



(RESEARCH ARTICLE)



Cytotoxic Effects of *Trifolium pratense*, *Baptisia australis*, and *Rubus idaeus* Extracts on CHO-K1 Cells

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Abstract

The cytotoxic effects of red clover, blue false indigo, and red raspberry plant extracts were evaluated on CHO-K1 cells. The hormone-dependent CHO-K1 cells are ovarian cells derived from Chinese hamsters. *Trifolium pratense* (red clover) extracts were obtained from the blossoms and leaves of the red clover plant. *Baptisia australis* (blue false indigo) extracts were obtained from the roots, stems, and leaves of the blue false indigo plant. *Rubus idaeus* (red raspberry) extracts were prepared from the fruits of the red raspberry plant. Two methods, soxhlet and microwave assisted extractions, were utilized and evaluated for their effectiveness in producing phyto extracts. Methanol was the solvent used in both methods. In all experiments, the CHO-K1 cell line was exposed to the different extracts for a period of 48 hours. An MTS assay was performed to evaluate the effectiveness of the cytotoxic capabilities of each extract at different concentrations on the CHO-K1 cell line. After the collection of all the data, the 1:1 combination of red clover and red raspberry extracts, obtained via soxhlet extraction, yielded the most potent cytotoxic effects on the CHO-K1 cell line.

Keywords: Soxhlet extraction; Microwave extraction; CHO-K1; Red clover; Red raspberry; Blue false indigo

1. Introduction

Ovarian cancer accounts for over 16,000 deaths in the US each year, making it the most lethal form of gynecologic cancer [1]. Ovarian cancer includes four histotypes: endometrioid, clear cell, mucinous, and papillary serous. This disease has a very poor prognosis because there is a lack of reliable screening tools. By the time of diagnosis, the disease has progressed to stage III, with less than a 39% survival rate in five years. Although new chemotherapeutic drugs and surgical procedures have been used to treat ovarian cancer, only small improvements in the overall survival rate have been recorded in the past 30-40 years [1].

Today's cancer treatments are physically and mentally taxing to a patient's body due to the side-effects. This occurs because the majority of drugs inflict unnecessary harm and destruction of healthy tissues when exposed to or given chemotherapeutic treatment. In an attempt to avoid administering generalized drugs, this study examines natural compounds which could be anticarcinogenic while avoiding deleterious side effects. Based on the scientific research of anticarcinogenic properties of red clover (*Trifolium pratense*), blue false indigo (*Baptisia australis*), and red raspberry (*Rubus idaeus*), extracts from these plants will be further evaluated in this study.

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Both *T. pratense* and *B. australis* are members of the *Fabaceae* family. Plants from this family are known to contain isoflavones and saponins. Several researchers established the potential effectiveness of *Zanthoxylum armatum* DC extracts and crude saponins against human breast cancer cell lines, MCF-7 and MDA-MB-468, and colorectal cancer cell line Caco-2 [2]. Women that were hormone-independent with cancer, versus, hormone-dependent with cancer, resulted in low to high benefits with treatments of red clover health supplements, respectively. Isoflavones found in red clover, are phytoestrogens, and possess anticancer properties. The isoflavones, formononetin and biochanin A, are both absorbed into the human body, then transformed to daidzein and genistein, respectively [3]. According to a lab technique called “chorioallantoic membrane assay”, the results show that daidzein, genistein, and biochanin A, contribute to red clover’s antiangiogenic and anti-inflammatory properties. Biochanin A also inhibits aromatase activity and expression [3]. The greatest contributor to red clover’s anticancer properties is the isoflavone formononetin, which has been extensively studied over the past decade. These studies offer more evidence of its potential as a strong candidate for cancer treatment [4]. Formononetin aided the apoptotic process and prevented proliferation in a multitude of cancers. Anticancer effects of formononetin were supported in studies in which breast, colorectal, and prostate cancer were analyzed. *In vivo* studies using formononetin, reported decreased tumor growth and metastasis in tested patients [5]. Formononetin exhibits strong antiproliferative effects and arrests the cell cycle’s oncogenic pathways. Additionally, formononetin also regulates transcription factors and growth-factor mediated oncogenic pathways [5]. Future research needs to be conducted to confirm whether or not red clover’s cancer fighting abilities are due to a select few of these flavonoids or if it is a synergistic effect provided by the presence of them as a whole. Red clover was studied as a complement to hormone replacement therapy. This extract contains isoflavones, mainly formononetin, which functions in treating menopausal disorders and reducing the risk of ovarian cancer due to high antioxidant properties [3].

Blue false indigo was tested *in vitro* against different cancer cell lines, including ovarian cancer to substantiate cytotoxic effects [6]. Evidence indicates that the anticancer effects of the blue false indigo plant are greatly due to their bioactive substances which include flavonoids, tannins, flavonols, polyphenols and saponins [6]. All of the different compounds within the blue false indigo plant are known to reduce cell proliferation in a variety of cancers. Heo, *et al.*, assessed the effects of indigo extractions on colon cancer, cervical cancer, liver cancer, breast cancer, gastric cancer, and laryngeal cancer cell lines, as well as healthy human renal cells [7].

As far as current research shows, ellagic acid is the prominent cancer fighting component found in red raspberries. Naturally found in red raspberries, as well as a variety of other fruits, ellagic acid is an antioxidant that exhibits anticancer effects within multiple cancer cell lines such as colorectal cancer, breast cancer, prostate cancer, non-small cell lung cancer (NSCLC), melanoma, bladder cancer, and ovarian cancer. The abilities of ellagic acid (EA) can be linked to two main attributes; its ability to be strongly antiproliferative, as well as apoptotic towards the cancer cell line tested. EA is a derivative of gallic acid, occurring in high levels within the fruits in which it is present. More specifically, these fruits are berries (strawberries, blackberries, red raspberries, and goji berries), pomegranates, and grapes. Tannins, and their sub-group of hydrolysable tannins, which includes ellagitannins, have received much attention in the past years with the evidence of their ability to fight a varying number of human diseases, including cancer, and their contribution to overall good health and well-being [8].

Furthermore, ellagic acid can inhibit metastasis, angiogenesis, and extra-cellular matrix invasion. EA effects all the necessary behaviors of a tumor’s attempt at survival and metastasis. EA may also increase tumor sensitivity to more mainstream cancer treatments such as chemotherapy and radiotherapy. Pure EA, its metabolites, and fruit juice containing ellagic acid are all under study and seem to be new to the face of cancer therapy. Within the studies that have been conducted, EA has been administered orally; however, an attempt to formulate a method with greater solubility will likely be available in the near future, if not already available [8]. EA is a polyphenol, found in a wide variety of fruits, vegetables, and some nuts. This polyphenolic compound comes from the ellagitannins family, which was first known from pomegranates for its antioxidant properties.

