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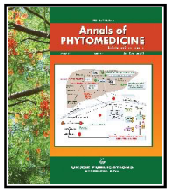

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Development and evaluation of *in vitro* and *in vivo* antioxidant activity of novel skincare formulation containing phytophospholipid complex

Jyotsna Anandrao Saonere◆, Madhuri A. Channawar, Nitin I. Kochar, Deepak Mohale, Anil V. Chandewar and S.L. Deore
Pataldhamal Wadhwani College of Pharmacy, Yavatmal-445001, Maharashtra, India

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Abstract

Skin is the outermost layer of the body, continuously exposed to the external environment, harmful UV radiation, chemicals, and air pollutants. Chronic UV radiation exposure induces the production of reactive oxygen species (ROS), and free radicals. Excessive production of free radicals accumulates in the skin and body signifies the oxidative stress. Oxidative stress destroys the antioxidant system of skin, results in number of harmful effects on the skin. Natural phenolic constituents of plant possess strong free radical scavenging and antioxidant potential, prevents the harmful effect of oxidative stress associated with UV radiation and promote the skin health. Low skin permeation of phenolics due to their polarity results in poor solubility, stability, and dermal penetration. Aim of this study was to formulate and evaluate the antioxidant activity of cream formulations containing phytophospholipid complexes of combination of phenolic fractions. Phytophospholipid complex was prepared by solvent evaporation method by incorporating a combination of fraction and soya phospholipid in different molar concentrations (1:1, 1:2, 2:2, 2:1).Various batches of novel skin care cream formulations were prepared by incorporating different concentrations (1%, 2%, 3%, 4%, 5%) of an optimized batch of phytophsopholipid complex. Prepared cream formulations are evaluated for pH, spreadability, viscosity, *in vitro* antioxidant effect, and *in vivo* antioxidant effect. The result of pharmaceutical evaluation of all batches of phytosomal cream formulations showed that pharmaceutical parameters are in the normal range. Optimum *in vitro* and *in vivo* antioxidant activity are found in novel skin care cream formulation (PCF5) as compared to the conventional cream formulation containing combination of extract.

1. Introduction

Early skin ageing, inflammation, skin cancer, erythema, and skin pigmentations are the outcomes of chronic exposure of skin to the UV radiation (UVR) of sunlight and the harmful effect of pollutants and chemicals (Khavkin and Ellis, 2011; Losquadro, 2017). Safe and effective skincare formulation is the need of the hour. Plants are an abundant source of secondary metabolites like alkaloids, flavonoids, phenolics, tannins, vitamins, and carotenoids (Jorge *et al.,* 2011; Fraternal *et al.*, 2011; Sabeena Arif *et al.,* 2022). Plant biomolecules play a crucial role in the management of free radicals-associated diseases and disorders (Csekes and Raèková, 2021; Khan and Mukhtar, 2018; Potbhare *et al.,* 2022). Chronic exposure to UV radiation induces the production of reactive oxygen species (ROS), free radicals, and excess free radicals accumulating in the skin and body signifies oxidative stress (Alkadi, 2020). Oxidative stress causes interruption and destruction of the protective cellular endogenous antioxidant system such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH), this results in a number of disorders such as ageing, cancer, coronary atherosclerosis, diabetes, ischemia, Alzheimer‘s, immune suppression, skin tanning, neurodegenerative disorders and skin cancer (Valko *et al.,* 2006; Zaric *et al.,* 2023). Plant polyphenolic biomolecules are the potential source of antioxidants, antimicrobials, anti-inflammatory, anticancer, antidiabetics, antiageing, and antiallergic which are attributed to their free radical scavenging effects. Plant phenolics are important bioactive in drug discovery and development, but the macromolecular size of phenolic biomolecules limits their therapeutic potential due to poor stability, solubility, and bioavailability, which creates hurdles in drug discovery and development (Mohideen, 2021; Singh *et al.,* 2021; Shen *et al.,* 2022). Microencapsulation of phenolic biomolecules increases their stability, solubility, bioavailability, and dermal penetration. Hence, the objectives of this study was to improve the therapeutic efficacy, stability, solubility, dermal penetration, and bioavailability of phenolic biomolecule, a novel stable phytophopholipid complex of combinations of phenolics were prepared and incorporated into cream formulations. Four plants are selected for this study, *Camellia sinensis, Glycyrrhiza glabra, Mesua ferrea, and Nelumbo nucifera*.

