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## Autophagy/ Apoptosis Induced by Geraniol Through HIF-1 $\alpha$ /BNIP3/Beclin-1 Signaling Pathway in A549 CoCl<sub>2</sub> Treated Cells

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Running title:

### Autophagy and Geraniol on A549 CoCl<sub>2</sub> Treated Cells

#### Abstract:

#### Background:

During cancer growth, hypoxia occurs along with autophagy as an adaptive response to overcome cellular stress. Geraniol is a natural isoprenoid known for its wide anticancer activity and autophagy induction in the cancer cell.

#### Purpose:

To investigate the antihypoxic potential of geraniol with the incidence of autophagy and apoptotic cell death in A549 CoCl<sub>2</sub>treated cells.

#### Methods:

A549 cells were incubated for 24 h with GE and CoCl<sub>2</sub> either alone or in combination. We examined the cytotoxicity and cell viability of GE either alone or in combination therapy using MTT and trypan blue assay. GE modulating effect was determined on lipid peroxidation, antioxidant capacity markers, gene expression levels of HIF-1 $\alpha$ , NF-kB, VEGF, autophagy factors in different groups, besides apoptotic bodies using AO/EB.

#### Results:

GE and CoCl<sub>2</sub>combination therapy downregulated the expression of HIF-1 $\alpha$  that suppressed A549 cell growth through downregulation of BNIP3 and Beclin-1 gene expression. This resulted in autophagy and

apoptotic cell death, in addition to the downregulation of NF- $\kappa$ B and VEGF expression. Also, GE treatment significantly reduced the oxidative stress markers and restored the antioxidant capacity.

**Conclusion:**

GE possesses an antihypoxic effect on A549 CoCl<sub>2</sub> treated cells and induces cell death via autophagy along with apoptosis through HIF-1 $\alpha$ /BNIP3/Beclin-1 signaling pathway.

**Key words:**

Hypoxia, Geraniol, HIF-1 $\alpha$ , Autophagy, BNIP3, Beclin-1, VEGF

**Abbreviation:**

GE, Geraniol; MDA, malondialdehyde; TAC, total antioxidant capacity; NF- $\kappa$ B, nuclear factor kappa-light-chain-enhancer of activated B cells; CoCl<sub>2</sub>, Cobalt chloride; HIF-1 $\alpha$ , hypoxia-inducible factor-1 alpha; BNIP3, Bcl-2 19KD interacting protein; Beclin-1, mammalian orthologue of yeast Atg6; VEGF, Vascular Endothelial Growth Factor.

## 1. Introduction

Lung cancer is one of the leading causes of death worldwide. This is due to the formation of wide areas of hypoxic tissue that reduce the response to radiotherapy and chemotherapy.<sup>1</sup> Therefore, developing anti-hypoxic drugs is the main aim of lung cancer therapy by targeting hypoxia, concurrent sequences of angiogenesis, invasiveness, and tumor growth.<sup>2</sup>

Hypoxia and necrotic areas are developed from insufficient oxygen supply during solid tumor growth.<sup>3</sup> Hypoxia inducible factor-1 (HIF-1) is the main cellular regulator during hypoxia, composed of oxygen regulated HIF-1  $\alpha$  subunit as well as the constitutively expressed HIF-1  $\beta$  subunit.<sup>4,5</sup> HIF-1  $\alpha$  role during hypoxia is to maintain blood, nutrients as well as energy resumption through the expression of regulatory genes controlling glycolysis, angiogenesis, and erythropoiesis.<sup>6,7</sup> Recent studies reported a bi-directional relation between HIF-1  $\alpha$  and NF- $\kappa$ B because HIF regulates the expression of NF- $\kappa$ B mRNA and vice versa.<sup>8</sup>

Autophagy is a self-eating and dynamic phenomenon that depends on lysosomal degradation of the damaged mitochondria, cytoplasm, lipids, and misfolded proteins back to their basic components with cellular recycling to preserve cellular homeostasis.<sup>9,10</sup> Autophagy is present at a low level under physiological conditions but increased under cellular stress ailments including hypoxia, and nutrient deficiency and ROS production.<sup>10-12</sup> Autophagy and apoptosis share many regulators and interactions like Beclin-1/Bcl-2,<sup>13</sup> and caspase/Beclin-1 cleavage.<sup>14</sup> During moderate hypoxic conditions, HIF-1 $\alpha$  induces activation of autophagy in a dependent manner and supplies cancer cells with cellular energy requirement for solid tumor proliferation.<sup>15,16</sup> However, severe hypoxia induces autophagy in HIF-1 $\alpha$  independent manner through involvement of the AMPK-mTOR and unfolded protein response pathways.<sup>15,17</sup> BNIP3 and Beclin-1 (Bcl-2 gene family) are cell death proteins that play an important role in hypoxia associated apoptosis, necrosis, and autophagy.<sup>18-20</sup> Thus, BNIP3 and Beclin-1 are markers for appraising autophagy.

Geraniol is a natural isoprenoid monoterpene derived from the essential oils of various aromatic plants including lemon, ginger, rose, and orange.<sup>21</sup> Previous *in vitro* and *in vivo* studies showed that GE possesses anti-microbial, anti-inflammatory, and anti-oxidant properties.<sup>22,23</sup>

Moreover, GE exerts an apoptotic, antitumor, and anti-angiogenic effect on various cancer cells,<sup>24–26</sup> besides being a potent inducer of autophagy.<sup>27</sup>

Taking these previous pharmacological effects into account, this study was designed to determine the protective effect of GE against CoCl<sub>2</sub> induced hypoxia associated autophagy on the A549 cell line and focused on the relation between HIF-1  $\alpha$ , autophagy, and apoptotic cell death.

## **2. Materials and methods**

### **2.1. Media and reagents:**

All chemicals and reagents were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated during this study. This study was achieved at the Cell Culture Research Unit on October 6 University (Cairo, Egypt).

### **2.2. Cell line and cell culture:**

Human lung adenocarcinoma epithelial A549 cells (ATCC, USA) were cultured according to the American Type Culture Collection protocol.<sup>28</sup> All experiments were repeated three times independently, and the data were represented as the mean  $\pm$  SD.

### **2.3. MTT assay:**

A549 cells were grown to 90% confluence then undergo trypsinization followed by counting with a hemocytometer. Afterward, A549 cells were seeded in 96 well tissue culture plates at  $10 \times 10^3$ /well in triplicates for 24 hours for cell attachment before further additions. The A549 cells were treated with various concentrations of GE (0.161 to 2.59 mM) and CoCl<sub>2</sub> (0.193 to 7.75mM) either alone or in combination then incubated for 24 hours.<sup>29,30</sup> After treatment, the standard 3,4,5-dimethylthiazol-2,5- diphenyl tetrazolium bromide (MTT) method was used to assess the cytotoxicity of GE alone or its combination with CoCl<sub>2</sub>.<sup>31</sup> The IC<sub>50</sub> concentration of GE and CoCl<sub>2</sub> either alone or in combination on the A549 cell line after 24 h were determined and used to assess other bioassays. The mean percentages of cell viability were detailed as mean  $\pm$  SD.

