ISOLATION OF ANTIMIGRATORY CHEMICAL CONSTITUENTS FROM *Curcuma caesia, Curcuma aeruginosa* RHIZOMES AND *Dioscorea bulbifera* TUBERS USING HUMAN BREAST CANCER CELL LINES

MD AL-AMIN

UNIVERSITI SAINS MALAYSIA

2021

ISOLATION OF ANTIMIGRATORY CHEMICAL CONSTITUENTS FROM *Curcuma caesia, Curcuma aeruginosa* RHIZOMES AND *Dioscorea bulbifera* TUBERS USING HUMAN BREAST CANCER CELL LINES

by

MD AL-AMIN

Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

April 2021

ACKNOWLEDGEMENT

Bismillahirrahmanirrahim,

In the name of Allah, the Most Gracious, Most Merciful. Alhamdulillah, all praise to Allah, the Most Gracious and Most Merciful for giving me strength, power, knowledge and patient to overcome all the challenges that life brings in my way.

I would like to express my sincere appreciation and gratitude to my supervisor Assoc. Prof. Dr. Salizawati Muhamad Salhimi for giving me the opportunity to undertake my research study with her and for all the guidance, supports, encouragements and excellent hospitality. I am very grateful to have her as my supervisor and feel very fortunate to study under her supervision.

My sincere appreciation goes to my co-supervisor, Assoc Prof. Dr. Melati Khairuddean, School of Chemical Sciences, USM, for her support to provide facility to conduct NMR experiments, valuable suggestions and critical comments regarding the data interpretation of NMR experiments.

My sincere appreciation also goes to my co-supervisor Dr. Siti Sarah Fazalul Rahiman for her valuable suggestions, critical comments, encouragements and supports.

My special thanks also go to Nagla Mustafa Eltayeb, Ph.D. student, Discipline of Pharmaceutical Chemistry, for her constant support regarding lab work and other academic and no- academic matters from the beginning of my enrolment until the end of the study. I am so happy and feel fortunate to have her throughout my study.

My sincere appreciation also goes to Prof. Dr. Chowdhury Faiz Hossain, Department of Pharmacy, East West University, Bangladesh for his consent to use *C. caesia* and *C. aeruginosa* rhizomes in my study and authentication of these species.

I also wish to offer my thanks to all those academic and technical staffs from School of Pharmaceutical Sciences and School of Chemical Sciences who have immensely helped me in this study.

I also extend my thanks to Assoc. Prof. Dr. Mohd Nazri Ismail, Analytical Biochemistry Research Centre (ABrC), USM, for his support to use Image Analyser and Malaysian Institute of Pharmaceuticals and Nutraceuticals (IPharm) for the agreement to run GC-MS analysis.

I would also like to express my gratitude to Mr. Abd. Rahim Abd. Rahman, Pahang, Malaysia for his help to provide *D. bulbifera* tubes as a gift to conduct research. I also like to express my thanks to Herbarium, Universiti Sains Malaysia and Bangladesh National Herbarium for identification and authentications of plant species.

The financial support from Universiti Sains Malaysia (USM) by RUI Grant (1001/PFARMASI/8011003), FRGS Grant (203 PFARMASI 6711764), USM Graduate Assistance scheme and Research Assistantship are gratefully acknowledged. This work would not have been possible without these sponsorships.

Last but certainly not least, I offer my gratitude to my family in my beloved country, Bangladesh, especially my mother, brother, sisters and brother-in-law's. I also wish to thank all my friends especially Mohd Shahrul Ridzuan Hamil for the valuable help in my research. Without my family and friends, it would have been an impossible task to complete the present work.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABTS	2,2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
ACN	Acetonitrile
ATCC	American type culture collection
Bax	Bcl-2 associated X-protein
Bcl-2	B-cell lymphoma 2
CD_3OD	Methanol-d_4
COSY	Homonuclear correlation spectroscopy
DEPT	Distortionless enhancement by polarization transfer
DHT	Dihydrotestosterone
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DMSO-d6	Dimethyl sulfoxide-d_6
DPPH	2,2-diphenyl-1-picrylhydrazyl
EAC	Enlich’s ascites carcinoma
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EI-MS	Electron Ionization Mass Spectrometer
ERK	Extracellular signal-regulated protein kinase
ER-α	Estrogen receptor-α
EtOAc	Ethyl acetate
EtOH	Ethanol
FBS	Fetal bovine serum
Fr.	Fraction
Frs.	Fractions

GC-MS	Gas chromatography–mass spectrometry
GC-MS-QTOF	Gas chromatography–mass spectrometry- quadrupole time-of-flight
HER-2	Human epidermal growth factor receptor- 2
HMBC	Heteronuclear multiple-bond correlation spectroscopy
HOCl	Hypochlorous acid
HPLC	High performance liquid chromatography
HPLC-HRMS	High performance liquid chromatography-high resolution mass spectrometry
HSQC	Heteronuclear single-quantum correlation spectroscopy
LC-MS-QTOF	Liquid chromatography–mass spectrometry- quadrupole time-of-flight
MCF-7	Michigan cancer foundation- 7
MDA-MB-231	M.D. Anderson metastatic breast cancer-231
MeOH	Methanol
MIC	Minimum inhibition concentrations
MMPs	Matrix metalloproteinases
MMP-2	Matrix metalloproteinase- 2
MMP-9	Matrix metalloproteinase- 9
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NOESY	Nuclear overhauser effect spectroscopy
PARP	Poly (ADP-ribose) poly-merase
PBS	Phosphate buffered saline
PDA	Photodiode array detector
PR	Progesterone receptor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

RP C-18	Reverse phase C-18
RP-Prep HPLC	Reverse phase-preparative high performance liquid chromatography
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SFr.	Subfraction
SOD	Superoxide dismutase
TLC	Thin layer chromatography
TNBC	Triple-negative breast cancer
VLC	Vacuum liquid chromatography
WHO	World health organization

LIST OF SYMBOLS

°C	Degree Celsius
d	Doublet
dd	Doublet of doublets
g	Gram
h	Hour
kg	Kilogram
L	Liter
m	Multiplet
mg	Milligram
mL	Milliliter
min	Minute
mm	Millimeter
nm	Nanometer
ppm	Parts per million
rpm	Revolution per minute
s	Singlet
v/v	Volume/volume
w/w	Weight/weight
µ	Micron
µg	Microgram
µL	Microliter
µM	Micromolar
%	Percentage

PEMENCILAN KOMPONEN KIMIA ANTI-MIGRASI DARIPADA RIZOM *Curcuma caesia* DAN *Curcuma aeruginosa*, SERTA UBI *Dioscorea bulbifera* MENGGUNAKAN TITISAN SEL KANSER PAYU DARA MANUSIA

