

Research Article

Cytotoxicity of Liquid Food Decontaminated by Spark Plasma

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Abstract

In the current study, the applicability of spark discharge for the decontamination of liquid food is investigated. While spark discharge has shown significant potential for liquid decontamination, food safety remains a primary concern for the adoption of any new method in food processing. In this work, the probable toxicity of the distillate following the spark plasma treatment was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT assay results for HepG2 cells showed no remarkable difference in cell viability between plasma-treated and untreated distillate. However, for Vero cells, a significant difference in cell viability was observed after 24 hours(h) of incubation. Aside from the group with an N(20)/D(80) culture media-to-distillate ratio, no significant differences in MTT assay results were observed after 48 hours of incubation. The discrepancy in cell viability observed in this specific group raises concerns regarding the method's suitability. Furthermore, inductively coupled plasma mass spectrometry (ICP-MS) results indicate that the concentration of certain trace essential elements, such as tungsten, increases in the distillate after treatment, exceeding the permissible limits for drinking water quality. Although spark discharge demonstrates significant bactericidal efficacy, its application for liquid food decontamination poses unacceptable safety risks.

Keywords: Pulsed discharge plasma, Cytotoxicity assessment, Elemental chemical analysis, MTT assay, ICP-MS

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1. Introduction

Water-containing substances possess a high potential for bacterial growth. Historically, the decontamination of liquid food and drinking water has been a major concern in the public health and food industries [1-4]. Even more recently, the importance of this issue has been escalated encountering with lack of potable water. Hence, many research groups try to develop efficient, cost-effective and

practical ways to decontaminate water and liquids. Filtering the water to remove contaminants physically like reverse osmosis [5], killing of bacteria using UV light [6], thermal processing [7], ozone injection or acidification [8] are some of the proposed methods for bacterial decontamination of water and liquid food. In this regard, decontamination of herbal distillates is one of the challenging issues in the beverage industry. Herbal distillates, which are widely used as flavorings, natural

beverages, and therapeutic drinks, are highly prone to microbial contamination due to their high water content and the complexity of their production and bottling processes, especially in traditional methods [9-11]. Furthermore, the presence of sensitive chemical components makes it difficult to select a most suited decontamination method. Along with the most common methods of thermal pasteurization and using chemical preservatives which can effect on the distillates nutritional and sensory properties, new alternative decontamination methods are being researched to minimize these negative side effects [12-16]. Pulsed discharge plasma is an innovative method for the decontamination of water and liquids in recent years. Several research groups, including Satoh et al. [17], Huang et al. [18], Lee et al. [19], Krishna Priya et al. [20] and Ohshima et al. [21] have reported successful bacterial inactivation in liquids using pulsed discharge plasma. These studies indicate that pulsed discharge plasma can effectively eliminate bacteria in water. Specifically, S. J. Lee et al. reported that the outer wall of *Escherichia coli* is damaged by the shockwaves generated by pulsed discharge plasma in water [19]. Despite these promising results, concerns remain regarding the potential negative effects of pulsed discharge plasma on the chemical, sensorial, and nutritional properties of water or liquid foods. In our previous study [22], we utilized pulsed discharge plasma to decontaminate peppermint (*Mentha piperita* L.) distillate from *Pseudomonas aeruginosa*. We investigated the effect of pulsed discharge plasma on the physicochemical properties of the distillate with sensitive compounds along with its bactericidal effects. Our results showed that the peppermint essential oil content decreased by approximately 4% during plasma processing, and the menthol content the primary component of the distillate was reduced by approximately 17%. Although change in pH, color and

peppermint essential oil of the distillate samples after the plasma treatment was negligible, safety concerns regarding its use in the food industry and for human consumption persist. Building upon this analysis, the present study aims to investigate the effects of spark plasma on the elemental chemical properties of distillate. As is well known, the generation of pulsed discharge plasma in a liquid results in shockwave formation, which can lead to electrode erosion. In this paper, we employed Inductively Coupled Plasma (ICP) analysis to determine the concentration of elements released into the plasma-treated samples. Furthermore, the cytotoxic potential of the treated samples was assessed using a colorimetric MTT cell viability assay. These evaluations provide deeper insight into the potential side effects of pulsed discharge plasma as a liquid disinfection method.

