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INVESTIGATION OF THE EFFECTS OF EXTRACTION POLARITY CHANGE ON THE BIOACTIVITY OF *Eruca Vesicaria*

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Abstract: *Eruca vesicaria* (arugula) plant is frequently used today as a daily food source that contains a rich variety of minerals and vitamins, especially vitamin C. It also contains a high percentage of phenolic compounds which are structures that plants develop to protect themselves from harmful organisms. Phenolic compounds found in plants are obtained by different extraction methods and have a high antioxidant effect. In this study, we report, anticancer activity extract from *Eruca vesicaria* against human prostate cancer cells (PC-3) in vitro. The phenolic substances contained in the plant were obtained in different concentrations by the extraction technique based on the polarity difference. HPLC and total phenol content were determined to perform extraction content analysis. According to in vitro MTT cell proliferation assay, it acted at high concentrations, regardless of polarity differences. The highest cytotoxic effect was observed in extract extracted with 50% ethanol concentration. It has been observed that it has an anticancer effect compared to the determination of total phenol content. Also, because of 24-hour MIC analysis, it shows antibacterial properties according to agent concentration. As a result of this study, it adds many new information to the literature, but also provides guidance for future research.

Keywords: *Eruca vesicaria*, Anticancer, Antibacterial, Phenolic contents, HPLC

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1. Introduction

Food consumption plays an important role in modulating certain degenerative processes that affect an organism's quality of life. The strongest evidence that vegetables and fruits are associated with a potential reduction in cancer risk comes from epidemiological studies (Gasper et al., 2007). Arugula species (*Eruca vesicaria*), which are among the vegetables, contain a range of phytochemicals that support health, including carotenoids, vitamin C, fibers and polyphenols (Bell and Wagstaff, 2019). *Eruca vesicaria* is thought to be an excellent source of antioxidants, such as phenolic compounds (Boyd et al., 2011). *Eruca vesicaria*, which grows in aquatic environments, is a plant that regulates its bioactive chemical content according to the amount of water in growing conditions (Bianco and Boari, 1996). *Eruca vesicaria* is a plant species rich in various minerals and vitamins, especially vitamin C. At the same time, this biochemical content gives the plant its own bitter taste and aroma (Bell and Wagstaff, 2019). The main bioactive chemical ingredients of the plant are phenolic compounds in general (Gillian, 2009). Phenolic compounds are an important group of bioactive chemicals that determine the taste, aroma and color of plants. In general, these substances are compounds that contain one or more hydroxyl groups in their aromatic ring. Structures with a single hydroxyl group are called phenols, and all phenolic compounds are thought to derive from this group. Nowadays, more than five thousand phenolic substances have been identified in plants (Bennett et al., 2006).

Developing technology and changing living conditions cause various diseases in today's world. The most important of these diseases is cancer. The effect of free radicals on the formation of cancer is among the topics frequently explored in scientific studies (Peroni et al., 2019). Antioxidant substances are used to prevent the formation of these free radicals or reduce the existing ones. In this context, plant-derived antioxidants and phenolic compounds serve as free radical scavengers with high reduction capacities (Jin et al., 2009, Allen and Tresini, 2000).

Various extraction methods are used to obtain these antioxidants provided from plants (Kim and Ishii, 2006). Productivity has also increased with the use of chemicals. However, research in the literature shows that comments are made on the results made with a single solvent. It is known that changing the polarity of the solvent will change the type and amount of the dissolved substance (Lamy et al., 2008; Bell et al., 2015).

The extraction of *eruca vesicaria* plant was done with 3 different polarity solvent groups. The phenolic change in the content of the obtained herbal extracts was determined by the ultra-pressure liquid chromatography

method (UHPLC) and spectroscopic analysis method. The content of matter was calculated by UHPLC method, and the total amount of phenol was determined by spectroscopic method. The effect of phenolic substance quantity changes on gram-positive and gram-negative bacteria was examined. Disc diffusion sensitivity test and microplate test were performed, where the presence of antibacterial property and how effective it would be if it existed were calculated. Finally, the anticancer effect on prostate cancer was measured using the MTT test.

