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Article

In Vitro Antiproliferative Activity and Selective Cytotoxicity of Sixteen South African *Euphorbia* Species Against Breast Cancer and Normal Cell Lines

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Abstract

The use of medicinal plants in traditional healthcare systems has a long history, particularly in regions with limited access to modern medical facilities. In South Africa, indigenous knowledge of plant-based remedies has been preserved through oral traditions, making these plants vital resources for local communities. This study investigates sixteen unexplored *Euphorbia* species for their potential in inhibiting breast cancer cell growth. Due to the small size of the plants, the entire plant was used for each species. A systematic approach was employed, including plant selection, sequential extraction using organic solvents, phytochemical screening, and in vitro cytotoxicity testing using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. High-performance liquid chromatography (HPLC) was used to identify primary metabolites. All sixteen species contained glycosides and triterpenoids. The ethyl acetate (EtOAc) extract of *Euphorbia ledienii*, dichloromethane (DCM) extract of *Euphorbia cooperi*, and DCM extract of *Euphorbia clavarioides* exhibited the highest inhibitory effects on Michigan Cancer Foundation-7 (MCF-7), and M.D. Anderson-Metastatic Breast-231 (MDA-MB-231) cell lines, with cytotoxic concentration (CC₅₀) values of 0.06 µg/mL, 0.07 µg/mL, and 0.23 µg/mL, respectively. *Euphorbia tirucalli* (DCM extract) and *Euphorbia cooperi* (hexane extract) displayed selectivity for MDA-MB231 cells, with selectivity index (SI) values of 49.40 and 84.03, respectively. *Euphorbia trigona*, *Euphorbia gorgonis*, *Euphorbia ledienii*, and *Euphorbia arabica* had similar distribution of secondary metabolites based on HPLC analysis. These findings suggest that certain *Euphorbia* species exhibit selective cytotoxicity against breast cancer cells while sparing normal cells, highlighting their potential for breast cancer therapy.

Keywords: *Euphorbia* species; phytochemicals; antiproliferation; selectivity index; breast cancer; cytotoxicity

1. Introduction

Breast cancer is the most frequently diagnosed cancer among women worldwide, with approximately 2.3 million new cases reported each year. This accounts for about 11.7% of all new cancer diagnoses globally [1]. This dire situation arises due to the diverse biological markers exhibited by different tumor types, rendering a universal treatment approach ineffective [2–4].

Chemotherapy is a widely employed cancer treatment that has limitations such as non-specific drug delivery and lack of selectivity, resulting in high toxicity to healthy cells [5–7]. These limitations hinder effective disease control. To address this, complementary therapeutic strategies like the use of

herbs and medicinal plants have gained attention. Many plants possess compounds that can enhance cancer treatment efficacy and mitigate toxicity [8]. Medicinal plants' advantages include their affordability, accessibility, and proven success in cancer treatment, prompting global research interest.

Most ethnobotanical assertions, however, have not yet been scientifically examined. Therefore, evaluating these plants for their potential as chemo-preventive and chemotherapeutic agents becomes crucial. For instance, some researchers have isolated and screened some of these medicinal plants for biological active compounds [9]. Some have further analyzed the pathways in which these compounds are able to combat cancer/tumor [10,11]. These isolated compounds can interfere with signals causing normal cells to transform into cancer cells or suppress cell progression [12]. So far, only a few approved anticancer agents (vincristine, irinotecan, etoposide, and paclitaxel) have been derived from plants and have played a crucial role in chemotherapy [13].

Numerous plants within the *Euphorbiaceae* family are commonly used in various parts of the world as traditional medicinal herbs and some of these plants have shown anticancer properties, as discussed in my recently published article [14,15]. However, the specific traditional uses of the selected 16 *Euphorbia* species in this study were not explored in detail, as the focus was on their cytotoxic potential against breast cancer cell line. These anticancer properties may be constituted by different secondary metabolites contained in these plants such as proteins (proteases, chitinases, oxidases and lectins), steroids, phenolic, cerebrosides, glycerol, flavonoids, and terpenes [16–18]. Antiproliferative activity and selectivity of *Euphorbia bupleurifolia* (*E. bupleurifolia*), *Euphorbia trigona* (*E. trigona*), *Euphorbia gorgonis* (*E. gorgonis*), *Euphorbia cooperi* (*E. cooperi*), *Euphorbia horrida* Var (*E. horrida* Var) and *Euphorbia horrida* Indigenous (*E. horrida* Indigenous), *Euphorbia polygona* (*E. polygona*), *Euphorbia enopla* (*E. enopla*), *Euphorbia Arabica* (*E. arabica*), *Euphorbia ammak* (*E. ammak*), *Euphorbia stellate* (*E. stellata*), *Euphorbia ferox* (*E. ferox*), *Euphorbia clavarioides* (*E. clavarioides*), *Euphorbia coerulescens* (*E. coerulescens*), *Euphorbia tirucalli* (*E. tirucalli*), and *Euphorbia ledienii* (*E. ledienii*) against MCF-7, MDA-MB231, and Vero cell lines were yet to be investigated. Hence, the present study was designed to investigate these sixteen *Euphorbia* species, seeking potential lead compounds capable of effectively and selectively inhibiting breast cancer cell growth.

2. Results

2.1. Phytochemical Investigation

Phytochemical analysis of sixteen *Euphorbia* plant species is summarized in Table 1. The results revealed the presence of glycosides and triterpenoids in all sixteen plants. Flavonoids and phytosterols were found in all the plant except *E. tirucalli*. Furthermore, *E. horrida* Var showed the presence of all 9 secondary metabolites. Alkaloids were present in 11 plants species namely; *E. ammak*, *E. ferox*, *E. bupleurifolia*, *E. cooperi*, *E. gorgonis*, *E. trigona*, *E. horrida* Var, *E. polygona*, *E. coerulescens*, *E. ledienii*, *E. clavarioides*. Tannins were present in 12 plants namely; *E. bupleurifolia*, *E. tirucalli*, *E. enopla*, *E. cooperi*, *E. arabica*, *E. horrida* Var, *E. horrida* indigenous, *E. polygona*, *E. coerulescens*, *E. ledienii*, and *E. clavarioides*.

Saponins were present in 8 plants namely; *E. polygona*, *E. tirucalli*, *E. coerulescens*, *E. bupleurifolia*, *E. enopla*, *E. gorgonis*, *E. horrida* Var, *E. horrida* indigenous. Alkaloids, saponins, and terpenoids which were present in *E. bupleurifolia*, *E. gorgonis*, *E. horrida* Var, *E. polygona* and *E. coerulescens* are all believed to have similar pharmacological effects, such as anticancer and antibacterial properties [19,20].

Table 1. Qualitative phytochemical analysis of sixteen selected *Euphorbia* plant species. Legend: (+) presence of phytochemical compounds; (-) absence of phytochemical compound.

Phytoconstituents	<i>Euphorbia</i> sp.																
	<i>E. ferox</i>	<i>E. bupleurifolia</i>	<i>E. tirucalli</i>	<i>E. ammak</i>	<i>E. enopla</i>	<i>E. cooperi</i>	<i>E. gorgonis</i>	<i>E. arabica</i>	<i>E. trigona</i>	<i>E. horrida</i>	<i>Var. E. horrida</i>	<i>indigenus</i>	<i>E. polygona</i>	<i>E. coerulescens</i>	<i>E. ledienii</i>	<i>E. stellata</i>	<i>E. clavaroides</i>
Phytosterols	+	+	-	+	+	+	+	+	+	+	+		+	+	+	+	+
Pentose	+	+	-	-	+	+	+	-	+	+	+		-	+	-	-	-
Tannins	-	+	+	-	+	+	-	+	-	+	+		+	+	+	-	+
Glycosides	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+
Triterpenoids	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+
Anthraquinones	+	-	-	+	+	+	-	+	+	+	+		-	-	-	-	+
Saponins	-	+	+	-	+	-	+	-	-	+	+		+	+	-	-	-
Flavonoids	+	+	-	+	+	+	+	+	+	+	+		+	+	+	+	+
Alkaloids	+	+	-	+	-	+	+	-	+	+	-		+	+	+	-	+

2.2. Cell Growth Inhibition

The cytotoxic effect of the 16 *Euphorbia* extracts against MCF-7, MDA-MB231, and Vero cell lines determined using the MTT assay was presented in Table 2. The results showed that some extracts inhibited the growth of the tested cell lines while others induced cell proliferation. This may be due to the lack of activity of some extracts against the cell lines. Others showed both proliferation at lower concentrations and inhibition at higher concentrations. This is known as a hormetic dose-response, where the extract or treatment substance has a stimulating effect at a low dose but becomes inhibitory at a higher dose [21]. The CC₅₀ values determined by non-linear regression of MTT assay dose-response data, is essential for evaluating the substance's cytotoxicity. The activity was further categorized using the following interpretation criteria: extracts with CC₅₀ values >100 µg/mL were considered inactive; <100–50 µg/mL indicated low activity; <50–10 µg/mL indicated moderate activity; <10–5 µg/mL reflected good activity; and extracts with CC₅₀ <5 µg/mL were regarded as having potent cytotoxicity. Among the tested species, *Euphorbia ledienii* (EtOAc extract), *Euphorbia cooperi* (DCM extract), and *Euphorbia clavarioides* (DCM extract) exhibited the most potent cytotoxic activity against MCF-7 and MDA-MB231 cell lines, with CC₅₀ values as low as 0.06 µg/mL and 0.006 µg/mL, respectively, and moderate to good selectivity indices. The standard drug (doxorubicin) used in this assay, along with their corresponding IC₅₀ values, are also shown in the Table 2.

Table 2. Cytotoxicity (IC₅₀ in µg/mL, n=5/6) of doxorubicin against MCF-7, MDA-MB231, and Vero cell lines. The dilutions were in triplicates.

Standard drug	IC ₅₀ (µg/mL) (µg/mL, n=5/6)								
	IC ₅₀	SD	R2	IC ₅₀	SD	R2	IC ₅₀	SD	R2
	MCF-7			MDA-MB-231			Vero		
Doxorubicin	4.40	13.61	0.909	20.80	26.96	0.8938	100.9	40.50	0.8790

To calculate the SI value, the study compared the CC₅₀ (concentration causing cytotoxicity in 50% of cells) values of the plant extract against two breast cancer cell lines, namely MCF-7 and MDA-MB231, with that of Vero cells (likely normal, non-cancerous cells). A SI value greater than 3 suggests that the plant extract has a significantly higher toxicity to the breast cancer cells compared to the normal Vero cells [22], indicating its potential selectivity for cancer cells.

