International Journal of Innovation and Applied Studies
ISSN 2028-9324 Vol. 9 No. 2 Nov. 2014, pp. 835-840

http://www.ijias.issr-journals.org/

Evaluation of post harvest quality for aflatoxin and microbial loads on the leaves of *Stevia rebaudiana Bertoni* cultivated in Odisha

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ABSTRACT: Herbs and farm produce are stored after harvest for different durations and have the potential to support microbial growth on them which can lead to shorter shelf life and enhancing food safety issues due to pathogens and allergens. The present study investigated the native microbial loads and aflatoxin contents on *Stevia rebaudiana Bertoni* leaf samples collected from different cultivar in Odisha during various seasonal harvesting processes. There is a clear indication that the total microbial populations in the leaf samples in the monsoon were significantly higher than summer and winter seasons. Therefore, different measures may be needed to handle and process these samples to minimize food safety risks of the product. Determination of AFB1 levels in stevia leaf samples by enzyme-linked immunosorbent assay (ELISA) procedure revealed that there is consistence presence of aflatoxin AFB1 in all most all samples collected from the region. There was no significant difference in contents within the different types of samples collected. The level of microbial population and aflatoxin contents of all the cultivars irrespective of cultivation cycle or duration needs to be reduced and appropriate post harvest measures needs to be taken for further application of the produce.

KEYWORDS: Shelf life, Microbiological quality, Allergens, Natural sweetener, Stevia leaves.

1 INTRODUCTION

Generally, plants constitute a major source of orthodox medicines and the presence of plant secondary metabolites has been attributed for most plants' therapeutic activities [1]. According to a World Health Organization (WHO) survey, about 70 – 80 % of the world's populations, particularly in the developing countries, rely on non-conventional medicines, mainly from herbal sources, for their primary healthcare [2]. As is widely acknowledged that increased consumption of sugar has resulted in several nutritional and medical problems including obesity, alternative sweetness have been entering the market [3]. Historically, low caloric sweeteners have been investigated to substitute sugar; one important class of low caloric sugar substitutes is known as a high intensity sweetener which is at least 50-100 times sweeter than sucrose [4]. Dried leaves of *Stevia rebaudiana* having more than 200 times sweet intensity, has increasingly been accepted as low-calorie sweetener or as dietary supplement of various food and beverages in many countries and therefore the worldwide demand for *S. rebaudiana* expected to increase many folds [5].According to the International Diabetes Federation (IDF) and the Madras Diabetes Research Foundation, India had 62.4 million people with type 2 diabetes in 2011, compared with 50.8 million in 2010 [6]. Due to this high potential demand, various Indian farmers have been encouraged to take up stevia cultivation as alternate method of livelihood [7]. However, consumption of unregulated natural produce has potential to become public

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health issue. Poor practices of cultivation, harvest, processing, storage and distribution can contribute to large variety of contaminations, defeating food safety and HACCP (Hazard Analysis and Critical Control Points) protocols. The safety and quality of raw medicinal plant materials and finished products depend on factors that may be classified as intrinsic (genetic) or extrinsic (environment, collection methods, cultivation, harvest, post harvest processing, transport and storage practice [8].Tropical climates with high temperature and humidity are suitable conditions for microbial contamination and the study area in the state of Odisha, India, is in one such climatic zone where stevia cultivation is being adopted as an alternate livelihood measure. Stevia plants in farm are exposed to a wide range of microbial contamination as a result of improper production process, extended drying times and poor storage conditions, stevia leaves being a very sensitive product for harboring micro organisms and accumulation of aflatoxin formation. The level of aflatoxin and pathogenic microbial population of contamination depends on cultivation condition and unsuitable processing conditions [9].The maximum limit for aflatoxin content - AFB1, should not exceed 5 µg/kg (ppb) in most food and feed products [10, 11, and 12].An understanding of the microbial population profile and aflatoxin content profile of various cultivars in the produce for the region can contribute to the successful production and processing of the herbal crop like stevia for public consumption. The profile will also reflect social economic attributes influencing the species traded and impact on trade due to critical quality parameters for effective resources management [13]. The current study is undertaken to evaluate the post harvest quality of the stevia leaves for microbial loads and aflatoxin content different cultivars on the leaves of *Stevia rebaudiana Bertoni* cultivated in the region of Odisha, India.

