EVALUATION OF CELL DEATH MECHANISMS INDUCED BY HYPERICIN AND ITS DERIVATIVE IN COLORECTAL CARCINOMA MODELS

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ABSTRACT
Colorectal cancer (CRC) is the third most malignancy and the fourth human killer. Photodynamic therapy (PDT) has emerged as a promising alternative to conventional cancer therapies. This study aimed to investigate the ability of Hypericin (HY) and its derivative (HAHY) as synthetic photosensitizers (PSs) to induce tumoridal effect in HCT-116 carcinoma models. Sixty balb/c male mice were transplanted with 5×10³ HCT-116 cells to induce carcinoma bearing mice model. The experimental animals were then divided into five groups; Group I (untreated), Group II (HY-PDT), Group III (HAHY-PDT), Group IV (HY-PDT+EGCG) and Group V (FOLFOX). Blood, liver and tumor mass samples were taken. Also, biochemical studies and MTT assay were performed. HY and HAHY-PDT had a significant cytotoxic effect on human HCT-116 cells. Also, a significant upregulation in gene expression of P53 and CYT-c in concentration dose dependent manner. Furthermore, a significant increase in IL-6 gene expression in group (2) higher than that in group (3). Whereas it decreased in group (5), and showed insignificant difference in group (4). Also, results showed decrease in VEGF gene expression in both group (3) and (4), while it showed significant increase in group (2) and slight increase in group (5). In conclusion, these PSs possess a strong cytotoxic and apoptotic effect on human HCT-116 cells. Using of EGCG along with HY-PDT improves the outcome. Also, HAHY-PDT can be used as an alternative to HY-PDT, where it showed tumor shutdown without metastasis besides its antitumor immunity.

KEYWORDS: Hypericin; Hexa Acetyl Hypericin; Colorectal cancer; Photodynamic therapy; Synthetic photosensitizers.

INTRODUCTION
Colorectal cancer (CRC) is known to be the third most frequent malignancy and the fourth most frequent cause of cancer-related deaths.[1] Development of CRC is attributed to a combination of genetic and environmental factors.[2] Surgical resection is the main curative option for patients with metastatic type of colon cancer. Unfortunately, not all patients are candidates for surgery.[3] For these patients, chemotherapy is often used. The most commonly used chemotherapy regimens include FOLFOX (5-fluourouracil/leucovorin and oxaliplatin).[4] FOLFOX has become part of the worldwide standard of care for the adjuvant and the palliative treatment of the disease.[5] The neurotoxicity observed during and after FOLFOX therapy is attributed to oxaliplatin.[6] These conventional methods result in poor quality of life, toxicity, lower overall survival rate, and resistance to treatment.[7]

Developing new strategies as well as novel agents with lower toxicity and higher efficacy become important.[8] Photodynamic therapy (PDT) is an effective therapeutic approach requiring a nontoxic photosensitizer (PS) drug, oxygen (O₂), and irradiation with an appropriate wavelength light, resulting in a selective destruction of tumor cells.[9] The evident advantages of PDT over conventional therapies are its selective targeting,[10] cheaper operation, less harmful side effects,[11] and reduced toxicity allowing repeated treatments[12] and non-immunosuppressive nature.[13]

Hypericin (HY), a naturally-occurring photosensitizer synthesized by Hypericum sp. (St. John’s Wort), has properties suitable for PDT.[14] It is a second-generation photosensitizer that has high efficiency in production of singlet oxygen and superoxide anions after irradiation with light wavelength around 600 nm,[15] and has low or no toxicity in the dark.[16] Several in vivo and in vitro investigations have confirmed the role of hypericin photodynamic therapy (HY-PDT) against tumor cell proliferation.[17] Although HY is localized in the endoplasmatic reticulum and Golgi apparatus, but not the mitochondrial,[14] rapid loss of mitochondrial membrane potential, subsequent cytochrome-c release, caspase-3...
activation and apoptosis occurred as a result of the photodynamic action of activated HY. TP53, a tumor suppressor gene, plays a central role in cellular responses and defense against DNA damage. P53 integrates signals as a result of different stimuli such as oxidative stress and hypoxia. Previous works evaluated cell death incidence evoked by HY-PDT in mut-p53 colon adenocarcinoma cells HT-29. The photosensitizing effects of HY are generally considered as oxygen-dependent. The primary action of HY-PDT is damage to the tumor vasculature where it causes oxidative stress, leading to hypoxia within the tumor tissue and triggers the release of angiogenic molecules, resulting in angiogenesis. This angiogenesis is mainly induced by up-regulating of cyclooxygenase 2 (COX-2) and releasing of proangiogenic factors such as vascular endothelial growth factor VEGF that promote endothelial cell proliferation, migration, and tube formation from existing vessels necessary for tumor growth, progression, and metastasis. Studies have demonstrated that high expression of VEGF observed in solid tumors is correlated with poor clinical outcome. Several groups have reported the up regulation of VEGF following PDT. Furthermore, epigallocatechin-3-gallate (EGCG), a natural inhibitor of COX-2, is a major flavonoid and accounts for half of green tea polyphenols. It possesses antiangiogenic, anticarcinogenic, antimetastatic and chemo-preventive effects. EGCG inhibits cell proliferation and induces apoptosis in colorectal cancer cells. In addition, inflammatory responses induced by reactive oxygen species (ROS) are believed to be the key priming event in the development of anti-tumor immunity. The phototoxic reaction following HY-PDT initiates the release of proinflammatory mediators by releasing of interleukin IL-6 in the tumor microenvironment which induce a strong inflammatory response. HY-PDT could also promote the accumulation of macrophages and neutrophils leading to tumor destruction. Overall, HY-PDT is considered vital for the activation of antitumor immunity. Collectively, this study aimed to test the tumoricidal efficacy and molecular mechanisms of HY-PDT, HexaAcetyl Hypericin (HAH)-PDT and EGCG along with HY-PDT against HCT-116 colorectal carcinoma models.

