

Identification and Qualitative Analysis of β -Sitosterol and Some Phytoestrogens in *In Vivo* and *In-Vitro* Samples of *Lepidium sativum*: A Semi-Arid Bone Healing Plant

Raghunandan S Nathawat, Preeti Mishra* and Vidya Patni

Plant Pathology, Tissue Culture and Biotechnology Laboratory, Department of Botany, University of Rajasthan, Jaipur, India

Abstract

Dry powder of different plant parts of *Lepidium sativum* (Garden Cress) are known to be used for treatment of fracture (bone healing) from ancient times. Seeds as *in vivo* plant part and callus as *in vitro* plant parts were chromatographically tested to identify and estimate β -sitosterol and various phytoestrogens. Plant-derived sterols and estrogens in tissues and oilseeds of *Lepidium sativum* were isolated by solvent extraction method. β -sitosterol, diadzein and formononetin were estimated qualitatively and quantitatively through TLC and HPTLC techniques. *In vitro* grown callus was compared biochemically with *in vivo* plant parts. CAMAG HPTLC was used and the developed plates were photo-documented using UV and white light. The amount of β -sitosterol was estimated to be about 0.20% w/w for seed powder and 0.024% w/w for callus powder. Daidzein and formononetin isolated from the samples of *Lepidium sativum* showed Rf values 0.73 and 0.67 and peak areas were 546.8 and 1033.5, respectively. Chloroform: methanol (8:2) gave excellent results in the present investigation for all samples. Results indicated that applied assay is accurate and reliable for the determination of phytoestrogens in the plant.

Keywords: *Lepidium sativum*; HPTLC; Diadzein; Formononetin

Abbreviations

β : Betasitosterol; Stg: Stigmasterol; Ln: Lanosterol; D: Daidzein; G: Genistein; Fr: Formononetin; Sp: Sprout; C: Callus; S: Seed

Introduction

The plant species *Lepidium sativum* L. belongs to family Brassicaceae (Synonym: *Nasturtium sativum* Medik) is also known as Garden Cress, Pepper Cress in English, Chandrasoora (/ˈtʃɑː.də.səˈrɑː, -ˈrɑː/), Chandrika (/ˈtʃɑː.də.rɪk.ə/) in Sanskrit, and vernacularly as Haim (/hæɪm/), Hin-Chansur (/hɪn ˈtʃɑː.n.sɜːr/) etc. It is cultivated throughout India and Tibet as a culinary vegetable and grows as a wild plant. It is also found distributed in North Africa, Palestine, Syria, Mesopotamia, Iran, ex-USSR, Southern and middle European Part, New Caledonia and in Australia and Indonesia. In Rajasthan (Mewar region, India), this plant has been studied under exploited fodder species [1]. The plant demonstrates weedy habit, grows on waste land and cultivated places. It is an erect, annual herb, up to 50 cm height. The plant extract is bitter, used as tonic, galactagogue, and aphrodisiac, in the treatment of dysentery, pain in abdomen, blood and skin disorders, injuries, tumors, eye diseases, asthma, cough, and bleeding piles. Seeds of *L. sativum* are useful in the treatment of fracture [2] and induce production of breast milk. Garden cress seed oil is rich in omega-3 fatty acid [3]. Seed powder has been proved useful in the treatment of fracture healing [4]. *Lepidium sativum* is one of the herbs mentioned in ancient scriptures of Ayurveda. Anti-osteoporotic effect of the plant has been discussed by many authors [5]. **Sudard** is an herbal formulation containing extracts of 11 medicinal plants including *Lepidium sativum* seeds. **Bio-Trib** is a synergic preparation including *L. sativum* roots-effective, safe and useful for successful treatment of a wide range of male sexual health disorders. **Delentigo** (made up of *Lepidium sativum* sprout extract) is an efficient and targeted solution for reducing age spots. **Flex**, **Laksha gogglu**, **painmukti** etc. are other formulations of *Lepidium*. Phytosterols and phytoestrogens are some important compounds present in higher quantities in this medicinal plant. Phytoestrogens have been suggested as cancer preventatives and as treatments for menopausal symptoms and osteoporosis. Phytoestrogens like Diadzein, formononetin containing diet can be useful for the prevention and treatment of many diseases including

osteoporosis [6]. The widely distributed C-29 sterols are formed from acetate via mevalonic acid pathway by higher plants. Phytosterols have been reported in many plants by several workers [4,7-9].

