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Formulation and evaluation of ointment for acne treatment

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Abstract

Acne is a common skin disease that occurs when hair follicles are clogged with dead skin cells or oil from the skin. Propionibacterium acne lives deep inside the cell that secrete digestive enzymes which damage the cell and trigger the inflammation. Manuka honey has anti-inflammatory qualities that helps to prevent redness and swelling. Tea tree oil is a natural alternative for curing acne. The phenol of gallic acid have anti-inflammatory, anti-fungal, antioxidant protection on skin cells against free radicals. Being bioflavonoid, Rutin used in cosmetics as an emollient. Curcumin has an antiseptic and pain-relieving property. This project aims to formulate and evaluate the antibacterial potential of ointment using herbal ingredient such as gallic acid, Rutin and curcumin. They were further subjected to *in vitro* turbidimetric assay test. The study was further extended to evaluate the minimal inhibitory concentration of formulated ointment.

Keywords: Propionibacterium acne; Gallic acid; Curcumin; Rutin; Ointment; Antibacterial activity; MIC

1. Introduction

The skin is the largest organ of the body almost covering 20 square feet. A good skin care is important to avoid acne. Blockage of sebaceous glands and colonisation with Proionobacterium acnes leads to acne. The site at which there is excess sebum, P. Acne grows unconditionally and block the skin pores. Propionibacterium acnes are present on our skin, when the favourable conditions exists, it can proliferates more rapidly and become a problem. This slow-growing bacterium feeds the sebum and produces an immune response which then leads to formation of skin inflammation and spots. Skin care regimen helps to remove excess oil, keeps pores clear, and can help speed healing of existing blemishes. Treatment of acne should be started as early as possible to minimise the risk of scarring and adverse psychological effects.

Acne Vulgaris is one of the most common skin disorders which when becomes severe it is better to consult the dermatologists to treat. It mainly affect adolescent, though may present at any age. Acne develops as there is blockage in the follicles. The naturally occurring Propionibacterium acnes can cause inflammation and as a result it also leaves a scar. Primary cause why people get acne is because of heredity and there are many other factors including lifestyle, stress and habits. Acne vulgaris is a common inflammatory skin condition. Although often perceived as a self-limited disease of adolescence, its prevalence remains high into. The factors which is involved in causing acnes: genetics, the menstrual cycle, anxiety and stress, hot and humid climates, using oil-based makeup, and squeezing pimples. No evidences are clear to reduce acne based on following diet. It can vary from mild to severe and can affect the skin of your face, back, shoulders and chest. Acne starts to develop when hair follicles in the skin get blocked with sebum and dead skin cells. It is characterized by blackheads or whiteheads, pimples, oily skin, and possible.

Topical preparations are the important therapy, and their actions helps in preventing the formation of new lesions.P.acnes are usually treated by consuming the Anti-biotics. Even though there are many types of acne treatment,

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the anti-acne ointments stay at the leading position which helps the skin to glow. The reason is fairly simple: they are easy to use and easy to obtain. Any milder forms of acne such as acne vulgaris, whiteheads, and blackheads perfectly respond to acne ointments.

It is common for acne creams or ointments containing benzoyl peroxide (bp) which causes a dry and stiff feeling of the skin. The topical agents available are benzoyl peroxide, antibiotics, azelaic acid, or retinoids. Benzoyl peroxide is bactericidal for P acnes and improves both inflammatory and non-inflammatory lesions. It is an oxidising agent that works by introducing oxygen into follicles, which then kills P acnes. Topical therapies, such as benzoyl peroxide (BP), retinoids and salicylic acid (SA), can cause skin irritation resulting in a lack of patient adherence. Dryness or skin irritation may cause barrier disruption of the stratum corneum leading to increased Trans-Epidermal Water Loss (TEWL) and production of inflammation.

