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TOPICAL HERBAL ANTIMICROBIAL FORMULATION CONTAINING *PROSOPIS JULIFLORA* METHANOL EXTRACT

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ABSTRACT

Defatted methanol extract obtained from *Prosopis juliflora* collected from Yemen was investigated for antimicrobial activity against different strains of bacteria and *Candida albicans* which were known to cause different types of skin infections. The minimum inhibitory concentration (MICs) was determined by microdilution assay. Defatted methanol extract of *P. juliflora* (MEPJ) exhibited strong activity against *S. aureus* and *S. pyogenes*, the main causative organisms of skin and soft tissue infections, with MIC values of 0.0156–0.0313 mg/ml and 0.0078 mg/ml, respectively. Topical formulations containing 4% w/w MEPJ were prepared using different bases. They were evaluated by agar diffusion assay against organisms under test in comparison with commercial antimicrobial products and different concentrations of MEPJ in phosphate buffer saline solution. Macrogol ointment, lutrol gel and emulgel prepared with MEPJ showed greater antimicrobial activities than cream and emulgel bases. Macrogol ointment, lutrol gel and emulgel containing 4%v/w MEPJ showed comparable inhibition zone to that of marketed products and to the MEPJ solutions. It was concluded that the satisfactory antimicrobial activities of defatted methanol extract of *P. juliflora* was achieved from macrogol ointment, lutrol gel and emulgel which could be used for further investigation.

KEYWORDS: *Prosopis juliflora;* Defatted methanolic extract; Antimicrobial activity; Topical Formulations; Lutrol gel.

INTRODUCTION

The genus *Prosopis* comprises 44 species distributed mainly in the arid and semiarid tropical and subtropical countries.[1]

P. juliflora is an important species for its high nitrogen-fixing potential in extremely dry areas and in drought seasons, and provides shelter and food to numerous species of animals which eat its nectar, pollen, leaves and fruits.[2] Many plants of *Prosopis* genus (Leguminosae) are recognized to be of medical properties and are used in folk medicine as astringents, in rheumatism and as remedies against scorpion stings and snake bites.[3] Products from this plant have also been used for human consumption in bread, biscuits, sweeties, syrups and liquors.[4]

Many alkaloids such as juliflorine, julifloricine and julifloridine, juliprosine, juliprosinine and juliflorinine, benzene insoluble alkaloidal fraction (containing 2 major and 3 minor alkaloids), analogous alkaloids such as 3'-oxojuliprosopine, secojuliprosopinol, 3-oxojuliprosine and 3'-oxo-juliprosine and julifloravizole have been isolated and their in vitro biological activities demonstrated.[5]

In vitro studies showed that *P. juliflora* has antibacterial, antifungal, antioxidant and anti-inflammatory activities.[2,3, 6, 7]

The objective of this study was to formulate and evaluate topical antimicrobial preparations containing deffated methanol extract of *P. juliflora*.

MATERIALS AND METHODS

Plant collection and preparation of the MEPJ

The leaves of *P. juliflora* were collected from Abyan, Zingibar in February 2008. The plant was identified in Botany Department, Faculty of Sciences, Sana'a University. A voucher specimen of the plant material (YMP-P2) has been deposited at the Pharmacognosy Department, Sana'a University, Yemen. The dried coarse powder from the dried leaves of *P. juliflora* was extracted successively in a soxhlet apparatus with petroleum spirit (BDH Chemical Ltd., England). The residue was removed by filtration. The dried residue was exhaustively re-extracted on a shaker with methanol 96% (Power oil, Egypt) (1:10 w/v ratio) for three hours. The extraction was repeated from three to five times. The methanol extracts were filtered over filter papers (Schleicherand Schuell ref. no. 31200) and then the filtrates

from each extraction were combined and concentrated in vacuo at 45°C and dried in an oven at 40°C until constant weight was obtained. The residues were stored at a freezer (-20°C) for further use.

