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Research Article

Induction of Autophagy in Human Myeloid and Lymphoid Leukaemia Cell Line by Using Polyphenols Alone and Combined with A Stander Chemotherapy

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Abstract: **Background:** Autophagy is a major catabolic mechanism that contributes to the degradation of dysfunctional or unnecessary components in cells. The main function of autophagy depends on the variant cellular contexts and inducers. The results of autophagy can stimulate either cell death or cell survival. Autophagy is known as a protective process for cell survival if it provides the cell with the necessary requirements and nutrients, and removes organelles that were damaged. In contrast, anti-cancer drugs are often able to activate autophagy and in this case, autophagy induces apoptotic cell death. The high level of polyphenols found in some fruits and vegetables has been reported to be potentially chemotherapeutic and having a considerable effect on haematological malignancies. **Objectives:** To explore the effect of a stander chemotherapy (Imatinib, Doxorubicin) , and/or polyphenols (emodin, rhein, apigenin and cis-stilbene) on inducing autophagy of myeloid and lymphoid leukaemia cells compared to non-tumour cells. **Methods:** one myeloid (K562), one lymphoid (CCRF-CEM), a leukaemia cell line and one non-tumour normal cell line (CD133) were treated with different doses of polyphenols. Using a CYTO-ID® green detection reagent and Hoechst 33342 nuclear stain to detect autophagy by fluorescent microscope. **Results:** Emodin, cis-stilbene, apigenin and rhein showed different levels of effect on inducing autophagy in K562 myeloid cells, CCRF-CEM lymphoid cells and CD133+ normal cells when these types of polyphenols used separately and combined with chemotherapy. **Conclusion:** Generally, the CCRF-CEM lymphoid leukaemia cell line was more sensitive to polyphenol treatments alone and when combined with Doxorubicin compared to the K562 myeloid leukaemia cell line and CD133+ non-tumour cells. These results suggest that polyphenols have different effects according to the type of cell and polyphenol. The variant effect between leukaemia cells and non-tumour cell suggests that polyphenols are a potentially therapeutic agent for leukaemia. Polyphenols can enhance the effect of chemotherapy and reduce the required dose to induce cell death in cancer cells.

Keywords: Induction of Autophagy, Imatinib, Doxorubicin, Polyphenols, Polyphenols combined with chemotherapy., Human Myeloid and Lymphoid Leukaemia Cell Line.

1. INTRODUCTION

Autophagy is a major catabolic mechanism that contributes to the degradation of dysfunctional or unnecessary components in cells. It is also known as macro autophagy, which is a dynamic and an inherently regulated mechanism related to the creation of auto phagosomes and cytoplasmic vesicles, with a double-membrane that engulf cellular constituents (JING, K., & LIM, K. 2012). The formation of auto-phagosomes begins with phagophores, also known as sequestering membranes and is followed by conjugating microtubule-associated protein (light chain 3) to the phagophores and controlling their elongation (XIE, Z. *et al.*, 2008). As the sequestering membrane expands, it engulfs cytoplasmic components that contain organelles such as the endoplasmic reticulum, aggregated proteins, mitochondria and foreign microorganisms (virus and bacteria). When the auto-phagosome is created, it is fused with lysosome to become autolysosome and degrades the cargo with LC3 and autophagy from the outer membrane. This process acts as a regulatory mechanism for maintaining a homeostatic balance in

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mammalian cells, by clearing injured organelles and reprocessing autophagy-derived nutrients (Figure 1) (JING, K., & LIM, K. 2012).

However, the main function of autophagy depends on the variant cellular contexts and inducers. The results of autophagy can stimulate either cell death or cell survival. For example, autophagy is known as a protective process for cell survival if it provides the cell with the necessary requirements and nutrients, and removes organelles that were damaged. In contrast, anti-cancer drugs are often able to activate autophagy and in this case, autophagy induces apoptotic cell death (Amaravadi, R. K. *et al.*, 2007; Song, K. S. *et al.*, 2008). Nonetheless, autophagy damage can delay or stop the development of cell death (Yuk, J. M. *et al.*, 2010), and in other instances, induce cell death by itself. Cell death enhanced by autophagy may be associated with depredating essential factors for cell survival (Yu, L. *et al.*, 2006).

