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Evaluating the Effects of Plasma Irradiation and *Stevia* on Gene Expression and Immune Responses in HepG2 Cells

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ABSTRACT

This research explores alternative cancer treatment options that minimize the severe side effects often associated with conventional therapies. Plasma medicine, a multidisciplinary field combining biology, clinical medicine, plasma physics, and chemistry, has emerged as a promising approach to cancer treatment. *Stevia* extracts, a natural sweetener derived from the *Stevia* plant, are known for their anti-cancer properties. The current study investigated the effects of gliding arc discharge (GAD) plasma and *Stevia* on the HepG2 cell line. Eight experimental groups were established: a control group, a *Stevia* extract group (17.5 µg/ml), GAD-exposed groups with three different exposure times (40, 60, and 80 seconds), and combined treatment groups of *Stevia* with each GAD exposure. The study evaluated cell viability, as well as the gene expression of P53 and Bcl2, and protein levels of PARP-1 and TNF-α. The results showed that the combined GAD treatment with 60 and 80 seconds of exposure was the most effective treatment and significantly reduced cell viability in the HepG2 cells. This treatment also increased the expression of P53, decreased the level of Bcl2, and inhibited the expression of PARP-1 and TNF-α proteins. In conclusion, this study suggests that the combination of GAD plasma exposure with *Stevia* can be a promising therapeutic strategy for certain types of cancer, acting through molecular mechanisms such as cell toxicity, tumor suppressor gene activation (P53), regulation of apoptotic genes (Bcl2), inhibition of DNA repair (PARP-1), and modulation of immune response (TNF-α).

Keywords: *Stevia*, Gliding arc plasma, Cancer, Bcl2, P53, Repair protein

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Introduction

Cancer encompasses a range of disorders where abnormal cells grow uncontrollably, invade surrounding tissues, or spread to other parts of the body, a process known as metastasis. Metastasis is a significant contributor to the majority of cancer-related deaths [1]. Hepatocellular carcinoma (HCC), the most common type of liver cancer, accounts for 75-85% of all liver cancer cases. In 2020, liver cancer ranked as the sixth most common cancer (841,000 cases) and the fourth leading cause of cancer-related deaths (782,000 cases) globally [2].

The treatment of cancer has long been a complex challenge. While traditional methods such as surgery, chemotherapy, and radiotherapy remain in use, recent advancements have introduced various innovative therapies, including nanoparticles, natural antioxidants, ablation therapy, stem cell therapy, targeted therapies, and chemo-dynamic therapies [3].

Plasma, the fourth state of matter, consists of a mixture of reactive species, including reactive oxygen species (ROS), reactive nitrogen species (RNS), protons, electrons, and other neutral particles generated when gas is exposed to an electric field [4]. Plasma has proven applications in medicine, including stimulating angiogenesis and cell proliferation at lower intensities, and inducing cell death, particularly in cancer cells, at higher intensities [5].

Gliding arc discharge (GAD) therapy represents a promising cancer treatment approach, selectively targeting cancer cells while sparing healthy ones. The reactive species generated during plasma treatment, such as ROS and RNS, play a pivotal role in killing cancer cells. Furthermore, GAD therapy exhibits anti-angiogenic, anti-inflammatory, and immune-modulatory properties, which may enhance its therapeutic potential. Combining GAD with other treatments like immunotherapy, chemotherapy, or radiation has also been shown to improve overall treatment outcomes [6].

This study investigates the combined effect of GAD treatment and *Stevia*, a natural plant extract known for its potential anti-cancer properties. *Stevia* contains carotenoids and polyphenols that influence normal cell functions, promote differentiation, and prevent cancer-related epigenetic dysfunctions. It has been found to reduce metastasis, decrease tumor growth, and enhance the effects of chemotherapy and radiotherapy [7]. *Stevia* Bertoni is recognized for its strong antioxidant, antibacterial, anti-diabetic, antiplatelet, anti-cariogenic, and anticancer activities [8].

