Estrogenic Potential of *Flemingia vestita* Benth Tubers in Ovariectomized Rat Model

Sunita Shailajan1*, Suman Kumaria2, Suhas Pednekar1, Sasikumar Menon1, Hiranjit Choudhury3 and Archana Matani1

1Herbal Research Lab, Ramnarain Ruia College, Matunga (East), Mumbai-400019, India.
2Department of Botany, North-Eastern Hill University, Umshing Mawkyrnoh, Shillong-793022, Meghalaya, India.
3Institute for Advanced Training and Research in Interdisciplinary Sciences, 194, Scheme No. 6, Road No. 15, Sion Koliwada, Sion (E), Mumbai-400022, India.

ABSTRACT

Objective: This study investigates the potential estrogenic activity of the ethanolic extract of *Flemingia vestita* Benth tubers using ovariectomized rat model. Materials and Methods: The ethanolic extract of *F. vestita* tubers has been standardized using validated HPLC method in terms of its genistein content (8.43 ± 0.06 mg/g of extract). Three to four week old young albino Wistar female rats were ovariectomized and treated for 14 days post ovariectomy with the standardized ethanolic extract at three different dose levels (100, 250, 500 mg/kg body weight) with a positive control of Estradiol valerate (1 mg/kg/day). The parameters evaluated were uterine weight, uterine glycogen, G6PDH, LDH, 17β-estradiol, progesterone, total cholesterol, triglycerides, HDL and histo architecture of uterus. Results: Treatment with the ethanolic extract of *F. vestita* tubers showed dose dependent increase in uterine weight, glycogen levels, G6PDH levels, estrogen and progesterone levels when compared with the ovariectomized control. Amongst three dose levels, high dose of plant extract showed significant increase in the uterine weight (p < 0.001), uterine glycogen content (p < 0.001), 17β estradiol and progesterone levels (p < 0.001), G6PDH and LDH levels (p < 0.001) as well as significant decrease in HDL and triglycerides levels (p < 0.001) compared to ovariectomized control. Histopathological evaluation of uteri sections revealed that the high dose of the plant show increase in the endometrial response as indicated by proliferation of endometrial glands and luminal epithelium of the ovariectomized rats. Conclusion: Thus, these data suggests that ethanolic extract (500 mg/kg body weight) of *F. vestita* tubers may exhibit good estrogenic activity in ovariectomized rat model.

Key words: Estrogenic activity, *Flemingia vestita*, Genistein, HPLC, Ovariectomized rats.

Address for Correspondence:
Dr. Sunita Shailajan, Associate Professor in Botany Herbal Research Lab, Ramnarain Ruia College, Matunga (East), Mumbai-400019, India.
Tel no: 022 24154390, Fax no: 022 24142480,
E-mail: sunitashailajan@gmail.com
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INTRODUCTION

Phytooestrogens, specifically isoflavones, are receiving great commercial interest at present. As these phytooestrogens have structural similarity to estrogenic steroids in human body, they mimic the effects of naturally occurring estrogenic compounds. Specifically the biological effects associated with ingesting isoflavones indicate that dietary supplements rich in these compounds might be useful for alleviating menopausal health concerns.1,2 Several medicinal plants have been evaluated for their estrogenic activity either individually or in the form of polyherbal combinations.3,4 There have been very few studies on the pharmacological validation of the plants that are used for the treatment of diseases in North-East India. One such plant is *Flemingia vestita* Benth (Soh-phlang, Fabaceae); an indigenous plant of Meghalaya, found practically throughout the Himalayas and Khasi Hills up to an elevation of 8,000 ft.5 The plant is known for its fleshy tubers which are scientifically evaluated against intestinal helminthes infections.6-11 These tubers are consumed by local people of Meghalaya to cure intestinal infections.12 These tubers possess major isoflavones like formononetin, diadzein, genistein, etc.13-15 Genistein is known to have a wide spectrum of biological activities,16 estrogenic activity being one of its most remarkable properties.17-18 Moreover, no research so far has been conducted to assess estrogenic potential of *F. vestita*. Hence, the present study aimed to evaluate the estrogenic activity of standardized extract of *F. vestita* tubers in ovariectomized rat model.

