

Original Article

Development of anti-acne gel formulation of anthraquinones rich fraction from *Rubia cordifolia* (Rubiaceae)

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Summary. Acne vulgaris, a common skin disorder speculated in the age group of 15-25 years begins with increased production of sebum followed by the attack of *Propionibacterium acne* (Kathryn *et al*). Most of the synthetic anti-acne drugs tend to exhibit mild to severe side effects along with peeling and darkening of skin, ultimately leading to social withdrawal. Hence, there arises a need to develop a safe and effective anti-acne formulation that would cure and also prevent recurrence of acne. Considering the fact that roots of *R. cordifolia* (Rubiaceae) are rich in anthraquinones characterized for their anti-inflammatory as well as wound healing property, (Singh, 2004) a gel formulation of anthraquinone rich fraction was developed and evaluated for its anti-acne potential using Cup plate diffusion method. A gel formulation containing 0.1 % of anthraquinone rich fraction exhibited optimum anti-acne activity against *P.acne, S.epidermidis, M.furfur* (zone of inhibition- 28.9, 20.4, 24.6 mm respectively) when compared with standard i.e. Clindamycin gel (zone of inhibition- 36.7, 35.3, 32.7 mm respectively). Thus anthraquinone rich fraction in a gel formulation is proved to have a better potential in treating acne.

Industrial relevance. *Rubia cordifolia*, often known as Common Madder, Indian Madder or *Manjistha* is highly recommended in skin diseases associated with edema and oozing (Yuangang Zu *et al*, 2010). The root powder was found to work well with ghee, for the medicament of acne. Used externally as a paste by itself or with honey, it heals inflammation and gives the skin an even tone and smoothness. Since the roots are claimed to be rich in anthraquinones, in this research work anthraquinone rich fraction from the roots *R. cordifolia* were extracted and formulated into an herbal gel and evaluated for its anti-acne activity. Topical gels are very useful as palliative products and prove to be economical and safe; hence it was decided to formulate a simple yet effective anti-acne gel of anthraquinone rich fraction of *R. cordifolia* with a carbomer as a best gelling agent.

Keywords: anti-acne, anthraquinone, gel, Rubia cordifolia, Propionibacterium acne, Staphylococcus epidermidis, Malassezia furfur.

INTRODUCTION

Acne vulgaris is a common skin condition, causing changes in pilosebaceous units (PSU) and skin structure consisting of a hair follicle and its associated sebaceous gland, via androgen stimulation. It is characterized by non-inflammatory follicular papules or comedones and by inflammatory papules, pustules and nodules in its more severe forms. The opportunistic bacteria *Propionibacterium acne* (*P.acne*) residing within the pilosebaceous follicle cause inflammation when exposed to the dermis with ruptured follicle (Christy C *et al*, 2009). The *P.acne* produces substances that promote inflammation, including chemotactic factors along with lipolytic and proteolytic enzymes. The enzyme hydrolytic action of *P.acne* converts triglycerides residing in the glands into free fatty acids that



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stimulate inflammation (Kaur P. et al, 2010) and edema that results into breakdown of the follicular wall. (Yuangang Zu et al, 2010)

Acne may also arise due to change in dietary habits, climate, allergy, mental stress and can cause embarrassment and depression leading to social withdrawal. (Mamgain R.K., 2000)

Many synthetic drugs like Benzoyl peroxide, antibiotics, anti-androgens are used to treat this disorder but exhibit several side effects like dryness of skin, dermatitis, bleaching cloth, darkening of skin and recurrence after withdrawal. (Said H.K., 2005)