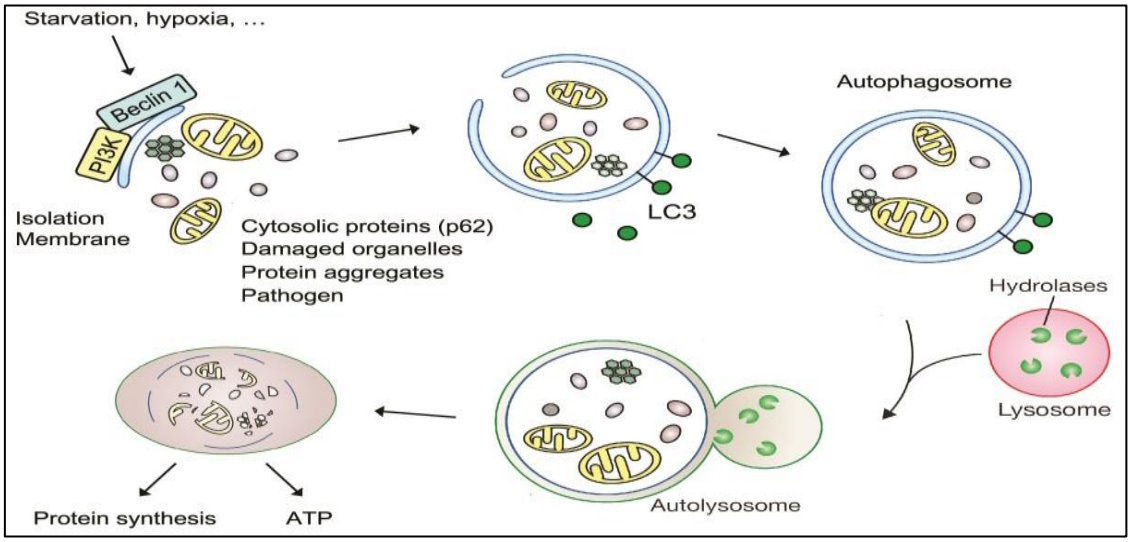

Figure1. The different stages of autophagy formation (DANIELLE, G. *et al.*, 2012**).**

Formation of the phagophore is the first step in autophagy, which conjugates with light chain 3 to control the elongation of the phagophore. The expansion of the isolation membrane (phagophore) leads to formation of the auto-phagosome, which can engulf cytoplasmic components containing organelles and foreign microorganisms (virus and bacteria). When the outer membrane of the auto-phagosome fuses with a lysosome (docking and fusion steps), it forms an auto-phagolysosome. Finally, the sequestered substance is destroyed inside the autophagolyosome (vesicle breakdown and degradation) and recycled.

The aim of this study was to determine the impact of polyphenols (emodin, apigenin, cis-stilbene and rhein) for inducing autophagy in one myeloid cell line (K562), one lymphoid cell line (CCRF-CEM) and one non-tumour normal cell line (CD133). The polyphenol doses and chemotherapy were then used separately and combined together to investigate if there was an antagonistic or synergistic effect for inducing autophagy.

2. METHODS AND MATERIALS:

2.1. Media preparation:

50 ml of RPMI media was removed into falcon tube. Then, 50 ml of fetal calf serum (FCS) was added, followed by 5 ml (1.5 mmol/L L-glutamine) and 5 ml 1 % penicillin/streptomycin to protect media from contamination by bacteria.

2.2. Cell Culture

Two leukaemia cell lines were obtained from the American Type Culture Collection (ATCC; Middlesex, U.K.). One myeloid cell lines: K562 (chronic myeloid leukaemia) (ATCC: CCL-243, Middlesex, UK) and one lymphoid cell line CCRF-CEM (acute lymphoblastic leukaemia) (ATCC: CCL-119, Middlesex, UK) together with the non-tumour (CD133) positive hematopoietic stem cells. These cell lines were maintained and cultured in RPMI 1640 medium (Invitrogen, Paisley, U.K.) supplemented with 10% (v/v) fetal bovine serum, 1.5 mmol/L L-glutamine, and 1 % penicillin/streptomycin (complete media) and then incubated in an atmosphere of 5% CO_2 at 37°C.

