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In vitro evaluation of polyherbal formulation (PHF-M1) used in the treatment of sub clinical bovine mastitis

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Abstract
The present study was conducted to explore the *in vitro* antimicrobial activity of polyherbal formulation (PHF-M1) used by local tribal healers against mastitis isolated pathogens. Scientific validation of traditional knowledge was carried out by standardizing prepared polyherbal formulation on the basis of Physico-chemical parameters, qualitative phytochemical screening of secondary plant metabolites and toxicity studies. However, safety, efficacy and quality test for herbal formulations are limited due to their complex nature. Further, the formulation was evaluated for *in vitro* antimicrobial activity against mastitis isolated pathogens which includes Gram-positive (*Staphylococcus aureus*, MRSA) and Gram negative (*Klebsiella pneumoniae, Escherichia coli* and *Pseudomonas aeroginosa*) organisms. Minimum Inhibitory Concentration (MIC) was measured. The results revealed that the polyherbal extract possesses considerable amount of antimicrobial inhibition against the tested strains and MIC values showed maximum activity even at low concentrations. These results suggest that the secondary metabolites might be responsible for the antimicrobial activity. This study provides in part scientific evidence for the use of polyherbal formulation as traditional remedy in the treatment of sub-clinical mastitis used by local healers of Wayanad district.

Keywords: Polyherbal formulation (PHF-M1), Physico-chemical parameters, qualitative phytochemical test, Minimum Inhibitory Concentration

1. Introduction
Mastitis is a multi-etiological complicated disease with no modest solution of its control causing heavy economic loss to the dairy industry and undesirable public health concern due to antibiotic residues. Mastitis may be caused due to contagious or environmental factors and divided into clinical (acute, subacute, peracute and chronic) and subclinical [1]. Economic loss due to subclinical mastitis was Rs. 6053.21crores per annum in 2001 whereas in 2012 it was Rs. 7165.51 crores per annum [2]. According to NAAS - 2013, there is 115 folds increase in the last 5 decades.

Antimicrobials of plant origin for mastitis have enormous therapeutic potential and being used since time immemorial. A polyherbal formulations consists of a selective combination of individual herbal ingredients that are formulated for a specific ailment or group of disease conditions. When herbs are combined together, they become more potent and effective within the body than single herb due to their activating or catalyzing influence upon one another. These combinations act as powerful catalysts with synergistic effects in order to activate individual healing energies which permeate the entire organism and reside in each and every cell in our body [3]**.** Traditional healers claim that their medicine is cheaper and more effective than modern medicine. In developing countries low income people such as farmers, people of small isolate villages and native communities use folk medicine for the treatment of common infections [4].

In view of above information the present study has been undertaken to assess the antimicrobial activity of polyherbal formulation containing herbs *viz., of Diploclisia glaucascens, Murraya koenigii*, *Ocimum tenuiflorum* and *Curcuma longa rhizome*.

2. Materials and methods
2.1.Collection, authentication and Preparation of formulation: The traditional knowledge of ingredients were collected from local traditional healers of Wayanad and the materials were collected at its natural stance and authenticated by MS Swaminathan Research foundation Wayanad district, Kerala (table no. 1). All the ingredients were shade dried, pulverized and mixed thoroughly in equal proportion.

A pinch of salt was added to the formulation, the entire preparation has been mixed with extract of red parboiled rice/ Kerala matta rice and allowed for shade drying. The formulation has been suspended in DMSO (as vehicle) and used for subsequent experimentation.

2.2. Physico-chemical character evaluation: Various physicochemical parameters like pH, moisture content, total ash value, foreign matter, total fat and crude protein followed by UV Fluorescence Analysis and Heavy Metal Test as per protocols suggested by Sindhu *et al.,* 2015 [5].

2.3. Qualitative phytochemical screening: The formulation (PHF-M1) was analyzed qualitatively for various phytochemical constituents as per standard procedures [6].

