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Comparing the efficacy of nanocurcumin and its bulk counterpart to understand the permeability - an *in vitro* approach

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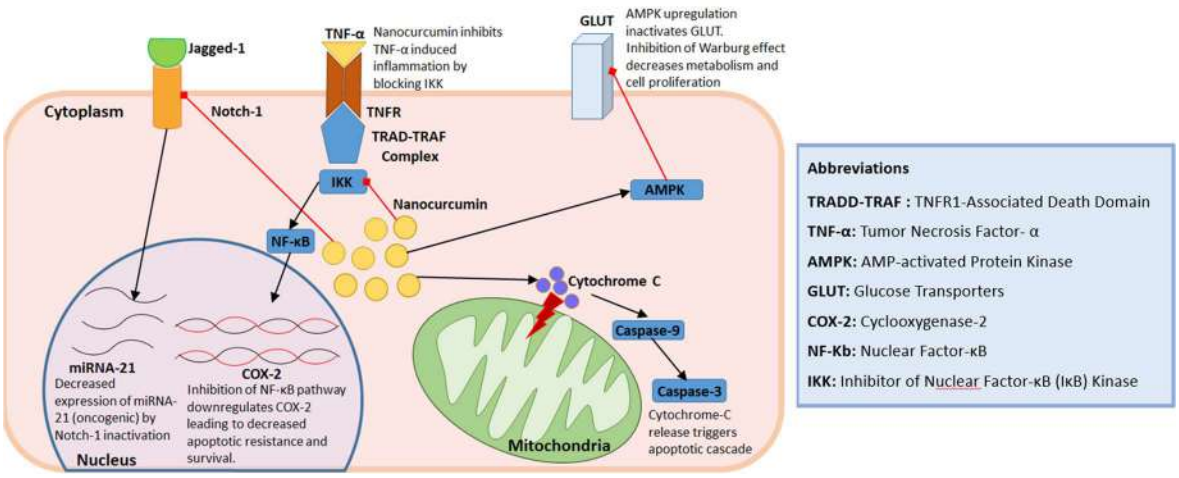
ABSTRACT



The anti-carcinogenic effects of curcumin have been enhanced by its use in the management of oral cancer when delivered orally, topically, as an adjuvant, and in combination with other drugs. However, the therapeutic effects of bulk curcumin are severely limited by a number of significant related issues including poor absorption and low bioavailability. This may be attributed to the poor permeability of bulk curcumin. The need for a smaller sized and smart molecule was accomplished by generating nanocurcumin. The present research study aimed at synthesis (chemical and green), characterization, and testing nanocurcumin on human lymphocytes, erythrocytes and OSCC cell lines and comparing its effects with bulk curcumin. The study has demonstrated the improved permeability of nanocurcumin compared to its bulk counterpart. Considering the high prevalence of oral cancer in India and the wide availability of the traditional curcumin, its cost effectiveness provides an excellent potential to nanoformulation of curcumin in cancer treatment.

Keywords: Nanocurcumin, Bulk-curcumin, Oral squamous cell carcinoma, In-vitro assay, Apoptotic effects

INTRODUCTION

Oral cancer (OC) is the sixth most common type of cancer worldwide, with over 77,000 new cases in India each year, accounting for nearly one-fourth of all global incidences.[1,2] For treating early-stage and locally advanced oral cancer, surgery and chemotherapy, and/or radiation therapy remain the 'gold standard'.[3] Despite major advances in cancer research and therapy, acute toxicity and long-term negative effects have prompted researchers to look for alternate approaches and natural sources such as plant polyphenols (e.g., curcumin).[4,5]

Curcumin (diferuloylmethane or 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), the bioactive ingredient of *Curcuma longa* (turmeric), is currently one of the most extensively studied compounds owing to its diverse pharmacological properties that include anti-oxidant, anti-inflammatory, anti-mutagenic, anti-bacterial, and anti-cancer properties.[6,7] Studies on the anti-cancer effects of curcumin have demonstrated and proved that it can reduce tumour size and inhibit tumour growth and spread by exerting anti-angiogenic effects, inducing apoptosis, and interfering with the cell proliferation

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cycle.[8–10] Cancer is in fact stated to be the primary target disease in over 37% of published curcumin research. Curcumin has established its role in haematological, gastrointestinal, genitourinary, thoracic, and head and neck malignancies through its different modulatory actions.[11,12]

