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Phytosteroids from tissue culture of *Allium cepa* L. and *Trachyspermum ammi* S prague.

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Production of secondary metabolites by cultured cells provides a particularly important benefit to manipulate and improve the production of desired compounds; thus biotechnological approaches to increase the concentrations of the metabolites are discussed. Present study deals with the production, isolation and identification of phytosterols from tissue culture of *Allium cepa* and from plant parts and tissue culture of *Trachyspermum ammi*. Steroidal analysis of plant parts showed the maximum amount of stigmasterol (0.240 mg/gdw) which was comparatively little less than that of the amount of β- sitosterol (0.295 mg/gdw) in the seeds of T. *ammi* . The maximum amount of stigmasterol was present in four weeks old tissue of T. *ammi* (0.249 mg/gdw) whereas the highest content of β-sitosterol was observed in six weeks old tissue of A. *cepa* (0.315 mg/gdw) However, lanosterol, was present only in the tissue of A. *cepa* which was maximum in six weeks old tissue (0.039 mg/gdw)

Keyword: *Allium cepa*, Liliaceae, *Trachyspermum ammi* Umbellifereae, Stigmasterol, Beta sitostewrol

1. Introduction

Phytosterols (referred to as plant sterol and stanol esters) are a group of naturally occurring compounds found in plant cell membranes. Because phytosterols are structurally similar to the body's cholesterol, when they are consumed they compete with cholesterol for absorption in the digestive system. As a result, cholesterol absorption is blocked, and blood cholesterol levels reduced. Throughout much of human evolution, it is likely that large amounts of plant foods were consumed. In addition to being rich in fiber and plant protein, the diets of our ancestors were also rich in phytosterols—plant-derived sterols that are similar in structure and function to cholesterol. There is increasing evidence that the reintroduction of plant foods providing phytosterols into the modern diet can improve serum lipid (cholesterol) profiles and reduce the risk of cardiovascular disease.

In the present study the callus of *Allium cepa* (Liliaceae) and Trachyspermum ammi (Umbellifereae) were successfully maintained in the laboratory and evaluated for their phytosterol content which is not well documented for these plants.

2. Material and Methods

Plant parts (seeds, stem and leaves) of *T. ammi* were collected from Haffkine campus. Plant parts were washed in running water, cut into small pieces, dried at 100^0 to inactivate enzymes and then dried at 60^0 till a constant weight was achieved. The tissue of *A. cepa* and *T. ammi* were

raised from the seedlings, grown and maintained by frequent subculturings of 6-8 weeks for twenty four months on fresh RT medium. The growth indices were calculated at the transfer age of the tissue at two, four, six and eight weeks. Three replicates in each case were examined and mean values taken into consideration.

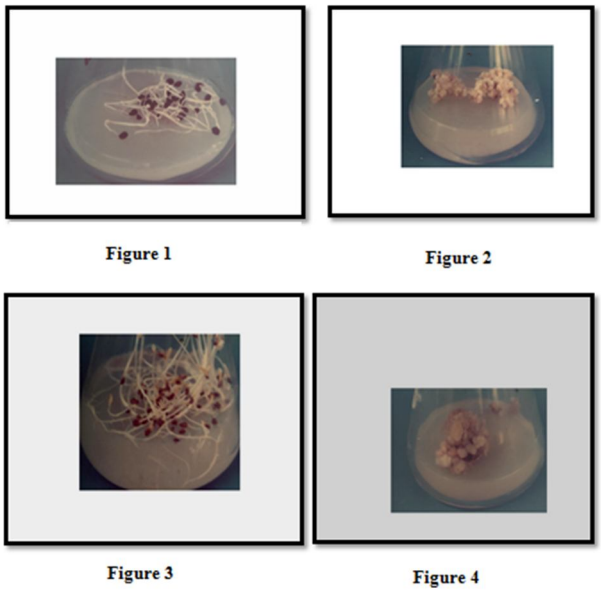

Fig 1-2: Showing the seed germination and developed callus of *Allium cepa* L.

