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Research Article

In Vitro Assessment of the Antioxidant, Antibacterial, Cytotoxic, and Hemolytic Activities of Hydroalcoholic *Eruca sativa* Leaf Extract from Najaf, Iraq

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Abstract

Background: *Eruca sativa* is a medical plant species famous for its various phytochemical components and biological activities. The objective of this study was to assess the antioxidant, antibacterial, cytotoxic, and hemolytic activities of a hydro-alcoholic extract obtained from the leaves of *E. sativa*, which have been collected from local markets of Najaf city, Iraq. **Methods:** Antioxidant activity was performed through the DPPH free radical scavenging test, while antibacterial activity was tested against *Escherichia coli* and methicillin resistant *Staphylococcus aureus* (MRSA) bacteria using the agar well diffusion technique. Cytotoxicity was done on the rhabdomyosarcoma (RD) cell line through the MTT test, and hemocompatibility was done by red blood cell hemolysis test. **Results:** 12.9% of crude extract was achieved. From the DPPH assay, it was found that the concentration-dependent antioxidant activity of the extract had various values for percent inhibition of the radical scavenging activity ranging between 49.6%-85.2%. On testing the antibacterial activity of the extract, it was found that it inhibited the growth of MRSA more than *Escherichia coli* with maximum inhibition zones of 13.5 and 8.5 mm, respectively. From the MTT test, it was found that the extract had concentration-dependent decrease in viability of the RD cell line with an IC_{50} value of 0.32 mg/ml. It could be said that no hemolysis of the red blood cells was detected at the various concentrations used in the experiment. **Conclusion:** The extract exhibited a high antioxidant effect and was able to achieve up to 85% of free radical scavenging. In addition, it had antibacterial effect on both *S. aureus* and *E. coli*. The extract had a cytotoxic effect on cancerous RD cells with IC_{50} of 0.32 mg/ml.

1. Introduction

Therapeutic potential of medicinal plants is gaining attention in recent times since about 60 percent of the medicines used to treat cancer originate from medicinal plants [1]. The most commonly used medicinal plant among all the edible plant species is *Eruca sativa* (Rocket), which belongs to the family of Brassicaceae and is largely consumed in Iraq [2], It has been known to show its medicinal potential for over two thousand years [3].

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Such features can be attributed to the presence of phytochemicals in rocket. Rocket leaves contain glucosinolates that upon hydrolysis yield isothiocyanates, which include erucin and sulforaphane together with flavonoids, phenolic acids, vitamins and minerals [4]. Erucin is known to trigger programmed cell death, arrest in cell cycle and mitochondrial depolarization along with disturbing the dynamics of microtubules [5].

Furthermore, cytotoxicity of extracts from *Eruca sativa (E. sativa)* against various types of human cancer cells has been reported along with low toxicity in animal studies [4]. Extracts from leaves have been found to exhibit similar growth inhibition against methicillin-resistant *Staphylococcus aureus* (MRSA) [6, 7].

Antioxidant extraction and its applicability in cancer treatment cannot be easily justified, however. Oxidative stress caused by free radicals and reactive oxygen species is associated with ageing, cancer, and inflammatory conditions [8]. In spite of the above-mentioned benefits, high concentrations of ROS may actually become detrimental for tumor cells, whereas in certain cases the consumption of antioxidants has resulted in a greater likelihood of tumor formation [9]. Indeed, antioxidants can be considered a two-faced agent [10], making it necessary to simultaneously determine antioxidant capacity, toxicity and safety of the agent.

Rhabdomyosarcoma (RMS) arises from skeletal muscle precursors [11], and accounts for the highest frequency of soft tissue sarcomas among children and adolescents at 4.5/1,000,000 per year [12], The outcome of relapsed disease is poor, with only 10-30% survival rates [13], thus stressing the necessity of developing new cytotoxic drugs and making the RD cell line a relevant test system.

However, studies which are conducted to examine the efficacy and biosafety in vitro using a combination of these parameters are very limited. Therefore, in the current study, a hydro-alcoholic leaf extract of *E. sativa* obtained from local markets of Najaf, Iraq was subjected to evaluation for the following parameters: (i) free radical scavenging potential through the use of the DPPH test; (ii) antibacterial activity against *E. coli* and MRSA; (iii) cytotoxicity against RD cells including IC_{50}; and (iv) hemolytic activity against human erythrocytes.

