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Atomic force microscopy correlates antimetastatic potentials of HepG2 cell line with its redox/energy status: effects of curcumin and *Khaya senegalensis*


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ABSTRACT

OBJECTIVE: The fatality of cancer is mostly dependent on the possibility of occurrence of metastasis. Thus, if the development of metastasis can be prevented through novel therapeutic strategies targeted against this process, then the success of cancer treatment will drastically increase. In this study, therefore, we evaluated the antimetastatic potentials of an extract of *Khaya senegalensis* and curcumin on the metastatic liver cell line HepG2, and also assessed the anticancer property of the extract.

METHODS: Cells were cultured and treated with graded concentrations of test substances for 24, 48, or 72 h with provisions made for negative controls. Treated cells were assessed as follows: nanotechnologically—atomic force microscopy (AFM) was used to determine cell stiffness; biochemically—cell cytotoxicity, glutathione level and adenosine triphosphate status, caspase activation and mitochondrial toxicity were considered; and microbiologically—a carrot disk assay was used to assess the anticancer property of the extract of *K. senegalensis*.

RESULTS: Curcumin and *K. senegalensis* increased the cell stiffness by 2.6- and 4.0-fold respectively, indicating their antimetastatic effects. Corresponding changes in redox (glutathione level) and energy (adenosine triphosphate) status of the cells were also demonstrated. Further mechanistic studies indicated that curcumin was not mitotoxic in HepG2 cells unlike the *K. senegalensis* extract. In addition, the extract potently inhibited the *Agrobacterium tumefaciens*-induced genetic transformation based on carrot disk assay.

CONCLUSION: Cell elasticity measurement data, using AFM, strongly suggested, for the first time, that both curcumin and the extract of *K. senegalensis* exhibited antimetastatic properties on HepG2 cells.

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1 Introduction

In the United States (US), 25% deaths is caused by cancer, making cancer a major public health challenge in the US, as well as in many other parts of the world.[1] Cancer is a group of diseases which are characterized by the uncontrolled growth and spread of abnormal cells. If this metastasis can be controlled, then the disease can easily be managed.[2] Moreover, in 2012, out of the 8.2 million global cancer related deaths, 745 000 were associated with liver cancer.[3]

The relevance of plants in the treatment of cancer dates back several decades,[4] as corroborated by the World Health Organization, which estimated that approximately 80% of the world’s inhabitants rely on traditional medicine for their primary health care.[5] Curcumin is a yellow-orange phytopolypheolic pigment derived from turmeric (Curcuma longa). It has been shown to have multiple anticancer effects, such as inhibition of proliferation, induction of apoptosis, inhibition of angiogenesis and DNA topoisomerase II.[6]

In addition, curcumin has been shown to have significant hepatoprotective activity against aflatoxin B1-induced liver injury, by lowering the levels of serum marker enzymes, lipid peroxidation and elevating the levels of reduced glutathione (GSH), superoxide dismutase, catalase and GSH peroxidase.[7] Further, the chemopreventive activity of curcumin had been demonstrated when administered before, during and after carcinogenic treatment as well as when administered during the promotion and progression phases of colon carcinogenesis in rats.[8]

Apart from the well-known chemopreventive agents, there are other medicinal plants with potential relevance in cancer treatment. Khaya senegalensis is a large tree commonly called the dry zone mahogany or African mahogany. It thrives mainly in the sub-Saharan savannah forests, growing up to 30 meters in height, and is regarded as the most popular medicinal meliaceous plant in African traditional remedies.[9] An extract of the stem bark is used extensively as a bitter tonic for the treatment of a variety of pro-inflammatory diseases, including cancer.[10–12] Furthermore, this extract has been used as a folk medicine for the treatment of diabetes, hypertension, jaundice and malaria, among other diseases.[13] Scientific reports on the chemical profile have shown that the stem bark extract contains scopoletin, scopolamine, limonoid, bitter principle, tannins, saponins and sterols.[14,15] Other researchers have determined the structures of the limonoids by using two-dimensional nuclear magnetic resonance spectroscopy and mass spectrometry.[16]

The high-precision measurement of nanomechanical properties of cancer cells has been made feasible through a number of biophysical (nanotechnological) methods, such as atomic force microscopy (AFM), microfluidic optical stretcher and magnetic tweezer system.[17–19] Originally invented in 1986 for high-resolution imaging purposes, the AFM is rapidly emerging as a powerful nanotechnological tool in cell biology for its unique capabilities as a nano-indenter to probe the dynamic viscoelastic material properties of living cells in culture.[20,21] As the only technique capable of real-time imaging of the surface of living cells in their native environment, this technique has found many applications in pharmacology, biotechnology, microbiology, structural and molecular biology, genetics and other biology-related fields.[22] Through AFM nanomechanical analysis, our laboratory has demonstrated that cell elasticity is highly correlated with metastatic potential; AFM nanomechanical profiling also has potential applications as a marker for cancer drug sensitivity.[23,24]

In this present work, we investigated the effects of treating HepG2, a metastatic liver carcinoma cell line, with an extract derived from the stem bark of K. senegalensis, using curcumin as a comparative phytochemical standard. The investigations were based on cellular morphological changes, adenosine triphosphate (ATP) status, GSH and oxidized glutathione (GSSG) levels, caspase activation, mitochondrial integrity and cell elasticity profiling by using AFM. This work begins to fill the knowledge gap in the relationship between cellular antioxidant/energy status and cell elasticity, a measure of metastatic potential. We report here that K. senegalensis hydroethanolic extract (K2S) induced antimetastatic effects on HepG2 cells by reducing the cellular energy and antioxidant status. Furthermore, from microbiological point of view, the anticancer activity of K2S is supported by its attenuation of genetic transformation induced by Agrobacterium tumefaciens L. This is a rod-shaped Gram-negative soil bacterium, also called Rhizobium radiobacter, which induces crown gall disease, a malignant tumor, occurring on the stems and leaves of infected plants.

