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PHYTOSTEROLS : NEW DIMENSION IN THE HORMONAL STEROIDAL DRUGS

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Phytosterols such as stigmasterol, ß - sitosterol and campesterol are considered promising naturally occuring raw material for the synthesis of hormonal steroidal drugs. Stigmasterol, ß - sitosterol and campesterol were isolated for the first time from fermented succulent bamboo shoot slices of *Bambusa balcooa* and *Dendrocalamus strictus* and subjected to microbial bioconversion which yielded a considerable amount of Androstadienedione (ADD) in the incubation mixture in presence of metabolic inhibitor (*a* - *a'* - dipyridyl).

Keywords : Androstadienedione; Bamboo; Biotransformation; Phytosterols.

Introduction

Compounds which can be converted into steroidal drugs are known as steroid drug precursors. Of these, phytosterols are considered potential raw materials for partial synthesis of pharmaceutically active steroids. Selective microbial cleavage of C-17 side chain of sterols to produce Androstenedione (AD) and Androstadienedione (ADD) which can serve as intermediates for the synthesis of active steroids are considered very promising[1]. Hormonal steroidal drugs takes a significant major role in animals as an oral contraceptive, anti-fertility and even employed in various gynaecological disorders. Thus, the increasing demand of steroidal drugs especially the corticosteroids and oral contraceptives in the market has resulted in the depletion of various natural resources. Hence, an alternative source for a starting material is imperative. The present paper offers fermented bamboo shoot slices as a potent source of phytosterols (stigmasterol, ß-sitosterol and campesterol) and its bioconversion into 17-ketosteroids (ADD) for use as precursors of hormonal steroidal drugs by the pharmaceutical industries.

Materials and Methods

Stigmasterol, ß-Sitosterol, Campesterol (authentic samples) and *a* - *a'* - dipyridyl were obtained from Sigma Co., USA. Fresh succulent shoot of bamboo (*Bambusa balcooa* and *Dendrocalamus strictus*) were collected from different localities of Manipur. For enrichment of sterols, the fresh succulent bamboo shoot slices were subjected to fermented samples of bamboo shoots (traditional way of fermentation) sold in the local market in the name of 'Soibum". After inoculation, the samples were kept in an incubator at 35±2°C for a period of two months.

To purify phytosterols, 100g of oven dried (at 60°C) and powdered fermented materials was refluxed with a solvent mixture of benzene, petrolium ether and 2N-ethanolic KOH (**10:5:1**) for 5h in a 1000 ml Ca Clevenzer apparatus. Further purification was done[2] and a partially purified form of stigmasterol, ß-sitosterol and campesterol were obtained from the crude phytosterols. These samples of isolated phytosterols (stigmasterol, ß-Sitosterol and Campesterol) were subjected to TLC[3] on silica gel-G (0.25 mm thick) plated (20X20 cm) using Hexane : Ethylacetate (**3:1**).

The melting point of the crystals (assumed to be stigmasterol, ß-sitosterol and campesterol) was determined. Further, the UV spectral analysis for the crystals obtained after preparative TLC3 as well as the authentic sample (Sigma Chemicals, U.S.A.) were measured from 225 nm to 400 nm on a Beckman - DU-64-Spectrophotometer.

The crystalline partially purified form of phytosterols obtained from the fermented succulent bamboo shoot were then subjected to microbial transformation using *Arthrobacter globiformis* (obtained from IMTECH, Chandigarh) for conversion of sterols into 17-Ketosteroids[4].

The medium on which the bacterium was grown for biotransformation contained (g/l) $NaH_2PO_4.2H_2O$, 3.0; KH_2PO_4, 3.0; NaCl, 0.2; $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_2O$, 0.3; $CaCl_2.2H_2O$, 0.1; $FeSO_4.7H_2O$, 0.001; Sodium molybdate, 0.0001. The pH was adjusted to 7.2. The nutrient medium containing phytosterol (0.1%) was homogenised in a waring blender for 10-15 min and then sterilised in an autoclave at 120°C for 15 min. The bacterium was allowed to grow in a 250 ml flask containing 50 ml of the medium on a reciprocal shaker (80 strokes/min) at 30±2°c for 24 h. A further addition of metabolic inhibitor (0.1% *a, a'*-dipyridyl) was done. The incubation was then carried out for 72h. At regular interval, flasks were withdrawn from the shaker and a representative sample (5 ml) of the fermentation broth was withdrawn from each flask and extracted with dried and redistilled ethylacetate (1:1v/v). Suitable aliquots of the extract were used for the estimation of residual sterol by Liebermann-Burchard reaction[5] and estimation of ADD by Zimmermann reaction[6].

