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Characterization of solvents and optimization of stability and solubility of bioactive compounds used in lymphoma cell culture treatments

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ABSTRACT

Phytochemicals represent one of the rising agents in cancer research today. They are recognized as one the most abundant bio-active compounds found in many different sources, specifically in plants. Their anti-cancer effects have been frequently explored and reported in various research studies. However, the bioavailability and solubility of phytochemicals still represent the major issue in in vivo and in vitro research. This report analyses the effects of three different phytochemicals, commonly used for survival interference in malignant cancer clones, including thymoquinone (TQ), curcumin, and quercetin in a model of diffuse large B cell lymphoma (DLBCL). In order to characterize the impact of solubility of these compounds to their bioactivity in the DLBCL model, three different but highly widespread solvents were used. Determination of an optimal compound - solvent association is warranted when assessing the stability and activity of phytochemicals in cells. The results of this study indicate the dose-dependent decrease in cellular viability, including the treatments with all combinations of substances and solvents. In addition, we demonstrated that the choice of the solvent greatly influenced solubility and the overall effect of the compound in cancer cells.

Keywords: Phytochemicals, cell culture, solubility, solvents

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1. Introduction

1.1. The origin of phytochemicals

The origin of phytochemicals (from Greek *phyto*, meaning "plant") can be traced back to the same origin of plants as well. In fact, many historical societies established their medicine and health practices on phytochemicals usage [1]. The most famous societies included the Chinese who have been using phytochemicals since 2800 BC and ancient Greece where Aristoteles and Hippocrates introduced plant medicine from Asia to Europe in the first century AD [2]. Fast forward to the 20th century, ever since the 1980s scientists have been focusing on identifying different chemicals from plants which are of great interest to human health [2]. Phytochemicals are chemicals produced by plants via primary or secondary metabolism [3]. Multiple studies suggest the beneficial effects of phytochemicals including the anti-proliferative and anti-angiogenic effects, regulation of nitric oxide, relaxation of blood vessels as well as the increase of blood flow [3]. Some of the notable examples of health benefits include carotenes which offer free radical protection [4], curcumin which blocks the carcinogens[4] and induces cell death in cancer cells as well as overall protection against DNA damage [3].

1.2. Solubility and bioavailability of phytochemicals

The solubility of bioactive compounds such as phytochemicals primarily depends on the solvent used as well as on temperature and pressure. Low aqueous solubility remains a problem for the pharmaceutical industry. It has been estimated that more than 40% of novel drugs developed by Big Pharma are practically insoluble in water. It remains a major difficulty, particularly for formulation scientists [5].

Another issue related to phytochemicals is its bioavailability. The term 'bioavailability' refers to the direct amount of ingested quantity of phytochemicals that can induce a beneficial effect in the target tissues[6]. The sole mechanism behind bioavailability is increasingly an issue in the drug development process [7]. These issues can be attributed to several different reasons such as various interactions between chemical and food components during processing, digestion, and/or absorption, etc. [7]. The most frequent causes of low oral bioavailability are attributed to poor solubility and low permeability.

1.3. Phytochemicals in cancer research

Cancer represents a group of many disorders that are predominantly based on the uncontrolled proliferation of mutated cells [8], an aberrant cell behavior [9]. Cancer cells multiply when regular cell proliferation signals are absent and cells are resistant to signals which trigger apoptosis [9]. Polyphenols have been identified as multi-beneficial therapeutic agents since they are known to inhibit cancer cell proliferation, modulate the activity of multiple cell signaling cascades, regulate the cell cycle, and platelet function[6]. These plant molecules, therefore, cause an overall decrease in tumor mass and also help protect the healthy cells from the adverse effects of usual cancer therapies [10]. A positive correlation between the intake of phytochemicals and disease prevention has been found to be closely related to dietary factors, approximately up to 90% at the highest times [11].

The stability of phytochemicals under certain conditions such as temperature, storage, and presence of supplements in culture media requires a constant update[6].Based on the wide literature review, we aimed to focus on three phytochemicals including thymoquinone, curcumin, and quercetin. The bioavailability of these substances is still under research in biomedicine and related disciplines. Association of a compound to an optimal solvent, in which it improves the stability of a compound, could in perspective provide more reliable data in cell culture models by proposing the most efficient ratio for a given compound/ solvent pair. Here we aimed to describe an experimental design that explores solubility and stability of chosen selected phytochemicals, commonly used for survival interference in malignant cancer clones.

