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CELL VIABILITY AND ANTITUMOR ACTIVITY OF PLANT PHENOLICS

Katerina Rouptiou[1], Rigini Papi[2], Amalia Aggeli[1] and Maria Liakopoulou-Kyriakides[3]*

[1]Laboratory of Biomedical Engineering, Department of Chemical Engineering, Aristotle University of Thessaloniki, Greece.
[2]Laboratory of Biochemistry, Department of Chemistry, Aristotle University of Thessaloniki, Greece.
[3]Department of Chemical Engineering, Aristotle University of Thessaloniki, Greece.

***Corresponding Author: Prof. Maria Liakopoulou-Kyriakides**
Department of Chemical Engineering, Aristotle University of Thessaloniki, Greece.

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ABSTRACT
Antioxidants protect cells from the damage caused by free radicals that are constantly generated in the human body, damaging tissues and leading to various diseases. Studies have shown that flavonoids reduce the risk of cardiovascular diseases, metabolic disorders, and other related diseases by reducing aggregation, acting as vasodilators in blood vessels. Here, extracts with major phenolics from the fruits of *Lycium barbarum* and *Hippophae rhamnoides*, *Punika granatum* reeds and wine marc as well as some pure phenolic acids and flavonoles were examined for their toxicity on mouse fibroblast cells L929 and two cancer cell lines, HeLa and MCF-7. Extracts at concentrations from 10 to 100 μg/ml of total phenolics were tested against the three cell lines using the MTT method. It was found that Goji berry and sea buckthorn phenolics affected cell proliferation of both HeLa and MCF-7 cell. Decrease on MCF-7 cell viability recorded by pomegranate while wine marc affected viability of HeLa cells. Possible apoptotic effect on HeLa and MCF-7 cells was also investigated using the multi-analyte profiling (xMAP) technology from Luminex.

KEYWORDS: *Punika granatum*, *Lycium barbarum*, *Hippophae rhamnoides*, wine marc, phenolic compounds, cell survival, L929, HeLa, MCF-7 cells, apoptosis.

INTRODUCTION
Phenolic compounds (phenolic acids and flavonoids) are known for their antioxidant properties and are widely distributed in fruits and vegetables. Antioxidants protect cells from the damage caused by free radicals. Free radicals are constantly generated in the human body resulting in extensive damage to tissues leading to various diseases such as cancer, Alzheimer's, renal diseases, cardiac abnormalities, etc.[1-4] Studies have shown that ingestion of flavonoids reduces the risk of cardiovascular diseases, metabolic disorders, and other related diseases by reducing aggregation, acting as vasodilators in blood vessels.[5-8] Thus, plants with antioxidant properties play vital functions in exhibiting beneficial effects and are used as an alternative source of medicine to mitigate diseases associated with oxidative stress and cancer.[4]

Hippophae rhamnoides L. commonly known as sea buckthorn, is a plant producing yellow-orange berries with bioactive constituents including lipids, polyphenols, carotenoids and vitamin C.[9-13] Sea buckthorn berries have been used in Tibetan and Mongolian medicines, for improving blood circulation and the function of the digestive system.[10,14-15] Since 1977, sea buckthorn has been listed in the Chinese Pharmacopeia as medicinal ingredient with his oil as raw material of many health products and cosmetics for a few decades. Since then, sea buckthorn oil is becoming more and more popular as special food supplement in Japan, Europe and North America. The most common among its phenolic compounds are ferulic acid, p-coumaric acid, ellagic acid, quercetin and kaempferol.[9]

Goji berry (*Lycium barbarum*) fruits are also known for their valuable properties and beneficial effect on human health. The fruits derived from *Lycium barbarum* have been used as food or medicine for more than 4000 years in China, which is the largest world producer of Goji berries yearly.[16-18] It is referred that berries have several pharmacological properties, such as antioxidant, anti-aging, neuroprotective, immunomodulatory and anti-Alzheimer's disease activities.[18] Traditionally, in China the herb is recommended for the nourishment of the liver and kidney and as an eye tonic.[16-18] Major phenolic constituent of the berries are myricetin, rutin, quercetin, kaempferol, quercetin, nicotiflorin, 7-O-β-d-glucopyranosyl-rutin, isorhamnetin 3-O-rutinoside.[19]

