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Black Cumin and Garlic Induced Cell Death in Hepatoma Cancer Cells (Huh-7)

Aliya I. Sani*, Farah Azhar

Department of Biochemistry, Ziauddin University, Karachi, Pakistan

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Abstract

***Background*:** Various plant products have been shown to limit proliferation in cancer cell lines by inducing apoptosis and cell cycle arrest.

***Objective*:** The study aimed to assess the growth inhibitory synergistic and solitary effects of black cumin and garlic on Hepatoma cell lines (Huh-7).

***Methods*:** Huh-7 hepatoma cells were treated with 10 g of ground black cumin, 10 g of ground garlic, individually, or in combination, and compared to water (control). After 32 hours, cell death was assessed using acridine orange staining under a fluorescent microscope. Cell necrosis was quantified by examining multiple high-intensity fields per slide.

***Results*:** Black cumin, garlic induced cell death in Huh-7. To determine alive/dead cell density, high intensity fields per slide, randomly selected after 32 hours of incubation. At magnification of 20x, the controls show 96.2% alive cells compared to 20.0% in garlic, 55.0% in black cumin and 50% in their mixture. However, at 40x magnification, 95.2% alive cells were seen in controls compared to only 20.0% in garlic, 18.0% in black cumin and 23.8% in their mixture.

***Conclusion*:** It is concluded that black cumin, garlic, and its mixture exhibit promising anticancer properties against hepatoma cells. In cases of individual cell death or death in limited number of cells, the apoptotic pathway may be involved. However, in cases where cell death was observed in group of cells as well as confluent areas containing dead cells, it is the process of necrosis that may be the mechanism behind the observed effects.

Keywords: Hepatoma cell lines, cell death, black cumin, garlic.

1. INTRODUCTION

Hepatocellular carcinoma (HCC) stands as a formidable global health challenge, marked by its high incidence rates and alarming mortality rates, especially in advanced stages [1]. Despite advancements in conventional treatments, the search for novel therapeutic approaches continues, driven by the need for more effective and less toxic alternatives [2]. In recent years, natural compounds have garnered considerable attention for their potential anticancer properties, with black cumin (Nigella sativa) and garlic (Allium sativum) emerging as noteworthy candidates due to their rich phytochemical composition and reported health benefits [3].

Among these, black cumin, and garlic have individually demonstrated cytotoxic effects against various cancer cell lines, including hepatoma cancer cells such as Huh-7. However, studies exploring the synergistic or additive effects of these natural substances on hepatoma cell viability remain limited.

* Address correspondence to this author at the Department of Biochemistry, Ziauddin University, Karachi, Pakistan; E-mail: aliya.sani@zu.edu.pk

Understanding the mechanisms by which black cumin, and garlic induce cell death in Huh-7 hepatoma cancer cells holds significant promise for the development of complementary or alternative therapeutic strategies against HCC.

Since, the natural products, through their synergistic and isolated effects, have been seen in combating cancer cells proliferation, therefore, this study was aimed to assess the cancer growth inhibition through synergistic and isolated inhibitory effects of black cumin, garlic and on Huh-7 *via* cell death induction.

2. METHODOLOGY

To study Individual and combined effect of certain herbal condiments with human hepatoma cell lines (Huh-7), two different herb/spices, black cumin and garlic used as normal food additives were checked for their cell death potential by comparing with the controls. Hepatoma cell lines (Huh-7) were kindly provided by the Aga Khan University (AKU). The cell lines were maintained in the cell culture laboratory of the AKU, Molecular Biology Laboratory. It was a descriptive, comparative, controlled *in-vitro* study. The black cumin and garlic were obtained from local market. Garlic was ground and dried at 37°C. Black cumin and garlic

were ground and made into powder. Mixture of all these ingredients was prepared by mixing 10 mg garlic and 5 mg of black cumin. A 24 well plate was used for growth of Huh-7. The growth of hepatoma cell lines (Huh-7) was checked by adding the additives, the cells were cultured according to given protocol [4]. These additives, individually and as mixture were added to these wells. To the controls water was added instead of spice. The cells were cultured according to the given protocol [5].

The flasks containing actively proliferating cells, which were at a confluence level of 80 to 90%, were chosen for the experiment. The cell culture medium included all necessary supplements for the specific cell line. Calcium- and Magnesium-Free Phosphate-Buffered Saline CMF-PBS (10 mL) and a trypsin solution of 0.1% were utilized. The experiment was conducted within a Laminar flow hood. Prior to subculturing, the cell cultures were inspected using an inverted phase contrast microscope (100 to 200x) to detect any signs of microbial contamination.

