Anticancer Activities of Solvent Fractions and of Sesquiterpene Lactones Isolated from Selected Ethiopian Traditional Medicinal Plants

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By

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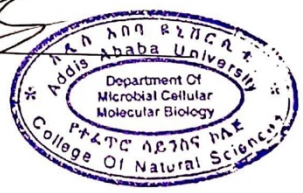

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Nigatu Tuasha Fissa, PhD Dissertation

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Abstract

The incidence and mortality of breast cancer in women is increasing worldwide. Breast cancer has high intertumoral and intratumoral heterogeneity. Cancer stem cells (CSCs) are one of the many subpopulations of cancer cells and are involved in tumor initiation, progression, evolution and metastasis, and are believed to be responsible for chemotherapeutic resistance. The present study investigated *in vitro* anticancer activities of extracts from seven medicinal plants traditionally used to treat breast cancer in Ethiopia. Chemical compounds were also isolated from the most potent bioactive fractions. The study determined cytotoxicity of the crude methanol extracts (80 %, v/v) and that of solvent fractions (1:1, v/v) of the most cytotoxic ones. Furthermore, the anti-CSCs potency of the bioactive solvent fractions and the isolated compounds were also investigated. Vacuum liquid chromatography, preparative thin layer chromatography, column chromatography and high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) were employed for the phytochemical characterization. Cytotoxicity of the phytochemicals against the human breast cancer cell lines, JIMT-1, HCC1937 and MCF-7 and the normal-like breast epithelial cell line, MCF-10A, was determined by a spectrophotometric MTT assay. To investigate specific activity against CSCs, flow cytometric determination of the aldehyde dehydrogenase (ALDH) positivity and determination of $CD44^{+}/CD24^{-}$ CSC subpopulations as well as the colony forming efficiency (CFE) in a serum-free soft agar

were performed. The effects of phytochemical treatment on tumor necrosis factor-α (TNF-α)-induced translocation of the nuclear factor-kappa B (NF-κB) to the nucleus and on cell migration in a wound healing assay were also investigated. The half maximal inhibitory concentrations (IC_{50}) were determined for the crude methanol extract of *Sideroxylon oxyacanthum* (0.09 µg/mL, MCF-7), *Clematis simensis* (0.17 µg/mL, HCC1937) and *Dovyalis abyssinica* (0.21 µg/mL, MCF-7). Ethyl acetate solvent fraction of *V. leopoldi* was the most cytotoxic against JIMT-1 cells (IC_{50} = 0.87 µg/mL). The bioactive solvent fractions of *V. leopoldi, S. oxyacanthum* and *C. simensis* showed dose dependent reduction in the $ALDH^+$ subpopulation of JIMT-1 cells. The chloroform fraction of *C. simensis* (80 µg/mL) completely blocked CFE in JIMT-1 cells. The bioactive solvent fractions of *V. leopoldi, S. oxyacanthum* and *C. simensis* significantly reduced cell migration ($P<0.05$). The ethyl acetate fraction of *V. leopoldi* (0.87 µg/mL) significantly reduced the number of NF-κB positive nuclei ($P<0.001$). Seven compounds - four sesquiterpene lactones and three flavonoids, were isolated from *V. leopoldi.* Among the sesquiterpene lactones, one was a novel compound ("Ndb21a2-P", $C_{20}H_{26}O_8$, M. Wt. = 394.42), which was highly cytotoxic (IC_{50} = 1.6 µM) to JIMT-1 cells. Whereas all sesquiterpene lactones significantly reduced cell migration ($P<0.05$), the compound Vernomenin was most effective ($P<0.005$) in reducing the $ALDH^+$ CSC subpopulation. Also, another isolate, 11ß,13-dihydrovernodalin, resulted in significant reduction in NF-κB positive nuclei ($P<0.05$). The study showed *V. leopoldi* to be the most cytotoxic medicinal plant to cancer cells and the sesquiterpene lactones isolated from it had desirable CSCs-specific activities. The study further showed the importance of systematic selection of traditional medicinal plants for anticancer experimental studies. In conclusion, it is recommended that an in-depth investigation into the chemotherapeutic potentials of the sesquiterpene

lactones from *V. leopoldi* and their structural analogues, be considered as this could augment the global breast cancer drug discovery research.

Key words: Bioactivity-guided fractionation, breast cancer, cancer stem cells, cytotoxicity, *in vitro*, MTT, sesquiterpene lactones, traditional medicine

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List of Abbreviations/Acronyms

ALDH	Aldehyde dehydrogenase
BRCA	Breast cancer genes
BSA	Bovine serum albumin
CAM	Complementary and alternative medicine
CC	Column chromatography
CD	Cluster of differentiation
CFE	Colony forming efficiency
CSCs	Cancer stem cells
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FMoH	(Ethiopian) Federal Ministry of Health
HCC_{1937}	Hamon Cancer Center
HER2	Human epidermal growth factor receptor 2
HR-ESI-MS	High-resolution electrospray ionization mass spectrometry
IARC	International Agency for Research on Cancer
IBC	Inflammatory breast cancer
IC_{50}	Half maximal (50%) inhibitory concentration
IHC	Immunohistochemistry
$MCF_{-7/10}$	Michigan Cancer Foundation
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
NBD	Nitrobenzoxadiazole
NF-κB	Nuclear factor-kappa B
PBS	Phosphate-buffered saline
PDT	Population doubling time
PR	Progesterone receptor
PTLC	Preparative thin layer chromatography
RPMI	Roswell Park Memorial Institute
TM	Traditional medicine

TNBC	Triple negative breast cancer
TNF-α	Tumor necrosis factor-α
VLC	Vacuum liquid chromatography
WHO	World Health Organization

1. Introduction

1.1. Overview of Cancer

Cancer is a potentially fatal disease where cells in the body grow and divide without normal control. In the history of medicine, cancer is one of the oldest diseases to be described (Ades *et al.*, 2017). The Egyptian papyrus of Edwin Smith, dated 1600 BC, is quoted to be one of the earliest manuscripts reporting on cancer treatment. However, the concept could have been copied possibly from a much older document aged 2500 to 3000 BC (van Middendorp *et al.*, 2010). The Greek physician Hippocrates created the term cancer around 400 BC from the word 'karkinos', the Greek term for crab or crayfish associated with the invasive behavior of the disease, touching and invading nearby tissues (Papavramidou *et al.*, 2010). Cancer is a group of diseases comprising a combination of genetic, metabolic and signaling aberrations, which leads to severe disruption of the normal homeostasis of cell growth and death (Long and Ryan, 2012). It is a highly lethal disease driven by huge genetic alterations taking place in normal cells of the body resulting in the accumulation of attributes like over-proliferation, evasion of apoptosis, sustained angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011).

Cancer constitutes an enormous burden globally despite the progress of medicine over the years (Font-Burgada *et al.*, 2016). Cancer is grouped as carcinoma (arises from the epithelial cells), sarcoma (tumor of connective or supportive tissues), lymphoma and myeloma (of the cells of the immune system), leukemia (starts in blood forming tissue) and brain and spinal cord cancers (of the central nervous system) based on the type of the cell and tissue it originated from (www.cancerresearchuk.org, 2017). According to

where they start in the body, there are at least 200 known forms of cancers with many subtypes (www.nih.gov, 2018).

Cancer is the second leading cause of death globally next to cardiovascular diseases and available estimates by GLOBOCAN (an International Agency for Research on Cancer - IARC) show that about 18.1 million new cancer cases and 9.6 million deaths occurred in 2018 (Bray *et al.*, 2018). One in six deaths worldwide is due to cancer (Ritchie, 2019). Lung and breast cancer accounted each for 2.09 million new cases in 2018 (Bray *et al.*, 2018; WHO, 2018). Colorectal and prostate cancers followed with 1.8 million and 1.28 million cases, respectively in 2018 (WHO, 2018). The most common causes of cancer deaths worldwide in 2018 include lung (1.76 million), colorectal (862, 000), stomach (783, 000), liver (782, 000) and breast (627, 000) cancers (WHO, 2018). Overall, 57 % of new cancer cases, 65 % of the cancer deaths and 48 % of the 5-year prevalent cancer cases occurred in the less developed regions of the world (Torre *et al.*, 2015). The occurrence of cancer is increasing because of the growth and aging of the population, as well as an increasing prevalence of established risk factors such as smoking, overweight, physical inactivity and changing reproductive patterns associated with urbanization and economic development (WHO, 2009; Torre *et al.*, 2015; www.who.int, 2019). A significant proportion of cancer burdens and deaths is attributed to modifiable lifestyle-related risk factors such as obesity, diet, physical activity, smoking and alcohol (Brenner *et al.*, 2016; Arriaga *et al.*, 2017). Though global cancer deaths are rising, earlier detection and/or improved treatments are two key factors contributing to improved five-year survival rates and reduction in age-adjusted cancer mortality in recent years (Ritchie, 2019).

In Africa, cancer is alarmingly becoming a critical public health problem. Cancer forms attributed to infectious agents, e.g., cervical cancer, liver cancer, Kaposi's sarcoma and urinary bladder cancer, are among the dominant types (Jemal *et al.*, 2012). According to the IARC, about 715,000 new cancer cases and 542,000 cancer deaths occurred in 2008 in Africa (Jemal *et al.*, 2012; McCormack and Schüz, 2012). The numbers are projected to be doubled by 2030 due to the aging and fast-growing population. The potential of being even higher has been imagined because of the adoption of behaviors and lifestyles associated with economic development (Jemal *et al.*, 2012; McCormack and Schüz, 2012; Parkin *et al.*, 2014). Due to this, prostate cancer in men and breast cancer in women have now become the most commonly diagnosed cancers in some parts of Africa (Ferlay *et al.*, 2010; Torre *et al.*, 2015).

Reports on the prevalence of cancer in Ethiopia are scanty and difficult to verify perhaps because oncology services are inadequate and national registry centers do not exist (Woldeamanuel *et al.*, 2013). However, the limited reports show that Kaposi's sarcoma, liver, prostate, cervical and breast cancer are the most common cancers in Ethiopia (Yarom *et al.*, 2015). According to the cancer country profile of the WHO, most prevalent forms among males are colorectal cancer, Kaposi's sarcoma, leukemia, lymphomas (non-Hodgkin) and prostate cancer in the order of listing. Cancer of the breast, cervix-uteri, ovary and colorectal tissue and leukemia, in the order given, are topping the list among females (WHO, 2014). A study at the radiotherapy center of Tikur Anbessa specialized hospital showed that breast cancer had a high prevalence (27.8 %) (Assefa, 2011). Leukemia (12.7 %) in males and breast cancer (24.4 %) in females were reported to have high mortality rates from total mortalities of 14,500 (males) and 26, 200 (females) in 2014 (WHO, 2014). The Ethiopian Federal Ministry

of Health (FMoH) report shows that the annual incidence of cancer in the country is around 60,960 and the mortality is over 44,000 per year, accounting for 5.8 % of the total national mortality (FMoH, 2015). In 2015, the FMoH set a National Cancer Control Plan for 2016-2020 to achieve a long-term goal of reducing cancer morbidity and mortality in Ethiopia (FMoH, 2015). The Addis Ababa City Cancer Registry (est. in 2011) is the only population-based cancer registry in the country which serves only a small proportion (~3.6 million, ~3.28 %) of the Ethiopian population (CSA, 2013). Breast cancer (31.5 %) in female and colorectal cancer (10.6 %) in male were the most common cancers from a total of 4139 newly diagnosed cases between 2012-2013 (Timotewos *et al.*, 2018). The data for the year 2015 also showed that breast cancer was the most common form of cancer (33 % in women and 23 % of all cancers) identified in Addis Ababa City Cancer Registry (Memirie *et al.*, 2018).

1.2. Breast Cancer

From all types of cancer forms described, breast cancer[1] is the most commonly diagnosed of all cancers and is the second overall cause of death among women both in the developed and less developed world (WHO, 2018). Breast cancer is a heterogenous disease both between individuals and within individuals characterized by high genotypic and phenotypic diversity (Turashvili and Brogi, 2017). Breast cancer, as many other cancers, contains a subpopulation of cancer cells called cancer stem cells (CSCs). The CSCs are tumorigenic multi-potential cells with dysregulated self-renewal properties and are thought to be involved in tumor initiation, progression, evolution and metastasis (Al-Hajj *et al.*, 2003; Chen *et al.*, 2013). Accumulation of knowledge over

[1] This dissertation is written based on the findings of female breast cancer research only. Male breast cancer is an uncommon form that comprises less than 1% of all breast cancers globally. Relatively little is known about the etiology of male breast cancer.

time has changed breast cancer from an incurable condition to a range of different diseases with specific molecular aberrations, clinical behaviors and patterns of response to systemic treatments (Ades *et al.*, 2017).

How cancer starts has become one of highly debatable topics (Hanahan and Weinberg, 2011). The breast epithelium consists of two layers of cells: luminal and basal epithelial cells in the inner and outer ductal layers, respectively (Visvader, 2009). Breast cancer originates from this breast tissue. Some breast cancers start in the ducts where milk is transported to the nipple (ductal cancers), while other breast cancers have their origin in the glands where the breast milk is generated (lobular cancers) (www.komen.org, 2017). Human breast cancer is a group of highly heterogeneous diseases with significant differences on many levels including histology, treatment response and patient survival outcomes (Perou, 2018). Its intertumoral heterogeneity includes clinical, histopathological (morphological), biomarker and genetic diversity; whereas the intratumor heterogeneity occur at morphologic, genomic, transcriptomic, proteomic and epigenetic levels (Turashvili and Brogi, 2017). The heterogeneity of breast cancers poses enormous challenges to diagnose and treat these solid tumors (Hutchinson, 2010). Five to ten percent of all breast cancers arise from germ-line mutations in breast cancer susceptibility genes (e.g., breast cancer genes 1 and 2 (BRCA1 and BRCA2), P53 and PTEN (phosphatase and tensin homolog)) and confer a high individual risk in developing hereditary breast cancer (Apostolou and Fostira, 2013).

1.2.1. Epidemiology and Risk Factors

Breast cancer comprises 25 % of all cancer cases in women both in the developed and less developed world (Torre *et al.*, 2015; WHO, 2018). It remains number one killer of

females in less developed countries while it is the second leading cause of cancer death among females in more developed countries, next to lung cancer (Torre *et al.*, 2015; Torre *et al.*, 2016). Around the world, there is no population of women with a truly low risk of developing breast cancer. Since the 2008 estimates by GLOBOCAN (IARC), both breast cancer incidence and mortality have increased by more than 20 % and 14 %, respectively (Burney *et al.*, 2014). The incidence rate of breast cancer varies from 19.3 per 100,000 women in eastern Africa to 89.7 per 100,000 women in western Europe (www.who.int, 2019). Mortality is relatively low in most of the lowest-incidence countries, but the likelihood that an individual dies of breast cancer is much higher (nearly 17 %) in low-incidence countries than in high-incidence countries (Tao *et al.*, 2015). The reasons for the differential survival are multiple and include cultural influences, stage of presentation and standards of healthcare (May, 2014). Availability of early detection methods and risk factors are the two leading reasons for international variation in breast cancer incidence rates (DeSantis *et al.*, 2014; Torre *et al.*, 2016).

Risk factors for breast cancer generally include being a female, early onset of menstruation, late onset of menopause, long menstrual history, use of oral contraceptives and never having children/having them later in life, age, family history, genetics, personal history of breast cancer, radiation to chest or face before age of 30, race/ethnicity, pregnancy and breastfeeding (Tao *et al.*, 2015; www.komen.org, 2017). Potentially avoidable risk factors encompass overweight/obesity, using hormone replacement therapy, drinking alcohol, smoking and lack of exercise (Chlebowski *et al.*, 2013; Font-Burgada *et al.*, 2016; www.komen.org, 2017). Certain kinds of noncancerous breast diseases (Clagett *et al.*, 2016), dense breasts (Engmann *et al.*, 2017), height (above average) (Warner *et al.*, 2016), low levels of vitamin D (Jacobs *et*

al., 2016), light exposure at night (Spivey, 2010) and exposure to multiple sources of polycyclic aromatic hydrocarbons from the environment (White *et al.*, 2016) are among the emerging risk factors. Consumption of grilled, barbecued, and smoked meats were reported as increasing risk for breast cancer and raising death risk for survivors (Parada *et al.*, 2017).

1.2.2. Classification of Breast Cancers

There is no simple way of classifying breast cancer mainly because of factors considered for its classification (Ostad and Parsa, 2011; Tao *et al.*, 2015). Histological grades, immunohistochemistry (IHC) and changes in gene expression are basis for conventional breast cancer classification nowadays (Hsiao *et al.*, 2010). Thus, among the routinely used classification parameters of breast cancer are tumor size, histological subtype and grade, lymph node status and expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (Nebbioso *et al.*, 2018). Nevertheless, the overlap between the IHC subtypes and those identified by molecular expression studies cannot be ruled out (Perou *et al.*, 2000; Goldhirsch *et al.*, 2013). To date, molecular profiling of breast cancer helps for more precise determination of the subtypes and better prediction of clinical outcome and response to therapy (Ostad and Parsa, 2011). Microarray kits, proteomics, single nucleotide polymorphisms, metabolomics and use of cancer epigenetics may further enrich breast cancer classification for better diagnosis, prognosis and therapeutics (Campbell and Polyak, 2007; Pecina-Slaus and Pecina, 2015; Nebbioso *et al.*, 2018).

Based on the available classification scheme, there are at least five intrinsic molecular subtypes of breast cancer based on gene expression patterns (Perou *et al.*, 2000; Sørlie

et al., 2001). They are luminal A ([ER^+|PR^+] $HER2^-KI\text{-}67^-$), luminal B ([ER^+|PR^+] $HER2^{+/-}KI\text{-}67^+$), HER2 over-expression ([ER^-PR^-] $HER2^+$), basal ([ER^-PR^-] $HER2^-$, basal $marker^+$) and normal-like ([ER^+|PR^+] $HER2^-KI\text{-}67^-$) subtypes (Dai *et al.*, 2015). Generally, ER positive tumors are more common than ER-negative tumors (DeSantis *et al.*, 2014; Tao *et al.*, 2015). The ER positive tumors are also often smaller and of low grade and lymph node negative (Cho, 2016; Torre *et al.*, 2016).

Luminal type A is characterized by cancers that express high levels of ER and PR, $HER2^-$ and have a low-grade histology (i.e. low Ki-67 index) and thus tend to grow slowly. Luminal type B is composed mostly of tumors with low levels of ER and PR expression and have a higher-grade histology (higher Ki-67 index), growing slightly faster than luminal A cancers and consistently show poorer treatment outcome when compared with that of luminal A (Tecalco-Cruz and Ramirez-Jarquin, 2017). The ER and/or PR statuses are important predictors of the response to hormonal therapy and chemotherapy and may be used in combination based on the status of KI-67 index (Ahn *et al.*, 2015).

$HER2^+$ cancers (about 20-25 % of all breast carcinomas) are distinguished by the amplification of HER2 (also called erb-b2 receptor tyrosine kinase 2), a transmembrane growth factor receptor and its protein product HER2 is the predictive marker and molecular target of anti-HER2 therapies (Perou *et al.*, 2000; Cho, 2016; Kim *et al.*, 2017). $HER2^+$ cancers show phenotypes of more aggressive disease with poor prognosis (Nuciforo *et al.*, 2015). In addition, they present shorter overall survival time and more likely develop visceral and central nervous system metastases than other $HER2^-$ subtypes (Leyland-Jones, 2009; Yardley *et al.*, 2014; Zhou *et al.*, 2016). The

recombinant antibody trastuzumab (Herceptin™) is the first Food and Drug Administration (FDA) approved drug (1998) that targets $HER2^+$ cancers and it functions by blocking the HER2 receptors from receiving growth signals (Slamon *et al.*, 2001). Although trastuzumab is currently used in combination with chemotherapy as a standard care for cancers overexpressing the HER2 protein, resistance to trastuzumab has become a frequent phenomenon (Claret and Vu, 2012).

The basal-like subtype is defined by a distinct gene-expression signature characterized by strong expression of basal markers such as cytokeratins 5, 6 and 17, and also encompasses a diverse group of tumors which are ER, PR and HER2 negative (Alluri and Newman, 2014). These tumors have high KI-67 expression and are highly proliferative on average (Badve *et al.*, 2011). This subtype has features such as aggressiveness, poor prognosis and a tendency to affect younger women and is more prevalent in African-American women (Alluri and Newman, 2014). Breast tumors that do not express either ER, PR, or HER2 are called triple negative breast cancers (TNBC) (Perou *et al.*, 2000). The majority of TNBC are of basal-like phenotype and the majority of tumors expressing basal markers are triple-negative. Nevertheless, not all basal-like cancers lack ER, PR and HER2 up on determination of gene expression profiles and conversely not all TNBC show a basal-like phenotype according to expression array analysis (Alluri and Newman, 2014). The majority of BRCA1-associated cancers are of TNBC phenotype (Macedo *et al.*, 2016). TNBC show a remarkable diversity in histologic patterns and subtypes (Lehmann *et al.*, 2015).

Normal-like tumors share the same status on the basic IHC markers with luminal A (i.e. ER^+, PR^+, $HER2^-$, low levels of KI-67). However, these tumors resemble normal breast

in the expression pattern of other proteins and have poor treatment outcome (Perou *et al.*, 2000; Dai *et al.*, 2015).

Due to heterogeneity of breast cancer and advancements made in research towards better prognosis, diagnosis and patient care, defining additional distinct subtypes is not uncommon. For instance, claudin-low TNBC (CL-TNBC) molecular subtype (the 'sixth' subtype) of breast cancer is a poorly described molecular subtype when compared to others (Dias *et al.*, 2017). The defining feature of these tumors is their low expression of many of claudin genes and thus their name (Perou, 2011). The claudins are involved in epithelial cell tight–tight junctions. These tumors also lack cell-cell junction proteins (e.g., E-cadherin) (Perou, 2011). Transcriptionally, they show high genomic instability and are highly undifferentiated (Matossian *et al.*, 2018). High rates of metastasis, recurrence and chemo-resistance are also their clinical features (Lehmann *et al.*, 2011). The subtype shows remarkable differences with respect to molecular and clinicopathological characteristics, clinical outcome and prognostic features, notably compared to basal-like subtype (Sabatier *et al.*, 2014). Although, these tumors have been recognized to preferentially display a triple-negative phenotype, only a minority of TNBC are claudin-low and are considered a distinct subtype (Perou, 2011; Dias *et al.*, 2017).

Of all breast cancer cases, about 1–5 % is known as inflammatory breast cancer (IBC) and about 7 % of all breast cancer-related deaths are attributed to this kind of cancer (Hance *et al.*, 2005). The accepted definition of IBC is "a clinicopathologic entity that is characterized by diffuse erythema and edema, often without an underlying palpable mass" (Greene *et al.*, 2002). The IBC is clinically and biologically distinct (Alvarez *et*

al., 2015) and is aggressive and the most lethal form due to its ability to progress quickly and the frequent presence of metastasis at diagnosis (Madden *et al.*, 2016). Majority of IBC patients have axillary lymph node involvement at diagnosis (Tao *et al.*, 2015). Although there is no specific molecular signature for IBC, the tumors are most commonly either HER2 over-expressing or triple negative (Bertucci *et al.*, 2010).

