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FORMULATION, CHARACTERIZATION AND ANTIMICROBIAL ANALYSIS OF CREAM CONTAINING *BERBERIS CALLIOBOTRYS* EXTRACT

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ABSTRACT

This study was aimed to formulate and evaluate the cream (W/O) containing methanolic extract of *Berberis calliobotrys.* Antibacterial potential of plant was use as a key to develop and characterize a dosage form. Extraction was carried out by maceration and minimum inhibitory concentration was determined by broth microdilution method. MIC values obtained when compared with amoxil as antibiotic control were 2.08, 2.07, 3.33, 2.08 and 2.5 of *B.subtilis, S.auerus, S.epidermidis, P.aeroginosa andE.coli* respectively.

W/O base was formulated by using paraffin oil, bees wax and tween 80 as emulsifier. Base was then formulated into cream with 5%, 7.5%, 10% and 15%concentrations of extract. Stability of both base and formulations were characterized by placing them at 8°C, 25°C, 40°C, 40°C +70%RH for 60 days. Different physicochemical parameters were determined at regular interval like the change in color, odor, phase separation, liquefaction, and pH. Both base and formulations were found to be stable. However formulations with 15% extract showed instability.

5%, 7.5% and 10% creams were evaluated for antibacterial activity by well diffusion method. When compared with standard result obtained for 10% formulation was significantly not

different with antimicrobial order *S. aureus*> *B. subtilis*> *P.aeruginosa*> *E.coli.* however *S.epidermidis*exhibited quite significant results at all concentration.

Permeation test was performed for 24 hrs by vertical franz diffusion cell. Percentage release obtained was 80% with flux of 0.186705ug/cm2/h.

Results showed that *Berberis calliobotrys*can be successfully incorporated in W/O emulsion for treatment of topical skin infections.

Keywords: *Berberis calliobotrys,* MIC, antibacterial activity, franz diffusion cell, W/O

INTRODUCTION

Humans are natural hosts for many microbial species that reside on the skin as normal flora. *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* are known as major cutaneous pathogens[1]. Most of the bacterial skin infections like Impetigo, Erysipelas [2], Cellulitis [3], Necrotizing fasciitis [4] , and Folliculitis [5] are caused by these cutaneous culprits.

Various medicinal plants have been evaluated for their antimicrobial activities and are being utilized as an alternate therapy for treatment of infectious diseases and these natural products have gained much importance during the last few decades due to their therapeutics benefits.[6].

Creams are frequently use as vehicle for drug delivery and plants can be use as natural remedy by incorporating them in this formulation. For dermatological therapy these topicals are prepared to exert their action on outer layer of skin at specific site [7]. Pakistan is rich with floral biodiversity and hence, in the current research an indigenous medicinal plant i.e. *Berberis calliobotrays* (*B. calliobotrays*) and its formulation (cream) has been investigated for its antimicrobial activity. *Berberis calliobotrys*, known commonly by name shin zaralga [8], Chowenj [9], Zarch, Karoski [10]. Plant is found in,Gilgit and Chitral, Pakistan and Afghanistan [11] and particularly in Gilgit-baldistan and CKNP [12]. Fruit of plant is use for fever after boiling it in water. For headache topical paste is prepared by mixing equal amount of rhizome (bark), butter and black pepper. For phyrangitis, bark decoction is prepared for gargles and for treating infection of GIT [10]. *B. calliobotrays* claimed to be use as anticonvulsant [9], anti-arthritic [13], anti inflammatory, antipyretic and analgesic [11].

MATERIAL AND METHOD

Chemical and solvents

Methanol, Potassium Dihydrogen Phosphate, Sodium Hydroxide and paraffin oil were obtaimed from Merck Chemicals, Germany. Mueller Hinton Agar and Mueller Hinton Broth were purchased from Oxoid, England. Beeswax from Sigma- Aldrich, U.S.A and Tween 80 was purchased from Riedel-deHaen, Germany.

Bacterial Strains

*Bacillus subtilis (*ATCC 6633), *Esherichia coli (*ATCC 8739) , *Pseudomonas aeruginosa (*ATCC 27853), *Staphylococcus aureus (*ATCC 25923), *Staphylococcus epidermidis (*ATCC 12228).