2.4. *In vitro* antimicrobial activity: Well in agar method has been carried out as per the method described by Mathabe *et al.,* (2006) [7] with minute modifications. The colonies of bacteria were grown in Mueller-Hinton broth (MHB) for specific OD at 600 nm to give a starting inoculum of $1X10^8$ bacteria / ml. A volume of 0.1 ml of the tested microorganisms (*E. coli, Staphylococcus aureus, Klebsiella spp, Pseudomonas aeruginosa and MRSA*) grown in nutrient broth (at 37ºC for 24 hrs, 10^8-10^9 cells/ml), was inoculated on Mueller- Hinton Agar (MHA) growth media, and then spread on the entire surface of the dish using a sterile spatula. A sterilized 5 mm borer was used to make holes in MHA plate. The bottom of the well was then sealed with molten soft agar. Minimum Inhibitory Concentration (MIC) of the crude herbal preparation against the test organisms were determined by preparing two fold serial dilutions to concentrations of 100%, 50%, 25% and 12.5%) of the test samples were loaded to the well along with vehicle control (DMSO) with streptomycin as standard. The plates that streaked with bacterial cultures were kept in an incubator at 37ºC for 24 hr. for growth of bacterial cultures after which the zone of inhibition was measured using Hi-Antibiotic Zone Scale. Each experiment was carried out on at least three separate occasions.

Determination of Zone of inhibition: The measurement of the diameter of zones of microbial growth inhibition surrounding wells containing various concentration of test substance, which are placed on the surface of a solid nutrient previously inoculated with culture of suitable microorganisms. Inhibition produced by the test drug was compared with that produced by known concentration of reference standard drug [8]. The lowest concentration (higher dilution) of the agent that produced no visible bacterial growth when compared with the control plate was regarded as the Zone of Inhibition –MIC [9]. Inhibition produced by the herbal formulations @ different concentrations were compared with that produced by known concentration of reference standard drug 8.

2.5. Acute toxicity study: Acute toxicity study was carried out according to the Organization of Economic Corporation Development Guidelines [10] guidelines along with the principles and criteria summarized in the Humane Endpoints Guidance Document [11].

2.6. Statistical analysis: Statistical technique of Duncan's multiple range test (DMRT) was used with the help of SPSS version 20 for assessment of mean comparison. The results are expressed in terms of mean ± standard deviation.

3. Results

The polyherbal formulation (PHF-M1) consisting of *Diploclisia glaucascens, Murraya koenigii, Ocimum tenuiflorum* and *Curcuma longa* were identified and authenticated by MS Swaminathan Research Foundation, Wayanad and specimen voucher has been deposited in the institute. Results of physicochemical properties were summarized in table no. 2. The fluorescence characteristic of formulation was recorded using high wavelength UV light (365 nm), low wavelength UV light (254 nm) and visible light. The results showed the presence of fluorescence activity in formulation (table no. 3). The results of qualitative phytochemical analysis showed the presence of alkaloids, flavonoids, tannins, phenolic compounds, saponins, carbohydrates, gums and mucilages and absence of glycosides, steroids and terpenoids (table no. 4).

3.1. Acute oral Toxicity: The results of the acute oral toxicity confirms that the crude herbal formulation (PHF-M1) possessed no toxicity at dose rate of 5, 50, 300 and 2000 mg/kg respectively. The animals were healthy, feed and water intake was normal. There was no behavioural changes during the entire period of study.

3.2. *In vitro* antimicrobial activity: Multiple doses of polyherbal formulation (PHF-M1) exhibited prominent MIC value ranging from 24±0.58 to 12.33±0.33 against mastitis causing pathogens (table no. 6).

4. Discussion

The antimicrobials obtained from plants are of much therapeutic potential and are effective in treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials [12]. According to WHO, standardization of medicinal plants having therapeutic potential is done to establish the identity and purity of herbal formulation [13]. The quality assessment of herbal formulation was done to justify their acceptability in modern system of medicine [14]. Concept of Polyherbalism helps to exploit therapeutic herbal strategy in combination of several medicinal herbs to achieve cumulative therapeutic effectiveness [15].

