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Anticancer, antioxidant and antimicrobial screening of extracts from selected medicinal plants from Oshikoto, Namibia

SECILIA KAENDA ILONGA[1,♥], MARTHA KANDAWA-SCHULZ[1], HESHAM EL-SAYED LOFTY[1], SYLVESTER LYANTAGAYE[2]

[1]Department of Chemistry and Biochemistry, Faculty of Science, University of Namibia. Private Bag 13301 Windhoek, Namibia. Tel.: +264 61 206 3465, Fax.: +264 61 206 3791, ♥email: vuahengo@unam.na

[2]Department of Molecular Biology and Biotechnology, Faculty of Science, University of Dar es Salaam. Uvumbuzi Rd., Mwalimu JK Nyerere campus University of Dar es Salaam. P.O. Box 35179, Dar es Salaam, Tanzania

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Abstract. *Ilonga SK, Kandawa-Schulz M, El-Sayed Lofty H, Lyantagaye S. 2018. Anticancer, antioxidant and antimicrobial screening of extracts from selected medicinal plants from Oshikoto, Namibia. Bioteknologi 15: 55-69.* Plants have been explored for years to treat ailments such as headaches, stomach-ache, diarrhea, tumors, wounds, and sexually transmitted diseases. *Heliotropium ciliatum*, *Ziziphus mucronata*, and *Gnidia polycephala* are traditionally utilized to treat tumors and wound-related illnesses. Tumors and persistent wounds can be an indication of cancer. Microbial wound infections can lead to fatal consequences, if unattended. This study evaluates the anticancer, antioxidant, and antimicrobial potential of extracts of these three medicinal plants. The leaves (*Z. mucronata*) and the aerial parts (*G. polycephala* and *H. ciliatum*) were ground and subsequently extracted with hexane, dichloromethane, ethanol and methanol, and water. The Brine shrimp lethality test (BST), 3- (4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromine (MTT) assay and APOPercentage^{TM} flow cytometry assay were employed to evaluate the anticancer potential of the extracts, whereas the antioxidant activity was evaluated by 1,1-diphenyl-picrylhydrazyl (DPPH) assay evaluated. The antimicrobial potential of the plant extracts was evaluated using the broth microdilution method against eight wound pathogens: *Escherichia coli, Candida albicans*, *Clostridium tetani*, Methicillin-resistant *Staphylococcus aureus*, *Mycobacterium terrae*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* and *Streptococcus A*. Methanol extracts of *Z. mucronata* and G. polycephala showed good antioxidant activity, compared to that of butylated hydroxyl toluene which served as control. High cytotoxicity is shown by dichloromethane and hexane extracts of *Z. mucronata* and *H. ciliatum*, as well as ethanol extracts of *H. ciliatum*, with LC50 values < 250 µg/mL. Meanwhile, water extracts showed the least cytotoxic activity. Dichloromethane extract of *Z. mucronata* also demonstrated a broad spectrum of antimicrobial activity, achieving MIC values ≤ 1 mg/mL against six out of eight tested microbes. The low toxicity of aqueous extracts of the three plants and the antimicrobial activity observed validates the use of these extracts in conventional medicine. Some extracts appear to be good sources of potential antioxidant and anticancer agents. However, further studies need to be conducted the isolation of potentially valuable drugs from these extracts.

Keywords: Anticancer, antioxidant, antimicrobial, plant extracts

INTRODUCTION

Since ancient times, plants have been a vital source of medicine. Early written reports on the utilization of plants as medicine appeared around 2600 BC when plants were used as medicine by Sumerians and Akkadians (Shoeb 2006). Since this finding, plants have been used to treat ailments such as toothaches, stomach aches, headaches, diarrhea, wounds, tumors, as well as sexually transmitted diseases (Van Wyk and Gericke 2000, Khaleeliah 2001; Von Koenen 2001; Wuyang 2008). Nevertheless, the potential of several plants as medicinal agents has not been fully characterized and established because most scientific studies carried out on plants only focused on specific diseases, thereby revealing a narrow spectrum of bioactive compounds. This limitation is often attributed to the limited resources, tedious work and time allocated for the research.

An example of this limitation is the screening of more than 35, 000 plants extract by the National Cancer Institute (NCI) of the United States in the 1960s which just targeted bioactive compounds for cancer. Mixtures with other medicinal abilities were left unexamined (Hostettmann et al. 1996). Screening of plants for medicinal purposes is crucial because plants are an indispensable source of lead and backbone compounds used in the synthesis of novel drugs in pharmaceutical industries (Potier et al. 1996). About 122 drugs were predicted to have been discovered through ethnobotanical leads of 94 plant species (Funnell et al. 2004). The screening of plant extracts by the NCI during the early 1960s shed light to the discovery of critical anticancer compounds such as Taxol, Camptothecin, and Vinblastine, which are used clinically in the treatment of cancer. Taxol, Camptothecin, and Vinblastine were isolated from extracts of *Camptotheca acuminata*, *Taxus brevifolia* and *Catharanthus roseus*, respectively (Sarkar et al. 1996). In Namibia, plants have been used among different indigenous communities to treat various diseases and ailments. However, the compounds responsible for the healing actions in most of the Namibian medicinal plants

are not yet investigated due to the lack of scientific studies and exposure.

Cancer is a disease caused by uncontrolled cell growth (Boyd, 1995). These cells proliferate uncontrollably and, in most cases, form lumps or tumors, and consequently interf with the normal functioning of the cells. In a particular case such as leukemia, the cancerous cells cause cell deformation which results in interference with normal blood functions without forming lumps. Not all tumors are malignant. Cells of cancerous and non-cancerous tumors differ in their ability to relocate to other parts of the body and invade healthy cells (Medical News Today 2009). Uncontrolled cell proliferation is primarily due to oxidative cell damage due to the presence of free radicals. Free radicals are incorporated into the body from the environment via exposure to cigarette smoke, products of incomplete combustion, ultraviolet radiation and are also generated in the body during the normal process of food breakdown (Tsang 2009). The anticancer compounds help prevent or slow down the development of cancer by regulating cell division and by killing cancerous cells (Wall and Wani 1995; Shoeb 2006). Meanwhile, by neutralizing the free radicals and repairing the oxidatively damaged cells, antioxidants can help prevent cancer (NCI 2004).

The objectives of the study were (i) To make crude extracts from the leaves of *Z. mucronata* and aerial parts of *H. ciliatum* and *G. polycephala,* (ii) To assess the anticancer and antioxidant activities of the extracts *Z. mucronata*, *H. ciliatum,* and *G. polycephala,* (iii) To assess antimicrobial activity of extracts of *Z. mucronata*, *H. ciliatum* and *G. polycephala* against eight wound pathogens.

MATERIALS AND METHODS

Preparation and extraction of plant material

Samples collection and preparation

Plant samples were obtained from Oshikoto, a region in northern Namibia in February 2010. The samples were collected from the leaves of *Ziziphus mucronata* and aerial parts of *Gnidia polycephala* and *Heliotropium ciliatum* collected. The fresh samples were cut in small pieces and air-dried in ambient temperature, then ground using a Waring® commercial heavy-duty blender to form a powder. The ground samples were sealed in a plastic bag at room temperature until use. **Figure 1** displays a map of Namibia pointing the location of the Oshikoto area.

Extraction

Hot water extraction

Aqueous extracts were done based on the method described elsewhere (Li et al. 2008) with little modifications. Briefly, 20 g of the powdered plant's sample was mixed with 200 mL of distilled water in a conical flask and incubated at 70 °C for 2 hours in a water bath. The mixture was cooled to room temperature before being filtered using Whatman filter paper (No. 1). The extracts were concentrated by heating in a water bath. These extracts were placed in a fridge until use.

Figure 1. The map of Namibia pointing the Oshikoto are, the sample collection site (Cheikhyoussef et al. 2011)

Organic extraction (Method 1)

Method for organic removal was modified from Goze *et al*. (2009), with slight modifications. Briefly, plant materials were extracted sequentially using hexane, dichloromethane, ethanol, and methanol, in the order of increasing polarities. Fifty grams of the powdered plant samples were soaked in 200 mL of the extraction solvent for three days, then filtered through Whatman® filter paper. Extraction per solvent was only performed once, and the residues were air-dried before further extraction with the next solvent.

