"PRODUCTION OF BIOSURFACTANT FROM *FLAVOBACTERIUM* SPECIES AND ITS EFFECT ON SELECTED FORMULATED DOSAGE FORMS"

Thesis submitted to

THE KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI
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For the award of the degree of

Doctor of Philosophy

In the Faculty of

Pharmacy

By

Mr. Udaykumar B Bolmal
(Registration No: KLE/Ph.D/10-11/DOUN10011)

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Under the Guidance of
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Thanks forever…..

MR. UDAYKUMAR. B BOLMAL

DATE:

PLACE:

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LIST OF ABBREVIATIONS

APIs:	Active Pharmaceutical Ingredients
ANOVA:	Analysis of Variance
Cm:	Centimeter
R^2 :	Coefficient of Regression
^{0}C:	Degree Celsius
DSC:	Differential Scanning Calorimeter
Fig:	Figure
FTIR:	Fourier Transform Infra-red Spectroscopy
>:	Greater
G:	Gram
h:	Hour
IP:	Indian pharmacopeia
IAEC:	Institutional Animal Ethical Committee
< :	Less
λmax:	Maximum UV absorption
mg:	Milligram
µg:	Micro gram
min:	Minute
ml:	Milliliter
n:	number of trial
%:	Percent
± :	Plus or minus
SD:	Standard Deviation
USP	United States Pharmacopoeia

UV	Ultra-Violet Spectroscopy
v/v	Volume by Volume
w/v	Weight by Volume
w/w	Weight by Weight

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ABSTRACT

Background: To overcome the various disadvantage of synthetic surfactant, a novel biodegradable, biocompatible effective in all pH range with temperature stability biosurfactant produced from the specified microorganisms. Biosurfactant application in the different conventional dosage forms containing poor water soluble drugs as solubility and permeability enhancing chemical agent were not been explored.

Objectives: The objective of the present study was production of biosurfactant from *Flavobacterium species* using economical industrial by product substrates. Study the effect of biosurfactant in Atovaquone tablet and Lornoxicam gel as solubility and permeability chemical enhancer

Methodology:

Phase-I: Biosurfactant was produced from the *Flavobacterium sp 2495 i*n Mineral salt media along with sugarcane molasses and waste fried oil, using factorial design to produce maximum yield. Extraction and partial characterization of biosurfactant was performed. Oral and dermal toxicity studies were performed as per OECD guidelines.

Phase-II: Atovaquone BCS IV anti-malarial drug. Binary and ternary solid dispersion technique was used for its enhancement of solubility. Best solid dispersed products were incorporated in the formulation of Atovaquone conventional tablet. Evaluation of Atovaquone prepared tablets were with respect to pre and post compression parameters. *In vivo* bioavailability was carried out on rabbit. Stability studies were also performed.

Phase-III: Lornoxicam BCS II NSID drug. Lornoxicam gel was prepared using biosurfactant as solubility and permeability chemical enhancer and compared with non ionic surfactant. Physicochemical evaluation of prepared Lornoxicam gel was

performed. *In -vitro* and *ex-vivo* permeation studies were conducted using egg membrane and rat skin respectively. In vivo anti-inflammatory study was performed using carrageenan paw edema model in rat. Modified short term stability study was conducted as per ICH guidelines.

Results: Biosynthesis of biosurfactant was carried out using *Flavobacterium sp*. MTCC 2495. Microorganisms were cultivated in the modified mineral salt media for enhanced propagation, there by yield. Sucrose and peptone as substrates in modified mineral salt media, bacteria produced the biosurfactant and it was confirmed by the emulsification tests. Optimization of substrates concentration was carried out using 3^2factorial design in laboratory scale in shake flask method. Three levels of sugar cane molasses and waste fried oil used as independent variables and dependent variables were surface tension and biosurfactant yield. Total nine runs were conducted with constant environmental conditions, like pH, temperature, aeration and duration. Dependent variables data were analyzed for its significance by ANOVA and p -value. Out nine runs, run number 3 was more significant than the other runs. From the overlay plot, design space was obtained for sugar cane molasses 5.2% and waste fried oil 5%. Pilot scale up was performed in the bioreactor of 2 liters capacity using molasses (5.2%)and waste fried oil(5%) as substrates maintained constant environmental conditions for 72 hrs. Total yield of the biosurfactant was 7.9 gms per liter from the solvent extraction of the broth. Partial chacterisation of the biosurfactant was confirmed by FTIR, TLC, DSC and UV method. CMC of biosurfactant was at 39.5 mg per liter.. Oral and acute dermal toxicity studies results indicated that biosurfactant was safe up to 2000mg per kg weight of rat.

In vitro dissolution profile of binary and ternary system of solid dispersion products showed 8.65% and 34.64% respectively. Precompression and post-compression

values of all atovaquone tablets formulations were within the specified limits. *In vitro* dissolution efficiency of F2 and F5 were 1.44 fold and 6.62 fold respectively, in accordance to the F1. *In vivo* study revealed that bioavailability of optimized formulation F5 was increased by 2.5 times and time to reach peak concentration was reduced to 1.4 h, in accordance to pure atovaquone suspension.

Physical-chemical properties of gels were within the acceptable limits. G1 formulation showed drug content (98.9%), spreadability (31.36gm/cm2), and viscosity (3853mPa.S) with yield value (220Pa.s). Drug permeation study by two methods resulted in an enhancement ratio was 0.856. *In vivo* anti-inflammatory activity of G1 and marked formulation exhibited 49.9% and 36% percentage of inhibition respectively. The short term stability studies conducted for 3 months revealed that at room temperature ($25^{o}C \pm 1^{o}C$ and RH 40%) optimized formulation was stable without much variation in drug content, viscosity, pH, and *in vitro* drug release rate.

Conclusion: Biosurfactant produced from the *Flavobacterium sp.* MTCC 2495 using industrial by product was proved to be promising commercial application for novel surfactant production. Biosurfactant also proved that it can be incorporated in to different dosage form containg the poor water soluble drug for its solubility and permeation catalyst, thus reduction in dose. But detail toxicological study to be performed for the acceptance by the regulatory authority as a novel excepient.

Keywords: Biosurfactant: Binary; Ternary; Solid dispersion; Atovaquone; Lornoxicam.

1. INTRODUCTION

1.1 BACKGROUND OF RESEARCH:

1.1.1 SURFACTANTS

Surfactants or surface-active agents are unique chemical entities containing the polar and non-polar groups, having the potential action on lowering the surface tension between two immiscible liquids or solid and liquid interfaces. Surfactant plays an important role as wetting agents, dispersants, emulsifiers, foaming agents depending upon the HLB values.

Surfactants are classified depending upon the polar head charges, like anionic, cationic, and Zwitterionic. No charges are called nonionic's. Anionic -Sodium dodecyl sulfate (SDS), ammonium lauryl sulfate, etc. Cationic Benzalkonium chloride & other alkyl trimethyl ammonium salts. Zwitterionic, Dodecyl betaine, Dodecyl dimethylamine oxide. Nonionic poly(propylene oxide), Alkyl poly(ethylene oxide), Polyoxamines, tween, and spans, etc. Nonionic surfactants have various pharmaceutical potential applications in different dosage form development and cosmetics[1]. Today's global surfactant market is above 17 million tons each year, and their demand is expected to rise by about 3.5 percent towards the end of the century. Almost all surfactants currently in use are chemically derived from petroleum[2]. The main disadvantages of synthetic surfactants are low biodegradability, skin dryness, and stability. The increases in awareness of ecosystem and environment scientists are framing strict eco- regulations leading to an alternative source of production of surfactant from microbes[3].

1.1.2. BIOSURFACTANTS

With increasing environmental awareness and emphasis on a sustainable society in harmony with the global environment, natural surfactants i.e. Biosurfactants have been becoming much important. Biosurfactants were first discovered as extracellular amphiphilic compounds in research into hydrocarbon fermentation, which started in the late 1960s. The Biosurfactants have been increasingly attracting attention in various fields as multifunctional materials for the new century[4]. Biosurfactants are a structurally diverse group of surface-active molecules biosynthesized by microorganisms, which are produced on the surface (Microbial cell surface) or excreted extracellular containing hydrophilic and hydrophobic moieties. In general Biosurfactants, the structure includes hydrophilic moiety (amino acids or peptide, mono, di-, or polysaccharides, etc.) and hydrophobic moiety such as saturated or unsaturated fatty acids, etc. The common microorganisms used in the production of biosurfactants are *Acinetonacterier* spices, *Bacillus* species, and *Candida* species The chemical structure of biosurfactant is dependent upon the substrate structure provided in the fermentation culture, thus the biosurfactant chemical structure elucidation is complex. The unique properties of Biosurfactants allow their use and have an advantage for replacements of chemically synthesized surfactants in various industrial operations.

1) Advantages [5,6,7,8]

There are many advantages of Biosurfactants as compared to their chemically synthesized counterparts, some of those are,

1. Structural diversity.

- Fatty acid, carbohydrate, and protein complex.

2. Biodegradability.

- Biosurfactants are easily metabolized by microorganisms.

3. Low toxicity.

- Biosurfactant showed lower toxicities as compared to the synthetics surfactants.

4. Biocompatibility and digestibility.

- A major application in cosmetics, pharmaceuticals and as a functional food additive.

5. Surface and interface activity.

- A low concentration of biosurfactants showed a 50% reduction in surface tension of water by following pure water. Interfacial tension of water/Hexadecane less than 1N/m by the high concentration of synthetic surfactant.

6. Stability.

- Biosurfactants are stable at a different temperature, pH and salinity.

7. Biosurfactants can be produced from economical raw materials.

- The carbon source may be from industrial waste or by-product of oil or petroleum or sugar or starch industries.

1.1.2.1. Classification [9, 10.]

Biosurfactants are categorized mainly by

1. Microbial origin.

2. Sources viz. marine microorganisms.

.3. Molecular weight.

1) On the microbial origin

The Biosurfactants producing organisms are distributed widely as in table (1) there are several reports that biosurfactants are biosynthesized by hydrocarbon-degrading microorganisms.

Table.1. List of Biosurfactants based on the microbial origin

Biosurfactants	Organisms
1.Glycolipids	
A] Rhamnolipids	*P. aeruginosa, Pseudomonas. sp*
B] Trehalolipids	*R. Mycobacterium sp.erythropolis, N. erythropolis*
C]Sophorolipids	*T.bombicola, T.apicol, T.petrophilum*
D]Cellobiolipids	*U.zeae, U.maydis*
2.Lipopeptides	
Lipoproteins.	
A] Peptide-lipid.	*Bacillus llicheniformis*
B]Serrawettin	*S. marcescens*
C]Viscosin	*Psedomonas fluorescens*
D]Surfactin	*Bacillus subtilis*
E]Subtilisin	*Bacillus subtilis*
F]Gramicidins	*Bacillus brevis*
G]Polymyxins.	*Bacillus polymyxa.*
3. Fatty Acids, Neutral lipids, and Phospholipids	
A] Fatty acids	*A. calcoaceticus*
B] Neutral lipids.	*A. calcoaceticus*
C] Mannan-lipid-protein	*C. tropicalis*
D]Liposan	*C .lipolytis*
E] Carbohydrate-protein-lipid	*C.fluorescens, D .polymorphis*
5. Particulate Biosurfactants	
A]. Vesicles and fimbriae	*A. calcoaceticus*
B].Whole Cells	*Variety of Bacteria*

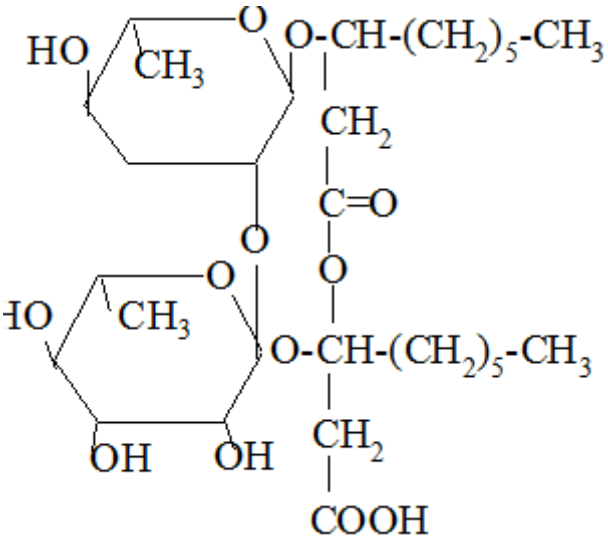
1. Glycolipids

Glycolipids are carbohydrates in combination with long-chain aliphatic acids or hydroxy aliphatic acids. The following are examples.

a) Rhamnolipids, b) Trealolipids c) Sophorolipids.

a) Rhamnolipid:

These glycolipids are characterized by the presence of one or two molecules of Rhamnose linked to two molecules of β-hydroxy decanoic acids.α-Rhamnosyl-L-rhamnosyl-β-hydroxydecanosyl-β-hydroxydecanoate.

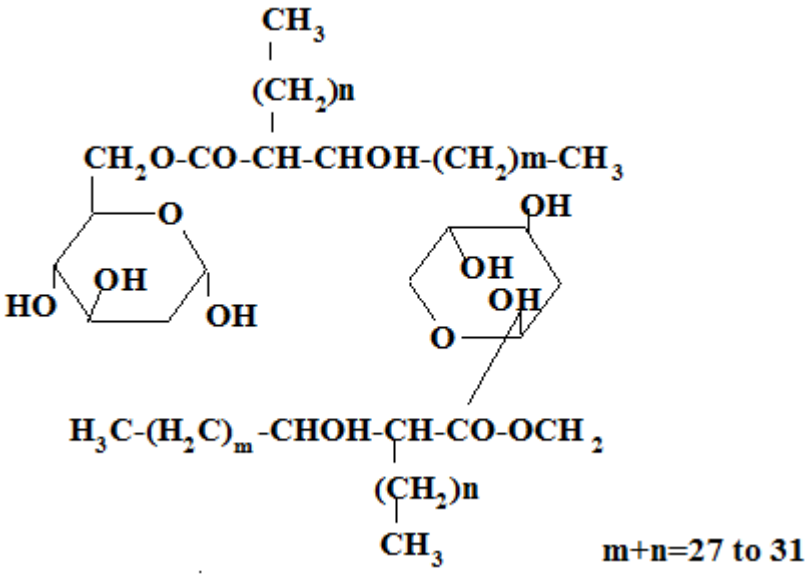


RAMNOLIPID

Some *Pseudomonas sp.* produce large quantities of glycolipids consisting of two molecules of –OH group of one of the acids involved in glycosidic linkage with reducing end of the ramose disaccharide, the –OH group of second acids involved in ester formation. The pure Rhamnolipid showed a critical micellar concentration CMC of 10 to 30 mg/L.

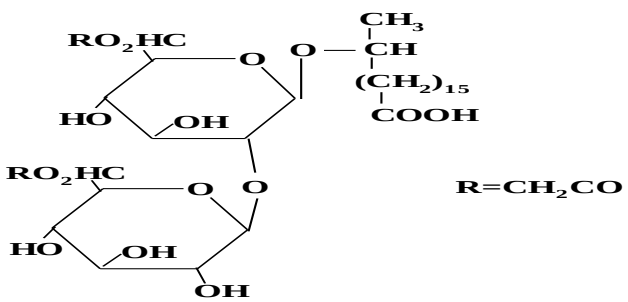
b) Trehalolipids:

These consist of disaccharide trehalose linked at C-6 and C-6' to mycolic acid. Mycolic acids are long-chain α-branched-β-Hydroxy fatty acids. Trehalolipids from different organisms have different sizes and structures of mycolic acid, which is associated with most species of *Mycobacterium*, *Nocardia*, *and Corynebacterium.* Trehalose lipids from *R. erythropolis* and *Arthrobacter sp.* lower the surface and interfacial tension in culture broth to 25 to 40 m N/m and 1 to 5 mN/m respectively.



TREHALOLIPID

c) Sophorolipids: These are mainly produced by yeasts such as *Torulopsis bombicola, T.petrophilum,* and *T.apicola,* consist of dimeric carbohydrates Sophorose linked to long-chain hydroxy fatty acids.

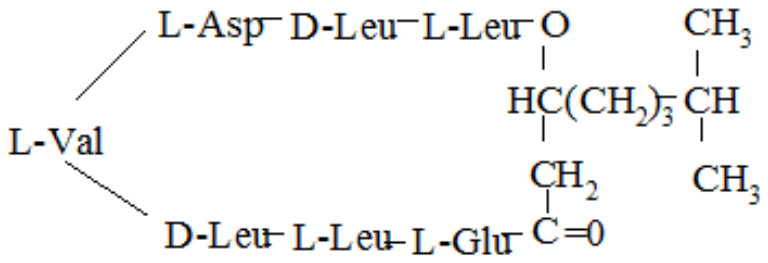


SOPHOROLIPID

Both lactonic and acidic sophorolipids lower the interfacial tension between n-hexadecane and water from 40 to 5 mN/m and show remarkable stability towards pH and temperature.

2. Lipopeptides and Lipoproteins:

Most of the cyclic lipopeptides including decapeptide antibiotics (gramicidin) and lipopeptides antibiotics (polymyxins), produced by *Bacillus brevis* and *B. polymyxa* respectively, possess remarkable surface-active property.

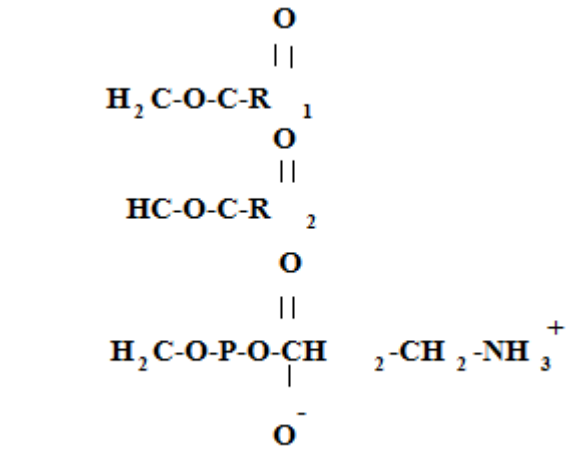


CYCLIC LIPOPEPTIDE

The cyclic lipopeptides surfactin, produced by *B. subtilis* ATCC 21332, is one of the most powerful biosurfactants; it lowers the surface tension from 72 to 2.79 m N/m at a concentration as low as 0.005%. *B. licheniformis produces* several biosurfactants which act synergistically and exhibits excellent temperature and pH and salt stability.

3. Fatty Acids, Phospholipids, and Neutral Lipids:

Several bacteria and yeast produce a large number of fatty acids and phospholipid surfactants. The quantitative production of phospholipids has also been detected in some *Aspergillus sp*. and *Thiobacillus thioxidans, Arthobacter strain* AK-19 and *P.erythropolis* grown on *n*-alkane caused a lowering of interfacial tension between water and hexadecane to less than 1 mN/m and a CMC of 30 mg /liter.



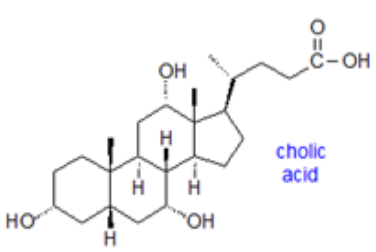
PHOSPHATIDY ETHANOLAMINE

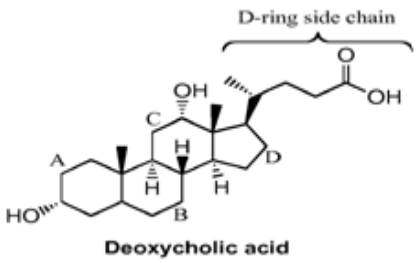
4. Polymeric Biosurfactant:

These consist of various types of polymeric Biosurfactants produced by different microorganisms (table-1). Emulsan is best studied polymeric Biosurfactant these are chemically Polysaccharide-Protein complex. It is produced by *Acinetobacter calcoaceticus,* chemically it consists of heteropolysaccharide backbone containing repeating trisaccharide of N-acetyl –D galactosamine, N-acetylgalactosamine uronic acid, and unidentified N-acetyl-amino-sugar.

Phospholipids and fatty acids:

This type of Bio surfactant has been produced by *Myroides* sp. SM 1 was found to produce bile acids; cholic acid, deoxycholic acid, and glycine conjugate when cultivated in Marine Broth.





2. Biosurfactants obtained from marine microorganisms:

Biosurfactants obtained from marine microorganisms and are grouped into different classes as shown in (Table.2).

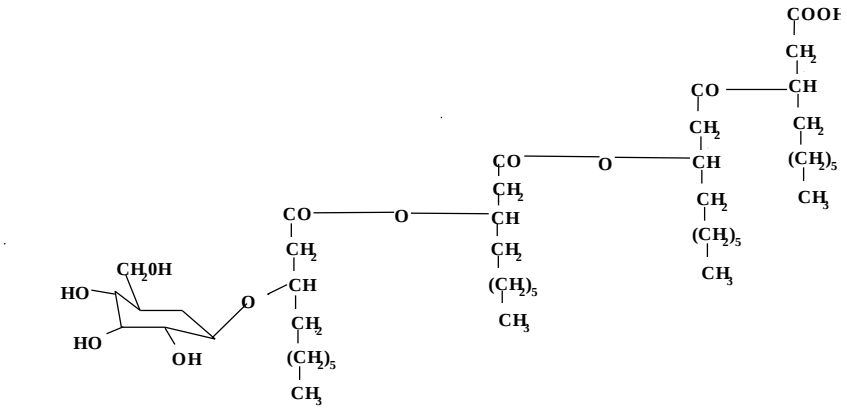
Table.2. List of marine microorganisms producing biosurfactant

Biosurfactants	**Organisms(Marine)**
Glycolipids	
A] Glucose lipids	*Alcanivorax borkumensis, Alcaligenes sp.*
B] Trehalose lipids	*Arthrobacter sp.*
Lipoproteins	
A] Ornithine lipids	*Myroides sp. SM1*
Phospholipids and fatty acids	
A]Bile acids	*Myroides sp. SM1*
Lipid-carbohydrate-protein	
A]Whole cells	Variety of bacteria

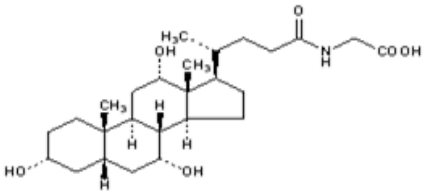
Glycolipids

Glycolipid Biosurfactants are carbohydrates containing long-chain aliphatic acids or hydroxy-aliphatic acids. Marine bacteria such as *Alcaligenes* sp. can produce glucose lipids. The lipophilic component consisting of four β-hydroxy-decanoic acids linked together by ester bonds is coupled glycosidically with C-1 of glucose.

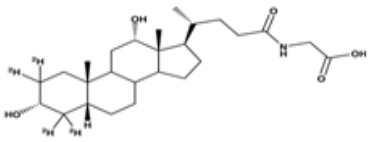
Alcanivorax borkumensis also produces an anionic glucose lipid with a tetramer oxyacyl side chain. The glycolipids extracted from the cell wall consist of this Biosurfactant N-terminally etherified with glycine. Ten different derivatives of lipid type have been identified. The varying chain length of one or two of four β-hydroxyl fatty acids (C_6, C_8 , and C_{10}) was reported.



structure of glucolipid from *Alcaligenes sp.*



Glycocholic acid



Glycodeoxycholic acid

Polymeric Biosurfactants: -

Polymeric Biosurfactants was produced by *Pseudomonas nautica.* The major constituents were proteins, carbohydrates, and lipids at the ratio of 35:63:2, respectively.

Particulate Biosurfactants:

Extracellular membrane vesicles partitioned hydrocarbons to form a microemulsion, which plays an important role in alkane uptake by microbial cells. In the case of *Sphinogomonas* sp. the cell surface of this strain was covered with extracellular vesicles when grown on polyaromatic hydrocarbons.

3. Classification of Biosurfactants based on their molecular weight:

a) Low –molecular weight biosurfactants:

The low-molecular-weight Biosurfactants are generally glycolipids or lipopeptides. The best-studied glycolipid emulsifiers; rhamnolipids, trehalolipids, and sophorolipids are the low-molecular-weight Biosurfactants.

Eg. *Streptomyces tendae* produces an extracellular hydrophobic peptide, referred to as streptofactin, which is a mixture of structurally related peptides, ranging in size from 1003 to 1127 Da.

b) High-molecular-weight biosurfactant:

High molecular weight biosurfactant includes exocellular polymeric surfactants composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixture of these biosurfactants. Alasan produced by *Acinetobacter radio-resist*ance is a complex of an anionic polysaccharide and protein with a molecular weight of approximately 1X 10^6 Da. eg. *A. calcoaceticus* A2 Produces an extracellular anionic polysaccharide surfactant of molecular mass 51.4kDa.

1.1.2.2. Biosynthesis of biosurfactant:

Exact biochemical pathways of biosynthesis of biosurfactants are not well established. Researchers have postulated the theory of biochemical pathway either by secretion or attaching to the cell wall of microbes during the fermentation process. In the fermentation process hydrophobic substrate contact with the cell wall of microbes and interact, the formation of emulsion with cell wall content thus passive diffusion of the substrate into the cell.

Styldark and Wagner state that the following are possibilities of biosynthesis of a hydrophilic and hydrophobic moiety of biosurfactant[11].

i) De novo synthesis of the hydrophilic and hydrophobic moiety.
ii) De novo synthesis of hydrophilic moiety and synthesis of hydrophobic moiety by precursor.
iii) De novo synthesis of hydrophilic moiety and hydrophobic moiety by substrate-dependent.
iv) Both hydrophobic and hydrophilic moieties are synthesized by substrate-dependent.

Regulation of biosynthesis of biosurfactant is dependent upon, induction, repression, nitrogen and multivalent ions.

Induction processes are extensive studies in the biosynthesis of sophorolipids using *Tourulopsis magnolia.* Also of fatty acid in the culture media, and the enhanced yield of sophorolipids was determined.

The repression mechanism also observed in the different strains of microbes. Decreased biosynthesis of biosurfactant in C lipolytic culture medium with addition of D-glucose, citric acid, and acetates. Addition of Nitrogen and multivalent elements like Ca^{++}, Mg^{++}, Fe^{++} (10 to 50µ mole per liter) an increase is the yield of Rhamnolipid biosurfactant in *Pseudomonas aeraginase* culture media with other environmental conditions were kept constant.

1.1.2.3. Raw materials for biosurfactant production: [12, 13]

Carbon source

Hydrophilic (glucose, glycerol, and mannitol)and hydrophobic (fatty acids, petroleum products) sources have been used for different microbes in the fermentation with suitable culture and environmental conditions

Hydrophobic sources

Vegetable oil, oil extract effluents, animal fat, waste fried oil, and dairy wastewater.

Hydrophilic source

Starchy effluents, soapstock, molasses, and corn steep liquor.

Nitrogen source

The general nitrogen source is urea, ammonium chloride sodium nitrate, glutamic acid, aspartic acid, and glycine. Lipopeptide biosurfactant was synthesized in the rich nitrogen source as compared to the other biosurfactant. In all types of biosurfactant production the ratio between C: N sources are kept in the range of 16:1 to 18:1 respectively.

Trace elements

Along with other raw material addition of trace elements like Ca^{++}, Mg^{++}, Fe^{++} showed a synergistic effect on the enhancement of yield of biosurfactant in the range of 16µmol to 35µmol concentration per liter.

Environmental conditions

The essential growth conditions for biosurfactant production are pH(in the range 5.5 to 8.5), temperature (30 to 35oc) agitation speed is in the range of 50 to 200 rpm depending upon the viscosity of the medium. Dissolved oxygen concentration in

the medium also essential requirement of propagation of the aerobic microbe. Aeriation concentration is maintained in the range of 30% to 60%.

1.1.2.4. Recovery of biosurfactant: [14]

Recovery of biosurfactant from culture media is complex process.speration of is solely dependent upon the polarity charge biosurfactant, its solubility in water and presence of biosurfactant in the microbes (intracellular, or in the cell wall) Most of the common methods of recoveries are batch process and continuous process.

a) Batch process

1) Ammonium precipitation method eg, emulsan,
2) Acetone precipitation method eg, Bioemusifier
3) Acid precipitation method eg, surfactin
4) Solvent extraction method (a mixture of organic solvents eg, sophorolipids and liposan.

b) Continuous process.

1) Centrifugation eg, Glycolipid
2) Adsorption(Charcoal or Amberlite resin) eg, lipopeptides glycolipids.
3) Foam separation and precipitation eg Surfactin
4) Tangential flow filtration Eg mixed biosurfactant
5) Ultra filtration (membrane filtration) eg Glycolipid.

1.1.2.5. Application of biosurfactants

Biosurfactants have several applications such as agriculture (soil wetting agents), commercial detergents, oil-refinery, petroleum industry, and food processing industries, medicine, pharmaceuticals and cosmetics. The application of biosurfactants in pharmaceuticals is very limited. Rhamnolipid is used as an antibacterial agent in the concentration of 16µg/ml to 32µg/ml.[15]

Daptomycin (Lipopeptides) for a skin infection caused by the methicillin-resistant *streptomyces roseoporas* sp. Daptomycin was approved in 2003 by USFDA to Cubist Pharmaceutical USA.[16]

Surfactin biosurfactant used in the preclinical study of insulin inhaler as a permeation enhancer in the rat model.[17]

Using surfactin, silver nanoparticles were stabilised and nanosuspension was stable fo r more than 6 months.[18, 19.]

Liposan is commonly used in the cosmetic and food industries for producing stable oil/water emulsions.[20]

1.2 REVIEW OF LITERATURE:

The following are the review of the literature report specifically concerning the different economic substrates used in the large scale production of biosurfactant.

E.Haba, et.al.[21] (2000) Reported that Rhamnolipid Biosurfactant has been produced by *Pseudomonas aeruginosa* 47T2 NCBI 40044 from waste frying oils. The medium was basal containing carbon and nitrogen ratio was 8, at pH 7.2, temperature 30^{o}c for 72hrs incubation. The surface-active agent was separated using the solvent extraction method and evaluated for the emulsification index and Critical micellar concentration using surface tension method. The total yield of biosurfactant was 0.34gms per liter.

Shyh-Yau Wang.[22] (2013) Reported that used oils obtained locally were adopted as the substrate for production of biosurfactant using *Flavobacterium sp isolated* from field samples under nonseptic conditions at pH 7.2, temperature 35^{o}C and rpm 80 for 72 hrs. Precipitation and purification were carried out using inorganic acid and chloroform-methanol (1:1) mixture. The organic layer was evaporated to dryness to get crude biosurfactant. Biosurfactant was subjected to surface tension reduction property and critical micellar concentration determination. Results were promising with maximum reduction in surface tension and at the lowest concentration of biosurfactant, CMC was observed.

Joshi S, et al. [23] (2013). Reported that production of biosurfactant from molasses and cheese whey as a source of substrate for Bacillus licheniformis k51 strain at optimum fermentation conditions for fermentation. Extraction and purification were performed with a conventional method. The produced biosurfactant was subjected to different evaluation parameters such as emulsification index and stability at various temperatures and salinity. Biosurfactant was stable for nine-day.

Waghmode, S et al. (2014).[24] Reported that from Bacillus subtilis isolated from garden soil been used in the production of biosurfactant. Substrates were coconut waste, soybean and

seasmanoil wastes in minimal salt media at 37 ^{o}C temperature for 48hrs. Biosurfactant was precipitated with HCl and purified with organic solvent chloroform and menthol (1:2) mixture, the organic layer was evaporated for dryness to get biosurfactant. Coconut waste showed good yield biosurfactant with an increase in the fold of the emulsification index by synthetic surfactant.

Luna, et al. (2015).[25] Reported that groundnut oil waste and corn steep liquor were used as the substrate for the production of biosurfactant using *Candida sphaerica* UCO 0995 in yeast mold media at 27^{0}C temperature, pH 7.3, rpm 150 for 144hrs in a bioreactor. The yield of biosurfactant was 12gm/liter. Produced biosurfactant showed good stability in the entire pH and salinity and emulsification index.

Almeda DG, et al. (2017).[26] Reported that the optimization of biosurfactant yield using *Candida tropicalis* UCP0996 with economic substrates, molasses, corn steep liquor waste and waste fried oil. Optimization of the yield of biosurfactant was carried out using response surface methodology. Concentrations of substrates and inoculum size were independent parameters and dependent parameters were yield and reduction in surface tension. Linear regression analysis was performed on the results, it was found that using 2% w/v of all substrate concentrations with 2.5%v/v inoculum size the yield of biosurfactant was 4.19 gms per liter and maximum reduction of surface tension 29.98mNm-1.

Chunyan, et al. (2018).[27] reported that the production of biosurfactant from kitchen waste oil using *Pseudomonas aeruginosa* at pH 7 and temperature 37 ^{0}C in agar peptone and electrolyte media for 36 hrs. Extraction and purification were performed using acidic precipitation and organic solvent respectively. The total yield of biosurfactant was 2grm per liter, having a good emulsifying index, reduction in surface tension and stability in salinity.

1.3 INTRODUCTION TO SOLID DISPERSION [28, 29.]

In 1961, Sekigiguchi and Obi introduced the solid dispersion concept using the water-soluble carrier and poorly water-soluble drug in solid-state. The main principle involved in the solid dispersion is to enhance the solubility of the poorly soluble drug, ultimately maximum bioavailability and dose reduction. At present, about 49% of new chemical entities are having the problem of solubility. The physical methods for enhancement of solubility of the poorly soluble drug are micronization, modification of the crystal habit, complexation, solubilization by surfactants, and drug dispersion in water soluble carriers. Solid solution, eutectic mixtures and solid dispersion. Among all solid dispersion methods, fusion and solvent evaporation techniques are often adopted processes in the research area and commercial production.

The key benefits of solid dispersion are decreased particlesize ranging from mi cro to molecular level, with free flow and wettability leads to improved solubility and increased bioavailability.

1.3.1. Ternary solid dispersion [30, 31.]

Solid dispersion is classified on the basis carrier used. first, second and third generation. The first generation mainly includes crystalline carriers like urea, sugar, maleic acid, benzoic acid, etc. the second generation contains synthetic polymers, eg PVP, HPMC, PEG. The third generation is the advance modification of the second generation with surfactant or surfactant alone. Commonly used surfactants are poloxamer, nonionic surfactant, Gelucire 44/41, etc.

Hiroyuki, et al.[32] (2018). Reported that the Atovaquone tablet was prepared by binary and ternary solid dispersion method using PVP K30 and self-emulsifying components respectively. Self emulsifying component consisted of campule MCM, Captex 300NF, and tween 20 with 2.5%, 2.5% and 5% respectively. Comparative *in vitro* dissolution

studies result revealed that the percentage of drug dissolution rate was increased by fivefold in accordance with the binary mixture. Globule size and drug particle size were evaluated using the zeta sizer and X-ray diffraction study respectively.

Tanja V, et al.[33] (2018). In this study enhancement of dissolution of poorly water-soluble carbamazepine drug was determined by using ternary solid dispersion using hydrophilic Kollidon VA64 polymer with adsorbent, Neusilin UFL2 carrier. D optimization tool was used with different concentrations as the independent variable and the dependent variable was dissolution rate. The profound effect of dissolution rate carbamazepine was observed by the ternary solid dispersion within 30 minutes. Optimized solid dispersion products were also subjected to DSC, FTIR, powder X-ray diffraction and polarizing light microscopy. Results revealed that carbamazepine was amorphous and thermodynamically stable in the monoclinic, metastable form (III).

Sontayal, et al.[34] (2017). In this study, it was aimed to enhance the dissolution rate and bioavailability of Manidipine by formulating ternary solid dispersion with D-α-tocopherol polyethylene glycol 1000 succinate with the hydrophilic co-povidone polymer. Melt and cold solidification methods were employed for the preparation of solid dispersion. Characterization of solid dispersion was performed using DSC, FTIR, and hot stage microscopy to evaluate the compatibility and homogeneity of the mixture. The best formulation also subjected to *in vivo* bioavailability using rats. Results of evaluated best ternary solid dispersion was that threefold enhanced dissolution and a fourfold increase in oral bioavailability with respect to AUC in accordance with a pure drug suspension.

Alhayali A, et al.[35] (2017). The aim of the study was to study the effect dissolution behavior of ezetimibe ternary solid dispersed product in fasted stimulate intestine and gastric fluids at 25^{o}C and 37^{o}C temperatures. Ternary solid dispersion was prepared by

PVPK30 and Polaxmer 188 by melt and spray drying methods. All batches were subjected to physical characterization, FTIR, DSC, SEM and powder X-rays diffraction. Best formulation from melt method of the ternary solid dispersed product showed that drug was in amorphous form, dissolution profile revealed that in fasting stimulated gastric fluid better solubility in accordance with the spray-dried method at both temperature. It was concluded that the ternary solid dispersion technique improves solubility and supersaturation of poorly water-soluble drugs.

Jin-Bin Liaoa, et al.[36] (2014). Investigated, by incorporation of poloxamer 188 and 407 grades of surfactants as solubility and dissolution rate enhancer in a solid dispersion of Patchouli alcohol; a water-insoluble bioactive compound. An immediate release pellets forms of Patchouli alcohol was prepared by the fusion method with quenching in the atoleine. Different concentrations of Poloaxmer 188 and 407 were prepared with patchouli alcohol and evaluated for compatibility studies, pallet sizes and in-vitro dissolution studies. In vitro dissolution studies revealed that Poloxmer 188 containing batches better drug release as compared to the 407 grades. Solid dispersion formulations containing both surfactants together showed a better dissolution rate in accordance with poloxamer 188 alone. Thus the technique of solid dispersion with surfactants was simple, cost-effective and commercially scalable.

Patel DM, et al.[37](2014) It was reported that fast dissolving Domperidone tablets were formulated by incorporation of ternary solid dispersion using poloxamer 188 and 407 with Gelucire, PVP k30, mannitol, PEG 6000, 4000 as binary phase, by fusion method. Best formulation from solid dispersion batches was incorporated in tablet formulation and prepared by the direct compression method. Solid dispersion formulations were evaluated for in vitro dissolution, FTIR and XRD studies. All the batches of Domperidone tablets were also evaluated for pre- post-compression parameters and

stability studies. Gelucire 50/20 with the poloxamer 188(2:1.5) showed fast disintegration and dissolution as compared to the other batches. The study was concluded that the incorporation of the ternary agent in the solid dispersion resulted in enhanced disintegration and dissolution rate of Domperidone tablets with acceptable stability.

1.4. PERMEATION ENHANCING AGENTS IN SEMISOLID DOSAGE FORM [38, 39.]

The transdermal route provides the local drug delivery with less concentration of drugs without many side effects as compared to the other route of drug administration. An outer most layer of the skin consists of stratum corneum, consists of a dead cell layer of corneocytes with varying thickness ranging from 10 microns to 40 microns. Stratum corneum is a major route for drug penetration as compared to the sweat gland and appendage routes but exerts retardation for penetration of drugs from the semisolid dosage form. Hence an improvement of enhanced permeation is a prime objective for the development of semisolid dosage form. Various techniques and penetration enhancing agents have been explored for the last two decades, such as physical approach, chemical approach and formulation approach. Among these, the chemical approach has major advantages over the other two approaches like easy, cost-effective and economical for commercial production with enhanced permeation. In chemical approach different penetrating agents have been explored such as propylene glycol, fatty acids (linseed oil), terpenes (Limonem) and surfactants (Tweens, spans, SLS). The main mechanism involved in penetration enhancement includes interaction with intercellular lipids disruptions and increased fluidity or loosening the intercellular tight junction and increasing the partitioning into stratum corneum or denaturing of the keratin layer.

Rong Y, et al.[40] (2018). Investigated the synergistic effect of two chemical permeation enhancing agents on ciprofloxacin poloxamer 407 gel through the tympanic membrane of chinchillas (rodent). Sodium dodecylsulphonate and limonene (FDA approved) were used as chemical permeation enhancing agents with varying concentrations. Ciprofloxacin gel was prepared using poloaxmer407 with varying concentrations of sodium dodecyl sulphonate and the limonene and ex vivo diffusion study was performed for 12 hrs and 24 hrs at 370C in Franz diffusion cell. Synergistic effect of sodium dodecyl sulphonate 1%w/w and limonene 2%w/w chemical permeation enhancing agents showed the four-fold increase in the flux rate of ciprofloxacin gel in accordance with higher concentrations chemical permeation enhancing agents. sodium dodecyl sulphonate act as softening and alters the lipid layer for drug diffusion and limonene increases the partition of drug molecule within the lipid layer thus synergetic effect was observed.

Zidan AS, et al.[41] (2017) The objective of the study was to evaluate the optimum concentration of isopropyl myristate, fatty acid permeation enhancing agent on human cadaver skin of testosterone –carbopol gel. Six concentrations of isopropyl myristate were used in the range of 0% to 3%. All formulated batches were characterized for pH, viscosities, and *in vitro* permeation studies. Formulated batches showed an acceptable pH and viscosities and uniform drug content. *In vitro* permeation data revealed that the 2% w/w isopropyl myristate content showed an 11fold increase in flux compared to 0% percentage of isopropyl myristate batch. Mass balance analysis resulted in uniform distribution of testosterone on the human skin. Based upon the all evaluated results, it was concluded that isopropyl myristate had a better permeation effect with an optimum concentration in the product.

Feng Xie, et al.[42] **(**2016). The application of camphor as a permeating agent for the transdermal drug delivery system was investigated. The drug selected for transdermal delivery were Indometacin, Lidocaine, Aspirin, Antiptrin, Tegafur and 5 fluorouracil having varying lipophilicities ranges from 3.8 to -0.95 (log p). All the drugs were dissolved in propylene glycol and water mixture (70:30) with and without camphor 3%w/v and luruocapram 3%w/v. Cytotoxicities for camphor were performed on cell line study by MTT assay and results revealed that low toxic. Invitro diffusion study was performed using rat abdomen skin. Effect of camphor and laurocapram as permeation enhancing agents was studied individually all with all selected drugs solutions. Results of *in-vitro* diffusion exhibited that for higher lop p-value drugs laurocapram showed better results of flux as compared to camphor, but for low log p-value drugs, camphor was better permeating agent because of high partition to stratum corneum, which was proved by the ATR FTIR graph. Thus suggests that camphor as good permeating agent for varying lipophilicities drugs in transdermal drug delivery system performance.

Akbhari J, et al.[43] **(2015).** Investigated the effect of rosemary essential oil in diclofenac sodium gel as a permeation enhancing agent. Diclofenac sodium gel was prepared from HPMC K4M and carbopol 934p as gelling agents with varying concentrations of rosemary oil (0.1%, 0.5% and 1.0%w/w). The antinociceptive effect was performed on mice by the tail-flick method. Diclofenac sodium gel containing rosemary oil in 0.5% and 1.0% showed increase analgesic effect after 25,30, and 35 minutes as compared to the reference gel in the tail-flick test. Rosemary oil proved to be an effective permeation enhancing agent in a topical gel.

Teixeira, et al. [44] (2014). Aim of the study was to investigate the effect of novel lysine base surfactant as a permeation enhancing agent for tetracaine (lipophilic) and

ropivacaine (hydrophilic) topical gel. Lysine based surfactants were synthesized (in - house) using different fatty acids ranging from C15 to C21. Synthesized lysine surfactants were subjected to its purity analysis by NMR DSC studies. Tetracaine and ropivacaine gels were prepared by using HPMC K15m as gelling agents and also with 0.01M lysine surfactants. Lysine surfactant-containing formulations were subjected for Cytotoxicities study using human epidermal keratinous cell-line. *In-vitro* diffusion study was conducted using young porcine skin for 24hrs. All results of evaluations proved that lysine base surfactants were pure, stable and non-toxic. Flux rate permeation for both drugs with C16 lysine surfactant showed promising results in accordance with other fatty acid lysine surfactant. It was also proved that hydrophilic ropivacaine drug can be permeated through the stratum corneum, this could be due to the effective partition of the drug by lysine surfactant.

1.5. JUSTIFICATION FOR THE STUDY [45,46]

All approved surfactants from various regulatory agencies are synthetic, mainly nonionic and cationic surfactants from the fatty acids base. These contain the polar and nonpolar head and tail respectively. Incorporation of surfactants in the formulations of BCS II and IV drugs for enhancement of solubility and permeability has been achieved successfully. From the review of literature, it was found that 0.5% to 20% of concentrations surfactants were incorporated in all drug delivery systems. The average net effect was on solubility and permeability was 1 to 5 fold and 1 to 4 fold increase in solubility and permeability of poorly soluble drugs respectively.

The disadvantages of synthetic surfactants are, [47, 48.]

- High concentration in drug delivery systems. (5% to 20%)
- The high concentration of surfactant (SLS) in the dosage form leads to the purgative effect. (Grisoflavin tablet)
- Dryness of skin and mild allergic reaction in cosmetic preparation.
- Partial biodegradable(fatty acid-base)
- Not stable in salinity
- Hazards to the environment.

Biosurfactants are exogenous secretions of specific microorganisms produced by fermentation technology using carbon and nitrogen source with optimum environmental conditions such as pH, temperature, etc. The microorganism is either yeast or bacterial, collected and cultured from different soils and marine sources in an appropriate media. Chemically biosurfactants are amphipathic molecule containing fatty acids as a base and conjugated with protein and carbohydrate molecule which resemble the endogenous component of human and animal body fluid composition.

Recent year's exploration of byproduct and wastes from different industries such as the sugar industry, whey industry and oil industry been utilized as a source of carbon and

nitrogen for production biosurfactant. There are 225 patents on the production of biosurfactants from different microorganisms with varying substrates.

Major advantages of biosurfactant over synthetic surfactants are

- Less or no toxicity.
- Biodegradable.
- Stable at all pH range, temperature, and salinity.
- Lowering surface tension and Critical micellar concentration in low concentration. (Less than 1%)
- Tailor-made genetically modified bacteria can produce specific biosurfactants with high yield.
- Broad functional properties like high emulsification index, corrosion inhibitors, viscosity reducer and oil recovery, etc.
- Specific biosurfactants showed therapeutic activities such as antifungal, antibacterial. (Peptides)
- In cosmetic industries, as ant wrinkle, good moisturizing agents. (Emulsan)
- In pharmaceutical industries

❖ Nanoparticle stabilizing agents

❖ Coating of implantable medical devices' to prevent biofilm formation.

