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Development and Evaluation of Nanoemulsion and Microsuspension Formulations of Curcuminoids for Lung Delivery with a Novel Approach to Understanding the Aerosol Performance of Nanoparticles

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Abstract:

Extensive research has demonstrated the potential effectiveness of curcumin against various diseases, including asthma and cancers. However, few studies have used liquid-based vehicles in the preparation of curcumin formulations. Therefore, the current study proposed the use of nanoemulsion and microsuspension formulations to prepare nebulised curcuminoid for lung delivery. Furthermore, this work expressed a new approach to understanding the aerosol performance of nanoparticles compared to microsuspension formulations. The genotoxicity of the formulations was also assessed. Curcuminoid nanoemulsion formulations were prepared in three concentrations (100, 250 and 500 µg/ml) using limonene and oleic acid as oil phases, while microsuspension solutions were prepared by suspending curcuminoid particles in isotonic solution (saline solution) of 0.02% Tween 80. The average fine particle fraction (FPF) and mass median aerodynamic diameter (MMAD) of the nebulised microsuspension formulations ranged from 26% and 7.1 µm to 40% and 5.7 µm, for 1000 µg/ml and 100 µg/ml respectively. In a comparison of the low and high drug concentrations of the nebulised nanoemulsion, the average FPF and MMAD of the nebulised nanoemulsion formulations prepared with limonene oil ranged from 50% and 4.6 µm to 45% and 5.6 µm, respectively; whereas the FPF and MMAD of the nebulised nanoemulsion prepared with oleic acid oil ranged from 46% and 4.9 µm to 44% and 5.6 µm, respectively. The aerosol performance of the microsuspension formulations were concentration dependent, while the nanoemulsion formulations did not appear to be dependent on the curcuminoids concentration. The performance and genotoxicity results of the formulations suggest the suitability of these preparations for further inhalation studies in animals.

Key words: Nanoemulsion, Microsuspension, Curcuminoids, Lung Delivery, Nebuliser Formulation, Genotoxicity.

1. Introduction

Curcuminoids are polyphenolic compounds that are extracted from rhizomes of turmeric [1]. The alcoholic extracts of turmeric contains three curcuminoids, namely curcumin (77%), demethoxycurcumin (18%) and bisdemethoxycurcumin (5%) [2]. Curcuminoids have strong anticancer activities [3-6, 47]. Also, several recent studies have reported the potential anti-asthmatic property of curcumin due to the anti-inflammatory of curcuminoids [7-9].

Inhalable curcumin particles has been recently reported in few studies as dry powder inhalers using different methods, such as supercritical CO2-assisted spray-drying [10,56], supercritical anti-solvent (SAS) precipitation[57], spray and freeze drying [11], mechanical milling followed by spray drying [12,48], nanocomposite particles [13], polymeric micelle based [14,47]. Several studies have reported the use of liquid-based vehicles (liposomes and Janus nanoparticles) in preparing curcumin formulations for potentially treating lung cancer. However, some of these studies (46, 49) have not considered the aerodynamic profile of curcumin particles. On the other hand, Manconi et al (50) reported an improvement of the curcumin deposition in the stages of a cascade impactor (≥50%) using chitosan coated liposomes. Additionally, Manca et al (51) reported that chitosan-glycerosomes may be used as lung delivery systems for curcumin, which improves curcumin's antioxidant and anti-inflammatory activity. However, further studies may be required to assess the safety of using chitosan as a carrier to lung therapeutics.

In general, microsuspension is the only approved solution formulation for inhaled neutral lipophilic drugs that are used for the treatment of asthma and chronic obstructive pulmonary disease (COPD) (55). Therefore, the nebulised formulations of the water-insoluble neutral compounds available in the market are in suspension form, such as budesonide microsuspension. However, several disadvantages of using microsuspension for inhalation have been reported, such as considerable drug concentration heterodispersity in the aerosol droplets [15], short drug-residence time in the lungs due to ciliary movement [16], limited bioavailability of the micronized drug compared to the nanoparticles, and variability in the drug deposition patterns when different nebulisers are used [17].

Therefore, curcuminoid nanoemulsion formulations have been considered and optimized for inhalation in this study. Besides, it was reported that curcumin's anti-inflammatory characteristics are improved in nanoemulsion formulations [18]. Oleic acid and limonene oils were used to prepare the nanoemulsion formulations, and Tween 80 was used as a surfactant. Oleic acid is approved by the FDA for use in respiratory preparations at a concentration of 0.28%. Tween 80 is also approved for use at 0.02%. In addition, it has been reported that

limonene does not cause respiratory irritation or symptoms related to the central nervous system when inhaled by human subjects (53), and it reduces airway inflammation in mice (54).

In this study, a curcuminoid suspension formulation was also prepared to examine the difference in the aerodynamic behaviour of nanoemulsion and suspension when nebulised. It is worth noting that no previous studies have conducted an in-vitro aerodynamic characterization of all the curcuminoid components, i.e. curcumin, demethoxycurcumin and bisdemethoxycurcumin.

However, nanoparticles have a potential harmful side effect in humans, they could be genotoxic [19, 20], which may be attributed to a direct interaction between the nanoparticles and genetic material, indirect damage from nanoparticle-induced reactive oxygen species, or by releasing toxic ions [21, 22]. Due to their unique size, nanoparticles have the ability to cross the cellular membrane and may reach the nucleus through diffusion across the nuclear membrane or transportation through the nuclear pore complex and direct interaction with DNA [23].

Genotoxicity tests of pharmaceutical products before commercialization are required by regulatory agencies worldwide [24]. Therefore, the in-vitro genotoxicity of the curcuminoid nanoemulsion was examined using single-gel electrophoresis (comet assay) on human lymphocyte cells.

This work proposes the preparation of a curcuminoid nanoemulsion using an extremely low amount of surfactant to avoid formulation toxicity, making the nanoemulsion safe for inhalation. Additionally, this study addresses a new approach to understanding the aerosol performance of nanoparticles compared to microsuspension formulations.

2. Methods

2.1 Formulation

Nanoemulsion Preparation:

Nanoemulsion formulations were prepared using different types of oil including limonene and oleic acid. Tween 80 and ethanol were used as surfactant and cosurfactant, respectively. (see table 1). The oil (limonene or oleic acid) containing the appropriate concentration of drug, surfactant (Tween 80) and cosurfactant (ethanol) were initially mixed and the aqueous phase was then added and mixed well in the mixture. The solution was then sonicated for 10 minutes to ensure that all ingredients had been mixed very well and the nanoemulsion is formed.

Loading capacity of nanoemulsion was studied by adding an excess amount of curcuminoid and then sonicated for 15 mins and left at room temperature for 24 hours. The nanoemulsion was then filtered using 0.45 µm syringe filter and diluted in a HPLC mobile phase then injected into HPLC.

Microsuspension Preparation

Saline solution was prepared by dissolving 0.9% of sodium chloride in ultrapure water. A sufficient amount of micronized curcuminoids was suspended in 0.2% (w/w) of Tween 80 and then diluted with saline solution to obtain a 0.02% of tween 80 with final concentration of 500, 250 and 100 µg/ml of curcuminoids. The suspension remained homogenous after shaking for the period of filling the nebulised chamber and during nebulisation time. The amount of Tween 80 was used according to FDA regulations.

2.2 Osmolality

The osmolality of the nanoemulsion samples was determined at room temperature using Advanced 3320 Micro-Osmometer (Model 3320). The Osmometer was calibrated using a standard solution (50 mOsm Calibration Standard). Samples were measured in triplicate and the mean was then calculated. Sodium chloride was used to adjust the osmolality of the samples.

