MJoSHT
Malaysian Journal of Science, Health & Technology
[mjosht.usim.edu.my]

Article

Cytotoxicity of VCO Microemulsion Developed Using Non-Ionic Surfactants Mixture against Normal L6 Cell and Optimisation its Formulation Containing Curcumin

Kharis Zahid[1], Nadia Halib[2], Ishak Ahmad[3], Zainah Adam[4], Razali Mirad[5]

[1]*Food Technology Research Centre, Malaysian Agricultural Research and Development Institute (MARDI), 43400 Serdang, Selangor, Malaysia*
E-mail: zkharis@mardi.gov.my

[2]*Department of Basic Sciences & Oral Biology, Faculty of Dentistry, Universiti Sains Islam Malaysia (USIM), 55100 Kuala Lumpur, Wilayah Persekutuan, Malaysia*
E-mail: nadia.halib@usim.edu.my

[3]*School of Chemical Sciences and Food Technology, Universiti Kebangsaan Malaysia (UKM), 43600 Bangi, Selangor, Malaysia*
E-mail: gading@ukm.edu.my

[4]*Medical Technology Division, Malaysian Nuclear Agency Bangi, 43000 Kajang, Selangor, Malaysia*
E-mail: zainah@nuclearmalaysia.gov.my

[5]*Agrobiodiversity and Environment Research Centre, Malaysian Agricultural Research and Development Institute (MARDI), 43400 Serdang, Selangor, Malaysia*
E-mail: zaley@mardi.gov.my

***Abstract*— The objective of the study was to evaluate the properties of curcumin formulated in Virgin Coconut Oil (VCO) microemulsion system developed using food-acceptable grade surfactants of Tween 80, Span 80 and Span 40. Cytotoxicity analysis was carried out toward VCO microemulsion using normal L6 cells *in vitro*. The microemulsion was formulated using VCO as the oil phase with non-ionic surfactants mixture consist of Tween 80, Span 80 and Span 40. Curcumin was incorporated into VCO microemulsion as a cold mixture under mild agitation to form curcumin microemulsion. The physical stability of curcumin microemulsion was investigated and its characterisations were further studied according to its appearance (visual), morphology, turbidity, pH, droplet size, viscosity, conductivity, and polydispersity index. Based on the results of optimisation studies, the final 0.1 % (w/w) of curcumin concentration was successfully loaded into VCO microemulsion system. This formulation had clear transparent yellow curcumin colour without phase separation. The results indicated that the curcumin microemulsion had an average particle size about 43.15 nm and good stability with zeta potential -0.24 mV. The curcumin micoemulsion morphology had spherical in shape when observed under transmission electron microscopy (TEM), the curcumin microemulsion was slightly acidic with pH value 5.28, the conductivity was 241.2 µS/cm and the viscosity was 8.5 cP.**

***Keywords*— Microemulsion; Curcumin; Virgin Coconut Oil (VCO); Non-ionic surfactants.**

I. INTRODUCTION

Curcumin is a natural compound of tumeric and extensive research has shown it contains remarkable anti-inflammatory activity. Curcumin is highly tolerated in term of safety and extremely safe for human consumption, even at high doses up to 8 g per day. These make curcumin compound a highly potential candidate to be developed as a therapeutic agent for many diseases. However, the use of curcumin is limited by its poor solubility and low bioavailability. Increase the dissolution rate and solubility of curcumin were among challenges to be overcome. Curcumin is a hydrophobic polyphenol compound and has very low solubility in water. It comes under class II drugs, which have low solubility and high permeability [1]. Therefore, in order to improve its solubility and bioavailability, curcumin should be formulated to facilitate its delivery when applied to the targeted area. Incorporation of curcumin into the appropriate delivery system using microemulsion aims to improve the absorption and raised its bioavailability due to high drug solubilisation capacity, which leads to high concentration gradients that allows drug diffusion ease, consequently enhanced the curcumin efficacy [2].

