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RESEARCH ARTICLE

Characterization of the methanolic seed extract of two medicinal plants, *Putranjiva roxburghii* and *Diplocyclos palmatus* and its effects on gonads of Albino Mice

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Abstract

An essential component of reproductive health, fertility, is prerequisite for social, economic and human development. However, a significant section of the population is still infertile with no child due to reproductive-related problems and infertility. Tackling these reproductive-associated problems with advanced clinical tools for the diagnosis of reproductive defects has been a huge task with about a quarter of clinical infertility cases being diagnosed as idiopathic. Despite tremendous advancements in synthetic drugs and modern medical science, traditional medicine is seeing rapid growth worldwide. The expanding perception among people about the potency and side effects of synthetic drugs has led them to be more dependent on natural product remedies for treating reproductive-related problems. *Putranjiva roxburghii* of Euphorbiaceae family and *Diplocyclos palmatus* of Cucurbitaceae family are two medicinal plants used by the local healers for various ailments such as gynaecological disorders, and fertility. Hence, methanol extracts of these two seeds were prepared and characterized by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Further, two experiments were carried out with the methanol extract of the seeds of these two medicinal plants at a dose of 100mg per kg body weight to observe its effect on the reproductive physiology of albino mice. Experimental mice (Male and female) of two age groups of 3-week-old (Experiment-I) and 8-week-old (Experiment-II) were administered with methanol extract (ME) dissolved in normal saline, while the controls for the respective male and female mice for the two experiments received normal saline for continuous 42 days. Mice were sacrificed at the interval of 14, 28 and 42 days after the treatment. The TLC analysis of the ME showed five fractions which were named as Spot1, Spot2, Spot3, Spot4 and Spot5. HPLC analysis showed the presence of three phytocompounds, i.e., flavonoids - Quercetin (peak 4.867), Tannic acid (peak 2.497) and Rutin (peak 3.440) in the ME of the seeds of the two medicinal plant species. Histology of the liver showed no toxicity at the administered dose in either of the male and female mice groups in both experiments. Histology of the testis showed an increased number of Sertoli cells, spermatozoa and Leydig cells with more vascularization and sperm count highly significant ($p<0.05$) in ME than NS-treated mice in both experiments. Ovarian histology in ME showed more substantial number of follicles in the stage of secondary antral follicle maturing towards the Graafian follicle with increased vascularization in both the experiments. This result is in consensus with the estrous cycle of the females where ME treated mice prolonged their cycle at the estrus phase (heat phase). The uterus histology also showed increased proliferation of uterine lumen with numerous epithelial glands in both the experiments. These changes observed may be due to the presence of the phytochemicals/flavonoids present in the plant extract, which might enhance the reproductive efficiency of the mice. Further extensive research along with a proper screening of phytosteroids of the methanol extract of the seeds of these two medicinal plants are needed for the declaration and formulation of fertility drugs from these two medicinal plants to provide hope for thousands of individuals dealing with reproductive and infertility issues.

Keywords: Reproduction; *Putranjiva roxburghii*; *Diplocyclos palmatus*; Methanol Extract; Quercetin; Tannic Acid; Rutin

1. Introduction

An essential component of reproductive health, fertility, is prerequisite for social, economic and human development contributing to public health (Paul et al., 2011). Today a healthy reproductive and sexual life is considered fundamental human rights for all and is protected by three bodies of law: human rights law, refugee law and humanitarian law. Better health is essential to social well-being and pleasure for the existence of species. However, health concern, a vital component for the existence of life, since the era of humankind has been an alarming issue, as a healthy population can live longer and contribute more to the society (Cedars et al., 2017).

But there is a significant section of the population which is still infertile with no child due to reproductive-related problems and infertility. Tackling these reproductive-associated problems even with advanced clinical tools for diagnosis of reproductive defects has been a huge task with about a quarter of clinical infertility cases being diagnosed as idiopathic (Matzuk and Lamb, 2002; Kamel, 2013). Thus, as of today, scientists after decades of serious obsession with the modern medicinal system have started looking at the ancient and primary healing systems like Ayurveda, Siddha and Unani.

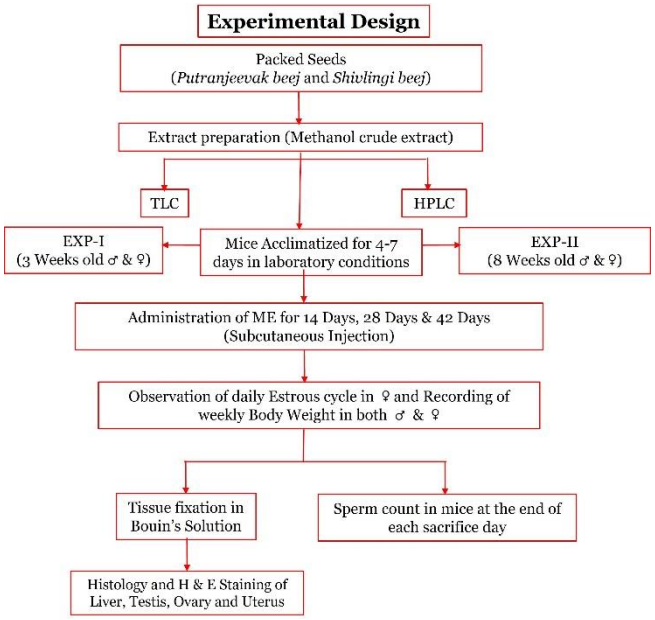


Figure 1: Flow Chart Showing the Experimental Design

The expanding perception among the people about the potency and side effect of synthetic drugs has led them to be more dependent on the natural product remedies with an increase in basic approaches towards nature for treating reproductive related problems using herbal medicines and therapies (Shankar and Ved, 2003). Despite tremendous advancement in synthetic drugs and modern medical science, traditional medicine is seeing rapid growth worldwide (Sandberg and Corrigan, 2001; Salim et al., 2008; Bussmann and Glenn, 2011). Plant with novel chemical entities since time immemorial have the potential to enhance the reproductive or fertility health in male and female with a potential for improved general health. Plant-derived phytochemicals or phytomedicines have recently shown a great promise in the treatment of infertility and reproductive abnormalities, due to the presence of numerous phytoestrogens and other beneficial compounds in the plants (Farnsworth et al., 1990). Herbal medications play a crucial role in health care programs, particularly in developing countries. Studies by several researchers using herbal medicines for the treatment of infertility and reproductive related issues have shown positive results such as improved sperm quality, sexual functions, libido and testosterone level, male retrograde ejaculation and sexual potency, impotence and erectile dysfunction in males; while metrorrhagia, infertility and aphrodisiac, dysmenorrhea or uterine contraction stimulation, etc. in females (Alahmadi, 2020). As of today, plant products are being used to address the health and reproduction-related issues across the world, specifically in developing countries like India.

Putranjiva roxburghii Wall. and *Diplocyclos palmatus* (L.) C. Jeffrey, two medicinal plants are believed to improve the reproductive efficacy and fertility rate in the human population. *P. roxburghii* Wall. (Syn. *Drypetes roxburghii* Wall.), is a deciduous plant commonly known as *putranjiva* of family Putranjivaceae or Euphorbiaceae. It is an evergreen tree of about 18 m tall with grey bark. The tree elliptic-oblong to ovate-lanceolate, unequal sided at the base, dark green and shining in appearance in leaves. Flowers are small; male flowers dense, rounded clusters, yellowish in color while female flowers are solitary or 2-3 together and green in color. Seeds being globose and white tomentose, stone pointed; rugose, very hard and ordinarily single are commonly called by name *putranjiva*. *P. roxburghii* is found in the wild or cultivated in almost all parts of India (Badole et al., 2011). The famous botanist William Roxburgh recognized the plant, and accordingly, the plant is named as *P. roxburghii* (Haldar et al., 2009). Roxburgh (1832) also explained the Sanskrit name of the tree "*pootranjeeva*", where *Putra* means a son and *Jeeva* means life. Child life tree, Lucky Bean Tree, Child's amulet tree and spurious wild olive are few of its common English names. It is widely grown all over Asia for its medicinal qualities, particularly in Indo China, India, Nepal, Thailand, Bangladesh, Myanmar and Sri Lanka (Phuphathanaphong and Chayamarit, 2006).The significant usefulness of *putranjiva* for antipyretics, anti-inflammatory and anti-rheumatic and also for gynaecological and fertility ailments have been reported (Gupta, 2016). Pharmacognostical analysis of leaves, fruits, stem and roots of *P. roxburghii* revealed the presence of various active polyphenolic compounds like glycosides, saponins, triterpenes, ellagic acid, gallic acid and flavonoids which may be associated with its many therapeutic properties (Garg and Mitra, 1968; Sengupta and Mukherjee, 1968). The plant is used as a drug for azoospermia, catarrh, and constipation and has been reported for its significant medicinal values. *Putranjiva* is referred to as uterine tonics and is believed to provide nutritional support to uterus and maintains endometrial health, normalize menstrual blood flow, and help to prepare the uterus by improving the thickness of endometrium for implantation (Samal, 2017).

