ISOLATION OF *STEVIOL GLYCOSIDES* FROM THE MIXTURE OF *S.REBAUDIANA* STEMS AND LEAVES USING ETHANOL AND ULTRASOUND TECHNIQUE

by

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ABSTRACT

The mixture of *s.rebaudiana* stems and leaves will be extracted to get the *stevioside*. *Stevioside* is a *diterpene steviol Glycoside* which is produced by conventional process. It is used to sweeten soft drink,soya sauce, yoghurt and other food. It also good for diabetic patients. This research objective is to extract the *steviol glycosides* from the mixture of *s.rebaudiana* stems and leaves using ultrasound extraction technique and find their applications. The process normally include the extraction, pre-treatment, separation, purification and refining. These processes will use ultrasound technique and followed by High performance liquid chromatography analysis (HPLC). Ultrasound extraction technique is one of the techniques to extract the mixture of *s.rebaudiana* stems and leaves before the sample of the the mixture of *s.rebaudiana* stems and leaves is analyzed by HPLC. The mixture of *s.rebaudiana* stems and leaves will be in dry and powder condition before it extracted by ultrasound extraction technique using ethanol as a solvent. Power level that used in the extraction is 280 W and the time range is between 10min to 50min at temperature range of 20^0C to 60^0C. After that the analysis was performed using High performance liquid chromatography (HPLC). Using 30 min, 40 ^{0}C and 80% ethanol is the most suitable time, temperature and percent of solvent used to break the analyte and matrix bond and produce the *steviol Glycoside* in optimum yields. From this research we know that ultrasound extraction technique is most common technique and most suitable in order to extract the *steviol glycosides* from the mixture of *s.rebaudiana* stems and leaves. The optimum yield can afforded at extraction time of 30 minutes, extraction temperature of 40 ^{0}C and the percentage of ethanol of 80%. Extraction kinetic of *steviol glycosides* approved the second-order kinetic yielding good R^2 values of 0.992.

PENGEKSTRAKAN *STEVIOL GLYCOSIDE* DARIPADA CAMPURAN BATANG DAN DAUN POKOK *S.REBAUDIANA* MENGGUNAKAN ETHANOL DAN TEKNIK ULTRASOUND

ABSTRAK

Campuran batang dan daun pokok *s.rebaudiana* akan diekstrakan untuk mendapatkan *stevioside*. *Stevioside* ialah diterpene *steviol glycoside* yang mana dihasilkan oleh proses konvensional. Ia digunakan untuk memaniskan minuman ringan, kicap, yogurt dan makanan lain. Ia juga bagus untuk pesakit diabetis. Objektif kajian ini ialah untuk mengekstrakan *steviol glycosides* daripada campuran batang dan daun pokok *s.rebaudiana* menggunakan teknik pengekstrakan ultrasound dan mencari aplikasinya. Kebiasaannya, ia melibatkan proses pengasingan, pra rawatan, penulenan dan penapisan. Proses-proses itu akan menggunakan teknik ultrasound dan diikuti dengan proses analisa menggunakan alat High performance liquid chromatography (HPLC). Teknik pengekstrakan ultrasound ialah salah satu teknik untuk mengekstrakan *steviol glycosides* daripada campuran batang dan daun *s.rebaudiana* sebelum dianalisa menggunakan alat HPLC. Campuran batang dan daun *s.rebaudiana* akan berada dalam keadaan kering dan debu sebelum diekstrakan oleh teknik pengekstrakan ultrasound dengan menggunakan ethanol sebagai bahan pelarut. Kuasa yang digunakan dalam pengekstrakan ialah 280 W dan dalam lingkungan masa antara 10 hingga 50 minit pada lingkungan suhu 20 hingga 60 ^{0}C. Selepas itu akan dianalisa oleh alat High Performance Liquid Chromatography (HPLC). Menggunakan 30 minit, 40 ^{0}C dan 80 % ethanol ialah masa, suhu dan peratus bahan pelarut yang digunakan dalam air yang paling sesuai untuk memecahkan ikatan analit dan matrik seterusnya menghasilkan *steviol glycosides* yang optimum. Daripada kajian ini kita tahu bahawa teknik pengekstrakan ultrasound ialah teknik yang lazim dan paling sesuai untuk mengekstrak *steviol glycosides* daripada campuran batang dan daun *s.rebaudiana*. Hasil *steviol glycosides* yang paling optimum boleh didapati pada masa 30 minit pengekstrakan, pada suhu pengekstrakan 40 ^{0}C dan pada 80 % peratusan ethanol yang digunakan dalam air sebagai bahan pelarut. Model ekstrakan kinetik oleh *steviol glycosides* menepati model kinetik kedua menghasilkan 0.992.

