Anticancer activity of *Illicium verum*, *Amomum subulatum* and *Parmotrema perlatum* extracts against MCF-7 human breast cancer cell line

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Abstract
Breast cancer remains one of the most widespread and lethal cancers among women worldwide, necessitating the development of more effective and less toxic therapeutic strategies. The study is aimed to assess the anticancer effect of combined extracts of Illicium verum, Amomum subulatum and Parmotrema perlatum on MCF-7 breast cancer cell lines using different assays. The ethanolic extracts of the spices were subjected to phytochemical analysis. In vitro antioxidant analysis was carried out to ascertain the effectiveness of combined extracts of spices compared to the individual extracts. The cytotoxicity of the combined extracts of I. verum, A. subulatum and P. perlatum was studied in the MCF-7 cell line by MTT, NRU and LDH assays. A variety of phytochemicals including phenolics, flavonoids, alkaloids, tannins and terpenoids were found to be present in the spice extracts. The combined extracts of the spices displayed significantly improved antioxidant activities in vitro when compared to the individual crude extracts.

The cytotoxicity studies on MCF-7 breast cancer cells indicate that the combined extracts of I. verum, A. subulatum and P. perlatum exhibited cytotoxic effect in a concentration-dependent manner where 300µg/ml, the highest concentration of the extract tested showed the highest cytotoxic potential on MTT assay. The results of the MTT assay correlated with NRU and LDH assays, all showing a significant cytotoxic effect of combined spice extracts against breast cancer. This could be due to the combined action of different phytochemicals present in the spice extracts that target multiple pathways involved in cancer pathogenesis. These findings suggest that the synergistic combination of Illicium verum, Amomum subulatum and Parmotrema perlatum holds promise as a potent therapeutic candidate for breast cancer treatment.

Keywords: Breast cancer, MCF-7 cell lines, Spices, Synergism, MTT assay.

Introduction

Cancer arises when abnormal cells in the body divide uncontrollably[33]. It is widely considered a global health problem that lacks a solution. In recent times, the rising prevalence of cancer has affected the physical, mental and social aspects of human life[4]. Breast cancer is rapidly becoming a major global health concern, ranking as the second most prominent cause of mortality among women. According to the World Health Organization (WHO), around 1 in 10 women globally suffers from breast cancer during lifetime[2]. A variety of risk factors namely race, ethnicity, genetic features, family history, lifestyle and environmental influences trigger cancer[12]. Breast cancer can be effectively treated if diagnosed early when it is still restricted to the breast or has only spread to the axillary lymph nodes. Advances in therapeutic strategies have improved the chances of a cure in 70-80% of the patients. However, advanced stages of breast cancer are considered untreatable with existing options[18].

Although extensive research is being done for cancer therapy, chemotherapy is the main available option which involves the use of cytotoxic drugs[24]. While chemotherapy is proven effective in some cases, it is often associated with side effects. The most common causes of treatment failure in maximum patients are resistance and adverse effects to chemotherapeutic drugs[33].

Medicinal plants and associated natural products have been employed for centuries by mankind for treating various disorders. Plants have been explored as sources of medicinally significant molecules with considerable therapeutic potential in treating various types of cancer[35]. The detection and isolation of anticancer phytochemicals vincristine and vinblastine from *Vinca rosea* are considered a significant achievement in anticancer agent research[26]. Plant-based medicines are increasingly becoming popular in developed as well as developing countries. The resurgence of community interest in alternative and complementary remedies is primarily due to the efficacy and safety of phytomedicines and also for their low cost and non-toxic properties[10].

Spices have been used as condiments for their flavor, color and taste for thousands of years. However, many spices are valued in folk medicine for their medicinal properties as they contain numerous bioactive compounds that offer a range of health benefits[38]. Research on the biological effects of spices shows that they possess a wide array of activities such as antioxidant, anticancer, anti-inflammatory and antimicrobial effects. Several spices can alter many cellular processes in cancer pathogenesis involving angiogenesis, cell division,

apoptosis and differentiation[21]. In the recent times, combining two different drugs has become a trend in treating human ailments, as it enhances drug efficacy by lowering the required dose and minimizing toxicity. This approach has also proven effective in overcoming the challenge of chemoresistance in cancer treatment[20].