### **2.4. Cell viability by the trypan blue dye exclusion assay:**

Cell viability of A549 cells in treated and untreated groups was determined by the trypan blue assay that depends on quantify live cells by labeling dead cells exclusively. The A549 cells were stained with 10 $\mu$ L trypan blue dye (0.4% solution) and to count unstained live and blue stained dead cells under a phase contrast microscope with a hemocytometer.<sup>32</sup> The mean percentages of live and dead cells per experiment were expressed as mean  $\pm$  SD of three autonomous experiments.

### **2.5. Apoptosis and necrosis Staining:**

Acridine orange/ethidium bromide staining (AO/EB) used to detect the mode of cell death.<sup>33</sup> The mean percentages of live, apoptotic, and necrotic cells per experiment were determined according to Zakaria and her colleagues and were expressed as mean  $\pm$  SD.<sup>34</sup>

### **2.6. Cell lysate preparation:**

The preparation of cell lysate was done according to Zakaria and her colleagues to be used in the analysis of oxidative stress and inflammatory markers.<sup>34</sup> Bicinchoninic acid used to determine the protein content of total cell lysate using bovine serum albumin as a standard, data not cited.<sup>35</sup>

### **2.7. Estimation of oxidative stress markers (MDA and TAC):**

Lipid peroxidation of cell lysate of treated and untreated groups was determined using malondialdehyde (MDA) as a representative of its final product using a commercially supplied kit (Biodiagnostic).<sup>36</sup> Also, the antioxidant capacity (TAC) of the cell lysate of different groups was assayed colorimetrically according to the protocol of the Biodiagnostic supplied kit.<sup>37</sup>

### **2.8. Gene expression analysis using quantitative real-time polymerase chain reaction (qRT-PCR):**

qRT-PCR was used to quantify the changes in HIF-1 $\alpha$ , NF- $\kappa$ B, VEGF, BNIP3, and Beclin-1 mRNA expression in different groups. In  $\Delta\Delta$ CT method, GAPDH mRNA was used as the internal control to detect the variation in the genes expression of HIF-1 $\alpha$ , NF- $\kappa$ B, VEGF, BNIP3, and Beclin-1 in all groups.<sup>38</sup> All primer sequences used in amplifying the HIF-1 $\alpha$ , NF- $\kappa$ B, VEGF, BNIP3, and Beclin-1 genes are listed in table 1.

**Table 1:**

Primer sequences used in qRT-PCR analysis

Target Gene	Primer Sequences
GAPDH	<b>F:</b> CTCTGATTTGGTCGTATTGGG <b>R:</b> TGGAAGATGGTGTATGGGATT
NF- $\kappa$ B	<b>F:</b> TGGTGCCTCACTGCTAACT <b>R:</b> GGATGCACTTCAGCTTCTGT
HIF-1 $\alpha$	<b>F:</b> ATCCATGTGACCATGAGGAAATG <b>R:</b> TCGGCTAGTTAGGGTACTTCTC
VEGF	<b>F:</b> AGGGCAGAATCATCACGAAGT <b>R:</b> AGGGTCTCGATTGGATGGCA
BNIP3	<b>F:</b> CAGGGCTCCTGGGTAGAACT <b>R:</b> CTACTIONCGTCCAGACTCATGC
Beclin-1	<b>F:</b> GGCTGAGAGACTGGATCAGG <b>R:</b> CTGCGTCTGGGCATAACG

### 2.9. Statistical analysis:

Experimental data were analyzed by Graph- Pad Prism (ISI®, USA) software (version 5) and expressed as mean  $\pm$  standard deviation (SD). One-way ANOVA followed by Tukey Kramer as a post hoc test was used to detect the variation between groups. A P-value of less than 0.05 was considered a statistically significant difference. (\*) is significant compared with the A549 cells, and (#) is significant compared with the CoCl<sub>2</sub> treated group.

## 3. Results:

### 3.1. Cytotoxicity of GE and CoCl<sub>2</sub> either alone or in combination

A significant decrease in cell viability in the CoCl<sub>2</sub>(hypoxic) and GE groups was observed compared to untreated cells and the calculated IC<sub>50</sub> was 6.2 mM and 2.59 mM subsequently, as shown in Fig. 1a-b. The effect of combined therapy was assessed on the viability of A549 using the IC<sub>50</sub> of CoCl<sub>2</sub> added to various concentrations of GE for 24 h. As shown in Fig. 1c, the combination therapy of CoCl<sub>2</sub> + 2.59 mM GE showed a significant reduction in cell viability (26 %  $\pm$  9) compared to untreated cells. Contrary to this, CoCl<sub>2</sub> + 1.29 mM of GE showed the least reduction in cell viability. Thus, the IC<sub>50</sub> value of GE (2.59 mM) and its diluted concentration  $\frac{1}{2}$  IC<sub>50</sub> 1.29 mM will be used in the combination therapy with IC<sub>50</sub> of CoCl<sub>2</sub> for 24 h in the following bioassays.

### 3.2. Trypan blue dye exclusion assay

As shown in Fig. 2, A549 cells treated with GE (2.59 and 1.29 mM) showed a significant decrease in the number of viable cells 48%  $\pm$  2.6, and 46%  $\pm$  3.2 respectively regarding that noticed in untreated cells (98%  $\pm$  0.6). Also, CoCl<sub>2</sub> A549 treated cells (6.2 mM) either alone or in combination, showed a major reduction in the number of viable cells (60%  $\pm$  2.6, 25%  $\pm$  3.8, and 32%  $\pm$  2.1 respectively) compared to that observed in untreated cells.

### 3.3. Apoptosis and necrosis staining

Under a fluorescence microscope, dual DNA staining by AO/EB dyes was used to determine the mode of cell death in untreated and treated A549 cell line. The untreated group showed no sign of cell death and most of the cells are viable with uniform green fluorescence color (Fig.3a-b). CoCl<sub>2</sub> treated group showed a high number of yellow to orange stained cells with a mean percentage of apoptotic cell death of 44% ± 1.5 compared to that noticed in untreated cells (93% ± 1) (Fig.3a-c). Groups treated with GE (2.59 and 1.29 mM) and CoCl<sub>2</sub> showed a significant percentage of apoptotic cell death (85% ± 2 and 75% ± 2.5 respectively) compared to that detected in CoCl<sub>2</sub> treated group as well as few necrotic cells that display orange to red nuclei (Fig.3d). Moreover, GE treated groups showed a meaningful percentage of apoptotic cells (50% ± 1 and 40% ± 1.5 respectively) with bright green to yellow color compared to untreated groups (Fig.3e).

### 3.4. Oxidative stress marker and antioxidant activity

Combined therapy of GE (2.59 and 1.29 mM) and CoCl<sub>2</sub> (6.2 mM) on A549 for 24 h showed a significant reduction in lipid peroxidation in terms of MDA level (1.9 ± 0.1 and 2.8 ± 0.6 respectively) compared to that observed in CoCl<sub>2</sub> treated cells (34.4 ± 0.75); MDA is an eminent detector for oxidative stress (Fig. 4a). On the other hand, total antioxidant capacity in untreated and treated groups was determined to detect the effect of ROS generation on antioxidant system. Combination therapy of GE (2.59 and 1.29 mM) and CoCl<sub>2</sub> as well as GE treated groups showed a significant elevation in the TAC levels (0.161 ± 0.01, 0.173 ± 0.007, 0.16 ± 0.003, and 0.169 ± 0.003 respectively) compared to that noticed in CoCl<sub>2</sub> treated cells (0.11 ± 0.01) as presented in Fig. 4b.

