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Development and characterization of anti-acne gel containing *Nymphaea nouchali* ethanolic extract

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Abstract
Nymphaea nouchali (Nymphaeaceae) possessing antibacterial properties are widely used in modern herbal medicines. *Propionibacterium acnes* has been recognized as a main target for medical treatment of acne since this bacterium promotes acne inflammation by inducing upregulation of proinflammatory cytokines production, resulting in an accumulation of neutrophils and oxygen-free radicals produced by neutrophils within acne lesion. Acne is the most common disorder treated by dermatologists. As many as >85% of all youngsters have some type of acne and 50% of them require medical treatment. It is an inflammatory disease of the pilosebaceous unit characterized by the formation of open and closed comedones, nodules, pustules, papules and cysts. In this study anti-acne gels were prepared using polymer Carbopol along with the extracts of plant *Nymphaea Nouchali*. The gel formulations were prepared in different concentrations of F1 to F6. The formulations were tested for the antiacne activity by well diffusion method against *Propionibacterium acnes.* Results showed that the gels were non-irritant, stable and posses anti-acne activity. The efficacy when tested with a standard was almost same to that of Clarithromycin. This suggests that *N. nouchali* has potential against acne causing bacteria and hence they can be used in topical anti-acne preparations and may address the antibiotic resistance of the bacteria.

Keywords: *Nymphaea nouchali, propionibacterium acnes,* carbopol

Introduction
Acne vulgaris, a chronic inflammatory disease of skin, could possibly have affected almost everyone at various points in their lives [1]. The inflammatory acne lesion, a crucial event of the disease often results in scarring and permanent mark [2-5]. There is a wide range of individual clinical expression with males tending to have more severe forms, the incidence is similar in males and females until mid-20s; thereafter acne is more prevalent in females, but the severity and frequency are markedly decreased [6]. Four main pathogenetic factors of acne include hyperproliferation of follicular epithelial cells, excess sebum production and colonization of *Propionibacterium acnes* and inflammation [2, 3]. *P. acnes* have been denoted as a predominant bacterium of acne due to its unique immunomodulatory effect which mainly induces the inflammatory process [7, 8]. Skin macrophages were directly induced by *P. acnes* heat-shock protein to produce several pro-inflammatory cytokines including interleukin-6 (IL-6) and neutrophils chemoattractants; interleukin-8 (IL-8), mainly stimulates neutrophils migration leading to acne lesion and pus formation [4, 7-9]. Neutrophils subsequently generate oxygen free radicals for killing the bacteria. However, excessive production of the free radicals, stimulated by *P. acnes*, leads to the leakage of the free radicals within extracellular space, which destroys follicular epithelium and accelerates progression of the inflammatory responses [10]. Therefore, *P. acnes* has been recognized as one of the main targets for acne treatment [1]. Nowadays, the attempts to find an alternative treatment for acne from natural resources have been considerably expanded every single year due to the antibiotic resistance of *P. acnes* and skin side effects, which might be occurred through the usage of conventional topical medicines [1, 11-14]. *Nymphaea nouchali* (synonym: *Nymphaea stellata*), is the national flower of Bangladesh. It is an aquatic rooting herb belongs to the family Nympheaceae generally found in lakes and ponds throughout the country [15]. It is commonly known as the white water lily. It is an important and well-known medicinal plant, widely used in Ayurveda and Siddha system of medicines for the treatment of diabetes, inflammation, liver disorders, urinary disorders and as a bitter tonic. The fruit of this plant is globose containing round, flaskshaped seeds. The seeds are used as stomachic and restorative. The seeds are also prescribed as diet for diabetes in the ayurvedic system of medicine [16]. Various secondary metabolites like sterols (nymphayol isolated from flower), alkaloids, saponins, tannins, and

flavonoids has been isolated from this plant and these metabolites may be responsible for antibacterial activities. It has been reported to use in treatment of diabetes, tumor, inflammation, liver and urinary disorders, menstruation problems, indigestion and also used as food by the local people [16-20]. There are many literatures reporting the ethno-medicinal values of *N. nouchali*, but there is little scientific proof for further using this plant commercially or in a more effective form. Therefore, an attempt was made to evaluate the antiacne activity of *N. nouchali* flower extracts against *P. acnes.*

Materials and Methods

Plant Materials

The flower of plant of *Nymphaea nouchali* was collected from rural area of Bhopal (M.P), India in the months of January 2017. The sample was identified by senior Botanist Dr. Pradeep Tiwari, Doctor Hari Singh Gour Vishwavidyalaya (M.P.) by comparing with the voucher specimen. Plant material (flower) selected for the study were washed thoroughly under running tap water and then were rinsed in distilled water; they were allowed to dry for some time at room temperature. Then the plant material was shade dried without any contamination for about 3 to 4 weeks. Dried plant material was grinded using electronic grinder. Powdered plant material was observed for their colour, odour, taste and texture. Dried plant material was packed in air tight container and stored for Phytochemical and biological studies.