Illicium verum, commonly known as star anise, is a fruit native to China and Vietnam and is extensively used both in cooking and traditional medicine. Medicinally, star anise is valued for its carminative, digestive, antispasmodic, expectorant, antioxidant and anti-inflammatory properties[27]. The bioactive compounds present in the fruit include monoterpenoids, flavonoids, limonene, α-pinene, α-terpinol, farnesol and safrol[39]. *Amomum subulatum*, commonly referred to as Black Cardamom, is commonly accessible and affordable and it contains a range of phytochemical compounds[13]. *Parmotrema perlatum* Huds, commonly known as Stone Flower, is a lichen found on old trees across hilly regions in Europe, North and South America and Asia. It is also cultivated in the mountainous areas of the Himalayas and Kashmir. Traditionally, *P. perlatum* has been utilized to treat various ailments including skin disorders, respiratory and pulmonary ailments and arthritis and is also employed as a spice[14].

In the current study, an effort was made to qualitatively analyze the phytochemical content of *I. verum, A. subulatum* and *P. perlatum*. Further, the synergistic *in vitro* antioxidant potential was assessed and also the anticancer activity of the combined *I. verum, A. subulatum* and *P. perlatum* extracts was studied in the MCF-7 cell line using MTT, NRU and LDH assays.

Material and Methods

Materials: The chemicals used were obtained from Sigma Aldrich and Hi Media. The MCF-7 breast cancer cell line was acquired from the National Centre for Cell Science, Pune.

Sample collection and processing: The spices namely *Illicium verum, Amomum subulatum* and *Parmotrema perlatum* were sourced from a local market in Coimbatore, Tamil Nadu. The spices *I. verum* and *A. subulatum* were identified and authenticated by BSI, TNAU Campus, Coimbatore, Tamil Nadu (Ref. BSI/SRC/5/23/2023/Tech-152 and Ref. BSI/SRC/5/23/2023/Tech-153). The spices were washed, shade-dried and finely powdered before being stored for future use.

Preparation of extracts: For extraction, the maceration technique was used where five grams of powdered spice materials were mixed with 50 ml of ethanol and incubated in a shaker incubator at 40°C for 48 hours, after which they were filtered and collected.

The collected extract was then evaporated to obtain dry extracts. The dry extract was stored at -20°C. To perform synergistic studies, 5ml (1mg/ml) of extracts of *I. verum, A. subulatum* and *P. perlatum* each, were mixed and taken for analysis.

Qualitative phytochemical analysis: The ethanolic extracts of *I. verum, A. subulatum* and *P. perlatum* were screened for the presence of phytochemicals like phenolics, flavonoids, terpenoids, alkaloids, carbohydrates, saponins, proteins, steroids, tannins, quinones, glycosides and oil and resins using standard reference methods[16,19,23].

***In vitro* antioxidant analysis**

Estimation of ferric reducing antioxidant power (FRAP) activity: 0.9 ml FRAP reagent was mixed with 90µl water and 10µl of the spice extracts. The reaction mixture was incubated at 37°C for 30 minutes and the absorbance was measured at 593 nm[6].

Estimation of DPPH radical scavenging activity: Spice extracts at different concentrations (250-1000µg) were taken and the volume was made to 100µl with methanol. 5 ml of 0.1 mM methanolic solution of DPPH was added. The absorbance of the sample was measured at 517 nm after incubating for 20 min at 27°C[8]. Ascorbic acid was used as a standard. The percentage radical scavenging activity of the sample was calculated using the following formula:

$$\% \text{ DPPH radical scavenging activity} = \frac{Abs\ (control) - Abs\ (test)}{Abs\ (control)} \times 100$$