2. Materials and Methods

2.1. Chemicals and equipment

The 2,2-Diphenyl-1-PicrylHydrazyl (DPPH) was purchased from Sigma-Aldrich (US), while the Mueller-Hinton agar (MHA) was obtained from Oxoid (UK). For bacteria dilutions, a densitometer known as DensiChek (bioMérieux, France) was utilized. Other pieces of equipment that were used in the experiment include: Incubator (Memmert, Germany), microanalytical balance (Sartorius, Germany), vortex (IKA, Germany), autoclave (Tuttnauer, Germany), microplate reader (TECAN, Austria), sterile swabs (Copan, Italy), syringe/syringe filter (Terumo, Japan).

2.2. Plant material

Fresh leaves of *E. Sativa* were obtained from the local markets, Najaf city, Iraq and identified by Taxonomist in the herbarium of the Biology Department, University of Kufa, Iraq.

2.3. Preparation of the hydroalcoholic extract

The drying process took place at room temperature after which the dried leaves were grounded to form powder with the use of an electrical grinder. The powdered material was then stored in clean sacks under refrigeration at 4 °C before use [14]. Extraction process involved use of 20% ethanol (ratio of ethanol/dw 20:80 v/v) at 1 g:3 mL of powder/solvent ratio and 30 minutes of homogenization using electric blender at room temperature. Filtration through gauze was carried out before drying at 40-45 °C [15]. Crude extract was stored at 4 °C before use. Extracted yield was obtained as described by [16]:

$$\text{Yield } (\%) = \left(\frac{W_2}{W_1}\right) \times 100$$

where, W_2 is the weight of the dry crude extract and W_1 is the initial weight of the dry plant powder.

2.4. DPPH radical-scavenging assay

To determine the antioxidant capacity of the plant extracts, the DPPH scavenging capacity method based on a modified previous published procedure [17] was used. In brief, the solutions of the plant extract were prepared by using different concentrations including 0.12, 0.25,0.5, and 1 mg/ml. For every concentration, 100 µL of extract was mixed with 100 µL of DPPH solution and 200 µL of DPPH solution in absence of plant extract for the blank, respectively, in a 96-well microplate. Then all of the plates were left in dark at room temperature for 30 minutes before measuring the absorbance values at wavelength of 514 nm by a microplate reader (TECAN, Grädig, Austria). The percentage of scavenging activity of DPPH was calculated by the formula as follows:

$$\text{Radical scavenging}(\%) = \left[\frac{(A)control - (A)sample}{(A)control}\right] \times 100$$

2.5. Hemolysis assay

Fresh human whole blood was obtained from voluntary blood donors in EDTA tubes. This study has been approved by the proper ethical committee, and informed consent has been taken from all subjects before the experiment. Plasma and buffy coat of blood were removed after spinning at 5000 rpm for 5 minutes. Washing of the red blood cells (RBCs) with PBS (pH 7.4) was done thrice. A 2% (v/v) RBC suspension was made. The extract was dissolved in 0.85% NaCl solution at concentrations of 1, 0.5, and 0.25 mg/mL. Same amounts of RBC

suspension and test solutions were added in the mixture. RBCs in PBS acted as a negative control, and RBCs in 0.1% Triton X-100 solution were used as a positive control. The mixtures were incubated at 37 °C for 30 minutes and then centrifuged for 5 minutes at 1500–1700 × g. Measurement of the absorbance of the supernatants was done at 540 nm wavelength. The % of hemolysis was determined by the equation:

$$\text{Hemolysis}\% = \left[\frac{A_{sample} - A_{neg}}{A_{pos} - A_{neg}}\right] \times 100$$

2.6. Antibacterial activity

A coarse extract of *Eruca sativa* was prepared by dissolving it in distilled water at 700, 800, and 900 mg/ml and then homogenizing it by mixing it in a vortex followed by sterilization through a syringe filter having a pore size of 0.22 µm [18]. Clinical strains of *E. coli* and MRSA bacteria were isolated from nutrient agar media at 37°C for 24 hours after which they were made as per the 0.5 McFarland scale that is, 1.5 x 10^8 CFU/mL [19]. Antimicrobial activity was determined using the agar well diffusion technique. Nutrient agar plates were inoculated with standardized suspensions of bacteria, and then wells having diameter 7 mm were prepared and filled with 75 µL of each concentration of the crude extract of *Eruca sativa* in each well along with an appropriate antibiotic used as positive control and distilled water as a negative control at 37°C for 24 hours [19, 20]. The inhibition zone was measured as the clear halo extending beyond the edge of the agar well.