Curcumin's anti-carcinogenic effects have been enhanced by its use in the management of OC when it is delivered orally, topically, as an adjuvant, and in combination with other drugs.[13,14] But the therapeutic effects of bulk curcumin are severely limited by a number of significant related issues, including poor absorption, low bioavailability, quick systemic elimination, and high metabolism. In an aqueous solution, curcumin degrades, with its rate being substantially slower at levels of pH< 7, resulting in less than 20% of total curcumin decomposing in 1 hour.[15] This adds to its brief half-life of twenty-eight hours, and studies have shown that bulk curcumin administered orally leads to approximately 40–75% of it being excreted in faeces.[16] A plausible explanation for low bioavailability would be bulk curcumin's high metabolic rates, which undergo significant metabolism in the liver and gastrointestinal tract, where it forms conjugates, reducing biodistribution.[17] The size of bulk curcumin is also a limitation, as substances with particle sizes > 1 µm are rapidly eliminated from the body and have a tendency to aggregate in physiological conditions rather than pass through capillaries for filtration.[18]

To substantiate its use, curcumin has to be delivered in a large amount for a prolonged period of time, as smaller doses of curcumin does not have an impact on apoptosis, cell integrity, cell shape, or proliferation.[19] Although the US Food and Drug Administration (FDA) classifies curcumin as 'Generally Recognized as Safe'(GRAS), a dosage response study found that curcumin in levels of 500–12000 mg caused diarrhoea, headaches, rashes, and yellow stools.[20] Epidemiologic findings imply a link between the extensive usage of dietary curcumin and the low prevalence of gastrointestinal mucosal malignancies in south-east Asia. Contrary to expectations, OC had the highest incidence rates in the same geographic areas without any discernible advantages from curcumin use.[21]

Nano formulations of curcumin have been created to address the drawbacks of bulk curcumin. Due to their submicron size, they enable access to inaccessible organs and tend to increase cellular uptake, thereby improving pharmacokinetics and half-life, both of which were issues with bulk curcumin. By increasing the cellular uptake of Nanocurcumin (NC), it triggers the apoptotic cell death through mitochondrial and caspase-dependent pathways and also inhibits TNF-α induced inflammation (Figure 9). While reducing tumor size and cell viability, NC strengthens curcumin's anti-carcinogenic effects against oral cancer cells.[22,23] Whilst pertaining to OC, the anatomical sites of occurrence (tongue and buccal mucosa being the most common followed by lip and palate) make them more accessible to nanoformulations. Nano formulations are one of the most efficient ways to increase permeability and solubilisation while dispensing small medication dosages directly into the lesion mediating 'Targeted Drug Delivery'.[24,25] *In vitro* permeation investigations using different nanocurcumin formulations on Franz diffusion cells and porcine oesophageal mucosa showed statistically significant results, indicating that nanocurcumin has good mucoadhesive qualities.[22] However, these results need to be validated by *in vivo* studies. In addition to its dynamic role in OC, curcumin has been used to treat radiation induced oral mucositis and oral potentially malignant disorders (OPMD), such as leukoplakia and oral submucous fibrosis (OSMF).[26] In the treatment of oral mucositis brought on by radio-chemotherapy, curcumin mouthwash was discovered to be superior to chlorhexidine mouthwash in terms of wound healing and patient compliance.[27] It is significant that nanocurcumin is essentially non-invasive in treating lesions and precancerous plaques in the oral cavity due to the ease of access.

The present study is an effort to evaluate the anti-carcinogenic efficacy of nanocurcumin (synthesized by chemical and green synthesis methods) as depicted in Figure 1. The study also compares the efficacy of bulk curcumin compared to that of nanocurcumin on oral squamous cell lines. This alternative drug delivery approach could possibly bring out nanocurcumin as an effective and promising agent for the treatment of OC.

EXPERIMENTAL PROTOCOLS

Preparation

Synthesis of bulk nanocurcumin:

Curcumin, (C.I- 75300) was purchased from Himedia Chemicals Ltd., India (RM 1449). 360 mg of curcumin was dissolved in 10 mL of distilled water.

Synthesis of nanocurcumin

Nanocurcumin was synthesized by solvent-antisolvent method 1.5g of curcumin was weighed and dissolved in 15mL of ethanol.[28] This solution was later made up to 150 mL using 135 mL of distilled water. The supernatant was transferred to a 30 mL crucible and dried in the muffle furnace at 200oC for 45 minutes with constant monitoring (checking for any colour changes). After complete drying, the nanocurcumin powder deposited at the sides and bottom of the crucible was collected using a spatula, and stored at room temperature until further analysis.