2. MATERIAL AND METHODS
2.1. Chemicals
HY and its derivative HAHY were prepared according to Amer et al. and references there in. Also, they were kindly obtained as gift from professor doctor Atef Amer (Fig.1). These materials were dissolved in sterile Dimethyl sulfoxide (DMSO) to prepare a stock solution that was stored at -20°C until further diluted 1x PBS shortly before injection into animal. FOLFOX was obtained from the National Cancer Institute (NCI), Cairo University. EGCG was obtained from the company of Now Foods (Bloomingdale, IL60108, USA, nowfoods.com).

Figure 1: Chemical structure of HY and HAHY and its Molecular weights: 504 and 756 respectively.

2.2. Mass spectrometry
The procedure was conducted in Micro Analytical center, Cairo University. The sample was analyzed by (C/GCMS solution) negative ion acquisition mode on DI Analysis Shimadzu QP-2010 Plus instrument. The program was operated as follows: start time: 0 min, end time: 10.00 min and the event time were held for 0.50 sec. The parameters for analysis were carried out using negative ion mode as follows: Ion source temperature 250 °C, Electron voltage 70eV and scan speed: 1666. Mass spectra were detected in the ESI negative ion mode between m/z 50–800. The peaks and spectra were processed using the Maslynx 4.1 software and tentatively identified by comparing its retention time (R) and mass spectrum with reported data.

2.3. In vitro PDT treatment
HY and HAHY were dissolved in DMSO and stored at -20°C, dilution was made using DMEM containing 0.1 % (v/v) DMSO as final concentration. HCT-116 cells
(5x10³) cells / ml were seeded on six well culture plate containing 2 ml DMEM, 24 hrs after incubation, the medium was removed and fresh medium containing various concentration of HY (0.5-1-2µg / ml) and HAHY (10-15-25 µg / ml) were added and incubated for 16 hrs in the dark and one well was seeded without drug as untreated control. After PDT treatment, cells were incubated in dark for 24 hrs at 37°C in humified atmosphere of 95%, 5% CO₂ until further analysis. The collected HCT-116 cells were used to study p53 and Cytochrome -c (CYT -c) genes expression using RT-PCR.

2.4. Cytotoxic effect on HCT-116 cell line using MTT assay
This cytotoxic activity test was conducted and determined in the Bioassay Cell Culture Laboratory, National Research Centre, Dokki, Egypt. The MTT assay is a colorimetric assay depend on reduction of yellow MTT (3- (4,5- dimethylthiazol-2-y) -2,5- diphenyl tetrazolium bromide) to purple formazan. Briefly, 10⁶ cells/well were treated with various concentrations of HY and HAHY for 16 hrs in dark followed by PDT. After 24hrs of incubation following PDT treatment, 2.5 µg/ml of MTT was added to each well and incubated at 37°C for 4hrs. The formazan crystals that were formed were dissolved by adding 200 µl/well of 10% Sodium dodecyl sulphate. A positive control was used that gives 100% lethality under the same conditions and the absorbance was read at 595 nm.

The percentage of change in viability was calculated according to the formula: (Reading of extract / Reading of negative control) -1) x 100.[30]

2.5. Induction of tumor bearing mice model
Sixty balb/c male mice (8-10 weeks old) weighting 25 g were obtained from the Animal House of ZSMRC, Zagazig University, Egypt. They were housed in a temperature-controlled and light-controlled room (12 h light/dark cycles) with free access to food and water. The human epithelial colorectal carcinoma cell line HCT-116 (purchased from VACSERA, Giza, Egypt) was used to establish the HCT-116 bearing mice model.[39] The cells were cultured as a monolayer in DMEM medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin and incubated at 37°C, 95% humidity, and 5% CO₂ (NUVE CO₂ incubator). The cells were examined under inverted microscope (Leica). Once confluent, the cell layer was washed with phosphate-buffered saline (PBS), trypsinized, and counted using a hemocytometer. Before inoculation, approximately 5x10⁵ cells were suspended in PBS and injected subcutaneously into the lower left flanks of the 60 mice.[40] All experimental procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee of the Zagazig University.