4.1. Physico-chemical property: The percentage of active chemical constituents in crude drugs is mentioned on air dried basis [16]. Total Ash is the residue remaining after removal of all moisture and organic material upon incineration [17]. Acid insoluble ash is the measure of amount of silica present, especially as sand and silicaceous earth [18]. Whereas, Crude fibre is the level of non-digestible carbohydrate and lignin, low level aids in absorption of glucose and fat [19]. Crude lipid is considered to be good source of energy and protects internal tissues and aids in multifarious cell processes [20]. Thus, the polyherbal formulation (PHF-M1) contains adequate nutritional value. The fluorescence characteristic of formulation was recorded using high wavelength UV light (365 nm), low wavelength UV light (254 nm) and visible light. The results showed the presence of fluorescence activity in formulation, which is important parameter for pharmacognostical evaluation & measure of analyzing crude drugs [21].

4.2. Anti-microbial activity of herbals: It was observed in the present study that PHF-M1 formulation at various

concentration inhibited the growth of all pathogenic bacteria tested. Preliminary phytochemical screening of PHF-M1 formulation showed the presence of a number of bioactive constituents such as alkaloids, flavonoids, tannins, phenolic compounds, Saponins, carbohydrates, gums and mucilages. The antimicrobial activity could be due to the presence of these phytoconstituents. Moreover, data of the present study also revealed that the plant extracts have *in vitro* broad spectrum antibacterial activity. The bacteriostatic and bactericidal activity could be ascribed to the presence of polyphenol compounds, which caused inhibition of a wide range of microorganisms. Phenol is well known as a chemical antiseptic [22]. Polyphenols, such as tannins and flavonoids, are of important antibacterial activity. The antimicrobial activity of flavonoids is due to their ability to complex with extracellular and soluble protein and to complex with bacterial cell wall while that of tannins may be related to their ability to inactivate microbial adhesions, enzymes and cell envelop proteins [23]. The results indicated that fraction inhibited both *Staphylococcus aureus* (Gram-positive) as well as Gram negative bacteria (*Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeroginosa*). This is further confirmed by the Zongo *et al.*, 2010 [24] that describe the high sensibility of Gram-positive bacteria towards plant extracts and their component. Djeussi *et al.*, 2013 [25] and Saranraj *et al.*, 2014 [26] reported that Gram-negative bacteria were more resistant to the plant-based organic extracts because the hydrophilic cell wall structure of Gram-negative is constituted essentially of a lipopolysaccharide that blocks the penetration of hydrophobic oil and avoids the accumulation of organic extracts in target cell membrane. According to a study conducted by Medini *et al.*, (2014) [27] a probable degree of lipophilicity might be responsible for the extracts being higher in activity than standard drugs used lipophilicity toxicity due to the interactions with the membrane constituents and their arrangement. Considering the above, Gram-positive bacteria should be more susceptible since they have only an outer peptidoglycans layer which is not an effective permeability barrier. However, inhibitory activity of the formulation against pathogenic bacterial strains mainly depends upon the multiplicity and magnitude of phytoconstituents present in them. Thus the antimicrobial activity of the formulation PHF-M1 can be attributed for the presence of secondary plant metabolites. This study provides in part scientific evidence for the use of polyherbal formulation as traditional remedy in the treatment of sub-clinical mastitis used by local healers of Wayanad district.

Table 1: composition of polyherbal formulation – (PHF-M1)

Botanical name	Weight	Parts used
Diploclisia glaucascens	50g	leaves
Murraya koenigii	50g	leaves
Ocimum tenuiflorum	50g	leaves
Curcuma longa	50g	rhizome
salt	To the required quantity	

Table 2: Physico-chemical character evaluation of (PHF-M1)

Sl No.	Parameters	Values
1	Moisture	70.31%
2	Dry matter	29.69%
3	Total ash	9.83%
4	Acid insoluble ash	0.67%
5	Total fat	14.94%
6	Crude protein	9.10%

Table 3: UV fluorescence analysis of (PHF-M1)