2.3 Particles size measurement

The particle sizes of the curcumin formulation samples for this study were determined using the Malvern Zetasizer Nano-ZS dynamic light scattering (DLS) instrument (Malvern Instruments, UK). A suitable volume of undiluted formulation solution was transferred into malvern cuvette cell. The mean particle size was determined from three measurements. Nanosphere™ (catalogue no. 3060A, mean diameter 59.0nm±2.5) and Duke Standards™ (catalogue no. 8050, diameter 500.0nm±0.02) were used to calibrate the Zetasizer's performance.

2.4 Viscosity

The viscosity of the samples was measured using vibrating viscometer (SV-10) at 25 °C. The sample was loaded into viscometer container (35 ml) and attached into the base of the instrument.

2.5 Aerosol output

The method reported by [25] was used to assess the aerosol output of the nanoemulsion using the Sidestream jet nebuliser. Each sample was run in triplicate (n=3); the mean and the SD were calculated. Among all samples the amount and the percentage of the inhaled, exhaled and remaining drug in the nebuliser chamber were calculated.

2.6 Aerodynamic diameter measurements and particles lungs depositions

Next Generation Impactor (NGI) was used to characterise the aerodynamic particles size of nebulised formulations. According to Pharmacopeia (USP 2012, Ph. Eur 2012), NGI was placed in a cooler system at 5°C for 30 mins before nebulisation. The jet Sidestream nebuliser was kept outside the cooler system and connected to the NGI by T-piece. The NGI was connected from the other side to a flow controller which was already attached to a vacuum pump. The flow rate was adjusted to be 15 L/min. The pump and the flow controller were switched on before starting the nebulisation. The nebuliser chamber was filled with 5 ml of nanoemulsion samples and run after switching the pump and the controller on. The nebulisation was stopped first after the sputtering sound is heard and then the controller and the pump. The NGI cups and the nebuliser chamber were then washed with 20 ml of the emodine solution (internal standard). Samples were assessed in triplicate and the nebulisation time for each one was recorded.

2.6.1 Data analysis

Copley Inhaler Testing Data Analysis Software (CITDAS) was used to identify the aerodynamic characteristics of the emitted dose. The fine particle dose (FPD) was the amount with particles that correspond to a size less than 5μm. The fine particle fraction % (FPF) was the FPD expressed as a percentage of the total amount deposited into the throat and stages of the cascade impactor (this is the dose exiting the mouthpiece) as well as expressed as a percentage of the nominal dose (label claim). The mass median aerodynamic diameter (MMAD) was the diameter corresponding to 50% undersize. The geometric standard deviation (GSD) was the square root for the size corresponding to 84.13% less than the stated size divided by the square root of the size for 15.87% (United States Pharmacopeia 2005).

2.7 Genotoxicity:

Comet assay is a simple and sensitive method for the detection of DNA breakage in individual cells [26]. This method was produced by Ostling and Johanson [26] and it has been developed further by Singh and Olive [27, 28].

It has been found that DNA damages (fragments) stretches from the nucleus, in the form of a comet, toward the anode in alkaline electrophoresis gel. The DNA migration is the function of the intensity of DNA breakage. Tail moment, a measure of tail length and the fraction of DNA in the Comet tail, was used as the arbitrary unit of assessment [29, 30]. Tail moment measures both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed / broken pieces of DNA (represented by the intensity of DNA in the tail)

$$Olive\ Tail\ Moment = (Tail.mean - Head.mean)\ X\ Tail\ \%\ DNA/100$$

The assay was carried out using lymphocytes cells because they are exposed to different environments within the body while travelling in the bloodstream and can therefore reflect DNA damage that has been induced by endogenous and exogenous genotoxins. These cells are excellent carriers to use in examining the genomic sensitivity of any cell as their sub-populations have a lengthy life spans and are capable of carrying mutagen-induced genetic aberrations for over 40 years [31]. In addition, the World Health Organisation/International Programme on Chemical Safety has reported that lymphocytes are suitable surrogate cells for cancer [32].Furthermore, Najafzadeh et al., reported that lymphocytes are not only suitable surrogates for cancer but for other disease states as chronic obstructive pulmonary disease (COPD) and asthma [45], because the DNA is the same in all the cells of an individual.

Comet assay was utilised to study the genotoxcity of nanoemulsion formulations of curcumnoids using the protocol that was reported by Tice [33]. All experiments of the genotoxicity study were conducted under the Human Tissue Authority License No. 1219 to School of Life Sciences.

Protocol of genotoxicity studies:

A glass slide was covered with 1 % normal melting point agarose (NMP) and left to dry overnight. 890 µl PRMI 1640 was added into Eppendorf tube, 10 µl of curcumin nanoemulsion (NE3, NE4, NE5, NE9, NE10 and NE11) was added to the cell media then 100 µl of whole blood sample was added to the previous mixture and incubated for 30 mins at 37 ˚C. The samples were moved to a centrifuge for 5 mins at 3000 rpm. 900 µl from the

supernatant was removed from the samples and 100 µl of 0.5 % low melting point of agarose (at 40 0C) was added to each sample. The cell pellets were disrupted gently and 100 µl of the suspended cells was transferred into previously coated glass slide with 1 % NMP and distributed uniformly by placing a cover slip, and left on ice for around 5 min. The cover slip was removed carefully and the slide was immersed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100, pH 10) at 4 ˚C for overnight. The slide then transferred into gel electrophoresis tank with cold alkaline buffer solution (300 mM NaOH, 1 mM EDTA, pH <13) and left for about 30 mins at 4 ˚C. The electrophoresis was conducted at 25 voltages and 300 mA for 30 min at 4 ˚C. The sample slides were rinsed thrice with neutralising buffer solution (400 mM Tris, pH 7.5) and left for 5 min. Ethidium bromide (60 µl, 20µg/ml) was added into the sample slides and a cover slip applied. After incubation at room temperature for 5 min the slides were examined with a fluorescent microscope equipped with CCD camera. A computerised image analysis system, Komet 4.0 (Kinetic Imaging, Liverpool, UK), was employed to measure the Comet parameters; the % Olive tail moment was then used for statistical analysis. The data was analysed using one-way ANOVA.

3. Results

3.1 Nanoemulsion preparation and optimization

Table 1 shows the visual observation of the nanoemulsion preparations. The limonene nanoemulsion was transparent and clear in all formulations. The oleic acid nanoemulsion was not formed with 0.8% of oleic acid as oil, so the oil percentage was reduced gradually to 0.3% to obtain a clear transparent nanoemulsion (Table 1). It was found that the minimum amount of surfactant that could form a nanoemulsion was 0.33% (NE5 & NE11). The cosurfactant (ethanol) also was reduced from 6.6% (in NE1 & NE7) to 0.25% (in NE5 & NE11). There were no changes in nanoemulsion appearance when the surfactant, cosurfactant and oil were reduced to the minimum (Table 1). However, the nanoemulsion did not form when reducing the percentage of the Tween 80 or ethanol less than 0.33% and 0.25%, respectively; this was due to insufficient amounts of surfactant and cosurfactant to solubilise the oil.

The loading capacity of curcuminoids in nanoemulsion formulations NE9, NE10 and NE11 was 500, 250 and 100µg/ml of curcuminoids, respectively. The loading capacity of NE3, NE4 and NE5 was 500, 250 and 100µg/ml, respectively.

3.2 Osmolality

The suitable osmolality of aerosol solutions should be between 130 and 500 mOsm/kg, and the formulation should also have permeant ions (such as chloride) in concentrations of 31mM to 300 mM to ensure the airway's tolerability of such a formulation [34].