2.3. Cell Feeding

The cells needed to be fed and split every three days, which they were split into 1:4 flasks each Friday and fed with fresh media every 48 to 72 h. This was performed by centrifuging the cells for 5 minutes at 1500 rpm, and then re-suspended in new fresh media. If large quantities of suspension cells are not required, cells were fed by discarding a part of cells and spent media in a clean bleach pot and 20 ml of fresh media was added (Do not overfill > 40ml). These were repeated Monday, Wednesday and on Friday, cell lines were split as described earlier.

2.4. Procedures to Prevent Contamination:

Rings, watches or any decorative ornaments on the arms were removed and a clean lab coat was worn before contacting with cells. Hands were then washed up and using antibacterial detergent to clean the fume hood surfaces including the bottom surface under the plates. Gloves were changed and sprayed after contact with surfaces outside the fume hood or when working with a different cell line. All equipment was sprayed with bactericidal spray before entering the fume hood.

2.5. Counting Cells

Cells were counted using the haemocytometer by calculating the total number of 5 small squares in the central square (4 in corner squares and one in the centre square). After the cells counted, they were then multiplied by 5 and by $1x10^4$ to represent cells in a 0.02mm-cubed volume and in 1000 cubic millilitre, respectively (cells number X 5 X 10^4). For example, $32x5x10^4$ =$160x10^4$, for 96 well plate $2.5X10^3$ cells per well was required; therefore, for 100 wells $2.5x10^5$cells were needed which $160X10^4$=$16x10^5$ /ml, and $16x10^5$ =1000 µl from this $1x10^5$=1000/16 =1 $x10^5$=62.5 µl. So,$2.5x10^5$ =$62.5x2.5^=$ $2.5x10^5$ = 156 µl from cell suspension was added to 9844 µl media to get 10000 µl for 96 wells, then 100 µl was pipetted to each well and treated with 5 µl polyphenols as described above.

2.6. Polyphenols Treatment Preparation:

Polyphenols (Emodin, Apigenin, Rhein and Cis-stilbene was prepared to the required concentration (0, 2, 10, 50 and 250µM) from an original master stock. The polyphenols master stocks for emodin and apigenin were prepared by dissolving and sterilizing 27.024 mg, in 100µl of 100 % ethanol (Sigma). Then, dissolved in 900 µl serum free media (SFM) (Invitrogen) to make the final concentration of 100mMol/ml with a 10% ethanol (100000 ulmol/ml). The apigenin has the same molecular weight as emodin, so it prepared by the same procedures. Rhien also was prepared by the same methods just 28.422 mg was weighted from rhein powder instead of 27.024 mg. Cis-Stilbene provided dissolved as a 96% pure solution in a glass bottle.

The polyphenols dosages were prepared according to the well plate was used. For 96 well 5 µl of polyphenol was added to 100 µl of cells suspension per well. This is 1:20 therefore, the stock needed to be X20 more concentrated. For 24 and 12 well plates that were used for apoptosis and autophagy experiments which 10 µl of polyphenols was added to 1ml cell suspension. This is a 1:100 therefore the stock needed to be X100 more concentrated which were prepared as follow:

- To make 0 µM, 1000 µl serum free media was taken =0 µlmol
- To make 2 µM, 2 µl of stock was added to 998 µl (SFM) =200 µlmol.
- To make 10 µM 10 µl of stock was added to 990 µl (SFM) =1000µlmol.
- To make 50 µM 50 µl of stock was added to 950 µl (SFM). = 5000 µlmol.
- To make 250 µM, 250 µl of stock was addedto 750 µl (SFM). =25000 µlmol.

