3. MATERIAL

3.1. Plant material

Present study was conducted on *Rheum emodi* and *Podophyllum hexandrum* and the work was carried out in the Division of Veterinary Biochemistry, Faculty of Veterinary Sciences and Animal Husbandry, SKUAST-Kashmir.

The rhizomes of both the plants *Rheum emodi* & *Podophyllum hexandrum* were collected in the month of May - June from Sonamarg area of Kashmir Valley at an elevation of 3000m and identified at the centre of Plant Taxonomy, Department of Botany, University of Kashmir under voucher no. (Kash-bot/KU/Rh-SB-1746). The plant material (rhizome) was dried in the shade at 30 ± 2°C. The dried rhizome was grounded into a fine powder using mortar and pestle and filtered through a sieve of 0.3 mm mesh size. The powder was extracted with water and methanol using a Soxhlet extractor (60-80°C). These extracts were concentrated using the rotary vacuum evaporator and then stored at 4°C for future use.

3.2 CHEMICALS

<table>
<thead>
<tr>
<th>Name of the chemical</th>
<th>Source</th>
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<tbody>
<tr>
<td>Potassium iodide</td>
<td>Merck</td>
</tr>
<tr>
<td>Bismuth nitrate</td>
<td>SRL</td>
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<tr>
<td>Ferric Chloride</td>
<td>Merck</td>
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<tr>
<td>Methanol</td>
<td>SRL</td>
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<tr>
<td>Sulphuric acid</td>
<td>Merck</td>
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<tr>
<td>Chloroform</td>
<td>SRL</td>
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<tr>
<td>Potassium tartrate</td>
<td>Loba Chemie</td>
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<tr>
<td>Acetic acid</td>
<td>Merck</td>
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<tr>
<td>Ammonia</td>
<td>Merck</td>
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<tr>
<td>Ninhydrin</td>
<td>SRL</td>
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<tr>
<td>Acetone</td>
<td>Merck</td>
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</tbody>
</table>
Magnesium Merck
Sodium hydroxide Merck
Sodium nitrite Merck
Sodium meta periodate Merck
Caffeine Bengal Chemicals
Hydrochloric acid Merck
Copper sulphate Merck
Aluminium chloride Merck
Rutin Merck
Sodium carbonate SRL
Tannic acid SRL
Gallic acid Merck
Diosgenin SRL
Cholestrol Merck

3.3. Phytochemical screening
Chemical tests were conducted on aqueous and methanolic extracts of *Rheum emodi* and *Podophyllum hexandrum* using standard protocols to identify various phytoconstituents as described by Sofowora 1993, Trease and Evans 1989, Herborne 1973.

**Test for Alkaloids:**

**Requirement:**

**Dragendorff’s reagent:** 0.5 g of bismuth nitrate was taken into a clean beaker. To this 10 ml of distilled water was added to make the suspension. 10 ml. of concentrated HCl was added to it with stirring. In another beaker 4g of potassium iodide (KI) was taken and little water was added to it and stirred till KI got completely dissolved. Both the solutions were mixed which resulted in the formation of dark orange solution (Dragendorff’s reagent).

**Procedure**
About 2ml of aqueous and methanolic extract of *Rheum emodi* and *Podophyllum hexandrum* were warmed separately with 2% Sulphuric acid for 2 minutes. It was filtered in test tube and few drops of Dragendorff’s reagent were added the presence of orange red precipitate indicated the presence of alkaloids.
Test for saponins:

Procedure:
About 2 ml. of the test extract was shaken with 5ml of distilled water and then heated to boil. Appearance of creamy miss of small bubbles (frothing) confirmed the presence of saponins.

Test for Tannin:

Requirement: ferric chloride (FeCl₃)

Procedure:
Small quantity of aqueous and methanolic extract of selected plants were individually mixed with 5ml of distilled water and heated on water bath. The mixture was filtered and then ferric chloride was added to the filtrate and observed for dark green solutions that indicated the presence of tannin.

Tests for sugars:

Requirement:
Fehling solution A: 7g of copper sulphate (CuSO₄) dissolved in distilled water containing 2 drops of conc. H₂SO₄
Fehling solution B: 35g of potassium tartrate (C₄H₄K₂O₆) and 12g of sodium hydroxide (NaOH) dissolved in 100ml of distilled water. Equal volumes of solution A and solution B were added.

Procedure:
Fehling’s test was followed for the detection of sugars. Equal volume of methanolic and aqueous extracts of each plant was added to the mixture of Fehling A and Fehling B solutions and heated for 5-10 min. in boiling water and allowed to cool thereafter. Depending upon the presence of amount of reducing sugar the change in the colour was observed from green to yellow to red, otherwise no color change would occur.

Test for Steroids:

Requirement: Acetic acid (CH₃COOH), H₂SO₄, (CHCl₃)

Procedure:
A small amount of aqueous/methanolic extract was dissolved in 1ml of chloroform and filtered. To the filtrate on ice, 1 ml of CH₃COOH was added and then few drops of concentrated H₂SO₄
were run down the sides of test tube. The appearance of blue, bluish-green or a rapid change from pink to blue colour indicated the presence of steroids.

