Unresponsiveness of *Enhalus acoroides* Ethanolic Extract on MDA-MB-231 Breast Cancer Cell Line

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Abstract: *Triple negative breast cancer (TNBC) is highly aggressive because it lacks estrogen (ER), progesterone (PR), and HER-2 hormone receptors. Recent anticancer research focuses on natural ingredients to reduce the toxicity of synthetic drugs. E. acoroides is known to contain bioactive compounds that can be cytotoxic to cancer cells. This study aimed to determine the toxicity, cytotoxic, and antiproliferative properties of E. acoroides ethanolic extract against TNBC cell line MDA-MB-231. The methods used includeed Phytochemical Test, Fourier-Transform Infrared Spectroscopy Analysis (FTIR) analysis, Antioxidant Activity Test DPPH (2,2-diphenyl-1-picrylhydrazyl), Toxicity Test with BSLT, Cytotoxic and Antiproliferative Test with WST-8. Based on phytochemical tests, E. acoroides ethanolic extract contained alkaloid, flavonoid, saponin, tannin, steroid, and phenolic. The BSLT results showed E. acoroides ethanolic extract has the LC_{50} value of 461.576 ppm, categorized as moderate toxic. Antioxidant activity of E. acoroides ethanolic extract indicated low with IC_{50} values of 858.43 ppm. Then, based on the cytotoxic and antiproliferative test, E. acoroides ethanolic extract has IC_{50} value of >2000 ppm respectively and do not have antiproliferative activity against MDA-MB-231 cells. These findings demonstrate the unresponsiveness of MDA-MB-231 cells to E. acoroides ethanolic extract, indicating limited anticancer potential against aggressive TNBC cell line.*

Keywords: *Anticancer, Enhalus acoroides, MDA-MB-231 cells, Triple Negative Breast Cancer (TNBC), Cytotoxic and Antiproliferative Test*

1. INTRODUCTION

Cancer is considered as one of the fatal diseases that has become a major health problem around the world. By 2022, there will be around 20 million cases with 9.7 million deaths worldwide. Breast cancer is ranked as the second most common cancer case. Age, gender, genetic factors, and hormones are among the factors that cause breast cancer (Aisha et al., 2025). The increasing number of cancer cases is not only caused by several factors but also due to late diagnosis so that the cancer has reached the final stage and is difficult to treat (Bray et al., 2024).

The characteristics of breast cancer can be recognized by abnormal cell growth in the epithelial tissue of the breast so that a lump will form (Ikhuoria and Bach, 2018). In general, cancer is caused by uncontrolled cell division that can spread to other tissues. Cancer cells that have spread to other normal tissues around them are called metastases (Jadhav and Babar et al., 2021).

TNBC is characterized by invasive cell growth, rapid progression, and is difficult to treat compared to other types of breast cancer (Gaol et al., 2023). This is because TNBC lacks receptors to estrogen, progesterone, and HER-2, making this type of cancer more aggressive and difficult to treat. The survival rate of TNBC patients reaches 4%-20%. Currently, treatment for TNBC is limited to surgery and chemotherapy (Pajewska et al., 2025).

E. acoroides is a type of seagrass that has an important role in the marine ecosystem. Currently, *E. acoroides* is widely used as an object of research because it has potential as an antibacterial, antifungal, antifouling, antioxidant, and anticancer (Dewi et al., 2017). Based on research conducted by Sami et al (2021), *E. acoroides* ethanolic extract has an IC_{50} value of 38.000 µg/mL against ABTS radicals which are considered very strong antioxidants. Besides its potential as an antioxidant, *E. acoroides* is also known to have potential as an anticancer. This was proven by Ahmed et al (2022) against HepG2 and MCF-7 cell lines. The 60% hydro-alcoholic extract of *E. acoroides* has an IC_{50} value of 112.20 µg/mL against HepG2 cell line and 101.60 µg/mL against MCF-7 cell line so it can be considered that *E. acoroides* is cytotoxic and has potential as an anticancer activity against liver and breast cancer.

