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RESEARCH ARTICLE ON ANTI-BACTERIAL EFFICACY OF ETHANOLIC EXTRACT OF *THYME* AND *CINNAMON* IN TREATMENT OF *ACNE VULGARIS*

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ABSTRACT

In current scenario, Herbal Formulations are being treatment of choice, as Allopathy drugs posing harmful side effects and additionally, microbes developing resistance to Allopathy drugs. Hence, the development of herbal formulation is going to be essential for enhancing the patient's quality of life, safety and avoiding harmful side effects. In this study, we have explored anti-bacterial activity of ethanolic extract of *Thymus vulgaris* and *Cinnamomum verum* against *Propionibacterium acnes*.*Thymus vulgaris* and *Cinnamomum verum*were macerated with aqueous-ethanolic solution to get extracts.The formulated proniosomal gel by using surfactants, lecithin, cholesterol with extracts. Formulations were evaluated for its physicochemical parameters and other parameters like TEM, DLS, *in vitro* study, optical microscopy. The efficacy of Thyme and Cinnamon was determined by evaluating zone of inhibition against standard Clindamycin.The formulated proniosomal gel was found effective against the acne and it was able to kill acne causing bacteria (*P.acnes*) and provide controlled release upto 24 hours.

KEYWORDS: Proniosomes, Thyme, Cinnamon, Propionibacterium acne, Dynamic light scattering (DLS), Transmission electron Microscopy (TEM).

INTRODUCTION

Acne is the most prominent disorder among adolescents with 15-25 years of age. It begins prominently at puberty age. Preferentially, it occurs on facial portion and includes back, shoulders, chest as non-facial parts. As per the statistics, it affects population from almost every age group i.e., approximately 85% of young people aged between 12-25 years, 8% of adults aged between 23-34 and 3% of elders aged between 35-44 across the world. Major factors responsible for acne include exposure to sunlight, less intake of water, dietary habits and stress. Other causes include hormonal imbalance, excessive production of sebum in sebaceous glands, depression and anxiety. Acne is characterized by inflammatory, non inflammatory lesions, Sebocyte differentiation and proliferation. Occurrence of papules, pustules, open and closed Comedones. *Propionibacterium acne* present on the skin significantly promotes acne lesions. Treatments for acne comprise of topical gels, creams and oral dosages forms. Reduced preference for Anti-acne treatments pose side effects and hence, antibiotic resistance while herbal medicines overcome it Propionibacterium acne is the anaerobic gram positive bacteria responsible for acne vulgaris. The bacterium promotes inflammation along with chemotactic factors, lipolytic and proteolytic enzymes in acne. The enzymes due to hydrolytic action convert triglycerides present in the glands into free acids that aggravate inflammation and edema. This further leads to breakdown of the follicular wall. The major reasons behind inflammation are higher sebum production, release of pro-inflammatory mediators and activity of the bacteria.[11,43]

Pathogenesis

The major etiology of acne includes high levels of sebum production, sebocyte differentiation and inflammation by virtue of Propionibacterium acne. The anaerobic gram positive bacteria is typically present on normal skin flora but grows rapidly on the areas of skin which are preferably block and have high levels of sebum. This causes inflammation and converts triglycerides into fatty acids which are presents in the glands. The bacterium also promotes Proinflammatory mediators and cytokines.[14]

The pathogenesis of *acne vulgaris* associated with multifactorial process involves follicular hyperkeratinization, obstruction and increased sebum production. *P.acnes* binds to immune receptors and stimulates inflammation. Microcomedones are caused by obstruction in the follicular orifice due to follicular hyperkeratinization and deposition of keratinocytes. Comedones can be classified as open and closed Comedones. Open Comedones are visible and easily

removable while closed Comedones are invisible. Moreover, some factors increase expression of IL-1alpha, K16 and filaggrin in conjuction with follicular hyperkeratinization and are possible contributing factors.[7]

Proniosomes have several distinctive features over conventional dosage forms. The vesicles are amphiphilic in nature and are capable of entrapping both hydrophilic and hydrophobic drugs.They are prepared mainly with non-ionic surfactants than cationic, anionic and ampholytic surfactants as non-ionic surfactants have capability to increase solubility and bioavailability of poorly soluble drugs.

Thyme and cinnamon having anti-inflammatory properties. Cinnamon contains variety of compounds such as cinnamyl acetate, eugenol, L-boronyl, caryophyllene oxide, terpenolyne, pinene and cymene while thyme contains thymol, carvacrol, linalool, apigenin, eugenol and rosmarinic acid. These constituents having different properties like- antiseptic, antibacterial, anti fungal, anti-oxidant and anti-microbial.