Diplocyclos palmatus commonly known as *shivlingi* (previously known as *Bryonia laciniosa*), is a medicinal plant belonging to Cucurbitaceae family (Balkrishna et al., 2021). It is one of the extremely crucial drugs in traditional system of medicine from ancient time. It is known as lollipop climber in English. It is a shrub found widely in India, Philippines and some parts of Africa (Kirtikar and Basu, 1988). In India it is distributed in Madhya Pradesh, Uttar Pradesh, Gujarat and Uttarakhand. It is an annual climber having bright red fruits, and it has been reported to be of high medicinal value. The seeds of *D. palmatus* are yellowish-brown, and since the upper surface of seeds has a morphology similar to that of *shivlinga* (Phallus of Lord Shiva in Hindu mythology) and hence called *"shivlingi"* (Panda, 2004). The presence of bryonin, saponin, punicic acid, goniothalamine, and glucomannan (Gowrikumar et al., 1981; Mosaddik et al., 2000; Saxena et al., 2004; Singh and Malviya, 2006) and pharmacological activities like analgesic, antipyretic, anticonvulsant, antimicrobial, cytotoxic, antiasthmatic, anti-inflammatory and antifertility has been reported for this particular plant species (Gupta et al., 2003; Ehsan et al., 2009). Seeds of *shivlingi* are used as a potential contraceptive when blended with *Putranjivi*, pepper, the Root bark of Vata (*Ficus bengalensis*), ginger (dry), and milk (Shukla et al., 2008). Further, *shivlingi beej* (*shivlingi* seed) is used for the treatment of female infertility. It is a uterine tonic for women suffering from infertility as it improves the chances of conception in them. It is also believed to enhance the fertility rate in humans when used in combination with *Putrajeevak beej* (*Putranjiva* seed) (Singh, 2017). The coadministration of powdered form of seeds of these two plants are considered to improve the reproductive efficiency in male and female human population and act as an aid to gynaecological disorders, as proposed by the firm, Patanjali and its Group.

Present work is thus focused on the characterization of the methanol extract of the seeds (*beej*) of the two medicinal plants, *P. roxburghii* and *D. palmatus* and its effect on the gonads of Albino Mice. No doubt several literatures have reported morphological features, phytochemical screening, medicinal benefits and the pharmacological profile of the individual plants by different researchers and scientists. However, as per our knowledge, there are no reports available on the coadministration of methanol extract of seeds of these two medicinal plants on reproductive aspects and its efficacy in animals. Hence, to validate the statement and to provide a baseline for future research, the present study was carried out.

2.Materials and methods

2.1. Preparation of extracts

Seeds of two medicinal plants, *P. roxburghii* (*putraveejak beej*) and *D. palmatus* (*shivlingi beej*) were collected and processed for Methanol Extraction (ME) as per standard protocol. Briefly, the *putrajeevak beej* and *shivlingi beej* were dried in an incubator at 37° C for 3 hours and grinded separately with the help of a mixer grinder to obtain the fine powdered form of the seeds. The powdered seeds were then stored in airtight containers until processed for methanol extraction. ME of the two medicinal plants were prepared as per the protocol of Beghashaw et al (2017) with slight modifications. Briefly, an equal amount of both the powdered seeds of the two medicinal plants, i.e., 100 gm each was weighed on a weighing machine and added in a clean and dry 3L conical flask. Methanol (2000ml) was

poured to the conical flask and continuously stirred on a magnetic stirrer from day 1 to day 7. The change in color of methanol from transparent to light green first and then to dark green was observed. The conical flask was covered with aluminum foil to prevent evaporation of the methanol. Continuous mixing of the mixture was done on a magnetic stirrer from the start of methanol extract until the termination of extraction of ME. The methanol content present in the conical flask was filtered with the help of Whatman's' filter paper on the 7th day, and the filtrate was collected in a 1000 ml beaker. After the collection of the filtrate, the mouth of the beaker was covered with muslin cloth and tightened with a rubber band. The beaker was kept undisturbed at room temperature until the final form of methanol extract (semi-solid or jelly-like) was obtained. The whole process of extract preparation was done at room temperature (25±2 °C).

2.2. *Storage of the methanol extracts (ME)*

The final form of ME was then collected in 50ml conical tubes or micro centrifuge tubes, and weight of ME in each tube was recorded individually. The mouths of the conical or micro centrifuge tubes were sealed with parafilm, the ME was stored at 4 °C for further use (characterization, analysis and use in experimental studies).

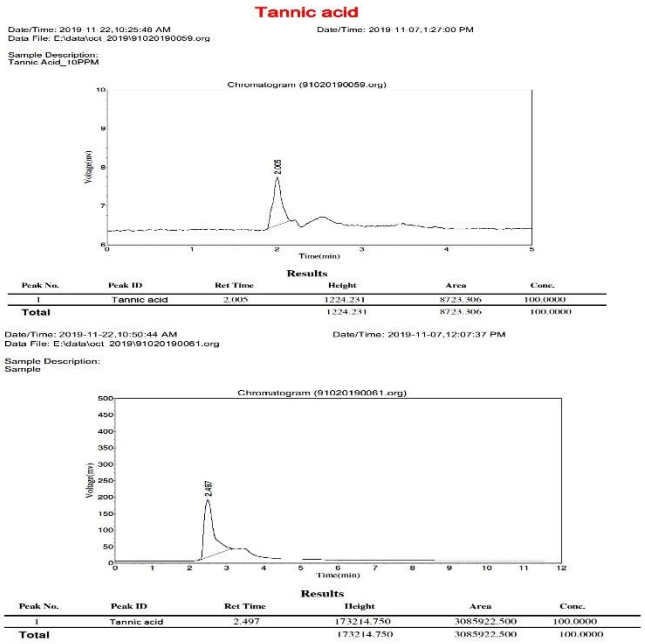


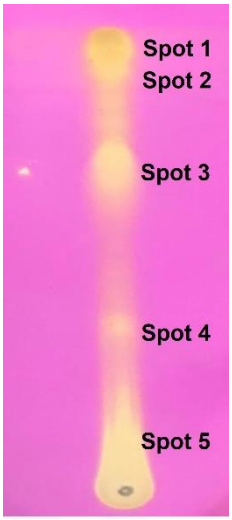
Figure 4: Typical Chromatogram of Tannic Acid along with its standard analysed by High-Performance Liquid Chromatography (HPLC)

2.3. *Thin Layer Chromatography (TLC)*

Thin Layer Chromatography or TLC is a chromatography technique for separation of the non-volatile mixture of molecules. This is carried out on an inert thin layer of solid sheet and consists of liquid as the mobile phase and solid surface as the stationary phase. TLC is typically performed using paper or commercially available glass, or plastic plates that are coated with a thin layer of adsorbent (TLC plates) (Meyers and Meyers, 2008). The ME of the seeds of two medicinal plants were separated by using a single solvent system of Kharat et al. (2016) with some modifications.

