with water at a temperature near to water's boiling point of 98°C. The extract was evaporated to reduce the water content. The water can isolate all sweet compounds (steviol glycosides) along with the principal compounds of Rebaudiana A and Stevioside as shown in figure (4.2.2). The extract was treated with n-Hexane to remove Stevia compound name Austroinulin and oily compounds. Further methanol was added to the remaining water extract. The combined solution of water and methanol increased the polarity as compared to chloroform so that while treating with chloroform at 61°C in a separating funnel, the lower contained other greasy materials, non-polar and colored compounds. Here the methanol helped in the separation of polar diterpenoids glycosides. An extract other than chloroform one was treated with n-butyl alcohol for the separation of the final required compounds as shown in figure 4.2.2 (G). The final extracted compounds underwent ethanolic treatment to get white to off-white crystals with less bitterness (Shamima *et al.*, 2019).

The extraction of compounds was followed by the process of biotransformation for that the extracted compounds were tested with the selected three different bacterial strains i.e., *Anoxybacillus, Bacillus licheniformis, and Bacillus stercoris* (previously isolated for better enzymatic activity specifically for β- glucosidase) to analyze the change in the compounds as the enzymes present inside the bacterial isolates utilize the compounds in the extract.

The process of biotransformation was important for the modification of compounds (Sultana, 2018). In this study, we have used M9 media with modification as in the literature, the ratio of glucose in M9 media was 20% while we used 5% as we were transforming the sugar moieties of Steviol glycosides by microorganisms and if a higher concentration of glucose was present in the media then the selected strains might utilize the glucose of media only and cause difficulty in the analysis of biotransformation of extract molecules. Therefore, by using 5% glucose, the microbial strain has more excess to the glucose attached with sweet compounds such that steviosides, steviol glycoside, rebaudiana A, or steviol. to support the hydrolysis type of biotransformation. According to the study of Van Hofwegen *et al.* (2016), M9 media (minimal salt media) is beneficial to use as it has a base formulation of salts and can be enhanced by different nutrients, carbon sources, as well as amino acids to support microbial strains (Van Hofwegen *et al.*, 2016).

Musa *et al.* (2014) had previously done transformation of stevioside specifically with the help of *L. citreum* with alternansurase specified activity by using MRS medium with sucrose to control the bitterness (Musa *et al.*, 2014). While over study was conducted with steviol glycosides in modified M9 medium with less glucose to focus on the maximum availability of steviol glycosides to bacterial isolates. The transformation of compounds structure during the process of biotransformation was analyzed after every 24 hours for three consecutive days by taking 1ml from the experiment and control which was further centrifuged to separate the bacterial species and other particles in a palate from the supernatant with the required compounds. The pH was analyzed to estimate the change occurring in the compounds by *Anoxybacillus, Bacillus licheniformis, and Bacillus stercoris* with time respectively. A previous study on the pH change indicated that during the fermentation of compounds by microbial strain the pH first drops and then increases as the strain utilized the compounds for its nutritional needs and produces organic acid (decreasing the pH) while after some time, they start utilizing the produced organic acids that cause an increase in pH. In this process, the pH before biotransformation was neutral at about 7 or slightly acidic i.e., near 6.5. The pH of our biotransformed solution became more acidic after 24 hours as the breakdown of glucose occurred as studied earlier by Marasinghege, (2020), the pH gradually changes during the process of transformation and fermentation (Marasinghege *et al.*, 2020). While after 48 hours it started increasing.

The physical analysis of experimentation was followed by the colorimetric changes in the conducted experiment of biotransformation indicating the alteration of compounds. The color of the extract became lighter during the process of biotransformation as compared to the control (only extract). This might be due to the breakdown of existing molecules into new compounds or might be isomerized into previous compounds that increase the acidity of the solution (BARBUT, 2010). These compounds exhibit polar behavior so, different polar solvents were selected for finding optimized extracting solvents through TLC. TLC (Thin Layer Chromatography) is an inexpensive and simple technique used for the separation of compounds present in the mixture. According to Bele *et al.*, (2011), it is a quick process that is mostly used in organic laboratories. It requires a very small amount of sample for the separation (less than 5 to 10 micrograms). It contains two phases stationary phase (for sample or mixture) which can be solid or liquid and a mobile phase (solvent for the separation of compounds). Mostly the compounds are invisible on the silica gel of TLC plates as in our case for which, Ultraviolet (UV) radiation box is used with different ranges based on the type of mixture or used stationary phase (Bele *et al.*, 2011). The compound's polarity is related to the retention factor which is a ratio of distance traveled by the stationary phase and mobile phase. The compounds that are less polar do not travel a large distance while more polar can move faster and cross a large distance. This helps more to identify the compounds. In the case of chloroform, water, and methanolic mobile phase, no proper separation took place as no separation was observed on the plate. while multiple spots have appeared on the TLC plate placed in ethyl acetate, acetone, and water-based mobile phase. When the extracts (S.E 1, S.E 2, and S.E 3) were compared with each other as well as with commercially available Stevia sweetener (SC), different Rf values were calculated based on what the main sweet compounds can be indicated easily in all. The values of compound 2 of commercial Stevia (0.742), and compound 4 of other Stevia extracts (S.E 1, S.E 2, and S.E 3 of 0.742) are important compounds while compounds 3 and 5 of S.E 1, S.E 2, and S.E 3 were also targeted compound for further structural analysis. While the TLC analysis after biotransformation indicated the presence of multiple compounds on the silica plates in the experimented sample as compared to the main non-transformed pure extract. The difference in the values of retention factors better differentiated the compounds. The value of the retention factor is related to the polarity of

compounds. According to Naknean and Meenune. (2010) the compounds with a more polar nature can adhere to absorbent and separated earlier near the baseline of the stationary phase on a silica plate with less R_f value while the less polar compounds have more R_f value (Naknean & Meenune, 2010).

