Arabian Journal of Chemistry (2016) 9, S1185–S1189

King Saud University

Arabian Journal of Chemistry

www.ksu.edu.sa
www.sciencedirect.com

ORIGINAL ARTICLE

Chemical composition of essential oil by different extraction methods and fatty acid analysis of the leaves of *Stevia Rebaudiana Bertoni*

A.B. Siddique [a], S.M. Mizanur Rahman [a], M. Amzad Hossain [b,*]

[a] *Department of Chemistry, University of Dhaka, Dhaka 1000, Bangladesh*
[b] *Chemistry Division, Atomic Energy Centre, GPO Box 164, Ramna, Dhaka-1000, Bangladesh*

Received 7 June 2011; accepted 7 January 2012
Available online 18 January 2012

KEYWORDS

Stevia Rebaudiana Bertoni;
Essential oil;
Hydro distillation;
Steam distillation;
Chemical constituents;
GC–MS

Abstract The chemical composition of essential oils of *Stevia Rebaudiana Bertoni* leaves grown in Bangladesh Sugarcane Research Institute, Pabna, Bangladesh and obtained by two different extraction methods (hydro distillation and steam distillation) were determined by gas chromatography–mass spectroscopy analyses (GC–MS). One hundred and twenty three peaks were obtained from hydro distillation where 62 compounds were identified as major compounds such as α-cadinol (2.98%), caryophyllene oxide (1.23%), (−)-spathulenol (2.21%) and β-guaiene (0.32%), and 50 peaks were obtained from the steam distillation where all the major compounds were present. After approximately 115 days the latter again ran the samples where only 4 peaks were identified of which none of these were constituents of essential oil in *Stevia Rebaudiana Bertoni*. The fatty acid analysis by GLC showed palmitic acid (86.50%) as the most abundant fatty acid in the leaves of *Stevia Rebaudiana Bertoni*.

1. Introduction

Stevia Rebaudiana Bertoni belongs to the family Compositae (Asteraceae) (Brandle et al., 1998; Midmore and Rank, 2007) that is indigenous to Paraguay and Brazil and is also cultivated in some region in Asia, Europe and Canada (Crammar and Ikan, 1987). Recently, cultivation of *Stevia Rebaudiana* has started in the subcontinent. The main sweet component in the leaves of the *Stevia Rebaudiana Bertoni* is stevioside (Brandle et al., 1998). It is 300 times sweeter than sucrose (Soejarto et al., 1982) and has recently gained importance as a natural noncaloric sweetener. Stevia leaf extracts are used in Japan, Korea and some other countries in South America to sweeten soft drinks, soju, soy sauce, yogurt and other foods, whereas in the United States it is used as dietary supplements and in Bangladesh as antidiabetic tea. *Stevia Rebaudiana Bertoni* sweetener extractives have been suggested to exert beneficial effect on human health including antihypertensive (Chan

* Corresponding author. Tel.: +968 92327578.
E-mail addresses: smmrdu@yahoo.com (S.M. Mizanur Rahman), dramzadh@gmail.com (M.A. Hossain).
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.arabjc.2012.01.004

et al., 2000; Lee et al., 2001), antihyperglycemic (Jeppesen et al., 2000), anticariogenic (Das et al., 1992) and anti-human rotavirus activities (Takahashi et al., 2001). It has also effects on the metabolism of glucose (Suanarunsawat and Chaiyabutr, 1997; Toskulkao et al., 1995) and renal function (Jutbha et al., 2000). The increasing importance of essential oils in various domains of human life (pharmacy, cosmetics as well as food and drink industries) has made this field very interesting for chemical investigations. It has reported (Viana and Metivier, 1980; Medicinales et al., 2001) that stevia is nutrient-rich, containing substantial amounts of protein, calcium, phosphorous and other important nutrients.

