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Research Article

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BIOCHEMICAL CHARACTERIZATION OF STEVIA ESSENTIAL OIL THROUGH DIFFERENT DRYING METHODS AND SOLVENTS

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ABSTRACT

Essential extracts from plants have been used as a food and medicinal cure since ancient times. Some plants have a good portion of carbohydrates e.g., stevia is being used as an excellent alternative to synthetic compounds *i.e.*, sugar. Therefore, it is important to chemically characterize these nutrients and study their various biological properties. This research aimed to extract essential oil from stevia plant by using Soxhlet apparatus and analysis of nutrients present in the oil were performed by high performance liquid chromatography system *i.e.*, HPLC. Two extraction solvents *i.e.*, hexane (60/40; v/v) and ethanol (70/30; v/v) were used to compare the concentration of bioactive compounds. The plant material was subjected to two drying methods *i.e.*, air dry and oven dry. 10 major compounds were identified as a result of the chemical characterization, in which steviol bioside *i.e.*, 317.01615 (m/z) and stevioside (803.2189 m/z) that is obtained from oven drying using ethanol as extraction solvent were slightly greater than steviol bioside *i.e.*, 315.2183 (m/z) and stevioside *i.e.*, 802.8531 (m/z) obtained from oven drying using hexane as extraction solvent. Likewise, plant material subjected to air dry with ethanol as extraction solvent resulted in greater yield of steviol bioside *i.e.*, 317.0717 (m/z) and stevioside *i.e.*, 803. 3179 (m/z) as compared to steviol bioside *i.e.*, 315.1104 (m/z) and stevioside (802.3185 m/z) that is obtained by air dry using hexane as a solvent. While using fresh leaves the concentrations of compounds *i.e.*, stevioside (799.2187 m/z), dulcoside A (787.3759 m/z) and steviol (639.4189 m/z) obtained by using ethanol were greater than concentration of compounds obtained by hexane. While Rebaudioside A (965.3213 m/z), Rebaudioside B (803.3717 m/z), Rebaudioside C (940.4276 m/z), Rebaudioside D (1066.4763 m/z), Rebaudioside E (964.4273 m/z) and Rebaudioside F (789.3857 m/z) obtained by using hexane as extraction solvent were greater as compared to concentration of compounds obtained by using ethanol. Based on these results, we can suggest that *Stevia rebaudiana* Bertoni can be a natural source of antioxidant, anti-inflammation and antimicrobial properties. It can also be considerd as a potential source of essential oil. These results suggest that the leaves *of S. rebaudiana* Bertoni can serve as therapeutic agents or in prevention of certain inflammatory diseases through their radical-scavenging capacities.

Keywords: *Stevia; Oil Extraction; Nutrients; Stevioside; Characterization.*

INTRODUCTION

Stevia rebaudiana Bertoni is a tiny perennial shrub native to Paraguay, Brazil, and Argentina in the Asteraceae (Compositae) family. For ages, indigenous peoples have utilized the leaves of this plant in remedies and to sweeten drinks such as mate i.e., a green herbal tea. (Samuel et al., 2018). Stevia, a natural sweetener has 200 species of herbs and shrubs from the Asteraceae family (Composite), however only *S. rebaudiana* has the highest level of sweetness among these species (Cosson et al., 2019). Stevia i.e., a perennial shrub has been successfully acclimatized to environmental conditions in several parts of Pakistan, particularly Punjab province, being a production pocket of 1.6-1.8 tons per acre (Chugtai et al., 2019). Currently, stevia cultivation in Pakistan is significantly being focused to constrict the use of conventional sweeteners i.e., sucrose from Sugar cane and Beetroot because most serious health problems,

such as diabetes and obesity, are becoming prevalent. However, sugar consumption cannot be eliminated from our daily lives since it is a necessary component of our food. In these circumstances, stevia with fewer negative impacts on human health is being explored. Moreover, the stevia extracts such as Stevia oil is gradually gaining limelight in medicinal and therapeutic fields. Nevertheless, in Pakistan no study has been carried out so far for its biochemical characterization in terms of nutritional and compositional factors.