The compound separation on the TLC plate is also related to the molecular weight of the compounds. Heavy molecules or compounds cannot travel a long distance on the TLC plate therefore, low Rf-valued compounds have a more molecular weight (Narayanan *et al.*, 2020). Compounds having brighter and thicker bands have more absorbance and molecular weight as in our conducted study, the TLC analysis of compounds transformed by *Bacillus stercoris* indicated the presence of more polar compounds with a more molecular weight representing a change in comparison to running control and non-biotransformed extracted compounds along.

Techniques such as FTIR was used to analyze chemical changes in the compounds. This technique helped in the final analysis of compounds along with the representation of change in the molecular structures. This study was based on the observation of changes in the steviol glycosides' wavelength concerning IR (infrared) spectroscopy. Chaturvedula *et al.*(2012) showed different stretches (peaks) in the structure of steviol glycosides which determined the presence of the OH (hydroxyl), sp^3-CH (alkane), C=O (carbonyl), C=C (alkene), and C-O-C (ether) groups, as well as the bending vibrations of -C=C (alkene) as done in the previous studies. The wider band between 3000-3500 cm^{-1} indicated the presence of OH group stretching, the peak at 1074 cm^{-1} determined the presence of ether group, while, stretching of alkene was noted between 1641-1662 cm^{-1}, alkane between 2918-2947 cm^{-1}, carbonyl groups between 1719-1728 cm^{-1}, ether groups between 1054-1070cm^{-1}, and alkene bending between 870-895cm^{-1} (Chaturvedula, Mubarak, and Prakash, 2012). The region between 1100-1200 cm^{-1} showed the glycosidic linkage which was absent in the biotransformed extract interpreting the breakage of the bond and the presence of bands at 1074-1077 cm^{-1} of ethers and change in the wideness of OH stretching above 3000 cm^{-1} indicated the formation of new glycosides. While the reduction in the bands between 2500-3000 indicated the changes in alkyl bond stretching in comparison with the study conducted by Chaturvedula et *al.* (2016). While the FTIR spectra of the

methanolic extract showed peaks of glycosidic bonds in the region of 1100-1200 cm^{-1}. There was a great change in the molecular structures of steviol glycosides after biotransformation but the changes can be confirmed by High-Performance Liquid Chromatography and LCMS as studied earlier (Chranioti *et al.*, 2016).

Stevioside could be present in a high amount. When the bacterial strains broke down the glucose from it, might be it added to Rebaudioside A, or new compounds were formed to improve the sweet taste and reduce the bitter aftertaste. It could also change the biological activities such that antioxidant potential could be observed through DPPH assay (antioxidant assay). It is based on the transfer of electrons that produces the purple color of 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical in methanol or ethanol that is reduced by an antioxidant molecule to the yellow-to-colorless solution. DPPH is a stable free radical at room temperature (Garcia *et al.*, 2012). This is a reaction between DPPH and a free-radical scavenger antioxidant that produces DPPHH having less absorbent than DPPH due to the lower hydrogen content. The color reduces as the number of electrons gathered rises (Baliyan *et al.*, 2022). According to the analysis of Periche *et al.* (2015), the drying treatment of stevia leaves showed an increase in the antioxidant potential of stevil glycosides but along with the presence of other flavonoids by taking gallic acid as a control. while in the current study, ascorbic acid was taken as a positive control to which the antioxidant potential of control and biotransformed sweet compounds were compared. Here the antioxidant potential was as 24-hour >48-hour>72-hour biotransformed samples. The antioxidant activity was checked in different proportions of 5µl, 10µl, and 20µl. The scavenging potential increased by 5µl>10µl>20µl but for 20µl 72-hour biotransformed at 58% as compared to the control having 21% of activity. which was increased than the activity of control (consisting of extract) at 46%. Our findings supported the influence of biotransformation on antioxidant activity and were in line with the results of the studies already described (Karimi *et al.*, 2014).

CONCLUSION

It was concluded from this research that:

- Methanol was a good extraction reagent than water for the isolation of sweet compounds from Stevia leaves.
- The isolated compounds were better transformed microbially with *Bacillus stercoris* at 40°C after 48 and 72 hours of incubations than the other two selected bacterial strains of *Anoxybacillus* and *Bacillus licheniformis* at 50°C and 40°C respectively.
- The control and biotransformed samples have altered compounds that were comparatively observed under the (**TLC**) Thin layer Chromatography techniques with optimized selected reagents solution of acetone, ethyl acetate, and water.
- A preferable FTIR analysis of samples indicated chemical changes in the extracted compounds after biotransformation.
- The antioxidant activity of biotransformed compounds was enhanced than the extracted compounds in the control.

FUTURE PROSPECTS

- The biotransformed compounds can be analyzed and purified by advanced HPLC techniques.
- Microbial transformation can be accomplished on a large scale by using large-scale fermenters.
- Specific biotransformation can be done directly with various enzymes.
- These purified compounds can further be tested for the enhancement of various biological activities such that antimicrobial properties, antioxidant, and antimutagenic activities.
- Recent studies with Stevia also allow the use of these sweet compounds in long-term infections therapies.
- After the successful biotransformation of compounds, these can be either directly used or in a purified form in industrial as well as pharmaceutical applications i.e., food, beverages, and medicines preparation for diabetic patients.