An extensive past literature survey revealed that the application of biosurfactant in conventional dosage forms as solubility and permeation enhancing agents for poorly water-soluble drugs, not been explored.

Hence the present study was aimed to produce biosurfactants from economic substrates like molasses and waste fried oil in lab-scale and further to pilot scale-up using *Flavobacterium* species 2495 MTCC. The effect of biosurfactant was studied by incorporation in the formulation of Atovaquone tablet for enhancement of drug

solubility and bioavailability and also in Lornoxicam gel as a chemical permeation enhancing agent.

1.5.1. CRITICAL JUSTIFICATION:

A) Microorganism:

- *Flavobacterium species* 2495 MTCC, non-virulent strain
- Stable at all pH, suitable for all substrates and high yield of biosurfactant.

B) Substrates:

- Molasses and waste fried oil.
- Cost reduction, reusable raw material and easy availability.

C) Optimization processes:

- Central composite design 3^2 levels and pilot scale up.(one liter)

D) Atovaquone tablet formulation:

- Atovaquone BSC IV drug, anti malarial agent.
- Ternary phase solid dispersion with biosurfactant.
- Dissolution rate and bioavailability studies.

E) Lornoxicam topical gel:

- Lornoxicam BCS II, NSAID.
- Solubility study with biosurfactant.
- *In vitro* permeation study.
- *In vivo* anti-inflammatory study

1.6. AIM OF THE STUDY:

Production of biosurfactant from *Flavobacterium* species and its applications in tablet and gel dosage forms as solubility and permeability enhancer respectively.

OBJECTIVES OF THE STUDY:

- Optimize biosurfactant production by the *Flavobacterium* species 2495 MTCC in a laboratory scale using Response surface methodology.
- Scale-up of biosurfactant production from the optimized lab-scale batch and characterization.
- Study the effect of biosurfactant in Atovaquone tablet using ternary solid dispersion technique and characterization *(in-vitro* and *in vivo* studies).
- Study the effect of biosurfactant as permeation enhancer in Lornoxicam gel and gels characterization.

1.6. PLAN OF WORK:-

Research work was carried out in three phases

Phase I: Biosynthesis of biosurfactant

1) Culturing and preliminary characterization of *Flavobacterium sp*. MTCC 2495.

a) Gram staining

b) Motility test: Hanging drop method

c) Oil spreading method

2) Laboratory scale biosynthesis of biosurfactant

a) Preparation of modified mineral salt medium

b) Cultivation of *Flavobacterium sp* 2495 in modified mineral salt medium

i) In processes control; Cell growth: Determination of biomass: emulsifying index.

3) Optimization of substrates concentration using 3^2 factorial designs.

a) Laboratory scale procedure.

b) In processes control: surface tension

4) Extraction and purification of biosurfactant: Solvent extraction method

5) Pilot plant scale-up of biosurfactants in the bioreactor

6) Partial characterization of biosurfactant

a) Determination of CMC of Biosurfactant.

b) UV Characterization. (Determination of λmax).

c) FTIR Characterization.

d) Differential scanning calorimeter

e) Thin Layer Chromatography characterization

7) Pharmacological studies of biosurfactant.

a) Oral toxicity study. b) Acute dermal toxicity study.

Phase II; Atovaquone tablet formulation with biosurfactant

1) Preformulation study of Atovaquone

a) Melting Point Determination

b) Solubility Determination

c) Standard Calibration Curve and λmax

2) Drug-excipients compatibility study

a)FT-IR spectroscopic studies

b) Differential scanning calorimetric study:

c) Method development and validation of HPLC for estimation of Atovaquone

3) Formulation of binary and ternary solid dispersion:

4) Evaluation of solid dispersion: Drug content. *In vitro* dissolution profile.

5) Atovaquone Tablet preparation: using the solid dispersed product

6) Evaluation of Atovaquone tablet

a) Precompression parameters

b) Post compression parameters

c) *In vitro* dissolution profile

d) Dissolution efficiency

7) *In vivo* bioavailability study: Pharmacokinetic parameters

8) Short term stability study

PHASE III: Lornoxicam gel formulation with biosurfactant

1) Preformulation of Lornoxicam

a) Melting point

b) Standard calibration curve and λmax

c) Solubility determination

2) Drug excipients compatibility study

a) FTIR spectroscopy study

.b) DSC study

3) Formulation of Lornoxicam gels:

4) Evaluation of Lornoxicam gels

a) pH of gels

b) Drug content

c) Rheological study

d) Spreadability study

e) Extrudability

f) Gel strength

5) *In vitro* permeation study

6) *Ex vivo* permeation study; Permeation data analysis: Drug release kinetics

7) *In vivo* anti-inflammatory study

8) Short term stability study

2. MATERIALS AND METHODS

2.1. Drug and excipients profiles

2.1.1. Atovaquone[49, 50.]

Description: Atovaquone is a dark yellow crystalline solid. Non hygroscopic, slightly bitter in taste. Practically insoluble in water. BCS IV

Structural formula:

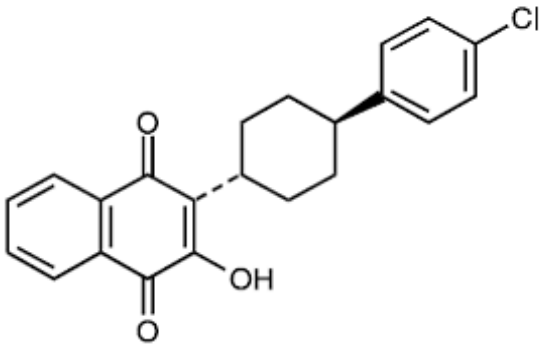


Fig 1: Chemical structure of Atovaquone

Chemical formula: 2-(trans-4-(p-Chlorophenyl) cyclohexyl)-3-hydroxy-1,4-naphthoquinone

Physical properties:

Appearance - Dark yellow colour, solid.

Molecular Formula - $C_{22}H_{19}ClO_3$

Molecular Weight - 366.837

Melting range - 216-219

Solubility - Soluble in methanol. In soluble in water. BCS IV.

Log P Value - 5.2

Category - Anti infective, anti malarial.

Dose: Adult dose 750mg. Pediatric dose 62.5 mg.

Mechanism of action:

In Plasmodium species, the site of action appears to be the cytochrome bc1 complex (Complex III). Complex metabolic enzymes are linked to the mitochondrial electron transport chain via ubiquinone. Inhibition of electron transport by atovaquone will result in indirect inhibition of these enzymes. The ultimate metabolic effects of such blockade may include inhibition of nucleic acid and ATP synthesis.

Pharmacokinetic parameters:

Absorption:

Bioavailability is 26% and 47% without food and with food respectively. It has 99.9% protein bond.

Biological half life is 2.2 to3.2 days.

Metabolism: Limited metabolism.

Excretion: Faecal 99% and urine 1%.

Market available dosage form:

Micronized oral suspension is available with 750 mg / 5ml.Tablet 250 mg.(GlaxoSmithKline)

2.1.2. Lornoxicam [51, 52, 53]

Description:

Lornoxicam is yellow in colour, amorphous solid, slightly hygroscopic, odourless, and slightly bitter in taste. It is in soluble in water.

Structural formula:

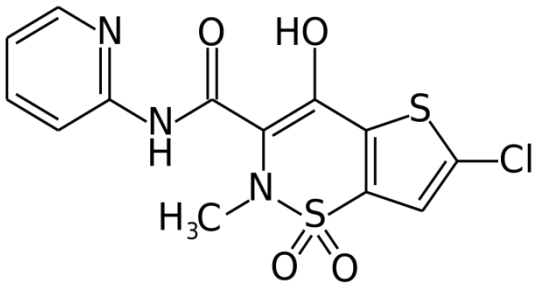


Fig 2: Chemical Structure of Lornoxicam

Chemical formula: $C_{13}H_{10}ClN_3O_4S_2$

6-chloro-4-hydroxy-2-methyl-1,1-dioxo-N-(pyridin-2-yl)-2H-1λ^6-thieno[2,3-e][1,2]thiazine-3-carboxamide

Physical properties:

Appearance	-	Dark yellow amorphous solid
Molecular Formula	-	$C_{13}H_{10}ClN_3O_4S_2$
Molecular Weight	-	371.42
Melting range	-	225-230 ^oC
Solubility	-	Soluble in 0.1N sodium hydroxide and phosphate buffer 7.4 pH.
Log P Value	-	2.62
Category	-	Non steroidal anti inflammatory drug (NSAID)

Mechanism of action: A Perfect balanced inhibition of COX1 and COX2 enzymes

Pharmacokinetic parameters:

It is a BCS II drug. Absorption is rapid from GIT. Cmax is 2.5hrs, no drug accumulation on repeated dosages. Biological half life is 3 to 5 hrs. Daily dose is either 4mg or 8 mg, twice a day. Drug is metabolized from liver through CYP2C9 to hydroxylated derivative without therapeutic effect and excreted through urine.

Route of drug administration: Oral and parental.

Marketed brands:

Camri tablet 4mg Zydus, Lornoxi tablet 4mg Hetero, Lorasid vial 8mg (piramal), Xilor tablet 8mg Sanifi(syntonic)

2.2 EXCIPIENT PROFILE

For Atovaquone tablet:

2.2.1 Hydroxy-propyl-methyl cellulose (HMPC) k – 100 m [54]

Non-proprietary Names-

BP: Hypromellose. **JP:** Hydroxypropyl methylcellulose

PhEur: Hypromellosum. **USP**: Hypromellose

Structural formula-

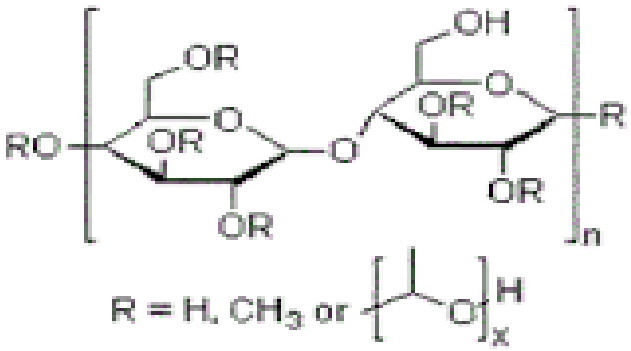


Fig 3: Chemical Structure of HPMC K100.

- **Chemical Name and CAS Registry Number –** Cellulose ,2-hydroxypropyl methyl ether [9004-65-3]
- **Empirical Formula** - $C_{56}H_{108}O_{30}$
- **Molecular weight**: 1261.45g/mole.

Functional Category

It acts as a Coating agent; film-former; rate-controlling polymer for Sustained release; stabilizing agent; suspending agent; tablet binder; viscosity increasing

agent.

Description

Hypromellose is an odourless and tasteless, white or creamy white fibrous or granular powder. It is also soluble in cold water, forming a viscous colloidal solution. It is also soluble in mixture of certain organic solvent with water in various ratios.

Pharmaceutical applications

a) Used as a suspending agent in suspensions.

b) Used as binders for tablet not less than 2%.

c) Film coating material of tablet 10%.

d) Viscosity graded in sustained release dosage form.

e) Acts an adhesive in plastic bandages.

2.2.2 Poly-vinyl pyrrolidine (PVP) K-30 [55]

Non-proprietary Names

BP: Povidone. **JP:** Povidone.**PhEu**r: Povidonum.**USP**: Povidone

Chemical Name and CAS Registry Number-

1-Ethenyl-2-pyrrolidinone homopolymer [9003-39-8].

Empirical Formula and Molecular Weight – (C_6H_9NO) n; 2500-3000000.

Structural Formula-

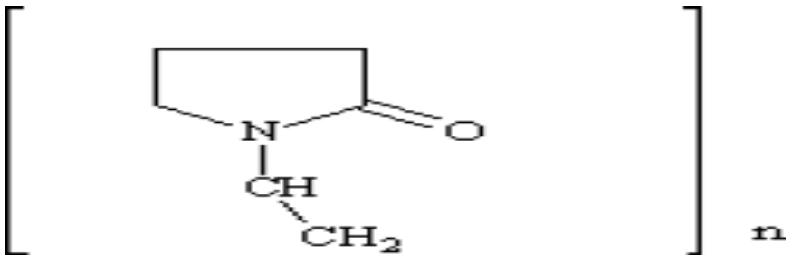


Fig 4: Chemical Structure of poly-vinyl pyrrolidine

The USP 28 describes Povidone as a synthetic polymer consisting of linear 1- vinyl-2-pyrrolidinone group .This has differentiating molecular weights based on different grades of polymer. It is characterized based on its viscosity in aqueous solution and expressed k-value in range of 10-120.

Functional Category

Acts as Pharmaceutical aid (Disintegrant; dissolution aid; suspending agent; tablet binder).

Use of Povidone

Sr. NO	Use	Concentration %
1.	Carrier for drug	10-25%
2.	Dispersing agent	Upto 5%
3.	Suspending agent and viscosity	Upto 5%
4.	Eye drops	2-10 %
5.	Tablet binder, tablet diluent or coating agent	0.5 to 5%

Description

Povidone occurs as a white or yellowish -white powder or flakes, odourless or almost odourless, hygroscopic powder.

Typical properties:-

Acidity/alkalinity:- pH = 4.0-7.0(5% w/v aqueous solution)

Bulk density: -0.29-0.39 g/cm^3 for Plasdone.

Tapped density: -0.39-0.54 g/cm^3 for Plasdone.

True density: -0.39-0.54 g/cm^3 for Plasdone.

Flowability: - 20g/s for Povidone K-15 16 g/s for Povidone K-29/32.

Melting point: Softens at 150°C.

Moisture content: Povidone is hygroscopic, significant amounts of moisture being absorbed at low relative humidities. Particle size distribution: Kollidon 25/30:90%, 50μm, 50%, 100μm, 5%, 200μm. Kollidon 90:90% 200μm, 95%250μm.

Solubility:

It is freely soluble in acids, chloroform, ethanol (95%), ketones, methanol, and water.. In water, the concentration of a solution is limited only by the viscosity of the resulting solution. It is practically insoluble in ether, hydrocarbons and mineral oil.

Pharmaceutical Applications –

Povidone is primarily used in solid dosage forms. Povidone solutions are used as binders in wet granulation for tablets. Used as a solubilizer in oral and parenteral formulations and as coating agents for tablets.

2.2.3 Lactose[56]

Non-proprietary Names –

BP: Lactose monohydrate. **Ph Eur**: Lacto sum monohydricum

JP: Lactose. **USPNF**: Lactose monohydrate.

Chemical Name and CAS Registry Number –

O-b-D-Galactopyranosyl-(114)-a-D glucopyranose monohydrate [64044-51-5].

Empirical Formula and Molecular Weight – $C_{12}H_{22}O_{11}.H_2O$, 360.31

Structural Formula

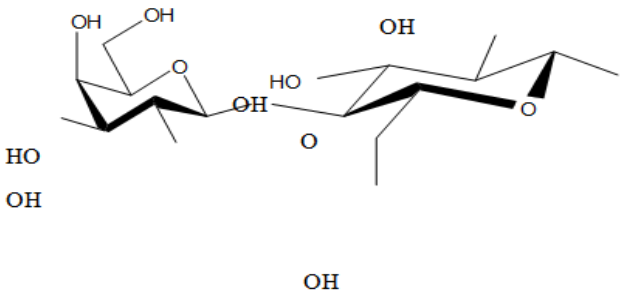


Fig 5: Chemical Structure of lactose

Functional Category

Binding agent; diluent for dry-powder inhalers; tablet binder; tablet and capsule diluent.

Description-

Lactose occurs as white to off-white crystalline particles or powder. Lactose is odourless and slightly sweet-tasting; a-lactose is approximately 20% as sweet as sucrose, while b-lactose is 40% as sweet.

Pharmaceutical Applications –

Lactose is used as a filler or diluent in tablets and capsules. Lactose is also used as a diluent in dry powder inhalation.

2.2.4. Poloxamer 407 [57]

Nonproprietary Names

BP: Poloxamer. PhEur: Poloxamera. USPNF: Poloxamer.

Chemical Name and CAS Registry Number

α-Hydro-ω-hydroxypoly (oxyethylene) poly (oxypropylene) poly (oxyethylene) block copolymer . **CAS NO**: 9003-11-6

Empirical Formula and Molecular Weight:

$HO(C_2H_4O)_a(C_3H_6O)_b(C_2H_4O)_aH$. For the Poloaxmer 407 the corresponding values for a, b are 101 and 56 respectively.

The average molecular weight: 9,840–14, 600.

Structural Formula:

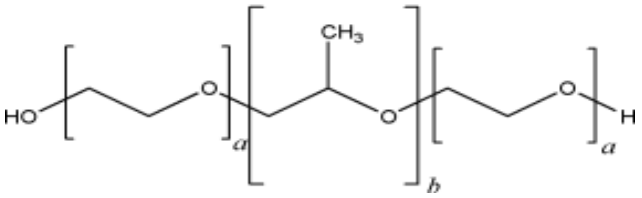


Fig 6: Chemical Structure of Poloxamer-407

Functional Category:

Emulsifying agent, dispersing agent, solubilizing agent, Co-emulsifying agent, wetting agent and tablet lubricant.

Description:

Poloxamer 407 generally occurs as white, waxy, free-flowing granules. It is practically odorless and tasteless.

Typical Properties:

Acidity/alkalinity: pH = 5.0–7.4 for a 2.5% w/v aqueous solution,

Cloud point **:** >100°C for a 1% w/v aqueous solution,

HLB **:** 18-23

Density **:** 1.06 g/cm^3 at 25°C,

Flash point **:** 260°C,

Flowability **:** Free flowing,

Melting point **:** 52–57°C

Moisture content: Poloaxmer generally contain less than 0.5% w/w water.

Solubility **:** Freely soluble in Water and 95 % Ethanol.

Applications in Pharmaceutical Formulation or Technology

Poloaxmer used as wetting agents in ointments and gels. Poloaxmer are used solubilizing and stabilizing agents and emulsifying agents in intravenous fat emulsions. Recently poloaxmer have found use in drug-delivery systems. Poloaxmer 407 is used in solutions for contact lens care.

2.2.5. Magnesium stearate [58]

Nonproprietary

Names:

BP: Magnesium sterate. JP: Magnesium stearate. PhEur: Magnesii stearas USPNF: Magnesium stearate.

Chemical Name and CAS Registry Number:

Octa-decanoic acid magnesium salt [557-04-0]

Empirical Formula and Molecular Weight: $C_{36}H_{70}$ Mg O_4 : 591.34

Structural Formula:

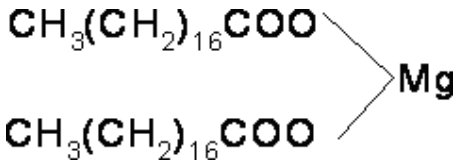


Fig 7: Chemical structure of magnesium stearate

Description:

Magnesium stearate is a very fine, light white, precipitated or milled, impalpable powder of low bulk density, having a faint odor of stearic acid and a characteristic taste. The powder is greasy to the touch and readily adheres to the skin.

Typical Properties

Crystalline forms: high-purity magnesium stearate has been isolated as a trihydrate, a dihydrate, and an anhydrate.

Density (bulk): 0.159 g/cm^3 Density (tapped): 0.286 g/cm^3 Density (true): 1.092 g/cm^3

Flash point: 250 ^{0}Ċ

Flow ability: Poorly flowing, cohesive powder.

Melting range: 117–150^{O}Ċ (commercial samples); 126– 130^{O}Ċ (high purity magnesium stearate).

Solubility: Practically insoluble in ethanol, ethanol (95%), ether and water; slightly soluble in warm benzene and warm ethanol (95%). Specific surface area: 1.6– 14.8m^2/g

Functional Category: Tablet and capsule lubricant.

Applications in Pharmaceutical Formulation:

Magnesium stearate is widely used in cosmetics, foods, and pharmaceutical formulations. It is primarily used as a lubricant in capsule and tablet manufacture at concentrations between 0.25% and 5.0% w/w. It is also used in barrier creams.

2.2.6 Talc [59]

Nonproprietary Names;

BP: Purified talc. JP: Talc .PhEur: Talcum

Chemical Name and CAS Registry Number: Talc:[14807-96-6]

Empirical Formula and Molecular Weight:

Talc is a purified, hydrated, magnesium silicate, approximating to the formula $Mg_6 (Si2O5)_4(OH)_4$. It may contain small, variable amounts of aluminum silicate and iron.

Structural Formula: $\mathbf{Mg_6(Si_2O_5)_4(OH)_4}$.

Typical Properties:

Acidity/alkalinity: pH = 7–10 for 20% w/v aqueous dispersion. **Hardness (Mohs):** 1.0–

1.5

Moisture content: talc absorbs insignificant amounts of water at 258C and relative humidities up to about 90%.

Particle size distribution: varies with the source and grade of material. Two typical grades are 99% through a 74 mm (#200 mesh) or 99% through a 44 mm (#325 mesh).

Refractive index: nD 20 = 1.54–1.59

Solubility: Practically insoluble in dilute acids and alkalis, organic solvents, and water.

Specific gravity: 2.7–2.8

Specific surface area: 2.41–2.42m^2/g

Functional Category;

Anticaking agent; glidant; tablet and capsule diluent; tablet and capsule lubricant.

Applications in Pharmaceutical Formulation or Technology:

Talc was once widely used in oral solid dosage formulations as a lubricant and diluent, although today it is less commonly used. However, it is widely used as a dissolution retardant in the development of controlled-release products.

FOR LORNOXICAM GEL

2.2.7 Sodium alginate[60]

Nonproprietary Names:

NF-Sodium alginate, BP-Sodium alginate. PhEur: Chitosani hydrochloridum

Chemical Name: Sodium alginate**,** it consists of sodium salt of Alginic acid, a polyuronic acid composed of a- D-Mannuronic acid carboxyl group of each unit is free while the aldehyde group is shielded by a glycoside linkage.

Empirical formula and Molecular Weight:

Sodium alginate consists chiefly of the sodium salt of alginic acid, which is a mixture of polyuronic acids composed of residues of Dmannuronic acid and L-guluronic acid.

Structural Formula

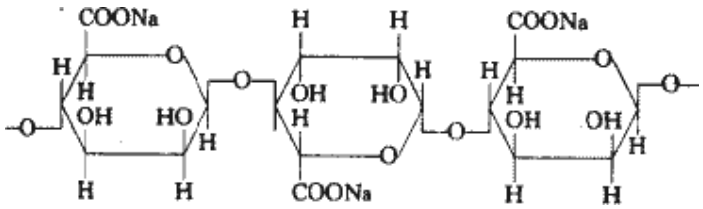


Fig 8: Chemical Structure of sodium alginate

The number and sequence of the Mannuronate and Glucuronate residues shown above vary in the naturally occurring alginate. The water molecules associated with the alginate molecule are not shown in the above structural formula.

Description: Sodium alginate occurs as an odorless and tasteless, white to pale Yellowish-brown colored powder.

Typical Properties:

Flow ability: Free flowing

Hygroscopicity: Highly hygroscopic.

Viscosity: Various grades of sodium alginate are commercially available that yield aqueous solutions of varying viscosity. Typically, a 1% w/v aqueous solution, at 20^{o}C, will have a viscosity of 20–400 mPas (20–400 cP). Above pH 10, viscosity decreases.

Acidity/alkalinity: pH=7.2 (1% w/v aqueous solution)

Solubility: Slowly soluble in water. It is insoluble in alcoholic and Hydroalcoholic solutions in which the alcohol content is greater than 30% by weight. It is also insoluble in other organic solvents and acids.

Functional Category: Stabilizing agent, suspending agent, tablet binder, tablet and capsule as disintegrants, viscosity increasing agent, coating agent, film-

forming agent mucoadhesive agent.

Applications in Pharmaceutical Formulation or Technology

Their application generally depends on the thickening, gel forming, and stabilizing properties. Sodium alginate can be used can be used as a binding and disintegrating agent in tablet, as a suspending agent and thickening agent in water- miscible gels, lotions and creams, and as a stabilizer for emulsion.

2.2.8 Calcium gluconate [61]

Nonproprietary Names.JP: Calcium Gluconate

PhEur: Calcium gluconate . USP-NF: Calcium Gluconate

3. Chemical Name and CAS Registry Number

Calcium bis((2R,3S,4R,5R)-2,3,4,5,6-pentahydroxyhexanoate)

Calcium D-gluconate (1:2) [299-28-5]. Monohydrate [18016-24-5].

4. Empirical Formula and Molecular Weight

$C_{12}H_{22}CaO_{14}$ 430.37

5. Structural Formula

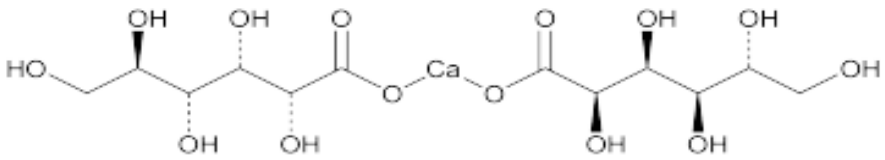


Fig. 9.Calcium gluconate structure

6. Functional Category: Mineral supplement polymer. Chelating agent.

8. Description: White, crystalline powder or granules

9. Typical Properties

Water Solubility- 44.2 mg/mL(Predicted Properties)

pH -Between 6,0 and 8,0 (5 % solution)

Density - 0.30-0.65 g/cm³

Melting point - 178 °C

Refractivity- 10

Polarizability- 49.11 $m^3 \cdot mol^{-1}$

pKa (Strongest Acidic)- 3.39

pKa (Strongest Basic) -3

2.2.9 Solutol® HS 15 [62]

Ph.Eur.: Macrogol 15 Hydroxystearate

USP : Polyoxyl 15 Hydroxystearate

Category: A non-ionic solubilizer

Chemical Nature:

SOLUTOL®HS 15 is an non-ionic solubilizer and emulsifying agent obtained by reacting 15 moles of ethylene oxide with one mole of 12-hydroxystearic acid.

Description:

SOLUTOL®HS 15 is a yellowish white paste at room temperature that becomes liquid at approx 30ºC.

The hydrophilic-Lipophilic balance lies between 14-16.

The critical micelle concentration (CMC) lies between 0.005 to 0.002%.

Composition:

SOLUTOL®HS 15 consist of polyglycol mono- and di-esters of 12-hydroxystearic acid (=lipophilic part) and of about 30% of free polyethylene glycol(=hydrophilic part). The free polyethylene glycol can be determined by HPLC. The main components of the lipophilic part have the following chemical structures: A small part of the 12-hydroxy group can be etherified with polyethylene glycol

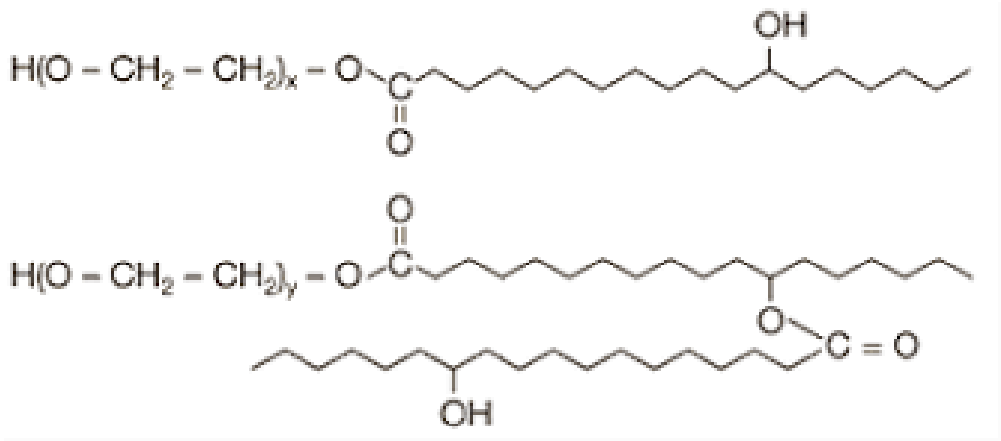


Figure 10: Chemical structure of lipophilic part of Solutol HS 15

Solubility:

Solutol HS 15 is soluble in water, ethanol and 2-propanol to form clear solutions. Its solubility in water decreases with increasing temperature. It is insoluble in liquid paraffin.

Applications:

The productis used for manufacturing aqueous parenteral preparations with vitamins A,D,K and E. For solubility enhancement of poor water solubile drug such as Nefidipine, Piroxicaim, Alfadolone and alfaxalone.

2.2.10 Methylparaben [63]

Nonproprietary Names

BP: Methyl hydroxybenzoate.JP: Methyl parahydroxybenzoate

PhEur: Methylis parahydroxybenzoas. USPNF: Methylparaben

Chemical Name and CAS Registry Number

Methyl-4-hydroxybenzoate [99-76-3]

Empirical Formula and Molecular Weight C_8H8O_3; 152.15.

Structural Formula

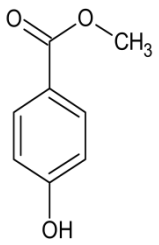


Fig 11: Chemical Structure of Methyl paraben

Functional Category: Antimicrobial preservative.

Applications in Pharmaceutical Formulation or Technology

Methylparaben is widely used as an antimicrobial preservative in cosmetics, food products, and pharmaceutical formulations.

Table .3. Uses of Methylparaben.

Use	Concentration (%)
IM, IV, SC injections	0.065–0.25
Inhalation solutions	0.025–0.07
Intradermal injections	0.10
Nasal solutions	0.033
Topical preparations	0.02–0.3
Ophthalmic preparations	0.015–0.2
Vaginal preparations	0.1–0.18
Rectal preparations	0.1–0.18
Oral solutions and suspensions	0.015–0.2

Description

Methylparaben occurs as colorless crystals or a white crystalline powder. It is odorless or almost odorless and has a slight burning taste.

Density (true): 1.352 g/cm^3

Dissociation constant: pKa = 8.4 at 22^{0}C

Melting point: 125–128^{0}C

2.2.11 Propylparaben [64]

Nonproprietary Names

BP: Propyl hydroxybenzoate. JP:Propyl parahydroxybenzoate

PhEur: Propylis parahydroxybenzoas. USPNF: Propyl paraben

Chemical Name and CAS Registry Number

Propyl 4-hydroxybenzoate [94-13-3]

Molecular weight: 180.2g/mol

Structural Formula

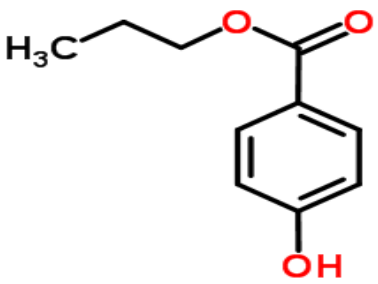


Fig: 12 chemical structure of propyl paraben

Functional Category Antimicrobial preservative.

Melting point: 95^{0}C to 96^{o}C

Density (bulk): 0.426 g/cm^3

Density (tapped): 0.706 g/cm^3

Density (true): 1.288 g/cm^3

Dissociation constant: pKa = 8.4 at 22^{0}C

Flash point: 140^{0}C

Table 4.List of Equipment's and Instruments

Sr. No.	Instrument Name	Model	Make
1.	Fermentor	B-lite	Sartorius B-lite. Mumbai
2.	Magnetic Stirrer	2-MLH	Remi Mumbai
3.	BOD Incubator	IC 10	IEC Pvt. Ltd Mumbai
4.	Freeze Dryer	Fd6	Lydol, Mumbai
5.	pH Meter	EQ- 610	Equip-Tronics
6.	Micropipettes	P100	Accupipipet, Capp
7.	High Speed Centrifuge.	Cure800plus	Sakova, Scientific Co.
8.	Laminar air flow		Klenzaides Bioclean
10.	UV-Visible double beam Spectrophotometer	Sican 2301	Inkarp Instrumentation Ltd Hydrabad
11.	Tablet Dissolution Test Apparatus	TD080	Electro lab, Mumbai
12	Tablet disintegration apparatus	USP ED2A	Electro lab, Mumbai
13	Vernier calliper	--	Mitutoyo, Japan
14	Monsanto hardness tester	--	Cambell Electronic Mumbai,
15	Friability test apparatus		Roche friabilitor
16.	FTIR	IRAffinity-1S	Shimadzu
17	Electronic Balance Model	AA-2200	Anamed Instruments Mumbai
18	Differential Scanning Calorimeter	DSC-60	Shimadzu
19	Distillation Assembly	2lt	Bhanu Scientific Instruments Co. Pvt. Ltd. Bangalore, India.

20	Hot air oven	T300	PSM industries.
21	Franz Diffusion Cell	V6B-02	PermeGear
22	Homogenizer	DV-III Ultra	Remi India
23	Probe Sonicator	Probe-400	Rivotek Mumbai
24	Autoclave	5LT	Emkay Ltd.Bangalore
25	High Performance Liquid Chromatography	SPD-M20A	Shimadzu
26	Compression machine	10station	Rimek Mini Press – I-Ahmedabad, India.
27	Humidity control oven	10ED	Lab Control, Mumbai
28	Orbital Shake Incubator	.6FL	Sakova, Scientific Co Mumbai

Table 5. List of Chemicals

Sr. No.	Chemicals	Supplied by,
1	*Flavobacterium* Sp 2495	Institute of Microbial Technology (IMTECH), Chandigarh, India.
2	Atovaquone USP	Matrix Lab Ltd Hyderabad
3	Lornoxicam IP	Hetero drug Hyderabad
4	Sugar cane molasses	Local sugar industry Sankeswar Belagavi
5	Waste fried oil	Local market
6	Lactose AR	Balji chemicals, Mumbai
7	Polyvinyl pyrrolidone (PVP) IP	Balji chemicals, Mumbai
8	Magnesium stearate IP	Balji chemicals, Mumbai
9	Talc IP	Balji chemicals, Mumbai
10	HPMC USP	Colorcon Ltd, Goa
11	Solutol HS 15	BASF India Ltd., Mumbai, India)
12	Polysorbate 80 USP	Balaji chemical Mumbai
13	Poloxamer 407	Sigma Aldrich. Mumbai
14	Ethanol 99.9%	Changshu Yangyuan Chemical China
15	Calcium Nitrate AR	Nice Mumbai
16	Potassium Dihydrogen Orthophosphate AR	Himedia Pvt Ltd. Mumbai
17	Disodium Hydrogen Phosphate LR	Himedia Pvt Ltd. Mumbai
18	Sodium chloride IP	Himedia laboratories pvt ltd ,Mumbai
19	Ammonium Chloride IP	Rankem Mumbai
20	Sodium hydroxide LR	Himedia laboratories pvt ltd ,Mumbai
21	Ammonium Sulphate LR	Rankem Mumbai
22	Potassium Chloride AR	Nice

23	Potassium Nitrate LR	Nice
24	Disodium hydrogen phosphate AR	Himedia Lab Pvt. Ltd-Mumbai
25	Peptone	Himedia Lab Pvt. Ltd-Mumbai
26	Beef extract	Himedia Lab Pvt. Ltd-Mumbai
27	Yeast extract	Himedia Lab Pvt. Ltd-Mumbai
28	Corn steep liquor	S.DFine Chemicals Pvt.Ltd.-Mumbai
29	Magnesium Sulphate	Rankem Mumbai
30	Magnesium Chloride	Rankem Mumbai
31	Ferrous Sulphate	Rankem Mumbai
32	Agar	Himedia Lab Pvt. Ltd-Mumbai
33	Calcium Chloride	Himedia Lab Pvt. Ltd-Mumbai
34	Zinc Sulphate	Rankem Mumbai
35	Copper Sulphate	Rankem Mumbai
36	Sodium Molybdate	Merk Mumbai
37	Potassium Hydroxide.	PCL keral
38	Urea	Himedia Lab Pvt. Ltd-Mumbai
39	Chloroform	Rankem Mumbai
40	Methanol	Rankem Mumbai

2.3. METHODS:

Phase I Biosynthesis of biosurfactant

2.3.1. Sub culturing and preliminary characterization of *Flavobacterium sp.* MTCC 2495.[65]

The *Flavobacterium* Sp. 2495 procured from MTCC Chandigarh was sub cultured in sterilized modified Wlkimoto medium contains, peptone 5gm per liter, agar 15 gms per liter, calcium nitrate 0.5gm per liter, sodium phosphate 2 gms per

liter, ferrous sulphate 0.5gm per liter, aseptically in to 6cm diameter petri dish at room temperature using laminar flow. Petri dishes were incubated at 32^{o}C in incubator for 24hrs.

2.3.1.1. Gram staining: [66, 67]

Subculture *flavanobaterium* sp 2945 was subjected for gram staining using following reagents and staining procedure (Smith AC et al 2016) was followed as mentioned below.

Reagents required:

- **Primary stain:** Two gms Crystal violet, 20mL 95% ethyl alcohol**,** 0.8 gms ammonium oxalate make up to 100mL with distilled water.
- **Gram's Iodine:** Two gms of potassium Iodide, one gm of Iodine dissolved in 100mL of distilled water.
- .**Decolourizer:** 50mL of ethanol and 50 mL of acetone.
- **Counter-stain:** 4.0 gms Safranin, 200 mL 95% ethanol, and 800 mL distilled water
- Microscopic slides were washed with soap and water to free from grease or oil substance and dried with hot air dryer
- With sterile loop, bacterial culture broth was smeared on the slide in circular motion with one centimeter diameter.
- Slide was air dried for one hour and warmed over the Bunsen burner until dried smeared was observed and no overheating was observed.
- Slide was kept on inclined glass rod stand; two drops of crystal violet were added over the smear and kept for 1 minute.
- Slide was washed with excesses of water. Gram's Iodine solution was added kept for one minute.

- Slide was washed with excesses of water, purple colour smear was observd.
- Coloured slide was rinsed with alcohol solution till no colure was eluted.
- Rinsed with water and safranin was added over the smear and kept for 45 seconds.
- Once again slide was washed with water and dried with whatman paper by gently pressing over it.
- Smear colour was observed under microscope with 40X magnification.
- Gr – ve bacteria stains red or pink colour. Gr+ bacteria stains blue or purple colour.

2.3.1.2. Motility test: Hanging drop method: [67]

[UK (NHS) Standards for investigation of microbiological. (Motility)]

- Central cavity microscopic slide was cleaned with tap water and dried with domestic dryer for 5 minutes.
- Applied petroleum jelly around the cavity of slide taking the care that cavity of slide was not stained.
- One drop of fresh bacterial culture broth was transferred in to the cavity of the slide with the help of sterile platinum loop.
- Cleaned and dry cover slip was taken and all the edges of the cover slip were coated with petroleum jelly in circular shape.
- Coated cover slip was inverted over the cavity slide slowly and gently pressed to seal the cavity.
- The slide is then clipped to the stage and examined under the low power objective of the microscope.
- Using the 4X objective, focus on the edge of the drop and observed for bacterial motility, slightly closing the iris diaphragm.

- Zigzag movement was observed bacteria are motile, no movement, non-motile.

2.3.1.3. Oil spreading method: [68]

Water (20ml) and 20µ liter of kerosene were mixed in petridish (12cm dia) *Flavobacterium* sp culture of 10µ liter was introduced over the surface of water. Oil spreading diameter was measured on the surface of the water. Interpretation of results was done as follows.

No spreading of oil on the surface of water as**" –Ve."**

Oil spreading diameter of 1 cm on the surface of water **"+ve."**

Oil spreading diameter of 2 cm on the surface of water **"++ve."**

Oil spreading diameter of 3 cm on the surface of water **"+++ve."**

2.3.2. Laboratory scale biosynthesis of biosurfactant:

2.3.2.1. Preparation of modified mineral salt medium.[69]

Table.6. Composition of modified mineral salt medium

	Solution A		Solution B	
SL No	Chemical	Quantity/liter	Chemical	Quantity/liter
1	$Ca(NO_3)_2$	0.050 g.	$FeSO_4.7\ H_2O$	150 mg
2	KCl	0.05 g.	$ZnSO_4.7H_2O$	150 mg
3	NaCl	1.0 g.	$MnSO_4.2\ H_2O$	150mg
4	$MgSO_4$	0.4 g .	$CoCl_2.6H_2O$	15mg
5	$CaCl_2.2H_2O$	0.05 g.	H_3BO_3	30 mg
6	$H_3\,PO_4$ (85%)	10ml.	$CuSO_4.5H_2O$	15 mg
7	Glucose	2%.	$NaMo_2O_4.2H_2O$	10 mg.
8	Peptone	0.3 g.	. pH was adjusted to 7.1 with KOH	
9	pH was adjusted to 7.1 with KOH			

Modified mineral salt medium was prepare from above composition of solution A and B in distilled water separately, sterilized in auto clave at 121^{o}C, 15 lb pressure for 45 minutes. Final modified mineral salt medium was prepared by mixing 10ml of solution B with one liter of solution A aseptically and stored 4^{o}C till further use.

2.3.2.2. Cultivation of *Flavobacterium sp* 2495 in modified mineral salt medium.[70, 71]

Subculture *Flavobacterium* sp 2495 was transferred (1gm) to the modified mineral salt medium (10ml) ascetically and vertex for 10 minutes in test tube. Bacterial suspension was mixed with 300ml of modified mineral salt medium in 500ml Erlenmeyer flask, supplemented with 5%w/v sucrose and 10%w/v peptone. Erlenmeyer flask containing bacterial suspension and media was incubated on rotary shaker at 32^{o}Cwith 100 rpm for 84hrs.cultivated bacterial suspension was stored at 4^{o}C till further use. All apparatus used in this preparation were previously sterilized in hot air oven at 220^{o}C for one hr.

In processes control

a) Cell growth: [72]

Cell growth was measured using UV Visible spectrophotomtericaly by pipetting out 2ml bacterial suspension and OD_{600} was determined for every 12 hrs interval. Constant or declined OD reading interpreted as cell growth was stopped.

b) Determination of biomass: [73]

It is a measurement of dry biomass of the cell growth from the cultivated medium. It is expressed in gms per liter. Ten ml of bacterial suspension was pipetted out and centrifuged at 5000x for 5 minutes. Sediment was collected and washed with distilled water dried at 60^{o}C for 1hr.Sediment was cooled to room temperature and

weighed. For every 12 hrs weight was determined till constant weight. Sink condition was maintained by replacing the fresh broth.

c) Emulsification index [74, 75].

Bacterial broth was filtered with whatman filter paper. Filtrate was used for determination of emulsifying index for liquid paraffin. Liquid paraffin (20ml) was triturated with 20ml of crude filtrate and 5 ml of tween 80 respectively. Emulsions were kept for 72hrs in measuring cylinder and observed for rate of separation.

Emulsifying index was determined from the following formula

$$\text{E index} = \frac{\text{H (emulsion layer)}}{\text{H (Total volume)}} \text{ x } 100$$

E index= Emulsifying index. H= Height.

2.3.2.3. Optimization of substrates concentration using 3^2 factorial designs[76, 77, 78.]

Lab scale production and optimization of substrate concentration was performed using 3^2factorial design (Design expert 9 software). Molasses and waste fried oil were used in the production of biosurfactant as independent variables with three levels. The dependent variable was yield of biosurfactant and surface tension. The three levels were -1, 0, and +1 as coded formulation for substrates expressed in %w/v. Coded values are shown the table.7.

Table7.Coded values and actual values for the independent Variables.

Sl no	Independent factors		Coded level			Dependent factors	
	X1 %w/v	X2 %w/v	-1	0	+1	Y1 Gm/liter	Y2 Dy/cm
1	Molasses	------	2	5	8	Biosurfactant yield	Surface tension
2	----	Waste fried oil	2	4	6		

Soft ware generated nine batches.

Table 8. Optimization of laboratory scale batches.

Run	Factor X1 Sugarcane molasses %	Factor X2 Fried waste oil %
1	8	4
2	2	6
3	5	6
4	2	2
5	8	2
6	8	6
7	5	4
8	2	4
9	5	2

2.3.2.3. Laboratory scale procedure:

Molasses and waste fried oils were sterilized in autoclave at 121^{o}C temperature with 15 lb pressure for 45 minutes. All nine batches were prepared by adding varying concentration of substrates in 500ml Erlenmeyer flask containg 400ml of modified mineral salt media and 25ml inoculum was transferred aseptically. Final solution pH was adjusted to 7.2 with potassium hydroxide. All flaks were kept for fermentation on orbital mechanical shaker at 32.5^{o}C temperature, 100rpm for 72hrs.

2.3.2.4. In processes control: surface tension [79]

The basic principle involved in the in- processes quality control of fermentation broth was determination surface tension before and intermediate intervals during the fermentation process. Biosurfactant concentration is inversely proportional to the surface tension of the broth. Surface tension was determined by drop count method using Stalgnometer. Surface tension was determined at Initial (O hr), intermediated (36hrs) and final intervals (72hrs) and density of media by specific gravity bottle. Surface tension was calculated using following formula.

$$V1 = \frac{N2\,\rho1}{N1\rho2}\ X\ V2 \qquad \text{Dy/cm}$$

V1 = Surface tension of broth

V2= Surface tension of water (72.5)

N2= Number of drops of water

N1= Number of drops of broth

ρ1 = Density of broth

ρ 2= Density of water

2.3.3. Extraction and purification of biosurfactant.[80, 81]

There are three common methods of extraction of biosurfactant from the fermented broth. These are acid precipitation method, ammonium sulphate

precipitation and solvent extraction.All three extraction processes were conducted on extra run batch no 3. It was found that solvent extraction method resulted in maximum yield comparatively.

2.3.3.1 Solvent extraction method

Solvents used in this method were methanol and chloform in the ratio of 1:2.

All the nine run batches of fermentation were filtered through what man filter paper no 41 under vacuum, biomass was dried, weighed and recorded. Solvent mixture 250ml was shaken with filtrate successively for three times in one liter conical flask and organic layer was separated using separating funnel. Pooled organic solvent was subjected for vacuum evaporator to get dry mass of biosurfactant and weighed. Yield of biosurfactant was recorded.

For all run batches, yield of biosurfactant and surface tension were calculated and subjected for optimization using Design expert 9 soft ware.