2 Materials and methods

2.1 Cell line and culture conditions
HepG2 (human liver carcinoma) cells were kindly
provided by Dr. Samuel French (Department of Pathology and Laboratory Medicine, University of California, Los Angeles, CA, USA). The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich, MO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma Aldrich, USA), 1% L-glutamine and 1% penicillin/streptomycin (Sigma Aldrich, MO, USA). The cells were cultured in a water-jacketed incubator at 37 °C, 5% CO2, 95% air, with renewal of medium every 2–3 days.

2.2 Plant source and identification

The stem bark extract used in this work was sourced from Lokoja, Nigeria by the kindness of Mr. and Mrs. J. O. A. Ohugbami, and identified by a plant taxonomist in the Department of Botany, University of Ibadan, Ibadan, Nigeria. A voucher specimen was deposited in the Herbarium of the same Department with reference number UIH-22403.

2.3 Preparation of extracts

The hydroethanol stem bark extract, K2S, used in this work was obtained according to the method already reported in our previous publication. The percentage yield of the extraction process was 7.94% (w/w).

2.4 Optimization of dimethyl sulfoxide concentration and cell density

HepG2 cells in 75 mL culture flasks were trypsinized with 3 mL of 0.05% trypsin solution (Sigma, WI, USA), and incubated for approximately 5 min at 37 °C. The trypsin was neutralized by addition of complete culture medium, before the cultures were centrifuged at 1 000 r/min (radius = 98 mm) for 5 min. Cells were harvested and resuspended in medium, to ensure a single cell suspension. Cells were then counted on a hemocytometer based on the trypsin blue dye exclusion method (0.4% trypsin blue; Sigma-Aldrich, St Louis, MO, USA) by adding 10 µL of trypsin blue solution to 10 µL of cell suspension (1:1, v/v). The cells were seeded at densities ranging from 500 to 6 000 cells per well in four replicates, using black, tissue culture treated, clear-bottom 384-multiwell plates. Cells were incubated for 24 h prior to treatment with varying concentrations (0.062 5%, 0.125%, 0.25%, 0.5% and 1.0%) of dimethyl sulfoxide (DMSO) solutions prepared with the complete medium. Cell cultures were then incubated for 24 h before staining with Hoechst 33342 (Hoechst) (Invitrogen, Eugene, OR, USA) and propidium iodide (PI) (Sigma, MO, USA), prepared in culture medium, at final concentrations of 5 and 10 µmol/L respectively for 1 h. Plates were imaged with an ImageXpress MicroXL high-content fluorescence microscope (Molecular Devices, Sunnyvale, CA, USA).

2.5 Cytotoxicity determination

A stock curcumin (Sigma, MO, USA) solution was prepared in 100% DMSO at 10 mg/mL, and sonicated to ensure total dissolution. K2S was prepared in 0.5% DMSO, sonicated and centrifuged at 13 500 r/min (radius = 11 mm) with the supernatant preserved for subsequent use. For the assays, curcumin and K2S were further diluted to appropriate concentrations in complete medium. In both cases, the final DMSO concentration did not exceed 0.25%. Having obtained an optimum DMSO concentration and cell density, cells were prepared at a cell density of 3 000 cells/well (this translates to 3.0 × 10^5 cells/mL) and then treated with curcumin (0.39, 0.78, 1.56, 3.13, 6.25, 12.5, and 25 μg/mL) and K2S (12.5, 25, 50, 100, 200, 400, and 800 μg/mL). Concentrations between curcumin and K2S treatments differ because the curcumin is a pure compound, and K2S is a crude extract. The treated cells were incubated for 72 h, with the effects of treatment being determined at 24-hour intervals. At each interval, the plates were stained and imaged with an ImageXpress MicroXL high-content fluorescence microscope.

2.6 Image acquisition and analysis

The ImageXpress® Micro Widefield High Content Screening System and its associated MetaXpress® High-content Image Acquisition and Analysis Software interface were utilized for Image acquisition and analysis. After dye addition and incubation (1 h), images were captured with two software-predetermined excitation/ emission filter sets; 380/535 nm for Hoechst and 555/645 nm for PI. Transmitted light and fluorescence images from each well were acquired using a 20× and 4× dry objective lenses, respectively. Under these settings and by plating HepG2 cells at the predetermined optimum cell densities indicated above, the system imaged from 100 to 400 cells per well and the images were analyzed using the MetaXpress® Analysis Software. To define nuclei as individual units, or regions of interest (ROIs), preprocessing filters and intensity thresholds were applied for image segmentation. Segmented images were subjected to data classification using the MetaXpress® Analysis Software. The percentage of dead cells was calculated from the total number of ROIs per well. Cell nuclei emitting fluorescence signal from both Hoechst and PI (fluorescence co-localization) were considered to be dead cells, while cells emitting only Hoechst signal were counted as live cells (Figure 1).