Fig 3-4: Showing the seed germination and developed callus of *Trachyspermum ammi*. Sprague

S. No.	Age in weeks	Growth Index	
		A.cepa	*T. ammi*
1	2	0.76	0.91
2	4	1.5	1.4
3	6	2.1	2.7
4	8	1.7	1.8

Table 1. Showing the growth index of *A.cepa* and *T. ammi*

2.1 Extraction Procedure

Each of the plant parts (seeds, stem and leaves) as well as the various tissue samples (2,4,6 and 8 weeks old)were dried, powdered, weighed and then separately subjected to soxhlet extraction in petroleum ether for 24 hr on a water bath for removing fatty acids. Each of the mixture was filtered and residual tissue masses were hydrolysed with 15% ethanolic hydrochloric acid (w/v) for 4 hr (Tomita et al., 1970)Each of the hydrolysed samples was filtered, the filtrate extracted in ethyl acetate separately and given continuous washings of distilled water till the pH was 7. The extract was passed over Na_2SO_4, for removing moisture, dried, taken up in chloroform and then analysed for steroidal content.

2.2 Qualitative Analysis

a. Thin-layer Chromatography (TLC)

Each of the extracts was applied on silica gel G coated and activated glass plates along with the standard samples of sterols (cholesterol, lanosterol, stigmasterol and B-sitosterol) The glass plates were then developed in an organic solvent misture of Hexane and acetone (80:20: Fazil and Hardman, 1968) and air dried. On spraying the developed plates with 50% H_2SO_4 three spots corresponging. to those of the standard of these of the standard samples and a stigmasterol (Rf 0.91; color grey) lanosterol (Rf 0.89; color, brown) and B-sitosterol (Rf. 0.85; color, purple) were observed in *A. cepa*. whereas two spots coinciding with those of the standard samples of β-sitosterol and stigmasterol were marked in case of *T. ammi* (Table 6.2) The developed plates were also sprayed with anisaldehyde reagent but 50% H_2SO_4 gave excellent results.

A few other solvent systems (Benzene : ethyl acetate: 85:15: Heble et al., 1968a; Benzene : ethylacetate 3:1: Kaul and Staba, 1968) were also use but Hexane and acetone gave excellent results in the present study. Ten replicates in each case were examined and the mean Rf values calculated.

b. Preparative Thin-layer Chromatography (PTLC)

Each of the extracts as also the standard samples of B-sitosterol, stigmasterol and lansterol were also applied on thickly coated silica gel and activated glass plates. The plates were developed as described above and a portion of the plates was sprayed with 50% H_2SO_4. Three spots corresponding to the standard samples of B-sitosterol, lanosterol and stigmasterol in case of A.cepa and two spots coinciding with those of the standard samples of B-sitosterol and stigmasterol were scrapped off alongwith silica gel from about 150 unsprayed plates. The isolated mixtures were separately extracted with chloroform. The various isolates were rechromatographed separately to test their purity. Each of the isolated purified compounds was crystallized by adding saturated acetone solution to which a few drops of methanol were added (Kaul and Staba. 1968). The crystals formed were removed from the mother liquor, washed twice and cold menthol and dried in vacuo. Each of crystallised compounds of all the samples was subjected to colorimetry (for quantitative estimation). mp (Thoshniwal Melting Point Apparatus, India) Infra-red spectral (Perkin-Elmer 337 Infra-red Spectrophotometer) studies and Gas-liquid chromatography (Perkin-Elmer OV-11 Gas Chromatograph) along with their respective standard reference sterols.

c. Gas-Liquid Charomatography

The Steroidal extracts of both the plant species were analysed along with their standard samples of β-sitosterol, stigmasterol and lanosterol by GLC equipped with a flame lionization detector and a stainless steel column containing SE-30, coated with 3% Gas chrom P. The operating temperature used for analysis was 300^0 Hydrogen was used as the carrier gas at a flow rate of 0.5 cm/min to ascertain the concentration of various steroidal compounds in the tissue.

2.3 Quantitative Analysis

Quantitative estimation of various identified sterols was carried out colormetrically with the help of a Spectrophotometer, following the method of Das and Benerjee (1980), which includes the preparation of a regression curve for each of the standard reference compound. A stock solution of each of the reference compounds (lanosterol, B-sitorsterol and stogmasterol) was prepared (in chloroform 500 mg/1) separately. From this six concentrations (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 ml) were prepared and spotted on silica gel G coated and activated glass plates. The plates were developed as described above and the plates were exposed to iodine vapours. Iodine positive spots were marked and heated to evaporate the excess of iodine.