Chemical Reagents

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India). All the chemicals and solvent used in this study were of analytical grade. The pathogenic microbes used in the current study are gram negative bacteria obtained from Microbial Culture collection, National Centre Forcell Science, Pune, Maharashtra, India.

Extraction Procedure

Defatting of Plant Material

Powdered Plant material (flower) *Nymphaea nouchali* was shade dried at room temperature. The shade dried flower was coarsely powdered and subjected to extraction with petroleum ether (60-80°C) in a soxhlet apparatus. The extraction was continued till the defatting of the material had taken place.

Extraction

80 g. of *Nymphaea nouchali* dried flower were successive extracted with various solvent (Chloroform, Ethyl Acetate, Ethanol and Aqueous) and using different drug: solvent ratios using hot continuous percolation for different time. The extracts were evaporated above their boiling points and stored in an air tight container free from any contamination until it was used. Finally the percentage yields were calculated of the dried extracts [21].

Formulating Anti-Acne Gel

Appropriate quantity of carbopol 934 was soaked in water for a period of 2 hours. Carbopol and rice bran wax was then neutralized by adding triethanolamine (TEA) with stirring. Then required amount of ethanolic extract was dissolved in appropriate and pre weighted amounts of propylene glycol and ethanol. Then solvent blend was transferred to carbopol and agitated for 20 min. The dispersion was then allowed to hydrate and swell for 60 min; finally pH was adjusted to 6.8-7.0 by adding 98% TEA until the desired pH value was reached. During pH adjustment, the mixture was stirred gently with a spatula until homogeneous gel was formed [22]. All the samples were allowed to equilibrate for 24 hours at room temperature prior to performing rheological measurements (Table 1).

Table 1: Formulation herbal gel F1 to F6

Ingredient (g)	F1 (w/w)	F2 (w/w)	F3 (w/w)	F4 (w/w)	F5 (w/w)	F6 (w/w)
Extract	1	1	1	1	1	1
Carbopol	0.1	0.2	0.3	0.4	0.5	0.6
Triethanolamine	0.2	0.2	0.2	0.2	0.2	0.2
Propylene Glycol	0.1	0.2	0.3	0.4	0.5	0.6
Methyl paraben	0.2	0.2	0.2	0.2	0.2	0.2
Propyl paraben	0.8	0.8	0.8	0.8	0.8	0.8
Propylene Glycol	qs.	qs.	qs.	qs.	qs.	qs.
Dist. water	100	100	100	100	100	100

Comparative evaluation of prepared gels

The psycho rheological gel was measured by Penetrometer. Three containers were filled carefully and completely with formulation, without forming air bubbles and stored at 25±0.5^{0}C for 24 characteristics were studied for topical gel formulations like colour, clogging, homogeneity and texture etc. Consistency or hardness of hrs. Test samples were placed on Penetrometer and position of spindle was adjusted as such that, its tip just touches the surface of sample. Penetrating object was released for 5 sec. Depth of penetration was measured. Same was repeated with remaining formulation. Extrudability study was performed by gel formulations were filled into aluminum collapsible tubes. The tubes were pressed by applying weight to extrude the material. Weight was measured which required to extrude the gel from collapsible tubes. An important criterion for gel is that it must possess good spreadability. Spreadability is a term expressed to denote the extent of area to which the gel readily spreads on application to skin. The therapeutic efficacy of a formulation also depends on its spreading value. A special apparatus has been designed to study the spreadability of the formulations. Spreadability is expressed in terms of time taken to slip a movable slides from another fixed slide placed in a frame with formulation under the application of a certain load. Lesser the time taken for the separation of two slides, better the spreadability. The experiment was repeated and the average of 6 such determinations was calculated for each gel formulation.

$$\text{Spreadability} = \frac{m.l}{t}$$

Where, S = Spreadability (gcm/sec)
m = weight tied to the upper slide (20 g)
l = length of glass slide (6 cm).
t = time taken is seconds.

pH of gel was determined by digital pH meter. Ten gram of gel was taken and the electrode was then dipped in to gel solution for 30 min until constant reading obtained. And constant reading was noted. The measurements of pH of each formulation were replicated three times. The viscosity of the prepared gel was determined using Brookfield digital viscometer. The viscosity was measured using spindle no. 6 at 10 rpm at ambient room temperature 25-30^{0}C. The sufficient quantity of gel was filled in appropriate wide mouth container. Wide mouth container use to allow spindle of the Viscometer inside of the container. Viscosity value was noted down after stable of reading. Samples of the gel were allowed to settle over 30 min at the constant room temperature before the measurements. The stability of the gels was tested using freeze thaw cycling method. The gels were subjected to a temperature of 4°C for 7 days, 25°C for 7 days and then at 40°C for 7 days. The gels were exposed to the ambient room temperature after each step and noted for synerisis, viscosity, and pH changes [23-26].

