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Original Article

# Nasal Delivery of Nanoemulsion Containing a Synergistic Combination of Curcumin and Gefitinib for Brain Tumor Targeting

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## ABSTRACT

**Objectives:** This study involves combination therapy of anticancer drugs (curcumin and gefitinib) as nanoemulsion and their application for treating human glioblastoma U373MG cell lines. **Materials and Methods:** Nanoemulsions containing the synergistic combination were prepared using a high-pressure homogenization technique. Nanoemulsion formulation was characterized for globule size, drug content, thermodynamic stability, zeta potential measurement and drug release. Histological studies were performed using isolated nasal mucosa of sheep. **Results:** The combination was found to be synergistic as they were more effective in inhibiting the growth or viability of the U373MG glioblastoma cells compared to using each drug individually. The lowest Combination Index (CI) value of 0.2 indicates a strong synergistic effect. A CI value less than 1 generally suggests synergy, meaning that the combined effect of the drugs is greater than what would be expected if they were simply additive. Both drug concentrations in the brain were significantly higher after IN administration of CRM-GFT-NE compared to IV administration of PDS. Drug targeting efficiency and direct nose-to-brain transport following intranasal administration of optimized CRM-GFT-NE were 1871.26±2.56 and 95.21±0.93 for CRM and 1807.95±5.11 and 93.52±0.63 for GFT respectively. **Conclusion:** The study used a passive targeting approach as nanocarriers to improve the delivery of CRM and GFT to the tumor cells. This suggests that the drugs were engineered or formulated to specifically target the cancer cells, potentially reducing dose-related side effects. The study also demonstrated a high percentage of nose-to-brain drug transport for both CRM and GFT. This implies that the optimized Nanoemulsions (NEs) used in the study effectively delivered the drugs to the Central Nervous System (CNS) via the intranasal route. This is crucial for treating glioblastoma, as it requires drugs to cross the blood-brain barrier and reach the tumor in the brain. In summary, this study indicates that the combination of curcumin and gefitinib has a strong synergistic effect in inhibiting glioblastoma cell growth. Additionally, the use of targeted drug delivery systems, specifically designed to transport the drugs to the brain, was successful in achieving effective CNS targeting. These findings suggest nanoemulsion formulation as a promising approach for the treatment of glioblastoma with reduced systemic side effects.

**Keywords:** Brain Targeting, Brain tumor, Curcumin, Gefitinib, Nanoemulsion, Nose to the brain.

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## INTRODUCTION

Single-drug treatments for brain tumors face challenges such as toxicity, tumor cell heterogeneity and the development of Multi-Drug Resistance (MDR). Combination therapy is essential to overcome these issues and provide a synergistic effect, increasing treatment efficacy while reducing toxicity. CRM enhances the cytotoxicity of GFT by downregulating nuclear factor-κB and Akt pathways, which helps reverse Multi-Drug Resistance (MDR). This combination approach targets multiple pathways, increasing the chances of a positive treatment response. An intranasal drug delivery system was designed to improve drug delivery to the Central Nervous System (CNS). This route helps bypass first-pass metabolism and increases drug bioavailability in the CNS.[1] Nanoemulsion, specifically the Oil-in-Water (o/w) type, was chosen as the drug delivery system due to its advantages. It allows lipophilic drugs like CRM and GFT to solubilize easily in the oily phase, promoting rapid absorption by facilitating prolonged contact with the mucosal membrane.[2,3] The results of the study indicate that the combination of CRM and GFT in a nanoemulsion for intranasal administration effectively targets the brain. This is a significant finding for the treatment of brain tumors, as it ensures that the drugs reach their intended

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site of action in the CNS. This study addresses the challenges of brain tumor treatment by using combination therapy with CRM and GFT. The choice of intranasal drug delivery, specifically in a nanoemulsion form, appears to be a successful strategy for enhancing drug delivery to the brain and achieving effective treatment outcomes while minimizing systemic side effects.

## MATERIALS AND METHODS

### Materials

Curcumin (CRM) was gifted by Sun Pure Extracts Ltd., located in Delhi, India. Gefitinib, another anticancer agent, was obtained from Khandelwal Industries Ltd., Mumbai, India. Oleic acid was obtained from Soofi Traders Ltd., located in Mumbai, India. Tween 80 and Polyethylene Glycol 400 (PEG 400) were obtained from Loba Chemie Ltd., located in Mumbai, India. All other reagents used in the study were of analytical grade, which typically implies a high level of purity and suitability for laboratory use.

### Methods

Synergistic combination analysis by using Sulforhodamine B (SRB) assay in human glioblastoma U373MG cell line.[4,5]

Astrocytoma-glioblastoma cells (U373MG) were cultured in 96-well plates at a seeding density of $1.0\times10^4$ cells per well. The culture medium used was Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 µg/mL penicillin, 200 µg/mL streptomycin and 2 mM L-glutamine. The cells were maintained under specific conditions: a humidified atmosphere with 5% $CO_2$, 37°C temperature, 95% air and 100% relative humidity. After seeding the cells, they were allowed to pre-incubate under the culture conditions described above for 24 hr before the addition of experimental drugs. This period allows the cells to adhere and stabilize. Both drugs (curcumin and gefitinib) were initially solubilized in Dimethyl Sulfoxide (DMSO) at 100 mg/mL concentration. These stock solutions were further diluted with doubled distilled water to achieve final concentrations of 1 mg/mL. Aliquots of the frozen drug concentrate (1 mg/mL) were thawed and diluted to various concentrations (10 µg/mL, 20 µg/mL, 40 µg/mL and 80 µg/mL) with a complete medium containing the test article. These diluted drug solutions were used for treatment. Ten microliters of the different drug dilutions were added to the corresponding microtiter wells, each of which already contained 90 µL of the cell culture medium. This resulted in the desired final drug concentrations in the wells. After adding the drugs, the microtiter plates were incubated under standard conditions for 48 hr. This incubation period allows the drugs to exert their effects on the cells. The assay was terminated by the addition of cold Trichloroacetic Acid (TCA) to the wells. The cells were stained with a Sulforhodamine B (SRB) solution. SRB is a dye used to assess cell density and viability. After staining, the plates were washed multiple times with 1% Acetic Acid (AA) to remove unbound dye. The bound SRB stain was eluted from the cells using a trizma base solution. The absorbance of the eluted dye was measured at a wavelength of 540 nm with a 690 nm reference wavelength. This measurement is used to quantify the effect of the drugs on cell growth. The percentage growth inhibition was calculated using the formula,

$$\text{Growth Inhibition } (\%)=[Ti/C]\times100 \quad \text{.....(1)}$$

Where, Ti-Test growth in percentage and C-Control growth.