Chromatographic analysis was performed on TLC plates pre-washed with methanol and activated at 110°C for 5 min before chromatography. The ME samples were spotted in the form of bands on silica gel pre-coated TLC aluminum plate 60 F254, (20 cm × 20 cm) with 200 µm thickness (E. Merck, Darmstadt, Germany). ME samples were dissolved in methanol, and a linear ascending development was carried out in a glass chamber saturated with the mobile phase. The mobile phase consisted of hexane: ethyl acetate (50:50, v/v) and 20ml was used per chromatography run. The optimized chamber saturation time with mobile phase was 30 min using saturation pads at room temperature (25 °C ± 2). The length of the ME chromatogram run was 80mm, and the run time was 40 min. Ultraviolet (UV) lamp emitting a continuous UV was used as a source of radiation. All determinations of the ME chromatogram were performed at ambient temperature with a detection wavelength of 254 nm. Distance travelled by each molecule was recorded, and Retention Fraction (Rf) was calculated with the help of following formula: The retention factor, or Rf, may be defined as the distance covered by the compound divided by the distance covered by the solvent.

2.4. *Purification and characterization of ME by High Performance Liquid Chromatography (HPLC)*



ME were reconstituted in HPLC grade methanol and processed for purification and characterization of unknown compounds present in the ME by HPLC with a UV detector (Kharat et al., 2016; Sowjanya et al., 2017). For purified extracts, retention time was determined by using HPLC system. The column used for chromatographic separation was C18 10 µm 100 A (250 × 4.6 mm). The samples were injected at a wavelength of 254 nm for flavonoids and 270 nm for tannin. The chromatographic separation was accomplished using mobile phase

Figure 2: Thin Layer Liquid Chromatography (TLC) of Methanol Extract of Seeds of the Two Medicinal Plants, *P. roxburghii* and *D. palmatus*. Analysis of TLC plate shows 5 spots in the run, designated as Spot 1, Spot 2, Spot 3, Spot 4 and Spot 5. Mobile Phase: 50% Ethyl Acetate + 50% Hexane and Visualized at ambient temperature with a detection wavelength of 254 nm.

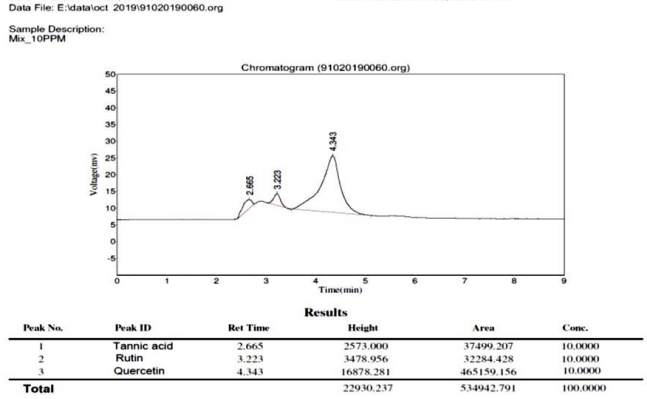


Figure 3: Typical Chromatogram of the mixed standards (Tannic Acid, Rutin and Quercetin) analysed by High-Performance Liquid Chromatography (HPLC).

methanol:0.1 % orthophosphoric acid (77:23), the vacuum pump was used for filtration and standard quercetin, rutin and tannic acid were used. The mobile phase was pumped at a flow rate of 1 ml/min at room temperature. Using the optimized chromatographic condition, a baseline was recorded.

2.5. *Animal maintenance*

Male (♂) and female (♀) mice (BalbC Strain) of around 3-week and 8-week age were used for the present study. Mice were purchased from Pasteur Institute, Meghalaya, Shillong, housed in the animal house of the Department of Zoology, Rajiv Gandhi University and a breeding colony was maintained to rear the animals for the present study. All mice were housed in polypropylene cages and maintained under conditions of 12L:12D light-dark cycle throughout the experiment. Ear-punching of the individual animal was done as per standard protocol (Dickie, 1975; Ingalis, 1980; Stark and Ostrow, 1991) with the help of ear punch for the identification of individual mice throughout the experiment. Animals were fed with standard mice diet and water ad libitum. All the animals were acclimatized to laboratory conditions for 4-7 days before the start of each experiment. All the experiments were performed as per the Animal Ethics Committee of the Rajiv Gandhi University and as per the guidelines within the

framework of the revised animals (Scientific Procedures) Act 2002 (CPCSEA) of Government of India.

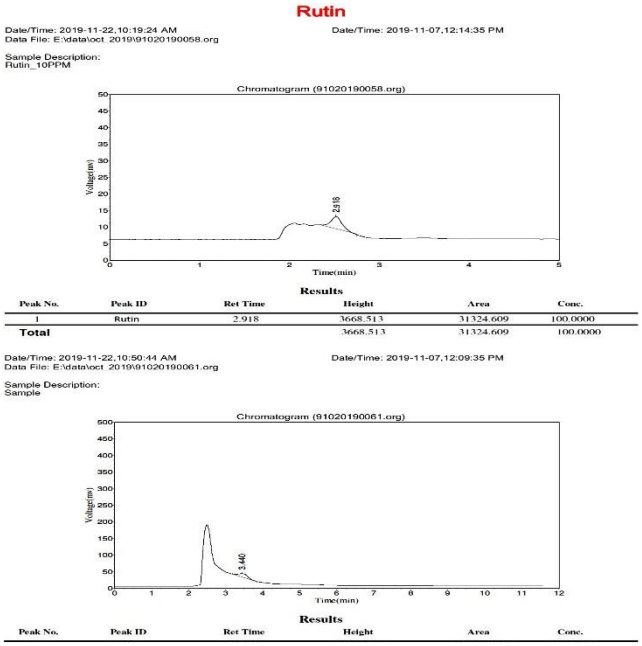


Figure 5: Typical Chromatogram of Rutin along with its standard analysed by High-Performance Liquid Chromatography (HPLC)

2.6. Two experiments were performed to achieve the objectives of the study

2.6.1. Experiment-I

Both Male (♂) and female (♀) mice (BalbC Strain) of around 3-week age were divided into two groups each, consisting of control (Vehicle-treated-NS Group) and Methanol Extract group (ME Group). The number of samples in each group was n=3/group in both male and female mice. Experimental male and female mice were administered with ME subcutaneously at a dose of 100 mg/kg bodyweight while the control male and female groups were administered with normal saline (NS) subcutaneously for three different durations, i.e., 14 Days (Short term), 28 Days (Midterm) and 42 Days (Long term). The vaginal smear of both NS and ME females were collected daily until the end of the experiment for the study of the estrous cycle of the animal. Weekly bodyweight of the animals were recorded. Animals (both male and female) were sacrificed after 14 Days, 28 Days and 42 Days of the study; tissues such as liver, testes, ovary and uterus were preserved in Bouins' Solution for further preparation of paraffin blocks, sectioning and haematoxylin-eosin staining. In the males, sperm count was performed at the end of the experiment as per the standard protocol.

2.6.2. Experiment-II

A similar experimental set up to that of Experiment-I was done in Experimental-II except that the age group of the mice was8-week-old. Vaginal smear was collected daily for the study of the estrous cycle of the animal, while the weekly body weight of animals of each group was also recorded. At the end of the experiment, i.e., 14 Days (Short term), 28 Days (Midterm), and 42 Days (Long term) of the study, animals were sacrificed; tissues such as the liver, testes, ovary, and uterus were preserved in Bouins' solution for 24 hrs for further processing of preparation of paraffin blocks, sectioning, and haematoxylin-eosin staining. In the males, sperm count was performed at the end of the experiment as per standard protocol (See Figure 1).

2.7. Estrous Cycle study

Estrous cyclicity was assessed by collecting the vaginal smears from all-female mice. In rodents such as rat and mice, the estrous cycle is completed within 4-5 days and the four stages of this cycle; Proestrus, estrus, metestrus and diestrus can easily be recognized and studied in the research laboratory with the help of a compound microscope taking vaginal smears.

