Annona muricata silver nanoparticles exhibit strong anticancer activities against cervical and prostate adenocarcinomas through regulation of *CASP9* and the *CXCL1/CXCR2* genes axis

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Abstract.
BACKGROUND: Green synthesized nanoparticles have been earmarked for use in nanomedicine including for the development of better anticancer drugs.
OBJECTIVE: The aim of this study was to undertake biochemical evaluation of anticancer activities of green synthesized silver nanoparticles (AgNPs) from ethanolic extracts of fruits (AgNPs-F) and leaves (AgNPs-L) of *Annona muricata*.
METHODS: Previously synthesized silver nanoparticles were used for the study. The effects of the AgNPs and 5-Fluorouracil were studied on PC3, HeLa and PNT1A cells. The resazurin, migration and colonogenic assays as well as qRT-PCR were employed.
RESULTS: The AgNPs-F displayed significant antiproliferative effects against HeLa cells with an IC_{50} of 38.58 μg/ml and PC3 cells with an IC_{50} of 48.17 μg/ml but selectively spared normal PNT1A cells (selectivity index of 7.8), in comparison with first line drug 5FU and AgNPs-L whose selectivity index were 3.56 and 2.26 respectively. The migration assay revealed potential inhibition of the metastatic activity of the cells by the AgNPs-F while the colonogenic assay indicated the permanent effect of the AgNPs-F on the cancer cells yet being reversible on the normal cells in contrast with 5FU and AgNPs-L. *CASP9* was significantly over expressed in all HeLa cells treated with the AgNPs-F (1.53-fold), AgNPs-L (1.52-fold) and 5FU (4.30-fold). *CXCL1* was under expressed in HeLa cells treated with AgNPs-F (0.69-fold) and AgNPs-L (0.58-fold) and over expressed in cells treated with 5FU (4.95-fold), but the difference was not statistically significant. *CXCR2* was significantly

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over expressed in HeLa cells treated with 5FU (8.66-fold) and AgNPs-F (1.12-fold) but under expressed in cells treated with AgNPs-L (0.76-fold).

CONCLUSIONS: Here we show that biosynthesized AgNPs especially AgNPs-F can be used in the development of novel and better anticancer drugs. The mechanism of action of the AgNPs involves activation of the intrinsic apoptosis pathway through upregulation of *CASP9* and concerted down regulation of the *CXCL1/ CXCR2* gene axis.

Keywords: Silver nanoparticles, HeLa, PC3, PNT1A, *CASP9*, *CXCL1/CXCR2*

Abbreviations

AgNPs	Silver Nanoparticles
AgNPs-F	Silver Nanoparticles from fruits of *Annona muricata*
AgNPs-L	Silver Nanoparticles from leaves of *Annona muricata*
A570	Absorbance at 570 nm
A600	Absorbance at 600 nm
5FU	5-Fluorouracil
CC_{50}	Cytotoxic concentration required to kill 50% of the normal cells
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl Sulfoxide
FCS	Fetal Calf Serum
IC_{50}	Inhibitory concentration required to kill 50% of the cancer cells
RPMI	Roswell Park Memorial Institute
SD	Standard Deviation

1. Introduction

Cancer is reported to rank second among the top causes of death worldwide and was responsible for 8.8 million deaths in 2015, a figure expected to rise if no immediate interventions are put in place [1]. Cervical cancer remains the top cause of morbidity and mortality in women diagnosed with cancer in Eastern Africa while prostate cancer is the most prevalent cancer type in men within the region [2, 3]. Currently, there is a limited choice of treatments for these cancers [2–6], and therefore the need to continue searching for more effective treatment regimens.

Among the hallmarks of human cancers is the intrinsic or acquired resistance to apoptosis. Cancer cells resistance to apoptosis is therefore a critical rationale behind treatment failure [7, 8]. *CASP9* gene encodes Caspase 9 enzyme which can undergo autoproteolytic digestion and activation by the apoptosome activating factor 1 which is critical in the earliest steps in the caspase activation cascade of the intrinsic pathway [9, 10].

Chemokines are a group of low-molecular-weight chemotactic cytokines that participate in various cellular processes such as embryogenesis, angiogenic activity, leukocyte migration and tumor growth and metastasis [11–13]. *CXCLs/CXCR2* signaling is important in both cancer and different inflammatory diseases. Because the increased expression of CXCR2 and neutrophil/monocyte migration is critical in chronic inflammation and cancer, the need to focus on using CXCR2 antagonists in cancer and related diseases drugs development remains eminent [12, 13]. *CXCL1* gene as well as its receptor *CXCR2* gene have been found to be highly associated with tumorigenesis, angiogenesis, and metastasis [11, 12].

In recent times, traditional medicine has taken an important place especially in developing countries where limited health services are available [14, 15]. *Annona muricata* of the Annonaceae family is

one plant that has a myriad of reported medicinal values and therapeutic potential [15–24]. *Annona muricata* is known as Soursop (English), Graviola (Portuguese), Guanábana (Latin American Spanish), Omusitafeli / Ekitafeli (Uganda), and other local indigenous names as has been enlisted [25, 26]. Various medicinal uses have been reported across the globe ranging from the use of leaves, bark, roots, fruits and seeds of *Annona muricata* [15, 16, 27–36, 17, 37–43, 18–24]. Despite the wide spread use of herbal products by the majority of sick people, it has been reported that most of the biologically active constituents of plant extracts, such as flavonoids, tannins, and terpenoids, are highly soluble in water, but have low absorption, because they are unable to cross the lipid membranes of the cells, have excessively high molecular size, or are poorly absorbed, resulting in loss of bioavailability and efficacy. Therefore some extracts despite having very good activity *in vitro* which are not reproducible *in vivo,* are not used clinically because of these obstacles [44]. It has therefore been widely proposed to combine herbal medicine with nanotechnology, because nanosystems can deliver the bioactive components at a sufficient concentration during the entire treatment period, directing them to the desired sites of action, and hence potentiating the action of the compounds, an aspect that conventional herbal treatments do not meet [44, 45]. To improve efficacy and bioavailability of plant derived medicinal products, green nanobiotechnology has been widely advocated for because of the various beneficial properties of nanosystems in delivery of bioactive components to target sites which conventional herbal treatments cannot meet [44, 45]. In line with the above, this study employed previously green synthesized AgNPs from ethanolic extracts of fruits and leaves of *Annona muricata* [46–48] to study their anticancer activities on different cancer cell lines. It specifically involved exploring the *in vitro* anticancer activities of the AgNPs as well as studying their effects on expression of Caspase 9 (*CASP9*), C-X-C motif chemokine ligand 1(*CXCL1*) and C-X-C motif chemokine receptor 2 (*CXCR2*) genes in cervical and prostate cancer cell lines.

