

*Full Length Research Paper*

# **Cytotoxicity of selected Ethiopian medicinal plants used in traditional breast cancer treatment against breast-derived cell lines**

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**Traditional medicine is widely practiced in Ethiopia. Here we investigate the toxicity of extracts of seven medicinal plants traditionally used to treat breast cancer in Ethiopia. These plants, *Sideroxylon oxyacanthum*, *Zanthoxylum chalybeum*, *Clematis simensis*, *Clematis longicauda*, *Dovyalis abyssinica*, *Vernonia leopoldi*, and *Clerodendrum myricoides*, were selected based on recommendations by traditional healers and on the frequency of use. After harvesting the plant material, the water content was determined and the powder was subjected to methanol extraction resulting in crude extracts which were tested for cytotoxicity in dose response assay. Then the methanol extract of the most toxic plants was subjected to further solvent-solvent fractionation to gain petroleum ether, hexane, chloroform, ethyl acetate, and water fractions and these were also tested for cytotoxicity in dose response assays. Extracts of *Z. chalybeum* and *C. myricoides* were not toxic. The crude extracts of *S. oxyacanthum*, *C. simensis*, and *D. abyssinica* showed cytotoxicity with half maximal inhibitory concentration 50% (IC<sub>50</sub>) below 1 µg/ml in the human breast cancer cell lines JIMT-1, MCF-7, and HCC1937. The ethyl acetate fraction of *V. leopoldi* was the most cytotoxic fraction of all fractions tested with an IC<sub>50</sub> of 0.87 µg/ml in JIMT-1 cells. The aqueous fraction of *S. oxyacanthum* and the chloroform fraction of *C. simensis* were also cytotoxic. In conclusion, our data show a wide difference in in vitro toxicity of medicinal plants used to treat breast cancer patients, which may guide the use of traditional medicine and the choice of plants for isolation of new compounds for cancer treatment.**

**Key words:** Cancer, Ethiopia, *in vitro* cytotoxicity, 3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide (MTT), traditional medicine.

## **INTRODUCTION**

Cancer is a group of diseases comprising a combination of genetic, metabolic, and signaling aberrations (Long and Ryan, 2012). It constitutes an enormous burden globally despite the progress of medicine over the years (Font-Burgada et al., 2016). As per the estimate by

GLOBOCAN, about 18.1 million new cancer cases and 9.6 million deaths occurred in 2018 (Bray et al., 2018). Lung cancer and breast cancer accounted for 2.09 million cases each and 1.76 and 0.63 million deaths, respectively (Bray et al., 2018). Overall, 57% of new

cancer cases, 65% of cancer-associated deaths, and 48% of the total number of diagnosed cancer cases occurred in the less developed regions of the world (Torre et al., 2016).

Traditional medicine (TM) has a long history of use in human ailment management systems globally and the World Health Organization promotes it as a source of less expensive and comprehensive medical care especially in developing countries (WHO, 2013). Accessibility, affordability, and acceptance by the community are core reasons for wider use of TM practices in Africa (Abdullahi, 2011). Ethiopia is a hub for different cultures and biodiversity and the medico-religious and historical accounts substantiate age-long TM usage (Kibebew, 2001).

Various sources reported that more than 70% of Ethiopians frequently use TM for their healthcare, where more than 95% of the TMs are sourced from plants (Kibebew, 2001; Teklay et al., 2013). The importance of identifying the active biological fractions and subsequently the active components in plants used in TM is substantiated by various reports and is a wide-open field of research nowadays (Fabricant and Farnsworth, 2001; Yuan et al., 2016).

The present study was carried out to assess the cytotoxicity of selected Ethiopian medicinal plants used in traditional breast cancer treatment. Using a dose response assay, we investigated the toxicity of crude and solvent-solvent fractions of selected plants in three human breast cancer cell lines and one normal-like human breast epithelial cell line.

## MATERIALS AND METHODS

### Selection of anticancer medicinal plants

Ethnobotanical reports from Ethiopia and judicious *in situ* investigations were used to select anti-cancer medicinal plants used for cancer treatment by traditional healers. Accordingly, medicinal plants widely used in different part of the country with special reference to breast cancer treatment were selected following analytical ethnobotanical tools (Tuasha et al., 2018a,b). The plant specimens were collected from Dalle district (Sidama Zone, Southern Nations, Nationalities and Peoples Regional State, southern Ethiopia) and from the Yayu forest biosphere (Illubabor Zone, Oromia Regional State, southwestern Ethiopia) in the month of June, 2015. Identification and authentication were done at the National Herbarium, Addis Ababa University, Addis Ababa, Ethiopia. The medicinal plants investigated are *Sideroxylon oxyacanthum* (Baill.) (Family: Sapotaceae), *Zanthoxylum chalybeum* Engl. (Family: Rutaceae), *Clematis simensis* Fresen. (Family: Ranunculaceae), *Clematis longicauda* Steud. ex A. Rich (Family: Ranunculaceae), *Dovyalis abyssinica* (A. Rich.) Warb. (Family: Flacourbitaceae), *Vernonia leopoldi* (Sch. Bip. ex Walp.) Vatke (Family: Asteraceae), and *Clerodendrum myricoides* (Hochst.)

