



Chloroform Fraction of *Mitracarpus hirtus* Induces p53-Mediated Cell Cycle Arrest and Apoptosis in MCF-7 Breast Cancer Cells

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Abstract

Background: Breast cancer remains a serious life-threatening disease to women worldwide, contributing to a high rate of mortality among women. A critical molecular event implicated in breast cancer progression is the dysregulation of the tumour suppressor gene p53, which plays a key role in DNA repair and the regulation of the cell cycle and proliferation of cells. *Mitracarpus hirtus*, traditionally used to treat various ailments, presents a largely untapped resource of agents with promising anti-cancer potential. This study examined the anti-breast cancer properties of the chloroform fraction of *M. hirtus*, with particular emphasis on the regulation of p53 upstream and downstream target genes.

Methods: The leaves of *M. hirtus* were extracted and fractionated using solvents with increasing polarity to obtain hexane, chloroform and water fractions. Anti-proliferative effects of chloroform fraction were evaluated on MCF-7 cells using a neutral red assay. Cell cycle progression and apoptosis were analysed using flow cytometry with PI and Annexin V-7AAD staining, respectively. Real-time PCR was used to assess the expression profiles of apoptotic-related genes.

Results: The results revealed a significant inhibition of MCF-7 cell growth by the chloroform fraction of *M. hirtus* ($IC_{50} = 9.54 \mu\text{g/mL}$). This fraction induced apoptosis in MCF-7 cells, increasing the population of cells in the early and late apoptotic stages. The pro-apoptotic genes p53, Bax, and Casp3 were upregulated, while the anti-apoptotic gene Bcl2 was downregulated. Additionally, UCK2 and STAT3 gene suppression was observed, with STAT3 showing the most significant change. The fraction also significantly inhibited cell cycle progression, causing an accumulation of cells in the G0/G1 phase.

Conclusion: Our study shows the ability of *M. hirtus* to inhibit breast cancer cell growth and induce apoptosis, presenting promising opportunities for further research in breast cancer treatment.

Introduction

Breast cancer remains a significant health challenge, responsible for high rates of mortality and morbidity annually.¹ This disease continues to be a significant life-threatening disease in Nigeria and other parts of the world, contributing to a high rate of mortality among women.² One of the critical biochemical pathways implicated in the growth and development of breast cancer involves the tumour suppressor protein p53. p53 is essential for regulating the cell cycle and apoptosis, primarily through the activation of p53, which in turn transactivates its target

genes, members of the Bcl-2 family such as Bcl-2 and Bax, as well as cytochrome c and caspases, leading to the induction of apoptosis.³ The activity of p53 is regulated through the p53-MDM2 signalling pathway, which responds to the activity of the uridine-cytidine kinase 2 (UCK2) enzyme. The role of the UCK2 gene in regulating this biochemical pathway has been documented in previous literature.⁴ The UCK2 gene is selectively overexpressed in breast cancer tissues but not in non-tumorous breast tissues.⁵ This gene is essential for cell survival and tumour proliferation as it sustains ribonucleic acid (RNA) and deoxyribonucleic

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acid (DNA) synthesis. Additionally, UCK2 promotes the proliferation of trophoblast cells by activating the signal transducer and activator of transcription 3 (STAT3) pathway, as described by Xia *et al.*⁶ Given the involvement of these biochemical pathways, they represent significant targets for breast cancer therapy.

Research on developing anti-cancer drugs from plants can also benefit from the evolutionary process of natural product biosynthesis. The evolutionary process can be advantageous in developing anti-cancer drugs as many promising agents are natural products biosynthesised by plants as a defence mechanism against various factors. These plant-derived treatments have historically played a crucial role in healthcare and continue to be widely used in breast cancer management.^{7,8} They are representing vital bioresources for discovering alternative anticancer agents. Furthermore, there has been growing interest in exploring natural compounds and phytochemicals as potential adjuvants or alternatives to conventional cancer treatment. In this regard, research into *Mitracarpus hirtus* presents a

promising opportunity for the discovery of new anticancer agents with potential for drug development.