Topical acne treatments, involving the ingredients particularly herbs and naturally derived compounds, have received considerable interest as they show less adverse effects than synthetic agents. Drugs derived from plants are used nowadays,since they are cheaper and safe and also have less side effects. Acne vulgaris drugs mostly possess adverse effects and therefore, medicinal plants might be considered as reliable sources for development of new drugs. Medicinal plants are also used for the treatment of acne. Phenolic compounds derived from plants have been shown to possess antibacterial activity.

Cetosteryl alcohol called as “wax” alcohol are fatty alcohols. They work as “emulsifiers” which helps to smoothen creams and ointments. They are used in ointments since they moisturize the skin. Fatty alcohols, which are absolutely non-irritating and can be exceptionally beneficial for skin. Works as an emollient, emulsifier, thickener and carrying agent for other ingredients contained in a cosmetic solution. It keeps the oil and water parts of an emulsion from separating, and gives products good spread ability. As a thickening agent and surfactant, it helps alter the viscosity and increase the foaming capacity of non-aqueous (i.e. lotions) and aqueous solutions (i.e. shampoo).

Liquid paraffin is a petroleum derivative that is also commonly defined as mineral oil. Liquid paraffin is often included in ointment formulations because it is believed to help the skin moisture. The skin has a natural barrier that prevents moisture loss and helps keep the skin supple. Liquid paraffin is sometimes formulated into skin care products to create a protective layer on the skin that helps retain hydration. Commonly, it creates a somewhat greasy, but smooth feeling on the skin, giving a sensation of moisture.

The objective of the study is to formulate ointment using Cetosteryl alcohol, liquid paraffin as base material along with natural ingredients such as Rutin, Gallic acid, Curcumin, Manuka honey and Tea tree oil for acne treatment. This study evaluates the anti-acne capacity of ointment with different formulations of above mentioned herbal ingredients. Knowledge developed from this study would be a good precursor for the application of natural ingredients for Acne treatment.

2. Material and methods

Poly Vinyl Alcohol (hot soluble), glycerol, xanthan gum, guar gum, ethanol, Cetosteryl alcohol, lauryl sodium sulphate, petroleum jelly, liquid paraffin, gallic acid, Rutin, curcumin, manuka honey, tea tree oil, nutrient agar, brain heart infusion broth, tetracycline, Propionibacterium acne (microbial suspension).

2.1. Collection and preservation of microbial suspension

The microbial suspension Propionibacterium acne was collected from MTTC (Microbial Type Culture Collection), Chandigarh, India. The collected microbial suspension was sub-cultured for further use. The glycerol stock for the culture was prepared for preservation method.

2.2. Preparation of biomaterial-based film using herbal ingredient

Poly Vinyl Alcohol (PVA) has good film forming and swelling properties. 7% of PVA(w/v) was dissolved in distilled water (v/v). To this mixture, herbal ingredients like Rutin, Curcumin and Gallic acid were added. Then, it was boiled in water bath at 70 °C for 6 hours. After it attains the homogenate form add 1% of glycerol which serves as a plasticizer. 7% of PVA (hot water soluble) were used to form a good film. The film was prepared in three different concentrations of 25%, 50% and 75%. 1.25 g of PVA and 1.25 mg of sample was added and boiled to mix the content.100 µl of 1% glycerol is added to the gel. Pour the gel into petri plates and allow it to dry. Remove the film from the plate when it dried out.

2.3. Disc diffusion method

It is the test of antibiotic sensitivity of bacteria which uses antibiotic disc. If the growth of the bacteria is stopped by the antibiotic, there remains an area on the wafer where bacteria do not grow. This is called the zone of inhibition. The fresh P.Acne culture suspensions were spread onto the nutrient agar plates. A disc of 6mm diameter was impregnated with the film containing extract. Tetracycline disk was used as a positive control. The plates are incubated at 37 ±2 ℃ for 24 hours. The zone of inhibition (ZOI) was measured in mm.