For 96 well plate 100 µl of cells was taken and treated with 5 µl of polyphenol, so there was a 1:20 dilution , therefore all stocks were 20 times more concentrated which were prepared by using the above concentration as follow:

- 20 µl of 200 µlmol stock was added to 80µl SFM to make 2µM stock = 40µlmol.
- 20µl of 1000µlmol stock was added to 80µl SFM to make 10µM stock = 200µlmol.
- 20µl of 5000µlmol stock was added to 80µl SFM to make 50µM stock = 1000µlmol.
- 20µl of 25000µlmol stock was added to 80µl SFM to make 250µM stock = 5000 µlmol.

2.7. Preparation of standard chemotherapy agents:

2.7.1. Imatinib:

14.7 mg of imatinib powder was dissolved in 1 ml sterile microbiology filtered distilled water. This made solution with 25 mmol solution. For 96 well plate, 1µMol concentration was needed out of the well. However, in well it should be 20 times stronger because 5 µl/ 100 µl this is a 1/20. This prepared by taking 4µl from super stock (25 mmol) and added to 4996 µl SFM with 10% ethanol to form 5 ml Imatinib with (1µMol) concentration.

2.7.2. Doxorubicin:

14.5 mg from doxorubicin powder was dissolved in 1 ml sterile microbiology filtered distilled water. This made solution with 25 mmol solution. For 96 well plate, 1µMol concentration were needed out of the well. However, in well it should be 20 times strong because 5 µl/ 100 µl this is a 1/20. This prepared by taking 5µl from super stock (25 mmol) and added to 4995 µl SFM with 10% ethanol to form 5 ml Doxorubicin with (1µMol) concentration.

2.8. Seeding the Cells:

Cells were seeded into 96-well plate (Fisher Scientific, Leicestershire, United Kingdom) at 2.5 X 10^3 cells per well and then treated with different concentration (0,2,10,50,250) µM of different types of polyphenols according to cell type. For example, emodin (Sigma, Poole, UK) was used for (CCRF-CEM, K562 and CD133), apigenin and cis-stilbene (Sigma, Poole, UK) were used for (K562 and CD133) and rhein (Sigma, Poole, UK) was used for (CCRF-CEM and CD133). All treatments were done in triplicate, and incubated at 37 ^{0}C for 24 hours. Then, 96 well plate and its contents were equilibrated at room temperature (RT) for 30 minutes. After that, 10 µl of reagent was added to each well and mixed well on an orbital shaker at 400 rpm to induce cell lysis and incubated for 10 minutes at RT to stabilize the luminescent signal. Then, 96 well

plat was read by using Wallace 1420 luminescence detector (PerkinElmer, Waltham, USA). Control was prepared with media without cells.

In a white 96 well plate standard curve of the luminescence was designed against cell number between 0 and 50000 cells per well to ensure luminescence was in the linear range. The density of cells that prepared was: 50,000; 25,000; 12,500; 6250; 3125 1562, 780 and 0. Then, IC_{50} was determined from a linear regression equation of each standard curve for each polyphenol with each cell line which is known as the treatment concentration at which 50% inhibition in cellular proliferation was detected which was performed to determine the effectiveness of treatments. The IC_{25} and IC_{10} were also calculated in order to provide treatment ranges for apoptosis detection.

2.9. Combination of Polyphenols with Chemotherapy:

After $IC_{50,}$ IC_{25} and IC_{10} were determined, cells were seeded into 96-well plate (Fisher Scientific, Leicestershire, United Kingdom) with 100 µl at 2.5 $x10^3$ cells per well as previously described and then treated with 5 µl of Imatinib for (K562 and CD133) or Doxorubicin for (CCRF-CEM and CD133) alone and combined with 5 µl of IC_{50}, IC_{25} and IC_{10} (LSD) of different types of polyphenols according to cell type as above. All treatments were done in triplicate, in three independent experiments and incubated at 37^oC for 24 hours. Then, completed as described above. This step was performed to see if there is any synergistic result for polyphenols when combined with chemotherapy.

2.10. Autophagy Assay

CYTO-ID® Autophagy Detection Kit has been optimized for measuring autophagy in live cells by using a novel dye that selectively marks autophagic vacuoles. This was detected by using fluorescence microscope. The best way of observing autophagic activity is by measuring the increased numbers of auto phagosomes in cells responding to induction. Monitoring autophagic flux provides a meaningful way the source of auto phagosomes. Rapamycin and starvation are known as inducer of autophagy (positive control), Chloroquine is a lysosome inhibitor. Rapamycin and Chloroquine were used as positive control.

