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# *In vitro and in vivo* assessment of polyherbal topical gel formulation for the treatment of acne vulgaris

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## Abstract

Anti-acne herbal formulations are used for the treatment of acne vulgaris with the added advantage of not producing adverse effects unlike synthetic drugs. Phytoconstituents present in methanolic extracts of *Camellia sinensis* (CSME), *Glycyrrhiza glabra* (GGME) and *Calendula officinalis* (COME) have antibacterial and antioxidant properties. Validated HPTLC fingerprinting confirmed the presence of myricetin (CSME), glycyrrhizin (GGME) and kaempferol (COME) in these extracts. Extracts loaded with carbopol® 940 were used for the preparation of herbal (F1-F3) and polyherbal gel (PHF), followed by evaluation for pH, viscosity, spreadability and *in vitro* antibacterial potential against *S. aureus*, *S. epidermidis* and *P.acnes*. Polyherbal gel formulation indicated antibacterial potential and was further assessed for skin permeation by gamma scintigraphy using hydrophilic radiotracer $^{99m}$Tc-DTPA and lipophilic radiotracer $^{99m}$Tc-MIBI. Significant permeation (78.11%) was observed with hydrophilic radiotracer labeled $^{99m}$Tc-DTPA-PHF and this suggested that the formulation was capable of sustained drug delivery for the treatment of moderate to severe type of acne.

**Keywords:** Acne, antimicrobial screening, gamma scintigraphy, HPTLC, polyherbal formulation

## Introduction

Acne vulgaris is a cutaneous disorder of multifactorial origin which manifests in the pilosebaceous follicle. It is characterized by open and closed comedones and inflammatory lesions like papules, pustules and nodules [1]. Micro-organisms like *Propionibacterium acnes*, *Staphylococcus aureus* and *Staphylococcus epidermidis* proliferate rapidly [2] leading to the development of acne. In clinical management of acne vulgaris, a considerable number of antibiotics and chemotherapeutic agents are available in the global market as topical or systemic treatment modalities [3]. Topical therapy is preferred as first-line treatment in mild acne whereas for moderate and severe type of acne, systemic therapy is required in addition to topical therapy. Herbal therapies on the other hand are gaining attention in comparison to existing formulations which cause enormous side effects like skin dryness, rashes, wrinkling, erythema, pruritis, skin eruption and development of resistance [4]. Several plants with antimicrobial and antioxidant activity such as *Ocimum gratissimum* [5], *Psidium* guajava [6], *Garcinia mangostana* [7] and *Humulus lupulus* L. were found to be effective [8] for prevention of acne vulgaris.

Our present study aims to explore medicinal plants for their anti-acne potential so as to bypass these side effects and to provide natural essence to the skin. Selection of common herbs namely *Camellia sinensis*, *Glycyrrhiza glabra* and *Calendula officinalis*, was based on findings that they possess many pharmacological attributes such as being antibacterial [9], antioxidant [10] and anti-inflammatory [11-13]. Green tea (*Camellia sinensis* Linn. Theaceae) contains large number of phytoconstituents like alkaloids (caffeine, theobromine, and theophylline), proteins, amino acids, minerals, sterols, enzymes, carbohydrates, lipids, phenolic acids, polyphenols, catechins, tannins and vitamins [14]. Green tea is a popular antioxidant because catechins present in it provide protection against diseases by contributing to the total antioxidant defense system along with antioxidant vitamins like C and E and enzymes like superoxide dismutase and catalase. Liquorice (*Glycyrrhiza glabra* Linn. Leguminosae) contains glycyrrhizin, glycyrrhizinic acid, glabrin A and B, glycyrrhetol, glabrolide, isoglabrolide, isoflavones, coumarins, triterpene sterols, liquiritin, isoliquiritin, flavones, chalcones and isoflavonoids such as glabridin [15]. It has been used as a demulcent, anti-tussive, laxative, sweetener, diuretic, antiarthritic, antibacterial [16], anti-

