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Study on Antimicrobial activity of Herbal Formulation

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Abstract

The present study was aimed to investigate antimicrobial potential of gel formulated with *Punica granatum* peel extract and *Glycyrrhiza glabra* root extract against two gram positive bacteria, two gram negative bacteria and a fungal strain. Different gels were formulated by varying the concentration of the extracts and evaluation was done by cup plate method for zone of inhibition and broth dilution method for MIC (Minimum Inhibitory Concentration) determination. The formulated gel was compared for antimicrobial activity with standard marketed silver sulfadiazine preparation. MIC range for *Punica granatum* pericarp and *Glycyrrhiza glabra* roots was found to be 0.05- 3.2 mg ml^{-1} and 5-25 mg ml^{-1} respectively. Formulation containing these extracts, showed significant zone of inhibition for 0.25%, 0.5%, 1%, 2%, 3% of which 1% showed maximum zone of inhibition (ranging from 30.5 to 37 mm) as compared to marketed preparation. The selected 1% gel formulation also showed good antifungal activity when compared to marketed preparation. Thus the present investigation revealed that the developed gel formulation has potential antimicrobial activity.

Key-Words: *Glycyrrhiza glabra, Punica granatum,* Zone of inhibition, Minimum inhibitory concentration, Antimicrobial activity

Introduction

Antimicrobial gels can be used in many advanced wound dressing products and as promising topical treatment for many other infectious diseases. Topical gels as wound healing treatment are popular because they are cost-effective, easy to use and comfortable for the patients.

Punica granatum L. (Pomegranate) belonging to family Punicaceae, consists of dried rind of the flower. This deciduous shrub is cultivated in Mediterranean regions, Southeast Asia, tropical Africa and in almost throughout India up to an altitude of 2000 m in the hills[1]. The potential therapeutic uses of pomegranate are wide-ranging including treatment and prevention of cancer especially breast and colon cancer[2-4], dental conditions[5, 6], protection from ultraviolet (UV) radiation[7], topical microbicide for HIV prevention[8] and in prevention of chronic periodontitis[9]. The tannins present in the extract of the fruit rind were found to be effective as antihelmintic[10], antibacterial[11-13], antifungal[14] and antiviral[15] activities. Ellagic acid present predominantly in pomegranate peel, mesocarp and arils extracts is responsible for its antibacterial activity[16, 17].

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Glycyrrhiza glabra L. (Licorice) belonging to family Fabaceae, consists of dried roots of the plant is native to Mediterranean region and central and southwest Asia. Glycyrrhizin is the main constituent in Licorice responsible for its anti-inflammatory activity; inhibits both cortisone degradation in the liver and generation of reactive oxygen species (O_2^-, H_2O_2, $\cdot OH$) by neutrophils. Since it has no effect on reactive oxygen species generated by cell free system, it does not act as a scavenger for these entities but decrease their generation by inhibiting neutrophil metabolism. Thus its anti-inflammatory activity can be used in the preparation of skin cosmetics and in treatment of dermatoses and pruritis[18]. Medicinally licorice is used in the treatment of menstrual cramps, menopause symptoms, upper respiratory track ailment, and hypoglycemia[19]. Studies on antibacterial activity of *Glycyrrhiza glabra* are reported where in glycyrrhizin, used as a vehicle in orally administered products inhibits the growth of some bacteria, as well as dental plaque formation[20]. Resistance of pathogens to synthetic drugs and antibiotics already in use makes search for plants with antimicrobial potential more important, as they can be used as substitute for synthetic antibiotics and drugs[21]. Therefore researchers are progressively turning their attention to folk medication, searching for new leads to develop better

drug against microbial infections. Keeping in mind the activity of both the herbs, this work was conducted to develop a gel formulation so as to get the synergistic activity as well as easy to use formulation.

Material and Methods

Plant material

The plants *Glycyrrhiza glabra* roots and *Punica granatum* pericarp were purchased from Yucca Enterprises, A-246, Antop Hill Warehousing Co., Barkat Ali Naka, Wadala (E) Mumbai and were authenticated by Dr. H.M. Pandit, Department of Botany, Khalsa College, Matunga, Mumbai. Specimen sample [*P. granatum* (3513a) and *G. glabra* (3513b)] was stored in their lab and voucher number was taken. The drugs were sun dried, coarsely powdered and stored in airtight container. All chemicals and reagents used were of LR grade.

Preparation of extracts

The individual drug weighing 50 g each of *Glycyrrhiza glabra* and *Punica granatum* were extracted with methanol by hot continuous percolation method in Soxhlet apparatus. The methanolic extracts were filtered, evaporated and dried under reduced pressure with rotary evaporator to get it in dry powdered form. The dried extract weighing 18.5 g of *Glycyrrhiza glabra* and 17 g of *Punica granatum* were used further for the formulation of gel.