In the present study the proniosomal gel was prepared separately for thyme and cinnamon (Drug) using coacervation phase separation method because of better extent of drug release, good entrapment efficiency and extraction of drug has been done by hydroalcoholic extraction method. Anti-microbial activity of thyme and cinnamon was tested against propionibacterium acne.To minimize the side effects of allopathic medicines and provide effective treatment for acne with fewer side effects

MATERIALS AND METHODS

Materials

Thyme and cinnamon was procured from Altitude Everydays (Certified Organic & Natural Products). Tween, cholesterol, DMSO was obtained from CDH chemical New Delhi.

Formulation design

Formulation design of proniosomal Gel (For both drugs (Thyme & Cinnamon).

Formulation	Drug(mg)	Ratio(C:L:S)	Cholesterol(mg)	Lecithin(mg)	Surfactant(mg)
F1	200	1:9:9	200	1800	1800
F2	200	1:4.5:4.5	200	900	900
F3	200	1:4.5:9	200	900	1800
F4	200	1:9:9	200	1800	1800

Thyme and cinnamon were used as potent drug in the formulation and both having anti-inflammatory and anti-microbial properties.

Methods

Preparation of Proniosomal Gel

The proniosomal gel was prepared separately for thyme and cinnamon (Drug) using coacervation phase separation method. Separate batches were prepared for both drugs. In the preparation of gel wide moth glass tube was used. Adding Drug (thyme and cinnamon) with surfactant, cholesterol and lecithin and they mixed with 500µL of absolute ethanol. Then opening of glass tube covered well to prevent loss of ethanol and tube was warmed in water bath at temperature 65ºC for 5min. After 5 min warming add 1.5ml of pH 7.4 phosphate buffer solutions was added and then this mixture again warmed for 2 min in the water bath due to this procedure clear solution was obtained. This niosomes dispersion was allowed to cool at room temperature until it converted to Gel. The obtained gel was stored in closed vessel.

Determination of MIC

Nutrient media was prepared with distilled water of prescribed quantity. The media was autoclaved at 15psi pressure at 121 degree Celsius for 15 minutes. Bacterial suspension was added to the sterilized media at suitable temperature.

The bacterial suspension inoculated freshly was added to test tubes in 1 ml of the quantity.

Dilutions of drugs .i.e., thyme and cinnamon were prepared in 10, 20, 30, 40, 50 µg/ml concentrations Dilutions of clindamycin, the standard drug, were prepared in a single concentration of 30µg/ml. 1 ml of above prepared concentrations of the three drugs weresubsequently added to the bacterial suspension prepared in triplicates The results for each drug were observed after 48 hours

Extraction of drugs

Hydroalcoholic Extraction Method

Dried powder of both the drugs extracted with hydroalcoholic extraction using organic solvent (petroleum ether). Desired Quantities of herbal drugs weighed and were individually added to the separate conical flask containing the ratio of 1:10 petroleum ether (solvent) content are allowed to kept for 36 hour at room temperature. After 36 hour contents (thyme and cinnamon) were filtered through eight layers of muslin cloth and after filteration samples were centrifuged at 5000rpm after centrifugation separated supernatant and put into the rotatary flask to evaporate the solvent and

collected the remaining sample in rotator flask. It should be kept in closed vessel.

Evaluation Method

Preparation of calibration Curve

The solubility of drug was checked and was found to be easily soluble in 2% ethanol.Stock solution of the drug was prepared in 2% ethanol of concentration 1mg per ml or say 1000mcg per ml. Subsequent dilutions were prepared with respective concentration 2, 4, 6, 8, 10 mcg per ml. To analyze the λ max of drugs in UV spectrophotometer, following the calibration of the equipment, the stock solution is scanned in 200-400 nm range. After obtaining the λ max of Thyme and Cinnamon, the above prepared dilutions are analyzed at the obtained λ max. Corresponding absorbance is plotted against the dilutions in Microsoft excel to obtain straight line equation of drug Thyme and Cinnamon in 2% ethanol and r value.

Evaluation of Proniosomal gel pH

The pH of the gel is measured through pH meter for each batch after incorporation of drug results are showed in table.

Drug entrapment efficiency

Accurately weighed 0.2 gm of proniosomal gel was taken in glass tube and 10 ml phosphate buffer (pH 7.4) was added. The aqueous suspension was sonicated for 10 min in a sonicator bath, followed by centrifugation at 9000rpm at 20ºC for 30 min. The supernatant was collected and assayed by UV method for un-entrapped thyme content at 239 nm. The percentage of drug encapsulation (EE %) was calculated by the following equation:

$$\text{Entrapment efficiency} = \frac{\text{Total amount of drug- un entrapped drug}}{\text{Total amount of drug}} * 100$$

Optical Microscopy

Optical microscopy was carried out to determine the morphological characters of proniosomal gel. Small amount of gel was placed on a cavity of glass slide and formation of vesicles was monitored at a magnification of 40X through an inverted microscope and for each slide of different formulation monitored and photographs were taken.