2.4. Turbidimetric assay

Turbidimetric measurement is a method of bacterial culture in which the bacteria is suspended in the liquid nutrient medium. The turbidity in the test tubes after incubation indicates growth of the desired bacteria. The turbidity of culture was measured using spectrophotometry at specific wavelength. Brain Heart Infusion broth were prepared and taken in test tube. Prepared stock culture(P.acne) was inoculated in all the tubes. The respective films were added to each tube and incubated overnight. Turbidity was measured and OD was estimated at 420 nm.

2.5. Ointment preparation

The composition that shown in the table 1 was weighed and melted using heating mantle at 65 ℃ for 5 minutes in a beaker. The constituents were stirred gently maintaining at temperature 65 ℃ for 5 minutes. To this sufficient quantity of ointment sample was added and mix well until a homogenous mass was obtained. Manuka Honey has the capacity to reduce redness and heals inflamed skin. The adequate amount of honey is added to the ointment sample. 250 µg/ml concentration of tree oil is added to the ointment sample. Tea tree oil provides excellent skin treatment for acne.

Table 1 Formulation of ointment

Emulsifying wax	**Amount**
Cetosteryl alcohol	1.8 g
sodium Lauryl Sulphate	0.2 g
Emulsifying ointment	2 g
Component	
Soft paraffin (petroleum jelly)	3.98 g
Liquid Paraffin	1.2 ml
Ointment sample	
Gallic acid	20 mg
Gallic acid and Rutin	20 mg
Gallic acid and Curcumin	20 mg
Honey	100 µg
Old tea tree oil	250 µg/ml

2.6. Well diffusion using spread plate method

Agar well diffusion method is widely used to evaluate an antimicrobial activity of product sample. The antimicrobial present in the product sample is allowed to diffuse out in the medium and interact in a plate freshly seeded with the test organism. The resulting zone of inhibition will be circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters.

- Nutrient Agar medium (100 ml): The medium was prepared by dissolving 2.8 g of commercially available nutrient agar in 100 ml of distilled water. The medium was sterilized by autoclaving at 15 lbs pressure at 121 ℃ for 15 minutes. The autoclaved medium was mixed well and poured onto the petri plates.
- Brain Heart Infusion broth (100 ml): 100 ml of broth was prepared by dissolving 3.7 g of brain heart infusion broth in distilled water. The prepared broth was sterilized by autoclaving at 15 lbs pressure at 121 ℃ for 15 minutes.

- Tetracycline disc (standard antimicrobial agent): Petri plates containing 20 ml medium (nutrient agar & brain heart infusion) was seeded with overnight culture of Propionibacterium acne. Well, was bored with help of sterile borer and 200 mg of ointment samples was loaded. The plates were incubated at 37 ℃ for 24 hours. Tetracycline was used as a positive control. The antimicrobial activity was assayed by measuring the diameter of inhibition zone formed around the well.

2.7. Dilution method (broth dilution)

Broth dilution testing provides both quantitative and qualitative results. It helps in establishing the level of resistance of a particular bacterial strain. Broth dilution can be done by two ways: macro dilution uses 1 ml volume of broth in standard test tubes whereas micro dilution uses 0.05 to 0.1 ml total broth and be performed in microliter plate.

2.8. Preparation of antibiotic stock solution

Antibiotic solution was prepared for formulated ointment. The amount needed and the diluent in which it dissolved was calculated. Prepare ointment stock solution at the concentration of 400 mg/ml.

2.9. Preparation of antibiotic dilution range

Use sterile test tube to conduct the test. 1ml of Brain heart infusion broth was added to 8 test tubes. Prepared stock (400 mg/ml) was added to the first tube to attain 200 mg/ml concentration. The samples were serially diluted to attain concentration from 200 mg/ml to 6.25 mg/ml. A minimum final volume of 1ml of each dilution is needed for the test.