This calculation assesses the extent to which the drugs inhibited cell growth compared to untreated control cells.

### Median-effect analysis

The median-effect analysis, as described by Chou and Talalay, is a method used to quantitatively assess the interaction between two drugs in combination therapy. In this case, it's applied to the combination of Curcumin (CRM) and Gefitinib (GFT). The Combination Index (CI) is a key parameter used to determine whether the effect of the combination is synergistic, additive, or antagonistic.[6,7]

$$\text{Combination Index (CI)}=(\text{Dose})1/(\text{Dose}\times)1+(\text{Dose})2/(\text{Dose}\times)2\text{.....(2)}$$

Where: CI: The combination index quantifies the drug interaction's nature.

(Dose)1 and (Dose)2: The doses of drug 1 (CRM) and drug 2 (GFT) in the combination.

(Dose x)1 and (Dose x)2: The doses of drug 1 and drug 2 as single drugs, i.e., the doses at which they would be used individually.

CI<1: Indicates synergism. The combination of the two drugs is more effective than would be expected if they were simply additive. In other words, they work together to produce a greater effect. CI=1: Suggests additivity. The combination has an effect like what would be expected if the drugs were simply added together. CI>1: Suggests antagonism. The combination is less effective than the sum of the effects of the individual drugs, indicating interference with each other's actions.

### Formulation of Nanoemulsion (NE)

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

The identification of an oil that, when combined with appropriate surfactants and cosurfactants, can form a stable emulsion for drug delivery or other applications. The selection is based on visual observations of clarity and stability during the mixing process, which can be indicative of the emulsion's potential for long-term stability and effectiveness in delivering drugs or other active ingredients. Initially an excess amount of CRM (Curcumin) and GFT (Gefitinib) into selected oils. The oils under consideration were oleic acid, ethyl oleate and clove oil. The mixture of oil and drugs was then mixed vigorously using a vortex mixer. This step

is crucial for ensuring that the drugs are well-dispersed in the oil phase. To evaluate the capability of the oils and surfactants to form an emulsion spontaneously, the selected oil was gradually added to a surfactant solution at a concentration of 20% (w/w). A vigorous vortex mixing was employed during this process. During the addition of the oil into the surfactant solution, the visual appearance of the mixture was observed. If a uniform clear solution was obtained, it suggests that the oil and surfactant are compatible and capable of forming a clear solution or stable emulsion. If the initial addition of oil resulted in a clear solution, more oil would be added until the solution became cloudy. The cloudiness indicates that the saturation point was reached, and further oil addition would lead to phase separation or emulsion destabilization. After achieving cloudiness, the surfactant/oil mixture was diluted with distilled water. Then, the cosurfactant was titrated incrementally into the system. The goal was to add more cosurfactant until the system turned clear again. The point at which the solution transitioned from cloudy to clear indicated the critical micelle formation, where the emulsion system would become stable.[8]

### Experimental design

To design the NE formulation, preliminary experiments revealed independent variables like oil volume (A), surfactant volume (B) and co-surfactant volume (C) during preparation (Table 1A). The main factors that affected the dependent variables such as droplet size, the content of CRM and the content of GFT. Central composite design CCRD-RSM (Design-Expert software, version 7, Stat-Ease, Inc., Minneapolis, Minnesota, USA) was applied to systemically investigate the influence of these three decisive independent variables on three dependent variables of the NE. All independent, coded and actual values of the variables of CCRD-RSM are given in (Table 1B). In this design, the best models such as the quadratic model can be selected due to the Analysis of Variance (ANOVA) F-value and $p$-value<0.05 which is statistically significant.[9,10]

### Preparation of nanoemulsion by spontaneous emulsification method

The oil phase, which includes oleic acid as well as the drugs CRM and GFT, is added to the aqueous phase. The aqueous phase consists of Tween 80 and PEG 400, which are surfactants often used to stabilize emulsions. These phases are mixed using a vortex mixer for 30 min. This initial mixing step helps in forming a pre-emulsion. After the pre-emulsion is formed, it is subjected to High-Pressure Homogenization (HPH, GEA Niro Soavi Homogenizer Panda) for 5 cycles. High-pressure homogenization is a mechanical process that involves forcing the emulsion through a small gap or nozzle under high pressure. This process helps in reducing the size of the dispersed oil droplets within the emulsion, leading to the formation of smaller droplets and achieving a more stable and finer nanoemulsion.[5]

## CHARACTERIZATIONS OF NANOEMULSION

### Droplet size analysis

The droplet size analysis was conducted using a photon correlation spectrophotometer known as the Zetasizer ZS 90, (Malvern Instruments Ltd., UK). To prepare the sample for analysis, a portion of the nanoemulsion formulation was diluted with double distilled water. The Zetasizer ZS 90 collects data on the intensity of scattered light as a function of time at a scattering angle of 90°. The analysis was conducted at a controlled temperature of 25°C. and from this data, it calculates the size distribution of the droplets in the nanoemulsion. the analysis provides information about the size distribution of the droplets, including the mean droplet size and the size distribution profile.[11]

### Drug content

Methanol is added to the NE to extract CRM and GFT from the formulation. Mixing was performed to ensure complete dissolution and extraction of the drugs into the methanol solvent. The extracted solution is then subjected to spectrophotometric analysis and is typically analysed at 423 nm and GFT is analysed at 254 nm using a UV-visible spectrophotometer (UV 1700, Shimadzu, Japan). The concentration of CRM and GFT in the methanolic extract is determined by comparing the absorbance values to a standard calibration curve.

### Zeta potential determination

Zeta potential analysis is performed using a Zetasizer instrument (Zetasizer ZS 90, Malvern Instruments Ltd., UK). Zeta potential is a measure of the electrostatic charge on the surface of particles or droplets in a colloid. It provides information about the stability and interaction of particles in dispersion. The instrument applies an electric field to the particles or droplets in the sample and measures the speed at which they move in response to the field. This movement is called electrophoresis. The zeta potential is calculated based on the electrophoretic mobility of the particles or droplets.[11]

### Differential Scanning Calorimetry (DSC)

The thermal analysis of samples, including pure CRM, GFT, physical mixture and CRM-GFT-loaded Nanoemulsions (NEs), was conducted using a Differential Scanning Calorimetry (DSC) instrument (Mettler-Toledo, Greifensee, Switzerland). The DSC analysis involved a controlled heating process with a heating rate of 10°C/min. The temperature range for analysis spanned from 30-300°C. This range allows for the observation of thermal transitions and changes in the samples within this temperature span. During the entire DSC measurement, the sample cell was continuously purged with nitrogen gas at a specified flow rate (40 mL/min), which helps prevent any oxidation or combustion of the samples during heating.