2.3.4. Pilot plant scale up of biosurfactants in bioreactor.[82, 83.]

Linear regression analysis was used to develop polynomial equations for the dependent variables, biosurfactant yield and surface tension. The optimized parameters from shaking flask methods scaled up on fermentor by using molasses and waste fried oil. Concentration of molasses and waste fried oil was selected from the design space of overlay plot. Bioreactor fermentor was set up for fermentation with one liter modified mineral salt broth containing 5.5% w/v molasses, 5.0 %w/v waste fried oil and 75ml of inoculum. Fermentation media, pH was maintained at 7.2 with potassium hydroxide. Aeration and temperature was maintained at 40% and $32.5^{O}C \pm 1^{o}C$ respectively. Initial rpm was kept at 200 for 30 hrs and it was reduce to 120 rpm up to the 72 hrs due to foam formation. In process quality control test of surface tension was performed every 12 hrs as described in lab scale flask shake

method. Extraction and purification method for biosurfactant was also adopted as described in the lab scale flask shake method. Total yield of biosurfactant was recorded.

Fermentor conditions:

Capacity 2 lts (Sartorius B-lite.)

Batch size: 1 liter

Temperature: 32.5^{o}C±1^{o}C

pH: 7.2±0.5

Aeration: 40%±1%

RPM: 200/100

Time: 72 hrs

Inoculums size: 75ml

Sugar cane molasses: 5.2%

Waste fried oil: 5.0%

Media: Modified mineral salt media 1lts

Fig. 13.Pilot scale up fermentor (Sartorius B-lite)

2.3.5. Partial chacterisation of biosurfactant.

2.3.5.1. Physical properties:

2.3.5.1.1. Determination of CMC of Biosurfactant [84]

- 2%w/stock solution of Biosurfactant was prepared in distilled water.
- From above stock solution 25ml serial dilutions were prepared in range of, 0%v/v,0.02%v/v,0.04%v/v,0.06%v/v,0.08%v/v,0.1%v/v,0.2%v/v,0.3%v/v,0.4%v/v,0.5%v/v.
- Density was determined by using specific gravity bottle and Surface tension was determined by using Stalgnometer.
- Then plot of graph of Surface tension Vs. the concentration of Surfactant was plotted. The point where the line becomes the constant that gives the CMC value.

2.3.5.1.2.. UV Characterization. (Determination of λmax) [84,85]

Biosurfactant solution was prepared in distilled water with concentration of 10µgm and 50 µgm. Maximum UV absorbance was determined using UV spectrophotometer, scanned from 200nm to 400nm.

2.3.5.1.3. FTIR Characterization [84, 85]

The infrared spectrum of the Biosurfactant was recorded using Fourier Transform Infra-Red spectrophotometer (Shimadzu IR affinity FTIR) in the rage of 4000-400 $cm.^{-1.}$ Graph was recorded and interpreted for important functional peaks.

2.3.5.1.4. Differential scanning calorimeter. [84, 85]

Differential scanning calorimeter was performed using the Metter Toled DSC810e instrument attached with intercooler and nitrogen purging. The sample was hermetically sealed in aluminum pan and heated at constant rate $20^{o}C$/min over range of $35^{o}C$ to $45^{o}C$, nitrogen gas was purged at the rate of 10ml/min. Graph was recorded.

2.3.5.2. Chemical properties:

2.3.5.2.1. Thin Layer Chromatography Characterization [84, 86]

The Biosurfactant was subjected for TLC characterization by using solvent system chloroform-methanol-acetic acid-water, 25:15:4:2. Silica gel plate (pre-coated 0.25mm) was preheated at 60^{o}C for one hour in hot air oven to remove the adsorbed moisture and cool to room temperature. Biosurfactant solution of 30%w/v in water was prepared and 25 µl to 30µl sample was applied on the base line of the pre coated plates. Plates were subjected for development with mobile phase in glass chamber for 5 hours. After the development the plates, one plate was sprayed with 1%α-napthol in sulphuric acid with ethanol(1:1) solvent acid and heated at 160^{o}C to 170^{o}C in oven for 15minutes, brown colour spot was marked and Rf value was calculated. To another plate, ninhydrine reagent (acetic acid) was sprayed

2.3.5.2.2. Identification of Fatty acids: [87]

The Biosurfactant was hydrolyzed with 6 M HCl at 110^{0}C for 20 hr and lipid moiety was subsequently separated by extraction with chloroform. Several drops of bromine water were added to extract. Disappearance of brown colour indicates that unsaturated fatty acid is present.

2.3.6. Pharmacological studies of biosurfactant.

2.3.6.1. Oral toxicity study.[88]

Biosurfactant acute oral toxicity test was performed on albumin rats as mentioned in the Organization for economic co-operation and development (OECD) guidelines, fixed dose procedure 420. Five female albinos wistar rats were used in the study having the average weight in the range of 150gms to 180gms.Fixed dose 2000mg per kilo weight was selected. Dose of biosurfactant was administered with respect body weight of each rat through oral gavage tube dissolved in 4 ml of distilled

water and administered in divided doses of 2 ml with 2hrs interval. All the animals were assessed with normal food and water during period of study. Animals were observed for following signs and symptom for 14 days. If any morbidity was observed, experiment was repeated with lower doses as mentioned in the OECD guidelines.

Signs and symptoms: a) skin and fur b) eyes c) body weight d) behavioral patterns

e) Salivation. f) Lethargy. g) Sleeps. h) Diarrhea. i) Coma tremors. j) Death.

Table 9.Acute oral toxicity study of biosurfactant

No. of animal	**Weight of animal /biosurfactant(mg)**	**Dose administered (ml)** (2hrs gap)
.1	158gms/316	2ml /2ml
.2	144gms/ 288	2ml /2ml
3	153gms/306	2ml /2ml
4	164gms/326	2ml/2ml
5	159gms/318	2ml/2ml

2.3.6.2. Acute dermal toxicity study [89]

Acute dermal toxicity test was performed as mentioned in the OECD guideline 402.Five albinos' wistar rats were selected with average weight in the range of 150 gms to 220 gms. Fixed area of approximately 3cmx 2cm area on the dorsal part was shaved carefully without affecting the skin layer. Biosurfactant dose 2000mg per kilo body weight was prepared in 2.5ml of distilled water applied on the specified surface area on four animals with sterilized cotton web and one animal was kept as control. Test animals were observed for following signs and symptoms for 14 days.

Signs and symptoms: a) reddening/ erythema b) oedema d) body weight c) food intake d) mortality.

Table 10. Biosurfactant dose application

No.of animal	Weight of animal /biosurfactant(mg)	Dose applied (ml)
1	138gms/276	2.5 ml
2	124gms/ 248	2.5 ml
3	143gms/286	2.5 ml
4	154gms/308	2.5 ml
5	139gms control	---

2.4. PHASE II

ATOVAQUONE TABLET FORMULATION WITH BIOSURFACTANT.

2.4.1. Preformulation study of atovaquone [90]

2.4.1.1 Melting Point Determination:

The melting point of Atovaquone was determined by capillary method using Thiele tube. The melting point was also confirmed by DSC (Shimadzu) study. Melting point range of Atovaquone is in the rage of 216 ^{o}C to 219 ^{o}C. (Drug bank)

2.4.1.2. Solubility Determination:

Saturation solubility study was performed with different solvents. Excesses quantity of drug was dissolved in 25ml of solvent in a conical flask and kept for 48 hrs in mechanical shaker at room temperature.5ml solution was pipetted out filter through the whatman filter paper no40 and drug content was analyzed through UV spectrophotometer (ShimadzuUV1800) at specified λmax.

2.4.1.3. Standard Calibration Curve and λmax.

1) Preparation of stock solution:

Accurately weighed 10 mg of Atovaquone drug was dissolved in 100ml of methanol in a volumetric flask. Serial dilutions were prepared to achieve the final concentration of 10µgm per ml with methanol.

2) Preparation of standard calibration curve solutions:

From the above stock solution serial dilutions was prepared with varying concentration raging from 2µg/ml to 10 µg/ml in a 10ml volumetric flask using methanol as a solvent (Beer's range1to10µg). 5µg/ml sample solution was scanned from 200nm to 400nm using UV spectrophotometer ((ShimadzuUV1800) absorbance maxima was recorded. Observed λmax was used for all other diluted solutions to get standard calibration curve. Abs V/s con graph was plotted. Linear regression equation was derived and R^2 valued recorded.

2.4.2.0. Drug-excipient compatibility study

2.4.2.1. FT-IR spectroscopic studies:

Physical mixture of drug and excipients were mixed in glass bottle with lid tight and kept for 15 days at room temperature. Physical mixture was subjected for compatibility study by Fourier transmission infrared spectrophotometer (FTIR) (Shimadzu Japan) KBr pellet method, scanned from 4000-400cm-1. Graphs were recorded, analyzed comparative compatibility study.

2.4.2.2. Differential scanning calorimetric study:

Drug and excipients compatibility studies was carried in DSC60 (Shimdzu) installed with intercooler system, using aluminum pan, hermetically sealed, endothermic /exothermic graphs were recorded. Heating processes was carried out at 10 ^{o}C/min rate and Nitrogen gas purging at 20ml/min. The heating range was selected from 50 ^{o}C to 300 ^{o}C according to the drug and excipients specified limits.

2.4.3. Development and validation of HPLC method for estimation of Atovaquone [91, 92, 93]

I) Instrument:

HPLC Model**:** Shimadzu LC-20

Fixed Capacity Loop**:** 20 µl

Detector**:** UV-20 AD (D2)

Column**:** TERMO Hypersil-Keystone, C8: 250x4.6mm, particle size-5µ.

Λmax: 254nm

Mode: Isocratic

Mobile phase: Acetonitrile: 0.1%acetic acid in Buffer (water) (80:20)

Injection volume: 20µl.

II) Preliminary study of Mobile phase:

Combination of acetonitrile and buffer solution of pH range from 1 to 8 was developed and studied for estimation of drug. The optimum ratio was found to be 80:20 of Acetonitrile and buffer solution (0.1% acetic acid) respectively. Solvents were HPLC grade and filtered through 0.45µ membrane filter before use.

III) Preparation of standard stock solution:

Atovaquone drug 10 mg was weighed and dissolved in the mobile phase consisting of acetonitrile and 0.1% acetic acid solution (80:20) in 100ml.Sonicated for

30 min to dissolve completely. In 10ml volumetric flask, serial dilutions were prepared in the range of 20µ/ml to 60µ/ml with mobile phase solvent.

IV) Preparation of sample solution:

Quantity equivalent to 10µ/ml Atovaquone drug solution was prepared in the mobile phase and filter through 0.45µ filter cartage piston. Solution was injected in to HPLC system and chromatogram was obtained in triplicate. Accurate amount of drug concentration was determined.

V) Chromatographic Conditions:

The mobile phase, Acetonitrile**:** 0.1% acetic acid pH 1.5 (80:20 v/v) pumped at a flow rate of 2 ml/min through the column TERMO Hypersil-Keystone, C8: 250x4.6mm, particle size-5µ.The mobile phase was degassed prior to use under vacuum by filtration through a 0.45µ membrane filter. Atovaquone showed good absorbance at 254nm, which was selected as wavelength for further analysis.

VI) Analysis of drug in spiked plasma solution:

Rabbit blood 2ml was collected from marginal vein of ear and centrifuged immediately at 6000xg for 15 minutes. Plasma was pipetted out mixed with mobile phase. 10mg of Atovaquone drug was dissolved with aid of sonicator and final volume was made up to 100ml in volumetric flask. Serial dilution was prepared in the range of 0.5µg/ml to 25µg/ml. The solution was filtered through Whatman Filter Paper No.42. The prepared sample solution was chromatographed for 6 minutes run time using mobile phase at 254 nm and a flow rate of 2 ml/min. From the peak area obtained in the chromatogram, the amounts of the drug were calculated by fitting peak area responses into the equation of the straight line representing the calibration curves for Atovaquone.

2.4.4. Formulation of binary and ternary solid dispersion: [94, 95, 96.]

Binary and ternary solid dispersion was prepared with drug and HPMC, dissolved in ethanol and water respectively with aid magnetic stirrer. Ethnolic drug solution was added to aqueous polymer solution with constant stirring for half an hour and solvent was evaporated on water bath at 60^{o}C for 5 hrs. Semi dried solid was dried in hot air oven for 3hrs, pulverized and passed through 60 ≠ mesh. Solid dispersed products were stored in air tight container till further use.

Ternary solid dispersed products were prepared by addition of surfactant and biosurfactant in the mixture of drug and polymer solutions with varying concentrations as shown in the table 11, and solid products were obtained with above mentioned procedure.

Table.11. Formulation of solid dispersion of Atovaquone

Ingredients	**Formulation code**							
	Unit	**S_0**	**S_1**	**S_2**	**S_3**	**S_4**	**S_5**	**S_6**	**S_7**
Atovaquone	mg	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5
HPMC	mg	----	125	125	125	125	125	125	125
Biosurfactant	mg	----	--	2	4	6	-	-	--
Poloxamer407	mg	----	--	--	-	-	10	20	30
Ethanol	ml	----	30	30	30	30	30	30	30

S_0 and S_1, pure drug and binary solid dispersion respectively.

2.4.4.1. Evaluation of solid dispersion:

Drug content.

All prepared solid dispersed products drug contents were determined by UV spectroscopy method. Dissolved 100mg of formulations S_1 to S_7 in volumetric flask with 0.1N hydrochloric acid pH1.2 stirred for one hr on magnetic stirrer, filtered through whatman filter paper 40 and analyzed drug content at 221nm λmax.

***In vitro* dissolution profile.**

Dissolution of formulated solid dispersed product was performed using USP dissolution apparatus (Electro lab India TD 80) paddle method with 0.1N HCl media (900ml) and temperature at 37^oC $\pm1^oC$ for one hour.Accurately100mg of all formulated batches were weighed and packed in teabag, tied to the paddle and run at 50 rpm. Specified intervals 5ml samples were withdrawn analyzed by UV spectra at λmax 221nm. Sink condition was maintained by replacing with fresh media.

2.4.5. Atovaquone Tablet preparation: using the solid dispersed product.[97]

Atovaquone tablets were prepared by two methods. F2, F3 and F1 formulations were physical mixture with and without surfactants respectively. F5; F6 and F4 were incorporation of ternary and binary solid dispersed products respectively.Table.12. Lactose as diluent, PVP as dry binder, Mg stearate and talc as lubricants were used in all formulated batches. Tablets were prepared by direct compression method using 9 mm punch, hardness 6kg/cm2 in 10 station rotary compression machine (M/s Karnavati). From S1, S_3 and S_7 solid dispersed products quantity equivalent to 62.5 mg of Atovaquone drug was incorporated.

Table.12. Formulation of Atovaquone tablets

Ingredients (mg)	Formulation code					
	F1	**F2**	**F3**	**F4**	**F5**	**F6**
Atovaquone	62.5	62.5	62.5	--	--	--
Solid dispersion system	---	---	---	S_1	S_3	S_7
Biosurfactant	--	4	--	--	--	--
Poloxamer407	---	--	30	--	--	--
Lactose	159.5	155.5	129.5	34.5	30.5	4.5
PVP	5	5	5	5	5	5
Mg stearate	1	1	1	1	1	1
Talc	2	2	2	2	2	2
Total weight	230	230	230	230	230	230

S1, Binary and S2, S3. Ternary solid dispersed products.

2.4.5.1. Evaluation of Atovaquone tablet: [98, 99,100.]

1. Precompression parameters:

Micrometric properties of lubricated powder of atovaquone were subjected to following parameters.

a) Bulk density and tapped density:

Quantity of accurately weighed powder (bulk) from each formula was introduced into a 25 ml measuring cylinder. After the initial volume was observed, the cylinder was tapped on hard surface from the height of 2.5 cm at 2 sec interval. The taping was continued until no further change in volume was noted. Untapped and tapped densities were calculated using following formula;

$$\text{UTD} = \frac{\text{weight of the powder}}{\text{final volume of the powder}}$$

$$\text{TD} = \frac{\text{weight of powder}}{\text{final volume of the powder}}$$

B) Carr's compressibility index:

Compressibility index was calculated using above data of UTD and TD with following formula:

$$\text{Compressibility index} = \frac{\text{TD} - \text{UTD}}{\text{TD}} \text{x} 100$$

c) Hausner's ratio:

Hausner's ratio is index of free flowing property of powder. Lower Hausner's ratio (<1.25) indicates better flow properties than higher ones (>1.25).

$$\text{Hausner ratio} = \frac{\rho 1}{\rho 2}$$

Where ρ_1 is tapped density and ρ_2 is bulk density

d) Angle of repose (θ):

Funnel method was used to determine the angle of repose. Weighed quantity of powder was introduced in to 5cm diameter funnel on graph paper. Funnel tip was adjusted to the apex of the heap of the powder. Experiment was repeated twice. Height of heap and diameter of the heap was measured. For good flow property of powder/granules θ t value should be in the rage of 25 θ to 30 θ. Following formula was used to calculate angle of repose θ

$$\tan^{-1} = \frac{\text{h}}{\text{r}}$$

h= height of the heap. r = radius of the pile

2) Post compression parameters:

a) Thickness and Diameter:

Thickness and diameters of all formulated batches tablets were measured using vernier calipers and recorded in triplicate.

b) Weight variation:

Randomly twenty tablets were selected from the all the batches, average weight and individual weights were determined using sensitive electronic balance. IP limits for 230mg cut weight is ±7.5 with two tablets deviation are permissible to pass the test.

c) Hardness:

Hardness of all formulated tablets was measured using Monsanto hardness tester. Randomly selected tablets were placed diagonally in the plunger socket and rotated till tablets breaks in two half. Hardness reading was recorded as kg/cm^2 from the scale.

d) Friability:

To determine the mechanical strength of the formulated tablets, friability test was performed using Roch friabilitor. Randomly20 tablets were selected and de-dusted and weighed. All tablets were placed in circular disc vertically and rotated for 5 minute with 25 rpm. After de dusting final weights were recorded. The % friability was calculated with following formula. IP limit is not more than 1%.

$$\text{percentage friability} = \frac{\text{Wt initial} - \text{Wt final}}{\text{Wt initial}} \text{X}100$$

e) Uniformity of drug content:

Ten tablets were from each batch and triturated in glass pestle and mortar to distribute the drug uniformly in the powder mass. Equivalently 62.5mg of drug, powder was weighed dissolved in to conical flask containg 40ml of methanol. Flask was kept for gyration in mechanical shaker for one hour at room temperature. Flask was warmed on water bath for half hour at 60^oC and filtered through whatman filter paper no 40.Filterate final volume was made up to 100ml in volumetric flask with methanol. Serial dilutions were prepared to achieve 5µg/ml concentration and

absorbance was recorded at 221 λmax using UV spectra (UV1800) drug content was determined from regression equation.

Disintegration test:

Disintegration test was performed for all the formulations of Atovaquone tablets as mentioned in the Indian pharmacopeia 2010 using Electro lab USP ED2A apparatus. Atovaquone tablets were introduced in to the disintegration basket and set for motion at 37^{o}C ±1^{o}C temperature using distilled water as disintegrating medium. Time was noted till whole tablet disintegrate and percolate to the media.

2.4.6. ***In vitro*** **dissolution profile:**

In vitro dissolution profile of Atovaquone tablets was carried out in USPXXIII (Electro lab) using basket type in 0.1NHCl, 900 ml media for one hour. The rpm and bath temperature was maintained at 50 and 37^{o}C±1^{o}C respectively. At prefixed intervals 5ml samples were withdrawn and replaced with fresh media for one hour. All samples drug content was analyzed by UV spectra at 221 λmax.

2.4.6.1 Dissolution efficiency: [101,102.]

The concept of dissolution efficiency (DE) was proposed by Khan and Rodes in 1975 is defined by

$$\mathrm{DEt\%} = \frac{\int_0^t \mathrm{Y.dt}}{\mathrm{Q100t}} \mathrm{X100}$$

Where Y is percentage of drug dissolved at any time t, Q100 is the100 percentage of dissolved product, DE is the area under the dissolution curve between time points 0 and t expressed as a percentage of the curve at maximum dissolution, Q100, over the same time period. In this study DE was found out at t=45 minutes using the dissolution profile data for all formulated batches.

2.4.7. *In vivo* bioavailability study: [103,104,105.]

Relative bioavailability and essential pharmacokinetic parameters were estimated from the best formulation of Atovaquone tablet (F5) and prepared oral conventional suspension of Atovaquone. *In vivo* experiment was performed after approval from Institutional animal ethic committee (Reg no221) Experiment was conducted in accordance with the committee for the purpose of control and supervision of experiments on animals (CPCSEA) guidelines. Six female albino rabbits (1.2-1.5kgs) were divided in to two groups equally. All animals were housed in polypropylene cages and maintained constant temperature ($25^oC \pm 3^oC$) and relative humidity (60±5%) for 24 hours before commencement of experiment. During the experiment period all animals were fed with normal feed and purified water. Group I and Group II animals were administered with best Atovaquone formulation (F5) and prepared conventional oral Atovaquone suspension respectively. Dose administered was 17mg per kg weight of the animals. All animals were fasted for 12hrs before administration of single dose through silicon oral tube. Blood samples (0.3ml) were collected from the marginal ear vein of rabbits at 0.5hr, 1hr, 3hr, 6hr and 12 hrs intervals in heparinized tube with sterilized needles. Plasma samples were collected from blood by centrifugation at 5000 X g for 15 minutes. Plasma was stored at -20^oC until analyzed. Drug content was determined through HPLC, mobile phase methanol and 0.1% acetic acid (80:20) ,flow rate 2ml/min, volume injected 20µl, 10 minute run time with 6 to 7 minute retention time .Drug effluent was monitored at 254λmax.Std calibration equation was used for calculate the drug concentration.

2.4.7.1 Pharmacokinetic parameters: [106]

The obtained data from *in vivo* study were subjected to student t -test and ANOVA for its significance. $p < 0.05$ was considered significant in all the tests.

Systat 13 software was used in the study. All data are expressed in mean±SEM. Primary PK parameters for calculation of bioavailability are AUC, Cmax and tmax. AUC was calculated from the trapezoidal rule, which signified the total exposure of drug in vivo. Cmax and tmax was estimated from the plasma- time profile normal graph.

2.4.8. Short term stability study: [107,108,109.]

Stability studies of best formulation were performed in accordance to the ICH guidelines Q1A (R2). Intermediate temperature 25^{o}C±2^{o}C /60RH±5% and accelerated temperature 40^{o}C±2^{o}C/75RH±5% conditions were selected for testing for 3 months in stability chamber (Lab India).Three batches of F5 batch (30 tablets each) were prepared and sealed in clean and dry glass bottle with absorbent wool on the top and sealed with pilferage proof aluminum caps. Formulated F5 batch was stored in the stability chamber for 3 months with specified temperature and relative humidity. Every month sample was withdrawn and in-process quality control tests were performed. Drug content, hardness and invitro dissolution profile were the in-processes control tests. In vitro dissolution study was conducted for one hr. Methodology adopted for all these tests were as per described early.

2.5. PHASE **III**

LORNOXICAM GEL FORMULATION WITH BIOSURFACTANT

2.5.1. Preformulation of Lornoxicam [110,111,112.]

2.5.1.1 Melting point:

Melting point of pure drug Lornoxicam was performed by capillary method by Thieles' tube. The observed melting point was confirmed by DSC study.

2.5.1.2. Standard calibration curve and λmax:

Accurately 10 mg of Lornoxicam was weighed and dissolved in buffer pH 6.8 in 100 ml volumetric flasks. Volume was made up with buffer solvent. Serial dilutions were prepared to get final concentration to the Beer's rage (2µg to 30µg per ml). 6µg/ml solution was scanned in UV spectrophotometer ranging from 200nm to 450nm. Maximum absorbance was considered as λmax. Serial diluted solutions absorbance was taken in the observed λmax. Linear regression equation was calculated and used in all further calculations.

2.5.1.3. Solubility determination:

Saturation solubility study of Lornoxicam was conducting in a varying concentration of biosurfactant and tween 80 by shake flask method in mechanical shaker at 50 rpm for 72 hrs and temperature at $37^{o}C \pm 2^{o}C$ with excess of drug. Sonicated for 10 minutes and filtered. Filtrate was diluted to specified concentration and absorbance was determined in UV spectrophotomer at λmax 377.5nm.

2.5.2. 0.Drug excipients compatibility study:

2.5.2.1. FTIR spectroscope study:

Drug and biosurfactant interaction study was performed using FTIR spectrometer by KBr pellet method. Drug and biosurfactant were mixed with KBr and triturated in glass mortar. Pellet was prepared in hydraulic press at 1000psi. Mixture was scanned from 4000 cm^{-1} to 400cm^{-1} and recoded the graph.

2.5.2.2. DSC study:

Drug and excipients compatibility studies was carried in DSC60 (Shimdzu) installed with intercooler system, using aluminum pan, hermetically sealed, endothermic /exothermic graphs were recorded. Heating processes was carried out at 10 ^{o}C/min rate and Nitrogen gas purging at 20ml/min. The heating range was selected from 50 ^{o}C to 300 ^{o}C according to the drug and excipients specified limits.

2.5.3 Formulation of Lornoxicam gels: [113,114,115.]

Lornoxicam gel was prepared(table.13) from natural gelling agent, sodium alginate in two concentrations by dispersing, in distilled water with aid of magnetic stirrer for 4hrs and kept for overnight for swelling. Lornoxicam was dissolved in biosurfactant and in mixture of tween 80 and solutol HS15 with water separately using magnetic stirrer for 3hrs.Presevatives was dissolved in warm water. Sodium alginate solution, Lornoxicam solutions and preservatives solution mixed using propeller for 2hr at 1000rpm.Calcium gluconate solution was prepared in hot (70^{o}C) water and added to the above mixture of drug, gelling and preservative solution. Stirred, using propeller at 2000 rpm for 1 hr.Formed gel was stored in glass air tight lid container at room temperature till further analysis.

Table 13. Formulation ingredients of Lornoxicam gel

Sno	Ingredients	Unit	G1	G2	G3	G4
1	Lornoxicam	gm	0.5	0.5	0.5	0.5
2	Sodium alginate	gm	5	5	6	6
3	Calcium gluconate	gm	0.05	0.05	0.08	0.08
4	Biosurfactant	gm	1	---	1	---
5	Tween 80	gm	---	1	----	1
6	Methyl paraben	gm	0.100	0.100	0.100	0.100
7	Propyl paraben	gm	0.050	0.050	0.050	0.050
8	Solutol HS15	ml	8.5	10	8.5	10
9	Distilled water qs	gm	100	100	100	100

2.5.4. Evaluation of Lornoxicam gels: [116,117,118.]

2.5.4.1 pH of gels:

All formulated batches of Lornoxicam gel; pH was measured using pH meter (Systronic) by dissolving one gram of gel in 20ml neutral water at room temperature. Calibration of pH meter was performed using standard pH solutions prepared from specified pH capsule.

2.5.4.2. Drug content:

Drug content of all the batches of Lornoxicam gel were determined from UV spectrophotometer. One gram of gel was dissolved in 100 ml of mixture of phosphate buffer pH 6.8 and methanol (80:20) in volumetric flask. Solution was sonicated for 20 minutes in sonicator at 40 Hz's. One ml of stock solution was diluted to 10ml with phosphate buffer pH 6.8 in volumetric flask solution absorbance was measured at 377.5 nm after filtration through the whatman filter paper no 40.

2.5.4.3. Rheological studies:

Approximately 0.5gm of Lornoxicam gels were placed on the plate of rheometer (Brookfield Capcalc V3build 20-0) and allowed for 10 minutes, to stabilize for constant room temperature ($25^{o}C \pm 2^{o}C$). Shearing rate was gradually increased from 5rpm to 50rpm, shearing stress was recorded. Yield values were calculated from the rheogram by plotting shear stress V/s shear rate. Viscosities of all formulated batches were recorded at 50 rpm.

2.5.4.4. Spreadability:

Lornoxicam gels spreadabilities were determined on glass plates. Two glass plates (12cm x12cm) cleaned and dried, on one glass plate 0.5gm gel was placed in the center and another glass plate was superimposed over other. Pressure was applied over top glass plate using 10gm weight and allowed for 10minutes.Time taken for

separation of two glass plates was found. Using following formula Spreadability was determined.

$$S = \frac{ML}{T}$$

S= Spreadability, gm.cm/second.

M= weight applied on the upper glass plate.gms.

L= Length of glass plate, cm.

T= time taken for separation, seconds.

2.5.4.5. Extrudability:

Extrudability was performed using collapsible aluminum ointment tube. Required quantity of gel was filled and bottom was sealed with crippling machine. Weight required to extrude 0.5 gm ribbon of gel in 10 second was noted. Area of exudates ribbon was calculated ($2\pi r^2+2\pi rh$) in cm^2. Following formula was used to calculate extrudability.

$$\text{Extrudability, } cm^2 = \frac{\text{Applied weight in gms}}{\text{Surface area of ribbon}}$$

2.5.4.6. Gel strength:

Gel strength was determined by in-house fabrication consisting of 100ml graduated beaker and penetrating T shape spindle, made up of glass rod. One end of the glass rod was fixed with glass slide (3cm x 12cm) and to the opposite end another small glass slide (1cm x 1cm) with adhesive as shown in the fig.14. Approximately 50gms of gel was placed in beaker kept for 10 minutes at room temperature. On the T shape glass spindle 25 gms weight was kept and allowed to penetrate in to the beaker containing gel. Time taken to penetrate spindle in to 3cm depth of gel was noted, and reported as gel strength in seconds.

2.4.5.6.1 In house gel strength apparatus validation procedure:

In house fabricated gel strength apparatus was validated using the 6.6 %w/v gelatin IP in warm water.50gms of gelatin gel was placed in 150mL beaker and kept for 10 minutes at room temperature.. T shape plunger with 25gms weight was placed over the surface of the gelatin gel. Time taken for penetrate 3 cm depth was noted. Average time taken for penetrate gel was found to be 92.33 seconds from triplicates.

Justification: The bloom strength of gelatin 6.6%w/v gel is in the range of 152 to 165 using penetrometer.(Shimdzu penetrometer product catalogue)

Fig.14.In house fabricated gel strength apparatus

2.5.4.7. *In vitro* permeation study: [119,120]

Preparation of egg membrane:

The outer egg shell consists of hard calcified layer of calcium carbonate, just below this layer is thin protein layer called Gallus domestious. Gallus domestious layer was removed from raw egg dipped in 15% hydrochloric acid for 8 hrs. All calcium carbonate was dissolved from egg shell was washed with distilled water and normal saline solution to remove the traces of HCl. Decalcified egg was carefully puncture with needle to expel the inside contents and washed with distilled water and followed by with normal saline successively. Cleaned egg membrane was stored in normal saline at 8^{o}C.

In vitro diffusion study was performed using modified Franz diffusion cell consisting of receptor cell with capacity of 40 ml and donor cell 5ml capacity. Diffusion experiment was conducted using egg membrane, cutting the appropriate circular size fixed in between the donor and receptor cells. Receptor cell and donor cell was filled with phosphate buffer pH 6.8: methanol (80:20) and one gram Lornoxicam gel respectively. Whole unit diffusion cell was kept on magnetic stirrer with bead. Temperature and rpm was maintained at $32^{o}C \pm 1^{o}C$ and 20 respectively. Warm water circulation was maintained from outside jacket of diffusion cell. Specified intervals 5 ml sample was withdrawn from the side vent with syringe and replaced with diffusion media to maintain the sink condition; experiment was conducted for 6 hrs. All samples were analyzed at λmax 377.5 nm with diffusion media as reference in UV spectrophotometer.

2.5.4.8. *Ex vivo* permeation study: [121,]

Permeation study was conducted using wistar rat abdomen skin in accordance to the OECD guidelines 428 (2004), after approval of Institutional animal ethical

committee (Reg no 221). Rat was euthanized with pentophenobarbitone injection, abdomen skin was carefully cut after shaving. Skin was washed with normal saline to remove adhered blood and warmed in distilled water for 5 minute at 60^{o}C. Epidermis with stratum corneum layer was carefully removed and stored in saline solution at 4^{o}C temperature. Modified Fraz diffusion cell was used for the study. Receptor cell volume was filled with 40ml Phosphate buffer pH 6.8: methanol (80:20), donor cell with one gram Lornoxicam gel. Rat skin was sandwiched between the cells and clamped. Whole unit was placed on magnetic stirrer at 32^{o}C±1^{o}C temperature and 15 rpm for 8 hrs. Specified intervals samples were withdrawn from the side port and analyzed the drug content at λ max 377.5 nm in UV spectrophotometer with reference as a receptor media.

2.5.4.9. Permeation data analysis: [121]

Permeation data analysis composed of flux of the drug, permeation coefficient and enhancement ratio. Flux is defined as the amount of drug permeated through the given cross section area of member at given time. Flux (J) is dependent on,

J=A.P. L. C.

A = Surface area of the permeate.

P = Partition coefficient of permeate between donor cell content and membrane.

L=Thickness of the permeating membrane.

C= Concentration of permeate in the donor cell.

General equation for flux,

$$\text{Flux} = \frac{\text{dm/dt}}{\text{A}}$$

dm/dt, fraction drug permeated through membrane at given time.

In this experiment data of flux was determined at steady state by plotting the graph cumulative amount of drug permeated against time and slope was calculated at linear line in the graph. Using the following formula Jss was calculated

$$\text{Jss} = \frac{\text{Slope}}{\text{A}}$$

Jss = steady state flux

A= Area of membrane for permeation of permant.

Permeability coefficient:

It is defined as the transport flux of permant through the membrane per unit driving force per unit membrane thickness.(Kp, cm^2/S))

$$\text{Kp} = \frac{\text{J}}{\text{Cd}}$$

Kp= permeability coefficient

J= flux

Cd= concentration of donor compartment

Enhancement ratio:

It is comparison between the high permeated to study the membrane to the low permeated membrane or effect of permeation enhancer used in the formulation. In this experiment following formula is used.

$$\text{Enhancement ratio} = \frac{\text{Jss of } ex\ vivo \text{ study}}{\text{Jss of } in\ vitro \text{ study}}$$

2.5.4.10. Drug release kinetics: [122,123.]

All the formulated Lornoxicam gels were subjected for kinetics and mechanism of drug release. The following models were applied.

Higuchi and Korsmeyer peppas, these models are mainly applied for the kinetics release study of semisolid preparation.

Higuchi (1961) simple equation is based on pseudo steady state application with following equation,

$$Q = A\sqrt{D(2C - 2Cs)Cs * t}$$

Q = Amount of drug release in t time per unit area.

C= Initial drug concentration.

Cs= Drug solubility in matrix media

D= diffusivity of drug molecule in the matrix.

For all calculation of drug release for semisolid and transdermal patches and other dosage for the simplified following equation is used.

$$Q = KH\sqrt{t}$$

Q= Amount of drug release in t time per unit area.

KH= Higuchi constant.

t= unit time.

The permeation data obtained were plotted against cumulative drug release against square root of time and linearity was observed (R^2).

Korsmeyer-peppas model or the power law (1983) proposed the prediction of drug release mechanism from the polymer. Korsmeyer-peppas model simplified equation is,

$$\frac{Mt}{M\infty} = kt^n$$

Mt/M∞= Fraction of drug release at time t

K= constant with respect to the structural and geometric chacteristics of dosage form

n= Release exponent.

All the release data of the experiment can be plotted as log cumulative percentage drug release to log time. Slope and linear regression were calculated.

Using the following values for ‘n’ mechanism of drug release was determined.

n= 0.5. Release is Fickian diffusion.

n= 0.5<n> 1. Non Fickian diffusion.

n = 0.89. Zero order

n = 1 Case II transport.

n = > 1 super case II transport.

For all formulations of Lornoxicam gels drug release data were fitted in to Graph prism software.

2.5.4.11. *In vivo* anti-inflammatory study: [124,125]

The anti-inflammatory activity of best formulation of Lornoxicam gel was performed on wistar rat and compared with marked formulation, after approval of Institutional animal ethical committee. Total nine animals were selected on body weight in the range of 150gm to 180gm (10 weeks).Animals were divided in to three groups of three animals each. Group I control, group II best formulation of Lornoxicam gel and group III for marked (pirox) gel.

All animals were housed in plastic cages and environment temperature was maintained at 24 ^{o}C±2^{o}C around 12hr/12hr light and dark cycle with free accessement of diet pellet with purified water. Prior to the experiment all animals were fasted for 8hrs but water was made available. Carrageenan 1%w/v solution was prepared in normal saline solution and 0.1 ml was administered in the sub plantar area of right hind paw for group I. After injection paw edema volume was measured using the plethysmography, reading was recorded as zero. Every half an hour readings were recorded up to the 6 hrs. Experiment was conducted on Group II and Group III adopting the above procedure but best formulation and marketed formulation were applied before 30 minutes intervals. For all animals volume was measured, recorded

and percentage of inhibition was calculated from the control group and treated groups by following formula,

$$\% \text{ of Inhibition} = \frac{C - T}{C} \text{ x } 100$$

C= mean inhibition of control group

T= mean inhibition of treated group.

2.5.4.12. Acute skin irritation study: [126,127.128]

Institutional animal ethical committee (REG no 221) approved the protocol of acute skin toxicity study of best formulated Lornoxicam gel on female wistar rats. All animals were rehabitulated.

Housing and feeding of animals:

Wistar rats were procured from the approved breeder. All animals were housed at room temperature (23 ^{o}C±2^{o}C) and optimum relative humidity (30% to 50%).lighting and dark cycle were maintained for 12 hrs respectively. Standard diet and purified water was provided throughout the day and night.

Procedure:

Five healthy animals were selected with respect to the body weight in the rage of 150gms to 180gms and skin of animals observed carefully for any minor cuts, wounds or any skin diseases. Selected animals were grouped in to two, Group I control containing two and group II containg three animals. All animals back side was shaved carefully with sterilized electrical razor to remove the fur with area of 2cm x 3cm.shaven area was cleaned with normal saline with sterile cotton wool and observed for intactness. Group I animals were applied with1ml of 0.1% formalin solution and group II animals were applied one gram of formulated Lornoxicam gel with aid of sterile cotton wool. Acute skin toxicity was observed for 72 hrs with interval of 1hr, 4hrs, 8hrs 12hrs, 24hrs and 72hrs. All observations were marked as for

as shown in the table .14. The score for treatment group and control group were then compared

Table.14. Erythema and edema measurement scale

Test	Skin reaction	score
Erythema	No erythema	0
	Very slight erythema	1
	Well define erythema	2
	Moderate severe erythema	3
	Sever erythema	4
Total possible erythema score		4
Edema	No edema	0
	Very slight edema	1
	Well defined edema	2
	Moderate to severe edema	3
	Severe edema	4
Total possible edema score		4
Total score for primary skin irritation test		8

2.5.4.13. Short term stability study: [129,130]

Short term stability test was conducted on best formulated Lornoxicam gel by modified ICH guideline for three months at room temperature and elevated temperature with relative humidities. Room temperature was 25^{o}C ±1^{o}C and RH 65%±5%. Accelerated temperature was 40^{o}C ±1^{o}C at RH 75% ±5%. Lornoxicam gel was filled in the plastic collapsible tube with nozzle sealed; bottom part of tube was sealed with hot plate crimpling machine. All the tubes were stored in stability chamber (Lab control) with respective temperature and humidity for three months. In processes quality control tests were conducted for every month for drug content, pH, viscosity and *in vitro* diffusion tests. The procedure for in process controls was performed as mentioned in the above method.

3. RESULTS

3.1 PHASE I

BIOSYNTHESIS OF BIOSURFACTANT

3.1.1. Gram staining:

Gram staining of procured *Flavobacterium sp*. MTCC 2495 showed the pink colour staining with rod shape colonies.(fig.16) Before staining bacteria were yellow in colours.(Fig.15) Hang drop test indicated that bacteria were motile.

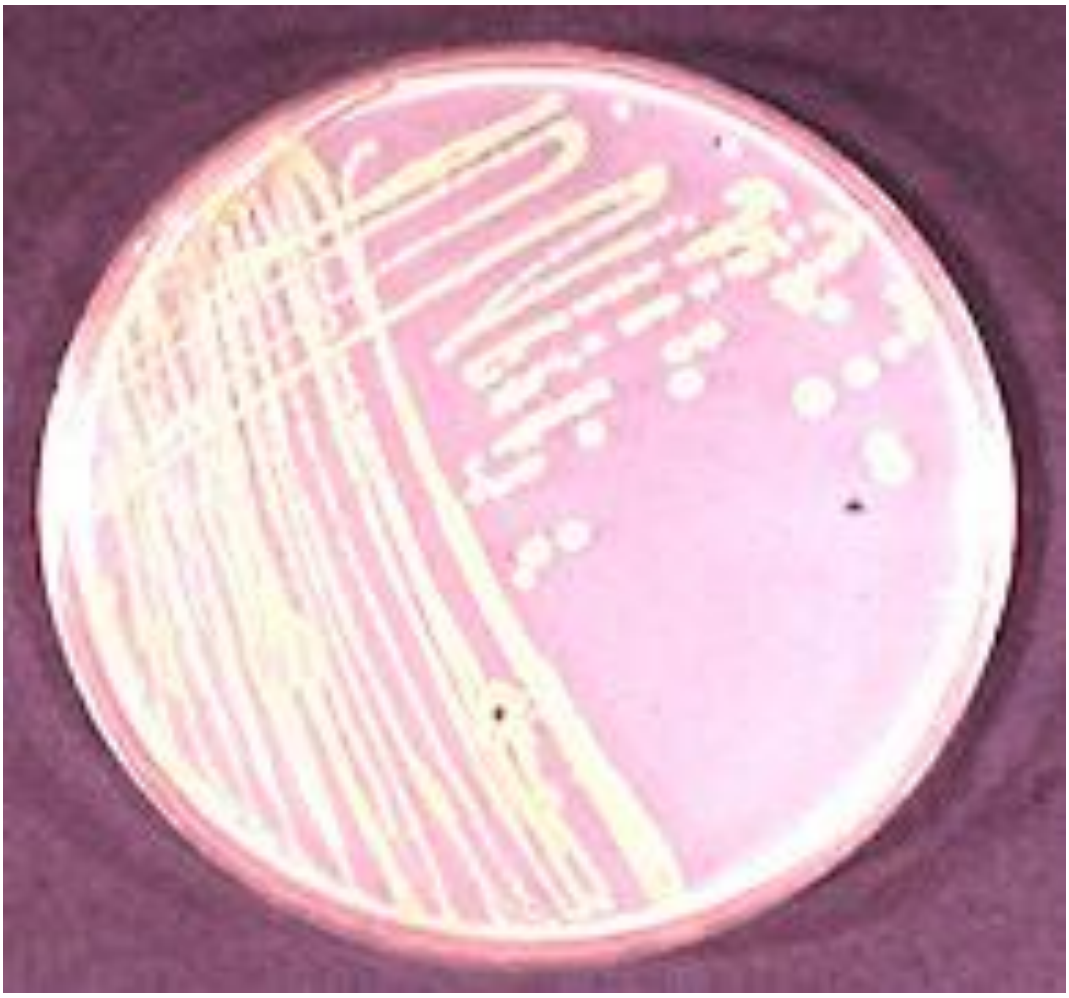


Fig. 15. **Sub cultured colonies of *Flavobacterium sp*. MTCC 2495**

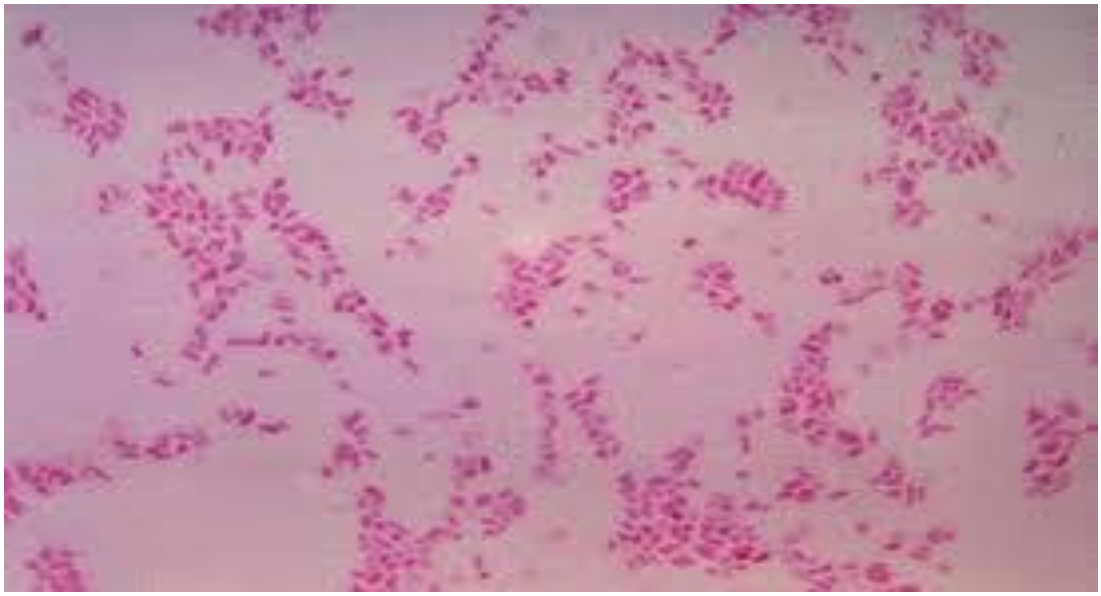


Fig.16. Microscopic image of gram staining of *Flavobacterium sp* MTCC 2495

3.1.2. Oil spreading method:

Oil spreading test in the petridish with water and kerosene diameter was measured in triplicate and average diameter was found in the range of 2.5cm to 3.1 cm.

3.1.3. Cultivation of *Flavobacterium sp* 2495 in modified mineral salt medium

In-process controls

a)Cell growth:

Results reveled that cell growth were gradually increased with respect to time and declined after specified interval. Results were shown in table.16 and fig.17.

