3

Experimental Procedure

This study was conducted in five phases. The first phase assessed the impact of environmental factors and lifestyle habits on semen parameters. The second phase was to study the *invitro* antioxidant, anti-inflammatory and cytoprotective properties of various extracts of dry and fresh petals of *R. indica.* In view with the results from phase II, the extracts were studied for their alleviating effects on steroidogenesis and spermatogenesis and their outcome on sperm quality in heat stress-induced male Wistar rats in phase III. Phase IV included the quantification of phytocompounds of *R. indica* petals extracts, followed by characterization of the extract using GC-MS analysis and HPTLC analysis. The protective role of ethanol and aqueous extract of *R. indica* petals on lifestyle-induced oxidative and heat stress on semen parameters was further validated using *in silico* approach in Phase V.

This following section briefs the experimental methods used in each phase of the study titled "*In vitro, in vivo* and *in silico* approaches to assess the protective effect of *Rosa indica* petals on male infertility".

PHASE I

3.1. Impact of environmental factors and the lifestyle habits on semen parameters

3.1.1. Study participants

A retrospective cross-sectional data was collected between February 2017 and November 2019, focusing on men of reproductive age who sought medical help for fertility issues while accompanied by their female partners at private hospitals in Coimbatore, Tamil Nadu. The Human Ethics Committee of Avinashilingam Institute for Home Science and Higher Education for Women (AUW/IHEC/BCBT- 1920/XPD-01) granted approval for this study. The study involved a retrospective analysis of environmental factors, lifestyle variables (nature of job, addiction), and semen parameters obtained from a sample of 299 participants. These data were then correlated with the criteria outlined in the WHO guidelines from 2021 (6^{th} Edition) to assess their impact on semen parameters.

In this study, the following exclusion criteria were applied: men who had illnesses, diabetes, neurological issues, or had undergone corrective surgeries related to reproductive problems were not included. The structured questionnaires included were environmental and lifestyle parameters such as age, sexual abstinence, date of sample collection, addictions (alcohol consumption, smoking) and nature of job (sitting hours).

3.1.2. Sample collection and semen analysis

The participants, by masturbation method, provided the semen samples into a sterile container for analysis. The analysis of the semen samples was carried out according to WHO's approved method. The semen parameters that were examined includes pH, volume, liquefaction, sperm concentration, motility and morphology (Appendix I, II and III).

3.1.3. Data analysis

The semen data were analysed using IBM Statistical Package for Social Sciences (SPSS) version 29.0.0.0 and the variables expressed as median and

Interquartile Range (IQR). The normality of the data was assessed using the Kolmogorov-Smirnov and Shapiro-Wilk tests. The results indicated that the data significantly deviated from a normal distribution, as the significance value was less than 0.05. The correlation analysis was performed using Spearman's correlation coefficient. Mann-Whitney test was performed to understand the association between semen parameters and life style and Kruskal-Walli's test was performed for season. A *p*-value of < 0.05 was considered statistically significant.

PHASE II

3.2. *In vitro* antioxidant, anti-inflammatory and cytoprotective effect of *R. indica* petal extracts

3.2.1. *Selection* of plant materia

Rosa indica is the perennial flower and in the Indian medicine system, the various rose preparations are used for various ailments. Fresh flowers of *R. indica* were procured from local market in Coimbatore. The rose plant was validated and authenticated by the "Institute of Forest Genetics and Tree Breeding, Coimbatore, Tamil Nadu and assigned an authentication number 924/FECC/ID/IFGTB/2020" (Plate 2). The scientific classification of the *R. indica* use for the present study are shown below.

Classification of *R. indica*

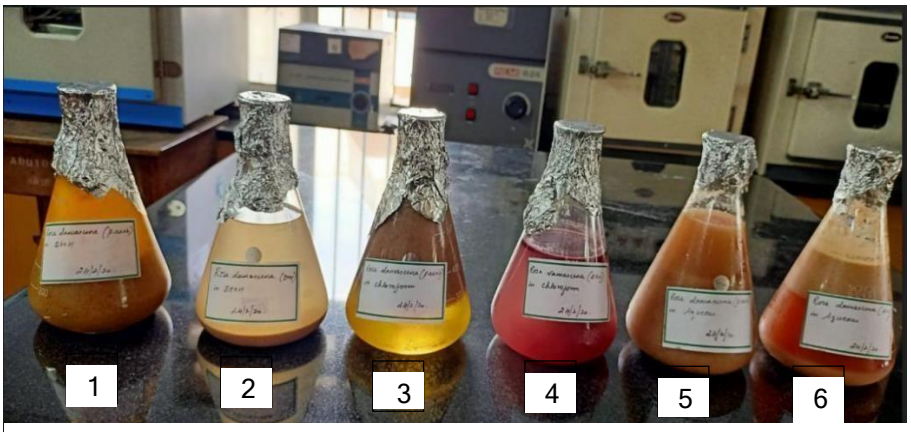
Class - Mangoliopsida
Subclass- Rosidae
Order - Rosales
Genus - *Rosa*
Species - *indica*

Plate 2

Rosa indica flower

3.2.2. Preparation of extract and screening for phytocompounds

Fresh flowers of *R. indica* were bought from the local flower market of Coimbatore, rinsed twice with tap water to eliminate the contaminants in the flowers and dried using blotting paper. Fresh and shade dried petals (100 g each) petals were extracted with 500 ml of respective solvents: double distilled water, 100% ethanol and 100% chloroform for the preparation of extract. After 48 hours of incubation at 4ºC the filtrate was collected by filtering the extract using cheese cloth. The extract was then lyophilized and maintained at 4ºC for further studies. The resultant solid extract was calculated to find the yield (Plate 3).



