

Anticancer Effects of Curcumin, Artemisinin, Genistein, and Resveratrol, and Vitamin C: Free Versus Liposomal Forms

Jerry T. Thornthwaite^{1*}, Hare R. Shah¹, Spencer R. England¹, Lee H. Roland¹, Seth P. Thibado², Thomas K. Ballard³, Brandon S. Goodman¹

¹Cancer Research Institute of West Tennessee, Henderson, TN, USA

²Union University Department of Chemistry, Jackson, TN, USA

³University of Tennessee Department of Biochemistry, Knoxville, TN, USA

Email: *jtt@criwt.com

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Abstract

Cancer prevention supplements, which also provide effective treatment with minimal side effects, are urgently needed. An accurate, fast assay system is described that reveals the ability of chemically defined products, such as curcumin, genistein, artemisinin, and vitamin C, to kill K562 Eryt-hroleukemic cells *in vitro*. In addition, curcumin and vitamin C were encapsulated into fatty acid micelles named MyCell-Enhanced-Nutraspheres™ (MEN) using all natural products. A unique viability stain, which allows the rapid staining of dead cells by membrane penetration using Propidium Iodide, was used to measure the cell viability by flow cytometry. Cell death by alteration of the cell membranes could be seen within 30 s of exposure to curcumin. The other free components required 0.5 - 70 h to see maximum killing, suggesting a more metabolic and/or apoptotic route of cancer cell destruction. Vitamin C up to 1×10^4 $\mu\text{mol}/\text{well}$ did not affect K562 cell viability. The vitamin C-MEN (3.2 nm diameter-60 mg/50 μL) showed an $\text{LD}_{50} = 133$ $\mu\text{mol}/\text{well} \pm 11$ SD ($n = 4$), which was over 75 times more potent than the free vitamin C. The curcumin-MEN (7.4 nm diameter-25 mg/50 μL) resulted in an $\text{LD}_{50} = 41.3$ $\mu\text{mol}/\text{well} \pm 5.6$ SD ($n = 8$) and represented a 264 fold increase in activity to destroy cancer cells. The goal is to develop water-soluble mixtures of anti-cancer compounds in the MEN with their high bioavailability (>90%) and without degradation in the stomach for preventing and curing cancer.

Keywords

Curcumin, Artemisinin, Cancer, Micelle, Natural

1. Introduction

Cancer is one of the major causes of death worldwide, and minimal progress has been accomplished in reducing its morbidity [1]. Cancer is caused by one or a combination of at least three factors: unhealthy diet, genetic predisposition, and the environment. Estimates by the American Cancer Society are that over 90% of all cancers are caused by lifestyle and may take as long as 20 - 30 years to develop. Current estimates from the American Cancer Society and from the International Union Against Cancer indicate that 12 million cases of cancer were diagnosed last year, with 7 million deaths worldwide; these numbers are expected to double in 15 years [2]. According to reports from the World Health Organization, more than 80% of world's populations depend on traditional medicine for their primary health care needs [3] [4] [5]. Plants have a long history of being used in the treatment of cancer, and it is noteworthy that over 60% of currently used anti-cancer agents come from natural sources [6] [7] [8] [9] [10].

Cancer prevention approaches prescribe natural/synthetic agent(s) with the aim to delay or disrupt the pathways and processes involved at multiple steps, such as the initiation, promotion, and progression of cancer [11]. Even our fresh fruits and vegetables are being depleted of essential minerals and vitamins, often necessitating supplementation [12]. Stress hormones such as adrenaline and cortisol suppress natural killer cell function [13] [14]. More people are living in large cities with its associated pollution, stress and poor eating habits. A partial solution may be that individuals need to make critical lifestyle changes if they want to live long, healthy lives. Nutritional supplements are being increasingly recognized as an essential part of a healthy lifestyle.

The beneficial effects of curcumin on cancer, cardiovascular disease, metabolic disorders, antioxidant capacity, inflammation, and neurological, liver and respiratory disorders have recently been reviewed [15]-[21]. This is in spite of the poor bioavailability of curcumin [22] [23]. Genistein [24], resveratrol [25] [26], artemisinin [27], and vitamin C [28] also have similar clinical importance but also suffer from low bioavailability.

This study presents importance of supplements using plant derived or synthesized, chemically pure compounds that show direct toxicity to the K562 cancer cells. These supplements include curcumin, genistein, resveratrol, artemisinin, and vitamin C. Furthermore, the greatly increased cytotoxicity of micelle-encapsulated curcumin and vitamin C will be compared to their free forms [29] [30] [31] [32] [33].

2. Materials and Methods

Cell Line and Media Production: Experiments were performed using a Chronic Myelogenous Leukemia K-652 cell line purchased from the American Type Culture Collection (ATCC).