(Family: Lamiaceae). The voucher specimens were deposited at the herbarium (NT014, NT012, NT037, NT072, NT017, NT073, and NT006, respectively). Except for the species *D. abyssinica* (stem bark) and the genus *Clematis* (whole aerial part), the leaf part of the medicinal plants was used. The two species, *S. oxyacanthum* and *C. longicauda* are endemic to the Ethiopian flora (Dagne, 2011), whereas, outside of Ethiopia, *V. leopoldi* is found only in Yemen (Marzouk and Abd Elhalim, 2016). Figure 1 shows images of some of the plants taken during specimen collection.

### Determination of the water content of the medicinal plants

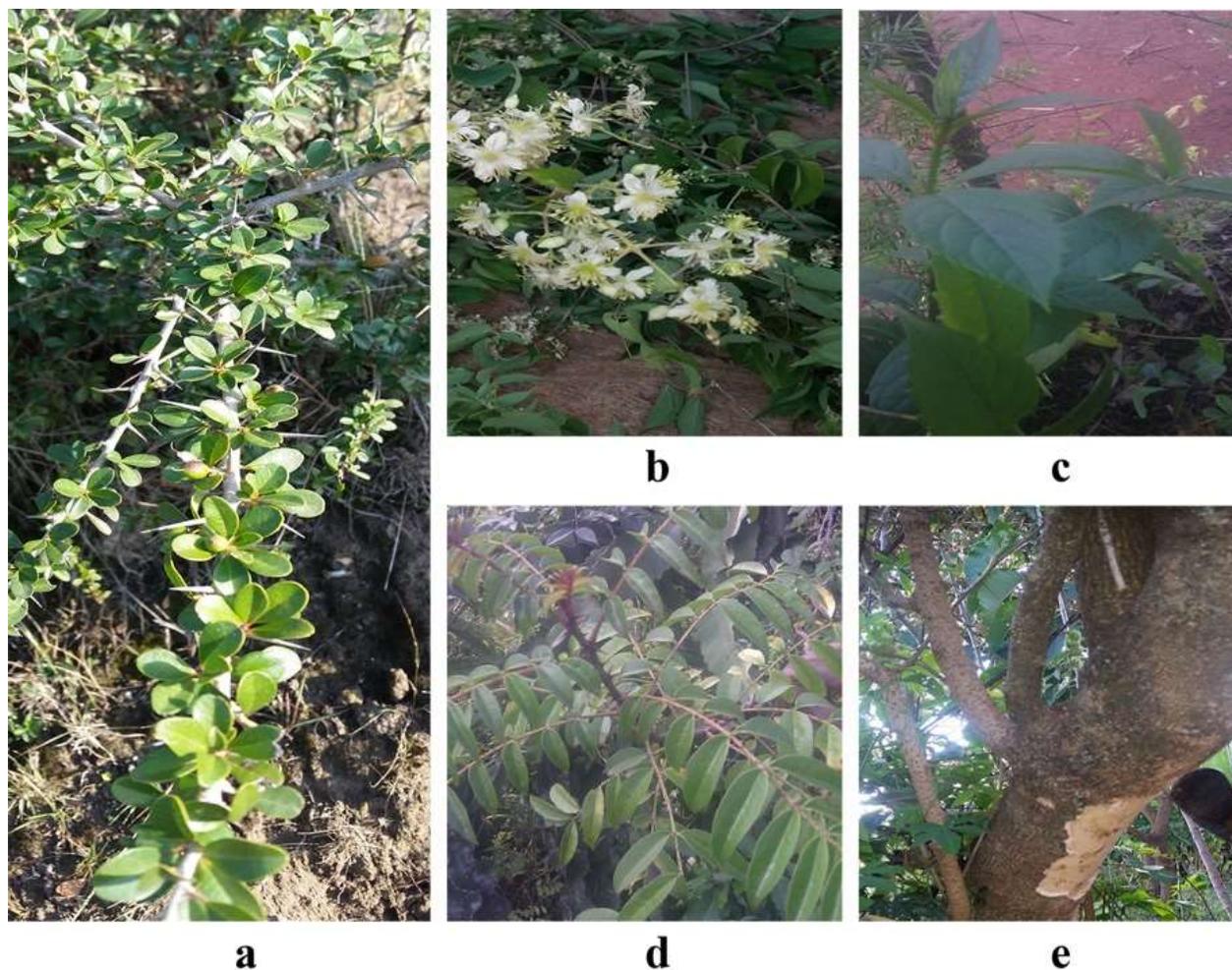
The water content of the medicinal plants was determined by estimating the weight loss up on drying in an air-ventilated open space at ambient temperature. The initial weight of the plant material was determined by weighing in the field during collection using a portable beam balance. After recording the initial weight, the plant material was thoroughly washed with tap water and rinsed with distilled water to remove adulterants and contaminants. The plant material was then dried in a shaded and ventilated open space. When dry, it was pounded and ground to finer powder. The fine powder was weighed and once again subjected to further drying. After repeated checking that a weight loss of not more than 0.25% took place between measurements, the final weight loss was determined by comparing the initial weight with the final weight. The drying process took approximately 15 days.

