A DISSERTATION ON

Improving product quality by Manoeuvring the extraction of Steviol glycosides from Stevia leaves

SUBMITTED TO THE
DEPARTMENT OF BIOENGINEERING
FACULTY OF ENGINEERING
INTEGRAL UNIVERSITY, LUCKNOW

IN PARTIAL FULFILMENT
FOR THE
DEGREE OF MASTER OF TECHNOLOGY
IN BIOTECHNOLOGY

BY
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UNDER THE SUPERVISION OF
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DECLARATION FORM

I, **Farhana Tarannum**, a student of **M.Tech Biotechnology** (2^{nd} Year/ 4^{th} Semester), Integral University have completed my six months dissertation work entitled **"Improving product quality by Manoeuvring the extraction of Steviol glycosides from Stevia leaves"** successfully from **Integral University** under the able guidance of **Er. Soban Ahmad Faridi.**

I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

Farhana Tarannum

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It is hereby certified that **Ms. Farhana Tarannum** (Enrollment Number 2000100841) has carried out the research work presented in this thesis entitled **"Improving product quality by Manoeuvring the extraction of Steviol glycosides from Stevia leaves"** for the award of **M.Tech Biotechnology** from Integral University, Lucknow under my supervision. The thesis embodies results of original work and studies carried out by the student herself and the contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University/Institution. The dissertation was a compulsory part of her **M.Tech Biotechnology** degree.

I wish her good luck and bright future.

Er. Soban Ahmad Faridi
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I wish her good luck and bright future.

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TO WHOM IT MAY CONCERN

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I wish her good luck and bright future.

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ACKNOWLEDGEMENT

First of all, I bow in reverence to the Almighty for blessing me with strong will power, patience and confidence, which helped me in completing the present work.

At the very outset I pay my warm thanks to our Honorable **Chancellor and Founder, Integral University, Lucknow. Prof. S.W Akhtar** for providing excellent infrastructure and Lab facilities at IIRC-7 Integral University, Lucknow. I am also grateful to Honorable **Vice Chancellor Integral University, Lucknow. Prof. Javed Musarrat** for his continuous motivation and a Special vote of thanks to **Pro chancellor, Dr. Nadeem Akhtar** for his encouragement and support.

I would like to express my special thanks to **Dr. (Er.) Alvina farooqui (Head, Department of Bioengineering)** for given me an opportunity to join the department laboratory and providing all the necessary facilities ever since I started my work.

I would I like to express my deep sense of gratitude to my supervisor **Er. Soban Ahmad Faridi (Assistant Professor),** internal advisor **Er. Adnan Ahmad (Assiatant Professor)**, and course coordinator **Dr. Salman Akhtar (Associate Professor)** for their invaluable guidance throughout the course of my dissertation work and academic session. It would have been impossible to complete this work in so short a time without her constant guidance.

I would like to thank respected dissertation coordinators **Dr. Roohi, Dr. Owais Yousuf, Er. Khwaja Osama** for their continuous support and help.

My acknowledgement will be incomplete if I do not mention **my parents and my family** with whose blessing; I was able to achieve my goal successfully. There are no words to express my feelings toward them. I silently acknowledge my debt to them.

I also like to acknowledge **Advanced Centre for Bioengineering and Bioinformatics (ACBB-IIRC-7)**, Integral University for providing facilities required for completion of my dissertation.

Date: **FARHANA TARANNUM**

LIST OF ABBREVATIONS

HPLC	High Performance Liquid Chromatography
NIRS	Near Infra Red Spectroscopy
SPE	Solid Phase Extraction
RSD	Relative Standard Deviation
SCFE	Super Critical Fluid Extraction
MAE	Microwave Assisted Extraction
TLC	Thin Layer Chromatography
EFSA	European Food Safety Committee
GRAS	Generally Recognized AsSafe

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1. Introduction

Stevia is sweet in nature and a native of Paraguay, thus it's known as the "sweet herb of Paraguay." it's also called honey leaf, sweet leaf, sweet herb, candy leaf, and honey Stevia, botanically famed as *Stevia rebaudiana* Bertoni (Family-Asteraceae) is a sweet herb. The leaves are delicate green and intensely sweet. The compounds within the leaves are known as stevioside and rebaudioside and they are more than two hundred times sweeter than sugar (Anon, 2004). Its leaves contain just about 100 percent of stevioside that are intensely sweet compounds. The leaves have been historically used since millenniums in Paraguay and Brazil to sweeten native teas, medicines and as a "sweet treat". There are currently over a hundred and fifty species of Stevia grown in world (Carakostas et al, 2008). Figure 1.1 shows a Stevia plantlet, planted in soil.

Even although there are more than two hundred species of the genus Stevia, only *S. rebaudiana* offers the sweetest essence (Savita et al, 2004). Stevia has a four-year life span and yields three to four crops per year, with an initial investment of 3.7 lakh ha^{-1}, a farmer will earn about 2 lakh each year, for four years. The yearly yields are often within the range of 7.41-9.88 tons ha^{-1}. The leaves of *Stevia rebaudiana* are sold at about 200 kg $^{-1}$, thus the dried leaves are therefore economically helpful to growers (Sharma and Chattopadhya, 2007). Stevia has been approved for many years in Brazil, Argentina, and Paraguay, similarly as in China, Korea, and Japan to sweeten soft drinks, soy sauce, yogurt, and different foods, whereas within the United States they are used as dietary supplements since 1995.Although Stevia has been in use in Asia and Europe for years, it was only within the past few years that it extremely started to capture attention in the Indian market as a healthy alternative, zero calorie, sweetener substitute to sugar.

Nor saccharin, neither calcium cyclamate, nor aspartame is present in Stevia and it is also calorie free. It is safe for diabetics, because it doesn't have an effect on blood glucose levels; it doesn't have the neurologic or renal side effects associated with some of the artificial sweeteners. The main advantage of stevioside over different sweeteners is that it is stable at 100°C (Buckenhuskers and Omran, 1997). Apart from this, Stevia is nutrient wealthy, containing substantial quantity of protein, Magnesium, Miocene, Riboflavin, Zinc, Chromium, Selenium, calcium & Phosphorus, besides Stevia also can be used as a household sweetener in preparation of most Indian sweets (Anon,

2004). A Stevia product has ample quantity of medicative usage and benefits for diabetic and blood pressure patients. Stevia sweetener extractives have been suggested to exert useful effects on human health, together with anti-hypertensive (Lee et al, 2001), antioxidant (Xi et al, 1998), anti-human rotavirus activities (Takahashi et al, 2001). Extraordinary antimicrobial activity of Stevia has conferred it as a potent non antibiotic pharmaceutical and an efficient food preservative (Ghosh et al, 2008). Reports have proven that it's safe for consumption with none health risks. Thereafter, Stevia emerged jointly of the most effective alternative sources of sweeteners (Savita et al, 2004).

Stevioside offers the impression of slightly bitter taste, whereas rebaudioside A contributes to the sweet taste (similar to sucrose) (Singh and Rao, 2005). Stevia is employed in form of fresh leaves, powder and liquid extract. Historically, it is used as dried leaf or fresh leaves directly, however it leaves the sediments in domestic cookery and wastage is more. So, the extraction of stevioside from Stevia leaves is more helpful and economical. The main sweet element within the leaves of *Stevia rebaudiana* is stevioside and totally different technologies are out there for extraction of stevioside. Some of them are extracted with hot water followed by separation, filtration, and crystallization and drying. Extraction with hot water includes the boiling the leaves in hot water to dissolve glycoside and filtering the liquid by precipitation. This filtered liquid is then concentrated and resin exchange is employed to separate the glycoside into high and low R-A fractions and crystallization and drying is done to get Stevia crystals. Boiling water extraction will attain 93-98% extraction of stevioside (Midmore and Rank, 2006). Hot-water treatment has been used as a classical extraction technique (Dacome et al, 2005). However, it ought to be noted that hot-water extraction is associated with long extraction time and high temperature.

Yoda et al, (2003) studied the supercritical fluid extraction and the kinetics of the glycosides from Stevia leaves. The results showed the yield was around 1.6%. Zhang et al, (2000) mentioned the use of membrane separation technology to manufacture Stevia extracts without residual taste. However, all these processes have complicated steps and there was no data regarding the consequences of extraction methodology on the contents of stevioside and rebaudioside A within the extracts obtained. In solvent extraction, totally different solvents are used to dissolve glycoside from leaves and this method is continual until we have a tendency to get miscella of

high glycoside content. Then this miscella is desolubilized, pure 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 and microwave assisted extraction have been used for extracting stevioside content. Microwave assisted extraction is gaining attention, primarily due to quicker extraction, reduced solvent, will increase recovery, saves time and energy consumption in comparison to typical ways of extraction. Bondarev et al, (2003) studied steviol glycoside content in numerous organs of *Stevia rebaudiana* and its dynamics during growth with HPLC. Kolb et al, (2001) developed an improved HPLC technique for quality management of stevioside and rebaudioside A contents in dried leaves of *Stevia rebaudiana*. These ways study one variable at a time, however in extraction method there are multiple independent variables affecting the extraction process. Therefore, it is necessary to use optimization techniques for improvement of completely different method parameters concerned in the extraction process of stevioside from Stevia leaves (Chaturvedula et al., 2011).