P53, a transcription factor, is a key regulator of cellular processes such as DNA repair, cell cycle arrest, and apoptosis, especially in response to genotoxic stress. P53 is frequently mutated in liver cancer, and it controls genes involved in tumor suppression, cell cycle regulation, and cellular metabolism [9, 10].

Bcl2, a protein involved in regulating apoptosis, is often over-expressed in cancers, preventing apoptosis and contributing to tumor survival. Targeting Bcl2 family proteins is an appealing strategy in cancer therapy due to their role in apoptosis regulation [11].

Poly (ADP) ribose polymerase (PARP) is another protein involved in DNA repair pathways, and its expression in liver cancer makes it a potential target for treatment. Inhibiting PARP could enhance the efficacy of existing cancer therapies [12].

Tumor necrosis factor-alpha (TNF-α) is a promising target for HCC treatment, as it can inhibit cancer cell proliferation and promote cell death. It is released by cytotoxic macrophages activated by plasma-induced reactive species, further making it a key player in cancer treatment [13-18].

This study aims to evaluate the effects of GAD plasma radiation and *Stevia* plant extract, mimicking radiotherapy and natural chemotherapy, on the HepG2 cell line, a model for liver cancer.

Materials and Methods

Cell line

The HepG2 liver cancer cell line, used in this study, was obtained from the genetic engineering unit at the National Research Center of Egypt. The cells were cultured in Eagle's minimum essential medium (EMEM) with 10% Fetal bovine serum (FBS), as per the supplier's recommendations. The cultures were maintained in a 37 °C incubator with 5% CO2 and the medium was replaced weekly.

Reagents and chemicals

The chemicals used included EMEM medium (ATCC), FBS (Thermo Fisher Scientific), dimethyl sulfoxide (DMSO) as a solubilizing agent, tri-fast reagent, trisNaCl buffer (used in blotting), tween 20 (emulsifier), non-fat dry milk (NFDM) (used for blocking), as well as primary antibodies such as anti-PARP-1, anti-TNF-α, and anti-β-actin. The study also utilized an HRP-conjugated secondary antibody and the RNeasy Mini Kit (Qiagen).

Treatment protocols

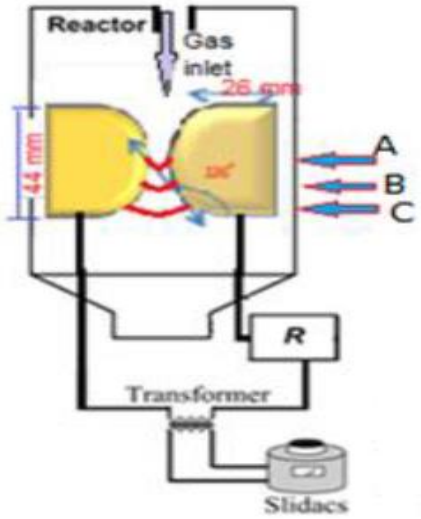
Stevia rebaudiana extract

Stevia plant extract, with a concentration of 17.5 µg/ml, was sourced from Al Memaar Home Company for Agricultural Development and Investment.

Gliding arc plasma radiation setup

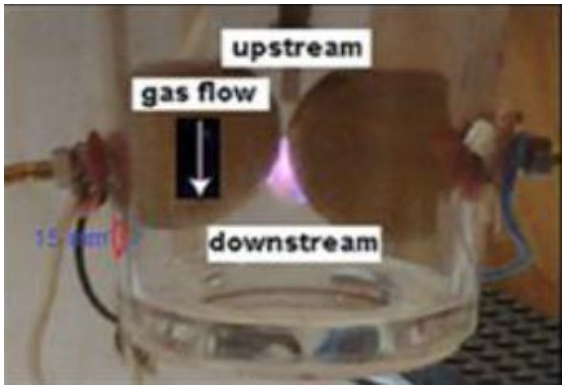
The GAD system used for the experiment involved two copper electrodes with identical specifications: 15 mm thick, 26 mm wide, and 44 mm long. The gap between the electrodes was 1 mm, and the arc angle was set to 120º. The electrodes were connected to an AC power supply (6.6 kV) through a 200 kΩ resistor to control the current. Voltage was managed via variable autotransformers (Slidacs).

