

Chemical evaluation of dietary herbal formulations consumed by pregnant women for sex selection of offspring

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RESEARCH ARTICLE

Abstract

Consumption of traditional indigenous preparations (IPs) comprising of herbal ingredients has been a dietary practice during pregnancy. In India, a similar practice for begetting a male child has gained popularity with time that reportedly contains phytoestrogen. In view of the potential harms caused, such as still births and congenital malformations and lack of genuine scientific evaluation of such IPs, the prevalent bioactives in them and their constituents collected from diverse geographical locations in India were evaluated for the chemical identities. The IPs were evaluated qualitatively for selected phytoestrogens (genistein, daidzein, formononetin and biochanin A), using thin layer chromatography (TLC) and reverse phase – high performance liquid chromatography (RP-HPLC) and quantified in case of positive presence. Presence of steroids was also checked through Liebermann-Burchard test. Of the 48 IPs collected from different Indian states, comprising mainly of four ingredients Shivalingi (*Bryonia laciniosa*), Majuphal (*Quercus infectoria*), Putrajeevak (*Putranjiva roxburghii*) and Nagkesar (*Mesua ferrea*), TLC chromatograms revealed the presence of phytoestrogens in more than half of the samples. Thirteen samples as well as the major ingredients of IPs exhibited peaks upon HPLC analysis with matching retention time; however their UV absorbance pattern did not match, possibly caused by $\lambda_{\max}$ shift due to combinatorial effect. The characteristic UV maxima 250-260 nm, 270-280 nm and 310-330 nm, indicate that IPs contain phenolic compounds (flavones and isoflavones) which was revealed through the fingerprint chromatograms. Shivalingi was found to contain daidzein and genistein. Two thirds of the samples showed presence of steroids. Most of the IPs used for sex selection and their major ingredients contained phytoestrogen and steroids. Their consumption during pregnancy could be of importance in terms of safety to both mother and fetus.

Keywords: indigenous dietary preparations, chemical analysis, phytoestrogens, steroids, sex selection

1. Introduction

Sex ratio is a social indicator to measure the extent of prevailing gender inequality. A skewed sex ratio in favour of males has been observed in India since decades. The conventional wisdom has always been that couples would prefer sons given a choice (Warade *et al.*, 2014). Various means adopted to have a male child include multiple pregnancies until the birth of a son, sex selective abortions, pre-conceptional techniques and post-conceptional intake

of herbal dietary formulations to beget a male child (Madan and Breuning, 2014). Herbal remedies are not usually recommended by medical professionals for pregnant women since unlike prescription drugs, natural herbs do not go through the same evaluation process by the FDA. Therefore, the quality and strength of herbal dietary supplements can vary between two batches of the same product and also between products from different manufacturers. Unwarranted use may lead to miscarriage, premature birth, uterine contractions, or injury to the fetus.

Intake of such formulations during post-conception includes oral contraceptive pills, progesterone analogues to confirm pregnancy, medications for medical ailments and 'sex selection' drugs (SSD) to bear male offsprings (Bandyopadhyay and Singh, 2007; Bhagat *et al.*, 2012; Manchanda *et al.*, 2011). SSDs are reported to be indigenous preparations (IPs) containing both herbal and non-herbal ingredients, directed to be consumed during the second month of pregnancy. Consumption of such preparations is prevalent in many parts of the country (Bhagat *et al.*, 2012; Manchanda *et al.*, 2011). The extent of their use varies from 7-46% as reported by different studies (Bhagat *et al.*, 2012; Neogi *et al.*, 2015a,b; Singh and Sinha, 2016). Existing studies have indicated that this could lead to birth defects including major malformations of internal organs like urogenital and renal malformation, trachea-oesophageal fistula, and visible defects like spina bifida, cleft lip/palate and imperforate anus (Neogi *et al.*, 2015a,b, 2016). Previous studies have reported that IP consumed during pregnancy for begetting a male child, contain elevated levels of phytoestrogens (isoflavones); presence of steroids in many of these were also postulated (Bandyopadhyay and Singh, 2007; Neogi *et al.*, 2015b).

Isoflavone phytoestrogens are plant-derived compounds that structurally or functionally mimic mammalian oestrogens and therefore they are considered to play an important role in the prevention of cancers, heart disease, menopausal symptoms and osteoporosis (Caserta *et al.*, 2008). But, they are also incriminated as potential endocrine disrupter chemicals, and therefore caution, in terms of quantity per day, should be exercised when taking them (Patil, 2014). It has been established that exposure to potent oestrogens *in utero* can have long-term adverse effects on human development and fertility in males and, more severely in female off springs (Bakker, 2004; Hughes, 2003), besides influencing the sex of the growing foetus.

