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Formulation and Evaluation of Topical Cream Enriched with Antimicrobial and Antioxidant Herbs

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Abstract

Aqueous extracts of liquorice (*Glycyrrhiza glabra* L.), mulberry (*Morus alba* L.) and turmeric (*Curcuma longa* L.) were used as the main active ingredients in formulated polyherbal topical cream along with other herbal aqueous extracts like neem (*Azadirachta indica* A. Juss*)*, basil (*Ocimum sanctum* L.*)*, crofton weed (*Ageratina adenophra* (Spreng.) King & H. Rob.*)*, mugwort (*Artemesia vulgaris* Mattf. Paati.*),* Indian gooseberry (*Emblica officinalis* Gaertn*)* and tea (*Camellia sinensis* L.*)* were also added. Sweet almond oil, clove oil and tea tree oil were used as oil phase. Vanilla extract was used for the fragrance. Using design expert software (Version 10.0.7, Stat-Ease Inc., Minneapolis, Minnesota), 17 different formulations(F1-F17) were designed by Box-Behnken Design(BBD) with percentage concentrations of liquorice, mulberry and turmeric as independent variables and antioxidant activity, occlusivity test, antimicrobial activity as response variables. The data were analyzed through polynomial equation statistically using a 3-factor, 3-level design. The pH, spreadability and sun protection factor (SPF) of different formulations were measured. It was found that all the formulations had acceptable pH and spreadability. F6 had the best antioxidant property at 500 μg/mL concentration with a value of 82.34% inhibition. The IC75 of the standard ascorbic acid was found to be 198.98 μg/mL. All formulations showed SPF value above 7 but F7 showed the highest SPF value of 12.600. The F7 had the best antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. All formulations showed satisfactory occlusion and stability at different temperature conditions (8°C, 25°C and 40°C). Furthermore, the invented formulation with the combination of the Nepalese herbs can be commercialized by contracting the market traders and manufactures in the future.

Keywords: Bioactivity, Box-Behnken design, Extracts, Medicinal plants, Polyherbal cream

Introduction

Herbal cosmetics have been the matter of great attraction mainly because they possess desirable metabolites to enhance the skin health (Gyawali & Poudel, 2022). The inclusion of herbal extracts in topical cream can minimize the skin damage caused by oxidative stress, which has been associated with delaying the aging process. Polyherbal skin care creams contain the different extracts intended for the topical application (Chavan et al., 2020). Such extracts are added to improve the variety of properties like antioxidant, anti-inflammatory, antiseptic, emollient, antiseborrheic, antikeratolytic, antibacterial and offer the corrective benefits (Goel et al., 2010; Kasprzak-Drozd et al., 2024; Yasheshwar et al., 2024). Addition of plant-based active ingredients such as alpha-hydroxy acid, retinoic acid, ferulic acid, ascorbic acid and coenzyme Q10 are common in beauty care products (Ayunin et al., 2022; Saini, 2011). Beneficial properties associated with the phenolic antioxidant phytochemicals has been proven for beauty and topical therapy (Lee et al., 2014; Saric & Sivamani 2016). The increasing demand of herbal cosmetics in today's world has raised the interest of researchers on cosmetics medicinal plants, their technologies, and formulations (Sathyaseelan et al., 2024).

Root of *Glycyrrhiza glabra* L. possesses good antioxidant and antiviral properties (Damle, M., 2014; Ju et al., 1989). Similarly, leaves of *Morus alba* L. as antioxidant and antimicrobial (Chang et al., 2021; Imran et al., 2010; Sohn et al., 2004), rhizomes of *Curcuma longa* L. as anti- inflammatory and sun burn repairmen (Heng, 2017), leaves of *Azadirachta indica* A. Juss as antioxidant, anti-inflammatory and wound healing (Alzohairy,

2016; Hossain et al., 2013) properties are already established through plethora of publication. Literature also demonstrates that the fruit of *Emblica officinalis* Gaertn is well known for antioxidant, skin lightening and antimicrobial activity (Pal et al., 2016), leaves of *Artemesia vulgaris* Mattf. Paati. and *Ageratina adenophra* (Spreng.) King & H. Rob. possesses an antibacterial property (Malik et al., 2019). The leaves of *Camellia sinensis* L. have successfully improved the inflammation and anti-wrinkle effect (Lee et al., 2014, Vishnoi et al., 2018). The use of locally available various medicinal plants in cream formulation along with almond oil, tea tree oil and clove oil could be an attempt to improve the quality of cream with the result that is effective, safe and non-irritating.

Materials and Methods

Chemicals and microorganisms

Chemicals used in the experiment i.e. stearic acid, tea tree oil, almond oil, cetyl alcohol, span 80, sodium hydroxide, vanilla extract, glycerin and tween 80 were pharmaceutical grade. The bacterial strains of *Escherichia coli* (ATCC-25922) and *Staphylococcus aureus* (ATCC-25923) were provided by the Department of Microbiology, Maharajgunj Medical Campus, Tribhuvan University, Nepal. Brain Heart Infusion (BHI) broth (BM0070, EO labs), Dehydrated Mannitol Salt Agar (CM0085B, Thermo Scientific™), MacConkey's agar (M081B, HiMedia™ Laboratories Pvt Ltd), Mueller-Hinton Agar (M173, HiMedia™ Laboratories Pvt Ltd) and 0.5 McFarland turbidity standards(R092, HiMedia™ Laboratories Pvt Ltd) were used. An analytical grade of hydrogen peroxide, phosphate saline buffer (pH 7.4), calcium hydroxide, ethanol and standard ascorbic acid were used.

Collection of herbal materials

Roots of *G. glabra*, rhizomes of *C. longa*, leaves of *C. sinensis*, buds of *S. aromaticum* and fruits of *E. officinalis* were purchased from local the market Kathmandu, Nepal. The leaves of *A. indica* were collected from Makwanpur, Nepal and the leaves of *M. alba* were collected from Kaski, Nepal. The leaves of *O. sanctum*, *A. vulgaris* and *A. adenophora* were collected from Kavre, Nepal. The plant materials were identified and authenticated by project supervisor, from Department of Pharmacy, Tribhuvan University, Nepal and cross verified using literatures (Baral & Kurmi, 2006; Department of Plant Resources [DPR], 2016.

Preparation of aqueous herbal extracts

Plant samples were cleaned and dried in shade for 7 days and grinded into fine powder by grinder (GX3, Bajaj Mixer, Bajaj Home Appliance Company, Solan H.P., India) separately. Except powder of *G. glabra , C. longa* and *M. alba* which concentrations were adjusted via Box Behnken Design (BBD), while 5.00 gm of others powdered herbals were taken separately on conical flask and each herb was macerated with 100 ml distilled water for 3 days with moderate shaking. After, herbal contents were filtered out using muslin cloth and filtrates were placed in beakers and labeled separately.

Extraction of clove oil

Total 500 gm clove buds were extracted in Clevenger apparatus on one cycle with the ratio of 1:5 clove and water for 4 hours. The 25 mL clove oil was collected in sterile vial and stored in refrigerator for further use. The hydrosol of clove buds were also stored after clove oil extraction. On 5 drops of clove oil 10 mL dilute calcium hydroxide was added, shaken vigorously for 5 minutes, a flocculent mass was seen and a slight yellow color was developed which confirmed the extract as clove oil as per Pharmaceutical and Medical Device Regulatory Science Society of Japan (PMRJ, 2022).