The tissue culture media was made by adding 5 mL of 100X penicillin-streptomycin (10,000 units penicillin with 10 mg of streptomycin/mL—Sigma-Aldrich), 5 mL of 200 mM sterile-filtered L-Glutamine (Sigma-Aldrich), 5 mL of

Cellgro sodium 100X bicarbonate solution (Cellgro), and 50 mL of fetal calf serum (Atlanta Biologics) to 500 mL of Minimum Essential Media, Alpha 1X, with Earle's salts without ribonucleotides, deoxyribonucleotides, and no L-glutamine (Cellgro).

Viability Stain: The viability stain used for analysis with the flow cytometer was developed by Dr. Jerry Thornthwaite. The viability stain, which uses a special medium and dye exclusion with Propidium Iodide (Sigma-Aldrich), was effective in measuring cell viability by showing a linear decrease in viability as the K562 cells were progressively subjected to a 56 C water bath (data not shown). Also, K562 cell viability was measured directly from cell cultures in which 100 μ L samples from the cell culture were added to 100 μ L of the viability stain. After incubation for five minutes at room temperature, the samples were suspended, incubated at room temperature for five min., and analyzed on the Accuri Flow Cytometer (BD Biosciences). Forward Light scatter was used to gate on the K562 cells and analyze the number of viable cells within the established control viability gate in the 585 ± 20 nm red channel. Typical control cultures with 95% - 99% viability were used to establish the boundary between the live cells, essentially without staining, and dead cells, which would take up the dye and fluoresce at larger channel numbers.

Figure 1 shows the measurement of cell viability using the viability stains. The K562 cells starting culture was 1×10^5 cells/mL. The right of the vertical line was used as the cutoff of viable (left) and non-viable (right) cells, which was established for the control cultures (95% - 97% viable). The control, viable PI population curve came to a baseline on the left of the curve, which was the beginning of the viable cell population fluorescent staining. The dead cells were to the right of the vertical line. At Day 6, the cells were 98% viable, with up to $1.5 - 2.0 \times 10^6$ cells/mL. At Day 10, dead cells were detected in the positive PI fluorescence portion of the histogram. By Days 14 - 18, two distinct populations of dead cells were seen, which showed the cells where the cell membrane was compromised (peak to the left of the vertical line) and cells where the nuclear membrane was compromised (peak to the right of the vertical line). This showed the PI had intercalated into the double-stranded nucleic acids, causing a very large increase in fluorescent yield. At Day 18, all of the cells were dead. There was over a 1000 fold increase in fluorescence, which is indicative of the high fluorescence yield due to the intercalation of the PI into the double stained nucleic acids.

Reagents: The highest chemical grades (97% - 99%) of curcumin, genistein, resveratrol, artemisinin, and vitamin C were used in these studies (Sigma-Aldrich). These reagents, except for the cell culture media soluble vitamin C, were prepared in 100X concentrations of pure Dimethyl Sulfoxide [DMSO] (Sigma-Aldrich) and diluted by a factor of 100 in the media sufficient enough to not have DMSO only being a factor in cell viability.

Encapsulated Reagents: Curcumin and Vitamin C MyCell-Enhanced-NutraNanoSpheres™ (MEN) had a concentration of 10.0% (w/v) or 2.5 mg/50 μ L for the curcumin

and 65 mg/50 μL for vitamin C.

Average Diameter Measurements of the MEN: The samples were diluted by volume in a ratio of 1:6 with DI Water and filtered by a 0.45 μm Nylon membrane to remove any dust contaminants. The Zetasizer ZSP (Malvern Instruments) was used with a backscattering angle of 173 degrees to measure the particle size by dynamic light scattering. A non-negative least squares algorithm was used to generate the size distribution by intensity, which indicated the diameter of the major population for the curcumin and vitamin C MEN. The intensity data was then converted to a mass or volume distribution to compare relative amounts of each size population, which indicated the percentage of the sample represented in the respective population.

Sample Preparation and Cell Counting: In all assays, viable cell counts were obtained by mixing 100 μL of a cell culture with 100 μL of viability stain. All samples were analyzed within an hour after room temperature incubation for at least 5 min. The percentage viability was stable for at least 2 h. A 10 μL portion

