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Chaulmoogra Oil Nanoemulsion and in Vitro Anti-Cancer Studies Using MCF 7 Cell Line

Seema Gadge[1], Priyanka Varade[2], Rini Punathil[3], Karthika Gouthaman[4], Jincy Joy[5]

[1]Assistant Professor, Department of Pharmaceutical Quality Assurance, NCRD's Sterling Institute of Pharmacy, Nerul, Navi Mumbai, Maharashtra, India-400706
Email: *seemaauti10[at]email.com*

[2]Assistant Professor, Department of Pharmaceutical Chemistry, MGM School of Pharmacy, Kamothe, Navi Mumbai, Maharashtra, India
Email: *priyankavarade2016[at]gmail.com*

[3]Assistant Professor, Department of Pharmaceutics, St. Wilfred's Institute of Pharmacy, Shedung, Panvel, Maharashtra, India-410206
Email: *rini.punathil[at]gmail.com*

[4]Assistant Professor, Department of Pharmaceutics, St. Wilfred's Institute of Pharmacy, Shedung, Panvel, Maharashtra, India-410206
Email: *nairkarthika212[at]gmail.com*

Abstract: ***Nanotechnology comprises technological developments on the nanometer range of usually 0.1 -100nm. The pharmaceutical developed on the basis of nanotechnology is referred to as "NANOPHARMACEUTICALS". Various nanopharmaceuticals currently being used or in the process of development are Nanoemulsions, Nanosuspensions, Nanospheres, Nanotubes, Nanoshells, Nanocapsules, lipid nanoparticles and dendrimers. Chaulmoogra oil Nanoemulsion was prepared by High pressure homogenisation technique. In this method, homogenisation involves the forcing of the emulsion under pressure through a valve having a narrow aperture. Nanoemulsions are thermodynamically and physically stable systems and are formed at a particular concentration of oil, surfactant and water, making them stable to phase separation, creaming or cracking. Thus, the formulations were tested for their physical stability using centrifugation, heating-cooling cycle and freeze-thaw cycle. The particle size of Chaulmoogra oil Nanoemulsion was found to be 200 – 800(d.nm). The Zeta potential values of the formulated Nanoemulsion were obtained as -0.0666mV and -0.0327mV. The viscosity of Nanoemulsions was found to be low as the consistency should be low for better application of the Nanoemulsion in topical administration. The formulated Chaulmoogra oil Nanoemulsion were screened for antimicrobial activity against both gram positive and gram negative organisms by cup-plate method. The Chaulmoogra oil Nanoemulsion exhibited a significant antioxidant activity against DPPH method at 10% V/V. The antioxidant activity of the samples showed a direct dose-response relationship. In-vitro cytotoxic activity against human breast cancer cell line MCF-7 was evaluated at different concentration (0.1% V/V, 1%V/V and 10% V/V) by MTT assay. The in-vitro screening of the formulated nanoemulsions showed potent cytotoxic activity against the human breast cancer cell line. The cytotoxicity analysis of the samples showed a direct dose-response relationship; cytotoxicity increased at higher concentration.***

Keywords: Chaulmoogra oil, Nanoemulsion, Viscosity, High pressure homogenizer, Brookfield viscometer.

1. Introduction

It comes the new technology, Nanotechnology which encompasses technological developments on the nanometer scale. The use of this robust technology in health care has developed tremendously since last few years towards the development of the so called 'nanomedicine'.