Prepare the inoculum by making direct broth suspension of Propionibacterium acne colonies from an 18 to 24 hours agar plate. Dilute the inoculum suspension in broth after the 15 minutes of preparation. After inoculation each tube contain approximately 5 X 10^5 CFU/ml.

Within 15 minutes after the inoculum has been standardized, add 1ml of adjusted inoculum to each tube containing 1 ml of ointment in dilution series. Microbial suspension was used as a positive control and ointment stock was used as negative control. Incubate the inoculated tubes at 36 ± 2 ℃ for 20 to 24 hours in an incubator. The lowest concentration at which completed inhibited (absence of visible bacterial growth) is noted as minimum inhibitory concentration.

2.10. Turbidimetry assay

Turbidimetric methods for determining the potency of antibiotics are inherently more accurate and more precise than comparable agar diffusion. The relationships between test organism and antibiotics were discussed. 100 ml of broth was prepared by dissolving 3.7 g of brain heart infusion broth in distilled water. The prepared broth was sterilized by autoclaving at 15 lbs pressure at 121 ℃ for 15 minutes. Use sterile test tubes to conduct the assay. 4 ml of broth is added to each test tube. A loop full of microbial suspension was added to the tubes. 400 mg/ml concentration of ointment sample was added to each tube. Incubate the tubes at 37±2 ℃ at incubator for a period of time. For every 4 hours of incubation observe the readings of sample using UV spectrophotometer at 420 nm.

3. Results and discussion

The natural ingredient has a beneficial effect on traditional Indian system of medicine. Various compound present in the herbal extract are responsible for this therapeutic effect. Acne vulgaris is an infectious disease that may be inflammatory or non-inflammatory. Herbal medicines are gaining increased popularity due to their advantages, such as better patient tolerance, long history of use, fewer side effects and being less expensive. Mostly tropical treatments are used to treat acne. However the response of patients is considerably different. Gallic acid, curcumin and Rutin are used alone or in combination with synthetic drugs to cure acne. Most importantly, other than treatment remedy, they might be accompanied with synthetic drug to reduce their side effect. In this study, gallic acid is used alone or synergy of gallic acid with Rutin and curcumin are used to treat acne.

3.1. Disc diffusion

Disc diffusion method of the bacterial culture was carried out for different bioactive compounds as depicted in the table 2. The disc diffusion method gives an estimate of zone of inhibition in the P.acnes that shown (figure 1). The susceptibility of the film was estimated by measuring the minimum and maximum inhibitory concentration. Curcumin measured as a slow release compound in the P.acnes and Rutin measured as a response with a minimum inhibition in the culture.

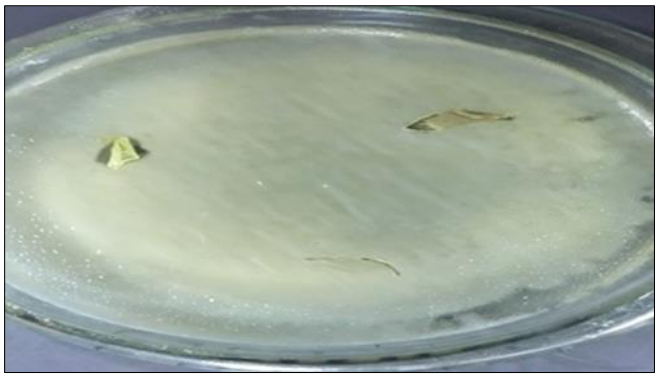


Figure 1 Antibacterial activity of formulated film against Propionibacterium acne

Table 2 Zone of inhibition for the antibacterial activity of formulation of film using bioactive compound

Bioactive compound film	With Glycerol (mm)	Without Glycerol (mm)
Rutin	1.56	1.39
Curcumin	0.8	0.62
Gallic acid	1.25	1.1

3.2. Turbidimetric assay

The turbidometric method estimates the number of bacteria in a liquid nutrient broth to measure the turbidity of a culture. Turbidity of the culture was measured at 420 nm, where the light passes through the medium and the cell numbers were calculated. The turbidity from different films is less compared to the control as depicted in the Table 3.