There are many very legitimate reasons for using stevia as a medicinal food. In spite of the prominence stevia has obtained as a flavour enhancer, it contains a variety of constituents besides the steviosides and rebaudiosides, including the nutrients specified above. Sterols, triterpenes, flavonoids, tannins, and an extremely rich volatile oil comprising rich proportions of aromatics, aldehyde, monoterpenes and sesquiterpenes have been reported from stevia. Essential Oil (EO) also called ethereal or volatile oils, are frequently referred to as the ''life force'' of plants. Unlike fatty oils, these ''essential'' oils are volatile, highly concentrated, substances extracted from leaves, flowers, stems, roots, seeds, barks, ruin or fruit rinds. Before such substances can be analysed, they have to be extracted from the material. At present different extraction techniques like distillation, effleurage, CO_2 extraction, expression and solvent extraction are applied for oil extraction from various plants. But conventional methods that are the most common methods usually used to extract essential oil from plants are the distillation methods like Steam-Distillation, Hydro-Distillation and Water–Steam-Distillation and solvent extraction methods are used (Suanarunsawat and Chaiyabutr, 1997; Toskulkao et al., 1995). *Stevia Rebaudiana Bertoni* leaves essential oil comprises a number of different types of complex constituents. So their separation and analysis is performed by gas chromatography–mass spectroscopy. During the last few years, gas chromatography–mass spectroscopy has been established as a fast efficient and relatively simple technique for separation and analysis of a mixture of volatile substances and is being extensively used by the perfumers (Viana and Metivier, 1980; Medicinales et al., 2001). The difference between the growth of population and the supplies of food particularly in the developing countries increased day by day. The search for easy sources of food is the major concern of all agencies so as to provide adequate food and improve the nutritional status of the population (Sankhala et al., 2005). Indigenous plants are the main sources of medicine and food. (Hill, 1952). A wide range of research has been conducted to document the nutritive value of foods (Rajalakshmi and Geeravani, 1990; Mohan and Janardhanan, 1995). Hence, the objective of the present study is to explore the untapped sources of good nutrition, as well as to identify the chemical composition of the essential oils in Bangladeshi *Stevia Rebaudiana Bertoni*. The main objective of the present work is to understand the atmospheric effect on dried and grinded powder leaves of *Stevia Rebaudiana Bertoni* as well as to compare steam-distillation with hydro-distillation; to identify and characterize extracted essential oil and fatty acids from the *Stevia Rebaudiana Bertoni* leaves using GC–MS.

2. Experimentals

2.1. Plant material

The green leaves of *Stevia Rebaudiana Bertoni* were collected from the Bangladesh Sugarcane Research Institute, Ishurdi, Pabna, Bangladesh. *Stevia rebaudiana Bertoni* (popularly called as Stevia and synonymously known as 'sugar substitute' belongs to family Asteraceae) leaves were washed, dried in an oven at 40 °C for 3 days, ground into powder. The leaves of this plant were harvested during the month of September, 2010. The leaves were collected at 2:00 pm–3:00 pm on September 2, 2010 and packed in polyethylene bags and stored at 4 °C until required. The plant was initially identified by morphological features and data base present in the library, Department of Botany, University of Dhaka, Bangladesh and a voucher specimen has been deposited at the Department of Biology, University of Dhaka, Bangladesh with voucher number 0147. Approximately 50 g of leaves were ground using a grinder (Blender 80115) for 20 s. The unfermented *Stevia rebaudiana Bertoni* leaves were kept in the oven at 40 °C and put in a desiccator for at least 24 h prior to analysis.

2.2. Drying and grinding

The green fresh leaves were allowed to dry in the open air and the dried leaves ground by a mortar. One part of the leaves powder was subjected to hydro distillation, 2nd part of the powder was subjected to fatty materials analysis and 3rd part was stored at room temperature for further analysis by steam distillation.

2.3. Materials

Aluminium chloride, potassium acetate, hydrochloric acid, sulphuric acid were obtained from Sigma–Aldrich. Solvents for extraction were ethanol, hexane, butanol, chloroform (reagent grade) obtained from Merck (Darmstadt, Germany). The water was purified from water distillation plants in Chemistry laboratory, Atomic Energy Centre, Ramna, Dhaka. All other chemicals were of analytical grade or GC grade. UV spectra UV–Visible spectra measurements were done using Spectro (Thermo Fisher Scientific, model 4001/4) spectrophotometer.

2.4. Analysis of fatty acid

Measured amount 100.0 mg of *Stevia* leaves sample was dissolved in *n*-hexane (50.0 ml) and refluxed about 30 min with 5% sodium bicarbonate solution (25.0 ml × 2).The mixture was allowed to stand for overnight and after separation the aqueous layer was analysed for free fatty acid (FFA) while the organic layer was analysed for bound fatty acid (BFA) (Sofowana, 1993).