Figure 1 presents the IC₅₀ values for doxorubicin against MCF-7, MDA-MB231, and Vero cell lines. MCF-7 cells were highly sensitive to doxorubicin, with a IC₅₀ value of 4.40 µg/mL, indicating strong toxicity against this cell line. Doxorubicin displayed moderate cytotoxicity against MDA-MB231 cells, with a IC₅₀ value of 20.80 µg/mL. In the case of Vero cells, it required a higher doxorubicin concentration of 100.9 µg/mL compared to MCF-7 and MDA-MB231 cells to achieve a 50% inhibition. These IC₅₀ values offer valuable insights into the cytotoxic effects of doxorubicin on these cell lines, highlighting variations in its potency and selectivity.

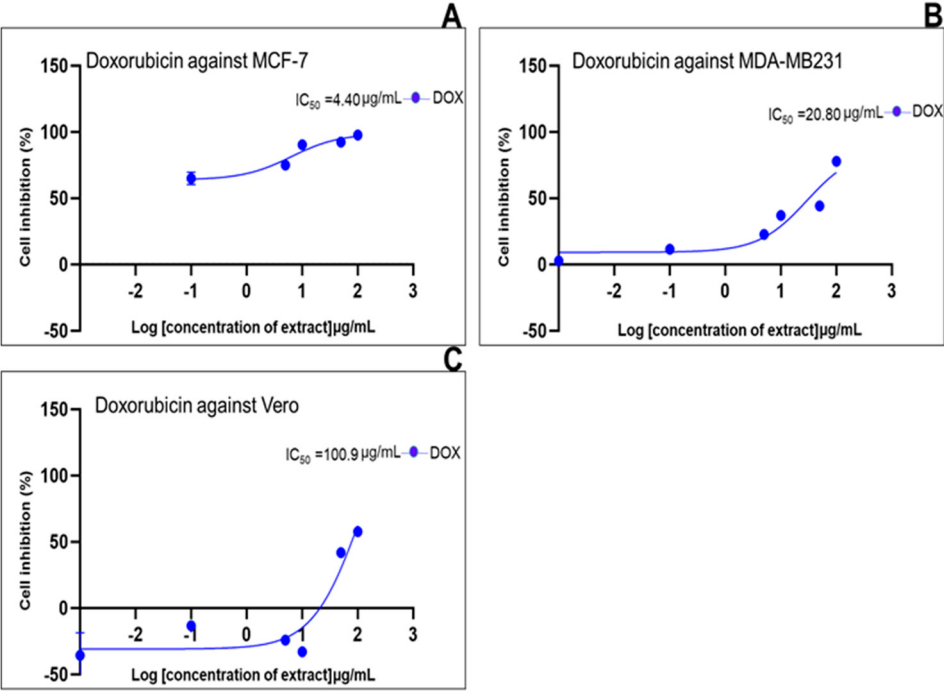


Figure 1. Anti-proliferative effect of doxorubicin on MCF-7 (A), MDA-MB-231 (B), and Vero (C) cell lines, assessed using the MTT assay. IC_{50} values were determined via nonlinear regression analysis. A concentration-dependent inhibitory effect was observed in (A) and (B), while (C) exhibited a hormetic dose-response. Data are presented as means $\pm$ SD from three independent experiments (n = 5 for A; n = 6 for B and C). DOX, doxorubicin.

The hexane (Hex) and dichloromethane (DCM) extracts from *E. bupleurifolia* exhibited varying inhibitory effects on MCF-7 and Vero cells. The CC_{50} values were 7.74 μg/mL (Hex) and >100 μg/mL (DCM) for MCF-7, and 4.67 μg/mL (Hex) and 1.18 μg/mL (DCM) for Vero cells, respectively (Figure 2 and Table S 1). However, the cytotoxic selectivity of *E. bupleurifolia* extracts showed that none of the active extracts had selectivity for the breast cancer cell line, as the SI value was lower than 3 (see Table S 1).

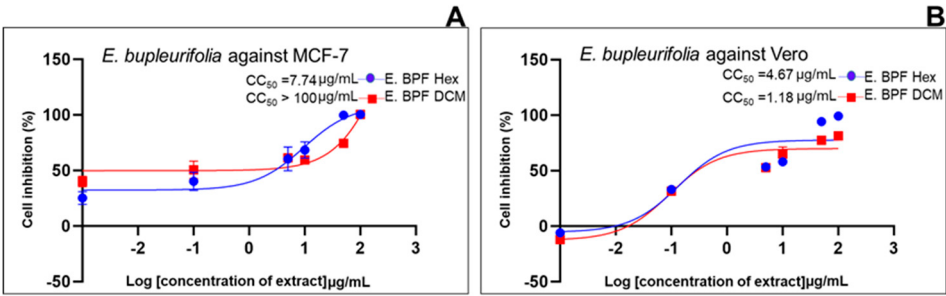


Figure 2. Anti-proliferative effect of *E. bupleurifolia* extracts on MCF-7 (A) and Vero (B) cell lines using the MTT assay. CC_{50} values were determined via nonlinear regression analysis. A concentration-dependent inhibitory effect was observed in (A), while (B) showed a hormetic dose-response. Data are means $\pm$ SD (n = 6). *E. bupleurifolia* hexane (E. BPF Hex); dichloromethane (E. BPF DCM).

The Hex and DCM extracts of *E. trigona* showed cell growth inhibition on MCF-7 cells with CC_{50} values of 7.55 μg/mL and 10.58 μg/mL (Figure 3 and Table S 1), indicating its moderate activity against MCF-7. The Hex extract showed cell growth inhibition of Vero cells with CC_{50} value of 12.11 μg/mL (Figure 3 and Table S 1), indicating moderate activity against the normal cell line. The DCM extract of *E. trigona* showed selectivity for the MCF-7 cell line with an SI value of 9.45 (Table S 1).

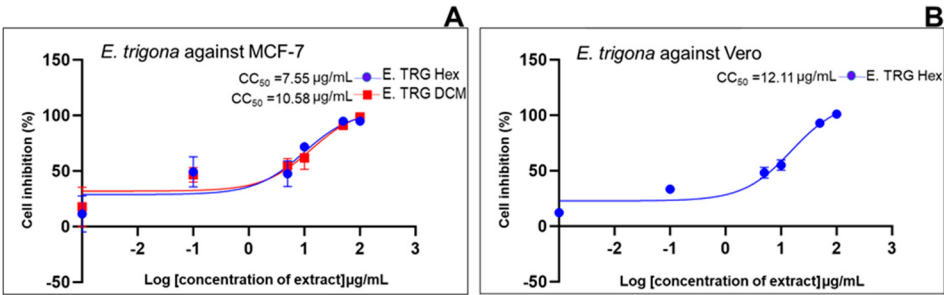


Figure 3. Anti-proliferative effect of *E. trigona* extracts on MCF-7 (A) and Vero (B) cell lines using the MTT assay. CC₅₀ values were determined via nonlinear regression analysis. Data are means ± SD (n = 6). *E. trigona* hexane (E. TRG Hex); dichloromethane (E. TRG DCM).

The Hex extract of *E. gorgonis* displayed the highest cell growth inhibition against MCF-7 and MDA-MB231 with CC₅₀ values of 1.16 µg/mL and 2.34 µg/mL (Figure 4 and Table S 1), demonstrating its potent activity against both cell lines. Moreover, the Hex extract of *E. gorgonis* exhibited selectivity for MCF-7 and MDA-MB231 cells with an SI values of 85.98 and 42.73 (Table S 1) respectively, affirming its efficacy and specificity.

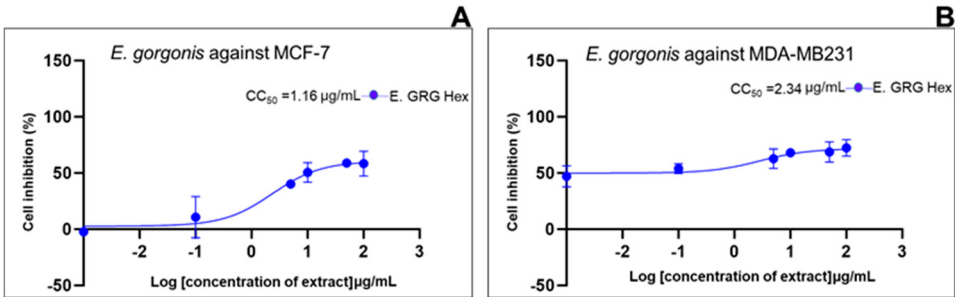


Figure 4. Anti-proliferative effect of *E. gorgonis* extracts on MCF-7 (A) and MDA-MB-231 (B) cell lines using the MTT assay. CC₅₀ values were determined via nonlinear regression analysis. Data are means ± SD (n = 6). *E. gorgonis* hexane (E. GRG Hex).

The Hex and DCM extracts of *E. polygona* demonstrated increased cell growth inhibition of MDA-MB231 cells with CC₅₀ value 17.84 µg/mL and 3.93 µg/mL (Figure 5 and Table S 1), indicating moderate to potent activity against the cell line, respectively. The Hex extract from *E. polygona* showed selectivity for MDA-MB231 cells, with an SI value of 5.60 and 25.44 (Table S 1).

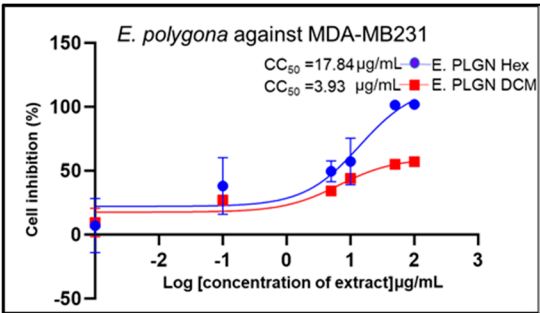


Figure 5. The anti-proliferative effect of doxorubicin (DOX) on MCF-7 (A), MDA-MB231 (B), and Vero (C) cell lines was assessed using the MTT assay. CC₅₀ values were evaluated via nonlinear regression analysis. Results indicated a concentration-dependent inhibitory effect for A and B, and a hormetic response for C. Data presented

as means ± SD from three independent experiments; n = 5 for A, n = 6 for B and C. *E. polygona* hexane (E. PLGN Hex); *E. polygona* dichloromethane (E. PLGN DCM).

All extracts from *E. horrida* Var showed low cell growth inhibition against MCF-7 and MDA-MB231. However, the DCM extract showed cell growth inhibition of Vero with CC₅₀ value of 6.89 µg/mL (Table S 1), indicating its moderate activity towards the normal healthy cells (Table S 1).

Furthermore, extracts from *E. horrida* Indigenous showed low inhibitory activity against MCF-7 cells and MDA-231 as well. The Hex and DCM extracts demonstrated cell growth inhibition against Vero cells with CC₅₀ values of 4.24 µg/mL and 5.42 µg/mL, indicating moderate to potent activity against the normal cell line, respectively (Table S1).

