Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(2): 248-256
Received: 02-01-2020
Accepted: 05-02-2020

Vidhi Bhatia
Assistant Professor,
Department of Pharmacognosy,
V.E.S. College of Pharmacy,
Mumbai, Maharashtra, India

Asmita Joyasar
M. Pharm V. E. S. College of Pharmacy, Mumbai,
Maharashtra, India

Corresponding Author:
Vidhi Bhatia
Assistant Professor,
Department of Pharmacognosy,
V.E.S. College of Pharmacy,
Mumbai, Maharashtra, India

Development of polyherbal anti acne gel formulation

Vidhi Bhatia and Asmita Joyasar

Abstract
Acne is the multifactorial chronic inflammatory disease of pilosebaceous gland. *Propionibacterium acnes* (*P. acne*) is considered as the major skin bacteria that causes the inflammation. The objectives of this study was to design a product to treat acne with herbal actives as herbal remedies are effective in treatment of various ailments with few or no adverse effects. Acne treatment is not only based on antimicrobial activity but also anti-oxidant, anti-inflammatory properties by which drug inhibit neutrophil migration and generation of Reactive Oxidative Species (ROS). For this purpose one essential oil and two herbal extract having anti-acne and anti-microbial properties were selected. These were incorporated into the gel base and *in vitro* antibacterial activities were studied against *P. acne* using agar diffusion method. All the formulations showed satisfactory antibacterial activity with. It was subjected for stress testing for 3months at different temperature. Thus it was concluded that formulated herbal anti acne gel can be effectively used for acne treatment.

Keywords: Acne, *propionibacterium acnes*, herbal gel, agar diffusion method

Introduction

Propionibacterium acne

On the skin surface, the microbial community is mostly consisted by bacteria belonging to the three main genera of Corynebacterium, Propionibacteria and staphylococci. Propionibacterium acnes belong to the human cutaneous propionibacterium along with Propionibacterium avidum, Propionibacterium granulosum, Propionibacterium innocuum and Propionibacterium propionicum. Historically, P. acnes has been designated as Bacillus acnes and Corynebacterium acnes and Corynebacterium parvum.

Acne lesions develop as the result of an inflammatory response to the bacterial colonization of the skin follicles that have become obstructed and swollen with sebum. Propionibacterium acnes bacteria digest triglycerides that are the principle component of sebum and release fatty acids, chemotactic factor, and other molecules that stimulate skin inflammation and the formation of pustules

Gels are semisolid dosage forms and are transparent to opaque. They contain high ratio of solvent to gelling agent. Gelling agents merge or entangle on dispersing in an appropriate solvent to form a three dimensional colloidal network structure. The fluid flow of solvent molecules by entrapment and immobilization get stopped due to the network. Gel formulation contains one essential oil that is lavender oil and two herbal actives such as turmeric and neem.

Materials and Method

Selection of herbal and essential oils

On the basis of extensive literature study, pure essential oil of lavender and two botanical herbs *Curcuma longa* (Turmeric) *and Azadirachta indica* (Neem) selected for the study as they are having antimicrobial activity.

Chemicals

Chemicals were obtained from Ranchem Laboratory Chemicals Pvt. Ltd., Himedia Laboratories Pvt. Ltd and Loba Chemie, Mumbai.

Preparation of extract

The fresh rhizomes of curcuma longa was washed and air dried at room temperature. The dried parts were powdered. The powdered drug was subjected to Soxhlet Extraction at 60 °C using methanol as the extracting solvent. The extraction method, solvents and the concentrations used were optimized as per yield obtained. The extract was stored in air-tight containers in refrigerator at 5 °C.

The fresh leaves of Azadirachta indica were washed and air dried at room temperature. The dried parts were powdered. The powdered drug was subjected to Soxhlet Extraction at 60 °C using chloroform as the extracting solvent. The extraction method, solvents and the concentrations used were optimized as per yield obtained. The extract was stored in air-tight containers in refrigerator at 5 °C.

Physical Parameter

Physical parameter of the herbal drugs were studied such as loss on drying, ash value, alcohol soluble extractive value and water soluble extractive values.

Phytochemical Screening: The extract were subjected to phytochemical screening separately using reagents and chemicals. Screening involved qualitative determination of alkaloid, glycoside, terpenoids, steroids volatile oil, flavonoids, tannins and phenolic compounds, carbohydrates and proteins. The extracts obtained from both the research drugs were subject to qualitative examination as per the Pharmacopoeia of India (IP).

