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Anti-proliferative effect of *Trifolium pratense* extract on Raji cell line in Burkitt's lymphoma

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ABSTRACT

Background & Aim: In folk medicine, red clover (*Trifolium pratense*) use to treat some diseases such as whooping cough, asthma, eczema, and some ophthalmic dysfunction. Due to its phytoestrogens and antioxidant-rich compounds, it is recommended to alleviate some cancers. This paper was conducted to study the anti-cancer and apoptosis effects of red clover extract (RCE) on the Raji cell line in Burkitt's lymphoma.

Experimental: Red clover lyophilized powder (CAS = 85085-25-2) with a fixed ratio of Formononetin, Biochanin A, Daidzein, and Genistein was acquired from Sigma Aldrich German company. Raji cells were obtained from the Pasteur Institute of Iran-Tehran. Raji cells were treated with different concentrations of RCE, and viable cells were measured at an interval of 24, 48, and 72 hours incubation in three days by MTT assay. Agarose gel electrophoresis was used to determine the DNA degradation. Annexin V-FITC/PI kit and flow cytometry assay were used to determine the percent of apoptotic and necrotic treated Raji cells. Also, expression changes in *cMYC*, *ID3*, and *P53* genes were measured by real-time PCR.

Results: Red clover extract can prevent in vitro proliferation of the Raji cells in a time and dose-dependent manner. RCE, as well as *c-MYC* gene suppression and induction of *ID3* and *P53* genes, enters the Raji cells in the apoptosis stages with an acceptable percentage and has complication cure potential in Burkitt's lymphoma.

Recommended applications/industries: The extract used in this study combines four components. The individual evaluation effects of Formononetin, Biochanin A, Daidzein, and Genistein on Raji cells are recommended.

1. Introduction

Lymphoma is a common name for lymph system cancers. This type of disease begins in lymph nodes or any other lymphatic tissues. Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL) are two main classes of this dysfunction. These classes are classified into more subtypes according to generation, proliferation, and initiation sources. Each sub-types needs different ways and methods for treatment (Caponetti *et al*., 2017; Mugnaini, 2016). Many types of lymphoma are curable using chemotherapy drugs and have a high rate of improvement. Medications for lymphoma included Adriamycin, Bleomycin, Vinblastine, Dacarbazine, Etoposide, Doxorubicin, Cyclophosphamide, Procarbazine, Prednisone, and

Rituximab. Depending on the type and stage of the disease, radiotherapy and a combination of these drugs are used. By using these components, side effects such as pulmonary and cardiovascular injuries might occur. Mortality in the early stage of HL is 5–10 %, and in severe cases, up to 30%. NHL in different settings has mortality up to 40% (Lewis *et al*., 2020; Potre *et al*., 2021). Burkitt's lymphoma (BL), one of the NHL subtypes especially, involves B-cell lymphocytes in the germinal center of these cells. BL characterizes by the Presence of Raji cells, modified B-cells, the first continuous human hematopoietic origin cell line (Pulvertaft *et al*., 1965).

BL occurs due to chromosomal translocation between immunoglobulin genes in 2, 22, and 14 chromosomes and the *c-MYC* gene in chromosome 8. In 80% of BL, translocations occur between the *c-MYC* gene and immunoglobulin H locus that produce (8:14) (q24:q23). In 15% of cases, the *c-MYC* gene translocates to the 2-q11 chromosome Kappa locus and 5% to the 22-q11 lambda locus. These changes result in the stimulation of *c-MYC* expression (Hu *et al*., 2007). Because of spatial proximity between the 8 and 14 chromosomes in B-cell lymphocytes, t(8:14) translocation often occurs in BL (Misteli, 2011; Roix *et al*., 2003). MYC proteins interfere in the pathogenesis of a large number and wide variety of human malignancies beyond leukemia and lymphoma (Wasylishen and Penn, 2010).