Dynamic light scattering (DLS)

Vesicular size distribution studies were evaluated by Dynamic light scattering method for both formulations with cinnamon and thyme. In this method 100 mg proniosomal gel was taken and mixed well with phosphate buffer (pH 7.4). This dispersion was sonicated for 10 min and samples were analyzed under DLS.

Rheology

Cone plate wells Brookfield Rheometer was used to determine the viscosity of gel. 0.5 gm gel was placed on the plates and viscosity of gel was determined at 25ºC with spindle speed of the viscometer rotated at 10 rpm .

Transmission electron Microscopy (TEM)

Thyme proniosomal gel was observed under TEM for its size distribution and surface morphology. A small amount of gel diluted with deionised water and mixed with glass rod then the proniosomal dispersion sonicated for 10 min.

A drop of diluted dispersion was placed to a carbon coated 400 mesh copper grid and is left for 1 min to settle on carbon substrate.Remaining sample on it removed by corner of filter paper, liquid get absorbed on filter paper.After that grid was twice rinsed with distilled water for 3-5 second and then a drop of 2% aqueous uranyl acetate is applied. Similarly remaining solution was adsorbed through the piece of filter paper and the sample is air dried.

In vitro Drug release

In vitro drug release study of thyme and cinnamon proniosomal gel were performed by using dialysis membrane with molecular weight cutoff of 10kDa. A volume containing a known amount of gel was placed in a dialysis bag, and both ends were tied to prevent any leakage. The bag was dipped into 200ml beaker of (pH 7.4) as release medium, and the medium was stirred continuously at 50 rpm, maintaining the temperature at 37º C ± 0.5º C. 5 ml of the sample was withdrawn at predetermined intervals (1,2,3,4,5,6,9,12,24) and immediately replaced with fresh medium. The samples were analyzed under UV spectrophotometer.

OBSERVATION

Phytochemical Screening- Thyme and cinnamon phytochemical screening test were performed which is showed in table 1.

Table 1: Results of Phytochemical screening of thyme and cinnamon.

Test	Thyme	Cinnamon
Alkaloids	-	_
Flavonoids	+	+
Fats and Fixed oils	+	+
Saponification	_	_
Triterpenoids and Steroids	+	+
Saponin	_	_
Glycosides	+	+
Tannins	+	+
Proteins	_	_
Carbohydrates	_	_

(+) Presence
(-) Absence

Optical microscopy

Proniosomal gel formulation present in the form of liquid crystalline state, under microscope this gel appeared spherical in shape.

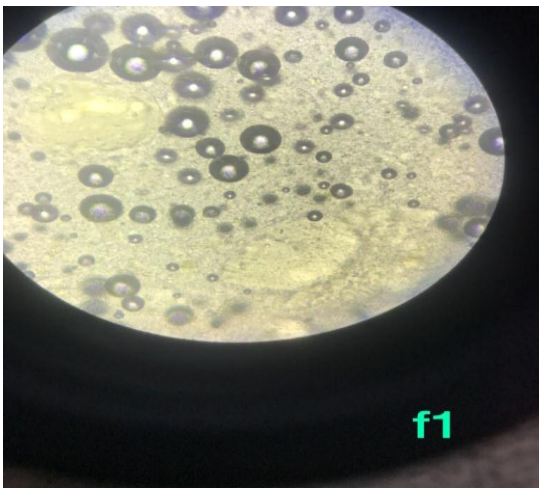


Figure 1: Proniosomes of thyme under optical microscope spherical in shape.

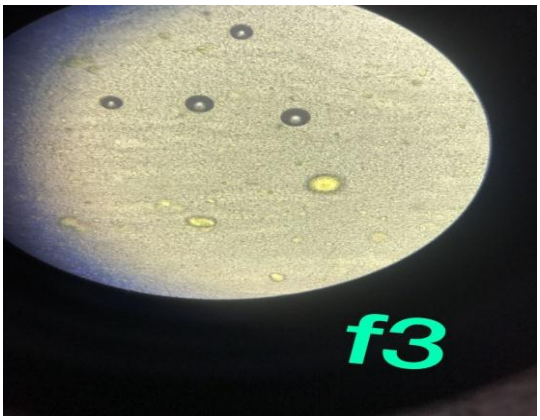


Figure 2: Proniosomes of cinnamon under optical microscope with uniform size range.

Transmission electron microscopy

Transmission electron microscopy images of best formulation of thyme and cinnamon prepared with Tween 20. Particle size of proniosomes may be a issue of prime importance. The surface morphology and size distribution of proniosomes were studied by TEM **(as shown in fig 3 & 4).**

The particle size ranged of thyme proniosomal gel was found to be 5.35-21.23nm and in cinnamon particle size ranged from 7.69-26.54nm.

