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Original Research Article

# Imatinib, Doxorubicin, and/or Polyphenols Inhibiting Cell Proliferation and Inducing Apoptosis in Human Myeloid and Lymphoid Leukaemia Cell Line

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## Abstract

***Background:*** Leukaemia is a complex form of blood malignancy characterized by a high mortality rate, despite significant improvement in cancer treatments. The consumption of fruits and vegetables are inversely related to the incidence and mortality of cancer, as a result of the high level of polyphenols found in some fruits that has been reported to be potentially chemotherapeutic and having a considerable effect on haematological malignancies. ***Objectives:*** To explore the effect of Imatinib, Doxorubicin, and/or polyphenols (emodin, rhein, apigenin and cis-stilbene) on the proliferation, and apoptosis 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 Imatinib, Doxorubicin, and/or different doses of polyphenols. The activity of leukaemia cell proliferation was assessed by Cell Titer-Glo® luminescent assay; the morphological changes of apoptosis, which includes DNA fragmentation and nuclear condensation, were detected by DAPI staining. ***Results:*** Emodin, cis-stilbene, apigenin and rhein showed different levels of effect on inhibition of ATP level and inducing apoptosis in K562 myeloid cells, CCRF-CEM lymphoid cells and CD133+ normal cells when these types of polyphenols used separately and combined with Imatinib or Doxorubicin. 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. ***Conclusion:*** 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:** Imatinib, Doxorubicin, Polyphenols, Inhibiting cell proliferation, Apoptosis, Myeloid and lymphoid leukaemia cell line.



## INTRODUCTION

Leukaemia is a complex form of blood cancer characterized by the rapid proliferation of abnormal white blood cells and the uncontrolled accumulation of these cells within the bone marrow and lymphoid tissue [1]. Cells grow abnormally as a result of a genetic defect; normal blood cells die, and are replaced by immortalised cancerous leukaemic cells [1, 2]. Leukaemia have been identified: acute lymphocytic leukaemia, chronic lymphocytic leukaemia, acute myelogenous leukaemia and chronic myelogenous leukaemia [3]. Leukaemia makes up approximately 3% of all incidence of cancer worldwide, with about 257 000 cases of leukaemia being diagnosed each year [4]. Leukaemia causes nearly 33% of all cancer deaths in children, where acute lymphoblastic leukaemia constitutes almost 55% of all types of blood cancer diagnosed among children younger than the age of five [4].

There are many types of treatments for treating leukaemia, such as chemotherapy, immunotherapy, radiotherapy and bone marrow transplant [5]. In addition, newer therapies have recently been used, for example, Imatinib, which is a type of tyrosine kinase inhibitor that works specifically as a target for the activated tyrosine kinase domain of the Bcr-AbL fusion gene, present in the majority of CML. Imatinib primarily interacts with DNA, but can also generate reactive oxygen species (ROS), which damage cell components [6]. Doxorubicin (DOX) is a powerful anthracycline antibiotic used against many human

neoplasms, including acute leukaemias, lymphomas and sarcomas [7]. It has anti-proliferative properties and as a cytostatic drug can reduce RNA synthesis and may produce reactive oxygen species, resulting in cells' death by DNA damaging; it can also intercalate with DNA [8].

It is important to discover new treatments that can enhance the survival rate among leukaemia patients. A particularly interesting area where this is concerned is the use of bioactive agents from natural sources [1]. For example, polyphenols and polyacetylenes, which are two groups of bioactive components that have demonstrated potential roles in cancer treatments [9]. Polyphenols are an essential part of the human diet, where flavonoid and phenolic acids show the majority of polyphenol existent in vegetables and fruits such as pomegranate. These have anti-carcinogenic impacts in *vivo* and in *vitro* by modulating vital mechanisms in cells that are related to carcinogenesis; for example, disturbing the cell cycle and the induction of apoptosis [10, 11]. Therefore, inhibition of the proliferation of tumour cells and apoptosis induction in these cells has become potential targets for cancer treatments [12].

Apoptosis, also known as programmed cell death, is a homeostatic mechanism that generally occurs during development and aging in order to keep cells in tissue [13]. Apoptosis can also act as a protective mechanism, for example, in immune response or if cells are damaged by toxin agents or diseases [14].

The present study aimed to explore the effects of Imatinib, Doxorubicin, and/or polyphenols (emodin, apigenin, cis-stilbene and rhein) on the inhibition of cell proliferation(ATP level) and for inducing apoptosis in one myeloid cell line (K562), one lymphoid cell line (CCRF-CEM) and one non-tumour normal cell line (CD133).

## MATERIALS AND METHODS

### Media Preparation

50 ml of RPMI media was removed into falcon tube. Then, 50 ml of fetal calf serum 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.

### 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.

### 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.

### 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 equipments were sprayed with bactericidal spray before entering the fume hood.

### 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.

### Polyphenols Treatment Preparation

Polyphenols (Emodin, Apigenin, Rhein and Cis-stilbene) were 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 plate 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 added to 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.

**Preparation of standard chemotherapy agents**

**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.

**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.

**Cell titter-Glo® Luminescent cell viability assay:**

The basic energy source for all metabolic mechanism is the adenosine triphosphate (ATP) which is found in intact cells. It works as a tool for the functional integrity of all living cells, which all cells require ATP to still alive and doing their function in a precise manner [15]. Disruption of cellular processes as a result of cell injury, chemical effects, deficiency of essential nutrients and oxygen, all of these cause a decrease in ATP levels in cells. Therefore, ATP measurement is important to study the viability of cells. Using ATP bioluminescence to measure cell proliferation has been used with leukaemia cells, which observe a significant correlation between ATP levels and number of cells [15].

