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7

Appendices

Appendix I

Semen collection

Semen analysis is the basic test performed to assess the male fertility.

Disposables

100 ml sterile sample container

Procedure

It was made sure that the serology of the participants was negative before starting the semen analysis

1. The sample container was labelled with male and female partner's name, age, ID number, date, days of abstinence.
2. The above details were recorded in the sample collection register with the participant's signature and consent.
3. The subjects were given clear instruction about genital hygiene and importance of the sample collection without any spillage while handing over the collection container.
4. It was made sure that there was no sample spillage while getting the sample
5. The time of sample collection and time of sample arrival to the lab was noted down in the register
6. The participants were instructed not to use spermicidal condoms, creams or lubricants before sample collection

Appendix II

Semen analysis (WHO 2021)

Disposables

1. 100 ml sterile sample container
2. 3 ml sterile pasteur pipette
3. Glass slide
4. Cover slip
5. Makler chamber

Equipment

1. Vertical laminar air flow
2. Test tube warmer
3. Automated pipette- 20 µl

Procedure

1. The container was left in the test tube warmer at 37℃ for at least 15 min after receiving the sample
2. Patient identification was checked before starting the procedure
3. Liquefaction time of the sample was registered (time of analysis-time of production). It should never exceed 60 min
4. Volume of the sample was determined with a graduated pipette
5. Viscosity was scored as low (if the sample fell from the glass rod drop by drop) or high (if the drop is > 2 cm long)
6. If the sample was too viscous, it was liquefied by aspirating it several times through a
7. 23 G needle. If the sample was still viscous, it was diluted by mixing the sample with HEPES/MOPS based buffer
8. Colour was either gray opalescent, yellow, transparent, white or red (in rare cases)
9. pH was assessed using pH paper (should be between 7.2 to 8.0)
10. Presence or absence of round cells was noted (if >$1x10^6$ /ml, microbiological test is recommended)
11. Presence of agglutination and/or aggregation was noted

12. After liquefaction, 10 µl of the well mixed sample was transferred to the center of the Makler chamber or a Neubaur chamber (if concentration was $<5x10^6$ M/ML)
13. To calculate the concentration in Makler chamber, 10 squares were counted thrice and an average was taken
14. To calculate the concentration in Neubauer chamber, the central 25 squares were counted and the number was divided by 100 to get the concentration in M/ML
15. While assessing the concentration, the motility was also determined according to their movement pattern and divided as A,B,C,D (A- Rapidly progressive, B- Slow progressive, C- Sluggish progressive, D- Non motile)
16. Morphology staining was done if the concentration of the sample was >1x106 m/ml (as per SOP for staining)

Appendix III

Sperm Morphology Analysis

Diff-Quik Staining

The kit contains a fixative solution, solution I (Eosin Y) an anionic dye and solution II (Thiazine) a cationic dye consisting of methylene blue and azure A.

Reagents required

1. Diff quik stain kit
2. Light mineral oil

Consumables required

1. Coupling jar
2. Glass slide

Procedure

1. Morphology analysis was performed after analysing the semen for motility and concentration
2. The liquified semen sample was prepared as a smear on the glass slide and allowed to dry

3. The dried smear was dipped in a jar containing fixative solution, excess solution bloat dried and allowed to air dry
4. The air dried, smeared sample was dipped for 1 min in solution I followed by 1 min in solution II
5. The slide was then rinsed gently with distilled water and observed under oil immersion
6. 100 spermatozoa were counted for their defects in head, neck, mid piece, tail and also for cytoplasmic droplets
7. Percentage of normal forms were calculated from the total counted sperm and defective sperm

Appendix IV

Preliminary phytochemical screening (Harborne *et al.,* 2010)

Detection of Alkaloids

A fraction of the extract was treated with Mayer's reagent (1.36g of mercuric chloride and 5g of potassium iodide in 100ml of distilled water) and observed for the formation of crean coloured precipitate.

Detection of phenols

To a fraction of theextract, 5% Fecl3 solution was added and obseved for the formation of deep blue colour

Detection of Flavanoids

To a fraction of the extract, a drop of 1N aqueous NaOH solution was added and observed for the formation of yellow orange colouration

Detection of tanin

For detection of treated with a few drops of lead solution. The formation of whit precipitate indicates the presence of tannin

Detection of saponins

In a teat tube,he presence of about 5ml of extract was added and a drop of sodium bicarbonate was added. The mixture was shaken vigorously and kept for 3 minutes. The formation of a honey comb like froth showed of saponins

Detection of terpenoids

5ml of extract was mixed with 2ml of chloroform and conc.H_2SO_4 (3m) was carefully added to form of layer. A reddish-brown colouration of the interface formed to show positive for the presence of terpenoids.

Detection of steroids

A portion of the extract was treated with 10ml chloroform and filtrated. To 2m of filtrate was treated with 2ml of acetic anhydride and conc. Sulphuric acid. Blue, green ring indicates presence of steroids

Test for phytosterols

To 1ml of plant extract, equal volume of chloroform and 3 drops of concentrated sulfuric acid were added. Formation of brown ring indicates the presence of steroids and formation of bluish green colour indicated the presence of phytosterols

Test for proteins

To 2 ml of each extract, 1 ml of 40% sodium hydroxide and few drops of 1% copper sulphate were added; formation of violet colour indicates the presence of peptide linkage molecules in the sample extract.

Test for carbohydrates

Take 1 ml of extract, add few drops of Molisch's reagent and then add 1 ml of concentrated sulphuric acid at the side of the tubes. The mixture was then allowed to stand for 2 to 3 minutes. Formation of red or dull violet colour indicates the presence of carbohydrates in the sample extract.

Appendix V
DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging assay (Mensor *et al*., 2001)

The scavenging ability of the Sample towards the relatively stable DPPH free radical was measured by the method of Mensor *et al*. (2001).

Principle

Antioxidants react with DPPH (2,2-diphenyl-1-picryl hydrazyl) and convert it to α,α'-diphenyl-β-picryl hydrazine by donating its OH group. This can be identified by the conversion of purple colour to yellow colour, which can be quantified at 518nm.

Reagents

1. DPPH – 2,2-diphenyl-1-picryl hydrazyl (0.3mM in methanol)
2. Methanol

Procedure

To 20-100µ*l* of the sample, 0.5ml of a methanolic solution of DPPH and 0.48 - 0.40ml of methanol were added (final volume made up to 1ml). The mixture was allowed to react at room temperature for 30 minutes in the dark. Methanol served as a blank and DPPH in methanol, without sample served as the positive control. After 30 minutes of incubation, the decolourization of the purple to yellow colour was measured at 518 nm.

Appendix VI

ABTS radical scavenging assay

(Shirwaikar *et al*., 2006)

Principle

The antioxidant effect of extracts were determined using ABTS radical cation decolorization assay according to the method of Shirwaikar *et al*. (2006). ABTS is a chromogen which changes into a coloured mono cation radical form (ABTS+) has an absorption peak at 745 nm. Antioxidants reduce (ABTS+) into its colourless form and the extent of decolourisation is proportional to the percent reduction of (ABTS+)

Reagents

1. ABTS - 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
2. Ammonium persulfate
3. Ethanol

Procedure

The ABTS radical cation (ABTS+) was produced by reacting ABTS solution (7mM) with (2.45mM) ammonium persulfate and the mixture was allowed to stand in dark at room temperature for 12 – 16 hours before use. To the Sample (20-100µ*l*), 0.3ml of ABTS solution was added and the final volume was made up to 1ml with ethanol. Ethanol served as a blank and ABTS in ethanol without sample served as the positive control The absorbance (A) was read at 745nm and the percentage inhibition was calculated as follows,

% scavenging activity = Ac-As/Ac×100

Where Ac is the absorbance of the control As is the absorbance of the sample

Appendix VII

Hydroxyl radical scavenging assay (Elizabeth and Rao 1990)

The effect of sample on oxidant-induced damage to deoxyribose *in vitro* was quantified as the amount of thiobarbituric acid reactive substances(TBARS) formed, according to the procedure explained by Elizabeth and Rao (1990).

Principle

The principle of the assay is the quantification of 2-deoxyribose degradation product, malondialdehyde, by its condensation with thiobarbituric acid.

Reagents

1. Deoxyribose (2.8 mM)
2. Ferric chloride (0.1mM)
3. EDTA (0.1mM)
4. H_2O_2 (1mM)
5. Ascorbate (0.1mM)
6. KH_2PO_4-KOH (final strength 20mM, pH 7.4)
7. Thiobarbituric acid (1%)

Procedure

The reaction mixture contained 0.1ml of deoxyribose, 0.1ml of ferric chloride, 0.1ml of EDTA, 0.1ml of H_2O_2, 0.1ml of ascorbate, 20-100 µl of sample and the final volume was made up to 1ml with KH_2PO_4-KOH buffer. The reaction mixture was incubated at 37°C for 1 hour. At the end of the incubation period, 1ml of thiobarbituric acid (TBA) was added and heated at 95°C for 20 minutes to develop the colour. After cooling, thiobarbituric acid reactive substances (TBARS) formation was measured spectrophotometrically at 532nm against an appropriate blank. The hydroxyl radical scavenging activity was determined by comparing the absorbance of the control with that of the fraction. The extent of TBARS production for the positive control (H_2O_2) was fixed at 100% and the relative percent TBARS was calculated for the treated groups.

Appendix VIII

Nitric oxide inhibition assay (Green *et al*., 1982)

The method reported by Green *et al*. (1982) was employed to test the inhibition of *in vitro* generation of nitric oxide by the test sample.