Table 3 Turbidimetric assays for formulated films

Formulated Films	Curcumin	Rutin	Gallic acid	Control
With Glycerol	1.25	1.1	1.22	1.35
Without Glycerol	1.32	1.17	1.25	

The film has good tensile strength and elasticity. By disc diffusion study, it was observed that, the drug diffusivity of film was very low. Thus, the formulation of peel able mask for acne was unsuccessful.

Hence, using same ingredients the ointment was formulated for acne therapy. The ointment was prepared by different formulation using manuka honey and tea tree oil. Manuka honey has the medicinal effect that acts as an anti-inflammatory and reduces the redness of skin. The old tea tree oil is used as a natural alternative for curing acne. In combination of tea tree oil with gallic acid exhibit the good result.

3.3. Evaluation of ointment

Table 4 Evaluation of ointment

Formulation	Color	pH
Gallic acid	whitish	6.75
Gallic acid + Rutin	Pale greenish	6.87
Gallic acid + Curcumin	yellowish	6.82

Three different ointments were formulated and physiochemical parameters such as color, odour and pH were evaluated. All the ointment has different characteristic odor and color that shown in the table 4. The pH of the ointment was range from 6.7 to 6.8. The antibacterial activity test showed that 12 mm zone of inhibition was obtained from the ointment prepared by synergy of gallic acid and Rutin. The other formulation did not show maximum inhibition.

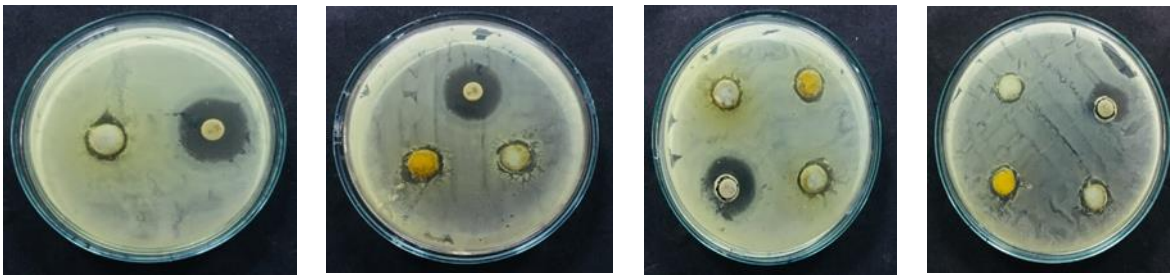
3.4. Antibacterial activity

The antibacterial potential of formulated ointment against Propionibacterium acne was determine using well diffusion method. The zone of inhibition for an entire ointment sample is showed in the table 5. It was observed that the zone of inhibition for all the sample was different and ranges from 6 to 12 mm. Maximum being for synergy of gallic acid with Rutin and minimum for synergy of gallic acid with curcumin using honey.

Table 5 Zone of inhibition for antibacterial activity of formulated ointment

Sample	**Zone of inhibition (mm)**			
	With honey	**Without honey**	**With tea tree oil**	**Control**
Gallic acid	8	10	11	14
Gallic acid + Rutin	9	12	`10	14
Gallic acid + curcumin	6	8	8	14

The antibacterial effect for prepared ointment is shown in fig 2. In general, gallic acid and Rutin showed the maximum antibacterial activity. The antibacterial activity of this sample due to presence of flavonoids and phenol. Flavonoids exhibit large number of biological activities of antibacterial, anti-inflammatory, antioxidant properties.



a B c d

Figure 2 Antibacterial activity for different type of formulated ointment. (A) ointment with gallic acid. (b) ointment with synergy of gallic acid & rutin and gallic acid & curcumin. (c) ointment with gallic acid, synergy of gallic acid & rutin, gallic acid & curcumin using honey. (d) ointment with gallic acid, synergy of gallic acid & rutin, gallic acid & curcumin using tea tree oil

3.5. Minimum inhibitory concentration test

The antibacterial activity of ointment against Propionibacterium acne was confirmed by minimum inhibitory concentration. The MIC test was carried out for the ointment that showed best antibacterial potential. The test was done in test tubes and the concentration ranges from 200 mg/ml to 6.25 mg/ml. The obtained result for MIC test was shown in the table 6.

