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Preparation and evaluation of herbo-mineral topical emulgel formulation in bovine clinical mastitis

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Abstract
The present study was conducted to prepare and evaluate the herbo-mineral emulgel formulation containing *Curcuma longa, Datura metel, Aloe vera and* and calcium hydroxide against bovine clinical mastitis. Extracts of selected plants were screened for phytochemicals. *In Vitro* antimicrobial screening of herbal extracts against isolated bacterial strains was performed by Agar disc-diffusion method. *In Vitro* antimicrobial activities of polyherbals (*Curcuma longa, Datura metel and Aloe vera)* and gentamicin sulphate (antibiotic agent) against *Staphylococcus aureus, Streptococcus agalactiae* and *Escherichia coli* isolates, alone and in combination were evaluated by minimum and fractional inhibitory concentrations study. Oil-in-water herbo-mineral emulsion was prepared using Cremophor EL and Tween 20. The optimized herbo-mineral emulsion was developed into emulgel using Carbopol 940 as a gelling agent and triethanolamine as alkalizer. Herbo-mineral emulgel formulations were evaluated for its pH, appearance, homogenecity, viscosity, spreadability, *ex vivo* permeation and stability. *In vivo* anti-inflammatory assay was done by carrageenan induced rat paw edema test. Acute dermal irritation study was conducted in rabbits. Clinical study was conducted in lactating cows to evaluate the efficacy herbo-mineral emulgel formulation against clinical mastitis. The phytochemicals screening showed the presence of alkaloids, flavonoids, glycosides, phenols, tannins, terpenoids, phlobatannins, saponins and carbohydrate compounds. Minimum and fractional inhibitory concentrations study showed that polyherbals in combination with gentamicin sulphate were able to provide stable synergistic therapeutic outcomes with higher efficacy in combating mastitis causing organisms in bovines. Based on *In Vitro* antimicrobial study, the extracts of *Aloe vera, Datura metel, Curcuma longa* and calcium hydroxide were taken in the ratio of ***2:1:1***:0.3 for the final formulation. The pH of emulgel (6.25± 0.30) was found to be in accordance with the range of udder skin pH. Herbo-mineral emulgel also illustrated efficient permeation (79.20 µL/cm2) through skin in 12 h. Viscosity (5720±85 cp), spreadability (10.02 ±0.12 g.cm/sec) indicated its suitability for topical application. Antimicrobial evaluation of polyherbal emulgel revealed broader zones of growth inhibitions against *E.coli* (16.35±0.58mm), *S. aureus* (19.23±1.05mm) and *S. agalactiae* (19.88±0.67mm). *In vivo* anti-inflammatory study showed that the anti-inflammatory effect of the herbo-mineral emulgel was significantly higher than the effect of Valdecoxib gel formulation and herbo-mineral formulation. Moreover, herbo-mineral emulgel was also found to be non-irritant. These parameters were consistent over 6 months. The Somatic cell counts and bacterial load in milk was significantly reduced in cows treated with herbo-mineral emulgel formulation. The results of clinical study indicated that the combined treatment i.e. herbo-mineral emulgel formulation topically along with systemic administration of gentamicin sulphate continuously for 5 days was most effective in the treatment of clinical mastitis as compared to herbo-mineral emulgel treatment alone or gentamicin sulphate treatment alone. From this study, it can also be concluded that the emulgel turned out to be a promising topical delivery of herbo-mineral containing *Curcuma longa, Datura metel, Aloe vera* and calcium hydroxide with enhanced therapeutic efficacy against clinical mastitis. Further, the herbo-mineral emulgel formulation will be an efficient and economically affordable formulation in the field to treat clinical mastitis immediately by farmers itself before getting help of veterinarians

Keywords: Cattle, Herbo-mineral, Emulgel, Bovine Mastitis, *Curcuma longa, Datura metel, Aloe vera, Calcium hydroxide*

Introduction
Bovine mastitis, an inflammation of the mammary gland, is a major disease challenge for the dairy industry worldwide. The economic losses due to mastitis have increased about 115 folds in the last five decades and presently the loss due to mastitis accounts to 982.72 million million USD per annum in India [1]. It is responsible for huge economic losses to dairy industries due to poor milk quality, reduced milk yield and increased expenditure on treatment and sometimes death due to the disease itself or through culling of affected cows [2, 3]. Clinical mastitis is easy to detect due to the appearance of visible signs associated with the disease.

Subclinical mastitis, however, is difficult to detect due to the absence of any visible signs and symptoms therefore, goes unnoticed most of the times and is responsible for heavy economic loss to dairy industry [4, 5]. However, micro-organisms and somatic cells were found in elevated in the milk of subclinical mastitis [6]. Subclinical mastitis was found more important in India (varying from 10 to 50% in cows and 5 to 20% in buffaloes) than clinical mastitis (1 to 10%). The incidence was highest in Purebred Holsteins and Jerseys and lowest in local cattle and buffaloes [7]. Further, the extensive and indiscriminate use of antibiotics in the treatment and control of mastitis may lead to undesirable presence of drug residues [8]. Given the seriousness of such problems, researchers, and clinicians have been trying to find effective therapeutic agents using alternative medicine.

The *Ayurvedic* literature *Sarangdhar Samhita* highlighted the concept of polyherbalism to achieve greater therapeutic efficacy. In the traditional system of Indian medicine, plant formulations and combined extracts of plants are chosen rather than individual ones. The herb-herb synergistic combinations produce a greater result, as compared to individual use of the plant. Certain pharmacological actions of active constituents of herbals are significant only when potentiated by that of other plants, but not evident when used alone [9]. Thus, the combinations of active phytochemical constituents of plants are efficient to achieve the desirable therapeutic effects. When combining the multiple herbs in a particular ratio, it gives a better therapeutic effect against both bacteria causing mastitis and in reducing inflammation of udder.

