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VARIATIONS IN STEVIOSIDE AND REBAUDIOSIDE-A CONTENT IN *STEVIA REBAUDIANA* UNDER DIFFERENT GROWING CONDITIONS

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Abstract- The effects of different growing conditions (open field and polyhouse) were investigated on stevioside and rebaudioside-A content of *Stevia rebaudiana* plant. The experimental results revealed that stevioside and rebaudioside-A content were maximum at pre-flowering growth phase followed by flowering and post-flowering phase under both experimental conditions. Leaves showed maximum stevioside (9.19%) and rebaudioside-A content (7.00%) under polyhouse and open field conditions, respectively. However, minimum stevioside and rebaudioside-A content (< 1.00%) was observed in green and woody stem.

Keywords- Glycosides, *Stevia*, polyhouse, open field conditions

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Introduction

Stevia comprises of approximately 200 species of herbs and shrubs belonging to the family *Asteraceae*. One of the representatives of the genus *Stevia* is *Stevia rebaudiana* Bertoni, previously named as *Eupatorium rebaudianum* Bertoni [1]. *Stevia rebaudiana*, the sweet herb of Paraguay, is becoming a major source of high potency sweetener having low calorific value [2]. Stevioside and rebaudioside-A are the two major steviol glycosides found in *S. rebaudiana*. Commercial interest in these steviol glycosides as sweeteners has been high for a long time. Leaf extract of *Stevia*, have been used from decades in soft drinks, yogurt, soy sauce and other foods in Japan, Korea and Brazil [3]. Stevioside and rebaudioside-A have beneficial effects on human health, including anti-hyperglycemic [4], anti-cancerous [5], anti-hypertensive [6], anti-rotavirus and cardiovascular actions [7], antioxidant [8] and anti-inflammatory [9].

Wide variation in the percentage of stevioside and other sweet diterpenoid glycosides has been reported in the leaves and other parts of *S. rebaudiana* and this has been ascribed to both genetic [10] and environmental factors [11]. Environment could be modified in regulated conditions to maximise the production of active compounds. Subjecting plant to such environmental conditions can result in change in levels of secondary metabolites. In a controlled environment system, it is possible to achieve high production of secondary metabolites within a very short period of cultivation [12]. Keeping in view this fact and increasing demand of this herbal drug, present study is an attempt to examine the presence of active chemical constituents in the plant samples collected from the open field and polyhouse conditions.

Materials and methods

Collection and post harvest approaches

The present study was conducted on the plants of *Stevia rebaudiana* grown under open field and polyhouse conditions at the experimental farm of the Department of Forest Products, Dr YS Parmar UHF, Nauni, Solan (Himachal Pradesh). Samples (leaves, green stem and woody stem) were collected separately from plants grown in both conditions at the pre-flowering, flowering and post-flowering stages. Collected samples were shade dried and then dried in oven at 60˚C temperature for 6 hours.

Quantitative estimation of active chemical constituents

The powdered samples (0.5 g) were refluxed with methanol (25 ml, 1 hr.) on the boiling water bath. The contents were then filtered and residue was further refluxed with methanol (25 ml, 1 hr.) for three times. Each time contents were filtered and all filtrates combined. At the end of fourth extraction all plant residues were non-sweet in taste. After combining all the filtrates of each sample, methanol was distilled off on a water bath.

Residue thus obtained was then dried under vacuum and warmed with dichloromethane (20 ml) on a water bath. After cooling, the contents were filtered and residue further extracted with dichloromethane to remove dichloromethane soluble less polar non-sweet compounds including colours. The dichloromethane insoluble residue was then dried under vacuum and dissolved in acetonitrile: water (80 : 20) mixture and final volume was made to 25 ml in a volumetric flask with the same solvent mixture. These plant extracts were then filtered through Millipore filter paper and 20-µl volume was injected into HPLC column and components were detected at λ_{max} 210 nm using variable length UV detector. For obtaining well separated peaks of two major sweet compounds i.e., stevioside [Fig-1] and rebaudioside-A [Fig-2], known weights of plant samples after initial repeated extraction with methanol were completely dried and then non-polar compounds and colours were removed through extraction with dichloromethane. Standard graphs drawn for the two sweet compounds (stevioside and rebaudioside-A) were found almost linear [Fig-3 and 4].