2. Materials and methods

2.1. Chemicals and reagents

RPMI1640, DMEM, Non-Essential Amino Acids, Penicillin/Streptomycin, Resazurin, and Trypsin-EDTA were procured from Solarbio (China Phosphate buffered saline (PBS), 5FU, FCS, and Dimethyl Sulfoxide (DMSO) were procured from Sigma Aldrich (Germany).

2.2. Silver nanoparticles

Previously prepared and characterized AgNPs from ethanolic extracts of fruits (AgNPs-F) and leaves (AgNPs-L) of *Annona muricata* were used for the study [46–48]. AgNPs-F used had an absorption maximum at 427 nm and were stable under different pH, Temperature and storage conditions. The AgNPs-F had an average crystalline size of 60.12 nm, a polydispersity index of 0.1235 and were spherical in nature. The functional groups responsible for the formation of the AgNPs included; Alkanes and alkyls, aldehydes and esters, nitro groups, alcohol groups, amines, amides, alkenes, acids and alkyl halides [46, 47]. On the other hand, AgNPs-L used had an absorption maximum at 429 nm and were stable under different pH, Temperature and storage conditions. The AgNPs-L had an average crystalline size of 87.36 nm, a polydispersity index of 0.16 and were spherical in nature. The functional groups responsible for the formation of the AgNPs included; Alkanes and alkyls, aldehydes and esters, nitro groups, alcohol groups, amines, amides, alkenes, acids and alkyl halides [47].

2.3. Cell culture

Three cell lines PC3, HeLa and PNT1A were used in the study. The PC-3 and HeLa cells were Prostate and Cervical adenocarcinomas respectively, while PNT1A cells were normal immortalized prostate cells. The cells were a gift from Dr Tim Forshew, University College London, UK.

Cells were grown in appropriate media containing L-Glutamine and supplemented with 1% Penicillin/Streptomycin, 1% Non-essential amino acids and 10% fetal calf serum (FCS). PC3 and PNT1A cells were propagated in RPMI 1640 while HeLa cells were propagated in DMEM. The cells were kept in a humified Carbon dioxide incubator (5% CO_2 and 95% humidity) at 37°C. Cells were passaged and used for the assays during the exponential phase at about 60–75% confluence.

2.4. Effects on cell viability using the resazurin assay

The effects of the AgNPs on the viability of the cells was determined using the Resazurin assay as previously described [49–52]. Exponentially growing cells were harvested, washed, counted using a haemocytometer and seeded in 96 well plates containing 0.5×10^4 Cells/well and incubated with 100 uL per well culture media and allowed to attach overnight. Seeding media was then removed. The attached cultured cells were then treated by adding of 100 μL of the treatments at concentrations of 200, 100, 50, 25, and 12.5 μg/mL (in culture media). DMSO alone in media was used as the solvent control blank (DMSO = 0.5%v/v). 5-FU was used as a reference positive control drug for cancer. The treated cells were then incubated in a humified CO_2 incubator at 37°C. 24 Hours later, 20 μl of 0.15 mg/ml in PBS resazurin solution was added to each of the wells and then incubated for 4 more hours.

The plates were then removed from the incubator and the absorbance signal was quickly measured at 570/600 nm (excitation/emission wavelengths), using a microplate reader (Infinite M1000, Tecan). Viable cells change the resazurin from blue (oxidized) to red (reduced) forms. The percentage cell viability was determined using the formula: % Viability = (Net absorbance of treated samples/Net absorbance of blank) × 100. The effect of the samples on the growth of the cells were then expressed in form of graphs of percentage cell viability against logarithm of concentration. Fifty percent of inhibitory concentration (IC_{50}) of each of the treatments were calculated from the growth inhibition curves. Selectivity index for each of the treatments was calculated.

All subsequent studies were undertaken on only HeLa and PNT1A cells, representing the cancerous and normal cell types respectively.

2.5. Cell migration assay

The effect of the AgNPs on cell migration were analyzed by use of the 2-dimensional cell culture wound closure assay as previously described [53–55]. Cells in the logarithmic phase of growth were harvested and about 5.0×10^4 cells/well were plated in 12 well plates and appropriate media added, with routine monitoring and change until the cells reached 95–100% confluence. In a sterile environment, a 100 μl pipette tip was used to press firmly against the top of the tissue culture plate and swiftly made a vertical wound down through the cell monolayer in each well. The media and cell debris were then carefully aspirated. 2 ml of culture media containing the different treatments at their predetermined IC_{50} values (AgNPsF-IC_{50}, AgNPsF-IC_{50}, 5FU- IC_{50}, and Blank – vehicle in media,) were then slowly added against the well walls, one column per treatment. For PNT1A cells, the treatments were at their predetermined CC_{50}.

Following the generation and inspection of the wound, as well as addition of the treatments, initial pictures were taken (Time 0) using an inverted microscope. The cell culture plates were then incubated. At several time points (6, 24, and 48 Hrs), the plates were removed from the incubator and placed

under an inverted microscope to take snapshot pictures and to check for wound closure. Wound closure was then analyzed and clearly presented as time series image captions against time. The treatments had different effects on the cell lines wound closure ability thus their effect on migration, metastasis and generally the regulation of cell motility.

2.6. The colony formation assay

A colonogenic assay was conducted to determine the ability of the cells to proliferate indefinitely and form colonies (effect on the replicative potential) upon drug removal [56, 57]. Cells in the logarithmic growth phase were harvested and seeded at 100-fold dilutions (1000 cells/well) from the normal plating density (1X 10^5 cells/well) in 6 well plates, allowed to attach and then treated with appropriate volumes of the AgNPs and controls at their predetermined IC_{50} values and incubated at $37°C$ in the growth chamber for 24 h. For PNT1A cells, the treatments were at their predetermined CC_{50} values.