Biochemical Composition of Stevia: Stevia leaves are high in a variety of compounds. There are nine essential amino acids among the chemicals: glutamate, aspartate, methionine, tyrosine, proline, alanine, isoleucine, lysine, and serine. (Mlambo et al., 2022). It is a nutrient-dense herb that contains a significant amount of protein, fibre, amino acids, sugars, lipids, essential oils, ascorbic acid, -carotene, riboflavin, thiamine, minerals (chromium, cobalt, magnesium, iron, potassium, phosphorus), and bioactive compounds such as austroinulin, sterebins A-H, nilacin, rebaudi oxide (Elhassaneen, 2019). Stevia was initially studied because of the steviosides, but in recent years, many research has concentrated on other useful components such as diterpene, triterpenes, sterols, pigments, and others, which account for 80%-90% of the dry leaves' composition, Carvacrol (isomer monoterpene phenol), caryophyllene (bicyclic sesquiterpene), caryophyllene oxide, and aromatic chemicals such as spatulenola, kardinola, -pinene, limonen, and iso-pinokarveola that demonstrate antioxidant, anti-inflammatory, and anti-bacterial action were identified in leaf extracts. (Putnik et al 2020). The major glycosides of stevia plants are stevioside and rebaudioside A (Bergs et al., 2012). The steviol (chemical backbone structure) of all diterpenoid glycosides isolated from *Stevia rebaudiana* is identical, with minor changes in the quantity of carbohydrates residues. Because of the expanding diabetic and obese populations in Europe they use sugar in food and drinks, this herbal sweetener has a high market potential (Ismail et al., 2020). Therefore, biochemical characterization of well-adapted and well-cultivated Stevia is urgently required to build a better nutritional framework that can help to explore its significant role in medical and nutritive fields. As a result, researchers have focused their attention on the antioxidant potential of essential oils.

Medicinal Properties of Stevia: The leaves of *S. rebaudiana* have several medical applications, including antibacterial, antiviral, antifungal, anti-hypertensive, anti-hyperglycaemic, anti-tumor, anti-inflammatory, anti-diarrheal, anti-human rotavirus activity, anti-HIV, hepatoprotective, and immune modulatory actions (Elhassaneen, 2019). Stevia extracts have been shown to offer significant health benefits, making them useful in the treatment of a wide range of chronic and nonchronic disorders, including diabetes, cardiovascular disease, cancer, renal disease, obesity, inflammatory bowel disease, and dental caries (Putnik et al., 2020). Furthermore, due to their benign properties, antioxidant chemicals found in edible plants have recently been advocated as food additives.

Stevia has been utilized as a non-caloric sweetener in dietary supplements. An unpleasant taste is also identified in human's herbal tea, natural sugar, and ayurvedic health system as anti-diabetic, anti-obesity, and anti-cholesterol. The antediluvian ayurvedic system of medicine provides several pasts on the use of *S. rebaudiana* . *S. rebaudiana* leaves have been recommended as a treatment for a variety of chronic and non-chronic disorders, including kidney disease, diabetes, cancer, obesity, inflammatory bowel disease, cardiovascular disease, and dental caries (Kumar et al., 2021). Although sweeteners that are artificial and non-caloric can be used to sweeten foods and beverages but cause serious health problems. As a result, it might be argued that using a natural, safe sweetener like stevia is still the best option, as it has gotten a lot of attention and acceptance from both scientists and consumers.