Based on literature survey [10, 11, 12, 13, 14, 15, 16, 17] and traditional use, *Curcuma longa*, *Datura metel* and *Aloe vera* were selected. In order to enhance the penetration of polyherbals through udder skin, calcium hydroxide was included along the selected polyherbals. The selected plants with antimicrobial and anti-inflammatory effects and calcium hydroxide were optimized to develop herbo-mineral formulation to achieve greater therapeutic efficacy for bovine clinical mastitis.

Emulgels delivery systems has considerable potential over conventional delivery systems by exhibiting jelly-like consistency and offer a wide utilization for dermatological products by virtue of their better applicability, thixotropic behaviour, greaseless nature, improved spreadability and controlled rheological properties relatively holding the properties of both emulsions and gels [18, 19]. By this technology, the optimized polyherbal drug with antimicrobial and anti-inflammatory properties can be delivered to the udder effectively and conveniently to get maximum therapeutic benefits. The polyherbal emulgel therapeutic kit can be utilized for easy application by farmers and women folk resulting in improved success rate in bovine clinical clinical mastitis when compared to conventional antimicrobial therapy. Hence, the present study is aimed to prepare and evaluate herbo-mineral emulgel formulations loaded with extracts of *Curcuma longa*, *Datura metel* and *Aloe vera* & calcium hydroxide and to assess its therapeutic efficacy against bovine clinical mastitis.

Materials and Methods

Collection of Plant

Fresh leaves of *Datura metel* and *Aloe vera* and rhizome of *Curcuma longa* were collected from Herbal garden (TANII Scheme), Veterinary College and Research Institute, Orathanadu, Tamilnadu, India. The taxonomic identities of plants were confirmed by Botanical Survey of India, Coimbatore, Tamilnadu, India. The reference herbarium specimen was deposited in TANII Scheme herbarium under the voucher number 10/SIF/EVHPR&D Centre/2019. The collected plants were washed with running tap water, air dried, homogenized to a fine powder and stored in air-tight container at 4°C.

Preparation of crude extracts

About 30 g of dried material of *Datura metel* and *Curcuma longa* were extracted with 100 ml of methanol using soxhlet apparatus. *Aloe vera* (30 g) was extracted with 100 ml of acetone. The extract was then concentrated using Rotary Evaporator (Buchi Rotavapor R-300) and the yielding final extract which was stored at 4°C for further usage.

Qualitative Phytochemical screening

Qualitative phytochemical screening was carried out to assess the bioactive components present in extracts of *Aloe vera*, *Datura metel and Curcuma longa* as described by Trease and Evans[20].

Antimicrobial activity

Test microorganisms: Bacterial strains used in this study were isolated from clinical cases of bovine mastitis brought to Veterinary Clinical Complex, Veterinary College and Research Institute, Orathanadu and maintained at Livestock Farm Complex, Veterinary College and Research Institute, Orathanadu. All the strains were confirmed by cultural and biochemical characteristics[21] and maintained in slants for further use.

Agar disc diffusion method: *In Vitro* antibacterial screening of herbal extracts against isolated bacterial strains was performed by Agar disc-diffusion method[22, 23]. The media (Mueller Hinton Agar No.2), along with the inoculum (108 cfu/ml) was poured into the Petri plate (Hi-Media). For the agar disc diffusion method, the disc (0.7 cm) (Hi-Media) was saturated with 100 µl of the test compound, allowed to dry and then placed on the upper layer of the seeded agar plate. Gentamicin was used as positive control antibacterial drug. Pure solvent was used as negative control. The plates were then incubated at 37 °C for 18–24 h. The diameter of the inhibition zone (mm) was measured. The results (zone of inhibition) were compared with the activity of the standard.

Preparation of herbo-mineral formulation

The different combinations of crude extracts of selected plants were tried to find out the most effective combination against *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus agalaectiae*. The crude extracts of plants were taken in different ratio randomly along with calcium hydroxide (0.3%) and the antimicrobial tests were carried out for all the combination of extracts. The most effective combination was then determined by comparing the results of the Zone of inhibition.

Combination of plant extract in different ratio.

Combination of extracts	*A.vera*	*D. metel*	*C. longa*	Calcium hydroxide
A	1	1	1	0.3
B	2	1	1	0.3
C	2	2	1	0.3
D	2	2	2	0.3
E	3	3	3	0.3

Determination of minimum and fractional inhibitory concentrations (MIC and FIC)

The minimum inhibitory concentrations (MICs) were determined in triplicates by the broth microdilution method as described by Andrew [24]. The concentrations ranged from 0.0078-1024 µg/ml for gentamicin and 8-8192 µg/ml for the extracts of *herbo-mineral (Aloe vera, Datura metel, Curcuma longa and Calcium hydrxoide-2:1:1:0.3 ratio)* against *Staphylococcus aureus, Streptococcus agalactiae* and *Escherichia coli*. The titre plates were inoculated with bacteria having 0.5 Macfarland turbidity (1), and incubated aerobically at 37°C for 24 hours

The fractional inhibitory concentration (FIC) index was used to quantify the synergistic interactions between the herbo-mineral and gentamicin against *Staphylococcus aureus, Streptococcus agalactiae* and *Escherichia coli.* The antimicrobial assays were performed using the checkerboard method[25] with gentamicin in combination with the herbo-mineral formulation. *Staphylococcus aureus, Streptococcus agalactiae* and *Escherichia coli* cultures were grown in the presence of polyherbals with the following concentrations: 1/ 8XMIC, 1/4XMIC, 1/2X MIC, 1XMIC, 2XMIC, and 4XMIC in combination with gentamicin, with concentrations ranging from 1/8X MIC to 4 XMIC. These experiments were conducted in the same manner as for the MIC determination in the susceptibility testing. The FIC index was calculated with the following formulas

$$FIC_{gentamicin} = \frac{\text{MIC of gentamicin in combination}}{\text{MIC of gentamicin alone}}$$

$$FIC_{polyherbal} = \frac{\text{MIC of polyherbal in combination}}{\text{MIC of polyherbal alone}}$$

$$\text{FIC index} = FIC_{gentamicin} + FIC_{polyherbal}$$

Where

FIC index values of less than 0.5 indicated synergy, 0.5–0.75 indicated partial synergy, 0.76–1 indicated an additive effect, and >2 indicated antagonism [25]