Preparation of *B. calliobotrays* extract

Collection and Extractionof plant

Berberis calliobotrys was collected from collected from Quetta. After washing and drying withstem and branches, dried plant was pulverized to powder in electric blender.Methanolic extractwas prepared by soaking 500g of powdered plant in 1500 ml of analytical grade methanol for seven days and sealed with aluminium foil. Macerated plant was filtered by several layers of muslin cloth and then by Whatman # 01 filter paper. Brownish colored extracts obtained was stored in tightly closed containers in refrigerator at 4°C[14].

PREPARATION AND CHARACTERIZATION OF CREAM

Preparation of cream

Ten bases were preparedwith different quantity of paraffin oil, beeswax and tween 80 as shown in Table 1. Both the oil phase (beeswax, paraffin oil and tween 80) and the aqueous phase was heated separately up to 75 ± 5 °C at the same time. The aqueous phase was added in the oily phase. W/O emulsion thus obtained was stirred mechanically at 2000rpm for 10 min for then reduced to 500 rpm for 5 min to cool down emulsion at room temperature [15, 16]. In order to prepare cream, ingredients were incorporated and desired amount of extract was added to make 5%, 7.5%, 10% and 15% cream.

Table 1: Composition of Base with different quantity of ingredients

Formulations w/w	Excipients				Results
100grams	Bees wax	Paraffin oil	Tween 80	Distilled warter	
1	8.89gm	20.74gm	5.5gms	q.s	No Emulsification
2	8.89gm	20.74gm	6 gms	q.s	No Emulsification
3	8.89gm	20.74gm	6.5gms	q.s	No Emulsification
4	8.89gm	20.74gm	7 gms	q.s	No Emulsification
5	8.89gm	20.74gm	8 gms	q.s	Light Emulsification
6	8.89gm	20.74gm	8.89 gms	q.s	Good
7	8.89gm	20.74gm	9.5gms	q.s	Greasy
8	8.89gm	20.74gm	10.5 gms	q.s	Oil separated
9	8.89gm	20.74gm	11 gms	q.s	Oil separated/Instable
10	8.89gm	20.74gm	11.5gms	q.s	Unstable

CHARACTERIZATION OF CREAM

STABILITY ANALYSIS OF CREAM

Bases and formulation were divided in four parts and stored at 25°C (room temperature), 8°C (in refrigerator, 40°C (in incubator) 40°C+75%RH (in incubator). All samples were evaluated for any change in physical characteristicslike appearance, color, concentration, phase separation and creamingat different time interval of 2 months [15]

Emulsion Type

Type of emulsion was determinedby diluting emulsion with oil and water.

Test for pH

Digital pH meter was used to determine pH. For this suspension of cream is prepared by dispersing 0.5 g of cream in 50 ml of water [17].

Centrifugation Test

Centrifugation test was performed by running 5g of sample in centrifugal tubes at 5000rpm for 10 minutes at 25°C [18].

ANTIBACTERIAL EVALUATION

Minimum Inhibitory Concentration of Extract.

Microdilution method was used.This method was carried out in sterile 96-well microtitre plate. 50 µl of nutrient broth and bacterial suspension was dispensed in the first row of microtitre plate (well 1 to 12). From the stock solution of extract 200mg/ml, 100 µl was dispensed to well 2. After mixing, 100 µl was removed from 2nd well and dispensed into 3rd well. This twofold dilution was done upto 11th well and 100 µl was discarded from last one. Sequential concentration obtained was 20mg/ml to 0.0195mg/ml. Well 1 kept as growth control (broth + inoculum) and Well 12 was kept as positive control or antibiotic control (broth + inoculum+ antibiotic). Amoxicillin (1mg/ml) was used as positive control [19]. Same procedure was done for all the strains and micropipette tip was discarded with each use. Plates were incubated for 24hours at 37°C and checked for resulting turbidity. A concentration where no more growth is visible as determined was MIC [20].

Preparation of inoculum

Inoculum was prepared by growth method. Sterile loop full of specific colony was transferred to Sterile Mueller Hinton broth. The broth was incubated at 37°C until turbidity visibly matched with that of 0.5 McFarland standard [21].