From the results in Table 2, it is clear that all of the nanoemulsion and suspension formulations (with curcuminoid concentrations of 500, 250 and 100 µg/ml) are in the ideal range for osmolality. The only formulations out of the accepted osmolality range for nebulisation are NE2 and NE8; this is attributed to a high amount of ethanol in the formulations. The ethanol forms 2.5% of the formulations NE2 and NE8. Therefore, these formulations with high osmolality and zero sodium chloride were excluded from further studies.

3.3 Viscosity

Viscosity is the resistance of the fluid to a flow; therefore, the viscosity is important in aerosol formulations. Ingredients of the formulations and concentration of the drugs may change the viscosity of the preparations; hence this could alter the aerosol output and the aerodynamic distribution of the particles [34, 35]. The results (Table 2) show that the viscosity of the nanoemulsion formulations with either limonene oil or oleic acid oil increases as the concentration of ingredients (oil, surfactant and cosurfactant) increases. The results of this study show that, as viscosity increases, MMAD increases, which negatively affects the aerosol performance. The relationship between the curcumin nanoemulsion formulations' viscosity and MMAD values was linear, with an R^2 of 96 for the formulation containing oleic acid and an R^2 of 95 for the formulation containing limonene. This finding agrees with the report from Mccallion et al (52), which states that droplet size is proportional to the viscosity of the nebuliser solution, and more viscous fluids have lower outputs. It has also been reported that it is impossible to nebulise highly viscous fluids (> 6cP) [35]. On the other hand. The suspension formulation did not show any increase in viscosity, this is because of the amount of surfactant used in each formulation, which was constant (0.02 % w/w).

3.4 Particle size analysis using Malvern (Zetasizer)

The particle size of the nanoemulsion prepared using limonene oil (NE3, NE4 and NE5) was smaller than the nanoemulsion produced by oleic acid (NE9, NE10 and NE11). The difference in the particle droplet size can be attributed to the difference in physicochemical properties of the oils (viscosity). The viscosity of limonene is about 0.923mPa at 25^0C [36], whereas the viscosity of oleic acid is 30mPa at 25^0C [37]. Increasing the viscosity of the fluid increases the resistance to the deformation of the particles, hence the oleic acid is more resistant to being deformed (dispersed into smaller droplet size) than limonene oil. The viscosity of oils has a significant effect on the droplet size of emulsions, as a less viscous oil produces a smaller droplet size of emulsion [38, 39]. The particle size of the microsuspension was about 1.6µm, which was the same as for micronized solid particles of curcuminoids that were prepared beforehand and then suspended in ultrapure water containing Tween 80 and 0.9% sodium chloride. The results on particle size of the nanoemulsion and suspension formulations are given in Table 3.

3.5 Aerosol output using the jet nebuliser

When comparing the nanoemulsion with the suspension formulations, (Table 4, Supplementary Tables 1 and 2), there is a significant increase in the performance of the curcuminoid nanoemulsion formulations (either with limonene or oleic acid oil) over the suspension preparations. In the suspension formulations, the percentage of the drug left in the nebuliser chamber was almost 50% of the delivered dose, whereas in the nanoemulsion it was about 30%. Also, the percentage of drug in the inhalation filter from the nanoemulsion preparation ranged from 33% to 37%, whereas in the suspension formulation it was about 21% to 27%.

3.6 Aerodynamic particle size characterization

Suspension formulations

The results of a low FPF and high MMAD indicate poor performance for a high drug concentration of suspension during nebulisation (Figures 1 & 2, Table 5). The results show that the suspension formulation containing curcuminoids at a concentration above 500 µg/ml is not suitable for inhalation due to low FPF (%) and high MMAD. These findings are in agreement with Amani's (34) results reported for commercial budesonides suspension using a jet nebuliser device. However, the same formulation with a lower drug concentration showed better performance in FPF (%) and MMAD.

Nanoemulsion formulations

The results of aerodynamic characterization of the nanoemulsion formulations using oleic acid and limonene for curcumin and demethoxycurcumin and bisdemethoxycurcumin (Tables 6 & 7) illustrate an improvement in FPF (%) and MMAD compared to suspension formulations. Figures 4 and 5 show that the variation in MMAD and FPF was very small throughout the nanoemulsion formulations for different curcuminoid concentrations.

3.7 Curcuminoid nanoemulsion genotoxicity study

Nanoemulsion of curcuminoid genotoxicity (DNA damage) has not been examined before. The genotoxicity of the optimised nanoemulsion formulations (NE3, NE4,NE5,NE9,NE10 and NE11) is presented in Figure 7. One-way ANOVA analysis (Supplementary Tables 3) reveals absence of any genotoxicity with any of the concentrations of limonene and oleic acid used in the current study. In fact the DNA damage observed was lower than that of the negative control indicating some genoprotective effect of the curcumin nanoemulsions.

4. Discussion

The study target was to prepare a nanoemulsion vehicle with the lowest possible amount of ingredients (i.e. surfactant, cosurfactant and oil). Therefore, each nanoemulsion component was decreased to the minimum. To date, all reported nanoemulsion formulations [40-42] have been prepared using a high concentration of surfactant (about 10% w/w of the formulation or above), which is not suitable for the lungs according to the FDA (US Food and Drug Administration). Whereas, in this work, the amount of Tween 80 (surfactant) used was ten to thirty times lower than the levels used in other reported work, which could be suitable and safer for the respiratory system.

The performance of aerosol output for the nanoemulsion was much better compared to suspension formulations. This finding is in agreement with previous studies done on nanoemulsion of budesonide [40]. The low FPF (%) and large MMAD obtained for the microsuspension formulations using Sidestream jet nebuliser as well as the large amount of the drug left in the nebuliser chamber show that the microsuspension type of formulations is inefficient for nebulisation of curcumin, compared with nanoemulsion formulations.

The authors of this manuscript proposed that the superior output performance of nanoemulsion over suspension is due to the fact that the particle size of the nanoemulsion is smaller than the particle size in the suspension formulation. Furthermore, the nanoemulsion

exists in liquid form and the nebulised droplet will be fully filled with a liquid form, rather than solid form (suspension). The results (Table 4, Supplementary Tables 1 and 2) also show an improvement in the inhalation rate of nanoemulsion formulations over suspension formulations, which are an important factor in regard to patient compliance, because of the duration required for the nebulised dose to be taken.

The enhanced performance of suspension formulations with low drug concentration could be due to the fact that nebulisers produce a droplet size ranging from 1 to 5 µm in diameter [43]. These droplets usually carry the drug particles during nebulisation. In our opinion, nebulised droplets at low drug concentrations carry the drug particles based on the actual size of the suspended drug particles (1.6 µm). For example, if the drug has a particle size of 1.6 µm, it will likely reside in a nebulised droplet size of 2 µm or above, as illustrated in Figure 3. However, at higher drug concentrations, as the number of drug particles increases, the nebulised droplets are forced to carry more drug particles within droplets. Consequently, more particles have to move at the same time inside the large droplets, which leads to particle agglomeration and hence increases their size (for example, if the individual particle has a size of 1.6 µm, the agglomerated size may be 2.5 µm or bigger). Therefore, the small droplets will remain unoccupied and free from the agglomerated drug particles. This theory could explain the poor aerosolized performance with low FPF and large MMAD of the formulated microsuspension with high drug concentrations. Similar aerosolized performance was reported by Hemmandez-Trejo [44]. They found the agglomeration of particles in a suspended solution to be a significant issue during nebulisation from a jet nebuliser.