C. sinensis is an evergreen shrub belonging to the family Theaceae. It contains an abundant amount of flavonoids and polyphenolic compounds used as antioxidants in the treatment of cancer, cardiovascular disease, ageing, skin rejuvenation, antiobesity, and antimicrobials. *G. glabra* belonging to the family Leguminosae, is commonly known as mulethi. It is an abundant source of saponin, flavonoids, and phenolic components used as anti-inflammatory, expectorant, antiulcer, asthma, bronchitis, antidiabetic, antioxidant,

Corresponding author: Ms. Jyotsna Anandrao Saonere
Pataldhamal Wadhwani College of Pharmacy, Yavatmal-445001, Maharashtra, India
E-mail: saonereja@gmail.com
Tel.: +91-9890922092

anticancer, antimalarial, antibacterial, antifungal, skin whitening, and antiallergic (Rana *et al.*, 2021). *M. ferrea* is a perennial tree belonging to the family Calophyllaceae. It is an abundant source of coumarin, flavonoids, proteins, and phenolics and has therapeutic potential such as antimicrobials, anti-inflammatory, antioxidant, asthma, antiallergic, antipyretic, estrogenic, hepatoprotective, antirheumatic, antiarthritic, urinary disorders, and skin disorders *N. nucifera* is a perennial aquatic plant belonging to the family Nelumbonaceae. Important constituents of *N. nucifera* are alkaloids, triterpenoids, flavonoids, polyphenols, and steroids, which possess anti-inflammatory, antimicrobial, hepatoprotective, diuretic properties, antidiabetic, antidiarrheal, anticancer, and antiviral.

2. Materials and Methods

All the selected plants materials (leaves of *C. sinensis,* roots of *G. glabra,* flower buds *of M. ferrea,* and seeds of *N. nucifera*) were purchased from the local crude drug shop and the taxonomic identification was confirmed by the Department of Pharmacognosy, PWCOP Yavatmal, Maharashtra, India.

2.1 Extraction

Each crude drug powder (about 200 g of each) was extracted by continuous hot extraction method using ethanol for 10 h filtered, and dried the extract. Dissolved dry extract in ethyl acetate, filtered and dried (Rana *et al.*, 2021).

2.2 Preparation of phytophospholipid complex

Phytophospholipid complex was prepared by solvent evaporation method. Different molar concentrations (1:1, 1:2, 2:2, 2:1) of combinations of extract and soya lecithin were taken. The required amount of mixture of soya lecithin and phenolic extract are dissolved in tetrahydrofuran, and stirred for 1 h at a temperature not exceeding 40°C. N-hexane was added to the thin film of the sample, continuously stirred until a monolayer of phospholipid formed, precipitated was obtained, filtered, collected and stored in an amber colored bottle at room temperature.

2.3 Preparation of novel cream formulation

Total five batches of cream formulation loaded with different concentrations (1%, 2%, 3%, 4%, and 5%) of an optimized batch of phytosomes containing combinations of extract (PCF1, PCF2, PCF3, PCF4, PCF5) and one batch of cream formulation loaded with a combination of extract (ECF) were prepared. The cream formulations were prepared by heating both the oil phase and water phase in separate beakers at a temperature 75°C in a water bath. Then aqueous phase was added to the oil phase dropwise with continuous stirring for 15-20 min until it became a homogeneous mixture. Glycerin was added while stirring as a moisturizing agent. The cream was stored in tightly closed containers. The composition of the cream formulation is shown in Table 1.

Table 1: Preparation of different batches of novel cream formulation

S. No.	Ingredients	PCF1	PCF2	PCF3	PCF4	PCF5	ECF
1	Castor oil	4 %	4 %	4 %	4%	4%	4%
2	Phytosomes	1 %	2 %	3 %	4 %	5 %	-
3	Combination of extract (5:5:5:5)	-	-	-	-	-	5%
4	Stearic acid	25 %	25 %	25 %	25 %	25 %	25 %
5	Cetyl alcohol	15 %	15 %	15 %	15 %	15 %	15 %
6	Glycerine	10 %	10 %	10 %	10 %	10 %	10 %
7	Methyl paraben	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%
8	Distilled water	25%	25%	25%	25%	25%	25%
9	Rose oil	0.5 %	0.5 %	0.5 %	0.5 %	0.5 %	0.5 %
10	Triethanolamine	q.s	q.s	q.s	q.s	q.s	q.s

2.4 Evaluation of novel cream formulation

2.4.1 Organoleptic evaluation

All the batches of novel cream formulations were evaluated with visual observation for the color, odor, texture and appearance. The outcomes of the test are given in the Table 3.

2.4. 2 Measurement of pH

The pH of all cream formulations were measured with the help of calibrated digital pH meter. The results are given in the Table 4.

2.4.3 Spreadability

Spreadability was determined by two glass slides and record the time (sec) taken by two glass slides to slip off the cream formulation placed in between the glass slides of uniform thickness, after heavy weight kept over the slides. The results are given in Table 4. Spreadability is calculated by the following formula:

$$S = M \times L/T$$

where, S = spreadability

M = mass applied on the glass slide

L = length of the diameter of the spreaded cream

T = time required to spread the formulation

2.4.4 Viscosity

The viscosity of all prepared cream formulations were determined by Brookfield viscometer at a 25°C temperature. The results are given in Table 4.