**Test for Flavonoid:**
**Requirement:** Methanol (CH$_3$OH), magnesium (Mg), HCl
**Procedure:**
4 ml of extract was taken and about 2ml of 50% methanol was added. The solution was warmed and metal magnesium was added. This was followed by addition of 5 to 6 drops of concentrated hydrochloric acid. Red coloration confirms the presence of flavanoids.

**Test for Glycoside:**
**Requirements:** H$_2$SO$_4$, CHCl$_3$, NH$_3$
**Procedure:**
(Borntrager’s Test): In a test tube containing 2 ml of extract 2 ml of dilute sulphuric acid was added and boiled for 5 min and filtered. To the filtrates equal volumes of chloroform was added and mixed properly. The organic layer was separated and ammonia was added to this. Appearance of pinkish red colour of the ammonia layer indicated the presence of glycosides.

**Test for Terpenoids:**
**Requirement:** CHCl$_3$, H$_2$SO$_4$
**Procedure:**
Crude aqueous/ methanolic extract of Rheum emodi and Podophyllum hexandrum was dissolved in 3 ml of chloroform in separate test tubes and then evaporated to dryness. To this 2 ml of conc. H$_2$SO$_4$ was added and heated about for 3 minutes. The appearance of grayish colour indicated the presence of terpenoids

**Test for Amino acid:**
**Requirement:** Ninhydrin reagent was prepared by dissolving 8 g of Ninhydrin in 100 ml of acetone
**Procedure:**
Test solution aqueous/methanolic of Rheum emodi and Podophyllum hexandrum was boiled with 0.2% Ninhydrin solution. The appearance purple color indicated the presence of amino acid.
**Test for Phenols**

**Requirement:** Ferric chloride

**Procedure:**
About 2 ml. aqueous/methanolic extract of tested plants were treated with 5% ferric chloride and observed for the formation of deep blue color which indicated the presence of phenol.

**Test for anthraquinones:**

**Requirements:** Hydrochloric acid, chloroform, ammonia

**Procedure:**
About 2 ml. of aqueous/methanolic extracts was boiled with 10% HCl for few minutes in a water bath. It was filtered and allowed to cool. Equal volume of CHCl₃ and filtrate were added. Few drops of 10% NH₃ were added to the mixture and heat. Appearance of rose-pink colour confirmed the presence of anthraquinones.

### 3.4 Quantitative determination of Phytochemicals

**Estimation of Alkaloids: (Singh and Sah 2006)**

- To 1.5 ml. of aqueous/methanolic extract distilled water was added to make final volume 10 ml. in a 25 ml. standard flasks.
- To this 1 ml. of 0.01 M sodium meta periodate (SPI) and 0.5 ml. of 0.1 M CH₃COOH was added and kept in boiling water bath for 10 minutes (min.).
- After this 2 ml. of 0.01 M 3-methyl 2-benzo thiazolinone hydrazone hydrochloride was added to each flask and boiled in water bath for 2 min.
- The flasks were cooled and made upto mark with double distilled water.
- The blue colour formed was read at 630nm with the help of UV/VIS spectrometer.
- Caffeine was used as a standard for drawing a calibration curve.

**Estimation of flavonoids (Zhishen et al., 2010)**

- To 0.1 ml of extract distilled water was added to make final volume 5 ml.
- To this 0.3 ml. of 5% sodium nitrite (NaNO₂) was added, 5 min. later 3 ml. of 10% aluminium chloride (AlCl₃) was added.
- 6 min. later 2 ml of 1 M sodium hydroxide was added and absorbance was measured at 510 nm with the help of UV/VIS spectrometer.
• Rutin was used as a standard for plotting a calibration curve.

**Estimation of Saponins: (Obadoni and Ochuko 2001)**
• Test extracts were dissolved in 80% methanol and 2 ml. of vanillin in ethanol was added and mixed well.
• To this 2 ml. of 72% H₂SO₄ was added, mixed well and heated on a water bath for 10 min.
• The absorbance was recorded at 544 nm with the help of UV/VIS spectrometer.
• Diosgenin was used as a standard for plotting a calibration curve.

**Estimation of Tannins (Graham 1992)**
• To 0.1 ml. test extract 6.9 ml. distilled water was added
• Then 1 ml. of 0.008 M potassium ferricyanide (C₆N₆FeK₃) and 1 ml. of FeCl₃ dissolved in 0.1 M was added and mixed well.
• The absorbance was recorded at 700 nm with the help of UV/VIS spectrometer.
• Tannic acid was used as a standard for plotting a calibration curve.

**Estimation of Phenolic Content (Singleton and Rosi 1965)**
• To 0.1 ml. of aqueous and methanol extract 3.9 ml. distilled water and 0.5 ml. of folin; reagent were added.
• The tubes were incubated at room temperature for 3 min.
• To this 2 ml. of 20% Na₂CO₃ was added and kept in boiling water bath for 1 min.
• The blue colour formed was recorded at 650 nm with the help of UV/VIS spectrometer.
• Gallic acid was used as a standard for plotting a calibration curve.