The last two decades a growing interest from scientists' targets to the recovery of nutraceutical or pharmaceutical products from food wastes as a part of recycling and improving environment. *Punica granatum* (Pomegranate) peels and winery wastes (marc and lees) are two wastes that have been reported to exert specific biological activities besides antioxidant properties due to their phenolic content.[20-26] The annual production of grapes worldwide is nearly 60.000.000 ton and mostly (80%) is used in winemaking thus resulting in large amounts of wastes. Wine marc (from red and white grapes) and lees contain valuable secondary by-products such as phenols, sugars and organic acids, whereas their free disposal increases the chemical and biochemical oxygen demand. Nowadays, winery wastes are considered as an alternative source for natural antioxidants for use in food and pharmaceutical industries. Fractions of isolated phenolics containing quercetin, kaempferol, ferrulic acid etc with high antioxidant activity as well as isolated compounds were reported to exert antiplatelet activity *in vitro* and anti-inflammatory activity against cyclooxygenases 1 and 2 (COX1 and COX2).[27-29] The antibacterial effect of wine wastes has been also reported.[30] In addition, pomegranate peel is a valuable and better source of bioactive compounds such as phenolic acids (hydroxycinnamic acid and hydrobenzoic acid), hydrolyzable tannins (ellagitannins, gallotannins, and gallagylesters), and flavonoids compared to the other parts of the pomegranate fruit.[30] Numerous studies have shown that the peel of the pomegranate is the component with the higher polyphenol content among the seeds, peel and juice.[20-21]

There is increasing evidence to support the hypothesis that free radical-mediated oxidative processes contribute to atherogenesis and specifically free radical damage results to pro-inflammatory response and finally to atherosclerosis.[31-32] Inflammatory response is mediated by cytokines, which induce the expression of adhesion molecules on the endothelial cell surface, such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1).[33] These molecules are involved in the endothelial recruitment of mononuclear cells. Adherence, followed by infiltration of mononuclear cells into the vascular wall, leads to scavenging of oxidized low-density lipoprotein (LDL), formation of lipid-laden foam cells and development or progression of the atherosclerotic plaque.

On the other, platelets play a key role in the atherosclerotic process. Platelet infiltration into the intima of arteries following endothelial damage contribute to the development of atherosclerotic lesions, and platelet aggregation at the site of atherosclerotic plaque rupture can increase the plaque size and lead to thrombus formation.[32] It is now recognized that free radical damage can impact upon the functionality of platelets and their contribution to the atherosclerotic process. Studies have shown that dietary polyphenols could reduce the risk of thrombosis, which is one of the leading causes for myocardial infarction, ischemic heart disease etc.[33]

In this study, we sought to examine and compare phenolic extracts, which from our previous studies had shown antiplatelet and anti-inflammatory activities, for their toxicity on three cell lines, mouse fibroblast L929 cells, HeLa and MCF-7 in an attempt to determine the limits of their positive effect to human life. The MTT method was used for cytotoxicity experiments and the Milliplex analysis kit to examine possible apoptotic effect of the extracts on the two cancer cell lines.[34]

MATERIALS AND METHODS

Materials

Dried Goji, sea buckthorn berries and pomegranate fresh fruits were obtained from the local market, Thessaloniki, Greece. Wine marc was obtained from a local winery during the period of wine production.

Total phenolic content

The content of total phenolic compounds (TPC), in all extracts, was determined using the Folin-Ciocalteu colorimetric method.[35] Gallic acid was used as a reference standard and the results were expressed as mg gallic acid per ml of water.