ABSTRAK

Curcuma caesia, *Curcuma aeruginosa* dan *Dioscorea bulbifera* digunakan secara tradisional dalam rawatan pelbagai penyakit manusia termasuk kanser. Kajian ini dijalankan bagi mengenalpasti bahan antimigrasi daripada rizom *C. caesia* dan *C. aeruginosa*, serta ubi *D. bulbifera* menggunakan titisan sel kanser payu dara. Komponen-komponen kimia daripada rizom *C. caesia* dan *C. aeruginosa* telah dipencilkan melalui fraksinasi. Ekstrak rizom *C. caesia* dan *C. aeruginosa* menunjukkan perencatan terhadap kebolehhidupan sel MCF-7 dan MDA-MB-231, bergantung kepekatan. Kajian lanjut menunjukkan fraksi heksana dan fraksi kloroform daripada rizom *C. caesia* serta fraksi kloroform daripada rizom *C. aeruginosa* adalah fraksi bioaktif yang merencat kebolehhidupan sel kanser payu dara. Pemisahan kromatografi yang dijalankan menemukan germakron (**1**), zerumbon (**2**), furanodienon (**3**), kurzerenon (**4**), kurkumenol (**5**), zederon (**6**), kurkumenon (**7**) dan dehidrokurdion (**8**) daripada fraksi heksana manakala kurkuminol G (**9**), kurkuzederon (**10**), (1S, 10S), (4S,5S)-germakron-1(10), 4-diepoksida (**11**), wenyujinin B (**12**), alismoksida (**13**), aerugidiol (**14**), zedoarolida B (**15**), zedoalakton B (**16**) zedoarondiol (**17**) dan isozedoarondiol (**18**) ditemukan daripada fraksi klorofom rizom *C. caesia*. Pemisahan kromatografi daripada fraksi kloroform *C. aeruginosa* menemukan germakron (**1**), furanodienon (**3**), kurzerenon (**4**), kurkumenol (**5**) dan zederon (**6**). Asai kebolehhidupan sel sebatian terpencil ini menunjukkan sebatian **1**-**4** dan **10** serta sebatian **1**, **3** dan **4** masing-masing merupakan sebatian bioaktif *C. caesia* dan *C.*

aeruginosa. Germakron (**1**) dan kurkuzederon (**10**) menunjukkan nilai IC_{50} masing-masing 246.3 dan 227.2 μM terhadap sel MDA-MB-231. Aktiviti germakron (**1**) dan kurkuzederon (**10**) ke atas migrasi sel MDA-MB-231 juga dikaji dan hasil kajian menunjukkan bahawa kedua-dua bahan ini menghasilkan aktiviti perencatan yang signifikan, bergantung kepekatan. Hasil kajian ini menunjukkan bahawa germakron (**1**) yang dipencilkan daripada rizom *C. caesia* dan *C. aeruginosa*, dan kurkuzederon (**10**) yang dipencilkan daripada rizom *C. aeruginosa* mempunyai aktiviti antimigrasi dalam sel kanser payu dara. Kesan ekstrak dan fraksi ubi *D. bulbifera* ke atas migrasi sel turut dikaji. Ekstrak, Fraksi 2 dan Fraksi 4 mempamerkan perencatan yang signifikan, bergantung kepekatan ke atas migrasi sel MDA-MB-231. Asai zimografi gelatin menunjukkan Fr.2 dan Fr.4 merencat migrasi sel melalui modulasi enzim MMP-2 dan MMP-9. Pemisahan kromatografi Fraksi 2 dan Fraksi 4 menghasilkan lusiantridin (**19**), flavantridin (**20**), 4,7-dihidroksi-2,3-dimetoksifenantrena (**21**), nudol (**22**), katekin (**23**) dan diosbulbin B (**24**). Hasil kajian ini mencadangkan bahawa aktiviti antimigrasi ubi *D. bulbifera* berkemungkinan terhasil daripada bahan **19**-**24**. Kesimpulannya, hasil dapatan kajian yang ditunjukkan dalam tesis ini secara kolektif, menyokong kegunaan tradisional *C. caesia*, *C. aeruginosa* dan *D. bulbifera* dan memberikan pemahaman tentang potensi kegunaan terapeutik komponen kimia dalam merawat kanser payu dara.

ISOLATION OF ANTIMIGRATORY CHEMICAL CONSTITUENTS FROM *Curcuma caesia, Curcuma aeruginosa* RHIZOMES AND *Dioscorea bulbifera* TUBERS USING HUMAN BREAST CANCER CELL LINES

ABSTRACT

Curcuma caesia, *Curcuma aeruginosa* and *Dioscorea bulbifera* are used traditionally for the treatment of human ailments including cancer. The present study was carried out to search for antimigratory compounds from *C. caesia*, *C. aeruginosa* rhizomes and *D. bulbifera* tubers using breast cancer cell lines. The chemical constituents from *C. caesia* and *C. aeruginosa* rhizomes have been isolated through fractionation. The extracts of *C. caesia* and *C. aeruginosa* rhizomes showed concentration-dependent inhibition of MCF-7 and MDA-MB-231 cells. Further study showed that hexane and chloroform fractions of *C. caesia* rhizomes and chloroform fraction of *C. aeruginosa* rhizomes are the bioactive fractions that significantly inhibit the viability of breast cancer cells. The chromatographic separation afforded germacrone (**1**), zerumbone (**2**), furanodienone (**3**), curzerenone (**4**), curcumenol (**5**), zederone (**6**), curcumenone (**7**) and dehydrocurdione (**8**) from hexane fraction and curcuminol G (**9**), curcuzederone (**10**), (1S, 10S), (4S,5S)-germacrone-1(10), 4-diepoxide (**11**), wenyujinin B (**12**), alismoxide (**13**), aerugidiol (**14**), zedoarolide B (**15**), zedoalactone B (**16**) zedoarondiol (**17**) and isozedoarondiol (**18**) from chloroform fraction of *C. caesia* rhizomes. The chromatographic separation afforded germacrone (**1**), furanodienone (**3**), curzerenone (**4**), curcumenol (**5**) and zederone (**6**) from chloroform fraction of *C. aeruginosa*. Cell viability assay of these isolated compounds further revealed that compounds **1**-**4** and **10**, and compounds **1**, **3** and **4** are the bioactive compounds of *C. caesia* and *C. aeruginosa*, respectively. Germacrone

(**1**) and curcuzederone (**10**) showed IC_{50} values of 246.3 and 227.2 μM against MDA-MB-231 cells, respectively. The activity of germacrone (**1**) and curcuzederone (**10**) on the migration of MDA-MB-231 cells was also investigated and the results showed that both compounds produced a significant concentration-dependent inhibitory activity. These results indicate that germacrone (**1**) isolated from *C. caesia* and *C. aeruginosa* rhizomes, and curcuzederone (**10**) isolated from *C. aeruginosa* rhizomes possess antimigratory activities in breast cancer cell lines. The effect of the extract and fractions of *D. bulbifera* tubers on cell migration was also investigated. The extract, Fr.2 and Fr. 4, showed significant inhibition in a concentration-dependent manner on the migration of MDA-MB-231 cells. Gelatin zymography assay showed that Fr.2 and Fr.4 inhibited cell migration through the modulation of MMP-2 and MMP-9 enzymes. The chromatographic separation of Frs. 2 and 4 yielded lusianthridin (**19**), flavanthridin (**20**), 4,7-dihydroxy-2,3-dimethoxyphenanthrene (**21**), nudol (**22**) catechin (**23**) and diosbulbin B (**24**). These results suggest that the antimigratory activity of *D. bulbifera* tubers is possibly from compounds **19**-**24**. In conclusion, the findings presented within this thesis collectively support the traditional uses of *C. caesia*, *C. aeruginosa* and *D. bulbifera* and provide an insight of the potential therapeutic use of their chemical constituents to treat breast cancer.