Principle

An aqueous solution of sodium nitroprusside spontaneously generates nitric oxide at physiological pH, which interacts with oxygen to produce nitrite ions, which are measured at 546 nm.

Reagents

1. Sodium nitroprusside (100mM)
2. Phosphate buffered saline (PBS) (pH 7.4)
3. Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride)

Procedure

Sodium nitroprusside (2.0ml), phosphate buffered saline (0.48- 0.40 ml) (final volume made up to 3ml) and the Sample (20-100µl) were mixed and incubated at 25ºC for 30 minutes. Griess reagent (0.5ml) was added and allowed to stand for 30 minutes. The control tube was prepared without the fraction. The absorbance of the pink coloured chromogen was read at 546nm against a reagent blank and the percent inhibition was calculated.

Appendix IX

Hydrogen peroxide radical scavenging activity (Ruch *et al*., 1989)

Principle

H_2O_2 scavenging activity was measured in terms of a decrease in the absorbance at 230nm spectrophotometrically.

Reagents

1. H_2O_2 (40mM in 0.1M phosphate buffer)
2. Phosphate buffer (0.1M, pH 7.4)

Procedure

The plant extract was diluted to a concentration of 10mg in 10µl. This extract (10µl corresponding to 10mg) was added to 0.6ml of H_2O_2 solution and the final volume was made up to 3ml with the same buffer. After 10 minutes, the absorbance values at 230nm of the reaction mixtures were recorded against a blank containing phosphate buffer without
H2O2 for each sample.

Appendix X

Reducing Power assay (Benzie and Strain, 1996) Principle

FRAP method is based on the reduction of Fe3+-tripyridyl-s-triazine (Fe3+ TPTZ) complex to ferrous form at low pH. This reduction is monitored by measuring the absorption change at 593 nm.

Reagents

1. Tripyridyltriazine (TPTZ) (10 mM)
2. Hydrochloric acid (40 mM)
3. Ferric chloride (20 mM)
4. Acetate buffer (300 mM, pH 3.6)

Procedure

The FRAP reagent contained 2.5 ml of a 10 mM TPTZ solution in 40 mM hydrochloric acid, 2.5 ml of 20 mM FeCl3.6H2O and 25 ml of 300 mM acetate buffer (pH 3.6). It was freshly prepared and warmed at 37°C. 900µl FRAP reagent was mixed with 90µl water and 10µl of the extract samples. The reaction mixture was incubated at 37°C for 30 minutes and the absorbance was measured at 593 nm.

Appendix XI

Anti-inflammatory activity (Mizushima *et al*., 1968)

Principle

The anti-inflammatory activity of *Rosa indica* wasstudied by using inhibition of albumin denaturation technique which was studied according to Mizushima *etal*., 1968 with minor modifications. The reaction mixture was consisting of test extract at different concentrations and 1% aqueous solution of bovine albumin fraction. pH of thereaction mixture was adjusted using small amount of 1 N HCL. The sample were incubated at 37℃ for 20 min. After cooling the samples, the turbidity was measured spectrophotometrically at 660 nm. The experiment was performed in triplicate.

Per cent inhibition of protein denaturation was calculated as follows

% inhibition = Control OD-sample OD X 100 / Control OD

Appendix XII

MTT assay (Igarashi and Miyazawa, 2001)

Principle

The MTT [2(4,4-dimethyl-2-tetrazoyl)-2,5-diphenyl tetrazolium] is converted into its formazon derivatives by living cells. The proportion of surviving cells can be determined by the MTT formazon produced, which is measured in a microtiter plate reader after solubilization with a suitable solvent like isopropanol.

Reagents

1. PBS
2. MTT (5g/ml in PBS)
3. Acid-propanaol (Isopropanol containing 0.4% of 0.04 N HCL)
4. HCL (0.04 N)

Procedure

The TM3 cells (mouse Leydig cells from testis of 13 days old mice) were exposed to the extracts of different concentrations, 50, 100, 150 and 200 μg/ml of aqueous, and ethanolic extracts of fresh and dry petals of *R. indica* and maintained at 37 ºC with 5% CO_2 until confluent. After that centrifuged at 1800 rpm for 10 min in a well-plate rotar (Plastocraft, India). The supernatant was discarded and the cells were treated with 50 µl of MTT and incubated at 37℃ for 3 hrs. At the end of the incubation period, the plates were centrifuged and the medium was removed. Then, the cells were washed with PBS carefully. The purple precipitate was solubilized using 200 µl of acid-propanol and incubated overnight in the dark. The absorbance values were recorded at 595 nm in an ELISA reader (Biorad, USA). The viability of the control cells was fixed as 100 % and the percentage viability of the cells in the other treatment groups were calculated relative to the control group.

Appendix XIII

Estimation of catalase (Luck, 1974)

Principle

The UV light absorption of hydrogen peroxide solution can be easily measured between 230 and 250nm. On decomposition of hydrogen peroxide by catalase, the absorption decreases with time. The enzyme activity could be arrived at from this decrease.

Reagents

1. Phosphate buffer (0.067M PH 7.0) - Dissolved 3.522g of KH_2PO_4 and 7.268g of $KHPO_4.2H_2O$ in distilled water and made up the volume to one litre.

2. Hydrogen peroxide – Phosphate buffer - Dissolved 0.16 ml of H_2O_2 10% W/V) to 100 ml phosphate buffer, prepared fresh. The absorbance of the solution should be about 0.5 at 240nm with 1 cm light path.

Procedure

Enzyme extract

The sample was homogenized in a prechilled mortar and pestle with M/150 phosphate buffer (assay buffer diluted 10 times) at 1 - 4°C and centrifuged. Stirred the sediment with cold phosphate buffer, allowed to stand in the cold with occasional shaking and then repeated the extraction once or twice. The extraction should not take more than 24 hr. The combined supernatants were used for the assay. Used fresh extract for assay.

Assay

Read against a control cuvette 3ml of H_2O_2 containing the enzyme solution as in the phosphate buffer (M/15). Pipetted into the experimental cuvette 3ml of H_2O_2 phosphate buffer. Mixed in 0.01-0.04ml sample with the glass or plastic rod flattened at one end. Noted the time it required for a decrease in absorbance from 0.45-0.4. This value was used for calculations. If 't' was more than 60 seconds, repeated the measurement with more concentrated solution of the sample.

Appendix XIV

Estimation of Glutathione peroxidase (Rotruck *et al.,* 1977)

Principle

A known amount of enzyme preparation was used to react with hydrogen peroxide in the presence of GSH for a specified time period when the screening was measured by the method of Ellman

Reagents

1. 0.4M Tris Buffer
2. 10µM sodium azide
3. 10% TCA
4. 0.4µM EDTA
5. 19µM hydrogen peroxide

Procedure

To 2ml of Tris buffer, 0.2ml of EDTA, 0.1ml of sodium azide and 0.5ml of plant extract were added following by 0.1ml hydrogen peroxide were added to the mixture, mixed well and incubated at 37℃ for 10 minutes along with the tube containing all the reagent expect sample. After 10 min the reaction was arrested by the addition of 0.5ml of 10% TCA centrifuged and supernatant was assayed for glutathione by the method of Ellman. The activities are expressed as µg GSH consumed/min/mg protein

Appendix XV

Estimation of Glutathione reductase Moron *et al*., (1979)

Principle

Reduced glutathione (GSH) was measured by its reaction with 5,5'- dithiobis-2- nitrobenzoic acid (DTNB) (Ellman's reaction) to give a yellow-coloured compound that absorbs at 412nm.

Reagents

1. TCA (5%)
2. TCA (25%)
3. Sodium phosphate buffer (0.2M, pH 8.0)
4. DTNB (0.6M in 0.2M sodium phosphate buffer)

Procedure

Preparation of plant extract

The petals of *Rosa indica* (0.5g) were ground with 2.5ml of 5% TCA. The precipitated protein was centrifuged at 1000rpm for 10 minutes. The homogenate was cooled on ice and the supernatant (0.1ml) was taken for the estimation of GSH.

Assay

The volume of different aliquots (0.2 to 1.0ml) was made up to 1ml with phosphate buffer. Freshly prepared DTNB (2ml) was added to the tubes and the intensity of the yellow colour was read at 412nm in a spectrophotometer after 10 minutes. A standard curve of GSH was prepared using concentrations ranging from 2 to 10 n moles of GSH. The concentration of GSH in the samples was calculated from this and the result were expressed as n moles GSH/g of extract.

Appendix XVI

Estimation of Superoxide dismutase (Misra and Fridovich, 1972)

Principle

The assay of SOD is based on the inhibition of formation of NADH phenazine methosulphate-nitroblue tetrazolium formazon, the extent of which can be assayed spectrophotometrically at 560nm.

Reagents

1. Sodium pyrophosphate buffer (0.025M, pH 8.3)
2. Phenazine methosulphate (PMS) (186µM)
3. Nitroblue tetrazolium (NBT) (300µM)
4. NADH (700µM)
5. Glacial acetic acid
6. n-butanol

Procedure Preparation of enzyme extract

Plant sample (0.5g) were ground with 3.0ml of sodium pyrophosphate buffer, centrifuged at 2000g for 10 minutes and the supernatant was used for the assay.

Assay

The assay mixture contained in a total volume of 3.0ml, 1.2ml of sodium pyrophosphate buffer, 0.1ml of PMS, 0.3ml of NBT, 0.2ml of enzyme preparations and 1.0 ml of water. NADH (0.2ml) was added to start the reaction.