2. Material and method

2.1. Experimental set-up

Figure 1 shows our experimental set-up schematically. This study was conducted following our previous investigation regarding the feasibility of using pulsed discharge plasma for decontamination of liquid foods including those containing sensitive chemical components [22]. Following the design used in our previous study, the setup includes four tungsten wire electrodes, each 120 mm in length and 0.5 mm in diameter. The tungsten wires are covered by ceramic tubes except for the 2 mm from the end and are placed equally separated in parallel on a disc shaped Teflon holder on a circle with a diameter of 40 mm. These wires are used as the high voltage electrodes. A tungsten ring is placed under the high voltage electrodes tip concentrically. It acts as the ground electrode in our set-up.

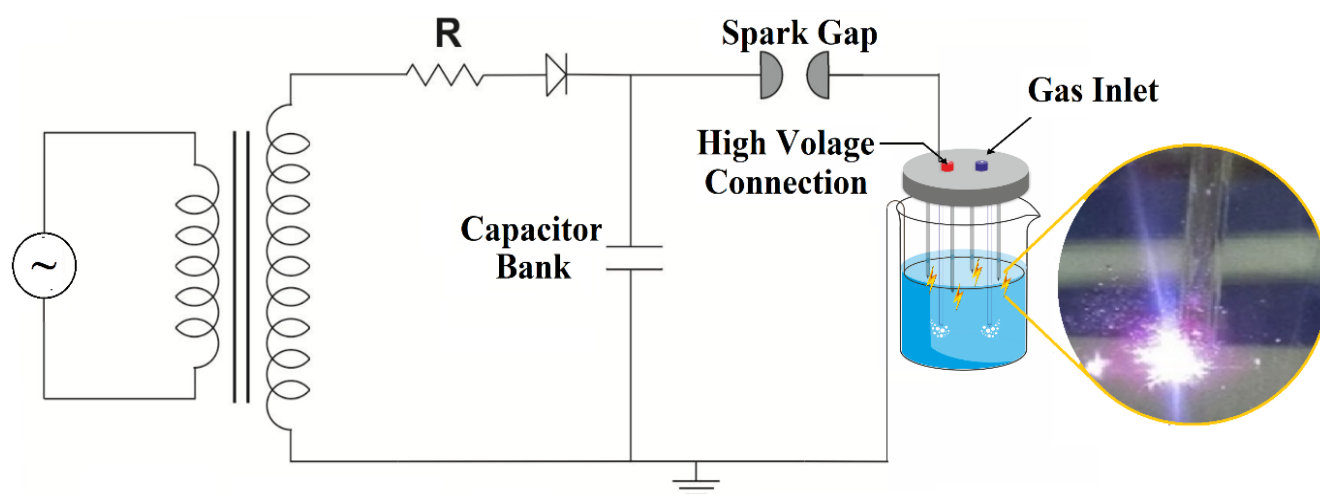


Figure 1. Schematic diagram used for decontamination of peppermint distillate by spark plasma

Electrodes are submersed in 350 mL peppermint distillate. Oxygen gas is bubbled into the distillate through eight gas exhaust tubes to homogenize the solution and facilitate plasma generation. Plasma is generated in the gas-liquid phase between the high-voltage electrodes and the ring-shaped ground electrode by applying a DC-pulsed high voltage with an amplitude of 20 kV across the spark gap. Figure 1 illustrates the spark discharge formed in the distillate when the spark gap is closed.

Contaminated alcohol-free peppermint distillate was supplied from an industrial company in Urmia, Iran. The distillate had been contaminated by *Pseudomonas aeruginosa* due to a failure in the production line. A 350 mL of originally contaminated peppermint distillate was treated applying pulse high voltage for 4 min. Based on results from our previous studies [15, 22], bacterial decontamination is expected to be complete following this process. Two 100 mL samples of both plasma-treated and untreated distillate were collected in separate glass containers for cytotoxicity testing and ICP-MS spectroscopy.