Extraction Methods of Essential Oils (EO): In order for the extracts and essential oils to be utilized safely by humans, the least toxic solvent and the safest extraction procedure are required for optimal phytochemical separation of natural sources of high-activity antioxidants to replace dangerous synthetic antioxidants. Supercritical fluid methods, solvents extraction, enzymatic extractions, ultrasound and microwaves are used as extraction procedures, which are followed by solvent-liquid-liquid extraction, purification using columns, ion exchange, nanofiltration, ultra-membranes crystallization, and fractional distillation (Rao et al., 2019). Despite significant improvements in extraction, producing low-impurity products that are scalable and need the least amount of solvents remains a challenge. Stevia has sparked economic and scientific attention due to its sweet and medicinal characteristics. This plant requires extremely little water i.e., 5 % of that required by sugarcane, that is now a big issue. The chemical makeup of stevia leaves varies depending on geographical location and cultivar (Khiraoui, 2017). The chemical composition of stevia active components is also affected by the drying and processing method (Gasmalla., 2014). Many studies have shown that stevia is the most common sugar substitute. Industrial plant biomass could also be a source of an essential oil (EO) that could be further useful in the development of new pesticidesEmerging technologies such as ultrasound-assisted crystallization, colloidal gas aphrons, isoelectric solubilization-precipitation, hydro

distillation and pressured microwave-assisted extraction are currently being used (Alvarez et al., 2019; Yuan et al., 2019).

MATERIAL AND METHODS

Procurement of Plant Material: Stevia plants of variety i.e., Honey Stevia were grown in pots at plant propagation unit at Horticulture research area Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi. The leaves from these plants were used in this study. The obtained leaves were taken to department laboratory for cleaning purpose where they were thoroughly washed by a disinfectant i.e., 70 % ethanol available in the laboratory so that any foreign matter, microbes, dust or any fibrous material was cleaned. Fresh green leaves were harvested from the whole plant by cutting the plant 5-10 cm from upper parts so that they can be subjected to drying process.

Drying and Grinding: The leaves were divided into two parts. Half of the leaves were dried in open air at 20-22℃ for 8 days and rest were subjected to oven dry at 50℃ for 5hrs. Once dried, leaves were grinded to powdered form by using a mortar and pestle or a lab grinding mill as shown in figure 1. The powdered leaves were packed in an airtight container for further oil extraction and chemical characterization. All the drying and grinding procedure took place in Department of Horticulture, PMAS-AAUR, Rawalpind