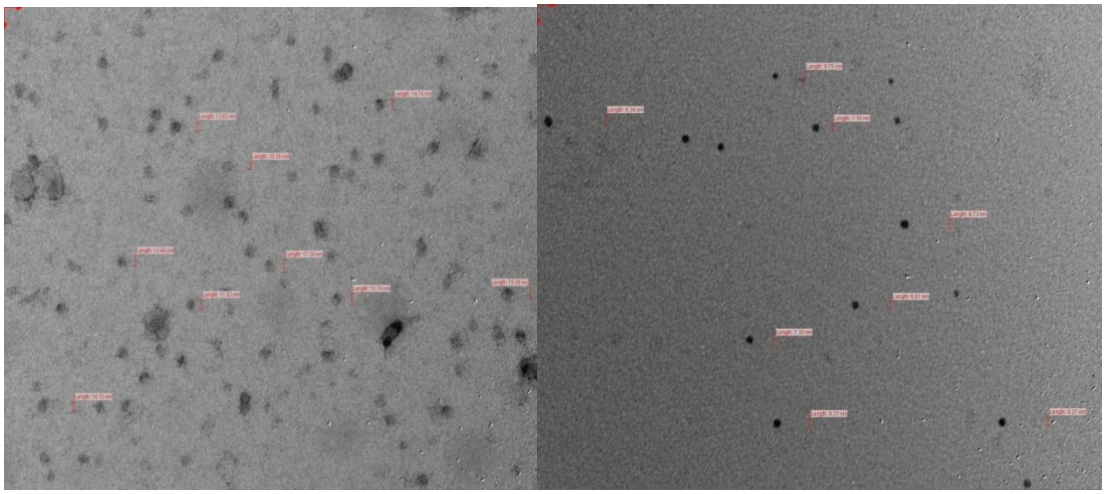


Fig. 3: TEM photographs of thyme loaded vesicular system prepared by Tween 20.

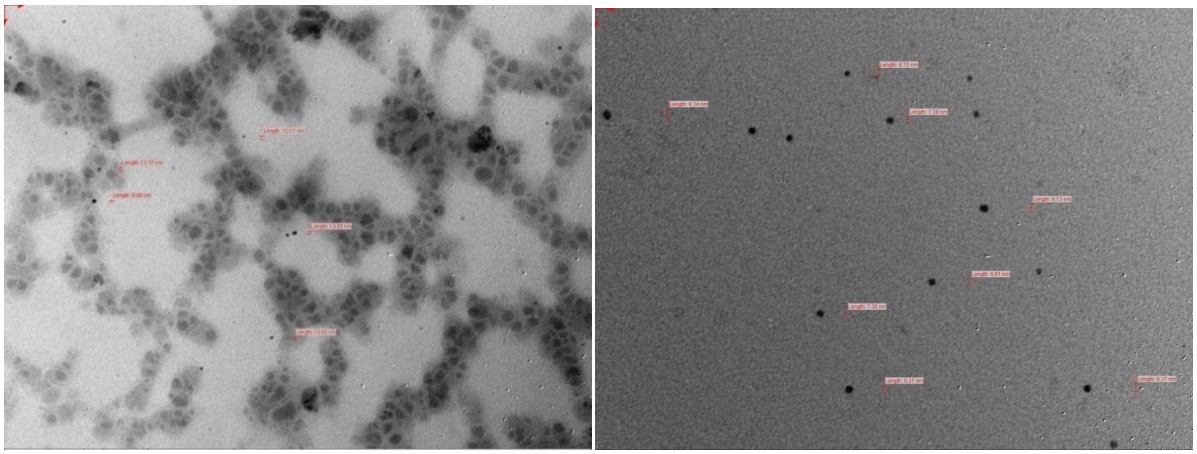
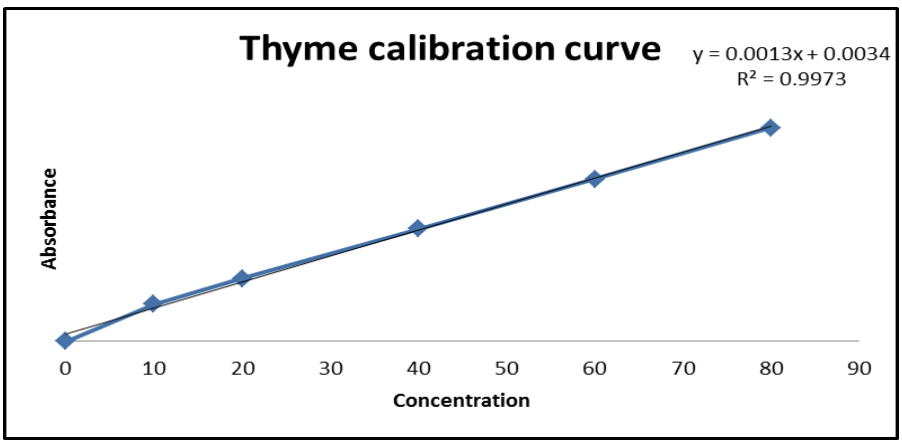


Fig. 4: TEM photographs of cinnamon loaded vesicular system prepared by Tween 20.

