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Production of Biosweetner from Stevia Rebaudiana and Feasibility of Incorporating it in Fruit Juice

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Abstract – **Biosweetner, a sugar substitute was extracted from the leaves of the plant species Stevia rebaudiana. Stevia leaves naturally contain a complex mixture of eight sweet diterpene glycosides including stevioside, steviolbioside, rebaudioside (A, B, C, D, E) and dulcoside. The main constituents present were glycosides such as stevioside, steviol and rebaudioside A and B. Fresh Stevia leaves contain a large amount of water 85%. Stevia leaves and steams having several nutritional components such as vitamins, carotenoids, flavonoids, anthocyanin and other phenolic compounds. Stevia is a flavor enhancer and it is 300 times sweeter than sucrose. Stevia is non calorie sweetener. It is stable on low pH. Stevioside can be used as medicine for treating diabetes patients, fighting obesity, preventing tooth decay, preventing throat cancer. Stevia attract to people on carbohydrate-controlled diets. Stevia leaves are dried and powdered by mills. Powdered stevia leaves extracted with different techniques and water as using solvent because it not produce harmful effect to products. Ultrasound assisted extraction condition: temperature 30 to 50℃, time 10 to 30 mins. Microwave assisted extraction condition: power 240 W, time 2 mins. Stevia extract collected and analysis for nutritional values. Glycosides level analysis by HPLC method in hydrophilic column. Crystals of Stevia extract were obtained using evaporative cooling technique. The stevia extract or crystals can be incorporated into the food products like cake, biscuits, soft drinks, and Milk products. In this study stevia extract incorporate in fruit juice.**

Keywords – **Crystallization, Extraction, Rebaudioside A, Steviol glycosides, Stevia rebaudiana.**

I. INTRODUCTION

Stevia, botanically known as stevia rebaudiana bertoni (Family: Asteraceae) is a sweet herb. The plant is native to tropical and subtropical regions of North America and South America. It is related to family of sunflower, marigold, etc. Stevia has an alternate leaf arrangement and herbaceous growth habit with flowers arranged in indeterminate heads. The leaves have been traditionally used for hundreds of year in both Brazil and Paraguay for local teas and medicines (Goyal et al 2010). There are nearby about 240 species of Stevia Genus. It is grown widely in countries like Brazil, Colombia, Paraguay and Venezuela. It grows 2-4 feet in height with slender, branched stems, and flourishes well all over the temperate, and some parts of tropical regions. Leaves of stevia plants are ready for first harvesting after four months of planting and subsequent harvesting can be done after every 3 to 4 months (Balwinder et al 2014).

Leaves of Stevia rebaudiana contains diterpene glycosides which sweet taste with in food products (Madan et al 2010). Diterpene glycoside does not have mutagenic, teratogenic, and carcinogenic effects. Stevioside and rebaudioside of Stevia are stable under wide range of temperatures and pH conditions in different food and pharmaceutical products .They do not alter the flavor and taste of a food product. Stevia is nutrient rich containing substantial amount of protein, magnesium, Miocene, riboflavin, zinc, chromium, selenium, calcium& phosphorus (Regina et al 2011). It is alternative for sugar, but it actually helps increasing insulin sensitivity, which is especially helpful for insulin-resistant diabetic people (Regev et al 2016).

Stevia is a medicinal plant that have at least one of their part (leaves, stems, barks, roots, tubers) are and flowers used for therapeutic purpose and active principles in medicinal plants plays a strategic role in the phytochemical investigation of crude plant extract and very important to their potential pharmacological effects (Pascual et al 2002). Medicinal plants are of great importance to the health of individuals and communities, and this plant lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, flavonoids, tannins, and phenolic compounds (Edeoga et al 2005).

It was approved by the U.S Food and Drug administration in 2008 to be sold as an ingredient to sweeten foods. The FDA deemed stevia “generally recognized as safe”. It is very sweet, the substance does have a bitter aftertaste. In comparision to artificial sweetners available in market, steviosides are 100% natural, zero calories, heat stable,

non-discoloring, and have no other side effects. It can be added to tea or coffee and cooked or baked. In India, prevalence of diabetes is rising rapidly, and more than half of the patients have poor glycemic control with vascular complications (Sheela et al 2014).