Estimation of total alkaloids content of MEPE[8]

One gram of MEPJ was weighed and 50 ml of distilled water and 25 ml of 1 N sulphuric acid was added to it, stirred for 15 min and extracted with chloroform (50mlx3) to remove pigments and other unwanted materials. The free alkaloids were then precipitated by the addition of excess 10% ammonia and separated by extraction with chloroform (50mlx3). The chloroform layers were passed over anhydrous sodium sulfate and evaporated to dryness. The residue was dried until a constant weight was obtained. The residue weight represented the total alkaloids content.

Antimicrobial assay

Staphylococcus aureus (ATCC 29737), *Staphylococcus epeidermedis* (ATCC 12228), *Escherichia coli* (ATCC 10536), *Klebsiella pneumonia* (ATCC 10031) and *Candida albicans* (ATCC 2091) were obtained from the National Drug Quality Control Laboratory, Sana'a. *Streptococcus pyogenes* (ATCC 19615), *Proteus vulgaris* (ATCC 13315) and *Staphylococcus aureus* (clinical isolate) were obtained from the National Center of Public Health Laboratories, Sana'a. All bacteria (except *S. pyogenes*) were maintained in nutrient broth (Fluka, Switzerland) containing 16% glycerol (Fluka, Switzerland) at -20°C while the fungus strain was maintained in Saboraud Dextrose broth (SDB) (Himedia, India) containing 16% glycerol at -20°. *S. pyogenes* was maintained in Brain Heart Infusion broth (BHI) (Himedia, India) containing 16% glycerol at -20°. Before testing all bacterial strains (except *S. pyogenes*) were sub-cultured on Mueller Hinton agar (MHA) (Scharlau, Spain) while the fungus strain was sub-cultured on Saboraud Dextrose agar (SDA) (Merck, Germany). *S. pyogenes* was sub-cultured on MHA supplemented with 5% blood. Colonies from overnight growth on appropriate agar plates were suspended in SDB, BHI, and Mueller Hinton broth (MHB) for *C. albicans*, *S. pyogenes* and the rest of test microorganisms, respectively to a turbidity that matches a 0.5 McFarland standard (10^8 CFU/ml for bacteria and 10^6 CFU/ml for the fungus). A portion of the 0.5 McFarland suspensions was diluted 1:100 in the same type of broth to be used in the determination of minimum inhibitory concentration (10^6 CFU /ml bacteria and 10^4 CFU/ml for the fungus).[9],[10]

Disc diffusion method

Disc diffusion method was employed for the determination of antimicrobial activities of the defatted methanolic extract.[10] Briefly, a suspension of the tested microorganism of a turbidity that matches a 0.5 McFarland standard was spread on the solid media plates. Whatman No. 1 sterile filter paper discs of 6 mm in diameter were impregnated with the stock solutions of the MEPJ (50 mg/ml in methanol) to give discs of 4 mg concentration. The discs were allowed to evaporate under sterile conditions. Negative controls were prepared using methanol. The prepared discs of extract, positive and negative controls were deposited on the surface of the inoculated agar plates. These plates, after staying for one hour in the refrigerator, were incubated at 37^{o} for 24 h for bacterial strains, 25^{o} for 48 h for the fungus. Ampicillin (10 µg/disc), gentamicin (10 µg/disc) and nystatin (100 units/disc) (all from Himedia, India) were used as positive controls for the Gram-positive bacteria, Gram-negative bacteria and the fungus, respectively.

Determination of minimum inhibitory concentration (MICs) minimum bactericidal/fungicidal concentration (MBC/MFC)

MEPJ was dissolved in DMSO (BDH Chemical Ltd., England) (400mg/500 µl) and diluted to 25 ml with MHB (for antibacterial test) or with SDB (for antifungal test). Broth microdilution assay was used to determine the MIC according to Ndi et al (2007)[11], concentrations ranged from 0.039 to 16mg/ml. MHB supplemented with 5% sheep serum was used for *S. pyogenes*. Positive controls were gentamicin and nystatin. The minimum bactericidal and fungicidal concentrations (MBC/MFC) were determined as described by Celiktas et al (2007).[12]

Preparation of topical formulations containing MEPJ

Preparation of MEPJ solution

Solutions of MEPJ of 0.25, 0.5, 1, 2 and 4% w/v were prepared in phosphate-buffered saline (PBS)[13] containing 1% DMSO.