The difference in the aerodynamic performance between the nanoemulsion and suspension formulations is attributed to the particle size of the drug in each formulation. Our theory for explaining this difference in aerodynamic performance between suspension and nanoemulsion formulations is that when the drug is in a suspension form, it will occupy the nebulised droplet based on the drug particle size. For example, if the drug particles are 1.6-2 µm, the drug will be carried in the larger nebulised droplet only, as is shown in Figure 6, therefore the smaller nebulised droplets will be free of the drugs. Consequently, the microsuspension formulations exhibit a lower FPF (%) and higher MMAD. In the case of nanoemulsion, the particles are very small in size (12 to 35 nm) and the particle size is more uniform, therefore all nebulised droplets will be fully filled with nanoparticles of the drug. For these reasons, nanoemulsion formulations exhibited much better fine particle fractions (FPF) and hence achieved deeper particle deposition compared to the microsuspension

formulation. Additionally, Amani et al., studied the performance of a nebulised nanoemulsion formulations containing budesonide in comparison to that of a microsuspension formulations of budesonide. The authors found the aerosolization performance of the nanoemulsion formulations to be superior to that of the microsuspension formulations because of the smaller mass median aerodynamic diameter (MMAD) and larger respirable fraction (FPF) values, and this agrees with our findings. However, Amani et al. did not discuss the reason for the improved performance of the nanoemulsion formulations. The theory we propose provides an explanation for the improved performance that was achieved with the nanoemulsion formulations than with the microsuspension formulations in both our study and the study reported by Amani et al. [40].

The data from the genotoxicity study indicates that the lymphocyte cells experienced no genotoxicity during treatment with the curcuminoid nanoemulsion formulations compared to the negative control. Garbuzenko et al. [49] supports these findings; however, the authors used a different technique to simultaneously encapsulate both curcumin and doxorubicin and did not assess the genotoxicity of the individual compounds. In addition, Garbuzenko et al. [49] evaporated the emulsion for 6 hrs to remove the organic solvent, which is a long process time. The Olive Tail moment in the curcuminoid nanoemulsion formulations was lower than in the negative control, indicating that the curcuminoid nanoemulsion formulations may have a protective effect and may be able to repair existing damages. Moreover, the DNA seems to be intact in the optimised formulations as no DNA comet was found (Figure 8).

5. Conclusion

The in-vitro aerosolized performance of nanoemulsion was superior to suspension formulations and was independent of drug concentration, whereas the performance of the suspension was drug concentration dependent. A theory of understanding the superlative aerosol performance of the nanoemulsion formulations over the microsuspension was provided and clearly explained.It was further supported by examining the physical properties (such as particle size) of the formulations of both types (nanoemulsion and microsuspension). Further investigations of this theory is in progress for aerosol nano-suspension formulations.

The nanoemulsion formulations prepared with limonene oil and oleic acid were found to be nontoxic at the curcuminoid doses that were used in the genotoxicity study. Therefore, this

could be an indication of the safety and suitability of the nanoemulsion formulation which could be extended to further investigations for both animals and humans.

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Figures:

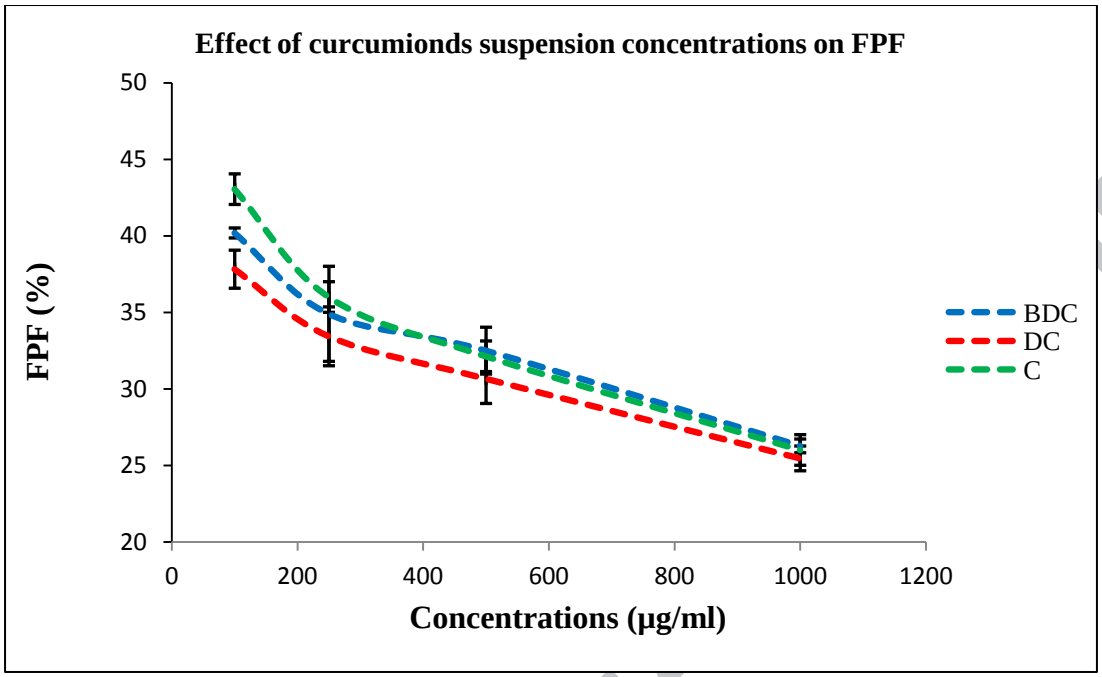


Figure 1. Relationship between the suspension concentrations and the fine particle dose fraction of curcuminoids.
BDC: Bisdemethoxycurcumin; DC: Demethoxycurcumin; C:Curcumin; FPF: Fine particle fraction

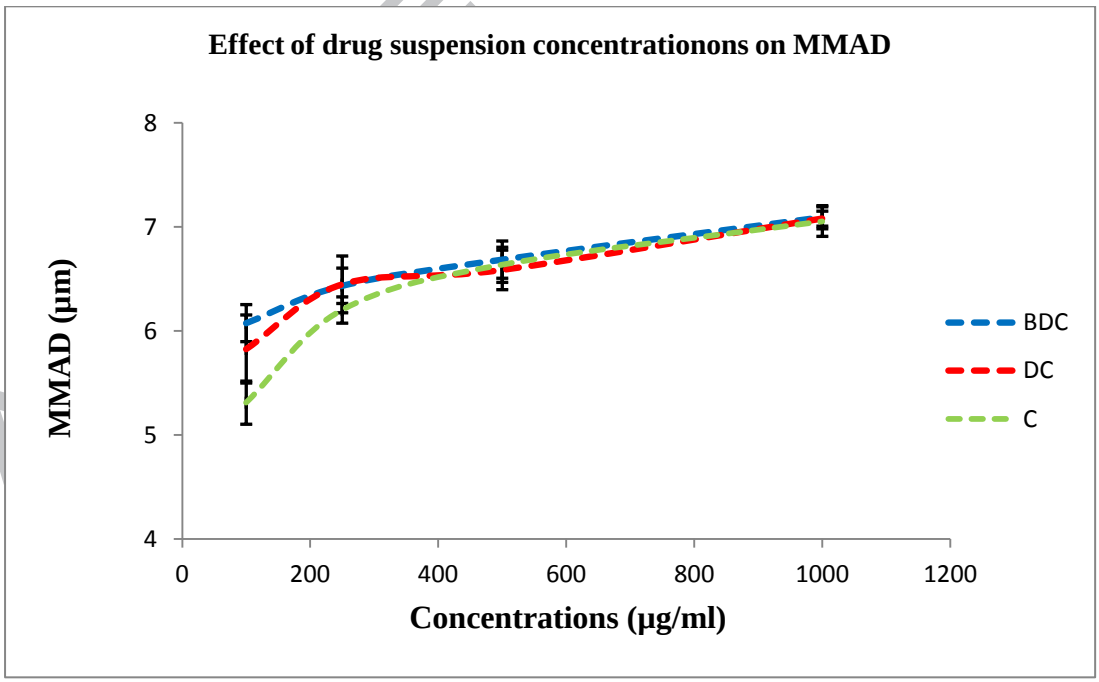


Figure 2. Relationship between the suspension concentrations and the mass median aerodynamic diameter (MMAD) of curcuminoids.