CHAPTER 1

INTRODUCTION

1.1 Breast Cancer

Despite the overwhelming progress in medical sciences, cancer is a major health problem and second leading cause of mortality worldwide. In 2018, the global estimated cancer-related deaths were 9.6 million. WHO reported that one among six deaths was due to cancer and 70 % cancer-related deaths have been reported from the low to middle-income countries (World Health Organization, 2018).

Breast cancer is a primary reason for cancer-related mortality among women. The global number of female deaths from breast cancer in 2018 was estimated to be 636,679 and the number of new female breast cancer cases was estimated to be 2,088,849, which was accounted as the second leading cancer type worldwide (Bray et al., 2018). In 2018, breast cancer was a dominant cancer type in Malaysia. The number of breast cancer cases was 7593, which ranked number one and accounted for 17.3 % of cancer related cases. The number of deaths from breast cancer was 2,894 which ranked two after lung cancer and accounted for 11.0 % of cancer related death in Malaysia (The Global Cancer Observatory, 2019). Breast cancer is a heterogeneous disease having comparatively diverse prognostic outcomes. It is divided into five major subgroups: Luminal A, Luminal B, Normal-like, HER2-enriched and triple negative breast cancer (TNBC) generally including basal-like subtype (Jia et al., 2019). Though the quality of treatments for most of the breast cancer subtypes have improved in developed countries with advanced healthcare delivery systems, over 20 % of the patients with negative expression of estrogen receptor-α (ER-α), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2) show aggressive behaviour and poor treatment outcomes. This subgroup of breast cancer patients is

defined as TNBC patients (Pan et al., 2012). Therefore, research currently focuses on the discovery of new drugs that could efficiently contribute more for the treatment of TNBC patients.

1.2 Cancer metastasis

Cancer metastasis is a multistep process which involves invasion and migration of cancer cells from the primary tumor into either blood or lymphatic vessels with the help of matrix metalloproteinases (MMPs). Thus, invasive breast cancer is the fundamental contributing factor of cancer-related mortality (Weng et al., 2010; Chun & Kim, 2013). Cancer metastasis involves several complex cell behaviors including migration, invasion, proliferation, adhesion, transition and intra/extravasation and epithelial-mesenchymal transition (Lingrand et al., 2020). The breast cancer cells become more prone to be metastatic and malignant when the cells start to degrade extracellular matrix (ECM). The degradation of ECM is caused by ECM degradation enzymes. Matrix metalloproteinases (MMPs) are the main ECM degrading enzymes and crucial mediators for the invasion and migration of tumor cells. Most of the MMPs are produced in latent forms and mainly function by catalytic removal of the pro-peptide domain. However, MMP-2 and MMP-9 are two unique ECM degrading MMPs due to the presence of fibronectin Type II in their catalytic domain (Lee et al., 2006). Many studies reported that MMP-2 and MMP-9 are major ECM-degrading proteolytic enzymes involved in cancer cell migration (Chun & Kim, 2013). Therefore, the suppression of the proteolytic activity of MMP-2 and MMP-9 is one of the crucial approaches to investigate molecular mechanism in breast cancer research.

1.3 Natural products

Natural products play a significant role for the treatment and prevention of numerous human diseases including infectious diseases, heart diseases, tumor and cancer. They usually derived from diverse sources including plants, bacteria, fungi, marine algae and animals. Natural products are the predominant source of new drugs and contribute for the discovery of new medicines through isolation of chemical constituents from natural sources, semi-synthesis of potent drug leads, total synthesis of bio-active compounds and modification of natural drug leads. The bioactive chemical constituents such as paclitaxel, doxorubicin, erythromycin, lovastatin, cyclosporin A, tacrolimus and amphotericin B are examples of natural products which are widely used as pharmaceutical products throughout the world (Chin et al., 2006; Calixto, 2019; Twilley & Lall, 2018).

Plants derived natural products contribute to 25 % of currently prescribed drugs worldwide (Calixto, 2019). At present, approximately, 121 marketed drugs had been obtained from plants source. According to WHO, almost 252 drugs are considered as essential drugs, among them 11 % derived directly from plants and the rest have been obtained by synthetic and semisynthetic approaches by considering the natural products as the main precursors (Rates, 2001; Calixto, 2019).

1.4 Plants derived anticancer agents

Plants have an immense contribution in anticancer drugs discovery. It is noteworthy to mention that 70 % of the anticancer drugs available in the market have been derived either directly from the plants or by modification of natural drug leads (Twilley & Lall, 2018). Vincristine, vinblastine, docetaxel, paclitaxel, topotecan, irinotecan and elliptinium are examples of anticancer drugs in the market have been isolated from the

plants. Whereas, vinorelbine, vindesine, flavopiridol and roscovitine are the synthetic and semisynthetic anticancer drug molecules, but the basis of these molecules are natural products (Cragg & Newman, 2005; Twilley & Lall, 2018). The intensive anticancer research had been started from 1950 when vinblastine was isolated from the Madagascar periwinkle collected from Jamaica. Vinblastine is one of the natural anticancer drugs available in the market for the treatment of Hodkin's disease, bladder cancer, lung cancer, brain cancer and testicular cancer. Vincristine is another useful anticancer agent of *Catharanthus roseus* for the treatment of acute lymphocytic and myeloid leukaemia, Hodgkin's lymphoma and lung cancer. The discovery of anticancer drugs from *Catharanthus roseus* leaves afforded minor quantity of vincristine and vinblastine. Therefore, these two molecules were further isolated by using cell culture, tissue culture and shoot culture of *Catharanthus roseus leaves*. However, the quantity from these techniques could not meet the requirements (Kumar et al., 2013; Twilley & Lall, 2018).

Paclitaxol, trade name Taxol, was first isolated in the 1960s from the bark of Pacific Yew tree. It is the most useful anticancer drug available in the market for Kaposi sarcoma, breast cancer and ovarian cancer. The success of pacitaxol in cancer treatment creates more interest among the researchers to develop new analogue which led to discovery of docetaxel, also known as taxotere. Docetaxol was launched in 1995 as a remedy for advanced non-small-cell lung carcinoma (NSCLC) that is refractory to primary therapy. It is also most widely used to treat advanced breast cancer, hormone refractory breast cancer, stomach cancer, non-small cell lung cancer and ovarian cancer (Kingston, 2009; Twilley & Lall, 2018*)*.

Camptothecin, isolated from *Camptotheca acuminate* Decne, is a drug advanced to clinical trials in the 1970s for new anticancer drug molecule. It has been

shown to produce severe bladder toxicity and, therefore was not approved as anticancer drug. However, the bioactivity of camptothecin has led researchers towards derivatization which yielded two potent camptothecin derivatives, topotecan and irinotecan. Topotecan has been approved as an anticancer drug for ovarian and small cell lung cancer, whereas irinotecan has been approved for treating colorectal cancer (Cragg & Newman, 2005; Twilley & Lall, 2018).