The system utilized a step-up transformer with a 1:30 ratio to amplify voltage. Nitrogen (N2) and argon (Ar) were used as gases, injected through a narrow tube placed between the electrodes, with the flow rate regulated by a pressure regulator. Voltage adjustments were made using a slide regulator, while high voltage was supplied through the transformer. The discharge frequency was set at 50 Hz. The current was measured using a Rogowski coil and clamp digital meter, with oscillograph data displayed on an oscilloscope **(Figure 1)**.



A: Gas Breakdown region
B: Quasi-equilibrum (thermal) plasma region
C: Non-equilibrum (non thermal) plasma region

a)



b)

Figure 1. a) gliding arc schematic and the electric scheme, and b) GAD plasma photo.

Experimental design

The study involved eight groups with the following treatments:

1. Group 1 (G1): Control group, with no treatment (HepG2 cell line).
2. Group 2 (G2): HepG2 cells treated with *Stevia* plant extract at a concentration of 17.5 µg/ml.
3. Group 3 (G3): HepG2 cells exposed to GAD for 40 seconds.
4. Group 4 (G4): HepG2 cells exposed to GAD for 60 seconds.
5. Group 5 (G5): HepG2 cells exposed to GAD for 80 seconds.
6. Group 6 (G6): HepG2 cells treated with *Stevia* extract, followed by exposure to GAD for 40 seconds.

7. Group 7 (G7): HepG2 cells treated with *Stevia* extract, followed by exposure to GAD for 60 seconds.
8. Group 8 (G8): HepG2 cells treated with *Stevia* extract, followed by exposure to GAD for 80 seconds.

After the treatments, the cells were cultured for three days. Then, an MTT assay was performed to assess cell viability.

Methods

MTT assay

Cell viability was calculated using the formula: Cell viability (%) = (Mean ODControl OD)×100\text{Cell viability (\%)} = \left(\frac{\text{Mean OD}}{\text{Control OD}} \right) \times 100Cell viability (%)=(Control ODMean OD)×100

Gene expression analysis

To assess the molecular effects of *Stevia* extract and GAD on the HepG2 cells, gene expression of the P53 and Bcl2 genes was analyzed. The primer sets for these genes are listed in **Table 1**. After collecting the treated and untreated cell samples, RNA extraction and RT-qPCR were performed to measure the expression levels of the target genes.

RNA extraction and cDNA synthesis

Total RNA from both control and treated cells was extracted using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions.

Real-time RT-qPCR

RT-qPCR was conducted using the SYBR Green PCR Master Mix (Fermentas, USA). The data were normalized to the GAPDH mRNA expression, which served as the housekeeping gene. The relative gene expression was calculated using the 2^-ΔΔCT method.

Table 1. Specific primer sequences used in RT-PCR

Primer	Direction	Sequences 5'-3'
P53	F	TAACAGTTCCTGCATGGGCGGC
	R	AGGACAGGCACAAACACGCACC
Bcl2	F	TTGTGGCCTTCTTTGAGTTCGGTG
	R	GGTGCCGGTTCAGGTACTCAGTCA
GAPDH	F	GTCTCCTCTGACTTCAACAGCG
	R	ACCACCCTGTTGCTGTAGCCAA

Western blotting (immunoblotting) and molecular assay (proteomics analysis)

The Western blotting technique was employed to analyze cell lysate samples under specific conditions [19-22]. After electrophoresis of proteins on SDS-PAGE, they were transferred to a Hybond™ nylon membrane (GE Healthcare) using a TE62 Standard Transfer Tank (Hoefer Inc.). The membrane was then incubated in a blocking solution for an hour at room temperature. β-actin was used as a reference protein for normalization.