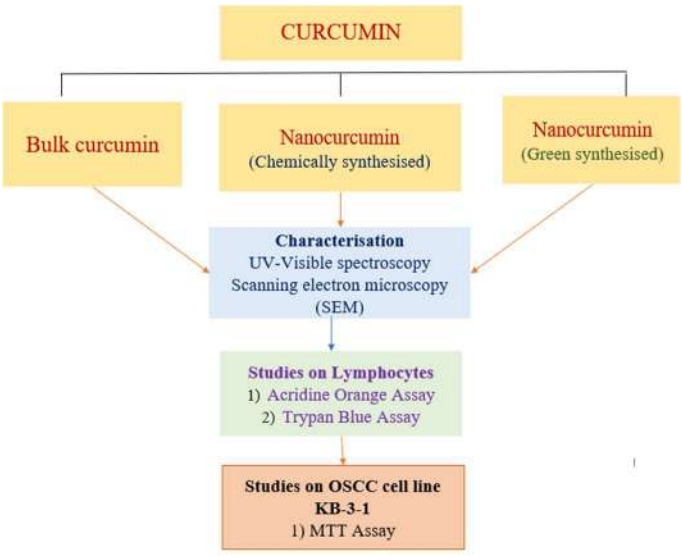

Figure 1. Experimental flowchart depicting the procedures of synthesis, characterization and various tests performed in this study.

Green synthesis of nanocurcumin:

Plant material collection and extract preparation

The rhizome of *C. longa* was collected from an agricultural farm in South India. The rhizome was cleaned and dried for a week under sunlight to remove moisture. The tuber was cut into small pieces, powdered in a blender and then sieved using a 20-mesh sieve to get a uniform size. The final sieved powder was used for all the further analysis. To make the extract, 2.5 g of the powder was mixed with 100 mL of sterile distilled water in a 500-mL Erlenmeyer flask and boiled for 5 minutes.

Green synthesis of nanocurcumin $AgNO_3$ from the rhizome extract: 5 mL of the extract was added to 500 mL of 1 mM aqueous $AgNO_3$ solution.

Characterization:

Bulk curcumin, chemical synthesized nanocurcumin and green synthesized nanocurcumin were characterization by UV spectrophotometry and scanning electron microscopy.

***In vitro* testing on *KB-3-1* Cell line:**

The oral cancer cell lines *KB-3-1* (Figure 2) were obtained from the NCCS in Pune, India. OSCC cell line KB-3-1 was used for *in vitro* cell testing. The cell line was cultured in DMEM along with 10% FBS and 1% of antibiotic cocktail (Streptomycin and Penicillin) and maintained at 5% CO_2 and 37°C. The flow of synthesis, characterization, and the various studies were performed for each of the following compounds namely, bulk curcumin; chemically synthesized nanocurcumin; and green synthesized nanocurcumin are shown in Figure 1.

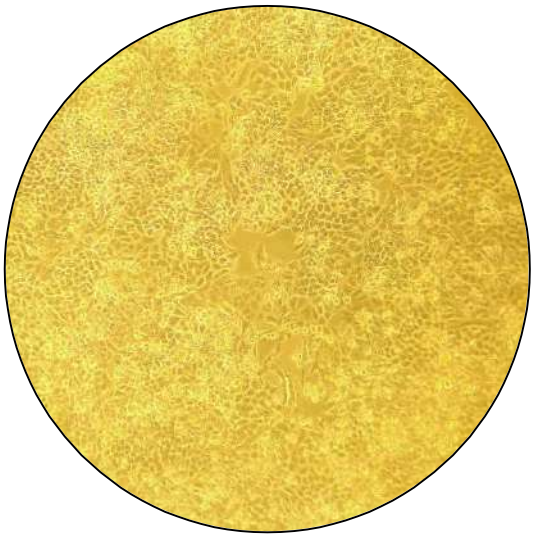

Figure 2. Oral Squamous Cell Carcinoma (OSCC) cell line *KB-3-1*

Uptake studies

Acridine orange assay (AO assay)

Apoptosis of lymphocytes induced by bulk and nanocurcumin was investigated by AO assay. Venous blood was drawn from healthy volunteer and exposed to 125 mM, 250 mM, and 500 mM bulk and nanocurcumin along with positive control (PC) and negative control (NC). Positive control involved the use of 6.25 µL of Benzaldehyde, whereas the negative control was distilled water. The microfuge tubes were incubated at 37°C for 2 hours. After incubation, the blood was layered on an equal amount of Ficoll (0.5mL) and centrifuged at 2500 rpm for 25 minutes. The buffy coat was removed and was washed with an equal volume of PBS until free from haemolytic residue. 8 µL of each sample was taken and to it 2 µL of AO dye was added. The sample was loaded onto the haemocytometer and cells (live and dead) were observed, counted and recorded.