There has been a vast amount of research conducted in the anticancer drugs discovery and it is obvious that natural products play a lead role for the isolation of anticancer drugs from natural sources and the development of potential novel agents from bioactive natural drug leads.

1.5 Problem statement, hypothesis and significance of the study

At present, the patients with TNBC are treated with conventional therapies such as surgery, radiotherapy, chemotherapy and targeted therapy. However, these types of tumours do not response well due to the migration and invasion of tumour cells to the distant sites with the help of matrix metalloproteinases (MMPs). Therefore, the survival rate of TNBC patients is still very poor. Thus, exploring new drug candidate that will prevent migration and invasion of tumour cells can give more time to conventional therapies and immune system to kill the cancer cells.

C. caesia, *C. aeruginosa* rhizomes and *D. bulbifera* tubers are widely used as traditional medicine for the treatment of tumor and cancer. Previous studies reported some pharmacological activities of these species and isolated some bioactive chemical constituents. However, no study is reported in the field of investigation and isolation of antimigratory chemical constituents from *C. caesia*, *C. aeruginosa* rhizomes and *D. bulbifera* tubers.

Based on the traditional uses and reported studies, *C. caesia*, *C aeruginosa* rhizomes and *D. bulbifera* tubes have been studied in the area of antimigratory activity since it has not been studied before even though the plants are widely used traditionally for the treatment of tumor and cancer. Thus, the present study will lead to discover new antimigratory drug leads for the treatment of triple-negative breast cancer patients and will provide the evidence-based support for the traditional uses of *C. caesia*, *C aeruginosa* rhizomes and *D. bulbifera* tubes for cancer treatment.

1.6 Objectives

C. caesia Roxb., *C. aeruginosa* Roxb. rhizomes and *D. bulbifera* tubers are widely used traditional and folk medicines for tumors and cancer in Asian countries. Therefore, the present study was carried out to fulfil the following objectives:

- To isolate antimigratory chemicals constituents from *C. caesia* and *C. aeruginosa* rhizomes by using triple-negative breast cancer cells MDA-MB-231.
- To evaluate anti-migratory activity of the crude extract, chromatographic fractions of *D. bulbifera* tubers against MDA-MB-231 cells.
- To investigate the effect of chromatographic fractions on MMP-2 and MMP-9 activities.
- To isolate the major chemical constituents from the antimigratory fractions by using chromatographic and spectroscopic techniques.

1.7 Limitations and problems of the study

Bioassay guided isolation of *D. bulbifera* tubers led to isolations of compounds **19-24**. The inhibitory effect of ethanolic extract and chromatographic fractions on MDA-MB-231 cell migration has been investigated. However, the activity of compounds **19-24** isolated from active fractions (Frs.2 and 4) was not evaluated on cancer cell line as the quantity of compounds **19-24** was insufficient for further studies on the biological activities.

The inhibitory activity of curzerenone (**4**) on the viability of breast cancer cell lines was investigated. However, the antimigratory activity of curzerenone (**4**) was not evaluated due to insufficient amount of curzerenone (**4**).

CHAPTER 2

LITERATURE REVIEW

2.1 Breast cancer cell lines

2.1.1 MCF-7 cell line

Michigan Cancer Foundation-7 (MCF-7) is the first hormone responsive breast cancer cell line developed from a 69-year-old female metastatic breast cancer patient (Levenson et al., 1997). MCF-7 is now a standard model of breast cancer cell line in most of the cancer laboratories worldwide. The MCF-7 cells are widely used in the estrogen receptor (ER) -positive breast cancer cell experiments and classified as ER-positive breast cancer cell line (Mughal et al., 2019). Even though, the cells were obtained from a metastatic patient, however, MCF-7 cells are non-invasive, less metastatic and poorly aggressive (Mughal et al., 2019). The cells are easy to culture in the lab and compatible for antihormone therapy resistance studies. Cancer research experiments using MCF-7 cells produced more published data than any other breast cancer cell line. Besides that, the majority of cancer research laboratories who are investigating the acquired antiestrogen drug resistance also utilize MCF-7 cells for their research. Since MCF-7 cells are so prevalent in breast cancer laboratories, therefore, these cells are the most commonly used estrogen receptor (ER) -positive cells which represent the early stage of breast cancer and widely used for the discovery of new molecules to treat breast cancer (Sweeney et al., 2012; Mughal et al., 2019; Levenson et al., 1997).

2.1.2 MDA-MB-231 cell line

MDA-MB-231 cell line is a highly invasive and metastatic cell line developed from a 51-year-old female metastatic breast adenocarcinoma patient (Lingrand et al., 2020). The name MDA stands for “M.D. Anderson” and MB stands for metastatic breast cancer. This cell line is PR, ER, and E-cadherin negative, expresses mutated P53 and lack of growth factor receptor HER2 (Pan et al., 2012). Therefore, MDA-MB-231 cell line is a TNBC cell line widely used to investigate late stage breast cancer. It represents 15 % of all breast cancer patients commonly found in younger and premenopausal women. As the patients with triple-negative breast cancer (TNBC) are mostly young, therefore, tumors get more time to become larger, migrate to distant sites and metastasize to the lungs, liver, brain and other organs. Although some of the anticancer drugs are in clinical trials, relevant targeted anticancer drug has not yet been discovered. Breast cancer patients mostly rely on traditional medicines and up to 60% of the survivors use herbal treatment despite the concern on safety and efficacy (Wu et al., 2018). Therefore, research currently focuses on the discovery of new drugs from traditionally useful natural products which could efficiently inhibit the invasion and migration of cancer cells and would be a practicable drug candidate for the treatment of TNBC patients (Wu et al., 2018; Pan et al., 2012; Lingrand et al., 2020).

2.2 The Dioscoreaceae family

The Dioscoreaceae is a monocycledonus family comprising 600-700 species and four genera, which are mostly found in tropical and subtropical areas (Mehrotra & Shukla, 2019). Some Dioscoreaceae species are most frequently used as traditional medicines to treat cancer and tumors in Asian countries. *Dioscorea collettii* var. *hypoglauca* is used for the treatment of cervical carcinoma, urinary bladder cancer and renal tumor

in traditional herbal medicines (Hu & Yao, 2002). *Dioscorea membrancea* is used as a remedy for liver cancer and cholangiocarcinoma in Thai traditional medicine (Thongdeeying et al., 2016). *Dioscorea bulbifera* is another widely used species of Dioscoreaceae for the treatment of tumors in Chinese traditional medicines and skin cancer by aboriginal people of North Queensland, Australia (Wang et al., 2009; Williams, 2013). It is also used as a medication of tumors by the local tribes of Southeast Asia. (Murray et al., 1984).

Diosbulbin B is a diterpene lactone isolated from *Dioscorea bulbifera* rhizomes as the major bioactive antitumor agent (Wang et al., 2012). Diosorealide B, a cytotoxic agent, is isolated from *Dioscorea membranaceae*. It showed cytotoxic activity against MCF-7 and MDA-MB-468 cells at the concentrations of 2.76 μM and 9.93 μM, respectively (Saekoo et al., 2011). The molecular study showed that diosorealide B increased the levels of P53, P21 and Bax as well as reduced Bcl-2 level of MCF-7 cells. It also caused apoptosis of MCF-7 cells through activation of caspase-7 and caspase-9 (Saekoo et al., 2011).