Thin layer Chromatography (TLC)

The extracts were subjected to the separation using different mobile phase based on the phytoconstituents present in them. TLC analysis was conducted on pre-coated silica gel 60F 254 TLC plates. The plates were visualizing in day light, in short UV and long UV. The Rf value is the "retardation factor" or "ratio-to-front" value expressed as a decimal fraction. The Rf value was calculated using following formula:

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent front}}$$

Procedure

Standard solution: 10mg of standards dissolved in 10ml of methanol and filter it to remove insoluble matter. The filtrates were used for spotting on silica gel plate.

Test solution: 0.5g of extract dissolved in 100ml of methanol and filters it to remove insoluble matter. The filtrates were used for spotting on silica gel plate.

Antibacterial study of extract (mic calculation)

Preparation of bacterial culture suspension

The acne causing bacterial species that were used for anti-bacterial studies was Propionibacterium acnes (MTCC No. 1951). The bacterial suspensions of the P. acne was prepared by using Mc Farland's standard method.

Preparation of Mc Farland's Standard

This method includes preparation of 1% w/v $BaCl_2$ solution and 1% w/v H_2SO_4 solution as stock solutions. For preparing Mc Farland's standard No. 1, measure 0.1 ml of 1% w/v BaCl2 solution and mix it to 9.9 ml of 1% w/v H_2SO_4 solution to give a slightly turbid solution of $BaSO_4$ which is used as a standard.

Preparation of BHI (brain heart infusion) broth HIMEDIA

Suspended 37.0gm in 1000ml of distilled water. Heat if necessary to dissolve the medium completely. Dispense in to conical flask. Sterilize by autoclaving at 15 lbs. pressure (121 °C) for 15 minutes. Note: for the best result, the medium should be used on the day it is prepared, otherwise it should be boiled or steamed for few minutes and then cooled before use.

Sample preparation: For Curcuma longa and Azadirachta indica Stock solutions (200mg/ml) of all three drugs were prepared with DMSO. (Solution A) From above solution 2ml is taken and diluted with 2ml of nutrient broth. (Solution I-100mg/ml) and further concentrations are made from data given below:

Table 1: Sample preparation for MIC calculation

	2ml	2ml	2ml	2ml	2ml	2ml	2ml	
Concentra-tion (mg/ml)	**200 (2ml)Stock solution(A)**	**100 (I)**	**50 (II)**	**25 (III)**	**12.5 (IV)**	**6.25 (V)**	**3.12 (VI)**	**Discard**
BHI broth	2ml	2ml	2ml	2ml	2ml	2ml	2ml	--
Culture of *P. acne*	0.2ml	0.2ml	0.2ml	0.2ml	0.2ml	0.2ml	0.2ml	--
Total quantity	4.2ml	4.2ml	4.2ml	4.2ml	4.2ml	4.2ml	4.2ml	--

To check the maximum and minimum growth of bacteria two control test were used. Along with above dilutions there were two control test such as positive control (presence of growth of microbes) and negative control (absence of microbial growth). Positive control contained 2ml BHI broth and 0.2ml culture where as negative control contained only 2ml BHI broth.

Drug-excipient compatibility study

Physical Mixture

Physical mixture of drug and excipient were evaluated for solid state compatibility under following condition.

Vials are kept at 25 °C/60% RH and 40 °C/75%RH for 1 month.

Physical mixtures were evaluated for colour change, moisture uptake and change in texture. Based upon result of this study excipient were selected for the formulation. The drug and various excipients were analysed and characterised using IR spectroscopic method using FT-IR spectroscopic method within the range 4000-500cm^{-1}.

Table 2: Drug-excipient compatibility study

Sr. no.	Drug excipients	*Rubia cordifolia*	*Curcuma longa*	*Azadirachta indica*
1.	Carbopol 934	1:1	1:1	1:1
2.	Propylene glycol	1:0.5	1:0.5	1:0.5
3.	Tween 80	1:0.25	1:0.25	1:0.25
4.	Methyl paraben	1:0.25	1:0.25	1:0.25
5.	Propyl paraben	1:0.25	1:0.25	1:0.25
6.	Xanthan gum	1:1	1:1	1:1
7.	Aloe Vera base	1:1	1:1	1:1

Formulation and development

The excipients selected for further developing a formulation was based on solubility and drug-excipient compatibility studies. A topical Poly-herbal formulation of Gel was

prepared. In the initial step, the base was optimized prior to addition of the extracts. The base was optimized by varying the concentration of the Gelling agent. The herbal gel of the optimized formulation was developed using a suitable polymer capable of modifying the rheological behavior. Different concentrations (0.5%, 0.75%, 1%, and 2%) of the gelling agent like Carbopol 934 were tried. The concentration of gelling agent was selected on the basis of compatibility with plant extracts, feel and ease of Spreadability. 0.5, 1.0, 1.5 and 2% w/w Carbopol 934 was swollen in the water. Triethanolamine (TEA) was used to adjust pH of the swollen gel matrices. Further, the drug (extracts) and essential oil were added to the optimized base and further subjected to evaluation.

