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The Effect of *Verbena officinalis* on Pro-Inflammatory and Anti-Inflammatory Responses in Prostate Cancer.

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ABSTRACT

Background: Verbena officinalis, a medicinal plant native to Jordan, is renowned for its antibacterial, antioxidant, and anticancer properties. However, its anti-inflammatory effects remain underexplored. This study aimed to investigate the anti-inflammatory potential of *Verbena officinalis* aqueous crude extract in PC3 prostate cancer cells, focusing on its ability to modulate inflammatory responses.

Methods: The anti-inflammatory activity of the aqueous extract was evaluated by measuring interleukin-6 (IL-6) production in response to bacterial lipopolysaccharide (LPS), a pro-inflammatory stimulus, and interleukin-10 (IL-10) secretion. Total polyphenol content was quantified using the Folin–Ciocalteu assay, while antioxidant activity was assessed through DPPH radical scavenging and tannic acid content analyses. Cytotoxicity was also evaluated to ensure the extract's safety.

Results: The aqueous extract exhibited the highest polyphenol content and demonstrated strong antioxidant properties, including a 45.5% radical scavenging activity and 7.7 mg/ml tannic acid content. At a concentration of 50,000 µg/ml selected to assess the maximal biological effect of the crude extract the extract significantly reduced LPS-induced IL-6 production at both protein and gene levels, while enhancing IL-10 secretion. This concentration was found to be non-cytotoxic, supporting its safety profile.

Conclusion: *Verbena officinalis* shows promise as a natural anti-inflammatory agent and potential preventive treatment for prostate cancer. The aqueous extract significantly reduced IL-6 secretion and decreased prostate cancer cell viability in a concentration-dependent manner, particularly at 50,000 µg/ml. The observed increase in IL-10 levels suggests a beneficial modulation of the inflammatory response. These findings warrant further investigation into its therapeutic potential.

Keywords: Anti-inflammation, IL-6, IL-10 , Prostate cancer, *Verbena officinalis.*

Introduction

Sarcopenia is the progressive and generalized skeletal muscle wasting and strength loss and is one of the most significant public health challenges of ageing populations worldwide. Muscle wasting is a significant, multifaceted condition associated with ageing, chronic Medicinal plants have been integral to human healthcare (1). Among them, *Verbena officinalis* also known as vervain, pigeons' grass, or herb of grace is a member of the Verbenaceae family valued for its rich bioactive compounds, making it important in drug discovery and development (2). This plant is cultivated in various countries, including Jordan, where it thrives in sunny areas with moist, well-drained soil (3). Traditionally, it has been used to treat gastric issues, thyroid disorders, respiratory ailments, and menstrual cramps (4–5).

Verbena officinalis is known for its diverse therapeutic properties, including antioxidant, antifungal, antibacterial, anticancer, and urolithiasis-preventive effects, due to its bioactive metabolites like flavonoids and essential oils (6–8). Some studies suggest its extracts may inhibit pro-inflammatory mediators (9) and reduce inflammation in animal models (10).

Chronic inflammation is a key contributor to the progression of many diseases, including prostate cancer. Pro-inflammatory cytokines such as interleukin-6 (IL-6) are major drivers of tumor development and progression. While *Verbena officinalis* is recognized for its general anti-inflammatory potential, its specific effects on IL-6 production particularly at the protein and gene expression levels—in prostate cancer cells remain poorly understood. This represents a clear gap in the literature.

With prostate cancer ranking as the second leading cause of cancer-related deaths among men globally (11), and accounting for 7.9% of all cancer cases in Jordan according to a 2022 Ministry of Health report (12), identifying natural agents that modulate inflammation is critically important. This study hypothesizes that *Verbena officinalis* aqueous extract can reduce inflammatory responses by

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decreasing IL-6 production at both the protein and gene expression levels in a prostate cancer cell model stimulated with bacterial lipopolysaccharide (LPS).