Separation of phytoestrogens by chromatography is particularly very useful, since it permits their identification, confirmation and/or quantification. The focus of this study was to simplify the extraction procedure and to qualitatively analyze various *in vivo* and *in vitro* parts of *Lepidium sativum* for relative determination of phytoestrogens.

Materials and Method

Seeds of mature plant of *Lepidium sativum* were collected locally from different regions of Rajasthan (India). *In vitro* grown hypocotyls of *Lepidium sativum* were inoculated after several rinses with sterile distilled water on MS medium supplemented with various combinations of growth regulators. Ideal medium for callus establishment through nodal stem segment explant was MS-medium supplemented with NAA (1.0 mg/l) in combination with BAP (5.0 mg/l). Callus tissues thus grown were harvested periodically (2, 4, 6 and 8 weeks), dried and calculated for growth indices separately. Five such replicates of seeds and callus were examined and the mean values were recorded.

Extraction procedure

Plant derived sterols in tissues and oilseeds of *Lepidium sativum* can be isolated by solvent extraction with diethyl ether followed by saponification and chromatographic purification to obtain total sterols.

***Corresponding author:** Preeti Mishra, Plant Pathology, Tissue Culture and Biotechnology Laboratory, Department of Botany, University of Rajasthan, Jaipur, India, Tel: +919414781210; E-mail: drpreetipathak@gmail.com

Received September 17, 2015; **Accepted** October 13, 2015; **Published** October 17, 2015

Citation: Nathawat RS, Mishra P, Patni V (2015) Identification and Qualitative Analysis of β -Sitosterol and Some Phytoestrogens in *In Vivo* and *In-Vitro* Samples of *Lepidium sativum*: A Semi-Arid Bone Healing Plant. Biochem Pharmacol (Los Angel) 4: 191. doi:10.4172/2167-0501.1000191

Copyright: © 2015 Nathawat RS, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

In vitro grown callus tissue and selected plant seeds were striped and milled before they were extracted by diethyl ether in a soxhlet extractor. After removal of solvent using evaporator, 30 ml of extracts were saponified with 9% alcoholic potassium hydroxide for 5 hrs. The resulting solution was then diluted two times by distilled water and extraction procedure followed five times with diethyle ether. The ether solution was dried to obtain unsaponifiable matters of plant seed oils and dried *in vitro* cultured callus. Five replicates were analyzed in each case. Identification of sterols was carried out by TLC, IR spectra, and HPTLC studies. Methanolic extracts of seed powder and callus were used in case of phytoestrogen studies.

Thin layer chromatography

The crude extracts were applied separately on silica gel 'G' coated and activated thin glass plates along with standard reference sample of sterols (β -sitosterol, stigmasterol, lanosterol, cholesterol). The plates were developed in different organic solvent mixtures of Benzene: Ethyl acetate (3:1) and Hexane: Acetone (8:2) [10]. The developed plates were air dried, sprayed with 50 % sulphuric acid or Anisaldehyde reagent and subsequently heated at 100°C for 10 minutes. To quantify phytoestrogens-diadzein, genistein and formononetin, different organic solvents like chloroform: methanol (8:2); chloroform: acetone: formic acid (75:16.5:8.5) were tested. Five to ten replicates were run and average Rf values were calculated for the standard and plant parts' extracts (*in vivo* and *in vitro*).

Preparative thin layer chromatography (PTLC)

The extracts along with standard reference β -sitosterol, stigmasterol, lanosterol, cholesterol were applied separately on thick (0.3 mm to 0.4 mm) silica gel 'G' coated and activated glass plates. The plates were developed in an organic solvent mixture of Hexane: Acetone (8:2). The developed plates were air dried and visualized by spraying 50% sulphuric acid and anisaldehyde reagent to mark the steroidal bands. Each of the mixture was eluted with chloroform, elutes were dried in vacuo, crystallized separately with acetone and methanol. The purified material was subjected for IR spectrum and HPTLC analysis.