2 MATERIALS AND METHODS

2.1 SAMPLES COLLECTION

The study was carried out for the local cultivars in the state Odisha, India, grown within latitude 19 – 22 ° north and longitude 83 – 87 ° east. A total of eight cultivars were collected that were cultivated during summer, monsoon and winter seasons of 2012 – 2014. The samples of different cultivars were recorded as sample 1 – 8 for the three identified harvesting seasons. For the purpose of further evaluations some of the samples were further segregates in sets of young and old leaves (old leaves harvested from bottom of the stems and young leaves are harvested from the top 4-6 leaves of the stem) and fresh and dried leaves. These were then processed and packed in sterile polypropylene pouches (0.02mm) sealed and stored at room temperature 25°C (± 5°C).

2.2 MICROBIOLOGICAL ANALYSIS

All samples were analysed for total viable count, moulds, yeasts, coliforms, *E. coli*, *Stephylococus aureus* by HPB Methods for the Microbiological Analysis of FoodsMFHPB-32, 33, 34, 35 [14], using 3M™ Petrifilm™ total plate count petrifilms, coliform count petrifilms, *E. coli* count petrifilms, *S. aureus* petrifilms and yeast and mold count petrifilms (3M Microbiology, St. Paul, MN, USA) according to the instructions of the manufacturer. All the solvents and other chemicals used unless otherwise specified were of analytical grade and the solutions were prepared with bi-distilled water.

Stevia leaves (2 g) were taken aseptically and diluted with 25 ml of 0.1% sterile peptone water. The samples were homogenized by stomacher at high speed for 2 min. Serial dilution were done in the range of 10^{-1} to 10^{-5}. Inoculated Petrifilm™ plates were incubated at 32°C for 48 h for aerobic bacteria count and 42°C for 24 h for *E. coli* count while *S. au*reus, yeast and mold were done as per the procedure stated in the manual.

2.3 DETERMINATION OF AFLATOXIN

Determination of AFB1 levels in stevia leaves were carried out by by enzyme-linked immunosorbent assay (ELISA) procedure. Determination of AFB1 was based on an ELISA using the AFB1 ELISA kit (Euro Proxima, Netherlands, Cat. No.: 5121AFB). Standard sample preparations were done according to the instructions of the kit. The kit was stored at 4°C and all the reagents were brought to room temperature, 2 hours before use. Stevia leaves (2 g) were taken aseptically and diluted with 25 ml of 0.1% sterile peptone water. The samples were homogenized by stomacher at high speed for 2 min. 50 µl of the AFB1 standards and samples were added to 96 wells microplates. 100 µl enzyme conjugate and 50 µl antibody were added to the wells respectively and incubated for 30 minutes at room temperature. At the end of the incubation the liquid was poured from the wells and washed 4 times with the washing buffer (1:10). 200 µl of the developing solution was added to the wells and incubated at room temperature for 20 minutes. 50 µl stop solution was then added to the wells and measured at 450 nm. AFB1 concentrations were calculated according to the guidelines of the kit using the responses of the different standard analyzed.

2.4 STATISTICAL ANALYSIS

After combing the data of each repetition and cultivars for total microbial count within each season during the two year period (2012 – 2014), the data were subjected to F test and single factor analysis of variance (ANOVA). Where the effect was significant in the ANOVA ($P < 0.05$) post-hoc analysis was performed for each paired samples using a Student's t-test for comparison between all mean values with the Bonferroni pair wise alpha to keep the family wise alpha value at 0.05. Student's t-test was also applied for comparing alfatoxin contents for young and old leaves, fresh harvested and dried leaves, and two consecutive summer seasons for two years. Significant differences were determined at $P < 0.05$ to establish significance level of 5%.

3 RESULTS AND DISCUSSION

3.1 MICROBIAL ANALYSIS

Figure 1 presents the microbial count results of the *Stevia rebaudiana Bertoni* when tested for total microbial population count, coliform count, *E. coli* count, Staphylococus count and yeast and mold counts in cfu/gm of sample tested. The figure also presents the content of aflatoxin AFB1 in microgram (mcg) /gm of sample tested. The results revealed that, there are apparent presence of microbial population which includes potential harmful coliform organisms, yeast and molds that can lead to enhanced food safety risk [10,11,12,15]. The contents of afflatoxin AFB1 is also a concern as the presence is above most regulatory limits for the products as per United States Food and Drugs Administration (USFDA) [16]. The findings clearly indicate the need for further detailed investigations for understanding the cultivars of the region to effectively address related issues.

