Materials and methods

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The plants selected for the study are locally available and are reported to possess good antimicrobial property in the literature.

Collection of plants

Plants used for the study were collected from kanjampatty (plain) and pasumalai (hills) throughout the year at regular intervals in order to find out the influence of geographical and seasonal variation on phytochemcial constituents of the plants.

Plants screened for preliminary studies

Plant name	Part used	Collected from
Allium sativum	Bulb	Kanjampatty
Andrographis paniculata	Leaves	Pasumalai hills
Annona squamosa	Leaves	Kanjampatty
Azardirachta indica	Leaves	Kanjampatty
Corallocarpus epigaeus	Tuber	Pasumalai hills
Lawsonia inermis	Leaves	Kanjampatty
Madhuca longifolia	Seeds	Pasumalai hills
Ocimum sanctum	Leaves	Kanjampatty
Pongamia pinnata	Bark	Kanjampatty

The collected plant materials were washed thoroughly, shade dried and extracted in soxhlet apparatus with different solvents like petroleum ether, benzene, chloroform, acetone, methanol and water. They were stored in refrigerator in air tight containers for further studies.

Screening of plants

Screening was done based on their antimicrobial properties. The plant extracts were tested against the fungal and bacterial pathogens collected from infected animals. Plants which gave good results were selected for further studies.

Collection and inoculation of pathogens

The skin scrapings and hair plucks of infected animals (including sheep, goat, cows and dogs) were collected manually with the help of sterilized scalpel and forceps. The samples were collected from different geographical regions of Tamilnadu such as Coimbatore, Pollachi, Madurai, Theni, Perambalur and Trichy. It was collected in sterile petriplates, sealed and kept in a cool pack and brought to the laboratory of veterinary hospital, kanjampatty in Tamil Nadu.

Inoculation and maintenance

The skin scrapings were directly inoculated into the Dermatophytes Test Medium (DTM) under aseptic condition. The cultures were incubated at 25^{o}C and kept under observation for 10-14 days.

Dermatophytes Test Medium (Taplin, 1969)

(Hi media product)

Ingredients	**gm/litre**
Papaic digest of soyabean meal	- 10
Glucose	- 10
Phenol red	- 0.2
Agar	- 20
pH (at 25°C)	- 5.5±0.2

Composition of Dermato supplement

Dermato supplement is a mixture of antibiotics available in the market in a vial (300 mg) which is sufficient for 500 ml medium.

Antibiotics	**Concentration**
Cycloheximide	- 250mg
Chlortetracycline	- 50mg
Gentamicin	- 50mg

Medium preparation

20 gm of DTM agar medium is suspended in 500 ml distilled water. Heated to boiling so as to dissolve the medium completely. It is sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes and allowed to cool to 50°C.

One vial of Dermato Supplement (300mg) is rehydrated by adding the content to 5 ml of 50% (v/v) acetone and mixed well. The rehydrated content was added to molten DTM agar medium (45-50°C) aseptically and mixed well before pouring into sterile petriplates.

DTM is a selective and differential medium used for detection and identification of dermatophytes. Papaic digest of soyabean meal provides nitrogenous and carbonaceous substances essential for growth. Glucose is the energy source. The pH indicator, phenol red is used to detect amine production. Cycloheximide inhibits most of the saprophytic fungi. Gentamicin inhibits gram-negative bacteria including *Pseudomonas* species while chlortetracycline inhibits a wide range of gram-positive and gram-negative bacteria. The microbial growth on the medium provides presumptive identification of dermatophytes. It helps in isolation and early recognition of members of the *Microsporum, Trichophyton* by means of the distinct colour change from yellow to red. Rapidly growing species affect complete colour change within 3 days while slow growers will change

colour in proportionately longer time. Non-Dermatophytes can be recognized by the absence of colour change.

Principle

A combination of three antimicrobial agents (cycloheximide, chlortetracycline and gentamicin) inhibits bacteria and saprophytic yeasts and moulds. Dermatophytes are presumptively identified based on gross morphology and the production of alkaline metabolites, which raise the pH and cause the phenol red indicator to change the color of the medium from yellow to pink-red.