Though preliminary results are available, a systematic chemical analysis of these samples has not been carried out thus far. Since SSDs are consumed during the most crucial period of embryo development, it is essential to probe further into the biochemical details of these preparations consumed. We envisaged that our initial investigations should be aimed in revealing the isoflavones, if any. The purpose of our study was to carry out chemical analyses in order to characterise the presence of and quantitate select phytoestrogens which have the possibility of causing a deleterious effect on the development of the embryo, and characterise other compounds that may fall under the same class. High levels of isoflavones are present in several herbal antifertility compounds (Kumar *et al.*, 2012), some of which are constituents of the IPs. Therefore, besides formulated IPs, we deemed pertinent to include the most common ingredients- Shivalingi (*Bryonia laciniosa*), Majuphal (*Quercus infectoria*), Putrajeevak (*Putranjiva roxburghii*),

Nagkesar (*Mesua ferrea*) (Neogi *et al.*, 2015a,b) used in preparing the IPs in the analysis. An approach to have an overall fingerprint of such herbal dietary formulations apart from the specific compounds like phytoestrogens was also attempted in view of some active principles that may still be unknown. The purpose was to identify three samples that could be subjected to toxicity studies in *in vitro* and *in vivo* studies.

2. Methods

Collection of indigenous preparations

Herbal dietary formulations intended for consumption during pregnancy or IPs for sex selection and their major ingredients – *Shivalingi*, *Majuphal*, *Putrajeevak* and *Nagkesar* were collected from different states located in northern part of India through snow balling technique. Forty eight samples were collected from different states in India; 27 from Punjab, 4 from Haryana, Uttar Pradesh and Chandigarh each, 1 from Madhya Pradesh and Chhatisgarh each, 2 from Delhi and 5 from Uttarakhand. Women who had recently delivered and different genres of people like '*pansari*' shop owners (grocers), local ayurvedic practitioners, rickshaw pullers, drivers and commoners were contacted to initiate the process of enquiry. Confidentiality of information was maintained throughout the process of collection and analysis. The collected samples were catalogued properly, stored in airtight containers then and later in desiccators in the laboratory, in dark at 28 °C. Out of 48 IP samples that were collected, categorisation into 18 groups was made out on the basis of redundancy of collected preparations and common ingredients reported. These samples were coded as IM 1-18 during the analysis.

Chemical analysis of indigenous preparations

All collected samples were tested for the presence of phytoestrogens, particularly for isoflavones and steroids. These were specifically chosen based on their propensity to affect the development of the embryo, from previous studies (Adams, 1995; Barnes, 2003; Batterham *et al.*, 1965; Cheng *et al.*, 1954; Hughes, 2003).

Solvents and standards

Pure phytoestrogen standards of four isoflavones (daidzein, genistein, formononetin and biochanin A) were purchased from Sigma Aldrich (St Louis, MO, USA). Analytical reagents used were of high-performance liquid chromatography (HPLC) grade and were purchased from Merck (Darmstadt, Germany).

The HPLC determinations were performed with Shimadzu Nexera instrument consisting of a binary pump LC-20 AD XR, PDA detector SPD-M20 A, a column oven CTO-10AS

VP and an autosampler Nexera XR SIL-20 AC. The system controlling and data acquisition were performed through LC-solution software (Shimadzu, Kyoto, Japan).

Sample preparation

Two separate extraction methodologies were employed. A simple methanolic extraction was carried out for fingerprinting analysis and an exclusive isoflavone detection extraction for identification and quantification of phytoestrogens.

- **Isoflavone extraction.** Extraction of finely grounded 1 gm of each 18 IP sample and seeds of the four major ingredients was carried out at 55 °C in an ultrasonic bath with a mixture of methanol and 2N HCl (80:20 v/v) as solvent for 1 hour and repeated thrice. This extract was then cooled and partitioned with diethyl ether thrice. The organic layer was separated out each time and concentrated. The dry extract was weighed (100 mg) and 1 ml of HPLC MeOH (methanol) was added for further analysis. The protocol was confirmed before by carrying out the extraction of isoflavones from soy seeds. The HPLC results had positive peaks for daidzein and genistein (Figure 1). Therefore, the protocol was adopted for extraction from IPs and the major ingredients (Beck, 1964; Carrao-Panizzi *et al.*, 2002; Lee *et al.*, 2004; Murphy *et al.*, 2009; Neogi *et al.*, 2015b; Orhan *et al.*, 2007).
- **Methanolic extraction.** Extraction of finely grounded 100 mg of each 18 IP sample and seeds of the four major ingredients was carried out at 55 °C in an ultrasonic bath with 5 ml methanol for half an hour and repeated three times. The extract was then cooled and concentrated (Shanker *et al.*, 2007). The dry extract was weighed (100 mg) and 1 ml of HPLC MeOH was added for further analysis.

Thin layer chromatography (TLC): qualitative test for presence of phytoestrogens

Hexane:ethyl acetate (5:5, v/v) was observed to be the best resolving solvent system on silica gel plates. All phytoestrogen standards (Sigma Aldrich) could be visualised as blue-white fluorescent spots under UV light (254 nm). Visualising agent used was vanillin-sulphuric acid reagent. The experiment was repeated at least thrice to confirm the initial authentication.