2. Materials and Methods

2.1. Reagent

Dulbecco's modified Eagle's minimal essential medium-high glucose (DMEM), fetal bovine serum (FBS), MTT dye (thiazolyl blue tetrazolium bromide), ethanol (%99), acetic acid and acetonitrile were supplied from Sigma-Aldrich (St Louis, MO, USA). Antibiotic/antimycotic solution was purchased from Biological Industries (Cromwell, CT, USA). Dimethyl sulfoxide (DMSO), sodium carbonate and Folin-Ciocalteu reagent were obtained from Merck (Darmstadt, Germany).

2.2. Extraction of Plant Materials

The *Eruca vesicaria* plant was taken and washed, the remains of agricultural medicine and soil were removed and dried at room temperature. *Eruca vesicaria* leaves were ground in a bench top mill. Ethanol was prepared in three different concentrations: 10%, 50% and 90%, then these were mixed with freshly ground *eruca vesicaria* in a ratio of 1:10 under magnetic stirring at room temperature for 24 hours. At the end of the required extraction time, the liquid extract was filtered with vacuum pump, and then ethanol content was removed by using rotary evaporator under vacuum at 45°C. The supernatant of aqueous extract was subjected to freeze-dried.

2.3. HPLC Analysis

High pressure liquid chromatography (HPLC) analysis was performed with a Thermo Scientific Ultimate 3000 HPLC. A reversed-phase column, ODS-2 HYPERSIL RP 18 with a 3-µm particle size (ThermoFisher Scientific, USA), was used at the flow rate of 1 mL min-1. Mobile phase gradient was performed by varying the proportion of solvent A (2.5% acetic acid) to solvent (100% acetonitrile) as follows: initial 1% B; linear gradient to 40% B in 40 minute. The samples were prepared at concentration of 50 mg/ml in 100% acetonitrile and injected sample volume was 20 µl. All solutions were filtered prior to injection through 0.20 µm membrane filters (Millipore, Bedford, MA, USA). The column temperature was at 25°C. The measurement was held at 254 nm.

2.4. Determination of Phenolic Content

Total phenolic content of *Eruca vesicaria* extract was determined by Folin-ciocalteu method (Slinkard and Singleton 1977). Folin-ciocalteu reagent was prepared by 1:10 dilution of stock solution. Sodium carbonate solution of 7% was prepared in distilled water. Gallic acid was used as standard in the calibration curve. *Eruca vesicaria* extract was dissolved in distilled water. 20 µl of each sample was mixed with 100 µl Folin-ciocalteu reagent and incubated for 2.5 min. Then 80 µl of sodium carbonate solution was added. The mixture was kept in dark for 1 hour. Samples were subjected to photometric measurement at 725 nm. The experiments were conducted in 3 replicates, and results were expressed as mg of gallic acid equivalents (GAE)/gr dry weight extract. The phenolic content was defined as gallic acid equivalents using the following linear equation 1 based on the calibration curve:

$$X = 0.99Y + 9.925 \times 10^{(-3)} \quad (1)$$

R2 = 0.9998, where X is the absorbance and Y is concentration as gallic acid equivalents (µg/g).

2.5. Minimal Inhibitory Concentration Assay

Stock solution was prepared with 100% DMSO at a rate of 1mg/ml from the extracts. Dilution was performed for each sample as shown in Table 1. L.monocytogenes, E. coli and 5% DMSO were used as a control group. Bacteria were subjected to serial dilutions. 96 well plates were used for measurement. At the end of all dilutions, 100 µL was first placed in each well in two sets of our diluted samples, then 100 µL was placed in each well from our diluted bacteria.