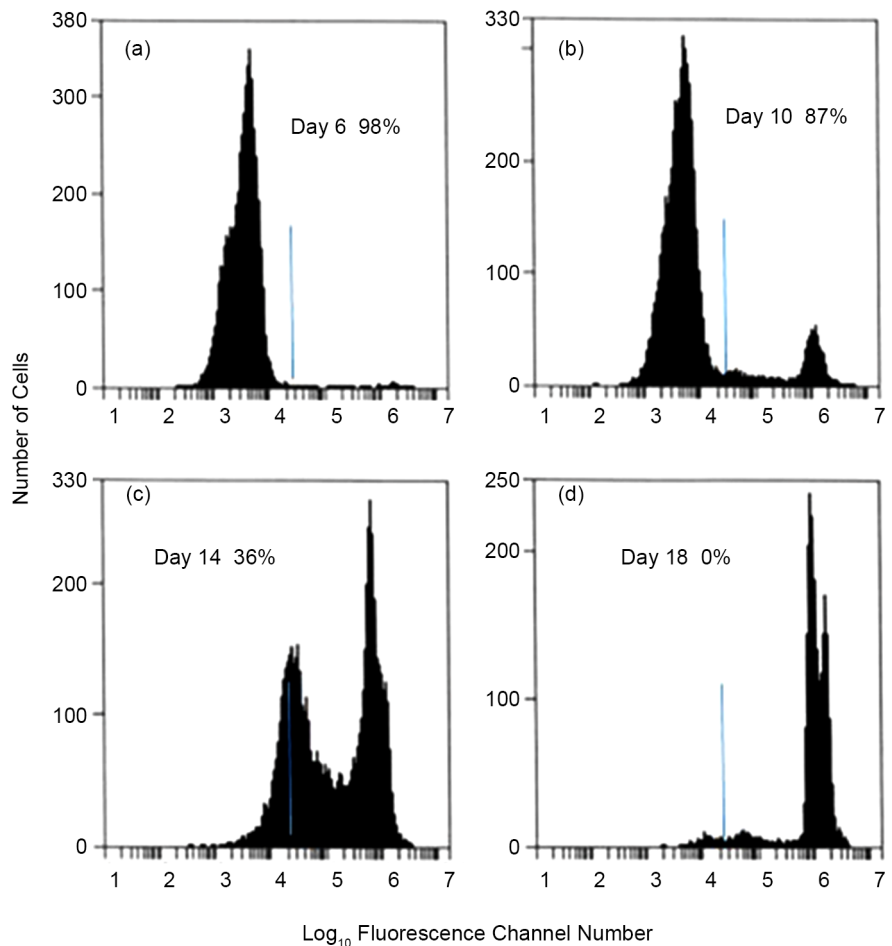


Figure 1. The effects on cell viability of K562 cancer cells vs time in culture showing increased cell death due to overcrowding and depleted nutrients. The vertical lines were used as the cutoff and to the right are the dead cells as measured by the increased uptake of the PI viability stain. The stained cell populations were gated on the light scatter cell population and analyzed in the red fluorescence channel.

of each sample was run through the Accuri C6 Flow Cytometer at a medium flow setting. The resulting number was multiplied by 200 to determine the number of viable cells/mL.

Procedures for Sterilization: All reagent samples were 0.22 μm sterile filtered and diluted with media in a sterile biologic safety cabinet.

Cell Growth Plate Preparation: The cells that were counted were then diluted with the media to a concentration of 1×10^5 viable cells/mL. A 500 μL portion of the cells was added to each well of the 48-well plate. The plates incubated in a Forma Scientific CO_2 water-jacketed incubator at a temperature of 37.2 C for 48 h to allow the cells to enter the exponential growth phase.

Addition of Compounds: After incubation for 48 h, the stock sample compounds were diluted accordingly, by a factor of two for up to eight dilutions. A 50 μL sample was added to each well, and up to six replicates of each dilution to the wells were prepared. 50 μL of cell culture media was added to each control well. Once finished, every well contained 550 μL . The plates were typically incubated for 48 h.

Cell Processing, Staining, and Analysis: Up to six replicates at each concentration, starting with the controls that were used to set the gates for viability, were suspended with a 500 μL pipet, and 100 μL portions were added to 2 mL 96 well analysis tubes. After all of the samples were added to the tubes, 100 μL of the viability stain were added using an 8-channel multipipetor, and the tray was shaken slightly and incubated for at least 5 min. All samples were analyzed within an hour after room temperature incubation for at least 5 min. The viability-stained cells were stable for at least 2 h at room temperature. A 10 μL portion of each sample was run through the Accuri C6 Flow Cytometer (BD Biosciences) using the fluorescence red channel (585 ± 20 nm) at a medium flow setting (200 - 300 cells/sec). Again the resulting number was multiplied by 200 to determine the number of viable cells/mL.

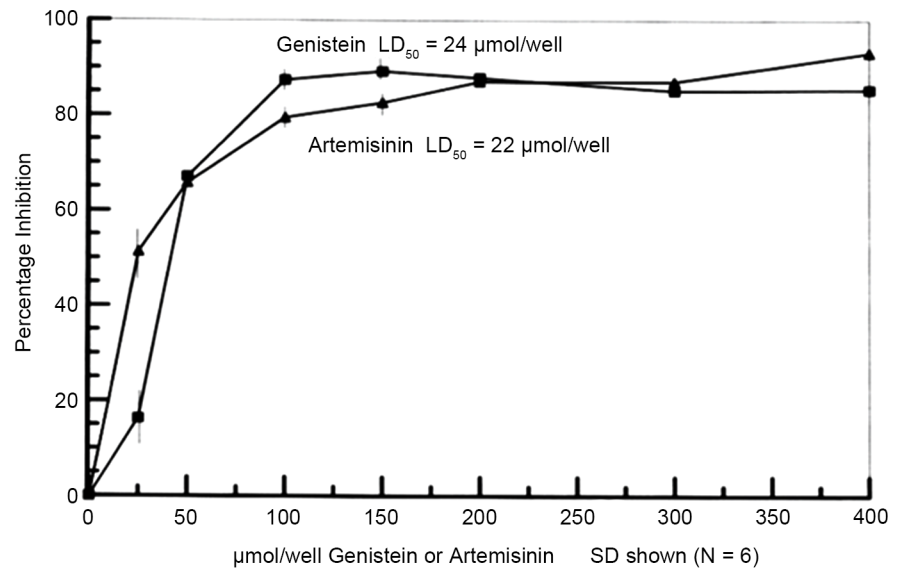
Percentage Cell Viability: Control cells were used to set the forward angle light scatter gate for the entire cell population gate for the cells less debris to the left of the scatter peak. The background fluorescent peak population of cells comprised the viable cells (95% - 97% viable). Any fluorescent cells to the right of the right baseline of the viable cell population comprised the dead cell population. The percentage viable cells were determined by dividing the number cells in the viable fluorescent cell population by the total number of viable + nonviable cells and multiplying by 100%.