A. Physical Evaluation

Color, physical appearance and homogeneity were tested by visual observation.

a) Homogeneity

After the formulations have been set in container, all developed formulations were tested for homogeneity by visual inspection.

b) pH

The pH of formulation was determined using a Digital pH meter. A volume of 1gm of gel was dissolved in 100ml of distilled water and stored for 2hrs. The measurement of pH was done. The pH of gel formulation was in range 5.5 to 7.5 which lies in normal range of the skin and would not produce any irritation.

c) Viscosity

The viscosity of gel formulation was determined using small volume Brookfield viscometer. The determination were carried out four times at 6, 12, 30 and 60 rpm and that reading was multiplied by the factor and mean of that were taken as final viscosity in centipoises.

Brookfield factor finder was used as follows:

Dial reading × factor = viscosity in centipoise

d) Spreadability

A volume of 20 g weight was tied to the upper slide carefully. The time taken for the upper slide to travel the distance of 6.0 cm and separated away from the lower slide under the influence of the weight was noted. The experiment was repeated by 3 times and the mean time taken for calculation. Spreadability was calculated using the following:

Formula: $S = M \times LT$

Where, S-Spreadability M-Weight tied to the upper slide (20 g), L-Length of the glass (6 cm),

T - Time taken in seconds.

B. Drug content

About 1gm of formulation was weighed and added to a 100ml volumetric flask with 30 ml of pH 5.5 phosphate buffer and shaken until all the gel has dispersed and dilute the solution to 100ml with pH 5.5 phosphate buffer. The solution was filtered. Further dilutions were made and measured the absorbance of the solution at three wavelength (λ max of 2 drugs at 260 and 423nm), using pH 5.5 phosphate buffer in reference cell. This can be estimated spectrophotometrically using UV/VIS spectrophotometer.

For the drug, content sample has taken from top, middle and bottom from container. The experiment was repeated for ten times for each batch and then average value was taken for the drug content calculation.

C. *In vitro* activity

Antibacterial study

The anti-bacterial activity of gel was carried out by Cup plate method.

Modified agar well diffusion method is used for the determination of antibacterial activity of different formulation. In this method, nutrient agar plates were seeded with 0.2ml of broth culture of P. acnes. The plates were allowed to dry for 1hr. A sterile 8 mm borer was used to cut four wells of equidistance in each of plates; 1g of formulations and marketed Clindamycin gel for comparison. Benzoyl peroxide gel was used as a positive control, and distil water was used as negative control were introduced in to the wells at randomly. The plates were incubated for 24hrs at 37 °C. The antibacterial activities were found out by measuring the diameter of zones of inhibition (in mm).

D. *In vitro* anti-inflammatory assay

Inhibition of albumin denaturation: The reaction mixture was consisting of test extracts at different concentration and 1% aqueous solution of bovine albumin fraction. pH of the reaction mixture was adjusted using small amount of 1N HCl. The sample was incubated at 37 °C for 20 minutes. And then heated at 57 °C for 20 minutes. After cooling the sample, the turbidity was measured spectrophotometrically at 660nm. The experiment was performed in triplicate. Percent inhibition of protein denaturation was calculated as follows:

$$\text{Percent inhibition} = \frac{(\text{Abs control} - \text{Abs sample}) \times 100}{\text{Abs control}}$$

E. *In vitro* diffusion study

In vitro diffusion study of the anti-acne gel formulation was done using the Franz diffusion cell. Franz diffusion cell has been the standard system used for the study of release of semi-solid drug formulations. 0.45µ dialyzing membrane was used. The media used for the *in vitro* diffusion was phosphate buffer pH 5.5. The dialyzing membrane was soaked in phosphate buffer 24 hrs. before use. The temperature was maintained constant at 32 °C. During study 5 ml sample was withdrawn and replaced with fresh solvent. The time interval was maintained as 15 minutes, 30 minutes, 1hr, and 2hrs up to 8 hrs. The drug concentration of receptor fluid was determined by UV spectrophotometer at 217, 260 and 423 nm. The correlation factor was included in the calculation for the drug loss during sampling. Thus, the amounts of drug permeation of all the formulations were calculated.

F. Stability study

The final formulation i.e. poly-herbal gel Formulation was subjected to stability studies for a duration of three months at various temperature and humidity conditions. 25 ±2 °C/60% ±5% RH, 40 ±2 °C/75% ±5% RH and Refrigeration temperature (4 °C) The samples was tested and evaluated initially and then at 15th day, 30th day, 60th day and 90th day from the day of commencement of the study.