### 3.5. Relative expression of HIF-1 $\alpha$ , NF- $\kappa$ B, VEGF, BNIP3, and Beclin-1 mRNA in different groups:

As shown in Figs. 5a-e, CoCl<sub>2</sub> treated group showed a significant upregulation in the mRNA expression levels of HIF-1 $\alpha$  (9.4 fold), NF- $\kappa$ B (11.74 folds), VEGF (8.46 folds), BNIP3 (5.3 folds), and Beclin-1 (2.7 folds) compared to untreated groups, indicating a significant incidence of hypoxia, autophagy as well as angiogenesis. On the other hand, Combined therapy of GE (2.59 and 1.29 mM) and CoCl<sub>2</sub> showed a significant downregulation in the mRNA expression levels HIF-1 $\alpha$  (6.4 and 7.15 folds respectively), NF- $\kappa$ B (5.1 and 7.4 folds respectively), VEGF (6.1 and 6.9 folds respectively), and BNIP3 (3.1 and 2.5 folds respectively) as well as a modest downregulation in the mRNA of Beclin-1 expression (2 and 1.9 folds respectively) compared to CoCl<sub>2</sub> treated group. It is worthwhile to mention that GE treated groups showed a significant upregulation in the mRNA expression levels of BNIP3 (1.95 and 2.05 folds respectively), and Beclin-1 (4.54 and 2.01 folds respectively) compared to that noticed in untreated groups.

## 4. Discussion

Exploration of antihypoxic agents from plant origin has drawn great attention these years in cancer research. From MTT results, GE induced cytotoxicity and loss of A549 cell viability at a concentration of 2.59 mM (IC<sub>50</sub> value). Yet, Galle and his colleagues reported that the IC<sub>50</sub> value of GE was 797.2  $\mu$ M.<sup>29</sup> Also, CoCl<sub>2</sub> is a hypoxic imitator agent that induces structural modification in the heme protein O<sub>2</sub> sensor that results in the generation of ROS, which provokes oxidative stress resulting in hypoxia-induced cytotoxicity.<sup>39</sup> In this study, the IC<sub>50</sub> value of CoCl<sub>2</sub> was 6.2 mM. But Mahey and her colleagues reported that 29.81 mg/l (231  $\mu$ M) is the IC<sub>50</sub> value of CoCl<sub>2</sub>.<sup>40</sup>

ROS formation in the mitochondria during hypoxia aggravates the oxidative stress, induces excessive autophagy, and disrupts the oxidant/antioxidant balance within the cells.<sup>41,42</sup> This study showed that the IC<sub>50</sub> value of CoCl<sub>2</sub> (6.2 mM) disrupted the oxidant/antioxidant balance in A549 cells, which results in the incidence of lipid peroxidation with a subsequent reduction in the TAC level. This finding further corroborates earlier observations of Tripathi et al.<sup>32</sup> as well as Mohamed et al.<sup>43</sup> GE (2.59 and 1.59 mM) possesses antioxidant capacity when combined with CoCl<sub>2</sub> by reducing the level

of MDA parallels with the elevation in the TAC level. This finding is similar to that of Khan and his colleagues.<sup>21</sup> The antioxidant activity of GE results from increasing endogenous antioxidant defense system through the elevation in glutathione peroxidase and superoxide dismutase activity via prompting their transcriptional up-regulation.<sup>22,44</sup>

HIF-1 $\alpha$  is released during hypoxia to maintain oxygen homeostasis besides the upregulation of transcriptional factor NF- $\kappa$ B; both are factors of stress response to tumor-associated hypoxia.<sup>45</sup> Recent studies reported a bi-directional relation between HIF-1  $\alpha$  and NF- $\kappa$ B because HIF regulates the expression of NF- $\kappa$ B mRNA and vice versa.<sup>8,45</sup> Also, HIF and NF- $\kappa$ B activate the expression of several genes controlling angiogenesis (VEGF), cytokines (TNF- $\alpha$ ), chemokines (IL-8), and cell death related proteins (Noxa and BNIP3).<sup>45</sup> Under stress condition such as hypoxia, NF- $\kappa$ B nexuses autophagy and inflammation through activation of mTOR, which in turn stimulates HIF-1  $\alpha$  and NF- $\kappa$ B transcriptional activation results in a sequence of hypoxic and inflammatory cascades that ends in autophagy and inflammation.<sup>46</sup> Together, these findings further support the results of our study where CoCl<sub>2</sub> treated cells showed a rapid upregulation in mRNA of HIF-1 $\alpha$ , NF- $\kappa$ B, VEGF, BNIP3, and Beclin-1 expression. Accordingly, these results indicated the rapid induction of hypoxia with angiogenesis that is concurrent with autophagy, and this agrees with other researchers using a similar approach.<sup>43,47,48</sup> The most consistent effect of CoCl<sub>2</sub> to accumulate HIF-1 $\alpha$  is through the direct binding of cobalt with oxygen-dependent degradation domain of HIF- $\alpha$ , followed by the suppression of hydroxylated HIF-1 $\alpha$  to be produced, as well as its interaction with von Hippel-Lindau protein.<sup>49</sup> Also, transcriptional activation of HIF-1 $\alpha$  induced by CoCl<sub>2</sub> results in the direct activation of BNIP3 (hypoxia response element), induced programmed cell death as well as regulates autophagy through hindering the binding between Bcl-2 and Beclin-1.<sup>50,51</sup>

GE is a natural product that blocks the modification of I $\kappa$ B which results in the inhibition of NF- $\kappa$ B activation as well as its transcriptional activation in TPA treated mouse skin.<sup>21</sup> Also, GE possesses a strong antiangiogenic, anti-inflammatory, antiproliferative, and apoptosis effect through the downregulation of VEGF, NF- $\kappa$ B, cyclin D1, PCNA, c-fos, p53 and Bcl-2 in buccal pouch carcinogenesis.<sup>52</sup> Moreover, GE suppresses the AKT signal with the activation of the AMPK pathway, followed by the suppression of mTOR phosphorylation that results in autophagy in PC-3 cells.<sup>53</sup> Furthermore, GE elevated the BNIP3 and BAX (Bcl-2 family members, apoptotic markers) as well as downregulated the cell cycle regulators (cyclins and CDKs) in PC-3 cells.<sup>53</sup> Inhibition of HIF-1 $\alpha$  can suppress autophagy by reducing the amount of LC3-II and LC3-I in oral squamous cell carcinoma.<sup>54</sup> Together, these findings further support the results of this study. Combination therapy of GE (2.59 and 1.59 mM) with CoCl<sub>2</sub> promotes the down-regulation of HIF-1 $\alpha$  and NF- $\kappa$ B expression that consequently downregulates the angiogenic expression factor (VEGF); reduction of autophagy was induced through the downregulation of BNIP3 with a modest downregulation in Beclin-1 expression. It is important to mention that GE treated groups showed a significant upregulation in BNIP3 and Beclin-1 expression indicates that the ability of GE to induce autophagy via the BNIP3/Beclin-1 signaling pathway. Our results corroborate the observations of Kim and his colleagues who reported that GE affects the expression of BNIP3 in PC-3 cells.<sup>53</sup> Still, there are no reports on the effect of GE on HIF-1 $\alpha$  in hypoxia and how it connected to autophagy through the BNIP3/Beclin-1 signaling pathway.