Procedure of Antibacterial Testing of Cream

In te inoculated plate, five wells of 6mm diameter were punched with the help of sterile borer. 0.1ml of 5%, 7.5% and 10% cream slurry (1mg/ml) was dispensed in

marked wells. Positive control (standard cream (1mg/ml) and negative control (base without active) was dispensed in remaining two wells and incubated for 24 hours at 37°C in incubator. zone of bacterial growth inhibition was determined by digital vernier caliper. Same procedure was carried out with all test strains and repeated thrice [22, 23].

RELEASE STUDIES

***Invitro* Permeation Test**

10% cream with maximum zone of inhibition was evaluated for its skin permeation by Vertical Franz diffusion cells with 1.767cm^2 surface area for diffusion. Both receptor and donor compartments were filled with phosphate buffer 7.6.1 g of cream was applied on membrane filter paper 0.45 micrometer pore size in both compartments. Magnetic bar (Teflon coated) was used in both compartments at 500 rpm. Temperature of whole apparatus is maintained at 37°C by water bath and peristaltic pump.

Samples were taken at different time intervals for 24 hours. Each time compartment was refilled by buffer used. Absorbance was measured spectrophotometrically at a wavelength of 279 nm against their respected blank[17].

STATISTICAL ANALYSIS

Data that is obtained in the test was evaluated and expressed as mean ± Standard deviation. To determine the difference in data, ANOVA was applied with 5% level of significance.

RESULTS AND DISCUSSION

Stability Testing

Stability of cream has been a paramount parameter, however evaluation of its stability is a critical step in its production [24]. A stable emulsion would have unchanged amount of dispersed phase in given amount of countinous phase, would retain its physicochemical properties and its elegancy throughout its shelf life [25].

Physical Evaluation

Emulsion base and formulation developed in this study were smooth in touch without any grittiness or foreign dust particles, thus easing their application on skin without irritation.

Color

Colorof base was white and that was unchanged throughout stability studies at all temperatures. 5% and 7.5% extract containing formulations were orange in color while 10% and 15 % formulation containing extract were amber orange in color due to presence of extract in greater quantity. Color of all formulations remained unchanged. This might be due to, colorless, nontoxic and non fluorescent nature of excipients including paraffin oil and tween 80 [26,27].

Growth of microorganism in formulation might also cause the change in color[28],since *Berberis calliobotrys* has been reported to have antimicrobial activity [9], so it might have inhibited growth of those microbial strains and resisted any color change of formulation.

Liquefaction

Greater viscosity of an emulsion greater will be its stability with improved shelf life [29]. Maintaining the viscosity of dispersed phase prevents creaming of emulsion according to stokes law [30]. Base showed no liquefaction at all storage specifications. In case of formulations, 5%, 7.5%, and 10% cream showed stability with minor liquefaction on 60^{th} day at 40°C and 40°C+75% RH. However, in 15% cream liquefaction was observed at 40°C+ 75% RH on 14^{th} day and at 40°C it was observed on 21^{st} day of study. Instability of 15% cream might be due to presence of excess of extract which disturbed the efficiency of emulsifier.

Phase Separation

Density of a dispersed phase usually leads to the separation of the two phases. Difference between densities of two phases also leads to separation of phases[31].

In the study, cream base and 5%, 7.5%, and 10% formulated creams have shown better results and no phase separation was observed except minor at 40°C and 40°C+ 75% RH. This might be attributed to excipients. Tween 80 improves emulsion stability by displacing surface protein and forms closely packed layer of surfactant surrounding the interface[32]. However, prominent separate phases were observed in 15% cream on 21^{st} at 40°C+ 75% RH and 28^{th} day at 40°C. This instability and phase separation of 15% cream was possibly due to high temperature or surfactant molecules may shift from interface and moves toward surface [33].One more reason of coalescence might be the greater energy of colliding particles than the energy that support dispersion [34].

Centrifugation Test

The effect of gravity on stability of cream was determined by centrifugation test. This test was performed at regular interval on base and formulations up to 60 days. Cream base and formulations showed compliance with accelerated study and no phase separation was observed. However, 15% cream showed instability from 21^{st} day to 60^{th} day at both 40°C + 75% RH and 40. This instability might be due to improper homogenization [34].