Identification

Dermatophytes were identified by the typical morphology and colour change in the medium within 10-14 days. Colour change after 14 days was disregarded since it may be due to fungal contamination. Morphologically varied fungal and bacterial cultures grown in DTM medium were isolated and cultured separately in Sabouraud Dextrose agar medium (SDA) and Muller Hinton Agar medium (MHA) respectively. Pure cultures of these dermatophytes were sent to Animal Disease Intelligent unit (ADIU), Madurai, Tamilnadu, India for identification and the mother culture was maintained at Veterinary Hospital, Kanjampatty (Po), Pollachi (Tk), Coimbatore (Dt), Tamil Nadu, India. Subcultures were made once in a month to maintain the mother culture.

Morphological characterization of fungal pathogens by Slide Culture technique

The morphological characters of fungal pathogens were determined by Slide culture technique (Onoins *et al*., 1986). Sterile Sabouraud Dextrose Agar block of 1 cm^2 was cut with the help of sterile tool (Scalpel/blade) and transferred to the centre of a sterile slides. The fungus was inoculated on agar block sides/cut ends and covered with a sterile cover glass. The slide was kept under moist condition by keeping it in 20% glycerol solution in the bottom of the plate. The fungus spreads

out from the sides of the agar block and tends to attach itself to the surface of both cover glass and slide. After desired stage of growth (after 7 days), cover glass was carefully removed from the agar block and mounted on separate slide with mounting fluid Lactophenol cotton blue. Then, the agar block was carefully removed from the slide and the fungal culture was mounted with lactophenol cotton blue.

Identification of bacterial pathogens

Bacterial pathogens were identified by following drop catalase test (Taylor and Achanzar, 1972). It is one of the most popular methods in clinical bacteriology because it requires small amount of organism and relies on a relatively uncomplicated technique. This protocol is used for the identification and differentiation of *Staphylococci sp* and *Streptococci sp.*

Protocol for identification of bacteria

Place a slide inside a petri dish and keep the petri dish cover available. The use of a petri dish is optional as the slide catalase can be properly performed without it. However, to limit catalase aerosols, which have been shown to carry viable bacterial cells, the use of a petri dish is strongly recommended. Using a sterile inoculating loop or wooden applicator stick, small amount of organism were collected from 18 to 24hour cultured colony and placed it on the microscope slide. Using a dropper or pipette, 1 drop of 3% H_2O_2 is placed over the organism on the slide. It was covered with the petri dish with a lid to limit aerosols and observe for immediate bubble formation. Bubbles were observed against a dark background enhances readability. Positive reactions were evident by immediate effervescence (bubble formation). No bubble formation (no catalase enzyme to hydrolyze the hydrogen peroxide) represents a catalase-negative reaction. *Staphylococcus sp* is catalase positive and *Streptococci sp* is catalase negative.

Pathogens identified

Fungi

1. *Aspergillus niger*
2. *Candida albicans*
3. *Curvularia geniculata*
4. *Geotrichum candidum*
5. *Microsporum gypseum*
6. *Penicillium sps*
7. *Rhizopus rhizopodoformi*
8. *Rhodotorula minuta*
9. *Trichophyton mentagrophytes*

Bacteria

1. *Staphylococcus sp*
2. *Streptococci sp.*

Antimicrobial studies

Well diffusion method was employed to study the antimicrobial properties of the screened plants. Different solvent extracts of the plants were tested against the isolated pathogens.

Media used

Sabouraud Dextrose Agar Medium (SDA) and Muller Hinton Agar Medium (MHA) were used for culturing fungi and bacteria respectively.

SDA medium composition (Cheesbrough, 1992)

Ingredients	**gm /litre**
Dextrose	- 40
Mycological, peptone	- 10
Agar	- 15
Final pH (at 25°C)	- 5.6±0.2

MHA medium composition (NCCLS, 2000)

Ingredients	**gm /litre**
Beef infusion	- 3
Casein acid hydrolysate	- 17.5
Starch	- 1.5
Agar	- 17
Final pH (at 25°C)	- 7.3±0.1

Sterilization

All glass wares were well sterilized in hot air oven at 180^{o}C for 3 hours. Instruments like forceps, inoculation needle were sterilized over flame after dipping in alcohol. Media were sterilized in pressure cooker for 20 minutes.

Source of inoculum

For bacteria, 24 hours old culture was used as an inoculum. A loopful of each bacterial culture was suspended in 5ml of sterile Muller Hinton Broth and shaken vigorously to get the uniform suspension.

For fungi, spore suspension was used. A loopful of sporulating culture of each fungus was suspended in 5ml of sterile distilled water and shaken vigorously.