Data Analysis: The data collected was then graphed using PSI-Plot. The data was graphed in the form of percentage inhibition vs. concentration of each component. To calculate percentage inhibition, the values for the viable cells/mL were incorporated into the equation, % Inhibition = $(1-X/Y) * 100\%$, where X was equal to the cells/mL in a particular well, and Y was the average number of cells/mL in the control. The mean of multiple replicates (4-6) \pm the Standard Deviation (SD) were then determined. LD_{50} is the concentration, either in nmol or μmol per well, of the supplement of interest that causes 50% cell death.

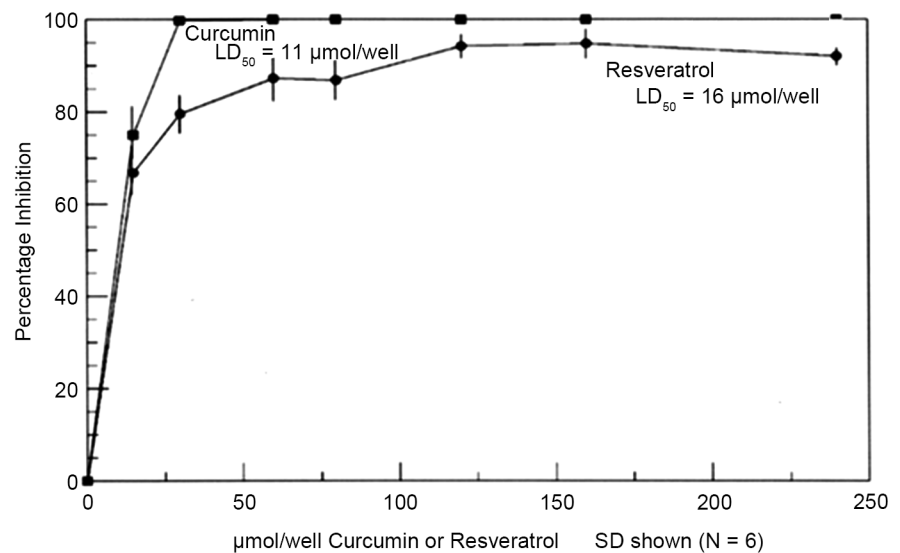
3. Results

Cytotoxicity of “Free” Supplements: Curcumin, Genistein, Resveratrol, Artemisinin and Vitamin C.

Figure 2(a) shows percentage of inhibition of cell growth when μmol additions of either genistein or artemisinin were added to the cell culture wells. The



(a)



(b)

Figure 2. (a) The percentage inhibition of the growth of K562 cells in the presence of Genistein or Artemisinin from 0 to 400 $\mu\text{mol}/500 \mu\text{L}$ after 48 h. The lethal dosages that kill half of the K562 cells (LD_{50}) are shown. SD bars ($n = 6$) are shown. The mean $\text{LD}_{50} \pm \text{SD}$ $\mu\text{mol}/\text{well}$ for eight experiments were $24.1 \pm 4.6 \text{ SD}$ for Genistein and 22.3 ± 5.8 for Artemisinin. (b) The percentage inhibition of the growth of K562 cells in the presence of Curcumin or Resveratrol from 0 to 250 $\mu\text{mol}/\text{well}$ after 48 h exposure. The lethal dosages that kill half of the K562 cells (LD_{50}) are shown. SD bars are shown ($n = 6$). The mean $\text{LD}_{50} \pm \text{SD}$ $\mu\text{mol}/\text{well}$ for eight experiments were $10.9 \pm 2.6 \text{ SD}$ for Curcumin and $15.9 \pm 6.7 \text{ SD}$ for Resveratrol.

lethal dosages in eight experiments, where the supplements kill 50% of the cancer cells (LD_{50}), for genistein was $24.1 \mu\text{mol/well} \pm 4.6 \text{ SD}$, while the LD_{50} for artemisinin was $22.3 \mu\text{mol/well} \pm 5.8 \text{ SD}$. In **Figure 2(b)**, the $LD_{50} = 15.9 \mu\text{mol/well} \pm 6.7 \text{ SD}$ for resveratrol and $10.9 \mu\text{mol/well} \pm 2.6 \text{ SD}$ for curcumin.