Research has shown that red raspberries pulp polysaccharides, termed RPP, exhibit anti-cancer effects. The raspberry pulp polysaccharides (RPP) were then tested against a cell line of melanoma within mice. B16F10 mouse melanoma cells were implanted subcutaneously into C57BL/6 mice in preparation for testing. The mice were given doses of 100 mg/kg, 200 mg/kg, and 400 mg/kg according to body weight. This dosage was maintained for two weeks. The RPP yielded significant results, showing inhibition of tumor growth by 7.56%, 24.32% and 59.95%, respectively. Additionally, RPP significantly increased the levels of TNF- α , IFN- γ , and IL-2 in the tumor-bearing mice in a dose-dependent manner. The increased secretion of TNF- α and IFN- γ might link to a stronger anti-tumor immune response by T cells; a topic warranting more attention in further research [9].

In this study our team undertakes a natural approach to treatment of ovarian cancer to minimize the harmful effects of toxic substances on the healthy cells of the body. Exposure of the CHO-K1 cell line to the red clover, blue false indigo, and red raspberry was examined.

2. Material and Methods

2.1. Cell Nutrition and Maintenance

CHO-K1 cells (85051005-1VL, Sigma-Aldrich Corp., St. Louis, MO, USA) were grown in T-75 cell culture flasks in an incubator at 37°C with flowing CO₂ at 5% concentration. The CHO-K1 cells were maintained with F-12K complete growth medium. The complete growth medium consisted of 500 mL of F-12K growth medium (ATCC® 30-2004, Manassas, VA), 50 mL of Fetal Bovine Serum (FBS) (15070063, VWR International, LLC, Suwannee, GA, USA), 500 µL of 10,000X HEPES (15630106, Thermo Fisher Scientific, Greenville, SC, USA) and 5 mL of Penicillin-Streptomycin (15070063, Thermo Fisher Scientific, Greenville, SC, USA). Following the addition of all components to the original 500 mL of F-12K medium, the mixture was poured into a vacuum filter (0.1 µm pore size) and run through to assure sterility. Every 48 hours, the complete growth medium was replaced. The old medium was removed and replaced with 25 mL of fresh medium. When the cells were approximately 80% confluent, they were split. The first step in this process was removing the old growth medium from the flask. Following this, 25 mL of phosphate buffered saline (PBS) (SH30256LS, Thermo Fisher Scientific, Greenville, SC, USA) was added to the culture flask to wash the cells. Once the PBS was in the culture flask, the flask was gently slid back and forth 80 times to ensure the cells were thoroughly washed, and then PBS was removed. Subsequently, 7 mL of trypsin (25200056, Thermo Fisher Scientific, Greenville, SC, USA) was added to the culture flask. Trypsin is added to unbind the cells from each other and away from the culture flask. While the trypsin was inside the culture flask, it was gently shaken back and forth 80 times. Trypsin was then removed, and the cell culture flask was placed into the incubator for 5 minutes. Following the 5-minute incubation, 25 mL of fresh growth medium was added to the culture flask. After the cells had been adequately suspended, the 25 mL volume was separated into 5 new culture flasks, with 5 mL in each. The new flasks containing only 5 mL of cells and media are brought up to a volume of 25 mL by adding 20 mL of complete growth medium.

3. Extraction Procedures

3.1. Soxhlet Extraction

In a typical soxhlet procedure [10], dried plants' parts, such as leaves, roots, and/or flowers were crushed, weighed, and placed in a cellulose thimble, which fit the soxhlet apparatus. Methanol (Thermo Fisher Scientific, about 300 mL) was used as the solvent for the extraction. On average, the process ran for 8 to 12 hours. Next, the solvent collecting in the thimble was almost colorless and the extraction was terminated. Methanol was removed using a rotary evaporator. The dark, sticky residue in the flask was scraped, transferred to a small vial, and weighed. It was stored in the freezer until used for the preparation of the stock solutions of extract in the proper media for the MTS assay.

3.2. Microwave Assisted Extraction

ETHOS™ X, microwave oven from the Milestone Inc. was used, with the infrared temperature probe, and set at the max power of 1600 W. The extraction was carried out in methanol, at the max temperature of 120 degrees Celsius for ten minutes, and under pressure, following the EPA Method 3546 [11]. Typically, plant material and methanol were added to the cylindrical glass tubes (about 20-30 mL). The glass tubes were then placed in the plastic sleeves of the vessels and tightly screwed on, before placing them symmetrically in the microwave oven rotor. The samples were kept for ten minutes at 120 degrees Celsius at the max power of 1600 W. After the heating period was completed, vessels were kept closed for at least 45 minutes to allow for cooling and only then were opened. The solvent was decanted and removed by rotary evaporation, the plant's residue was weighed and stored in the refrigerator until further use.

4. Preparation of the Extract's Solutions in Complete Medium

Extract preparation of *Trifolium pratense*, started by weighing 0.1g of the dried extract and aseptically placing the extract into a 50 mL centrifuge tube. Complete medium for the corresponding cell line was added to bring the final volume to 10 mL. The mixture was then vortexed into the solution. The same procedure was performed for *Baptisia australis* as well as *Rubus idaeus* to make the other extract solutions. Until used, all extracts were stored in the refrigerator at 4°C.

In addition, three separate combinations (of 1:1 mass ratio mixture) of two different phytoextracts were prepared. To begin, 0.05 g of *T. pratense* and 0.05 g of *R. idaeus* were weighed. Then, each sample was placed in a 50 mL centrifuge tube, along with the complete medium for the corresponding cell line, the volume was brought to 10 mL. The mixture was vortexed into solution and refrigerated until use. The same procedure was performed for a 1:1 mixture of *R. idaeus* and *B. australis* and a 1:1 mixture of *B. australis* and *T. pratense* to make the other extract solutions.

5. Plate Preparation

The cultured CHO-K1 cells were plated at a concentration of 1.0×10^5 cells/mL at a volume of 100 μ L in each well for columns 3-11 in a 96 well plate. The plate was incubated for 48 hours at 37°C, at 5% CO₂. Following the 48-hour incubation period, the wells were all emptied onto a sterilized paper towel. The wells were washed with 100 μ L of PBS and then emptied, followed by adding the extract mixture dilutions. Beginning with column one; this column contained only medium (M). Column two contained medium and extract only (ME). Columns one, two, and eleven were negative controls depicting no contamination in the medium or extract, and cells with normal proliferation free of contaminants, respectively. Column three contained cells with medium and hydrogen peroxide (H₂O₂) at 3% in a 1:1 ratio (MCH₂O₂), with a final concentration of 1.5% H₂O₂. Column three was our positive control, resulting in cell death. Columns 4-10 contained cells with the different plant extract dilutions. Columns 4-10 contained cells and extract in concentrations of 1000 μ g/mL, 500 μ g/mL, 100 μ g/mL, 50 μ g/mL, 10 μ g/mL, 5 μ g/mL, and 1 μ g/mL, respectively. The same dilutions of extract were made for red raspberry, red clover, and blue false indigo, as well as the 1:1 red clover/red raspberry, 1:1 red clover/blue false indigo, 1:1 red raspberry/ blue false indigo mixtures. Duplicate plates were made in the same fashion. Following 48 hours of incubation, the plates were tested with an MTS assay to measure cell viability.