Toxicity activity and anticancer potential of a compound can be proven by BSLT. Toxicity test with BSLT method is based on the death of *Artemia salina* by 50% of the total population after given the compound or lethal concentration 50 (LC_{50}). Toxicity test with BSLT have been widely carried out to test a compound known to have potential as an anticancer (Aksono et al., 2022).

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Based on that, it is necessary to prove the anticancer activity contained in *E. acoroides* through toxicity, cytotoxic, and antiproliferative test against MDA-MB-231 cells which is TNBC cell lines.

2. MATERIALS AND METHODS

2.1 Plant Collection

E. acoroides was obtained from Ketapang Beach, Pesawaran Regency, Lampung Province and then identified and determined in The Botany Laboratory, Faculty of Mathematics and Natural Sciences, University of Lampung.

2.2 Plant Extraction

E. acoroides was cleaned using running water. Then, air-dried for ± 3 days. After drying, *E. acoroides* was pulverized to form simplisia. The maceration process was carried out with 96% ethanol solvent in a ratio of 1:10 form 3x24 hours. Then, filtered using filter paper to separate the macerate and filtrate. The filtrate concentrated with rotary evaporator at 78.7°C until a thick extract is formed (Khasana et al., 2025).

2.3 Phytochemical Test

Phytochemical test based on research conducted by Kartikasari et al (2022)

- **Alkaloid**
  A total of 0.5 mL of extract added with 5 drops of chloroform then 5 drops of Mayer reagent.
- **Flavonoid**
  A total of 0.5 mL of extract added with 0.5 g Mg powder then 0.5 mL HCl.
- **Steroid and Triterpenoid**
  A total of 0.5 mL of extract added with 2 mL chloroform then 3 mL H_2SO_4.
- **Tannin**
  A total of 0.5 mL of extract added with 3 drops of $FeCl_3$ 10%.
- **Saponin**
  A total of 0.5 mL of extract added with 5 mL aquadest then shake for about 30 seconds.
- **Phenolics**
  A total of 1 mL of extract added with 3 drops of $FeCl_3$ 2%

2.4 FTIR Analysis

A total of 0.0020 g of *E. acoroides* ethanolic extract and 0.1980 g of KBr were weighed and mashed then molded into a thin plate (transparent). Then, read using FTIR Spectroscopy tool. The resulting chromatogram was then compared with the IR table and previous research (Sari et al., 2018).

2.5 Antioxidant Activity Test with DPPH

A 2000 ppm stock solution was prepared by dissolving 200 mg of *E. acoroides* ethanolic extract in 100 mL of methanol. From this stock, a series of concentrations (250; 500; 750; 1000; and 1250 ppm) were prepared, each tested in triplicate. Separately, 4 mg of DPPH powder was dissolved in 100 mL of methanol to obtain a 40 ppm DPPH solution. The DPPH solution was stored in an aluminum foil – wrapped volumetric flask at low temperature to maintain its stability. Ascorbic acid was used as positive control at concentrations of 2; 4; 6; 8; and 10 ppm, each with triplicate.

For antioxidant assay, 2 mL of 40 ppm DPPH solution was added into aluminum foil – wrapped test tubes, followed by the addition of each 2 mL of *E. acoroides* ethanolic extract. The absorbance was measured using a UV-Vis spectrophotometer.

2.6 Toxicity Test with BSLT

A total of 100 mg of *Artemia salina* eggs were put into the dark part of the aquarium and hatched for 48 hours. Then, 400 mg of *E. acoroides* ethanolic extract were dissolved in 200 mL of 70% ethanol to prepare 2000 ppm stock solution. For toxicity test, each test tube was filled with 1 mL of seawater and one drop of yeast solution (prepared by dissolving 3 mg of yeast in 5 mL of seawater) as a food source. Subsequently, 10 *Artemia salina* larvae were added into each test tube and seawater was added to a total volume of 5 mL to obtain concentrations of 62.5; 125; 250; 500; 1000; and 2000 ppm. The test was performed in five replicates, incubated for 24 hours, and the mortality of *Artemia salina* larvae was calculated.