Stevia is a generic term for a food ingredient derived from the plant Stevia rebaudiana (Carakostas, 2008). Stevia is also known as sweet leaf or sugar leaf due to the high levels of sweetener found in its leaves (Kansaf, 2004). Stevia has numerous industrial applications in food and beverages, as well as medical applications such as low uric acid treatment, anaesthetic, and anti-inflammatory properties (Jayaraman et al., 2008). According to Gardana et al. (2003), the extraction of Stevia sweetener has beneficial effects on human health, including antioxidant, carcinogenic, and anti-human rotavirus. Previously, several toxicological studies were conducted to confirm the possible mutagenic and genotoxic effect of Stevia extracts on bacterial cells and mammalian species. Stevia leaf extract contains a complex mixture of compounds, including glycosides such as stevioside and rebaudioside A. The residual flavour associated with Stevia extracts is caused in part by the glycoside and other compounds such as terpenes (Guzen et al., 2002).

Glycosides are organic compounds that have both a sugar component and no sugar component. The sugar component of hydrolysis is known as glycone, and the non-sugar

component is known as aglycone (Elkin, 1997). Rhamnose, fructose, glucose, xylose, and arabinose are examples of glycone constituents.

Stevioside is a diterpene glycoside found in S. rebaudiana (Genus, 2003). Based on the hydrolysis of stevioside with crude hesperidinase, a simple enzymatic method for determining stevioside from S. rebaudiana is described. The reaction is then monitored for glucose production using a glucose oxidase-peroxidase-2 system (Mizukami et al., 1982).

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Numerous scientific organizations and specialists, notably the EFSA and most recently, Magnuson et al., have evaluated the absorption, digestion, and defecation of steviol glycosides in great detail (Magnuson BA et al., 2016). Steviol glycosides pass through the upper gastro-intestinal system undigested. The steviol backbone is retained, absorbed systemically, glucuronidated in the liver, and eliminated through urine in humans and faeces in rats. They are only hydrolyzed or destroyed when they come into contact with bacteria in the colon that cleave the glycosidic bonds, eliminating the glycon (Magnuson BA et al., 2016).

Studies conducted in vitro reveal that the glycosidic linkages found in stevioside cannot be hydrolyzed by human saliva, salivary-amylase, pancreatin, pepsin, and pancreatic-amylase, as well as jejuna brush border enzyme from mice, rats, and hamsters. Therefore, stevioside can be transformed into steviol by the gut micro-biota of humans, rats and hamsters (Hutapea AM et al., 1997).

Reb A and stevioside were both entirely hydrolyzed to steviol in 10 and 24 hours, respectively, when they were incubated with human faecal bacteria. It is a zero-calorie sweetener since the released glycon is not absorbed and is likely used as an energy source by the gut microorganisms soon. Steviol is not metabolised by gut bacteria and is instead absorbed from the colon, according to an in vitro model of the intestinal barrier, which has demonstrated that the transport of Reb A and stevioside through the mono layers is

very low, whereas the transport of steviol is very high. Through the action of their glycosidase, Bactericides species are principally in charge of the hydrolysis of steviol glycosides in the stomach (Gardana C et al., 2003).

Fig 1.1 *Stevia rebaudiana* plant.

2. Review of Literature

The literature associated with review of the current study has been reviewed as under: the foremost favored method for isolation of glycosides involves four steps; liquid or solvent extraction, ion exchange precipitation or coagulation with filtration, then crystallization and drying. Ahmed and Dobberstein (1982) extracted stevioside and rebaudioside A and C from the dried leaves of *S. rebaudiana* in a micro soxhlet equipment. They determined that chloroform/methanol provided best results, compared to chloroform.

Potential sweetening agents of plant origin and field look for sweet-tasting Stevia species were studied by Soejarto et al, (1983). Field work in Paraguay, Peru, Colombia and Mexico States, as well as field organoleptic tests and interviews, was administered in search of sweet tasting Stevia species. The information obtained showed that leaves of no different Stevia species studied possessed a potent sweet taste like that of *Stevia rebaudiana* leaves. Nishiyama et al, (1992) used the close to Infrared coefficient qualitative analysis (NIRS) for the analysis of stevioside in *Stevia rebaudiana* leaves with same accuracy as obtained by HPLC. The leaves were extracted with close to boiling water and therefore subjected to HPLC analysis. For NIRS analysis, leaves were ground employing a cyclone mill fitted with 1.0 mm screen, NIRS standardization was developed from 64 samples covering the vary of stevioside commonly found in *Stevia rebaudiana* leaves (4-13%). The result prompt that NIRS was a particular and simple methodology for routine stevioside determinations in *Stevia rebaudiana*. Extraction of stevioside, rebaudioside A and C and dulcoside was additionally performed by super critical fluid extraction methodology exploiting carbon dioxide, water and methanol as modifier by Liu et al, (1997). The extraction conditions were optimized and extraction potency of over 88% was obtained. Such extraction technique has been gaining quality as are associated analytical tool as a result of their fast, easy and cheap result delivery.

The stevioside content in plant material and food samples determined using HPLC by Bovanova et al, (1998), similarly, HPLC methodology for determination of sweet-tasting stevioside within the leaves of the plant of *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 utilizing boiling water and a solid-phase extraction (SPE) matrix. 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 variance (RSD) ≤ 3.3%). The chromatographic separations, intended, were accomplished and the boundaries of determination of STS were 5 μg/ml for stevia leaf extract and tea sample whereas it had been 8μg/ml for the juice sample.

Selectivity of compound adsorbent in adsorptive separations of Stevia diterpene glycosides was studied by Chen et al, (1998). Some hydrophobic (including each, the nonionic and polar) and hydrophilic compound adsorbents were designed and synthesized, and their sorption properties and sorption mechanism toward Stevia glycosides were studied in complete detail. The skeleton structure and polarity of the resins had resulted on the sorption capability and also the selectivity properties throughout the sorption of stevioside and rebaudioside A. A sweetener with high rebaudioside content was isolated by targeting the sorption property of the polar resins.

Steviol glycosides were extracted by super critical fluid extraction (SCFE) methodology exploiting carbon dioxide 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 bars, 16°C, below this condition, the total yield was 3.4% and the standard of the glycoside fraction with reference to its capability as sweetener was higher for SCFE extract as compared to extract obtained by standard methods. The extraction curves were well represented by Lack extended model (Pasquel et al, 2000).

Zhang et al (2000) studied the method of extraction and purification of sweeteners with reduced range of unit operations and minimization or elimination of chemical usage as well as organic solvents. Water was very effective for extracting glycosides at chosen pH and temperature. It had been additionally shown that a multistage membrane extraction method can be successfully be able to concentrate glycosidic sweeteners and bitter tasting components can be washed out from the sweetener concentrate by using Nano-filtration method. Supercritical fluid extraction and liquid chromatographic-electro spray mass spectroscopic analysis of

stevioside from *Stevia rebaudiana* leaves was studied by Choi et al, (2002). In developing an alternative extraction methodology for stevioside exploitation SCFE, the result of temperature, pressure, and proportion of modifier was evaluated on the extraction yield. Though adequate extractability was not obtained by pure carbon dioxide under any conditions of temperature and pressure, the addition of a modifier dramatically improved the extraction yield of stevioside, creating its equivalent to organic solvent extraction. Among the modifiers evaluated, the mixture of methyl alcohol 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 times of standard organic extraction. Additionally, to raising the extraction yield, SFE clearly provided a better purity of stevioside within the final extract.

The estimation of glycosides from *Stevia rebaudiana* was studied by Kovylyaeva et al, (2007). A proposal was given in which new strategy was highlighted for the isolation of rebaudioside A and C and glycosides stevioside from the *Stevia rebaudiana* leaves. In plants grown in Ukraine (Crimea) and Russia (Voronezh Oblast), the glycoside levels consisted of 0.3-1.3% (Rebaudioside A and C) and 5-6% (stevioside) as per according to HPLC.

The comparison of two totally different solvents methanol versus water was studied by Pol et al, (2007). They studied that the pressurized fluid extraction using water or methanol was utilized for the extraction of stevioside from *Stevia rebaudiana* Bertoni. The extraction technique was optimized in terms of temperature and time period. The analysis of extracts was done by liquid chromatography followed by ultraviolet (UV) and mass-spectrometric (MS) detections.

