OPTIMIZATION OF AQUEOUS EXTRACT OF STEVIA LEAVES AND ISOLATION OF STEVIOSIDE FROM THE EXTRACT

A thesis

by

MD. MILLATUN MOMIN
Student ID.: 1805109

Session: 2018-19

MASTERS OF SCIENCE (MS)

in

BIOCHEMISTRY AND MOLECULAR BIOLOGY

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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Submitted to the Department of Biochemistry and Molecular Biology
Hajee Mohammad Danesh Science and Technology University, Dinajpur
Bangladesh, in partial fulfillment of the requirement for the degree of

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TO MY

BELOVED PARENTS

Acknowledgement

All praises for "Almighty Allah" the Omnipresent, Omnipotent, Omniscient, most gracious, most merciful and the supreme ruler of the universe Who has blessed me with life, time and energy and enable me to complete this research work and manuscript for the degree of Master of Science (MS) in Biochemistry and Molecular Biology.

I would like to thank with heartfelt respect and the profound gratitude and indebtedness to my respected teacher and research supervisor Dr. Md. Yeasin Prodhan, Assistant Professor, Department of Biochemistry and Molecular Biology, Hajee Mohammad Danesh Science and Technology University, Dinajpur for her invaluable guidance, helpful comments, suggestions, inspiration, providing facilities and supports needed throughout the research project.

I would like to thank with profound respect, gratitude and indebtedness to my co-supervisor Dr. Mst. Nur-E-Nazmun Nahar, Associate Professor, Department of Biochemistry and Molecular Biology, Hajee Mohammad Danesh Science and Technology University, Dinajpur for his kind help, cordial guidance, valuable advice, helpful comments, constructive criticism, effective instructions and continuous encouragements to accomplish the research work as well preparation of thesis.

The door to Dr. Prodhan and Dr. Mst. Nur-E-Nazmun Nahar office were always open whenever I faced any problem or had a question about my research work or thesis writing. They consistently allowed me to do my own work, but steered me in the right direction whenever they thought I needed it.

I would also like to thank all honorable teachers of the Department of Biochemistry and Molecular Biology, Hajee Mohammad Danesh Science and Technology University, Dinajpur for their valuable advice and continuous encouragement to accomplish the research work as well as preparation of this thesis.

I would like to thank the Institute of Research and Training, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh for funding to conduct my research work.

Last but not the least, I would like to thank my Parents Md. Jamal Uddin, Most. Late Minara Begume and my elder brother Md. Monjurul Hasan for supporting me throughout my life.

The Author

June, 2019

ABSTRACT

A sweet component of the stevia plant (*Stevia rebaudiana*), stevioside, is used as a natural sweetener in the world. Stevia leaf contains well-characterized steviol glycosides such as stevioside which is 250 to 300 times sweeter than sucrose. Stevia has a negligible effect on blood glucose. The long-term use of artificial sweeteners such as cyclamate and saccharine have deleterious side effect but stevia is the safest alternative and is being preferred by the diabetic patients all over the world. In developed countries, pure stevioside is used as a food additive. The preparation of pure stevioside is based on complex chemical extraction process which is costly. Stevioside is water soluble and it can be extracted by water. Therefore, here we aimed to prepare a suitable aqueous extract of stevia leaf for using in the foods. Aqueous extraction of stevia is a non-chemical and economically feasible method. To do this, we selected aqueous extract based on different extraction conditions. In general, stevia extract gives a bitter taste due to the presence of pigments in the extracts. The release of stevioside and the release of pigments in the extract depend on the forms of leaf, time, temperature, and leaf volume etc. Firstly, we prepared stevia aqueous extract from dry whole leaf, leaf powder, and fresh leaf. We measured the pigment contents (total chlorophyll and carotenoid) of the extract and tested the sweetness sensitization on tongue of the extracts. Results showed that dry whole leaf released the less pigments in the extract. We also prepared the stevia extract to different time exposures such as 20 min, 40 min, 60 min, 80 min and 100 min. We determined the pigment contents of the extract and tested the sweetness sensitization on tongue of the extracts. We found that leaf extract prepared at 40 min contained less pigments and more sweetness on tongue. Next, we prepared the stevia aqueous extract exposed to different temperatures such as 20^0C, 40^0C, 60^0C, 80^0C and 100^0C. We measured the pigment contents of the extract and tested the sweetness sensitization on tongue. We found that leaf extract prepared at 60^0C contained less pigments and more sweetness on tongue. We also prepared the stevia extract using different solvent volumes such as 20 ml, 40 ml, 60 ml, 80 ml and 100 ml. The results of pigment analysis and sweetness sensitization on tongue test revealed that leaf and solvent ratio 1:40 was the best. Based on the above results we concluded that water extraction of stevia dry whole leaf at 60^0C for 40 min using leaf: solvent (1:40) were the best aqueous extraction method. In a second attempt, we isolated stevioside from our optimized-aqueous extract. Then we use isobutanol to reduce the impurities of the extracts and we found white powder at the end. To confirm the presence of stevioside in the extracts we conducted FTIR analysis of white powder. FTIR analysis revealed the similar peaks of our white powder and the standard. However, some impurities were present in isolated stevioside. Therefore, further purification is needed to get more purified stevioside.

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ABBEREVIATIONS

%	= Percent
µM	= Micro molar
^{0}C	= Degree Celsius
A	= Absorbance
Agric.	= Agriculture
Chl (a+b)	= Total chlorophyll
Chl	= Chlorophyll
Chl a	= Chlorophyll a
Chl b	= Chlorophyll b
cm	= Centimeter
cm^{-1}	= Per centimeter
e.g.	= For example
et al.	= and others
FTIR	= Fourier-transform infrared spectroscopy
HSTU	= Hajee Mohammad Danesh Science and Technology University
i.e.	= In other words
Int.	= International
J.	= Journal
km	= Kilometer
mg/L	= Mili Gram per liter.
Min	= Minute
ml	= Milliliter
NIRS	= Near-Infrared reflectance spectroscopy
Res.	= Research
RSD	= Relative Standard Deviation
SA	= Stevia anisostemma
SD	= Stevia dianthoidea

ABBEREVIATIONS (con't.)

SGs	= Steviol glycosides
SL	= Stevia Leaf
SM	= steviamicrantha
SP	= Stevia Phlebophylla
SPE	= solid-phase extraction
SRB	= Stevia rebaudiana Bertoni
Univ.	= University
wt.	= Weight
μg g⁻1 DW	= Microgram per gram dry weight

CHAPTER I

INTRODUCTION

Stevia (*Stevia rebaudiana Bertoni)* is a sweet herb indigenous to Paraguay. The stevia leaves are sweet in the test. The plant contains a mixture of eight diterpene glycosides commonly called steviol glycosides. The concentration of which depends on the genetic constitution of an individual organism and environment, Brandle and Telmer, et al., (2007). Additionally, the sweetener should fulfill the requirements relating to non-toxic nature, sugar-like taste profile, low calorific value, heat, and pH stability. In recent years, there has been considerable interest in stevia-based natural sweeteners that possess many desired qualities Leung and Foster, et al., (1996).

This natural sweetness has been up to be 300 times sweeter than sucrose on weight basis, Richman et al., (1999). Stevia leaves contain an average of 10% of stevioside. Stevia leaves have been using for hundreds of years in Paraguay and Brazil to sweeten local teas and traditional medicines. Stevia also is known as a honey leaf, sweet leaf, sweet herb, and candy leaves Carakostas et al., (2008). Stevia is more than 200 species of the genus stevia, only *S. rebaudiana* gives the sweetest essence Savita et al., (2004). Stevia has been approved for several years in Brazil, Argentina, and Paraguay, as well as in China, Korea, and Japan to sweeten soft drinks, soy sauce, yogurt, and other foods. The United States used as a dietary supplement since 1995. Stevia has been used in Asia and Europe for recent years. Only in the past couple of years that it has really started to capture attention in the Indian market as a healthy alternative sweetener to sugar. Stevia has no calcium cyclamate, no saccharin, no aspartame, and no calories. So it is safe for diabetics and does not affect blood sugar levels. It does not have the neurological or renal side effects associated with some of the artificial sweeteners. The main advantage of stevioside, that is

stable at 100°C, Buckenhuskers and Omran, et al., (1997). As a natural non-caloric sweetener, the stevioside has been used to a variety of foods including seafood, pickled vegetables, dessert items, soft drinks, and confectionary for decades, Geuns, et al., (2004). The leaves of the plant have been used for centuries in the treatment of diabetes as traditional medicine. In recent years especially in developing countries, where diet sensible consumers look for a natural non-caloric sweetener as an alternative to chemical sweeteners. Several experimental studies of natural sweetener on animals and human, the volunteers have indicated the anti-hyperglycemic and insulinotropic effects of stevioside. Chan et al., (1998); Hsieh et al., (2003); Jeppesen et al., (2002). Human physiology cannot metabolize the sweet glycosides that present in stevia leaves. Therefore they are eliminated from the body as a non-caloric absorption Mantovaneli et al., (2004). Several studies support the non-toxic effect of stevioside and it has positive functions on cell metabolism Gregersen et al., (2004); Abdullah et al., (2004). Stevia products have a sufficient amount of medicinal usage and advantages for diabetic and high blood pressure patients. The extractives sweetener have been suggested to bring beneficial effects on human health, including antihypertensive Lee et al., (2001), antioxidant, Xi et al., (1998), anti-human rotavirus activities Takahashi et al., (2001). Surprising antimicrobial activity of Stevia has shown as a potent nonantibiotic pharmaceutical and an efficient food preservative Ghosh et al., (2008). At present, the most common high-intensity sweeteners products in the world market are made of synthetic compounds. A frequent metallic after taste of such synthetic sweeteners do not provide the real taste of sugar as well as some types of synthetic sweeteners such as saccharin; it is associated with the effective risk of cancer of the bladder, when they are used high quantity. Several toxicological studies have been shown that when stevioside used as a sweetener, stevioside does not have mutagenic, teratogenic or carcinogenic effects and no allergic reaction has been observed. Diabetic persons with hyperglycemia can use

“Stevioside” or stevia leaves as alternative natural sweetener Din et al., (2006). It is used for the treatment of cancer in various conditions Yasukawa et al., (2002) and also used for the treatment of diabetes, obesity, hypertension, fatigue, depression and in cosmetic and dental preparation Dyrskog et al., (2004).

Stevia is used in form of fresh leaf, dried and powder leaves. Different technologies and methods are available for extraction of stevioside from the stevia leaves. Two of them are extraction with hot water and extraction with chemicals. Extraction with hot water includes, boiling the leaves in hot water to dissolve glycoside and filtering the liquid part by precipitation. Boiling water extraction can achieve 93-98% extraction of stevioside Midmore and Rank et al., (2006). Hot-water treatment has been used as a classical extraction method Dacome et al., (2005). In solvent (methanol) extraction, different solvents are used to extraction of glycoside from leaves. Solvent extraction will be repeated till to get several of high glycoside content. Then this high glycoside is purified and separated to get clear glycoside. For crystals and powder, this liquid is crystallized and dehydrated, Nikolai et al., (2001).

Modern extraction techniques such as pressurized fluid extraction, pressurized hot water extraction, supercritical fluid extraction, methanol and iso -butyl alcohol extraction have been used for extracting stevioside content Bondarev et al., (2003). There are a number of modern technological methods to get refining the processes that registered internationally, for the production of pure stevioside commercially. Methanol has been used in most extraction and purification process as a solvent, probably to improve extraction efficiency. This study was conducted to optimize a liquid and chemical extraction process of stevioside from stevia leaves. So this study was conducted to reduce the extraction and purification cost and time and also help to get optimize time and temperature for the extraction process.

Objectives:

- To develop an easy stevioside extraction procedure.
- To find out what kind of leaves gives proper sweetness from stevia leaves.
- To optimize proper time, temperature and solvent volume for stevioside extraction.
- To determine pigment (total chlorophyll and carotenoid) content in stevia leaf extracts.
- To isolate stevioside from the extract.