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LIST OF ABBREVIATIONS

HPLC	High Performance Liquid Chromatography
CH_3OH	Ethanol
mg	Miligrams
C_2H_3N	Acetonitrile
min	Minutes
ml	Milliliter
Mg/mL	Milligram/mililiter
s.rebaudiana	Stevia rebaudiana
t	Time
v/v	Volume/volume

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LIST OF SYMBOLS

^{o}C	Degree celcius
C_e	Equilibrium concentration
Ct	Concentration at certain time
C_o	Initial concentration
h	initial extraction rate
k	Second-order extraction rate constant
k_{OBS}	Observation constant rate of first order
M1	Molarity
R^2	Correlation coefficients
V1	Volume
W	Watt
%	Percentage

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Steviol glycosides are originating in *s.rebaudiana* plant. It having up to 300 times sweeter than sugar. *Stevioside and rebaudioside A* are a main low-calorie *steviol glycosidse* from *s.rebaudiana* plant. (Jaitak et. al, 2008). *Stevioside* is better in term of taste quality because *Stevioside* is less bitter and sweeter than *rebaudioside A*. (Brandle and Telmer, 2007). *S.rebaudiana bertoni* plant initially can found in Brazil and Paraguay rain forest. But now it can also found in Malaysia especially in Pahang and Melaka. Among 150 stevia species,the only one with significant sweet tasting properties is *s.rebaudiana* (Jaitak et. al, 2008).

As we know,before this many research about *s.rebaudiana* leaves are done to extract the *steviol glycosides*. So its stems become a agricultural waste since it is not used to extract the *steviol glycosides*. Because of that this research have been done to extract *steviol glycosides* from the mixtures of *s.rebaudiana* stems and leaves. Furthermore in Malaysia we lack the source to produce sugar. Previous, we use sugar cane to produce sugar in Malaysia.

Therefore if we investigate weather in the mixtures of *s.rebaudiana* stems and leaves can afford higer yield of *steviol glycosides*, we can solve two problem at once. First, we can solve the problem of the source of sugar and second we can reduce the agricultural waste which is came from *s.rebaudiana* stems. Thus,it also can safe the budget in produce sugar since we use the waste.

In order to extract *steviol glycosides* from the mixtures of *s.rebaudiana* stems and leaves, we use ultrasound technique. Then, High Perfomance Liquid Chromatography(HPLC) will be use to analyzed the content. Extraction process is selected in this research because it is quite selective, effective, and able to separate *steviol glycosides* from the mixtures of *s.rebaudiana* stems and leaves. Actually, there are several technique to extract *steviol glycosidse* from the mixtures of *s.rebaudiana* stems and leaves other than ultrasound technique which is conventional extraction method and ultrasound-assisted extraction method. Ultrasound-assisted extraction technique allows faster extraction, decreased uses of solvent and higher recovery. In term of yield,time and energy consumption it also more effective than other method (Jaitak et. al, 2009). The process by which ultrasound energy is used to heat solvents in contact with solid samples and to partition compounds of interest from the sample into the solvent is known as ultrasound-assisted extraction method (Hayat et. al, 2009).

Using this method can result in a yield increase in shorter time using less solvent at the same temperature. Choose a solvent in which their target analyte is soluble because more ultrasound energy can be absorb if use solvent with high dielectic constant. The polarity of the solvent is very important in ultrasound-assisted technique.(Proestos and Komaitis, 2007). With the purpose to identify,quantify and purify the individual components of the mixture,

High Perfomance Liquid Chromatography(HPLC) is used to seperate a mixtures of compound in analytical chemistry and biochemistry.

S.rebaudiana extracts have been used for sweetening soft drinks such as diet coke,soju,soy sauce,dried seafood,candies,ice cream,chewing gum,yoghurt,and as well as in toothpaste and mouthwash in Japan,Korea,and Brazil.(Erkucuk et. al, 2009). Serious side effect also may occured if stevia is taken in high doses. *Stevia* and *stevioside* have no effect on mammalian reproduction or fertility, are safe for use as sweeteners and that they are acceptable for both diabetic and phenylketonuria patients.(Brandle and Telmer, 2007). Toxicological studies have shown that *stevioside* does not have mutagenic, teratogenic or carcinogenic effects. Likewise, allergic reactions have not been observed when it is used as a sweetener.(Lemus et.al, 2011).