Mitracarpus hirtus (L.) DC. (Figure 1) is a plant representing an untapped resource of agents with significant potential for anti-cancer activity. The plant is a common weed growing wildly across various Northern regions of Nigeria. It is frequently used for its medicinal properties with its juice commonly applied in the treatment of eczema. *M. hirtus* has shown promising health-promoting properties, but scientific research on it is limited in the literature. Various solvent extracts of this plant have demonstrated antioxidant and enzyme inhibitory activities against AChE, tyrosinase, α -amylase, and α -glucosidase enzymes.⁹ Phytochemical constituents recently isolated from this plant have proven effective against *Helicobacter pylori*.^{10,11} Our recent study has reported its ability as a valuable resource for agents with significant anti-proliferative activity against various cancer cell lines, including breast cancer.¹²

The research question is whether or not the chloroform

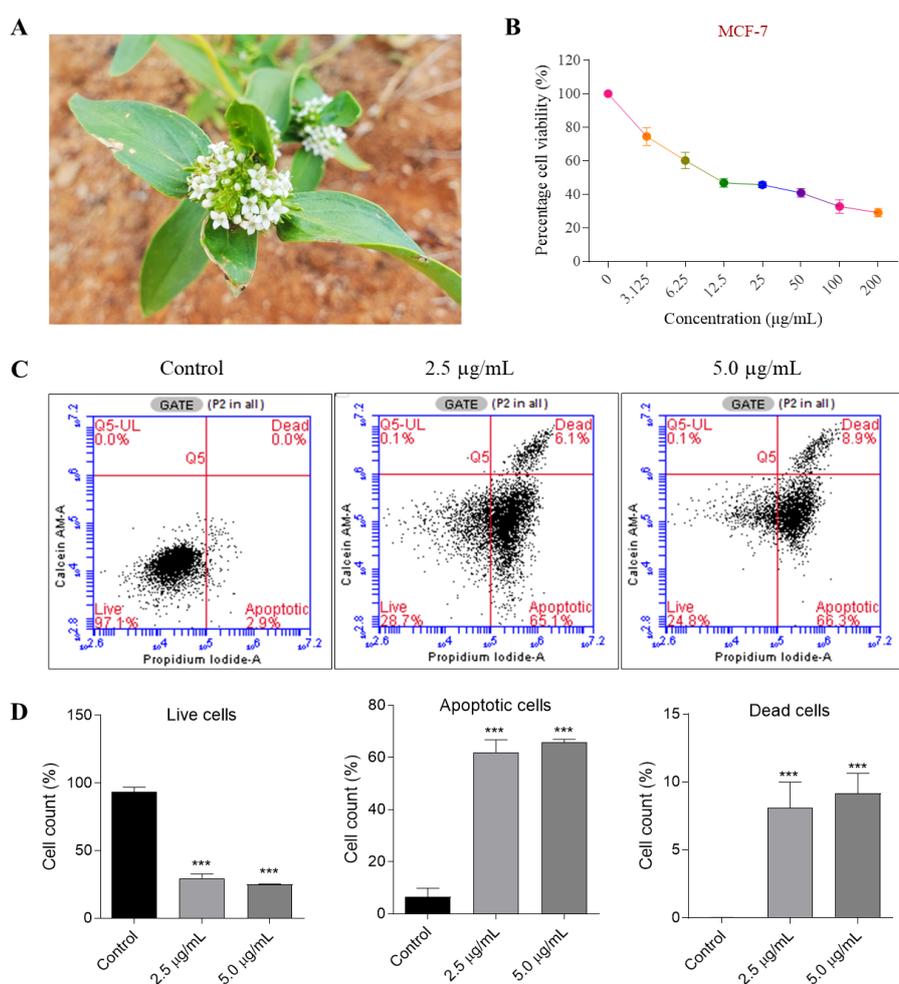


Figure 1. A. An image of *M. hirtus* captured at the Botanic Garden of the Department of Pharmacognosy and Ethnopharmacy at Usmanu Danfodiyo University, Sokoto. B. MCF-7 cells exposed to different concentrations of chloroform fraction of *M. hirtus*. C. Flow cytometry evaluation of apoptosis induction by the chloroform fraction of *M. hirtus* in MCF-7 cells treated with concentrations of 2.5 and 5.0 $\mu\text{g/mL}$ for 72 hours. D. Percentage of cell population in MCF-7 cells distributed among live, apoptotic, and dead cells. Data are presented as Mean \pm SD; *** p <0.001 compared to control cells.

fraction of *M. hirtus* induces p53-mediated cell cycle arrest and apoptosis of breast cancer cells through the regulation of upstream and downstream target genes. This study examined the anti-breast cancer properties of the fraction, focusing on its potential as a source of new, effective and safer anti-cancer agents.