***In-vitro* drug release studies using the prehydrated cellophane membrane**

Preparation of cellophane membrane for the diffusion studies

The cellophane membrane approximately 25 cm x 2cm was taken and washed in the running water. It was then soaked in distilled water for 24 hours, before used for diffusion studies to remove glycerin present on it and was mounted on the diffusion cell for further studies.

Diffusion Studies

The in-vitro diffusion of drug from the different gel preparations were studied using the classical standard cylindrical tube fabricated in the laboratory; a simple modification of the cell is a glass tube of 15mm internal diameter and 100 mm height. The diffusion cell membrane was placed with one gram of the formulation and was tied securely to one open end of the tube, the other end was kept remain open. The cell was inverted and immersed slightly in 250 ml of beaker containing freshly prepared phosphate buffer pH 7.4, as a receptor media and the system was maintained for 2 hrs at 37± 0.5^o C. The media was stirred using magnetic stirrer. Aliquots, each of 5 ml volume were withdrawn periodically at predetermined time interval of 15, 30, 45, 60, 90, 120 min and replaced by an equal volume of the receptor medium. The aliquots were suitably diluted with the phosphate buffer pH 7.4 and 1 ml of 2% $AlCl_3$ solution was added and allowed to stand for 60 min at room temperature; absorbance was measured at 420 nm analyzed by UV-Vis spectrophotometer.

Determination of antimicrobial activity of herbal anti-acne gels against acne causing microorganisms

Preparation of plates

After sterilization, the nutrient agar in flask was immediately poured (20 ml/ plate) into sterile Petri dishes on plane surface. The poured plates were left at room temperature to solidify and incubate at 37^{o}C overnight to check the sterility of plates. The plates were dried at 50^{o}C for 30 minutes before use.

Revival of the bacterial cultures

The Bacterial cultures used in the study were obtained in lyophilized form. With the help aseptic techniques the lyophilized cultures are inoculated in sterile nutrient broth than incubated for 24 hours at 37^{o}C. After incubation the growth is observed in the form of turbidity. These broth cultures were further inoculated on to the agar plates with loop full of bacteria and further incubated for next 24 hours at 37^{o}C to obtain the pure culture and stored as stocks that are to be used in further research work.

Antimicrobial sensitivity

The antimicrobial sensitivity test is employed on to the all the bacteria used under present study with ethanolic extract of *Nymphaea nouchali* for this experiment 6 mm diameter well with stock of 100 mg/ml of extract separately then dried in aseptic conditions. A nutrient agar plate is seeded with particular bacteria with the help of spread plate technique prior and left for 5 minutes. Now culture plates then incubated for 24 hours at 37^{o}C. After incubation, plates were observed to see the sensitivity of extracts towards test bacteriums at particular concentration in the form zone of inhibition [27].

Antibiogram studies

Broth cultures of the pure culture isolates of those test microorganisms which are sensitive towards the 100 mg/ml concentration of Phyto extract used in present study were prepared by transferring a loop of culture into sterile nutrient broth and incubated at 37^{o}C for 24-48hours. A loop full was taken from these broths and seeded onto sterile nutrient agar plates through sterile cotton swab to develop diffused heavy lawn culture. The well diffusion method was used to determine the antibacterial activity of the extracts prepared from the *Nymphaea nouchali* using standard procedure. There were 3 concentration used which are 25, 50 and 100 mg/ml for antibiogram studies. The plates were incubated at 37^{o}C for 24 hr. and then examined for clear zones of inhibition around the wells with particular concentration of drug.

Results and Discussion

The color of the formulations was brownish yellow and the intensity of the color increased with the increase in concentration of the extract in the gel. This might be due to the brownish yellow color of the combined extracts. The viscosity and the pH of the formulations are given in Table 2. The pH value of the products varied from 6.95 to 7.03. The results of the spreadability test was given in Table 2 and observed that F1 with 13.45±2.5g·cm/s possess a better spreadability than other formulations. Extrudability study was performed by gel formulations were filled into aluminum collapsible tubes, all formulation have good Extrudability Table 2. The data of *in-vitro* release of formulations was applied on different drug release kinetic model to determine release kinetic profile results are given in table 3-6. The efficacy of the anti-acne gels from herbal extracts is shown in Table 7. The anti-acne gels could inhibit the growth of the microorganisms that inhabit acnes and all the formulations exhibited comparatively less efficacy to standard drug but formulation 100mg/ml showed almost equal efficacy to Clindamycin. Hence, it is considered best for preparation of antiacne gels.

