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Phytosterols production in *Moringa oleifera in vitro* cultures

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Abstract
Phytosterols are a group of steroid alcohols, naturally occurring in plants. Sterols are known to be starting materials for a number of plant steroids, which are used in pharmaceuticals and industries. The phytosterols have been found effective in treating high cholesterol as the plant sterols compete for absorption sites with cholesterol. β-sitosterol and stigmasterol are major phytosterols in most plants. Sitosterol and its related compound, sitosterolin decreases cholesterol absorption and helps to modulate immune function, inflammation and pain levels through its effects on controlling the production of inflammatory cytokines. This modulation of cytokine production and activity may help to control allergies and reduce prostate enlargement.
Medicinally useful plant *Moringa oleifera* of family Moringaceae was studied *in vivo* and *in vitro* for its sterols. For *in vitro* studies unorganized tissue was established on MS medium supplemented with various combinations and concentrations of growth hormones.
Callus (unorganized tissues) of *Moringa oleifera* was achieved on MS medium supplemented with 1.5 mg /L BAP + 1.5 mg /L 2, 4 D with germinated seed as explant. Tissue was maintained and multiplied for six months under controlled conditions. Plant parts (separately) and unorganized tissues of the selected plant were separately analyzed for qualitative and quantitative estimation of sterols. β-sitosterol and stigmasterol were identified and estimated in *Moringa oleifera* plant parts as well as in unorganized tissue. Amount of sterols was higher in unorganized tissues than plant parts.

Keywords: Phytosterols, β-sitosterol, Stigmasterol, growth index, unorganized tissue

1. Introduction
Plants are capable of synthesizing variety of low molecular weight organic compounds, called secondary metabolites usually with very unique and complex structures. Secondary metabolites are compounds biosynthetically derived from the primary metabolites and may be restricted to a particular taxonomic group. The role of secondary product has been rather ambiguous and earlier these were thought to be first waste materials. Now days, plant secondary metabolites are seen as tremendous source of pharmacological value for scientific and clinical research. Their biological activities have high therapeutic value, applicable in health care, drug development and synthesis of beneficial compounds.
Steroids are economically useful class of secondary metabolites. Steroids are waxy, soapy or greasy in texture, more soluble in oil than water. It includes sterols and sapogenins. The best known animal sterol cholesterol is the human. Among phytosterols, the most common ones are β-sitosterol, campsterol and stigmasterol. Researches have shown that phytosterols are beneficial in treating various diseases and are essential nutrients that ensure optimal functioning of the body's defense mechanisms [4, 6, 7, 15]. The phytosterols have been found effective in treating high cholesterol as the plant sterols compete for absorption sites with cholesterol, they thus reduce the amount of cholesterol absorbed. Phytosterols also contributes to the anti-inflammatory effect and are used to treat enlarged prostates and prostatitis
Moringa oleifera (family- Moringaceae) has been selected for *in vivo* and *in vitro* study for its sterols [1-3, 9-10]. The main objective of this study is to encourage attempts to commercially produce plant products from plant tissue culture [11, 14].

2. Material and Methods
Fresh plant parts of *M.oleifera* were collected from different local areas of Bikaner. Plant parts were separated, dried in shade and analyzed for phytosterols. Five replicates were taken for each plant part and mean value was calculated.
Unorganized cultures with profuse callusing were established using seeds as explants, on Murashige and Skoogs medium supplemented with 1.5 mg/L BAP+1.5mg/L 2,4-D in *M. oleifera.* These cultures were maintained for a period of six months by frequent subculturing at interval of 6 to 8 weeks at 26± 1 ˚C, 55% relative humidity and diffused light conditions (3000 lux). The growth indices (GI) were calculated at different time intervals of 2,4,6,8 and 10 weeks using the formula given below.

$$GI=\frac{\text{Final fresh weight of tissue - Initial fresh weight of tissue}}{\text{Initial fresh weight of tissue}}$$

Unorganized tissues were harvested at their maximum growth indices *i.e.* eighth week. Tissues were dried at 100 ˚C for 15 minutes to inactivate enzymes followed by 60 ˚C till constant weight was achieved. Five such replicates were analyzed for calculating mean value.
Plant parts as well as tissue samples were analyzed separately for their phytosterols content following the procedure of Kaul and Staba (1968) [8].