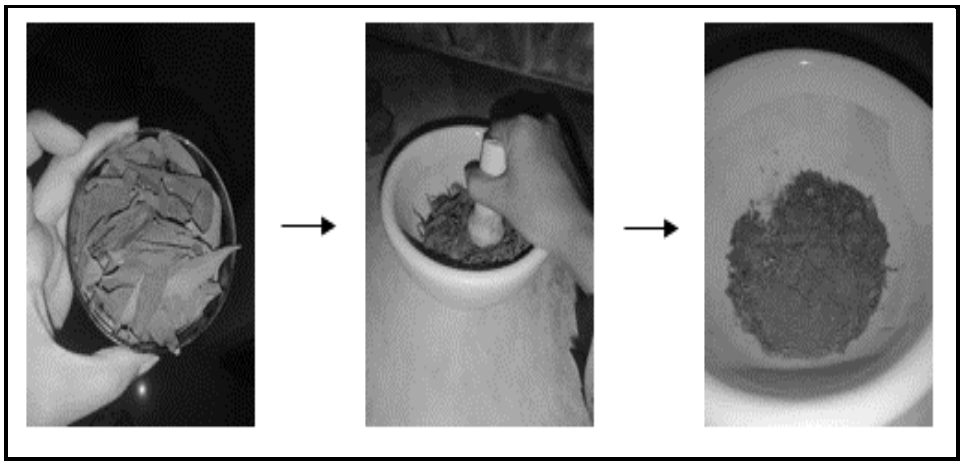

Fig. 1: Drying and grinding process of stevia leaves

Working of Soxhlet Apparatus: Soxhlet apparatus was used for the extraction procedure. Two extraction solvents i.e., ethanol and hexane were subjected into the Soxhlet apparatus each time for oven and air-dried leaves. 10g of powdered leaves were placed in a thimble and sunk in the condensate solvent from the boiling container in an extraction chamber. The solvent reached its maximum level during heating i.e., 80℃ in the thimble during heating, and then trilled into the boiling flask via the syphon duct, extracting the bioactive components into the solvent reservoir. The menstruum i.e., solvents (ethanol and hexane) were present in a flask with a circular bottom. Through the side tube, the vapours reached the condenser, where they condensed and dripped into the extractor. It percolated through the medication, dissolving the soluble components before falling into the flask. The entire extract drained out into the flask through the syphon tube when the level of menstruum in the extractor climbed over the syphon mark. The procedure was repeated for a total of 4-5 cycles until the extraction was completed (Sharma and Shoab, 2020). The intended product was received after intervals and then filtered properly for further analysis as shown in figure**.**

HPLC Analysis*:* In HPLC, a sample mixture or analyte in a solvent i.e., oil extract also known as the mobile phase was pumped through a column with chromatographic packing material at high pressure i.e., stationary phase. A moving carrier gas stream of helium or nitrogen transported the sample. The interaction between the stationary phase, the molecules being analyzed, and the solvent, or solvents utilized, affected retention time of sample. Because the analytes had distinct polarity, the sample interacted with the two phases at different rates as it went through the column. Analytes with the least interaction with the stationary phase and the most interaction with the mobile phase escaped the column first. HPLC is capable of separating and identifying chemicals in any material that can be dissolved in a liquid at trace quantities as low as parts per trillion.