Formulation experiment design

A 3-factor, 3-level design was prepared by Box Behnken Design to evaluate quadratic response surfaces and constructing second order polynomial models using Design Expert software (Version 10.0.7, Stat-Ease Inc., Minneapolis, Minnesota). The 17 run formulations were designed based on the different combination of various levels of independent variables while the responses were the dependent variables. The polynomial equation

generated by this experimental design is given as

$Y_o = b_o + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$;

Where, Y_o is the dependent variable; b_o is the intercept; b_1 to b_{33} are the regression coefficients computed from the observed experimental values of Y; and X_1, X_2 and X_3 are the coded levels of independent variables. The terms X_1, X_2 and X_i^2 (i = 1, 2, or 3) represent the interaction and quadratic terms respectively (Chaudhary et al., 2011).

Preliminary trials

Preliminary trials were carried out to determine the optimal formulation of the base. Stearic acid and cetyl alcohol in concentration of 6.00 gm and 2.00 gm respectively for the formulation of 50.00 gm cream made the base too viscous and thick. In concentration of stearic acid 3.00 gm and cetyl alcohol 1.00 gm gave the thin and runny base. When the stearic acid and cetyl alcohol were used in concentration of 4.00 gm and 1.50 gm, the formulation was found to be desirable and stable and hence it was chosen for the formulation of base.

Table 1: Variables in polyherbal cream using Box-Behnken design

Factor	Levels used (Actual coded)		
	Low (-1)	**Medium (0)**	**High (+1)**
Independent variables			
X_1=Liquorice extract concentration (w/v %)	10.00	15.00	20.00
X_2=Mulberry extract concentration (w/v %)	10.00	15.00	20.00
X_3=Turmeric extract concentration (w/v %)	5.00	7.50	10.00
Dependent variables	Response polynomial equation		
Y_1=Antioxidant activity (%)	$+72.44+7.28*X_1+5.62*X_2+3.04*X_3-0.22*X_1X_2-0.27*X_1X_3-O.84*X_2X_3+ 3.52*X_1^2-2.41*X_2^2-2.72X_3^2$.		
Y_2= Occlusivity test (%)	$+43.00+1.57*X_1+0.38*X_2+1.90*X_3-0.31*X_1X_2+1.25*X_1X_3+ 0.95*X_2X_3- 1.40*X_1^2-0.85*X_2^2-1.60X_3^2$.		
Y_3=Antimicrobial activity(ZOI in mm)	$+19.00+1.50*X_1+1.13*X_2+3.38*X_3+0.00*X_1X_2+0.00*X_1X_3+0.25*X_2X_3$ $-0.13*X_1^2-1.38*X_2^2-0.87X_3^2$(for *E.coli*). $+15.88+1.12\ X_1+0.87X_2+2.75X_3$(for *S.aureus*).		

Table 2: Formulation design for polyherbal cream using Box-Behnken design (BBD)

Formulation	Concentration of Liquorice (w/v %)	Concentration of Mulberry (w/v %)	Concentration of Turmeric (w/v %)
1	10.00	15.00	5.00
2	15.00	20.00	5.00
3*	15.00	15.00	7.50
4	15.00	10.00	10.00
5	10.00	15.00	10.00
6	20.00	20.00	7.50
7	20.00	15.00	10.00
8*	15.00	15.00	7.50
9	20.00	15.00	5.00
10	10.00	20.00	7.50
11	15.00	10.00	5.00
12*	15.00	15.00	7.50
13*	15.00	15.00	7.50
14*	15.00	15.00	7.50
15	15.00	20.00	10.00
16	20.00	10.00	7.50
17	10.00	10.00	7.50

Note : * Formulation containing same concentration of independent variables

Formulation of polyherbal topical cream

Stearic acid, cetyl alcohol, span 80 and almond oil were added in a beaker and heated upto 75^{0}C on a water bath (Navyun water bath) for oily phase. Aqueous extracts of the herbal, glycerin, tween 80 along with clove hydrosol were heated upto 75ºC on a water bath (Navyun water bath) in separate beaker. Oil phase was added to aqueous phase at 75ºC and blended by hand stirrer (Hand-001, Generic) at 1300 rpm for first 15 minutes and at 900 rpm for next 15 minutes along with the addition of few drops of NaOH (1N). As the cream reached the room temperature clove oil, tea tree oil and vanilla extract were added (Table 3) (Soumya et al. 2020)..

Evaluation of cream

Organoleptic characteristics: All formulations were tested for physical appearance, color, texture, phase separation and homogeneity. These characteristics were evaluated by visual observation (Chen et al., 2016).

pH value measurement: 5±0.01 gm of each formulation was dispersed in 45 mL of deionized water and the pH was determined using a calibrated pH meter (HI 2210 pH meter) at 25°C. A skin acceptable P^H value is 4-6 (Mali et al., 2015).

Spreadability test: Total 0.50 gm cream was placed in a circle having diameter of 1 cm marked over on a glass plate previously and a second glass plate was placed such that the cream was pressed between both plates by weight of 250.00 gm which was kept on the upper glass plate for 2 minutes. The increase in the diameter (cm) due to spreading of the cream was recorded (Sanghi et al., 2016).

Hydrogen peroxide (H_2O_2) scavenging activity: Phosphate saline buffer (pH 7.4) was used to prepare hydrogen peroxide solution and different

Table 3: Composition of polyherbal topical cream

Ingredients	Quantity (w/w %)
Oil Phase(Phase 1)	
Stearic acid	8.00
Cetyl alcohol	3.00
Sweet almond oil	4.00
Tea tree oil	0.20
Span 80	0.90
Aqueous phase(Phase 2)	
Aqueous extract of *M.alba L.**	4.00
Aqueous extract of *C.longa L.**	4.00
Aqueous extract of *G.glabra L.**	4.00
Aqueous extract of *O.sanctum L.***	3.00
Aqueous extract of *A. vulgaris*Mattf. Paati**	3.00
Aqueous extract of *A. adenophora*(Spreng.) King & H. Rob. **	3.00
Aqueous extract of *A. indica*L.**	3.00
Aqueous extract of *E. officinalis*Gaertn**	3.00
Aqueous extract of *C. sinensis*L.**	3.00
Glycerin	10.00
Tween 80	2.90
Hydrosol of Clove buds	q.s to 100 gm
Phase 3	
Clove oil	0.20
Sodium Hydroxide(1N)	4.00
Vanilla extract	4.00

Note: *4.00 gm of independent variables concentrations from BBD (Table 2) was used. For instance, for formulation F7 preparation, 4.00 gm of 20.00 gm powdered Liquorice roots (X_1) extracted on 100 mL distilled water, 4.00 gm of 15.00 gm powdered Mulberry leaves (X_2) extracted on 100 mL distilled water and 4.00 gm of 10.00 gm powdered Turmeric rhizomes (X_3) extracted on 100 mL distilled water were used.