Preparation of topical formulation

The following topical formulations containing 4% w/v *P. juliflora* defatted methanol extract were prepared:

A macrogol ointment[14]**:** macrogol-4000 40% and macrogol-400 60%; **A hydrophilic petrolatum**[15]**:** cholesterol 3%, stearyl alcohol 3%, white wax 8% and white petrolatum 86%; **A cetomacrogol emulsifying ointment**[16]**:** cetomacrogol emulsifying wax 30%, white

soft paraffin 50% and liquid paraffin 20%; **A vanishing cream**[17]**:** stearic acid13%, stearyl alcohol 1%, cetyl alcohol 1%, glycerin 10%, potassium hydroxide 0.9% and purified water to 100%. **A cream base, o/w**[17]**:** stearyl alcohol 15%, bees wax 8%, sorbitanmonooleate 1.25%, sorbitol solution (70%) 7.5%, polysorbate-80 3.75% and purified water to 100%); A **w/o base**[18]**:** liquid paraffin 15%, Vaseline 37.5%, cetyl alcohol 18%, cetomacrogol 1000 4.5% and purified water to 100%; **A hand lotion**[19]**:** mineral oil 5%, stearic acid 2.5%, glyceryl monostearate 2.5%, lanolin 1%, glycerol 2%, triethanolamine 1% and purified water to 100%; **A lutrol F127 gel**[20]**:** lutrol F127 20%, propylene glycol 20% and purified water to 100%; **A carbopol emulgel with modification**[21]**:** carbopol-915P 1%, liquid paraffin 5%, span 80 3%, tween-20 1%, propylene glycol 5%, ethanol 2.5% and purified water to 100%. **A lutrol F127 emulgel**[20]**:** propylene glycol 15% liquid paraffin 10%, lutrol F127 20% and purified water to 100%.

Different additives were used to stabilize the formulations. All formulations were listed in [table 1]. MEPJ was separately incorporated into the already prepared bases at the room temperature by levigation method. The control formulations did not contain the plant material. The strength of the final product of MEPJ was prepared to be 4% w/v (equivalent to 0.428% total alkaloids).

In-vitro antimicrobial activity of topical formulations

P. juliflora methanolic extract (MEPJ) (4%w/w) made up separately in different semisolid preparations and MEPJ in PBS were tested for antimicrobial activity against the tested microorganisms using an agar well diffusion method.[22]

The following commercial topical products were used as positive controls: Furazina® ointment (nitrofurantoine; 0.2% w/w) (International Pharma, Italy), Foban® ointment (sodium fusidate; 2% w/w) (HOE Pharmaceuticals, Malaysia), Gentamicin® ointment and cream (0.3% w/w) (MEDICO ABS, Syria), Tetracycline® ointment (3% w/w) (Julphar, U.A.E), Fucidin® cream (frusidic acid; 2% w/w) (LEO, Denmark), Canestal® cream (clotrimazole; 2% w/w) (Alpha Aleppo Pharmaceuticals, Syria) and Kenazol® cream (ketoconazole; 2% w/w) (Domina, Syria).

PBS containing 1% DMSO and the formulation bases were used as negative controls. The tested microorganisms and the media were prepared in the same way as mentioned above. The antimicrobial activity was evaluated by measuring the zone of inhibition in mm.

RESULTS AND DISCUSSION

Several alkaloids (i.e. juliflorine, julifloricine and julifloridine, juliprosinine and juliflorinine) were isolated from *P. juliflora* leaves and their antimicrobial activities were demonstrated.[3,6,7] Therefore, the antimicrobial activity of *P. juliflora* might be attributed to a specific chemical compound (its alkaloids content), as confirmed by the phytochemical screening, or each class of compounds exerted synergistic effect to give the observed biological activity.

Since alkaloids were the most interesting pharmacologically active compounds of *P. juliflora* leaves, MEPJ was standardized in its alkaloids content. The content of total alkaloids of MEPJ was 10.69%. Standardization; adjusting the herbal drug preparation to a defined content of a constituent or group of substances with known therapeutic activity, is the first step for the establishment of consistent biological effect, a consistent chemical profile, or simply a quality assurance program for production and manufacturing.[23]

Recently, various extracts of plants have gained a particular concern as sources of natural antimicrobial because of the resistance to antibiotics that some microorganisms have acquired.[24] The antimicrobial activity in terms of zone of inhibition was presented in Table 2.