The membrane was incubated overnight at 4 °C with primary antibodies against PARP-1 and TNF-α (Abcam, ab32378; ab255275), along with an anti-β-actin primary antibody (Abcam, ab8227). The membrane was washed with blotting buffer for 30-60 minutes, undergoing at least five changes of the buffer at room temperature.

After washing, the membrane was exposed to horseradish peroxidase (HRP)-conjugated secondary antibody for one hour at room temperature. The antibody concentration was optimized between 0.05 and 2.0 µg/mL to achieve a strong signal and reduce background. Following this, the membrane was washed again for 30-60 minutes. Data analysis was conducted using the Gel Documentation system (Geldocit, UVP, England) and TotalLab analysis software (www.tatallab.com, ver. 1.0.1) [23].

Statistical analysis

The data were subjected to statistical analysis with CoStat software to evaluate the significance of differences between the samples. CoStat version 6.400 was used for the analysis (copyright © 2022-2008 COHORT SOFTWARE, Monterey, CA, USA) [24].

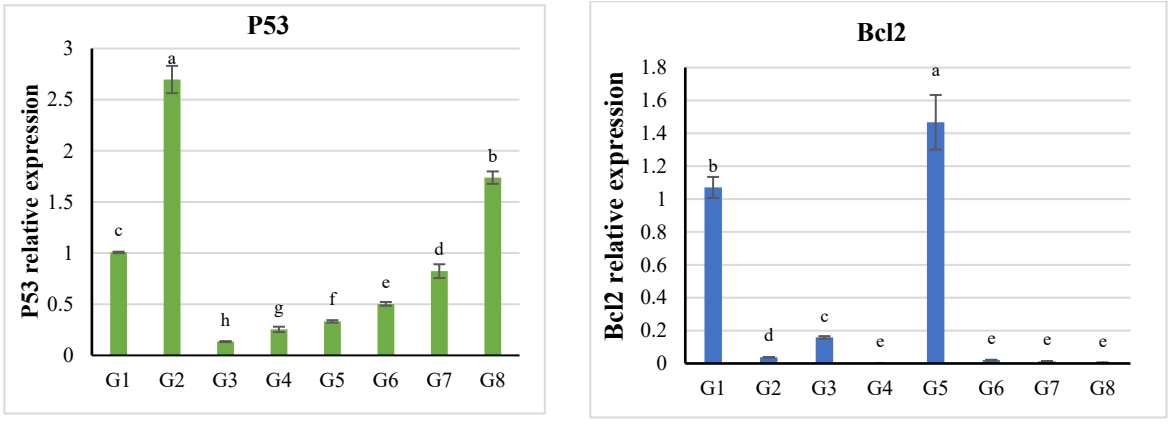
Results and Discussion

The data indicated a marked decrease in cell viability in all treatment groups compared to the control (G1). The most notable decrease in viability was observed in G7, which combined *Stevia* treatment with 60 seconds of gliding arc plasma exposure, resulting in a viability rate of 26%. The cell viability percentages for the other groups were as follows when compared to G1: G2 (34%), G6 (42%), G8 (43%), G4 (61%), G5 (63%), and G3 (74%) **(Table 2)**.

Table 2. Cell viability % in HepG2 cell line treated with *Stevia* and/or gliding arc plasma radiation (40, 60, 80 Sec)

Groups	Cell viability (%)
Group (1): Control.	90 ± 3^{a}
Group (2): *Stevia* extract.	34 ± 1^{f}
Group (3): Plasma exposure at 40 Sec.	74 ± 3^{b}
Group (4): Plasma exposure at 60 Sec.	61 ± 2^{d}
Group (5): Plasma exposure at 80 Sec.	63 ± 0^{c}
Group (6): Combination of *Stevia* extract and plasma exposure at 40 Sec.	42 ± 2^{e}
Group (7): Combination of *Stevia* extract and plasma exposure at 60 Sec.	26 ± 1^{g}
Group (8): Combination of *Stevia* extract and plasma exposure at 80 Sec.	43 ± 1^{e}