BDC: Bisdemethoxycurcumin; DC: Demethoxycurcumin; C:Curcumin; FPF: Fine particle fraction.

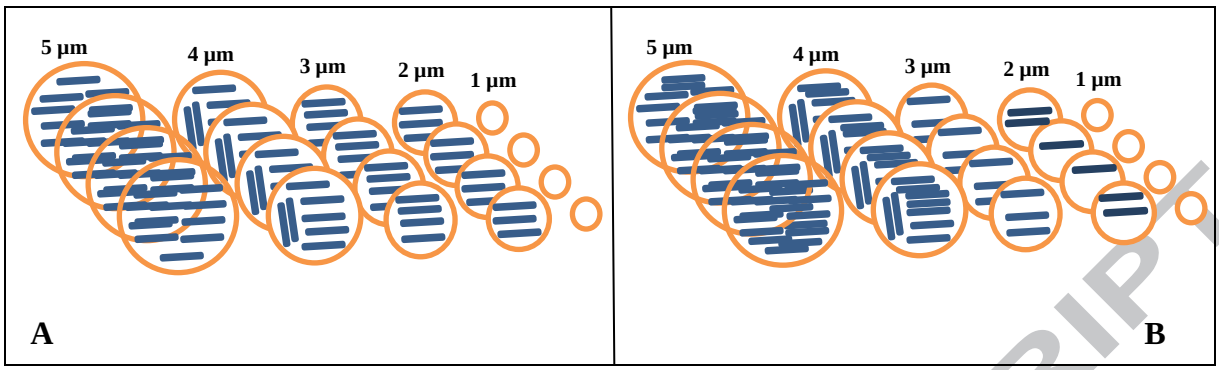


Figure 3. Nebulised droplets from microsuspension formulations at low drug concentration (A) and at high drug concentration (B)

: Nebulised droplets.
: Suspended drug particles (1.7 µm).

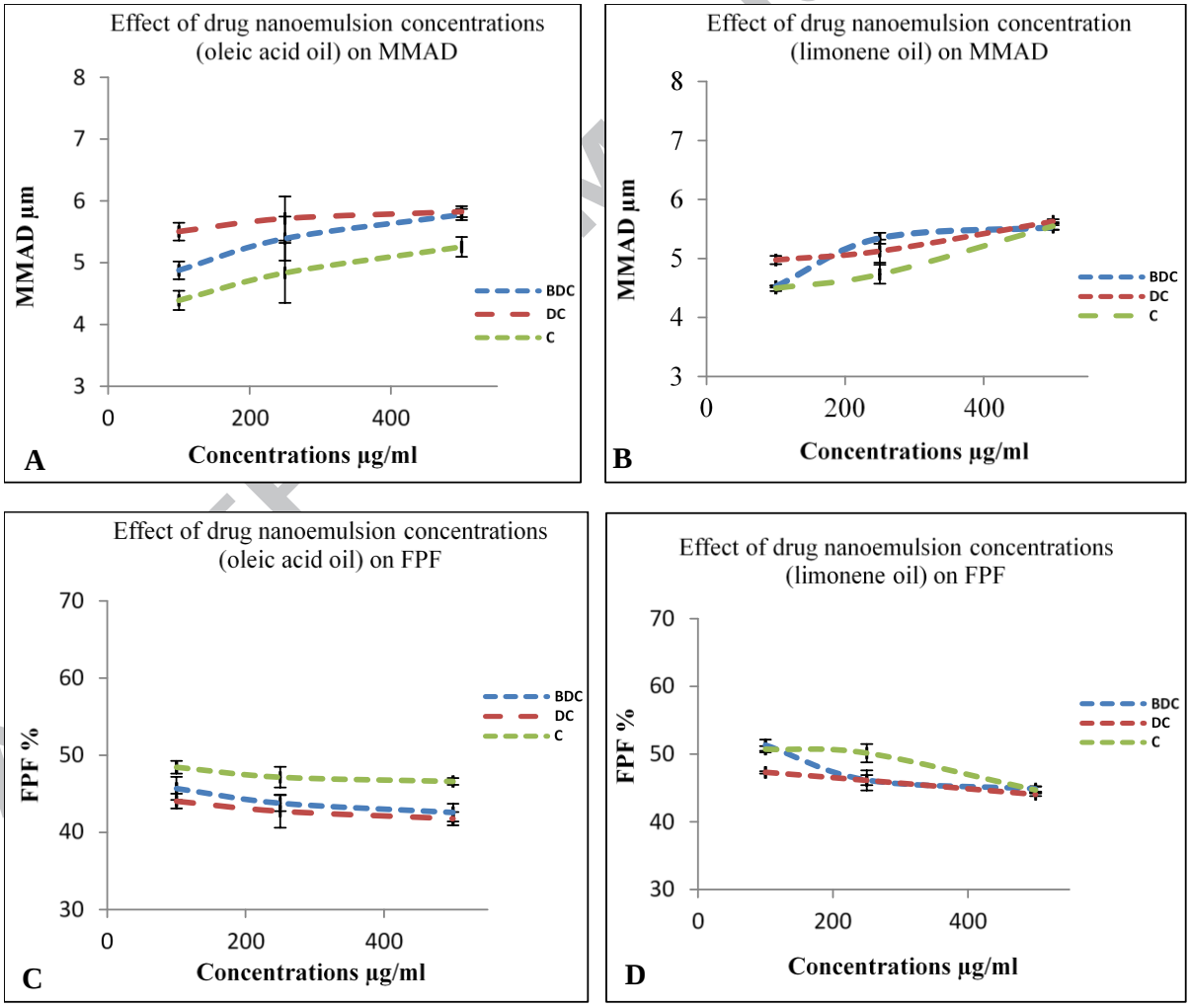


Figure 4. Relationship between drug nanoemulsion concentrations and FPF and MMAD of curcuminoids. A: MMAD with oleic acid (NE9, NE10, NE11); B: MMAD with limonene (NE3, NE4, NE5); C: FPF with oleic acid (NE9, NE10, NE11); D: FPF with limonene (NE3,

NE4, NE5).BDC: Bisdemethoxycurcumin; DC: Demethoxycurcumin; C:Curcumin; FPF: Fine particle fraction