Autophagy and apoptosis share many regulators and interactions like Beclin-1/Bcl-2,<sup>13</sup> and caspase/Beclin-1 cleavage.<sup>14</sup> Apoptosis induced by insufficient and excessive autophagy that resulted from the accumulation of autophagic vacuoles.<sup>55</sup> Also, inhibition of autophagy was accompanied by apoptotic cell death in colorectal cancer cells.<sup>56</sup> This study showed that CoCl<sub>2</sub> treated group induced a significant increase in the percentage of apoptotic cells compared to that of necrotic cells as well as upregulation in the expression levels of BNIP3 and Beclin-1. Cobalt ion possesses a cytotoxic activity and induces hypoxia followed by the upregulation and downregulation of apoptotic and anti-apoptotic

proteins subsequently, which stimulates apoptotic cell death.<sup>32</sup> Also, Chen et al showed that CoCl<sub>2</sub> upregulates the HIF-1 $\alpha$  as well as autophagic proteins (LC3, BNIP3, and Beclin-1) in the C2C12-hypoxic model.<sup>51</sup> These reports and our current results confirmed the link between CoCl<sub>2</sub>, hypoxia, autophagy signaling, and apoptotic cell death.

Moreover, GE in the combination therapy with CoCl<sub>2</sub> showed a significantly higher percentage of apoptotic cells and a down regulation in the BNIP3/Beclin-1 level. Also, GE alone showed a significant percentage of apoptotic cells, an upregulation in the BNIP3/Beclin-1 level. Our results corroborate the observations of Kim and his colleagues who reported the GE induced autophagy and apoptotic cell death in PC-3 cell.<sup>53</sup> BNIP3 (Bcl-2 19KD interacting protein) is a mitochondrial outer membrane protein that is activated under hypoxia by the action of HIF-1 to clear the impaired mitochondria; therefore driving autophagy by acting as a mitophagy receptor.<sup>50</sup> Furthermore, BNIP3 can avert the connection between Beclin-1 and Bcl-2, freeing Beclin-1 to hasten the autophagic process through autophagosome formation. This in turn induces apoptosis by increasing mitochondrial membrane permeability.<sup>18-20</sup> Also, Beclin-1 overexpression by an anticancer drug resulted in apoptotic cell death in cervical cancer cells.<sup>57</sup>

## 5. Conclusion

In this study, we assessed the antihypoxic role of GE (2.59 and 1.29 mM) against CoCl<sub>2</sub> induced hypoxia in the A549 cell line which relies on restoring pro-oxidant / antioxidant equilibrium. Also, GE modulates the inflammatory and angiogenic markers through downregulation of the expression levels of NF- $\kappa$ B and VEGF that results from the upregulation of HIF-1 $\alpha$  during hypoxia. Additionally, GE reduces autophagy in combination therapy by downregulating the expression levels of BNIP3 and Beclin-1, associated with an elevation in apoptotic cell death through HIF-1 $\alpha$  signaling pathways. Further experiments will be needed to explore the mechanism of GE in controlling hypoxia-associated autophagy at the molecular and cellular levels in different cell types.

## 6. Study highlights:

### Current knowledge:

GE exerts an apoptotic, antitumor, and anti-angiogenic effect on various cancer cells, besides being a potent inducer of autophagy. No previous study mentioned the relation between GE and HIF-1 $\alpha$  in hypoxia and how it connected to autophagy through the BNIP3/ Beclin-1 signaling pathway.

### New findings:

- GE reduces the oxidative stress induced by CoCl<sub>2</sub> in A549 cells by reducing MDA and increasing TAC content.
- GE and CoCl<sub>2</sub> combination therapy downregulate the expression of HIF-1 $\alpha$  that in turn suppresses the A549 cell growth through downregulation of BNIP3 and Beclin-1 gene expression, results in autophagy and apoptotic cell death.
- GE and CoCl<sub>2</sub> combination therapy reduce the expression levels of inflammatory (NF- $\kappa$ B) and angiogenic factors (VEGF).

## 7. Declarations:

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Conflict of interest: The authors declare no potential conflicts of interest.

Author contributions: Dina M. Abo El-Ella designed experiments, performed the experiments, wrote the paper, revised and finalized the manuscript.

Ethical Issues: Not applicable.

## 8. References:

1. Salem A, Asselin MC, Reymen B, Jackson A, Lambin P, West CML, et al. Targeting Hypoxia to Improve Non-Small Cell Lung Cancer Outcome. *J Natl Cancer Inst* 2018;110(1). doi:10.1093/jnci/djx160
2. Lin SH, Koong AC. Breathing New Life Into Hypoxia-Targeted Therapies for Non-Small Cell Lung Cancer. *J Natl Cancer Inst* 2018;110(1). doi:10.1093/jnci/djx163
3. Baskaran R, Kalaiselvi P, Huang C-Y, Padma VV. Neferine, a bisbenzylisoquinoline alkaloid, offers protection against cobalt chloride-mediated hypoxia-induced oxidative stress in muscle cells. *Integr Med Res* 2015;4(4):231-241. doi:10.1016/j.imr.2015.09.002
4. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O<sub>2</sub> tension. *Proc Natl Acad Sci U S A* 1995;92(12):5510-5514. doi:10.1073/pnas.92.12.5510
5. Semenza GL. HIF-1 and mechanisms of hypoxia sensing. *Curr Opin Cell Biol* 2001;13(2):167-171. doi:10.1016/S0955-0674(00)00194-0
6. Semenza GL, Roth PH, Fang HM, Wang GL. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J Biol Chem* 1994;269(38):23757-23763.
7. Maltepe E, Simon MC. Oxygen, genes, and development: An analysis of the role of hypoxic gene regulation during murine vascular development. *J Mol Med* 1998;76(6):391-401. doi:10.1007/s001090050231
8. D'Ignazio L, Batié M, Rocha S. Hypoxia and inflammation in cancer, focus on HIF and NF- $\kappa$ B. *Biomedicines* 2017;5(2). doi:10.3390/biomedicines5020021
9. Zhang H, Chen Z. Autophagy and Cell Death: Antitumor Drugs Targeting Autophagy. In: Muhtasib HG, Editor. *Programmed Cell Death*. IntechOpen; 2019. P. 1-21.
10. Denton D, Xu T, Kumar S. Autophagy as a pro-death pathway. *Immunol Cell Biol* 2015;93(1):35-42. doi:10.1038/icb.2014.85
11. Wu H-M, Jiang Z-F, Ding P-S, Shao L-J, Liu R-Y. Hypoxia-induced autophagy mediates cisplatin resistance in lung cancer cells. *Sci Rep* 2015;5:12291. doi:10.1038/srep12291
12. Park SY, Sun EG, Lee Y, Kim MS, Kim JH, Kim WJ, et al. Autophagy induction plays a protective role against hypoxic stress in human dental pulp cells. *J Cell Biochem* 2018;119(2):1992-2002. doi:10.1002/jcb.26360
13. Liang XH, Kleeman LK, Jiang HH, Gordon G, Goldman JE, Berry G, et al. Protection against Fatal Sindbis Virus Encephalitis by Beclin, a Novel Bcl-2-Interacting Protein. *J Virol* 1998;72(11):8586-8596.
14. Wirawan E, Vande Walle L, Kersse K, Cornelis S, Claeherhout S, Vanoverberghe I, et al. Caspase-mediated cleavage of Beclin-1 inactivates Beclin-1-induced autophagy and enhances apoptosis by promoting the release of proapoptotic factors from mitochondria. *Cell Death Dis* 2010;1(1):e18. doi:10.1038/cddis.2009.16