Since the discovery of microemulsion, numerous applications have been explored in various fields of studies and its potential as a carrier has attracted many researchers to utilize it as a tool for compound delivery [3].

Microemulsion drug delivery system is a novel and versatile approach for overcoming the formulation difficulties of drugs with poor aqueous solubility. A right combination between surfactant, oil and water spontaneously cause microemulsion formation which appears as single optically isotropic liquid with a particle size within the range of 10-100 nm [4]. It has high thermodynamic and kinetic stability, low viscosity and optical transparency that suitable for solubility, dissolution and absorption enhancement of liphophilic compound [5]. Due to its great stability, microemulsion offers a better system as solution for compounds delivery in the body [6].

In this study, a preferred microemulsion system using Virgin Coconut Oil (VCO) as oil phase was used to formulate curcumin microemulsion. A VCO microemulsion system based on [7] was selected to load with curcumin to obtain curcumin microemulsion. No such study and publication being reported so far on curcumin formulation using this microemulsion system. The optimum and stable curcumin microemulsion obtained will be characterised and evaluated for its safety and suitability to use as curcumin carrier.

II. THE MATERIAL AND METHOD

A. Chemicals

Curcumin powder of 95.18% purity was purchased from MERCK (Billerica, MA USA). Tween 80 was purchased from Amresco, whereas Span 80 and Span 40 were purchased from Nacalai tesque (Kyoto, Japan). Trypsin and fetal bovine serum (FBS) were purchased from Gibco, whereas MTT (3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, dimethyl sulfoxide (DMSO) and isopropyl myristate (IPM) were purchased from Sigma-Aldrich Chemical. Virgin coconut oil was obtained from local stores.

B. Cell Culture

The L6 cells were purchased from the cancer cell lines, obtained from the American Type Culture Collection (Rockville, MD, USA). The culture medium for L6 cells were purchased from Millipore and Gibco, respectively. The L6 cells were cultured in DMEM supplemented with 5% heat-inactivated fetal bovine serum (FBS) and a 1% antibiotic solution (100 U/ml penicillin G and 0.1 mg/ml streptomycin). The cell was maintained at 37 °C in a humidified incubator containing 5% CO_2.

C. Microemulsions Preparation

Two microemulsion systems were prepared based on different oil phase which were VCO microemulsion and Isopropyl Myristate (IPM) microemulsion. The VCO microemulsion was prepared by titration method using non-ionic surfactants combination of Tween 80:Span 80:Span 40 (Smix) with the ratio of 90:3.33:6.67 and the proportion between VCO, Smix and water was 4:20:76. The mixture was mildly heated and stirred until a transparent and isotropic microemulsion was obtained. The preparation of IPM microemulsion was achieved using Tween 80 and PEG-400 formulated at 6% isopropyl myristate (IPM), 30% Tween 80 and 10% PEG-400 and 54% water respectively as described in [8].

D. MTT (Cytotoxicity) Assay for L6 Treated with Microemulsions.

The L6 cells were used for cytotoxicity tests of the microemulsions. The confluent cell culture was trypsinised and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM containing 10% FBS. The cells were plated on 96-well flat bottomed plates, with each well at a density of 1×10^5 cells, and incubated for 24 hours at 37 °C in the CO_2 incubator. To each well of the 96-well microtitre plate, about 100 µl of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, the media of the confluent attached cell was flicked off and replace with new media containing test microemulsions at different concentration. The plates were then incubated at 37 °C for 72 hours in 5% CO_2 atmosphere. The cytotoxicity effect was evaluated by the MTT assay. The absorbance was measured using a microplate reader at a wavelength of 570 nm. The absorbance values obtained were then converted to percentage of cell viability [9] and determined by the following equation:

$$\text{Cell viability (\%)} = T/C\times100 \quad (1)$$

Where T/C is the test treatment over control (T/C) values [10]-[11]. The experiments were repeated at least three times for all microemulsion samples tested.