Materials and Methods

This study aims to investigate the potential anti-inflammatory effects of *Verbena officinalis* crude extract through an *in vitro* PC3 cell culture experiment. PC3 cells were selected to introduce novelty to the study, as no prior research in Jordan has examined this cell line under comparable experimental conditions.

Verbena officinalis whole plant parts (excluding roots) were purchased in dried form from various local markets in Jordan between July and November 2023. A sample size of 10 g, representing the average daily consumption as advised by local herbalists, was used. The dried parts were washed with distilled water, air-dried, and ground into a fine powder. The powder was soaked in methanol, ethanol, acetone, and distilled water (24 hours at room temperature). Extracts were separated by centrifugation.

Each extraction was performed in triplicate (technical replicates). Total polyphenol content (TPC) in each extract was determined to identify the solvent yielding the highest content.

Determination of Total Polyphenols

TPC was assessed using the Folin–Ciocalteu assay, following the method by Kupina et al. (13). Absorbance was measured at 765 nm, and results were calculated against gallic acid standards. All measurements were done in triplicate (technical replicates).

Cell Culture Techniques

Human prostate adenocarcinoma PC3 cells were cultured under aseptic conditions as per Baust et al. (14), in RPMI 1640 Supplemented with 10% fetal bovine serum (FBS), at 37°C and 5% CO. The culture medium was changed every 48 hours

Experiments were conducted when cells reached 70–80% confluency. All treatments were performed using three independent biological replicates, each with triplicate technical replicates.

WST-1 Cell Viability Assay

Cell proliferation was assessed using the Water-soluble Tetrazolium WST-1 assay according to Präbst et al. (15). PC3 cells were treated with 1, 500, and 50,000 µg/ml concentrations of aqueous extract. After 24 hours, WST-1 reagent was added, and absorbance was read at 450 nm. Data were averaged from three biological replicates, each analyzed in triplicate.

ELISA: Inflammatory Cytokine Quantification

IL-6 (Pro-inflammatory Cytokine)

PC3 cells (60–70% confluence) were treated with 1, 500, and 50,000 µg/ml of *Verbena officinalis* extract, with or without lipopolysaccharide (LPS), and incubated for 24 hours. Supernatants were collected and IL-6 levels quantified using a commercial Human IL-6 ELISA kit. Experiments were performed in technical triplicate.

IL-10 (Anti-inflammatory Cytokine)

A similar procedure was followed for IL-10. After extract and LPS treatment, IL-10 secretion was measured using a Human IL-10 ELISA kit. Results were based on technical triplicates per sample.

Gene Expression Analysis of IL-6

RNA Extraction and Quality Assessment

Following treatment with 1, 500, or 50,000 µg/ml aqueous extract for 24 hours, total RNA was extracted using an RNA isolation kit. RNA purity was evaluated using a Nanodrop spectrophotometer at 260/280 nm. Samples with ratios between 1.8 and 2.1 were considered acceptable.

RT-qPCR for IL-6 Expression

Using purified RNA, cDNA was synthesized and amplified via RT-qPCR using the CFX Opus 96 Real-Time PCR System. GAPDH served as the housekeeping gene. All qPCR analyses were performed using technical triplicates.

DPPH Antioxidant Assay

The radical scavenging capacity of the aqueous extract was evaluated using the DPPH assay. Absorbance was measured at 515 nm. Results were calculated from triplicate measurements.

Tannic Acid Content Analysis

Total tannins were quantified using the Folin-Ciocalteu method according to Polumackanycz et al. (16). Absorbance was read at 725 nm, and results expressed as mg tannic acid equivalent (TAE)/ml. Each sample was analyzed in triplicate.

Statistical analysis

GraphPad Preism version 10.4.1 (San Digo, USA) was used to perform statistical analysis. one-way ANOVA analysis of varience followed by Tukey's multiple comparison post-test were applied.