Extraction of Phenolic compounds

Selective Ultra sound-assisted extraction of the phenolic compounds was performed as previously reported with several minor modifications. For the two dried fruits (Goji and sea buckthorn berries) the same procedure for phenol extraction was followed.[14,27] Thus, certain amount of them with ethanol-water (70:30 v/v) was homogenized, put in an ultrasound apparatus (3x15 min), centrifuged after that and evaporated to dryness. The resulted solid material was diluted with water and its phenolic content was estimated using Folin-Ciocalteu colorimetric method.[35] Pomegranate fresh fruits (3-4 pieces) were peeled off and the collected peels were weighted, homogenized with alcohol and extraction was performed in ultrasound apparatus as above. After centrifugation of the mixture, alcohol solution was removed in a vacuum pump, following the same procedure for determination of total phenolic compounds. The wine marc from red wines, collected in the period of wine production, washed with cold water first to remove sugars and then dried in the oven at low temperature for 24 h and kept in a fridge until use. Then, refrigerated wine marc, at certain amount, was homogenized with ethanol and total phenolics were measured following the described procedure.

Cytotoxicity experiments

The potential cytotoxicity of the four phenolic extracts and specific phenolic acids and flavonoids such as ferullic, caffeic acids, myricetin, quercetin and kaempherol, constituents of the examined plant extracts on L929, HeLa MCF-7 cells was assessed using the 3-

(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[34] The MTT method records data using a plate reader. MTT and other similar assay methods include the use of different classes of colorimetric tetrazolium reagents, resazurin reduction and protease substrates generating a fluorescent signal, the luminogenic ATP assay, and a novel real-time assay to monitor live cells for days in culture. The assays are based on measurement of a marker activity associated with viable cell number. A variety of tetrazolium compounds have been used to detect viable cells such as MTT, MTS, XTT, and WST-1.[34,36,37] Viable cells with active metabolism convert MTT into formazan (Scheme 1), a purple colored product with an absorbance maximum at 570 nm. When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells. The exact cellular mechanism of MTT reduction into formazan involves reaction with NADH or similar reducing molecules that transfer electrons to MTT. The absorbance at 570 nm was determined using a Power wave XS plate reader (Biotek).

Apoptosis on cancer cell lines

Immortalized HeLa and MCF-7 cancer cell lines were used to test the possible apoptotic effect of the mentioned phenolic extracts. All extracts were tested at concentrations ranging from 10 to 100 μg/ml total phenolics. In particularly, phenolic extracts at these concentrations were added in a culture of 10^6 cancer cells and their apoptotic activity was investigated using multi-analyte profiling (xMAP) technology from Luminex. The MILLIPLEX® Early Phase Apoptosis 7-plex Signaling kit was used to detect changes in phosphorylated Akt (Ser473), JNK (Thr183/Tyr185), Bad (Ser112), Bcl-2 (Ser70), p53 (Ser46), active Caspase-8 (Asp384) and active Caspase-9 (Asp315) in cell lysates as described in the user guide (Cat. No. 48-669MAG, Millipore/Merck). Early phase apoptosis associated with phenolic extract treatment for 24 h as compared to no-treatment controls were determined using Luminex multiplex technology with a MagPix instrument. Cells were lysed in MILLIPLEX MAP Lysis buffer supplemented with protease inhibitor cocktail (Sigma) and, after BCA protein determination, 20 μg of total proteins were added to each well. All samples were run in duplicate and changes in MFI between treated cells and non-treated control were analyzed.

RESULTS AND DISCUSSION

Cell-based assays are often used for screening several compounds to determine if the test molecules have effects on cell proliferation or show direct cytotoxic effects that eventually lead to cell death. The effect of phenolic extracts on the viability of the three cell lines (L929, HeLa, MCF-7) was investigated using the MTT colorimetric method.[34] The MTT assay determines the functional state of mitochondria, indicating cell viability. A mitochondrial dehydrogenase enzyme in living cells reduces yellow tetrazolium MTT salt to blue MTT formazan, which is precipitated in uninjured cells.