Method of inoculation

Sterilized petriplates containing sterile medium was swabbed with selected microorganisms in such a way so as to get a thorough coverage of uniform thick lawn of growth of the inoculum.

Well diffusion method

After swabbing the cultures, wells of 6 mm diameter were created with a sterile cork borer. To this, 20-100µl (µg / µl) of plant extracts were dispensed and the petriplates were carefully sealed and incubated.

Method of incubation

Fungal cultures were incubated at 25^{o}C for 48 hours and bacteria at 37^{o}C for 24 hours. Triplicates were maintained for each experiment. Fresh cultures were used for the analysis. The performance of plant extracts were determined by the zone of inhibition.

Minimum Inhibitory Concentration

Minimum Inhibitory concentration is the lowest concentration of an antimicrobial agent that inhibit the growth of microorganism after overnight incubation. The method adopted was tube dilution method (Savitha and Rathnavijaya, 2011). Serial dilution of methanol extracts of three plants ranging from 1-100 µg were made in the fungal and bacterial broth. The pathogens were inoculated to these broth and incubated. The tubes were scored for growth. The minimum concentration in which there was no growth of pathogens was recorded as MIC value. Triplicates were maintained.

Pharmacognostical studies

After preliminary screenings, plants with good antimicrobial properties were selected for further pharmacognostical studies. They are,

1. *Andrographis paniculata* - Acanthaceae
2. *Lawsonia inermis* - Lythraceae
3. *Madhuca longifolia* - Sapotaceae

Preparation of powder

The plant materials were shade dried, powdered and stored in air tight containers for further analysis.

Extraction procedure

The powder of selected plant materials were successively extracted with different solvents in a soxhlet apparatus.

Solvents used

Petroleum ether (70^{o} C)

Benzene (75^{o} C)

Chloroform (75^{o} C)

Acetone (55^{o} C)

Methanol (70^{o} C)

Water (100 ^{o} C)

Preparation of plant extracts for TLC, HPLC, antioxidant and antimicrobial studies

The selected plant materials were extracted with required amount of methanol in a soxhlet apparatus. Then the extract was completely dried by keeping it in hot air oven at 45^{o}C. The dried extracts were used for further studies like TLC, HPLC, antioxidant and antimicrobial studies.

Physico Chemical analysis

Analytical method

The powders of selected plant materials were used for calculating the physico-chemical parameters like loss on drying, total ash, acid insoluble ash, water soluble ash and percentage of extractive value.

Loss on Drying

Freshly collected and pre-weighed samples were dried in Hot air oven at 40^{o}C until it reaches a constant weight.

Total ash

3 gm of plant powders were ignited in an electric muffle furnace at 100^{o}C in silica crucible until the sample reaches a constant weight.

Acid –insoluble ash values

Total ash obtained was heated by adding 25 ml of diluted hydrochloric acid for 10 minutes. It was then filtered in ashless filter paper (Whatmann No.1) and the residue was incinerated in the furnace to get a constant weight.

Water soluble ash

The same procedure was adapted for the water soluble ash.

Extractive values

The powdered materials were extracted with different solvents like petroleum ether, benzene, chloroform, acetone, methanol and water in a soxhlet apparatus. The extracts were concentrated and the extractive values were calculated on dry weight basis.

Fluorescent analysis

The fluorescent behavior of the plant materials were analyzed by Chase and Pratt (1949) method. Powdered plant materials treated with different solvents and their illuminations were observed under ordinary and Ultra-violet light conditions.

Qualitative phytochemical analysis

Qualitative phytochemical analysis was done by using the procedure of Kokate *et al.*, (2005). Presence of alkaloids, flavonoids, glycosides, terpenoids, tannins, phenols, fixed oils, fats, gums, mucilage and saponins were analyzed qualitatively.

Alkaloids

The selected plant powders were extracted with different solvent, and dissolved in diluted sulphuric acid and filtered. The filtrate was then treated with Mayer's and Wagner's reagents separately. Appearance of cream and reddish brown precipitate respectively indicates the presence of alkaloids.

Glycosides

Plant extracts treated with Fehling's reagent and Benedict's reagent separately. Appearance of brownish red colour and green precipitate respectively indicates the presence of glycosides.

Flavonoids

Plant extracts treated with ferric chloride, formation of brown precipitate indicates the presence of flavonoids.