The Hex and MeOH extracts of *E. ferox* showed cell growth inhibition of MDA-MB231 with CC₅₀ value of 16.79 µg/mL and 6.37 µg/mL, indicating moderate activity against the cell line (Figure 6 and Table S 1). Both the Hex and MeOH of *E. ferox* extracts showed selectivity for MDA-MB231 cell line, with an SI values of 5.95 and 15.69, respectively (Table S 1).

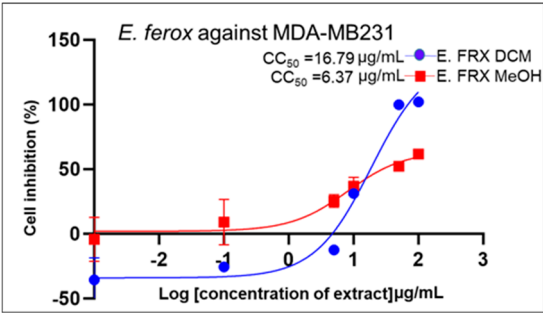


Figure 6. The anti-proliferative effect of *E. ferox* extract on the MDA-MB231 cell line was evaluated using the MTT assay, revealing concentration-dependent inhibitory effects. CC₅₀ values were determined through nonlinear regression analysis, with results expressed as means ± SD from three independent experiments (n = 6). *E. ferox* dichloromethane (E. FRX DCM; *E. ferox* methanol (E. FRX MeOH).

The Hex and DCM extract of *E. clavarioides* exhibited increased cell growth inhibition of MCF-7 and MDA-MB231 with CC₅₀ value of 0.85 µg/mL, 0.23 µg/mL and 1.26 µg/mL (Figure 7 and Table S 1), indicating its potent activity against the two breast cancer cell lines, respectively. Furthermore, the DCM extract exhibited inhibitory activity of Vero cells, with CC₅₀ values of 1.82 µg/mL (Figure 7 and Table S 1), indicating potent activity against the normal cell line, respectively. The Hex and DCM extracts of *E. clavarioides* displayed higher selectivity for MCF-7 with an SI values of 7.91 and >100, respectively (Table S 1).

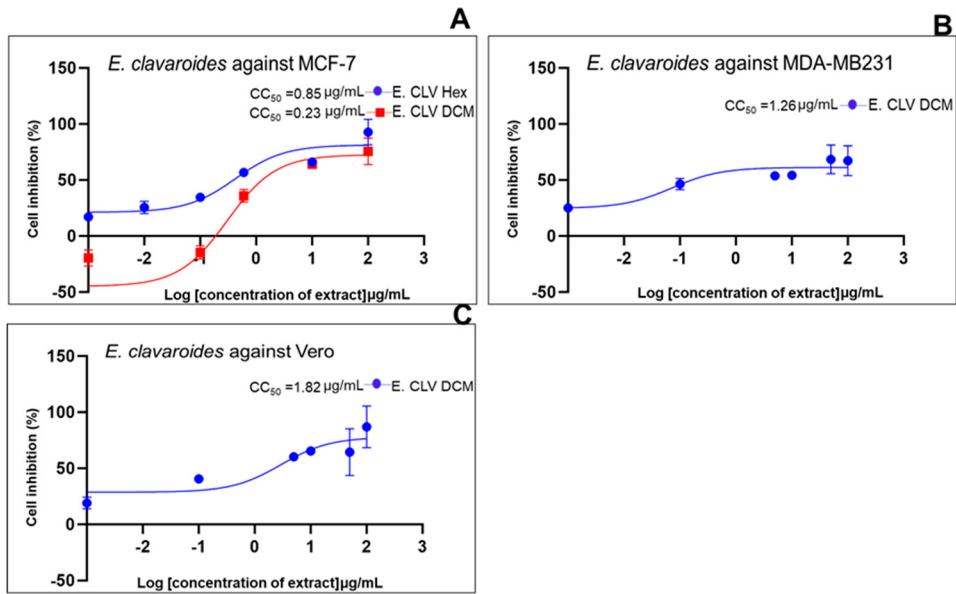


Figure 7. The anti-proliferative effects of *E. clavaroides* extracts on MCF-7 (A), MDA-MB231 (B), and Vero (C) cell lines were evaluated using the MTT assay, with CC₅₀ values determined via nonlinear regression analysis. The results demonstrated a hormetic dose-response in MCF-7 and a concentration-dependent inhibitory effect in MDA-MB231 and Vero cells. Data are presented as means ± SD from three independent experiments (n = 6). *E. clavaroides* hexane (E. CLV Hex); *E. clavaroides* dichloromethane (E. CLV DCM).

The Hex and DCM extracts of *E. ammak* exhibited increased cell growth inhibition of MCF-7 and MDA-MB231 with CC₅₀ values of 1.17 µg/mL, 0.27 µg/mL and 4.32 µg/mL (Figure 8 and Table S 1), indicating potent activity against the two breast cancer cell lines. Furthermore, the Hex and DCM extract showed cell growth inhibition of Vero with CC₅₀ value of 6.49 µg/mL and >100 µg/mL (Figure 8 and Table S 1), indicating moderate to low activity against the cell line, respectively. Based on the SI values, the Hex and DCM extracts of *E. ammak* showed selectivity for MCF-7 cells, with an SI values of 5.54 and > 100, respectively (Table S 1).

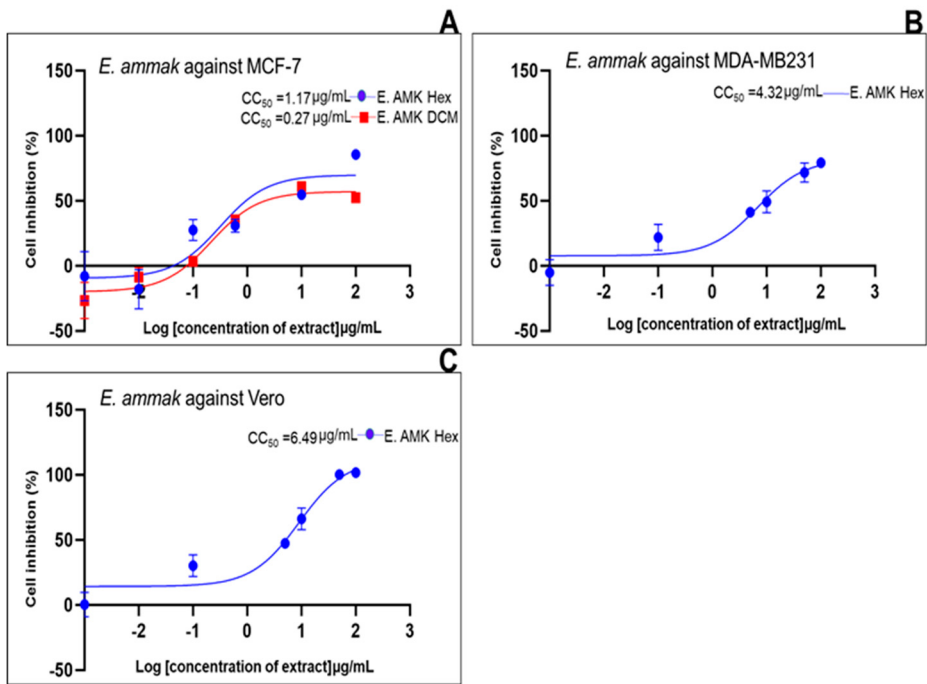


Figure 8. The anti-proliferative effects of *E. ammak* extracts on breast cancer cell lines MCF-7 (A) and MDA-MB231 (B), as well as the Vero cell line (C), were evaluated using the MTT assay, with CC₅₀ values determined

via nonlinear regression analysis. Results indicate a hormetic dose-response effect on MCF-7 (A) and concentration-dependent inhibitory effects on MDA-MB231 (B) and Vero (C). Data are presented as means $\pm$ SD from three independent experiments (n = 6). *E. ammak* hexane (E. AMK Hex); *E. ammak* dichloromethane (E. AMK DCM).

The Hex and DCM extracts from *E. enopla* exhibited cell growth inhibition of MCF-7 and MDA-MB231 with CC₅₀ values of 2.14 μ g/mL, 0.07 μ g/mL, 1.12 μ g/mL and 3.21 μ g/mL (Figure 9 and Table S 1), indicating potent activity towards both cells, respectively. The Hex and DCM extract exhibited cell growth inhibition of Vero with CC₅₀ value of 8.65 μ g/mL and >100 μ g/mL (Figure 9 and Table S 1), indicating potent to low activity against Vero, respectively. The Hex and DCM extracts of *E. enopla* showed selectivity for MCF-7 and MDA-MB231 cells, with high SI values of 4.04, > 100, 7.72 and 31.15, respectively (Table S 1).

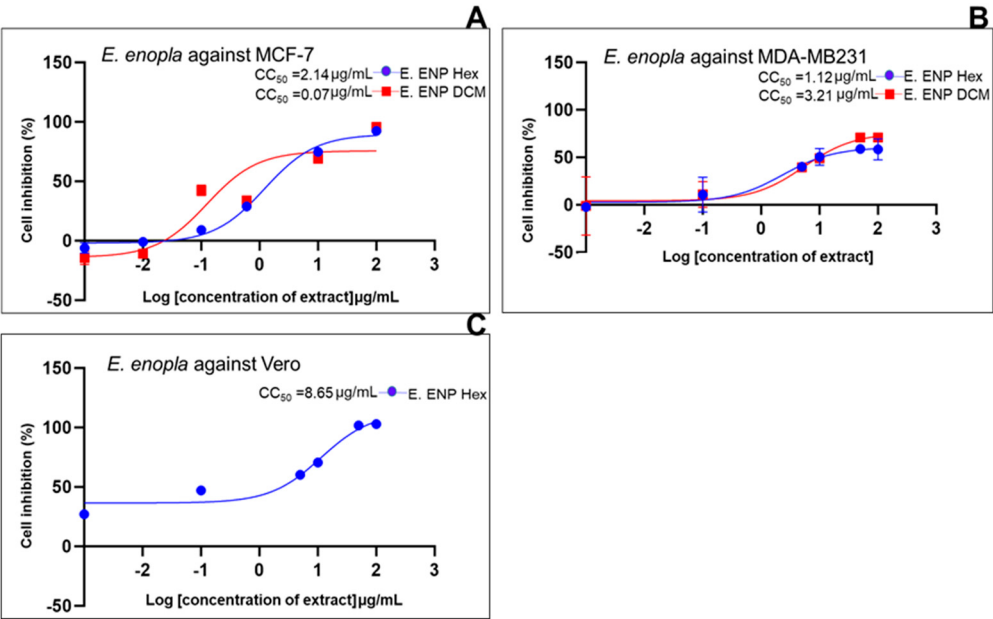


Figure 9. The anti-proliferative effects of *E. enopla* extracts on MCF-7 and MDA-MB231 cell lines, as well as Vero cells, were evaluated using the MTT assay, with CC₅₀ values determined via nonlinear regression analysis. The results demonstrated a hormetic dose-response effect in the cancer cell lines (MCF-7 and MDA-MB231) and a concentration-dependent inhibitory effect on Vero cells. Data are expressed as means $\pm$ SD from three independent experiments (n = 6). *E. enopla* hexane (E. ENP Hex); *E. enopla* dichloromethane (E. ENP DCM).