2.7 Sample preparation and AFM measurements

Cells (3.0 × 10^5 cells/mL) were cultured in 60 mm Petri dishes for 24 h before treatment with either curcumin or K2S for another 24 h. All cells (n = 20) subjected to measurements in each of the experiments were initially confirmed to be viable by their attachment to the culture substrate; apoptotic/dead cells were found floating in the medium. All unhealthy cells were thus excluded from AFM
analyses. All measurements were conducted using Bioscope Catalyst AFM (Bruker Instruments, Santa Barbara, CA, USA), with a combined inverted optical/confocal microscope (Zeiss, Corp, Thornwood, NY, USA). This combination, having a motorized stage, along with AFM software permits lateral positioning of the cell nucleus with submicron precision; therefore, we did not expect to see topographical variations or substrate effects in our indentation analysis. AFM nanomechanical measurements were collected in contact mode, using sharpened silicon nitride cantilevers with experimentally determined spring constants of 0.05 N/m and a tip radius of < 20 nm. All measurements were performed at a constant approach and retract velocity of 4.15 µm/s, with the loading rate being 2.0 µN/s. The measurements were obtained at 37 °C maintained by LakeShore 331 temperature controller (Veeco Digital Instruments, USA); force measurements were recorded at 1 Hz and a loading force of 1 nN was used. Force-displacement curves were obtained on each cell and converted to force-indentation curves. This allows for the subsequent determination of the local cell surface elasticity or “stiffness” (Young’s modulus, $E$) by fitting the curves based on Sneddon model, which describes the indentation of an elastic sample using a conical indenter.\textsuperscript{[23,24,26,27]}

2.8 ATP assay

ATP levels were measured using CellTiter-Glo Luminous Cell Viability Assay (Promega Corporation, Madison, WI, USA). Cells were plated at 1 500 per well, in triplicate, in white 384-multiwell plates, and allowed to attach for 24 h. Plates were incubated for a further 24 h after addition of experimental treatments (vehicle or test substance) in a volume of 12.5 µL culture medium. Then a volume of CellTiter-Glo reagent equal to the volume of each well was added. After 10 min, luminescence was read using a Wallac plate reader (Perkin-Elmer, USA), and signal intensity was calculated relative to in-plate DMSO control wells.

2.9 Glutathione assay

Reduced-oxidized GSH levels were determined by using the luminescence-based GSH/GSSG-Glo™ Assay reagents (Promega, USA). Cells were plated at 3 000 per well, in a volume of 25 µL culture medium, in white 384-multiwell plates and allowed to attach for 24 h. After treatments (vehicle or test substances) in a volume of 25 µL culture medium, plates were incubated for 24 h. Subsequently, the cell culture medium/treatment was removed and plates were treated according to manufacturer’s instructions to determine the levels of total oxidized GSH. After 15 min of incubation to stabilize the signal, luminescence was read using a Wallac plate reader. GSH/GSSG ratios were calculated directly from relative luminescent unit measurements.

2.10 Caspase 3/7 activity

Caspase 3/7 activity was assessed with the luminescent Caspase-Glo™ 3/7 Assay reagents (Promega, USA). Cells were plated at 1 500 per well, in a volume of 12.5 µL culture

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Figure 1  Staining of cells
Sequential steps in the use of Hoechst and propidium iodide (PI) to determine cytotoxicity of treatments on HepG2 cells using 384-well plates.
medium, in white 384-multiwell plates and allowed to attach for 24 h. After treatments (vehicle or test substances), in a volume of 12.5 μL culture medium, plates were incubated for 24 h. Then 25 μL of Caspase-Glo™ 3/7 reagent was added to each well. Contents of wells were mixed using a plate shaker at 1 300–1 500 r/min for 30 s and the plates were incubated at room temperature for 1 h before measuring luminescence on an Analyst GT multimode reader (Molecular Devices Corporation, Sunnyvale, CA, USA).

2.11 Mitochondrial toxicity testing

The Mitochondrial ToxGlo™ Assay (Promega, USA) used here is a cell-based assay that employs multiplexed chemistry for predicting potential mitochondrial dysfunction due to xenobiotic exposure. The assay is based on the differential measurement of biomarkers associated with changes in cell membrane integrity and cellular ATP levels relative to vehicle-treated control cells. A volume of culture medium (10 μL) containing approximately 1 500 cells was plated on opaque-walled, black, tissue culture plates, compatible with fluorescent and luminescent measurements (clear bottoms). As the assay was attachment-dependent, cells were incubated for 24 h to allow adhesion to the multiwells. The cells were then treated with 10 μL of sample and incubated for 24 h. Using an electronic multichannel pipette, 5 μL of 5× cytotoxicity reagent was added to each well and mixed briefly (1 min) by orbital shaking (1 300 r/min) to ensure reagent/sample homogeneity and to mitigate possible reagent/sample partitioning. The sample was then incubated at 37 °C for 30 min and fluorescence was measured with a rhodamine 110 filter set at 485 nm excitation /525 nm emission using a Flexstation 3 microplate reader (Molecular Devices, Sunnyvale, CA). Thereafter, the assay plate was equilibrated to room temperature (10 min) and 25 μL of ATP detection reagent was added to each well. The plate was mixed by orbital shaking (1 300 r/min) for 3 min and luminescence was measured with a Wallac plate reader.