The purpose of this study is to determine the *steviol glycosides* extracted from the mixtures of *s.rebaudiana* stems and leaves which can give advantages to country to reduce agriculture waste and have more sources in produce sugar in the country. Another purpose is to determine weather the mixtures of *s.rebaudiana* stems and leaves can increase yield of *steviol glycosides* or not.

1.2 Problem Statement.

Nowadays, the productions of sugar in Malaysia not as active as before due to lack of sugar cane to produce sugar. As we know, many times happen in Malaysia where the consumer only can buy two pack of sugar in one time for one person due to production of the sugar decrease. Many scientists did a research to extract sugar from other material other than sugar cane. The industrial use *s.rebaudiana* leaves to extract *steviol glycoside* which is use as a natural sweetener.

Currently, their extraction from *s.rebaudiana* leaves has attracted many scientist interests to use them as natural sweetener. Its stems did not used. So in order to avoid its stems become an agriculture waste this research is done to extract *steviol glycoside*s in the mixture of *s.rebaudiana* stems and leaves and analyzed how much *steviol glycoside* in them. As we know in *s.rebaudiana* plant has eight sweet components known as *steviol glycosides*. Among eights component, *stevioside* is the most exhaustively studied (Puri et. al, 2011).

Till now no mixture of *s.rebaudiana* stems and leaves have been done yet to produce *steviol glycoside*. So, this research is done in order to overcome this matter. As we know, only the leaves of the *s.rebaudiana* used to extract the *steviol glycoside*.

Up to now, low cost process to seperate *steviol glycosides* from the mixtures of stems and leaves still being investigate. If this matter can be solved, the cost to extract *steviol glycoside* from the mixture of *s.rebaudiana* stems and leaves can be reduced. Automatically, the price of this product from this extraction process also can be reduced.

1.3 Objectives.

- To separate *steviol glycoside* from the mixture of *s.rebaudiana* stems and leaves using ultrasound-assisted extraction method and ethanol due to its easy method. The mixture of *s.rebaudiana* stems and leaves is added in the mixture of ethanol and water to extract using ultrasound-assisted extraction method.
- To find the kinetic of *steviol glycoside* separation from the mixture of *s.rebaudiana* stems and leaves using ultrasound extraction method. Kinetic is important in extraction because it evaluated the value of optimum extraction capacity using related equations.
- To study the effects of various parameter on extraction yield and extraction kinetic. Some parameters were varied to get the results such as extraction time, extraction temperature and the percentage of ethanol in water. Five reading were taken for each parameter to evaluate them.

1.4 Scopes of the Study.

- This study was done to separate the *steviol glycosides* from the mixture of *s.rebaudiana* stems and leaves. This extraction of the *steviol glycosides* from the mixture of *s.rebaudiana* stems and leaves was observed in term of its extraction yield using optimum condition determined.
- Analyzing extraction processes that using ultrasound-assisted extraction method due to it lower energy consumption, lower consumption of solvents and higher extraction efficiency

- Observation the effect of some parameters (time,temperature and percentage of solvent in water) to the separation of *steviol glycoside* from the mixture of *s.rebaudiana* stems and leaves. The parameters were observed to find which value of parameter can give the optimum extraction yield.
- Determination concentration of *steviol glycoside* in the mixture *of s.rebaudiana* leaves and stems by analyzing the result using High Performance Liquid Chromatography. The manipulated variables for this study are extraction temperature (20, 30, 40, 50, and 60 ^{0}C), the percentage of ethanol in water (20, 40, 60,80, and 100 %) and extraction time (10, 20, 30,40, and 50 minutes).
- Determination of the kinetic model of separation of *steviol glycoside* from the mixture of *s.rebaudiana* stems and leaves.