2.2. MTT assay procedure

One of the most important issues is whether the plasma processing induces toxicity in the treated substances. To address this, cytotoxicity tests were performed. In vitro cytotoxicity tests provide a biological evaluation to identify the potential toxicity of a substance. Although in vitro methods have limitations compared to in vivo or animal testing, they provide crucial data and valuable results for safety assessments. These tests utilize tissue cells to study the effects of substances on cell growth, reproduction, and morphology. Among various in vitro methods, the MTT assay is a widely used colorimetric method for measuring cell metabolic activity and the number of viable cells [23]. In this study HepG2 and normal Vero cell lines were used as the cell models. To investigate the effects of two types of distillates (plasma-treated and untreated distillates) on the growth and proliferation of Vero and HepG2 cell, the cells were treated with different concentration of two types of distillates and results were compared. HepG2 and Vero cell lines were provided from National Cell Bank (Pasteur Institute, Tehran, Iran). Both cells were seeded at the density of 6000 cells/well in 24 well polystyrene cell culture plates and cultured in complete Dulbecco's modified Eagle's medium-F12 (DMEM-F12; Sigma-Aldrich) supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 100 × non-essential amino acids (NEAA), 100 U/mL penicillin, 100 mg/mL streptomycin and maintained at 37 °C in a 5% humidified CO₂ incubator. After 24 hours, culture media was removed and two types

of distillates were added in ratio of 0, 20, 60, 80 and 100 % to culture media. After 24 h and 48 h of cell incubation at 37 °C with 5% CO₂, cell cytotoxicity in different conditions was evaluated by MTT assay. Briefly, culture media was removed and then 100 µL of media containing 10 µL of MTT solution (5 mg/mL-Sigma) was added to each well. After 4 h, medium was carefully removed and 100 µL DMSO (Sigma) was added and agitated for 15 min to dissolve the violet Formosans crystals. Finally, the absorbance of the solution was measured at a wavelength of 570 nm using the micro plate spectrophotometer system (BioTek, USA).

2.3. ICP assessment

One of the most important elemental analysis techniques is ICP spectroscopy. This method analyzes the elemental composition of samples with a high speed and sensitivity. In this method, the samples are sprayed into high-temperature argon plasma and converted into atoms and ions. Combining this technique with mass spectrometer (ICP-MS) can identify compounds and elements in the sample with a high accuracy [24]. In this study, ICP-MS was used to determine whether applying of electrical discharge between the two electrodes in our experimental condition causes release of the electrode material into the distillate and if it happens, how much is the corrosion of the electrode. Analysis was performed using Perkin-Elmer Company ICP-MS spectrometer (ELAN6100 DRCa, USA) and concentration of 53 elements in the plasma-treated samples was determined.

2.4. Statistical analyses

Statistical analysis of MTT assay was performed using SPSS 23.0 (SPSS Inc., USA). An independent samples t-test was used to compare the cell viability in the plasma-treated and untreated peppermint distillate as the cells culture media. Furthermore, a one-way Analysis of Variance (ANOVA) was utilized to compare the mean value between the plasma-treated and untreated peppermint distillate groups. Significant differences between mean values were identified with a confidence levels of $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

3. Results and discussion

3.1. MTT assay

To investigate the potential toxicity of the plasma-treated distillate, MTT assay was performed using Vero and HepG2 cell lines. The Vero cell line (African green monkey kidney cells) and HepG2 (human liver cancer cell

line) were selected as biological models because the kidney and liver are primary organs that likely affected by the ingestion of liquids.

Figure 2 show MTT assay results of untreated and plasma-treated samples for Vero cell lines. From the diagrams, one can deduce several points about the cell growth under different experimental conditions as follows. First, increase in the distillate concentration would contribute to significant decrease in cell growth in both untreated and plasma-treated distillate groups. In the diagrams, different ratios of nutrient media (N)-to-Distillate (D) were examined. It shows that regardless of plasma treatment, cell growth decreased as the proportion of distillate increased (i.e., a lower N/D ratio). The effect of the plasma treatment on the distillate toxicity can be easily found by comparing the cell viability across the different N/D ratios. Except for the first (N(100)) and last (D(100)) groups, all experimental groups showed a meaningful difference between the cell viability in the plasma-treated and untreated distillate after 24 h incubation. The impact of plasma-treated distillate on Vero cell line cytotoxicity was most pronounced in ratio of N(20)/D(80) of culture media-to-distillates ($P < 0.001$).