CHAPTER- II

REVIEW OF LITERATURE

2.1 Background

The most favored process for optimization of aqueous extract of stevia leaf and isolation of stevioside involved two steps; aqueous and methanol extraction then purification and isolation of stevioside from stevia leaf Ahmed and Dobberstein et al., (1992). They observed methanol extraction provided the best results for purification of stevioside than aqueous extracted. Potentially the natural sweetening agents of plant origin and field search for sweet-tasting stevia species were studied by Soejarto et al., (1983). It is quite possible that further research may develop standard methods for stevioside extraction and purification according to Nishiyama et al., (1992). The sweet compounds of stevia were first isolated in 1909. The chemical structure of the stevioside was introduced in 1952 as a diterpene glycoside. The leaves contain a complex mixture, including stevioside, steviolbioside, rebaudioside (A, B, C, D, and E) and dulcoside A. Rebaudioside- A is the sweet taste, most stable, and it is less bitter than stevioside. Rebaudioside -E is as sweet as stevioside, and rebaudioside -D is as sweet as rebaudioside -A, while the other glycosides are less sweet than stevioside Cramer and Ikan et al., (1987). According to Liu et al., (1997), the extraction of stevioside, rebaudioside - A, stevioside - C and dulcoside was also performed by supercritical fluid extraction method using Co_2 and methanol as a modifier. The liquid extraction conditions were optimized and extraction efficiency was obtained, such an extraction technique has been gaining popularity as an analytical tool because it is rapid, simple and less expensive in terms of solvent extraction. In solvent extraction, methanol was preferable able to better stevioside extraction ability than liquid extraction from the stevia leaves. When isobutanol was treated, stevioside purity was increased

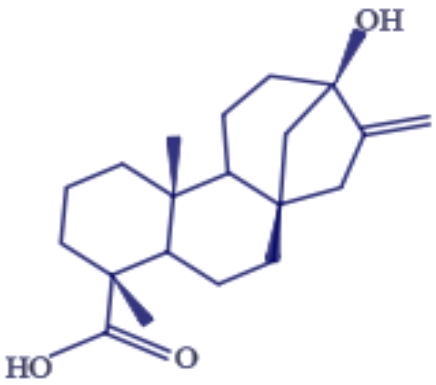
respectively than water extraction. This study was conducted to “Optimization of liquid extract and purification of the chemical extract of stevioside from stevia leaves”. Research work was conducted in “Hajee Mohammad Danesh Science and Technology University” Department of “Biochemistry and Molecular Biology” stevia research field. In this study, several stevia leaves such as the dried whole leaf, fresh leaf, and powder leaf were used for extraction and obtained which leaves given the best intensity of sweetness sensitization on tongue taste at the same time carried low pigments (total chlorophyll and carotenoid) content. This study also conducted optimized the time, temperature and solvent volume. The dry whole leaf gave the proper extraction and isolation. At the same time giving best intensity of sweetness and low pigment content. FTIR (Fourier-transform infrared spectroscopy) was used for the analysis of stevioside in stevia leaves extracts.

2.2 History

Stevia rebaudiana is an herb of the 950 genera of Asteraceae family Yadav et al., (2011). Centuries ago Paraguay natives used the leaves of this small, herbaceous, semi-bushy, perennial shrub to sweeten their bitter drinks. Dr. Moises Santiago Bertoni discovered this plant in 1888 at Paraguay. In 1905, the plant was scientifically named as *S. rebaudiana* after a paraguayan chemist Dr. Rebaudi. It was reported that there are around 150 species within the Stevia family including Stevia dianthoidea, Stevia Phlebophylla, Stevia anisostemma, Stevia bertholdii, stevia crenata, stevia enigmatica, stevia eupatoria, stevialemmonii, steviamicrantha, stevia ovata, stevia plummerae, *S. rebaudiana*, stevia salicifolia, stevia serrata and stevia viscida with all plants being sweet but rebaudiana having the highest sweetness levels. It is also known sweet herb, sweet leaf, honey leaf Carakostas et al., (2008). It is widely used in many parts of the world as sweetener and grown commercially in Central America, Korea, Paraguay, Brazil etc. Gupta et al., (2013).Two

French chemists in 1931 isolated the glycosides which is secondary metabolites responsible for the sweet taste of stevia Bridel and Lavielle., (1931).The chemical structure was established in 1952 as a diterpene glycoside. The leaves of stevia contain a natural complex mixture of eight sweet diterpene glycosides, including isosteviol, stevioside, rebaudiosides (A, B, C, D, E, F), steviolbio-side and dulcoside A Gupta et al., (2013). Steviol glycosides (SGs), stevioside and rebaudioside A are the major metabolites and these compounds are 250 to 300 times as sweet as sucrose Bondarev et al., (2001) , pH-stable, heat-stable, not fermentable Abdullateef et al., (2012) and possess health promoting potential. Along with sweetness, stevia has some bitter aftertaste due to the presence of some essential oils, tannins and flavonoids Phillips and Peter., et al., (1987). The stevia leaves have sensory and functional properties superior to those of many other high-potency sweeteners and is likely to become a major source of natural sweetener for the growing food market Goyal et al., (2010). *S. rebaudiana* has many medical applications like antimicrobial Kumar et al., (2008), antiviral Kedik et al., (2009) antifungal Abrahamson and Silva et al., (2008), anti-hypertensive Chen et al., (2008), anti-hyperglycaemic Gupta et al., (2013), anti-tumour, anti-inflammatory, anti-diarrhoeal, diuretic, anti-human rota-virus activities,anti-HIV Nakamura et al., (1998), hepatoprotective and immunomodulatory effects Gupta et al., (2013).

2.3 The chemistry of the diterpene glycoside sweeteners



IUPAC name:	Kaur-16-en-18-oic acid
Mol. formula:	$C_{20}H_{30}O_3$
Molar mass:	318.4504 g/mol

Figure- 2.1: Steviol, the basic building block of stevia's glycosides

The sweet diterpene glycosides of stevia have been the subject of a number of Treatment of this substance with the digestive juice of a snail yielded three moles of glucose and one mole of steviol, while acid hydrolysis gave isosteviol Bridel and Lavieille et al., (1931). Isosteviol was also obtained when steviol was heated in dilute sulfuric acid. The structure, stereochemistry and absolute configuration of steviol and isosteviol were established, through a series of chemical reactions and correlations over 20 years after the pioneering work of Bridel and Lavieille. The diterpene known as steviol is the aglycone of stevia's sweet glycosides, which are constructed by replacing steviol's bottom hydrogen atom with glucose (forming an ester), and replacing the top hydrogen atom with combinations of glucose and rhamnose.

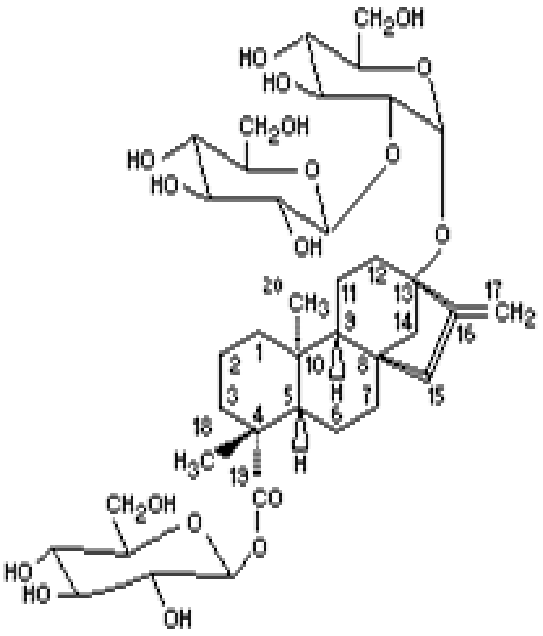


Figure -2.2: Chemical structure of stevioside

Subsequent studies have led to the isolation of seven other sweet glycosides of steviol. In terms of weight fraction, the four major steviol glycosides found in the stevia plant tissue are:

- 5⟷ 10% stevioside
- 2⟷ 4% rebaudioside A
- 1 ⟷2% rebaudioside C
- ½⟷1% dulcoside A

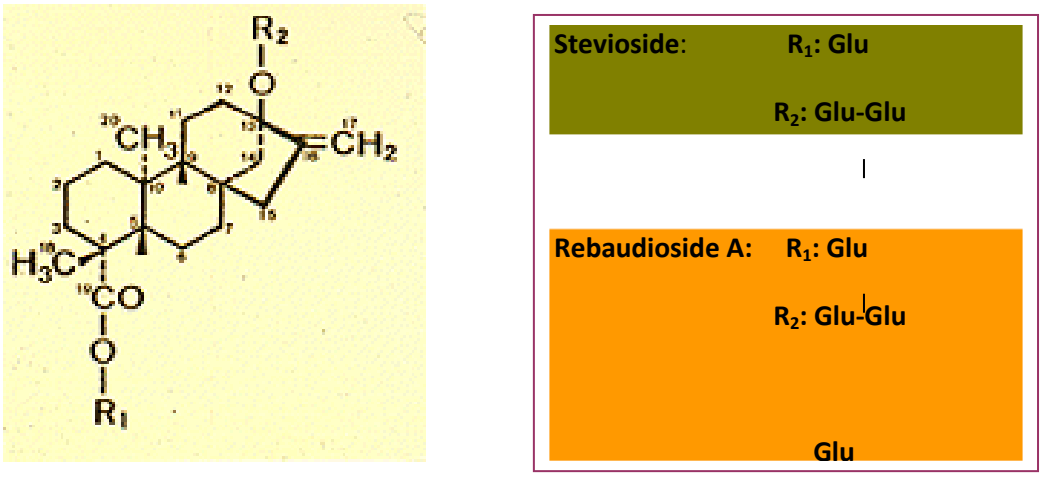


Figure- 2.3: Chemical structures of the steviol glycosides

2.4 Optimization of process and parameters for stevioside extract

Pigment and sweetness sensitization intensity on the tongue of different forms of stevia leaf then pigment and sweetness sensitization intensity on the tongue of a different time, temperature and solvent volume of stevia leaf extract. The extraction of stevioside was adopted to be a system affected by two dependent variables such as liquid and chemical extraction. The liquid and chemical extraction including purification and identification of stevioside by FTIR analysis.

2.5 Collection and Preparation of stevia leaves

The stevia seedling was obtained from BRAC, Gazipur, Bangladesh and then cultivated in HSTU farm for the experiment to be continued. The stevia plants that propagate the maximum growth stage were harvested by cutting the leaves from the stevia plant. The fresh leaves were collected from the HSTU stevia farm and the brown and yellow leaves were removed and washed in clean water and spread on trays covered with a soft cotton cloth to remove the excess of water. The plants were also dried in direct sunlight for 48 h. The dried stevia leaves were collected and an essential amount of dried leaves were crushed with hands.

2.6 Aqueous extract of stevia and pigment release and sweetness sensitization intensity

2.6.1 Pigment release and sweetness sensitization intensity of different form of stevia leaf

One gram dried stevia leaves, fresh leaves and powder leaves of stevia were taken three different containers and soaked in 25 ml of water (each container) and heated underwater bath. The different solution was filtered with Whatman-4 and the separated solution was stored in three different funnels for pigment and sweetness sensitization intensity on tongue taste in a different solution. After completing pigment and sweetness sensitization intensity on tongue taste it needs to determine the concentration of total pigment in three different solutions. According to wettest et al., (1959), 85% acetone was mixed with every solution in dark bottle and left at room temperature for 15 hours. After 15 hours, the solutions were filtered on glass wool into a 100 ml volumetric flask. The absorbance of the solutions is measured at 440 nm, 644 nm, and 662 nm

using a spectrophotometer. A blank experiment using 85% acetone was carryed out. The total pigment contents were calculated using the following equations:

Chlorophyll A (mg/L) = (9.784 × E662) – (0.99 × E664).

Chlorophyll B (mg/L) = (21.426 × E664) – (4.65 × E662).

Total chlorophyll (mg/L) = (9.784 × E662) – (0.99 × E664) + (21.426 × E664) – (4.65 × E662).

Total carotenoids (mg/L) = (4.695 × E440) – 0.369 (chl. A + chl. B).