1.4. Thymoquinone

Thymoquinone (TQ) is a common active biological substance of the volatile oil of black seed [12]. The recognized biological functions of TQ include initiation of apoptosis, cell cycle arrest, reactive oxygen species (ROS) generation, immune system propelling, prevention of metastatic, and control of angiogenic events. Moreover, TQ has been shown to lower the side effects which occur as the consequence of traditional anticancer therapy such as chemotherapy [12]. TQ is soluble in organic solvents at approximately 0.5 mg/ml in a 1:1 ratio solution of ethanol and PBS (pH 7.2) [13]. The solubility of TQ in ethanol is approximately 16 mg/ml and in DSMO it is around 14 mg/ml [14]. For maximum solubility, TQ should firstly be dissolved in ethanol and then diluted with the buffer chosen in the study [14].

Many studies have reported the anticancer effect of TQ in different cancer models, including prostate cancer [15], colon cancer [16], and breast cancer [17]. Among others, TQ showed efficiency in decreasing cell viability in hematological malignancies including leukemia and lymphoma[18][19]. Many cell cycle assays usually showed inhibition of tumor cell proliferation even at the smallest dose of TQ, such as 25 μM solutions [13].

1.5. Curcumin

Curcumin is a constituent of yellow powder extracted from the roots of *Curcuma longa Linn*, known as turmeric [20]. It is commonly found in dietary supplements, flavoring for foods, beverages, and cosmetics

[21]. Curcumin is a natural phenolic compound, and as such is known as a potent anti-tumor agent with various anti-oxidant and anti-inflammatory properties [15]. Curcumin affects cancer cell proliferation by acting on different phases of the cell cycle and promoting apoptosis [21]. Its solubility is proposed to be 10 mg/ml in ethanol and >11 mg/L in DSMO as well as 0.5 M in NaOH after which it is immediately diluted in PBS[22].

Combinatorial treatments with curcumin and other anti-cancer drugs were discussed in many studies. For instance, one study described the range of curcumin concentrations 0.5- 8.0 g/day for 7 days with the addition of therapeutics for the treatment of breast cancer and 5 g for chronic myeloid leukemia (CML) in a 6 week period, including imatinib[23] or doxorubicin, a drug used for leukemia, lung, brain, prostate, ovarian and breast cancer [24]. This study demonstrated improved treatment efficiency upon the combination of doxorubicin and 4mg/kg curcumin when compared to doxorubicin alone [24]. Leukemia cells were shown to be highly sensitive to curcumin treatment. This is particularly important due to the fact that yearly almost 500,000 people are diagnosed with leukemia and moiety dies at a rather quick rate [25]. Moreover, curcumin was found to inhibit the cancer growth rate and apoptosis in T-cell leukemia lineages by causing DNA damage and initiating cancer cell apoptosis [25].

1.6. Quercetin

Quercetin is a plant flavanol representing the vast majority of the flavonoid group of phytochemicals. Commonly found in different foods such as apples, grapes, black, and green tea, it serves as a natural lining of protection against cancer activity [26]. Known quercetin anti-cancer effects include cell cycle arrest, and promoting apoptosis and autophagy [26]. In the discussion of generalized parameters of solubility, quercetin was found to be best dissolved in solvents of approximately 2 mg/ml concentration in ethanol and 30 mg/ml concentration in DMSO [27]. Most of the studies regarding quercetin are related to its antitumor activity at high concentrations, which usually range from 25 μM to 200 μM[28]. Research shows that peak concentrations of quercetin in the blood usually lead to 10-25 μM concentration [27]. Different types of studies also investigated the cytotoxic effects of quercetin in ovarian cancer, where it was found that quercetin does not harm healthy cells, and as such is toxic only to the malignant cells [28]. In virtually all of the cancer cells of the ovarian lineage, there was a high level of inhibited proliferation and induced apoptosis as well as particularly the induced cell cycle arrest [29].

For the purpose of our study, we have chosen three different solvents, DMSO, PBS, and ethanol to test the solubility and stability of the above-mentioned phytochemicals.