The aqueous portion was acidified with 2 M sulphuric acid (pH 2.5) and the mixture was further treated with hexane (25.0 ml × 3). The hexane fraction was dried over anhydrous sodium sulphate, filtered and evaporated to dryness. After adding 2.0 mL of borontrifluoride–methanol (BF_3–MeOH) complex to the dry sample the mixture was refluxed on a boiling water bath for 6–10 min. The mixture was then evaporated

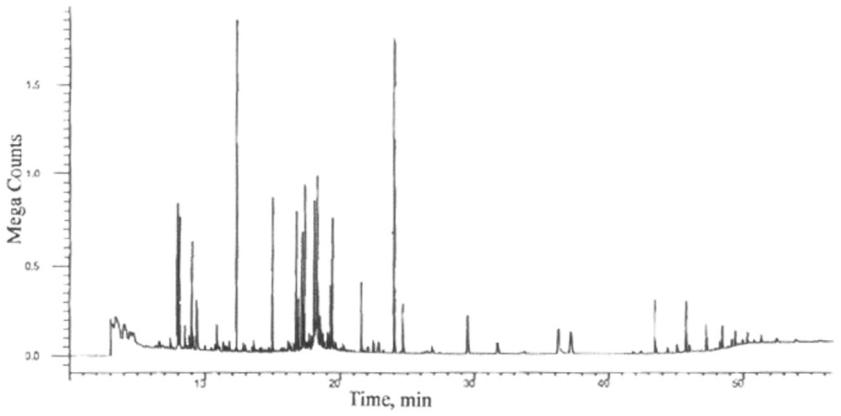

Figure 1 A typical chromatogram of the constituents of hydrodistillate oil of *Stevia Rebaudiana Bertoni* leaves.

in a rotavapor to dryness and transferred into a small separatory funnel containing a little water (6.0 mL). The mixture was shaken vigorously followed by further treatment with hexane. The aqueous layer was discarded. The hexane part containing the methyl esters of fatty acids was made free from water by adding anhydrous sodium sulphate. The solution was filtered and the filtrate was concentrated for the analysis of free fatty acids by GLC.

Similarly, the organic portion was taken in a pear shaped flask and was treated with methanolic sodium hydroxide (0.5 M, 10.0 mL) followed by refluxing in a boiling water bath for 30 min. After concentrating the mixture under reduced pressure a small proportion of water was added to the mixture and transferred to a separating funnel to settle down. The non-saponified materials were separated from the saponified portion (aqueous layer) by extraction with hexane and the aqueous layer was acidified with the addition of 2 M sulphuric acid to pH 2.5. After treating the acidic mixture with *n*-hexane the organic part was collected in a conical flask and made free from water by adding with anhydrous sodium sulphate and filtered. Finally, the filtrate was methylated as before and concentrated and analysed for bound fatty acids by GLC (Shimadzu 9A, Column-BP-50, Detector-FID, 170 °C-1 min/4–270 °C-30 min).

2.5. Extraction and isolation of essential oil

2.5.1. Hydro distillation

Two hundred and fifty grams of the air-dried leaves of *Stevia Rebaudiana Bertoni* was subjected to hydro distillation for 3 h using a Clevenger type apparatus. Sodium chloride (1 g) and 20 mL of dichloromethane was added with the aqueous distillate in a separating funnel and shaking was continued for 40 min and allowed to stand for 15 min. The organic layer was separated and concentrated to 5 mL under reduced pressure. The oils dissolved in the organic layer were dried over anhydrous sodium sulphate and preserved in a sealed vial at 4 °C until further analysis. The hydrodistillate oil was subjected to GC–MS.

2.5.2. Steam distillation

Two hundred and fifty grams of the air-dried leaves of *Stevia Rebaudiana Bertoni* was subjected to *Steam distillation* for 3 h using a steam distillation apparatus. Sodium chloride (1 g) and 40 mL of dichloromethane was added with the aqueous distillate in a separating funnel and shaking was continued for 40 min and allowed to stand for 15 min. The organic layer was separated and concentrated to 6 mL under reduced pressure. The oils dissolved in the organic layer were dried over anhydrous sodium sulphate and preserved in a vial at room temperature without sealing by Para film for few days. The volume of the organic layer content oil was reduced to 4 mL followed by storing in a glass vials in a tin at 4 °C. It is observed that very little gummy material was formed within two weeks. So the organic layer was dried again over anhydrous sodium sulphate, filtered and the clear brownish oil was subjected to GC–MS.

2.6. Analysis of the essential oils by GC–MS

The GC–MS analysis of the essential oil samples of *Stevia Rebaudiana Bertoni* was performed using a Varian GC–MS (Model Varian CP 3800, USA) equipped with a VF-5 fused silica capillary column (30 m × 0.25 i.d. mm. film thickness 0.25 μm, Varian, USA). For GC–MS detection, an electron ionisation system with ionisation energy of 70 eV was used. Helium gas was used as a carrier gas at a constant flow rate

Table 1 Chemical composition of the essential oils of *Stevia Rebaudiana Bertoni* obtained by two different methods.