The CellTiter-Glo® Luminescent cell viability assay is known as a homogeneous method used to show live cells by identifying the number of live cells in culture depend on quantification of ATP level which signal the presence of metabolically active cells. The homogenous results in cell lysis and formation of luminescent signal proportional to the amount of ATP existing and the amount of ATP proportional directly with the cell number [16]. This assay based on the production of signal or light that is released by the reaction of luciferase with the ATP in cells. The luciferase reaction is shown below which mono-oxygenation of luciferin is catalysed by luciferase in the presence of Mg2+, ATP and molecular oxygen.

$$ATP + Luciferin + O_2 \longrightarrow Oxyluciferin + AMP + PP_I + CO_2 + LIGHT$$

## METHODS

**Preparing the Reagent**

Ten ml of CellTiter-Glo® Buffer was transported to the bottle containing Cell Titer-Glo® Substrate to reconstitute the lyophilized enzyme/ substrate mixture and form CellTiter-Glo® Reagent. After that, reagent was mixed gently by vortex for 10 minutes.

**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.

**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.

**Cell titter-Glo® Luminescent cell viability assay**

**Apoptosis investigation by DAPI Staining**

**Preparation of DAPI stain**

- 4% paraformaldehyde was prepared by adding 20g of paraformaldehyde to 500ml of PBS. This was done in the fume cupboard on a warm plate in order to dissolve it.
- 10 ug/ml DAPI stain was prepared by taking 10 µl of DAPI that was provided as a 1mg/ml stock and added to 490µl of distilled water.

**Plating out cells for DAPI staining**

Cells were seeded in 12-well plates (Fisher Scientific, Loughborough, U.K.) at a cell density of 0.5 x $10^6$ cells per well and treated with dose ranges between $IC_{10}$ and $IC_{50}$ for each polyphenol as determined from the CellTiter-Glo® assay. In addition, the cells that were appeared synergistic effects on inhibition of the cell ATP levels when treated with polyphenols combined with chemotherapy. These were also treated with chemotherapy (Imatinib or Doxorubicin) alone and combined with $IC_{50}$, $IC_{25}$ and $IC_{10}$ for 24 hours in this step as previously described. Then, Apoptosis was assessed using the morphological assessment of DAPI stained cells (Sigma, Poole, UK).

**Procedures**

After cells were seeded in 12-well plates (Fisher Scientific, Loughborough, U.K.) at a cell density of 0.5 x 106 cells per well and treated with polyphenol. Cells were incubated at 37 ºC with 5% CO2 for 24 h. Then, cells were transferred to a labelled Eppendorf tubes and centrifuged for 3min at 2000 rpm. Then, cells were re-suspended in 500 µl of 4% paraformaldehyde. After the fixation step, cells were transferred on slides by a 5 min cytospin at 1000 rpm (Shandon Cytospin 3 centrifuge). Slides were then dried at room temperature and 50 µl of DAPI stain (10 ug/ml) (Sigma) was added to cells for 5 minutes in the dark at RT. After that, excess DAPI stain was removed and drop of immersion oil was added and cover slip was applied and sealed with nail varnish. An Olympus, BX60 fluorescence microscope at magnification 400 was used to assess the morphology of cell nuclei. Pictures were taken by Q-capture-pro 8.0 (UVP Bio Imaging Systems) and saved on memory stick.

## RESULTS

**Standard curve of luminescence against the number of myeloid and lymphoid leukaemia cells and non-tumour cells**

Titration curves for K562 myeloid cells, CCRF-CEM lymphoid cells and CD133+ normal cells were performed by using CellTiter-Glo® assay to determine the optimal number of cells and to ensure it correlated with the ATP level and make it possible to cover luminescent values of cell number after treatments. The results (Figure 1) showed that the R-squared values obtained from the three cell lines were 0.90, 0.98 and 0.97, respectively. This indicated that differences between the observed values were small and unbiased. This means that the data were closer. Additionally, linear regression was calculated using an equation that was used to minimize the distance between the fitted line and all data points.

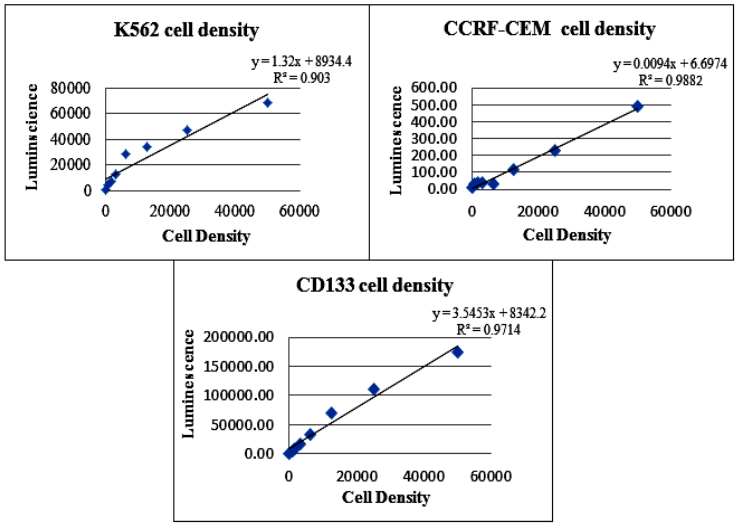



**Fig-1: Different cell densities for the K562 myeloid cell line, CCRF-CEM lymphoid cell line and CD133 normal cells, which were evaluated using a CellTiter-Glo® assay**

**Effect of polyphenol treatments on ATP levels of the K562 myeloid cell line, CCRF-CEM lymphoid cell line and CD133 non-tumour normal cell line**

The effect of four polyphenols (Emodin, Cis-Stilbene, Apigenin and Rhein) on the cellular proliferation of the myeloid cell line (K562), lymphoid cell line (CCRF-CEM) and non-tumour normal progenitor cell line (CD133+) showed different reduction levels for cells' ATP levels (Figure 2). Figure-2A shows the effect of three polyphenols (emodin, apigenin and cis-stilbene) on the ATP level of K562 myeloid cell lines. The K562 cells showed some resistance to polyphenol treatments and there was only a decrease in cell ATP levels when treated with emodin and cis-stilbene at 50µM and 250µM, to about 53% and 56%, respectively($p$ value <0.05). In contrast, when these cells were treated with apigenin, there was an increase in cell ATP levels.