In the literature review it was found that *Rubia cordifolia*, *Manjistha* a species of flowering plant in the coffee family, Rubiaceae is considered to be one of the most valuable herbs in Ayurvedic medicine and has been largely used by physicians since ancient times internally as well as externally for most of the skin ailments (Hazra *et al*, 2003). The roots of *Manjistha* were used for medicinal purpose. Externally *Manjistha* is highly recommended in skin diseases associated with edema and oozing. The wounds and ulcers dressed with *Manjistha ghrata* heal promptly and get dried up and are well cleansed. The root powder was found to work well, with ghee, for the medicament of acne (Joshan R.S., 2010). Used externally as a paste by itself or with honey, it heals inflammation and gives the skin an even tone and smoothness. (Chadha Y. R, 2005). Considering the fact that the roots are rich in anthraquinones and could be responsible for the above mentioned activities the efforts were made to separate the anthraquinones rich fraction from *Rubia cordifolia* roots and incorporate it into a topical gel with the purpose to develop a safe, effective and cheaper remedy for healing acne.



Rubia cordifolia leaves and roots

MATERIAL AND METHODS

Collection of Plant material: The plant *Rubia cordifolia* distributed throughout India, was collected from the dense forest of Bhima Shankar and authentified by Botanical Survey of India, Pune. **No.BSI/WRC/Tech/2009/661**

Extraction : Dried powder of roots was extracted by continuous hot extraction (soxhlet) method using ethanol (95%). The extract obtained was concentrated and then subjected to phytochemical screening.

100g ethanolic extract was refluxed with 100 ml 5% HCl solution for 1 hour. The anthraquinone fraction were later separated with (100 x 3 ml) ether and concentrated to give a yield of 7.3% in semisolid form. (Mukherjee P.K, Singh R & Geetanjali, 2004)

Phytochemical screening of extract:

Table 1. Phytochemical screening of ethanolic extract of roots (Khandewal, K.R., 2005)

Phytochemical	Test
Alkaloids	Dragendroff's test, Mayer's test
Carbohydrates	Molish test
Glycosides	Borntager's test
Flavonoids	Shinoda test
Saponins	Foam test
Protiens & Amino acids	Biuret test, Ninhydrin test
Tannins	FeCl3 test, Lead acetate test
Fixed oils & Fats	Saponification test
Mucilage	Ruthenium red test

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Characterization of anthraquinone rich fraction: TLC for anthraquinones

Stationary Phase: Silica Gel.

Mobile Phase: Ethyl acetate: Methanol: Water (80:23.7:20).

Visualizing agent: 5% ethanolic KOH, Detection: A red colour spot was observed at solvent front. (Wagner H & Bladt S)

HPTLC. Solutions of the extracts in concentration of 200 ppm were prepared and were spotted on HPTLC plates. Stationary Phase: Silica gel Plates.

Mobile Phase: Glacial acetic acid: Toluene: Water. (50:40:10)

Instrument: T Mag HPTLC.

Software: WINCATS.

Detection: At 254 nm.

Chamber Saturation: 10 minutes.

Development distance: 80 mm.

Scanner: Camag Scanner.

Development mode: Ascending technique.

Formulation of gels. Method of preparation: Required quantity of Carbomer 940 was soaked in some amount of distilled water for 2 to 3 hrs. (Phase I). Required quantity of anthraquinone was dissolved in the small amount of Propylene glycol propyl paraben and methyl paraben were later added (Phase II).

Phase I and II were mixed and adjusted to a pH of 6.8-7.4 with drop wise addition of triethanolamine. The remaining quantity of distilled water was then added to make up the final 100gm weight. Stir the formulation with mechanical stirrer to homogenize the formulation. (Table No 1) (Waghmare N *et al*, 2011)

Inquadianta	Formulation Codes				
ingreatents	G ₁	G ₂	G ₃		
Anthraquinones	0.02%	0.05%	0.1%		
Carbomer 940	0.9%	0.9%	0.9%		
Propylene glycol	20%	20%	20%		
Propyl paraben	0.08%	0.08%	0.08%		
Methyl paraben	0.2%	0.2%	0.2%		
Triethanolamine	q.s.	q.s.	q.s.		
Water	q.s.	q.s.	q.s.		