5.1. 96-Well Cell Plating, Addition of Extracts, and MTS Assay

First, the medium was removed from one of the T-75 culture flasks that contained the CHO-K1 cells. Phosphate buffered saline (PBS) (25 mL) was added to the flask to remove protective agents from the cells. The T-75 tissue culture flask was gently slid back and forth. PBS was then removed, and 15 mL of trypsin was added. Once again, the T-75 tissue culture flask was delicately moved back and forth. Trypsin was removed and the cells were incubated for 5 minutes at 37°C. Fresh medium (25 mL) was added to each of the T-75 culture flasks to remove attached cells. The same procedure was repeated for each of the next series flasks.

Subsequently, the 25 mL of medium/cell solution were removed and placed into a 50 mL centrifuge tube. Then, from this tube 20 μ L of medium/cell solution were removed and placed into a micro centrifuge tube. Trypan blue (20 μ L) was added and gently aspirated to mix. This mixture (10 μ L) was loaded into each side of a hemocytometer chamber, resulting in two cell counts. Once a cell count was obtained, the concentration was adjusted to inoculate 1×10^5 cells/mL to each 96-well plate. After a 48-hour incubation period the medium was removed from each 96-well plate decanting it onto a paper towel leaving the adhered cells attached to the bottom of the 96-well plate. Medium and other components were added to wells of columns 1-11 for a final volume of 100 μ L/well and incubated for 48 hours at 37°C, at 5% flowing CO₂. Following the 48 hours of cellular exposure to the various dilutions of plant extracts and controls, a reservoir was filled with 24 mL of fresh medium and 5.4 mL of MTS solution (CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS), Promega, Madison, WI, USA), and mixed well under light sensitive conditions. After the 96-well plates were taken out of the incubator, the solutions were removed from the plate by decanting it onto a paper towel. Each well was washed with 100 μ L of PBS. The MTS mixture (120 μ L) was added to wells in columns 1-11 using a multi-channel pipette. The plate was then incubated for 30 minutes at 37°C, at 5% CO₂. The absorbance of each plate was read on an ELISA plate reader for 2 hours, at 30-minute intervals, at 450 nm. For the other two extracts, as well as the three 1:1 mixture, the same procedure was applied.

5.2. Analysis

To ensure the results are significantly different from each other, the Bonferroni adjustment was used to compensate for a Type I error. Thus, the results of all figures use an $\alpha = 0.05 / 22 = 0.00227$ for each graph and result to indicate a true significant difference.

6. Results and Discussion

The red clover extracts incorporating the soxhlet method resulted in significant cytotoxic effects on the CHO-K1 cells at the 50 μ g/mL concentration and above (Fig. 1). Comparing the 50 μ g/mL extract concentration to the controls of medium (M), medium with extract at 1000 μ g/mL (ME 1000) (not significantly different), medium with cells and

hydrogen peroxide (MCH₂O₂), and medium with cells (MC) alone were significantly different with p-values of 0.00057, 0.00704, 0.000255, and 0.000145, respectively.

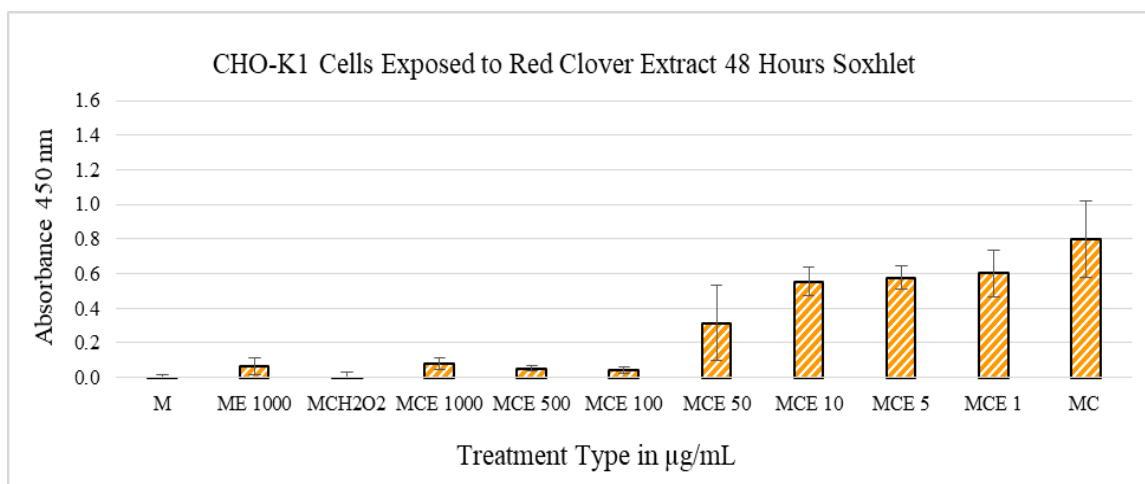


Figure 1 Cytotoxic effects of red clover exposed to CHO-K1 cell line for 48 hours was measured at an absorbance of 450 nm (soxhlet extraction method). Comparing the 50 µg/mL concentration to the controls of medium (M), medium and extract (ME 1000) (not significantly different), control, medium with cells and hydrogen peroxide (MCH₂O₂), and medium with cells (MC) alone, were significantly different with p-values of 0.00057, 0.00704, 0.000255, and 0.000145, respectively

The red clover extract made with the microwave assisted extraction method resulted in significant cytotoxic effects on the CHO-K1 cells at the 100 µg/mL concentrations and above (Fig 2). Comparing the MCE 100 µg/mL data to the medium (M) control, medium and extract (ME 1000) control, and hydrogen peroxide medium with cells (MCH₂O₂) were not significantly different, (p values of 0.00342, 0.01027, and 0.00356, respectively, while medium with cells (MC) control yielded significantly different results with a p-value of 0.00041.