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inflammatory [12], anti-acne [17], aphrodisiac, antimitotic, estrogenic, antioxidant, antineoplastic, anticholinergic and antiulcer agent [18] in traditional systems of medicine. Various studies have demonstrated that liquorice blocks the activity of 3-β-hydroxysteroid dehydrogenase (3HSD), 17-hydroxysteroid dehydrogenase (17HSD) and 17-20 lyase. Interestingly, it stimulates the activity of aromatase. All these enzymes are also involved in the synthesis or metabolism of androgens and estrogens. It can also affect the ratio 5 /5β ring. 5 -reductase affects the conversion process of testosterone into dihydrotestosterone, which is the most active hormonal mediator involved in the pathogenesis of acne vulgaris [19]. Pot marigold (*Calendula officinalis* Linn. Asteraceae) contains triterpenoids namely ψ-taraxasterol, faradiol-3-*O*-palmitate and faradiol-3-*O*-myristate and exhibited remarkable anti-inflammatory effect against TPA- induced inflammation and croton oil induced mouse oedema [20]. Literature revealed that the extract of the petals was found to be more potent antioxidant than the flower head during lipid peroxidation assay and indicated that flavonoids were responsible for this action [10].

Among the skin care formulations, single-phase gel is extensively used for cosmetic products due to its aesthetic appearance [21].Moreover, organic macromolecules are uniformly distributed throughout a liquid in such a manner that no apparent boundaries exist between the dispersed macromolecules and the liquid [22]. An ideal formulation for acne should spread easily and leave minimal residue or oiliness as it is meant for large hairy surfaces like the chest and the back.Carbopol®940 used for the formulation is an excellent viscosity builder even at low concentration and does not support microbial growth. In addition, it provides good plastic flow properties with significant yield value. Propylene glycol is a water-miscible co-solvent for carbopol®940 and acts as a preservative, humectant, plasticizer or stabilizer in a variety of pharmaceutical formulations [23]. Its penetration enhancement capability has attributed to increased transdermal flux of many drugs [24].

In our previous work we had screened methanolic extracts for phytochemical, antibacterial and antioxidant potential [27, 32] .The main objective of the present study is to prepare and evaluate herbal gel formulations loaded with methanolic extracts of *Camellia sinensis*, *Glycyrrhiza glabra* and *Calendula officinalis* and to assess for *in vitro* antimicrobial and *in vivo* skin permeation using hydrophilic and lipophilic radiotracers by gamma scintigraphy. The results will be useful in designing specific, novel and effective herbal anti-acne formulation for cosmetic and dermatological application with the aim to prevent the adverse effects of existing non herbal formulations.

# Materials and Methods

## Materials

*Camellia sinensis*, *Glycyrrhiza glabra* and *Calendula officinalis* were collected from medicinal gardens and authorized herbal stores in New Delhi and authenticated by taxonomists of National Institute of Science Communication and Information Resources, New Delhi, India and a sample was submitted in the department for future reference (Authentication voucher no: NISCAIR/ RHM/consult/2008-09/978/09). Clindamycin phosphate (purity 98.58%) was obtained as gift sample from Sri Ram Institute of Industrial Research, New Delhi. Methanol (Thomas baker), Carbopol®940 (Hi-media), propylene glycol (Sd-fine), catechin, myricetin, glycyrrhizin, kaempferol (Sigma Aldrich Chemicals). Radiotracers $^{99m}$Tc-DTPA and $^{99m}$Tc-MIBI were procured from Regional Center for Radiopharmaceuticals (northern region), Board of Radiation and Isotope Technology (BRIT), Department of Atomic Energy, India. All the chemicals and reagents used in the study were of analytical grade. Double distilled water was used for all experiments.

## Methods

### Preparation and evaluation of extracts

Extraction of dried leaves of *Camellia sinensis*, roots and stolons of *Glycyrrhiza glabra* and flowers of *Calendula officinalis* was done by a continual hot extraction method, using methanol. Various phytochemical tests for alkaloids, carbohydrates, flavonoids, steroids, volatile oils, tannins, phenolic compounds, proteins and amino acids, saponins, aromatic acids, triterpenoids, gum and mucilage were performed for the extracts [25]. Methanolic extracts of *Camellia sinensis* (CSME), *Glycyrrhiza glabra* (GGME) and *Calendula officinalis* (COME) were tested for *in vitro* antibacterial activity against aerobic bacteria, *Staphylococcus aureus* (MTCC 96), *Staphylococcus epidermidis* (MTCC 2639) and anaerobic bacteria *Propionibacterium acnes* (MTCC *1951), using agar disc diffusion method [26, 27] followed by determination of minimum inhibitory concentration (MIC) [27, 28] and minimum bactericidal concentration (MBC) [27, 29]. Total polyphenolic content [30] and antioxidant potential of the extracts was assessed by DPPH antioxidant assay and compared with standard ascorbic acid [31, 32].