Estimation of hydrogen peroxide radical scavenging activity: Hydrogen peroxide solution (40 mM) was prepared in phosphate buffer (50 mM, pH 7.4). 1 ml of the sample at different concentrations (250- 1000 µg/mL) in methanol was mixed with 0.6 ml of 40mM hydrogen peroxide, incubated for 10 minutes and the absorbance was read at 230 nm against a blank solution[30]. Ascorbic acid was used as a standard. The percentage of hydrogen peroxide scavenging activity is calculated using the following formula:

$$\% \ H_2O_2 \text{ scavenged} = \frac{Abs\ (control) - Abs\ (test)}{Abs\ (control)} \times 100$$

***In vitro* anticancer activity**

Cell lines and culture medium: The human breast cancer cell line (MCF-7) was sourced from the National Centre for Cell Science (NCCS), Pune and grown in Eagle's minimum essential medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37°C, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly and the culture medium was changed twice a week.

Cytotoxicity assessment by MTT assay: The *in vitro* cytotoxicity of the combined spice extracts was investigated using MCF-7 cells by MTT assay[25]. The cell lines were treated with combined spice extract at concentrations of 50, 100, 150, 200, 250 and 300µg/ml. After incubating for 24 h,

15µl of MTT (5mg/ml) was added to each well and incubated for 4 h at 37°C. The supernatant was removed and the formed formazan crystals were solubilized in DMSO and the absorbance was recorded at 550nm.

Cytotoxicity assessment by NRU assay: The cytotoxicity of the synergistic plant extract was evaluated by NRU assay[9]. The cell lines were treated with synergistic spice extract at concentrations ranging from 50, 100, 150, 200, 250 to 300µg/ml. After 24 h incubation, the wells were washed with PBS and neutral red solution (50 µg/mL) was added to each well and incubated for further 3 h. The cells were washed with 1% calcium chloride and 0.5% formaldehyde solution after which 50% ethanol and 1% acetic acid solution were added to each well for the dye extraction. The absorbance was recorded at 550 nm.

Cytotoxicity assessment by LDH assay: LDH assay was performed to study cytotoxicity by assessing the membrane integrity using kit method[9]. Cells were treated with synergistic spice extract at concentrations 50, 100, 150, 200, 250 to 300µg/ml and incubated for 24 h. The culture medium was aspirated and centrifuged at 4000 rpm for 5 minutes. 0.1 ml of the supernatant was added to a 96-well plate with 0.1 ml reaction mixture and incubated for 30 minutes in the dark. The absorbance of the sample was recorded using a microplate reader at 490 nm.

Results and Discussion

Breast cancer remains one of the most prevalent cancers worldwide, particularly affecting women and accounting for a significant number of cancer-related deaths. Despite advances in chemotherapy, radiotherapy and hormone treatments, resistance to these conventional therapies often leads to treatment failure, recurrence and metastasis[5]. This has spurred increased interest in alternative therapies, particularly those derived from natural sources. Natural products, including plant extracts, have been valued since ancient times for their therapeutic properties, with various cultures using herbs and spices for disease prevention and healing[15]. In recent years, the World Health Organization (WHO) has highlighted the need for integrating traditional medicine including plant-based therapies, into modern healthcare systems, recognizing their potential to complement standard cancer treatments[36]. Spices are especially significant due to their bioactive compounds, which possess antioxidant, anti-inflammatory and anticancer properties[34]. These natural compounds could offer novel, less toxic treatment options, providing a complementary approach to combat breast cancer while minimizing side effects associated with conventional therapies. In this study, an attempt was made to assess the anticancer effects of combined extracts of spices on MCF-7 breast cancer cell lines.

Qualitative phytochemical analysis: The ethanolic extracts of *Illicium verum, Amomum subulatum* and *Parmotrema perlatum* were examined for phytochemicals including phenolics, terpenoids, alkaloids, carbohydrates, saponins, steroids, tannins, flavonoids, quinones, glycosides, proteins and oil and resins. Proteins were not present in all three spice extracts. Oils and resins were also absent in the *P. perlatum* extract. The phytochemical screening results are given in table 1. Studies have pointed out the incidence of alkaloids, flavonoids, terpenoids and tannins in these three spices, supporting the results of the present study[3,22].