Table.15. Cell growth of *flavobacterium* sp 2495 with respect to time

Time	Turbidity= 2.303 X A/L= OD
0	0.04
12	0.23
24	0.52
36	0.63
48	1.34
60	1.62
72	1.72
84	1.21

A= Absorbance. L = cell path length.(1cm)

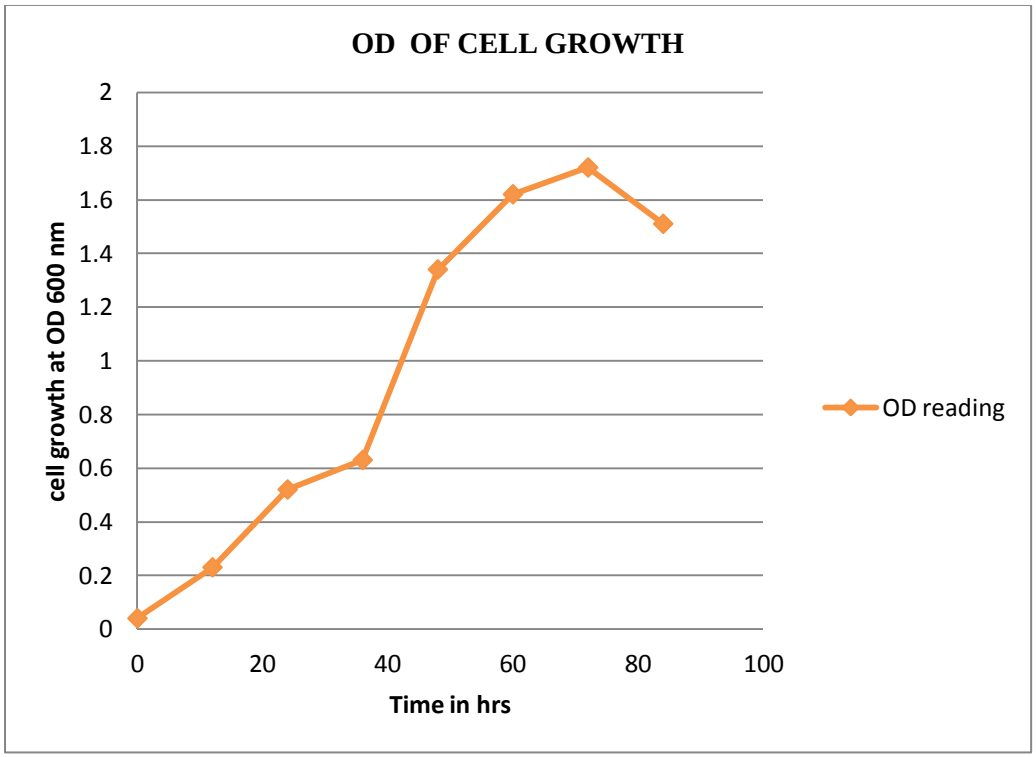


Fig. 17. Graphical representation of *flavobacterium sp* cell growth.

b) Determination of biomass:

Biomass was determined with specified intervals by dry weight basis. Results of biomass weights were tabulated in table. 16.

Table.16. Biomass weight of *flavobacterium sp* 2495

Time(hr)	Weight gm/100ml
0	0
12	0.12
24	0.31
36	0.52
48	0.77
60	0.81
72	0.98
84	0.76

c) Emulsifying index

The separated layer volume for biosurfactant and Tween 80 emulsions with liquid paraffin was found 5ml and 26.5ml respectively. Emulsifying index was for biosurfactant emulsion was 5% and for Tween 80 was 26.6%. Emulsifying index percentage is inversely proportional to the stability of emulsion. In Biosurfactant emulsion the percentage was 5% and had maximum stability was observed. From the Fig 18. it was evident that the biosurfactant containing emulsion had least separated layer.

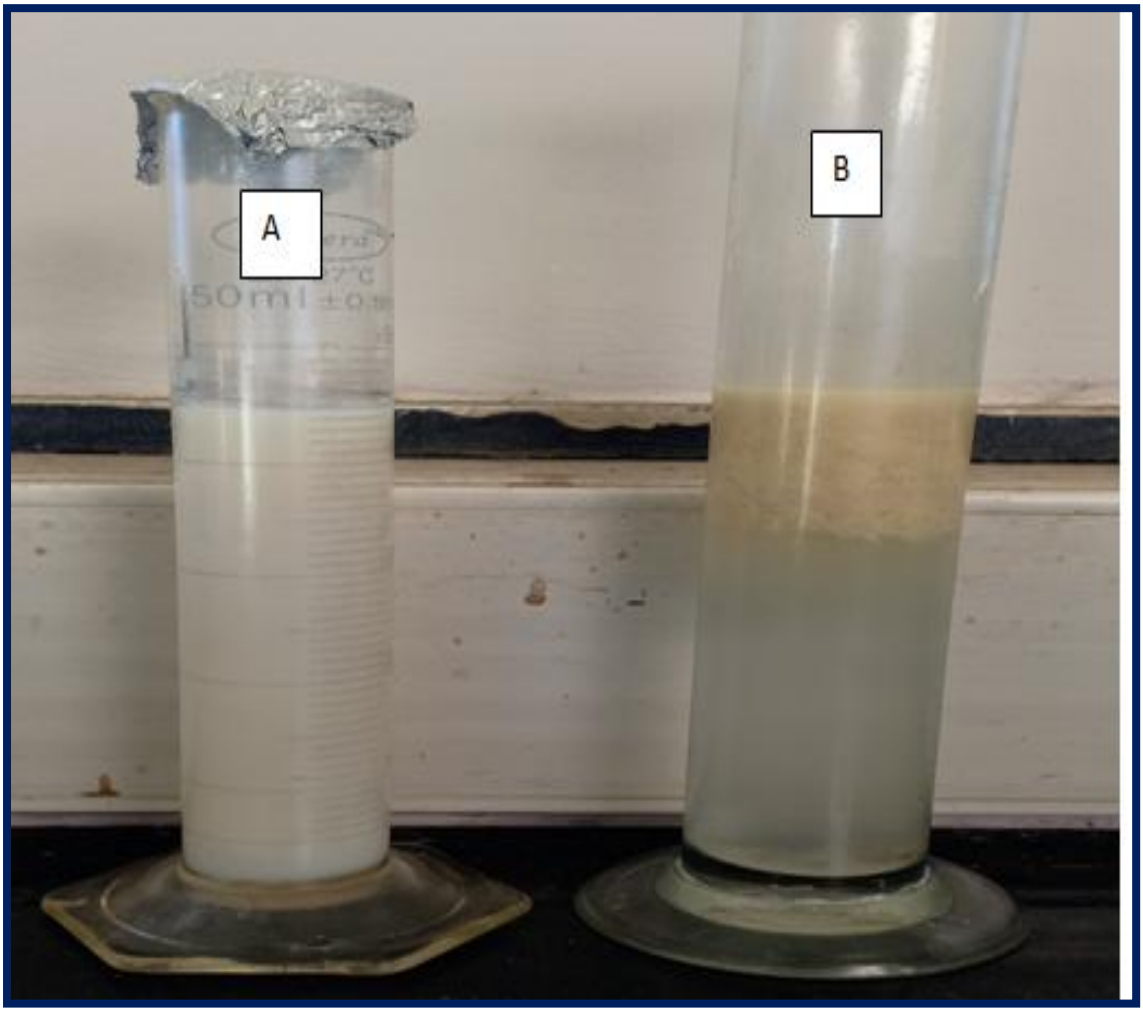


Fig.18. Comparative stability of paraffin emulsion with a) biosurfactant b) nonionic surfactant.

3.1.4. Optimization of substrates concentration using 3^2 factorial designs.

Sugar cane molasses and waste fried oil were used for laboratory scale for biosynthesis of biosurfactant. Optimum concentration of substrate was determined from factorial design by selecting the three levels of substrates concentrations and biosurfactant yield and surface tension as dependent factors. Under 3^2 factorial designs, soft ware generated nine runs. Dependent factors were determined by experimentally and determined data were further analyzed with respect to independent factors for correlation and interactions. Biosurfactant yield and surface tension data were shown in the table.17.

Table. 17. Dependent variables parameter's responses data

Run	Factor 1 Sugarcane molasses %	Factor 2 Waste fried oil %	Response 1 Surface tension dy/cm	Response 2 Yield of Bio surfactant gm/500ml
1	8	2	34.5	2.77
2	8	6	34.9	2.33
3	2	2	28.2	3.90
4	8	4	35.5	2.10
5	5	6	33.4	2.90
6	5	2	33.9	2.75
7	2	4	32.9	2.90
8	2	6	34.2	2.78
9	5	4	33.5	2.89

Final equation in terms of coded factors surface tension and biosurfactant yield are

Surface tension= $+33.44+1.60\ A +0.9833\ B -1.40\ AB+0.845A^2 -0.3585B^2$

Yield of Biosurfactant=$+2.81+0.3967A -0.2350\ B+0.7356AB- 0.8652A^2+0.5487B^2$

Table. 18. Statistical data of Dependent factors (ANOVA)

Source	Surface tension				Biosurfactant yield			
	R^2	P value ≤0.05	Std D(±)	Pure error	R^2	P value ≤0.05	Std D(±)	Pure error
Model								
A-sugar cane molasses	0.8047	0.0214	1.19	0.18	0.8537	0.0415	0.3365	0.06
B-Waste fried oil	0.7625	0.031			0.7820	0.0371		

The application of factorial design was to evaluate the optimum concentrations of substrates to be used in the scale up batches, to achieve the maximum yield of the biosurfactant with respect to decreased surface tension value.

On critical evaluation of linear and polynomial equations of surface tension revealed that positive coefficients,A,A^2showed synergetic effect and negative coefficient AB, B^2 an antagonistic effect R^2 found to be 0.8047 indicated that effect of independents variables was 80 percentage. P value was less than 0.05 with 90% confidential interval. Pure error indicates that reproducibility of the experiment.

Similarly with biosurfactant yield synergistic coefficients were A, AB and B^2 and antagonistic coefficient were B, A^2. R^2factor value was 0.8537 indicative of 85 percentages of independent variable contribution on dependent variables. Pure error factor was 0.06; state that good reproducibility of the experiment.

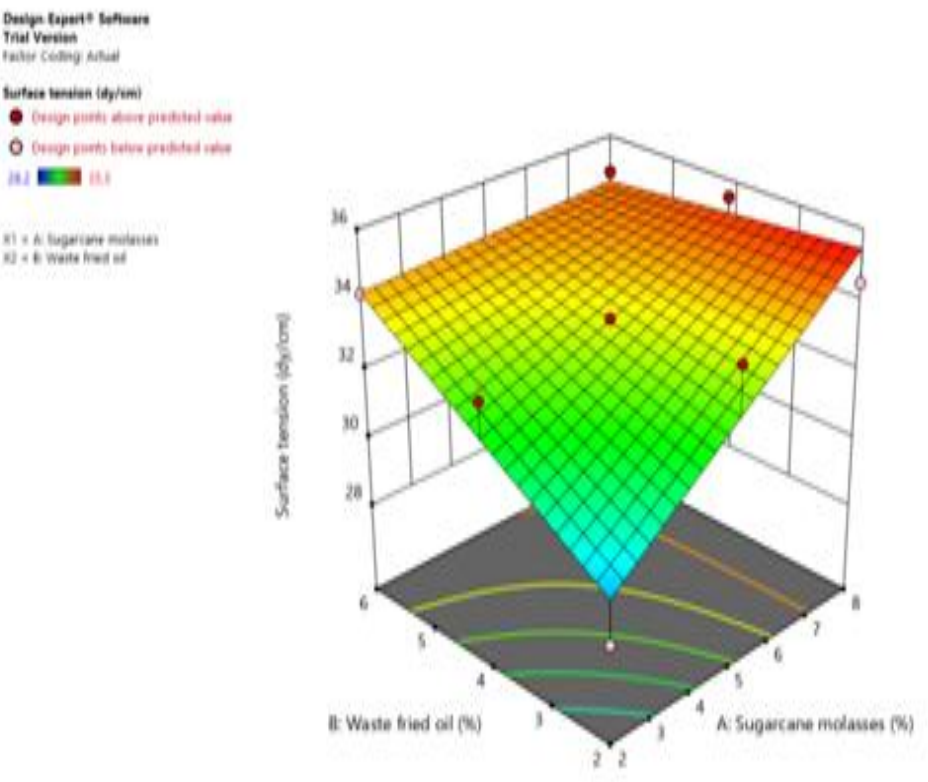


Fig.19. Contour plot for surface tension response.

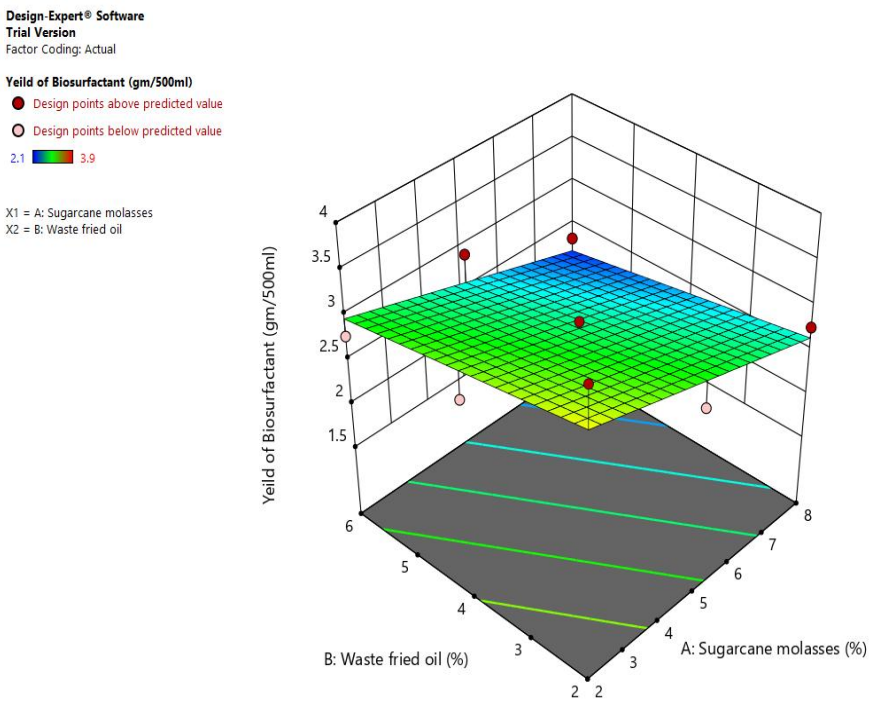


Fig.20. Contour plot for response biosurfactant yield

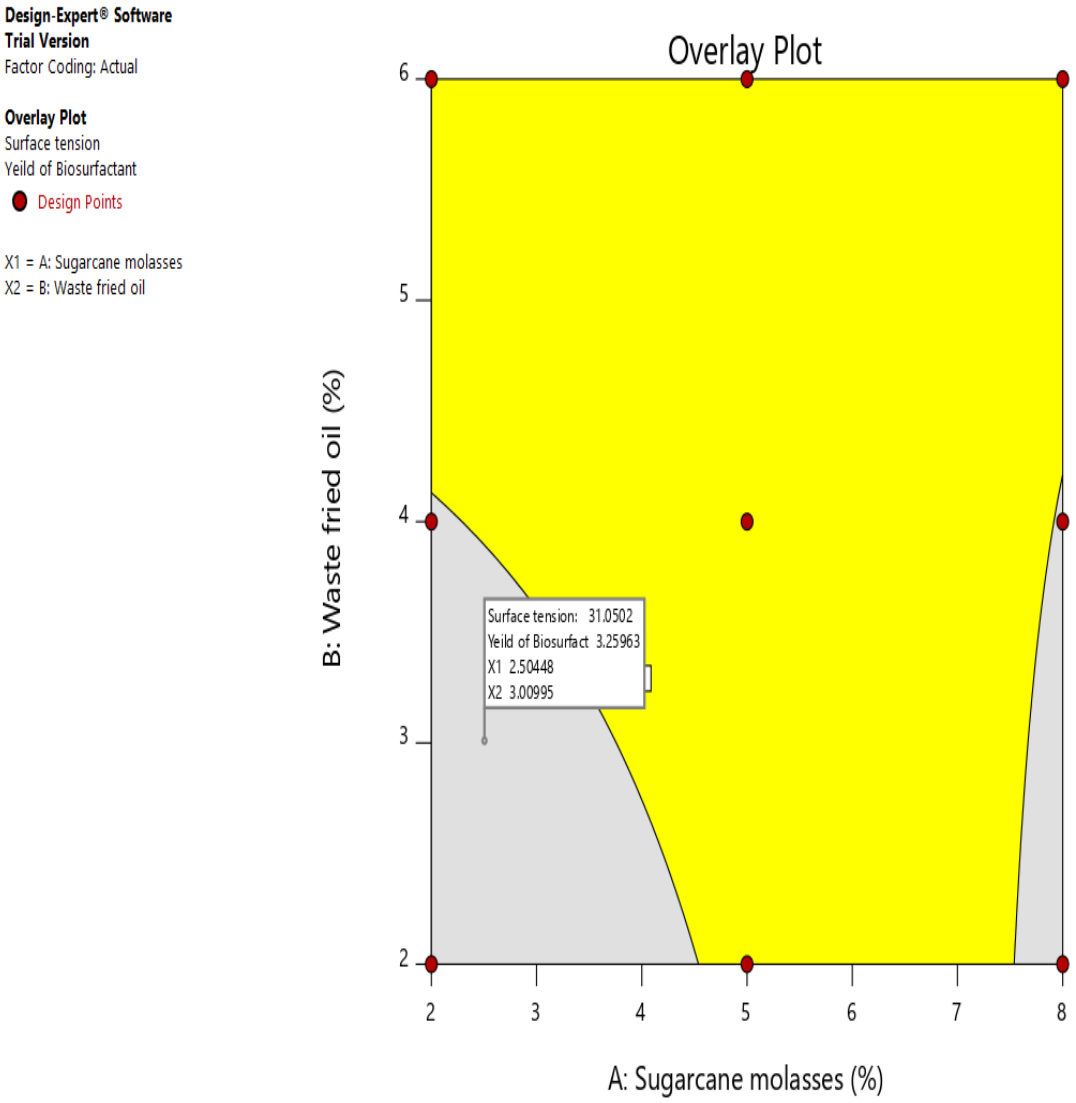


Fig. 21. Design space overlay plot

From the design space graph optimum concentration of sugar cane molasses and waste fried oil concentrations were determined for scale up process in bioreactor fermentor.

Table 19. Overlay plot limits of substrate concentrations

Sugar cane molasses		**Waste fried oil**	
Lower limit	Upper limit	Lower limits	Upper limits
4.5%	8%	4.1%	6%

Table. 20. Biofermentor scale up batch substrates concentration

Biofermentor batch concentrations	
Sugar cane molasses	**Waste fried oil**
5.2%	5.0%

3.1.5. In processes control: surface tension

Surface tension parameter was selected as in process control for lab scale biosynthesis of biosurfactant. Surface tension of fermenting media is inversely proportional to the yield of biosurfactant. Surface tension of the media were measured at specified intervals and recorded as shown in the table 22.

Table.21. In processes control of fermented broth

Time hrs	Experimental runs Surface tension [dy/cm]								
	1TB	2 TB	3 TB	4 TB	5 TB	6 TB	7 TB	8 TB	9 TB
1	90.8 ±0.91	92.6 ±1.05	91.5 ±0.95	90.45 ±0.99	91.65 ±0.88	92.12 ±0.98	91.65 ±0.94	90.89 ±0.95	90.88 ±0.67
36	65.2 ±1.4	66.12 ±1.02	55.32 ±1.52	66.01 ±0.95	65.24 ±0.76	64.32 ±1.08	63.14 ±0.99	62.56 ±0.78	60.12 ±0.98
72	34.5 ±0.99	34.9 ±1.5	28.2 ±0.98	35.52 ±0.87	33.4 ±0.99	33.98 ±1.12	32.94 ±0.98	34.20 ±0.89	33.53 ±0.87

TB = Trial batch. Mean±SD (n=3)

3.1.6. PILOT PLANT SCALE UP PRODUCTION OF BIOSURFACTANTS

Biosynthesis of biosurfactant from *flavanobacteria sp* in modified mineral salt media under specified environmental conditions was scale up in Sartous- B liter fermentor with optimized substrates concentrations for 72hrs.The biofermentor consist of automatic regulator for pH, RPM and aeration system An in-process quality control parameter was introduced to evaluate the bacterial cell growth and production of biosurfactant during the fermentation period. Relative surface tension was determined from 1hrs to 72hrs with intermediate predetermined intervals. Surface tension results are tabulated in table.22. Total yield of the biosurfactant after solvent extraction process was **7.9 gms** per one liter batch.

Table.22. In processes control of scale up batch

Time in hrs	Surface tension dy/cm.
1	88..3±0.64
12	79.5±0.98
24	64.8±0.73
333 36	45.7±0.97
48	40.6±.1.02
60	31.4±0.86
72	28.6±0.88

Mean±SD (n=3)

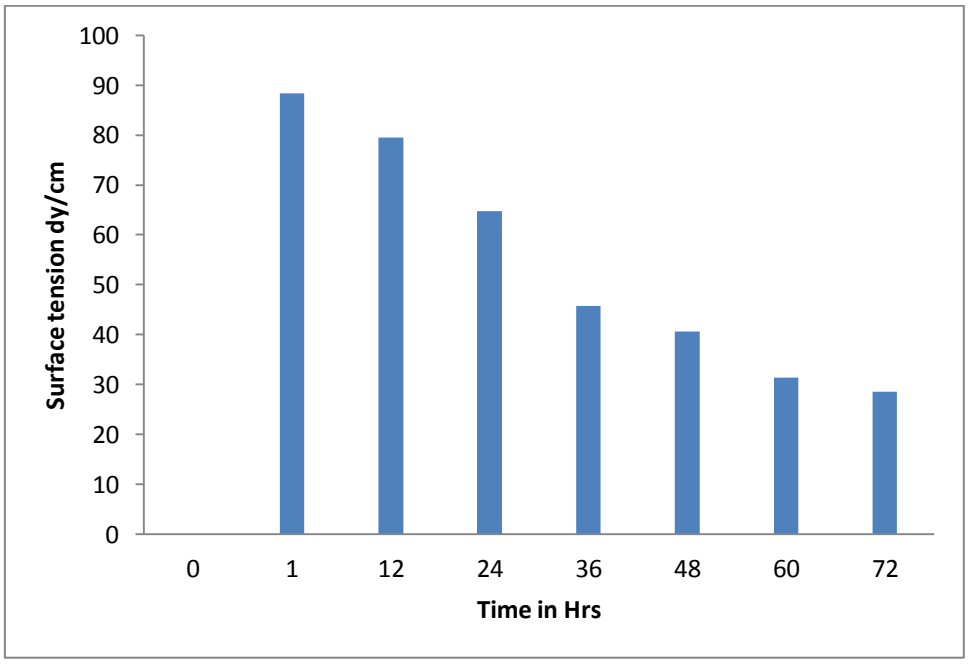


Fig. 22. Surface tension of scale up batch

3.1.7. PARTIAL CHACTERISATION OF BIOSURFACTANT

3.1.7.1. Determination of CMC of Biosurfactant

Critical micellar concentration of biosurfactant was determined from relative surface tension method. From the different concentrations of biosurfactant solutions in water surface tension was determined. Critical micellar concentration point was found from the graph as shown in the fig.21.It was found that surface tension constant at 39.5 mg per liter concentration.

Table.23.In process surface tension data

S.No	Concentration of Biosurfactant in water %w/v	Surface tension dy/cm
1	0.0005	71.3±0.98
2	0.0010	65.5±0.88
3	0.0015	60.53±1.02
4	0.0020	58.4±0.93
5	0.0025	49.7±1.05
6	0.0030	43.6±0.90
7	0.0035	38.5±1.02
8	0.0040	31.5±.0.99
9	0.0045	29.65±0.89
10	0.0050	28.9±1.06
11	0.0055	28.60±.0.86
12	0.0060	28.2±1.02

Mean± SD (n=3)

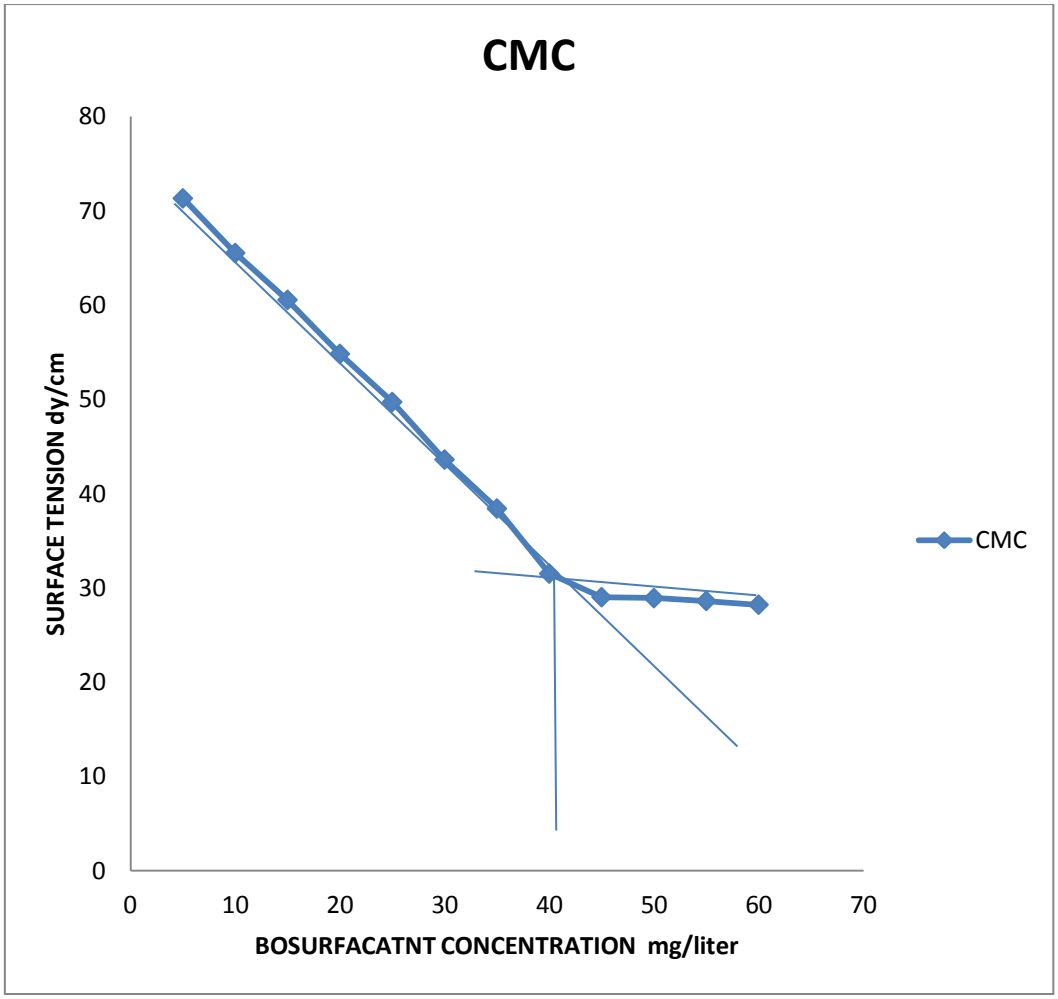


Fig.23. Graphical determination of CMC

3.1.7.2 UV Characterization. (Determination of λmax)

Maximum absorbance of biosurfactant solution in water was determined using UV spectroscopy (Shimdzu 1900). Scanned results were recorded, as shown in fig.22. Maximum absorbance was found at 312.6 nm.

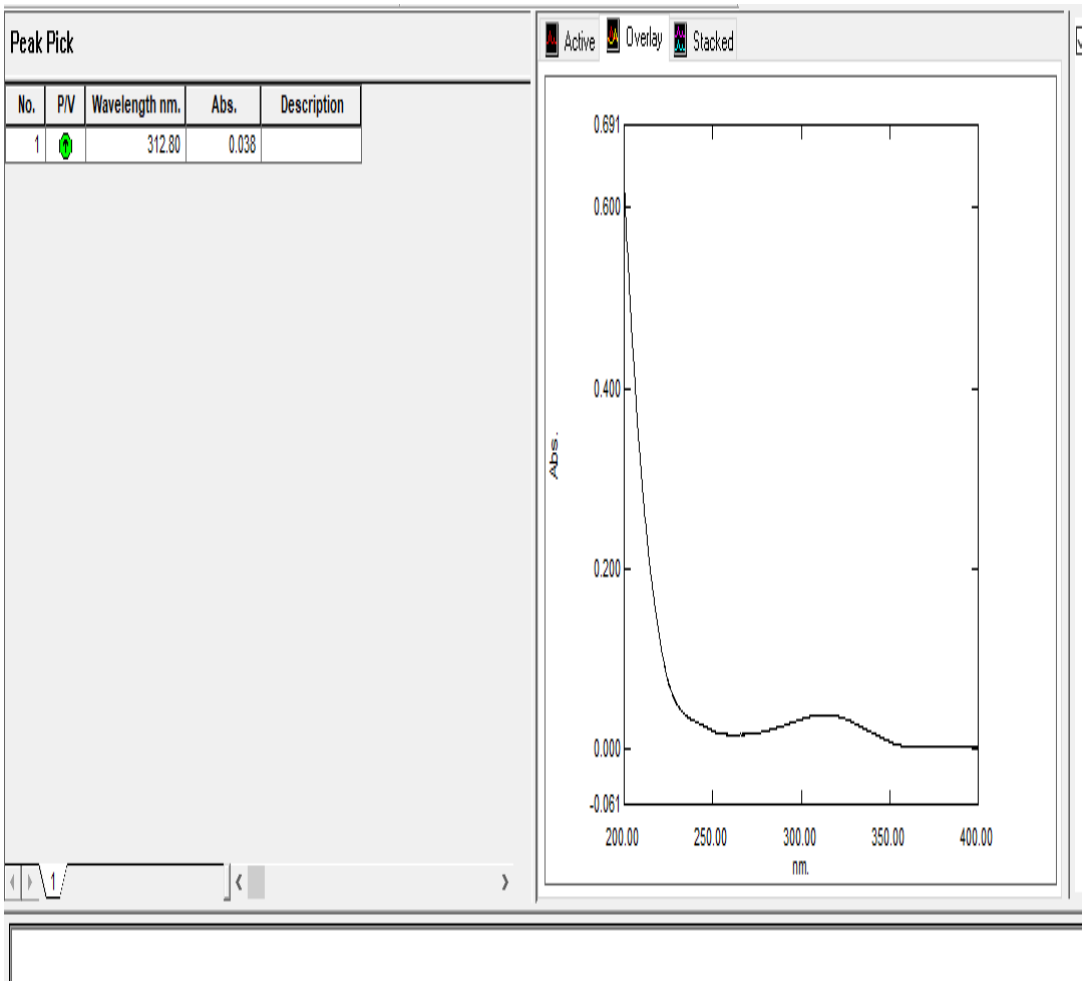


Fig.24. Absorbance maxima of Biosurfactant by UV spectroscopy

3.1.7.3. FTIR Characterization

The interpretation of FTIR graph of biosurfactant peaks were performed by comparing the standards peaks rages with respect to the graph peaks and possible functional groups were predicted approximately. The major peaks at 3421.5 represents the stretching of aliphatic amines and amides, 3007 represents the amino salts of carboxylic acid, 2855 carboxylic acid stretch, 1744 C=O stretch of lipid ester. The details peaks wavelength and significance were shown in the table 24.

Table.24. Functional groups of Biosurfactant and peaks numbers

Frequency, cm–1 range	Bond cm-^{1}	Interpretation
3200-3650	3421.5	Stretching of aliphatic amines and amide
2700-3300	3007.6	Amino salts of carboxylic acid
2500-3300	2855	Carboxylic acid
1650-1780	1744.6	C=O stretching of lipid ester

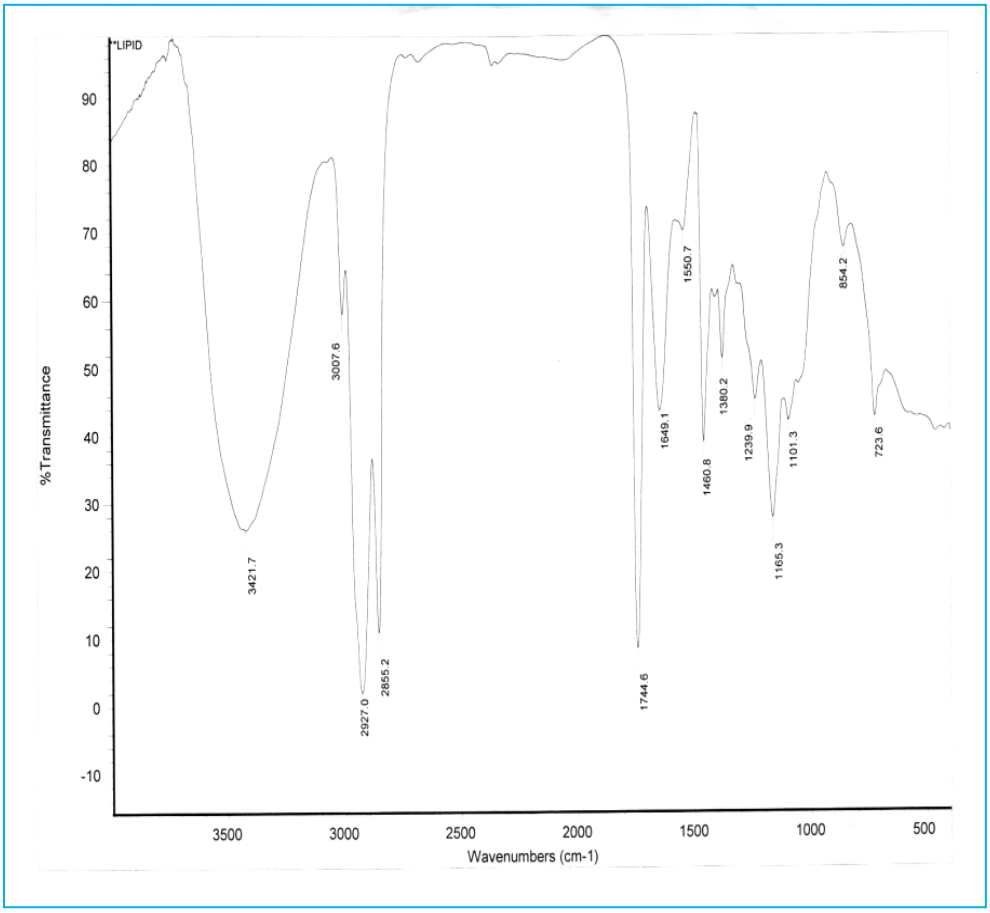


Fig.25. FTIR Spectrum of Biosurfactant

3.1.7.4 Differential scanning calorimeter.

The melting point (MP) of the Biosurfactant was at 109.2^{0}C in DSC endothermic peak as shown in .Fig.24.

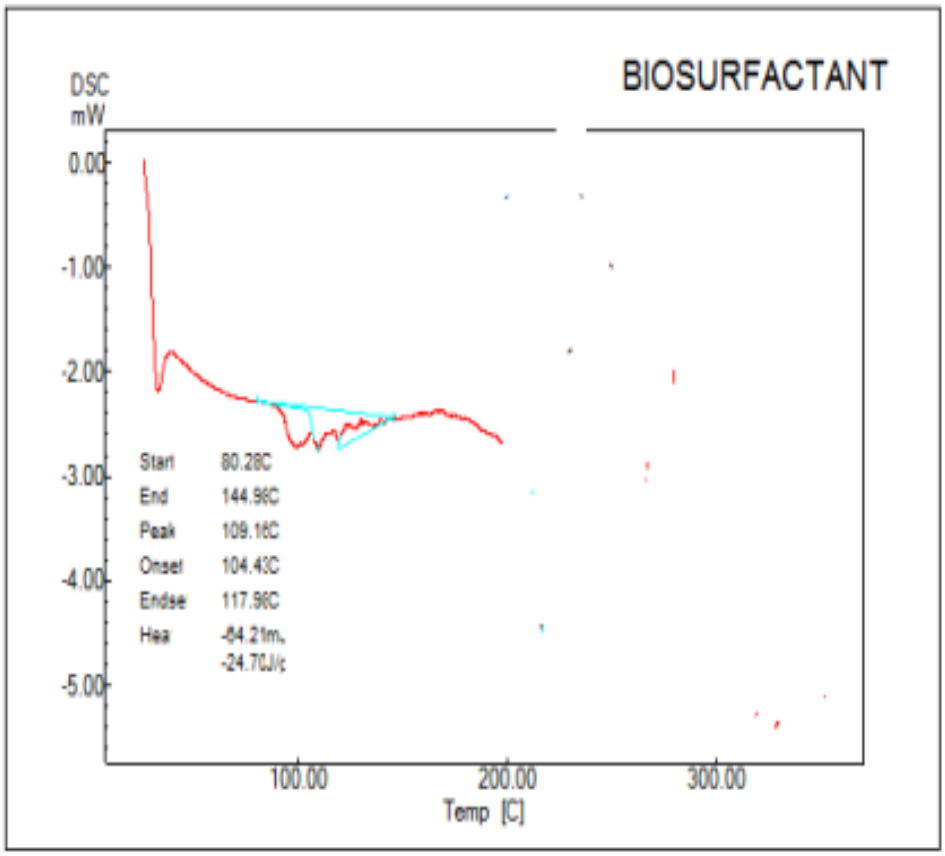


Fig.26. DSC Thermograms of Biosurfactant

3.1.7.5. Thin Layer Chromatography characterization

Two plates were developed with biosurfactant samples separately and colour was developed with respective reagents, Rf values were calculated. It was found that for carbohydrate and amino acid were 0.28 and 0.21 respectively. Fig 27.

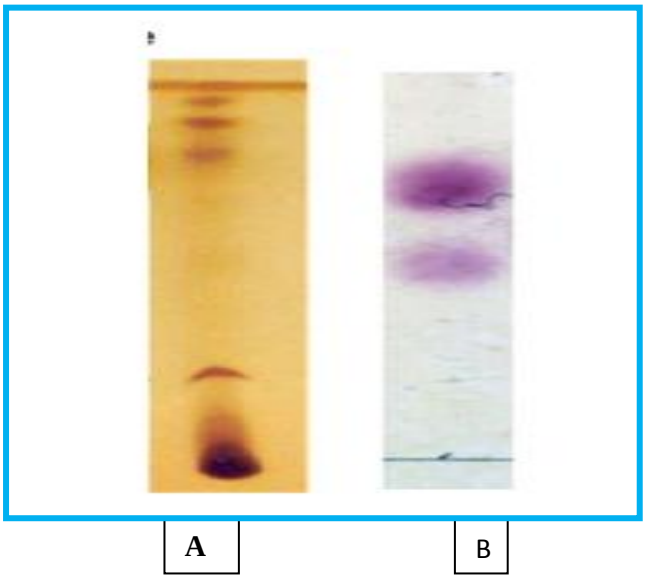


Fig.27.Thin layer chromatogram of biosurfactant [A]carbohydrate,[B] amino acid

3.1.8. Pharmacological studies of biosurfactant

3.1.8.1. Oral toxicity study.

Animals were administered with 2000mg/kg body weight. All animals were observed for mortality and behavioral changes, excretion of urine and faeces etc, for 14days .Major observation were recorded as shown in the table 25

Table.25. Observation data of acute oral toxicity

Observation										
Duration	**24hrs**					**14th day**				
Rat no	1	2	3	4	5	1	2	3	4	5
Water intake	20ml	24ml	20ml	20ml	24ml	24ml	20ml	20ml	24ml	24ml
Fecal nature	N	N	N	N	N	N	N	N	N	N
Food intake	9.81 gm	10.21 gm	8.28 gm	9.81 gm	10.21 gm	10.4 gm	11.10 gm	10.8 gm	10.4 gm	11.10 gm
Morbidity	No death	No death	No death	No death	No death	No death	No death	No death	No death	No death

N =normal consistency

3.1.8.2. Acute dermal toxicity study

Acute dermal toxicity study was performed using four female albino wistar rats and one as a control. All animals were observed for following signs and symptoms for 14 days as shown in the table.27 and fig.26.

Table.26. Dose administration data for acute dermal toxicity

No. of animal	Weight of animal/biosurfactant(mg)	Dose applied (ml)
Rat no.1	138gms/276	2.5 ml
Rat no.2	124gms/ 248	2.5 ml
Rat no.3	143gms/286	2.5 ml
Rat no4	154gms/308	2.5 ml
Rat no5	139gms control	----

Table.27. Acute dermal toxicity observation data

Observation										
Duration	**24hrs**					**14th day**				
Rat no	1	2	3	4	5 control	1	2	3	4	5 control
Erythema	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
oedema	N	N	N	N	N	N	N	N	N	N
Body weight/gms	138	125	143	154	139	142	134	148	167	152
Skin appearance	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Morbidity	No death	No death	No death	No death	No death	No death	No death	No death	No death	No death

-Ve= No signs of Erythema, N= no oedema, NR= normal without scare or lesion.

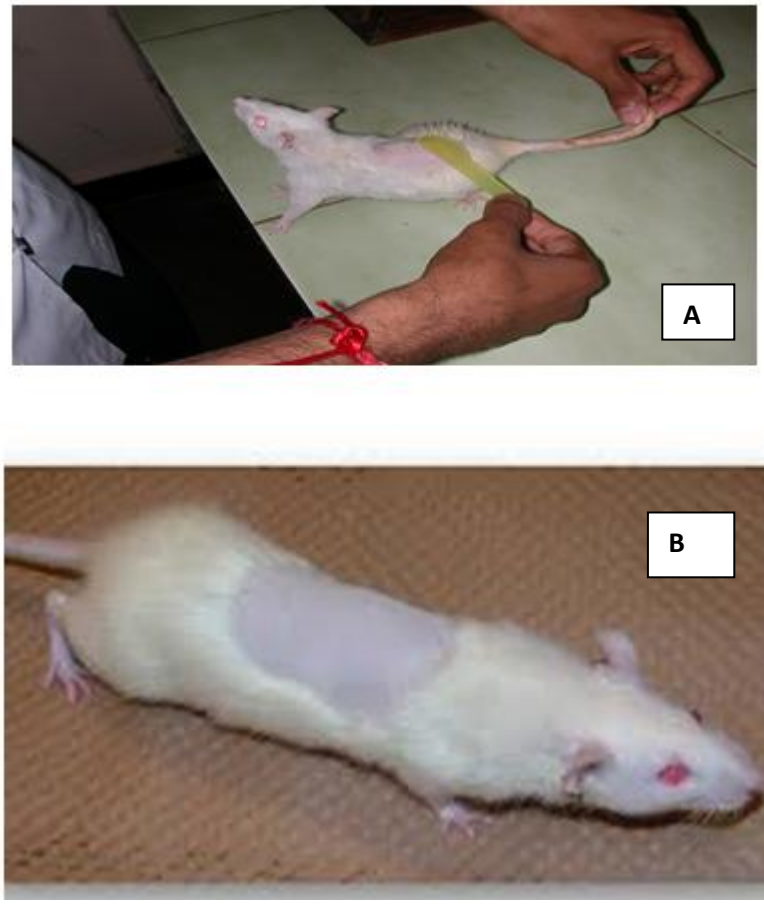


Fig.28.Photos of dermal toxicity study [A] 24hrs [B] 14th day

3.2. PHASE II ATOVAQUONE TABLET FORMULATION WITH BIOSURFACTANT.

3.2. Preformulation study of Atovaquone

3.2.1. Melting Point Determination:

Melting point was determined from capillary Thiele tube was 216.2°C, similarly confirmed by DSC. The pharmacopieal range is 216°C to 219°C

3.2.2. Solubility Determination:

Saturation solubility of Atovaquone was determined in 1.2 pH buffer, 6.8phosphate buffer, ethanol and isopropyl alcohol. It was found that Atovaquone was very sparingly soluble in buffer solutions and slightly soluble in organic solvents but mixture of isopropyl alcohol and phosphate buffer pH 8 (40: 20), solubility was increased. All solvents solubility data were shown in the table. 28.

Table.28. Solubility profile of Atovaquone

pH buffer 1.2 mg/ml	**Phosphate buffer 6.8** mg/ml	**Methanol** mg/ml	**Isopropyl alcohol** mg/ml	**Isopropyl alcohol, buffer pH 8(40:20)** mg/ml
0.0008±0.98	0.0027±1.02	0.9±0.81	0.96±0.78	2.3±0.56

Mean± SD (n=3)

3.2.3. Standard Calibration Curve and λmax.

Standard calibration curve was plotted from average of triplicates absorbance readings. A linear regression graph was drawn and regression coefficient R^2 was calculated, it was found that R^2 was 0.9965. Prior to the standard calibration absorbance, to determine the λmax of Atovaquone, 2µg/ml and 8µg/ml of atovaquone drug concentrations were scanned from 200nm to 400nm. It was found that 221nm fig. 27.