### Preparation of herbal gel formulations

Carbopol® 940 gel base was prepared by hydrating Carbopol® 940 (2%) in propylene glycol (30%) and distilled water q.s. (100%) for 24 h followed by stirring with double bladed mixer at 200 rpm for 10 min. Methanolic extracts (1%-5% w/w) were used for optimization to form stable gel formulations. Optimized formulations F1 (CSME), F2 (GGME), F3 (COME) and polyherbal gel (PHF) containing the mixture of these three extracts solution were subsequently mixed with carbopol gel base (control) using spatula until homogenous stable herbal gels were formed. Exact composition of the herbal gels (Patent application reference no: 688/DEL/2012) is not disclosed [33]. The gels were then transferred into clear glass vials and gel base served as negative control.

## Characterization of herbal gel formulations

## Determination of pH and viscosity

Herbal gel formulations were diluted with distilled water (1:10) and pH was measured using Lab India pH meter (model Pico).Viscosity of herbal gel formulations was determined using Brookfield Viscometer (Brookfield Engineering Laboratories, USA) with spindle # C 50-1 having a speed of 50 rpm . All the measurements were done in triplicate at room temperature [34].

## Determination of Spreadability

Spreadability (g.cm/sec) is expressed in terms of time taken in seconds by two slides to slip off from the gel placed between them, under certain load [35]. The standardized weight tied
on the upper plate was 20 g and length of the glass slide was 6 cm. The lesser the time taken for separation of the two slides, the better is the spreadability. The spreadability was calculated by using the following formula:
Spreadability = (Weight Length) / Time

## In vitro antimicrobial evaluation of gel formulations

Herbal gel formulations were tested for antibacterial activity against test organisms namely *S. aureus*, *S. epidermidis* and *P. acnes* using modified agar well diffusion method [36].
Aerobic bacteria: *Staphylococcus aureus* (MTCC 96), *Staphylococcus epidermidis* (MTCC 2639) and anaerobic bacteria: *Propionibacterium acnes* (MTCC *1951) were obtained from the Microbial Type Culture Collection Centre, Institute of Microbial Technology, Chandigarh. In this method, nutrient agar plates and reinforced clostridial agar (RCA) were seeded with 100µl of standardized bacterial suspension [$5x10^5$ CFU/ml]. After optimization of dose, 200 mg of gel dispersion was poured into the wells. Standard clindamycin (1% w/w) in-house gel (CLN) was used as positive control. The plates were then incubated at optimum temperature conditions and antibacterial activity was evaluated by measuring the diameter of zones of inhibition (mm) including cup size. The experiments were repeated three times.

## Estimation of phytoconstituents in PHF using validated HPTLC method

Phytoconstituents were estimated by spotting 5 µl of (10 mg/ml MeOH) markers (catechin, myricetin, glycyrrhizin, and kaempferol) and PHF (100 mg/ml MeOH) on pre-coated TLC plates of silica gel 60GF254 of 10 x10 cm size (Merck KGA, Germany). Chromatogram was developed in Camag Twin Trough glass chamber of 10 x 10 cm with stainless steel lid equilibrated with optimized mobile phase. Application rate was maintained at 10 µl/min using Linomate-V applicator (automatic TLC applicator, Camag, Switzerland) [37]. Chromatographic plates were air-dried, observed under UV chamber (Camag UV chamber-3, model no. 022.9120) and scanned using densitometer at 254 nm (Camag TLC Scanner-3, model No. 027.6480). $R_f$ values and the percentage of the phytoconstituents present in PHF gel formulation were determined using the formula:
[% w/w = Sample Area x std wt x sample dilution x potency / Standard Area x std dilution x sample wt]