Table 1. Dilution rates of *Eruca vesicaria* extracts of 10%, 50% and 90% with medium

Concentration	Media	Agent	Total Volume
0.5 mg/ml	960 µL	240 µL	1200 µL
0.25 mg/ml	500 µL	500 µL	1000 µL
0.125 mg/ml	300 µL	300 µL	600 µL

2.6. Cell Culture

The human prostate cancer cell line PC-3 (ATCC CRL-1453) and human embryonic kidney cell line HEK-293 (ATCC CRL-1573) were used in this study and were maintained in DMEM supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution. Then, cells were maintained at 37 °C and 5% CO_2 in an incubator (Nuve EC 160). Cells were subculture every 48 hours.

2.7. Cytotoxic Activity Determination by MTT

The MTT cell proliferation assay was performed to observe the viability of PC-3 cells treated with *Eruca vesicaria* extracts (Eroglu et al., 2020). Cytotoxicity of *eruca vesicaria* extract was evaluated by the MTT (tetrazolium (3-{4,5-dimethylthiazol-2-yl}-2,5-diphenyl tetrazolium bromide) assay. Extract was dissolved and diluted serially in DMEM with FBS(10%) and filter-sterilized. PC-3 cells and HEK-293 cells were incubated in an incubator at 37°C and 5% CO_2. PC-3 cells and HEK-293 cells were grown in the absence or presence of various concentration of *eruca vesicaria* for 24, 48 and 72 hours. Then the cells were exposed to MTT for 3 h in dark at

37°C. Mitochondrial hydrogenases in the viable cells reduce MTT into formazan crystals which can be dissolved in DMSO. Average absorbance value was measured at differences between 690 nm and 570 nm. Cell viability was calculated by using below formula:

Cell viability:100% × ((Average absorbance value of treated cells)/(Average absorbance value of control cells))

3. Results

3.1. Extraction

Extraction processes were performed under constant stirring and at room temperature for 24 hours (Fig. 1A). Color changes were observed depending on the alcohol concentration as desired (Fig. 1B). The resulting extracts were kept inside the glass tubes at +4°C for at least 24 hours so that the chloroplast and chlorophyll residues inside them would collapse to the bottom. Liquid phases separated from chlorophyll and chloroplasts were transferred into lyophilization bottles (Fig. 1B).

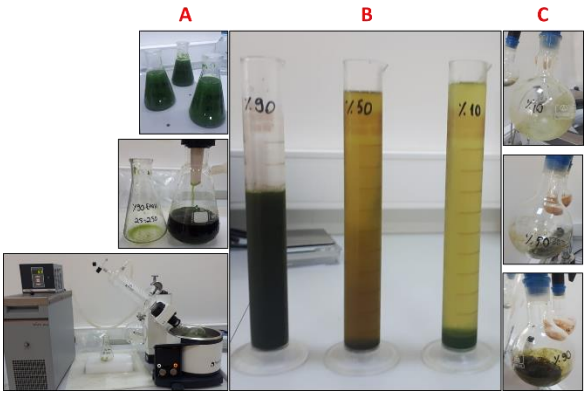

Figure 1. All extraction processes, respectively. A) Sample preparation, filtering and rotary evaporator, B) Color change according to the concentration of ethanol, 90%, 50% and 10% respectively, C) Freeze-dried process (Lyophilization).

Percentage of the extraction yield (25 gram of dry leaves of *eruca vesicaria* was used for extraction) after lyophilization were measured at 18.12% yield for a sample extracted with 10% ethanol, 21.18% yield for a sample extracted with 50% ethanol, and 19.02% yield for a sample extracted with 90% ethanol (Fig. 1C). The structure of the 10% extraction was in the form of powder, while the 90% extraction was obtained in a denser structure. The reason for this is the rate at which phenolic compounds in the content of *eruca vesicaria* are obtained depending on the alcohol concentration. After the extraction processes have been successfully carried out, the characterization phase has been initiated.

3.2. Effect of Extraction Parameters on Total Phenol Content (TPC)

The extracts were analyzed for their total phenolic content by Folin-ciocalteu method. The results can be seen in Table 2 as milligram Gallic acid equivalent per gram dry weight of extract.