The DCM extract of *E. stellata* displayed cell growth inhibition of MCF-7 and MDA-MB231 with CC₅₀ values of 9.06 μ g/mL and 3.92 μ g/mL (Figure 10 and Table S 1), indicating moderate to potent activity against the breast cancer cell lines, respectively. Despite the fact that DCM extract displayed considerable inhibitory activity of MCF-7 and MDA-MB231, this extract did not demonstrate any selectivity for any of the breast cancer cell lines.

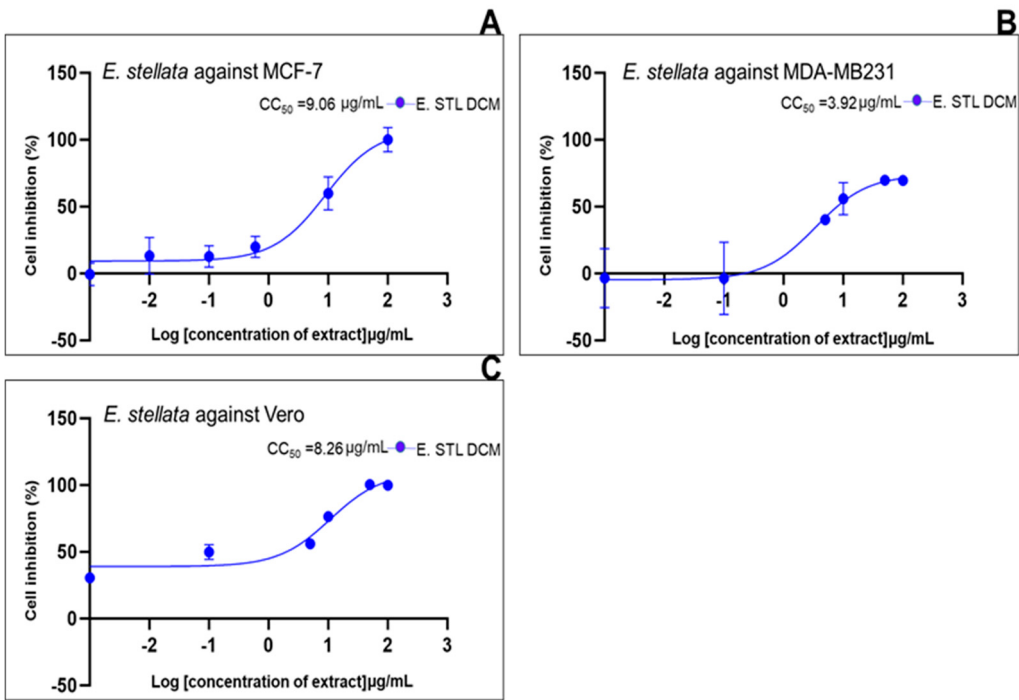


Figure 10. The anti-proliferative effect of *E. stellata* extracts on MCF-7, MDA-MB-231, and Vero cell lines was assessed using the MTT assay to determine CC50 values. Nonlinear regression analysis was employed to calculate these values. The results indicated a concentration-dependent inhibitory effect of the *E. stellata* extracts. Data are presented as means ± SD from three independent experiments (n = 6). *E. stellata* dichloromethane (E. STL DCM).

The Hex and EtOAc extracts of *E. ledienii* showed cell growth inhibition of MCF-7 with CC₅₀ values of 0.90 µg/mL and 0.07 µg/mL (Figure 11 and Table S 1), indicating potent activity towards MCF-7, respectively. The Hex and EtOAc extracts demonstrated cell growth inhibition of Vero cells with CC₅₀ value of 4.23 µg/mL and >100 µg/mL (Figure 11 and Table S 1), respectively. The Hex and EtOAc extracts of *E. ledienii* showed selectivity for MCF-7, with an SI value of 4.7 and > 100, respectively (Table S 1).

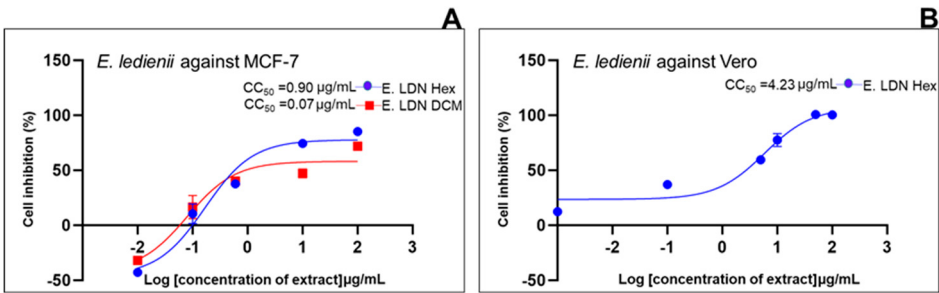


Figure 11. The anti-proliferative effects of *E. ledienii* extracts were evaluated against MCF-7 (A) and Vero (B) cell lines using the MTT assay. The CC50 values for each cell line were determined through nonlinear regression analysis. The results demonstrated a hormetic dose-response effect for MCF-7 cells and a concentration-dependent inhibitory effect for Vero cells. Data represent means ± SD from three independent experiments, with n = 5 for MCF-7 and n = 6 for Vero. *E. ledienii* hexane (E. LDN Hex); *E. ledienii* dichloromethane (E. LDN DCM).

All tested extracts of *E. coerulescenes* exhibited low cell growth inhibition of MCF-7 and MDA-MB231, while the DCM extract inhibited the cell growth of Vero cells. None of the extracts from *E. coerulescenes* demonstrated selectivity for MCF-7 and MDA-MB231.

The DCM extract of *E. arabica* demonstrated cell growth inhibition of MCF-7 with CC₅₀ value of 2.70 µg/mL (Figure 12 and Table S 1), indicating potent activity against MCF-7 cells. Furthermore, the Hex and DCM extracts exhibited cell growth inhibition of Vero with CC₅₀ values of 51.34 µg/mL, and 13.01 µg/mL, indicating its weak to moderate activity against the normal healthy cells (Figure 12 and Table S 1), respectively. The DCM extract of *E. arabica* showed selectivity for MCF-7 cells with an SI value of 4.81 (Table S 1).

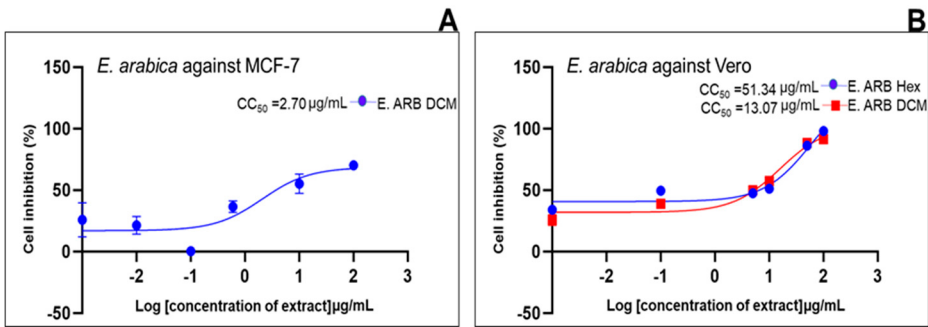


Figure 12. The anti-proliferative effects of *E. arabica* extracts on MCF-7 (A) and Vero (B) cell lines was assessed using the MTT assay, with CC₅₀ values determined through nonlinear regression analysis. Results indicate a concentration-dependent inhibitory effect of *E. arabica* extracts, with values expressed as means ± SD from three independent experiments (n = 6). *E. arabica* hexane (E. ARB Hex); *E. arabica* dichloromethane (E. ARB DCM).

The Hex and DCM extract of *E. cooperi* showed cell growth inhibition of MCF-7 and MDA-MB231 cells with CC₅₀ value of 4.80 µg/mL, 15.57 µg/mL, 2.02 µg/mL and 0.006 µg/mL (Figure 13 and Table S 1), indicating moderate to potent activity against the breast cancer cell lines, respectively. Moreover, the Hex and DCM extracts of *E. cooperi* was selectivity for MCF-7 and MDA-MB231 with SI values of 20.83, 6.42, 49,40 and >100 (Figure 13 and Table S 1), respectively. These SI values show considerable selectivity.

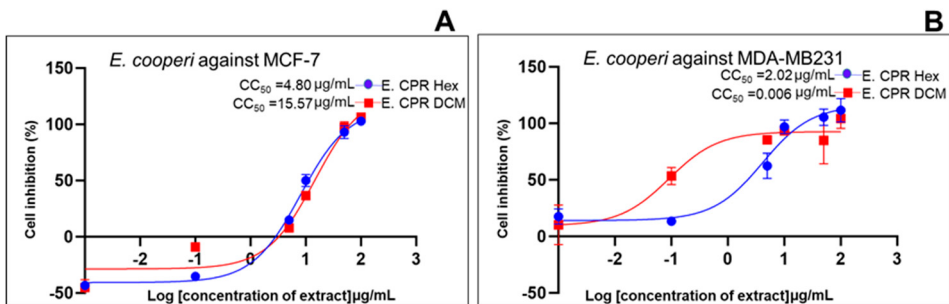


Figure 13. The anti-proliferative effects of *E. cooperi* extracts on MCF-7 (A) and MDA-MB-231 (B) cell lines were assessed using the MTT assay, and CC₅₀ values were determined. The MTT assay results revealed a hormetic dose-response effect (A) and a concentration-dependent inhibitory effect (B) of *E. cooperi* extracts. Data are presented as mean ± SD from three independent experiments, with n = 6. *E. cooperi* hexane (E. CPR Hex); *E. cooperi* dichloromethane (E. CPR DCM).

The Hex and DCM extracts of *E. tirucalli* exhibited cell growth inhibition against MCF-7 and MDA-MB231 cells with CC₅₀ values of 9.77 µg/mL, 7.19 µg/mL, 0.94 µg/mL and 1,19 µg/mL (Figure 14 and Table S 1), demonstrating potent activity against the breast cancer cell lines, respectively. The Hex and DCM extract of *E. tirucalli* displayed significantly higher selectivity for MCF-7 and MDA-MB231 with SI values of 10.23, 13.90, 84.03 and >100, respectively (Table S 1).