HPTLC

Determination of Curcumin in formulation

Identification of Curcumin in the Formulation was performed. Using the mobile phase Toluene: Ethyl Acetate: Methanol:

Formic Acid (8:2:1:0.5 v/v/v/v). The method was found to be satisfactory. The Same Method was used for Quantification of Curcumin in the Formula.

HPTLC parameter for *Curcuma longa*

Table 3: HPTLC parameter

i)	Sample Preparation	Sample was weighed 500mg and 5ml of water was added this solution was sonicated using a ultrasonication for 30 min. Then the Solution was filtered and the filtration ws used as solutuin for Chromatography
ii)	Stationary phase	TLC silica gel 60 F254
iii)	Standard Preparation	10 mg of Provided Standard was weighed and 10 ml of methanol was added.
iv)	Mobile phase	Toluene: Ethyl Acetate: Methanol : Formic Acid (8:2:1:0.5 v/v/v/v)
v)	Development chamber	Saturated chamber with whatmann filter paper no 1 Developing Distance 70mm from the lower edge.
vii)	Documentation	At 254nm
Viii)	Chromatogram	At 393nm

Result and Discussions

Pre-formulation

Physical parameter

The physical parameters of drug were evaluated to assess drug purity and identity. Following are the results for the evaluated physical parameters.

Table 4: Results for physical parameter of extracts

Drugs / parameter	*Curcuma longa*	*Azadirachta indica*
Description	golden yellow color	Greenish Brown with Acrid and bitter taste colour powder
Loss on drying (%)	7.98	6.63
Ash value (%)	4.78	4.16
Alcohol soluble extractive value	92.61%	90.52 %
Water soluble extractive value	51.78%	80.83%

Remarks

1. Both drugs having good alcohol soluble extractive values whereas curcuma longa having less water soluble extractive value.
2. Both extracts showed ash values less than 5%, indicating that the extracts were pure and free from any impurities

Phytochemical Screening

The extracts were subjected to phytochemical screening separately using reagents and chemicals.

Table 5: Results for phytochemical screening of extracts

Sr. No.	Tests	*Curcuma longa*	*Azadiracta indica*
Test for oils	Sudan red test	Present	Absent
Test for Tannin	Ferric chloride test	Absent	Present
Test for Glycoside	1. Keller-Kiliani Test:	Absent	Absent
	2. Bontrager's test:	Absent	Absent
Test for Saponin	Foam test	Absent	Present
Test for steroids:	1. Salkowaski test:	Absent	Absent

Antibacterial study of extract (MIC calculation)