### Extraction and fractionation of the medicinal plants

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 (MeOH) in H<sub>2</sub>O in an Erlenmeyer flask. The suspension was macerated by shaking on a rotary water bath shaker (DZK-2, Shanghai, China) (120 routes per minute) for 72 h 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 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 MeOH extract was weighed and allowed to completely dissolve in 250 ml of 10% MeOH in Millipore H<sub>2</sub>O 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 h which resulted in an aqueous and an n-hexane layer. The n-hexane phase 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. 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 and an aqueous phase. All the fractions were

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**Figure 1.** Images of some of the medicinal plants taken during specimen collection (a - leaves of *S. oxyacanthum*, b - aerial part of *C. simensis*, c - leaves of *C. myricoides*, d – leaves of *Z. chalybeum*, and e - debarking of *D. abyssinica*).

concentrated and freeze-dried as described above.

A slightly modified partitioning procedure was followed to obtain fractions of *C. longicauda* and *V. leopoldi*. Briefly, 100 g of fine powder of each plant was dissolved in 500 ml of 90% MeOH in Millipore H<sub>2</sub>O and then the solution was subjected to maceration in a shaking water bath (DZK-2, Shanghai, China) for 12 h at ambient temperature. Filtration and concentration of the crude extract was performed as described above. The dried crude extract was weighed and fractionated with petroleum ether and then by ethyl acetate to give 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).

#### Cell lines and culturing 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 cytotoxicity experiments. The JIMT-1 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 human ductal breast carcinoma cell line HCC1937, was sourced from a 24 years old patient (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 DMEM/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, 1 mM non-essential amino acids, 10 µg/ml insulin, 1 mM L-glutamine, and 100 U/ml penicillin/100 µg/ml streptomycin. The HCC1937 cells were

**Table 1.** Estimation of water content of the medicinal plants.

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

cultured in RPMI 1640 medium (VWR) supplemented with 10% heat-inactivated FBS, 1 mM non-essential amino acids, 10 µg/ml insulin, 20 ng/ml epidermal growth factor (Sigma-Aldrich), and 100 U/ml penicillin/100 µg/ml streptomycin. The MCF-10A cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1 mM non-essential amino acids, 10 µg/ml insulin, 20 ng/ml epidermal growth factor, 50 ng/ml cholera toxin (Sigma-Aldrich), 250 ng/ml hydrocortisone (Sigma-Aldrich), and 100 U/ml penicillin/100 µg/ml streptomycin. All cell lines were kept at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The cells were seeded at different densities. Accordingly, JIMT-1 cells were seeded at 1.5×10<sup>4</sup> cells/cm<sup>2</sup>, both MCF-7 and HCC1937 at 2×10<sup>4</sup> cells/cm<sup>2</sup>, and MCF-10A cells were seeded at 10<sup>4</sup> cells/cm<sup>2</sup>. Tissue culture vessels of the appropriate size were used with the volume of medium about 0.2-0.3 ml per cm<sup>2</sup>.

#### MTT dose response assay

The yellow soluble tetrazolium salt 3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) is reduced by metabolically active cells to insoluble formazan crystals (Mosmann, 1983) and this *in vitro* assay is a widely accepted model in a 96-well format to obtain dose response curves and half maximal inhibitory concentration 50% (IC<sub>50</sub>). Briefly, confluent cells were detached by trypsinization 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 h before addition of the extracts/fractions.

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% MeOH and were allowed to dissolve completely. Then, the highest used concentration of 20 µg/ml with 4% MeOH in PBS (crude extracts) and 5 mg/ml with 2% MeOH in PBS (fractionated extracts) were prepared and sterile-filtered using 0.22 µm filters followed by serial dilutions to the lowest concentration of 0.01 µg/ml (crude extracts) and 0.001 mg/ml (fractionated extracts). Then, 20 µl of the serially-diluted methanolic 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% MeOH in PBS (crude extracts) or 2% MeOH in PBS (fractionated extracts). Thus, the final MeOH concentration in all wells was 0.4% (crude extracts) or 0.2% (fractionated extracts). The plates were then incubated for 72 h before MTT addition.