Table 2. Total phenol content of *eruca vesicaria* extract prepared at different extraction conditions

Sample	TPC (mg Gallic Acid equivalent/ gr DW)
10% *eruca vesicaria*	63,49
50% *eruca vesicaria*	67,01
90% *eruca vesicaria*	57,66

Total phenol analysis is done to each component and shows how much phenolic compound is in its content. TPC does not give the content of the phenolic compound, it indicates the proportion in which the amount of phenolic is found (Elsadek et al., 2021). Total phenol analysis in three different concentrations was performed for plant extract analysis. It was analyzed in samples that were extracted with the highest 50% and lowest 90% alcohol concentration. This is because phenolic compounds in the *eruca vesicaria* plant were extracted with 10% and 50% ethanol concentrations, while all phenols were obtained and 90% were not sufficient for all phenols. This could be understandable because the polarity of the solvent had a high influence on the solubility of the phenolic compounds. The phenolic content of *eruca vesicaria* consists of substances with polar properties.

3.3. HPLC Analysis of the Extract

The HPLC method is used to analyze which type of phenolic compound is in the content of the resulting component. In our experiment, the aim is that the extraction process performed at different concentrations affects the types of phenolic compounds to be obtained. Our measurements were carried out at a flow rate of 1 mL min-1 with reverse phase column at 254 nm.

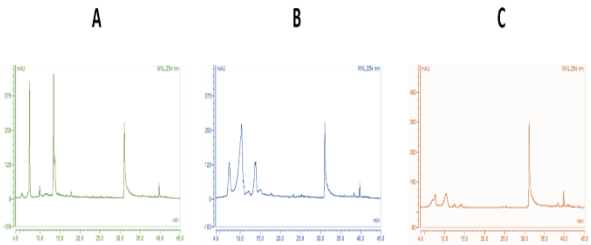

Figure 2. HPLC results of *eruca vesicaria* extracted with 10% (A), 50% (B) and 90% (C) ethanol, respectively. Each of them has been solved in its own solution and subjected to chromatography.

The compositional differences between the extracts which caused the diversity in the total phenol contents were investigated and confirmed by HPLC analysis. HPLC chromatograms showed consistency with total phenolic content results. Although there are proportional differences between the samples, it is observed that the same phenolic compounds are obtained. Phenol intake rates varied depending on alcohol concentration. Similarities have been observed between substances clinging to the column depending on polarity. The first

substance in the samples to come out of the column and reach the detector is the first peak, that is, the drift rate is high, the retention time is short. At the last peak, the drift rate is slow, and the retention time is longer. Due to the polarity difference of the solvent at the extraction stage, Figure 2A and Figure 2C observed field differences in the peaks, although the peaks were read at the same minute. The reason for this is the concentration of alcohol in the extraction stage.

3.4. MIC Experiment

Dilution methods are used to detect MIC values of antimicrobial agents. These methods are reference methods for antimicrobial sensitivity tests. Dilution tests are based on visible reproduction in Microplate wells or on the surface of agar containing dilutions of antimicrobial agent. The lowest concentration of antimicrobial agents, in which the visible reproduction of a microorganism is inhibited, is determined as "Minimal inhibitory concentration (MIC)" (Chiemchaisri et al., 2021; Xia et al., 2021).

The MIC test is a more effective method to determine whether the compound is antibacterial. Bacterial growth is studied depending on the time. Plant extracts prepared in different concentrations have been tested on *L. monocytogenes* and *E. coli* bacteria.