However, in the case of the CCRF-CEM lymphoid cell line (Figure-2B), emodin and rhein had a greater effect, with an $IC_{50}$ value of 50µM and 250 µM, respectively. Emodin significantly reduced cell proliferation in the non-tumour CD133+ cell line, with an $IC_{50}$ value of 10 µM. Rhein also showed a moderate effect but did not reach 50% inhibition. Apigenin and cis-stilbene did not show any effect on ATP levels in non-tumour control cells ($p$<0.05) (Figure-2C).

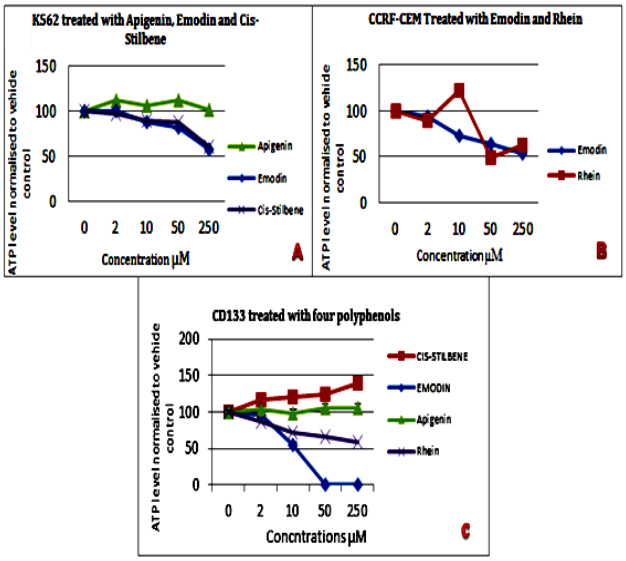



**Fig-2: The effect of four polyphenols (emodin, cis-stilbene, apigenin and rhein) on the ATP levels of a myeloid cell line (K562), lymphoid cell line (CCRF-CEM) and non-tumour normal progenitor cell line (CD133).This was assessed by CellTiter-Glo® assay. These three types of cells were treated with different concentrations of polyphenols (0, 2, 10, 50, and 250µM) for 24 hours. Data was normalised to vehicle control, which was evaluated at 100% cell viability after calculating the average of triplicate wells, and using a linear regression equation for each standard curve and for each polyphenol with each cell line**

Overall, from the above results, it can be seen that emodin had effective proliferation inhibition in all leukaemia cells, with a slightly better effect on lymphoid than myeloid cells. Although determining $IC_{50}$ where 50% of inhibition in cellular proliferation was detected had been expected, the obtained results were unexpected, and $IC_{50}$ was only detected in CCRF-CEM. Therefore, $IC_{50}$, $IC_{25}$ and the lowest significant dose (LSD) of polyphenols were required for the next step. These different dosages were obtained from a previous study (Table-1) [20].

**Table-1: The $IC_{50}$, $IC_{25}$ and the lowest dose ($IC_{10}$) of polyphenols that were obtained from a previous study [20]**

| Cell Type | | Polyphenols IC50, IC25 and IC10 (lowest dose ) µM | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | EMODIN | | | Cis-Stilbene | | | Apigenin | | | Rhein | | |
| | | IC50 | IC25 | IC0 | IC50 | IC25 | IC10 | IC50 | IC25 | IC10 | IC50 | IC25 | IC10 |
| **Myeloid cell line** | K562 | 13 | 8 | 2 | 53 | 28 | 2 | 350 | 180 | 10 | - | - | - |
| **Lymphoid cell line** | CCRF-CEM | 22 | 12 | 2 | - | - | - | - | - | - | 140 | 95 | 50 |
| **Non-tumour cell line** | CD133+ | 150 | 65 | 30 | 650 | 325 | 130 | 600 | 300 | 120 | 380 | 190 | 76 |

**Effect of chemotherapy and polyphenols alone and combined on the ATP levels of leukaemia cells lines after 24h treatments**

The results obtained from the effect of Imatinib (IM) and Doxorubicin (Dox) when used alone and in combination with $IC_{50}$, $IC_{25}$, and $IC_{10}$ of polyphenols (emodin, apigenin, cis-stilbene and rhein) (Table **1**) on the ATP levels of myeloid (K562) and lymphoid (CCRF-CEM) leukaemia cell lines, as well as a non-tumour (CD133) cell line, showed an additive effect in K562 cells, which was treated with emodin and cis-stilbene. In CD133 cells, this was treated with rhein. The reduction of ATP in K562 was 0% when treated with Imatinib alone and was between 40% and 70 % when treated with different dosages of emodin and cis-stilbene alone; when this cell line was treated with a combination of Imatinib and emodin and cis-stilbene, the inhibition level was also 0%. The same effect was indicated when CD133 was treated with Doxorubicin alone and combined with rhein.

CCRF-CEM cells that were treated with emodin and rhein indicated synergistic effects when combined with Doxorubicin. These cells showed a decrease in ATP levels when treated with Doxorubicin alone to about 65% and when treated with $IC_{50}$, $IC_{25}$, and $IC_{10}$ of emodin and rhein, the decrease was between 40% and 70% for emodin and about 75 % for rhein. However, there was a significant decrease in the ATP levels of cells when treated with Doxorubicin combined with emodin and rhein to about 5% and 0%, respectively.

However, K562 treated with apigenin and CD133 treated by emodin showed competitive antagonistic effects when combined with Imatinib, where the ATP level of these two types of cells showed a relative increase. An antagonistic effect was also shown in CD133 cells treated with apigenin and cis-stilbene when combined with Imatinib, resulting in a significant increase in the ATP levels of this type of cell (Figure-3).