MTT (Sigma-Aldrich) was dissolved in PBS to a concentration of 5 mg/ml in PBS, the solution was sterile-filtered, and stored at -20°C wrapped with aluminum foil to protect from light exposure. After 72 h 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<sub>2</sub> incubator for 1 h. 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<sub>50</sub> 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, and the mean IC<sub>50</sub> ± SD was calculated.

## RESULTS

### Water content and estimation of extract yield

Based on the weight measurements before and after drying, we determined the weight loss and obtained a measure of water content to get an estimate of how much plant material would be needed for future use (Table 1). The water content was highest in the leaves of *C. myricoides* (61.3%) and lowest in the stem bark of *D. abyssinica* (38.9%).

In Table 2, we present the percent methanolic crude extract and solvent-solvent fraction yield for the most cytotoxic anticancer medicinal plants. Accordingly, high yield was obtained from solvent with high polarity (that is, aqueous phase) for *S. oxyacanthum* and *C. simensis*. Similarly, proportionally high yield was obtained from the more polar solvent for the medicinal plant *V. leopoldi* (Table 2).

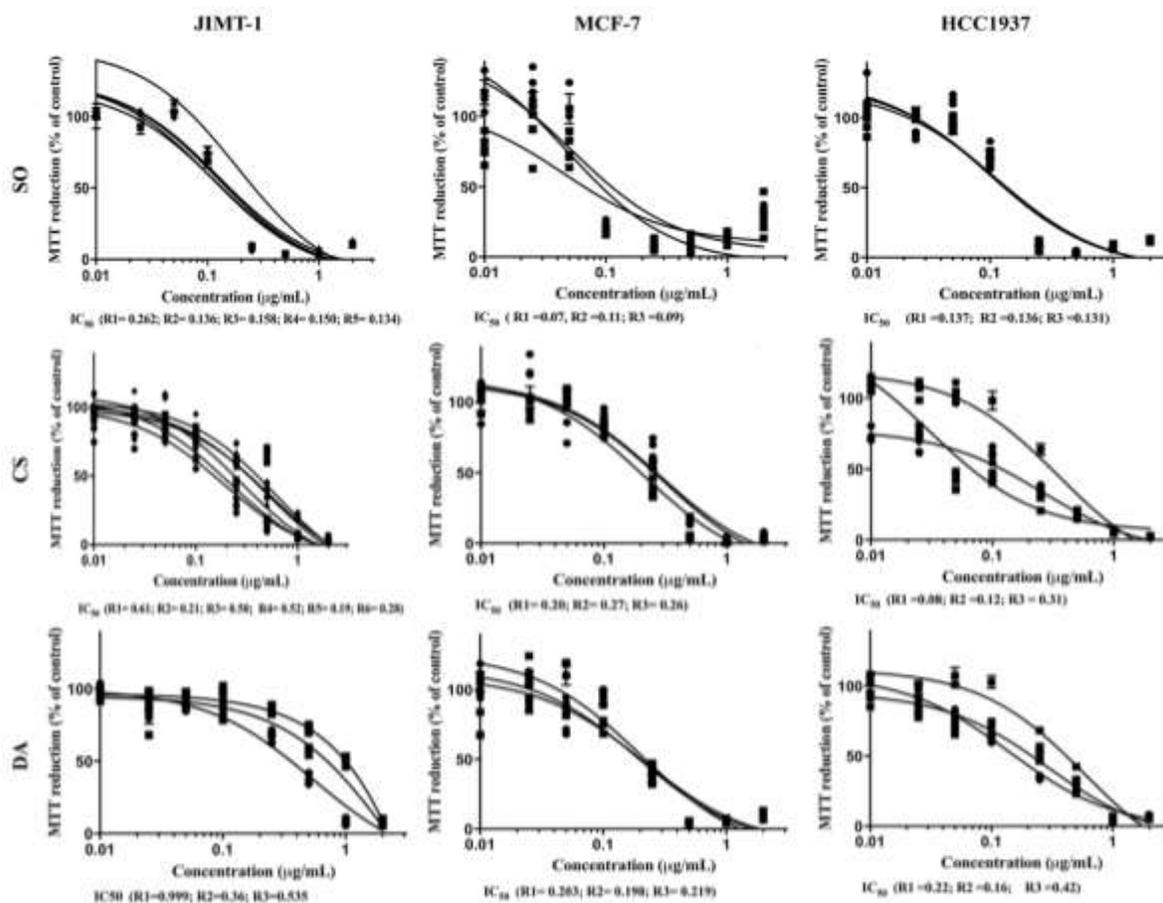
### Traditionally used anticancer medicinal plants show dose dependent cytotoxicity

We initiated our study by investigating the toxicity of crude methanolic extracts to identify plants for further analysis. An MTT assay was used to determine the overall basal toxicity profiles of the crude MeOH extracts in the breast-derived cell lines. Thus, IC<sub>50</sub> values were obtained after treating the cell lines for 72 h with different methanolic crude extracts (Figure 2 and Table 3). The crude extract of *S. oxyacanthum* was found to be the