2.10.1. Reagent preparation

2.10.1.1. Rapamycin

Rapamycin was provided lyophilized, so it was re-suspended in 50 µl of DMSO. This formed 500 µM stock solutions.

2.10.1.2. Chloroquine

Chloroquine was also supplied lyophilised (7.5 µmoles) which was centrifuged to gather the material at the bottom of the tube and reconstituted in 125 µl deionized water for 60 mM stock solution.

2.10.1.3. .1x assay buffer

1 ml from 10x assay buffer that was provided was taken and added to 9 ml deionized water.

2.10.1.4. -CYTO-ID® Green detection reagent

This was prepared by taking 1 ml of 1x assay buffer, then 2 µl of cyto-ID® green detection reagent and 1 µl of Hoechst 33342 nuclear stain were added and mixed well.

2.10.2. Procedure

The autophagy assay was performed for cells that showed synergistic effects of polyphenols when combined with chemotherapy.

24 well plates were prepared for each cell line at 1 x 106 cells per well. 1 ml of 1 x 106 cell were pipetted to each well and treated with 10 µl of IC50, IC25, and IC10 of polyphenols, chemotherapy agents and combination of chemotherapies with polyphenols in the cell that showed synergistic results. In addition, the CYTO-ID® Autophagy Detection Kit was pipetted to four well as follow: 10 DMSO, Rapamycin, Chloroquine, and Rapamycin and Chloroquine together in four wells. Then, incubated at 370C with 5% CO2 for 18 hours. Then, cells were collected in Eppendorf tubes and centrifuged at 2000 rpm for 3 min. After that, cells were washed twice with 1x Assay Buffer. After the supernatant was removed, about 100 µl of Microscopy Dual Detection Reagent solution was added to each tube and re-suspended by pipette. The samples were after that covered with foil to protect them from the light and incubated at 370C for 30 min. Another time cells were washed twice with 1x assay Buffer and added 4% paraformaldehyde for 20min to fix cells. Cells were then washed again three times using 1x Assay Buffer. Finally, the supernatant was removed and pellet re-suspended in 100 µl of 1x Assay Buffer. Then, one drop was applied on the microscope slides and a drop of immersion oil was added and coverslip was also applied and sealed with nail varnish.

Slides were analysed by fluorescent microscopy (Olympus, BX60). Cells were analysed by fluorescent microscopy using magnification 400. Pictures were taken by Q capture-pro 8.0 (UVP Bio Imaging Systems) and saved on memory stick.

3. RESULTS

3.1. Effect of combining chemotherapy and polyphenols on the induction of Autophagy in leukaemia cell lines after 24h treatments

Cell lines that showed an additive or synergistic effect on the inhibition of ATP levels when treated with IC_{50}, IC_{25} and IC_{10} of polyphenols combined with chemotherapy (Imatinib or Doxorubicin) were also investigated for their effect on the induction of autophagy. After treatments with the selected polyphenols/chemotherapy combination treatments, cells were stained using a CYTO-ID® green detection reagent and Hoechst 33342 nuclear staining, and

examined by florescent microscope, and images were captured. A DMSO negative control and Rapamycin and Chloroquine positive control were also included. By looking to the images that were collected and comparing them to the positive and negative controls, it was concluded that K562 showed autophagic induction when treated with 0.05µM of Imatinib and when Imatinib was combined with the IC_{25} of emodin and cis-stilbene (8 µM and 28 µM) respectively. CCRF-CEM cell lines that were treated with 0.05µM of Doxorubicin also showed the induction of autophagy, which is indicated by the arrow. In contrast, CD133 cells did not indicate any autophagic induction following treatment (Figure 2).