***In vitro* antioxidant analysis:** The spice extracts were analyzed for their antioxidant activities *in vitro.* The combined as well as crude spice extracts in different concentrations ranging from 250, 500, 750 and 1000 µg/ml were used. To prepare combined extracts of spices, 5ml (1mg/ml) of extracts of *I. verum, A. subulatum* and *P. perlatum* each were mixed and taken for analysis.

Ferric reducing antioxidant power (FRAP) assay: FRAP method relies on the capability of an antioxidant molecule to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions[31]. The FRAP activity of the spice extracts is presented in table 2.

Table 1
Phytoconstituents present in the spice extracts

Phytochemical	*Illicium verum*	*Amomum subulatum*	*Parmotrema perlatum*
Phenolics	+	+	+
Flavonoids	+	+	+
Terpenoids	+	+	+
Alkaloids	+	+	+
Carbohydrates	+	+	+
Saponins	+	+	+
Proteins	-	-	-
Steroids	+	+	+
Tannins	+	+	+
Quinones	+	+	+
Glycosides	+	+	+
Oil and Resins	+	+	-

+ indicates presence, - indicates absence

FRAP assay indicated that the synergistic spice extracts had a higher FRAP value of about 101.01 ± 2.2 mmol (Fe (II)/g sample compared to crude extracts. *I. verum* extract had a FRAP value of about 59.9 ± 2.1 mmol (Fe (II)/g sample followed by *A. subulatum* which had a value of about 58.94 ± 3.75 mmol (Fe (II)/g sample. *P. perlatum* had the lowest value of about 68.03 ± 1.1 mmol (Fe (II)/g sample compared to other extracts. The standard antioxidant, ascorbic acid had a FRAP value of about 127.7 ± 1.73 mmol (Fe (II)/g sample extract. The results indicate that the antioxidants present in the spice extracts exhibited reducing power by reducing Fe^{3+} to Fe^{2+} ions.

DPPH Radical Scavenging Activity: DPPH radical scavenging activity measures the capacity of antioxidants like phenolics in plant extracts to react with and reduce DPPH radical[5]. Once reduced, the dark purple hue in the solution is transformed to colorless or light yellow. The results indicate that combined spice extract had the highest radical scavenging activity at 83.43% at 1000 µg/ml. Among the crude extracts, *A. subulatum* had the highest with 75.48% followed by *I. verum* at 72.43% and *P. perlatum* at 66.78% at 1000 µg/ml. The results of the DPPH radical scavenging activity are presented in figure 1.

Table 2
FRAP assay of spice extracts

Extract	FRAP* (mmol (Fe (II)/g sample)
Illicium verum	59.9 ± 2.1
Amomum subulatum	58.94 ± 3.75
Parmotrema perlatum	68.03 ± 1.1
Combined extract	101.01 ± 2.2
Ascorbic acid	127.7 ± 1.73