2.6.2 Pigment release and sweetness sensitization intensity of different time, temperature and solvent volume extracts of stevia leaf

One gram of dried stevia leaves was taken in five different containers and soaked in 25 ml of water (each container) and heated underwater bath with different time such as 20 min, 40 min, 60 min, 80 min, and 100 min. The different solution was filtered with Whatman-4 and the separated solution was stored in five different funnels for pigment and sweetness sensitization intensity on tongue taste in a different solution. After completing pigment and sweetness sensitization intensity on tongue taste it needs to determine the concentration of total pigment in different solutions. According to wettest et al., (1959), 85% acetone was mixed with every extract solution in a dark bottle and left at room temperature for 15 hours. After 15 hours, the solutions were filtered on glass wool into a 100 ml volumetric flask. The absorbance of the solutions was measured at 440 nm, 644 nm, and 662 nm using a spectrophotometer. A blank experiment using 85% acetone was carried out. The total pigment contents were calculated using the following equations:

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Total carotenoids (mg/L) = (4.695 × E440) – 0.369 (chl. A + chl. B).

According to Jeffrey and Humphrey et al., (1975), Two grams of stevia leaves were extracted repeatedly with ethanol to give a final volume of 75 ml. The ethanol was evaporated and the resultant slurry was made up of water. The ethanolic extract of the leaf was labeled ET. For the SC extract ($Na2CO_3$ precipitated), 2 g of leaf were soaked in sufficient 20% $Na2CO_3$ solution and heated in an oven at 100°C for 10 min. The soaked leaf was extracted with ethanol as described above. The ET, SC and CO extracts were lyophilized on a Christ alpha 1–2 CD Plus lyophilized. One ml of 90% acetone was added to lyophilized samples and mixed well. The samples were then centrifuged at 10,000 rpm for 10 min at ambient temperature (25 ± 1°C). The samples were transferred from centrifuge tubes into cuvettes. A Hitachi SP-10 spectrophotometer (Hitachi, Japan) was used to measure the absorbance of the sample extract at 750, 664, 647, 630 nm against a 90% acetone blank. The concentration of chlorophyll a, b and c were calculated according to the equations Jeffrey and Humphrey et al., (1975):

Chlorophyll a = (11.85 × (E664 − E750) − 1.54 × (E647 − E750) − 0.08 (E630−E750))

Chlorophyll b = (−5.43 × (E664 − E750) + 21.03 × (E647 − E750) − 2.66 (E630−E750))

Chlorophyll c = (−1.67 × (E664 − E750) − 7.60 × (E647 − E750) + 24.52 (E630−E750))

2.7 Extraction and purification of stevioside

2.7.1 Extraction and purification of aqueous extract

Dried 5 g of stevia leaf were soaked in 100 ml of water and properly dissolved. The accumulated solutions were heated on a water bath for 40 minutes and the temperature with 60°C occasional shaking and stirring. The time and temperature were obtained by using stopwatch and thermometer (°C). The solution was filtered on Whatman No.4 for three times. The filtrate transferred to a separating funnel.

According to Nishiyama et al., (1992) used the Near-Infrared reflectance spectroscopy (NIRS) for the analysis of stevioside in *stevia rebaudiana* leaves with the same accuracy as obtained by HPLC. The leaves were extracted with near-boiling water and then subjected to HPLC analysis. For NIRS analysis, leaves were ground using a cyclone mill fitted with 1.0 mm screen, NIRS calibration was developed from 64 samples covering the range of stevioside normally found in *Stevia rebaudiana* leaves (4-13%). The result suggested that NIRS was a precise and simple method for routine stevioside determinations in *S. rebaudiana*.

Extraction of stevioside, rebaudioside A and C and dulcoside was also performed by supercritical fluid extraction method using CO_2 and methanol as a modifier by Liu et al., (1997). The extraction conditions were optimized and

the extraction efficiency of more than 88% was obtained. Such an extraction technique has been gaining popularity as an analytical tool because it is rapid, simple and less expensive in terms of solvent.

The stevioside content in plant material and food samples was determined by HPLC. An HPLC method determination of sweet-tasting stevioside in the leaves of the plant for the *Stevia rebaudiana* and in some beverages (e.g. tea, orange juice) was developed. The pre-separation procedure consisted of extraction of sweet-tasting stevioside from the plant material using boiling water and solid-phase extraction (SPE). Recovery rates of the SPE for the analyzed matrices ranged from 92.8% to 97.8% (for concentrations of STS of 105, 210 and 300 μg/ml; Relative Standard Deviation (RSD) ≤ 3.3%).

The selectivity of polymer adsorbent in adsorptive separations of stevia, diterpene glycosides were studied by Chen et al., (2008). Some hydrophobic (including both the nonpolar and polar) and hydrophilic polymer adsorbents were designed and synthesized, and their adsorption properties and adsorption mechanism toward stevia glycosides were studied in great detail. The skeleton structure and polarity of the resins had an effect on the adsorption capacity and the selectivity properties during the adsorption of stevioside and rebaudioside A. A sweetener with high rebaudioside A content was isolated by using the adsorption selectivity of the polar resins.

Stevia glycosides were extracted by supercritical fluid extraction (SCFE) method using CO_2 as solvent and water/ethanol as co-solvent. The mean total yield for SCFE treatment was 3.0%. The yields of stevia glycosides for SCFE with co-solvent were below 0.50%, except at 120 bar, 16°C. The overall extraction curves were well described by the Lack of extended model Pasquel et al., (2000).

Zhang et al., (2000) studied the process of extraction and refining of sweeteners with a reduced number of unit operations and minimization or elimination of chemical the usage including organic solvents. Water was very effective for extracting glycosides at selected pH and temperature. It was also shown that a multistage membrane process was successfully able to concentrate glycoside sweeteners and bitter-tasting components were washed out from the sweetener concentrate in the Nano filtration process.

Supercritical fluid extraction and liquid chromatographic-electrospray mass spectrometric analysis of stevioside from stevia rebaudiana leaves was studied by Choi et al., (2002). In developing an alternative extraction method for stevioside using SCFE, the effect of temperature, pressure, and percentage of modifiers were evaluated on the extraction yield. Although sufficient extractability was not obtained by pure CO_2 under any conditions of temperature and pressure, the addition of a modifier dramatically improved the extraction yield of stevioside, making it comparable to organic solvent extraction. Among the modifiers evaluated, the mixture of methanol and water showed greater extraction efficiency than the others. The extraction yield by CO_2-methanol-water (80:16:4) was found to be 150% of conventional organic extraction. In addition to improving the extraction yield, SFE obviously provided a higher purity of stevioside in the final extract. The estimation of glycosides from Stevia rebaudiana was studied by Kovylyaeva et al., (2007). The comparison of two different solvents methanol versus water was studied by Pol et al., (2007). They studied that the pressurized fluid extraction using water or methanol was employed for the extraction of stevioside from *stevia rebaudiana* Bertoni. The extraction method was optimized in terms of temperature and duration. Extracts were analyzed by liquid chromatography followed by ultraviolet (UV) and mass-spectrometric (MS) detections. The thermal degradation of stevioside was the same in both solvents within the

range 70–160°C. Methanol showed better extraction ability for isolation of stevioside from *Stevia rebaudiana* leaves than water within the range 110–160°C.

Extraction by conventional, ultrasound and microwave-assisted extraction techniques using methanol, ethanol, and water as single solvents as well as in binary mixtures were studied by Jaitak et al., (2009). Conventional cold extraction was performed at 25°C for 12 h while ultrasound extraction was carried out at a temperature of 35 ± 5°C for 30 min. Microwave-assisted extraction (MAE) was carried out at a power level of 80 W for 1min at 50°C. MAE yielded 8.64 and 2.34% of stevioside and rebaudioside A, respectively, while conventional and ultrasound techniques yielded 6.54 and 1.20%, and 4.20 and 1.98% of stevioside and Rebaudioside-A respectively.

Extraction of stevia by three methods, first by hot water (65°C) at different ratios of leaves to water (1:15 – 1:75) was studied by Abou-Arab et al., (2010). The optimum ratio was 1:35 in which the maximum stevioside content was obtained (7.53%), recovery of stevioside was 80.21%. The second method, extraction by methanol at a ratio of 4:1 methanol/leaves, the recovery was 94.9%. The third method of extraction by a mixture of methanol/water (4:1), the recovery was 92.34%.

Inamake et al., (2010) attempted to isolate stevioside from the dried leaves of Stevia in its purest form. Isolated stevioside was purified, analyzed & characterized by using various chromatographic & analytical methods including Thin-layer chromatography (TLC), UV, Fourier transform infrared spectroscopy (FTIR), Nuclear magnetic resonance spectroscopy (NMR) and HPLC methods. The Rf value for TLC was 0.32, λmax of UV spectra was obtained at 333 nm and HPLC showed a sharp peak with 1.958 min retention time. The isolated

stevioside was also compared with standard stevioside with all analytical methods.

According to Rai et al., (2012), Hot water extraction process was used for the extraction of stevioside from dry stevia leaves. The independent variables were, leaf to water ratio (1:5 to 1:20), heating time (10 to 120 min), and temperature (30 to 90) ^{o}C. The combined effects of these independent variables on the extracted stevioside concentration and color of the extract were studied. For optimizing the extraction process, a central composite rotatable design in combination with response surface methodology was used. Significant regression models with a coefficient of determination greater than 0.90 were established to study the effect of independent variables on the responses. The optimum conditions are the temperature of water: 70^{o}C, time of heating: 56 min and leaf to water ratio: 1:20 (g: mL). Stevia sweetener (Stevioside) was extracted from the dried ground leaves of stevia plant by using water, methanol and ethanol extraction. The dried ground leaves were mixed with hot water (65°C) at a different percentage of powder leaves/water ratio of 1:45 (w/v). Stevia leaves were extracted by using hot water for 3 h. The crude extract containing Stevioside was filtered through Whatman No. 1filter paper. It was named as filtrate Abou-Arab et al., (2010).

2.7.2 Extraction and purification of methanol extract

Chemical extracts one of the most popular methods for extraction of stevioside from stevia leaves. Dried Stevia leaves were extracted by using methanol according to the method of Nikolai et al., (2001). Methanol was added to dried leaves at ratio (20:1 v/w) and remained for 7 h, then filtered through Whatman No. 4 filter paper and separated the desired solution and discarded the plant impurities.

According to Jaitak et al., (2009), Methanol showed better extraction ability for isolation of stevioside from *Stevia rebaudiana* leaves than water within the range 110–160°C. Extraction by conventional, ultrasound and microwave-assisted extraction techniques using methanol, ethanol, and water as single solvents as well as in binary mixtures. Conventional cold extraction was performed at 25°C for 12 h while ultrasound extraction was carried out at a temperature of 35 ± 5°C for 30 min. Microwave-assisted extraction (MAE) was carried out at a power level of 80 W for 1min at 50°C. MAE yielded 8.64 and 2.34% of stevioside and rebaudioside A, respectively, while conventional and ultrasound techniques yielded 6.54 and 1.20%, and 4.20 and 1.98% of stevioside and Rebaudioside-A respectively. Extraction of stevia by three methods, first by hot water (65°C) at different ratios of leaves to water (1:15 – 1:75) was studied by Abou-Arab et al., (2010). Dried ground stevia leaves were extracted by using methanol. Methanol was added to ground leaves at ratio (4:1 v/w) and remained for 7 h, then filtered through Whatman No. 4 filter paper. The filtrate containing the solvent was evaporated to dryness by using a rotary evaporator at 45°C. The residue was washed with ether and then extracted with butanol (three times). The organic phase was evaporated, and the residue was recrystallized at -5°C overnight and purity during extraction and purification steps were determined by determination of pigments.