To assess time-dependent cytotoxicity of distillate, cell viability was also measured after 48 h of incubation. In the group N(100) (no distillate) an increase in cell number was

observed between 24 h and 48 h, consistent with normal cell proliferation. In addition to this typical effect, incubation time also influenced the cytotoxic properties of the distillates.

For the N(80)/D(20) and N(60)/D(40) ratios, no meaningful difference was observed between the plasma-treated and untreated distillates growth rate after 48 h ($P < 0.05$), suggesting that distillate toxicity may decrease over time.

This may be attributed to the ability of surviving cells to mitigate stress from plasma-generated oxidative agents by producing protective enzymes between 24 and 48 h.

Furthermore, the degradation or neutralization of these chemically unstable plasma agents within the biological media may reduce their effective concentration below the threshold required for cellular dysfunction. However, for the N(20)/D(80) culture media-to-distillate ratio, a significant difference in cell viability between the plasma-treated and untreated distillate persisted even after 48 h ($P < 0.001$). Notably, the influence of incubation time was more pronounced in the plasma-treated groups than in the untreated groups.

The significant decrease in cellular viability observed at 24 h in the plasma-treated groups was partially compensated for by 48 h, resulting in no significant difference in cell viability for the N(20)/D(80) and N(60)/D(40) ratios.