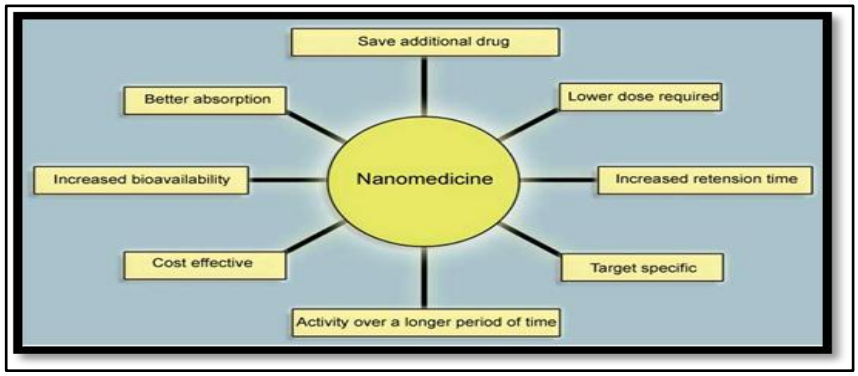

Figure 1: Nanomedicine in Pharmaceutical Application

Nanotechnology comprises technological developments on the nanometer range of usually 0.1 -100nm. The pharmaceutical developed on the basis of nanotechnology is referred to as "NANOPHARMACEUTICALS". Various nanopharmaceuticals currently being used or in the process of development are Nanoemulsions, Nanosuspensions, Nanospheres, Nanotubes, Nanoshells, Nanocapsules, lipid nanoparticles and dendrimers.[1] Among these nanocarriers, nanoemulsion are under extensive investigation as drug

carriers for improving the delivery of drugs in general and for chemotherapeutics in particular.

Nanoparticulate drug delivery systems show a promising approach to obtain desirable drug like properties by altering the Biopharmaceutics and pharmacokinetics properties of the molecule. Apart from the advantages of enhancing potential for systemic administration, nanoparticulate drug delivery systems can also be used for site-specific delivery, thus alleviating unwanted toxicity due to nonspecific distribution, improve patient compliance and provide favourable clinical outcomes.

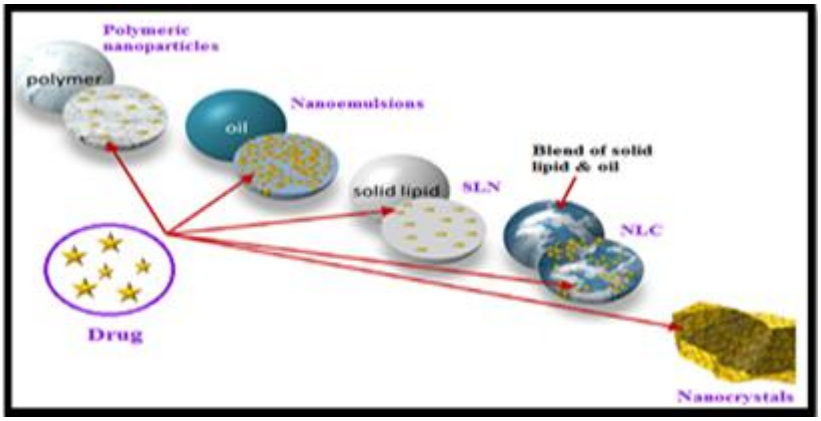

Figure 2: Nanopharmaceuticals

2. Materials and Methods

2.1 Formulation of chaulmoogra oil nanoemulsion

The nanoemulsions were prepared by mixing the oil with the surfactant/ co-surfactant mixture before adding the required amount of water. And then the mixture was undergone High pressure homogeniser for 20 minutes.

Table 1: Selected Nanoemulsion formulations [%(V/V)]

Components	F1	F2	F3
Chaulmoogra oil	10	10	25
Tween 20 (surfactant)	5	10	25
Ethanol (co-surfactant)	5	-	25
Water	80	80	25

2.2 Thermodynamic stability studies

The physical stability of a nanoemulsion formulation depends upon its preparation and mixing ratios of oil phase and aqueous phase. In addition, poor formulation can lead to phase separation, affecting not only formulation performance but also visual appearance. Thermodynamic stability tests were performed to overcome metastable formulation.

- **Centrifugation**
  Selected formulations were centrifuged at 3500 rpm for 30 minutes. The formulations that did not show any phase separation were taken for heating and cooling cycle.
- **Heating and cooling cycle**
  Six cycles between refrigerator temperature (4°C) and 45°C with storage at each temperature of not less than 48 hours were done. The formulations which were stable at these temperatures were subjected to freeze thaw cycle test.
- **Freeze- thaw cycle test**
  Three freeze thaw cycle test were done for the formulation between -21°C and 25°C. The formulations that survived thermodynamic stability tests were selected for further study.