**Estimation of Sterols (Libermann-Burchard method)**
• To 1 ml of test extract chloroform was added to make the volume upto 5 ml. in a test tube.
• To this 2 ml. of Libermann-Buchard reagent (0.5 ml. of conc. H₂SO₄ in 10 ml. of acetic anhydride) was added and mixed well.
• The tubes were covered with black paper and kept under dark for 15 min.
• The green colour complex formed was measured at 640 nm with the help of UV/VIS spectrometer.
• Cholestrol was used as a standard for plotting a calibration curve.
3.5. Test microorganisms
The test organisms were supplied by Department of Microbiology, Government Medical College Srinagar. The bacteria strains used in the study were *Bacillus megaterium* MTCC 1684 and *Psedomonas aeruginosa* MTCC 3541 two fungi: *Fusarium solani* and *Aspergillus flavus* were used in the study.

Preparation of inoculum
Stock cultures were maintained at 4°C on slopes of nutrient agar. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of Mueller-Hinton broth (MHB) for bacteria and Sabouraud dextrose broth (SDB) for fungi that were incubated without agitation for 24 h at 37°C and 25°C respectively. To 5ml of MHB and SDB, 0.2 ml of culture was inoculated and incubated till it reached the turbidity equal to that of the standard 0.5 McFarland solution (McFarland 1907) at 600 nm which is equivalent to $10^6$– $10^8$ CFU/ml.

Antimicrobial assay
Disc diffusion method
Bauer et al., 1966 was followed for disc diffusion assay. *In vitro* antimicrobial activity was screened by using Mueller Hinton Agar (MHA) obtained from Himedia. The MHA plates were prepared by pouring 15 ml of molten media into sterile petriplates. The plates were allowed to solidify for 5 min and 0.1 % inoculum suspension was swabbed uniformly and the inoculum was allowed to dry for 5 min. The same procedure has been followed for the fungi using Sabouraud dextrose agar. The different concentrations of extracts (1, 2 and 4 mg/disc) were loaded on 5 mm sterile individual discs. The loaded discs were placed on the surface of medium and the compound was allowed to diffuse for 5 min and the plates were kept for incubation at 37°C for 24 h. Negative control was prepared using respective solvent. Erythromycin and tetracycllin (10μg/disc) were used as positive control. At the end of incubation, inhibition zones formed around the disc were measured with transparent ruler in millimeter. These studies were performed in triplicate.

Antifungal Assay
Extracts of *Podophyllum hexandrum* and *Rheum emodi* prepared with methanol (MERCK) were used to test their antifungal activity. Antifungal activity was demonstrated using a modification
of the method originally developed by Bauer et al which is widely used for the antimicrobial susceptibility testing. Liquid nutrient potato dextrose agar media and the petri plates were sterilized by autoclaving at 120°C for 30 minutes. Under septic conditions in the laminar airflow chamber, about 20 ml of the agar medium was dispensed into each petriplate to yield a uniform depth of 4mm. After solidification of the media, the fungal strains were swabbed on the surface of the plates. Whatmann no.1 filter paper was cut into small discs of diameter 0.4cm and autoclaved. The discs were dipped into the different plant extracts of each four concentrations namely 2.5mg/ml, 5.0mg/ml, 7.5mg/ml and 10.0mg/ml. The dipped discs were placed on the appropriate swabbed petriplates such as that each petriplate have the four concentrations of each plant extract. Amphotericin B and noxafil were used as the standard drugs. It was then kept in incubator maintaining the temperature at about 25°C for 48 hours and then the zones of inhibition were measured in mm.

3.6 Minimum Inhibitory Concentration (MIC) Asssay

The MIC method was applied on extracts that proved their high efficacy against microorganisms by the disk diffusion (Bauer et al., 1966) method. The highest dilution of a plant extract that still retains an inhibitory effect against the growth of a microorganism is known as MIC (Misra and Dixit 1978). Selected plant extracts were subjected to a serial dilution (25 mg/ml to 0.37 mg/ml) using sterile nutrient broth medium as a diluent. In a 96-well titre plate 20 μl of an individual microorganism and 20 μl of selected plant extract were loaded and inoculated at 37°C for 24 h. The highest dilution of the plant extract that retained its inhibitory effect resulting in no growth (absence of turbidity) of a microorganism is recorded as the MIC value of the extract. A control experiment was run in parallel to study the impact of the solvent alone (without plant extracts) on growth of the four test organisms. Methanol was diluted in a similar pattern with sterile nutrient broth followed by inoculation and incubation.

3.7 Statistical analysis

All the determinations were carried out in triplicates. The results were expressed as mean ± SE and mean values were plotted in all figures. The level of significance was expressed using Students’t-Test. All the analysis was carried out using GraphPad Prism 5 software.