REFERENCES

1. Amien, S., Aji, D. N., & Mamluatul, T. (2020). Kecepatan Multiplikasi Tunas Tiga Aksesi Stevia (Stevia rebaudiana (Bertoni)) Secara In Vitro. *Kultivasi*, *19*(3). https://doi.org/10.24198/kultivasi.v19i3.29468
2. Chong Saw Peng, Akil, M. bin, & Bahari, N. binti. (2020). SEPARATION OF STEVIOL GLYCOSIDES FROM Stevia rebaudiana USING DIFFERENT AQUEOUS EXTRACTION TECHNIQUES. *Borneo International Journal of Biotechnology (BIJB)*, 77. https://doi.org/10.51200/bijb.vi.1240
3. Formigoni, M., Milani, P.G., da Silva Avíncola, A., dos Santos, V.J., Benossi, L., Dacome, A.S., Pilau, E.J. and da Costa, S.C. (2018). Pretreatment with ethanol as an alternative to improve steviol glycosides extraction and purification from a new variety of Stevia. *Food Chemistry*, [online] 241, pp.452–459. doi:10.1016/j.foodchem.2017.09.022.
4. Howlader, M. M. S., Ahmed, S. R., Kubra, K., & Bhuiyan, M. K. H. (2016). Biochemical and phytochemical evaluation of Stevia rebaudiana. *Asian Journal of Medical and Biological Research*, *2*(1), 121–130. https://doi.org/10.3329/ajmbr.v2i1.27577
5. https://www.globenewswire.com/news-release/2019/05/28/1853758/0/en/Stevia-Use-In-Food-and-Beverages-Accelerated-Significantly-in-2018.html
6. Mäkinen, K. K. (2016). Gastrointestinal Disturbances Associated with the Consumption of Sugar Alcohols with Special Consideration of Xylitol: Scientific Review and Instructions for Dentists and Other Health-Care Professionals. *International Journal of Dentistry*, *2016*, 1–16. https://doi.org/10.1155/2016/5967907
7. Musa, A., Miao, M., Zhang, T., & Jiang, B. (2014). Biotransformation of stevioside by Leuconostoc citreum SK24.002 alternansucrase acceptor reaction. *Food Chemistry*, *146*, 23–29. https://doi.org/10.1016/j.foodchem.2013.09.010
8. Myint, K. zar, Wu, K., Xia, Y., Fan, Y., Shen, J., Zhang, P., & Gu, J. (2020). Polyphenols from Stevia rebaudiana (Bertoni) leaves and their functional

properties. *Journal of Food Science*, *85*(2), 240–248. https://doi.org/10.1111/1750-3841.15017

9. O'Connor, R. (2020, October 12). *Benefits of Biotransformation in Building Innovative Food Products*. INewtrition - Raphaelle O'Connor. https://inewtrition.com/biotransformation-to-innovate-in-food/#:~:text=The%20benefits%20of%20biotransformation%20are
10. Pang, M. D., Goossens, G. H., & Blaak, E. E. (2020). The Impact of Artificial Sweeteners on Body Weight Control and Glucose Homeostasis. *Frontiers in Nutrition*, *7*, 598340. https://doi.org/10.3389/fnut.2020.598340
11. Purkayastha, S., Markosyan, A., Prakash, I., Bhusari, S., Pugh, G., Lynch, B., & Roberts, A. (2016). Steviol glycosides in purified Stevia leaf extract sharing the same metabolic fate. *Regulatory Toxicology and Pharmacology*, *77*, 125–133. https://doi.org/10.1016/j.yrtph.2016.02.015
12. Ray, J., Kumar, S., Laor, D., Shereen, N., Nwamaghinna, F., Thomson, A., Perez, J., Soni, L., & McFarlane, S. (2020). Effects of Stevia Rebaudiana on Glucose Homeostasis, Blood Pressure and Inflammation: A Critical Review of Past and Current Research Evidence. *International Journal of Clinical Research & Trials*, *5*(1). https://doi.org/10.15344/2456-8007/2020/142
13. Ruiz-Ruiz, J. C., Moguel-Ordoñez, Y. B., & Segura-Campos, M. R. (2017). Biological activity of *Stevia rebaudiana* Bertoni and their relationship to health. *Critical Reviews in Food Science and Nutrition*, *57*(12), 2680–2690. https://doi.org/10.1080/10408398.2015.1072083
14. Stevia Market | 2021 - 26 | Industry Share, Size, Growth - Mordor Intelligence. (2018). Retrieved from www.mordorintelligence.com website: https://www.mordorintelligence.com/industry-reports/Stevia-market
15. Sultana, N., & Saify, Z. S. (2012). Enzymatic biotransformation of terpenes as bioactive agents. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *28*(6), 1113–1128. https://doi.org/10.3109/14756366.2012.727411
16. TEJO, V. K., ADIYOGA, B., & ANANINGSIH, V. K. (2013). Utilization of Stevia rebaudina & Its Health Benefits. *International Conference on Environment and Health "Integrating Research Community Outreach and Service*

Learning." http://repository.unika.ac.id/15950/

17. Teo, C. C., Tan, S. N., Yong, J. W. H., Hew, C. S., & Ong, E. S. (2009). Validation of green-solvent extraction combined with chromatographic chemical fingerprint to evaluate quality of*Stevia rebaudiana*Bertoni. *Journal of Separation Science*, *32*(4), 613–622. https://doi.org/10.1002/jssc.200800552
18. Wang, Y., Chen, L., Li, Y., Li, Y., Yan, M., Chen, K., Hao, N., & Xu, L. (2016). Efficient enzymatic production of rebaudioside A from stevioside. *Bioscience, Biotechnology, and Biochemistry*, *80*(1), 67–73. https://doi.org/10.1080/09168451.2015.1072457
19. Ahmad, J., Khan, I., Blundell, R., Azzopardi, J., & Mahomoodally, M. F. (2020). Stevia rebaudiana Bertoni.: an updated review of its health benefits, industrial applications and safety. *Trends in Food Science & Technology*, *100*, 177–189. https://doi.org/10.1016/j.tifs.2020.04.030
20. Ahmad, U., Ahmad, R. S., Arshad, M. S., Mushtaq, Z., Hussain, S. M., & Hameed, A. (2018). Antihyperlipidemic efficacy of aqueous extract of Stevia rebaudiana Bertoni in albino rats. *Lipids in Health and Disease*, *17*, 175. https://doi.org/10.1186/s12944-018-0810-9
21. Ahmed, S., Shah, P., & Ahmed, O. (2021). *Biochemistry, Lipids*. PubMed; StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK525952
22. AL-Ishaq, R. K., Liskova, A., Kubatka, P., & Büsselberg, D. (2021). Enzymatic Metabolism of Flavonoids by Gut Microbiota and Its Impact on Gastrointestinal Cancer. *Cancers*, *13*(16), 3934. https://doi.org/10.3390/cancers13163934
23. Al-Taweel, S., Azzam, C., Khaled, K., & Abdel-Aziz, R. (2021). IMPROVEMENT OF STEVIA (Stevia rebaudiana BERTONI) AND STEVIOL GLYCOSIDE THROUGH TRADITIONAL BREEDING AND BIOTECHNOLOGICAL APPROACHES. *SABRAO Journal of Breeding and Genetics*, *53*(1), 88–111. http://sabraojournal.org/wp-content/uploads/2021/03/SABRAO-J-Breed-Genet-53-1-88-111-AL-TAWEEL-Review.pdf
24. Anvari, M., & Khayati, G. (2016). Separation and purification of rebaudioside A from extract of *Stevia Rebaudiana* leaves by macroporous adsorption resins. *Polish Journal of Chemical Technology*, *18*(1), 127–132. https://doi.org/10.1515/pjct-