Reverse phase-high pressure liquid chromatography: final identification and quantitative test for presence of phytoestrogens

- **Isoflavone detection.** The HPLC analyses were performed using a reverse phase column Zorbax 2.1 mm × 100 mm, 3.5 µm (Agilent, Blacksburg, VA, USA). Detector used was UV-VIS (ultraviolet-visible) variable wavelength detector while analysis was carried out at 260 nm and 380 nm. Mobile phases used were 0.1% H₃PO₄ in distilled water and 0.1% H₃PO₄ in ACN (acetonitrile). Separation was achieved by using a gradient system, programmed to increase from 20% to 80% of 0.1% H₃PO₄ ACN for first 25 minutes and then decrease from 80 to 20% of 0.1% H₃PO₄ ACN in last five minutes and finally saturating the column with initial conditions for ten minutes at a flow rate of 0.5 ml/minute. Injection volume was set to 10 µl with a column temperature of 35 °C (David and Huber, 2001).
- **Fingerprinting of presumptive samples.** The HPLC analyses were performed using a reverse phase column Symmetry C18 4.6 mm × 250 mm, 5 µm (Waters, Milford, MA, USA). Mobile phases used were distilled water and ACN. A flow rate of 1.0 ml/min was maintained throughout the run. Injection volume used was 20 µl. Detection was done at 260 nm. Separation was achieved by using a gradient system programmed as 0 min 5% B, 10 min 15% B, 35 min 75% B, 50 min 85% B, 55 min

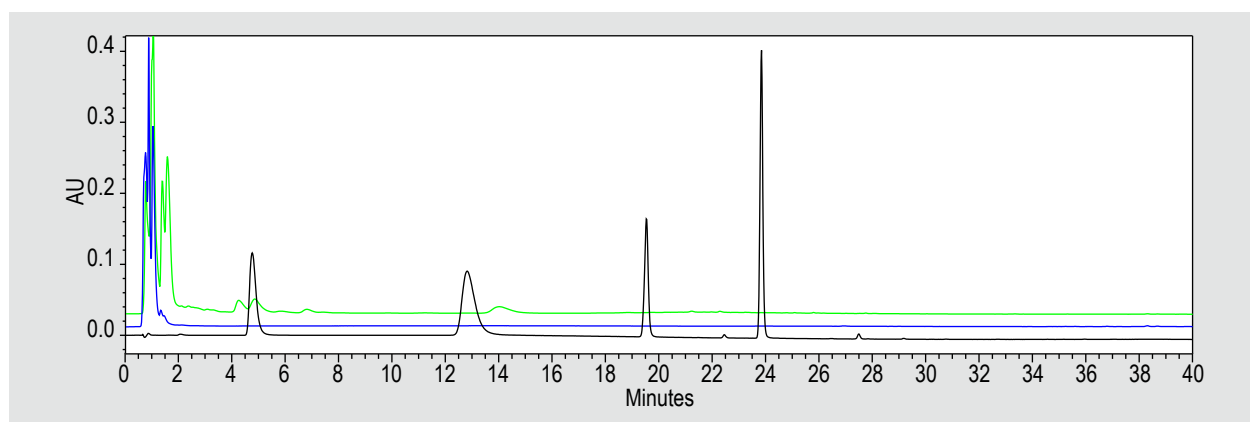


Figure 1. Methanol (100%) extraction (green) and ethanol (70%) extraction (blue) of isoflavones from soy seeds with acid digestion at 260 nm compared with standards mix (black).

95% B, 60 min 5% B and finally holding it for the last 5 minutes to again saturate the column with initial conditions (Shanker *et al.*, 2007).

Liebermann-Burchard test: primary test for detection of steroids

Few drops of acetic anhydride and 1 ml of concentrated H_2SO_4 (sulphuric acid) were added to the methanolic extract of each IP sample in a test tube and a formation of reddish brown ring at the layer junction confirmed the presence of steroids (Burke *et al.*, 1974; Campbell and Shawn, 2005).

The TLC results obtained for the IP samples and their major ingredients were confirmed by HPLC analysis. The samples with the peaks with retention time (RT) same as that of the standards, were confirmed by matching their UV pattern. Quantitation from HPLC peak area indicated the approximate quantities of each phytoestrogen. The results have been presented as the proportion of samples detected positive for phytoestrogens. Samples that were found strongly positive for the presence of concerned compounds were selected for *in vivo* studies. Quantification of steroids was however, not done.

Ethical considerations

This study was a subset of a larger study funded by the Department of Science and Technology under the Science for Equity Empowerment and Development division, for which ethics clearance was already sought from Indian Institute of Public Health Institutional Ethics Committee. Standard and good laboratory practices were adhered to during conduct of the study.

3. Results

Eighteen subgroups of a total of 48 IPs, used for sex selection and consumed by pregnant women, were made based on the redundancy of material, mostly similar ingredients and texture. Four major ingredients of such IP, Shivalingi, Majuphal, Putrajeevak and Nagkesar were also included in the analysis separately.