**3.00 gm of individual 5.00 gm powdered herbals macerated on 100 mL distilled water was used.

concentration of three solutions i.e., standard, test and control were prepared for determining percentage of the hydrogen peroxide scavenging activity by the samples and the standard compounds which absorbances are measured at 230 nm, given by Ruch et al., 1989, Rajasree et al., 2012 and Singh et al., 2012.

$$Percentage\ Scavenged[H2O2] = \frac{Ac - As}{Ac} x100$$

Where,

A_c= Absorbance of control sample and A_s= Absorbance of cream sample

For standard solution, different concentrations (100, 200, 300, 400, 500 µg/mL) of the ascorbic acid was prepared in phosphate buffer (pH 7.4). Total 3.4 mL of each solution of ascorbic acid was mixed with 0.5 mL of 40 mM H_2O_2 solution. After 10 minutes, absorbance of different concentrations of ascorbic acid solutions was measured at 230 nm in Ultraviolet-Visible spectrophotometer (Shimazdu UV-01800) against phosphate buffer (pH 7.4) as blank. For control solution, 3.4 mL of phosphate buffer (pH 7.4) solution was mixed with 600 µL of 40 mM H_2O_2 solution. For test solution, cream 1 mg/mL in methanol was prepared. Then different concentration of the creams (100, 200, 300, 400, 500) µg/mL were prepared in phosphate buffer (pH 7.4). Total 3.4 mL of each solution were mixed with 600 µl of 40 mM H_2O_2 solution. After 10 minutes, absorbances of different concentrations of the cream samples were measured at 230nm in Ultraviolet-Visible spectrophotometer (Shimazdu UV-01800) against phosphate buffer (pH 7.4).

Sun protection factor determination: Total 1.00 gm of all formulations of the cream were weighed, transferred to a 100 mL volumetric flask, diluted to volume with ethanol, followed by sonication on ultrasonic bath sonicator (K410HTDP, AnonKia) for 5 minutes and then filtered through cotton. Each 5.0 mL aliquot was transferred to 50 mL volumetric flask and diluted to volume by ethanol separately. Again each 5.0 mL aliquot was transferred to 25 mL volumetric flask and then volume made up to mark by adding ethanol. The absorption data were then measured in the range of 290 nm to 320 nm with every 5 nm wavelength interval by Ultraviolet-Visible spectrophotometer (Shimazdu UV-01800). 3 determinations were made at each point. Table 4 shows universal spectrophotometric erythmogenic effect of radiation followed by the application of Mansur equation (Dutraet al., 2004; Mansur et al., 1986) given by,

$$SPF_{spectrophotometric} = CFx\sum_{290}^{320} EE(\lambda)xI(\lambda)xAbs(\lambda)$$

Where,

CF = correction factors taken as 10 and determined by UV spectrophotometry so that a standard sunscreen formulation containing 8% homosalate presented a SPF value of 4. EE (λ) = erythmogenic effect of radiation with associated wavelength (λ) and Abs (λ) = spectrophotometric absorbance value at associated wavelength (λ). The values of EE (λ) x I (λ) is constant (shown in Table 4).The SPF value greater than 4 blocks 75% of UV radiation.

Table 4: Normalized function used in calculation of SPF

Wavelength(λ)	Normalized function[EE (λ) x I (λ)]
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180
Total	**1**

***In vitro* occlusivity test:** Total 10.00 gm of distilled water was placed on Beaker (Height 4.60 cm, diameter 3.20 cm) and the open end was closed by Whatman filter paper (0.45 pore size) on the upper surface of which 0.20 gm of each cream sample was evenly distributed. Negative control sample was made only by covering open end of 10.00 gm distilled water containing beaker (Height 4.6 cm, diameter 3.2 cm) only by Whatman filter paper (0.45 pore size). These beakers were then placed at 37±2 °C/60%±5% RH for 48 hours and occlusion factor F(%) was calculated as (Maru et al., 2018). Triplicate data were taken.

F (%) = (A-B)/A x100, where

A=Water flux through filter on negative control sample (% water loss), B= Water flux through filter when covered by cream sample (% water loss)

Antimicrobial activity of cream: Total 1 gm of the cream formulations were aseptically added on 20 mL of Brain Heart Infusion (BHA) (BM0070, EO labs) broth and incubated at 37ºC for 48 hours on Incubator (Thermo Scientific). 50 µL of aliquots of the overnight cultures were then streaked on surfaces of Dehydrated Mannitol Salt Agar (MSA) (CM0085B, Thermo Fisher Scientific™) and MacConkey's agar (M081B, HiMedia™ Laboratories Pvt. Ltd.) separately. Such nutrients agar plates were incubated at 37ºC for 24 hours (Zeitounet al., 2015). The two bacterial strains, *E. coli* (ATCC-25922) and *S. aureus* (ATCC-25923), were separately cultured on sterilized Muller-Hinton Agar (MHA) plates (M173, HiMedia™ Laboratories Pvt. Ltd) at 37°C for 24 hrs by using streak plate method maintaining log phase of bacterial growth. Bacterial suspensions from cultured colonies were prepared and then turbidly were adjusted by visual inspection with 0.5 McFarland turbidity standards (R092, HiMedia™ Laboratories Pvt. Ltd) and adjusted bacterial suspensions were used as inocula within 15 minutes where a sterile cotton swab was dipped and streaked over the surface of sterilized MHA plates by lawn culture method. Sterile Kirby Bauer disks were taken, impregnated with formulation samples and control (Boroplus™ antiseptic cream) formulation at concentration of 100mg/mL with normal saline and allowed to diffuse for 15 minutes on previously prepared MHA plates. The MHA plates were then incubated in inverted position in an Incubator (Thermo Scientific) for 24 hours at 37ºC.The diameter of each formulation was measured by a measuring scale and taken as zone of inhibition.

Stability study: Different cream formulations stability were studied using freeze-thaw cycling method where creams were exposed to extreme temperatures (4ºC, 25ºC and 40ºC) for 7 days and such formulations were thawed at room temperature. The significant changes in color, phase separation, odor and pH were noted (Sekar & Halim, 2017).

Data analysis: All data were taken in triplicate. Data were analyzed by SPSS 16.0.0. For different formulation designations, design expert software (Version 10.0.7, Stat-Ease Inc., Minneapolis, Minnesota) was used.

Results and Discussion

Organoleptic characteristics of formulations

All formulations (F1-F17) exhibited the same organoleptic characteristics and result is presented in Table 5. Cream samples were within the acceptable organoleptic characteristics. This finding suggested that, the prepared cream possesses an acceptable basic property which is in agreement with the previous finding (Inoue et al., 2014).

Table 5: Organoleptic characteristics for polyherbal formulations

Organoleptic parameter	Observation
Color	Creamy white
Odor	Mild vanilla
Texture	Smooth
Phase separation	None
Grittiness	None

pH, spreadability and in vitro occlusivity of formulations

All formulations had acceptable pH value, spreadability and occlusivity in vitro (Table 6). pH (at 25°C) of all formulations (F1-F17) were found to be in range 5.52 to 5.91. Spreadability of all prepared formulations was found to be ranging from 5.30 cm to 6.10 cm and occlusivity (%) was ranged from 37.00 to 44.82. This all parameters were expressed in Table 6. Most creams feathered in a pH range of 5.00 to 5.20 indicating that they were moderately stable to slightly unstable emulsions (Bolling et al., 2005). The results show that formula no. 9 had good spreadability (average spreadability 6.10±2.00) as compared with other formula and considered as a satisfactory (Sabale et al., 2011).

