



Università degli Studi
“G. d’Annunzio”
Chieti-Pescara



Ph.D. Course in
"Biomolecular and Pharmaceutical Sciences"

Co-tutelle thesis with University of Córdoba, (Spain)

Ph.D. Course in **“Biomedicine”**

CYCLE XXXVII

Ph.D. Thesis

**Protective effects of aged black garlic aqueous
extract in different diseases**

Scientific/Disciplinary Sector (SDS) BIOS-11

Coordinator

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List of publications

This Doctoral Thesis is based on the original research articles listed below, which will be referred in the text by their Roman numerals.

Article I: Lucia Recinella[#], Maria Loreta Libero[#], Valentina Citi, Annalisa Chiavaroli^{*}, Alma Martelli^{*}, Roberta Foligni, Cinzia Mannozi, Alessandra Acquaviva, Simonetta Di Simone, Vincenzo Calderone, Giustino Orlando, Claudio Ferrante, Serena Veschi, Anna Piro, Luigi Menghini, Luigi Brunetti and Sheila Leone. [#]Co-first authors; ^{*}Corresponding authors.

Anti-Inflammatory and Vasorelaxant Effects Induced by an Aqueous Aged Black Garlic Extract Supplemented with Vitamins D, C, and B12 on Cardiovascular System.

2023. *Foods* 12(7):1558. doi: 10.3390/foods12071558

[JIF: 4.7, 38/173 (Q1), FOOD SCIENCE & TECHNOLOGY (SCIE)]

Article II: Maria Loreta Libero[#], Elena Lucarini[#], Lucia Recinella^{*}, Clara Ciampi, Serena Veschi, Anna Piro, Annalisa Chiavaroli, Alessandra Acquaviva, Nilofar Nilofar, Giustino Orlando, Daniele Generali, Carla Ghelardini, Lorenzo di Cesare Mannelli, Antonio J. Montero-Hidalgo, Raúl M. Luque, Claudio Ferrante, Luigi Menghini, Simonetta Cristina di Simone, Luigi Brunetti, Sheila Leone. [#]Co-first authors; ^{*}Corresponding author.

Anti-inflammatory and anti-hyperalgesic effects induced by an aqueous aged black garlic extract in rodent models of ulcerative colitis and colitis-associated visceral pain.

2024. *Phytotherapy Research* 38, 4177–4188, doi:10.1002/ptr.8270.

[JIF: 6.1, 26/354 (Q1), PHARMACOLOGY & PHARMACY (SCIE)]

Article III: Maria Loreta Libero[#], Antonio J. Montero-Hidalgo[#], Lucia Recinella^{*}, Raúl M. Luque^{*}, Daniele Generali, Alessandra Acquaviva, Giustino Orlando, Claudio Ferrante, Luigi Menghini, Simonetta Cristina Di Simone, Nilofar Nilofar, Annalisa Chiavaroli, Luigi Brunetti and Sheila Leone. [#]Co-first authors; ^{*}Corresponding authors.

The protective effects of an aged black garlic water extract on the prostate.

2024. *Nutrients* 16, 3025, doi:10.3390/nu16173025

[JIF: 4.8, 18/114 (Q1), NUTRITION & DIETETICS (SCIE)]

Abstract

Questa Tesi di Dottorato indaga il potenziale terapeutico dell'estratto acquoso di aglio nero invecchiato (ABGE) attraverso un approccio multidisciplinare, concentrandosi sui suoi effetti nei contesti cardiovascolare, gastrointestinale e oncologico. La ricerca è articolata in quattro sezioni, che corrispondono ai risultati sperimentali riportati in tre articoli scientifici pubblicati e a un'analisi integrativa dei meccanismi d'azione dell'ABGE.

La prima sezione evidenzia la composizione fitochimica dell'ABGE, con particolare enfasi sul suo elevato contenuto di polifenoli, in particolare acido gallico e catechine, che contribuiscono alle sue proprietà antiossidanti e antinfiammatorie. Gli studi cardiovascolari dimostrano la capacità dell'ABGE di proteggere i tessuti cardiaci riducendo lo stress ossidativo e l'infiammazione, suggerendo un potenziale terapeutico per le malattie cardiovascolari.

La seconda sezione esplora il ruolo dell'ABGE nella salute gastrointestinale, in particolare nei modelli di colite ulcerosa e dolore viscerale. I risultati mostrano che l'ABGE sopprime significativamente i marcatori pro-infiammatori e allevia l'iper-algesia, supportando la sua applicazione nel trattamento delle malattie infiammatorie intestinali e del dolore cronico associato.

La terza sezione si concentra sulle proprietà antiproliferative dell'ABGE nei confronti del carcinoma prostatico. L'estratto inibisce efficacemente la crescita tumorale e la migrazione cellulare nelle linee di carcinoma prostatico androgeno-dipendenti e indipendenti, modulando vie di segnalazione chiave come MAPK, AKT e JAK/STAT. Questi risultati evidenziano il potenziale dell'ABGE come terapia complementare in oncologia.

La quarta sezione integra questi risultati, proponendo un'ipotesi unificata secondo cui l'ABGE esercita i suoi effetti terapeutici attraverso meccanismi interconnessi che agiscono sull'infiammazione, sullo stress ossidativo e sulla proliferazione cellulare. Questa analisi sottolinea la versatilità dell'ABGE come agente terapeutico e il suo potenziale utilizzo nella prevenzione e gestione delle malattie croniche.

In conclusione, questa tesi dimostra il vasto potenziale terapeutico dell'ABGE, supportato da rigorose evidenze sperimentali e pubblicato in riviste di alto impatto. Sebbene siano necessari ulteriori studi clinici, i risultati forniscono una solida base per lo sviluppo dell'ABGE come agente versatile ed efficace per affrontare condizioni infiammatorie croniche e oncologiche.

Resumen

Esta Tesis Doctoral investiga el potencial terapéutico del extracto acuoso de ajo negro envejecido (ABGE) mediante un enfoque multidisciplinario, centrado en sus efectos en los contextos cardiovascular, gastrointestinal y oncológico. La investigación se estructura en cuatro secciones, correspondientes a los resultados experimentales presentados en tres artículos científicos publicados y un análisis integrado de los mecanismos de acción del ABGE.

La primera sección destaca la composición fitoquímica del ABGE, enfatizando su alto contenido de polifenoles, en particular ácido gálico y catequinas, que contribuyen a sus propiedades antioxidantes y antiinflamatorias. Los estudios cardiovasculares muestran que el ABGE protege los tejidos cardíacos al reducir el estrés oxidativo y la inflamación, lo que sugiere su potencial terapéutico en enfermedades cardiovasculares.

La segunda sección explora el papel del ABGE en la salud gastrointestinal, específicamente en modelos de colitis ulcerosa y dolor visceral. Los resultados demuestran que el ABGE suprime significativamente los marcadores proinflamatorios y alivia la hiperalgesia, apoyando su aplicación en el tratamiento de enfermedades inflamatorias intestinales y el dolor crónico asociado.

La tercera sección se centra en las propiedades antiproliferativas del ABGE en el cáncer de próstata. El extracto inhibe eficazmente el crecimiento tumoral y la migración celular en líneas de cáncer de próstata dependientes e independientes de andrógenos, modulando vías clave de señalización como MAPK, AKT y JAK/STAT. Estos hallazgos resaltan el potencial del ABGE como terapia complementaria en oncología.

La cuarta sección integra estos hallazgos, proponiendo una hipótesis unificada en la que el ABGE ejerce sus efectos terapéuticos mediante mecanismos interconectados que actúan sobre la inflamación, el estrés oxidativo y la proliferación celular. Este análisis subraya la versatilidad del ABGE como agente terapéutico y su potencial uso en la prevención y gestión de enfermedades crónicas.

En conclusión, esta tesis demuestra el amplio potencial terapéutico del ABGE, respaldado por evidencias experimentales rigurosas y publicado en revistas de alto impacto. Aunque se requieren estudios clínicos adicionales, los resultados ofrecen una base sólida para el desarrollo del ABGE como un agente versátil y eficaz para abordar condiciones inflamatorias crónicas y oncológicas.

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1. Introduction

1.1 *Inflammation*

Inflammation is an essential and complex biological response of the immune system to harmful stimuli such as infections, trauma, or irritants (Chen et al., 2018). An acute inflammation plays a critical role in defending body (Chen et al., 2018). When this process persists for months and years, it can become the focal point of many chronic and degenerative diseases, turning into a double-edged sword (Gupta et al., 2018). Chronic inflammation, in fact, is associated with a wide range of conditions, including cardiovascular diseases, cancer, chronic inflammatory bowel diseases (IBD), and prostatitis. Its prolonged presence induces a constant state of oxidative and immune stress, which promotes tissue degeneration and pathological remodeling (Pahwa et al., 2023).

The growing importance of studying chronic inflammation is evidenced by the increasing global incidence of chronic diseases with inflammatory basis (Furman et al., 2019). Conditions such as atherosclerosis, cancer, and autoimmune diseases are known as the leading causes of morbidity and mortality in the Western world (Global Health Estimates: Estimates for SDG Indicators, 2020). Understanding the mechanisms underlying inflammation as well as research of effective strategies in modulating the inflammatory response are therefore of crucial importance for preventing and treating these diseases.

In recent years, scientific research has focused on natural agents that could modulate inflammation in a safe and effective way. Among these, aged black garlic (ABG) has attracted particular attention (Kurowska et al., 2023; Nunes et al., 2020; Gonfa et al., 2023). ABG is obtained through a fermentation and aging process of raw garlic at high temperatures and under controlled humidity conditions. This process modifies the chemical properties of garlic, increasing the concentration of bioactive compounds with potent antioxidant and anti-inflammatory properties (Ahmed et al., 2021). This treatment converts unstable sulfur compounds, such as allicin, into more stable substances like S-allylcysteine, a potent antioxidant. Additionally, the process significantly increases the

concentration of polyphenols and flavonoids, which give black garlic its strong antioxidant potential (Bae et al., 2015; Zhang et al., 2016; Sun et al., 2018).

Black garlic has shown promising therapeutic properties in a wide range of chronic and degenerative inflammatory diseases, positioning itself as a potential candidate for the integrative management of many inflammatory conditions (Stępień et al., 2024; Ahmed et al., 2021).

1.1.1 Inflammation and cardiovascular system

Cardiovascular diseases are the leading cause of mortality worldwide (Lindstrom et al., 2022), and chronic inflammation is considered one of the key factors in the development of these conditions (Sorriento et al., 2019). In this context, atherosclerosis is a condition characterized by the accumulation of lipids, calcium, and immune cells (mainly macrophages) in the arterial walls, leading to the formation of atherosclerotic plaques (Henein et al., 2022). If these plaques become unstable, they may rupture, causing the formation of clots that can block blood flow and lead to heart attacks or strokes (Badimon et al., 2012).

The inflammatory process in the arteries is triggered by traditional risk factors such as hypertension, hypercholesterolemia, and smoking, which damage the endothelial cells lining the arteries (Kong et al., 2022). These damages induce a local inflammatory response that involves the recruitment of monocytes and macrophages and the accumulation of oxidized lipoproteins, which further promote inflammation (Moore et al., 2013).

ABG has emerged as a potential protective agent against cardiovascular diseases due to its ability to reduce inflammatory response and oxidative stress, two key factors in the atherosclerotic process (García-Villalón et al., 2016). Preclinical studies on animal models, which will be discussed in this thesis, have shown that ABG can significantly reduce levels of cyclooxygenase (COX)-2, tumor necrosis factor (TNF)- α , interleukin (IL)-6, nuclear factor-kB (NF-kB), and inducible nitric oxide

synthase (iNOS) on mouse heart specimens. These findings suggests that ABG may exert a protective effect against the development and progression of atherosclerosis, making it a promising candidate for the prevention of inflammation-based cardiovascular diseases.

In a study on induced atherosclerosis models, black garlic led to a significant reduction in the size of atherosclerotic plaques as well as in arterial wall thickness, associated with a decrease in systemic inflammation (Czompa et al., 2018). Additionally, black garlic was found able to improve antioxidant capacity, protecting endothelial cells from oxidative damage and promoting the release of nitric oxide (NO), a powerful vasodilator improving blood circulation (Czompa et al., 2018).

1.1.2 Inflammation and Cancer

Chronic inflammation has long been recognized as one of the key factors in carcinogenesis (Multhoff et al., 2012). Numerous studies have demonstrated that prolonged inflammation can promote the neoplastic transformation of cells through mechanisms such as increased oxidative stress, DNA damage, and the stimulation of cell proliferation (Kay et al., 2019). The inflammatory environment also provides signals that favor angiogenesis, the growth and survival of cancer cells, as well as their ability to metastasize (Liu et al., 2023).

A clear example of the association between inflammation and cancer is colorectal carcinoma, which often develops in patients with chronic inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease (Kim et al., 2014; Laredo et al., 2023). In these cases, chronic intestinal inflammation generates a pro-tumor microenvironment characterized by the activation of pro-inflammatory signaling pathways, such as the NF- κ B pathway, which promotes the proliferation of epithelial cells and the survival of cancer cells (Luo et al., 2017).

ABG has shown significant potential as an anti-cancer agent due to its ability to modulate inflammation and inhibit cancer cell growth (Dong et al., 2014; Unal et al., 2024). *In vitro* studies

have highlighted that ABG can inhibit the proliferation of colon, breast, and stomach tumor cell lines (Dong et al., 2014; Yang et al., 2023; Wang et al., 2012). The molecular mechanism through which black garlic exerts these effects isn't clear. In particular, it seems to reduce the expression of pro-inflammatory cytokines, growth factors, and anti-apoptotic proteins involved in tumor progression (Yang et al., 2023; Libero et al., 2024).

Moreover, black garlic was shown to induce apoptosis in colon cancer cells through the inhibition of the PI3K/Akt pathway (Dong et al., 2014). These preclinical results support the use of black garlic as a potential adjuvant in anti-cancer therapy, particularly in cancers associated with chronic inflammation.

1.1.3 Inflammation and Inflammatory Bowel Diseases (IBDs)

Chronic inflammatory bowel diseases (IBDs), which include Crohn's disease and ulcerative colitis, are characterized by persistent inflammation of the gastrointestinal tract, leading to debilitating symptoms and, in many cases, serious complications such as colon cancer (Muzammil et al., 2023). The pathogenesis of IBD is multifactorial, and involves a combination of genetic, environmental, and immunological factors contributing to the onset of chronic inflammation (Loddo et al., 2015).

Gut microbiota plays a key role in regulating intestinal inflammation. In individuals with IBD, a condition known as dysbiosis is observed, where the normal composition of the gut microbiome is altered, contributing to the perpetuation of inflammation (Khan et al., 2019). Patients with IBD often display an increase in pathogenic bacterial species and a decrease in beneficial bacterial species that regulate immune responses. This imbalance leads to the continuous activation of both the innate and adaptive immune systems, with the release of inflammatory cytokines such as IL-1 β , TNF- α , and IL-6, which further damage the intestinal barrier (Santana et al., 2022).

ABG has shown a protective effect against intestinal inflammation in experimental models of IBD. In particular, studies on animal models have demonstrated that ABG can significantly reduce

gene expression levels of pro-inflammatory cytokines in colon *ex vivo* (Libero et al., 2024).

Moreover, ABG was shown to reduce tissue damage and improve mucosal regeneration in the intestine (Libero et al., 2024), on a mouse model of induced colitis.

1.1.4 Inflammation, Prostatitis, and Prostate Cancer

Chronic prostatitis is a common condition in men that can significantly impact quality of life and, in some cases, contribute to long-term complications, including prostate cancer (Oseni et al., 2023). Although a direct causal link between prostatitis and prostate cancer has not been definitively proven, many studies suggest that chronic inflammation of the prostate may create favorable conditions for neoplastic transformation (Sfanos et al., 2012).

The prostate microenvironment, in the presence of chronic inflammation, becomes highly reactive, with increased levels of reactive oxygen species (ROS) and oxidative damage to the DNA of prostate cells (Khandrika et al., 2009). In fact, chronic prostatitis is characterized by persistent episodes of inflammation in the prostate gland, which may be associated with bacterial infections or non-infectious factors such as oxidative stress, hormonal imbalance, and immunological abnormalities. If left untreated, chronic prostate inflammation can promote a perpetual cycle of tissue damage and regeneration, gradually leading to a pre-cancerous condition (He et al., 2023; Khandrika et al., 2009; Fibbi et al., 2010).

ABG, although not yet extensively studied in the context of prostatitis and prostate cancer, offers significant potential as an anti-inflammatory and antioxidant agent (Ahmed et al., 2021). ABG could help to reduce chronic prostate inflammation and potentially lower the risk of long-term damage associated with carcinogenesis. For this reason, in this thesis, I aim to study the potential protective effects induced by an aged black garlic extract (ABGE) on prostate inflammation and prostate cancer.

1.2 Aged Black Garlic: A Biochemical and Nutraceutical Overview

From a nutraceutical perspective, ABG offers numerous advantages over fresh garlic. Not only does it have a sweeter and less pungent flavor, making it more tolerable for daily use, but it also has a biochemical profile that makes it particularly suitable for the prevention and treatment of inflammatory and degenerative diseases. The bioactive compounds in black garlic, such as thiosulfinates, cysteine acid derivatives, and phenolic compounds, have been shown to be effective in countering oxidative stress, reducing inflammation, and improving immune function (Song et al. 2020; Sasmaz et al., 2022; Kim et al., 2012; Garcia-Villalón et al., 2016; Venkatesh, 2018).

Numerous studies have confirmed that ABG has a much higher antioxidant profile compared to fresh garlic (Sasmaz et al., 2022). For instance, S-allylcysteine is highly bioavailable and stable, allowing for an effective reduction in oxidative stress and lipid peroxidation, which are key mechanisms in the development of chronic inflammatory diseases (Colín-González et al., 2012).

Research on the use of ABG as a nutraceutical was primarily focused on three areas in this thesis: cardiovascular health, cancer prevention, and the management of inflammatory diseases. Preclinical studies that we will show in this thesis have provided encouraging results, demonstrating that incorporating black garlic into the diet can have beneficial effects on various inflammatory conditions, improving endothelial function, and reducing the incidence of inflammation-related chronic diseases.

2 Aim of the study

This Doctoral Thesis aims to investigate the potential beneficial properties of aged black garlic aqueous extract (ABGE) and its potential use in various pathologies characterized by chronic inflammation, visceral pain, tumor proliferation, and cardiovascular disorders. The specific objectives of the present study can be divided into four main areas:

1) Evaluation of the cardioprotective potential of ABGE

- **Objective:** to explore the protective effects of ABGE on the cardiovascular system, with a focus on its ability to reduce heart tissue inflammation and induce vasorelaxant effects in aortic smooth muscle cells.
- **Hypothesis:** ABGE, due to its high content of polyphenols such as gallic acid and catechins, can reduce the levels of pro-inflammatory mediators and improve vascular function by promoting the bioavailability of H₂S.

2) Investigation of the anti-inflammatory activities of ABGE in intestinal tract

- **Objective:** to study the efficacy of ABGE in reducing chronic colon inflammation and modulating visceral pain in models of ulcerative colitis. In particular, we aimed to determine whether ABGE can reduce levels of pro-inflammatory cytokines and alleviate pain associated with these conditions.
- **Hypothesis:** ABGE has the potential to modulate intestinal inflammatory pathways and reduce visceral pain associated with colitis, offering a promising alternative for treating chronic pain in inflammatory bowel diseases.

3) Determination of anti-tumor properties of ABGE in prostate cancer

- **Objective:** to determine the anti-proliferative effects of ABGE on different prostate cancer cell models, both androgen-dependent and androgen-independent, by analyzing its impact on critical signaling pathways involved in tumor progression (including MAPK, AKT, JAK/STAT).
- **Hypothesis:** ABGE, through its ability to modulate cellular signaling pathways and reduce tumor cell proliferation, could represent a potential therapeutic agent for the prevention and treatment of prostate cancer.

4) Develop an integrated understanding of ABGE's mechanisms of action

- **Objective:** Connect the results obtained from various models (cardiovascular, gastrointestinal, and oncological) to build a comprehensive understanding of the mechanisms through which ABGE acts on multiple pathological conditions, highlighting its potential use in prevention and as an adjuvant in treatments for chronic diseases.
- **Hypothesis:** ABGE can operate through interconnected mechanisms that modulate inflammation, pain, and cellular proliferation, suggesting its use in both prevention and as an adjuvant in the treatment of chronic inflammatory and oncological diseases.

3 Results and general discussion

The results obtained in the present Doctoral Thesis and their discussion have been divided into four sections (I, II, III, and IV) and presented in three independent scientific manuscripts published in top-ranking journals within the fields of “Food Science & Technology”, “Pharmacology & Pharmacy”, and “Nutrition & Dietetics”. The fourth section represents an integration of the published results, proposing a hypothesis on the mechanisms through which black garlic extract (ABGE) may act across multiple physiological and pathological systems. This section suggests that ABGE, with its anti-inflammatory, antioxidant, and anti-proliferative properties, may modulate various cellular and molecular processes, offering potential as a versatile therapeutic agent in a range of chronic conditions. The three accepted manuscripts can be found at the end of the Doctoral Thesis.

3.1 Section I – Aged Black Garlic Water Extract (ABGE): phytochemical composition and its role on the cardiovascular system.

ABGE is rich in polyphenol compounds such as gallic acid and catechins. These components exhibit anti-inflammatory and antioxidant effects on the cardiovascular system.

The ABGE showed a polyphenol content of 21.91 mg GAE/g of dry matter, a result comparable to previous studies on conventional and organic black garlic, which reported values between 13.64 and 17.24 mg GAE/g of dry matter (Najman et al., 2021). It was observed that the polyphenol content in black garlic is higher than in fresh garlic, likely due to factors such as the aging process, temperature, and humidity (Medina et al., 2019).

An HPLC-DAD-MS analysis identified 12 phenolic compounds in ABGE, with gallic acid and catechin being the main components. These results partially align with previous studies that found more compounds in black garlic than fresh garlic (Najman et al., 2021; Medina et al., 2019). It was known that different cultivars express varying levels of phenolic compounds depending on cultivation methods. Additionally, several studies have confirmed that black garlic possesses a wide range of

pharmacological activities, including hypolipidemic, anticancer, and cardiovascular effects, attributed to its anti-inflammatory and antioxidant properties (Ahmed et al., 2021).

To evaluate the toxicity and pharmacological effects of ABGE, its action on H9c2 rat cardiomyoblast cells was analyzed, both in basal conditions and after treatment with LPS, a known inflammation inducer. ABGE did not affect cell viability under basal conditions but was able to counteract the cytotoxicity induced by LPS, suggesting a protective effect against inflammation. Preclinical and clinical studies have demonstrated the critical role of inflammation and oxidative stress in the development of cardiovascular diseases, such as hypertension, diastolic dysfunction, and ischemia/reperfusion injury (García et al., 2017).

In an *ex vivo* model using LPS-treated mouse heart tissue, ABGE (1-100 µg/mL) significantly reduced the levels of various pro-inflammatory and pro-oxidant mediators, including COX-2, TNF- α , IL-6, NF-kB, and iNOS, without showing a dose-dependent relationship. The antioxidant and anti-inflammatory properties induced by ABGE could be related to polyphenolic composition of ABGE, particularly gallic acid and catechin. In agreement, gallic acid has been shown to inhibit prostaglandin E2 and IL-6 production and reduce oxidative stress in rat liver mitochondria (Dutta M. et al., 2019). Catechin is also known for its cardioprotective properties, as demonstrated by its ability to reduce inflammation in animal models of coronary heart disease (Tu S. et al., 2018).

Next, the effects of a formulation composed of ABGE (100 µg/mL) and vitamins [Vitamin B12 (1 µg/mL), Vitamin C (10 µg/mL) and vitamin D (1 µg/mL)] were tested on H9c2 cells, both pretreated and not pretreated with LPS. Neither the formulation nor the vitamins alone affected cell viability under basal conditions. However, both the formulation and the vitamins, were able to reduce the LPS-induced cytotoxicity. Furthermore, the formulation proved to be more effective than the vitamins alone in reducing the gene expression of inflammatory markers such as IL-6, TNF- α , and NF-kB. Recent studies have highlighted the critical role of vitamin D in the pathogenesis of cardiovascular diseases, due to its ability to reduce oxidative stress and inflammation (Renke G. et al., 2023). Vitamin C has also

shown antioxidant effects, related to both the prevention and treatment of cardiovascular diseases, while vitamin B12 has been demonstrated to reduce inflammation by modulating the expression of NF-kB and IL-6.

In summary, ABGE demonstrated significant antioxidant and anti-inflammatory properties, primarily attributed to its high polyphenol content, especially gallic acid and catechin. These bioactive compounds were shown to exert cardioprotective effects by inhibiting key pro-inflammatory and pro-oxidant mediators, as evidenced by their ability to counteract the cytotoxicity induced by LPS in cardiomyoblast cells. Moreover, the combination of ABGE with vitamins B12, C, and D further enhanced these protective effects, particularly in reducing the gene expression of pro-inflammatory markers such as IL-6, TNF- α , and NF-kB. Altogether, these results suggest a potential therapeutic role for ABGE, either alone or in combination with essential vitamins, in the prevention and treatment of cardiovascular diseases linked to inflammation and oxidative stress. Further in-depth studies, including clinical trials, are needed to validate these findings and explore the full pharmacological potential of black garlic in cardiovascular health.

3.2 Section II – ABGE exhibits anti-inflammatory and anti-hyperalgesic effects in rodent models of ulcerative colitis and colitis-related visceral pain.

Previous studies showed that ABGE was rich in polyphenols, for this reason, its potential effect has also been proposed on the gastrointestinal system.

This study investigated the anti-inflammatory and anti-hyperalgesic effects induced by ABGE using rodent models of ulcerative colitis and colitis-associated visceral pain. The primary objective was to evaluate the potential protective effect of ABGE in alleviating intestinal inflammation and persistent pain associated with colitis.