2.4.5 Phase separation test

Cream formulations were transferred to the container and closed with tight lead. Containers containing respective cream formulations were maintained at 25-100°C temperature in dark conditions. Phase separation was monitored for 24 h formulations were kept for 30 days and observed for phase separation. The results are given in Table 4.

2.4. 6 Drug content

The drug content of all formulations was analyzed by the supernatant solution diluted with mobile phase and analyzed by UV spectroscopy 730 nm. The results are given in Table 4.

2.4.7 Stability study

According to the ICH guideline, stability study was performed. Optimized batch PCF5 and ECF were kept at different temperature 25°C ± 2°C/65% RH ± 5% RH, 40°C ± 2°C/75% RH ± 5% RH and 4°C ± 2°C for three month and physicochemical parameter was evaluated. No significant change was observed in physicochemical parameters.

2.4.8 Fourier transform infrared spectroscopy

FTIR is a powerful method for structural analysis and compatibility study of active components and additives. The formation of phytophospholipid complex and compatibility of phenolic bioactive with phospholipid and other additives were conducted using Fourier transform infrared spectroscopy. A small quantity of samples (optimized batch of phytosomes and cream formulation) was placed below the probe and spectral scanning was performed in the range between 4000 and 400 cm^{-1} and interpreted for the functional group at their respective wave number (Das and Kalita, 2014). Results are shown in Figures 2 and 3.

2.4.9 *In vitro* drug release

The *in vitro* drug release study of an optimized batch of cream formulations (PCF5) and (ECF) was performed by using Franz diffusion cells with an effective diffusion area of 3.14 cm^2. The artificial membrane was soaked in phosphate buffer solution (PBS 7.4) for 1 h The receptor compartment was filled with (PBS 7.4) maintained at 37°C ± 1°C. Both cream solutions (PCF5, ECF) containing 100 mg of formulation were filled in the donor compartment. Withdrawn the samples periodically (1 h, 2 h, 4 h, 6 h, 6 h, 8 h,10 h, and 12 h) from the receptor compartment, replacing with the same amount of fresh PBS solution. Drug content was determined by spectrophotometer (Patel, 2009; Verma and Utreja, 2018). Results are shown in Figure 4.

2.5 Therapeutic evaluation

2.5.1 *In vitro* antioxidant study by DPPH method

In vitro antioxidant activity of an optimized batch of cream formulation loaded with phytosomes containing combinations of extract (PCF5) and cream formulation loaded with extract (ECF) was determined by DPPH method. Change in the absorbance was measured at 517 nm. Dilution of sample was done (10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml) to which 1ml of DPPH solution was added, incubated for 30 min at 37°C in dark. After 30 min, the absorbance was measured. Ethanol was used as a blank. The experiment was performed in triplicate. Outcomes of the antioxidant study are shown in Figure 5.

The percentage of the DPPH radical scavenging is calculated by the following equation:

% scavenging activity = [(Ac-As) / Ac] x 100

where, Ac = Absorbance of control

As = Absorbance of sample

2.5.2 *In vivo* antioxidant study

Antioxidant study was performed on albino westar rats of either sex. The rats were housed in polypropylene cages as per standard animal houses conditions such as temperature (25 ± 2°C), relative humidity (60 ± 5%) and 12 h dark/light cycle as per CPCSEA (Committee of control and supervision of experiments on animals) regulations. Experiment was performed after due permission from IAEC (Institutional Animal Ethics Committee) permission letter No. 650/PO/RE/S-2002/2022/CPCSEA/34.

For antioxidant study, animals (wistar rats) were divided into different group each group consists of 6 animals (n = 6) as shown in Table 1.

Table 2: Groups of animal for antioxidant study

S. No.	Name of group	Treatment
1	Group1 (Control)	Neither irradiated nor treated with cream formulation
2	Group2	UVB irradiated
3	Group3	UVB irradiated + Base cream formulation BCF
4	Group4	UVB irradiated + Extract cream formulation ECF
5	Group 5	UVB irradiated + Phytosomal cream formulation (PCF5)