The cells in each well were then washed with PBS 3 times (to remove the treatments) and replaced with regular culture medium. The cells were then incubated, with routine media replenishment until colonies had fully recovered and formed in the wells with the blank. At the end of the experiment, surviving colonies were stained with 0.4% crystal violet (Sigma) in 50% methanol, and the number of colonies (each consisting of at least 50 cells) were then counted in an inverted microscope [56, 57]. Results were presented as mean number of colonies per treatment. At least 3 replicates per treatment were done.

2.7. RNA extraction

Exponentially growing cells at a confluence of about 70% in T75 flasks were exposed to the IC_{50} of the different treatments for 24 hours. Total RNA was extracted from each of the samples using the DirectZol kit (Zymo Research, USA) according to manufacturer's instructions. Briefly, cells were prepared in lysis buffer, and then purified by directly adding them to the Zymo-Spin™ II Column. This was spun and washed after which the RNA was eluted. The purity, quality, quantity and integrity of the extracted RNA was determined by resolution using a 1% agarose gel electrophoresis and nanodrop spectrophotometry (Nanodrop 2000C Spectrophotometer – Thermo Scientific).

2.8. cDNA synthesis

Approximately 100 ng/μl of RNA was used for cDNA synthesis using FIREScript RT cDNA synthesis kit (Solis BioDyne, Estonia) according to manufacturer's instructions. A 20 μl reaction volume contained 10 μl of template RNA (100 ng/ μl), 1 μl oligo (dT) primer (100 μM), 0.5 μl dNTP mix (20 mM), 2 μl 10 × RT reaction buffer with DTT, 1 μl FIREScript RT, 0.5 μl RNase inhibitor (40 U/μl) and 5 μl nuclease free water. Using SimpliAmp™ thermal cycler (Applied Biosystems, USA), the reverse transcription process was carried out at $50°C$ for 60 minutes followed by enzyme inactivation at $85°C$ for 5 minutes. The synthesized cDNA was assessed for quality and quantity using a nanodrop (Nanodrop 2000C Spectrophotometer – Thermo Scientific).

2.9. Primers design for genes of interest

Genes assayed included *CASP9, CXCL1* and *CXCR2*. *GAPDH* was used as a house keeper to set baseline. Primers for use for qPCR were designed using NCBI's Primer Blast tool. The reference sequence for each of the Homo sapiens gene were retrieved from GenBank and the FASTA sequences format used to design the primers. The primers were set to have a PCR product in the size range between

Table 1
Primers for qRT-PCR

Gene		Sequence	Annealing Temp. / $^\circ$ C	PCR product size / bp	NCBI Ref Seq.
GAPDH	F	GCTCCCACCTTTCTCATCCA	61	139	NC_000012.12
	R	TACTCCCCACATCACCCCTCTA			
CASP9	F	TAAGCAGGAGATGAACAAAGGAAGA	61	172	NC_000001.11
	R	GAAATGGGGGAGACAAGGTGAGA			
CXCLI	F	CAGTAGGACAAACAGCAACAGGT	61	90	NC_000004.12
	R	TCTTTTAGGAATGGGGGTGGGG			
CXCR2	F	GCCACTCCAATAACAGCAGGT	61	131	NC_000002.12
	R	GCTTCTACACTTCATCACCCC			

75 – 250 bps, % GC content of between 40 – 55%, as well as self-complementarity of not more than 2. The selected primer sequences for each of the genes were run on dry PCR using the Sequence Manipulation Suite Bioinformatics tools (SMS Bioinformatics) in order to confirm the products size and the sequences of the PCR products.

The studied genes, their corresponding primer sets, annealing temperatures the expected PCR product sizes as well as are as their NCBI GenBank Reference sequences are outlined in Table 1. The primers were manufactured by Macrogen Inc. South Korea.

2.10. qRT-PCR analysis and relative gene expression

Quantitative real-time polymerase chain reaction was used to assess the effect of the treatments on the expression of the selected genes. The qRT-PCR reaction mix were set up with EvaGreen® qPCR Mix (Solis-BioDyne, Estonia) according to manufacturer's protocol. A 20 μl reaction comprised of 4 μl of the Master Mix, 0.5 μl of each of forward and reverse primer set (10 μM), 5 μl cDNA (50 ng/ μl) and 10 μl nuclease-free water. Each reaction was conducted in triplicates. The qRT-PCR was carried out in a Light Cycler® 96 instrument (Roche Diagnostics GmbH, Germany) with the following cycling program: 95°C for 720 s followed by 50 cycles of 95°C for 15 s, 61°C (annealing) for 20 seconds and 72°C for 20 seconds. The above program was used for all the primers.

The threshold cycle (Ct) was calculated. The melting curve was examined for each reaction to confirm that only desired PCR products were amplified and to rule out possibility of primer-dimers contributing to the amplification signal. To confirm that only one single PCR amplicon of expected product size was produced, a 4 % agarose gel electrophoresis was run.

Relative quantification of specific mRNA levels was performed using the comparative $2^{-\Delta\Delta Ct}$ method [58]. *GAPDH* was used as the reference gene against which the expression values were normalized. The expression levels were presented as n-fold differences relative to the controls/ calibrator.

2.11. Statistical analysis

The data points represent the average of at least 3 independent experiments. The data was presented using Microsoft Office, Excel software and expressed as mean $\pm$ SD. Statistical package for social scientists, IBM version 21 was used by employing the ANOVA technique to observe the significance between the groups. All analyses were conducted at $p < 0.05$.

Table 2

Summary of IC_{50} values of the different treatments corresponding to the cell lines used

Cell line	IC_{50} (µg/ml) / CC_{50} (µg/ml)		
	AgNPs-F	AgNPs-L	5FU
HeLa	38.58	57.63	10.38
PC3	48.17	47.58	235.9
PNT1A	375.68	112.29	840.37

Table 3

Selectivity index of the AgNPs and 5FU

Cell line	IC_{50} (µg/ml) / CC_{50} (µg/ml)		
	AgNPs-F	AgNPs-L	5FU
PC3	48.17	47.68	235.9
PNT1A	375.68	112.29	840.37
Selectivity Index (SI) = (CC_{50} / IC_{50})	7.80	2.36	3.56

CC_{50} – Cytotoxic concentration required to kill 50% of the normal cells; and IC_{50} – Inhibitory concentration required to kill 50% of the cancer cells.