Name of the compounds	Retention time RT	Area (%)
Hydro distillate oil		
Cyclopentasiloxane	9.372	0.88
Geranyl vinyl ether	11.778	0.14
Cyclohexasiloxane	12.318	4.40
Indole	12.778	0.17
Caryophyllene	14.715	0.08
(−)-Spathulenol	17.195	2.21
Caryophyllene oxide	17.282	1.23
Santalol, cis alpha pyranomenthol	17.527	0.07
β-Guaiene	17.975	0.32
Ledene oxide-(II)	17.735	0.21
α-Cadinol	18.292	2.98
Aristolene epoxide	19.657	0.18
Steam distillate oil		
Silanediol	3.540	4.49
Cyclopentasiloxane	9.343	7.00
Cyclohexasiloxane	12.270	7.10
Estra-1,3,5(10)-trien-17-ones	19.365	1.92

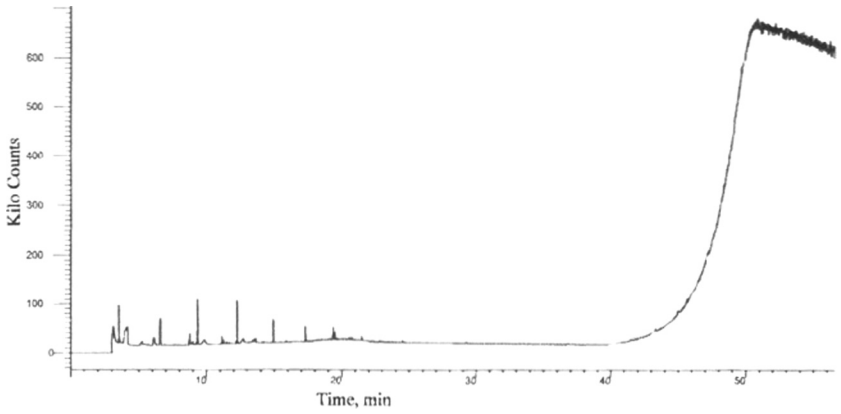

Figure 2 A typical chromatogram of the constituents of Steam distillate oil of *Stevia Rebaudiana Bertoni* leaves.

Table 2 Relative percentages of free and bound fatty acids in hexane extract of *Stevia Rebaudiana Bertoni* leaves.

Fatty acids	Retention time (RT)	Relative percentage
FFA		
Palmitic acid	12.57	86.50
Stearic acid	15.56	2.20
Linoleic acid	33.14	3.26
BFA		
Palmitic acid	12.61	50.55
6-Octadecenoic acid	29.5	7.28

of 1 ml min^{-1}. Injector and mass transfer line temperature were set at 250 °C and 300 °C, respectively. The oven temperature was programmed from 50 °C to 200 °C at 8 °C min^{-1} and then held isothermally for 20 min and finally raised to 300 °C at 10 °C min^{-1}. Diluted samples (1/100 v/v, in methanol) of 0.2 µl were manually injected in the split less mode. Identification of compounds of the essential oil was based on GC retention time on VF-5 capillary column, computer matching of mass spectra with standards (Mainlab, Replib and Tutorial data of GC–MS systems).

3. Results and discussion

3.1. Physical properties

Colour of the essential oil obtained by methods E-1and E-2 both was transparent but E-I is colourless while brownish and the *Refractive index* was found to be 1.42 and 1.5 at the temperatures 31.4 °C and 30.5 °C, respectively.

3.2. Chemical composition of essential oils (E-1)

The GC–MS total ion chromatogram (TIC) of the essential oils from the leaves of *Stevia Rebaudiana Bertoni* is shown in Fig. 1. The major compounds shown in Table 1. identified in the oils, were (−)-spathulenol, caryophyllene oxide, caryophyllene, ledene oxide-(II), β-guaiene, geranyl vinyl ether, tricyclo [5.2.2.0(1,6)] undecan-3-ol, indole, aristolene epoxide, 1,2,3,5,6, 7,8,8a-octahydro-1,4-dione and 2,6,6-trimethyl-2-cyclohexene-1,4-dione, respectively (Table 1).

3.3. Chemical composition of essential oils (E-II)

The GC–MS total ion chromatogram (TIC) of the essential oils from the leaves of *Stevia Rebaudiana Bertoni* is shown in Fig. 2. The all matches compounds shown in Table 2 identified in the oils were silanediol, cyclopentasiloxane, cyclohexasiloxane and estra-1,3,5(10)-trien-17-ones (Table 1).