MATERIALS AND METHODS

Plant materials

Fresh tubers of *F. vestita* were collected from Shillong (Meghalaya) and authenticated from Department of Botany, North-East Hill University, Shillong. The tubers were thoroughly washed, cleaned and shade dried for a week. The materials were then packed in absorbent paper; oven dried at 37°C for three days, powdered using a mixer-grinder and sieved (BSS mesh number 85).

Chemicals and reagents

Analytical grade solvents were purchased from Merck Specialities, Mumbai, India. Progynova™ (Estradiol valerate tablets) was procured from Zyudus Healthcare. Ketamine Hydrochloride injection was procured from Neon Laboratories Ltd. The estrogen and progesterone kits were obtained from Eticon Endo Surgery (Mexico). Healex plus spray was procured from Shreya Life Sciences Pvt. Ltd. Surgical staplers were obtained from Eticon Endo Surgery (Mexico).

Preparation of ethanolic extract and standardization

Fine powder of *F. vestita* tubers (300 g) was extracted with ethanol (1500 mL, 5X) by refluxing on heating mantle (30 %) for 5 h. The mixture was filtered through Whatmann filter No. 1 and the filtrate was evaporated under reduced pressure using a rota evaporator at 45°C. The extract was standardized in terms of genistein content using validated HPLC method.19 Separation was achieved on Cosmosil C18-column (150 mm x 4.6 mm, 5.0 μm) using mobile phase 0.025 M phosphate buffer (in water): acetonitrile (68: 32, v/v, pH 2.4) delivered at a flow rate of 1 mL/min. The peaks were recorded at 261 nm using PDA detector (MD- 1510). The presence of genistein in the extract was also confirmed using LC-MS technique. Mass spectrometric analysis was performed on an API
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Applied Biosystems Hybrid Q- Trap API 2000 Mass Spectrometer (AB- MDS Sciex, Toronto, Canada) equipped with an electrospray ionization source (ESI).

Animals

Albino Wistar female rats weighing 150-180 g and 180-220 g were used for safety and estrogenic study, respectively. Animals were obtained from Hawkine Biopharmaceuticals, Parel and were housed in polypropylene cages under regulated temperature of 22 ± 3°C, relative humidity of 60 ± 5 % and 12 h light-dark cycle. Animals were provided free access to standard water and food supplied by Amrut Feed. The experimental procedures and protocol for the safety (AMM-130626-01) and efficacy (AMM-130626-02) study were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of Ramnarain Ruia College, Mumbai (CPCSEA/315).

Safety evaluation

The safety of the standardized ethanolic extract of F. vestita was evaluated in female albino Wistar rats at a dose of 2000 mg/kg as per OECD guidelines No. 420.28 The animals were randomly grouped into three groups of five animals each. Group I served as normal control received Distilled water, Group II served as vehicle control received 1 % CMC and Group III received ethanolic extract of F. vestita tubers. After dosing, the animals were observed after every 30 min till 2 h for immediate toxicity, periodically during the first 48 h and then daily for 14 days. Toxicity was evaluated on the basis of mortality, daily food and water intake, change in body weight and general behavioral changes.

Experimental design for evaluating estrogenic potential

Estrogenic potential of standardized extract of F. vestita was evaluated in ovariectomized rats at three different doses (100 mg/kg, 250 mg/kg and 500 mg/kg, body weight respectively). Histopathological parameters, uterine weight, glycogen content and biochemical parameters like 17-βestradiol, progesterone, lactate dehydrogenase (LDH), glucose-6-phosphatase (G6PDH), total cholesterol, triglycerides and High-density lipoprotein (HDL) were used to evaluate the estrogenic potential.

Vaginal smear cytology was performed for the determination of rat estrous cycle phases and to ensure the regular cycles. Vaginal secretions were examined from all the animals every morning for 15 days.21-22 Rats showing diestrous phase (leukocyte cells) were selected for the procedure of ovariectomy.