## In vivo permeation studies of polyherbal gel formulation

Healthy, young, male New Zealand white rabbits weighing 2.5-3.0 kg were used for this study with prior approval of study protocol (INM/IAEC/2010/07/007) from Institutional Animal Ethical Committee of Institute of Nuclear Medicine and Allied Sciences (INMAS), Delhi. The rabbits were not allowed to consume food during the study period or during the preceding 24 hours. The dorsal area of the trunk was shaved with clippers 24 h before the beginning of the activity. Six squares of 1.5 inches were drawn on back of rabbit, and the skin was scarred with a lancet. Three healthy rabbits were used at each time interval. For assessment of *in vivo* skin permeation of PHF, rabbits were subjected to gamma scintigraphy studies. Hydrophilic radiotracer $^{99m}$ Tc-DTPA (diethylenetriamine pentaacetic acid) and lipophilic radiotracer $^{99m}$ Tc-MIBI (methoxy isobutyl isonitrile) were procured from Regional Centre for Radiopharmaceuticals (northern region), Board of Radiation and Isotope Technology (BRIT), Bhabha Atomic Research Centre (BARC), Mumbai, India. The radiolabeled [38] PHF gel formulation (200 mg) was applied to the skin of rabbits and static images (180 sec/image) during pre and post wash of topical applications of PHF at different time intervals (2, 4 and 24h) were taken using SPECT-CT, Symbia (Siemens, USA). Pre-wash and post-wash radioactivity was measured in kilo counts per seconds (Kcts/sec).
Scintigraphy images of pre-wash and post-wash were analyzed using region of interest (ROI) with software to quantify net activity of permeation of gel through skin using the formula: Percentage permeation = (100 x post-wash counts) / pre-wash counts.

## Statistical Analysis

The data was subjected to ANOVA using the SPSS software version 10.1. A confidence limit of P 0.05 was fixed for interpretation of the results.

# Results and Discussion

In recent years, during treatment of acne vulgaris, emphasis has been given to develop a formulation which can be applied directly to the lesions without causing side effects.
Hence medicinal plant extracts with antibacterial and antioxidant potential were utilized for the development of topical gel formulation and assessment of *in vivo* skin permeation was the main approach of this study

## Evaluation of extracts

Methanolic extract of *Glycyrrhiza glabra* indicated the highest percentage yield (20.11) and phytochemical screening confirmed the presence of the following constituents: (alkaloids, flavonoids, terpenoids and tannins) in CSME, (carbohydrate, glycosides, flavonoids, saponins, terpenes and sterol) in GGME and (flavonoids, saponins and terpenoids) in COME. Although there is lack of literature evidence for antimicrobial activity of these medicinal plants against acne causing bacteria but in our study, *in vitro* antimicrobial screening using clindamycin phosphate as a positive control clearly indicated that CSME, GGME and COME show promising antimicrobial activity against test organisms. Highest zone of inhibition (17.8 ± 0.016 mm) was observed for CSME against *S. epidermidis.* The lowest MICs against *S. epidermidis* (0.625 mg/ml), *S. aureus* (1.25 mg/ml) and *P. acnes* (1.25 mg/ml) were recorded in CSME. Similarly lowest MBCs against *S. aureus* (2.5 mg/ml), *S. epidermidis* (2.5 mg/ml) and *P. acnes* (2.5 mg/ml) were also observed in CSME [27]. In recent years, during treatment of acne vulgaris, emphasis has been given to toxicity related with oxidative stress because the rate of generation of ROS is found to be more than the rate of its removal. Free radical scavenging activity of methanolic extracts of *C. sinensis*, *G. glabra* and *C. officinalis* was determined when DPPH radical was used as a substrate and significant DPPH radical scavenging activity ($IC_{50}$ = 44.03 ± 1.784 µg/ml) was observed in CSME followed by GGME ($IC_{50}$ = 51.07 ± 3.050 µg/ml) and COME ($IC_{50}$ = 111.96 ± 1.129 µg/ml). Antioxidant properties of medicinal plants have been shown to be due to the presence of high content of phenolic compounds [32, 39].To support this fact, total phenolic content was assessed in terms of mg GAE/g and appreciably high amount (78.94 ± 6.40 mg GAE/g) was observed [32]. The assessment of *in vitro* antibacterial and antioxidant properties confirmed that these plants can be a good natural source in the treatment of acne. Hence topical herbal gels were prepared and assessed for *in vitro* antibacterial and *in vivo* skin permeation studies.

## Characterization of herbal gel formulations

The topical approach is effective because the formulation is applied directly to the lesions and the medicinal plants are less likely to cause side effects. Herbal gel formulations were assessed for homogeneity, pH, viscosity, spreadability and *in vitro* antimicrobial activity. The visual inspection indicated that there were no lumps in the formulation and pH of gel formulations was found near to the pH value of skin which clearly indicated that the formulations were compatible with skin and were unlikely to exert any pH effects on pH-sensitive human skin (Table 1). The intrinsic viscosity for PHF was 6535±61 cp with spreadability value of 8.2±0.11 g.cm/sec.