II. MATERIALS AND METHODS

1. Raw materials

Stevia rebaudiana leaves were purchased from seller (Amazon). The purchased leaves stalks were removed manually. The dried leaves pulverized to fine powder, sieved and packed in air tight container for subsequent analysis. All solvents and chemicals used were of analytical grade.

2. Methodology

Methodologies adopted in their study are provided as under.

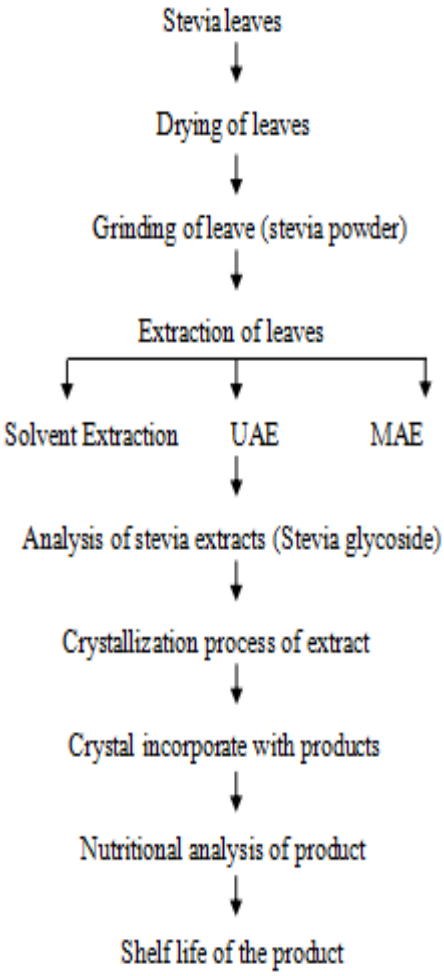

Fig .1.Process flow chart for biosweetner production.

3. Drying of leaves

The drying conditions applied in fresh Stevia leaves have a great impact on the extraction of steviol glycosides and antioxidants. In general, the yield of these compounds was affected in different ways according to the drying conditions (hot air drying at 100 ˚C and 180 ˚C, freeze drying and shade drying). The drying conditions produced an important increase in antioxidant capacity but an important decrease in the principal steviol glycoside (stevioside) which diminished with all treatments, especially with hot air at 100 ˚C. For this compound, there were no significant differences between the other treatments, although shade drying produced the highest values of this compound.

Drying was carried out at 50, 60 and 70 °C within 3 – 6 h depending on the drying temperature. Each temperature was repeated 4 times to get better estimation of the drying rate and moisture reduction. The best drying temperature was found to be at 50 °C to maintain the physical and chemical content of the leaves. With the pilot scale dryer, more than 7 h was required to reduce the moisture content of the Stevia leaves from 80% to 3 – 5%. Drying at 50 °C produced leaves with brighter and greener color compared to drying at higher temperatures (Samsudin et al 2013).

4. Moisture analysis of stevia powder

5 g of the stevia leaf powder is weighed accurately and placed in oven for 1 hour at 130oC. After drying the sample was cooled in the desiccators. This process of drying and cooling was repeated until the difference between two consecutive weights (taken at 30 minute interval) be less than 1mg.

5. Extraction of stevia leaves

Solvents (water and 70% ethanol) for conventional solvent extraction of green stevia powder were used. The extraction with water as a solvent was carried out as follows: 500 mg of the stevia sample was placed in a flask and 200 ml of distilled water (100°C) was added. The mixture was left for 24 h with agitation on the magnetic mixer. After extraction was completed, the extracts obtained were vacuum- filtered and used for chemical analysis. Extraction with ethanol was performed according to (Lavini et al 2008) with some modifications: 500 mg of the stevia sample was placed in a flask and 200 ml of 70% (v/v) ethanol was added. The mixture was kept in a water bath at 70°C for 30 min with stirring. The extracts obtained were vacuum-filtered. The prepared extract was used for further analysis.

Extraction of bioactive compounds was performed using ultrasonic bath consisting of stainless steel tank operating at a fixed frequency of 40 KHz with digital timer and temperature controller. For extraction, 1g of powdered setvia leaves were fed in to a volumetric beaker and mixed with various amount of methanol, ethanol, water (30-70% w/v) for various solvent to feed ratios (1:10-1:30 ml/g). The beaker was covered with aluminium foil to prevent loss of the solvent. Extracts were filtered through filter cloth and stored at refrigerated condition for further analysis.