Organic extraction (Method 2)

Plant materials were subsequently extracted with hexane, dichloromethane, ethanol, and methanol in order of increasing polarities, as described above. Approximately 25-35 g of the dry powdered plant materials were obtained, with 150 mL of organic solvents in a Soxhlet apparatus for six hours each. The residues were also air-dried before extraction with the next solvent. All natural extracts were concentrated in a rotary evaporator and later left in the fume hood for a few days to dry off the excess solvent. The extracts were placed in a fridge until use.

Bioassays

Antioxidant bioassay – DPPH radical scavenging activity

DPPH radical-scavenging method measured the inhibition effects of the extract on a free radical DPPH (Goze et al. 2009). Two milliliters of different concentrations (25, 50, 75, 100 and 500 μg/mL) of the plant extracts in methanol was added into 2 mL of DPPH solution, shaken vigorously, and allowed to stand for 30 minutes before determining the absorbance with a UV spectrophotometer at 517 nm. One of the dissolving solvents (methanol), without plant extract, was applied as a negative control. Butylated hydroxyl toluene (BHT) was used as a positive control. The inhibition effects of the

extract on free radical DPPH were expressed in the formula below:

% inhibition = [($A_{control}$- A_{sample})/$A_{control}$] × 100, where $A_{control}$ is the absorbance of the

control and A_{sample} is the absorbance of the sample.

Anticancer bioassay – Cytotoxicity assay using BST

The BST assay was carried out according to the previous method (Meyer et al. 1982) with some modifications (Silva et al. 2005). The shrimp were grown in a small tank that contained two compartments filled with artificial seawater. The shrimp eggs were placed in the covered chamber. A lamp was installed over the open side of the tank to attract hatched shrimps via perforations in the partition wall. The shrimp were grown for 48 hours until mature, now being called nauplii. All plant extracts were dissolved in 2 mL of DMSO. Artificial seawater made by dissolving 3.8 g of sea salt in 1 L of distilled water was added to mesh up 5 mL of total volume. Appropriate volumes of the resulting solution were transferred to tubes, in duplicate, with 5 mL of saline solution containing ten nauplii each to make the final sample concentrations of 24, 40, 80, 120 and 240 µg/mL. The negative control contained brine shrimp, artificial seawater and 0.6% DMSO, under the same conditions without the addition of plant extracts. The tubes were incubated under light for 24 hours, and the number of dead or surviving brine shrimps per tube was recorded. A graph depicting the percentage mortality against logarithm concentration was plotted using a computer program Microsoft Excel. The lethal level causing 50 % mortality (LC_{50}) was determined by taking the anti-logarithm of logarithm concentration corresponding to 50% mortality. An LC_{50} value higher than 1000 µg/mL were considered to represent an inactive extract.

Anticancer potential of the extracts against cancerous cell

Cell lines and cell culture

Small intestinal fetal tissue (H4), Colon adenocarcinoma (Caco-2), Chinese hamster ovary (CHO), non-tumorigenic immortalized human diploid fibroblasts (KMST-6), lung carcinoma (H157), and human cervical cancer (HeLa) cell lines were used to investigate the anticancer potential of the extracts of medicinal plants. Assays using Caco-2 and H4 cell lines were done at the Department of Microbiology, Biochemistry, Molecular Biology, and Biotechnology, University of Maribor, Slovenia. Meanwhile, the APOP*ercentage*™ assays using CHO, H157, HeLa, and KMST-6 cell lines were performed at the Department of Biotechnology, Western Cape University, South Africa. The cells were seeded in 100 mL conical flasks in a selected medium, supplemented as listed in Table 1 and were incubated in a humidified 5% CO_2 incubator at 37ºC. After reaching ~90% confluency, they were disassociated with trypsin and cells counted before plating.

Cell trypsinization and subculturing

Cells were ready for trypsinization when they had reached ~90% confluency after 2-4 days of incubation. The cell monolayer was rinsed with 2 mL of a trypsin-EDTA solution, then incubated with 1 mL of trypsin to allow for cells detachment from the flask surface. Fresh media (9 ml) was added to stop, and the cell suspension was pooled by centrifugation at 800 rpm at 22 ºC for 5 minutes. The pellet was re-suspended in ten mL of fresh media with the addition of FBS (Table 1). One-fifth of the resuspended cells (2 mL) solution was seeded into a new culture 100 mL flask to which 8 mL of media was added for subculturing. The cells were then incubated at 37 ºC in the 5% CO_2 incubator. The remaining cells were seeded into plates and used for the experiments.

Cell counting

The trypan blue staining technique determined the cell viability. The cell suspension was mixed with 0.1% trypan blue at ratio 1:9 in an Eppendorf tube. A drop of this suspension was transferred to a sealed hematocytometer. The number of live cells inside the 25 squares was calculated under a light microscope, by following the rule of counting on only two sides of the square for cells trapped between the squares. For accuracy in calculations, the number of cells counted had to be between 10 and 25. If the cell number was more than 25 cells in the 25 squares, the cell solution was diluted with 4- (2-hydroxyethyl)-piperazine-1-ethanesulfonic acid - buffered saline solution (HEPES-BSS) and the cells were counted again. The cell concentration (the number of cells per mL of cell suspension) was calculated using the following formula:

Number of cells/mL cell suspension = number of cells counted $\times 10^6$/25 squares

Cell plating

From the concentration of cells obtained from the step above, the cells were diluted to the desired concentration with fresh media. For the cell growth inhibition assay, the cells were seeded in 96-well plates at a density of 111 cells/mL and for the inhibitionMTT assay at 6×10^4 cells/mL. Cells were seeded in 12- well plates at a density of 2.5×10^4 cells/mL for the APOPercentage™ apoptosis assay. One-hundred µL of the cell suspension was delivered into each well using a multichannel pipette in 96-well plates. The cell suspension was transferred into a sterile petri dish which was shaken regularly before drawing out the cell suspension. For a 12-well plate, 1 mL of the cell suspension was poured into each well. The plates were incubated for 24 hours at 37 ºC in a 5% CO_2 incubator or until they reached 90% confluency before treatment with plant extracts.

Testing plant extracts for anticancer activity

Cell growth inhibition assay

Caco-2 and H4 cells suspensions were seeded into 96-well plates. The outlying wells of the plates, i.e., rows A and H and columns 1 and 12, were supplied with 100 µL of distilled water. The inner wells were provided with 100 µL of cell solution (Figure 2). The plates were incubated at 37 oC for 24 hours at 5% CO_2. The cells were treated with plant extracts on the next day, by replacing the media with fresh media containing plant extracts.

Table 1. Growth media and supplements for the cell lines used.

Cell line	Species	Media	Supplements
H4	Human	DMEM	5% Foetal Bovine Serum, 100 U/mL Penicillin, 100 µg /mL Streptomycin, 2 mM L-glutamine
Caco-2	Human	DMEM	5% Foetal Bovine Serum, 100 U/mL Penicillin, 100 µg /mL Streptomycin, 2 mM L-glutamine
CHO	Chinese Hamster	F-12Hams	10% Foetal Bovine Serum, 50 U/mL Penicillin, 50 µg /mL Streptomycin
H157	Human	DMEM	10% Foetal Bovine Serum, 50 U/mL Penicillin, 50 µg /mL Streptomycin
HeLa	Human	DMEM	10% Foetal Bovine Serum, 50 U/mL Penicillin, 50 µg /mL Streptomycin
KMST-6	Human	DMEM	10% Foetal Bovine Serum, 50 U/mL Penicillin, 50 µg /mL Streptomycin

Figure 2. The treatment of cells in the 96-well plate for the growth inhibition assay.

The extracts were dissolved in DMSO to generate a stock solution of 50 mg/mL. Row B was filled with control solution. Eleven microliters of plant extract stock solution were supplied into well in row C, performing a 1: 10 serial dilution through to well in row G. The experiment was conducted in 5 replicates (Figure 2). The plates were incubated at 5% CO_2, 37 ºC for eight days, where cell growth was visible under a light microscope. After that, the media was removed, and the cells were treated with crystal violet for 5 minutes. Under running tap water, the crystal violet was washed off; then the plates were dried on a towel paper overnight. We counted the number of colonies observed in wells under the light microscope. The highest concentration, in which some selective toxicity against Caco-2 cells was observed (500 µg/mL), was used as the highest concentration in the MTT assay. Only extracts which exhibited selective toxicity towards Caco-2 cell lines were further used for the cytotoxicity (MTT) assay.

