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Cytotoxicity and Anti-cancer Effects of *Hibiscus sabdariffa* Leaf Extracts on Triple Negative Breast Cancer (TNBC) Cell Lines

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ABSTRACT

Hibiscus sabdariffa belongs to the family of Malvaceae and is widely cultivated and used in traditional medicine for its antioxidant, anti-inflammatory, anti-obesity, and anti-cancer properties. This study aims to investigate the effects of *H. sabdariffa* leaf extracts using the brine shrimp Lethality Test (BSLT) and MTT assay against Triple-Negative Breast Cancer (TNBC) cell lines: MCF7 (invasive ductal carcinoma), MDA-MB-231 (adenocarcinoma), Hs578T (invasive ductal carcinoma), and SKBr3 (invasive ductal carcinoma). The Brine Shrimp Lethality test (BSLT) revealed significant cytotoxicity across all extracts, with mortality ranging from 27 to 30, where 30 brine shrimp larvae were used. The methanol extract exhibited the highest cytotoxic activity, indicating a higher concentration of potent cytotoxic compounds compared to the ethyl acetate and n-hexane extracts. The LC_{50} values for n-hexane, ethyl acetate, and methanol extracts are 72.26, 51.19, and 50.61 µL, respectively. The MTT assay was performed to assess the anti-cancer potential of the extracts. The assay revealed different IC_{50} values of the most potent extract on the cell line: MCF7 (n-hexane IC_{50}-77µM, etoposide IC_{50}-68µM), Hs578T (methanol IC_{50}-66µM, etoposide IC_{50}78µM), SKBr3 (methanol IC_{50}-96µM, etoposide IC_{50}-70µM), and MDA-MB-231 (ethyl acetate IC_{50}-67µM, etoposide IC_{50}-69µM). These results suggest that the methanol extract exhibits strong anti-cancer activity against the Hs578T cell line, which outperformed the standard drug etoposide. Overall, *Hibiscus sabdariffa* leaf extracts showed significant medicinal properties and potential as a source of anti-cancer therapy for pharmaceutical development.

KEYWORDS: Cytotoxicity, Anti-Cancer Effects, TNBC, *Hibiscus sabdariffa* Leaves.

1. INTRODUCTION

Cancer is a critical health condition worldwide. Among various cancers, breast cancer is the most prevalent type and the second most common cause of death in women around the globe.[1] The World Health Organization reported 2.3 million women were diagnosed with breast cancer in 2020, and 685,000 deaths were recorded globally, making it the world's most prevalent cancer in the past five years.[2] Triple-Negative Breast Cancer (TNBC) is known as inflammatory and invasive breast cancer. It is resistant to therapies and occurs in 15 to 20% with a lower survival rate. TNBC lacks the three most common markers used for targeted therapies: estrogen receptors (ER), progesterone receptors (PR), and HER2, which are all negative. It is more common in younger women and often affects those with a BRCA1 gene mutation.[3] *H. sabdariffa* exhibits a wide range of biological activities, including antibacterial, antifungal, antiviral, anticancer, immunomodulatory, antioxidant, smooth muscle relaxant, gastrointestinal anti-inflammatory, wound healing, and cardiovascular protective effects.[4] *H. sabdariffa* aqueous extract (HSE) on a human breast adenocarcinoma cell line (MCF-7) and normal foreskin fibroblast cells (HFFF), apoptosis induction was assessed at a concentration of 0.5 mg/mL; the extract reduced the viability of MCF-7 cells to below 50% after 72 hours of incubation, demonstrating notable cytotoxic effects.[5] Despite *H. sabdariffa's* ethnomedicinal uses, there is limited literature on its cytotoxicity properties. This research aims at investigating the cytotoxicity of crude extracts from *H. sabdariffa* leaf against triple-negative breast cancer (TNBC) cell lines.