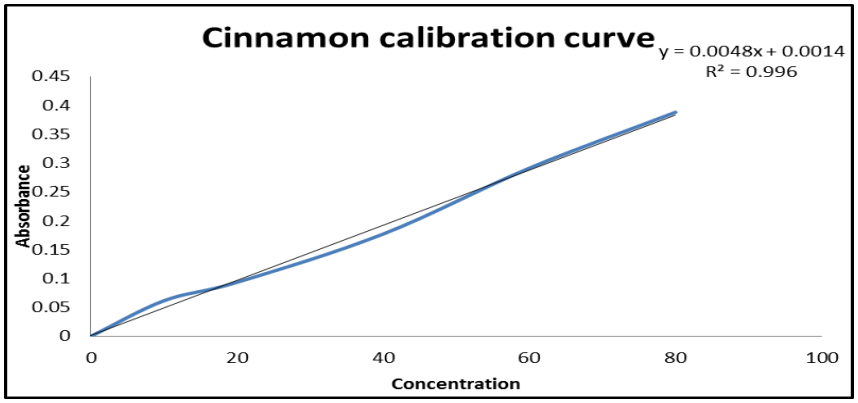
Calibration curve

The λ max of thyme and cinnamon in ethanol was determined to be at 239nm. The λ max was chosen 239 nm it was found as the wave length, where maximum absorbance peak was observed.

Table 5: Calibration curve for estimation thyme.



Graph 5: Standard curve of thyme.



Graph 6: Standard curve of cinnamon.

pH of proniosomal gel

Table 7: PH of thyme proniosomal gel.

Formulation	pH
F1	6.7
F2	6.6
F3	6.5
F4	7.2

Table 8:PH of Cinnamon proniosomal gel.

Formulation	pH
F1	6.9
F2	6.4
F3	6.3
F4	6.5

Dynamic light scattering (DLS)

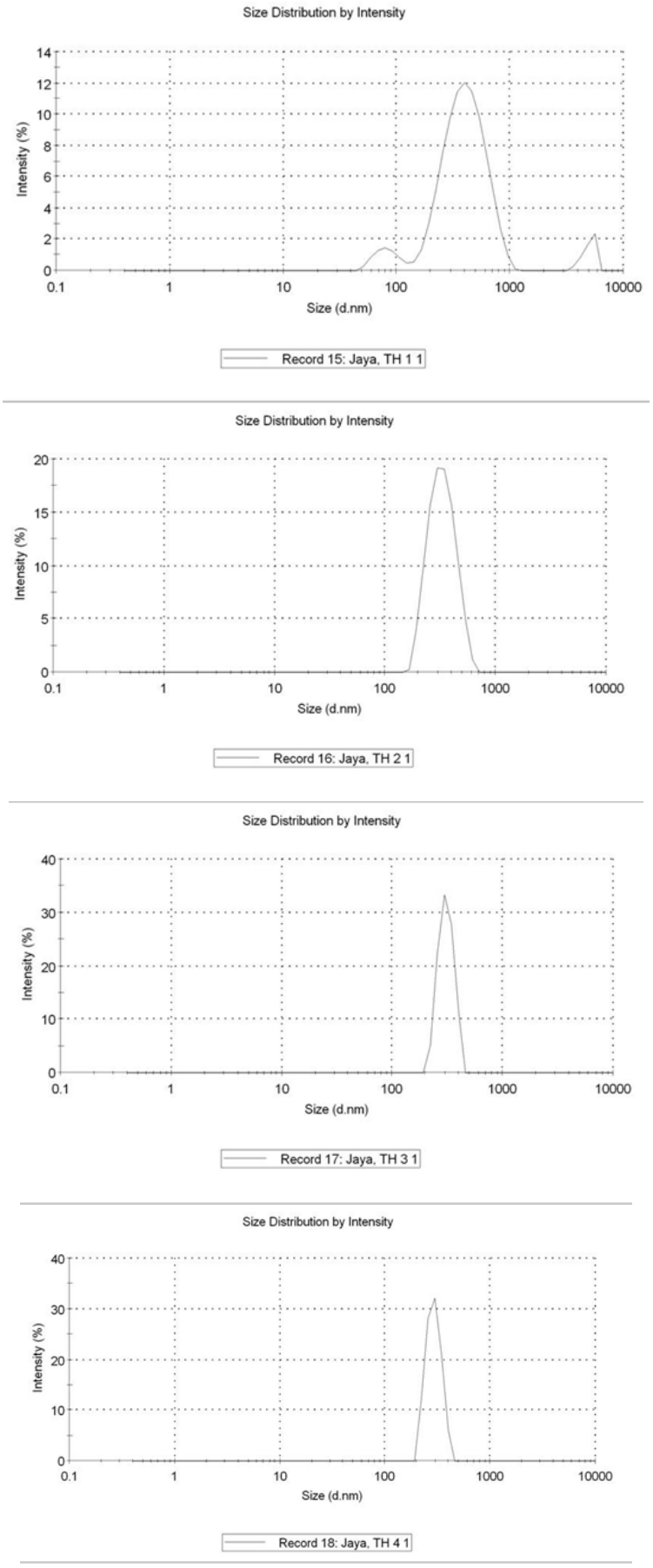
Dynamic light scattering were carried out with all the batches of proniosomal gel of both the drugs. Results of DLS shown in table 9 & 10.