Trypan blue assay (TB assay)

The viability of lymphocytes induced by bulk and nanocurcumin was investigated using the TB assay. Venous blood was drawn from a healthy volunteer and exposed to 125 mM, 250 mM, and 500 mM bulk and nanocurcumin along with positive control (PC) and negative control (NC). The microfuge tubes were then incubated at 37 °C for 2 hours. After incubation, the blood was layered on an equal amount of Ficoll (0.5mL) and centrifuged at 2500 rpm for 25 minutes. The buffy coat was removed and was washed with an equal volume of PBS until free from haemolytic residue. 10 µL of TB dye was added to10 µl of each sample concentration. The sample was loaded onto the haemocytometer and cells (live and dead) were observed, counted, and recorded.

Viability was calculated using the formula:

$$\text{Viability (\%)} = \frac{\text{Total no. of viable cells}}{\text{Total no. of cells}} \text{ x } 100$$

Haemolytic assay

Lysis of erythrocytes on exposure to bulk and nanocurcumin was tested using a haemolytic assay.[29] 3 mL of venous blood was drawn and centrifuged at 1000 rpm for 10 minutes. 0.5 mL of the pellet was diluted with 49.5 mL of PBS to form a 1% RBC suspension. 1.5 mL of the 1% RBC suspension was tested with different concentrations of nanocurcumin (250, 500, and 1000 µL) in microfuge tubes. The tubes were incubated at 37°C for 2 hours and centrifuged at 1000 rpm for 10 minutes. The supernatant was transferred to a 96-well plate and spectrophotometric absorbance was noted at 545 nm. Percentage of haemolysis was calculated using the formula:

$$\text{Haemolysis (\%)} = \frac{\text{OD of test sample-OD of negative control}}{\text{OD of positive control-OD of negative control}} \text{ x } 100$$

MTT Assay

The cytotoxicity of bulk, chemically and green synthesized nanocurcumin was performed by using MTT assay on OSCC *KB 3-1* cells. In this method, OSCC *KB 3-1* cells (approximately 1.2 x 104 cells/well) were seeded in a 96 well plate and incubated to achieve confluency. Cells were washed and 100 µL of defined concentrations of the chemically synthesized nanocurcumin (62.5, 125, 250, 500 µg/mL) was added and incubated for 24 hours. Freshly prepared MTT was added and incubated at 37ºC for 4-6 hours. This test was repeated for green synthesized and bulk curcumin.

$$\text{Viability (\%)} = \frac{\text{Total no. of viable cells}}{\text{Total no. of cells}} \text{ X } 100$$

$$\text{Cytotoxicity \%} = 100 - \text{Viability \%}$$

RESULTS AND DISCUSSION

Characterization

Scanning electron microscopy (SEM)

The results of UV/VIS spectrophotometry and SEM analysis used to characterize bulk and nanoparticles are described in Table 1, Figure 3, and Figure 4.