Phenolic extracts were added to culture medium with L929 cells and incubated for 24, 48 and 72 hrs. In all cases, cell survival was over 80% at low concentrations (10-20 μg /ml) at 24 h of culture. Figure 1 depicts the effect of Goji berry's and wine marc's extracts on L929 cell culture at concentrations ranging from 10 to 100 μg /ml total phenolics. The cytotoxic effects of the plant extracts on the viability of the L929 cell lines are presented as percent cell viability. On the other, sea buckthorn berries and to a lesser extent wine marc did not present any significant toxicity at the same concentrations tested at 24hrs of cultivation (data not shown). Interesting, cell viability was decreased at 48 h of cell culture with all plant phenolic extracts and even more at 72 h. Pomegranate and Goji berry extracts decreased cell viability significantly after 48 hrs of cultivation, whereas with the other two extracts (wine marc and sea buckthorn) L929 cell viability, affected to a lesser extent. Figure 2 shows the differences in the percentage L929 cell viability in the presence of pomegranate extract at 24 and 48 h of culture. The decrease of cell viability after prolong culture in the presence of specific phenolic extracts may be attributed to several reasons besides further toxicity of phenolic's metabolic products in the culture medium.

The results indicated that positive effects could be obtained working with polyphenols at appropriate concentration range. It was found, that exposure time is also critical besides the concentrations of polyphenols. Therefore, the dose and composition of polyphenols should be investigated further for secure and healthy application.[40] A noticeable DNA damage induced in mice spleen cells has been recorded when higher concentrations of catechin were used.[38]

Preliminary experiments with higher concentrations of phenolic extracts and pure compounds (50 to 500 μg/ml) recorded higher values of cell toxicity. Indicatively, cell L929 viability in the presence of myricetin, caffeic acid and quercetin three major constituents of the extracts and other fruit and vegetables with multiple biological effects on human health, is given in Figure 3. It was found that myricetin, at concentration of 50 μM decreased L929 cell proliferation only 20% compared to other pure phenolic compounds tested, which recorded higher percentages ranging from 24-to 56% for caffeic acid and quercetin respectively, at the same concentration.

This was a necessary toxicity experiment in order to approach proper concentration of phenolic extracts to use in subsequent experimental studies without causing harm to the cells before hand. Literature regarding toxicological effects from human consumption and the width of nontoxic doses is rather limited.

The above extracts were also examined for their toxicity on two cancer cell lines, HeLa and MCF-7 at the same concentrations (10-100 μg/ml). All extracts affected cell viability with variations regarding cell type. As in all other cases time of culture was also significant. Here, 48hrs of culture found to be most appropriate time to evaluate the effect of phenolics on the toxicity of cell lines studied.

Figure 4 shows HeLa cell survival % in the presence of phenolic extracts from Goji berries at 24 and 48 h of culture. All plant extracts tested on MCF-7 cells did not cause any toxicity at low concentrations (10-50 μg/ml). Decrease in cell toxicity was recorded at higher concentrations. With Goji berry's extract (100 μg/mL) at 24 h cell viability was 80% and at 48 h decreased to 60%. Interesting, sea buckthorn extract at the same concentration of total phenolics, also resulted in significant decrease of MCF-7 viability from 60% at 24 h to about 30% at 48 h of culture (Table 1). Based on IC_{50} values calculated for the extracts tested against the two cancer cell lines at 48 h of culture, Goji and sea buckthorn berries had better effect on both cell lines (Table 2). Pomegranate peel extract had better effect on MCF-7 cells while wine marc on HeLa cells. The anticancer effect of pomegranate peel extracts has been reported.[39] The dietary use of pomegranate peels has been also reported to improving quality of meat and meat products.[40,41]

Extracts were also investigated for their possible apoptotic effect, at various concentrations (10 to 50 μg/mL) on immortalized HeLa and MCF-7 cancer cells, using the MILLIPLEX® 7-plex Early Apoptosis Magnetic Bead Kit (Cat. No.48-669MAG). The kit can be used for the simultaneous quantification of the following seven analytes: Akt/PKB, Bad, Bcl-2, caspase-8 and 9, Jank/sark1 and p53. Goji berry and pomegranate recorded significant increase of Bad and caspase 9 with a smaller increase of Jink and p53 apoptosis biomarkers (Figure 5).