Table 1: Growth Indices of Static Cultures of *M. Oleifera* at Different Age Intervals

Age of Tissue	2 Weeks	4 Weeks	6 Weeks	8 Weeks	10 Weeks	12 Weeks
Growth index of unorganized tissue	0.92±0.06	1.86±0.08	5.01±0.13	8.34±0.35	8.02±0.27	6.68±0.18

Mean value of five replicates ± SD

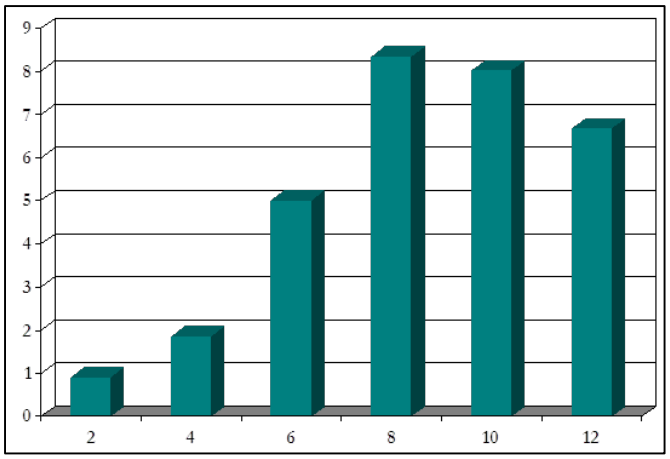

Fig 1: Growth Indices of static cultures of *M. Oleifera* at different age intervals

2.1 Extraction Procedure

Tissue samples were hydrolyzed with 30% (v/v) hydrochloric acid (2 gm/20 ml) for 4 hours on water bath. The hydrolyzed test samples were washed separately with distilled water till the filtrate attained pH 7.0. Test samples so obtained were dried at 60 ˚C for eight hours and soxhlet extracted in benzene (200 ml) for twenty four hours separately. Benzene extracts of various test samples were dried separately *in vacuo* and taken up in chloroform for further analysis.

2.2 Qualitative Analysis

1. Thin Layer Chromatography (TLC)

Each of the crude extract was applied separately on silica gel 'G' coated and activated thin (0.2-0.3 mm) glass plates along with the standard reference samples of sterols (β-sitosterol, campesterol, lanosterol and stigmasterol). The plates were developed in an organic solvent mixture of hexane and acetone (80:20 v/v), air dried, sprayed with 50% sulphuric acid and subsequently heated at 100 ˚C for 10 minutes. Two purple coloured spots coinciding with those of standard samples of β-sitosterol (Rf 0.60) and stigmasterol (Rf 0.64) were observed in all the samples.

2. Preparative Thin Layer Chromatography (PTLC)

Each of the extract along with the standard reference sterols was applied separately on thickly coated (0.3-0.4mm) silica gel 'G' activated glass plates. The plates were developed in an organic solvent mixture of hexane and acetone (80:20). The developed plates were air dried and visualized under UV light. The two fluorescent spots (Rf 0.60 and Rf 0.64) corresponding with those of the standard reference samples of β-sitosterol and stigmasterol respectively were separately marked and collected along with silica gel from unsprayed plates. Each of the mixtures was then eluted with chloroform, elutes dried *in vacuo* and crystallized separately with acetone and methanol.

The crystallized isolates from all the samples tested were subjected to colorimetry (for quantitative estimation), melting point (melting point apparatus Toshniwal, India) and Infra-red spectral (Perkin-Elmer, 337, Grating, Infra-red spectrophotometer, using nujol or potassium bromide pellets) studies along with their respective standard reference sterols.

3. Quantitative Estimation

Quantitative estimation of β-sitosterol and stigmasterol test samples was carried out using the method of Das and Banerjee (1980) [5]. Stock solution of β-sitosterol and stigmasterol in chloroform (500µg/ml) was separately prepared. From this soloution 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 plates. The plates were developed in a solvent system of hexane: acetone (80:20). Such developed chromatograms were air dried and exposed to iodine vapours. Iodine positive spots were marked and heated to evaporate excess of iodine. The spots were scrapped along with silica gel and each was eluted with 5 ml of chloroform in test tubes. Each of the tubes was centrifuged, supernatants were collected, evaporated to dryness and proceeded further. To the dried samples, 3 ml of glacial acetic acid was added and shaken on a vortex mixer at room temperature for 1 minute and then immersed in crushed ice. 2 ml of freshly prepared chromogenic reagent (0.5 ml of 5% anhydrous ferric chloride in glacial acetic acid and 100 ml of concentrated sulphuric acid; Klyne, 1965) was added dropwise at 0 ˚C to the frozen samples and mixed thoroughly. All the reaction mixtures were then incubated at 40 ˚C for 30 minutes and their optical density was determined using a spectronic-20 colorimeter (Bausch and Lomb) set at 540 nm against a blank solution (3 ml glacial acetic acid and 2 ml of chromogenic reagent). For every concentration, five such replicates were run and average optical density was plotted against their respective concentrations to complete a regression curve which followed the Beer's law.