According to Inamake et al., (2010), the optimum ratio was 1:35 in which the maximum stevioside content was obtained (7.53%), recovery of stevioside was 80.21%. The second method, extraction by methanol at a ratio of 4:1 methanol/leaves, the recovery was 94.9%. The third method of extraction by a mixture of methanol/water (4:1), the recovery was 92.34%. According to Rajab R et al., (2009), the leaves were powdered and extraction of sweet glycosides was carried out with methanol. Powdered leaf material (250 g) was refluxed with 2l methanol for 1 h in a round bottom flask on a hot water bath. The contents were then cooled and filtered under vacuum. The plant residue was further refluxed with methanol three times and each time the filtrates were collected. All the filtrates were combined and the solvent was distilled off on a hot water bath. The residue thus obtained was completely dried under vacuum and then it was refluxed (1 h) with chloroform (500 ml) on a hot water bath. After cooling, the chloroform layer was decanted and collected in a separate flask. The process was repeated until there was no color in chloroform. The chloroform insoluble part was dissolved in methanol (250 ml) by gentle heating and kept overnight in the refrigerator. The settled mass was filtered and washed with methanol. The main filtrate was combined with methanol washings and distilled in a water bath. The residue thus obtained was vacuum dried and dissolved in distilled water (400 ml) and repeatedly extracted (200 ml×5) with n-butyl alcohol in a separating funnel. All the n-butyl alcohol layers (upper layers) were combined and the solvent was distilled off in a rotavap to give a light brown residue. The residue was dissolved in methanol and run on a TLC plate to check the presence of sweet glycosides. The use of methanol even though it is all removed pigment from the final product of Stevia extracts as natural and GRAS (generally regarded as safe) by US FDA (United State Food and Drug Administration). Vibrational spectroscopy methods have been developed to determine “rebaudioside A” and “stevioside”.

2.8 Acceptable daily intake of stevia

Glycosides with Rebaudioside A and Stevioside as Principal Components) as appears to have an adequate daily intake (ADI) of 25 mg/kg (Chatsudthipong and Muanprasat et al., (2009) (following 100-fold safety factor, commonly seen in ADI values) in rats which are around 7.9 mg/kg in humans Xili et al., (1992) also calculated the acceptable daily intake (ADI) of stevioside which is 7.9 mg/kg body weight.

2.9 Uses of stevia

The leaves are used to prepare sauces but are best in herbal teas and for direct consumption. Stevia is mainly used as a sweetener and flavor enhancer in the food and beverage industry. Fresh leaves have a mild licorice flavor. The stevia plant is widely grown for its sweet leaves and medical value. Medical research has also shown the possible benefits of stevia in treating obesity and high blood pressure Duke, J.A et al., (1997).

2.9.1 Medicinal Uses

According to World Health Organization (WHO) findings, stevia regulates blood pressure, fights cavities, induces pancreas to produce more insulin, and acts as a bactericidal agent. No negative clinical reports have appeared. It also showed the antibacterial, antiseptic, anti-inflammatory, anti-fertility, hypotensive, diuretic and cardiotonic properties. The compound obtained from stevia is considered to be the best alternative sweetener source for diabetes patients Begovich et al., (2004) and Hossain et al., (2017).

2.9.2 Glucoregulation

Steviol glycosides have an enhancing effect on insulin secretion by directly acting on β-cells without altering the K^+ - ATP channel activity and cAMP level in the islets, thus documenting stevioside and steviol as potent antihyperglycemic agents Begovich et al., (2004); Gupta et al., (2013); Rooke et al., (2011). Stevioside also enhances glucose-stimulated insulin secretion, but does not affect fasting insulinemia Sondergaard et al., (2003). Stevioside has the ability to increase insulin effect on cell membranes, increase insulin production Shivanna et al., (2013).

2.9.3 Blood pressure regulation

Stevia can be used as a heart tonic to normalize blood pressure levels, to regulate heartbeat, and for other cardiopulmonary indications. In humans, a hot water extract of the leaf has been shown to lower both systolic and diastolic blood pressure Gardana et al., (2010); Atteh et al., (2008), improves cell regeneration and blood coagulation Lemus-Mondaca et al., (2012). The use of stevioside results in a clinically significant hypotensive effect in spontaneously hypertensive rats, without adversely affecting their heart rates or serum catecholamine levels (Chan et al., (1998); Markovié et al., (2008).

2.9.4 Cancer

Stevioside, the stevia leaf aglycones, steviol and is steviol, inhibit tumor promotion by blocking Epstein-Barr virus early antigen (EBV-EA) induction as well as by reducing tumor formation in the two-stage mouse skin carcinogenesis model following sequential exposure to 7,12-dimethylbenz anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) (Brahmachari *et al.,* 2011). The hydrolysis product of stevioside is steviol, potently inhibits DNA replication and human cancer cell growth in vitro (with LD50 values of 84 to 167 μMol). Rebaudioside-A was evaluated for mutagenic potential in cultured

human lymphocytes, results showed that pre-neoplastic or neoplastic lesions development was not enhanced in urinary bladders by stevioside while studies performed with the dose-effect of bladder carcinogenicity of N-nitrosobutyl-N-(4-hydroxybutyl) amine. Sandford et al., (1980); Guest et al., (2006).

2.9.5 Renal function

Globally, there are nearly 70 million people with kidney disease of varying severity levels. The main function of the kidney is to maintain homeostatic balance with respect to fluids, electrolytes, and organic solutes both the normal and hypertensive rats. Steviol and its analogs represent promising natural plant-based drug candidates for the treatment of polycystic kidney disease by stevia Chughtai and Farhan et al., (2017).

2.9.6 Obesity

Obesity is the most common nutritional disorder; it is a state of excess accumulation of fat in the body. In clinical terms, obesity is a condition of excess body weight more than 20% above the ideal body weight. Overweight and obesity are a major risk factor associated with a wide number of health problems including hypertension, hyperlipidemia, diabetes, surgical risks, pulmonary and renal problems, pregnancy complications and certain types of cancer Després and Lemieux et al., (2006). Stevia can be used in place of sugar as they provide fewer calories per gram than sugar which is not completely absorbed by the digestive system. Consumption of stevia leaves and extracts reduce the craving for sweet and fatty foods and are useful in the weight loss program Anton et al., (2010).

CHAPTER-III

MATERIALS AND METHODS

The study was conducted in the laboratories, Department of "Biochemistry and Molecular Biology", "Hajee Mohammad Danesh Science and Technology University", Dinajpur, Bangladesh. Healthy plants of Stevia, maintained in the experimental stevia farm of "HSTU", were selected for the study. The aqueous extract was prepared from the dried leaves. For the extraction and purification, the fresh mature leaves were collected from healthy stevia plants prior to flowering.

Figure-3.1: Stevia plant growing in HSTU farm.

Materials

The stevia seedling was obtained from BRAC, Gazipur, Bangladesh and then cultivated in “HSTU” farm for the experiment to be continued.

Reagents

Water, methanol, ether, acetone, and isobutanol, were used for extraction, purification, and identification of stevioside from different extract.

Stevioside sugar standards

The standard of stevioside was obtained from a commercial company, Japan (Zhejiang Green World Bio-tech Engineering Co., Ltd).

3.1 Optimization of process and parameters

The experimental design for “Optimization of liquid extract and purification of the chemical extract of stevioside from stevia leaves”. At first, pigment and sweetness sensitization intensity on the tongue of a different form of stevia leaf then pigment and sweetness sensitization intensity on the tongue of a different time, temperature and solvent volume of stevia leaf. The extraction of stevioside was adopted to be a system affected by two dependent variables such as liquid and chemical extraction. The liquid and chemical extraction including purification and identification of stevioside by FTIR analysis.

3.2 Preparation of stevia leaves

The fresh leaves were collected from the HSTU stevia farm and the brown and yellow leaves were removed and washed in clean water and spread on trays covered with a soft cotton cloth to remove the excess of water. The plants were also dried in direct sunlight for 48 h. The dried stevia leaves were collected and the essential amount of dried leaves was crushed with hands.

(a) Fresh leaves (b) Dried leaves (c) Powder leaves

Figure-3.2: Preparation of stevia leaves

3.3 Preparation of stevia leaves extract based on different form

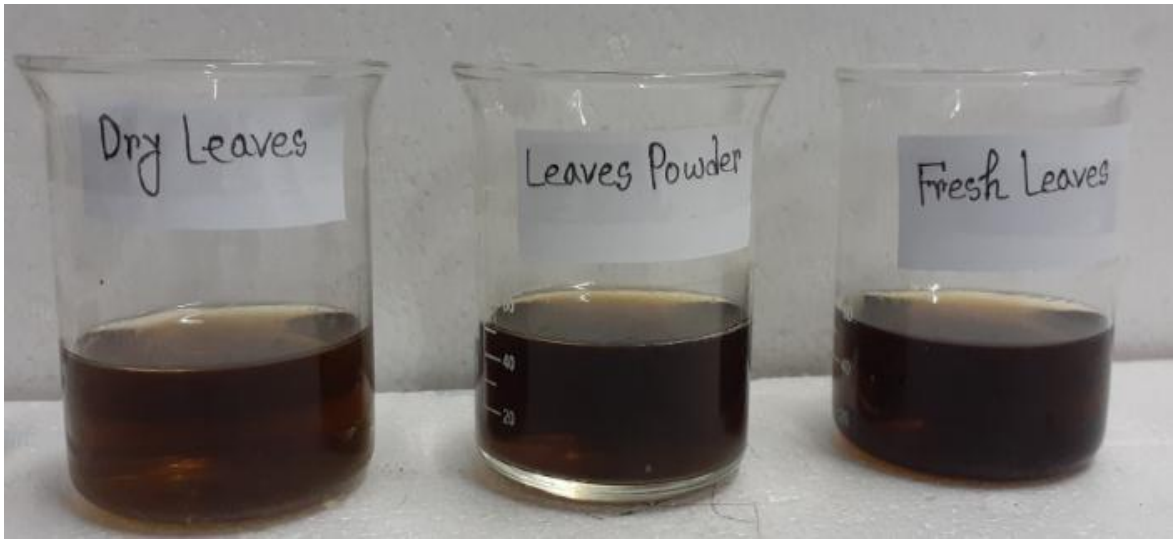


Figure-3.3: Different form of stevia leaves extract

Dried whole leaves, fresh leaves and leaves powder of stevia were taken three different containers and soaked in 1:40 (leaf: water) each container and heated under water bath. The different solutions were filtered with Whatman-4 and the separated solution was stored in three different funnels for pigment and sweetness sensitization intensity on the tongue taste. For statistical analysis of sweetness sensitization on the tongue taste different solution were taken. In this case, 9 point hedonic rating test was performing to assays the degree of acceptability of this solution. Three different solutions were presented to 20 panellists as randomly the taste panellists were asked to rate the solution as a 9 point hedonic scale for sweetness. The overall acceptability with the ratings of 7-9 best sweetness sensitization taste, 4-6 for good sweetness sensitization taste and 1-3 very low sweetness sensitizations on the tongue taste. Point hedonic test method as recommended by Joshi et al., (2006) was used for the purpose of sweetness sensitization on the tongue taste. After completing pigment and sweetness sensitization intensity on the tongue taste it needs to determine the

concentration of total pigment in three different solutions. According to (wettest et al., 1959), 85% acetone was mixed with every solution in a dark bottle and left at room temperature for 15 hours. After 15 hours, the solutions were filtered on glass wool into a 100 ml volumetric flask. The absorbance of the solutions were measured at 440 nm, 644 nm, and 662 nm using a spectrophotometer. A blank experiment using 85% acetone was carried out. The total pigment contents were calculated using the following equations:

Chlorophyll A (mg/L) = (9.784 × E662) – (0.99 × E664).

Chlorophyll B (mg/L) = (21.426 × E664) – (4.65 × E662).

Total chlorophyll (mg/L) = (9.784 × E662) – (0.99 × E664) + (21.426 × E664) – (4.65 × E662).

Total carotenoids (mg/L) = (4.695 × E440) – 0.369 (chl. A + chl. B).