For the *ex vivo* experimental models, rat colon samples treated with lipopolysaccharide (LPS) were used to replicate ulcerative colitis. The results demonstrated that ABGE significantly suppressed the gene expression of several pro-inflammatory biomarkers, including IL-1 β , IL-6, TNF- α , and NF- κ B, thus suggesting its anti-inflammatory effect.

For the *in vivo* experiments, visceral pain was induced in rats through the intrarectal injection of 2,4- dinitrobenzenesulfonic acid (DNBS), a well-established model for studying colitis and associated visceral pain. The acute administration of ABGE exhibited a dose-dependent efficacy in reducing post-inflammatory visceral pain. Specifically, at the highest dose of 1 g/kg, the extract significantly decreased both the nociceptive response and abdominal contraction measured by electromyography during colorectal distension in DNBS-treated rats.

The beneficial effects observed with the use of ABGE could be attributed to its polyphenolic composition, with a significant role played by gallic acid and catechin. These compounds are known for their antioxidant and anti-inflammatory properties and may modulate the activity of various cellular pathways involved in inflammatory processes and the neuronal sensitization of visceral pain. ABGE's ability to reduce the activation of pro-inflammatory mediators suggests its potential use as an adjuvant treatment for managing chronic pain associated with colitis and other inflammatory bowel conditions.

In conclusion, the study's findings indicate that ABGE could represent a promising strategy to modulate colonic inflammatory processes and alleviate visceral pain associated with colitis, offering a potential therapeutic alternative with a favorable safety profile compared to conventional therapies. Further studies are needed to confirm these effects and to better understand the molecular mechanisms underlying ABGE's anti-inflammatory and analgesic properties.

3.3 Section III – ABGE exhibits protective effect in prostate cancer.

In the present study we aimed to determine the protective effects of ABGE on prostate inflammation and its potential anti-cancer properties against different prostate cancer cell models. The primary aim was to assess the anti-inflammatory and anti-proliferative capabilities of ABGE in both *ex vivo* models using mouse prostate specimens, and *in vitro* models using different prostate cancer cell lines.

In the *ex vivo* experiments, inflammation was induced in the prostate tissues using *Escherichia coli* lipopolysaccharide (LPS). ABGE demonstrated a significant reduction in the gene expression of key pro-inflammatory markers, including COX-2, NF- κ B, TNF- α , and IL-6, with the most pronounced effects observed at a concentration of 1000 μ g/mL. This suggests that ABGE has potent anti-inflammatory effects, which could be linked to its polyphenolic components, such as catechin and gallic acid.

In the *in vitro* studies, ABGE was tested on both androgen-dependent (LNCaP) and androgen-independent (PC-3) prostate cancer cell lines. ABGE significantly inhibited cell proliferation, colony formation, and migration in these cell lines in a dose-dependent manner. The most substantial effects were observed at the highest concentration of 1000 μ g/mL, wherein ABGE also reduced tumorsphere formation in the PC-3 cells, indicating its effectiveness in limiting cancer cell proliferation and potential metastasis.

The further explored the molecular mechanisms underlying ABGE's effects, we analyzed its impact on the activity of several signaling pathways, including MAPK, AKT, JAK/STAT, and TGF- β . ABGE was found to modulate these pathways by reducing the phosphorylation of key proteins involved in cell survival and proliferation, such as ERK1/2, JNK, and mTOR. Additionally, ABGE enhanced the activation of proteins like p53 and AMPK, which are known to suppress cancer progression.

The findings indicate that ABGE has promising anti-inflammatory and anti-cancer properties that could potentially be harnessed for managing prostate cancer. In particular, the polyphenolic

content of ABGE, with particular regard to catechin and gallic acid, appears to play a critical role in its bioactivity. Therefore, these results support further investigation into the use of ABGE as a therapeutic agent or dietary supplement for prostate health and cancer prevention. However, additional in vivo studies are necessary to validate these effects and understand the precise mechanisms of action of ABGE in cancer prevention and treatment.

3.4 Section IV – Integrated Mechanistic Insights: Connecting ABGE’s Multi-Pathway Actions Across Chronic Diseases.

The findings from studies on ABGE highlight a synergistic action involving multiple pathological systems, confirming its efficacy as a modulator across different conditions of inflammation, oxidative stress, and cellular proliferation. In cardiovascular, gastrointestinal, and oncological contexts, ABGE acts primarily through bioactive compounds, such as gallic acid and catechins, which modulate cellular and molecular pathways common to the pathological processes of chronic inflammation, oxidative damage, and uncontrolled cell growth.

ABGE’s anti-inflammatory action, demonstrated by the gene expression reduction of pro-inflammatory markers (e.g., TNF- α , IL-6, and NF-kB) in both cardiac and intestinal models, suggests that ABGE can mitigate systemic and localized inflammatory responses, thereby protecting tissues from oxidative stress and reducing the risk of cellular dysfunction associated with chronic diseases. This modulation of the inflammatory response, combined with the antioxidant effects of the polyphenols present in ABGE, contributes to cardiovascular system protection against inflammation-related diseases such as atherosclerosis and heart failure.

Similarly, results from gastrointestinal models of colitis and visceral pain indicate that ABGE effectively reduces hyperalgesia and pain sensitivity, showing therapeutic potential as an analgesic in inflammatory bowel contexts. Finally, ABGE’s antiproliferative action against prostate cancer cells, mainly mediated by the inhibition of MAPK and NF-kB signaling pathways and the activation of tumor

suppressor proteins like p53, suggests that ABGE may help limit tumor growth and metastatic spread. In summary, the results of this Doctoral Thesis demonstrate that ABGE exerts a multifactorial action through interconnected mechanisms that modulate inflammation, cellular proliferation, and pain response. **Therefore, this versatility suggests the potential use of ABGE as both a preventive agent and as an adjuvant in treatments for chronic inflammatory and oncological diseases, with favorable prospects for clinical studies to further explore its efficacy and safety.**

4 General conclusions

This Doctoral Thesis provides significant insights into the therapeutic potential of ABGE across multiple pathological conditions, including cardiovascular health, inflammation, visceral pain, and tumor proliferation. The findings of this research suggest that ABGE is a promising natural agent that could be used for both preventive measures and as adjuvant therapy in managing different chronic diseases. Specifically, the following conclusions are derived from the previous independent sections included of this Doctoral Thesis:

4.1 Cardiovascular health

The study demonstrated that ABGE exerts cardioprotective effects by reducing inflammation and oxidative stress in heart tissues. Moreover, the incorporation of vitamins D, C, and B12 enhances the anti-inflammatory effects of ABGE and triggers a vasorelaxative effect. These results underline ABGE's potential in preventing and treating cardiovascular diseases, especially those linked to chronic inflammation, such as hypertension and atherosclerosis.

4.2 Colon inflammation and visceral pain

ABGE has shown a remarkable ability to reduce colon inflammation and modulate visceral pain in animal models of ulcerative colitis. By suppressing gene expression of pro-inflammatory cytokines such as IL-1 β , IL-6, TNF- α , and NF- κ B, ABGE acts as both an anti-inflammatory and analgesic agent. Based on its dual action, ABGE could be proposed as a natural therapeutic alternative or complement for managing both inflammation and chronic pain in gastrointestinal diseases.

4.3 Anti-tumor effects in prostate cancer

ABGE exerts strong anti-proliferative effects in prostate cancer cells. By inhibiting key signaling pathways, including MAPK, AKT, and JAK/STAT, ABGE effectively suppressed tumor cell growth

and migration in both androgen-dependent and androgen-independent prostate cancer cell models. These findings underscore the potential of ABGE as a complementary therapy in cancer treatment, with particular interest in prostate cancer.

A valuable direction for future research would be to investigate the efficacy of black garlic when combined with conventional or integrative therapies for prostatitis and prostate cancer. Specifically, studies should focus on black garlic's capacity to modulate distinct inflammatory and oncological biomarkers and to examine its impact on the prostate microenvironment in both preclinical and clinical models. This approach could provide critical insights into black garlic's role in enhancing therapeutic outcomes in prostate health and cancer management.

4.4 Integrated mechanisms of action

One of the most significant outcomes of this research is the understanding that ABGE operates through interconnected mechanisms across different tissues. Interestingly, in the cardiovascular system, intestinal tract, or cancerous tissues, ABGE reduces both inflammatory responses and oxidative stress, and influences cell proliferation, thus suggesting its potential as a valuable therapeutic agent that could be applied in a variety of relevant medical fields.

4.5 Future Perspectives

While the results of this study are promising, further research is needed to translate these findings into clinical applications. Therefore, clinical trials will be essential to confirm the efficacy of ABGE in human subjects, as well as to investigate its long-term safety and potential interactions with conventional therapies. Additionally, exploring other disease models and refining the understanding of the molecular mechanisms behind ABGE's action could open new clinical avenues for its use in personalized medicine.

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6 Original articles

Article I



Anti-Inflammatory and Vasorelaxant Effects Induced by an Aqueous Aged Black Garlic Extract Supplemented with Vitamins D, C, and B12 on Cardiovascular System

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Abstract: Multiple studies demonstrated biological activities of aged black garlic, including anti-inflammatory, antioxidant, and cardioprotective effects. We aimed to investigate the protective effects of an aged black garlic water extract (ABGE) alone or in association with multivitamins consisting of combined Vitamins D, C, and B12, on mouse heart specimens exposed to E. coli lipopolysaccharide (LPS). Moreover, we studied the hydrogen sulphide (H₂S) releasing properties and the membrane hyperpolarization effect of the Formulation composed by ABGE and multivitamins, using Human Aortic Smooth Muscle Cells (HASMCs). ABGE, vitamins D and C, and the Formulation suppressed LPS-induced gene expression of cyclooxygenase (COX)-2, tumor necrosis factor (TNF)- α , interleukin (IL)-6, nuclear factor-kB (NF-kB), and inducible nitric oxide synthase (iNOS) on mouse heart specimens. The beneficial effects induced by the extract could be related to the pattern of polyphenolic composition, with particular regard to gallic acid and catechin. The Formulation also increased fluorescence values compared to the vehicle, and it caused a significant membrane hyperpolarization of HASMCs compared to ABGE. To conclude, our present findings showed that ABGE, alone and in association with multivitamins, exhibited protective effects on mouse heart. Moreover, the Formulation increased intracellular H₂S formation, further suggesting its potential use on cardiovascular disease.

Keywords: aged black garlic; hydrogen sulfide; oxidative stress; inflammation

1. Introduction

Cardiovascular disease (CVD) represents the main cause of mortality all over the world. In this context, it has been demonstrated that inflammation and oxidative stress play a pivotal role in the development of CVD such as heart failure, acute coronary syndromes, atherosclerosis, and hypertension [1–4]. In agreement, an association between alterations in levels of pro-inflammatory and pro-oxidant markers and CVD has been found [5]. Various herbal extracts, particularly in pharmacological associations, were shown to be effective in decreasing the burden of inflammation and oxidative stress [6–9]. Moreover, hydrogen sulfide (H₂S), a gaseous molecule, is critically involved in CVD. To this end, several

preclinical and clinical studies suggested its protective role in heart failure, myocardial infarction, and hypertension [10].

Aged black garlic (ABG), obtained from fresh garlic (*Allium sativum* L.) and fermented under high temperatures (60–90 °C) and high humidity (80–90%) for a specific time period, exerts beneficial effects in various experimental paradigms. In particular, ABG induced multiple biological activities, including antioxidant, antiallergic, antidiabetic, anti-inflammatory, cardiovascular, hepatoprotective, neuroprotective, and anticarcinogenic effects [11–19]. The protective effects induced by aged garlic on different types of CVD have been demonstrated in several studies [20]. In agreement, aged garlic was found able to decrease high blood pressure in humans [21]. Furthermore, black garlic extract was found able to modify serum levels of triglycerides and cholesterol [12]. Various phytoconstituents, including phenolics, S-allyl cysteine (SAC), and hydroxycinnamic acid derivatives were found in BG, with respect to raw garlic [12,13].

The beneficial properties of garlic in CVD have been hypothesized to be related to polyphenolic compounds and SAC [22]. Multiple studies showed significant differences in the total phenolic content of black and fresh garlic. In particular, the content in phenolic compounds is 5–8-times higher in black garlic than that in fresh garlic [23].

The maintenance of cardiovascular health is also attributed to vitamins, such as vitamin B 12, vitamin C, and vitamin D [24].

In particular, vitamin D supplementation improves the cardiac function [25], while vitamin C supplementation, thanks to its antioxidant properties, is effective in the prevention or treatment of several cardiovascular diseases [26]. Furthermore, vitamin B12 deficiency promotes the onset of different CVD, including myocardial infarction, stroke, and other circulatory health problems [27].

The present study aims to investigate the potential antioxidant and anti-inflammatory effects of an ABG water extract (ABGE), alone or in association with multivitamins consisting of the combined Vitamin D, C, and B12 Formulation on mouse heart specimens exposed to *Escherichia coli* lipopolysaccharide (LPS), a known proinflammatory agent. In this context, we evaluated gene expression of various biomarkers involved in inflammation and oxidative stress, including cyclooxygenase (COX)-2, tumor necrosis factor (TNF)- α , interleukin (IL)-6, nuclear factor-kB (NF-kB), and inducible nitric oxide synthase (iNOS). In addition, we studied the H₂S releasing properties and the membrane hyperpolarization effect of ABGE, as well as the Formulation composed by ABGE and multivitamins using Human Aortic Smooth Muscle Cells (HASMCs).

The ABGE was also investigated in order to identify and quantify the polyphenolic content using high-performance liquid chromatography coupled with a photo diode array detector (HPLC-DAD) analytical method.

2. Materials and Methods

2.1. Preparation of ABGE

ABG cloves were supplied as dried material by il Grappolo S.r.l. (Soliera, Modena, Italy). Preparation of ABGE was performed as previously reported [28,29]. The detailed protocol is enclosed as supplementary materials.

2.2. Total Polyphenol Content of ABGE

Total polyphenol content was determined according to the Folin-Ciocalteu method, as described in Savini et al. (2017) [30] with some modifications. The detailed protocol related to total polyphenol content of ABGE is described in the Supplementary Materials Section.

2.3. HPLC-DAD-MS Analysis of Phenolic Compounds

The extract was analyzed for phenol quantitative determination using a reversed-phase HPLC-DAD-MS in gradient elution mode [31]. The details of the analysis are reported in Supplementary Materials (Tables S1 and S2).

2.4. Toxicological and Pharmacological Studies

2.4.1. Cell Line

H9c2 cells (rat cardiomyoblasts, ATTC, Rockville, MD, USA) were maintained in DMEM (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS), 1% of 100 unit/mL penicillin, and 100 mg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in T75 red cap tissue culture flasks, at 37 °C in a humidified atmosphere of 5% CO₂.

2.4.2. Cell Viability Assay

Cell viability was evaluated by MTT assay [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (Sigma, St. Louis, MO, USA), as previously described [32]. Briefly, H9c2 cell line was seeded in 96-well plates (5 × 10³ cells/well) and it was pretreated with 10 µg/mL LPS for 24 h. Subsequently, both LPS-pretreated and not LPS-pretreated H9c2 cells were exposed to ABGE at various concentrations (1–100 µg/mL), or with vehicle (control) for a further 48 h. On the basis of results, we then performed a second set of experiments to evaluate the effects induced by the Formulation [ABGE (100 µg/mL) + Vitamin B12 (1 µg/mL) + Vitamin C (10 µg/mL) + Vitamin D (1 µg/mL)] and the vitamins alone [Vitamin B12 (1 µg/mL), Vitamin C (10 µg/mL) and vitamin D (1 µg/mL)] on H9c2 cell viability in both LPS- and not LPS-pretreatment. The detailed protocol is described in the Supplementary Materials section.

2.4.3. Ex Vivo Studies

Adult C57/BL6 male mice (3-month-old, weight 20–25 g) were housed in Plexiglas cages (2–4 animals per cage; 55 × 33 × 19 cm) and maintained under standard laboratory conditions (21 ± 2 °C; 55 ± 5% humidity) on a 14/10 h light/dark cycle, with ad libitum access to water and normal laboratory chow (RMH-B diet, Arie Blok animal feed, Woerden, the Netherlands). Housing conditions and experimentation procedures were strictly in agreement with the European Community ethical regulations (EU Directive no. 63/2010) on the care of animals for scientific research. According to the recognized principles of “Replacement, Refinement and Reduction in Animals in Research”, heart specimens were obtained as residual material from vehicle-treated animals randomized in our previous experiments, approved by the local ethical committee (‘G. d’Annunzio’ University, Chieti, Italy) and Italian Health Ministry (Project no. 885/2018-PR).

After collection, isolated heart specimens were maintained in a humidified incubator with 5% CO₂ at 37 °C for 4 h (incubation period) in a RPMI buffer with added bacterial LPS (10 µg/mL), as previously described [33,34]. During the incubation period, the tissues were treated with ABGE (1 µg/mL, 10 µg/mL, 100 µg/mL), the Formulation [ABGE (10 µg/mL) + Vitamin B12 (1 µg/mL) + Vitamin C (10 µg/mL) + Vitamin D (1 µg/mL)], and the vitamins alone [Vitamin B12 (1 µg/mL), Vitamin C (10 µg/mL) and vitamin D (1 µg/mL)].

Extraction of total RNA was performed from the heart specimens using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), in agreement with the manufacturer’s protocol. Contaminating DNA was removed using 2 units of RNase-free DNase 1 (DNA-free kit, Ambion, Austin, TX, USA). Determination of gene expression of COX-2, IL-6, NF-κB, TNF-α, and iNOS was performed by quantitative real-time PCR using TaqMan probe-based chemistry, as previously reported [7,35]. The detailed protocol is described in the Supplementary Materials Section.

2.4.4. Cell Line

HASMCs were cultured in Medium 231 (Life Technologies, Carlsbad, CA, USA) supplemented with a Smooth Muscle Growth Supplement (SMGS, Life Technologies, Carlsbad, CA, USA) and 1% of 100 units/mL penicillin and 100 mg/mL streptomycin (Sigma Aldrich, St. Louis, MO, USA) in tissue culture flasks at 37 °C in a humidified atmosphere and 5% CO₂, as previously described [36,37]. Cells were split 1:2 twice a week and used until passage 18.

2.4.5. Evaluation of H₂S Release on HASMCs

After 24 h, to allow cell attachment, the medium was replaced and cells were incubated for 30 min in the buffer standard (HEPES 20 mM, NaCl 120 mM, KCl 2 mM, CaCl₂·2H₂O 2 mM, MgCl₂·6H₂O 1 mM, Glucose 5 mM, pH 7.4, at room temperature), as previously described [36,37]. The detailed experimental procedure is reported in the Supplementary Materials Section.

2.4.6. Evaluation of the Membrane Hyperpolarizing Effects on HASMCs

After 24 h to allow cell attachment, the medium was replaced and cells were incubated for 1 h in the buffer standard containing the bisoxonol dye bis-(1,3-dibutylbarbituric acid) DiBac4(3) (Sigma Aldrich, St. Louis, MO, USA) 2.5 µM [38]. NS1619 (Sigma-Aldrich, St. Louis, MO, USA) 10 µM, a BK_{Ca} channel opener, was used as a reference drug. The ABGE (1–100 µg/mL), or the Formulation and the vitamins alone (Vitamin B12 1 µg/mL, Vitamin C 10 µg/mL and Vitamin D 1 µg/mL), were added to the cells, and the trends of fluorescence were followed for 35 min. The relative fluorescence decrease, linked to hyperpolarizing effects, was recorded every 2.5 min and was calculated as previously reported [38]. Six different experiments ($n = 6$) were performed.

2.5. Statistical Analysis

The data were analyzed by the licensed software GraphPad Prism version 6.0 (Graph-pad Software Inc., San Diego, CA, USA). Analysis of means $\pm$ SEM for each experimental group was performed by one-way analysis of variance (ANOVA), followed by either the Newman-Keuls multiple comparison post hoc test or by the Bonferroni post hoc test [39]. The level of significance was set to 0.05. The Tukey-Kramer's Honest Significant Difference (HSD) test was used to compare the mean polyphenol contents of the extracts.

3. Results and Discussion

3.1. Total Polyphenol Content of ABGE

The ABGE provided a yield equal to 21.91 mg GAE/g DM in phenolic components [extraction yields of polyphenolic compounds obtained in ABGE (mg GAE/g DM): means $\pm$ SEM, 21.91 $\pm$ 1.07]. In our experiments, the ABGE showed a yield comparable to those reported by Najman et al. [35] (2021). Water extracts from conventional and organic black garlic have shown a content in polyphenolic components between 13.64 and 17.24 mg GAE/g DM [40]. In particular, a higher content in polyphenols was shown in black compared to fresh garlic, which was suggested to be dependent on various factors, including the garlic aging process (time, temperature, and relative humidity) [40].

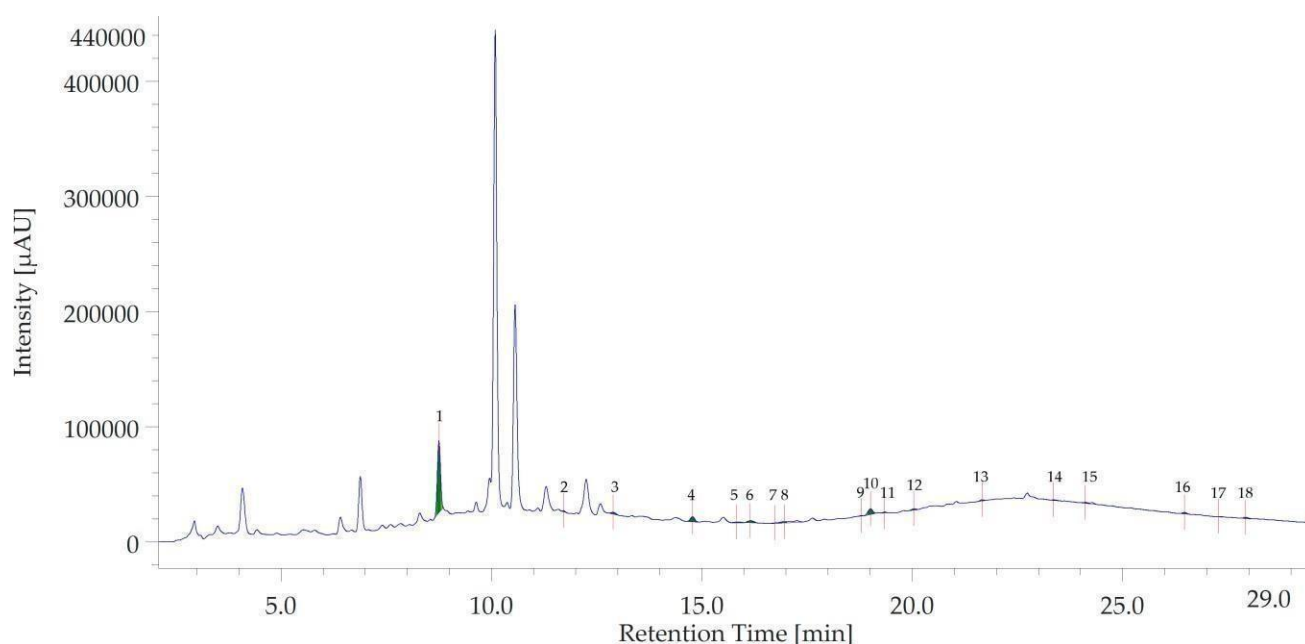
3.2. HPLC-DAD-MS Analysis

The retention times, m/z ratio, as well as quantity (µg/mL) of the investigated phenolic compounds in ABGE are reported in Table 1. In this context, a total of 12 compounds were identified at a wavelength of 254 nm. Gallic acid (#1) and catechin (#4) were the prominent phytochemicals, as shown in Figure 1.

Results are only in part comparable to those reported in the literature [41]. A study performed by Moreno-Ortega and collaborators [42] (2020) has found an increase in phenolic compounds, such as gallic acid and epigallocatechin gallate, in black compared to fresh garlic. In addition, it is well known that each cultivar expresses a different analytes content dependent on cultivation methods. Different studies have confirmed that the bioactive compounds of ABG possess a wide range of pharmacological activities, such as hypolipidemic, anticancer, and cardiovascular effects [43], which have been suggested to be mainly due to its anti-inflammatory and antioxidant properties.

Table 1. Retention times, m/z ratio, as well as quantity (g/mL) of the investigated phenolic compounds in ABGE.

Peak Name	tR	m/z (Positive Ion)	Quantity ($\mu\text{g/mL}$)
Gallic Acid	8.80	171.12	24.495
3-Hydroxytyrosol	11.71	155.16	1.713
Caftaric acid	12.93	313.23	1.773
Catechin	14.80	291.26	30.877
Gentisic acid	15.82	155.12	1.322
4-Hydroxybenzoic acid	16.20	193.12	n/a
Loganic acid	16.60	377.36	n/a
Chlorogenic acid	16.81	355.31	0.561
Vanillic acid	18.60	169.14	n/a
Caffeic acid	19.00	181.16	1.153
Epicatechin	19.41	291.26	1.338
Syringic acid	21.80	183.17	1.724
p-Coumaric acid	23.06	165.16	n/a
t-Ferulic acid	24.00	195.18	n/a
Benzoic acid	26.38	123.12	3.891
Rutin	27.16	611.52	n/a
Resveratrol	27.70	229.25	1.058

**Figure 1.** HPLC-DAD chromatogram of aged black garlic water extract (ABGE). The chromatographic analysis showed the presence of 12 phytochemicals: gallic acid (peak #1), 3-hydroxytyrosol (peak #2), caftaric acid (peak #3), catechin (peak #4), gentisic acid (peak #5), loganic acid (peak #7), chlorogenic acid (peak #8), caffeic acid (peak #10), epicatechin (peak #11), syringaldehyde (peak #13), benzoic acid (peak #16), and resveratrol (peak #18).