2. MATERIALS AND METHODS

2.1 Plant Collection and Identification

The fresh leaves of *H. sabdariffa* were collected randomly over a period of 5 days, verified, and authenticated (ABU02768) at the herbarium, Botany Section of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Kaduna, Nigeria.

2.2 Sample Collection for Bioassay Analysis

In the Brine Shrimp Lethality (BSLT) assays, *Artemia salina* shrimp eggs were collected, and four TripleNegative Breast Cancer (TNBC) cell lines were used in the study, namely invasive ductal carcinoma
(MCF7), adenocarcinoma (MDA-MB-231), invasive ductal carcinoma (Hs578T), and invasive ductal

carcinoma (SKBr3), which were collected from the cell-culture laboratory, Centre for Natural Product Discovery, School of Biochemical Science, Liverpool John Moores University (LJMU), United Kingdom.

2.3 Brine Shrimp Lethality Test (BSLT) Assays Procedure

Brine shrimp (*Artemia salina*) eggs were hatched in seawater for 48 hours to obtain larvae. Extracts (0.2 g) were dissolved in 2 ml of solvent of extraction, and concentrations of 1000, 100, and 10 µg/ml were prepared in triplicate with controls. Ten larvae were introduced into each vial, and survival was recorded after 24 hours. LC_{50} values were calculated using Finney probit analysis software.

2.4 MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) Assay procedure

Extract concentrations of 1.0, 0.5, and 0.25 mg/ml were prepared by dissolving measured amounts in 2 ml of extraction solvent, evaporating to dryness, re-dissolving in a drop of DMSO, and making up to 2 ml with distilled water. Cells were washed with phosphate buffer saline (PBS), harvested by trypsinization, plated in 96-well plates, and incubated at 37°C under 5% CO_2 for 24 hours. All concentrations of plant extracts were in triplicate on the same cell batch. Growth of tumor cells was quantitated by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5diphenyl-2H-tetrazolium bromide (MTT) to a blue formazan product.

2.5 Extraction and Fractionation

The freshly collected leaves of *H. sabdariffa* were carefully separated and air-dried at room temperature and pulverized using a ball mill machine (Gilson Company INC). Cold extraction by the maceration method was used. A portion (500 g) of the powdered sample was soaked in 2000 ml of n-hexane, ethyl acetate, and methanol successively and allowed to stand for seven days, then decanted and filtered with 20 cm filtered paper and concentrated using a rotary evaporator at 30°C to 60°C (86°F to 140°F) and 10-50 mbar. The extraction yields were calculated.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Brine-Shrimp Lethality Test Results.

The lethal concentration of n-hexane, ethyl acetate, and methanol extracts of *H. sabdariffa* indicates its toxicity against brine shrimp larvae. The lower the LC_{50} value, the higher the toxicity showed in table 1 and figure.

Table 1: LC_{50} (µg/ml) of the Crude Extracts of *H. Sabdariffa* Leaves.

CRUDE EXTRACTS	SOLVENT OF EXTRACTION	LC_{50} (µg/ml)
001JN	n-Hexane	72.26 (83.79-491.63)
002JE	Ethyl Acetate	51.19 (42.13-558.11)
003JM	Methanol	50.61 (43.18-458.12)