MEPJ exhibited antibacterial activity against *S. aureus* strains, *S. epeidermedis, S. pyogenes, E coli, K. pneumonia* and *P. vulgaris* which was in accordance with that observed by Sathiya et al (2008) who tried ethanolic extract.[25] The antimicrobial activity of MEPJ was in agreement with that of julifloricine, an alkaloid isolated from *P. juliflora* leaves.[6] The antifungal activity of MEPJ against *C. albicans* was in a good agreement with that observed by Aqeel et al (1989)[6] who found that julifloricine, an alkaloid isolated from *P. juliflora* leaves was responsible for antifungal activity.

The principal quantitative measures of the in vitro activity of antibiotics and plant extracts with antimicrobial potentials were the MIC and the MBC/MFC.[26] Generally, dilution methods were suitable for assaying polar and non-polar extracts or compounds for determination of MIC and MBC/MFC-values.[27] Aligiannis et al (2001)[28], proposed a classification for plant materials, based on MIC values like this: strong inhibitors with MIC values up to 0.5 mg/ml; moderate inhibitors with MIC values between 0.6 and 1.5 mg/ml and weak inhibitors with MIC values above 1.6 mg/ml. The data in Table 3 indicated varying

level of antimicrobial activity against the investigated cutaneous pathogens. *P. juliflora* showed a maximum antimicrobial activity against Gram-positive bacteria (MIC values 0.0078–0.0625 mg/ml) while *P. vulgaris* and *C. albicans* (MIC value 1 mg/ml) were the least sensitive organisms. In accordance with the aforementioned classification, it was recorded that *P. juliflora* defatted methanol extract was a strong inhibitor against *S. aureus, S. epeidermedis, S. pyogenes, E. coli* and *K. pneumonia* and was a moderate inhibitor against *P. vulgaris* and *C. albicans* . The majority of MBC/MFC values were similar to or within a twofold dilution of the MIC values which indicated a fungicidal effect of *P. juliflora*, on *C. albicans* and bactericidal effects of extract on *S. aureus* (isolate), *S. epeidermedis, E. coli* and *K. pneumonia*.

The results of antimicrobial activity of MEPJ in phosphate-buffered saline (PBS) obtained from the well diffusion assay showed that there has been an increasing effect on bacterial/fungal growth inhibition with increasing concentration of the extract in PBS. *P. juliflora* extract showed good inhibitory activity on almost all the tested bacteria. It has been found that among all the tested organisms, the Gram-positive bacteria strain (*S. aureus*) was found to be more susceptible to the plant extract as demonstrated by inhibition zone which ranged between 19 and 25 mm. *P. juliflora* concentration of 1mg/ml showed activity against all the tested organisms. Results were expressed in Table 4.

Concerning the antimicrobial activities of the various topical preparations containing MEPJ by using agar diffusion method in terms of zone of inhibition was presented in Table 5. The antimicrobial activity of the MEPJ from its various formulations can be ranked in the following descending order: lutrol F127 gel ≈ lutrol F127 emulgel ≈ macrogol ointment > cream base, o/w ≈ cetomacrogol emulsifying ointment > w/o base ≈ carbopol emulgel > hydrophilic petrolatum > hand lotion ≈ vanishing cream.

The higher antibacterial activity of *P. juliflora* in lutrol F127 gel and lutrol F127 emulgel **might be probably** due to lack of interaction between the non-polar polymeric network of lutrol F127 and *P. juliflora* components. Lutrol F127 possesses properties which appear to make it suitable for use in the formulation of topical dosage forms; these include relative low toxicity, high compatibility with other drugs, ability to form clear gels in aqueous media, ease of application, cosmetic appearance and good drug release characteristics.[29]

The higher antibacterial activity of the macrogol blend ointment preparations might be related to the inherent activity of the bland macrogol blend bases.[30] On the other hand, the reduction of antimicrobial efficacy of *P. juliflora* extract in ointment and cream bases might be ascribed to the reduction of the amount of freely available active components that form complex with the different phases of the cream or solubilized in the oily environment of the ointment.[31]