1.5 Rationale and Significance.

In this experiment, *steviol glycosides* will be extracted from the mixture of *s.rebaudiana* stems and leaves. The research of production of *steviol glycosides* from the mixture of *s.rebaudiana* stems and leaves have been made in order to use the whole plant of *s.rebaudiana* instead of it leaves only. Before this, many researchers have been made to extract *steviol glycosides* from it leaves. Because of that its stems has been agricultural waste. From that, it will give positive impact like help to reduce agricultural waste, can reduce health disease, renewable source and environmental product since *steviol glycosides* produce by the mixtures of *s.rebaudiana* stems and leaves have beneficial effects on human health. For industrial player, this research will give alternative solutions to generate more of sugar production. This *steviol glycosides* also can be use to sweeten soft drinks, soju, soy sauce,

yoghurt and other foods. Another purpose to do this research is to find the kinetic of *steviol glycosides* separation from the mixtures of *s.rebaudiana* stems and leaves.

CHAPTER 2

LITERATURE REVIEW

2.1 *Steviol Glycoside* in the Mixtures of S.*Rebaudiana* Leaves and Stems and the Applications.

Only the species *rebaudiana* and *phlebophylla* produce *steviol glycosides* among the 230 species in the genus *Stevia*. The amount of sugar units bonded to the *steviol aglycone* will increase if the sweetness of *rebaudiosides* increases but at the same time their content in the plant material will be deacreases. (Lemus et. al, 2011).

At least eights *steviol glycosides* will be accumulated in the leaves of *s.rebaudiana bertoni*. By enzymatic and chemical procedure *stevioside* has been transformed to *rebaudioside A* (Puri et. al, 2011). *Stevia* and *stevioside* are safe for use as sweeteners because they have no effect on mammalian reproduction or fertility and they are acceptable for both diabetic and phenylketonuria patients (Brandle and Telmer, 2007). According to those reviews, clinical proof emerges that recommended *stevioside* can reduce blood glucose levels in type II diabetics and blood pressure in mildly hypertensive patients. Both leaves and

stems contain *stevioside* and *rebaudioside-A* but it proved that in stems, the *steviol glycosides* content is lower than in the leaves. (Bondarev et. al, 2001).

2.2 Ultrasound-Assisted Extraction Technique.

Extraction is one of the methods used to obtain *steviol glycoside* from the mixture of s.rebaudiana stems and leaves. There are various extraction technique to extract *steviol glycosides* from the mixture of s.rebaudiana stems and leaves such as Conventional solvent extraction (CSE), microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE).

Ultrasound-assisted extraction (UAE) is one of the new extraction method to extract *steviol glycosides* from the mixture of *s.rebaudiana* stems and leaves. This method has been developed to reduce the extraction time and improve the extraction yield (Goula, 2012). UAE has lower energy consumption, lower consumption of solvents, higher extraction efficiency and higher level of automation and is preferable to CSE (Ying et. al, 2011). Increasing extraction yield, reducing solvent usage, economizing power consumption and shortening extraction time also the advantages of UAE (Zou et. al, 2009).

Ultrasound-assisted extraction method also used to extract many other materials not only can be applied to extract *steviol glycoside* from *s.rebaudiana* leaves, stems or flowers. For example, this method also used to extract *anthocyanins* from grape skins. The extraction of *anthocyanins* from grape skins using ultrasound extraction method was carried out under different extraction conditions. (Liazid et .al, 2010)

2.3 Other Method to Extract *Steviol Glycosides* from Stevia Rebaudiana Leaves.

Up to now, several extraction techniques have been reported for the extraction of *steviol glycoside* from *s.rebaudiana* plants other than ultrasound-assisted extraction technique like conventional extraction technique and ultrasound-assisted extraction technique. Among all method ultrasound-assisted extraction method afforded highest yield of *stevioside* and *rebaudioside-A* but ultrasound energy in MAE is a non-ionising radiation. The radiation causes motion of molecule and rotation of dipoles to heat solvents to promote targeted compounds to move from the sample matrix into the solvent. However, the radiation does not induce changes in molecular structure (Ying et. al, 2011). Solvent extraction method also can be used to extract *steviol glycosides* from *s.rebaudiana* leaves. The powdered sample will be extracted in ethanol using Erlenmeyer flasks in a shaking hot-water bath for 30min at 70^0C. For *stevioside*, solvent extraction method was indicative of the efficiency in extraction of *rebaudioside C* and *rebaudioside A*. (Hearn and Subedi, 2008). But among all method CSE take highest extraction time to extact *steviol glycoside* (Jaitak et. al, 2009). Other than that, supercritical CO_2 extraction also can be used to extract *steviol glycoside* from *s.rebaudiana* leaves. The extractor volume was 100mL, thus it was filled with about 30g of ground *S.rebaudiana* leaves. The independent variables were temperature, pressure and co-solvent ratio. (Erkucuk et. al, 2009).