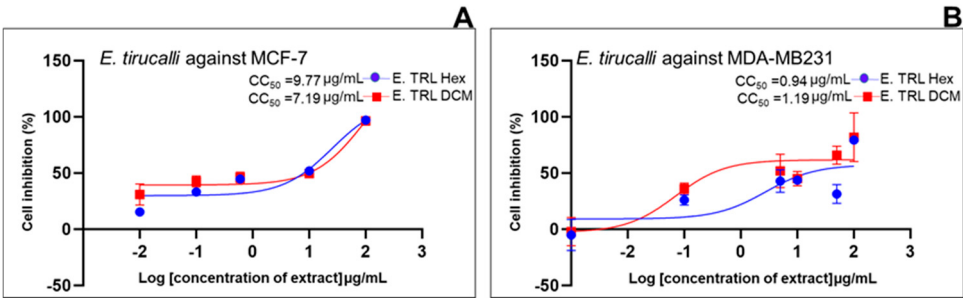


Figure 14. The anti-proliferative effects of *E. tirucalli* extracts on MCF-7 (A) and MDA-MB231 (B) breast cancer cell lines using MTT assay. Results indicate a concentration-dependent inhibition, with CC₅₀ values determined through nonlinear regression analysis. Data are presented as means ± SD from three independent experiments, n = 5 for (A), and n= 6 (B). *E. tirucalli* hexane (E. TRL Hex); *E. tirucalli* dichloromethane (E. TRL DCM).

2.3. Identification of Key Metabolites from Eleven Selective Euphorbia Extracts

The utilization of HPLC has proven to be instrumental in the identification of primary metabolites. Within the study, eleven distinct plant extracts were identified, each demonstrating a unique selectivity for the breast cancer cell lines MCF-7 and MDA-MB231. The chromatograms, derived was presented in Figures 15 to 25. Within each active plant extract, distinctive characteristic peaks of significance were readily visible. These peaks likely correspond to secondary metabolites responsible for the observed cytotoxicity. However, due to resource constraints, neither isolation nor tentative identification of these bioactive compounds was performed. This represents a limitation of the present study, as the specific metabolites contributing to the activity remain unknown. Future studies will be conducted aiming to isolate and characterize these compounds to better understand their mechanisms of action.

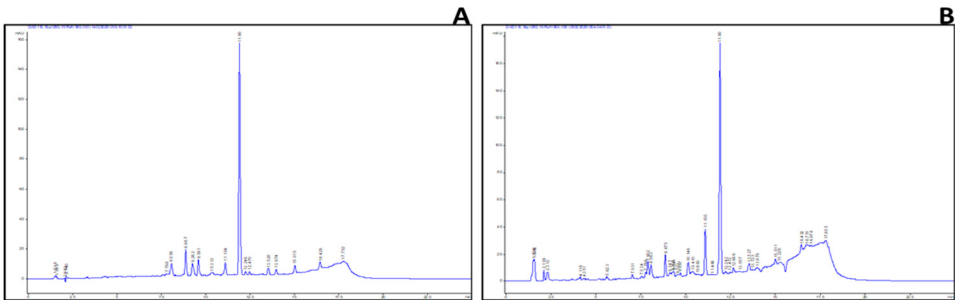


Figure 15. HPLC chromatograms of selective extracts of *E. clavarioides* (A) Hex and (B) DCM for MCF-7.

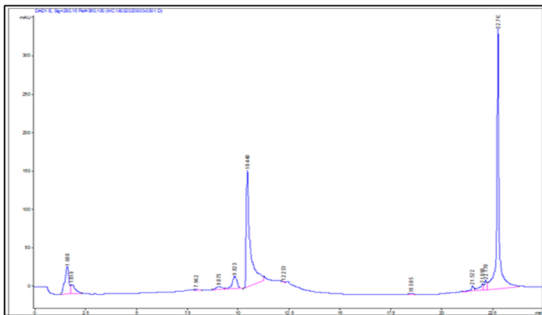


Figure 16. HPLC chromatograms of selective extract of *E. trigona* DCM for MCF-7.

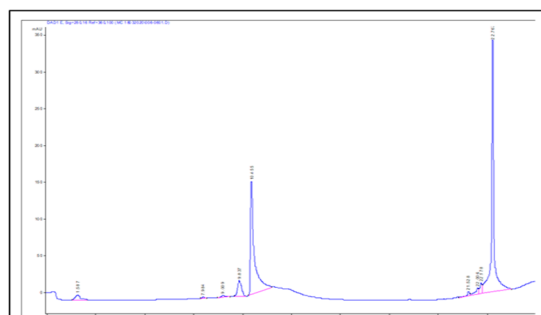


Figure 17. HPLC chromatograms of selective extract of *E. gorgonis* Hex for MCF-7 and MDA-MB231.

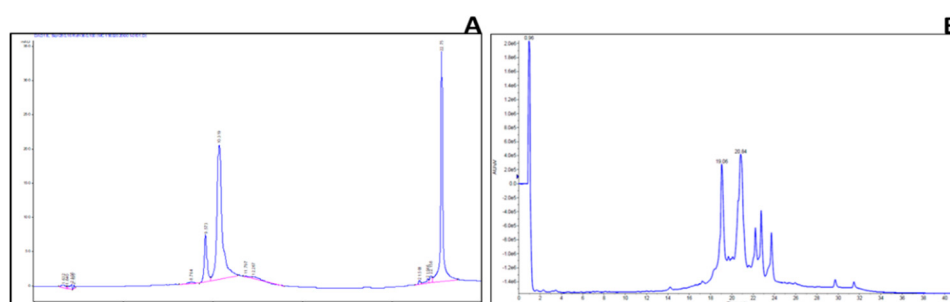


Figure 18. HPLC chromatograms of selective extracts of *E. ledienii* (A) Hex and (B) EtoAC for MCF-7 and MDA-MB231.

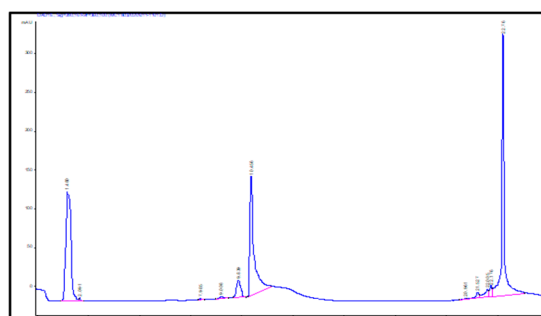


Figure 19. HPLC chromatograms of selective extract *E. arabica* DCM for MFC-7 and MDA-MB231.

The analysis indicated that the two extracts from *E. clavarioides* exhibited distinct chemical profile at across retention times, which ranged from 1.51 to 17.52 minutes. Notably, significant peaks were identified at retention times of 8.86, 11.10, and 11.90 minutes, as shown in Figure 15. Similarly, *E. trigona* DCM, *E. gorgonis* Hex, *E. ledienii* Hex, and *E. arabica* DCM exhibited parallel peaks spanning a retention time of 1.50 to 22.76 minutes, with prominent peaks at 1.58, 9.83, 10.45, and 22.76 minutes for all four extracts (Figure 16-19). This shared profile highlights the presence of common phytochemicals such as phytosterols, glycosides, triterpenoids, and flavonoids among these *Euphorbia* species.

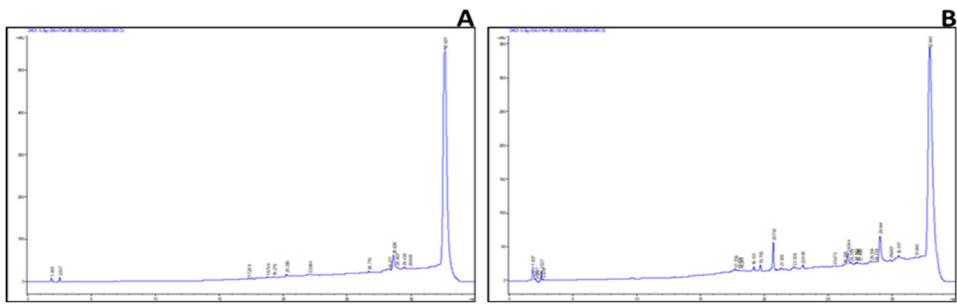


Figure 20. HPLC chromatograms of selective extracts of *E. cooperi* (A) Hex and (B) DCM for MCF-7 and MDA-MB231.

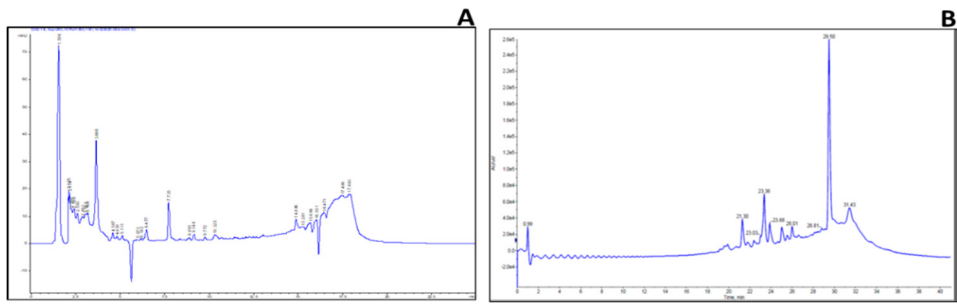


Figure 21. HPLC chromatograms of selective extracts of *E. ammak* (A) Hex and (B) DCM for MCF-7.

Noteworthy observations were made in the extracts from *E. cooperi*, with peaks manifesting across a range of 1.80 to 32.90 minutes. Specifically, significant peaks were evident at 20.70, 26.54, 28.6, 29.04, and 32.90 minutes, with a more noticeable presence in the *E. cooperi* DCM extract (Figure 20). Similarly, *E. ammak* Hex exhibited peaks within a retention time of 1.55 to 17.89 minutes, with major peaks recorded at 1.55, 2.40, 3.66, 7.73, and 14.88 minutes. Conversely, *E. ammak* DCM featured peaks from 0.99 to 31.43 minutes, with major peaks at 0.99, 21.30, 23.36, and 29.50 minutes (Figure 21).

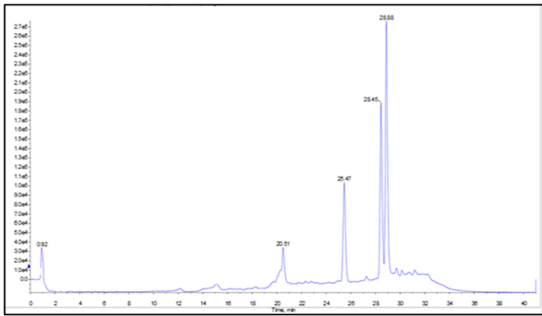


Figure 22. HPLC chromatograms of selective extract *E. ferox* DCM.

