Research Article

Available online through
www.ijrap.net

IJRAP
ISSN 2229-3566

# PHYTOSTEROLS FROM *IN VIVO AND IN VITRO* CULTURES OF TWO MEDICINAL PLANTS VIZ. *ADHATODA VASICA* AND *AGERATUM CONYZOIDES*

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Received on: 14/04/2011 Revised on: 20/05/2011 Accepted on: 11/06/2011

**ABSTRACT**
In the present study two medicinally important plants viz. *Adhatoda vasica* and *Ageratum conyzoides* extensively used in Ayurvedic preparation of medicines were analyzed for their sterols contents. Induction of callus in these two plants was carried out by using different explants on MS media supplemented with various concentrations and combinations of growth regulators. In *Adhatoda vasica*, stock callus developed from nodal segment on MS-medium supplemented with BAP (0.5 mg/l) and NAA (2.5 mg/l) whereas in case of *Ageratum conyzoides* stock callus was developed from internodal segment on MS medium supplemented with 2,4-D (3.0mg/l) and BAP (0.5 mg/l). The calli so formed were maintained on MS medium for 18 months by frequent subculturimgs after every 4-5 weeks and then analyzed for their sterol contents and compared with their contents present in plant parts of both the plants. Presence of β-sitosterol and stigmasterol in various tissue samples and plant parts of *Adhatoda vasica* and *Ageratum conyzoides* were confirmed by Co. TLC, mp and IR spectral studies.
**KEY WORDS**: *Adhatoda vasica*, *Ageratum conyzoides,* Tissue culture, Phytosterols

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## INTRODUCTION

*Adhatoda vasica* and *Ageratum conyzoides* are two important medicinal plants which are extensively used in Ayurvedic medicines. They are highly demand able in pharmaceutical industries. Sterols are the triterpenoid isoperoxides which are characterized by a 3β-monohydroxy per hydro-1, 2-cyclopentanophenenthrene ring system. Over 4000 triterpenoids have been characterized but only around 300 sterols and closely related compounds occur in plants. These are listed by Akihisia *et al.*[1]. Sterols are often estimated by a fatty acid at the C–3 hydroxyl group. Steryl esters have been reported to be in membranes[2] and also in the soluble cellular fraction[3]. In general, most plants produce sterols that are alkylated at C-24 as typified by stigmasterol and β-sitosterol. Cholesterol rarely represents a few percent of phytosterol mixtures.

A combination of chromatography, mass spectrometry and NMR is used for the purification and identification of sterols[4]. Sterols are the starting material for the biosynthesis of plant steroids[5]. Sterols have been isolated from large number of plant species and probably occur in all angiosperms and gymnosperms. The common phytosterols reported from plants are β- sitosterol, stigmasterol and campesterol. Phytosterols have always been a fascinating subject of study because of their diversified physiological and pharmacological effects on animals. Since β-sitosterol is an active ingredient of *Adhatoda vasica* and *Ageratum conyzoides,* phytosterols were studied in both the plant parts (root, stem and leaves) of these species along with *in vitro* tissue cultures. (2, 4, 6 and 8 week old tissues).

## MATERIAL AND METHODS

### *In vivo* plant parts

Various mature plant parts (stem, leaves and roots) of *Adhatoda vasica* and *Ageratum conyzoides* were collected locally from nurseries. These were washed with tap water to remove dust and dried in shade.

### *In vitro* tissue culture

Nodal stem explants of *Adhatoda vasica* and *Ageratum conyzoides* were sterilized with 0.1% mercuric chloride solution and inoculated after several rinses with sterile distilled water onto Murashige and Skoog's (MS)[6] medium supplemented with BAP (0.5 mg/l) and NAA (2.5mg/l). BAP (0.5 mg/l) and 2,4-D (3.0 mg/l) and Callus appeared on the medium after 20-25 days. The

unorganized callus tissue of *Adhatoda vasica and Ageratum conyzoides* maintained for 18 months by frequent subculturing after every 6-8 weeks onto fresh medium were transferred to fresh MS-medium of the same composition. Tissues thus established were harvested periodically (after 2, 4, 6 and 8 weeks), dried and their growth indices calculated separately. (Five replicates of each of the tissue samples were examined and the mean values recorded.