2.5.2.1 Exposure to UV radiation

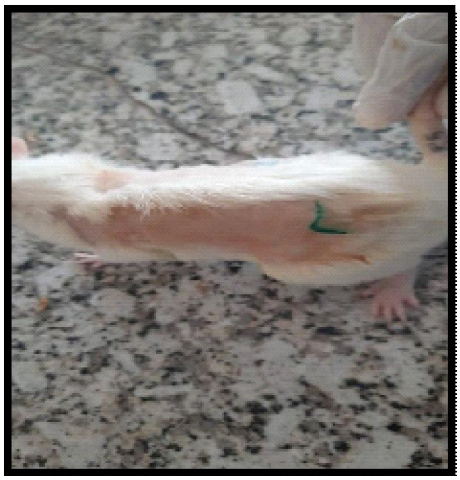
Before one day of the study, the hair on the back portion of rats was removed using an electrical clipper (~2 cm^2 area). About 100 mg/cm^2 of the cream formulations were applied on the rat skin by spreading evenly on the hair removal area on the next day. The rats were irradiated with UV-radiation by using UV light 360 nm with an energy output of 52.5 mJ /cm^2 for 30 min.

2.5.2.2 Biochemical study

Chronic exposure of UV radiation induces formation of free radicals, accumulation of free radicals results in formation oxidative stress in epidermal and dermal tissues in skin, causes depletion of dermal antioxidant enzymes such as superoxide dismutase (SOD), glutathione (GSH), malondialdehyde, and catalase (CAT) and protein oxidation

in skin. The histopathological studies and biochemical analysis is the important tool to assess the antioxidant efficacy of the prepared novel formulations. After UV irradiation, the rats were anaesthetized (with diethyl ether in anesthesia chamber). Excised the treated skin, washed with phosphate buffer saline, and removed the excess of adherent tissues and fascia. The skin was cut into small pieces followed by homogenization in tissue homogenizer using phosphate buffer. Centrifuged tissue homogenate at 5000 rpm for 15 min and stored in deep freeze for biochemical parameter estimation like antioxidant enzymes catalase, total glutathione (GSH) and superoxide dismutase (SOD) . Biochemical study was analyzed by autoanalyser and results are shown in Figure 6.

A: Before application of cream



B: After application cream

Figure1: ***In vivo*** **antioxidant study on rat.**

3. Results

All the prepared cream formulation was evaluated for physicochemical and pharmaceutical evaluations. Outcomes of results are given in Tables 3 and 4.

3.1 Pharmaceutical evaluation

Table 3: Organoleptic evaluation of cream formulation

S. No.	Parameters	PF1	PF2	PF3	PF4	PF5	ECF
1	Color	Faint brownish	Faint brownish	Brownish	Brownish	Brownish	Faint creamish
2	Odor	Pleasant	Pleasant	Pleasant	Pleasant	Pleasant	Pleasant
3	Texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
4	Appearance	Semi-solid	Semi-solid	Semi-solid	Semi-solid	Semi-solid	Semi-solid

Table 4: Pharmaceutical evaluations of different batches of cream formulation

S. No.	Parameters	PCF1	PCF2	PCF3	PCF4	PCF5	ECF
1	Spreadability (g cm/sec)	22.11± 0.02	21.09 ± 0.001	22.35 ± 0.005	23.03 ± 0.01	23.10 ± 0.01	22.68 ± 0.001
2	pH	5.9 ± 0.005	6.2 ± 0.001	6.7 ± 0.005	6.5 ± 0.005	6.5 ± 0.005	6.4 ± 0.001
3	Viscosity cp	22344 ± 01	22032 ± 0.01	21870 ± 0.005	21510 ± 0.01	22012 ± 0.01	22877 ± 0.02
4	Drug content	91.27 ± 0.005	92.10 ± 0.01	92.22 ± 0.01	93.30 ± 0.005	94.27 ± 0.005	93.09 ± 0.001
5	Skin irritancy	Nil	Nil	Nil	Nil	Nil	Nil
6	Phase separation	No phase separation	No phase separation	No phase Separation	No phase separation	No phase separation	No phase separation

The values are expressed as mean ± SD (n=3). SD: standard deviation.

3.2 FT-IR compatibility study

Compatibility study of optimized batch of phytophopsholipid complex and optimized batch of cream formulation containing phytophospholipid complex of combination of extract (PCF) was conducted on FT-IR spectroscopy. Results of FT-IR compatibility study is shown in Figures 2 and 3.

