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Abstract. Cancer is a global emergency that needs instant intervention. Breast cancer is the second most common cancer after lung and the most common cancer amongst women. Current treatments are linked with adverse side effects, treatment failure, and cancer relapse. Photodynamic therapy (PDT) is one of the emerging cancer treatment options that is highly selective and specific towards cancer cells. Consequently, the use of gold nanoparticles (AuNP) further enhances the efficacy of PDT. In this study, gold-nanoparticle (AuNP) conjugated Hypericin (Hyp) mediated PDT was used for the treatment of MCF-7 human breast cancer cells by inducing cell death, in vitro. Cellular responses after 24 h post-PDT, and at 10 J/cm² were observed. The morphological changes, viability, cytotoxicity, and cell death mechanism analysis by Annexin V/PI staining was performed. The results showed activation of the apoptotic pathway with characteristic morphological features and biochemical responses of dying cells. Hence this study provided an insight into the application of advanced PDT in breast cancer treatment by actively targeting the apoptotic cell death pathway in vitro.

1. Introduction
Breast cancer begins when there is an uncontrolled growth of breast cells, leading to lump formation, inflammation, and metastasis to other parts of the body. Several different types of breast cancers have been identified. Such as those that begin from the ducts of the breast referred to as ductal carcinoma in situ or the ones that are in the lobules of the breast known as lobular carcinoma in situ [1]. Another uncommon yet increasing form of breast cancer is inflammatory breast cancer which is characterized by the warm, red, and swollen appearance of the breast. Breast cancer is a heterogeneous disease, and its treatment varies depending on the location of the tumour and whether it is benign or malignant. Current treatments are associated with several undesirable side effects such as poor prognosis, poor survival rate, drug resistance, and cytotoxicity to normal cells resulting in poor quality of life. In Africa, the five-year survival rate is less or equal to 60% for low and middle-income countries [2].

Photodynamic therapy (PDT) is a novel therapy due to its selective cytotoxicity and much reduced invasive quality [3]. It utilizes the use of a Photosensitiser (PS), visible light at a specific wavelength, as well as molecular oxygen to abolish cancer cells and disease causing bacteria [4]. It has the potential to meaningfully improve the quality of life and lengthen the survival rates in cancer patients. In PDT the PS plays a vital role in cancer cell destruction by entering the cell and activation with a specific wavelength light. Some PSs like hypericin, are hydrophobic and require a carrier molecule such as nanoparticles to enter the cell. Thus, increasing their movement across the cell membranes as a result of
increased aqueous solubility, stability, and bioavailability [5, 6]. PS and nanoparticle conjugates have proven to improve PDT efficiency. Using gold nanoparticles Portilho et al., 2013 observed an increased triplet lifetime of PS than unconjugated PS [7]. Some studies have demonstrated increased PS drug delivery and preservation using nanoparticles [8]. Nanoparticles have also been observed to disguise the PS from biological barricades and enzymes, resulting in improved cellular uptake with augmented ROS synthesis [9].

2. Materials and Methods
Hypericin (Sigma-Aldrich, 1MG-56690) was prepared in Dimethyl sulfoxide (DMSO), to a final stock concentration of 0.02 M. It was then conjugated with gold nanoparticles (AuNPs) (Sigma-Aldrich, 1ML-765457) by sonication for 2 h at room temperature. UV-Vis Spectrophotometry was used to determine the activation wavelength of Hypericin, AuNP, and the conjugate from 200 – 800 nm wavelengths. Dynamic Light Scattering (DLS), Zeta potential, and Fourier-transform infrared (FTIR) spectroscopy were used to characterize the conjugate. MCF-7 Breast cancer cells were commercially purchased from the ATCC, (ATCC® HTB-22™) and seeded in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% Foetal Bovine Serum, 1% amphotericin B, 1% pen-strep and incubated at 37 °C, 85% humidity and 5% CO₂. For PDT experiments, 3×10^5 cells were seeded in 3.4 cm^2 cell culture plates and treated with Hypericin-AuNP conjugate at concentrations of 3.8, 7.6, and 15.2 µM per plate. After 12 h of incubation to allow maximum absorption of the PS by the cells, the cells were washed 3 times using pre-warmed Hank’s Balanced Salt Solution (HBSS) to eliminate unabsorbed PS. The treated cells were then irradiated using a 594 nm diode laser (Oriel Corporation), supplied by the National Laser Center (NLC) of South Africa. Cells were irradiated at a fluence of 10 J/cm² as shown in table 1 below.