E. Preparation of Curcumin Microemulsion

A cold preparation was used to formulate curcumin microemulsion by incorporating curcumin into VCO microemulsion. An appropriate amount of curcumin was slowly added into VCO microemulsion followed by sonication and gentle shaking for the mixture complete mixing and homogeneous solubilization. The mixture was then left to reach equilibrium and then a clear isotropic microemulsion containing curcumin was obtained. A desired maximum concentration of curcumin was obtained by dissolving the curcumin to a final 0.1% (w/w) concentration or 1 mg/g. No phase separation was observed after the addition of the curcumin. The curcumin microemulsion was then kept at 25 °C for further use.

F. Thermodynamic Stability of Curcumin Microemulsion

The curcumin microemulsion was examined for thermodynamic stability through several tests including centrifugation, heating-cooling cycle and freeze-thaw cycle based in [12]. Centrifugation test was carried out at 3500 rpm for 30 minutes. Then heating-cooling cycle was performed between the refrigerator temperature of 4 °C and 45 °C for 24 hours. Finally, the curcumin microemulsion sample was taken for freeze-thaw cycle test at temperature between −21 and +25 °C. Those curcumin microemulsion formulations that did not show any phase separations, creaming or cracking was thermodynamically stable and selected for further studies.

G. UHPLC-DAD Analysis of Curcumin

All samples were analysed by Agilent UHPLC System using a reverse-phase C-18 Kinetex column (100 mm length, 4.6 mm diameter) for estimation of curcumin content. The UHPLC conditions used were as follows, mobile phase 60 (0.1% formic acid): 40 (acetonitrile) and a gradient elution programme was performed as shown in Table 1 with a flow rate adjusted to 0.3 ml per minute. Meanwhile the detector was set at 426 nm and 2 µl of the sample was injected for every run. The curcumin concentration in microemulsion was determined and quantified by comparing the integrated peak areas with that of an internal standard. Each experiment was replicated three times.

TABLE I
GRADIENT ELUTION METHOD

Minutes	**H_2O/0.1%CHOOH (%)**	**CH_3CN (%)**
0.00	60.00	40.00
6.00	5.00	95.00
9.00	5.00	95.00
10.00	60.00	40.00

H. Physical Characterisation of Curcumin Microemulsion

Curcumin was loaded into VCO microemulsion as in [13]. Various amounts of curcumin in increasing concentration (ranging from 0.05% - 0.20%) were loaded into VCO microemulsion in order to investigate solubilizing capacity and encapsulation efficacy on curcumin in VCO microemulsion. The mixture was sonicated and gently shaken to complete solubilisation. After a while, the undissolved curcumin was removed by centrifugation at 11832 rpm for 10 minutes and their Encapsulation Efficiency (EE%) were quantified by Agilent UHPLC-DAD with C-18 Kinetex column (100 mm length, 4.6 mm diameter) at 426 nm after appropriate dilution with ethanol. The standard curve of curcumin in ethanol at concentrations between 20 µg/ml and 100 µg/ml was used for calculation. The analysis was performed in triplicate and the Encapsulation Efficiency (EE%) was calculated as follows:

$$\text{Encapsulation Efficiency (EE \%)} = (Wt/Wi) \times 100\ \%$$

Where Wt is the total amount of curcumin in the microemulsion suspension and Wi is the total quantity of curcumin added initially during preparation.

1) Visual Appearance: Appearance of the curcumin microemulsion was observed visually whether the system is turbid or translucent or transparent.

2) Measurement of pH: The pH of curcumin microemulsion formulations was determined by using a digital pH meter. The measurement of pH of each formulation was done in triplicate and average values were calculated.

3) Turbidity: Turbidity analysis of the formulated microemulsion was carried out by measuring the transmittance of undiluted samples at 600 nm (λmax) using a PowerWave HTMicroplate (Biotek) UV-visible spectrophotometer. The reading was taken in triplicate.