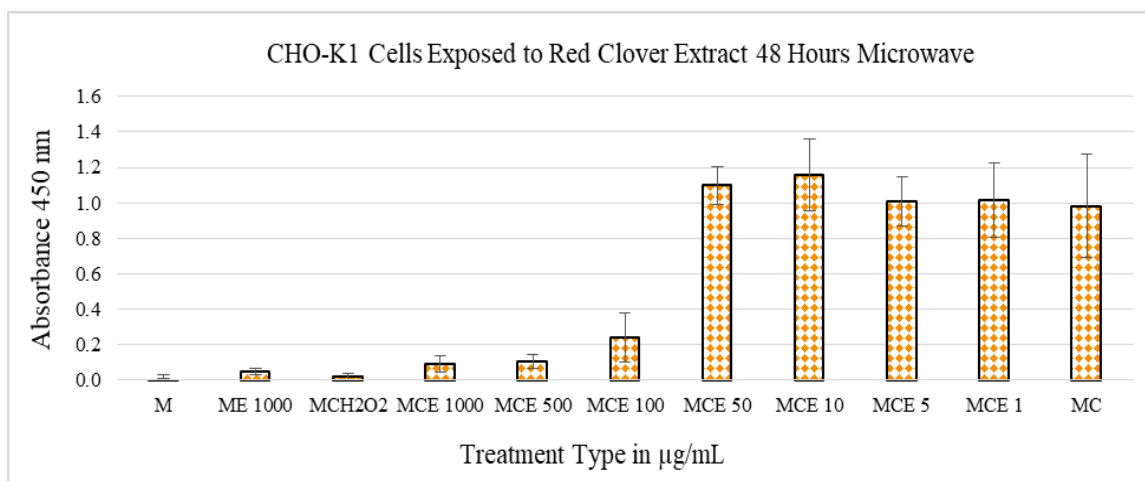


Figure 2 Cytotoxic effects of red clover at an absorbance of 450 nm on CHO-K1 cell line exposed for 48 hours (microwave assisted extraction method). Comparing the MCE 100 µg/mL data to the medium (M) control, medium and extract (ME 1000) control, and hydrogen peroxide medium with cells (MCH₂O₂) were not significantly different, (p values of 0.00342, 0.01027, and 0.00356, respectively, while medium with cells (MC) control yielded significantly different results with a p-value of 0.00041

The red raspberry extract incorporating the soxhlet method resulted in cytotoxic effects on the CHO-K1 cells at the 50 µg/mL concentration and above (Fig. 3). Comparing the 50 µg/mL extract concentration to the controls of medium (M), medium with extract at 1000 µg/mL (ME 1000), medium with cells and hydrogen peroxide (MCH₂O₂), and medium with cells (MC) alone were significantly different with p-values of 1.6934×10^{-7} , 9.0597×10^{-10} , 2.2687×10^{-7} , 6.7257×10^{-6} , respectively.

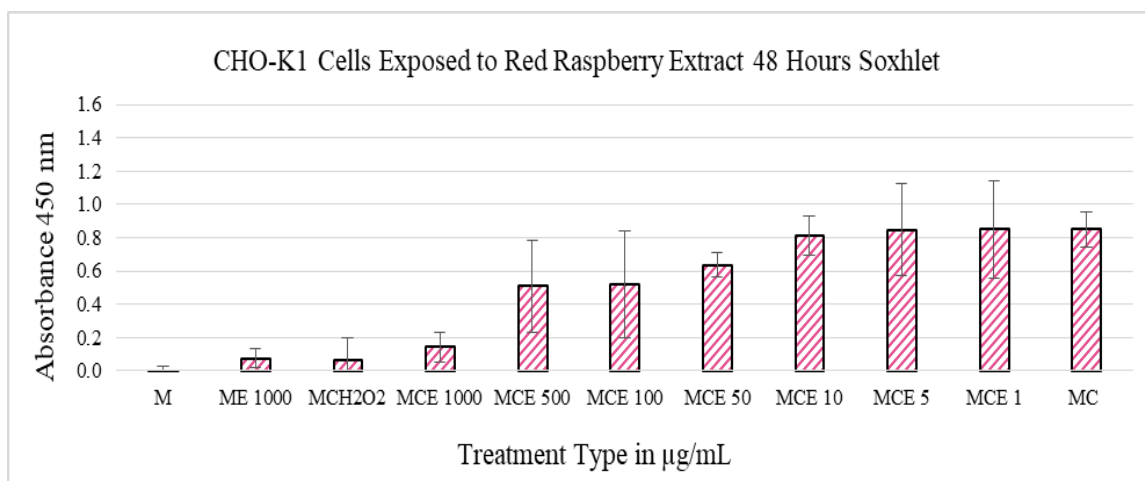


Figure 3 Cytotoxic effects of red raspberry extract exposed to CHO-K1 cell line for 48 hours was measured at an absorbance of 450 nm (soxhlet extraction method). Comparing the 50 $\mu\text{g}/\text{mL}$ extract concentration to the controls of medium (M), medium with extract at 1000 $\mu\text{g}/\text{mL}$ (ME 1000), medium with cells and hydrogen peroxide (MCH_2O_2), and medium with cells (MC) alone were significantly different with p-values of 1.6934×10^{-7} , 9.0597×10^{-10} , 2.2687×10^{-7} , 6.7257×10^{-6} , respectively

The red raspberry extract incorporating the microwave assisted extraction method resulted in no cytotoxic effects on the CHO-K1 cells at the 1000 $\mu\text{g}/\text{mL}$ concentration and below (Fig. 4). Comparing the 1000 $\mu\text{g}/\text{mL}$ extract concentration to the controls of medium (M), medium with extract at 1000 $\mu\text{g}/\text{mL}$ (ME 1000), were significantly different. However, medium with cells and hydrogen peroxide (MCH_2O_2), and medium with cells (MC) alone were not significantly different. All p-values in order are 2.4525×10^{-11} , 1.1588×10^{-9} , 0.1608, 0.6664, respectively.