MYC is a bHLHLZ (basic helix–loop–helix leucine zipper) protein and a part of the "Proximal MYC Network" (PMN). The PMN acts as a center in the nucleus, integrating signals from diverse upstream signaling pathways and regulating the expression of many target genes necessary for cell cycle progression. Members of the PMN dimerize and bind to DNA through a conserved bHLHLZ domain. Dimerization of MYC and MAX (MYC-associated factor X) is needed for DNA Binding (Beaulieu *et al*., 2020).

Next-generation sequencing (NGS) studies have shown that the inhibitor of DNA binding 3 (*ID3*) mutated in BL *ID3* encodes for a helix-loop-helix (HLH) protein that lacks an essential DNA-binding domain and inhibits other HLH proteins from DNA binding by heterodimerization. Incidence of *ID3* mutations in BL reports between 34% and 68%. *ID3* mutations resulted in increased expression of *TCF3* targets and promote growth, survival, and cell cycle regulation by activating B-cell receptor signaling. ID3 mutant proteins are less or utterly ineffective in inhibiting *TCF3* (Rohde *et al*., 2017).

In BL, alterations of the P53 tumor suppressor are also relatively frequent. The P53 tumor suppressor is a transcriptional factor controlling the expression of multiple target genes. This protein regulates many cellular processes, such as cell cycle, apoptosis, senescence, and genome stability (Smardova *et al*., 2008).

Red clover is a legume, like soy, which considers phytoestrogens. Chinese and Russian folk healers have used red clover to treat respiratory problems such as asthma and bronchitis. Native American healers recommended red clover for pertussis and cancer. Some experts believe that red clover accelerates wound healing and alleviates psoriasis. Red clover isoflavones have phytoestrogen properties and affect bone metabolism, serum lipid levels, and arterial problems (Nelsen *et al*., 2002). The main bioactive red clover isoflavones are Formononetin and Biochanin A, with smaller concentrations of Daidzein and Genistein (Kucukboyaci *et al*., 2013).

In this study anti-proliferative effect of red clover (*Trifolium pratense*) extract on Raji cell line in BL was evaluated.

2. Materials and Methods

2.1. Plant extracts preparation

Red clover lyophilized powder (CAS = 85085-25-2) with a fixed ratio of Formononetin, Biochanin A, Daidzein, and Genistein was acquired from Sigma Aldrich German company. 100 mg of red clover extract was solved in 1 mL DMSO, and 9 mL deionized sterile water was added. The concentration of this stock was 10 mg/mL or 10000 µg/mL. This stock was diluted in RPMI (Roswell Park Memorial Institute) culture medium with 10% FBS (Fetal Bovine Serum) by 1/100, 1/50, 1/33, 1/25, and 1/20 to obtain 100, 200, 300, 400, and 500 µg/mL concentrations. RPMI culture mediums without red clover extract wad used as control.

2.2. Cell culture and MTT assay

Raji cells were obtained from the Pasteur Institute of Iran-Tehran. Raji cells were cultured according to the German Cell Lines Service (CLS) protocol. The molecular biology method was carried out for the MTT

assay (Meerloo *et al*., 2015). After defrosting, Raji cells were seeded in 25 mL flasks containing a 10 mL RPMI culture medium with 10% FBS. Viability was assessed by Trypan blue method. Cultured Raji cells were poured into a falcon tube, and the tube was centrifuged at 300 RPM (Revolutions Per Minute) for 5 minutes. The supernatant was discharged, and 1 mL fresh medium with 10% FBS was added to prepared cell suspension. 50 µL of cell suspension mixed with the equal volume of 0.25% Trypan blue dye and microscopic cell counting by Neobar slid was done. In this method, cells with a smooth membrane and unstained nuclei are alive, and cells with a wrinkled membrane and stained nuclei are dead. Total cells are counted in 1 mL, and the percent of viable cells was defined by:

Percent of viable cells=Viable cells count/ total cells count × 100

In this way, when the viability reached about 80%, an MTT assay was done. After preparing Raji cell suspension, 40000 cells in each well of the 96-well plate were seeded with different concentrations (0, 100, 200, 300, 400, and 500 µg/mL) of RCE. The volume of the cell suspension in each well was 100 µL and plates were incubated at 37° C and 5% CO_2. After 24, 48, and 72 hours, 50 µL MTT reagent was added and set for 3 hours. Then 100 µL DMSO was added to dissolve formazan crystals, and the Elisa plate reader measured optic density at 570 nm. For each concentration, three wells were considered, and the percent of viable cells was calculated by:

Percent of growth relative to control = (Mean OD of treated wells/ Mean OD of control wells) × 100

2.3. DNA fragmentation (Agarose gel electrophoresis)

Fragmentation of DNA to 180-200 bp is a sign of apoptosis. The DNA ladder method was used to visualize the degradation products of DNA. About 5×10^6 Raji cells in each of the 6-well plate containing 2 mL RPMI medium with 10% FBS and antibiotics were treated for 6 hours, and then different concentrations (0, 100, 200, 400, and 500 µg/mL) of RCE was added. After 24 hours, the wells were washed twice with PBS (phosphate buffered saline) and collected in tubes with 500µL of lysing buffer and 10 µL of protein kinase-K and incubated for 12 hours at 50°C. After adding 50 µL saturated NaCl (5 M), tubes were refrigerated at 4°C for 10 min and then centrifuged for 12 min at 11,000 rpm. The supernatant was transferred to the microtubule, and 1 mL of pure ethanol was added to each microtubule. The samples were centrifuged again for 15 min at 11000 rpm, and after complete removal of ethanol, about 1 mL of 70% ethanol was added and shacked well. Samples were centrifuged again for 10 min. After thoroughly removing ethanol, the samples were allowed to dry at room temperature for 20 min. Finally, 5 µL of DNA loading buffer was added and DNA samples were run in 1.5% agarose gel dry wells in TAE (Tris-Acetate-EDTA**)** containing 0.5 µg/mL ethidium bromide. Using a Gene Flash gel documentation system, DNA fragments are visualized with ultraviolet illumination (Green and Sambrook, 2019).

2.4. Flow cytometry

The flow cytometry technique and the Annexing V-FITC/PI kit were used to evaluate cell apoptosis and necrosis. Raji cells were treated with 250 µg/mL RCE, and three times with 24 hours intervals were assayed. Annexing V is one of the intercellular proteins with an affinity for phosphatidylserine (PS). Phosphatidylserine is a membrane phospholipid that is located in the inner layer of the plasma membrane in intact cells. In the early stage of apoptosis, PS translocates to the outer layer of the plasma membrane. Annexin V, labeled with fluorescein isothiocyanate (FITC), can specifically bind to PS, and apoptotic cells will be marked. Also, in order to separate apoptotic and necrotic cells, propidium iodide (PI) is used. PI is a fluorescent dye that binds to DNA. It is commonly used to evaluate cell viability or DNA content in cell cycle analysis. Early apoptotic cells will exclude PI, while late-stage apoptotic cells and necrotic cells will stain positively due to the passage of this dye into the nucleus and binding to DNA. The flow cytometry apparatus detect these two kinds of marked cells, and the results will be signed in a dot plot graph (Rieger *et al.*, 2011).

2.5. Real-time PCR

In real-time PCR (RT-PCR), gene expression evaluates based on the cycle threshold (Ct) (Yuan *et al.*, 2006). Relative expression levels, known as fold change, were determined by:

Δct = sampl Ct – *GAPDH* Ct
$\Delta\Delta ct$ = sampl Ct – control Ct
Fold change = $2^{-\Delta\Delta ct}$

The expression of *c-Myc*, *ID3*, and *P53* genes, was measured by RT-PCR, and *GAPDH* was used as the housekeeping gene. 1×10^6 Raji cells were seeded into 6-well plates containing 2 mL RPMI medium with 10% FBS. The concentration of RCE was 250µg/mL, determined by MTT assay as IC50 for 24h treatment. The exact number of Raji cells seeded without RCE as control. After 24 hours, total RNA was obtained separately from the treated and control cells by Gene All kit. The German Sigma Aldrich cDNA synthesis kit used the RNAs to make cDNA. Primers were designed by Gene Runner software and checked by the Blast program at NCBI prepared by Sina Clone Company. Sequences of primers are presented in Table 1.