**Extraction procedure**

The tissues grown for different time intervals along with plant parts were collected separately. Tissues and plant parts were dried at 105°C for 15 minutes in order to inactivate enzymes and then at 60°C till a constant weight was achieved. Each of the samples was powdered in a mortar and then refluxed in 30% hydrochloric acid (v/v) for 4 hours. Each hydrolyzed sample was washed with cold water and filtered till the pH of the filtrate was 7. Each sample was then dried at 60°C for 8 hours and then soxhlet extracted with benzene for TLC (Thin layer chromatography). The steroidal contents of the tissue samples were calculated (mg/gdw) at different intervals and compared with their respective growth indices. Extracts were then dried *in vacuo*. Ten to fifteen replicates were analyzed in each case. Identification of sterols was carried out by TLC, melting points and infra red studies.

**Thin layer chromatography (TLC)**

Thin glass plates (20 × 20 cm) were coated (0.2 mm to 0.3 mm) with silica gel G (Kiesgel G; 30 gm/60 ml. of distilled water) and dried at room temperature. The coated plates were activated in an oven for 30 min at 100°C and cooled. Benzene extracts of tissue and plant parts along with the standards (β-sitosterol, stigmasterol, lanosterol, cholesterol) were spotted at 1cm. intervals on the staining line of chromatograms. The solutions were added in increments by means of micropipette so as to keep the spot size less than 3 mm in diameter. The plates were then placed in developing tanks, to which had been added, at least one hour prior to use, 120 ml. of solvent. The end walls of the tanks were lined with strips of filter paper freshly saturated with the solvent and the tank lids were sealed with vacuum grease. Development was allowed to proceed until the solvent front had risen 10-12 cm beyond the original spots. The plates were then removed from the tanks and allowed to air dry. The spots were detected by spraying the chromatogram with either 50% sulphuric acid or anisaldehyde reagent[7] and heating the plates at 110°C for 10 min. Many solvent combinations were examined (n hexane- acetone, 8:2[8;] Benzene- ethyl acetate, 85:15[9]; benzene- ethyl acetate 3:1[7] for developing the chromatograms on silica gel G, the best of these was found to be n-hexane-acetone (8:2)[8] for the present work. Fifteen to twenty replicates were run and average $R_f$ values were calculated for the standard, as well as tissues and plant parts extracts. It was noted that plates developed in unsaturated tanks did not give reproducible $R_f$ values whereas plates developed in saturated tanks gave values with the normal accepted tolerance (± 0.02 $R_f$ units) for work on TLC.

**Preparative thin layer chromatography (PTLC)**

Silica gel G coated thin layer glass plates (0.4 mm to 0.5 mm; 4.5 g/80 ml. distilled water) were activated at 100°C for 30 minutes and developed in methanol to remove any fluorescent impurities[7]. The benzene extracts of tissues and plant parts as well as standard β-sitosterol, stigmasterol, lanosterol, cholesterol) were applied 1 cm above the edge of the plates and developed in an organic solvent mixture (n-hexane-acetone, 80:20). The portion of plate, coating the standard mixture and the extract was sprayed with 50% sulphuric acid to mark the steroidal bands. The various unsprayed bands from about 200 plates were collected and extracted separately with benzene. The various extracted bands (sterols) were re-chromatographed to test their purity. The IR spectrum and melting point of each of the isolated compounds was taken and compared with those of standards.

**Quantitative estimation**

Quantitative estimation of identified sterols was carried out calorimetrically with the help of a spectrophotometer following the method of Das and Banerjee[10] which includes the preparation of regression curve for each of the reference compounds.

A stock solution of each of the reference compounds (β-sitosterol, stigmasterol and cholesterol) in chloroform (500 µg/ml.) was separately prepared. 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 coated and activated plates (PLC).

The plates were developed in a solvent mixture of n hexane- acetone (8:2). Such developed chromatograms were air dried and exposed to iodine vapors. Iodine positive spots were marked and heated to evaporate excess of iodine.

The spots were separately scrapped along with silica gel and such elutes were taken with 5 ml of chloroform in test tubes. Each of the tubes was centrifuged, supernatants taken, solution evaporated to dryness and processed further.