Cytotoxicity assay

Concerning the slow growth of Caco-2 cells, only H4 cells were used for this assay. Extracts that displayed selective toxicity against Caco-2 in the previous test were used in this assay. The cells were seeded and incubated as mentioned in the method section. Before the test, the cell monolayer was washed two times with 200 µL of sterile PBS. One hundred and twenty microliters of DMEM only supplemented with L-glutamine (no FBS, no indicator) was added.

Nine microliters of the extract stock solution (50 mg/mL) were mixed with 291 µL of DMEM (no phenol red) media to prepare working solution of 1.5 mg/mL. Row A served as a control. To row B, 60 µL of extract solution was applied (making the starting concentration of 500 µg/mL). A 1: 4 serial dilution was made through to wells in row H, always discarding 60 µL from the last well to afford a final volume of 120 µL per well. The experiment was done in triplicate, and the plates were incubated for 24 hours. The effects of DMSO as the extract dissolving solvent was also tested, which was treated similarly as the plant extracts.

After 24 hours, two sets of 40 µL of the incubation media from two wells were moved into sterile 96 well plates in a sequence corresponding to that of the original dish, and these were later used for the H_2O_2 and NO assay. The leftover media was discarded, and the cells were used for the MTT assay. The three tests were performed following the provided protocol as briefly described below.

MTT assay

First, the cell monolayer was rinsed with PBS. We added 220 µL of a mixture DMEM (only supplemented with L-glutamine) and 5 mg/mL MTT solution (at the ratio 10: 1). The plates were incubated at 5% CO_2 and 37 ºC for 5 hours to develop a purple formazan color. Then, the media was discarded, and the plates were air-dried on a towel paper overnight. One hundred microliters of 0.04% HCl in isopropanol was poured to each well to dissolve the formazan. The plates were shaken on a rotating shaker for 5 minutes, before being incubated at 5% CO_2 and 37 ºC for 20 minutes. The absorbance was read at 570 nm with background wavelength set at 630 nm.

NO assay

To the forty µL of the overnight incubation media, we added forty µL of Griess reagent. The plates were gently shaken on a shaker for 20 minutes, and the absorbance was read at 540 nm.

H_2O_2 assay

To the forty µL of the overnight media, forty µL of 0.01% H_2O_2 was added, followed by the addition of 100 µL of TMB-H_2O mixture (at ratio 1:1). The control was made by mixing 50 µL of 0.001% H_2O_2, 50 µL of 0.01% peroxidase and 100 µL mixture of 1:1 TMB and H_2O_2 in one well. The mixture was placed on a shaker for 20 minutes before measuring the absorbance at 450 nm.

APOPercentage^{TM} assay (Flow cytometric analysis of apoptosis)

Preparation of extracts. Plant extracts (20 mg) were dissolved in 200 µL DMSO, then 800 µL of an appropriate media was mixed to make a stock solution of 20 mg/mL.

From the stock, 2.5 and 5.0 mg/mL of working solutions were prepared by diluting the stock solution with the media. The working solution was filtered through a filter paper with pore size: 0.45 µm.

APOPercentage^{TM} assay. Chinese hamster ovary (CHO), human cervical cancer (HeLa), lung carcinoma (H157), and non-tumorigenic immortalized fibroblasts (KMST6) cell lines were utilized in this assay. When the cells in the culture flask had grown to 90% confluency, cells were trypsinized, and the pellet was resuspended in complete media to make a cell density of 2.5×10^4 cells per mL. These cells were seeded in 12-well tissue culture plates, 1 mL per well and incubated at 37°C for 24 hours.

Following this step, the media was replaced with working extract solution at 2.5 and 5.0 mg/mL. Complete media without plant extract served as a negative control, whereas 150 µM ceramide was used as a positive control. The cells were incubated again at 37 ºC and 5 % CO_2 for 24 hours. The negative control cells were applied to aid in adequately distinguishing healthy cells from apoptotic cells. Floating (apoptotic) cells were moved to 15 mL centrifuge tubes, and the adherent cells were trypsinized and added to tubes containing floating cells. The cells were centrifuged at 300 x *g* for 3 minutes to obtain a pellet, washed twice with PBS by centrifuging at 300 x *g* for 3 minutes for each wash. At the end of the last roll, the cell pellet was resuspended in the residual PBS added with 250 µL APOP*ercentage*™ dye (a 1: 160 dilution in complete media). The cells were incubated for 30 minutes. After that, 500 µL of PBS was added to the tube, and the cell mixture was spun down for 5 minutes at 300 x g to obtain a cell pellet. The pellet was rinsed one more time with PBS, resuspended in 400 µL of PBS and analyzed using a FASCan^{TM} (Becton Dickson) instrument equipped with a 488 nm Argon Laser as a light source within an hour. The acquisition was made by setting forward scatter (FSC), and side scatters (SSC) on a log scale dot plot to differentiate the population of cells and debris. On a linear histogram dot plot, APOP*ercentage*™ (FL-3 channel) was calculated against the relative cell numbers. Negative control cells were applied to set the cells in the negative quadrant before measurement of all samples. A minimum of 10, 000 cells per sample were obtained and analyzed using CELLQUEST Pro software by setting the non-stained (untreated) cell population in the first quadrant (10^1) of the forward side scatter histogram dot plot and cells which appeared in the second (10^2) or the third quadrant (10^3) were regarded as APOP*ercentage* ™ positive (apoptotic/necrotic) cells.

Antimicrobial test

We assessed antimicrobial activity of the selected medicinal plants according to method established earlier with few modifications (Chingwaru et al. 2011). Briefly, *M. terrae*, *E. coli*, *Streptococcus* A, *P. aeruginosa*, C. tetani, MRSA and *S. epidermidis* were cultivated in nutrient broth (10 mL), and *C. albicans* in yeast extract peptone glucose (YEPG) broth (10 mL) for 24 hours at 37 oC. The optical density of the microbial suspension was determined at 600 nm, and the microbial suspension was diluted with media to yield microbial suspensions of 1× 103 colony forming unit (CFU)/mL for bacteria and 1× 103 CFU/mL for yeast, which was then used for the experiments. Before the addition of the microbe, the microbial suspension was shaken to distribute the microbes evenly.

Plant extracts were dissolved in DMSO to prepare a stock solution of 50 mg/mL. The extracts were diluted with nutrient broth for bacteria (or YEPG for *C. albicans*) to make a working solution at a concentration of 20 mg/mL for *E. coli* and *M. terrae*; and 5 mg/mL for all the other microbes. In a 96-well plate, forty µL of the appropriate media was filled into each well. To the first well, 40 µL of working extract solution was given and a 1:2 serial dilution was carried out through to the last well, always discarding 40 µL from the previous well. Twenty microliters of the bacterial suspension (1 ×105 CFU/mL) or yeast (1 ×103 CFU/mL) was added. The plates were incubated at 37 oC. Measurement of the microbial growth was done at 600 nm every hour within the first 10 hours and at the end of the experiment, i.e., at 24 hours. DMSO and appropriate growth media were used as negative control whereas gentamycin (4 mg/mL) and streptomycin (10 mg/mL) were utilized as a positive control. Data was set in triplicate, and the absorbances were expressed as mean ± standard deviation. The minimum inhibition concentration (MIC) was determined as the lowest concentration which inhibited the growth of the microbe.

RESULTS AND DISCUSSION

Natural products extraction

Depending on the method, some native plant products could be successfully extracted during the extraction process (Table 2). As described earlier, method 1 is room temperature extraction, and method 2 is the Soxhlet extraction. The weight of starting material used for these two extraction methods is about 50 g for method 1 and ranged between 25-35 g for method 2. All yields were showed as yield fraction, i.e., yield fraction = amount of extract/amount of starting material to allow for statistical comparison. The percentage yields were shown as means ± standard deviation and are shown in Table 2.A-D. Although the yield of the Soxhlet extraction was slightly higher than that obtained from room temperature extraction, the yield per extraction solvent were not statistically significant except for ethanol extract of *H. ciliatum* (t= -5.533, p=0.005), hexane extracts of *G. polycephala* (t= -4.708, p=0.009) and hexane and methanol extracts of *Z. mucronata*. The yield accumulation from the two extraction methods, however, were statistically significant, with p values of 0.001 (*Z. mucronata*), 0.004 (*G. polycephala*) and 0.005 (*H. ciliatum*). Since the yields per extraction solvent for all plants were not statistically significant with a few exceptions, only extracts of method one were used in the assays for this experiment. Codes of these extracts were shown in Table 3 and were referred to by these codes throughout this document.