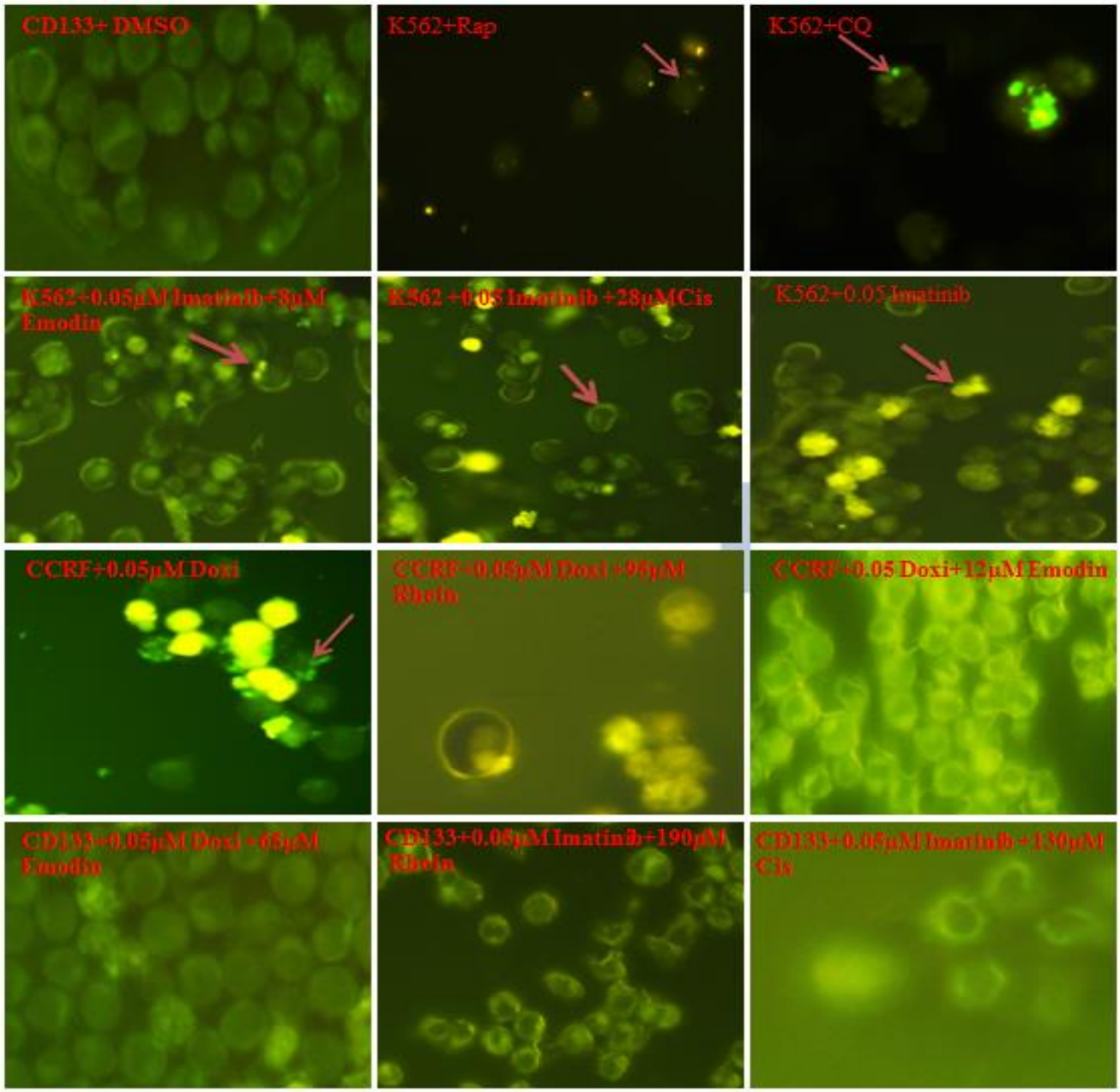

Figure 2. Atypical example of autophagic induction in K562 myeloid leukaemia cells, CCRF-CEM lymphoid leukaemia cells and the CD133+ non-tumour cells following treatment combining polyphenols and chemotherapy for 24h and stained using a CYTO-ID® green detection reagent and Hoechst 33342 nuclear staining. DMSO was used as a negative control, while Rapamycin, Rapamycin and Chloroquine were used as positive controls. The results were determined by comparing images of treated cells to the negative and positive controls.