The presence of selected plant isoflavones i.e. daidzein, genistein, formononetin and biochanin A was explored using TLC, which presumptively showed the presence of phytoestrogens in more than half of the samples. It indicated the presence of daidzein in IM1, IM2, IM6, IM8, IM9, IM10, IM12, IM14 (8 out of 18), Shivalingi, Majuphal and Nagkesar (3 out of 4), genistein in IM4, IM7, IM9, IM10, IM13 & IM17 (6 out of 18) and formononetin in IM4, IM11 (2 out of 18) and Majuphal (1 out of 4) (Figure 2). Though retardation factor (Rf) of respective standards matched

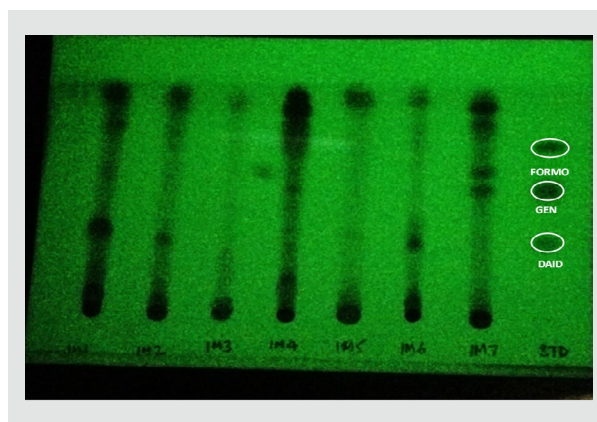


Figure 2. Thin layer chromatography spots of phytoestrogen standards mixture on the extreme right and isoflavonic compounds in indigenous preparations samples on the left as observed under UV light.

in the samples yet in order to confirm the findings, HPLC analysis was carried out.

The samples were analyzed using previously reported HPLC procedure. A representative HPLC chromatogram with peaks of the four selected isoflavone standards and their respective UV absorbance pattern is depicted in Figure 3 and 4 respectively.

The HPLC analysis of samples prepared by isoflavones extraction method indicated the presence of isoflavones but not the select phytoestrogen standards in particular. However, in order to investigate the presence of other phytochemicals, these samples were further analyzed for their fingerprint development (Figure 5). The fingerprinting analysis indicated that out of the 18 IP samples and the four major ingredients, many samples had common pattern of peaks with characteristic UV-Vis spectrum. These common peaks (encircled in the chromatogram) and their characteristic UV-Vis spectra (inset) are provided in the Figure 5. Shivalingi exhibited confirmatory presence of daidzein and genistein (Figure 6).

Though the other samples did not have the presence of the select standard isoflavones, yet they had one or more major ingredients common in all samples. Secondly, the characteristic UV maxima 250-260 nm and 270-280 nm and in some samples 310-330 nm, further indicate that the IP samples and other major ingredients having presence of phenolic compounds qualitatively belong to flavones and isoflavones, since the flavones class of compounds produces 3 maxima lying at 254, 280 and 320 nm while isoflavones produce intense absorption at 250-260 nm.

Of the 18 IP samples analysed for characterisation through fingerprinting, 13 exhibited strong identities to phytoestrogens Shivalingi showed the presence of the

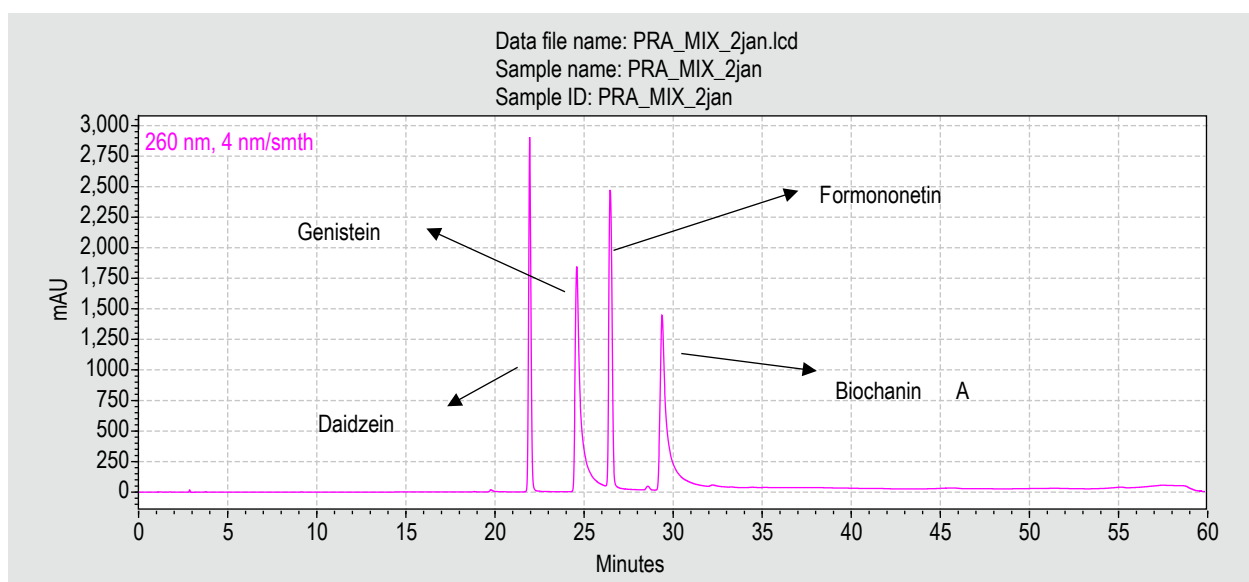


Figure 3. High performance liquid chromatography chromatogram with peaks of the four selected isoflavone standards.