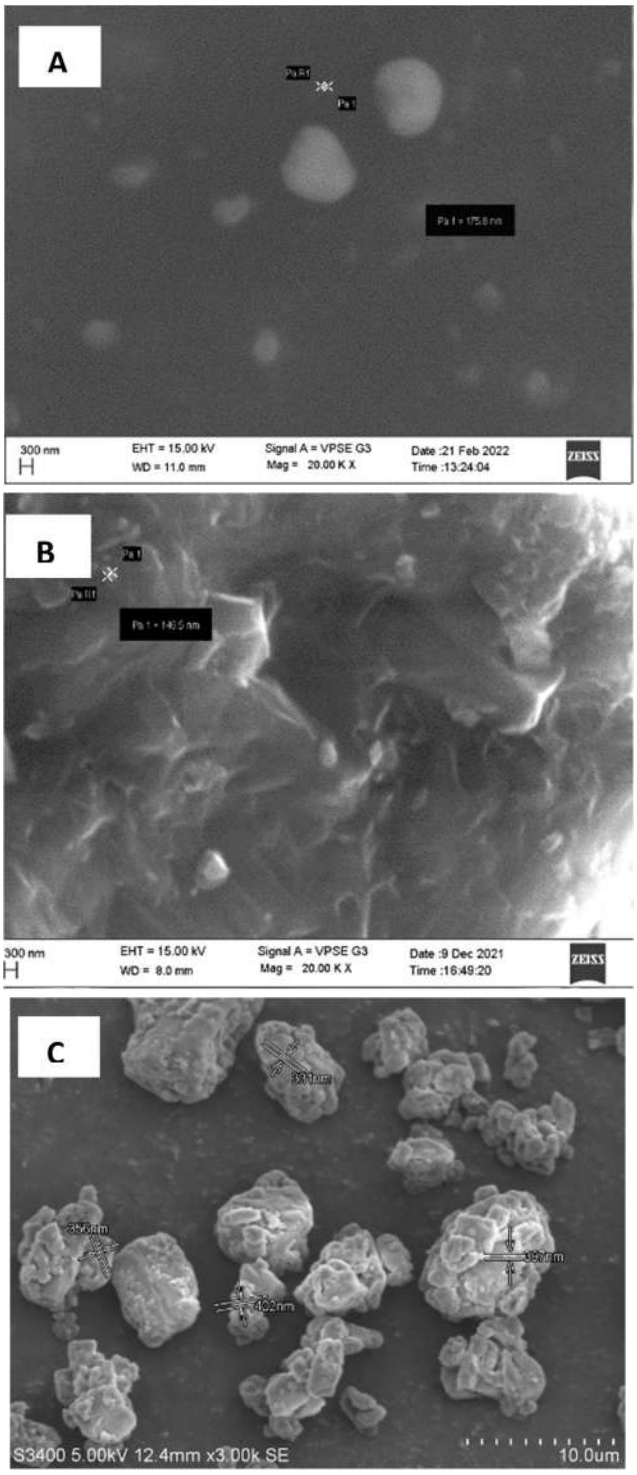

Figure 3. Results of SEM characterization of A) bulk curcumin, B), chemically synthesized nanocurcumin, and C) green synthesized nanocurcumin.

Table 1. Comparative analysis of the results of SEM and UV/VIS characterization across all the three compounds:

	Bulk curcumin	***Chemically synthesized nanocurcumin***	***Green synthesized nanocurcumin***
SEM	402 nm	146.5 nm	175.8 nm
UV-Vis. (426-nm. Abs.)	0	0.410	0.424

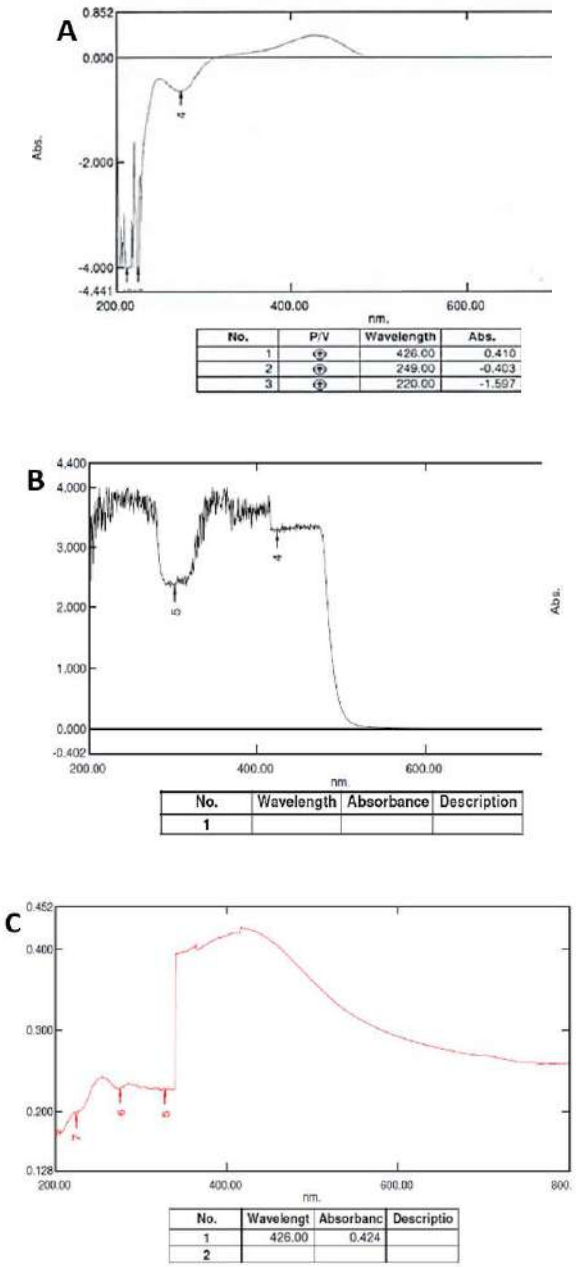

Figure 4. Results of UV visible spectroscopy for A) bulk curcumin, B) chemically synthesized nanocurcumin, and C) green synthesized nanocurcumin.

Uptake studies

Acridine orange assay

The results of the Acridine Orange assay show a steady decline in viability of cells and increased apoptosis with increase in concentration of nanocurcumin as seen in Figure 5.