Test for pH

The pH of a typical formulation like creams affects the absorption and penetration of through stratum corneum to inner layers of

skin [23]. pH of freshly prepared cream base was 5.67. it decrease with the passage of time. In the case of formulation, 5%, 7.5%, 10% freshly prepared cream has pH value 5.72, 5.82, and 5.88 respectively. All of these formulations showed minor fluctuations in whole study duration. 5% cream has shown the value of pH at 60th day 5.23, 5.30, 527 and 5.15 at 8°C, 25 °C, 40 °C and 40°C+ 75% RH respectively. In case of 7.5% cream, pH decreased from 5.82 to 5.31, 5.34, 5.47, and 5.43 on 60th day at 40°C+ 75%RH, 40 °C, 25 °C, and 8°C respectively. In 10% cream, pH declined more and value dropped to 4.89, 498, 5.07, and 5.11 on 60th day at 40°C+ 75%RH, 40 °C, 25 °C, and 8°C respectively.

As a whole, pH of both base and formulations ranges from 5 to 6 approximately which lies within range of human skin pH i.e. 4.5-6 [35]. Decrease in pH might be due to acidic metabolites of plant extract which has been released from the extract with time. Moreover oxidation of paraffin oil into organic acid and aldehyde might be the reason for lowering of the Ph of cream to acidic nature [36, 37].