Preparation of herbo-mineral topical emulgel

Oil-in-water (O/W) emulsion were prepared using a mixture of Cremophor EL and herbo-mineral formulation as dispersed oil phase and Tween 20 containing distilled water as the continuous aqueous phase[18]. The oil phase was obtained by mixing herbo-mineral formulation (4%) and Cremophor EL (10%). The oil phase was added drop wise to the aqueous phase consisting of Tween 20 (5%) and was continuously stirred for 15 min. The optimized emulsion was slowly dispersed with gelling agent Carbopol 940 (0.75%) with constant stirring to avoid air bubbles. This dispersion was neutralized with alkalizer triethanolamine to obtain an adequate semisolid formulation. Methyl paraben was added as a preservative. Using the same method, blank emulgels was prepared.

Characterization of herbo-mineral emulgels

Appearance and pH determination: The pH of herbo-mineral emulgels was determined using digital pH meter (Mettler Toledo, LE-438) at 25^{0}C by the dispersion of an aliquot of formulation in ultrapure water (10% w/v). Experiments were performed in triplicate. The colour, homogenecity and consistency of polyherbal emulgels were evaluated based on physical examination.

Viscosity: The viscosity of polyherbal emulgels was measured using Brookfield Viscometer (Brookfield Engineering Laboratories, USA) at 25ºC. The viscosity was recorded using t-92 number spindle at rotational speeds of 10 rpm. Experiments were performed in triplicate.

.*Spreadability studies***:** As per Jain *et al* [26]. spreadability of polyherbal emulgels was measured using two different glass slides (7.5×2.5 cm). The first slide was bound with wooden frame. On top of the first glass slide, 1 g of polyherbal emulgel was placed, and second glass slide was placed over first glass side. Furthermore, 100 g weight was imposed over second glass top. Due to overweight, entrapped air between the sandwiched polyherbal emulgels was removed. Using thread and progressive weights, the second glass slide was pulled up to pre-set distance of 7.0 cm. The time (second) and weight (g) required to mobilized second slide up to 7 cm was measured. The spreadability can be calculated using following formula:

Spread ability =M×L/T

Where

M is the weight (g) tied to the second slide.

L is the length of the glass slide.

T is the time taken to separate two glass sides

***In vivo* anti-inflammatory assay**

Carrageenan-induced rat paw edema: This experiment was conducted as described by Oliveira, *et al* [27]. For the experiment, the male Wistar rats (150–175 g) were divided into four groups (n = 6). The first group treated with plain emulgel base, while the second group was treated with 1% Valdecoxib gel. The third group was group was applied with herbo-mineral formulation and the fourth group was administered with the herbo-mineral emulgel. Approximately 50 µL of a 1% suspension of carrageenan in saline was prepared 1 h before each experiment and was injected into the plantar side of right hind paw of rat. 0.2 g of polyherbal emulgel was applied to the plantar surface of the hind paw by gently rubbing 50 times with the index finger. Rats in the Group I, II, III received the plain emulgel, 0.2 g Valdecoxib gel and herbo-mineral formulation, respectively in the same way was used as a herbo-mineral emulgel. Paw volume was measured immediately after carrageenan injection and at1, 2, 3 and 4 hrs intervals after the administration of carrageenan by using a Digital plethysmometer (Orchid Scientific)**.**

Dermal irritation study: Skin irritation study was conducted for herbo-mineral emulgel in rabbits (New Zealand White with a average body weight of 1300 g) in accordance with the OECD Guidelines 404[28] "Acute dermal irritation/corrosion". The animal facility was maintained at 22 °C–24 °C, a relative humidity of 55% ± 10%, and a 12 h light/dark cycle at 160–290 lx throughout the experiment. Animals were kept under acclimatization for eight days before application. Approximately 24 hours before the test, fur was removed by closely clipping the dorsal area of the trunk on either side of spinal cord of the animals. Care was taken to avoid abrading the skin, and an only animal with healthy, intact skin was used. The test substance (herbo-mineral emulgel) was be applied in a single dose (0.5 g) to the skin (approximately 6 cm^2) of an experimental animal and covered with a gauze patch (right side of the trunk). Untreated skin areas (left side) of the test animal

served as the control. Rabbits were exposed to the test drug for period of one hour. The degree of irritation/corrosion was observed and scored (as per the Table below) at specified intervals and was further described in order to provide a complete evaluation of the effects. In addition to the observation of irritation, all local toxic effects, such as defatting of the skin, and any systemic adverse effects (e.g., effects on clinical signs of toxicity and body weight), were fully recorded. The skin patch of the each rabbit was closely observed and recorded twice a day. Data was recorded at interval of 24hr, 48hr and 72hr after patch removal.

Grading of Skin Reactions

Grading for Erythema and Eschar Formation		Grading for Oedema Formation	
No erythema	0	No oedema	0
Very slight erythema (barely perceptible)	1	Very slight oedema (barely perceptible)	1
Well defined erythema	2	Slight oedema (edges of area well defined by definite raising)	2
Moderate to severe erythema	3	Moderate oedema (raised approximately 1 mm)	3
Severe erythema to eschar formation preventing grading of erythema	4	Severe oedema (raised more than 1 mm and extending beyond area of exposure)	4

Ex vivo permeation studies

Ex vivo permeation studies were carried out (as per Reichling *et al*.) [29] by USP type II dissolution system (LabIndia, Dissolution apparatus) with enhancer cell, flask (200 ml) and cellulose acetate membrane (14,000 KDa). The receptor compartment was filled with phosphate buffer (pH 7.4) to make and was constantly stirred at 100 rpm paddle speed. The temperature of the receptor medium was maintained at 37°C. About 3 g polyherbal emulgel and conventional polyherbal formulations was placed in the donor enhancer cell. Cellulose acetate membrane was placed over the sample and backed by a hollow circular plate. The system was allowed to run for 30 min.