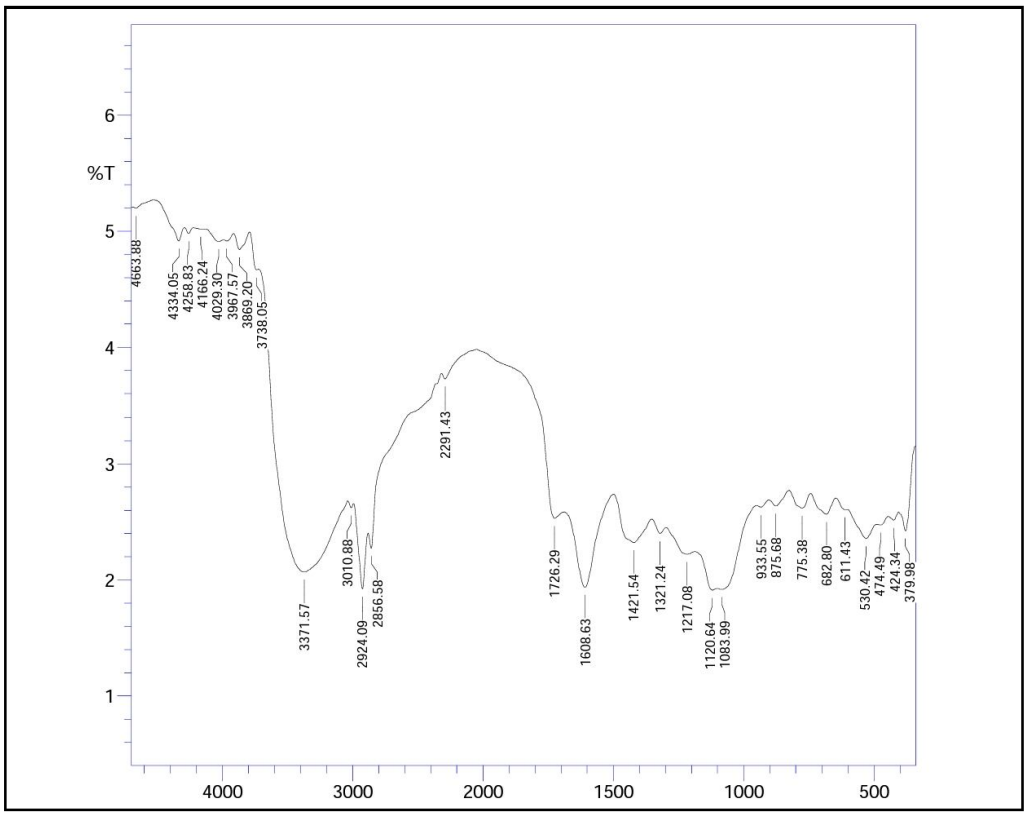


Figure 2: FTIR spectrum of optimized batch of phytophospholipid complex.

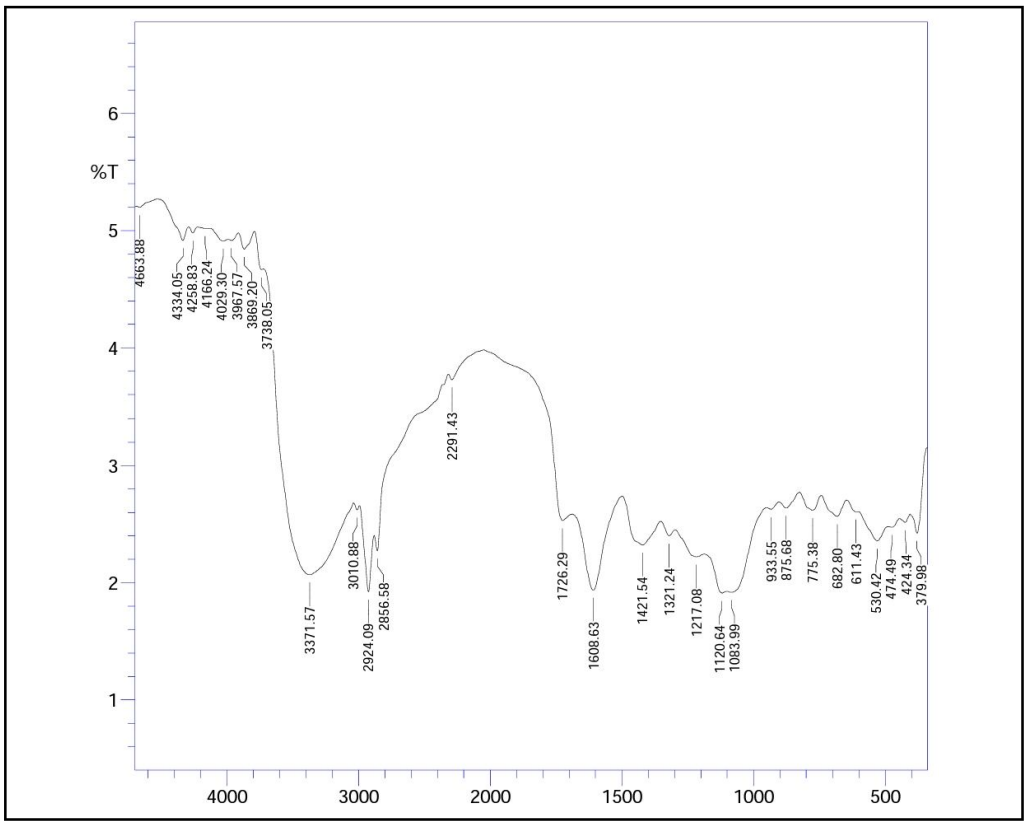


Figure 3: FTIR spectrum of optimized cream formulation (PCF5).