2.12 Carrot disk genetic transformation assay (anti-tumor activity)

Antitumor activity of K2S was determined by using the carrot disk bioassay with minor modifications.[28] Carrot (Daucas carota L.) samples were sterilized with 2% Clorox for 5 min, followed by the formation of disks by cutting into sections of approximately equal dimension of 5 mm × 8 mm (thickness × radius). The disks were subsequently submerged in 2% Clorox for 30 min and then rinsed thrice in autoclaved distilled water at 15-minute intervals to remove excess bleach. Using sterile forceps, the disks were initially blotted on a set of sterile filter papers and then transferred to a piece of sterile filter paper (Whatman) already placed in a sterile and labelled Petri dish. A fresh plate of A. tumefaciens (strain A348) was kindly provided from the laboratory of Dr. Maskit Maymon. The identity of the strain was verified by using PCR-based 16s rRNA gene sequence analysis. This Agrobacterium strain did not possess drug resistance markers. It was cultured in Luria Bertani medium and kept in a G25 controlled environment incubator shaker (New Brunswick Scientific Co, Edison, NJ, USA) at 28 °C for 3 d. The culture was centrifuged at 2 500 r/min (radius = 50 mm) for 10 min and the growth medium (supernatant) was discarded. The bacterial cells were resuspended in Murashige and Skoog (MS) medium and the absorbance was measured at 600 nm, using a NanoDrop ND 1000 spectrophotometer (Wilmington, DE, USA). Having prepared K2S at final concentrations of 100, 200, 400 and 800 μg/mL in autoclaved distilled water, the center of each disk was treated according to the following order:

a) 25 μL of solvent was dispensed on all the negative control disks, followed by 25 μL of MS medium;

b) 25 μL of solvent was dispensed on all the positive control disks, then 25 μL of A. tumefaciens inoculum was overlaid;

c) 25 μL of graded solutions of K2S was dispensed on the test disks followed by overlaying with 25 μL of A. tumefaciens inoculum.

Petri dishes were sealed with Parafilm and incubated at 28 °C. The disks were checked daily for the development of young galls (tumors) from the meristematic tissue around the central vascular system.

2.13 Statistical analysis

Data were analyzed using a graphing and analysis software, GraphPad Prism 5.0 (GraphPad Software Inc., San Diego CA, USA). The results were expressed as mean ± standard deviation and 50% cytotoxic concentration (CC50) values were obtained from linear regression plots. The normality test was based on the D’Agostino-Pearson method. One-way analysis of variance was used to assess differences among group means (student’s t test, where applicable); if significant differences were found (P < 0.05), the post-hoc Tukey-Kramer multiple comparison test was used.

3 Results

3.1 Optimization of HepG2 cells by Hoechst and PI staining depends on concentration of DMSO and cell density

An extensive literature search showed that virtually no work has been carried out involving the titration of the common organic solvent, DMSO, with HepG2 cells using 384-multiwell plates in the presence of fluorescent staining dyes, Hoechst and PI. For all the concentrations of DMSO tested, the cytotoxicity level did not exceed...
5%. Cell density affected viability, and at the highest cell density (6 000 cells/well), the percentage of dead cells for even the negative control was almost the same as in the highest DMSO concentration (1%) applied to the lowest cell density (500 cells/well). At a cell density of approximately 3 000 cells/well and a DMSO concentration of 0.25% there was not much difference in percent dead cells compared to the negative control, in which cells were treated with medium only (Figure 2). Moreover, using a DMSO concentration of 0.25% made it possible to dissolve higher concentrations of the test substances. Hence, we opted for this cell density and DMSO concentration for the downstream experiments.

3.2 Cytotoxic effects and phenotypic changes induced by curcumin and K2S

Over the range of concentrations used in the experiment, it was observed that treatment of HepG2 cells with lower concentrations (0.39, 0.78, 1.56, 3.13 and 6.26 µg/mL) of curcumin did not induce any significant increase in percent dead cells over a period of 72 h, when assessed at 24-hour intervals. Whereas, at higher concentrations (12.5 and 25 µg/mL), there was a marked increase in cell death. The highest concentration consistently resulted in dead cell counts of above 50% throughout the time of incubation; treatment with the second greatest concentration (12.5 µg/mL) resulted in an increase in percent dead cells over time. K2S treatments resulted in a concentration- and time-dependent increase in percentage of dead cells; fluorescence interference resulted in an apparent decrease in percentage of dead cells at the highest treatment concentrations. On one hand, curcumin had a CC50 of 16.24 µg/mL after 24 h of treatment, which did not change significantly for the remaining 48 h of incubation (Figures 3 and 4). On the other hand, K2S had a CC50 of 83.92 µg/mL after 24 h, but the dead cell proportion increased significantly (P < 0.05) over the following 48 h in a consistent manner (Figures 4 and 5). Treatment of HepG2 cells with either curcumin or K2S induced almost no morphological changes at lower concentrations relative to the negative control. However, as the concentrations of treatment increased, cell viability was affected, which is evident from the rounded morphology of some cells as shown in Figures 3 and 5.

3.3 Changes in cell and nuclear areas induced by curcumin and K2S

Induction of cell death, either through apoptotic pathway or necrosis, is associated with corresponding changes in the shapes and sizes of affected cells; hence, we assessed these changes in response to treatment of HepG2 cells with curcumin or K2S. After 24 h of treatment, both curcumin and K2S independently induced a decrease in the overall nuclear size in a concentration-dependent manner. The extract showed a greater effect. DMSO did not significantly affect the nuclei total area (NTA) at each of the respective

Figure 2  Optimization of cell density and DMSO concentration
Values of cell death (%) in response to treatment of different densities of HepG2 cells with correspondingly varying concentrations of dimethyl sulfoxide (DMSO).
Figure 3  Morphological changes in HepG2 cells after 24 h of treatment with curcumin
These images were visualized with a transmitted light microscope (ImageXpress MicroXL, Molecular Devices) with 20× magnification. In the graph, concentrations were converted into the corresponding logarithm values for an improved representation. The sequential final concentrations of dimethyl sulfoxide (DMSO) from the lowest to the highest are 0.003 9%, 0.007 8%, 0.015 6%, 0.031 2%, 0.062 5%, 0.125% and 0.25%. The arrow indicates a dead cell.