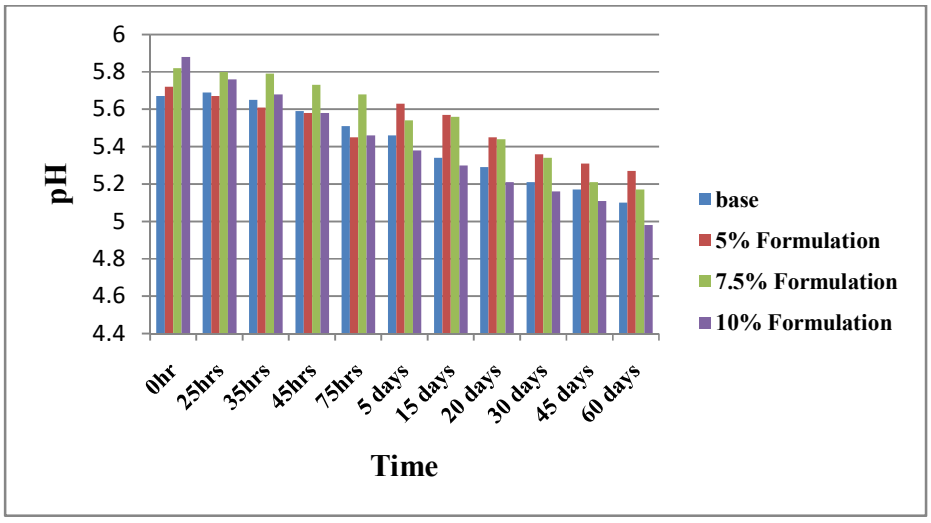


Figure: 1 pH values of base and formulations kept at 8°C

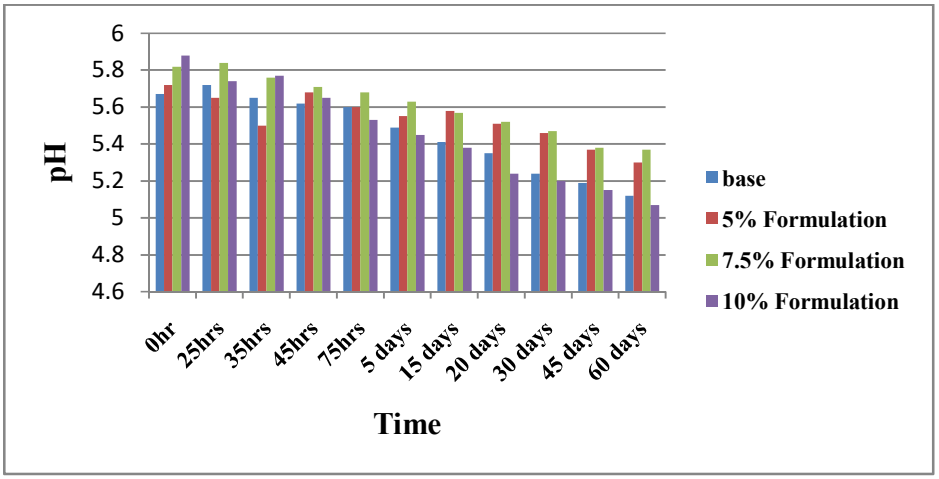


Figure: 2 pH values of base and formulations kept at 25°C

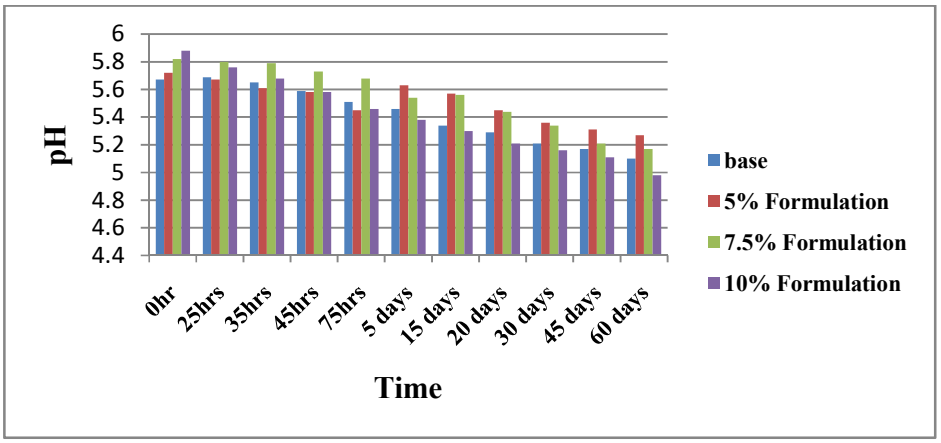


Figure: 3 pH values of base and formulations kept at 40°C

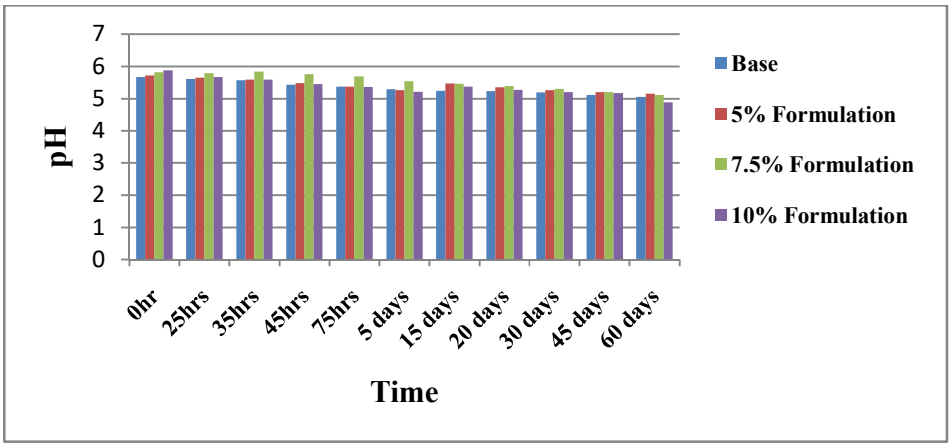


Figure 4: pH values of base and formulations kept at 40°C± 75%RH

ANTIMICROBIAL EVALUATION

Minimum Inhibitory Concentration of Extract

The plant was evaluated for its anti bacterial effect using, minimum inhibitory (MIC). MIC values determined were 2.08, 2.08, 2.07, 3.33 and 2.5 against *B. subtilis, P.aeruginosa, S. aureus, S. epidermidis* and *E.coli* respectively (Figure 5). The outcomes of the study for MIC against *B. subtilis, P.aeruginosa,* and *S. aureus* were in concurrence with the prior investigations [9]. *B. subtilis* showed inhibition at concentration 2.08 which was same as reported, whereas MIC values for *P.aeruginosa* and *S. aureus* were 2.08 and 2.07 respectively, which were comparable to the reported value i.e. 2.06 and 2.05 respectively [9]. This variation in MIC values was possibly due to change in environmental condition, source of plant collection or experimental condition. MIC values against *S. epidermidis* and *E.coli* has been reported first time from this plant species, which shows concurrence with the antibacterial study on *Juniperus oxycedrus* [38].

The least MIC value demonstrated that low concentration of extract was required to inhibit the bacterial strains. The greater values of MIC showed that extract has low antibacterial action. It has been evident from the results that methanolic extract of *B.calliobotrys* is more effective for infections caused by *S. aureus* [39].