**Table 2.** Percent yield of the crude and solvent fractions of the three most cytotoxic medicinal plants.

The extract/solvent fraction	Net yield (%) <sup>‡</sup>
<i>S. oxyacanthum</i> (MeOH crude)	20.6
hexane	2.3
chloroform	5.1
ethyl acetate	10.1
aqueous	67.9
<i>C. simensis</i> (MeOH crude)	19.2
hexane	11.7
chloroform	17.0
ethyl acetate	3.2
aqueous	26.0
<i>V. leopoldi</i> (MeOH crude)	29.4
petroleum ether	23.5
ethyl acetate	46.3

<sup>‡</sup> The crude yield was calculated from the initial dried plant material used for extraction as described in Methods section (i.e., 500 g for *S. oxyacanthum* and *C. simensis*; and 100 g for *V. leopoldi*).



**Figure 2.** Dose response curves obtained after treatment of the three breast cancer cell lines JIMT-1, MCF-7, and HCC1937 with methanolic extracts of Ethiopian medicinal plants. SO, Methanolic crude extract of *S. oxyacanthum*; CS, methanolic crude extract of *C. simensis*; DA, methanolic crude extract of *D. abyssinica*. Note: Each curve represents one experiment with n=6 wells in each point. The values in parenthesis show IC<sub>50</sub> values in the different replicates (R). The cells were treated for 72 hours before evaluation using an MTT assay.

**Table 3.** The IC<sub>50</sub> (µg/ml) values obtained using an MTT assay after treating breast cancer cell lines with crude MeOH extracts of selected Ethiopian medicinal plants used in traditional breast cancer treatment.

Crude MeOH extract	Cell line		
	JIMT-1	MCF-7	HCC1937
<i>S. oxyacanthum</i>	0.17 ± 0.05 <sup>a</sup>	0.09 ± 0.02	0.13 ± 0.003
<i>C. simensis</i>	0.27 ± 0.01	0.24 ± 0.04	0.17 ± 0.12
<i>D. abyssinica</i>	0.63 ± 0.33	0.21 ± 0.01	0.27 ± 0.14
<i>C. myricoides</i>	NA <sup>b</sup>	0.74 <sup>c</sup>	NA <sup>d</sup>
<i>Z. chalybeum</i>	NA <sup>b</sup>	NA <sup>b</sup>	NA <sup>d</sup>

<sup>a</sup>The number of 96-well assays run for each plant extract is found in Figure 1 as each assay generates one curve. Unless specified, replicates are ≥ 3 96-well plate independent assays with n = 6 wells in each assay. The data are presented as mean ± SD. <sup>b</sup>NA = not applicable as no IC<sub>50</sub> was found and no toxicity at the highest concentration of 2 µg/ml. <sup>c</sup>Mean of two replicates. <sup>d</sup>The MTT test was not carried out in this cell line since no toxicity was found in the other two.

**Table 4.** The IC<sub>50</sub> (µg/ml) values obtained using an MTT assay after treating two breast cancer cell lines (JIMT-1 and MCF-7) and one normal-like breast epithelial cell line (MCF-10A) with solvent-solvent fractions of Ethiopian traditional medicinal plants.