*Values are Mean ± SD of triplicates

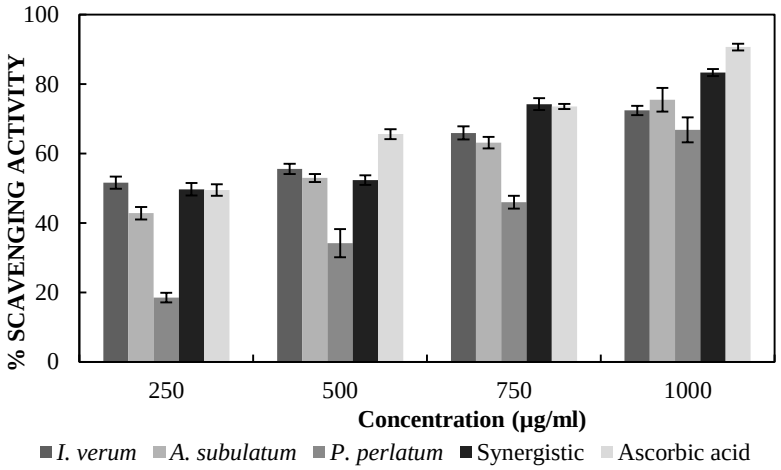

Figure 1: DPPH radical scavenging activity of spice extracts

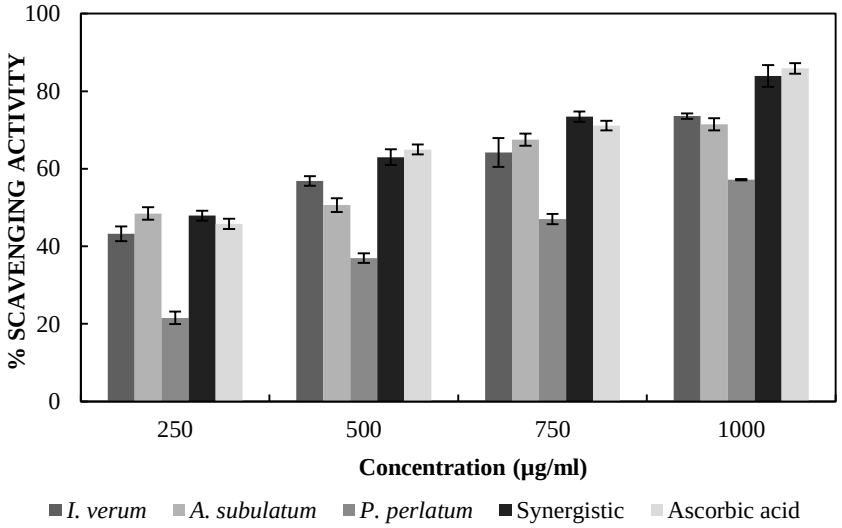

Figure 2: Hydrogen peroxide radical scavenging activity of spice extracts

H_2O_2 Radical Scavenging Activity: The H_2O_2 scavenging activity of the spice extracts is presented in figure 2. The H_2O_2 scavenging activity of the extracts was the highest in the combined extract with 83.91% at 1000 µg/ml followed by *I. verum* at 73.57%, *A. subulatum* at 71.44% and *P. perlatum* at 57.19% at 1000 µg/ml. Hydrogen peroxide, formed in biological systems, is quickly decomposed to oxygen and water. The process may also create hydroxyl radicals ($OH^{\cdot}$), initiating lipid peroxidation and causing damage to DNA. The spice extracts exhibited H_2O_2 scavenging potential which might be due to the phytochemicals that can neutralize hydroxyl radicals into water[7].

***In vitro* cytotoxicity assays:** The combination of two or more different drugs has shown to be effective in treating human ailments, as drug efficacy can be improved by limiting toxicity and reducing dosage. This combination therapy is also effective in overcoming chemoresistance[37]. This is relevant to this study as the above antioxidant and anti-inflammatory analysis shows that the combined ethanolic extracts of spices *I. verum, A. subulatum* and *P. perlatum* had a better antioxidant and anti-inflammatory capacity than the crude individual extracts.

Hence further *in vitro* cytotoxicity analysis was accomplished to examine the anticancer ability of the combined extracts of *I. verum, A. subulatum* and *P. perlatum* in MCF-7 cell lines by different cytotoxicity assays.

MTT assay: The cytotoxicity of synergistic ethanolic extract of spices was determined in MCF-7 cell lines at 50, 100, 150, 200, 250 and 300µg/ml concentrations. MTT assay is an easy, sensitive and reliable approach for measuring the cytotoxic effects of plant extracts. The assay measures the formation of insoluble purple formazan from mitochondrial dehydrogenase in live cells. The determination of mitochondrial dehydrogenase activity of live cells reflects the number of live cells[1]. Cell viability analysis indicated that the combined spice extract caused cytotoxicity of the MCF-7 cell lines in a concentration-dependent way with an IC_{50} value of about 47.10 µg/ml.

The results show that incubation of MCF-7 cells for 24 hours with the combined spice extract is toxic to the cells. The findings of the MTT assay are presented in figures 3a and 3b. Previous studies on the anticancer properties of *I. verum* and *A. subulatum* on MCF-7 cell lines also indicate that the extracts exert cytotoxicity, inhibition of cell growth and apoptosis[28,32]. These findings support the results of the current study and further, this study presents the anticancer effects of combined extract of *I. verum, A. subulatum* and *P. perlatum* on breast cancer cell lines.