15. Fang Y, Tan J, Zhang Q. Signaling pathways and mechanisms of hypoxia-induced autophagy in the animal cells. *Cell Biol Int* 2015;39(8):891-898. doi:10.1002/cbin.10463
16. Denko NC. Hypoxia, HIF1 and glucose metabolism in the solid tumour. *Nat Rev Cancer* 2008;8(9):705-713. doi:10.1038/nrc2468
17. Mazure NM, Pouyssegur J. Hypoxia-induced autophagy: Cell death or cell survival? *Curr Opin Cell Biol* 2010;22(2):177-180. doi:10.1016/j.ceb.2009.11.015
18. Eng CH, Abraham RT. The autophagy conundrum in cancer: Influence of tumorigenic metabolic reprogramming. *Oncogene* 2011;30(47):4687-4696. doi:10.1038/onc.2011.220
19. Lin YF, Chiu IJ, Cheng FY, Lee YH, Wang YJ, Hsu YH, et al. The role of hypoxia-inducible factor-1 $\alpha$  in zinc oxide nanoparticle-induced nephrotoxicity in vitro and in vivo. *Part Fibre Toxicol* 2016;13(1):1-14. doi:10.1186/s12989-016-0163-3
20. Zhang J, Ney PA. Role of BNIP3 and NIX in cell death, autophagy, and mitophagy. *Cell Death Differ* 2009;16(7):939-946. doi:10.1038/cdd.2009.16
21. Khan AQ, Khan R, Qamar W, Lateef A, Rehman MU, Tahir M, et al. Geraniol attenuates 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced oxidative stress and inflammation in mouse skin: Possible role of p38 MAP Kinase and NF- $\kappa$ B. *Exp Mol Pathol* 2013;94(3):419-429. doi:10.1016/j.yexmp.2013.01.006
22. Tiwari M, Kakkar P. Plant derived antioxidants - Geraniol and camphene protect rat alveolar macrophages against t-BHP induced oxidative stress. *Toxicol Vitro* 2009;23(2):295-301. doi:10.1016/j.tiv.2008.12.014
23. Solórzano-Santos F, Miranda-Navales MG. Essential oils from aromatic herbs as antimicrobial agents. *Curr Opin Biotechnol* 2012;23(2):136-141. doi:10.1016/j.copbio.2011.08.005
24. Wittig C, Scheuer C, Parakenings J, Menger MD, Laschke MW. Geraniol Suppresses Angiogenesis by Downregulating Vascular Endothelial Growth Factor (VEGF)/VEGFR-2 Signaling. *PLoS One* 2015;10(7):e0131946. doi:10.1371/journal.pone.0131946
25. Zhang Z, Zhao S, Li X, Zhuo X, Zhang W, Nie Q, et al. Amentoflavone Inhibits Osteoclastogenesis and Wear Debris-Induced Osteolysis via Suppressing NF- $\kappa$ B and MAPKs Signaling Pathways. *Planta Med* 2018;84(11):759-767. doi:10.1055/s-0043-124594
26. Qi F, Yan Q, Zheng Z, Liu J, Chen Y, Zhang G. Geraniol and geranyl acetate induce potent anticancer effects in colon cancer Colo-205 cells by inducing apoptosis, DNA damage and cell cycle arrest. *JBUON* 2018;23(2):346-352.
27. Kim SH, Park EJ, Lee CR, Chun JN, Cho NH, Kim IG, et al. Geraniol induces cooperative interaction of apoptosis and autophagy to elicit cell death in PC-3 prostate cancer cells. *Int J Oncol* 2012;40(5):1683-1690. doi:10.3892/ijo.2011.1318
28. Culturing A549  
Cells. [https://www.nanopartikel.info/files/methodik/VIGO/cell\\_culture\\_A549\\_hic\\_V1-1.pdf](https://www.nanopartikel.info/files/methodik/VIGO/cell_culture_A549_hic_V1-1.pdf).  
Accessed 14 January 2014.
29. Galle M, Crespo R, Kladniew BR, Villegas SM, Polo M, de Bravo MG. Suppression by geraniol

- of the growth of A549 human lung adenocarcinoma cells and inhibition of the mevalonate pathway in culture and in vivo: Potential use in cancer chemotherapy. *Nutr Cancer* 2014;66(5):888-895. doi:10.1080/01635581.2014.916320
30. Wu D, Yotnda P. Induction and testing of hypoxia in cell culture. *J Vis Exp* 2011;(54). doi:10.3791/2899
31. Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods* 1989;119(2):203-210. doi:10.1016/0022-1759(89)90397-9
32. Tripathi VK, Subramaniyan SA, Hwang I. Molecular and Cellular Response of Co-cultured Cells toward Cobalt Chloride (CoCl<sub>2</sub>)-Induced Hypoxia. *ACS Omega* 2019;4(25):20882-20893. doi:10.1021/acsomega.9b01474
33. Giuliano M, Lauricella M, Vassallo E, Carabillò M, Vento R, Tesoriere G. Induction of apoptosis in human retinoblastoma cells by topoisomerase inhibitors. *Investig Ophthalmol Vis Sci* 1998;39(8):1300-1311.
34. Zakaria S, Gamal-Eldeen AM, El-Daly SM, Saleh S. Synergistic apoptotic effect of Doxil® and aminolevulinic acid-based photodynamic therapy on human breast adenocarcinoma cells. *Photodiagnosis Photodyn Ther* 2014;11(2):227-238. doi:10.1016/j.pdpdt.2014.03.001
35. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985;150(1):76-85. doi:10.1016/0003-2697(85)90442-7
36. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979;95(2):351-358. doi:10.1016/0003-2697(79)90738-3
37. Koracevic D, Koracevic G, Djordjevic V, Andrejevic S, Cosic V. Method for the measurement of antioxidant activity in human fluids. *J Clin Pathol* 2001;54(5):356-361. doi:10.1136/jcp.54.5.356
38. Yuan JS, Reed A, Chen F, Stewart CN Jr. Statistical analysis of real-time PCR data. *BMC Bioinformatics* 2006;7:85-97. doi:10.1186/1471-2105-7-85
39. Pialoux V, Mounier R, Rock E, Mazur A, Schmitt L, Richalet JP, et al. Effects of acute hypoxic exposure on prooxidant/antioxidant balance in elite endurance athletes. *Int J Sports Med* 2009;30(2):87-93. doi:10.1055/s-0028-1103284
40. Mahey S, Kumar R, Arora R, Mahajan J, Arora S, Bhardwaj R, et al. Effect of cobalt(II) chloride hexahydrate on some human cancer cell lines. *Springerplus* 2016;5:930-944. doi:10.1186/s40064-016-2405-0
41. Scherz-Shouval R, Elazar Z. Regulation of autophagy by ROS: Physiology and pathology. *Trends Biochem Sci* 2011;36(1):30-38. doi:10.1016/j.tibs.2010.07.007
42. Devasagayam TP, Tilak JC, Bloor KK, Sane KS, Ghaskadbi SS, Lele RD. Free radicals and antioxidants in human health: Current status and future prospects. *J Assoc Physicians India* 2004;52:794-804.
43. Abdel-Rahman Mohamed A, M.M.Metwally M, Khalil SR, Salem GA, Ali HA. *Moringa oleifera*