<table>
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<tr>
<th>Table 1. Laser Parameters for PDT</th>
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<td>Variable</td>
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Following irradiation, cells were placed in the incubator for 24h and the morphology was checked using an inverted light microscope (Wirsam, Olympus CKX41). The cell proliferation assay was done using the CellTiter-Glo Luminescence Cell Viability Assay (Promega, G7570) to check the amount of ATP present in the cells. The luminescence signal produced from the conversion of ATP to adenosine monophosphate (AMP) by the enzyme luciferase was read out in relative light units (RLU) on the Victor-3 multi-plate reader (Perkin Elmer, VICTOR Nivo Multimode Microplate Reader). The signal is directly proportional to the amount of ATP in the cells, which represents the number of metabolically active cells. Cellular cytotoxicity was analyzed using the spectrophotometric analysis of Lactate Dehydrogenase, LDH at 490 nm ((Perkin Elmer, VICTOR Nivo Multimode Microplate Reader). The CytoTox 96® Non-Radioactive Cytotoxicity Assay (Anatech: Promega, PRG1780) was utilized to detect the amount of LDH in the media. Annexin V- fluorescein isothiocyanate (FITC) apoptosis detection kit1 (BD Biosciences, BD Pharmingen™) was used to analyze the apoptotic or necrotic cell death pathways and the flow cytometric analysis was performed using the BD Accuri C6 (BD
All experiments were repeated three-times (n=3). The SSPS software version 27 was used for statistical analysis. Dunnett with a Confidence interval of 0.95% was used between the control (cells only) and experimental groups at significance levels $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***)

3. Results

The results indicated damage to Hyp-AuNP PDT treated cells compared to the untreated control cells. The morphology images (Figure 1) distinctly show the rounding up of cells and detachment from the culture dish surface. The higher the concentration of the conjugate, more number of cells showed rounding up, detachment from the culture dish, and thus cell death in PDT treated cells. Cellular responses also showed a dose-dependent cell death. LDH levels were increased with the increasing concentration of conjugate ($p<0.01$) in experimental PDT groups (3.8, 7.6 and 15.2 µM at 10 J/cm²) (Figure 2) while ATP levels decreased when compared to the untreated cells ($p<0.05$). There was no significant difference when control cells were compared to the Hyp-AuNP conjugate only treated group (Figure 3). The Annexin V/PI flow cytometry results (Figure 4) indicate the early and late apoptotic cell death after the treatments.

![Figure 1. Morphology of MCF-7 cells pre- and post-PDT at 594 nm.](image-url)
Figure 2. Lactate dehydrogenase hydrogenase (LDH) cytotoxicity assay indicated a significant ($p<0.01$) ** increase in LDH levels in Hyp-AuNP PDT treated cells compared to the control cells.

Figure 3. ATP Luminescence of cells indicating the rate of proliferation. Control cells in comparison to PDT treated groups showed a significant ($p<0.05$)* decrease in ATP proliferation after 24 h. No significance was observed when control cells were compared with groups that only received the Hyp-AuNP conjugate.
Figure 4. Annexin V/PI Flow cytometry results demonstrating early and late stage apoptosis at 24h post irradiation, where early apoptosis is shown as a percentage of the total population in the lower right quadrant and late-stage apoptosis shown as a percentage in the upper right quadrant. Plots a, c and e are non-irradiated control groups, while plots b, d and f are the PDT treated experimental groups.

4. Discussion and Conclusion
PDT prompts cell death by the production of reactive oxygen species resulting in oxidative damage to the cells, which results in cell death via apoptosis, necrosis, or autophagy [10]. In this study, the varying concentrations of the nano-PS conjugate resulted in significant photodamage to cells following laser irradiation at 594 nm. The morphology of conjugate PDT treated cells showed distinct damage compared to the untreated cells. This was further confirmed by the significantly decreased levels of ATP in proliferation assay results which indicated the decrease in cell proliferation in comparison to the cells only and cells treated with the nano-conjugate alone. There was an increase in the LDH levels in treated cells as compared to the untreated cells this indicates the damage to the cell membrane in treated cells following PDT and incubation at 24 h. The Annexin V/PI results indicate the oxidative cell damage in treated cells was due to the induction of early and late apoptosis.
Several studies have demonstrated the effects of Hypericin in PDT [11, 12]. However, its hydrophobicity has always been an issue, leading to the reduced cellular uptake and localization due to reduced movement through the cellular membrane of cancer cells. Conjugation of Hypericin to nanoparticles proves to be more effective and leading to an enhanced PDT efficacy than when used alone [13]. Hyp-AuNPs conjugate has demonstrated the desired therapeutic effects in MCF-7 cells in *in-vitro*. However, more studies need to be performed on normal breast cells to ensure the specificity of the treatment with no side effects to normal cells.

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**References**