Two antioxidant compounds showed no inhibition of tumor growth. N-acetyl Cysteine up to $400 \mu\text{mol}$ (not shown) and vitamin C up to $10,000 \mu\text{mol}$ (not shown), were not cytotoxic against the K562 cancer cells.

The **Greatly Enhanced Anticancer Effects of Encapsulating Curcumin and Vitamin C in the MyCell-Enhanced-NanoSpheres™ (MEN)**. The curcumin and vitamin C MEN had a concentration $2.5 \text{ mg}/50 \mu\text{L}$ for the curcumin and $65 \text{ mg}/50 \mu\text{L}$ for vitamin C. Measurements of their respective LD_{50} determinations showed a greatly enhanced potent activity when these compounds were encapsulated in the liposomal structure.

Figure 3 shows the size distributions for the curcumin and vitamin C MEN samples, respectively. The samples were diluted by volume in a ratio of 1:6 with DI Water and filtered by a $0.45 \mu\text{m}$ Nylon membrane to remove any dust contaminants. These samples were run on the Malvern Zetasizer ZSP with a back-scattering angle of 173 degrees to measure the particle size by dynamic light

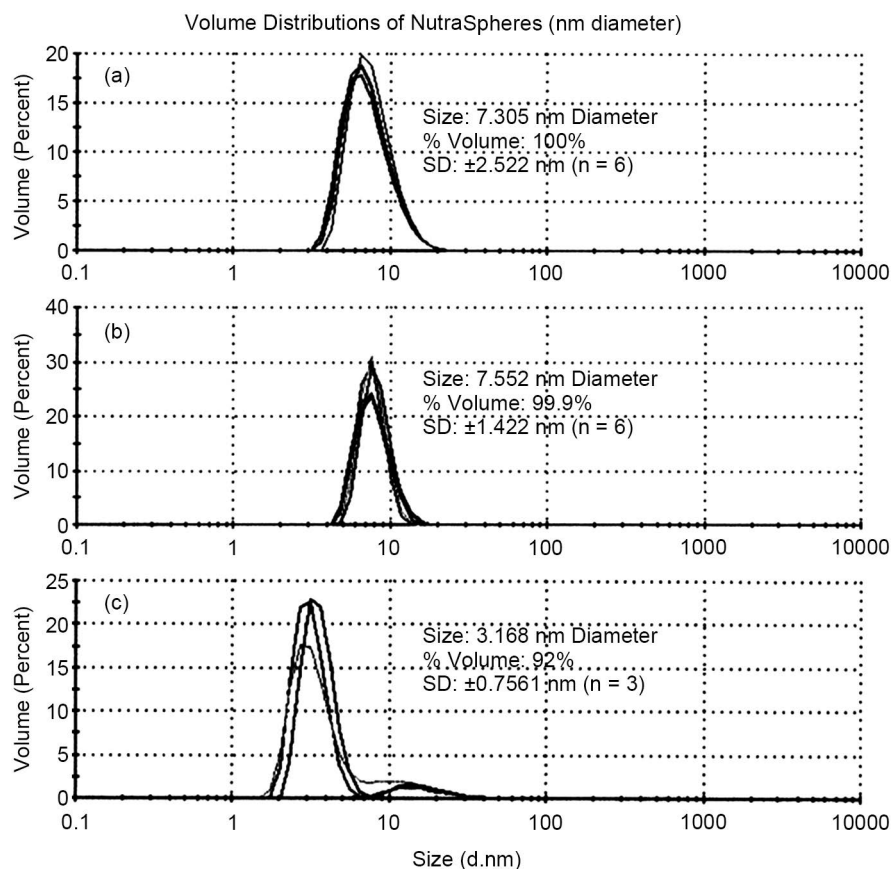
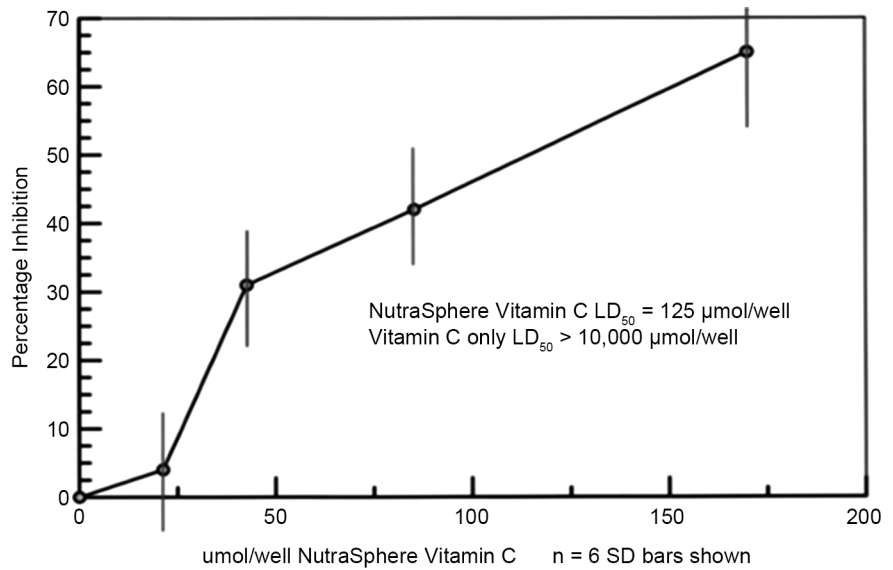