## In vitro antimicrobial evaluation of herbal gel formulations

Agar well diffusion method was used for screening the antimicrobial potential of herbal gel formulations. The results clearly indicated that amongst herbal and polyherbal formulations, PHF showed promising synergistic antibacterial activity against all the three test strains. Highest zone of inhibition was observed against *S.epidermidis* (18.47±0.222 mm) followed by *P.acnes* (16.87±0.294 mm). Lowest zone of inhibition was observed against *S. aureus* (16.13±0.180 mm) (Figure 1). Data was found to be significant ($P < 0.001$) when compared with control. Presence of epigallocatechin gallate and epicatechin in *C. sinensis* is generally believed to be responsible for the bactericidal action [40]. Antibacterial and anti-inflammatory effect of *G. glabra* is attributed to the presence of glycyrrhizin and its hydrolysis product, glycyrrhetinic acid [12]. It has also been reported that triterpenoids, flavonoids and kaempferol in *C. officinalis* are known to provide anti-inflammatory activity. Furthermore, antibacterial activity against *P. acnes* is believed to be due to the esters of faradiol-3-myristic acid, faradiol-3-palmitic acid and 4-taraxasterol present in *C. officinalis* [41].

## HPTLC analysis of polyherbal gel formulation

HPTLC fingerprinting was done after optimization of mobile phase for confirming the presence of active phytoconstituents responsible for antimicrobial action. HPTLC analysis enabled monitoring of densitograms of PHF at various wavelengths. Results clearly indicated that in HPTLC densitograms there were many peaks which were not detectable but the peaks of important phytoconstituents responsible for anti-acne were well resolved and supported the antimicrobial activity data of these extracts against acne causing organisms. Rf values confirming the presence of catechin, myricetin, glycyrrhizin and kaempferol in PHF, were recorded and percentage content of the identified phytoconstituents was calculated (Table 2).

## In vivo skin permeation

Development of new drug formulation always requires extensive studies in terms of *in vivo* evaluation. Gamma scintigraphy provides rapid and complementary information that often cannot be obtained by other methodologies. It is very important to choose the proper radionuclide. $^{99m}$Tc (technetium) is used very often as this has optimal characteristics of half-life (6 h) and allows images to be obtained with high efficiency at low doses [42]. It emits gamma rays which have relatively low energy as compared to and β rays and so it leads to no serious health hazards to the workers. Furthermore, incorporation of hydrophilic and lipophilic radiotracers into drug formulations enables the determination of permeation of topical gels [43]. It has been reported that stratum corneum is a major barrier for penetration of topical antibiotics such as clindamycin, into and through the skin. Generally, sebaceous glands release sebum into follicular canal creating lipoidal environment and restrict the follicular penetration of clindamycin phosphate [44]. In this present study, it was observed that $^{99m}$Tc-MIBI labeled PHF formulation showed constant increase in percentage permeation (Table 3) whereas $^{99m}$Tc-DTPA labeled PHF formulation initially decreased percentage permeation and later showed a significant rise (Figure 2). This could be due to the

fact that initially skin could not recognize the new molecule because of hydrophilicity and after 4 h once the new molecule got recognized, the skin responded very well and showed 78.11% permeation (Figure 3). PHF gel contained three methanolic extracts having polar fraction
which indicated that hydrophilic components were exhibiting better permeation in subcutaneous tissue which in turn increased their concentration in blood.
It is likely that the cosolvent propylene glycol was able to increase the concentration of both the permeant and the enhancer in stratum corneum [45]. Results clearly indicated that topical polyherbal gel formulation could be potentially effective in the treatment of acne, due to continuous release for a longer time. Though both the labeled formulations showed different permeation patterns but higher percentage permeation was observed with gel formulation labeled with hydrophilic radiotracer $^{99m}$Tc-DTPA.