The assay mixture was incubated at 30ºC for 90 seconds and the reaction was stopped by the addition of 1.0ml of glacial acetic acid. n-butanol (4ml) was added to the above mixture, allowed to stand for 10 minutes and then centrifuged at 2000g for 5 minutes. The intensity of the chromogen in the butanol layer was measured at 560nm against butanol as blank. The system devoid of enzyme served as control. One unit of enzyme activity is defined as the amount of enzyme causing a 50% reduction in NBT oxidation/minute.

Appendix XVII

Estimation of lipid peroxidation (Ohkawa *et al*., 1979)

The tissues were homogenized in10% (w/v) 0.1 M Tris-HCl buffer (pH 7.5) (24). One ml of the homogenate was combined with 2 ml of TCA-TBA-HCl reagent (15% trichloroacetic acid (TCA) and 0.375% thiobarbituric acid (TBA) in 0.25 N HCl) and boiled for 15 min. Precipitate was removed after cooling by centrifugation at 1000g for 10 min and absorbance of the sample was read at 535 nm against a blank without tissue homogenate. The levels of MDA were calculated using extinction coefficient calculation. The values are expressed as nmoles of MDA/ min/ mg protein

Appendix XVIII

Estimation of total alkaloids

Aim

To estimate the amount of alkaloid content in the given samples

Materials

1. Standard- Weight 10mg of pure caffeine and dissolved inn 25ml of 20% acetic acid A.R dilute as aliquots. Further 10 times with 20% acetic acid. Thus, solution contains 40µg/ml
2. 20% acetic acid
3. Chloroform

4. Anhydrous Na_2SO_4
5. Acetate buffer pH 4.8

Preparation

5.4 g of sodium acetate and 2.66ml of glacial acetic acid in 100ml double distilled water

Procedure

1. Into 5 test tubes pipette out 1,2,3,4,5 ml of 40µg/ml solution.
2. The volume of each was made up to 5ml with acetic acid
3. To each test tube 5ml of acetic buffer and 1 ml of methyl orange were added
4. After shaking for 10 sec 5ml of chloroform was added
5. The separated were stopped and shaken for 3 minutes
6. After standing for a few minutes chloroform layer were with drawn into dry test tubes, dried with small amount anhydrous Na_2SO_4
7. Absorbance read on a spectrophotometer at 420 nm using 10mm cells
8. From the reading standard curve was constructed.

Calculation

Express the amount in mg/g or 100 ml of sample

Appendix XIX

Estimation of flavanoids (Zhishen *et al.,* 1999)

Principle

Flavonoids react with vanillin to produce a coloured product that can be measured spectrophotometrically at 360nm.

Reagents

1. Vanillin reagent (1% vanillin in 70% sulphuric acid)
2. Sulphuric acid (70%)

3. Methanol : water (2:1)
4. Methanol : water (1:1)
5. Hexane
6. Catechin standard (110µg/ 1ml)

Procedure Preparation of plant extract

The *Rosa indica* petals were weighed (0.5g) and divided equally into two parts. One part was extracted with methanol : water (2:1) and other part was extracted with methanol : water in the ratio of 1:1. These homogenates were allowed to stand over night. The next day, the two extracts were combined and evaporated to 1/3rd the original volume. The resultant aqueous extract was cleared of low polarity contaminants by extracting with hexane. The solvent-extracted aqueous layer containing the bulk of the flavonoids was then concentrated.

Estimation

An aliquot of the plant extract was pipetted out and evaporated to dryness. Aliquots of the standard (0.2 to 1.0ml) were taken in test tubes and made up to 1.0ml with distilled water. Vanillin reagent (4ml) was added to all the test tubes and heated in a boiling water bath for 15 minutes. The absorbance was measured at 340nm after cooling. The values are expressed as mg flavonoids/g extract.

Appendix XX

Estimation of total phenols (Malick and Singh, 1980)

Principle

Phenol reacts with phosphomolybdic acid in Folin – ciocalteau reagent alkaline medium and produce blue coloured comples (molybdenum blue), which is read in a spectrometer at 650nm.

Reagent

1. 80% ethanol
2. Diluted Folin – ciocalteau reagent
3. 20% Sodium carbonate
4. Stock solution: 100 mg of catechol was made up with 100ml of distilled water
5. Working standard: 10ml of stock solution was diluted in 100ml of distilled water. 1.0ml of this contains 100µg of catechol

Procedure

One gram of sample was homogenized using 20 ml of 80% ethanol. The homogenate was centrifuged at 10,000rpm for 20 minutes. The supernatant was saved. The residue was reextracted with 10ml 0f 80% ethanol, centrifuged and collected the supernatant and evaporated to dryness. The residue was dissolved in known volume of distilled water (50ml) and 2.0m was taken in the experiment. A working standard of 0.5 to 2.5ml catechol solution was corresponding to 50-250µg of catechol were pipetted out into a series of test tubes. The volume was made upto 2.5ml with water. To all the tubes added 0.5ml of dilted Folin – ciocalteau reagent. After 3 minutes, added 2.0m of 20% Na_2CO_3 soution to each tube and mixed thoroughly.

The tubes were placed in a boiling water oth for exactly one minute. Cooled and measured at 650nm against of ca reagent blank. Calculate the amount of polyphenol present in the sample used to regression value.

Appendix XXI

Estimation of Phytosterols (Araujolbcolva *et al.,* 2013)

Principle

The phytosterol present in the sample reacts with Libermann burchard reagent to give a blue colour product which is measured at 640 nm. The intensity of colour developed is directly proportional to the amount of phytosterol present in the sample.

Materials

1. Liberman burchard reagent (acetic anhydrate and sulfuric acid)

2. 0.5 ml of H_2SO_4 dissolved in 10.0 ml of acetic anhydride and kept in ice Standard: 10.0 mg of cholesterol dissolved in 10.0 ml of chloroform. 1 ml contains 1 mg of sterol.

Procedure

1. Pipette out 0.5, 1.0, 1.5, 2.0 and 2.5 ml of standard into a series of test tubes and 0.2 ml of the sample extract into unknown test tube.
2. Add 2.0 ml of Libermann burchard reagent to all the tubes
3. Make up equal volume in all the tubes with chloroform
4. Cover the tubes with carbon paper and incubate at room temperature in dark for 30 minutes. A green colour developed was read at 640 nm.

INSILICO ANALYSIS

Appendix XXII

Docking score and Glide energy for the phytocompounds against COX2

A. Aqueous fresh petal extract against

S.No	Pubchem ID	Glide gscore	glide energy
1	5280863	-9.455	-30.051
2	7311	-7.973	-25.134
3	9590244	-7.902	-28.514
4	238273	-7.754	-22.912
5	24699	-7.542	-27.246
6	569173	-7.178	-28.48
7	244	-6.978	-25.191
8	6054	-6.804	-23.614
9	10393	-6.757	-23.473
10	6781	-6.73	-23.822
11	439710	-6.157	-19.976
12	7654	-5.911	-27.216
13	445070	-5.863	-24.02
14	2724705	-5.751	-22.602

15	528664	-5.621	-10.558
16	5281	-4.799	-15.22
17	5365842	-4.454	-20.663
18	985	-3.802	-18.262
19	67147	-3.27	-20.843
20	536145	-3.219	-19.245
21	679	-3.014	-15.892
22	5280934	-1.413	-31.452

B. Aqueous dry petal extract against

S.No	Pubchem ID	Glide gscore	Glide energy
1	5280863	-9.455	-30.051
2	7149	-8.276	-21.934
3	7311	-7.973	-25.134
4	9590244	-7.908	-28.568
5	570611	-7.561	-8.594
6	578393	-7.304	-10.963
7	244	-6.978	-25.191
8	6054	-6.886	-23.358
9	644176	-6.837	-28.058
10	1057	-6.776	-23.893
11	10393	-6.621	-25.402
12	439710	-6.103	-19.206
13	2724705	-5.751	-22.602
14	5281	-4.218	-13.667
15	679	-3.014	-15.892

C. Ethanolic fresh petal extract

S.No	Pubchem ID	Glide gscore	Glide energy
1	5280863	-9.455	-30.051
2	7311	-7.973	-25.134
3	578393	-7.412	-12.016

4	535560	-7.184	-40.126
5	21160048	-6.962	-27.56
6	6054	-6.886	-23.358
7	439710	-6.157	-19.976
8	537255	-5.975	-21.711
9	7083742	-5.767	-30.163
10	2724705	-5.681	-21.188

D. Ethanolic dry petal extract

S.No	Pubchem ID	Glide gscore	Glide energy
1	5280863	-9.243	-30.263
2	7311	-7.973	-25.134
3	578393	-7.412	-12.016
4	535560	-7.184	-40.126
5	21160048	-6.962	-27.56
6	6054	-6.886	-23.358
7	439710	-6.157	-19.976
8	537255	-5.975	-21.711
9	7083742	-5.767	-30.163
10	2724705	-5.681	-21.188
13	7149	-8.276	-24.089
14	7311	-7.973	-25.134
15	18950	-7.749	-25.761
16	101715	-7.168	-26.88
17	244	-6.978	-25.191
18	644176	-6.837	-28.058
19	6054	-6.804	-23.614
20	9231	-6.801	-20.884
21	1057	-6.776	-23.893
22	10393	-6.757	-23.473
23	643820	-6.66	-27.265
24	5364713	-6.654	-25.614
25	7463	-6.596	-20.512