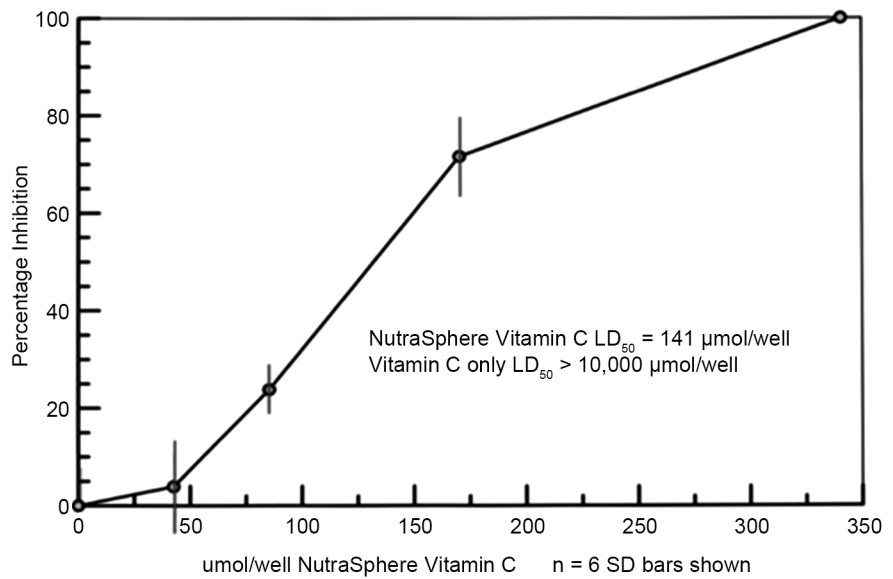
Figure 3. Volume distribution of the NutraNanoSpheres™ using the Zetasizer ZSP (Malvern Instruments) to measure the sphere sizes in nm diameter. Samples were run as six replicates. Standard deviation (SD) and mean diameters are shown. (a), (b) Representative Curcumin and (c) Vitamin C size distributions are shown.

scattering. A non-negative least squares algorithm was used to generate the size distribution by intensity. **Figure 3(a), Figure 3(b)** reveals the diameter measurements ($7.552 \pm 7.303SD$ nm) for the curcumin MEN for two separate preparations, while the mean diameter for a typical preparation for MEN vitamin C is 3.168 nm is shown in **Figure 3(c)**.

The vitamin C MEN (3.2 nm diameter-65 mg/50 μ L) showed a mean $LD_{50} = 133 \mu\text{mol/well} \pm 11 SD$ ($n = 4$) was greater than 75 times more potent than the free vitamin C, which showed no inhibition of K562 cell viability to at least 10^4 nm/well. This can be seen in **Figure 4(a), Figure 4(b)** where two typical Percentage Inhibition vitamin C MEN curves are shown.



(a)



(b)

Figure 4. The percentage Inhibition of the growth of K562 cells in the presence of NutraNanoSphere™ Vitamin C from (a) 0 to 200 and (b) 0 to 350 $\mu\text{mol/well}$. The $LD_{50} = 125$ and $141 \mu\text{mol/well}$ levels example data are shown.

The curcumin MEN (7 nm diameter-25 mg/50 μ L) resulted in a mean $LD_{50} = 41.3$ nmol/well ± 5.6 SD ($n = 8$). Two representative percentage inhibition experiments are shown in **Figure 5**. In **Figure 1(b)** the average LD_{50} for the free curcumin was 10,900 nm/mL, while the MEN curcumin averaged LD_{50} was 41.3 nmol/well. The ratio of the LD_{50} values (10,900/41.3) revealed there was a 264-fold increase in toxicity when the curcumin was encapsulated.

In **Figure 6**, cancer cell death by alteration of the cell membranes could be seen within 30 s of exposure to the MEN curcumin. The free curcumin showed a similar rapid killing (not shown). The killing process was probably near instantaneous, since the time from the addition of the viability stain and analyzing on the flow cytometer was about 30 s.