3.3 In *vitro* drug release

In vitro drug release of optimized batch of cream formulation containing phytophospholipid complex of combination of extract and cream formulation containing combination of extract was performed by Franz diffusion cells. The results of *in vitro* drug release are given in Figure 4.

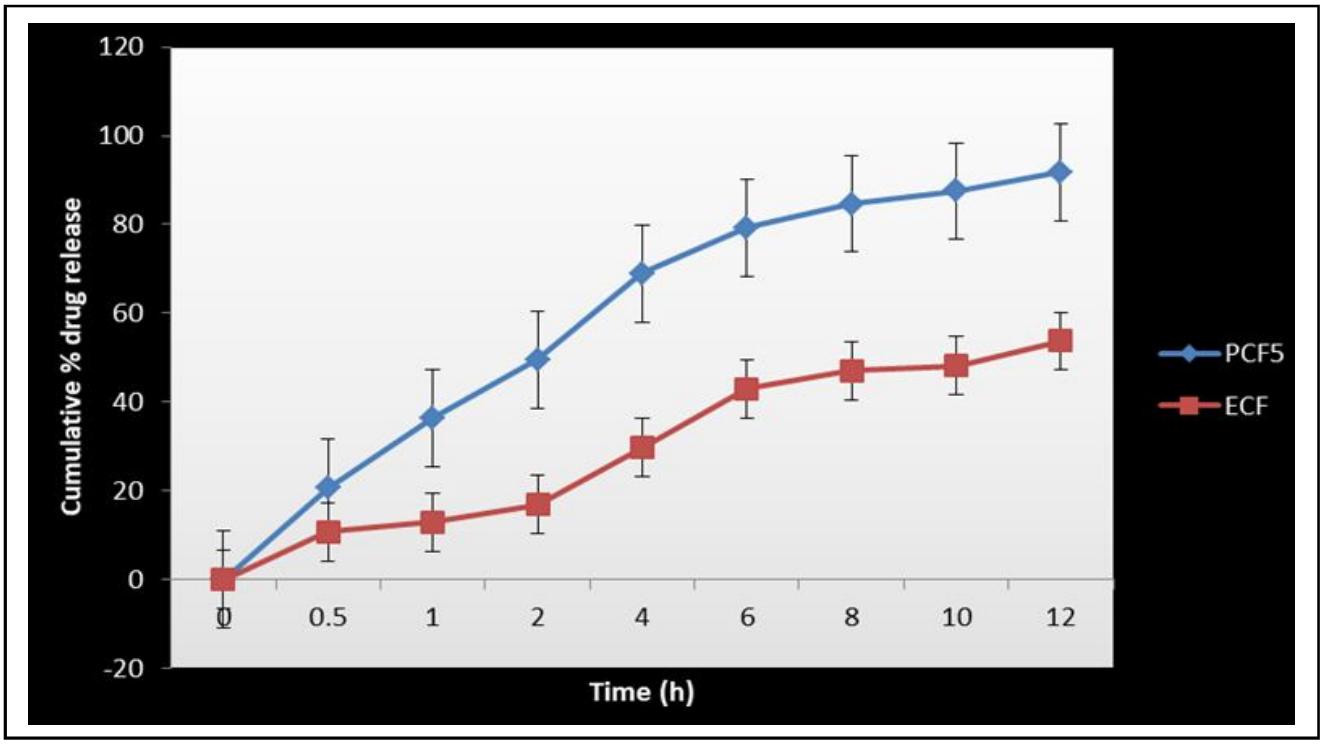


Figure 4: *In vitro* drug release of PCF5 and ECF cream formulation.

3.4 *In vitro* antioxidant study

Percentage free radical scavenging activity of optimized cream formulation containing phytophospholipid complex containing combination of extract and cream formulation containing combination of extract was determined by DPPH method. Results are given in Figure 5.