Albino Wistar female rats were randomized into six groups of six animals each. Animals from all the groups were ovariectomized (OVX) except the rats from the Group I that served as sham-operated control. In the sham-operated control, the ovaries were exposed and kept for the healing period of 14 days pre dosing. During the healing period, the animals were observed for any abnormal behavior or side effects. The animals were then randomized in 5 groups (except sham-operated control) on the basis of 17-βestradiol and progesterone levels. Animals showing reduced levels of estrogen and progesterone were then orally administered with their respective test samples as per dosage regimen for 14 consecutive days. The experimental groups were as follows:

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<tr>
<th>Group no.</th>
<th>Group details</th>
<th>Code</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>I</td>
<td>Normal control (Sham operated)</td>
<td>NC</td>
<td>1 % CMC (Carboxymethylcellulose)</td>
</tr>
<tr>
<td>II</td>
<td>Ovariectomized control</td>
<td>O VX</td>
<td>1 % CMC</td>
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<tr>
<td>III</td>
<td>Estradiol valerate</td>
<td>EV</td>
<td>1 mg/kg body weight</td>
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<tr>
<td>IV</td>
<td>Low dose of Flemingia vestita extract</td>
<td>FVL</td>
<td>100 mg/kg body weight</td>
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<tr>
<td>V</td>
<td>Medium dose of Flemingia vestita extract</td>
<td>FVM</td>
<td>250 mg/kg body weight</td>
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<tr>
<td>VI</td>
<td>High dose of Flemingia vestita extract</td>
<td>FVH</td>
<td>500 mg/kg body weight</td>
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Cage side observations including general behavioral changes, daily food and water consumption and daily weight changes were observed during the healing period of 14 days and after dosing for 14 days.

Blood collection procedure

Blood sample was withdrawn from all the animals prior to dosing (Day 0) and on day 15 (post dosing) by retro orbital plexus technique using heparinised capillaries. The whole blood, serum and plasma samples collected during the study were stored at -20°C until the determination of biochemical parameters.

Histopathological evaluation

The animals were sacrificed by cervical dislocation after blood collection. The left and right uterus of all the animals were removed after sacrifice, rinsed in saline, blotted and weighed. The weight of the uterus was expressed as mg/100 g body weight. A portion of uterus was taken for the estimation of glycogen by anthrone method and remaining was fixed in 10 % neutral buffered formalin and processed by paraffin technique. Sections of 5 μm thickness were cut and stained by routine hematoxylin-eosin (H&E) method for histopathological evaluation.27

Evaluation of Biochemical parameters

Serum samples were used to determine the 17-β estradiol and progesterone levels along with the estimation of LDH levels. The whole blood sample was used to estimate G6PDH levels whereas plasma samples were used to determine total cholesterol, HDL and triglycerides levels. The standard kits were used to evaluate biochemical parameters like 17-βestradiol, progesterone, G6PDH, LDH, total cholesterol, HDL and triglycerides levels.

Statistical analysis

Values are expressed as Mean ± SEM, n = 6. Graph Pad Prism5 software (version 5.03) was used for the statistical analysis of data. Data was analyzed using ANOVA followed by Dunnett’s test. Values of p < 0.05 were considered significant.

RESULTS AND DISCUSSION

Tubers of Flemingia vestita Benth has been reported to possess phytoestrogens such as formononetin, paeonlactone, diadzein and genistein.21 Recently, much interest has been paid to phytoestrogens for their potential health benefits in counteracting menopausal symptoms and in lowering the incidence of hormone dependent diseases.26 Genistein is one such phytoestrogen with a wide variety of pharmacological effects in animal cells, including tyrosine kinase inhibition, and dietary genistein ingestion has been linked, through epidemiological and animal model studies, with a
range of potential health beneficial effects. These include chemoprevention of breast and prostate cancers, cardiovascular disease and post-menopausal ailments. Hence, genistein was selected as a bioactive marker for evaluating the estrogenic potential of *F. vestita* tubers using ovariectomized rat model.