Following the standard cell harvesting procedure, cell culturing was performed with the necessary additives. The 24-well plate, employed for the cell culturing process was labeled accordingly before seeding. Slide covers were placed in all wells to serve as a foundation for cell growth. Hepatoma cells were seeded in the presence of minimum essential medium at a density of 0.05 x 106 cells per well of the 24-well cell culture plates (Invitrogen, Carlsbad, CA) along with 10µl of the prepared sample containing black cumin and garlic.

After 32 hours, the cells were examined using acridine orange dye under an immuno-florescent microscope (inverted phase contrast microscope) and multiple fields were documented. Two to four fields of higher intensity were randomly chosen per slide to determine the density of dead cells, and this process was repeated at least 10 times.

3. RESULTS

This study presented the comparative analysis of cell death potential of various treatments, including control, black cumin (BC), garlic, and a combination of black cumin and garlic (BC+garlic), on hepatoma cells at different magnifications (20x and 40x). The data includes the number of total cells counted, as well as the proportion of cells classified as alive or dead, along with corresponding p-values for statistical significance.

At both 20x and 40x magnifications, the control group exhibited a significantly higher percentage of alive cells (96% and 95%, respectively) compared to dead cells. Black Cumin (BC) treatment results indicated a marked decrease in cell viability following treatment with black cumin. At 20x magnification, the proportion of alive cells dropped to 55%, while at 40x magnification, it decreased to 18% (Fig. **1**). Conversely, there was a substantial increase in the percentage of dead cells, reaching 81.2% at 40x magnification. On the other hand, garlic treatment also resulted in a notable decrease in cell viability, with only 20% of cells remaining alive at 20x magnification and at 40x magnification as well. Conversely, the majority of cells (77-80%) were classified as dead, indicating a potent cytotoxic effect of garlic on hepatoma cells. Additionally, the combination treatment (BC+garlic) resulted in an intermediate effect (24% at 40x-50% at 20x) on cell viability compared to individual treatments. The percentage of alive cells was 49% at 20x and 76.2% at 40x (Table **1**).

Table 1: Black Cumin and Garlic effects observed on hepatoma cells at 20x and 40x magnification.

	Selected Field	**Hepatoma Cells at 20x**			**p- value**	**Hepatoma Cells at 40x**			**p- value**
		Total Cells	Alive n (%)	Dead n (%)		Cells	Alive n (%)	Dead n (%)	
Controls	10	405	390(96.2)	15(3.8)	<0.001	207	197(95.2)	10(4.8)	<0.001
Black Cumin (BC)	9	881	486(55)	401(49)		154	29(18.8)	125(81.2)	
Garlic	6	495	100(20.2)	385(77.8)		126	30(23.8)	96(76.2)	
BC+Garlic	10	272	137(50.8)	135(49.2)		100	20(20)	80(80)	

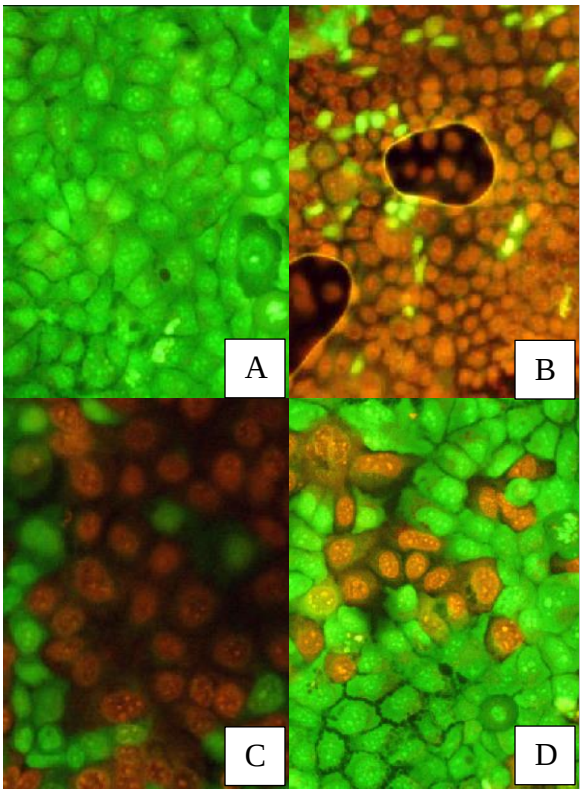

Figure 1: Pattern of cell-death induced by black cumin, garlic and mixture of both compared to controls at 20x magnification. (**A**): Control (**B**): Garlic (**C**): Black Cumin (**D**): Mixture.

3. DISCUSSION

This recently research has focused on plants' ability to induce cell death in cancer cells to inhibit their proliferation and immortal cell lines have been crucial in studying this phenomenon. Tumor cells often evade apoptosis, enabling unrestricted growth. Alterations in the Hedgehog (Hh) pathway genes, implicated in embryonic development, have been associated with hepatocellular cancer (HCC). The loss of HHIP expression may contribute to HCC progression [5, 6]. The plant extracts exhibit the ability to induce cell death in malignant cells, suggesting modulation of gene expression or activation of cell death programs [7].