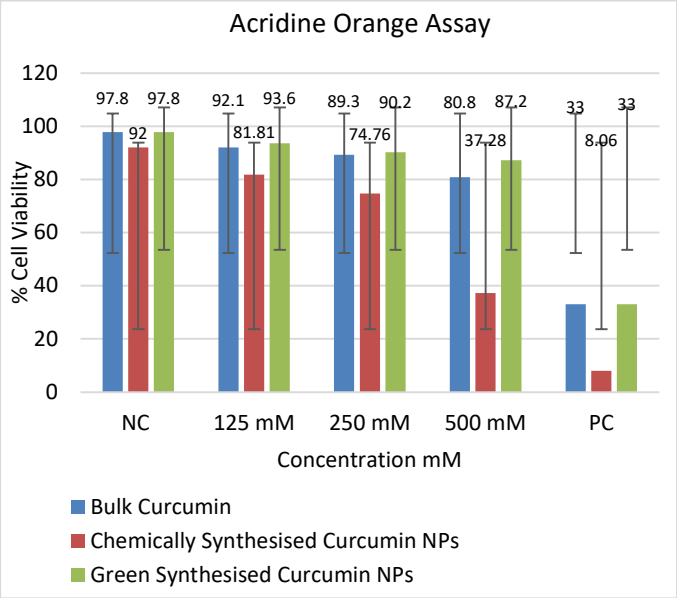

Figure 5. Comparative analysis of percentage cell viability by acridine orange staining for A) bulk curcumin, B) chemically synthesized nanocurcumin, and C) green synthesized nanocurcumin exposure on human lymphocytes.

Trypan blue assay

The Trypan Blue assay results show that as the concentration of nanocurcumin increases, cell viability decreases steadily as in Figure 6.

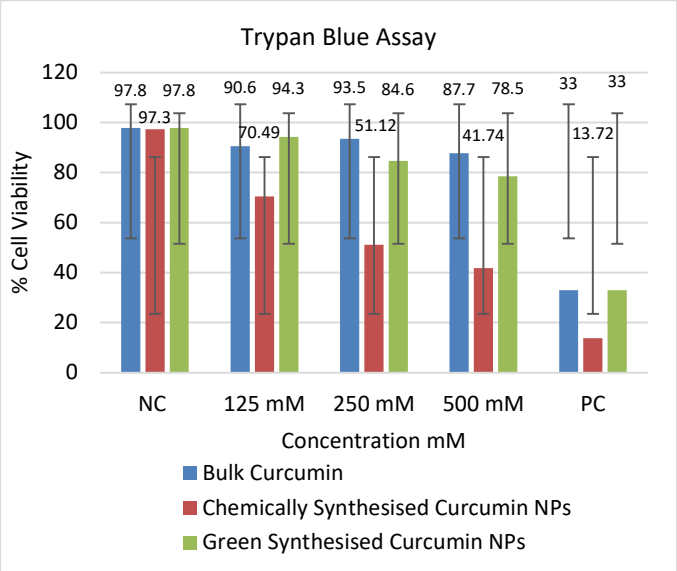

Figure 6. Comparative analysis of percentage cell viability by trypan blue staining for A) bulk curcumin, B) chemically synthesized nanocurcumin, and C) green synthesized nanocurcumin exposure on human lymphocytes.

Haemolytic assay

Table 2. Comparative analysis of percentage haemolysis for chemically synthesized nanocurcumin exposure on human erythrocytes

S.No.	Volume of Exposure	OD at 545nm	Percentage of haemolysis
1.	H1 - 250 µL	0.062	0.60%
2.	H2 - 500 µL	0.065	0.75%
3.	H3 - 1000 µL	0.067	0.85%
4.	PC (6.25 µL Benzaldehyde)	2.048	100%
5.	NC	0.050	0%

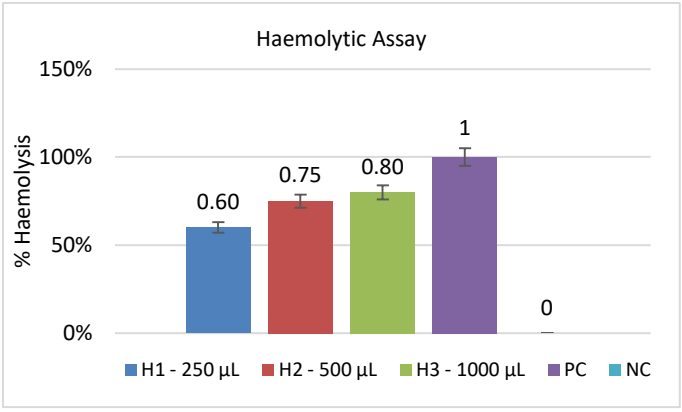

Figure 7. Comparative analysis of percentage haemolysis for chemically synthesized nanocurcumin exposure on human erythrocytes.