Table 2. Comparative analysis of extraction yield of different extracts of the three medicinal plants using cold extraction by soaking for three days (for method 1) and Soxhlet extraction for six hours (for method 2). The yield per extraction solvent per plant are displayed in Table 2.A (*G. polycephala*), Table 2.B (*H. ciliatum*) and Table 2.C (*Z. mucronata*). Table 2.D presented the pooled yields per plant. * Values were the mean ± standard deviation of three replicates.

Extract	Percentage yield (%)	
	Method 1*	Method 2*
A. *G. polycephala*		
Hexane	1.90±0.31	3.42±0.47
Dichloromethane	2.27±0.25	2.95±0.55
Ethanol	2.51±1.09	4.14±0.47
Methanol	5.77±0.52	12.02±1.94
B. *H. ciliatum*		
Hexane	1.10±0.19	1.23±0.68
Dichloromethane	1.03±0.32	1.27±0.12
Ethanol	1.06±0.36	2.68±0.35
Methanol	2.70±0.12	4.26±1.16
C. *Z. mucronata*		
Hexane	1.50±0.10	3.35±0.36
Dichloromethane	1.57±0.16	1.88±0.86
Ethanol	4.98±0.09	14.2±0.16
Methanol	9.70±0.92	12.9±1.3

D. The pooled yields per plant

Plant	Cumulative percentage yield (%)	
	Method 1*	Method 2*
Z. mucronata	17.74±1.20	32.34±2.16
H. ciliatum	5.88±0.52	9.45±1.01
G. polycephala	12.45±1.44	22.53±2.64

Table 3. Codes assigned to hexane, dichloromethane, ethanol, methanol, and water extracts of *G. polycephala, Z. mucronata,* and *H. ciliatum.*

Plant	Hexane	Dichloromethane
Z. mucronata	ZH	ZD
G. polycephala	GH	GD
H. ciliatum	HH	HD

Table 4. IC50 values (µg/mL) for the DPPH scavenging activity.

Extract	IC_{50} (µg/mL)
BHT	29.92
GM	50.20
GE	140.68
GH	> 1000
HM	357.59
HE	343.81
ZM	45.19
ZE	82.68

Antioxidant activity: DPPH assay

The ethanol and methanol extracts of the three traditional medicinal plants were examined for their ability to scavenge DPPH radical. These extracts demonstrated dose-dependent DPPH scavenging activities. Methanol extracts tend to show higher antioxidant activity compared to ethanol extracts (Figure 3-5). The DPPH scavenging activity for the methanol extract of GM and ZM was comparable to the activity of the synthetic antioxidant BHT, although ZM showed slightly higher activity than that of GM (Figure 6). For all extracts, as indicated in Figure 6 and 7, *Z. mucronata* showed the highest DPPH scavenging activity, followed by *G. polycephala*, while *H. ciliatum* is showing the least activity. The concentration of the extracts that were able to scavenge at least 50% of the DPPH dye (IC50) was measured. ZM and GM showed the most active with low IC50 of 45.19 µg/mL and 50.20 µg/mL respectively. *Z. mucronata* exhibited the most DPPH radical scavenging activity among the three plants with both extracts showing IC50 < 85 µg/mL and *H. ciliatum* possessed the least DPPH radical scavenging activity with IC50 for both extracts > 300 µg/mL. The results are summarized in Table 4.

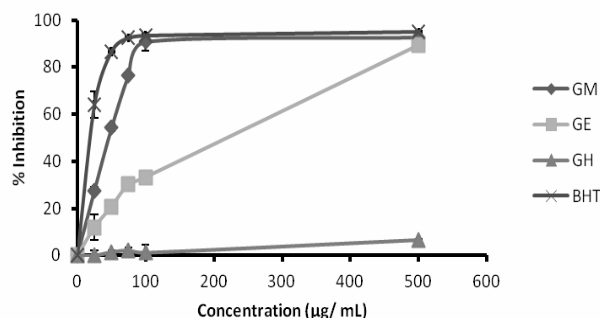


Figure 3. DPPH scavenging activity of GE, GM, and GH extracts as compared to that of BHT.

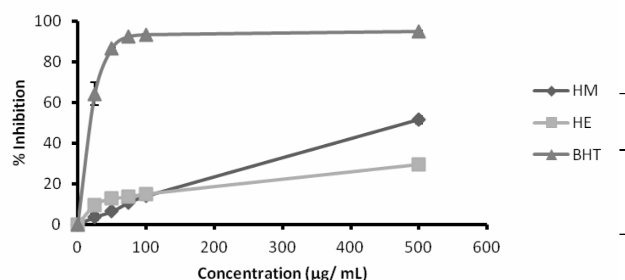


Figure 4. DPPH scavenging activity of HE and HM extracts as compared to that of BHT.

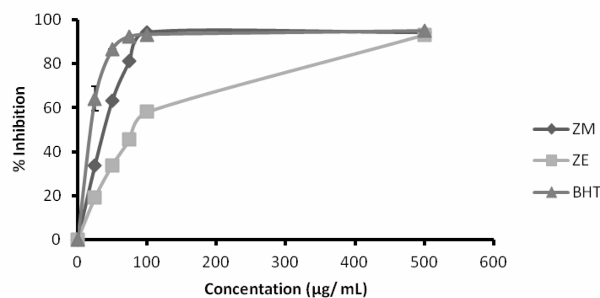


Figure 5. DPPH scavenging activity of ZE and ZM extracts as compared to that of BHT.

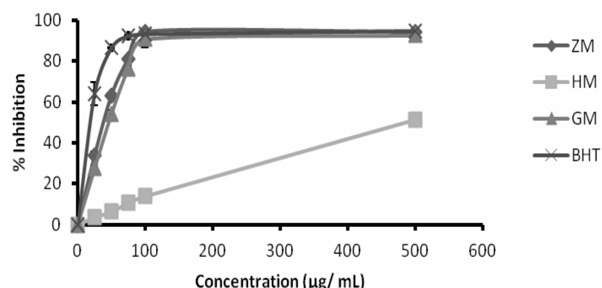


Figure 6. DPPH scavenging activity of methanol extracts of the three indigenous plants.

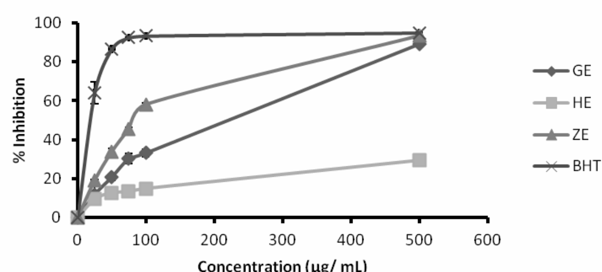


Figure 7. DPPH scavenging activity of ethanol extracts for the three indigenous plants.

Anticancer activity

Brine shrimp lethality test

None of ZM, ZW, ZE, and GM extracts were active, showing lethal concentration to at least 50 % (LC50) of the *A. salina* larvae at a concentration higher than 1000 µg/mL. An LC50 value of ≥ 1000 µg/mL was applied as inactive and extracts which showed this score were classified safe to the tested organism. *H. ciliatum* demonstrated the most toxicity with LC50 for the hexane, ethanol, and methanol extracts < 200 µg/mL. Other extracts that exhibited an LC50 < 200 µg/mL are hexane and dichloromethane extracts of *Z. mucronata*. Extracts of *G. polycephala* fell in the moderate toxicity range with LC50 values for ethanol, dichloromethane, and hexane extract all above 250 µg/mL (Table 5).

Table 5. Brine shrimp activity of extracts of the three selected medicinal plants. LC= Lethal concentration; LCL= Lower confidence limit; UCL= Upper confidence limit; *= No activity (mortality) up to the maximum tested concentration (240 µg/mL); # = LC50 ≥1000 µg/mL.

Extract	LC_{50} (µg/mL)	95% Fiducial Limit (µg/mL) LCL — UCL
ZW	#	#
ZM	#	#
ZE	*	*
ZD	165.64	44.50—616.43
ZH	198.54	119.04—331.12
GM	*	*
GE	264.46	48.39—1445.34
GD	590.85	181.862—442.95
GH	729.27	100.945—268.53
HM	169.71	51.52—558.99
HE	142.67	54.723—71.93
HD	230.27	40.863—76.46
HH	131.84	94.991—82.99

Cell growth inhibition assay

We identified GH, GE, GD, GM, ZD, ZE, ZM, HH, HE and HM after screening for selective activity against cancerous Caco-2 cells and unhealthy H4 cells. These extracts were used in the cytotoxicity assay. The influence of plant extracts on the proliferation of the two cell types is shown in Figure 8 below. The plant extracts dilutions were d1 is a 1:10 dilution, d2 is 1:100 dilution, d3 is 1:1000, d4 is 1:10 000, d5 is a 1: 100 000 dilution. The extracts which showed selected toxicity towards Caco-2 cells are listed in Table 6.