Table 2: Characterization of herbal formulation F1 to F6

Parameters	Formulation					
	F1	F2	F3	F4	F5	F6
Physical Appearance	Translucent, white, smooth on application	Translucent, white, smooth on application	Translucent, white, smooth on application	Translucent, white, smooth on application	Translucent, white, smooth on application	Translucent, white, smooth on application
pH	6.95±0.3	6.85±0.1	7±0.3	7.03±0.2	7.03±0.2	7.0±0.2
Viscosity (CP)	32458±4.5	32569±10.5	33568±10.1	34548±2.5	34987±4.2	35548±4.2
Spreadability (gm.cm/sec)	13.45±2.5	12.15±0.42	11.25±1.35	11.05± 2.5	10.15± 2.45	9.23± 2.35
Extrudability	+++	+++	+++	+++	+++	+++

Table 3: Zero order release kinetics of formulation F1 to F6

S. No.	Time (min)	% Cumulative drug release					
		F1	F2	F3	F4	F5	F6
1	0	0	0	0	0	0	0
2	15	15.25±1.23	17.15±0.19	19.25±0.35	23.45±0.58	26.56±1.43	32.45±0.15
3	30	38.56±1.03	40.56±0.54	43.25±0.67	55.58±1.68	60.25±2.34	68.89±2.13
4	45	48.89±1.32	52.25±1.05	55.69±1.48	63.56±2.69	75.56±2.16	82.56±1.45
5	60	55.45±2.08	57.89±2.23	62.02±1.65	68.89±3.64	83.25±3.99	95.56±2.75
6	90	68.78±2.19	73.56±2.67	80.56±1.32	89.98±3.16	94.58±2.32	98.89±3.56
7	120	82.56±1.43	85.45±2.38	93.32±2.67	96.56±2.18	99.89±3.56	98.79±2.43

N=3

Table 4: First order release kinetics data

S No.	Tim (min)	Log % Cum. drug remain to be release					
		F1	F2	F3	F4	F5	F6
1	0	0	0	0	0	0	0
2	15	1.928	1.918	1.907	1.884	1.866	1.830
3	30	1.788	1.774	1.754	1.648	1.599	1.493
4	45	1.709	1.679	1.647	1.562	1.388	1.242
5	60	1.649	1.624	1.580	1.493	1.224	0.647
6	90	1.494	1.422	1.289	1.001	0.734	0.045
7	120	1.242	1.163	0.825	0.537	-0.959	0.083

Table 5: Higuchi matrix release kinetics data

S No	Root Time	% Cumulative drug release					
		F1	F2	F3	F4	F5	F6
1	0	0	0	0	0	0	0
2	3.873	15.25	17.15	19.25	23.45	26.56	32.45
3	5.477	38.56	40.56	43.25	55.58	60.25	68.89
4	6.708	48.89	52.25	55.69	63.56	75.56	82.56
5	7.746	55.45	57.89	62.02	68.89	83.25	95.56
6	9.487	68.78	73.56	80.56	89.98	94.58	98.89
7	10.954	82.56	85.45	93.32	96.56	99.89	98.79

Table 6: Korsmeyer-Peppas release kinetics data

S No.	Log T	Log % Cumulative drug release					
		F1	F2	F3	F4	F5	F6
1	0	0	0	0	0	0	0
2	1.176	1.928	1.918	1.907	1.884	1.866	1.830
3	1.477	1.788	1.774	1.754	1.648	1.599	1.493
4	1.653	1.709	1.679	1.647	1.562	1.388	1.242
5	1.778	1.649	1.624	1.580	1.493	1.224	0.647
6	1.954	1.494	1.422	1.289	1.001	0.734	0.045
7	2.079	1.242	1.163	0.825	0.537	-0.959	0.083

Table 7: Anti-acne efficacy of formulations.

S. N.	Formulation	Zone of inhibition*		
		(30µg/ml)	(20µg/ml)	(10µg/ml)
1	F	16±0.86	15±0.28	12±0.57
2	Standard	19±0.86	16±0.57	13±0.28

*Mean of triplicate readings.

Conclusion

This study aimed at developing herbal gels for antiacne treatment using extracts of *Nymphaea nouchali* in an aqueous based Carbopol gel system. Six formulations of the gel were prepared by varying the proportions of polymers and evaluated for their physicochemical properties, like pH, spreadability, viscosity and microbial assay. Based on these tests, formulation containing 100mg/ml was selected as the best formulation. The microbial assay of all the formulations demonstrated better inhibitory activity against *Propionibacterium acnes, Pseudomonas aeruginosa, Pseudomonas plecoglossicida, E. Coli, Klebsiellaoxytoca, Salmonella bongori* and stood competitive to the standard marketed formulation. It was concluded that the present research might hopefully bring advancement in the treatment of acnes using herbs as well as in developing herbal formulations for safe and effective management of diseases.

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