The vaginal smears were taken and evenly spread on a clean, dry glass slide with the help of earbuds soaked completely in 0.05M phosphate-buffered saline (PBS). The slides were then allowed to dry at room temperature (RT). Methanol (1-2 drops) was then added to the slides and again left to dry at RT. Few drops of Giemsa stain was added to the slides and kept for 10 minutes. Thereafter, the extra stains were removed by washing the slides with the help of distilled water. The slides were again allowed to dry, later it was observed under the microscope. All the stages were recorded daily for each individual female mouse. Protocol of Byers et al (2012) was followed for this study with slight modifications.

2.8. Sperm count

Spermatozoa were obtained from the cauda epididymis. After exposing the epididymis, the cauda epididymis was transected at the point of origin of the vas deferens at the distal end and at the boundary between the corpus and cauda epididymis at the proximal end. The tissue was placed in a watch glass containing 0.5 ml of normal saline (NS) maintained at 37 °C. The tissue was minced

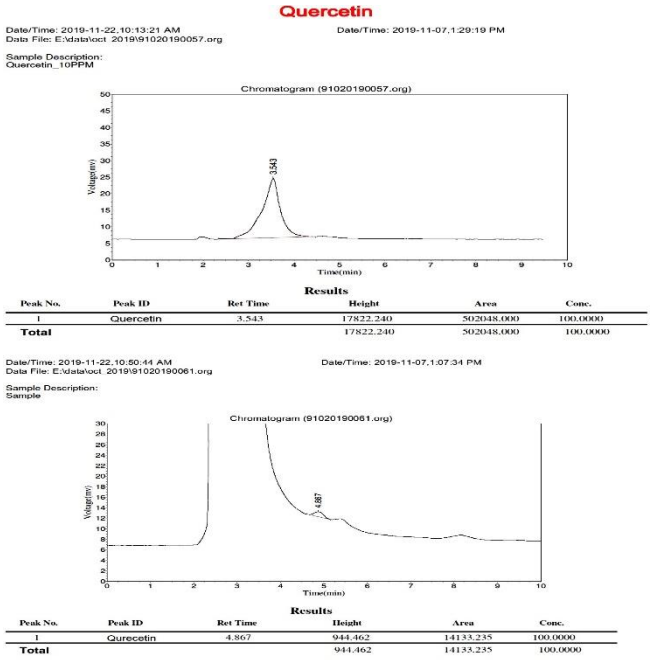


Figure 6: Typical Chromatogram of Quercetin along with its standard analyzed by High-Performance Liquid Chromatography (HPLC)

carefully with the help of fine forceps and scissors to ensure the extrusion of spermatozoa from the cauda epididymis. The tissue fraction was then removed by forceps or needles, and the suspension was used for sperm analysis following the protocols of Singh and Chakravarty (2003) and Alshahrani et al (2017).

Briefly, a Haemocytometer (Fein optic, Jena, Germany) with improved Newbauer chamber was employed for counting the spermatozoa. A 20-fold dilution was made by mixing the sperm suspension with the spermicidal solution (NaHCO :4g + Phenol: 1 g in 100 ml of distilled water). The dilution was made using a white blooded pipette; the sperm suspension was drawn to the 0.5 mark halfway up the stem and the spermicidal solution subsequently to the mark at the top of the bubble chamber. The preparation was then thoroughly mixed, and one drop of it was added to both sides of the haemocytometer. The spermatozoa were allowed to settle optimally by keeping the haemocytometer in a humid (wet) chamber for 30 minutes. The humid chamber was constructed by placing a wet sponge inside a faintly airtight icebox. The number of spermatozoa was counted in the four corner squares of the haemocytometer under a microscope at 400X when spermatozoa crossed the lines of the joins. Only those at the top and right-hand sides of the squares were

counted. Spermatozoa on both sides of the haemocytometer were counted, and the average number was recorded.

Concentration of spermatozoa = Average number of spermatozoa counted (N) X Multiplication factor (10,000) X Dilution factor (20)
= N X 10,000 X 20 Spermatozoa
= N X 0.2 X 10^6 Spermatozoa

2.9. *Histology, Haematoxylin and Eosin*

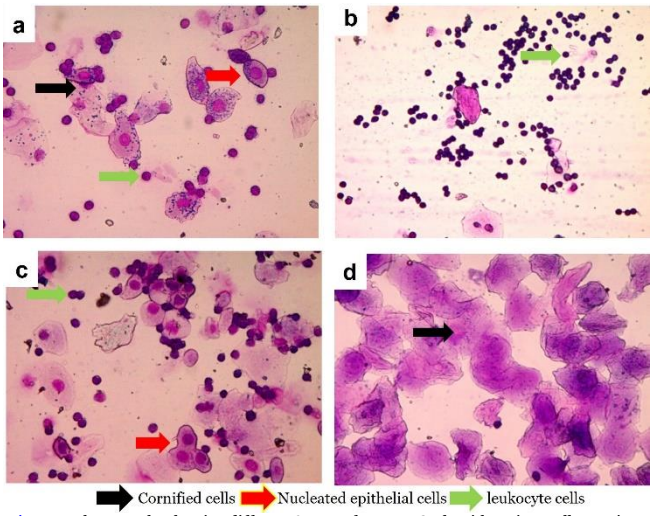


Figure 7: Photographs showing different Stages of Estrous Cycle with various cell types i.e., a. Metestrus (Nucleated epithelial cells, cornified cells & leukocytes), b. Diestrus (leucocyte cells), c. Proestrus (nucleated epithelial cells) and d. Estrus (cornified cells) in female mice as visualized by Giemsa Stain (Photographs= 400X Magnification).

Liver, Testes, ovary and uterus of all the animals were fixed in Bouin's fluid (Bouin, 1897), for histological studies.
Following fixation, the tissues were dehydrated in an ascending series of ethanol, cleared in xylene and then embedded in paraffin wax. Serial sections were cut at a thickness of 6 µm using a Leica semi-motorized rotary microtome (Model Leica RM 2145, Leica Biosystems, Germany). The sections were mounted on ethanol cleaned glass slides coated with 5% Gelatin and were kept in a hot-air oven at 37 °C overnight to dry. Sections were deparaffinized in xylene and were hydrated in a descending ethanol series. The sections were then stained with Ehrlich's haematoxylin and eosin (H/E) (Ehrlich, 1886) to study the general organization and structural changes in the tissue. The stained sections were dehydrated in an ascending series of ethanol, cleared in xylene, and mounted in Kirkpatrick and Lendrum's Distrene Dibutylphthalate Xylene (DPX).
Observations were made on a Carl Zeiss Axioscope AX10 Microscope (Carl Zeiss Promenade 10, Jena, Germany). The results were recorded using an AxiocamERc 5s CCD camera (Carl Zeiss Promenade 10, Jena, Germany) for automatic microphotography on an HP 280 G2 Computer with ZEN-2012-Imaging Software Version 8.0.0.

2.10. Statistical analysis

All the data are represented as Mean ± SEM. The data were analyzed by t-test for statistical comparison between the groups at a confidence limit of 95%.

3.Results

3.1. *Thin layer chromatography (TLC) and High-Performance Liquid Chromatography (HPLC)*

The ME obtained was analyzed through TLC and HPLC. TLC analysis of the ME showed 5 fractions labeled as (Spot 1-5 with Rf Value in the range from 0.08-0.16) (Figure 2). Further analysis of the TLC fractions is under process and investigation.

Further, HPLC analysis of the ME showed that it had three compounds (Figure 3), namely Tannic Acid (Figure 4), Rutin (Figure 5) and Quercetin (Figure 6), which have been proven to have a role in reproduction. The compounds showed a peak of 2.497 (Tannic Acid) (Figure 4), 3.440 (Rutin) (Figure 5), and 4.867 (Quercetin) (Figure 6).