To determine the permeation of drug, 2 ml samples were withdrawn at regular intervals (0, 5, 10, 20, 30, 45, 60, 90 min, and 2, 4, 6, 8, 10 and 12 h) from the receiver solution and the same amount of fresh receiver solution was added to maintain the volume constant. Since this formulation was a mixture herbals, curcumin was taken as key active compound to measure the permeation ability of the herbo-mineral formulation. Curcumin concentration in the released samples were measured spectrophotometrically at 423nm using a UV spectrophotometer. The permeation experiments were carried out in triplicate. The permeation profiles were calculated by plotting the average cumulative amount of drug permeated per unit surface area as a function of time

Stability test

The stability test of herbo-mineral emulgel stored in closed glass containers was performed for a period of 6 months at room temperature. Samples were collected at predetermined intervals, 0, 4^{th}, 8^{th}, 12^{th}, $16^{th,}$ 20^{th} and 24^{th} week and their physicochemical parameters such as colour, homogenecity, phase separation, viscosity, spreadability and pH were evaluated.

Clinical study

Experimental design: The study was carried out in cows affected with subclinical mastitis at Livestock Farm Complex, Veterinary College and Research Institute, Orathanadu. Cows with clinical signs of swollen udder, hot, hard and painful to touch, altered milk by the presence of clots, flakes, or discoloured serum and sometime blood was selected for the study. The selected cows were randomly divided into six groups as detailed below;

Experimental design

Groups	Drug	No of animals	Route of administration
I	Conventional antimicrobial therapy	Minimum of 8	Intramammary application of gentamicin sulphate 150 mg four times daily for 5 days The drug was injected into affected mammary quarter after antisepsis of the teat tips with 70% alcohol.
II	Herbo-mineral formulation	Minimum of 8	Topical application, Four times daily on udder for 5 days
III	Herbo-mineral emulgel formulation	Minimum of 8	Topical application, Four times daily on udder for 5 days
IV	Gentamicin sulphate injection	Minimum of 8	Gentamicin @ 2.5 mg/kg bwt i/m twice daily for 5 days
V	Herbo-mineral formulation and Gentamicin sulphate	Minimum of 8	Herbo-mineral formulation – Topical application – four times daily for 5 days & Gentamicin sulphate @ 2.5 mg/kg bwt i/m twice daily for 5 days
VI	Herbo-mineral emulgel formulation and Gentamicin sulphate	Minimum of 8	Herbo-mineral gel formulation – Topical application– four times daily for 5 days and Gentamicin @ 2.5 mg/kg bwt i/m - twice daily for 5 days

Collection of milk samples: Udder of affected cow was thoroughly washed with potassium permanganate solution (1:1000) and the teats were wiped with 70% ethyl alcohol before sample collection. A volume of 300 ml of milk from each cow was collected in sterile container. Milk was collected before treatment (day 0) and thereafter on day 5 and 21 postlast treatment

Bacteriological culture study: Bacteriological culture study was conducted in the milk samples on day 0 (before treatment) and thereafter on day 5 and 21 postlast treatment to obtain the bacterial load (colony forming unit (CFU)/ml).

Milk Somatic Cell Count (SCC): Milk somatic cell count was determined on day 0 (before treatment) and thereafter on day 5 and 21 postlast treatment to in the collected milk sample using an electronic Somatic Cell Counter

Statistical Analysis

The data obtained were analyzed using a Statistical Package for Social Sciences.

All values are expressed as their mean±S.D. The value of $p < 0.05$ is considered statistically significant

Results and Discussion

Plants extraction yield percentage

The ethanobotanical data of the selected plants and their extract percentage yield are given in Table 1. 1.56 to 4.87 g. The highest yield of plant extract was obtained from *Aloe vera* (17.8%±0.29) followed by *Curcuma longa* (15.78%±0.23) while *Datura metel* (14.73%±0.18) gave the lowest extract yield percentage.

Table 1: Ethnobotanical data of of selected plants and their extract yield percentage

Plant	Family	Local name	Plant part used	Extract yield (%) (±SD)
Aloe vera	Asphodelaceae	Katralai	Leaves	17.80±0.29
Datura metel	Solanaceae	Oomathai	Leaves	14.73±0.18
Curcuma longa	Zingiberaceae	Turmeric	Rhizome	15.78±0.23

Qualitative Phytochemical Screening

The result of phytochemical screening of extracts of *Aloe vera*, *Datura metel* and *Curcuma longa* is presented in Table 2. The preliminary screening for the phytochemicals showed the presence of phytoconstituents *viz*. alkaloids, flavonoids, glycosides, phenols, tannins, terpenoids, phlobatannins, saponins and carbohydrate compounds.

Table 2. Qualitative phytochemical screening of extracts

S. No	Phytochemicals	Extract of *C. longa*	Extract of *A. vera*	Extract of *D. metel*
1	Alkaloids	Positive	Positive	Positive
2	Glycosides	Positive	Negative	Negative
4	Phenols	Positive	Negative	Negative
5	Tannins	Negative	Positive	Positive
6	Phlobatannins	Negative	Negative	Positive
8	Flavonoids	Positive	Positive	Positive
9	Terpenoids	Positive	Positive	Positive
10	Saponins	Negative	Positive	Positive
11	Carbohydrates	Positive	Positive	Negative

Preparation of herbo-mineral formulation

Antibacterial and anti-inflammatory effect of *Aloe vera* [11, 12, 30, 31], *Datura metel* [13, 32, 33, 17] and *Curcum* longa [34, 35, 36] were supported to select for this study. Venigalla *et al.* [37] reported that the antibacterial activity of Ca (OH)2 attributed due to its ability to dissociate into hydroxyl ions which causes damage to bacterial cytoplasmic membrane, protein denaturation and damage to DNA. Luo *et al*. stated that hydroxide-releasing agent like calcium hydroxide in any patch or formulation had ability to increase the flux of the drug through the body surface. The findings of Venigalla *et al*. (2015) [37] and Luo et al [38] supported to include 0.3% Ca (OH) 2 with polyherbals in order to increase antibacterial activity and to enhance penetration of polyherbals through udder skin. Zone of inhibition obtained by the 5 different combination ratio are given in the Table 4. Of the 5 different combinations tried, the combination B was selected as the most effective one based on the *In Vitro* antimicrobial efficacy. Crude phytochemical extracts *Aloe vera*, *Datura metel*, *Curcuma longa and mineral Calcium hydroxide* were taken in the ratio of ***2:1:1***:0.3 for the final formulation development.