## *In vitro* drug release

The *in vitro* release study of an optimized batch of nanoemulsion was performed using a Franz diffusion cell assembly (Electrolab, India), which had a diameter of 2.0 cm and a capacity of 25 mL. A dialysis membrane with an average molecular weight cutoff of 12,000-14,000 Da was used. The receptor compartment of the diffusion cell was filled with pH 6.4 phosphate buffer saline containing 2% methanol. The temperature of the experiment was maintained at 37±0.5°C. The donor chamber was loaded with nanoemulsion equivalent to 5 mg of each CRM and GFT. Samples were periodically withdrawn at 30, 60, 120, 180 and 240 min from the receptor compartment over 4 hr. After each withdrawal, the receptor compartment was replenished with an equal amount of fresh pH 6.4 phosphate buffer saline with 2% methanol. The samples collected from the receptor compartment were analyzed using High-Performance Liquid Chromatography (HPLC) at 242 nm. This wavelength is suitable for detecting the presence and quantifying the concentration of CRM and GFT.[12]

## Drug release mechanism

To study the drug Release Mechanism from (CRM-GFT-NE) the drug release data were fitted in the Korsmeyer-Peppa's equation. The logarithmic plot of the cumulative percentage of drug released vs. log time gives the release exponent n and K value from the slope of the straight line and y-intercept respectively $Mt/M\infty=Kt^n$.

## Histopathological studies

The histopathological studies conducted using isolated sheep nasal mucosa is aimed at assessing the effects of various treatments on nasal tissue. Three pieces of sheep nasal mucosa (S1, S2 and S3) with uniform thickness were selected and mounted onto Franz diffusion cells. Mucosa S1, S2 and S3 were subjected to their respective treatments. S1 received the negative control treatment (PBS), S2 received the nanoemulsion treatment (CRM-GFT-NE) and S3 received the positive control treatment (isopropyl alcohol). The treatments were applied for duration of 6 hr. After the treatment period, all samples were thoroughly washed with double distilled water to remove any residual substances and were stained with hematoxylin and eosin. The stained tissue sections were examined under an optical microscope. Images of the tissue sections were captured using the optical microscope. These images can provide visual evidence of any observed histological changes.[12]

**Table 1A: Independent variables along with their code, levels and respective droplet size (nm), drug content of CRM and GFT (%) of different batches of CRM-GFT-NE (*n*=3). These results are mean±standard deviation.**

| Code | A (mL) | B (mL) | C (mL) | Droplet size (nm) | Drug content of CRM (%) | Drug content of GFT (%) |
|---|---|---|---|---|---|---|
| F1 | 2.00 | 10.00 | 6.00 | 55.2±0.75 | 80.2±0.87 | 79.3±0.11 |
| F2 | 2.00 | 20.00 | 6.00 | 53.7±0.17 | 89.3±0.94 | 87.2±0.31 |
| F3 | 3.00 | 6.59 | 4.00 | 49.3±0.40 | 86.3±0.88 | 87.2±0.70 |
| F4 | 1.32 | 15.00 | 4.00 | 52.4±0.24 | 83.4±0.93 | 84.5±0.46 |
| F5 | 3.00 | 15.00 | 7.36 | 51.7±0.12 | 77.4±0.87 | 78.3±0.49 |
| F6 | 4.00 | 10.00 | 2.00 | 52.4±0.23 | 82.1±0.90 | 81.2±0.67 |
| F7 | 3.00 | 15.00 | 4.00 | 47.6±0.12 | 91.3±0.87 | 90.1±0.71 |
| F8 | 3.00 | 15.00 | 4.00 | 47.6±0.17 | 91.3±0.89 | 90.1±0.63 |
| F9 | 2.00 | 20.00 | 2.00 | 64.7±0.17 | 84.2±0.88 | 85.6±0.82 |
| F10 | 3.00 | 15.00 | 0.64 | 59.5±0.35 | 72.5±0.87 | 75.2±0.86 |
| F11 | 3.00 | 23.41 | 4.00 | 63.9±6.35 | 83.1±0.79 | 82.3±0.94 |
| F12 | 3.00 | 15.00 | 4.00 | 47.6±0.23 | 91.3±0.88 | 90.1±0.91 |
| F13 | 4.00 | 10.00 | 6.00 | 61.9±1.00 | 83.9±0.80 | 85.3±1.40 |
| F14 | 4.68 | 15.00 | 4.00 | 54.8±0.17 | 90.8±0.87 | 89.8±0.47 |
| F15 | 3.00 | 15.00 | 4.00 | 47.6±0.92 | 91.3±0.88 | 90.1±0.91 |
| F16 | 4.00 | 20.00 | 2.00 | 72.4±0.42 | 85.2±0.94 | 83.9±0.47 |
| F17 | 3.00 | 15.00 | 4.00 | 47.6±0.35 | 91.3±0.89 | 90.1±0.91 |
| F18 | 3.00 | 15.00 | 4.00 | 47.6±0.35 | 91.3±0.89 | 90.1±0.91 |
| F19 | 2.00 | 10.00 | 2.00 | 54.1±0.23 | 85.1±0.88 | 83.3±0.47 |
| F20 | 4.00 | 20.00 | 6.00 | 57.4±0.29 | 87.1±0.88 | 88.2±0.13 |

## Brain-distribution studies

The brain distribution study in accordance with the guidelines and ethical approvals using male Wistar albino rats to assess the distribution of drugs (CRM and GFT) following Intranasal (IN) and Intravenous (IV) administration. The study followed guidelines approved by CPCSEA and animal protocol approved by the Institutional Animal Ethics Committee (IAEC) of RCPIPER, Shirpur and had a specific registration number (651/PO/ReBi/S/02/CPCSEA). The study was divided into two groups, with each group consisting of 6 rats. Group I: Rats in this group received 100 µL of the formulation (CRM-GFT-NE) containing 5 mg of each CRM and GFT via Intranasal (IN) administration. Group II: Rats in this group received 100 µL of the Plain Drug Suspension (PDS) containing 5 mg of each CRM and GFT via Intravenous (IV) administration. For IN administration in Group I, a micropipette attached to LDPE tubing with a 0.1 mm internal diameter was used. The rats were held in a slanted position during administration. For IV administration in Group II, a tuberculin syringe (1 mL) was used. Blood samples were collected at specific time intervals (15, 30, 60, 90, 120 min) post-administration. These samples were anticoagulated with heparin. After blood collection, the rats were decapitated, and their skulls were cut open. The brain tissue was carefully excised and quickly rinsed with saline. Brain tissue samples were homogenized with saline in a tissue homogenizer. Plasma samples and brain homogenates were stored at -70°C in a deep freezer until further analysis. High-Performance Liquid Chromatography (HPLC) analysis was performed to quantify the levels of CRM and GFT in both plasma samples and brain homogenates.[8,9]

## Sample processing

To 100 µL of brain homogenate or 100 µL of plasma sample, 100 µL IS Hydrochlorothiazide (20 µg/mL) and add extraction solvent 2 mL of acetonitrile was spiked and vortex mixture for 20 min. This sample was ultracentrifuge at 10,000 rpm for 10 min. The supernatant layer was collected and 20 µL was injected into the HPLC system and the whole procedure was carried out at room temperature.