3.3. Toxicological and Pharmacological Studies

In the first series of experiments, we tested the effects of the ABGE (1–100 $\mu\text{g/mL}$) on the viability of cardiomyoblast (H9c2) cells. The experiments have been conducted both in basal conditions and after LPS-treatment for inducing an inflammatory status, *in vitro*. ABGE (1–100 $\mu\text{g/mL}$) did not alter H9c2 cell viability in basal conditions (Figure 2a). On the other hand, when H9c2 cells were treated with LPS, their viability was reduced, but ABGE (1–100 $\mu\text{g/mL}$) was able to revert the cytotoxicity (Figure 2b).

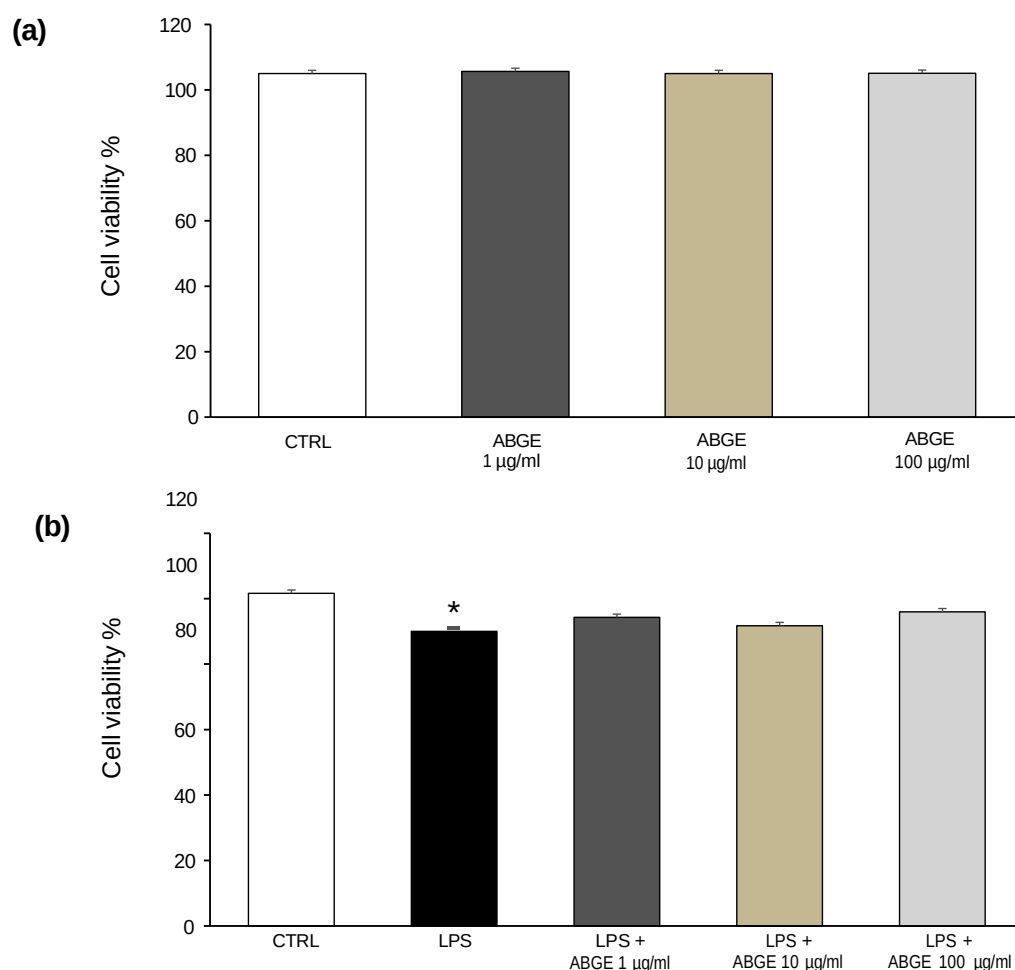


Figure 2. MTT assay of H9c2 cells exposed to aged black garlic water extract (ABGE) (1, 10, and 100 µg/mL) for 48 h, in basal (a) and after LPS pre-treatment (b) conditions. Data are reported as means $\pm$ SEM. ANOVA, Newman-Keuls multiple comparison post hoc test, * $p < 0.05$ vs. CTRL.

In particular, preclinical and clinical evidence has demonstrated that inflammation and oxidative stress play a crucial role in various CVD, including hypertension, fibrosis, diastolic dysfunction, left ventricular hypertrophy, heart failure, and ischemia/reperfusion damage [44].

Therefore, we investigated the protective effects induced by ABGE (1–100 µg/mL) in mouse heart specimens stimulated with LPS, which represents a validated model to study the modulatory activities of herbal extracts and drugs on inflammatory pathways and oxidative stress [33,34]. In particular, we evaluated the effects of ABGE (1–100 µg/mL) on pro-inflammatory and pro-oxidant mediators, such as COX-2, TNF- α , IL-6, NF- κ B, and iNOS mRNA levels on isolated LPS-stimulated heart specimens, by RT-PCR analysis. This demonstrates the involvement of NF- κ B in the transcription of various proinflammatory cytokines, such as TNF- α , and IL-6 [45], whose involvement in mediating cardiac dysfunction is well known [46].

In our ex vivo model, we observed that ABGE (10 and 100 µg/mL) significantly inhibited all markers investigated without showing a dose-dependent relationship (Figure 3a–e). In this context, polyphenol compounds have been suggested to induce cardioprotective effects by inhibiting oxidative stress and inflammation, as confirmed by a recently published study [7,47–51]. In particular, the beneficial activities induced by ABGE could be related to the pattern of polyphenolic composition, with particular regard to gallic acid and catechin. Accordingly, BenSaad and collaborators [52] (2017) reported that gallic acid inhibited LPS-induced prostaglandin E₂ and IL-6 production in RAW264.7 cells. Gallic acid was hypothesized to be able to exert a protective effect on rat liver mitochondria

by reducing oxidative stress induced by bisphenol A in ex vivo studies [53]. In addition, gallic acid pretreatment decreased levels of cardiac marker enzymes, including troponin T, which has been hypothesized to be involved in the myocardial damage reduction in rats [54]. Cardioprotective activities of catechins are also well known [55]. In particular, catechin administration attenuated coronary heart disease in a rat model by suppressing inflammation [56].

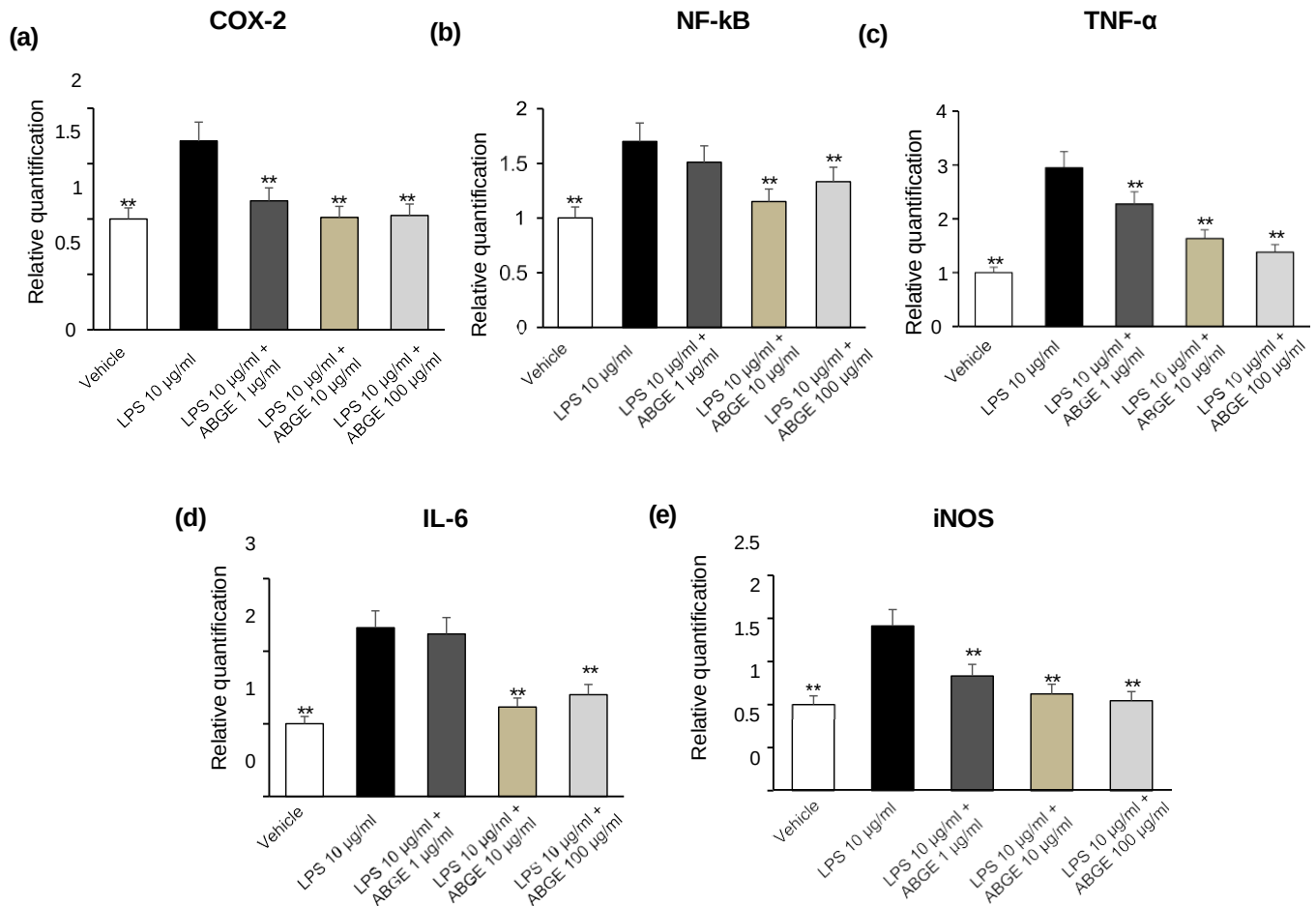


Figure 3. Effects of aged black garlic water extract (ABGE) (1, 10, and 100 µg/mL) on LPS-induced cyclooxygenase-2 (COX-2) (a), nuclear factor kB (NF-kB) (b), tumor necrosis factor α (TNF- α) (c), interleukin (IL)-6 (d), and inducible nitric oxide synthase (iNOS) (e) gene expression (RQ, relative quantification) in mouse heart specimens. Data are reported as means $\pm$ SEM. ANOVA, Newman-Keuls multiple comparison post hoc test. ** $p < 0.005$ vs. LPS.

Moreover, catechin, as well as being known for its antioxidant activities, has been described as an anti-inflammatory agent, being able to inhibit COX-2 expression [57,58].

Furthermore, black garlic was found to exert stronger antioxidant activity than fresh garlic, as confirmed by in vivo and in vitro experiments [41].

On the basis of these results, we performed a second series of experiments, aimed at evaluating the effects of the Formulation [ABGE (100 µg/mL) + Vitamin B12 (1 µg/mL) + Vitamin C (10 µg/mL) + Vitamin D (1 µg/mL)] on the viability of LPS-pretreated and not LPS-pretreated H9c2 cells. The results were compared with vitamins alone [Vitamin B12 (1 µg/mL), Vitamin C (10 µg/mL), and vitamin D (1 µg/mL)]. Our findings showed that the Formulation and the vitamins alone did not modify H9c2 cell viability in basal conditions (Figure 4a). In addition, the Formulation and the vitamins alone were able to contrast the cytotoxicity induced by LPS in H9c2 cells (Figure 4b).

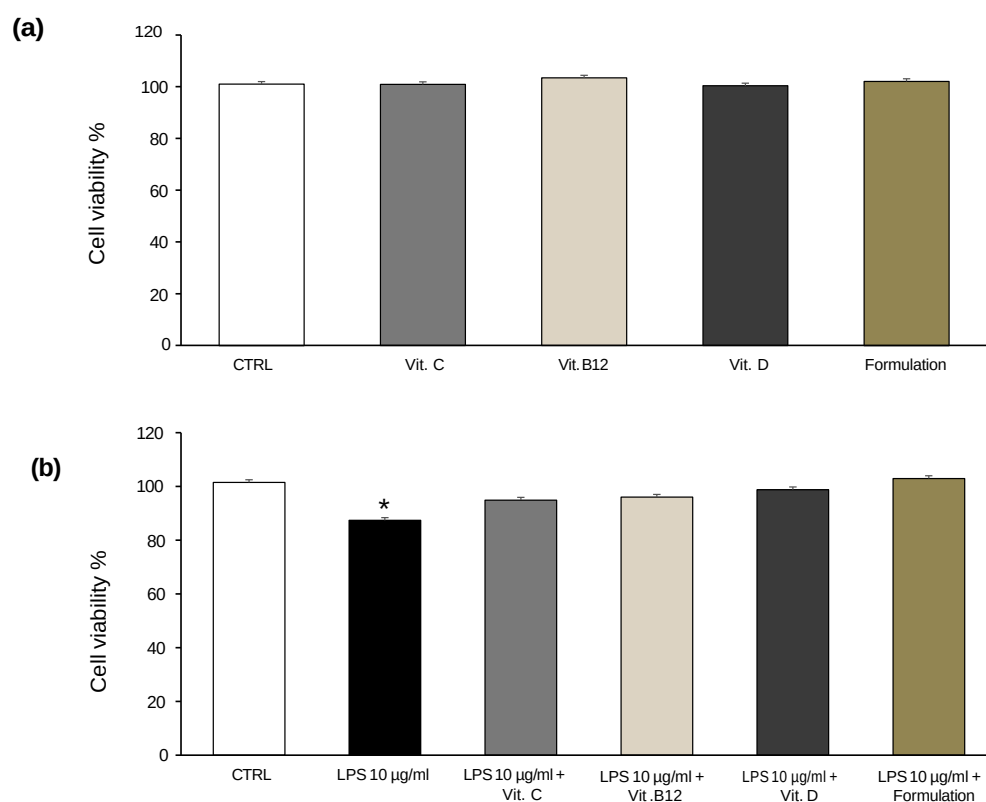


Figure 4. MTT assay of H9c2 cells exposed to Vitamin B12 (1 µg/mL), Vitamin C (10 µg/mL), vitamin D (1 µg/mL), and the Formulation [ABGE (100 µg/mL) + Vitamin B12 (1 µg/mL) + Vitamin C (10 µg/mL) + Vitamin D (1 µg/mL)] for 48 h in basal (a) and after LPS pre-treatment (b) conditions. Data are reported as means $\pm$ SEM. ANOVA, Newman-Keuls multiple comparison post hoc test
* $p < 0.05$ vs. CTRL group.

Thereafter, we investigated the effects induced by the Formulation and the vitamins alone on COX-2, TNF- α , NF- κ B, IL-6, and iNOS mRNA levels in mouse heart specimens treated with LPS.

As shown in Figure 5a–e, vitamins C and D as well the Formulation reduced gene expression of almost all markers tested in our ex vivo study. In particular, the Formulation was more effective than vitamins alone in blunting LPS-induced gene expression of IL-6, TNF- α , and NF- κ B. Recent studies reported that vitamin D represents one of the mediators playing a pivotal role in the pathogenesis of CVD [59]. In agreement, vitamin D supplementation was able to decrease inflammation and oxidative stress [59,60], confirming its pivotal role in heart tissue. In particular, TNF- α and IL-6 secretion was decreased by vitamin D in monocytes and macrophages [61]. Vitamin D also exerts various potent antioxidant effects by downregulating intracellular oxidative stress-related protein oxidation, lipid peroxidation, and DNA damage [62].

Similarly, an inverse correlation between vitamin C supplementation and the risk of CVD has been suggested in various observational studies [63,64]. In this regard, the antioxidant effects of vitamin C have been shown to be involved in both prevention and treatment of CVD [65]. In particular, Ellulu [62] (2017) showed that vitamin C protected against oxidative stress via its effect on nitric oxide release as well as alleviating inflammation by down-regulating IL-6, TNF- α , and NF- κ B mRNA levels [66]. As for vitamin B12, its deficiency can cause hyperhomocysteinemia, an independent risk factor for CVD [27]. Moreover, an association between vitamin B12 deficiency and increased incidence of inflammation and associated metabolic complications has been demonstrated by a number of studies [67,68]. Our present findings showed that vitamin B12 decreased LPS-induced gene expression of NF- κ B, IL-6, and iNOS. In agreement, Birch and collaborators [69]

(2009) showed that vitamin B12 decreased NF- κ B levels, which could represent a signaling molecule of vitamin B12 deficiency. Moreover, vitamin B12 was able to suppress IL-6 production, in vitro. Weinberg et al. [70] (2009) also reported that vitamin B12 is involved in the modulation of NOS function and NO synthesis in vivo.

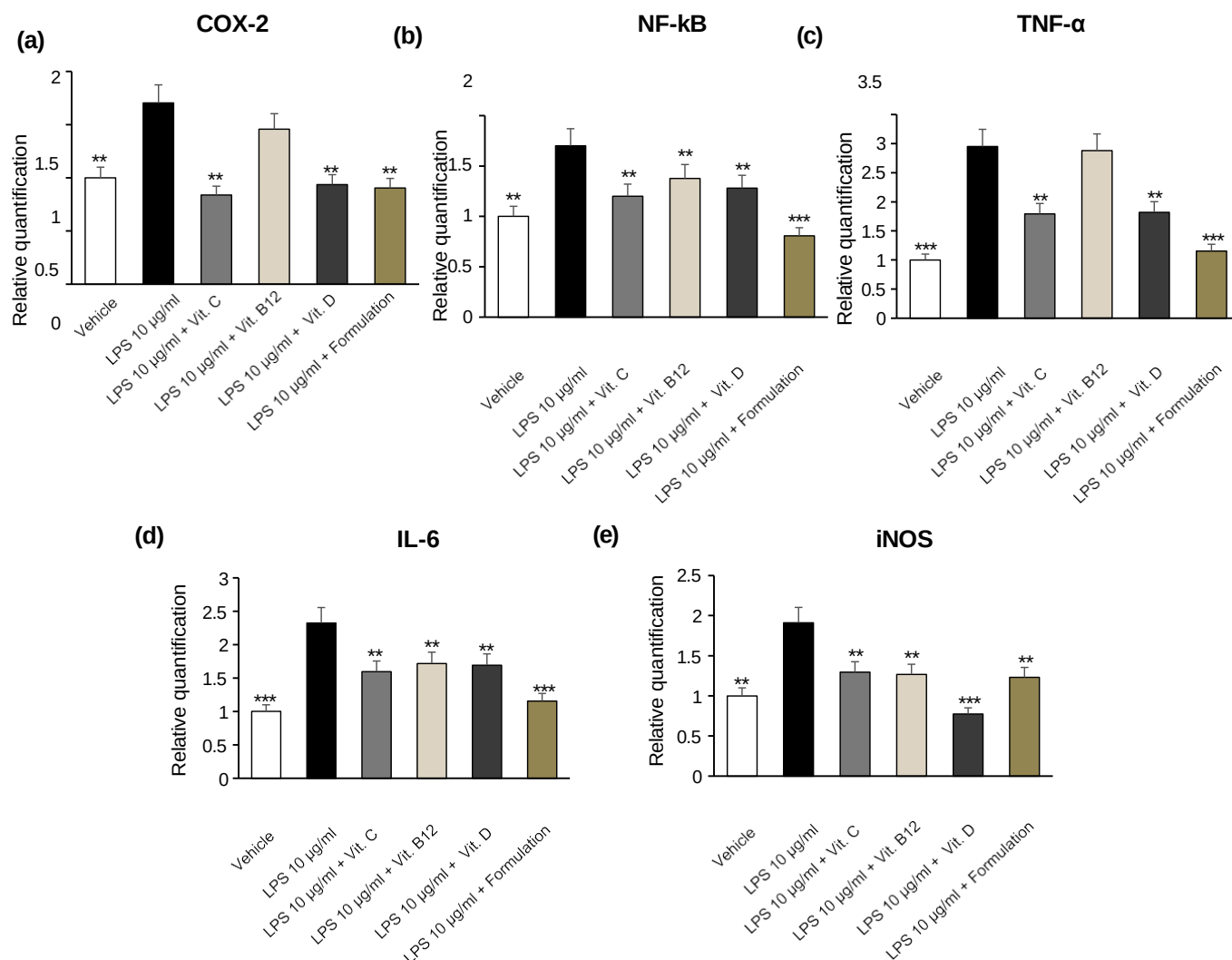
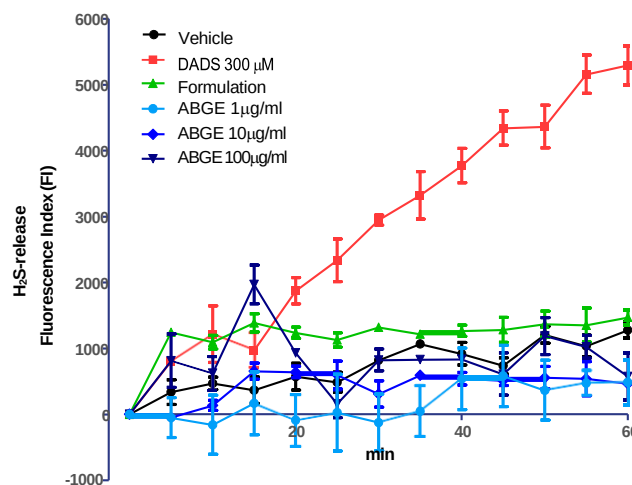


Figure 5. Effects of the Formulation [ABGE (100 µg/mL) + Vitamin B12 (1 µg/mL) + Vitamin C (10 µg/mL) + Vitamin D (1 µg/mL)] and the vitamins alone [Vitamin B12 (1 µg/mL), Vitamin C (10 µg/mL), Vitamin D (1 µg/mL)] on LPS-induced cyclooxygenase-2 (COX-2) (a), nuclear factor κ B (NF- κ B) (b), tumor necrosis factor- α (TNF- α) (c), interleukin (IL)-6 (d) and inducible nitric oxide synthase (iNOS) (e) gene expression (RQ, relative quantification) in mouse colon specimens. Data are reported as means $\pm$ SEM. ANOVA, Newman-Keuls multiple comparison post hoc test ** $p < 0.005$, *** $p < 0.001$ vs. LPS.

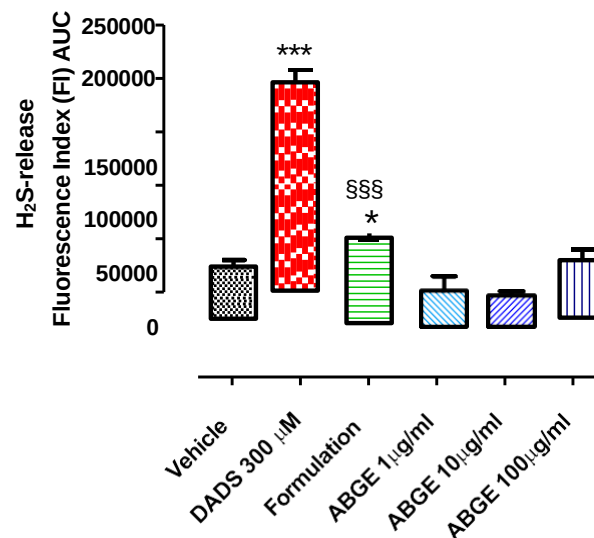
3.4. Evaluation of H₂S Release in HASMCs

H₂S has been suggested to be able to modulate many pathways related to cardiovascular pathophysiology [71]. In addition, it is one of the most important biological mediators involved in different pathological processes, where inflammation plays a predominant role, including CVD [72]. H₂S is known to be critically involved in garlic-induced cardioprotective effects [73–75]. In this context, H₂S was shown to play a key role in preventing the progression of cardiac hypertrophy to heart failure [76]. Considering the inhibitory effects induced by both ABGE (1–100 µg/mL) and the Formulation [ABGE (100 µg/mL) supplemented with Vitamin B12 (1 µg/mL) + Vitamin C (10 µg/mL) + Vitamin D (1 µg/mL)] on the investigated markers of inflammation and oxidative stress in our study, we also evaluated their potential effects on H₂S releasing properties using cultured HASMCs. DADS (300 µM) was used as a known H₂S releasing molecule and significantly increased the fluorescence index, thus indicating the intracellular H₂S formation (Figure 6a,b). We

showed that ABGE did not determine significant H₂S formation into the cells with respect to the vehicle (Figure 6a,b). Our findings are in agreement with those of Leitao et al. (2022) [77], showing that improvement of microvascular reactivity induced by aged garlic extract was not mediated by H₂S in older adults at CVD risk. Interestingly, the Formulation significantly increased fluorescence values compared to the vehicle, reflecting the H₂S formation inside the cells. In this context, we speculate that the presence of the vitamins into the Formulation allows the garlic extract to more easily cross the cell membrane and release H₂S. In this regard, B vitamins could act as cofactors of enzymes playing a key role in the sulfur network and modulate H₂S production [78]. Accordingly, Wilinski et al. [79] (2012) showed that vitamin D increased H₂S levels in a number of mouse organs, including the heart [79].



(a)



(b)

Figure 6. Fluorometric recording of H₂S-release inside HASMCs. (a) Time course of the fluorometric recordings of H₂S released by vehicle, ABGE (1–100 µg/mL), Formulation [ABGE (100 µg/mL) + Vitamin B12 (1 µg/mL) + Vitamin C (10 µg/mL) + Vitamin D (1 µg/mL)] and DADS 300 µM, during 60 min of observation: the increase in H₂S is expressed as FI. (b) The histograms show the total amount of H₂S released by vehicle, ABGE (1–100 µg/mL), Formulation [ABGE (100 µg/mL) + Vitamin B12 (1 µg/mL) + Vitamin C (10 µg/mL) + Vitamin D (1 µg/mL)] and DADS 300 µM in the 60 min of observation time, expressed as AUC. The vertical bars represent SEM, six different experiments were performed, each with six replicates (n = 6). ANOVA, Bonferroni post hoc test * $p < 0.05$, *** $p < 0.001$ vs. vehicle; §§§ $p < 0.001$ vs. ABGE.