Apoptosis is an essential physiological process that plays a crucial role in normal development and homeostatic mechanisms of multicellular organisms. Cell death can happen as a result of structural damage, or as a sequence of programmed cellular processes known as apoptosis. There have been several methods for detecting apoptosis. The most commonly described biomarkers of apoptosis can be measured in tissues and blood using a variety of technology platforms such as ELISA, flow cytometry, PCR, DNA. Apoptosis biomarkers include caspaces 2,3, 5, 8, and 9, Bcl-2, Bad, p53, Akt and JNK.[42,43] As it is reported two major apoptotic pathways have been described in mammalian cells: the mitochondria directed intrinsic pathway and the extrinsic (death-receptor-mediated) pathway. The extrinsic pathway, triggered by ligands binding plasma membrane death receptors, leads to activation of initiator caspase 8.[42] In certain cell types, extrinsic pathway activates effector caspases, such as caspase 3, whereas in others (and in most cancer cells) caspase 8 can amplify death signalling by engaging the intrinsic pathway which is controlled by pro- and anti-apoptotic Bcl-2 family proteins where, upon an apoptotic stimulus, changes in protein interactions at the mitochondrial surface determine the release of cytochrome *c*. Cytosolic cytochrome *c* activates the apoptosome complex, initiator caspase 9 and the effector caspases. The apoptotic effect of garcinol and curcumin (plant flavonoids) on human leukemia cells through cytochrome c and activation of caspases has been also reported.[44] Bcl-2 family members play a pivotal role in the regulation of apoptosis as they reside upstream of irreversible cellular damage and focus at the level of mitochondria.[43]

Table 1: Survival % of MCF-7 cells in the presence of the extracts.

Extracts	Concentration μg/ml total phenolics	MCF-7 cell survival % (24h)*	MCF-7 cell survival % (48h)*
Goji berries	50	87.70	77.96
	100	82.23	60.36
Sea buckthorn berries	50	93.25	73.65
	100	62.60	33.45
Pomegranate reed	50	99.70	98.25
	100	87.86	79.95
Wine marc	50	97.95	97.62
	100	100.00	96.90

*All values reported here are the means of three different measurements.

Table 2: IC_{50} values (mg/mL) of phenolics tested against HeLa and MCF-7 at 48 h.

Phenolic Extract	HeLa cells IC_{50} values (mg/ml)	MCF-7 cells IC_{50} values (mg/ml)
Goji berries	0.080	0.120
Pomegranate reed	1.028	0.222
Sea buckthorn berries	0.168	0.073
Wine marc	0.260	1.833

Legends to Figures

Figure 1. L929 cell viability % in the presence of wine marc and Goji berry's phenolic extracts at 24 h

Figure 2. L929 cell viability % in the presence of pomegranate phenolic extracts at 24 and 48 h of culture

Figure 3. L929 cell viability % in the presence of myricetin, caffeic acid and quercetin at 24 h of culture

Figure 4. HeLa cell survival in the presence of phenolic extracts from Goji berries, at 24 (blue circles) and 48 h (red circles) of culture.

Figure 5. Multiplex analysis of HeLa cells early phase apoptosis after 24h treatment with various concentrations of Goji berry and pomegranate phenolic extracts. The MILLIPLEX® Early Phase Apoptosis 7-plex Signaling kit (Cat. No. 48-669MAG, Millipore/Merck) was used and the Median Fluorescence Intensity (MFI) was determined against 20 µg of total protein.