1.2.3. Biology and Biomarkers of Breast CSCs

Stem cells are undifferentiated cells defined by their properties of self-renewal and potency to differentiate. Based on this, they are classified hierarchically ranging from totipotent, to pluripotent, to multipotent and are rare in nature (Li *et al.*, 2017). The CSCs, first identified in acute myeloid leukemia, are defined by their unlimited self-renewal ability and their capacity to initiate and maintain malignancy (Lapidot *et al.*, 1994; Relation *et al.*, 2017). Breast cancers contain CSCs and these cells are thought to be involved in tumor initiation, progression, evolution and metastasis (Chen *et al.*, 2013). CSCs, typically constitute 1-5 % of the cancer cells (could be as high as 11-35 % in breast cancer) (Korkaya *et al.*, 2008; Gaur *et al.*, 2011). Characteristically, these cells share many features in common with normal stem cells and are slow-dividing, have a lower ability to undergo apoptosis and a higher ability to repair DNA damage (Reya *et al.*, 2001; Morrison *et al.*, 2008). Breast CSCs were the first CSCs prospectively demonstrated in human solid tumors (Al-Hajj *et al.*, 2003) and therefore are among the best characterized. Correlation between epithelial to mesenchymal transition (EMT) and CSCs was reported and CSCs displaying mesenchymal characteristics are resistant to chemotherapy (Wu *et al.*, 2016). During the EMT process, epithelial cancer cells acquire molecular alterations resulting in loss of epithelial features and gain mesenchymal features including disruption of intracellular

tight junctions and loss of cell-cell contact, of which one cause is the loss of E-cadherin. This transformation results in cancer cell invasion and migration, promoting the reconstitution of metastatic colonies at distant sites (Bill and Christofori, 2015; Wu *et al.*, 2016). Intrinsically resistant to chemo- and radiotherapy, CSCs are considered responsible for recurrence of the disease after treatment (Shah and Allegrucci, 2012).

Phenotypic markers for breast CSCs include epithelial cell adhesion molecule/epithelial specific antigen (EPCAM/ESA$^+$), CD44$^+$, CD24$^-$, CD133 and integrin β1 (Al-Hajj *et al.*, 2003; Horimoto *et al.*, 2016) and hedgehog-gli and high aldehyde dehydrogenase (ALDH$^+$) activity (Ginestier *et al.*, 2007). The majority of these molecular markers can be investigated, but the level of scrutiny differs per marker (Medema, 2013). Cells with the phenotypic pattern of CD44$^+$/CD24$^-$/ALDH$^+$ were reported to be more tumorigenic, metastatic, invasive and more migratory, and their presence in a tumor is associated with poor clinical outcome and decreased patient survival (Charafe-Jauffret *et al.*, 2010; Velasco-Velázquez *et al.*, 2011).

The nuclear factor-κB (NF- κB) is a transcription factor that controls multiple cellular processes and in most breast cancer tumors, it is activated constitutively and plays critical roles in cell survival, proliferation, inflammation and immunity (Hayden and Ghosh, 2008). NF-κB is found in the cytoplasm in an inactive form associated with the inhibitor of NF-κB (IκB) (Chaturvedi *et al.*, 2011). When released from the inhibitor, NF-κB is translocated into the nucleus and activates gene transcription by binding to sequence-specific targets in DNA (Gilmore and Garbati, 2010). The transcriptional activation of genes associated with cell proliferation, angiogenesis, metastasis and suppression of apoptosis appears to lie at the heart of the ability of NF-κB to promote

cancer therapy resistance (Shostak and Chariot, 2011; Xia *et al.*, 2014). In addition, decrease in the rate of apoptosis and increased dysregulation of the cell cycle helps a tumor to proliferate and sustain in the body (Hanahan and Weinberg, 2000). Hence, selective elimination of proliferating tumor cells through apoptosis or induction of quiescence is a feasible approach to cancer treatment (Schwartz and Shah, 2005; Dickson and Schwartz, 2009). So, searching agents that specifically affect CSCs, affect cell migration, arrest the cell cycle and block any of the signaling pathways is highly desirable and could be instrumental to achieve durable clinical responses. To this end, a number of small-molecules and their analogues are screened for their specificity and selectivity towards breast CSCs (Naujokat and Steinhart, 2012; Huang *et al.*, 2016).

A detoxifying enzyme ALDH is responsible for the oxidation of retinol (vitamin A) to retinoic acid and this step is crucial for the early differentiation of stem cells (Chute *et al.*, 2006). In cancer research, $ALDH^+$-based identification of CSCs has become a powerful tool because of its ability to separate viable subpopulations associated with an enhanced ALDH activity (Taylor and Jabbarzadeh, 2017).

The interaction of CSCs with their nurturing microenvironment (niche) is implicated as sources of the intra-tumor heterogeneity and support the survival of the CSCs, and CSCs proactively remodel their microenvironment (Rich, 2016; Prager *et al.*, 2019). Destroying this microenvironment is recently considered to be one of the therapeutic targets to kill CSCs (Shen *et al.*, 2016). Generally, in view of conventional breast cancer therapy, virtually all cells, both extensively proliferating and metastasized ones, must be eliminated to cure the disease. In contrast to this approach, the CSC model proposes

that the growth and progression of many cancers are driven by the small subpopulation of CSCs and thus this subpopulation must be targeted (Reya *et al.*, 2001).

1.2.4. Breast Cancer Treatment Options

For more than a century, cancer treatment has been largely based on the premise that cancer cells are homogeneously distinct from their normal counterparts and that it is this difference that could therapeutically be targeted (Yang *et al.*, 2014). Breast cancer treatment, as it stands today, is selected according to the 'group of patients' a specific individual 'fits in' (Ades *et al.*, 2017). Some breast cancer patients may be treated with only one treatment while others may require a combination of treatments. Breast cancer treatment includes local therapies (surgery and radiotherapy) and systemic (chemotherapy, hormonal therapy and targeted therapy) (Miller *et al.*, 2016). Cancer immunotherapy is an additional edge to fight breast cancer, especially highly inflamed subtypes including TNBC, though it is at its infancy (Nathan and Schmid, 2017). In addition to their modest therapeutic efficacies and the occurrence of resistance, all treatment approaches contribute to the burden of side effects experienced by patients (Cleeland *et al.*, 2012; Pareja *et al.*, 2017). Besides, treatment outcomes are largely affected by the time of detection, diagnosis and initiation of cancer therapy and advanced stage cancer presentation at diagnosis is usually correlated with poor survival outcome (Caplan, 2014).

1.2.4.1. Surgical Management

Surgery is considered as the primary treatment for early-stage breast cancer and many patients can be totally cured (Chalasani, 2017). Different types of breast surgery could be done for different reasons. Breast-conserving surgery, also called lumpectomy,

quadrantectomy, partial mastectomy, or segmental mastectomy, is the removal of as much of the cancer as possible or it could be a complete removal of a breast (mastectomy). This is decided based on the status of a breast tumors spreading to the lymph nodes in the arm pit (Harris *et al.*, 2012; www.cancer.org, 2019).

1.2.4.2. Radiotherapy

Treatment with high-energy rays (e.g., x-rays) or particles that destroy cancer cells is radiation therapy. External beam radiation or internal radiation (brachytherapy) could be employed (www.cancer.org, 2019). Its severe side effects include a damage to noncancerous tissue and other site-dependent effects (Cleeland *et al.*, 2012).

1.2.4.3. Chemotherapy

Chemotherapy (also known as cytotoxic therapy) is treatment with cancer-killing drugs. The drugs can be given intravenously or orally and are most effective when combinations are used. It is the primary established systemic treatment for patients with TNBC in both early and advanced-stages (Bianchini *et al.*, 2016) and advanced stages of other breast cancer sub-types (www.cancer.org, 2019). Chemotherapy acts systemically and therefore, also damages healthy cells. Associated side effects of chemotherapy include central and peripheral neurotoxicity, cardiotoxicity, gastrointestinal toxicity and immune suppression (Cleeland *et al.*, 2012). Chemotherapy is also used to kill any cancer cells that might have been left behind after surgery **(**adjuvant chemotherapy) or to shrink the tumor so it can be removed with less extensive surgery (neoadjuvant chemotherapy).

1.2.4.4. Hormonal Therapy

Hormonal therapy is a systemic therapy and it is used to treat women with hormone receptor positive breast cancer after surgery (adjuvant therapy) to help reduce the risk of recurrence (www.cancer.org, 2019). Hormonal therapy is mandatory for all patients with hormone receptor-positive breast neoplasms (Miller *et al.*, 2016). It works either by stopping estrogen from stimulating breast cancer cells to grow or by stopping estrogen production or lowering estrogen levels (e.g., tamoxifen or aromatase inhibitors (Tremont *et al.*, 2017; www.cancer.org, 2019).

1.2.4.5. Targeted Therapy

Targeted therapy is a newly evolving science. It serves as the foundation of precision medicine, which uses information about a tumor's DNA profile to identify additional treatment options. It is a more effective treatment that can attack specific breast cancer cells causing less harm to normal cells and its goal is to interfere with specific characteristics (e.g., genes or proteins) involved in tumor growth (www.breastcancer.org, 2019; www.nationalbreastcancer.org, 2019). Currently used such therapies include apoptosis inducers, angiogenesis inhibitors, signal transduction inhibitors, gene expression modulators and toxin delivery molecules among others (Henderson, 2017). Identification of tumor-specific molecular landscapes of a patient is central to the success of targeted therapy (Siravegna *et al.*, 2017).

1.2.4.6. Immunotherapy

Cancer immunotherapy refers to any treatment that modifies and/or enhances the patient's immune system to fight cancer and is believed to pave innovative paths toward truly personalized medicine (Nathan and Schmid, 2017; Kelly, 2018). The approach is

based on drugs that can manipulate components of the immune system and methods to genetically engineer patients' own T-lymphocytes to recognize and attack their tumors (Kelly, 2018). The programed cell death 1/programed cell death 1 ligand 1 pathway plays a crucial role in cancer immunotherapy (Wang *et al.*, 2017). Patients with TNBC, the most inflamed breast cancer subtype with a relatively high level of tumor infiltrating lymphocytes, are believed to benefit from immunotherapy compared to non-inflammatory cancers such as ER positive and HER-2 negative breast cancer (Disis and Stanton, 2017; Nathan and Schmid, 2017). Tumors can evade T-cells attack by expressing surface ligands that engage inhibitory receptors on tumor-specific T-cells and induce immune tolerance (Freeman *et al.*, 2000). The FDA approved an anti-PD-L1 antibody called atezolizumab (Tecentriq®), a checkpoint inhibitor immunotherapy drug, for the first time in March 2019 as a frontline combination treatment with nab-paclitaxel (Abraxane) for PD-L1–positive TNBC (www.fda.gov, 2019).

1.3. Perspectives on the Development of New Classes of Anticancer Agents

The development of any new drug is an expensive, time taking and risky process (Chen *et al.*, 2018). When adding up post-approval research and development costs, the total cost of any new approved drug is estimated to be 2.87 billion USD (DiMasi *et al.*, 2016). It is estimated that only one out of thousands of compounds screened in pre-clinical research passes all the clinical tests and becomes a drug (Saadi and White, 2014). Meanwhile, of the total experimental drugs entering the clinical trials, approval rate is estimated to be around 12 % (DiMasi *et al.*, 2016).

Despite the innovation of anticancer therapeutic agents in clinical use amidst all the challenges, the world is in persistent need of new approaches with better patient care

and ultimate cure. Among the recent paradigms, designing therapeutic agents against the clonogenic core of the tumor, the CSCs, is one of the models actively considered (Kitambi and Chandrasekar, 2011). The model is not older than 25 years. However, the inability to precisely identify and isolate the CSCs has become a challenge to the model and has resulted in mounting clinical failures. This in turn is forcing researchers to revise the approach (Garber, 2018). However, the model is expected to bear fruits, once the limitations of the model are addressed, and remains a potential target for the breakthrough in cancer therapeutics (Saygin *et al.*, 2018).

The ultimate goal for cancer therapy is to find drugs that are totally selective for cancer cells. In line with this selective inhibitors of histone acetyltransferases (KAT6A and KAT6B), which can permanently put cancer cells to sleep, are discovered, highlighting a new paradigm in the field of cancer therapeutics (Baell *et al.*, 2018). Conventional cancer treatments cause irreversible DNA damage in cancer cells and also affect healthy cells. This new class of drugs target specific proteins and puts cancer cells into a form of cell senescence (Baell *et al.*, 2018).

In addition, exploiting genetic similarities between tumors could save lives and give additional weaponry. The FDA approved an oral drug called Lynparza™ (olaparib), a poly-ADP-ribose polymerase (PARP) inhibitor, specifically to treat BRCA mutated, HER2-negative metastatic breast cancer for the first time on Jan 12, 2018. Previously, this class of drugs have been in use for BRCA mutated ovarian cancer and their efficacy in breast cancer would mean there is a potential to develop drugs that target the underlying genetic causes of cancer. Talzenna™ (talazoparib) was also approved later by FDA in October 2018 to treat the same breast cancer subtype (www.cancer.org,

2018; www.fda.gov, 2018). On the areas where eradicating the cancer, such as metastatic cancers, using conventional surgical or chemoradiotherapeutic strategies is not possible, engineered stem cells can be used as novel delivery tools by homing to and targeting both primary and metastatic tumor foci. In addition, they can also be used as viral vectors and nanoparticle carriers to enhance primary therapeutic efficacies and relieve treatment side effects (Zhang *et al.,* 2017).

1.4. Traditional Medicine as a Source of Cancer Therapeutic Agents

Global inequity in primary healthcare coverage has forced mankind to use ancient alternative medical practices in order to save lives. Of note is the use of plant-based traditional healing practices sustained for millennia in different parts of the world (Benzie and Wachtel-Galor, 2011). Complementary and alternative medicine (CAM) is the term used for medical products and practices that are not part of standard medical care (WHO, 2013). Herbal treatment (phytomedicine, phytotherapy, or botanical medicine) is the oldest used system of CAM in the world with more than 2,000 years history (Ma *et al.*, 2011). It is a medicine made exclusively from plant parts such as roots, bark, flowers, seeds, fruits, leaves, or branches and is used in all societies and common to numerous cultures including Asian, African, European and American (WHO, 2013). There are various types of herbal medicines that spring from different cultures around the world, however, they vary in the way they are prepared and in treatment approaches (Olaku and White, 2011).

CAM use is common amongst cancer patients particularly breast cancer patients being the most likely users (Morris *et al.*, 2000). Cancer patients may turn to CAM use for a number of reasons. These include, a hunt for anything that would help to avoid the

unfavorable outcome of the disease, lessening side effects of chemotherapy, manage the symptoms, or to treat conditions unrelated to their cancer (Smith *et al.*, 2014; Berretta *et al.*, 2017). In addition, rising drug costs and economic insufficiency are other driving forces towards the use of traditional medicine (TM) (Dukes *et al.*, 2003; WHO, 2013). Herbal remedies are assumed by the general public to be safe, cause less complications and are less likely to cause dependency (Olaku and White, 2011). Most cancer patients combine herbal remedies with conventional therapy in the hope of enhancing the effect of conventional medicine (Richardson *et al.*, 2004). Its role in providing sense of control or psychological boosting of being actively involved in treatment was also implicated (Vickers *et al.*, 2006).

Various reports have shown that the clinical use of traditional herbal medicine improved disease symptoms and quality of life, reduced chemo/radiotherapy induced side effects and resulted in tumor size reduction (Ernst, 1998; Molassiotis *et al.*, 2005; Li *et al.*, 2013). They also support conventional cancer treatments and prevent recurrence (Shahid, 2013). In addition, anti-angiogenesis effects, prevention of tumor recurrence and assisting the body's immune system to battle cancer have been documented with the use of TM (Shahid, 2013; Levitsky and Dembitsky, 2015).

Many of the alkaloid chemicals derived from plants are associated with cytotoxic effects and have potent activities that target human diseases, including cancer (Howes, 2018). According to the FDA, about 40 % of the approved drugs and 74% of those used in cancer therapeutics are either sourced from natural compounds or derived from them (Seca and Pinto, 2018). Extraction and fractionation are, thus, the starting steps in research involving traditional medicinal plants aiming at isolation and purification of chemical constituents.

Drug resistance to the chemotherapeutics employed in cancer treatment remains a stern challenge these days. Most often, this situation leads to the use of two or more drugs in combinations and transfer of a patient to a different drug regimen (Fridlender *et al.*, 2015; Ahmed *et al.*, 2017). Besides, the chemotherapeutics currently in use cause severe side effects (e.g., high toxicities and off-target effects) among the users resulting in dramatical reduction in the quality of life and thus restricting their clinical use (Fridlender *et al.*, 2015; Iqbal *et al.*, 2017). New strategies are, therefore, needed to acquire lasting cure against cancer with reduced side effects. To this end, plant-based TM practices and applications are dependable sources for the search of lead materials for preclinical work with the goal to be used in clinical medicine today (Fabricant and Farnsworth, 2001).

On the other hand, though a substantial number of cancer patients take herbal medicines at any time during cancer treatment (first or then in their disease history), there is no robust evidence base for understanding what these CAMs do to the users (Damery *et al.*, 2011). Some herbal medicines may cause serious effects by their own and/or by interacting with cancer treatments in use (Gratus *et al.*, 2009). In addition, it may also render the therapeutic outcome of the conventional drug unpredictable and may result in consequences with unacceptable toxicities (Tascilar *et al.*, 2006). For health-care professionals, an understanding of the self-medication behaviors (e.g., use of CAM for instance) of the patients is essential to help them avoid such unwanted pharmacological interactions (Damery *et al.*, 2011). In most of the cases, the use of CAM is not based on empirical evidence and thus the field requires validating studies to a higher extent (Damery *et al.*, 2011; Smith *et al.*, 2014).

1.5. The Experimental Plants

Ethnobotanical reports from the country (Tuasha *et al.*, 2018a) and judicious *in situ* investigations were used to select anticancer medicinal plants for this study. The literature sources have revealed that there are over 117 species of anticancer medicinal plants distributed in 102 genera and 57 families across the country (Tuasha *et al.*, 2018b). In addition, ethnobotanical research conducted in Dalle district, Sidama where TM is an integral part of primary healthcare revealed priority anticancer medicinal plants used by traditional healers of the area (Tuasha *et al.*, 2018a). Based on these sources, anticancer medicinal plants widely used in different part of the country and those with comparative advantages were identified using analytical ethnobotanical tools. The plants were collected from sites described in Table 1. The plants were identified and authenticated by a taxonomist (Provide the name) and a voucher specimen were deposited in the National Herbarium, Addis Ababa University for future reference.

Table 1. The anticancer medicinal plants studied and their distribution in the Ethiopian flora

Scientific name [Family]	Local/Vernacular name	Collection site	Distribution in Ethiopia [Alt. range]
Sideroxylon oxyacanthum (Baill.) [Sapotaceae]*	Bunguude (Sid)	Dalle District, Sidama, SNNPR	TU GD, SU, AR, BA, HA [1250–2800]
Zanthoxylum chalybeum Engl. [Rutaceae]	Gadda (Oro/Sid)	Dalle District, Sidama, SNNPR	GG, BA, HA [900–1550]
Clematis simensis Fresen. [Ranunculaceae]	Ye Azo Hareg (Amh); Feetii/Hidda feetii (Orm); Fiide (Sid)	Dalle District, Sidama, SNNPR	GD, TU, WU, GJ, SU, AR, WG, KF, GG, SD, BA, HA [1500–3350]
Clematis longicauda Steud. ex A.Rich. [Ranunculaceae]*	Alaya/Elaya, Nech Ye Azo Hareg (Amh); Feetii (Oro)	Yayu (Jimma area), Oromiya Regional State	GD, GD/GJ, GJ, SU, WG, KF, IL, SD [1350–3300 m]
Dovyalis abyssinica (A. Rich.) Warb. [Flacourbitaceae]	Koshim (Amh); Shiilo (Sid)	Dalle District, Sidama, SNNPR	TU, GD, GJ, WU, SU, AR, GG, SD, BA, HA [1700–3000]
Clerodendrum myricoides (Hochst.) Vatke [Lamiaceae]	Misrichi (Amh) Mardhisiisaa (Oro) Ma'niisa (Sid)	Dalle District, Sidama, SNNPR	TU, GD, WU, SU, AR, WG, IL, KF, GG, SD, HA [700–2600]
Vernonia leopoldi (Sch. Bip. ex Walp.) Vatke [Asteraceae]**	Merara kitel/ Chibo (Amh)	Yayu (Jimma area), Oromiya Regional State	TU, GD, GJ, WU, SU, WG, KF, HA, GG [1850–2850]

Abbreviations: TU - Tigray region above 1000 m contour; AF - Afar region below 1000 m contour 895 to Eritrean border in the east and Harerge border in the south; WU - Welo region above 1000 m contour; GD - Gondar region; WG - Welega region; KF - Kefa region; AR - Arsi region; BA - Bale region; GJ - Gojam region; IL - I1ubabor region; GG - Gamo Gofa region; SU - Shewa region above 1000 m contour; SD - Sidamo region; HA - Harerge region

Note: * - Endemic species; **Outside Ethiopia, it was reported from Yemen. The voucher specimens were deposited in the herbarium (NT014, NT012, NT037, NT072, NT017, NT006 and NT073).

1.6. Description of the Medicinal Plants

1.6.1. *Sideroxylon oxyacanthum*

S. oxyacanthum Baill. (Family: Sapotaceae) is a spiny shrub or small tree, often with stunted growth and rounded crown, up to 10 m tall. It carries densely clustered leaves and axillary flowers, with a 1-4 cm long spine that normally develops at the base of each of these short shoots (Hedberg *et al.*, 2003). The genus is represented by two species: *S. oxyacanthum* Baill. and *S. mascatense* (A. DC) Pennington (Hedberg *et al.*, 2003). Some reports have shown that the plant species is used as a traditional medicinal plant in different part of the country. For instance, fresh leaves were reported to be used for casting out an evil spirit (Yineger *et al.*, 2008), treat eye infections (Hedberg *et al.*, 2003) and as an anticancer agent (Tuasha *et al.*, 2018a). Raw fruits are known as edible in southwestern and central part of the country (Lulekal *et al.*, 2011; Seyoum *et al.*, 2015).

1.6.2. *Zanthoxylum chalybeum*

Z. chalybeum Engl. (Family: Rutaceae) is a deciduous spiny shrub/tree and can grow from 1.5-10 m tall. The genus *Zanthoxylum* is represented by three species and the other two are *Z. leprieurii* Guill. & Perr and *Z. usambarense* (Engl.) Kokwaro (Hedberg and Edwards, 1989). Zanthoxylum means (in Greek), 'yellow wood' ['xanthos' means yellow and 'xylon' means wood]. Its origin and distribution spans from Ethiopia and Somalia south to eastern Botswana and Zimbabwe. The plant is widely used in traditional medical practices and is harvested from the wild. Different part of the plant including stem and root bark (frequently used part of a plant), hot and peppery fruits and leaves are commonly harvested. A wide range of human ailments traditionally treated from a remedy preparation of the plant include malaria, sickle cell disease, tuberculosis, pneumonia, colds, ulcers, sore throat, tonsillitis, urticaria (hives), tumors,

measles, abdominal pain, diarrhea, intestinal worms, bilharzia, amoebas, general body pain, female sterility, venereal diseases, uterine fibroids, fainting, dizziness, headache and others (www.combonimissionaries.co.uk, 2017; www.prota4u.org, 2019). The major bioactive principles of the plant are alkaloids (www.prota4u.org, 2019). In Ethiopia, it is reported that the plant is used for the treatment of various human ailments including cancer, more specifically breast cancer (Regassa, 2013; Kewessa *et al.*, 2015; Tuasha *et al.*, 2018a).