Table 6: pH value measurement, spreadability test and *in vitro* occlusivity test results for different polyherbal creams (F1-F17) (Values are expressed as mean ± SD. N=3)

Formulation	pH (at 25°C)	Spreadability(cm)	Occlusivity(%) (mean ±SD)
F1	5.81±0.01	5.50±1.00	38.33±1.53
F2	5.83±0.02	5.40±1.00	37.00±2.21
F3*	5.58±0.02	5.30±2.00	43.00±1.46
F4	5.79±0.01	5.60±3.00	42.00±1.60
F5	5.91±0.03	6.00±2.00	37.50±0.91
F6	5.84±0.02	6.00±0.00	41.88±0.33
F7	5.73±0.01	5.80±1.00	44.00±1.22
F9	**5.75±0.03**	**6.10±2.00**	**39.81±2.21**
F10	5.88±0.02	5.80±0.00	40.23±1.65
F11	5.63±0.01	5.70±1.00	38.00±1.22
F15	5.63±0.02	5.90±0.00	44.82±1.56
F16	5.59±0.00	5.50±0.00	41.89±1.76
F17	5.52±0.01	5.70±1.00	39.00±2.12

Note : *F3, F8, F12, F13 and F14 formulations were same as designed by Box Behnken Design (BBD)

Table 7: Hydrogen peroxide (H_2O_2) scavenging activity of various polyherbal formulations (F1-F17)

Formulation	Inhibition percentage (Absorbance)				
	100 µg/mL (mean±SD)	200 µg/mL (mean±SD)	300 µg/mL (mean±SD)	400 µg/mL (mean±SD)	500 µg/mL (mean±SD)
F1	27.60±0.18	36.17±0.10	45.32±1.03	52.66±0.05	55.75±0.10
F2	56.09±0.14	59.96±0.16	62.65±0.13	65.14±0.16	69.94±0.11
F3*	65.54±0.17	65.78±0.13	68.52±0.08	71.16±0.10	72.44±0.17
F4	45.03±0.25	52.03±0.15	60.20±0.10	61.38±0.13	66.37±0.15
F5	26.57±0.09	36.07±0.14	57.76±0.12	57.75±0.09	61.32±0.12
F6	62.57±0.12	68.28±0.11	73.27±0.11	78.13±0.11	82.34±0.09
F7	61.38±0.09	63.63±0.11	66.32±0.15	72.44±0.13	76.10±0.10
F9	59.03±0.19	61.38±0.17	65.73±0.13	66.37±0.10	71.61±0.13
F10	31.13±0.18	45.86±0.13	55.94±0.09	60.35±0.12	65.73±0.14
F11	28.14±0.15	36.36±0.14	45.57±0.11	53.98±0.13	57.56±0.16
F15	62.65±0.08	66.27±0.15	69.60±0.12	72.05±0.10	75.37±0.14
F16	51.58±0.16	57.75±0.12	60.64±0.11	61.90±0.11	67.74±0.15
F17	25.74±0.10	31.27±0.13	35.34±0.17	45.47±0.14	53.50±0.08
Standard	70.04±0.11	74.98±0.12	80.32±0.11	85.26±0.09	90.25±0.13

Note: *F3, F8, F12, F13 and F14 formulations were same as designed by Box Behnken Design (BBD)

Hydrogen peroxide (H_2O_2) scavenging activity

For different formulations (F1-F17) the inhibition percentage were found to be ranging from 53.5% to 82.34% at 500 µg/mL concentration. The inhibition percentage of standard ascorbic acid was found to be 90.25% (Table 7).

Among different formulations F6 and F15 formulations showed best linear regression fit line means R^2 value were 0.9971 and 0.9962 with linearity equations y (% inhibition) =0.0494x [Concentration (µg/mL)] + 58.101 and y (% inhibition) =0.0312x [Concentration (µg/mL)] + 59.822 respectively.

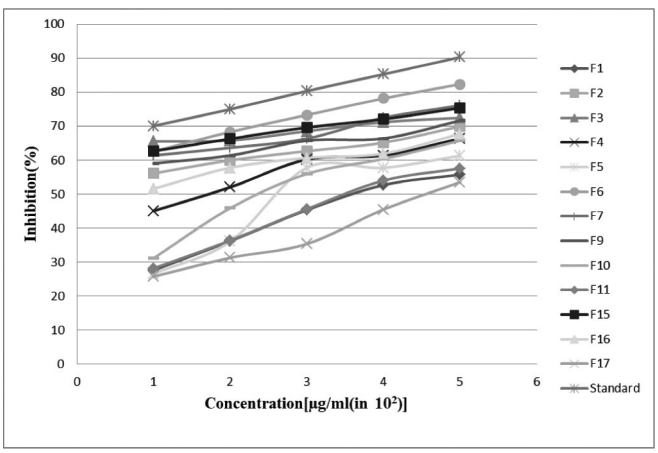

Figure 1: Hydrogen peroxide (H_2O_2) scavenging activity of various polyherbal formulations (F1-F17) showing IC75%

The standard ascorbic acid solution showed R^2 value 0.998 with linear regression equation, y (% inhibition) =0.0507x [Concentration (µg/mL)] + 64.968. Since, IC_{75} (µg/mL) value for F6 was 325.605 µg/mL and for F15 was 489.723 µg/mL, F6 polyherbal cream formulation showed more antioxidant property than others with comparison to ascorbic acid (IC_{75}=198.986 µg/mL) (Table 7).

The antioxidant herbs are mixed in the cream which exhibited the antioxidant property. Some plants used for cutaneous wound treatment have flavonoid compounds. Similar finding on the protective effect of mulberry extract on B[a] P-induced cytotoxicity in human keratinocytes was also supporting to present finding (McGhie, 2006; Woo et al., 2017). Several properties such as anti-inflammatory, anti-wrinkle are associated with the phenolic antioxidant phytochemicals found in our study and also associated with previous findings (Lee et al., 2014; Saric & Sivamani 2016). Similarly basil and curcuma also possesses a broad spectrum of biological potentialities, of which anti-inflammatory and cardio-protective effects are most often investigated (Goel et al., 2010; Kasprzak-Drozd et al., 2024).

Sun protection factor (SPF) determination

Absorbances of different formulations (F1-F17) at different wavelengths (290 nm, 295 nm, 300 nm, 305 nm, 310 nm, 315 nm and 320 nm) were shown in Table 8. All formulations had showed SPF value above 7 and were found to be ranging from 7.20 to 12.60 but F7 showed the highest SPF value 12.60. This result supports us to utilize it into modern cosmetic formulations. Hence it can be concluded that used extracts and oils have the good SPF values, a finding that will be helpful in the selection of perfumes during the formulation of sunscreens (Kaur & Saraf, 2010).

