An Innovative Breast Cancer Treatment Using Turmeric Leaf Oil Encapsulated Nanoemulgels

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ABSTRACT

There are several uses for turmeric oil made from the leaves in the food and pharmaceutical industries. The effectiveness of turmeric leaf oil in treating cancer has been documented in numerous trials. The best technique to encapsulate it is via nanoemulsion technology, which can overcome its drawbacks including instability and a hydrophobic character that inhibits its biological activity. The objective of this research was to develop a nanoemulgel containing ethanol as a co-surfactant, tween 80 as a surfactant, and turmeric leaf oil (TLO) as a surfactant in order to describe it and evaluate its anticancer potential against the MCF-7 (human breast cancer) cell line. With an entrapment efficiency range of 67.72–85.13 percent, the mean particle size was 175.4 nm. According to the results of the in-vitro cytotoxicity study, TLO nanoemulgel had 28.56% cell viability after 24 hours and an IC50 value of 59.84 g/ml.

KEYWORDS: Turmeric Leaf Oil, MCF-7

INTRODUCTION

Breast cancer is the most common tumour in women population, with an incidence of 11.6 % and a mortality rate of 6.6 %. Although chemotherapy is regarded as one of the main strategies for breast cancer treatment, several disadvantages including, multidrug resistance, adverse side effects and low tumour cell specificity, often result in poor treatment efficacy.[1]Nanotechnology is a multidisciplinary approach which entails the development and utilization of different systems on a non-metric scale. The formulation of nanoemulsion that is able to decrease the droplet size in the emulsion, which can promote very small droplet diameter, increased physical stability, high bioavailability and optical transparency.[2] Being an oily preparation, the therapeutic efficacy of the oil has also been addressed in many such nanoemulsions (NE), in which the oily phase itself is therapeutically active. Since the NE system has the droplet size in a nano range from 20 to 200 nm. The nanosized particles leading to greater interfacial area would influence the transport properties of the drug, increasing the absorption and distribution, enhancing bioavailability, protection from toxicity, enhancement of stability and therapeutic activity and protection from chemical decomposition by their thermodynamic stability and small droplet size.[3] The challenges of nanoemulsion are low viscosity, low spreadability and low

retention time when intended for topical application. These issues are being addressed by incorporating the NE into gel system. For example, nanoemulsion of Mentha piperita essential oil was formulated and its anti- cancer effects was studied.[4] A nanoemulgel is a reservoir that is formed on the addition of a nanoemulsion to the hydrogel matrix, such as Carbopol, which increases the thickness, reduces the interfacial tension and develops stability.

The investigation of essential oil anticancer properties has received more attention. Curcuma longa L. (Zingiberaceae) is a perennial plant. The extensive reports in the literature about the rhizomes of C. longa have revealed various pharmacological activities. After harvesting the rhizome parts, aerial parts of this plant are considered as waste product. However, several available publications on the biological properties of aerial parts of C. longa have indicated that the turmeric aerial parts are beneficial for health.[5]Further investigations states that turmeric leaf oil can be used for cancer treatment.[6]In breast cancer, some subtypes have been proposed based on hormone receptor (HR) and human epidermal growth factor receptor (HER2). Luminal A (HR+, HER2) is the most common subtype of breast cancer. In this study, the anticancer effect of turmeric leaf oil loaded nanoemulgel on human breast cancer cell line, MCF-7 has been studied.[7] In this present study, we have formulated TLO nanoemulgel by ultrasonication. Additionally, we used an in vitro system to evaluate the anticancer activity of TLO nanoemulgel on MCF-7 human breast cancer cells.

MATERIALS AND METHOD

Materials

Turmeric leaf oil (TLO) was purchased from Master Organics, Uttarkhand. Tween 80, Ethanol and Triethanolamine was purchased from Nice chemicals Private Limited, Chennai. DMEM medium, Fetal Bovine Serum (FBS) and antibiotic solution were from Gibco (USA), DMSO (Dimethyl sulfoxide) and MTT (3-4,5 dimethylthiazol-2yl-2,5-diphenyl tetrazolium bromide) (5 mg/ml) wereprovided by Sigma, (USA), 1X PBS was from Himedia, (India). 96 well tissue culture plates and wash beakers were from Tarson (India). MCF-7 Cells (Human Breast Cancer Cells) was purchased from NCCS, Pune.