Table.29. Parameters of Calibration Curve

Parameters	UV method observations
λmax (nm)	221
Beer's law limits (µg/ml)	2-20
Regression equation	Y= 0.0845+0.012
Correlation coefficient (R^2)	$R^{2=}$ 0.9965

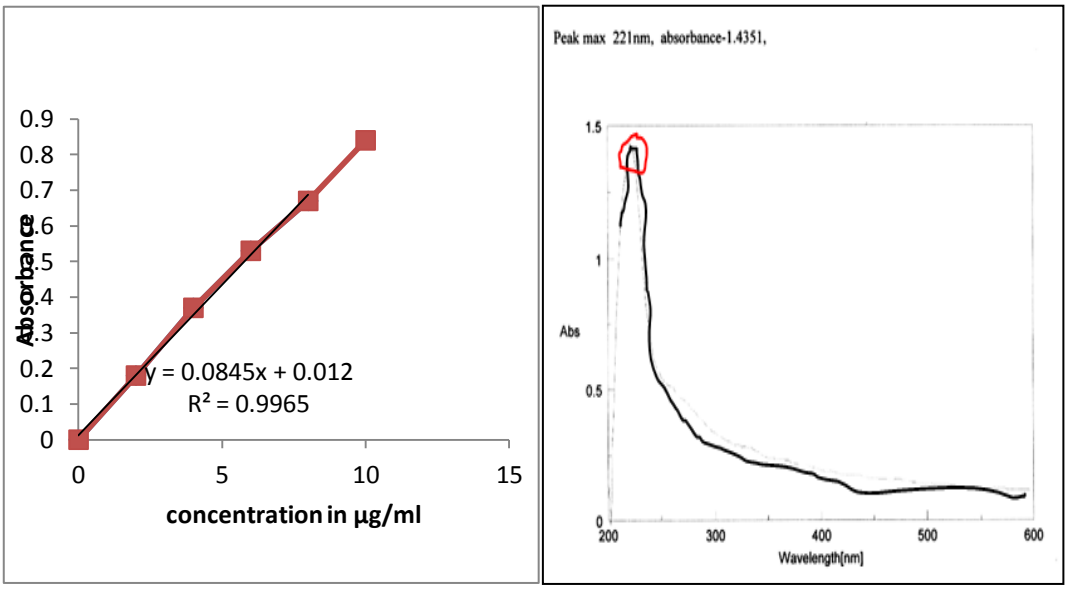


Fig. 29.Standard calibration curve of Atovaquone and λmax

3.2.4. Drug-excepient compatibility study

3.2.4.1. FT-IR spectroscopic studies:

The FTIR spectra of pure Atovaquone and physical mixture with biosurfactant samples were recorded and functional groups were interpreted in accordance with the structure of drug. Important peaks were enlisted from both IR spectra with respect functional groups, shown in the table 30 and fig 28, 29.

Table. 30. Comparative interpretation of peaks wave length

Functional groups	Pure drug cm^{-1}	In physical mixture Drug cm^{-1}
C-O stretch	3375	3375.5
Aromatic C=O stretch	1658	1662
Aromatic C-H stretch	2924	2920
C-Cl	729.0	729.09

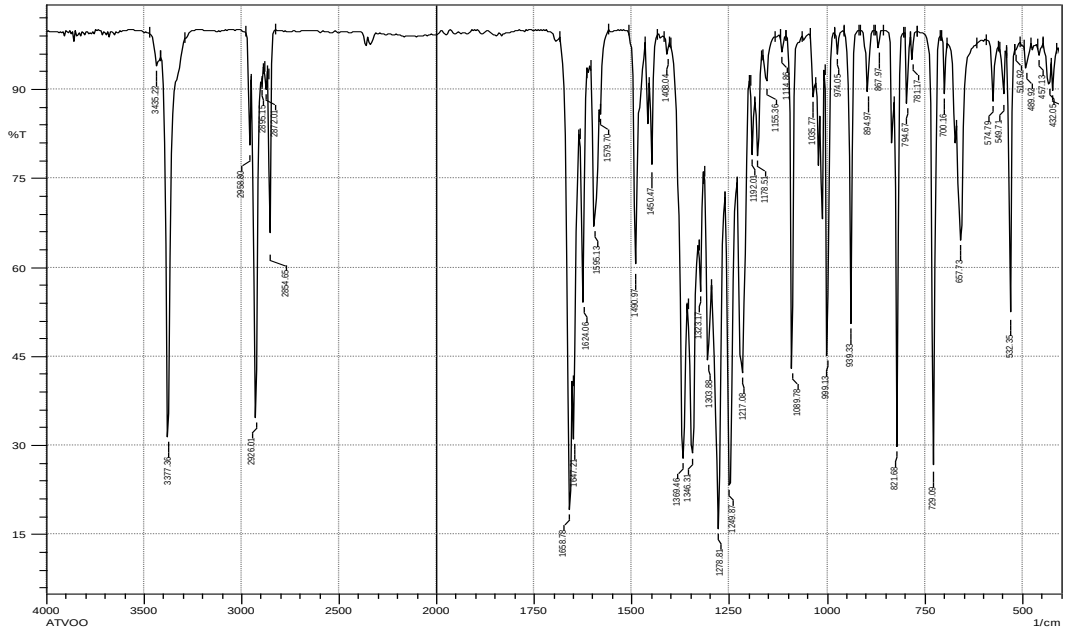


Fig. 30.Atovaquone pure drug FTIR spectra

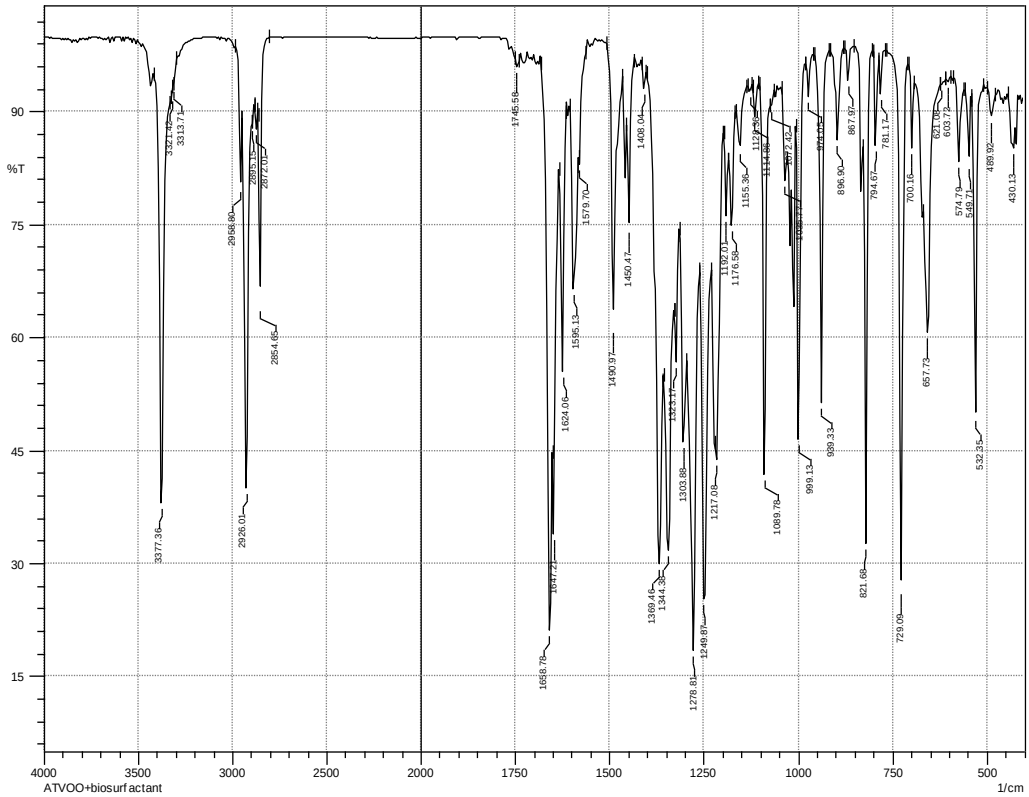


Fig. 31.Atovaquone and biosurfactant mixture FTIR spectra

3.2.4..2. Differential scanning calorimetric study:

DSC thermograms were obtained for the Atovaquone (Fig 30) and drug-polymers mixture (Fig 31). Atovaquone and biosurfactant showed sharp endothermic peak at 216 ^{0}C and 109 ^{0}C which indicates the MP of the drug and biosurfactant respectively. The thermograms for drug-polymers mixture showed characteristic MP of drug at 216 ^{0}C i.e., there is no significant change in the endothermic peak of drug. This indicates that the drug has not undergone any physical or chemical changes when mixed and stored.

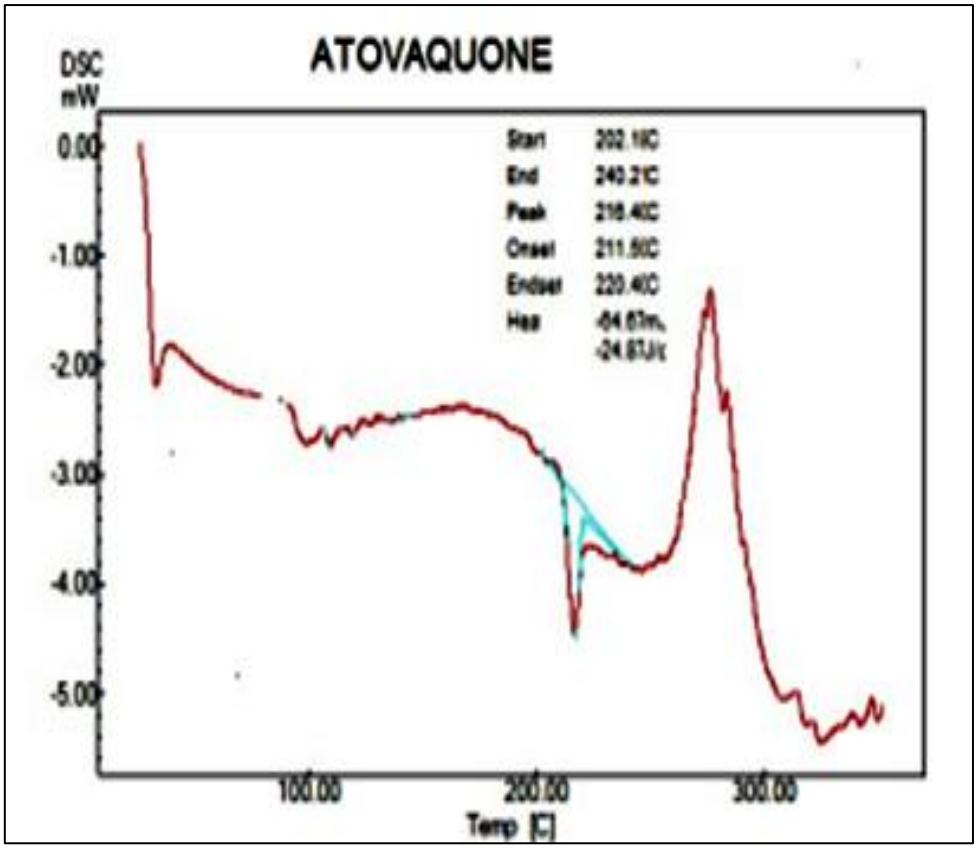


Fig. 32.DSC thermograms of pure Atovaquone

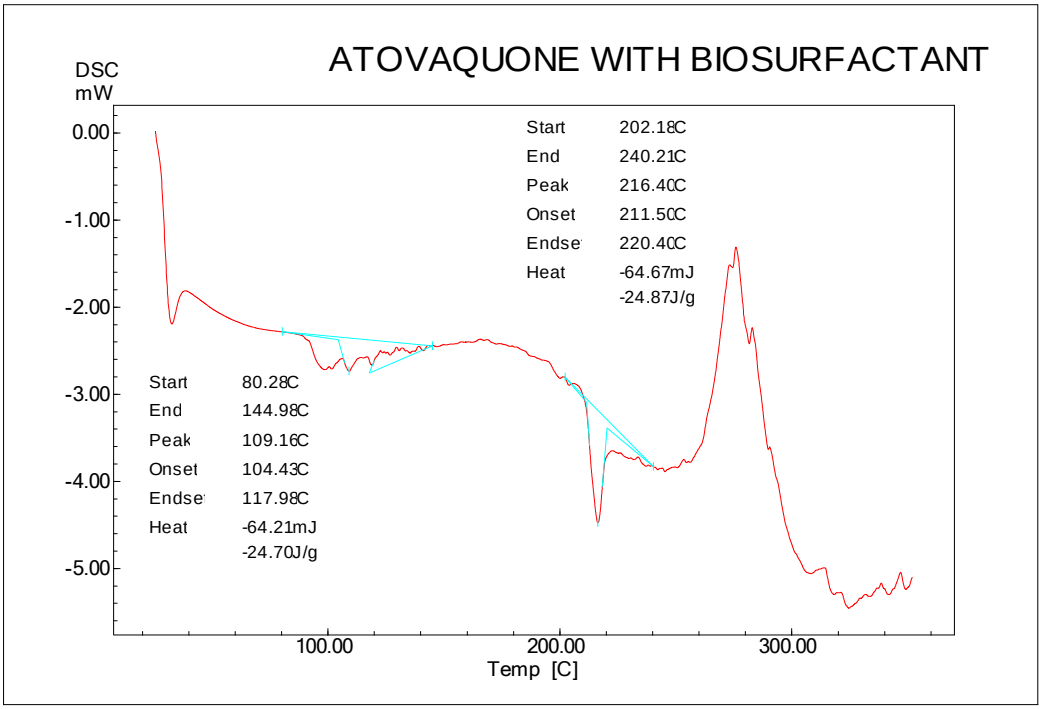


Fig.33.DSC thermograms of Atovaquone and biosurfactant

3.2.5. HPLC METHOD FOR ESIMATION OF ATOVAQUONE

Maximum Absorbances of Atovaquone with a retention time of 6.2 min at 254 nm, further analysis of all samples were conducted at 254nm. The standard calibration curve to quantify Atovaquone was found to be linear having 0.9946 of correlation coefficient (over the range of 0.5-30µg/ml) in plasma as depicted in table 31 and fig 34. Fig 32 and 33 represents the typical peaks for Atovaquone in mobile phase and plasma sample respectively.

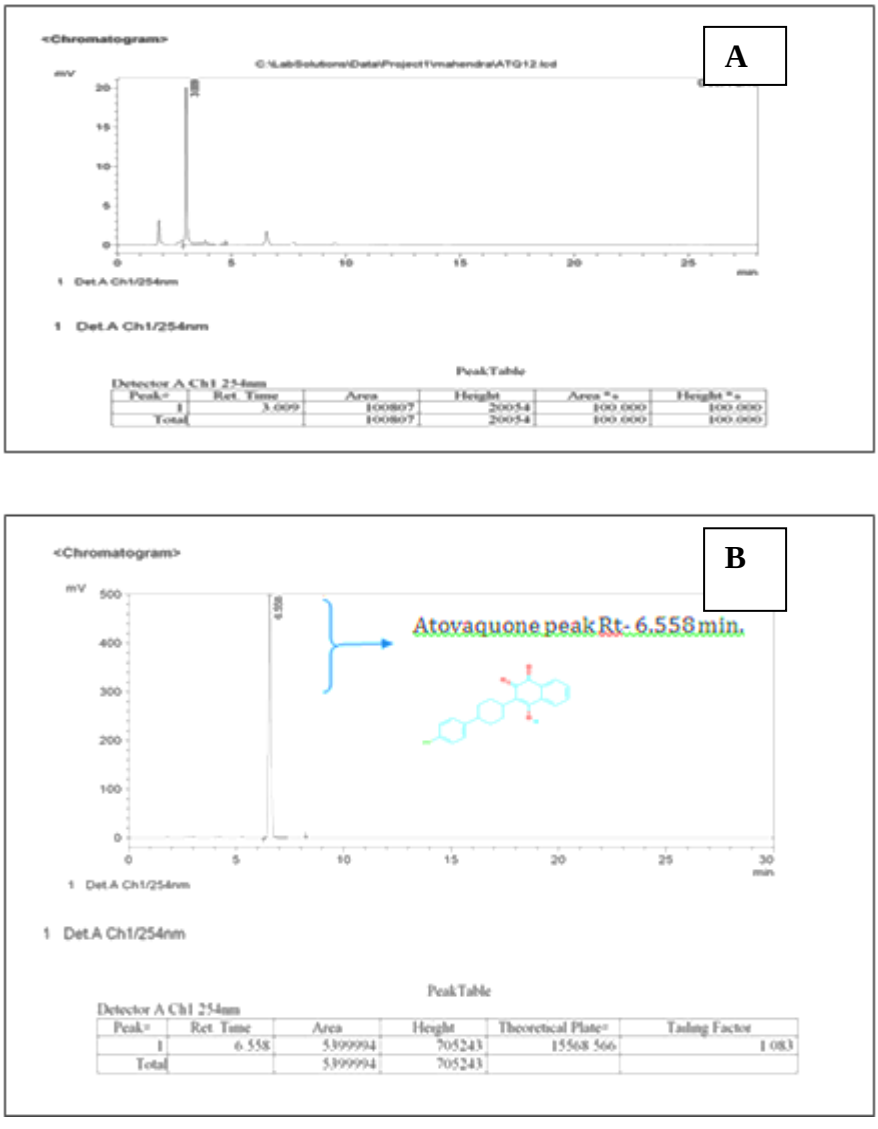


Fig.34. A) Chromatogram of blank mobile phase B) Chromatograph of Atovaquone in mobile phase

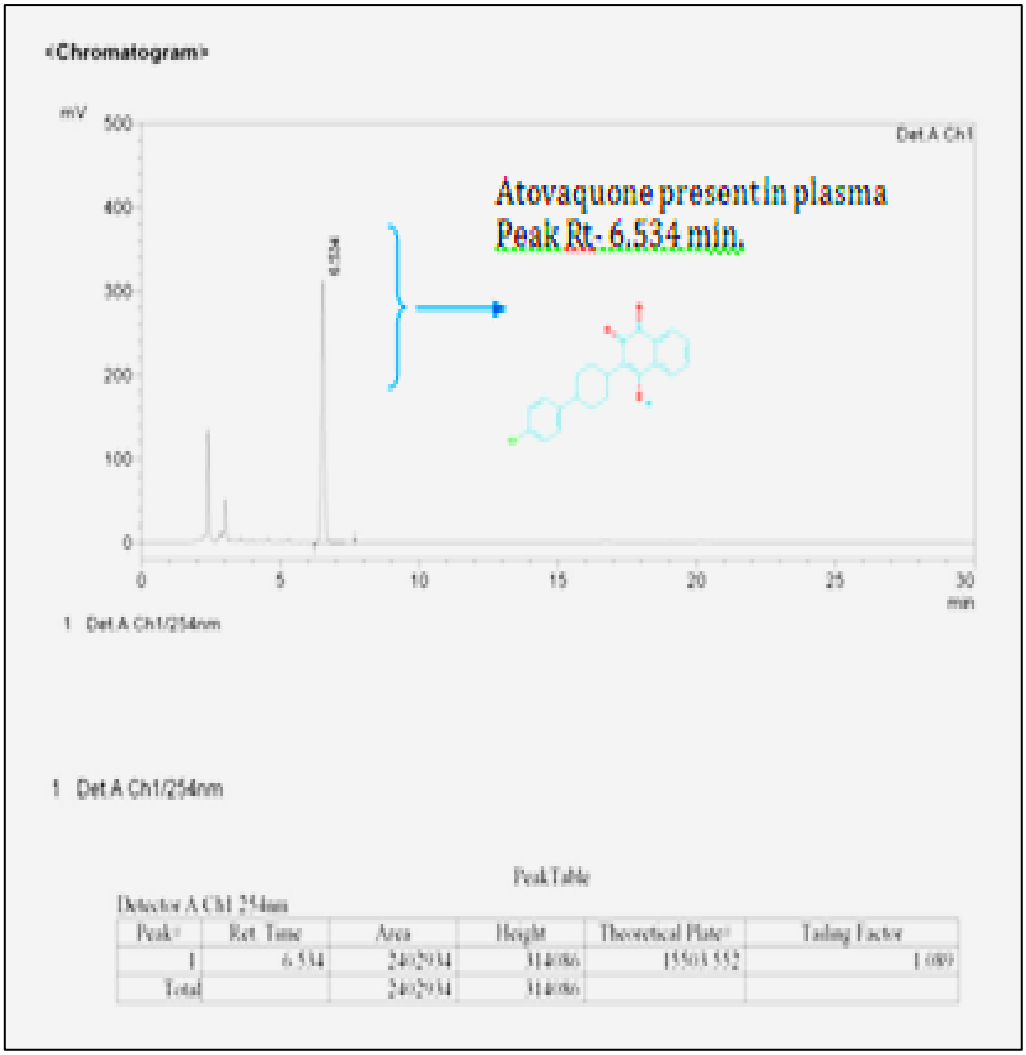


Fig .35. Chromatogram of Atovaquone (15µg/ml) in rabbit plasma.

Table.31. Standard calibration curve values of Atovaquone in rabbit plasma

Concentration (µg/ml)	Mean Peak Area
0	0
0.5	150549
1.0	185259
2	351649
5	648925
10	983974
25	2450678

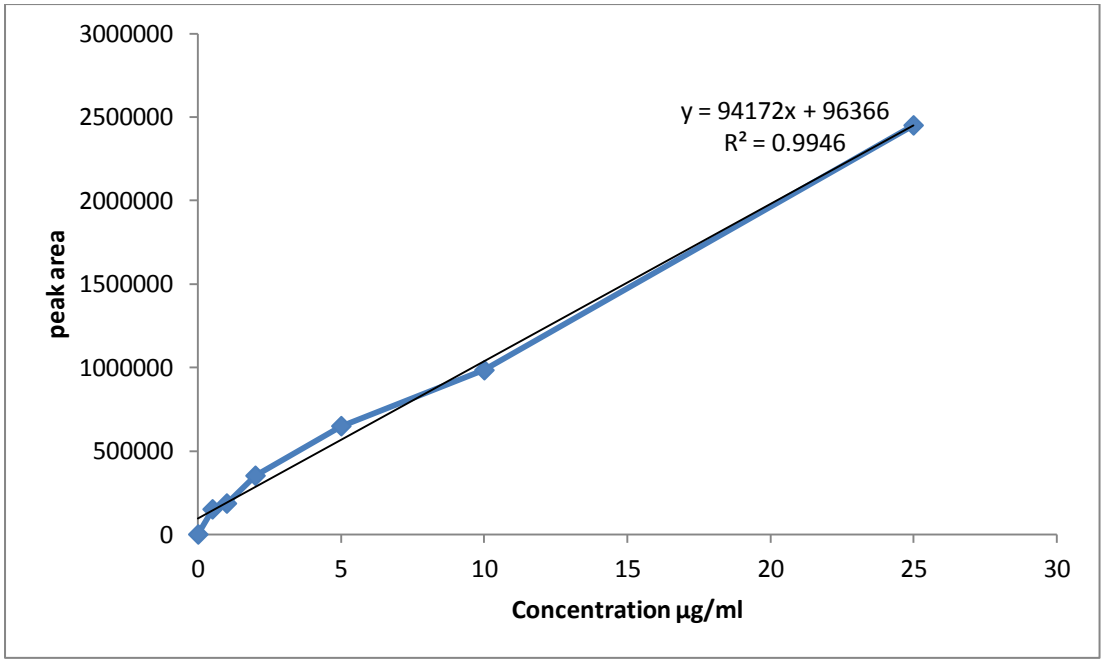


Fig.36.Standard calibration curve graph of Atovaquone in rabbit plasma

3.2.6. Evaluation of solid dispersion:

3.2.6.1. Drug content.

Atovaquone drug content in all formulated solid dispersion products (S1 to S7) were in the range of 96.8% to the 98.5% as shown in the table 32.

Table.32. Drug content of solid dispersed products

S_1	S_2	S_3	S_4	S_5	S_6	S_7
97.5% ±0.4%	96.8% ±0.6%	97.2% ±0.4%	98.01% ±0.6%	97.2% ±0.56%	96.9% ±0.7%	98.5% ±0.5%

Mean±SD (n=3)

3.2.6.2. *In vitro* dissolution profile.

Dissolution profile of all solid dispersed products ($S_1$to S_7) and pure drug (S_0) were performed for one hr interval, in acidic pH, using paddle method. Results of average triplicates were shown in the table 33 and fig 35.Comparative dissolution profile of binary and ternary solid dispersion batches were shown in fig 37.

Table.33 Dissolution profile of solid dispersed products of Atovaquone for 1hr

S_0	S_1	S_2	S_3	S_4	S_5	S_6	S_7
3.65 ±6.8	8.68 ±6.5	19.14 ±5.8	34.64 ±6.8	36.23 ±5.8	10.32 ±4.9	12.45 ±6.1	17.35 ±5.2

Mean±SD (n=3)

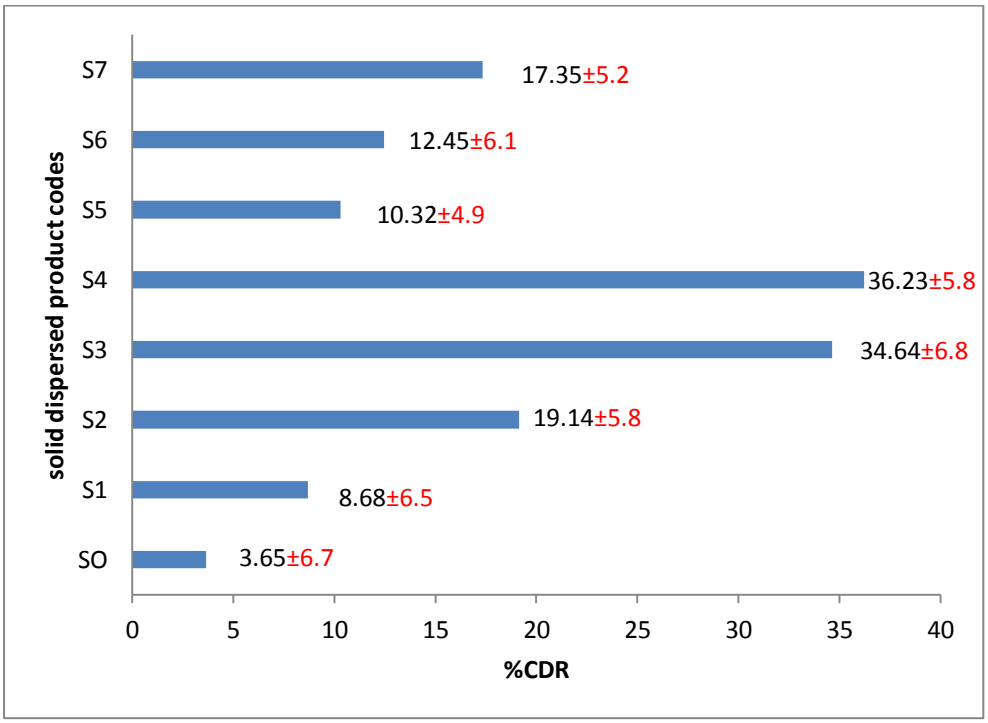


Fig 37. Graphical representation of dissolution profile of solid dispersions

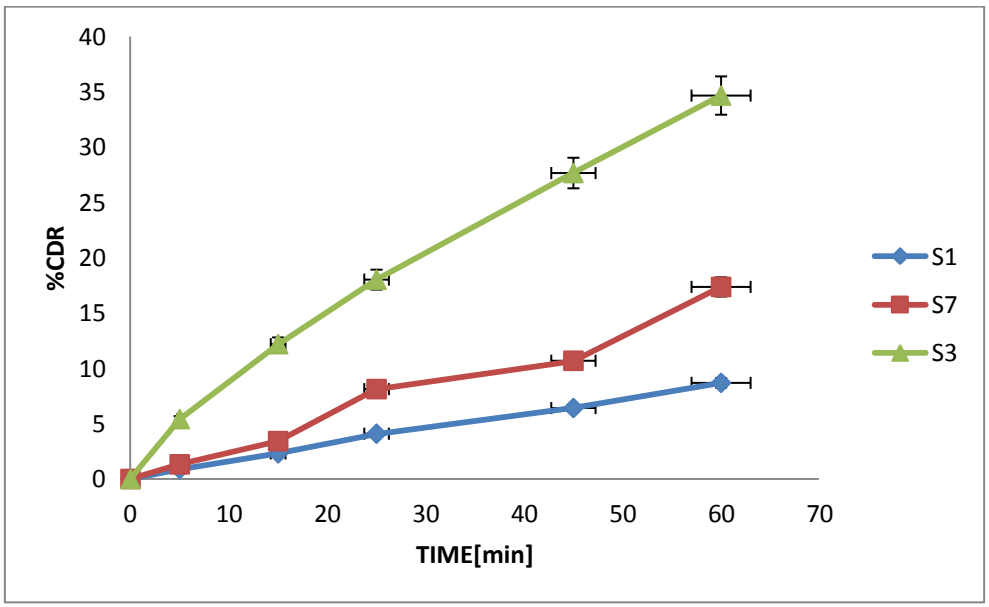


Fig 38. Comparative dissolution profile of binary and ternary solid dispersion

3.2.7. Evaluation of Atovaquone tablet:

3.2.7. 1. Precompression parameters:

3.2.7.2Bulk density and tapped density Carr's compressibility index:

Hausner's ratio

Lubricated powder of all formulated Atovaquone tablets (F_1 to F_6) were subjected for bulk density, tapped density by measuring cylinder method. The data obtained from bulk and tapped densities been used for calculation of Carr's index and Hausner's ratio. All results were tabulated in table 34.

Table.34. Pre-compression micromeritics parameters of Atovaquone

Formulation code	Bulk density gm/cm^3	Tapped density gm/cm^3	Carr's index %	Hausner's ratio
F1	0.48 ±0.04	0.56 ±0.07	10.14 ±0.02	1.16 ±0.01
F2	0.49 ±0.03	0.55 ±0.05	10.90 ±0.05	1.12 ±0.01
F3	0.47 ±0.04	0.54 ±0.07	12.96 ±0.04	1.14 ±0.07
F4	0.46 ±0.05	0.53 ±0.04	13.20 ±0.03	1.15 ±04.
F5	0.48 ±0.03	0.54 ±0.05	11.11 ±0.06	1.13 ±0.06
F6	0.45 ±0.04	0.52 ±0.04	13.46 ±0.04	1.15 ±0.02

Mean±SD (n=3)

3.2.7.3. Angle of repose (θ):

Formulated solid dispersed products were subjected to angle of repose by funnel method, to evaluate its free flowing properties. Results of angle of repose were tabulated in table 35.

Table 35. Angle of repose of Atovaquone tablets

Angle of repose	F1	F2	F3	F4	F5	F6
θ	25.6 ±1.08	26.71 ±1.23	27.13 ±1.34	26.4 ±1.21	27.74 ±1.10	28.20 ±1.43

Mean±SD (n=3)

3.2.7.4. Post compression parameters:

3.2.7.4.1 Thickness. Diameter. Weight variation. Hardness. Friability Uniformity of drug content:

Thickness, diameter and hardness are the non pharmacopieal standards', friability; weight variation and uniformity of drug content were as per the pharmacopieal standards (IP). All evaluated parameters values were tabulated in the table 36.

Table.36.—Post compression parameters of Atovaquone tablets

Formulation code	Thickness mm	Diameter mm	Hardness Kg/cm^2	Friability %	Weight variation mg	Uniformity of drug content %
F1	3.5 ±0.5	9.1 ±0.0	3.3 ±0.02	0.32 ±0.01	230 ±1.5	98.22 ±0.9
F2	3.5 ±0.6	9.1 ±0.0	3.5 ±0.03	0.31 ±0.05	230 ±1.2	97.2 ±1.02
F3	3.4 ±0.4	9.1 ±0.0	3.4 ±0.02	0.39 ±0.03	230 ±1.8	98.5 ±1.65
F4	3.6 ±0.3	9.1 ±0.0	3.3 ±0.05	0.29 ±0.04	230 ±1.8	97.65 ±1.68
F5	3.6 ±0.6	9.1 ±0.0	3.4 ±0.02	0.31 ±0.05	230 ±1.7	97.53 ±1.7
F6	3.7 ±0.2	9.1 ±0.0	3.4 ±0.05	0.29 ±0.04	230 ±1.6	98.25 ±1.8

Mean ±SD (n=6)

3.2.7.4.2. In vitro disintegration test:

Disintegration time of Atovaquone prepared tablets was determined in Electro lab USP ED2A tablet disintegration test machine using water as test fluid at $37^{o}C \pm 1^{o}C$ temperature. Mean values from triplicates were tabulated in table 37.

Table.37. Disintegration time data of Atovaquone tablets

Formulation code ⇨	F1	F2	F3	F4	F5	F6
Time in minutes	7.50 ±0.08	4.5 ±0.06	5.6 ±0.07	3.9 ±0.04	2.84 ±0.05	3.5 ±0.03

Mean±SD (n=3)

3.2.7.4.3. *In vitro* dissolution profile:

The dissolution profile of all formulated Atovaquone tablets were conducted for one hr in acidic medium using USP dissolution apparatus II in triplicate .The average values of dissolution data were mentioned in the table.38 and graphically represented in fig.37.

Table.38. Dissolution profile data of Atovaquone tablets

Time minutes	F1 %CDR	F2 %CDR	F3 %CDR	F4 %CDR	F5 %CDR	F6 %CDR
0	0	0	0	0	0	0
5	0.488 ±0.50	0.716 ±0.41	0.567 ±0.21	0.875 ±0.32	1.385 ±0.54	0.981 ±0.45
10	1.087 ±0.64	1.624 ±0.60	1.164 ±0.54	1.881 ±0.52	3.655 ±0.61	2.440 ±0.76
15	2.146 ±0.43	2.703 ±0.41	2.365 ±0.61	2.86 ±0.42	7.801 ±0.81	3.71 ±0.83
20	2.817 ±0.54	3.319 ±0.30	3.184 ±0.35	3.45 ±0.51	10.501 ±0.76	5.600 ±0.56
30	3.341 ±0.34	4.983 ±0.41	3.543 ±0.43	5.466 ±0.51	18.629 ±0.67	10.407 ±0.75
45	4.619 ±0.42	6.255 ±0.21	4.827 ±0.62	8.60 ±0.38	28.017 ±0.81	15.724 ±0.84
60	5.460 ±0.51	9.003 ±0.51	7.273 ±0.43	10.95 ±0.71	37.653 ±0.64	18.684 ±0.63

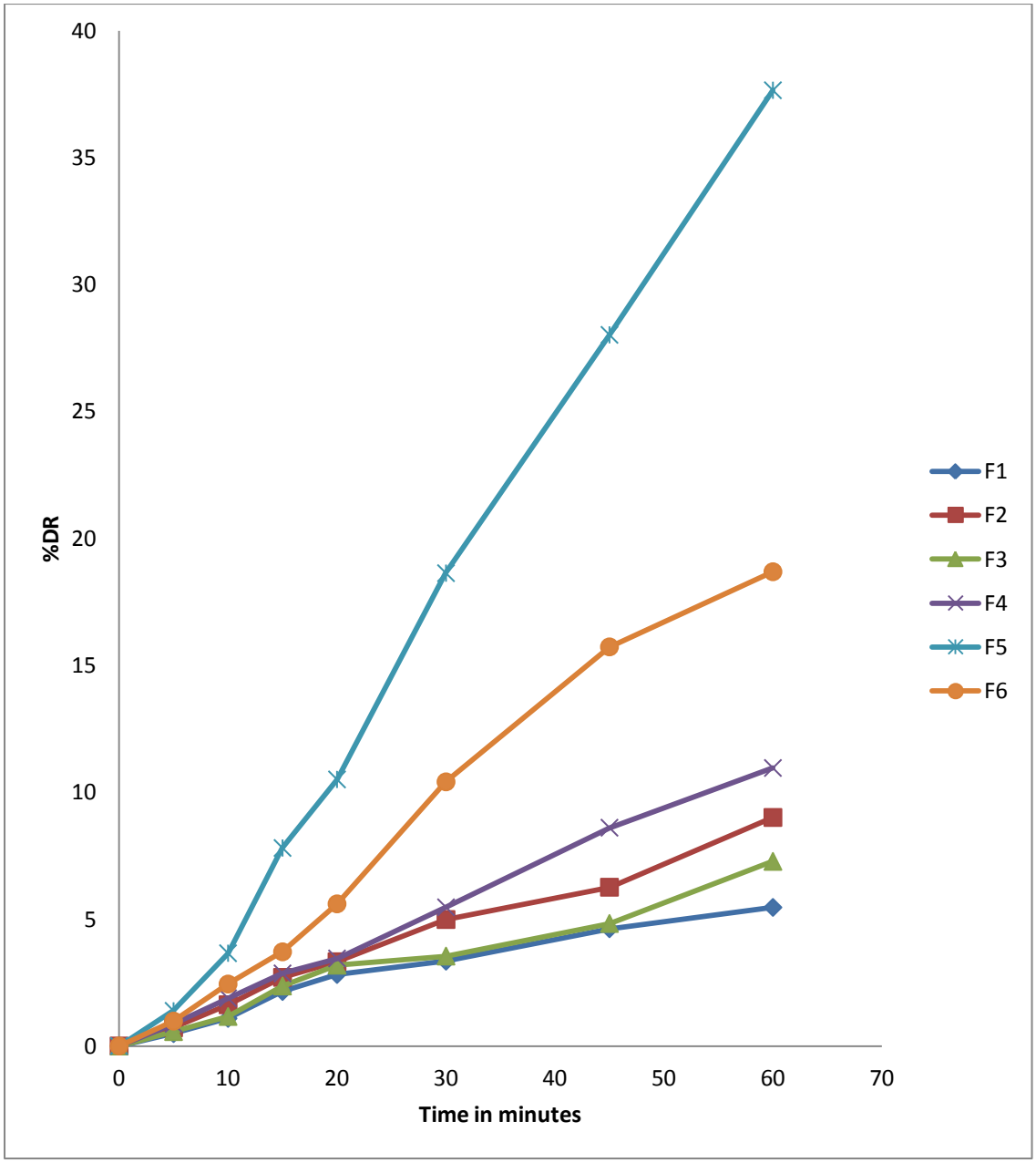


Fig.39. Graphical representation of Atovaquone tablets dissolution profile.

3.2.7.4.4. Dissolution efficiency:

Dissolution efficiency at 45 minutes was determined as proposed by Khan and Rhode, the number of fold increase in (%) dissolution rate was calculated with respect to pure drug's (%) dissolution rate (Table 39.Fig 38)

Table.39. Percentage dissolution efficiency at 45 of Atovaquone tablets

Formulation code ⇨	F1	F2	F3	F4	F5	F6
%DE45E	5.02	7.2	6.45	9.75	33.25	17.65
No of fold increase in dissolution	---	1.44	1.28	1.94	6.62	3.51

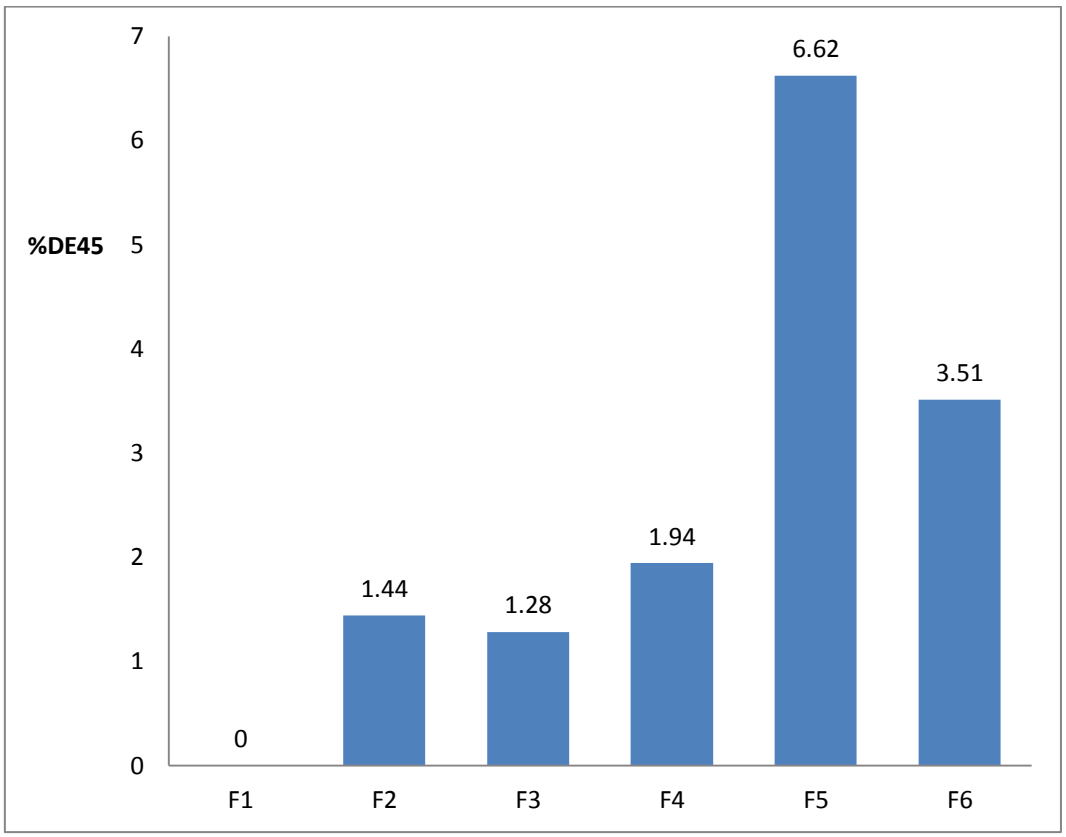


Fig.40. Graphical representation of % DE45

3.2.8. *In vivo* bioavailability study:

Raw data collected from the in vivo studies were subjected for model fitting to calculate the pharmacokinetic parameter: Cmax, tmax, and relative bioavailability. Table 40 shows the average values of plasma concentration with respect to time and Table 41, shows the calculated values of Cmax, tmax and AUC.

The relative bioavailability was found to be 2.771 with respect to oral suspension (fig39). All data were subjected for student t test for its significance; p value was set at less than 0.05.

Table 40. Plasma time profile data of *in vivo* study

F5 µg/ml	Time hrs	Oral suspension µg/ml
4.8±12.62	0.5	1.23±26.32
12.5±13.02	1.0	2.35±21.89
24.35±17.62	3.0	4.8±19.65
29.36±20.56	5.0	7.6±35.62
25.76±24.32	6.0	10.35±34.12
19.48±22.78	12	6.84±29.32

Mean ±SD (n=3)

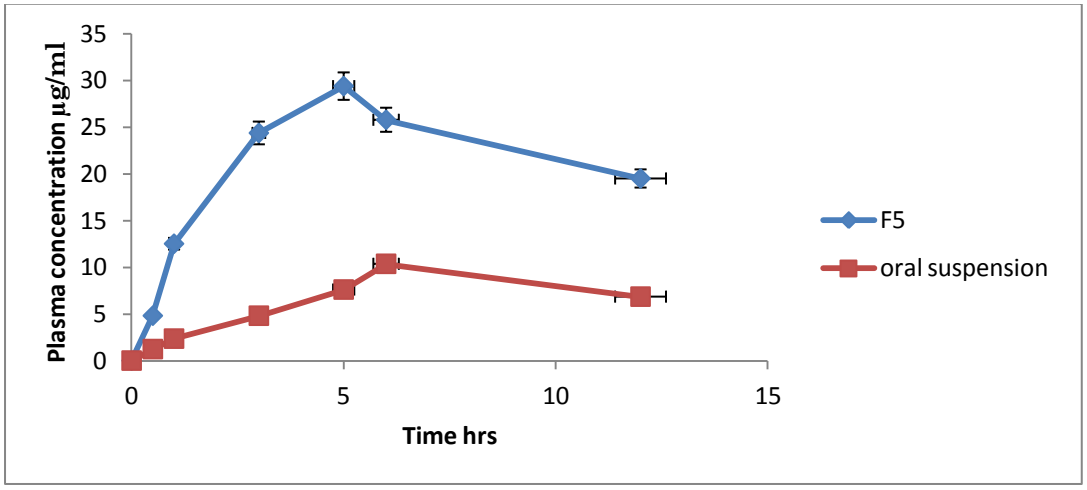


Fig.41.Graphical representation of plasma time profile data

Table.41.PK parameters for F5 and oral suspension of Atovaquone.

Pharmacokinetic parameters					
Cmax µg/ml		**t max hr**		**AUC µg/ml/hr**	
F5F5	**oral suspension**	**F5**	**oral suspension**	**F5**	**oral suspension**
30.12 ±15.62	10.56 ±12.51	5.1 ±13.62	6.1 ±14.32	240.73 ±25.56	88.72 ±25.32

Mean ±SD (n=3)

3.2.9. Short term stability study:

Modified Short term stability study in accordance with ICH guidelines for best formulation F5 was subjected at room temperature and accelerated stability studies for three months. The specific evaluation parameters were analyzed and all obtained result values were tabulated in the table.42. Drug content, hardness and invitro dissolution study were the in process stability parameters.

Table. 42. Modified short term stability study of F5 formulation

Short term stability study								
Temp/RH	**25°C±2°C/60RH±5%**				**40°C±2°C/75RH±5%**			
Month	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**
QC test								
Drug content %	97.72 ±0.95	97.34 ±1.05	97.16 ±0.86	97.05 ±0.92	97.72 ±0.75	97.01 ±0.83	96.58 ±0.91	95.02 ±0.94
Hardness kg/cm^2	3.3 ±0.05	3.2 ±0.04	3.2 ±0.04	3.1 ±0.04	3.3 ±0.03	3.1 ±0.02	2.90 ±0.03	2.50 ±0.01
%CDR(1hr)	37.72 ±1.05	37.64 ±0.91	37.02 ±0.83	37.02 ±1.05	37.72 ±1.02	37.05 ±1.05	36.82 ±1.03	36.02 ±1.08

Mean±SD (n=3)

3.3. PHASE III

FORMULATION OF LORNOXICAM GEL WITH BIOSURFACTANT

3.3.1. Preformulation of Lornoxicam

3.3.1.1. Melting point:

Lornoxicam melting point was determined by Thieles tube capillary method; was found to be 228.5^{o}C. The pharmacopieal standard rage is 225^{o}C to 230^{o}C.

3.3.1.2. Standard calibration curve and λmax:

Standard calibration curve was plotted from the Lornoxicam standard solution prepared from phosphate buffer at 6.8 pH. The Beer's range was 4µg/ml to 20µg/ml concentrations. Table 43 and fig 41 shows the absorbance reading in triplicates and graph of standard calibration curve with R^2 value respectively. Maximum absorbance of Lornoxicam was found at 376nm with 8µg/ml concentration as depicted in the fig.40.