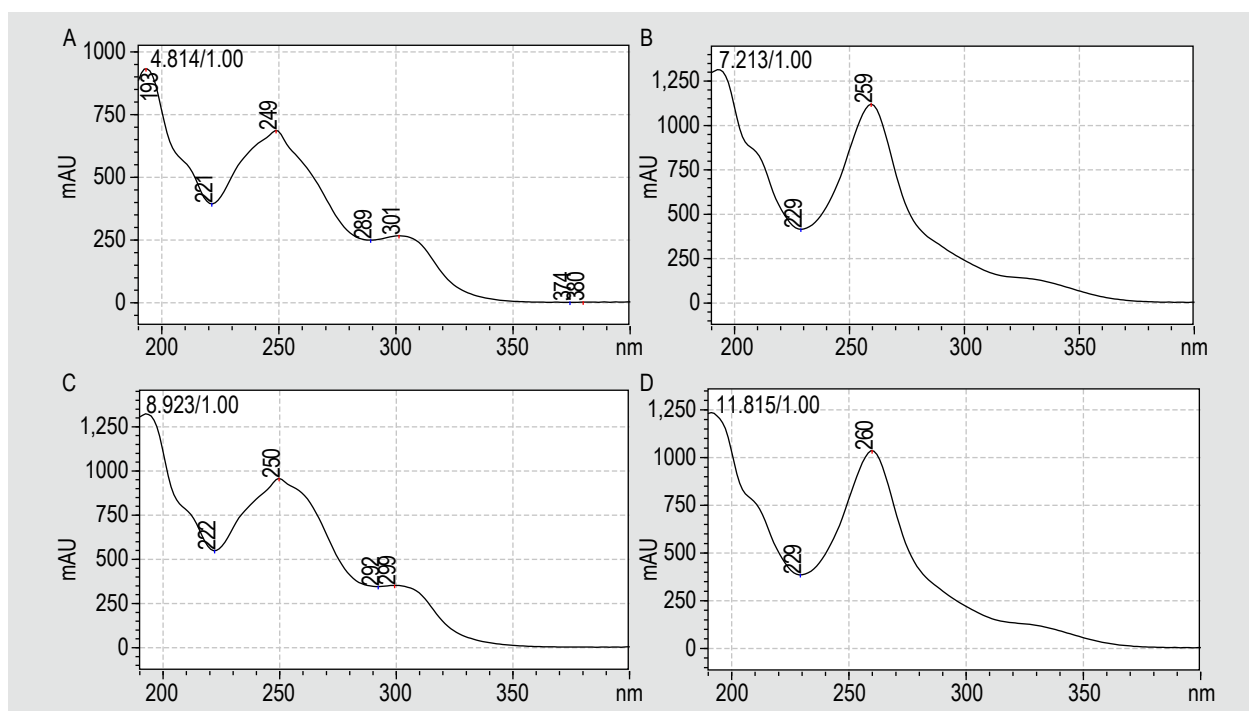


Figure 4. UV absorbance pattern of (A) daidzein, (B) genistein, (C) formononetin and (D) biochanin A.

two select standard phytoestrogens used, daidzein and genistein. The area % calculated for daidzein and genistein present in Shivalingi fingerprinting extract were 3.09 and 1.25 respectively. However, the quantitative estimation of other flavones and isoflavones present in the SSDs could not be determined.

Of the 18 samples analyzed for preliminary detection of steroids, 12 showed presence of steroids (Figure 7).

Shivalingi and Putrajeevak seeds also gave positive results for steroids. (Figure 8).

Based on the analysis, we identified three samples that were found to be most rich in flavones and isoflavones (based on the abundance of peaks in HPLC) and steroids and these were considered further for toxicity study in an alternate animal model (*Caenorhabditis elegans*).