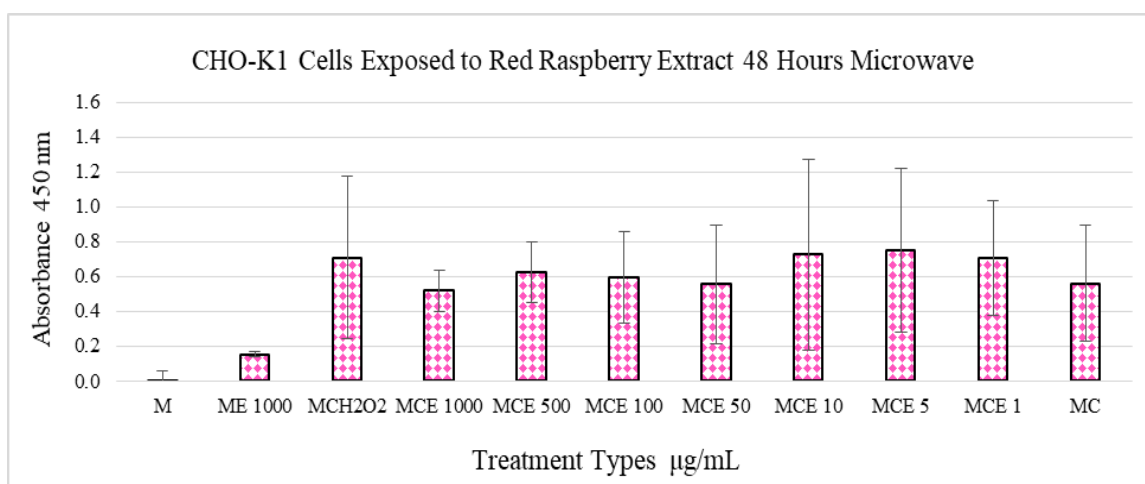


Figure 4 Cytotoxic effects of red raspberry extract exposed to CHO-K1 cell line for 48 hours was measured at an absorbance of 450 nm (microwave extraction method). Comparing the 1000 $\mu\text{g}/\text{mL}$ extract concentration to the controls of medium (M), medium with extract at 1000 $\mu\text{g}/\text{mL}$ (ME 1000), were significantly different. However, medium with cells and hydrogen peroxide (MCH_2O_2), and medium with cells (MC) alone were not significantly different. All p-values in order are 2.4525×10^{-11} , 1.1588×10^{-9} , 0.1608, 0.6664, respectively

The blue false indigo extract incorporating the soxhlet extraction method resulted in cytotoxic effects on the CHO-K1 cells at the 50 $\mu\text{g}/\text{mL}$ concentration and above (Fig. 5). Comparing the 50 $\mu\text{g}/\text{mL}$ extract concentration to the controls of medium (M), medium with extract at 1000 $\mu\text{g}/\text{mL}$ (ME 1000), medium with cells and hydrogen peroxide (MCH_2O_2), and medium with cells (MC) alone were significantly different with p-values of 4.5412×10^{-6} , 3.8440×10^{-5} , 4.4576×10^{-7} , 7.029×10^{-5} , respectively.

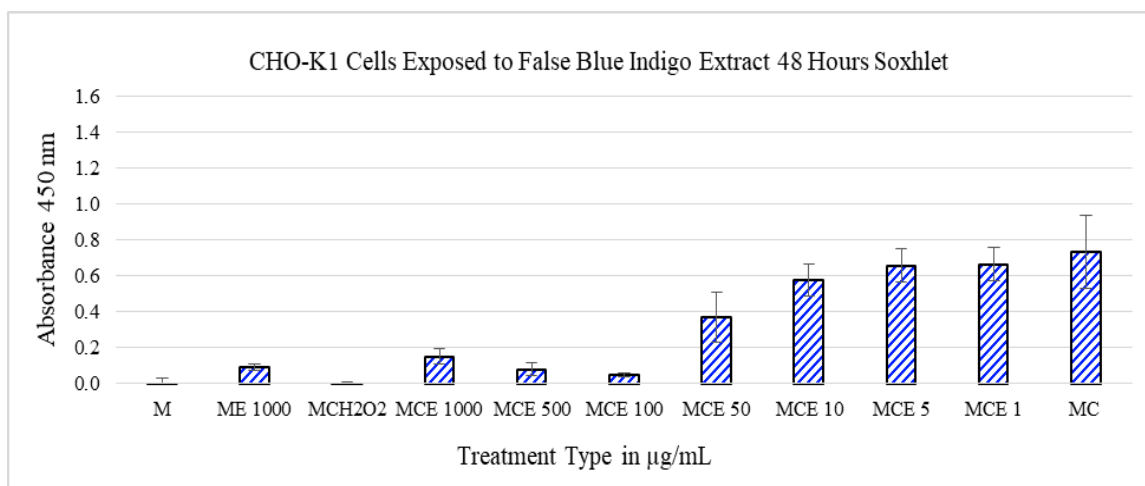


Figure 5 Cytotoxic effects of blue false indigo extract exposed to CHO-K1 cell line for 48 hours was measured at an absorbance of 450 nm (soxhlet extraction method). Comparing the 50 µg/mL extract concentration to the controls of medium (M), medium with extract at 1000 µg/mL (ME 1000), medium with cells and hydrogen peroxide (MCH₂O₂), and medium with cells (MC) alone were significantly different with p-values of 4.5412×10^{-6} , 3.8440×10^{-5} , 4.4576×10^{-7} , 7.029×10^{-5} , respectively

The false Blue False Indigo extract incorporating the microwave assisted extraction method resulted in cytotoxic effects on the CHO-K1 cells at the 1000 µg/mL concentration and above (Fig. 6). Comparing the 1000 µg/mL extract concentration to the controls of medium (M), medium with extract at 1000 µg/mL (ME 1000), medium with cells and hydrogen peroxide (MCH₂O₂), and medium with cells (MC) alone were significantly different with p-values of 2.4608×10^{-6} , 2.4621×10^{-6} , 3.7992×10^{-6} , 2.469×10^{-7} , respectively.

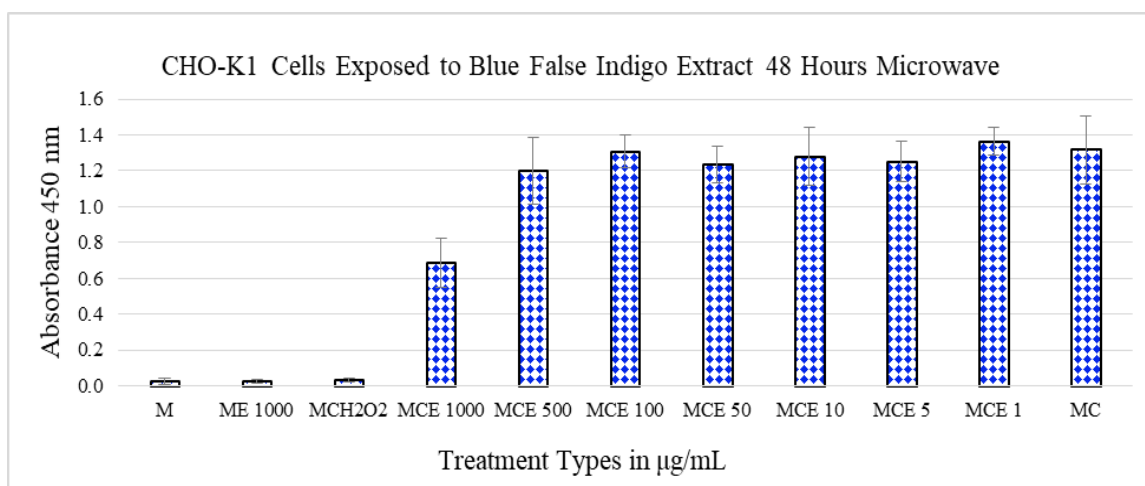


Figure 6 Cytotoxic effects of blue false indigo extract exposed to CHO-K1 cell line for 48 hours was measured at an absorbance of 450 nm (microwave assisted extraction method). Comparing the 1000 µg/mL extract concentration to the controls of medium (M), medium with extract at 1000 µg/mL (ME 1000), medium with cells and hydrogen peroxide (MCH₂O₂), and medium with cells (MC) alone were significantly different with p-values of 4.5412×10^{-6} , 3.8440×10^{-5} , 4.4576×10^{-7} , 7.029×10^{-5} , respectively

The 1:1 mixture of red clover-red raspberry extracts made via the soxhlet method showed the greatest cytotoxic effects on the CHO-K1 cells at the 50 µg/mL concentration or higher concentrations (Fig. 7). Comparing the 50 µg/mL extract concentration to the controls of medium (M), and medium with cells and hydrogen peroxide (MCH₂O₂), were not significantly different, however, medium with extract at 1000 µg/mL (ME 1000), and medium with cells (MC) alone were significantly different. All p-values in order are 0.5762, 0.1157, 0.0093, 0.00088, respectively.