An important finding of our study is that the Formulation tested has increased the release of H_2S , suggesting its potential role on CVD, including hypertension, thanks to its vasodilatation action [80]. In addition to its vasoprotective effects, H_2S could be critically involved in the pathogenesis of hypertension-related vascular dysfunction through its effects on blood pressure regulation, too, as well as inflammation [81–84].

3.5. Evaluation of Membrane Hyperpolarization of HASMCs

In addition, the effects of ABGE and the Formulation were evaluated on the membrane potential of cultured HASMCs. We showed that ABGE (1–100 $\mu\text{g/mL}$) did not modify membrane hyperpolarization. On the other hand, we showed that the Formulation caused a significant membrane hyperpolarization of HASMCs compared to ABGE (1–100 $\mu\text{g/mL}$) (Figure 7).

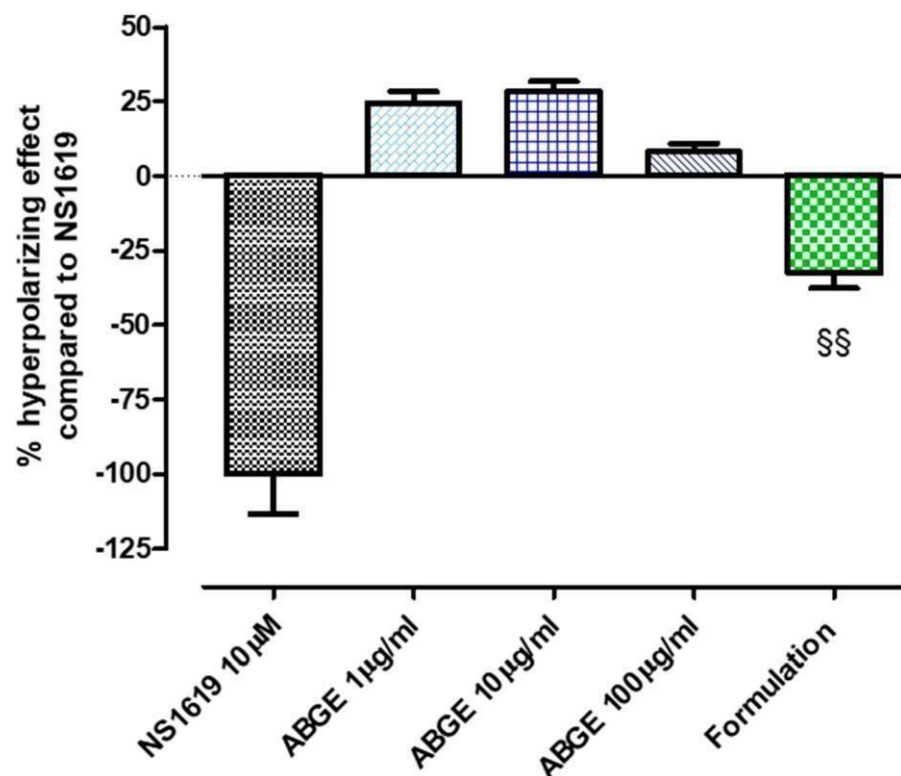


Figure 7. Hyperpolarizing effects in HASMCs. % hyperpolarization calculated as mean of changes in HASMCs membrane potential, followed for 35 min, induced by ABGE (1–100 $\mu\text{g/mL}$) or the Formulation [ABGE (100 $\mu\text{g/mL}$) + Vitamin B12 (1 $\mu\text{g/mL}$) + Vitamin C (10 $\mu\text{g/mL}$) + Vitamin D (1 $\mu\text{g/mL}$)]. Data are expressed as mean $\pm$ SEM, reported as % of the hyperpolarizing effect evoked by (1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazole-2-one (NS1619). Six different experiments were performed, each with six replicates ($n = 6$). The §§ indicates a significant difference from the effect evoked by ABGE (ANOVA, Bonferroni post hoc test §§ $p < 0.01$).

These results seem to suggest that the hyperpolarization is a consequence of the ability of the Formulation to release H_2S . Indeed, it is well known that compounds able to release H_2S , also called as H_2S -donors, exhibited the property to induce vascular smooth muscle hyperpolarization through the activation of different subtypes of potassium channels [36,85].

Experimental and clinical studies showed that ABG was able to exert beneficial effects on cardiometabolic alterations, which are usually related to metabolic syndrome [86,87]. Accordingly, Amor and collaborators [22] (2019) showed an improvement of metabolic syndrome following ABG treatment in rats [22]. In this context, the aging process was suggested to enhance the activity of bioactive compounds, including S-allylcysteine and

S-allylmercaptocysteine, whose cardioprotective effects are well known [88,89]. It is also well known that black garlic shows a reduced content of allicin when subjected to high temperatures during the production phase [87]. Moreover, Bradley and collaborators [73] (2016) suggested that allicin and alliin could not be the main bioactive compounds involved in the cardioprotective effects induced by aged garlic.

The content of phytochemicals in garlic has also been reported to be dependent on environmental, genetic, and agronomic factors [90].

Interestingly, gallic acid was found able to induce hyperpolarization of the cell membranes and excitation of muscles by binding to glutamate-gated chloride channels [91]. Furthermore, catechins have also been suggested to display inhibitory effects on voltage-dependent Ca^{2+} channels involving, albeit partially, membrane hyperpolarization deriving from the opening of K^{+} channels [92]. Finally, we hypothesized that the beneficial effects of the Formulation are due to the presence of gallic acid, catechin, and vitamins.

In conclusion, our results showed that ABGE, alone and in association with multi-vitamins consisting of combined Vitamins D, C, and B12, exhibited protective effects, as confirmed by the inhibitory activities on multiple inflammatory and oxidative stress-related pathways on mouse heart specimens exposed to LPS. These effects could be related, at least in part, to the ABGE content in polyphenolic compounds, with particular regards to gallic acid and catechin. Moreover, the Formulation increased intracellular H_2S formation, and caused a significant membrane hyperpolarization of HASMCs, further suggesting its potential use on CVD. In this context, we speculate that the presence of the vitamins in the Formulation allows the garlic extract to more easily cross the cell membrane and release H_2S . However, further studies using independent experimental paradigms are necessary to accurately evaluate the in vivo activity.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/foods12071558/s1>, Table S1. Gradient Elution Conditions; Table S2. MS analysis conditions.

Author Contributions: Conceptualization, L.R., L.B., V.C. (Valentina Citi), A.C., A.M. and S.L.; methodology, A.C., R.F., C.M., M.L.L., A.A., S.D.S. and S.V.; software, G.O., C.F. and L.M.; validation, G.O., C.F. and L.M.; formal Analysis, L.R., A.C., R.F., C.M., M.L.L., A.A., S.D.S., S.V. and A.P.; investigation, A.C., M.L.L., A.A., S.D.S., G.O., C.F., S.V. and A.P.; resources, L.R., A.C., L.B. and S.L.; data Curation, L.R., A.C., V.C., A.M., V.C. (Vincenzo Calderone) and S.L.; writing—original draft preparation, L.R., M.L.L., A.M., A.C., A.M. and S.L.; writing—review and editing, L.R., A.C., A.M., V.C. (Valentina Citi), L.B. and S.L.; visualization, V.C. (Vincenzo Calderone) and R.F.; supervision, L.B.; project administration, L.R., A.C., A.M., L.B. and S.L.; funding acquisition, L.R., L.B. and S.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Supplementary materials

Article I

Anti-inflammatory and vasorelaxant effects induced by an aqueous aged black garlic extract supplemented with vitamins D, C and B12 on cardiovascular system.

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2. Materials and Methods

2.1 Preparation of ABGE

ABG cloves were supplied as dried material by il Grappolo S.r.l. (Soliera, Modena, Italy). 10 g of garlic cloves were crushed with a utility garlic crusher by hand, and the juice and debris of the garlic were collected in a centrifuge tube by pouring 10 mL of water onto the crusher.

After having been shaken 10 times, the tube was placed in a Trans-sonic T460 ultrasonic bath supplied by Elma (Singen, Germany) for 15 min at room temperature and then centrifuged twice at 4000 rpm for 10 min each time [1-2]. The supernatant was filtered, until chemical analyses were performed.

2.2 Total polyphenol content of ABGE

Total polyphenol content was determined according to the Folin-Ciocalteu method, as described in Savini et al. [3] (2017) with some modifications. Briefly, in a 50 ml volumetric flask, 1 ml of extract was added to 0.5 ml of Folin-Ciocalteu reagent 2 N (Sigma-Aldrich, St. Louis, MO), 2 ml of sodium carbonate (Sigma-Aldrich, St. Louis, MO) 15% w/v solution, and made up to volume with water. The reaction mixture was allowed to stand for 2 hours before the absorbance at 765 nm was measured against a blank of reagents and solvents. A set of working

solutions (50, 100, 300, 500, and 700 ppm) of gallic acid (Sigma-Aldrich, Milan, Italy) was used for the calibration curve. The total amount of the phenolic compounds was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight.

2.3 HPLC-DAD-MS analysis of phenolic compounds

The extract was analyzed for phenol quantitative determination using a reversed-phase HPLC-DAD-MS in gradient elution mode [4]. The separation was conducted within 60 min of the chromatographic run, starting from the following separation conditions: 97 % water with 0.1 % formic acid, 3 % methanol with 0.1 % formic acid. The separation was performed on an Infinity lab Poroshell 120-SB reverse phase column (C18, 150 × 4.6 mm i.d., 2.7 µm; Agilent, Santa Clara, CA, USA). Column temperature was set at 30 °C. Quantitative determination of phenolic compounds was performed via a DAD detector. Quantification was done through 7-point calibration curves, with linearity coefficients (R^2) > 0.999, in the concentration range 2–140 µg/mL. All standards were purchased from Sigma Aldrich (Milan, Italy), and have a purity ≥ 95 %. The limits of detection were lower than 1 µg/mL for all assayed analytes. The area under the curve from HPLC chromatograms was used to quantify the analyte concentrations in the extract. The extracts were also qualitatively analyzed using an expression compact mass spectrometer (Advion, Ithaca, NY, USA) in negative and positive ion mode (m/z scan mode: 100–1200) [4]. MS signal identification was realized through comparison with a standard solution and MS spectra present in the MassBankEurope database. The details of the analysis are reported in supplementary materials (Tables S1-S2).

Table S1. Gradient Elution Conditions

TIME (Min.)	COMPOSITION A% (Water+Formic acid 0.1%)	COMPOSITION B% (Methanol+Formic acid 0.1%)	FLOW (mL/min)
1.00	97.0	3.0	0.600
5.00	77.0	23.0	0.600
12.00	73.0	27.0	0.600
18.00	57.0	43.0	0.600
25.00	52.0	48.0	0.600
32.00	50.0	50.0	0.600
34.00	50.0	50.0	0.600
37.00	35.0	65.0	0.600
40.00	5.0	95.0	0.600
47.00	5.0	95.0	0.600
48.00	97.0	3.0	0.600
60.00	97.0	3.0	0.600

Table S2. MS analysis conditions

Ion Source Parameters (APCI Positive):

Capillary Temperature = 250 °C

Capillary Voltage = 140 V
Source Voltage Offset = 30 V
Source Voltage Span = 10 V
APCI Source:
Source Gas Temperature = 250 °C
Transfer Line Temperature = 100 °C
APCI Corona Discharge = 3 µA
Tune Parameters Voltages:
Extraction Electrode = 9
Hexapole Bias = 8
Hexapole RF Offset = 5
Hexapole RF Span = 150
Ion Energy Offset = -1.2
Ion Energy Span = -0.4
Resolution Offset = -0.02
Resolution Span = -1.7
Detector gain = 1200

2.4 Toxicological and pharmacological studies

2.4.1. Cell line

H9c2 cells (rat cardiomyoblasts, ATTC, USA) were maintained in DMEM (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS), 1% of 100 unit/ml penicillin and 100 mg/mL streptomycin (Sigma-Aldrich, USA) in T75 red cap tissue culture flasks, at 37 °C in a humidified atmosphere of 5% CO₂.

2.4.2. Cell viability assay

Cell viability was evaluated by MTT assay [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (Sigma, St. Louis, MO, USA) as previously described [5]. Briefly, H9c2 cell line was seeded in 96-well plates (5x10³ cells/well) and it was pretreated with 10 µg/ml LPS for 24 hours. Subsequently, both LPS-pretreated and not LPS-pretreated H9c2 cells were exposed to ABGE at various concentrations (1-100 µg/ml), or with vehicle (control) for a further 48 hours. Then, on the basis of results, we performed a second set of experiments to evaluate the effects induced by the Formulation [ABGE (100 µg/ml) + Vitamin B12 (1 µg/ml) + Vitamin C (10 µg/ml) + Vitamin D (1 µg/ml)] and the vitamins alone [Vitamin B12 (1 µg/ml), Vitamin C (10 µg/ml) and vitamin D (1 µg/ml)] on H9c2 cell viability in both LPS- and not LPS-pretreatment.

After treatment, the MTT solution was added to each well and incubated at 37 °C for at least 3 hours, until purple formazan crystals were formed. In order to dissolve the precipitate, the culture medium was replaced with dimethyl sulfoxide (DMSO, Euroclone). Absorbance of each well was quantified at 540 and 690 nm, using a Synergy H1 microplate reader (BioTek Instruments Inc., Winooski, VT, USA) [5].

2.4.3. *Ex vivo* studies

Adult C57/BL6 male mice (3-month-old, weight 20–25 g) were housed in Plexiglas cages (2–4 animals per cage; 55 cm x 33 cm x 19 cm) and maintained under standard laboratory conditions (21 ± 2 °C; 55 ± 5% humidity) on a 14/10 h light/dark cycle, with ad libitum access to water and food. Housing conditions and experimentation procedures were strictly in agreement with the European Community ethical regulations (EU Directive no. 26/2014) on the care of animals for scientific research. In agreement with the recognized principles of “Replacement, Refinement and Reduction in Animals in Research”, heart specimens were obtained as residual material from vehicle-treated mice randomized in our previous experiments, approved by local ethical committee

('G. d'Annunzio' University, Chieti, Italy) and Italian Health Ministry (Project no. 885/2018-PR).

After collection, isolated heart specimens were maintained in a humidified incubator with 5% CO₂ at 37 °C for 4 h (incubation period), in RPMI buffer with added bacterial LPS (10 µg/mL), as previously described [6-7]. During the incubation period, the tissues were challenged with scalar concentrations of ABGE (1µg/ml, 10 µg/ml, 100 µg/ml), the Formulation [ABGE (10 µg/ml) + Vitamin B12 (1 µg/ml) + Vitamin C (10 µg/ml) + Vitamin D (1 µg/ml)] and the vitamins alone [Vitamin B12 (1 µg/ml), Vitamin C (10 µg/ml) and vitamin D (1µg/ml)].

Total RNA was extracted from the heart specimens using TRI Reagent (Sigma–Aldrich, St. Louis, MO, USA), according to the manufacturer's protocol. Contaminating DNA was removed using 2 units of RNase-free DNase 1 (DNA-free kit, Ambion, Austin, TX, USA). The RNA concentration was quantified at 260 nm by spectrophotometer reading (BioPhotometer, Eppendorf, Hamburg, Germany) and its purity was assessed by the ratio at 260 and 280 nm readings. The quality of the extracted RNA samples was also determined by electrophoresis through agarose gels and staining with ethidium bromide, under UV light. One microgram of total RNA extracted from each sample in a 20 µL reaction volume was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc., Monza, Italy). Reactions were incubated in a 2720 Thermal Cycler (Thermo Fisher Scientific Inc., Monza, Italy) initially at 25 °C for 10 min, then at 37 °C for 120 min, and finally at 85 °C for 5 s. Gene expression of COX-2, IL-6, NF-kB, TNF-α and iNOS was determined by quantitative real-time PCR using TaqMan probe-based chemistry, as previously described [8]. PCR primers and TaqMan probes, including β-actin used as the housekeeping gene, were purchased from Thermo Fisher Scientific Inc. (Assays-on-Demand Gene Expression Products, Mm00478374_m1 for COX-2 gene, Mm00443258_m1 for TNF-α gene, Mm00446190_m1 for IL-6 gene, Mm00476361_m1 for NF-kB gene, Mm00440502_m1 for iNOS gene, Mm00607939_s1 for β-actin gene). The real-time PCR was carried out in triplicate for each cDNA sample in relation to each of the investigated genes. Data were elaborated with the Sequence Detection System (SDS) software version 2.3 (Thermo Fisher Scientific Inc.). Gene expression was relatively quantified by the comparative 2^{-ΔΔCt} method [9].

2.4.4. Cell line

HASMCs were cultured in Medium 231 (Life technologies) supplemented with Smooth Muscle Growth Supplement (SMGS, Life Technologies) and 1% of 100 units/ml penicillin and 100 mg/ml streptomycin (Sigma Aldrich) in tissue culture flasks at 37 °C in a humidified atmosphere and 5% CO₂. HASMCs were cultured up to about 90% confluence and 24 h before the experiment cells were seeded onto a 96-well black plate, clear bottom pre-coated with gelatin 1% (from porcine skin, Sigma Aldrich), at density of 72 × 10³ per well for the evaluation of the membrane hyperpolarizing effects, at density of 30 × 10³ per well for the evaluation of H₂S release. Cells were split 1:2 twice a week and used until passage 18.

2.4.5. Evaluation of H₂S release on HASMCs

After 24 h to allow cell attachment, the medium was replaced and cells were incubated for 30 min in the buffer standard (HEPES 20 mM, NaCl 120 mM, KCl 2 mM, CaCl₂·2H₂O 2 mM, MgCl₂·6H₂O 1 mM, Glucose 5 mM, pH 7.4, at room temperature) containing the fluorescent dye WSP-1 (Washington State Probe-1, 3'-methoxy-3-oxo-3H-spiro(isobenzofuran-1,9'-xanthen)-6'-yl2(pyridin-2-yl)disulfanyl) benzoate, Cayman Chemical) at the concentration of 100 µM [10].

Then the supernatant was removed and replaced with a solution of the ABGE (1 µg/ml, 10 µg/ml, 100 µg/ml), or the Formulation 10 µg/ml, or DADS 300 µM (used as reference drugs as well-known H₂S-donor), all dissolved in buffer standard containing dimethylsulfoxide (DMSO) 0,1% (vehicle). When WSP-1 reacts with H₂S, it releases a fluorophore detectable with a spectrofluorometer at excitation and emission wavelengths of 465-515 nm [11]. The increasing of fluorescence (expressed as fluorescence index=FI) was monitored for 60 min, using a spectrofluorometer (EnSpire, Perkin Elmer). The obtained curves were analyzed to obtain the area under the curve (AUC).

2.4.6. Evaluation of the membrane hyperpolarizing effects on HASMCs

After 24 h to allow cell attachment, the medium was replaced and cells were incubated for 1 h in the buffer standard (HEPES 20 mM, NaCl 120 mM, KCl 2 mM, CaCl₂·2H₂O 2 mM, MgCl₂·6H₂O 1 mM, Glucose 5 mM, pH 7.4, at room temperature) containing the bisoxonol dye bis-(1,3-dibutylbarbituric acid) DiBac4(3) (Sigma Aldrich) 2.5 µM [12]. This membrane potential-sensitive dye DiBac4(3) measures the cell membrane potential, shuffling between cellular and extracellular fluids in a membrane potential-dependent manner (following the Nernst laws). In particular, an increase of fluorescence, corresponding to an inward flow of the dye, reflects a membrane depolarization; in contrast, a decrease in fluorescence, due to an outward flow of the dye, is linked to membrane hyperpolarization. The spectrofluorometric recording is carried out at excitation and emission wavelengths of 488 and 520 nm, respectively (Multiwells reader, Enspire, PerkinElmer). NS1619 (Sigma-Aldrich) 10 µM, a BK_{Ca} channel opener, was used as reference drug. The ABGE (1-100 µg/ml), or the Formulation and the vitamins alone (Vitamin B12 1 µg/ml, Vitamin C 10 µg/ml and Vitamin D 1 µg/ml) were added to the cells, and the trends of fluorescence was followed for 35 min. The relative fluorescence decrease, linked to hyperpolarizing effects, was recorded every 2.5 min and was calculated as (F_t-F₀)/F₀ where F₀ is the basal fluorescence before the addition of the tested compounds, and F_t is the fluorescence at time t after their administration. Moreover, the mean of the hyperpolarizing effect was calculated. Data are expressed as a % of the hyperpolarizing effects induced by NS1619 10 µM. Six different experiments (n = 6) were performed.

2.5 Statistical analysis

The software GraphPad Prism version 6.0 (Graphpad Software Inc., San Diego, CA, USA) was used to perform analysis of the data. Means ± SEM were determined for each experimental group and analyzed by one-way analysis of variance (ANOVA), followed by Newman-Keuls multiple comparison post hoc test and by Bonferroni post hoc test. The limit of statistically significant differences between mean values was set at p-value < 0.05. The number of animals randomized for each experimental group was calculated on the basis of the "Resource Equation" $N = (E + T)/T$ ($10 \leq E \leq 20$) [13]. The Tukey-Kramer's Honest Significant Difference (HSD) test was used to compare the mean polyphenol contents of the extracts. The level of significance was set to 0.05.

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Article II

RESEARCH ARTICLE

WILEY

Anti-inflammatory and anti-hyperalgesic effects induced by an aqueous aged black garlic extract in rodent models of ulcerative colitis and colitis-associated visceral pain

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Abstract

Inflammatory bowel disease (IBD) is a morbid condition characterized by relapsing-remitting inflammation of the colon, accompanied by persistent gut dysmotility and abdominal pain. Different reports demonstrated biological activities of aged black garlic (ABG), including anti-inflammatory and antioxidant effects. We aimed to investigate beneficial effects exerted by ABGE on colon inflammation by using ex vivo and in vivo experimental models. We investigated the anti-inflammatory effects of an ABG water extract (ABGE) on rat colon specimens exposed to *E. coli* lipopolysaccharide (LPS), a known ex vivo experimental model of ulcerative colitis. We determined gene expression of various biomarkers involved in inflammation, including interleukin (IL)-1 β , IL-6, nuclear factor-kB (NF-kB), tumor necrosis factor (TNF)- α . Moreover, we studied the acute effects of ABGE on visceral pain associated with colitis induced by 2,4-dinitrobenzene sulfonic acid (DNBS) injection in rats. ABGE suppressed LPS-induced gene expression of IL-1 β , IL-6, NF-kB, and TNF- α . In addition, the acute administration of ABGE (0.03–1 g kg⁻¹) dose-dependently relieved post-inflammatory visceral pain, with the higher dose (1 g kg⁻¹) able to significantly reduce both the behavioral nociceptive response and the entity of abdominal contraction (assessed by electromyography) in response to colorectal distension after the acute administration in DNBS-treated rats. Present findings showed that ABGE could represent a potential strategy for treatment of colitis-associated inflammatory process and visceral pain. The beneficial effects induced by the extract could be related to the pattern of polyphenolic composition, with particular regard to gallic acid and catechin.

KEYWORDS

aged black garlic, colon, inflammation, oxidative stress, pain

1 | INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic and multifactorial inflammatory condition of the gastrointestinal tract, characterized by unbalanced and increased immune response to external stimuli (Achitei et al., 2013). As a consequence of this condition, enteric mucosa produces various pro-inflammatory biomarkers, such as cytokines and prostaglandins, as well as reactive oxygen species (ROS), which reinforce the inflammatory status, thus contributing to tissue functional impairment (Kang et al., 2011a; Kruidenier et al., 2003). An increasing number of studies reported that up to 60% of patients with diagnosis of IBD frequently show abdominal pain. Abdominal pain perception and pathogenesis involve several mechanisms. In patients affected by IBD, chronic abdominal pain is sustained by a sensitization of primary neurons induced by inflammation, immune response derangement, and dysbiosis in the gut microbiota, which involves the maintenance of a low-grade inflammation within the gut (Bakshi et al., 2021; Coates et al., 2021; Colombel et al., 2019; Lucarini et al., 2022; Srinath et al., 2012). Together these mechanisms contribute to the multifactorial nature of post-inflammatory visceral pain, combining inflammatory as well as neuropathic aspects, that make it difficult to treat (Takahashi et al., 2021).

Current therapeutic strategies for IBD display several disadvantages such as numerous side effects, ineffectiveness in counteracting pain establishment and persistence, as well as weak responsiveness (Choi et al., 2017; Zeitz et al., 2016). In this context, the identification of new pharmacological and non-pharmacological approaches is necessary. Noteworthy, herbal extracts have long been reported to contrast IBD-related oxidative stress and inflammatory response (Chung et al., 2007; Lenoir et al., 2012; Recinella et al., 2022). Garlic was shown to possess protective effects against ulcerative colitis (Harisa et al., 2009). Accordingly, we recently showed that a hydroalcoholic and a water extract of garlic (*Allium sativum* L.) exert beneficial effects in colon, suggesting their possible role in preventing and managing ulcerative colitis (Recinella et al., 2022).