1.6.3. *Clematis simensis*

C. simensis Fresen. (Family: Ranunculaceae) is a woody climber under the genus Clematis, which is represented by over 250 species in the world. Four species (*C. longicauda* Steud. ex A. Rich., *C. hirsuta* Perro & Guill., *C. burgensis* Engl. and *C. simensis* Fresen.) are reported from the flora of Ethiopia (Edwards *et al.*, 2000). The species is mostly found in ecotones and disturbed habitats, particularly forest edges and bushland, rocky hillsides and old lava beds with shrubby vegetation. It is found growing with many different species. In Ethiopian TM, the species is claimed to be used for the treatment of various ailments including cancers, leprosy, fever, various skin diseases, headache, common cold, hemorrhoids and eczema (Pankhurst, 1998; Wubetu *et al.*, 2017; Tuasha *et al.*, 2018a).

1.6.4. *Clematis longicauda*

C. longicauda Steud. ex A. Rich. (Family: Ranunculaceae) is found in most parts of the country growing in open montane forest and forest borders, along roads, streams, on fences and in woodland associations (Edwards *et al.*, 2000; Dagne, 2011). The traditional use of the species include the treatment of itching skin (Giday *et al.*, 2010). Antimicrobial and wound healing activities of *C. longicauda* were also reported (Hawaze *et al.*, 2012; Hawaze *et al.*, 2013).

The genus *Clematis* is rich in triterpene saponins, alkaloids, flavonoids, lignans, steroids, coumarins, macrocyclic compounds, phenolic glycosides, anemonin and volatile oils as major classes of chemical constituents (Sun and Yang, 2009; Chawla *et al.*, 2012).

1.6.5. *Dovyalis abyssinica*

D. abyssinica (A. Rich.) Warb. (Family: Flacourtiaceae) is an indigenous evergreen, dioecious and spiny shrub/small tree of about 6-10 m in height. The genus Dovyalis is represented by three species (*D. caffra* (Hook. f & Harv.) Hook. f*, D. verrucosa* (Hochst.) Warb. and *D. abyssinica* (A. Rich.) Warb.) in the flora of Ethiopia (Edwards *et al.*, 2000). Ecology of *D. abyssinica* ranges from Ethiopia, Somalia and Socotra south to Malawi in upland rainforest, dry evergreen forest, on riverbanks and sometimes in more open wooded grassland. The plant has a small, round and orange colored edible fruit with a thin, tender skin and a juicy flesh (Edwards *et al.*, 2000). Traditional medicinal use of the species includes treatment of hemorrhoid (Chekole *et al.*, 2015) and cancer (Abate, 1989; Enyew *et al.*, 2014; Tuasha *et al.*, 2018a).

1.6.6. *Clerodendrum myricoides*

C. myricoides (Hochst.) Vatke (Family: Lamiaceae) is a perennial herb (Hedberg, 2006). In the flora region, the genus Clerodendrum is represented by eight species. These are *C. cephalanthum Olivo, Clerodendrum*. sp., *C. johnstonii Olivo, C. umbellatum Poir.*, *C. alatum Gurke*, *C. myricoides (Hochst.) Vatke*, *C. robecchii Chiov.* and *C. acerbianum (Vis.) Benth.* & *Hook* (Hedberg, 2006). *C. myricoides* species is widely distributed in the Ethiopian flora. Various traditional medicinal uses of the species were reported. Casting out an evil spirit (Yineger *et al.*, 2008; Teklay *et al.*, 2013; Araya *et al.*, 2015; Chekole *et al.*, 2015), cancer (Tuasha *et al.,* 2018a), treating snake bite (Hedberg, 2006; Teklay *et al.*, 2013), malaria (Asnake *et al.*, 2016), diarrhea

(Kefalew *et al.*, 2015), arthritis/rheumatism, conjunctivitis and trachoma (Araya *et al.*, 2015), 'almaz balechira' (viral infection) (Teklehaymanot *et al.*, 2007), coughs, headaches and abdominal pains.

1.6.7. *Vernonia leopoldi*

V. leopoldi (Sch. Bip. ex Walp.) Vatke (Family: Asteraceae) is a shrub or rarely woody herb, covered with soft hairs and grows up to 0.5-2.5 m in height (Hedberg *et al.*, 2004; Dagne, 2011) under the tribe Vernonieae Casso (Hedberg *et al.*, 2004). In the Ethiopian flora area, there are seven genera with Vernonia having the largest number of species. The genus Vernonia is represented by about 49 species excluding imperfectly known taxa (Hedberg *et al.*, 2004) and it encompasses herbs, shrubs, or small trees. The growth habitat of *V. leopoldi* encompass forest margins, Acacia wooded grassland with scrub of Maytenus*, Rosa abyssinica* and Carissa on shallow soil, often in ravines, roadside thickets and wasteland (Hedberg *et al.*, 2004). The traditional medicinal use of the plant include cancer treatment (Abera, 2003; Wabe *et al.*, 2011).

Outside Ethiopia, *V. leopoldi* is found only in Yemen (known as *V. leopoldii*) (Marzouk and Abd Elhalim, 2016). Its traditional medicinal uses in Yemen include the treatment of cough, colic diseases and skin diseases (Marzouk and Abd Elhalim, 2016). In 2009, a crude extract of *V. leopoldii* was reported to have growth inhibitory effect in different cancer-derived cell lines with IC_{50} values ranging from 34.2 to 41.5 µg/mL (Mothana *et al.*, 2009). Qualitatively, it was reported that the plant contains phytochemicals such as terpenoids, phytosterols and flavonoids (Mothana *et al.*, 2009).

A number of studies with species from the genus Vernonia have reported cytotoxicity at varying concentrations. The crude methanol extract of the stem of *V. divaricata* had

IC_{50} of 10.1 µg/mL in HL-60, 12.6 µg/mL in MCF-7 and 9.9 µg/mL in, PC-3 cells (Lowe *et al.*, 2014). The extracts of *V. condensate* resulted in a dose- and time-dependent cytotoxicity (9 mg/mL – 26 mg/mL) against various cancer cells (Thomas *et al.*, 2016). The acetone extract of *V. guineensis* was also reported to show high toxicity against ten different cell lines with IC_{50} values ranging from 4-26 mg/mL (Toyang *et al.*, 2013). A report of potent cytotoxicity in colon adenocarcinoma cells (HT29) and hepatoma cells (HepG2) with the extracts of *V. cinerea* (Khay *et al.*, 2012) were few among others.

The Asteraceae (Compositae) family, the sunflower family where the genus Vernonia belongs, is known for large proportion of sesquiterpenoids as their major chemical constituents. Sesquiterpenoids are natural products with 15 carbons in the backbone and they are widely found in leaves, fruits, or roots of many plants (Davis and Croteau, 2000). The sesquiterpene lactones are sub-class of sesquiterpenoids and they are typical secondary metabolites of several plant families including Acanthaceae, Anacardiaceae, Apiaceae, Asteraceae, Euphorbiaceae, Lauraceae, Magnoliaceae, Menispermaceae, Rutaceae and Winteraceae (Zhang *et al.*, 2005). The sesquiterpene lactones are found in almost all genera of Asteraceae, notably in Vernonia, Tanacetum, Helenium, Ambrosia, Arnica and Artemisia (Kreuger *et al.*, 2012). The compounds are bitter, colorless substances, with lipophilic character and are thermolabile and less volatile (Ivanescu *et al.*, 2015).

More than 5000 structurally different sesquiterpene lactones are known so far with a proportionally high number (over 3000) isolated from Asteraceae (Wu *et al.*, 2006; Chadwick *et al.*, 2013; Ivanescu *et al.*, 2015; Shoaib *et al.*, 2017). A wide variety of

biological and pharmacological activities of the sesquiterpene lactones have been described (Chaturvedi, 2011; Ivanescu *et al.*, 2015). The potential of many sesquiterpene lactones for use in the treatment of cancer and cardiovascular disease have received large interest (Chukwujekwu *et al.*, 2009; Chadwick *et al.*, 2013; Shoaib *et al.*, 2017; Sotillo *et al.*, 2017) and recently anticancer activities have been reported (Chaturvedi *et al.*, 2015). The α-methylene-γ-lactone group found in sesquiterpene lactones is correlated with many of their pharmacological actions (Chadwick *et al.*, 2013; Burlec *et al.*, 2017). Studies involving structure-activity relationships indicated that rapid Michael type of addition takes place when cytotoxic sesquiterpene lactones react with thiols, such as free sulfhydryl groups of, mainly, cystine in proteins (Chaturvedi, 2011; Ivanescu *et al.*, 2015). According to these studies, selective alkylation of growth regulatory biological macromolecules (e.g. enzymes) result in the inhibition of tumor growth through cascades which lead to apoptosis. Factors including molecular geometry, lipophilicity and chemical environment of the target sulfhydryl group affect the activities of sesquiterpene lactones (Chaturvedi, 2011).

1.7. Statement of the Problem

Except surgery, almost any cancer therapeutics can result in resistance and growing evidence asserts that the source of resistance are the CSCs (Krause *et al.*, 2017; Peters, 2018). Besides, conventional chemotherapeutics have undesirable off-target actions and many times only target the bulk cancer cells leaving the CSCs unharmed whereby the remaining CSCs reconstitute the tumor after the standard treatment regime. With all the limitations and severe side effects of existing therapeutics, searching for an alternative therapeutic in general and anti-CSC agent in particular is essential. Though Ethiopia has immense potential of anticancer TMs, few or none of them have been

investigated for cytotoxicity profiling and pursued for potential lead molecules for anticancer agents.

1.8. Rationale

These days, much of the breast cancer therapeutic research is focusing on the CSCs, the main causes of tumor maintenance and sustainability. The CSC model proposes that the growth and progression of many cancers are driven by this small subpopulations of CSCs (Gangopadhyay *et al.*, 2013). Cell migration, one of the features of CSCs, gives rise to cancer metastases which results in tumor initiation at different sites of the body (Chambers *et al.*, 2002).

Historically, TM has a central role in contributing valuable anticancer drugs (Gordaliza, 2007; Fridlender *et al.*, 2015; Seca and Pinto, 2018). Among which are the well-known paclitaxel (Taxol®) (from *Taxus brevifolia* Nutt.) (Wani *et al.*, 1971) approved by the FDA for the treatment of woman with epithelial ovarian cancer in 1992 (Rowinsky and Donehower, 1995) and vincristine (from *Catharanthus roseus* (L.) G. Don) which was approved for clinical use in 1963 (Evans *et al.*, 1963). Ethiopia is a country with immense cultural and biological diversity and TM has been practiced for time immemorial. Experimental findings are rarely reported from the medicinal plants of Ethiopia that are traditionally used in cancer treatment and cytotoxicity profiles of the most commonly reported ones are virtually absent. Therefore, the present study has a rationale to present *in vitro* cytotoxicity profiles for selected and widely used traditional medicinal plants and pursue a bioactivity-guided experimentation to search for a biologically active agents and investigate anti-breast CSC activities.

1.9. Hypothesis

The present study was initiated with the assumption that systematically selected medicinal plants traditionally used in cancer treatment in Ethiopia, will be cytotoxic against various breast cancer-derived cell lines and would lead to biologically active small molecules with selective actions against breast CSCs.

1.10. Research Questions and Objectives of the Study

1.10.1. Research Questions

Based on the rationale and research hypothesis stated above, the objectives for the study were derived from the following questions.

1. Are selected Ethiopian medicinal plants traditionally used in cancer treatment cytotoxic at very low doses?
2. Would solvent-solvent fractions show any anti-CSCs activities *in vitro*?
3. Does bioactivity guided fractionation lead to biologically active principle with anticancer activities?

1.10.2. General Objective

The general objective was to investigate *in vitro* anti-CSC activities of selected Ethiopian medicinal plants traditionally used in breast cancer treatment and isolate biologically active principle(s) guided by the bioactivities.

1.10.3. Specific Objectives

- ✓ Conduct *in vitro* cytotoxicity dose response assay with methanolic crude extracts and determine the half maximal inhibitory concentration (IC_{50}) values in breast cancer cell lines and normal-like breast epithelial cell line
- ✓ Conduct *in vitro* cytotoxicity dose response assay with the solvent-solvent fractions of the selected medicinal plants and determine the IC_{50} values in the representative breast cancer cell lines and normal-like breast epithelial cell line
- ✓ Evaluate the selective anti-CSC activities of the most cytotoxic solvent fractions of the medicinal plants
- ✓ Investigate the mechanism of actions of the most cytotoxic solvent fractions of the medicinal plants
- ✓ Isolate and chemically characterize the active biological ingredients from the most cytotoxic solvent fractions of the medicinal plants
- ✓ Determine the basal cytotoxicity of the purified compounds and investigate anti-breast CSC activities

2. Materials and Methods

2.1. Drying and Determination of Water Content

The water content of the medicinal plants was determined by estimating the weight loss up on drying in an air-ventilated open room at ambient temperature. The initial weight of the plant material was determined by weighing at the field during collection of the plants using a portable beam balance. After recording the initial weight, the plant material was thoroughly washed with tap water and then rinsed with distilled water to remove contaminants at ambient room temperature. The drying process took place in a plant drying room of the Biomedical Research Laboratory of the department of Microbial, Cellular and Molecular Biology, Addis Ababa University. The plant material was allowed to dry naturally in a shaded, ventilated open space away from exposure of sunlight. It was promptly checked if the dried material could be pounded and ground to finer powder forms. Before measuring the final weight of the plant material, the fine powder was weighed and once again subjected to further drying. After checking a weight loss of not more than 0.25 %, the final weight was measured and expressed in percentage of the initial weight. Finally, the plant materials were ground to a finer powder at ambient room temperature using a pestle and an electronic mill and then sieved with a mesh. All the materials used were at their cleaner forms. The powdered plant materials were stored, protected from light at the Biomedical Science laboratory in dark bottles until extraction.

2.2. Extraction and Fractionation

Fine powder (500 g) of each medicinal plant was subjected to extraction. Accordingly, 100 g of the powder was suspended in 500 ml of 80 % methanol in an Erlenmeyer flask.

The suspension was macerated using a rotary water bath shaker (DZK-2, Shanghai, China) (120 routes per minute) for 72 hours at ambient temperature. Thereafter, the liquid and solid phases were separated by filtration, initially using cotton cloth while squeezing gently. Subsequently, three consecutive filtrations were performed using Whatman filter paper №1 (Whatman LTD, England) at ambient temperature. Removal of the solvent and concentration was performed using a rotary vacuum evaporator (BÜCHI-Germany) under reduced pressure at 45° C. The concentrated extract was then freeze-dried by lyophilization (CHRIST, Alpha 2-4 LDplus, Osterode, Germany).

The crude extract of the most potent medicinal plants was then further fractionated according to the following procedure. Solvent-solvent (1:1, v/v) fractionation was performed based on the polarity of the solvents. Accordingly, the dried crude methanol extracts were weighed and allowed to completely dissolve in 250 ml of distilled H_2O containing methanol (10 %) in an Erlenmeyer flask. A separation funnel was used for the partitioning and 250 ml of n-hexane (100 %) was added. It was then sealed with a stopper and the stopcock was tightly closed. The solution was then gently mixed in the funnel before letting it partition for 1 hour which resulted in an aqueous and an n-hexane layer. The n-hexane phase *(Fraction 1)* was carefully collected. The volume of the aqueous phase was determined and an equal volume of chloroform (100 %) was added. The same procedure as described above was followed resulting in the collection of a chloroform phase *(Fraction 2)*. The volume of the aqueous phase was determined and mixed with an equal volume of ethyl acetate (100 %). The same procedure was followed resulting in the collection of an ethyl acetate phase *(Fraction 3)* and the remaining aqueous phase was taken as aqueous fraction *(Fraction 4)*. All the fractions were freeze-dried as described above.

A slightly modified extraction procedure was followed to obtain fractions of *C. longicauda* and *V. leopoldi.* Briefly, 50 g of fine powder of each plant was dissolved in 250 ml of 90 % methanol and then the solution was subjected to maceration on a shaking water bath (DZK-2, Shanghai, China) for 12 hours at ambient temperature. Filtration and concentration of the crude extract was performed as described above. The dried crude extract of each medicinal plant was weighed and fractionated with petroleum-ether and then ethyl acetate to obtain the petroleum-ether and ethyl acetate fractions, respectively. The fractions were freeze dried as described above and stored at -20° C until use. All chemicals used for the extraction and partitioning process were purchased from Sigma-Aldrich (St. Louise, MO, USA) and were of analytic grade.

2.3. Bioactivity-guided Isolation of Compounds

Following the investigations of anti-CSCs bioactivities with the most cytotoxic solvent fractions of the medicinal plants (*V. leopoldi, C. simensis* and *S. oxyacanthum*), the isolation of compounds was carried out.

2.3.1. Extraction and Isolation of Compounds from *V. leopoldi*

Powdered dry leaves of *V. leopoldi* (1 kg) was extracted by maceration at room temperature in 95 % ethanol (EtOH) (5 L, 7 days). The solvent was removed under reduced pressure to yield a semisolid residue (145.2 g). After being suspended in 1.2 L of a mixture of H_2O-methanol (8:2, v/v), the solution was successively extracted with heptane (three times, 2:1, v/v) and ethyl acetate (three times, 1:1, v/v). The ethyl acetate extract (46.3 g) was subjected to vacuum liquid chromatography (VLC) on silica gel, using step gradient of heptane−ethyl acetate (1:0 to 0:1, v/v), afforded eighteen

fractions (A-R). Fraction N (24.1 g) was subjected once more to VLC on silica gel, using a step gradient of heptane−ethyl acetate (8:2 to 0:1, v/v) to yield five sub-fractions (Na−Ne). The sub-fraction Nc (2.2 g) was subjected to sephadex LH-20. It was eluted with methanol and afforded four fractions (Nca-Ncd). Fraction Nca afforded compound 1 **(1)** and fraction Ncc (60 mg) was separated by preparative thin layer chromatography (PTLC) to give compound 2 **(2)** and a mixture of compound 2 and compound 3 **(3)**. Sub-fraction Nd (17.2 g) was subjected to VLC on silica gel eluted with heptane−ethyl acetate (8:2 to 0:1, v/v) to give 2 sub-fractions (Nda and Ndb). Fraction Ndb (11.5 g) was eluted with heptane−ethyl acetate (1:1, v/v) afforded two fractions (Ndb1 and Ndb2). A fraction Ndb1 was identified as compound 4 **(4)**. From Ndb2 (7.75 g), a portion (2.20 g) was purified using sephadex LH 20 eluted with methanol and then silica gel column chromatography (CC) with heptane−ethyl acetate (4:6 to 0:100, v/v) afforded compound 5 **(5)**, compound 6 **(6)** and compound 7 **(7)**.

2.3.2. Extraction and Isolation of Compounds from *C. simensis*

Dried powder of the whole aerial part of *C. simensis* (1 kg) was extracted by maceration at room temperature in 95 % ethanol (5 L, 5 days). The solvent was removed under reduced pressure and yielded a semisolid residue (168 g). After being suspended in 1 L of a mixture of H_2O-methanol (8:2, v/v), the solution was successively extracted with heptane, chloroform and ethyl acetate, resulting in 3 extracts weighing 58.5 g, 2.4 g and 12.4 g, respectively. The heptane extract (58.5 g) was subjected to phytochemical study after confirming that this was the active solvent fraction in a dose-response assay. Using VLC on silica gel and step gradient of heptane−ethyl acetate (1:0 to 0:1, v/v), ten fractions were obtained (A-J). The fractions were examined by TLC.

2.3.3. HR-ESI-MS Confirmation of a New Compound

Nuclear magnetic resonance (NMR) spectra were recorded at room temperature on a Bruker Avance II 500 MHz spectrometer. The spectra were recorded in deuterated chloroform ($CDCl_3$) and deuterated dimethyl sulphoxide (DMSO-d_6) and the solvent signals (7.27/77.0 and 2.50/39.51) were used as a reference. VLC separation was carried out on Merck Silica gel 60G, while CC was performed using Silica gel 60 (230–400 mesh, Merck) and gel permeation on Sephadex LH-20 (GE Healthcare). Chromatograms were visualized under a UV lamp at 254 nm then spraying with vanillin followed by heating. PTLC was run on 20 cm × 20 cm glass-coated plates (1 mm thickness, Analtech). High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) was performed in a Waters Q-TOF Micro system spectrometer (using H_3PO_4 for calibration and as internal standard).

2.4. Cell Lines and Culture Conditions

The human breast cancer cell lines JIMT-1 (population doubling time (PDT) ≈ 24 h), HCC1937 (PDT ≈ 35 h), MCF-7 (PDT ≈ 35 h) and one normal-like cell line (MCF-10A, PDT ≈ 15 h) were used for the cell culturing assay experiments. The JIMT-1, a human ductal breast carcinoma cell line, was established from a pleural metastasis of a 62-year old patient with breast cancer who was clinically resistant to trastuzumab (Tanner *et al.*, 2004). The HCC1937, human ductal breast carcinoma cell line, was sourced from infiltrating ductal primary breast tumor (grade III) from a 24 years old patient with a germ-line BRCA1 mutation (Tomlinson *et al.*, 1998). The MCF-7 cell line was derived from a pleural effusion of a 69 years old female with an epithelial breast adenocarcinoma (Soule *et al.*, 1973). The cell lines JIMT-1, MCF-7 and HCC1937 represent HER2 positive, luminal A and basal-like breast cancer sub-groups,

respectively. The MCF-7 (HTB-22) and HCC1937 (CRL-2336) cancer cell lines as well as the human normal-like breast epithelial cell line MCF-10A (CRL-10317) were purchased from American Type Culture Collection (Manassas, VA, USA). The JIMT-1 cell line (ACC589) was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

JIMT-1 cells were routinely cultured in Dulbecco's Modified Eagle Medium/Ham's F-12 medium (VWR, Lund, Sweden) supplemented with 10 % fetal bovine serum (FBS) (VWR), 1 mM non-essential amino acids (VWR), 10 μg/mL insulin (Sigma-Aldrich, Stockholm, Sweden), 1 mM L-glutamine (VWR) and 100 U/mL penicillin/100 μg/mL streptomycin (VWR). The MCF-7 cells were cultured in RPMI 1640 medium supplemented with 10 % heat-inactivated FBS (VWR), 1 mM non-essential amino acids (VWR), 10 μg/mL insulin (Sigma-Aldrich), 1 mM L-glutamine (VWR) and 100 U/mL penicillin/100 μg/mL streptomycin (VWR). The HCC1937 cells were cultured in RPMI 1640 medium (VWR) supplemented with 10 % heat-inactivated FBS (VWR), 1 mM non-essential amino acids (VWR), 10 μg/mL insulin (Sigma-Aldrich), 20 ng/mL epidermal growth factor (Sigma-Aldrich) and 100 U/mL penicillin/-100 μg/mL streptomycin (VWR). The MCF-10A cells were cultured in RPMI 1640 medium (VWR) supplemented with 10 % heat-inactivated FBS (VWR), 1 mM non-essential amino acids (VWR), 10 μg/mL insulin (Sigma-Aldrich), 20 ng/mL epidermal growth factor (Sigma-Aldrich), 50 ng/mL cholera toxin (Sigma-Aldrich), 250 ng/mL hydrocortisone (Sigma-Aldrich) and 100 U/mL penicillin/100 μg/mL streptomycin (VWR). All cell lines were kept at 37˚ C in a humidified incubator with 5 % CO_2.