2.3 Characterization of chaulmoogra oil nanoemulsion:

Particle size distribution and zeta potential of chaulmoogra oil nanoemulsion
The average hydrodynamic diameter and Polydispersity index (PDI) of the formulated nanoemulsion were determined by dynamic light scattering (DLS) analysis using Zetasizer Nano ZS90 (Malvern Instruments Limited, UK), 1 ml Chaulmoogra oil nanoemulsion sample dispersion was placed in disposable cuvettes for particle size measurements. Each experiment was conducted in triplicate. The electrophoretic mobility (Zeta potential) measurements were made using the MalvenZetasizer (Nano ZS90, Malvern Instruments) at 25°C.

2.4 Viscosity

The viscosities of the formulated nanoemulsions were measured at different shear rates at different temperatures using Brookfield type rotary viscometer. The temperature of the sample was maintained at room temperature and the sample for the measurement was immersed in the sample holder before testing. The S61 spindle was used at 100 rpm and the viscosity was measured.

2.5 Determination of antibacterial activity: Media used in the study

Media: Nutrient agar
Nutrient broth gelled by the addition of 2% agar (bacteriological grade)

Ingredients

Agar	: 17.5g/L
Peptone	: 300g/L
Sodium chloride	: 1.5g/L
Yeast extract	: 2.0g/L
Beef extract	: 1.0g/L

Final pH at 25^0c : 7.4±0.2

Preparation:
The ingredients were dissolved in distilled water with the aid of heat and pH was adjusted to7.2-7.6 using dilute alkali or acid.

Sterilization
Nutrient agar were transferred to conical flask plugged with nonabsorbent cotton and then autoclaved at a pressure of 15psi for not less than 15 minutes.

Culture used
The strains of Bacillius subtilis NCIM 2025, Staphylococcus aureus NCIM 5021, Escherichia coli NCIM 2911 and Pseudomonas aeruginosa NCIM 5029 were procured from National Collection of Industrial Microorganisms, Pune and stored in the pharmaceutical Microbiology and biotechnology Laboratory, St.James College of Pharmaceutical Sciences, Chalakudy, Kerala.
Samples used: Chaulmoogra oil (Standard) and Nanoemulsion formulations
Concentration used: 10µl.

Maintenance of culture
The selected strains were confirmed for their purity and identify by gram staining method by their characteristics biochemical reactions. The strains were preserved by sub culturing them periodically by nutrient agar slants and storing them under frozen condition. For the study fresh 24 hours broth cultures were used after standardization of the culture.

Antibacterial screening by Cup- plate method:
Preparation of agar plates.The Petri dishes which measured around 8.5 cm diameters and 2 cm thickness were selected after sterilizing by dry heat in an oven. Base layer was obtained by pouring around 20–30 ml of Nutrient Agar solution to obtain a thickness of 4 mm. It was then kept for solidification.

Experimental Procedure
- The sterile borer was used to prepare three cups of 8 mm diameter, in the medium of each Petri dish. One cup was made for standard drug; its zone of inhibition was measured to compare with the zone of inhibition of the test sample
- Underneath of the petridish, standard was labeled as "standard", Nanoemulsion formulation F1 was labeled as "NE1" and Nanoemulsion formulation F2 was labeled as "NE2".
- Standard, NE1 and NE2 samples were introduced with the help of a micropipette at a concentration of 10 µL in each well.
- All the plates were kept at 4°C for effective diffusion of the standard drug and samples NE1 and NE2.
- Then, they were incubated at 37 ± 1°C for 24 hours.
- The presence of definite zones around the cup of any size indicated antibacterial activity.
- The diameter of the zone of inhibition was measured and recorded

2.6 *In vitro* antioxidant activity of Chaulmoogra oil Nanoemulsion

The antioxidant activity was determined using the 2, 2-diphenyl-1-picryl hydrazyl (DPPH) free – radical scavenging method. Free radical scavenging activity of all the formulated nanoemulsions were determined by DPPH assay method and Curcumin used as standard.

Chemicals used
- 2.2-diphenyl-1-picryl hydrazyl (DPPH)
- Methanol
- Curcumin

Preparation of solutions:

Preparation of 1000µg/mL stock solution of nanoemulsion
10mg of the formulation were taken and dissolved in methanol. The volume was made up to 10mL with methanol.