1. Ethanolic extract of fresh petals
2. Ethanolic extract of dry petals
3. Chloroform extract of fresh petals
4. Chloroform extract of dry petals
5. Aqueous extract of fresh petals
6. Aqueous extract of dry petals

Plate 3: Extracts of *R. indica* petals

The aqueous, ethanolic and chloroform extracts of fresh and dry petals of *R. indica* was qualitatively screened for phytocompounds including sugars, alkaloids, flavonoids, phenols, sterols, anthocyanins, tannins, saponins, proteins and volatile oils by following the standard procedures - Appendix IV.

3.2.3. Evaluation of antioxidant activity of the extracts

20-100 µl of 1 mg/mL concentration of the extracts of dry and fresh petals of *R. indica* were studied for their *in vitro* antioxidant, anti-inflammatory and cytoprotective properties using standard procedure.

Antioxidant activity

1.	DPPH	Mensor *et al.,* (2006)	Appendix V
2.	ABTS	Shirwaikar *et al.,* (2006)	Appendix VI
3.	Hydroxyl radical	Elizabeth and Rao (1990)	Appendix VII
4.	Nitric oxide radical	Green *et al.,* (1982)	Appendix VIII
5.	Hydrogen peroxide radical	Ruch *et al.,* (2989)	Appendix IX
6.	Reducing power	Oyaizu (1986)	Appendix X

3.2.4. Anti-inflammatory activity Mizushima *et al.,* (1986) Appendix XI

3.2.5. Cytoprotective activity

The MTT assay [3(4,4-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was used to measure cellular metabolic activity as an indicatory of cell viability. The TM3 Cell lines (mouse Leydig cells derived from 11-13 day mouse testis) procured from NCCS, Pune were exposed to different concentrations (50, 100, 150, 200 and 250 μg/ml) of the aqueous and ethanolic extracts of fresh and dry petals of *R. indica* separately, to determine the optimal dose and cell viability as described by Igarashi and Miyazawa (Appendix XII).

PHASE III

3.3 In vivo studies on the alleviating effect of various extracts of fresh and dry petals of R. indica on heat stress-induced male Wistar rats

3.3.1. Maintenance and grouping of experimental animals

The study was conducted on three months old male Wistar rats with the mean weight of 302 ± 8.14 g. The rats were maintained in a controlled hygienic environment at room temperature with the constant 12 h light/dark schedule. They were fed with standard healthy diet *ad libitum.* All the experiments were conducted after obtaining animal ethics clearance (approval number: COPSRIPMS/IAEC/PHD/PROJECT/001/2020-2021). Thirty animals were used for the present study in 5 groups (6 in each group) for 28 days (7 days for acclimatization and 21 days for study). Heat stress was induced by immersing hind legs, tail and scrotum in 43°C thermostatically controlled water bath for 30 min for

6 consecutive days from day 8 of the treatment. In order to assess the effectiveness of the plant extract as an antioxidant and anti-inflammatory potential, it is crucial to have a positive control for comparison. Quercetin, a well-established antioxidant with documented anti-inflammatory properties, was employed as the positive control in this study.

The experimental groups are as follows:

Group I	Feed only (control)
Group II	Heat stress induced (negative control)
Group III	Heat stress induced + 50 mg/kg quercetin (Positive control)
Group IV	Heat stress induced + 200 mg/kg of ethanolic extract
Group V	Heat stress induced + 200 mg/kg of aqueous extract

After sacrificing the animals under ketamine, I.P 100 mg/kg on 28th day the following studies including serum testosterone, testis weight, semen parameters, histopathology of the testis and *in vivo* antioxidant assays including CAT, GSH, GPx, SOD and MDA were performed.

3.3.2. Estimation of body weight and testis weight of the animals

The animals were weighed before and after the experiment (28th day of the study). Testis was dissected and subsequently weighed.

3.3.3. Estimation of serum testosterone

The collection of blood from the heart was done through cardiac puncture and subsequently, the blood was centrifuged at 3000 rpm for 10 minutes at 4°C. The resulting serum was used to measure the level of testosterone (T) using a specific ELISA kit for rats from ROCHE, by following the manufacturer's instructions.

3.3.4. Analysis of semen parameters

Cauda epididymis was dissected and minced in MOPs buffer to release the sperm to study the sperm quality including sperm concentration (M/ml),

progressive motility (%) and normal forms (%) using inverted microscope as given in Appendix III.