NANOEMULSION FORMULATION

The nanoemulsion of Turmeric leaf oil was formed using the oil, water and surfactant and co-surfactant. The non-ionic surfactant used in this study– Tween 80– exhibits high solubility in essential oils and coalescence with the essential oil droplets, which boosts the stability of the system. Preparation of the nanoemulsions in this study was a twofold process, where the initial step was formation of emulsions by mixing the three key components, namely – oil, water and surfactant, at varying oil: surfactant ratios – 1:1, 1:2, and 1:3. Another set of formulations with key components oil, surfactant,co-surfactant (ethanol) and water at varying oil: surfactant: co-surfactant ratios– 1:1:1, 1:2:1, and 1:3:1. They were mixed using a magnetic stirrer rotating at a speed of 500 rpm for 30 min. These emulsions were then made into their respective nanoemulsions using a probe sonicator.[8]

Formulation of TLO Nanoemulgel

Carbopol 940 was selected as a gelling agent and was dispersed in small amount of water and allowed to swell overnight. Triethanolamine was added in drops under magnetic stirring to form nanoemulgel.

Table 1: Ingredients of nanoemulsion formulation

S.NO	FORMULATION	RATIO	OIL (ml)	SURFACTANT (ml)	COSURFACTANT (ml)	WATER (ml)
1.	F1	1:1:1	6	6	6	82
2.	F2	1:2:1	6	12	6	76
3.	F3	1:3:1	6	18	6	70
4.	F4	1:1	6	6	-	88
5.	F5	1:2	6	12	-	82
6.	F6	1:3	6	18	-	76

NANOEMULSION AND NANOEMULGEL CHARACTERIZATION

Optical Transparency

Optical transparency of the formulations was determined by inspecting the samples in clear and transparent container under the presence of good light against reflection into the eyes and viewed against black and white illuminated background.[9]

Thermodynamic Stability Studies

Thermodynamic stability studies are often carried out in two steps.

- Firstly, heating-cooling cycle, which is performed for observing any influence on the stability of Nanoemulsion by varying temperature conditions. Nanoemulsion is exposed to six cycles between 4°C (freeze temperature) and 40°C by storing the formulation at each temperature for not less than 48 h. The formulations which are stable at these temperatures are further chosen for centrifugation studies.
- Secondly, centrifugation study in which the formulated nanoemulsion are centrifuged at 3000 rpm for 30 minutes and observed for phase separation or creaming or cracking.[10]

Dilution test

Test for dilution was performed in order to observe for the phase inversion of the nanoemulsion. For this 1 ml of optimized nanoemulsion was diluted in 10 ml of water and observed for phase inversion.[10]

Particle Size Determination

The average mean diameter and size distribution of loaded nanoparticles is found by Dynamic Light Scattering method using Malvern zeta sizer at 25°C.The dried nanoemulsion were dispersed in water to obtain proper light scattering intensity for mustard oil nanoemulsion.[11]

Morphological studies of nanoemulsion

The nanoemulsion was subjected for microscopic examination y scanning electron microscopy (SEM) for characterizing size and shape.

Measurement of pH

Since the formulation was a topical formulation to be applied to the skin, therefore pH measurement was essential to ensure nonirritating nature of the formulation. The pH of the formulation was

determined at ambient temperature with digital pH meter. 2.5 gm of the formulation was accurately weighed and dispersed in 25ml of distilled water. The pH of this dispersion was measured by using digital pH meter.[12]

Spreadability

The formulated nanoemulgel spreadability was measured by determining the spreading diameter of preparation between the two glass plates after 1 min. A 400 mg nanoemulgel was weighed and placed on a 1 cm premarked circle on a glass plate and the second plate was placed on over it when weight was increased on the upper plate, and increased diameter of the gel was noted and calculated by using the following equation:

$S = m \times l / t$

Where,

S = spreadability of nanoemulgel,

m = weight placed on the upper glass plate,

l = length upper of the glass plate and

t = time taken.[12]

Entrapment efficiency

About 3ml of nanoemulsion was centrifuged for 1 hour. The entrapment efficiency (EE) was determined by measuring the amount of active ingredient in water layer obtained after centrifugation. The EE was calculated using the equation,

EE% = (Total concentration – concentration in water layer) / Total concentration x 100 [13]