**Standardized ethanolic extract of *F. vestita***

The ethanolic extract of the plant was standardized in terms of its genistein content (8.43 ± 0.05 mg/g) using a validated HPLC method reported previously. The HPLC chromatograms of genistein and ethanolic extract of *F. vestita* are shown in Figure 1. The presence of genistein in the ethanolic extract of *F. vestita* tubers was also confirmed by using LC-MS technique.

The molecular mass of genistein is 270.241. As the mass spectrometric analysis was carried out at negative ionization mode, the mass of genistein obtained was found to be 269.8 m/z. The peak corresponding to genistein was observed at m/z 269.8 in the Full scan Q1 MS spectrum of the ethanolic extract of *F. vestita* tubers confirming its presence (Figure 2).

**Safety evaluation**

The data of the acute oral toxicity studies on medicinal plants is necessary in order to increase the confidence in its safety to human, particularly for use in the development of pharmaceuticals. Oral administration of the ethanolic extract of *F. vestita* tubers at a dose of 2000 mg/kg body weight did not show any toxic effect or mortality. Also, no significant change in the body weight, food intake and water intake of the animals was observed compared to the animals of control group. Hence, the extract had a wide margin of safety for oral use in rats (Data not shown).

**Evaluation of estrogenic potential**

Cage side observations during the efficacy study were found to be normal with no sign of abdominal dullness, subcutaneous slug, opacity and discharge for eyes and breathing abnormality.

**Vaginal Cellular Differentiation**

The estrous cycle is characterized by cyclical changes in uterus, ovaries, vaginal mucosa as well as behavioral and hormonal changes. Vaginal cytology assay is particularly used to determine the estrogenic activity of the synthetic estrogens and phytoestrogens as they induce the cornification of epithelial cells. After ovariectomy, all the rats had leukocytes population affirming complete removal of ovaries. Vaginal smears of OVX rats did not show any cornification proving the absence of endogenous estrogens. Vaginal cells showed maturation in response to Estradiol valerate in 5 days during the 15 day exposure period. Similar observations were reported by Cordial et al., 2006 using 17β-Estradiol at a dose of 50µg/kg bd. wt. FVH induced significant cornification of cells when compared to OVX control on 9th day whereas FVM and FVL induced cornification of epithelial cells after 10th and 13th day of the exposure period. There are many preclinical and clinical studies reported on the effects of phytoestrogens on vaginal cytology maturation. In our study, the cornification of vaginal smear in OVX rats treated with *F. vestita* tubers was found to be dose dependent which can be attributed to its estrogenic potential.

**Effect of ethanolic extract of *F. vestita* tubers on 17β-estradiol and Progesterone level**

Ovarian hormones, estrogen and progesterone govern the primary changes in the uterine tissue. All the animals after ovariectomy procedure showed significant decrease (p < 0.001) in the 17β-estradiol and progesterone levels compared to the animals of normal control group before dosing indicating complete removal of ovaries. After treatment for 14 days, animals treated with estradiol valerate showed a significant increase (p < 0.001) in the 17β-estradiol and progesterone levels compared to the animals of OVX control group. Similarly, animals treated with all the three doses of plant extract showed significant increase (p < 0.001) in 17β-estradiol and progesterone levels when compared with the OVX control. Amongst all the treatment doses, FVH showed maximum increase in the 17β-estradiol and progesterone levels indicating its dose dependent estrogenic potential (Table 1).

**Effect of ethanolic extract of *F. vestita* tubers on uterine weight**

Uteri undergo innumerable physiological and biochemical changes under the influence of ovarian hormones such as estrogen and progesterone. Administration of estrogenic substances to ovariectomized rats often leads to proliferative changes in the uterine endometrium. Animals in OVX control showed significant decrease (p < 0.001) in the uterine weight when compared with the normal control due to the loss of ovaries. Administration of estradiol valerate showed a significant (p < 0.001) increase in the uterine weight when compared with the OVX control. Animals treated with FVL did not show significant increase in the uterine weight when compared with the OVX control. Animals treated with FVH and FVM showed significant increase (p < 0.001) in the uterine weight when compared with OVX control (Table 2). Similar results have been reported earlier wherein plant extracts of estrogenic nature increased uterine weight in ovariectomized rats. Estrogenic potency and efficacy have traditionally been expressed in terms of uterotrophic effects in ovariectomized female rats. In this study, the increase in uterine wet weight was successive and gradual with the increase in dose of the extract of *F. vestita*.