3.4 Preparation of stevia leaf extract based on different time.

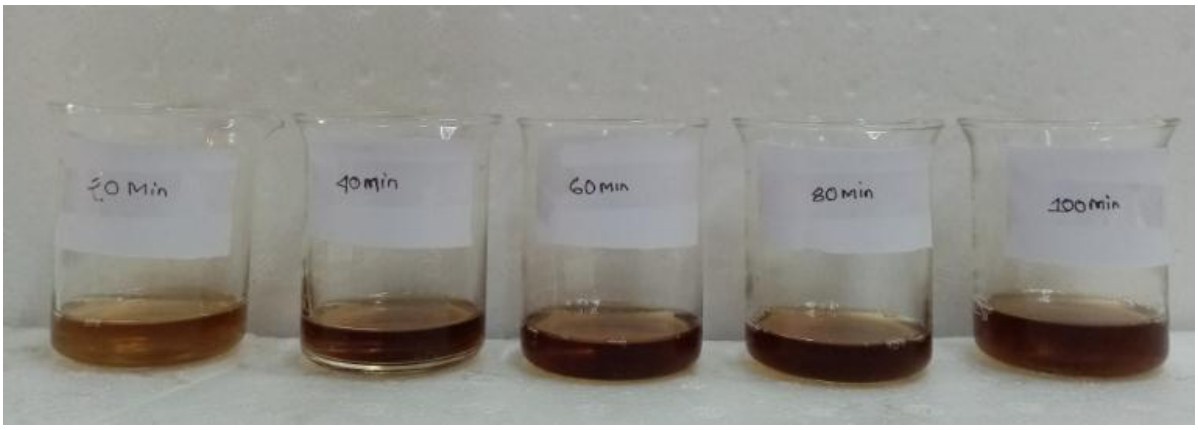


Figure-3.4: Pigment and sweetness sensitization intensity on the tongue of different time of stevia leaf

Dried stevia leaves was taken five different containers and soaked in 1:40 (leaf: water) each container and heated underwater bath with different time such as 20 min, 40 min, 60 min, 80 min, and 100 min. The different solution was filtered with Whatman-4 and the separated solutions were stored in five different funnels for pigment and sweetness sensitization intensity on tongue taste in a different solution. For statistical analysis of sweetness sensitization on the tongue taste different solutions were taken. In this case, 9 point hedonic rating test was performing to assays the degree of acceptability of this solution. The five different solutions were presented to 20 panellists as randomly the taste panellists were asked to rate the solution as a 9 point hedonic scale for sweetness. The overall acceptability with the ratings of 7-9 best sweetness sensitization taste, 4-6 for good sweetness sensitization taste and 1-3 very low sweetness sensitizations on the tongue taste. Point hedonic test method as recommended by Joshi et al., (2006) was used for the purpose of sweetness sensitization on the tongue taste. After completing pigment and sweetness

sensitization intensity on tongue taste it needs to determine the concentration of total pigment in different solutions. According to wettest et al., (1959), 85% acetone was mixed with every extract solution in a dark bottle and left at room temperature for 15 hours. After 15 hours, the solutions were filtered on glass wool into a 100 ml volumetric flask. The absorbance of the solutions were measured at 440 nm, 644 nm, and 662 nm using a spectrophotometer. A blank experiment using 85% acetone was carried out. The total pigment contents were calculated using the following equations:

Chlorophyll A (mg/L) = (9.784 × E662) – (0.99 × E664).

Chlorophyll B (mg/L) = (21.426 × E664) – (4.65 × E662).

Total chlorophyll (mg/L) = (9.784 × E662) – (0.99 × E664) + (21.426 × E664) – (4.65 × E662).

Total carotenoids (mg/L) = (4.695 × E440) – 0.369 (chl. A + chl. B).

3.5 Preparation of stevia leaf extract based on different temperature.

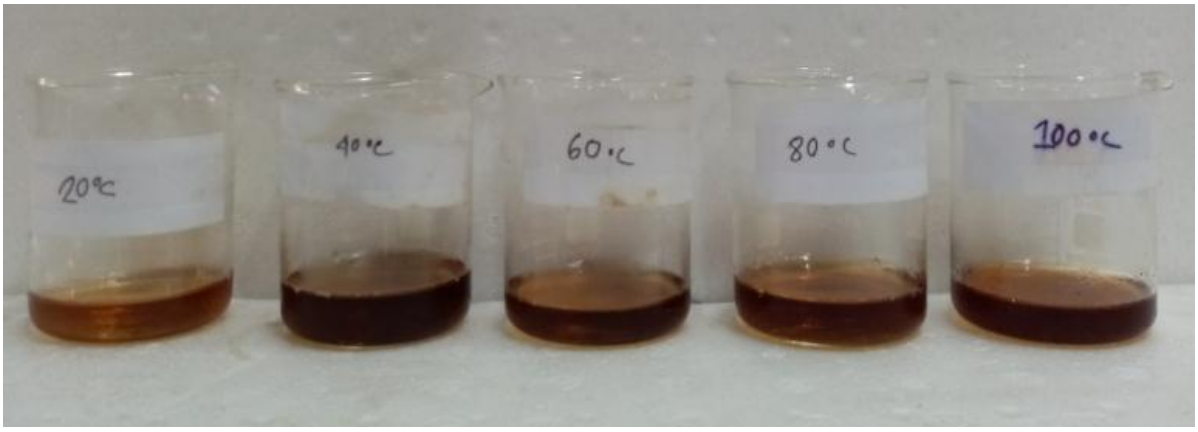


Figure-3.5: Pigment and sweetness sensitization intensity on the tongue of different temperature of stevia leaf

Dried stevia leaves were taken five different containers and soaked in 1:40 (leaf: water) each container and heated underwater bath with different temperature such as 20°C, 40°C, 60°C, 80°C, and 100°C. The different solutions were filtered with The Whatman-4 and the separated solutions were stored in five different funnels for pigment and sweetness sensitization intensity on tongue tastes in a different solution. For statistical analysis of sweetness sensitization on the tongue taste different solution were taken. In this case, 9 point hedonic rating test was performing to assay the degree of acceptability of this solution. The five different solutions were presented to 20 panellists as randomly the taste panellists were asked to rate the solution as a 9 point hedonic scale for sweetness. The overall acceptability with the ratings of 7-9 best sweetness sensitization taste, 4-6 for good sweetness sensitization taste and 1-3 very low sweetness sensitizations on the tongue taste. Point hedonic test method as recommended by Joshi et al., (2006) was used for the purpose of sweetness sensitization on the tongue taste. After completing pigment and sweetness sensitization intensity on tongue taste it needs to determine the concentration of

total pigment in different solutions. According to (wettest *et al.,* 1959), 85% acetone was mixed with every extract solution in a dark bottle and left at room temperature for 15 hours. After 15 hours, the solutions were filtered on glass wool into a 100 ml volumetric flask. The absorbance of the solutions were measured at 440 nm, 644 nm, and 662 nm using a spectrophotometer. A blank experiment using 85% acetone was carried out. The total pigment contents were calculated using the following equations:

Chlorophyll A (mg/L) = (9.784 × E662) – (0.99 × E664).

Chlorophyll B (mg/L) = (21.426 × E664) – (4.65 × E662).

Total chlorophyll (mg/L) = (9.784 × E662) – (0.99 × E664) + (21.426 × E664) – (4.65 × E662).

Total carotenoids (mg/L) = (4.695 × E440) – 0.369 (chl. A + chl. B).

3.6 Preparation of stevia leaf extract based on different volume.

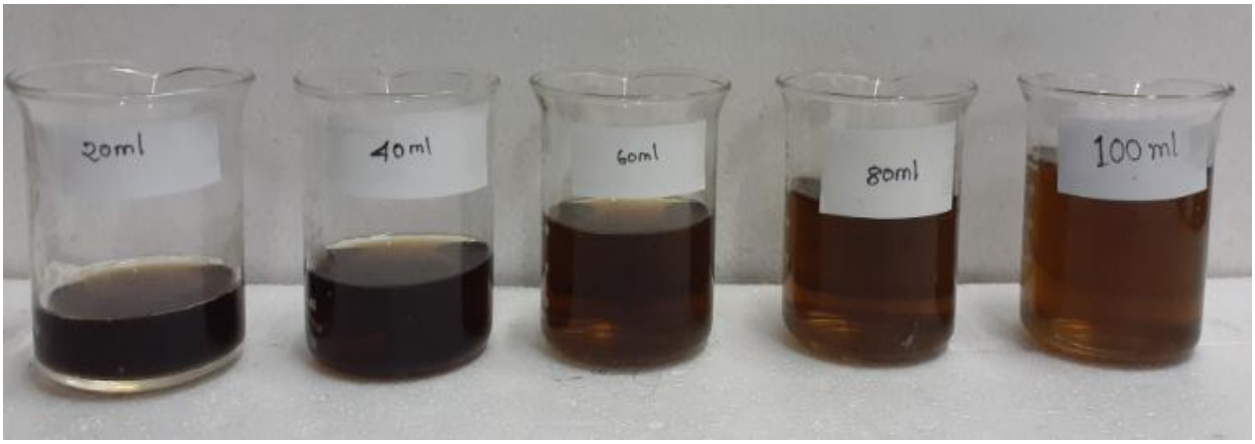


Figure-3.6 Pigment and sweetness sensitization intensity on the tongue of different solvent volume of stevia leaf

0.5 grams of dried stevia leaves were taken in five different containers and soaked in 20 ml, 40 ml, 60 ml, 80 ml and 100 ml of water and heated underwater bath in 60°C for 40 min. The different solution was filtered with Whatman-4 and the separated solution was stored in five different funnels for pigment and sweetness sensitization intensity on tongue taste in different solution. For statistical analysis of sweetness sensitization on the tongue taste different solution were taken. In this case, 9 point hedonic rating test was performing to assays the degree of acceptability of this solution. The five different solutions were presented to 20 panellists as randomly the taste panellists were asked to rate the solution as a 9 point hedonic scale for sweetness. The overall acceptability with the ratings of 7-9 best sweetness sensitization taste, 4-6 for good sweetness sensitization taste and 1-3 very low sweetness sensitizations on the tongue taste. Point hedonic test method as recommended by Joshi et al., (2006) was used for the purpose of sweetness sensitization on the tongue taste. After completing pigment and sweetness sensitization intensity on tongue taste it needs to determine the concentration of

total pigment in different solutions. According to Wettest et al., (1959), 85% acetone was mixed with every extract solution in a dark bottle and left at room temperature for 15 hours. After 15 hours, the solutions were filtered on glass wool into a 100 ml volumetric flask. The absorbance of the solutions were measured at 440 nm, 644 nm, and 662 nm using a spectrophotometer. A blank experiment using 85% acetone was carried out. The total pigment contents were calculated using the following equations:

Chlorophyll A (mg/L) = (9.784 × E662) – (0.99 × E664).

Chlorophyll B (mg/L) = (21.426 × E664) – (4.65 × E662).

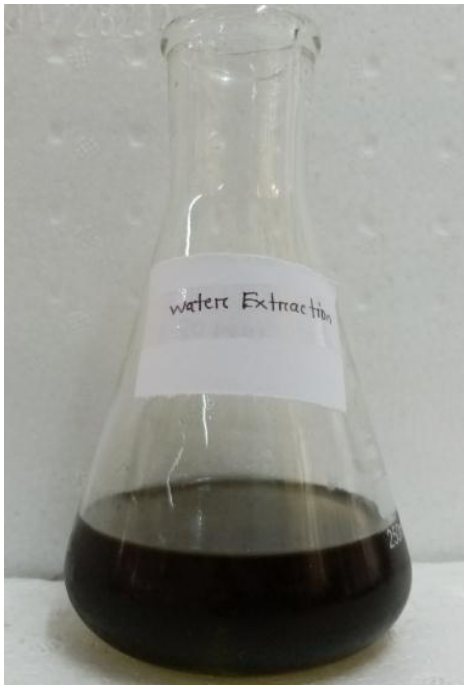
Total chlorophyll (mg/L) = (9.784 × E662) – (0.99 × E664) + (21.426 × E664) – (4.65 × E662).

Total carotenoids (mg/L) = (4.695 × E440) – 0.369 (chl. A + chl. B).

3.7 Extraction and purification of stevioside

3.7.1 Aqueous extract of stevioside

Dried stevia leaves were soaked in 1:40 (leaf: water) and properly dissolved. The accumulated solution was heated on a water bath for 40 minutes and the temperature with 60°C occasional shaking and stirring. The time and temperature were maintained by using stopwatch and thermometer (°C). The solution was filtered on Whatman No.4 for three times. The filtrate solution was transferred to a separating funnel.