The crude extracts obtained from various plant parts and culture samples were separately dissolved in chloroform and spotted along with reference β-sitosterol and stigmasterol on silica gel 'G' coated and activated glass plates and developed in a solvent system of hexane: acetone (80:20). Two spots coinciding with those of the authentic samples of β-sitosterol and stigmasterol were marked in all the respective samples. Each of these spots were eluted and extracted as described earlier. Elutes were dried, taken up in 5 ml of chloroform and processed further as mentioned above. The concentration of respective sterols present in various tissue samples of *M. oleifera* was calculated (in mg/g.d.w.) by comparing the

optical density of the experimental sample with the regression curves of β-sitosterol and stigmasterol separately. Five such replicates were examined in each case and their mean values were determined.

3. Results

All the plant parts and unorganized tissues of *M. oleifera* showed the presence of β-sitosterol and stigmasterol. As far as the amount of sterols estimated quantitatively in different plant parts of *M. oleifera*.

In *in vivo* studies the amount of β -sitosterol and stigmasterol was maximum in leaflets and minimum in flowers. Total amount of sterols was maximum in leaflets (15.16 mg/100g.d.w.) and minimum in flowers (11.54 mg/100g.d.w).

In *in vitro* studies showed that unorganized tissue contained higher amount of total sterol (15.31 mg/100g.d.w.) than maximum observed in leaflets (15.16 mg/100g.d.w.).

Table 2

Sterols	Plant Parts (*In Vivo*)				*In Vitro*
	Rachis	Leaflets	Flowers	Fruits	Callus (8 weeks)
β-sitosterol	8.13±0.06	9.73±0.07	7.70±0.06	7.87±0.04	β-sitosterol = 9.85±0.06
Stigmasterol	4.17±0.05	5.33±0.05	3.90±0.04	3.93±0.03	Stigmasterol= 5.46±0.04
Total sterol	12.30±0.05	15.16±0.05	11.60±0.05	11.80±0.04	Total sterol = 15.31±0.06

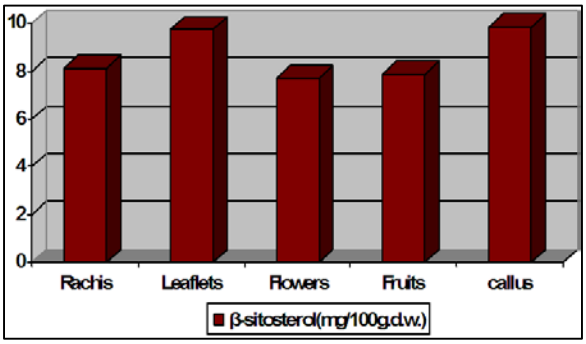

Fig 2

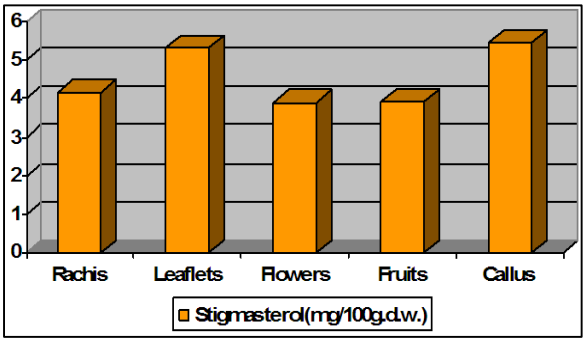

Fig 3

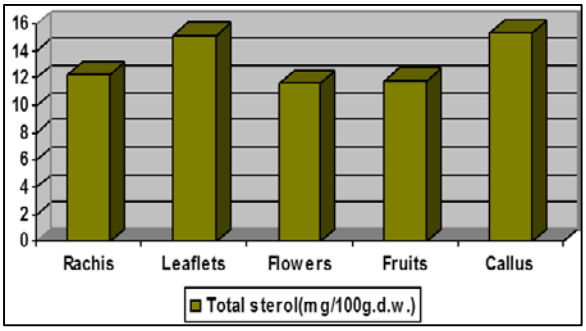

Fig 4

4. Discussion

Moringa oleifera is highly valued plant, with impressive range of medicinal uses and high nutritional value. A plethora of traditional medicine references attest to its curative power, and scientific validation of these popular uses is developing to support at least some of the claims *Moringa* preparations known to have antibiotic, antitrypanosomal, hypotensive, antispasmodic, antiulcer, anti-inflammatory, hypocholesterolemic, and hypoglycemic activities as cited in the scientific literature. Further purification of compounds can be done and the compounds may be subjected for animal studies. Present study showed that phytosterols which are considered as precursors of growth and reproductive hormones are present in sufficient amount in all plant parts as well as tissue cultures of *M. oleifera.* Depending upon the needs amount of sterols can be further increased in tissue culture by supplementing MS medium with various precursors of sterols.

5. Conclusion

Thus it can be concluded that phytosterols are present in sufficient amount in all plant parts as well as tissue cultures of *M. oleifera.* Tissue cultures of *M. oleifera* retain the potential to synthesize steroids in fair amount.

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