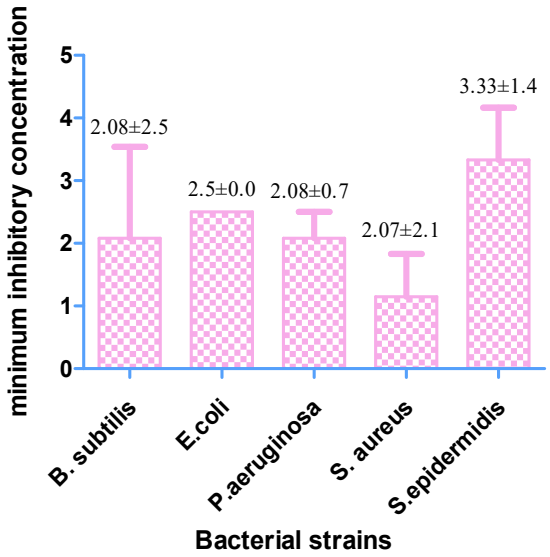


Figure 5: MIC value of of methanolic extract of *B. calliobotrys* on bacterial strains

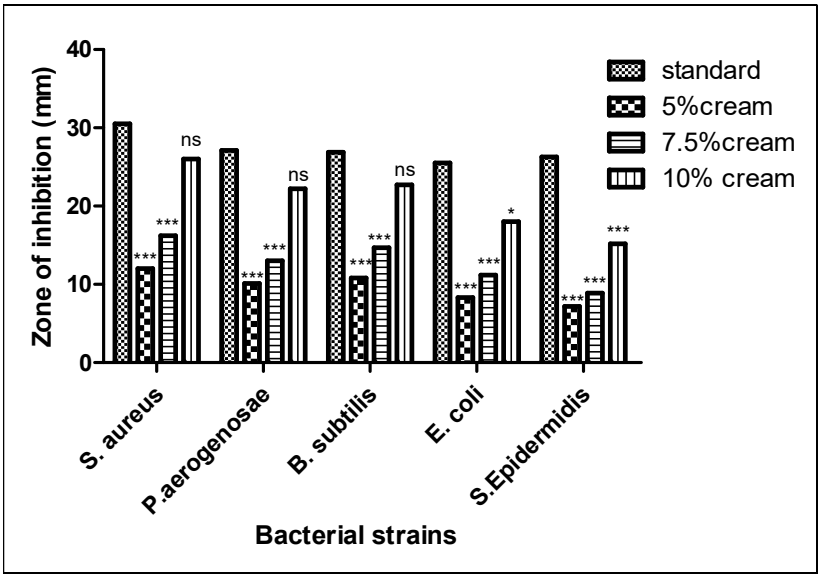
Antibacterial Activity of Cream

Cream was evaluated against *B. subtilis, P.aeruginosa, S. aureus, S. epidermidis* and *E.coli.* Bacterial strains used are less sensitive to 5% and 7.5% formulation and showed significant $p<0.001$ results as compare to standard cream. 5 % and 7.5 % formulation has lower activity (Table 2) because of presence of smaller amount of extract [17]. At low concentration, significant difference might be due to presence of tween 80. Which is supposed to bind to phenolic components of extract (which exert antibacterial activity), thus in turn at low concentration of phenolic compounds bacterial inhibition is retarted [40]. The formulation containing 10% of extract exhibited non significant results against*B. subtilis, P.aeruginosa* and *S. aureus*(Table 2). This result has been supported by the study of handali *et al.,* on anti-bacterial cream containing *Oxalis corniculata* which demonstrated thatwith increase in concentration of extract, in cream antibacterial acticvity also [41] similar results were shown by Dahalan *et al*., in his study on evaluation of antibacterial activity of cream containing extract of *Andrographis paniculata* [42] and from study of Ajala *et al*., in evaluating antibacterial cream containing extract of *Phyllanthus amarus* [23] . Order of sensitivity of bacterial strains for methanolic extract of*B. calliobotrys* is *S. aureus> B. subtilis> P.aeruginosa> E.coli> S. epidermidis* (Figure 6). Significant sensitivity of *S. aureus* is a matter of interest, because this microorganism might infect deep into skin and do not get off by single rubbing or washing [43] and adherent property of cream helps in diffusing the extract for long duration [44].