Deltonin is a steroidal saponin isolated from *Dioscorea zingiberensis* Wright. It showed cytotoxic effect against A549, MDA-MB-231, LL/2, Skov3, B16, PC-3 and C-26 cell lines. The study of molecular mechanism against C26 cells showed that deltonin induces apoptosis by the release of cytochrome C, depolarization of mitochondrial membrane potential and generation of reactive oxygen species (ROS) (Shu et.al., 2011). Deltonin also increased Bax expression, suppressed Bcl-2 expression and induced activation of caspase-3, caspase-9 and Poly (ADP-ribose) Poly-merase (PARP) (Shu et.al., 2011). Zingiberensis saponin is another steroidal saponin isolated from *Dioscorea zingiberensis* WRIGHT. It showed cytotoxic activity against A549, LL2, SK-OV-3, B16, PC-3, HEK293 and C-26 cell lines. Zingiberensis

saponin showed effect on C26 cells and induced apoptosis by the activation of caspase-3, caspase-9, PARP, suppressed Bcl-2 expression and increased Bax expression (Tong et al., 2012).

Although some species of Dioscoreaceae are widely used as traditional medicines for cancer and tumors treatment, a limited number of compounds which showed effect on cancer cells have been isolated so far from Dioscoreaceae. Therefore, some species could be a potential source to discover more bioactive anticancer drug leads for late-stage cancer treatment.

2.3 The Zingiberaceae family

Zingiberaceae is one of the most preferable family for centuries in cooking and cosmetics. Many species of Zingiberaceae are globally used as traditional and folk medicines for the treatment of numerous diseases including inflammation, ulcer, wounds, heart diseases, diabetes, cancer and tumors. Some species are more frequently used in the Asian countries as the traditional medicines for a vast number of human diseases including cancer and heart diseases (Padalia et al., 2018). There are many investigations conducted to discover potent bioactive chemical constituents from some common species including *Zingiber officinale* and *Zingiber zrumbet* from the genus of *Zinger* and *Curcuma longa*, *Curcuma aeruginosa* and *Curcuma amada* from the genus of *Curcuma* (Jatoi et al., 2007; Singh et al., 2012; Pour et al., 2014; Salehi et al., 2019; Widyowati & Agil, 2018;). There are still other species of both genus *Curcuma* and *Zingiber* which are extensively used traditionally, but very few studies have been reported.

Zerumbone is a potent anticancer agent and first isolated from *Zingiber zerumbet (*Matthes et al., 1980). It is also isolated from some other species of genus

Curcuma and *Zingiber* including *Z. montanum, Zingiber roseum, C. longa, C. amada* and *C. zedoaria* (Al-Amin et al., 2012; Al-Amin et. al, 2019; Sun et al., 2017). Zerumbone has been shown to inhibit the viability of A293 (Kidney cancer), H-1299 (Lung cancer), COLO205, LS174T, L-8174, L-5189. COLO320DM (Colon cancer), MCF-7 (Breast cancer), GBM8401 (Brain cancer), CEMSS, WEH-3B, KBM-5 (Blood cancer), non-malignant chang Liver, HepG2 (Liver cancer) and MDBK cell lines (Kalantari et al., 2017). It increases HepG2 cell apoptosis shown by the down regulation of Bcl-2 protein expression and up regulation of Bax protein expression (Sakinah et al., 2007). The inhibitory activity on CXCl12 induced MCF-7 cell invasion showed significant inhibition of cell invasion by zerumbone (Sung et al., 2008). The inhibitory activity of zerumbone (**2**) on MDA-MB-231 cell migration and invasion with 20 ng/mL Interleukin-1β (IL-1β) showed that this compound suppressed IL-1β induced cell invasion and migration through the modulation of IL-8 (Han et al., 2014).

Germacrone, a sesquiterpenoid, was isolated from some species of Zingiberaceae (Siddique et al., 2019; Sun et al., 2017). It inhibited MDA-MB-231 and MCF-7 cells proliferation. The molecular study showed that germacrone increased lactase dehydrogenase (LDH) release, induced mitochondrial membrane potential ($\Delta\Psi_m$) depolarization and cell cycle arrest at G0/G1 phase for MDA-MB-231 cells and G2/M phase for MCF-7 cell line. It induced apoptosis through mitochondria-mediated caspase pathway and enhanced caspase-3,7,9 and PARP cleavage (Zhong et al., 2011). Another study revealed that germacrone inhibited the proliferation of MCF-7 cells. The study of molecular mechanism stated that germacrone suppressed ERα-mediated gene expression at the transcriptional level and inhibited the recruitment of ERα on the estrogen response element. Germacrone increased the antitumor activity of 5-fluorouracil and methotrexate in combination therapy and significantly inhibited the

migration of ER-α-positive MCF-7 cells in a preliminary scratch assay (Lim et al., 2016)

Furanodienone is another sesquiterpenoid of *Rhizoma curcuma* and isolated recently from *Z. montanum*. The studies reported that it is a potential drug lead for ER-α-positive breast cancer therapy. However, it is more sensitive to ER-α-positive MCF-7 and T47 cells and less sensitive to ER-α-negative MDA-MB-231 cells (Lu et al., 2012). Moreover, extensive studies of curcumin and its derivatives have been conducted on various cancer cells and showed promising anticancer activities (Allegra et al., 2017).

Even though, zerumbone, germacrone, furanodienone and curcumin are potent anticancer drugs which had been isolated from some species of Zingiberaceae, there are still many medicinally useful Zingiberaceae species widely used for tumors and cancer in Asian countries. Those species could be a potential source of new anticancer drugs to minimize the deaths from late-stage breast cancer.

2.4 *Curcuma caesia* Roxb.

Figure 2.1 *Curcuma caesia* Roxb. leaves and rhizomes

Curcuma caesia is a species of Zingiberaceae family and belongs to genus *Curcuma*. The accepted name is *Curcuma caesia* Roxb. (Synonym: *Curcuma kuchoor* Royle). The common names are black turmeric or black zedoary. The species is native to India especially in Indian Himalayan region and most commonly found in northern district, Panchagarh, of Bangladesh (Karmakar et al., 2011). *Curcuma caesia* is also common in Indonesia, Thailand and Malaysia. The local name of *C. caesia* in Malaysia is kunyit hitam (Zuraida, 2013; Vairappan et al., 2013; Nawi et al., 2014). The species can grow in the places where the plant gets enough sunlight. Flood, muddy and wet soil can hinder the growth of this plant, therefore the species is vulnerable to the places where the soil is muddy, wet and affected by floods. The brown colour line in the middle of the leaf is the unique characteristic to distinguish *C. caesia* from other species of genus *Curcuma*. The middle of the rhizomes are blue coloured and therefore the species is called black turmeric. The rhizomes are bitter taste and different from *Curcuma longa*.