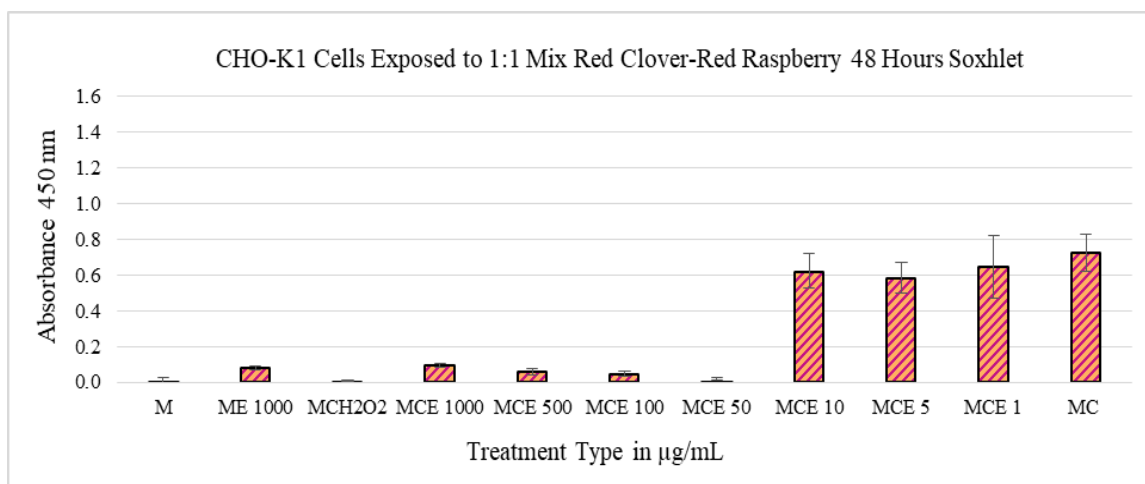


Figure 7 Cytotoxic effects of 1:1 mixture of red clover-red raspberry extracts at an absorbance of 450 nm exposed to CHO-K1 cell line for 48 hours (soxhlet extraction method). Comparing the 50 µg/mL extract concentration to the controls of medium (M), and medium with cells and hydrogen peroxide (MCH₂O₂), were not significantly different, however, medium with extract at 1000 µg/mL (ME 1000), and medium with cells (MC) alone were significantly different. All p-values in order are 0.5762, 0.1157, 0.0093, 0.00088, respectively

The 1:1 mixture of red clover-red raspberry extracts made via the microwave assisted extraction method showed the greatest cytotoxic effects on the CHO-K1 cells at the 1 µg/mL concentration or higher concentrations (Fig. 8). Comparing the 500 µg/mL extract concentration to the controls of medium (M), medium with extract at 1000 µg/mL (ME 1000), medium with cells and hydrogen peroxide (MCH₂O₂), and medium with cells (MC) alone were significantly different with p-values of 6.5027×10^{-6} , 6.6764×10^{-6} , 5.6809×10^{-5} , 3.5473×10^{-5} , respectively.



Figure 8 Cytotoxic effects of 1:1 mixture of red clover-red raspberry at an absorbance of 450 nm on CHO-K1 cell line for 48 hours (microwave assisted extraction method). Comparing the 500 µg/mL extract concentration to the controls of medium (M), medium with extract at 1000 µg/mL (ME 1000), medium with cells and hydrogen peroxide (MCH₂O₂), and medium with cells (MC) alone were significantly different with p-values of 6.5027×10^{-6} , 6.6764×10^{-6} , 5.6809×10^{-5} , 3.5473×10^{-5} , respectively

The 1:1 mixture of blue false indigo-red clover extracts made via the soxhlet method showed the greatest cytotoxic effects on the CHO-K1 cells at the 50 µg/mL concentration or higher concentrations (Fig. 7). Comparing the 50 µg/mL extract concentration to the controls of medium (M), and medium with cells and hydrogen peroxide (MCH₂O₂), were not significantly different, however, medium with extract at 1000 µg/mL (ME 1000), and medium with cells (MC) alone were significantly different. All p-values in order are 6.5628×10^{-6} , 0.29768, 8.33183×10^{-7} , 2.90672×10^{-5} , respectively.

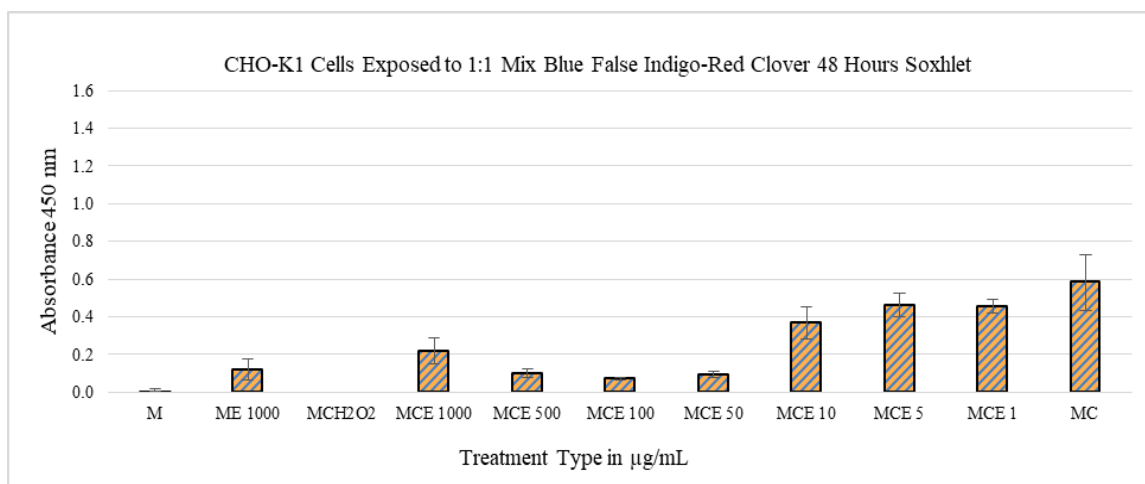


Figure 9 Cytotoxic effects of a 1:1 mix of blue false indigo and red clover extracts exposed to CHO-K1 cell line for 48 hours at an absorbance of 450 nm (soxhlet extraction method). Comparing the 50 µg/mL extract concentration to the controls of medium (M), and medium with cells and hydrogen peroxide (MCH₂O₂), were not significantly different, however, medium with extract at 1000 µg/mL (ME 1000), and medium with cells (MC) alone were significantly different. All p-values in order are 6.5628×10^{-6} , 0.29768 , 8.33183×10^{-7} , 2.90672×10^{-5} , respectively