**Effect of ethanolic extract of *F. vestita* tubers on uterine glycogen content**

The energy source for female reproductive system is glycogen and estrogens have been reported to increase the hexose transport into the rat uterus thereby increasing the synthesis of glycogen in the uterus. The decrease in levels of uterine glycogen in the OVX control may be due to the estrogen deficiency. Animals treated with the plant extract showed...
Table 1: Effect of F. vestita extract at three different doses and estradiol valerate on serum 17-β estradiol and progesterone levels after ovariec-
tomy at 0 day and 15th day

<table>
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<tr>
<th>Treatment groups</th>
<th>17-β estradiol (pg/mL)</th>
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<td>0 day</td>
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|                   |                        |                      |
|                   | 0 day                  |                      |
|                   | 11.02 ± 0.77***        | 11.52 ± 0.14***      |
|                   | 0.81 ± 0.01***         | 0.95 ± 0.01***       |
|                   | 2.88 ± 0.10***         | 3.29 ± 0.01***       |
|                   | 7.04 ± 0.05f           | 8.49 ± 0.04f         |
|                   | 22.83 ± 0.10***        | 25.15 ± 0.26**       |
|                   | 0.79 ± 0.01’’          | 0.33 ± 0.01’’        |
|                   | 4.55 ± 0.05f           | 8.33 ± 0.05f         |
|                   | 12.90 ± 0.05f          | 14.20 ± 0.05f        |

Table 2: Effect of F. vestita extract at three different doses and estradiol valerate on uterine weight, glycogen content, G6PDH, LDH, cholesterol, HDL and triglycerides levels in ovariec-
tomized rats

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<tr>
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<th>Uterus weight (mg/100g)</th>
<th>Glycogen content (mg/g)</th>
<th>G6PDH (Ug/Hb)</th>
<th>LDH (IU/L)</th>
<th>Cholesterol (mg/dL)</th>
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Effect of estradiol valerate on cholesterol, HDL and triglycerides

Significant increase (p < 0.001) in the cholesterol, HDL and triglycerides levels in OVX control were observed when compared to normal control group. Animals treated with the estradiol valerate showed significant decrease (p<0.001) in the levels of these markers as compared to OVX control. Significant reduction in the levels of cholesterol (p < 0.01), HDL (p < 0.001) and triglycerides (p < 0.001) were observed in the rats treated with FVH as compared to the OVX control group (Table 2).

Effect of estradiol valerate on uterine G6PDH and LDH levels

G6PDH is the first enzyme in the pentose phosphate pathway which provides pentose phosphates and reducing equivalents in the form of NADPH. Estrogen is known to have a direct influence on glycogen synthesis and is a potent stimulator of G6PDH and LDH activities in the uterus of OVX rats. The OVX control showed significant decrease (p < 0.001) in the G6PDH and LDH levels as compared to the normal control. Significant increase (p < 0.001) in the levels of G6PDH and LDH (except G6PDH level in low dose; p < 0.05) was observed in the animals treated with all the three doses of plant extract and estradiol valerate when compared with the OVX control (Table 2).

Significant increase (p < 0.001) in the G6PDH levels low in premenopausal women. Postmenopausal women lose this protection because of dramatic decrease in estrogen levels as a result of natural atrophy of the ovaries.

Effect of ethanolic extract of F. vestita tubers on Cholesterol, HDL and Triglycerides

Estrogen has been reported as a beneficial factor in preventing cardiovascular diseases like atherosclerosis by keeping plasma cholesterol levels low in premenopausal women. Postmenopausal women lose this protection because of dramatic decrease in estrogen levels as a result of natural atrophy of the ovaries.