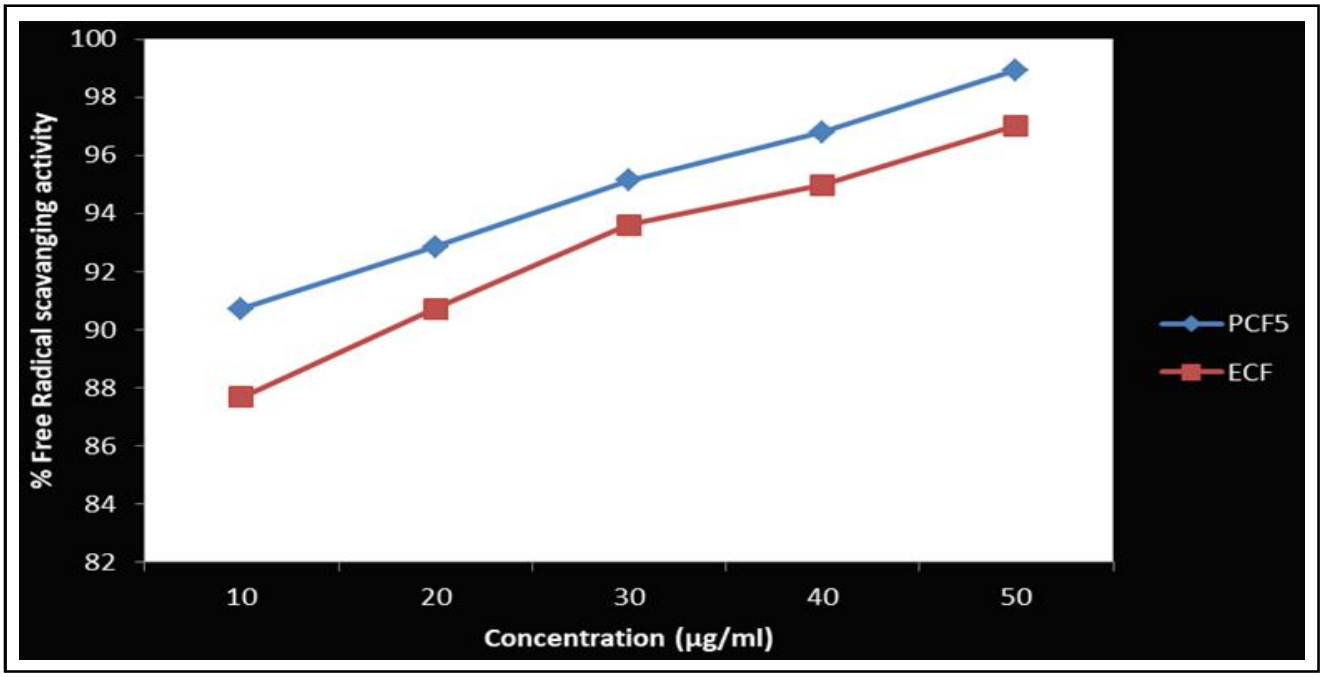


Figure 5: % FRA of PCF5 and ECF.

3.5 *In vivo* antioxidant study

In vivo antioxidant study was performed on albino westar rats. Animals were divided into the five group, each group consists of six animals (n=6) [group1: neither irradiated nor treated with cream formulation, group 2: UVB irradiated (irradiated with UV-B radiation), group3: application of base cream formulation BCF and UVB irradiated, group 4: application of cream formulation containing combination of extract ECF and UVB irradiated, group 5: application of cream formulation containing phytophopsholipid complex of combination of extract PCF5 and UVB irradiated]. Result of biochemical studies are shown in Figure 6.

