

# Formulation of Resveratrol Nanoemulsion by Phase Inversion Technique and Evaluation of Anti-Cancer Activity on Human Colon Cancer Cell Lines

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## ABSTRACT

Colorectal cancer is in third place among the most usually detected cancer worldwide. Many natural compounds have been proved for its efficacy in the treatment of different types of carcinomas. Resveratrol a polyphenolic phytoalexin found in several sources including grapes, berries, etc. Like most of the polyphenols resveratrol also exhibits poor aqueous solubility and hence bioavailability. In this study nanoemulsion of resveratrol has been formulated using a low energy technique (phase inversion composition) using capryol as oil phase. The optimized formulations were evaluated for size of nanodroplets, zeta potential as well as *in vitro* drug release. The resveratrol nanoemulsion was evaluated on HCT-166 cell lines to study the inhibitory effect on the viability of human colorectal cancer cell and compared with resveratrol suspension and blank nanoemulsions. Transmission electron microscopy and dynamic light scattering revealed the globule size of nanoemulsion was  $113.6 \pm 18.21$  nm with a zetapotential of  $-15.12 \pm 1.028$ . *In vitro* release profile showed above 80% release within 6 hr. The results of the cell line studies showed that the developed resveratrol nanoemulsion was cytotoxic to the colon cancer cells while non-toxic to the non-tumor cells. Hence, the developed resveratrol nanoemulsion was proved to be a promising approach for colon cancer management.

**Key words:** Resveratrol, Lung cancer, HCT-166 cells, Nanoemulsion, Phytochemicals.

## INTRODUCTION

Lung, colorectal and breast cancer are the most frequent lethal types of cancers worldwide. As per cancer statistics 2020, colorectal carcinoma is the second most significant cause of death related to cancer in United States.<sup>1,4</sup> Current chemotherapy methods for treating cancers are associated with major toxicities. Therefore, naturally occurring plant products, which are non-toxic are favored as compared to synthetic agents. Resveratrol is such naturally occurring dietary compound, which proved to be a potential compound for the treatment of colon cancer. Resveratrol is a polyphenolic compound mainly found in grape skin berries etc. function as a chemotherapeutic agent against colorectal carcinomas and many other types of cancers. Resveratrol chemically 3,4',5-trihydroxy-*trans*-stilbene occur in high

levels in grape skin up to 100  $\mu\text{g/g}$  and in red wine up to 20 mg/L.

It possesses wide-ranging biological properties including cardioprotective, anti-platelet aggregation, immunomodulatory as well as neuroprotective actions. The discovery of anti-cancer effect of resveratrol in colon cancer as well as other carcinomas aroused a great enthusiasm in grapes and related dietary supplements. This polyphenol has proved its efficacy in the management of cancer in different stages of carcinogenesis due to its capability to arrest cell-cycle and triggering of apoptosis.<sup>5-9</sup> Many studies have found that resveratrol could induce tumor cell apoptosis by mitochondrial dependent pathways which that partly depended on Bax conformational changes as well as cellular redistribution.<sup>10,11</sup> Some studies

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have proved that Fas-mediated pathways can contribute to killing of cells which are subjected to different stimulus like anti-cancer agents and immune modulatory molecule in a ligand-dependent or -independent manner.<sup>10,12</sup>

Like most of the polyphenols, resveratrol belong to the class-2 of the BCS (Biopharmaceutics Classification System) hence exhibits poor aqueous solubility and bioavailability.<sup>13</sup> There are extensive researches are going on to augment the solubility and hence the bioavailability of this natural compound for maximizing its therapeutic potential. The nanotechnology related formulation development strategies seems to be efficient in enhancing the bioavailability. Nanotechnology utilized carriers have been highly researched *in-vitro* and also *in-vivo* to discover the impacts on bioavailability as well as efficacy. Which include nanosuspensions,<sup>14</sup> polymeris nanoparticles,<sup>15-17</sup> polymeric hydrogels,<sup>18</sup> self-nanoemulsifying drug delivery systems,<sup>15,19</sup> liposomes,<sup>20</sup> solid lipid nanocarriers,<sup>21</sup> protein based nanocarriers<sup>22</sup> and cyclodextrin complexes.<sup>23</sup> Among these approaches the most prevalent one is the encapsulation or incorporation of the drug into inactive lipid carrier like oil, surfactant dispersions, micro/nanoemulsions etc. Amongst these nanoemulsions can enhance the bioavailability by improving the solubility of lipophilic bioactive very efficiently.<sup>24,25</sup> Nanoemulsions have generally droplet size less than 200 nm and are stable isotropic systems of two immiscible liquid phases formulated with the help of surfactants.<sup>26,27</sup>