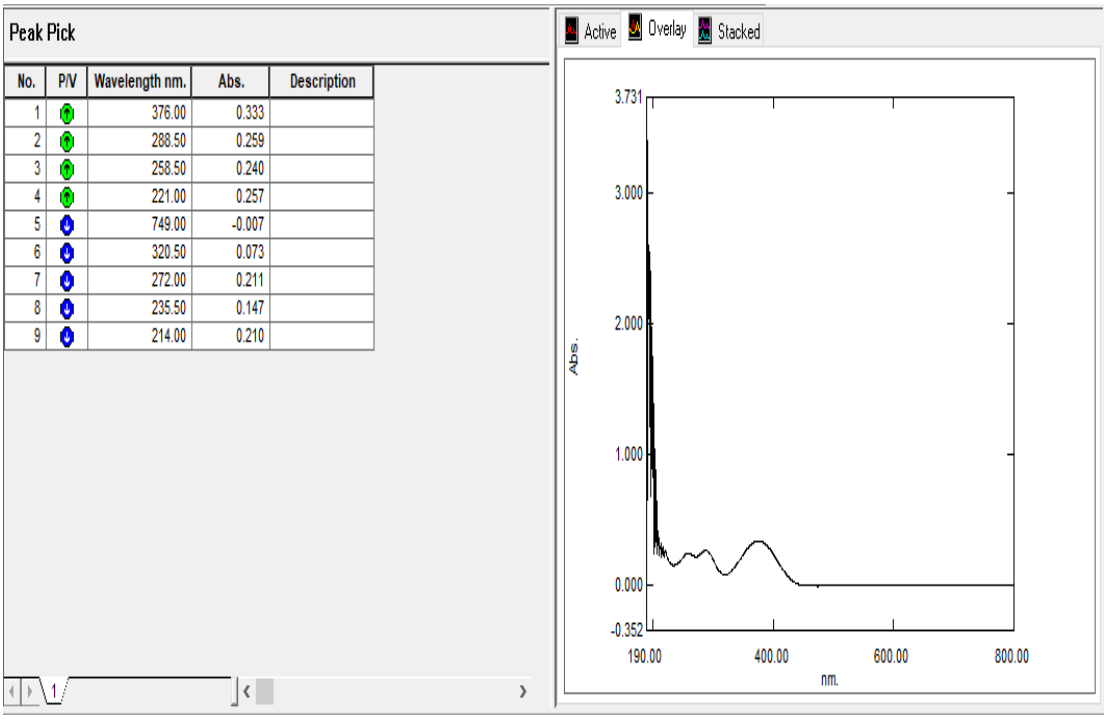


Fig.42.Maximum absorbance of Lornoxicam at pH 6.8 buffer

Table.43. Lornoxicam absorbance data at 376nm.

Concentration (mcg/ml)	Absorbance
0.00	0.00± 0.000
4.00	0.156± 0.004
8.00	0.312± 0.003
12.00	0.461± 0.002
16.00	0.608± 0.006
20.00	0.761± 0.005

Mean±SD (n=3)

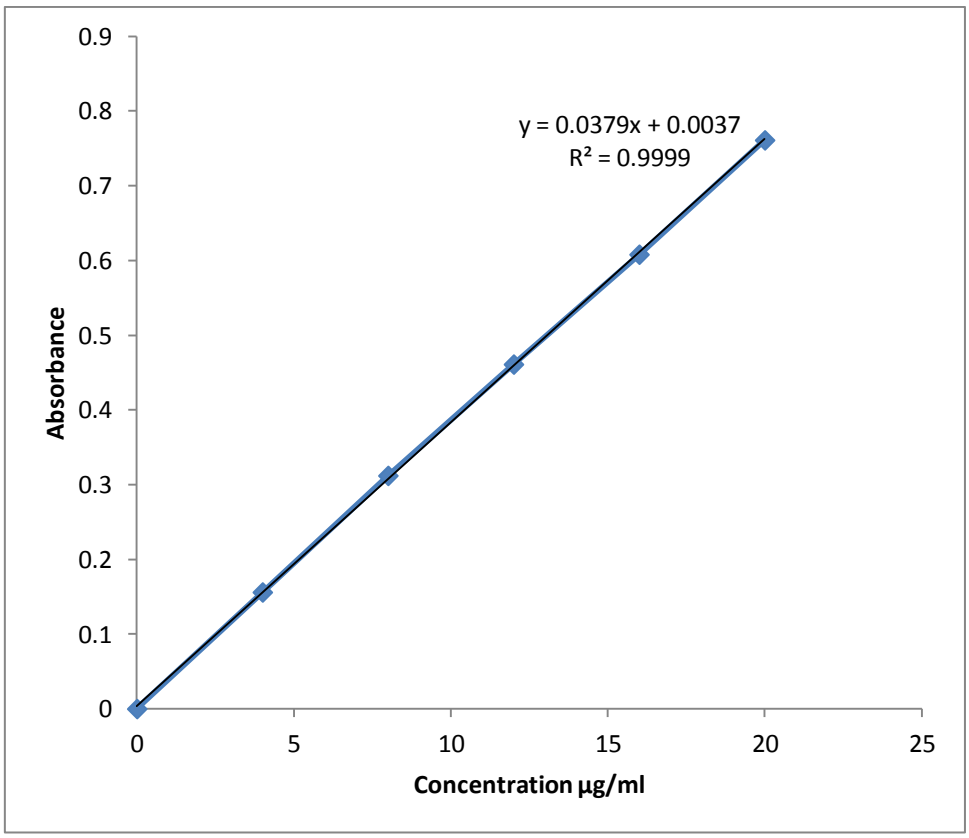


Fig.43.—Standard calibration curve of Lornoxicam

3.3.1.3. Solubility determination:

Saturation solubility studies of Lornoxicam were conducted with biosurfactant and tween 80 solutions with following concentrations (table 44). Drug contents of all samples were determined in triplicates at 376nm by UV spectroscopy.

Table.44. Solubility profile of Lornoxicam in biosurfactant and Tween 80

Biosurfactant %w/v	Amount of drug dissolved mg/ml	Tween 80w/v	Amount of drug dissolved mg/ml	Solutol HS 15 mg/ml
0.1	0.0068±0.08	2.0	0.0024±0.05	56±0.02
1.0	0.015±0.05	4.0	0.0076±0.03	
1.5	0.0564±0.04	6.0	0.0145±0.05	
2.0	0.0975±0.02	8.0	0.0165±0.04	
2.5	0.1465±0.04	10	0.0225±0.02	
3.0	0.1489±0.05	12	0.0232±0.03	
2.5% ,1ml(25mg)=0.1465mg drug		10% ,1ml(100mg)= 0.0225mg drug		

Mean±SD (n=3)

3.3.2. Drug excipients compatibility study

3.3.2.1. FTIR spectroscope study:

Comparative and compatibility evaluation of FTIR graph of pure and physical mixture of Lornoxicam were shown in Fig 42 and Fig 43 respectively. The significant functional group's peaks from pure drug were identified with its wave numbers and compare for same peaks in the physical mixture graphs. All peaks results were tabulated in the table 45.

Table.45. Comparative FTIR peaks of pure and physical mixture of Lornoxicam

Functional groups	Pure drug Lornoxicam	Physical mixture Lornoxicam
Aromatic CH stretch or NH	3066.82	3066.82
C=O stretch of carboxamide group	1647.21	1710.86
C-C aromatic	1589.34	1597.06
C-H bending (aliphatic)	1379.10	1379.10
C=N stretch	1327.03	1327.03
SO2N stretch	1147.65	1147.65
C-S stretch	1041.56	1041.56
C-Cl stretch	831.32	831.32

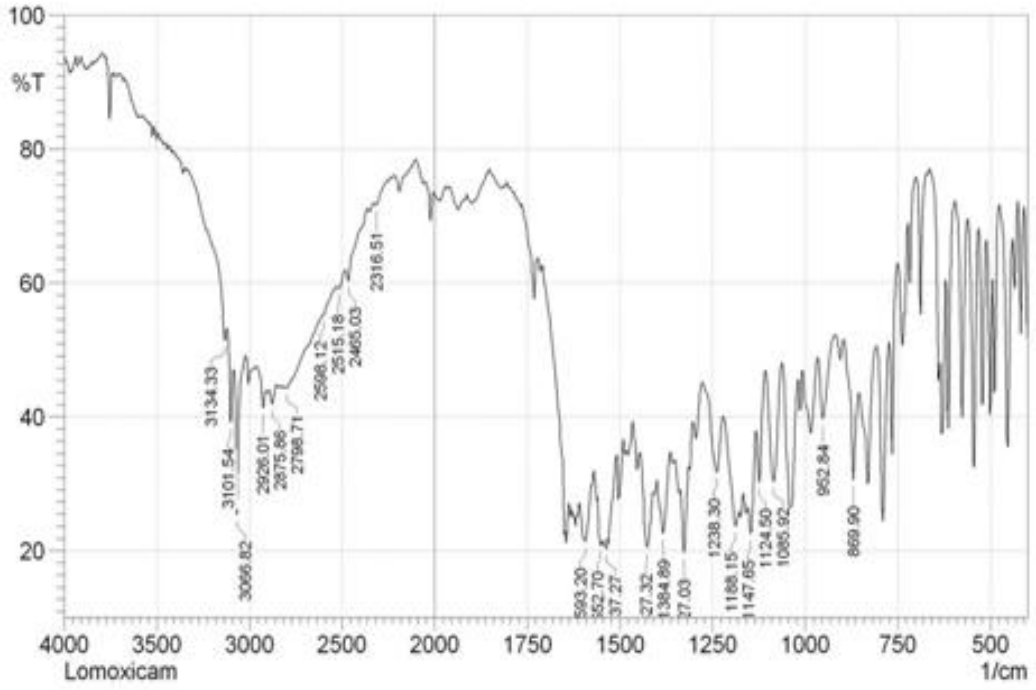


Fig.44.Lornoxicam pure drug FTIR graph

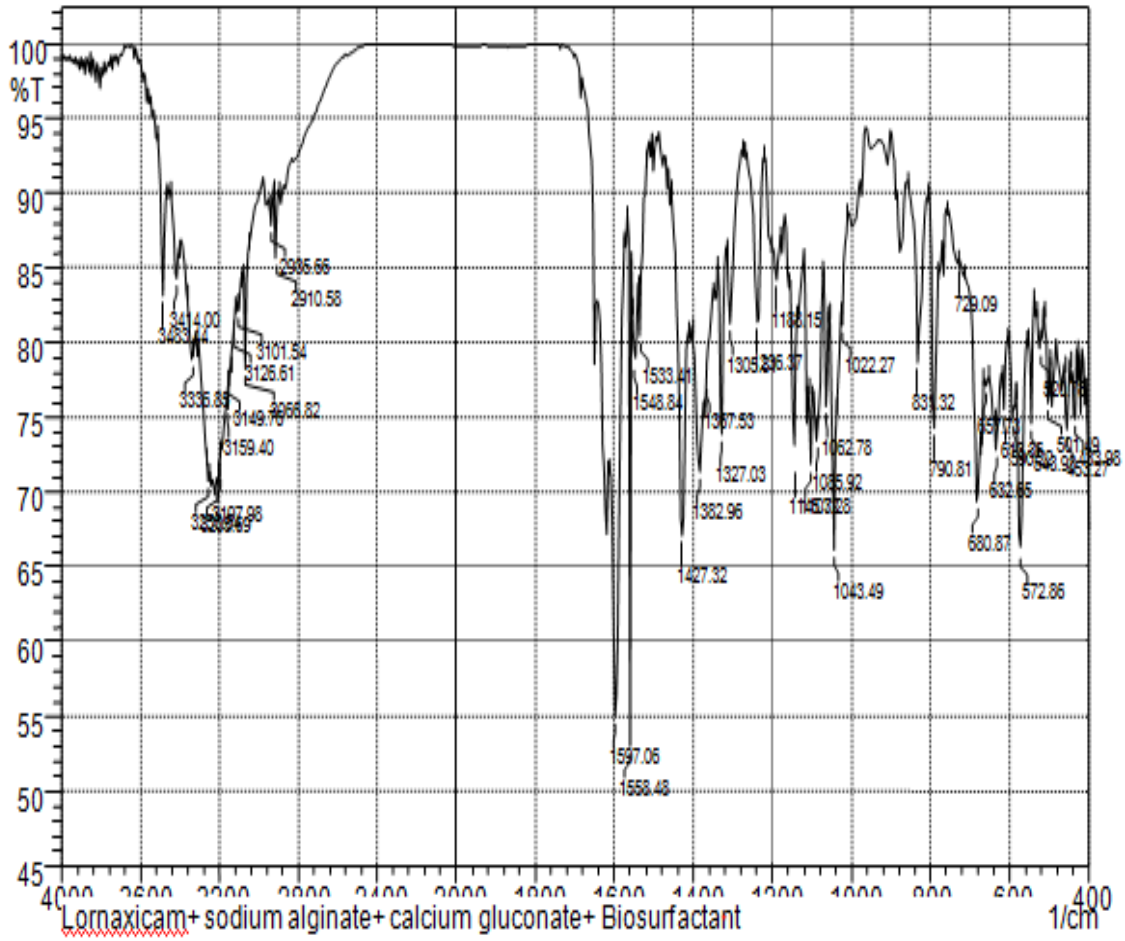


Fig.45. Physical mixture of Lornoxicam and biosurfactant FTIR graph

3.3.2.2. DSC study:

Thermograms obtained from DSC for pure Lornoxicam and physical mixture of Lornoxicam and biosurfactant were shown in fig.45 and fig46 respectively. The maximum peak of pure Lornoxicam was observed at 230.2^{o}C.In physical mixture maximum peak was observed for Lornoxicam was at 256.6^{o}C.For biosurfactant it was observed at 93.7^{o}C in physical mixture but it was observed for biosurfactant alone at 109^{o}C from the fig.24. (page103)

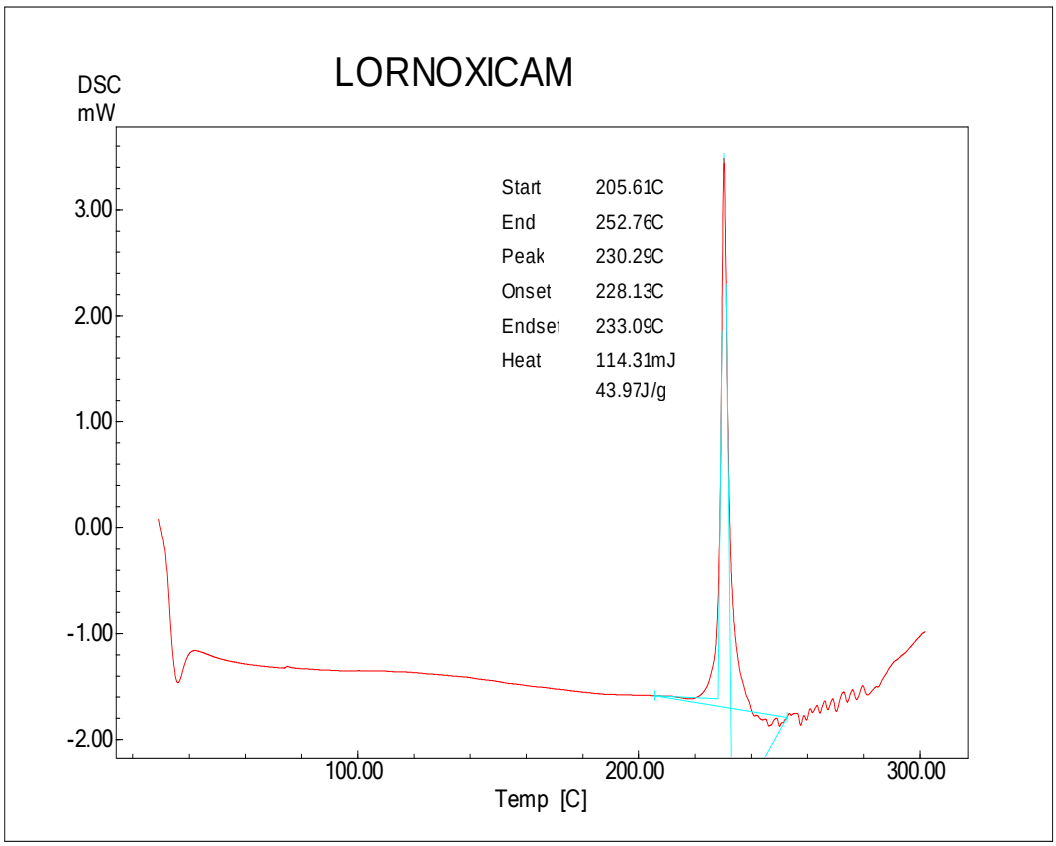


Fig. 46.DSC of pure Lornoxicam

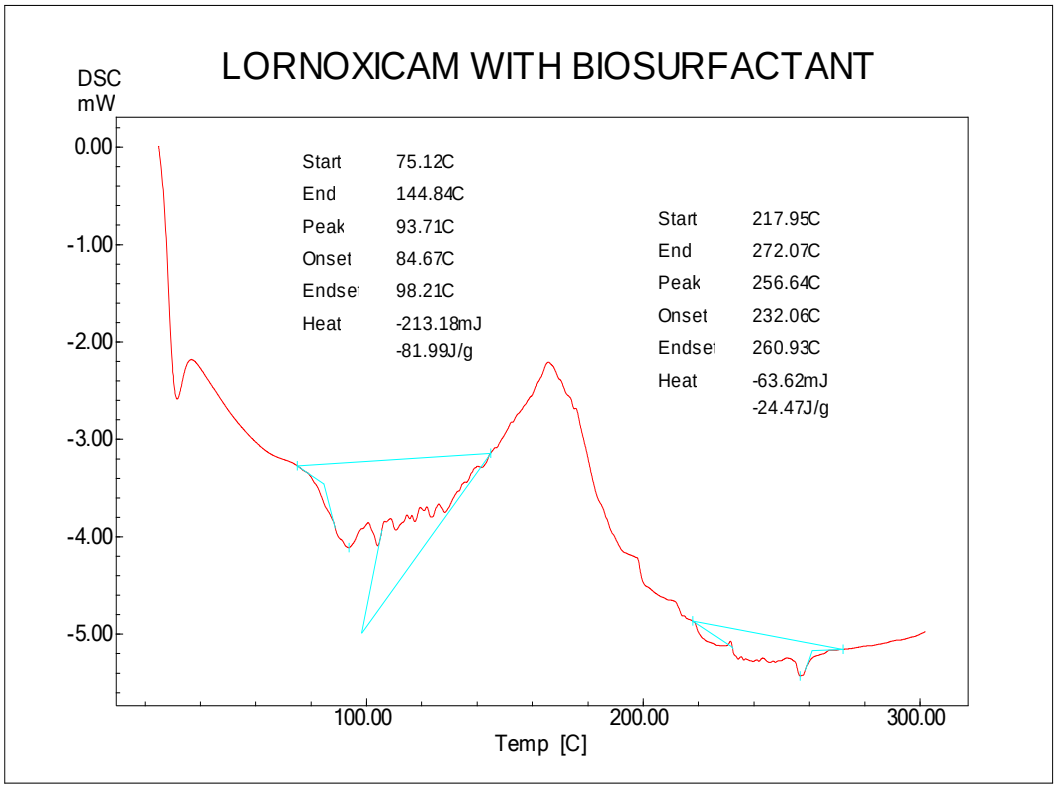


Fig. 47.DSC of Lornoxicam with biosurfactant

3.3.3 Evaluation of Lornoxicam gels:

3.3.3.1. pH of gels:

All formulated gels of Lornoxicam pH were determined and recorded in triplicates with mean in the table 46

Table.46. pH of Lornoxicam gels

Formulation code	G_1	G_2	G_3	G_4
pH	5.81±0.02	6.1±0.03	5.85±0.01	6.61±0.04

Mean±Sd (n=3)

3.3.3.2. Drug content:

Formulations of Lornoxicam gels from G_1 to G_4 drug content were shown in the table 47.

Table.47. Drug contents of Lornoxicam gels

Formulation code	**G_1**	**G_2**	**G_3**	**G_4**
Drug content %	98.23± 1.05	97.64±0.988	98.34±1.04	98.74±0.990

Mean±SD (n=3)

3.3.3.3. Rheological studies:

Viscosities of all formulations of Lornoxicam gels were determined by cup and plate rheometer values were tabulated in the table 48. Fig 46, graphical representation of all the Lornoxicam gels rheogram. Yield values were determined by the graphs of shearing stress against shearing rate as shown in the fig. 47. Formulation G1, G4 and G2, G4 were containing low and high concentration of solutol HS 15 respectively.

Table.48.Viscosity and yield value of Lornoxicam gels

Formulations	**Viscosity Pa.s /50 rpm**	**Yield stress Pa**
G_1	3563 ± 43.89	220 ±20
G_2	3100 ±46.22	205 ±18
G_3	3895 ±55.71	103 ±12
G_4	3656±67.35	125 ±10

Mean±SD (n=3)

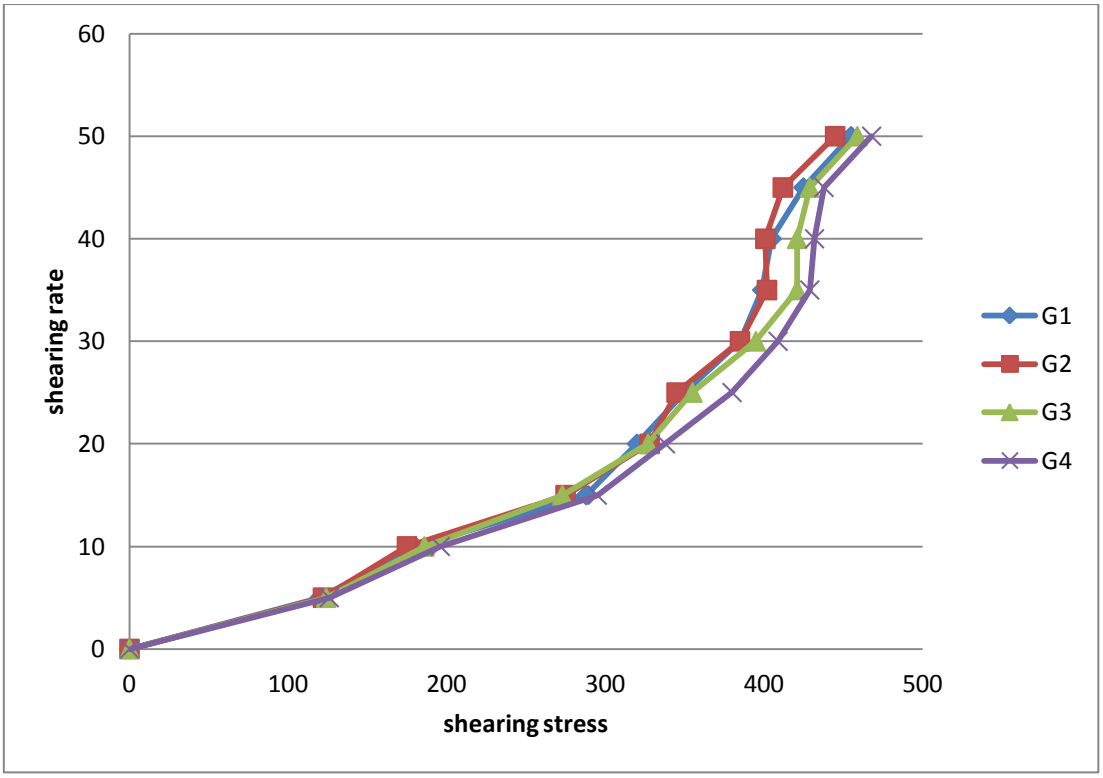


Fig.48.Rheogram of Lornoxicam gels

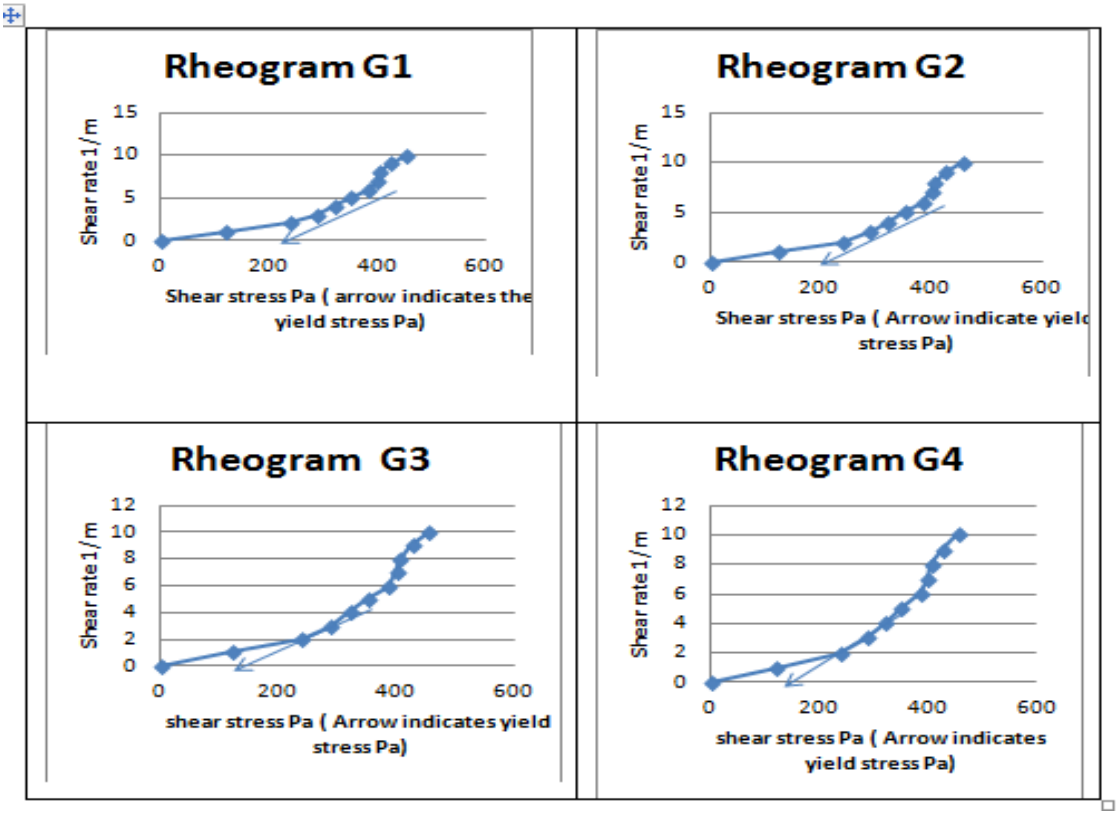


Fig.49.Yield value of Lornoxicam gel formulation

3.3.3.4 Spreadability, Extrudability and Gel strength:

Obtained results of Spreadability, extrudability and gel strength of all formulated Lornoxicam gels were tabulated in table. 49.

Table.49. Physical properties of Lornoxicam gels

Formulation code	Spreadability ($gm./cm^2$)	Extrudability (g.cm)	Gel strength (Sec)
G_1	26.91± 0.25	15.15±0.27	88.0±3.05
G_2	21.02±0.12	13.51±0.34	86.0±.3.21
G_3	17.10±0.21	10.43±0.26	65.0±3.51
G_4	16.03±0.31	09.21±0.39	73.0±3.46

Mean±SD (n=3)

3.3.3.5. *In vitro* permeation study:

In vitro permeation experiments results of all Lornoxicam gel formulations were tabulated in the table 50, with respect to the 1hr, 3hrs and 6 hrs but in fig.48, all the intervals have been depicted. All data were analyzed for using student t test ($p < 0.05$) for its significance.

Table.50. In vitro permeation study of Lornoxicam gels

Time hrs	G1 %CDR	G2 %CDR	G3 %CDR	G4 %CDR
1	25.86±0.57	19.89 ± 0.85	19.56±0.93	16.17 ±0.72
3	51.59±0.86	33.49 ± 0.68	40.06 ±0.89	31.49 ±0.59
6	82.63±0.73	73.37 ± 0.77	76.23± 0.65	67.23±0.92

Mean±SD (n=3)

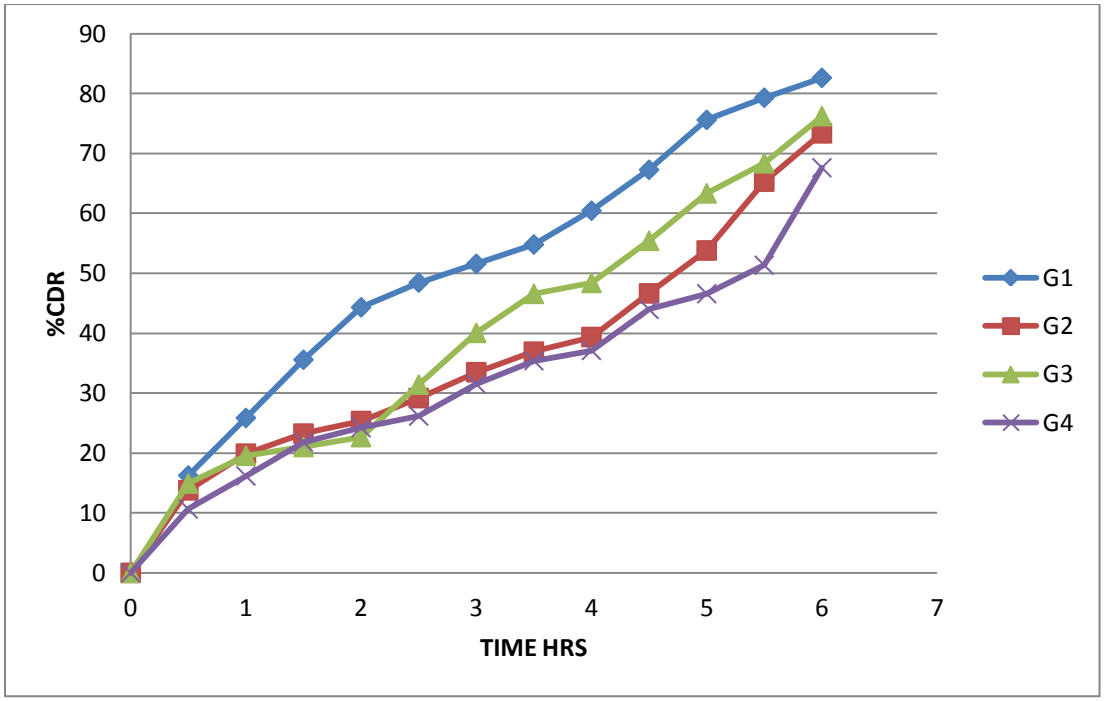


Fig. 50.Graphical representation of *in vitro* permeation study

(egg membrane)

3.3.3.6. Ex vivo permeation study:

Ex vivo permeation study using rat skin results of all the Lornoxicam gels were depicted in the fig 49. The percentage cumulative drug releases for 1 hr, 3hrs and 6 hrs were tabulated in the table .51. All data were analyzed for using student t test ($p < 0.005$) for its significance.

Table.51. *Ex vivo* permeation study data

Time hrs	G1 %CDR	G2 %CDR	G3 %CDR	G4 %CDR
1	6.85±0.65	4.30±0.72	5.86±0.67	3.94±0.61
3	28.38±0.81	18.46±0.91	26.48±0.87	17.38±0.78
6	68.35±0.79	46.65±0.78	63.46±0.84	43.31±0.62

Mean±SD (n=3)

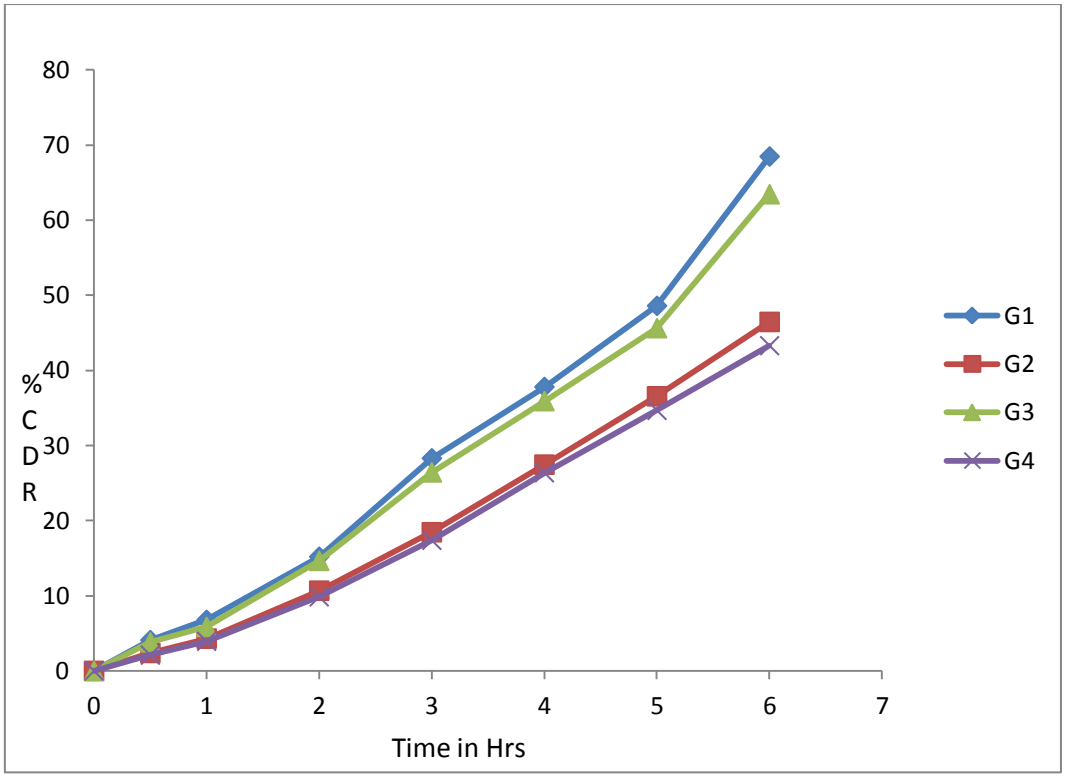


Fig.51. Graphical representation of *ex vivo* permeation study. (Rat skin)

3.3.3.7. Permeation data analysis:

Using the invitro and ex vivo data the significant factors like flux, permeability coefficient and enhancement ratio was calculated. Calculated results were shown in the table.52. A comparative flux of gels between the egg membrane and rat skin was also shown in the fig.50.

Table. 52. Comparison permeation data between egg membrane and rat skin

	Egg membrane		Rat skin		
F. code	**Flux Jss µm/cm^2.h**	**Permeability coefficient Kp.cm^2/hr 10^{-6}**	**Flux Jss µm/cm^2.h**	**Permeability coefficient Kp.cm^2/hr 10^{-6}**	**Enhancement ratio**
G1	4.49±2.34	8.98±1.86	3.82±1.41	7.64±1.25	0.85
G2	3.61±0.98	7.22±1.25	2.73±2.11	5.46±1.65	0.75
G3	4.08±1.45	8.16±0.75	3.23±1.65	7.05±2.84	0.79
G4	3.39±0.85	6.78±0.89	2.41±2.74	4.80±2.22	0.72

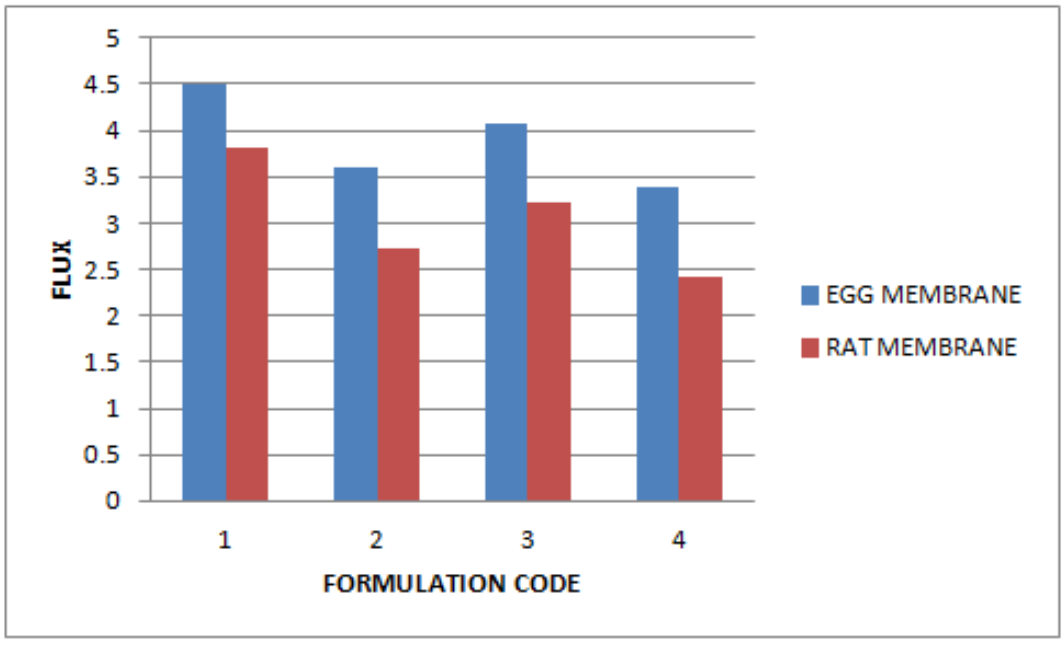


Fig. 52. Comparative flux between egg membrane and rat skin

3.3.3.8. Drug release kinetics:

The data obtained from the *in vitro* and *ex vivo* permeation studies of Lornoxicam gels were subjected for different release kinetic models to assess the release mechanism. All Lornoxicam gels kinetic model fitting graphs were plotted in excel sheet and R values were determined. Table 53 and 54 were the best fit model from egg membrane and rat skin respectively.

Table.53. Release kinetic model fitting of egg membrane

	Egg membrane		**Correlation coefficient [R]**		
Formulation code	**zero order**	**First order**	**Higuchi**	**Korsmeyer-Peppas**	**Best fit model**
G1	0.685	0.795	0.966	0.865	Higuchi
G2	0.780	0.776	0.973	0.850	Higuchi
G3	0.769	0.769	0.978	0.901	Higuchi
G4	0.789	0.765	0.968	0.845	Higuchi

Table.54. Release kinetic model fitting of rat skin

	Rat skin Correlation coefficient [R]				
Formulation code	**zero order**	**First order**	**Higuchi**	**Korsmeyer-Peppas**	**Best fit model**
G1	-0.685	0.595	0.986	0.675	Higuchi
G2	0.780	0.476	0.963	0.550	Higuchi
G3	-0.469	0.561	0.984	0.801	Higuchi
G4	0.789	0.663	0.978	0.745	Higuchi

3.3.4. *In vivo* anti-inflammatory study:

Anti inflammatory efficacy of best formulation of Lornoxicam and marketed preparation were evaluated on model paw edema of rat by administering carrageenan. Obtained results were compared with marketed product (Piroxy).The edema volume was measured and percentage inhibition was calculated for 6hrs.The significance of data was analyzed using student t test at $p<0.005$.All the results were tabulated in table 55 and depicted in fig 51.

Table.55. Percentage inhibition data

Percentage of Inhibition		
Duration hr	**G1**	**Marketed formulation**
0.5	4.5±0.21	1.87±0.18
1	8.95±0.16	3.04±0.17
1.5	10.25±0.23	3.61±0.18
2	12.43±0.27	4.06±0.16
2.5	17.52±0.19	6.54±0.24
3	22.51±0.13	8.76±0.16
3.5	28.75±0.21	14.05±0.14
4	37.84±0.15	18.94±0.13
4.5	40.32±0.13	23.84±0.24
5	43.08±0.24	26.81±0.12
5.5	47.61±0.22	29.79±0.26
6	51.07±0.19	33.5±0.23

Mean±Sd(n=3)

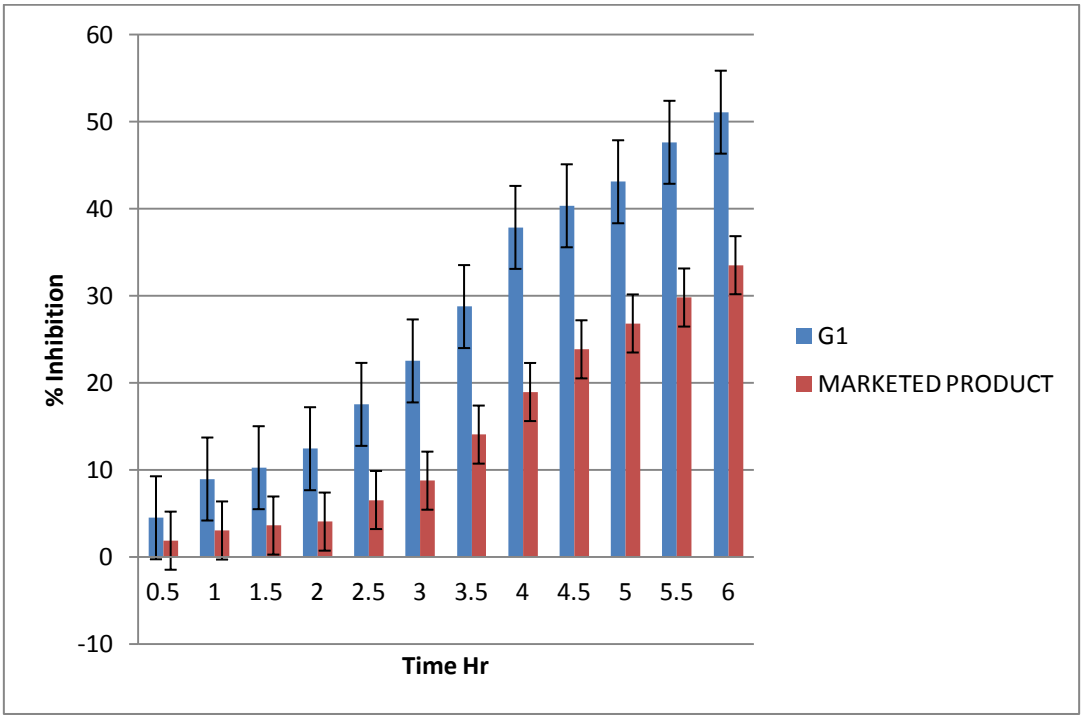


Fig.53. Percentage of inhibition of G1 and marketed formulation

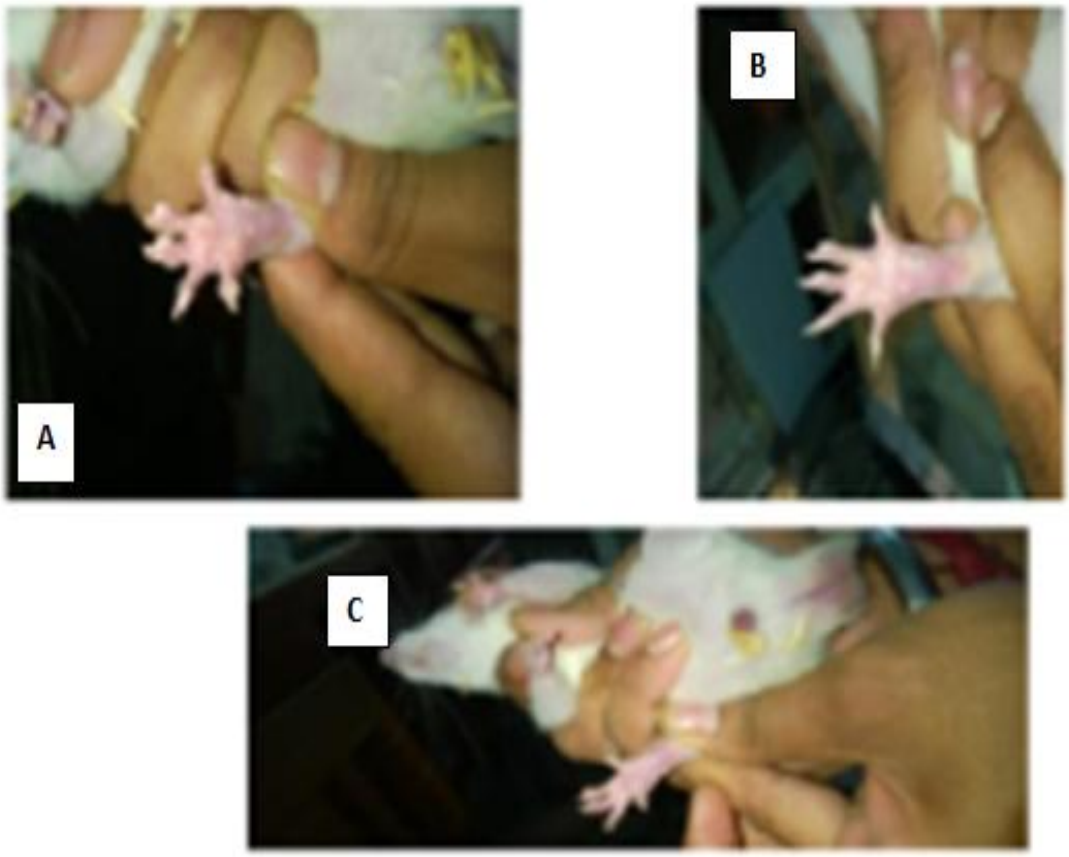


Fig.54. Paw edema [A] control group [B] G1 formulation [C] marketed formulation

3.3.5. Acute skin irritation study:

Acute skin irritation test was conducted on rats with positive control and samples of G1, marketed formulation. The positive control skin irritation scale was drawn on the bases of the erythema and edema observed during the studies and same was shown in table.57. Similarly with G1 and marketed formulations were shown in table 56.

Table.56. Observed erythema and edema data of skin irritation test.

Test	Skin reaction	Score
Erythema	No erythema	0
	Very slight erythema	0
	Well define erythema	0
	Moderate severe erythema	0
	Sever erythema	0
Total possible erythema score		0
Edema	No edema	0
	Very slight edema	0
	Well defined edema	0
	Moderate to severe edema	0
	Severe edema	0
Total possible edema score		0
Total score for primary skin irritation test		0

Table . 57. Positive control skin irritation reactions and score

Test	Positive control Skin reaction	Score
Erythema	No erythema	
	Very slight erythema	
	Well define erythema	
	Moderate severe erythema	3
	Sever erythema	0
Total possible erythema score		3
Edema	No edema	
	Very slight edema	
	Well defined edema	3
	Moderate to severe edema	
	Severe edema	
Total possible edema score		3
Total score for primary skin irritation test		6

3.3.6. Short term stability study:

Short term stability was conducted in accordance to the ICH guideline but slight modifications with duration of study by keeping the temperature and relative humidity similar with ICH (Q1B). In this study room temperature [25^{o}C ±2^{o}C and RH 65% ±5%] and accelerated temperature [40^{o}C ±2^{o}C at RH 75%±5%] were selected for three month. Periodically in process tests were performed for every month with respect to pH, drug content, viscosity and in vitro diffusion study for G1 formulation. In process parameters data were shown in the table 58 and table 59 for room temperature and accelerated temperature respectively.