Thermal degradation of stevioside was similar in each solvents among the range 70–160°C, methanol showed higher extraction ability for isolation of stevioside from Stevia rebaudiana leaves than water among the range 110–160°C.

Stevia rebaudiana Bertoni plants grown up *in vitro* and *ex vitro* were investigated by Rajasekaran et al, (2007) for variation within the profile of stevioside in their leaves, shoots, root and flower. Esterification and chemical reaction were used to extract stevioside, evaporation to xerotes and dissolved in methanol for measurement by HPLC. The HPLC analysis and separation profiles indicated the presence of eight legendary sweet diterpene glycosides. Leaves which were only 30 days old gave

the best records for stevioside content (64.80 g/kg dried plant material) and in vitro (0.99g, Rebaudioside A /kg dried leaves plant material).

Wang et al (2007) applied Microwave-assisted extraction (MAE) for pectin extraction from the dried apple pomace and response surface methodology (RSM) was used in the optimization of the results of parameters of extraction on the yield of pectin. Four independent variables like extraction time (min), pH scale of HCl solution, solid; liquid quantitative relation and microwave power (W). The optimum conditions were determined and tri dimensional response surfaces were plotted from the mathematical models.

The F-test and p-value indicated that the extraction time as well as pH scale of HCl solution had extremely important impacts on the response worth conjointly the quadratic of microwave power also displayed vital effect, followed by the interaction effects of pH scale and solid:liquid quantitative relation. The best and most suitable condition for the extraction of pectin was, the pH should be 1.01, microwave power should be 499.4W, solid liquid quantitative relation should be 0.069 and extraction time should be minimum 20.8 min, considering the potential of the experiment. Application of MAE within the extraction from dried apple pomace dramatically reduced extraction time. The optimum expected pectin yield of 0.315 g from the dried apple pomace (2 g) was obtained. Such an agreement between experimental and expected yields was obtained.

The plastid ultrastructure, the production of steviol glycosides and photosynthetic equipment activities in *Stevia rebaudianain vivo* as well as *in vitro* were studied by Lady Gin et al, (2008). The buildup of steviol glycosides (SGs) in cells of *Stevia rebaudiana* Bertoni *in vivo* as well as *in vitro* was associated with the extent of the growth of the membrane system of chloroplasts and therefore the content of photosynthetic pigments. Chloroplasts of the in vitro plants, not like those of the intact plants, had poorly developed membrane system. Leaves of *in vivo* plants accumulated larger quantity of the pigments than leaves of the *in vitro* plants. The callus tissue fully grown within the dark contained simply trace amounts of the pigments.

Leaves of the intact and also the *in vitro* plants didn't exhibit any important variations in photosynthetic O_2 evolution rate. However, photosynthetic O_2 evolution rate within

the callus cells was a lot of less than that within the differentiated plant cells. The *in vitro* cell cultures containing simply proplastids didn't much manufacture SGs. However, when transferring these cultures within the light, each the formation of chloroplasts and also the production of SGs in them were detected.

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

Extraction of Stevia by three strategies, firstly, by hot water (65ºC) at completely different ratios of leaves to water (1:15 – 1:75) was studied by Abou-Arab et al, (2010). The optimum ratio was 1:35 during which the most stevioside content was obtained (7.53%), recovery of stevioside was 80.21%. The second technique, extraction by methanol at ratio of 4:1 methanol/leaves, the recovery was 94.9%.The third technique of extraction by mixture of methanol/water (4:1), the recovery was 92.34%.

Inamake et al, (2010) tried to isolate stevioside from the dried leaves of Stevia in its purest type. Isolated stevioside was purified, analyzed with the help of numerous chromatographic& analytical ways together with thin layer chromatography (TLC), UV, Fourier transform infrared spectrometry (FTIR), nuclear magnetic resonance spectrometry (NMR) and HPLC ways. 0.32 was the R_F value for TLC, λ_{max} of UV spectra was obtained at 333 nm and the sharp peak showed by the HPLC was with 1.958 min retention time. The isolated stevioside was conjointly compared with standard stevioside with all analytical ways.

The nutrient composition of cultivated Stevia leaves and also the influence of polyphenols and plant pigments on sensory and inhibitor properties of leaf extracts was studied by (Kaushik et al, 2010; Castro-Muñoz et al., 2022).The leaf and its extract though sweet have a bitter taste also that precludes industrial satisfactoriness. The composition of the leaf mirrored a high nutritious value and polyphenol concentration

averaging 4.15% by weight of dried leaf. Presence of polyphenols influenced the satisfactoriness of the sweeteners marginally, whereas chlorophyll was found unacceptable in any of the extracts. The antioxidant activity of the extracts was synergistic onceit had been mixed with coffee and juice. Complete purification of Stevia leaf extracts to get pure glycosides isn't necessary for it to become a commercially acceptable sweetener.

The stability of the natural sweetener stevioside throughout completely different process and storage conditions was studied by Kroyer (2010). Incubation of the solid sweetener stevioside at elevated temperatures for 1 hour showed sensible stability up to 120°C, whereas at temperatures surpassing 140°C forced decomposition was detected. In aqueous solutions stevioside is outstanding stable during a pH scale range 2–10 below thermal treatment up to 80°C. However, below sturdy acidic conditions (pH= 1) a big decrease within the stevioside concentration was detected.

Liu et al (2010) maximized the yield of total carbohydrates from *Stevia rebaudiana* Bertoni, response surface methodology (RSM) was used to optimize the ultrasound assisted extraction condition. The results indicated the best extraction conditions were associate degree extraction temperature of 68ºC, a sonic power of 60 Watt associate degreed an extraction time of 32 min. By the use of the ultrasound-assisted extraction, the yield of extracts redoubled by an element of 1.5 at the lower extraction temperature (68ºC) and also the extraction time (32 min) considerably shortened compared with that of classical extraction. The parts analysis of crude extracts unconcealed that the relative quantity of rebaudioside A redoubled within the ultrasound assisted extracts as compared with extracts obtained by classical method, and also the ultrasound assisted extracts had higher quality.

The improved HPLC methodology for the analysis of the key steviol glycosides in leaves of *Stevia rebaudiana* was studied by Rieck et al, (2010). A straightforward reversed-phase high-performance liquid chromatographic methodology has been developed for the determination of the key steviol glycosides, the diterpene sweeteners derived from *Stevia rebaudiana*. The strategy relies on a water extraction step and a solid-phase extraction (SPE) clean-up. The applicability of this methodology was demonstrated within the analysis of stevioside and rebaudioside A from Stevia plants grown up in 2totally different areas in European nation. Stevioside

and rebaudioside A contents showed statistically important variations (f and t-test) between the 2 harvests. However, the overall concentrations (>12%) and also the quantitative relation of stevioside to rebaudioside A (6:4) were kind of like those found within the countries from which *Stevia rebaudiana* originates. Supported a comparison of yields from totally different harvests, we have a tendency to mentioned whether or not *Stevia rebaudiana* may be economically grownup within the temperate zones of the northern European hemisphere.

A new improved method of extraction of steviosides from the Stevia leaves was established Rao et al (2012) during which the dry treated leaves were grounded, defatted, and extracted through pressurized hot water extractor (PHWE), followed by purification of the sweet glycosides through ultra (UF) and Nano (NF) membrane filtration in getting high (98.2%) quality steviosides. This method established "green" methodology for isolation of prime quality steviol glycosides, with improved final yield is 10.1% from 11 ml of crude leaf extract and discovered the improved organoleptic and biological activity (antioxidant). Therefore the strategy confirms a straightforward, cheap and eco-friendly method in getting pure steviosides by easy extraction and membrane purification method for isolation of steviosides with improved organoleptic activity.

Steviol glycosides (stevioside and rebaudioside), which are heat-and pH-resistant, 30 to 150 times sweeter than table sugar, and not fermentable, are the active ingredients. The excessive consumption of sugar is becoming increasingly risky because it leads to numerous health issues, particularly diabetes. To protect the health of customers, a replacement sweetener must be created. In the recovery process known as solid-liquid extraction, specific components of a solid material are recovered by the extracting solvent. Steviol glycosides from Stevia plants have been extracted using a variety of techniques. One of these entails environmentally friendly glucose extraction using ethanol and water (Muthusamy et al., 2019).

Table sugar is typically used as the primary sweetening agent in the food sector. Customers are becoming more interested in natural source sweeteners based on Stevia. Additionally, the sweetener is risk-free for consumption, has nearly little calories, is unaffected by heat and pH changes, and tastes just like sugar. The daisy family includes the herb Stevia, which has a pleasant flavour (Planas G et al., 1968).