2.7 Cytotoxic Test with WST-8

A 100.000 ppm stock solution of *E. acoroides* ethanolic extract was made by dissolving 100 mg of extract in 1 mL of DMSO 100% and water (1:1). Stock solution was diluted to obtain concentrations of 62.5; 125; 250; 500; 1000; and 2000 ppm. Moreover, Cisplatin as positive control was made by dissolving 2 mg in 2 mL of DMSO 100% to make 2000 ppm stock solution. Then diluted to concentrations of 3.125; 6.25; 12.5; 25; 50; and 100 ppm.

A total of 50 µL of *E. acoroides* ethanolic extract were added to wells containing 50 µL of cell suspension. The plate was then incubated in CO_2 incubator for 24 hours. After incubation, the contents of each well were carefully removed to minimized measurement bias, and 100 µL of complete RPMI medium was added. Subsequently, 10 µL of CCK-8 reagent was added to each well, followed by a 2 hours incubation in CO_2 incubator. The absorbance was then measured using UV-Vis Spectrophotometer at wavelengths of 450 nm and 620 nm, in accordance with the CCK-8 reagent protocol (Amalia et al., 2025).

2.8 Antiproliferative Test with WST-8

A total of 50 µL of *E. acoroides* ethanolic extract were added to wells containing 50 µL of cell suspension. The plate was then incubated in CO_2 incubator for 24, 48, and 72 hours. After incubation, the contents of each well were carefully removed to

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minimized measurement bias, and 100 µL of complete RPMI medium was added. Subsequently, 10 µL of CCK-8 reagent was added to each well, followed by 2 hours incubation in CO_2 incubator. The absorbance was then measured using UV-Vis Spectrophotometer at wavelengths of 450 nm and 620 nm, in accordance with the CCK-8 reagent protocol (Amalia et al., 2025).

3. Results and Discussions

3.1 Phytochemical Test

The results of phytochemical test that have been carried out on *E. acoroides* ethanolic extract, can be seen in Table 1.

Table 1. Phytochemical test of *E. acoroides*

Compounds	Results
Alkaloid	++
Flavonoid	+
Tannin	++
Steroid	-
Terpenoid	+
Saponin	+
Phenolic	+

Note: ++: strong positive; +: positive, -: negative

Based on phytochemical test of *E. acoroides* ethanolic extract shown in Table 1. The extract was found to contain alkaloids, flavonoids, tannins, terpenoids, saponins, and phenolic. A study by Mahmiah et al (2023) on *E. acoroides* from East Nusa Tenggara similiarly reported the presence of alkaloids, flavonoids, saponins, terpenoids, steroids, and polyphenols. Furthermore, GC/MS analysis by Mettwally et al (2025) revealed that *E. acoroides* ethanolic extract contained sucrose (53.57%), fructose (7.21%), talose (5.82%), glucose (0.27%), organic acids (6.24%), lipids (4.46%), sterols (0.42%), phenolic acids (0.17%), and nitrogen-containing compounds (9.57%).

Variations in secondary metabolite composition can be influenced by several factors, including species, variety, season, environmental conditions, cultivation site, processing and storage methods, temperature, sunlight exposure, rainfall, and soil characteristics (Julyasih, 2022).

E. acoroides is recognized for its potential antioxidant and anticancer properties due to the content of bioactive compounds (Shaffai et al., 2023). Flavonoids targeting topoisomerase, protein kinases, and angiogenesis, as well as by inducing apoptosis of cancer cells (Shah et al., 2023). Saponins inducing cell cycle arrest at the G0/G1 or G2/M1 phases (Elekofehinti et al., 2021). Alkaloids and Phenolics activating the p53 tumor suppressor gene, promoting pro-apoptotic proteins, and regulating telomere length (Vakili et al., 2020). Terpenoids suppress early tumorigenesis by inhibiting cell cycle progression (Kamran et al., 2022). Tannins target signaling pathways involved in cancer progression and inhibit epithelial-to-mesenchymal transition (EMT) (Kleszcz et al., 2023).

3.2 FTIR Analysis

The spectrum of FTIR analysis that has been carried out on *E. acoroides* ethanolic extract can be seen in Figure 1.