3.2. *Estrous cycle study*

To prove the efficacy of the methanol extract on reproduction, two (three) experiments were performed by administrating ME to albino Mice (male and female, 8 and 3 weeks old) at a dose of 100 mg per kg body weight for 14, 28 and 42 days. There was no significant change

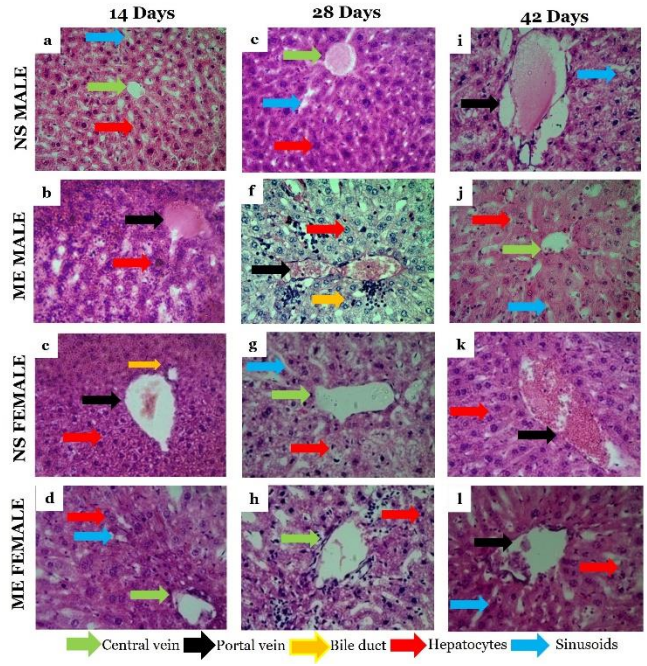


Figure 8: Photographs of T.S. of Liver of both Male and Female mice (3-week old) treated with Methanol Extract (ME) of Seeds of Two Medicinal Plants, *P. roxburghii* and *D.palmatus* at a dose of 100mg/kg body weight for 14 (a, b, c and d), 28 (e, f, g and h) and 42 (i, j, k and l) days. No significant change in histoarchitecture of the liver in both male and female mice were observed when compared to their respective controls. (Photographs = 400X Magnification).

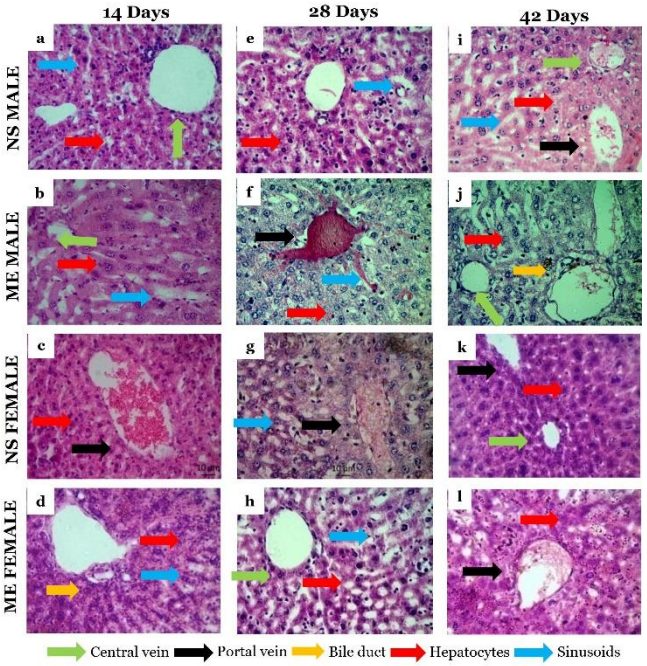


Figure 9: Photographs of T.S. of Liver of both Male and Female mice (8-week old) treated with Methanol Extract (ME) of Seeds of Two Medicinal Plants, *P. roxburghii* and *D. palmatus* at a dose of100mg/kg body weight for 14 (a, b, c and d), 28 (e, f, g and h) and 42 (i, j, k and l) days. No significant change in histoarchitecture of the liver in both male and female mice were observed when compared to their respective controls. (Photographs=400X Magnification).

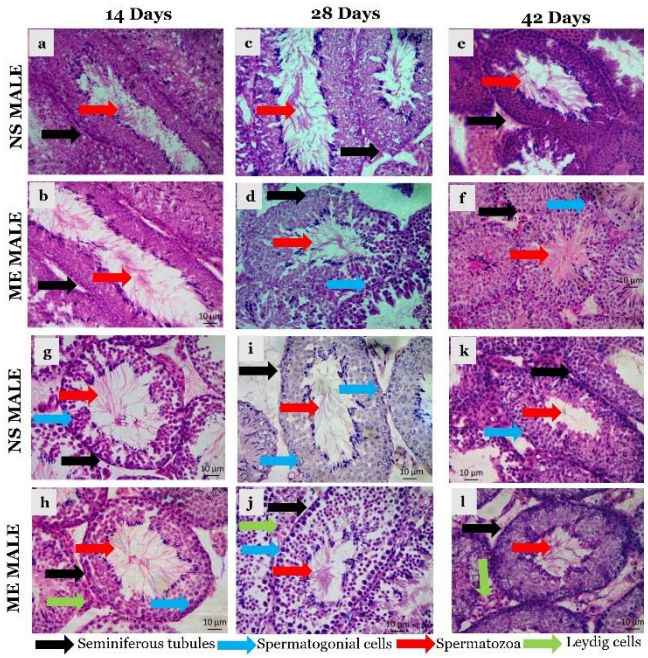


Figure 10: Photographs of T.S. of Testes of Male mice: 3-week old (a, b, c, d, e & f) & 8-weeks old (g, h, i, j, k & l) treated with Methanol Extract (ME) of Seeds of Two Medicinal Plants, *P. roxburghii* and *D. palmatus* at a dose of 100mg/kg body weight for 14 (a, b, g and h), 28 (c, d, i and j) and 42 (e, f, k and l) days. Note the increased number of Sertoli cells, interstitial cells, spermatozoa and Leydig cells with more vascularization in ME treated mice than their respective vehicle-treated (NS) male mice (Photographs = 400X Magnification).

in the body weight (recorded weekly) of vehicle-treated, and ME treated male and female mice groups in both the experiments conducted. In experiment-I, daily vaginal smears of female mice were collected from 6th week onwards until the end of the experiment. In contrast, in experiment II, daily vaginal smears of female mice were collected from the day 01 of the experiment to study the various phases of estrous cycle in vehicle treated and ME treated groups in female mice in both the experiments. The present study showed a prolonged increase in the occurrence of estrus phase ME treated groups in comparison to the NS groups. This may be due to the reproductive values by phytocompounds present in ME of the plants.

Estrous cycle, the reproductive cycle or the breeding cycle of sexually matured female mice last for about 4 days. Unlike menstrual cycle, bleeding doesn't occur during the estrous cycle while the renewal of endometrial surface epithelium is observed. These variations of endometrial surface epithelium and the stages of the estrous cycle can be monitored by studying the cell types through vaginal smear. Based on the cyclic changes, the estrous cycle may be categorized into four phases: Diestrus, Proestrus, Estrus phase (Heat phase) and Metestrus. The characterization of each stage is based on the presence of different cell types observed in the vaginal smear: epithelial cells, cornified cells and leukocytes. Metestrus is characterized by the presence of a mixture of cells, i.e., epithelial cells, cornified cells and leukocytes; diestrus by the presence of leucocyte cells, proestrus mostly by the presence of epithelial or nucleated cells and estrus by the presence of cornified cells only (Figure 7).

3.3. *Liver histoarchitecture*

Further, to rule out any toxicological effect of the ME extract on the body physiology of the mice, liver histology was performed in each experiment. The T.S. of the liver showed no change in its organizational structure in both vehicle-treated (NS), and ME treated male and female mice (Figures 8 and 9). The sections of the liver in all the groups had healthy hepatocytes and showed many lobules, with each lobule separated by connective tissue, the Glisson's capsule. Further, many hepatic plates radiating from the central vein and separated by hepatic sinusoids were also observed in all the groups.