Table 2: Zone of inhibition (millimeters) of standard and formulations containing 5%, 7.5% and 10% extract

Microbial strains	Standard (mm)	5% Formulation (mm)	7.5% Formulation (mm)	10% Formulation (mm)
S. *aureus*	**30.5±0.3**	**12.0±0.2**	**16.2±0.2**	**26.0±0.3**
P.aerogenosae	**27.1±0.3**	**10.1±0.2**	**13.0±0.3**	**22.2±0.2**
B. subtilis	**25.5±0.2**	**10.8±0.1**	**14.7±0.2**	**22.7±0.2**
E. coli	**26.9±0.8**	**8.3±0.4**	**11.2±0.3**	**18.0±0.3**
S.Epidermidis	**26.3±0.3**	**7.2±0.1**	**8.9±0.3**	**15.2±0.3**

Values are expressed as Mean± S.D (n= 3)



*****= highly significant, ns= non significant, *= significant**

Figure: 6: Zone of inhibition of formulations in comparison with standard

PERMEATION TEST

Permeation test has been usually performed for topical preparations to evaluate release of active drug from formulation. The affinity of active constituent with base and viscosity of topical formulation are the two factors that could affect the release of active from formulation [45, 46]. 10% cream (formulation with 10% extract) was tested for its *in-vitro* release profile by using Franz diffusion cell. About 60% of the total active constituenthas crossed membrane in 6 hours, however extended studied were carried out to evaluate the release pattern for 24 hours. Drug release profile showed 80% release after 24 hours with 0.186705 ug/cm^2/h flux (Figure 7). Rate of drug release per unit time (flux) was calculated by using Fick's law of diffusion as expressed in equation 1 [47].

J= Kp. Cd……Eq 1

Where,

J= flux, Kp= Permeability coefficient, Cd= concentration in donor compartment.

Release profile of the formulation in this study was in correspondent with release pattern of herbal cream documented by [48]with percentage release of 74%. The release profile of current formulation was supported by results of sandhya *et al*., showed 72% release over 12hrs [49]. The delayed release pattern might be due to presence of hydroxyl group on cellulose membrane that absorb the solvent from both donor and receptor cell and thus increase thickness and alter partition coefficient [50, 48].

Cellulose membrane used is considered best for the test as it do not contain any leachable substance that interact with the analytical

procedure. Synthetically prepared membranes are more preferable because of their simplicity and easy availability [51] and also these membranes have rate limiting characteristics like skin [52].

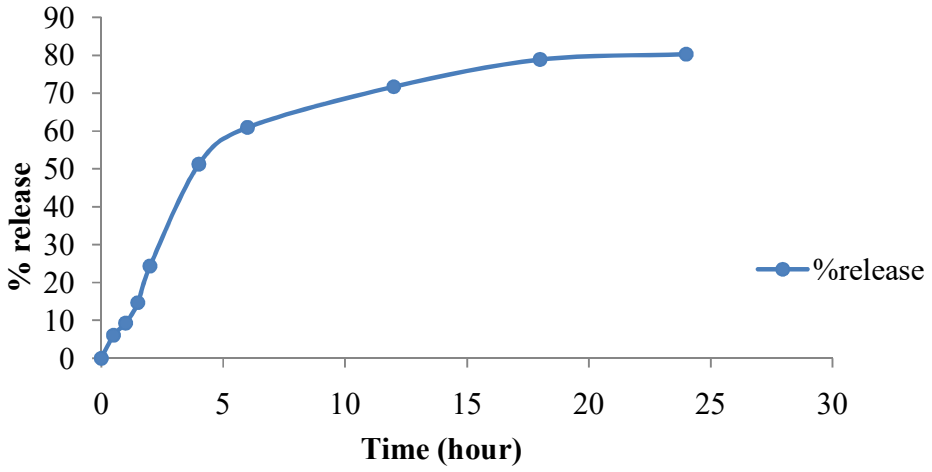


Figure 7: Percentage release of *Berberis calliobotrys* from 10% formulation

CONCLUSION

Cream prepared in this study showed adequate stability and permeability profile with the significant antibacterial activity. It can be concluded from the study that this herbal cream can be used to treat topical bacterial infections that will help to overcome antibiotic generated resistance. To increase efficacy of active there is need to extend this study *invivo* and evaluate more uses of plant with noval drug delivery system.There is further need to investigate themore potential uses of plant and separate its constituents for maximum result.

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