Table 2. Formulation of gels

Determination of anti-acne activity. A) Collection and preservation of the cultures . All culture were obtained from IMTECH (Institute of Microbial Technology), Chandigarh, The microorganism were grown using Brain Heart Infusion Broth (Hi media Laboratories limited, Mumbai).

Table 3. Standard Protocols followed for collection and preservation of microbial cultures

Standards	P.acne	S. epidermidis	M. furfur
MTCC no.	1951	3615	1374
Growth condition	Anaerobic	Aerobic	Aerobic
Temperature	37 ⁰ C	37 ⁰ C	37 ⁰ C
Incubation time	48 Hrs	48 Hrs	48 Hrs
Subculture	15 days	15 days	15 days

Tween 80: 1% and Thioglycollic acid: 0.03% was added as a reducing agent.

B) Standardization of Cultures: The culture of above bacteria's was standardized by spectrophotometric method using Mc Farland turbidity standard. The test organism used was grown on the plates of specified medium for specified time. The inoculum suspension was prepared by picking 5 colonies of at least 1 mm diameter and suspending the material in 5 ml sterile 0.85% sodium chloride to match that 0.5 McFarland turbidity standard. This produced a cell suspension containing 1×10^6 to 5×10^6 cells per ml, which was diluted in the ratio 1:100 with the desired test medium to provide starting inoculums of 1×10^4 to 1×10^4 cells per ml. (Baueg A.W., 1996)

Anti-acne gel formulation from Rubia cordifolia

C) Determination of Minimum Inhibitory Concentration by Broth dilution Method: Cultures of each aforesaid bacterium were prepared separately in an aseptic area. The medium was poured in the test tubes and sterilized by autoclave using 15 lb pressure at 121° C for 30 min. Using sterile pipettes exact amount of extract was added as indicated in the Table 3 to obtain a final volume of 10 ml. The tubes were then inoculated with 0.05 ml of the standardized culture and further incubated at 37° C for specified period of time and observed for any microbial growth in form of turbidity. The test procedure was repeated to check the reproducibility of the results. The lower concentration that inhibited the microbial growth was taken as the Minimum Inhibitory Concentration (MIC). Clindamycin was used as reference standard. (Thiem B & Grosslinka O., 2003, Islam M. A. *et al*, 2008, Yuangang Zu *et al*, 2010)

Amount of anthraquinone fraction/ml	Amount of medium (ml)	Total volume (ml)	Conc. of anthraquinone fraction in final solution (mg/ml)
0.1	9.9	10	0.1
0.2	9.8	10	0.2
0.3	9.7	10	0.3
0.4	9.6	10	0.4
0.5	9.5	10	0.5
0.6	9.4	10	0.6
0.7	9.3	10	0.7
0.8	9.2	10	0.8
0.9	9.1	10	0.9
1.0	9.0	10	1.0

Table 4. Protocol for evaluation of MIC by broth dilution method

D) Preparation and sterilization of Test Samples: Solutions containing different concentrations 100, 200, 500, μ g/ml respectively of isolated anthraquinone as well as 0.02%, 0.05%, 0.1% of anthraquinones gel formulation was prepared and sterilized in autoclave using 15 lb pressure at 121°C for 30 min.

E) Cup plate diffusion method: Anti-acne susceptibility was done by cup plate diffusion method. The cup plate diffusion method involves sterilization of Petri plates, seeding of medium, inoculation and incubation. The plates were sterilized by dry heat in an oven at 160° C for one hour. Broth for each bacterial agar was prepared and sterilized by autoclaving. Molten agar (30ml) was poured in each Petri plate. The test tubes were cooled upto 50° C, and then 20ml of molten agar from each test tube was added in sterile Petri plates aseptically and kept to solidify. Standard cultures of microbes were poured on top of the agar in the plates. After solidifying, wells of 8mm were bored aseptically using sterile cork borer. The agar plugs were taken out carefully so as not to disturb the surrounding medium. The holes were filled completely with desired extract. The plates were kept in an incubator at 37° C for specified time. After this Petri plates were observed for the antibacterial activity by measuring the zone of inhibition using a zone reader. Clindamycin gel was used as a standard. (Barry A.L., 1995, Joshan R.S. et al, 2010)