## Chromatographic conditions

The chromatographic conditions for the analysis of drugs (CRM and GFT) and the internal standard (Hydrochlorothiazide) using High-Performance Liquid Chromatography (HPLC). A specific column packed with 5 µM C18 stationary phase 150 mm in length and 4 mm in diameter was used. The mobile phase consisted of acetonitrile and water with 0.1% formic acid. The ratio of acetonitrile to water in the mobile phase was 30:70 (v/v). The mobile phase was pumped through the column at a flow rate of 0.2 mL/min. Detection of the compounds (CRM, GFT and Hydrochlorothiazide) was performed at a wavelength of 242 nm. The chromatographic separation was performed at ambient temperature.[13]

## Data analysis

The non-compartmental model was used to calculate various pharmacokinetic parameters and the specific indexes used to evaluate brain targeting via nasal administration. $C_{max}$ and $T_{max}$ were directly computed from the concentration vs. time plot. The trapezoidal method was employed to calculate the concentration-time curve ($AUC_{0\to t}$). Kinetica5® software, (Thermo Fisher Scientific Inc.,) was used for data analysis and pharmacokinetic parameters calculation.

To evaluate brain targeting via nasal administration, you used the following two indexes:

Drug Targeting Efficiency (DTE) represents a time-average partitioning ratio between drug concentrations in the brain and plasma calculated as below.

$$\% \text{ DTE}=(\text{AUC brain/AUC plasma}) \text{ in}/(\text{AUC brain/AUC plasma}) \text{ iv}\times 100 \ldots\ldots\ldots (3)$$

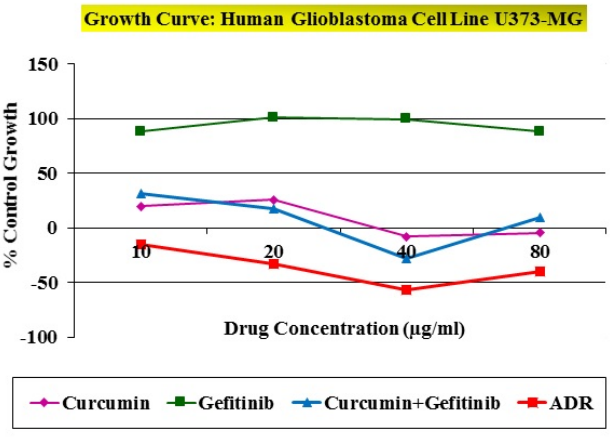


**Figure 1:** Growth curve: Human astrocytoma-Glioblastoma cell line U373MG.

**Table 1B: Coded values of variables.**

| Symbol | Variables | Coded values of variables | |
|---|---|---|---|
| | | (+) | (-) |
| A | Oil volume in mL. | 4 | 2 |
| B | Surfactant volume in mL. | 20 | 10 |
| C | Co-surfactant volume in mL. | 6 | 2 |

**Table 2: Summary of regression analysis results for responses P, Q and R and analysis of variance for droplet size and drug content.**

| Parameter | DF | SS | MS | F | *p* value | $R^2$ | SD | %CV |
|---|---|---|---|---|---|---|---|---|
| **Droplet Size (P)** | | | | | | | | |
| Model | 9 | 867.11 | 96.34 | 14.92 | 0.0001 Significant | 0.9307 | 2.54 | 4.67 |
| Residual | 10 | 64.55 | 6.45 | - | - | - | | |
| Total | 19 | 931.67 | - | - | - | - | | |
| **Drug content of CRM (Q)** | | | | | | | | |
| Model | 9 | 467.25 | 51.91 | 5.83 | 0.0055 Significant | 0.8399 | 2.98 | 3.47 |
| Residual | 10 | 89.03 | 8.90 | - | - | - | | |
| Total | 19 | 556.29 | - | - | - | | | |
| **Drug content of GFT (R)** | | | | | | | | |
| Model | 9 | 352.63 | 39.18 | 6.54 | 0.0035 Significant | 0.8548 | 2.45 | 2.86 |
| Residual | 10 | 59.87 | 5.98 | - | - | - | | |
| Total | 19 | 412.50 | - | - | - | - | | |

DF: Degrees of freedom; SS: Sum of square; MS: Mean sum of square; F: Fischer's ratio, *p* value, Probability value; SD: Standard deviation; %CV: Coefficient of variation.

Direct Transport Percentage (DTP) is used to clarify the extent of direct transport of the drug from the nose to the brain, calculated using the formula below.

$$\% \text{ DTP}=[(\text{Bin-Bx})/\text{Bin}]\times 100 \ldots\ldots\ldots\ldots (4)$$

Where Bin is the brain AUC following intranasal administration, Bx is the brain AUC fraction contributed by systemic circulation through the Blood-Brain Barrier (BBB) following intranasal administration, B i.v. is the brain AUC following intravenous administration, P i.n. is the plasma AUC following intranasal administration and P i.v. is the plasma AUC following intravenous administration.

## RESULTS AND DISCUSSION

### Synergistic combination analysis by using Sulforhodamine B (SRB) assay in human glioblastoma U373MG cell line

The study compared the effects of CRM, GFT, CRM+GFT and a standard drug (aureomycin) on cell viability using the Sulforhodamine B (SRB) assay. It was observed that CRM+GFT produced higher inhibition of the U373MG cells at various concentrations compared to individual CRM and GFT treatments.