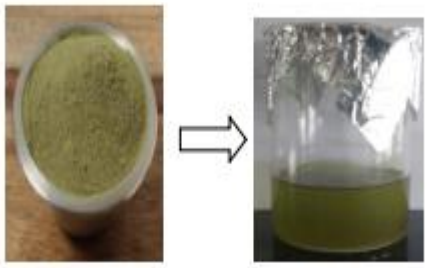

Fig .2. Stevia Powder Fig 3.Stevia Extract

Microwave drier works at the frequency of 2450MHz was used to extract Polyphenols from stevia leaves with the maximum of 800W microwave power. This microwave drier can be used in combination of air and vacuum. An accurately weighed 1g of stevia powder sample was loaded into a 500ml beaker and mixed with various amount of methanol, ethanol, water and sealed. The extraction was conducted using microwave as a heat source. After completion of extraction process, the extracts were immediately cooled to room temperature, filtered and stored for quantitative analysis. The parameters investigated include solvent concentration (30-70% w/v); the ratio of solvent to material (1:10 to 1:30 ml/g); power of extraction (160-360W); extraction time (60-180 sec).

6. Crystallization of stevia extract

Stevia leaf extract is taken from the round bottom flask and top of the flask tightly sealed. A thermometer inserted in to the flask was used to measure the temperature. A conical flask tightly sealed with cork and fitted a vacuum pump with Round bottom flask using a tube. This whole setup placed in the hot plate. By inducing vacuum evaporation crystals were obtained.

7. Stevia sugar incorporation in juice

The potential to process stevia without any chemical treatment during production should enhance the acceptance of stevioside as a "green" sweetener. We have recently observed that purified stevioside from S. rebaudiana showed reduction in number of pathogenic bacteria. These food borne pathogenic bacteria such as Bacillus cereus, Klebsiella pneumonia, and Pseudomonas aeruginosa are the root cause of many food-borne diseases such as enteric fever, diarrhea etc. (Puri and Sharma, 2011). It is anticipated that usage of stevioside in various food formulations herbal tea, bakery, confectionary items, tooth paste, mouth refreshers, candies, chewing gums etc. may protect from mentioned bacterium.

In this study we incorporate stevia crystals and extract in juices. Concentration will be varying for juices and stevia crystals and extract. Stevia sugar and juice concentration vary from 25, 50, 75 and 100%. Flavor, taste, color, sweet acceptability and overall acceptability determined by sensory analysis method.

8. Nutritional analysis of Product

Nutritional analysis checks with the juice for before incorporation of stevia extracts and also checks with after incorporation of stevia extract. Three main nutritional analyses done by the juice like Polyphenols, flavonoids and antioxidants.

9. Determination of total Polyphenol content

The phenolic compounds were isolated; its concentration was estimated by the Folin ciocalteau assay with absorbance at 765nm ozcan et al 2006. A sample of 0.1 ml was mixed with 6.4 ml of distilled water. To this mixture 0.5 ml of Folin's reagent and 3 ml of 10 % anhydrous sodium carbonate solution were added and the final mixture was kept in water bath at 40℃ for 30 minutes for color development. Results were reported as mg of gallic acid equivalent per g of dry sample.

10. Determination of total flavonoid content

Total flavonoids content was determined spectrophotometrically using a method suggested by Marinova et al 2005. 0.1 ml of the extract was mixed with 4 ml of distilled water and subsequently with 0.3 ml of 5 % sodium nitrite solution. After 5 min, 0.3 ml of 5% aluminium chloride was added and allowed to stand for further 6 min; then sodiumhydroxide solution (1M, 2 ml) was added to this mixture. Finally, total volume was made upto 10 ml by using distilled water. Then, the mixture was properly mixed and the absorbance was measured against blanck at 510 nm. Results were reported as mg Quercetin equivalent per 100g of samples.