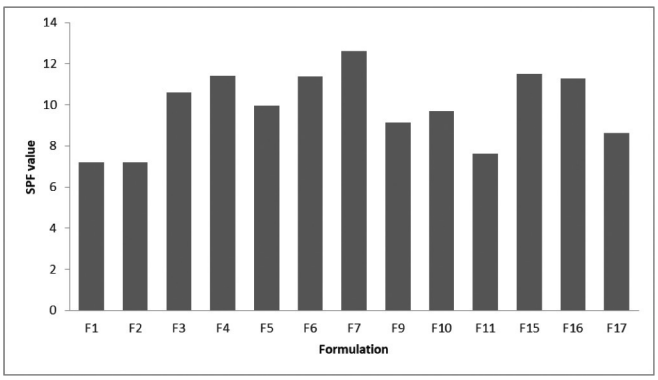

Figure 2: Calculated SPF value of various polyherbal formulations (F1-F17)

Antimicrobial activity

Zone of inhibition against *E. coli* by the prepared formulations were found to be ranging from 12 to 23 mm while the control product (Boroplus) showed the inhibition of 27mm. Also, zone of inhibition against *S. aureus* by the prepared formulations were found to be within the range of 12mm to 20 mm while that of Boroplus showed inhibition of 24 mm.

Table 8: Absorbance of different formulations for SPF value calculation

Formula No.	Absorbance						
	290 nm	295 nm	300 nm	305 nm	310 nm	315 nm	320 nm
F1	1.567±0.001	1.312±0.012	0.913±0.092	0.712±0.035	0.404±0.093	0.215±0.067	0.115±0.059
F2	1.563±0.001	1.204±0.005	0.813±0.082	0.763±0.022	0.512±0.035	0.217±0.038	0.110±0.005
F3	1.789±0.015	1.601±0.011	1.342±0.056	1.105±0.061	0.804±0.085	0.612±0.025	0.430±0.011
F4	1.813±0.095	1.658±0.056	1.407±0.150	1.112±0.185	0.806±0.092	0.605±0.059	0.437±0.025
F5	1.867±0.022	1.607±0.034	1.312±0.031	0.905±0.023	0.612±0.054	0.506±0.012	0.312±0.024
F6	1.800±0.003	1.559±0.020	1.302±0.008	1.107±0.012	0.998±0.035	0.617±0.010	0.567±0.009
F7	1.867±0.010	1.742±0.017	1.503±0.019	1.205±0.016	1.001±0.025	0.805±0.019	0.512±0.012
F9	1.500±0.001	1.312±0.004	1.106±0.003	0.857±0.015	0.732±0.018	0.512±0.210	0.312±0.013
F10	1.506±0.015	1.347±0.013	1.102±0.010	0.987±0.009	0.767±0.003	0.543±0.011	0.417±0.015
F11	1.653±0.018	1.206±0.007	0.983±0.008	0.793±0.009	0.406±0.016	0.227±0.020	0.109±0.013
F15	1.803±0.006	1.612±0.054	1.423±0.043	1.105±0.019	0.906±0.019	0.516±0.016	0.432±0.014
F16	1.800±0.017	1.500±0.012	1.289±0.018	1.102±0.023	0.987±0.010	0.647±0.017	0.423±0.010
F17	1.467±0.019	1.212±0.009	1.008±0.003	0.898±0.006	0.612±0.008	0.432±0.014	0.367±0.019

F7 had highest zone of inhibition (Figure 1).This study suggested that most of prepared formulations had moderate potential for antimicrobial activity except F7, where high potential antimicrobial activity were seen explaining herbals combination on topical delivery could be used to treat common skin conditions (El-Gied et al., 2015).

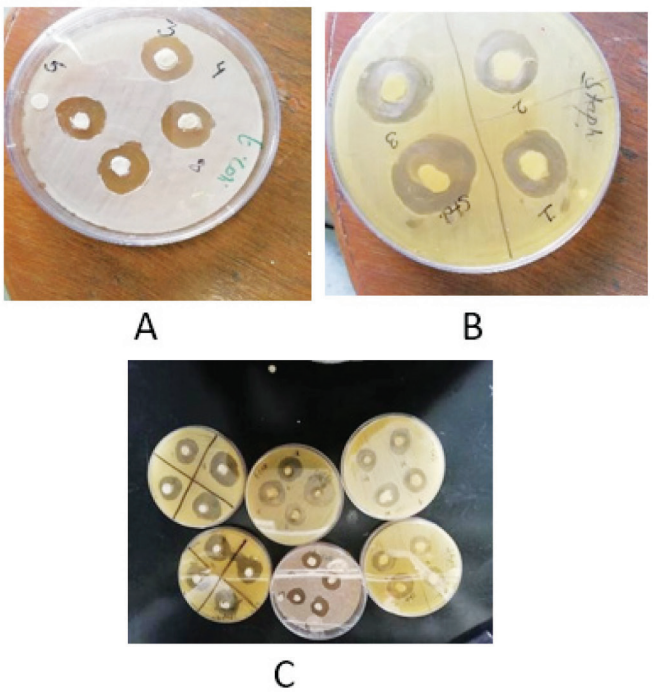

Figure 3: (A) zone of inhibition showing for *E. coli*; **(B)** zone of inhibition showing for *S. aureus;* **(C)** zone of inhibition showing for all formulations (F1-F17) for *E. coli* and *S. aureus*

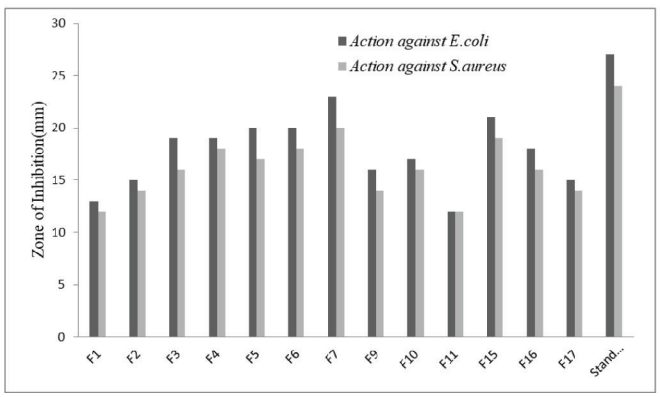

Figure 4: Zone of inhibition showing for formulations (F1-F17) for *E. coli* and *S. aureus*

Stability study

There were no significant changes on color, phase separation and odor in different polyherbal formulated creams. However, when the formulations were exposed at the different temperatures, the pH wass lightly influenced at different acceptable degrees (Figure 3). No significant visible changes was occurred during study time period showed that selected cream base was found to be stable with different herbal concentration designs (Sovová et al., 2023).