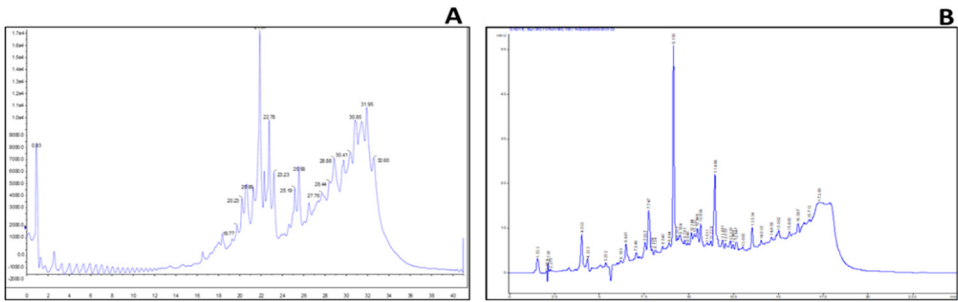


Figure 23. HPLC chromatograms of selective extract of *E. polygona* (A) Hex and (B) DCM for MDA-MB231.

Further investigation unveiled that *E. ferox* displayed peaks over a retention time range of 0.92 to 28.88 minutes, with major peaks at 0.92, 20.51, 25.47, 28.45, and 28.88 minutes (Figure 22). *E. polygona* Hex displayed peaks from 0.93 to 32.60 minutes, prominently at 0.93, 21.91, 22.78, 25.58, and 31.95 minutes. In contrast, the DCM extract of *E. polygona* displayed peaks spanning 1.53 to 17.28 minutes, with major peaks observed at 1.53, 4.03, 7.74, 9.15, 11.46, and 13.54 minutes (Figure 23).

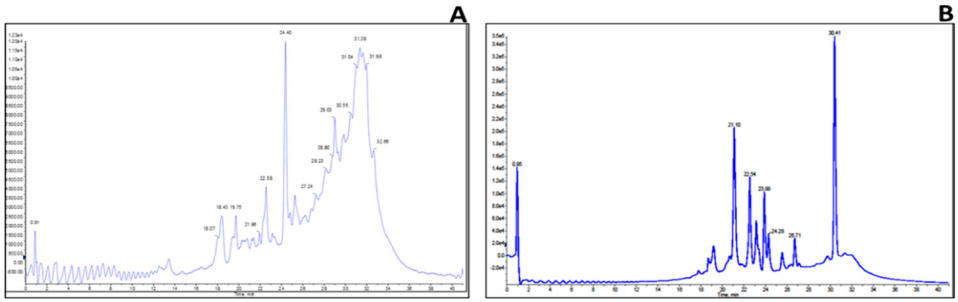


Figure 24. HPLC chromatograms of selective extract *E. enopla* (A) Hex (B) DCM for MFC-7 and MDA-MB231.

Remarkably similar profiles occurred for *E. enopla* Hex and *E. enopla* DCM extracts. The former displayed peaks from 0.91 to 32.66 minutes, featuring major peaks at 0.91, 18.43, 22.58, 24.40, and 29.03 minutes. Similarly, the DCM extract of *E. enopla* displayed peaks within a retention time of 0.95 to 30.41 minutes, notably at 0.95, 21.10, 22.54, 23.88, 26.71, and 30.41 minutes (Figure 24).

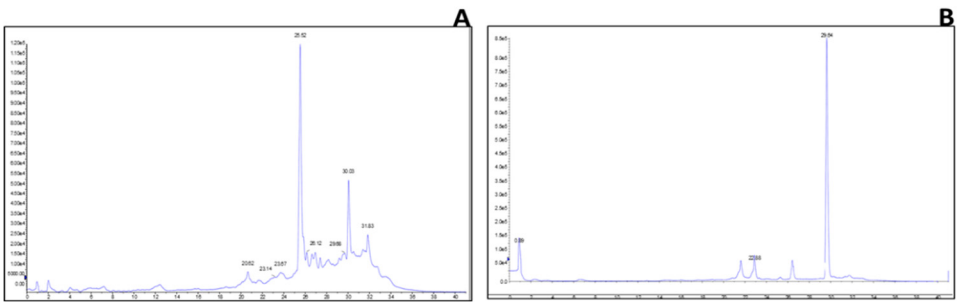


Figure 25. HPLC chromatograms of selective extract *E. tirucalli* (A) Hex (B) DCM for MFC-7 and MDA-MB231.

In the context of *E. tirucalli* Hex, peaks emerged between 20.62 and 31.83 minutes, with major peaks at 20.62, 25.52, and 30.02 minutes. *E. tirucalli* DCM, on the other hand, exhibited peaks over a retention time span of 0.89 to 29.64 minutes, featuring major peaks at 0.89, 22.88, and 29.64 minutes (Figure 25).

3. Discussion

The present study focused on evaluating the phytochemical profiles, cytotoxic activity, and selectivity of sixteen *Euphorbia* plant species against the breast cancer cell lines MCF-7 and MDA-MB231, as well as the normal Vero cells. The results offer valuable insights into the potential anticancer properties of these plant extracts and shed light on their underlying mechanisms. The phytochemical analysis revealed the presence of various secondary metabolites across the studied *Euphorbia* species.

Glycosides and triterpenoids were found ubiquitously, indicating their wide distribution within this plant group. The presence of these phytochemicals is in support with studies by Shi et al., 2008 [16] which noted the common secondary metabolites found in the *Euphorbiaceae* family which were also present in all the selected plants in this study. It has also been shown that these two phytochemicals also possess anticancer properties [23]. Furthermore, triterpenoid have proven to possess medicinal properties such as anticarcinogenic, antimalarial, anti-ulcer, hepatocidal, antimicrobial activity, antimalarial and anti-viral [24–27]. Munro et al., 2015 [28] demonstrated that the tetracyclic triterpene euphol derived from *E. tirucalli* latex has anticancer properties. Arnidiol is a compound that belongs to the class of pentacyclic triterpenoid diols. It is a natural substance that has been the subject of several studies in the field of cancer research. A study conducted by Hu et al., 2020 [29] to investigate the effects of arnidiol on human breast cancer cells known as MDA-MB-231 cells. The study found that arnidiol was able to induce apoptosis or programmed cell death in these cells. This was achieved through the process of mitochondrial translocation of Drp1 and cofilin, which are proteins that play important roles in cell division and movement. The researchers also observed a correlation between the induction of apoptosis and the reduction of PARP expression, which is a protein involved in DNA repair, and the increase in caspase-3 cleavage, which is a marker of apoptosis.

Stevioside is a naturally occurring diterpenoid glycoside that is commonly found in the leaves of the *Stevia rebaudiana* (Bertoni) Bertoni plant. It has gained significant attention in recent years due to its potential health benefits. A study conducted by Paul et al., 2012 [30] revealed that stevioside can reduce MMP (mitochondrial membrane potential) and activate the mitochondrial-mediated apoptotic pathway in MCF-7 cells. This indicates that stevioside can effectively induce apoptosis, which is a process of programmed cell death that occurs in damaged or abnormal cells. The study further suggests that stevioside has the potential to become a valuable therapeutic agent for cancer treatment.

Flavonoids and phytosterols were almost universally present, except in *E. tirucalli*. This suggests that these compounds might play a significant role in the biological activity of these plants. Flavonoids were used to treat various ailments as they possess medicinal properties such as anti-inflammatory activity, enzyme inhibition, antimicrobial activity, estrogenic activity, antiallergic activity and antioxidant activity [28,31–33]. Mai et al., 2017 [34] reported that *E. helioscopia* which had high concentration of flavonoids exhibited cytotoxic activity against a triple negative breast cancer cell line. Zhu and Xue, 2019 [35] conducted a study where they investigated the effect of kaempferol, a flavonoid, on MDA-MB-231 cells. They found that kaempferol induces apoptosis in these cells through a mechanism that involves increasing the expression of cleaved caspase-9/3 and p-ATM. Moreover, in a study conducted by Zhang et al., 2018[36], three flavonoids were isolated from *Tephrosia kirilowii* (Turcz. ex DC.) Holub - isorhamnetin, genkwanin, and acacetin. These flavonoids were found to have a significant impact on MDA-MB-231 cells. They suppressed cell growth, induced apoptosis, and downregulated the PI3K/AKT/mTOR/p70S6K/ULK signaling pathway. They did this by stimulating the apoptotic pathways, leading to the programmed death of the MDA-MB-231 cancer cells. The downregulation of the PI3K/AKT/mTOR/p70S6K/ULK signaling pathway by these flavonoids is also significant. This pathway is known to play a crucial role in cell survival and growth. The flavonoids were able to suppress this pathway, leading to decreased cell growth and increased sensitivity to apoptosis.

Additionally, phytosterols are considered to have a number of bioactive properties that have a variety of health benefits, including anti-inflammatory, antioxidative, and anticarcinogenic properties, as well as the ability to reduce cholesterol [37,38]. A mixture of phytosterols were able to inhibit tumor development in various cancer forms, including cholangiocarcinoma and breast cancer, at physiological doses [39,40]. Alkaloids were detected in a considerable number of species, further emphasizing the chemical diversity within the *Euphorbia* genus. It is not surprising that alkaloids were found in almost all selected plants, because the presence of alkaloids in *Euphorbiaceae* species has been documented in many studies [41–44]. Extracts from plants containing alkaloids have been used as prescription medicines for many years, and due to the presence of alkaloids they owe their potent effects. Morphine which is currently used as an analgesic was the first alkaloid to be obtained from opium poppy [45]. Furthermore, other alkaloids such as vinblastine, quinine, morphine, atropine, nicotine, caffeine, ephedrine and strychnine are also used in production of medicine [46]. Alkaloids have a number of physiological effects on humans, such as antibacterial, antimitotic, anti-inflammatory, local anesthetic, hypnotic, antitumor activity and several others [47]. Furthermore, they are also of great interest to organic chemists, biologists, biochemists, pharmacologist and pharmacists, typically from plants rather than animals [48]. Berberine is a naturally occurring isoquinoline alkaloid isolated from *Cotridis rhizoma*. Zhao and colleagues [49] investigated the effect of berberine on triple-negative breast cancer (TNBC) cells. Their findings revealed that berberine activated caspase-9/CytoC-mediated apoptosis in TNBC cells, leading to a significant suppression of cell proliferation both in vitro and in vivo. Tetrandrine is a naturally occurring alkaloid that has shown great potential as an anticancer agent. A study conducted by Bhagya et al. in 2019[50] found that tetrandrine, which was extracted from *Cyclea peltata* (Lam.) Hook. f. and Thomson, demonstrated cytotoxic and apoptotic effects on MDA-MB-231 cells. The mechanism of action was attributed to an increase in the levels of reactive oxygen species (ROS) and caspase-8/9/3.