It is critical for the production of steviol glycosides to extract and purify them from plant material in a way that yields a high purity of unaltered molecules. Dehydration or drying of plant material is required as a first step to prevent microbial development and biochemical changes. Common dehydration techniques include freeze drying, convection drying, vacuuming, microwave drying, infrared drying, sun drying, and shadow drying. The drying conditions used have a significant impact on the amount of total and specific steviol glycosides extracted from fresh Stevia leaves. Dehydration or drying of plant material is required as a first step to prevent microbial development and biochemical changes. Shade drying has been claimed to be the least harsh treatment for all steviol glycosides (Periche et al., 2015).

However, drying in the shade increases the risk of contamination, which reduces the quality of the organic material. A variety of traditional and cutting-edge technologies are used to extract steviol glycosides, including heat extraction and maceration, high pressure and high temperature, radiation, chromatographic, electrical voltage, and ultrasound methods. Depending on the solvent and method used, the extraction yields obtained using these technologies range between 2 and 35 percent. Applying these technologies to large-scale production is difficult due to the low percentages and vast volumes of organic solvents that are eliminated later in the refining process. Fibroblast technologies are now being proposed as one of the efficient methods of extracting Steviol glycosides. These techniques, which include microfiltration, ultrafiltration, and nanofiltration, are still in the early stages of development (Díaz-Montes et al., 2020).

Semi permeable membranes are commonly used in pressure-driven membrane processes as a protective layer for both the movement of chemicals inside a solution and the Membrane processes have many advantages, including low energy consumption, shorter extraction times, improved dispersibility, adaptability, productivity increases, and ease of leveling. Furthermore, because successful extraction requires only a small amount of chemical solvent, membrane techniques are friendly to the environment. Steviol glycosides are particularly interested in this because of their intended use in the pharmaceutical and food industries. According to the most recent data, the removal efficiency of stevioside and rebaudioside A, as well as their cleanness, are now in the spectrum of 19 to 90 percent and 32 to 98 percent, respectively, when using integrated membrane methods.

The value of the extraction yield is affected by a number of variables, including operational factors, inherent membrane characteristics, and pretreatment procedures (Melis M et al., 1999). Last but not least, it's critical to take into account the potential for using a "green" solvent to maximize the extraction of steviol glycosides. These solvents are distinguished by minimal toxicity, simple accessibility, ease of reusability, and great efficacy. Additionally, the development of "green" solvents for the extraction of significant trade chemicals is becoming an increasingly essential area of research due to legislative considerations and the changing attitude toward environmental issues. Although it seems that water, an all-purpose solvent, is sufficient for the extraction of steviol glycosides, it cannot be completely ruled out that the recovery of these compounds can benefit from the use of other "green" solvents (Castro-Muñoz, R et al., 2022).

Table 2.1: Physical properties of stevioside.

Matrix	**Physical properties of stevioside**
Chemical abstract Name	Kaur-16-en-18-oic acid, 13(2-0-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy)-β-D-glucopyranosyl ester,(4α)-(9CI)
Others Names	1H-2,10a - Ethanophenathrene, Kaur-16-en-18-oic acid Derivestevioside (6CI, 7CI), α-G-sweet, steviosin.
Molecular formula	C_{38} H_{60} O_{18}
Molecular weight	804.88
Melting point	196-198 ºC
Solubility	Water, ethanol, dioxane, not in methanol.
Storage temperature	Store at 4 ºC, in dark places.
PKa	12.52±0.70,most acidic
Toxicity	Non toxic
Polarity	Polar
Optical rotation	-39.3º in water
Wave length maximum	200 mm

3. Aim and Objectives

1. To evaluate extraction of Steviol glycosides using different solvents.
2. To analyze the extraction method for desired product quality.

4. Materials and methods

Procurement of leaves

The green leaves of the morita variety were procured from local supplier.

Drying of leaves

Drying was carried out in different batches. The leaves were placed in Hot Air Oven at 50ºC until similar consecutive weights were achieved. Drying plots were studied by the obtained data of weights.

Grinding of leaves

The dried Stevia leaves were subjected to grinding in a 600 watt grinder to make fine Stevia powder, comprising of different particle size.

Size screening

The grinded Stevia leaves were screened for different particle size using ASTM standard sieves for the separation of fine material from coarse material by passing the Stevia powder through different mesh size. The screened particles of different sizes were segregated in different air tight food grade containers, for further defatting and extraction steps.

Soxhlet Apparatus

Soxhlet apparatus is an equipment to be used in extraction with organic solvent (such as ether, hexane, alcohol, or benzene) or inorganic solvent. Consisting of a vertical glass cylindrical extraction tube that has each a siphon tube and a vapor tube, that is fitted to a condenser at the top end and at its lower finish to a flask in order that the solvent could also be distilled from the flask into the condenser wherefrom it flows back to the cylindrical tube and siphons over into the flask to be distilled once more**.**

It mainly consists of three parts – a percolator (boiler), solvent is circulated by the boiler. A thimble, the solvent which is to be extracted is retained in thimble, and a siphon mechanism, which empties the thimble periodically. A pictorial representation of the soxhlet apparatus is shown in Figure 3.1.

The extraction procedure: The compound whose extraction is to be done is placed inside the thimble. Then the loading of thimble is done into the soxhlet extractor. The distillation flask is filled with the solvent. Heating element is used for providing heat

to the flask, which is placed on the heating mantle. Then the soxhlet extractor is fitted on top of the flask. The condenser is then fitted over the extractor.

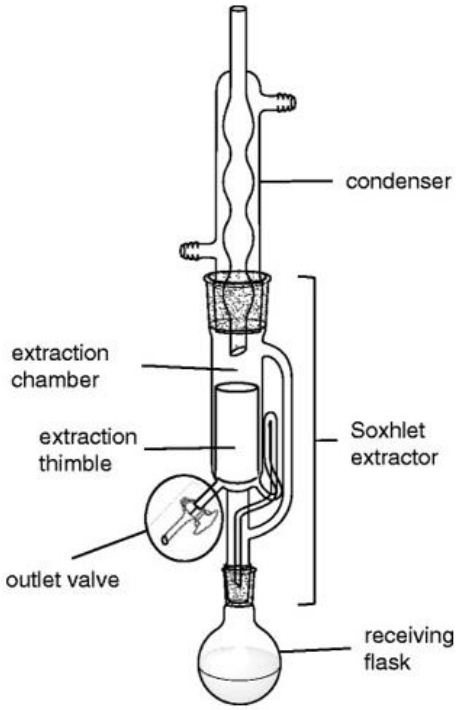

Fig. 3.1 Soxhlet Apparatus

Defatting of leaves sample

First of all, we have to dry the material and remove moisture in order to facilitate entry of the organic solvent, because moisture restricts the entry of organic solvent. Fat is soluble in organic solvent and insoluble in water, because of this, we use organic solvents like hexane. Hexanes have the ability to solubilize fat. Later the fat is collected by evaporating the solvent.

The dried, grinded leaf samples were weighed and packed in packets of blotting papers and wrapped in gauze bandages, these were placed in the soxhlet thimble and 300 ml hexane was poured into it. The heating mantle was operated at 40% duty cycle. The condenser was connected to a manually prepared chiller setup as shown in figure 3.2 below. The defatting process was continued until the solvent was completely colorless. To recover solvent, after siphon-off, the sample packets were removed and the soxhlet apparatus was operated and most of the hexane was recovered and used in later extractions and the extracted fat was collected and its residual hexane was left for evaporation in a pre weighted petri-dish and hence, weight of fat collected was

determined. Figure 3.2 shows extraction of fatty components from Stevia leaf powder using hexane. The figure shows the process near its end point.

Fig. 3.2 Defatting of Stevia using hexane

Methanolic extraction of steviol glycoside

After defatting, the entire samples were dried in a hot air oven at 50 Cº to remove the remaining residual hexane. Then the soxhlet apparatus was kept in a solution of chromic acid for 30 minutes and then rinsed with tap water. Then the soxhlet extractor was dried in a hot air oven at 50ºC. Weight 0.1% and 2% w/v of dried sample, giving a sample/solvent ratio of 1:1000 and 1:50 w/v, respectively.

The sample was placed in the thimble. After that the thimble was placed in the soxhlet extractor. A clean 500 ml round bottom flask was taken and filled with 300 ml of 100% methanol. Whole setting was placed on a heating mantle and methanol was allowed to boil. The extraction process was continued till several hours, almost 16-24 hours. Then the whole setting was allowed to cool down. The condenser and thimble was removed. And all the extract was collected in the round bottom flask. Figure 3.3 shows the setup towards the end of operation.