Preparation of herbo-mineral topical gel

The emulgel base without herbal extract was transparent and had good viscosity. The colour was changed to dark yellow adding the herbal extract and viscosity also slightly decreased due to addition of extract. Since, herbo-mineral formulation was itself having calcium hydroxide (pH-12.2), emulgel formulation required less quantity of triethanolamine for neutralization. Wang and Fang[39] reported that emulgel formulation could be used as better topical drug delivery systems over present systems. The authors stated that emulgel based formulation had several favourable properties for dermatological use such as being thixotropic, greaseless, easily spreadable, easily removable, emollient, nonstaining, long shelf life, bio-friendly, transparent and pleasing appearance.

Characterization of herbo-mineral gels

The detail of characteristics of herbo-mineral emulgels is given in Table 3. The visual inspection indicated that there were no lumps in the formulation. The pH of emulgel was 6.25± 0.30 which was found near to the pH value of udder skin (4.5 to 7.0). It clearly indicated that the formulations were compatible and were unlikely to exert any unwanted effects on pH-sensitive udder skin. The viscosity of herbo-mineral emulgels was found to be 5720±85 cp with spreadability value of 10.02 ±0.12 g.cm/sec. An increase in the viscosity was witnessed with increasing concentrations of the polymer carbopol 940 and alkalizer, which may be due to enhanced cross linking of the polymer at alkaline to neutral pH. Viscosity, being a substantial factor in topical semisolid formulations, greatly influences the spreadability and the overall elegance of the formulation. Viscosity is also known to be inversely proportional to sedimentation/creaming, thereby, affecting the stability of the formulation[40]

Table 3: Physical appearance, pH, viscosity and spreadability of polyherbal gel (n=3)

Parameters	0 day	4th week	8th week	12th week	16th week	20th week	24th week
Colour	Dark yellow	Dark yellow	Dark yellow	Dark yellow	Dark yellow	Dark yellow	Dark yellow
Homogenecity	Good	Good	Good	Good	Good	Good	Good
Phase separation	None	None	None	None	None	None	None
Viscosity±SD (cp)	5720±85	5702±59	5700±71	5689±64	56880±52	5674±62	5667±49
Spreadability±SD (g.cm/sec)	10.02 ±0.12	9.98±0.08	9.96±0.09	9.90±0.09	9.82±0.08	9.79±0.08	9.73±0.07
pH ±SD	6.25± 0.30	6.13± 0.25	6.12± 0.25	6.05± 0.21	6.01± 0.20	5.98± 0.27	5.95± 0.27

Antimicrobial activity

Staphylococcus aureus, Streptococcus agalactiae and *Escherichia coli were* isolated from clinical cases of bovine mastitis reared at Livestock Farm Complex, Veterinary College and Research Institute, Orathanadu. Herbo-mineral formulation showed promising synergistic antibacterial activity against all the three test strains (Table 4). Highest zone of inhibition was observed against *S. agalactiae* (19.88±0.67 mm) followed by *S. aureus* (19.23±1.05mm). Lowest zone of inhibition was observed against *E. coli* (16.35±0.58 mm)

(Table 4). *In Vitro* antimicrobial screening using gentamicin as a positive control clearly indicated that *Aloe vera*, *Datura metel*, *Curcuma longa* show promising antimicrobial activity against test organisms. The reports of the previous studies supported the findings of the present study. Gupta *et al*[48] reported that *C. longa* extract produced broad spectrum antimicrobial effect by causing morphological deformity, with partial lack of the cytoplasmic membrane, which leads to cell disruption. Chainani-Wu, *et al.* [35] found that the bioactive compound curcumin isolated from *Curcum longa* showed bactericidal activity. Lawrence *et al.* [30] reported that Pyrocatechol, Cinnamic acid, *p*-coumaric acid and Ascorbic acid were the important active compounds in the *Aloe vera* exhibiting maximum antibacterial activity. Pawar et al[11] reported that *Aloe vera* leaf extract shown bactreiocidal effect against *Staphylococcus aureus* at 1 to 3 mg// ml. Noreen et al.[12] found that extract of Aloe vera showed cidal effect against *Escherichia coli, Streptococcus sp., Staphylococcus aureus, Salmonella typhi and Bacillus Subtilus*. Okwu and Igara [32], isolated a β-carboline alkaloid (1, 7 dihydroxy-1- methyl 6, 8 dimethoxy β -carboline) from *D. metel* (leaves) with prominent antibacterial effect. Dhiman *et al.* [44]. reported that *Datura metel* were active against *Escherichia coli, Staphylococcus aureus* and *Bacillus subtilis, Candida allbicans* and *Aspergillus niger.*

Table 4: Zone of inhibition of the combined extract of plant extracts (n=3)

Extracts combination No.	*Zone of inhibition ±SD (in mm)*		
	E.coli	***S. aureus***	***S. agalactiae***
A	10.51±0.66	12.83±0.60	13.07±0.81
B	16.35±0.58	19.23±1.05	19.88±0.67
C	13.62±0.78	17.39±*0*.97	18.00±0.69
D	15.11±1.01	16.30±0.89	14.88±0.71
E	12.12±0.56	12.96±0.86	14.85±0.63