11. Determination of antioxidant capacity

Antioxidant capacity was determined according to the method suggested by Braca et al 2002. A solution was prepared by dissolving 2 mg of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) in 100 ml of methanol. About 3 ml of prepared solution was mixed with 1 ml of sample extract and the mixture was kept in dark place for about 30 min, thereby the absorbance was read at 517 nm against in blank. Methanol (1ml) with 3 ml DPPH solution (0.002% w/v in methanol) was used as the blank.

DPPH radical scavenging activity (%) = [(Ablank – Asample)/ Ablank] ×100

Where Asample is the absorbance of the solution when the extracts have been added at different concentration and Ablank is the absorbance of the blank solution.

12. Determination of glycoside content

The sample, 1g of dried leaves of Stevia rebaudiana is ground and extracted with EtOH 70% (w/w) in Erlenmeyer flasks by shaking for 30min at 70 ˚C in water bath. After the extract was cooled, it was filtered and analyzed by HPLC using an NH2 column (250×4.6mm) and a mixture of acetonitrile/water (80:20, v/v) is employed as mobile phase. The pH of the mixture is

maintained as 5 using acetic acid. The measurements were carried out in the UV range of 210nm.

III. RESULT AND DISCUSSION

1. Moisture content of stevia leaf powderMoisture
analyzer and Hot air oven method are used to analysis the moisture content of stevia leaf. Moisture analyzer: sample taken is (3g), temperature (100℃) and moisture content (9.57 % w.b). Hot air oven method: initially sample taken (5g), temperature (105℃) for 3 hrs and moisture content (11.86% w.b).

2. Extraction of UAE
Box Behnken design in response surface methodology was selected to describe the effect of three independent variables on the yield of bioactive compounds. The main factors influencing extraction efficiency are solvent to feed ratio, extraction temperature, and extraction time, the ethanol concentration were selected as independent variables that should be optimized for the extraction of total phenolic compounds and antioxidant capacity.

3. Effect of temperature on response variables for UAE

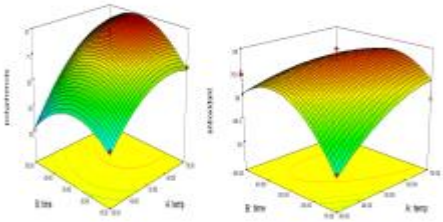

Fig.4.Effect of temperature on response variables (TPC, TAC) for UAE.

For extraction of bioactive compounds by ultrasound assisted extraction, temperature is varied from 40 to 60ºC. The increased extraction of total polyphenol content and total antioxidant content was observed for ultrasonic temperature from 60ºC (Figure 4). A further increase in sonication temperature to 70ºC showed declines in TPC and TAC yields, possibly due to the increase in extraction temperature above the boiling point of the solvent there occurs saturation of solvent leading to a decrease in the concentration gradient of extracted compounds. An excessive temperature causes degradation of bioactive compounds.

4. Effect of time on response variables for UAE

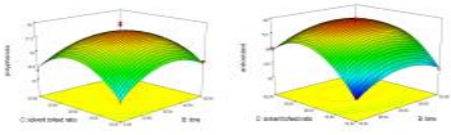

Fig.5. Effect of time on response variables (TPC, TAC) for UAE.

For extraction of bioactive compounds assisted by ultrasound extraction, time is varied for 10min, 30 min and 50min. The increased extractions of total polyphenol content and total
antioxidant content were observed for ultrasonic time from10 min to 30.39 min (Figure 5). A further increase in sonication time to 50 min showed declines in TPC and TAC yields, due to the reaction heat increase in extraction time, solvent saturation occurs which leading to a decrease in the concentration gradient of extracted compounds. These results can be explained as the effects of acoustic cavitation and rupture the plant cells, which causes the intensification of mass transfer and thus closed interaction between the solvent and the plant tissues. Along with the increase of extraction time, all the plant cells will be completely cracked by acoustic cavitation effect, and the extraction yield will increase within certain time duration. An excessive sonic time is known to cause heating of the extraction solvent, resulting in degradation of bioactive compounds. The extraction condition that yield high phenolic compound showed the strong antioxidant activity.
Effect of solvent to feed ratio on response variables for UAE

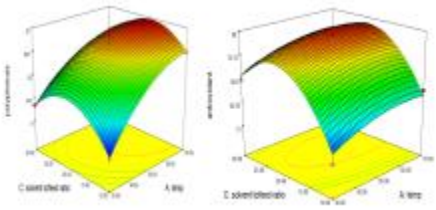

Fig.6.Effect of solvent to feed ratio on response variables (TPC, TAC) for UAE.