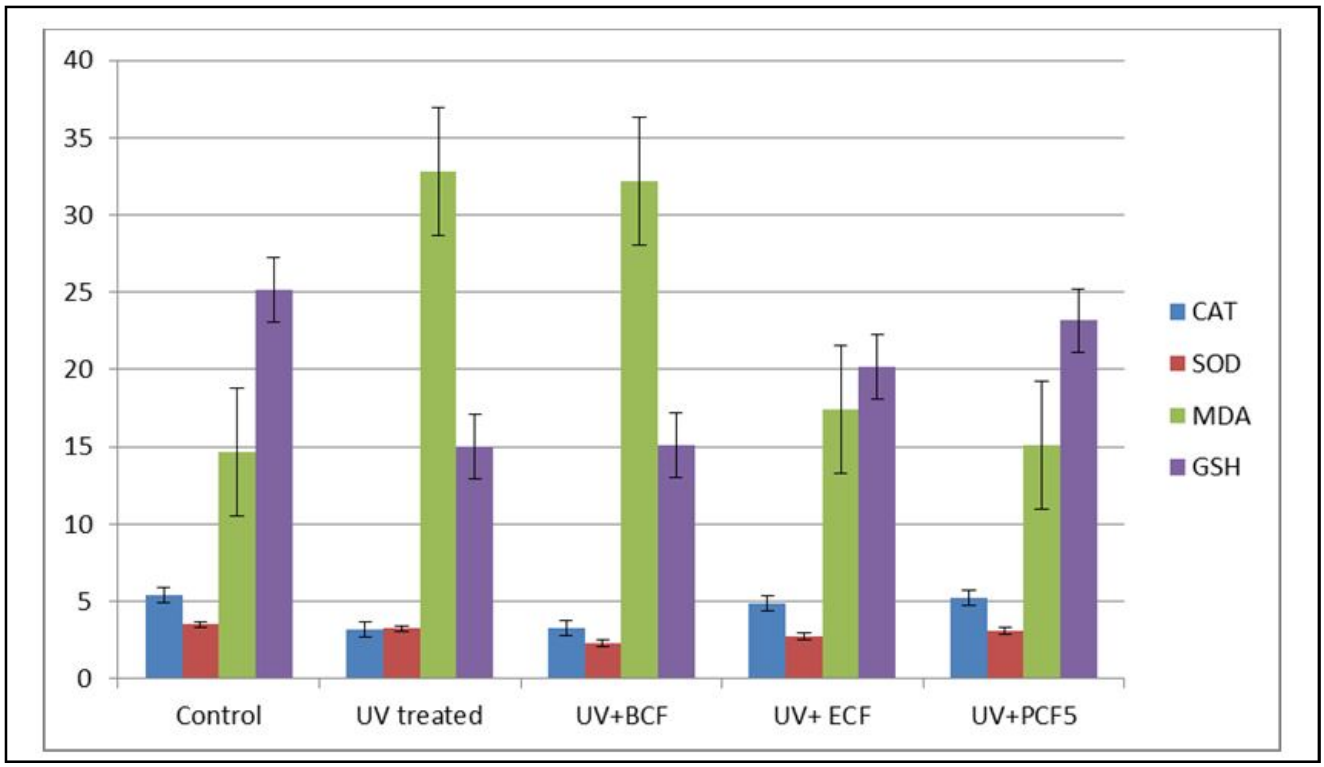


Figure 6: *In vivo* antioxidant result of PCF5 and ECF.

4. Discussion

Results of pharmaceutical evaluation revealed that pharmaceutical parameters such as pH, spreadability, viscosity of all cream formulation was found in the required range. Optimum phenolic content was found in PCF5. IR spectra of phytophospholipid complex containing combination of extract showed O-H peak at 3371.57cm^{-1}, C-H peak at 3010.88 cm^{-1} and 2924.09 cm^{-1}, O =C=O peak at 2291.43 cm^{-1}, C=O peak at 1726.29 cm^{-1} no significant changes was observed in O-H stretching, C-H stretching, O=C=O stretching and C=C stretching of functional groups extracts. This indicates that, stable phytophospholipid complex of combination of extract and soya phospholipid was formed, no structural changes observed in phytosomes due to interaction. IR spectra of optimized cream formulation containing phytosomes (PCF5), showed the C-H stretching, C-H bending, O-H bending in the range of 3300 to 2500 cm^{-}1 found similar to that of the phytosomes IR spectrum. Thus, no significant drug interaction induced structural changes observed in cream formulation. This indicates that the phytosomal complex of combination of extract found compatible with cream excipients. *In vitro* drug release study of cream formulation loaded with phytosomal complex of combination of phenolic extract (PCF5) was compare with cream loaded with extract (ECF). Highest drug permeation was found with novel cream formulation (PCF5) than the conventional cream formulation. Free radical scavenging activity (FRA) was found maximum at a concentration 50 µg/ml. Highest % FRA 98.23% was found for novel cream formulation (PCF5). *In vivo* antioxidant biochemical analysis result showed that biological antioxidant system of skin is significantly affected due to the harmful effect of UV mediated ROS, about 40% of reduction was found in biochemical constituents of UV irradiated rat skin. Remarkable improvement was seen in biochemical constituents of skin after the application of cream formulation loaded with phytophospholipid complex containing combination of extract (PCF5) when compared with standard marketed cream formulation and UVR group. This study concluded that the developed novel cream formulation are able to overcome the issue of solubility, stability, bioavailability and dermal penetration which would improve the therapeutic potential of phenolic extract than the conventional cream formulation.

5. Conclusion

All phenolic content plants were selected for the preparation of novel drug delivery system. Phytophospholipid complex of phenolic fractions were prepared and incorporated in cream formulations, total 6 formulation were prepared and evaluated for pharmaceutical parameters, *in vitro* drug release, *in vitro* and *in vivo* antioxidant study. Pharmaceutical parameter of all cream formulation was found in the required range. Optimum phenolic content and *in vitro* drug release was found in (PCF5) cream formulation loaded with phytophopholipid complex containing combination of extract. Remarkable improvement was seen in biochemical constituents of

skin after the application of cream formulation loaded with phytosomes containing combination of extract (PCF5) when compared with standard marketed formulation and UVR group. This study concluded that the developed novel cream formulation is able to overcome the issue of solubility, stability, bioavailability and dermal penetration which would improve the therapeutic potential of extracts.

Conflict of interest

The authors declare no conflict of interest relevant to this article.

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