However, as the proliferation of any two different cell lines differs, it makes identification of which extracts are active against Caco-2 cells but not H4 cells, quite tricky. Also, it is difficult to keep the same concentration of diluted cell solution, and only some of the transferred cells survive and proliferate, thus, making it tough to compare entire colonies acquired with different cell lines. The spectrophotometric method is also not reliable as much of the absorbance recorded is somewhat because of the dye attached to the plate walls than the dye retained by the cell colonies.

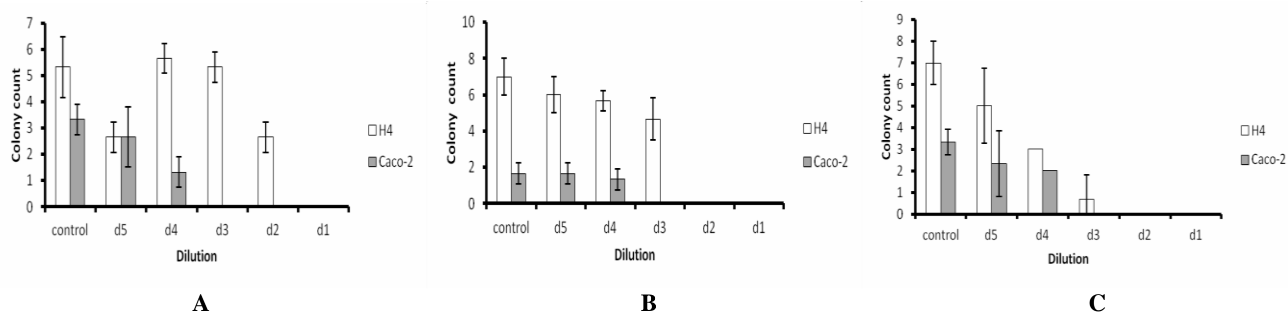


Figure 8. The proliferation of H4 and Caco-2 cell lines against GE (A), ZD (B) and HH (C).

Table 6. Plant extracts which showed selective toxicity towards the Caco-2 cell line.

Plant	Hexane	Dichloro-methane	Ethanol	Methanol
Z. mucronata	✗	✓	✓	✓
G. polycephala	✓	✓	✓	✓
H. ciliatum	✓	✗	✓	✓

Table 8. Anti-proliferation activity of selected plant extracts against H4 cells.

Extract	IC_{50} (µg/mL)
GM	180.91
GE	264.04
GD	126.95
GH	160.37
HM	145.73
HE	116.80
HH	119.34
ZE	658.40
ZD	126.73

MTT assay

Chinese hamster ovary cells

Water extracts showed no activity at a concentration of 2.5 mg/mL, while at a concentration of 5 mg/mL, the proliferation of CHO cell lines declined by 25% for GW and 55% for ZW (see Figure 9).

H4 cells

Figure 10 illustrates plant extracts which exhibited anti-proliferation activity in a dose-dependent manner. IC50 was determined by plotting concentration against % cell survival. Table 7 shows HE and HH were the most active with IC50 of 116.80 µg/mL and 119.34 µg/mL respectively. Whereas, GE and ZE showed the least activity with IC50 values of 264.04 µg/mL and 658.40 µg/mL, respectively.

NO assay

Cells did not produce a significant amount of NO after treated with various concentrations of the plant extracts, except at the lowest dilution factor (Figure 11).

H_2O_2 assay

H4 cells treated with different levels of plant extracts did not produce a significant amount of H_2O_2 because the absorbances of cells treated with the extracts were like that of the DMSO (control), except at the highest concentration tested (Figure 12).

APOPercantageTM assay

This assay evaluates the apoptotic effect of extracted samples and to evaluate its dose-response activity. Apoptosis also is known as programmed cell death is a process characterized by cell shrinkage, membrane blebbing and nuclear condensation (Chinkwo 2005). The cells treated with plant extracts showed morphological changes such as cell shrinkage and disintegration, compared to the cells in control. Cells of the negative control were more intact and maintained their shape as compared to cells treated with an apoptotic agent, ceramide, thus reveals the apoptotic activity of some of the tested extracts. An example of cell morphology observations is illustrated in Figure 13.

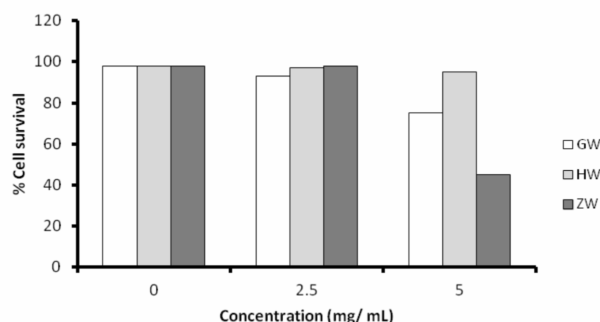
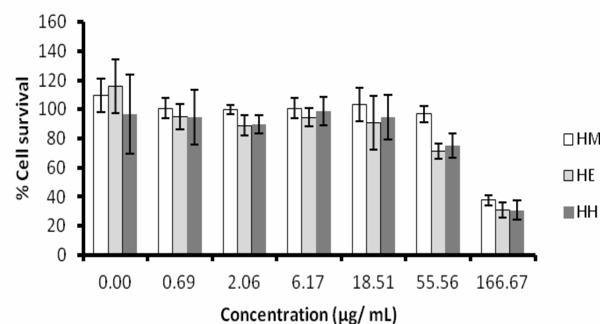
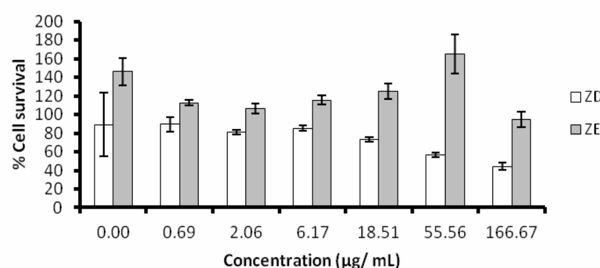


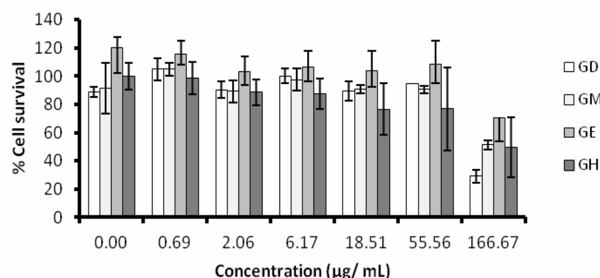
Figure 9. The effects of water extracts of selected traditional medicinal plants on the proliferation of CHO cells



A

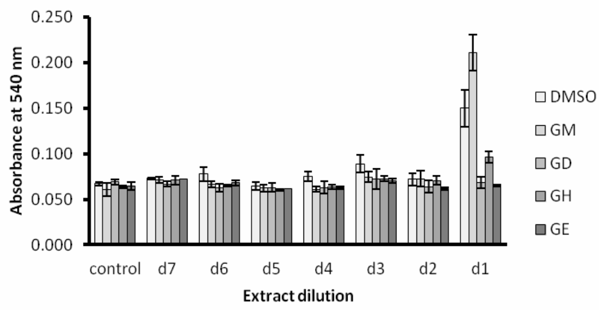


B

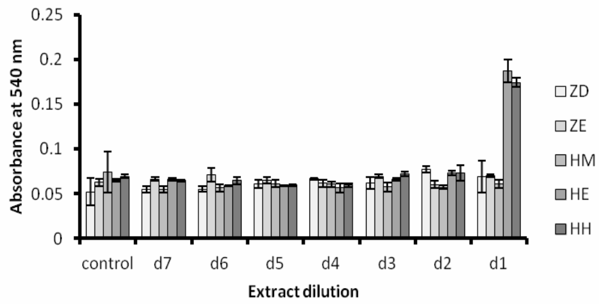


C

Figure 10. The effects of different extracts on the proliferation of H4 cells, *H. ciliatum* (A), *Z. ucronate* (B) and *G. polycephala* (C)