HPTLC studies

The TLC spots corresponding to sterols were eluted by methanol (1/1 v/v). The separation was made on HPTLC system equipped with a sample applicator device CAMAG lino mate 5, CAMAG twin through chamber, CAMAG TLC scanner and integration software (Win CATS), stationary phase for the sample was pre washed HPTLC silica gel plates 60 F254, mobile phase for the samples was Hexane: Diethyl Ether (6.5:3.5). The developed bands were analyzed on 520 nm (β -sitosterol) and on 380 nm (Diadzein and formononetin). Peaks of standard sterols were compared with the peaks of *in vitro* callus tissue and with the peaks of *in vivo* plant parts. The plates were photo-documented by using UV and white light.

Results

Qualitative analysis showed the presence of β -sitosterol in all *in vivo* (seeds) and *in vitro* (callus) samples of *Lepidium sativum*. The developed TLC plates showed coloured spots for isolated β -sitosterol of seeds (Rf- 0.62) and callus (Rf-0.63) samples which coincided with that of the reference β -sitosterols (Rf- 0.64, purple). The Rf value of standard β -sitosterol in HPTLC analysis was found to be 0.18 and peak area recorded was 3580.50. Callus and seed extracts of the plant showed Rf value 0.18 and 0.20 respectively; which coincided with standard Rf value and peak area was 176.10 and 1449.1 respectively. The amount of

β -sitosterol was estimated to be about 0.20 % w/w for seed powder and 0.024 % for callus powder (Figure 1A-1D). The 3D spectra of all tracks scanned at 520 nm are shown in (Figure 2).

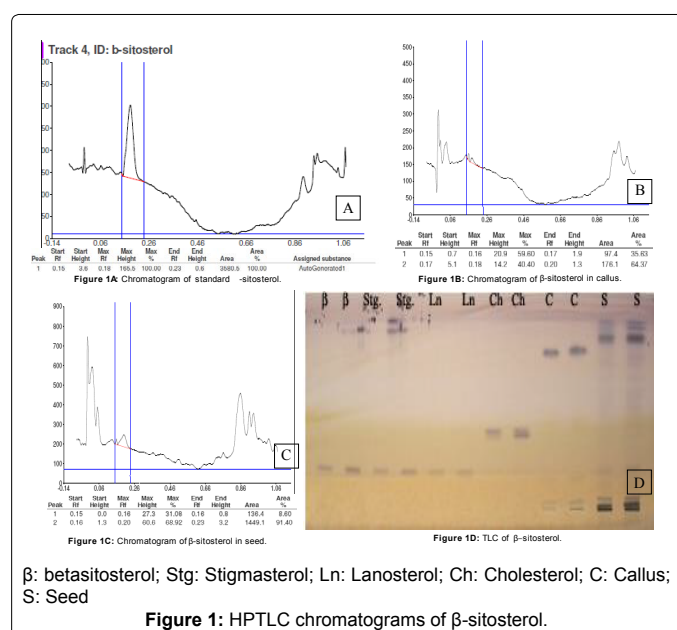
Daidzein and formononetin isolated from the samples of *Lepidium sativum* showed Rf value 0.73 (for seeds) and 0.67 (for callus) and peak area was 546.8 and 1033.5 respectively (Figure 3A-3C) (Figure 4A-4D). Chloroform: methanol (8:2) gave excellent results in the present investigation for all samples. During HPTLC analysis the amount of daidzein was estimated to be about 18.81 % w/w in seed and minimum in callus (9.99 % w/w) by using the HPTLC values. The 3D spectra of all tracks scanned at 380 nm (Figure 5).