Methods

Plant material and extraction

Plant material was collected in August 2023 from the vicinity of Usmanu Danfodiyo University Teaching Hospital, Sokoto, Nigeria, and air-dried at room temperature. The plant specimens were authenticated at the Herbarium of the Department of Pharmacognosy and Ethnopharmacy, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University Sokoto, Nigeria, using the existing herbarium database, and assigned a voucher number (PCG/UDUS/Rubi/0002). Subsequently, the dried leaves were pulverized into a fine powder. The resulting crude powder was then subjected to cold maceration for 72 hours using 70% methanol. The crude methanol extract was then concentrated, and successive partitioning was carried out with solvents of increasing polarity as described.¹² The concentrated crude methanol extract was initially dissolved in 250 mL of distilled water and then sequentially partitioned with equal volumes of hexane and chloroform. This process yielded crude hexane, chloroform, and water fractions. The resulting crude fractions were concentrated, and the chloroform fraction was selected for anti-proliferative studies based on our previous studies.¹²

Cell lines and cultures

The MCF-7 breast cancer cell line was purchased from AddexBio (San Diego, CA, USA). The cell was maintained in RPMI medium (Cytiva, US), supplemented with 10% fetal bovine serum (FBS) (Cytiva, US) and 1% penicillin-streptomycin antibiotics (Life Technologies, US). The cells were maintained in suitable culture conditions in a humidified CO₂ incubator. Routine subculturing was performed when the cell confluence reached 70%–80% using 0.25% trypsin-EDTA (Cytiva, US).

Neutral red uptake assay

The effects of the chloroform fraction of *M. hirtus* were evaluated using the Neutral Red uptake assay as previously described¹³, with slight modifications. Briefly, a cell suspension with a concentration of approximately 1×10^5 cells/mL was seeded in a 96-well plate with each well containing 100 μ L of a cell density of 1×10^4 cells. The plate was incubated overnight under suitable culture conditions to ensure cell attachment. Subsequently, cells were exposed to the fraction dissolved in dimethylsulfoxide (DMSO) for 72 h at various concentrations ranging from 200 to 3.125 μ g/mL in a serial dilution setup. Following exposure, cells were washed with phosphate-buffered saline (PBS) and then incubated with a neutral red medium for 2 h. After another PBS rinse, a destain solution was added to each

well-containing cell. The plate was gently stirred for 10 min, and the colour development was monitored and quantified at 540 nm. The IC₅₀ concentration was determined by extrapolating from a dose-response inhibition curve.

Calcein AM/Propidium Iodide staining for analysis of apoptosis

The apoptotic effects of the chloroform fraction of *M. hirtus* were evaluated using Calcein AM/PI (Bridgen, China) double staining. Briefly, cells were seeded into a 6-well plate at a density of 1×10^5 cells/mL and incubated until they formed a 70 to 80% confluent monolayer in suitable culture conditions. The plate was then incubated for 72 h with (2.5 and 5 μ g/mL) or without the test fraction. Subsequently, cells were harvested and rinsed 3 times with PBS. The cells were readjusted to 1.5×10^5 cells/mL and suspended in 100 μ L of assay solution comprising solutions A (Calcein AM) and B (Propidium Iodide) prepared in PBS and then incubated at room temperature in the dark for 15 min. Following incubation, the cells were analysed at 10,000 events per sample using the BD Accuri C6 flow cytometer (BD Bioscience, USA).

DNA staining for analysis of cell cycle progression

Cell cycle progression analysis was performed using the BD Cyclestest Plus DNA kit (BD Bioscience, USA). Briefly, cells were seeded into a 6-well plate at a density of 1×10^5 cells/mL and incubated until they formed a 70 to 80% confluent monolayer in suitable culture conditions. The plate was then incubated for 72 h with (2.5 and 5 μ g/mL) or without the test fraction. Subsequently, cells were harvested and washed with ice-cold PBS. The cell pellet was then resuspended in buffer solution and gently vortexed for a few seconds. Following this, cells were incubated for 10 min at room temperature in the presence of solution A. Solution B was then added, mixed by gentle tapping, and allowed to incubate for an additional 10 min at room temperature. Next, ice-cold solution C was added, gently mixed and incubated in the dark for 10 min at 8°C. Finally, the cells were analysed at 30,000 events per sample using the BD Accuri C6 flow cytometer (BD Bioscience, USA).