Figure 1 was presented to show the percentage inhibition of the U373MG cell line when treated with different drug concentrations of CRM, GFT, CRM+GFT and aureomycin. The graph illustrated that CRM+GFT had a notable inhibitory effect, particularly at a concentration of 10 µg/mL. When CRM and GFT were administered individually, GFT alone was less effective, with a GI50 value of more than 10 µg/mL, indicating higher cellular viability and less cytotoxicity (Figure 1). However, the combination of CRM-GFT exhibited GI50 values below 10 µg/mL, suggesting reduced cellular viability and higher cytotoxicity. This indicates the effectiveness of CRM and GFT in combination against U373MG cells. The study was designed to investigate the synergistic effects of CRM and GFT in combination therapy for cancer treatment. The combination (CRM-GFT) demonstrated a synergistic effect on glioblastoma cell lines, as evidenced by lower GI50 values compared to individual CRM and GFT treatments. This implies that the combination of CRM and GFT together is more effective in inhibiting cell growth than when used separately. The result of the study underscores the potential of combining CRM and GFT for the treatment of glioblastoma.[14] The combination therapy appeared to have a synergistic effect, leading to greater cytotoxicity and reduced cellular viability compared to individual drug treatments. This finding suggests a promising approach for improving the treatment of glioblastoma by using a combination of these two drugs.[15]

### Synergistic effect confirmation

To quantitatively confirm the synergistic effect, the researchers calculated Combination Index (CI) values for various CRM-GFT combinations in relation to cell viability. CI values are a quantitative measure of the interaction between two drugs in combination. The CI values were found to be reduced (CI<1) when CRM and GFT were combined. This reduction in CI indicates a synergistic effect. When CRM and GFT were used in combination at 100 µg/mL and 10 µg/mL concentrations, the CI value was 0.2, clearly indicating a synergistic interaction (CI<1). The study reveals that CRM-GFT-based combinations demonstrated an even higher synergistic effect, with CI values consistently less than 1. This indicates that the combination of CRM and GFT was more effective in inhibiting the growth of U373MG cells compared to when these drugs were used individually or in different combinations. Importantly, the CRM-GFT combination showed the lowest CI values with CI<1. This suggests that this combination had the most optimal synergistic effect for the treatment of human glioblastoma U373MG cell lines. The study provides quantitative

evidence of the synergistic effect of CRM and GFT when used in combination for the treatment of glioblastoma. The combination consistently resulted in CI values below 1, indicating a synergistic interaction and optimal therapeutic potential for targeting U373MG cells.[15,16]

## Formulation of Nanoemulsion (NE)

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

The solubility of CRM and GFT in combination was tested in various oils. It was found that their solubility was highest in oleic acid. Specifically, the solubility of CRM in oleic acid was 4.42±0.39 mg/mL, while the solubility of GFT was 3.60±0.45 mg/mL. This suggests that oleic acid is an excellent choice as the oil phase for nanoemulsion. Tween 80 was chosen among the surfactants tested because it solubilized the maximum quantity of oleic acid. Tween 80 is commonly used as a surfactant in pharmaceutical formulations due to its ability to stabilize emulsions and improve solubility. Polyethylene glycol 400 (PEG 400) was selected as the cosurfactant. PEG 400 is known for its ability to reduce interfacial tension, which is essential for forming stable nanoemulsions.[10] It helps to maintain the dispersion of oil droplets in the aqueous phase. The selection of oleic acid as the oil phase, Tween 80 as the surfactant and PEG 400 as the cosurfactant suggests that these components were chosen based on their ability to create a stable nanoemulsion for delivering the combination of CRM and GFT. This optimized formulation is likely to enhance the solubility and bioavailability of these drugs, which is crucial for effective drug delivery in cancer treatment.

## Experimental design

Responses observed for twenty formulations prepared were fitted to various models using Design-Expert® software 7.0 (Design-Expert software, version 7, Stat-Ease, Inc., Minneapolis, Minnesota, USA). CCRD-RSM methodology offers to investigate a high number of variables at different levels with a limited number of experiments. The polynomial equation shows the relationship between independent variables and response variables such as droplet size (P), the content of CRM (Q) and the content of GFT (R) respectively. All values of $R^2$, SD and % coefficient of variation and ANOVA are depicted in Table 2.

$$P=47.60+1.50*A+3.60*B+2.09*C+0.80*A*B+0.55*A*C-4.57*B*C+2.81*A2+3.87*B2+3.51*C2$$

$$Q=91.33+1.12*A+0.67*B+0.89*C-0.24*A*B+0.44*A*C+1.26*B*C-0.59*A2-1.79*B2-5.24*C2$$

$$R=90.10+1.13*A+0.55*B+0.82*C-0.57*A*B+1.35*A*C+0.73*B*C-0.38*A2-1.59*B2-4.41vC2$$

Where, P=Droplet Size (nm), Q=Drug content of CRM (%), R=Drug content of GFT (%). A=Oil volume, B=Surfactant volume and C=Co-surfactant volume.

## Response surface plots

The response surface plot showing the effect of volume of oil and surfactant volume on droplet size (A) and the response surface plot showing the effect of volume of oil and co-Surfactant volume on droplet size (B) was presented in Figure 2A. response surface showing the effect of oil and surfactant volume on % drug content of CRM (C), response surface showing the effect of oil and co-surfactant volume on % drug content of CRM (D) and response surface showing the effect of oil and surfactant volume on % content of GFT (E), response surface showing the effect of oil and co-surfactant volume on % drug of GFT (F) was presented in Figure 2A.

## Effect of oil, surfactant and co-surfactant volume on droplet size and drug content

The droplet size was found to decrease with an increase in the oil volume and droplet size was found to decrease with an increase in the volume of surfactant and co-surfactant. The content of CRM and GFT was found to increase with an increase in the oil volume and the content of CRM and GFT was found to decrease with an increase in the volume of surfactant and co-surfactant. The volume of oil, surfactant and co-surfactant show a significant effect on droplet size and drug content of CRM and GFT (Figure 2B).

## Characterizations of Nanoemulsion (NE)

### *Droplet size analysis*

The study measured the size of the oil droplets in the prepared nanoemulsion formulations. This information is important because it indicates the stability and uniformity of the formulation. The results, along with the Polydispersity Indices (PDI), are presented in Figure 3A. The droplet size of F12 was found to be the smallest (47.60±0.59 nm), suggesting fine emulsion stability. The polydispersity index of F12 was the lowest (0.149±0.01), indicating a more uniform distribution of droplet sizes. The diameter of the dispersed oil droplets in the optimized NE (F12) was much smaller than 50 nm. This size is considered suitable for intranasal administration, especially for achieving brain targeting. Smaller droplets are advantageous for this purpose because they are more likely to pass through the blood-brain barrier and reach their intended site of action within the central nervous system. The study achieved a stable and suitable nanoemulsion formulation for intranasal administration of curcumin and gefitinib.