3.4. Chemical composition of the fatty acids

There were five fatty acids identified by comparison with the fatty acid methyl ester standards. Palmitic acid was found to be the major compound with the relative percentage of 86.50% whereas stearic acid as the least abundant with a percentage of 2.20%. The results are shown in Table 2.

4. Conclusion

This is to conclude that the stevia leaves may be used as the source of palmitic acid. The TIC of hydrodistillate oil shows some valuable constituents of essential oil such as (−)-spathulenol, caryophyllene oxide, caryophyllene, ledene oxide-(II), β-guaiene, geranyl vinyl ether, indole and aristolene epoxide where TIC of steam distillate oil shows only four matches compounds silanediol, cyclopentasiloxane, cyclohexasiloxane and estra-1,3, 5(10)-trien-17-ones. Cyclopentasiloxane and cyclohexasiloxane may appeared from contaminants of the equipments (GC–MS) because these two compounds were present in Hydro distillate oil whereas Silanediol and estra-1,3,5(10)-trien-17-ones were present in the blank experiment. There is no report on natural occurrence of the last two products. Therefore, the main conclusion of the present study is that such a type of result observed in the case of steam distillation is due to unsafe storage of leaves powder or essential oils after extraction because most of the constituents were volatile and decomposed followed by air oxidation which appears as turbid and gummy materials.

Acknowledgements

We are grateful to Dr. M. Ali, Chief Scientific Officer and Head, Chemistry Division, Atomic Energy Centre, Ramna,

Dhaka for his continuous encouragement during the work and providing all laboratory facilities. We also thank Mr. Zahidul Islam and Mr. Ayub Ali for their help in the preparation the samples.

References

Brandle, J.E., Starratt, A.N., Gijzen, G., 1998. Can. J. Plant Sci. 78, 527–532.

Chan, P., Tomlinson, B., Chen, Y., Liu, J., Heish, J., Cheng, J., 2000. Br. J. Clin. Pharmacol. 50, 215–222.

Crammar, B., Ikan, A., 1987. Progress in the chemistry and properties of the rebaudiosides. In: Grenby, T.H. (Ed.), . In: Developments Sweeteners, vol. 45. Elsevierv Applied Science, London, Uk.

Das, S., Das, A.K., Murfhy, R.A., Punwani, I.C., Nasution, N.P., Kinghom, A.D., 1992. Caries Res. 26, 363–365.

Hill, A.F., 1952. A Textbook of Useful Plants and Plant Products, second ed. McGarw-Hill Book Company Inc., New York.

Jeppesen, P.B., Gregersen, S., Poulsen, C.R., Hermansen, K., 2000. Metabolism 49, 208–216.

Jutbha, P., Toskulkao, C., Chatsudthipong, V., 2000. Can. J. Physiol. Pharmacol. 78, 737–742.

Lee, C.N., Wong, K., Liu, J., Chen, Y., Chen, J., Chan, P., 2001. Plant Med. 67, 796–803.

Hierbas, Medicinales, Caa Jhee. Bulletin, 2001.Centro de Promocion de las Exportaciones, Ministerio de Industria Y Comercio, Paraguay, pp. 39–55.

Midmore, D. J., Rank, A. H. 2007. A new rural industry-stavia-to replace imported chemical sweeteners. A Report for the Rural Industries Research and Development Cooperation, Australia, RIRDC Project No. UCQ-16A, 2002, pp. 1–3.

Mohan, V.R., Janardhanan, K., 1995. J. Food Sci. Technol. 32, 465–471.

Rajalakshmi, P., Geeravani, P., 1990. J. Food Sci. Technol. 27, 260–265.

Sankhala, A., Sankhala, A.K., Bhatnagar, B.H., Singh, A., 2005. J. Food Sci. Technol. 42, 446–452.

Soejarto, D.D., Kinghorn, A.D., Farnsworth, N.R., 1982. J. Nat. Prod. 45, 590–594.

Sofowana, A., 1993. Medicinal Plants and Traditional Medicine in Africa. Spectrum Books Ltd., Ibadan, Nigeria, 191–289.

Suanarunsawat, T., Chaiyabutr, N., 1997. Can. J. Physiol. Pharmacol. 75, 976–978.

Takahashi, K., Matsuda, M., Oashi, K., Yaniguchi, K., Nakagomi, O., Abe, Y., Mori, S., Sato, S., Okuyani, K., Shigeta, S., 2001. Antiviral Res. 49, 15–19.

Toskulkao, C., Sutheerawatananon, M., Wanichanon, C., Saitongdee, P., Suttagit, M., 1995. J. Nutr. Sci. Vitaminol. 41, 105–112.

Viana, A.M., Metivier, J., 1980. Ann. Bot. 45, 469–475.