Results

Levels of Total Polyphenols in Verbena Officinalis Crude Extracts

The polyphenolic content of *Verbena officinalis* was assessed using the Folin-Ciocalteu method, showing significant antioxidant properties Figure 1. Distilled water was the most effective solvent for extracting polyphenols, particularly within the first 24 hours, yielding 163,620.54 µg GAE/L significantly higher than methanol (132,277.63 µg GAE/L), ethanol (47,517.41 µg GAE/L), and acetone (43,032.65 µg GAE/L). Over 48 hours, the total polyphenols in distilled water decreased to 921.43 µg GAE/L, slightly higher than methanol (891.77 µg GAE/L) but far exceeding acetone and ethanol. After 72 hours, distilled water still proved to be the most effective yielding (917.93 µg GAE/L), compared to other solvents, though the yield plateaued, showing no additional benefit from prolonged soaking. These findings highlight that distilled water efficiently extracts bioactive compounds, with most extraction occurring within the first 24 hours. Extending the soaking time beyond this period offers minimal additional benefit.

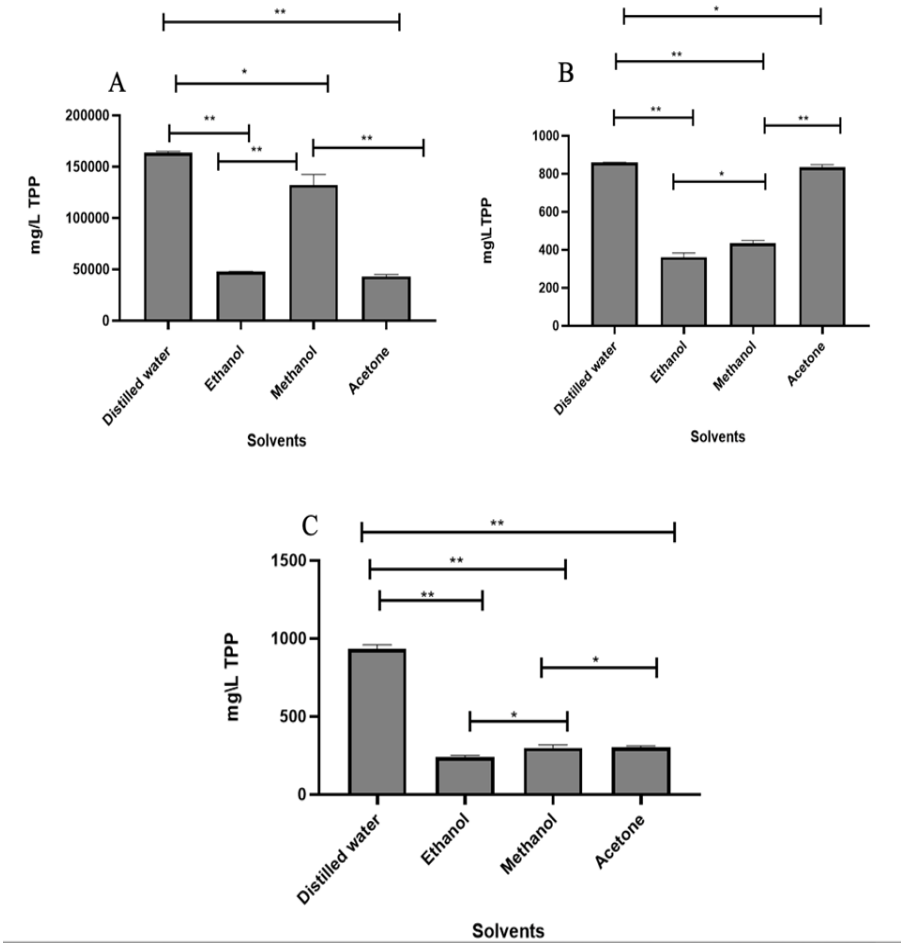

Figure 1. Levels of total polyphenols in crude extracts of *Verbena officinalis*. Total polyphenols were measured in crude extracts prepared with distilled water, ethanol, methanol and acetone after 24 hours (A), 48 (B) and 72 (C) hours. aqueous crude extract have the highest content of polyphenols compared to the crude extracts prepared with other solvents. The results represent means ± SD of three biological replicates. Data represent the mean ± standard deviation (SD) from six biological replicates. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison post-test. $P \leq 0.05$ was considered statistically significant.