**Effects of polyphenol treatments on apoptosis induction in leukaemia cell lines**

Four polyphenols showed significant results in the induction of apoptosis after cell lines were stained by DAPI stain and images were captured by Captured-pro 8.0. Two hundred cells were counted (live and apoptotic) and the percentage of apoptotic cells was calculated. All four polyphenols induced considerably higher levels of apoptosis in the K562 myeloid cell line and CCRF-CEM lymphoid cell line, compared to the CD133 non-tumour cell line ($p<0.05$) (Figure 11). Emodin was the most effective polyphenol at inducing apoptosis in K562 with an $AP_{50}$ value 200µM; in CCRF-CEM, AP50 was >250 µM; CD133 was less effected than either the lymphoid and myeloid leukaemia cell lines, where apoptotic percentage was less than 10%. Apigenin and cis-stilbene also showed considerable effects on the K562 cell line compared to the CD133 non-tumour cell line, where the percentage of apoptosis was about 45% in K562 compared to about 20% in CD133 non-tumour cells. Rhein induced a significant level of apoptosis in the CCRF-CEM cell to about 40% compared to the normal cells. The two types of leukaemia cell lines showed different sensitivities to polyphenol treatments (Figure-4). The different morphological changes of apoptosis were demonstrated on lymphoid and myeloid leukaemia cell lines following treatment with different types of polyphenols for 24h and were investigated using a fluorescent microscope after staining by DAPI stain; images were captured and are shown in Figure-5.

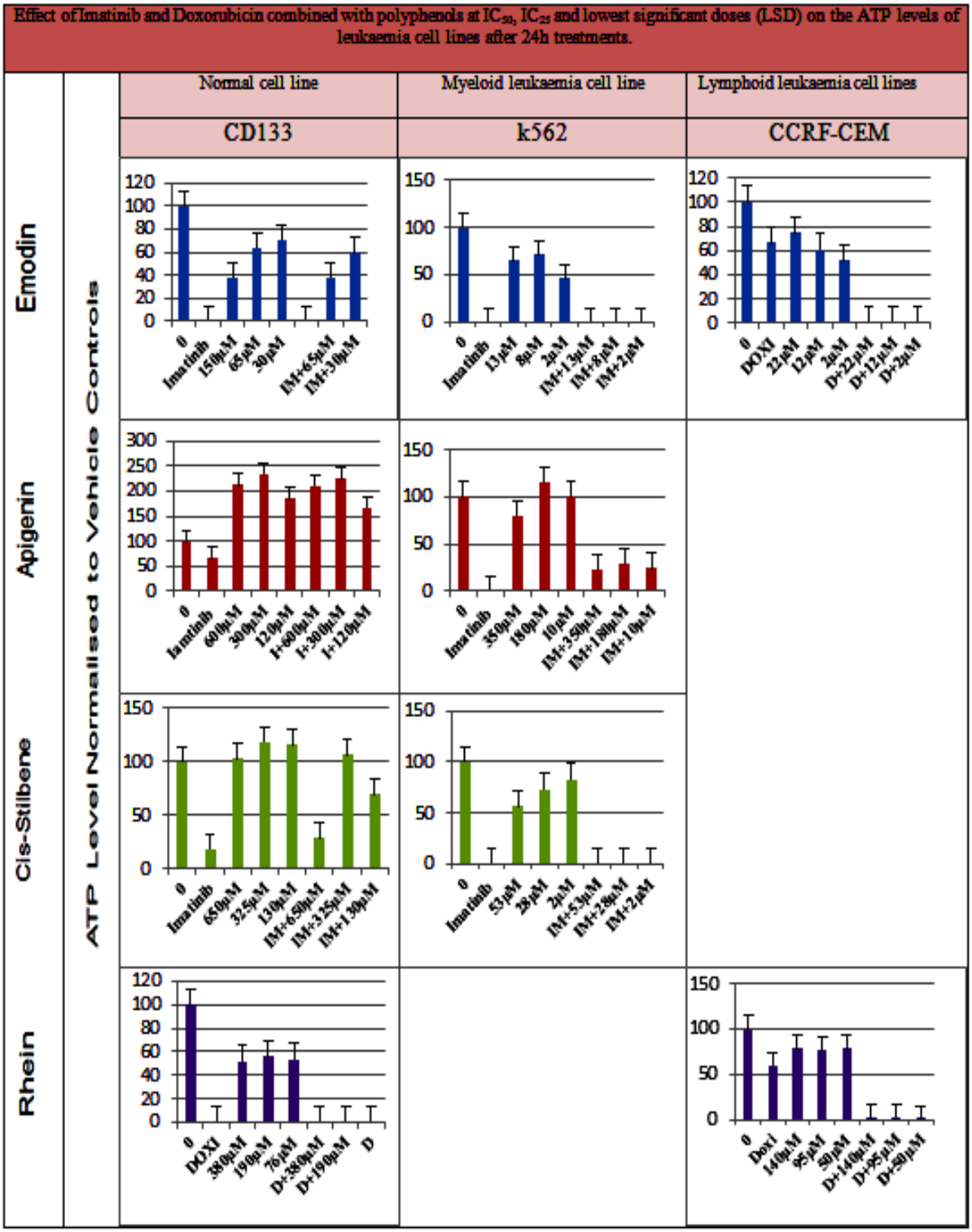



**Fig-3: The effect of Imatinib (IM)and Doxorubicin (Dox) when used in combination with polyphenols (emodin, apigenin, cis-stilbene and rhein) on the ATP levels of myeloid and lymphoid leukaemia cell lines and a non-tumour cell line. This was evaluated by CellTiter-Glo assay. Cells were treated with Imatinib (IM) or Doxorubicin (Dox) and polyphenols alone and combined together for 24 hours using $IC_{50}$, $IC_{25}$, and $IC_{10}$ of polyphenols, which were obtained from a previous study (Table-1). The date was normalised to the vehicle control, which was assigned 100% cell viability. The bars on the left show the vehicle controls and treatments alone, Imatinib or Doxorubicin alone, then IC50, IC25 and IC10 of polyphenols alone and combined in the last three bars. Some results showed a significant additive effect and synergistic effect, while others showed competitive antagonistic effects and antagonistic effects**