2.6. In vivo treatment protocols
Two weeks after inoculation, different treatment protocols have started and continued for 4 weeks. The animals were randomly assigned to five different groups (n=12) for treatment: Group 1; control (mice with untreated tumor). Group 2; treated intraperitoneally (IP) with 5mg/kg B.wt HY.[41] Group 3; treated IP with 5mg/kg B.wt HAHY. Group 4; treated IP with 2 mg/kg B.wt HY[28], in combination IP with 50 mg/kg B.W EGCG.[42] Group 5; treated with (IP) 6mg/kg oxaliplatin and after 2hrs with 50mg/kg B.wt (5-Fu) + 90mg/kg B.wt leucovorin.[43] The experimental animals in each of the treated groups (2, 3, 4), 2 hrs after injection, were irradiated for 5 min[41] by exposure of the tumor area of mice to 7L18w/30 fluorescent lamps for 5 days a week.[44]

2.7. Sampling
At the end of the experimental period, all animals were sacrificed and venous blood samples were collected from the retroorbital plexus on EDTA, tumor mass and liver tissue samples were taken for biochemical and histological studies.

2.8. RNA extraction, cDNA synthesis and real time RT-PCR
Total RNA was extracted from the culture collected cells, from the tumor tissue homogenate and from whole blood of mice bearing HCT-116 cell using PureLink® RNA Mini Kit purchased from Ambion by life technologies by Thermo Scientific, Catalog numbers: 12183018A following the manufacture instructions. cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit purchased from Thermo Scientific, code 40374966. Real time PCR amplification was performed using Maxima SYBR Green qPCR Master Mix (2X) kit purchased from Thermo scientific, catalog #K0251. PCR reactions were incubated for 10 min at 95 °C as initial denaturation and polymerase activation, after which the target was amplified with 45 cycles for 15 sec at 95°C as denaturation and 30 sec at 51 °C as annealing and 30 sec 72 °C as extension, this program was operated by Stratagene Mx3005P. The amount of target gene expression levels was quantified using the formula 2^-∆∆ct.[45] The gene expression level was normalized to GAPDH. The primers used were purchased from Invitrogen Thermo Scientific as shown in Table 1.[38-46]
Table 1: Specific primers for examined genes.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
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<tr>
<td>hCYT-C</td>
<td>5'-AGTGTTCACCAGTGCCACACCG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TCCTTCTCCCAGAATGATGCCTTG-3'</td>
</tr>
<tr>
<td>hGADPH</td>
<td>5'-AAGGTGAAAGTGGAGTCAAC-3'</td>
</tr>
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<td></td>
<td>5'-GGGTCTATTGAGTGGCAAACATA-3'</td>
</tr>
<tr>
<td>hP53</td>
<td>5'-ACTTGTGGCTCTTGAAAAGCTAC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GATGGCAAGAATCTTTGGAACA-3'</td>
</tr>
<tr>
<td>mVEGF</td>
<td>5'-GTGAGGTGTTATGATGTGGG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-ACGGTCTTGCGAGTTAAACCTG-3'</td>
</tr>
<tr>
<td>mIL-6</td>
<td>5'-CAGCAAGAGATCCATCCAG-3'</td>
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<tr>
<td></td>
<td>5'-AGTGGTATAGACAAGTCTGTTGG-3'</td>
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<tr>
<td>mGADPH</td>
<td>5'-TGCCCCTCCGTTCTTACCAC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GAGTTGCTTGGAAAGCTGCA-3'</td>
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2.9. Histological and immunohistochemical study

Tumor mass and liver from each animal was carefully dissected and the specimens were immediately immersed in 10% neutral buffered formaline for 48 hrs before being processed to prepare 5-μm-thick paraffin sections and stained with hematoxylin and eosin (H & E). The immunohistochemical staining occurred by using Cytokeratin7 to investigate the origin of this study tumor cell in both liver and lymph node of untreated group which were bearing to HCT-116 cell line. Slides were viewed by using Labomed, Labo America, Inc. USA microscope.[50]

2.10. Statistical analysis

Data were statistically analyzed using the software SPSS. One-way ANOVA was used to analyse the variance, and dunken post hoc test Multiple Comparison test was used to compare the genes expression levels in all the groups. Data were mean± SEM, *P<0.05, **P<0.001, ***P<0.001 and ns>0.05.