2016-0019

25. Ashwell, M. (2015). Stevia, Nature's Zero-Calorie Sustainable Sweetener. *Nutrition Today*, *50*(3), 129–134. https://doi.org/10.1097/nt.0000000000000094
26. Baliyan, S., Mukherjee, R., Priyadarshini, A., Vibhuti, A., Gupta, A., Pandey, R. P., & Chang, C.-M. (2022). Determination of Antioxidants by DPPH Radical Scavenging Activity and Quantitative Phytochemical Analysis of Ficus religiosa. *Molecules*, *27*(4), 1326. https://doi.org/10.3390/molecules27041326
27. Barbet-Massin, C., Giuliano, S., Alletto, L., Daydé, J., & Berger, M. (2015). Towards a semi-perennial culture of Stevia rebaudiana (Bertoni) Bertoni under temperate climate: effects of genotype, environment and plant age on steviol glycoside content and composition. *Genetic Resources and Crop Evolution*, *63*(4), 685–694. https://doi.org/10.1007/s10722-015-0276-9
28. BARBUT, S. (2010). COLOR DEVELOPMENT DURING NATURAL FERMENTATION AND CHEMICAL ACIDIFICATION OF SALAMI-TYPE PRODUCTS. *Journal of Muscle Foods*, *21*(3), 499–508. https://doi.org/10.1111/j.1745-4573.2009.00198.x
29. Basharat, S., Huang, Z., Gong, M., Lv, X., Ahmed, A., Hussain, I., Li, J., Du, G., & Liu, L. (2021). A review on current conventional and biotechnical approaches to enhance biosynthesis of steviol glycosides in Stevia rebaudiana. *Chinese Journal of Chemical Engineering*, *30*, 92–104. https://doi.org/10.1016/j.cjche.2020.10.018
30. Bele, A., Khale, A., Archana, M. and Bele, A. (2011). AN OVERVIEW ON THIN LAYER CHROMATOGRAPHY. *IJPSR*, [online] 2(2), pp.256–267. Available at: https://citeseerx.ist.psu.edu/document?repid=rep1&type=pdf&doi=974c337508d22ee7219c0cafb2ed4d4db2c1c630.
31. Bender, C. (2018). Stevia Rebaudiana's Antioxidant Properties. *Reference Series in Phytochemistry*, 349–375. https://doi.org/10.1007/978-3-319-27027-2_6
32. Bernal, J., Mendiola, J., Ibáñez, E., & Cifuentes, A. (2011). Advanced analysis of nutraceuticals. *Journal of Pharmaceutical and Biomedical Analysis*. https://doi.org/10.1016/j.jpba.2010.11.033
33. Boniface, P. K., & Ferreira, E. I. (2019). Flavonoids as efficient scaffolds: Recent

trends for malaria, leishmaniasis, Chagas disease, and dengue. *Phytotherapy Research*, *33*(10), 2473–2517. https://doi.org/10.1002/ptr.6383

34. Boniface, P. K., & Ferreira, E. I. (2019). Flavonoids as efficient scaffolds: Recent trends for malaria, leishmaniasis, Chagas disease, and dengue. *Phytotherapy Research*, *33*(10), 2473–2517. https://doi.org/10.1002/ptr.6383
35. Borgo, J., Laurella, L. C., Martini, F., Catalán, C. A. N., & Sülsen, V. P. (2021). Stevia Genus: Phytochemistry and Biological Activities Update. *Molecules*, *26*(9), 2733. https://doi.org/10.3390/molecules26092733
36. Cantabella, D., Piqueras, A., Acosta-Motos, J. R., Bernal-Vicente, A., Hernández, J. A., & Díaz-Vivancos, P. (2017). Salt-tolerance mechanisms induced in Stevia rebaudiana Bertoni: Effects on mineral nutrition, antioxidative metabolism and steviol glycoside content. *Plant Physiology and Biochemistry*, *115*, 484–496. https://doi.org/10.1016/j.plaphy.2017.04.023
37. Ceunen, S. and Geuns, J.M.C. (2013). Steviol Glycosides: Chemical Diversity, Metabolism, and Function. *Journal of Natural Products*, 76(6), pp.1201–1228. doi:https://doi.org/10.1021/np400203b.
38. Chaturvedula, V. S. P., Clos, J. F., Rhea, J., Milanowski, D., Mocek, U., DuBois, G. E., & Prakash, I. (2011). Minor diterpenoid glycosides from the leaves of Stevia rebaudiana. *Phytochemistry Letters*, *4*(3), 209–212. https://doi.org/10.1016/j.phytol.2011.01.002
39. Chaturvedula, V.S.P., Mubarak, C. and Prakash, I. (2012). IR Spectral Analysis of Diterpene Glycosides Isolated from Stevia rebaudiana. *Food and Nutrition Sciences*, 03(10), pp.1467–1471. doi:https://doi.org/10.4236/fns.2012.310191.
40. Chong Saw Peng, Akil, M. bin, & Bahari, N. binti. (2020). SEPARATION OF STEVIOL GLYCOSIDES FROM Stevia rebaudiana USING DIFFERENT AQUEOUS EXTRACTION TECHNIQUES. *Borneo International Journal of Biotechnology (BIJB)*, 77. https://doi.org/10.51200/bijb.vi.1240
41. Chranioti, C., Chanioti, S., & Tzia, C. (2016). Comparison of spray, freeze and oven drying as a means of reducing bitter aftertaste of steviol glycosides (derived from Stevia rebaudiana Bertoni plant) – Evaluation of the final products. *Food Chemistry*, *190*, 1151–1158. https://doi.org/10.1016/j.foodchem.2015.06.083