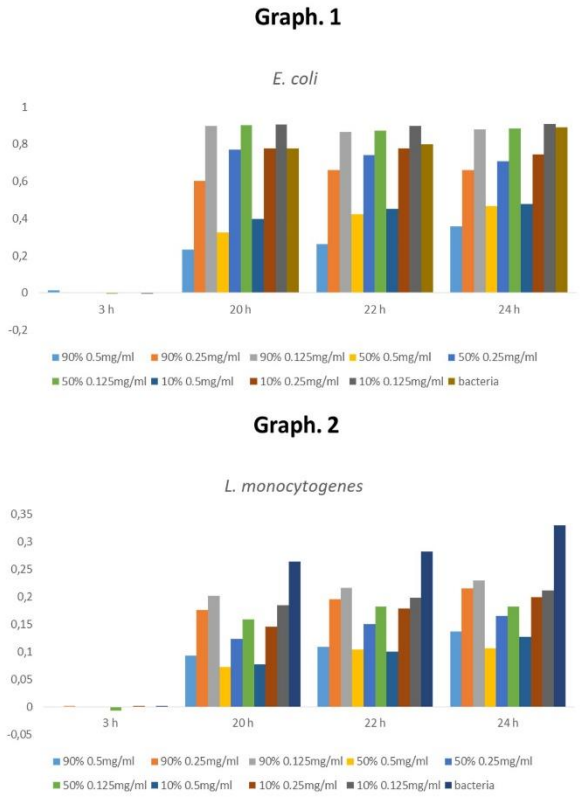

Figure 3. The charts show the results of the MIC analysis method in 3, 20, 22 and 24 hours, respectively. Graph 1.) Measurements taken for *E. coli* according to concentration differences and time changes, Graph 2.) Measurements taken for *L. monocytogenes* according to concentration differences and time changes.

The most bacterial deaths were observed in a sample prepared with 5 mg/ml of 90% for *E. coli* (Fig.3 Graph 1). In 5 mg/ml concentrations of 50% and 10% samples were observed close results at the end of 24 hours. The control group shows the normal growth rate of the bacterium, and our plant extracts of different concentrations have been observed to inhibit growth for the *E. coli*. Samples prepared with 0.125 mg/ml for *E. coli* were not effective, this may be due to the high resistance of the bacterium or low concentration of the agent.

For *L. monocytogenes* (Fig.3 Graph 2), the most bacterial deaths were the sample prepared with 5 mg/ml of 50%. At the end of 24 hours, there was also death at 5 mg/ml concentrations of 90% and 10% samples. Plant extract was more effective in this type of bacteria, while the control group was the normal growth rate of the bacteria, the samples extracted in different concentrations and prepared in different concentrations were all less than the normal growth rate of the bacterium and showed antibacterial effect for the *L. monocytogenes*.

3.5. MTT Cell Proliferation Assay

The MTT viability test was performed to observe how the extractions affected human prostate cancer cells for cytotoxicity analysis. The goal is to test whether the *eruca vesicaria* plant has an anti-carcinogenic effect. For this purpose, PC-3, a prostate cancer cell, and HEK-293, a healthy kidney cell, were used as a control group. In Fig.4, MTT assay was performed with different concentrations of prepared agents of the *eruca vesicaria* plant extracted according to the polarity change.

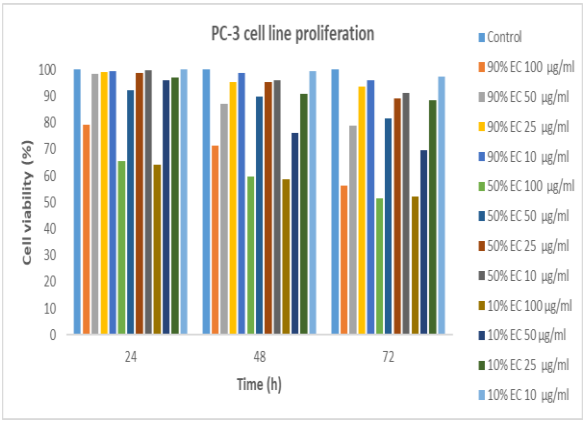

Figure 4. MTT test result on prostate cancer cell line PC3 according to time and concentration change of extracted plant according to time. In figure extraction conditions ethanol concentration of 10%, 50% and 90%, and their different concentration of extract. Human embryonic kidney cell line HEK-293 was used for control group.