3. RESULTS

3.1. Mass Spectrometry Result

Mass spectrometry can be used as a detector and combined with chromatography. The major advantage of this technique is its selectivity and its capability to give information about the structure of the tested compound. From this point of view the analysis of synthetic Hexa Acetyl Hypericin (HAHY) is carried out using this advanced analytical technique of chromatography coupled to electrospray ionization mass spectroscopy (C/GCMS) in negative mode to confirm its structure and Molecular weight. This type of scan displays one and two masses unit higher than the initial mass of the tested compounds (M+1) and (M+2). The x-axis represents the expanding mass-to-charge (m/z) ratio whereas the y-axis shows the relative abundance of each ion which is associated to the number of times that an ion of that m/z ratio hits the detector. (Fig.2) shows the total ion MS spectrum of HAHY. The molecular ions (M+1) at m/z 757 and (M+2) at m/z 758 were displayed by HAHY in the negative ion mode. The fragment at m/z743 (M+1-CH₂) was observed. The fragment at m/z 674 (M+2-2CH₃=CH=O) resulted from the loss of the diacetyl groups and represents tetraacetyl Hypericin. The fragment at m/z 364 (M+1-CH₃) was observed. The fragment at m/z 504 (M-6CH₃=CH=O) resulted from the loss of the hexaacetyl groups and represents Hypericin. Product ions of m/z 252 and 142 were also detected which represents Emodine and the compound resulted from losing C₇H₈O from Emodin respectively. The base peak at relative intensity 100% was detected at m/z 57.

These results proved that the tested compound is Hexa Acetyl Hypericin and it has the molecular weight equal to 756.

Figure 2: Fragment ions MS spectrum of HAHY.
3.2. **Invitro Model Results**

3.2.1. **Cytotoxic effect of HY and HAHY - PDT on HCT-116 cell line**

The MTT assay was performed to assess the rate of proliferation of HCT-116 cells after treatment with varying concentrations of HY and HAHY-PDT ranged between 0.78 to 100 µg/ml. The result showed that the current PSs inhibited the growth of HCT-116 cells in a concentration-dependent manner. At the concentration of 2.03 µg/ml for HY, 50% viability was detected during the 48 h treatment, whereas maximum cytotoxicity LC$_{50}$ was observed at a concentration of 5.5 µg/ml. Whereas at the concentration of 52.3 µg/ml for HY derivative, 50% viability was detected, and maximum cytotoxicity LC$_{90}$ was observed at a concentration of 82.5 µg/ml as showed in table (2) and (Fig.3).

<table>
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<tr>
<th>Sample Code</th>
<th>LC$_{50}$ (µg/ml)</th>
<th>LC$_{90}$ (µg/ml)</th>
<th>Remarks</th>
</tr>
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<tr>
<td>HY-PDT treated HCT116 cells</td>
<td>2.03</td>
<td>5.5</td>
<td>100% at 100ppm</td>
</tr>
<tr>
<td>HAHY-PDT treated HCT-116 cells</td>
<td>52.3</td>
<td>82.5</td>
<td>95.2% at 100ppm</td>
</tr>
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LC$_{50}$: Lethal concentration of the sample which causes the death of 50% of cells in 48 hrs. LC$_{90}$: Lethal concentration of the sample which causes the death of 90% of cells in 48 hrs.

Figure 3: The effect of different concentration of HY (A) and its derivative (B) on HCT 116 cell line viability by using MTT assay.

3.2.2. **Effect of HY and HAHY-PDT on p53 and CYT-c genes expression using RT-PCR**

The results of genes expression showed increase in the level of P53 gene expression in HCT 116 cells by increasing the concentration of all treated groups compared with the untreated group. While, CYT-c gene expression was significantly elevated with increasing the concentration in all treated groups except the concentration of 10 µg/ml HY derivative-PDT in HCT 116 cells (figure4).

Figure 4: P53 (A) and CYT-c (B) genes expression of vitro model in untreated, HY (0.25, 0.5 and 1 µg/ml) and HY derivative (10, 15 and 25 µg/ml) treated HCT 116 cell line, gene expression analysis was performed at 24 post PDT treatments. Data are mean ±S.E.M. statistically significant difference were labeled as *P<0.05, **P<0.001, ***P<0.0001 and ns>0.05, ns is non-significant.
3.3. Invivo Model Results

3.3.1. HY and HAHY mediated PDT lead to up regulation of IL-6 in HCT-116 bearing mice resulting in anti-tumor immunity

There was a highly significant increase in IL-6 expression of tumor mass in group (2). While, there were slight significant increase in group (3). There were a highly significant increase in IL-6 expression of blood samples in group (2), while there were non-significant difference in group (3) compared with untreated group (control) as shown in figure (5B and C).

3.3.2. Effect of HY and HAHY mediated PDT on VEGF in HCT-116 bearing mice

There was a highly significant increase in VEGF expression of tumor mass in group (2). While, there were slight significant decrease in group (3) treated with the same dose with HAHY-PDT compared with untreated group as shown in figure (5A).

3.3.3. Effect of EGCG treated along with HY-PDT as anti-angiogenesis on VEGF expression and as anti-inflammatory on IL-6 expression

In present study, we used EGCG along with HY-PDT. Our results showed significant decrease in VEGF of tumor mass. While this group showed non-significant difference in IL-6 of tumor mass. Whereas there were a significant decrease in IL-6 of blood samples of this group in comparison with control group as shown in figure (5A-C).