The extracts treated with 1 gm of magnesium powder and 1ml of concentrated hydrochloric acid, formation of orange colour indicates the presence of flavonoids.

Tannins and Phenols

Plant extracts were taken in two separate test tubes and treated with 5% ferric chloride and 10% lead acetate. Appearance of blue colour with ferric chloride or precipitation with lead acetate, indicates the presence of phenols and tannins respectively.

Fixed oils and Fat

Plant extracts treated with 0.5N alcoholic potassium hydroxide along with a drop of phenolphthalein and heated on water bath for few minutes, formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

Saponins

1ml of the extract is diluted with distilled water and made up to 20ml and shaken in a test tube for 15 minutes, formation of foamy layer indicated the presence of saponins.

Terpenoids

Plant extracts shaken with few drops of antimony trichloride solution, appearance of blue colour precipitate indicates the presence of terpenoids.

Quinones

Few drops of sodium hydroxide was mixed with the plant extracts and shaken vigorously. Blue green or red colour indicates the presence of quinone.

Steroids

2ml of acetic anhydride is mixed with 2 ml of Conc.sulphuric acid and this mixture is added to the plant extract. Colour changes from violet to blue green indicates the presence of steroids.

TLC studies

TLC studies were done to determine the active principles of the selected plants.

Preparation of plates

Silica gel was used as an adsorbent. Slurry was prepared with distilled water and spread uniformly (0.3mm thickness) with the help of an applicator on clean dry glass plates. Plates were allowed to dry at room temperature and activated in an oven at 120^{o}C for 30 minutes.

Preparation of plant extracts

Dried plant powder was extracted with chloroform and methanol in a soxhlet apparatus. The extracts were condensed and used for TLC studies.

Development of chromatogram

Name of the plant	Extracts	Mobile phase used
A.paniculata	Chloroform and methanol	Chloroform: Methanol: Ethyl acetate (80:10:10)
L.inermis	Chloroform and methanol	Toluene: Ethyl acetate: Acetic acid (5:4:1)
M.longifolia	Chloroform and methanol	Methanol: formic acid (70:30)

Chromatograms were developed by one way ascending TLC with respective mobile phase. The spotted plates were kept inside the chamber and immediately closed with the lid. The solvent was allowed to travel up on the plate until to reach the desired level (10 - 15 cm). The plates were then removed from the chamber, solvent front was marked and dried at room temperature. The number and position of the various chemical constituents of the extracts were detected by keeping it under UV light and their Rf values were calculated.

HPLC studies

Methanol extracts of selected plant materials were used for HPLC analysis. The system used for analysis was Shimadzu SP 20A model with C-18 column. The injector volume was 20µl. Each sample was chromatographed twice and the data were reported.

Parameters	*Andrographis paniculata*	*Lawsonia inermis*	*Madhuca longifolia*
Mobile phase	Methanol: Water 65:35	Methanol:Water 70:30	Acetonitril:Water 60:40
Wavelength	223 nm	254 nm	254 nm
Flow rate	1ml/min	1ml/min	1ml/min
Run time	10 min	10 min	10 min
Standard used	Andrographolide	Lawsone	--

Estimation of Andrographolide from *A. paniculata*

Andrographolide was estimated in fresh and dried leaves of *A.paniculata* following the method of Azhar Ali farooq and Sree Ramu, (2001).

10gm of coarsely powdered leaf samples (fresh and dried) were extracted with 200ml of ethanol (95%) in a soxhlet apparatus for six hours. The extract was concentrated to 50 ml. To that 30 ml of distilled water was added. The solution was extracted with 25 ml of benzene twice and benzene layer was removed. Again 20ml distilled water was added to the extract. The combined aqueous solution was then extracted with 25 ml of ethyl acetate thrice which was sufficient to extract the andrographolide completely. The combined ethyl acetate extract was then treated with anhydrous sodium sulphate and filtered through a cotton mesh. The cotton mesh was again washed with 5ml ethyl acetate. The collected filtrate was dried by keeping in water bath until to reach a constant weight. The values were expressed in percentage.

Estimation of Lawsone from *L. inermis*

Lawsone content was estimated by the method of Pawan Porwal *et al.*, 2011. 50 gm of crushed leaves (both fresh and dried) of *L. inermis* were taken and extracted by agitated with 200ml of 20% sodium carbonate solution for 2 hours. The content was filtered and again the plant material was agitated with 100ml of sodium bicarbonate solution for 1 hour and filtered. Then the two extracts were pooled together and acidified with diluted sulphuric acid and allowed to stand for 10 min. To this, sufficient quantity of aluminium hydroxide was added and again it was acidified with diluted hydrochloric acid. Finally it was treated with two successive volume of 40ml benzene and filtered. The filtrate was evaporated to get yellowish brown crystal of Lawsone.