Figure 4  Cytotoxic effects of treatment
These data show a concentration- and time-dependent assessment of cytotoxicity resulting from treatment of HepG2 cells with curcumin or extract (K2S). The concentrations were converted into the corresponding logarithm values for improved graphical representations. The sequential final concentrations of dimethyl sulfoxide (DMSO) from the lowest to the highest are 0.003 9%, 0.007 8%, 0.015 6%, 0.031 2%, 0.062 5%, 0.125% and 0.25%. The arrow indicates a dead cell.
incubation times. However, as was expected for the negative control, there was a time-dependent increase in NTA over the time of incubation. Treatment with K2S markedly reduced the nuclear size throughout the incubation period (Figure 6A–C), even at lower concentrations, whereas treatment with curcumin did not affect the nuclear size at the lower concentrations relative to the negative control. To gain more insight into the mechanism of cell death induced by the treatments, we considered the changes in the sizes of the nuclei of compromised cells that were stained with both Hoechst and PI. It was observed that K2S, at almost all the concentrations and throughout the period of treatment, led to a significant increase in the nuclear area of compromised cells relative to the negative control. To gain more insight into the mechanism of cell death induced by the treatments, we considered the changes in the sizes of the nuclei of compromised cells that were stained with both Hoechst and PI. It was observed that K2S, at almost all the concentrations and throughout the period of treatment, led to a significant increase in the nuclear area of compromised cells relative to the negative control. The nuclear area of cells whose deaths were induced by curcumin showed almost the same features as the negative control, except at its highest concentration (Figure 6D–F). After treatment, the nuclear size of viable cells (stained only by Hoechst) exhibited almost the same trend in overall nuclear size. The extract significantly reduced the nuclear area over the 72 h of treatment, beginning at the first measurement interval (Figure 6G–I).

3.4 The proliferation of HepG2 cells is influenced by treatment with either curcumin or K2S

As expected, the negative control increased total cell count in a time-dependent manner. K2S possessed antiproliferative activity against HepG2 cells at almost all concentrations considered in this study and throughout the time of exposure to treatment. Curcumin showed a biphasic activity, promoting cell growth at lower concentrations and antiproliferative at higher concentrations of treatment. However, the antiproliferative effects of both K2S and curcumin increased in a time-dependent manner (Figure 7).

3.5 Curcumin and K2S possess antimetastatic potential from cell elasticity measurements using AFM

To assess the possible antimetastatic effects of treatments, we measured the Young’s modulus of the untreated cells in comparison to the treated cells.
Figure 7 Antiproliferative effects of curcumin and K2S
Numerical changes associated with treatment of HepG2 cells with curcumin (Cur) or extract (K2S) followed up for 3 d. The sequential final concentrations of dimethyl sulfoxide (DMSO) from the lowest to the highest are 0.003 9%, 0.007 8%, 0.015 6%, 0.031 2%, 0.062 5%, 0.125% and 0.25%.

Figure 6 Changes in nuclear areas induced by curcumin and K2S
Changes in nuclear size associated with treatment of HepG2 cells with curcumin (Cur) or extract (K2S) followed up for 3 d. The sequential final concentrations of dimethyl sulfoxide (DMSO) from the lowest to the highest are 0.003 9%, 0.007 8%, 0.015 6%, 0.031 2%, 0.062 5%, 0.125% and 0.25%.
The negative control (Figure 8A), which was exposed to culture medium alone, had a mean stiffness value of 
(1.33 ± 0.034) kPa (n = 124), while the average Young’s modulus values for curcumin treatment at graded 
concentrations of 0.78 and 1.56 µg/mL were (2.06 ± 0.07) kPa (n = 84) and (3.40 ± 0.26) kPa (n = 143), respectively 
(Figure 8 B and D). K2S caused the Young’s modulus of HepG2 cells to increase to (2.01 ± 0.09) kPa (n = 95) and 
(5.03 ± 0.34) kPa (n = 156) at treatment concentrations of 25 and 50 µg/mL, respectively (Figure 8C and E). 
These data demonstrate the high metastatic capability of HepG2 cells by way of the low Young’s modulus of 
the untreated negative control. Both curcumin and K2S increased the Young’s moduli of the cells in a dose-
dependent manner. However, the histograms in Figure 8 show that K2S had a greater effect on the elasticity of 
the cells as shown by the higher value of the average Young’s modulus. In addition, treatment of HepG2 cells 
with curcumin and K2S resulted in an approximately 2.6- and 4.0-fold increase in Young’s modulus values of 
viable cells, respectively, at the higher concentrations. In all, treatment with either curcumin or K2S resulted in 
significant increases in cell stiffness (P < 0.001).