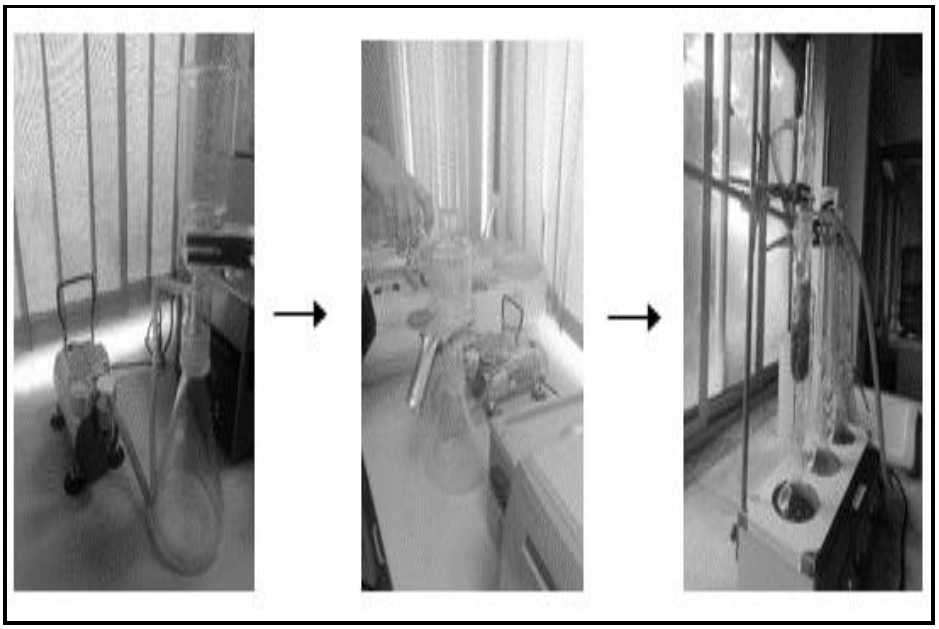

Fig. 2: Oil Extraction of stevia leaves

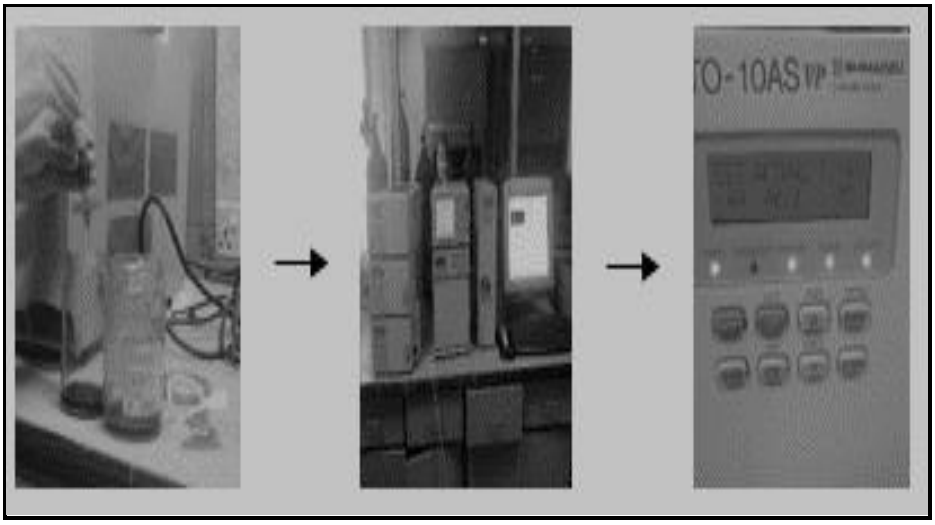

Fig. 3: HPLC analysis of essential oil for biochemical characterization

STATISTICAL ANALYSIS: Data were analyzed through ANOVA (Anaylysis of Variance) using the Statistix 8.1 software. Triplicate analysis were performed on all samples under the completely randomized design. A mean and standard deviation were calculated for the composition analysis. While the difference between the mean values were determined by using LSD (least significant difference) at 5%.

RESULTS

Comparison of ethanol and hexane after air drying: Table 1 depicts the concentration of different biochemical compounds extracted as a result of air drying method by using ethanol and hexane as a solvent. 10 µl sample was subjected for HPLC analysis for 30 min run time, with column temperature 50 ℃ with flow 1.0ml/min. Both solvents used in the study resulted in significant difference in the chemical composition of stevia leaves obtained by both air drying method. By using ethanol solvent highest concentration of Rebaudioside D (1127.47 m/z) was obtained followed by Rebaudioside E (965.42 m/z) at par with Rebaudioside A. While the lowest concentration was found in steviol bioside (317.07 m/z).By using hexane solvent, Rebaudioside D was found maximum (1120.47 m/z) while the lowest content was observed in Steviol bioside (315.1104 m/z).

Table 1: Concentration of compounds obtained by using ethanol and hexane as extraction solvent after air drying method

Compounds	**Ethanol (m/z)**	**Hexane (m/z)**	**Difference (%)**
Steviol bioside	317.07±1.99i	315.1104±0.48h	0.620486
Stevioside	803.32±0.11f	802.3185±0.67e	0.124486
Rebaudioside A	965.42±0.11b	966.4231±0.11b	-0.10349
Rebaudioside B	803.37±0.11e	803.3707±0.11e	0
Rebaudioside C	949.84±1.11c	949.4286±0.54c	0.043596
Rebaudioside D	1127.47±0.11a	1120.476±0.67a	0.622789
Rebaudioside E	965.42±1.11b	966.4231±0.11b	-0.10349
Rebaudioside F	935.41±0.57d	935.428±0.11d	-0.00161
Dulcoside A	787.37±0.67g	788.5873±1.11f	-0.15375
Steviol	641.31±0.08h	642.3179±0.10g	-0.15581