2.12. Ethics approval

The study was cleared by the PAUSTI board of examiners, registration number MB400-0007/17, Uganda National Council for Science and Technology (NS 43ES) as well as the Jomo Kenyatta University of Agriculture and Technology Institutional Ethics Review Committee (Ref. no: JKU/2/4/896B).
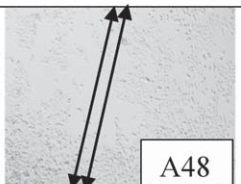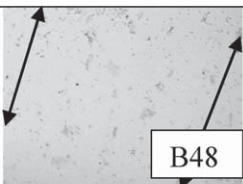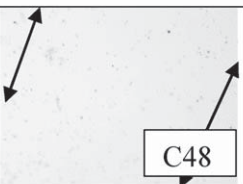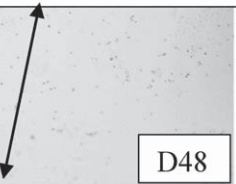
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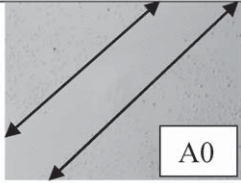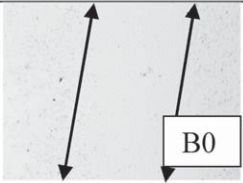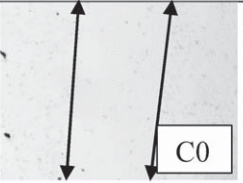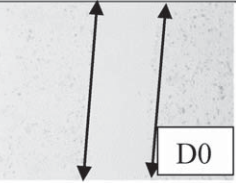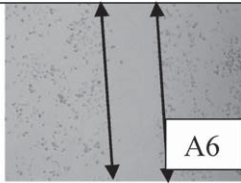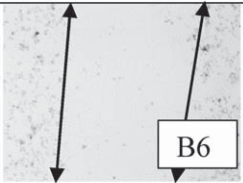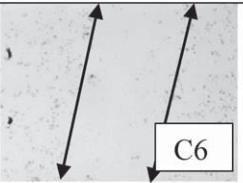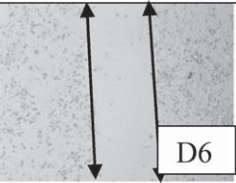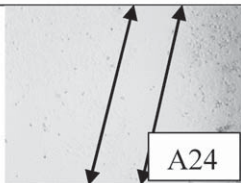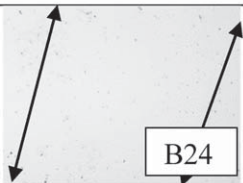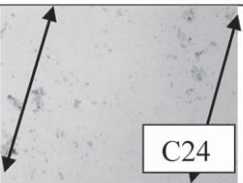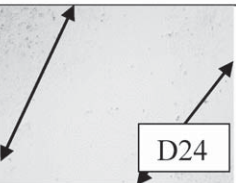
3.1. Resazurin metabolic assay cytotoxicity results

There was strong registered antiproliferative activity of the tested drugs on the different cell lines as shown in Table 2. Graphs for computations of the IC_{50}s and CC_{50}s are available as supplementary material. Additionally, it is evident that there was some cytotoxic activity of all the AgNPs tested and the 5FU on the normal cells. The AgNPs-L had the highest cytotoxic activity compared to the AgNPs-F and 5FU. In contrast, AgNPs-F and the standard anticancer drug 5FU had a lower cytotoxic activity on the normal cells, implying their potential high selectivity for cancer cells compared to normal cells. The AgNPs-L may not be very selective, but this has to be confirmed from the selectivity index studies. It is very critical to note that selectivity is a very key factor in cancer drugs development.

3.2. Selectivity index of the AgNPs

From the results in Table 3, it was revealed that AgNPs-F had the highest selectivity index of 7.8. AgNPs-L and 5FU had very low selectivity index, and thus their higher toxicity to non-target cells.

Table 4

Table showing the wound healing activity on HeLa cell line

3.3. Migration assay

The arrows denote boundaries of the wound regions at both ends of the monolayer with the gap in between the arrows indicating the wound. A0, A6, A24, and A48 in Table 4 represent the effects of the blank on the wound healing of HeLa cells. As shown, there was a significant increase in wound closure with time. Within 48 hours from the start of the experiment, almost the entire wound had been closed and a monolayer generally re-established. B0, B6, B24 and B48 represent the effects of the AgNPs-F on the wound closure. There was no closure of the wound observed throughout the treatment period. on the contrary, the wound/zone of clearance increased in size probably due to the effect on the treatment on the cells over time. This must have been as a result of the dying of the cells coupled with the inhibition of the pathway for production of proteins responsible for the adhesion as well as motility.

Similarly, the effects of AgNPs-L (represented by images C0, C6, C24 and C48) as well as that of 5FU (represented by images D0, D6, D24, and D48), inhibited the wound closure process and initiated

increased cell clearance and loss of adherence and motility as explained for AgNPs-F above. This loss of ability to adhere and migrate as well as increased cell death is of great importance as it helps interfere with the process of metastasis in the cancerous cells and thus preventing further spread of the tumors.