The 1:1 mixture of blue false indigo-red clover extracts made via the microwave assisted extraction method showed the greatest cytotoxic effects on the CHO-K1 cells at the 1000 µg/mL concentration (Fig. 10). Comparing the 1000 µg/mL extract concentration to the controls of medium (M), medium with extract at 1000 µg/mL (ME 1000), medium with cells and hydrogen peroxide (MCH₂O₂), and medium with cells (MC) alone were significantly different with p-values of 2.86347×10^{-6} , 4.56641×10^{-6} , 3.10889×10^{-6} , 0.0003303 , respectively.

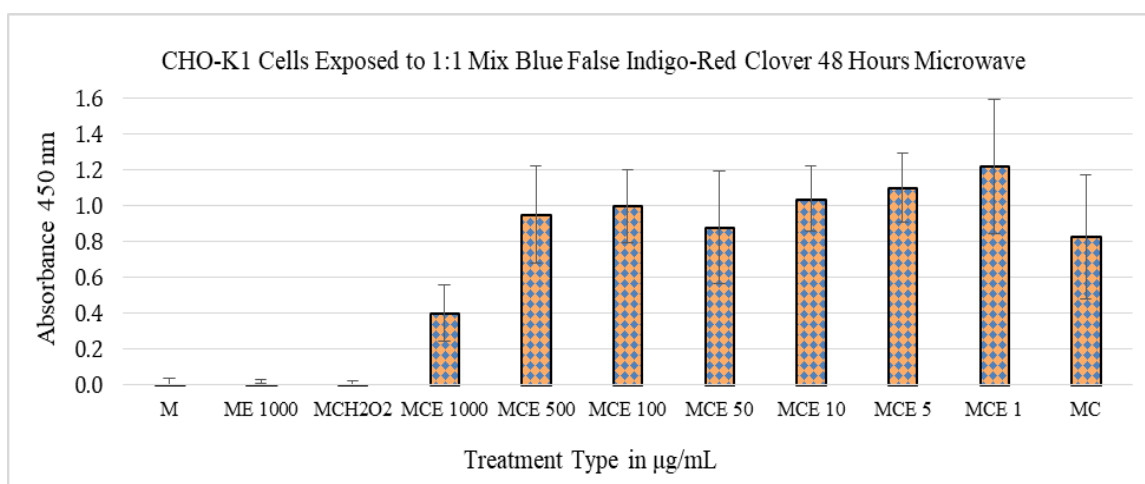


Figure 10 Cytotoxic effects of 1:1 mixture of blue false indigo-red clover exposed to CHO-K1 cell line for 48 hours and read at 450 nm, (microwave assisted extraction method). Comparing the 1000 µg/mL extract concentration to the controls of medium (M), medium with extract at 1000 µg/mL (ME 1000), medium with cells and hydrogen peroxide (MCH₂O₂), and medium with cells (MC) alone were significantly different with p-values of 2.86347×10^{-6} , 4.56641×10^{-6} , 3.10889×10^{-6} , 0.0003303 , respectively

Using the soxhlet method of extraction, the 1:1 blue false indigo-red raspberry extract applied to the CHO-K1 cell line for 48 hours, showed significant difference to the controls. Comparing the 100 µg/mL, blue false indigo extract, with the 100 µg/mL concentration and greater to controls of media (M), media and extract (ME 1000) (not significantly different), medium-cells and hydrogen peroxide (MCH₂O₂), media and cells (MC), with p-values of 8.6258×10^{-8} , 0.00266 , 1.710×10^{-7} , 2.5611×10^{-12} , respectively (Fig.11).

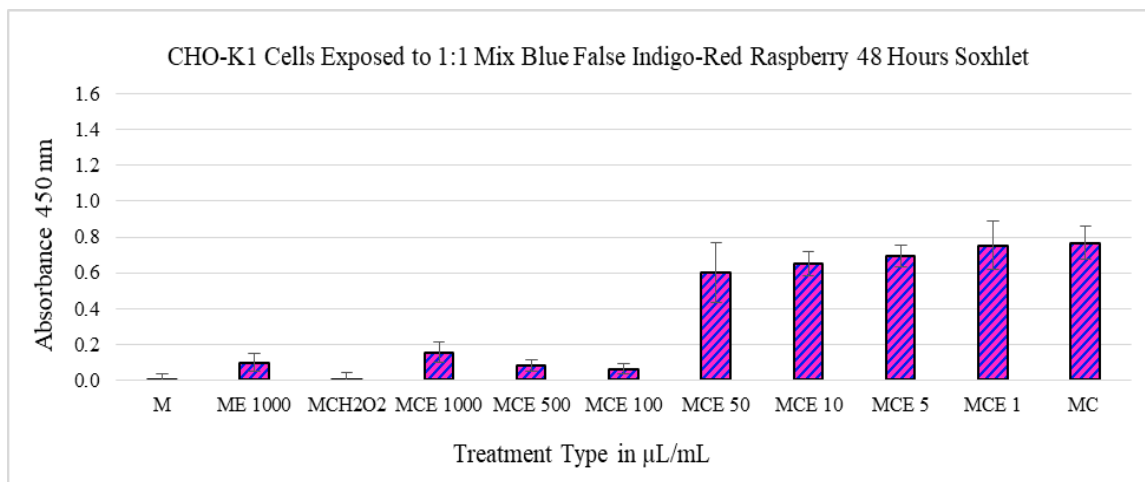


Figure 11 Cytotoxic effects of 1:1 mixture blue false indigo-red raspberry exposed to CHO-K1 cell line at an absorbance of 450 nm for 48 hours (soxhlet extraction method). Comparing the 100 µg/mL, blue false indigo extract, with the 100 µg/mL concentration and greater to controls of media (M), media and extract (ME 1000) (not significantly different), medium-cells and hydrogen peroxide (MCH₂O₂), media and cells (MC), with p-values of 8.6258 x 10⁻⁸, 0.00266, 1.710 x 10⁻⁷, 2.5611 x 10⁻¹², respectively

The 1:1 mixture of blue false indigo and red raspberry using the microwave extraction method showed a significant difference at the 1000 µg/mL concentration. Comparing the 1000 µg/mL concentration to the controls of media (M), media and extract (ME 1000), cells and hydrogen peroxide (MCH₂O₂), media and cells (MC), only the cells with hydrogen peroxide was not significantly different. All p-values in order 0.2320, 0.14959, 0.2263, and 0.00391, respectively (Fig.12).