Tannins, known for their antioxidant properties, were also detected in several species. The presence of these diverse compounds aligns with the potential bioactivity of *Euphorbia* plants against cancer cells. The presence of this phytochemicals is in agreement with Aleksandrov et al., 2019 [51], who reported larger number of tannins in *E. hirta*. Fraga-Corral et al., 2021 [52] reported that topical tannin treatments help to flush all the irritants out of the skin, can also be used as an anti-inflammatory agent and use their antihemorrhagic and antiseptic ability in the treatment of burns and other wounds. In other studies, tannins were utilized in treatment of different diseases as they have been reported to possess high antioxidants, free radical scavenging activity, antimicrobial, antivirals and in cancer chemotherapy [53–57]. Saponins were also detected in some plants investigated. Bigoniya and Rana, 2009 [58] reported the presence of saponins in *E. neriifolia*. Furthermore, saponins were also reported in *E. paralias* and *E. terracina* [59]. The presence of saponins in the above plants is unsurprising, as it is clear that most of the *Euphorbiaceae* has it. Moreover, they also possess pharmacological and medicinal properties such as cell membrane permeability, hemolytic activity, antiviral, antifungal, anti-inflammatory and antiallergic [60–62]. Furthermore, they have promising uses in the treatment of cancer based on their ability to suppress angiogenesis, decreased invasiveness of cells, cell cycles arrest and apoptosis induction [63]. Along with other antitumor medicines, saponins have been added to enhance their cytotoxicity in tumor treatment [64,65].

The cytotoxicity assays, yielded intriguing findings. The extracts from various *Euphorbia* species displayed a spectrum of effects on the tested cell lines. Some extracts exhibited potent cell growth inhibition, while others demonstrated little or no activity. This variability could be attributed to differences in chemical composition, concentrations of active compounds, and the mechanisms underlying their actions. Notably, certain extracts were inactive against the cell lines, which might suggest their lack of efficacy or different modes of action. Extracts from *E. clavarioides*, *E. gorgonis*, and *E. tirucalli* showed notably low CC₅₀ values, indicating strong inhibition of cancer cell growth. In the current study, the hexane and DCM extracts of *E. tirucalli* demonstrated potent antiproliferative activity against breast cancer cells, with CC₅₀ values of 9.77 µg/mL and 7.19 µg/mL against MCF-7,

and 0.94 $\mu\text{g/mL}$ and 1.19 $\mu\text{g/mL}$ against MDA-MB-231, respectively. These values compare favorably with the IC_{50} values reported by Choene and Motadi (2016) [66], who found 15 $\mu\text{g/mL}$ for MCF-7 and 30 $\mu\text{g/mL}$ for MDA-MB-231 using a butanol extract. These findings highlight the potential of these extracts as promising candidates for further investigation and potential development into therapeutic agents. This similarity supports the reliability of the current findings, implying that these extracts possess potent antiproliferative potential against the specific cancer cell lines. The co-occurrence between the two studies strengthens the notion that these extracts could serve as promising candidates for further exploration and potential development into therapeutic agents for breast cancer treatment. In contrast, *E. tirucalli* was found to be inactive against MCF-7 cells in the study by El-Hawary et al. (2020) [67], with no significant cytotoxicity observed against HEPG2 and CACO2 cell lines. These differences may reflect variations in plant origin, solvent polarity, extraction protocols, or assay conditions. Nevertheless, the consistent antiproliferative activity observed in both the current study and that of Choene and Motadi underscores the therapeutic potential of *E. tirucalli* extracts, particularly in breast cancer treatment. In the current study, *E. trigona* hexane and DCM extracts exhibited moderate antiproliferative activity against MCF-7 cells, with CC_{50} values of 7.55 and 10.58 $\mu\text{g/mL}$, respectively. These results align with the IC_{50} value of 16.1 μM reported by El-Hawary et al. (2020) [67], confirming the moderate cytotoxic potential of *E. trigona* against MCF-7 cells. Additionally, El-Hawary et al. reported cytotoxicity against CACO2 cells ($\text{IC}_{50} = 15.6 \mu\text{M}$), further supporting its broader anticancer potential. This consistency supports the theory that *E. trigona* extracts may indeed have biologically relevant activities against breast cancer cells. Additionally, the absence of antiproliferative effects in *E. horrida* extracts against all tested cell lines in the current study is consistent with the findings reported by El-Hawary et al., 2020 [67], indicating that *E. horrida* extracts might lack significant cytotoxic effects against the selected breast cancer cell lines. Additionally, *E. horrida* did not demonstrate any notable cytotoxic effects on HEPG2 and CACO2 cells [67]. This uniformity in results, despite the differences between studies, highlights the strength of the conclusions.

Furthermore, several extracts exhibited strong selectivity toward breast cancer cell lines, as indicated by their high selectivity index (SI) values. Notably, *E. gorgonis* hexane extract showed exceptional selectivity for both MCF-7 (SI = 85.98) and MDA-MB231 (SI = 42.73) cells. Similarly, *E. tirucalli* and *E. cooperi* extracts displayed potent selectivity for MDA-MB231, with SI values reaching up to >100. Other species such as *E. clavarioides*, *E. ammak*, *E. enopla*, *E. ledienii*, *E. polygona*, *E. arabica*, and *E. ferox* also showed notable selectivity for MCF-7 and/or MDA-MB231, with SI values ranging from 4.04 to >100. In contrast, *E. bupleurifolia* extracts did not demonstrate selectivity, with SI values below 3. These findings support the idea that certain *Euphorbia* species contain bioactive compounds that preferentially target breast cancer cells, particularly triple-negative subtypes, and may serve as promising candidates for anticancer drug development with minimized off-target effects.

The HPLC analysis further revealed the complexity of the phytochemical profiles within the tested *Euphorbia* extracts. Common peaks observed across multiple species indicate shared compounds, such as phytosterols, glycosides, triterpenoids, and flavonoids. This suggests a certain level of structural similarity in these phytochemicals, which may contribute to their observed bioactivities. However, the presence of distinct peaks in specific species indicates individuality in their chemical composition, which might explain the differential activities observed in the cytotoxicity assays. However, the current study faced limitations due to resource constraints, which prevented both the isolation and preliminary identification of these bioactive compounds. Consequently, the specific metabolites responsible for the observed activity remain undetermined.

4. Materials and Methods

4.1. Materials

Hexane, dichloromethane, ethyl acetate, and methanol used for plant extraction were purchased from Merck (South Africa). Human breast cancer cell lines (MCF-7 and MDA-MB231) and the normal Vero cell line were obtained from Cellonex (South Africa). Cell culture reagents, including Dulbecco’s Modified Eagle Medium (DMEM), trypan blue dye, and MTT salt, were sourced from Sigma-Aldrich (South Africa).

4.2. Plant Collection and Processing

In March of 2017, 16 distinct species of *Euphorbia* plants were utilized. All plant material was sourced from a reputable commercial supplier, Griep Nursery, located in Pretoria, South Africa (GPS coordinates: 25°47’3’’S, 28°18’58’’E). We interacted with Mr Kotie Retief a representative at Griep Nursery, who facilitated the acquisition of these plants. The *Euphorbia* species were identified by the expert horticulturists at Griep Nursery. Due to the confidence in the accuracy of the identification, no voucher specimens were deposited in a herbarium. As all plant material was obtained from a commercial nursery, no wild collection was undertaken, and thus, no permits were required. This complies with the Nagoya Protocol and the Biodiversity Act 10 of 2004, of South Africa, as well as our institutional guidelines. No plants were sourced from the wild, and therefore, no collection permits were necessary. Due to the size of the plants, it was not feasible to isolate their roots, stems, and leaves, thus the entire plant was utilized for the study. For further details regarding the commercially acquired *Euphorbia* plant species, please refer to Table 3.

Table 3. List of 16 selected *Euphorbia* plant species used in this study.

Plant species	Abbreviation
<i>Euphorbia ferox</i>	<i>E. ferox</i>
<i>Euphorbia bupleurifolia</i>	<i>E. bupleurifolia</i>
<i>Euphorbia tirucalli</i>	<i>E. tirucalli</i>
<i>Euphorbia clavarioides</i>	<i>E. clavarioides</i>
<i>Euphorbia polygona</i>	<i>E. polygona</i>
<i>Euphorbia gorgonis</i>	<i>E. gorgonis</i>
<i>Euphorbia enopla</i>	<i>E. enopla</i>
<i>Euphorbia cooperi</i>	<i>E. cooperi</i>
<i>Euphorbia ammak</i>	<i>E. ammak</i>
<i>Euphorbia coerulescens</i>	<i>E. coerulescens</i>
<i>Euphorbia trigona</i>	<i>E. trigona</i>
<i>Euphorbia horrida</i> Var	<i>E. horrida</i> Var
<i>Euphorbia horrida</i> Indigenous	<i>E. horrida</i> ind
<i>Euphorbia arabica</i>	<i>E. arabica</i>
<i>Euphorbia stellata</i>	<i>E. stellata</i>
<i>Euphorbia ledienii</i>	<i>E. ledienii</i>

The plants were rinsed with tap water upon arrival, cut into smaller pieces, air-dried for 14 days, and ground into fine powder. To extract different components from various *Euphorbia* species, 10 g each of their powdered material was sequentially extracted with hexane, dichloromethane, ethyl acetate, acetone, and methanol in increasing order of polarity. The resulting extracts were subjected to a 48-hour shake at 37°C on an OrbiShake shaker (Labotec) before being filtered through Whatman filter paper. This process was repeated twice, and the resulting filtrates were concentrated under reduced pressure at 45°C using a rotary vacuum evaporator (Buchi Labotech, Switzerland). The extracts were then dried under the LabAire fume hood and stored at 4°C for future use.

4.3. Phytochemical Analysis

A comprehensive qualitative screening was conducted on sixteen species of *Euphorbia* to evaluate their secondary metabolites, including alkaloids, glycosides, phytosterols, pentose, anthraquinones, flavonoids, phenols, saponins, triterpenoids, and tannins. The methodology followed the protocols established by Yadav and Agarwala (2011) with minor modifications as detailed below. Each test relied on visual observations, specifically changes in color or the formation of precipitates following the introduction of designated reagents.

Detection of Phytosterols

Approximately 0.05 g of powdered plant material was extracted with 10 mL of chloroform. Subsequently, 1 mL of concentrated sulfuric acid (H_2SO_4) was carefully added along the sides of the test tube to form a separate layer. The development of a reddish-brown coloration in the chloroform layer indicated the presence of phytosterols.

