RJC

RASĀYAN *J. Chem.*
Vol. 15 | No. 3 |1619-1625| July - September | 2022
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

PHYTOCHEMICAL CONSTITUENT AND *In-vitro* CYTOTOXIC ACTIVITY OF *Hibiscus sabdariffa* L. CALYX FRACTION ON HUMAN BREAST CANCER CELL LINE MDA-MB-231

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ABSTRACT

Hibiscus sabdariffa L. is a medicinal plant that has various pharmacological activities. One of the activities of *Hibiscus sabdariffa* L. is the immunomodulatory activity closely connected with the anticancer activities. This study aimed to test the cytotoxic activity of *Hibiscus sabdariffa* L. Calyx Fractions against breast cancer cells (MDA-MB-231), determine the active fractions, and analyze the compounds of the active fraction of *Hibiscus sabdariffa* L. with LC-MS. The *Hibiscus sabdariffa* L.Calyx was fractionated using vacuum liquid chromatography. Meanwhile, the cytotoxic activity was examined using the MTT method. The test results discovered that the Fraction A of *Hibiscus sabdariffa* L. Calyx is the active fraction with a score of IC50 187.89 µg/ml. The identification of Fraction A's chemical compounds using LC-MS discovered three identified compounds:tert-butyl-4-methoxy phenol Nigakilactone H and Stigmastan-3,6-dione. Since these compounds potentially function as active cytotoxic compounds,it is suggested that further studies isolate the active compounds from Fraction A of *Hibiscus sabdariffa* L.

Keywords: *Hibiscus sabdariffa* L., Cytotoxic, Fraction, Breast cancer, MDA-MB-231.

RASĀYAN *J. Chem.*, Vol. 15, No.3, 2022

INTRODUCTION

Cancer has become the second largest cause of death globally after heart disease. Cancer caused 8.8 million deaths in 2015. Of some cancer types, breast cancer ranked the sixth position with 571,000 deaths. Breast cancer is one of the leading causes of death in women, there are 1 million cases of breast cancer every year, and a half end in death[1]. Natural compounds have been found in various medicinal plants.[2] These compounds have some activities, such as antioxidant, cytotoxic, anti-inflammatory, antimutagenic, and anticancer.[3] Natural compounds in medicinal plants can boost the immune system, prevent cancer cells by inhibiting angiogenesis, increase detoxification, prevent the increase of toxic substances in the body, and neutralize free radicals that cause DNA mutations. Herbal medicine with antioxidant and anticancer properties can be used as complementary medicine in the prevention and treatment of cancer.[4] *Hibiscus sabdariffa* L. is a medicinal plant that has various pharmacological activities, such as antioxidant, antihypercholesterolaemic, antinociceptive, antipyretic, diuretic, hepatoprotective, antidiabetic, and antimicrobial activities.[5,6] Moreover, *Hibiscus sabdariffa* L. Calyx has secondary metabolism activities, such as organic acids, anthocyanins, flavonoids, mucilage, pectin, and carbohydrates.[6] The primary compound of *Hibiscus sabdariffa* L. Calyx is Hibiscus Acid, which has many activities. A previous study has shown that *Hibiscus sabdariffa* L. Calyx extract and fraction have immunomodulatory activity.[7] The immunomodulatory activity very closely relates to anticancer activities. Immune systems kill the cancer cells in the body, and thus, the increasing immune systems escalate the anticancer efficacy.

EXPERIMENTAL

Ethanol, Chloroform, Silica Gel GF 254 (Merk®), ethyl acetate cisplatin, DMSO, HCl, PBS, Fetal bovine serum, MTT, and ethyl acetate. All standardized chemicals are purchased at local stores.

http://doi.org/10.31788/RJC.2022.1536694

Cell Culture

The human breast cancer line (MDA-MB-231) was a cell culture from the Laboratory of Cell and Molecular Biology of Universitas Padjadjaran. cells were cultured in an incubator at 37 °C with 5% CO_2 in a DMEM-high glucose medium containing 10% FBS (Fetal Bovine Serum).

Plant Materials and Fractionation

Hibiscus sabdariffa L. Calyx was collected in a local area determined at the Department of Biology, Halu Oloeo University. The Calyx was dried and macerated using ethanol. The extracts were then partitioned using liquid-liquid extraction to remove the glycosides. The ethyl acetate extract was then separated by vacuum liquid chromatography (VLC) with gradient solvents of hexane, ethyl acetate, and methanol. Fraction results produce 20 Fractions.