The spots were separately scrapped off along with silica gen and such eluates were taken up in 5 ml of chloroform in test tubes. Each of the tubes was centrifuged, supernatants taken and evaporated to dryness. To each of the dried samples, 3 ml of glacial acetic acid was added

and shaken vigorously at room temperature for 1 min, then placed in freezer. To this frozen sample, 2 ml of freshly prepared chromogenic reagent (0.5 ml of 0.5% anhydrous ferric chloride in glacial acetic acid and 100 ml of concentrated H_2SO_4 ; Klyne, 1965) was added drop wise at 0^0 and mixed thoroughly. Each of the reaction mixtures was incubated at 40^0 for 30 min and optical density was read on a Spectronic-20 Colorimeter (Bausch and Lomb) set as 540 nm against a blank (3 ml of glacial acetic acid and 2 ml of chromogenic reagent). Five such replicates were run for each of the concentrations and average optical density was plotted against their respective concentration to compute a regression curve which followed the Beer's law.

Each of the extracts was dissolved in chloroform and was spotted along with the standard reference samples of βsitosterol, stigmasterol and lanosterol on silica gel coated and activated glass plates which were developed as described earlier. Three spots coinciding with those of the authentic sample of β-sitosterol, stigmasterol and lanosterol were marked. Each of these eluates were dried taken up in 5 ml of chlololform and were worked out as described above. Concentrations of β-sitosterol, stigmasterol and lanosterol were calculated (mg/gdw) by computing the optical density of the experimental sample with the regression curve of the standard reference sample of β-sitosterol, stigmasterol and lanosterol. Three such replicates were examined in each case and mean values calculated. Six weeks old tissues of *A. cepa* and *T. ammi* were extracted and analysed with reference samples of B-sitosterol, stigmasterol and lanosterol by GLC.

PLANT	PHYTOSTEROL	RF VALUE IN HAXENE: ACETONE 8:2	COLOR AFTER SPRAYING WITH 50%H_2SO_4	COLOR IN UV
A.CEPA	βSITOSTEROL	95	PURPLE	DL RED
	STIGMASTEROL	85	GREY	DKBROWN
	LANOSTEROL	89	YELLOW	GREEN
T.AMMI	βSITOSTEROL	95	PURPLE	DL-RED
	STIGMASTEROL	85	GREY	DKBROWN

PLANT	PLANT PARTS USED	AGE IN WEEK	GROWTH INDEX	STIGMASTEROL	LANOSTEROL	βSITOSTEROLMG/GDW
T.AMMI	SEED	-		0.240	-	0.295
	LEAVES	-		0.014	-	0.012
	STEM	-		0.224	-	0.019
		2	0.91	0.13	-	0.012
		4	1.4	0.249	-	0.027
		6	2.7	0.17	-	0.107
		8	1.8	0.061		0.09
		2	0.76	0.045	0.015	0.081
A.CEPA		4	1.5	0.075	0.025	0.12
		6	2.1	0.025	0.039	0.315
		8	1.7	0.018	0.023	0.14

Table. 2: showing the steroidal content in *Allium cepa* and *Trachyspermum ammi*

3. Results and Discussion

The maximum growth index was observed in six weeks old tissues of *T. ammi* (2.7) followed by six weeks oldtissue of *A. cepa* (2.1) The growth index increased in a linear fashion up to a period of six weeks which then gradually declined (Table 6.2; Fig. 6.1). The chromatographic analysis showed the presence of two phytosterols corresponding to those of the standard samples of β-sitosterol (Rf. 0.85: colour, purple) and stigmasterol (Rf. 0.91; colour, grey) in *T. ammi*, whereas the presence of three spots coinciding