3.6 K2S affects ATP status of HepG2

Another biochemical parameter that we measured while investigating possible mechanisms of curcumin and K2S 

![Figure 8](image-url) **Figure 8** Histograms of atomic force microscopy nanomechanical assessment of the effects of treatment of HepG2 cells for 24 h with curcumin or extract (K2S)

An average of 20 cells was considered for each experiment with force measurements recorded at 1 Hz with a loading force and rate of 1 nN and 2.0 µN/s respectively. In all, treatment with either curcumin or K2S results in significant increase in cell stiffness (P < 0.001).
activity is the level of cellular ATP. The results here (Figure 9) correlate well with the GSH status; curcumin did not affect the ATP levels in cells, except at the highest two concentrations. However, K2S reduced ATP levels in all treatment concentrations, almost completely depleting the ATP levels at the highest concentration of treatment. As expected, at all concentrations, there was no significant change for the negative control.

3.7 Depletion in the antioxidant status with curcumin or K2S

Based on the above results and to correlate the observed effects of treatment of HepG2 cells with antioxidant status, we decided to determine the GSH levels in comparison with the GSSG levels (Figure 10); this is because depletion of GSH could precede the onset of apoptosis. We found out that curcumin did not significantly affect the levels of GSH as much as K2S did. Curcumin had a very small effect on GSH levels at the lower treatment concentrations, but had a greater effect at its highest concentration. However, K2S consistently increased GSSG levels in a concentration-associated trend from the lowest to the highest concentration, relative to the negative control.

3.8 Activities of caspase-3/7 are induced by curcumin and K2S

One of the hallmarks of apoptosis is caspase activation. To assess the relevance of apoptosis in the cell death induced by various concentrations of curcumin or K2S, we determined the overall activities of caspases-3 and -7. Figure 11 shows a two-phased pattern in the induction of caspase activities with both the lowest and highest concentrations displaying reduced activities.

3.9 Curcumin proves not being mitotoxic while K2S displays mitotoxicity

To have a better understanding of the mechanism of cell death induced by curcumin or K2S, we evaluated the mitotoxicity potentials of the two treatments. Based on the trends displayed in Figure 12, curcumin shows no significant change in either cytotoxicity or ATP levels, while K2S exhibited an almost-constant level of cytotoxicity accompanied by declining ATP levels after treatment for 24 h.
3.10 K2S mitigates against genetic transformation induced by A. tumefaciens

The genetic integrity of the A. tumefaciens (strain A348) that we used was verified using PCR-based 16S rRNA gene sequence analysis; its tumor-inducing capability was also confirmed through pilot assays. Categorically, this strain induced tumors on the carrot disks and thus we proceeded to assess the effect of treatment with K2S. At the lowest concentration of K2S treatment, the bacterium induced tumors that were morphologically different from those induced in the positive control group. At higher concentrations of K2S, as seen in Figure 13, there was no induction of tumors. Also, as expected, there was no tumor formation on the negative control disks.

![Figure 13 Carrot disk antitumor assay](image)

Figure 13 Carrot disk antitumor assay
Initial determination of tumor-inducing potential of A348 strain of A. tumefaciens (A). The whitish outgrowth confirms genetic transformation induced by the bacterium. Carrot disks inoculated with A348 strain of A. tumefaciens were cotreated with graded concentrations (µg/mL) of extract (K2S) (B). Twenty-three days after inoculation with the bacterium (C). Higher magnification of tumors induced on a positive control carrot disk (D) and a disk having the least concentration of treatment with extract (E). Marked disk in (A) indicates negative control. A, B and C with 4× magnification; D and E with 400× magnification.

4 Discussion

Hepatocellular carcinoma (HCC) was once reported to be the most common malignancy in Nigerian males. Approximately 35,660 new cases of liver cancer, including intrahepatic bile duct cancers, were expected to occur in the US during 2015, around 75% of which will be HCC.\[29,30\] The fatality of cancer is mostly dependent on the occurrence of metastasis, which itself is a product of several interconnected processes, including cell proliferation, cell adhesion, migration, invasion into the surrounding tissue and angiogenesis. The appearance of metastases in organs distant from the primary tumor is the most destructive feature of cancer.\[31\] Thus, if the development of metastasis can be contained through novel therapeutic strategies targeted against this process, then successful cancer treatment can be achieved. Plant derived compounds, such as Vinca alkaloids (vinblastine and vincristine), curcumin, camptothecin derivatives (topotecan and irinotecan), and derivatives of epipodophyllotoxin (etoposide and teniposide), have been clinically important as anticancer agents. Our main focus in this study was to assess the anticancer/antimetastatic potentials of treating HepG2 cells with curcumin in comparison with K2S. HepG2 cells are malignant cells of a well-differentiated HCC derived from a 15-year-old Caucasian male.\[32\]

By natural affinity, without any scientific basis, ancient Chinese and Indian systems of medicine have long incorporated turmeric into their regimens. Aside from being used for medicinal purposes, turmeric is also used as a spice and a pigment. Turmeric is derived from the rhizomes of Curcuma longa and its bright yellow color is mainly due to the presence of fat-soluble, polyphenolic pigments, known as curcuminoids, with curcumin being the most abundant curcuminoid and also considered to be the most active component in turmeric.\[33\] The various biological attributes associated with curcumin\[6–8\] make it a good candidate comparison to those of K2S, in order to confirm the presence of potent bioactive phytochemicals in the extract.