Fig. 3.3 Extraction of steviol glycosides by using 100% methanol

Extraction of steviol glycosides in 20% Methanol

Extraction of another sample was carried out by using 20% methanol in soxhlet apparatus. The setup is shown in figure 3.4.Again the soxhlet extractor was dried in a hot air oven at 50ºC. Weigh 0.1% and 2% w/v of dried sample. The sample was placed in the thimble. After that the thimble was placed in the soxhlet extractor.

A clean 500 ml round bottom flask was taken and filled with 300 ml of 20% methanol. Whole setting was placed on a heating mantle and methanol was allowed to boil. The extraction process was continued till several hours, this time almost up to 48 hours.

Then the whole setting was allowed to cool down. The condenser and thimble were removed, and all the extract was collected in the round bottom flask. Figure 3.4 shows the setup towards the end of operation.

Fig.3.4 Extraction of steviol glycosides using 20% methanol

Preparation of standard curve for steviol glycosides

For estimation of steviol glycoside content in extracts, standard curve for steviol glycoside was prepared by using commercially available steviol glycoside product Stevia®. The standard curve was prepared on a UV-Vis spectrophotometer.

Determination of color (A_{420})

Color (A_{420}) was measured in order to analyze the concentration of extract in different samples. In crude Stevia extract there are many kinds of pigments and it is difficult to characterize the decolonization capacity for each pigment quantitatively. The visible absorption spectrum of the transparent solution of crude extracts is usually tested by a spectrophotometer. There are strong absorption peaks at 420 nm (Markosyan and Yerevan, 2013). Therefore, color of sample was measured in terms of optical absorbance (A_{420}) at a wavelength of 420 nm using UV-Vis spectrophotometer.

Complete process flow diagram :

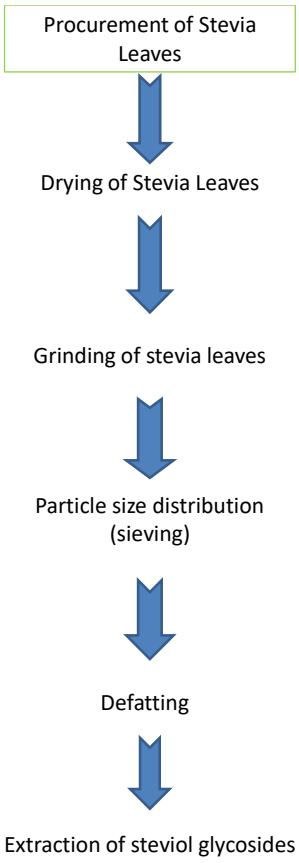

Fig 3.5 The complete process flow diagram.

5. RESULTS AND DISCUSSION

The standard curve for estimation of steviol glycosides was obtained at 210 nm, using a UV-Vis spectrophotometer as shown in Figure 4.1.

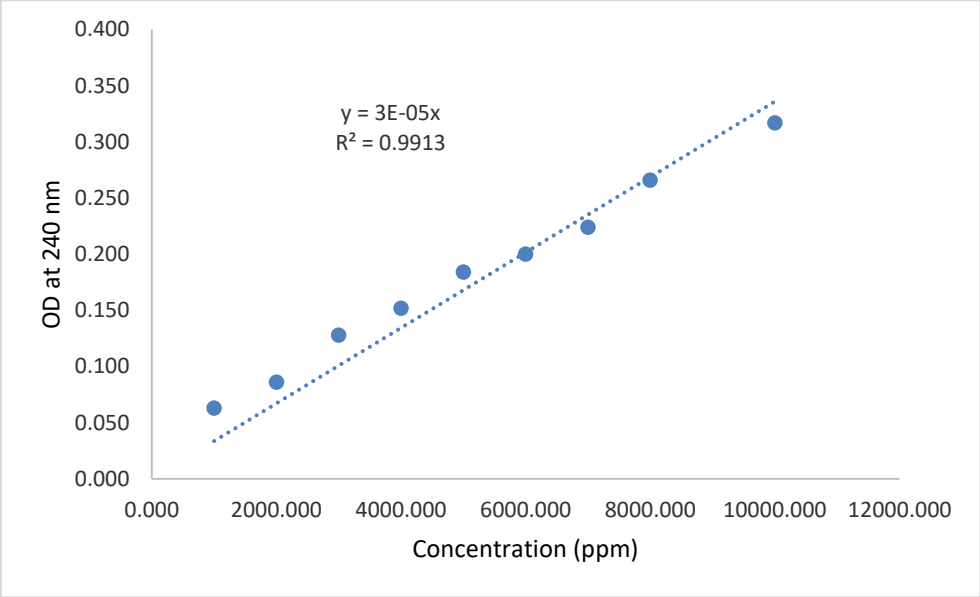

Fig 4.1 Standard curve for estimation of steviol glycosides.

The equation obtained from the standard curve was used to calculate the concentration of steviol glycosides in the extracts.

$$A_{240} = 0.00003C$$

Where, 'A_{240}' is the absorbance at 240 nm and 'C' is the corresponding concentration of steviol glycosides.

Drying of leaves

The leaves were placed in Hot Air Oven at 50ºC until similar consecutive weights were achieved. Since 410 g of leaves was a huge amount to be adjusted in the hot air oven, therefore batches of approximately 100 g were dried at a time. Each batch almost took about 8 hours to dry.

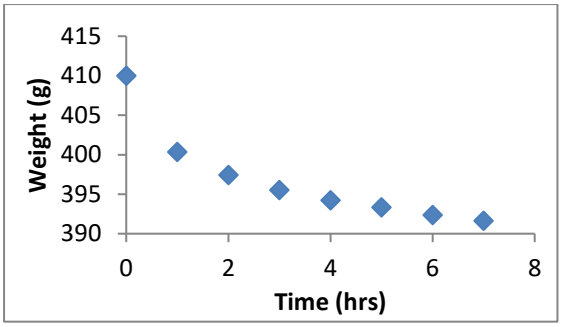

Fig 4.2 Graph showing the weight profile during the drying operation

Grinding and Sieving

Particles were grinded in a mixer grinder to a fine powder. The leaf powder was further screened according to its particle size by using ASTM sieves. Hence the powder particles were segregated in 3 particle sizes, according to screen size, i.e. 600-300 µm, 300-150 µm and <150 µm, respectively. The different sized particles were screened and stored separately in air tight containers. Figure 4.3 shows the three different particle sizes.

(a) (b) (c)

Fig 4.3 The screened powder of different particle sizes (a) 600-300 µm (b) 300-150 µm (c) <150 µm.

Defatting of Leaf powder

Defatting was carried out using soxhlet extraction apparatus with hexane. 119 g of leaf powder was taken in batches with maximum weight 30 g. The total fat recovered after defatting of all samples was about 2.8 g, which is about 2.4% of the dry leaf weight. This is in accordance with the reports mentioned elsewhere in literature (Gupta et al., 2013).

Extraction in Methanol

After defatting, the leaf powder was subjected to extraction using soxhlet apparatus. Extraction in methanol was studied by estimating optical density of the extract at 240 nm for steviol glycoside content and 420 nm for color of the extract. The extraction was done in 100% methanol (LR grade). The results obtained were calculated using the standard curve equation, by substituting the OD obtained at 240 nm of each extract and calculating the corresponding concentration of steviol glycosides.

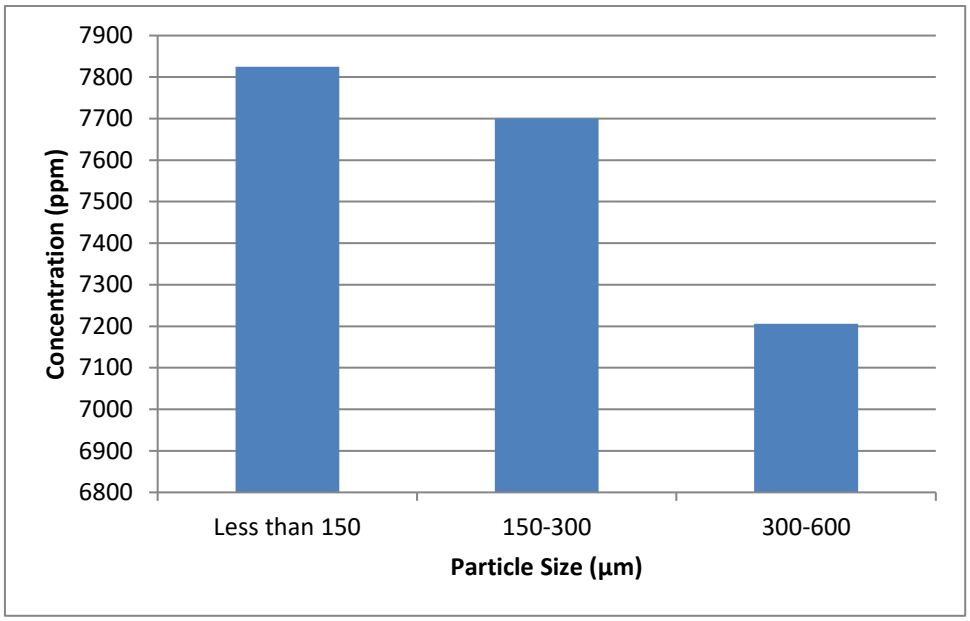

Fig 4.4 Extract concentration profile obtained with different particle size and 0.1% w/v particle to solvent ratio in 100% methanol.