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Histopathological evaluation

The uterine histology of the sham-operated rats showed single layered columnar epithelial cells (LE), numerous and irregular endometrial glands (EG). The compact arrangement of the myometrium (M) and perimetrium (P) layer were also observed. The uterine section of OVX control rats revealed changes characterized by atrophy of the uterus and more intracellular spaces. There was noteworthy decrease in the number of endometrial glands (EG) with disappearance of lumen (LE) at some places, disorganization of epithelial cell lining and poor vascularity.

This condition was recovered by FVL which showed histological changes like hydrometra, epithelial proliferation and endometrial glandular hyperplasia. Animals treated with estradiol valerate showed intact luminal epithelium with low columnar cells and regular distribution of mitotic places, disorganization of epithelial cell lining and poor vascularity.
figures. Endometrial layer showed increase in the number of endometrial glands as well as developed vascular layer and connective tissue. However, FVH was effective than OVX control, FVL and FVM and caused changes in the uterus which exhibited hypertrophy of stromal and endometrial glandular cells, epithelial hyperplasia and increased vascularity. Endometrial layer was well developed with more number of endometrial glands lined with simple columnar epithelium. FVH was found to be more effective on proliferation of endometrial glands than on the luminal epithelium (Figure 3). An increased mitotic activity of uterine tissues was clearly evident in the animals treated with high dose of plant extract indicating its estrogenic nature. 49

Estrogenic potential of the ethanolic extract of _F. vestita_ tubers at three dose levels was evaluated in OVX female albino Wistar rat model in terms of biochemical markers such as estrogen, progesterone, LDH, G6PDH, cholesterol, HDL, triglycerides, urethane weight and uterine glycogen content. Marked recovery of these biochemical markers were observed in the animals treated with _F. vestita_ tubers as compared to OVX control group indicating estrogenic potential of this plant and results were at par with the estradiol valerate treatment. The improved histarchitecture of the uterus was observed in histopathological evaluation of animals treated with the ethanolic extract of _F. vestita_ tubers as compared to the animals of OVX control group. This indicates the ability of plant to proliferate luminal epithelium and endometrial cells. Thus, the estrogenic activity shown by ethanolic extract of _F. vestita_ tubers can be attributed to the presence of phytooestrogens like genistein.

From the above observations it can be concluded that the standardized ethanolic extract of _F. vestita_ tubers at high dose level showed the promising effects on the physical, histological and biochemical parameters of the uterine tissue.

**CONCLUSION**

Findings of the current study, therefore suggest that the extract could form a basis for phytotherapeutic preparations which might help raise the market value of _F. vestita_ tubers. Such plants can also be used as an alternative therapy for menopausal syndrome.

**ACKNOWLEDGEMENT**

We acknowledge the financial assistance from the Department of Biotechnology, Government of India under Twinning Program with North East Hill University (Sanction No. BT/250/NE/TPB/2011).

**ABBREVIATION USED**


**REFERENCES**

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PICTORIAL ABSTRACT

SUMMARY

- The ethanolic extract of F. vestita tubers was standardized in terms of genistein content (8.43 ± 0.06 mg/g of extract) using validated HPLC method.
- Presence of genistein in the ethanolic extract of F. vestita tubers was confirmed using LC/MS technique (m/z ratio: 269.8).
- The standardized extract of F. vestita tubers was found to be safe in albino Wistar rats at a dose of 2000 mg/kg body weight using OECD Guideline no. 420.
- Estrogenic potential of F. vestita tubers extract at three dose levels was evaluated in ovariectomized rat model.

ABOUT AUTHOR

Dr. Sunita Shailajan: Is an Associate Professor in Botany, Incharge of Herbal Research Lab and Scientist Incharge of Animal Testing Centre at Ramnarain Ruia College, University of Mumbai, India. She is recognized research guide in the subject of Botany, Bioanalytical Sciences, Biotechnology and Applied Biology. Her specialization is in the area of standardization of herbal raw materials and ASU formulations, instrumentation (HPTLC and HPLC), bioanalytical method validation, pharmacokinetic studies. She has more than 90 scientific publications and made presentations in various National and International conferences in India and overseas. She has been working as Principal Investigator for Government funded projects.