4) Conductivity Measurement: The conductivity of microemulsion formulations in the absence or loaded with curcumin was determined at 25±2 °C using an electrochemical analyser (CONSORT model C933) connected with conductivity electrode. Each microemulsion formulation of 10 ml was placed in a test tube and the electrode was totally immersed in equilibrium curcumin microemulsion until a stable measurement was achieved. The experiments were carried out in triplicate for each sample.

5) Viscosity: The viscosity of microemulsion formulations in the absence or loaded with curcumin was examined as such without dilution using Anton Paar rheometer (model Physica MCR 301) equipped with measuring cone spindle CP 25-2 at 25 °C. All analysis parameters were controlled using Rheoplus Software. The determination of viscosity was carried out in triplicate.

6) Droplet Size and Polydispersity Index (PDI): Measurement of droplet size and polydispersity index (PDI) of formulated microemulsion was determined by dynamic light scattering (DLS) method using Melvern Particle Size Analyzer (model Nano ZS). Light scattering was monitored at 25 °C under measurement angle 173° backscatter and photo diode with standard 633 nm laser. The dispersed microemulsion formulations were measured without any dilution. About 1.5 ml of microemulsion formulations were pipeted into a cuvette for determination of microemulsion globule size and its PDI. The measurements were carried out in triplicates.

7) Determination of Zeta Potential: The instrument of Melvern Particle Size Analyzer (model Nano ZS) also was used for zeta potential determination. The zeta potential of microemulsion droplet was measured at 25 °C. The electrophoretic mobility (mm/s) of the particles was converted to the zeta potential by in-built software based on Helmholtz-Smoluchowski equation. Measurements were performed using small volume disposable folded capillary cells. About 1 ml of microemulsion formulations were pipeted into disposable folded capillary cells for turbid or translucent or transparent.

8) Transmission Electron Micrographys (TEM): Visualization of morphology and microstructure of curcumin microemulsions was characterized by TEM. To perform TEM observations, a dilution of curcumin microemulsions were prepared and sonicated for a minute before the examination. Then a drop of the diluted solution was placed on copper grid and was allowed to dry to remove excess water. After air drying, the morphology of curcumin microemulsion droplet was observed by CM12 Philips transmission electron microscope.

9) Statistical Analysis: All measurements were performed at least three replication of samples and the data obtained for different formulations were analysed by one way analysis of variance *(ANOVA).*

III. RESULTS AND DISCUSSION

A. Formation of VCO and IPM Microemulsion

VCO and IPM microemulsions were successfully prepared as described in the methods. All microemulsions were allowed to place at ambient temperature overnight. Visual observation made on both VCO and IPM microemulsion showed as a clear transparent and isotropic solution. However, the IPM microemulsion was more viscous as compared to VCO microemulsion. This was due to different water content and effect of components in VCO and IPM microemulsion. Microemulsion formulation characteristic should have lower viscosity for less resistance to flow, easy handling and hassle-free administration [14]-[15].

B. Cytotoxicity Effect of VCO and IPM Microemulsion on L6 Cell

The cytotoxicity results of VCO and IPM microemulsions are shown in Fig. 1 and expressed as % cell viability after incubated at maximum 48 hours. The effect of VCO microemulsion and IPM microemulsion on normal L6 cells line has been determined by MTT assay.