The highest concentration of steviol glycosides found in 100% methanolic extract with 1:1000 (0.1% w/v) particle to solvent w/v ratio, was reported in particles with size less than 150 µm, as shown in figure 4.4.

The color of the extract was estimated by absorbance at 420 nm, as reported in literature and mentioned earlier in the previous chapter 3 of materials and methods. Figure 4.5 shows the absorbance profile obtained at 420 nm of 100% methanolic extract with 1:1000 (0.1% w/v) particle to solvent w/v ratio.

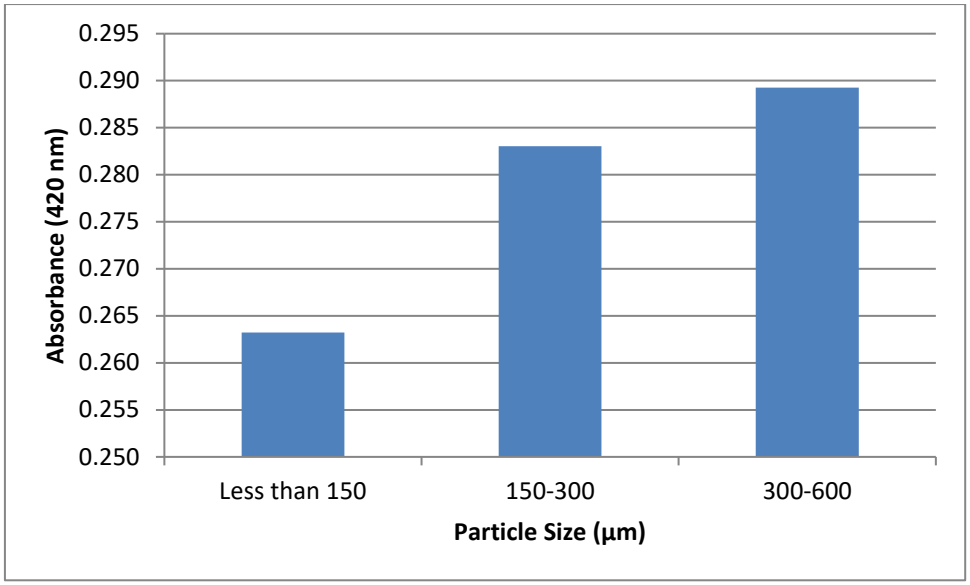

Fig 4.5 Extract color profile obtained with different particle size and 0.1% w/v particle to solvent ratio in 100% methanol.

The color profile was opposite to that of steviol glycoside content profile, suggesting that when color of extract increases the steviol glycoside content decreases. Extract from particles with the smallest size had the highest glycoside content and *vice versa*, suggesting an inverse relationship between the two factors.

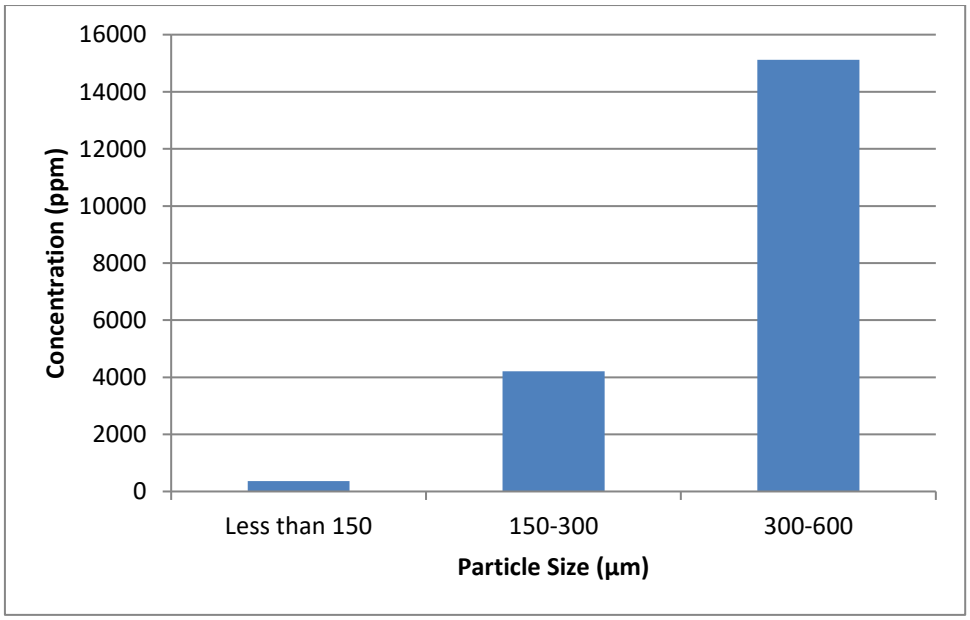

Fig 4.6 Extract concentration profile obtained with different particle size and 2% w/v particle to solvent ratio in 100% methanol.

Figure 4.6 shows the effect of increasing particle to solvent w/v percentage. The highest concentration of steviol glycosides found in 100% methanolic extract with 1:50 (2% w/v) particle to solvent w/v ratio, was reported in particles with size in the range of 300-600 µm. Figure 4.7 shows the obtained color profile.

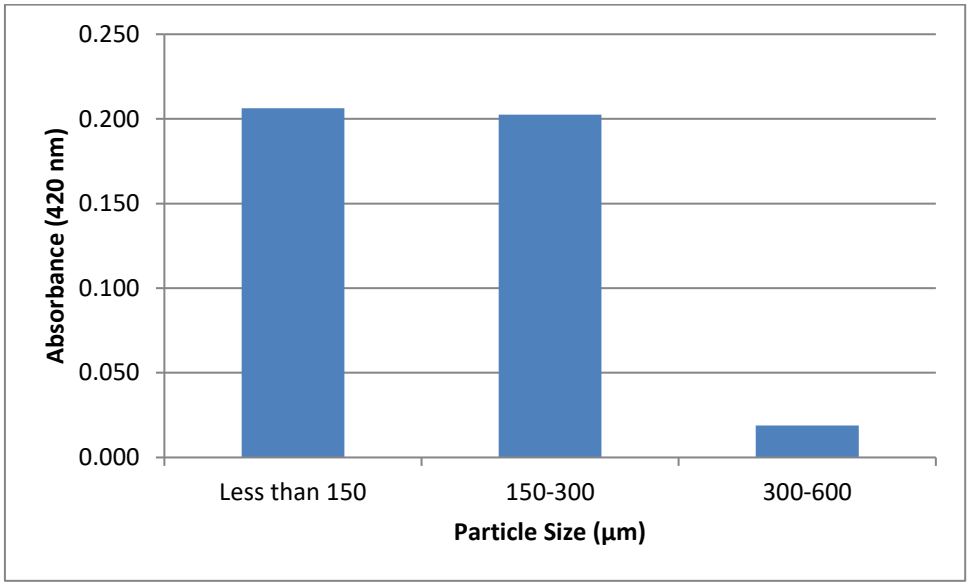

Fig 4.7 Extract color profile obtained with different particle size and 2% w/v particle to solvent ratio in 100% methanol.

The color profile showed similar inverse relationship with the glycoside content profile in both 100% and 20% methanolic extracts.

Extraction in 20% Methanol

After defatting, the leaf powder was subjected to extraction using soxhlet apparatus. Extraction in methanol was studied by estimating optical density of the extract at 240 nm for steviol glycoside content and 420 nm for color of the extract. The extraction was done in 20% methanol (LR grade). The results obtained were calculated using the standard curve equation, by substituting the OD obtained at 240 nm of each extract and calculating the corresponding concentration of steviol glycosides.