The significance levels varied from (a) to (g) when compared with G1, as indicated by the statistical analysis using CoStat. **Figure 2** illustrates the relative expression of the P53 gene, which decreased from (a) to (h) across the groups. The highest expression values for the P53 gene were observed in G2 and G8.
On the other hand, the expression of the Bcl2 gene followed a declining order from (a) to (e). Significant reductions in Bcl2 expression were observed in all groups, except for G5, where the levels remained unaffected.



a) b)

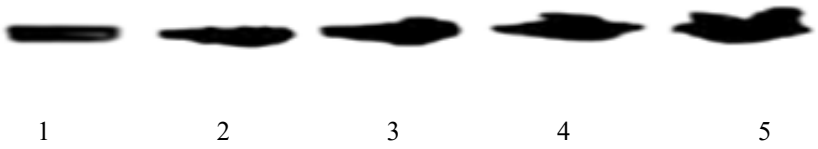
Figure 2. Levels of P53 and Bcl2 genes relative expressions in HepG2 cell line treated with *Stevia* and/or gliding arc plasma radiation (40, 60, 80 Sec)

PARP-1 is a critical epigenetic protein involved in the regulation of transcription and gene expression repair, while tumor necrosis factor-α (TNF-α) is a key cytokine in tumor-related pathways. The impact of *Stevia* extract and gliding arc plasma exposure on these proteins was assessed using Western Immunoblotting techniques, with the results analyzed and represented by a computerized dendrogram for gel analysis.
The group designations for the PARP-1 and TNF-α lanes are shown in **Table 3**.

Table 3. The group designations for the PARP-1 and TNF-α lanes.

Lane	**1**	**2**	**3**	**4**	**5**
Group	Control	40 seconds	60 seconds	80 seconds	*Stevia* extract

Figure 3 illustrates the PARP-1 protein expression bands across all groups. The highest expression levels were observed in lanes 4 and 5, while the lowest levels were seen in lanes 2 and 3, as compared to the control in lane 1.



1 2 3 4 5

Figure 3. The bands of all groups for PARP-1 protein expression.

The PARP-1 protein expression levels, based on the dendrogram analysis, were recorded as 78.65, 64.70, 70.65, 90.44, and 99.25 for lanes 1, 2, 3, 4, and 5, respectively **(Table 4)**.

Table 4. Expression levels of PARP-1 protein in HepG2 cell line with four treatments

Lane	**1 (%)**	**2 (%)**	**3 (%)**	**4 (%)**	**5 (%)**
PARP-1 expression	78.65	64.70	70.65	90.44	99.25

Figure 4 depicts the protein bands for TNF-α expression across all groups, with the highest expression observed in lane 2 and the lowest in lane 5 when compared to the control in lane 1.

1 2 3 4 5

Figure 4. TNF-α protein expression level for HepG2 cell line protein with four treatments.

The TNF-α protein expression levels, determined via the dendrogram, were recorded as 79.91, 81.91, 78.04, 75.21, and 67.75 for lanes 1, 2, 3, 4, and 5, respectively **(Table 5)**.

Table 5. TNF-α protein expression levels in HepG2 cell line for four treatments

Lane	**1 (%)**	**2 (%)**	**3 (%)**	**4 (%)**	**5 (%)**
TNF-α expression	79.91	81.91	78.04	75.21	67.75

For β-actin protein, which was used as a reference, the expression levels were 100% for all lanes.

The definition of cancer is complex and should consider a variety of factors including checkpoints, chromosomal instabilities, chemical influences, physical factors, psychological stress, and immune system failure. It is critical to take all these elements into account when addressing cancer, its treatment, and its evaluation. The epigenetic factor, particularly the physical nature of DNA that regulates gene expression without altering sequences, plays a key role in these processes [25].

Cell lines are commonly used in medical research, particularly for drug discovery and cancer studies. These cell lines provide an unlimited supply of biological material, and when maintained under proper conditions, they preserve most of the genetic characteristics of the original cancer [26].