Evaluation of Gel (Barry A.W., 1983, Sanjay et al, 2007, Lachman L et al). The following parameters were evaluated for each formulation;

- 1. Appearance
- 2. pH
- 3. Viscosity determination:

A plate and cone Brookfield viscometer (model CAP 2000+2) was used to determine viscosity (cp). Sample gel was placed on the centre of plate and the viscosity was measured at 37° C.

4. Spreadability:

Spreadability of the gel formulations was determined by measuring the spreading diameter of 0.1 g of gel between two horizontal plates (7.0 cm \times 2.5 cm) after one min against the standardized weight tied on the upper plate (25 g).

5. In- vitro Diffusion Study with help of Keshary-Chien (K-C) Diffusion Cell:

A Keshary-Chien (K-C) diffusion cell with a receiver compartment volume of 27 ml, diameter of 2.6 cm and effective diffusion area of 5.31 cm² was used in this study.

RESULTS *Phytochemical Screening:*

Sr.	Phytochemicals	Test	Res
No.	-		ult
1.	Alkaloids	Dragendroff's test,	+
		Mayer's test	+
2.	Carbohydrates	Molish test	+
3.	Glycosides	Borntager's test	+
	(anthraquinones)		
4.	Flavonoids	Shinoda test	+
5.	Saponins	Foam test	-
6.	Protiens & Amino acids	Biuret test,	-
		Ninhydrin test	-
7.	Tannins	FeCl3 test,	-
		Lead acetate test	-
8.	Fixed oils & Fats	Saponification test	-
9.	Mucilage	Ruthenium red test	-

 Table 5: Phytochemical Screening of anthraquinone rich fraction:

Characterization of anthraquinone rich fraction:



Figure 1. TLC for anthraquinones



Figure 2. HPTLC profile of anthraquinone rich fraction

Minimum inhibitory concentration (MIC):

Table 6. N	/inimum i	nhibitory	v conce	ntration	for	P.acne,	S.epide	rmidis :	and M.furfur
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S. No.	P. acne	S. epidermidis	M. furfur
MIC	1 mg/ml	1 mg/ml	1 mg/ml

Evaluation of zone of inhibition:

Table 7. Zone of inhibition for anthraquinone rich fraction and its gel based formulations

Test Samples	Zone of inhibition (mm)*for <i>P. acne</i>	Zone of inhibition (mm)*for <i>S</i> . <i>epidermidis</i>	Zone of inhibition (mm)* for <i>M. furfur</i>
anthraquinones fraction – (100µg/ml)	20.6	21.4	15.6
anthraquinones fraction-(200µg/ml)	26.8	31.4	19.4
anthraquinones fraction – (500µg/ml)	34.6	34.7	32.7
$G_1 - (0.02\%)$	17.4	14.8	17.3
$G_2 - (0.05\%)$	21.6	18.6	21.5
$G_3 - (0.1\%)$	28.2	20.4	24.6
Clindamycin 1% gel	36.7	35.3	32.7
Dummy gel	No Zone found	No Zone found	No Zone found



Figure. 3A

Figure. 3B

Figure. 3C



Figure. 3D

Figure. 3E

Figure. 3F

Figure. 3. A-C: Zone of inhibition of 100, 200 & 500 µg anthraquinones fraction against *S. epidermidis* D-F: Zone of inhibition of 0.02, 0.05, 0.1 % gel, dummy and clindamycin gel against *S. epidermidis*

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Figure 4A

Figure 4B



Figure 4C

Figure 4D

Figure 4E

Figure. 4. A-B: Zone of inhibition of 100, 200 & 500 µg anthraquinones fraction against *P. acne* C-E: Zone of inhibition of 0.02, 0.05, 0.1 % gel, dummy and clindamycin gel against *P. acne*