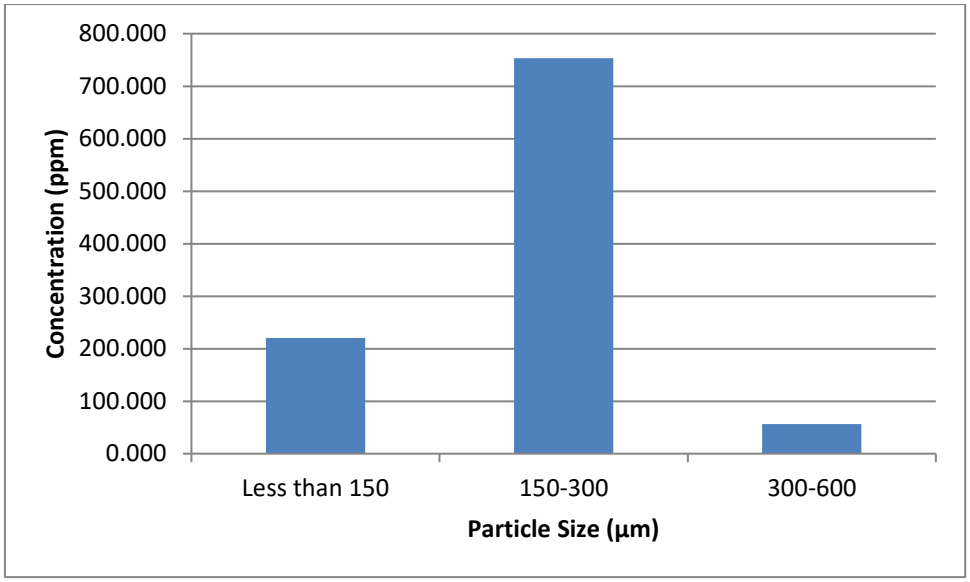

Fig 4.8 Extract concentration profile obtained with different particle size and 0.1% w/v particle to solvent ratio in 20% methanol.

The highest concentration of steviol glycosides found in 20% methanolic extract with 1:1000 (0.1% w/v) particle to solvent w/v ratio, was reported in particles with size in the range of 150-300 µm, as shown in figure 4.8.

Figure 4.9 shows the absorbance profile obtained at 420 nm of 20% methanolic extract with 1:1000 (0.1% w/v) particle to solvent w/v ratio.

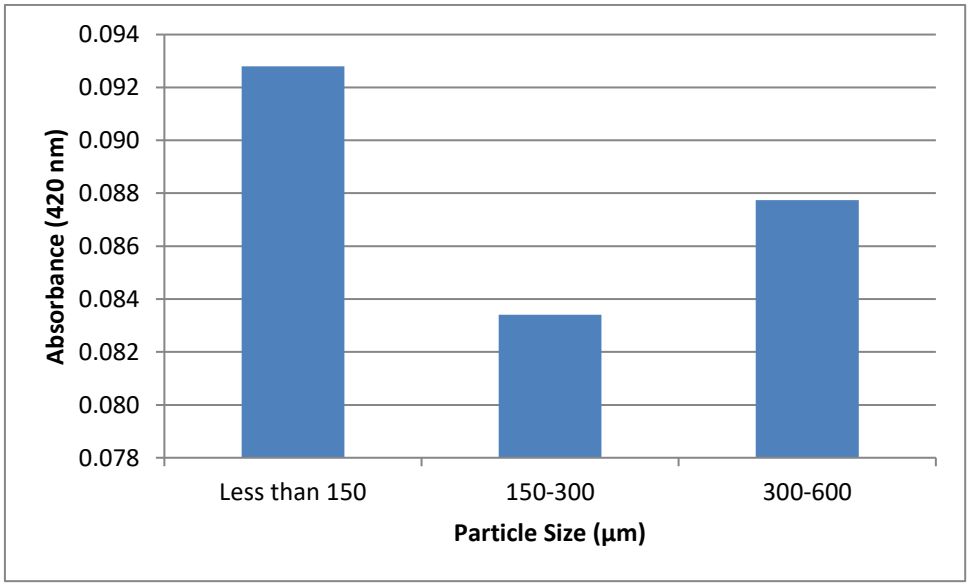

Fig 4.9 Extract color profile obtained with different particle size and 0.1% w/v particle to solvent ratio in 20% methanol.

The inverse relationship between color profile and concentration profile has not been strictly followed here, as, although the minimum absorbance has been obtained for the particle size range 150-300 µm, for which the extract concentration was maximum, but, the next highest concentration of glycosides was observed in particle size less than 150µm and the next lowest absorbance has been obtained for particle size ranging 300-600µm, which is contrary to the inverse relationship.

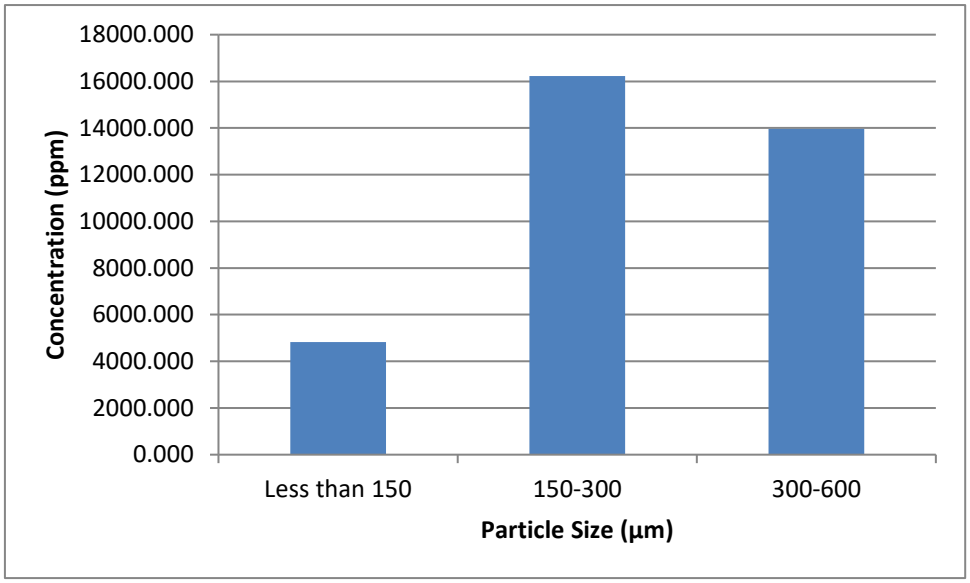

Fig 4.10 Extract concentration profile obtained with different particle size and 2% w/v particle to solvent ratio in 20% methanol.

Figure 4.10 shows the effect of increasing particle to solvent w/v percentage. The highest concentration of steviol glycosides found in 20% methanolic extract with 1:50 (2% w/v) particle to solvent w/v ratio, was reported in particles with size in the range of 150-300µm, but, with not much significant difference in concentration from that of 300-600µm. Figure 4.11 shows the obtained color profile.

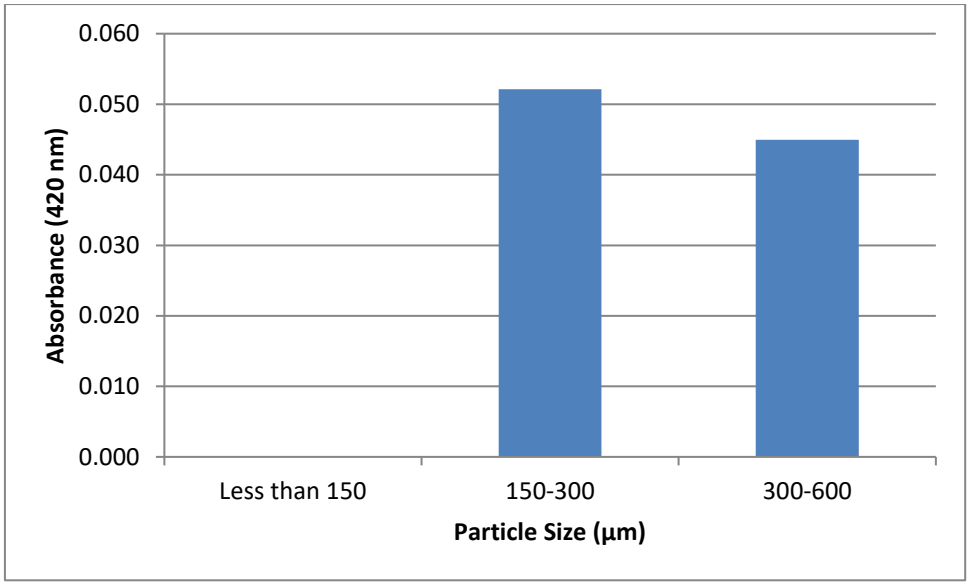

Fig 4.11 Extract color profile obtained with different particle size and 2% w/v particle to solvent ratio in 20% methanol.

Although the color profile did not show the inverse relationship with the glycoside concentration profile,yet, the values of absorbance are too low.

6. CONCLUSIONS

The extraction of steviol glycosides from *Stevia rebaudiana* leaves can be summarized by the following figures.