42. Ebbeling, C. B., Feldman, H. A., Steltz, S. K., Quinn, N. L., Robinson, L. M., & Ludwig, D. S. (2020). Effects of Sugar-Sweetened, Artificially Sweetened, and Unsweetened Beverages on Cardiometabolic Risk Factors, Body Composition, and Sweet Taste Preference: A Randomized Controlled Trial. *Journal of the American Heart Association*, *9*(15). https://doi.org/10.1161/jaha.119.015668
43. Eggleston, G. (2019). History of Sugar and Sweeteners. *ACS Symposium Series*, 63–74. https://doi.org/10.1021/bk-2019-1314.ch005
44. Enright, R., Miljkovic, N., Alvarado, J.L., Kim, K. and Rose, J.W. (2014). Dropwise Condensation on Micro- and Nanostructured Surfaces. *Nanoscale and Microscale Thermophysical Engineering*, 18(3), pp.223–250. doi:10.1080/15567265.2013.862889.
45. Fatima, S. (2017). PRELIMINARY PHYTOCHEMICAL SCREENING AND HPTLC FINGERPRINT ANALYSIS OF CHLOROFORM EXTRACT OF POLYHERBAL FORMULATION. *World Journal of Pharmacy and Pharmaceutical Sciences*, 2389–2395. https://doi.org/10.20959/wjpps20178-9780
46. Garcia, E. J., Oldoni, T. L. C., Alencar, S. M. de, Reis, A., Loguercio, A. D., & Grande, R. H. M. (2012). Antioxidant activity by DPPH assay of potential solutions to be applied on bleached teeth. *Brazilian Dental Journal*, *23*(1), 22–27. https://doi.org/10.1590/s0103-64402012000100004
47. Gasmalla, M., Yang, R., Amadou, I., & Hua, X. (2014). Nutritional Composition of *Stevia rebaudiana* Bertoni Leaf: Effect of Drying Method. *Tropical Journal of Pharmaceutical Research*, *13*(1), 61. https://doi.org/10.4314/tjpr.v13i1.9
48. George Thompson, A. M., Iancu, C. V., Nguyen, T. T. H., Kim, D., & Choe, J. (2015). Inhibition of human GLUT1 and GLUT5 by plant carbohydrate products; insights into transport specificity. *Scientific Reports*, *5*(1). https://doi.org/10.1038/srep12804
49. Gerwig, G. J., te Poele, E. M., Dijkhuizen, L., & Kamerling, J. P. (2016, January 1). *Chapter One - Stevia Glycosides: Chemical and Enzymatic Modifications of Their Carbohydrate Moieties to Improve the Sweet-Tasting Quality* (D. C. Baker, Ed.). ScienceDirect; Academic Press. https://www.sciencedirect.com/science/article/abs/pii/S0065231816300014

50. Goyal, S. K., Samsher, & Goyal, R. K. (2009). Stevia (Stevia rebaudiana) a bio-sweetener: a review. *International Journal of Food Sciences and Nutrition*, *61*(1), 1–10. https://doi.org/10.3109/09637480903193049

51. Grillo, A., Salvi, L., Coruzzi, P., Salvi, P., & Parati, G. (2019). Sodium Intake and Hypertension. *Nutrients*, *11*(9). https://doi.org/10.3390/nu11091970

52. GUPTA, E., PURWAR, S., SUNDARAM, S., TRIPATHI, P., & RAI, G. (2016). Stevioside and Rebaudioside A – Predominant Ent-Kaurene Diterpene Glycosides of Therapeutic Potential: a Review. *Czech Journal of Food Sciences*, *34*(No. 4), 281–299. https://doi.org/10.17221/335/2015-cjfs

53. Hajihashemi, S. (2017). Physiological, biochemical, antioxidant and growth characterizations of gibberellin and paclobutrazol-treated sweet leaf (Stevia rebaudiana B.) herb. *Journal of Plant Biochemistry and Biotechnology*. https://doi.org/10.1007/s13562-017-0428-4

54. Hettinga, K. A. (2019, January 1). *Chapter 6 - Lactose in the dairy production chain* (M. Paques & C. Lindner, Eds.). ScienceDirect; Academic Press. https://www.sciencedirect.com/science/article/pii/B9780128117200000064

55. Huang, X.-Y., Fu, J.-F., & Di, D.-L. (2010). Preparative isolation and purification of steviol glycosides from Stevia rebaudiana Bertoni using high-speed counter-current chromatography. *Separation and Purification Technology*, *71*(2), 220–224. https://doi.org/10.1016/j.seppur.2009.11.025

56. Ibrahim, N. A., El-Gengaihi, S., Motawe, H., & Riad, S. A. (2006). Phytochemical and biological investigation of Stevia rebaudiana Bertoni; 1-labdane-type diterpene. *European Food Research and Technology*, *224*(4), 483–488. https://doi.org/10.1007/s00217-006-0400-3

57. Jaitak, V., Bandna, B. S., & 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. https://doi.org/10.1002/pca.1120

58. Jo, H.-J., Kim, J.-H., Kim, Y.-N., Seo, P.-W., Kim, C.-Y., Kim, J.-W., Yu, H., Cheon, H., Yeol Lee, E., Kim, J.-S., & Park, J.-B. (2022). Glyoxylate carboligase-based whole-cell biotransformation of formaldehyde into ethylene glycol via

glycolaldehyde. *Green Chemistry*, *24*(1), 218–226. https://doi.org/10.1039/D1GC03549E