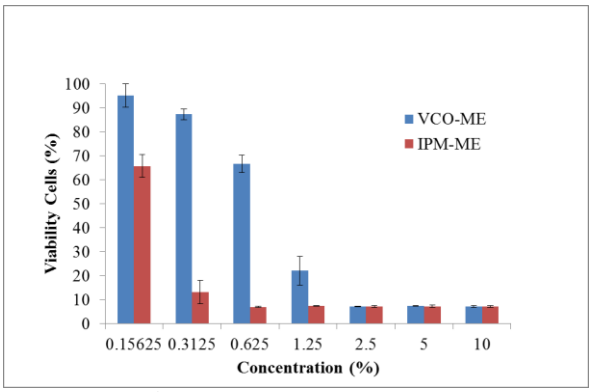

Fig. 1 Cytotoxicity of VCO and IPM Microemulsion on Normal L6 Cells

Results from analysis demonstrated adverse inhibition effect on L6 cells was shown in IPM microemulsion. Whereas VCO microemulsion display minimal inhibition on L6 cells. When VCO microemulsion at concentration 0.15625% were applied to the cell culture medium the % cell viability of L6 cell was about 95.15%. In other hand IPM microemulsion exhibited lower % cell viability about 65.80% when similar concentration of IPM microemulsion (0.15625%) was applied. The inhibition effect of IPM microemulsion was more pronounced where the % cell viability was reduced to 13.13% only when the concentration was increased to 0.3125%. The greater inhibition rate was observed with increasing of microemulsion concentration. At high concentration above 0.625%, the inhibition rate of both VCO and IPM microemulsion were almost similar. These findings suggest that VCO microemulsion is more suitable for use as a carrier to load with curcumin.

C. Curcumin Loading Capacity into VCO Microemulsion

Further characterization of curcumin microemulsions by DLS regards to their droplet size, PDI and zeta potential are

The curcumin was added into VCO microemulsion in increasing manner starting with the lowest concentration at 0.05% (w/w). The results found that as the amount of curcumin added increased, the curcumin concentration in VCO microemulsion was also increased in a dose-dependent manner. The maximum capacity of curcumin loading in VCO microemulsion was 0.1% (w/w) and the encapsulation efficiency was 99.96%. Any additional amount of curcumin above 0.1% (w/w) will cause the encapsulation efficiency of curcumin loading reduced. An addition of curcumin at 0.2% (w/w) in VCO microemulsion caused phase separation in curcumin microemulsion and the remaining curcumin will settle down at the bottom layer as shown in Fig. 2.

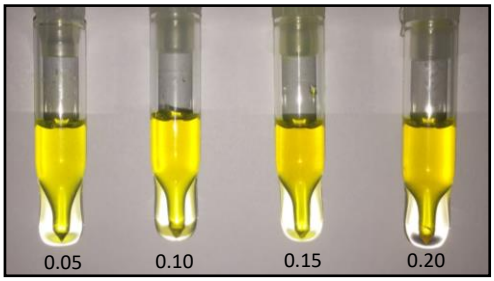

Fig. 2 Curcumin microemulsion at different concentration of curcumin

D. Curcumin Microemulsion Characteristics

The appearance of curcumin microemulsion formulations (0.05%, 0.1% and 0.15%) were both uniform and transparent with a clear orange yellow colour. Thermodynamic stability studies demonstrated that all curcumin microemulsion formulations were stable after gone through centrifugation, heating-cooling cycle and freeze-thaw cycle test. No phase separation, creaming or cracking were observed in all curcumin microemulsion formulations. Therefore, all curcumin formulations were then subjected for further characterisation. The characteristics of blank and curcumin microemulsion included the pH, % transmittance, viscosity and conductivity are presented as in Table II. The results showed that the amount of curcumin loaded had an effect on pH, turbidity viscosity and conductivity of the curcumin microemulsion. Viscosity and conductivity were increased while % transmittance and pH of curcumin microemulsion decreased with the increase amount of curcumin. The pH of curcumin microemulsions was around 5 which were beneficial to the curcumin stability. Many studies reported that curcumin degradation occurred in a pH-dependant manner, with faster reactions at neutral to basic conditions [16].