Determination of minimum and fractional inhibitory concentrations (MIC and FIC)

The FIC index value of *polyherbals* obtained in the study indicated synergistic effect against *Staphylococcus aureus and Streptococcus agalactiae*, while additive effect against *Escherichia coli* (Table 5). The result demonstrated a potentiated antibacterial effect of gentamicin against *Staphylococcus aureus, Streptococcus agalactiae* and *Escherichia coli*. The results demonstrated that polyherbals was able to prolong and potentiate the bactericidal activity of gentamicin. Similar effect was observed with quercetin in combination with fusidic acid, minocycline and rifampicin [37]. The synergistic effects could be observed in as early as 4 hours post inoculation, with maximum effects observed at 24 hours of incubation. Therefore, polyherbals in combination with gentamicin (antibiotics) were able to provide stable therapeutic outcomes with higher efficacy in terms of killing rate throughout 24 hours. These synergistic therapeutic pairs could be useful in combating mastitis causing organisms (*Staphylococcus aureus, Streptococcus agalactiae and Escherichia coli)* in bovines.

Table 5: Fractional inhibitory concentration (FIC) index of polyherbals for *S.aureus, S.agalactiae* and *E.coli* in combination with gentamicin sulphate

Plant etraxts	***Staphylococcus aureus***		***Streptococcus agalactiae***		***Escherichia coli***	
	FIC index	Outcome	FIC index	Outcome	FIC index	Outcome
Polyherbals (Aloe vera, Datura metel, Curcuma longa-2:1:1ratio)	0.35	Synergy	0.45	synergy	0.85	Additive

***In vivo* anti-inflammatory assay**

Carrageenan-induced rat paw edema: The results of anti-inflammatory activity after topical application of herbo-mineral emulgel presented in Table 6.The results showed that the anti-inflammatory effect of the herbo-mineral emulgel was significantly higher than the effect of Valdecoxib gel formulation (Group II) and herbo-mineral formulation (Group III). The herbo-mineral formulation (Group III) was also produced equivalent anti-inflammatory effect as that of Valdecoxib gel formulation (Group II) up to 3h of study. The results are indicating the enhanced *in vivo* permeation ability of the emulgel based formulation than conventional formulation or drug solution. The results of anti-inflammatory effects of these herbal plants are in agreement with findings of previous study. The anti-inflammatory activity of *Aloe vera* gel has been revealed by a number of *In Vitro* and *in vivo* studies through bradykinase activity. The peptidase bradykinase was isolated from aloe and shown to break down the bradykinin, an inflammatory substance that induces pain. Che *et al.* isolated a novel anti-inflammatory compound, C-glucosyl chromone from *Aloe vera* gel extracts. They reported that *Aloe vera* inhibits the cyclooxygenase pathway and reduces prostaglandin E2 production from arachidonic acid. Sahu *et al.* [43] suggested that the sterol isolated from the *Aloe vera* which included campesterol, *β*-sitosterol, lupeol, and cholesterol were responsible for the anti-inflammatory action. They stated that *Aloe vera* extract (5.0% leaf homogenate) decreased inflammation by 48% in a rat adjuvant-induced inflammatory model. Monira and Munan[36] reported that the crushed leaves of *D. metel* were used to relieve pain. Parveen *et al*. [13] informed that extracts of *Datura* leaves exhibited significant anti-inflammatory activity comparable to the diclofenac sodium against carrageenan-induced rat paw edema. Chainani (2003) found that the curcumin present in *C.longa* inhibited a number of different molecules involved in inflammation including phospholipase, lipooxygenase, COX-2, leukotrienes, thromboxane, prostaglandins, nitric oxide, collagenase, elastase, hyaluronidase, MCP-1, interferon-inducible protein, tumor necrosis factor, and interleukin-12. Ravindran [36] (2010) reported that bisdemethylcurcumin isolated from *C.longa* responsible for potent anti-inflammatory effect

Table 6: Effect of polyherbal emulgel on carrageenan induced paw edema

Group	Reduction of edema (ml) (mean±S.E.)				
	0 h	**1 h**	**2 h**	**3 h**	**4 h**
Group I	0.23±0.02	0.43±0.01	0.66±0.02	0.75±0.01	0.79±0.01
Group II	0.19±0.01	0.31±0.01	0.36±0.01	0.39±0.02	0.41±0.01
Group III	0.22±0.01	0.33±0.02	0.38±0.01	0.40±0.02	0.44±0.02
Group IV	0.21±0.01	0.30±0.02	0.34±0.02	0.38±0.02	0.39±0.02

Ex vivo permeation studies

The herbo-mineral emulgel and conventional herbo-mineral formulation were characterized for *ex vivo* permeation profiles and the results are shown in Fig. 1. The cumulative amount of drug permeated at the end of 12 h was found to be 79.2 µL and 37.9 µL for herbo-mineral emulgel and conventional herbo-mineral formulation, respectively. From this result, it was evident that the permeation of herbo-mineral emulgel was higher than conventional herbo-mineral formulation.

The important criteria of topical formulation are that the drug should cross many layers of the skin before it executes its therapeutic action at target site. From this result, it was evident that the permeation of herbo-mineral emulgel was higher than conventional herbo-mineral formulation. The results are in agreement with the earlier studies [40] demonstrating the enhanced *In Vitro* permeation ability of the emulgel based formulation than conventional formulation or drug solution. These emulgel formulations were known to reduce the surface tension between vehicle and skin as a function of their contact to the skin lipids prompting faster permeation [18]. The emulgel formulation possessed appreciable permeation potential without incorporation of any chemical enhancers which are habitually irritants, as Tween 80 had been shown to be an effective permeation enhancer significantly reducing the barrier of stratum corneum and increasing the diffusion coefficient of drug in skin. Based on these facts, emulgel exerted a higher permeation rate for the topical release of herbo-mineral than conventional formulation. Also, there was better permeation with decrease in viscosity, as emulgel exhibited lesser viscosity compared to conventional gel [41].