Phytochemical Analysis

Hibiscus sabdariffa L. Calyx fractions were analyzed for their compounds using the standard method of Liquid Chromatography-Mass Spectroscopy (LC-MS). The compounds were identified by comparing the Wiley library database.

Cytotoxic Assay

Cytotoxic assay of anti-breast cancer conducted by MTT method. MDA-MB-231 breast cancer cell was incubated plates well plate for 24 hours. After that, extract and control were added and incubated for 48 hours. The extract was assayed at concentration of 1000, 500, 250, 125, 62.5, 32.25, 15.625, and 7.8125 µg/ml, Cisplatin 1 µg/ml is applied as a positive control. Then cell was isolated with 1X PBS and incubated with 100 µl of 0.5 mg mg/ml MTT at 37°C. After 30 minutes, reagent stopper (10% SDS) in HCl 0, 01 N was added as much as 100 µl to each well. Formazan MTT solution was measured its absorbance at 596 nm wavelength using microplate reader. Absorbance value measured was applied for percentage of viability cell calculated by the following formula:

$$\% \text{ Viability} = \frac{\text{Average Absorbance of Sample}}{\text{Average Absorbance of Control}} \text{ x } 100\%$$

RESULTS AND DISCUSSION

Extraction and Fractionation

The extraction of *Hibiscus sabdariffa* L. Calyx using the maceration and ethanol method produced 42.5 g of the condensed extract (yields of 8.2%). The extract was then extracted liquidly using the ethyl acetate solvent and produced extract for 20.75 g. Then, the extract was fractionated using vacuum liquid chromatography (VLC). The fractionation produced seven fractions: A (0.19 g), B (0.37 g), C (1.76 g), D (0.87 g), E (3.81 g), F (2.24 g), and G (1.99 g).

Cytotoxic Assay

The cytotoxic activity of *Hibiscus sabdariffa* L. Calyx fraction was tested against human breast cancer cell line (MDA-MB-231) using the MTT method. The test results are presented in Table 1-8 and Figures 1-8. Meanwhile, the IC_{50} values of each fraction are presented in Table-9. The test results showed that all fractions at high concentrations (500 µg/ml and 1000 µg/ml) caused 50% MDA-MB-231 cancer cell death. The IC50 values of the fractions A, B, C, D, E, F, G, and cisplatin (positive control) respectively were 187.89 µg/ml, 328.68 µg/ml, 481.22 µg/ml, 449.01 µg/ml, 479.57 µg/ml, 492.93 µg/ml, 492.93 µg/ml, and 3.25 µg/ml. The sample's cytotoxic activity was tested against cancer cells using criteria to categorize the activities by exploring the IC_{50} values of the activity NCI and Geran *et al.*[8] categorized cytotoxic activities into four groups: IC50 ≤ 20 µg/ml = highly active, IC50 of 21 - 200 µg/ml = moderately active, IC50 201 - 500 µg/ml = weakly active, and IC50 > 501 µg/ml = inactive.

Based on these categories, the cytotoxic activities of Fraction A were considered moderately active, the fraction B-G was weakly active, and the cisplatin was highly active. Fraction A was an active fraction of *Hibiscus sabdariffa* L. Calyx functioning as cytotoxic activity against MDA-MB-231 cancer cells. Although the activities of Fraction A were not as many as those of cisplatin, this ownership was considered enough because Fraction A still had some compounds. In contrast, cisplatin did not because it was an active compound.

Tabel-1: Cytotoxic activity of *Hibiscus sabdariffa* L. Calyx Fraction A

Concentration (µg/ml)	Cell Viability (%)
1000	0.00
500	3.29 ± 1.04
250	33.68 ± 1.51
125	65.32 ± 1.10
62.5	70.73 ± 1.49
31.25	74.63 ± 2.67
15.625	73.38 ± 2.62
7.8125	79.30 ± 3.20

Tabel-2: Cytotoxic activity of *Hibiscus sabdariffa* L. Calyx Fraction B

Concentration (µg/ml)	Cell Viability (%)
1000	0.0000
500	30.31 ± 0.11
250	59,34 ± 2.27
125	68.98 ± 1.01
62.5	69.42 ± 4.43
31.25	71.40 ± 0.71
15.625	70.95 ± 2.16
7.8125	79.49 ± 1.87