Percentages of stevioside and rebaudioside-A were calculated using the following formula:

$$\text{Sweet compound (\%)} = \frac{\text{Test area}}{\text{Standard area}} \times \frac{\text{Weight of standard compound}}{\text{Standard compound dilution}} \times \frac{\text{Test sample dilution}}{\text{Test sample weight}} \times 100$$

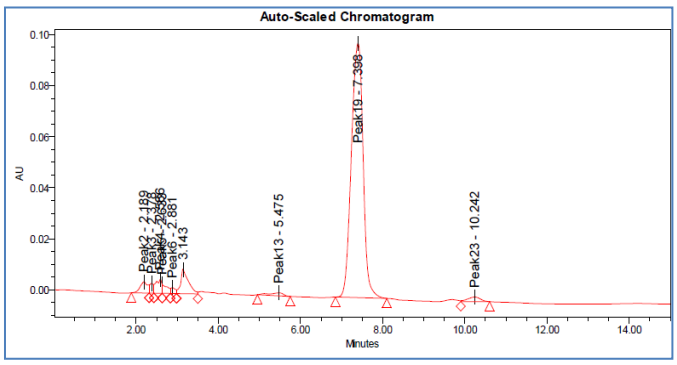

Fig-1 HPLC Chromatogram of reference stevioside (400ppm)

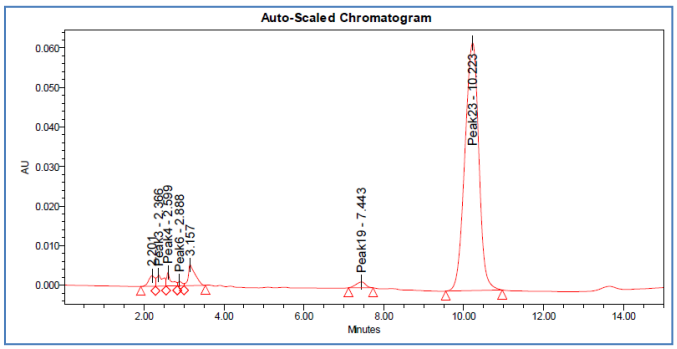

Fig-2 HPLC Chromatogram of reference rebaudioside-A (400ppm)

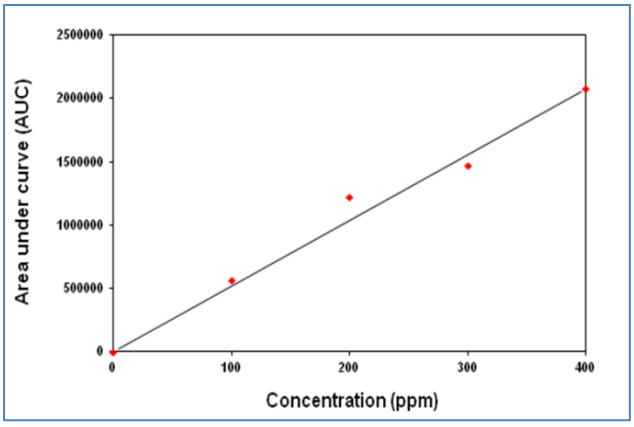

Fig-3 Standard graph of stevioside

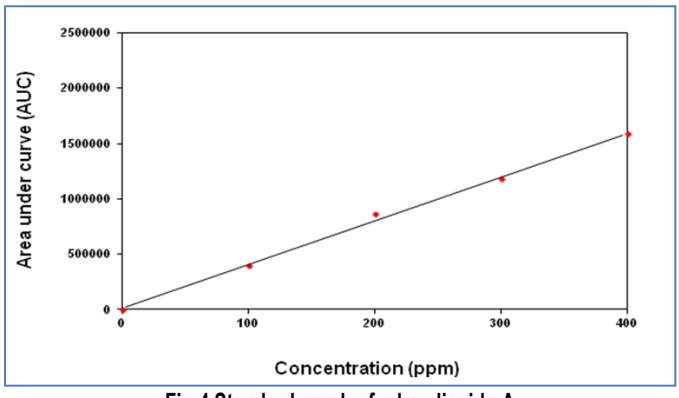

Fig-4 Standard graph of rebaudioside-A

Results

Stevioside and rebaudioside-A contents in different plant parts during different growth stages of plants grown under open field conditions

Stevioside and rebaudioside-A contents in different plant parts (leaves, green stem and Woody stem) of plants grown in the open field at pre-flowering, flowering and post-flowering stages were found significant [Table-1]. Stevioside content was observed maximum in the leaves and minimum in the woody stem in all the three harvesting stages. In leaves, stevioside content was found maximum in pre-flowering stage (8.20%), which decreased to 4.56 per cent in post-flowering stage. In case of stems (green and woody) though the stevioside contents were far less (<1.00 %) than of leaves, but the contents were more in the flowering stage (green stem: 0.82%, woody stem: 0.33%) in comparison to pre-flowering (green stem: 0.72%, woody stem: 0.22%) and post-flowering stages (green stem: 0.53%, woody stem: 0.21%).