Aged black garlic (ABG) is obtained by processing fresh garlic under controlled high temperature (70–90°C) and humidity (70%–90%) involving intrinsic chemical reactions with spontaneous fermentation. ABG has been suggested to exert multiple biological properties, such as antioxidant, anti-inflammatory, anticancer, cardiovascular protection, hepatoprotective, neuroprotective, anti-obesity, anti-allergy, immunomodulation, and hepatoprotection (Javed & Ahmed, 2022; Jeong et al., 2016; Mathew & Biju, 2008; Najman et al., 2021; Shin et al., 2014; Vinayagam et al., 2023). Interestingly, bioactive compounds, including polyphenols, organosulfur, and S-allyl cysteine, are critically involved in beneficial effects induced by ABG (Kodera et al., 2002; Sasmaz et al., 2022). After processing, ABG content in phenolic compounds is 5–8 folds higher compared to fresh garlic, resulting in higher antioxidant activity than fresh garlic (Kim et al., 2013). Moreover, a wide body of evidence suggested protective effects of polyphenols against various oxidative stress- and inflammation-related diseases, including cardiovascular diseases, IBD and cancer (Biasi et al., 2011; Das & Das, 2010; Kang, Kugathasan, et al., 2011a; Kang, Shin, et al., 2011b; Singh et al., 2008). Accordingly, our previous study showed that an ABG water extract (ABGE) exhibited anti-inflammatory and antioxidant properties on mouse heart specimens

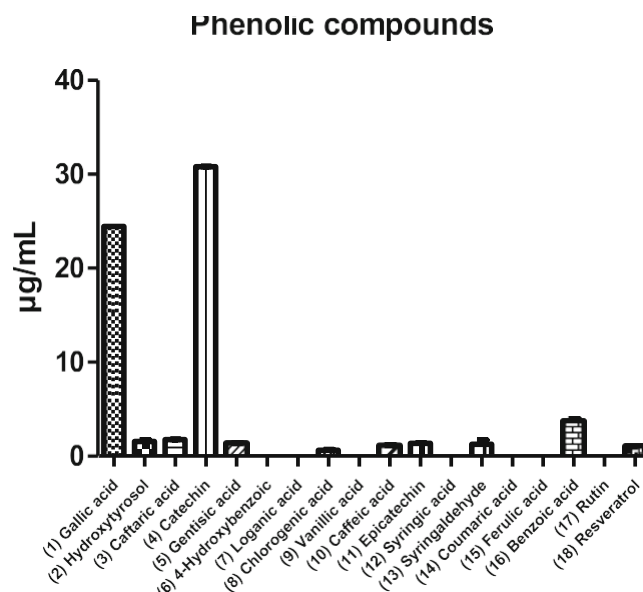


FIGURE 1 Identified phenolic compounds in aged black garlic water extract (ABGE), by high-performance liquid chromatography coupled with a photo diode array detector (HPLC-DAD) analytical method. Gallic acid (#1) and catechin (#4) were the prominent phytochemicals (Recinella et al., 2023).

exposed to LPS, which have been suggested to be related, at least in part, to the ABGE content in polyphenolic compounds, with particular regards to gallic acid and catechin. In this context, the polyphenolic content in the extract was previously quantified using high-performance liquid chromatography coupled with a photo diode array detector (HPLC-DAD) analytical method. In particular, a total of 12 compounds were identified at a wavelength of 254 nm, with gallic acid (#1) and catechin (#4) being the prominent phytochemicals (Figure 1) (Recinella et al., 2023).

Based on these findings, we aimed to investigate potential protective effects exerted by ABGE on colon damage caused by inflammation by using both ex vivo and in vivo experimental models. We evaluated the potential anti-inflammatory effects induced by ABGE, on a validated ex vivo experimental model of ulcerative colitis constituted by rat colon specimens exposed to *Escherichia coli* lipopolysaccharide (LPS) (Recinella et al., 2020; Recinella et al., 2022). In this context, we assessed gene expression of various biomarkers involved in inflammation, including interleukin (IL)-1 β , IL-6, nuclear factor-kB (NF-kB), and tumor necrosis factor (TNF)- α . In addition, we used the experimental model of colitis induced by the intrarectal injection of 2,4-dinitrobenzenesulfonic acid (DNBS) (Lucarini et al., 2020), to evaluate the potential beneficial effects of ABGE on colitis-associated persistent visceral pain in rats.

2 | MATERIALS AND METHODS

2.1 | Preparation of ABGE

ABG cloves were supplied as dried material by il Grappolo S.r.l. (Soliera, Modena, Italy). 10 g of garlic cloves were crushed with a

utility garlic crusher by hand, and the juice and debris of the garlic were collected in a centrifuge tube by pouring 10 ml of water onto the crusher. After having been shaken 10 times, the tube was placed in a Trans-sonic T460 ultrasonic bath supplied by Elma (Singen, Germany) for 15 min at room temperature and then centrifuged twice at 4000 rpm for 10 min each time (Fujisawa, Suma, Origuchi, Kumagai, et al., 2008a; Fujisawa, Suma, Origuchi, Seki, & Ariga, 2008b). The supernatant was filtered, until chemical analyses were performed.

3 | TOXICOLOGICAL AND PHARMACOLOGICAL STUDIES

3.1 | Cell lines and treatments

Colorectal cancer cell line SW-480 (ATCC, Manassas, VA) were cultured in RPMI1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO), 1% Pen/Strep (EuroClone, Milan, Italy) and 1% L-glutamine (EuroClone, Milan, Italy). Human fibroblast HFF-1 cell line (ATCC, Manassas, VA) was cultured in DMEM high glucose (4.5 g/L; Sigma, St. Louis, MO), supplemented with 15% FBS, 1% Pen/Strep and 1% L-glutamine. Both cell lines were maintained in a humidified incubator at 37°C, 5% CO₂.

3.2 | Cell viability assay

Cell viability was evaluated by MTT assay [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (Sigma, St. Louis, MO), as previously described (Veschi et al., 2018). Briefly, SW-480 and HFF-1 cell lines were seeded in 96-well plates (5 × 10³ cells/well) and were treated the following day with ABGE at various concentrations (1–1000 µg/ml), or with vehicle (control). After 72 h of treatment, the MTT solution was added to each well and incubated at 37°C for at least 3 h, until purple formazan crystals were formed. In order to dissolve the precipitate, the culture medium was replaced with dimethyl sulfoxide (DMSO, Euroclone, Milan, Italy). Absorbance of each well was quantified at 540 and 690 nm, using a Synergy H1 microplate reader (BioTek Instruments Inc., Winooski, VT). IC50 values were calculated using the CompuSyn software.

3.3 | Animals

Adult Sprague-Dawley male rats (200–250 g, Envigo, Varese, Italy) were housed in Plexiglass cages (40 cm × 25 cm × 15 cm), in colony rooms (22 ± 1°C; 60% humidity), on a 12 h/12 h light/dark cycle (light phase: 07:00–19:00 h), with free access to tap water and food, 24 h/day throughout the study, with no fasting periods. Rats were fed with a standard laboratory diet.

Colon specimens were obtained from rats treated with the vehicle from our previous experiments, approved by Local Ethical Committee (University “G. d’Annunzio” of Chieti-Pescara) and Italian Health Ministry (Italian Health Ministry Authorization N. 880, delivered on

24 August 2015). All animal manipulations were performed according to the Directive 2010/63/EU of the European parliament and of the European Union council (September 22, 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the described experiments was obtained from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines (McGrath & Lilley, 2015). All efforts were made to minimize animal suffering and to reduce the number of animals used.

3.3.1 | Ex vivo studies

Rats were sacrificed by CO₂ inhalation (100% CO₂ at a flow rate of 20% of the chamber volume per min), and colon specimens were immediately collected and maintained in a humidified incubator with 5% CO₂ at 37°C for 4 h, in RPMI buffer (Sigma, St. Louis, MO) with added bacterial LPS (Sigma-Aldrich, St. Louis, MO) (10 µg/ml) (incubation period) (Recinella et al., 2019; Recinella et al., 2020). During the incubation period, colon specimens were treated with scalar concentrations of ABGE (1 µg/ml, 50 µg/ml, 500 µg/ml).

After collection, total RNA was extracted from the colon specimens using TRI Reagent (Sigma-Aldrich, St. Louis, MO), according to the manufacturer's protocol. Contaminating DNA was removed using 2 units of RNase-free DNase 1 (DNA-free kit, Ambion, Austin, TX). The RNA concentration was quantified at 260 nm by spectrophotometer reading (BioPhotometer, Eppendorf, Hamburg, Germany) and its purity was assessed by the ratio at 260 and 280 nm readings. The quality of the extracted RNA samples was also determined by electrophoresis through agarose gels and staining with ethidium bromide, under UV light. One microgram of total RNA extracted from each sample in a 20 µl reaction volume was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc., Monza, Italy). Reactions were incubated in a 2720 Thermal Cycler (Thermo Fisher Scientific Inc., Monza, Italy) initially at 25°C for 10 min, then at 37°C for 120 min, and finally at 85°C for 5 s. Gene expression of IL-1β, IL-6, NF-κB, and TNF-α was determined by quantitative real-time PCR using TaqMan probe-based chemistry, as previously described (Recinella et al., 2021). PCR primers and TaqMan probes were purchased from Thermo Fisher Scientific Inc. (Assays-on-Demand Gene Expression Products, Rn00580432_m1 for IL-1β gene, Rn01410330_m1 for IL-6 gene, Rn01399572_m1 for NF-κB gene, Rn99999017_m1 for TNF-α gene). β-actin (Thermo Fisher Scientific Inc., Monza, Italy, Part No. 4352340E) was used as the housekeeping gene. The real-time PCR was carried out in triplicate for each cDNA sample in relation to each of the investigated genes. Data were elaborated with the Sequence Detection System (SDS) software version 2.3 (Thermo Fisher Scientific Inc., Monza, Italy). Gene expression was relatively quantified by the comparative 2^{−ΔΔCt} method (Livak & Schmittgen, 2001).

3.4 | In vivo studies

3.4.1 | Induction of colitis and drug administration

Colitis has been induced in rats in conformity with the method described by Lucarini et al. (2020). During a brief period of anesthesia with isoflurane (2%; VIRBAC S.r.l., Milan, Italy), inhaled into animals by means of a Compact Gas Anesthesia System (UGO Basile, Varese, Italy), 30 mg of 2,4-dinitrobenzenesulfonic acid (DNBS; Sigma-Aldrich, Milan, Italy) dissolved in 0.25 ml of 50% ethanol was intrarectally injected using a polyethylene PE-60 catheter inserted 8 cm proximal to the anus. Further, 0.25 ml of saline solution (0.9% NaCl; Sigma-Aldrich, Milan, Italy) was injected in control rats.

On day 14 after DNBS injection, subgroups of animals affected by colitis-associated visceral pain were orally administered with ABGE (0.03–1 g kg⁻¹; 10 ml kg⁻¹) or vehicle (10 ml kg⁻¹). Control group was administered with the vehicle (10 ml kg⁻¹ per os). Visceral sensitivity was assessed 30 and 60 min after the acute administration of the extract at different doses or the vehicle. The dose dependence of the acute pain-relieving effect was studied.

3.4.2 | Assessment of visceral sensitivity by abdominal withdrawal response (AWR)

Visceral sensitivity to colorectal distension (CRD) was assessed via Abdominal Withdrawal Reflex (AWR) measurement using a semi-quantitative score as described previously in conscious animals (Lucarini et al., 2020). Briefly, rats were anesthetized with isoflurane (2%; VIRBAC S.r.l., Milan, Italy), by means of a Compact Gas Anesthesia System (UGO Basile, Varese, Italy), and a lubricated latex balloon (length: 4.5 cm), attached to polyethylene tubing, assembled to an embolectomy catheter and connected to a syringe filled with water were inserted through the anus into the rectum and descending colon of adult rats. The tubing was taped to the tail to hold the balloon in place. Then rats were allowed to recover from the anaesthesia for 15 min. AWR measurement consisted of visual observation of animal responses to graded CRD (0.5, 1, 2, and 3 ml) by a blinded observers who assigned a scores: No behavioral response to colorectal distension (0); Immobile during colorectal distension and occasional head clinching at stimulus onset (1); Mild contraction of the abdominal muscles but absence of abdomen lifting from the platform (2); Observed strong contraction of the abdominal muscles and lifting of the abdomen off the platform (3); Arching of the body and lifting of the pelvic structures and scrotum (4).

3.4.3 | Assessment of visceral sensitivity by Viscero-motor response

The objective measure of visceral sensitivity in animals involved assessing the visceromotor response (VMR) to CRD. Two electromyographic electrodes were surgically implanted into the external oblique abdominal muscle under deep anesthesia (isoflurane 2%; VIRBAC S.r.

l., Milan, Italy), inhaled into animals by means of a Compact Gas Anesthesia System (UGO Basile, Varese, Italy) and externally positioned dorsally. The evaluation of visceromotor response took place under light anesthesia (isoflurane 1.5%). A lubricated latex balloon (length:

4.5 cm), attached to an embolectomy catheter and connected to a syringe filled with water, was employed for colorectal distension. The balloon was introduced into the colon, positioned 6.5 cm from the anus, and filled with incremental volumes of water (0.5, 1, 2, and 3 ml). The electromyographic electrodes were connected to a data acquisition system, and the corresponding signal generated by colorectal stimulation was recorded, amplified, and filtered (Animal Bio Amp; ADInstruments, Oxford, United Kingdom), digitized (PowerLab 4/35; ADInstruments), analyzed, and quantified using LabChart 8 (ADInstruments). To quantify the VMR magnitude at each distension volume, the area under the curve 30 s before the distension was subtracted from the area under the curve during the balloon distension (30 s), with responses expressed as a percentage increase from the baseline. The time interval between two consecutive distensions was set at 5 min.

3.5 | Statistical analysis

Animals were randomly assigned to the experimental groups. Behavioral measurements were performed on six animals for each treatment, performed in two experimental sets. The experimental procedures were performed by a researcher blinded to the treatment. Results from *ex vivo* and *in vivo* studies were expressed as means $\pm$ SEM. The analysis of variance (ANOVA) was performed by one-way ANOVA with Bonferroni's significant difference procedure used for post-hoc comparisons. *p* values of <0.05 were considered significant. Data were analyzed using the "Origin 9" software (OriginLab, Northampton, MA).

The number of animals randomized for each experimental group was calculated on the basis of the "Resource Equation" $N = (E + T)/T$ ($10 \leq E \leq 20$) (Charan and Kantharia, 2013).

4 | RESULTS AND DISCUSSION

4.1 | Toxicological and pharmacological studies

In the first set of experiments, we tested the effect of the ABGE (1–1000 μ g/ml) on viability of normal HFF-1 fibroblast cells. Notably, ABGE exhibited no toxicity against normal HFF-1 cells, as shown in the dose response curve (Figure 2). These findings are consistent with a recent study of ours, showing that the same extract did not affect viability of cardiomyoblast (H9c2) cells in basal conditions (Recinella et al., 2023). Moreover, ABGE was tested on SW-480 cells for evaluating the effects on cell viability. In the present study, the aged black garlic extract (50–1000 μ g/ml) was effective to exert a significant reduction in colon cancer SW-480 cell viability, in basal conditions (Figure 3). In this context, an aged garlic extract was previously shown

FIGURE 2 Effect of aged black garlic water extract (ABGE) on viability of normal HFF-1 fibroblast cells. Cell viability was assessed by MTT assay after incubation for 72 h of HFF-1 cells with ABGE (1–1000 µg/ml), or with vehicle (control). Data shown are the means ± SEM of two independent experiments with quintuplicate determinations.

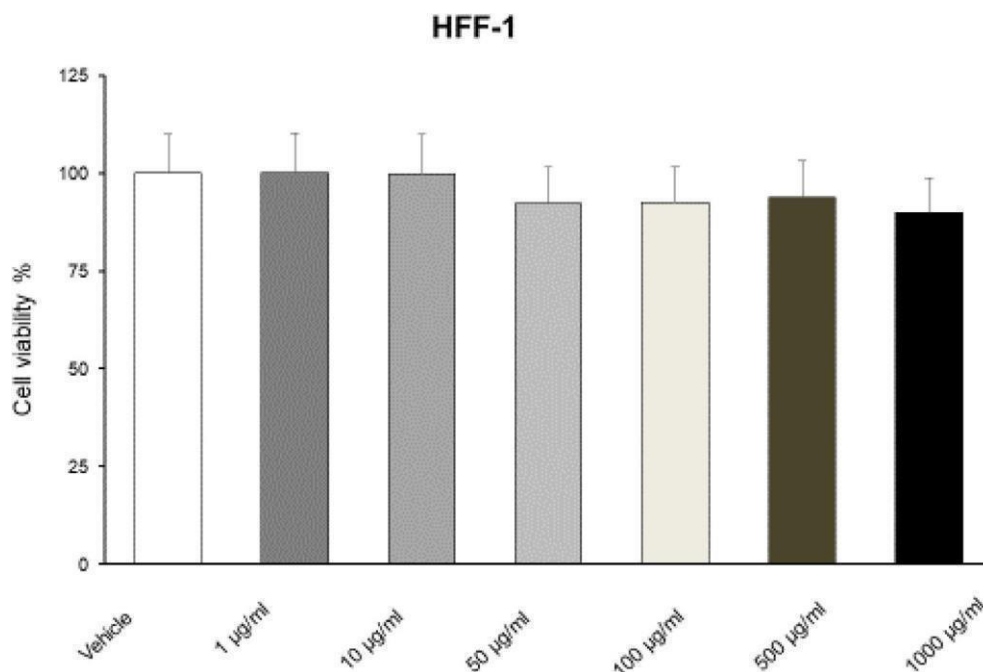
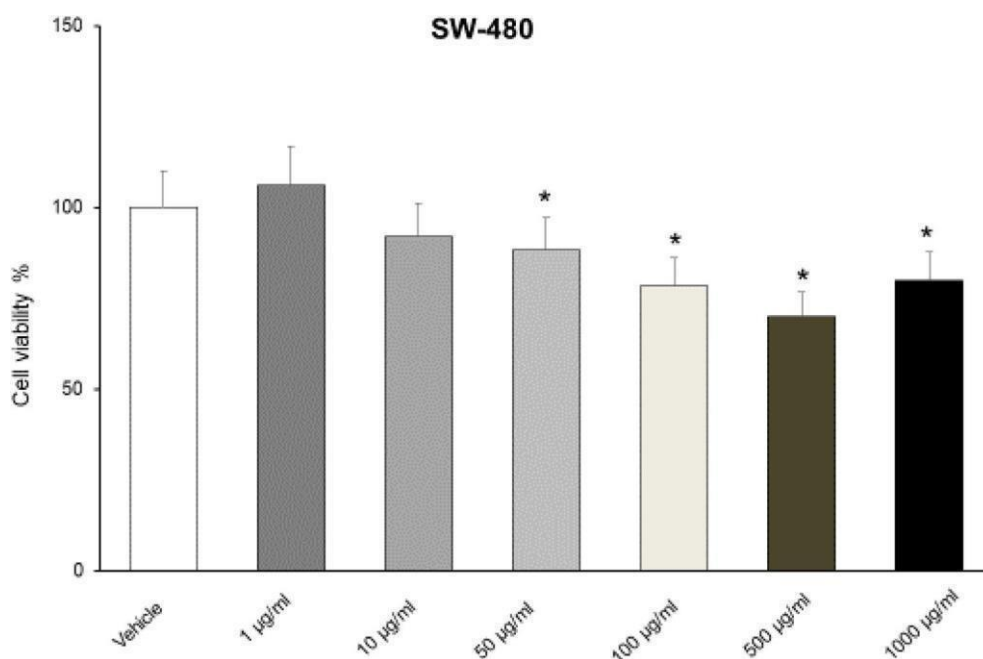


FIGURE 3 Effect of aged black garlic water extract (ABGE) on viability of colorectal cancer cell line SW-480. Cell viability was assessed by MTT assay after incubation for 72 h of SW-480 cell line with ABGE (1–1000 µg/ml), or with vehicle (control). Data shown are the means ± SEM of two independent experiments with quintuplicate determinations. ANOVA, * $p < 0.05$ vs. vehicle.



to exert antiproliferative effects on colorectal carcinoma cells HT-29, SW-480 and SW-620, suggesting its potential role in chemoprevention of colorectal cancer (Matsuura et al., 2006). Previous studies also demonstrated the capability of catechins to induce apoptosis in SW-480 cells (Cordero-Herrera et al., 2013; Kim et al., 2012). Similarly, gallic acid was found able to inhibit proliferation and induce apoptosis of colon cancer HCT-116 and HT-29 cells (Lin et al., 2021). In particular, the beneficial activities induced by ABGE could be related to the pattern of polyphenolic composition, with particular regard to gallic acid and catechin.

A wide body of evidence suggested the critical role played by inflammation in ulcerative colitis (Kang et al., 2011b; Kruidenier et al., 2003).

In this context, we also evaluated the potential beneficial activities induced by ABGE (1–500 µg/ml) in rat colon specimens stimulated with LPS, which represents a validated model of ulcerative colitis (Recinella et al., 2020; Recinella et al., 2022). We aimed to investigate the effects of ABGE on pro-inflammatory mediators, such

as TNF- α , IL-1 β , IL-6, and NF- κ B gene expression on isolated LPS-stimulated colon specimens, by RT-PCR analysis. In our ex-vivo model,

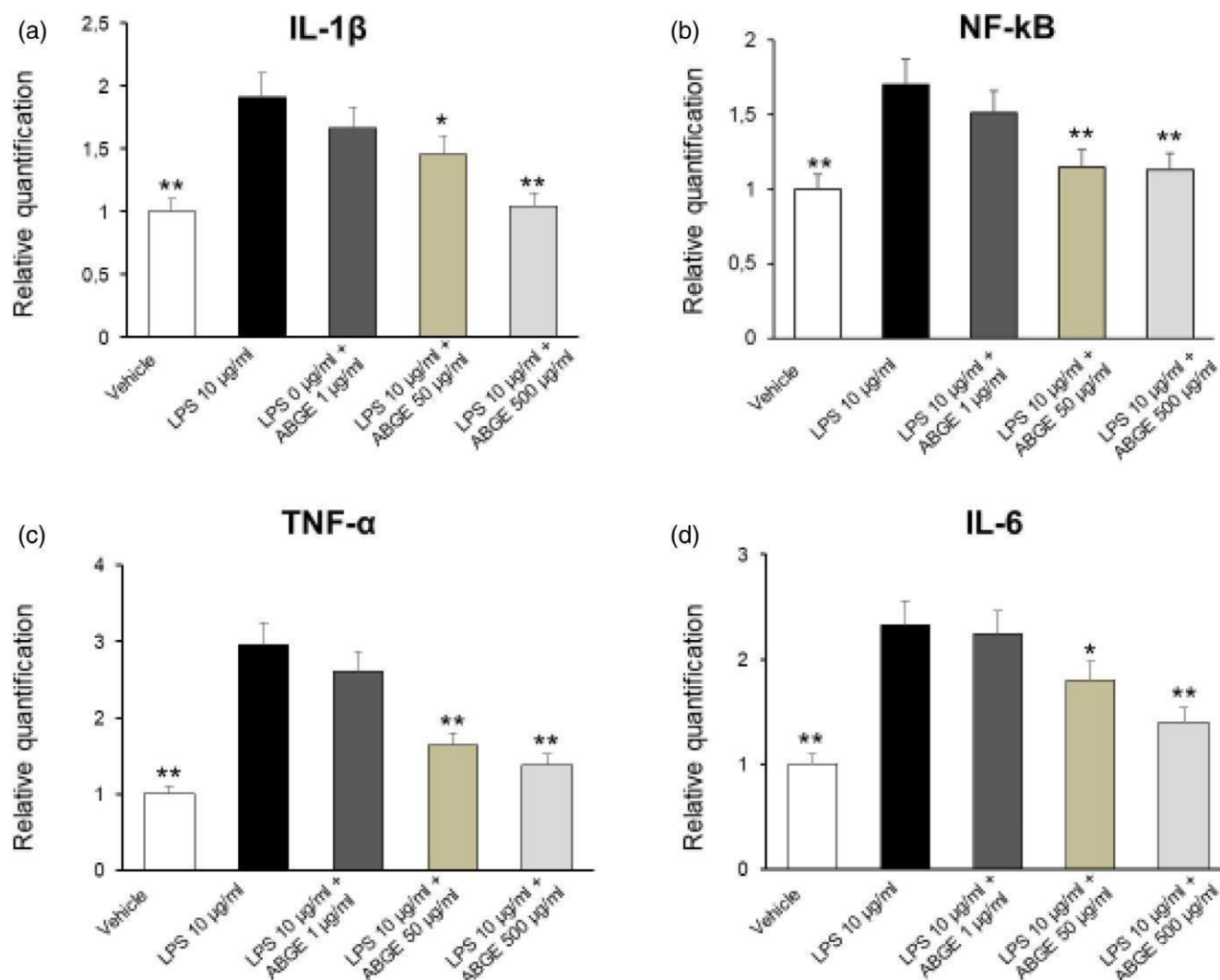


FIGURE 4 Effects of aged black garlic water extract (ABGE) (1, 50, and 500 μ g/ml) on LPS-induced interleukin (IL)-1 β (a), nuclear factor κ B (NF- κ B) (b), tumor necrosis factor- α (TNF- α) (c) and IL-6 (d) gene expression (RQ, relative quantification), in rat colon specimens ($n = 6$ for each experimental group). Data shown are the means $\pm$ SEM of two independent experiments with triplicate determinations. ANOVA, * $p < 0.05$ and ** $p < 0.005$ vs. LPS.

we showed that ABGE (50 and 500 μ g/ml) was able to reduce gene expression of all markers investigated without showing a dose-dependent relationship (Figure 4a-d).