Preliminary phytochemical screening

The methanolic extracts were subjected to qualitative test for the identification of various plant constituents[22].

Development of formulation

Ingredients used for the formulation of gel are given in Table no. 1. A water soluble gel was prepared using the dried methanol extract of Pomegranate peels and Licorice roots. Carbopol 940 was dispersed in distilled water (Methyl Paraben 0.15% and Propyl Paraben 0.02%) and glycerin overnight. Weighed quantities of extract of *Glycyrrhiza glabra* and *Punica granatum* was dissolved in propylene glycol and was added to the polymer dispersion. The mixture obtained was finally mixed to obtain a hydro gel. Remaining quantity of distilled water was added and neutralized to pH 7 with Triethanolamine by constant stirring for 10 minutes. Formulation was prepared by varying extract ratio by 0.25%, 0.5%, 1%, 2% and 3%; finally gel was packed in collapsible tubes.

Microorganisms

For anti-bacterial study gram positive bacteria such as *Staphylococcus aureus (*Strain No.NCIM 2079, ATCC NO 6538), *Bacillus subtilis* (Strain No. NCIM 2063, ATCC NO 6633) and gram negative bacteria like *Pseudomonas aeruginosa* (Strain No.NCIM 5031, ATCC NO 25619), *Klebsiella pneumonia* (Strain No. NCIM 2957, ATCC NO 10031) were used in the study. All the bacterial strains were grown and maintained on nutrient agar slants for 24 hours and *Candida albicans* was grown on Sabouraud's Dextrose Agar slants for 48-72 hours. All the microorganisms were confirmed by staining technique.

Standardization of culture

McFarland standards were used as a reference to adjust the turbidity of bacterial suspension so that the number of bacteria will be within a given range. Original McFarland standards were obtained by mixing specified amounts of barium chloride and sulfuric acid together. Mixing the two compounds forms a barium sulfate precipitate, which causes turbidity in the solution. A 0.5 McFarland standard was prepared by mixing 0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2.2H_2O$) with 9.95 mL of 1% sulfuric acid (H_2SO_4). The standard can be compared visually to a suspension of bacteria in sterile saline or nutrient broth. If the bacterial suspension is too turbid, it can be diluted with more diluents. If the suspension is not turbid enough, more bacteria can be added. The culture was standardized by spectrophotometric method using McFarland turbidity standard[23]. The inoculums suspension was prepared by picking 5 colonies of at least 1 mm diameter and suspending the material in 10 ml sterile 0.85% NaCl solution. The turbidity of resulting cell suspension measured at 540 nm was adjusted with saline solution to match that of 0.5 McFarland turbidity standard. This produced cell suspension containing $1x10^6 – 5x10^6$ cell ml^{-1}, which was then diluted 1:100 with desired test medium (Nutrient broth for bacterial culture and Sabouraud's Dextrose broth for fungal culture) to provide inoculums of $1x10^4$- $5x10^4$ cells ml^{-1}.

Antibacterial and antifungal assay

Antibacterial and antifungal activity was checked by agar well diffusion method[24]. For antibacterial assay, the individual drug extract was studied using Nutrient broth by Broth dilution and the gel formulation using Nutrient agar by agar well diffusion method. The formulated gel was then compared with the marketed Licorice preparation (Bleminor®, Himalaya Product) and marketed silver sulfadiazine preparation (Soframycin®) for the antimicrobial activity.

Determination of Minimum Inhibitory Concentration (MIC)

MIC of individual extracts of Pomegranate rind and Licorice roots were determined by nutrient broth using broth dilution method[25]. The bacterial suspension was used as positive control and nutrient broth was used as negative control. The MIC is recorded as lowest concentration of drug, which can inhibit the growth and

show clear fluid without turbidity after 24 hours of incubation at 37ºC. The values of MIC determination were compared against Streptomycin standard. The results of MIC determination are mentioned in Table no. 2.

Determination of Zone of inhibition

Zone of inhibition was carried out for different formulations by cup- plate method to evaluate the antibacterial activity by using nutrient agar against gram negative microorganisms such as *Klebsiella pneumonia, Pseudomonas aeruginosa* and gram positive microorganisms such as *Bacilus subtilis and Staphylococus aureus.* The plates were incubated for 24 hours at 37ºC. Placebo gel was without the drugs. Formulation containing both extracts showed significant zone of inhibition for 0.25%, 0.5%, 1%, 2% and 3% of which 1% showed maximum inhibition ranging from 30.5 mm to 37 mm and compared against marketed Licorice preparation (Bleminor®) and Marketed silver sulfadiazine preparation (Soframycin®). The results of zone of inhibition determination are mentioned in Table no. 3.