**Table 1: pH, viscosity and spreadability of herbal gel formulations**

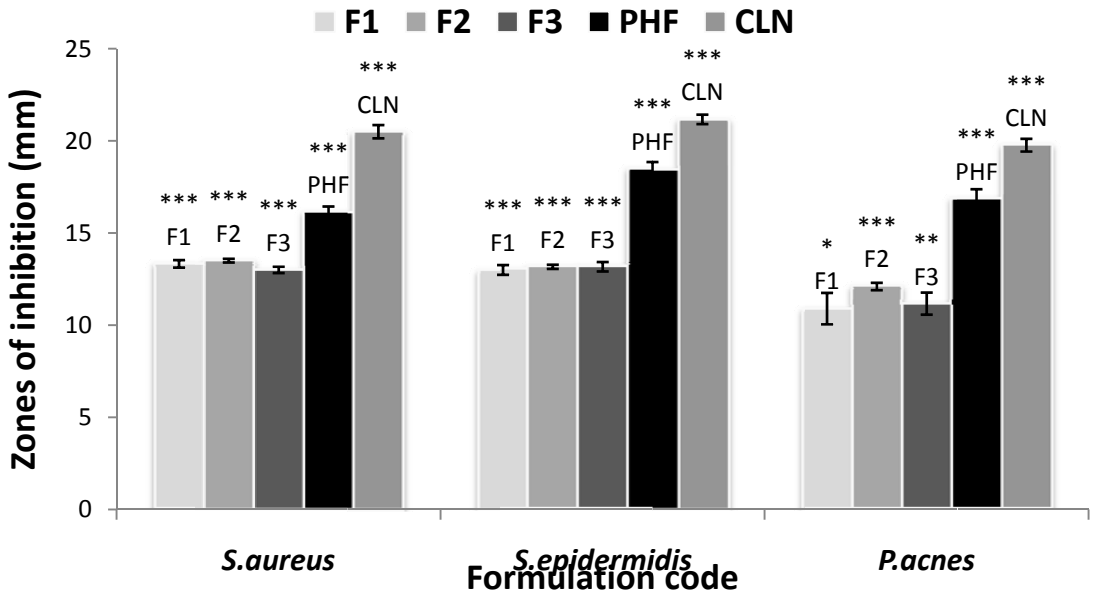
| Formulation Code | pH±SD (n=3) | Viscosity (cp)±SD (n=3) | Spreadibility±SD (n=3) (g.cm/sec) |
|---|---|---|---|
| F 1 | 5.43±0.09 | 6702±55 | 7.9±0.08 |
| F 2 | 5.55±0.06 | 6899±45 | 7.7±0.06 |
| F 3 | 5.48±0.03 | 6087±70 | 8.8±0.13 |
| PHF | 5.39±0.02 | 6535±61 | 8.2±0.11 |

**Table 2: Estimation of phytoconstituents in polyherbal gel formulation using validated HPTLC method**

| Phytoconstituent | Mobile phase | (%w /w) | Rf value |
|---|---|---|---|
| Catechin | Toluene: Ethyl Acetate: Formic Acid (10:8:1) | 0.0902 | 0.22 |
| Myricetin | Toluene: Ethyl Acetate: Formic Acid(10:3:1) | 0.512 | 0.08 |
| Glycyrrhizin | Pet ether: Benzene: Ethyl Acetate: Glacial Acetic Acid (4:8:2.8:0.2) | 2.263 | 0.38 |
| Kaempferol | Toluene: Ethyl Acetate: Formic Acid (10:8:1) | 0.0196 | 0.35 |

**Table 3. Percentage permeation of $^{99m}$Tc-DTPA labeled PHF and $^{99m}$Tc-MIBI labeled PHF formulations**

| Time (h) | Pre-wash (Kcts/sec) | | Post-wash (Kcts/sec) | | % Permeation | |
|---|---|---|---|---|---|---|
| | $^{99m}$Tc-DTPA | $^{99m}$Tc-MIBI | $^{99m}$Tc-DTPA | $^{99m}$Tc-MIBI | $^{99m}$Tc-DTPA (Mean±SD) | $^{99m}$Tc-MIBI (Mean±SD) |
| 2 | 119618 | 3902.949 | 60876 | 12499.97 | 50.89201± 6.562 | 37.61855± 11.322 |
| 4 | 80317 | 14346.75 | 34828.67 | 10747.02 | 43.364± 9.436 | 53.93182± 9.269 |
| 24 | 12080 | 13726 | 9436 | 8344.333 | 78.11258± 1.348 | 60.79217± 0.927 |




* P .05; ** P 0.01; *** P 0.001

Figure 1: *In vitro* antimicrobial activity of herbal and polyherbal gel formulations