Scanning Electron Microscopic (SEM) analysis of selected plant materials

Powdered materials of the three plants were subjected to SEM analysis for nano particles. The work was done at BU- DRDO Centre for Life sciences in Bharathiar University, Coimbatore, Tamil Nadu, India. The instrument used for the analysis is 7EI QUANTA 200, Icon Analytical. Sample was loaded in double sided conducting carbon type. It was then subjected to gold coating for 60 sec. the gold coated samples were taken for analysis.

Antioxidant studies

Antioxidant property of the plant materials were determined by reducing power assay method (Savitha and Rathnavijaya, 2011). Plant extracts were prepared by using methanol and water ranging from 20µg-100µg/ml. Various concentrations of ascorbic acid (from 20-100µg/ml) were used as standard reference.

Procedure

1 ml of plant extract with different concentrations (20, 40, 60, 80 and 100µg/ml) were mixed with 2.5ml of 0.2M phosphate buffer (pH 6.6) and

2.5 ml of potassium ferricyanide. The mixture was incubated at 50^{o}C for 20 minutes. To this 2.0 ml of TCA was added to the mixture and centrifuged at 3000 rpm for 10 minutes. From the upper layer, 2.5ml of solution was taken and mixed with 2.5 ml of distilled water and 5ml of ferric chloride solution (1:1:2). The absorbance was measured at 700nm in UV- Visible spectrophotometer. Phosphate buffer used as blank solution. Increased absorbance signified the increase of reducing power.

***In vitro* studies**

Antimicrobial studies of methanol and water extracts of selected plant materials were tested against the pathogens.

Plant materials were collected during the months of June – August because the plants were observed to possess higher phytochemical constituent during this period. The plant materials were extracted with methanol and water using soxhlet apparatus. Different concentration and combination of these extracts were used for antimicrobial studies.

Povidone iodine solution, candid cream, ketoconozole (100µg) and itraconozole (100µg) were used as positive control for fungi and ampicillin (30µg) used as positive control for bacteria. Methanol used as a negative control for both bacteria and fungi.

***In vivo* studies of plant extracts**

After testing the efficacy of the three plants extracts under *in vitro* condition, the best concentration and combinations were standardized and used for *in vivo* studies.

Drugs used for *in vivo* studies

Plant extracts	Name of the plant	Concentrations used (µg/ml)				
Single	*Andrographis paniculata*	100	200	300	400	500
	Lawsonia inermis	100	200	300	400	500
	Madhuca longifolia	100	200	300	400	500
Combination (1:1)	*A.paniculata* : *L.inermis*	--	--	--	--	500
	A.paniculata : *M.longifolia*	--	--	--	--	500
	L.inermis : *M.longifolia*	--	--	--	--	500
1:1:1	*A.paniculata* : *L.inermis* : *M.longifolia*	--	--	--	--	500
Control	Povidone iodine ointment (100 µg)	--	--	--	--	--
	Candid cream (100 µg)	--	--	--	--	--

In vivo studies were conducted on infected cows at kanjampatti village, pollachi, Coimbatore district, Tamilnadu. The standardized combinations were sprayed ad-libidum over the infected regions of the animals using sprayer twice a day (morning and evening).

Different concentrations (100-500µg/ml) of the plant extracts were used as single drug. The plant extracts with the concentration 500µg/ml showed quick recovery within 8 days and thus this concentration was used for further studies.

The combinations of the plant extracts were made at 2 and 3 combinations as given in the table above. Standard antifungal drugs Povidone iodine and candid cream were used as control. The number of days the plant extracts took to cure the infection was recorded. The combination of drug which cured the disease faster was selected for the preparation of herbal ointment.

Preparation of herbal formulations

The formulation of the selected plants were used to cure skin diseases of domestic animals like cow, dog, horse, sheep and goat. This is the first time these plants were used in combination to treat skin diseases.

Formulation – I - Traditional method

The ointment was prepared following the method detailed in Gunapadam (Thiyagarajan, 1992).