Results and Discussion

The results of phytochemical screening of the extracts showed the presence of Phenols and Tannins, Reducing sugar and Carbohydrates in *P.granatum* extract and Flavonoids, Saponins and Coumarin Glycosides in *G.glabra* extract. MIC of *Punica granatum* pericarp ranges from 0.05- 3.2 mg ml^{-1} and for *Glycyrrhiza glabra* roots MIC ranges from 5-25 mg ml^{-1}, which was compared against Streptomycin (standard). Formulation containing both extracts showed significant zone of inhibition for 0.25%, 0.5%, 1%, 2% and 3% of which 1% showed maximum inhibition ranging from 30.5 mm to 37 mm and compared against marketed Licorice preparation (Bleminor®) and marketed Silver Sulfadiazine preparation (Soframycin®). Zone of inhibition of the developed formulation was more than marketed preparations. 1% formulation showed maximum zone of inhibition as compared to other formulations. After 1% there was no significant inhibition and therefore 1% is selected as the final concentration. The developed 1% gel formulation showed good antifungal activity when compared with marketed preparations. Marketed Licorice preparation was ineffective against gram positive bacteria, however formulated gel of both *Punica granatum* and *Glycyrrhiza glabra* extracts was found to be effective against gram positive and gram negative bacteria's as well as a potent antifungal agent. 1% of formulated gel showed maximum anti-microbial activity compared to the marketed formulation. Our findings suggest that, herbal preparations have great potential against pathogenic microbes and can be used as antimicrobial agent for treatment of various infectious diseases.

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Table 1: Development of Formulation

Ingredients	Quantity taken per 100 g (in grams)				
	1 (0.25%)	**2 (0.5%)**	**3 (1%)**	**4 (2%)**	**5 (3%)**
Glycyrrhiza glabra extract	0.125	0.25	0.5	1	1.5
Punica granatum extract	0.125	0.25	0.5	1	1.5
Carbopol 940	1	1	1	1	1
Propylene Glycol 200	20	20	20	20	20
Glycerin	5	5	5	5	5
Methyl Paraben	0.15	0.15	0.15	0.15	0.15
Propyl Paraben	0.02	0.02	0.02	0.02	0.02
Tri ethanolamine	QS	QS	QS	QS	QS
Purified water	100	100	100	100	100

Table 2: MIC for Pomegranate and Licorice extract

Drug concentration	*Staphylococcus aureus*	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Klebsiella pneumonia*
***Punica granatum* extract**				
0.05	+	+	+	+
0.1	+	+	-	+
0.2	-	+	-	-
0.4	-	-	-	-
0.8	-	-	-	-
1.6	-	-	-	-
3.2	-	-	-	-
***Glycyrrhiza glabra* extract**				
1	+	+	-	+
5	+	+	-	-
10	+	-	-	-
15	-	-	-	-
20	-	-	-	-
25	-	-	-	-

+: Presence of growth; - : Absence of growth

Table 3: Zone of Inhibition for formulation

Micro organisms	**Preparations**	**Zone of Inhibition (mm)***				
		0.25%	**0.5%**	**1%**	**2%**	**3%**
Staphylococcus Aureus	Gel formulation	-	30	34	37	40
	Marketed Silver Sulfadiazine Preparation	20	19.5	21	19	20
	Marketed Licorice preparation	-	-	-	-	-
Bacillus subtilis	Gel formulation	18	28	30.5	34	35.5
	Marketed Silver Sulfadiazine Preparation	16	15	15.5	16	14
	Marketed Licorice preparation	-	-	-	-	-
Klebsiella pneumonia	Gel formulation	29	35	37	40	43
	Marketed Silver Sulfadiazine Preparation	22	21	22.5	22	21.5
	Marketed Licorice preparation	20	21.5	21	21	22
Pseudomonas aeruginosa	Gel formulation	28	31	35	37	38
	Marketed Silver Sulfadiazine Preparation	22	21	22.5	23	22
	Marketed Licorice preparation	23	24	23	23.5	23
Candida albicans	Gel formulation	-	-	35	-	-
	Marketed Silver Sulfadiazine Preparation	-	-	20	-	-
	Marketed Licorice preparation	-	-	22	-	-

* Results are the means of values in triplicate.