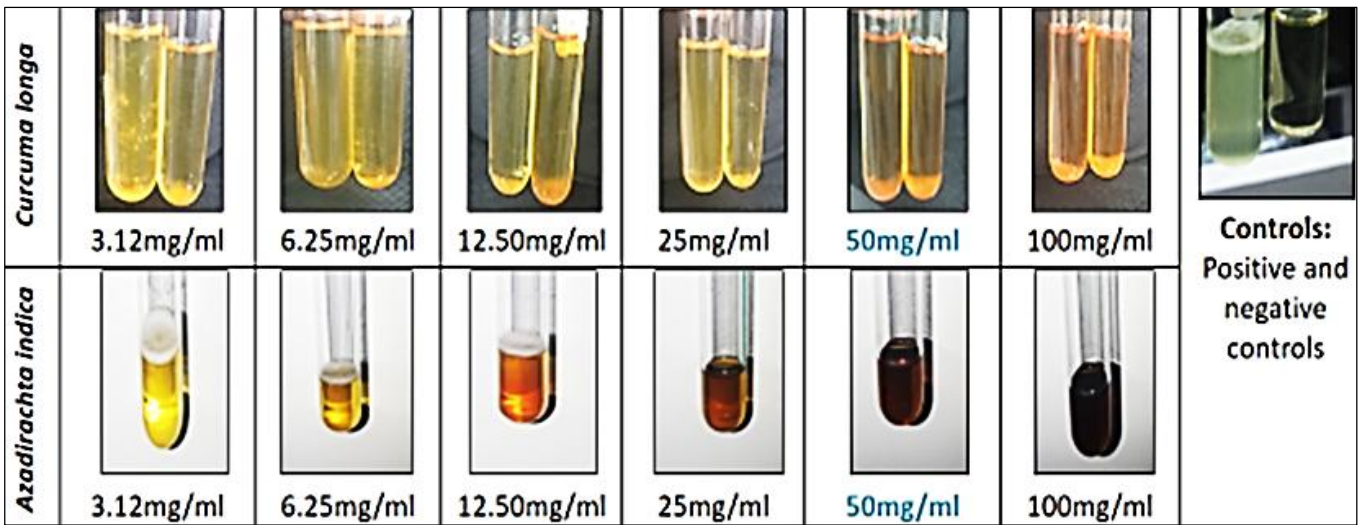


Fig 1: Minimum Inhibitory Concentration by double dilution method

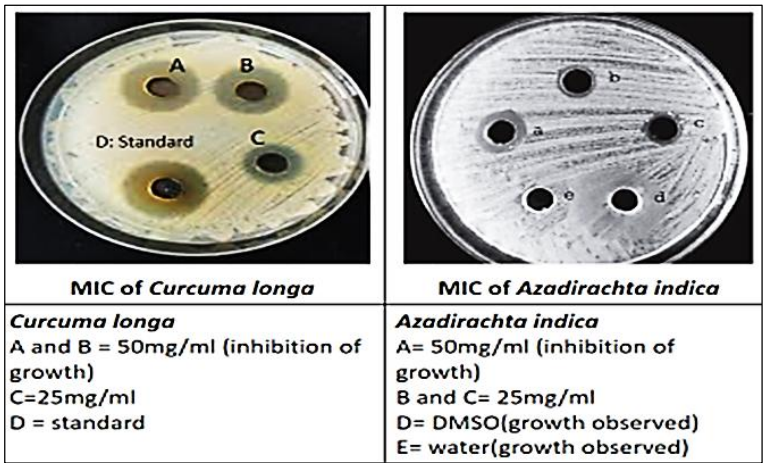
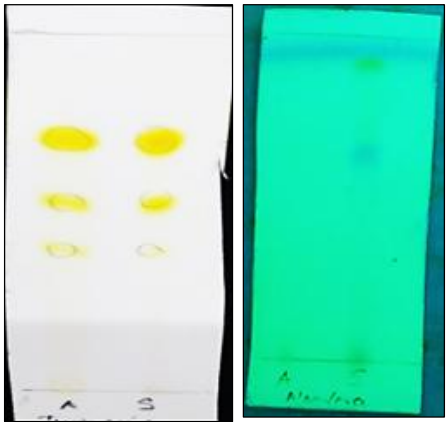


Fig 2: Thin layer chromatography (TLC)

Table 6: Mobile phase and Rf value of extract

Drugs	***Curcuma longa***		***Azadirachta indica***	
Standards	**Curcumin**		**Azadirachtin**	
Mobile phase	Chloroform : methanol 9.5 : 5		Diethyl ether : acetone 8.1:1.9	
Rf value	**Standard**	**Sample**	**Standard**	**Sample**
	0.7	0.71	0.74	0.78



Curcuma longa ***Azadirachta indica***

Fig 3: Pictorial representation of TLC plate scanned under the UV light

Formulation and development

Table 7: (Formulation trials with different concentration of Propylene Glycol and Tween 80)

Formulation	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**
Curcuma longa (gm)	0.75	0.75	0.75	0.75	0.75	0.75
Azadirachta indica(gm)	1	1	1	1	1	1
Carbopol 934	2%	2%	2%	2%	2%	2%
Propylene glycol	20%	15%	10%	20%	15%	10%
Tween 80	1%	1%	1%	1.5%	1.5%	1.5%
Propyl paraben	0.2%	0.2%	0.2%	0.2%	0.2%	0.2%
Methyl paraben	0.08%	0.08%	0.08%	0.08%	0.08%	0.08%
Triethanolamine	Quantity sufficient to pH5.5					
Lavender oil	0.1%					
Water	q.s. to 20ml					

Evaluation

Table 8: (Evaluation of Formulation trial batches)

Runs	Colour	Appearance	pH	Viscosity (cp)	Spreadability (gm.cm/min)	Drug content	
						Curcuma longa	Azadirachta indica
F1.	Orange	Translucent	5.8	18.452	24.21	96.4 ±1.25	57.12 ± 0.52
F2.	Orange	Translucent	5.5	19.26	24.01	87.25 ±0.08	82.79 ± 0.74
F3.	Dark orange	Translucent	6.2	24.123	24.69	90.14 ±1.15	87.71 ±1.04
F4.	Dark orange	Translucent	6.3	18.43	24.56	81.63 ±2.51	69.45±1.40
F5	orange	Translucent	5.3	20.17	23.74	92.97 ±0.81	89.17 ±0.70
F6	Orange	Opaque	5.0	14.32	21.24	98.63 ±2.51	72.45±1.49

In vitro antimicrobial activity

Disc Diffusion Method

The antibacterial activity of different formulations was determined by modified agar well diffusion method.