- extract attenuates the CoCl<sub>2</sub> induced hypoxia of rat's brain: Expression pattern of HIF-1 $\alpha$ , NF- $\kappa$ B, MAO and EPO. *Biomed Pharmacother* 2019;109:1688-1697. doi:10.1016/j.biopha.2018.11.019
44. Khan AQ, Khan R, Qamar W, Lateef A, Rehman MU, Tahir M, et al. Geraniol attenuates 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced oxidative stress and inflammation in mouse skin: possible role of p38 MAP Kinase and NF- $\kappa$ B. *Exp Mol Pathol* 2013;94(3):419-429. doi:10.1016/j.yexmp.2013.01.006
45. D'Ignazio L, Bandarra D, Rocha S. NF- $\kappa$ B and HIF crosstalk in immune responses. *FEBS J* 2016;283(3):413-424. doi:10.1111/febs.13578
46. Ravanan P, Srikumar IF, Talwar P. Autophagy: The spotlight for cellular stress responses. *Life Sci* 2017;188:53-67. doi:10.1016/j.lfs.2017.08.029
47. Li Q, Ma R, Zhang M. CoCl<sub>2</sub> increases the expression of hypoxic markers HIF-1 $\alpha$ , VEGF and CXCR4 in breast cancer MCF-7 cells. *Oncol Lett* 2018;15(1):1119-1124. doi:10.3892/ol.2017.7369
48. Hsu HL, Liao PL, Cheng YW, Huang SH, Wu CH, Li CH, et al. Chloramphenicol induces autophagy and inhibits the hypoxia inducible factor-1 alpha pathway in non-small cell lung cancer cells *Int J Mol Sci.* 2019;20(1). doi:10.3390/ijms20010157
49. Yuan Y, Hilliard G, Ferguson T, Millhorn DE. Cobalt inhibits the interaction between hypoxia-inducible factor-alpha and von Hippel-Lindau protein by direct binding to hypoxia-inducible factor-alpha. *J Biol Chem* 2003;278(18):15911-15916. doi:10.1074/jbc.M300463200
50. Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, Pouyssegur J, et al. Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. *Mol Cell Biol* 2009;29(10):2570-2581. doi:10.1128/MCB.00166-09
51. Chen R, Jiang T, She Y, Xu J, Li C, Zhou S, et al. Effects of Cobalt Chloride, a Hypoxia-Mimetic Agent, on Autophagy and Atrophy in Skeletal C2C12 Myotubes. *Biomed Res Int* 2017;7097580. doi:10.1155/2017/7097580
52. Vinothkumar V, Manoharan S, Sindhu G, Nirmal MR, Vetrichelvi V. Geraniol modulates cell proliferation, apoptosis, inflammation, and angiogenesis during 7,12-dimethylbenz[a]anthracene-induced hamster buccal pouch carcinogenesis. *Mol Cell Biochem* 2012;369(1-2):17-25. doi:10.1007/s11010-012-1364-1
53. Kim SH, Bae HC, Park EJ, Lee CR, Kim BJ, Lee S, et al. Geraniol inhibits prostate cancer growth by targeting cell cycle and apoptosis pathways. *Biochem Biophys Res Commun* 2011;407(1):129-134. doi:10.1016/j.bbrc.2011.02.124
54. Li Y-N, Hu J-A, Wang H-M. Inhibition of HIF-1 $\alpha$  Affects Autophagy Mediated Glycosylation in Oral Squamous Cell Carcinoma Cells. *Dis Markers* 2015;239479. doi:10.1155/2015/239479
55. Chen Y, Klionsky DJ. The regulation of autophagy - Unanswered questions. *J Cell Sci* 2011;124(2):161-170. doi:10.1242/jcs.064576
56. Zhuo J, Wang X. Combination of targeting CD24 and inhibiting autophagy suppresses the proliferation and enhances the apoptosis of colorectal cancer cells. *Mol Med Rep* 2019;20(1):539-548. doi:10.3892/mmr.2019.10288

57. Sun Y, Liu JH, Jin L, Lin SM, Yang Y, Sui YX, et al. Over-expression of the Beclin1 gene upregulates chemosensitivity to anti-cancer drugs by enhancing therapy-induced apoptosis in cervix squamous carcinoma CaSki cells. *Cancer Lett* 2010;294(2):204-210. doi:10.1016/j.canlet.2010.02.001