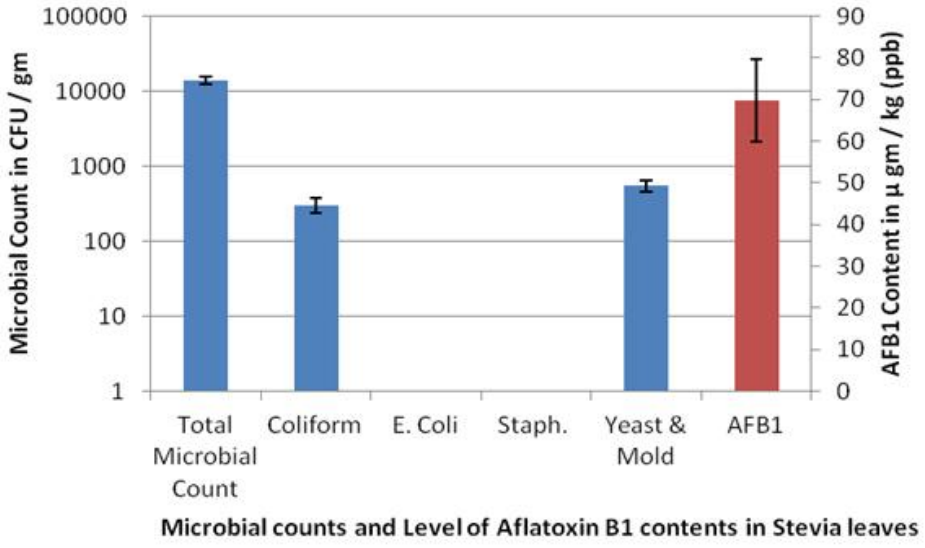

Fig. 1.Mean of microbial populations and aflatoxin contents in the dried leaves of Stevia rebaudiana Bertoni. (Error bars indicate standard error-SEM)

Comparative study to evaluate the variability in microbial population in samples of two consecutive summer seasons and also the variability between freshly harvested leaf samples and the regular dry samples were carried out. T-Test of the both the types of sample pairs were done assuming unequal variances and the summary of the results are as presented in the Table 1. With a two-tail (inequality) t- test, the t Stat > -t Critical two-tail or t Stat < t Critical two-tail, therefore, we cannot reject the null hypothesis. (Critical t-value is 4.3 and is greater than t-stat -2.1).Therefore, we conclude the observed difference between the sample means *2812 (*±2036*) & 7198 (±469)* for year to year count for same season and *13260 (±2642) &, 11341 (±2245)* for fresh and dry sample collections were not convincing enough to say that they differ significantly. This is further confirmed by the fact that the two-tailed P-value was 0.17 and 0.87 respectively, which is more than 0.05; therefore the differences between these values are not significant and the means for all the tests can be considered as equal.

Table 1. Two-tail (inequality) t- test results for total microbial population for samples of two consecutive summer seasons and for freshly harvested leaf samples and the regular dry samples

	Summer of 2013 & 2014	*Fresh & Dry*
μ -Mean (SEM)	*2812 (*±2036*) &7198 (±469)*	*13260 (±2642) &, 11341 (±2245)*
df	2	17
t Stat	-2.1	-0.17
P(T<=t) two-tail	0.17	0.87
t Critical two-tail	4.3	2.11

T-Test of the samples of young leaves and older leaves were done assuming unequal variances and the summary of the results are as presented in the Table 1. As per the results, with a two-tail (inequality) t- test, the t Stat > -t Critical two-tail or t Stat < t Critical two-tail, therefore, we cannot reject the null hypothesis. Therefore, we conclude the observed difference between the young and old leaf sample means for each seasonal were not convincing enough to say that they differ significantly. This is further confirmed by the fact that the two-tailed P-value was 0.28, 0.15, 0.62 and 0.58 for summer, monsoon, and winter for cumulative annual respectively, which is more than 0.05; therefore the differences between these values are not significant and the means for all the tests can be considered as equal.

Table 2. Two-tail (inequality) t- test results for total microbial population for samples of two types of samples collected from the farm young leaves and old leaves during the all seasonal harvesting cycles

	Young & Old leaves of Summer	*Young & Old leaves of Monsoon*	*Young & Old leaves of Winter*
df	10	6	6
t Stat	-1.1	1.6	0.5
P(T<=t) two-tail	0.3	0.2	0.6
t Critical two-tail	2.2	2.4	2.4

Comparative analysis to understand the variance in microbial population in samples of three different harvested seasons was carried out and the ANOVA results are presented in Table 3. The results indicate F > F crit (23.25445 > 3.4668), and P - value is extremely small. Therefore, null hypothesis rejected (P < 0.05) Thus, the test is significant at α = 5%.and microbial count differences between seasons are highly significant.

Table 3. ANOVA results for comparing total microbial population of three different seasons (summer, monsoon and winter)

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.58E+09	2	7.9E+08	23.25445	4.73E-06	3.4668
Within Groups	7.13E+08	21	33958749			
Total	2.29E+09	23				

To further identify the significant differences between the mean of total microbial counts, simple student's t-test was carried out to identify the season responsible for variation in the data. The results of three pair of seasonal t-test are presented in the Table 4. As can be seen the means of summer and winter the P value is > 0.05, indicating their means do not differ significantly where as summer & monsoon and monsoon and winter resulted in P < 0.05 and therefore, means differ significantly. This suggests the monsoon session's microbial population is different from that of summer and winter while the microbial population in summer and winter seasons were same. Similar higher load of microbial populations have been reported and is in consistence with studies conducted by Gasmalla et al on *Stevia rebaudiana Bertonie* [17] and other similar herbs [18]. Efforts are being undertaken to minimize microbial load by physico chemical treatments [19, 20].

Table 4. Students' t-test results of paired samples for comparison between individual seasons mean values and identifying the significant different season's means

	Summer & Winter	*Summer & Monsoon*	*Monsoon & Winter*
μ -Mean (SEM)	6320.8 (± 690.4) & 8240.6 (± 2913.2)	6320.8(± 690.4) & 24015.6 (± 1179.8)	24015.6 (± 1179.8) & 8240.6 (± 2913.2)
df	8.0	24.0	9.0
t Stat	-0.6	-12.9	5.0
P(T<=t) two-tail	0.5	2.57E-12	7.20E-04
t Critical two-tail	2.3	2.1	2.3

3.2 AFLATOXIN AFB1 ANALYSIS

The relative impact on possible aflatoxin AFB1 accumulation was evaluated for the two seasonal hasted samples (summer and monsoon) having significant difference in microbial. t -Test of the both the types of sample pairs were done assuming unequal variances and the summary of the results are as presented in the Table 1. It is evident from the two-tail (inequality) t- test, the t Stat > -t Critical two-tail or t Stat < t Critical two-tail, therefore, we cannot reject the null hypothesis. (Critical t-value is 2.1 and is greater than t-stat -1.4).Therefore, we conclude the observed difference between the sample means 65.4 (±3.3) & 70.3 (±1.1) for two different seasons summer and monsoon were significantly different. This is further confirmed by the fact that the two-tailed P-value is 0.2, which is greater than 0.05; therefore the differences between the means for all the two seasons can be considered as equal. The tropical weather conditions which prevail during most part of the year in India can favour fungal growth and mycotoxin production [18, 20]. Various physicochemical measures may be undertaken to decontaminate aflatoxins to render the produce safe for consumption [10, 11, and 19].

Table 5. F Test and Two-tail (inequality) t- test results for aflatoxin AFB1 contents of samples collected during the two different seasons having highly significant microbial populations (summer, monsoon)

	Summer	*Monsoon*
Mean	65.4	70.3
SEM	3.3	1.1
F	16.9	
F Critical one-tail	3.5	
df	17.0	
t Stat	-1.4	
P(T<=t) two-tail	0.2	
t Critical two-tail	2.1	

4 CONCLUSION

The presence of microbial populations and aflatoxins in the leaves of *Stevia rebaudiana Bertoni* were evident which included some of the possible pathogenic indicator organisms and detectable aflatoxin AFB1 contents.Pathogens like *E. coli* count, *Staphylococus aureus* were bit detected in the entire sample tested. The contents of aflatoxin AFB1 is a concern as its presence is above most regulatory limits for the products as per FDA. The finding clearly suggests the need for further detailed investigations for understanding the sampling methods and evaluating different cultivars of the region to effectively address related issues to enhance the product quality and shelf life of the produce. There was no significant difference in microbial population in samples of two consecutive summer seasons and therefore year to year variability is not a significant than other related parameters, though this may be validated with further studies. The microbial population between freshly harvested leaf samples and the regular dry samples were similar suggesting the collection of different fresh / dried leaf sample for the study will not impact the outcome significantly. The effect of harvesting age of the leaves do not have any significant impact on the microbial load as means for both types of leaves can be considered statistically equal. There is a clear indication that the total microbial population in the leaf samples in the monsoon were significantly higher than summer and winter seasons. Therefore, different measures may be needed to handle and process these samples to minimise food safety risks of the product as has been suggested by Idu *et al* [17]. Though the presence aflatoxin AFB1 content in all the samples collected during different seasons over a year were significant, there is no significant deference in contents within the different samples collected. The level of microbial population and aflatoxin contents of all the cultivars irrespective of

cultivation cycle or duration needs to be reduced and appropriate post harvest measures needs to be taken for further application of the produce.

ACKNOWLEDGMENT

This study was financially supported by the Board of Research in Nuclear Sciences (BRNS) project No.2012/34/43/BRNS/1041, Department of Atomic Energy (DAE) Govt. of India and Shailja's Organic Products Pvt. Ltd. Bhubaneswar, India.

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