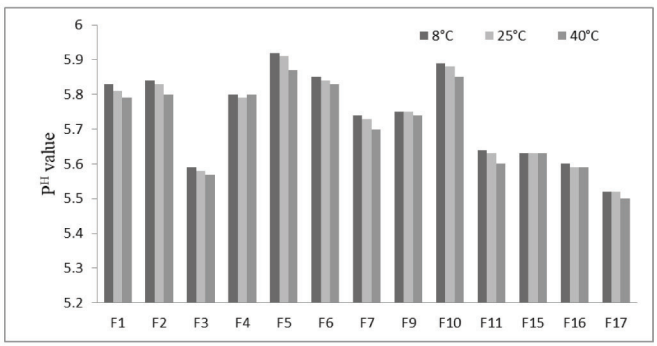

Figure 5: Effect of formulations (F1-F17) pH due to various temperatures (8°C, 25°C and 40°C) for 7 days

Experimental analysis

Fitting of data to the model: The independent variables were X_1=Liquorice extract concentration (w/v %), X_2=Mulberry extract concentration (w/v %) and X_3=Turmeric extract concentration (w/v %) where response variables were Y_1=Antioxidant activity (%), Y_2= Occlusivity (%) and Y_3=Antimicrobial activity (ZOI in mm) as suggested by Box Behnken Design (BBD)(3 factor, 3 level design) using Design Expert software (Version 10.0.7, Stat-Ease Inc., Minneapolis, Minnesota).The response polynomial equation for antioxidant optimum formulation activity (%) was Y_1=+72.44+7.28* X_1+5.62*X_2+3.04*X_3-0.22*X_1X_2-0.27*X_1X_3-O.84*X_2X_3+ 3.52*X_1^2-2.41*X_2^2-2.72X_3^2 where model was significant with p value <0.0001.The mean was found to be 68.37 with standard deviation of 0.75 and coefficient of variance (CV%) 1.10. The R- squared value was 0.9955; the predicted R^2 was 0.9281 which was in reasonable agreement with adjusted R^2 of 0.9897. Adequate precision showed signal to noise ratio. The adequate precision of this model was 44.814.Now, the response polynomial equation for occlusive optimum formulation test (%) wasY_2=+43.00+1.57*X_1+0.38*X_2+1.90 *X_3-0.31*X_1X_2+1.25*X_1X_3+0.95*X_2X_3-1.40*X_1^2-0.85*X_2^2-1.60$X_3^2$where model implied that the model was significant with p value <0.0001. The

mean was found to be 41.14 with standard deviation of 1.22 and CV% 2.97. The R- squared value was 0.8910. Adequate precision showed signal to noise ratio. The adequate precision of this model was 7.379. Also, the response polynomial equations for antimicrobial optimum formulation test (mm) for *E. coli* and *S. aureus* were Y_3^1=+19.00+1.50*X_1+1.13 *X_2+3.38*X_3+0.00*X_1X_2+0.00*X_1X_3+0.25*X_2X_3-0.13*X_1^2-1.38*X_2^2-0.87X_3^2 and Y_3^2=+15.88+1.12 X_1+0.87X_2+2.75X_3 respectively, where for Y_3^1, mode was significant with $p < 0.0001$. The mean was found to be 17.88 with standard deviation of 0.19 and CV% 1.06. The R- square value was 0.9981. Adequate precision value 75.030 showed signal to noise ratio and for Y_3^2 model was significant with p value <0.0001. The mean was found to be 15.88 with standard deviation of 0.28 and CV% 1.76. The R- squared value was 0.9870. Adequate precision value 57.187 showed signal to noise ratio.

Contour plots and response surface: For antioxidant response (Y_1), when liquorice aqueous extract concentration (X_1) and mulberry aqueous extract concentration (X_2) were increased Y_1 also increased rapidly but for Turmeric aqueous extract concentration(X_3), Y_1 increased slowly and get saturated action on Y1 when X_3 ≥7.2% as shown by Figures, suggesting that high aqueous extract concentration of mulberry and liquorice with moderate aqueous extract concentration of turmeric believed to show better antioxidant property- as in case of F6 composition (shown in Table 2) which showed experimentally high antioxidant property. Also, the concentration of turmeric extract had affected highly on antimicrobial property than other extracts concentration as shown by flat curve line in Figure 9. These different counter plot designs were performed, based on extracts concentration variation X_1, X_2 and X_3, for the determination of the model adequacy for cream formulations (F1-F17) to give maximum antioxidant (Y_1) and antimicrobial activity (Y_3) with acceptable cream occlusive (Y_2) property (Abd-El-Aziz et al., 2022).

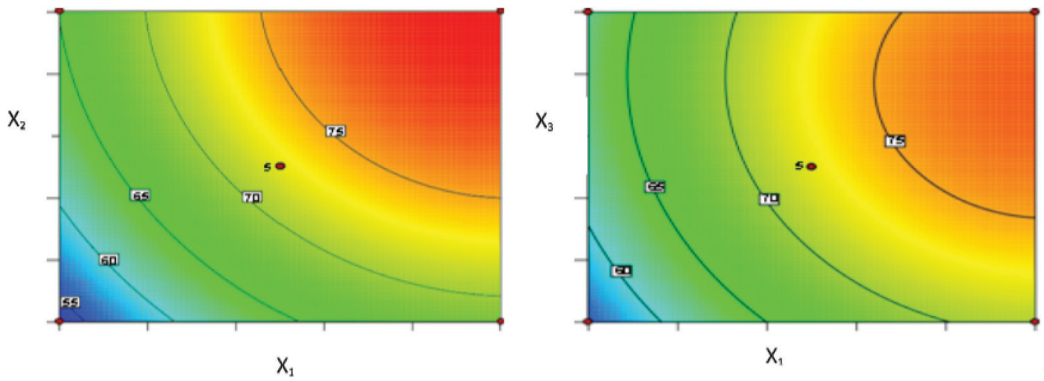

Figure 6: Contour plot of antioxidant activity (Y_1) vs liquorice concentration (X_1) and mulberry concentration (X_2)

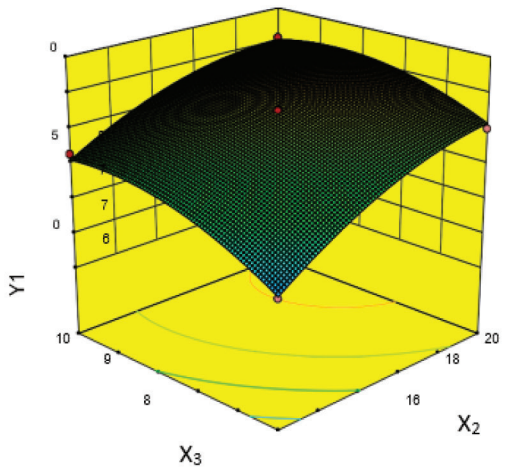

Figure 7: 3D plot of antioxidant activity (Y_1) vs mulberry extract concentration (X_2) and turmeric extract concentration (X_3)

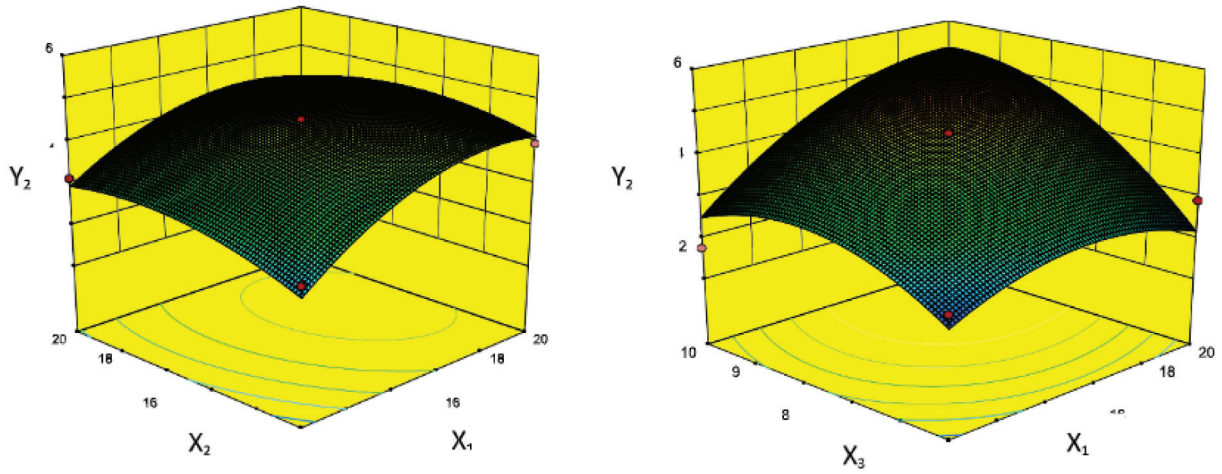

Figure 8: The X_1, X_2 and X_3 effect on % of occlusivity (Y_2), where Y_2 increased slowly with increase in X_1, X_2 and X_3 respectively