Tabel-3: Cytotoxic activity of *Hibiscus sabdariffa* L. Calyx Fraction C

Concentration (µg/ml)	Cell Viability (%)
1000	0.00
500	50.06 ± 0.69
250	79.03 ± 0.55
125	82.66 ± 1.56
62.5	82.05 ± 3.51
31.25	79.65 ± 2.10
15.625	79.94 ± 2.65
7.8125	81.71 ± 0.19

Tabel-4: Cytotoxic activity of *Hibiscus sabdariffa* L. Calyx Fraction D

Concentration (µg/ml)	Cell Viability (%)
1000	0.00
500	40.97 ± 1.32
250	68.48 ± 3.77
125	81.61 ± 4.45
62.5	81.08 ± 0.43
31.25	81.90 ± 2.35
15.625	86.08 ± 2.15
7.8125	89.18 ± 1,38

Tabel-5: Cytotoxic activity of *Hibiscus sabdariffa* L. Calyx Fraction E

Concentration (µg/ml)	Cell Viability (%)
1000	0.00
500	43.08 ± 2.99
250	77.49 ± 2.81
125	81.00 ± 3.47
62.5	78.87 ± 3.41
31.25	78.38 ± 6.16
15.625	77.43 ± 2.64
7.8125	80.81 ± 7.03

Tabel-6: Cytotoxic activity of *Hibiscus sabdariffa* L. Calyx Fraction F

Concentration (µg/ml)	Cell Viability (%)
1000	8.95 ± 0.29
500	47.40 ± 1.75
250	78.56 ± 2.58
125	82.16 ± 1.73
62.5	83.62 ± 1.64
31.25	79.93 ± 0.87
15.625	82.05 ± 2.77
7.8125	89.09 ± 2.82

Tabel-7: Cytotoxic activity of *Hibiscus sabdariffa* L. Calyx Fraction G

Concentration (µg/ml)	Cell Viability (%)
1000	11.98 ± 0.65
500	40.37 ± 1.58
250	75.36 ± 3.52
125	80.86 ± 2.32
62.5	79.38 ± 2.43
31.25	80.05 ± 4.60
15.625	78.34 ± 2.20
7.8125	79.08 ± 3.30

Tabel-8: Cytotoxic activity of Cisplatin

Concentration (µg/ml)	Cell Viability (%)
200	0.00
100	0.00
50	0.00
25	12.40 ± 5.86
12.5	18.42 ± 0.58
6.25	20.46 ± 1.63
3.125	44.45 ± 6.45
1.5625	72.49 ± 2.63