1.7. DMSO

Dimethyl sulfoxide (DMSO) is a clear odorless liquid [30], economically produced as a by-product of the paper industry [31]. It is a colorless liquid that performs as a polar solvent able to dissolve a large range of both polar and non-polar substances [32]. It is generally accepted that DMSO below 10% of concentration is considered nontoxic [33][34]. However, its cytotoxicity can vary between the cell lines, media, or incubation time [31]. Due to its useful and ambiguous properties, DMSO is used as a solvent in a vast majority of experimental studies [31].

1.8. PBS

Phosphate-buffered saline (PBS) is a type of isotonic buffer, commonly used in biological research [28]. The mechanism behind PBS involves performing a simulation of normal human homeostatic conditions including osmolarity, pH, and ion concentrations. PBS has many uses in research because it is isotonic and non-toxic to most cells [35].

1.9. Ethanol

In addition to its nature in commercial use, ethanol (EtOH) is used in the medical and research area as a solvent in cell culture [33]. Usually, it is necessary to be dissolved 1000 times or more in order to make viable concentrations used for experimental studies [33]. The solvent usage varies in each study, therefore the

concentrations can range from 0.01% to 5% from which the optimum solvent concentration can be found [33]. The general rule is using a concentration that will cause less toxicity to the cell cultures. Lower concentrations are always preferred, but, this is once again related to the importance of the stock concentration [33].

2. Material and methods

2.1. Cell culture

DHL-4 cell line was generously given by Eugenio Gaudio, PhD (Institute for Oncology Research, Bellinzona, Switzerland). Cells were cultured in RPMI- 1640 basal medium (Sigma Aldrich, USA), supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 1mM sodium pyruvate, and 1% of non-essential amino acid α-Glutamine (Sigma Aldrich, USA). Cell culture was grown in suspension and maintained at optimal conditions in a humidified atmosphere (95%), 5% CO_2 at 37°C. Mycoplasma contamination was tested in cultured cells by LookOut Mycoplasma qPCR Detection Kit (Sigma Aldrich, USA).

2.2. Substances and solvents

Thymoquinone (TQ), curcumin, and quercetin were purchased from Sigma Aldrich, USA (Table 1). Three concentrations of each substance were prepared using three different solvents including phosphate-buffered saline (PBS; Fisher Bioreagents, USA), dimethyl sulfoxide (DMSO; Sigma Aldrich, USA), and ethanol (EtOH; Honeywell, Germany). Prior to usage, PBS 10x was diluted in sterile dH_2O to obtain 1x concentration, whereas DMSO and ethanol were used without dilution in dH_2O. Substances were diluted in each solvent at the final volume of 1 ml to obtain 100 mM stock solutions.

Table 1. Overview of substances

Characteristics	Thymoquinone	Curcumin	Quercetin
Molecular Weight	164.20 g/mol	368.38 g/mol	302.24 g/mol
Appearance	White to Dark Yellow and Faint Orange to Orange and Faint Brown to Brown and Orange-Brown and Brown-Orange	Yellow to Orange, powder	Yellow, powder
Purity (HPLC)	> 98.0 %	> 65 %	> 95 %
Formula	$C_{10}H_{12}O_2$	$C_{21}H_{20}O_6$	$C_{15}H_{10}O_7$

Information obtained from Sigma Aldrich, USA.

2.3. Evaluation of solution stability at different temperatures

In order to test the stability of substances in all solvents, stock solutions were stored at different temperatures including 37 °C, room temperature (RT) (18-20 °C), and 4 °C for 48 hours. The formation of precipitates and/or crystals in solutions was followed twice in the period of 48h.

2.4. Evaluation of solution stability in the cell culture medium

To further test the stability of each solution, stock concentrations were diluted in RPMI-1640 basal cell culture medium (Sigma Aldrich, USA), supplemented as mentioned above. The solutions were left in the cell culture incubator at 37 °C and after 48 hours of incubation, the formation of precipitates was observed.

2.5. Evaluation of substances on cell metabolic activity

Cells were plated at an optimum seeding density of $2.5x10^4$ cells/well in triplicates in a 96-well plate and treated with doses of 100 mM, 50 mM, and 10 mM concentrations of TQ, curcumin, and quercetin dissolved in DMSO, PBS, and EtOH. Upon 48h incubation, 10 µl of Cell Counting Kit-8 (CCK-8) (Bimake, USA) was added to each well[36]. Plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 2-3 h. Cells incubated with solvents only were used as a control and RPMI-1640 (100 µl/well) was used as a blank. The absorbance values were measured at 450 nm and the reference wavelength 620 nm in a Multiscan FC microplate reader (Thermo Fisher Scientific, USA). The results are represented as a percentage relative to the negative control that was set as 100% of viability. Evaluation of effect was obtained by linear inhibition curve using Prism GraphPad software, version 8.