Discussion

β -sitosterols have antifungal, antibacterial and anti-inflammatory activity [11,12]. In the present investigation Chloroform: methanol (8:2) combination showed best results and exhibited coherence with the results of [13,14]. HPTLC technique with other biochemical assays supplemented evidence to confirm the presence of some bioactive compounds, which have provided the plants an important position in folkloric usage [15] In the present study, quantitative and qualitative biochemical methods can be employed as a stability indicator [16] that can be used for the routine quality control analysis and quantitative determination of β -sitosterol from *Lepidium sativum*. Results obtained by HPTLC for diadzein and formononetin were coincided with the results of [17-19]. Although originally identified in cementum, PTPLA/CAP is very effective at inducing bone repair and healing and therefore this novel molecule has a great potential to be used for mineralized tissue bioengineering and tissue regeneration studied by [20]. *Lepidium sativum* seeds have been used in traditional folk medicine to heal fractured bones especially in cases of glucocorticoids induced osteoporosis [21,22]. Results thus obtained provided a chromatographic fingerprint of specific phytochemicals and are suitable for identify and purify raw materials from such medicinal plants.

Conclusion

These results indicate that, the assay is accurate and reliable for the determination of phytoestrogens in *Lepidium sativum*. This technique



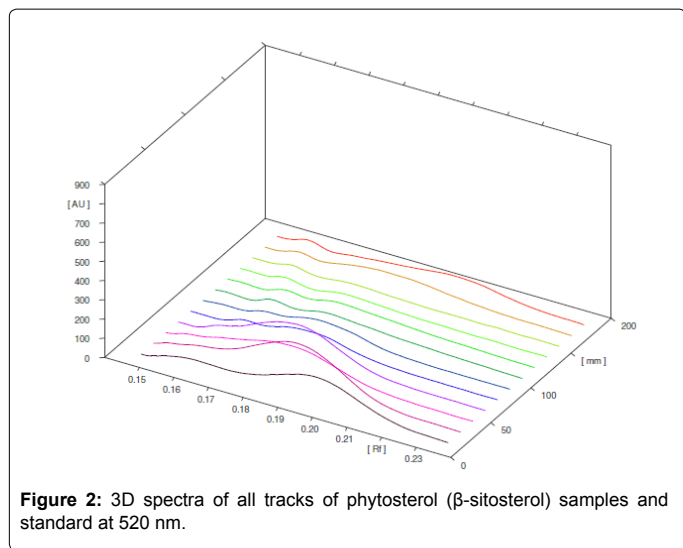


Figure 2: 3D spectra of all tracks of phytosterol (β -sitosterol) samples and standard at 520 nm.

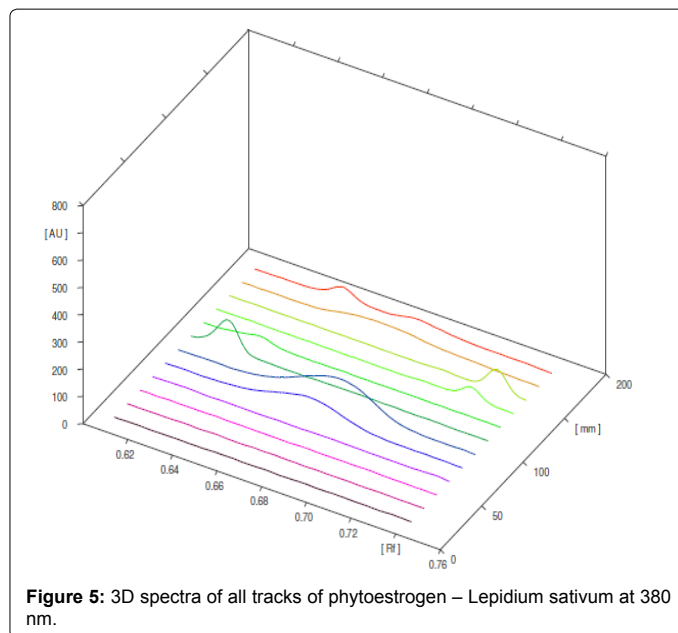


Figure 5: 3D spectra of all tracks of phytoestrogen – *Lepidium sativum* at 380 nm.