Results and Discussion

The total phytosterols estimated in 60 days old fermented samples was much higher both in *Bambusa balcooa* (0.60% dry wt) and *Dendrocalamus strictus* (0.42% dry wt) as compared to that in its fresh samples (0.18 and 0.14% of dry wt respectively) as in Table 1. The dry matter content was also higher in *Bambusa balcooa* (13.3%) than that of *Dendrocalamus strictus* (10.7%). The total phytosterols content is found to be higher in *Bambusa balcooa* than that of *Dendrocalamus strictus* (Table 1).

The Co-Chromatography of the crude phytosterols (isolated from fermented samples) with standard samples revealed the presence of ß-sitosterol, stigmasterol and campesterol. The Rf value of each spot was calculated as in Table 2. The TLC study showed the presence of at least four phytosterols tentatively identified as ß-sitosterol, stigmasterol and campesterol and one unidentified phytosterol.

The UV spectra of the peaks for the authentic samples were found at 247nm for ß-sitosterol, 244nm for stigmasterol and 243 nm for campesterol. The purified samples of phytosterols extracted from fermented bamboo shoots also showed similar peaks.

The phytosterols (ß-sitosterol, stigmasterol and campesterol) isolated from the fermented bamboo shoots was used for microbial conversion into Androstadienedione (ADD) using *Arthrobacter globiformis*, which yielded a considerable amount of

Table 1. Levels of total phytosterols in the succulant shoot samples of different species of bamboo.

Name of the Species of bamboo	Dry Matter Content %	Concentration of Phytosterols (%dry wt.)	
		Fresh delicate shoot apex	Fermented shoot slices (60 days old)
Bambusa balcooa	13.3	0.18 ± 0.01*	0.60 ± 0.04
Dendrocalamus strictus	10.7	0.14 ± 0.06	0.42 ± 0.02

* Standard error of the mean (n = 3)

Table 2. Rf values of different spots separated on TLC plate separated with Hexane and ethylacetate solvent pairs. The chromatogram was run at 30°C for 80 min and for development of spots the plates were sprayed with Lieberman - Burchard reagent followed by heating in an oven at 80°C for 30 min.

Solvent pair	Spots position	Rf value	Possible phytosterols using standard samples on co-chromatography
Hexane : Ethylacetate (3:1)	1st (lower most)	0.0428	Unidentified
	2nd	0.320	ß-sitosterol
	3rd	0.732	Stigmasterol
	4th	0.814	Campesterol

Table 3. Production of ADD from various phytosterols by biotransformation using *Arthrobacter globiformis* in the presence of metabolic inhibitor, (*a*, *a*'-dipyridyl).

Initial conc. of phytosterols (0.1%)	Conc. of inhibitor (0.1%)	Concentration of 17-Ketosteriods (ADD) [mg/ml] Incubation period (h)						
		4	8	12	24	36	48	72
β-Sitosterol	Zero	0.13 ± 0.02*	0.16 ± 0.02	0.16 ± 0.03	0.18 ± 0.02	0.10 ± 0.02	0.03 ± 0.01	0.01 ± 0.001
β-Sitosterol	Dipyridyl	0.15 ± 0.01	0.20 ± 0.02	0.28 ± 0.01	0.47 ± 0.12	0.19 ± 0.01	0.09 ± 0.01	0.08 ± 0.003
Surface floating crystals	Zero	0.09 ± 0.01	0.09 ± 0.01	0.11 ± 0.02	0.14 ± 0.02	0.06 ± 0.01	0.02 ± 0.003	0.02 ± 0.003
Surface floating crystals	Dipyridyl	0.09 ± 0.01	0.15 ± 0.02	0.16 ± 0.02	0.18 ± 0.01	0.15 ± 0.01	0.08 ± 0.02	0.02 ± 0.01
Side wall sticking crystals	Zero	0.11 ± 0.01	0.19 ± 0.02	0.18 ± 0.01	0.17 ± 0.01	0.09 ± 0.003	0.03 ± 0.01	0.01 ± 0.001
Side wall sticking crystals	Dipyridyl	0.19 ± 0.02	0.21 ± 0.02	0.21 ± 0.01	0.53 ± 0.12	0.20 ± 0.02	0.04 ± 0.02	0.02 ± 0.01
Bottom residue	Zero	0.11 ± 0.01	0.09 ± 0.01	0.15 ± 0.02	0.09 ± 0.01	0.08 ± 0.01	0.01 ± 0.01	0.002 ± 0.001
Bottom residue	Dipyridyl	0.11 ± 0.003	0.12 ± 0.06	0.12 ± 0.01	0.15 ± 0.02	0.18 ± 0.003	0.05 ± 0.01	0.01 ± 0.005