2.6. Evaluation of substance on cell viability

Cells were plated at an optimum seeding density of $2.5x10^4$ cells/well in triplicates in a 96-well plate and treated with three different concentrations prepared in three solvents. Plates were incubated at 37°C in a humidified atmosphere with 5% CO2 for 48 h. Cells incubated with solvents only were used as a control. In order to confirm the effect of treatments, the trypan blue exclusion test was performed to determine the number of viable cells in each treated sample. Cells were mixed and stained with 0.4% filtered trypan blue solution (GibcoTM Life Technologies, USA) at a 1:1 ratio and counted with Countess II FL Automated Cell Counter (Thermo Fisher Scientific, USA). The results are represented as a percentage relative to the negative control that was set as 100% of viability. Evaluation of effect was obtained by linear inhibition curve using Prism GraphPad software, version 8.

2.7. Statistical analysis

Homogeneity of variances was tested by Levene's test. Tukey's post hoc test was performed where Levene's test indicated homogeneity of variances ($p>0.05$), whereas the Games-Howell post hoc test was used in case of non-homogeneity of variances ($p<0.05$). $p<0.05$ was considered as the level of statistical significance. Statistical analysis and graph preparation were done by GraphPad PRISM software, version 8.3.

3. Results

One of the primary aims of using three different stock solutions in this study is to find at which concentrations substances would dissolve the best in chosen solvents. As shown in Figure 1, the solubility of the tested substances was the most efficient in DMSO, when compared to PBS and EtOH. Furthermore, PBS has been evaluated as a poor solvent, being unable to dissolve any substance. TQ could not be dissolved in PBS, whereas quercetin formed an opaque solution with undissolved particles. TQ was completely dissolved in ethanol, while curcumin and quercetin formed precipitants even after vigorous mixing and vortexing.
However, in preparation of 10 mM and 1mM stock concentrations, the smallest concentrations of 1 mMhave demonstrated the best solubility across all solvents in the case of curcumin and quercetin. TQ was not soluble in PBS at any stock concentration, while it was completely dissolved in DMSO and EtOH.

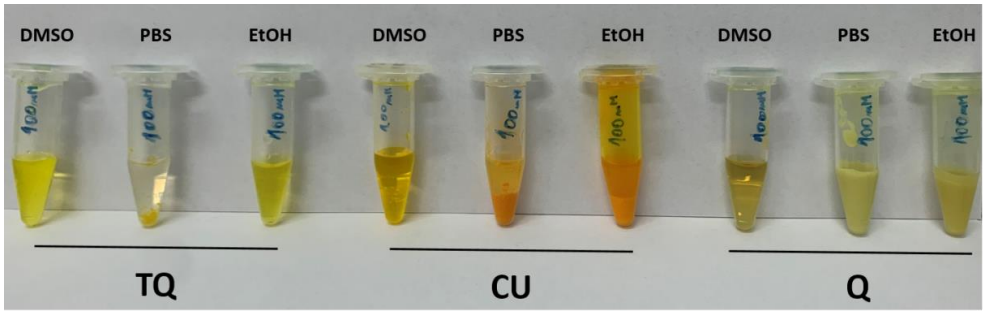

Figure 1. Stock solutions at 100 mM in DMSO, PBS and EtOH. TQ – thymoquinone; CU – curcumin; Q - quercetin

3.1. Evaluation of solution stability at different temperatures

All stock solutions were incubated at three different temperatures, 4°C, RT (18-25°C), and 37°C for 48h. Changes in the solubility of compounds were not observed upon different temperature conditions (Supplementary Table 1). The smallest concentrations were dissolved in all solvents at the RT, with an exception of TQ in PBS. Overall, we have noticed no difference between the highest and lowest temperatures of stock solutions. The solvents performed consistently across all temperatures where precipitates,non-cleared solutions, and non-homogeneity were present at higher and less at lower concentrations. PBS did not perform well at any temperature whereas DMSO was the best solvent at any temperature. EtOH dissolved substances at lower concentrations only across all temperatures.

3.2. Evaluation of substances on cell viability

Prior to the cell treatment, the stability of all 1mM stock solutions in the RPMI-1640 cell culture medium in the incubator for 48 h had been investigated. No precipitate or crystal formation in all substances except for TQ in PBS had been observed.