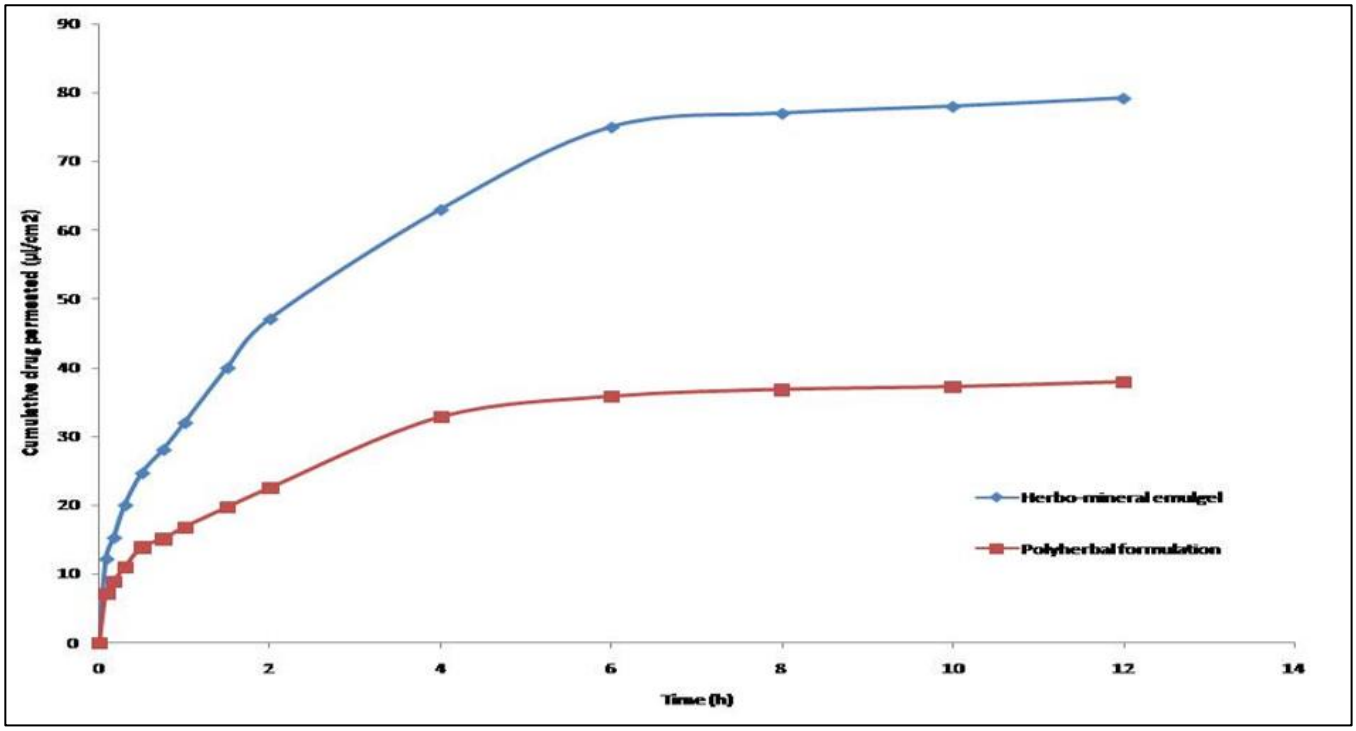

Fig 1: Comparative *ex vivo* permeation of herbo-mineral emulgel and herbo-mineral formulation

Dermal irritation study

The results obtained from the dermal irritation studies showed that herbo-mineral emulgel was non-irritant in nature. No irritation symptoms like erythema and edema appeared on rabbits up to 24 h, hence considered being non-irritant to skin at the applied dose. The blank emulgel was also found to produce no irritation on rabbits upto 24h period. These findings confirmed that the excipients used in the development of emulgel were topically non-irritant when used in the specified amount.

Stability studies

Colour, homogenecity, viscosity, spreadability and pH of emulgel were found to be consistent with no signs of separation and deterioration over a period of 24 weeks (Table 5). This indicated the reproducibility of the physical and chemical parameters ensuring consistent quality of the developed herbo-mineral over a period of 24 weeks. Colour, phase separation and homogenecity exhibited no significant changes under storage at room temperature. The viscosity and pH values indicated the stability of emulgel under room temperature.

Clinical study

The response to treatment with herbo-mineral emulgel in mastitis is given in the Table 7. Animals in the Group IV, V and VI were responded for treatment. But, recovery from clinical mastitis in Group VI cows was quick compared to Group IV and V. The treatment with antibiotic gentamicin alone in group V shown recovery on 15.11 days with SCC (481±0.35X10^3/ml) and bacterial load (3.6±0.55 X10^3/ml) on 21st day, while in combination therapy, it was was achieved within 13.22 days. The combined treatment i.e. herbo-mineral emulgel formulation along with systemic administration of gentamicin sulphate was most effective in the treatment of clinical mastitis as compared to herbal treatment alone. The results of the clinical improvement were correlated with the results of somatic cell counts and bacterial load to assess the therapeutic effect of herbo-mineral emugel formulation. The SCC (Table 8) and bacterial load (Table 9) was significantly reduced in Group IV, V and VI, but cows in Group VI were shown complete recovery on 21st day. In group I, SCC on day 0 was 1410 x10^3 /ml which was significantly ($P < 0.05$) increased to 1634 x x10^3 /ml on 5th day after conventional intramammary antimicrobial therapy. Since, the condition was worsening, treatment with conventional intramammary

antimicrobial therapy was withdrawn and the animals in this group were treated with systemic antimicrobials. In Group II, SCC was non-significantly reduced on 5th day. The reduction of SCC was noticed only one animal. Topical application of herbo-mineral formulation did not show clinical improvement in the remaining animals of Group II. Hence, they were withdrawn from the clinical trial and treated with systemic antimicrobial therapy. Even though, SCC was significantly reduced in all other groups of animals, the cows in Group VI were shown complete recovery on 21st day. Overall increase in milk yield was high in Group VI animals treated with herbo-mineral emulgel formulation along with gentamicin.