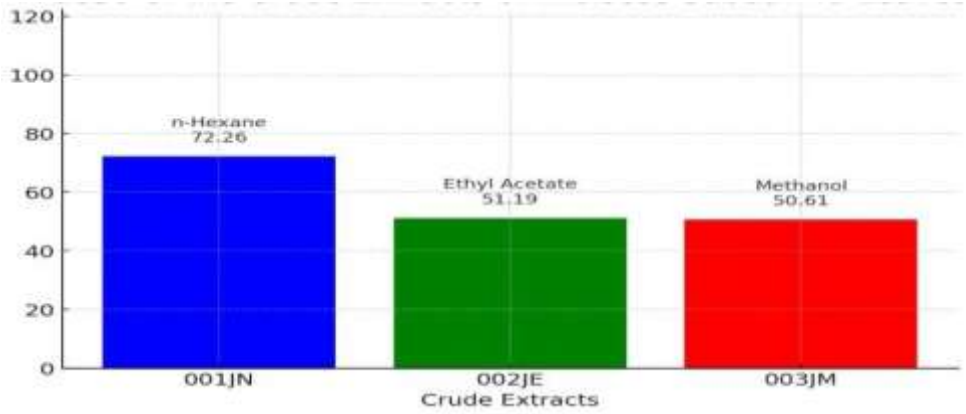

Figure 1: A bar chart showing LC_{50} of extracts from *H. sabdariffa* Leaf.

3.1.2 MTT Assay of N-Hexane, Ethyl Acetate, and Methanol Extracts.

Table 2a is the inhibitory activity of the extracts from *H. sabdariffa* Leaf against TNBC cell lines (Cell proliferation activity). The inhibition increases as the concentration of extracts increase on the four cell lines however; same action was noted on the control etoposide.

Table 2a: Inhibitory Activity of the extracts from *H. sabdariffa* Leaf against TNBC cell lines (Cell proliferation activity).

	Concentration µ/m	MCF7	H_S578T	SKBr3	MDAMB-231
001JN	0.25	246± 2.37	234.1 ± 1.273	306.6 ± 2.571	360.3 ± 2.144
	0.5	158.0± 2.370	149.0± 2.226	265.0 ± 2.372	268.0± 2.155
	1.0	77.3± 2.519	90.3± 1.511	176.3 ± 2.271	109.3± 3.132
002JE	0.25	197.6 ± 2.453	282.2± 2.401	227.2 ± 2.102	281.7± 2.140
	0.5	140.7± 2.519	171.3± 2.360	201.1 ± 2.305	199.0± 2.142
	1.0	98.5± 2.352	100.2± 2.488	124.5± 2.472	67.2± 2.243
002JM	0.25	287.4 ± 2.271	233.4± 2.461	211.4± 2.412	210.1± 2.230
	0.5	156.0 ± 2.361	153.0± 2.981	175.0± 2.312	172.2± 2.145
	1.0	107.1± 1.283	66.1± 2.743	96 .1± 2.320	78.4± 2.167
Etoposide	0.25	144.3± 2.471	144.3± 2.471	144.3± 2.471	144.3± 2.471
	0.5	105.7± 2.471	105.7± 2.471	105.7± 2.471	105.7± 2.471
	1.0	68.9± 2.471	78.9± 2.471	70.2± 2.471	68.9± 2.471

Table 2b presents the inhibitory activity of n-hexane, ethyl acetate and methanol extracts from *H. sabdariffa* leaves. The inhibitory activity compared well with the standard drug etoposide, revealing its potency. The lower the value of IC_{50} µ/ml the higher the potency of the extract against the four cell lines as shown in table 2b and figure 2.

Table 2b: IC_{50}µ/ml Values of extracts from *H. sabdariffa* Leaves.

Crude extracts	MCF7	H_S578T	SKBr3	MDAMB-231
001JN	77	90	>100	>100
002JE	98	100	>100	67
003JM	>100	66	96	78
Etoposide	68	78	70	69