Table 9: DLS Report of Thyme.

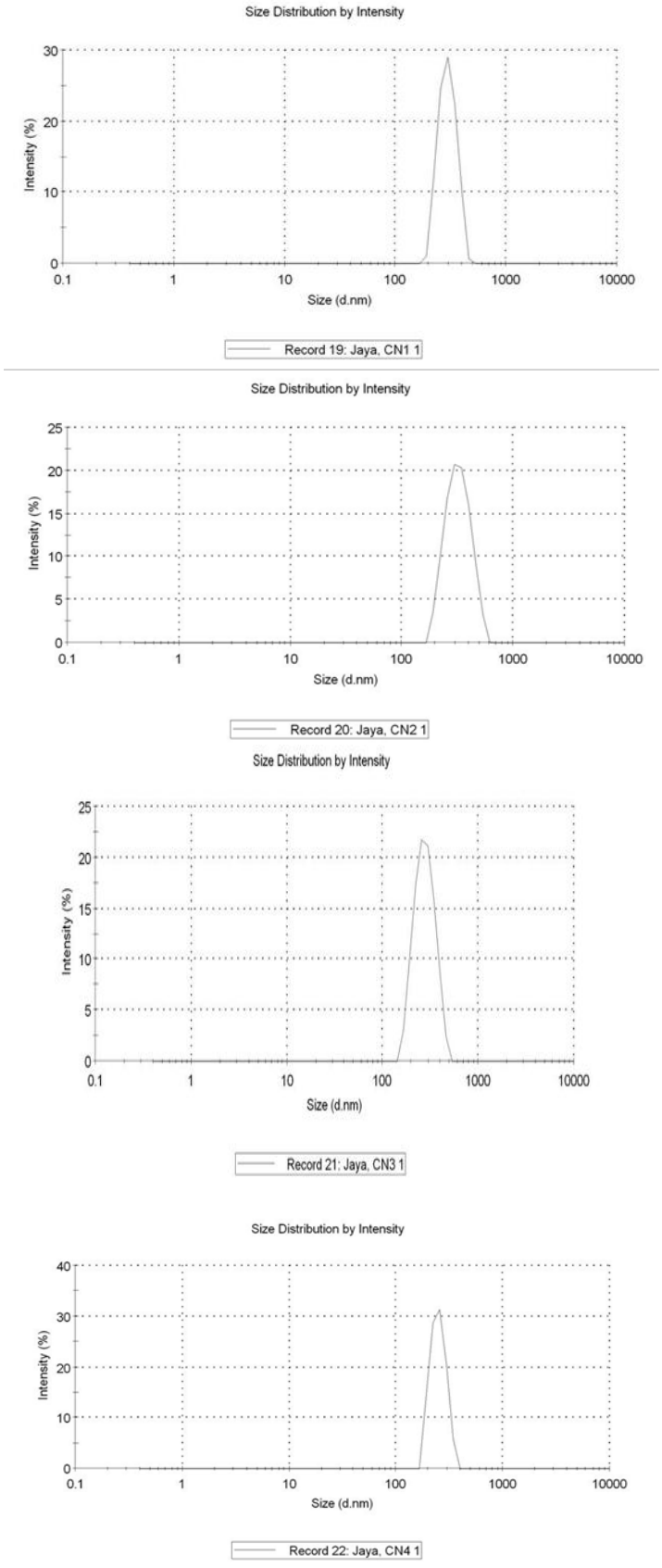
S. No	PDI(polydispersity index)	Vesicle size Diameter(nm)
1	0.439	421.0
2	0.111	333.8
3	0.770	307.0
4	0.632	290.8

Table 10: DLS Report of cinnamon.