with those of the standard samples of β-sitosterol (Rf. 0.85; Colour, Purple) Stigmasterol(Rf. 0.91; Colour, grey) and lanosterol (Rf. 0.87; Colour, Purple) were observed in *A. cepa* (Table 6.1). Further confirmation of the isolated compounds was done by mp (B-sitosterol, 139-140^0; stigmasterol 114-115^0; and lanosterol 143-144^0), superimposable IR spectra and Gas-liquid chromatography of the isolated and their respective standard compounds standard compounds (Fig. 6.2) Steroidal analysis of plant parts showed the maximum amount of stigmasterol (0.240 mg/gdw) which was comparatively little less than that of the amount of β- sitosterol (0.295 mg/gdw) in the seeds of *T.ammi* (Table 6.2; Fig. 6.1) The maximum amount of stigmasterol was present in four weeks old tissue of *T.ammi* (0.249 mg/gdw) whereas the highest content of β- sitosterol was observed in six weeks old tissue of *A. cepa* (0.315 mg/gdw) However, lanosterol, was present only in the tissue of *A. cepa* which was maximum in six weeks old tissue (0.039 mg/gdw).

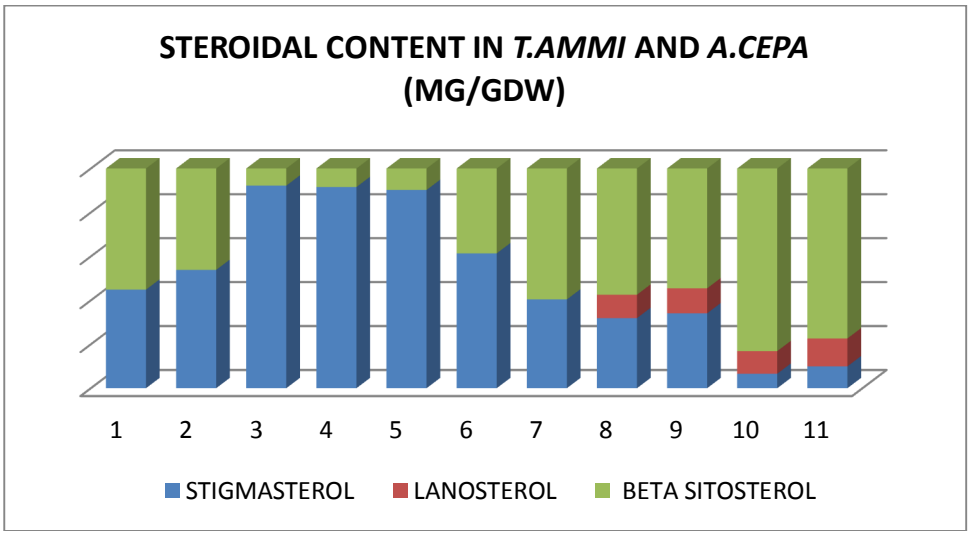

FIG. 2: Graph showing the steroidal content in *A.cepa* and *T.ammi*

Phytosterol such as β-sitosterol and stigmasterol have been reported in tissue cultures of Artemisea scoparia, *Datura metel*, *Emblica officinalis* and *Trigonella foenum-graecum* (Khanna, 1987) and βsitosterol and stigmasterol in the tissue of *C. arietinum*, *S. indicum* and *Momordia charantia* (Khanna, 1984). Only β-sitosterol and stigmasterol.It has been reported in tissue culture of *S. xanthocarpum* (Heble et al., 1968) *Helianthus annus* (Sharma, 1975), *S. aviculare* (Gaur, 1978) and *Trigonella corniculata* (Jain, 1979) .Lanosterol, stigmasterol and β-sitosterol along with cholesterol have been observed in tissue of *Sesamum indicum* (Jain and Khanna, 1973) and *M. Charantia* (Khanna and Mohan, 1973) Smoczklewicz et. al., (1982) have reported βsitosterol from bulbs of *Allium cepa*. Whereas Cholesterol, campesterol. β-sitosterol and stigmasterol have been identified from the bulbs of *A. sativum* (Stoyamo et.al., 1981) Claus et. al., (1980) and Catalano et. al., (1983) have shown the presence of β-sitosterol and stigmasterol in umbelliferous vegetables.

In the present study, however, three phytosterols as β-sitosterol , stigmasterol and lanosterol have been observed in the tissue of *A. cepa* whereas only two phytosterols, β-sitosterol , stigmasterol have been identified from plant parts and tissue culture of T. *ammi*.