26	579336	-6.477	-13.721
27	5365675	-6.453	-25.387
28	537255	-6.436	-22.823
29	5369468	-6.422	-31.748
30	5280934	-6.309	-21.277
31	8122	-5.889	-29.555
32	5363633	-5.762	-21.471
33	5367460	-5.752	-17.189
34	2724705	-5.751	-22.602
35	537255	-5.636	-21.517
36	5281	-5.437	-19.217
37	537255	-5.437	-25.049
38	6421916	-5.187	-27.057
39	537255	-5.076	-27.601
40	3893	-4.641	-23.791
41	1529845	-4.43	-27.395
42	1.35E+08	-4.351	-36.334
43	985	-4.252	-8.939
44	5364560	-4.006	-16.661
45	91697237	-3.985	-9.386
46	12366	-3.981	-22.905
47	88422390	-3.948	-9.028
48	24624	-3.726	-21.849
49	91703390	-3.106	-43.262
50	5365075	-3.036	-20.49
51	1.35E+08	-2.773	-30.008
52	1.35E+08	-2.665	-5.09
53	5280863	-2.271	-27.605
54	28962	-1.535	-26.976

Docking score and Glide energy for the phytocompounds against Androgen Receptor

A. Aqueous extract of fresh petals

S.No	Pubchem ID	Glide gscore	Glide energy
1	5280863	-9.226	-31.719
2	7311	-8.125	-23.466
3	244	-7.141	-24.117
4	6054	-6.757	-26.249
5	439710	-6.597	-30.730
6	6781	-6.578	-38.604
7	7654	-6.462	-28.962
8	10393	-6.308	-29.395
9	2724705	-6.099	-22.873
10	679	-4.695	-17.139
13	536145	-3.384	-20.307
14	445070	-3.358	-28.854
15	5280934	-2.955	-31.196
16	67147	-2.569	-30.589
17	5365842	-1.866	-24.999

B. Aqueous extract of dry petals

S.No	Pubchem ID	Glide gscore	Glide energy
1	5280863	-9.226	-31.719
2	578393	-8.221	-18.007
3	7311	-8.125	-23.466
4	244	-7.141	-24.117
5	1057	-6.845	-29.522
6	6054	-6.757	-26.249
7	7149	-6.725	-26.802
8	439710	-6.597	-30.730
9	10393	-6.308	-29.395
10	2724705	-6.099	-22.873
11	679	-4.695	-17.139
12	644176	-4.390	-29.550

C. **Ethanolic extract of fresh petals**

S.No	Pubchem ID	Glide gscore	Glide energy
1	5280863	-9.226	-31.719
2	578393	-8.221	-18.007
3	7311	-8.125	-23.466
4	538432	-7.304	-26.557
5	6054	-6.757	-26.249
6	439710	-6.597	-30.730
7	2724705	-6.099	-22.873
8	7083742	-5.982	-24.199

D. **Ethanolic extract of dry petals**

S.No	Pubchem ID	Glide gscore	Glide energy
1	5280863	-9.226	-31.719
2	5369468	-8.649	-39.211
3	7311	-8.125	-23.466
4	9231	-7.551	-25.106
5	7463	-7.203	-23.136
6	101715	-7.185	-36.993
7	244	-7.141	-24.117
8	579336	-7.091	-27.719
9	1057	-6.845	-29.522
10	6054	-6.757	-26.249
11	7149	-6.725	-26.802
12	1529845	-6.558	-28.977
13	10393	-6.308	-29.395
14	18950	-6.307	-32.097
15	2724705	-6.099	-22.873
16	643820	-5.841	-28.105

17	644176	-4.390	-29.550
18	24624	-3.951	-26.098
19	3893	-3.853	-28.650
20	5280934	-2.955	-31.196
21	5363633	-2.743	-34.433

Docking score and Glide energy for the phytocompounds against AKT1

A. **Aqueous extract of fresh petals**

S.No	Pubchem ID	Glide gscore	*Glide energy*
1	439710	-5.580	*-25.619*
2	5280863	-5.560	*-37.117*
3	2724705	-5.185	*-23.446*
4	7311	-5.184	*-24.556*
5	10393	-4.772	*-23.416*
6	528664	-4.517	*-30.527*
7	244	-4.443	*-18.735*
8	9590244	-4.232	*-44.851*
9	7654	-4.023	*-25.177*
10	6781	-3.831	*-33.114*
11	536571	-3.645	*-38.301*
12	679	-3.632	*-13.494*
13	6452096	-3.543	*-38.317*
14	536452	-3.530	*-42.257*
15	6054	-3.483	*-18.313*
16	569173	-3.204	*-41.529*
17	24699	-3.198	*-40.968*
18	134832752	-3.100	*-39.045*
19	457801	-3.091	*-31.013*
20	238273	-2.947	*-29.648*
21	536145	-2.759	*-16.798*
22	445070	-1.963	*-28.938*
23	292285	-1.902	*-27.102*
24	5281877	-0.621	*-39.880*
25	67147	-0.273	*-26.080*
26	5365842	0.067	*-21.504*
27	5281	0.441	*-32.635*

28	5280934	0.495	*-31.936*
29	985	1.126	*-22.939*

B. **Aqueous extract of dry petals**

S.No	Pubchem ID	Glide gscore	Glide energy
1	57387363	-6.693	-64.712
2	439710	-5.580	-25.619
3	5280863	-5.560	-37.117
4	2724705	-5.185	-23.446
5	7311	-5.184	-24.556
6	1057	-4.885	-21.135
7	10393	-4.772	-23.416
8	578393	-4.788	-40.905
9	7149	-4.460	-23.811
10	244	-4.443	-18.735
11	9590244	-4.232	-44.851
12	553617	-4.073	-46.235
13	627381	-4.023	-32.652
14	679	-3.632	-13.494
15	6452096	-3.543	-38.317
16	545669	-3.540	-40.995
17	6054	-3.483	-18.313
18	570611	-3.316	-30.100
19	644176	-3.174	-24.408
20	292285	-1.902	-27.102
21	5281	0.441	-32.635

C. **Ethanolic extract of fresh petals**

S.No	Pubchem ID	Glide gscore	Glide energy
1	7083742	-5.691	-46.139
2	439710	-5.580	-25.619
3	5280863	-5.560	-37.117
4	2724705	-5.185	-23.446
5	7311	-5.184	-24.556
6	91699518	-4.960	-51.137
7	578393	-4.788	-40.905
8	624530	-4.816	-25.440
9	21160048	-3.561	-44.591
10	6054	-3.483	-18.313
11	538432	-3.425	-30.208
12	535560	-3.311	-41.925
13	11683	-3.272	-32.142
14	541562	-2.986	-36.972
15	91697197	-2.837	-40.240
16	537255	-2.775	-31.450

D. **Ethanolic extract of dry petals against**

S.No	Pubchem ID	Glide gscore	Glide energy
1	135398658	-6.200	-53.423
2	5280863	-5.560	-37.117
3	18950	-5.377	-25.887
4	1529845	-5.261	-24.989
5	2724705	-5.185	-23.446
6	7311	-5.184	-24.556
7	7463	-5.174	-17.271
8	9231	-5.081	-19.189
9	1057	-4.885	-21.135
10	10393	-4.772	-23.416
11	101715	-4.761	-25.571
12	7149	-4.460	-23.811
13	244	-4.443	-18.735

14	582634	-4.052	-39.357
15	5369468	-3.985	-26.310
16	8343	-3.970	-39.748
17	550132	-3.941	-31.077
18	554350	-3.817	-31.625
19	5375868	-3.733	-33.734
20	577916	-3.690	-36.235
21	643820	-3.611	-21.419
22	259803	-3.606	-35.718
23	6421916	-3.583	-47.030
24	6452096	-3.543	-38.317
25	6054	-3.483	-18.313
26	91697237	-3.462	-29.715
27	5367746	-3.406	-40.743
28	500213	-3.406	-32.105
29	65252	-3.404	-32.022
30	579336	-3.386	-19.306
31	101616676	-3.311	-34.886
32	225688	-3.179	-34.592
33	173183	-3.178	-31.310
34	644176	-3.174	-24.408
35	457801	-3.091	-31.013
36	91703390	-3.056	-41.543
37	5281326	-3.039	-30.355
38	541562	-2.986	-36.972
39	99615	-2.984	-34.083
40	537255	-2.775	-31.450
41	8122	-2.696	-36.983
42	91694967	-2.393	-34.197
43	88422390	-2.391	-31.958
44	5367460	-2.225	-30.340
45	5364713	-2.015	-28.895
46	24624	-1.936	-22.319
47	292285	-1.902	-27.102
48	5365675	-1.867	-30.021
49	5363633	0.433	-25.383
50	5281	0.441	-32.635
51	5280934	0.495	-31.936
52	3893	0.514	-24.028
53	28962	0.751	-26.362

54	985	1.126	-22.939
55	5364560	1.490	-25.307
56	12366	1.793	-26.738

Docking score and Glide energy for the phytocompounds with StAR
A. Aqueous extract of fresh petals against

S.No	Pubchem ID	Glide gscore	Glide energy
1	9590244	-6.419	-52.877
2	7311	-6.379	-27.432
3	6452096	-5.530	-28.541
4	2724705	-5.418	-25.744
5	439710	-5.341	-26.432
6	244	-5.261	-20.824
7	238273	-5.137	-42.747
8	457801	-4.833	-21.590
9	5281877	-4.823	-43.138
10	5280863	-4.846	-42.583
11	6054	-4.685	-22.680
12	10393	-4.652	-24.732
13	7654	-4.542	-25.200
14	679	-4.426	-17.087
15	569173	-4.206	-37.313
16	528664	-3.975	-30.765
17	292285	-3.873	-34.120
18	6781	-3.745	-31.885
19	24699	-3.429	-41.560
20	445070	-2.924	-27.051
21	536145	-2.507	-19.293
22	5280934	-0.788	-31.745
23	67147	-0.667	-25.282
24	985	0.314	-28.664
25	5365842	0.813	-23.898
26	5281	0.888	-25.955