Human embryonic kidney cells were used to study the cytotoxicity activity of extraction. The results showed that the agent at each concentration does not kill HEK-293 cells. The bar shown in blue in Figure 4, called control, represents HEK-293 cells. Since no death due to the extract was observed in the experiments performed here, it is shown as a single bar to simplify the graph. In

Figure 4, cell viability was measured according to the concentration change of the plant extracted with different concentrations of ethanol at 24, 48 and 72 hours. At the lowest concentrations, no results were achieved, the cancer cells continued to grow. But PC3 cell line deaths were observed in measurements taken in the first 24 hours at 100 µg/ml and 50 µg/mL. By the end of 72 hours, there was half death. This means that measurements made in high concentrations create an anti-carcinogenic effect through the cancer line. Cytotoxic evaluation of *eruca vesicaria* for 24, 48 and 72 hour was compared with total phenol content. The cell viability test is similar according to the phenol amounts obtained in proportion to the total phenol analysis and HPLC analysis. It has been observed in this study that extracts having higher total phenol content leads to cytotoxic activity on fibroblast cells, in accordance with the theory. Cytotoxicity activity and total phenol content is found as closely related with the extraction parameters such as extraction time, extraction medium system and temperature. It can be concluded that optimization of extraction plays a key role in properties of the extract, that is also relevant with its.

4. Discussion

The present study aimed to optimize the extraction process of *Eruca vesicaria* and evaluate its effects on total phenolic content (TPC), phenolic compound composition, antimicrobial activity, and cytotoxicity. Our findings revealed a distinct correlation between the ethanol concentration used in the extraction process and the yield, phenolic content, and biological activities of the extracts. The results suggest that ethanol concentration significantly impacts the solubility of phenolic compounds, influencing the extract's overall properties.

The extraction yields varied with ethanol concentration, with the 50% ethanol extraction yielding the highest (21.18%), followed by 90% (19.02%) and 10% (18.12%). This outcome reflects the solubility behavior of polar phenolic compounds, which are more effectively extracted in a moderately polar solvent such as 50% ethanol. The lower yield at 90% ethanol suggests a reduced efficiency in extracting polar phenolic compounds, likely due to the decreased polarity of the solvent. This observation aligns with previous studies, where optimal extraction conditions for phenolic compounds were found to depend on the balance between solvent polarity and phenolic solubility (Elsadek et al., 2021).

TPC analysis corroborated these findings, with the 50% ethanol extract showing the highest phenolic content (67.01 mg GAE/g DW), while the 90% ethanol extract exhibited the lowest (57.66 mg GAE/g DW). The observed decrease in TPC at higher ethanol concentrations suggests that while a more nonpolar solvent may enhance the extraction of some compounds, it is less efficient for phenolic extraction. This trend reflects the polarity-dependent solubility of phenolic compounds, which are primarily polar in nature. It is well-established that ethanol-water mixtures provide a more balanced polarity, thereby facilitating a higher extraction of phenolics (Xia et al., 2021).

HPLC analysis further elucidated the effect of ethanol concentration on the types of phenolic compounds extracted. While all extracts displayed similar phenolic profiles, the peak intensities varied, indicating that ethanol concentration influences the relative amounts of different phenolics. Notably, the 50% ethanol extract had a more balanced phenolic composition, as seen in the chromatographic peaks. This suggests that an intermediate solvent polarity optimally extracts a broader range of phenolic compounds. The longer retention times in the 10% and 90% ethanol extracts may also indicate that solvent polarity impacts the interaction of phenolics with the column, leading to varied retention behavior depending on the compound's structure and polarity.