Sl. No.	Combinations	High wavelength UV (365 nm)	Low wavelength UV (254 nm)	Visible light
1	Powder (P) as such	Brown	Dark brown	Dark brown
2	P+ Concentrated H_2So_4	Brown	Brown	Brown
3	P+ Conc. Hcl	Brown	Brown	Brown
4	P+ Conc. $Hn0_3$	Green	Green	Dark Green
5	P+ Dil. Hcl	Green	Green	Green
6	P+ Dil. H_2S0_4	Brown	Brown	Brown
7	P+ Dil. NH_3	Brown	Brown	Brown
8	P+ Dil. $HN0_3$	Green	Green	Green
9	P+ 10% Picric acid	Light Green	Green	Light Green
10	P+ Pet. Ether	Brown	Light Brown	Brown
11	P+$FeCl_3$	Black	Blackish Green	Dark Green
12	P+ 5% H_20_2	Dark Green	Dark Green	Dark Green
13	P+ Acetic acid	Dark Green	Brown	Light Brown
14	P+CCl_4	Black	Blackish Green	Green
15	P+ $AgN0_3$	Green	Dark Green	Dark Green
16	P+ Xylene	Dark Green	Blackish Green	Green
17	P+ Methanol	Black	Dark Green	Yellowish Green
18	P+ Acq. Iodine	Dark Brown	Dark Brown	Brownish Yellow
19	P+ Benedict's reagent	Black	Dark Brown	Brown
20	P+ Biuret's reagent	Dark Green	Dark Green	Dark Green
21	P+ Mayer's reagent	Green	Black	Dark Green
22	P+ Dragendroff's reagent	Green	Blackish Green	Dark Green
23	P+ Barfoed's reagent	Black	Blackish Green	Bluish Green
24	P+ Wagner's reagent	Black	Yellowish green	Light Green
25	P+ Hager's reagent	Black	Yellowish Green	Yellowish Green

Table 4: Qualitative phytochemical analysis of (PHF-M1)

Sl. No.	Test for Phytoconstituents		(PHF-M1)
1	Alkaloids	Mayer's test	P
		Wagner's test	P
		Hager's test	P
		Dragendroff's test	P
2	Flavonoids	Ferric chloride test	P
		Lead acetate test	P
3	Glycosides	Sodium hydroxide reagent test	A
		Benedict's test	A
4	Steroids	Salkowski test	A
		Leiberman Burchardt test	A
5	Tannins	Ferric chloride test	P
		Gelatin test	P
6	Phenolic compounds		P
7	Diterpenes	Salkowski test	A
		Leiberman Burchardt test	A
8	Saponins		P
9	Gums and mucilages		P
10	Carbohydrates – Molish's test		P

Table 5: Heavy metal test of formulation PHF-M1

Sl. No.	Heavy metal		Result
1	Cadmium	NH_40H test	Absent
		Potassium Ferro-cyanide test	Absent
2	Bismuth	H_2S test	Absent
		NH40H test	Absent
3	Lead	Dil. HCl test	Absent
		KI test	Absent
4	Foreign matter		Nil
5	Pesticide residue		Nil
6	Microbial contamination		Nil
7	Radioactive contamination		Nil

Table 6: Antimicrobial activity of Polyherbal formulation (PHF-M1) at various concentration**.**

Concentration	Streptomycin	100%	50%	25%	12.5%
S. aureus	28.33 ± 0.33^{a}	22.67 ± 0.33^{c}	19.33 ± 0.33^{c}	17.33 ± 0.33^{c}	14.67 ± 0.33^{c}
MRSA	26 ± 0.33^{a}	21 ± 0.58^{d}	19 ± 0.58^{c}	17 ± 0.58^{c}	15 ± 0.58^{c}
E. coli	35 ± 0.35^{a}	24 ± 0.58^{b}	20.67 ± 0.33^{b}	19 ± 0.58^{b}	17.33 ± 0.33^{b}
K. pneumonia	25 ± 0.12^{a}	19.67 ± 0.33^{e}	17.33 ± 0.33^{d}	15.33 ± 0.33^{d}	14.33 ± 0.33^{c}
P. aeroginosa	22.67 ± 0.33^{a}	15.67 ± 0.33^{f}	14.67 ± 0.33^{e}	13.67 ± 0.33^{e}	12.33 ± 0.33^{d}
P-value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

$P < 0.05$, expressed as mean ± S.E.

Means having same small letters as superscript indicate treatment are homogenous within rows and means having same capital letters as superscript indicate treatment are homogenous within a column. Means with at least one common superscript (a-c) for rows and (A-F) for columns do not differ significantly at 5 % level.

5. Conflict of interest

We declare that we have no conflict of interest.

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7. Ethical matter

This research work does not use any laboratory animals.

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