B. **Aqueous extract of dry petals against StAR protein**

S.No	Pubchem ID	Glide gscore	Glide energy
1	9590244	-6.419	-52.877
2	7311	-6.379	-27.432
3	1057	-5.846	-23.361
4	578393	-5.686	-50.749
5	6452096	-5.530	-28.541
6	2724705	-5.418	-25.744
7	439710	-5.341	-26.432
8	244	-5.261	-20.824
9	7149	-5.195	-24.108
10	627381	-4.949	-31.416
11	57387363	-4.841	-50.172
12	5280863	-4.846	-42.583
13	6054	-4.685	-22.680
14	10393	-4.652	-24.732
15	679	-4.426	-17.087
16	644176	-4.099	-30.038
17	570611	-3.938	-37.533
18	292285	-3.873	-34.120
19	5281	0.888	-25.955

c. **Ethanolic extract of fresh petals**

S.No	Pubchem ID	Glide gscore	Glide energy
1	7311	-6.379	-27.432
2	1057	-5.846	-23.361
3	582634	-5.755	-47.489
4	8343	-5.560	-48.060
5	2724705	-5.418	-25.744
6	5365675	-5.332	-41.146
7	101715	-5.309	-28.876
8	244	-5.261	-20.824
9	9231	-5.250	-21.380
10	135398658	-6.306	-53.018
11	7149	-5.195	-24.108

12	18950	-5.183	-28.759
13	1529845	-5.154	-26.790
14	5369468	-5.074	-33.248
15	91697237	-4.932	-41.373
16	7463	-4.928	-20.413
17	554350	-4.886	-22.154
18	5280863	-4.846	-42.583
19	6054	-4.685	-22.680
20	10393	-4.652	-24.732
21	579336	-4.368	-23.521
22	577916	-4.159	-23.309
23	644176	-4.099	-30.038
24	537255	-4.057	-42.275
25	5367460	-3.809	-38.140
26	643820	-3.232	-24.464
27	24624	-2.720	-23.745
28	5364713	-2.610	-36.727
29	550132	-2.498	-37.132
30	5363633	-0.791	-33.151
31	5280934	-0.788	-31.745
32	28962	-0.516	-35.922
33	12366	-0.287	-35.987
34	3893	-0.109	-27.359
35	985	0.314	-28.664

D. **Ethanolic extract of dry petals**

S.No	Pubchem ID	Glide gscore	Glide energy
1	7311	-6.379	-27.432
2	1057	-5.846	-23.361
3	582634	-5.755	-47.489
4	8343	-5.560	-48.060
5	2724705	-5.418	-25.744

6	5365675	-5.332	-41.146
7	101715	-5.309	-28.876
8	244	-5.261	-20.824
9	9231	-5.250	-21.380
10	135398658	-6.306	-53.018
11	7149	-5.195	-24.108
12	18950	-5.183	-28.759
13	1529845	-5.154	-26.790
14	5369468	-5.074	-33.248
15	91697237	-4.932	-41.373
16	7463	-4.928	-20.413
17	554350	-4.886	-22.154
18	5280863	-4.846	-42.583
19	6054	-4.685	-22.680
20	10393	-4.652	-24.732
21	579336	-4.368	-23.521
22	577916	-4.159	-23.309
23	644176	-4.099	-30.038
24	537255	-4.057	-42.275
25	5367460	-3.809	-38.140
26	643820	-3.232	-24.464
27	24624	-2.720	-23.745
28	5364713	-2.610	-36.727
29	550132	-2.498	-37.132
30	5363633	-0.791	-33.151
31	5280934	-0.788	-31.745
32	28962	-0.516	-35.922
33	12366	-0.287	-35.987
34	3893	-0.109	-27.359
35	985	0.314	-28.664

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वन आनुवंशिकी एवं वृक्ष प्रजनन संस्थान

(भारतीय वानिकी अनुसंधान एवं शिक्षा परिषद्)
(पर्यावरण एवं वन मंत्रालय, भारत सरकार का एक स्वायत्त निकाय)
पि.बी.नं. 1061 कोयम्बत्तूर 641 002 तमिलनाडु, भारत

INSTITUTE OF FOREST GENETICS AND TREE BREEDING

INDIAN COUNCIL OF FORESTRY RESEARCH & EDUCATION

(An autonomous Body of Ministry of Environment & Forests, Govt. of India)
P.B. No. 1061, R.S. Puram HPO., Coimbatore - 641 002, Tamil Nadu, India

No. 924 /FECC/ ID/ IFGTB/ 2020 Date: 03.03.2020

CERTIFICATE

This is to certify that the plant specimen brought by **Ms. Nithya Sethumadhavan**, Ph D scholar, Dept. of Biotechnology, Biochemistry and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, is identified and authenticated as *Rosa indica* L. belonging to Rosaceae family.

03/03/2020

C. KUNHIKANNAN

Dr. C.KUNHIKANNAN
Scientist G
Forest Ecology & Climate Change Division
Institute of Forest Genetics & Tree Breeding
Coimbatore - 641 002.

Avinashilingam Institute for Home Science and Higher Education for Women
(Deemed to be University under Category 'A' by MHRD, Estd. u/s 3 of UGC Act, 1956)
Re-accredited with 'A+' grade by NAAC, Recognised by UGC under Section 12 B
Coimbatore – 641 043, Tamil Nadu, India

Certificate

This is to certify that the project entitled **"Study on the Androgenic effect of *Rosa damascena* extract on Heat stress induced infertility in male wistar rats"** has been approved by the IAEC having IAEC approval No **AIW:IAEC.2020:BC:01**

Authorized by	Name	Signature	Date
Chairman:	Dr. S. KOWSALYA	S. Kowsalya	19/02/2020
Member Secretary:	Dr. R. NIRMALADEVI		19/02/2020
Main Nominee of CPCSEA:	Dr. C. GUNASEKARAN		19.02.2020

Institutional Animal Ethics Committee
Reg. No: 523/02/b/CPCSEA
19.05.02
Coimbatore - 641

APPENDIX – L2

Avinashilingam Institute for Home Science and Higher Education for Women
(Deemed to be University Estd. u/s 3 of UGC Act 1956, Category A by MHRD)
Re-accredited with A++ Grade by NAAC. Recognised by UGC Under Section 12 B
Coimbatore - 641 043. Tamil Nadu. India

(Item No. 5 of Check List) Details of Research Publications

S. No.	Article	Journal	Other Details Vol. / No. / Page No. / Year	Published in UGC-CARE / Scopus Indexed/ Web of Science (*List of Journals in that category including the particular Journal to be attached)
1	Preliminary phytochemical screening and Antimicrobial study of Dry & fresh Aqu, Ethanolic & chloroform Extracts of R. indica petals	The Indian Journal of Nutrition & Dietetics	59(3):285-293 Jul-Sep 2022.	UGC-CARE List I.
2	Alleviating effect of R. indica petal extracts on heat stress Induced male infertility	Research journal Of Agricultural sciences- An International Journal	13(4): 1117-1120 2022.	UGC-CARE List I.
3				
4				

*Proof of list of Journals from Internet to be attached along with copies of reprints.

Scholar : Nithya. S.

Supervisor : N.S.

Checked

By:

5/8/2022

HoD / Dean

The articles published by the scholar Mrs. Nithya Sethumadhavan are in UGC care list Group I.

① The Indian Journal of Nutrition & Dietetics and

② Research Journal of Agricultural sciences are available in UGC care list Group I as of today 05.08.2022

(Dr. T. Thilagavathi)
Asst. Librarian

The Indian Journal of Nutrition and Dietetics
ISSN: 0022-3174; eISSN: 2348-621X www.ijndonline.org

DOI:10.21048/IJND.2022.59.3.29440

Priliminary Phytochemical Screening and Antimicrobial Study of Dry and Fresh Aqueous, Ethanolic and Chloroform Extracts of *Rosa Indica* Petals

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Abstract

Plants are a valuable source of a multitude of secondary metabolites which have been used as medicine since times. Most unarguable merit of using plants as a source of medicine is that they are low priced and readily available with very minimal side effects. The present study deals with the comparative analysis of dry and fresh aqueous, ethanolic and chloroform extracts of Rosa indica petals for its phytochemical screening. The results confirmed the presence of almost all tested secondary metabolites in aqueous and ethanolic extracts, whereas chloroform extract showed only the presence of saponins. Further study was proceeded only with dry and fresh aqueous and ethanolic extracts of Rosa indica petals for its antimicrobial activity against Gram negative bacteria including Escherichia coli (ATCC NO: 25922), Pseudomonas aeruginosa (ATCC NO: 27853) and gram positive bacteria including Staphylococcus aureus (ATCC NO: 43300), Enterococcus faecalis (ATCC NO: 29212) and Candida albicans (ATCC NO: 24433) which is an opportunistic pathogenic yeast. Both dry and fresh ethanolic extracts showed a comparatively higher zone of inhibition for all the microbes. Aqueous dry and fresh extracts showed zone of inhibition for Staphylococcus aureus and Enterococcus faecalis, where as none of the extracts showed antimicrobial activity against Candida albicans. Results from the above study suggest that the ethanolic extract of Rosa indica petal are an encouraging

alternative to some of the antibacterial drugs. This in turn would potentially be used as natural source to treat various multi drug resistant bacterial strains.