Real-time qPCR analysis of mRNA expression

Gene expression analysis was conducted to assess the relative expression levels of relevant genes involved in cancer cell proliferation. Purified total RNA was extracted from MCF-7 cells, with or without the test fraction, using the HiYield Total RNA Mini Kit (RBC Real Biotech, Taiwan) following the manufacturer's instructions. The extracted RNA templates were used for RT-qPCR analysis using the One-step NZYSpeedy RT-qPCR Green kit (nzytech, Lisbon, Portugal) on a Rotor-gene Q machine (QIAGEN, Düsseldorf, Germany). Reaction mixtures containing master mix, forward and reverse primers, NZYRT mix, template, and nuclease-free water were prepared and subjected to real-time PCR. Thermal cycling conditions included reverse transcription at 50 °C for 20

min, polymerase activation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 5 sec and annealing/extension at 55 °C for 30 sec. Primer sequences used for amplification of RNA fragments are provided in Table 1. Relative mRNA expression levels were quantified using the 2^{-ΔΔCT} method with GAPDH serving as the reference gene. Three independent biological replicates were performed for robust analysis.

Data analysis

The data obtained from the experiments were analysed using GraphPad Prism 9.0 software (GraphPad Software, San Diego, USA). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test to compare treatment groups with the control group. Results are presented as mean ± SD and statistical significance was determined at $p < 0.05$.

Results and Discussion

Chloroform fraction of *M. hirtus* induced the proliferation of MCF-7 cells

The anticancer effects of the chloroform fraction of *M. hirtus* were evaluated using a dose-response inhibition curve. Our previous studies have shown that the chloroform fraction of *M. hirtus* leaves exhibits remarkable cytotoxic effects with low IC₅₀ concentrations against various cancer cell lines, including breast cancer.¹² In this study, the IC₅₀ value extrapolated from the dose-response inhibition curve (Figure 1B) was determined to be 9.54 µg/mL. This result reaffirms the potent inhibitory effect of *M. hirtus* against breast cancer cells, with its cytotoxic effect occurring at relatively low concentrations. According to the National Cancer Institute (NCI), plant extracts with IC₅₀ values below 30 µg/mL are classified as having significant anticancer potential.¹⁴ The observed IC₅₀ value of the *M. hirtus* chloroform fraction falls well within this range, which confirms its strong cytotoxic activity against breast cancer cells.

Table 1. Sequences specific to the primers used for amplifying RNA fragments.

Primer name	Sequence (5'-3')
p53_F	CCAGAAAACCTACCAGGGCA
p53_R	GAATGCAAGAAGCCCAGACG
Bcl2_F	CCTGTGGATGACTGAGTACC
Bcl2_R	GAGACAGCCAGGAGAAATCA
Bax_F	CCCGAGAGGTCTTTTCCGAG
Bax_R	CCAGCCCATGATGGTTCTGAT
Casp3_F	TTCATTATTCAGGCCTGCCGAGG
Casp3_R	TTCTGACAGGCCATGTCATCCTCA
Uck2_F	CGGCGAGCCCTTCTTATAG
Uck2_R	ATTGTCAAAGGCATCCGGGT
Stat3_F	ACCAGCAGTATAGCCGCTTC
Stat3_R	GCCACAATCCGGGCAATCT
Gapdh_F	GTCTCCTCTGACTTCAACAGC
Gapdh_R	ACCACCCTGTTGCTGTAGCCAA