The constituent polyherbal emulgel extracts *Aloe vera*, *Datura metel*, *Curcuma longa* and calcium hydroxide are well known to possess antimicrobial and anti-inflammatory activities [34, 30, 11, 12, 13, 44, 36]. These properties may be responsible for normalize the SCC and bacterial load of the milk, thus leading to increase milk yield in clinical mastitic animals. Nair *et al.* [42] utilized freshly prepared fine paste of *Curcuma longa*, *Aloe vera and* calcium hydroxide to treat bovine mastitis successfully. The authors suggested to use fresh preparation six times per day until cure. It is practically very difficult for the farmers to collect fresh plant materials and prepare recipe every time. Hence, the herbo-mineral emulgel formulation will be an efficient and economically affordable formulation for the farmers and women folk to treat clinical mastitis immediately.

Table 7: Response to treatment with herbo-mineral gel, herbo-mineral formulation and enrofloxacin (n=8)

Group	Response to treatment
I	No clinical improvement Instead condition was worsen Animals were treated with conventional antimicrobials parenterally 5th day onwards
II	Slight reduction on swelling Pain on palpation was reduced Because of unwillingness by owners, 5 animals were treated with conventional antimicrobials parenterally 5th day onwards One cow was continued with herbo-mineral formulation, but did not show improvement
III	Reduction on swelling Slight change in the milk of affected quarter
IV	Animals in these group were responded for treatment But recovery (in terms of reduction in swelling, normal milk) was quick in VI (13.22 days) and V (15.11 days) compared to group IV (18.77 days)
V	
VI	

Table 8: Milk Somatic Cell Count (Mean± SE) in different treatment groups at different intervals

Groups	Drug	SCC		
		0 day	5th day	21st day
I	Conventional antimicrobial therapy	1479±0.53X10^3/ml	1670±0.50X10^3/ml	-
II	Herbo-mineral formulation	1430±0.33X10^3/ml	1308±0.83X10^3/ml	1132X10^3/ml (Single animal)
III	Herbo-mineral emulgel formulation	1511±0.70X10^3/ml	1221±0.65X10^3/ml	831±0.34X10^3/ml
IV	Gentamicin suphate	1326±0.53X10^3/ml	921±0.35X10^3/ml	481±0.35X10^3/ml
V	Herbo-mineral formulation and Gentamicin suphate	1426±0.4110^3/ml	860±0.60X10^3/ml	373±0.60X10^3/ml
VI	Herbo-mineral gel formulation and Gentamicin suphate	1526±0.22X10^3/ml	621±0.74X10^3/ml	312±0.60X10^3/ml

Table 9: Total bacterial load (Mean± SE) in different treatment groups at different intervals

Groups	Drug	Total bacterial load (cfu/ml)		
		0	5th	21st
I	Conventional antimicrobial therapy	5.1±0.72X10^6	4.7±0.72X10^7	-
II	Herbo-mineral formulation	5.8±0.53 X10^6	2.1±0.67 X10^5	1.01 X10^5
III	Herbo-mineral emlgel formulation	9.1±0.45 X10^{6}l	6.1±0.70 X10^5	4.1±0.33 X10^4
IV	Gentamicin sulphate	5.2±0.71 X10^6	4.1±0.43 X10^4	3.6±0.55 X10^3
V	Herbo-mineral formulation and Gentamicin sulphate	7.3±0.83 X10^6	3.4±0.53 X10^4	2.93±0.49 X10^3
VI	Herbo-mineral gel formulation and Gentamicin sulphate	8.8±0.66 X10^6	2.1±0.56 X10^4	1.3±0.54 X10^3

Conclusion

In the present study *In Vitro* antibacterial and *in vivo* anti-inflammatory studies, confirm the efficacy of herbo-mineral formulation comprising *Curcuma longa*, *Datura metel*, *Aloe vera* and calcium hydroxide in the ratio of *2:1:1*:0.3 against bovine clinical mastitis. Minimum and fractional inhibitory concentrations study showed that herbo-mineral in combination with gentamicin (antibiotics) were able to provide stable synergistic therapeutic outcomes with higher efficacy in terms of killing rate throughout 24 hours in combating mastitis causing organisms in bovines. The herbo-mineral formulation was developed as a potential and an alternative drug delivery tool via emulgel topical route with aesthetic appearance, good homogenecity and no signs of phase separation. The developed emulgel was shown adequate pH, viscosity, and spreadability. The ex *vivo* permeation study data showed that, highest amount of herbo-mineral was permeated by emulgel formulation. The results of acute dermal irritation studies showed that herbo-mineral emulgel was non-irritant in nature. Colour, homogenecity, viscosity, spreadability and pH of emulgel were found to be consistent with no signs of separation and deterioration over a period of 24 weeks. Hence development of topical emulgel formulation with herbo-mineral synergistic effect is a very promising approach for the treatment of clinical mastitis. The improvement in milk yield, reduction in somatic cell count and bacterial load after the treatment indicated that

herbo-mineral topical emulgel therapy along with gentamicin sulphate systemically eliminates udder infection in clinical mastitis quickly. Hence, the combined treatment i.e. herbo-mineral emulgel formulation along with systemic administration of gentamicin sulphate continuously for 5 days was most effective in the treatment of clinical mastitis as compared to herbo-mineral emulgel treatment alone. Hence, herbal treatment along with standard treatment was most effective in the amelioration of pH, somatic cell count and bacterial load as compared to standard treatment or herbal treatment when given alone. Also, the herbo-mineral emulgel formulation will be an efficient and economically affordable formulation in the field to treat clinical mastitis immediately by farmers itself before getting help of veterinarians

Conflict of Interests

The authors state no conflict of interests.

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