The arrows denote boundaries of the wound regions at both ends of the monolayer, with the gap in between the arrows indicating the wound. P0, P6, P24, and P48 in Table 5 represent the effects of the blank on the wound healing of PNT1A normal cells at the CC_{50} values. As shown, there was an initiation in wound closure with time as shown by the cells' movement towards the centre of the wound. Within 48 hours from the start of the experiment, there was a greater advance towards the re-establishment of monolayer, though the speed was not as fast. Probably due to the slow growth properties of the cells. On the other hand, Q0, Q6, Q24 and Q48 represent the effects of the AgNPs-F on the wound closure. There was no closure of the wound observed throughout the treatment period. on the contrary, the wound/zone of clearance remained static probably due to the effect on the treatment on the cells over time. There was no wound closure as well as no increase in the zone of clearance. This must have been as a result the inhibition of the pathway for production of proteins responsible for the adhesion as well as motility. This is an indication that even at CC_{50} values, the AgNPs-F have a lower cytotoxic effect on normal cells, as evidenced by the maintenance of the monolayer at uncleared zones.
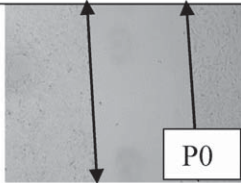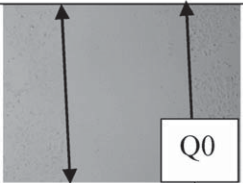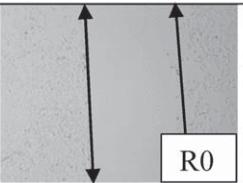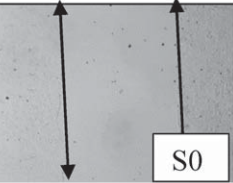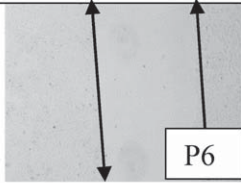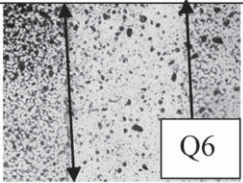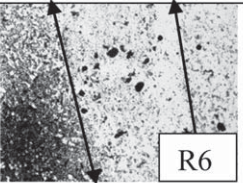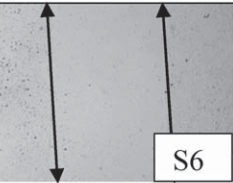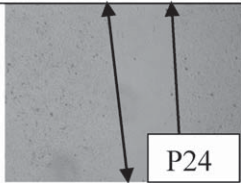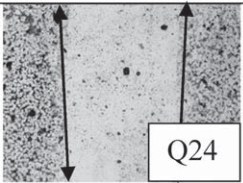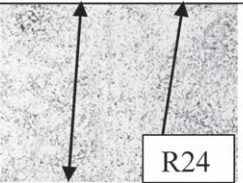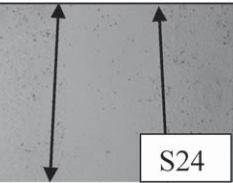
Conversely, the effects of AgNPs-L (represented by images R0, R6, R24 and R48) showed no wound closure coupled with an increase in the size of the zone of clearance. This could be due to the double effect of the toxic nature of the particles on the cells as well as their effects on the inhibition of the pathways responsible for the production of proteins critical in motility. The effects of 5FU (represented by images S0, S6, S24, and S48), show that they inhibited the wound closure process and initiated increased cell clearance and loss of adherence and motility as explained for AgNPs-L above. This loss of ability to adhere and migrate as well as increased cell death is of great importance as it helps shows that the 5FU and AgNPs-L could have adverse non-selective effects on the normal cells in the body, even if they are highly cytotoxic to the cancer cells. Selective cytotoxicity is very key in cancer drugs development.

3.4. Colony formation assay

Figure 1 shows the results for colony formation and survival assay. It is evident that the AgNPs-F had the highest effect on the colony survival of the HeLa cells at 0% recovery post treatment. These were closely followed by 5FU (0.5%) and AgNPs-L (4.27%). Similarly, the treatments which had the lowest effect on the PNT1A normal cells were the AgNPs-F at 40.81% recovery. The 5FU and AgNPs-L were highly toxic to the normal cells as implied by their very low recovery rates of 20.66% and 6.81% respectively. These resul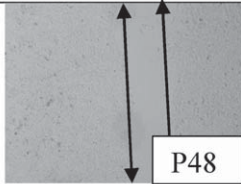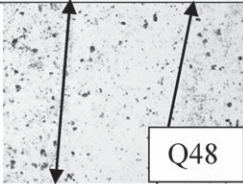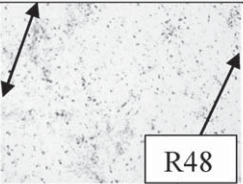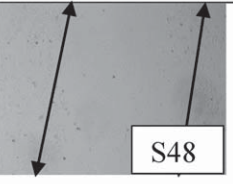ts are further in line with the earlier observation in relation to the selectivity index of the treatments where AgNPs-F (7.8) had the highest selectivity index followed by 5FU (3.56) and AgNPs-L (2.36) respectively.

Figure 2 shows the relative gene expression in HeLa cells in response to the different treatments. Letters on top of each box indicate mean separations according to Tukey's HSD test ($\leq$0.05). Means followed by the same letter are not significantly different. At $P = 0.03$, *CASP9* was significantly up regulated in HeLa cells subjected to all the 3 treatments of AgNPs-F (1.53-fold), AgNPs-L (1.52-fold) and 5FU (4.30-fold). *CXCL1* was down regulated in both AgNPs-F (0.69-fold) and AgNPs-L (0.58-fold) treated HeLa cells and up regulated in 5FU (4.95-fold) treated cells. The up regulation and down regulation was however not statistically significant at $P = 0.130$. At $P < 0.001$, *CXCR2* was significantly down regulated in AgNPs-L (0.64-fold) treated HeLa cells and significantly up regulated in both AgNPs-F (1.12-fold) and 5FU (8.66-fold) treated cells. The up regulation in AgNPs-F and

Table 5
Table showing the wound healing activity on PNT1A cell line at the CC_{50} values

Time (Hrs)	Blank	AgNPs-F	AgNPs-L	5FU
0	P0	Q0	R0	S0
6	P6	Q6	R6	S6
24	P24	Q24	R24	S24
48	P48	Q48	R48	S48