As a first step to have reliable and reproducible results in the subsequent assays, we determined the optimum concentration of DMSO and cell density. Our results showed that, within the cell culture conditions used, DMSO was not significantly cytotoxic to HepG2 cells as the percentage of dead cell did not exceed 5%. HepG2 cells were treated for 72 h to access the possible acute or subacute toxicity of K2S in comparison with curcumin. The toxicity profile, over the period of incubation, indicated that the cytotoxicity of K2S was initiated upon treatment; this was more consistent than curcumin, which showed a gradual increase in the number of compromised cells. From this, we inferred that the extract possessed a sustained acute toxicity on HepG2 cells, while curcumin was subacute at the concentrations of treatment. This may suggest that K2S activity was both necrotic and apoptotic, while curcumin was principally apoptotic in inducing cell death. These mechanisms of cell death were further validated by observations that the compromised cells, positively stained by PI, induced by K2S treatment, displayed a significantly higher nuclear area than those
in the negative control. It should be noted that one of the morphological features of necrosis is cell swelling, whereas apoptosis is associated with cell shrinkage and chromatin condensation.\cite{33}

Scientific observations over the years have clearly shown that there are factors that regulate nuclear size and influence the karyoplasmic ratio.\cite{21} It has also long been reported that the earliest cells to enter DNA synthesis (S phase) are those with the largest nuclei, whereas cells with the smallest nuclei are among the latest.\cite{33} This implies that nuclear size correlates with the maximum rate at which cells could enter S phase which is an indication of cell proliferation/growth. The NTA indicates the overall combination of the area occupied by the genetic materials of both dead and live cells. An increase in NTA denotes either an increase in cell proliferation/growth or the induction of necrosis based on its associated cell-swelling. A decrease in NTA means either inhibition of cell growth/proliferation or an apoptotic cell population. Treatment of HepG2 cells with DMSO resulted in an increase in NTA in a time-dependent manner due to cell proliferation/growth. The increase in NTA induced by curcumin may be due to its cell-growth inducing capability at lower concentrations as at 72 h; at higher concentrations, curcumin was growth-inhibitory/death-inducing. However, the results obtained from K2S treatment showed consistent inhibition of the genetic material synthesis, which suggests growth-inhibition or cell-death induction. The positive nuclei total area (PNTA) measures the nuclear/genetic size of dead cells. A decrease in PNTA denotes cell death induced principally by apoptosis, while an increase indicates necrosis. Considering the significant increase in PNTA induced by K2S, it may be inferred that the extract was highly necrotic to the cancerous liver cells. Curcumin was also necrotic at high concentrations. Lastly, the negative nuclei total area (NTTA) is a parameter that measures the genetic/nuclear size of live cells. An increase in NNTA implies cell growth/proliferation while a decrease in NNTA signifies growth inhibition. In this work, treatment with K2S resulted in a reduction in the nuclear size of PI-negative (viable) cells through the 72-hour incubation. Thus the rate of cell division (proliferation) was negatively affected. This may simply explain the basis for the antiproliferative activity of both curcumin and K2S, with K2S significantly inducing more reduction in NNTA than curcumin.

Cell death via the apoptotic pathway involves the disintegration of the cytoskeleton, which should result in reduced cell stiffness. Moreover, research efforts have already proven the relevance of robust cytoskeletal structures to increased cell stiffness values.\cite{321} It can therefore be confirmed that the cells considered for Young’s modulus (elasticity) measurements in this work were not apoptotic cells, but rather viable cells that have undergone cytoskeletal remodeling, resulting in the observed increase in cell stiffness. The nanomechanical stiffness (Young’s modulus) results from live cells that we obtained confirm with previous reports from our laboratory demonstrating that metastatic cancer cells have low Young’s modulus values with small standard deviations.\cite{25,30} Our AFM cell elasticity measurements further conform with earlier reports on apparent stiffness of mammalian cells, which typically range between 1 and 100 kPa; both curcumin and K2S increased the cell stiffness values of HepG2 cells.\cite{37–39} Moreover, studies involving the use of actin depolymerizers have clearly demonstrated drastic reductions in cell stiffness, but no reductions in cell stiffness with depolymerizers of microtubules.\cite{40–44} From the biochemical point of view, the mechanical integrity of a cell is established via the dynamic interplay of networks of structural, cross-linking and signalling molecules. Hence, changes in these networks directly affect the mechanical properties of cells. The identification and characterization of healthy and diseased cells through cell stiffness measurements are becoming increasingly common, possibly because of its reliability. Basically, as an alternative to identification through changes in morphology, measurement of cell stiffness has been proposed as a candidate for cancer cell detection, since cancerous cells are considerably softer than healthy cells. In addition, literature review has demonstrated that under a change in external conditions, the elasticity of a cell membrane changes more dramatically than the morphology of the cell. Besides, the softness associated with cancer cells has been linked to the deformability of the cytoskeleton, which plays a vital role in metastasis.\cite{45–50}

Under physiological conditions, a seesaw imbalance in the levels of oxidants and reductants with a tendency toward oxidants results in oxidative stress, but this situation has been countered through various antioxidant defense systems. One of the main regulatory components of these systems is glutathione. GSH, a redox-active tripeptide, is an endogenous reducing agent that is found in abundance and throughout the cell.\cite{33} A drop in GSH levels, which correspondingly leads to an increase in GSSG levels, also results in an increase in the levels of reactive oxygen species (ROS). However, raised ROS levels have been reported to precede the induction of the apoptotic process.\cite{52} Our results corroborated earlier reports on treatment of HepG2 cells with an effective, but low concentrations of curcumin. Curcumin preserved the GSH levels in HepG2 cells and even slightly increased the GSH levels above the levels in the negative control, at the lowest concentration applied in this work.\cite{35,34} Curcumin, thus, did not entirely induce oxidative stress in HepG2
cells within the concentrations considered; this is in sharp contrast to the activity of K2S. Among other factors, induction of oxidative stress through the depletion of antioxidant status seems to account for the sustained acute toxicity induced in HepG2 cells by K2S.