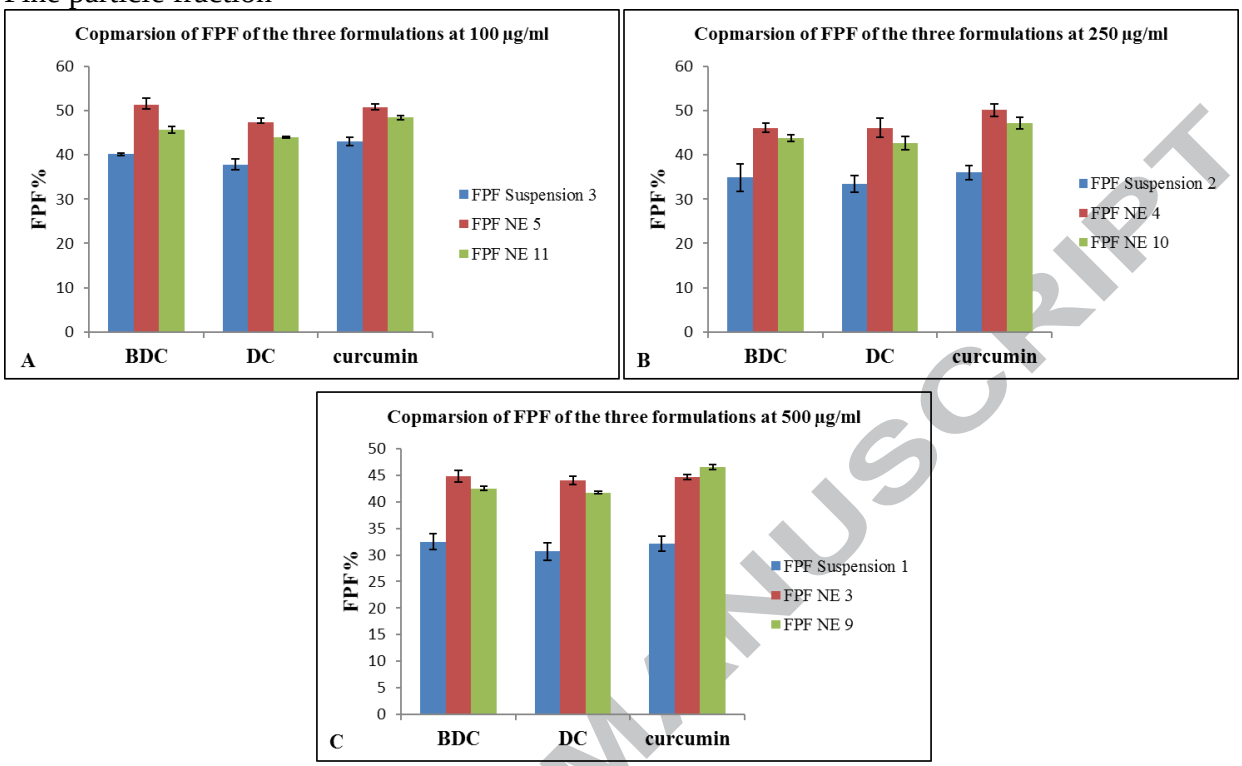


Figure 5. Comparison of fine particle fraction (FPF) between nanoemulsion formulations and suspension for different concentration of curcuminoids (100, 250,500 µg/ml). **A**: suspension 3, NE5 and NE11 for curcuminoids of 100µg/ml; **B**: suspension 2, NE4 and NE 10 for curcuminoids of 250µg/ml; **C**: suspension 1, NE3 and NE9 for curcuminoids of 500µg/ml. BDC: Bisdemethoxycurcumin; DC: Demethoxycurcumin; C:Curcumin

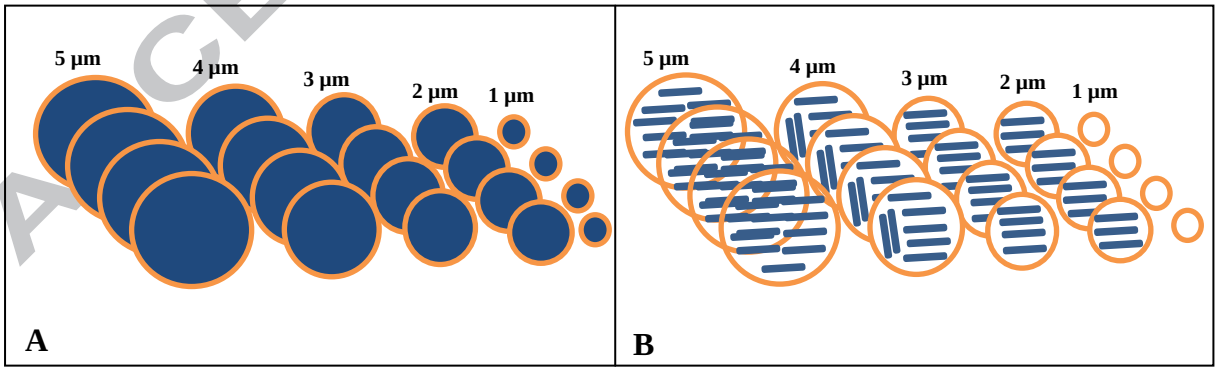
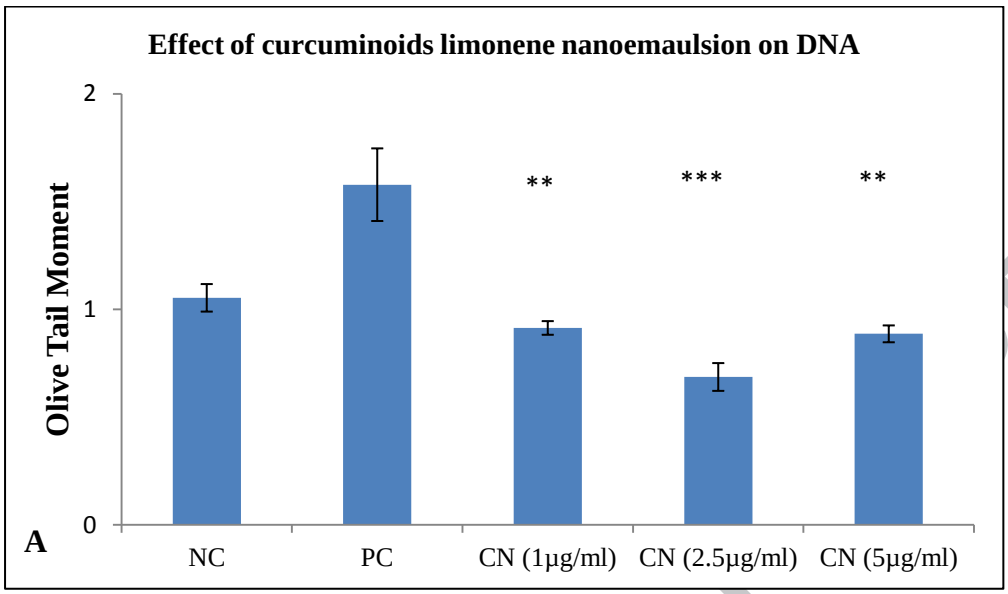


Figure 6. Nebulised droplets from nanoemulsion formulation (A) and from microsuspension formulation (B).

: Nebulised droplets.
: Nanoemulsion particles with the drug (35-12 nm)
: Suspended drug particles (1.7 µm).