2.7. Cytotoxicity assay (MTT)

To evaluate the cytotoxic effect of *Eruca sativa* extract on the RD cell line, the MTT method was performed. RD cells were cultured as a single layer in 96-well microplates and treated with increasing amounts of *Eruca sativa* extract (0.001, 0.01, 0.025, 0.05, 0.1, 0.2, and 0.4 mg/ml) in three replicates (volume of the solution was 100 µl per well). After 24 hours incubation, 10 µl of the MTT reagent solution (solution was prepared at 5 mg/ml in phosphate buffer saline) was added into each well and plates were further incubated for 4 more hours in 37 °C conditions. Then the culture medium was aspirated, and 200 µl of DMSO (v/v 1:1) was added per well to dissolve formazan crystals. Gentle mixing of plates was carried out for 5 minutes, and absorbance was measured at the wavelength of 490 nm using microplate ELISA-reader.

2.8. Statistical analysis

All experiments were carried out in triplicates and were replicated independently on three different occasions. The data have been expressed as mean ± SE. All the statistical analysis was done using IBM SPSS Statistics Version 21. For comparing the differences among the groups, one-way ANOVA test has been used, with $P \leq 0.05$ considered statistically significant.

3. Results

3.1. Extraction Yield

The extraction yield was calculated to evaluate the efficiency of the solvent used Table 1.

Table 1: Extraction Yield Calculation Data of *Eruca sativa*

Parameter	Value / Unit
Initial Weight of Dry Powder	31.0 g
Total Weight of Crude Extract	4.0 g
Final Extraction Yield %	12.9 %

The extraction results of *Eruca sativa* show an efficiency of 12.9%. The findings from the DPPH assay clearly depicted that there was a concentration-dependent improvement of the antioxidant capacity, which is shown by the reduction in absorbance and the percentage of radicals scavenged from 49.6% at 0.12 mg/mL to 85.2% at 1 mg/mL Table 2.

Table 2: Antioxidant assay results

Sample Name	Concentration	Absorbency	Scavenging %
1	0.12 mg/ml	0.399	49.608
2	0.25 mg/ml	0.327	58.723
3	0.5 mg/ml	0.236	70.177
4	1 mg/ml	0.117	85.209

The hemolysis assay results showed that all tested sample concentrations caused no red blood cell lysis, with a hemolysis percentage of 0%, comparable to the negative control. In contrast, the positive control exhibited 100% hemolysis Figure 1.

The cytotoxic activity of the hydroalcoholic extract of *Eruca sativa* leaves was assessed against the RD cancer cell line using the MTT assay at concentrations ranging from 0.001 to 0.4 mg/ml. A gradual decrease in absorbance values was observed with increasing extract concentration, indicating reduced cell viability. The percentage of growth inhibition increased progressively from 0% at 0.001 mg/mL to 17.36%, 24.04%, 15.13%, and 24.11% at 0.01, 0.025, 0.05, and 0.1 mg/mL, respectively. Higher inhibitory effects were recorded at 0.2

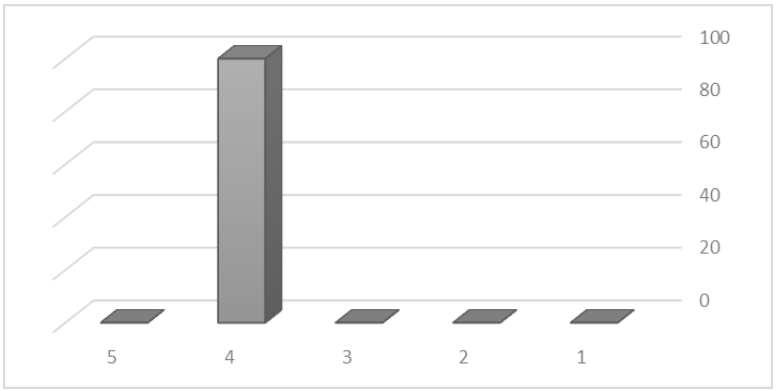


Figure 1: Hemolysis result of the tested sample

mg/mL (49.30%), 0.4 mg/mL (60.89%). The half-maximal inhibitory concentration (IC_{50}) of the extract was calculated to be 0.32 mg/ml, with a maximum inhibition zone of 8.5 mm at the same concentration Table 3, Figure 2.