The antimicrobial activity of MEPJ in different semisolid preparations in term of inhibition zone size comparing with activity of MEPJ in PBS indicated that the zone size for 4%w/w PJE in macrogol ointment, lutrol F127 gel and lutrol F127 emulgel was comparable to zone size obtained by 0.5%w/v MEPJ in PBS solution for all tested Gram-positive bacteria (*S. aureus* strains, *S. epeidermedis* and *S. pyogenes*) while the zone size for 4%w/w PJE in cream base, o/w was comparable to zone size obtained by 0.25%v/v PJE in PBS solution for the same bacteria.

According to the size of inhibition zones, the zone size for 4%w/w PJE in macrogol ointment, lutrol F127 gel and lutrol F127 emulgel was nearly closed to zone size obtained by Gentamicin® ointment 0.3% for *S. aureus* strains, and *S. epeidermedis* while it showed higher activity than Tetracycline® ointment 3% against the less susceptible *S. aureus* clinical strain. The zone size for 4%w/w PJE in macrogol ointment, lutrol F127 gel and lutrol F127 emulgel was comparable to zone size obtained by the tested commercial skin antibiotics (except nitrofurazone 0.2%) against *S. pyogenes*.

It was concluded that, the prepared macrogol ointment, lutrol gel and emulgel containing MEPJ showed broad antimicrobial activity as compared to the marketed antibiotic products and therefore further evaluation should be advised.

Table 1: Topical formulations of MEPJ

Ingredients	Quantity (w/w%), formulation code									
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
P. juliflora methanolic extract	4	4	4	4	4	4	4	4	4	4
Vitamin E	0.075	0.075	0.075	0.075	0.075	0.075	0.075	-	0.075	0.075
Citric acid	1	1	1	-	-	-	-	-	-	-
Propylene glycol	2	2	2	2	2	2	2	20	-	-
Carbopol				0.18	0.18	-	0.18	-	1	-
EDTA disodium	-	-	-	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Triethanolamine	-	-	-	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Methyl paraben	-	-	-	0.2	0.2	0.1	0.2	-	0.2	0.2
Propyl paraben	-	-	-	0.02	0.02	0.01	0.02	-	0.02	0.02

Lutrol F127	-	-	-	-	-	-	-	20	-	20
Macrogol ointment (q.s)	100	-	-	-	-	-	-	-	-	-
Hydrophilic petrolatum (q.s)	-	100	-	-	-	-	-	-	-	-
Cetomacrogol Emulsifying ointment (q.s)	-	-	100	-	-	-	-	-	-	-
Vanishing cream (q.s)	-	-	-	100	-	-	-	-	-	-
Cream base, o/w (q.s)	-	-	-	-	100	-	-	-	-	-
w/o base q.s)	-	-	-	-	-	100	-	-	-	-
Hand lotion (q.s)	-	-	-	-	-	-	100	-	-	-
Lutrol F127 gel [Purified Water (q.s)]	-	-	-	-	-	-	-	100	-	-
Carbopol emulgel. (q.s)	-	-	-	-	-	-	-	-	100	
Lutro l F127 emulgel (q.s)	-	-	-	-	-	-	-	-	-	100

Table 2: Antimicrobial activity of MEPJ (4mg/disc), and standard antibiotic discs against the tested microorganisms evaluated by agar diffusion method (n= 3)

Test samples	Inhibition zones diameter* (mm)							
	S.a	S.a[a]	S.e	St.p	E.c	K.p	P.v	C.a
MEPJ	25±1.0	23±0.0	19±0.0	24±0.0	22±2.0	20±0.0	17±0.6	14±1.2
Ampicillin** 10µg/disc	33±0.6	14±0.0	25±3.5	35±1.2	n.t	n.t	n.t	n.t
Gentamicin** 10µg/disc	n.t	n.t	n.t	n.t	22±0.0	26±1.5	30±0.0	n.t
Nystatin** 100 units/disc	n.t	n.t	n.t	n.t	n.t	n.t	n.t	22±0.6

* = including the diameter of the disc (6mm), S.a = *Staphylococcus aureus*, S.a[a] = *Staphylococcus aureus* (isolate), S.e =*Staphylococcus epeidermedis*, St.p = *Streptococcus pyogenes*, E.c = *Escherichia coli*, K.p = *Klebsiella pneumonia*, P.v = *Proteus vulgaris*, C.a = *Candida albicans*, ** = positive controls, n.t= not tested. Each value was the main ± SD of three determinations. Note: Zone of inhibition was 0.00 in negative control (methanol) against all the tested microorganisms.