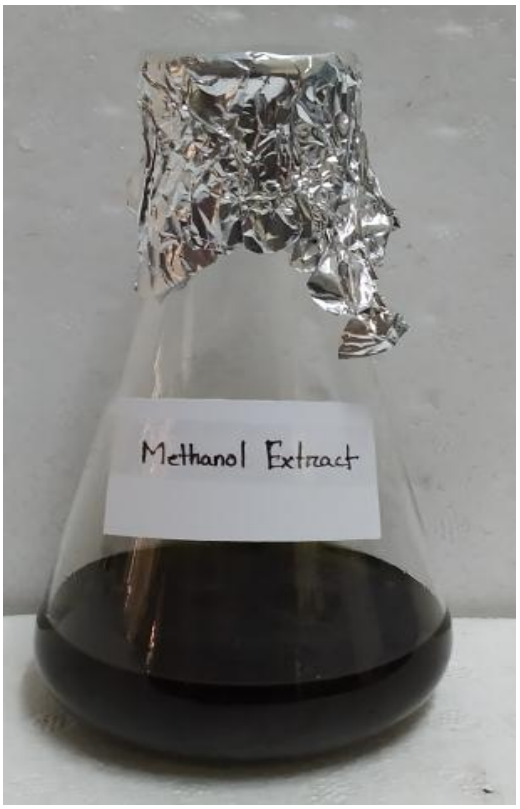


Aqueous extraction

Figure-3.7.1: Aqueous extract of stevioside.

3.7.2 Methanol extract of stevioside

Methanol extract is one of the most popular methods for extraction of stevioside from stevia leaves. Dried stevia leaf was extract by using methanol according to the method of Nikolai et al., (2001). Methanol was added to dried stevia leaf at ratio (40:1 v/w) and remains for 7 h, then the extract filter in Whatman No. 4 filter paper and separate the filtrate solution and discarded the plant impurities from the extract.



Methanol Extraction

Figure-3.7.2: Methanol extract of stevioside

3.7.3 Purification of stevioside from aqueous extract.

The separated solution was filtrated at three times with whatman-4 filter paper and transferred to a separating funnel. The 140 ml of isobutanol was added to separating funnel. The isobutanol was carried out the stevioside from the solution. The organic extracted was separated and evaporated under the water bath for 30 minutes and stored in refrigerator for overnight. White color powder was sedimented at the bottom. The aqueous extraction efficiencies were achievable of stevioside and so produce "natural product" according to Nishiyama et al., (1991).

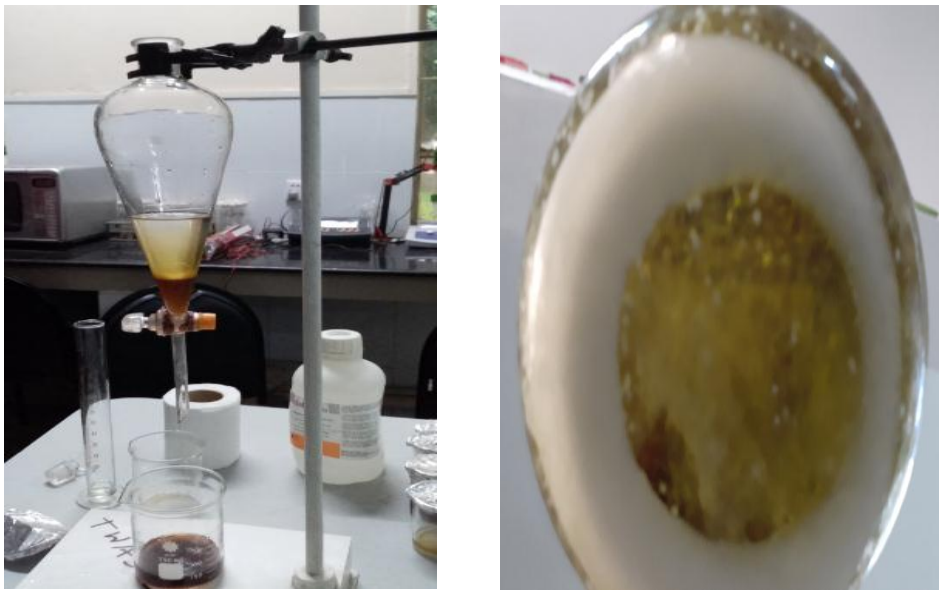


Figure-3.7.3: Purification of aqueous extract stevioside.

3.7.4 Purification of stevioside from methanol extract

The filtrate solvent was evaporated until dryness by using a water bath at 40°C. The residue was washed with ether in three times and then extracted with isobutanol. Isobutanol carry out the stevioside from the extracted. The organic phase was store in refrigerator (-5°C) for overnight. Methanol extract improves efficiency and facilitates the partial separation of individual stevioside Brandle et al., (1998).

Figure-3.7.4: Purification of methanol extract

3.8 Confirmation of stevioside in the extracts by FTIR spectrum analysis

The organic extract was separated, and the solution filtered with whatman -4 filter paper and concentrated underwater bath and evaporated until the solution become white colour turbid. The turbid was collected and prepared for the identification of stevioside by FTIR spectroscopy. Rohman et al., (2015) and Rohman et al., (2011).

Figure-3.8: Confirmation of stevioside from different extracts

3.9 Statistical Analysis

The statistical analysis (The effects of pigments released and sweetness sensitization intensity of stevia leaves extract) was analyzed through one way ANOVA method by 'SPSS' (Statistical Package for the Social Science) software. All data were expressed as the mean value and standard deviation of total measurements for 3 replicates where significant differences were performed by using Fisher's protected LSD test at $\rho \leq 0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

The present investigation was carried out in the Department of "Biochemistry and Molecular Biology". Stevioside is a natural sweet component and used as a natural sweetener in the world. Stevia leaf contains well-characterized steviol glycosides such as stevioside which is 250 to 300 times sweeter than sucrose. Stevia was extracted from dried stevia leaf by using different methods. Different processes and parameters such as the effect of different forms of stevia leaves on pigment release and sweetness sensitization intensity on the tongue taste from stevia leaf extract. The release of stevioside and the release of pigments in the extract depend on the forms of leaf, time, temperature, and leaf volume etc. The isolation of stevioside was conducted in aqueous and methanol extracts. Then we use isobutanol to reduce the impurities of the extracts and we found white powder at the end. To confirm the presence of stevioside in the extracts we conducted FTIR analysis of white powder.

Table-4.1: Effect of different forms of stevia leaf on pigment release and sweetness sensitization intensity on the tongue of stevia leaf extract

Parameters	Dry whole leaf	Leaf powder	Fresh leaf
Total chlorophyll (mg/L)	3.93 ± 0.028^{b}	4.023 ± 0.005^{a}	4.106 ± 0.058^{a}
Carotenoid content	2.96 ± 0.123^{b}	3.11 ± 0.006^{a}	3.335 ± 0.0682^{a}
Intensity of Sweetness sensitization on tongue	9.4 ± 0.251^{a}	6.932 ± 0.388^{b}	2.732 ± 0.389^{c}

According to the table- 4.1, the dry whole leaf, leaf powder, and fresh leaf were used for the analysis of different forms of stevia leaf on pigment release and sweetness sensitization intensity on the tongue of stevia leaf extract. In this study, the dry leaf extract contained total pigment (total chlorophyll and carotenoid) content was 3.93 mg/l and 2.96 mg/l, in leaf powder extract contained total pigment (total chlorophyll and carotenoid) content was 4.023 mg/l and 3.11 mg/l and fresh leaf extracted contained total pigment (total chlorophyll and carotenoids) content was 4.106 mg/l and 3.335 mg/l. The highest pigment was found in fresh leaf extract at the same time it gave the low intensity of sweetness sensitization. The lowest total pigment was found in dry whole leaf extract but at the same time it gave the best intensity of sweetness sensitization on the tongue taste.

However, the dry whole leaf released less pigment and gave the best intensity of sweetness sensitization on the tongue taste than the other two leaves (leaf powder and fresh leaf) extracts.

Table-4.2: Effect of time on pigment release and sweetness sensitization intensity on tongue of stevia leaf extract

Parameters	20 min	40 min	60 min	80 min	100 min
Total chlorophyll (mg/L)	3.470±0.443^{d}	3.930±0.017^{c}	4.797±0.040^{b}	5.246±0.024^{a}	5.580±0.01^{a}
Carotenoid content (mg/l)	2.585±0.134^{d}	2.962±0.125^{c}	3.241±0.021^{b}	3.424±0.012^{a}	3.418±0.067^{a}
Intensity of sweetness sensitization on tongue	2.260±0.434^{d}	8.994±0.223^{a}	5.734±0.280^{b}	3.932±0.685^{c}	2.666±0.47^{d}

According to the table- 4.2, the 20 min extract contained total pigment (total chlorophyll and carotenoid) content was 3.470 mg/l and 2.585 mg/l, the 40 min extract contained total pigment (total chlorophyll and carotenoid) content was 3.930 mg/l and 2.962 mg/l, the 60 min extract contained total pigment (total chlorophyll and carotenoid) content was 4.797 mg/l and 3.241 mg/l, the 80 min extract contained total pigment (the total chlorophyll and carotenoid) content was 5.24 mg/l and 3.424 mg/l and finally the 100 min extract contained total pigment (total chlorophyll and carotenoid) content was 5.580 mg/l and 3.418 mg/l.

In this study, the highest pigment (total chlorophyll, and carotenoid) content was found when the extraction time was 100 min but the 100 min extract contained less intensity of sweetness sensitization and the lowest pigment (total chlorophyll and carotenoid) content was found when the extraction time was 20 min but the 20 min extract also contained less intensity of sweetness sensitization. The higher the intensity of sweetness sensitization was found when the extraction time was 40 min and at same time the pigment (total chlorophyll and carotenoid) content was also low.

However, the 40 min extraction time given the best intensity of sweetness sensitization on tongue taste and at the same time gave the low pigment (total chlorophyll and carotenoid) concentration than different extracts conditions.

Table-4.3: Effect of temperature on pigment release and sweetness sensitization intensity on tongue of stevia leaf extract

Parameters	20°C	40°C	60°C	80°C	100°C
Total chlorophyll (mg/L)	2.417±0.068^{e}	3.992±0.038^{d}	4.919±0.059^{c}	6.130±0.145^{b}	8.739±0.263^{a}
Carotenoid content (mg/l)	0.576±0.083^{c}	0.723±0.011^{c}	0.934±0.085^{c}	1.749±0.044^{b}	2.581±0.597^{a}
Intensity of Sweetness sensitization on tongue	1.198±0.181^{e}	3.734±0.760^{c}	8.934±0.548^{a}	7.000±0.233^{b}	2.800±0.447^{d}

According to the table- 4.3, the 20°C temperature extract contained total pigment (total chlorophyll and carotenoid) content was 2.417 mg/l and 0.576 mg/l, the 40°C temperature extract contained (total chlorophyll and carotenoid) content was 3.992 mg/l and 0.723 mg/l, the 60°C temperature extract contained total pigment (total chlorophyll and carotenoid) content was 4.919 mg/l and 0.934 mg/l, the 80°C temperature extract contained total pigment (total chlorophyll and carotenoid) content was 6.130 mg/l and 1.749 mg/l and finally, the 100°C temperature extract contained total pigment (total chlorophyll and carotenoid) content was 8.739 mg/l and 2.581 mg/l.

In this study, the highest pigment (total chlorophyll and carotenoid) content was found when the extraction temperature was 100°C but the 100°C temperature extract contained less intensity of sweetness sensitization. The lowest pigment (total chlorophyll and carotenoid) content was found when the extraction temperature was 20°C but at the same time 20°C temperature contained low intensity of sweetness sensitization. The higher intensity of sweetness sensitization was found on when the extraction temperature was 60°C and the pigment (total chlorophyll and carotenoid) content was also lower than 80°C and 100°C. The 40°C temperature extraction also carried the intensity of sweetness sensitization and low pigment (total chlorophyll and carotenoid) content but 60°C temperature given the best intensity of sweetness sensitization on the tongue taste rather than 40°C temperature.