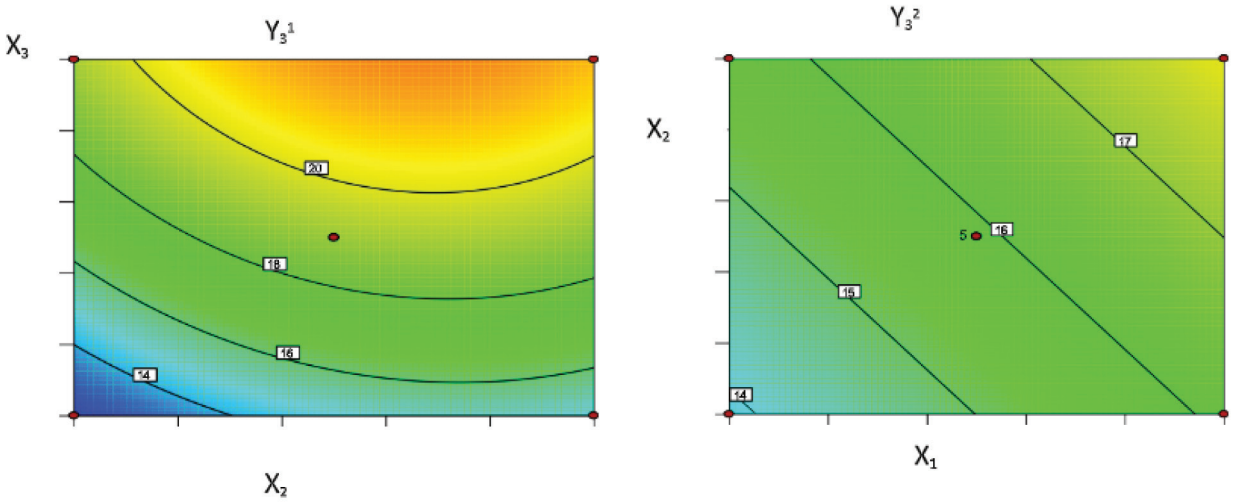

Figure 9: Contour plot of antimicrobial activity for *S. aureus* (Y_3^2) vs liquorice extract concentration (X_1) and mulberry extract concentration (X_2)

Conclusion

Polyherbal topical cream was designed and developed by Box Behnken Design (3 factor 3 level design) using Design Expert software (Version 10.0.7, Stat-Ease Inc., Minneapolis, Minnesota). The R-square value of each parameters were near to 1.00 and thus showed that the variation in the concentration of active constituents (X_1, X_2 and X_3) were fairly responsible for the change in responses (Y_1, Y_2 and Y_3^1, Y_3^2). This study showed that the formulations had mainly good antioxidant, sunscreen property and antibacterial properties and can be used instead of synthetic cosmetic creams for topical application.

Author Contributions

S K.C. performed laboratory work, D Basyal, R Gyawali supervised the work, A Acharya prepared draft manuscript and R Gyawali technically edited and finalized the publication.

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References

Abd-El-Aziz, N. M., Hifnawy, M. S., El-Ashmawy, A. A., Lotfy, R. A., & Younis, I. Y. (2022). Application of Box-Behnken design for optimization of phenolics extraction from *Leontodon hispidulus* in relation to its antioxidant, anti-inflammatory and cytotoxic activities. *Scientific Reports*, *12*(1).

Alzohairy, M. A. (2016). Therapeutics role of *Azadirachta indica* (neem) and their active constituents in diseases prevention and treatment. *Evidence-based Complementary and Alternative Medicine*, *2016* (11), 1-11.

Ayunin Q., Miatmoko A., Soeratri W., Erawati T., Susanto J., & Legowo D. (2022). Improving the anti-ageing activity of coenzyme Q10 through protransfersome-loaded emulgel. *Sci Rep*., *12*(1), 906.

Baral, S. R. & Kurmi, P. P. (2006). *A compendium of medicinal plants in Nepal*. Rachana Sharma.

Bolling, J. C., Duncan, S. E., Eigel, W. N., & Waterman, K. M. (2005). Processing effects on physicochemical properties of creams formulated with modified milk fat. *Journal of dairy science, 88*(4), 1342-1351.

Chang, B., Koo, B., & Kim, S. (2021). Pharmacological activities for *Morus alba* L., focusing on the immunostimulatory property from the fruit aqueous extract. *Foods*, *10*(8), 1966.

Chaudhary, H., Kohli, K., Amin, S., Rathee, P., & Kumar, V. (2011). Optimization and formulation design of gels of diclofenac and curcumin for transdermal drug delivery by Box-Behnken statistical design. *Journal of Pharmaceutical Sciences*, *100*(2), 580-593.

Chavan, P., Kalshetti, M., & Navindgikar, N. (2020). Formulation and evaluation of polyherbal cream. *International Journal of Current Pharmaceutical Research*, *12*(4), 75-77.

Chen, M. X., Alexander, K. S., & Baki, G. (2016). Formulation and evaluation of antibacterial creams and gels containing metal ions for topical application. *Journal of Pharmaceutics*, *2016*, 110.

Damle, M. (2014). *Glycyrrhiza glabra* L. (Liquorice)-A potent medicinal herb. *International Journal of Herbal Medicine*, *2*, 132-136.

Department of Plant Resources.(2016). *Medicinal plants of Nepal (Revised)*.

Dutra, E. A., Da Costa Oliveira, D. a. G., Kedor-Hackmann, E. R. M., & Santoro, M. I. R. M. (2004). Determination of sun protection factor (SPF) of sunscreens by ultraviolet spectrophotometry. RBCF. *Revista Brasileira De Ciências Farmacêuticas*, *40*(3), 381-385.

El-Gied, A. a. A., Abdelkareem, A. M., & Hamedelniel, E. I. (2015). Investigation of cream and ointment on antimicrobial activity of *Mangifera indica* extract. *Journal of Advanced Pharmaceutical Technology and Research, 6*(2), 53.

Goel, A., Kumar, S., Singh, D. K., & Bhatia, A. K. (2010). Wound healing potential of *Ocimum sanctum* Linn. with induction of tumor necrosis factor-alpha. *Indian journal of experimental biology*, *48*(4), 402-406.

Gyawali, R., & Paudel, P. N. (2022). Herbal remedies in cosmeceuticals formulation: A review on Nepalese perspectives. *Annapurna Journal of Health Sciences*, *2*(1), 59-65.

Heng, M. C. (2017). Topical curcumin: A review of mechanisms and uses in dermatology. *International Journal of Dermatology and Clinical Research*, *3*(1), 010-017.