Treatments	Blank	AgNPs-F	AgNPs-L	5FU
HeLa cells	100	0	0.04	0.01
PNT1A cells	100	40.81	0.07	0.21
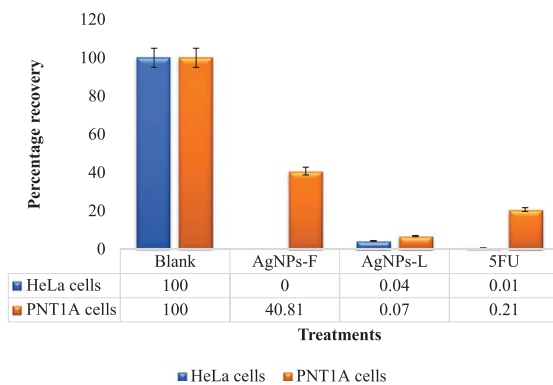
Fig. 1. Comparative colony formation assay results.
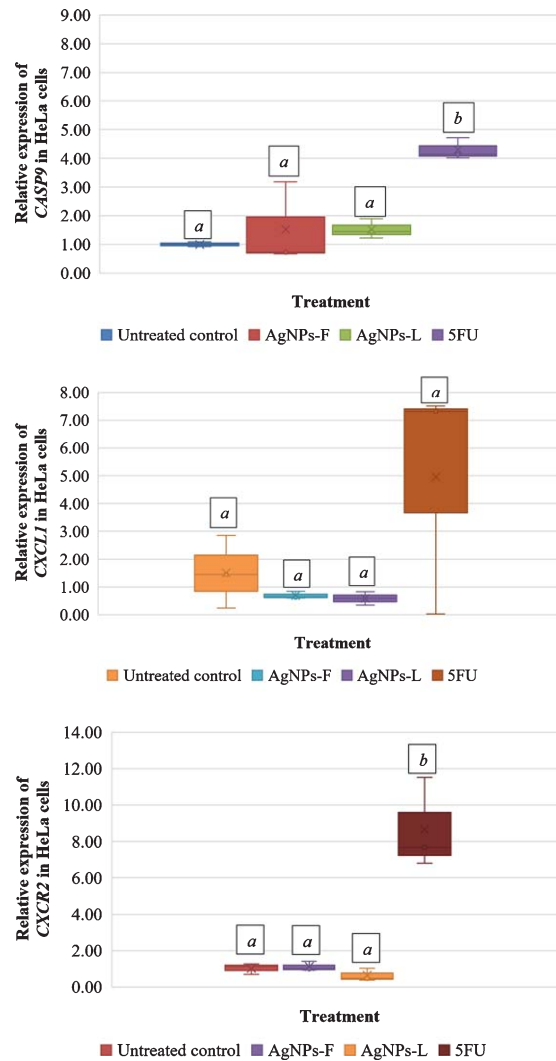
Fig. 2. Relative gene expression in HeLa cells in response to the different treatments.

down regulation of AgNPs-L treated cells was not statistically significantly different from the control. The up regulation of *CXCR2* in 5FU treated cells was significantly different from the control.

Figure 3 shows the relative gene expression in PNT1A cells exposed to different treatments. Letters on top of each box indicate mean separations according to Tukey's HSD test (≤ 0.05). Means followed by the same letter are not significantly different. At $P < 0.001$, exposure of the cells to AgNPs-F, AgNPs-L and 5FU resulted into a significant up regulation of *CASP9* at 1.37-fold, 16.78-fold and 1.36-fold respectively. However, the differences in the expression of *CAPS9* in cells exposed to AgNPs-F and 5FU was not significant in relation to the control. Expressions in AgNPs-L exposed cells was significantly different from the control.

At $P < 0.001$, *CXCL1* was significantly up regulated in PNT1A cells for all treatments of AgNPs-F (7.17-fold), AgNPs-L (85.96-fold) and 5FU (6.25-fold). There was an unusually very high up regulation of *CXCR2* in cells exposed to AgNPs-L. At $P < 0.001$, *CXCR2* was significantly down regulated in PNT1A cells exposed to both AgNPs-F (0.66-fold) and 5FU (0.54-fold), but was significantly up regulated in cells exposed to AgNPs-L (20.53-fold) just as was observed with *CXCL1*. There was no
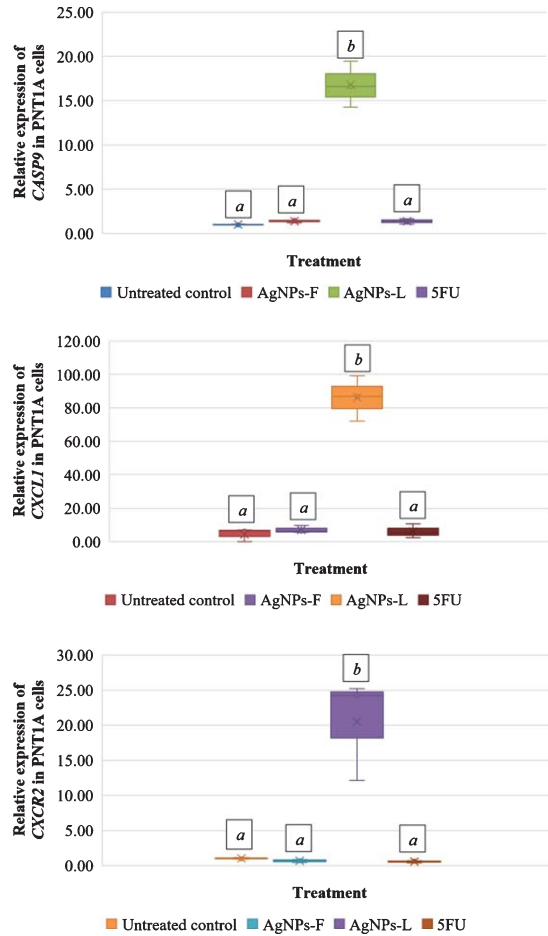
Fig. 3. Relative gene expression in PNT1A cells exposed to different treatments.
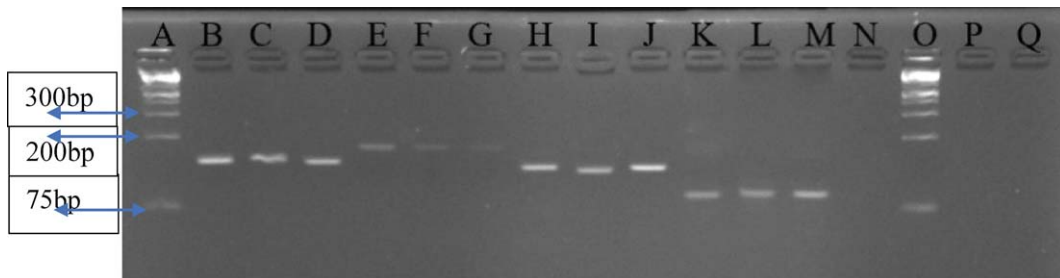
Fig. 4. A 4% Agarose gel analysis for selected amplicons of qRT-PCR products per gene. Lane A and O – 1 kb plus Ladder; Lanes B, C, D –*GAPDH* (139 kb); Lanes E, F, G –*CASP9* (172 bp); Lanes H, I, J –*CXCR2* (131 bp); Lanes K, L, M –*CXCL1* (90 bp); N – Negative control.

significant difference between the down regulation of *CXCR2* in the AgNPs-F and 5FU treated cells relative to the control. There was a significant up regulation of *CXCR2* in the cells exposed to AgNPs-L relative to the control.