To each of the dried samples, 3 ml of glacial acetic acid was added and shaken on a vortex mixture at room temperature for one minute and then placed in a 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 sulphuric acid) was added dropwise at 0°C and mixed thoroughly. Each of the reaction mixture was incubated at 40°C for 30 minutes and optical density of each of the test samples was read on a spectrophotometer (UV-VIS, Systronic) set at 540 nm. against a blank (3 ml glacial acetic acid and 2 ml of chromogenic reagent).

Five such replicates were run for each of the concentrations to minimize the standard deviation and average OD was plotted against their respective concentrations to complete a regression curve which followed the Beer's law.

Each of the extracts dissolved in chloroform was spotted along with β-sitosterol and stigmasterol on silica gel coated and activated glass plates and developed in an organic mixture of n-hexane-acetone (8:2). Two spots coinciding with those of the authentic samples of β-sitosterol and stigmasterol were marked in case of various extracts of plants species. Each of these elutes was dried, taken up in 5 ml of chloroform and processed as described above.

Concentrations of β-sitosterol, stigmasterol and cholesterol were calculated (mg/gdw. and %) separately by comparing the O.D. of the experimental samples with the regression curves of the standard reference samples of β-sitosterol, stigmasterol and cholesterol. Five such replicates were examined in each case and mean value recorded.

## RESULT AND DISCUSSION

Presence of β-sitosterol and stigmasterol in various tissue samples and plant parts of *Adhatoda vasica* and *Ageratum conyzoides* were confirmed by Co. TLC (β-sitosterol, Rf 0.95, purple; stigmasterol, Rf 0.89 grey); m.p. (β-sitosterol 139-140°C, stigmasterol, 142-144°C) (Table-1); superimposable IR spectra of the isolated and the authenetic samples of each of the sterols. Presence of Cholesterol could not be detected in *in vitro* tissue cultures of *Adhatoda vasica and Ageratum conyzoides* whereas its presence was detected in *in vivo* plant parts of both the plant species. Cholesterol was confirmed in *in vivo* plant parts by Co. TLC (cholesterol Rf 0.88, Reddish pink; m.p. 114-115°C) and superimposable IR spectra of the isolated and the authentic samples of cholesterol.

Maximum growth index (6.22) was observed in six week old tissues of *Adhatoda vasica* (Table-2) and slightly lower in *Ageratum conyzoides* (Table-4). Total and individual amount of sterols (β-sitosterol (0.272) and stigmasterol (0.167) (total amount – 0.439) were found to be higher in 6 week old tissue of *Adhatoda vasica* and in *Ageratum conyzoides* (Table-2 and 4). Both the amount of sterols (total amount – 0.432, β-sitosterols, 0.270, and stigmasterol 0.162 mg/g.d.w) were found to be higher in 6 weeks old tissue. Whereas it was observed that total and individual amount of sterols were slightly more in six week old tissue of *Adhatoda vasica* as compared to *Ageratum conyzoides* (6 week old tissue)..

When total amount of steroidal contents of *in vivo* plant parts of *Adhatoda vasica* compared with *Ageratum conyzoides* (*in vivo* plant parts). Then it was found that the total amount of sterol content was higher in stem (0.0876%) followed by leaves (0.0665%), roots (0.0550%) of *Adhatoda vasica.* Whereas in case of *in vivo* plant parts of *Ageratum conyzoides* it was observed slightly higher in stem (0.0868%) followed by leaves (0.0656), and roots (0.0533).

Total amount of steroidal contents were found to be slightly higher *in vivo* plant parts of *Adhatoda vasica* as compared to *Ageratum conyzoides* (*in vivo* plant parts) (Table-3 and 5). Individual amounts of β-sitosterol and stigmasterol were also maximum in stem (0.0222%, 0.0392%) followed by leaf (0.0122%, 0.0232%) and roots (0.0114%, 0.0211) of *Adhatoda vasica* as compared to *Ageratum conyzoides*, stem (0.0220%, 0.0389), leaves (0.0119%, 0.0230) and roots (0.0111%, 0.0200)..