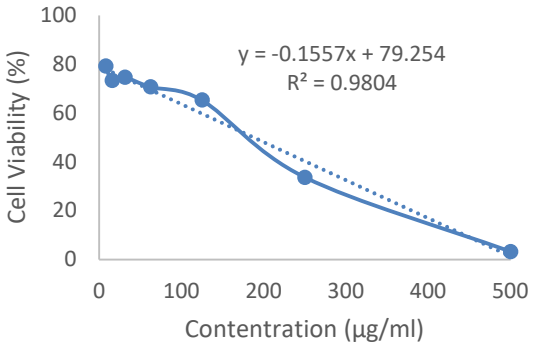

Fig.-1: Cytotoxic activity of *Hibiscus sabdariffa* L. Calyx Fraction A

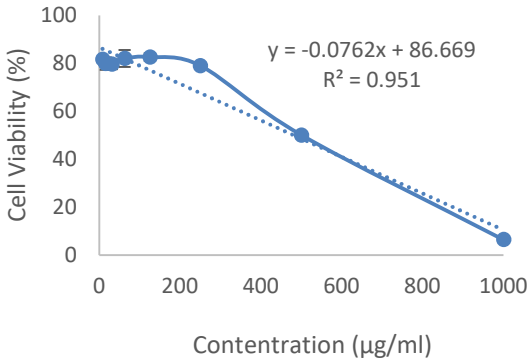

Fig.-2: Cytotoxic activity of *Hibiscus sabdariffa* L. Calyx Fraction B

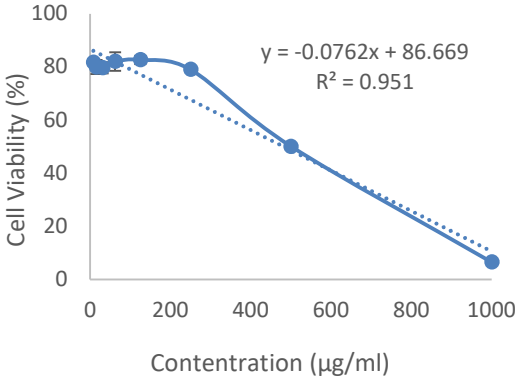

Fig.-3: Cytotoxic activity of *Hibiscus sabdariffa* L. Calyx Fraction C

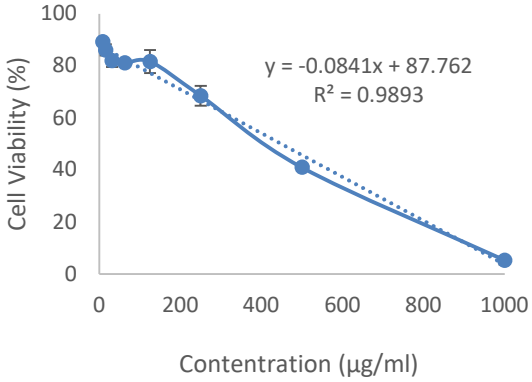

Fig.-4: Cytotoxic activity of *Hibiscus sabdariffa* L. Calyx Fraction D

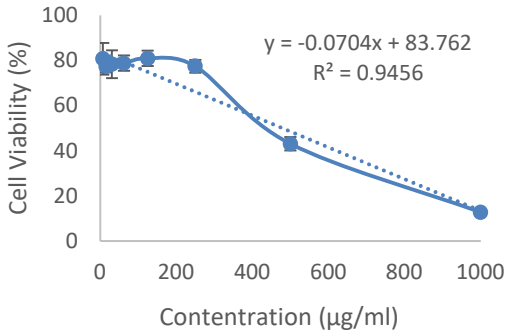

Fig.-5: Cytotoxic activity of *Hibiscus sabdariffa* L. Calyx Fraction E

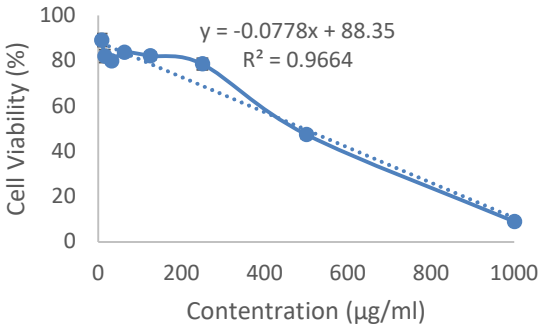

Fig.-6: Cytotoxic activity of *Hibiscus sabdariffa* L. Calyx Fraction F

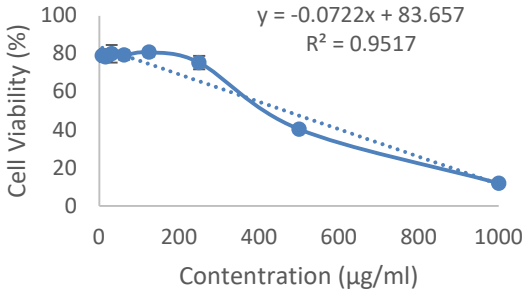

Fig.-7: Cytotoxic activity of *Hibiscus sabdariffa* L. Calyx Fraction G

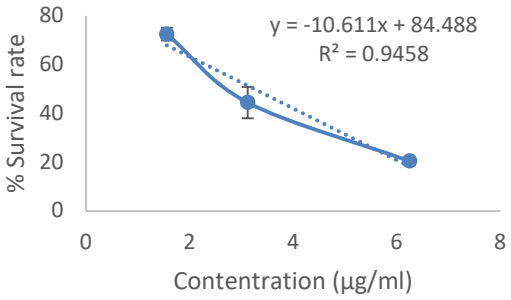

Fig.-8: Cytotoxic activity of Cisplatin

Phytochemicals Analysis

Fraction A as an active fraction of *Hibiscus sabdariffa* L. Calyx had cytotoxic activities against MDA-MB-231 cancer cells, and the chemical compounds of Fraction A were identified using the LC-MS. The results of identifying chemical compounds of Fraction A are presented in Table-9. The LC-MS identified five compounds but only identified the compound names of three of them: 3-tert-butyl-4-methoxyphenol, Nigakilactone H, and Stigmastan-3,6-dione.