The cells were seeded at different densities. Accordingly, JIMT-1 cells were seeded at 1.5×10^4 cells/cm^2, both MCF-7 and HCC1937 at 2×10^4 cells/cm^2 and MCF-10A cells were seeded at 10^4 cells/cm^2. Tissue culture vessels of the appropriate size were used with the volume of medium about 0.2–0.3 ml per cm^2. The cells were tested for mycoplasma and were found to be negative (Eurofins GATC Biotech, Konstanz, Germany).

2.5. MTT Assay

Measurement of cell viability and proliferation are the basis for numerous *in vitro* assays of a cell population's response to external factors. The yellow soluble tetrazolium salt 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) is reduced by metabolically active cells to insoluble formazan crystals inside the cells (Mosmann, 1983) and nowadays this *in vitro* assay is a widely accepted method to obtain dose response curves in a 96-well format from which IC_{50} values are derived. Briefly, confluent cells were detached by trypsinization (0.05 % Trypsin-EDTA) and counted in a hemocytometer. Cells of the different cell lines were seeded at the recommended densities (described above) in 180 µL of medium into the wells of 96-well plates and then allowed to attach for 24 hours before addition of the extracts/solvent fractions/compounds.

For the treatments, stock solutions of 1 mg/mL (crude methanol extracts) and 250 mg/mL or 500 mg/mL (fractionated extracts) were prepared in 100 % methanol and were allowed to dissolve completely. Then, the higher concentrations of 20 µg/mL with 4 % methanol in PBS (crude extracts) and 5 mg/mL with 2 % methanol in PBS (fractionated extracts) were prepared and sterile-filtered using 0.22 µm filters followed

by serial dilutions to the lowest concentrations of 0.01 µg/mL (crude extracts) and 0.001 mg/mL (solvent fractions). Then, 20 µL of the serially-diluted methanol crude extracts or fractionated extracts were added to obtain the desired concentrations in the wells of the 96-well plates. The controls received 20 µL 4 % methanol in PBS (crude extracts) or 2 % methanol in PBS (fractionated extracts). Thus, the final methanol concentration in all wells was 0.4 % (crude extracts) or 0.2 % (fractionated extracts). For the compounds, the stock solution of each was prepared as 50 mM in 100 % DMSO. After it was completely dissolved, it was diluted 50x in PBS so that the maximum concentration became 1000 µM in 2 % DMSO. Then, it was serially diluted to eight different concentrations (1000 µM, 500 µM, 100 µM, 75 µM, 50 µM, 10 µM, 1 µM and 0.1 µM) and diluted 1/10 into 96-well plates. The controls received 20 µL 2 % DMSO in PBS. The plates were then incubated for 72 hours before MTT addition.

MTT (Sigma-Aldrich) was dissolved in PBS to a concentration of 5 mg/mL, the solution was sterile-filtered and stored at -20° C wrapped with aluminum foil to protect from light exposure. After 72 hours of incubation, 20 µL of the MTT solution was added to the wells and the 96 well plates were wrapped with aluminum foil and returned to the CO_2 incubator for 1 hour. The formazan crystals were dissolved by adding 100 µL of 100 % DMSO to each well. To dissolve the precipitates, the plates were allowed to gently swirl at room temperature for 10-15 minutes. Then the absorbance was read at 540 nm using a Labsystems iEMS Reader MF (Labsystems Oy, Helsinki, Finland) and using DeltaSoft II v.4.14 software (Biometallics Inc., Princeton, NJ, USA). The percent of control was calculated as absorbance units in the presence of the extracts/fractions as percentage of that in the control and thus the dose response curves were drawn and IC_{50} values were obtained using the GraphPad Prism software (San

Diego, CA, USA) version 7.02. The dose-response experiments were performed several times (3 to 6) for each extract/fraction/compound and the mean IC_{50} ± SD was calculated.

2.6. Breast CSC Population Estimation by an ALDH Assay

The ALDH assay was performed using the JIMT-1 cells. The cells were seeded in Petri dishes at the density described above and incubated for 24 hours. The test solvent fractions were added to their final concentrations of dose ranges between IC_{25} and IC_{50} values, whereas the sesquiterpene lactones were added at 2 µM concentration. The control groups were treated with 0.005 % methanol in PBS (0.005 % DMSO in PBS, for the compounds) (i.e. the same final methanol/DMSO concentration as for the solvent fractions/compounds). The cells were then incubated for 72 hours. Accutase™ (Stem Cell Technologies, Grenoble, France) was used to detach the cells and the detached cells were collected in PBS containing 1 % FBS and kept on ice. The cells were counted using a hemocytometer. The ALDEFLUOR™ kit (Stem Cell Technologies, Grenoble, France) procedures as provided in the manufacturer's protocol were followed. Briefly, two test tubes (250,000 cells/tube) were prepared for each $ALDH^+$ assay. The specific ALDH inhibitor, diethylaminobenzaldehyde, was added to one of the test tubes, which served as a negative control. The ALDH substrate BODIPY-aminoacetaldehyde was then added to both tubes and incubated for 45 minutes at 37˚C. After incubation, the cells were centrifuged and the pelleted cells were re-suspended in 250 µl assay buffer. The BD Accuri C6 Flowcytometer (BD Biosciences) was used to analyze the $ALDH^+$ cell population. Diethylaminobenzaldehyde-treated cells were assayed first and used as a control to set

the $ALDH^+$ region for each parallel sample. The data were evaluated using the CFlow software.

2.7. Colony Forming Efficiency Assay

The effect of fractions on the colony forming efficiency (CFE) was evaluated using a serum-free soft agar assay that supports colony formation of CSCs. The JIMT-1 cells were seeded in Petri dishes at the density described above and incubated for 24 hours before addition of the solvent fractions. The solvent fractions were added to the final concentrations of dose ranges (0.63 µg/mL to 80 µg/mL IC_{25} and IC_{50} included), while the controls were treated with 0.005 % methanol in PBS. The Petri dishes were then incubated at 37˚C in a humidified incubator with 5 % CO_2 in air for 72 hours. The cells were detached by treatment with Accutase for 10 minutes at 37˚C and kept on ice while counting using a hemocytometer. Human mammary epithelial cells basal medium (CC-4136 kit) (Cambrex, Walkersville, Maryland, USA), supplemented with 20 ng/mL epidermal growth factor (Sigma-Aldrich), 250 ng/mL hydrocortisone (Sigma-Aldrich), 10 mg/mL insulin (Sigma-Aldrich), B-27 supplement (Thermo Fisher Scientific), 20 ng/mL basic fibroblast growth factor (R&D Systems, Minneapolis, MN, USA), 100 U/mL penicillin/100 µg/mL streptomycin (VWR) was used to prepare cell suspension for the CFE assay. The medium was heated to 42˚C and mixed with agar to a final concentration of 0.4 % and the counted cells were added to a final concentration of 1000 cells/mL. Then, 500 µL of this mixture was added to the inner wells of hydrophobic 48-well plates and the outer rows of the plates were filled with 1 mL PBS to minimize evaporation. The plates were wrapped in saran wrap and incubated at 37˚C in a humidified incubator with 5 % CO_2 in air for 14 days. The colonies were counted visually in an inverted phase contrast microscope using the 10x objective.

2.8. Cell Migration Assay

The JIMT-1 cells were seeded at high density (~125,000 cells/cm^2) in Petri dishes and incubated at 37˚C in a humidified incubator with 5% CO_2 in air for 24 hours to allow them to attach and form a confluent layer of cells. Then, the medium was removed and three parallel scratch wound areas (left, middle and right side of the dish) were made in the cell layer using a sterile 200 µL pipette tip, following the previously established protocol (Rodriguez *et al.*, 2005). This cell layer was washed twice with PBS and serum-free medium was added to minimize the wound healing process that would result from cell proliferation. The respective solvent fractions were added to the final concentration of their IC_{50}, the average of IC_{25} and IC_{50}, or IC_{25} values while the controls were treated with 0.005 % methanol. The zero hour (time 0) photographic images were taken immediately before incubation at 37°C in CO_2 incubator. The wound area was photographed at 24, 48 and 72 hours after time zero. For the sesquiterpene lactones, treatment was performed using a final concentration of 2 µM and images were taken at 0 and 72 hours. To estimate the wound closure, the scratch areas were measured with ImageJ 1.47v software (https://imagej.nih.gov). The wound closure was calculated as percentage of the wound at time zero.

2.9. Inhibition of NF-κB Translocation

The effect of the fractions on the tumor necrosis factor-α (TNF-α) induced translocation of NF-κB to the cell nucleus was evaluated. For this experiment, JIMT-1 cells were seeded (0.1 x 10^6 cells/well) in 12-well plates with a round glass cover slip in each of the wells. The cells were then allowed to attach to the cover slips by incubating at 37°C in the CO_2 incubator for 48 hours. After incubation, the cover slips were transferred to the wells of another 12-well plate containing 800 µL medium supplemented with 0.1

% FBS and then incubated in the CO_2 incubator for 20 minutes to stabilize the temperature. The solvent fractions were added to the final concentration of their IC_{50} values (*C. simensis,* 80 µg/mL and *V. leopoldi,* 0.87 µg/mL) and at 52 µg/mL for *S. oxyacanthum* and the plates were returned to the incubator for 1 hour. For the sesquiterpene lactones, stock solution of 400 µM was prepared and were tested at 2.5 µM and 10 µM. Following this, TNF-α (25 ng/mL) was added and incubation continued for another 40 minutes. Then, the medium was removed and the cells were fixed in 3.7 % formaldehyde in PBS and incubated for 15 minutes at 37°C and stored at 4°C in 2 mL PBS. Blocking buffer (1 % bovine serum albumin (BSA) and 1 % Tween 20 in PBS) was used to prevent nonspecific binding and to permeabilize the cells. This was done for 1 hour at room temperature and the samples were washed with PBS twice. Then, rabbit anti-p65/NF-κB primary antibody (ab76311) (Abcam, Cambridge, MA, USA) (diluted 1:250 in blocking buffer) was added and the samples were incubated for 1 hour at room temperature while shaking gently. After washing twice with PBS, Alexa 488 anti-rabbit-conjugated secondary antibody (Molecular Probes, Inc., Eugene, USA) (diluted 1:500 in blocking buffer) was added. The plate was covered with aluminum foil and the samples incubated for 1 hour at room temperature while shaking gently. Finally, the cover slips were mounted on glass slides using Mowiol (Sigma-Aldrich) as mounting medium. The samples were kept at 4°C protected from light. The imaging of the slides was done using Olympus/Nikon epifluorescence microscope (Olympus Optical Co. Ltd., Japan) equipped with a digital camera (Nikon Imaging Japan Inc., Japan).

2.10. Estimation of $CD44^+/CD24^-$ CSC Subpopulation of JIMT-1 Cells

The JIMT-1 cells were used in the assay analysing CD44 and CD24 expression by flow cytometry. The cells were seeded in Petri dishes at the density described above and incubated for 24 hours. Then the cells were treated with 2 μM (final concentration) sesquiterpene lactones and the control groups were treated with 0.01 % DMSO in PBS. The cells were then incubated for 72 hours. Accutase™ was used to detach the cells, and the detached cells were collected in PBS containing 1 % FBS and counted using a hemocytometer. The test tubes were kept on ice. Then, 200 000 cells per sample were incubated with the monoclonal antibodies (CD44-fluorescein isothiocyanate-conjugated (1:100) (clone G44-26) and CD24-phycoerythrin-conjugated (1:50) (clone ML5)) and phycoerythrin-conjugated or fluorescein isothiocyanate-conjugated mouse IgG1 isotype controls (MOPC-21) for 15 minutes on ice. All the monoclonal antibodies were purchased from BD Biosciences. Then, the cells were washed with cold PBS containing 1% FBS before analysis in the flow cytometer. CFlow software was used to evaluate the data. The data are expressed as % of control.

2.11. Effects of the Sesquiterpene Lactones on the Cell Cycle Phase Distribution

The effect of the sesquiterpene lactones on the cell cycle phase distribution was evaluated by flow cytometry after staining with propidium iodide (Sigma-Aldrich). The JIMT-1 cells were seeded in Petri dishes at the density described above and incubated for 24 hours. Then the cells were treated with a final concentration of 2 μM sesquiterpene lactones and the control groups were treated with 0.01 % DMSO in PBS. The cells were then incubated for 72 hours. Accutase™ was used to detach the cells and the detached cells were collected in PBS containing 1 % FBS and counted using a hemocytometer. After pelleting, the cells were fixed in ice-cold 70 % ethanol for 30

minutes and stored at -20°C. After washing with PBS, PBS containing 100 µg/mL propidium iodide, 0.6 % NP-40 and 100 µg/mL ribonuclease A (Sigma-Aldrich) was added to the cells and incubated overnight at 4°C. After the samples were vortexed and triturated, they were transferred to the appropriate test tubes and evaluated using a BD Accuri C6 Flowcytometer.

2.12. Uptake and Localization of Vernolepin-NBD Conjugate

To investigate the uptake and localization of the sesquiterpene lactones inside the cell, fluorophore nitrobenzoxadiazole (NBD) was conjugated to vernolepin **(5)**, one of the four sesquiterpene lactones isolated from *V. leopoldi.* After evaluating preservation of the activity of the compound by the MTT assay, the JIMT-1 cells were seeded (0.35 x 10^6 cells) in a Petri dish containing 2 mL regular medium and incubated for 48 hours. The vernolepin-NBD conjugate was then added to a final concentration of 10 µM, under dark condition. Then, phase contrast and immunofluorescence images were taken at 40x by an inverted phase-contrast microscope equipped with a light source for fluorescence 1, 5, 10, 15, 30, 45, 60 and 120 minutes and 24, 48 and 72 hours following addition of the NBD-conjugated compound, and the uptake and localization were evaluated.

2.13. Statistical Analysis

Nonlinear regression analysis was used to calculate the IC_{50} values for all dose-response curves. All the results are shown as mean ± SD. The data were statistically analyzed by one-way analysis of variance (ANOVA) followed by post hoc Dunnett's test and $P< 0.05$, CI = 95 % was considered statistically significant.

2.14. Ethics Approval

The study was approved by the Institutional Review Board of the College of Natural and Computational Science (CNS-IRB), Addis Ababa University (IRB/022/2016). Additionally, Armauer Hansen Research Institute/All Africa Leprosy Rehabilitation and Training Hospital (AHRI/ALERT) Ethics Review Committee also granted an approval (Project Registration №: PO19/16). The material transfer agreement (MTA) was granted by the Ethiopian Biodiversity Institute (EBI) to ship the plant material and conduct scientific research.

3. Results

3.1. Water Content and Yield

Following weight measurements before and after drying, the weight loss was estimated and a measure of water content was obtained to get an estimate of how much plant material would be needed for future use. Whilst a highest water content was found in the leaves of *C. myricoides* (61.3 %), that of the stem bark of *D. abyssinica* was the lowest (38.9 %) (Table 2).

Table 2. Estimation of water content after drying the medicinal plants under ventilated condition

The medicinal plant	Part used	Weight at collection (g)	Final dry weight (g)	Water content (%)
S. oxyacanthum	Leaf	1200	720	40.0
Z. chalybeum	Leaf	1500	820	45.3
C. simensis	Whole aerial part	1200	610	49.2
C. longicauda	Whole aerial part	850	445	47.6
D. abyssinica	Stem bark	900	550	38.9
V. leopoldi	Leaf	1000	415	58.5
C. myricoides	Leaf	750	290	61.3

Percent yield of the methanol extracts and fractions was depicted in Table 2. A very good yield was obtained from all the crude extracts except with *C. myricoides,* where it was found to be quite low. The yield from the fractions appeared to depend on the nature of solvent used for fractionation. The aqueous fraction provided a maximum yield in cases of *S. oxyacanthum* and *C. simensis*, while the petroleum ether and ethyl acetate fraction with *C. longicauda* and *V. leopoldi*, respectively.

Table 3. Percent yield of the crude and solvent fractions of the medicinal plants

The extract/solvent fraction	Net yield (%) ‡
S. oxyacanthum (80 % methanol)	20.6
hexane	2.3
chloroform	5.1
ethyl acetate	10.1
aqueous	67.9
C. simensis (80 % methanol)	19.2
hexane	11.7
chloroform	17.0
ethyl acetate	3.2
aqueous	26.0
V. leopoldi (90 % methanol)	29.4
petroleum ether	23.5
ethyl acetate	46.3
C. longicauda (90 % methanol)	17.3
petroleum ether	44.3
ethyl acetate	18.6
D. abyssinica (80 % methanol)	13.9
C. myricoides (80 % methanol)	4.0
Z. chalybeum (80 % methanol)	25.33

‡ The net yield was calculated from the initial dried plant material used for extraction (i.e., 500 g for *S. oxyacanthum* and *C. simensis*; and 100 g for *V. leopoldi and C. longicauda).*

3.2. MTT Assay

The study was initiated by determining the cytotoxicity of crude extracts. MTT assay was used to determine $IC_{50,}$ the overall basal toxicity profiles, of the crude extracts in the breast-derived cell lines. The crude extract of *S. oxyacanthum* was found to be the most cytotoxic against all cell lines used, where the lowest concentration of 0.09 μg/mL was recorded in the MCF-7 cell line. Extracts from *Z. chalybeum* and *C. myricoides*

were not toxic in JIMT-1 and MCF-7 cells at the maximum concentration used (2 μg/mL) (Table 4 and Appendix 1).

Table 4. **IC_{50} (μg/mL) values of the crude extracts against JIMT-1, MCF-7 and HCC1937 cell lines**

Crude extract	Cell line		
	JIMT-1	**MCF-7**	**HCC1937**
S. oxyacanthum	0.15 ± 0.01	0.09 ± 0.02	0.13 ± 0.003
C. simensis	0.44 ± 0.20	0.24 ± 0.04	0.17 ± 0.12
D. abyssinica	0.63 ± 0.33	0.21 ± 0.01	0.27 ± 0.14
C. myricoides	NC [a]	0.74 [b]	- [c]
Z. chalybeum	NC [a]	NC [a]	- [c]

[a] NC = not cytotoxic at the highest treatment concentration of 2 μg/mL. [b] Mean of two replicates. [c] The MTT test was not carried out on this cell line since no high toxicity was found in the other cell lines.

After determining the IC_{50} values, the medicinal plants with the lowest IC_{50} values were selected for solvent-solvent fractionation for further testing and bioactivity-guided isolation of compounds. Based on reports of wider use among the herbal medicine practitioners and endemicity, solvent-solvent fractions of two medicinal plants, *V. leopoldi* and *C. longicauda,* were further included in the study. Furthermore, the normal-like cell line, MCF-10A, was included and the HCC1937 breast cancer cell line was omitted. Of the solvent fractions tested, the aqueous fraction of *S. oxyacanthum*, the chloroform fraction of *C. simensis* and the ethyl acetate fraction of *V. leopoldi* were toxic at low concentrations. The ethyl acetate fraction of *V. leopoldi* was the most cytotoxic medicinal plant with an IC_{50} value of 0.87 μg/mL on JIMT-1 breast cancer cell line (Table 5). Representative dose response curves are shown in Appendix 2.

Table 5. **IC_{50} (μg/mL) values of fractions on JIMT-1, MCF-7, and MCF-10A cell lines**

Solvent fractions	Cell line		
	JIMT-1	**MCF-7**	**MCF-10A**
S. oxyacanthum (aqueous)	69 ± 2	49 ± 2.6	80 ± 2
S. oxyacanthum (hexane)	NC [a]	- [b]	- [b]
S. oxyacanthum (chloroform)	694 ± 20	NC [a]	- [b]
S. oxyacanthum (ethyl acetate)	660 ± 44	240 ± 16	- [b]
C. simensis (chloroform)	80 ± 19	190 ± 70	97 ± 9
C. simensis (hexane)	NC [a]	- [b]	- [b]
C. simensis (ethyl acetate)	NC [a]	NC [a]	- [b]
C. simensis (aqueous)	858 ± 190	NC [a]	- [b]
V. leopoldi (ethyl acetate)	0.87 ± 0.2	3.5 [c]	1.72 [c]
V. leopoldi (petroleum ether)	80 [c]	NC [a]	- [b]
C. longicauda (ethyl acetate)	70 [c]	NC [a]	- [b]
C. longicauda (petroleum ether)	170 [c]	NC [a]	- [b]

Note: [a] NC = not cytotoxic at the highest concentration used. [b] The MTT test was not carried out on this cell line since it is not toxic at low doses in the other cell lines. [c] Mean of two replicates.

3.3. Reduction of the $ALDH^+$ Subpopulations of JIMT-1 Cells

One of the phenotypic markers for breast CSCs is an enhanced ALDH activity and thus the fraction of $ALDH^+$ cells was estimated using the ALDEFLUOR™ assay. Treatment with two different concentrations (69 μg/mL and 52 μg/mL) of the aqueous fraction of *S. oxyacanthum* for 72 hours significantly reduced the CSC population of JIMT-1 cells ($p<0.001$). However, treatment with IC_{25} concentration (20 μg/mL) did not result in reduction of the CSC population. Treatment with the chloroform fraction of *C. simensis* and ethyl acetate fraction of *V. leopoldi* at IC_{50} values (80 μg/mL and 0.87 μg/mL, respectively) also resulted in a significant reduction of the CSC population ($p<0.05$) (Figure 1). Representative cytograms of the flowcytometric analysis of the CSC population of JIMT-1 cells are shown in Figure 2.

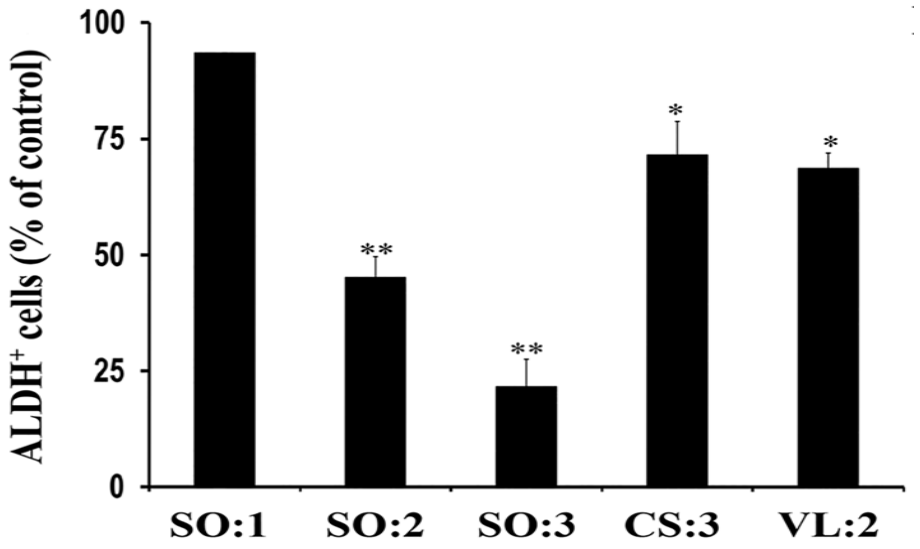


Figure 1. ALDH$^+$ population of JIMT-1 following treatment with the solvent fractions of *S. oxyacanthum, C. simensis* and *V. leopoldi*: evaluation was performed using flow cytometry.

SO, aqueous fraction of *S. oxyacanthum* and SO:1, SO:2 and SO:3 represent treatment concentrations of 20 µg/mL, 52 µg/mL and 69 µg/mL, respectively; CS3, chloroform fraction of *C. simensis* 80 µg/mL; VL2, ethyl acetate fraction of *V. leopoldi* 0.87 µg/mL. *p<0.05; *p<0.001; CI: 95 %; all values were compared to the control.