Chloroform fraction of *M. hirtus* induced apoptosis in MCF-7 cells

The anti-proliferative effect of the chloroform fraction of *M. hirtus* was further validated through flow cytometry analysis to assess its apoptosis-inducing potential. In the apoptosis assay (Figure 1C), the results revealed a significant ($p < 0.001$) reduction in the population of live cells, decreasing from 93.49% in the control group to 29.67% and 24.94% at 2.5 µg/mL and 5 µg/mL, respectively. Concurrently, there was a significant ($p < 0.001$) increase in the proportion of apoptotic cells, increasing from 6.49% in the control group to 62.13% and 65.87% at the respective concentrations. The percentage of dead cells also increased significantly ($p < 0.001$), from 0.01% in the control to 8.09% and 9.17% at 2.5 µg/mL and 5 µg/mL, respectively. These findings align with previous observations of apoptotic cell death as evidenced by phase-contrast inverted microscopy, following exposure of MCF-7 cells to the chloroform fraction of the plant extract.¹² Additionally, the genomic DNA fragmentation patterns observed in earlier studies further corroborate the flow cytometry data, which strengthens the ability of the plant to induce apoptosis. This dose-dependent increase in apoptosis indicates that the fraction effectively triggers key mechanisms of programmed cell death, potentially involving p53-mediated signalling pathways and the activation of intrinsic apoptotic processes.

Chloroform fraction of *M. hirtus* modulates pro- and anti-apoptotic genes in MCF-7 cells

To confirm the involvement of p53-mediated signalling pathways, the expression levels of key apoptotic and survival genes were analysed following treatment with the chloroform fraction of *M. hirtus*. At a concentration of 2.5 µg/mL, p53 and the pro-apoptotic gene Bax were slightly upregulated with log fold changes of 0.2 each, however, these changes were not statistically significant ($p > 0.05$) (Figure 2). In contrast, the anti-apoptotic gene Bcl2 was significantly ($p < 0.01$) downregulated by a fold change of 1.4, which indicates a suppression of survival pathways involving these genes associated with mitochondrial apoptotic signalling.¹⁵ Caspase-3 (Casp3), a central effector in apoptosis,⁴ showed a significant ($p < 0.05$) upregulation with a fold change of 2.1, suggesting active induction of the apoptotic cascade. At the higher concentration of 5 µg/mL, the modulation of gene expression was more prominent. The p53 gene was significantly ($p < 0.05$) upregulated by a fold change of 1.6, while Bax increased by 1.0, maintaining the activation of pro-apoptotic signals. Bcl2 expression was further suppressed by a fold change of 1.7, and Casp3 exhibited a significant ($p < 0.01$) upregulation of 2.4, confirming the increased induction of apoptosis. Additionally, genes associated with cancer cell survival and proliferation, such as UCK2^{3,16} and STAT3,¹⁷ were downregulated at a concentration of 2.5 µg/mL with log fold changes of 0.6 and 0.8, respectively. At 5 µg/mL, STAT3 expression was significantly ($p < 0.001$)