2.4 Kinetic Model

The solid–liquid extraction process can be considered as the reverse of an adsorption operation, therefore the bases of the adsorption kinetic equations can be applied to solid–liquid extraction and the second-order law was found to give the best fits for the extraction rate. The general second- order model can be written as Equation 2.1 (Goula, 2012).

$$\frac{dCt}{dt} = k.(Ce - Ct) \tag{2.1}$$

where k is the second-order extraction rate constant (L/g min), Ce is the equilibrium concentration of *steviol glycosides* in the liquid extract (g/L) (extraction capacity), and Ct is the stevia concentration (g/L) in the liquid extract at a given extraction time t. The integrated rate law for a second-order extraction under the boundary conditions t = 0 to t and Ct = 0 to Ct, can be written as an Equation 2.2. or a linearized Equation 2.3 (Goula, 2012).

$$Ct = \frac{k.t.Ce^2}{1+k.t.Ce} \tag{2.2}$$

Or

$$\frac{t}{Ct} = \frac{1}{kCe^2} + \frac{t}{Ce} = \frac{1}{h} + \frac{1}{Ce} \tag{2.3}$$

where h is the initial extraction rate (g/L min) when t approaches 0 as Equation 2.4.

$$h = k.Ce^2 \tag{2.4}$$

CHAPTER 3

MATERIALS & METHODS

3.1 Materials.

This study main material is rubber the mixture of s.rebaudiana leaves and stems which come from Pahang agriculture plantation area. The chemicals involved in this study are ethanol and acetinitrile. Standard solution of *steviol glycoside* was used to analyze the extraction yield using High Performance Liquid Chromatography. Water also used to mix with ethanol in order to produce the percentage of ethanol in water parameter.