In figure 6 it can be seen that an increase in temperature from 30 to 60 ℃ with solvent to feed ratio of 1:10 to1:20 showed the enhanced total polyphenol and total antioxidant content. While with a further increase in temperature over 70 ℃ and solvent to feed ratio over 1:30 showed a gradual decline in the yield of phenolic compounds. This could be explained by the fact that a larger solvent volume can dissolve plant constituents more efficiently leading to an enhancement of the extraction yield. However, if the solution is very dilute, an extra quantity of solvent would not lead to a sufficient increase in the temperature difference and there would be limited enhancement in extraction yield. Zhao et al (2007) reported the results of decreased extraction with increased volume of solvent.

5. Stevioside and rebaudioside analysis by HPLC
Stevia rebaudiana Bertoni leaves were crushed and approximately 0.1 g of the powder was weighed into glass vial. 1g stevia powder dissolved with mobile phase (acetonitrile + water 70:30) and extraction carried out 50℃ for 30 mins. The contents of the vial were

centrifuged and the supernatant diluted 10 times with 30% water and 70% Acetonitrile mix. Five micro liters of the sample solutions were injected for analysis.

Table -1:stevioside and rebaudioside level in stevia leaves

Sweetening components	Amount (%)
Stevioside	25.14
Rebaudioside	45.97

6. Storage stability of fruit juice

Storage stability of fruit juice can be calculated based on these parameters like pH, acidity, color and Brix. Nutritional analysis calculated 30 days. There is no change in nutritional quality of fruit juice.

Table 2 Storage stability of fruit juice

Parameters	Day 1	Day 10	Day 20	Day 30
pH	4.01	3.87	3.65	3.41
Acidity (%)	0.02	0.029	0.035	0.052
Color	4.59	3.56	2.78	2.13
\□□"	41	44	48	51

7. Sensory profile of product

Sensory analysis or sensory evaluation is a scientific discipline that applies principles of experimental design and statistical analysis to the use of human senses (sight, smell, taste, touch and hearing) for the purposes of evaluating consumer products. The discipline requires panels of human assessors, on whom the products are tested and recording the responses made by them. By By applying statistical techniques to the results it is possible to make inferences and insights about the products under test.

ANOVA is useful for product optimization. Statistical analysis based on the consumer review of product. In this study choosing 10 panelists and give the stevia incorporation of juice and analysis the juice give rating. 9 point hedonic scale using for sensory analysis.

Table – 3 ANOVA for Stevia extract incorporation of fruit juices.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	6.08	9	0.6756	2.075085	0.05857	2.152607
Columns	0.68	4	0.17	0.522184	0.719989	2.633532
Error	11.72	36	0.3256			
Total	18.48	49				

IV. CONCLUSION

The Preliminary study on ultrasound extraction of stevia rebaudiana leaves using ethanol, methanol and water as solvents was conducted. The extraction of bioactive compounds from the leaves of stevia rebaudiana and the optimal extraction conditions were obtained by applying response surface methodology. The optimal conditions of UAE were extraction time for 30 min, solvent to feed ratio1:20 ml/g and temperature 50 ℃ with the yield of TPC 69.61 mg/100g of sample and TAC 82.4%.

The stevia leaf extracts analysis by HPLC method. Stevia rebaudiana powder 1g dissolved with mobile phase (acetonitrile + water 70:30) and extraction carried out 50℃ for 30 mins. The contents of the vial were centrifuged and the supernatant diluted 10 times with 30% water and 70% Acetonitrile mix. Five micro liters of the sample solutions were injected for analysis.

Amount of stevioside and rebaudioside content present in the leaves are 25.14% and 45.97%.

Stevia extract incorporation is fruit juice. The optimized products were obtained using Response Surface methodology. The optimized values of the factors are 100 ml fruit juice, 30 ml stevia extract, 0.1 g citric acid. The product is obtained with the goals of minimum pH and titratable acidity, maximum sensory acceptability. Storage of fruit juice under refrigeration condition at 35 °F. Shelf life of juice taken based on the analytical methods like pH, acidity, color and TSS.

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