More accuracy was obtained by repeating samples three times and amplifications calculated by Mean fold changes ± standard deviation (SD).

Table 1. Forward and reverse primer nucleotide sequences and melting temperature points.

Gene	F primer Sequence 5'→3'	R primer Sequence 5'→3'	F-TM	R-TM
GAPDH	AGGGCTGCTTTTAACTCTGG	CCCCACTTGATTTTGGAGGG	60.21	60.01
P53	GCCCAACAACACCAGCTCCT	CCTGGGCATCCTTGAGTTCC	60.36	60.41
ID3	GACTACATTCTCGACCTGC	TCGTTGGAGATGACAAGTT	60.17	60.09
C-MYC	GGGAAGAAGCCAGTTCAGATC	GGAAGCAAGGAGAGTTTGTAG	60.54	60.73

2.6. Statistical analysis

In this study, SPSS (Statistical Package for the Social Sciences) software was used, and data were analyzed based on the experimental design with three separate assays. The student's t-test was used to compare groups in experiments, and the p-value <0.05 was statistically significant. The fold changes of gene expression were calculated by cycle threshold (Ct) data obtained from RT-PCR.

3. Results and discussion

3.1. MTT assay results

Evaluation of Raji cells viability by using RCE at different concentrations for three-time of 24, 48, and 72 hours is shown in Table 2. The table view shows that the inhibitory effect of RCE on Raji cell proliferation has a time and dose-dependent manner. IC_{50} for 24h incubation time is 258±6.23, while in 48h and 72h are 176±8.24 and 127±11.26, respectively.

Table 2. Inhibitory effects of red clover extract on Raji cells.

Concentration of red clover extracts (µg/mL)	Inhibition (%)		
	24 h	48 h	72 h
0 (untreated)	5.01±0.4	5.27±0.6	6.39±1.41
100	21.13±1.52	30.14±1.98**	45.53±2.78
200	42.25±2.10*	55.06±2.54	68.25±2.89*
300	67.91±3.07*	35.28±2.93	77.34±3.01
400	73.76±2.81**	80.17±3.12*	86.47±3.36
500	84.30±3.14	85.39±03.47	90.01±3.86*
IC50	258±6.23	176±8.24	127±11.26

Values expressed as mean ± standard deviation (n = 3). In each column, * represent *P*<0.05, and ** represent *P*<0.01 compared with untreated cells.

3.2. DNA fragmentation results

Apoptosis or programmed cell death plays an important role in the genesis of many physiological and pathological processes. Detecting the signs of apoptosis in the study of cellular metabolism is very important. DNA fragmentation is one of the most specific findings in apoptosis (Majtnerová and Roušar, 2018). In this study, Raji cells were treated for 48 hours with different concentrations of RCE (0, 100, 200. 400, 500 µg/mL), and DNA fragmentation was analyzed by DNA ladder assay technique. Agarose gel electrophoresis results (Figure 1) showed that RCE destructed the DNA into small pieces. Fragmented bands of DNA increased by increasing the RCE concentration. These results confirm the ability of this extract to induce apoptosis in Raji cells.