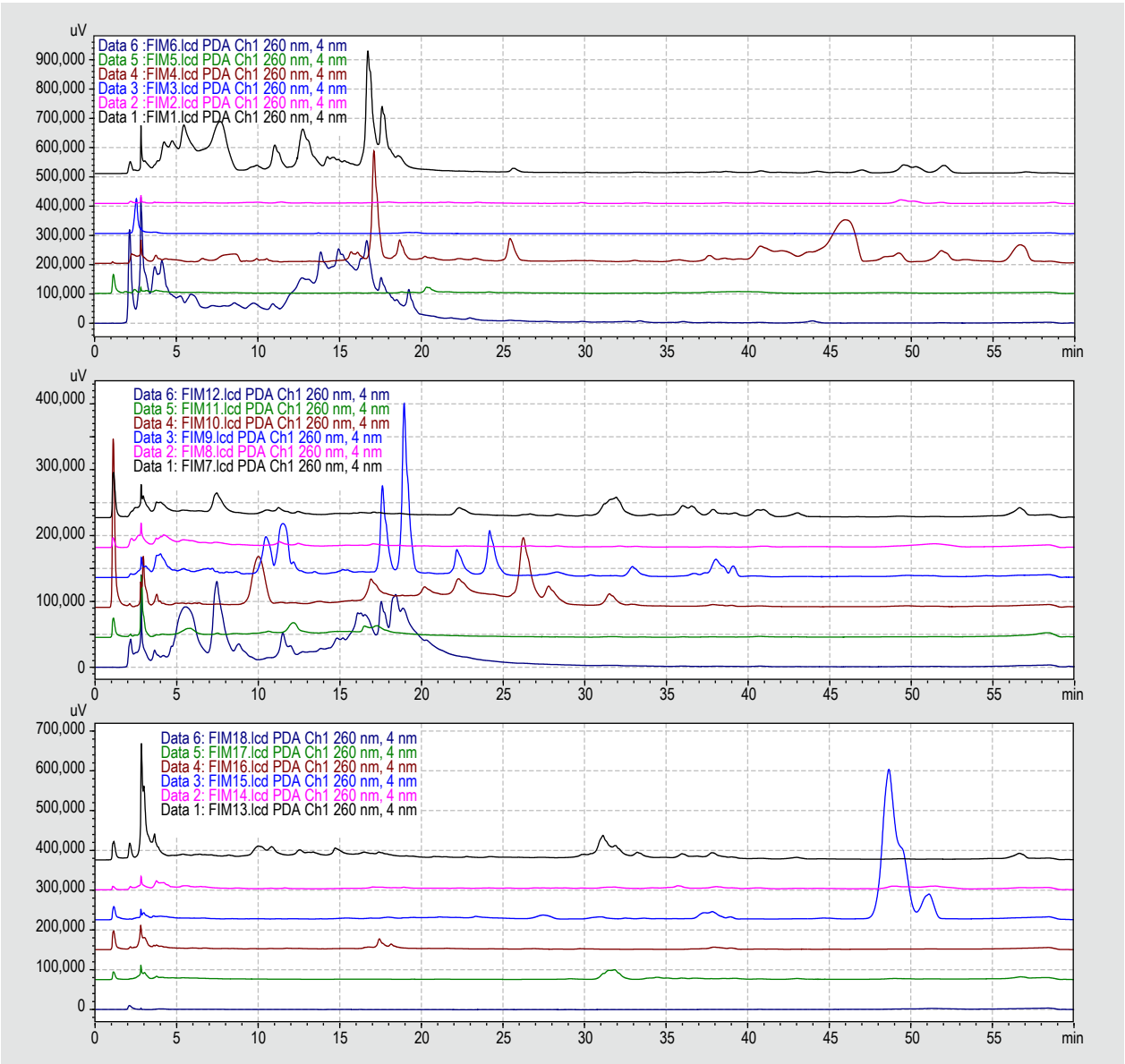


Figure 5. Comparative fingerprinting chromatograms of all the 18 samples, peaks with common retention time and UV absorbance pattern highlighted in red.

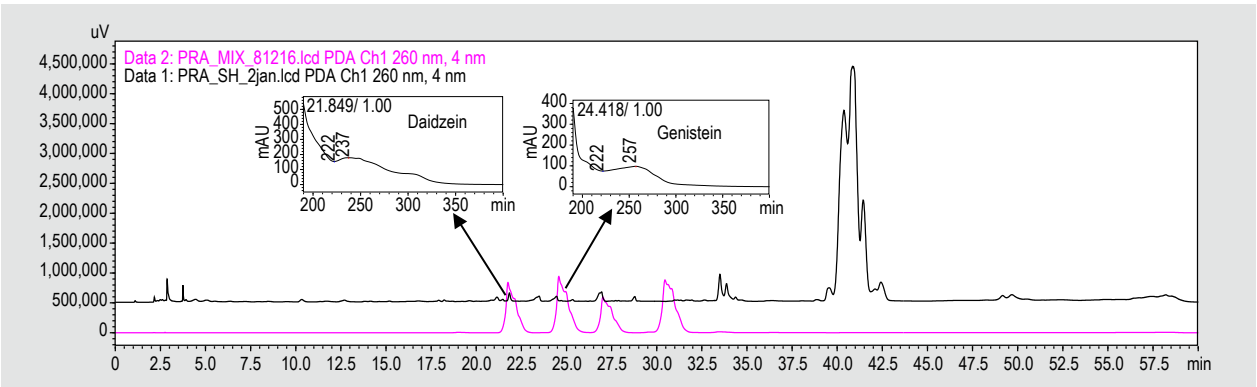


Figure 6. Comparative high performance liquid chromatography chromatogram of *Bryonia* (Shivalingi) extract and phytoestrogen standard mixture.



Figure 7. Positive Liebermann-Burchard test (brown ring formation) found in preliminary detection of steroid for an indigenous preparations sample.