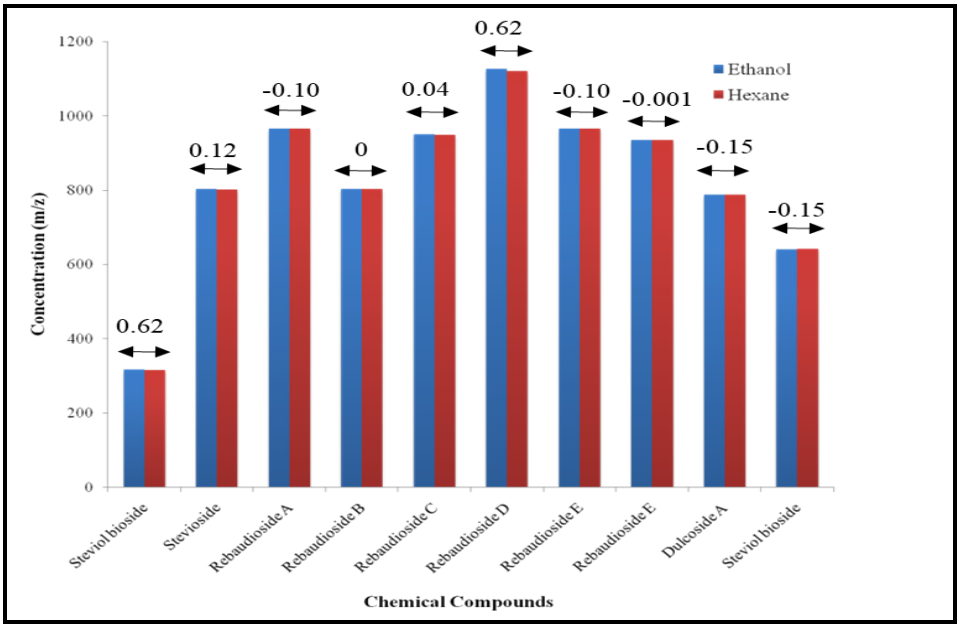

Figure 4: Comparison of concentration by using two different solvents

Comparison of ethanol and hexane after oven drying: The results regarding the impact of oven drying method by using ethanol and hexane as a solvent on the Composition of stevia leaves are shown in table 2. 10 µl sample was subjected for HPLC analysis for 30 min run time, with column temperature 50 ℃ with flow 1.0ml/min. The results suggest a slight difference in concentrations obtained by using ethanol and hexane. Highest concentration of Rebaudioside D was obtained by using both ethanol and hexane solvent while the lowest concentration was recorded in Steviol bioside by using both ethanol and hexane solvent.

Table 2: Concentration of compounds obtained by using ethanol and hexane as extraction solvent after oven drying method

Compounds	**Ethanol (m/z)**	**Hexane (m/z)**	**Difference (%)**
Steviol bioside	317.06±1.99 h	315.22 ±0.48 h	0.583033
Stevioside	803.22±0.12e	802.85±0.67e	0.045552
Rebaudioside A	964.42± 0.67b	966.42± 0.11b	-0.20652
Rebaudioside B	803.87±0.30 e	803.37±0.11e	0.062218
Rebaudioside C	949.28± 1.15c	949.42± 0.54 c	-0.01498
Rebaudioside D	1127.47± 0.11a	1120.48±0.67a	0.621718
Rebaudioside E	966.22± 1.11b	965.42± 0.67b	0.082852
Rebaudioside F	935.51±0.60d	936.42±0.67d	-0.09714
Dulcoside A	787.28±0.65f	718.42±0.11f	9.147074
Steviol	681.32±0.08g	652.32±0.18g	4.349195