Keywords: *Rosa indica, aqueous, ethanolic, phytochemicals, antimicrobial activity*

Introduction

Plants are a well known source of modern medicine. From ancient times, humans have utilized plants for the treatment or prevention of various diseases, leading to the dawn of traditional medicine[1]. Medicinal plants have been used in folk medicine by Asian and African population and many plants are consumed for their health benefits in both developed and developing nations. They can readily be administered orally as a part of dietary intake[2]. According to WHO, some countries still rely on plant based treatment as their main source of medicine and developing countries are utilizing the benefits of naturally sourced compounds for therapeutic purpose.

Plants are considered as a natural source of modern medicine, since they are potentially rich in phytochemical compounds like steroids, terpenoids, glycosides, glycoprotein, protein, alkaloids, flavanoids, phenols etc. Plants are widely used in developing countries to treat various infectious diseases. Infections in humans are caused by pathogens[3]. Gram negative bacteria like *Escherichia coli, Pseudomonas aeruginosa* are associated with food poisoning. Gram positive bacteria including *Enterococcus faecalis,* can cause a range of illnesses, from minor skin infections to life threatening diseases like bacteremia, pneumonia, sepsis, endocarditis, meningitis etc. These diseases are treated with antibiotics. The

widespread use of antibiotics might lead to antibiotic resistance[4].

Rosa indica is a perennial flower shrub of the genus Rosa. It belong to family Rosaceae, which contains herbs, shrubs or trees that are rhizomatous, thorny or

climbing[5,6]. The leaves of rose are alternative and pinnately compound. They are sharply toothed oval shaped leaflets. The fruit of plant is fleshy, edible (rose hip) which ripens in late summer. Roses are rich source of vitamin C and are used for making various herbal preparations[7]. Ethnopharmocologically, roses have been used to treat various eye diseases and heart diseases[8].

In the Indian system of medicine, various rose preparations are used as astringent, tonic, mild laxative, antibacterial agent, in treating sore throat, enlarged tonsils, gall stones and also a vehicle for the preparation of other medicines[9].

A study in 2017 reported the antimicrobial effect of ethanolic extract of *Rosa indica* on *E.coli, S. aureus, Salmonella sp, K.pneumonia* and *Vibrio* sp[10]. However, there is a lack of study pertaining to the medicinal uses of aqueous, ethanolic and chloroform extracts of *R.indica.* Considering these informations, this article explores the phytocompounds and antibacterial and antifungal activity of aqueous, ethanolic and chloroform extracts of *R. indica.* which can be considered as a candidate to treat various multi drug resistant strains.

Material and methods

Chemicals

Solutes and solvents of analytical grade were used in assays carried out in the present study.

Plant material

R. indica flowers were collected from local areas of Coimbatore, Tamil Nadu and was authenticated by the Head, Institute of Forest Genetics and Tree Breeding, Coimbatore with authentication number 924/FECC/ID/IFGTB/2020

Preparation of sample

The cleaned fresh petals of *R.indica* weighing 300 g was shade dried for two weeks. Pre weighed 100 g of each fresh and dried petals were extracted with 500 ml of double distilled water, 100% ethanol and chloroform respectively using mortar and pestle and incubated at 4°C for 48 hours. After 48 hours each extract was filtered using cheese cloth and the filtrates lyophilized to get a coarse powder. The extract was stored at 4°C to carry out further studies.

Methods

Preliminary phytochemical screening

Preliminary phytochemical screening was carried out for aqueous, ethanolic and chloroform extracts of dry and fresh *Rosa indica* petals using the standard methods [11-14].

Test for sugars

Benedict's test- Few drops of plant extract was treated with Benedict's reagent and heated gently. Formation of orange red coloured precipitate indicated the presence of reducing sugars.

Molisch's test- To 1 ml of the extract few drops of Molisch's reagent and 1 ml of concentrated sulphuric acid was added along the sides of the test tube and the mixture allowed to stand for 2 to 3 minutes. Formation of red or dull violet colour indicated the presence of carbohydrates.

Fehling's test- Few drops of Fehling's reagent was added to the dry extract and tubes kept in boiling water bath. Change in colour from deep blue to a red precipitate confirmed the presence of sugars

Tests for alkaloids

Mayer's test- Plant extracts were treated with Mayer's reagent (potassium mercuric iodide). Formation of yellow coloured precipitate indicated the presence of alkaloids

Wagner's test- A fraction of extract was treated with Wagner's reagent (1.2 g of iodine and 2 g of potassiun iodide in 100 ml of water) and observed for colour change. Formation of reddish brown precipitate indicated the presence of alkaloids

Dragendorff's test- An aliquot of extract was treated with Dragendorff's reagent (solution A- 0.7 g bismuth nitrate in 2 ml glacial acetic acid and 8 ml of water, solution B-4 g potassium iodide in 10 ml glacial acetic acid and 20 ml water. Solution A and B was mixed and diluted to 100 ml with distilled water). Formation of reddish orange precipitate indicated the presence of alkaloids

Test for phenols

Ferric chloride test- To a fraction of extract, 5% ferric chloride was added. Formation of deep blue colour indicated the presence of phenols.

Test for flavanoids

Lead acetate test- A fraction of extract was treated with 10% lead acetate solution and observed for the formation of white precipitate which indicated the presence of flavanoids.

Aluminium chloride test- To 1 ml of the extract 2 drops of 1% aluminium chloride was added and observed for the formation of yellow colouration which indicated the presence of flavanoids.

Test for diterpenes

Plant extracts were treated with 2 ml of chloroform and 2 ml of sulphuric acid and observed for the formation of brown ring at the junction which indicated the presence of diterpenes.

Test for triterpenes

Salkowski's test was performed to identify the presence of triterpenes, where

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5 ml of the extracts were mixed with 2 ml of chloroform and 3 ml of sulphuric acid carefully. Reddish brown colouration of the interface indicated the presence of triterpenes.

Test for primary amines

To the boiled and cooled extract was added 1 ml of sodium hydroxide and observed for the formation of pink colouration which indicated the presence of primary amines.

Test for secondary amines

To the boiled and cooled plant extract was added 1 ml of sodium hydroxide and 1 ml of hydrogen chloride. Formation of dark pink to violet colour indicated the presence of secondary amines.

Test for phenols

Ferric chloride test- To a fraction of the extract, 5% ferric chloride was added and watched for the formation of deep blue colour which indicated the presence of phenols.

Test for saponins

Foam test was performed according to Sofowora, (1993) to identify the presence of saponins. 0.5 ml of extracts was shaken with 1 ml of water. If foam produced was persistant for 10 min, it indicated the presence of saponins.

Test for tannins

Extracts of *Indica*

Plant extracts were treated with a few drops of lead solution. Formation of white precipitate indicated the presence of tannins.

Test for proteins

Ninhydrin test- 0.25% w/v ninhydrin was added to the extract and boiled for few minutes. Blue colouration of the mixture indicated the presence of protein.

Test for volatile oils

A 2 ml of extract was shaked with 0.1 ml of dilute sodium hydroxide and small quantity of dilute hydrochloric acid. Formation of white precipitate indicated the presence of volatile acid.

Test for glycosides

To 2 ml of the extracts were added 2 ml of chloroform and 2 ml of sulphuric acid. Formation of reddish brown colour indicated the presence of glycosides.

Test for anthocyanins

Test for primary and secondary amines was performed and observed for the colour change. Colour formation indicated the presence of anthocyanins.

Test for phytosterols

Lieberman-Burchard's test- Plant extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled to which concentrated sulphuric acid was added. Formation of brown ring at the junction indicated the presence of phytosterols.

Antimicrobial study

Antibacterial and antifungal activity of the plant extract were analysed by agar well diffusion method against gram negative bacteria including *Escherichia coli*, *Pseudomonas aeruginosa* and gram positive bacteria including *Staphylococcus aureus*, *Enterococcus faecalis* and *Candida albicans.* About 20 ml of nutrient agar was poured into the petriplates and allowed to solidify for 30 min. The test organisms were seeded (0.2 ml : 10^7-10^8 cells/ml) into sterile molten media, 10 mm wells were punched into which 10 mg/ml and 20 mg/ml of the extracts were loaded. Approved positive controls were used. Wells loaded with double distilled water was used as negative control. Plates were incubated at 37^0C for 24-48 hours in upright position and observed for clear and distinct

zone of inhibition surrounding the well[15]. Potato Dextrose Agar (PDA) was used to culture *Candida albicans.*

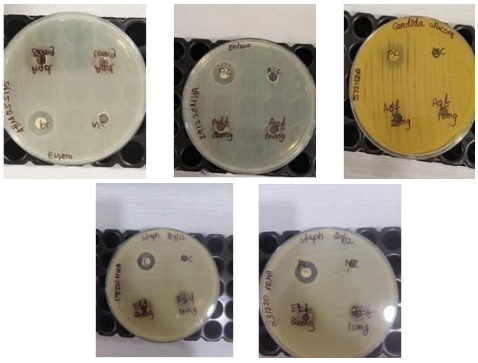
Results and Discussion

From Table I, it was clear that both dry and fresh aqueous and ethanolic extracts of *Rosa indica* petals are rich in secondary metabolites which make them a potential source of natural medicine. However the chloroform extract did not show the presence of any Phyto compounds tested, due to which it was not further analysed for its antimicrobial activity. From

TABLE I

Preliminary Phytochemical Screening of Dry and Fresh Aqueous, Ethanolic and Chloroform Extracts of *R.indica* Petals