IBD has been hypothesized to be related to the activation of NF- κ B pathways, resulting in increased expression of pro-inflammatory markers, leading to abdominal pain, diarrhea, bleeding, along with various extra-intestinal manifestations (Laurindo et al., 2023). In this context, various phytochemicals, including catechins, have been shown to modulate various inflammation targets, such as TNF- α , COX-2, IL-1 β , and IL-6 (Iqbal et al., 2022; Qazi et al., 2022). In particular, catechins were found able to revert histological lesions and decrease expression of cytokines, such as TNF- α and IL-6, as well as antioxidant markers, including SOD, GSH-Px, and CAT, and MDA in a mouse model of DSS-induced colitis (Zhang et al., 2016). Furthermore, our present findings are in agreement with a previous study of ours showing that a water garlic extract exerted anti-inflammatory and antioxidant

effects on isolated mouse colon specimens treated ex vivo with LPS, which have been suggested to be related, at least in part, to its polyphenolic composition, with particular regards to catechin (Recinella et al., 2022). Interestingly, a wide body of evidence found that gallic acid was effective in inhibiting inflammatory process in animal models of experimental colitis (Li et al., 2019; Pandurangan et al., 2015; Shree et al., 2020; Zhu et al., 2019). In particular, Shree and collaborators (2019) suggested that gallic acid could revert 1,2-dimethylhydrazine-induced ulcerative colitis through inhibition of NF- κ B signaling pathway. More recently, Yu et al. (2023) reported that gallic acid inhibited NLRP3 inflammasome in DSS-induced ulcerative colitis, in mice, further suggesting potential anti-inflammatory activity of herbal preparations containing gallic acid in ulcerative colitis. Accordingly, BenSaad et al. (2017) reported that gallic acid suppressed LPS-induced prostaglandin E₂ and IL-6 production in RAW264.7 cells. Taken together, our present findings suggest that the beneficial effects induced by the extract could be related to the pattern of

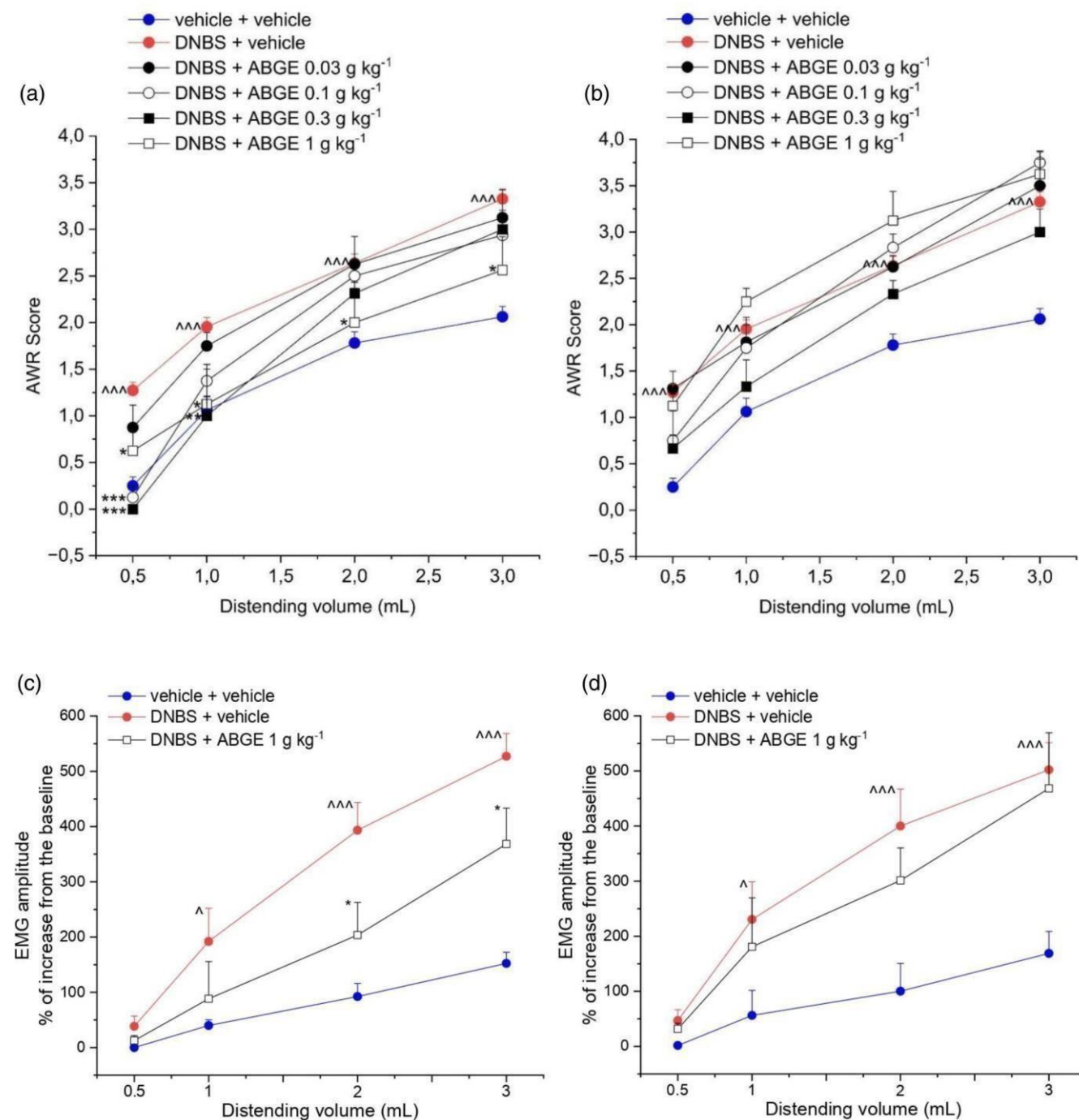


FIGURE 5 Effect of the acute administration of aged black garlic water extract (ABGE) on visceral pain induced by 2,4-Dinitrobenzenesulfonic acid (DNBS) in rats. Visceral pain was assessed by evaluating the Abdominal Withdrawal Reflex (AWR) score (0–4; a and b) and the Viscero-Motor Response (VMR) (c and d) of animals in response to Colo-Rectal Distension (CRD) (0.5–3 mL), 30 min (a and c) and 60 min (b and d) after the oral administration of the ABGE (0.03–1 g kg⁻¹). Behavioral tests were performed 14 days after DNBS injection. Each value is the mean ± SEM of six rats per each group, performed in two experimental sets. ANOVA, ^^^*p* < 0.001 vs. vehicle + vehicle treated animals. **p* < 0.05 and ****p* < 0.001 vs. DNBS + vehicle treated animals.

polyphenolic composition, with particular regard to gallic acid and catechin. However, various phytoconstituents, including phenolics, S-allyl cysteine (SAC), and hydroxycinnamic acid derivatives were also found in BG, with respect to raw garlic (Javed & Ahmed, 2022; Vinayagam et al., 2023).

On the basis of these results, we performed a second set of experiments, with the aim to investigate the effects of ABGE (0.03–1 g kg⁻¹ p.o.) on visceral pain induced by colitis after DNBS injection.

4.2 | Anti-hyperalgesic efficacy of ABGE against colitis-associated visceral pain

The effect of the acute administration of ABGE on visceral pain induced by colitis was evaluated 14 days after DNBS injection (Figure 5), when colon hypersensitivity persists despite tissue healing (Lucarini et al., 2020). Visceral pain was monitored in the animals by assigning a score to their abdominal withdrawal response (AWR; 0–4) to colorectal distension (CRD; 0.5–3 ml). 14 days after colitis induction, the AWR elicited by the distension with 0.5–3 ml was significantly higher in DNBS-treated animals compared to the controls (vehicle + vehicle treated group; Figure 5a,b; $p < 0.001$ for each volume tested). The acute administration of black garlic aqueous extract (ABGE 0.03–1 g kg^{−1} p.o.) dose-dependently reduced visceral hypersensitivity induced by DNBS in rats. This effect peaked 30 min after administration (Figure 5a) and began to subside after 60 min (Figure 5b). In particular, the dose of 1 g kg^{−1} significantly reduced animals' AWR response to CRD back to the value of controls (vehicle + vehicle treated group; $p < 0.05$ for 0.5–3 ml; Figure 5a). ABGE (0.1–0.3 g kg^{−1}) was partially effective, significantly reducing the AWR only at the stimuli 0.5 ml ($p < 0.05$) and 1 ml ($p < 0.05$), while the lowest dose (0.03 g kg^{−1}) was ineffective (Figure 5a). The fully effective dose (1 g kg^{−1}) was also tested for the evaluation of EMG abdominal muscle response (VMR) in response to CRD. The entity of VMR resulted greater in animals treated with DNBS compared to healthy controls after the application of the same protocol of CRD (Figure 5c,d). 30 min after the injection, the VMR was overall lower in DNBS-treated animals administered with ABGE 1 g kg^{−1} in comparison to that of DNBS-treated animals receiving vehicle, though the effect resulted statistically significant at higher distension volumes (2 and 3 ml; $p < 0.05$; Figure 5c). The effect on VMR was no longer present 60 min after administration (Figure 5d).

The response to rectal distention testing in patients with irritable bowel syndrome have been demonstrated to be a predictive value of pain sensory thresholds (Bouin et al., 2002). Similarly, AWR and VMR assessment to colorectal distension provide a quantitative measure of animal's intestinal sensitivity and a faithful predictor of therapy efficacy at clinical level (Camilleri et al., 2017; Lucarini et al., 2020). The higher dose of ABGE (1 g kg^{−1}) was able to significantly reduce both AWR and VMR entity in response to CRD in DNBS-treated animals, thus it is expected that it might effectively relieve persistent visceral hypersensitivity in patients remitting from IBD. The management of post-inflammatory pain still represents an unmet clinical issue because of the lack of effective and safe therapeutic approaches. Although different types of approaches are currently used to manage pain in patients with different gut disease, most of interventions displayed a limited effectiveness in IBD patients (Baillie et al., 2023; Camilleri & Boeckstaens, 2023; Swierczynski et al., 2023).

Several studies focused on the potential role of pro-inflammatory cytokines such as TNF- α , IL-6, IL-1 β , as well as NF- κ B on pain processing and associated these cytokines with chronic pain conditions (Murphy et al., 1999; Woolf et al., 1997; Xu et al., 1997). Besides their role in modulation of neuronal expression of neuropeptides (Klein

et al., 1997), there is evidence that certain cytokines, such as IL-6 and TNF- α , are critically involved in the neuroplastic response of sensory neurons to inflammatory insult, as well as in microglial and astrocytic activation (Ramer et al., 1998). In agreement, a direct relationship between baseline tissue inflammation and systemic TNF- α and IL-6, with worsen of pain over 5 years, has been reported in clinical studies (Hosnijeh et al., 2015; Stannus et al., 2013).

Antinociceptive effect of garlic and its organosulphur compounds in preclinical models and clinical assays to treat different diseases have been recently reviewed, but direct evidence of pain-relieving effects of garlic on colitis-associated pain were not proven (Hernández-Cruz et al., 2022). Antinociceptive properties exerted by garlic were consistently demonstrated in chronic inflammatory diseases in humans, including knee rheumatoid arthritis and osteoarthritis (Dehghani et al., 2018; Moosavian et al., 2020). Regarding the mechanisms of action, the antioxidant and anti-inflammatory properties, including the ability to block the synthesis pro-inflammatory cytokines, are likely involved in effects of garlic constituents on pain. In particular, the anti-hyperalgesic activities induced by ABGE might be related to its polyphenolic content, with particular regards to catechin and gallic acid. Indeed, polyphenols, beyond to the therapeutic benefits on IBD pathophysiology (Fan et al., 2017), play beneficial effects on pain, as confirmed by clinical and preclinical studies (Hernández-Cruz et al., 2022; Šir, 2022). A potential role of catechin in treatment and management of neuropathic pain has been recently suggested in rats, including an effective decrease of both hyperalgesia and allodynia. The same authors hypothesized that these effects could

be related to inhibition of NF- κ B activation and reduction of TNF- α , IL-6, and IL- β levels in the rat brain (Foudah et al., 2022). These results are in line with previous studies showing that catechin was able to suppress NF- κ B (Suhail et al., 2022), and the expression of NF- κ B-regulated pro-inflammatory cytokines (Noll et al., 2013; Vazquez Prieto et al., 2015). Anyway, gallic acid has been reported to exert anti-hyperalgesic effects (Sohrabi et al., 2021; Yang et al., 2021) in neuropathic pain conditions which are not responsive to anti-inflammatory drugs, suggesting further mechanisms of action. Indeed, recent evidence reported gallic acid efficacy against comorbid visceral pain and depression may be mediated by the negative modulation of P2X7 in the central nervous system (Wen et al., 2022), which has implication in both inflammation and pain (Hu et al., 2022).

Aside from its therapeutic uses in traditional medicine, garlic has a wide use as a spice in cooking, thus overall, garlic consumption is considered safe for humans, though some side effects have been reported after ingestion of raw garlic in high doses on an empty stomach by sensitive people (El-Saber Batiha et al., 2020). In the present work, any toxic effect was observed in rats treated with ABGE (0.03–1 g kg^{−1}), in a range of doses which might be translated in 0.3–1 g day in humans. The recommended daily dose for the therapeutic use of garlic is 2–4 g of crushed raw garlic or equivalent in alliin content (ESCOP MONOGRAPHS. *The Scientific Foundation for Herbal Medicinal Products*. ESCOP; Devon, PA: 2019. *Allii sativi bulbosus* Garlic). In this regard, it is also important to consider that the manufacturing conditions of black garlic and the procedure of extraction change the

chemical composition of raw garlic, which has a major impact on its biological properties and on its toxicological profile. For instance, during the preparation of aged garlic extracts, the odorous, harsh, and irritating compounds of garlic are naturally transformed into stable and safe sulfur compounds (Ribeiro et al., 2021). Accordingly, different toxicological studies have confirmed the safety of aged garlic (Ansary et al., 2020; Miraghajani et al., 2018). Nevertheless, further studies are needed to assess the effectiveness and the safety of a repeated treatment with ABGE and to elucidate its pharmacodynamic profile.

In conclusion, our present findings showed that ABGE could represent a potential adjuvant strategy to defuse colitis-associated inflammatory process, as well as to manage resulting persistent visceral pain. The anti-inflammatory and anti-hyperalgesic effects induced by ABGE are likely attributable to its content in polyphenolic compounds, with particular regards to gallic acid and catechin, though other constituents in the extract might support ABGE beneficial properties.

AUTHOR CONTRIBUTIONS

Maria Loretta Libero: Conceptualization; data curation; methodology; resources; writing - original draft; writing - review and editing. Elena Lucarini: Data curation; formal analysis; investigation; methodology; resources; visualization; writing - original draft. Lucia Recinella: Conceptualization; data curation; funding acquisition; methodology; project administration; resources; writing - original draft; writing - review and editing. Clara Ciampi: Formal analysis; investigation. Serena Veschi: Formal analysis; investigation. Anna Piro: Formal analysis; investigation. Annalisa Chiavaroli: Formal analysis; investigation. Alessandra Acquaviva: Formal analysis; investigation. Nilofar Nilofar: Formal analysis; investigation. Giustino Orlando: Formal analysis; investigation; software; validation. Daniele Generali: Formal analysis; investigation. Carla Ghelardini: Formal analysis; investigation. Lorenzo di Cesare Mannelli: Formal analysis; investigation; writing - original draft. Antonio J. Montero-Hidalgo: Formal analysis; investigation. Raúl M. Luque: Formal analysis; investigation. Claudio Ferrante: Formal analysis; investigation; software; validation. Luigi Menghini: Formal analysis; investigation; software; validation. Simona Cristina di Simone: Formal analysis; investigation. Luigi Brunetti: Methodology; project administration; resources; writing - original draft. Sheila Leone: Conceptualization; data curation; funding acquisition; methodology; project administration; resources; supervision; visualization; writing - original draft; writing - review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no financial/commercial conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Article III



The Protective Effects of an Aged Black Garlic Water Extract on the Prostate

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Abstract: Chronic inflammation is a recognized risk factor for various cancers, including prostate cancer (PCa). We aim to explore the potential protective effects of aged black garlic extract (ABGE) against inflammation-induced prostate damage and its impact on prostate cancer cell lines. We used an ex vivo model of inflammation induced by Escherichia coli lipopolysaccharide (LPS) on C57BL/6 male mouse prostate specimens to investigate the anti-inflammatory properties of ABGE. The gene expression levels of pro-inflammatory biomarkers (*COX-2*, *NF-κB*, and *TNF-α*, *IL-6*) were measured. Additionally, we evaluated ABGE’s therapeutic effects on the prostate cancer cell lines through in vitro functional assays, including colony formation, tumorsphere formation, migration assays, and phosphorylation arrays to assess the signaling pathways (MAPK, AKT, JAK/STAT, and TGF-β). ABGE demonstrated significant anti-inflammatory and antioxidant effects in preclinical models, partly attributed to its polyphenolic content, notably catechin and gallic acid. In the ex vivo model, ABGE reduced the gene expression levels of *COX-2*, *NF-κB*, *TNF-α*, and *IL-6*. The in vitro studies showed that ABGE inhibited cell proliferation, colony and tumorsphere formation, and cell migration in the prostate cancer cells, suggesting its potential as a therapeutic agent. ABGE exhibits promising anti-inflammatory and anti-cancer properties, supporting further investigation into ABGE as a potential agent for managing inflammation and prostate cancer.

Keywords: ABGE; prostate; inflammation; cancer

1. Introduction

Prostatitis is a prostate gland inflammation, which encompasses a range of disorders, such as acute and chronic bacterial prostatitis. These conditions can be caused by bacterial infections, immune responses, or non-infectious factors such as trauma or stress [1].

In particular, acute prostatic inflammation in mice induced an epithelial transformation, named proliferative inflammatory atrophy, which could promote prostatic intraepithelial neoplasia [1].

In this context, chronic inflammation is often linked with the process of carcinogenesis and is recognized as both a hallmark and a potential risk factor for various cancers [2]. Specifically, for prostate cancer (PCa), chronic inflammation is suggested as a bridge between environmental factors and tumor development [3–5].

Numerous studies have explored the relationship between prostate gland abnormalities and the inflammatory process, showing a strong prevalence of mild chronic inflammation in PCa [6].

Chronic inflammation can create a microenvironment conducive to carcinogenesis by producing pro-inflammatory cytokines, reactive oxygen species, and DNA damage [7].

In line with this, NLRP3 inflammasome is critically involved in PCa aggressiveness [8].

Altogether, the presented evidence indicates the proficient pro-oncogenic role of certain inflammatory processes in PCa [9,10].

Various biomarkers, such as tumor necrosis factor (TNF)- α , nuclear factor (NF)- κ B, interleukin (IL)-6, and cyclooxygenase (COX)-2, play a critical role in inflammatory responses. In particular, Baud and their collaborators (2001) reported that TNF- α is a potent pro-inflammatory cytokine whose involvement in inflammation, cell proliferation, differentiation, and apoptosis is well known. Increased serum levels of pro-inflammatory markers such as TNF- α are related to accelerated progression and a poor prognosis in PCa [11,12].

Furthermore, NF- κ B is essential for regulating both the innate and adaptive immune responses, particularly in inflammation. Besides its role in the survival and activation of immune cells, NF- κ B stimulates the release of pro-inflammatory genes, including cytokines and chemokines, and regulates inflammasome activity. Moreover, the dysregulation of NF- κ B contributes to various inflammatory diseases, including rheumatic diseases and asthma [13,14]. Interestingly, a wide body of evidence suggested that NF- κ B activation, as well as various signals linked to inflammation, are well known to be involved in the modulation of PCa malignancy [15]. In particular, NF- κ B activation exerts modulatory effects on the expression of the cytokines and factors involved in cancer development and progression, including IL-6 [15]. Moreover, the activation of IL-6 signaling was found to induce growth, proliferative activity, and the migration of PCa cells [16].

COX-2 is also critically involved in carcinogenesis in various tissues, including breasts and lungs, as well as the prostate [17].

Various studies suggested the potential activity of a number of herbal extracts commonly used in traditional medicine as well as natural compounds exhibiting an innovative action mode as a possible remedy for PCa [18,19].

In this context, aged black garlic (ABG) has garnered attention for its bioactive compound profile and biological activities [20].

ABG is produced by fermenting fresh garlic at controlled high humidity (80–90%) and temperature (60–90 °C) conditions over several weeks. As previously reported [20], the temperature and humidity conditions of the thermal treatment chosen during ABG production are strongly involved in the quality of ABG.

This process alters garlic's organoleptic properties, making it sweeter and less pungent, and increases the concentration of bioactive compounds, such as S-allylcysteine, polyphenols, and flavonoids [21]. These compounds were found to be able to exert various beneficial effects, including the suppression of cell proliferative activity, as well as the stimulation of apoptosis and the modulation of the cell cycle, all of which are relevant in cancer prevention and treatment [22,23].

Interestingly, in the previous studies of ours, a water extract of ABG (ABGE) showed anti-inflammatory and antioxidant effects in preclinical models [24,25]. In particular, the protective effects induced by ABGE were suggested to be partly related to the polyphenolic content in the same extract, notably catechin and gallic acid [24,25]. We previously performed the quantification of polyphenolic content in the extract using high-performance liquid chromatography coupled with a photo diode array detector (HPLC-DAD) analytical method. In particular, various compounds were identified in ABGE, with gallic acid and catechin being the more representative phytochemicals [26].

This research aims to explore the potential benefits of ABGE on inflammation and prostate cancer. Building on these findings, we sought to explore the potential protective effects of ABGE against inflammation-induced prostate damage using an ex vivo experimental model, as well as its impact on prostate cancer cell lines through in vitro studies. We investigated the anti-inflammatory properties of ABGE using a well-established ex vivo model of inflammation composed of mouse prostate specimens exposed to *Escherichia coli* lipopolysaccharide (LPS) [26,27]. In this setting, we examined the gene expression levels of the key pro-inflammatory biomarkers, including COX-2, NF- κ B, TNF- α , and IL-6. Furthermore, we assessed the potential therapeutic effects of ABGE on the prostate cancer cell lines using in vitro experimental models through functional parameters (colony formation, tumorsphere formation, and a migration assay) and molecular studies to evaluate the potential involvement of different signaling pathways, such as mitogen-activated protein kinase (MAPK), protein kinase B (AKT), Janus kinases/the signal transducer and activator of transcription proteins (JAK/STAT), and transforming growth factor (TGF- β).

2. Materials and Methods

2.1. Extraction and Sample Preparation of ABGE

Dried ABG cloves were provided by il Grappolo S.r.l. (Soliera, Modena, Italy). The preparation of ABGE followed the method described in the previous studies [24,28,29].

A detailed protocol is included in the Supplementary Materials.

2.2. Ex Vivo Studies

Adult C57BL/6 male mice (3 months old, weight 20–25 g, n = 25) were housed and maintained as described in the Supplementary Materials Section. The housing conditions and experimentation procedures were strictly in agreement with the European Community ethical regulations (EU Directive no. 26/2014) for the care of animals for scientific research. In agreement with the recognized principles of “Replacement, Refinement and Reduction in Animals in Research”, prostate specimens were obtained as residual materials from the vehicle-treated mice randomized in our previous experiments, approved by the local ethical committee (‘G. d’Annunzio’ University, Chieti, Italy) and the Italian Health Ministry (Project no. 885/2018-PR).

Mouse sacrifice was performed by CO₂ inhalation (100% CO₂ at a flow rate of 20% of the chamber volume per min). After collection, the isolated prostate specimens were maintained in a humidified incubator with 5% CO₂ at 37 °C for 4 h, as previously described [26,30] and reported in the Supplementary Materials Section.

Total RNA was extracted from the prostate specimens using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer’s protocol. The gene expression of COX-2, NF- κ B, TNF- α , and iNOS was quantified by real-time PCR with TaqMan probe-based chemistry, as previously described [27,31,32]. The detailed protocol can be found in the Supplementary Materials Section.

2.3. Cell Culture

Cell lines from control prostate (PNT-2), androgen-dependent PCa (LNCaP), and androgen-independent PCa (PC-3) (American Type Culture Collection, Manassas, VA, USA) were maintained in a humidified incubator with 5% CO₂ at 37 °C following the manufacturer’s guidelines as previously outlined [33,34] (Supplementary Materials Section).

2.4. Cell Proliferation

Cell proliferation was evaluated using resazurin reagent (Canvax Biotech, Cordoba, Spain) [33]. Cell proliferation was measured at the start and after 24, 48, and 72 h of treatment (Supplementary Materials Section).

2.5. Clonogenic Assay

A clonogenic assay was performed on the LNCaP and PC-3 PCa cells treated with 1000 µg/mL of ABGE and incubated for 10 days. The results were expressed as a percentage of the number of colonies relative to the control [34] (Supplementary Materials Section).

2.6. Tumorsphere Formation

The tumorsphere formation assay was conducted as previously described on LNCaP and PC-3 [35,36]. A minimum of three experiments with two replicates for each condition were performed. The results are expressed as a percentage of tumorsphere area relative to the control [35] (Supplementary Materials Section).

2.7. Cell Migration Assay

Cell migration was assessed using a wound healing assay as previously detailed [33,35,36]. The results are presented as the percentage of the migration rate relative to the control. A minimum of three experiments with three replicates for each condition were performed. This experiment was conducted using PC-3 cell lines, but not LNCaP cells due to their lower migration capacity (Supplementary Materials Section).

2.8. Phosphorylation Array

Protein extracts from the LNCaP cells were collected in lysis buffer from 6-well plates after 24 h of treatment with 1000 µg/mL ABGE. The determination of protein content was conducted using a Pierce BCA Protein assay (ThermoFisher Scientific, Madrid, Spain) and adjusted with assay buffer. The data were normalized following the manufacturer's instructions. In brief, the membranes designed for the semi-quantitative detection of 55 phosphorylated human proteins, which are part of the MAPK, AKT, JAK/STAT, and TGF-β signaling pathways, were incubated with blocking buffer for 30 min at 25 °C. The array spots' densitometric analysis was performed using ImageJ software (version number 1.54j), with positive control spots used for normalization. The results are expressed as the log2 Fold Change in each protein signal relative to the control signal, with a log2 Fold Change of 0.2 set as the threshold [37] (Supplementary Materials Section).

2.9. Statistical Analysis

To calculate sample size, we performed power analysis by using G*Power 3.1.9.4 software (effect size = 0.6, α = 0.05, power = 0.85) [38]. As for the ex vivo evaluations, the experimental procedures were performed by a researcher blinded to the treatment. All experiments were conducted at least three times independently ($n \geq 3$). The results from ex vivo and in vitro studies are expressed as means $\pm$ SEM. Statistical differences between the two groups were evaluated using either an unpaired parametric *t*-test or a nonparametric Mann–Whitney U test, depending on normality as determined by a Kolmogorov–Smirnov test. For comparisons involving more than two groups, a One-Way ANOVA was employed. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA).