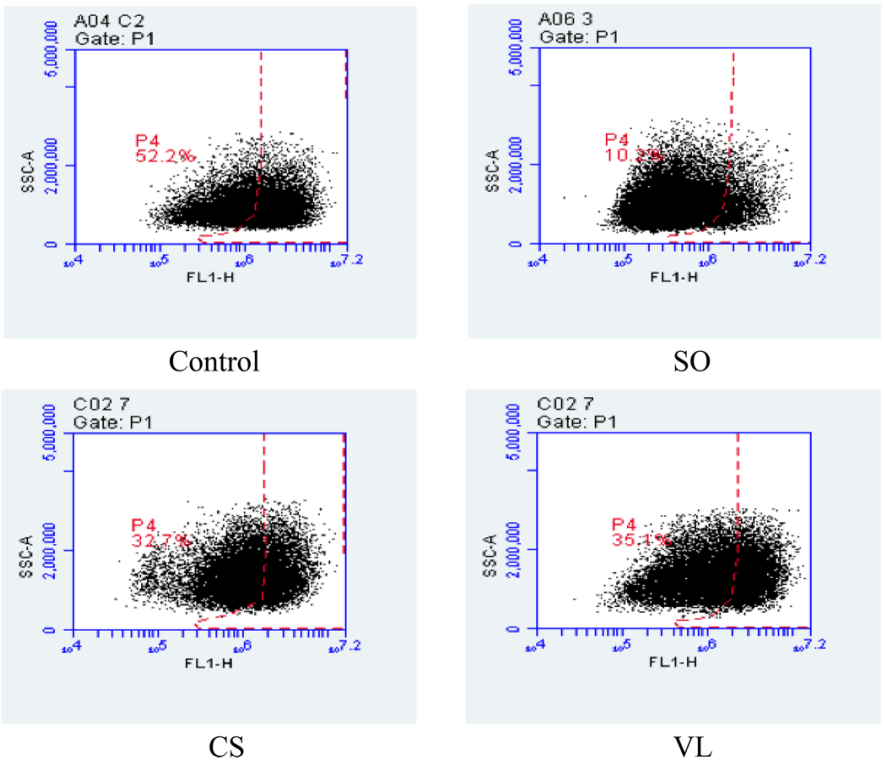


Figure 2. Representative cytograms of the flow cytometric analysis of ALDH$^+$ cells of JIMT-1 cells treated with the solvent fractions of *S. oxyacanthum, C. simensis* and *V. leopoldi.*

The percentage in each figure represents the proportion of ALDH$^+$ cells (found in the outlined red region) in relation to all cells; SO, aqueous fraction of *S. oxyacanthum* (69 µg/mL); CS, chloroform fraction of *C. simensis* (80 µg/mL); VL, ethyl acetate fraction of *V. leopoldi* (0.87 µg/mL).

3.4. Colony Forming Efficiency Assay

The CFE in serum free medium containing soft agar is a functional assay used to investigate the survival of cells with stem-like properties (Dontu *et al.*, 2003). Treatment with the chloroform fraction of *C. simensis* at the IC_{50} value (80 µg/mL) resulted in 100 % prevention of colony formation of JIMT-1 cells. Treatment with solvent fractions of *V. leopoldi, S. oxyacanthum* and *C. simensis* significantly reduced CFE in a dose-dependent manner compared to the control (Figure 3). In addition to reducing the number of colonies, treatment with these solvent fractions resulted in smaller colonies than in the control (Figure 4).

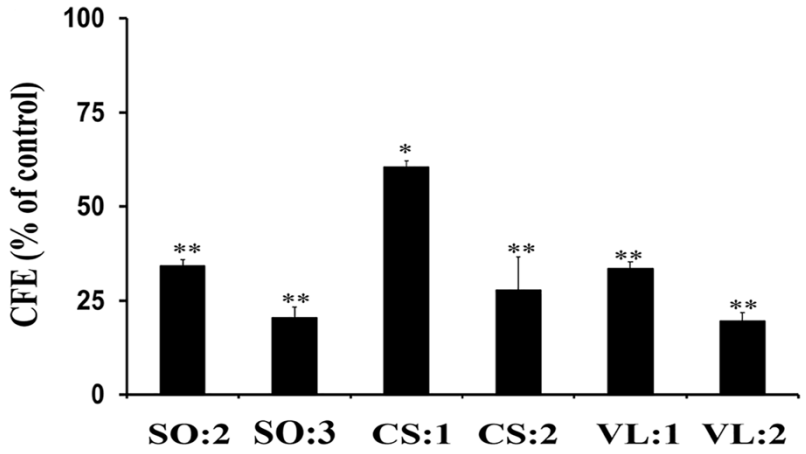


Figure 3. **Colony Forming Efficiency of JIMT-1 cells treated with the solvent fractions of *S. oxyacanthum, C. simensis* and *V. leopoldi.***
Values are expressed as percent of control; SO, aqueous fraction of *S. oxyacanthum* and SO:2 and SO:3 represent treatment concentrations 52 µg/mL and 69 µg/mL, respectively. CS, chloroform fraction of *C. simensis* and CS:1 and CS:2 represent concentrations 15 µg/mL and 33.7 µg/mL, respectively; VL, ethyl acetate fraction of *V. leopoldi* and VL:1 and VL:2 represent concentrations 0.63 µg/mL and 0.87 µg/mL, respectively; *p<0.05; **p<0.001; CI: 95 % confidence interval; all values were compared to the control.

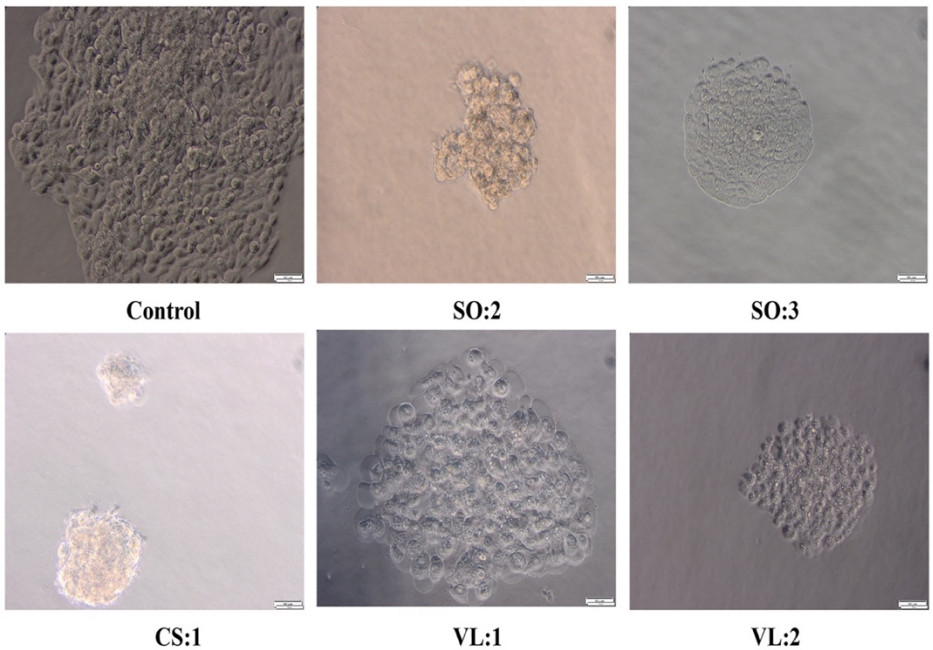


Figure 4. **Representative images showing the effects of solvent fractions of *S. oxyacanthum, C. simensis* and *V. leopoldi* on colony forming efficiency of JIMT-1 cells.**
SO, aqueous fraction of *S. oxyacanthum* and SO:2 and SO:3 represent treatment concentrations 52 µg/mL and 69 µg/mL, respectively; CS1, chloroform fraction of *C. simensis* 15 µg/mL. VL, ethyl acetate fraction of *V. leopoldi* and VL:1 and VL:2 represent concentrations of 0.63 µg/mL and 0.87 µg/mL, respectively; Image bars = 50 µm.

3.5. Effect of Solvent Fractions on Cell Migration

In this experiment, the aqueous fraction of *S. oxyacanthum* (52 µg/mL), the chloroform fraction of *C. simensis* (80 µg/mL) and the ethyl acetate fraction of *V. leopoldi* (0.44 µg/mL) were evaluated in a wound healing assay. For *V. leopoldi* and *S. oxyacanthum* concentrations lower than the IC_{50} was selected based on the observation of high number of cell death with the treatment at IC_{50}, interfering with the bioassay. Based on the results of the wound closure defined as 0 % closure at 0 hour for each sample, JIMT-1 cells treated with *C. simensis* were stopped and unable to migrate after incubation for 24 hours where about only 27.5 % wound closure was recorded (Figure 5). In comparison with the control, this inhibition was statistically significant ($P<0.05$). The *S. oxyacanthum*-treated cells attained the closure of 30.8 % of the wound area at 48 hours and the inhibition was statistically significant ($P<0.001$). The *V. leopoldi*-treated cells attained about 55.1 % wound area closure after incubation for 72 hours. In the controls, the wound closure was around 81 % after 72 hours of incubation and thus the inhibition attained in *V. leopoldi*-treated cells was statistically significant ($P<0.001$) (Figure 5).

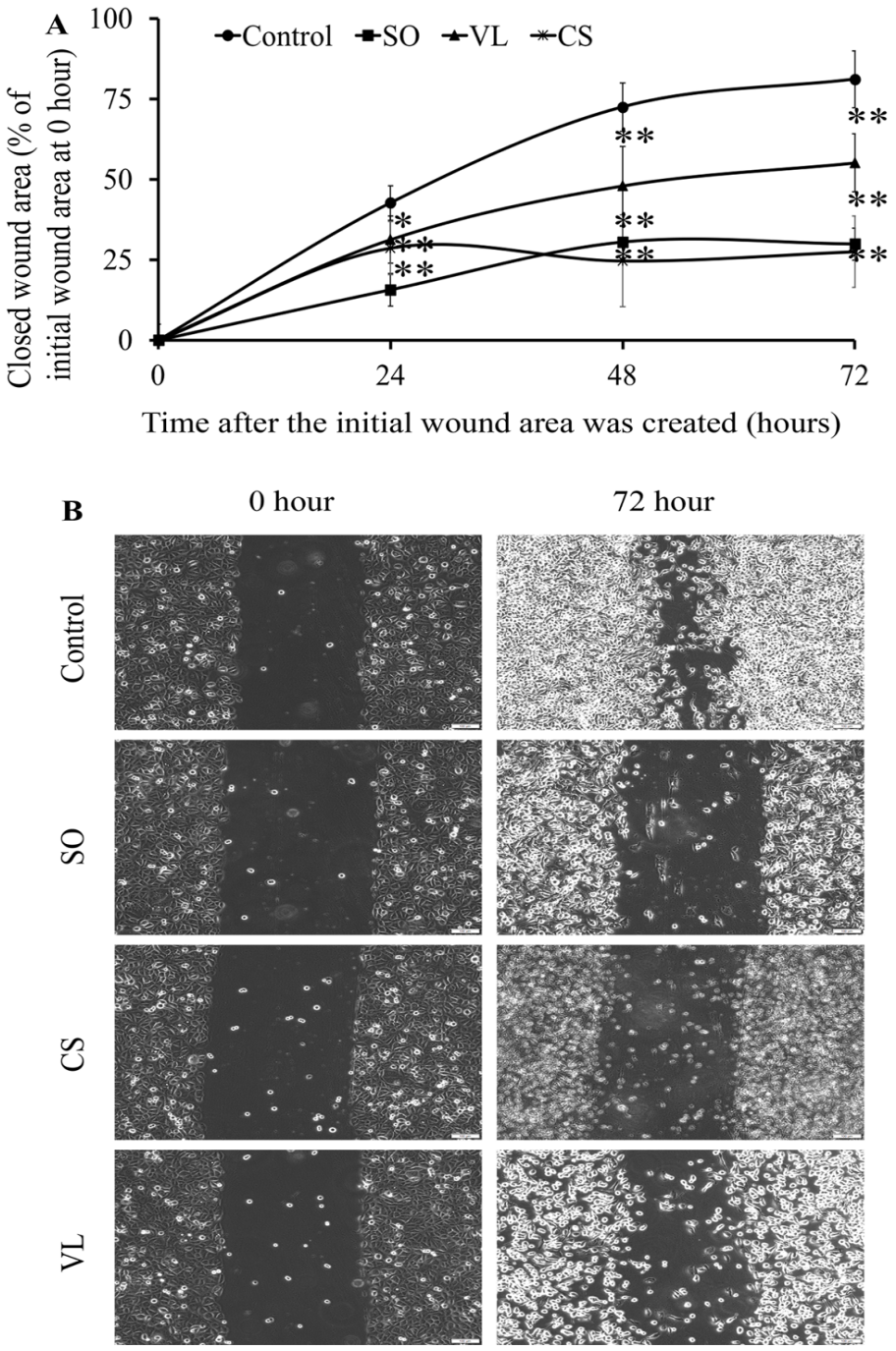


Figure 5. **The effect of solvent fractions of *S. oxyacanthum, C. simensis* and *V. leopoldi* on JIMT-1 cell migration using a scratch wound healing assay.**
SO, aqueous fraction of *S. oxyacanthum*; CS, chloroform fraction of *C. simensis*; VL, ethyl acetate fraction of *V. leopoldi*. Panel A - Wound closure (at 0, 24, 48 and 72 hours after the scratch). Panel B - Representative images taken with an inverted phase contrast microscope. **p*<0.05; ***p*<0.001; CI: 95 %; all the values were compared against the control. Image bars = 100 μm.

3.6. Inhibition of NF-κB Translocation by Treatment with the Solvent Fractions

In the experiment, a pro-inflammatory cytokine, TNF-α, was used to activate the pathway and induce translocation of NF-κB from the cytoplasm to the nucleus (Hayden and Ghosh, 2004). If NF-κB is blocked by an inhibitor, there will be no significant nuclear translocation after TNF-α treatment. In control, less than 3 % of the nuclei were positive for NF-κB and the number of positive nuclei increased to 99 % after TNF-α treatment (Figure 6). When the cells were treated with the ethyl acetate fraction of *V. leopoldi* (0.87 μg/mL) only 11 % of the nuclei were NF-κB positive, i.e. a significant reduction compared to TNF-α treatment alone ($p<0.001$). Treatment with the aqueous fraction of *S. oxyacanthum* also reduced the number of NF-κB positive nuclei but to a lower extent compared to the ethyl acetate fraction of *V. leopoldi* ($p<0.05$). Treatment with the chloroform fraction of *C. simensis* did not reduce the TNF-α-induced NF-κB translocation (Figure 6).

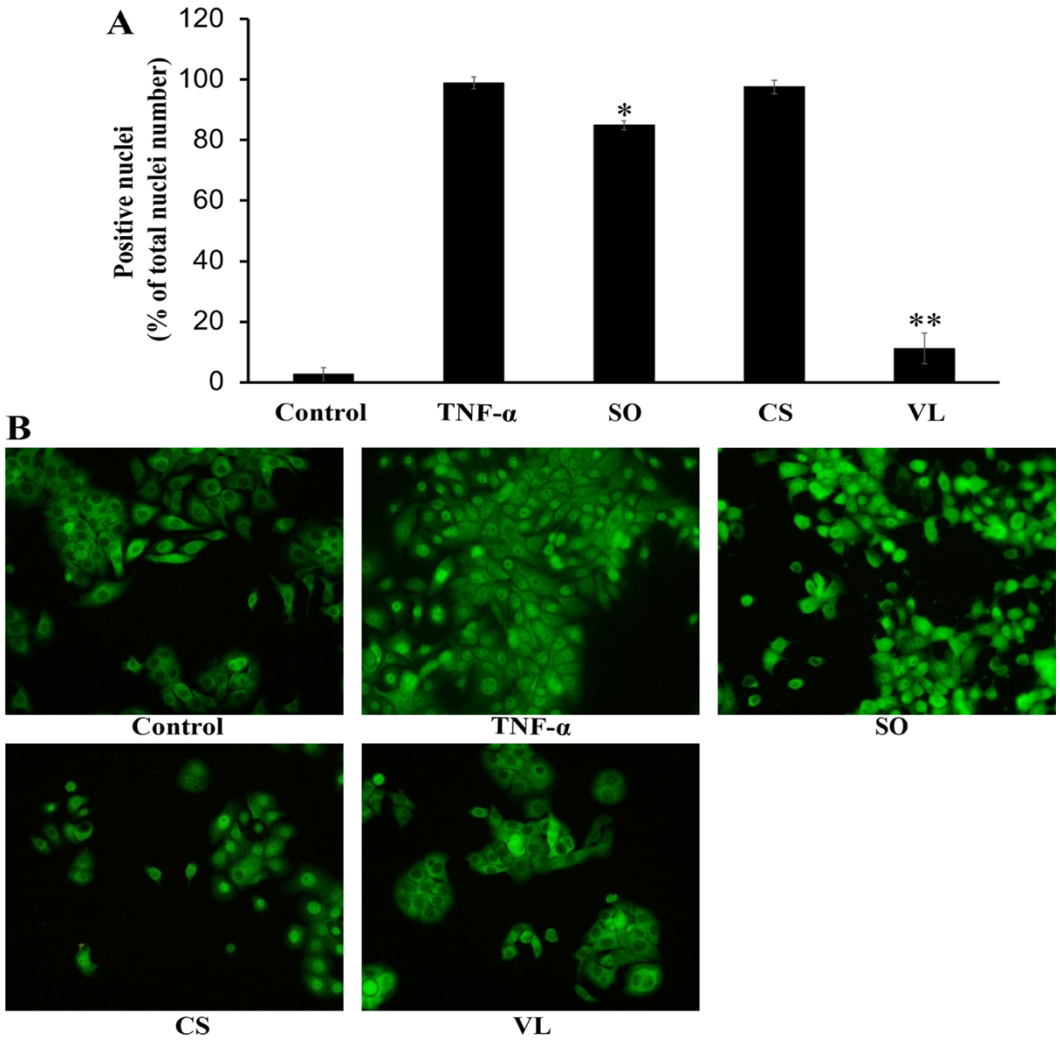


Figure 6. **Effect of the solvent fractions on TNF-α-induced nuclear translocation of NF-κB in JIMT-1 cells.**
Percent of positive nuclei in % of total nuclei number (A); Representative images from independent experiments for each treatment (B); SO, aqueous fraction of *S. oxyacanthum*; CS, chloroform fraction of *C. simensis;* VL, ethyl acetate fraction of *V. leopoldi*; All images were taken with a 40x objective. *p<0.05; **p<0.001; CI: 95 % confidence interval; all the values were compared with TNF-α treatment.

3.7. Isolated Compounds and their Biological Activities

3.7.1. Isolated compounds from *V. leopoldi*

Seven compounds were isolated from the ethyl acetate fraction of *V. leopoldi* (three flavonoids and four sesquiterpene lactones) (Table 6 and Figure 7). A compound vernomenin **(1)**, a sesquiterpene lactone, was isolated first in the successive extractions. The flavonoids apigenin **(2)** and eriodyctiol **(3)** were then isolated. Another flavonoid,

luteolin **(4)** was then obtained as a yellow precipitate and the final elution yielded the last three sesquiterpene lactone: vernolepin **(5)**, 11,13-dihydrovernodalin **(6)** and a novel compound Ndb21a2-P **(7)**. The proportion of these three compounds **(5**, **6** and **7)** in the initial ethanol extract is estimated to 1.71 %, 0.05 % and 0.02 %, respectively. Compound **(7)** was obtained as a clear oil. The ^{C}NMR and ^{H}NMR spectra are shown in the appendices section (Appendix 5 and 6). Vernomenin **(1)**, vernolepin **(5)** and 11ß,13-dihydrovernodalin **(6)** have previously been isolated from other plant groups, but were isolated from *V. leopoldi* for the first time. The relative proportion of the sesquiterpene lactones was higher than that of the flavonoids (Table 6).

Table 6. The compounds isolated from the bioactive solvent fraction of *V. leopoldi*

Compound designation	**Name of the compound**	**Chemical formula, MW**	**Yield (%)**
1	Vernomenin, SL [a]	$C_{15}H_{16}O_5$, 276.29	0.52
2	Apigenin, F [b]	$C_{15}H_{10}O_5$, 270.24	0.012
3	Eriodyctiol, F	$C_{15}H_{12}O_6$, 288.26	0.0008
4	Luteolin, F	$C_{15}H_{10}O_6$, 286.24	0.02
5	Vernolepin, SL	$C_{15}H_{16}O_5$, 276.29	1.71
6	11ß,13-dihydrovernodalin, SL	$C_{19}H_{22}O_7$, 362.38	0.05
7	Ndb21a2-P, SL [c]	$C_{20}H_{26}O_8$, 394.42	0.08

[a]SL = Sesquiterpene lactone; [b]F = Flavonoid; [c]Novel compound. The numbers in bold indicate the order of elution of the compounds.

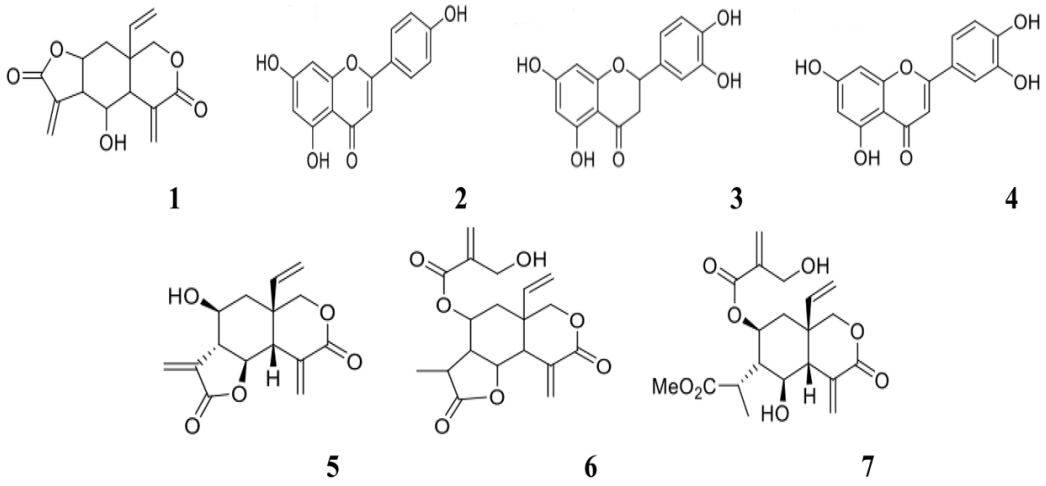
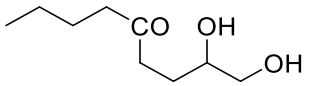
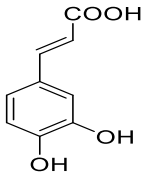
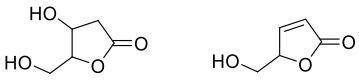
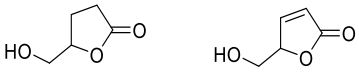
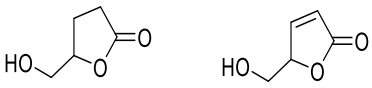
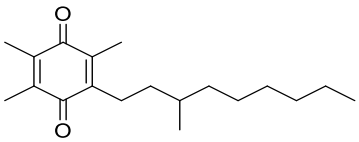
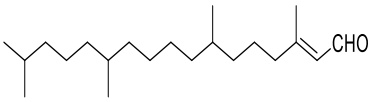


Figure 7. Molecular structures of the sesquiterpene lactones and the flavonoids isolated from the ethyl acetate fraction of *V. leopoldi*.
1 = Vernomenin; 2 = Apigenin; 3 = Eriodyctiol; 4 = Luteolin; 5 = Vernolepin; 6 = 11ß,13-dihydrovernodalin; 7 = Ndb21a2-P.