Cholesterol could not be detected in *in vitro* static tissue cultures of investigated plant of *Adhatoda vasica* and *Ageratum conyzoides* but it was detected in the plant parts stem, leaves and roots. Total amount of cholesterol was found to be slightly higher *in vivo* plant parts of *Adhatoda vasica* (0.0798%) in comparison to *Ageratum conyzoides* (0.0788%) (Table-3 and 5).

## ACKNOWLEDGEMENT

The authors would like to acknowledge with thanks to Head, Department of Botany, University of Rajasthan, Jaipur for providing all the facilities in the department to carry out the present research work.

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**Table1: Confirmation of sterols through Rf, Colour after spraying and melting point (°C)**

| Name of the sterols | Relative front (Rf) | Colour after spraying | Melting point mp°C |
|---|---|---|---|
| β-sitosterol | 0.95 | Purple | 139-140°C |
| Stigmasterol | 0.89 | Grey | 142-144°C |
| Cholesterol | 0.88 | Reddish pink | 114-115°C |

**Table 2: Sterols from static tissue cultures of *Adhatoda vasica***

| Age of tissue weeks | GI | β-sitosterol | | Stigmasterol | | Total sterol | |
|---|---|---|---|---|---|---|---|
| | | mg/g dw. | % | mg/g dw. | % | mg/g dw. | % |
| | 1.55 | 0.257 | 0.0257 | 0.150 | 0.0150 | 0.407 | 0.0407 |
| 4 | 3.27 | 0.262 | 0.0262 | 0.157 | 0.0157 | 0.419 | 0.0419 |
| **6** | **6.22** | **0.272** | **0.0272** | **0.167** | **0.0167** | **0.439** | **0.0439** |
| 8 | 4.44 | 0.267 | 0.0267 | 0.162 | 0.0162 | 0.429 | 0.0429 |

**Table 3 : Sterols from *in vivo* plant parts of *Adhatoda vasica***

| Parts used | β-sitosterol | | Stigmasterol | | Cholesterol | | Total sterol | |
|---|---|---|---|---|---|---|---|---|
| | mg/g dw. | % | mg/g dw. | % | mg/g dw. | % | mg/g dw. | % |
| Stem | 0.222 | 0.0222 | 0.392 | 0.0392 | 0.262 | 0.0262 | 0.876 | 0.0876 |
| Leaves | 0.122 | 0.0122 | 0.232 | 0.0232 | 0.311 | 0.0311 | 0.665 | 0.0665 |
| Roots | 0.114 | 0.0114 | 0.211 | 0.0211 | 0.225 | 0.0225 | 0.550 | 0.0550 |

**Table 4: Sterols from static tissue cultures of *Ageratum conyzoides***

| Age of tissue weeks | GI | β-sitosterol | | Stigmasterol | | Total sterol | |
|---|---|---|---|---|---|---|---|
| | | mg/g dw. | % | mg/g dw. | % | mg/g dw. | % |
| 2 | 1.53 | 0.255 | 0.0253 | 0.148 | 0.0148 | 0.403 | 0.0403 |
| 4 | 3.26 | 0.258 | 0.0258 | 0.151 | 0.0151 | 0.409 | 0.0409 |
| 6 | 6.18 | 0.270 | 0.0270 | 0.162 | 0.0162 | 0.432 | 0.0432 |
| 8 | 4.40 | 0.261 | 0.0261 | 0.0155 | 0.0155 | 0.848 | 0.0848 |

**Table 5: Sterols from *in vivo* plant parts of *Ageratum conyzoides***

| Parts used | β-sitosterol | | Stigmasterol | | Cholesterol | | Total sterol | |
|---|---|---|---|---|---|---|---|---|
| | Mg/g dw. | % | mg/g dw. | % | mg/g dw. | % | mg/g dw. | % |
| Stem | 0.220 | 0.0220 | 0.389 | 0.0389 | 0.259 | 0.0259 | 0.868 | 0.0868 |
| Leaves | 0.119 | 0.0119 | 0.230 | 0.0230 | 0.307 | 0.0307 | 0.656 | 0.0656 |
| Roots | 0.111 | 0.111 | 0.200 | 0.0200 | 0.222 | 0.0222 | 0.533 | 0.0533 |

Source of support: Nil, Conflict of interest: None Declared