* Standard error of the mean (n=3)

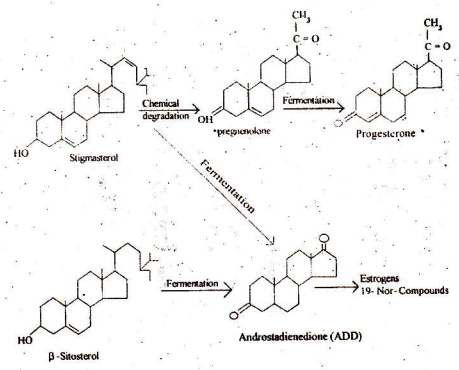

Fig. 1 Conversion of Stigmasterol and ß-Sitosterol into progesterone and Estrogens - 19 - Nor-compounds.

ADD in the incubation mixture in presence of metabolic inhibitor *a-a'*-dipyridyl. The level of ADD yield so obtained was very close to the levels obtained by using the standerd sample of ß-sitosterol. Gradual accumulation of ADD was more or less maintained for a prolonged period of incubation (72h) but declined later as in Table 3.

Diosgenin used as the starting material for corticosteroids and spironolactone (diuretics) is well known but not for the hormones and oral contraceptives[7]. However, due to increasing prices, uncertainty of continued availability of this drug precursor and on the other hand, use of oral contraceptive and anabolic steroid increases significantly, an extensive source for suitable raw materials other than diosgenin as a starting material for the synthesis of oral contraceptives and anabolic steroid is imperative to meet the sudden drastic increased demand of the said drugs in the market at low cost. According to Martin[1], phytosterols such as stigmasterol, ß-sitosterol and campesterol are considered potential raw materials for the synthesis of pharmaceutically active hormonal steroidal drugs. Among phytosterols, stigmasterols accounts for 15% of the total steroid precursors used in the world[8] and sitosterols find use as precursor for estrogens and progestogens besides diuretics[7] as in Fig. 1. Stigmasterol is an important starting material for bioconversion to biologically active steroidal drugs. Because of the presence of a double bond in the side chain, it is relatively easy to remove the side chain for conversion to steroid hormones[9]. Stigmasterol can be converted to ADD through biological fermentation[7,10] which can be further converted to pregnenolone acetate and produce progesterone in excellent yield.

To conclude in the paper, the authors, specifically, would like to mention that the naturally occuring phytosterols such as stigmasterol, ß-sitosterol and campesterol isolated for the first time from the fermented succulent bamboo shoot slices of *Bambusa balcooa*[11] and *Dendrocalamus strictus* are the most promising raw material for the synthesis of hormonal steroidal drugs. Stigmasterol is almost as versatile as diosgenin and can be converted into different groups of steroid (as in Fig. 1) and being a by-product precursor, the stigmasterol-based steroid drugs will be cheaper and can afford than those base on diosgenin as the raw material is being abundantly and freely available in almost unlimited amount throughout the contry as the non-conventional source of phytosterols in the fermented bamboo shoots. Such a research finding of conversion of phytosterols isolated from these raw material to ADD may be a profound advantage for those biotechnology based pharmaceutical industries.

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