Effect of *Verbena officinalis* on growth of PC3 Cells

The effect of *Verbena officinalis* aqueous extracts on PC3 cell growth was assessed using the WST-1 assay. Results indicated that the extract did not significantly affect cell viability at lower concentrations (1 and 500 µg/ml). However, at the highest concentration (50,000 µg/ml), a marked reduction in viability was observed. While this decrease suggests an inhibitory effect on cell proliferation, it did not indicate overt cytotoxicity, as cell morphology and adherence remained largely unaffected. This finding implies that *Verbena officinalis* may exert anti-proliferative effects at higher concentrations, potentially through stress-related mechanisms rather than direct cell death. Figure 2 illustrates the impact of *Verbena officinalis* on PC3 cell viability across different concentrations.

Levels of the anti-inflammatory IL-10 protein secretion

This study investigated the impact of *Verbena officinalis* extract on IL-10 secretion in PC3 cells, with and without LPS stimulation. Results showed a concentration-dependent increase in IL-10 levels, with the highest concentration (50,000 µg/ml) significantly elevating IL-10 compared to untreated controls, indicating a strong anti-inflammatory effect. Lower concentrations (1 and 500 µg/ml) induced only slight, statistically insignificant increases in IL-10. These findings suggest that while low doses have minimal anti-inflammatory effects, the extract at higher concentrations has the potential to promote anti-inflammatory pathways effectively as shown in Figure 3.

Levels of the pro-inflammatory IL-6 protein secretion

The effect of *Verbena officinalis* crude aqueous extract on IL-6 protein secretion was evaluated to understand its impact on inflammatory responses in PC3 cells. LPS at 500 ng/ml significantly increased IL-6 levels, triggering an inflammatory response. The extract at concentrations of 1, 500, and 50,000 µg/ml reduced basal IL-6 secretion in a dose-dependent manner. When treated with LPS, the extract effectively protected the cells, significantly reducing IL-6 secretion compared to LPS-induced levels. The highest concentration (50,000 µg/ml) reduced IL-6 to levels similar to the control cells, highlighting the extract's potential anti-inflammatory effect Figure 4.

Measurement of IL-6 gene expression

The impact of *Verbena officinalis* on IL-6 gene expression was investigated. The results in Figure 5 demonstrated that the highest concentration of 50,000 µg/ml significantly downregulated IL-6 gene expression, suggesting an anti-inflammatory effect. In contrast, the lower concentrations of 1 and 500 µg/ml did not significantly alter IL-6 expression compared to the control.