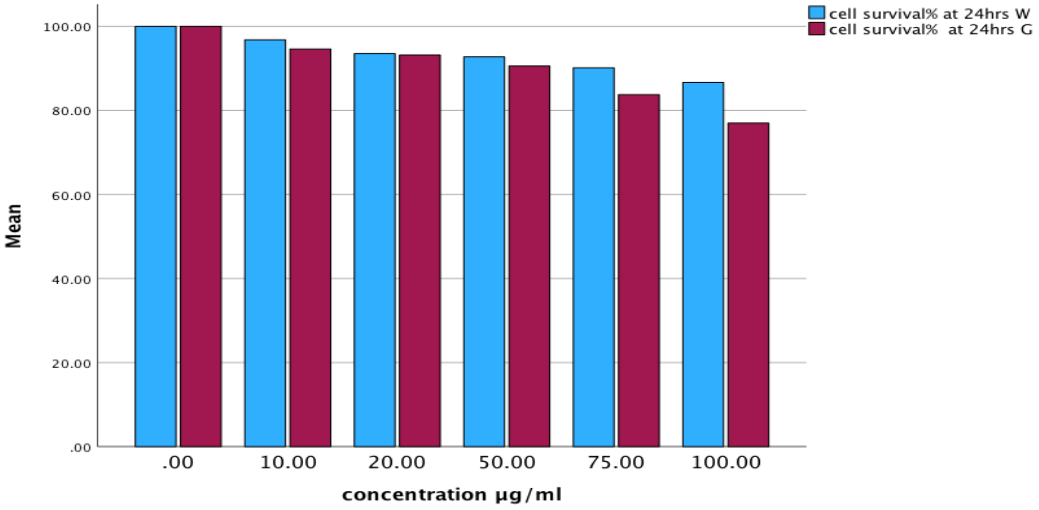


Fig.1.

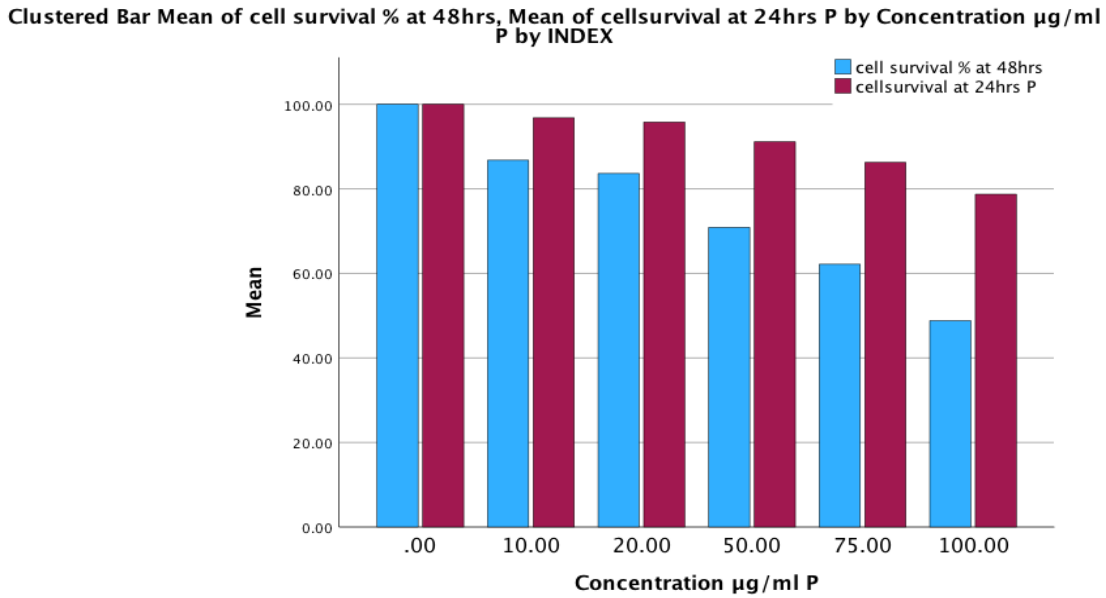


Figure 2.