Preparation of 0.1% V/V, 1% V/V and 10% V/V nanoemulsion.
From the above solution 1.0mL aliquot was transferred to a 10mL standard flask and the volume was made up to 10mL with methanol for 10%v/v solution. From the stock solution 1.0mL and 0.1mL aliquots were transferred to a 100mL standard flask and the volume was made up to 100mL with methanol for 1%v/v and 0.1%v/v solution.

Preparation of 0.2mM DPPH solution
0.00789g of DPPH was taken in a 100mL standard flask and dissolved in 100mL of methanol. The final volume was made up to 100ml with methanol.

Preparation of 0.1% V/V, 1% V/V and 10% V/V standard solution:
10 mg of curcumin was taken in a 10mL standard flask and dissolved in methanol. The volume was made up to 10mL with methanol. From this solution 1.0mL aliquot was transferred to a 10mL standard flask and the volume was made up to 10mL with methanol for 10%v/v solution. From the stock solution 1.0mL and 0.1mL aliquots were transferred to a 100mL standard flask and the volume was made up to 100mL with methanol for 1%v/v and 0.1%v/v solution.

Procedure for the evaluation of antioxidant activity
1.5ml of 2.0mM of DPPH solution was added to 1.5mL of different concentration of the extract solution. Another series of solution were prepared by taking 1.5mL of methanol and 1.5ml of different concentrations of extract solution. The above solutions were allowed to react at room temperature for 30min. after 30 min the absorbance values were measured at 517nm. Curcumin was used as the reference standard.

Percentage of scavenging activity was calculated by using the following formula.

$$\% \text{ scavenging} = \frac{(Ab+As)-Am}{Ab} \times 100$$

Ab= absorbance of 1.5mL of DPPH +1.5mL of methanol at 517nm.
Am= absorbance of 1.5mL of DPPH +1.5mL of extract solution at 517nm.
As= absorbance of 1.5mL of metanol+1.5mL of extract solution at 517nm.

2.7 *In vitro* cytotoxicity of chaulmoogra oil nanoemulsion

MTT assay: The 3-(4,5 – dimethylthiazol-2,5-diphenyltetrazolium bromide) dye reduction assay was conducted to diagnose the cyto-toxic activity of the formulated nanoemulsion. MCF7 cells were plated onto 48 wells plates, 18 hours before the commencement of the test. Growth medium used was DMEM with 10% Fetal Bovine Serum. The plates were incubated in an animal cell culture incubator, maintained at 37^0C with 5% carbon dioxide. The wells achieved 70% confluency at the time of testing. The original growth medium in the 48 well plates was removed and the samples prepared above were added to the wells. The plates were returned to the incubator for 96 hours. At the end of 96 hours, the media in the wells were carefully removed and fresh complete growth media was added. To each well MTT solution (5 mg/ml of MTT dissolved in PBS) was added and replaced in the incubator for 3 hours. After 3 hours, the medium was carefully removed from the wells, and DMSO was added to each well and kept on a rocking platform for efficient mixing and extraction of formazan dye from the cells by DMSO. After 30 minutes, the absorbance of the DMSO was measured at 570 nm, in a multi-well spectrophotometer.

The average absorbance of the "control" wells was taken as 100% and all other absorbance values were calculated based on this and plotted on the graph.

3. Results and Discussion

3.1 Chaulmoogra oil nanoemulsion

Chaulmoogra oil Nanoemulsion was prepared by High pressure homogenisation technique. In this method, homogenisation involves the forcing of the emulsion under pressure through a valve having a narrow aperture. The homogenizer instrument was operated at 15,000 bar pressure to produce the Chaulmoogra oil Nanoemulsion (CNE). Therefore, this study indicated that homogenisation method is cost effective and could be suitable for large scale production.

3.2 Thermodynamic stability studies of chaulmoogra oil nanoemulsion

Nanoemulsions are thermodynamically and physically stable systems and are formed at a particular concentration of oil, surfactant and water, making them stable to phase separation, creaming or cracking. Thus, the formulations were tested for their physical stability using centrifugation, heating-cooling cycle and freeze-thaw cycle. Only those formulations which survive the thermodynamic stability tests were selected for further study.