Considering the indispensability of ATP in living cells, the results we obtained showed that curcumin treatment did not affect ATP status in the HCC cells except at necrotic concentrations. As for the HepG2 cells treated with K2S, ATP depletion seemed to have played a crucial role in compromising the viability of the cells. Our results demonstrated that both ATP and GSH levels exhibited the same trends as a result of treatments, and this validated the fact that synthesis of GSH is dependent on the availability of ATP.\footnote{55}

The cytoskeleton provides structural support to eukaryotic cells.\footnote{56} It has long been established that actin, in particular, responds to external forces through deformation and rearrangement.\footnote{57,58} Polymerization of the cytoskeletal components, microtubules and microfilaments (actins), involves guanosine triphosphate (GTP) and ATP respectively, which are hydrolyzed when these proteins polymerize. Globular actin, which is the most profuse cellular protein, polymerizes to form filamentous actin by binding to ATP.\footnote{59} Hence, the significant decrease in ATP levels, which correspond with a significant increase in cell stiffness, would likely be due to the utilization of ATP molecules during actin polymerization. It can therefore be inferred that nucleotide hydrolysis is the price the cells must pay to allow rapid remodelling of these cytoskeletal elements. However, it should be noted that ATP and GTP are energetically equivalent, as ATP can easily be converted to GTP in a reaction catalyzed by nucleoside diphosphate kinase.\footnote{60} This implies that cytoskeletal remodelling, among other factors, such as biosynthesis of GSH, may account for the decrease in ATP levels especially as a result of treatment with K2S. It should also be noted that the cells considered for AFM elasticity measurements were viable and nonoxidatively stressed, as evident from the GSH levels obtained at the concentrations of test substances.

Meanwhile, associated with apoptotic mode of cell death is the activation of executioner/effect caspases. Apoptosis is an energy-dependent process and a decrease in cells with ATP below critical levels, which prevents the execution of apoptosis and promotes cell death via necrosis. When the ATP levels are high, apoptosis can occur as there is sufficient amount of energy to activate the cascade of events associated with caspases.\footnote{61} The data we obtained here indicate a two-phased pattern, which is principally due to sustained apoptosis until the threshold concentrations are reached, at which point the depletion in ATP levels is significant. Concentrations of either curcumin or K2S beyond the threshold concentration result in necrosis as the principal mode of cell death and therefore, the levels of caspases dropped remarkably.

On the mechanistic approach, curcumin displayed the trend of a nonmitochondrial toxin, whereas K2S exhibited the traits of a mitochondrial toxin. Among other factors, this confirmed that the cell death induced by curcumin is not essentially due to cellular energy depletion as it is for K2S.\footnote{62}

To further delve into the anticancer activity of \textit{K. senegalensis}, we assessed it from the microbiological point of view. Tumor induction in plants by \textit{A. tumefaciens} is mediated by the presence of a large (200 kbp in length) tumor-inducing (Ti) plasmid or pTi and the initiation of a conjugation tube by the bacterium linking it to a plant cell.\footnote{63,64} Upon bacterial infection of a wounded plant, the defense response from the infected plant, in form of phenolic chemicals, leads to the activation of virulence genes on the Ti-plasmid. The aftermath of this stimulates the processing, transfer and ultimate integration of a segmental portion of the Ti-plasmid into a semirandom location in the genome of the plant cell. This segment, which is about 20–23 kbp in length, is designated as transferred DNA (T-DNA). The Ti plasmid also contains all the genes necessary to transfer T-DNA to the plant cell, but many strains of \textit{A. tumefaciens} do not contain a pTi; hence the necessity for us is to initially validate the tumor-inducing capability of the A348 strain, having already confirmed the presence of 16S rRNA. The mechanisms by which \textit{A. tumefaciens} inserts materials into plant cells by type IV secretion system (T4SS) are very similar to the mechanisms used by animal pathogens to insert materials (usually proteins) into human cells.\footnote{65,66} As a typical example, the induction of gastric carcinogenesis involves the delivery of CagA into gastric epithelial cells, which is mediated through T4SS by \textit{Helicobacter pylori}. Similarly, \textit{Bordetella pertussis}, which causes whooping cough, secretes the pertussis toxin partly through the type IV system, just like \textit{Legionella pneumophila}, the causative agent of Legionellosis (Legionnaires’ disease), which utilizes a type IVB secretion system to translocate numerous effector proteins into its eukaryotic host.\footnote{67,68} Therefore, the K2S’s attenuation of the genetic transformation that normally leads to tumor induction, in this work, further reinforced its anticancer property and it may be an indication of the potential of K2S to prevent diseases caused by the aforementioned microbes in humans.

\section*{5 Conclusions}

In conclusion, the results we obtained in this work have shown that curcumin possesses antiproliferative
activity. Our results demonstrate, for the first time, the antiproliferative activity of a hydroethanol extract of *K. senegalensis* on a metastatic cell line derived from human liver hepatoma. Also, our cell elasticity measurement data, using AFM, strongly suggest, for the first time, that both curcumin and K2S exhibit antimetastatic properties. The pro-oxidant activity of the extract in liver cancer cells, as shown here, warrants further research using normal liver cells.

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