3.5. qRT-PCR products electrophoresis results for selected amplicons

Figure 4 shows the qRT-PCR products electrophoresis results for selected amplicons.

4. Discussion

The current study shows that both the AgNPs and 5FU had strong cytotoxic activities against HeLa cells with IC_{50} values of 38.58, 57.63 and 10.38 µg/ml for AgNPs-F, AgNPs-L and 5FU respectively. Similarly, treatment of PC3 cells with the AgNPs and 5FU resulted in strong activities by the AgNPs with IC_{50} values of 48.17 and 47.68 µg/ml for AgNPs-F and AgNPs-L respectively compared to 5FU with an IC_{50} of 235.9 µg/ml. Whereas they did not use AgNPs in their studies, these results are in line with earlier studies that showed that *Annona muricata* extracts had strong cytotoxic activities against different cell lines [15, 26, 59]. The results are also in resonance with those from a study that used green synthesized AgNPs from *Agrimoniae herba* extracts against a human lung carcinoma cell line [60]. These results support the fact that AgNPs from ethanolic extracts of *Annona muricata* could be strong anticancer agents.

The AgNPs-F had a higher selectivity index compared to the standard drug 5FU. This implies that the AgNPs-F are safer to be used than the 5FU. Selectivity of drugs has been outlined as a critical hallmarks of any successful treatment regimen. From the current study with a selectivity index of 7.8, we do ascertain that the AgNPs-F would provide safer drugs. A higher activity on the cancerous cells while sparing the normal cells is very important. Comparatively, AgNPs-L had a much lower selectivity index than standard drug 5FU. From the current results therefore, it is evident that AgNPs-L are much more toxic than the 5FU, though within the same selectivity index range.

Metastasis is the main cause of cancer complications with 90% of deaths from solid tumors being ascribed to metastatic effects [61]. The need for novel agents that avoid metastatic spreading is therefore imperative for development of new antimetastatic drugs. The results of the migration assay in the HeLa cells indicate that the blank did not have any inhibitory activity of the migration of the cells to re-establish the mono layer. This is expected as there wasn't any treatment added to these cells. In comparison, the marked inhibitory activity of the AgNPs and 5FU on the migration of the cells as evidenced by the failure for the cells to cover the wound as well as evident increase in the size of the wound and zone of clearance indicated that they have an effect on genes responsible for motility, invasion, inflammation, chemoattraction among others. When the ability of the cells to re-establish communication and contact is inhibited, it indicates that they can no longer be in position to move and thus directly impact on their metastatic ability [61]. These results were further be supported by gene expression studies as reported in the subsequent sections of this thesis. When metastatic cells cannot spread, then there is hope that the treatment can be a better drug.

In regard to the migration ability of the PNT1A normal cells, whereas there was initiation of wound closure observed in the plates treated with the blank, there was inhibition of the process by the AgNPs-F, AgNPs-L as well as the 5FU. Other than the inhibition of the wound closure, there wasn't any other effect shown by the AgNPs-F. Comparatively, the 5FU and AgNPs-L, in addition to inhibiting the wound closure process, led to a partial increase in the size of the wound or zone of clearance. This observed phenomenon is probably due to the cytotoxic effect of the standard drug 5FU and AgNPs-L on normal cells that led to their continued death. This observation is in sync with the selectivity index results described much earlier, where the AgNPs-L and 5FU had relatively very low selectivity indices. These result therefore re-affirm the importance of selectivity of the drugs on the cells. Whereas all the treatments showed relatively high CC_{50s}, the 5FU and AgNPs-L turned out to be more cytotoxic compared to the AgNPs-F, making the AgNPs-F a better drug of choice, once utilized.

The main importance of the colony survival assay is to assess the ability of the cells to re-establish their proliferative potential following withdraw of the treatment. Many major therapies used in the cancer treatment many a time result into resurgence of the cancers after the treatments withdrawn, a key factor in treatment failure as cells revert to adaption of the original hallmarks including sustaining proliferative signaling, evasion of growth suppressors, resistance of cell death, activation of replicative immortality [8]. In the current study, relative to the treatments with the blank, insights into the colony survival of the cells were elucidated. For HeLa cells, withdraw of the treatments resulted into 0% recovery for AgNPs-F, 0.5% recovery for 5FU and 4.17% recovery for the AgNPs-L. This therefore implies that the effect of the AgNPs-F on the HeLa cell lines is more less permanent with no chance of re-establishment of the cancer upon withdraw of the treatment. Comparatively, the observed 0.5% and 4.17 % recovery in the cells treated with 5FU and AgNPs-L respectively indicated that their effects are not permanent and therefore leaves some room for surviving cells to re-establish the cancer. This is a confirmation that AgNPs-F show better anticancer activity compared to the first line drug 5FU and AgNPs-L, and therefore would be a greater alternative.

Following withdraw of the drugs in the PNT1A normal cells, a different trend was observed. Relative to the blank, withdraw of the treatments resulted into 40.81 % recovery for AgNPs-F, 20.66 % recovery for 5FU, and 6.8 % recovery for AgNPs-L. This therefore implies that the effect of the AgNPs-F on the normal cell lines is temporary and cells can easily re-adopt and survive upon withdraw of the treatment. On the other hand, the observed 20 % recovery in the cells treated with 5FU indicated that whereas its effect is not very permanent on the normal cells, there is an almost 50% chances that cells will not survive exposure to 5FU compared to when they are exposed to the AgNPs-F. The same trend observed for 5FU applies to treatments with AgNPs-L. These results therefore further confirm that AgNPs-F show better selectivity for normal body cells compared to the standard drug 5FU and AgNPs-L, and therefore would be a greater alternative.