59. Kakigi, Y., Suzuki, T., Icho, T., Uyama, A., & Mochizuki, N. (2013). Classification of Stevia sweeteners in soft drinks using liquid chromatography and time-of-flight mass spectrometry. *Food Additives & Contaminants: Part A*, *30*(12), 2043–2049. https://doi.org/10.1080/19440049.2013.843101
60. Karimi, M., Ahmadi, A., Hashemi, J., Abbasi, A., & Angelini, L. G. (2014). Effect of two plant growth retardants on steviol glycosides content and antioxidant capacity in Stevia (Stevia rebaudiana Bertoni). *Acta Physiologiae Plantarum*, *36*(5), 1211–1219. https://doi.org/10.1007/s11738-014-1498-8
61. Kaushik, R., Narayanan, P., Vasudevan, V., Muthukumaran, G., & Usha, A. (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. https://doi.org/10.1007/s13197-010-0011-7
62. Khattab, S. N., Massoud, M. I., Abd El-Razek, A. M., & El-Faham, A. (2017). Physico-chemical and sensory characteristics of steviolbioside synthesized from stevioside and its application in fruit drinks and food. *Journal of Food Science and Technology*, *54*(1), 185–195. https://doi.org/10.1007/s13197-016-2450-2
63. Kim, M. J., Zheng, J., Liao, M. H., & Jang, I. (2018). Overexpression of Sr UGT 76G1 in Stevia alters major steviol glycosides composition towards improved quality. *Plant Biotechnology Journal*, *17*(6), 1037–1047. https://doi.org/10.1111/pbi.13035
64. Koubaa, M., Roselló-Soto, E., Šic Žlabur, J., Režek Jambrak, A., Brnčić, M., Grimi, N., Boussetta, N., & Barba, F. J. (2015). Current and New Insights in the Sustainable and Green Recovery of Nutritionally Valuable Compounds from Stevia rebaudiana Bertoni. *Journal of Agricultural and Food Chemistry*, *63*(31), 6835–6846. https://doi.org/10.1021/acs.jafc.5b01994
65. Kumari, N., Ranac, R. C., Sharma, Y. P., & Kumar, S. (2017). Extraction, Purification, and Analysis of Sweet Compounds in Stevia rebaudiana Bertoni using Chromatographic Techniques. *Indian Journal of Pharmaceutical Sciences*, *79*(4).

https://doi.org/10.4172/pharmaceutical-sciences.1000270

66. Lall, S. P. (2022, January 1). *Chapter 6 - The minerals* (R. W. Hardy & S. J. Kaushik, Eds.). ScienceDirect; Academic Press. https://www.sciencedirect.com/science/article/pii/B9780128195871000057
67. Lemus-Mondaca, R., Vega-Gálvez, A., Zura-Bravo, L. and Ah-Hen, K. (2012). Stevia rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. *Food Chemistry*, 132(3), pp.1121–1132. doi:https://doi.org/10.1016/j.foodchem.2011.11.140.
68. Lemus-Mondaca, R., Vega-Gálvez, A., Zura-Bravo, L., & Ah-Hen, K. (2012). Stevia rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. *Food Chemistry*, *132*(3), 1121–1132. https://doi.org/10.1016/j.foodchem.2011.11.140
69. Lemus-Mondaca, R., Vega-Gálvez, A., Zura-Bravo, L., & Ah-Hen, K. (2012). Stevia rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. *Food Chemistry*, *132*(3), 1121–1132. https://doi.org/10.1016/j.foodchem.2011.11.140
70. Madeira Junior, J. V., Teixeira, C. B., & Macedo, G. A. (2013). Biotransformation and bioconversion of phenolic compounds obtainment: an overview. *Critical Reviews in Biotechnology*, *35*(1), 75–81. https://doi.org/10.3109/07388551.2013.803020
71. Mahmud, S., Akter, S., Jahan, I., Khan, S., Khaleque, A., & Islam, S. (2015). Comparative analyses of stevioside between fresh leaves and in-vitro derived callus tissue from Stevia rebaudiana Bert. using HPLC. *Bangladesh Journal of Scientific and Industrial Research*, *49*(4), 199–204. https://doi.org/10.3329/bjsir.v49i4.22621
72. Marasinghege, C., Rackemann, D., & Doherty, W. (2020). *Impacts of superheated steam on juice degradation and evaporator performance* (P. Allsopp, Ed.). Eprints.qut.edu.au; Australian Society of Sugar Cane Technologists - ASSCT. https://eprints.qut.edu.au/205169/
73. Martínez-Rojo, E., Cariño-Cortés, R., Berumen, L. C., García-Alcocer, G., &

Escobar-Cabrera, J. (2020). Stevia Eupatoria and Stevia Pilosa Extracts Inhibit the Proliferation and Migration of Prostate Cancer Cells. *Medicina*, *56*(2), 90. https://doi.org/10.3390/medicina56020090

74. Mazumdar, S. (2020). Sugar and Society in China: Peasants, Technology, and the World Market. In *Google Books*. BRILL. https://books.google.com.pk/books?hl=en&lr=&id=rfYFEAAAQBAJ&oi=fnd&pg=PR3&dq=history+of+sugar+uses&ots=8vEsQoHn7b&sig=IMyBlCnh-N3VRpT1alBFNFeLGmk&redir_esc=y#v=onepage&q=history%20of%20sugar%20uses&f=false
75. Meynet, P., Davenport, R.J. and Fenner, K. (2020). Understanding the Dependence of Micropollutant Biotransformation Rates on Short-Term Temperature Shifts. *Environmental Science & Technology*, 54(19), pp.12214–12225. doi:10.1021/acs.est.0c04017.
76. Moongngarm, A., Sriharboot, N., Loypimai, P., & Moontree, T. (2022). Ohmic heating assisted water extraction of steviol glycosides and phytochemicals from Stevia rebaudiana leaves. *LWT*, *154*, 112798. https://doi.org/10.1016/j.lwt.2021.112798
77. Mroczyńska, M., & Libudzisz, Z. (2010). Beta-glucuronidase and beta-glucosidase activity of Lactobacillus and Enterococcus isolated from human feces. *Polish Journal of Microbiology / Polskie Towarzystwo Mikrobiologow = The Polish Society of Microbiologists*, *59*(4), 265–269.
78. Naknean, P., & Meenune, M. (2010). *International Food Research Journal*, *17*, 23–34. http://ifrj.upm.edu.my/17%20(01)%202010/(3)%20IFRJ-2010-23-34%20Mutita%20Thailand.pdf
79. Narayanan, M., Kumarasamy, S., Ranganathan, M., Kandasamy, S., Kandasamy, G., & Gnanavel, K. (2020). Enzyme and metabolites attained in degradation of chemical pesticides ß Cypermethrin by Bacillus cereus. *Materials Today: Proceedings*, *33*, 3640–3645. https://doi.org/10.1016/j.matpr.2020.05.722
80. Naumoff, D. G. (2011). Hierarchical classification of glycoside hydrolases. *Biochemistry (Moscow)*, *76*(6), 622–635. https://doi.org/10.1134/S0006297911060022