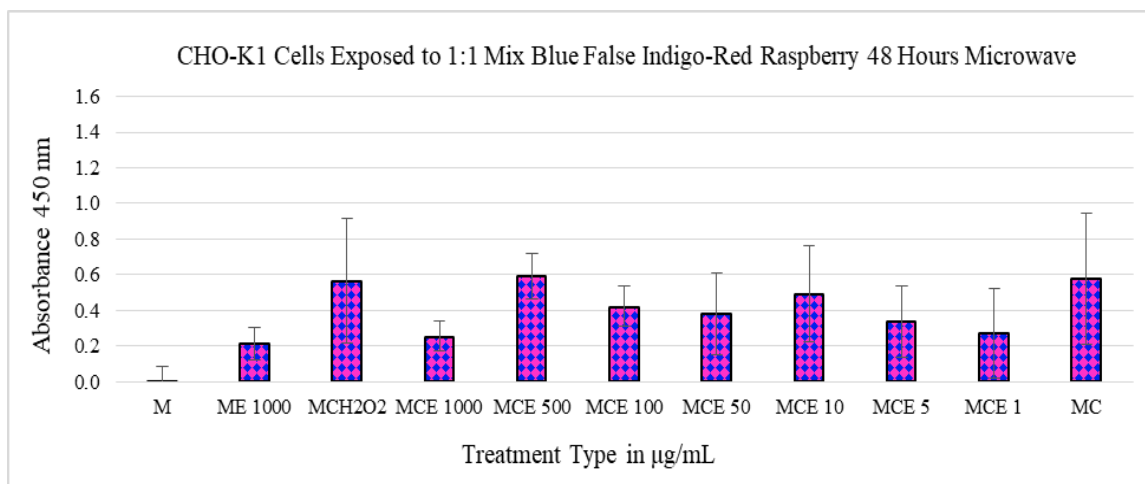


Figure 12 Cytotoxic effects of 1:1 mixture of blue false indigo-red raspberry exposed to CHO-K1 cell line at an absorbance of 450 nm for 48 hours (microwave assisted extraction method). Comparing the 1000 µg/mL concentration to the controls of media (M), media and extract (ME 1000), cells and hydrogen peroxide (MCH₂O₂), media and cells (MC), only the cells with hydrogen peroxide was not significantly different. All p-values in order 0.2320, 0.14959, 0.2263, and 0.00391, respectively

To summarize the 12 different graphical data figures, the table below, (Table 1) describes the minimum inhibitory concentration tested of the different plant extract types, either alone or in combination, to cells alone grown in medium. Two different methods of extraction were evaluated, the soxhlet extraction method and the microwave assisted extraction method.

Table 1 Minimum inhibitory concentration of plant extract types to cells alone comparing the soxhlet extraction method to the microwave assisted extraction method

Extract Type	Soxhlet $\mu\text{g/mL}$	Microwave $\mu\text{g/mL}$
Red Clover	50	100
Red Raspberry	50	None significantly different
Blue False Indigo	50	1000
Red Clover-Red Raspberry	50	500
Blue False Indigo-Red Clover	50	1000
Blue False Indigo-Red Raspberry	100	None significantly different

7. Conclusion

The results were quite different between the two extraction methods. Examining the microwave extraction method, the red raspberries alone and the combination of blue false indigo and red raspberry extracts yielded no cytotoxic activity against the CHO-K1 cells at the 1000 $\mu\text{g/mL}$ concentration. Continuing with the microwave assisted extraction method, the blue false indigo alone, blue false indigo and red clover combined, all showed cytotoxic activity at the 1000 $\mu\text{g/mL}$ concentration against the CHO-K1 cells. However, the microwave extracted red clover and red raspberries combined resulted in a cytotoxic effect on the CHO-K1 cell line at 500 $\mu\text{g/mL}$, while the red clover alone exhibited significant cytotoxic effects against the CHO-K1 cells at 100 $\mu\text{g/mL}$ concentration.

The soxhlet extraction method yielded better results than the microwave assisted extraction method. The blue false indigo and red raspberry combination extract showed significant cytotoxicity to the CHO-K1 cells at 100 $\mu\text{g/mL}$. The soxhlet extraction method in red clover alone, red raspberry alone, blue false indigo alone, the combined red clover-red raspberry, and the combined blue false indigo-red clover resulted in significant cytotoxicity against the CHO-K1 cells at the 50 $\mu\text{g/mL}$ concentration. This data indicates that the phytochemicals and antioxidants within these extracts should be further investigated. The red clover alone with the microwave assisted extraction method and the blue false indigo-red raspberry combined extract from the soxhlet extraction method both yielded cytotoxic effects against the CHO-K1 cells at 100 $\mu\text{g/mL}$ concentration. However, the greatest cytotoxic effects came from the extracts produced via soxhlet assisted extraction.

The lowest concentration of phytoextracts that had the highest cytotoxic effect on CHO-K1 cell line was red clover alone, red raspberry alone, blue false indigo alone, the combined red clover-red raspberry, and the combined blue false indigo-red clover resulted in significant toxicity against the CHO-K1 cells at the 50 $\mu\text{g/mL}$ concentration.

The best results between the soxhlet and microwave methods, was determined to be the soxhlet method. The microwave assisted extraction is shorter than the soxhlet (10 minutes versus 12 hours); however, soxhlet temperatures correspond to the boiling point of methanol 65°C, which is significantly lower than 120°C for microwave assisted extraction. Therefore, the data supports the fact that at the higher temperature, the decomposition of biologically important components of the extracts may occur. It is highly recommended that all components in these extracts should be identified and tested on the cell lines separately in future experiments.

Since the soxhlet extracted red clover alone, red raspberry alone, blue false indigo alone, the combined red clover-red raspberry, and the combined blue false indigo-red clover yielded the best results with a minimal concentration of phytoextracts, we recommend that these combinations be tested on a variety of ovarian cell lines. Further evaluation of different ovarian cancer cell lines with our recommended extraction combination and method could potentially identify a novel carcinogenic drug to treat ovarian cancer.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

Ryan Deweese, Ryan Hunter, Connor Davey, Christina Stacy, Dorota Abramovitch, and Donna Weinbrenner all declare they have no conflict of interest. Diana Ivankovic received the funding from the Dabo's All In Team Foundation and has no conflict of interest.

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