S. No.	PDI (polydispersity index)	Vesicle size Diameter(nm)
1	0.223	296.9
2	0.139	325.8
3	0.070	279.4
4	0.439	249.3



Graph 6: DLS graph of thyme proniosomal gel showing particle size range.



Graph 7: DLS graph of cinnamon proniosomal gel showing particle size range.

Entrapment efficiency

Tween 20 was used as a surfactant in the formulation of thyme and cinnamon proniosomal gel.

The % Encapsulation efficiency of the formulations are listed below in Table 10 & 11.

Table 10: Percentage Encapsulation efficiency values of Thyme formulation.

Formulations	%Encapsulation Efficiency
F1	80.9
F2	63.9
F3	73.72
F4	79.1

Table 11: Percentage Encapsulation efficiency values of Cinnamon formulation.

Formulation	%Encapsulation Efficiency
F1	90.2
F2	90.5
F3	88.41
F4	86.4

Rheological properties

The viscosity of proniosomal gels were carried out at 20rpm. From the result it was observed that the gel formulation showed good spreadability and viscosity as shown table 12 & 13.

Table 12: Results of Viscosity of thyme proniosomal gel at 20rpm.

Formulations	Rpm	Viscosity (cps)
F1	**20**	**0.5432**
F2	**20**	**0.3452**
F3	**20**	**0.7005**
F4	**20**	**0.6784**

Table 13: Result of viscosity of cinnamon proniosomal gel at 20rpm.

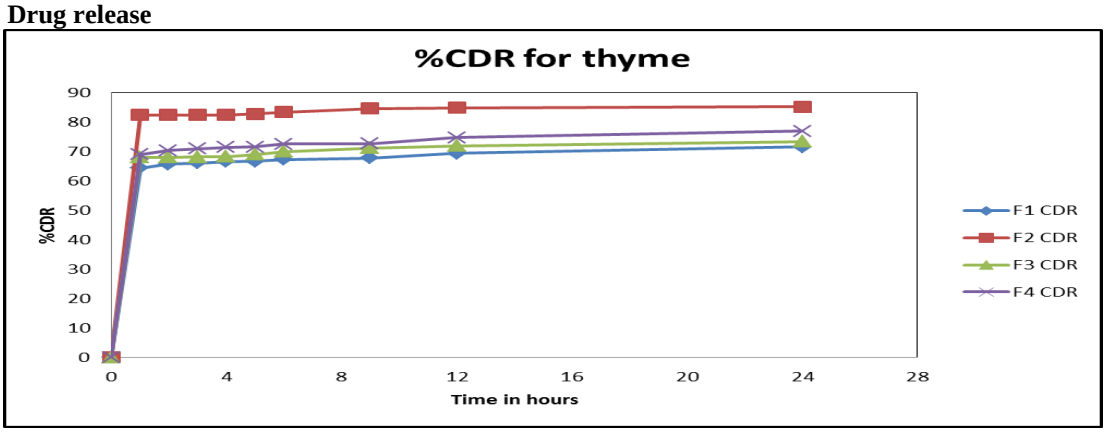
Formulations	Rpm	Viscosity
F1	**20**	**0.8148**
F2	**20**	**0.3605**
F3	**20**	**0.3456**
F4	**20**	**0.4356**

***In Vitro* Drug release**

Percentage drug release from the proniosomal gel was monitored within a time interval of 24 hours. The release data of thyme proniosomal gel was found to in the ranged from 71-85%. Figure- shows the cumulative amount of thyme transferred from proniosomal formulations to the receptor compartment (phosphate buffer pH 7.4). Formulation F2, containing the ratio (1:4.5:4.5) is showing more in vitro drug release i.e., 85.20% among all the formulations. The release rate is also dependent on membrane fluidity, as a function of either acyl chain length and saturation or cholesterol content. In cinnamon gel cumulative release was found to 66-88%. From the graph it was cleared that F2 formulation showed highest release i.e., 88.48% among all the formulations.

Table 14: Drug release data of thyme proniosomal gel in phosphate buffer pH7.4 upto 24hrs.

Time(hour)	F1 CDR	F2 CDR	F3 CDR	F4 CDR
0	0	0	0	0
1	64.42	82.38	67.98	69.06
2	65.68	82.40	68.07	70.34
3	66.04	82.43	68.18	70.96
4	66.62	82.48	68.26	71.45
5	66.70	82.74	69.05	71.54
6	67.35	83.40	69.99	72.56
9	67.74	84.61	71.18	72.65
12	69.40	84.85	71.88	74.78
24	71.61	85.20	73.44	76.98



Graph 8: In vitro % release of thyme with cholesterol: lecithin ratio. In order to treat the skin disorder, long treatment is required with extended release pattern.

Table 15: Drug release data of cinnamon proniosomal gel in phosphate buffer pH 7.4.