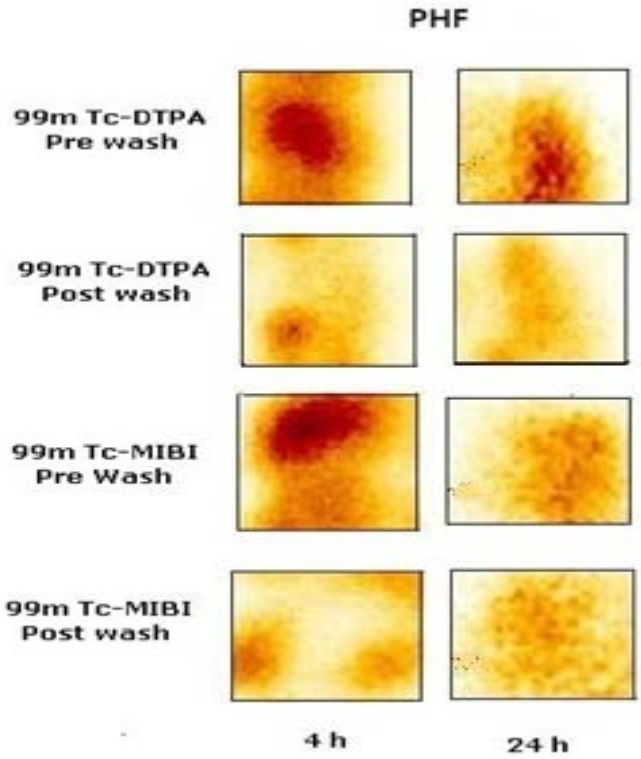



Figure 2: Static images for permeation of $^{99m}$Tc-DTPA labeled PHF and $^{99m}$Tc-MIBI labeled PHF formulations after 4h and 24h

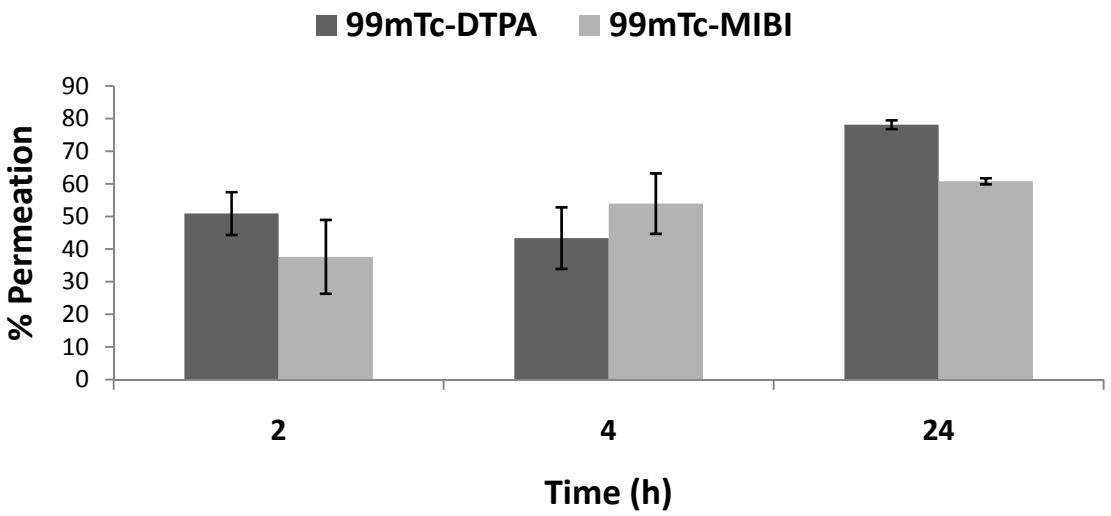



**Figure 3: Percentage permeation of $^{99m}$Tc-MIBI-PHF and $^{99m}$Tc-MIBI-PHF labeled formulations**

## Conclusion

Acne vulgaris is a common skin disorder and many formulations are available in the global market but existing non herbal formulations cause many side effects. Moreover development of antibiotic resistance in acne causing organisms has been rising steadily since the 1980s. Hence development of polyherbal topical formulation with synergistic effect is a very promising approach for its treatment. In the present study *in vitro* antibacterial and *in vivo* skin permeation studies confirm the efficacy of polyherbal gel comprising of three methanolic extracts. It could be theorized that the developed topical polyherbal gel is suitable for the treatment of moderate to severe type of acne.

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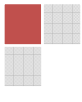

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