81. Obodovskiy, I. (2019, January 1). *Chapter 33 - Basics of Biochemistry* (I. Obodovskiy, Ed.). ScienceDirect; Elsevier. https://www.sciencedirect.com/science/article/pii/B9780444639790000331

82. Ohta, M., Sasa, S., Inoue, A., Tamai, T., Fujita, I., Morita, K., & Matsuura, F. (2010). Characterization of Novel Steviol Glycosides from Leaves of Stevia rebaudiana Morita. *Journal of Applied Glycoscience*, *57*(3), 199–209. https://doi.org/10.5458/jag.57.199

83. Pallarés, Á., Carrasco, G., Nava, Y., Pallarés, O., Pérez, I., Rifá, R. and Rodríguez, M. (2015). Effectiveness and safety of Stevia rebaudiana dried leaves as an adjuvant in the short-term treatment of type 2 diabetes: A randomized, controlled, cross-over and double-blinded trial. *JMPHTR*, [online] 3, pp.16–26. Available at: http://www.bluepenjournals.org/jmphtr/pdf/2015/June/Pallares_et_al.pdf

84. Perera, W. H., & McChesney, J. D. (2021). Approaches toward the Separation, Modification, Identification and Scale up Purification of Tetracyclic Diterpene Glycosides from Stevia rebaudiana (Bertoni) Bertoni. *Molecules*, *26*(7), 1915. https://doi.org/10.3390/molecules26071915

85. Peteliuk, V., Rybchuk, L., Bayliak, M., Storey, K. B., & Lushchak, O. (2021). Natural sweetener Stevia rebaudiana: Functionalities, health benefits and potential risks. *EXCLI Journal*, *20*, 1412–1430. https://doi.org/10.17179/excli2021-4211

86. Phang-Lyn, S. and Llerena, V.A. (2020). *Biochemistry, Biotransformation*. [online] PubMed. Available at: https://www.ncbi.nlm.nih.gov/books/NBK544353/.

87. Pól, J., Varaďová OstráE., 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. https://doi.org/10.1007/s00216-007-1404-y

88. Prakash, I., Chaturvedula, V. S. P., & Markosyan, A. (2014). Structural Characterization of the Degradation Products of a Minor Natural Sweet Diterpene Glycoside Rebaudioside M under Acidic Conditions. *International Journal of Molecular Sciences*, *15*(1), 1014–1025. https://doi.org/10.3390/ijms15011014

89. Ravindran, S., & Basu, S. (2013). Significance of Biotransformation in Drug Discovery and Development. *Journal of Biotechnology & Biomaterials, s13*. https://doi.org/10.4172/2155-952x.s13-005

90. Rogers, P. J., Hogenkamp, P. S., de Graaf, C., Higgs, S., Lluch, A., Ness, A. R., Penfold, C., Perry, R., Putz, P., Yeomans, M. R., & Mela, D. J. (2015). Does low-energy sweetener consumption affect energy intake and body weight? A systematic review, including meta-analyses, of the evidence from human and animal studies. *International Journal of Obesity*, *40*(3), 381–394. https://doi.org/10.1038/ijo.2015.177

91. Ruiz-Ojeda, F. J., Plaza-Díaz, J., Sáez-Lara, M. J., & Gil, A. (2019). Effects of Sweeteners on the Gut Microbiota: A Review of Experimental Studies and Clinical Trials. *Advances in Nutrition*, *10*(suppl_1), S31–S48. https://doi.org/10.1093/advances/nmy037

92. Savita, S. M., Sheela, K., Sunanda, S., Shankar, A. G., & Ramakrishna, P. (2004). Stevia rebaudiana – A Functional Component for Food Industry. *Journal of Human Ecology*, *15*(4), 261–264. https://doi.org/10.1080/09709274.2004.11905703

93. Schmiermund, T. (2022). pH Value. *The Chemistry Knowledge for Firefighters*, 231–249. https://doi.org/10.1007/978-3-662-64423-2_14

94. Segura-Campos, M., Barbosa-Martín, E., Matus-Basto, Á., Cabrera-Amaro, D., Murguía-Olmedo, M., Moguel-Ordoñez, Y., & Betancur-Ancona, D. (2014). Comparison of Chemical and Functional Properties of Stevia rebaudiana (Bertoni) Varieties Cultivated in Mexican Southeast. *American Journal of Plant Sciences*, *5*(3), 286–293. https://doi.org/10.4236/ajps.2014.53039

95. Shamima, K. A. A., Hoque, Md. A., & Bhuiyan, M. N. I. (2019). Isolation of Stevioside and related compounds from two types of Stevia rebaudiana (Bertoni) species from Bangladesh. *International Journal of Chemistry, Mathematics and Physics*, *3*(5), 95–99. https://doi.org/10.22161/ijcmp.3.5.1

96. Shivanna, N., Naika, M., Khanum, F., & Kaul, V. K. (2013). Antioxidant, anti-diabetic and renal protective properties of Stevia rebaudiana. *Journal of Diabetes and Its Complications*, *27*(2), 103–113. https://doi.org/10.1016/j.jdiacomp.2012.10.001

97. Shukla, S., Mehta, A., Mehta, P., & Bajpai, V. K. (2012). Antioxidant ability and total phenolic content of aqueous leaf extract of Stevia rebaudiana Bert. *Experimental and Toxicologic Pathology*, *64*(7-8), 807–811. https://doi.org/10.1016/j.etp.2011.02.002

98. Shukla, S., Mehta, A., Mehta, P., & Bajpai, V. K. (2012). Antioxidant ability and total phenolic content of aqueous leaf extract of Stevia rebaudiana Bert. *Experimental and Toxicologic Pathology*, *64*(7-8), 807–811. https://doi.org/10.1016/j.etp.2011.02.002

99. Singh, R. (2017). Microbial Biotransformation: A Process for Chemical Alterations. *Journal of Bacteriology & Mycology: Open Access*, [online] 4(2). doi:10.15406/jbmoa.2017.04.00085.