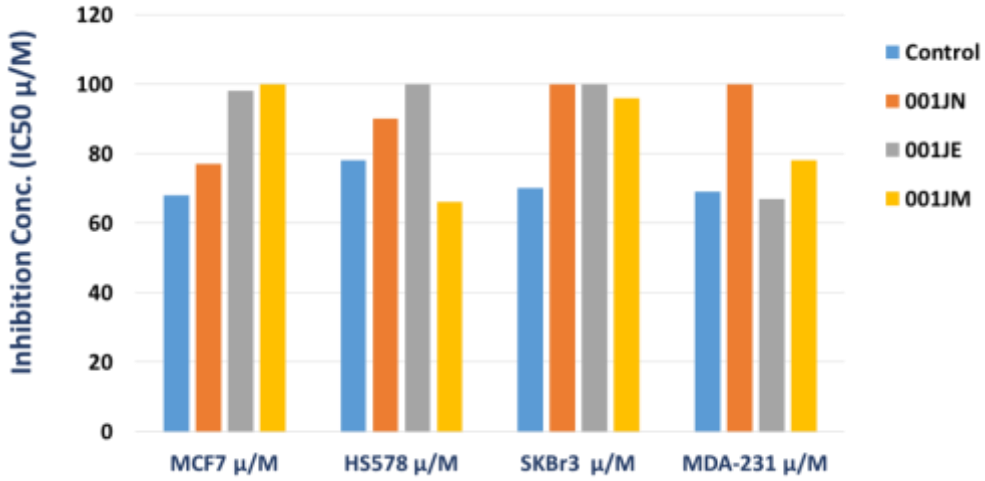

Figure 2: A bar graph of IC_{50} µ/ml Values of Extracts from *H. sabdariffa* leaves

3.2 Discussion

The Brine Shrimp Lethality Test (Table 1) showed high cytotoxicity across all extracts, with 27–30 mortality where 30 brine shrimp larvae were used. The methanol extract exhibited the highest cytotoxic activity, indicating potent cytotoxic compounds compared to ethyl acetate and n-hexane crude extract. The inhibition of methanol from Table 2b exhibited the cytotoxic effect of IC_{50} = 66 µM against the H_S578T cell line, outperforming etoposide (IC_{50} = 78 µM), and moderate activity of IC_{50} = 96 µM against the SKBr3 cell line; however, it was less potent than etoposide (IC_{50} = 70 µM). N-Hexane extract showed moderate activity (IC_{50} = 77 µM) against the MCF7 cell line; however, it was less potent than etoposide (IC_{50} = 68 µM), the standard drug. While ethyl acetate extract was most toxic (IC_{50} = 67 µM) against the MDA-MB-231 cell line, it was slightly more potent than etoposide (IC_{50} = 69 µM), the chemotherapy drug used as the standard. The cytotoxic effects of *H. sabdariffa* aqueous extract (HSE) on a human breast adenocarcinoma cell line (MCF-7) and normal foreskin fibroblast cells (HFFF) and apoptosis induction were assessed at a concentration of 0.5 mg/ml; the extract reduced the viability of MCF-7 cells to below 50% after 72 hours of incubation, demonstrating notable cytotoxic effects.[5] The cytotoxic activity of *H. sabdariffa* results showed IC_{50} 187.89 µg/ml against breast cancer cells (MDAMB-231).[6] *H. sabdariffa* Linn flowers revealed cytotoxic activity with IC_{50} values of 719.28 µg/mL ethyl acetate and 906.57 µg/mL n-hexane, indicating weak cytotoxic activity.[7] In related research, the 95% ethanoic extract of *H. sabdariffa* dried leaves (HSDE95) demonstrated potent cytotoxicity with an IC_{50} of 8.58±0.68 µg/mL.[8] The cytotoxic potential of *H. sabdariffa* leaves showed an average IC_{50} of 43.48 µg/mL, indicating significant activity [9]

4. CONCLUSION

The study evaluated the cytotoxic effects of *H. sabdariffa* leaf extracts using n-hexane, ethyl acetate, and methanol extract against the Brine-Shrimp Lethality Test (BSLT) and four breast cancer cell lines (MCF7, MDA-MB-231, Hs578T, and SKBr3), comparing their efficacy to the standard chemotherapy drug etoposide. These findings highlight methanol extract's superior activity against H_S578T and SKBr3 cell lines, ethyl acetate's competitive efficacy against MDA-MB-231, and n-hexane's potency against

MCF7. These may warrant further investigation for their potential in future breast cancer treatment strategies.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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