3.3.4. Effect of FOLFOX on VEGF and IL-6 genes expression in HCT-116 bearing mice

There were a little significant decrease in IL-6 gene of tumor mass in group (5) treated with FOLFOX with non-significant difference in IL-6 gene of blood samples. Whereas there were a little significant increase in VEGF gene of tumor mass of this group in comparison with untreated group as shown in figure (5A-C).

![Figure 5: VEGF (A), IL-6 (B) genes expression of tumor mass and IL-6 (C) of Blood in untreated and treated groups of HCT-116 cell line bearing mice. Data are mean ± S.E.M. Statistically significant difference were labeled as *P<0.05, **P<0.001, ***P<0.0001 and ns >0.05, ns is non-significant.](image)

3.4. Immunohistochemistry Results

In group (1), immunohistochemically (IHC), both liver and L.N of this group showed strong positive reaction with Cytokeratin 7 as brown coloration occupying the cytoplasm of the metastatic cells of both liver and L.N. denoting an epithelial origin of such tumor cells confirming that the detected tumor in this organs was HCT-116 colorectal carcinoma (Fig.6 A and B).

![Figure 6: Metastasis of HCT-116 cell line in liver and lymph node of the untreated group (A&B). A Photomicrograph of immunostained section for Cytokeratin 7 of control mice lymph node H&E x400. B. A Photomicrograph of immunostained section for Cytokeratin 7 of control mice liver H&E x400.](image)

3.5. Histopathological Results

In group (1), H&E staining showed that Examined sections from subcutaneous lymph node (L.N.) appeared with replacement of nodal architecture by groups and nests of atypical large polygonal epithelial cells with intact nodal capsule (Fig.7A). Liver sections of this
group showed dilated congested central vein and sinusoids; scattered hepatocytes showing hepatocellular dysplasia; and scattered foci of malignant epithelial cells mainly at the surface showing hyperchromatic nuclei and increased mitosis and mild lobular inflammatory infiltrate (Fig. 8A).

In group (2), H&E staining showed that L.N sections presented as normal lymphoid structures free of neoplastic metastasis also, replacement of lymphoid tissue by polymorphous infiltrate of non-specific inflammatory cells (Fig. 7B). Liver sections of this group revealed focal vascular metastasis of atypical epithelial cells, portal and interstitial chronic inflammatory cell aggregation of round cells, kuffer cells hypertrophy and degenerative changes in some hepatocytes and replacement of portal triad by non-specific inflammatory cells (Fig. 8B).

In group (3), H&E staining showed that L.N. revealed normal cortical and medullary structures. No tumor cells could be observed. (Fig. 7C). Liver section of this group revealed congested portal blood vessels inflammatory cells infiltration and mild biliary proliferation. No tumor cells could be detected (Fig. 8C).

In group (4), H&E staining showed that L.N revealed cortical and medullary lymphoid hyperplasia with predominant plasma cell reaction (Fig. 7D). Liver section of this group revealed Lymphocytic hepatitis as demonstrated by aggregation of moderate number of lymphocytes in the portal triade with mild biliary proliferation (Fig. 8D).

In group (5), H&E staining showed that L.N was in normal cortical and medullary structures free from any tumor metastasis (Fig. 7E). Liver sections of this group revealed normal hepatic parenchyma. No evidence of tumor metastasis or liver degenerative changes or mild to moderate hepatitis could be detected (Fig. 8E).

Figure 7: Effect of HY, HAHY, HY+EGCG and FOLFOX on lymph node morphometry of different treated groups (A – E). A. Photomicrograph of control mice lymph node showing: replacement of nodal architecture by groups and nests of atypical large polygonal epithelial cells (black arrow), H&E x400. B. Photomicrograph of HY treated mice lymph node showing distorted nodal architecture and desmoplastic reaction with no evidence of neoplastic infiltration (black arrow); H&E x 400. C. Photomicrograph of HAHY treated mice lymph node showing replacement of lymphoid tissue by polymorphous infiltrate of non-specific inflammatory cells mainly; H&E x400. D. Photomicrograph of HY+EGCG treated mice lymph node showing cortical and medullary lymphoid hyperplasia with predominant plasma cell reaction (black arrow) H&E x400. E. Photomicrograph FOLFOX treated mice lymph node showing normal cortical and medullary structures free from any tumor metastasis H&E x400.
4. DISCUSSION
Colorectal cancer is the second most common cancer among men and the third most common cancer among women worldwide. It causes cancer-related deaths every year. Surgical resection is the only potential cure. Post-operative adjuvant chemotherapy has been demonstrated to improve outcomes.

FOLFOX became the standard adjuvant regimen for stage III colon cancer treatment, but leads to significant cost increase and toxicity. In particular, oxaliplatin-induced neurotoxicity, Peripheral neuropathy was reported for 92.1% of patients receiving treatments. Thus, there is dire need to investigate alternative strategies for CRC treatment.