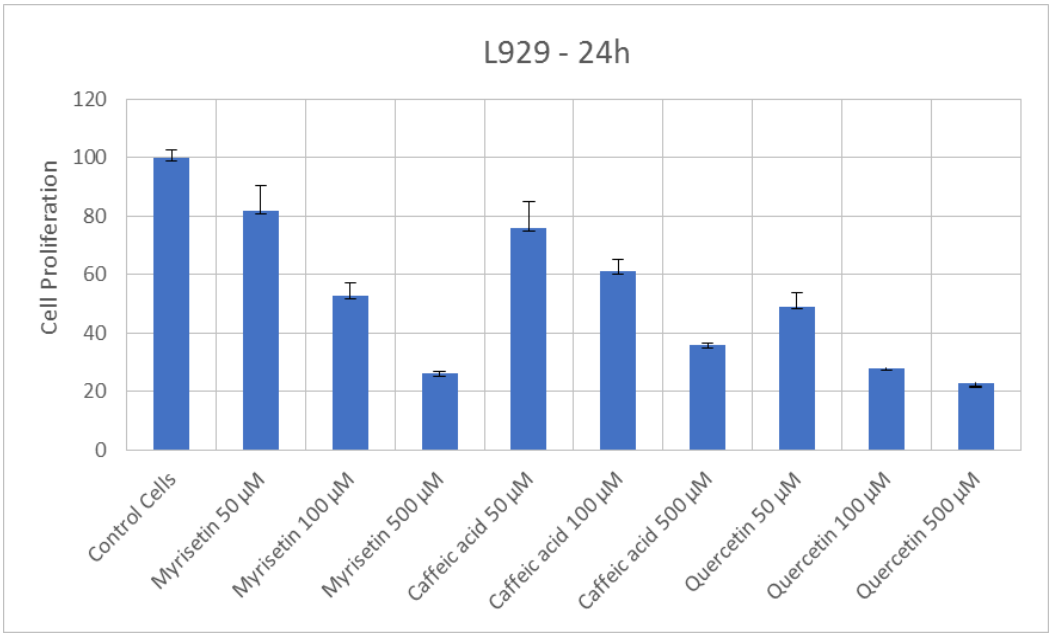


Figure 3.

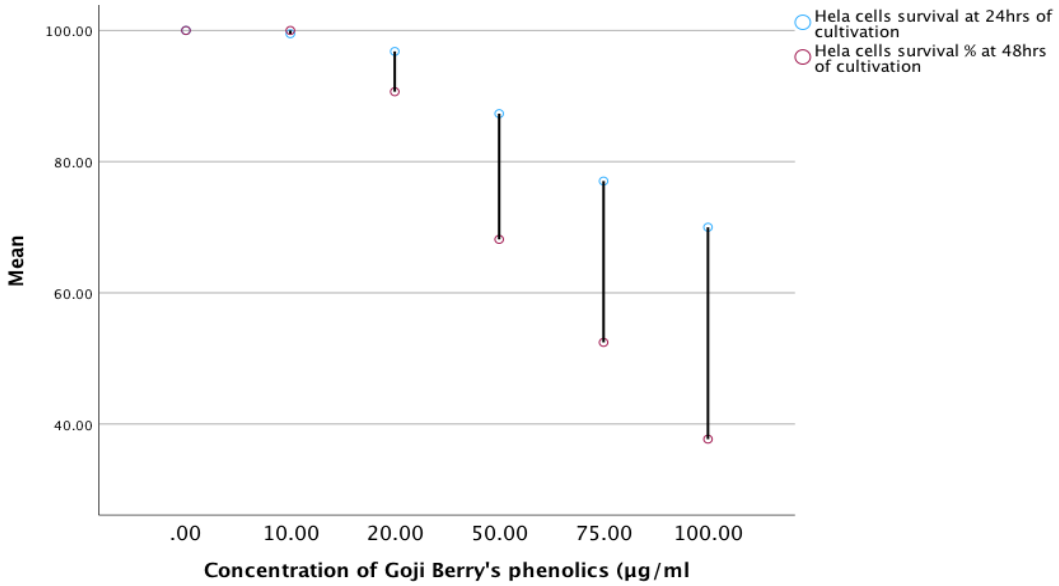


Figure 4.