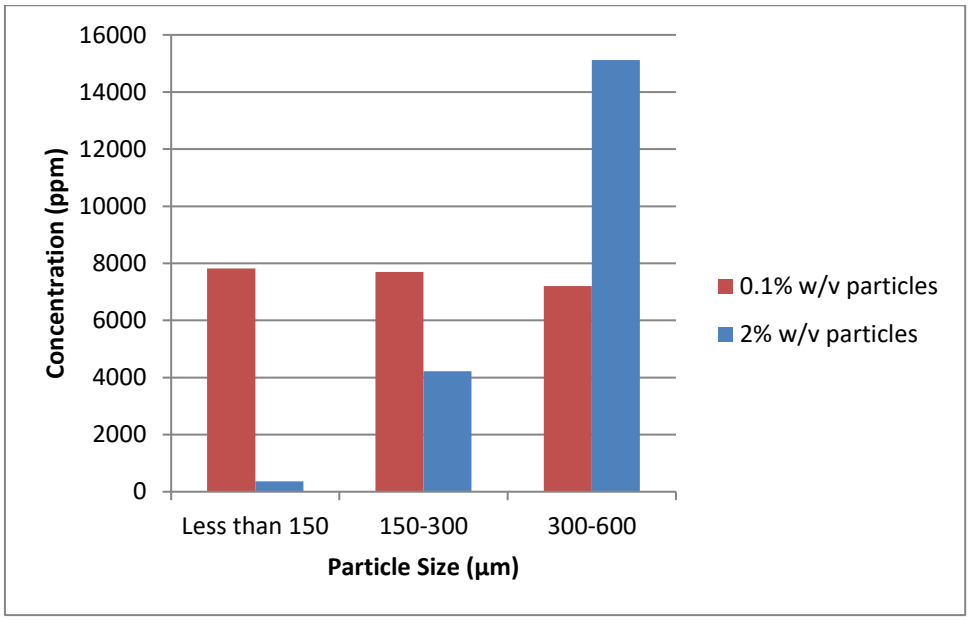

Fig 5.1 Comparison of extract concentrations in different particle size and particle to solvent ratios for 100% methanol as solvent.

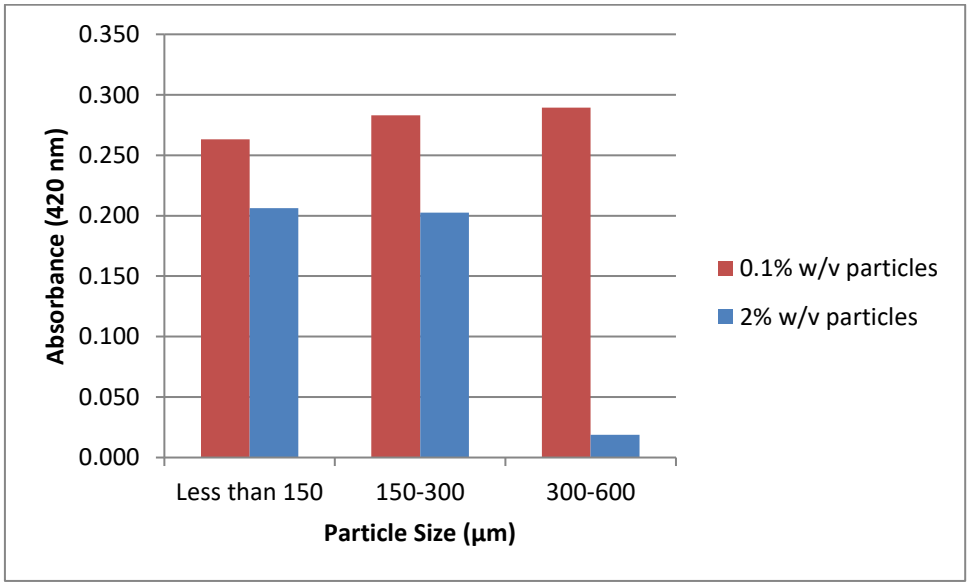

Fig 5.2 Comparison of color concentrations in different particle size and particle to solvent ratios for 100% methanol as solvent.

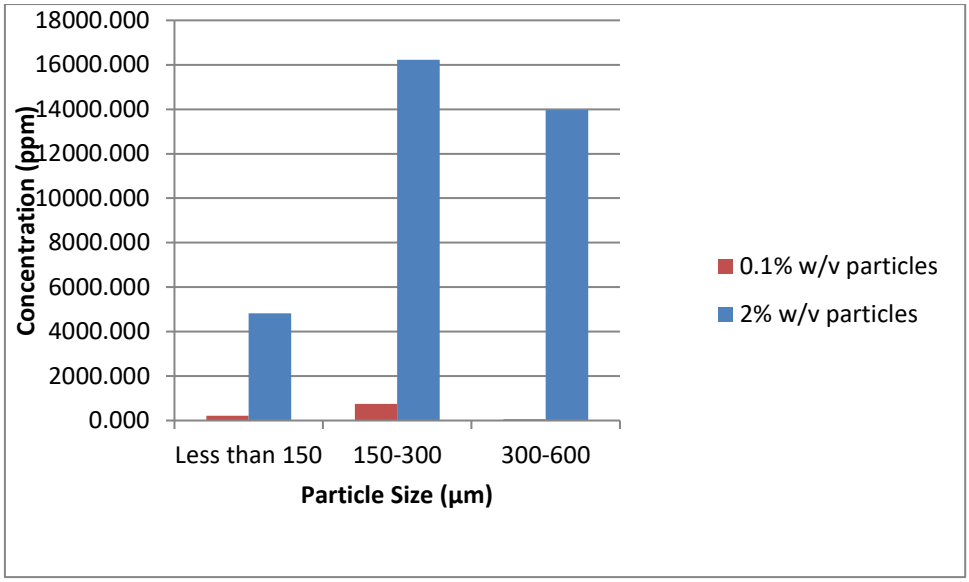

Fig 5.3 Comparison of extract concentrations in different particle size and particle to solvent ratios for 20% methanol as solvent.

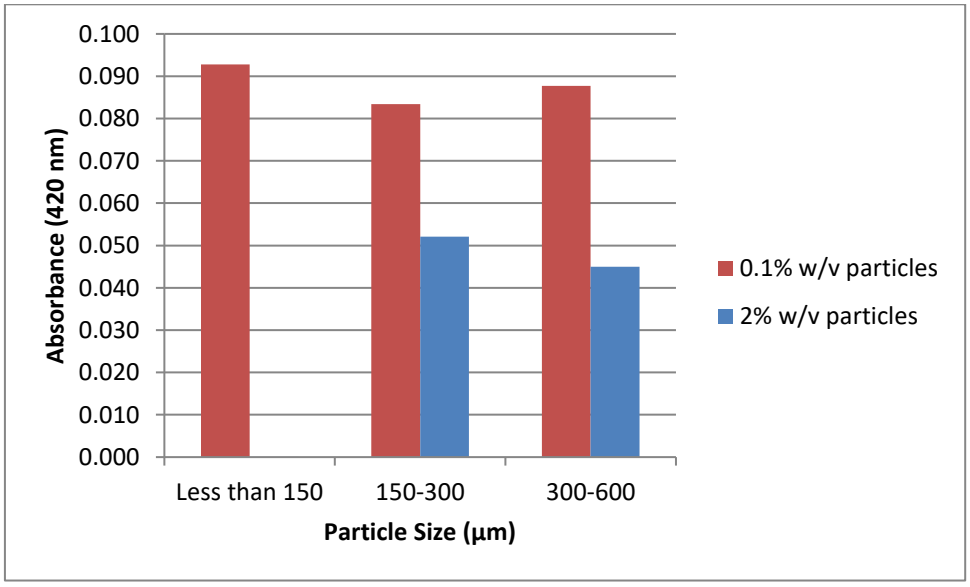

Fig 5.4 Comparison of color concentrations in different particle size and particle to solvent ratios for 20% methanol as solvent.

Extraction from very fine particles have shown very poor results, which might be due to the fact that fine particles coalesce and create a solid mass which in turn would offer large mass transfer hindrances, whereas, extractions from larger particles gave better yields. 2% particle to solvent ratio was found to give better yield in most of the extractions. 20% methanol as solvent provided more clear extracts with better yields than 100% methanol, hence decolorization would not be required. In present study, the best results obtained

was in 20% methanolic extract with 2% w/v particle to solvent ratio with 150-300 µm particle size.

Further study is required to establish more suitable concentration of solvent and particle to solvent ratio. Extraction using soxhlet needs to be modified in a way so that particles do not form solid blocks and offer mass transfer resistance. If the mass transfer resistance is elevated, then the finer particles hold a greater promise due to enhanced surface to volume ratio and thus greater chances of increased extraction efficiency. Other factors and pretreatment steps like enzymatic treatment of leaf samples and/or sonication, microwave, etc. could be studied.

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