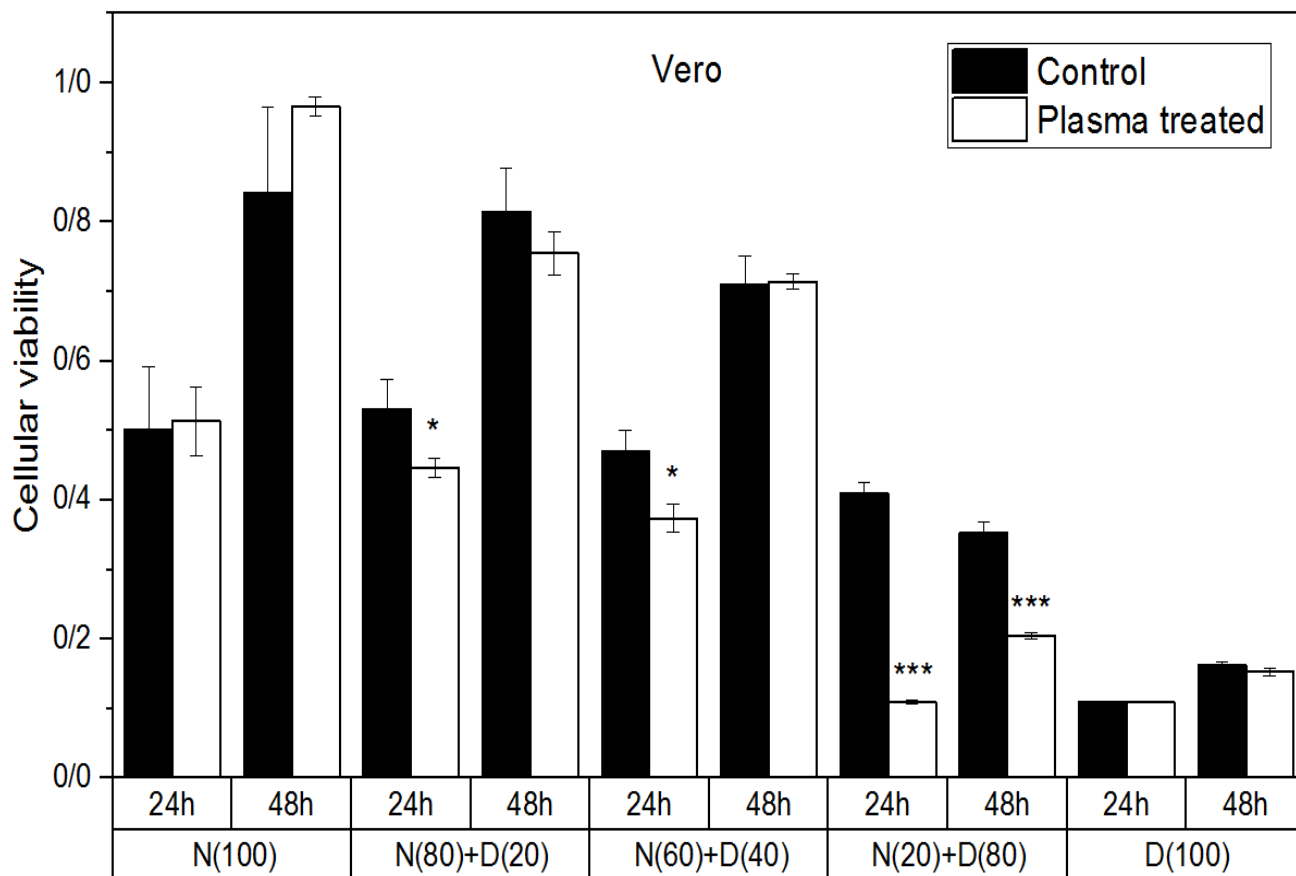


Figure 2. Comparison of MTT assay results between the control and plasma-treated samples for Vero cell line(*: $P < 0.05$,***: $P < 0.001$)

Figure 3 presents MTT assay results of untreated and plasma-treated samples for HepG2 cell line. Many of the observations made with Vero cell line were also observed in the HepG2 cells. An increase in distillate concentration resulted in a marked decrease in cell viability in both the untreated and plasma-treated groups. Contrary to the Vero cells, no significant difference in cell viability was observed for HepG2 cells cultured in untreated versus plasma-treated distillates at any of the evaluated concentrations after 24 h of incubation. This is expected, as HepG2 hepatocarcinoma cells exhibit greater resistance to oxidative stress than normal Vero cells. However, similar to the Vero cell line, incubation time significantly affected the growth levels of both groups. The impact of incubation time was more significant in the plasma-treated groups, particularly at the N(20)/D(80) and N(60)/D(40) ratios, relative to the untreated groups.

3.2. ICP assessment

Table 1 presents the concentrations and permissible limits of 53 major and trace elements in the peppermint distillate before and after plasma treatment. The results indicate an increase in the content of several elements following the plasma processing, including Ca(3.68 mg/L), Mg(0.58 mg/L), Na(6.17 mg/L), S(1.48 mg/L), Si(0.90 mg/L), Ba(4.06 µg/L), Sc(1.91 µg/L), Se(1.88 µg/L), Cu(8.11 µg/L), Sn(2.28 µg/L), Sr(49.23 µg/L), Mo(0.34 µg/L), W(2485.58 µg/L), Ni(12.67 µg/L), Zn(259.17 µg/L). These data provide evidence of electrode material release

into the distillate during plasma processing. Specifically, the corrosion of the electrode material during the 4 minutes plasma treatment released 2485.58 µg/L of tungsten into the peppermint distillate. According to the literature, chemical elements in living systems can be classified in two categories of essential and non-essential elements. Major and trace essential elements, are vital for biological functions such as growth and reproduction. Na, Mg, K, Ca, P, S and Cl are some examples of the major essential elements. Trace essential elements, such as Mn, Fe, Co, Ni, Cu, Zn, Mo, I and Se are also necessary for vital activity of living systems, however they can be toxic above a critical concentration (Hariri et al. 2015). Non-essential elements such as Li, V, Cr, B, F and Si have no known function in living systems and some non-essential elements such as Cd, Pb, As and Hg are toxic even at very low concentrations. The pulsed discharge plasma treatment did not significantly alter the concentrations of non-essential or highly toxic elements. Among the trace essential elements tungsten (W) showed the most substantial increase following the plasma treatment. According to the available document World Health Organization (WHO) has not published any guidelines on the maximum allowable concentration limit of tungsten in drinking water yet, however Russia considered a limit of 0.05 mg/L [26], and the Soviet Union established a 50 ppb limit for tungsten in drinking water. Hence concentrations of tungsten in the peppermint distillate after the pulsed plasma treatment is above the permission limit for drinking water quality.