Hossain, M. A., Al-Toubi, W. A., Weli, A. M., Al-Riyami, Q. A., & Al-Sabahi, J. N. (2013). Identification and characterization of chemical compounds in different crude extracts from leaves of Omani neem. *Journal of Taibah University for Science*, *7*(4), 181-188.

Imran, M., Khan, H., Shah, M., Khan, R., & Khan, F. (2010). Chemical composition and antioxidant activity of certain Morus species. *Journal of Zhejiang University SCIENCE B*, *11*(12), 973-980.

Inoue, Y., Suzuki, K., Maeda, R., Shimura, A., Murata, I., & Kanamoto, I. (2014). Evaluation of formulation properties and skin penetration in the same additive-containing formulation. *Results in pharma sciences, 4*, 42-49.

Ju, H. S., Li, X. J., Zhao, B. L., Han, Z. W., & Xin, W. J. (1989). *Yao xue xue bao = Acta pHarmaceutica Sinica, 24*(11), 807-812.

Kasprzak-Drozd, K., Niziρski, P., Hawry³, A., Gancarz, M., Hawry³, D., Oliwa, W., Pa³ka, M., Markowska, J., & Oniszczuk, A. (2024). Potential of Curcumin in the Management of Skin Diseases. *International journal of molecular sciences, 25*(7), 3617.

Kaur C.D., & Saraf S. (2010). *In vitro* sun protection factor determination of herbal oils used in cosmetics. *Pharmacognosy Res. 2*(1), 22-25.

Lee K.O., Kim S.N., & Kim Y.C. (2014). Anti-wrinkle effects of water extracts of teas in hairless mouse. *Toxicol Res*., *30*(4), 283-289.

Mali, A. S., Karekar, P., & Yadav, A. V. (2015). Formulation and evaluation of multipurpose herbal cream. *International journal of science and research, 4*(11), 1495-1497.

Malik, S., de Mesquita, L. S. S., Silva, C. R., de Mesquita, J. W. C., de Sá Rocha, E., Bose, J., Abiri, R., de Maria Silva Figueiredo, P., & Costa-Júnior, L. M. (2019). Chemical profile and biological activities of essential oil from *Artemisia vulgaris* L. cultivated in Brazil. *Pharmaceuticals (Basel, Switzerland*), *12*(2), 49.

Mansur, J. S., Breder, M. N. R., Mansur, M. C. A., & Azulay, R. D. (1986). Determinação do fator de proteção solar por espectrofotometria. *An Bras Dermatol Rio de Janeiro, 61*, 121-124.

Maru, A. D., & Lahoti, S. R. (2018). Formulation and evaluation of moisturizing cream containing sunflower wax. *Int J Pharm Pharm Sci*, *10*(11), 54-59.

McGhie, T. K., Rowan, D. R., & Edwards, P.J. (2006). Structural identification of two major anthocyanin components of boysenberry by NMR spectroscopy. *J Agric Food Chem*. *54,* 8756-61.

Pal, S., Aku, R., & Mahajon, B. (2016). Medicinal uses of amalaki (Indian gooseberry): a historical review. *International Journal of Science and Research Methodology*, *5*(1). 536- 548.

Pharmaceutical and Medical Device Regulatory Science Society of Japan. (2022). *Japanese Pharmacopoeia* (18th ed.).

Rajasree, P. H., Vishwanad, V., Cherian, M., Eldhose, J., & Singh, R. (2012). Formulation and evaluation of antiseptic polyherbal ointment. *International Journal of Pharmacy & Life Sciences*, *3*(10).

Ruch, R. J., Cheng, S., & Klaunig, J. E. (1989). Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis*, *10*(6), 1003-1008.

Sabale, V., Kunjwani, H., & Sabale, P. (2011). Formulation and *in vitro* evaluation of the topical antiageing preparation of the fruit of *Benincasa hispida*. *Journal of Ayurveda and integrative medicine*, *2*(3), 124-128.

Saini, R. (2011). Coenzyme Q10: The essential nutrient. *Journal of pharmacy & bioallied sciences*, *3*(3), 466-467.

Sanghi, D. K., & Tiwle, R. (2016). Formulation and characterization of herbal face wash/scruber. *European Journal of Pharmaceutical and Medical Research*, *3*(11), 274-278.

Saric S., & Sivamani, R. (2014). Polyphenols and sunburn. *Int J Mol Sci.*, *17*(9), 1521.

Sathyaseelan, S., Rao, B. H. , & Anushmati S. (2024). Cosmeceuticals: A transit state from synthetic to natural. *Indian J Pharmacol.*, *56*(1), 42-51.

Sekar, M., & Halim, F. (2017). Formulation and evaluation of natural anti-acne cream containing *Syzygium samarangense* fruits extract. *Annual Research & Review in Biology*, *17*(3), 1-7.

Singh, J., Singh, R., & Kumar, S. (2012). Comparing of antioxidant and H_2O_2 induced free radical scavenging activity of *Sesbania grandiflora* and *Acacia nilotica* plants. *Journal of Scientific & Innovative Research, 1*(2), 51-59.

Sohn, H., Son, K., Kwon, C., Kwon, G., & Kang, S. (2004). Antimicrobial and cytotoxic activity of 18 prenylated flavonoids isolated from medicinal plants: *Morus alba* L., *Morus mongolica* Schneider, *Broussnetia papyrifera* (L.) Vent, *Sophora flavescens* Ait and *Echinosophora koreensis* Nakai. *Phytomedicine*, *11*(7-8), 666–672.

Soumya, A., Rasheed, S., & Manjunath, S. (2020). Formulation and evaluation of herbal cream containing extracts of *Murraya koenigii* and *Cajanus cajan*. *Int. J. Res. Phytochem. Pharmacol. Sci. 1*, 58-63.

Sovová, S., Megyesi, Š. L., Mudroňová, D., Mudroň, P., Harvanová, J., Rendošová, M., Goga, M., Tkáčiková, Ľ., & Vargová, Z. (2023). Antibacterial activity, quality and stability study of creams with new potential silver(I) complexes and *in vivo* case report. *Acta Pharmaceutica, 73*(2), 311-323.

Vishnoi, H., Bodla, R., & Kant, R. (2018). Green tea (*Camellia sinensis*) and its antioxidant property: a review. *International Journal of Pharmaceutical Sciences and Research*, *9*(5).

Woo, H., Lee, J., Park, D., & Jung, E. (2017). Protective Effect of Mulberry (Morus alba L.) extract against Benzo[a]pyrene induced skin damage through inhibition of aryl hydrocarbon receptor signaling. *Journal of agricultural and food chemistry*, *65*(50), 10925-10932.

Yasheshwar, N., Gaurav, N., Ekkbal, R., Gupta, P., & Gyawali, R. (2024). System Pharmacological approach to investigate and validate multitargeted and therapeutic effect of furocoumarins of *Apium graveolens* L. for treatment of kidney disease. *Advances in Pharmacological and Pharmaceutical Sciences*, *2024* (3), 1-13.

Zeitoun, H., Kassem, M., Raafat, D., AbouShlieb, H., & Fanaki, N. (2015). Microbiological testing of pharmaceuticals and cosmetics in Egypt. *BMC microbiology, 15*, 1-13.