The current results demonstrate the potent cytotoxic effects of black cumin and garlic on hepatoma cells, leading to a significant decrease in cell viability and increased cell death. Additionally, the combination of black cumin and garlic showed promising results, indicating potential synergistic interactions in inducing cell death.

The observed cell death pattern in our research involved the presence of large groups of confluent cells with detachment. This indicates impact of these substances caused harm to the structural elements and denaturation of enzymes, linking this occurrence to the cell death pattern seen in necrosis. Additionally, we also noted cell death in individual cells and clusters of cells. Therefore, the pattern observed is individual cell death within groups of cells, characteristic of apoptosis, while necrosis represents the gross and histological manifestation of cell death resulting from irreversible external damage. Necrotic cells lack membrane integrity, leading to the leakage of their contents. The morphological features of necrosis stem from protein denaturation and enzymatic breakdown within the cell [8]. It is widely recognized that apoptosis and necrosis can coexist, sharing common features and mechanisms, indicating the involvement of both processes of cell demise - necrosis and apoptosis [9].

Black cumin, also known as black seed or Nigella sativa, has a rich history in traditional medicine, attributed to its diverse health benefits. Its active constituent, thymoquinone, has gained attention for its potential anticancer effects. Various studies have highlighted the cytotoxic impact of thymoquinone on different cancer cell lines, including hepatoma cells, breast cancer, lung, ovary and colonic cancer [10]. Thymoquinone's (TQ) ability to induce apoptosis, inhibit cell proliferation, and disrupt cancer-related cellular signaling pathways has been noted [11]. One of the studies reported the increased expression of microRNA-16 and microRNA-375 in HepG2 and Huh7 cells after treating with doxorubicin and TQ, this led to activated caspases, low anti-apoptotic proteins, and increased pro-apoptotic proteins. Moreover, TQ anti-inflammatory, antiangiogenic and antioxidant properties may contribute to its anticancer effects by modulating the tumor microenvironment and mitigating oxidative stress-induced damage [12, 13].

Garlic is a commonly used culinary herb with many recognized health benefits. It contains compounds like allicin, diallyl sulfide, and diallyl disulfide, which are reported to have anticancer effects. It has been observed that compounds found in garlic can alter the progression of the cell cycle, hinder metastatic potential, and enhance the effectiveness of standard chemotherapy medications. Moreover, allicin has shown inhibitory growth effect inhibited on human mammary, endometrial, and colon cancer cells in vitro models [14, 15]. The apoptotic pathways are instigated by allicin via activating caspases, enhancing mitochondrial membrane permeability, and generating reactive oxygen species (ROS), suggesting their potential as adjunctive therapies for HCC [16].

The synergistic interactions between black cumin and garlic in inducing cell death in cancer cells have also been explored. Preclinical studies have demonstrated heightened cytotoxicity and apoptosis when combining black cumin and garlic extracts, compared to individual treatments. These synergistic effects may be attributed to complementary mechanisms of action, including the simultaneous activation of multiple apoptotic pathways, inhibition of pro-survival signaling pathways, and augmentation of oxidative stress-induced cell damage. Furthermore, the combination of black cumin and garlic extracts has been observed to mitigate drug resistance mechanisms and enhance the effects of conventional chemotherapy drugs in cancer cells, suggesting their potential as complementary therapies for HCC [17].

One of the major limitations of this study was lack of evaluation of apoptotic markers like Bcl-2, Bad, Bax due to limited resources.

Despite promising preclinical evidence, several challenges remain in translating the anticancer effects of black cumin and garlic into clinical practice. Variability in the composition and bioavailability of active compounds in black cumin and garlic extracts poses a significant challenge, impacting their efficacy and safety profiles. Standardization of extraction methods and quality control measures are crucial to ensure consistent and reproducible outcomes across studies. Additionally, further preclinical investigations are warranted to determine the optimal dosage, treatment duration, and potential side effects of black cumin and garlic extracts in HCC models before progressing to clinical trials.

4. CONCLUSION

Black cumin, garlic and its mixture exhibit promising anticancer properties against hepatoma cells, including Huh-7 cells through diverse mechanisms inducing cell death.

Based on our observations during current study, we have evidence to conclude that in cases of individual cell death or death in limited number of cells, this apoptotic pathway must have been involved. However, in cases where we have demonstrated cell death in group of cells as well as confluent areas containing dead cells, it is the process of necrosis that may be the mechanism behind the observed effects. Although further investigation is warranted to elucidate the underlying mechanisms.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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AUTHOR'S CONTRIBUTION

AS: Proposed the research question and wrote the manuscript.
FA: Performed the data entry and analyzed the results.

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