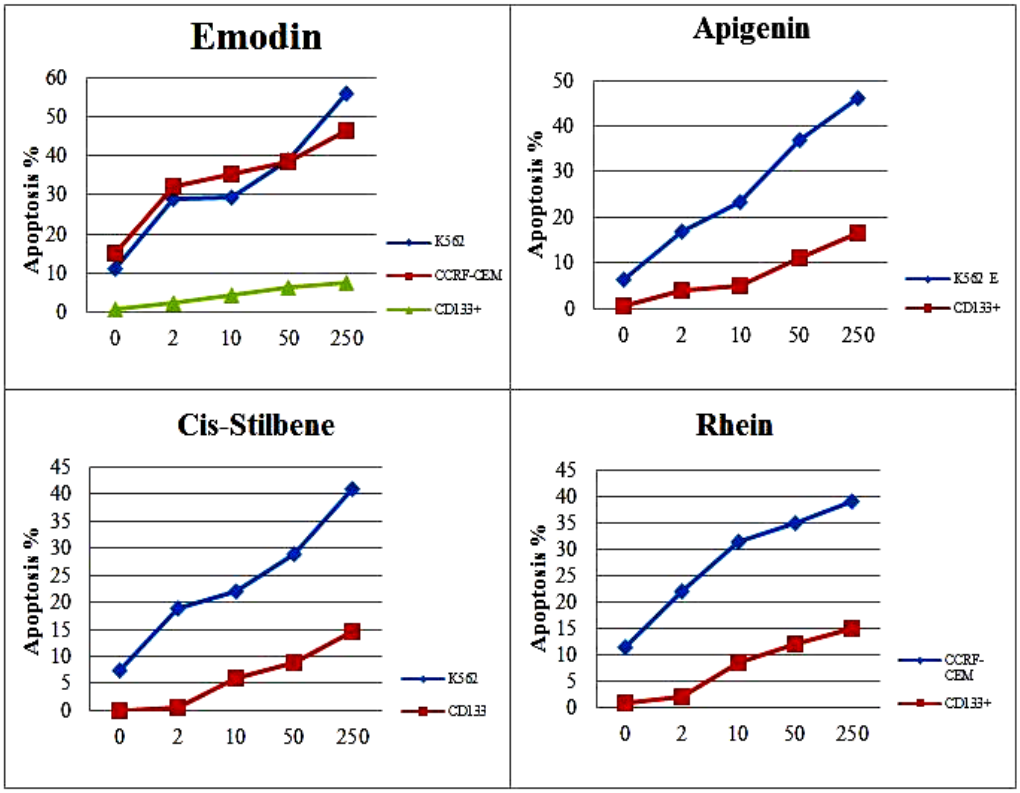
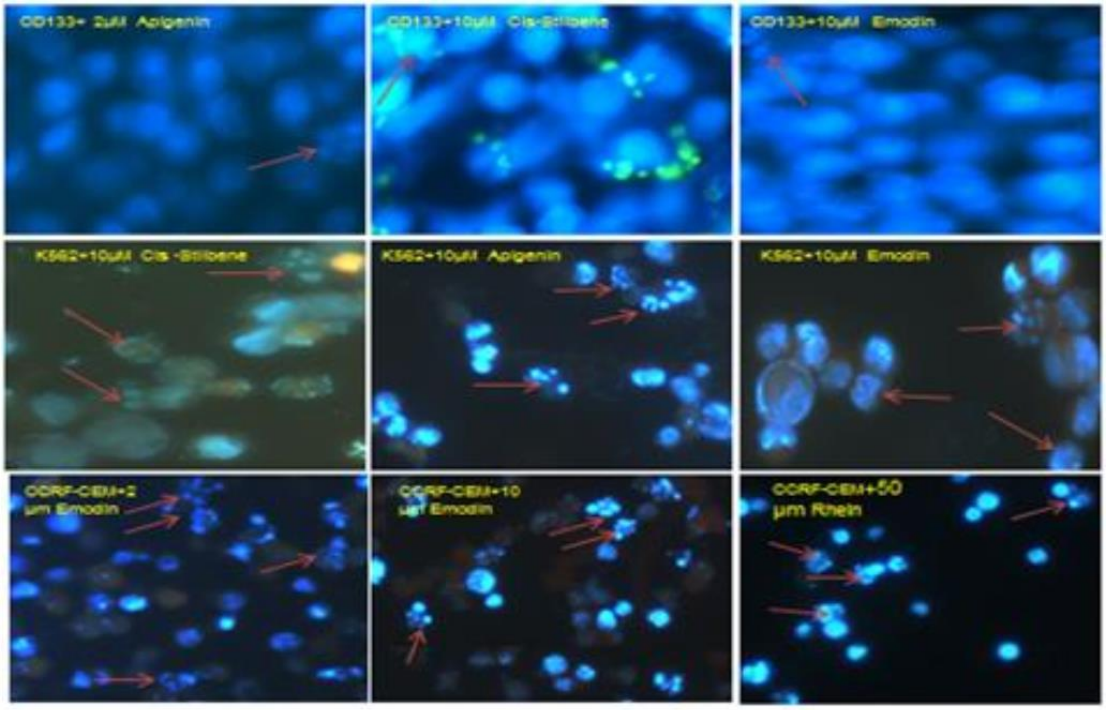
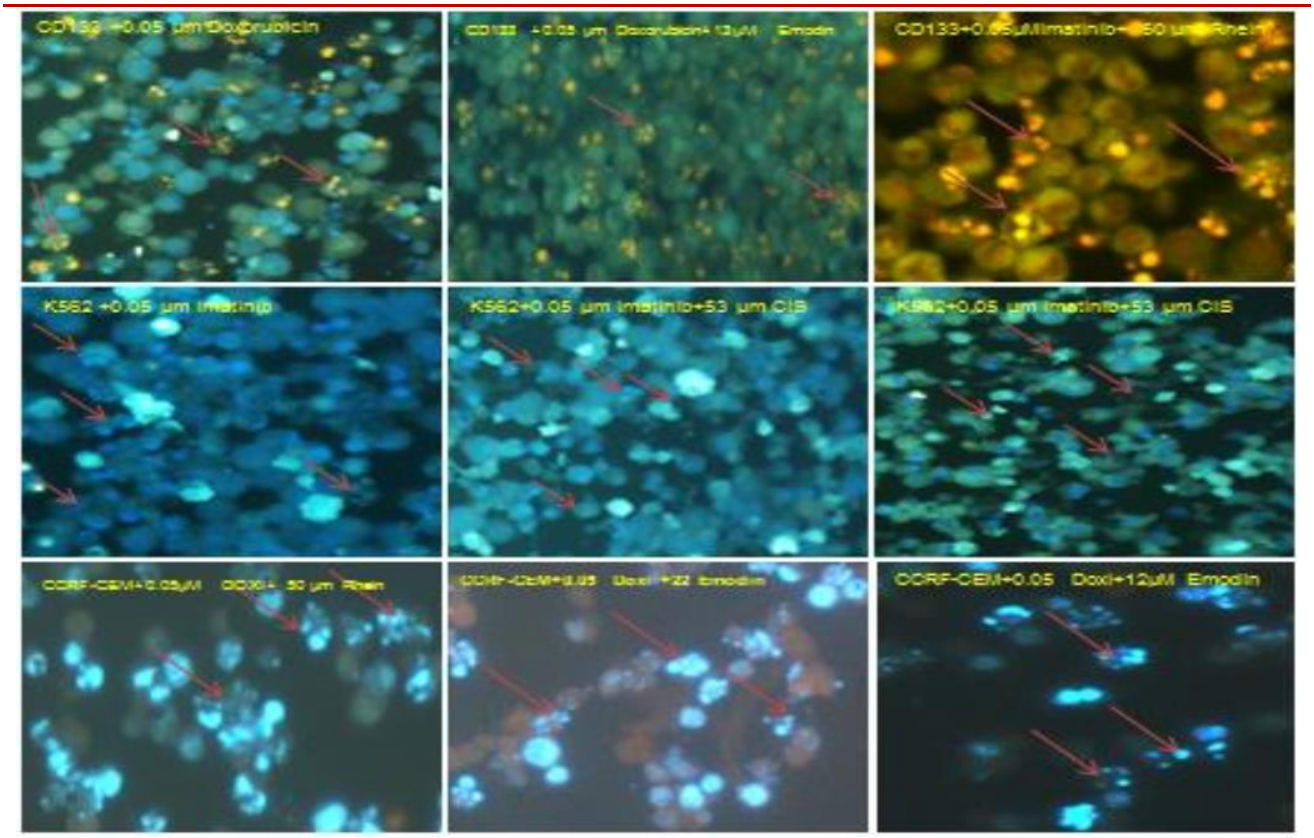