The average stevioside content was found maximum in leaves (6.49%) and minimum in woody stem (0.25%) irrespective of plant growth stage. In different plant growth stages, average stevioside content in leaves, green stem and woody stem was observed maximum (3.05 %) at pre-flowering stage being at par with flowering stage (2.62%) while, minimum was recorded in post-flowering stage (1.77%).

Similarly, rebaudioside-A content was also found maximum in the leaves and minimum in woody stem in all the plant harvesting stages. In leaves, rebaudioside-A content was found maximum in pre-flowering stage (7.00%), which decreased to 3.21% in post-flowering stage [Table-2]. Though both green and woody stems showed less than 1.00 per cent rebaudioside-A contents, however, the contents were about 2-6 times more in green stem than in woody stem. Green stems showed maximum rebaudioside-A content in flowering stage (0.93%) than in pre-flowering (0.85%) and post-flowering (0.45%) stages. In woody stem rebaudioside-A content increased from 0.15 % in pre-flowering stage to 0.23 % in post-flowering stage. The average rebaudioside-A content in the these harvesting stages was found maximum in leaves (4.53%) and minimum in woody stem (0.19%). In the different plant growth stages, the average rebaudioside-A content of the three aerial plant parts (leaves, green stem and woody stem) was observed maximum in pre-flowering stage (2.67%) and minimum in post-flowering stage (1.29%).

Table-1 *Effect of stage of harvest on stevioside content (%) in different plant parts of S. rebaudiana grown in open field conditions*

Stages	Leaves	Green stem	Woody stem	Mean
Pre-flowering	8.20(2.86)*	0.72(0.85)	0.22(0.47)	3.05(1.39)
Flowering	6.71(2.59)	0.82(0.90)	0.33(0.57)	2.62(1.36)
Post-flowering	4.56(2.13)	0.53(0.73)	0.21(0.46)	1.77(1.11)
Mean	6.49(2.53)	0.69(0.83)	0.25(0.50)	
	Stages	Plant parts	Stages ×plant parts	
$CD_{0.05}$	0.04	0.04	0.08	
SE±	0.02	0.02	0.04	

*Figures in the parentheses are square root transformed values

Table-2 *Effect of stage of harvest on rebaudioside-A content (%) in different plant parts of S. rebaudiana grown in open field conditions*

Stages	Leaves	Green stem	Woody stem	Mean
Pre-flowering	7.00(2.64)*	0.85(0.92)	0.15(0.38)	2.67(1.32)
Flowering	3.39(1.84)	0.93(0.96)	0.18(0.43)	1.50(1.08)
Post-flowering	3.21(1.79)	0.45(0.67)	0.23(0.48)	1.29(0.98)
Mean	4.53(2.09)	0.74(0.85)	0.19(0.43)	
	Stages	Plant parts	Stages× plant parts	
$CD_{0.05}$	0.05	0.05	0.08	
SE±	0.02	0.02	0.04	

*Figures in the parentheses are square root transformed values

Variation in stevioside and rebaudioside-A contents in plants grown under polyhouse conditions

Overall profile of stevioside content analysed in the three plant parts showed that maximum stevioside content was found in leaves and minimum in woody stem. Both green and woody stem had less than 1.00 per cent stevioside content. In leaves, stevioside content was found maximum in pre-flowering stage (9.19%) which decreased to 4.77 per cent in post-flowering stage [Table-3]. In case of green stem, the maximum stevioside content was observed in pre-flowering stage