Fig 4: Zone of Inhibition of Final Formulation and Standard

Table 9: Zone of Inhibition of Final Formulation and Standard

Sample	Zone	Observation
Standard	56mm	Sensitive
Gel sample	32mm	Resistant

In vitro anti-inflammatory assay

Inhibition of albumin denaturation

Table 10: (Zone of Inhibition of Final Formulation at different concentration levels)

Concentration (ppm)	%inhibition	
	Formulation	Standard -Aspirin
20	18.43±0.08	51.41±0.01
40	25.71±0.05	54.15±0.015
60	38.60±0.05	64.79±0.04
80	51.84±0.18	69.91±0.071
100	61.56±0.47	77.14±0.036

Maximum inhibition of 61% was observed at 100 µg/ml. Aspirin, a standard anti-inflammation drug showed the maximum inhibition 77% at the concentration of 100µg/ml compared with control.

Stability Study: There was no significant change in any of evaluated parameter for the specified period of time. The drug content was also found to be within the specified limits with no significant change observed. Hence, it can be concluded that the formulation is stable at the specific conditions of temperature and humidity.

HPTLC

a) Identification

Table 11: Sample Application Layout

Track	Application position	Application volume	Vial	
1	15.0mm	2.0 µl	1	Standard
2	38.3mm	5.0 µl	1	Standard
3	61.6mm	2.0 µl	2	Sample
4	84.9mm	5.0 µl	2	Sample

Result

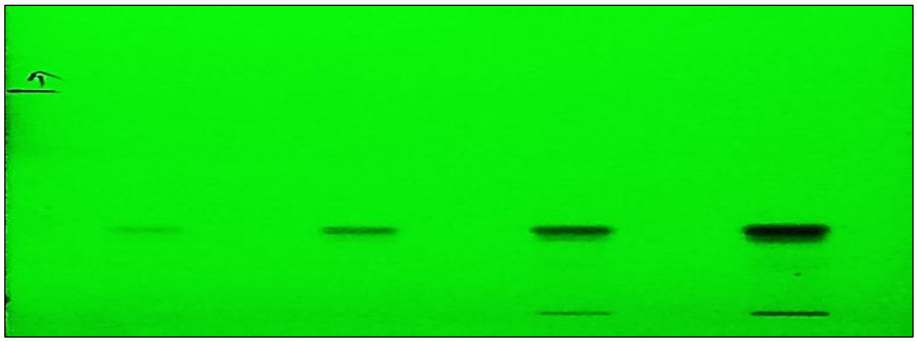


Fig 5: HPTLC analysis of Curcumin at 254nm

Chromatogram

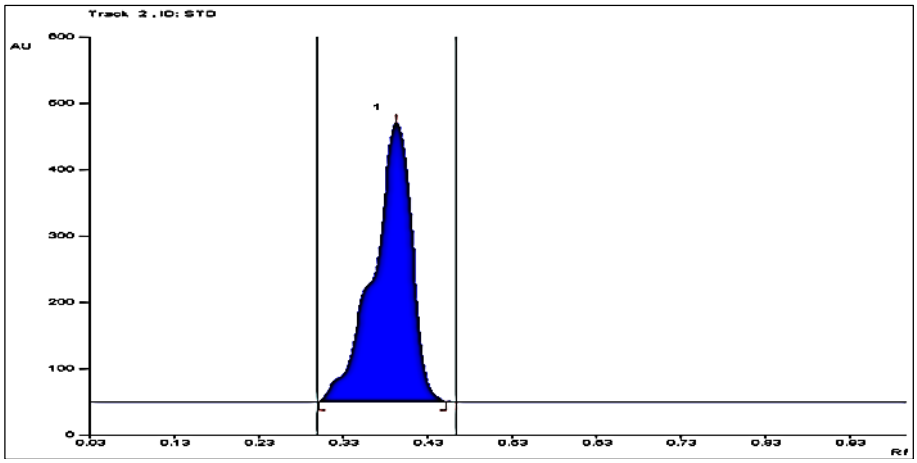


Fig 6: chromatogram of standard

Table 12: Chromatographic parameter

Peak	Start position	Start height	Max position	Max height	MAX %	End position	End height	Area	Area%
1	0.30Rf	0.7AU	0.04Rf	422.0AU	100.00%	0.45Rf	1.5AU	14172.9AU	100.0%