4. DISCUSSION

Nonetheless, the results of this study are consistent with existing evidence on the anticancer properties of polyphenol. A number of research studies have identified the polyphenols apigenin (Zhu, Y. *et al.*, 2013; Shukla, S. *et al.*, 2014), cis-stilbene (Mahbub, A. *et al.*, 2013; Chen, Y. C. *et al.*, 2002), and rhein (Mahbub, A. *et al.*, 2013; Duraipandiyan, V. *et al.*, 2012; Bounda, G. A. *et al.*, 2015) as having anti-proliferative properties.

Polyphenols have equally been shown in several studies to exhibit synergistic and antagonistic effects with chemotherapeutics (Mertens-Talcott,S.U., & Percival, S.S. 2005; Lecumberri, E. *et al.,* 2013).

In this study, results indicated that when 13µM (IC_{50}) of emodin and 53µM (IC_{50}) of Cis-Stilbene were combined with the chemotherapeutic Imatinib, a 50% increase in cell death of K562 myeloid cells was recorded. Similar results were observed when 150µM (IC_{50}) of emodin and 140µM (IC_{50}) of rhein were administered alongside the chemotherapeutic Doxorubicin to CCRF-CEM lymphoid cells, which showed a 60% increase in cell death. Likewise, autophagy was observed in K562 myeloid cells and CCRF-CEM lymphoid cells when treated with 0.05 µM of Imatinib and Doxorubicin respectively and also in K562 when 8µM (IC_{25}) of emodin and 28µM (IC_{25}) of Cis-Stilbene were combined with the chemotherapeutic Imatinib.

According to some researchers (Arora, A., & Scholar, E. M. 2005; Han, W. *et al.,* 2011), Imatinib inhibits cancer cell growth and proliferation by blocking BCR-ABL kinase, down regulating the MAP kinase and PI3K/Akt pathways and the activation of RSK kinases. It thus promotes emodin and cis-stilbene-induced apoptosis by aggravating cell proliferation in myeloid leukaemia cells. Likewise, the mechanism of action of Doxorubicin involves the up regulation of the production of ROS which triggers autophagy and apoptotic pathways (Thorn, C. F. *et al.,* 2011; Tacar, O. *et al.,* 2013).

A study by Jia *et al.,* (2009) demonstrated that some types of polyphenols are able to induce autophagy in a chronic myeloid leukemia cell line via down regulation of the Bcl-2 protein. These actions were shown when the potent inhibitor of autophagosome–lysosome fusion, bafilomycin A1 and pan-caspase inhibitor zVAD-FMK blocked the cell death. In another study on prostate cancer cells, curcumin was exposed to induce autophagy cell death by down regulation of another important Bcl-2 family member, Bcl-xL. Curcumin did not stimulate the cleavage of procaspase-8, -9, -3, or -7 or PARP however cause appearance of the LC3B-II isoform and led to increase the number of autophagosomes (Teiten, M. H. *et al.,* 2011).

CONCLUSION

It can be deduced from the results of this study that cancer cells display variable sensitivity to the anti-cancer activity of different polyphenols and this depended on the type of leukaemia cell lineage (myeloid *vs* lymphoid). It has been shown that lymphoid cell line (CCRF-CEM) was more sensitive to polyphenols than myeloid cell line (K562). The anti-cancer activities of polyphenols have been shown to be significantly improved by chemotherapeutic in this in similar studies which also induced autophagy in leukaemia cells. This suggests that the molecular mechanism of action of polyphenols may differ according to the type of polyphenols and cell lines. It was proved in this study that polyphenols have the capability to synergize the effect of chemotherapeutic drugs and decrease the dosage required to induce autophagy in cancer cells. However, more research is still required to improve current understanding of the action of polyphenols alone and polyphenol-chemotherapeutic interactions to advance anti-proliferative and autophagic activities in cancer cells. Future studies may be required to investigate the exact mechanism of action of polyphenols in induction of autophagy cell death in leukaemia and findings of pharmacokinetic and pharmacodynamics properties of polyphenols.

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