The MIC assay demonstrated significant antimicrobial activity of the *Eruca vesicaria* extracts, particularly against L. monocytogenes and E. coli. The most potent antibacterial effect was observed in the 90% ethanol extract at a concentration of 5 mg/mL for E. coli and in the 50% ethanol extract for L. monocytogenes. This suggests that while 90% ethanol may not efficiently extract total phenolics, it is effective in isolating specific bioactive compounds with potent antibacterial activity. The differential antimicrobial effects observed between the two bacteria further emphasize the complexity of the bioactive compounds in *Eruca vesicaria*, where specific compounds may exhibit varying degrees of activity depending on the target microorganism. The lower efficacy observed at lower concentrations indicates that a threshold concentration of bioactive compounds is necessary for antimicrobial action.

In terms of cytotoxicity, the MTT assay revealed promising anticancer potential of the *Eruca vesicaria* extracts. The 50% and 90% ethanol extracts exhibited significant cytotoxic effects on prostate cancer cells (PC3), with a dose- and time-dependent reduction in cell viability. The highest cytotoxicity was observed in the 50% ethanol extract, which correlates with its higher TPC, and phenolic diversity as revealed by HPLC. These findings suggest that the phenolic compounds extracted at 50% ethanol play a critical role in the extract's anticancer activity. The similarity between the phenolic content and cytotoxicity results aligns with previous studies, where higher phenolic content has been associated with enhanced cytotoxic and antioxidant activities (Chiemchaisri et al., 2021).

The cytotoxic effect on PC3 cells contrasts with the minimal impact on HEK-293 cells, indicating selective anticancer activity. The bar shown in blue in Figure 4, called control, represents HEK-293 cells. Since no death due to the extract was observed in the experiments performed here, it is shown as a single bar to simplify the graph. This selectivity suggests that the phenolic

compounds in *Eruca vesicaria* could serve as potential chemo preventive agents, targeting cancer cells while sparing normal cells. The time-dependent increase in cytotoxicity highlights the importance of both extract concentration and exposure time in maximizing anticancer efficacy.

In conclusion, our study demonstrates the critical role of ethanol concentration in modulating the extraction efficiency, phenolic content, and biological activities of Eruca vesicaria extracts. The 50% ethanol extract consistently showed superior performance across all assays, suggesting it as the optimal extraction condition for maximizing bioactive phenolic yield and biological efficacy. Future studies should focus on further characterizing the individual phenolic compounds responsible for the observed antimicrobial and anticancer activities and exploring their mechanisms of action. The optimization of extraction parameters holds significant promise for enhancing the therapeutic potential of *Eruca vesicaria* in bioengineering applications.

5. Conclusion

In the studies, the extraction of *Eruca vesicaria* was done with 3 different polarity solvent groups. Firstly, standardization of extraction was done. These were made in the form of ethanol concentrations of 10%, 50% and 90% over the same period and under the same ambient conditions. Phenolic substance change in the content of the obtained plant extracts was measured by HPLC. The results show that phenolics were obtained in the same but different proportions. Total phenol quantities were determined by spectrophotometric method. A different result was obtained according to the amount of concentration, which was a proportional result compared to the HPLC results. HPLC analyses showed the differences in the contents of extracts obtained with changing extraction conditions. At the same time, the effect of this phenolic change on gram-positive and gram-negative bacteria has been tested on different bacteria using the microplate test. As a result, the presence of antibacterial properties of plant extract in different concentrations was tested. Although the concentration of extraction had little effect on antibacterial property, concentrations of agents prepared in different proportions showed antibacterial property. All the agents prepared in high concentrations killed the bacteria. Finally, the anti-carcinogenic effect on prostate cancer was measured using the MTT test. The plant extracted at a high alcohol concentration showed an effect on PC3, a prostate cancer line, when prepared with a high concentration. Direct proportional results were obtained compared to total phenol determination and microplate studies. Samples prepared in high concentration were measured to have anticancer and antibacterial properties.

Author Contributions

The percentage of the author contributions is presented below. The author reviewed and approved the final version of the manuscript.

	M.E.U.
C	100
D	100
S	100
DCP	100
DAI	100
L	100
W	100
CR	100
SR	100
PM	100
FA	100

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The author declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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