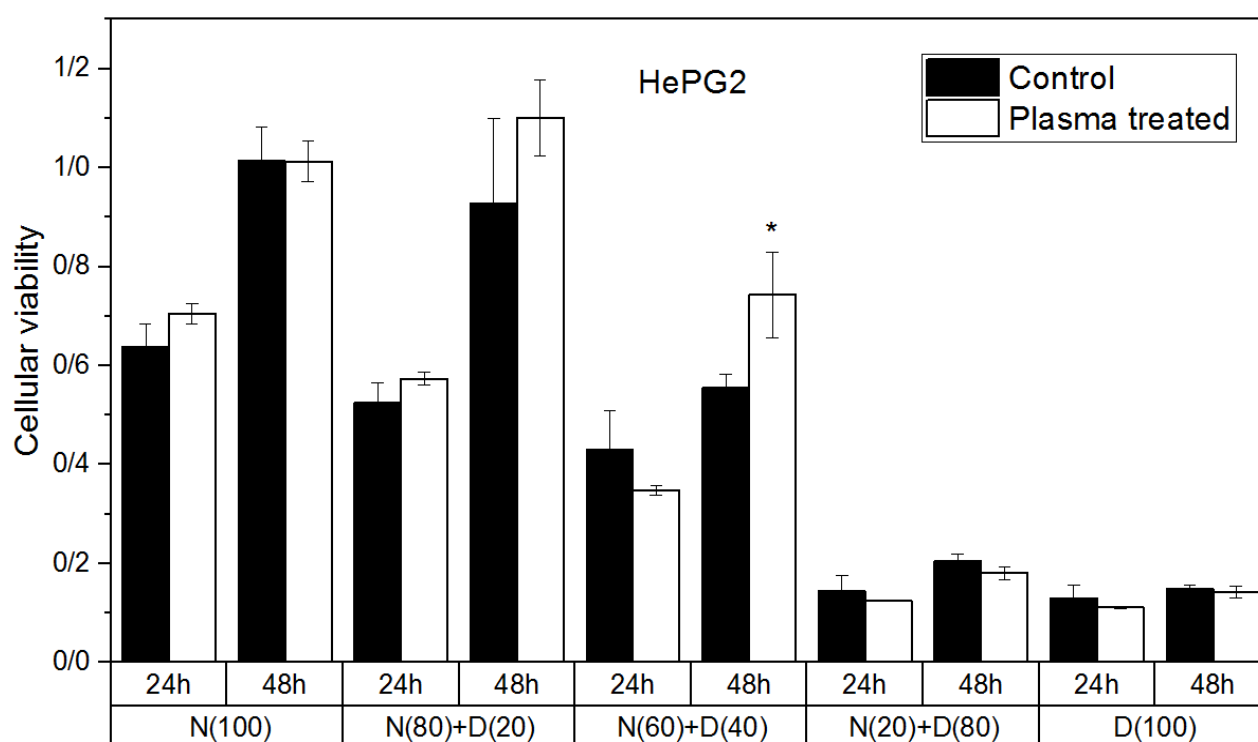


Figure 3. MTT assay results of the control and plasma-treated samples for HepG2 cell line(*:P<0.05)

Table 1. Concentrations of 53 elements in peppermint distillate after plasma treatment