3.4. *Testis histology and sperm count*

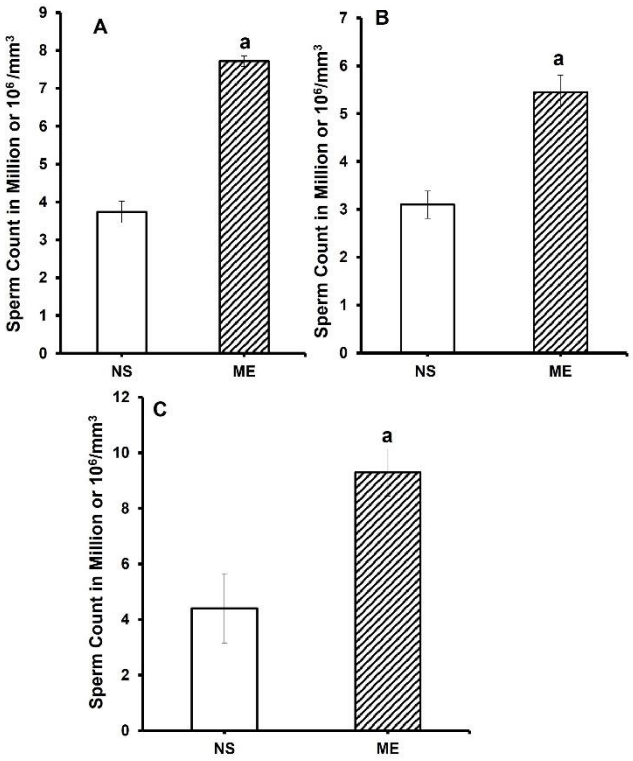


Figure 11: Sperm Count of 3-week old Male mice treated with Methanol Extract (ME) of Seeds of Two Medicinal Plants, *P. roxburghii* and *D. palmatus* at a dose of 100mg/kg body weight for 14 (A), 28 (B) and 42 (C) days and sacrificed at the end of the treatment period. Note the significant increase (bars bearing the alphabet 'a') in the number of sperm count in ME treated mice than their respective vehicle-treated (NS) male mice ($P<0.05$).

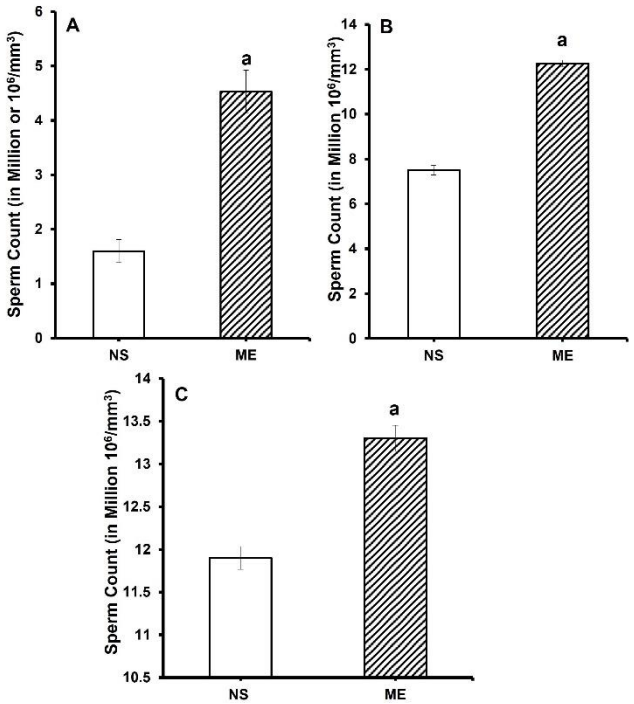


Figure 12: Sperm Count of 8-week old Male mice treated with Methanol Extract (ME) of Seeds of Two Medicinal Plants, *P. roxburghii* and *D. palmatus* at a dose of 100mg/kg body weight for 14 (A), 28 (B) and 42 (C) days and sacrificed at the end of the treatment period. Note the significant increase (bars bearing the alphabet 'a') in the number of sperm count in ME treated mice than their respective vehicle-treated (NS) male mice ($P<0.05$).

The primary reproductive organ of male, testis consists of series of tubules producing testosterone and sperm-producing cells covered by multi-layer tunica vasculosa, tunica albuginea and tunica vaginalis. Testis consists of numerous lobules giving an oval shape to the testis.

Epithelial supporting cells, Sertoli cells acting as hemato-testicular barrier nourishes the growing sperms. Located adjacent to seminiferous tubules interstitial Leydig cells produce various hormones, androgen and testosterone required for the male reproductive development. In experiment-I (3-week-old male mice), T.S. of the testis, in general, after 14, 28 and 42 days of treatment with ME treatment showed an increased number of Sertoli cells, spermatozoa and Leydig cells with more vascularization than vehicle-treated (NS) male mice (Figure 10 a, b, c, d, e and f).

Further, in experiment-I, sperm count was significantly higher ($P<0.05$) in ME administered male mice than vehicle-treated male mice after 14, 28 and 42 days of treatment (Figure 11 A, B and C). Sperm count in experiment-II also showed a similar result with significantly higher ($P<0.05$) count in ME administered male mice than vehicle-treated male mice after 14, 28 and 42 days of treatment (Figure 12 A, B and C). A similar result was also observed in the treated group of experiment-II (8-week-old male mice) with an increased number of Sertoli cells, spermatozoa and Leydig cells with more vascularization than vehicle-treated (NS) male mice after 14, 28 and 42 days of ME and NS treatment (Figure 10 g, h, i, j, k and l).

3.5. *Ovarian and uterus histology*

The female reproductive organ, the ovary, is surrounded by the germinal epithelium layer, tunica albuginea. It consists of an inner medulla comprising of several ovarian follicles at different stages (primary, secondary, and Graafian follicles) and an outer cortex. These follicles secrete hormones, estrogen, and progesterone, that influence stages of the estrous and menstrual cycle in females. In both the experiments, Experiment-I and -II, increased follicular development was observed in ovarian histology after 14, 28,and 42 days of ME treatment in female mice when compared to vehicle-treated (NS) female mice (experiment-I, 3-week-old female mice - Figure 13 a, b, c, d, e and f) and experiment-II, 8-week-old female mice (Figure 13 g, h, i, j, k and l). The uterus is a major female hormone-responsive reproductive organ of humans and of most other mammals. The fetus develops within the uterus during gestation. In general, the T.S. of uterus tissue showed the following histological structure: three layers which are endometrium which is slaughtered out during the reproductive cycle (inner layer), myometrium is the smooth muscle of the uterus (middle layer) and perimetrium is the serous layer (outer layer) with endothelial glands. The T.S. of the uterus in both the experiments showed a similar result as that of T.S. of the ovary with increased proliferation of uterine lumen and numerous epithelial glands in ME-treated female mice than vehicle-treated (NS) female mice uterus (experiment-I, 3-week-old female mice -Figure 14 a, b, c, d, e and f and experiment-II, 8-week old female mice - Figure 14 g, h, i, j, k and l).

4. Discussion

Seeds of two medicinal plants, *P. roxburghii* and *D. palmatus*, with potency to enhance reproductive efficacy in humans, were selected for the present study. The co-administration of powdered form of seeds of these two medicinal plants is believed to enhance the reproductive efficiency in humans (male and female) and act as an aid to gynecological disorders. However, scanty literature is available to prove the above claim; hence, the present study was designed to characterize the ME of the seeds of these two plants and investigate the role of the ME on the reproductive efficiency of male and female mice. The characterization of the methanol seed extracts of these two medicinal plants shows the presence of phytochemicals or phytocompounds, i.e., Tannic Acid, Rutin and Quercetin, besides many other compounds which need to be characterized as observed in the result of the TLC. It is speculated that the fractions obtained through TLC may contain other estrogenic and non-estrogenic phytocompounds, which may be helpful in treating reproductive disorders. However, characterization and analysis of the TLC fractions are under process and this opens up an avenue for future study. There was also a significant rise in sperm count in the males and maintained estrus phase of estrous cycle in the females in the treated groups compared to their controls. Thus, it may be assumed that the ME may lead to an increase in the levels of steroid hormones testosterone and estradiol in male and females, thus enhancing the reproductive ability of the animals.