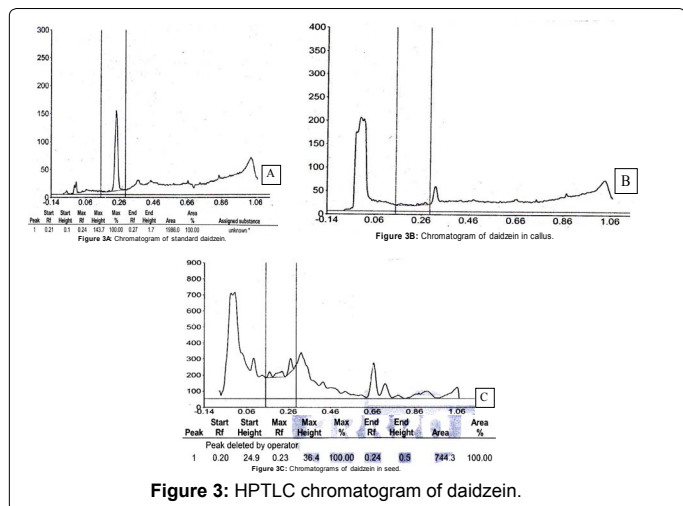
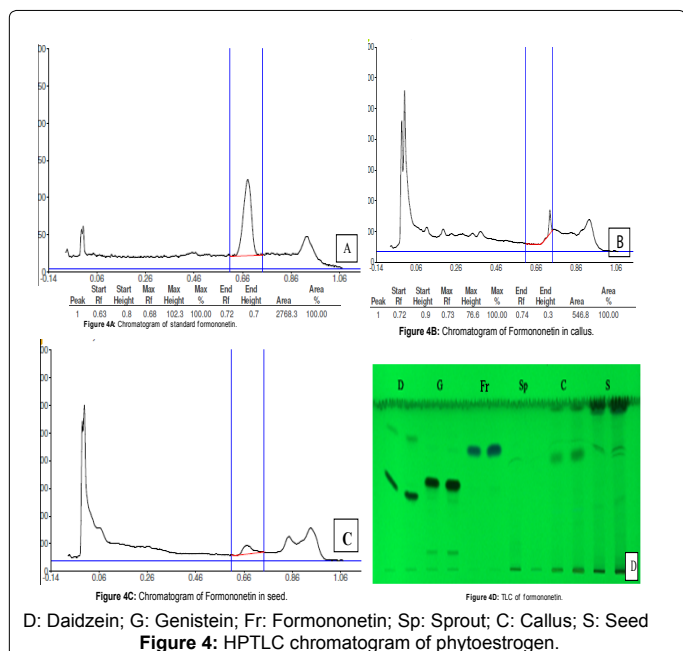


Figure 3: HPTLC chromatogram of daidzein.



D: Daidzein; G: Genistein; Fr: Formononetin; Sp: Sprout; C: Callus; S: Seed
Figure 4: HPTLC chromatogram of phytoestrogen.

greatly simplifies the analysis of phytoestrogens. The HPTLC method provided a quick and easy approach for detection and quantitation of biomarker β -sitosterol in *Lepidium sativum*.

Acknowledgements

We are thankful to Rajasthan State Department of Science and Technology for the financial support to complete the research project, the part of which is presented here.