Photodynamic therapy (PDT) is a cancer treatment modality exploiting non-toxic photosensitizer preferentially localized in tumor tissue and its targeted activation with light, leading to subsequent reactive oxygen species production causing photochemically induced cell death through three tumoricidal mechanism; direct cytotoxic, shutdown of tumor vasculature and antitumor immune response. Hypericin (HY), derived from the plant Hypericum perforatum, has been used in PDT because of its selective accumulation in cancer cells, high-quantum yields, and low cytotoxicity. An antiproliferative effect of HY was reported in several in vitro and in vivo investigations in colon cancer cells.

For these reasons, we shed light on the therapeutic effect of HY-PDT with different concentrations in vitro and in vivo HCT-116 models. This study differs from others where, we used for the first time HY derivative (HAHY) as a synthetic PS, and compares between its therapeutic effect and that of HY and also compares between their effects and that of FOLFOX in HCT-116 bearing mice model. This study investigated if the current PSs could induce the three known tumoricidal mechanisms of PDT in HCT-116 models.

The choice of HY-PDT was according to Paszkoc et al. Yoo and Ha, who mentioned that Several PSs including HY, are in advanced stages of clinical trials or in the approval phase for the treatment of several malignant diseases. PDT has been approved in several countries for treatment of bladder, head and neck, lung, breast, esophageal, cervical and pancreatic cancers.

Furthermore, because tumor tissues have properties such as decrease of pH value and presence of high lipid content this contribute to preferential accumulation of PSs in tumors via the enhanced permeability retention (EPR) effect and so is passively absorbed, so their selective uptake can be achieved, promoting PDT induced tumor destruction with only slight healthy tissue damage. Additionally, HY has a high affinity for cancer cells due to the high content of low density lipoprotein (LDL) receptors in cancer cells which enhances uptake of the PS.
The present study revealed the cytotoxic effect of current PSs on HCT-116 cell line using MTT assay confirming the first tumoricidal mechanism of PDT using HY and HAHY. These results were supported by the experiment of Blank et al. who concluded that, HY-PDT had effective cytotoxic activity at low dose against Murine C 26 colon carcinoma using MTT assay which showed that LC50 was 1µM. Also, supported by Sačková et al. who approved the antiproliferative effects of HY-PDT in CRC cells.

Our results were in agreement with Mikes et al. who revealed a dose dependent reaction to HY-PDT which was significant in almost all groups comparing to untreated control when examined the in vitro cytotoxic activity of HY-PDT on HCT-116 cell lines by MTT assay. Another reports showed the powerful cytotoxic effect of HY on HT-29 and Caco-2 cell lines by MTT assay.

The mechanism of this selective cytotoxic and apoptotic effect of current PSs was studied through detection of the expression of some apoptotic genes such as P53 and CYT-c. The results showed that the mRNA expression levels of these genes were significantly increased according to their concentrations which indicate the apoptotic effect of them on HCT-116 cell line.

Multiple studies confirmed our results. Sanovíc et al. revealed that oxidation by type I and type II photochemical reaction-derived ROS, which leading to oxidative stress and, in case of excessive damage or stress, cell death via apoptosis where this agreed with our results because our PSs generated ROS by type I and type II photochemical reaction.

Turrens proved that photoactived ROS in mitochondria induced the release of CYT-c which further activates pro-apoptotic caspases. Another study was occurred by Liu et al. showed that HY-PDT resulted in the loss of mitochondria membrane integrity leading to the release of CYT-C into the cytosol which interacts with apoptosis protease - activating factor-1 to form apopotsome, that activates caspase 9 and subsequently caspase 3.

Lastly our results were in agreement with Du et al. who reported that HY-PDT induced ROS which release Ca2+ from endoplasmic reticulum and losing of the integrity of mitochondrial membrane and releasing of CYT-C leading to tumor cell death via mitochondrial apoptotic pathway.

In our vitro model, we studied also the P53 gene expression, our results showed significant increase in all treated groups with increasing the concentration of HY and its derivative mediated-PDT, our results agreed with Fisher et al., Tong et al. and Hajri et al. where they all found that PDT-treated cells with various sensitizers up regulated the P53, but PDT-induced cell death or apoptosis didn't show a significant dependence on P53.

Firstly, in our vivo results, we successes to establish metastasized xenografted model by transplanting 5x10³ HCT-116 cells to balb/c mice as shown in and confirmed this with H&E and immunohistochemical investigations in L.N. and liver sections of this group as shown in (Fig.6A and B) which showed strong positive reaction with Cytokeratin 7 denoting an epithelial origin of such tumor cells confirming that the detected tumor in this organs was HCT -116 colorectal carcinoma and this results were in agreement with Rajput et al. who proved that HCT-116 cells have an epithelial morphology and can metastasize in xenograft models.