(0.92%), which was gradually declined to 0.70 per cent in post-flowering stage, whereas in woody stem the stevioside content lied in a very narrow range of 0.27 per cent in pre-flowering stage to 0.21 per cent in flowering stage. Average stevioside content in the three harvesting stages was found 7.06 per cent for leaves, 0.83 per cent for green stem and 0.24 per cent for woody stem. The average stevioside content of leaves, green stem and woody stem portion was found maximum in pre-flowering stage (3.46%) followed by flowering (2.76 %) and post-flowering (1.91%) stages. Like stevioside, rebaudioside-A content was also found maximum in the leaves than in green and woody stems. In leaves rebaudioside-A content was observed maximum in pre-flowering stage (5.51%) which gradually decreased with further plant growth and development to 3.31 per cent in post-flowering stage [Table-4]. In green stem, rebaudioside-A content decreased from 1.07 per cent in pre-flowering stage to 0.27 per cent in post-flowering stage. In woody stem portion rebaudioside-A content varied from 0.09 per cent in pre-flowering stage to 0.39 per cent in post-flowering stage. Average value of rebaudioside-A in the three harvesting stages (pre-flowering, flowering and post-flowering) was found maximum in the leaves (4.77%) followed by green stem (0.63%) and woody stem (0.21%). The average value of rebaudioside-A in leaves, green stem and woody stem was found maximum in pre-flowering stage (2.22 %) which reduced to 2.06 per cent in flowering and 1.32 per cent in post-flowering stages.

Table-3 *Effect of stage of harvest on stevioside content (%) in different plant parts of S. rebaudiana grown in polyhouse conditions*

Stages	Leaves	Green stem	Woody stem	Mean
Pre-flowering	9.19(3.03)*	0.92(0.96)	0.27(0.52)	3.46(1.50)
Flowering	7.22(2.69)	0.86(0.93)	0.21(0.45)	2.76(1.36)
Post-flowering	4.77(2.18)	0.70(0.84)	0.25(0.50)	1.91(1.17)
Mean	7.06(2.63)	0.83(0.91)	0.24(0.49)	
	Stages	Plant parts	Stages ×plant parts	
$CD_{0.05}$	0.02	0.02	0.03	
SE±	0.01	0.01	0.01	

*Figures in the parentheses are square root transformed values

Table-4 *Effect of stage of harvest on rebaudioside-A content (%) in different plant parts of S. rebaudiana grown in polyhouse conditions*

Stages	Leaves	Green stem	Woody stem	Mean
Pre-flowering	5.51(2.35)*	1.07(1.03)	0.09(0.29)	2.22(1.23)
Flowering	5.48(2.34)	0.55(0.74)	0.14(0.38)	2.06(1.15)
Post-flowering	3.31(1.82)	0.27(0.52)	0.39(0.56)	1.32(0.96)
Mean	4.77(2.17)	0.63(0.76)	0.21(0.41)	
	Stages	Plant parts	Stages× plant parts	
$CD_{0.05}$	0.12	0.12	0.21	
SE±	0.06	0.06	0.10	

*Figures in the parentheses are square root transformed values

Discussion

In the present study, stevioside and rebaudioside-A content was found maximum in the leaves [Table 1-4] followed by green stem and lastly in the woody stem at pre-flowerimg growth stage (flower bud stage in July month). Similarly, other workers [13-14] claimed that plants should be collected at the time of first flower appearance because during flowering the stevioside content diminishes. However, considering the growing conditions (open field and polyhouse) stevioside per cent was maximum (9.19%) in plants grown under polyhouse than in open field condition (8.20%). While, rebaudioside-A content was recorded maximum (7.00%) in open field grown plants (5.51%). In conformity with the results, [15-16] found more picrotin and picrotoxin content and aconitine content in *Picrorrhiza kurroa* and *Aconitum heterophyllum* and *A. balfourii* plants, respectively grown under polyhouse conditions. Plants grown under controlled environment conditions can be easily exposed to changing light irradiance which can alter the chemical composition of the plant. In addition, secondary metabolites are prone to diurnal fluctuations [17]. Earlier workers [18] had also reported more stevioside per cent in the *Stevia rebaudiana* plants grown from the seeds kept under long day conditions (16 hours light). Similarly, there exists evidence that the synthesis of terpenes is affected by environmental factors such as photoperiod and light intensity [19-23].

Conclusion

From the present study, it is evident that harvesting stages as well as different plant parts have prominent effect on the stevioside and rebaudioside-A content. So. It is advisable to collect the leaves of *Stevia rebaudiana* before the flowering. As far as growing conditions are concerned, stevioside content was found more in polyhouse conditions, while rebaudioside-A content was reported maximum under open field conditions.

Conflict of Interest: None declared

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