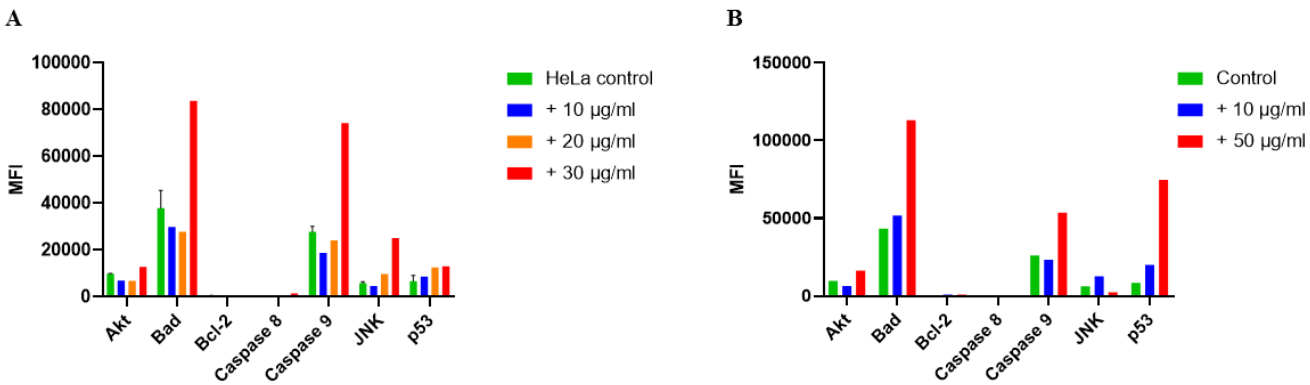
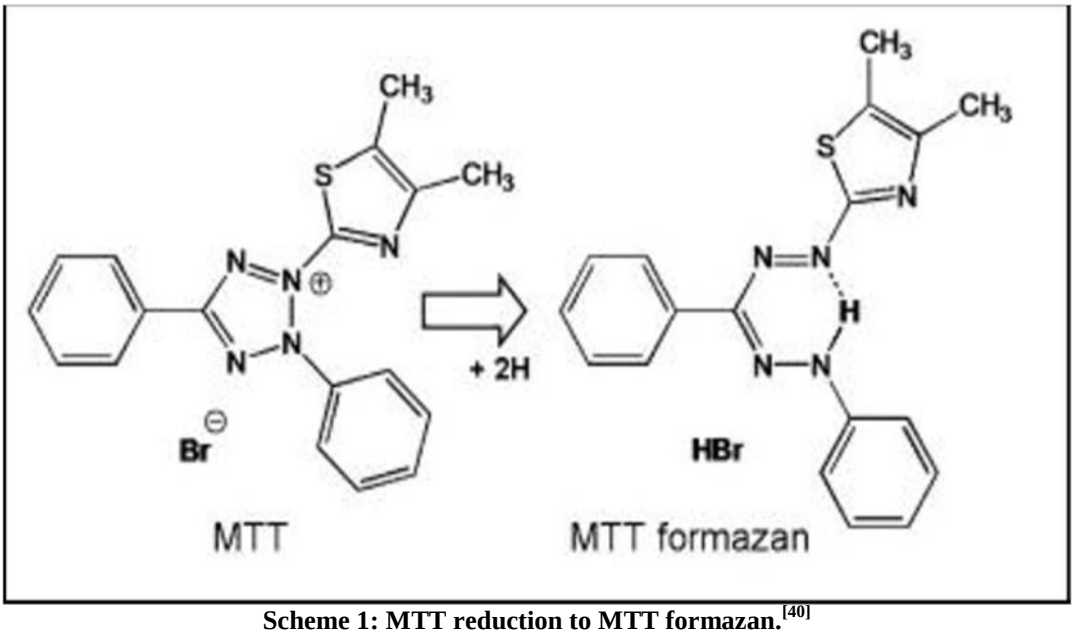


Figure 5.



Scheme 1: MTT reduction to MTT formazan.[40]

CONCLUSION

Plant extracts contain polyphenols with many biological activities besides their high antioxidant properties. Studies have shown their beneficial effect to human health without giving a range of the daily need in phenolic uptake through consumption of fruit and vegetables. Toxicologial studies here with L929 cells, indicated that phenolic extracts could be nontoxic at low concentrations. HeLa and MCF-7 cell toxicity caused by the extracts varied as it was expected due to the different phenolics contained in each extract and exposure time. Further clarification is needed regarding consumption of such products and their supplements, since overdose or interaction with medical treatments may have negative results. Similarly, risk/benefit studies may be necessary when these are going to be used as health promoting or possible anticancer drugs.

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