**Fig-4: The effect of polyphenols (emodin, cis-stilbene, apigenin and rhein) on the induction of apoptosis in a K562 myeloid cell line, CCRF-CEM lymphoid cell line and in non-tumour normal progenitor cells (CD133). Cells were treated with different concentrations of each polyphenol for 24 hours, then stained by DAPI stain and images were captured by Captured-pro 8.0. Two hundred cells were counted (live and apoptotic) and the percentage of apoptotic cells were calculated using Excel**




**(A)**




**(B)**

**Fig-5: Atypical example of morphological changes brought on by apoptosis, inducted after treatment by polyphenols for 24h and stained using DAPI stain. (A) Shows the K562 myeloid leukaemia cells, the CCRF-CEM lymphoid leukaemia cells and the CD133+ non-tumour cells after treatment with different concentrations of different types of polyphenols and stained by DAPI staining; pictures were taken using a florescent microscope; (B) shows the effect of chemotherapy combined with $IC_{50}$, $IC_{25}$ and $IC_{10}$ of polyphenols detected by CellTiter-Glo® and showed a synergistic effect. Apoptotic cells were identified by intensely stained nuclei, condensation of the chromatin and shrinkage in the cell wall. Cells became irregular in shape and apoptotic bodies formed. An example of apoptotic cells is indicated by the red arrow**

**Effect of the combination of chemotherapy and polyphenols on the induction of apoptosis in leukaemia cell lines after 24h treatments**

Cell lines that showed an additive or synergistic effect on the inhibition of ATP cell levels when treated with $IC_{50}$, $IC_{25}$ and $IC_{10}$ of polyphenols combined with chemotherapy (Imatinib or Doxorubicin) were also treated to measure the effect of these dosages on the induction of apoptosis. The K562 myeloid cell line showed an additive effect on cell ATP levels when treated with $IC_{50}$, $IC_{25}$ and $IC_{10}$ of emodin and cis-stilbene when combined with Imatinib. This combination also increased the percentage of apoptotic cells, which was about 50% of apoptosis in cells treated with Imatinib combined with 53µM ($IC_{50}$) of cis-stilbene (Figure-6A) or with 13µM ($IC_{50}$) of emodin (Figure-6B). The CCRF-CEM lymphoid cell line that showed synergistic results in cell proliferation when treated with $IC_{50}$, $IC_{25}$ and $IC_{10}$ of emodin and rhein when combined with Doxorubicin also demonstrated a significant effect on the percentage of apoptosis, which reached approximately 60% when 150µM ($IC_{50}$) of emodin combined with Doxorubicin was used (Figure-6C), or with 140µM of rhein (Figure-6D). There was also a similar effect for $IC_{25}$ and low doses of polyphenols on these two types of cells. However, emodin demonstrated a greater toxicity in lymphoid leukaemia cells (CCRF-CEM) than in myeloid leukaemia cells (K562).