After treatment with 10 mM, 50 mM, and 100 mM concentrations of substances in all three solvents, we have observed a dose-dependent decrease in relative cell viability (Figure 2). Since all concentrations were unique across the solvents that also gave us an insight into how solvents might affect cell viability. Trypan blue assay has shown that substances dissolved in DMSO had the strongest effect on cell viability compared to EtOH and PBS (Table 2).

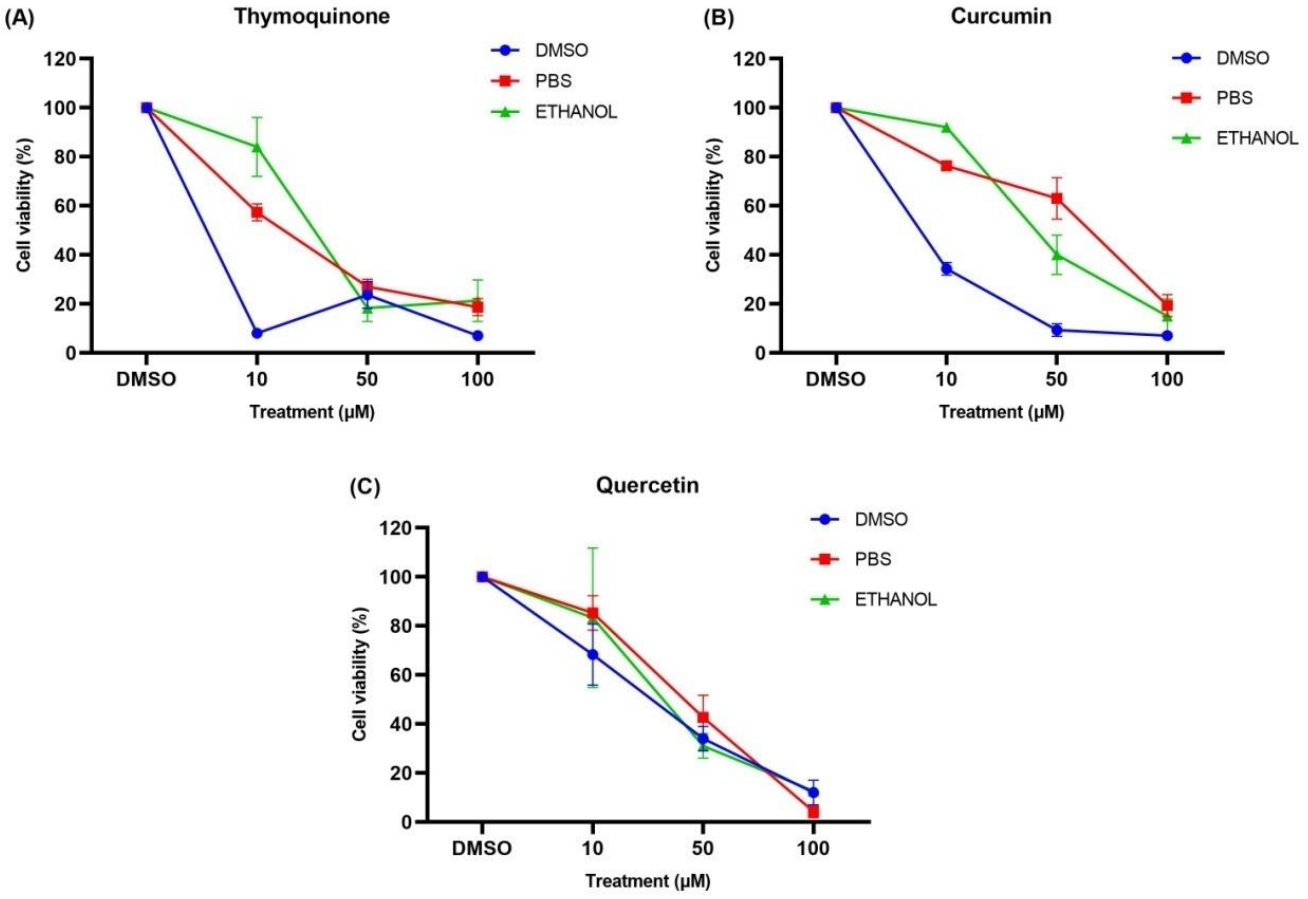

Figure 2. The cell viability analyzed by trypan blue assay after the treatment with TQ (A), Curcumin (B), and Quercetin (C) dissolved in DMSO, PBS, and EtOH