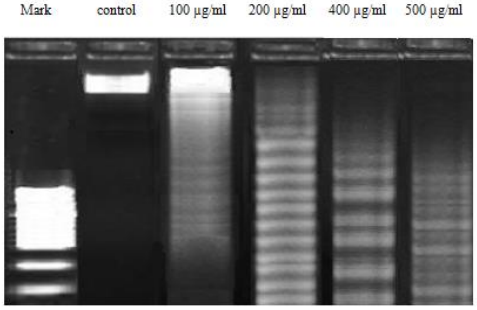

Figure 1. DNA fragmentation of Raji cells after 48 h treatment with red clover extract (0, 100, 200, 400, and 500 µg/mL)

3.3. Flow cytometry results

Flow cytometry studies have shown that cell growth and proliferation in treated Raji cells with RCE were inhibited by apoptosis-inducing. Raji cells were treated

with 250 µg/mL RCE, and a flow cytometry assay using Annexin V-FITC/PI kit was done after 24, 48, and 72 hours of incubation. Untreated Raji cells were used as control. Assay repeating showed that 250 µg/mL of RCE was the optimum concentration to separate the apoptotic and necrotic cells, and using less than 250 µg/mL resulted in aggregation of cells in the Q3 region of the graph, and in higher concentrations, most of the cells located in Q1 (Figure 2). By using the 250 µg/mL RCE, distribution in four regions of the graph is suitable for separating the apoptotic or necrotic cells. The graph of this assay has been shown in Figure 2.