MTT assay

In the present study, the proliferative index of green synthesized, chemically synthesized nanocurcumin were compared to that of

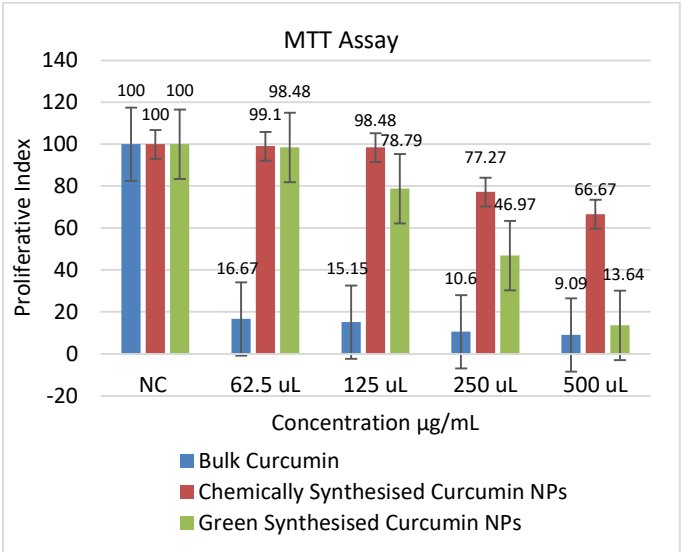

Figure 8. Comparative analysis of proliferative index of A) bulk curcumin, B) chemically synthesized nanocurcumin, and C) green synthesized nanocurcumin exposure on KB-3-1 cell-line.

bulk curcumin. It was observed that cell death increased with an increase in the concentration of nanocurcumin. The results show dose-dependent cytotoxicity of OSCC after 24 hours of treatment at concentrations of 62.5, 125, 250, and 500 µg/mL. (Figure 8) It was significant to note that green synthesized and chemically synthesized nanocurcumin were found to be more cytotoxic to the OSCC than bulk curcumin which can be attributed to the small size of the nanocurcumin thus increasing its uptake and hence better efficiency.

Chemically synthesized nanocurcumin was prepared by the solvent anti-solvent method as it effectively produced particles in the nano-range.[28] The green synthesis followed the protocol previously standardised for the production of silver nanoparticles from rhizome extracts for testing on colorectal cancer.[37] Bulk curcumin suspension was produced by directly mixing it with distilled water and filtering the residual components. Characterisation results revealed a distinct reduction in size as reflected in SEM analysis and confirmed by UV visual spectrophotometry which was supported with previous literature. Three concentrations, 125, 250, and 500 mM of chemically synthesized and green synthesized nanocurcumin were tested for uptake by Acridine Orange and Trypan Blue assay on human lymphocytes.

Their effect on human erythrocytes was assessed by haemolysis assay and its effect on the proliferative index was tested on Oral Squamous Cell Carcinoma cell line using MTT assay.

The Acridine Orange and Trypan Blue assay demonstrated, decline in viability which may be due to apoptosis induced by increasing concentrations of nanocurcumin. Trypan Blue and Acridine Orange Assay was performed according to standard methods. The highest concentration of 500 Mm showed distinct drop in cell viability in chemically synthesized compared to green synthesized nanocurcumin. This may be attributed to the smaller size of chemically synthesized nanocurcumin (146.5 nm) compared to green synthesized nanocurcumin (175.8 nm).[30]

The results of haemolytic assay performed on human erythrocytes revealed negligible haemolysis across the three test concentrations. This may be attributed to the increased uptake of nanocurcumin by erythrocytes owing to their smaller size and better permeability. Hence they offer a great potential towards treatment of metastatic cancer involving angiogenesis and leukemic related neoplasia.

Another flavonoid naringenin, derived from citrus fruits has demonstrated similar anti-cancer properties.[31,32] Multiple studies have proved the anti-cancer property of naringenin-based nanoparticles. A recent study on OSCC rat models revealed its ability to specifically target and eliminate tumour formation in the buccal cavity. It also showed better activity compared to bulk naringenin and thus presents great potential in the field of targeted tumour therapy.[33,34]

Apart from natural compounds, molecules like iron, silver NPs have also shown anticancer activity especially in OSCC. In case of such inorganic NPs, they are coated with a mixture of organic molecules and behave as carriers for anti-cancer drugs. Iron-oxide NPs are most commonly coated with fatty acids, paclitaxel and doxorubicin in the treatment of OSCC.[34]