Further, it may also be hypothesized that the ME of the seeds of these two medicinal plants contains beneficial phytoestrogens (estrogenic and nonestrogenic compounds) which may help histoarchitectural changes in the animals such as increased follicular development in the ovary, the higher proliferation of uterine lumen and the elevated number of Leydig cells in testis. To justify the statement, a chemical literature survey of *P. roxburghii* and *D. palmatus* revealed the presence of saponins (Hariharan, 1974), bioflavonoids (Garg and Mitra, 1971; Varshney et al., 1973), terpenoids (Sengupta et al., 1968; Chopra et al., 1969) and sterols (Chopra et al., 1968), which are helpful in combating reproductive disorders. The phytochemical analysis of *Putranjiva* sps. showed the presence of tannins, saponin, steroid, alkaloids, carbohydrates, clycosides, phenols, fixed oils, coumarins, sterols, terpenoids, starch, calcium oxalate crystals and flavonoids (Chinmaya et al., 2009; Minj and Britto, 2017; Emasushan and Britto, 2018). Moreover, the plant, *P. roxburghii* has a crucial role to play in reproduction as it has been proven as anti-oxidant, febrifuge, anti-inflammatory, biofuel, herbal preservative and a trypsin inhibitor (Supriya et al., 2017). The cytotoxicity study of methanol extract of seeds of *P. roxburghii* revealed the presence of phenols, alkaloids, saponins, steroids, flavonoids and glycosides. The extract showed cytotoxicity with lethal dose 50 (LC_{50}) of 427.74 µg/ml in brine shrimp lethality assay (Raghavendra et al., 2010). *P. roxburghii* has also been referred to as uterine tonic in the available literature as it is believed to provide nutritional support to uterus and maintains endometrial health, normalize menstrual blood flow and help to prepare the uterus by improving the thickness of endometrium for implantation (Rajurkar et al., 2018). Moreover, the plant has been reported to modulate ovarian insufficiency, relieve anxiety, likewise enrich and assist in rebuilding the natural equilibrium of female hormone, which are very much important proceeding, through and following pregnancy. Rahman and Akter (2013) reported that the seeds of *Putranjiva* if given in powdered form along with milk at a dose of 1-3 g help in enhancing the sperm count in males and maintaining the fetus in pregnant females. The use of this plant species as antipyretics, anti-rheumatic and as an anti-inflammatory and its role in fertility and gynecological ailments have also been discussed and mentioned in the Ayurveda (Gupta, 2016).

Likewise, the phytochemical screenings of *D. palmatus* has also been carried out to some extent and have identified the presence of steroids (β-sito sterol) content in the plant product (Swapna et al., 2014). Traditionally *D. palmatus* have been used as an antipyretic, analgesic, anti-inflammatory, anti-microbial (Swapna et al., 2014). Further phytochemical characterization of petroleum, ether and benzene leaf extracts of this plant species have shown the presence of tannins, triterpenoids, phenols, anthraquinones and flavonoids besides carbohydrates, proteins and amino acids, alkaloids, glycosides etc. (Vijayashalini et al., 2016). The metabolites present in the plant extract showed a positive response to steroids and sterols by petroleum extract, and no response to steroids and sterols was reported from benzene extract (Vijayashalini et al., 2016). In rats, oral administration of different doses of ethanol seed extract of this plant for28 days has been shown to have significantly increased the sperm count and spermatogenesis in the testes with a simultaneous increase in the level of serum luteinizing hormone and testosterone (Chauhan and Dixit, 2010). This suggests that the seed of *D. palmatus* (*shivlingi beej*) has an effect on the hypothalamo-hypophyseal-gonadal (HPG) axis along with androgenic activity in the animals (Chauhan and Dixit, 2010). Further, it has also been demonstrated that the seeds of the *shivlingi beej*, when used in combination with root bark of *Vata* (*Ficus bengalensis*), pepper, *putrajivi*, ginger (dry) and milk may act as a potential contraceptive (Shukla et al., 2008). *Shivlingi* seeds have also been described to show abortifacient action if consumed with sugar and milk, *bhawda, amala* or administered in combination with an identical amount of *ashwagandha* roots (Patil and Bhaskar, 2006). It is also postulated that *putranjeevak beej* if administered in combination with *shivingi beej* is an excellent fertility enhancer herb.

Plant-derived antioxidants, such as free radicals are an aid to animal reproduction, suggesting their advantageous or harmful effects in animal reproduction, including semen functions, spermatogenesis, estrous cycles, ovary functions, endometrium, embryo development, ovulation, and pregnancy (Zhong and Zhou, 2013). Natural antioxidants have lower side effects than those of synthetic

antioxidants; hence plants or their extracts have been extensively utilized in animals. Among plant molecules, quercetin has been increasingly used in experimental studies (Pu et al., 2007). Quercetin acts as a prooxidant or an antioxidant depending on its concentration which is suggestive of its hermetic behavior with lower doses displaying mostly antioxidant properties (Watjen et al., 2005). It has also been reported that quercetin counters the cytotoxic effect in Sertoli-germ cell cultures due to atrazine (Abarikwu et al., 2012). Similarly, quercetin also prevents the oxidative damage caused by diethylstilbestrol to spermatogenic cells in hamsters (Li et al., 2010). However, contradictory to the above, quercetin administration to male mice have also been reported to increase the lipid peroxidation and generation of reactive oxygen species in the testis with a simultaneous decrease in sperm count and motility of sperm in a dose-dependent manner (Ranawat et al., 2013). In vitro and in vivo experiments with quercetin and its effect on male fertility are controversial, with reports of impairment of male fertility both in humans and animal models (Aravindakshan et al., 1985; Khanduja et al., 2001; Ranawat et al., 2013) or its utilization as an alternative drug for the therapy of male infertility - improvement of the sperm quality in rats (Taepongsorat et al., 2008). Moreover, quercetin in combination with FSH has been reported to increase the release of testosterone (T) in cultured granulosa cells of porcine ovary (Sirotkin et al., 2019).

Quercetin supplemented diet (QU-HS) or non-supplemented (HS) diet on follicle population, apoptosis, in vitro maturation rate and quality of oocytes were also evaluated in New Zealand White heat stress (HS) exposed female rabbits. The results showed higher follicle number, retrieved oocytes and A-grade oocytes in QU-HS compared to the HS diet. It was also observed that quercetin helps in improving

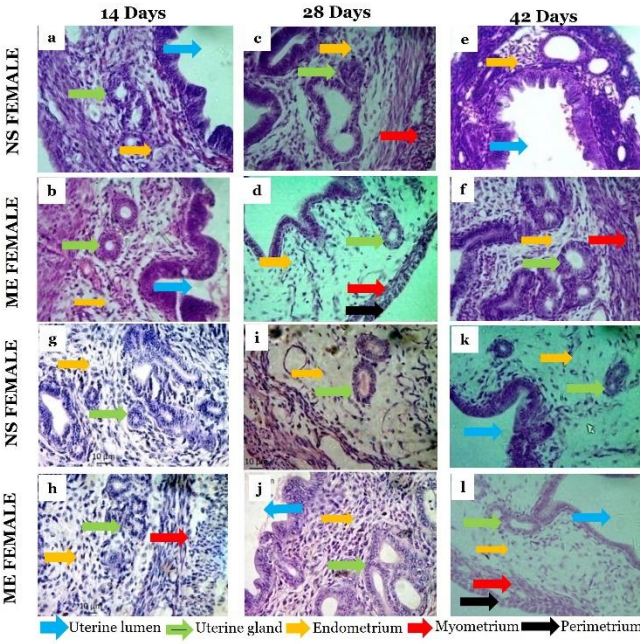


Figure 14: Photographs of T.S. of Uterus of 3-week old (a, b, c, d, e & f) and 8-week old (g, h, i, j, k & l) Female mice treated with Methanol Extract (ME) of Seeds of Two Medicinal Plants, *P. roxburghii* and *D. palmatus* at a dose of 100mg/kg body weight for 14 (a, b, g and h), 28 (c d, i and j) and 42 (e, f, k and l) days. Note the increased proliferation of uterine lumen with numerous epithelial glands after 14, 28 and 42 days of ME treatment in female mice than their respective vehicle-treated (NS) female mice. (Photographs = 400X Magnification).