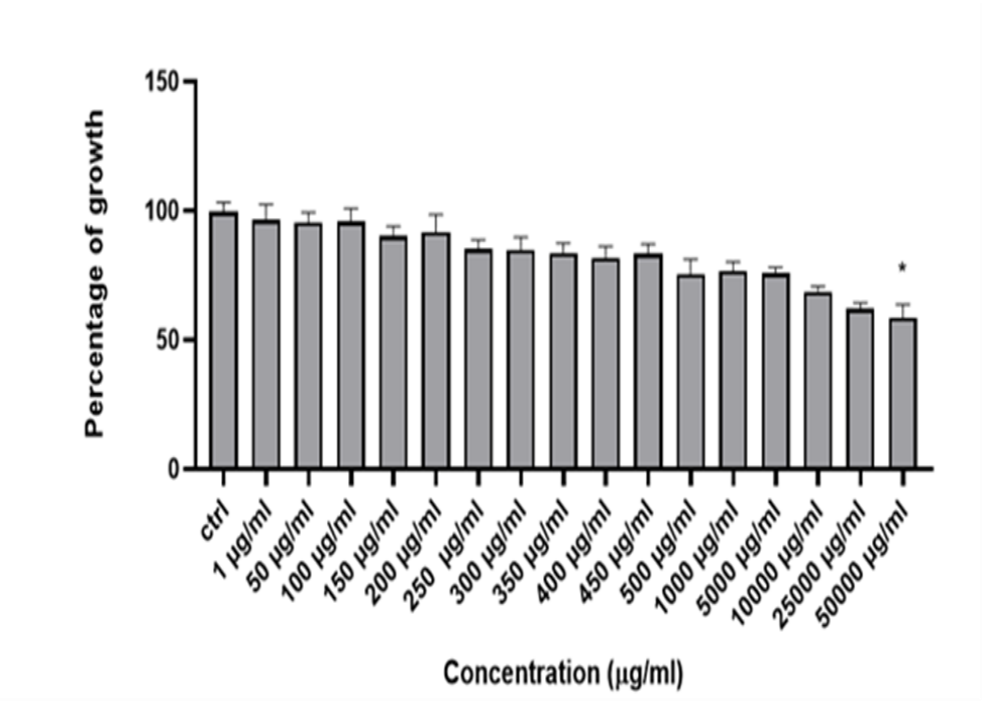

Figure 2. Effect of Verbena officinalis on growth of PC3 cells. Percentage of growth in response to Verbena officinalis at different concentrations (1 -50,000 µg/ml) was measured using WST-1 assay. The highest concentration of Verbena officinalis (50,000 µg/ml) has led to a decrease in % of cells growth to around 58.9%. Data represent the mean ± standard deviation (SD) of three biological replicates. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison post-test. P ≤ 0.05 and P ≤ 0.01 indicate statistically significant differences compared to the control.

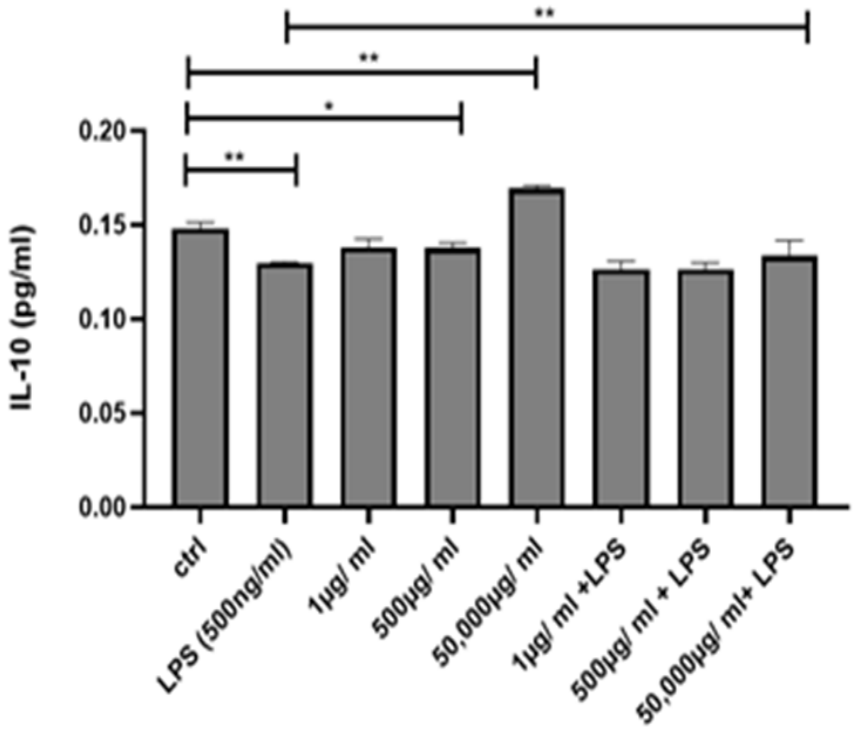

Figure 3. The levels of IL-10 protein secretion in PC3 cells in response to *Verbena officinalis* aqueous crude extract. *Verbena officinalis* aqueous extract at 500 and 50,000 µg/ml significantly increased IL-10 levels compared to the control. Data represent mean ±SD of three biological replicates. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison post-test. *P≤0.05, **P ≤ 0.001 compared to the control.