Table 2. Statistical analysis performed on trypan blue results in DHL-4 cell line after 48 h treatment

Substance	Treatment	Adjusted p value*		
		DMSO vs. PBS	DMSO vs. EtOH	PBS vs. EtOH
Thymoquinone	Control	>0.9999	>0.9999	>0.9999
	10 mM	<0.0001	<0.0001	<0.0001
	50 mM	0.7090	0.4233	0.1176
	100 mM	0.0267	0.0061	0.8015
Curcumin	Control	>0.9999	>0.9999	>0.9999
	10 mM	<0.0001	<0.0001	0.0005
	50 mM	<0.0001	<0.0001	<0.0001
	100 mM	0.052	0.812	0.450
Quercetin	Control	>0.9999	>0.9999	>0.9999
	10 mM	0.1113	0.1746	0.9670
	50 mM	0.5414	0.9274	0.3369
	100 mM	0.6169	0.9963	0.5664

*p values obtained by Tukey's post hoc test

3.3. Evaluation of substances on cell metabolic activity

To determine if substances decrease cell metabolic activity, we have performed WST-8 assay after 48h of incubation. As expected, in all substances dissolved in different solvents, we have observed a dose-dependent decrease (Figure 3). Results have indicated that treatment with substances in EtOH had a more prominent and significant effect (Table 3). Interestingly, we have seen that substances dissolved in PBS and DMSO had quite similar effects, without significant difference, except curcumin treatment at 100 mM and quercetin treatment at 10 mM ($p<0.001$ and $p=0.0011$, respectfully).

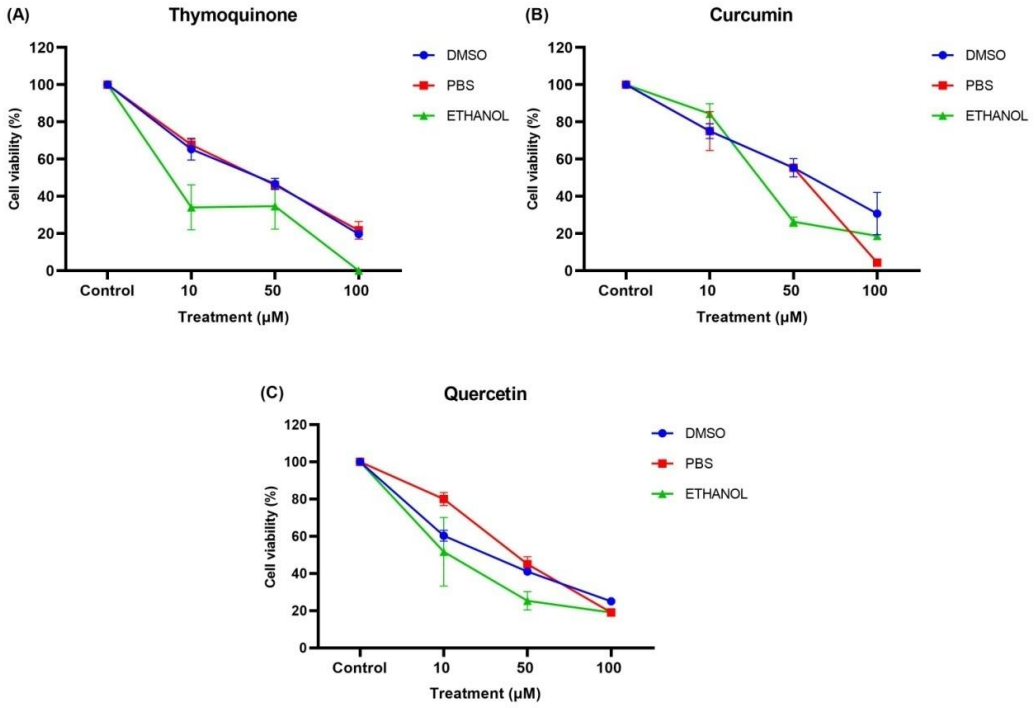

Figure 3: The growth inhibition curve of TQ (A), Curcumin (B), and Quercetin (C) dissolved in PBS, DMSO, and EtOH

Lavene's normality test showed that all samples were normally distributed ($p>0.05$), and we used the Games-Howell post hoc test to analyze all treatments.