3. Results and Discussion

In the present study, we aimed to study the potential effects of ABGE on proliferation, colony formation, tumor spheroid formation, cell migration, and the phosphorylation array in three prostate cell lines: PNT-2, LNCaP, and PC-3.

3.1. Ex Vivo Studies

Considering the critical role of chronic inflammation in PCa, we first investigated the potential beneficial activities exerted by ABGE (10–1000 µg/mL) as a validated experimental model of inflammation [24,39]. We studied the effects of ABGE on the gene expression of pro-inflammatory mediators, including COX-2, *NF-κB*, *TNF-α*, and *IL-6*, on isolated LPS-stimulated prostate specimens by RT-PCR. In this context, we found that ABGE

(10–1000 $\mu\text{g/mL}$) was able to inhibit gene expression of all the markers investigated, with 1000 $\mu\text{g/mL}$ being the most effective dose (Figure 1a–d).

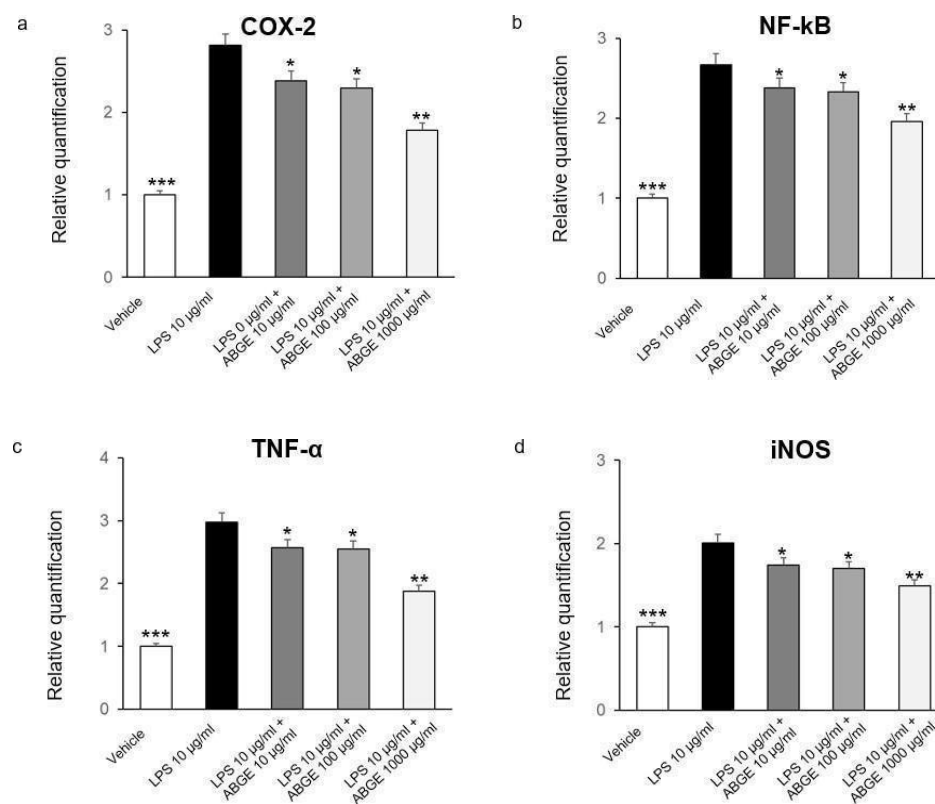


Figure 1. Effects of aged black garlic water extract (ABGE) (10–1000 $\mu\text{g/mL}$) on LPS-induced cyclooxygenase (COX)-2 (a), nuclear factor kappa (NF- κ) B (b), tumor necrosis factor (TNF)- α (c), and interleukin (IL)-6 (d) gene expression (RQ, relative quantification) in mouse prostate specimens. Data shown are means $\pm$ SEM of two independent experiments with triplicate determinations. ANOVA, * $p < 0.05$, ** $p < 0.005$; and *** $p < 0.001$ vs. LPS.

Various pro-inflammatory markers were shown to be implicated in prostatic inflammation. In this context, different phytochemicals, such as catechins, were found to modulate a number of inflammation targets, including TNF- α , COX-2, and interleukins. In the previous studies, we demonstrated that ABGE induced protective activities on colon and heart tissues treated ex vivo with LPS, which have been hypothesized to be related, at least partially, to its polyphenolic composition, with particular regards to gallic acid and catechin [24,25]. Accordingly, BenSaad et al. (2017) found that gallic acid suppressed the LPS-induced production of prostaglandin E2 and IL-6 in RAW264.7 cells [40].

Furthermore, we previously found that ABGE (1 g kg^{-1}) exerted protective effects in rats in vivo [25] in a dose which could be translated to 1 g day in humans.

3.2. Cell Proliferation in Basal Conditions

Cell proliferation was measured after 24, 48, and 72 h of the treatment with ABGE (10, 100, 500, and 1000 $\mu\text{g/mL}$) in basal conditions. The non-tumor prostate cell line PNT-2 was used as the control cell line. In the control prostate line PNT-2, ABGE (10–1000 $\mu\text{g/mL}$) did not affect cell proliferation more compared to that of the control group at any concentration at the different times. In agreement, we previously reported that ABGE did not modify the viability of cardiomyoblast (H9c2) cells or the human fibroblast HFF-1 cell line [24,25] (Figure 2a).

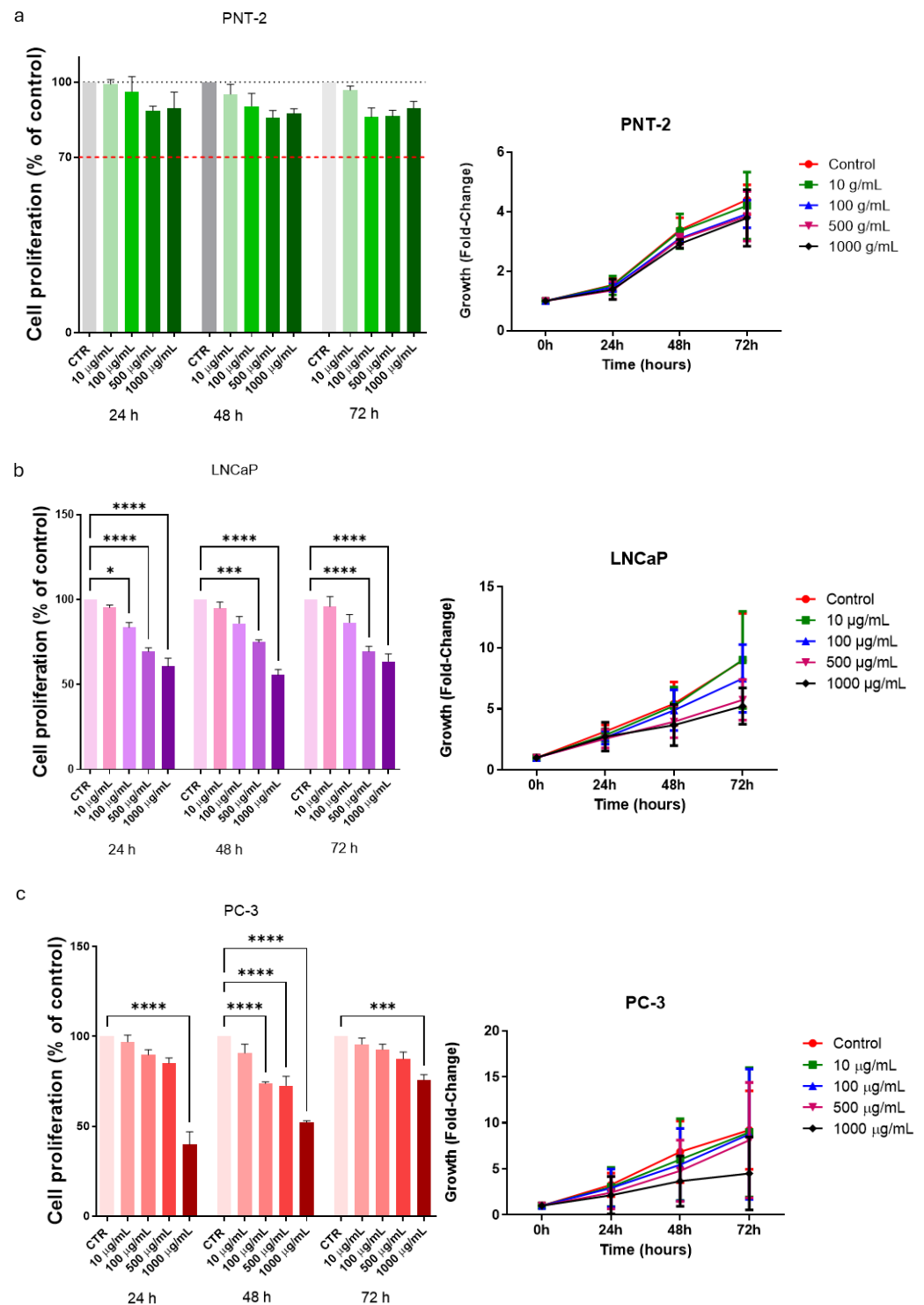


Figure 2. Effects of aged black garlic water extract (ABGE) on cell proliferation of control prostate (PNT-2) (a), androgen-dependent (LNCaP) (b), and androgen-independent (PC-3) (c) prostate cancer (PCa) cells. Cell proliferation and growth were evaluated by resazurin reagent after incubation for 24, 48, and 72 h of PNT-2, LNCaP, and PC-3 cell lines with ABGE at different concentrations (10, 100, 500, and 1000 µg/mL) or vehicle. Data shown are means $\pm$ SEM of 3 independent experiments with 3 replicates of each condition. ANOVA, * $p < 0.05$, *** $p = 0.001$, **** $p < 0.0001$ vs. vehicle.

The LNCaP cell line is derived from lymph node metastasis specimens of individuals with prostate cancer [41].

It retains the characteristics of prostate cancer tumor cytology as well as its early differentiation function, which represents the early androgen-dependent notable features of prostate cancer.

On the other hand, ABGE (10–1000 µg/mL) was able to significantly suppress LNCaP cell proliferation. Interestingly, the inhibitory effect on cell proliferation was dose-dependent, with a greater reduction at higher concentrations and longer exposure times (Figure 2b). Figure 2b shows inhibitory effects in cell proliferation after just 24 h, starting at a concentration of 100 µg/mL. The inhibition induced by the extract is also confirmed following 48 and 72 h of treatment at 500 and 1000 µg/mL concentrations.

The PC-3 cell line was isolated from human prostate cancer bone metastases with a low differentiation degree [42] and represents an androgen-independent prostate cancer cell with moderate metastatic potential in the absence of endogenous androgen receptors. Similarly, the PC-3 cell line showed a significant reduction in cell proliferation following the treatment with ABGE (10–1000 µg/mL) compared to that of the control, with a greater decrease at higher concentrations (Figure 2c), thus confirming its antiproliferative activity also against androgen-independent cancer cells. After 48 h, we showed a significant decrease in cell proliferation starting from 100 µg/mL.

The mechanism underlying the inhibition of cell proliferation induced by ABGE is not yet clear. Dong and their collaborators (2014) demonstrated that an alcohol extract of ABG inhibited the growth of HT129 colon cancer cells probably by the inhibition of the PI3K/Akt pathway [22].

Additionally, Wang and their collaborators (2012) have demonstrated that aged black garlic water extract can inhibit the growth of gastric cancer cells in both in vitro and in vivo [23].

Moreover, an aged black garlic water extract showed dose-dependent apoptosis in human gastric cancer cell lines [23]. Notably, the extract in the prostate did not induce apoptosis in the LNCaP cells (Figure S1, Supplementary Materials).

Meanwhile, in vivo study highlighted the anti-cancer properties of the extract, including the inhibition of tumor growth in mice with tumors. The researchers proposed that the anti-cancer effects of the aged black garlic extract might be due to its antioxidant and immunomodulatory characteristics [23].

Multiple studies have indicated that black garlic possesses anti-tumor properties by inhibiting cell proliferation in both colon and gastric cancers. Jikihara et al. (2014) have performed an experiment using aged garlic extract on F344 rats and DLD-1 human colon cancer cells. The findings revealed antiproliferative effects in both adenoma and adenocarcinoma lesions [43].

3.3. Colony Formation

The analysis of the colony-forming ability of the LNCaP and PC-3 cell lines was performed after the treatment with ABGE (1000 µg/mL) or the vehicle. The highest concentration was chosen because it proved to be the most effective, while remaining biocompatible. In the LNCaP cell line (Figure 3a), the treatment with 1000 µg/mL of ABGE significantly reduced number of colonies formed compared to that of the control, suggesting that ABGE is effective in decreasing the long-term proliferative capacity of androgen-dependent cancer cells. Similarly, in the PC-3 cell line, the treatment with ABGE (1000 µg/mL) (Figure 3b) led to more inhibitory effects in colony formation compared to those of the control, further supporting the potential activity of the extract as an anti-tumor agent. These effects might be due to the presence of polyphenolic compounds in ABGE. In agreement, Jang et al. (2020) demonstrated that gallic acid can inhibit colony formation in various cancer cell lines [40].

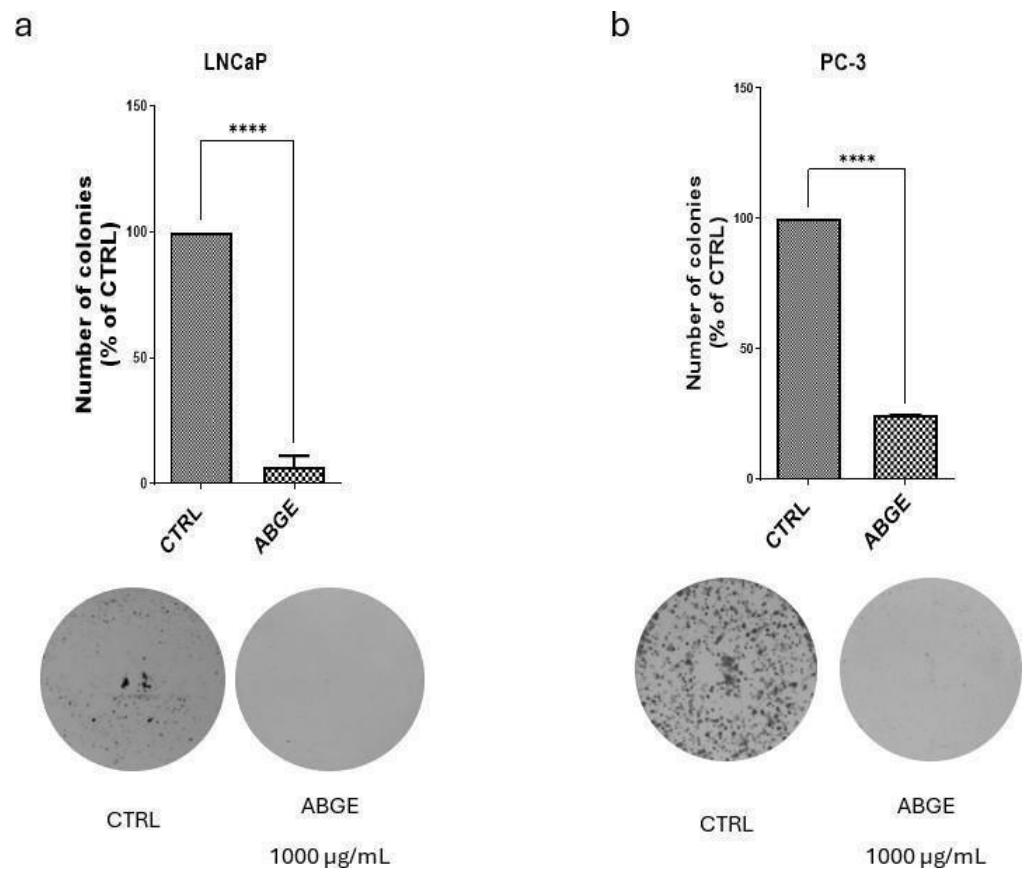


Figure 3. Effects of aged black garlic water extract (ABGE) on colony formation of LNCaP (a) and PC-3 (b) cell lines in response to ABGE at 1000 µg/mL or vehicle. Data shown are means $\pm$ SEM of 3 independent experiments with 3 replicates of each condition. ANOVA, **** $p < 0.0001$ vs. vehicle.

3.4. Tumor Spheroid Formation

Tumor spheroid formation was assessed by measuring the number of spheroids after the treatment with ABGE (1000 µg/mL) or the vehicle in the LNCaP and PC-3 cell lines. In the LNCaP cells (Figure 4a), the treatment with ABGE (1000 µg/mL) did not significantly affect the number of spheroids, which remained unchanged compared to that of the vehicle. However, our present findings also showed that in the PC-3 cells (Figure 4b), ABGE (1000 µg/mL) significantly reduced the number of spheroids compared to that of the control, suggesting that ABGE impedes the proliferation of cancer cells.

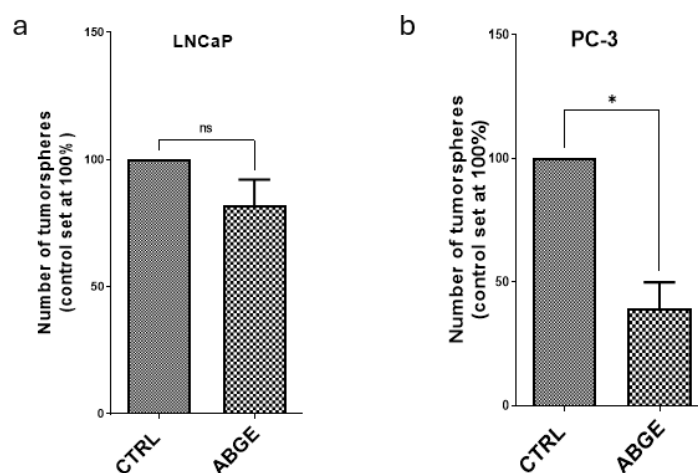


Figure 4. Cont.

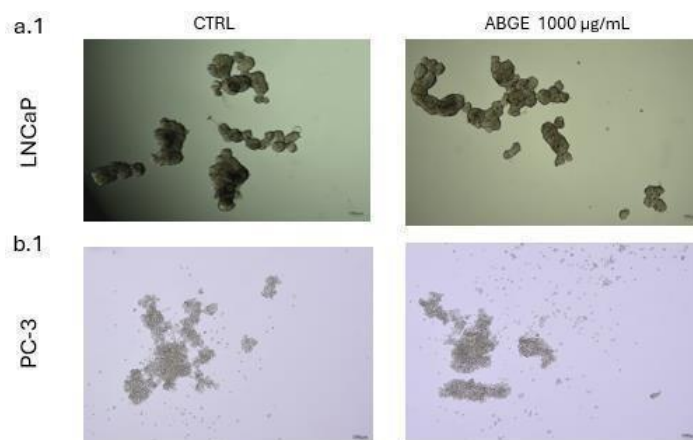


Figure 4. Effects of aged black garlic water extract (ABGE) on tumor spheroid formation of LNCaP (a,a.1) and PC-3 (b,b.1) cell line with ABGE at 1000 µg/mL or vehicle. Data shown are means $\pm$ SEM of 3 independent experiments with 3 replicates of each condition. ANOVA, * $p < 0.05$ vs. vehicle.

3.5. Migration Assay

The cell migration assay was conducted only on the PC-3 cell line because the morphology of LNCaP cells does not allow for accurate migration assessment. The treatment with 1000 µg/mL of ABGE more significantly reduced the cell migration rate compared to that of the control after 24 h of incubation. The reduction in cell migration in PC-3 (Figure 5) suggests that ABGE may also limit the capacity of cancer cells to spread. In agreement, recently, ABG (dissolved in 0.9% normal saline) extract was found able to impede cell migration in breast cancer cells [44].

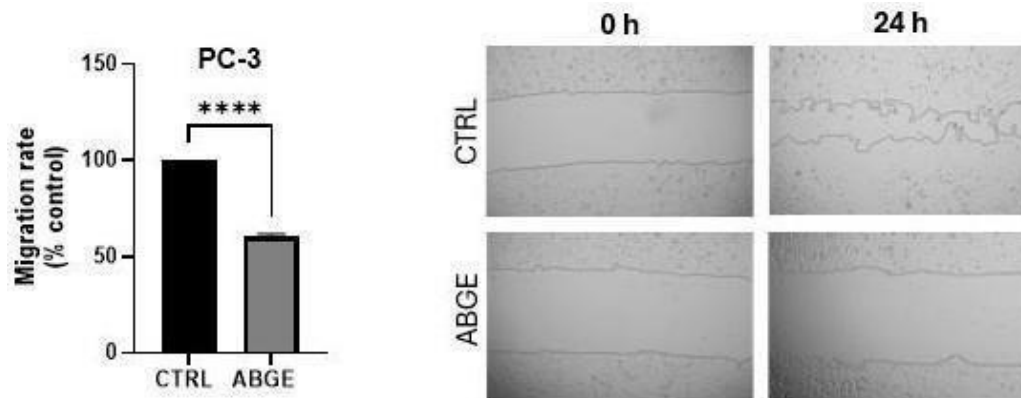


Figure 5. Effects of aged black garlic water extract (ABGE) on migration of PC-3 cell line with ABGE at 1000 µg/mL or vehicle for 24 h. Data shown are means $\pm$ SEM of 3 independent experiments with 3 replicates of each condition. ANOVA, **** $p < 0.0001$ vs. vehicle.

3.6. Cell Proliferation after LPS Pre-Treatment

Considering the previously found effects of ABGE into a pro-inflammatory cell context [18], we then decided to evaluate its potential interaction with LPS. We studied the effects of ABGE (10–1000 µg/mL) on LPS-treated cell proliferation in the PC-3 line (Figure 6), which was chosen for its higher aggressiveness compared to that of the LNCaP cell line, as supported by previous studies [45]. In this context, Xu and their collaborators (2021) showed that LPS combined with ATP significantly increased the proliferation and migration of PC-3 cells, reducing apoptosis. This effect was related to the stimulation of the NLRP3/caspase-1 inflammasome, hypothesizing that inflammation plays a crucial role in prostate cancer progression [8]. Interestingly, our data indicate that the LPS pre-treatment may sensitize the PCa cells to ABGE (Figure 6).

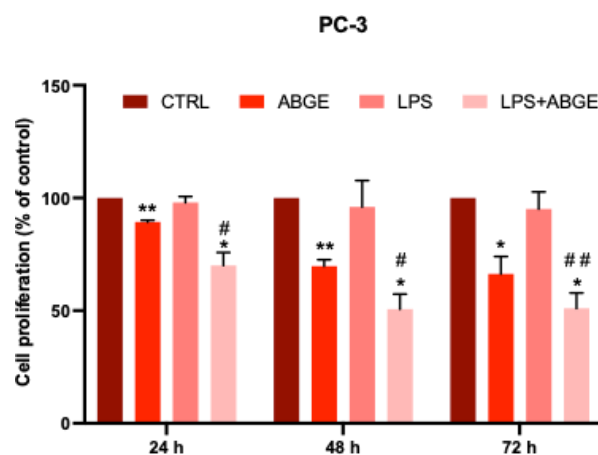


Figure 6. Effects of aged black garlic water extract (ABGE) in combination to LPS on cell proliferation of androgen-independent PC-3 cells. Cell proliferation was evaluated by resazurin reagent after incubation for 24, 48, and 72 h of PC-3 cell line with ABGE at 1000 µg/mL or vehicle. Data shown are means ± SEM of 3 independent experiments with 3 replicates of each condition. ANOVA, * $p < 0.05$, ** $p < 0.005$ vs. vehicle. ANOVA, # $p < 0.05$, ## $p < 0.005$ vs. ABGE.

Gallic acid and catechin, which are the main components of ABGE, as previously shown [24,25], are well known to suppress, proliferate, and stimulate the apoptosis of PCa cells [39,40].

Moreover, a previous study showed that gallic acid decreased the viability of PCa cell lines, but not normal cells' viability [46]. In agreement, we could suggest the potential involvement of polyphenolic compounds, with particular regards to gallic acid and catechin, due to the beneficial effects induced by ABGE on PCa proliferation.

3.7. Phosphorylation Array

A wide body of evidence shows that the MAPK, AKT, JAK/STAT, and TGF-β pathways play a key role in cell proliferation, survival, apoptosis, and growth [23,39–41]. In our study, we analyzed the phosphorylation of the key proteins that participate in these signaling pathways in response to the treatment with ABGE (1000 µg/mL) or the vehicle using a phosphorylation array.

Specifically, the MAPK signaling pathway showed significant modulation in response to the treatment with ABGE (Figure 7). Proteins such as ERK1/2 and JNK showed reduced phosphorylation, suggesting that ABGE (1000 µg/mL) could inhibit these signaling pathways. In this context, the MAPK signaling pathway is critically related to cell proliferation and survival [47]. Furthermore, the reduced phosphorylation of ERK1/2 (T202/Y204) and JNK (T183/Y185) suggests reduced cell proliferation and the potential inhibition of the apoptotic response [48].

p53 is involved in regulation of cell growth, DNA repair, survival, cycle, autophagy, senescence, and apoptosis [49,50].

After the injection of knockdown of ribosomal S6 protein kinases (RSK) 1 and RSK2 in mouse femurs, there was a reduction in osteolytic lesions in the PC3 cells compared to those in the control cells [51].

In our present study, we found that ABGE increased the quantity of LPS-treated p53, while decreased the RSK2 phosphorylation levels (Figure 7), which could be related to the anti-cancer properties of the extract.

The role of AKT signaling pathway in cell survival and growth is also well known [23]. ABGE (1000 µg/mL) showed variable effects on the phosphorylation of both the AKT and downstream proteins (Figure 8).