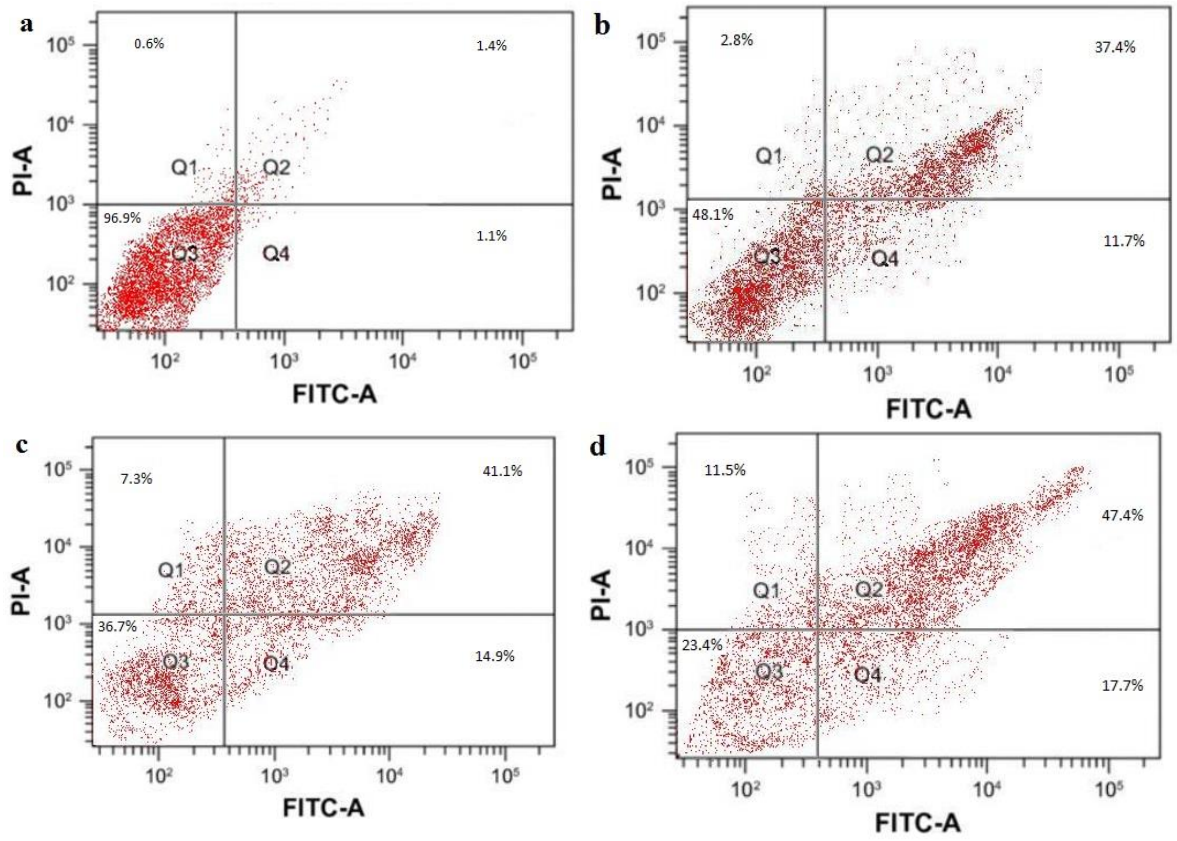

Figure 2. Flow cytometry graph of Raji cells treated with 250 µg/mL red clover extract. a) untreated b) 24 h treated c) 48 h treated and d) 72 h treated cells. Q1= necrosis, Q2=late apoptosis, Q3= -viable, Q4=early apoptosis.

Figure 2 illustrates the graph of one flow cytometry run as an example to explain cell distribution in four parts of the graph. Table 3 summarizes the mean percentage of three assays in each part of the graph. The percentages of viable, early apoptosis, late apoptosis, and necrosis after three days with an interval of 24 h are summarized in Table 3.

Table 3. Percentages of viable, early apoptosis, late apoptosis and necrosis Raji cells treated with 250 µg/mL of red clover extract.

	Without treatment (%)	24 h treatment (%)	48 h treatment (%)	72 h treatment (%)
Viable cells	96.61±2.83	48.17±3.14*	36.73±2.86	23.41±1.42
Early apoptosis	1.14±0.07**	11.72±1.35	14.92±1.74	17.76±1.26
Late apoptosis	1.46±0.23	37.49±2.34*	41.16±3.49	47.43±4.15*
Secondary necrosis	0.62±0.11**	2.87±0.53	7.32±1.14	11.59±1.63

Values expressed as mean ± standard deviation (n = 3). In each column, * represent $P<0.05$, and ** represent $P<0.01$ compared with untreated cells.

According to Table 3, treating Raji cells with 250 µg/mL RCE (three times at 24 hours intervals) percentage of viable cells decreased from 96.61±2.83% to 23.41±1.42%, and early apoptosis increased from 1.14±0.07 to 17.76±1.26. The percentage of late apoptosis and secondary necrosis increased from 1.46±0.23 to 47.43±4.15 and 0.62±0.11 to 11.59±1.63, respectively. RCE induced apoptosis in Raji cells with an acceptable rate in a time-depending manner.

3.4. RT-PCR results

BL occurs due to chromosomal translocation and stimulation of c-*MYC* expression (Hu *et al.*, 2007). Suppression of *ID3*, which acts as an inhibitor, occurs in BL (Rohde *et al.*, 2017).

Mutation of *P53* tumor suppressor in BL leads to disease progression. Mutant P53 proteins have no regulatory role in the cell cycle (Smardova *et al.*, 2008). RT-PCR showed that treating Raji cells by RCE could inhibit the *cMYC* expression and induce the *ID3* and *P53* genes. These genomic changes in treated Raji cells with RCE lead to force cells entering to apoptosis pathway. In the condition of this study, the fold changes of *cMYC*, *ID3*, and *P53* are 0.68, 2.6, and 1.63, respectively. Figure 3 illustrates the fold changes of these three genes in treated Raji cells by 250 µg/mL of RCE for 24 hours.

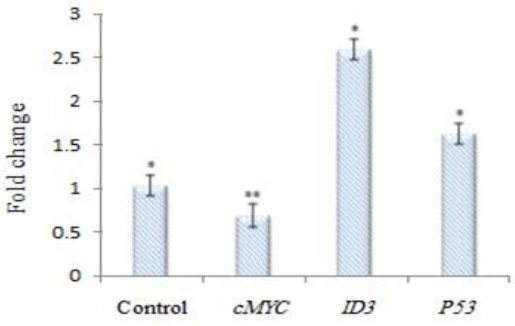

Figure 3. Expression of *cMYC*, *ID3*, and *p53* genes in treated Raji cell treated by 250 µg/mL RCE for 24 hours.
The data are expressed as mean ± SD of three independent experiments. * represent $P<0.05$, **represent $P<0.01$ compared with untreated cells.