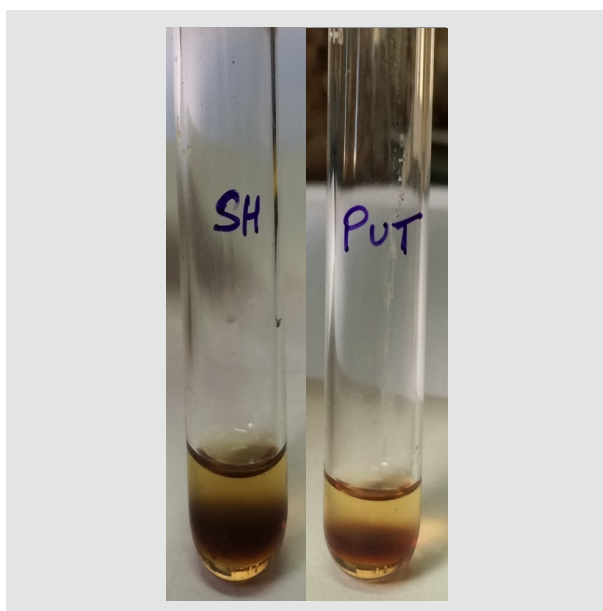


Figure 8. Positive Liebermann-Burchard test (brown ring formation) found in preliminary detection of steroid for Shivalingi and Putrajeevak seeds.

4. Discussion

The results of the present study indicated that 13 out of 18 IP samples (72%) along with all the major ingredients analysed composed of isoflavones. Of the four ingredients, Shivalingi showed the presence of daidzein and genistein. Around two thirds (12) of the 18 (67%) IPs, and Shivalingi and Putrajeevak seeds among the ingredients, contained steroids. These observations are in line with two previous studies where similar findings were reported i.e. high isoflavone concentrations (Jain, 2014; Sandhu, 2010).

The association of phytoestrogens in diet, especially during pregnancy has profound adverse effects on mammalian development and fertility, these observations have been reported in animals consuming phytoestrogen-rich plants (clover) and developing abnormal plasma concentrations of endogenous hormones with subsequent loss of fertility, termed as 'Clover Disease' (Bennett *et al.*, 1946; Moersch *et al.*, 1967; Obst and Seamark, 1975; Prasanth *et al.*, 2016). A study indicated that high concentrations of daidzein and genistein in commercial feed, together with the limited ability of captive female cheetahs to deactivate these compounds metabolically, resulted in veno-occlusive disease and reproductive failure. Fertility problems in the females of mammalian species such as the sheep, cow, guinea pig, rabbit and mouse have also been reported (Adams, 1995; Batterham *et al.*, 1965; Cheng *et al.*, 1954). These effects raised concerns that dietary phytoestrogens might have similar effects on fertility and development in other species including humans.

It was important to analyse the IPs since these are used for several other purposes apart from sex selection. All the four ingredients present in the IPs are among the herbs well studied and reported for their use in reproductive conditions. Shivalingi is used in Ayurvedic medicine traditionally as a sexuality enhancer and is sometimes recommended to preserve youth during aging (perhaps as a testament to its aphrodisiac effects) (Patel, 2012). The galls of Majuphal are used as a home remedy for sore throat and chronic diarrhoea in both rural and urban areas since ages. It is especially recommended for female reproductive disorders like extra growths and prolapsed uterus and vaginal tightening (Dhiman, 2006). Putrajeevak plant is reported to be cooling, pungent, aphrodisiac, laxative, diuretic and prevents abortion. The seeds are known to strengthen the uterine musculature and help a woman get pregnant (Abhimayu *et al.*, 2015; Singh, 2017). Nagkesar offers compounds which are haemostatic and anti-inflammatory in nature and thus its extract is used for treating blood disorders such as epistaxis, menorrhagia, piles, and metrorrhagia. It helps in reproductive issues as well since it exhibits some aphrodisiac properties (Sharma, 2017).

Experimental animal studies have demonstrated *in utero* phytoestrogens exposure to cause potential adverse effects on male offspring development, and more severely pronounced, in female offspring (Hughes, 2003). A critical review of the available studies on effects of phytoestrogens indicates that an oral isoflavone dose of 50 mg/d (± 0.7 mg/kg bw/d) should be considered safe in most population groups (Barnes, 2003).

In contrast to the dietary intake of phytoestrogens recommended, an earlier qualitative and quantitative analysis study conducted on similar 'sex selection'

formulations reported to be strongly positive (63%) for most isoflavone phytoestrogens (Neogi *et al.*, 2015a). Moreover, the total quantity consumed by the pregnant woman represented many-fold increase over that recommended for dietary intake (Neogi *et al.*, 2015a; Jain, 2014). In the same study as reported by the authors, the samples revealed the presence of daidzein and genistein (Neogi *et al.*, 2015a). Confirmation was based on a comparison of the RTs of the peaks in the sample extracts and the standards of genistein, daidzein and formononetin.

In the current study, UV spectroscopy analysis was also performed. Both RT of the peaks as well as UV absorbance pattern were analysed. As it appeared that many samples had peaks at the same RT as that of the selected standards, but their respective UV absorbance pattern did not match. Due to this, those peaks could only be categorised as phenolic compounds but not particularly the four selected ones. This may be attributed to the shift in the λ_{max} of the flavonoid bands possibly due to interaction/ interference with the other phenolic groups present causing deprotonation of OH groups (Alam *et al.*, 2017). In other words, the shift in the UV pattern may be attributed to the complex nature of matrix, resulting in the alteration of absorption maxima. It has been reported that stability of herbal preparations show a wide variation; herbal products are often prone to deterioration, especially during storage, leading to loss of active component, production of metabolites with no activity and, in extreme cases, production of toxic metabolites (Thakur *et al.*, 2011).