From the table, positive sign represents the growth of microbes whereas negative sign indicates the inhibition of microbes. The best MIC 100 mg/ml was shown by gallic acid, synergy of gallic acid & Rutin, gallic acid& curcumin, gallic acid using honey, gallic acid, synergy of gallic acid & Rutin with tea tree oil when compared to others. From the study, gallic acid that showed good MIC was used as an active agent in the preparation of ointment.

Table 6 MIC of ointment sample against Propionibacterium acne

Tubes	Sample concentration (mg/ml)	A	B	C	D	E	F	G	H	I
1	200	-	-	-	+	+	-	-	-	+
2	100	-	-	-	-	+	+	-	-	+
3	50	+	-	+	+	+	+	+	+	+
4	25	+	+	+	+	+	+	+	+	+
5	12.5	+	+	+	+	+	+	+	+	+
6	6.25	+	+	+	+	+	+	+	+	+

A – Gallic acid, B – Gallic acid & Rutin, C – Gallic acid & Curcumin, D – Gallic acid using honey, E - Gallic acid & Rutin using honey, F – Gallic acid & Curcumin using honey, G – Gallic acid using tea tree oil, H – Gallic acid & Rutin using tea tree oil, I – Gallic acid & Curcumin using tea tree oil.

3.6. Turbidimetric assay

The turbidimetric assay was carried out for all types of formulated ointment. The reading was observed in UV spectrophotometer at 420 nm for an interval of time. The absorbance value was shown in the table 7.

Table 7 Turbidimetric assay test

TIME	ABSORBANCE (420 nm)			
	Control	Gallic acid	Gallic acid + Rutin	Gallic acid + Curcumin
0	1.58	1.34	1.42	1.53
4	1.65	1.54	1.58	1.6
8	1.72	1.57	1.52	1.65
12	1.79	1.62	1.59	1.73
24	1.84	1.65	1.62	1.8

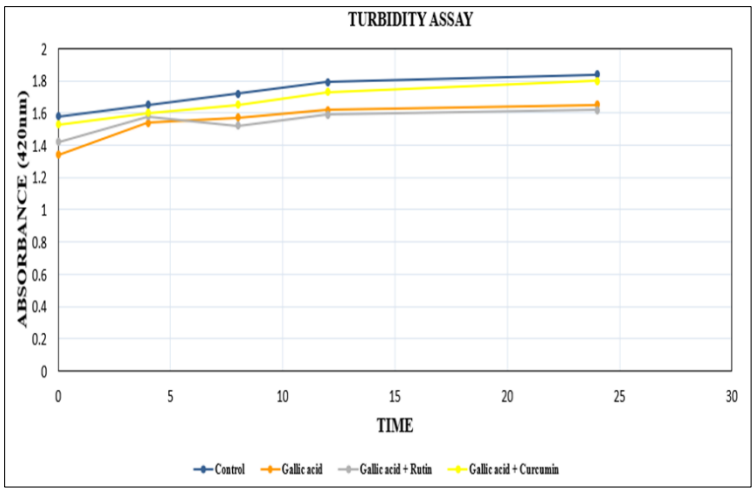


Figure 3 Turbidimetric assay test for different kind of formulated ointment

From the above shown figure, the growth rate of P.acne were gradually increased from 0th hour to 24th hours. The absorbance was noted at every four hours of interval. Comparatively, the gallic acid showed greater inhibition at zeroth hour. In comparison with growth rate of P.acne in control, synergy of gallic acid & Rutin exhibit the maximum inhibition after 24 hours of inoculation.