Table.58. Data of optimize formulation G1 at 25^{o}C±1^{o}C and RH 65%± 5%

Duration	pH	Viscosity Pa.S	Drug content %	*In vitro* release studies %CDR
0 month	6.7±02	3565 ±45.71	99.5±2.31	82.23± 2.15
1st month	6.6±0.3	3465±38.61	98.6±2.54	79.64±2.38
2nd month	6.5±0.3	3478±41.2	98.5±2.98	79.52±2.48
3rd month	6.5±0.2	3465±38.3	98.9±3.01	78.01±2.57

Mean ± SD (n=3)

Table.59. Data of optimize formulation G1 at 40^{o}C ±1^{o}C at RH 75%± 5%

Duration	pH	Viscosity Pa.S	Drug content %	*In vitro* release studies %CDR
0 month	6.7±02	3565 ±45.71	99.5±2.31	82.23± 3.15
1st month	6.6±0.3	3265±38.61	98.6±2.54	76.64±1.08
2nd month	6.3±0.3	3228±41.2	97.5±2.98	75.52±2.98
3rd month	6.3±0.2	3215±38.3	95.9±3.01	74.01±2.12

Mean ± SD n=3

4.0. DISCUSSION

4.1. PHASE I BIOSYNTHESIS OF BIOSURFACTANT

4.1.1. Sub culturing and preliminary characterization of *flavobacterium sp*. MTCC 2495.

In the present study, the *Flavobacterium sp*. MTCC 2495 bacteria were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India. Sub-cultured in the mineral salt media, gram staining, and motility test results revealed that bacteria were rod shape with yellow colour and pink colour before and after gram staining respectively. Procured bacteria were gram –ve and motile. The oil spreading technique results showed that there was an average 2.8cm diameter indicated bacteria produces biosurfactant. The bacteria were cultivated in the modified mineral salt media for maximum propagation by adding peptone and trace elements into the mineral salt media by keeping other constituents constant. Laboratory scale cultivated bacteria in modified mineral salt with sucrose and peptone as substrates were accessed for the growth phases, the maximum growth of the bacteria was observed during the 30hrs to 72hrs (fig17), which was also reflected in the results of biomass estimation. From these results, it was concluded that the total duration for fermentation to achieve maximum propagation was 72hrs and adopted in all further studies. The preliminary test of emulsifying index results indicated that *Flavobacterium sp* broth filtrate had shown the optimum stability of emulsion as compared to the synthetic surfactant tween 80(Sobrinho,etal.2013)(fig18).The emulsifying index confirmed that *Flavobacterium sp* produces the biosurfactant in the specified media and other environmental conditions.

4.1.2. Optimization of substrates concentration using 3^2 factorial designs

The factorial design matrix 3^2 results revealed that from the nine runs of laboratory-scale fermentation with varying the concentration of sugar cane molasses and waste fried oil, there was the synergistic and antagonistic effect of substrates on dependent variables. The dependent variables were surface tension and biosurfactant yield.

The optimum conditions for maximum biosurfactant yield and lowest surface tension were found in the Run 3. Run 3 consist of a low concentration of both substrates in constant environmental conditions. ANOVA was performed to analyses the significance and acceptance of the model. It can be concluded from Table 18 that the linear and quadratic terms and interactions were statistically significant $p < 0.05$. The pure error from both models was very low indicated the reproducibility of the experiment. The contour plot from fig 19 and 20, for surface tension and yield of biosurfactant respectively, showed that as the percentage concentration of waste fried oil was increased the surface tension did not decrease or indirectly yield of the biosurfactant was also decreased. But as the percentage of concentration of sugar cane molasses was increased there was an optimum decrease in the surface tension, indirectly an increase in the yield of biosurfactant. These results conclude that a high concentration of substrates does not stimulate the yield of biosurfactant (Dane G A, et al2017). Optimization of substrate concentrations was obtained from the overlay plot from the trial run batches as shown in fig 21. The design space predicted for sugar cane molasses concentration was in the rage of 4.5% to 8% and for waste fried oil was

in the range 4.1 to 6.0%. Estimation of surface tension as in-process control during the fermentation resulted that, the total duration required for fermentation was 72hrs. To confirm the efficacy of optimized run from the laboratory scale, the pilot scale-up was conducted in a bioreactor of 2-liter capacity using the intermediate concentrations of substrates. Sugarcane molasses and waste fried oil concentrations were 5.2% and %.5.0% respectively. The pilot scale-up batch resulted in a **7.9 gms** biosurfactant yield. The increases in the yield of biosurfactant could be attributing factors like better control of aeration, agitation and temperature. The solvent extraction method was more adequate process for maximum yield of biosurfactant (Ibrahim MB, et al. 2014)

4.1.3. Partial characterization of biosurfactant

Critical micelle concentration is a unique physical property of surfactant, above the certain concentration in the bulk phase surfactant micelle or aggregation occurs and further addition of surfactant goes into micelle formation. Reported synthetic surfactants showed the CMC in the rage of 1% to 15%. The CMC of biosurfactant was found from fig 23, 39.5 mg per liter or 0.036%w/v. (fig23). Reduction of Surface tension of bulk phase was found to be at the lowest concentration of biosurfactant and thus it enhances the solubility of poorly water-soluble drugs. UV maximum absorbance of biosurfactant was at 312.5nm, this study was essential for determining any interference with other drugs used in this study. FTIR graph (fig.25) indicated the essential functional groups present in the biosurfactant chemical structure. The structure of the biosurfactant constituted with lipid (1744.5 cm^{-1}), the amino acid (3421cm^{-1}) and carbohydrate (3007.6 cm^{-1}). From the review of literature it was reported that major peaks wave numbers for glycolipids were 2927cm-1, 2850cm-1, 1728cm-1, 1074cm-1 and 1000cm-1. (Bharati, et al.2011)

TLC study also confirmed the presence of carbohydrate and amino acid moieties. The melting point of biosurfactant (fig.26) found at 109.2^{o}C indicative of the low molecular weight of biosurfactant.

4.1.4. Pharmacological studies of biosurfactant

Oral toxicity and acute dermal toxicity studies were conducted for 14 days. No morbidity was observed in both studies. In oral toxicity experiment's animals were observed for water and food intake it was found that no variation in quantities of food and water and also fecal texture and colour was normal (Table.25, 26). Acute dermal toxicity study also indicated that no erythema and no edema with normal food and water intake every day. These studies concluded that biosynthesized biosurfactant was safe for oral and dermal use.

4.2 PHASE II: ATOVAQUONE TABLET FORMULATION WITH BIOSURFACTANT

4.2.1. Preformulation study of Atovaquone

The melting point of Atovaquone was 216.3^{o}C and within the specified range. The purity of the sample drug received was pure. Atovaquone drug belongs to the BCS class IV. The solubility of the drug was higher in a mixture of isopropyl alcohol and phosphate buffer pH 8(40:20) as compared to the aqueous and non-aqueous solvents. The hierarchy of solubility of the drug was Isopropyl alcohol: Phosphate buffer >isopropyl alcohol >methanol > phosphate buffer 6.8 > acidic buffer. The maximum UV absorbance of the drug was found at 221nm.From the standard calibration curve: regression analysis R^2 value was nearer to 1(0.995) due to the drug

Beer's range and appropriate solvent system selection. Drug and excipient compatibility study through the FTIR technique led to the comparative compatibility of important functional group peaks from the pure drug and physical mixture was intact without major deviations. A comparative correlation was observed between the capillary tube method (216^{o}C) and DSC study (216.2^{o}C) for the melting point for Atovaquone and biosurfactant. It was observed that drugs and biosurfactants were compatible with the physical mixture (fig 31). These studies concluded that drugs and biosurfactants were compatible and stable. HPLC method developed for Atovaquone in the mobile phase and rabbit plasma was validated as mentioned in the ICH Q2 (R1). Validation parameters were linearity, accuracy, precision and repeatability. The calibration curve was linear in the limits of 0.5µg to 25µg with R^2 (0.994) lower limit of detection was found at 0.5µ/ml, UV detected at 254nm.Estimated results were found to be within acceptable ranges, so the validated procedure was applied for *in vivo* study of the drug in further study.

4.2.2. Solid dispersion of Atovaquone

Solid dispersion of drug with HPMC of binary and ternary formulations was prepared and evaluated. Two sets of solid dispersion formulations were evaluated for the effect of biosurfactant in the enhancement of solubility of the drug in accordance with the synthetic poloaxmer 407 surfactant. The binary solid dispersion (S1) enhancement was 2.33 fold with respect to pure drug. (S0).The ternary solid dispersion of biosurfactant with the increase in concentration from 2mg to 6mg resulted in 2.2 to 4 fold improvement in dissolution rate, in accordance with the binary system, but poloaxmer 407 with higher concentration, showed maximum 2 fold improvement in the dissolution rate in accordance with binary. The mechanism

of enhanced solubility of drugs with biosurfactant could be the maximum penetration and wettability of biosurfactants into the very poor soluble drug. In binary solid dispersion even though the drug was dispersed in, molecular level or nano-level solubility was enhanced by two-fold only. Biosurfactant concentration was used (S3) 4mg and 6mg(S4) concentrations but the obtained dissolution rate was not significant, so in further study 4mg concentration, solid dispersion was used.

4.2.3. Formulation and evaluation of Atovaquone tablet

A total of six formulations of Atovaquone tablets were prepared. Three batches from by physical mixture containing with and without surfactant (F1, F2, F3) and from solid dispersion: binary and ternary (F4.F5, F6). The main purpose of these two sets of the technique was to evaluate significant differences in dissolution rate between them. For industrial easy scale-up and economical purpose physical mixture technique is preferred. The results obtained from all the six formulations with respect to pre-compression parameters were within the acceptable ranges. In post-compression parameters, results were acceptable for the friability and hardness of tables were having good mechanical strength, which can withstand the mechanical shocks at the time of packing and transport. Disintegration time for all tablets of the drug was in the rage of 2.84 minute to 7.50 minutes but within the pharmacopeia range. Formulation batches containing surfactants in physical mixture resulted in a slow disintegration process as compared to the binary and ternary solid dispersed products. The effect of biosurfactant in the physical mixture and ternary system was found to be more significant in comparison with poloaxmer 407. *In vitro* dissolution media for Atovaquone tablet prescribed in USP is a mixture of isopropyl alcohol 40% with potassium hydrogen phosphate pH 8. In this study, 0.1HCl pH 1.2 was used to

mimic the *in vivo* biological fluid to evaluate its efficacy. The formulated batches from the physical mixture of the drug showed a dissolution rate in the rage from5.4% to 9.0 %.But the binary and ternary system of drug formulation batches were in the range from 10.95% to 37.65%. The maximum dissolution rate was observed in the formulation F5 in comparison with other formulated batches. The improvement in dissolution rate at 45 minutes was calculated in accordance with Khan and Rhode procedure, Physical mixture batches of drug-containing biosurfactant and polaxamer a407 showed 1.44 and 1.23 fold increase in the dissolution rate respectively. Binary system batch DE_{45} showed 1.94 fold. Ternary system the DE_{45} was with biosurfactant 6.6 folds and poloxamer 407 3.51 folds. The attributing factors for enhancement of dissolution efficiency of F5 could be molecular dispersion of drug within the hydrophilic polymer matrix along with biosurfactant coating or entrapment of drug within the biosurfactant. F5 Atovaquone tablet was selected as the best formulation for further study. Primary pharmacokinetic parameters for bioavailability study are AUC, Cmax and tmax. AUC represents the total amount of Atovaquone absorbed from the dose administered. An increase in AUC and Cmax, of F5, formulated batch in accordance with the oral drug suspension, 2.71fold and 3 fold respectively. Tmax of the F5 was shortened by one hr. (fig41) Attributing factors could be the enhanced dissolution of the drug in the biological fluid and loosening the tight junctions of the internal lining of the intestine by the biosurfactant. Modified short term stability study with reference to ICH Q1c. Formulated F5 batch was more stable at room temperature with RH as compared to the accelerated temperature with RH due to the differences found in the results of drug content; hardness and percentage of drug release on 3rd month were 2.7%, 19%, and 2.69% respectively.

PHASE III:LORNOXICAM GEL FORMULATION WITH BIOSURFACTANT

4.3.1. Preformulation of Lornoxicam

Preformulation of the drug is an essential component of robust and stable formulation development of dosage form. The melting point of Lornoxicam was 228.5^{o}C. Indicated the purity of the sample received. The maximum UV absorbance was found at 376nm in (phosphate) buffer pH alkali (6.7). The obtained standard calibration curve was followed by the Beer-Lambert law and the regression coefficient R^2 nearer to 1(0.999). The solubility of the drug in 2.5% biosurfactant solution was found significant in comparison with the tween 80 solutions. Attributing factors for the increase in solubility could be wettability and reduction in surface tension of the bulk phase by the biosurfactant. In Solutol HS 15 solubility of the drug was found maximum (56mg/ml) and used as co-solvent in the further formulation. FTIR graphs for a pure and physical mixture of the drug were found to compatible because essential functional groups of API and in the physical mixture were C=N stretch, C-S stretch SO_{2-} N stretch and C-Cl stretch wave numbers matching respectively. The exothermic peak of pure Lornoxicam was changed into an endothermic peak in the physical mixture at 230.2^{o}C and 256.6^{o}C respectively. Similarly for biosurfactant in pure and in the physical mixture was 109^{o}C and 93.7^{o}C respectively. There was an alteration in the melting point of drug and biosurfactant from the pure form and in the physical mixture could be moisture absorption of the drug from the biosurfactant, thus resulted in a reduction in the melting point of biosurfactant and increase in the drug.

4.3.2. Formulation and evaluation of Lornoxicam gel

Lornoxicam gel formulations were prepared by sodium alginate as a gelling agent, calcium gluconate activator for gelling, methyl and propyl parabens as preservatives, solutol HS 15 as co-solvent. Drug, biosurfactant and tween 80 were primary ingredients.Solutol HS15 concentration was more in the drug gel of tween80 since the solubility of the drug in tween 80 was low. All four batches of Lornoxicam gels were evaluated for physic-chemical properties. the pH of all gel batches was in the rage of 5.51 to 6.61, which was acceptable for the topical application because skin pH is in the range of 4.5 to 5.6. Content of drug of all the formulation was in the limits of 97.64% to 98.74%, indicated the equal distribution of the drug in a gel base. Rheological properties of formulated gels are essential in the industrial applications, includes manufacturing, filing and storage. Major parameters of the rheological study were yield stress and viscosity. Yield value is defined as the lowest shearing stress required producing flow, below this point gels act as solid. Generally, yield value for semisolid to be high because the flow will retard when the product is stored at 90^0 degrees or inverted position. The yield value is independent of viscosity. All batches of drug gels showed plastic flow. The yield values of the gels were in the range of 103 to 220 Pa, this due to the concentrations of sodium alginate and solutol HS15. The high yield value was observed in G1, which consists of optimum concentration of gelling agent and H bonding between the polymer. The viscosity of all the drug gels was in the range of 3100 to 3656Pa.s at 50 rpm. Lower viscosity was observed in the G2 batch in comparison with the G1 batch due to the Solutol HS15 concentration. Similarly, the viscosity of the G4 batch was low in comparison with the G3 batch. From the literature survey, the optimum viscosity for stable semisolid dosage form

was in the rage 3000 to 6000 Pa.s at 25 to 50 rpm. The spreadability and extrudability of all formulated gel's results were correlating with viscosity results. Viscosity is inversionally proportional to the spreadability and extrudability of the gel. Low viscosity gels resulted in high spreadability and extrudability and vise versa. G1, G3 formulation batches were low viscosity and G2 and G4 were high viscosity gels. Gel strength was measured with penetration time for specific depth in a gel matrix, it is dependent on the gel; consistency, polymer chemical rigidity and fluidity. Gel strength was measured in seconds and more in the high viscosity formulated drug gels batches compared to the low viscosity batches. From the *in-vitro* permeation study, the cumulative percentage of drug release all formulated gels were in the range of from 67.23% to 82.63% for 6hrs. Egg membrane was used as semi-permeable to mimic partial with *in vivo* system. G1 and G4 batches showed the highest and lowest drug release respectively. Retarded rate release of the drug could be the viscosity of the gels and also proved the theory of molecular diffusion of gels from Max A Lauffer. Ratskin study results; the percentage of drug diffusion for all formulation batches varied from 43.33% to 68.33%. The variation of drug release could be the effect of biosurfactant, which act as good permeation enhancers as compared to the nonionic surfactant. The biosurfactant containing formulations (G1 and G3) showed an increase in the transdermal flux as compared to the nonionic surfactant (G3 and G4) a possible explanation for this finding could be due to the surface-active agents can increase the rat membrane fluidity increasing the drug diffusion through the rat membrane. The results of % drug diffusion from the egg membrane and rat skin study, it was revealed that the low percentage of drug release from rat membrane could be due to the stratum corneum layer and absent in the egg membrane. The above explanation is also justified from the data presented in the transdermal flux and

permeability coefficient in table 50. The enhancement ratio for G1 formulation was high as compared to the other formulation batches; the possible explanation could be the optimum acceptable physicochemical parameters of G1 with biosurfactant. In vitro and ex vivo percentage, drug release data were fit in the various kinetic model to assess the release phenomenon. It was observed that all batches of gels were obeyed Higuchi kinetics or diffusion model from the correlation coefficient R-value. The G1 formulation was adjudged as the best formulation batch for the next study.

In vivo anti-inflammatory experiment was performed in Wistar rats with three groups. The literature review indicated that Lornoxicam is the best choice of drug for the treatment of arthritis and inflammation of tissues, which is administered orally but topically marketed formulations are not available to date. The best formulation of Lornoxicam gel (G1) was comparatively evaluated for its anti-inflammatory activity with a market formulation containing piroxicam (0.5%) for 6hrs. The anti-inflammatory activity from the paw edema study indicated the percentage of inhibition for the best formulation was 1.5 fold in accordance with the marketed formulation. The enhancement of the percentage of inhibition for best formulation could be overcoming the permeation retardant of the skin layer (stratum corneum) by loosening the corneocytes fibers or increasing the intercellular gap of stratum corneum and fluidity. Acute skin irritation study with positive control and treated control groups were evaluated for the erythema and edema signs with respect to table 15. The best formulation (G1) had shown no evidence of erythema and edema mainly in 2hrs and subsequent up to72hrs. The observed positive control group was had erythema and moderate edema. The total primary skin irritation score was 6 for the positive control group and the treated control group with 0 scores. The best-

formulated drug gel and its compositions were compatible with the skin matrix and its secretions.

ICH guideline specified the duration of stability period for room temperature and accelerated is 6 months and 12 months respectively. A modified stability study in accordance with ICH was three months. During the stability study, in-process quality assessments were performed; content of drug, pH, viscosity and in vitro drug diffusion. At room temperature $25^{o}C \pm 1^{o}C$ and RH 65%± 5%, for three months, drug content, pH, and viscosity parameters were not deviating more than 1% from the initial data. The percentage of drug release was 5% less; this could be due to the drug content deviation. Accelerated stability study data for three months the pH, viscosity, content of drug and in vitro drug diffusion were 0.5%, 3.2%, 4.0% and 9.97 % deviation from the initial values respectively. It was concluded from the stability study that the optimum storage temperature for gel was room temperature

Phase I

Biosynthesis of biosurfactant was carried out using *Flavobacterium sp*. MTCC 2495 procured from the IMTECH Chandigarh after the preliminary characterization of microorganisms. Microorganisms were cultivated in the modified mineral salt media for enhanced propagation, thereby yield. Sucrose and peptone as substrates in modified mineral salt media, bacteria produced the biosurfactant and it was confirmed by the emulsification tests. The total duration required for complete the fermentation was determined by the in-process check of cell growth.

Optimization of substrates concentration was carried out using 3^2factorial design in the laboratory scale in the shake flask method. Three levels of sugar cane molasses and waste fried oil used as independent variables and dependent variables were surface tension and biosurfactant yield. A total of nine runs were conducted with constant environmental conditions, like pH, temperature, aeration and duration. Dependent variables data were analyzed for its significance by ANOVA and p-value. Out nine runs, run 3 was more significant than the other runs. From the overlay plot, design space was obtained for sugar cane molasses 5.2% and waste fried oil 5%. Pilot scale-up was performed in the bioreactor of 2 liters capacity using molasses (5.2%)and waste fried oil(5%) as substrates maintained constant environmental conditions for 72 hrs. The yield of the biosurfactant was monitored by a decrease in the surface tension of broth. The total yield of the biosurfactant was 7.9 gms per liter from the solvent extraction of the broth. Partial characterization of the biosurfactant was confirmed by FTIR, TLC, DSC and UV method. The CMC of biosurfactant was at 39.5 mg per liter. Biosurfactant animal toxicity study was performed on rats in accordance with OECD guidelines. Oral and acute dermal toxicity studies results indicated that biosurfactant was safe up to 2000mg per kg weight of rat.

Phase II

The application of biosurfactant in pharmaceutical dosage form was successfully proved in Atovaquone tablets formulation. Drug tablets were prepared by the incorporation of the ternary solid dispersed product with biosurfactant. Preformulation study of Atovaquone was carried out; melting point, solubility study, DSC, FTIR, indicated that the drug was pure and excipients used were compatible and stable. Developed and validated method of HPLC for the drug in spiked plasma sample was found to be linear, robust. Solid dispersion technique proved to be more significant in the enhancement of drug dissolution in the acidic media, but specified media is isopropyl alcohol 40% in potassium hydrogen phosphate pH 8. Ternary system of biosurfactant with drug-enhanced dissolution rate 3 fold in comparison with synthetic surfactant and pure drug. Atovaquone tablets were formulated by two processing techniques; by physical mixture and incorporation of solid dispersed products. The entire formulated tablets were passed the pharmacopeial standards. The dissolution rate of the ternary system of drug and biosurfactant tablets showed a higher percentage of drug release, 6 fold. Relative bioavailability of best formulation (F5) was found to be 2.7 in accordance with the oral suspension of drugs. Cmax and tmax were 3 fold increased and 1hr less respectively. Stability study data predicted that the best formulation of drug tablets was stable at room temperature.

Phase III

Lornoxicam gel was prepared and evaluated with biosurfactant as solubility and permeability enhancer successfully. Preformulation study of the pure drug; melting point, solubility, maximum UV absorbance, standard calibration curve, FTIR and DSC studies results were in specified limits. Drug and biosurfactant compatibility and stability was confirmed by DSC and FTIR study. Drug gels were formulated with a gelling agent in two concentrations with biosurfactant and tween 80. Physicochemical parameters for drug gels; pH, drug uniformity, rheological study, spreadability, extrudability, and gel strength were evaluated. the pH of gels was compatible with the skin pH. The drug content in the drug gels was uniformly distributed. Yield stress and extrudability and gel strength of all drug gels were in the optimum acceptable ranges. Viscosity was more than 3000Pa.s for all gels with good physical stability without phase separation. *Invitro* and *ex vivo* permeation of drugs were significant for gels containing biosurfactant. The enhancement ratio proved that the incorporation of biosurfactant in gel act as good chemical permeation enhancer. Fit data in kinetic models obeyed the Higuchi model of drug release. Efficacy of biosurfactant was proved by *in vivo* anti-inflammatory study with the best formulation and marketed formulation, resulted in approximately 2 fold in inhibition activity by the best formulation batch of biosurfactant. Formulated drug gel was safe for application on the skin without irritation. Drug gel was stable at room temperature for three months without charge in the physicochemical characteristics.

6. Conclusion

Biosurfactant biosynthesis was carried out with *Flavobacterium sp*. MTCC 2495 in M-M salt media. Bacterial propagation and production of biosurfactant was accessed in the preliminary stage successfully. Economical substrates were selected for production of biosurfactant in the laboratory scale using sugar cane molasses and waste fried oil. The concentrations of substrates were optimized using 3^2 factorial designs, three levels of independent factors and two dependents factors were selected with objective that maximum yield of biosurfactant. Total nine experimental runs were conducted with sugar cane molasses and waste fried oil, dependent parameters as broth surface tension and biosurfactant yield. Statistical tool was applied for the dependent factors for its significance ANOVA, p value less than 0.05. Design space was drawn and selected the optimum substrates concentration for pilot plant scale up using sugar cane molasses and waste fried oil. Pilot plant scale up was performed in the 2 liters bioreactor with substrates under controlled environmental conditions. Biosurfactant was partitioned from the broth by solvent extraction process and partial chacterization was biosurfactant. FTIR, DSC, TLC studies confirmed the low molecular biosurfactant.

Application of biosurfactant as solubility and permeation enhancers for BCS II and IV drugs in two different dosage forms was studied. Atovaquone drug belongs to BCS IV, formulation of its tablets using solid dispersion technique; binary and ternary systems. Dissolution rate of drugs from the binary and ternary systems of solid dispersion proved to be significant increase in accordance with pure drug. Between binary and ternary system of drug, ternary system containing biosurfactant was higher dissolution rate in comparison with synthetic surfactant. Atovaquone tablets formulated using the ternary system of solid dispersion and also with physical mixture

of excepients. Drug tablets containing ternary system of biosurfactant resulted in significant increase dissolution rate and bioavailability by *in vitro* and *in vivo* study respectively. Use of biosurfactant in Atovaquone tablet was proved to be good solubility and bioavailability enhancer, since drug is low soluble and low permeable.

Lornoxicam gels were prepared by incorporation of biosurfactant as solubility and permeability enhancers. Formulated drug gels were studied for effective permeation and solubility, in comparison with synthetic nonionic surfactant, Lornoxicam included in the BCS II. Solubility of drug in biosurfactant was 20% more than that of tween 80 in low concentration. Effective permeability of drug through the skin of the rat also proved that biosurfactant act as chemical permeation enhancer for topical preparation.

From the above two dosage forms studies, biosurfactant was a novel biocompatible and biodegradable microbial surfactant, effective in low concentrations and stable. However its detail study on chemistry, manufacturing, toxicity study is required to be approved as essential microbial surfactant excepient in pharmaceutical dosage form by the regulatory authorities.

LIMITATIONS AND FURTHER SCOPE FOR THE STUDY

In this research study, a preliminary investigation on the biosynthesis of biosurfactant was performed. Biosurfactant applications in different selected dosage forms were also studied. However, it needs a detailed study on medium scale up (10lts) process for optimization of substrates concentrations. Optimum purification processed to be developed to characterize the exact structure of biosurfactant and yield. Detail oral and dermal toxicity studies to perform to acquire regulatory approval.

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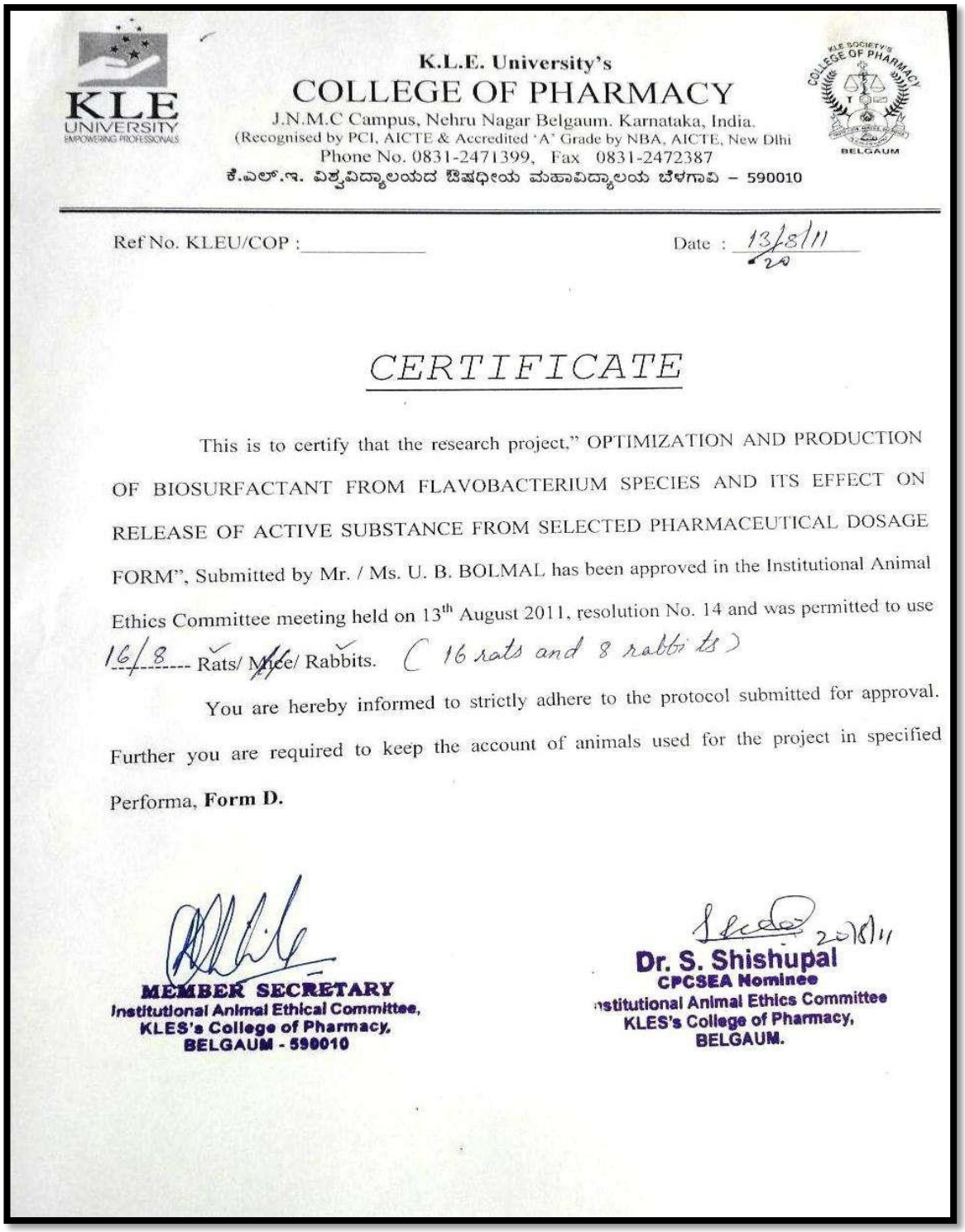
FUTURE SCOPE:

- Application of biosurfactant in different dosage forms containing poorly water soluble drugs to be explored. (Wetting agent, solubilizing agent, etc.)
- Application in specific type cosmetic formulation.(Shampoo, face creams etc.)
- Application of biosurfactant in Nano particle stabilization.



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CERTIFICATE

This is to certify that the research project," OPTIMIZATION AND PRODUCTION OF BIOSURFACTANT FROM FLAVOBACTERIUM SPECIES AND ITS EFFECT ON RELEASE OF ACTIVE SUBSTANCE FROM SELECTED PHARMACEUTICAL DOSAGE FORM", Submitted by Mr. / Ms. U. B. BOLMAL has been approved in the Institutional Animal Ethics Committee meeting held on 13th August 2011, resolution No. 14 and was permitted to use 16/8 Rats/ ~~Mice~~/ Rabbits. (16 rats and 8 rabbits)

You are hereby informed to strictly adhere to the protocol submitted for approval. Further you are required to keep the account of animals used for the project in specified Performa, **Form D.**

MEMBER SECRETARY
Institutional Animal Ethical Committee,
KLES's College of Pharmacy,
BELGAUM - 590010

20/8/11
Dr. S. Shishupal
CPCSEA Nominee
Institutional Animal Ethics Committee
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BELGAUM.

CERTIFICATE OF ATTENDANCE

This is to certify

Dr/Prof/Mr/Mrs UDAYKUMAR BOLMAL

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RESEARCH ARTICLE

Enhanced Solubility and Percutaneous Permeability of Lornoxicam gel using Biosurfactant.

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ABSTRACT:

Objective: Lornoxicam is the anti inflammatory agent under BCS II. Oral use for the chronic condition may cause peptic ulcer. Topical gel formulation is an alternative route of administration to overcome the shortcoming. The present study was aimed at enhancement of solubility and permeation of Lornoxicam using a novel biosurfactant from biotechnology product and compared with a nonionic surfactant (Tween 80).

Methods: Topical gel was prepared by sodium alginate and calcium gluconate as a gelling agent and complexing agents in different concentrations respectively. The drug was solublised with biosurfactant and Tween 80.

Results: Gels were evaluated for its physical-chemical properties. *In vitro* and *ex vivo* permeation studies were carried out through egg membrane and rat skin respectively.G1 formulation showed drug content (98,9%), spreadability (31.36gm/cm^2), viscosity (3853mPa.S) with yield value (220Pa.s). Drug permeation study by two methods resulted in an enhancement ratio was 0.856. *In vivo* anti-inflammatory activity of G1 and marked formulation exhibited 49.9% and 36% percentage of inhibition respectively. The short term stability studies conducted for 3 months revealed that at room temperature (25^{o}c±1^{o}c and RH 40%) optimized formulation was stable without much variation in drug content, viscosity, pH, and *in vitro* drug release rate.

Conclusion: Lornoxicam gel was promising to be used for topical application with natural polymer base preparation .

KEYWORDS: Lornoxicam, Biosurfactant, nonionic surfactant, natural gelling agent, permeability.

1. INTRODUCTION:

The solubility of BCS II drugs is a major challenge to the formulation scientist. Novel solubilization technologies have been developed from past two decades. The majority of the processes are not commercially viable due to the cost, sophisticated equipment, time and manpower. Use of synthetic surfactants of different classes, anionic, cationic, nonionic and amphiphilic in different dosage forms are available in the market.

Nonionic surfactants are the major role in solubilization poor water soluble drug.The average concentration of nonionic surfactant was used in the different formulation is ranging from 2% to 20%. It is reported that synthetic surfactant supper from biodegradability, GIT irritation and skin sensitization [1]

Biosurfactants are a new class of surface active agents' biosynthesized from yeast, bacteria and filamentous fungi using carbon and nitrogen source; such as waste fried oil, molasses, expressed oil nuts and peptone, ammonium chloride respectively. Biosurfactants are classified into Glycolipids, Rhamnolipids, Sophorolipids, Trehalolipids, Lipoproteins and Lipopeptides etc. The advantages of biosurfactant are biodegradability, low toxicity, biocompatible, easy production with economical raw materials, stability in all pH and specificity. Biosurfactant have numerous uses in pharmaceuticals, for solubilization of lipophilic drugs in aqueous media, as components of emulsion or surfactant self-assembly vehicles for oral and transdermal drug delivery agents to improve drug

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absorption and as penetration enhancer, due to its very low critical micelle concentration (CMC). [2] In the present research, an attempt was made to produce biosurfactant from the *flavnobacterium* species using economical substrates and purified [2]. Enhancements of Solubility and permeation properties of biosurfactant were evaluated by selecting model the drug Lornoxicam. Lornoxicam is a potent NSAID drug. It is practically water insoluble drug classified under the BCS II. The oral dose is 8 to 16mg daily divided doses and topically 0.5% w/w act as anti-inflammatory through Cox I & II inhibitor. Common side effect of the drug is GIT irritation hence the topical application of the drug in the gel form is preferred.[3] Solubilization of Lornoxicam is enhanced by biosurfactant and incorporated into gel base using sodium alginate as the gelling agent to achieve the uniform distribution of the drug. Gels were evaluated for its physicochemical properties and *in vitro* permeation study. *Ex vivo* permeation and *in vivo* anti-inflammatory activities were also studied for the effectiveness of biosurfactant as a permeation enhancer.

2 MATERIAL AND METHODS:

Lornoxicam procured from Optimus Drug Pvt Ltd Hyderabad gift sample. Sodium alginate, methyl parbens, Propyl paraben and tween 80 purchased from Hi-media Lab Ltd Mumbai. Solutol HS 15 USP BASF Germany. Calcium gluconate from Forbes Pharmaceutical Pvt Ltd Mumbai. All other chemicals were AR grades.

In- house biosynthesis of biosurfactant:[4,5]
Biosurfactant was produced from the biotechnology processes in the fermentor (Sartorius B Lite) using *Flavobacterium* Sp. 2495 in a modified mineral salt media. Molasses 2%, waste fried oil 5%, and 1% peptone, 0.5% ammonium chloride as carbon and nitrogen source respectively. Environmental conditions, pH at 7.4, temperature 35±1ºC aeration 40% and rpm 450.Duration of fermentation was 90 hrs. The fermented fluid was separated by solvent extraction using methanol-water mixture (1:2). Purification was carried out using 5 N HCl by precipitation method. The product was dried at 40ºC for 5hrs and passed through 60 mesh. Stored in the airtight container at room temperature. Skin irritation test was performed on the albumin rat as per OECD guidelines. It was found nonirritant.

Solubilization of Lornaxicam [6,7]
Excesses of Lornoxicam was added into 0.1%w/v to 2.5%w/v of biosurfactant and 2%w/v to 10%w/v Polysorbate 80 in 20ml conical flask respectively and stirred in the mechanical shaker for 24 hr at 10 rpm. Sonicated for 10 minutes, filtered, the filtrate is diluted with phosphate buffer in the Beer's range, drug content was determined using UV-Visible spectroscopy (Shemazid) at λmax 307 nm. Solubilization was carried in triplicate.

Drug compatibility study:
Drug excipients compatibility studies were performed to identify an interaction between drug and excipients used.IR spectra of drug and excipients mixture were carried out from 4000cm^{-1} to 400cm^{-1}. (Shimadzu FTIR spectra)

Preparation of gel:[8]
Sodium alginate was used as the gelling agent in different concentrations. Polymer was dispersed in distilled water without lumps, kept for 24hrs for hydration. Lornoxicam was dissolved in of Solutol HS15, Biosurfactant and Tween 80 was added with constant stirring using propeller stirrer. Calcium gluconate was dissolved in hot water (50ºC) and added to the gelling mixture and stirred for 30 minutes. Aqueous solutions of preservatives were added to the gel [Table no2]. The final weight of gel was adjusted using phosphate buffer pH 6.8 stirred for 20 minutes and stored in airtight container at room temperature until further analysis.

Evaluation of gels:

pH:
pH of all formulated gels was determined using calibrated pH meter (Systroinic MK 6) All the reading were recorded in triplicate for three days and average reading was calculated.

Drug content:
Gels (1gm) were dissolved in the mixture of phosphate buffer pH 6.8 and methanol (1:1), stirred for 10min. one ml sample was diluted to 10ml using the same solution and filter through What man filter paper(No 40). Absorbance was measured at λmax 307 nm using UV-Visible spectroscopy (Shemazdu)

Rheological and apparent viscosity determination: [9]
The rheological determination was carried out using Brookfield Capcalc V3.0Build20-0.The system was equipped with cone and plate with plate diameter 40mm. About 0.5 gms of the sample was placed in the plate, allowed to set for constant temperature (25.0±0.1^{0}C) for 5 minutes. Shear stress was determined by the gradual increase in rpm and recorded. Rheogram was plotted by the shear rate on the y axis and shear stress on the x axis. Yield stress was calculated by fitting the in to the mathematical model Bingham.

Spreadability: [10]
The spreadability was performed on two glass plates (12cmx12cm).500mg of the gel was placed on one slide over it another slide was placed. 1grm of weight was placed on an upper slide for 5 min. weight was removed and time taken for separation of the slide was noted.

Following formula was applied for calculation of spreadability.

S = ML/T
S = spread ability gm.cm/sec.
M = Weight tied on the upper slide.
L = Length of glass slide, cm.
T= Time taken to separate the two slides in sec

Extrudability:[11]
Formulated gel was filled in an aluminum tube (10gms) and the bottom is sealed. The Extrudability of the formulations were determined in terms of weights in grams required to extrude a 0.5 cm. ribbon of gel in 10 seconds. The measurement was done in triplicate.

$$\text{Extrudability} = \frac{\text{Applied weight to extrude emugel from tube (g)}}{\text{Area (in cm}^2\text{)}}$$

Gel strength: [12]
It was measured by the method reported by Jaiswal et al. An in-house modified apparatus was fabricated (Fig no2). The 50gms formulation was placed in 100ml graduated cylinder at ambient temperature. 35 grams weight was placed on the T-bar, allowed to penetrate up to 5cm. The time required to penetration was noted and reported as gel strength.

Fig: 2 In-house fabrication for gel strength measurement.

***In vitro* diffusion study: [13]**
The experiment was conducted in Franz diffusion cell of 40ml capacity. Pretreated egg membrane was fixed in between the donor and receptor cells. The receiver contains phosphate buffer pH 6.8 and methanol [1:1] solution. Lornoxicam gel (1gm) was applied on the egg membrane and donor compartment was clamped. Water circulation was maintained at 37±1ºC with 20 rpm on the magnetic stirrer.1ml sample was withdrawn with the syringe and replace with fresh PBS solution to maintain the sink condition. Samples were analyzed by UV spectroscopy at λmax 307 nm. The study was conducted for 6 hrs in triplicate.

***Ex-vivo* permeation study:[10]**
The *ex vivo* drug permeation study was carried out in Franz diffusion cell using wistar rat abdomen skin (IAEC, CPCSEA NO: 221) Clean, shaven, washed with saline abdomen rat skin was clamped between the donor and receiver compartment. Receiver compartment contains PBS buffer pH 6.8 and methanol (1:1) as dissolution medium. Lornoxicam 1 gram gel was spread on the upper skin layer. The temperature of the dissolution medium was maintained at 37±1ºC with 20 rpm on the magnetic stirrer.1ml sample was withdrawn with the syringe and replace with fresh PBS solution to maintain the sink condition. Samples were analyzed by UV spectroscopy at λmax 307 nm. The study was conducted for 6 hrs in triplicate.

Permeation Data Analysis:
The cumulative % drug release was plotted against time for all the formulations. Permeation rate (drug flux) at steady state (Jss)was determined by dividing the slope of the linear portion of the graph by the area of the diffusion cell. The permeation coefficient (Kp) was calculated by dividing Jss by the initial concentration of drug in the donor cell (Co)

$$Kp = {}^{Jss}/_{co}$$

$$\text{Enhancement ratio was determined by} = \frac{\textbf{Jss of rat skin}}{\textbf{Jss of egg membrane}}$$

Drug release kinetics:
To understand the drug release kinetics of Lornoxicam gel, drug release data were fitted in different models of release kinetic using PCP Disso V3 software (Pune. India). To know which mathematical model best fits the obtained release profile

Skin irritation test:[10]
Skin irritation test was performed in six male Wistar rats. The animals were divided into two groups standard and test respectively containing three each. The dorsal side of the skin was shaven ($6cm^2$) one day before the commencement of the experiment. The standard group was applied with 0.8% formalin. The test group was applied with optimized gel. All group animals were observed for erythrema, swelling or reddening at the end of 24 hrs. The score was given accordingly and analyzed for as per Draize table.

***In vivo* anti-inflammatory study:[14]**
The experiment was conducted on male albino rats (150 to 200gms 10 weeks) by carrageenan induced paw edema model. Nine rats were taken, divided into three groups. Group, I control receiving 1% carrageenan in saline. Group II received standard marketed formulation (Piroxy gel). Group III optimized formulation with 1% carrageenan saline. Carrageenan saline injection of 0.1 ml was given to the plantar site of the right-hand paw to all groups, but group II and group III received the injection after 30 minutes of application of marked gel and optimized gel respectively. The volume of paw

edema was measured using plethysmography immediately after injection and taken as zero reading. The volume was measured at different intervals up to 6 hrs and % inhibition of paw edema in the drug-treated group was compared with carrageen control group and calculated according to the formula.

$$\% \text{ Inhibition edema} = \frac{(C-T)}{C} \times 100$$

C= mean volume of edema for control
T= mean volume of edema for test.

Stability study:
Stability studies were conducted at 25ºC ±1ºC and65% RH 40%and 40ºC ±1oc at RH 75% in stability chamber (Lab control). Duration of stability study was for 3 months. Lornoxicam gel was filled in 10gms aluminum tubes with cap stored in the stability chamber. Every month gels were analyzed for the pH, Viscosity, drug content, and *in vitro* release.

Statistical analysis:
Data were expressed as mean ±SD. Differences were considered statistically significant at $p < 0.05$. Statistical analyses were performed using GraphPad Prism 4.01 software.