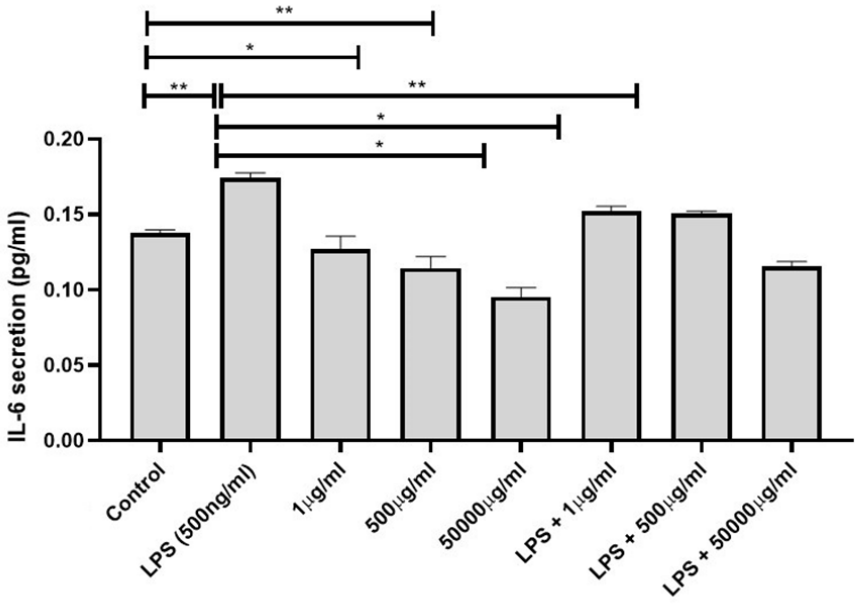

Figure 4. The levels of IL-6 protein secretion in PC3 cells in response to aqueous crude extract of *Verbena officinalis*. LPS (500 ng/ml) significantly increased IL-6 levels compared to control cells ($P < 0.05$). *Verbena officinalis* extract at 500 and 50,000µg/ml significantly reduced IL-6 secretion after 24 hours. *Verbena officinalis* aqueous extract significantly decreased LPS-induced IL-6 secretion. The results represent mean ±SD of three biological replicates. Statistical analysis was conducted using a one-way ANOVA followed by Tukey's multiple comparison. * P≤0.05, **P≤0.01.