Priliminary Phytochemical Screening	Aqueous		Ethanol		Chloroform	
	Dry extract	Fresh extract	Dry extract	Fresh extract	Dry extract	Fresh extract
Sugars						
Molisch test	+	+	-	-	-	+
Fehling's	-	-	+	+	-	-
Benedict's test	+	+	+	+	-	-
Alkaloids						
Mayer's test	-	-	-	-	-	-
Wagner's test	-	-	+	+	+	+
Phenols	+	+	+	+	-	-
Flavanoids						
Lead acetate	-	-	+	+	-	-
Sterols and steroids						
Diterpenes	-	-	+	+	-	-
Triterpenes	-	-	+	+	-	-
Anthocyanins Primary amine	+	-	+	+	-	-
Secondary amine	-	-	+	+	-	-
Tannin	+	+	+	+	-	-
Saponin Proteins and aminacids						
Ninhydrin	+	+	-	+	-	-
Volatile oils	-	-	-	-	+	-



Figures 1-5

Antibacterial activity of extracts of *R. Indica* Petals

Extracts of *Indica*

TABLE II

Zone of Inhibition of Dry and Fresh Aqueous and Ethanolic Extracts of R.indica Petals

Organism	Positive control	Aqueous	Dry	Aqueous	Fresh	Ethanol	Dry	Etthanol	Fresh
		20 mg/ml	10 mg/ml	20 mg/ml	10 mg/ml	20 mg/ml	10 mg/ml	20 mg/ml	10 mg/ml
E.Coli	Meropenem (25 mm)	0	0	0	0	15*	13	13	11
S.aureus	Vancomycin (14 mm)	0	0	12	10	15*	14	14	12
P.aurogenosa	Meropenem (23 mm)	0	0	0	0	20*	18	22*	18
E.Fecalis	Vancomycin (14 mm)	0	0	11	10	18*	14	14	11
C.albicans	Ketocanazole (18 mm)	0	0	0	0	0	0	0	0

* - Extract having the maximum zone of inhibition (in mm)

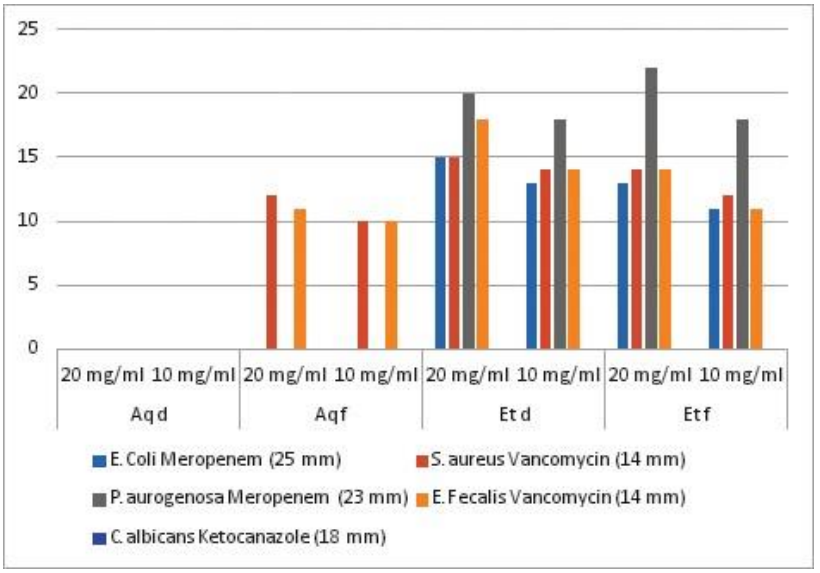


Figure 6

Comparative antimicrobial activity of dry and fresh aqueous and ethanolic extracts of *R. indica* petals

Table II, Figures 1-5 and 6 it can be concluded that dry and fresh Ethanolic extracts had maximum antibacterial activity as confirmed by the maximum zone of inhibitions, fresh aqueous extract was comparatively effective against *S.aureus* and *E.fecalis* whereas dry aqueous extract did not exhibit antibacterial activity. Also none of the extracts were effective against *C.albicans* which is a commonly acquired fungal infection.

Conclusion

Various preparations of rose have long been used for treating diseases. However there are no much articles to witness the efficacy of *Rosa indica* as a natural medicine in alleviating heat stress induced male infertility. From the above study it is clear that dry and fresh ethanolic extracts of *R.indica* petals can be used as an effective antibacterial drug. Also further the study will be proceeded to analyse the *in vitro* and *in vivo* antioxidant capacity of the dry and fresh aqueous and ethanolic extracts which would make them a good candidate for treating male infertility due to heat stress.

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Conflict of interests

Authors declare that there are no conflicts of interest.

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Alleviating Effect of Rosa indica Petal Extracts on Heat Stress Induced Male Infertility

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Full Length Research A

Alleviating Effect of *Rosa indica* Petal Extracts on Heat Stress Induced Male Infertility

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ABSTRACT

Rosa indica is a perennial flower shrub which belongs to the family Rosaceae and genus rosa. From ancient times various rose preparations are used to treat sore throat, enlarged tonsils, as an antibacterial agent and there are few studies on its fertility enhancing properties too. The present study aims to evaluate the effect of aqueous and ethanolic extracts of *Rosa indica* petals on the serum levels of testosterone (ng/ml) and thus the semen parameters including Concentration (M/ML), motility (%) and morphology (%) on heat stress induced male wistar rats. In this experimental study, 30 adult male Wistar rats were used. The rats were randomly divided into 5 groups. First group was control (only with compressed food and adequate water), second group was Negative control (heat stressed), third group was positive control (heat stressed + treated with Quercetin), fourth and fifth group was heat stressed with 200 mg/kg of ethanolic and aqueous extracts respectively. After 21 days of treatment, blood samples were drawn after euthanasia by heart puncture from all the animals to measure the serum testosterone levels and Epididymal sperm was extracted to analyse various parameters. The collected data were analysed using ANOVA and found to be statistically significant and as follows. The group receiving heat treatment with ethanolic extract exhibited higher testosterone levels followed by increased semen concentration, motility and morphology. From this study it is apparent that the ethanolic extract of *Rosa indica* petals alleviate heat stress induced male infertility.

***Key words*:** Heat stress, Male infertility, *Rosa indica*, Sperm parameters, Testosterone

Fertility has drastically decreased in the last two decades, especially in men. Heat stress is one of the main factors responsible for decreased spermatogenesis due to oxidative stress [1]. Higher testicular temperature leads to increase in metabolism as a result decrease in spermatogenesis, increase in sperm damage in mice [1], men [2], bull [3-6] and ram [7-8]. Increase in ROS or decrease in antioxidant levels could happen after exposure to chronic heat stress. Antioxidant response would be immediate in acute situation, accomplished mainly by protein activation and on the other hand by gene activation and translation of few proteins in chronic conditions. Now a days, plants are a reliable source of alternate medicine due to various reasons like easy access, low cost, low side effects with sustainable effectiveness. Several plants are used to treat various diseases, including that of the reproductive system [9]. Plants are a rich source of modern medicine due to their abundance in phytochemicals like flavanoid, alkaloid, steroids,

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terpenoids, glycosides, phenols, glycoprotein etc. [10] which neutralises free radicles and toxins [11].

Rosa indica, a perennial shrub are a rich source of Vit c, anthocyanin, cyanidin 3,5 diglydiglycoside, kaempferol, arbinoside, galactoside, citronellol, terpenes, quercetin, geranoil [12-14] which makes it a candidate for treating various diseases [15]. Various rose preparations are used as tonics, astringents, laxatives, medicine to treat sore throat, tonsilitis, gall stones and also as an antibacterial agent [16]. Numerous studies shows that damask rose serves to decrease stress, relieve depression and trigger joy by stimulating central nervous system [17]. However very few studies have been carried out in context to the medicinal properties of *Rosa indica*. The aim of this study was to evaluate the alleviating effect of *Rosa indica* petal extracts on chronic heat stress induced male infertility.

MATERIALS AND METHODS

Chemicals

Solutes and solvents of analytical grade were used to carry out the present study.

Plant material

Rosa indica flowers collected from the local areas of Coimbatore, Tamil Nadu, was authenticated by the Head,

Institute of Forest Genetics and Tree breeding, Coimbatore with authentication number 924/FECC/ID/IFGTB/2020.

Preparation of sample

The cleaned fresh petals of *R. indica* weighed to 100 g each was shade dried for 2 weeks. Pre weighed 100 g each of the petals were extracted with 500 ml of double distilled water and 100% ethanol respectively using mortar and pestle and incubated at 4℃ for 48 hours. After 48 hours each extract was filtered using cheese cloth and the filtrates lyophilized to get a coarse powder. The extract obtained was stored at 4℃ to carry out further studies.

Animals

After obtaining the Institutional animal ethical clearance from Ramakrishna College of Pharmacy, Coimbatore with approval number (COPSRIPMS/ IAEC/ PHD/ PROJECT/001/ 2020-2021), the study was conducted on 3 months old male Wistar rats weighing 180-240 g. Animals were housed under standard lab conditions of 12 hrs light and dark with free access to compressed food and water. The treatment was started after 7 days of acclimatization.

Experimental design

3 months old male Wistar rats each weighing 180-240 g were randomly allocated to 5 groups, each containing 6 animals. The study group is as follows:

Group 1- Control - Water and Food only
Group II- Negative control- Heat treatment
Group III- Positive control- Heat treatment + 50 mg/kg Quercetin
Group IV- Heat treatment + 200 mg/kg Aqueous extract
Group V- Heat treatment + 200mg/kg Ethanolic extract

Heat stress was induced from day 8 of treatment for 6 consecutive days by immersing hindlegs, tail and scrotum in 43℃ thermostatically controlled water bath for 30 minutes (Naushin). Both the extracts and Quercetin were dissolved in

0.5% carboxy methyl cellulose and administered through intra gastric gavage. After 21 days of treatment animals were euthanised using 100 mg/kg ketamine intra peritoneally and Blood collected immediately by cardiac puncture. Epididymis was removed and sperm collected from the cauda epididymis using MOPS buffer.