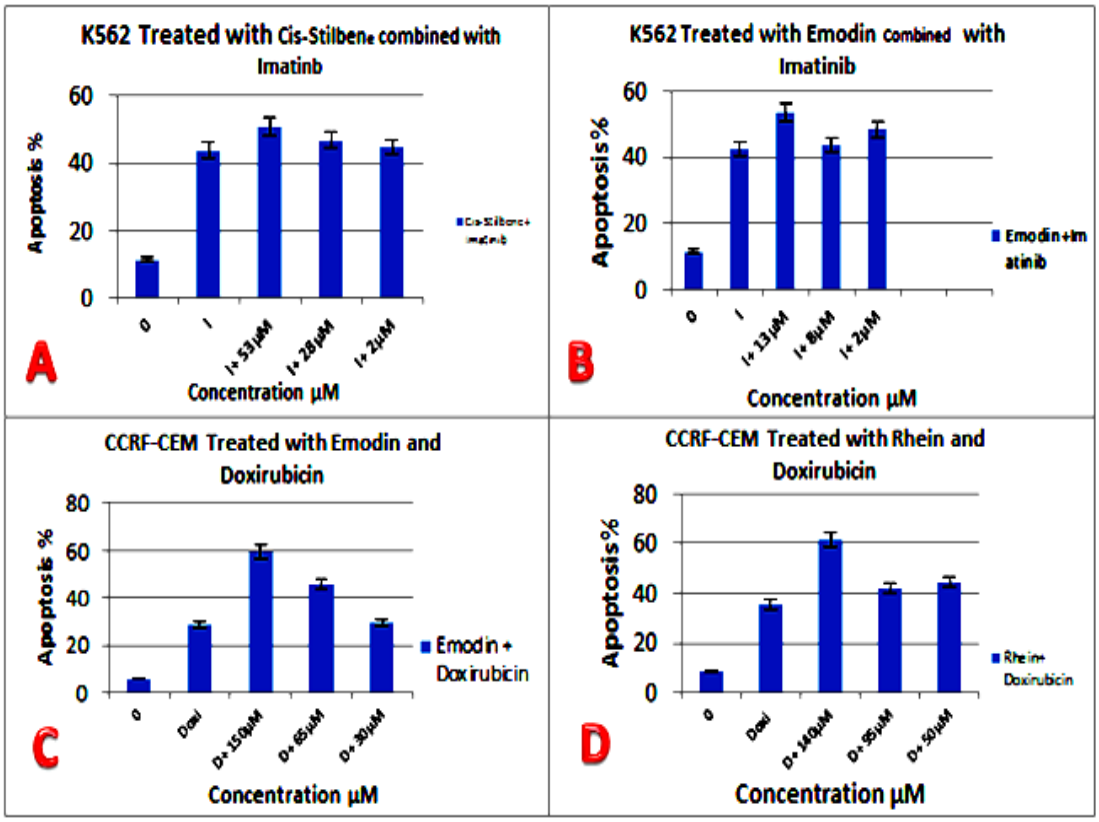



**Fig-1: The effect of chemotherapy alone and combined with $IC_{50}$, $IC_{25}$ and $IC_{10}$ polyphenols on the induction of apoptosis in leukaemia cell lines that showed synergistic or additive effects in the inhibition of ATP cell proliferation after treatment for 24h. Emodin and cis-stilbene also showed additive results on the induction of apoptosis in the K562 cell line when combined with Imatinib, which for $IC_{50}$, $IC_{25}$ and $IC_{10}$ (as determined by CellTiter-Glo® assay) were 53, 28, and 2µM of cis-stilbene and 13, 8 and 2µM of emodin. Emodin and rhein also showed a significant effect on the induction of apoptosis in the CCRF-CEM lymphoid leukaemia cell line when $IC_{50}$, $IC_{25}$ and $IC_{10}$ of these two polyphenols were combined with Doxorubicin. IC50, IC25 and IC10 of emodin were 150, 65 and 30µM and 140, 95 and 50µM of rhein**

## DISCUSSION

This study investigated the anti-proliferative (measured as ATP levels) and pro-apoptotic actions of the polyphenols emodin, apigenin, cis-stilbene and rhein on human myeloid (K562) and lymphoid leukaemia cell lines (CCRF-CEM) and non-tumor control cells (CD133). A study was made of the effect of these polyphenols alone and when used in combination with two Imatinib or Doxorubicin.

The study results revealed that the polyphenol emodin was the most effective polyphenol at inhibiting cell ATP levels and inducing apoptosis in both leukaemic cell lines in a dose dependent manner, whilst it had a lesser effect on the non-tumour control cells (CD133+); in which apoptosis was 10% or less. It could be deduced from the study results that emodin exhibits significant anti-proliferative and pro-apoptotic activities that appear to be more pronounced in lymphoid cells than in myeloid leukaemia cells. These results corroborate existing knowledge of the anti-cancer qualities of emodin [17-20]. However, current understanding of the molecular mechanism of emodin is still evolving. In a study by Su *et al.*, [18], the apoptotic effect of emodin on human lung adenocarcinoma (A549) cells and the mechanism of action were investigated. They reported that emodin-induced apoptosis of A549 cells was initiated by oxidative damage that disrupts ERK and AKT signaling pathway, mitochondrial functions as well as Bcl-2 and Bax regulation. Conversely, emodin also promotes the expression of mitochondrial cytochrome C and caspase activation, all of which leads to apoptosis [18]. In another study by Chen *et al.*, [17] that examined the apoptotic effect of emodin on human promyelocytic leukemia cells (HL-60). Chen and his colleagues reported that emodin-induced apoptosis in HL-60 cells via the stimulation of the caspase 3 cascade; however, ROS production was unhindered by emodin activity [17]. These studies suggest that the molecular mechanism of emodin induced apoptosis is likely to be mediated via a number of pathways. In a recent study by Ma *et al.*, [19] the apoptotic effect of emodin on human colon cancer cells (LS1034) was investigated. The results of the study showed that emodin initiated LS1034 cell proliferation via the arrest of G2/M phase

of the cell cycle. It also induced apoptosis of LS1034 cells in a dose dependant manner via the up regulation of ROS and $Ca^{2+}$ expression, activation of caspase-9 and caspase-3 and dysregulation of mitochondrial function [19].