The relative up regulation of the *CASP9* in all the treated HeLa cells is a direct indication of the involvement and activation of the intrinsic apoptotic pathway machinery as one of the mechanisms of action of the treatments under study. It is worth appreciating that *CASP9* is the gene for the upstream caspase for the intrinsic pathway of apoptosis [9, 10, 62–64]. Since apoptosis is one of the mechanisms of cells death, its activation due to exposure of the cancer cells to the treatments is a great indication of the effectiveness of the AgNPs and 5FU on the cytotoxicity of the cells and thus confirm the earlier observations of the resazurin assay in measurement of cytotoxicity. These are further supported by the *CASP9* expression levels where 5FU that had the lowest IC_{50} (10.38 μg/ml) also had the highest relative expression (4.30-fold), followed by AgNPs-F (IC_{50} of 38.58 μg/ml) at 1.53-fold and finally AgNPs-L (IC_{50} 57.63 μg/ml) had the lowest expression levels at 1.52-fold. These observations are in accordance with earlier studies which reported that re-establishment of apoptosis is very vital in overcoming the immortal nature and other hallmark of cancer cells [62, 65].

Comparatively, there was up regulation of *CASP9* in the PNT1A cells exposed to the AgNPs-F and 5FU, with the individual relative expression levels of 1.37-fold and 1.36-fold respectively, but the difference was not significant from the controls, implying that the up regulation of the gene was not very significantly different from the control and therefore the apoptosis not pronounced in these treatments thus increased selectivity [65]. For AgNPs-L, the relative expression levels of *CASP9* at 16.78-fold is an indication of a very pronounced apoptosis on the normal cells, and thus an affirmation of the more toxic nature and non-selectivity of the AgNPs-L on the on the normal cells. The observed high toxicity of the AgNPs-L could be attributed to the original source of the phytochemicals from the leaves which were shown to be composed of various secondary metabolites, some of which highly toxic like cardiac glycosides [15, 26], which consequently affects the green synthesis process and resultant activities [47]. The evidence presented in relation to the *CASP9* regulation of apoptosis corresponds with the earlier observations in regards to the selectivity indices of the AgNPs-F, 5FU and AgNPs-L.

Down regulation of *CXCL1* in HeLa cells treated with AgNPs-F and AgNPs-L is in sync with the observed effects with the migration assay, where cells could not re-establish the mono layer due to inhibition of the genes involved in migration and thus invasion of the cells to other sites. The mechanism of action therefore, as earlier on proposed, involves downregulation of *CXCL1*, a gene heavily related to cancer cells migration and inflammation. Inhibition of migration has been proposed as one of the most effective ways to manage the effects of metastatic tumors by localization [61, 66]. On the other hand, the up regulation of the *CXCL1* in HeLa cells treated with 5FU could be due the different mechanism of action of the drug that may not solely be dependent on *CXCL1*. Furthermore, up regulation of *CXCL1* in the HeLa cells treated with 5FU presents evidence on the tumorigenicity nature of the treatment as it increases expression of the genes responsible for the development, inflammation and invasion of the cancer cells. This observation could explain as to why many 5FU based treatment regimens may not be very effective in managing different cancers. In comparison with the synthesized AgNPs, there is clear evidence that the AgNPs are much better than 5FU in regards to down regulating one of the most critical chemokines in cancer sustenance. The difference could further be attributed to the very small size of the AgNPs which have been shown to be in the range of 55 nm – 86 nm, which can therefore directly reach target sites within the DNA and thus exert their effect, in contrast to 5FU and other antimetabolite-based treatments.

The up regulation of the *CXCL1* gene in PNT1A cells exposed to the different treatments is due to the fact that being normal immortalized cells, the effects of the treatments were not expected to be at a level of inhibiting normal chemotactic and growth activities in these cells. This revelation is also in sync with the earlier studies observed during the migration assays where there was evidence of initiation for re-establishment of wound closure. Normal immortalized cells have been designed to mimic the hall marks of cancer and thus are expected to continue growth and communication just like cancerous cells. Down regulation of some of the key genes involved in the chemo-activities would directly imply high toxicity levels of the treatments under study. These reported results therefore support our earlier reported observations on selectivity in normal cells.

Down regulation and near constant expression of *CXCR*2 gene in HeLa cells treated AgNPs-L and AgNPs-F respectively is directly due to the reduced activity of the CXCR2 protein since its corresponding ligands such as *CXCL1* was already under expressed. The concerted under and expression of the *CXCR2* gene further depends on a number of other factors since it is not solely specific to the *CXCL1*. Nevertheless, the observed phenomenon of down regulation in the HeLa cells treated with the AgNPs helps clarify on the underlying mechanisms of action. It is therefore suggested that AgNPs exert their effect via the downregulation of the *CXCL1/CXCR2* gene axis thereby inhibiting the action of the proteins profoundly associated with tumorigenesis, angiogenesis, and metastasis as previously described [11, 12, 66, 67].

5. Conclusions

The study presents evidence that the biosynthesized AgNPs, especially AgNPs-F (as supported by their higher selectivity index of 7.8), can be used in the development of novel and better anticancer drugs. The mechanism of action of the AgNPs involves potential activation of the intrinsic apoptosis pathway through upregulation of *CASP9* and concerted down regulation of the *CXCL1/ CXCR2* genes axis. To explain the recorded differences, further studies exploring the reasoning for the difference in the anti-cancer activity between AgNPs-L and AgNPs-F need to be undertaken.

Acknowledgments

Author contributions

CONCEPTION: YG, HAE, GM and FW
DATA CURATION: All authors
ANALYSIS OF DATA: All authors
PREPARATION OF THE MANUSCRIPT: All authors
REVISION FOR IMPORTANT INTELLECTUAL CONTENT: All authors
FUNDING ACQUISITION: YG, ENM, GM and FW

Conflict of interest

Part of the work reported in this manuscript has been filed for a grant of patent and utility model at the African Regional Intellectual Property Organization (ARIPO) under the title: "Synthesis of Silver Nanoparticles from Extracts of *Annona muricata* and Use Thereof". ARIPO Patent Application number: AP/P/2019/011514; ARIPO Utility Model Application number: AP/U/2020/000179. The above information notwithstanding, we further declare that the patent application do not in any way affect the outcome of this manuscript submission.

Availability of data and materials

Sufficient data associated with this research and enough to draw the results and conclusions has been provided within the manuscript. However, all datasets (from cytotoxicity studies) not included in this manuscript have been previously made available and published as a data article in Data in Brief Journal (https://doi.org/10.1016/j.dib.2019.104442) [68].

Supplementary material

The supplementary material is available in the electronic version of this article: https://dx.doi.org/10.3233/TUB-200058.

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