3.7.2. Isolated compounds from *C. simensis*

From the bioactive fraction of *C. simensis,* ketones, an aldehyde, a caffeic acid, many fatty acids, triglycerides and alkanes were isolated. The compounds were isolated in very small amounts (Table 7).

Table 7. Compounds isolated from the bioactive fraction of *C. simensis*

Compounds isolated from *C. simensis*			
Code[§]	**Amount**	**Structures/Description**	**Name**
ZE-45-GB4-6-7a-1B	1.4 mg		1,2-dihydroxy-nonan-5-one, $C_9H_{18}O_3$
ZE-45-H15-16	4.3 mg		3,4-dihydroxycinnamic acid, (Caffeic acid), $C_9H_8O_4$
ZE-45-IB-10-11	5.8 mg (1:1)		4-hydroxy-5-(hydroxymethyl)dihydrofuran-2(3*H*)-one, $C_5H_8O_4$ and 5-(Hydroxymethyl)-2(5H)-furanone, $C_5H_6O_3$
ZE-45-IB2-7-8-A	2.9 mg (1:1)		5-(hydroxymethyl)dihydrofuran-2(3*H*)-one, $C_5H_8O_3$ and 5-(Hydroxymethyl)-2(5H)-furanone, $C_5H_6O_3$
ZE-45-IB2-7-8-B	2.0 mg (1: 0.5)		5-(hydroxymethyl)dihydrofuran-2(3*H*)-one, $C_5H_8O_3$ and 5-(Hydroxymethyl)-2(5H)-furanone, $C_5H_6O_3$
ZE-45-Da-3-6-C-10-11	3.7 mg		2,3,5-trimethyl-6-(3-methylnonyl)cyclohexa-2,5-diene-1,4-dione, $C_{19}H_{30}O_2$
ZE-45-Da2-2	2.6 mg	Mixture of triglycerides, fatty acids and alkanes	
ZE-45-Da2-4-6	2.1 mg	Triglycerides with mixed saturated and unsaturated fatty acids	
ZE-45-Da2-20-21	1.4 mg	Mixture of saturated and unsaturated fatty acids	
ZE-45-Da-7-11aA	0.9 mg		(*E*)-3,7,12,16-tetramethylheptadec-2-enal, $C_{21}H_{40}O$

[§]The elucidation of the structures of the compounds coded ZE-45-Da-7-11aB (2.7 mg), ZE-45-Da-3-6-C-7-8 (7.6 mg) and ZE-45-H21 (1.6 mg) could not be completed whereas ZE-45-B (4.8 mg) needed further purification.

3.7.3. Cytotoxicity of the Sesquiterpene Lactones and Flavonoids Isolated from *V. leopoldi*

The basal cytotoxicity of the sesquiterpene lactones isolated from *V. leopoldi* was determined using an MTT assay. The MTT assay was conducted using the JIMT-1, MCF-7 and MCF-10A cell lines. The four sesquiterpene lactones were found to have similar cytotoxicity in the JIMT-1 and MCF-10A cell lines, while the MCF-7 cell line was less sensitive (Table 8 and Appendix 3).

Table 8. IC_{50} (µM) values of the sesquiterpene lactones isolated from the ethyl acetate fraction of *V. leopoldi*

Cell lines	Vernomenin	Vernolepin	11ß,13-Dihydrovernodalin	Ndb21a2-P
JIMT-1	2.2 ± 0.9	1.7 ± 0.3	2.2 ± 0.8	1.6 ± 0.05
MCF-7	42.9 ± 5.4	15.9 ± 0.6	4.9 ± 0.3	3.9 ± 0.7
MCF-10A	1.6 ± 0.1	2.3 ± 0.2	2.0 ± 0.5	2.2 ± 0.9

The novel compound had the lowest IC_{50} value of 1.6 µM in JIMT-1 cells. The flavonoids isolated from *V. leopoldi* were not cytotoxic up to a concentration of 75 µM (Figure 8).

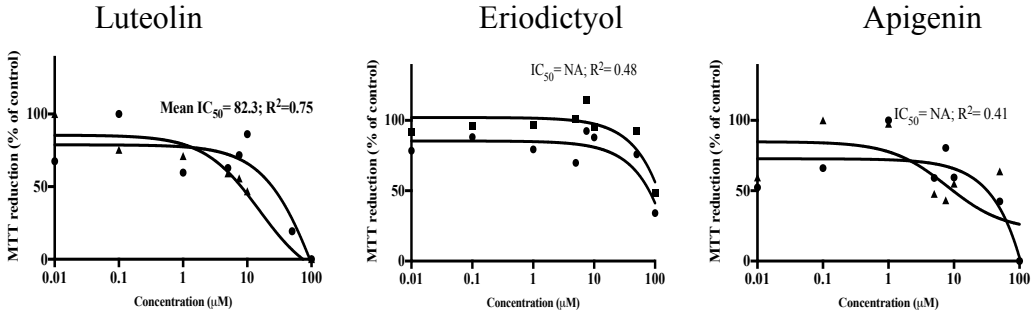


Figure 8. Dose response curves obtained after treatment of JIMT-1 cells with the flavonoids isolated from the ethyl acetate fraction of *V. leopoldi.*

3.7.4. Estimation of the CSCs Population using ALDH$^+$ as a Marker

The effect of sesquiterpene lactones isolated from *V. leopoldi* on ALDH$^+$ subpopulation of the JIMT-1 cells was evaluated following the ALDEFLUOR™ assay. Treatment with vernomenin **(1)** highly reduced the ALDH$^+$ CSC subpopulation (p<0.005). Treatment with the other three sesquiterpene lactones also resulted in a statistically significant reduction of the ALDH$^+$ CSC subpopulation (p<0.05) (Figure 9). Representative cytograms of the ALDEFLOUR assay are shown in Figure 10. Although the ALDH$^+$ CSCs subpopulation decreased after treatment with the sesquiterpene lactones, determination of cell surface markers (CD44$^+$/CD24$^-$) did not show any apparent difference with treatment (only cytograms obtained from the flow cytometry are presented) (Figure 11).

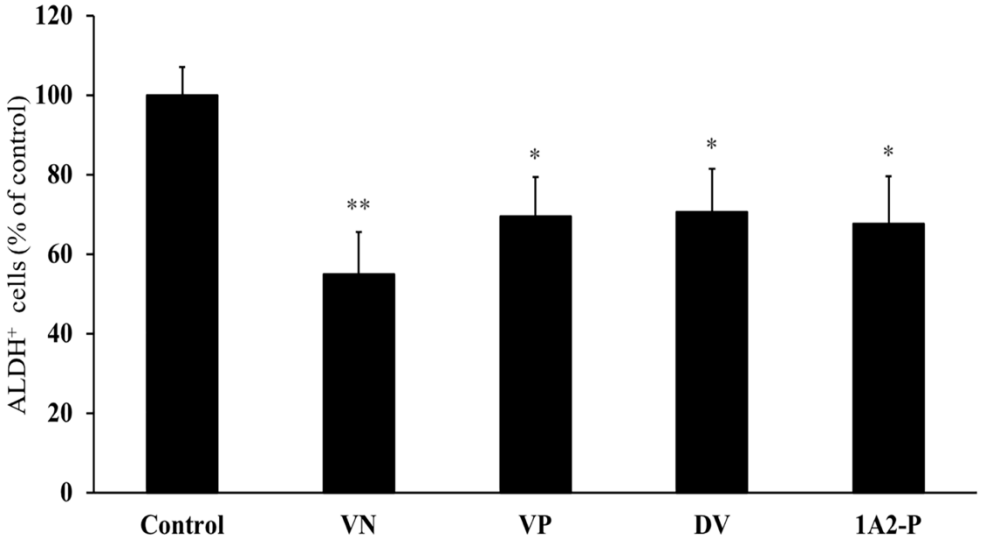


Figure 9. Reduction of ALDH$^+$ subpopulation of JIMT-1 cells following treatment with sesquiterpene lactones isolated from *V. leopoldi.*
VN, Vernomenin; VP, Vernolepin; DV, 11ß,13-dihydrovernodalin; 1a2P, Ndb21a2-P. *p<0.05; **p<0.005; CI: 95 %; all values were compared against the control.

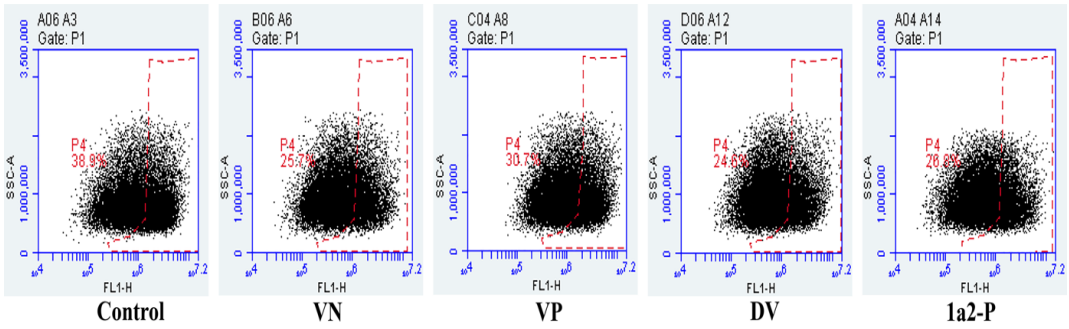


Figure 10. Representative cytograms of ALDH$^+$ CSC subpopulation of JIMT-1 cells obtained after treatment with sesquiterpene lactones isolated from *V. leopoldi*.

VN, Vernomenin; VP, Vernolepin; DV, 11ß,13-dihydrovernodalin; 1a2P, Ndb21a2-P. The percentage in each figure represents the proportion of ALDH$^+$ cells (found in the outlined red region) in relation to all cells.

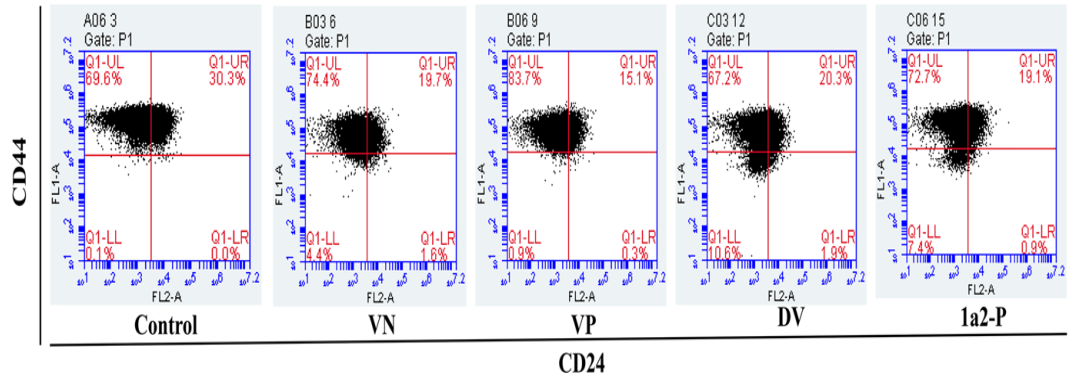


Figure 11. Representative cytograms of cell surface markers, CD44 and CD24, of JIMT-1 cells after treatment with sesquiterpene lactones isolated from *V. leopoldi* obtained using flow cytometry.

VN, Vernomenin; VP, Vernolepin; DV, 11ß,13-dihydrovernodalin; 1a2P, Ndb21a2-P.

3.7.5. Effects of the Sesquiterpene Lactones on Cell Cycle Phase Distribution

The effect of sesquiterpene lactones isolated from *V. leopoldi* on the inhibition of proliferation through the induction of cell cycle arrest was evaluated with flow cytometry. Treatment with the compounds did not show significant alteration in the cell cycle phase distribution in relation to the control after treatment with 2 μM for 72 hours (Figure 12).

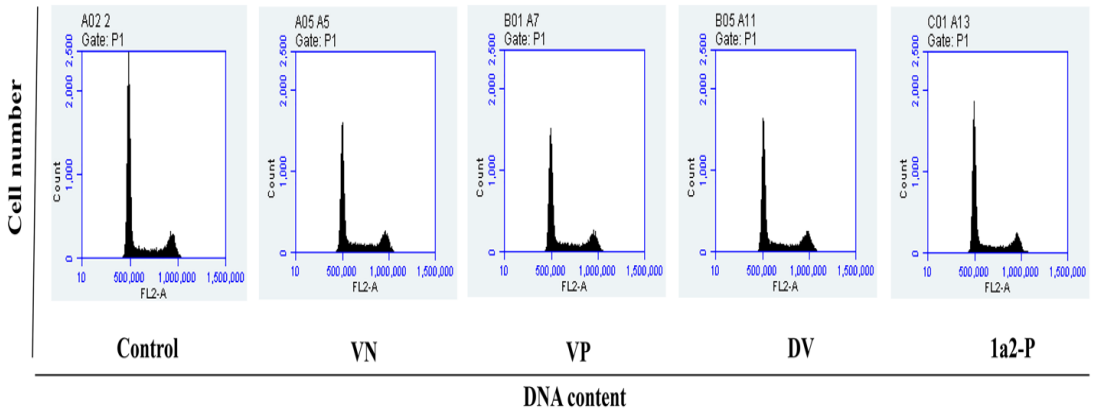


Figure 12. Flow cytometric determination of cell cycle phase distribution of JIMT-1 cells after treatment with sesquiterpene lactones isolated from *V. leopoldi*.
VN, Vernomenin; VP, Vernolepin; DV, 11ß,13-dihydrovernodalin; 1a2P, Ndb21a2-P.

3.7.6. Effect of the Sesquiterpene Lactones on Cell Migration

Based on the results of wound closure defined as 0 % closure at 0 hour for each sample, all the compounds inhibited cell migration after incubation for 72 hours ($p<0.05$). The most effective compound in inhibiting the cell migration was 11ß,13-dihydrovernodalin **(6)** (29.92%) (Figure 13).

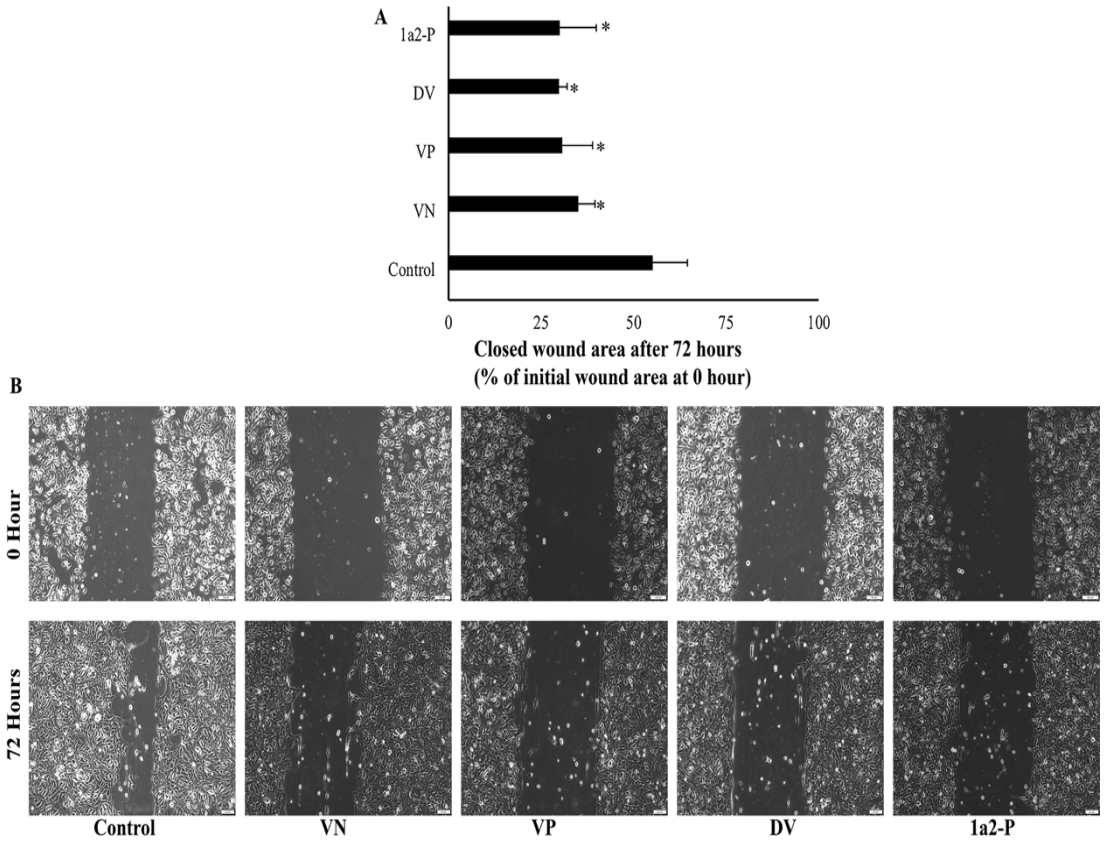


Figure 13. The effect of sesquiterpene lactones isolated from *V. leopoldi* on JIMT-1 cell migration.
VN, Vernomenin; VP, Vernolepin; DV, 11ß,13-dihydrovernodalin; 1a2P, Ndb21a2-P. Wound closure at 72 hours after the scratch (A). Representative images taken with an inverted phase contrast microscope (B). *p< 0.05; CI: 95 %; all the values were compared against the control. Image bar = 100 μm.

3.7.7. Effects of the Sesquiterpene Lactones on NF-κB Translocation

Using JIMT-1 cells, the inhibitive effect of the compounds on the TNF-α-induced NF-κB nuclear translocation was evaluated. At 10 μM concentration, 11ß,13-dihydrovernodalin **(6)** resulted in a significant reduction in the NF-κB positive nuclei compared to TNF-α treatment (%) (p<0.05) (Figure 14).

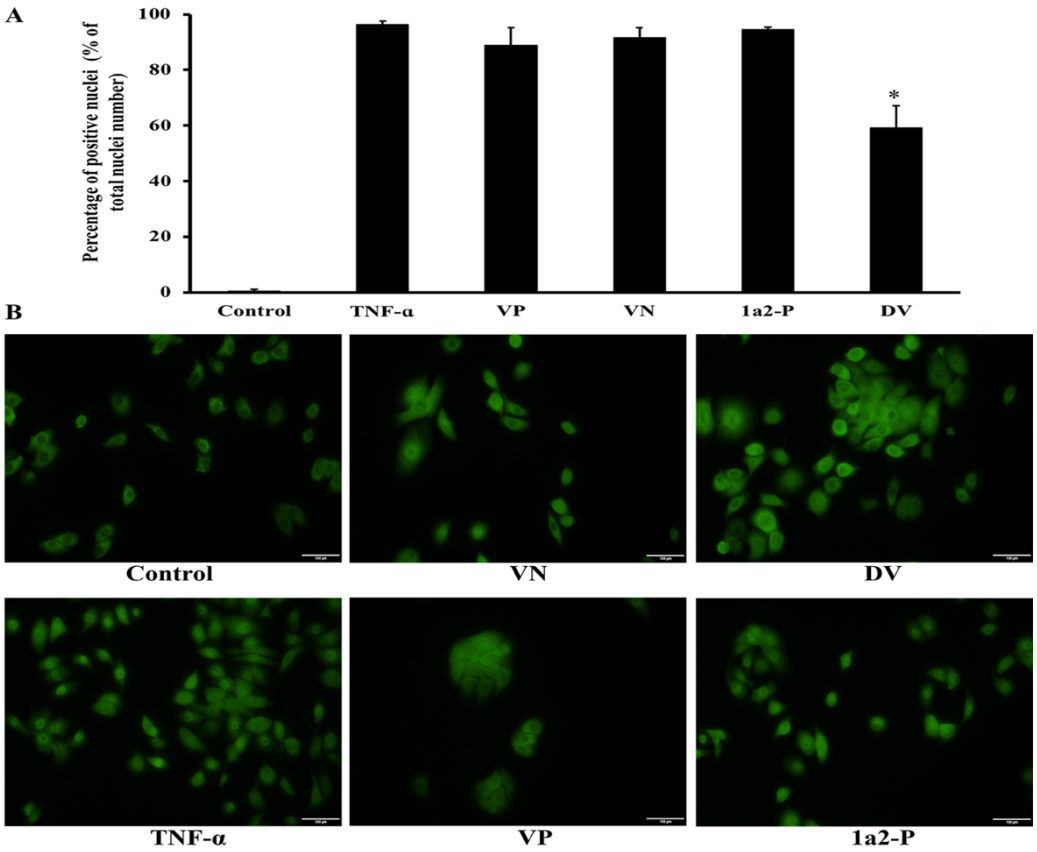


Figure 14. **The effect of treating JIMT-1 cells with sesquiterpene lactones isolated from *V. leopoldi* on TNF-α-induced translocation of NF-κB to the cell nucleus.** VN, Vernomenin; VP, Vernolepin; DV, 11ß,13-dihydrovernodalin; 1a2P, Ndb21a2-P. Note: Panel A- Positive nuclei in % of total nuclei. Panel B - Representative images from independent experiments for each treatment. The scale bars denote 100 µm. *P< 0.05; CI: 95 %; all the values were compared against TNF-α treatment.

3.7.8. Uptake and Localization of Vernolepin-NBD Conjugate

The cytotoxic activity of vernolepin-NBD conjugate ($C_{25}H_{24}N_4O_{10}$, M Wt. 540.49) was maintained in JIMT-1 cells, however, with an increased IC_{50} (=20 µM), compared to vernolepin alone (IC_{50} = 1.7 µM) (Figure 15). The uptake and localization study showed that the compound is taken up by JIMT-1 cells within 60 seconds and the intensity in the cells increases up to 2 hours after addition of vernolepin-NBD to the cell culture medium (Figure 16). The fluorescence was found in the entire cell. However, the intensity decreased after incubation with vernolepin-NBD for 24, 48 and 72 hours (Figure 16).

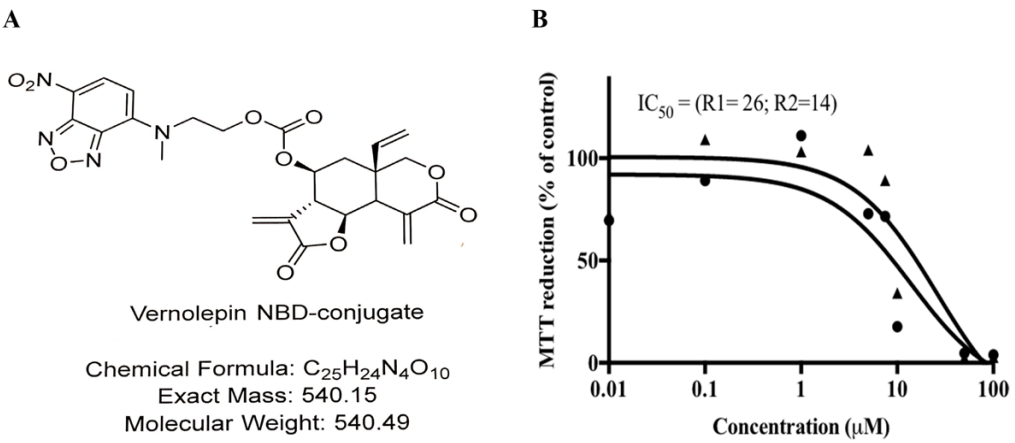


Figure 15. Vernolepin-NBD conjugate (A) and a dose response curves obtained after treatment of JIMT-1 cells with Vernolepin-NBD conjugate for 72 hours (B).