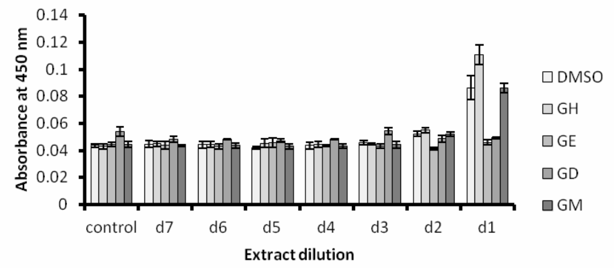


A

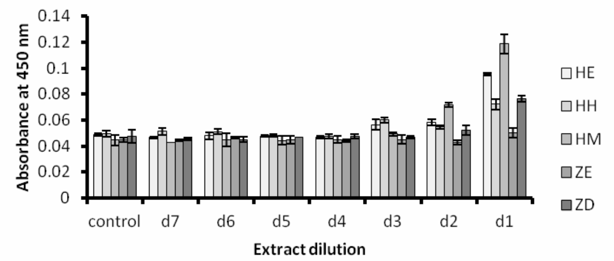


B

Figure 11. The release of NO by H4 cells treated with different levels of plant extracts, (A) extracts of *G. polycephala* and (B) *Z. ucronate* and *H. ciliatum.*



A



B

Figure 12. The release of H_2O_2 by H4 cells treated with different concentrations of plant extract, (A) *G. polycephala*, and (B) *Z. ucronate* and *H. ciliatum.*

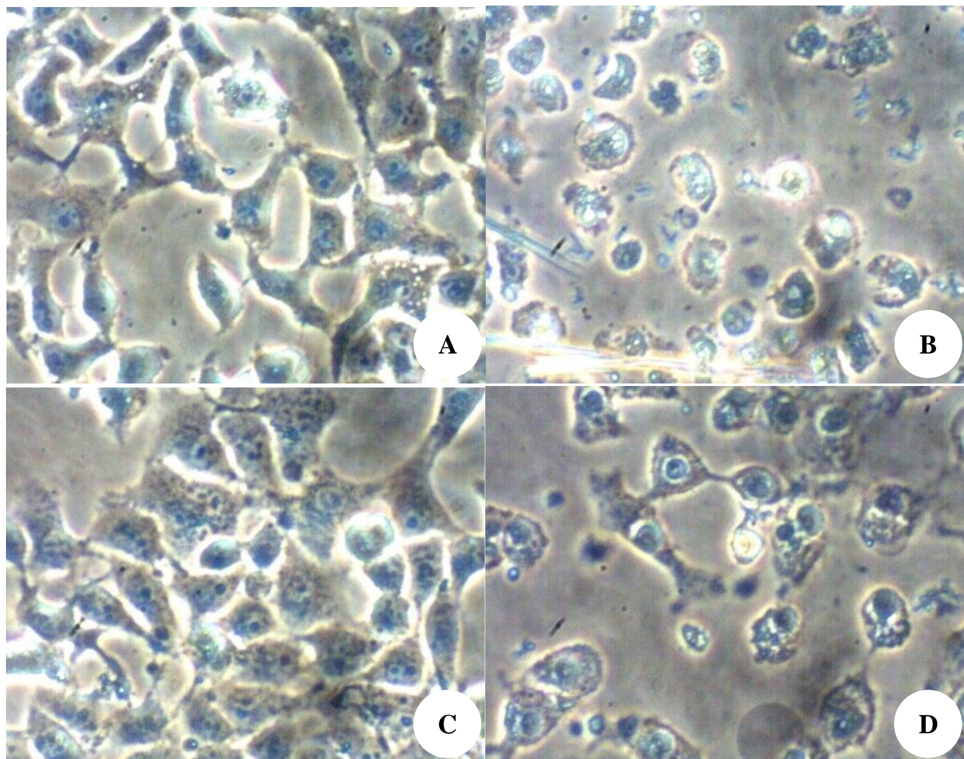


Figure 13. Morphology of HeLa cells treated with 2.5 mg/mL plant extracts. (A) Above, an untreated control, (B) cells treated with 150 µM ceramide, (C) cells treated with ZH and (D) cells treated with ZM.

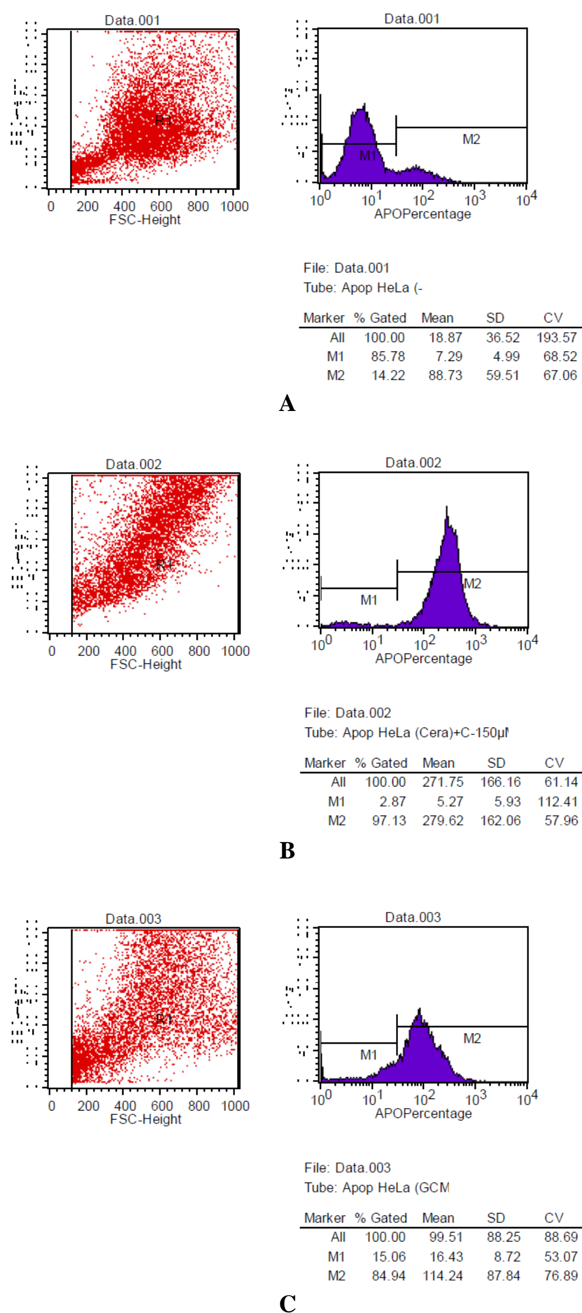


Figure 14. Forward and side scatter and histogram analysis of HeLa cells stained with APOPercentage™ dye. (A) Untreated cells/control, (B) cells treated with 150 µM ceramide and (C) cells treated with 2.5 mg/mL of GM.

As stated in the materials and methods, APOPercentage™ assay was done by setting forward scatter (FSC), and side scatters (SSC) on a log scale dot plot to differentiate a population of cells and debris. On a linear histogram dot plot, APOPercentageä (FL-3 channel) was examined against relative cell numbers (Figure 14).

Plant extracts commonly exhibited apoptotic activity in a dose-dependent manner and reacted differently towards different cell lines. HM was highly active at a 2.5 mg/mL against H157 and KMST-6 cells causing more than 90% cell death (99.15 % and 95.77 %, respectively) and even a higher percentage cell death was observed at 5.0 mg/mL (99.76 % and 99.06 % respectively). The percentage of cell death caused by GM ranged from 58.46 % against H157 and 61.54% against KMST-6. When the extract concentration was increased to 5.0 mg/mL (Figure 14-15), it showed increased cell death population by 78.52 % for H157 and 66.30 % for KMST-6. The LC50 for various extracts tested could not be determined because most extracts exhibited over than 50 % cell death at the lowest concentration examined (2.5 mg/mL). The water extracts for the three medicinal plants were the least active since they caused less than 10 % cell death at the highest level (Figure 15).

The results on the apoptotic activities of different extracts on HeLa cells revealed that, at a level of 2.5 mg/mL, all extracts (except GH, HD, and ZH) were highly apoptotic, killing more than 50 % of the cells, and some causing more than 90 % cell death. This activity is comparable to that of a conventional drug ceramide (150 µM) (Figure 16). The apoptotic percentage of GH, HD, and ZH were of 20.62 %, 42.25 %, and 11.06 % respectively.