2.4.1 Traditional medicinal uses

C. caesia rhizomes are traditionally used as medication for piles, asthma, fever, haemorrhoids, leprosy, wounds, anthelmintic, aphrodisiac, menstrual disorder, vomiting, gonorrhoeal discharges, inflammation and cancer in the Himalayan region of India, Nepal, and Northern districts of Bangladesh (Karmakar et al., 2013). The rhizomes are used in India to treat patients with epilepsy, bronchitis, dysentery, diarrhoea, cough, leukoderma and tumor (Karmakar et al., 2011a; Karmakar et al., 2011b). *C. caesia* rhizomes are dried, crushed to powder, mixed with water to make paste and used to cure bruises, contusions and rheumatic pain. The mixture of dried rhizomes and leaves powder is used to treat impotency, fertility, toothache and allergies. The decoction of fresh rhizomes is used for the treatment of diarrhoea and stomach-ache. The dried powdered rhizomes are mixed with water to form paste and applied on the skin to treat scorpion bite and snake bite. The paste of fresh rhizomes is used to treat rheumatic arthritis and applicable to control bleeding. The fibrous roots of *C. caesia* are dried, grounded to powder, mixed with water and used to relieve gastric disorders (Das et al., 2013).

2.4.2 Pharmacological studies

2.4.2(a) Antitumor activity

The methanolic extract of *C. caesia* rhizomes was investigated for the antitumor activity against Enrlich's ascites carcinoma (EAC)-induced mice. *C. caesia* rhizomes extract showed direct cytotoxic effect and the IC_{50} values were calculated to be 90.70 ± 8.37 μg/mL. The antitumor activity was also evaluated by different parameters consisting of tumor weight, tumor volume, percentage increase in life span (% ILS), viable/nonviable tumor cell count, haematological and biochemical parameters. The

extract significantly reduced tumor weight, tumor volume, number of viable tumor cells and the percentage of increased life span were 57.14 % and 88.09 %. In the observation of haematological and biochemical parameters, the extract significantly ($p < 0.01$) decreased red blood cell count, haemoglobin level, levels of liver functional enzymes and the total protein content at the concentrations of 50 and 100 mg/kg. Previous study has also reported that the methanolic extract showed direct cytotoxic effect on EAC induced mice (Karmakar et al., 2013).

2.4.2(b) Antioxidant activity

The effect of the methanolic extract of *C. caesia* rhizomes on reactive nitrogen species (RNS) and reactive oxygen species (ROS) was reported using different *in-vitro* methods. The study described DPPH scavenging, peroxinitrite, superoxide radical scavenging, hydroxyl radical scavenging and hypochlorous acid (HOCl) scavenging activities of the extract of *C. caesia* rhizomes. The IC_{50} values for DPPH, peroxinitrite and superoxide radical scavenging activities were calculated to be 94.03 ± 0.67 µg/mL, 252.53 ± 5.31 µg/mL and 68.10 ± 1.24 µg/mL, respectively. The IC_{50} values in the hydroxyl radical and HOCl scavenging activities were determined to be 21.01 ± 1.78 µg/mL and 182.40 ± 4.63 µg/mL, respectively. The extract also significantly inhibited lipid peroxidation and the IC_{50} value was reported to be 61.93 ± 2.05 µg/mL (Karmakar et al., 2011a). The *in-vivo* antioxidant activity of methanolic extract was also investigated on EAC-induced mice (Karmakar et al., 2013). The extract significantly increased total protein content in both liver and kidney in EAC induced mice at 50 mg/kg and 200 mg/kg, which strongly corroborates the antioxidant property of *C. caesia* rhizomes (Karmakar et al., 2013).

2.4.2(c) Antimutagenic activity

The antimutagenic activity of *C. caesia* rhizomes aqueous, methanolic and ethanolic extracts were evaluated against the indirect acting mutagen cyclophosphamide using TA98 and TA100 strains of *Salmonella typhimurium*. The extracts showed strong antimutagenic activity at the selected three concentrations of 50, 500 and 5000 µg/mL. The most potent activity was shown by the ethanolic extract. In TA98 and TA100 strains of *Salmonella typhimurium*, the ethanolic extract showed 97.21 % and 90.30 % reduction of mutagenicity, respectively, in the presence of S9 at the maximum concentration of 5000 µg/mL. The report concluded that all the extracts (ethanolic extract, methanolic extract and aqueous extract) showed strong antimutagenic activity against indirect acting mutagen cyclophosphamide (Devi et. al., 2015).

2.4.2(d) Neuropharmacological activities

The methanolic extract was assayed for the neuropharmacological activities which included analgesic, locomotor, anticonvulsant and muscle relaxant activities by using experimental mice models. The extract significantly inhibited acetic acid induced writhing in mice at the concentrations of 50 and 100 mg/kg. The extract also significantly increased mean reaction time of 60 mins observation period in mice tail flick method at the concentrations of 50 and 100 mg/kg. In locomotion study, the extract significantly decreased motor activity in a concentration-dependent manner. In convulsion study, the extract showed concentration-dependent reduction of convulsion by increasing onset of convulsion, percentage of protection and by recovering the mice to survival stage. The extract also significantly decreased fall off time on muscle relaxant activity in mice, which indicated strong muscle relaxant activity of *C. caesia* rhizomes (Karmakar et al., 2011b).

2.4.3 Phytochemical studies

The phytochemical studies of *C. caesia* rhizomes reported monoterpenoids and sesquiterpenoids as the major chemical constituents of *C. caesia* rhizomes. The identification of chemical components from the essential oil of *C. caesia* rhizomes yielded numerous volatile constituents which are listed in Table 2.1. The major volatile components from the essential oils were reported to be camphor (28.3 %), eucalyptol (16.43%), tropolone (15.86 %), ar-tumerone (12.3%) and (*Z*)-β-ocimene (Mukunthan et al., 2014; Borah et al., 2019; Pandey & Chowdhury, 2003; Chaturvedi et al., 2019).

One study reported the isolation of nine chemicals constituents as the non-volatile compounds of *C. caesia* rhizomes. This study isolated germacrone (**1**), curcumenone (**2**), zederone (**3**), aerugidiol (**4**), curcumenol (**5**), furanodiene (**6**), germacrane-4,5-epoxide (**7**), curzerenone (**8**) and isofuranodienone (**9**) (Vairappan et al., 2013). Another study reported germacrone(**1**), curcumenone (**2**), zederone (**3**), curcumenol (**5**), furanodiene (**6**), germacrane-4,5-epoxide (**7**), curzerenone (**8**), isofuranodienone (**9**), glechomanolide (**10**), furanodienone (**11**), and β-sitosterol-α-D-glucoside (**12**) from *C. caesia* rhizomes (Devi, 2014).