## Zeta Potential Determination

The zeta potential of your optimized nanoemulsion (F12) is -35.7±0.11 mV (Figure 3B). This value is negative, indicating that the particles or droplets in your nanoemulsion are negatively charged. A negative zeta potential suggests that there is a strong electrostatic repulsion between the particles or droplets in your

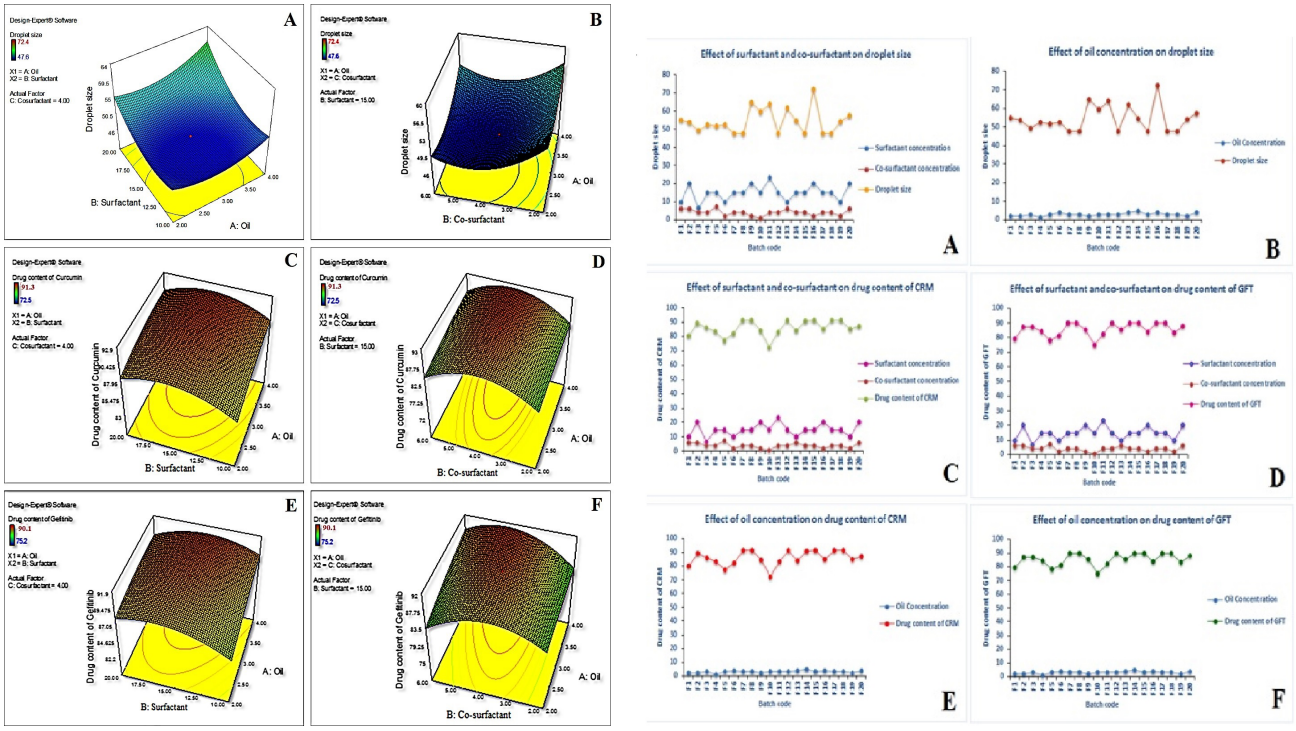


**Figure 2A and 2B:** 2A) Response Surface plots 2B) Effect of surfactant and co-surfactant concentration and concentration on droplet size (A) &(B), content of CRM (C) & (D), content of GFT (F) & (G).

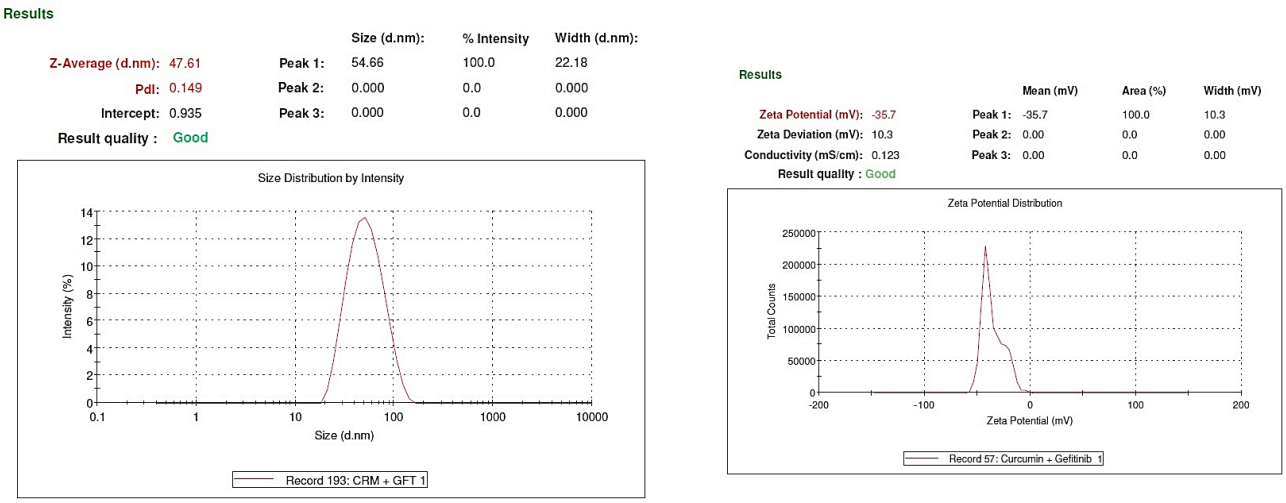


**Figure 3:** A: Particle size determination of optimized formulation, B: Zeta potential measurement of optimized formulation.

nanoemulsion. This repulsion helps prevent the particles from coming close together and agglomerating, which is essential for the long-term stability of the dispersion.

## Differential Scanning Calorimetry (DSC)

The physical mixture of the drug (CRM-GFT) exhibited thermal behavior like that of pure CRM (Figure 4A). This suggests that the drug did not form new crystalline structures or undergo significant interactions with CRM in the physical mixture. The Nanoemulsion formulation (NE) did not show any distinct peaks in the DSC thermogram under the studied temperature range. This lack of peaks suggests that the drug (CRM-GFT) in the nanoemulsion remained in a molecularly dispersed or amorphous state rather than forming crystalline structures.