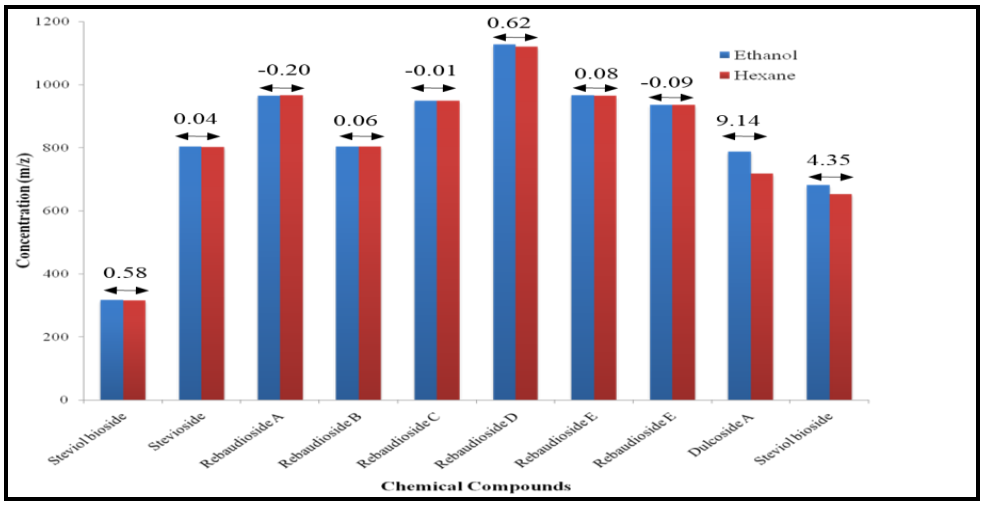

Figure. 5: Comparison of concentration by using two different solvents

Comparison of ethanol and hexane for fresh leaves: 10 µl sample was subjected for HPLC analysis for 30 min run time, with column temperature 50 ℃ with flow 1.0ml/min. Very slight difference in concentrations were observed by using ethanol and hexane. The proximate chemical composition of fresh Stevia rebaudiana leaf extracted by using ethanol and hexane solvent is shown in Table 3. The results exhibited slightly significant ($p \leq 0.05$) values in steviol bioside, Stevioside, Rebaudioside A, Rebaudioside B, Rebaudioside C, Rebaudioside D, Rebaudioside E, Rebaudioside F, Dulcoside A and Steviol of fresh stevia leaves. The composition data indicate that the fresh Stevia leaves has higher Rebaudioside D content (1066 m/z and 1118.22 m/z) lowest Stviol bioside content (310.82 m/z and 315.32 m/z).

Table 3: Concentration of compounds obtained by using ethanol and hexane as extraction solvent for fresh leaves

Compounds	Ethanol (m/z)	Hexane (m/z)	Difference (%)
Steviol bioside	310.82± 0.67i	315.32 ±0.56i	-1.438
Stevioside	799.22 ±1.05e	787.22 ±0.11f	1.512
Rebaudioside A	965.32 ±0.11b	964.32 ±1.22b	0.103
Rebaudioside B	803.37 ±0.11d	803.37 ±0.11d	0
Rebaudioside C	940.42± 0.99c	941.43 ±1.11c	-0.106
Rebaudioside D	1066.47±0.61a	1118.22 ±0.10a	-4.736
Rebaudioside E	964.43 ±1.25b	965.32 ±0.64b	-0.092
Rebaudioside F	789.38 ±0.11f	789.47± 0.30e	-0.011
Dulcoside A	787.37± 0.67g	718.37 ±0.11g	9.164
Steviol	639.42 ±0.79h	639.42 ±1.75h	0.0002