Time (hour)	F1 CDR	F2 CDR	F3 CDR	F4 CDR
0	0	0	0	0
1	72.4	85.97	74.14	64.59
2	72.60	86.06	74.45	64.71
3	72.79	86.19	74.79	64.87
4	73.14	86.35	74.94	65.00
5	73.35	86.51	74.04	65.39
6	73.69	86.78	75.39	65.61
9	73.98	87.65	75.52	65.89
12	74.45	88.22	75.95	66.28
24	75.04	88.48	76.38	66.54

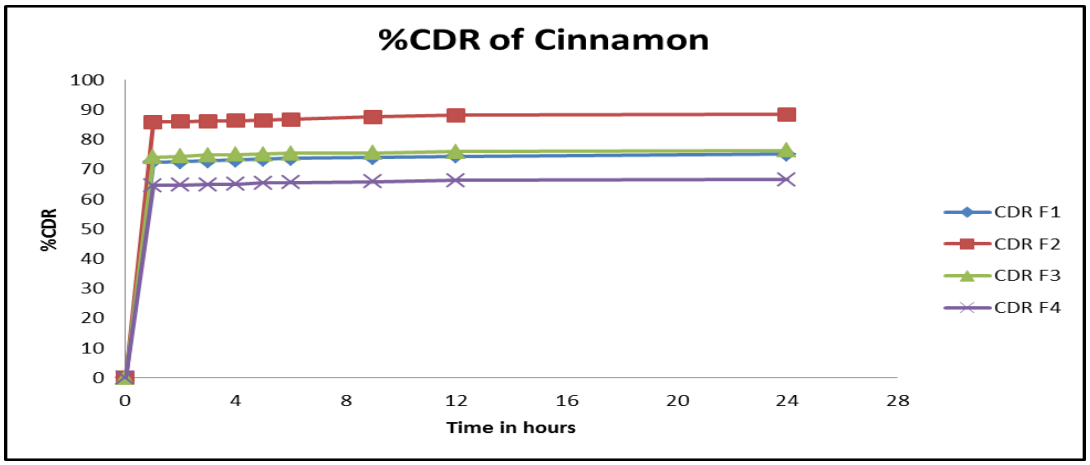


Figure 9: % Cumulative drug release of cinnamon proniosomal gel.

Antimicrobial study

Antimicrobial study were carried out with *P.acnes*using dilutions of both the drugs i.e. 10,20,30,40 & 50µg/ml and compared with standard drug Clindamycin (30µg/ml). Both the drugs are used in combination (1:1) also.

The MIC values of the *Thymus vulgaris* and *Cinnamomum Zeylanicum*extracts against the tested bacteria are shown in Table-- . The result showed that *T.vulgaris* and *C.Zeylanicum*gave significant MIC against P.acnes. So, these two plant extracts can be used for anti-acne topical formulation.

Table 16: Results of antimicrobial study of thyme and cinnamon using P.acnes.

S.NO	Drug	10µg/ml	20 µg/ml	30 µg/ml	40 µg/ml	50 µg/ml
1	**Thyme**	+	+	_	_	_
2	**Thyme**	+	+	_	_	_
3	**Thyme**	-	+	_	_	_
4	**Cinnamon**	+	+	_	_	_
5	**Cinnamon**	+	_	+	_	_
6	**Cinnamon**	+	+	_	_	_
7	**Combination (1:1)**	_	+	_	_	_
8	**Standard(clindamycin) 30 µg/ml**	+	+	+	-	-

(-) Turbidity absence

(+) Turbidity presence

RESULT AND DISCUSSION

Preformulation studies showed the absorption maxima for thyme and cinnamon at 239nm and phytochemical screening were carried out for both the drugs to determine the presence of chemical constituents. Antimicrobial study carried out with bacteria P.acnes to evaluate the potency of thyme and cinnamon as a antimicrobial agent. Proniosomal gels were prepared by coacervation Phase separation method. The formulations were studied for physical characterstics like determination of PH and rheological properties and found to be within the acceptable limits as indicated in results.

It was observed that the gel formulations showed good spreadability and viscosity. Determination of vesicle size

of thyme and cinnamon through DLS was found to be 290.8-421nm and 249.3-325.8nm respectively. Size was reduced when the dispersion was sonicated. Transmission electron microscopy was performed for surface morphology and size distribution and vesicle size of thyme and cinnamon was found to be 5.39-21.3nm and 7.69-26.54nm respectively. The Morphology of thyme and cinnamon proniosomal gel was studied using electron microscopy revealed that niosomes were spherical in shape as shown figure. In vitro diffusion studies of proniosomal gel showed cumulative percentage release for thyme is 71-85% and for cinnamon is 66-88% in 24 hour.

CONCLUSION

The above results indicated that the proniosomal gel could be formulated for controlled release of thyme and cinnamon for acne treatment. The formulations would be used for controlled release once a day.

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