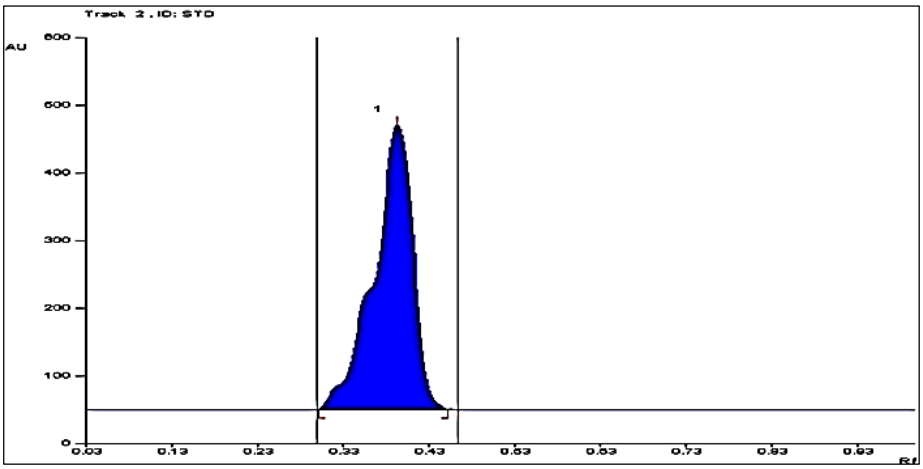


Fig 7: Chromatogram of sample

Table 13: Chromatographic parameter

Peak	Start position	Start height	Max position	Max height	MAX %	End position	End height	Area	Area%
1	0.30Rf	0.8AU	0.04Rf	566.8AU	100.00%	0.46Rf	0.3AU	22385.3AU	100.0%

Spectral data of all the tracks combined

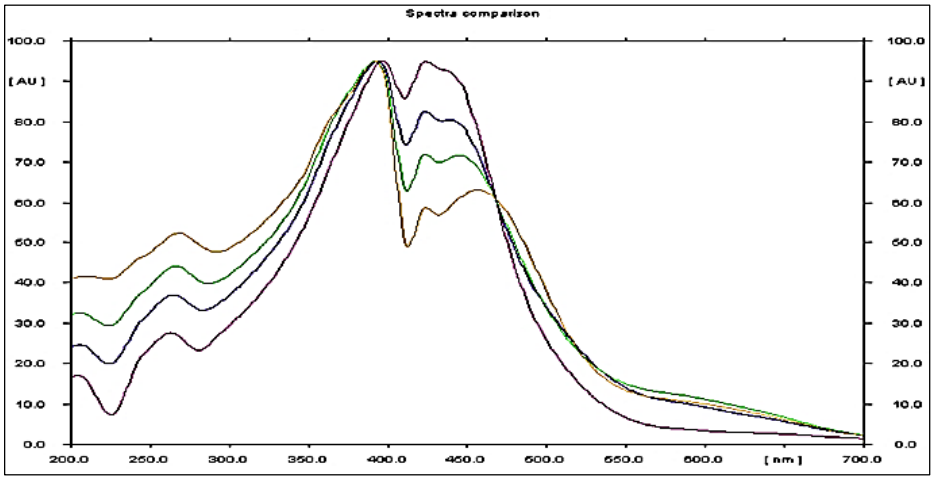


Fig 8: Spectral data of all the tracks combined

a) Inference

1. From the image and scan data it was confirmed that there is a band coinciding with that of standards rf in the sample.
2. From the Spectral analysis it was confirmed that the band in the sample and that of standard was of same as the pattern was matching completely. The Maximum absorbance was found to be 393nm
3. Thus the further analysis including quantification and method validation was performed with same wavelength 393nm.

b) Quantification

Table 14: Sample Application Layout

Track	Application position	Application volume	vial	
1	15.0mm	5.0 µl	1	Standard
2	29.0mm	5.0 µl	1	Standard
3	43.0mm	5.0 µl	1	Standard
4	57.0mm	2.0 µl	2	Sample
5	71.0mm	2.0 µl	2	Sample
6	85.0mm	2.0 µl	2	Sample