Figure.5. A-C: Zone of inhibition of 100, 200, 500 µg anthraquinone fraction; 0.02, 0.05, 0.1 % gel, dummy and clindamycin gel against *M.furfur*

Formulation code	Color	Appearance	рН	Viscosity (cp)	Spreadability (gm.mm/min)
G ₁	Light orange	Translucent	6.94	24.937	23.67
G ₂	Orange	Translucent	7.08	23.062	24.12
G3	Dark orange	Translucent	7.18	16.500	25.67

Table 8. Evaluation of gels

Times	Absorbance at 289.4 nm Gels of anthraquinone rich fraction and Clindamycin					
(minutes)	G ₁	G ₂	G ₃	Clindamycin		
5	0.0041	0.0084	0.0174	0.0747		
10	0.0048	0.0102	0.0224	0.1047		
20	0.0054	0.0119	0.0241	0.1658		
30	0.0059	0.0135	0.0274	0.2014		
40	0.0065	0.0141	0.0301	0.2658		
50	0.0070	0.0152	0.0325	0.2980		
60	0.0075	0.0159	0.0358	0.3247		

Table 9. Drug release of anthradulinone fich fraction and Clindamycin get at 289.4 nm	Table 9. Drug release	of anthraquinone rich	fraction and Clindamy	cin gel at 289.4 nm:
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DISCUSSION

The cultures of *P.acne, S.epidermidis* and *M.furfur* procured from IMTECH, Chandigarh were standardized by spectroscopic method using Mc Farland turbidity standard and subjected for the determination of MIC using standard protocol. The lowest concentration that inhibited the microbial growth (MIC) was 1mg/ml showing a clear solution indicating complete inhibition of microbes. Anthraquinone rich fraction (100, 200, 500 µg/ml) and its gel based formulations (0.02, 0.05 & 0.1 %) was subjected to anti-acne screening using Cup-plate diffusion method. Anthraquinone rich fraction at 500 µg/ml exhibited optimum zone of inhibition of 34.6, 34.7, 19.4 mm against *P.acne, S.epidermidis* and *M.furfur* respectively. Similarly the zone of inhibition for herbal gel containing 0.1% of anthraquinone fraction was found to be 28.2, 20.4, 24.6 mm against *P.acne, S.epidermidis* and *M.furfur* respectively, as against to that obtained for the standard Clindamycin gel (36.7, 35.3, 32.7 mm respectively), which were significantly comparable suggesting their anti-acne potential. (Table 7, Figure 3-5)

The gels were further subjected to pharmaceutical evaluation using various parameters like pH, viscosity, spreadability and *in-vitro* diffusion studies.

The pH of all the formulations was ranging from 6.85 to 7.41. The viscosity in aqueous dispersion of carbomer 940 was found to be 15-25 cp. Formulations showed good spreadability between 23-25 gm.mm/min. (Table 8)

The in-vitro studies were performed to estimate the amount of drug released from the skin in specific period of time compared with the standard Clindamycin gel spectrophotometricaly. It was found that after 60 min, absorbance for 0.1 % gel was 0.03583 which depicted linear correlation of drug release with increasing time. (Table 9)

CONCLUSION

Anthraquinones rich fraction in concentration of 500μ g/ml exhibited significant anti-acne activity. Also a gel formulation G₃ with 0.1% anthraquinone fraction was found to be effective against *P. acne*. The gel formulations also complied with standards of various pharmaceutical parameters. Thus the present research work suggests that anthraquinone gel formulation holds a tremendous potential against acne and can prove to be a safe and efficacious remedy for treating this dermatological disorder. However an elaborate protocol for the clinical trials is needed to be designed and implemented to check the anti-acne activity on human volunteers.

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CONFLICT-OF-INTEREST STATEMENT

The authors report no Conflict-of-Interest. The authors alone are responsible for the content and writing of the paper.

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