In the present study a nanoemulsion of resveratrol is formulated by a phase inversion technique which does not require high energy for the preparation. After characterization, the nanoemulsion is evaluated on HCT -166 human colon cancer cell lines for cell viability assay and the result was compared with blank nanoemulsion and resveratrol suspension. To the best of our understanding no research has been published on resveratrol nanoemulsion to prove the anticancer action on human colon cancer cell line.

## MATERIALS AND METHODS

Resveratrol, capryol 90, olive oil, labrafil, tween 20, tween 80, triacetin, PEG 400, span 20, ethanol and propylene glycol were obtained from Sigma (Sigma Aldrich-Germany). All chemicals used were of analytical grade and commercially available.

### Selection of oil, surfactant and co-surfactant

Determination of solubility of drug in different components of nanoemulsion is important to ensure

maximum solubility and stability. To assess the solubility of resveratrol, extra amount of drug incorporated in fixed amount of different oils and surfactants and mixed by vortex shaker for 5 min and then kept in a shaking water bath (GFL- Burgwedel, Germany) for 72 h (37°C). The solutions were centrifuged using a laboratory centrifuge (NF 800R NUVE, Turkey) for 30 min (5000 rpm). The upper portion were taken and methanol was added to dilute the sample and amount of resveratrol solubilized was determined at 306 nm using UV-Visible spectrophotometer.

### Preparation of nanoemulsion

Nanoemulsion was prepared by using phase inversion composition method. First resveratrol was solubilized in Capryol 90. Then surfactant (Tween 20) and cosurfactant (PEG 400) were mixed to form Smix and different quantities of this mixture was added to fixed amount of oil and mixed well with vortex shaker. Then water was added slowly and mixed continuously using vortex mixer.<sup>28-30</sup>

### Characterization of optimized nanoemulsion

#### Droplet size and PDI

Zetasizer Nano was used to establish the droplet size of nanoemulsion (Malvern Instruments Limited-Worcestershire, UK). Dynamic light scattering technique was the principle behind. Nanoemulsion was diluted 100 times before experiment so as to prevent multiple scattering effects. All the measurements were done in triplicate.

#### Zeta potential

Zeta potential of the formulations was measured by Zetasizer Nano (Malvern Instruments Limited, Worcestershire, UK). The surface charge was calculated as an average of three readings.

#### Transmission electron microscopy (TEM)

The morphology of resveratrol nanoemulsion formulation was illustrated by TEM as per previous published methods.<sup>22</sup> A drop of the nanoemulsion sample after suitable dilution was placed directly on copper grid. Before analysis, the staining was done for 30 sec with phosphotungstic acid (2%) and finally air dried.

#### In vitro drug release

Amount of resveratrol released from the developed nanoemulsion was compared with a resveratrol suspension. Suspension of resveratrol was formulated by suspending resveratrol in 1% sodium carboxy methyl cellulose in water. The final concentration of resveratrol in suspension was 8 mg/ml. Drug release

from developed resveratrol nanoemulsion and suspension were determined. Formulations were filled in the cellulose membrane tubing (molecular cut off 12000Da, Sigma -Aldrich, USA) with the help of a clip. The filled bags were tied on the paddles of USP dissolution apparatus type 2. 900 ml of pH 6.8 phosphate buffer used as dissolution medium and instrument was operated at 50 rpm. Samples were withdrawn (5ml) at 0, 0.5, 1, 2, 4, 6, and 8h for measuring the drug release and same amount of fresh medium was returned to recompense the volume loss. Resveratrol content was measured from the samples at 306 nm in a UV-Visible spectrophotometer (UV-2600 Shimadzu). The drug released from the nanoemulsion was compared with resveratrol suspension.