TABLE II
CHARACTERISTICS OF CURCUMIN MICROEMULSION WITH DIFFERENT CURCUMIN PERCENTAGE LOADED

Curcumin Micro-emulsion (%)	pH value	Transmit-tance (%)	Viscosity (cps)	Conductivity (µS/cm)
Unloaded	5.51a	95.07a	7.47a	172.00d
0.05	5.35b	94.91a	7.99a	223.00c
0.1	5.28b	94.84a	8.50a	240.97b
0.15	5.03c	89.95b	10.61a	255.37a

presented as in Table III. Results of TEM confirmed the morphology of curcumin microemulsion were appeared spherical in shape and uniform without aggregation.

TABLE III
PARTICLE SIZE, POLYDISPERSITY INDEX (PDI) AND ZETA POTENTIAL OF CURCUMIN MICROEMULSION FORMULATIONS

Curcumin Microemulsion (%)	**Particle size (nm)**	**Polydispersity Index (PDI)**	**Zeta Potential (mV)**
Unloaded	19.29c	0.19c	-2.15b
0.05	40.95b	0.65a	-0.61a
0.1	43.15b	0.70a	-0.24a
0.15	224.87a	0.47b	-0.88a

The incorporation of curcumin into VCO microemulsion caused the droplet size increased as compared to curcumin-free (VCO) microemulsion. However, the incorporation of 0.15% curcumin into VCO microemulsion had a greater effect on droplet size which was 224.87 ± 2.76 nm. The size was bigger than normal range within 10-100 nm. Therefore, the curcumin microemulsion at 0.15% was excluded and taken out. Whereas curcumin microemulsion at 0.05% and 0.1% showed reasonably increase on droplet size ranged within 10-100 nm. The droplet size of 0.05% and 0.1% curcumin microemulsion were 40.95 ± 0.26 nm and 43.15 ± 1.22 nm, respectively. All curcumin microemulsion formulations were stabled and showed negative zeta potential. Increased in curcumin microemulsion droplet size would cause by the tendency of curcumin to localize at interface and may partially move the surfactants from the interface, thereby increasing the size [13]. The TEM image of curcumin microemulsion is shown in Fig. 3.

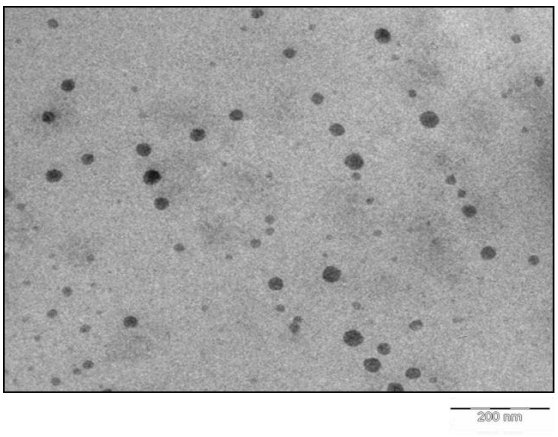

Fig. 3 Morphology of curcumin microemulsion observed using CM12 Philips transmission electron microscope.

IV. CONCLUSION

Curcumin microemulsion was successfully formulated by incorporation of curcumin into VCO microemulsion produced using combination of Tween 80, Span 80 and Span 40. The VCO microemulsion exhibited minimal cytotoxic on L6 cells Therefore, the use of VCO microemulsion was a good candidate as a vehicle to formulate a safe curcumin microemulsion. This study has successfully formulated curcumin microemulsion with desirable characteristics. The optimum of curcumin microemulsion was obtained at 0.1% curcumin loading. Based on the results, it was found that 0.1% curcumin microemulsion showed a micro droplet sized below 100 nm with reasonable PDI that could be used to enhance the curcumin bioavailability and avoid curcumin degradation.

REFERENCES

[1] K. Maiti, K. Mukherjee, A. Gantait, B. P. Saha, and P. K. Mukherjee, "Curcumin-phospholipid-complex: Preparation, therapeutic evaluation and pharmacokinetic study in rats," *International Journal of Pharmaceutics*, vol. 330, pp. 155-163, 2007.