References

- Gokavi SS, Malleshi NG, Guo M (2004) Chemical composition of garden cress (*Lepidium sativum*) seeds and its fractions and use of bran as a functional ingredient. *Plant Foods Hum Nutr* 59: 105-111.
- Krishnaraju AV, Rao TVN, Dodda S, Vanisree M, Tsay HS, et al. (2005) Assessment of bioactivity of Indian medicinal plant using brin shrimp (*Artemia salina*) lethality assay. *Int. J. Appl. Sci Eng* 3: 125-134.
- Umesha SS, Manohar RS, Indiramma AR, Naidu AK (2015) Enrichment of biscuits with microencapsulated omega-3 fatty acid (Alpha-linolenic acid) rich Garden cress (*Lepidium sativum*) seed oil: Physical, sensory and storage quality characteristics of biscuits. *LWT- Food Sci. Tech* 62: 654-661.
- MilovanoviÄ M, Banjac N, RadoviÄ BV (2009) Functional food: rare herbs, seeds and vegetable oils as sources of flavors and phytosterols. *J Agri Sci* 54: 80-93.
- Juma Ab (2007) The effects of *Lepidium sativum* seeds on fracture-induced healing in rabbits. *MedGenMed* 9: 23.
- Sirotkin AV, Harrath AH (2014) Phytoestrogens and their effects. *Eur J Pharmacol* 741: 230-236.
- Shirwaikar A, Khan S, Kamariya YH, Patel BD, Gajera FP, et al. (2010) Medicinal plants for the management of post-menopausal osteoporosis: A review. *The Open Bone J* 2: 1-13.
- Ye JC, Chang WC, Hsieh DJY, Hsiao MW (2010) Extraction and analysis of β -sitosterol in herbal medicines. *J Med Plants Res* 7: 522-527.
- Shareef H, Rizwani GF, Haq MH, Ahmad S, Zahid H, et al. (2012) Tocopherol and phytosterol profile of *Sesbania grandiflora* (Linn.) seed oil. *J Med Plants Res* 6: 3478-3481.
- Senthilmanickam J, Bhavani AL, Venkatramlingam K, Chandra G (2012) The role of 2, 4-D and NAA in callus induction of *Achyranthes aspera* and its secondary metabolite studies. *Jamonline (J. Atoms Mol.)* 2: 232-243.
- Sathishkumar T, Baskar R, Rajeshkumar M (2012) In vitro antibacterial and antifungal activities of *Tabernaemontana heyneana* Wall. Leaves. *J Appl. Pharm. Sci* 2: 107-111.

12. Jasuja ND, Choudhary J, Sharama P, Sharma N, Joshi SC, et al. (2012) A review on bioactive compounds and medicinal uses of *Commiphora mukul*. *J. Plant Sci* 7: 113-137.
13. Liew R, Macleod KT, Collins P (2003) Novel stimulatory actions of the phytoestrogen genistein: Effects on the gain of cardiac excitation-contraction coupling. *FASEB J* 17: 1307-1309.
14. Ercetin TG, Toker M Kartal, Colgecen H, Toker MC (2012) *In vitro* isoflavonoid production and analysis in natural tetraploid *Trifolium pratense* (red clover) calluses. *Rev Bras Farmacogn* 22: 964-970.
15. Mariswamy Y, Gnaraj E, Antonisamy JM (2011) Chromatographic fingerprint analysis on flavonoids constituents of the medicinally important plant *Aerva lenata* L. by HPTLC technique. *Asia-Pac. J. Trop. Biomed* S8-S12.
16. Genetea G, Hymete A, Bekhi AAA (2012) Development and validation of HPTLC assay method for simultaneous quantification of hydrocortisone and clotrimazole in cream and applying for stability indicating test. *J chil chem Soc* 57: 1199-1203.
17. Fang C, Wan X, Jiang C, Cao H (2005) Comparison of HPTLC and HPLC for determination of isoflavonoids in several kudzu samples. *J. Plant Chromatogr* 18: 73-77.
18. Shirke SS, Jadhav SR, Jagtap AG (2008) Methanolic extract of *Cuminum cyminum* inhibits ovariectomy-induced bone loss in rats. *Exp Biol Med (Maywood)* 233: 1403-1410.
19. Rakesh SU, Patil PR, Salunkhe VR, Dhale PN, Burade KB (2009) HPTLC method for quantitative determination of quercetin in hydroalcoholic extract of dried flower of *Nymphaea stellata* willd. *Inter J Chem Tech Res* 1: 931-936.
20. Montoya G, Arenas J, Romo E, Zeichner-David M, Alvarez M, et al. (2014) Human recombinant cementum attachment protein (hrPTPLa/CAP) promotes hydroxyapatite crystal formation *in vitro* and bone healing *in vivo*. *Bone* 69: 154-64.
21. Elshal MF, Almalki AL, Hussein HK, Khan JA (2013) Synergistic antiosteoporotic effect of *Lepidium sativum* and alendronate in glucocorticoid-induced osteoporosis in Wistar rats. *Afr J Tradit Complement Altern Med* 10: 267-73.
22. Wu YB, Zheng CJ, Qin LP, Sun LN, Han T, et al. (2009) Antiosteoporotic activity of anthraquinones from *Morinda officinalis* on osteoblasts and osteoclasts. *Molecules* 14: 573-583.