In-vitro diffusion study

The in-vitro release rate of all the formulations of the nanoemulgel were evaluated by open ended tube through using phosphate buffer pH 7.4 as diffusion medium upto 8 hours. Egg membrane was soaked in phosphate buffer pH 7.4 for 24 hrs prior to use. 1g of the formulation was placed in the treated membrane and was tied in one end of the tube and then immersed in the receptor compartment containing 400 ml of pH 7.4 buffer solution and was stirred at 100 ± 10 rpm and maintained at 37℃ ± 2℃. Every hour for 8 hours, 5 ml aliquots are removed each hour from diffusion medium and replaced with same amount of freshly prepared phosphate buffer pH 7.4. The samples were analyzed spectrophotometrically at 245 nm using Shimadzu 1700 Double beam Spectrophotometer.[14]

Drug release kinetics

The mechanism of TLO release from nanoemulgel was studied by fitting diffusion data of formulation in the following model dependent kinetics such as Zero order, First order, Higuchi and Korsmeyer - Peppas equation.[15]

CYTOTOXICITY STUDIES

MTT assay

MCF-7 cells were used to assess the TLO sample for in vitro cytotoxicity using the MTT assay. Briefly, the cultured MCF-7 Cells were harvested by trypsinization, pooled in a 15 ml tube. Then, the cells were plated at a density of 1×105 cells/ml cells/well (200 µL) into the 96-well tissue culture plate in a

DMEM medium containing 10 % FBS and 1% antibiotic solution for 24-48 hour at 37°C. The wells were rinsed with sterile PBS and treated with different concentrations of the TLO sample in a serum-free DMEM medium. Each sample was replicated in triples and the cells were incubated at 37°C in a humidified 5% CO2 incubator for 24 h. After incubation, MTT (20 µL of 5 mg/ml) was added into each well and the cells were incubated for another 2-4 h until purple precipitates were clearly visible under an inverted microscope. Finally, the medium together with MTT (220 µL) were aspirated off the wells and washed with 1X PBS (200 µl). Furthermore, to dissolve formazan crystals, DMSO (100 µL) was added and the plate was shaken for 5 min. The absorbance for each well was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, USA) and the percentage cell viability and IC50 value was calculated using Graph Pad Prism 6.0 software (USA).[16,17]

RESULTS

Table 2: Thermodynamic stability studies of formulated nanoemulsion

Formulationcode	Heat- coolingcycle	Centrifugation
F1	Instable	Instable
F2	Stable	Instable
F3	Stable	Instable
F4	Instable	Instable
F5	Stable	Stable
F6	Stable	Stable

Dilution test

Dilution test was performed on all six formulations and is visually checked for phase separation and clarity. F6 did not show any sign of phase inversion and any kind of precipitation.

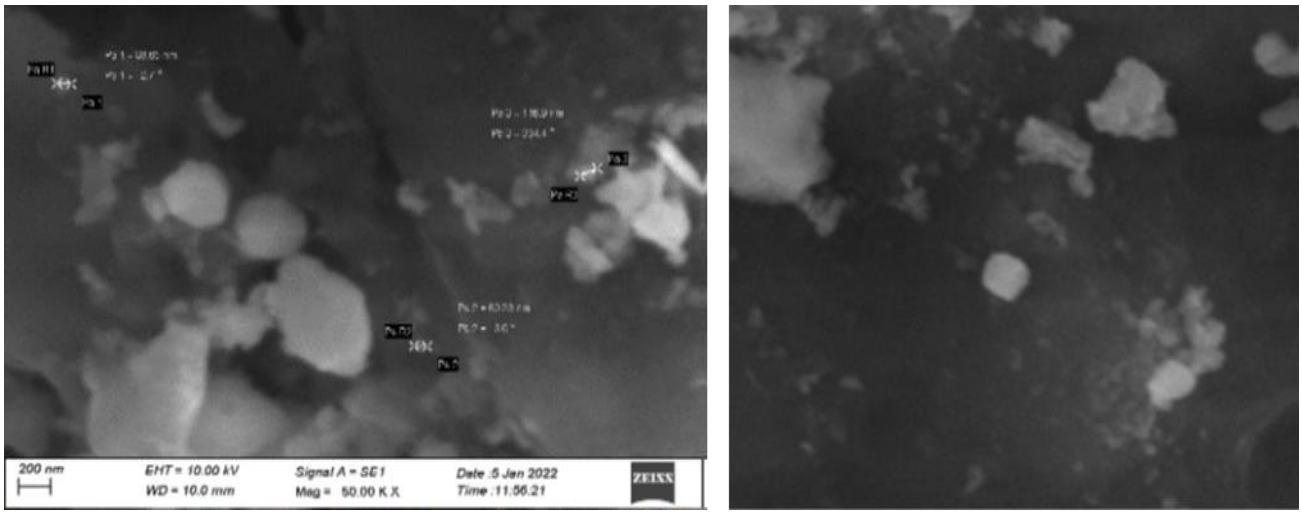

Fig 1: SEM images of F6 nanoemulsion

Table 3: Optical transparency, pH, Spreadability, Stability and % entrapment efficiency of all formulated nanoemulsion and nanoemulgel.

FORMULATION CODE	OPTICAL TRANSPARENCY	pH		SPREADABILITY (cm)	STABILITY	ENTRAPMENT EFFICIENCY %
		NE	**NEG**			
F1	Transparent	5.5	5.6	6.5	Instable	67.72
F2	Transparent	5.5	5.8	6.2	Instable	76.23
F3	Transparent	5.9	6.2	5.5	Instable	76.21
F4	Transparent	5.4	5.6	6.5	Instable	80.41
F5	Transparent	5.9	5.6	6.5	Stable	83.55
F6	Transparent	6.2	6.4	6.8	Stable	85. 13

Table 4: Percentage release of nanoemulgel through diffusion (F1-F6)

Formulation code	Percentage release
F1	75.88±0.02
F2	75.88±0.02
F3	75.05±0.35
F4	75.04±0.02
F5	69.65±0.52
F6	89.98±0.22

Table 5: Release Kinetics of optimized formulation F6

Formulation code	Zero order	Higuchi	Peppas		First
	r^2	r^2	r^2	N	r^2
F6	0.9848	0.8527	0.9844	1.125	0.8349

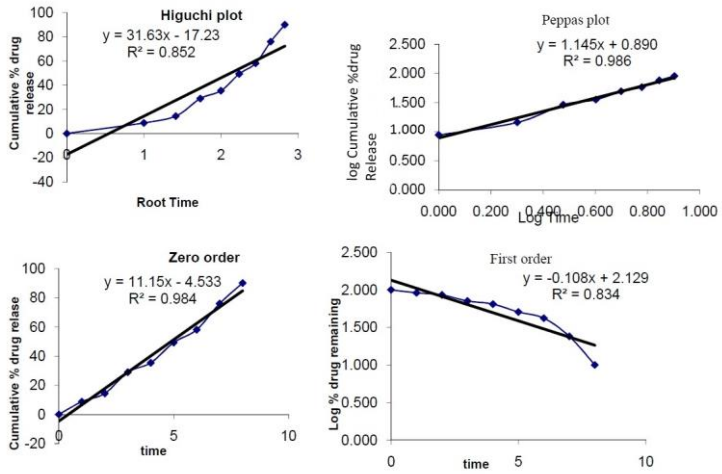

Fig 2: Release kinetics of F6 formulation

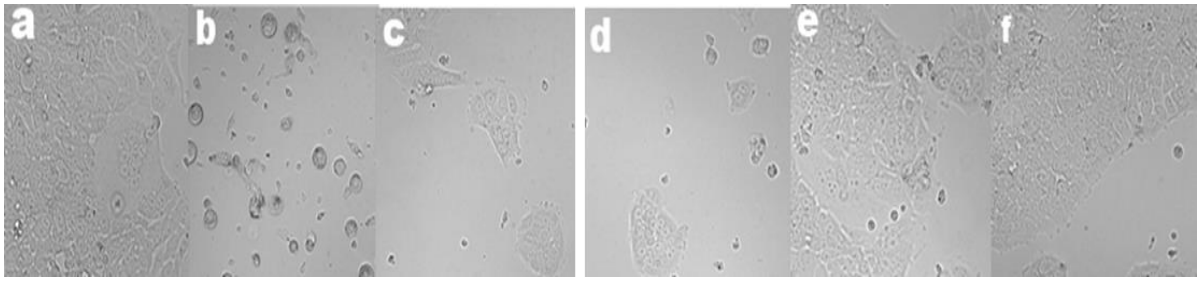

Fig 3: Images showing cell viability with F6 formulation (500, 400, 300, 100, 80, 10 µg/ml)

DISCUSSION

The leaves of Curcuma longa are considered as waste product during post-harvest operations. Traditionally, the leaves are extensively used in culinary preparations, they are aromatic and contains essential oil. The physiochemical characteristics of the turmeric leaf extract demonstrates its functional effects such as anti-cancer, antioxidant, antifungal, skin whitening and antibacterial activities. Turmeric leaves have a wide surface area and volume.

Table 6: Tested sample evaluation value

S. No	Tested sample concentration (µg/ml)	Cell viability (%)(in triplicates)			Mean Value (%)
1.	Control	100	100	100	100
2.	500 µg/ml	29.4643	29.3495	26.8817	28.565159
3.	400 µg/ml	32.1429	33.1316	33.4869	32.920473
4.	300 µg/ml	43.006	44.6293	44.7005	44.111921
5.	100 µg/ml	56.25	56.8835	56.9892	56.707586
6.	80 µg/ml	61.3095	65.9607	64.2089	63.826366
7.	60 µg/ml	67.1131	68.3812	70.1997	68.564676
8.	40 µg/ml	73.0655	73.9788	74.5008	73.848355
9.	20 µg/ml	84.9702	86.0817	87.0968	86.049569
10.	10 µg/ml	88.244	89.5613	90.4762	89.42717

The turmeric oil yield of leaves is high when compared to rhizome.[18] These effects are primarily attributed to curcumin, total phenolic compounds and flavonoids found in turmeric leaves. The cost of oil preparation is less in leaves when compared to rhizome. [19] Nanoemulsion is helpful in enhancing skin permeation. Moreover, it has become a solution for loading hydrophobic compounds in water soluble bases for long term stability.[20] Nanoemulgel is used as a carrier for topical application of turmeric leaf oil due to its permeation properties. The nanoemulgel formulation containing multicomponent of oil, S_{mix} and carbopol 940 (F1-F3) and oil, surfactant and carbopol 940(F4-F6) are formulated(Table 1). Based on the physiochemical studies, rheological studies such as particle size(175.4 nm), morphology(spherical),entrapment efficiency ranging from 67.72-85.13%, pH (5.5-6.4) and in-vitro release studies (89.98±0.22%), nanoemulsion prepared by ultrasonication was thus used to formulate optimized formulation. F6, being optimized formulation, was selected that contained oil: surfactant ratio 1:3 was subjected to particle size analysis and morphology studies. The particle size gave desired nanometer of 175.4 nm (Table 3). Moreover, Tween 80 is generally regarded as safe for use in pharmaceuticals and as a food surfactant. Our results demonstrated that an increase

in the concentration of surfactant used in the NE preparation decreased droplet size.[21] The SEM images (Fig 1)showed spherical structure and uniformity of droplet size exhibited by PDI value to be 0.475 (Table 3), proper entrapment efficiency (85.13%) and release rate (89.98 ± 0.22%) after 8 hours (Table 5). Entrapment efficiency and percentage release rate were increased as a function of drug: surfactant with gelling agent ratio.The results of the release kinetics elucidated from Korsmeyer-peppas equation shows that the release exponent values (n) for F6 was found to be 1.125 for F6 (Table 5), this indicates Non-fickian diffusion model(super case II). The F6 showed most satisfactory Zero order release (R2= 0.9848) which describes that the drug release rate is independent of its concentration whereas dependent on the composition of nanoemulgel.

The cytotoxicity studies of TLO nanoemulgel with mean droplet size of 175.6 nm was evaluated against human breast cancer cell line for an inhibition period of 24 hours. Exposure of the MCF-7 cells to TLO nanoemulgel led to gross cytological modifications, such as cell shrinkage. The IC50 value of F6 was found to be 59.84 µg/ml for 24 hours. The cell viability of F6 at different concentration was determined (Table 6). At 500 µg/ml the cancer cells was mostly disrupted and the cell viability was about 28.56 %.In addition, our results suggest that the Tween 80 may have improved solubilization of the hydrophobic TLO compounds, which enhanced permeability across the cancer cell membranes and facilitated apoptosis. This study provides evidence for the anti-tumor growth properties of TLO-NEG with respect to breast cancer. Thus, TLO-NEG may effectively inhibit breast cancer cell proliferation.

CONCLUSION

In this study, formulation and its anti-cancer activity validation on TLO nanoemulgel were carried out for the first time. TLO nanoemulgel suggests that knowledge of traditional medicinal systems can be used to bio prospect, identify and develop new sources of medicine that are safe and efficient. Here, we have formulated nanoemulsion of TLO that varied in size, based on the ratio of oil and surfactant. Increasing the amount of surfactant concentration in the TLO nanoemulsion decreases the droplet size. We demonstrate that TLO nanoemulgel reduces cell viability and alters nuclear morphology of the cancer cells. This significant reduction of cancer cell viability suggests that the nanoemulsion easily penetrates the cell membrane to induce cell death. Our results suggest that TLO nanoemulgel induced destruction in MCF-7 cancer cells yet further in-vivo studies are required for testing its effectiveness and topical applicability.

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