[2] H. K. Nguyen, and V. T Tran, "Formulation of microemulsion-based gel for skin delivery of curcmin," in *Proc. PHARMA INDOCHINA'13, 2013,* pp. 105-110, 2013.

[3] D. C. Maria, M. Evgenia, Y. Anan, X. Aristotelis, and P. Vassiliki, "Formulation and characterization of food-grade microemulsions as carriers of natural phenolic antioxidants," *Colloids and Surfaces A: Physicochemical and Engineering Aspects,* vol. 483, pp. 130-136, 2015.

[4] J. Flanagan, and H. Singh, "Microemulsions: a potential delivery system for bioactivein food," *Critical Reviews in Food Science and Nutrition*, vol. 4, pp. 221–237, 2006.

[5] Y. H. Tsai, Y. H. Hsieh, Y. B. Huang, J. S. Chang, and C. T. Huang, "Microemulsions for intravesical delivery of gemcitabine," *Chemical and Pharmaceutical Bulletin*, vol. 58(11), pp. 1461-1465, 2010.

[6] C. R. Laili, S. Hamdan, and N. Kamaruddin, "Weight Loss in Microemulsion with Eugenol Oil," *Oriental Journal of Chemistry*, vol. 32, pp. 2701-2706, 2016.

[7] S. Yuwanti, S. Raharjo, P. Hastuti, and Supriyadi, "Formulasi mikroemulsi minyak dalam air (o/w) yang stabil menggunakan kombinasi tiga surfaktan non ionik dengan nilai HLB rendah, tinggi, dan sedang," *Agritech*, vol. 31(1), pp. 21-29, 2011a.

[8] R. Verma, G. N. Darwhekar, A. Gupta, and P., Sharma, "Design and development of microemulsion drug delivery system of felodipine for improvement of oral bioavailability," *International Journal of Pharmacy and Life Sciences*, vol. 8(4), pp. 5511-5517, 2017.

[9] X. Wang, S. Yuan, J. Wang, P. Lin, G.Liu, Y. Lu, J. Zhang, W. Wang, and Y. Wei. "Anticancer activity of litchi fruit pericarp extract against human breast cancer *in vitro* and *in vivo," Toxicolology and Applied Pharmacology*, 215(2), pp.m168-178, 2006.

[10] D. M. Coder, "Studies of cell function," *Current Protocols in Cytometry*, 1997.

[11] V. Fischer, J. Marcus, D. Touraud, O. Diat, and W. Kunz, "Toward surfactant-free and water-free microemulsions," *Journal of Colloid and Interface Science*, vol. 453, pp. 186–193, 2015.

[12] V. P. Chavda, M. M. Soniwala, and J. R. Chavda. "Formulation development and evaluation of mefenamic acid microemulsion," *Indian Journal of Research in Pharmacy and Biotechnology*, vol. 3(4), pp. 2320-3471, 2015.

[13] M. C. Bergonzi, R. Hamdouch, F. Mazzacuva, B. Isacchi, and A. R. Bilia, "Optimization, characterization and in vitro evaluation of curcumin microemulsions," *Food Science and Technology*, vol.59, pp. 148-155, 2014.

[14] M. R. Patel, R. B. Patel, J. R. Parikh, A. B. Solanki, and B. G. Patel, "Effect of formulation components on the *in vitro* permeation of microemulsion drug delivery system of Fluconazole," *AAPS PharmSciTech*, vol. 10, pp. 917-923, 2009.

[15] N. Sharma, V. Antil, and S. Jain, "Microemulsion: A review," *Asian Journal of Pharmaceutical Research and Development*, vol. 1(2), pp. 23–36, 2013.

[16] Y. Xiao, X. Chen, L. Yang, X. Zhu, L. Zou, F. Meng, and Q. Ping "Preparation and oral bioavailability study of curcuminoid-loaded microemulsion," *Journal of Agricultural and Food Chemistry*, vol. 61, pp. 3654−3660, 2013.