the follicular development with a smaller number of apoptotic granulosa cells, and maintains the oocyte competence in HS rabbits (Naseer et al., 2017). The beneficial action of quercetin on health has also been reported due to its antiproliferative, anticarcinogenic, cardio-protective properties, antioxidant, antiaging and anti-inflammatory properties (Chen et al., 2010; Anand et al., 2016; Sharma et al., 2018). However, there is little knowledge on the nature and mechanism of quercetin's influence on reproduction, and the available data is debatable. The stimulatory effects of quercetin on ovarian function have been noted in numerous articles. In the mouse (Shu et al., 2011; Beazley and Nurminskaja, 2016) and rabbit (Beazley and Nurminskaja, 2016) feeding with quercetin decreased ovarian

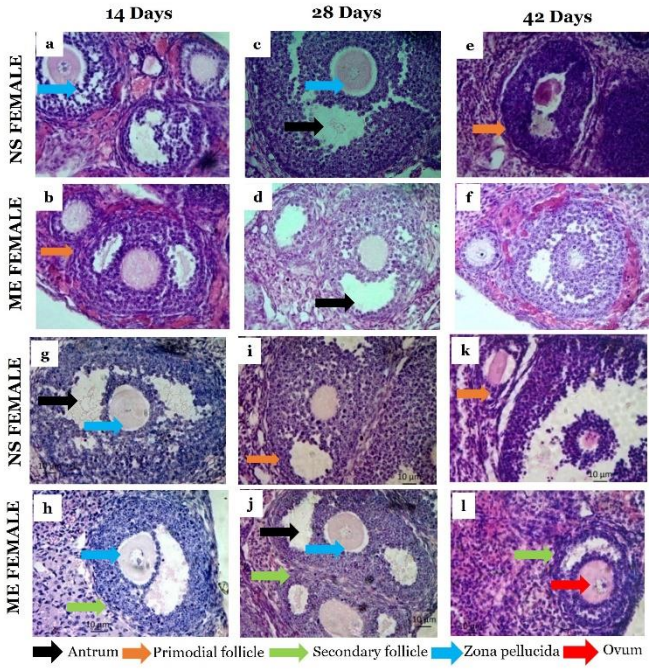


Figure 13: Photographs of T.S. of Ovary of 3-week old (a, b, c, d, e & f) and 8-week old (g, h, i, j, k & l) Female mice treated with Methanol Extract (ME) of Seeds of Two Medicinal Plants, *P. roxburghii* and *D. palmatus* at a dose of 100mg/kg body weight for 14 (a, b, g and h), 28 (c, d, i and j) and 42 (e, f, k and l) days. Note the increased follicular development in ovarian histology after 14, 28 and 42 days of ME treatment in female mice than their respective vehicle-treated (NS) female mice. (Photographs = 400X Magnification).

cells apoptosis, promoted ovarian cell proliferation, improved ovarian folliculogenesis, and boosted ovarian weight, oocyte quality and litter size (Nasser et al., 2017). Other research, however, revealed quercetin suppresses ovarian function. Old mice fed with quercetin showed increased ovarian follicular atresia, decreased ovarian follicle development and ovulation, altered gonadotropin secretion, and smaller litter size (Shu et al., 2011).

According to in vitro research, quercetin may directly decrease the activity of the aromatase enzyme and the release of progesterone (P4) in cultured ovarian granulosa cells (Santini et al., 2009). Some reports suggest the role of quercetin in directly suppressing granulosa cell steroidogenesis. Quercetin also has beneficial effects on sperm viability, motility and serum total testosterone in diabetic rats (Khaki et al., 2010). However, studies by Nass-Arden and Breitbart (1990) and Khanduja et al (2001) on sperm obtained from healthy volunteers have shown that quercetin impaired the viability and motility of sperm in vitro. There are conflicting reports on whether quercetin has a pro-oxidant or antioxidant effect on male reproduction and fertility (Ranawat et al., 2013). Some authors reported that quercetin has a deleterious effect on male reproductive function through its pro-oxidant effect (Farombi et al., 2013; Ranawat et al., 2013) and the other mechanism of action (Khanduja et al., 2001); and many other researchers reported quercetin to have improvement effect on male reproductive dysfunction utilizing its antioxidant activity (Ciftci et al., 2012; Kanter et al., 2012; Moretti et al., 2012; Zribi et al., 2012) and its other effects (Taepongsorat et al., 2008; Abarikwu et al., 2012).

Tannins and its function in reproductive regulation are not well understood yet. Tannins including tannic acid have antioxidant (Koleckar et al., 2008; Serrano et al., 2009; Hamiza et al., 2012), antimicrobial (Buzzini et al., 2008; Serrano et al., 2009; Payne et al., 2013) antiviral (Buzzini et al., 2008; Serrano et al., 2009), anti-inflammatory (Holderness et al., 2008) and anti-tumour (Sun et al., 2012; Chang and Wang, 2013) properties. The application of tannic acid on human is restricted because it can form insoluble complexes with precipitate proteins, alkaloids, glycosides, and heavy metals. However, scientists have proven the role of tannic acid in many biological functions which have applications to improve human health, including treatment of ulcers, burns, haemorrhoids, stomatitis, tonsillitis, and pharyngitis. Rutin, an important flavonoid in the pharmaceutical industry, acts as an essential antioxidative, neuroprotective, immunomodulatory properties, antitumor, anti-

inflammatory, antidiarrheal, vasodilator, anti-ulcer, antimutagenic, anti-diabetic, anti-adipogenic and antibacterial, because of its pharmacological effects (Buszewski et al., 1993; Chua, 2013). Although the strong anti-oxidative capacity, particularly for excellent scavenging activity of rutin has been proven by numerous studies (Chua, 2013), its availability in protecting male reproductive function as an antioxidant is still unknown known (Atanassova and Bagdassarian, 2009; Ugusman et al., 2014). Protective effects of rutin against reproductive toxicity have also been confirmed by previous studies (Abarikwu et al., 2013).

5. Conclusion

In the present study, the administration of the ME of *Putranjiva roxburghii* seeds (*putranjeevak beej)* and *Diplocyclos palmatus* seeds (*shivlingi beej)* at the dose of 100mg per kg body weight on 3-week and 8-week-old albino mice showed no toxicity on hepato-architecture. At the same time, it showed positive response to the treatment in the testis, ovarian and uterine histology with increased spermatogenesis, sperm count, ovarian and uterine development. Prolonged maintenance of estrus phase of the estrous cycle in the ME group was observed, which may be attributed to the increase in the levels of estradiol in the females. The observed positive changes of the ME extract in both male and female mice may be due to the presence of the phytocompounds (phytosteroids or flavonoids) in the ME of the seeds of the two selected medicinal plants, thus helping the animals in enhancing the reproductive efficiency. However, further extensive research to characterize the compounds present in the ME of the seeds of the two selected medicinal plants and its physiological effect in both the male and female mice needs to be ascertained. Proper screening of phytocompounds and its impact is mandatory before the formulation of fertility drug to provide hope for thousands of individuals dealing with infertility issues. This work may also be a model study for further investigations for researchers planning to work in this field and may be a reference of hope for patients depending on herbal plants to treat a number of reproductive related ailments. The present findings provide some evidence for the potential application of these two medicinal plants, *P. roxburghii* and *D. palmatus* for the treatment of reproductive-related health issues.

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Authors' Contributions

PB, MY and PK conceptualized the work. PB and HK performed all the experiments, while SK performed the TLC and HPLC analysis. PB, MY, PK and BL analysed the results. PB, MY and PK wrote the first draft of the manuscript. MY, PK and BL edited the manuscript. All the authors read and approved the final version of the manuscript. PK supervised the work.

Conflict of interests

The authors have no conflict of interest.

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