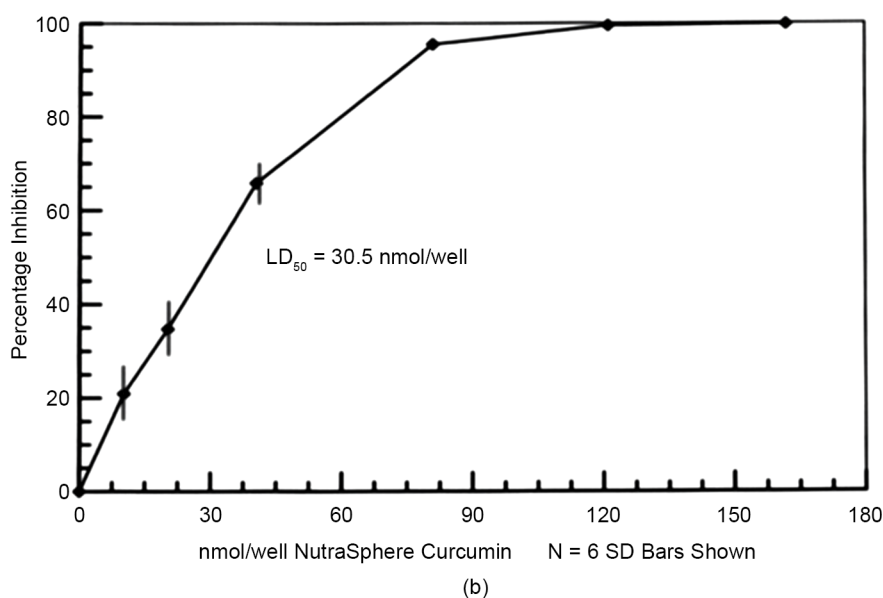
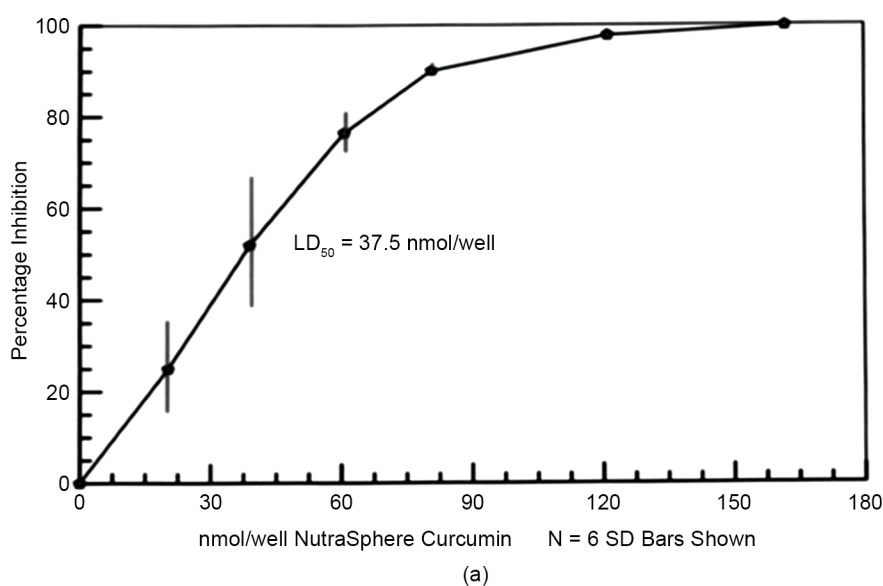


Figure 5. The percentage Inhibition of the growth of K562 cells in the presence of NutraNanoSphere™ Curcumin from 0 to 180 μ mol/well. The $LD_{50} = 37.5$ and 30.5 nmol/well levels example data are shown.

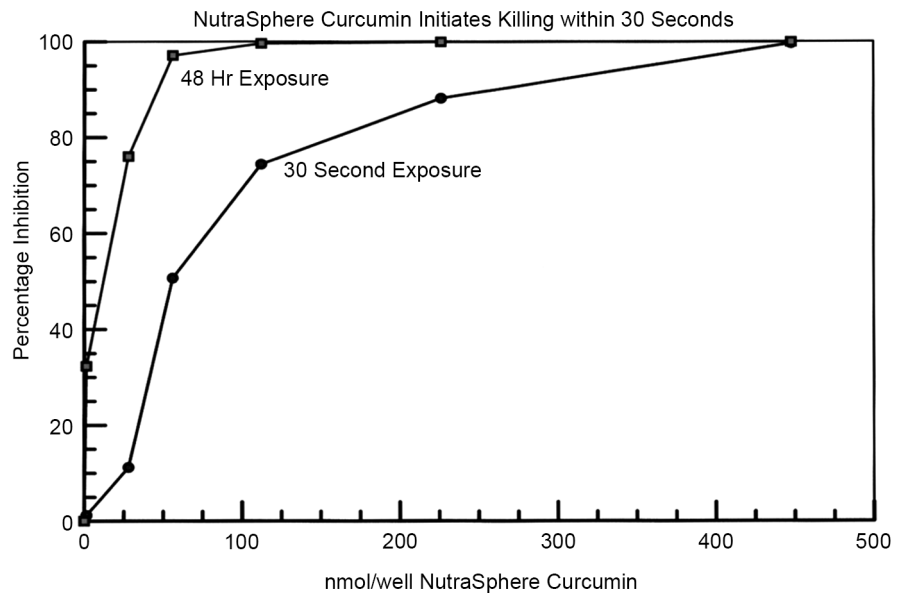


Figure 6. The percentage inhibition of the growth of K562 Cells in the presence of NutraNanoSphere™ Curcumin from 0 to 450 nmol/well. The LD₅₀ values per well were 20 nmol after a 48 hr exposure and 50 nmol for a 30 sec exposure. The viability of the K562 cancer cells dramatically diminishes as early as 30 seconds of exposure to the NutraNanoSphere™ Curcumin.