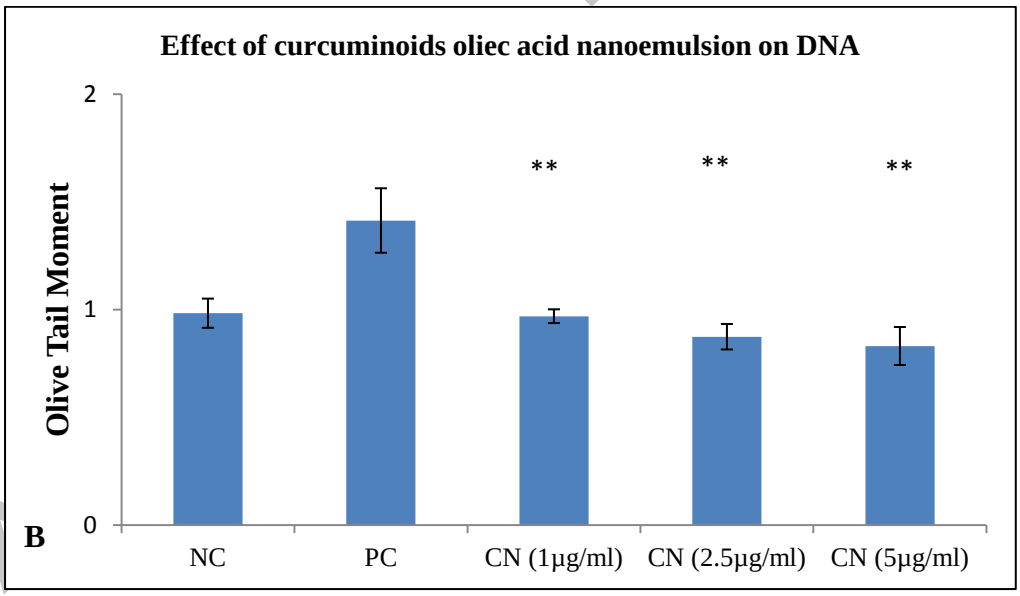


Figure 7. Effect of curcuminoids nanoemulsion formulations on lymphocyte's DNA. A: limonene oil (NE3, NE4, NE5); B: oleic acid oil (NE9, NE10, NE11).

Positive control: Hydrogen peroxides (50µg/ml), NC: negative control, PC : positive control, CN: curcuminoids nanoemulsion

Negative control: No drug or reagents used, For more details see the protocol (method, section 2.7)

Significance:

P value <0.05= *
P value <0.01= **
P value <0.001= ***

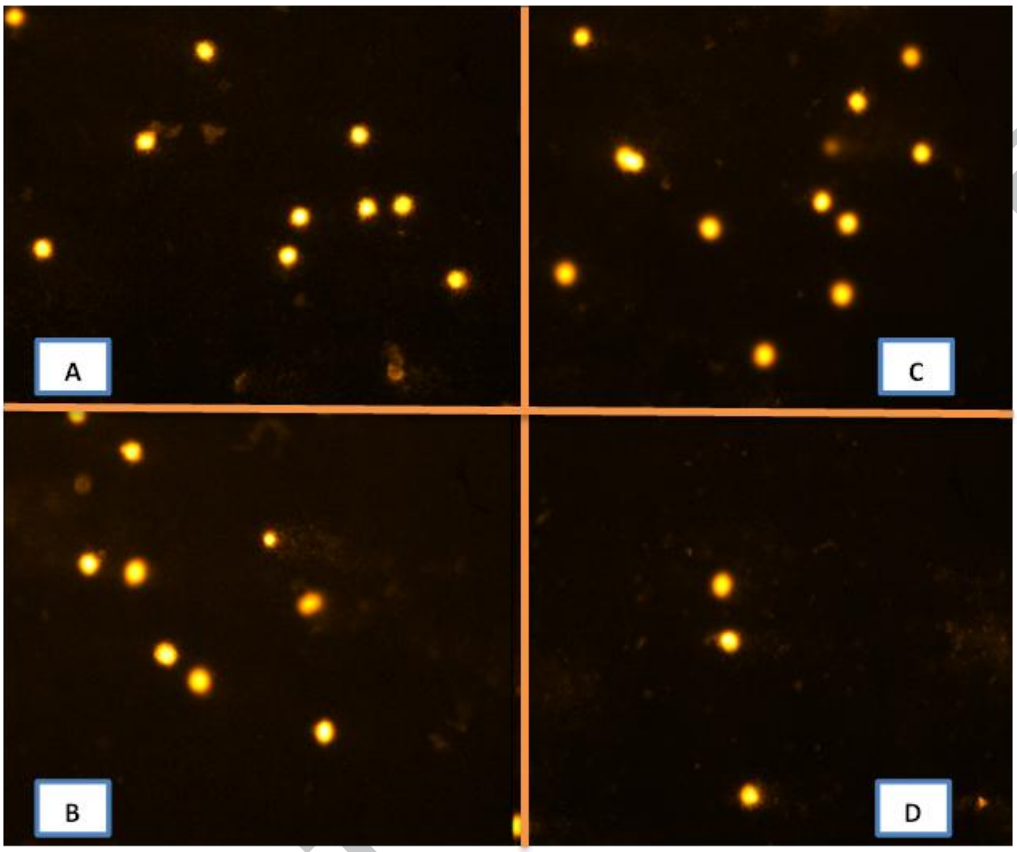


Figure 8. Lymphocyte DNA under a fluorescence microscope after treatment with curcuminoids nanoemulsion formulations, A & C: treated with curcuminoids nanoemulsion using oleic acid oil at concentration 1 & 5 µg/ml (NE9, NE11). B &D: treated with curcuminoids nanoemulsion using limonene oil at concentration 1 & 5 µg/ml (NE3, NE5).

Image magnification=X20

List of Tables:

Table 1. Nanoemulsion compositions (NE: Nanoemulsion)

Formulations	**Surfactant% (w/w)**	**Co-surfactant % (w/w)**	**Oil % (w/w)**	**Water % (w/w)**	**Visual observation**
NE1	Tween 80 (3.3)	Ethanol (6.6)	Limonene (0.8)	Water (89.4)	Clear
NE2	Tween 80 (3.3)	Ethanol (2.5)	Limonene (0.8)	Water (93.4)	Clear
NE3	Tween 80 (1.6)	Ethanol (1.2)	Limonene (0.4)	Water (96.7)	Clear
NE 4	Tween 80 (0.8)	Ethanol (0.62)	Limonene (0.2)	Water (98.4)	Clear
NE5	Tween 80 (0.3)	Ethanol (0.2)	Limonene (0.1)	Water (99.2)	Clear
NE6	Tween 80 (3.3)	Ethanol (6.6)	Oleic acid (0.8)	Water (89.3)	Turbid
NE7	Tween 80 (3.3)	Ethanol (6.6)	Oleic acid (0.3)	Water (89.8)	Clear
NE8	Tween 80 (3.3)	Ethanol (2.5)	Oleic acid (0.3)	Water (93.9)	Clear
NE9	Tween 80 (1.6)	Ethanol (1.2)	Oleic acid (0.1)	Water (96.9)	Clear
NE10	Tween 80 (0.8)	Ethanol (0.6)	Oleic acid (0.07)	Water (98.4)	Clear
NE11	Tween 80 (0.3)	Ethanol (0.2)	Oleic acid (0.03)	Water (99.4)	Clear

NE: Nanoemulsion

Table 2. Osmolality and viscosity results for the nanoemulsion and suspension preparations

Formulations	Concentration of NaCl (mM)	Osmolality (mOsm/kg)	Viscosity (mPas)	pH
NE 2 (1000µg/ml)	0	600 ± 3	--	--
NE3 (500µg/ml)	35	350 ± 2	1.2	7
NE4 (250µg/ml)	95	345 ± 3	1.1	7
NE5 (100µg/ml)	150	340 ± 5	1.1	6
NE8 (1000µg/ml)	0	600 ± 4	--	--
NE9 (500µg/ml)	35	353 ± 4	1.2	7
NE10 (250µg/ml)	95	343 ± 6	1.7	7
NE11 (100µg/ml)	150	336 ± 2	1.1	6
Suspension1 (500µg/ml)	160	286 ± 4	1.1	6
Suspension 2 (250µg/ml)	160	286 ± 3	1.1	5
Suspension 3 (100µg/ml)	160	284 ± 3	1.1	5

Table 3. Particle size results and polydispersity index for nanoemulsion and suspension formulations using Malvern Zetasizer