The present study revealed also the second and third tumoricidal mechanism of PDT using HY and HAHY as synthetic PSs through detection of VEGF and IL-6 gene expression in tumor mass and examination of H&E sections in tumor mass and liver tissues of HCT-116 bearing mice. Our results showed significant increase of VEGF gene expression in tumor mass of group (2) as a result of hypoxia with some metastasis in the liver of this group. This results may be explained by the fact that the current PSs after PDT induced oxygen shortage within treated tissue because of the consumption of oxygen during PDT on the tissue microvasculature which leading to severe tissue hypoxia and anoxia which is considered a second tumoricidal mechanism of PDT in which the induction of local hypoxia in the irradiated tumor bulk as result of O2 depletion in consequence to the O2 → O2 or O2 conversion and oxidation of biomolecules during PDT and also the shutdown of tumor vasculature after PDT cells under hypoxic stress may switch to an adaptive response by inducing a hypoxia-inducible factor such as HIF-1α, up regulating COX-2 and releasing of pro-angiogenic signals such as VEGF which increases migration and differentiation. Our result confirmed with H&E investigation of liver section of this group which showed in (Fig 8B) some focal vascular metastasis of atypical epithelial cells whereas its lymph node section showed normal lymphoid structure free of neoplastic metastasis in (Fig.7B).

Multiple studies confirmed our results: Chen et al. showed that HY-PDT induced tumor vasculature damage which occurs due to the consumption of oxygen during PDT causing oxygen shortage within the treated tumor and nutrient deprivation.

Chen et al. showed that the majority of second-generation photosensitizers localize primarily in endothelial cells as well as tumor cells that line the tumor vasculature, this studies agreed with our results for inducing hypoxia where our drugs are second - generation and endothelial photosensitizers.

Ferrario and Gomer reported that PDT produced significant increases in VEGF within treated lesions.
indicating that hypoxia within tumors plays a major role in angiogenesis.

However, our results showed that VEGF gene decreased in group (3) treated with HAHY-PDT and also, it showed significant decrease in group (4) treated with HY-PDT in combination with EGCG as COX-2 inhibitors. Whereas in group (5) treated with FOLFOX showed slight increase in comparison with untreated group. This results confirmed with H&E investigation for the last three groups (3), (4), (5), where they showed no evidence of tumor metastasis in lymph node or liver as demonstrated in (Fig.7C-E and Fig.8C-E). This result proved that combination of EGCG with HY-PDT improves its outcome in HCT-116 bearing mice by blocking the VEGF signaling pathway and inhibiting metastasis to eventually achieve long-term tumor control.

Our results are in agreement with Ferrario et al.\textsuperscript{[74]} and Makowski et al.\textsuperscript{[75]} who reported that blocking of COX-2 with celecoxib showed increased survival of mice in which various tumor cell lines were xenografted.

Furthermore, our results agreed with Ferrario et al.\textsuperscript{[76-77]} who mentioned that enhancement of PDT with NSAIDS resulting in decrease the cell survival in variety of tumor cell lines with a reduction in the levels of PGE2 and proangiogenesis factor VEGF.

Our results supported with Bhuvaneswari et al.\textsuperscript{[28]} who showed that the use of celebrex as NSAIDS, along with HY-PDT down regulated the expression of VEGF suggesting that using of angiogenesis inhibitor can improve HY-PDT in nasopharyngeal carcinoma.

Lastly Bhuvaneswari et al.\textsuperscript{[78]} obtained similar positive results when they used mAb against VEGF (Avastin, bevacizumab) in combination with hypericin-PDT on mice bladder carcinoma tumors.

Regarding to EGCG, In recent years, several studies are in agreement with our results where Siddiqui et al.\textsuperscript{[79]}; Honicke et al.\textsuperscript{[80]} and Kwak et al.\textsuperscript{[81]} all demonstrated that EGCG inhibits biomarkers associated with angiogenesis (e.g. VEGF, angiopoietin1 and 2), invasion and metastasis (MMP-2, 3, and 9). Furthermore, we are in agreement with Wu et al.\textsuperscript{[82]} who concluded that EGCG improved the performance of chemotherapy in reducing tumor weight and/or volume in xenograft models.

Thus, the inhibition of COX-2 activity with natural inhibitor as EGCG that used in our study could be a valuable intervention strategy for PDT to reduce tumor cell survival and potentially reduce the proangiogenic effects induced by PGE2.

Our in vivo results also, represented that HY and HAHY-PDT could induce antitumor immuno response, where our experiments have clearly shown that HY and HAHY-PDT with the same dose leads to increased release of IL-6 gene in the tumor microenvironment which serves as an inducer of chronic inflammation in addition to eliciting specific cellular immune responses to damaged cells. In group (2), IL-6 gene expression was higher than in group (3). There were a highly significant increase in IL-6 expression of blood samples in group (2), while there were non-significant difference in group (3), whereas in group (4) treated with HY-PDT + EGCG showed non-significant difference in IL-6 in tumor mass with significant decrease in IL-6 of blood samples. While in group (5) treated with FolFox showed slight decrease in IL-6 in tumor mass with non-significant difference in IL-6 gene of blood samples in comparison with untreated group. we could explain these results by the facts proved previously that PDT induced inflammatory reaction which contribute to tumor vascular damage where phototoxic damage in tumor blood vessels provide proinflammatory signals lead to the contraction of endothelial cells and exposure of the basement membrane in the vessel wall.\textsuperscript{[83]}