### Cell culture

HCT166 (human colorectal cancer cell line) cells were obtained from the American Type Culture Collection (ATCC). HCT166 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) and supplemented with FBS (fetal bovine serum 10%), 100 I.U./mL of penicillin, and 100 µg/mL streptomycin. All these reagents were purchased from Sigma -Aldrich, USA. The cells were grown in a humidified incubator in 5% CO<sub>2</sub> at 37°C.

### In-vitro cytotoxicity analysis

The anticancer property of resveratrol nanoemulsion was studied using a previously described method. To determine the toxicity of optimized formulation and suspension toward cultured cells MTT assay was used. 96-well plates were used to seed the cells and allowed to grow confluence of 70 to 80%. The HCT166 cells were added with various concentrations (20, 30, 40 µM) of formulations for 24 hr with DMSO. After removing the media, 10 µl of sterile and filtered 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) in phosphate buffered saline (5 mg/mL) was put in to each well to have a final concentration of 0.4 mg/mL. After 3h of re-incubation of the plates for allowing the metabolism of MTT by viable cells to form insoluble formazan crystals, the media and unconverted MTT were aspirated. Then 150 µl of DMSO was put in to each well for ensuring complete solubilization of formazan. The absorbance were noted on a BioTek Synergy H1 microplate reader at 490 nm.<sup>31,32</sup> The formula given below was used to calculate percentage of inhibition.

% Cell viability = (Mean absorbance of control -Mean absorbance of treated cells)/ Mean absorbance of control × 100

### Cell Morphological Analysis

Resveratrol nanoemulsion (F), blank nanoemulsion (B) and resveratrol suspension (S) at different concentrations (20, 30, 40 µM) tested on HCT166 cells (seeding density  $2.5 \times 10^3$  cells/well) in 24-well plates. Treated as well as untreated cells were then cultured in 5% CO<sub>2</sub> (37°C) in an incubator for 24 hr. Microscopic images were captured after experimental procedure to analyze any visible changes in cell morphology.

### Statistical Analysis

The mean ± standard deviation of three replicates for individual assays are presented in data. SPSS 22.0 software was used determine the statistical significance. \* $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$  indicates statistical significance in comparison with the untreated control cells.

## RESULTS AND DISCUSSION

### Formulation of resveratrol nanoemulsion

Nanoemulsions were formulated by a low energy technique using phase inversion composition technique. The free energy required to form nanoemulsion is highly dependent on the extent to which the surfactant reduces or lessens the surface tension between oil and water interface. It also depends on the changes in dispersion entropy. Hence a negative free energy of formation is accomplished when great decrease in the surface tension is supplemented by major beneficial entropic change. Therefore in these conditions the process of nanoemulsification will be spontaneous and the resultant dispersion will be stable.<sup>29</sup> Based on the solubility and miscibility study capryol 90 was selected as oil, tween 20 as surfactant and PEG 400 as cosurfactant (Figure 1). Different trials have been done with varying ratios oil and surfactant. Different ratios of Tween 20 and PEG400 also tried (Table 1). Formula-

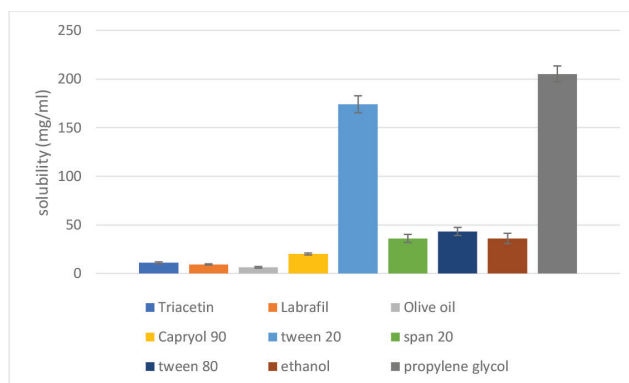


Figure 1: Resveratrol solubility in different oils and surfactants.

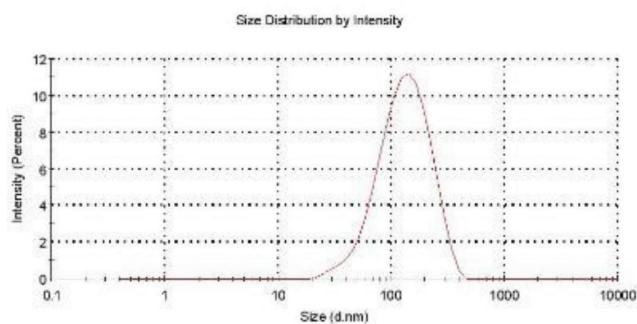


Figure 2: Size distribution pattern for globule size of the developed nanoemulsion.

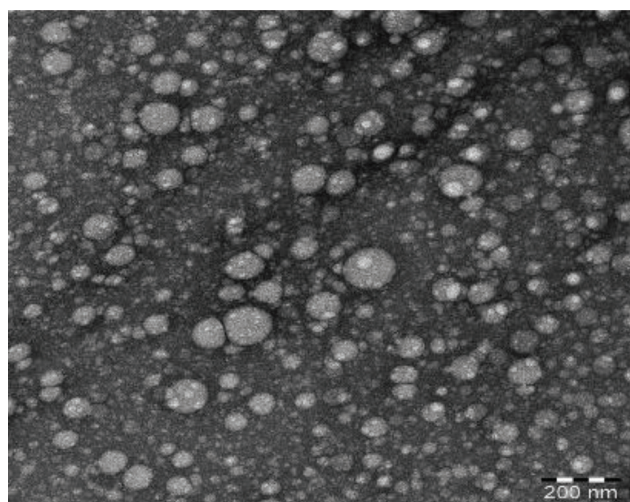


Figure 4: TEM image of the optimized formulation.

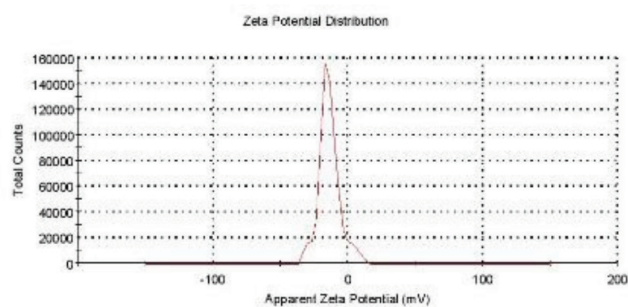


Figure 3: Zeta potential distribution of developed nanoemulsion.

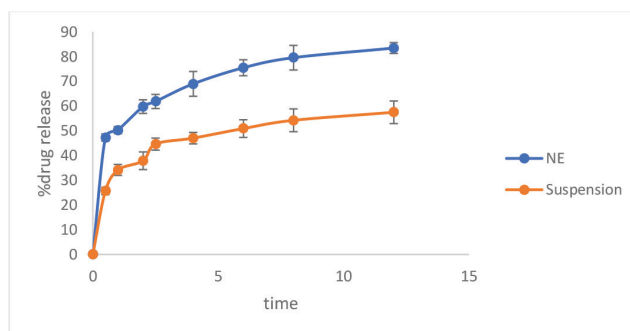


Figure 5: Percentage release of resveratrol from nanoemulsion and suspension.

Table 1: Different formulation trials and results.				
Formulation	Tween 20: PEG400	Oil: Smix	Droplet size (nm)	PDI
F1	1:0.5	1:0.25	321.5±21.74	0.535±0.04
F2	1:0.5	1:0.5	273.1±30.21	0.619±0.02
F3	1:0.5	1:1	210.3±19.3	0.415±0.09
F4	1:1	1:0.25	189.8±17.93	0.311±0.07
F5	1:1	1:0.5	113.6 ±18.21	0.205±0.06
F6	1:1	1:1	110.2±17.12	0.237±0.02

tions F5 and F6 with minimum droplet size and PDI size were selected for further studies.

**Characterization of nanoemulsion**

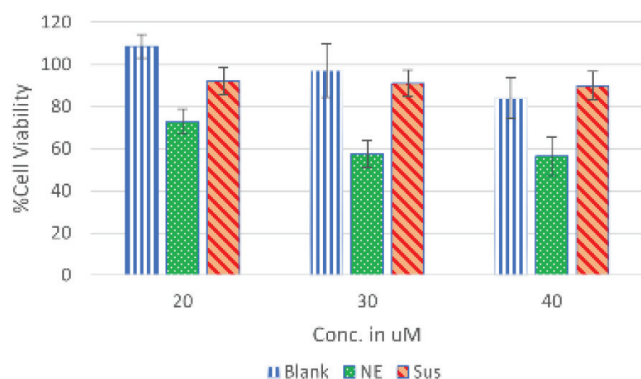
**Droplet size and PDI**

The mean droplet size of developed resveratrol nano-emulsions were 113.6 ±18.21 nm for the formulation F5 and 110.2±17.12 for the formulation F6 with a polydispersity index of 0.205±0.06 for F5 and 0.237±0.02

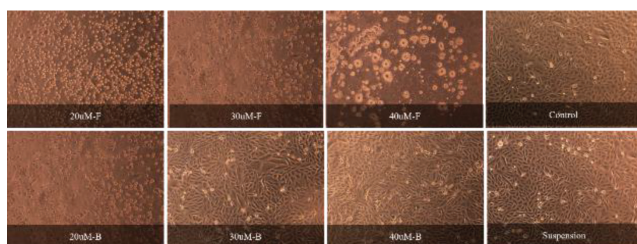
for F6. Figure 2 shows the size distribution pattern of formulation F5. The results were comparable to other published reports of similar systems.<sup>33</sup>

**Zeta potential**

The developed resveratrol nanoemulsions showed a zeta potential of -15.12±1.028 mV for formulation F5 (Figure 3) and -14.34±2.011 mV for formulation F6. Sufficient electrokinetic stability can be expected for



**Figure 6: Activity of control, resveratrol nanoemulsion (NE), blank nanoemulsion (Blank) and efavirenz suspension (Suspension) at different concentration on the cell viability of HCT-166 cancer cells.**



**Figure 7: Cell morphology of treated HCT-166 cells.**

a zeta potential value of above 15mV either negative or positive charge.<sup>30</sup>

### Transmission electron micrograph

The TEM image revealed a uniform spherical morphology with globule size near 100 nm for the resveratrol nanoemulsion (Figure 4). This result was similar with the droplet size found by zeta sizer measurement. Also, the morphology of oil droplets was comparable to that of reported nanoemulsions formulated by phase inversion composition technique.

### Drug release

The drug release from the developed resveratrol nanoemulsion was significantly greater as compared with resveratrol suspension (Figure 5). The release study was carried out in 0.1N HCl for the first two hr and then in pH 6.8 Phosphate buffer till 8 hr. Nearly 60% of resveratrol released from both the formulation F5 and F6 in the first 2h, while only 37.87±3.56 % from suspension. After 8 h nanoemulsion showed 83.43± 2.2 % release and suspension showed only 57.46 ±4.5 % drug release. The very small size of droplets in nanoemulsion improved the resveratrol release by increasing the solubility is the reason for higher percentage of release as compared to suspension.<sup>34,35</sup> Formulation F5 and F6

did not showed a significant difference in the percentage drug release, formulation F5 was selector for further studies since the percentage of surface tent is comparatively less as compared to formulation F6. Therefore, formulation F5 was selected as the optimized formulation and it contains 2ml of capryol 90, 0.5 ml of Tween 20, 0.5 ml of PEG 400 and 3 ml of water with 5mg/ml of resveratrol.

### In vitro anticancer activity on HCT-166 cells

To investigate the cytotoxic effect on HCT-116 cells, the cells were treated with an array of concentration (20, 30, 40  $\mu$ M) of resveratrol nanoemulsion, blank nanoemulsion, resveratrol suspension and control, and then the cell viability was measured after 48 hr. The *in-vitro* results showed that resveratrol nanoemulsion decreased the HCT-116 cells viability (Figure 6) in a dose-dependent manner.

From the results, it was observed that NE at 30 and 40  $\mu$ M are highly significant as compared to 20 $\mu$ M which is non-significant. The blank formulations did not showed any effect on cell viability at all the three selected concentrations. In our study it was observed that resveratrol seems to be selective for tumor cells evidenced by the very minimal toxicity against normal cells (Figure 7). Previous studies have reported that polyphenolic compounds possess anti-cancer effects metastasis and angiogenesis in various cancer cell.<sup>36,37</sup> Based on previous studies, the cytotoxic effect of resveratrol was ascribed to different molecular mechanisms in favor of anticancer activity are the signaling pathways linked to extracellular growth factors and receptor tyrosine kinases; cell metabolism and formation of multi-protein complexes; genome instability and also cell proliferation.<sup>38,39</sup> Other mechanisms include inflammation, immune surveillance and also apoptosis. It can also behave as a blocking agent by interrupting the transformation of procarcinogen to carcinogen, by inhibiting the monooxygenase cytochrome P450 isoenzyme.<sup>38</sup> It was evidenced that resveratrol can halt the cell cycle at S phase in various cancer cells, such as breast, colon and pancreas.<sup>40-43</sup> So the results prove that resveratrol nanoemulsion can effectively inhibit tumor cell growth in colon cancer cells *in vitro*. This study proved the effectiveness of resveratrol nanoemulsion in colon cancer cell lines for the first time.

### CONCLUSION

In this study we formulated and characterized a resveratrol nanoemulsion formulation by a simple phase inversion technique. The developed nanoemulsion was proved to

be very effective against in killing of HCT-166 colon cancer cells. Our study proved the dose dependent effect of resveratrol on killing of colon cancer cells, so we recommend adequate intake of resveratrol containing food or food supplements can prevent or manage colon cancer.

## CONFLICT OF INTEREST

The author declares no conflict of interest.

## ABBREVIATIONS

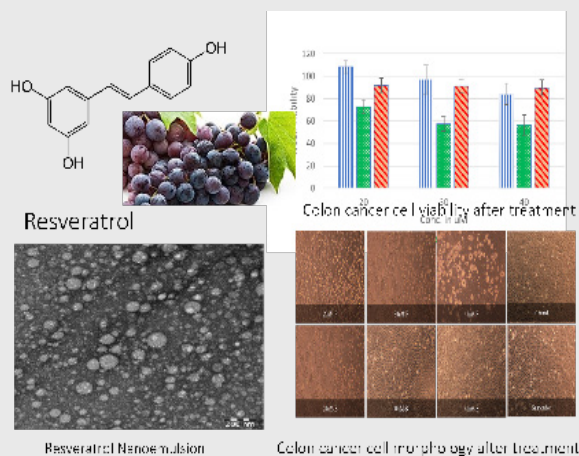
**PEG:** Polyethylene Glycol; **HCT-166:** Human colorectal cancer cell line; **NE:** Nanoemulsion.

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## PICTORIAL ABSTRACT



## SUMMARY

- Resveratrol has been proved to be beneficial in the management of Colorectal cancer.
- The poor aqueous solubility of resveratrol is improved by nanoemulsion formulation.
- The developed nanoemulsion was proved to be very effective against in killing of HCT-166 colon cancer cells.
- So adequate intake of resveratrol can prevent or manage colon cancer.

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