Serum testosterone measurement

Serum was collected by centrifuging blood at 2000 rpm for 10 minutes and stored at -20℃ till further study. Serum testosterone was measured using Electrochemiluminescence immunoassay (ECLIA) kit by Roche Diagnostics GmbH, Mannheim as per manufacturer's instruction.

Sperm analysis

The cauda epididymis was dissected from all the study animals, washed with Sodium bicarbonate buffer, several incisions made using scalpel blade in the epididymal tail, gentle pressure applied and incubated for 15 minutes at 37℃ to allow the spermatozoa to swim out from the reproductive duct (Nichi m from evaluating lasting 28). After incubation the following parameters were studied including sperm concentration, motility and morphology using improved Neubauer hemocytometer by diluting sperm suspension in the diluting solution (50g/L Sodium bicarbonate) in distilled water (1:20). 10 µl of the diluted sample was put into the Neubauer hemocytometer and number of sperm were counted under a light microscope (Nikon Ts100, Tokyo, Japan) and expressed as Million/ml. Sperm motility (as Rapidly progressive, Slow progressive and sluggish) was counted and expressed as % motility. Sperm morphology was assessed from air dried smear prepared by mixing 10 µl of the sperm sample with equal amount of Eosin Nigrosin stain under oil immersion and expressed as % normal forms.

Statistical analysis

The data obtained were analysed using one way ANOVA and a $P<0.05$ was considered statistically significant

RESULTS AND DISCUSSION

Testosterone level

The total testosterone (ng/ml) was highest in the positive control group (animals with heat treatment + Quercetin) followed by heat stressed animals treated with 200 mg/kg ethanolic extract and then by heat stressed animals treated with 200 mg/kg aqueous extract with a significant P value of 0.01.

Epididymal sperm parameters

Heat stressed animals treated with 200mg/kg ethanolic extract and 200 mg aqueous extract showed a comparably higher sperm parameters including sperm concentration, motility and morphology to the group treated with quercetin. The group treated with heat stress alone had least semen parameters which could be witnessed from the graphs below:

) with a significant $P<0.01$

Table 1 Testosterone level (ng/m

	Mean(ng/ml)	St D	Std Error	F stat	p value
Control	0.53	0.15	0.087	59.88	0.01
Negative control	0.46	0.22	0.128		
Positive control	1.88***	0.12	0.069		

Aqueous extract	0.69	0.1	0.057
Ethanolic extract	1.63**	0.11	0.068

***Highest level of testosterone; * ᵏComparably higher level

Table 2 Sperm concentration (%) with ignificant P<0.05

	Mean(ng/ml)	St D	Std Error	F stat	p value
Control	7.2	0.8367	0.3742	72.34	0.05
Negative control	4.8	0.8367	0.3742		
Positive control	11.8***	0.8367	0.3742		
Aqueous extract	5.6	0.5477	0.2449		
Ethanolic extract	10.2**	0.8367	0.3742		

***Highest level of testosterone **Comparably higher level

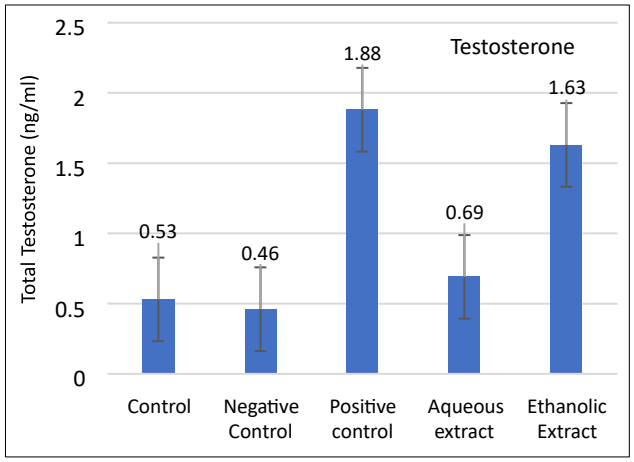

Fig 1 Testosterone levels (ng/ml).
Positive control (Quercetin) showing the highest testosterone levels followed by ethanolic extract treated group

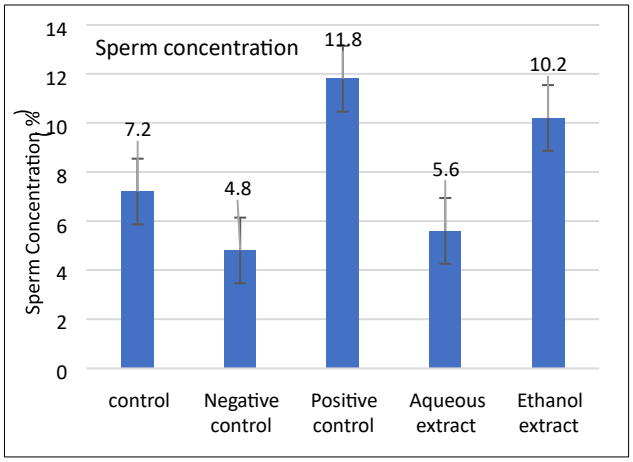

Fig 2 Sperm Concentration (m/ml).
Positive control showing the highest sperm concentration followed by ethanolic extract treated group

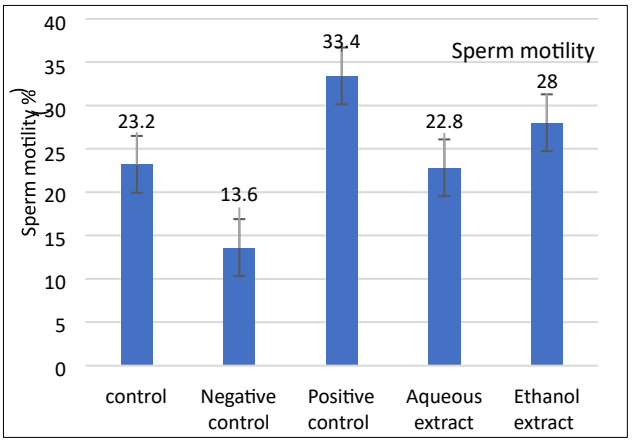

Fig 3 Sperm motility (%). Positive control showing the highest % of sperm motility followed by ethanolic extract treated group

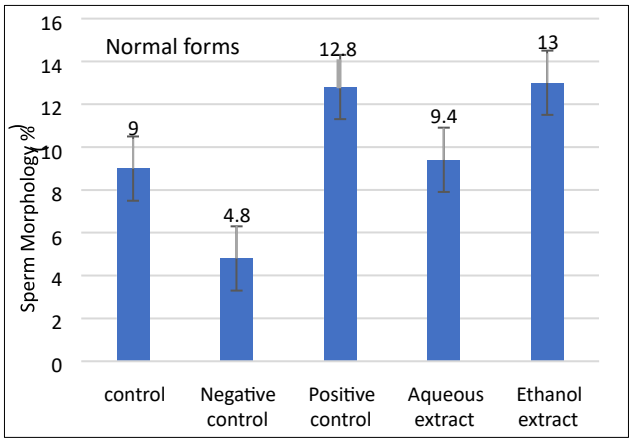

Fig 4 Sperm morphology (%). Ethanolic extract treated group showing the highest % of normal forms followed by positive control

Table 3 Sperm motility (%) with a significant P<0.01

	Mean(ng/ml)	St D	Std Error	F stat	p value
Control	23.2	1.6432	0.7348	99.25	0.01
Negative control	13.6	1.6733	0.7483		
Positive control	33.4***	1.6733	0.7483		
Aqueous extract	22.8	2.0494	0.9165		
Ethanolic extract	28**	1	0.4472		

***Highest level of testosterone
**Comparably higher level

rms (%) with a significant P<0.05

Table 4 Normal f

	Mean(ng/ml)	St D	Std Error	F stat	p value
Control	9	2.1213	0.9487	19.1498	0.05
Negative control	4.8	1.3038	0.5831		
Positive control	12.8	1.6432	0.7348		
Aqueous extract	9.4	1.5166	0.6782		
Ethanolic extract	13	1.8708	0.8367		

***Highest level of testosterone **Comparably higher level

The present study shows that heat stress damaged the *Rosa indica* petals, rich in antioxidants reduces oxidative stress testicular structures including the leydig cells thus exhibiting reduction in the serum total testosterone level and semen parameters. Whereas the oral administration of ethanolic extract of *ROSA indica* petals alleviated the effect of heat stress on the animals and thus by improving the spermatogenesis. From the above results it could be justified that the ethanolic extract of

in testes, increase the leydig cell activity thus by increasing the serum testosterone and there by spermatogenesis.

CONCLUSIO N

It could be concluded that *Rosa indica* is a best candidate and a cost effective alternate to treat male infertility caused due to oxidative stress as a result of increased testicular heat.

Acknowledgement

Ethical approval: *Author declares that the Institutional ethical approval number was obtained and mentioned COPSRIPMS/ IAEC/ PHD/ PROJECT/001/ 2020-2021. Department of Pharmacology, Sri Ramakrishna College of Pharmacy, Coimbatore, Tamil Nadu for extending his help to conduct animal study.*

Conflict of interest: *Authors declare that there are no conflicts of interest.*

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