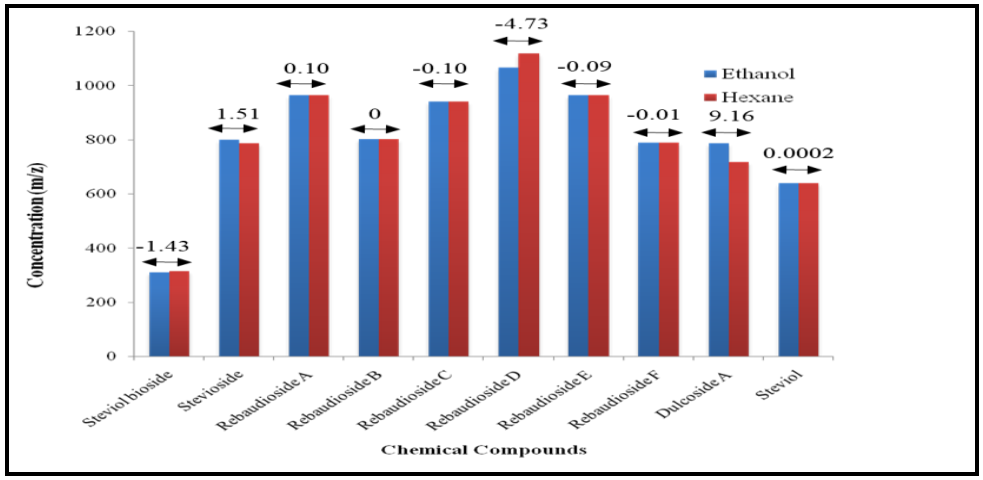

Figure. 6: Comparison of concentration by using two different solvents

DISCUSSION

Ethanol and hexane after air drying: The concentrations of compounds i.e., steviol bioside (317.0717 m/z), stevioside (803.3179 m/z), Rebaudioside C (949.8426 m/z) and D (1127.476 m/z) obtained by using ethanol were greater than concentration of compounds obtained by using hexane solvent. While concentrations of rebaudioside A (966.4231m/z), rebaudioside E (966.4231 m/z), rebaudioside F (935.428 m/z), Dulcoside A (788.5873m/z) and steviol (642.3179 m/z) obtained by hexane solvent were greater than concentration of compounds obtained by ethanol solvent.

Ethanol and hexane after oven drying: The concentrations of compounds i.e., steviol bioside (317.0615 m/z), stevioside (803.2189 m/z), Rebaudioside B (803.8707 m/z), Rebaudioside E (966.2235 m/z), rebaudioside D (1127.476 m/z), dulcoside A (787.2859 m/z) and steviol (681.3182 m/z), obtained by using ethanol were greater than concentration of compounds obtained by using hexane solvent. While concentrations of rebaudioside A (966.4231 m/z), rebaudioside C (949.4286 m/z), rebaudioside F (936.422 m/z) obtained by hexane solvent were greater than concentration of compounds obtained by ethanol solvent.

From the above results, ethanol has shown better extraction results as compared to hexane after oven drying. On the other hand, hexane has shown better results as compared to ethanol after air drying.

Ethanol and hexane after for fresh leaves: The concentrations of compounds i.e., stevioside (799.2187 m/z), dulcoside A (787.3759 m/z) and steviol (639.4189 m/z) obtained by using ethanol were greater than concentration of compounds obtained by hexane. While Rebaudioside A (965.3213 m/z), Rebaudioside B (803.3717 m/z), Rebaudioside C (940.4276 m/z), Rebaudioside D (1066.4763 m/z), Rebaudioside E (964.4273 m/z) and Rebaudioside F (789.3857 m/z) obtained by using hexane as extraction solvent were greater as compared to concentration of compounds obtained by using ethanol.

Thus, whole oil extraction procedure was practiced to obtain active botanical elements of stevia, which are it's "vital force". They are an essential liquid version of a plant that allow its beneficial chemicals to reach the bloodstream faster than they would if the plant is consumed as a whole. Therefore, when a plant is exposed to a solvent, a part of the plant material components dissolves, resulting it in an herbal extract. These solvents can be used as preservatives or as chemicals that aid in the breakdown and release of plant cells' contents. The evaluation and chemical characterization of nutrient composition of these essential oils from plant i.e., stevia being used in the present study can help to determine the medicinal properties and which nutrients are responsible for it. Moreover, if there is any change in nature of essential oil extracted from stevia, it can also be determined from the experiment of extraction process. Furthermore, it will give an optimum approach to be used to discover various components and probable interactions between these nutrients to generate a set of ideal experimental circumstances.

CONCLUSION

In this work, the phytochemicals of the leaves and essential oil of *Stevia rebaudiana* Bertoni were examined. We may infer from these findings that *S. rebaudiana* Bertoni may have natural antioxidant, anti-inflammation, and antibacterial capabilities. Additionally, it may serve as a source of essential oils. These findings imply that the radical-scavenging abilities of the leaves of *S. rebaudiana* Bertoni can be used as therapeutic agents or in the prevention of some inflammatory diseases linked to an increase in NO production. In addition to being used as sugar plants, it can also be used according to its constituents for a variety of cosmetic, medicinal, and pharmacological goods as a supplement. It stands for a highly appealing economic alternative in that regard.

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