Element	Unit	Concentration			Element	Unit	Concentration		
		After	Before	Permissible limits			After	Before	Permissible limits
Al	mg/L	0.01	0.01	0.2*	La	ug/L	<1.00	<1.00	N.A.
Ca	mg/L	3.68	1.83	75*	Lu	ug/L	<0.10	<0.10	N.A.
Fe	mg/L	<0.01	<0.01	0.3*	Mo	ug/L	0.34	0.19	70*
K	mg/L	0.20	0.20	10*	Nb	ug/L	<0.10	<0.10	N.A.
Li	mg/L	<0.01	<0.01	N.A.	Nd	ug/L	<0.50	<0.50	N.A.
Mg	mg/L	0.58	0.23	30*	Ni	ug/L	12.67	<0.10	20*
Mn	mg/L	<0.01	<0.01	0.4*	Pb	ug/L	<0.10	<0.10	10*
Na	mg/L	6.17	5.02	<20*	Pr	ug/L	<0.10	<0.10	N.A.
P	mg/L	<0.01	<0.01	N.A.	Rb	ug/L	<1.00	<1.00	N.A.
S	mg/L	1.48	<0.10	N.A.	Sb	ug/L	<1.00	<1.00	5*
Si	mg/L	0.90	0.15	N.A.	Sc	ug/L	1.91	<0.50	N.A.
Ti	mg/L	<0.01	<0.01	N.A.	Se	ug/L	1.88	1.75	10*
Ag	ug/L	<0.05	<0.05	N.A.	Sm	ug/L	<0.10	<0.10	N.A.
As	ug/L	<1.00	<1.00	10*	Sn	ug/L	2.28	1.69	N.A.
Ba	ug/L	4.06	<0.10	700*	Sr	ug/L	49.23	<0.10	4200**
Be	ug/L	<0.20	<0.20	N.A.	Ta	ug/L	<0.10	<0.10	N.A.
Bi	ug/L	<0.1	<0.1	N.A.	Tb	ug/L	<0.1	<0.10	N.A.
Cd	ug/L	<0.10	<0.10	3*	Te	ug/L	<0.1	<0.10	N.A.
Ce	ug/L	<0.10	<0.10	N.A.	Th	ug/L	0.10	0.10	N.A.
Co	ug/L	<1.00	<1.00	N.A.	Tl	ug/L	<0.10	<0.10	N.A.
Cr	ug/L	<1.00	<1.00	50*	U	ug/L	<1.00	<1.00	N.A.
Cs	ug/L	<0.10	<0.10	N.A.	V	ug/L	<1.00	<1.00	N.A.
Cu	ug/L	8.11	7.30	2000*	W	ug/L	2485.58	<0.10	50***
Dy	ug/L	<0.10	<0.10	N.A.	Y	ug/L	<1.00	<1.00	N.A.
Eu	ug/L	<0.10	<0.10	N.A.	Yb	ug/L	<0.10	<0.10	N.A.
Gd	ug/L	<0.50	<0.50	N.A.	Zn	ug/L	259.17	245.70	3000*
In	ug/L	<0.50	<0.50	N.A.					

N.A. Not available (No official limit exists for drinking water quality), *[3], **[25], ***[26, 27]

4. Conclusions

In this study, we evaluated the suitability of pulsed discharge plasma as a candidate for liquid decontamination. Building upon our previous work, it was shown that pulsed plasma discharge can decontaminate peppermint distillate well.

Assessment of physicochemical properties of distillate after the treatment by pulsed discharge plasma showed it may be considered as an alternative decontamination method of herbal distillate with large amount of sensitive compounds. No change in pH and distillate color and a maximum reduction of 17% in distillate essential oil [22], keep this method as a promising alternative for liquid decontamination.

However, the present study provides a more critical assessment of its safety.

In this study, the probable toxicity of distillate after the plasma treatment was investigated by MTT assay. In addition, the elemental chemical composition of the treated material was studied using ICP-MS method. The MTT assay results indicate that the toxicity induced by the treatment is significant at 24 h post-treatment, although this

toxicity appears to decrease over time. Furthermore, elemental analysis revealed an increase in certain elements most notably tungsten within the distillate. This contamination is attributed to electrode erosion and equipment corrosion caused by the shockwaves generated during the discharge process.

Although no WHO guidelines exist for tungsten in water, the recorded concentration (2485.58 µg/L) far surpasses the Russian/Soviet limit of 50 µg/L. In conclusion, while pulsed discharge plasma is highly effective for microbial inactivation, its application in liquid food decontamination is hindered by the risk of heavy metal contamination, specifically tungsten. To ensure food safety, future efforts must address the removal of these liberated elements from the treated medium.

Abbreviations

MTT:3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide tetrazolium

ICP: Inductively coupled plasma

ICP-MS: Inductively coupled plasma mass spectrometry

WHO: World Health Organization

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Authors Contribution

HG was project administration and work supervision, NNS and HG conceived, NNS, AM and MD carried out the experiments, SK organized MTT test methodology, NNS and SK wrote the manuscript and all authors read and approved the manuscript.

Availability of data and materials

All data presented in this article are available on request.

Conflict of interests

The authors declare that there is no conflict of interest to this work.

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