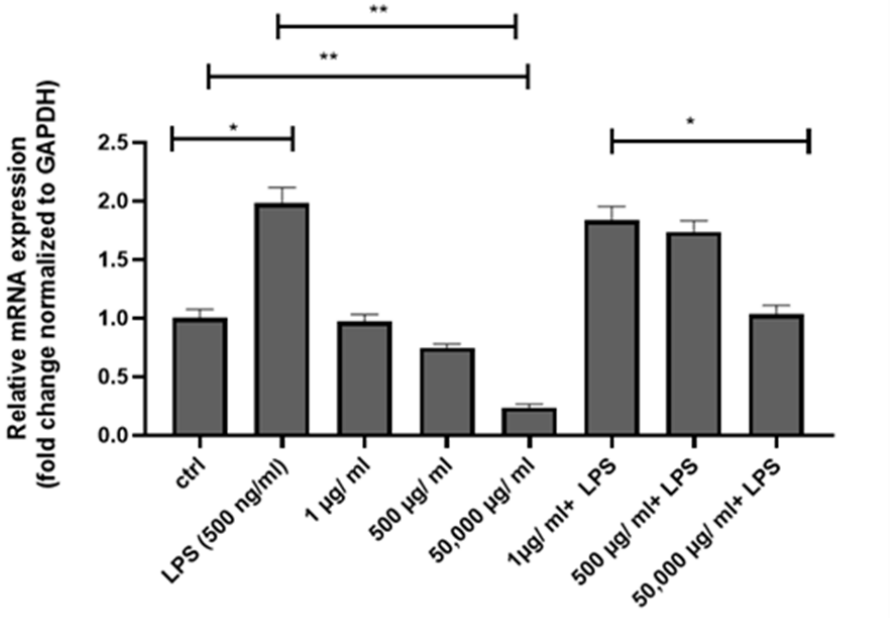

Figure 5. IL-6 gene expression in response to *Verbena officinalis* aqueous extract. Aqueous crude extract of Verbena officinalis significantly decreased LPS-induced IL-6 gene expression after 24 hours. Gene expression was measured using RT-PCR. The results represent mean ±SD of three biological replicates. Statistical analysis was conducted using a one-way ANOVA followed by Tukey's multiple comparison. *P≤0.05, **P≤0.01.

Levels of DPPH *Verbena officinalis* aqueous extracts

The DPPH assay revealed that the aqueous extract of *Verbena officinalis* exhibited a 45.5% free radical scavenging activity, indicating moderate antioxidant potential. This suggests the extract can neutralize nearly half of the free radicals, potentially offering protection against oxidative stress, highlighting the presence of bioactive compounds that contribute to its antioxidant effects.

Tannic acid measurements in *Verbena officinalis* aqueous extracts

Tannic acid, a prominent polyphenol, is well-known for its antioxidant and anti-inflammatory properties and serves as a key indicator of the quality and therapeutic potential of plant extracts. The results revealed that the aqueous extract of *Verbena officinalis* contains 7.7 µg/ml of tannic acid. The concentration of tannic acid observed suggests its role in enhancing the antioxidant and potentially anti-inflammatory effects of the extract, emphasize its significance in the plant's overall medicinal value.

Discussion

Variations in total phenolic content (TPC) levels in *Verbena officinalis* can be influenced by factors such as extraction methods, solvents, and plant parts analyzed. Accurate TPC measurement is crucial for understanding the bioactive properties and health benefits of the plant. In particular, the TPC values for *Verbena officinalis* in this study align closely with those reported by Polumackanycz e*t al.,* (16), who identified TPC values ethanolic extract 104.64 mg GAE/g Dry weight, which is comparable to our observed value of 99.7 mg GAE/g dry weight (represented in the results as 299.2 GAE mg/L).

There is limited research on the effects of *Verbena officinalis* extracts specifically on PC3 cells, with most studies focusing on less aggressive cancer cell lines, such as those from colon or breast cancers (17) which require lower concentrations of plant. Previous study by Dai *et al.* (10) have shown that *Verbena officinalis* extracts exhibit cytotoxic effects on cancer cell lines, with significant anti-proliferative effects at lower doses on less aggressive cell lines, such as colon cancer. In contrast, our study on PC3 prostate cancer cells, which are highly aggressive, found a much higher IC50 value of 50,000 µg/ml, indicating that PC3 cells are more resistant to the extract and require higher doses to inhibit growth. This discrepancy likely reflects the more resilient and proliferative nature of PC3 cells compared to less aggressive lines.

IL-10 was found to be the most potent anti-inflammatory cytokine and has a role in enabling cancer immune surveillance and tumor rejection (18). However, studies specifically examining the relationship between *Verbena officinalis* aqueous extracts, IL-10 levels, and PC3 prostate cancer cells are limited. While the plant has shown anti-inflammatory and immunomodulatory effects, its impact on IL-10 regulation in aggressive prostate cancer models remains unexplored. Further research is needed to understand these mechanisms and evaluate the therapeutic potential of *Verbena officinalis* in managing inflammation-related cancer progression.