3.7. FTIR analysis

Fourier-transform infrared spectroscopy (FTIR) is a technique used to obtain an infrared spectrum of adsorption or emission of a solid, liquid or gas. It is an effective analytical instrument for detecting functional group and characterizing covalent bonding information. FTIR for gallic acid was analysed and generated peak were shown in the fig 6. Generally, the phenol group has an absorption ranges from 3600 to 3100 cm-1 due to hydrogen-bonded O-H stretch. The FTIR spectrum for gallic acid shows the following characteristic peaks: 3757.33cm^{-1}; 2850.79 cm^{-1}; 2324.22 cm^{-1};1797.66 cm^{-1}; 1462.04 cm^{-1}; 1377.17 cm^{-1}; 989.48 cm^{-1}; 721.38 cm^{-1} respectively. The 2850.79 cm^{-1} peak is specific to H-C-H asymmetric & symmetric frequency (CH2 stretching frequency). The peak having the value of 2324.22 cm^{-1} represent hydrogen-bonded O-H stretch. 1462.04 cm^{-1} peak correspond to the C-C=C stretch which is specific for aromatic rings. Similarly characteristics FTIR peaks of curcumin and rutin was observed and plotted using origin software.

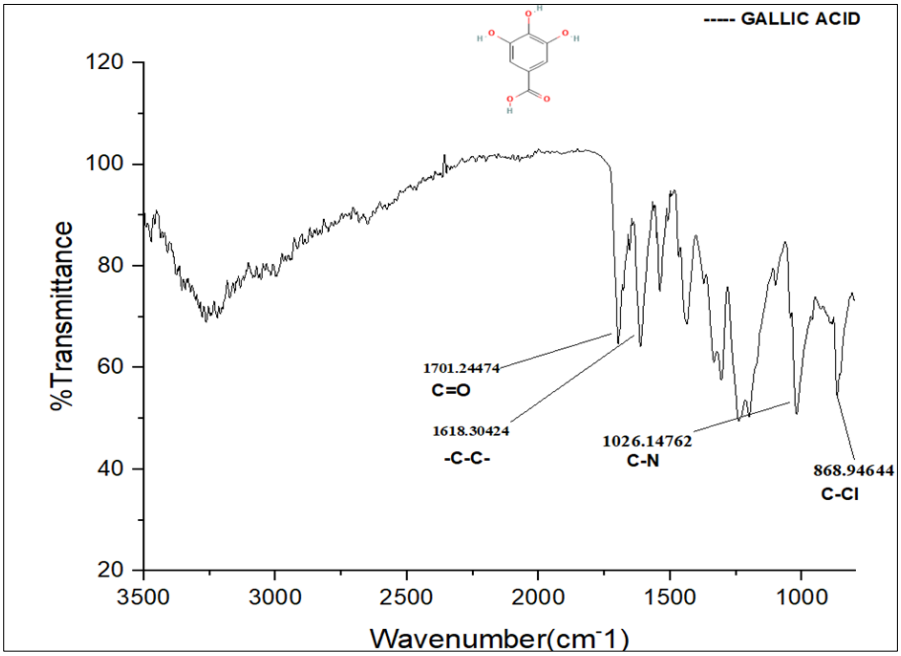


Figure 4 FTIR generated peak for gallic acid ointment sample

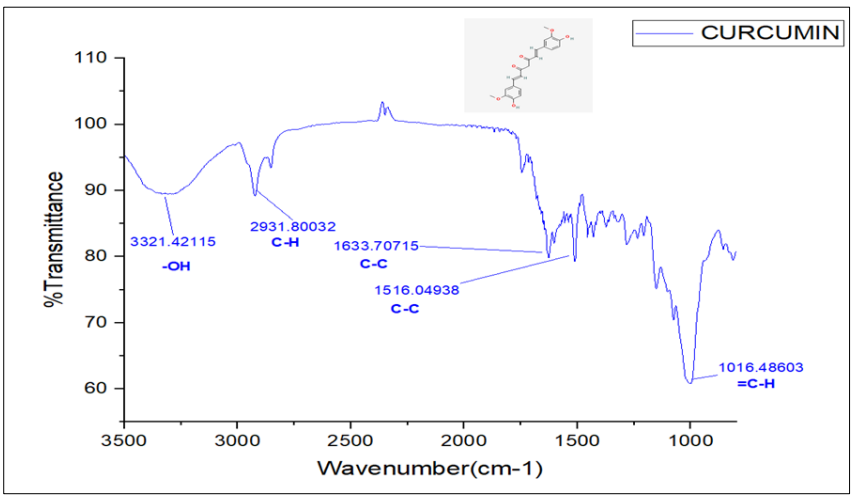


Figure 5 FTIR generated peak for curcumin ointment sample

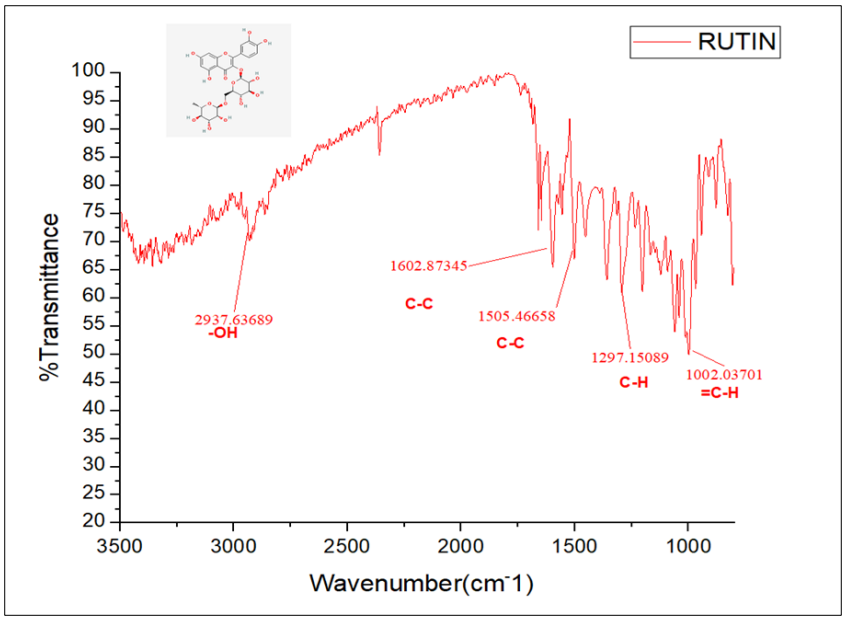


Figure 6 FTIR generated peak for rutin ointment sample

4. Conclusion

The ointment with synergy of gallic acid & Rutin formulation can be concluded as a effective one among the other two formulation. The gallic acid shows the maximum inhibition in following test such as antibacterial activity, minimum inhibitory concentration test and turbidimetric assay. The result of FTIR confirm the presence of phenols and flavonoids groups in the ointment. Antibacterial potential of gallic acid & Rutin may be because of flavonoid constituents present in it. Hence, gallic acid is a potent source of anti-acne and anti-inflammatory property. Being low economic cost of gallic acid, it could pave the way for cheap future medicine for acne treatment. Further concentration optimization studies with gallic acid and rutin will be performed in future for effective antibacterial and healing of acne scars.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest.

Author Contribution

Iswaryalakshmi Saravanabavan and Aarushi Pradeep carried out experiments of this research like gummy bear making ointment formulation; and contributed to writing, experimentation, result reporting and original draft preparation. Veerabhuvaneshwari Veerichetty participated in the analysis MIC test and conceptualization, methodology planning, research supervision and data analysis of results and manuscript preparation.

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