Plasma radiation has been recognized as an effective treatment for heat-sensitive materials and tissues. It has shown promise in treating various cancers, including breast, ovarian, pancreatic, melanoma, glioblastoma, osteosarcoma, and cervical cancer. The main mechanism by which plasma works is through the dissipation of

energy, either chemically or via radiation. Plasma exposure in vivo has not shown adverse effects in animals or small human clinical studies [25].

In both preclinical and clinical settings, natural substances are often used in cancer treatment, and combining these substances with GAD plasma treatment may enhance the therapeutic effects. Natural products can target multiple pathways, such as inducing apoptosis or inhibiting cell division, making them highly effective in counteracting the biological complications associated with cancer and offering valuable resources for chemoprevention [27, 28]. *Stevia* contains a variety of bioactive compounds such as glycosides, flavonoids, and fatty acids, which contribute to its wide range of biological effects. These compounds enable *Stevia* to have chemotherapeutic properties, treat polycystic kidney disease, stimulate insulin production in diabetics, and offer strong antibacterial, antioxidant, and immunomodulatory effects [29].

The current study evaluates the cell viability and anti-cancer and anti-inflammatory effects of GAD plasma irradiation and/or *Stevia* extract on Hepatocellular carcinoma (HepG2) cell lines.

Table 2 illustrates a significant reduction in cell viability for all treatment groups with *Stevia* and/or gliding arc plasma exposure (G2, G3, G4, G5, G6, G7, and G8) when compared to the control group (G1). The most notable decrease in cell viability was observed in the combined treatment groups (G6, G7, and G8), with the lowest viability recorded in G7 (*Stevia* and 60-second plasma exposure), showing a viability of just 26%.

In this study, two genes—P53 and Bcl2—were chosen for their roles in regulating the death and proliferation of malignant cells. The P53 gene, classified as a tumor suppressor, plays a critical role in programmed cell death (apoptosis) and maintaining genomic stability. It is often referred to as the "guardian of the genome" due to its role in regulating apoptosis, the cell cycle, and overall genomic integrity [30].

Apoptosis is an essential process for maintaining cellular health, but cancer cells often evade apoptosis by over-expressing anti-apoptotic proteins like Mcl-1, Bcl-xL, and Bcl2. These proteins are becoming promising targets for cancer therapies [31].

Figure 2 shows the relative gene expression values for P53 and Bcl2. The highest expression levels of the P53 gene were found in G2 (*Stevia* alone) and G8 (*Stevia* and 80-second plasma exposure). On the other hand, Bcl2 gene expression was significantly reduced in all treatment groups compared to the control, except for G5 (80-second plasma exposure).

The observed variations in cell death (HepG2 cell line) and gene transcription levels may be attributed to the different mechanisms of action of *Stevia* and plasma. While *Stevia* operates chemically, plasma works physically through its radical-induced effects.

Poly ADP-ribose polymerases (PARPs) are enzymes involved in DNA repair, recombination, replication, and transcription. Inhibiting PARP activity can increase the sensitivity of tumors to DNA-damaging agents. PARP inhibitors are thus being explored for their potential in cancer therapy [32].

Recent research has focused on targeting mutations in DNA repair mechanisms, including genes and PARPs associated with homologous recombination repair, to protect cells from DNA-damaging agents like radiation and chemotherapy [33].

The present study aimed to assess how each treatment—*Stevia* or plasma radiation—impacts the inhibition of DNA repair processes in cancer cells. **Table 4** shows that plasma radiation, particularly at doses of 40 and 60 seconds, is more effective than *Stevia* in inhibiting PARP activity. These results suggest that plasma radiation may act as an epigenetic agent, controlling the transcription process and influencing gene repair mechanisms [25].

The study also examined the effect of TNF-α protein expression in HepG2 cancer cells, which can promote cell growth and proliferation. **Table 5** indicates that *Stevia* and plasma radiation treatments (particularly at 80 and 60 seconds) were the most effective in reducing TNF-α overproduction, likely through their modulation of various signaling pathways [34].