NRU assay: NRU assay assesses the integrity of lysosomes of live cells. The dye neural red is accumulated in the lysosomes of live cells whereas the dead or damaged cells do not take up the dye. The absorbance of the dye from the cells is relative to the percentage of living cells[11]. The neutral red uptake assay in MCF-7 cell lines was performed with the synergistic spice extract at different concentrations (50-300µg/ml). Cell viability analysis showed that the synergistic spice extract exhibits cytotoxicity in a concentration-dependent way with an IC_{50} value of 53.10 µg/ml similar to the findings of the MTT assay. The findings of the NRU assay are presented in figure 4.

LDH assay: The membrane damage in MCF-7 cells after treatment with combined spice extract was evaluated by assessing the release of lactate dehydrogenase (LDH). It is a cytoplasmic enzyme that converts pyruvate to lactate and is released from the cells when the cell membrane is damaged. Therefore, the measurement of LDH activity provides information about the percentage of dead cells[29]. The control cells released less LDH compared to combined spice extract-treated cells. The treatment with combined spice extract led to an increase in the release of LDH in a dose-dependent way showing membrane damage and possible necrosis. As the concentration of spice extract increased, the release of lactate dehydrogenase from the cells increased. The findings of the LDH assay are presented in figure 5.

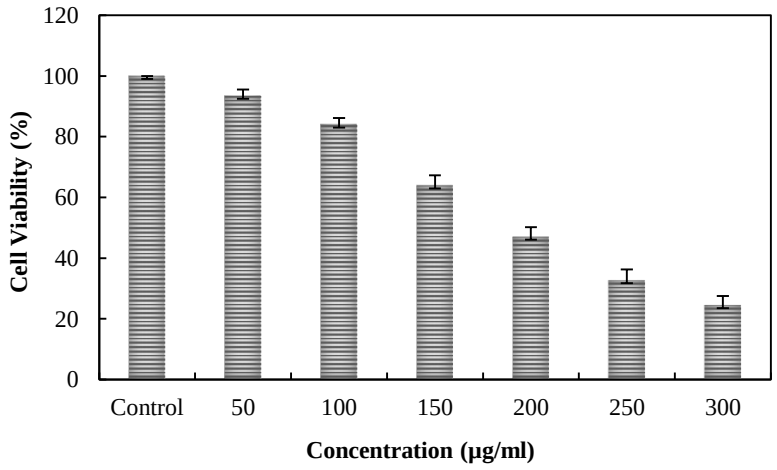

Figure 3a: MTT assay of synergistic spice extracts on MCF-7 cell lines

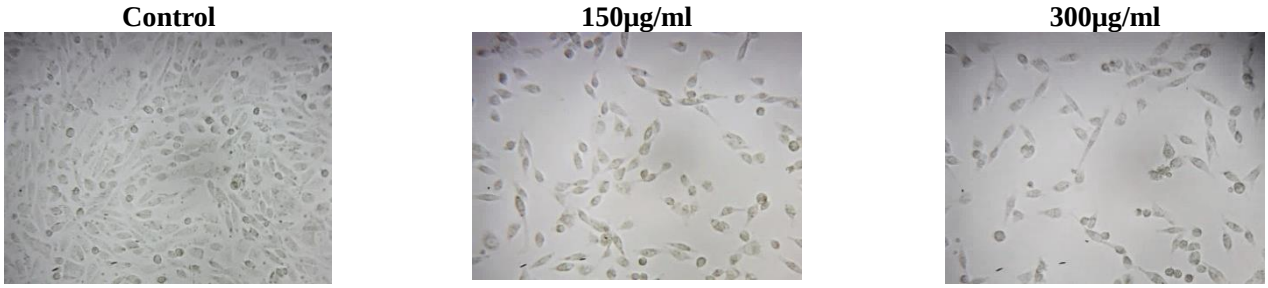

Figure 3b: Viable cell counts and morphological changes in control and synergistic spice extract treated MCF-7 cells after 24h at concentrations 150 and 300 µg/ml