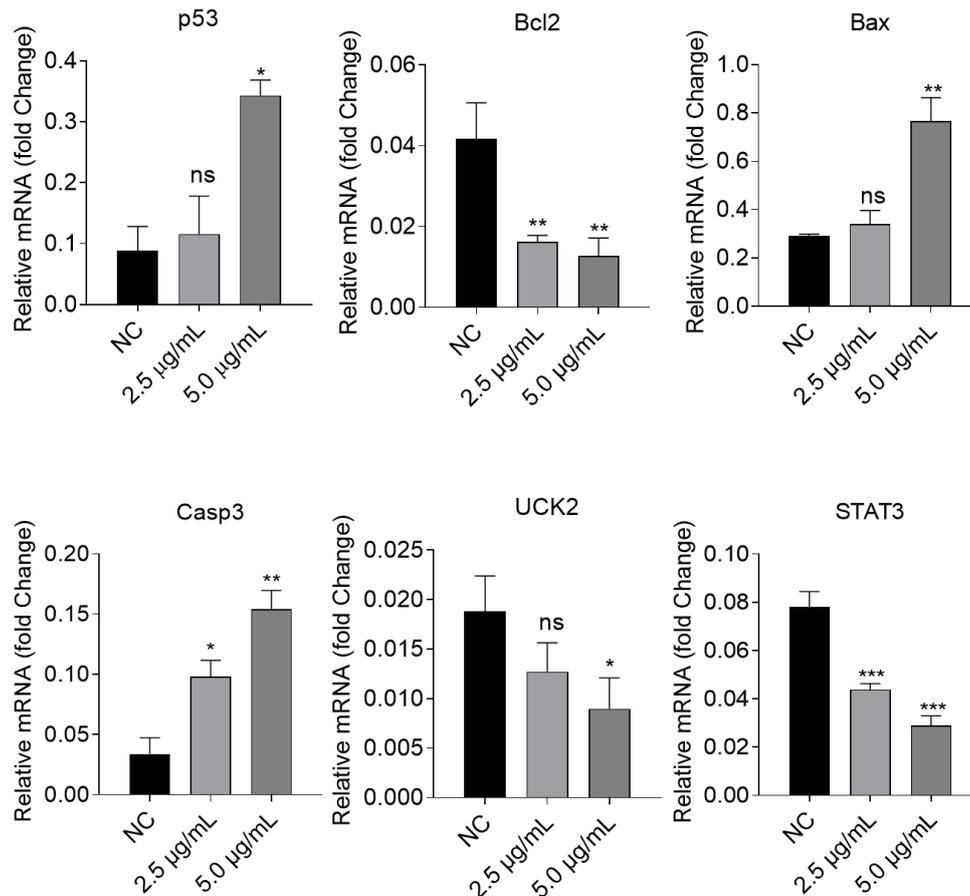


Figure 2. Effects of chloroform fraction of *M. hirtus* on the relative mRNA expression levels of p53, Bcl2, Bax, Casp3, UCK2, and STAT3 genes in MCF-7 cells treated with concentrations of 2.5 and 5.0 µg/mL for 72 hours. Data are presented as Mean±SD; ns: non-significant ($p>0.05$); * $p<0.05$; ** $p<0.01$; *** $p<0.001$ compared to control cells.

reduced by a fold change of 1.4 and UCK2 expression was significantly ($p<0.05$) downregulated by 1.0, maintaining a dose-dependent suppression of cell survival mechanisms. These results exhibited a dose-dependent modulation of apoptotic and survival genes by the chloroform fraction of *M. hirtus*. The upregulation of p53, Bax and Casp3, alongside the suppression of Bcl2, STAT3, and UCK2, demonstrate the extract's potential to induce apoptosis through p53-mediated pathways and triggering p53-dependent cell cycle arrest.¹⁸

Chloroform fraction of *M. hirtus* induced cell cycle arrest in MCF-7 cells

To evaluate the effect of the chloroform fraction of *M. hirtus* on cell cycle distribution, flow cytometry analysis was performed on MCF-7 cells treated with the extract (Figure 3). The data revealed a dose-dependent arrest in the G0/G1 phase, accompanied by a reduction in cells in the S and G2/M phases. In the control group, 66.2% of cells were in the G0/G1 phase, with 21.4% and 9.2% in the S and G2/M phases, respectively. Treatment with 2.5 µg/mL of the chloroform fraction significantly ($p<0.01$) increased the G0/G1 population to 74.0%, while the S phase population was reduced to 18.7%, and the G2/M phase

population reduced significantly ($p<0.05$) to 4.0%. At a higher concentration of 5 µg/mL, the accumulation of cells in the G0/G1 phase further increased to 82.3%, suggesting a significant block at this checkpoint. Concurrently, the percentage of cells in the S and G2/M phases reduced further to 14.1% and 4.2%, respectively.

These findings indicate that the chloroform fraction of *M. hirtus* effectively induces cell cycle arrest at the G0/G1 phase, thereby inhibiting cell proliferation. The observed G0/G1 phase arrest indicates that the chloroform fraction arrests cell cycle progression, likely through mechanisms involving p53 activation and downstream signalling pathways that regulate cell cycle checkpoints.¹⁹ The concurrent decrease in S phase cells suggests a reduction in DNA synthesis activity, while the suppression of G2/M phase populations implies that mitotic entry and progression are being effectively inhibited. This observation suggests that the chloroform fraction of *M. hirtus* may interfere with DNA synthesis and cell division processes, leading to cell cycle arrest. Such inhibition of cell cycle progression is a hallmark of many anticancer agents.^{20,21} Collectively, these findings highlight the therapeutic potential of *M. hirtus* in targeting breast cancer and warrant further investigation into its molecular mechanisms and