Table 2: Thermodynamic stability studies of formulated Nanoemulsions

	F1	F2	F3
Centrifugation	✓	✓	✓
Heating and cooling cycle	✓	✓	-
Freeze – thaw cycle	✓	✓	-

The thermodynamic stability studies showed that the F1 and F2 formulations were physically stable than the F3 formulations.

3.3 Particle size distribution and Zeta potential of chaulmoogra oil nanoemulsion:

The particle size of Chaulmoogra oil Nanoemulsion was found to be 200 – 800(d.nm). Nanogrinding is a critical process which used to obtained appropriate particle size reduction and stability of nanoemulsion. The mean particle size and width of the particle size distribution are important characterization parameters as they govern the saturation solubility, dissolution velocity and physical stability.

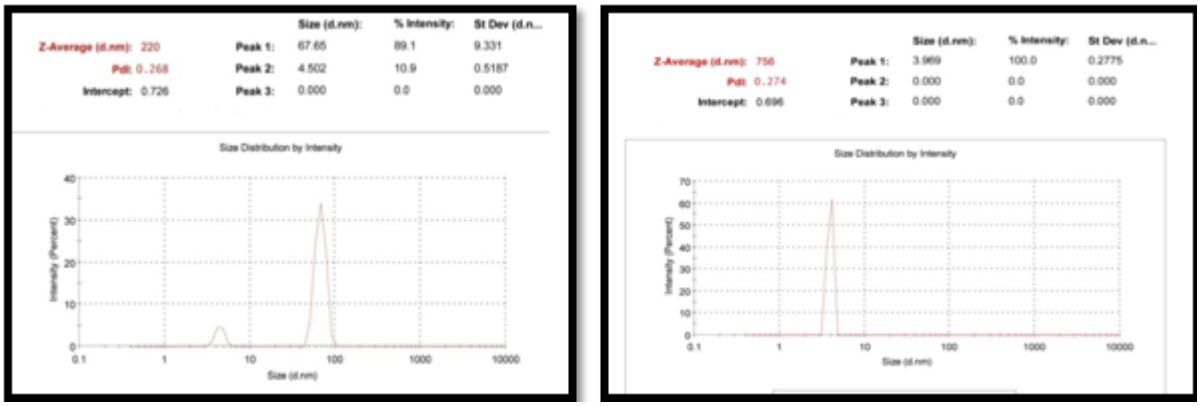

Figure 3: The particle size distribution of F1 & F2 formulation was found to be 220 & 756d.nm

The PDI value obtained is a measure for width size distribution and ranges from 0 to 1. The values near to zero indicate monodispersed particles whereas values >0.5signifies a very broad size distribution. The PDI values obtained were 0.268 and 0.274 of F1 and F2 formulations respectively. The PDI remained below 0.3 which reflects their relative homogeneity. The Zeta potential is caused by the net electric charge contained within the region bounded

by the slipping plane, and also depends on the location of the plane. The determination of Zeta potential of a nanoemulsion is essential as it gives an idea about the physical stability. The Zeta potential values of the formulated Nanoemulsion were obtained as -0.0666mV and -0.0327mV.