In estrogen receptor expression, BL is similar to hormone-dependent, such as ER-positive breast cancer, and involves estrogen in its development and growth. ERα and ERβ are the two kinds of detected estrogen receptors. ERβ can interact with or inhibit ERα function. In order to apply the "inhibition of cell growth" function to cancer treatment, several specific ERβ agonists have been synthesized and tested for effectiveness in cancer treatment (Omoto and Iwase, 2015). ERβ, as a member of the nuclear receptor superfamily, shows potent tumor suppressive activities in many cancers. Raji cells dominantly have ERβ expression, and Diarylpropionitrile, an ERβ agonist, is valid for activating this receptor and reducing the dissemination of grafted BL tumors. Phytoestrogens' structural resemblance to 17 β-estradiol allows their binding to ERβ isoform predominantly, and therefore, expression of genes connected with elevated proliferation, motility, and invasiveness of cancer cells may be downregulated (Yakimchuk *et al.*, 2014; Zaklos *et al.*, 2020). The antitumor effect of RCE has been shown on the MCF-7 breast cancer cell line (Mannella, 2012; Kianinodeh *et al.*, 2017). The synergetic effects of RCE with Doxorubicin in xenograft mice are shown in triple-negative breast cancer (Akbaribazm *et al.*, 2020)

Burkitt lymphoma is usually treated with chemotherapy drugs and a targeted immunotherapy drug called Rituximab. This drug is a chimeric antibody that recognizes the human CD20 molecule and is indicated for treating non-Hodgkin lymphomas and other CD20-positive malignant cells. The CD20 molecule is a non-glycosylated protein expressed mainly on the surface of mature B-lymphocytes except in plasma cells (Dorvignit *et al.*, 2012; Pierpont *et al.*, 2018). Cross-linking of CD20 by antibodies (such as Rituximab) induces a redistribution of CD20 molecules to specialized microdomains at the plasma membrane known as lipid rafts. Stimulation of the B-cell receptor induces depletion of intracellular calcium stores. Calcium depletion results in the activation of calcium channels at the plasma membrane. Calcium-dependent signaling involves processes such as transcriptional control, cell cycle progression, or apoptosis (Janas *et al.*, 2005). Rituximab causes rapid and nearly complete depletion of peripheral B cells, which often do not replace for approximately six months (Chen and Cohen, 2012).

Without B-cells, the body would not be as effective at fighting some common bacteria and viruses. It would lack the long-lasting "memory antibody" function that is typical after recovering from an infection or being immunized against a specific infectious invader (Mallick, 2022).

Eliminating Rituximab or decreasing its usage in Burkitt's lymphoma treatment will prevent B-cell depletion and its side-affects. Currently, the replacement of Rituximab needs more investigations, but as mentioned above, RCE suppresses the Raji cells' growth and could be considered as an agent that reduces the use of Rituximab.

4. Conclusion

The effectiveness of a component for cancer therapy depends on its mechanism of the action on malignant cells. If the mechanism is necrosis, it may injure healthy cells in the body. If the mechanism is apoptosis-inducing, it can be considered as a drug because of the difference in metabolism between cancerous and healthy cells. In this study, DNA fragmentation and flow cytometry results have shown that the effective mechanism of the red clover extract to prevent Ragi cells proliferation is apoptosis-induction. Also, RT-PCR findings have shown that *cMYC*, the primary gene overexpression in Burkitt's lymphoma, is suppressed by red clover extract, and *ID3* and *P53* genes have favorite regulations to confront the disease. These changes led to the entering of Raji cells in the apoptosis pathway. Also, This study showed that red clover phytoestrogens could prevent Raji cell growth, and hormone therapy may be considered an adjuvant treatment. Finally, red clover extract has complication cure potential in Burkitt's lymphoma. The extract used in this study consists of four components. So, individual evaluation effects of Formononetin, Biochanin A, Daidzein, and Genistein on Raji cells are recommended.

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