Method of preparation

Fresh leaves of the selected plant materials were ground with the help of mechanical grinder without adding water and filtered through a muslin cloth to get clear extract for the preparation of ointment.

The mixture of leaf extracts along with madhuca oil were boiled together till to get uniform mixture. Paraffin wax was added and the content was transferred to a wide mouthed container.

Formulation – II- Industrial method

This ointment was prepared following the modified method of Jarupa Viyoch *et al*., (2003) at Department of Pharmacy, Karpagam college of Pharmacy, Coimbatore, Tamilnadu, India

Method of preparation

The method of preparation was similar to that of traditional method of ointment preparation. The difference is instead of paraffin wax bee wax was used as oil base. Borax was used as a preservative. After preparation the ointment was transferred to wide mouthed bottle under molten state and sealed after cooling.

Storage

The ointments were stored in air tight containers under 4^{o}C.

Evaluation parameters of the herbal formulations

Physical evaluation

State, colour, odour, texture, appearance was observed and evaluated.

pH determination

pH was determined by mixing the formulation with water and measured in digital pH meter (Panigrahi *et al.*, 1997).

Homogeneity

Homogeneity was determined by visual examination. The formulations were examined for the appearance of lumps (Panigrahi *et al.*, 1997)

Viscosity

The viscosity of ointments was tested in Red wood viscometer (No.1 model) instrument at 90^{o}C. Experiments done in triplicate and average values were recorded (Sayyad *et al.*, 2012).

Determination of spreadability (Pattanayak *et al.*, 2011)

For determination of spreadability, excess of sample was applied in between two glass slides and was compressed to uniform thickness by placing weight (10 gm) for 5 minutes. The time required to move the upper glass slide over the lower slide was taken as a measure of spreadability

$$S = \frac{m \times L}{T}$$

S = Spreadability

m= weight on upper slide

L= length moved on glass slide

T= time taken

Determination of Stability

The stability study was carried out by storing the ointments at different temperature conditions (4^{o}C, 25^{o}C and 37^{o}C) for 3 months. The evaluation

parameters mentioned above were studied at different time intervals (15, 30, 60 and 90th day) (Mohanta *et al.*, 2007)

Primary skin irritation test

The irritation test was made on intact skin of animals (Pooja Banerjee *et al.*, 2009). Hair from the back of animals of 5cm^2 was shaved and the ointments were applied over the site. It was observed upto 48 hours for erythema and/or odema. If there is no symptoms like erythema or odema the ointments can be used for further studies.

Heavy Metal studies

The selected three plant materials collected along with the soil adhere to the root and the ointments were used for heavy metals analysis. The digested samples were used to analyze the heavy metals like lead, chromium, nickel and cadmium using Atomic Absorption Spectrometer (Model - Perkin Elmer AAnalyst 200).

Sample digestion

Preparation of Triacid mixture

Conc. Nitric acid, Sulphuric acid and Perchloric acid were mixed together at the ratio of 9:2:1.

Digestion process

0.5 gm of the samples were taken in 100ml conical flask, 10-15 ml of triacid mixture was added and the mouth of the conical flask was covered with a funnel and kept overnight. Then the next day, digested content was heated in a hot plate until yellow fumes disappeared and clear solution obtained. After that, the solution was made upto 50ml with distilled water and filtered using Whatmann no. 1 filter paper. The filtrate was used for heavy metal analysis.

Clinical studies (Ali Haydar Kirmizigul *et al.*, 2012)

The animals having fungal borne skin diseases were identified from geographical regions like Coimbatore, Pollachi, Madurai, Theni, Perambalur and

Trichy. The disease was identified based on the symptoms like alopecia, loss of hair, grayish-white, crusty raised extensive lesion over the infected area. The animals were isolated and grouped according the clinical status categorized based on size and number of lesions. The lesions were most commonly found on the head, neck, chest, back and dorsal side. The animals were fed as usual.

Clinical status	Condition
Mild (+)	2-4 lesions with size of 1-2 cm in diameter, occured in head, neck and other parts of the body.
Moderate (++)	4-8 lesions with size of 2-4 cm in diameter, occured in head, neck and other parts of the body.
Severe (+++)	More than 8 lesions with 4-6 cm in diameter, ocurred in head, neck and other parts of the body.

The herbal ointments were applied ad-libidum over the infected regions and kept under observation till recovery. The recovery was confirmed by the disappearance of symptoms and animals regaining the normal intake of food and other habits.