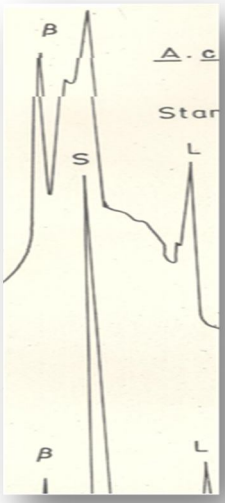

Fig 3: GLC curve of isolated and standard β-sitosterol , stigmasterol and lanosterol from *Allium cepa* L. tissue culture.

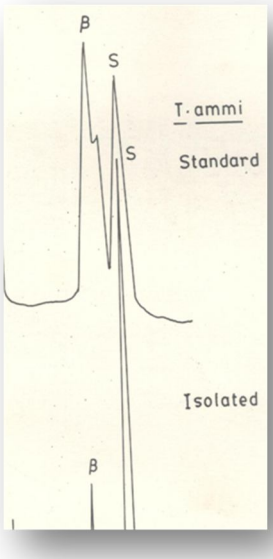

Fig 4: GLC curve of isolated and standard β-sitosterol , stigmasterol from *Trachysoermum ammi* Sprague.

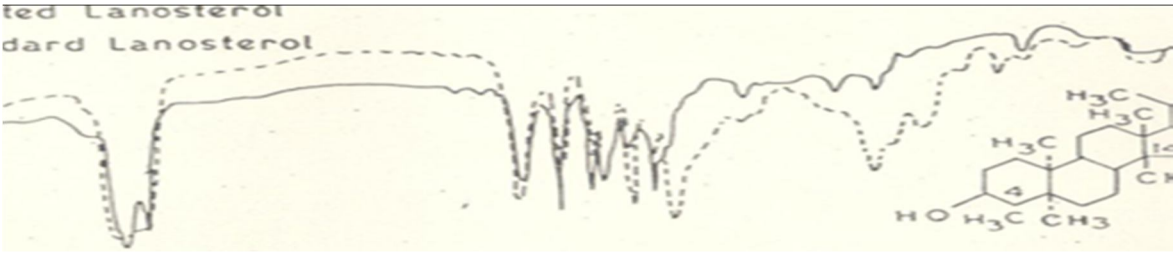

Fig 5: Infra-red spectra of isolated and standard lanosterol

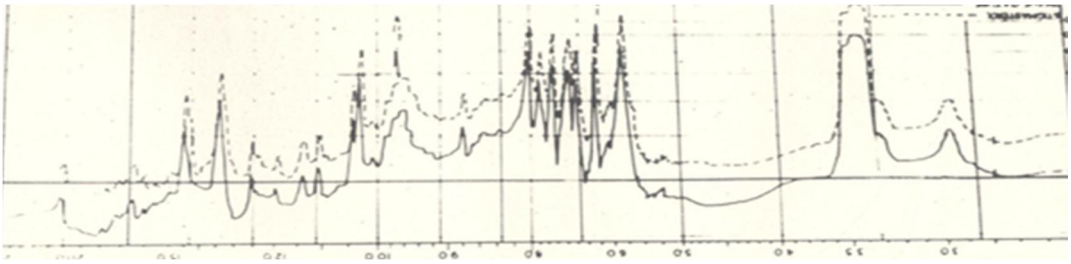

Fig 6. Infra-red spectra of isolated and standard stigmasterol.

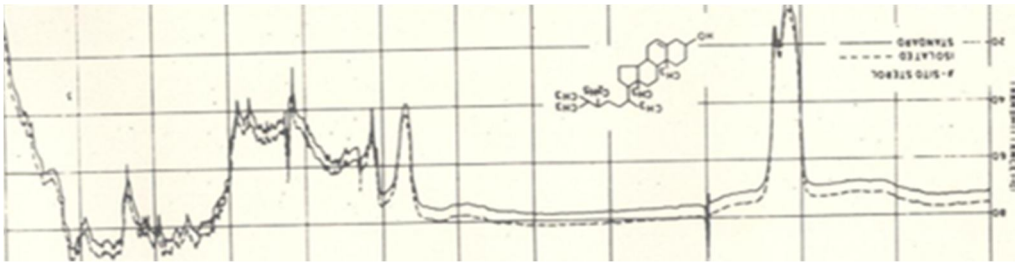

Fig.7. Infra-red spectra of isolated and standard β-sitosterol.

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