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

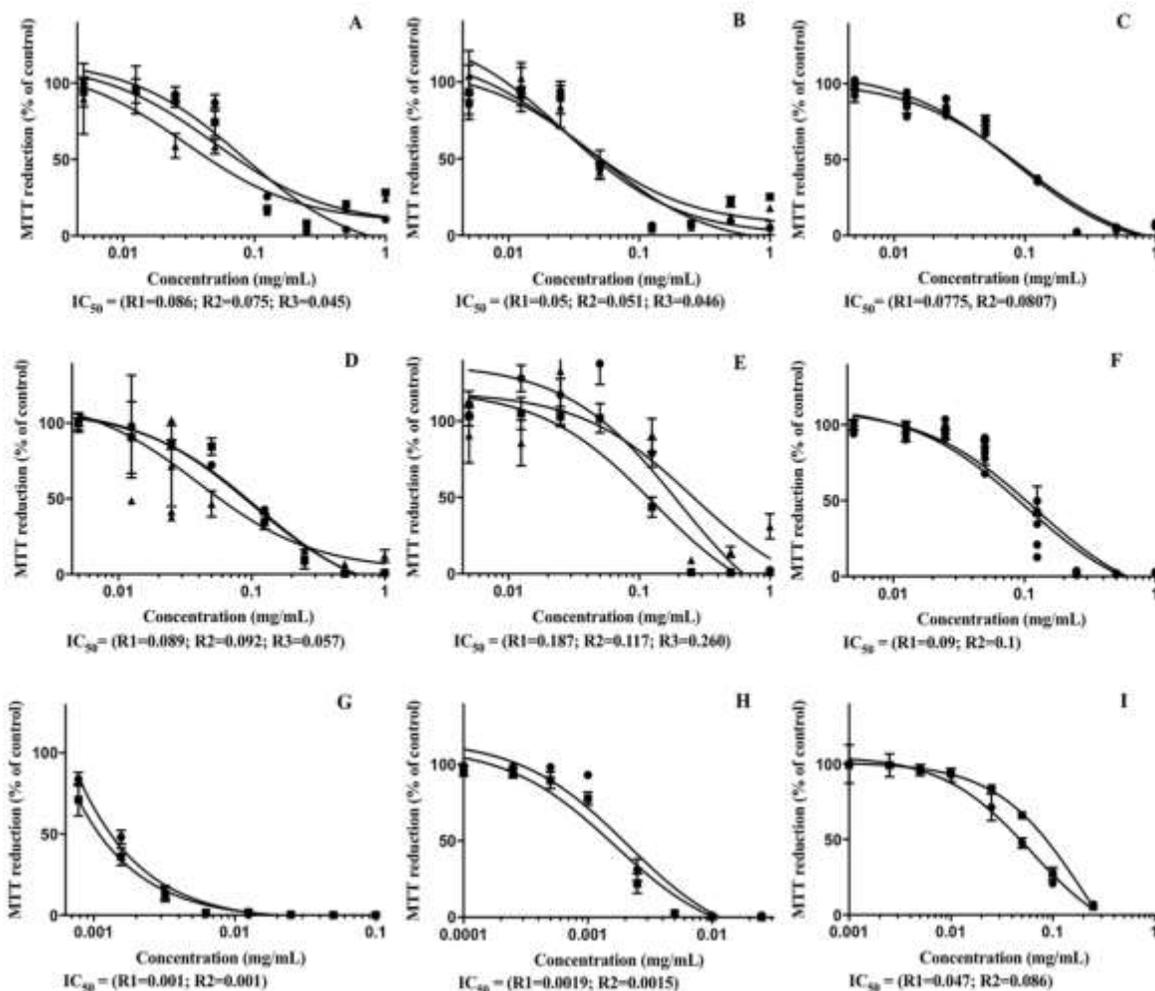
<sup>a</sup>The number of 96-well assays run for each fraction is found in Figure 2 as each assay generates one curve. Unless specified, replicates are ≥ 3 96-well plate independent assays with n = 6 wells in each assay. The data are presented as mean ± SD. <sup>b</sup>NA = not applicable as no IC<sub>50</sub> was found and no toxicity at the highest concentration of 1 mg/mL. <sup>c</sup>The MTT test was not carried out in this cell line since no desirable level of toxicity was found in the other two. <sup>d</sup>Mean of two replicates.

most cytotoxic against all the cell lines used where the lowest concentration of 0.09 µg/ml was recorded in the MCF-7 cell line. MeOH extracts of *C. simensis* and *D. abyssinica* were also highly cytotoxic. Extracts from *Z. chalybeum* and *C. myricoides* (except against MCF-7; 0.74 µg/ml, N=2) did not give IC<sub>50</sub> values at the maximum concentration of 2 µg/ml (Table 3).

After the IC<sub>50</sub> values of the crude extracts were determined, *S. oxyacanthum* and *C. simensis* were selected for solvent-solvent fractionation for further testing. Based on reports of wider use among the herbal medicine practitioners, the plants, *V. leopoldi* and *C. longicauda* were included for the solvent-solvent fractionation. For this further study, we decided to include the normal-like MCF-10A and omitted the HCC1937 breast cancer cell line since the MCF-7 and JIMT-1 cells

resulted in quite similar IC<sub>50</sub> in the crude extract testing (Table 3).

Table 4 shows the obtained IC<sub>50</sub> values and Figure 3 the dose response curves of fractions that resulted in the lowest IC<sub>50</sub> values. Table 4 shows that not all fractions were toxic and that specific fractions were toxic in the respective plants. Also, it is clear that the fractions are less toxic than the crude MeOH extract. Nevertheless, the aqueous fraction of *S. oxyacanthum*, the chloroform fraction of *C. simensis*, and the ethyl acetate fraction of *V. leopoldi* were found to be toxic at low concentrations. The ethyl acetate fraction of *V. leopoldi* was the most cytotoxic fraction with an IC<sub>50</sub> value at the concentration of 0.87 µg/ml in JIMT-1 breast cancer cell (Table 4). Notably, the IC<sub>50</sub> values of MCF-10A cells were higher than those of the JIMT-1 cells, which is not as clear when