Table 3: Effect of *Eruca sativa* leaf extract on RD cell viability (MTT assay)

Conc. mg/ml	Absorbance (Mean± SEM)	% Inhibition	IC_{50} mg/ml	R2
0.001	0.968 ± 0.000577	0		
0.01	0.83967±.175667	17.36256		
0.025	0.77567±.190958	24.04315		
0.05	0.86100±.008327	15.1357	0.32	0.8296
0.1	0.77500±.026502	24.11273		
0.2	0.53387±.212168	49.30411		
0.4	0.42267±.012032	60.89074		

The antibacterial activity of *Eruca sativa* extract was evaluated against two bacterial strains. The results showed that the extract exhibited a dose-dependent inhibitory effect. The highest zone of inhibition was recorded against *S. aureus* (MRSA) at a concentration of 900, reaching an average of 13.5 mm. In contrast, *E. coli* showed lower sensitivity to the extract, with a maximum inhibition zone of 8.5 mm at the same concentration, Table 4.

Table 4: Antibacterial activity of *Eruca sativa* extract against *E. coli* and *S. aureus* measured by inhibition halo width (mm)

Bacterial Species	Concentration mg/ml	Isolation 1 mm	Isolation 2 Mm	Average
E. coli	900	8	9	8.5
E. coli	800	6	0	3
E. coli	700	5	0	2.5
S. aureus	900	14	13	13.5
S. aureus	800	10	12	11
S. aureus	700	10	5	7.5

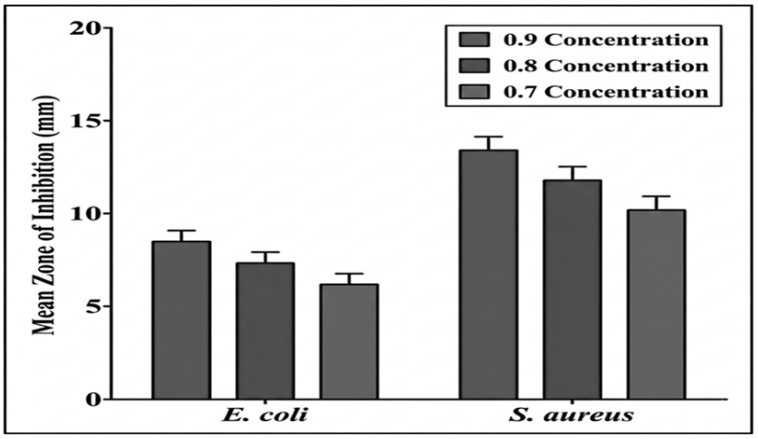


Figure 2: Mean zone of inhibition (mm) of *Eruca sativa* extract against *E. coli* and *S. aureus* at different concentrations

As can be seen from the results provided in Table 4, the antibacterial effect of *Eruca sativa* extract against *E. coli* and MRSA is clearly dosage-related and the latter is more susceptible to the extract. At the dosage of 900 mg/ml, the extract demonstrated high inhibitory action, namely the zone of inhibition measured 13.5 mm for MRSA as compared to 8.5 mm for *E. coli*.

4. Discussion

The extraction results of *Eruca sativa* show an efficiency of 12.9%, suggesting a highly efficient solubility of the solvent used for the extraction of the biologically active components of the plant. It is consistent with the framework of the phytochemical approach offered by [21] who highlighted the abundant presence of the secondary metabolites in Rocket plants. In this case, a relatively high yield can be considered evidence of the perfect environment of extraction along with a high-quality source material. Such significant yield of the crude extract can be helpful for evaluating its biological activity.

The findings show that the hydroalcoholic extract from the leaves of *Eruca sativa* possesses a remarkable amount of antioxidant potential in a dose dependent manner, and at the highest dose level (1mg/ml), the highest level of inhibition of radical species was witnessed. The above properties are primarily due to the presence of phenolics and flavonoids in a large amount that act as reducing agents for stabilization. The above findings agree with the research carried out by [22], which established a strong correlation between the radical scavenging activity and the presence of quercetin glycosides in *Eruca sativa* leaves.

Furthermore, the effectiveness of the combination solvent system ensured the extraction of a wider range of secondary metabolites compared to mono-solvents. This is in accordance with [23] findings where it was demonstrated that TPC and antioxidative activity is better in extracts made from alcoholic solvents. The results have proven the importance of *Eruca sativa* from a medicinal standpoint and confirm the results of the study by [24] that showed isothiocyanates' important role in cellular protection from oxidative stress. Therefore, this particular extract can serve as an effective natural agent in pharmaceutical research. High scavenging ability (85.2%) has confirmed the significant impact of *E. sativa* as an antioxidant source.

The hemolysis assay demonstrated that the assay material had a high level of hemocompatibility because there was no RBC lysis seen at any of the concentrations of 1, 0.5, and 0.25 mg/mL tested. The percent of hemolysis was maintained at 0% compared to the negative control, and the positive control showed a hemolysis rate of 100%, which confirms the reliability of the assay [25].

According to international standards concerning hemocompatibility, materials causing less than 2-5% hemolysis can be considered non-hemolytic and therefore suitable for use in contact with blood [25, 26]. Lack of hemolysis means that the test material did not cause any membrane damage to the RBC [26]. Therefore, it can be concluded that the test material is safe for the red blood cells, and it has great potential in biomedical or pharmaceutical applications. For complete clinical safety, more in vivo studies on hemocompatibility are recommended. The lack of hemolytic activity means that the extract is completely safe and bio-compatible with human blood.

The present research explores the biological activity of *Eruca sativa*; thus, the hydroalcoholic leaf extract of *Eruca sativa* showed high cytotoxic activity towards the RD cancer cell line. This cytotoxic effect was shown in a clear dose-dependent way due to the decreasing absorbance values, which means reducing cell viability. Thus, growth inhibition percentages increased from 0% at the lowest concentration of the extract (0.001 mg/mL) to 0.4 mg/ml. A strong correlation between the extract concentration and inhibition level can be seen in the regression analysis data, since R2 = 0.8296. IC_{50} of the extract was equal to 0.32 mg/mL, and it can be considered as a low value, which confirms a high cytotoxic activity of the extract.

Thus, the obtained results indicate the presence of bioactive substances in the *Eruca sativa* leaves, which possess high anti-cancer activity regarding muscle cancer cells. This biological activity is usually associated with phytochemicals of the Brassicaceae family. Glucosinolates and their derivatives (erucin, sulforaphane) are well-known for inducing apoptosis through caspase cascade activation, for causing cell cycle arrest (mainly in the G2/M phase), and for increasing ROS levels [27].

As a result, it is possible to assume that bioactive compounds contained in arugula, such as isothiocyanates and phenolics, identified by [28] can better permeate through the Gram-positive bacteria cell wall. It is consistent with the results obtained by [6], according to which *Eruca sativa* promotes important changes in metabolic processes in MRSA that impede its development. Moreover, a steep drop in the zone of inhibition for *E. coli* at dosages of 800 mg/ml and 700 mg/ml (to an average of 3 mm and 2.5 mm correspondingly) may indicate that there is a threshold close to the Minimum Inhibitory Concentration (MIC) for Gram-negative strains. The results obtained coincide with the conclusions made by [7] about high antimicrobial properties of arugula leaves.

5. Conclusion

The extract exhibited a high antioxidant effect and was able to achieve up to 85% of free radical scavenging. In addition, it had antibacterial effect on both *Staphylococcus aureus* and *Escherichia coli*. The extract had a cytotoxic effect on cancerous RD cells with IC_{50} of 0.32 mg/ml.

Article Information

Ethical Approval: This study was reviewed and approved by the Institutional Ethics Committee of the Faculty of Medicine, University of Kufa, Najaf, Iraq.

Informed Consent: Written informed consent was obtained from all voluntary blood donors prior to sample collection.

Data Availability: The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request

Conflict of Interest: The authors declare that they have no conflict of interest.

Funding: No external funding was received for this study.

Author Contributions: Z.K.L. - Conceptualization, Methodology, Formal Analysis, Writing – Original Draft, Writing – Review & Editing, Supervision, Project Administration; R.A.Z.A. - Conceptualization, Methodology, Formal Analysis, Writing – Original Draft, Writing

– Review & Editing; A.R.H. - Methodology, Investigation, Resources; S.R.M. - Methodology, Investigation, Resources; Z.M.A. - Data Curation, Resources; K.A.A. - Data Curation, Resources.

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