Table 3. Statistical analysis performed on WST-8 assay in DHL-4 cell line after 48 h treatment

Substance	Treatment	Adjusted p-value*		
		DMSO vs. PBS	DMSO vs. EtOH	PBS vs. EtOH
Thymoquinone	Control	>0.9999	>0.9999	>0.9999
	10 mM	0.8689	<0.0001	<0.0001
	50 mM	0.9885	0.0398	0.0541
	100 mM	0.9017	0.0008	0.0003
Curcumin	Control	>0.9999	>0.9999	>0.9999
	10 mM	>0.9999	0.0930	0.0930
	50 mM	>0.9999	<0.0001	<0.0001
	100 mM	<0.0001	0.0249	0.0070
Quercetin	Control	>0.9999	>0.9999	>0.9999
	10 mM	0.0011	0.1851	<0.0001
	50 mM	0.6828	0.0084	0.0011
	100 mM	0.4315	0.4315	>0.9999

*p values obtained by Tukey's post hoc test

4. Discussion

This study aimed to evaluate the efficiency of each substance within a specific solvent. Generally speaking, EtOH was shown to be the most efficient solvent across all concentrations. However, its reaction across all substrates was strong, demonstrating the need for the presence of PBS or water for dilutions.

Moreover, an important feature to consider is the effect of different storage temperatures on the solubility of all compounds. This further indicates that temperature has a slight or no effect on solubility when its range is from 4 ˚C to 37˚C. It was reported that the prolonged effects of solvents on compounds, which showed that a longer period of time is required for the solvents to have a great effect on the solubility of compounds [37]. We have previously demonstrated the importance of optimal sampling technique and choice of a sample for successful downstream applications [38], [39].

In this study, we aimed to determine the most effective concentrations of substances at which they are dissolved the best and have the greatest anti-tumor effects on the cancer cell line. This was elucidated by treating the cancer cell lines with three different concentrations of substances dissolved in different solvents. In this case, DMSO performed with the most desirable outcome, whilst PBS performed with unwanted outcomes. Other studies also showed a prominent effect of DMSO on cell lines, such as a study done by PolokaFerk and Barbara D., which showed inhibitory effects of DMSO on the metabolic activity of human melanoma cancer cell lines [40]. Others showed that the dosage of DMSO concentration had great importance on adverse events [41]. In terms of concentrations alone, 1mM showed the best solubility across all solvents, and particularly in curcumin and quercetin. Regarding the cell viability, the higher concentrations caused a dose-dependent decrease in cell viability. The idea that DMSO is surpassing solvent can be further supported by various studies done on cancer models including myeloblastic leukemia [42], glioblastoma [43], or myeloid leukemia [31]. Further research demonstrated that dissolving quercetin in DMSO diluted with PBS was more favorable compared to quercetin being dissolved in DMSO alone [23]. Likewise, analyses demonstrated that it is advisable to use organic solvents such as DMSO in *in vitro* experiments to obtain more credible results, but also suggesting the mixing of different solvents in right rations for improved solubility [44].

Finally, the results showed the lowest cell viability for the highest concentrations across all samples present when compared to the controls. Therefore, the results of this study indicate the dose-dependent decrease in cell viability, considering the treatments with all combinations of substances and solvents.

5. Conclusion

The present study was designed to evaluate the stability and effects of thymoquinone, curcumin, and quercetin dissolved in the three most commonly used solvents. Solubility of substances in DMSO, PBS, and EtOH was particularly emphasized. Effects of differently prepared and stored substances were reflected through the treatment of DLBCL cell line, as shown by the differences in cell viability and metabolic activity upon the treatment. As previously mentioned, DMSO was shown to be the best solvent at all concentrations of phytochemicals, whereas phytochemicals could not be fully dissolved in PBS. Ethanol was shown to be a powerful solvent for 10 mM and 1 mM concentrations, whereas at higher concentrations its potency was not demonstrated. Upon the treatment, a dose-dependent decrease in cell viability was consistently observed in all prepared solutions.DMSO, as a solvent showed the greatest effects on the reduction of cell viability when compared to PBS and EtOH. Future prospective studies regarding the solubility of compounds and its effect on intracellular interactions are required to properly select the solvent and optimize solvent-compound ratio.

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