Table 3: Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs/ MFC) of MEPJ against the tested microorganisms.

Test	Antimicrobial activity (% w/v)							
	S.a	S.a[a]	S.e	St.p	E.c	K.p	P.v	C.a
MIC	0.0156	0.0313	0.0625	0.0078	0.125	0.125	1	1
MBC/MFC	0.0156	0.0625	0.0625	0.125	0.125	0.125	>4	1

Table 4: Antimicrobial activity profile of MEPJ in phosphate-buffered saline (PBS) solution against the tested microorganisms using agar-well diffusion method

* MEPJ Concentration (%w/v)	Inhibition zones diameter (mm)							
	S.a	S.a[a]	S.e	St.p	E.c	K.p	P.v	Ca
4	25	25	21	29	24.5	19	18	16
2	23.5	24	20	27	23	17	16.5	12
1	22	22	18	25	21	17	14	9.5
0.5	20.5	20	15	23	19	16	11	-
0.25	19	19	13	21	16.5	15	-	-

* MEPJ solution prepared in PBS and 1% DMSO. Each value was the mean of duplicate.

Table 5: Antimicrobial activity of semisolid formulations containing 4% (w/w) MEPJ

No.	Test sample	Inhibition zones diameter (mm)							
		S.a	S.a[a]	S.e	St.p	E.c	K.p	P.v	C.a
F1	Macrogol ointment	21.5	22.5	16.5	23	17.5	18	18	12
F2	Hydrophilic petrolatum	13	12.5	-	n.t	11	11	-	-
F3	Cetomacrogol emulsifying ointment	17	18	9	n.t	13	10	-	-
F4	Vanishing cream	9	10	-	n.t	-	-	-	-
F5	Cream base, o/w	18.5	18	13	n.t	15	11	-	9
F6	w/o base	15	15	-	n.t	12	10	-	-
F7	Hand lotion	11.5	10	-	n.t	-	8	-	-
F8	Lutrol F127 gel	23	23	15.5	21	20	17	14	17
F9	Carbopol emulgel.	14	15	-	n.t	11	9	-	-
F10	Lutrol F127 emulgel	22	23	15	20	19.5	16	15.5	15
1	Gentamicin® cream 0.3 %	28	28	23	21	26	22	30	n.t
2	Fucidin® cream (fusidic acid 2%)	34	31	23	20	n.t	n.t	n.t	n.t
3	Gentamicin® ointment 0.3 %	20	16	19	-	22	19	25	n.t
4	Foban® ointment (sodium fusidate 2%)	32	30	33	17	n.t	n.t	n.t	n.t
5	Furazina®ointment (nitrofurazone 0.2%)	28	27	38	36	29	25	26	n.t
6	Tetracycline® ointment 3%	29	9	10	20	18	19	24	n.t
7	Kenazol®cream (ketoconazole 2%)	n.t	n.t	n.t	n.t	n.t	n.t	n.t	15
8	Canestal® cream (clotrimazole 2%)	n.t	n.t	n.t	n.t	n.t	n.t	n.t	23

Each value was the main of duplicate. S.a = *Staphylococcus aureus*, Sa[a] = *Staphylococcus aureus* (isolate), S.e =*Staphylococcus epeidermedis*, St.p = *Streptococcus pyogenes*, E.c = *Escherichia coli*, K.p = *Klebsiella pneumonia*, P.v = *Proteus vulgaris*, C.a = *Candida albicans*,- = no inhibition, n.t = not tested. Note: Zone of inhibition was 0.00 in negative control (corresponding bases) against all the tested microorganisms except macrogol ointment base which gave halos of 8-10 mm in diameter against all organisms except *C. albicans*.

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