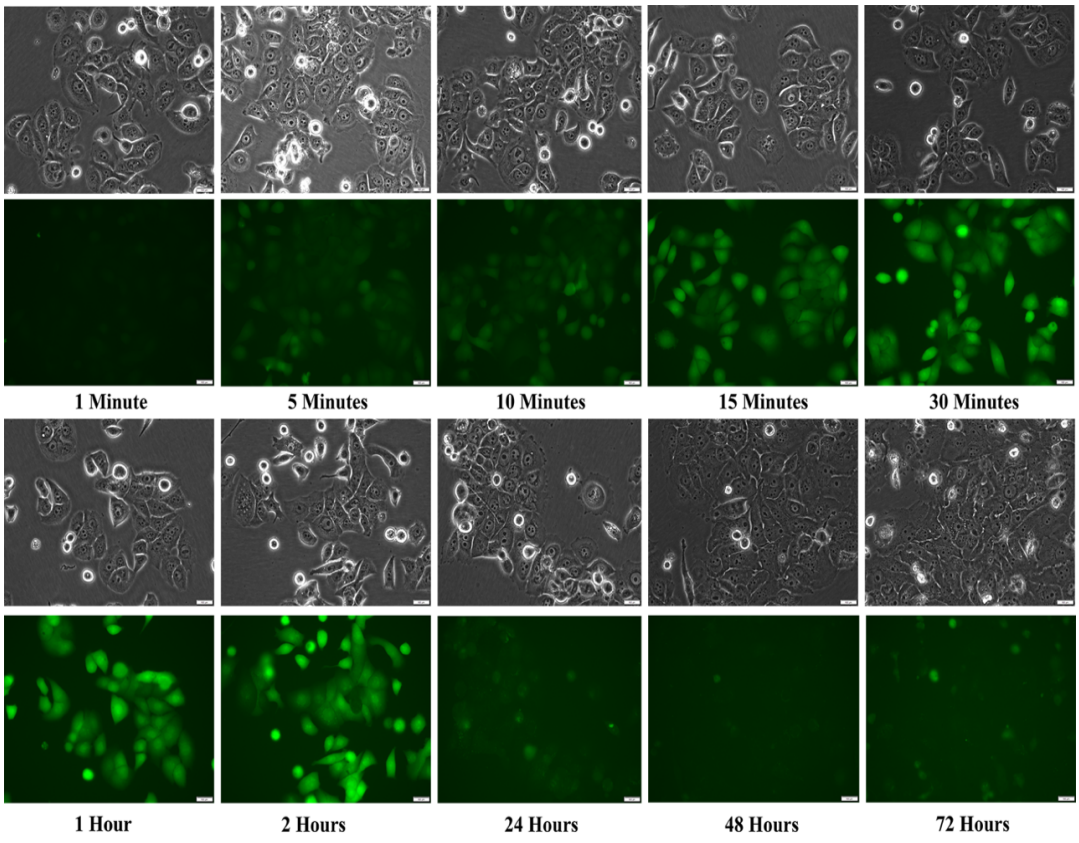


Figure 16. Uptake of vernolepin-NBD conjugate in JIMT-1 cells evaluated using phase contrast and immunofluorescence microscope.
The scale bars denote 100 μm.

4. Discussion

The present study has provided evidence for a cautious interpretation of *in situ* findings as latter dose response experimental assays had failed to confirm the information provided by some TM practitioners. For example, the plant *Z. chalybeum* is one of the frequently cited anticancer medicinal plants from different parts of Ethiopia and was a choice of several traditional healers during *in situ* investigations (Regassa, 2013; Kewessa *et al.*, 2015; Tuasha *et al.*, 2018a). However, although it is possible that its metabolites may exert toxicity in the traditional medical practice, its methanol crude extract was not cytotoxic to cancer cell lines for up to a maximum concentration of 2 μg/mL in an *in vitro* cell culture assay. On the other hand, the plant *S. oxyacanthum,* whose crude methanol extract was highly cytotoxic (IC_{50} as low as 0.09 μg/mL on MCF-7 cells), was not reported widely across the country, but was selected based upon the recommendation of TM practitioners from one locality during the *in situ* investigation (Tuasha *et al.*, 2018a). Therefore, considering the inherent secrecy of traditional medicinal knowledge among the practitioners, different factors must be taken into consideration during the selection phase of the medicinal plants for experimental investigation.

The loss of water content upon drying was measured to help estimate the amount of raw plant materials collected for future traditional medicinal uses. The traditional healers tend to use fresh plant material for the preparation of the remedy because of the perception that the freshly collected material is more efficacious than material in dried form (Teklay *et al.,* 2013; Tuasha *et al.*, 2018a). The water content can vary seasonally, from plant to plant and also across its parts. This was evident in the present study whereby as high as 61.3 % water content was recorded for the leaves of *C. myricoides*

and as low as 38.9 % for stem bark of *D. abyssinica* collected in the same season. Collection of large amounts of fresh plant materials every time, especially the root and the bark of a plant, poses threat to the medicinal plants and therefore a wise utilization would be saving the dried materials for future use (Teklay et al., 2013).

Consistent with earlier reports about various biological activities (Hao *et al.*, 2013; Kırmızıbekmez *et al.*, 2018) of the genus Clematis, the chloroform fraction of *C. simensis* was most cytotoxic (80 µg/mL on JIMT-1 cells) against all the breast cancer cell lines tested. Since the phytochemistry of its chloroform fraction was reported to be rich in flavonoids, the cytotoxic activities of the fraction may be related to these chemical constituents (Karimi *et al.*, 2017).

The significant reduction of $ALDH^+$ CSC subpopulation following treatment with the aqueous fraction of *S. oxyacanthum,* the chloroform fraction of *C. simensis*, or the ethyl acetate fraction of *V. leopoldi* is supported by the earlier report based on flow cytometry (Huang *et al.*, 2009), which showed the retention of fluorescent BODIPY-aminoacetate produced upon oxidation within the cells expressing high ALDH activity. This may imply that these plants used in TM can reduce the metastatic burden of cancer by specifically targeting the CSC subpopulation (Larzabal *et al.*, 2013).

The inhibition of colony formation in JIMT-1 cells that resulted in over 60 % reduction of CFE upon treatment with 15 µg/mL of chloroform fraction of *C. simensis* shows the inhibition of the unlimited self-renewal property and the capacity to initiate and maintain malignancy of the CSCs (Lapidot *et al.*, 1994; Relation *et al.*, 2017). The dose dependent reduction in the number of colonies and reduced sizes of the colonies in

comparison to the controls may indicate that the aqueous fraction of *S. oxyacanthum,* the chloroform fraction of *C. simensis*, or the ethyl acetate fraction of *V. leopoldi* have inhibitory effect against the features involved in maintaining the stemness of the CSCs.

In agreement with the present finding that showed the aqueous fraction of *S. oxyacanthum,* the chloroform fraction of *C. simensis*, or the ethyl acetate fraction of *V. leopoldi* had significantly affected cell migration, various studies reported plant extracts with similar effects in various cancer cell lines, including MCF-7 and MDA-MB-231 cell lines (Wu *et al.*, 2013; Chowdhury *et al.*, 2017). Since cell migration involves the transition of epithelial cells into motile mesenchymal cells (Lamouille *et al.*, 2014) and thus promotes CSC migration, contributing to the reconstitution of metastatic cancer at distant sites (Bill and Christofori, 2015; Wu *et al.*, 2016), the present finding implicates that the bioactive fractions may contribute to reducing the metastatic burden of breast cancer.

The NF-κB is important for CSC function and the significant reduction of the number of NF-κB positive nuclei of TNF-α-treated cells compared to TNF-α treatment alone by ethyl acetate fraction of *V. leopoldi* implies that the inhibitory effect of the solvent fraction on CSC may partly be exerted through an effect on NF-κB. This can be an indication that the molecular mechanism involved in CSC maintenance may have been affected.

All four sesquiterpene lactones isolated from *V. leopoldi* in the present study were highly cytotoxic when compared to the lanostane-type triterpene (lanost-3β, 23*S*-dihydroxy-22(31)-ene) isolated from the aerial part of the plant *V. leopoldii* from

Yemen in 2016 (IC_{50} values ranging from 25.7 to 58.8 μM) (Marzouk and Abd Elhalim, 2016).

According to available literature and database sources, the novel sesquiterpene lactone isolated from *V. leopoldi* and designated "Ndb21a2-P" ($C_{20}H_{26}O_8$, 394.42 g/mol) is closely related to a compound called vernodalol ($C_{20}H_{24}O_8$; 392.404 g/mol). The compound vernodalol was isolated from various plant species in the Asteraceae family including *V. amygdalina* (Ganjian *et al.*, 1983; Erasto *et al.*, 2006) and *Centratherum anthelminticum* (Wu *et al.*, 2018). Vernodalol has a double bond at C_{11}-C_{13} position whereas Ndb21a2-P **(7)** is reduced at this position. Here it is shown that Ndb21a2-P **(7)** is highly cytotoxic to breast cancer cell lines in dose response experiments (the lowest IC_{50}, 1.6 μM, JIMT-1). The IC_{50} for vernodalol was reported to be between 16.4 and 23.8 μM in NB4, GK-1a and HL-60 cell lines derived from acute promyelocytic leukemia (APL) (Wu *et al.*, 2018). The same report showed that the compound vernodalol induces dose dependent G2/M growth arrest, apoptosis, through the mitochondrial pathway and inhibits activation of the PI3K/Akt/mTOR signaling pathway (Wu *et al.*, 2018). From the structural similarity between vernodalol and Ndb21a2-P **(7)**, it can be inferred that they may have similar anticancer activities. Worth noting, however, is that much higher concentrations of vernodalol were used (25, 75 and 100 μM) for anticancer activity, compared to the concentrations used for Ndb21a2-P **(7)** (2 μM). Other reported effects of vernodalol include its use as an insect antifeedant (Ganjian *et al.*, 1983), to treat malaria (Kraft *et al.*, 2003; Pedersen *et al.*, 2009), trypanosomiasis (Kimani *et al.*, 2017) and has antioxidant activity (Erasto *et al.*, 2007).

The other known sesquiterpene lactones vernomenin **(1)**, vernolepin **(5)** and 11ß,13-dihydrovernodalin **(6)**, isolated from *V. leopoldi* in the present study, were also found to be cytotoxic in various breast cancer cell lines at low doses. Vernomenin **(1)** was isolated from *V. hymenolepis* (Kupchan *et al.,* 1968) and a range of biological activities including anti-trypanosomal (Grieco *et al.*, 1977; Kimani *et al.*, 2017) and cytotoxicity against human pharyngeal carcinoma (KB) cells (Kupchan *et al.*, 1968) were reported. It was also reported to be widely used in Chinese TM in various formulations (Lili *et al.*, 2011).

Vernolepin **(5)** was isolated from *V. hymenolepis* A. Rich (Kupchan *et al.*, 1968) and similar to the present finding, had high cytotoxicity against several cell lines - L6 cell line (Kimani *et al.*, 2017), SK-MEL, KB, BT-549, SK-OV-3, VERO and LLC-PK_1 cell lines (Ahmed *et al.*, 2018) and a broad antitumoral effects (Kupchan *et al.*, 1968; Jisaka *et al.*, 1993). Its other biological activities reported include antitrypanosomal (Kimani *et al.*, 2017), antimicrobial (Jisaka *et al.*, 1993) and platelet antiaggregating (Laekeman *et al.*, 1985). Therefore, these reports support the present finding that Vernolepin **(5)** has important biological activities and is worth considering in further in-depth investigations with possible structural analogue developments. The finding that Vernolepin **(5)** did not show significant blocking effect on the TNF-α-induced translocation of NF-κB to the nucleus up to the dose of 10 µM, indicates that there may be another mechanism of action than through NF-κB for its activity. For example, the STAT3 signaling pathway could be a possible mechanism since this pathway has also been shown to be inhibited by sesquiterpene lactones (Yeh *et al.*, 2013; Mehmood *et al.*, 2017).

The compound 11ß,13-dihydrovernodalin **(6)**, first isolated from the leaf of *V. amygdalina* (Ganjian *et al.*, 1983), is found in various plants in the family Asteraceae (Laekeman *et al.*, 1983; Kraft *et al.*, 2003; Chukwujekwu *et al.*, 2009) has a strong anti-plasmodial (Kraft *et al.*, 2003; Pedersen *et al.*, 2009), anti-trypanosomal (Kimani *et al.*, 2017) and antifeedant (Ganjian *et al.*, 1983) activities. In the present study, 11ß,13-dihydrovernodalin **(6)** was found to be highly cytotoxic in all breast cancer-derived cell lines tested, a finding which is corroborated by reports elsewhere (Ganjian *et al.*, 1983; Ahmed *et al.*, 2018). In addition to its high cytotoxicity, there was a statistically significant reduction in TNF-α-induced translocation of NF-κB to the cell nucleus after treatment. To date, the bioactivity of 11ß,13-dihydrovernodalin **(6)** in blocking the TNF-α-induced translocation of NF-κB has not been reported. However, inhibition of NF-kB activity was reported from NF-kB luciferase activity estimation with a reporter gene (*κ*B luciferase plasmid) assay (Ahmed *et al.*, 2018). The inhibition of NF-kB translocation testifies that the compound has mechanistic anti-inflammatory effects and also affects the maintenance of the CSC population (Burlec *et al.*, 2017; Ahmed *et al.*, 2018). Thus, it is considered as a promising compound for the development of new chemotherapeutic/chemopreventive agent (Chaturvedi, 2011). Therefore, the present finding suggests that 11ß,13-dihydrovernodalin **(6)** may have valuable anticancer attributes.

The significant reduction of $ALDH^+$ CSC subpopulation of JIMT-1 cells by sesquiterpene lactones isolated from *V. leopoldi* indicated their selective inhibition of CSCs. However, $CD44^+/CD24^{-/low}$ surface marker-based estimate of CSCs did not correlate with the reduction of intracellular $ALDH^+$ CSC marker. Substantiating this finding, the $ALDH1^+$ cells are more common in HER2-overexpressing and

basal/epithelial breast cancers, while $CD44^{+}/CD24^{-/low}$ phenotype is more associated with basal-like breast cancers (Korkaya *et al.*, 2008; Ricardo *et al.*, 2011). Although, validation studies of a CSC subpopulation with $CD44^{+}/CD24^{-/low}$ and $ALDH^{+}$ phenotypes together is long standing, there could be significantly low rate of overlap between the two marker groups (Al-Hajj *et al.*, 2003; Ginestier *et al.*, 2007; Liu *et al.*, 2014). Although there are reports revealing gene expression profiles associating CD44 with ALDH1, evidence exists showing that there is potential interconversion between $CD44^{+}/CD24^{-}$ and $CD44^{+}/CD24^{+}$ phenotypes due to the plasticity of CSCs, revealing distinct differentiation among same cancer subtypes (Jaggupilli and Elkord, 2012). In addition, the surface markers may also not refer to the same cell from which the CSCs originated (Shipitsin and Polyak, 2008). The reduction in the CSC subpopulation, however, can be taken as a prime reason to consider these sesquiterpene lactones for further cancer research.

The data in the present study show that the sesquiterpene lactones significantly inhibited the cell migration, which indicates that they may have a role in reducing a metastatic burden of cancer disease. Since CSCs possess more mesenchymal character than cancer cells, the inhibition observed here may signify the role of the sesquiterpene lactones to affect the CSCs subpopulation of JIMT-1 cells (Hale *et al.*, 2012; Sotillo *et al.*, 2017).

Although Vernodalol, a sesquiterpene lactone, which only differs from the novel compound Ndb21a2-P **(7)** by a double bond, inhibited cell proliferation through G_2/M growth arrest at concentrations as high as 25 μM to 100 μM (Wu *et al.*, 2018), the cell cycle phase distribution was not altered by treatment with 2 μM sesquiterpene lactones isolated from *V. leopoldi.* However, from existing knowledge that structurally similar

compounds usually show very close bioactivities, the lack of cell cycle phase alteration in the present study could be due to the low concentrations investigated in the present study (Chaturvedi, 2011).

Although the anticancer potential of the flavonoids has been widely described (Batra and Sharma, 2013; Angulo *et al.*, 2017), the flavonoids isolated from the ethyl acetate fraction of *V. leopoldi* were found to be less cytotoxic in JIMT-1 cells in comparison with the sesquiterpene lactones. However, the additive effects of flavonoids cannot be ruled out for potentiating anticancer cell effect (Nijveldt *et al.*, 2001).

The present study demonstrated, for the first time, that Nitrobenzoxadiazole (NBD) can be conjugated with the sesquiterpene lactones for uptake and intracellular localization studies. Unlike a previous report where salinomycin-NBD reporter was accumulated in specific organelles (e.g. endoplasmic reticulum) (Huang *et al.*, 2018), specific accumulation could not, however, be delineated in the present study, though preliminary observations indicated preferential accumulation. The observation that the intensity was decreasing after 24 hours may indicate the degradation or excretion of the compound by xenobiotic export pump.

One of the limitations of the present study is that only *V. leopoldi,* out of the three medicinal plants with most active solvent fractions, was fully characterized chemically and assayed with selected protocols. The chemical characterization of the active fractions of two medicinal plants, *C. simensis* and *S. oxyacanthum*, could not be completed due to a number of factors encountered during the study period. Additionally, there were large degrees of impurities and interferences with fatty acids, triglycerides and alkanes in the active fraction of *C. simensis* and this limited the

possibility of selecting compounds of interest for bioassays. Lastly, the complexity and time-consuming nature of chemical characterization in relation to the highly polar compounds in the aqueous phase of *S. oxyacanthum,* deterred the completion of this part of the study.

5. Conclusions and Recommendations

5.1. Conclusions

The following conclusions can be drawn from the present study.

- ✓ Not every medicinal plant frequently reported to be used in TM has an effect against cancer cells in subsequent bioassays.
- ✓ Out of the seven medicinal plants initially considered for the bioassays, *V. leopoldi*, *C. simensis* and *S. oxyacanthum* showed high cytotoxicity against various breast cancer cell lines and a normal-like breast epithelial cell line. Among these, *V. leopoldi* was the most cytotoxic.
- ✓ Overall, JIMT-1 cell line was more susceptible to treatment with the solvent fractions of the three cytotoxic medicinal plants; and the MCF-7 cell line was more refractory to the treatments.
- ✓ The ethyl acetate fraction of *V. leopoldi* was a prime solvent fraction for compound characterization because of its: inhibition of TNF-α-induced translocation of NF-κB to the target cell nucleus; high cytotoxicity recorded in a dose response assay; and its high anti-CSC effect.
- ✓ The bioactivity-guided fractionation of ethyl acetate fraction of *V. leopoldi* yielded seven compounds (four sesquiterpene lactones with one novel compound and three flavonoids), among which the sesquiterpene lactones were highly cytotoxic against all the cell lines used except MCF-7, which was relatively more refractory.
- ✓ All the sesquiterpene lactones significantly reduced the $ALDH^+$ CSC subpopulation of JIMT-1 cells and also resulted in significant reduction in cell migration, indicating that the compounds are targeting the CSCs.

- ✓ Among the sesquiterpene lactones isolated from *V. leopoldi*, only 11ß,13-dihydrovernodalin significantly reduced the TNF-α-induced translocation of NF-κB.
- ✓ The high cytotoxicity and CSC-specific activities of the sesquiterpene lactones make the compounds likely candidates for further in-depth anticancer treatment evaluation.

5.2. Recommendations

- ✓ The initial phase for the choice of medicinal plants for the identification of potential anticancer agents must include personal communication with TM practitioners in a community, ethnomedicinal surveys among communities rich in TM knowledge and review of the literature to maximize the likelihood of success for the experimental investigations.
- ✓ *In vitro* bioassays are demanding and cannot be easily conducted in resource-poor settings and this limits the conduct of such studies. Therefore, work must be done on simpler bench-top aseptic techniques, such as brine shrimp lethality assay and potato disc assay (crown gall tumor inhibition) to prescreen potential cytotoxic plants before an in-depth *in vitro* bioassay studies.
- ✓ More in-depth bioassays will be required to discriminate between specific anticancer cell effects from normal cell cytotoxicity of the sesquiterpene lactones. This should include structural analogues of each sesquiterpene lactone tested in the present study.
- ✓ Further studies must be carried out to clearly show the localization of vernolepin and other sesquiterpene lactones within the target cell. These could possibly include confocal microscopy imaging and differential labeling of organelles.
- ✓ Based on the data on cytotoxicity of the aqueous fraction of *S. oxyacanthum*, it is worth considering the isolation of compounds and conducting further anti-CSC studies. The same may be recommended to complete chemical characterization of the bioactive solvent fraction of *C. simensis.*

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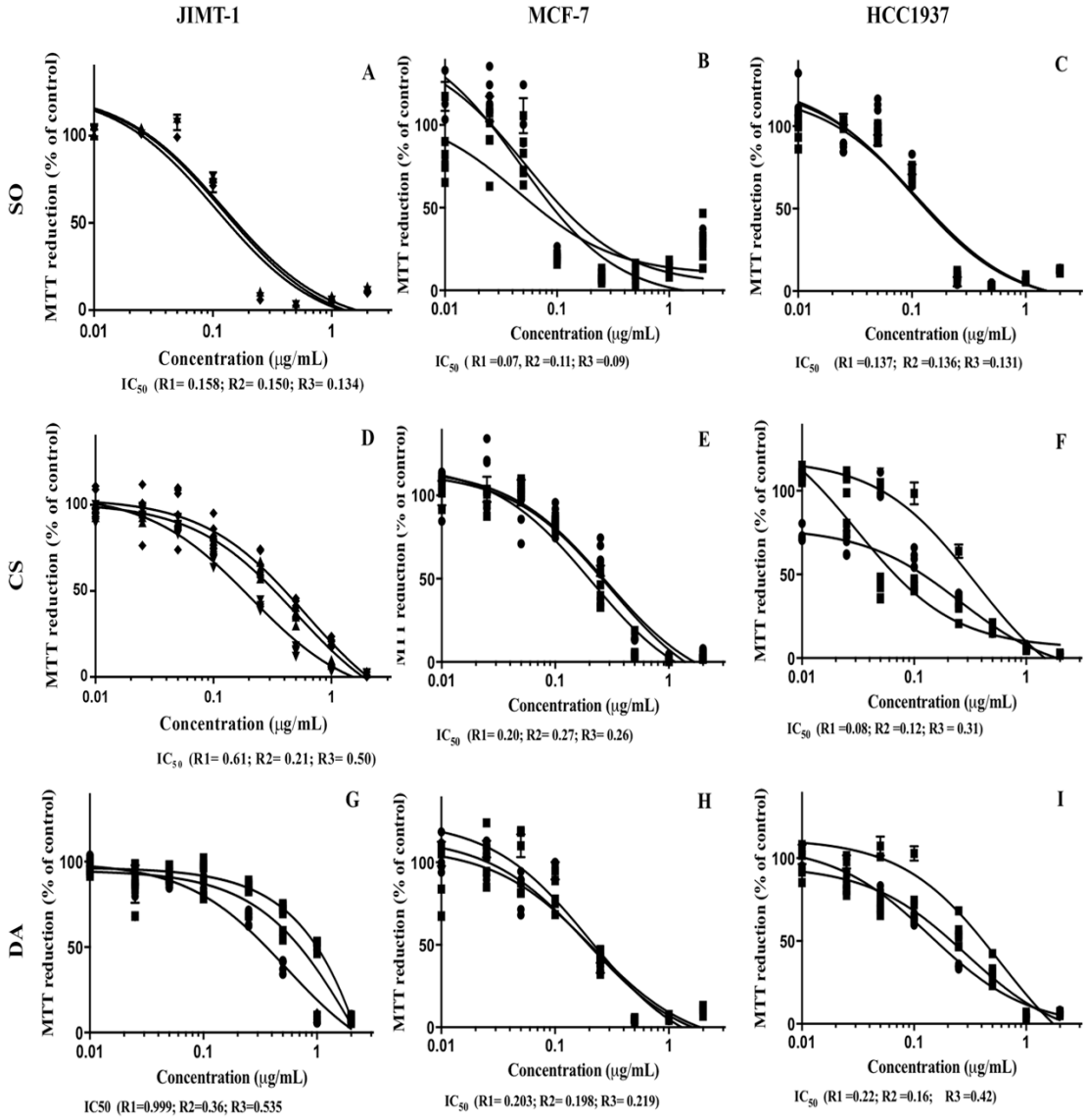
Appendices