**Figure 3.** Dose response curves obtained after treatment of the breast cancer cell lines JIMT-1 and MCF-7 and the normal-like breast epithelial cell line MCF-10A with solvent-solvent fractions of Ethiopian traditional medicinal plants. The images show the following dose response curves: the aqueous fraction of *S. oxyacanthum* of JIMT-1 (A), MCF-7 (B), and MCF-10A (C) cell lines; the chloroform fraction of *C. simensis* of JIMT-1 (D), MCF-7 (E), and MCF-10A (F) cell lines; the ethyl acetate fraction of *V. leopoldi* of JIMT-1 (G) and MCF-10A (H); and the ethyl acetate fraction of *C. longicauda* of the JIMT-1 cell line (I). Note: Each curve represents one experiment with  $n=6$  wells in each point. The x-axis is mg/mL. The values in parenthesis show IC<sub>50</sub> values in the different repeats (R). The cells were treated for 72 hours before evaluation using an MTT assay.

comparing the IC<sub>50</sub> values of MCF-10A and MCF-7. This may be caused by differences in gene expression between JIMT-1 cells and MCF-7 cells (Figure 3).

## DISCUSSION

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. Ethiopia is one of the ancient civilizations in Africa and the traditional herbal medicine is widely practiced across the country (Tuasha

et al., 2018b). Cancer patients prefer to visit traditional healers over conventional therapeutics for a number of reasons. Mainly, lack of awareness among the majority in rural regions results in stigma and cancer patients avoid public presentations. In addition, rising drug costs and economic insufficiency are other driving forces towards the use of TM (WHO, 2013). These days, the chemotherapeutics employed to treat cancer are challenged by cancer recurrence (Ahmed et al., 2017). New strategies are, therefore, needed to acquire lasting cure against cancer. To this end, plant-based TM practices and applications are dependable sources for the search for new lead materials for preclinical work with the goal to be used in clinical medicine today

(Fabricant and Farnsworth, 2001). Extraction and fractionation are the starting steps in research involving traditional medicinal plants aiming at isolation and purification of chemical constituents.

The TM is commonly used by cancer patients and breast cancer patients are the most likely users (Vardy et al., 2013). The assumptions for its wide use lies in that it is safe, causes less complications, and is less likely to cause dependency (Olaku and White, 2011). Most of the users usually combine TM with the conventional drugs hoping it boosts the effects (Richardson et al., 2004). 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 (Molassiotis et al., 2005). In addition, anti-angiogenesis effects, prevention of tumor recurrence, and assisting the body's immune system to battle cancer have been documented in conjunction with TM (Shahid, 2013; Levitsky and Dembitsky, 2015).

In the present study, selected Ethiopian medicinal plants implicated for use in traditional breast cancer treatment were investigated. According to various reports in Ethiopia, these plants are traditionally used against a range of human ailments in addition to cancer. For instance, *Z. chalybeum* is used to treat malaria, sickle cell disease, tuberculosis, pneumonia, colds, ulcers, sore throat, tonsillitis, urticaria (hives), measles, abdominal pain, diarrhea, intestinal worms, bilharzia, amoebiasis, general body pain, female sterility, venereal diseases, uterine fibroids, fainting, dizziness, and headache (<http://www.combonimissionaries.co.uk>, 2017). Some species from the genus *Clematis* are used for the treatment of leprosy, fever, various skin diseases, headache, common cold, hemorrhoids, and eczema (Wubetu et al., 2017). *D. abyssinica* was reported for its use in the treatment of hemorrhoids (Chekole et al., 2015) and the use of *C. myricoides* include casting out an evil spirit (Araya et al., 2015; Chekole et al., 2015), treating snake bites (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*' (herpes zoster) (Teklehaymanot et al., 2007) coughs, headaches, and abdominal pains. For various human ailments, different parts of the medicinal plants with various modes of preparation are employed.

Though this should be interpreted cautiously, the results of the present study indicate that some medicinal plants selected for breast cancer treatment by TM practitioners may not have the sought efficacy. For instance, the species *Z. chalybeum* is one of the frequently cited medicinal plants from different part of Ethiopia and it is also identified as a choice of traditional healers during the in situ investigation (Regassa, 2013; Kewessa et al., 2015; Tuasha et al.,

2018a). In cell culture, the MeOH extract was not cytotoxic up to the maximum concentration of 2 µg/ml, although it is possible that its metabolites may exert toxicity in the traditional medical practices. In the used cell culture systems there is no biotransformation. On the other hand, the species *S. oxyacanthum* (IC50 as low as 0.09 µg/ml in MCF-7 cells of crude methanol extract) was not widely reported across the country and yet selected based upon the recommendation of TM practitioners during the in situ investigation (Tuasha et al., 2018a). Also, the results reported underline the need to consider many factors during the selection phase of the medicinal plants for experimental investigation.

The traditional healers, use fresh plant material for the remedy preparation because of the perception that the freshly collected material is more efficacious than material in dried forms. They use water as the main solvent for the extraction. The amount of the plant material initially collected is not consistent across the traditional healers and the amount used for extraction in water varies considerably. Nevertheless, practitioners consider different factors before deciding the dose given to the patient (Teklay et al., 2013). The water content is usually high for harvested medicinal plant materials and this is in agreement with the present study where as high as 61.3% water content was recorded for *C. myricoides* (Poós and Varju, 2017). There is variation in the water content between different plants and also between different parts of a plant. This implies that for medicinal plants with high water content, a relatively large amount of the plant material has to be harvested. Collection of large amounts of plant materials, especially the root and the bark of a plant, poses unequivocal threat to the sustainability of the medicinal plants and thus needs conservational utilization (Teklay et al., 2013).