3.2 Overall Methodology Flow Chart.

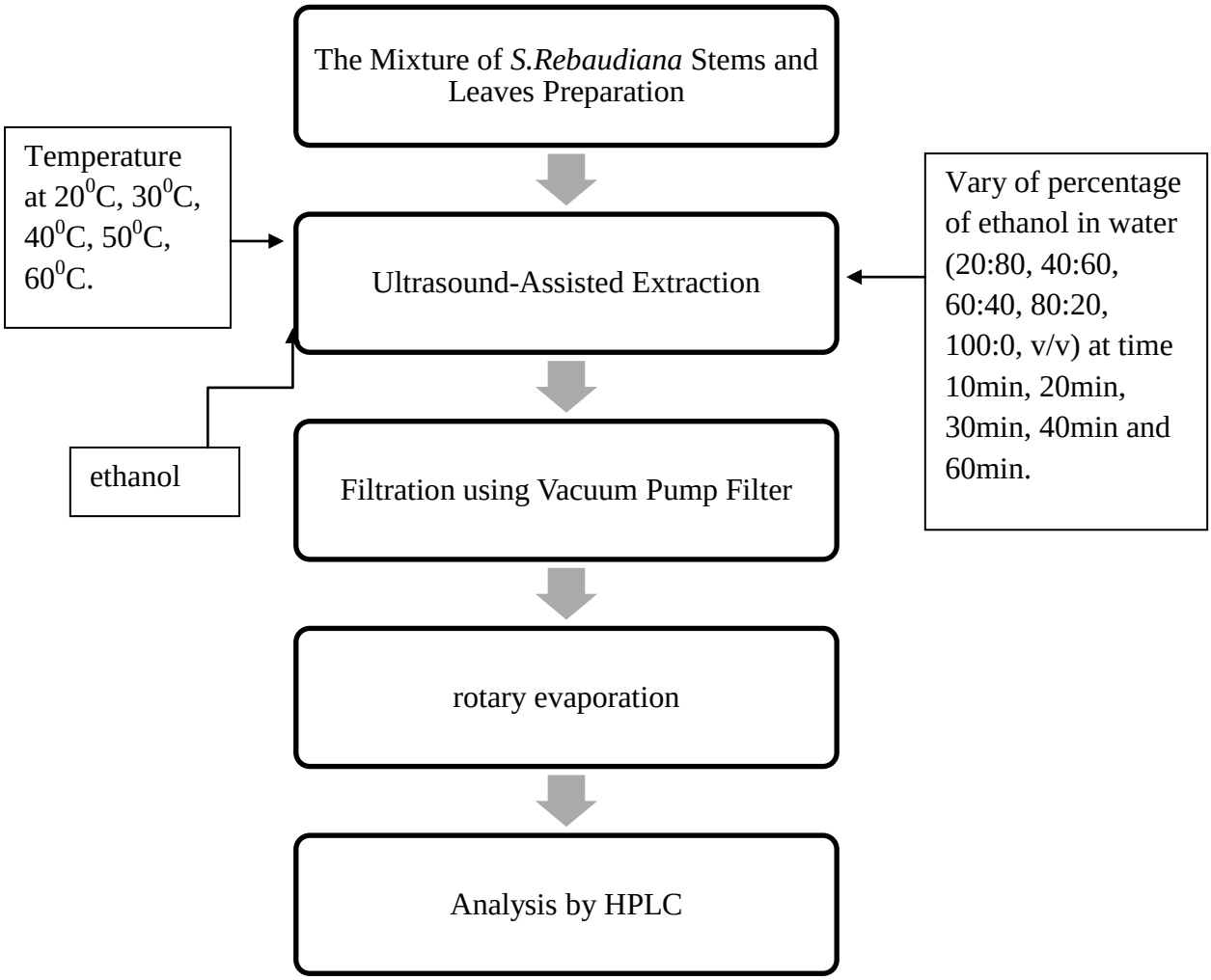

Figure 3.1: Overall flow chart for experimental methodology.

3.3 Experimental Methodology.

3.3.1 The Mixture of *S.Rebaudiana* Stems and Leaves Preparation

The mixtures of *s.rebaudiana* stems and leaves were dried in open shade. They were milled to 60 mesh powders using a grinder and were stored at ambient temperature.

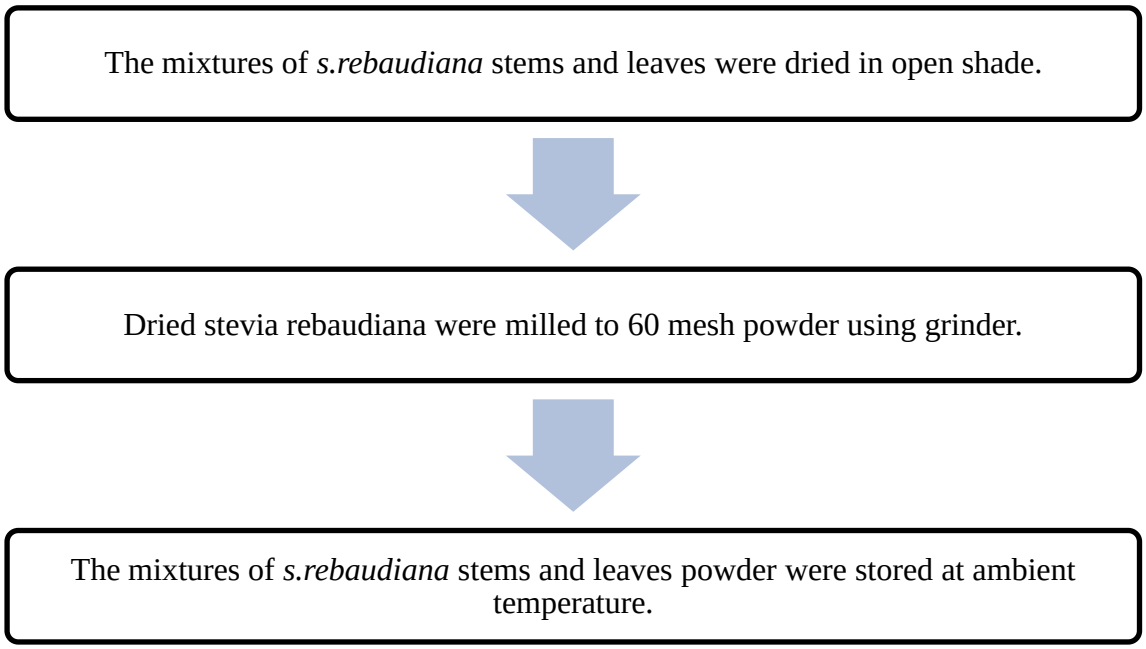

Figure 3.2: Flow diagram for the preparation of the mixtures of *s.rebaudiana* stems and leaves