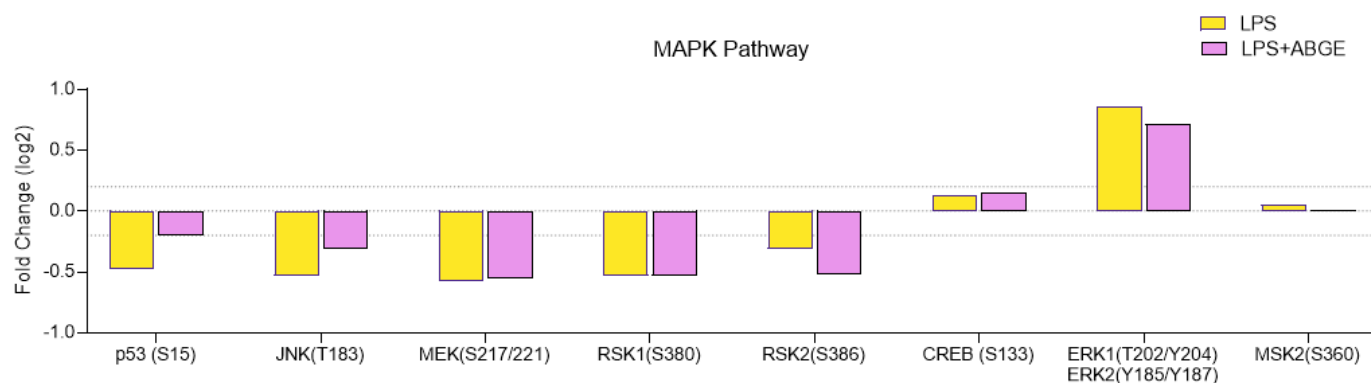


Figure 7. The MAPK signaling pathway in the phosphoprotein array in response to 24 h treatment of 1000 µg/mL ABGE. The log2 Fold Change in the phosphorylation protein level in comparison with that for the control condition (threshold: log2 Fold Change = 0.2).

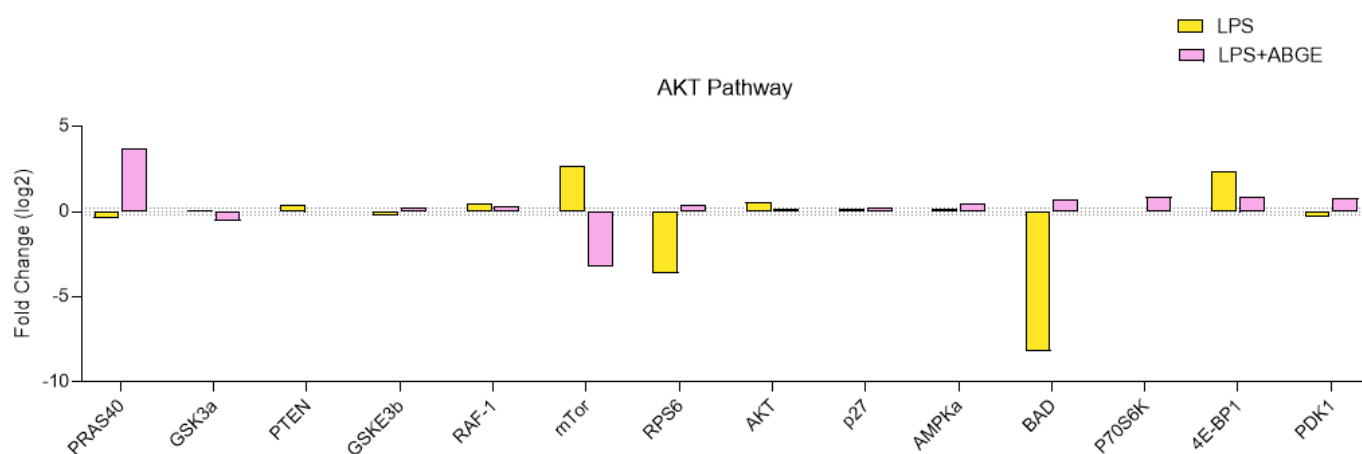


Figure 8. The AKT signaling pathway in the phosphoprotein array in response to 24 h treatment of 1000 µg/mL ABGE. The log2 Fold Change in the phosphorylation protein level in comparison with that for the control condition (threshold: log2 Fold Change = 0.2).

The reduced phosphorylation of mTOR suggests decreased protein synthesis and cell growth [52]. mTOR activation has been shown to induce the phosphorylation of many substrates, such as eukaryotic translation initiation factor 4E (eIF4E)-binding proteins (4E-BP1), and mTOR kinase inhibitors have been reported to block p4E-BP1 [53].

Our findings show that ABGE reduced the LPS-treated mTOR and 4E-BP1 phosphorylation levels. In agreement, considering that high levels of 4E-BP1 have been measured in prostate cancer cells, we can speculate that mTOR and 4E-BP1 could be involved, at least in part, in the beneficial effects induced by ABGE [54].

Moreover, ABGE also lowers the LPS-treated levels of glycogen synthase kinase (GSK)-3, phosphatase, tensin-homolog in chromosome 10 (PTEN), and serine/threonine kinase Raf-1 (RAF-1), which are involved in cancer development and progression [55–57].

On the other hand, ABGE increased the LPS-treated levels of p27 and AMP-activated protein kinase (AMPK), which possess a well-known suppressor role in carcinogenesis [58,59].

The JAK/STAT signaling pathway plays a key role in numerous essential biological processes, such as differentiation, cell proliferation, immune regulation, and apoptosis [48].

The inactivation of Src induced a reduction in the migration and growth in PCa cell lines [60,61].

In addition, the reduced phosphorylation of STAT1, STAT2, STAT3, and STAT5 (Figure 9) indicates a potential decrease in proliferative signaling and cancer development [62–65].

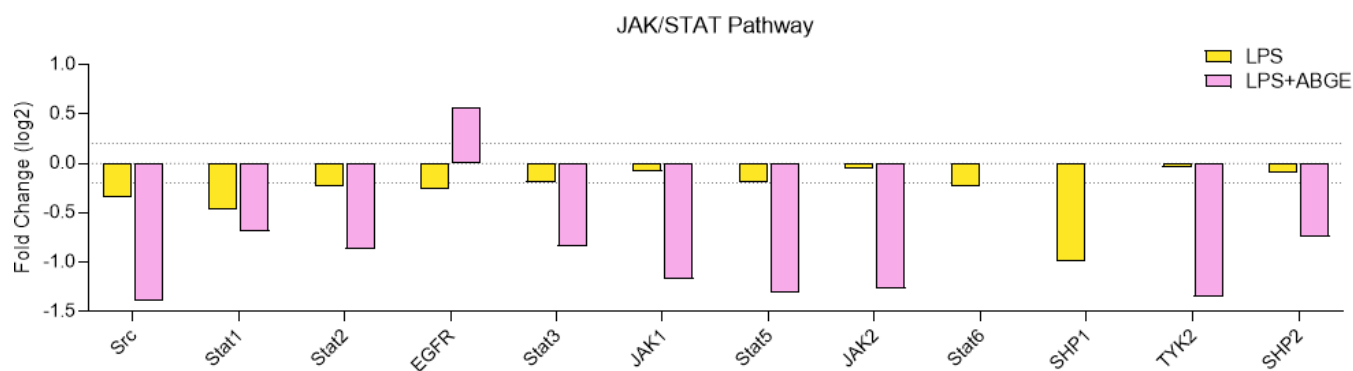


Figure 9. The JAK/STAT signaling pathway in the phosphoprotein array in response to 24 h treatment of 1000 $\mu\text{g/mL}$ ABGE. The log₂ Fold Change in the phosphorylation protein level in comparison with that for the control condition (threshold: log₂ Fold Change = 0.2).

Moreover, the decreased phosphorylation of JAK1 and JAK2 suggests decreased signal transduction promoting prostate cancer cell proliferation and survival [66,67].

In agreement, TYK2 signaling promotes the invasiveness of prostate cancer cells [68].

Accordingly, the involvement of SHP2 in several cancer-related processes has been reported [69].

Actually, our findings, showing that ABGE decreased the LPS-treated Src, STAT1, STAT2, STAT3, STAT5, JAK1, JAK 2, TYK2, and SHP2 phosphorylation levels, could suggest the potential protective role of the extract in PC-3 cells.

Regarding the TGF- β pathway (Figure 10) involved in cell growth regulation and tumor progression [49], we showed that ABGE (1000 $\mu\text{g/mL}$) decreased the phosphorylation of SMAD1, suggesting reduced TGF- β signaling, which may be associated with reduced cell invasiveness [70].

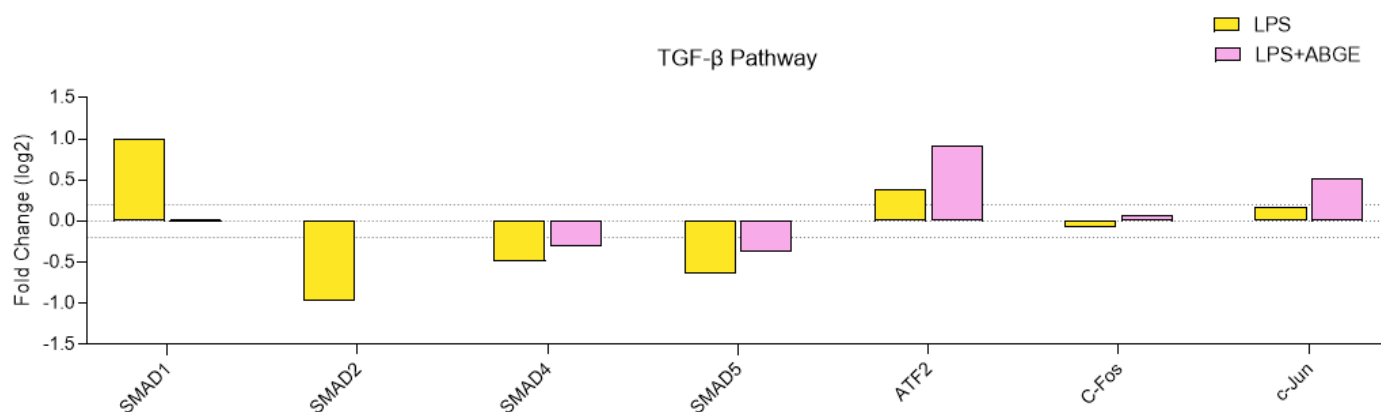


Figure 10. The TGF- β pathway in the phosphoprotein array in response to 24 h treatment of 1000 $\mu\text{g/mL}$ ABGE. The log₂ Fold Change in the phosphorylation protein level in comparison with that for the control condition (threshold: log₂ Fold Change = 0.2).

The previous studies reported a correlation between SMAD2 and SMAD4, which are involved in the inhibition of cell growth [71].

ATF2 has been found as a tumor promoter in various human cancers, such as prostate cancer [72].

Furthermore, c-Jun or c-Fos overexpression has been directly related with PCa cell line invasiveness, and the phosphorylated c-Jun levels are high in PCa [73].

In our study, we showed that ABGE decreased the quantity of LPS-treated SMAD1, while it increased the SMAD2, SMAD4, ATF2, c-Jun, and c-Fos phosphorylation levels, further confirming the potential protective role of the extract.

In the literature, there are not many studies about the effects of ABGE on the prostate; thus, its mechanisms are not particularly well known.

4. Conclusions

In conclusion, our results showed the potential anti-inflammatory and anti-proliferative effects of ABGE on prostate cancer. In this context, ABGE reduced the gene expression of the different biomarkers involved in inflammatory response, such as *COX-2*, *TNF- α* , *IL-6*, and *NF- κ B*, also modulating relevant signaling pathways, including AKT, MAPK, TGF- β , and JAK/STAT. Furthermore, we performed different in vitro assays, where ABGE had beneficial effects on both the prostate cancer lines. Therefore, our results suggest that ABGE might be potentially used as a diet supplement for health promotion and a source of bio-organic compounds with antitumor properties in PCa.

A limitation of our study is that we have not evaluated specific targeting, as well as the signaling pathways modulating the potential anti-inflammatory and anti-cancer effects of the extract. However, further studies are needed in the future to accurately evaluate the in vivo activity of ABGE in reducing inflammation and cancer, as well as its potential negative effects on the body.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/nu16173025/s1>. Figure S1: Apoptosis induction in response to ABGE (1000 μ g/mL) in LNCaP cells. Annexin V positive cells were considered as apoptotic. Annexin positivity was determined by flow cytometry (LSRFortessa SORP) with FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Jose, CA, USA; #556547) at 10 μ g/mL in PBS. References [24,27–29,31–38] are cited in the supplementary materials.

Author Contributions: Conceptualization, M.L.L., L.R., R.M.L. and S.L.; methodology, M.L.L., A.J.M.-H., L.R., R.M.L., L.B. and S.L.; software, G.O., C.F. and L.M.; validation, G.O., C.F. and L.M.; formal analysis, M.L.L., A.J.M.-H., D.G., A.A., G.O., C.F., L.M., S.C.D.S., N.N. and A.C.; investigation, M.L.L., A.J.M.-H., D.G., A.A., G.O., C.F., L.M., S.C.D.S., N.N. and A.C.; resources, M.L.L., A.J.M.-H., L.R., R.M.L., L.B. and S.L.; data curation, M.L.L., A.J.M.-H., L.R., R.M.L. and S.L.; writing—original draft preparation, M.L.L., L.R., L.B. and S.L.; writing—review and editing, M.L.L., A.J.M.-H., L.R., R.M.L. and S.L.; visualization, M.L.L., A.J.M.-H., L.R., R.M.L. and S.L.; supervision, L.R., R.M.L. and S.L.; project administration, L.R., R.M.L., L.B. and S.L.; funding acquisition, L.R., A.J.M.-H., R.M.L., L.B. and S.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The housing conditions and experimentation procedures were strictly in agreement with the European Community ethical regulations (EU Directive no. 26/2014) on the care of animals for scientific research. In agreement with the recognized principles of “Replacement, Refinement and Reduction in Animals in Research”, the prostate specimens were obtained as residual materials from the vehicle-treated mice randomized in our previous experiments, approved on 23 November 2018 by the local ethical committee (‘G. d’Annunzio’ University, Chieti, Italy) and the Italian Health Ministry (Project no. 885/2018-PR).

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article and Supplementary Materials, further inquiries can be directed to the corresponding authors request.

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Supplementary materials

Article III



Supplementary Materials

The Protective Effects of an Aged Black Garlic Water Extract on the Prostate

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Abstract: Chronic inflammation is a recognized risk factor for various cancers, including prostate cancer (PCa). We aim to explore the potential protective effects of aged black garlic extract (ABGE) against inflammation-induced prostate damage and its impact on prostate cancer cell lines. We used an ex vivo model of inflammation induced by *Escherichia coli* lipopolysaccharide (LPS) on C57BL/6 male mouse prostate specimens to investigate the anti-inflammatory properties of ABGE. Gene expression levels of pro-inflammatory biomarkers (*COX-2*, *NF-κB*, *TNF-α*, *IL-6*) were measured. Additionally, we evaluated ABGE's therapeutic effects on prostate cancer cell lines through in vitro functional assays, including colony formation, tumorsphere formation, migration assays, and phosphorylation arrays to assess signaling pathways (MAPK, AKT, JAK/STAT, TGF-β). ABGE demonstrated significant anti-inflammatory and antioxidant effects in preclinical models, partly attributed to its polyphenolic content, notably catechin and gallic acid. In the ex vivo model, ABGE reduced gene expression levels of *COX-2*, *NF-κB*, *TNF-α*, and *IL-6*. In vitro studies showed that ABGE inhibited cell proliferation, induced apoptosis, and modulated the cell cycle in prostate cancer cells, suggesting its potential as a therapeutic agent. ABGE exhibits promising anti-inflammatory and anti-cancer properties, supporting further investigation into ABGE as a potential agent for managing inflammation and prostate cancer.

Keywords: ABGE; prostate; inflammation; cancer

1. Materials and Methods

1.1. Extraction and Sample Preparation of ABGE

ABG cloves were provided in dried form by Il Grappolo S.r.l. (Soliera, Modena, Italy). 10 grams of garlic cloves were manually crushed using a garlic press, and the resulting juice and debris were collected in a centrifuge tube by rinsing the crusher with 10 mL of

water. After shaking the tube 10 times, it was placed in a Trans-sonic T460 ultrasonic bath from Elma (Singen, Germany) for 15 minutes at room temperature. Subsequently, the mixture was centrifuged twice at 4000 rpm for 10 minutes each [1–3]. The supernatant was then filtered and stored until further analysis.

1.2. Ex Vivo Studies

Adult C57BL/6 male mice (3-month-old, weight 20–25 g, $n = 25$) were housed in Plexiglas cages (2–4 animals per cage; 55 cm x 33 cm x 19 cm) and maintained under standard laboratory conditions (21 ± 2 °C; $55 \pm 5\%$ humidity) on a 14/10 h light/dark cycle, with ad libitum access to water and food. Housing conditions and experimentation procedures were strictly in agreement with the European Community ethical regulations (EU Directive no. 26/2014) on the care of animals for scientific research. In agreement with the recognized principles of “Replacement, Refinement and Reduction in Animals in Research”, prostate specimens were obtained as residual material from vehicle-treated mice randomized in our previous experiments, approved by local ethical committee (‘G. d’Annunzio’ University, Chieti, Italy) and Italian Health Ministry (Project no. 885/2018-PR).

Mice sacrifice was performed by CO₂ inhalation (100% CO₂ at a flow rate of 20% of the chamber volume per min). After collection, isolated prostate specimens were maintained in a humidified incubator with 5% CO₂ at 37 °C for 4 h (incubation period), in RPMI buffer with added bacterial LPS (10 µg/mL), as previously described [3–4]. During the incubation period, the tissues were challenged with scalar concentrations of ABGE (10–1000 µg/ml) or vehicle (controls) ($n = 5$ for each experimental group).

Total RNA extraction from the single prostate specimen was performed using TRI Reagent (Sigma–Aldrich, St. Louis, MO, USA), in agreement with the manufacturer’s protocol. One microgram of total RNA extracted from each sample in a 20 µL reaction volume was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc., Monza, Italy). Reactions were incubated in a 2720 Thermal Cycler (Thermo Fisher Scientific Inc., Monza, Italy) initially at 25 °C for 10 min, then at 37 °C for 120 min, and finally at 85 °C for 5 s. Gene expression of COX-2, NF-κB, TNF-α, and iNOS was measured by quantitative real-time PCR using TaqMan probe-based chemistry, as previously described [5–6]. PCR primers and TaqMan probes, including β-actin used as the housekeeping gene, were purchased from Thermo Fisher Scientific Inc. (Assays-on-Demand Gene Expression Products, Mm00478374_m1 for COX-2 gene, Mm00443258_m1 for TNF-α gene, Mm00476361_m1 for NF-κB gene, Mm00446190_m1 for IL-6 gene, Mm00607939_s1 for β-actin gene). The real-time PCR was carried out in triplicate for each cDNA sample in relation to each of the investigated genes. Data were elaborated with the Sequence Detection System (SDS) software version 2.3 (Thermo Fisher Scientific Inc.). Relative quantification of gene expression was performed by the comparative $2^{-\Delta\Delta Ct}$ method [7].

1.3. Cell Culture

Cell lines from control prostate (PNT-2), androgen-dependent PCa (LNCaP), and androgen-independent PCa (PC-3) were sourced from the American Type Culture Collection (Manassas, VA, USA) and maintained in a humidified incubator with 5% CO₂ at 37 °C, following the manufacturer’s guidelines as previously outlined [8–9]. To verify the authenticity of these cell lines, short tandem repeat (STR) sequence analysis was conducted using the GenePrint 10 System (Promega, Barcelona, Spain), and the absence of mycoplasma contamination was confirmed by PCR, as previously described [8].

1.4. Cell Proliferation

Cell proliferation was evaluated using resazurin reagent (Canvax Biotech, Cordoba, Spain), as previously described [8]. In brief, 3000 to 5000 cells were plated per well in 96-

well plates, at 5% FBS and then treated with varying concentrations (10–1000 µg/mL) of ABGE or vehicle for 24, 48, and 72 hours. Cell proliferation was measured at the start and after 24, 48, and 72 hours of treatment by recording fluorescence intensity (560 nm excitation and 590 nm emission) following a 3-hour incubation with 10% resazurin using the FlexStation III system (Molecular Devices, Sunnyvale, CA, USA). The results were expressed as a percentage of fluorescence intensity relative to the control (vehicle-treated). A minimum of three experiments with three replicates for each condition were conducted.

1.5. Clonogenic Assay

To evaluate the clonogenic capacity of LNCaP and PC-3 PCa cells, 2000 cells were seeded into 6-well plates, treated with 1000 µg/mL of ABGE, and incubated for 10 days. Following the incubation period, the medium was removed, and the colonies were washed with PBS, stained with a crystal violet solution (6% glutaraldehyde, 0.5% crystal violet) for 30 minutes, then rinsed and allowed to air dry. A minimum of three experiments with two replicates for each condition were conducted. The number of colonies was counted using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The results were expressed as a percentage of the number of colonies relative to the control [8].

1.6. Tumorsphere Formation

The tumorsphere formation assay was conducted as previously described [9–10]. Briefly, 2000 cells per well were seeded in Corning Costar 24-well ultra-low attachment plates (Merck, Madrid, Spain) with DMEM F-12 medium supplemented with 20 ng/mL EGF (Sigma-Aldrich, Madrid, Spain). After 24 hours, 1000 µg/mL ABGE was added. The area of tumorspheres was measured after 14 days of incubation using ImageJ software. A minimum of three experiments with two replicates for each condition were conducted. Results were expressed as a percentage of tumorsphere area relative to the control [9].

1.7. Cell Migration Assay

Cell migration was assessed using a wound healing assay as previously detailed [7, 9–10]. In summary, 50,000 cells were seeded in 96-well plates and cultured until they reached confluence. Wounds were created using the IncuCyte WoundMaker (Essen BioScience, Ann Arbor, MI, USA) following the manufacturer's protocol. The wells were then rinsed with PBS and the cells were treated overnight with 1000 µg/mL of ABGE in a serum-free medium. Images of the wounds were captured immediately after wounding (0 h) and after 24 hours of treatment. Wound healing was quantified as the ratio of the scratched areas at the initial and final timepoints, analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Results were presented as the percentage of the migration rate relative to the control. A minimum of three experiments with three replicates for each condition were performed. This experiment was conducted using PC-3 cell lines but not LNCaP cells due to their lower migration capacity.

1.8. Phosphorylation Array

Protein extracts from LNCaP cells were collected in lysis buffer from 6-well plates after 24 hours of treatment with 1000 µg/mL ABGE. A phosphorylation pathway profiling array was conducted using the Human Phosphorylation Pathway Profiling Array C55 kit #AAH-PPP-1–8, according to the manufacturer's instructions (Raybiotech, Inc., Peachtree Corners, GA, USA). Protein content was determined by Pierce BCA Protein assay (ThermoFisher Scientific) and adjusted with assay buffer. Data were normalized following manufacturer's instructions. Briefly, membranes designed for the semi-quantitative detection of 55 phosphorylated human proteins, which are part of the MAPK, AKT, JAK/STAT, and TGF-β signaling pathways, were incubated with blocking buffer for 30 minutes at 25 °C. Subsequently, the membranes were incubated overnight at 4 °C with 1

mL of a 4-fold dilution of pooled LNCaP cell lysates ($n = 3$). After washing, the membranes were treated with a detection antibody cocktail for 2 hours at 25 °C, followed by an incubation with horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibody for another 2 hours at 25 °C. Signals were detected using an ECL reagent with a BioRad Universal Hood II chemiluminescence detection system (BioRad Laboratories, Hercules, CA, USA). The array spots' densitometric analysis was performed using ImageJ software, with positive control spots used for normalization. Results were expressed as the log2 fold change of each protein signal relative to the control signal, with a log2 fold change of 0.2 set as the threshold [11].

1.9. Statistical Analysis

To calculate sample size, we performed a power analysis by using G*Power 3.1.9.4 software (effect size = 0.6, $\alpha = 0.05$, power = 0.85) [12]. As for ex vivo evaluations, the experimental procedures were performed by a researcher blinded to the treatment. All experiments were conducted at least three times independently ($n \geq 3$). Results from ex vivo and in vivo studies were expressed as means $\pm$ SEM. Statistical differences between two groups were evaluated using either an unpaired parametric t-test or a nonparametric Mann–Whitney U test, depending on normality as determined by the Kolmogorov–Smirnov test. For comparisons involving more than two groups, a One-Way ANOVA was employed. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA).

2. Results

2.1. Apoptosis Assay

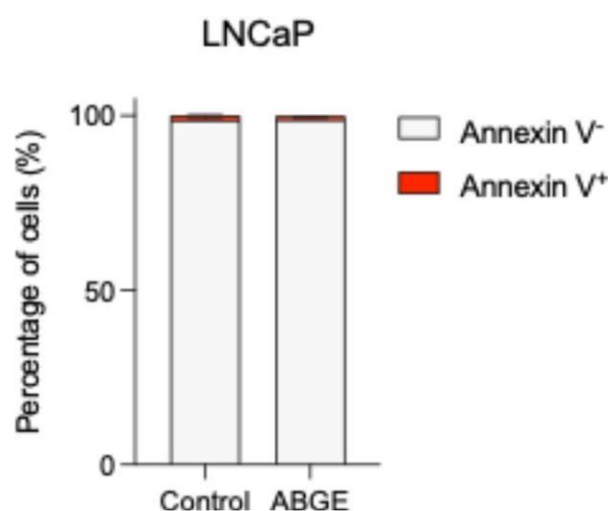


Figure S1. Apoptosis induction in response to ABGE (1000 $\mu\text{g/mL}$) in LNCaP cells. Annexin V positive cells were considered as apoptotic. Annexin positivity was determined by flow cytometry (LSRFortessa SORP) with FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Jose, CA; #556547) at 10 $\mu\text{g/mL}$ in PBS.

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