4. Discussion

The ability to inhibit the viability of the K562 cancer cells was greatly enhanced by the fatty acid micelle encapsulation of curcumin and vitamin C in the form of NutraNanoSpheres™ (MEN). The clinical goal is to develop water-soluble mixtures of MEN with their high bioavailability (>90%) and without degradation in the stomach and intestines for the prevention and treatment of cancer.

In the case of vitamin C, no inhibition of cancer cell growth was seen with the “free” vitamin C up to 1×10^4 $\mu\text{mol}/\text{well}$. The solubility of vitamin C is 1 g/3 mL of water or 1.89 M [34] [35]. However, there was concern that going much above 1×10^4 $\mu\text{mol}/\text{well}$ would cause osmotic toxicity to the K562 cells. The encapsulated MEN vitamin C allows for the measurement of vitamin C toxicity against the K562 cells at a LD₅₀ = 133 nmol/well average, which makes the anti-cancer toxic measurements even possible.

The LD₅₀ of curcumin in the “free” form was 10,900 nmol/well (Figure 2(b)) compared to the curcumin MEN (Figure 5), which was averaged only 41.3 nmol/well. The LD₅₀ = 41.3 nmol/well is equal to 41.3 nmol/550 μL well volume, which in turn is 75.1 μM . The average 70 kg human has about 4.7 liters of blood [36]. In order to have an equivalent LD₅₀ = 75.1 μM in 4.7 L of blood, one would need at least 353 μmol of curcumin. The MEN contain 65 mg of curcumin per drop (50 μL) or 65 mg/0.36838 mg/mol = 176.4 μmol . Therefore, to provide an LD₅₀ would require (353 $\mu\text{mol}/176.4$ μmol) = 2.0 drops of the curcumin MEN assuming 100% bioavailability, which is not too far from the 90.2% that appears in the serum within 30 min. in patients orally ingesting the encapsulated curcu-

min as reported by the manufacturer.

The free and MEN curcumin showed complete cell destruction (zero viable cells out of 7×10^5 viable cells in the control wells) at the $LD_{50} = 90\text{-}120$ $\mu\text{mol}/\text{well}$ as early as 20 h in culture. Cell death by alteration of the cell membranes could be seen within 30 s of exposure to curcumin (Figure 6). The other components required at least 48 h for maximum killing, suggesting a more metabolic and/or apoptotic route of destruction using the other free supplements.

The freeform vitamin C did not affect the K562 cell viability. However, the MEN-vitamin C exhibited a greatly enhanced cytotoxicity against cancer cells. The same was seen for the MEN-curcumin. High dose MEN-curcumin above 1 - 2 mL per day may be very effective in treating advanced cancers, and warrant clinical trials, because of its relatively low cytotoxicity to normal cells.

Genistein, resveratrol, and artemisinin may also be encapsulated in the MEN and be used to produce a “cocktail” of components that would show synergism or additive effects with different mechanisms of action, which include metabolic, apoptotic, and direct cytotoxicity for the prevention and treatment of cancer. These supplements may also show a significant increase in their anti-cancer activity, as seen with MEN curcumin and vitamin C.

5. Conclusions

In conclusion, these supplements are safe and effective in combination, especially in the MEN form, to provide a simple, water-soluble droplet formulation, which could be added to water or juice and taken on a daily basis. The high bioavailability that results from encapsulation would assure effective dosages to be determined in clinical trials for both the prevention and treatment of cancer.

The MEN Vitamin C and Curcumin presented in this paper have functions that affect virtually every aspect of cellular development. For example, vitamin C has been shown to be a cofactor for methylcytosine dioxygenases, which are enzymes for DNA demethylation, an important crossroad in epigenetic regulation [37] [38]. Curcumin is a complementary therapy that may be helpful for the treatment of a variety of diseases because of its anti-inflammatory, antiangiogenic, antioxidant, and antiproliferative effects [39] [40] [41].

Our next goal is to encapsulate the other free supplements that show anti-cancer activity, such as genistein, resveratrol, and artemisinin. Therefore, the possibility of being able to use a combination of MEN supplement types for cancer preventative and treatment protocols will revolutionize our options in treating cancer. The advantages of using this proposed MEN cancer formulation include safety, efficacy without immunosuppression and actual facilitation of stimulation of the Natural Killer Cell activity [42] using curcumin [43] [44], genistein [45], resveratrol [46] [47] [48] [49] and at least a general immune stimulatory role with artemisinin and its derivatives [50] [51]. By simply adding MEN supplement drops together in a cancer prevention or treatment protocol in a drinkable form, would eliminate individuals having to swallow pills and greatly enhance bioavailability.

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