This study also showed that cis-stilbene treatment of K562 myeloid cell lines resulted in the inhibition of cell ATP level in dose dependent manner ( *P* value <0.05). However, this effect was not seen when K562 myeloid cell lines were treated with apigenin, were there were no effects on ATP levels.

However, in the CCRF-CEM lymphoid cells when treated with rhein there was a considerable decrease in cell ATP levels, although this was not in a dose dependent manner.

Alternatively in the CD133 non-tumour control cells lines the ATP levels were not affected by either apigenin or cis-stilbene treatment. The research results therefore suggest that polyphenols cis-stilbene and rhein were associated with the inhibition of theATP levels of K562 myeloid cells and CCRF-CEM lymphoid cells respectively but nature of their influence was unclear. On the other hand, the polyphenol apigenin did not dysregulate the ATP levels of any of the leukaemic cells. However, results of apoptosis investigations by DAPI staining showed that all three polyphenols (emodin, cis-stilbene and rhein) induced statistically significant levels of apoptosis in K562 myeloid cell line and (emodin and rhein) in CCRF-CEM lymphoid cell line compared to CD133 non-tumour cells line ($p< 0.05$).

However it is worth pointing out that the $IC_{50}$ and $AP_{50}$ values for the polyphenols could not be determined during the course of the research and as such the values from a similar research by Mahbub *et al*., [20] were used in this study. The difficulty in determining the $IC_{50}$ and $AP_{50}$ values is likely due to statistical or procedural errors during the experiment.

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 [20-22], cis-stilbene [20, 23] and rhein [20, 24-26] as having anti-proliferative and pro-apoptotic properties.

A study by Chan *et al*., [27] investigated the anticancer effects of apigenin on squamous cell carcinoma cell lines (SCC25 and A431). They reported that apigenin inhibits the proliferation of SCC25 and A431 cells and triggered apoptosis of SCC25 cells by up-regulating the TNF-R and TRAIL-R signaling pathways as well as by disrupting Bcl-2 regulation.

Similarly Zhu *et al.*, [21] also reported that apigenin inhibited the proliferation of T24 bladder cancer cells proportional to increasing concentration and time, via the arrest of the G2/M phase of the cell cycle and apoptosis. They argue that apigenin induced-apoptosis was associated with the dysregulation of PI3K/Akt pathway and Bcl-2 mediated –caspase dependent pathway, as well as with the upregulation of caspase-3 activity and the cleavage of Poly ADP ribose polymerase (PARP) [21].

Similar research has underlined the anti-cancer properties and molecular activities of the polyphenol rhein. Duraipandiyan *et al.*, [24] examined the anticancer activity of rhein on human colon cancer cell line COLO 320 DM and normal cell line VERO. They reported that rhein induced anti-proliferative and apoptotic effect on the cancer cells COLO 320 DM but not towards the normal VERO cells. Duraipandiyan and his colleagues reported that rhein-induced apoptosis was associated with the activation of p53/p21 signaling pathway, disruption of mitochondrial oxidative phosphorylation and glycolysis pathway [24]. In a related study by Bounda *et al.*, [26] rhein induced apoptosis in human hepatic cell line (HL-7702) was associated with a number of changes in cell morphology molecular signaling. Bounda and his colleagues suggest that the molecular mechanism of rhein induced apoptosis involved the dysregulation of the mitochondrial membrane and lipid metabolism alongside the up regulation of ROS, Cytochrome-c expression and PUMA, Apaf-1, and caspase-9 and -3 activities [26].

Likewise, the anticancer properties and apoptotic mechanism of the polyphenol cis-stilbene has also been the subject of some research. Nguyen *et al*., [28] examined the anti-tumour activities of trimethoxy-cis-stilbene (TMS) on chronic hepatitis C virus (HCV)-induced hepato cellular carcinoma (HCC) cells. The results showed that TMS exhibited inhibition the proliferation and promoting the apoptosis of HCC cells. According to Nguyen and others, the molecular mechanism of TCC anti-proliferative effect was associated with the arrest of the G2/M phase of the cell cycle, while it induced apoptosis via the up regulation of p21(Cip1/Waf1) and the disruption of AKT signaling pathway.

Polyphenols have equally been shown in several studies to exhibit synergistic and antagonistic effects with chemotherapeutics [29-32].

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, auto

phage 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.

The synergistic and antagonistic apoptotic effects observed between the polyphenols (emodin and cis-stilbene) and Imatinib and Doxorubicin is very likely due to the fact that both polyphenols and chemotherapeutics induce apoptosis via similar pathways. According to some researchers [33-35]. 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 apoptotic pathways [36, 37], which compliments rhein-induced apoptosis. A study by Jia *et al.*, [38] demonstrated that some types of polyphenols are able to induce apoptosis in a chronic myeloid leukaemia cell line via down regulation of the Bcl-2 protein.

## CONCLUSION

It can be deduced from the results of this project 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. 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 apoptosis 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 pro-apoptotic in cancer cells. Future studies may be required to investigate the findings of pharmacokinetic and pharmacodynamics properties of polyphenols.

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