In our previous study, the analysis of IPs was carried out immediately following their collection since the samples were collected from the same state. It must be noted that the process of procurement of a reasonable number of IPs from various states of the country in adequate quantities for a meaningful analysis took a lot of time; collection was based on inputs from local people, and reaching the most appropriate source for collection of samples from diverse locations necessitated storage. Moreover, the actual formulation history is often not disclosed by the quacks who are responsible for preparation as well as distribution of the IPs. The complex nature of the IPs analysed poses constraints in chemical analysis and in deciphering the specific isoflavones. Although storage of IPs prior to the analysis in our laboratory involved adequate conditions, we believe a time lapse initially might have affected the UV pattern, as opposed to the previous study which could be conducted with freshly collected samples. Since the set of IP samples analysed in the two studies are entirely different with regard to place of collection and sources from where those were procured, it is difficult to interpret whether the constituents were different or they had undergone some changes with time.

The comparative fingerprinting chromatogram analysis revealed common peaks of unknown compounds in many IP samples. The consistent pattern of peaks lay within the UV maxima between 250–280 nm, implying presence of isoflavanoids arising from the A ring (240–285 nm) and other unknown compounds (Alam *et al.*, 2017). This eventually opened up the possibility of presence of other compounds that commonly appeared in those samples.

The preliminary test confirming the presence of steroids was a major observation of the study. One of the most common ingredient of such IP samples, *Bryonia*, has been reported for presence of phytosterols in previous literature (Tiwari and Gupta, 1958). Besides, it is well documented that feathers of birds contain corticosterone and testosterone (Bortolotti *et al.*, 2008; Lee *et al.*, 2012). The presence of steroids in the IP samples is indicative of material of animal origin which was traced to feathers of peacocks, in most IPs, a possible confirmation of the origin of steroids in these samples. The accumulation of steroids in feathers of birds has been reported and the origin of steroids other than isoflavones may be attributed to peacock feathers. Although characterisation of class and source of steroids could not be elucidated explicitly in the current study, it would be definitely worthwhile to carry out further investigations aimed at investigating the mechanism of specific components important for causing toxicity.

We envisaged that daidzein and genistein as likely components to be present in the IPs and the ingredients. These phytoestrogens are known to interact with the classical oestrogen receptors, ER α and ER β . This affect signalling pathways including non-genomic signalling mediated by oxidative stress pathways, tyrosine kinases, nuclear factor- kappa B and extracellular signal regulated kinases. Additionally, phytoestrogens serve as ligands for peroxisome proliferator activated receptors. They can interfere with the activities of DNA and alter epigenetics. Besides, phytoestrogens lower endogenous oestrogen levels by inhibiting the production of oestradiol (Jefferson *et al.*, 2012). Keeping this mechanistic viewpoint in mind, a parallel toxicity study of these two select pure phytoestrogens was carried out using the worm *C. elegans*. The latter model is rapid and reliable for assessing toxicity and is recommended as a predictive toxicity model. The worm assay presented gross abnormalities in the reproductive and developmental behaviour of *C. elegans* as reflected by the physiological parameters such as egg laying capacity, egg viability and progeny count. A dose of 12.5 $\mu\text{g/ml}$ was selected as optimum through dose fixation assay, which was then followed by survival assay for the select dose. With pure phytoestrogens survival of 90 and 70% worms respectively was noted upon treatment for up to 96 hours. In the reproductive assay, brood size was significantly affected ($P < 0.05$). The mean eggs and progenies in the vehicle control group for three consecutive days

were 715 and 709 while the mean number of eggs in the daidzein and genistein extract group was only 438 and 339 respectively. The progeny count was even lower for the phytoestrogen groups, 427 and 337 for daidzein and genistein respectively. It is important to note that although the dosage was defined as per the administration pattern of such formulations, the dose intake by pregnant women is predominantly multiple times of the dose defined as optimum in (alternate) animal models, which evidently had significant reproductive toxicity.

The results corroborated well with observations of IP sample extracts on the worms (data not shown) confirming the reproductive toxicity. Overall, the results of the present study substantiated the presence of high levels of isoflavones phytoestrogens along with animal steroids which significantly affect the reproductive toxicity. Whether other chemical constituents accord a synergistic effect with isoflavones and steroids in these IP formulations remains to be seen, the findings of this study imply a potential concern for health of the mother and foetus and calls for immediate intervention, regulatory reforms to safeguard pregnant women consuming the formulations.

5. Conclusions

More than 67% of the IPs used for sex selection which were evaluated contained isoflavones and steroids. For characterisation and better evaluation of other potential compounds likely to be present in the IP, advanced analytical tools like gas chromatography-mass spectrometry will be important (Gryniewicz *et al.*, 2005). Also, corroboration of *in vivo* experiments using established higher mammalian models like rodents should provide strong evidence and scientific explanation on the outcomes of consumption of these samples.

Availability of data and materials

The data and materials are available with the research team and will be made available on request.

Competing interests

The authors do not report any competing interest.

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