As a result, the circulating neutrophils and platelets are attracted and attached to the lesions leading to a progressive impairment of vascular function and release of various inflammatory mediators including leukocyte chemoattractants, cytokines, growth factors and other immunoregulators.\textsuperscript{[84]} The hallmark of inflammatory process is the accumulation of inflammatory cells including neutrophils, mast cells monocytes/macrophages attracted by the release of potent mediators from PDT treated tumor following neutrophil invasion is the arrival of mast cells.\textsuperscript{[85]}

Our results in group (2) confirmed by non-specific inflammation in its H&E investigation (Fig.7 B) and, that of its derivative in group (3) also confirmed with some non-specific inflammation in H&E investigation (Fig.7C) which proved that the HY derivative induced slower immuno response than that induced in group (2) treated with the same dose with HY-PDT. These results supported with Korbelik and Cecic,\textsuperscript{[86]} who reported that Monocytes/macrophages are another class nonspecific immune effect cells that contribute to antitumor effect of PDT Our results are in agreement with previously published findings that have established the increased presence of IL-6 in the tumor microenvironment following PDT; Moor,\textsuperscript{[87]} reported that PDT could increase the permeability of the local vasculature through proinflammatory cytokine production leading to diapedesis of circulating inflammatory leukocytes to the site of inflammation. Also, Gollnick et al.\textsuperscript{[88]} reported that investigations on human cervical carcinoma cells have provided evidence that PDT could increase IL-6 expression following activation of activator protein-1.

Furthermore Gabay,\textsuperscript{[89]} reported that PDT could induce certain heat-shock proteins, such as, heat-shock protein and heat-shock protein 90 that facilitate the recruitment of tumor-specific cytolytic T cells. Kabingu,\textsuperscript{[90]} proved the third tumoricidal mechanism of PDT that involves...
the induction of antitumor immune response, where the tumor cell death that occurs directly from photochemical damage or as a result of vascular shutdown-mediated hypoxia/anoxia and hyponutrition is the key precursor event for the antitumor immune response.

Thong et al.\(^\text{[91]}\) reported that the major advantage of the PDT-triggered on immunological pathways is that these pathways can trigger an antitumor immune response mediated by antigen-specific T-cells against distant tumor cells that were not subjected to PDT. Trisciuoglio et al.\(^\text{[92]}\) had a concrete evidence to support our findings that HY-PDT induces changes in the tumor microenvironment by up regulating IL-6 causing chronic inflammation, respectively, culminating in tumor cell death.

Firczuk et al.\(^\text{[93]}\) reported that IL-6 as proinflammatory cytokine caused a rapid and strong inflammatory reaction. These processes together with the release of histamine and serotonin from damaged vasculature cause infiltration of the tumour site by diverse populations of immune cells (neutrophils, mast cells and macrophages) that become activated and engaged in tumour cell destruction. Moreover, Zhao et al.\(^\text{[94]}\) had provided evidence that IL-6 could be up regulated following PDT directed at subcutaneous tumors.

He et al.\(^\text{[95]}\) showed that PDT causes oxidative stress that induces protective responses. Which includes expression of heat shock proteins (HSPs), transcription factors such as nuclear factor κB (NF-κB) and activator protein 1 (AP-1) then induce expression of immunoregulatory and proinflammatory proteins such as interleukins IL-6.

Lastly Barathan et al.\(^\text{[96]}\) showed that IL-6 was significantly increased HepG2 cell treated with 1µg/ml HY-PDT resulting in cell death which provide additional hints for the existence of alternative mechanism of antitumor immunity in hepatocellular carcinoma.

5. CONCLUSIONS
In summary, there can be no doubt that FOLFOX has increased survival of colorectal cancer patients with metastatic disease, but still has neurotoxicity as a side effect. The current PSs have a strong cytotoxic and apoptotic effect on human HCT-116 cells. Combining HY-PDT and EGCG is a promising approach for cancer therapy, in agreement with previous preclinical studies involving angiogenesis inhibitor combination therapies. Also, HAHY-PDT can be used as an alternative to HY-PDT, where it showed tumor shutdown without metastasis besides its antitumor immunity.

6. Recommendation
Since the colon can be easily accessed via the rectum opening of the large intestine using an endoscope. Thus, colonoscopy endoscopes could be used to directly deliver PSs drugs (HY and HAHY) to target tumor regions, as well as administer the required wavelength of laser irradiation light to activate a PS drug. So, this form of oncological PDT treatment for CRC tumors is possible.

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AUTHOR CONTRIBUTIONS
All authors performed the experiments, analyzed the data, interpreted the results of the experiments, and drafted the manuscript.

Ethics statement
All animal experiments were approved by the ZSMRC, Faculty of Medicine, Zagazig University. Animal Research Ethics and were done in accordance with their guidelines for Animal Care.

6. REFERENCES