Antimicrobial activity

The antimicrobial activity exhibited by the plant extracts depended on the dose and length of exposure, thus, enabling the determination of the MIC of each extract against the pathogens tested in this study. Also, the antimicrobial activities exhibited by different plant extracts were pathogen-specific as they showed different antimicrobial activity against different pathogens. The MIC values shown by different extracts against different pathogens are presented in Table 9.

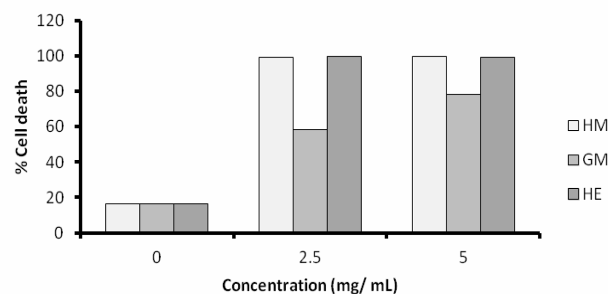


Figure 15. The effects of selected traditional medicinal plant extracts on H157 cells.

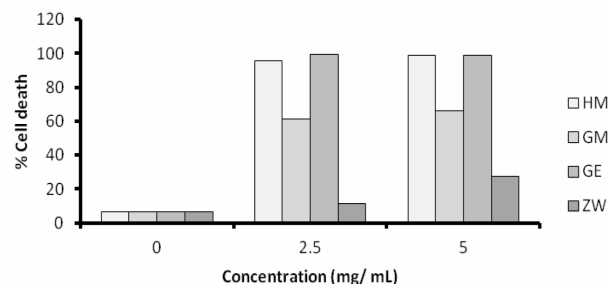


Figure 16. The effects of selected traditional medicinal plant extract on KMST-6.

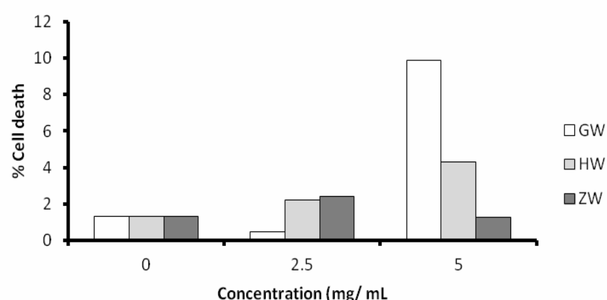


Figure 17. The effects of water extracts of traditional medicinal plants on the proliferation of CHO cells.

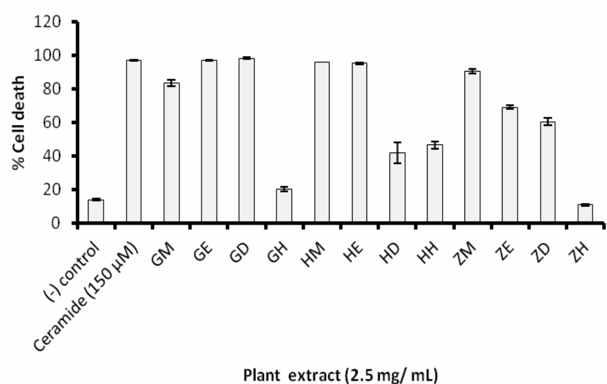


Figure 18. Apoptotic effects of different extracts on HeLa cells.

A comparison of the MIC of various plant extracts and those of the antibiotics (gentamycin, and streptomycin) for the different test organisms, demonstrated that the plant extracts were generally less active. Some extracts, however, showed stronger antimicrobial activity than the conventional drug streptomycin against some of the microorganisms tested. Plant extracts were less active against *M. terrae* than the two antibiotics. HH, HD and ZD (showing MIC < 0.156 mg/mL) exhibited the highest activity against *M. terrae* and GE showed the least activity against the same microbe (MIC = 20 mg/mL) compared to gentamycin and streptomycin (0.03125 and 0.07813 mg/mL). As compared to gentamycin and streptomycin, HM, and GD (MIC 5.0 mg/mL each) show similar activity compared to streptomycin. ZE and ZM (MIC 2.5 mg/mL each) were more effective than streptomycin. The remaining extracts were less active, exhibiting MIC ≥ 10.0 mg/mL.

All extracts showed better results against *P. aeruginosa*, except HD, HE and HH compared to streptomycin, with all exhibiting MIC values ≤ 1.25 mg/mL compared to 5.0 mg/mL recorded for streptomycin. The same is correct about the activity of the extracts against *S. aureus,* through some extracts activity was comparable to that of streptomycin. All extracts showed more potent antimicrobial activity than streptomycin against MRSA (MIC of ≤ 1.25 mg/mL and 2.5 mg/mL respectively). ZD showed better activity than gentamycin. All extracts demonstrated some antimicrobial activity against C. tetani and *S. epidermidis* with MIC values ≤ 2.5 mg/mL. Although, in all cases they were less effective than gentamycin and streptomycin MIC < 0.03125 and < 0.07813 mg/mL against C. tetani and *S. epidermidis*.

Extracts were grouped as being good, moderately good, moderate, or poor antimicrobial agent using the following criteria to ease interpretation (Gibbons 2004; Ríos and Recio 2005 in Suliman 2010):

MIC ≤ 1 mg/ml: good antimicrobial activity
MIC > 1mg/ml or < 4 mg/ml: moderately good antimicrobial activity
MIC = 4 mg/ml or < 6 mg/ml: moderate antimicrobial activity
MIC ≥ 6 mg/ml: poor antimicrobial activity

Table 9. The minimum inhibition concentrations (MIC, mg/mL) of different extracts of *Z. mucronata*, *H. ciliatum* and *G. polycephala* on different pathogens. Note: MT: M. terrae, PA: *P. aeruginosa*, SA: *Streptococcus* A, EC: *E. coli*, CA: *C. albicans*, MRSA: Methicillin-Resistant *Staphylococcus aureus*, CT: C. tetani, SE: *S. epidermidis.*

Extract	MT	EC	PA	SA	CA	MRSA	CT	SE
ZD	<0.156	10	0.625	0.625	>5.0	0.078	0.313	0.313
ZE	0.313	2.5	1.25	1.25	2.5	0.625	0.625	1.25
ZH	5.0	10.0	1.25	1.25	1.25	1.25	1.25	1.25
ZM	5.0	2,5	1.25	1.25	2.5	0,625	0.625	1.25
GD	2.5	5.0	0.313	0.313	2.5	0.313	1.25	0.313
GE	20.0	10.0	0.313	0.313	2.5	0.313	0.625	0.625
GH	1.25	10.0	0.625	0.625	2.5	0.625	0.625	1.25
GM	2.5	10.0	1.25	1.25	2.5	1.25	1.25	0.625
HD	<0.156	10.0	>5.0	1.25	1.25	1.25	1.25	0.313
HE	1.25	10.0	>5.0	1.25	1.25	0.625	1.25	0.625
HH	<0.156	>20	>5.0	1.25	1.25	1.25	1.25	2.5
HM	2.5	5.0	0.625	1.25	2.5	>5.0	1.25	0.625
Gentamycin	<0.0313	<0.0313	0.25	0.25	1.0	0.5	<0.0313	<0.0313
Streptomycin	<0.0781	5.0	5.0	1.25	5.0	2.5	<0.0781	<0.0781

ZD extract, for example, demonstrated fair antimicrobial activity against MRSA, *P. aeruginosa* and S. aureus, but poor antimicrobial activity against *E. coli*. ZE showed good antimicrobial activity against MRSA, *M. terrae* and *C. tetani* and moderately good antimicrobial activity against all other pathogens. ZH exhibited good antimicrobial activity against all pathogens except for M. terrae, and *E. coli* where it exhibited moderate and poor antimicrobial activity respectively. ZM displayed good antimicrobial activity against MRSA and C. tetani; moderately good antimicrobial activity against *C. albicans*, *S. epidermidis*, and *E. coli* and moderate activity against *M. terrae*. The antimicrobial activities of all extracts against all microorganisms tested were less effective than the conventional antibiotic gentamycin, except for ZD against MRSA (MIC 0.078 mg/mL).

Discussion

Extract yield

Soxhlet extraction method yielded slightly higher as compared to the cold extraction method, though not statistically different. The high yield obtained with the former method is attributed to the increase in temperature of the extraction solvent because the solubility of solute increases with increased temperature (Chang 1998). Nevertheless, the risk of destroying valuable substances increases with temperature.