In our study, the cytotoxicity of the fractions was not directly related with the yield of the extract. For instance, though the material obtained from the aqueous phase of *C. simensis* was the most abundant, the most cytotoxic fraction was the chloroform fraction indicating that the activity would be related to biological constituent(s) soluble in less polar solvents. In the case of *S. oxyacanthum*, however, disproportionately large quantity yield is obtained in the aqueous fraction which is also the most cytotoxic fraction. In this case, the activity in the aqueous phase is very closely related with the traditional medicine preparation. This finding supports the claim of traditional healers that the plant *S. oxyacanthum* is considered to be the most effective remedy. Generally, since the crude extraction mimicked the way traditional remedies were prepared, it is justifiable that bioactive fractions are contained in their preparations.

Based on the solvent-solvent fractionation, the ethyl acetate fraction of *V. leopoldi* was found to be the most

cytotoxic fraction across the cell lines used (JIMT-1 = 0.87 µg/ml, MCF-7 = 3.5 µg/ml and MCF-10A = 1.72 µg/ml). To our knowledge, this is the first in vitro cytotoxicity report of its kind for the solvent fractions of *V. leopoldi*. A number of studies with other species from the genera reported cytotoxicity at varying concentrations. For instance, the crude MeOH extract of the stem of *V. divaricata* yielded the IC<sub>50</sub> values of 10.1, 12.6, and 9.9 µg/ml in HL-60, MCF-7, and PC-3 cells, respectively (Lowe et al., 2014). In addition, dose- and time-dependent cytotoxicity (ranging 9-26 mg/ml) against various cancer cells was reported for the extracts of *V. condensata* (Thomas et al., 2016). *Vernonia cinerea* was also reported to show potent cytotoxicity against colon adenocarcinoma cells (HT29) and hepatoma cells (HepG2) (Khay et al., 2012). The acetone extract of *V. guineensis* was also reported to show high toxicity against ten different cell lines with the IC<sub>50</sub> values ranging 4-26 mg/mL (Toyang et al., 2013). These findings indicate the potential of the plants in the genus *Vernonia* to have anticancer activities. Therefore, our report of cytotoxicity at low concentrations suggests the use of this species as a source for the isolation of defined compounds for investigation of bioactivity against cancer cells.

The genus *Clematis* has received large interest in conventional medicine and investigations on different species have revealed various biological activities (Hao et al., 2013; Kırmızıbekmez et al., 2018). We here report the chloroform fraction of *C. simensis* to be cytotoxic against all the breast cancer cell lines tested. Literature sources show that 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). Phytochemistry of the chloroform fraction within the genus was reported to be rich in carbohydrates and flavonoids and thus the cytotoxic activities of this fraction could be related to these chemical constituents (Karimi et al., 2017).

Generally, the crude methanol extracts showed higher toxicity than the individual fractions since it contains all the cytotoxic components. This is in line with previous reports where cytotoxicity of crude extracts and fractions have been evaluated (Rasoanaivo et al., 2011; Tantengco and Jacinto, 2015; Mtunzi et al., 2017).

## Conclusion

According to the present study, the crude extracts/fractions of some traditionally used medicinal plants have shown desired cytotoxic effects against various breast cancer-derived cell lines. The ethyl acetate fraction of *V. leopoldi* was found to be the most cytotoxic of those

tested. In addition, the aqueous fraction of *S. oxyacanthum* and the chloroform fraction of *C. simensis* were found to be highly cytotoxic at low concentrations against all the cell lines used. The slightly higher IC<sub>50</sub> values found for MCF-10A cells (especially in comparison to JIMT-1 cells) should be exploited in further studies. One of the limitations of the present study is that we were unable to exactly relate the cytotoxicity findings to the use of these plants in TM, since it is not possible to obtain accurate information of how much is used in the preparation of remedies by traditional healers. However, the fact that toxicity was found at low concentrations of some fractions, suggests cautionary TM dosage and guides isolation and characterization of biologically active compounds.

## Ethics approval and consent to participate

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 granted an approval (Project Registration №: PO19/16). The material transfer agreement (MTA) was granted from the Ethiopian Biodiversity Institute (EBI) to ship the genetic material and conduct scientific research.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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## ABBREVIATIONS

**DMSO**, Dimethyl sulfoxide; **FBS**, fetal bovine serum; **IC<sub>50</sub>**, half maximal inhibitory concentration 50%; **MTT**, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide; **PDT**, population doubling time; **TM**, traditional medicine.

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