In conclusion, the findings suggest that both gliding arc plasma and *Stevia* may serve as promising tools for cancer treatment, especially when combined. These treatments may target a variety of pathways and mechanisms to combat cancer growth and promote anti-inflammatory effects, as will be further discussed.

The application of plasma to biological cellular structures generates numerous reactive species, which interact with cells and influence cellular redox signaling. This results in changes to receptor function, activation of cell cycle arrest, DNA damage-induced activation of P53, and subsequent P53-dependent apoptosis, among other effects on various cells [35, 36].

Plasma can be categorized by its applications, with plasma produced at moderate temperatures and atmospheric pressures being suitable for plasma-assisted medicine. GAD (gliding arc discharge) is a type of plasma that uses

an electric field as its excitation source. There are various methods for generating GAD, including dielectric barrier discharge (DBD), glow, and gliding or corona discharge. The main advantages of GAD include its low cost, controllability, and ease of turning the system on and off [37].

Finding an effective cancer treatment is a significant challenge due to the diverse nature of cancers, with each requiring a specific treatment approach. Chemotherapy is widely used but often comes with many side effects, and certain cancers may develop resistance to it. To address these challenges, researchers are exploring new treatments, such as GAD, which works similarly to radiation and chemotherapy. GAD produces ions, electrons, and radicals that have anti-cancer properties and can destroy malignant cells [6].

Research on different types of plasma and their effects on both healthy and cancerous cells has demonstrated that atmospheric non-thermal plasma and gliding arc plasma exposure can aid in tissue repair, treat various diseases, and be particularly effective for treating drug-resistant tumors [38, 39]. Plasma exposure can cause chromosomal instability, induce mitotic checkpoint arrest, and lead to cell death through necrosis and apoptosis. Furthermore, plasma affects the transcription of anti-cancer genes like Bcl2, caspase-3, and P53, while enhancing immune responses, including TNF-α and interleukins [40]. Plasma can also alter the cancer cell microenvironment by stopping cell spreading, lysing cells, and disrupting cell-to-cell communication [41].

A major challenge in cancer treatment is avoiding damage to healthy cells. The immune system interacts with tumor cells in a complex manner, sometimes promoting tumor growth and at other times inhibiting it. This process, known as cancer immunoediting, consists of three stages: elimination, equilibrium, and escape. GAD aids in the immunoediting process, promoting the regulation of cancer cells [42].

In this study, exposure of HepG2 cells to GAD inhibited DNA repair mechanisms and prevented cancer cell metastasis. This was achieved through the regulation of protein expressions of cytokines and immune mediators, especially after exposure times of 60 and 80 seconds. TNF-α plays a central role in cancer-related inflammation, and treatment with TNF-α antibodies significantly reduced pro-inflammatory cytokine levels, which suggests therapeutic potential for Hepatocellular carcinoma (HCC). Plasma irradiation effectively reduced these inflammatory factors [13].

Further research has explored how plasma influences cancer treatment through both oxidative stress (via free radicals) and epigenetic factors, particularly through the resonance of electrons [25].

Stevia, a plant known for its high levels of rebaudioside and stevioside glycosides, has also shown promise in cancer treatment. Stevioside is a potent apoptosis inducer that triggers cell death through the generation of reactive oxygen species (ROS). This process leads to mitochondrial-mediated apoptosis, enhancing the expression of apoptotic proteins such as caspase-9, Bcl2, and Bax [43].

Conclusion

Based on the findings from the literature and our results, we concluded that medical applications of plasma, when combined with natural products like stevioside and other *Stevia* compounds, exhibit significant anticancer effects in cultured HepG2 cell lines. These effects are achieved through multiple mechanisms, including the inhibition of DNA synthesis, suppression of cell viability, and the induction of apoptosis via the mitochondrial apoptotic pathway.

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Conflict of Interest: None

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Ethics Statement: None

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