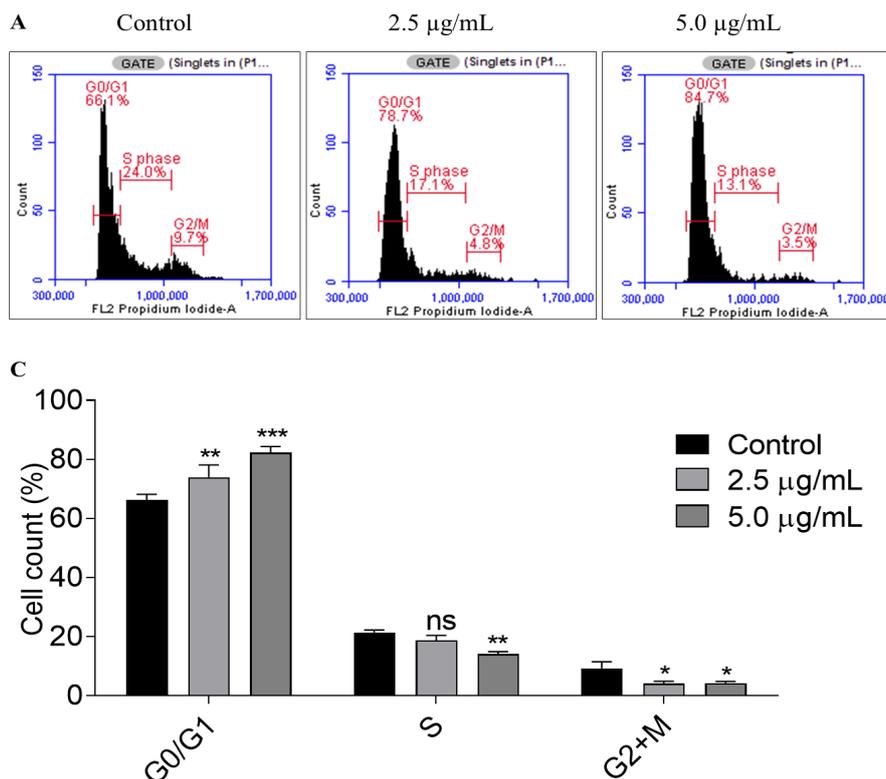


Figure 3. A. Evaluation of the effects of the chloroform fraction of *M. hirtus* on different phases of DNA content in MCF-7 cells treated with concentrations of 2.5 and 5.0 µg/mL for 72 hours. B. Percentage of DNA content in MCF-7 cells distributed across different phases of the cell cycle. Data are presented as Mean±SD; ns: non-significant ($p>0.05$); * $p<0.05$; ** $p<0.01$; *** $p<0.001$ compared to control cells.

clinical applications. This study demonstrates the cytotoxic effects of the chloroform fraction of *M. hirtus* on MCF-7 breast cancer cells, indicating its potential as a natural source of anticancer agents. However, as the experiments were conducted *in vitro*, further validation in animal models is necessary to better understand its effectiveness *in vivo*. The use of a single time point (72 hours) limits the assessment of dose-response dynamics over a broader time frame. Additionally, while the study focus on the p53-mediated pathway, other molecular mechanisms and comprehensive identification of bioactive constituents responsible for the the observed effects require further investigation. Future research should aim to address these limitations to refine our understanding of the therapeutic potential of *M. hirtus*.

Conclusion

In conclusion, the chloroform fraction of *M. hirtus* inhibits the growth and progression of MCF-7 cells through p53-mediated cell cycle arrest and apoptosis induction. Our data suggest that *M. hirtus* is a promising natural resource for the discovery of anti-breast cancer agents, presenting promising opportunities for further research in breast cancer treatment. Further investigation into the specific molecular targets and pathways implicated in cancer cell proliferation would be essential for elucidating the full therapeutic potential of the plant extract, hence, providing important therapeutic strategies against breast cancer.

Author Contributions

Ibrahim Malami: Conceptualization, Methodology, Project Administration, Formal Analysis, Data Curation, Resources, Validation, Supervision, Writing - Review & Editing. **Ramlatu Yusuf Hussaini:** Resources, Investigation, Methodology, Data curation, Writing - Original Draft. **Zakiyyah Yakubu Yahaya Ibrahim:** Validation, Writing - Review & Editing. **Ibrahim Babangida Abubakar:** Validation, Writing - Review & Editing. **Aliyu Muhammad:** Validation, Writing - Review & Editing.

Conflict of Interest

The authors report no conflicts of interest.

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