Detection of Pentoses

Two grams of powdered plant material were mixed with 40 mL of distilled water and filtered. To 2 mL of the filtrate, 2 mL of hydrochloric acid (HCl) containing a pinch of phloroglucinol was added. The mixture was heated on a hot plate for 5 minutes. The appearance of a red coloration indicated the presence of pentose sugars.

Detection of Tannins

About 0.5 g of powdered plant material was boiled in 20 mL of distilled water and filtered. A few drops of 0.1% ferric chloride (FeCl_3) solution were added to the filtrate. The formation of a brownish-green or blue-black coloration suggested the presence of tannins.

Detection of Glycosides

A total of 0.5 g of powdered sample was mixed with 2 mL of glacial acetic acid, followed by the addition of one drop of 0.1% ferric chloride solution. Carefully, 1 mL of concentrated H_2SO_4 was added to the mixture. A color change from violet to blue to green indicated the presence of glycosides.

Detection of Triterpenoids

Two grams of dried, powdered plant material were extracted with 1 mL of chloroform. To this, 3 mL of concentrated H_2SO_4 was carefully added to form a separate layer. A reddish-brown coloration at the interface confirmed the presence of triterpenoids.

Detection of Anthraquinones

One gram of plant material was boiled in 12 mL of 10% HCl for 5 minutes and filtered. Once cooled, 10 mL of chloroform was added to the filtrate and shaken. The chloroform layer was transferred to a clean tube, and 10 mL of 10% ammonia solution was added. A rose-pink coloration in the upper layer indicated the presence of anthraquinones.

Detection of Saponins

Half a gram (0.5 g) of the powdered material was boiled in 5 mL of distilled water and filtered. To 3 mL of the filtrate, an additional 3 mL of distilled water was added and shaken vigorously for 5 minutes. The formation of a stable froth indicated the presence of saponins.

Detection of Flavonoids

Approximately 0.5 g of powdered sample was mixed with 10 mL of ethyl acetate and heated for 3 minutes. The mixture was filtered, and 1 mL of dilute ammonia solution was added to 4 mL of the filtrate. A yellow precipitate indicated the presence of flavonoids.

Detection of Alkaloids

About 0.2 g of powdered plant material was extracted with 2 mL of 1% hydrochloric acid and filtered. To the filtrate, 1 mL of Mayer's reagent was added, followed by a few drops of Dragendorff's reagent. The appearance of a creamy white or orange precipitate was indicative of alkaloids.

4.4. Antiproliferation and Cytotoxic Screening of Euphorbia Extracts

4.4.1. Cell Culture

Two cell lines belonging to human breast cancer, namely MCF-7 and MDA-MB231, along with a green monkey kidney cell line named Vero, were procured from Cellonex in South Africa. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) that was supplemented with 10% fetal bovine serum (FBS), and were placed in a 5% CO₂ incubator that was humidified and kept at a temperature of 37°C. The cells were sub-cultured once they attained a confluency level of 90%.

4.4.2. MTT Assay

After cells had reached the desired confluency of 90% as observed under a microscope, they were rinsed with 10 mL of PBS and harvested using trypsin. The concentration of the live cells was evaluated using typhan blue dye under a cell counter, countess FL, life technology. A total of 1 x 10⁵ cells/mL were seeded into each well in a 96-well microtiter plate and incubated under the same condition for 24 hours to allow attachment.

All cells were treated with 100 µl of a series of dilutions (100-0.1µg/mL) and were in triplicates for 48 hours. Wells with untreated cells served as negative control. Doxorubicin drug was used as positive control. Cell viability was conducted based on tetrazolium dye (MTT assay) method with slight modification [28].

4.4.3. CC₅₀ Determination

Graph Prism 9 software was utilized to analyze the data, with CC₅₀ values being determined through non-linear regression plotting. To improve data visualization, stabilize variances, and normalize data distributions, Prism graphs were generated using log-transformed values. The toxicity of the extracts was assessed using the CC₅₀ standard criteria established by Indrayanto et al. [69], as shown in Table 4.

Table 4. CC₅₀ criteria for cytotoxicity.

CC ₅₀ (µg/mL)	Status
>100 µg/mL	Inactive
<100 ≥ 50 µg/mL	Low activity
< 50 µg/mL ≥10 µg/mL	Moderate activity
< 10 µg/mL ≥ 5 µg/mL	Good activity
<5 µg/mL	Potent activity

4.4.4. Selectivity Index

The potential of an extract to inhibit growth of cancerous cells without harming normal cells was evaluated by calculating the selectivity index (SI) of the potent extracts. This was determined by comparing the antiproliferative activity (CC₅₀) of the plant extract against two breast cancer cells (MCF-7 and MDA-MB231) with that from the kidney of an African green monkey cell line (Vero). A SI value greater than 3 indicates potential of safer therapy [70]. For the purpose of calculating SI, extracts that had a growth inhibition of less than 50% and which had an CC₅₀ greater than 100 were taken as 100.

Selectivity index= IC₅₀ calculated for normal cells/ CC₅₀ calculated for cancer cells

4.4.5. High-Performance Liquid Chromatography (HPLC)

The HPLC-diode array detection analysis was performed on the extracts using an Agilent 1100 series (Agilent, Waldbronn, Germany) instrument equipped with photo diode array, autosampler, column thermostat and degasser. A Phenomenex: Luna 5 µm C18 (2) (150 × 4.6 mm; 5 µm particle

size) column was used as the stationary phase. Water containing 0.1% of formic acid (A) and acetonitrile (B) served as mobile phases at a flow rate of 1 ml/min. Gradient elution was applied as shown on Table 5:

Table 5. HPLC gradient elution.

Time (min)	% Solvent A (0.1% formic acid in H ₂ O)	% Solvent B (Acetonitrile)
0	95	5
10	90	10
20	70	30
30	50	50
40	95	5
41	STOP	

Temperature was set to 30 °C for column. Extracts were dissolved in HPLC grade methanol (2 mg/mL) and the injection volume was 20.0 µL. Chromatograms were recorded at 254 nm. The elucidation of the chemical structures of the compounds depicted by peaks on the chromatograms falls outside of the scope of this study.

4.4.6. Statistical Analysis

The results are presented as mean ± SD of three independent experimental measurements that are used to calculate the inhibitory concentrations. Graph Pad prism 9 software was used to calculate the IC₅₀/CC₅₀ values.

5. Conclusions

This is the first study of its kind to report on antiproliferative and selectivity of *Euphorbia species* investigated in this study against MCF-7, MDA-MB231 and Vero cell lines. The findings of this study contribute to the understanding of *Euphorbia* plant species as potential sources of anticancer agents. The presence of various bioactive compounds suggests their pharmacological relevance. Further research could involve isolating and characterizing these compounds to identify key bioactive constituents responsible for the observed effects. Mechanistic studies could elucidate how these compounds interact with cancer cells and potentially modulate critical pathways involved in cell growth and proliferation. Correlating the identified metabolites with their observed cytotoxic and selective effects could pave the way for targeted drug development. This information might also guide the selection of appropriate extraction methods, yielding higher concentrations of active compounds.

This study highlights the diverse chemical composition and potential anticancer activities of *Euphorbia* plant species. The combination of phytochemical analysis and cytotoxicity assays provides a valuable foundation for further research aimed at connecting the therapeutic potential of these plants in breast cancer treatment.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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Abbreviations

The following abbreviations are used in this manuscript:

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide	(MTT) assay
High-performance liquid chromatography	HPLC
Ethyl acetate	EtOAc
Dichloromethane	DCM
Hexane	Hex
cytotoxic concentration	CC ₅₀
selectivity index	SI
<i>Euphorbia bupleurifolia</i>	<i>E. bupleurifolia</i>
<i>Euphorbia trigona</i>	<i>E. trigona</i>
<i>Euphorbia gorgonis</i>	<i>E. gorgonis</i>
<i>Euphorbia cooperi</i>	<i>E. cooperi</i>
<i>Euphorbia horrida</i> Var	<i>E. horrida</i> Var
<i>Euphorbia horrida</i> Indigenous	<i>E. horrida</i> Indigenous
<i>Euphorbia polygona</i>	<i>E. polygona</i>
<i>Euphorbia enopla</i>	<i>E. enopla</i>
<i>Euphorbia arabica</i>	<i>E. arabica</i>
<i>Euphorbia ammak</i>	<i>E. ammak</i>
<i>Euphorbia stellata</i>	<i>E. stellata</i>
<i>Euphorbia ferox</i>	<i>E. ferox</i>
<i>Euphorbia clavarioides</i>	<i>E. clavarioides</i>
<i>Euphorbia coerulescens</i>	<i>E. coerulescens</i>
<i>Euphorbia tirucalli</i>	<i>E. tirucalli</i>
<i>Euphorbia ledienii</i>	<i>E. ledienii</i>
<i>E. bupleurifolia</i> dichloromethane	E.BPF DCM
<i>E. trigona</i> hexane	E. TRG Hex
<i>E. trigona</i> dichloromethane -E. TRG DCM	
<i>E. gorgonis</i> hexane	E. TRG Hex
<i>E. polygona</i> hexane	E. PLGN Hex
<i>E. polygona</i> dichloromethane	E. PLGN DCM
<i>E. ferox</i> dichloromethane	E. FRX DCM
<i>E. clavarioides</i> hexane	E. CLV Hex
<i>E. clavarioides</i> dichloromethane	E. CLV DCM
<i>E. ammak</i> dichloromethane	E. AMK DCM
<i>E. ammak</i> hexane	E. AMK Hex
<i>E. ferox</i> methanol	E. FRX MeOH
<i>E. enopla</i> hexane	E. ENP Hex
<i>E. enopla</i> dichloromethane	E. ENP DCM
<i>E. ledienii</i> hexane	E. LDN Hex
<i>E. stellata</i> dichloromethane	E. STL DCM
<i>E. ledienii</i> dichloromethane	E. LDN DCM
<i>E. arabica</i> hexane	E. ARB Hex
<i>E. arabica</i> dichloromethane	E. ARB DCM
<i>E. cooperi</i> hexane	E. CPR Hex
<i>E. cooperi</i> dichloromethane	E. CPR DCM
<i>E. tirucalli</i> hexane	E. TRL Hex
<i>E. tirucalli</i> dichloromethane	E. TRL DCM
<i>E. bupleurifolia</i> hexane	E. BPF Hex
Triple-negative breast cancer	TNBC
Reactive oxygen species	ROS

Dulbecco's modified Eagle's medium	DMEM
Fetal bovine serum	FBS
Standard deviation	SD
Doxorubicin	DOX

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