Numerous studies have proved that nanoformulations of curcumin have better bio-availability compared to its bulk counterpart. Approaching nanosize could be accomplished by encapsulating curcumin within a nanocarrier, modifying the surface chemistry or by directly reducing particulate size.[35] For example, anti-bacterial creams formulated from nanocurcumin have shown better efficacy compared bulk. The nano-version showed a 40%

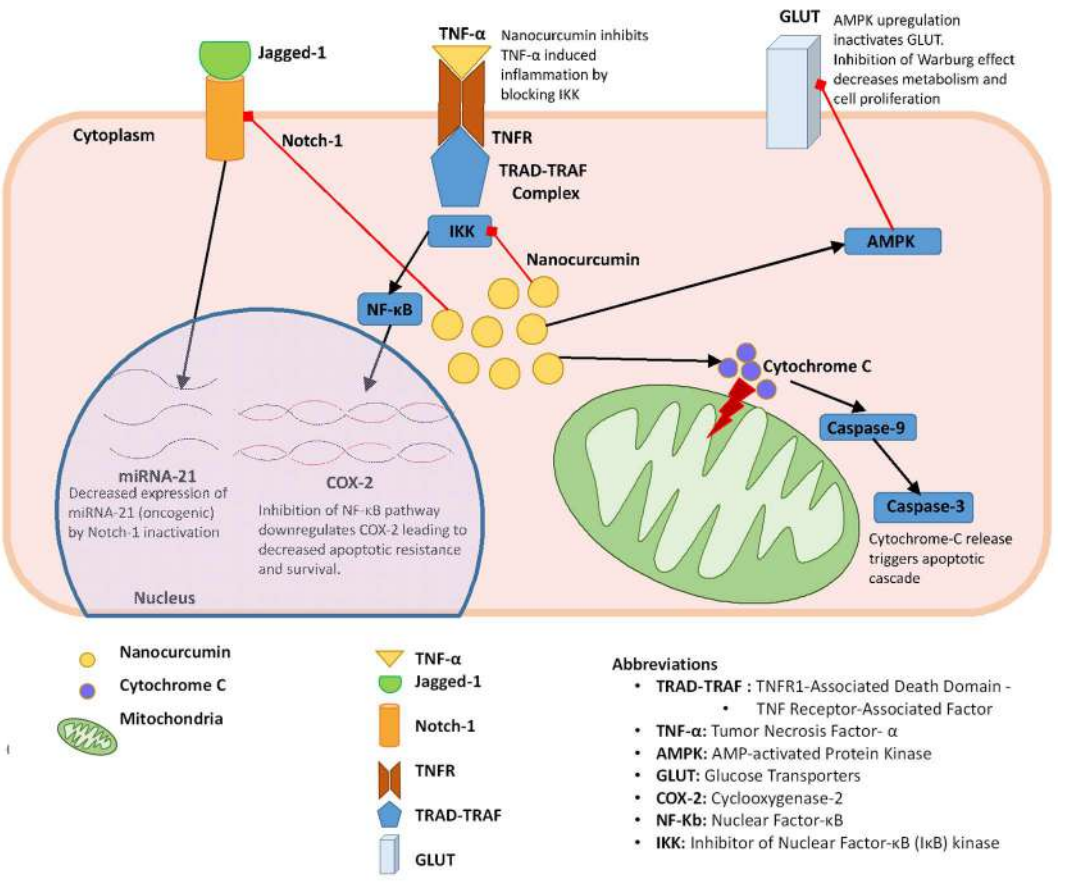

Figure 9. Nanocurcumin action in cells.

increase in anti-bacterial activity in both gram positive and gram negative strains.[36]

The effect of chemically synthesized and green synthesized nanocurcumin demonstrated increased proliferative index compared to the bulk. The poor proliferative index of bulk is attributed to its large size and poor permeability. Also, chemically synthesized nanocurcumin demonstrated better proliferative index compared to green synthesized which due to a three-fold decrease compared to bulk (Figure 9). This clearly demonstrates better uptake of nanocurcumin by cell lines and its application towards adjuvant chemotherapy and radiotherapy for the treatment of oral cancer.[36,37]

CONCLUSION

The results of the assays demonstrate the cellular uptake and effect of bulk and nanocurcumin. When compared to the bulk, the effect of chemically and green synthesized nanocurcumin revealed an elevated proliferative index. Bulk curcumin has lower proliferative index due to its size and permeability. This study provides better insights towards understanding permeability of nanoformulations for the treatment of oral cancer. The results are qualitative, inclusion of advanced molecular tools and flow cytometric assay will be able to provide quantitative data along with understanding the possible mechanisms involved in the permeability of nanocurcumin.

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Conflict of Interest: All authors declare that they have no conflict of interest.

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