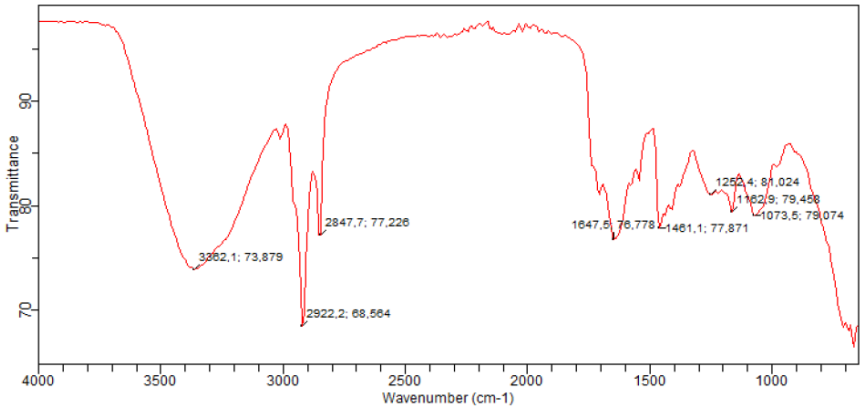

Figure 1. FTIR spectrum of *E. acoroides* ethanolic extract

The FTIR spectrum of the *E. acoroides* ethanolic extract (Figure 1) shows a broad peak at 3362 cm^{-1}, indicating O-H stretching and a sharper band suggesting N-H groups. Peaks at 2922.2 cm^{-1} and 2847.7 cm^{-1} correspond to aliphatic C-H stretching of CH_2 and CH_3 groups (Nandiyanto et al., 2023), consistent with findings by Cordova et al (2022).

A peak at 1647.5 cm^{-1} indicating C=O and C=C bonds, while absorption around 1461.1 cm^{-1} represents C-H bending typical of alkyl or lipid groups. Similar results were reported by Pharmawati and Wrasiati (2020), who identified conjugated C=C C-O bonds and secondary alcohols near these regions.

In the fingerprint region, peaks at 1252.4 cm^{-1}, 1162.9 cm^{-1}, and 1073.5 cm^{-1} correspond to C-O, C-O-C, and C-N functional groups, indicating the presence of alcohols, glycosidic bonds, and

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polysaccharides (Nandiyanto et al., 2023). These peaks suggest a high carbohydrate content in *E. acoroides* ethanolic extract (Cordova et al., 2022).

3.3. Antioxidant Activity Test with DPPH

Based on the antioxidant activity test of *E. acoroides* ethanolic extract and Ascorbic acid as positive control, the comparison of IC_{50} values can be seen in Figure 2.

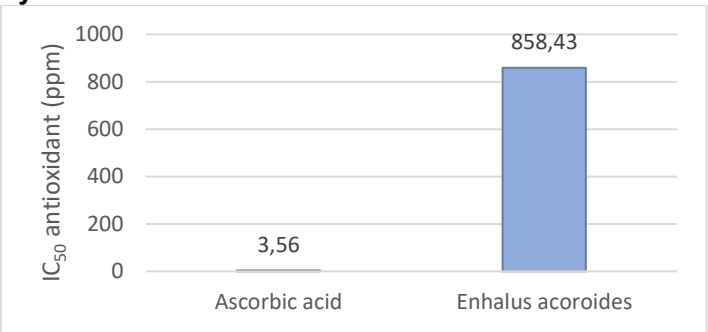

Figure 2. IC_{50} value comparison chart

After calculating the IC_{50} value, it can be seen that the *E. acoroides* is categorized as weak antioxidants. Meanwhile, Ascorbic acid has very strong antioxidant activity. It is known that the *E. acoroides* ethanolic extract and Ascorbic acid have an IC_{50} value of 858.43 ppm and 3.56 ppm, respectively.

Low antioxidant activity in *E. acoroides* ethanolic extract is in line with research conducted by Bareta et al (2025) that *E. acoroides* ethanolic extract has an IC_{50} value of 747.78 ppm which also has low antioxidant activity. There are several factors that affect the results of antioxidant activity, such as physical and chemical conditions in seagrass habitats, less than optimal treatment, the wavelength used when conducting spectrophotometry, the concentration series used, and the polarity of the solution used during the maceration process (Bareta et al., 2025). The content of bioactive compounds, such as phenolics or flavonoids in the extract is related to antioxidant activity. The higher content of phenolics or flavonoids compounds, the stronger antioxidant activity (Ahmed et al., 2022).

Ascorbic acid has a high antioxidant activity because it works by donating its hydrogen atoms (H^+) to free radicals, such as DPPH, and is converted into a stable non-radical form (Njus et al., 2020). Ascorbic acid is known to have a low redox potential. The lower the redox potential of a compound, the easier it is to release electrons so that it is more reactive to free radicals (Furdak et al., 2025).

3.4 Toxicity Test with BSLT

The results of toxicity test with BSLT that have been carried out on *E. acoroides* ethanolic extract can be seen in Table 2.

Table 2. Toxicity test on *E. acoroides* ethanolic extract

Concentration (ppm)	Mean±St.dev Mortality	Mortality (%)	LC_{50} (ppm)
0	0.0±0.00[a]	0	
62.5	0.0±0.00[a]	0	
125	0.2±0.44[a]	2	
250	1.0±0.70[b]	1	461.576
500	4.6±1,14[c]	46	
1000	10.0±0.00[d]	100	
2000	10.0±0.00[d]	100	

Note: a, b, c, and d indicate significant differences

Based on Table 2, the results show that the highest average larval mortality in *E. acoroides* ethanolic extracts, is at a concentration of 1000 ppm and 2000 ppm with an LC_{50} value of 461.576 ppm. Previous research using *E. acoroides* methanolic extract had an LC_{50} value of 404.488 ppm (Orno and Rantesalu, 2020). Toxic compounds enter through *Artemia salina* mouth and are absorbed into the digestive tract through the cell membrane. The compound can spread quickly throughout the larval body which still has a simple anatomical structure and also due to changes in the concentration gradient between the inside and outside of the cell so that it can interfere with the metabolic processes within the body (Setianingsih et al., 2023). Bioactive compounds, such as alkaloids, once inside the larval body will interfere with the stimulation of taste so that the larvae cannot recognize their

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food until they die of starvation (Sugrani et al., 2024).

3.5 Cytotoxic Test with WST-8

The results of the cytotoxic test with WST-8 conducted on MDA-MB-231 cells using *E. acoroides* ethanolic extract can be seen in Table 3.

Table 3. Cytotoxic test against MDA-MB-231 cells using *E. acoroides* ethanolic extract

Compound	Concentration (ppm)	Viability (%)	IC_{50}
E. acoroides	62.5	110.478	>2000
	125	112.197	
	250	110.226	
	500	100.135	
	1000	95.668	
	2000	84.087	
Cisplatin	3.125	64.751	3.6
	6.25	53.631	
	12.5	40.922	
	25	25.515	
	50	16.382	
	100	3.588	

E. acoroides ethanolic extract against MDA-MB-231 cells with 24 hours incubation time, there is no concentration in the range of 62.5-2000 ppm that causes 50% inhibition of MDA-MB-231 cell viability. Therefore, the IC_{50} value considered to be >2000 ppm. In contrast, Cisplatin showed an IC_{50} value of 3.6 ppm with the lowest percentage of cell viability at 100 ppm, which was 3.588%.

Based on the study by Shaffai et al (2023), *E. acoroides* ethanolic extract showed an IC_{50} value of 1000 µg/mL against MDA-MB-231 cells after 48 hours of incubation. Similiary, Prajoko et al (2024) reported that *E. acoroides* ethanolic extract had a LD_{50} value that was far from effective in killing MDA-MB-231 cells, requiring a much higher dose compared to Doxorubicin (IC_{50} = 550.888 µg/mL).

Other studies also show the low sensitivity of MDA-MB-231 cells. For example, Tarap stem (*Artocarpus odoratissimus*) ethanolic extract exhibited a high IC_{50} value of 1607.302 µg/mL after 24 hours of incubation in MDA-MB-231, whereas the same extract showed strong cytotoxicity in MCF-7 cells with an IC_{50} of 3.4 µg/mL (Rachmi et al., 2023). This difference relates to the distinct characteristics of the two breast cancer cell lines: MCF-7 cells are less aggressive, non-invasive, more differentiated, and tend to form tight clusters, while MDA-MB-231 cells are highly aggressive, invasive, and commonly used to study metastasis and drug resistance (Gest et al., 2013).

MDA-MB-231 cells are known to survive under hypoxic conditions, exhibit high Epithelial-to-Mesenchymal Transition (EMT) activity, and show strong resistance to chemotherapeutic agents such as doxorubicin and carboplatin, particularly when cultured as 3D spheroids (Huang et al., 2020). In contrast, MCF-7 cells express estrogen (ER^+) and epidermal growth factor (EGF) receptors, while MDA-MB-231 cells lack these hormone receptors. These differences contributed to the varied responses observed in cytotoxic and antiproliferative assays (Rachmi et al., 2023).

3.6 Antiproliferative Test with WST-8

Antiproliferative test were carried out using *E. acoroides* ethanolic extract to evaluate the ability to inhibit the growth of MDA-MB-231 cells at 24, 48, and 72 hours of incubation. The antiproliferative effect is shown by the doubling time value, which indicates how long the cells need to double in number. The results of the antiproliferative test on MDA-MB-231 cells are presented in Table 4.

Table 4. Antiproliferative test on MDA-MB-231 cells using *E. acoroides* ethanolic extract

Compound	Concentration (ppm)	y = ax+b	R	Doubling Time (hours)
E. acoroides	62.5	224097x – 100000	1	45
	125	216536x – 98269	0.9996	45
	250	220629x – 100000	0.9999	45
	500	216319x – 98167	0.9997	45
	1000	198184x – 89190	1	45
	2000	154217x – 67724	0.9999	44

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Cisplatin	3.125	48557x – 178874	0.9266	368
	6.25	46665x – 173029	0.9526	371
	12.5	37189x – 133896	0.9513	360
	25	29554x – 105302	0.9462	356
	50	22256x – 76564	0.9419	344
	100	14348x – 46810	0.9737	326
Cell control	0	185918x – 83157	1	45

In the *E. acoroides* ethanolic extract, the doubling time values at all concentrations were similar to the cell control, ranging from 44-45 hours. This indicates that the extract did not increase the doubling time, even at the highest concentration of 2000 ppm. Although the correlation (R) values were close to 1, reflecting consistent cell growth data, they do not indicate growth inhibition. Thus, the *E. acoroides* ethanolic extract showed no significant antiproliferative effect on MDA-MB-231 cells within the tested concentration range.

In contrast, Cisplatin showed doubling time values that differed markedly from the cell control. The doubling time decreased from 368 hours at 3.125 ppm to 326 hours at 100 ppm. This decrease does not reflect an acceleration of proliferation but instead indicates a reduction in cell population. The antiproliferative and cytotoxic effects of Cisplatin were showing a dose-dependent effect.

The antiproliferative activity of *E. acoroides* ethanolic extract against non-aggressive cells, such as MCF-7 cells, is still moderate. In contrast, MDA-MB-231 cells are highly aggressive, and even with 48 hours of incubation, the extract shows an IC_{50} value of 1000 µg/mL (Shaffai et al., 2023).

Malignant cancer cells like MDA-MB-231 can change the way their cells metabolize, which is by increasing glycolysis despite sufficient oxygen availability, leading to excessive lactate accumulation or known as the Warburg effect. This result in acidification of the tumor microenvironment, promoting extracellular matrix degradation and enhancing cancer cell invasion and metastasis (Chen and Chen, 2025). Although *E. acoroides* can inhibit the HER2/EGFR/HIF-1α signaling pathway, its actual effectiveness is strongly influenced by bioavailability, which refers to the rate and extent of absorption of bioactive compounds or drugs into the body (Rein et al., 2013).

4. CONCLUSIONS

The study indicates that *E. acoroides* ethanolic extract shows moderate toxicity in preliminary screening but did not show meaningful cytotoxic or antiproliferative activity against MDA-MB-231 TNBC cells. These results indicate that, within the tested concentration range, the extract has limited effectiveness against aggressive cancer cell lines, highlighting the need for further investigation into its active compounds and potential mechanisms of action.

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