3 RESULTS AND DISCUSSION:

Solubility analysis:
Solubility studies of Lornoxicam with 0.1% to 2.5%w/v biosurfactant were in the range of 0.68mg/ml to 14.68mg/ml and with 2% to 10%w/v Polysorbate 80 were in the range of 0.44mg/ml to 2.25mg/ml. There were 7 fold increases in the solubility with the difference of 5% concentration of polysorbate 80. This could be due to the amphiphilic nature of the biosurfactant.SolutolHS15 solubility was 56mg/ml which was added to compensate the fraction of undissolved Lornoxicam. **(**Table no 1)

Table 1: Solubility of Lornoxicam

Biosurfactant %w/v	Concentration of drug dissolved mg/ml	Polysorbate80 %w/v	Concentration of drug dissolved mg/ml
0.1	0.68±0.01	2	0.44±0.07
1	1.81±0.03	4	0.76±0.01
1.5	6.41±0.03	6	1.45±.02
2	9.76±0.09	8	1.65±0.05
2.5	14.68±0.02	10	2.25±0.08

Mean ± SD. n=3

Drug compatibility study:
Results of IR spectra of pure drug Lornoxicam and with the physical mixture of excipients were interpreted for prominent functional groups of Lornoxicam structure. It was observed that presence of absorption peak at 3068 cm^{-1} due to the presence of C=C stretching, 1592 cm^{-1} for primary and secondary amines and amides, 831.7 cm^{-1} for aromatic group, 1041.3 cm^{-1} for S=O stretching. It concludes that drug is present in free form than the reacting form.(Fig no1)

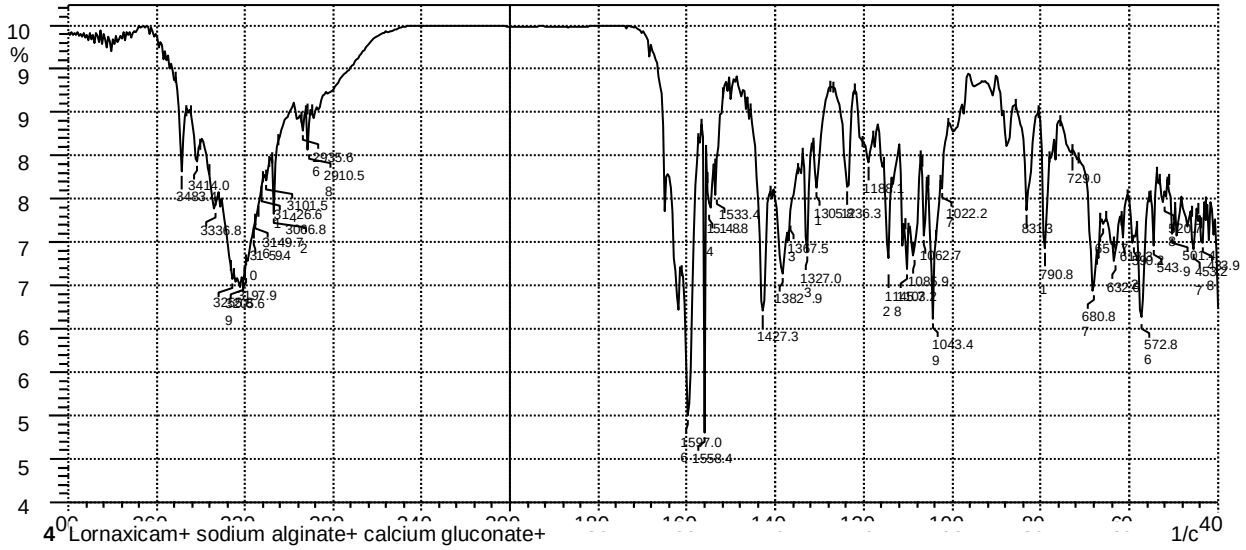


Fig :1 FTIR graph of Lornoxicam an + sodium alginate + Calcium gluconate + Biosurfactant.

Preparation of gel:
The concentration of Lornoxicam was 0.5%w/v in the gel. Solutol HS 15 was added in different proportions to solubilises the fraction of undissolved drug in the gel. Calcium gluconate is used as the complexing agent for sodium alginate and moisturizing agent. Methyl and Propyl Parabens were preservatives. All the formulations are shown in the table no 2.

Evaluation of gels:

Physical examination and pH:

The prepared Lornoxicam gels were viscous, yellow colour preparation with smooth and homogeneous appearance.

All the prepared formulations pHs were in the range of 6.62 to 6.72.Compatible with skin components. [Table no 3]

Table 2: Formulations of gels.

Sno	Ingredients	Unit	G1	G2	G3	G4
1	Lornoxicam	gm	0.5	0.5	0.5	0.5
2	Sodium alginate	gm	5	5	6	6
3	Calcium gluconate	gm	0.05	0.05	0.08	0.08
4	2.5 % w/v Biosurfactant	ml	10	---	10	---
5	10% w/v Polysorbate 80	ml	---	40	---	40
6	Solutol HS 15	ml	5	10	5	10

Drug content:

Formulations G1 to G4 drug contents were to be in the range of 97.89% to 98.50 % indicative of uniform distribution and within the compendial limits. [Table 3]

Spreadability:

Topical gel formulations of lornoxicam were studied for its Spreadability property in order to determine the ease of application of gel onto the skin surface

The values of spreadability of all the formulations were in the range of 17.1 to 23.02g cm/sec High value indicate the spreadability is more with less shearing.[Table 3]

Extrudability:

The Extrudability of the prepared gel formulations were found to be between 14.43gm to 17.51gm. For formulations containing the high concentration of Solutol HS 15, the extrudability was found to be more. [Table 3]

Gel strength:

Gel strength of all the prepared formulations was in the ranges of 65.66sec to 88.33 Sec. Results indicate that all gels are having good integrity and homogeneity. [Table 3]

Table . 3: Physicochemical evaluations.

Formulation code	pH	% Drug content	Spreadability (gm./cm^2)	Extrudability g.cm	Gel strength Sec
G1	6.71± 04	98.34± 2.31	21.91± 0.25	16.15± 0.27	73± 3.46
G2	6.62± 05	97.84± 2.45	26.02± 0.12	17.51± 0.34	65.66± 3.51
G3	6.71± 03	98.13± 1.54	17.10±0. 21	14.43± 0.26	86±. 3.21
G4	6.60± 02	98.50± 1.89	22.03± 0.31	15.21± 0.39	88.33± 3.05

Mean ± SD. n=3

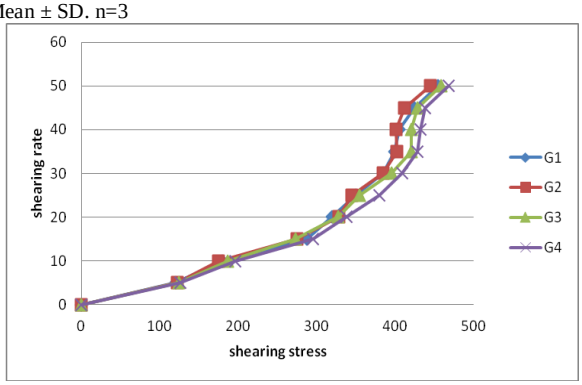
Rheological and apparent viscosity determination:

The rheological characteristics of prepared gels were essential in the technical application including manufacturing, material pumping, filling, and storage. Yield value is defined as minimum shear stress required to flow and below this point, the material will behave as solid. The pharmaceutical and cosmetic preparations should have high yield value so that they do not flow out of the container when placed up down position or in transport. The apparent viscosity and the yield values of G1 to G4 formulations are shown in the table no 4 and fig no 3. G1 formulation showed the highest yield value with optimum apparent viscosity as compared to the G2, G3, G4 formulations. The attributing factors might be the Solutol HS 15. G2 and G4formulation contain 10% of Solutol HS15 as compared to the G1 and G3 (5%). The higher value of viscosity showed in G3 and G4 as compared to the G1 and G2 due to the increase in the concentration of gelling polymer

Table: 4 Viscosity and Yield value

Formulations	Apparent Viscosity mPa.s	Yield stress Pa
G1	3563 ± 43.89	220 ±20
G2	3100 ±46.22	205 ±18
G3	3895 ±45.71	103 ±12
G4	3656±67.35	125 ±10

Mean ± SD. n=3



Fig; 3 Rheogramm of gels

***In vitro* and *Ex-vivo* diffusion study:**

In vitro release of drug from the semisolid dosage form gives an idea of a number of free drug molecules available for partitioning into stratum corneum. A study conducted by two methods, results revealed that higher percentage of drug release through the egg membrane was found as that of abdomen rat skin, it could be due to the absence of stratum corneum layer in egg membrane. Permeation of drug molecules through the animal skin model of all gels was dependent on apparent viscosity, permeation enhancers and concentration of gelling polymers. All these three parameters have been studied results are tabulated in table no5.There was an increase in the percentage of drug release for low viscous gels

(G1 and G2) as compared to the high viscous gels (G3 and G4). Lauffer's molecular diffusion theory states that diffusion coefficient of solute is inversely proportional to the volume occupied by the gel-forming agent. This statement is true with G3 and G4 formulations results. Ex Vivo study results revealed that biosurfactant act as good permeation enhancers as compared to the nonionic surfactant. The biosurfactant containing formulations (G1 and G3) showed the increase in the transdermal flux as compared to the nonionic surfactant (G3 and G4) A possible explanation for this finding could be due to the surface active agents can increase the membrane fluidity increasing the drug diffusion through the membrane. All formulated gels were fit into kinetic modeling and followed the diffusion model.

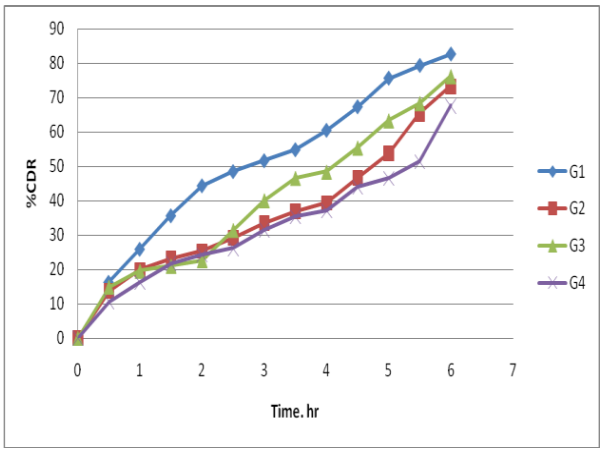


Fig 4: In vitro egg membrane permeation studies.

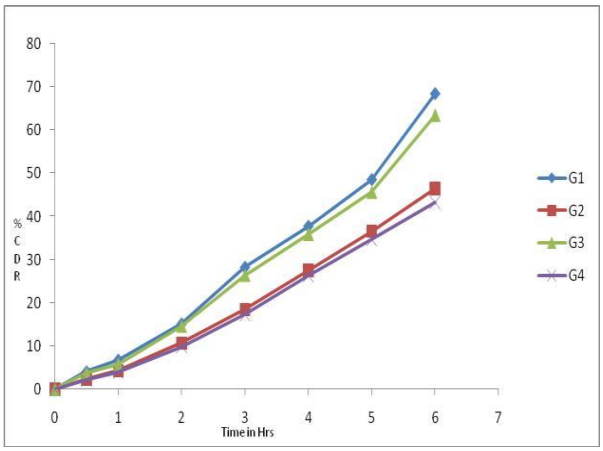


Fig:5 *Ex-vivo* permeation study.

Skin irritation test:

The test group showed no irritation but standard group moderate erythema and mild edema were observed. The score given for test groups zero and the standard group was 2.6.From the results obtained from the skin irritation test optimized formulation G1 containing biosurfactant was nonirritant.

***In vivo* anti-inflammatory study:**

Test and standard formulations exhibited the anti-inflammatory effect for 2.5 hrs and percentage inhibition of standard and test groups were 34.6% and 49.48% respectively. It concludes that the optimized formulation G1 has the better therapeutic effect of decreasing paw edema volume of the Wistar rat as compared to standard formulation. This could be due the effectiveness of biosurfactant as permeation enhancer.

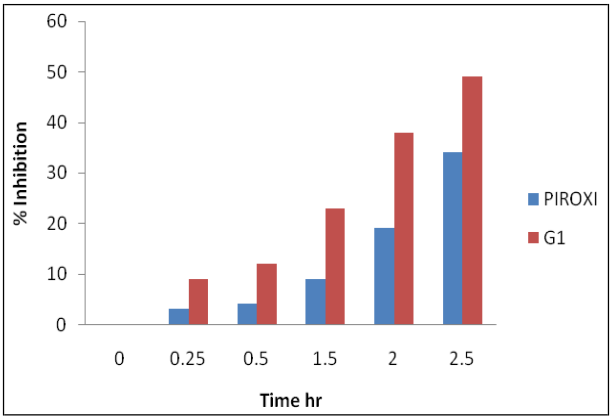


Fig: 6 In vivo anti-inflammatory paw edema studies

Stability study:

Formulation G1 showed no changes in colour and appearance after three months of storage at room temperature and elevated the temperature. The pH, apparent viscosity, drug content and percentage of drug release showed no much variation in all the mentioned parameter at room temperature. But at elevated temperature, there was an average 10 percentage deviation in all parameters from the initial values. The results are tabulated in the table no 6 and 7 indicative of G1 formulation is stable at room temperature for three months

Table 5: Comparative permeation studies data

Formulation	Flux Egg membrane Jss µm/cm^2.h	Permeability coefficient Egg membrane Kp.10^{-6}	Flux *Ex-vivo* rat membrane Jss µm/cm^2.h	Permeability coefficient rat membrane Kp.10^{-6}	Enhancement ratio
G1	4.49±2.34	8.98±1.86	3.82±1.41	7.64±1.25	0.85
G2	3.61±0.98	7.22±1.25	2.73±2.11	5.46±1.65	0.75
G3	4.08±1.45	8.16±0.75	3.23±1.65	7.05±2.84	0.79
G4	3.39±0.85	6.78±0.89	2.41±2.74	4.80±2.22	0.72

Mean ± SD n=3

Table:6 For optimized formulation G1 at 25ºc ±1ºc and RH 40%

Duration	pH	Viscosity Pa.S	Drug content %	*In vitro* release studies (%CDR)
0 month	6.7±02	3565 ±45.71	99.5±2.31	82.23± 2.15
1st month	6.6±0.3	3465±38.61	98.6±2.54	79.64±2.38
2nd month	6.5±0.3	3478±41.2	98.5±2.98	79.52±2.48
3rd month	6.5±0.2	3465±38.3	98.9±3.01	75.01±2.57

Mean ± SD n=3

Table: 7 for optimized formulation G1 at 40ºc ±1oc at RH 65%

Duration	pH	Viscosity Pa.S	Drug content %	*In vitro* release studies %CDR
0 month	6.7±02	3565±45.71	99.5±2.31	82.23± 3.15
1st month	6.6±0.3	3265±38.61	98.6±2.54	76.64±1.08
2nd month	6.3±0.3	3228±41.2	97.5±2.98	75.52±2.98
3rd month	6.3±0.2	3215±38.3	96.9±3.01	74.01±2.12

Mean ± SD n=3

4 CONCLUSION:

Solubility studies of lornoxicam using the in-house biosynthesized biosurfactant showed maximum solubility with the lowest concentration (2.5%). There were no interactions between the ingredients selected for the formulation of Lornoxicam hydrogel by FTIR. Good consistency, uniform drug distribution, easily applicable with minimum shear and compatible skin pH were achieved for the optimized formulation G1.In vitro and *ex vivo* permeation studies proved that the incorporation of biosurfactant in the Lornoxicam hydrogel act as solublising agent and permeation enhancer with enhancement ratio 0.85 for the G1 formulation. Carrageenan-induced paw edema test revealed the anti-inflammatory activity of G1 formulation was 49%inhibition as compared with 34% inhibition of marketed formulation. The ideal temperature for storage of G1 formulation was at room temperature as compared to the elevated (40/^{O}C±1/65 RH). It can be concluded that biosurfactant is the good solublising agent as well as the permeation enhancer for the topical drug delivery containing the water in- soluble drugs. However, further preclinical and clinical studies are required.

5. ACKNOWLEDGMENTS:

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6. LIST OF ABBREVIATIONS AND SYMBOLS:

Symbols	
w	Weight
v	Volume
λmax	Lambda max
nm	Nanometer
ml	Milliliter
gr	Gram
mg	Milligram
%	percentage
pH	Power of hydrogen

Abbreviations	
BCS	Biopharmaceutical classification system
FTIR	Fourier tramission infrared spectroscopy
Pa S	Pascal second
GIT	Gastro intestinal trac
CMC	Critical micelle concentration
COX	Cyclooxygenase enzyme
OECD	Organisation for Economic Co-operation and Development
Jss	Transdermal flux
SD	Standard deviation
RH	Relative humidity
Max	Maximum

7. CONFLICT OF INTEREST:

There is no conflict of interest.

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Original Article

FORMULATION OF ATOVAQUONE TABLET USING BIOSURFACTANT IN A TERNARY SOLID DISPERSION SYSTEM: *IN VITRO* AND *IN VIVO* EVALUATION

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ABSTRACT

Objective: The goal of the present investigation was to improve the solubility and bioavailability of atovaquone tablet, using in-house biosynthesized biosurfactant in the ternary system of solid dispersion containing hydrophilic polymers with varying concentrations of biosurfactant. Atovaquone is an anti-malarial agent and belongs to biopharmaceutical classification system class IV.

Methods: The solid dispersion of binary and ternary mixture was prepared using hydroxyl propyl methyl cellulose (HPMC) and biosurfactant respectively by a solvent evaporation method. All the atovaquone tablet formulations were prepared by incorporation of physical mixture, binary and ternary solid dispersed products with excipients by direct compression method. Pre-compression and post-compression parameters of atovaquone tablets were evaluated. *In vivo* bioavailability study was performed using female albino rabbits.

Results: *In vitro* dissolution profile of binary and ternary system of solid dispersion products showed 8.65% and 34.64% respectively. Precompression and post-compression values of all atovaquone tablets formulations were within the specified limits. *In vitro* dissolution efficiency of F2 and F5 were 1.44 fold and 6.62 fold respectively, in accordance to the F1. *In vivo* study revealed that bioavailability of optimized formulation F5 was increased by 2.5 times and time to reach peak concentration was reduced to 1.4 h, in accordance to pure atovaquone suspension.

Conclusion: Potential application of biosurfactant in the solid dosage form of atovaquone tablet was proved for enhanced dissolution rate and bioavailability of atovaquone for malaria treatment.

Keywords: Atovaquone, Solid dispersion, Physical mixture, Ternary system, Biosurfactant, Bioavailability

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INTRODUCTION

Atovaquone is unique naphthoquinone with a broad-spectrum antiprotozoal agent, belongs to the biopharmaceutical classification system (BCS) class IV and exhibits low and variable oral bioavailability (46%) due to its poor aqueous solubility (less than 0.25µg/ml) [1]. Enhancement of dissolution and thereby its bioavailability is a primary requisite of an oral drug delivery of atovaquone. Numerous technologies have been developed for enhanced solubility of poorly water-soluble drug such as micronization, salt-formation, solid dispersion, complexation etc. Solid dispersion using a water-soluble carrier system is the most commercially viable technology applicable in recent years. The ternary system of solid dispersion is the addition of surfactants in a binary system and also plays essential processes of modification for maximum solubility of the drug in the aqueous fluid [2].

Nonionic surfactants were used for enhancement of solubility of poorly water-soluble drug in the range of 5%w/w to 20%w/w. Synthetic surfactants are non-biodegradable. In recent years novel biotechnological products are produced from various micro-organisms through fermentation. Biosurfactants are produced from specific microorganisms with economic substrates such as waste fried oils, molasses etc, in a suitable media and environmental conditions. Major advantages of biosurfactants over the synthetic surfactants are, at low concentration maximum solubility of poorly soluble drugs, stable in all pH range, very less toxicity and biodegradability. This is due to its unique chemical composition with fatty acid, monosaccharide and amino acids [3, 4].

From the literature survey, it was revealed that the potential application of biosurfactant has not been explored as a solubility modulator and permeability catalyst for in-soluble and less permeable drugs. In this study, atovaquone drug was selected as model drug for its enhancement of solubility and permeability. The aim of our present study was to formulate atovaquone tablet of the physical mixture, binary and ternary solid dispersion system using water-soluble carrier hydroxyl propyl methyl cellulose (HPMC) with biosurfactant and compare its dissolution rate and bioavailability with poloaxmer 407 a nonionic surfactant.

MATERIALS AND METHODS

Materials

Gift sample of atovaquone was procured from Matrix Lab Ltd Hyderabad. Poloaxmer 407 from venous ethoxy ether Pvt Ltd, Goa. Hydroxypropyl methyl cellulose (HPMC) from Colorcon industry, Goa. Lactose, polyvinyl pyrrolidone (PVP), magnesium stearate and talc from Balji chemicals, Mumbai. All other chemicals were from SD fine chem India (AR grade).

Methods

In-house biosynthesis of biosurfactant

Biosurfactant was produced from the biotechnology processes using Flavobacterium Sp 2495(Chandigarh) in a modified mineral salt media. Molasses 5%w/v, waste fried oil 5%w/v and 1%w/v peptone, 0.5%w/v ammonium chloride were used as carbon and nitrogen source respectively. Trace elements were ferric chloride and vitamin B6. Fermentation conditions were maintained at pH at 7.4, temperature 32±1 °C, aeration 40% and rpm 150. Duration of fermentation was 72 h (sorotus lite fermentor). The fermented fluid was separated by solvent extraction using a methanol-water mixture (1:2). Purification was carried out using 5N hydrochloric acid (HCl) by the precipitation method. The product was dried at 40 °C for 3 h and passed through 60 mesh and stored in an airtight container at room temperature. An oral toxicity study was performed on albumin rats as per organization for economic co-operation and development (OECD) guidelines. It was found that 2000 mg per kg body weight of albumin rats was safe without mortality and toxicity. The yield of biosurfactant was 2.5g per liter [5].

Preformulation studies

Compatibility studies

Compatibility study of drug and excipients was carried out through fourier transmission infrared spectrophotometer (FTIR) (Shimadzu Japan) using potassium bromide (KBr) probe technique, scanned from 4000-400 cm-1. Graphs were recorded and analyzed for comparatively for compatibility studies.

The solid dispersion of atovaquone with HPMC and bio surfactant

Solvent evaporation technique was adopted for the preparation of the solid dispersion product. Atovaquone was dissolved in ethanol. Hydroxyl propyl methyl cellulose was dissolved in water in the ratio 1:2 (drug: polymer) using a magnetic stirrer. The drug solution was added to the aqueous hydroxyl propyl methyl cellulose solution with constant stirring. The ternary system was prepared by adding various concentrations of surfactants to the drug-polymer solution as shown in the table 1. The mixture was evaporated on 8 cm diameter china dish for 48 h at room temperature. The solid mass obtained was dried at 40 °C in the oven for 4 h, pulverized, sieved through 60 mesh and stored in an airtight container until use [6, 7].

Table 1: Solid dispersion of atovaquone with hpmc and surfactants

Ingredients	Formulation code							
	Unit	S0	S1	S2	S3	S4	S5	S6	S7
Atovaquone	mg	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5
HPMC	mg	----	125	125	125	125	125	125	125
Biosurfactant	mg	----	--	2	4	6	-	-	---
Poloxamer407	mg	----	--	--	-	-	10	20	30
Ethanol	ml	----	30	30	30	30	30	30	30

Preparation of atovaquone tablet using the solid dispersed product

Compressed tablets containing physical mixture and solid dispersed product with and without surfactants were prepared separately by direct compression method. Diluent lactose, dry binder polyvinyl pyrrolidone, and lubricants talc, magnesium stearate were used as excipients. All ingredients were sieved through 40 mesh and blended with lubricants and compressed in 10 stations rotary machine (M/s Karnavati) at 6 kg/cm2 hardness using 9 mm flat punches. All the formulation batches were shown in the table 2.

Table 2: Formulation of atovaquone tablets

Ingredients (mg)	Formulation code					
	F1	F2	F3	F4	F5	F6
Atovaquone	62.5	62.5	62.5	--	--	--
Solid dispersion system	---	---	---	S1	S3	S7
Biosurfactant	--	4	--	--	--	--
Poloxamer407	---	--	30	--	--	--
Lactose	159.5	155.5	129.5	34.5	30.5	4.5
PVP	5	5	5	5	5	5
Mg stearate	1	1	1	1	1	1
Talc	2	2	2	2	2	2
Total weight	230	230	230	230	230	230

S1, S3, S7. Solid dispersed products

Evaluation of atovaquone solid dispersed products

***In vitro* dissolution**

All solid dispersion products and pure drug were subjected for dissolution profile using USP dissolution apparatus (Electro lab India TD 80) at 50 rpm in 1.2 pH buffer media for 1 h. Sample equivalent to 62.5 mg of atovaquone was placed in the tea bag and tied to the paddle. Aliquot samples were withdrawn from 900 ml at specified intervals and analyzed using uv-vis spectrophotometer (UV 1900 Shimadzu Japan) at λmax 220 nm under sink condition [8].

Evaluation of atovaquone tablets

Pre-compression

All the formulation of atovaquone lubricated powder blends was subjected for micromeritics properties using conventional methods. Bulk density and tapped density was determined by using measuring cylinder method. Hausner's ratios, Carr's index, were determined using the bulk density and tapped density data. An angle of repose was determined by conventional funnel method [9].

Post-compression

All batches of atovaquone tablets were subjected to non-pharmacopieal and pharmacopieal tests. Tablet thickness, hardness, was determined using a vernier caliper and Monsanto hardness tester respectively. Friability, disintegration test was performed as per Indian pharmacopeia (IP 2014) [10].

***In vitro* dissolution rate study**

The dissolution rate of atovaquone tablets prepared was studied in 900 ml of acidic buffer of pH 1.2 using USP dissolution apparatus type I at 50 rpm and the 37±1 °C temperature was maintained throughout the study. Aliquot samples of dissolution media (5 ml) were withdrawn through a filter (0.45µ) syringe at different intervals of time up 60 min. All the samples were suitably diluted and assayed at λmax 220 nm and sink condition was maintained [11, 12].

Dissolution efficiency

The concept of dissolution efficiency (DE) was proposed by Khan and Rodes in 1975 is defined by

$$DEt\% = \frac{\int_0^t Y.dt}{Q100t} X100$$

Where Y is the percentage of drug dissolved at any time t, Q100 is the100 percentage of dissolved product, DE is the area under the dissolution curve between time points 0 and t expressed as a percentage of the curve at maximum dissolution, Q100, over the same time period [13].

***In vivo* bioavailability study**

Experiments were conducted in compliance with the committee for the purpose of control and supervision of experiments on animals (CPCSEA) and experimental protocol approval from institutional animal's ethics of our institution (Registered No221). Female albino rabbits (1.2 to 1.5 kg) were used in the study. Animals were divided into two groups. Each group containing three rabbits was housed in polypropylene cages in a room with the controlled temperature of 25±3 °C and relative humidity (RH) 60±5% in 12 h light/12 h night cycle. The animals were fed with a standard pelleted diet and purified water ad libitum throughout the experiment. Group I and group II received atovaquone (25 mg/kg) oral suspension and optimized atovaquone tablet formulation (F5) respectively, through oral silicon tube after 12 h fasting. Blood samples were collected from the marginal vein of the ear of rabbits at an interval

0.5 h, 1 h, 3 h, 6 h, 12 h in a heparinized tube. Plasma samples were obtained by centrifuged at 5000Xg for 10 min and stored at-20 °C until analyzed. All the plasma samples were analyzed for drug content through high-performance liquid chromatography (HPLC SPD-M20A Shimadzu) with the following conditions. Chromatographic separation was developed for the quantitative serum level of atovaquone using column C18. Mobile phase consists of 0.1%v/v formic acid and methanol in the ratio of 20:80. The aqueous phase was eluted at a flow rate of 1 ml/min, volume injected was 20 µl and retention time was 7 to 8 min with the 15 min run time. The effluent was monitored at UV 254 nm. Standard calibration was constructed by spike method using a different concentration of drug in serum. All other chromatographic separation conditions were kept constant [14].

Pharmacokinetic analysis of data

The obtained data were expressed as mean±SEM. Student t-test and ANOVA were used to analyze and compare the results p< 0.05 was considered significant in all the tests. Systat 13 software was used in the study.

Maximum peak plasma concentration (Cmax) and time to reach maximum plasma concentration (t max) were obtained from the plasma concentration v/s time graph, area under the curve (AUC) calculated from the trapezoidal rule [15].

RESULTS AND DISCUSSION

Drug and excipients compatibility study

The FTIR graph interpretation of atovaquone and excipients showed the characteristics peaks of atovaquone at 3375 cm-1(C-O stretch),1658 cm-1(aromatic C=O stretch),729.09 cm-1(C-Cl stretch),2924(aromatic C-H stretch) were retained. No significant differences were observed, indicative of compatibility with drug and excipients. (fig. 1)

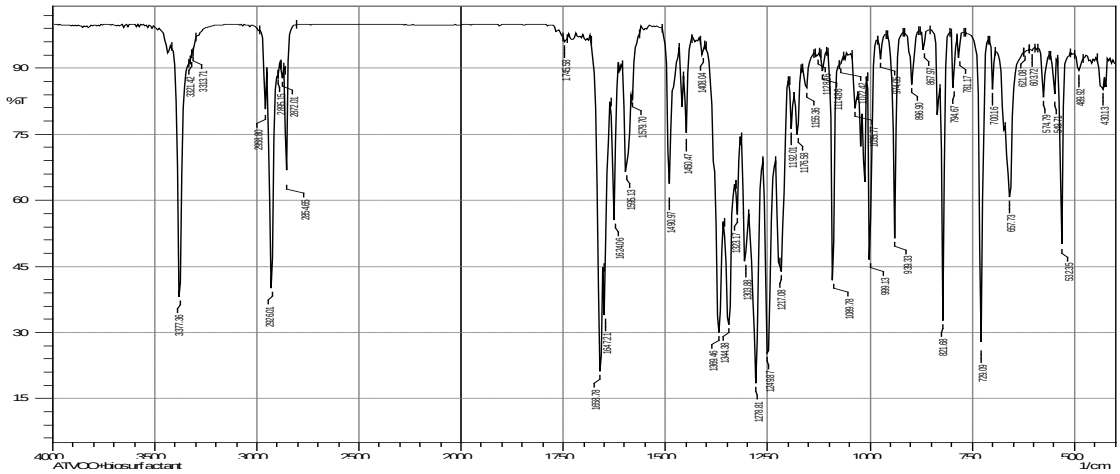

Fig. 1: FTIR of atovaquone and excipients

Solid dispersion of atovaquone

The dissolution medium in United State pharmacopeia (USP) for atovaquone drug is isopropyl alcohol 40% v/v with potassium hydrogen phosphate pH 8. In the present study 0.1N hydrochloric acid pH 1.2 dissolution medium was used to mimic the biological fluid. All the formulated solid dispersion batches, dissolution rate profiles were shown in the fig. 2. Batch S1 showed two fold increases in dissolution rate in accordance with batch S0. All the formulation batches of ternary systems of biosurfactant showed an average two-fold increase in the dissolution rate as compared to all batches of poloaxmer 407. Enhanced percentage of dissolution rate of atovaquone drug could be the effective penetration and wettability by biosurfactant, a similar study was reported by Swati S *et al.* [16]. Dissolution rates of batch S3 and S4 did not show significant differences in the percentage of drug release. Hence S3 formulation was chosen for further study. A comparative dissolution profile of binary and ternary systems of solid dispersion is shown in fig. 3.

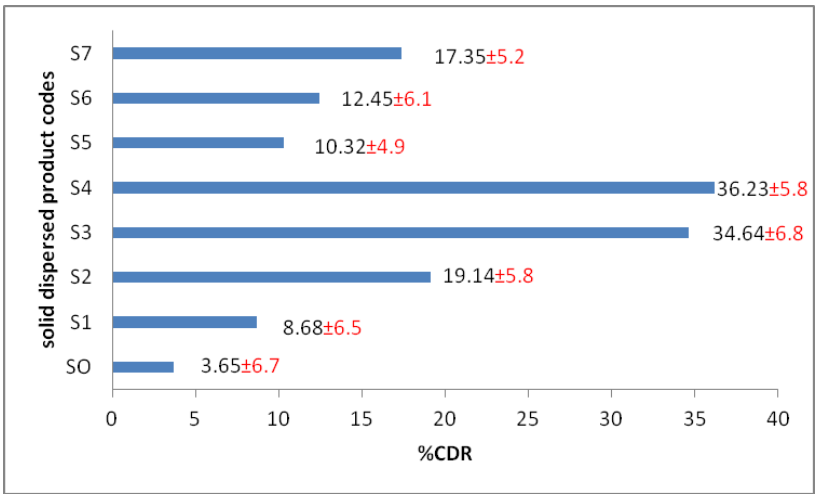

Fig. 2: Comparative dissolution profile of solid dispersed products from S0 to S7 for 1h. (n=3)(mean±SD)

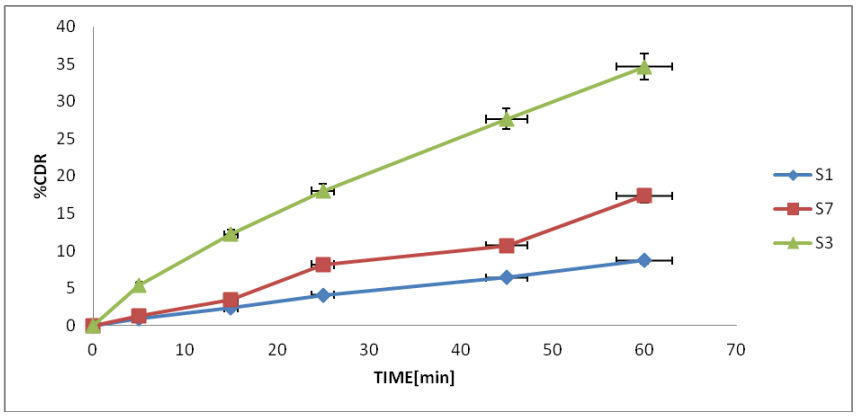

Fig. 3: Comparative dissolution profile of binary (S1) and ternary system of solid dispersion (S3, S7). (n=3) (mean±SD)

Atovaquone tablet evaluation

Atovaquone tablets formulations were prepared from the physical mixture (F1, F2, F3) and solid dispersed product blends of binary (F4) and ternary systems (F5, F6) using the dry granulation method. These two processing steps (physical mixture and solid dispersion) were adapted to atovaquone tablet preparation, to evaluate the appropriate method of addition of surfactants to achieve an enhanced dissolution rate of atovaquone.

Pre-compression

All the formulated atovaquone formulation powder blends were subjected for micromeritics properties. The compressibility index, Hausner's ratio, and angle of repose were within the specified limits of 10.8% to 12.19%, 1.11 to 1.27 and 25.60 to 28.30 respectively. From table 3 results indicates that all formulations were having a good free flowing and compressible properties.

Table 3: Micrometric properties of lubricated powder of atovaquone

Formulation code	Bulk density* (g/cm^3)	Tapped density* (g/cm^3)	Compressibility index (%)*	Hausner's ratio*	Angle of repose*
F1	0.49±0.02	0.55±0.02	12.19±0.03	1.13±0.08	25.60±2.07
F2	0.48±0.01	0.54±0.04	10.83±0.04	1.11±0.07	26.71±0.57
F3	0.47±0.09	0.55±0.03	10.89±0.04	1.12±0.04	27.13±0.75
F4	0.48±0.08	0.53±0.02	13.01±0.01	1.13±0.04	26.40±0.43
F5	0.47±0.01	0.53±0.02	11.70±0.07	1.10±0.02	27.58±0.35
F6	0.48±0.03	0.53±0.04	10.93±0.09	1.13±0.09	28.30±0.01

*mean±SD (n=6)

Table 4: Post-compression parameters

Code	Weight variation * (mg)	Thickness (mm) *	Hardness (kg/cm^2)*	Friability (%)*	Drug Content (%)*	Disintegration time (min)*
F1	230±2.01	3.5±0.5	3.3±0.35	0.32±0.091	98.22±0.50	7.50±0.01
F2	230±3.04	3.5±0.6	3.5±0.50	0.39±0.015	96.70±1.06	5.5.±0.04
F3	230±3.25	3.5±0.5	3.3±0.35	0.36±0.028	99.05±0.43	6.60±0.25
F4	230±2.02	4.5±0.5	3.4±0.40	0.19±0.041	98.03±0.37	6.30±0.02
F5	230±3.05	4.25±0.5	3.3±0.35	0.21±0.028	97.45±0.43	4.50±0.05
F6	230±3.04	3.35±0.5	3.40±0.40	0.25±0.013	98.25±0.37	5.50±0.04

*mean±SD (n=6)

Post-compression

Pharmacopieal and non–pharmacopieal tests for atovaquone tablets results were shown in the table 4. Weight variation test, thickness, hardness, friability, and drug content results were within the specified limits. Results of disintegration test for batch F2 and F5 indicated that addition of biosurfactant in the ternary system can improve the disintegration time. Similar results were also reported by Shaji A *et al.* [17].

***In vitro* dissolution profile**

In vitro dissolution of a physical blend of atovaquone tablets F1, F2 and F3 showed 5.7%, 9.0% and 7.23% drug release respectively. Binary and ternary blend system atovaquone tablets, F4, F5 and F6 showed 10.95%, 37.65% and 18.64% respectively (fig. 4). The numbers of fold increase in dissolution efficiency for F2, F3, F4, F5, and F6 were 1.44, 1.28, 1.94, 6.62, and 3.51 respectively (table 5). *In vitro* dissolution profile revealed that physical mixture blend(F2) and the ternary system blend (F5) of biosurfactant showed 2 fold and 6.6 fold an increase in the percentage of dissolution rate respectively, calculated at DE45. From these results conclude that the most effective step for the addition of surfactant in the tablet formulation was in the ternary system. Mohamed FK *et al.* [18] reported that addition of surfactant in ternary solid dispersion system showed an increased in dissolution rate of less water-soluble drug.

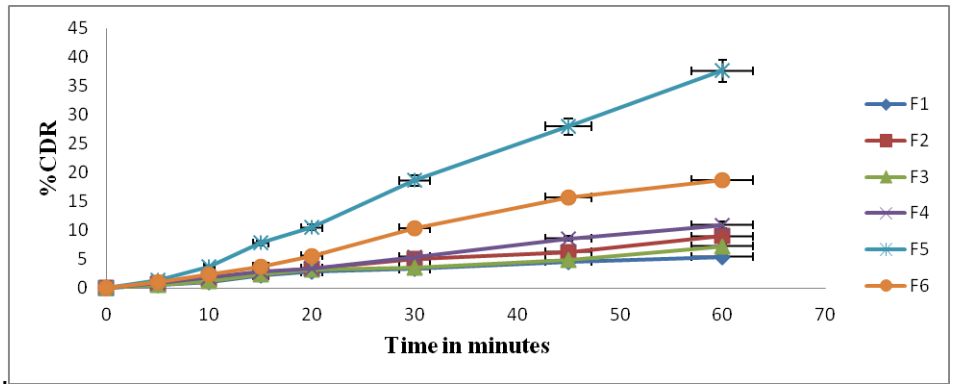

Fig. 4: *In vitro* dissolution of atovaquone tablet F1 to F6. (n=3) (mean±SD)

Table 5: Dissolution efficiency of F1 to F6 (n=3) (mean±SD)

Formulation	F1	F2	F3	F4	F5	F6
DE45	5.02±5.3	7.2±6.8	6.45±7.2	9.75±5.8	33.25±4.8	17.65±5.6
No of fold increase in dissolution rate	---	1.44	1.28	1.94	6.62	3.51

***In vivo* study**

From *in vivo* data, model-independent pharmacokinetic parameters, Cmax, tmax, and AUC were summarized in the table 6. The mean AUC of F5 and oral suspension were 240.73 µg/ml/h and 88.71 µg/ml/h respectively. Formulation F5 exhibited a significant increase Cmax: 3 folds and tmax was shortened by 1.4h in accordance to oral suspension (fig. 5). Formulation strategy for BCS class IV drug has been met with moderate success by incorporation of biosurfactant in the atovaquone dosage form. The beneficial effect of biosurfactant could be enhanced solubility-dissolution kinetics is the probable driving force behind the improved pharmacokinetic properties and effective softening of tight junction of the intestinal cell lining. Improved bioavailability study was reported by Borhardev *et al.* [19].

Table 6: *In vivo* study of F5 and oral suspension

PK parameters	Oral suspension*	F5*
Cmax µg/ml	10.56±12.51	30.12±14.32
t max h	6.5±14.32	5.1±13.58
AUC µg/ml/h	88.71±23.17	240.73±24.56

*mean±SD (n=3)

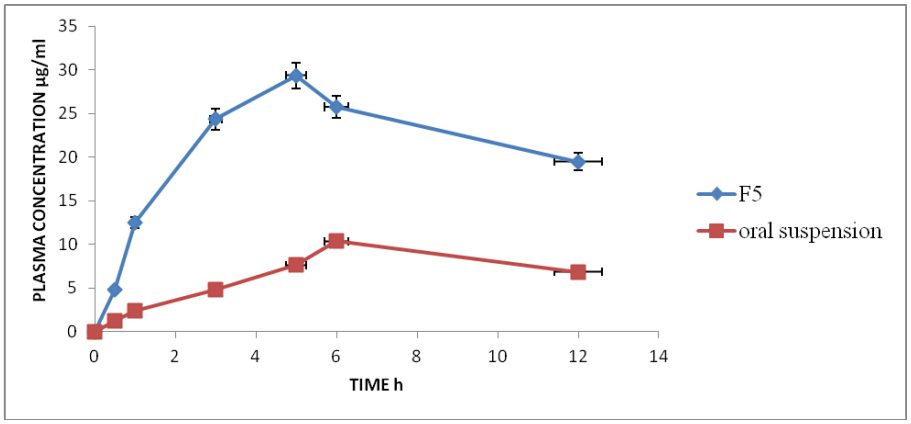

Fig. 5: *In vivo* study of F5 and atovaquone suspension. (n=3) (mean±SD)

CONCLUSION

The main objective of the present study was to evaluate the effect of biosurfactant on dissolution rate and bioavailability of BCS class IV drug. Atovaquone was selected as a model drug. Formulations of a solid dispersion in a binary and ternary system with biosurfactant and synthetic surfactants were prepared and compared. The ternary system was proved to be better than the binary system for enhancement of dissolution rate. Atovaquone tablets prepared from physical mixture blend and solid dispersed products. Evaluated results indicated that addition of biosurfactant in the ternary system better than the physical mixture. *In vivo* study indicated the biosurfactant can also act as bioenhancer for BCS class IV drugs. However, detailed toxicology and stability studies are needed for the biosurfactant to be approved from regulatory authorities as the surface active agent.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest

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"Production of Biosurfactant from *Flavobacterium* species and its effect on selected formulated dosage forms''

An errata submitted to

KLE Academy of Higher Education and Research, Belagavi

Accredited **'A' Grade** by NAAC (2nd Cycle) Placed in 'A' Category by MHRD (GoI)

[Deemed-to-be-University u/s 3 of the UGC Act, 1956 vide Government of India Notification No.F.9-19/2000-U.3(A)]

Under the guidance of Prof. (Dr.) Pramod J Hurkadale

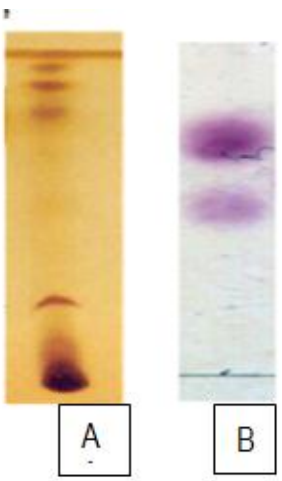
In the Faculty of Pharmacy

By Mr. U B Bolmal.
Registration No: KLE/Ph.D/10-11/DOUN10011

September 2020

Errata submission of the thesis:

Observations	Clarifications / corrected as	Corrected thesis copy page number
1) In hanging drop method, the methodology is very brief and not clear.	1) Hang drop method: [UK (NHS) Standards for investigation of microbiological. (Motility)] ➢ Central cavity microscopic slide was cleaned with tap water and dried with domestic dryer for 5 minutes. ➢ Applied petroleum jelly around the cavity of slide taking the care that cavity of slide was not stained. ➢ One drop of fresh bacterial culture broth was transferred in to the cavity of the slide with the help of sterile platinum loop. ➢ Cleaned and dry cover slip was taken and all the edges of the cover slip were coated with petroleum jelly in circular shape. ➢ Coated cover slip was inverted over the cavity slide slowly and gently pressed to seal the cavity. ➢ The slide is then clipped to the stage and examined under the low power objective of the microscope. ➢ Using the 4X objective, focus on the edge of the drop and observed for bacterial motility, slightly closing the iris diaphragm. ➢ Zigzag movement was observed bacteria are motile, no movement, non-motile.	55
2) In Grams staining technique, duration of staining is not as per the standard procedure.	2) Gram staining procedure: Subculture *flavanobaterium* sp 2945 was subjected for gram staining using following reagents and Staining procedure (Smith AC et al 2016) was followed as mentioned below. **Reagents required:** ➢ **Primary stain:** 2 gms Crystal violet, 20mL 95% ethyl alcohol**,** 0.8 gms ammonium oxalate make up to 100mL with distilled water. ➢ **Gram's iodine:** 2 g potassium iodide, 1 g Iodine crystals, and 100 mL distilled water. **Decolourizer:** 50mL of ethanol and 50 mL of acetone. ➢ **Counter-stain:** 4.0 g Safranin, 200 mL 95% ethanol, and 800 mL distilled water ➢ Microscopic slides were washed with soap and water to free from grease or oil substance and dried with hot air dryer ➢ With sterile loop, bacterial culture broth was smeared on the slide in circular motion with one centimeter diameter. ➢ Slide was air dried for one hour and warmed over the Bunsen burner until dried smeared was observed and no overheating was observed.	53-54

	➢ Slide was kept on inclined glass rod stand; two drops of crystal violet were added over the smear and kept for 1 minute. ➢ Slide was washed with excesses of water. Gram's Iodine solution was added kept for one minute. ➢ Slide was washed with excesses of water, purple colour smear was observed. ➢ Coloured slide was rinsed with alcohol solution till no colure was eluted. ➢ Rinsed with water and safranin was added over the smear and kept for 45 seconds. ➢ Once again slide was washed with water and dried with whatman paper by gently pressing over it. ➢ Smear colour was observed under microscope with 40X magnification. ➢ Gr – ve bacteria stains red or pink colour. Gr+ bacteria stains blue or purple colour.	
3) In section 2.5.4.6. Gel strength; in house fabricated gel strength apparatus method is not validated.	3) In house fabricated gel strength apparatus was validated using the 6.6 %w/v gelatin IP in warm water.50gms of gelatin gel was placed in 150mL beaker and kept for 10 minutes at room temperature. T -shape plunger with 25gms weight was placed over the surface of the gelatin gel. Time taken for penetrate 3 cm depth was noted. Average time taken for penetrate gel was found to be 92.33 seconds from triplicates. **Justification:** The bloom strength of gelatin 6.6%w/v gel is in the range of 152 to 165 using penetrometer.(Shimdzu penetrometer product catalogue)	83
4) Many photographs lack clarity. E.g. As per the Fig. 27 TLC chromatogram is not valid	Fig 27: Resolved  	108

5) Fig. 28 Dermal toxicity photograph is not clear	Fig: 28 Resolved 	111
	Following figure sizes are increased for clarity: Fig. 13 Fig: 14 Fig: 15 Fig: 16 Fig:18	 63 83 91 92 95

Name & Signature of Research Scholar

Name & Signature of Guide

Date: