Original Research Article

Formulation and evaluation of microemulsion of curcumin in thymol-menthol carrier system.

Comment [FPB1]: All Active words should start with capital letters

ABSTRACT

Curcumin is a phytochemical obtained from rhizomes of curcuma longa. It has been proven clinically and has many therapeutic applications. Being BCS Class IV drug, it has poor bioavailability owing to no solubility in water practically and poor permeability. Literature survey revealed that no attempt is yet done by dissolving curcumin in eutectic mixture of menthol & thymol. Curcumin dissolved in mixture of thymol & menthol to reasonable extent.100 mg of curcumin in 1 g of thymol and 0.6 g of menthol mixture was finalized as product mixture. The solution of curcumin in eutectic mixture being oily liquid, it gave the idea for development for the microemulsion .Hence the present work was done with an objective to formulate and evaluate the microemulsion of curcumin in thymol-menthol carrier system. Microemulsion system with eutectic mixture of thymol and menthol was chosen as oil phase and carrier for curcumin, tween 80 as surfactant and ethanol as co-surfactant.Ternary phase diagrams were constructed to obtain the optimum concentration range of oil phase, surfactant and co-surfactant. The microemulsion of 100 mg curcumin containing 4.95 % oil phase, 33.39 % surfactant, 11.13% co-surfactant,50:50% of water was optimum. Microemulsion of curcumin was prepared by water titration method and evaluated for globule size, drug content, pH,viscosity ,conductivity and *In* vitro drug release study. The *ex*-vivo permeation study for microemulsion of curcumin was carried out on excised mice skin usingfranz diffusion apparatus. The cytotoxicity study for thymol, menthol was performed on healthy L929 murine fibroblast cell line. Anticancer activity of thymol-menthol eutectic mixture alone and with curcumin was performed on Hela cell lines with promising results. The formulation microemulsion of curcumin developed under this study was found to be stable and effective with promising anticancer cell line study.

Comment [FPB2]: Expurge- take to literature or methodology

Comment [FPB3]: Present the result of the anticancer activity on the abstract

Comment [FPB4]: Report the result here accordingly.

Keywords: Curcumin, thymol, menthol eutectic mixture, ternary phase system, microemulsion of curcumin, cytotoxicity, anticancer cell line study.

INTRODUCTION

The microemulsion term is applied to asystem prepared by emulsifying oil in an aqueous surfactant and then adding a fourth component known as co-surfactant which is generally intermediate chain length alcohol like butanol, isopropyl alcohol, pentanol. [1]Curcumin is BCS class IV drug with severe limitations of solubility in water and permeability across biological membranes. This restricts the use of curcumin as a drug in clinical practice. Curcumin is reported to have around 10-15 pharmacological activities but clinically this becomes a challenge to make curcumin bioavailable. Currently only oral dosage forms of curcumin are availablewith very high dose (4-5gm in a day).This shows that there is wide scope to develop effective formulation of curcumin with increased bioavailability and decreased dose. Literature survey done under this study shows there is lots of research done and is going on in the area of development of successful drug delivery system for curcumin. The concept of hydrotropy was one of the not much tried effort with curcumin, The concept of eutectic mixture of thymol and menthol as excipients seemed untouched area yet.There had been various reports that various phenolic compounds can act as good hydrotropic agents and also may be beneficial as permeation enhancers. 1gm thymol formed eutectic mixture with 0.6 gm of menthol and resulted into clear oily liquid at room temperature. The increasing amount of curcumin was dissolved into this eutectic mixture to get maximum of 100 mg of curcumin in 1.6 gm of eutectic mixture.This was basic clear liquid mixture further used in design and development of microemulsions

MATERIALS AND METHODS-

Materials:

Curcumin (purity 90%) was purchased from Amsar Goa Pvt.Ltd.India.Thymol crystals (AR)and Menthol crystals(AR)was purchased from Research Lab Fine Chem. India. Tween 80 (AR)was obtained from Research Lab Fine Chem. IndiaEthanol (AR)was obtained from Research Lab Fine Chem. India, Citric acid (AR)was obtained from Research Lab Fine, India

Comment [FPB5]: Indicate the city in India as well as the trade name and company of the products accordingly.

Methods:

Drug excipient compatibility studies:

The drug excipient compatibility was done by studying the FTIR(IR Affinity -1 SHIMADZU)[2,20], DSC(Perkin Elmer DSC 40000), NMR(Bruker) and TLC[3](Chloroform: Ethanol: Glacial acetic acid=95:5:1)of the curcumin, thymol-menthol eutectic mixture and mixture of thymol-menthol and curcumin to determine the compatibility and interaction with each other.

Anticancer activity of thymol-menthol eutectic mixture:

TMC and TM were sterilized by 30 minutes UV exposure inside bio-safety cabinet. A stock solution was made in DMSO, later it was diluted using complete DMEM media. The concentrations were 100, 120, 140, 160, 180, 200 µg/mL. Healthy L929 murine fibroblast cell lines (passage number 61/62) were maintained using complete DMEM. 10,000 cells were plated in each well of a 96 well plate. It was incubated in 5% CO_2 at 37°C for 1 day. At 1d samples of above concentrations were added in triplicates. After 1d incubation in 5% CO_2 at 37°C, 100 µl of 200 µM resazurin solution (in complete DMEM media) was added. It was incubated for 6 hours in 5% CO_2 at 37°C. From each well 100 µl media was taken out and read in a plate reader (excitation 530 to 560 nm and emission at 590 nm).

Comment [FPB6]: Reference the method used for anticancer activity of thymol-menthol eutectic mixture with date show any modification made.

Cyto-toxicity study of thymol-menthol:

TMC and TM were sterilized by 30 minutes UV exposure inside biosafety cabinet. A stock solution was made in DMSO, later it was diluted using complete DMEM media. The concentrations were 100, 120, 140, 160, 180, 200 µg/mL.Healthy L929 murine fibroblast cell lines (passage number 61/62) were maintained using complete DMEM. 10,000 cells were plated in each well of a 96 well plate. It was incubated in 5% CO_2 at 37°C for 1 day. At 1d samples of above concentrations were added in triplicates. After 1d incubation in 5% CO_2 at 37°C, 100 µl of 200 µM resazurin solution (in complete DMEM media) was added. It was incubated for 6 hours in 5% CO_2 at 37°C. From each well 100 µl media was taken out and read in a plate reader (excitation 530 to 560 nm and emission at 590 nm).

Comment [FPB7]: Quote author reference for this method you used and indicate any modification. Or are you the author of this method of analysis.

Solubility of curcumin in various oils:

2ml of sunflower oil was taken in test tube (25^{o}C)to which curcumin was added and sonicated until no more curcumin is dissolved ,Further more quantity of curcumin was added to make it 100mg ,mixture was sonicated to 15 min covered with aluminium foil and kept over night,later this mixture was centrifuge to get clear supernatant which was removed

without disturbing settled curcumin at bottom. The settled curcumin was filtered and washed with ample of water and dried, The weight of dry curcumin obtained was subtracted from 100mg.that determined solubility of curcumin in Sunflower oil,similar experiment was carried out using linseed oil and Thymol- menthol eutectic mixture[4,5].

Comment [FPB8]: Here does not need citation rather method of analysis established by an author which you should reference. Indicate any modification. Remove the citation used for comparison or use it as the author method for your experimentation

Screening of surfactants:

Two types of surfactants were screened for microemulsion formulation. Which included Tween 60 and Tween 80. 2.5 ml of 15% v/v surfactant solution was prepared in water, and 4µL of oil was added with micropipette with vigorous vortexing. If one-phase clear solution was obtained, the addition of the oil was repeated until the solution became cloudy [5,6].

Comment [FPB9]: Reframe the sentence as "The method adopted by ..... was used for screening of surfactant". Add your modification if any!

Screening of co-surfactants:

Tween 80 was combined with two solubilizers as co-surfactants namely, ethanol, and propylene glycol. At a fixed surtactant mixture (Smix) ratio of 1:1,thepseudoternary phase diagrams were constructed. Nine different combinations(B1 to B9) in different weight ratios of oil an Smix, 1:9, 2:8, 3:7, 4:6, 5:5,6:4, 7:3, 8:2, 9:1 were utilized so that maximum ratios were covered to delineate the boundaries of phases precisely formed in the phase diagrams[7,8].

Comment [FPB10]: Do same as indicated in screening of surfactants

Construction of pseudo-ternary phase Diagram:

Form solubility studies of curcumin in various oils and thymol-menthol eutectic mixture, (TMC);TMC,tween 80 and ethanol were selected as oil, surfactant and co-surfactant respectively for preparation of microemulsion. Microemulsion region was identified by constructing pseudo ternary phase diagram containing different proportion of surfactant: co-surfactant (S/CoS) (1:1, 2:1 and 3:1), and oil , water[7]. S/CoS mix and oil were mixed in ratio of 1:9, 2:8, 3:7, 4:6,5:5, 6:4,7:3,8:2,9:1[7] . To the resultant mixtures, water was added drop wise till the first sign of turbidity in order to identity the end point and after equilibrium; if the system became clear then the water addition was continued.

Physicochemical characterization of curcumin loaded microemulsion:

Percentage drug content of the optimised batch B1,of microemulsion (ME) was determined at 420nm using methanol as solvent [6,13]. Viscosity of ME was measured using Brookfield viscometer at room temperature with spindle no.63[16,17].Electrical conductivity of ME was measured using conductivity meter 306 at ambient temperature [18]and pH of ME was measured using digital pH meter[8,13].Droplet size and zeta potential distribution was measured using (Malvern NANOZS-90,U.S.)[14,8]. ME was centrifuged at 15000 rpm for period of 15 min. and examined for any change in phase seperation and optical transparency[6,12]. PDI was calculated[15,8].

Comment [FPB11]: Develop each physical parameters analysed with a sub title indicating the method of analysis adopted with reference

Scanning electron microscopy:

Scanning electron microscopy (SEM) was used to characterize microstructure of emulsions. SEM of samples were measured using (Jeol JSM-6510, USA)[18].

***In*-vitro drug release study:**

The release of curcumin from curcumin +water and from microemulsion formulation was compared. Release of drug from microemulsion employed a dialysis bag to study grugrelease..It was first activated using release medium. Phosphate buffer of pH 7.4 was used as release medium. The dialysis bag was suspended in a beaker containing 200 ml of phosphate buffer solution which was kept on magnetic stirrer. For overall experiment temperature of 37^{o}C was maintained, 5 ml formulation (ME)and curcumin +water mixture contatining same quantity of curcumin was transferred to dialysis bag.1ml sample was removed from the beaker containing phosphate buffer at time interval of 1 hr,2 hr ,3 hr ,4hr ,5hr ,6hr ,7hr ,8hr,22 hr,23 hr,24hr and was diluted to 10ml with methanol and absorbance was noted at 420nm[16].

***Ex-Vivo* Study:**

Preparation of skin for Ex-vivo permeation study:

Mice Skin: Swiss albino mice weighing 80-100 gm were selected for preliminary permeation study and the study was conducted with the approval of institutional animal ethical committee. The mice were sacrificed using anesthetic ether. Then the hair from their abdominal region were removed using animal hair clipper, and, subsequently, full thickness

of skin was harvested. The fatty layer, adhering to the dermis side, was removed by surgical scalpel.

Comment [FPB12]: Indication the author referenced for this method of preparation of skin for ex-vivo permeation study

Procedure for permeation study:

Ex vivo skin permeation studies were carried out using Franz diffusion cell. The cell consists of two chambers, the donor and the receptor compartment with a diffusion area of 1.43 cm^2. The donor compartment was open at the top and was exposed to atmosphere. The excised mice skin was mounted between the compartments of the diffusion cells with stratum corneum facing the donor compartment and clamped into position. Magnetic stirrer bars were added to the receptor chambers and filled with the receptor phase. Phosphate buffer saline, pH 7.4, was used as receptor medium. The entire setup was placed over magnetic stirrer , and the temperature was maintained at 37± 0.5°C.The skin sections were initially left in the franz cells for 2 hours in order to facilitate hydration of the excised skin . After this period,1 ml of ME(B1) formulation was applied onto the surface of the skin.1 ml of medium was collected from receptor compartment at 1 hr,2 hr ,3 hr ,4hr ,5hr ,6hr ,7hr ,8hr,22 hr,23 hr,24 hr intervals In 24 hrs study period and replaced with the same amount of fresh buffer. The amount of permeated drug was estimated using UV spectrophotometer by measuring absorbance at 420 nm [19].

Anticancer activity of microemulsion:

Samples (ME and Curcumin) were sterilized by 30 minutes UV exposure inside the bio-safety cabinet. Samples were initially dissolved in sterile DMSO and further dilutions (5, 10, 15, 20 µg/100 mL) were carrier out using complete DMEM. HeLa cervical cancer cell lines (passage number 89/90) were maintained using complete DMEM. 10,000 cells were plated in each well of a 96 well plate. It was incubated in 5% CO_2 at 37°C for 1 day. At 1d, samples of above concentrations were added in triplicates. After 1d incubation in 5% CO_2 at 37°C, 100 µl of 200 µM resazurin solution (in complete DMEM media) was added. It was incubated for 6 hours in 5% CO_2 at 37°C. Entire plate was read in a plate reader (excitation 530 to 560 nm and emission at 590 nm).

Comment [FPB13]: No author referenced for this method of analysis. Indicate the meaning of your abbreviations.

Result and discussion:

FTIR:

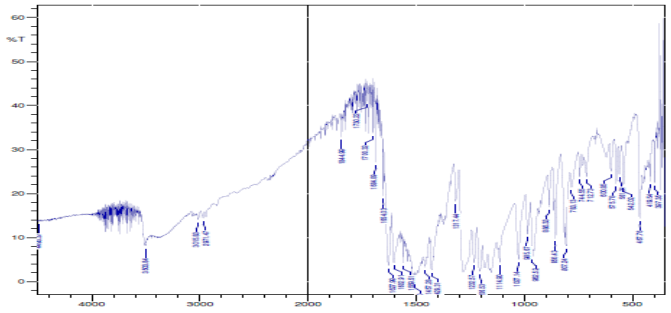


Fig.1-IR spectra of Curcumin

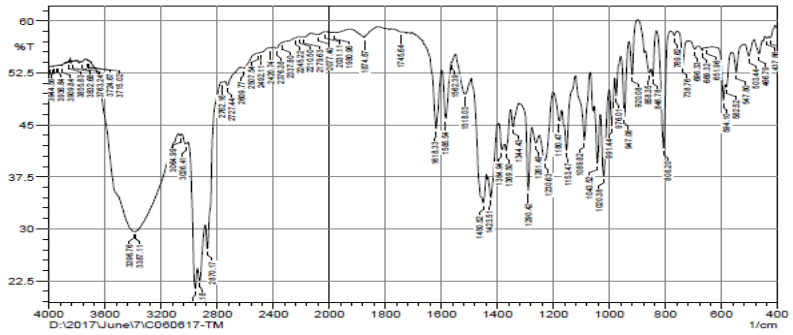


Fig.2- IR spectra of Thymol+ Menthol

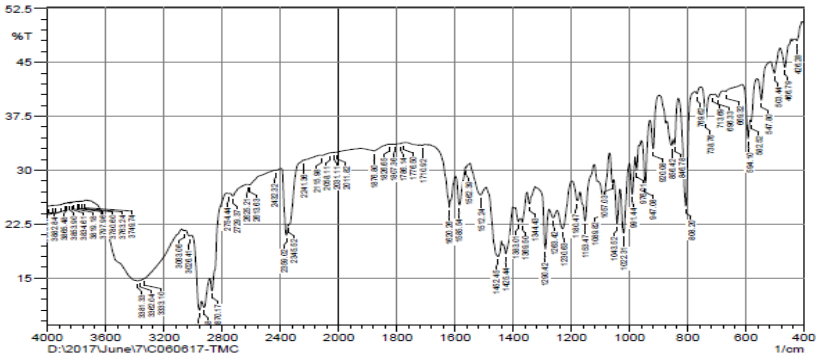


Fig.3-IR spectra of Thymol+ Menthol+ Curcumin

In fig.1,2,3 All reported frequencies are found identical in TM sample and TMC sample. The thymol+ menthol forms eutectic mixture that showed decrease in intensity of peak but not vanished, that showed physical interaction occure between thymol and menthol, But in TMC there was no chemical interaction was found between TM eutectic mixture and curcumin.

DSC Study:

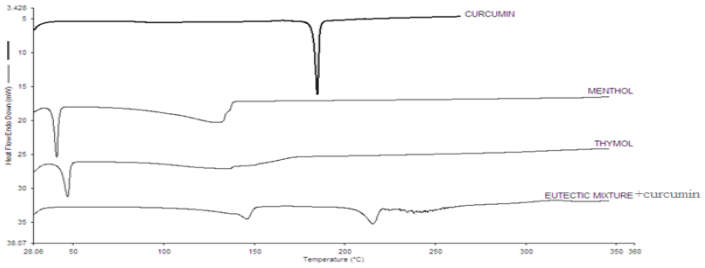
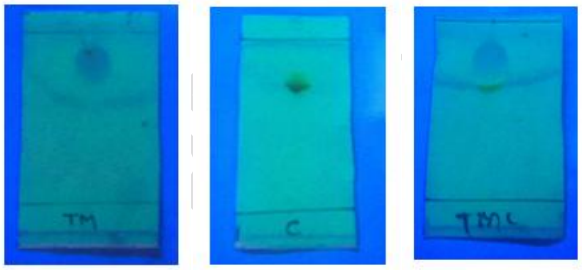


Fig.4-DSC Overlay

All melting points of Thymol , menthol and curcumin vanished in eutectic mixture because Thymol + menthol melt at room temperature when mixed being eutectic and curcumin is dissolved in it. In the Eutectic mixture+ curcumin, the endotherm of melting points of Thymol, menthol and curcumin are not seen ,beacause thymol and menthol melted into which curcumin is dissolved[Fig.4].

Thin layer Chromatography:



Thymol-Menthol EM

(R_f =0.8)

Curcumin

(R_f =0.6)

Thymol-menthol +curcumin

(R_fTM =0.8)

(R_fC =0.6)

Fig.5- TLC Images of different component

Comment [FPB14]: Use plate instead of figure for TLC photographed images.

As shown in fig.5 the R_f value of TM was found to be 0.8 which is different from R_f values of thymol and menthol that clearly showed that the thymol and menthol forms the physical mixture (eutectic mixture) at the ratio of 1:0.6. In the TLC of TMC the spot of TM and curcumin was observed at different $R_f$value showed that there was no chemical interaction found between curcumin and eutectic mixture TM.

NMR Study:

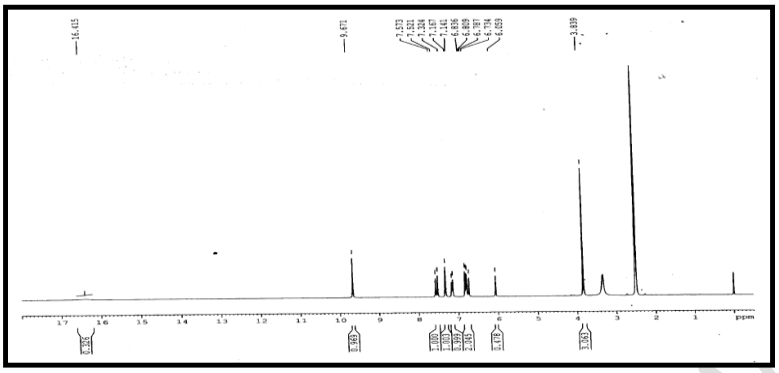


Fig.6-NMR of Curcumin

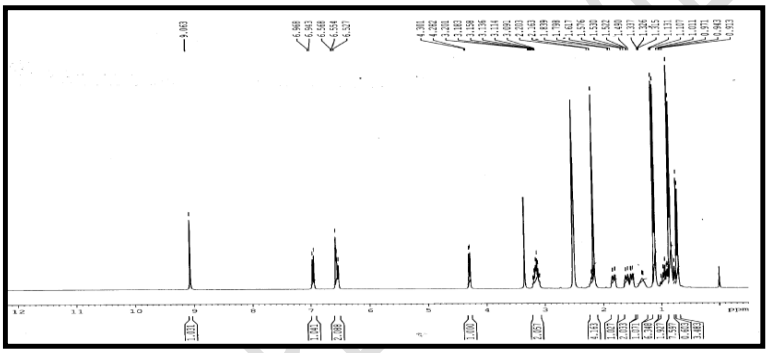


Fig.7-NMR of Thymol : Menthol

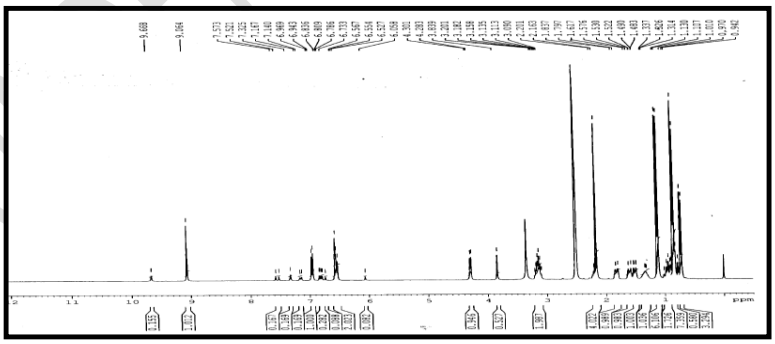


Fig.8-NMR of Thymol: Menthol : Curcumin

Anticancer activity of thymol-menthol eutectic mixture and TMC:

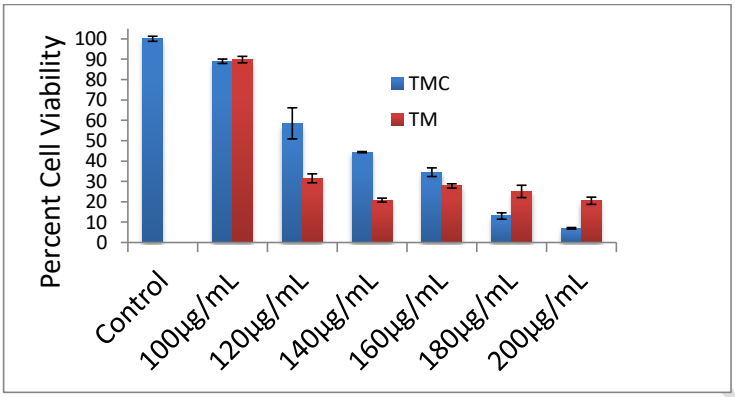


Fig.9-Anticancer activity of TM and TMC

The anti cancer activity of Thymol : Menthol eutectic mixture can be clearly seen which does not increase beyond 120µg/ml concentration and anti cancer activity of curcumin along with thymol 44and menthol increases from 100µg/ml to 120µg/ml in which concentration of curcumin is 5.8µg/ml to 11.8µg/ml. The scope of using thymol and menthol as an excipient has its threshold limit to 120µg/ml in development of suppository this was taken into consideration.

It implies that curcumin is potential anti- cancer agent and its response is quantitative as anti-cancer agent. Whereas thymol and menthol can be used in maximum 120 µg/ml concentration 45and thus it can potentiate the anti- cancer response of curcumin. It was also indicated in the cyto-toxicity pre-formulation study that thymol and menthol can be used till 120 µg/ml concentration and beyond this it proves cyto-toxic.

Comment [FPB15]: Maintain one type of font as according to the authors guideline for publication.

Cyto-toxicity study of TM eutectic mixture and TMC:

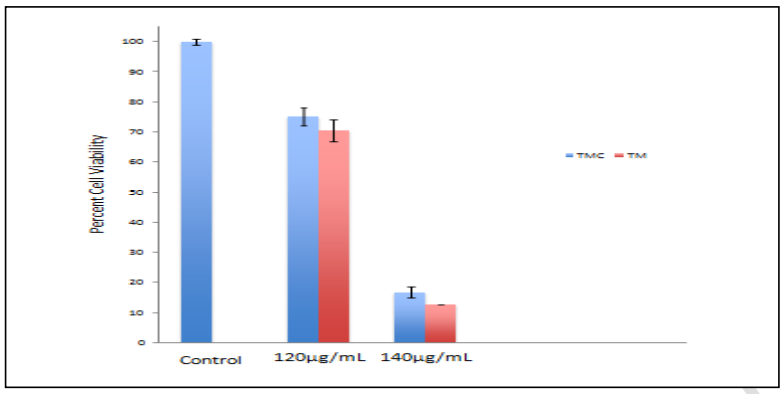


Fig.10-Cyto-toxicity study of TM and TMC

It was necessary to carry out cyto-toxicity study especially for thymol-menthol eutectic mixture being phenolic compounds. The maximum effective concentration of thymol and menthol in anticancer study is 120 µg/ml, so cyto-toxicity study was carried out for this concentration. In this study it has seen that thymol and menthol in concentration 120 µg/ml is safe to be used but proves cyto-toxic in concentration 140 µg/ml[Fig.10].

Comment [FPB16]: Recast and rewrite your grammar for better understanding. Compare your analysis with an already established study or studies. Reference your argument on the maximum effective concentration of thymol and menthol in anticancer study.

Screening of oils:

Table 1 – Solubility of Curcumin in Different oils

Sr. No.	Oils	Saturation Solubility of curcumin(mg/ml)
1	Sunflower Oil	32.5
2	Linseed Oil	26
3	Thymol-Menthol Eutectic Mixture(1:0.6)	62.5

Comment [FPB17]: Remove gridlines and bold the headings.

Screening of Surfactant:

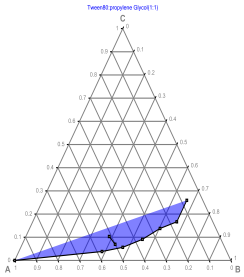
Table 2 –Miscibility of oily mixture of thymol and menthol with Tween 80 andTween 60

Surfactant	Oil (T:M)%
Tween 80	31.25
Tween 60	18.75

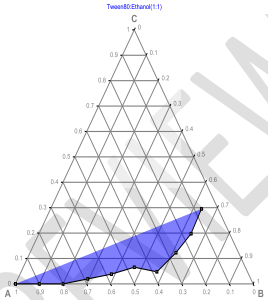
Comment [FPB18]: Remove gridlines and bold headings

Screening of co-Surfactant:

Figure 11 presents the pseudo-ternary Phase diagrams constructed for thymol-menthol eutectic mixture (oil phase), water, tween 80 (surfactant), and different co-surfactant at a fixed ratio of S:CoS 1:1.based on area of microemulsion formation in ternary phase diagram, ethanol was selected and used for further studies as co-surfactant.

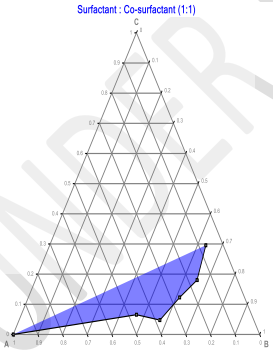


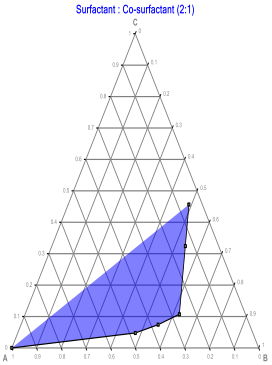
Phase diagram of Tween 80: Propylene Glycol



Phase diagram of tween 80: Ethanol

Fig.11-Pseudo-ternary phase diagram of microemulsion composed of T:M mix , tween 80 , water and co-surfactant a)propylene glycol ; b) Ethanol





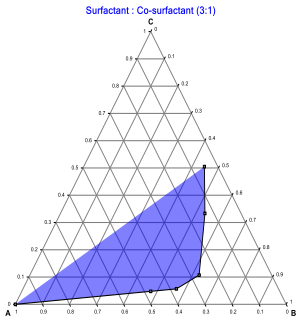


Fig.12-Pseudoternary Phase diagram using T:M eutectic mixture as oil, Tween 80 as surfactant, Ethanol as co-surfactant and water(Tween80:Ethanol=1:1,2:1,3:1)

Table 3 –**Formulation design of microemulsion containing curcumin with 3:1 ratio(S:Co-S mix)**

Batch code	Curcumin (mg)	(Oil phase)T:M Eutectic mix (1:0.6) %	Tween 80 %	Ethanol %	Water %
B1	100	4.95	33.39	11.13	50.50
B2	100	13.5	40.14	13.36	33
B3	100	27	46.89	15.63	10.3
B4	100	38	42	14	6
B5	100	47	35.25	11.75	6
B6	100	57	28.5	9.5	5
B7	100	68	21.75	7.25	3
B8	100	78.4	14.7	4.9	2
B9	100	90	7.5	2.5	0

Comment [FPB19]: Remove gridlines

Physicochemical parameters of ME:

Table 4 –**Composition of optimised microemulsionB1**

Component	B1
Curcumin(mg)	100
TM eutectic mixture%	4.95
Tween 80%	33.39
Ethanol%	11.13
Water%	50.50

TM=Thymol-menthol eutectic mixture

Table 5 –**Drug Content**

Batch code	Drug content in %(n=3, ±S.D)
B1	74.6±0.0060
B2	41.84±0.0095
B3	30.75±0.0084
B4	21.70±0.0118
B5	19.59±0.0108
B6	9.94±0.0077
B7	12.17±0.0084
B8	9.34±0.0051
B9	6.19±0.008

Batch B1 was found to be optimised batch beacause it consumes more amount of water and more percentage of drug content as compared it with rest 8 batches. So, batch B1 used for further study.

Table 6 –Physicochemical parameters ME (B1)

Parameter	B1
% Drug content	74.6±0.0060
pH	6.8±0.0816
Globule size(nm)	131.54
Conductivity(ms/cm)	8.7±0.25
Viscocity	76.66±3.39
Zeta potential(mV)	-0.57
Optical transparency	Transparent
Phase seperation	No Phase separation

The microemulsion should have good physical stability which was examined by phase seperation/flocculation and optical transparency. This can be achieved when zeta potential values are negative. The pH, viscosity ,conductivity, globule size and zeta potential of prepared formulations are shown in table 6.Result of globule size indicated that smallest globule size with the PDI 0.2802,Which is close to zero indicating that the ME(B1) had uniform globule size. The pH of ME is within the normal range of 6-6.8.The conductivity of

the results confirmed the formation of solution type ME with water in continuous phase. Viscosity of ME was found to be 76.66cps.Zeta potential was negative which indicated stability of formulation as there was less chances of globules aggregation. After centrifugation cycle it was found that ME was stable and no seperation was seen which indicate centrifugation stability. The ME remained clear and transparent even after a month of storage. The *In*-vitro drug release and *Ex*-vivo permeation study was performed with optimised microemulsion which showed promising results.

Globule size measurement:

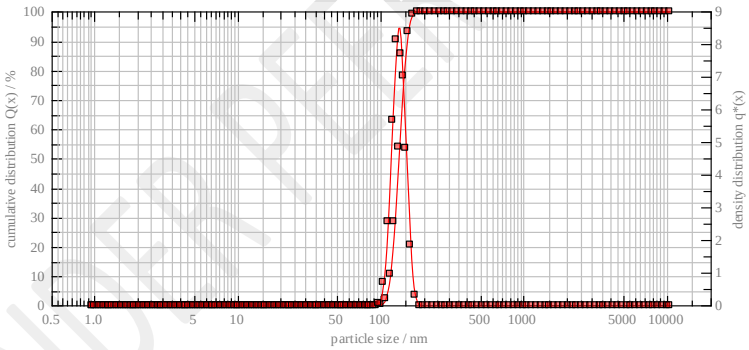


Fig.13-Droplet size distribution of microemulsion for batch B1

Zeta potential:

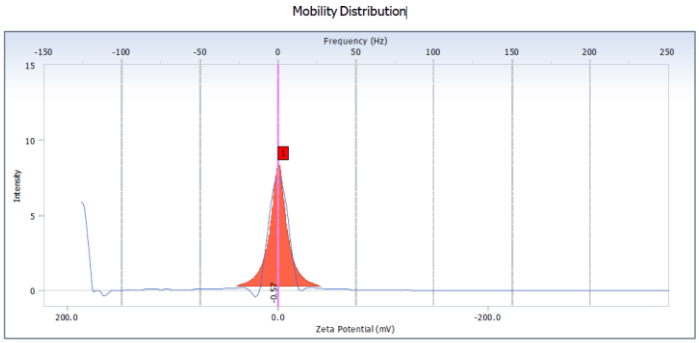


Fig.14-Zeta potential of microemulsion of batch B1

SEM study:

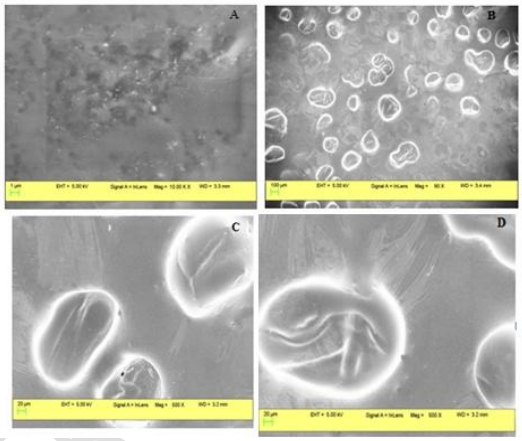


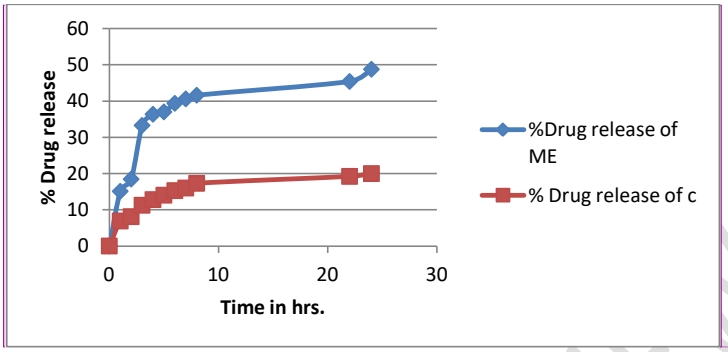
Fig.15-SEM images of batch B1 microemulsion

Batch B1 contains more amount of water compared to other batches. At water concentration 50.50 % w/w, globular structures were observed as given in figure [Fig.15]. Image A showed the surface structure of the microemulsion where Image B,C,D showed the globular structure present in microemulsion[18].

In-vitro Drug release studies:

The result of drug release study are shown in figure no.16. The in vitro study of microemulsion formulation and curcumin + waterwere compared with each other. It can be seen that curcumin released from microemulsion formulation and curcumin +water are different. As seen in figure nearly 48.75 % of drug was released from microemulsion

formulation and 19.97 % of drug was released from curcumin + water after 24 hours. The *in*-vitro drug release study showed that drug released at a faster rate from the microemulsion (ME) system than from the curcumin + water (C)[16].



Comment [FPB20]: Remove gridlines

Fig.16-Comparative *in*-vitro drug release profile of curcumin( ) for microemulsion (ME) and

( )for curcumin + water.

***Ex*-Vivo permeation study:**The *Ex*-Vivo permeation of curcumin was carried out for microemulsion and for the curcumin dissolved in water as refence. After 24 hr, the amount of curcumin permeated from microemulsion formulation and from water + curcumin was compared that given in figure17 [19].

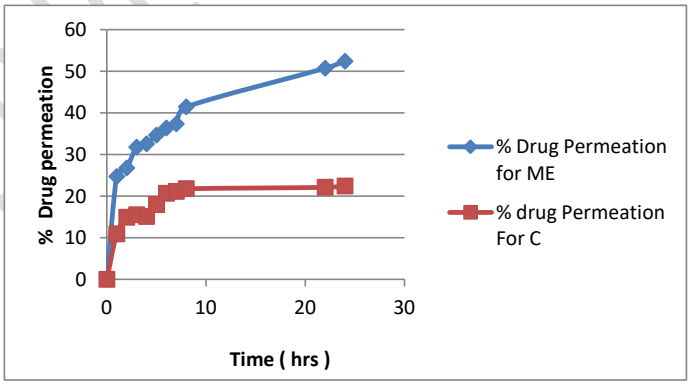


Fig.17-Comparative *ex*-vivo permeation profile of curcumin through mice skin ( ) for microemulsion (ME) and ( ) for curcumin + water.

Anticancer activity of microemulsion formulations:

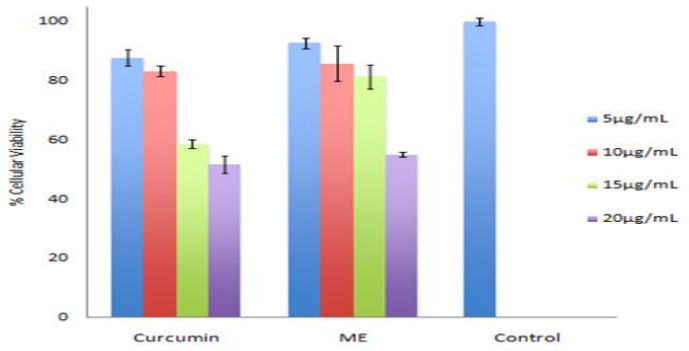


Fig.18-Anticancer activity of microemulsion on ------ cell lines

The microemulsion was diluted to get concentration of curcumin in the range of 5 µg/ml, 10µg/ml, 15 µg/ml, 20 µg/ml and compared with standard curcumin in same concentration range. The result indicates that microemulsion is successful in achieving anticancer activity as compared with curcumin as standard. The anticancer activity of microemulsion of curcumin in 20 µg/ml concentration was found to be 55%, Invivo better results will be obtained with ME of curcumin than curcumin alone as it has poor bioavailability .

Conclusion:

Curcumin microemulsion was prepared in thymol-menthol eutectic mixture carrier as novel technique to improve solubility and permeability of curcumin. The preformulation studies done clearly indicate good compatibility of drug with the thymol menthol carrier system.The cytotoxicity study was done on thymol – menthol(TM) as a preformulation aspect to ascertain the toxicity of these two excipients to be tried.It was found that TM was not cytotoxic in 120ug/mLconcentration and thus was safe to be used in concentration below 120ug/mL.Batch B1 was optimised ----------. The average globule size of microemulsion was found to be 131.54 nm, zeta potential was found to be -0.57 mV. The drug content in microemulsion batch B1 was determined and found to be 74.6%.Permeability study across mice skin *ex*-vivo model showed 52.38% permeability compared with curcumin alone which was 22.41%. So permeability of curcumin is enhanced substantially with microemulsion made in thymol and menthol, owing to fact that thymol and menthol act as good permeation enhancers. The *In* vitro drug release study of microemulsion was compared with curcumin alone.About 48.75% of curcumin was released from microemulsion showed increase in release rate. Curcumin in microemulsion of thymol menthol carrier system showed promising anticancer activity,the extension of this research can be *in*-vivo bioavailability study of the same.

Comment [FPB21]: Liquid line means???????

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Comment [FPB22]: Eliminate some of the et al in the reference and replace with authors especially where the authors are not much.

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