Table. 2.1 Characterization of the volatile constituents in the essential oil of *C. caesia* rhizomes:

Number	Volatile constituents	Number	Volatile constituents
1	Camphor	48	Isobomeol
2	Cis-α-copaene-8-ol	49	Terpinen-4-ol
3	Borneol	50	1,8-cineole
4	α-selinene	51	Verrucarol
5	β-selinene	52	Endo-borneol
6	6-methyl-2(1*H*)-pteridinone	53	2-nonanol
7	Bicycle[3.1.0] hexan-3-one	54	Bornyl ester
8	6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronapthalene-2ol	55	δ-elemene
9	4-(dimethylamino)-3,5-dimethyl-phenol	56	β-elemene

10	4-dimethylamino-benzoic acid	57	γ-elemene
11	Ledol	58	Dibutyl phthalate
12	Elemenone	59	Boldione
13	β-elemenone	60	Himbaccol
14	Eucalyptol	61	α –acorenol
15	1,3,3-trimethyl-2-(3-methyl-2-methylene-3-butenylidene)-(2Z)	62	Coniferol
16	1,1,4,4-tetramethyl-2,3-tetralindione	63	Curzerene
17	6-isopropylidene-bicyclo[3.1.0]hexane	64	3,7(11)-selinadiene
18	α-terpineol	65	β-copaene
19	Cadinene	66	α-santonin
20	δ-cadinene	67	Dimethylandrostanolone
21	β-guaiene	68	ar-turmerone
22	Caryophyllene	69	Acorenol
23	Caryophyllene oxide	70	Arglabin
24	δ1(9)-2-Octalone	71	Cycloartenol acetate
25	Rosifoliol	72	Rotundene
26	Occidentalol	73	alpha bulnesene
27	Globulol	74	Isoaromadendrene epoxide
28	Cyclohexanol	75	Costunolide
29	2,7 dimethyl oxepine	76	α-eudesmol
30	α-bulnesene	77	Isobornyl acetate
31	Spathulenol	78	β-sitosterol
32	Megastigmatrienone	79	Methenolone
33	Confertin	80	Germacrene D
34	Naphthalene	81	1-nonadecene
35	tau-cadinol	82	cedr-8-en-13-ol
36	Geranyl-alpha-terpinene	83	(-)-spathulenol
37	T-muurolol	84	Germacrone
38	1,1'-butadiynylenedicyclohexanol	85	Arglabin
39	Tetrahydroisoquinoline	86	Dimethandrostanolone
40	Xanthinin	87	5-alpha-dihydroprogesterone
41	Ivalin	88	alpha –acorenol
42	Cucurbitacin b	89	Himbaccol
43	Isofuranogermacrene	90	β-selinenol
44	Germacrone	91	1-heptatriacotanol
45	Saussurea lactone	92	Ledene oxide-(1)
46	Methyl stearolate	93	1,1'-butadiynylenedicyclohexanol
47	Yelleral	94	Tetrahydroisoquinoline

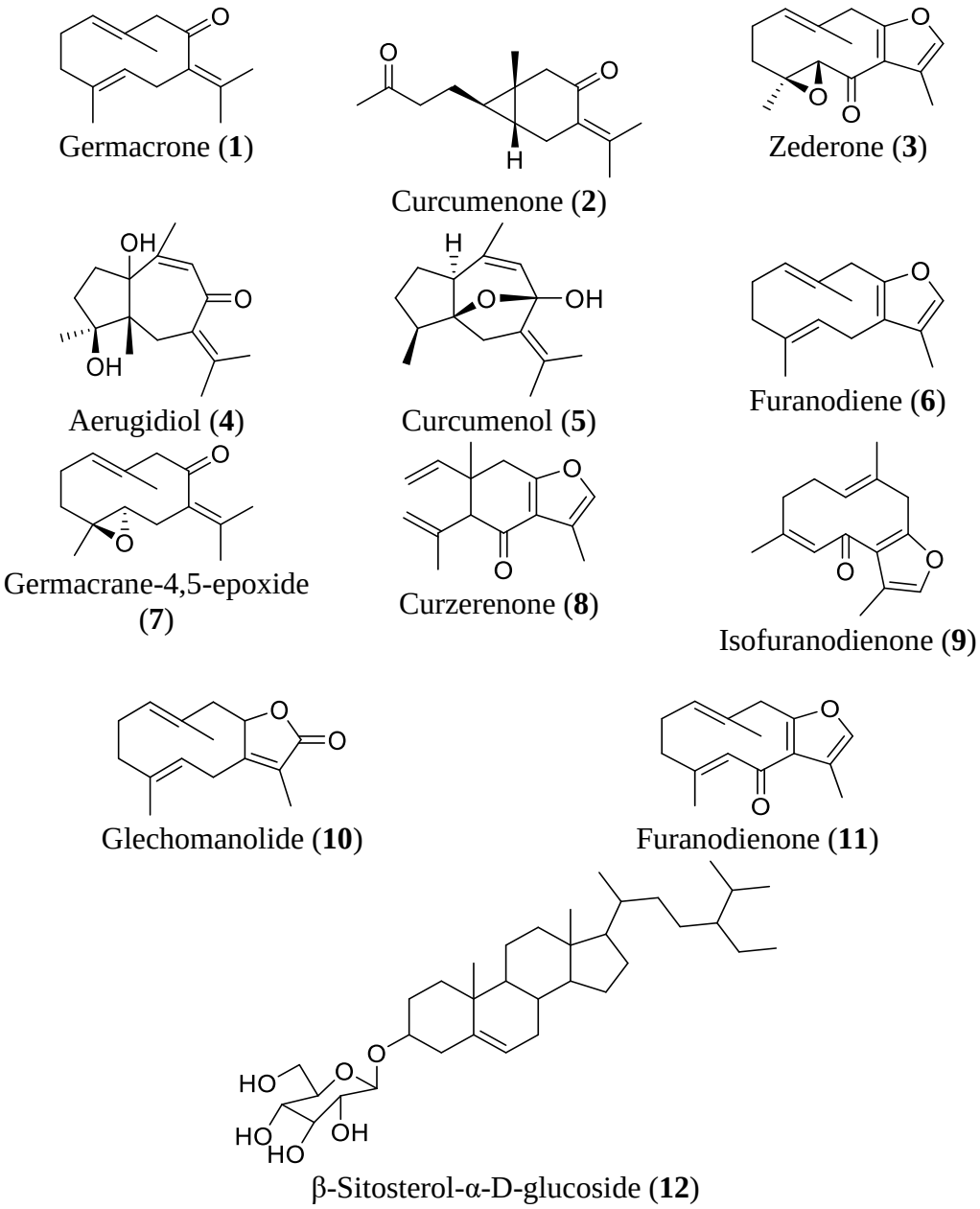

Figure 2.2 Chemical constituents from *C. caesia* Roxb. rhizomes.

2.5 *Curcuma aeruginosa* Roxb.

Figure 2.3 *Curcuma aeruginosa* Roxb. leaves and rhizomes

Curcuma aeruginosa Roxb., a perennial plant, is most commonly found in Malaysia, Indonesia, Bangladesh and India (Nawi et al., 2014; Hossain et al., 2015). The local names are temu hitam in Malaysia and kathali holud in the northern district, Panchagarh, Bangladesh (Hossain et al., 2015; Hanum et al., 1999). The leafy stems can grow up to 300 cm from the rhizomes which can develop up to 16 cm long and 3 cm wide. The rhizomes are bitter taste and blue to yellowish in colour. The leaf also produces a brown colour line in the middle of the leaf, identical to *C. caesia*. The plant is usually grown in the wild region and the people collect them from the wild to use as traditional medicine. *C. aeruginosa* is also cultivated in Malaysia as an ornament and sometimes used in food.