## *In vitro* drug release

For CRM (one of the drugs), the release from the optimized NE (F12) was significantly higher (95.84%) compared to PDS

(52.62%) at the end of 4 hr. For GFT (the other drug), a similar trend was observed, with the release from the optimized NE (F12) being higher (94.02%) than from PDS (43.02%) after 4 hr (Figure 4B). The release profiles for both CRM and GFT were analysed using first-order release kinetics. This means that the drug release can be described by a first-order rate equation, which suggests that the release rate is directly proportional to the amount of drug remaining to be released. The high $R^2$ values (0.9979 for CRM and 0.9962 for GFT) indicate a good fit of the release data to the 1st-order model, suggesting that the release of both drugs follows this kinetic pattern. The Korsmeyer-Peppas equation is often used to describe drug release from polymeric matrices and is associated with different release mechanisms. The release exponent (n-value) obtained was 0.85 for CRM and 0.87 for GFT. These values suggest that the drug release mechanism is an anomalous non-Fickian diffusion mechanism and is characteristic of systems where both drug diffusion and polymer relaxation contribute to the release process, indicating complex release behaviour.

## Histopathological examination

The examination of the negative control mucosa (S1) showed no significant changes in the structure of normal nasal mucosa. The positive control mucosa (S3), treated with IPA, exhibited damage to the mucosal structure, as expected. However, the key observation is that the mucosa treated with your nanoemulsion formulation (S2) did not show significant alterations in the nasal epithelium structure (Figure 5A). There were no signs of destructive effects on the nasal mucosa caused by the CRM-GFT-NE formulation. Based on these histological examinations, it can be concluded that your nanoemulsion formulation appears to be safe for nasal administration. The lack of significant structural changes or destructive effects on the nasal mucosa indicates that the

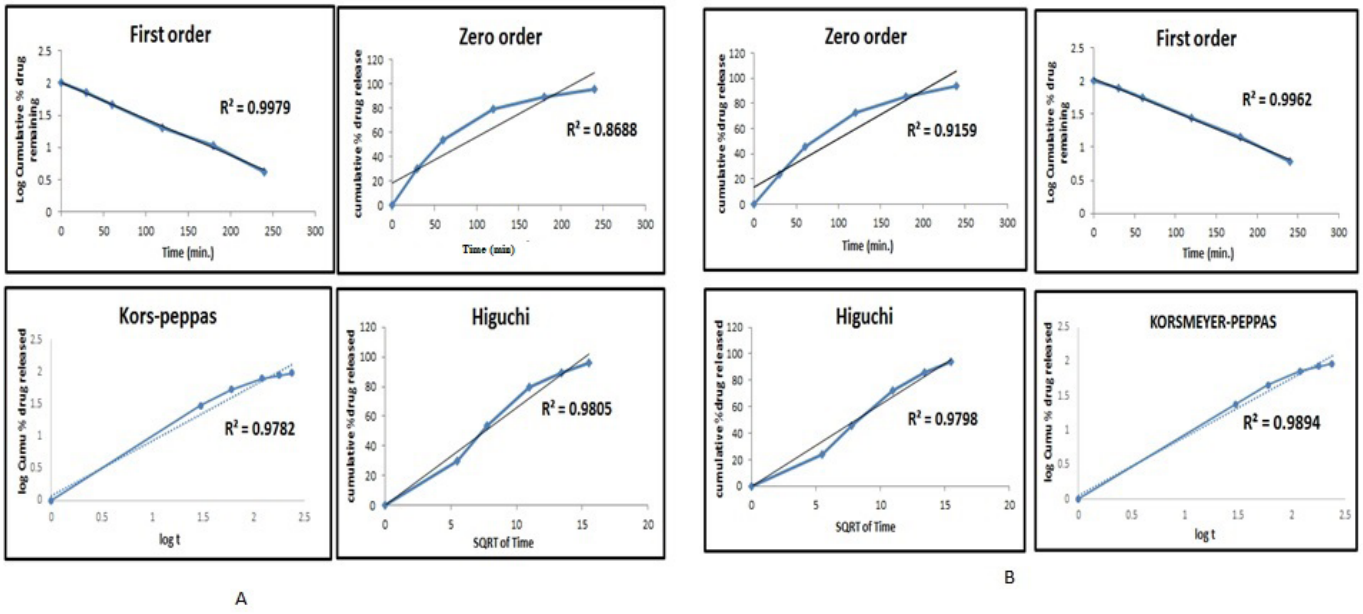


**Figure 4:** A: Differential Scanning Calorimetry thermograms, B: Release kinetics of Curcumin (A) and Gefitinib (B) from nanoemulsion.

**Table 3: Pharmacokinetics parameters of CRM-GFT-NE (F12) following Intranasal (IN) and Intravenous (IV) administration.**

| Formulation and route of administration | Drug | Organ/ Tissue | $C_{max}$±SD (ng/mL) | $T_{max}$ (min) | $AUC_{0-120}$ (ng/mL) |
|---|---|---|---|---|---|
| Nanoemulsion (Nasal) | CRM | Brain | 8328.67±995.05 | 15 | 675797±23173.10 |
| Nanoemulsion (Nasal) | GFT | Brain | 8323.04±936.33 | 15 | 622531.3±61159.11 |
| PDS (intra venous) | CRM | Brain | 462.73±37.82 | 15 | 27534.16±472.58 |
| PDS (intra venous) | GFT | Brain | 574.81±54.51 | 15 | 21692.93±849.34 |
| Nanoemulsion (Nasal) | CRM | Plasma | 5507.48±541.84 | 15 | 424861±10717.33 |
| Nanoemulsion (Nasal) | GFT | Plasma | 4283.12±312.60 | 15 | 345858±50645.87 |
| PDS (intra venous) | CRM | Plasma | 3957.38±656.85 | 15 | 209954.7±47409.92 |
| PDS (intra venous) | GFT | Plasma | 3750.643±76.279 | 15 | 219359.3±10742 |

PDS: Plain drug suspension.

