

Theme: 3.2. Food and Pharmaceuticals

Evaluation of phytosterols from juvenile shoots of an edible bamboo

***Dendrocalamus hamiltonii* Arn. Ex Munro**

M. Ingudam¹, K. Sarangthem^{1*}, C. Nirmala² and M.S. Bisht³

1. Department of Life Sciences, Manipur University, Canchipur, Imphal-795003, India

2. Department of Botany, Panjab University, Chandigarh, India

3. Center for Science Education, North Eastern Hill University, Shillong, Meghalaya, India

*Corresponding author – kananbala_s@rediffmail.com

Abstract

Bamboo shoots are regarded as potential sources of phytosterols. Young shoots of bamboo are a potential source of phytosterols especially β -Sitosterol (beta-sitosterol) and can be used as dietary supplements as they are reported to lower cholesterol levels by inhibiting the absorption of cholesterol from the small intestine. Fresh bamboo shoots of *Dendrocalamus hamiltonii* were assessed of its phytosterol content and were subjected to fermentation which resulted in an enrichment of phytosterols from 0.19 % to 0.78 % dry wt, as compared to that of fresh one. Further, extraction and purification of the crude phytosterols were done to isolate different phytosterols. The results showed the presence of β -sitosterol, campesterol and stigmasterol in the bamboo shoot. Further β -Sitosterol were isolated from the fermented products of bamboo shoot slices of *Dendrocalamus hamiltonii* and quantitative analysis were conducted using Thin Layer Chromatography (TLC) and high performance liquid chromatography (HPLC). The aim of the study is to utilize bamboo shoot as potential sources of β -Sitosterol and to facilitate dietary recommendations and promote comprehensive utilization of bamboo shoot resources in pharmaceutical, nutraceutical and food industries

Key words: β -Sitosterol(beta-sitosterol) / bamboo shoots/ *Dendrocalamus hamiltonii*

Introduction

Phytosterols are a group of naturally occurring lipophilic steroid alcohols found in plants. Recently there is considerable interest in beta-sitosterol as dietary supplements as they are reported to lower cholesterol levels by inhibiting the absorption of cholesterol from the small intestine (Vanhanen et al.1993; Ostlund et al. 2003) and also have a positive impact on cardiovascular diseases (Patel and Thompson,2006;

Weingartner et al. 2009). β -Sitosterol (beta-sitosterol) is one of several phytosterols with chemical structures similar to that of cholesterol. β -sitosterol has been shown to possess beneficial effects against a wide variety of human ailments. β -sitosterol is being studied for its potential to reduce benign prostatic hyperplasia (Awad et al. 2008 ;Wilt, et al. 2000; kim et al. 2012) and blood cholesterol levels (Rudkowska et al. 2008). Its close resemblance to cholesterol allows it to be incorporated into mammalian cellular membrane thereby blocking the absorption of cholesterol in intestine (Awad et al. 2008) and plasma (Frank et al.2005). β -sitosterol also normalizes blood sugar and insulin levels in Type-II diabetics. It slows the rise of blood glucose levels by down regulation of glucose-6-phosphatase by releasing insulin and helps delay age related worsening of glucose tolerance and onset of Type- II diabetes(Awad and Fink 2000; Frank et al. 2005)). It also improves the liver function activity (Zak et al. 2005), thus reducing prostate and colon-cancer cell and lympholytic leukemia (Von Holtz et al. 1998; Awad and Fink 2000).

Over the last few decades, natural bioactive compounds with potential for the treatment and prevention of human diseases have attracted much attention in many laboratories and industries. Moreover, the increased price and demand for phytosterols in recent years has resulted in the depletion of traditional raw phytosterols from by-products in vegetable oil refining and the wood pulp industries (Leong et al. 2011). Therefore, an alternative source for a starting material is imperative. In this context presence of phytosterols in bamboo had been reported (Sarangthem and Srivastava 1997; Lu et al. 2009; Nirmala et al. 2014; Ingudam and Sarangthem 2016).

Bamboo shoots have a long history of being used as a source of both food and medicine. Moreover, presence of high amount of phytosterols in bamboo shoots (Sarangthem and Singh 2003) makes this food item precursor of many pharmaceutically active products. Though, bamboo shoots are regarded as potential sources of phytosterols., it is crucial to get accurate quantitative data on the distribution of β -sitosterol in bamboo species. Hence, the aim of the study was to evaluate freshly harvested bamboo shoots and its fermented products as sources of β -Sitosterol (beta-sitosterol) in order to facilitate dietary recommendations and comprehensive utilization of bamboo shoot resources.

Materials and Methods

The emerging young fresh succulent bamboo shoots of *Dendrocalamus hamiltonii* Nees & Arn.ex Munro were collected during peak sprouting season (May-August) from different district of Manipur, India. Collection of these bamboo shoots were made in the afternoon and processed the same day. The

outermost scale portions of the fresh succulent bamboo shoots and the inner soft delicate shoots which are edible were taken out and kept separately for the experiment.

Analysis of total phytosterol

The delicate bamboo shoot apex was sliced and oven dried at $60^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 12h. The dried samples of the delicate shoot apex were then crushed to powder form. The powder was used for determination of total phytosterols using Liebermann-Burchard reaction (Katayama et al. 1974).

Fermentation

Traditional method of fermentation

The fermented products of the bamboo shoot slices locally called 'soibum' is prepared traditionally by storing thin slices of fresh succulent and soft bamboo shoots in certain containers/chambers for 2-3 months. The fermented chambers are either made of bamboo planks or roasted earthen pots. The inner surface of bamboo chambers are lined with banana leaves and a thin polythene sheets. The upper surface is sealed with polythene sheet and weights are then put on top for proper pressing. At the initial stage of fermentation the exudates is leached/drained out of the tilted side of the bamboo plank chamber. After fermentation is completed, which is indicated by the smell, colour and texture, soibum can be stored up to one year. For the present studies traditionally fermented bamboo shoot slices of *Dendrocalamus hamiltonii* fermented samples of 90days old were taken as the research samples.

Laboratory fermentation

Fermentation was also carried out in the laboratory by a modified form of the traditional method of fermentation which involves inoculating thin slices of succulent bamboo shoots of *Dendrocalamus hamiltonii* with the exudates obtained from already fermented slices of bamboo shoots sold in the local market in the name of soibum After inoculation the samples were kept in an incubator at $30^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for a period of 90 days, during which the period of fermentation took place. The whole process of fermentation (from slicing the shoot till sealing the polythene bag) was undertaken under sterile condition in a laminar flow chamber. Weekly interval analyses on the changes in the level of total phytosterols were carried out during fermentation using Liebermann Burchard reaction. The 90 days old fermented bamboo shoot slices ample was taken for extraction of crude phytosterols

Extraction of phytosterols

For extraction of phytosterol, the fermented bamboo shoot slices of *Dendrocalamus hamiltonii* (90-day-old fermentations) were taken and dried in an oven at 60°C, grinded and sieving with the help of mesh 100. The powdered form obtained was used for the extraction. Extraction was done in a 1 litre Soxhlet apparatus using benzene, petroleum ether and 2N ethanolic KOH (10:5:1) as the refluxing solvent (Sarangthem and Srivastava 1997). It yielded a highly concentrated extract, after decantation, and drying off the solvent at 60°C followed by keeping at room temperature, yielded a soft cake. The soft cake were then refluxed further with acetonitrile for selective solubilisation of phytosterol and kept for further analysis.

Analysis of β -sitosterol

After selective solubilization of the crude phytosterols with acetonitrile, the fractions were then subjected to Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) was performed with the crystalline fraction sticking to the wall of the container

Thin layer chromatography analysis

The partially purified phytosterols were then subjected to TLC (Stahl 1969). The TLC analysis was performed on silica gel-G plates (0.25mm thick, 20x20 cm) using different solvent pairs Benzene: Diethyl ether (7:3) and Hexane: Ethyl acetate (3:1). Detecting reagent were acetic anhydride and sulphuric acid (30:1).

For obtaining crystallized form of the phytosterols isolated from fermented shoot samples, preparative TLC was conducted. The phytosterols (tentatively identified as β -Sitosterol) resolved on TLC and confirmed with standard samples were scraped and eluted in chloroform for analysis.

The UV spectral analysis for the crystals obtained after preparative TLC (Stahl 1969) as well as control authentic samples of β -Sitosterol (Sigma Chemicals, USA) were measured from 225 to 400 nm a spectrophotometer. Further analysis of IR, NMR and Mass spectral analysis were done at CDRI, Lucknow for confirmation of the compound in comparison with control authentic samples of β -Sitosterol (Sigma Chemicals, USA). Separation, identification and quantification of the extracted β -Sitosterols were performed using a High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) analysis

β -sitosterol Standard ((Sigma Chemicals, USA).) and the extracted β -Sitosterols samples were dissolved in methanol separately and kept for the analysis. HPLC analysis of the extracted samples and standard β -sitosterol were carried out in a HPLC Agilent 1260 system with DAD detector. 20 μ l of sample was injected (Rheodyne injector, USA) into a Zorbax Eclipse Plus C-8 column with dimension -4.6mx150mm and bead size of 5micron with a flow rate of 1ml/min and detection wavelength of 210nm (DAD1 Detector). Methanol/water (75:25) was used as the mobile phase

Results and discussion

The level of total phytosterols in fresh bamboo shoot samples of *Dendrocalamus hamiltonii* was found to be 0.19% on dry weight basis. High concentration of phytosterols was also found in the discarded culm sheath or the outermost scale of the bamboo shoots (0.17%) as shown in Table 1. This results shows a correlation with the results obtained earlier where bamboo shoots of the different twelve bamboo species were screened for their phytosterols content and it shows a varying content of phytosterols. The highest concentration was obtained with *Dendrocalamus hamiltonii* bamboo shoot as reported by Ingudam and Sarangthem 2016. Hence this species was taken up for the phytosterols extraction.

Table 1. Total phytosterols in fresh succulent shoot samples and culm sheath

Species	Phytosterols in shoot apex (% dry wt.)	Phytosterols in outermost scale (% dry wt)
<i>Dendrocalamus hamiltonii</i>	0.19 \pm 0.01	0.17 \pm 0.01

Data presented as mean \pm SD.

In the fermented shoots, there was an increasing trend in the concentration of total phytosterols from the initial stage of fermentation (0.19% on dry wt .basis) to 90 days (0.78 % on dry weight basis as represented in Table 2. The level of phytosterols increases to four times or more in fermented bamboo slices with 90 days (0.78 %). The increase in the level of phytosterols in the fermented samples may be due to anaerobic digestion by microorganisms that cause degradation of the organic matter and resulted in the enrichment of phytosterols (Sarangthem and Srivastava 1997).

Table 2: Change in the level of total phytosterols at different stage of fermentation

Name of the species	Concentration of total phytosterol (% dry wt.)							
	Fermentation period (days)							
	0	7	14	21	28	35	60	90
<i>Dendrocalamus hamiltonii</i>	0.19	0.26	0.25	0.25	0.27	0.37	0.36	0.78
	±0.01	±0.02	±0.02	±0.03	±0.01	±0.02	±0.05	±0.03

Data presented as mean ± SD.

For extraction of phytosterol, the 90-days-old fermented shoot samples which had the highest phytosterols accumulation were used. Extraction was done in a 1litre Soxhlet apparatus using benzene, petroleum ether and 2N ethanolic KOH (10:5:1) as the refluxing solvent (Sarangthem and Srivastava, 1997). It yielded a highly concentrated extract, after decantation, and drying off the solvent at 60°C followed by keeping at room temperature, yielded 17g soft cake (average of three replicas) as shown in Table 3. The above soft cake when refluxed further with acetonitrile for the selective solubilisation of phytosterol yielded three types of precipitates after cooling. These precipitates were further purified and the weight determined as mentioned below

- i) A fine crystalline ppt. on the upper surface of the solution(0.3g)
- ii) A crystalline ppt. sticking to the wall of the container (0.7g)
- iii) Amorphous sticky ppt. at the bottom of the beaker (3.08g)

The data are represented in table 3.

Table 3. Partially purified fractions of phytosterols obtained from further purification of solubilised fractions with Acetonitrile.

Amt. of dry sample (g)	Amt. of soft cake (g)	Amt. of solubilised fractions (g)	Weight of partially purified phytosterols in different fractions(g)		
			Surface layer fraction	Side wall fraction	Bottom residue
100	17±1.85*	8± 0.05	0.3±0.03	0.70±0.05	3.08±0.85

Data presented as mean ± SD.

After selective solubilization of the crude phytosterols with acetonitrile, the crude fractions were then subjected to TLC. The TLC plate, developed with Lieberman-Burchard reagent, showed four spots with

two of the Solvent Pairs (I and II). Of these four spots, one was light green colour just above the spotting point and remaining three were violet coloured and clearly visible in the heated condition and became light coloured at room temperature. The co-chromatography with standard samples revealed presence of Campesterol, β -Sitosterol and Stigmasterol with all-the two solvent pairs. The lowermost spot, in-the Solvent-Pairs I and II, could not be identified. The Rf values were calculated for each spot separated with each solvent pairs and are shown in Table-4. Further purification and characterization of these spots were conducted. In addition to qualitative detection, TLC also provides semi quantitative information on the major active constituents of a crude compound and is therefore suitable for monitoring the identity and purity of crude compound (Mukherjee 2002).

Table 4. Rf value of different spots on TLC plate separated with different solvent pairs. The chromatogram was run at 25°C for 90minute and for development of spots the plates were sprayed with Lieberman- Burchard reagent followed by heating in oven at 80°C for 30min.

Solvent pairs	Spots position	Rf value	Possible phytosterols using standard samples on Co-Chromatography
I Benzene: Diethyl ether (7:3)	1st (Lowermost)	0.699 \pm 0.07	Unidentified
	2nd	0.769 \pm 0.02	β -Sitosterol
	3rd	0.833 \pm 0.04	Stigmasterol
	4th	0.872 \pm 0.03	Campesterol
II. Hexane: Ethyl acetate (3:1).	1st (Lowermost)	0.0428 \pm 0.04	Unidentified
	2nd	0.320 \pm 0.01	β -Sitosterol
	3rd	0.732 \pm 0.07	Stigmasterol
	4th	0.814 \pm 0.09	Campesterol

Data presented as mean \pm SD.

Melting point of the side wall fraction of phytosterols (identified as β -Sitosterol) was found to be 140°C. The UV spectral analysis of the authentic sample (β -Sitosterol) and the side wall fractions showed

similar peaks. The IR spectral data and the mass spectra of the compound showed similarity with those obtained of the authentic samples of β -sitosterol (Sigma Chemicals)

β -sitosterol was quantitatively evaluated in bamboo shoots samples of *Dendrocalamus hamiltonii* by HPLC analysis. Standard β -sitosterol showed a peak at 11.686 minute retention time (Figure 1). The β -sitosterol extracted from the bamboo shoots of *D. hamiltonii* were resolved with retention time of 11.7002 minute (Figure2) and calculations were done by chemstation -3 giving an amount of 0.37154 μ g/ μ l of β -sitosterol.

Chromatograms

Figure 1. HPLC-DAD chromatogram of sample β -Sitosterol

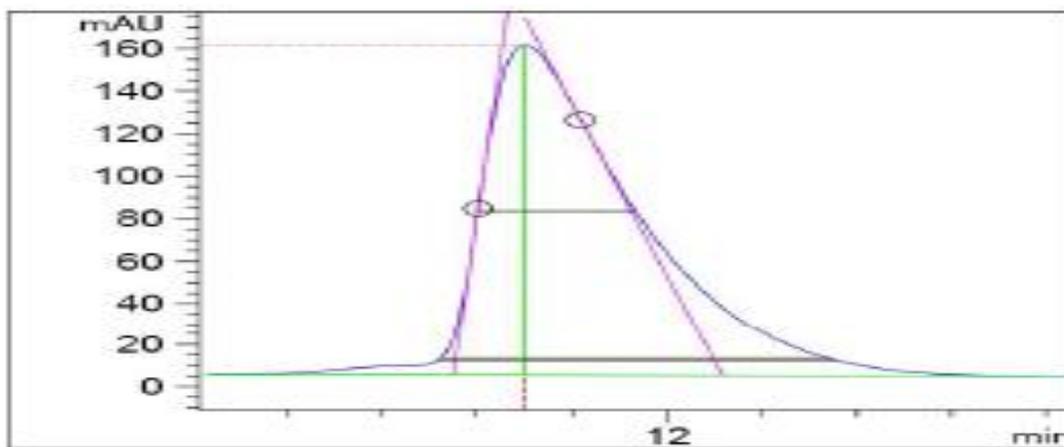
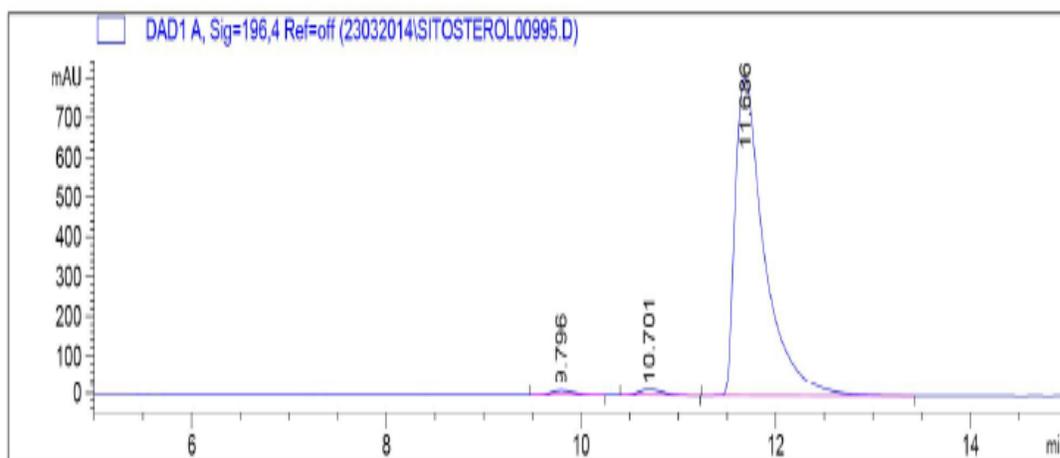


Figure 2. Shows quantitative analysis with retention time of 11.7002 and with sufficient resolution to allow for accurate quantitation

Conclusions

The present study has concentrated on the assessment of relative amounts of the phytosterol (β -sitosterol) extracted from the bamboo shoots of *Dendrocalamus hamiltonii*. The present work attempts at utilising bamboo shoot which are easily available in huge quantity as raw material for the isolation of β -sitosterol at large scale so as to facilitate dietary recommendations and comprehensive utilization of bamboo shoot resources in pharmaceutical, nutraceutical and food industries

Acknowledgements

The authors are thankful to the Dept. of Biotechnology (DBT), Govt. of India (.BT/475/ NE/ TBP / 2013), for providing financial support to carry out the research work.

References

Awad, A.B.; Fink, C.S. 2000. Phytosterols as anticancer dietary components: evidence and mechanism of action. *Journal of Nutrition*, 130,2127- 2130

Awad, A.B.; Barta, S.L.; Fink, C.S.; Bradford, P.G. 2008. Beta-sitosterol enhances tamoxifen effectiveness on breast cancer cells by affecting ceramide metabolism. *Mol Nutritional Food Research*, 52,419-426.

Frank, N.; Andrews, F.M.; Elliott, S.B.; Lew, J.I Boston, R.C. 2005 Effects of rice bran oil on plasma lipid concentrations, lipoprotein composition, and glucose dynamics in mares. *Journal of Animal Sciences*, 83,2509-2518.

Ingudam, M.; Sarangthem, K. 2016. Phytosterols Content in Different Bamboo Species of Manipur, India. *International Journal of current advance in Microbiology and Applied Sciences*, 5(7), 943-948. doi: <http://dx.doi.org/10.20546/ijcmas.2016.507.106>

Katayama, M.; Holl, C.T.; Chen, N.C.; Hirota, T.; Kiribuchi, J. and Sumahashi, S. 1974. A method for fractional determination of soybean in four class by florisil column chromatography. *Agricultural Biological Chemistry*, 38, 1661.

Kim, T. H.; Lim, H. J.; Kim, M. S.; Lee, M. S. 2012. Dietary supplements for benign prostatic hyperplasia: An overview of systematic reviews. *Maturitas*. 73 (3): 180-5. doi:10.1016/j.maturitas.

2012.07.007 (<https://doi.org/10.1016/j.maturitas.2012.07.007>). PMID 22883375 (<https://www.ncbi.nlm.nih.gov/pubmed/22883375>).

Leong, W. F.; Lai, O. M.; Long, K.; Che Man, Y. B.; Misran, M.; & Tan, C. P. 2011. Food Chemistry, 129, 71–83.

Lu, B.; Ren, Y.; Zhang, Y.; Gong, J. 2009. Effects of genetic variability, parts and seasons on the sterol content and composition in bamboo shoots. Food Chemistry, 112, 1016–1021.

Mukherjee, P.K.2002. Quality control of herbal drugs: An approach to evaluation of botanicals. 1st ed. New Delhi; Business Horizons 28p

Patel, M.D.; Thompson, P.D. 2006. Phytosterols and Vascular Disease. Atherosclerosis , 186 (1), 12–19.

Nirmala, C.; Bisht, M.S.; Laishram, M. 2014. Bioactive compounds in bamboo shoots: health benefits and prospects for developing functional foods. International Journal of Food Science and Technology, 49, 1425–1431.

Ostlund, R.E.; Racette, S.B.; Stenson, W.F. 2003. Inhibition of Cholesterol Absorption by Phytosterol-Replete Wheat Germ Compared With Phytosterol-Depleted Wheat Germ. American Journal of Clinical Nutrition, 77 (6), 1385–1589.

Rudkowska I; AbuMweis ,S.S.; Nicolle ,C.; Jones ,P.J .2008. Cholesterol-lowering efficacy of plant sterols in low-fat yogurt consumed as a snack or with a meal .Journal American Coll Nutr. 27 (5): 588–95. doi:10.1080/07315724.2008.10719742 (<https://doi.org/10.1080/07315724.2008.10719742>). PMID 18845709 (<https://www.ncbi.nlm.nih.gov/pubmed/18845709>).

Sarangthem, K. ; Srivastava, R.C. 1997. Enrichment of phytosterols level in fermenting sample of succulent shoots of *Dendrocalamus* species grown in Manipur. National. Academy. Science. letters ,20(5&6), 64-67.

Sarangthem, K.; Singh. T.N. 2003. Microbial bioconversion of metabolites from fermented succulent bamboo shoots into phytosterols. Current Science, 84(12), 1544–1547.

Stahl, E.1969. Thin Layer Chromatography .Acad. Press, New York;p339-341

Vanhanen, H.T.; Blomqvist, S.; Ehnholm, C.; Hyvonen, M.; Jauhiainen, M.; Torstila, I.; Miettinen, T.A.1993. Serum cholesterol, cholesterol precursors, and plant sterols in hypercholesterolemic subjects with different apoE phenotypes during dietary sitostanol ester treatment, *Journal of Lipid Research*, 34,1535–1544.

Von Holtz, R.L.; Fink, C.S.; Awad, A.B. 1998. Beta-sitosterol activates the sphingomyelin cycle and induces apoptosis in LNCaP human prostate cancer cells. *Nutrition Cancer*, 32,8-12.

Weingärtner O. et al.2009. Controversial Role of Plant Sterol Esters in the Management of Hypercholesterolaemia. *European Heart Journal*, 30, 404–409.

Wilt, T.; Ishani, A; MacDonald, R; Stark, G; Mulrow, C; Lau, J .2000. Beta-sitosterols for benign prostatic hyperplasia. *The Cochrane Library* (2): CD001043. doi:10.1002/14651858.CD001043 (<https://doi.org/10.1002/14651858.CD001043>). PMID 10796740 (<https://www.ncbi.nlm.nih.gov/pubmed/10796740>).

Zak, A.; Vecka, M.; Tvrzicka, E.; Hruby, M.; Novak, F.; Papezova, H. et al. 2005. Composition of plasma fatty acids and non-cholesterol sterols in *Anorexia nervosa*. *Physiology Research*, 54, 443-51.