IL-6 protein secretion measurements are commonly used to assess the anti-inflammatory and immune-modulating effects of herbal plant extracts, as IL-6 is a pro-inflammatory cytokine overexpressed in cancerous and inflammatory environments, including prostate cancer (19). Inhibition of IL-6 secretion suggests potential therapeutic properties, particularly in controlling inflammation and cancer cell growth (20). While *Verbena officinalis* has been studied for its anti-inflammatory effects, particularly due to its flavonoids and phenolic compounds, research on its direct impact on IL-6 secretion, especially in cancer models like PC3 cells, is limited. Most studies focus on broader inflammatory models or its antioxidant properties. A study by Khalaf and colleagues (2015) showed that *Verbena officinalis* extract reduced inflammatory cytokines, including IL-6, in a mouse model of influenza infection (21). It also enhanced natural killer (NK) cell activity, with Verbenalin identified as a key bioactive compound that improved NK cell killing efficiency. This study highlights the anti-inflammatory effects of *Verbena officinalis*, suggesting that it may help reduce cancer progression by mitigating inflammation and enhancing immune functions. These findings emphasize the need for further research into *Verbena officinalis* as a potential therapeutic agent for cancer prevention and therapy.

In this study, *Verbena officinalis* demonstrated a higher DPPH radical scavenging activity of 45.5%, compared to Khalaf and colleagues (2015) who reported a 23.63% for the same plant species. This discrepancy is likely due to the different extraction solvents used: Khalaf and colleagues (2015) employed 96% ethanol, while we used distilled water. Ethanol, being more polar, may extract a different spectrum of antioxidant compounds (22), whereas distilled water may preserve certain stable phenolic compounds, leading to higher radical scavenging activity (23). The choice of methanol versus water could impact the extraction of bioactive molecules, highlighting the importance of considering both plant species and extraction methods when comparing antioxidant activities across studies (24-25).

Tannic acid, known for its potent antioxidant properties, is associated with various health benefits, *Verbena officinalis* exhibited low levels of tannic acid (7.7 µg/ml), with no prior research specifically assessing its tannic acid content. This minimal level found in *Verbena officinalis* suggest that this plant may have a distinct polyphenolic profile compared to plants with higher tannic acid content. The lack of specific data on tannic acid in *Verbena officinalis* highlights a gap in understanding its chemical composition and antioxidant potential. This finding aligns with research indicating that plant species have diverse polyphenolic profiles influenced by genetic, environmental, and methodological factors (26). While *Verbena officinalis* may not be a major source of tannic acid, its overall antioxidant profile and other beneficial compounds warrant further investigation.

In this study, an aqueous extract of *Verbena officinalis* was prepared by steeping 10 grams of the plant material in 100 ml of water, mimicking traditional household methods. The crude extract contains a complex mixture of bioactive compounds, including iridoids (such as verbenalin and hastatoside), flavonoids (such as apigenin and luteolin), and phenolic acids (such as rosmarinic and chlorogenic acids), which may work synergistically to produce the observed effects. Nevertheless, the precise mechanisms behind these effects are still not fully understood and require further research.

Conclusion

In conclusion, *Verbena officinalis* shows promise as a natural anti-inflammatory agent and a potential preventive treatment for prostate cancer. The study demonstrated that its aqueous extracts significantly reduced IL-6 secretion in prostate cancer cells, particularly at the high concentration of 50,000 µg/ml, while also decreasing cell viability in a concentration-dependent manner. The increased IL-10 secretion at this concentration suggests a favorable shift in the inflammatory response. However, the use of such a high concentration may limit its practical therapeutic application, highlighting the need to identify and isolate active compounds responsible for these effects. Future research should focus on evaluating lower, pharmacologically relevant doses and exploring *in vivo* models to better assess clinical potential. Despite these limitations, *Verbena officinalis* remains a promising candidate for further exploration in cancer prevention and inflammation modulation. Our results show that *Verbena officinalis* aqueous extract has anti-inflammatory and anticancer effects on PC3 cells, though high concentrations are needed. Future studies should isolate active compounds, determine safe and effective doses, explore mechanisms, and evaluate efficacy in vivo.

Declaration of interest

None

Data sharing

Data will be provided upon request

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Ethical Compliance Statement

This study involved in vitro experiments using established human prostate cancer cell lines and did not involve human participants or animal subjects. All experimental procedures were conducted in accordance with institutional guidelines and adhered to internationally recognized ethical standards for laboratory research.

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