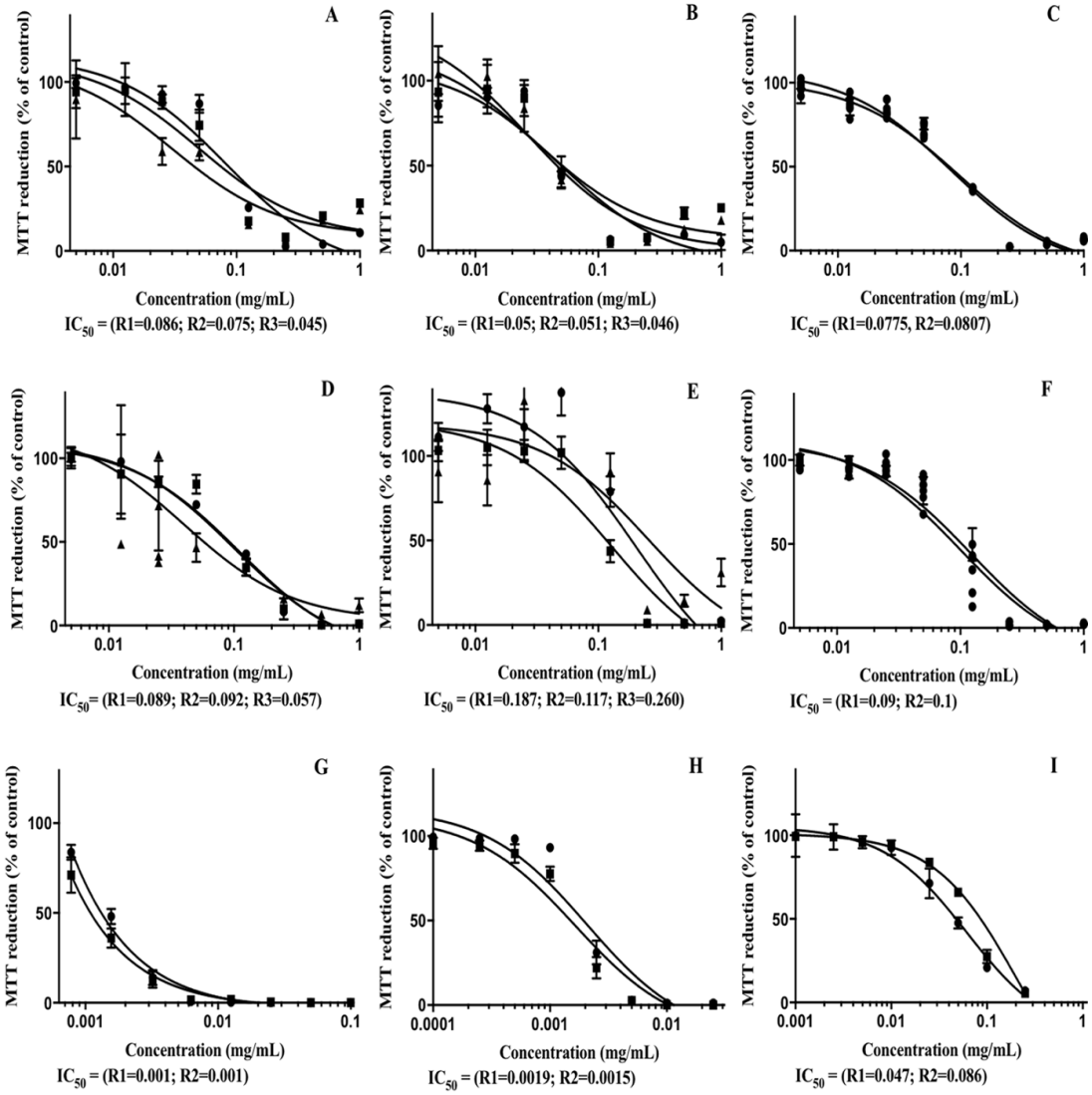
Appendix 1. Figures showing dose-response experiments with the methanolic crude extracts of *S. oxyacanthum, C. simensis* and *D. abyssinica*



Representative dose response curves obtained after treatment of the three breast cancer cell lines JIMT-1, MCF-7 and HCC1937 with methanolic crude extracts of *S. oxyacanthum, C. simensis* and *D. abyssinica.*

SO, methanolic crude extract of *S. oxyacanthum*; CS, methanolic crude extract of *C. simensis*; DA, methanolic crude extract of *D. abyssinica*. Each curve represents one experiment with n=6 wells in each point. The values in parenthesis show IC_{50} values of the different repeats (R).

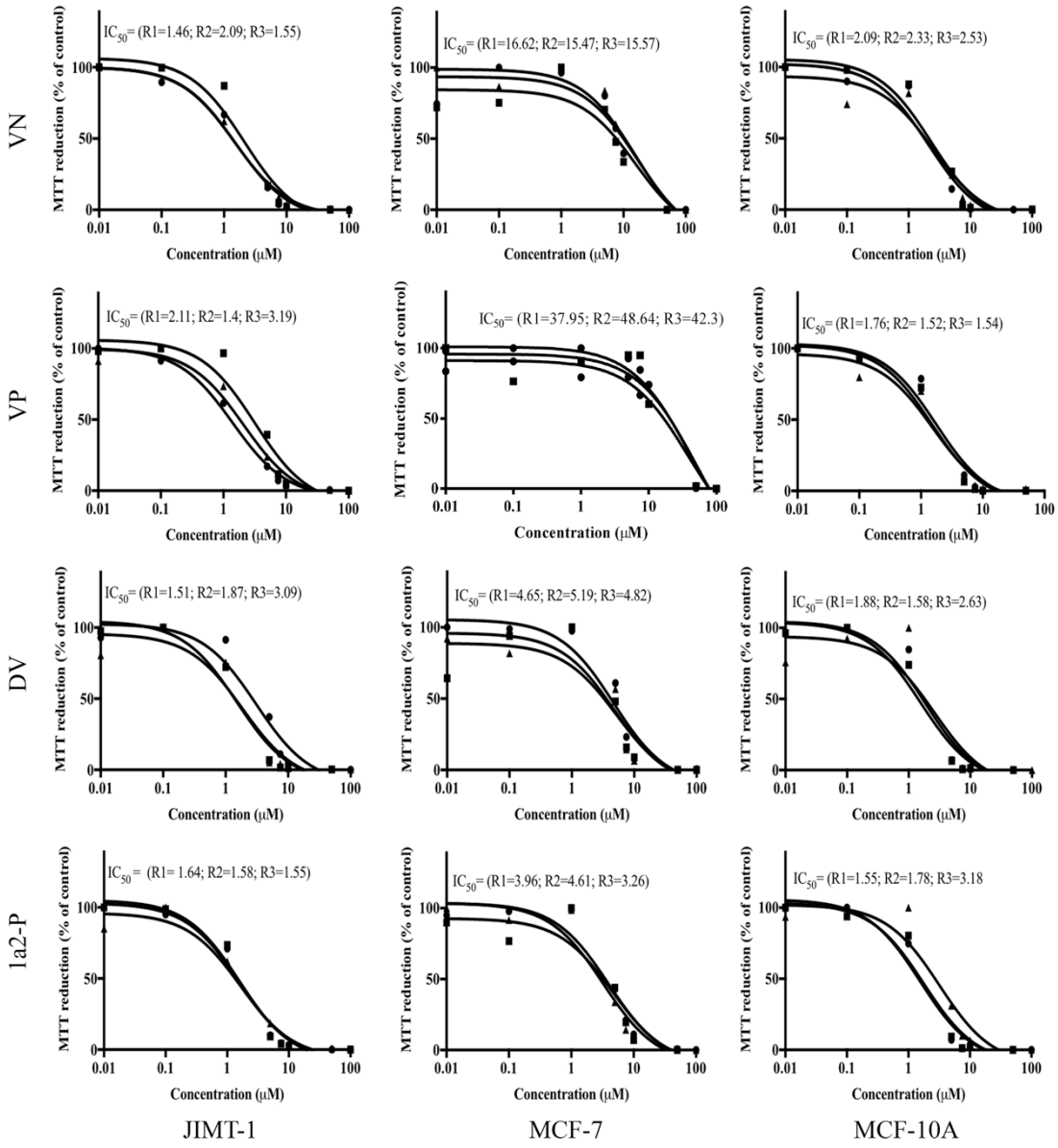
Appendix 2. Figures showing dose-response experiments with the selected bioactive solvent fractions of *S. oxyacanthum, C. simensis, C. longicauda* and *V. leopoldi*



Representative dose response curves obtained after treatment of JIMT-1 and MCF-7 breast cancer cells and normal like MCF-10A breast epithelial cells with solvent fractions of *S. oxyacanthum, C. simensis, C. longicauda* and *V. leopoldi*

S. oxyacanthum aqueous fraction treatment of JIMT-1 (A), MCF-7 (B) and MCF-10A (C) cells; *C. simensis* chloroform fraction treatment of JIMT-1 (D), MCF-7 (E) and MCF-10A (F) cells; *V. leopoldi* ethyl acetate fraction treatment of JIMT-1 (G) and MCF-10A (H) cells; *C. longicauda* ethyl acetate fraction treatment of JIMT-1 (I) cells. Each curve represents one experiment with 6 test wells in each point.

Appendix 3. Figures showing dose-response experiments with the sesquiterpene lactones isolated from the ethyl acetate fraction of *V. leopoldi*



Dose response curves obtained after treatment of JIMT-1 and MCF-7 breast cancer cells and normal like MCF-10A breast epithelial cells with the sesquiterpene lactones isolated from the ethyl acetate fraction of *V. leopoldi*

VN, Vernomenin; VP, Vernolepin; DV, 11ß,13-dihydrovernodalin; 1a2P, Ndb21a2-P.

Appendix 4. Table data for the graphs presented in the Results section

4.1. $ALDH^+$ population estimation of JIMT-1 cells by Aldefluor+ in percent (%) of control

Treatment (N)	Ratio of $ALDH^+$ population (Mean ± SD)	$ALDH^+$ % of control (Mean ± SD)
Control (N=3)	49 ± 5.21	100 ± 5.25
SO (69 µg/mL) (N=3)	10.33 ± 1.78	21.68 ± 5.87
SO (52 µg/mL) (N=3)	22.27 ± 2.21	45.07 ± 4.48
SO (28 µg/mL) (N=2)	40.95	93.45
CS (80 µg/mL) (N=3)	34.6 ± 2.16	71.48 ± 7.31
VL (0.87 µg/mL) (N=3)	33.9 ± 1.66	68.63 ± 3.37

SO, Aqueous fraction of *S. oxyacanthum;* VL, Ethyl acetate fraction of *V. leopoldi;* CS, Chloroform fraction of *C. simensis*

4.2. Effect of the solvent fractions of the Ethiopian traditional medicinal plants on the colony forming efficiency of JIMT-1 cells

Treatment (N*)	Mean ± SD (% of control)
Control	36.74 ± 6.2 (100)
SO (69 µg/mL)	7.5 ± 2.9 (20.42)
SO (52 µg/mL)	12.56 ± 1.7 (34.2)
VL (0.87 µg/mL)	7.18 ± 2.3 (19.5)
VL (0.63 µg/mL)	12.31 ± 1.8 (33.5)
CS (33.7 µg/mL)	11.11 ± 9 (27.68)
CS (15 µg/mL)	24.27 ± 1.7 (60.47)

N*=Replicates in triplicate; SO, aqueous fraction of *S. oxyacanthum*; VL, ethyl acetate fraction of *V. leopoldi*; CS, chloroform fraction of *C. simensis*

4.3. Mean values of the wound area closure by a scratch wound healing assay using JIMT-1 cells

Time of measurement	Control (% of closed wound area)	SO (% of closed wound area)	CS (% of closed wound area)	VL (% of closed wound area)
0 hour	0	0	0	0
24 hours	42.69 ± 5.23	15.67 ± 7.3	27.5±8.29	31.27 ± 11.2
48 hours	72.5 ± 7.54	30.80 ± 12.3	24.64±14.34	48.0 ± 9.23
72 hours	81.15 ± 8.79	29.91 ± 9.0	27.5±11.16	55.1 ± 10.8

The values are from N=3 independent assays with 9 wound areas for each. SO, aqueous fraction of *S. oxyacanthum;* CS, chloroform fraction of *C. simensis;* VL, ethyl acetate fraction of V. *leopoldi*

4.4. Summary of the translocation inhibition efficiency of NF- κB estimated from randomly selected immunofluorescent images assayed using JIMT-1 cells

Treatments	Rate an NF-κB not translocated	Rate an NF-κB translocated	Translocation inhibition efficiency (%)	Translocation inhibition efficiency Mean (SD)
Control	98	0	100	
	131	5	96.32	97.35 (2.3)
	90	4	95.74	
TNF-α	0	85	0	
	3	90	3.22	1.1 (1.9)
	0	111	0	
S.	14	75	15.73	
oxyacanthum	15	78	16.13	15.1 (1.5)
	11	71	13.41	
C. simensis	1	85	1.17	
	1	82	1.20	2.43 (2.2)
	3	58	4.92	
V. leopoldi	68	9	88.31	
	48	3	94.12	88.8 (5.1)
	47	9	83.93	

For each treatment three randomly selected immunofluorescent images from the left, middle and right areas of independent studies (taken at 40x using an Olympus/Nikon epifluorescence microscope) were counted to calculate the NF- κB translocation efficiency.

4.5. ALDH$^+$ population estimation of JIMT-1 cells by Aldefluor$^+$ in percent (%) of control for sesquiterpene lactones

Treatment (N=3)	Ratio of ALDH$^+$ population (Mean ± SD)	ALDH$^+$ % of control (Mean ± SD)
Control	38.5	100 ± 7.2
Vernomenin	21.1	54.9 ± 10.8
Vernolepin	26.7	69.5 ± 10
11ß,13-dihydrovernodalin	27.2	70.7± 10.8
Ndb21a2-P	26.1	67.8 ± 11.8

4.6. Mean values of the wound area closure by a scratch wound healing assay using JIMT-1 cells

Treatment	Control	VN	VP	DV	1a2-P
R1	45.77	39.51	22.39	27.91	19.96
R2	64.57	30.37	38.84	31.96	39.92
R3	55.20	35.02	30.59	29.90	29.95
Mean	55.18	34.97	30.61	29.92	29.94
SD	9.40	4.57	8.23	2.03	9.98

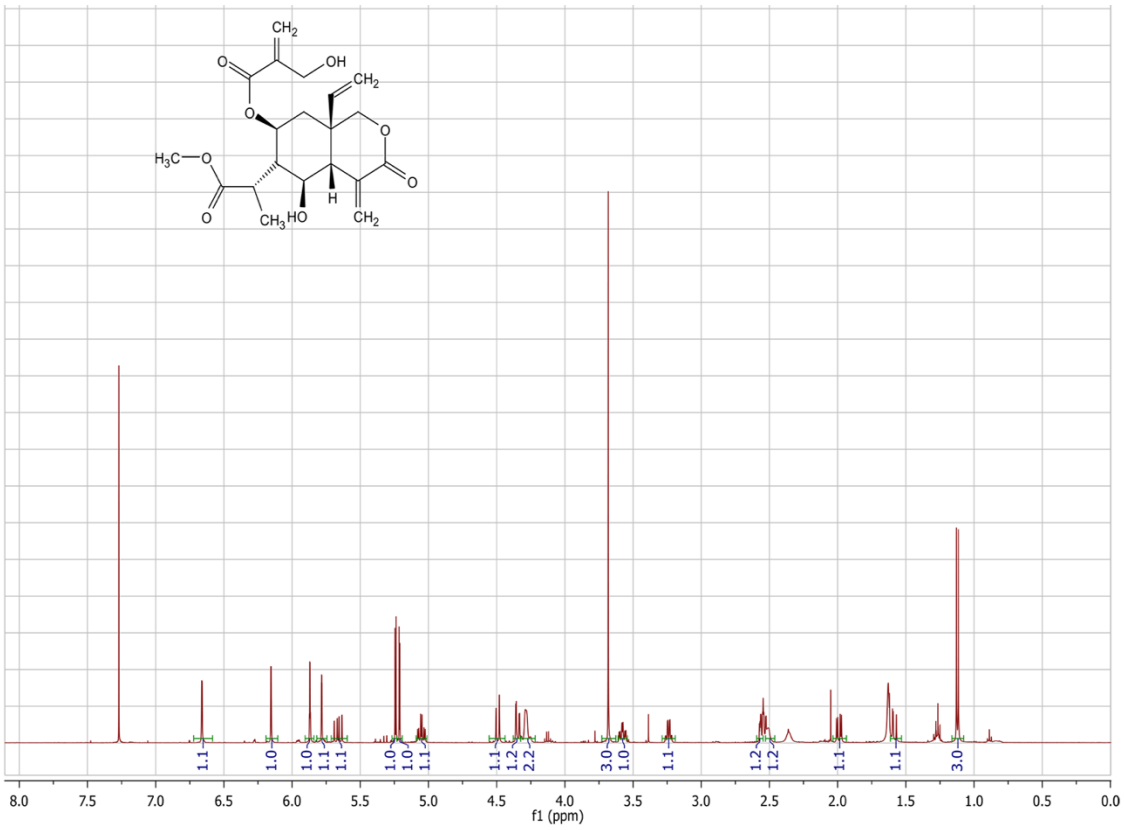
The values are from N=3 independent assays with 9 wound areas for each. VN, Vernomenin; VP, Vernolepin; DV, 11ß,13-dihydrovernodalin; and 1a2P, Ndb21a2-P

4.7. The translocation inhibition efficiency of NF- κB by the sesquiterpene lactones from *V. leopoldi*

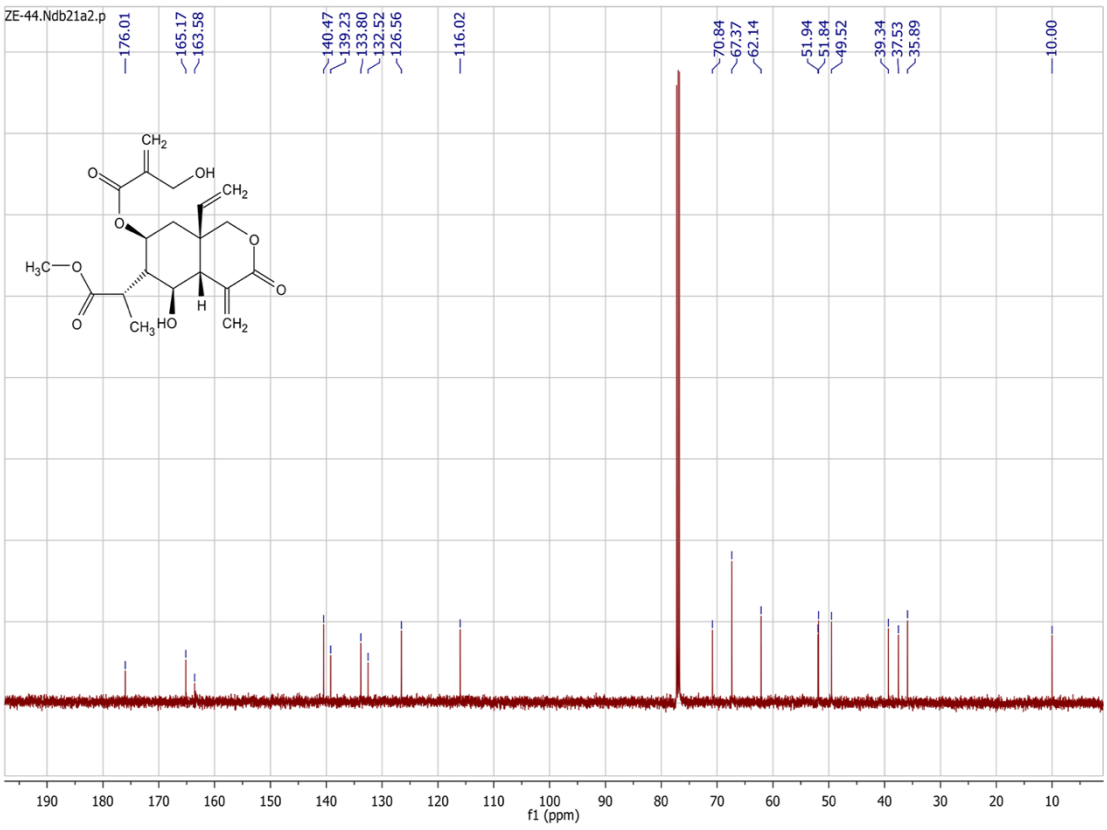
Treatments	Rate an NF-κB not translocated	Rate an NF-κB translocated	Translocation inhibition efficiency (%)	Translocation inhibition efficiency Mean (SD)
Control	76	1	98.7	
	69	0	100.0	99.6 (0.75)
	55	0	100.0	
TNF-α	2	70	2.8	
	3	91	3.2	3.7 (1.23)
	4	75	5.1	
Vernolepin	5	40	11.1	
	10	47	17.5	11.2 (6.3)
	3	58	4.92	
Vernomenin	4	39	9.3	
	6	46	11.5	8.4 (3.6)
	3	64	4.5	
Ndb21a2-P	4	85	4.5	
	5	73	6.4	5.5 (0.95)
	3	50	5.6	
11ß,13-dihydrovernodalin	34	47	42.0	
	31	33	48.4	40.9 (8.05)
	24	50	32.4	

For each treatment, 6 randomly selected immunofluorescent images of independent experiments (taken at 40x using an Olympus/Nikon epifluorescence microscope) were counted to calculate the percentage of positive nuclei.

Appendix 5. ^{H}NMR spectra of the new compound Ndb21a2-P, ($C_{20}H_{26}O_8$, M. Wt. = 394.42)



Appendix 6. ^{C}NMR spectra for the new compound Ndb21a2-P ($C_{20}H_{26}O_8$, M. Wt. = 394.42)



Declaration

I, the undersigned, declare that the dissertation is my original work, has not been presented for degrees in any other university and all sources of material used for the dissertation have been duly acknowledged.

Name: *Nigatu Tuasha Fissa (GSR/1340/06)*

Signature: _____

Date: *July 21, 2020*

Place: *College of Natural Sciences, AAU*