2.5.1 Traditional medicinal uses

C. aeruginosa rhizomes are popular as folk medicine to relieve rheumatic pain and inflammation in the northern districts of Bangladesh (Hossain et al., 2015]. The rhizomes are used as traditionally medicine for the treatment of diarrhoea and colic. They are used by women to treat uterine pain, uterine inflammation and postpartum

complications (Thaina et al., 2009). In Indonesia, *C. aeruginosa* is a useful traditional medicinal plant to treat piles, gonorrhoea, prolapsed uterus and used as antidote to poisons. This plant prevents breast feeding children from diseases that are transmitted through mother's milk. The plant was used as an anti-flatulent and to treat colic in Indochina. The rhizomes are used in Singaporean universal tonic (called ubat jamu) as a medicine for asthma and cough. The local Malaysian people also use *C. aeruginosa* to treat colic and skin diseases including scurvy and pruritis (Global Information Hub on Integrated Medicine, 2016b).

2.5.2 Pharmacological studies

2.5.2(a) Anti-androgenic activity

Suphrom et al. (2012) assayed antiandrogenic activity of the crude hexane extract by using rat liver 5-α-reductase and human prostate cancer cells LNCaP. The crude extract showed 72.8 ± 2.6 % enzymatic inhibition against the conversion of testosterone to dihydrotestosterone (DHT). The cell viability assay of the crude extract showed no effect against LNCaP cells. The study selected low concentrations (0.00001-10 mg/mL) of the crude extract due to the solubility problem in culture medium. The isolation of chemical constituents from hexane extract afforded germacrone, zederone, dehydrocurdione, curcumenol, zedoarodiol and isocurcumenol. Germacrone showed the most potent enzymatic inhibition against the conversion of testosterone to DHT. The cell viability assay showed 30 % inhibition of LNCaP cells by germacrone at a concentration of 40 μM. Zederone showed concentration-dependent inhibition of LNCaP cell line. The androgen receptor binding assay showed no binding of germacrone to androgen receptor which disclosed strong anti-androgenic

activity. The study stated that the anti-androgenic activity of germacone is possibly through the suppression of 5-α-reductase activity.

2.5.2(b) Antitumor activity

Kirana et al. (2003) studied the antitumor property of the ethanolic extract against HT-29, MCF-7 and skin fibroblasts SF3169 cell lines. The crude extract inhibited the viability of HT-29 and MCF-7 cells and the IC_{50} values in MTT assay were calculated to be 119 ± 5.8 and 103.8 ± 16.5 µg/mL, respectively. However, the crude extract exhibited an IC_{50} value of more than 150 µg/mL against SF3169 cells. The study stated that *C. aeruginosa* rhizomes are selective to cancer cell lines.

Atun et al. (2016) evaluated the inhibitory activity of methanol, hexane and chloroform fractions on the viability of cervical carcinoma CaSki and Hela S3, breast cancer cell lines MCF-7 and T-47D and normal Vero cells. The methanol extract showed the IC_{50} value of 95.73 ± 3.03 µg/mL against CaSki cell line. The hexane fraction exhibited the IC_{50} values of 69.47 ± 2.16 µg/mL and 66.02 ± 0.45 µg/mL against MCF-7 and CaSki cells, respectively. The IC_{50} values of chloroform fraction were 92.60 ± 4.10 µg/mL and 94.87 ± 1.94 µg/mL against MCF-7 and CaSki cell lines, respectively. However, more than 500 µg/mL IC_{50} value was observed against Vero cells by all three factions. This study also highlighted selective activity of *C. aeruginosa* rhizomes against different cancer cells.

2.5.2(c) Antioxidant activity

Superoxide dismutase (SOD) is an antioxidant enzyme and has long been applied in cosmetic and chemical industries and used for the purposes of medical treatments. Moon-ai et al. (2012) investigated SOD activity of *C. aeruginosa* rhizomes. The study

fractionated *C. aeruginosa* rhizomes to the crude homogenate and ammonium sulfate cut fractions. The fractions showed SOD activity in a significant level. Diethylaminoethyl cellulose ion exchange chromatography, superdex 75 gel filtration chromatography and sequential ammonium sulfate precipitation were used to enrich SOD enzyme. The study stated that the enriched SOD was weakly simulated by hydrogen peroxide, Mn^{2+} and Fe^{2+}, but was insensitive to hydrogen peroxide and potassium cyanide inhibition. The enriched SOD also showed concentration-dependent inhibition of lipopolysaccharide-induced nitrite oxide production in mouse RA 264.7 cell line.

2.5.2(d) Antinociceptive activity

The *in-vivo* antinociceptive activity of *C. aeruginosa* rhizomes methanolic extract has been reported. The bioassay-guided study led to the purification and isolation of germacrone as the antinociceptive principle from the methanolic extract of *C. aeruginosa* rhizomes. The crude extract showed significant and concentration-dependent inhibition of acetic acid-induced writhing and formalin-induced licking in mice, which indicated central and peripheral antinociceptive activity. Germacrone showed potent central and peripheral antinociceptive activity by the reduction of acetic acid-induced writhing and formalin-induced licking in mice (Hossain et al., 2015b).

2.5.2(e) Anti-inflammatory activity

The anti-inflammatory activity of the crude ethanol extract was conducted using the erythrocyte membrane stabilization activity and carrageenan-induced paw oedema assay. In the erythrocyte membrane stabilization activity, the ethanol extract showed an EC_{50} value of 47.8 ± 1.6 mg/mL. In contrast, the positive control indomethacin

showed an EC_{50} value of 26.4 ± 2.9 mg/mL. Moreover, the crude extract exhibited significant and concentration-dependent reduction of carrageenan induced paw oedema at the concentrations of 100, 200 and 400 mg/kg, when compared to control. The anti-inflammatory activity of the crude extract was identical to that of positive control. The study prognosticated that the crude extract of *C. aeruginosa* extract may inhibit the discharge of inflammatory chemical mediator that increase vascular permeability. The study also stated that curcumin and germacrone might be the possible anti-inflammatory chemical constituents of *C. aeruginosa* rhizomes (Paramita, 2019).

2.5.2(f) Antimicrobial activity

Philip et al. (2009) investigated the antimicrobial activity of *C. aeruginosa* rhizomes at University of Malaya. The authors collected *C. aeruginosa* rhizomes from Jogjakarta, Indonesia in 2006 and extracted with four different solvents (hexane, EtOAc, MeOH and water). The antimicrobial activity of four extracts were conducted against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus* using the disc diffusion method. The methanol extract showed 7.0 mm inhibition zone against *Pseudomonas aeruginosa* at the highest concentration of 500 mg/mL. The hexane extract showed 7.2 mm and 7.5 mm inhibition zone against *Pseudomonas aeruginosa* at the selected two concentration of 50 mg/mL and 500 mg/mL, respectively, and 7.5 mm inhibition zone against *Staphylococcus aureus* at the maximum concentration of 500 mg/mL. The ethyl acetate extract showed 7.0 mm and 9.0 mm inhibition zone against *Bacillus subtilis* at the selected concentrations of 50 mg/mL and 500 mg/mL, respectively and 7.8 mm and 6.7 mm inhibition zone against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, respectively, at 500 mg/mL.