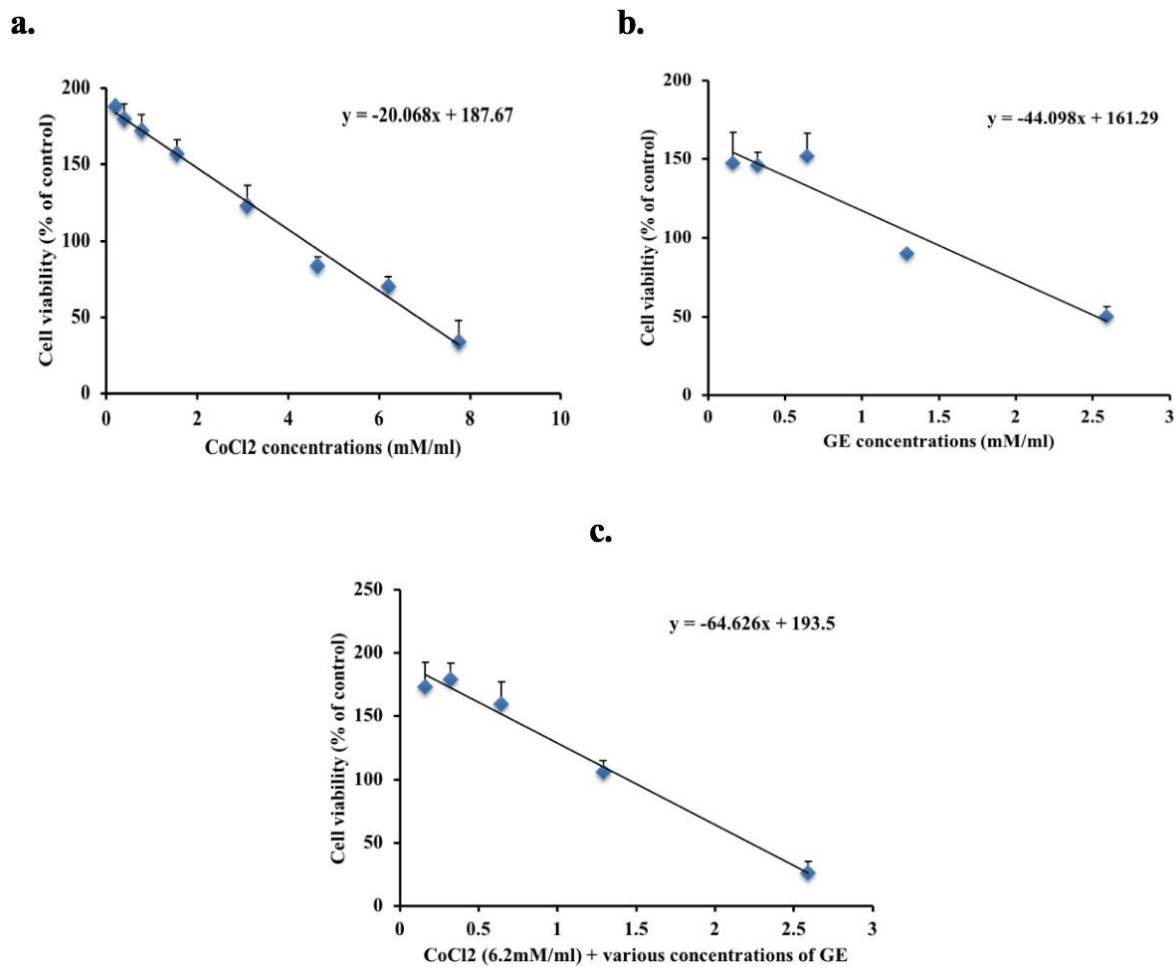


Fig1

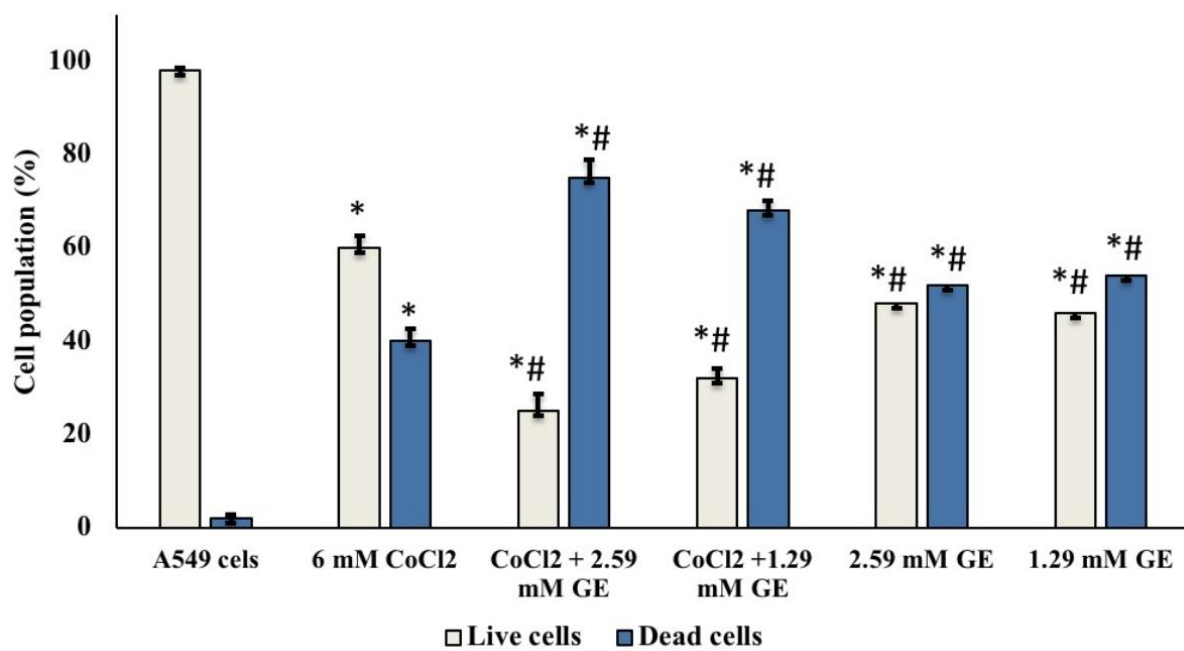


Fig2

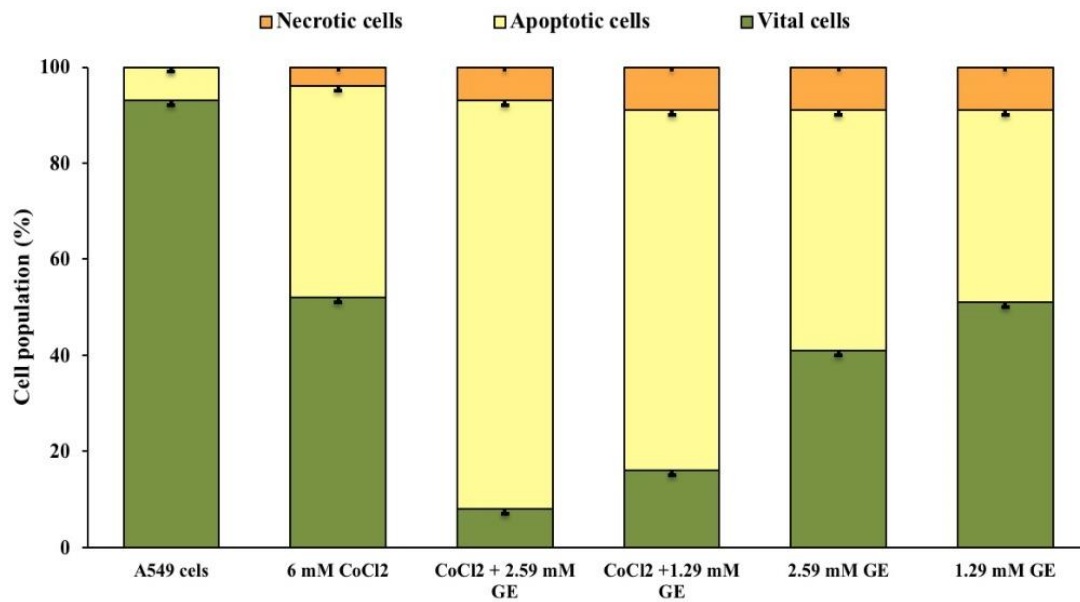
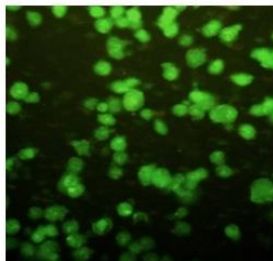
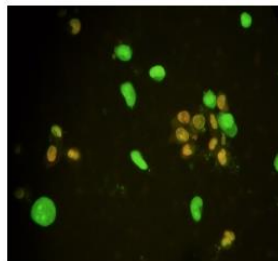
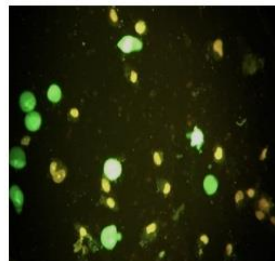
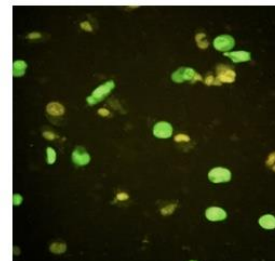
**a.****b. Untreated A549 Cells****c. 6.2 mM CoCl<sub>2</sub>****d. Combination therapy****e. 2.59 & 1.29 mM GE**