Formulations	**Particle size (nm) ± SD**	**PDI**
NE3 (500µg/ml)	13 ± 5	0.1
NE4 (250µg/ml)	13 ± 3	0.1
NE5 (100µg/ml)	12 ± 4	0.1
NE9 (500µg/ml)	39 ± 25	0.2
NE10 (250µg/ml)	33 ± 18	0.2
NE11 (100µg/ml)	31 ± 15	0.2
Suspension 1 (500µg/ml)	1650 ± 1015	0.2
Suspension 2 (250µg/ml)	1650 ± 1015	0.2
Suspension 3 (100µg/ml)	1650 ± 1015	0.2

Table 4. Aerosol output data of curcumin (C) that was nebulised from jet nebuliser at dose of 100, 250 and 500 µg/ml of 5 ml of curcuminiods (n=3)

Form	Conct of curcuminoid)	% Inhal	% Exhal	% Cham	% Con	Neb time (min)	Inhal rate (%drug/min)	Exhal rate (%drug/min)
NE 5	100 µg/ml (contains 5 µg/ml of C)	34	31	33	1.4	13	2.6	2.4
NE 11		37	27	31	2.7	13	2.9	2.1
Suspension 3		28	21	49	1.7	15	1.9	1.4
NE 4	250 µg/ml (contains 12.5 µg/ml of C)	33	36	29	1.4	13	2.6	2.8
NE 10		31	36	30	3.1	12	2.5	2.9
Suspension 2		27	27	44	2.6	15	1.9	1.8
NE 3	500 µg/ml (contains 25 µg/ml of C)	34	35	30	1.9	13	2.5	2.6
NE 9		37	34	27	2.4	13	2.9	2.7
Suspension 1		21	23	54	1.9	15	1.4	1.5

Form: formulations; Conct: concentration; Exhal: Exhalation; Inhal: inhalation; Cham: chamber; Con: connector; Neb: nebulisation

Table 5. The mean (n=3) of the aerodynamic data of suspension formulations using jet nebuliser at flow 15 L/min

Drug	Formulation	FPF %	MMAD (µm)	GSD	FPD (µg)
Bisdemethoxycurcumin	Suspension 3 (100µg/ml)	40	5.8	2.1	12
	Suspension 2 (250µg/ml)	35	6.5	2.2	17
	Suspension 1 (500µg/ml)	33	6.6	2.0	34
	Suspension 4 (1000 g/ml)	26	7.1	2.0	43
Demethoxycurcumin	Suspension 3 (100µg/ml)	38	6.1	2.2	18
	Suspension 2 (250µg/ml)	33	6.4	2.1	40
	Suspension 1 (500µg/ml)	31	6.7	2.0	99
	Suspension 4 (1000 g/ml)	25	7.1	2.0	109
Curcumin	Suspension 3 (100µg/ml)	43	5.3	2.6	101
	Suspension 2 (250µg/ml)	36	6.2	2.2	152
	Suspension 1 (500µg/ml)	32	6.6	2.1	369
	Suspension 4 (1000 g/ml)	26	7.1	2.0	379

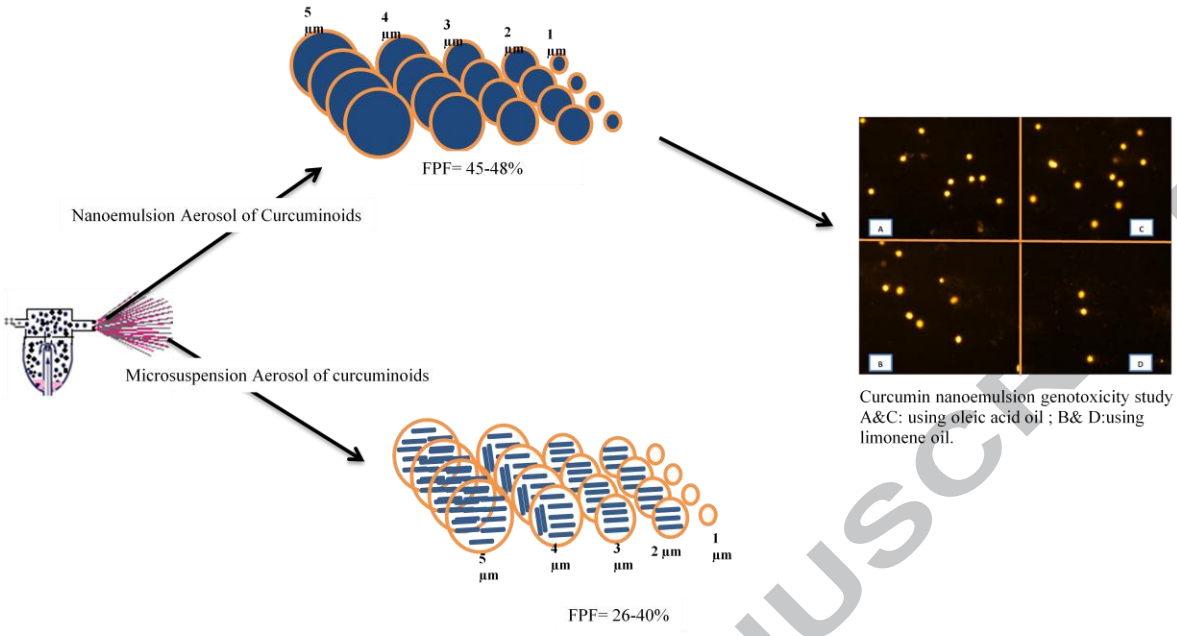
FPF %*: the percentage of Fine Particle Fraction. MMAD*: Mass Median Aerodynamic Diameter. GSD*: Geometric Standard Deviation. FPD*: Fine Particle Dose.

Table 6. The mean (n=3) of the aerodynamic characterisation of curcuminoids nanoemulsion with oleic acid oil formulations using Jet nebuliser at flow 15L/min

Drug	Formulations	FPF %	MMAD (µm)	GSD	FPD (µg)
Bisdemethoxycurcumin	NE 11(100µg/ml)	46	4.8	2.3	12
	NE 10 (250µg/ml)	44	5.3	2.3	16
	NE 9 (500µg/ml)	43	5.7	2.1	42
Demethoxycurcumin	NE 11(100µg/ml)	44	5.5	2.4	31
	NE 10 (250µg/ml)	43	5.7	2.5	41
	NE 9 (500µg/ml)	42	5.8	2.1	149
curcumin	NE 11(100µg/ml)	48	4.3	2.6	161
	NE 10 (250µg/ml)	47	4.8	2.8	182
	NE 9 (500µg/ml)	47	5.2	2.1	585

Table 7. The mean (n=3) of the aerodynamic characterisation of curcuminoids nanoemulsion with limonene oil formulations using Jet nebuliser at flow 15L/m

Drug	Formulations	FPF %	MMAD (µm)	GSD	FPD (µg)
Bisdemethoxycurcumin	NE 5 (100µg/ml)	51	4.5	2.7	17
	NE4 (250µg/ml)	46	5.3	2.3	21
	NE 3 (500µg/ml)	45	5.5	2.2	44
Demethoxycurcumin	NE 5 (100µg/ml)	47	4.9	2.2	40
	NE4 (250µg/ml)	46	5.1	2.1	76
	NE 3 (500µg/ml)	44	5.6	2.1	155
Curcumin	NE 5 (100µg/ml)	51	4.5	2.4	174
	NE4 (250µg/ml)	50	4.7	2.2	300
	NE 3 (500µg/ml)	45	5.5	2.2	587



Graphical Abstract