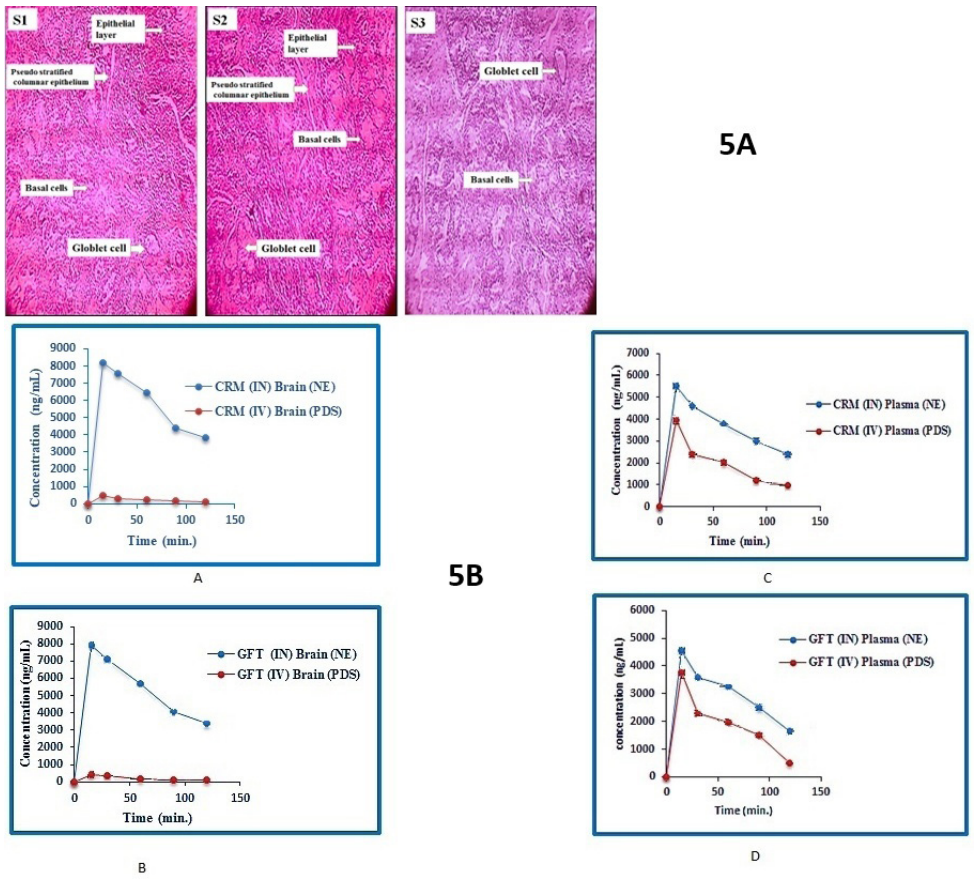


**Figure 5:** A: Photomicrogph histological examination of nasal mucosa after 6 h exposure of (S1, negative control) PBS pH 6.4; (S2) drug-loaded nanoemulsion; (S3, positive control) IPA and B: Brain concentration-Time profiles of CRM (A) and GFT (B), Plasma concentration-Time profiles of CRM(C) and GFT (D).

**Table 4: Drug targeting efficiency and direct nose-to-brain transport following intranasal administration of optimized CRM-GFT-NE (F12).**

| Name of Drug | % DTE | % DTP |
|---|---|---|
| CRM | 1871.26±2.56 | 95.21±0.93 |
| GFT | 1807.95±5.11 | 93.52±0.63 |

DTE: Drug targeting efficiency; DTP: Direct transport percentage.

formulation does not harm the nasal tissue. This is an important finding for assessing the safety of the formulation for potential use in nasal drug delivery.[12]

## Pharmacokinetics and Brain-distribution Studies

The concentration profiles of both CRM and GFT in the brain and plasma were measured after IN and IV administrations. After IN administration, both CRM and GFT showed an initial absorption phase with maximum concentrations achieved after about 15 min in the brain. Interestingly, the drug concentrations in the brain were significantly higher after IN administration of CRM-GFT-NE compared to IV administration of PDS for both CRM and GFT (Figure 5B).

Like the brain, the plasma concentration profiles showed an initial absorption phase with maximum concentrations reached after 15 min following IN administration. The drug concentrations in plasma were also found to be higher after IN administration of CRM-GFT-NE compared to IV administration of PDS for both CRM and GFT (Table 3). The study introduced the terms % DTP (percentage of drugs directly transported to the brain via the olfactory pathway) and % DTE (percentage of drugs transported to the brain). CRM-GFT-NE exhibited the highest % DTE and % DTP values for both CRM and GFT, indicating better brain targeting efficiency. The results suggest that the enhanced brain concentration and nasal bioavailability of CRM and GFT were achieved through direct nose-to-brain transport via the olfactory pathway when administered as CRM-GFT-NE. The findings of this investigation demonstrate that the nanoemulsion formulation (CRM-GFT-NE) facilitates the direct transport of CRM and GFT to the Central Nervous System (CNS) following intranasal administration. This results in significantly higher drug concentrations in the brain compared to IV administration of plain drug solutions. The use of % DTP and % DTE metrics further supports the enhanced brain targeting efficiency of

CRM-GFT-NE, particularly through the olfactory region of the nasal cavity (Table 4). These results suggest the potential of CRM-GFT-NE as a promising approach for improving the delivery and bioavailability of CRM and GFT to the brain.

## CONCLUSION

The study suggests that combining CRM and GFT in a nanoemulsion for intranasal administration could be an effective and promising strategy for targeting and treating brain tumors, especially glioblastoma. The synergistic effect observed in the study, along with the potential for targeted delivery to CNS, makes this approach particularly valuable for further exploration and development in the field of brain tumor therapy. However, further research and clinical studies will be needed to validate these findings and assess the safety and efficacy of this treatment approach in humans.

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## ABBREVIATIONS

**CRM:** Curcumin; **GFT:** Gefitinib; **NE:** Nanoemulsion; **BT:** Brain Targeting; **IN:** Intranasal; **IV:** Intravenous; **DTE:** Drug Targeting Efficiency; **DTP:** Direct Transport Percentage; **CI:** Combination Index; **SRB:** Sulforhodamine B; **DSC:** Differential Scanning Calorimetry; **HPLC:** High-Performance Liquid Chromatography; **PBS:** Phosphate Buffer Saline; **FBS:** Fetal Bovine Serum; **DMEM:** Dulbecco's Modified Eagle's Medium; **MDR:** Multi-Drug Resistance; **ZP:** Zeta Potential; **TCA:** Trichloroacetic Acid; **AA:** Acetic Acid; **AG:** Analytical Grade; **H&E:** Hematoxylin and Eosin; **CNS:** Central Nervous System; **BBB:** Blood-Brain Barrier; **PGI:** Percent Growth Inhibition; **PDR:** Percent Drug Release; **RSM:** Response Surface Methodology; **ANOVA:** Analysis of Variance; **PDI:** Polydispersity Index; **ACN:** Acetonitrile; **N2:** Nitrogen Gas; **OM:** Optical Microscope; **WAR:** Wistar Albino Rats; **TAPR:** Time Average Partitioning Ratio; **CCL:** Cancer Cell Lines; **GBM:** Glioblastoma; **AS:** Astrocytoma; **DC:** Drug Content; **DR:** Drug Release; **DC:** Drug Concentration; **CV:** Cell Viability.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## SUMMARY

The present study provides a detailed study involving the development of nanoemulsions containing the combination of curcumin and gefitinib for nose-to-brain delivery in the management of glioblastoma. This study presents a detailed account of formulation and in vitro as well as in vivo studies of developed nanoemulsion formulations.

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