Fig3

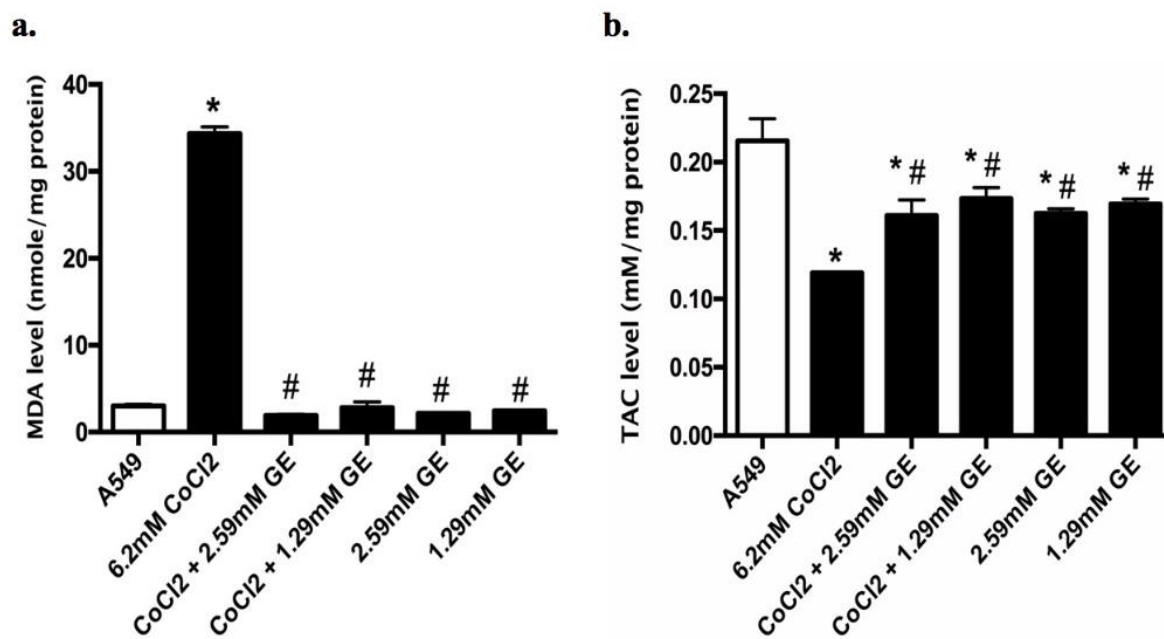


Fig4

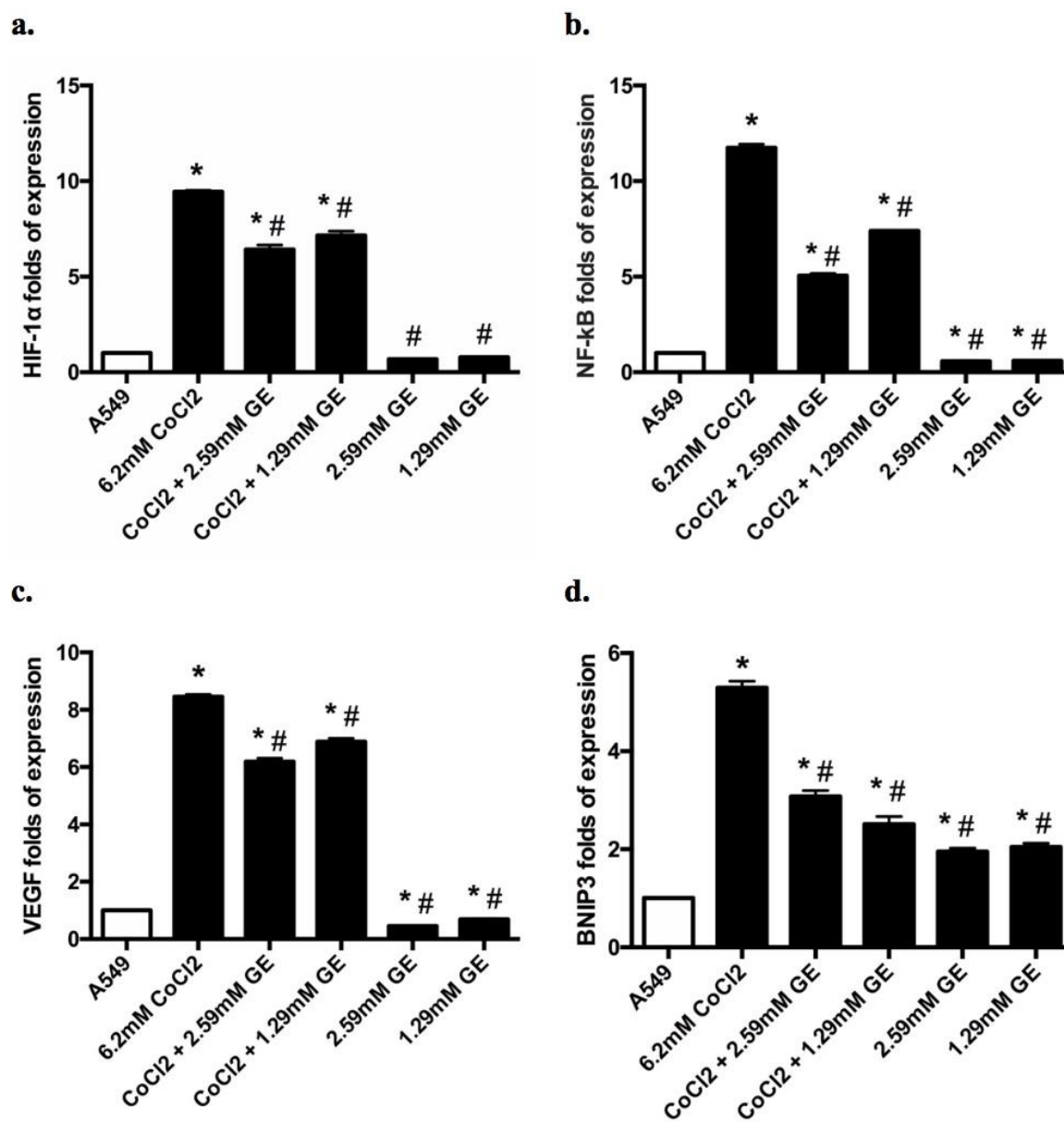


Fig5