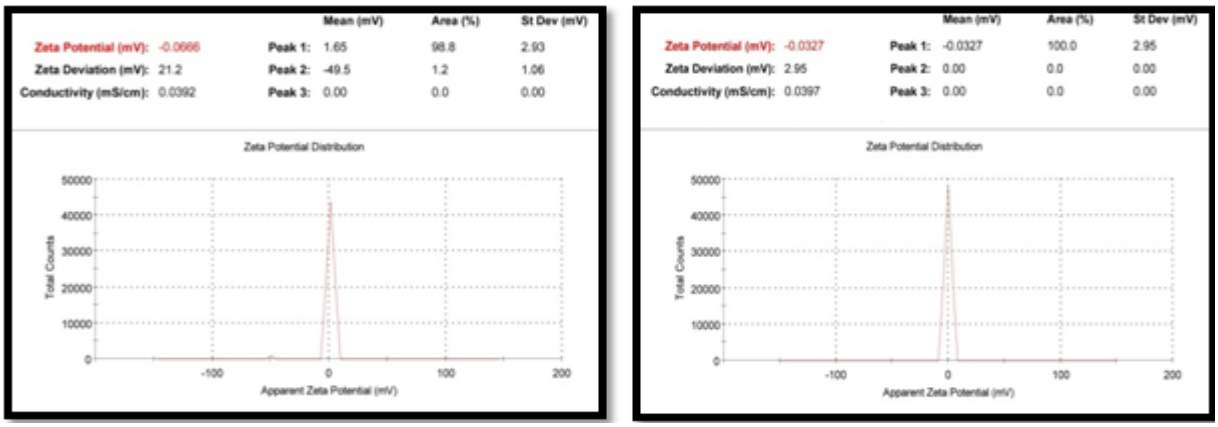

Figure 4: The zeta potential of the F1 & F2 formulation was found to be -0.0666 & -0.0327 mV

3.4 Viscosity

The formulations showed similar viscosity measurement. This is because the compositions of the formulations did not very much. The viscosity was found to be low.

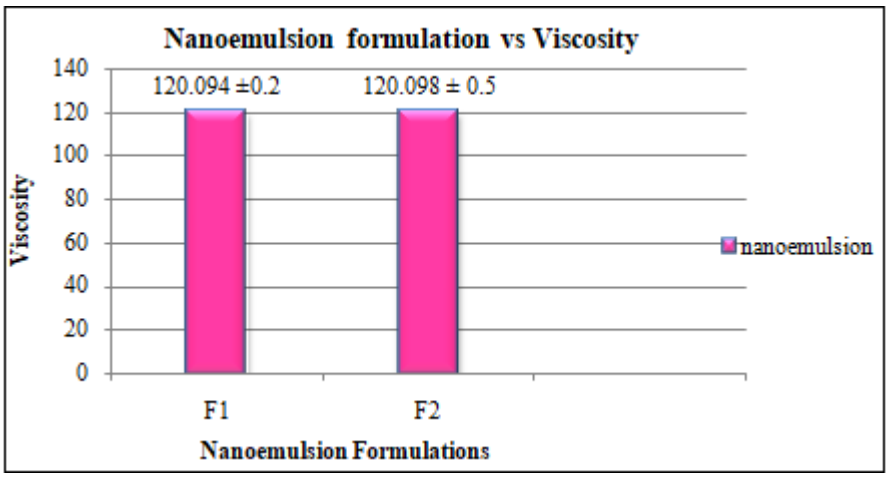

Figure 5: Viscosity study of Nanoemulsion formulations

The viscosity of Nanoemulsions was found to be low as the consistency should be low for better application of the Nanoemulsion in topical administration.

3.5 Anti- microbial studies of chaulmoogra oil nanoemulsion

The formulated Chaulmoogra oil Nanoemulsion were screened for antimicrobial activity against both gram positive and gram negative organisms by cup-plate method.

Gram positive organism	Gram negative organism
Pseudomonas aeruginosa NCIM 5029	*Pseudomonas aeruginosa* NCIM 5029
Escherichia coli NCIM 2911	*Escherichia coli* NCIM 2911
Staphylococcus aureus NCIM 5021	
Bacillus subtilis NCIM 2025	

Table 3: Zone of inhibition for nanoemulsion formulations

	Inhibition zone (in mm)			
	P. aeruginosa	*E.coli*	*S. aureus*	*B. subtilis*
Standard (Chaulmoogra oil)	21	19	15	15
F1	20	18	14	14
F2	18	15	13	10

Table 4: Percentage of antimicrobial potency

	Percentage of antimicrobial Potency			
	P. aeroginosa	*E.coli*	*S. aeurus*	*B. subtilis*
F1	99.26	99.18	98.96	98.96
F2	97.69	96.41	97.85	94.01

Percentage Potency = Antilog 2.0 + a log I

Where, $\mathbf{a} = \frac{(U1+U2)-(S1+S2)}{(U1-U2)+(S1-S2)}$

The F1 and F2 formulation showed considerable antimicrobial activity against the gram positive and gram negative bacteria. The percentage of antimicrobial potency for standard, chaulmoogra oil was considered to be 100%.

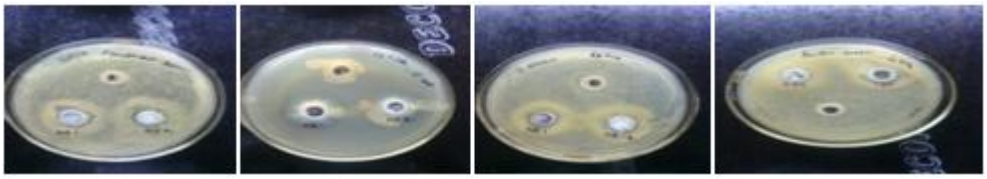

Figure 6: Effect of Nanoemulsion against gram positive bacteria (P. aeroginosa and E. coli) and gram negative bacteria (S. aeurus and B. Subtilis)

3.6 Antioxidant activity of chaulmoogra oil nanoemulsion

Antioxidant activity can be evaluated by scavenging of the stable DPPH radical. This model is extensively used as it is less time consuming than the other methods. DPPH can accept an electron and hydrogen radical and can be converted into a stable diamagnetic molecule. DPPH contain an odd electron, and so it has a strong absorption at 5171nm. When this electron becomes pairs off, the absorption decreases stoichiometric ally with respect to the electron taken up. Such change in the absorbance produced in this reaction has been widely applied to assess the capacity of numerous molecules to act as free radical scavengers.

Table 5: Antioxidant activity of Curcumin by DPPH method

Extract	Concentration (% V/V)	Absorbance of Curcumin + DPPH (517nm)	Absorbance of Curcumin+ methanol (517nm)	% Scavenging
Curcumin	0.1	0.280	0.013	73.08
	1	0.138	0.015	87.06
	10	0.073	0.019	94.55

Table 6: Antioxidant activity of formulated Nanoemulsions

	Concentration (%V/V)	Absorbance of NE + DPPH (517nm)	Absorbance of NE + Methanol (517nm)	% Scavenging
F1	0.1	0.232	0.054	82.05
	1	0.105	0.059	95.36
	10	0.092	0.063	97.88
F2	0.1	0.243	0.056	81.14
	1	0.105	0.058	95.26
	10	0.097	0.064	96.67

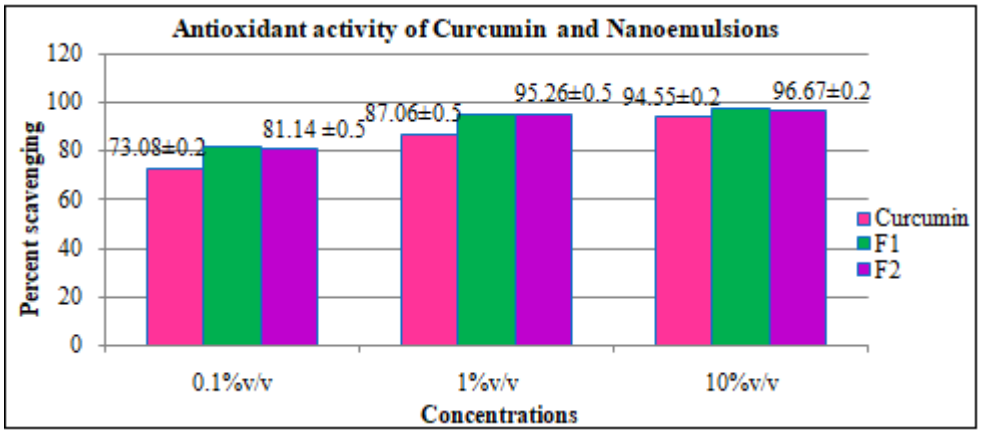

Figure 7: Antioxidant activity of Nanoemulsion formulations compared with Curcumin

The Chaulmoogra oil Nanoemulsion exhibited a significant antioxidant activity against DPPH method at 10% V/V. The antioxidant activity of the samples showed a direct dose-response relationship.