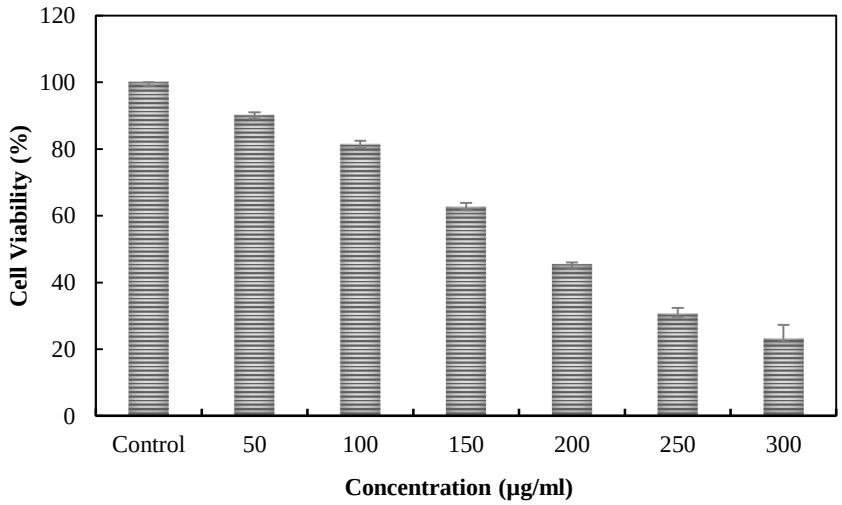

Figure 4: NRU assay of synergistic spice extracts on MCF-7 cell lines

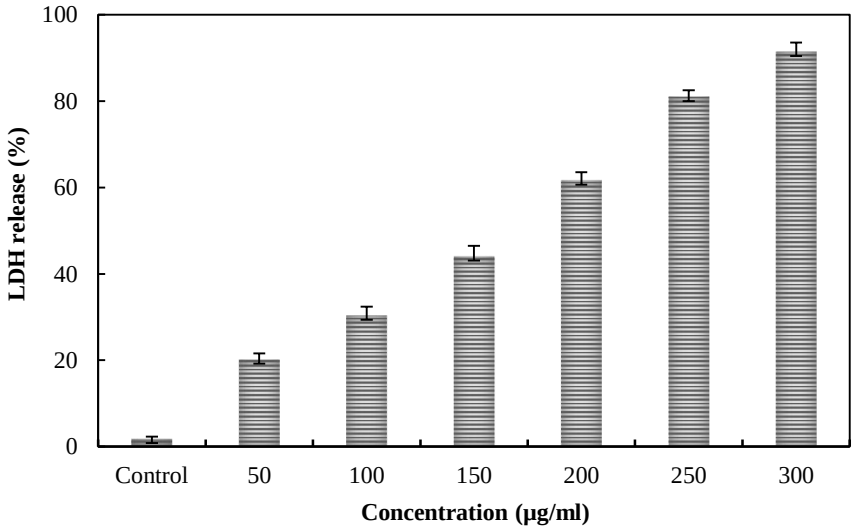

Figure 5: LDH assay of synergistic spice extracts on MCF-7 cell lines

Conclusion

The current research underscores the significant potential of combining *Illicium verum, Amomum subulatum and Parmotrema perlatum* extracts in the management of breast cancer, specifically targeting the MCF-7 cell line. The observed synergistic anticancer activity suggests that these spice extracts work together to inhibit cancer cells more effectively and present a promising approach to cancer therapy. This synergy could be accredited to the diverse range of phytochemicals present in each spices which may target multiple pathways involved in cancer cell survival and growth.

The findings provide a foundation for further exploration into the molecular mechanisms underlying this synergy and open avenues for developing novel therapeutic strategies

that integrate these botanical extracts into breast cancer treatment. Future research should focus on the molecular mechanisms underlying this synergy. Additionally, i*n vivo* studies are also needed to validate these findings as well as to ensure their effectiveness in biological systems.

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