100. Singh, R. (2017). Microbial Biotransformation: A Process for Chemical Alterations. *Journal of Bacteriology & Mycology: Open Access*, *4*(2). https://doi.org/10.15406/jbmoa.2017.04.00085

101. Slavin, J., & Carlson, J. (2014). Carbohydrates. *Advances in Nutrition*, *5*(6), 760–761. https://doi.org/10.3945/an.114.006163

102. Słoczyńska, K., Powroźnik, B., Pękala, E., & Waszkielewicz, A. M. (2014). Antimutagenic compounds and their possible mechanisms of action. *Journal of Applied Genetics*, *55*(2), 273–285. https://doi.org/10.1007/s13353-014-0198-9

103. *Stevia Market Size, Trends and Global Forecast To 2032*. (2022). Www.thebusinessresearchcompany.com. Retrieved February 5, 2023, from https://www.thebusinessresearchcompany.com/report/Stevia-global-market-report#:~:text=The%20global%20Stevia%20market%20size

104. Sultana, N. (2018). Microbial biotransformation of bioactive and clinically useful steroids and some salient features of steroids and biotransformation. *Steroids*, *136*, 76–92. https://doi.org/10.1016/j.steroids.2018.01.007

105. Tresserra-Rimbau, A., Rimm, E.B., Medina-Remón, A., Martínez-González, M.A., López-Sabater, M.C., Covas, M.I., Corella, D., Salas-Salvadó, J., Gómez-Gracia, E., Lapetra, J., Arós, F., Fiol, M., Ros, E., Serra-Majem, L., Pintó, X., Muñoz, M.A., Gea, A., Ruiz-Gutiérrez, V., Estruch, R. and Lamuela-Raventós,

R.M. (2014). Polyphenol intake and mortality risk: a re-analysis of the PREDIMED trial. *BMC Medicine*, 12(1). doi:10.1186/1741-7015-12-77.

106. Urban, J. D., Carakostas, M. C., & Taylor, S. L. (2015). Steviol glycoside safety: Are highly purified steviol glycoside sweeteners food allergens? *Food and Chemical Toxicology*, *75*, 71–78. https://doi.org/10.1016/j.fct.2014.11.011

107. Van Hofwegen, D. J., Hovde, C. J., & Minnich, S. A. (2016). Rapid Evolution of Citrate Utilization by Escherichia coli by Direct Selection RequirescitTanddctA. *Journal of Bacteriology*, *198*(7), 1022–1034. https://doi.org/10.1128/jb.00831-15

108. Vanzan, L., Sklias, A., Herceg, Z., & Murr, R. (2017, January 1). *Chapter 3 - Mechanisms of Histone Modifications* (T. O. Tollefsbol, Ed.). ScienceDirect; Academic Press. https://www.sciencedirect.com/science/article/pii/B9780128053881000031

109. Verma, M., Kumar, N., & Kumar Verma, M. (2019). A review on the pharmacological properties and medicinal use of Stevia rebaudiana. ~ *371* ~ *the Pharma Innovation Journal*, *8*(1), 371–374. https://www.thepharmajournal.com/archives/2019/vol8issue1/PartG/8-1-124-152.pdf

110. Wang, M., Li, H., Xu, F., Gao, X., Li, J., Xu, S., Zhang, D., Wu, X., Xu, J., Hua, H., & Li, D. (2018). Diterpenoid lead stevioside and its hydrolysis products steviol and isosteviol: Biological activity and structural modification. *European Journal of Medicinal Chemistry*, *156*, 885–906. https://doi.org/10.1016/j.ejmech.2018.07.052

111. Ware, M. (2018). *Antioxidants: Health benefits and nutritional information*. Www.medicalnewstoday.com. https://www.medicalnewstoday.com/articles/301506

112. Well, C., Frank, O., & Hofmann, T. (2013). Quantitation of Sweet Steviol Glycosides by Means of a HILIC-MS/MS-SIDA Approach. *Journal of Agricultural and Food Chemistry*, *61*(47), 11312–11320. https://doi.org/10.1021/jf404018g

113. Wölwer-Rieck, U. (2012). The Leaves of Stevia rebaudiana (Bertoni), Their Constituents and the Analyses Thereof: A Review. *Journal of Agricultural and*

Food Chemistry, *60*(4), 886–895. https://doi.org/10.1021/jf2044907

114. YALÇIN, V., TORUN, H., EROĞLU, E., & USTA, E. Ü. (2022). Physiological and Biochemical Effects of Drought Stress in Stevia (Stevia rebaudiana Bertoni). *Düzce Üniversitesi Bilim ve Teknoloji Dergisi*, 1165–1176. https://doi.org/10.29130/dubited.1109502

115. Yan, Z., Cao, X., Yang, X., Yang, S., Xu, L., Jiang, X., & Xiao, M. (2021). A Novel β-Glucosidase From Chryseobacterium scophthalmum 1433 for Efficient Rubusoside Production From Stevioside. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.744914

116. Zang, H., Wang, H., Miao, L., Cheng, Y., Zhang, Y., Liu, Y., Sun, S., Wang, Y. and Li, C. (2020). Carboxylesterase, a de-esterification enzyme, catalyzes the degradation of chlorimuron-ethyl in Rhodococcus erythropolis D310-1. *Journal of Hazardous Materials*, 387, p.121684. doi:10.1016/j.jhazmat.2019.121684.

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