However, the 60°C temperature gave the best intensity of sweetness sensitization on tongue taste and at the same time 60°C temperature given low pigment (total chlorophyll and carotenoid) concentration than different extracts conditions.

Table-4.4: Effect of solvent volume on pigment release and sweetness sensitization intensity on the tongue of stevia leaf extract

Parameters	20 ml	40 ml	60 ml	80 ml	100 ml
Total chlorophyll (mg/L)	2.532±0.096[a]	2.375±0.009[b]	1.866±0.055[c]	1.637±0.01531[d]	1.089±0.032[e]
Carotenoid content (mg/l)	1.717±0.017[a]	1.479±0.037[b]	1.019±0.012[c]	0.897±0.025[d]	0.569±0.014[e]
Intensity of Sweetness sensitization on tongue	8.732±0.547[a]	5.398±0.547[b]	3.668±0.23[c]	2.332±0.237[d]	.835±0.191[e]

According to table-4.4, the 20 ml solvent ratio contained total pigment (total chlorophyll and carotenoid) content was 2.532 mg/l and 1.717 mg/l, the 40 ml solvent ratio contained pigment (total chlorophyll and carotenoid) content were 2.375 mg/l and 1.479 mg/l, the 60 ml the solvent ratio contained total pigment (total chlorophyll and Carotenoid) content was 1.866 mg/l and 1.019 mg/l, the 80 ml solvent volume contained total pigment (total chlorophyll and Carotenoid) content were 1.637 mg/l and 0.897 mg/l and the100 ml of solvent volume contained total pigment (total chlorophyll and carotenoid) content was 1.089 mg/l and 0.569 mg/l. The highest pigment (total chlorophyll and carotenoid) content was found on 20 ml solvent ratio.

However, the highest pigment (total chlorophyll and carotenoid) content was found in 20 ml and 40 ml solvent ratio at the same time its gave the high intensity of sweetness sensitization of the tongue taste but 20 ml solvent volume given more intensity of sweetness than 40 ml solvent volume.

4.1 Isolation of stevioside from aqueous extract

After the overnight white color powder was sediment in the bottom. The white color powder was collected and evaporated using a water bath until dryness. The dried powder contained stevioside and some other impurities. The white color powders indicate it contained stevioside and dark color impurities were indicated the plant impurities.

Purification Purity of final stevioside

Figure-4.5: Isolation of stevioside from aqueous extract

According to this figure, this isolated stevioside (white color powder) was not 100% purified but partially purified form. Further purification process will have been suggested to gets more purified form of stevioside.

However, the aqueous extraction is a classical, low cost and easy method for extraction of stevioside from stevia leaves rather than other methods. When isobutanol was treated, stevioside purity was increased.

4.2 Isolation of stevioside from methanol extract

According to figure 4.6, after the overnight white color powder was sediment at the bottom and some solvent were shown in the upper portion. The solvent was removed carefully and again placed in the refrigerator overnight. After overnight, the white color sediment was shaking low heat under the water bath until removing the remaining liquid portion. When the removing was completed the white color powder and very little amount of impurities were shown there. The white color powder was indicated the presence of stevioside and little dark color indicate the presence of impurities.

Figure-4.6: Isolation of stevioside from methanol extract

According to Jaroslav et al., (2007), it could be noticed that, after methanol extraction (20:1 v/w), when isobutanol was treated stevioside purity was increased respectively.

However, methanol was shown the better stevioside extraction ability than aqueous extract.

4.3 Confirmation of stevioside by FTIR analysis

4.3.1 Confirmation of aqueous isolated stevioside by FTIR analysis

Isolated white color powder contained stevioside and it was confirmed by FTIR analysis (model: Perkin Elmer spectrum ir version 10.6.0).

In this figure, the FTIR spectroscopy given two absorption peaks, one of the standard stevioside and another was isolated stevioside. FTIR spectroscopy confirmed that, this isolated white powder contained stevioside. The blue color curve indicated the standard stevioside and red color indicated the isolated stevioside.

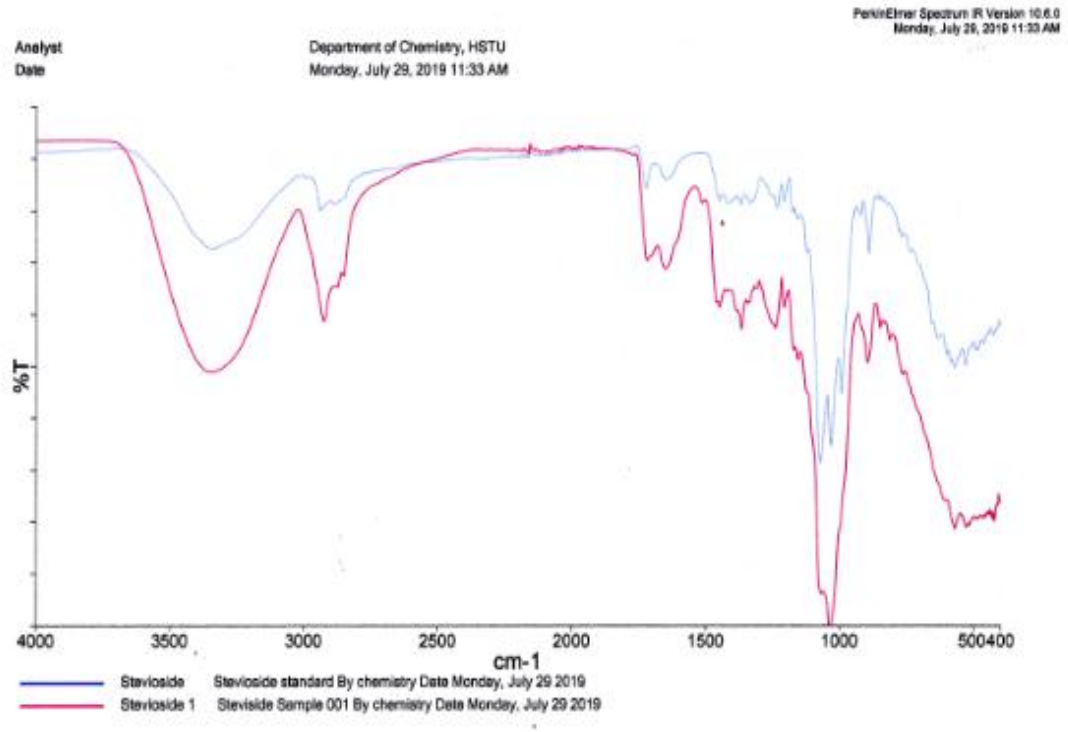


Figure-4.3.1: Absorption peaks of standard and isolated stevioside by FTIR analysis

Table-4.3.1: Absorption of FTIR peaks values of aqueous extract

Model: Perkin Elmer spectrum ir version 10.6.0

List of peak area/ Height

Peak number	X (cm^{-1})	Y (% T)
1	3348.66	68.14
2	2925.17	75.10
3	1718.75	83.28
4	1651.40	82.16
5	1447.34	76.96
6	1366.99	74.00
7	1238.39	74.10
8	1205.96	79.91
9	1033.55	32.78
10	844.68	69.45
11	555.98	45.98
12	523.64	47.14
13	419.06	47.98

In this above table shown that, both absorption peaks start at the same area 3348 cm^{-1}, which indicated it contained alcohol and phenol. The second peaks were started at 2925 cm^{-1} and indicated it contained alkenes. The third peaks were started at 1718.75 cm^{-1} and indicated the ketone and carboxylic acid. The fourth peaks were started at 1651cm^{-1}, which indicated the amino acid. The fifth peaks were started at 1447 cm^{-1} and indicated the aromatics, alkanes. The six peaks were started at 1368 cm^{-1} and indicated the nitro compound. The seven peaks were started at 1238 cm^{-1} and indicated the nitro compound and alkyl halide. The tenth peaks were started at 1033 cm^{-1} and indicated the carboxylic acids and aliphatic amines. The last peaks were started at 523 cm^{-1} and it indicated the disulfide.

4.3.2 Confirmation of methanol isolated stevioside by FTIR analysis

Methanol isolated white color powder contained stevioside and it was confirmed by FTIR analysis. The FTIR spectroscopy confirmed that, this methanol isolated white color powder contained stevioside. But it also contained some other chemical components such as alcohol, phenols, amino acids, alkanes, aromatics compounds, carboxylic acids, aliphatic amines. FTIR spectroscopy obtained a total of 13 peaks.

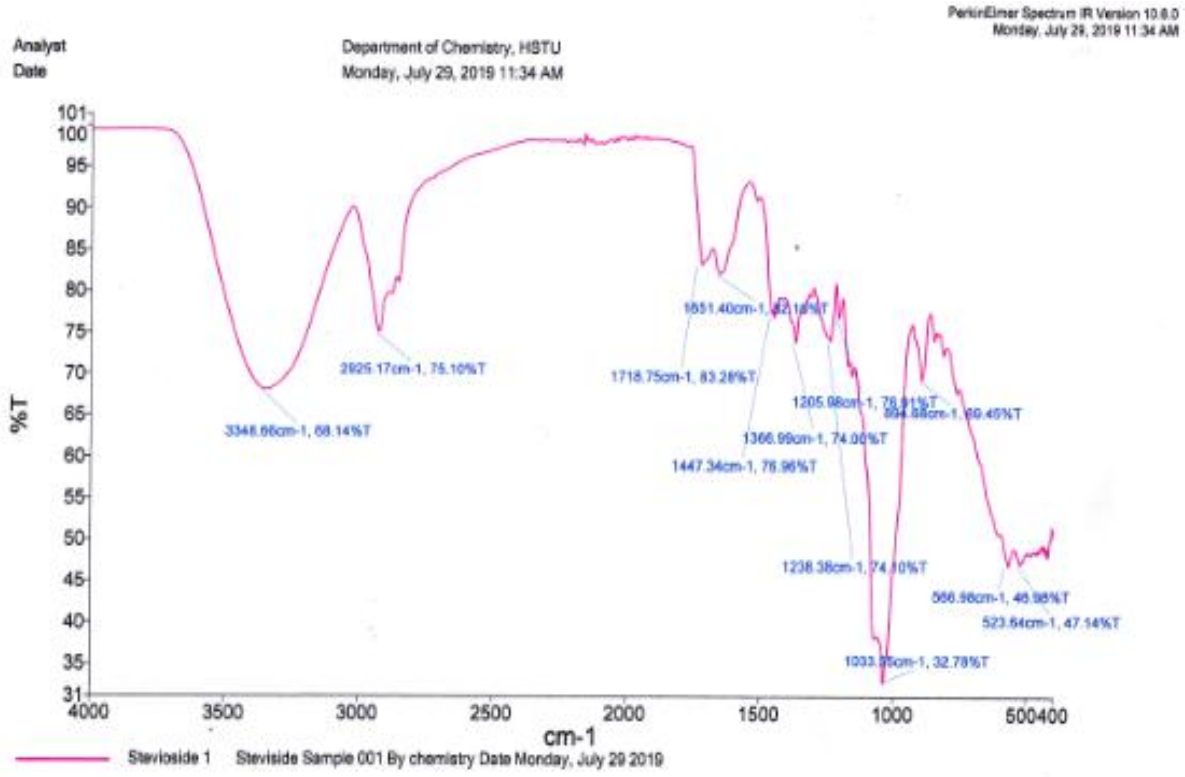


Figure -4.3.2: Absorption peaks of methanol isolated stevioside by FTIR

Table-4.3.2 Absorption of FTIR peaks values of methanol isolation

Model: Perkin Elmer spectrum ir version 10.6.0

List of peak area/ Height

Peak number	X (cm^{-1})	Y (% T)
1	3348.66	68.14
2	2925.17	75.10
3	1718.75	83.28
4	1651.40	82.16
5	1447.34	76.96
6	1366.99	74.00
7	1238.39	74.10
8	1205.96	79.91
9	1033.55	32.78
10	844.68	69.45
11	555.98	45.98
12	523.64	47.14
13	419.06	47.98

In this FTIR analysis, the first absorption peak was started at 3348 cm^{-1}, which indicated it contained phenol. The second peak was started at 2925 cm^{-1} and indicated the alkenes. The third peak was started at 1718.75 cm^{-1} and indicated the ketone and carboxylic acid. The fourth peak was started at 1651 cm^{-1} and indicated the amino acid. The fifth peak was started at1447 cm^{-1} and indicated aromatics, alkanes. The six peaks started at 1368 cm^{-1} and indicated the nitro compound. The seven peaks started at 1238 cm^{-1} and indicated nitro compound and Alkyl halid. The tenth peak was started at 1033 cm^{-1} and indicated the carboxylic acids and aliphatic amines. The last peak was started at 523 cm^{1} and indicated the disulfide groups.