Result

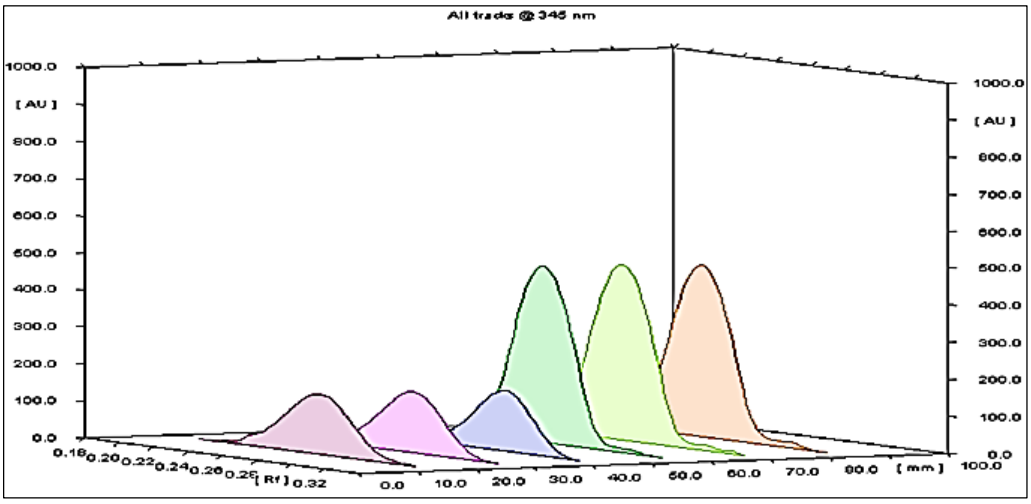


Fig 9: 3D data scan

Concenteration of the Standard Applied was 0.1mg/ml					
Thus 5µl was applied, hence On Plate Concenteration is 0.5µg					
Concenteration(µg)	Area		Area(Sample)	Unknown Concn	Average
0.5	13954.9		20855.4	0.75	
0.5	14116.6		21071.4	0.75	0.76µg
0.5	13377.2		21860.9	0.8	
Sample Concenteration was 10mg/ml					
Hence 2µl was applied then on plate concenteration is 20µg					
Hence 0.76µg of Curcumin is Present in 20µg of Sample					
Hence 3.8% is the Concenteration od Curcumin in the Formulation					

References

1. Perry AL, Lambert PA. Propionibacterium Acnes. Lett. Appl. Microbiol. 2006; 42(3):185-188. https://doi.org/10.1111/j.1472-765X.2006.01866.x.
2. Webster GF. The Pathophysiology and Principles of Acne Treatment *-. 2005; 3(7):228-233.
3. Kligman AM. Pathogenesis of Acne Vulgaris. Arch. Dermatol. 2011; 98(1):53. https://doi.org/10.1001/archderm.1968.01610130059013.
4. Khan N, Karodi R, Siddiqui A, Thube S, Rub R. Development of Anti-Acne Gel Formulation of Anthraquinones Rich Fraction from Rubia Cordifolia (Rubiaceae). Int. J Appl. Res. Nat. Prod. 2011; 4(4):28-36.
5. Pawar H. Phytochemical Evaluation and Curcumin Content Determination of Turmeric Rhizomes Collected From Bhandara District of Maharashtra (India). Med. Chem. (Los. Angeles). 2014; 4(8):588-591. https://doi.org/10.4172/2161-0444.1000198.
6. Nikam S. Anti-Acne Gel of Isotretinoin: Formulation and Evaluation. Asian J Pharm. Clin. Res. 2017; 10(11):257. https://doi.org/10.22159/ajpcr.2017.v10i11.19614.
7. Sinica DP, Awale KB, Shah RR, Kondawar MS, Birnale A, Sangli VC. Pelagia Research Library Preparation and Evaluation of Herbal Gel Containing Clerodendrum Serratum for Anti-Inflammatory Activity. 2014; 5(4):47-54.
8. MMV. Identification and Chemical Characterization of Azadirachta Indica Leaf Extracts through Thin Layer Chromatography. Int. J Res. Eng. Technol. 2016; 05(02):117-122. https://doi.org/10.15623/ijret.2016.0502021.
9. Tripathi YC. Chemical Constituents of Leaves of Azadirachta Indica, 2018, 1996.
10. Cebrián R, Arévalo S, Rubiño S, Arias-Santiago S, Rojo MD, Montalbán-López M *et al.* Control of Propionibacterium Acnes by Natural Antimicrobial

Substances: Role of the Bacteriocin AS-48 and Lysozyme. Sci. Rep. 2018; 8(1):1-11. https://doi.org/10.1038/s41598-018-29580-7.
11. Mishra H, Sharma K, Singhai S. Characterization of Azadirachtin from Ethanolic Extract of Leaves of Azadirachta Indica. 2012; 5(1):199-201.
12. Josephine Leno Jenita J, Madhusudhan NT, Wilso N, Manjula D, SBK. World Journal of Pharmaceutical Research formulation development and evaluation of. World J Pharm. Res. 2012; 1(2):207-215.
13. Gorle AM, Patil SS. Evaluation of Antioxidant and Antiacne Property of Rubia Cordifolia. 2010; 1(3):59-63.
14. Agnihotri S, Wakode S, Agnihotri A. Formulation and Evaluation of Herbal Antiacne Gel of Myrica Esculenta. Asian J Pharm Clin Res. 2016; 9(4):109-113.