3.3.5. Assessment of *in vivo* antioxidants activity of the extracts

In vivo antioxidants in testis tissue homogenate were studied from different group of animals.

Catalase	Luck, 1974	Appendix XIII
Glutathione peroxidase	Rotruck *et al*. (1977)	Appendix XIV
Glutathione reductase	Moron *et al*. (1979)	Appendix XV
Superoxide dismutase	Misra & Fridovich (1972)	Appendix XVI
Lipid peroxidation	Ohkawa*et al*. (1979)	Appendix XVII

3.3.6. Histopathology analysis

Testis excised from the experimental animals were preserved in 10% formalin and fixed in paraffin for histopathology studies and viewed under light microscope under the magnification of 10X and 40 X. The microtome sections of 6 µm thickness of the testis samples from different groups were stained with haematoxylin-eosin for histopathological observation according to Culling, (1974).

PHASE IV

3.4. Quantification and validation of phytocompounds of *R. indica* petal extracts

3.4.1. Quantification of phytocompounds

The quantification of alkaloids, flavonoids, phenolics and phytosterols of the studied extracts of fresh and dry petals of *R. indica* were analysed by following standard procedures as given in Appendix XVIII, XIX, XX and XXI.

3.4.2. HPTLC analysis

The phytochemical profiling of all the extracts of *R. indica* fresh and dry petal extracts were performed using CAMAG Linomat 5 HPTLC system with silica gel 60 F 254 as stationary phase. Mobile phase was toluene : ethyl acetate : diethylamine for alkaloid; ethyl acetate : formic acid : acetic acid : water for

flavonoids; cyclohexane : ethyl acetate : formic acid for phenol; n-hexane : ethyl acetate for terpenoids along with the standards cochicine, rutin, gallic acid, oleanolic acid respectively and identified at different wavelengths.

3.4.3. GC-MS analysis

The extracts of *R. indica* were subjected to GC-MS analysis using the Agilent GC-MS system. 1 µl of 1mg/mL of different extracts were injected into a gas chromatography column. The compounds were identified with peak area and retention time.

PHASE V

3.5. *In silico* studies on the interaction of various phytocompounds on selected targets

3.5.1. Selection of targets

Cyclooxygenase-2 (COX-2) is important for the production of prostaglandin, which are hormone-like substances that play a role in inflammation and pain (Gambera *et al.,* 2007). Heat stress can cause an increase in the level of COX-2 in the body. Inhibiting COX-2 activity can reduce inflammation and pain. The three-dimensional structure of the COX-2 with PDB ID: 5IKT with the resolution of 2.54 Å (Orlando and Malkowski, 2016) was selected for the present study.

Androgen receptor (AR) plays a crucial role in male fertility by regulating the development and function of the male reproductive system. In the testis androgen receptor activation promotes spermatogenesis as well as the maturation and function of sperm cells (Kumar *et al*., 2016). Heat stress reduce androgen receptor expression. Exposure to high temperatures can lead to heat shock protein (HSP) expression which can interfere with androgen receptor signaling (Chen *et al*., 2019). The three- dimensional structure of the AR with PDB ID: 5VO4 with the resolution of 2.35 Å was downloaded from PDB database for molecular docking studies (Unwalla *et al*., 2017).

AKT1 is also known as protein kinase B, important for various cellular process such as growth, proliferation, differentiation and survival. AKT1 is also involved in the regulation of testosterone and regulates the activity of steroidogenic acute regulatory protein (StAR) which is necessary for the

spermatogenesis. The PDB structure 6HHF of AKT1 with the resolution of 2.5 Å was selected for the current study (Landel *et al*., 2020).

StAR protein is necessary for the transport of cholesterol into the mitochondria of Leydig cells in the testis. Further, induce the production of testosterone level. The StAR protein with PDB ID 3P0L was selected for the evaluation interaction of phytocompounds with target proteins (Thorsell *et al*., 2011).

3.5.2. Protein preparation

Before performing molecular docking against phytocompounds, the target protein was pre-processed using Protein Preparation Wizard of Schrödinger's suite 2022-4. The following process were done during protein preparation, namely, addition of hydrogen atom, assigning partial charges, using OPLS-2005 force field, protons ion states and restrained minimization in vacuum.

3.5.3. Ligand preparation

The three-dimensional structure of compounds identified through GC-MS profiling of *R.* indica petal extracts were downloaded from PubChem database. All the compounds were prepared using LigPrep (Schrödinger suite 2022-4) before performing docking process against the target protein.

3.5.4. ADMET studies

The pharmacokinetic and the pharmacodynamic properties of the phytocompounds were analysed using Schrödinger suite 2022-4 module.

3.5.5. Molecular docking of phytocompounds with target proteins

The molecular docking study was carried using Glide (Grid based ligand), a Schrödinger suite 2022-4 module. Glide will identify the possible location of the ligand within the reactive site region of the target and the interaction of the phytocompounds against target protein validate the steroidogenic and anti-inflammatory properties (Appendix XXII).

3.6. Statistical examination

The data was subjected to statistical analysis using one-way analysis of variance (ANOVA) to determine its significance at *p*-value <0.05.