CHAPTER V

SUMMARY AND CONCLUSION

The stevia leaves are sweet in taste and the plant contains a mixture of diterpene glycosides including stevioside, stebiolbioside, rebaudioside- A, B, C, D, E commonly called as steviol glycosides. Stevia leaf contains well-characterized steviol glycosides such as stevioside which is 250 to 300 times sweeter than sucrose. Stevioside is using as a natural sweetener in the world. The long-term use of artificial sweeteners such as cyclamate and saccharine have deleterious side effect but stevia is the safest alternative and is being preferred by the diabetic patients all over the world. Stevioside has been suggested to bring several beneficial effects on human health including antihypertensive, antioxidant, anti-human rotavirus activities, anti-microbial activities, anti-fungal activities. Stevia has a negligible effect on blood glucose. Diabetic persons with hyperglycemia can use “stevioside” as an alternative natural sweetener and it also used a substitute for sucrose, for treatment of diabetes mellitus, obesity, and hypertension. Purification of stevioside is a complex, costly and time consuming process. So, in this case we selected aqueous extract because aqueous extract is a classical, low cost and easily extracting method for stevioside from stevia leaves. Therefore, here we aimed to prepare a suitable aqueous extract of stevia leaf to use in the foods. Aqueous extract of stevia is a non-chemical and economically feasible method. To do this, we selected aqueous extract based on different extraction conditions. In general, stevia extract gives a bitter taste due to the presence of pigments in the extracts. The release of stevioside and the release of pigments in the extract depend on the forms of leaf, time, temperature, and leaf volume etc. Firstly, we prepared stevia aqueous extract from dry whole leaf, leaf powder, and fresh leaf. We measured the pigment contents (total chlorophyll and carotenoid) of the extract and tested the sweetness sensitization on the tongue of the extracts. In dry leaf extract,

pigment (total chlorophyll and carotenoid) contents were found 3.93 mg/l and 2.96 mg/l. In leaf powder extract pigment contents were found 4.023 mg/l and 3.11 mg/l and fresh leaf extract pigment content were found 4.106 mg/l and 3.335 mg/l. The highest pigment (total chlorophyll and carotenoid) contents were found in fresh leaf and leaf powder extracted at the same time they contained low intensity of sweetness sensitization. The lowest pigment (total chlorophyll and carotenoid) contents were found in dry whole leaf extracted at the same time dry leaf extract found high intensity of sweetness sensitization on the tongue. Therefore, we selected dry whole leaf for optimize time, temperature and solvent ratio. This process also conducted based on the pigment release and intensity of sweetness sensitization on tongue taste. The highest pigment (total chlorophyll and carotenoid) content was found when extract time was 60 min, 80 min and 100 min at the same time intensity of sweetness sensitization was low. Lowest pigment (total chlorophyll and carotenoid) content was found when extracts time was 20 min and 40 min but 20 min contained low intensity of sweetness. 40 min extract contained low pigment content at the same time it contained the high intensity of sweetness sensitization.

At temperature, the highest pigment (total chlorophyll and carotenoid) was found when temperatures were 80˚C and 100˚C but intensity of sweetness sensitization was low. The lowest pigment (total chlorophyll and carotenoid) contents were found when temperature was 20˚C but intensity of sweetness sensitization was low. The lowest pigment content and high intensity of sweetness sensitization were found in 40˚C and 60˚C temperatures but 60˚C temperature extract gave the best intensity of sweetness sensitization than 40˚C.

In solvent ratio, the highest pigment (total chlorophyll and carotenoid) content was found in 20 ml solvent at the same time this volume contained high intensity of sweetness. In 40 ml, 60 ml, 80 ml and 100 ml contained low

pigment contents but at the same time it contained low intensity of sweetness because water volume gradually increased. Pigment (total chlorophyll and carotenoid) content was measured based on above result. The pigment content was measured by spectrophotometer using 440, 644 and 662 nm.

We used two different types of extracts, our optimized aqueous extract and methanol extract. In aqueous extract, water has been used for extraction and purification was done by isobutanol. Isobutanol has been used a solvent to carry stevioside from the extract. Aqueous extraction is a classical, low cost and easy method for extraction of stevioside from stevia leaves.

In methanol extraction, methanol has been used for the extraction and ether has been used for removed of impurities from methanol extract. Generally, methanol shows better stevioside extraction ability than aqueous extraction. When isobutanol was treated, stevioside purity was increased. After purification, very little amount of impurities contained this extract. After extraction and purification, we isolated stevioside from this extracts. In aqueous and methanol isolation, we found white color powder at the end.

To confirm the presence of stevioside in the extracts we conducted FTIR analysis of white powder. FTIR analysis revealed the similar peaks of our white powder and the standard. However, some impurities were present in isolated stevioside. Therefore, further purification is needed to get more purified stevioside.

The present investigation reviewing above mentioned the results obtained. In this study, it could be concluded that dry stevia leaf extract given a good effort on pigment released and sweetness sensitization intensity on tongue taste rather than leaf powder and fresh leaf. In dry leaf extract, pigment (total chlorophyll and carotenoid) content was found 3.93 mg/l and 2.96 mg/l and at the same time given best intensity of sweetness sensitization on the tongue. In leaf powder and

fresh leaf extract release high pigment at the same time it given low intensity of sweetness sensitization on the tongue. The optimize extract time was 40 min, temperature was 60^0C and solvent volume was 1:40. The aqueous stevioside extraction method was a simple, easily and cheapest extraction method than methanol extraction. In methanol extract, shown better stevioside extraction and purification ability than aqueous extract. When isobutanol was treated stevioside purity was increased. This purified extract contained white color powder but also contained very little amount of impurities. This isolated white color powder was not 100% purified. To confirm the presence of stevioside in the extracts we conducted FTIR analysis of white powder. FTIR analysis revealed the similar peaks of our white powder and the standard. However, some impurities were present in isolated stevioside. Therefore, further purification is needed to get more purified stevioside.

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APPENDICES

Appendix I: Total chlorophyll contents of different forms of stevia leaf extracts.

Parameters	Dry whole leaf	Leaf powder	Fresh leaf
Total chlorophyll (mg/L)	3.95	4.02	4.05
	3.92	4.02	4.10
	3.92	4.03	4.17
Average	3.93	4.023	4.106
Std.	0.028	0.005	0.058
DMRT	d	d	d
Intensity of Sweetness sensitization on tongue	9.4 ± 0.251^{a}	6.932 ± 0.388^{b}	2.732 ± 0.389^{c}

Appendix II: Carotenoid contents of different forms of stevia leaf extracts.

Parameters	Dry whole leaf	Leaf powder	Fresh leaf
Carotenoid content (mg/l)	3.09	3.11	3.26
	2.95	3.10	3.39
	2.85	3.11	3.36
Average	2.96	3.11	3.335
Std.	0.123	0.006	0.0682
DMRT	c	c	c
Intensity of Sweetness sensitization on tongue	9.4 ± 0.251^{a}	6.932 ± 0.388^{b}	2.732 ± 0.389^{c}

Appendix III: Total chlorophyll contents of different time of dry whole stevia leaf extract.

	20 min	**40 min**	**60 min**	**80 min**	**100 min**
Total chlorophyll (mg/L)	3.98	3.95	4.76	5.27	5.59
	3.18	3.92	4.84	5.24	5.58
	3.25	3.92	4.79	5.22	5.57
Average	3.470	3.930	4.797	5.246	5.580
Std.	0.443	0.017	0.040	0.024	0.010
DMRT	d	c	b	a	a
Intensity of Sweetness sensitization on tongue	2.260±.434 d	8.994±.223a	5.734±.280b	3.9320±.685c	2.666±.472d

Appendix IV: Carotenoid contents of different time of dry whole stevia leaf extract

Parameters	**20 min**	**40 min**	**60 min**	**80 min**	**100 min**
Carotenoid content (mg/L)	2.72	3.09	3.26	3.43	3.50
	2.59	2.95	3.25	3.41	3.38
	2.45	2.84	3.22	3.43	3.37
Average	2.585	2.962	3.241	3.424	3.418
Std.	0.134	0.125	0.021	0.012	0.067
DMRT	d	c	b	a	a
Intensity of Sweetness sensitization on tongue	2.260±.434d	8.994±.223a	5.734±.280b	3.9320±.685c	2.666±.472d

Appendix V: Total chlorophyll contents of different temperature of dry whole stevia leaf extract.

Parameters	**20°C**	**40°C**	**60°C**	**80°C**	**100°C**
Total chlorophyll (mg/L)	2.41	4.03	4.84	5.95	8.50
	2.53	3.96	4.88	6.16	9.02
	2.42	3.97	4.96	6.22	8.86
Average	2.417	3.992	4.919	6.130	8.739
Std.	0.068	0.038	0.059	0.145	0.263
DMRT	e	d	c	b	a
Intensity of Sweetness sensitization on tongue	1.198 ± 0.181^{e}	3.734 ± 0.760^{c}	8.934 ± 0.548^{a}	7.000 ± 0.233^{b}	2.800 ± 0.447^{d}

Appendix VI: Carotenoid contents of different temperature of dry whole stevia leaf extract.

Parameters	**20˚C**	**40˚C**	**60˚C**	**80˚C**	**100˚C**
Carotenoid content (mg/L)	0.67	0.73	0.98	1.80	1.92
	0.55	0.71	0.99	1.71	2.75
	0.51	0.73	0.84	1.74	3.08
Average	0.576	0.723	0.934	1.749	2.581
Std.	0.083	0.011	0.085	0.044	0.597
DMRT	c	c	c	b	a
Intensity of Sweetness sensitization on tongue	1.198 ± 0.181^{e}	3.734 ± 0.760^{c}	8.934 ± 0.548^{a}	7.000 ± 0.233^{b}	2.800 ± 0.447^{d}

Appendix VII: Total chlorophyll content on the tongue of dry whole leaf with different solvent ratio (leaf: water) of stevia leaf extract.

Parameters	**20 ml**	**40 ml**	**60 ml**	**80 ml**	**100 ml**
Total chlorophyll (mg/L)	2.63	2.38	1.91	1.65	1.12
	2.53	2.37	1.81	1.62	1.09
	2.44	2.37	1.88	1.64	1.06
Average	2.532	2.3747	1.866	1.6373	1.0893
Std.	0.09606	0.00862	0.05534	0.01531	0.03201
DMRT	a	b	c	d	e
Intensity of Sweetness sensitization on tongue	8.732 ± 0.547^{a}	5.398 ± 0.547^{b}	3.668 ± 0.23^{c}	2.332 ± 0.237^{d}	$.835\pm0.191^{e}$

Appendix VIII: Carotenoid content on the tongue of dry whole leaf with different solvent ratio (leaf: water) of stevia leaf extract.

Parameters	**20 ml**	**40 ml**	**60 ml**	**80 ml**	**100 ml**
Carotenoid content (mg/L)	1.70	1.46	1.03	0.89	0.58
	1.73	1.52	1.01	0.88	0.57
	1.73	1.45	1.02	0.93	0.56
Average	1.717	1.479	1.019	0.897	0.569
Std.	0.017	0.037	0.012	0.025	0.014
DMRT	a	b	c	d	e
Intensity of Sweetness sensitization on tongue	8.732 ± 0.547^{a}	5.398 ± 0.547^{b}	3.668 ± 0.23^{c}	2.332 ± 0.237^{d}	$.835\pm0.191^{e}$