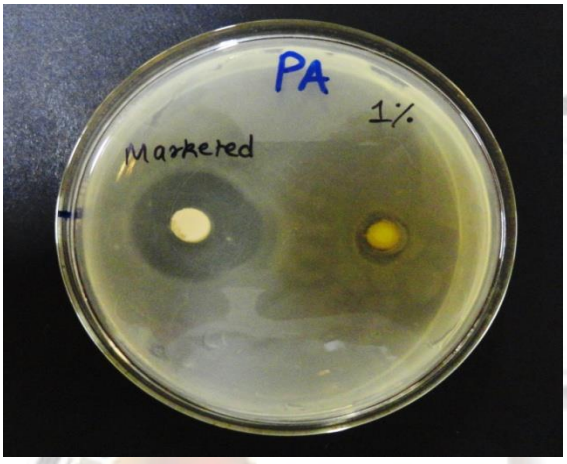

Fig.1: Zone of inhibition - 1% gel against *Pseudomonas aeruginosa*

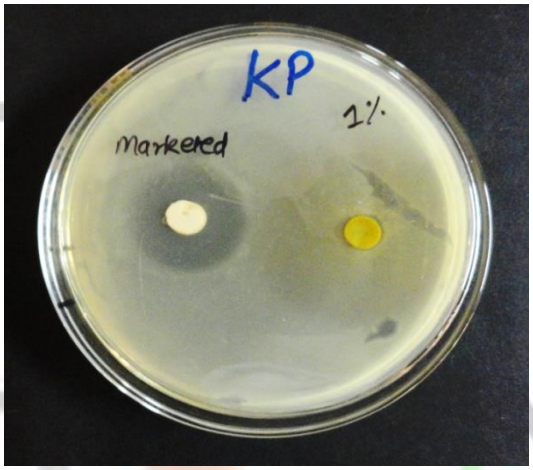

Fig.2: Zone of inhibition - 1% gel against *Klebsiella pneumonia*

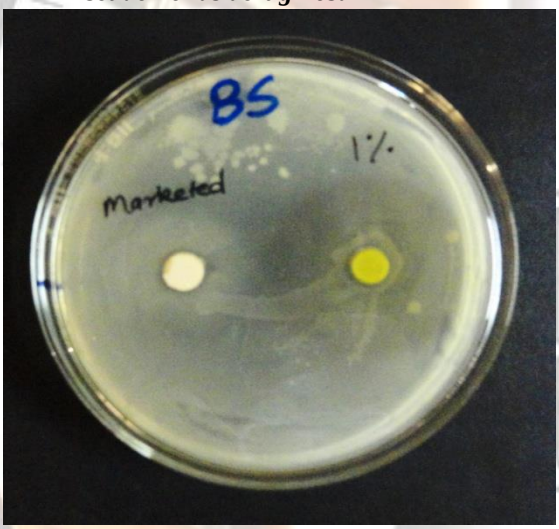

Fig.3: Zone of inhibition - 1% gel against *Bacillus subtilis*

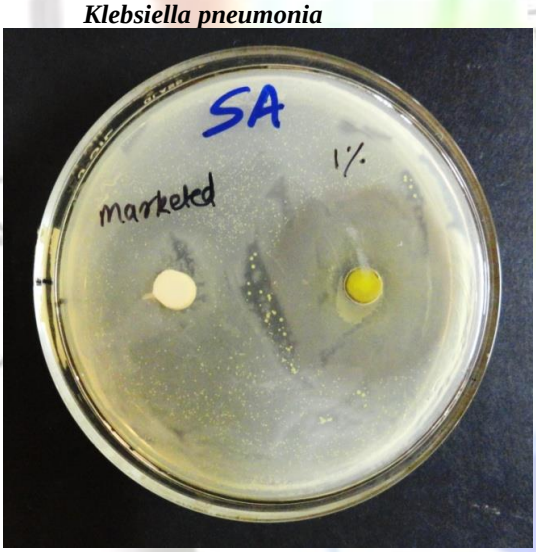

Fig.4: Zone of inhibition - 1% gel against *Staphylococcus aureus*

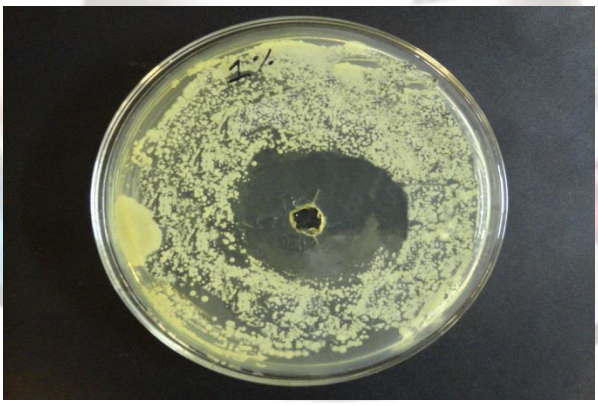

Fig.5: Zone of inhibition- 1% gel against *Candida albicans*