3.7 *Invitro* cytotoxicity studies of chaulmoogra oil nanoemulsion

In-vitro cytotoxic activity against human breast cancer cell line MCF-7 was evaluated at different concentration (0.1% V/V, 1%V/V and 10% V/V) by MTT assay. The *in-vitro* screening of the formulated nanoemulsions showed potent cytotoxic activity against the human breast cancer cell line. The cytotoxicity analysis of the samples showed a direct dose-response relationship; cytotoxicity increased at higher concentration. The average absorbance of the "control" wells was taken as 100% and all other absorbance values were calculated based on this and plotted on the graph.

Table 7: % Cytotoxicity of formulated nanoemulsions

	Concentration (%V/V)	Percentage Cytotoxicity
F1	0.1	7.93
	1	7.95
	10	13.89
F2	0.1	7.94
	1	7.92
	10	14.62

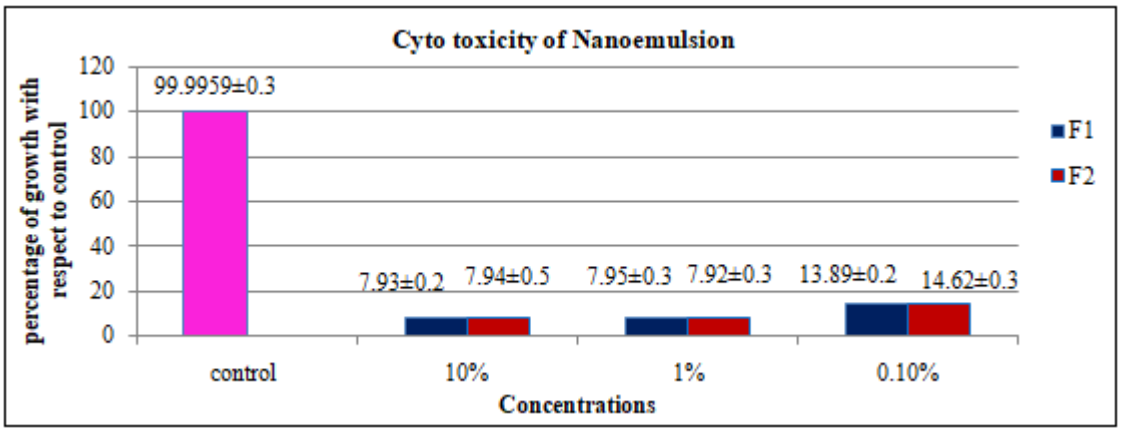

Figure 8: % Cytotoxicity of formulated nanoemulsions with respect to control

The average absorbance of the "control" wells was taken as 100% and all other absorbance values were calculated based on this and plotted on the graph. Formulated Nanoemulsions of 10% v/v showed potent cytotoxicity.

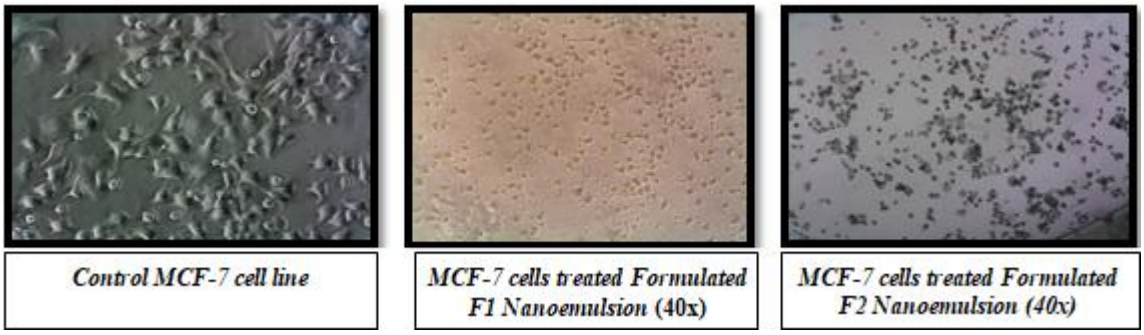

Figure 9: *In vitro* cytotoxicity study of formulated nanoemulsions (F1 and F2) with control

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