Tabel-9: Phytochemicals Analysis of *Hibiscus sabdariffa* L. Calyx Fraction

No	Compound name	Retention Time (RT) (min)	Compound Mass m/z
1	3-Tert-butyl-4-methoxyphenol	7.82	181.1221
2	Nigakilactone H	9.55	425.2153
3	Stigmastan-3,6-dione	10.38	429.3720
4	Candidate Mass C45H84O15	10.85	887.5691
5	Candidate Mass C45H84O16	10.77	903.5645

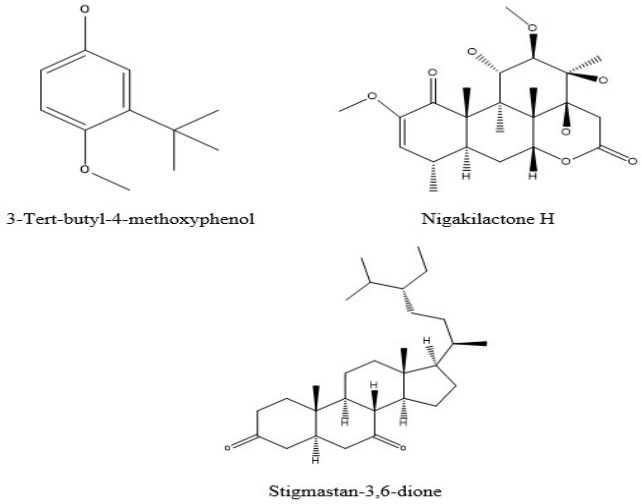

Fig.-9: Identified compound of *Hibiscus sabdariffa* L. Calyx Fraction A

The Stigmastan-3,6-dione compound was reported as one of the compounds in *Hibiscus sabdariffa* L.[9] The 3-tert-butyl-4-methoxyphenol compound was a compound in another species of Hibiscus genus, *Hibiscus tiliaceus* L.[10] However, no study has reported Nigakilactone H. compounds in *Hibiscus sabdariffa* L. Calyx. The methoxyphenol derivatives were reported to have cytotoxic activity. Kamadatu[11] reports that methoxyphenol compounds have cytotoxic activities against MCF-7 breast cancer cells tested by the MTT method. Meanwhile, Kadoma *et al.*[12] contend that methoxyphenol compounds and their derivatives are active against RAW264.7 cancer cells. Phenol compounds can inhibit cancer cell proliferation through apoptosis in intrinsic and extrinsic pathways.[13] Phenol compounds in the intrinsic pathway of the mitochondrial intermembrane space can inhibit NF-kB-dependent signals associated with proliferation and survival.[14] This condition causes cell cycle arrest through p53 upregulation[1516], stabilizes and activates the p53 tumor suppressor gene[16], downregulates the expression of Bcl-2, and the anti-death protein Bcl-XL, and supports the induction of apoptosis through activation of the activity of multiple caspases and cytochromes. -c (cyt-c).[13,17] Nigakilactone H is classified as a triterpenoid compound. These compounds have cytotoxic activities against various cancer cells, such as prostate cancer, melanoma, ovarian carcinoma, sarcoma, kidney cancer, leukemia cancer of the peripheral nervous system, colon cancer, lung cancer, breast cancer, and cervical carcinoma.[18] Moreover, the triterpenoid compounds can induce apoptosis by upregulating Bax and p53. Bax and Bcl-2 are the main proteins in the intrinsic pathway of apoptosis.[19] Stigmastan-3,6-dione are included in the group of steroid compounds. Steroid compounds are cytotoxic against P-388 murine leukemia cells, human T- lymphoblastic leukemia, Human Lung Carcinoma (A549), and Human Liver Cancer Cell (HepG2).[20–23] Moreover, steroid compounds have cytotoxic working mechanisms by inducing apoptosis.[24] The results of LC-MS revealed that Fraction A still had two

unidentified compounds. Many potential-active compounds that can become cytotoxic against human breast cancer cells of the MDA-MB-231 line have not been examined. Therefore, further research is necessarily conducted to isolate active compounds of *Hibiscus sabdariffa* L. Fraction A. Calyx.

CONCLUSION

Cytotoxic activities of *Hibiscus sabdariffa* L. Calyx faction signified that Fraction A was an active fraction with IC50 at 187.89 µg/ml. The identification of Fraction A's chemical compounds using LC-MS discovered three identified compounds: tert-butyl-4-methoxyphenol, Nigakilactone H, and Stigmastan-3,6-dione. Since these identified compounds potentially become active cytotoxic compounds, further research is suggested to isolate the active compounds of *Hibiscus sabdariffa* L. Fraction A.

ACKNOWLEDGEMENT

The author would like to thank Padjajaran University and Mandala Waluya University for providing supports in the implementation of this study.

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[RJC-6694/2021]