Antioxidant activity (DPPH assay)

The DPPH assay assesses the antioxidant activity of the extracts. DPPH is a stable free radical, which receives an electron or hydrogen radical to form a stable diamagnetic molecule that is widely employed to investigate radical scavenging activity. An antioxidant will react with the DPPH radicals (Prasad et al. 2009). In this assay, *Z. mucronata* exhibited the most activity, followed by *G. polycephala* whereas *H. ciliatum* showed the lowest activity. Methanol extracts proved to be more potent antioxidants than the corresponding ethanol extracts in all cases (Figure 3-7). A pattern of increasing antioxidant activity with increasing polarity of the solvent has been studied (Goze et al. 2009). The polar methanol solvent easily polyphenols and phenolic compounds which are also polar. Polyphenols and phenolic compounds are great antioxidants (Goze et al. 2009). Flavonoids, a subclass of the class of the phenolic compound was tentatively identified from the leaves of *Z. mucronata*, which justifies the high antioxidant activity described for the methanol extract of *Z. mucronata* (Suliman 2010). The presence of carbon-carbon double bond, carbonyl, and free hydroxyl groups contribute to the free radical scavenging activities of scavengers since they can donate either hydrogen radical or an electron to other radicals. By doing so, the scavenger forms a more stable radical (Solomons and Fryhle 2008).

Anticancer activity

A few essays were employed in the assessment of the anticancer activity of the extracts of the three medicinal plants, namely: the BST, MTT, NO, H_2O_2 and APOPercentage™ assay. These widely used cytotoxic and anticancer assays employed in the preliminary examination of toxicity, detection of toxins and evaluating the anticancer potential of compounds or extracts. The assays are reasonably reliable, and the results obtained are an accurate reflection of the possibility of the test compounds as anticancer agents. The NO and H_2O_2 assays produced no significant activity from all the extracts tested in this study (Figure 11-12). These results suggest that no H_2O_2 were released during cell death or that these compounds were excreted in low amounts at the concentrations of extracts tested. We compared the performance of different extracts in the three anticancer assays: BST, MTT, and APOPercentage™ assays. ZD, HE and HM exhibited excellent anticancer activity in all the three experiments. HD, ZW, GW and HW extracts were inactive against cancer in all the three tests performed. GM, GD, ZM showed negative results in the BST test but positive results in the other two tests. ZE, GE only showed positive results in the APOPercentage™ assay. HH and ZH displayed positive results for the BST and MTT assay and adverse effects in the APOPercentage™ assay.

Water extracts of the three medicinal plants demonstrated low anticancer activity. The low cytotoxic potential of the aqueous extracts is of great significance for their conventional use in the treatment of various disorders other than cancer (Uddin et al. 2009). Aqueous extracts are administered in conventional medicine. Because they are of low toxicity, their use in traditional medicine is justified as the extracts can provide toxicity enough to cure a particular ailment but not high enough to intoxicate the cells.

Anticancer activity was observed for at least one of extracts of the three medicinal plants, in at least one anticancer test. Antioxidant activity was also reported for some of these extracts. Plant anticancer and antioxidant compounds are secondary metabolites, compounds which can be restricted to specific taxonomic groups be it, family, genus, or species according to (Balandrin et al.,1985). Although no published work could be referred for the three medicinal plants on the exact work done in this study, there have been reports on studies of other species in the same genus and on different topics regarding these plants. Biological activity and chemical composition on species belonging to the same group as the plants under study had been published. Assuming the production of secondary metabolites is restricted to the genus level, the conclusion concerning the observations made in this study can be drawn.

Most plant-based secondary metabolites are phenolic compounds, alkaloids, flavonoids, and tannins (Gupta et al. 2004; Wong et al. 2006; Uddin et al. 2009). These natural products possess large pharmacological properties including cytotoxic and cancer chemopreventive effects. Flavonoids, steroids, and triterpenoids particularly exert multiple biological effects due to their antioxidant and free radical scavenging abilities (Gupta et al. 2004). Reports have shown antioxidant and anticancer activity associated with a variety of classes such as polyphenols, flavonoids, and catechins (Uddin et al. 2009).

The literature supports the results of this study. Numerous alkaloids, flavonoids, and anthocyanins were

isolated from *Z. ucronate* (Suliman 2010). Anticancer activity of *Z. jujube* against HepG2 cells has also been reported (Huang et al. 2007). Some triterpenoid acids isolated from *Ziziphus jujube* showed moderate anticancer activity against some cell models (HT-29, HepG-2, and NCI-H460) using the MTT assay (Guo et al. 2011). Pyrrolizidine alkaloids and indicine-n-oxide were reported for H. indicum and other Heliotropium species (Spjut, 1985; Velasco et al. 2005). Methanol extracts of *H. zeylanicum* also possessed anticancer and antioxidant activity against Ehrlich ascites carcinoma cells in Swiss albino mice in vivo (Kandasamy et al. 2005). Some Gnidia species contain diterpene esters and coumarins which possessed antitumor activity (van Wyk et al. 1997, van Wyk and Gericke 2000). All these works are in support of the findings of this study.

The exact mechanism behind the biological activity is not known, but speculations have been made. Polyphenolic compounds might inhibit cancer cells progression by xenobiotic metabolizing enzymes that alter metabolic activation of potential carcinogens. While some flavonoids could also change hormone production and inhibit aromatase to prevent the progression of cancer cells. In another theory, the mechanism of action of anticancer activity of phenolics could be done by disrupting cellular division during mitosis at the telophase stage. Phenolics reduce the amount of cellular protein, mitotic index, and formation colony during cell proliferation. The more the number of hydroxyl groups in the phenolics, the higher is the antioxidant activity. The presence of a 4-carbonyl group of the flavonoid molecule also plays roles to the anticancer activity. Also, the presence of 2, 3-double bond in the flavonoid molecules correlates with mitochondrial damage and cancer cell death (Prasad et al. 2009). Some extracts of traditional medicinal plants studied here have the potential to yield useful antioxidant and anticancer drugs. However, further studies need to be conducted the isolation of potentially valuable drugs from these extracts.

Antimicrobial activity

The method used here method entails two criteria: the first being the number of plant extracts that showed excellent antimicrobial activity, i.e., MIC value of ≤ 1 mg/mL and the second is the average MIC value obtained by the plant extracts against a specific pathogen (Table 10) (Suliman 2010). Seven extracts out of the twelve tested plant extracts showed excellent antimicrobial activity against MRSA and *S. epidermidis* (MIC value ≤ 1 mg/mL), making the two pathogens the most sensitive. *P. aeruginosa* and *C. tetani* were the second sensitive as good antimicrobial activity (MIC value ≤ 1 mg/mL) against these pathogens were acquired in five of the twelve extracts tested. *C. albicans* and *E. coli* were the least sensitive as no good antimicrobial activity was received against these pathogens by the plant extracts tested.

About the average MIC values, the lowest average MIC value (0.91 mg/mL) was demonstrated against *S. epidermidis*, followed by *C. tetani*, *Streptococcus* A and MRSA showing average MIC values of 0.96 mg/mL, 0.99 mg/mL, and 1.10 mg/mL respectively. *C. albicans*, *M. terrae*, and *E. coli* were the least sensitive (Table 10).

Extract that showed the best antimicrobial activity

We determined which extract exhibited the best antimicrobial activity against the microorganisms tested using the method described by Suliman (2010). Two criteria were adopted in this method: first, determining the lowest MIC value of each extract and the pathogen against which this MIC value. Second, determining the number of pathogens against which the extract obtained a MIC value of ≤ 1 mg/mL. The criteria summarize the pathogens against which the plant extracts obtained good antimicrobial activity.

ZD exhibited the lowest MIC value (0.078 mg/mL) among all extracts tested against MRSA. HD and HH acquired the second lowest MIC values (<0.156 mg/mL) against M. terrae. The next lowest MIC value (0.3125 mg/mL) was shown by ZE, GE, and GD against M. terrae, *Streptococcus* A, *P. aeruginosa*, MRSA or *S. epidermidis*. For all extracts, a MIC value ≤ 1 mg/mL was obtained against at least one of the pathogens, except for ZH extract which lowest MIC value was 1.25 mg/mL. This result makes many of the extracts screened good antimicrobial agents against pathogens.

About the second criterion which was the number of pathogens against which the plant extracts obtained MIC value ≤ 1 mg/mL, ZD possessed the broadest- spectrum activity, exhibiting good antimicrobial activity against six of the eight test pathogens. GE followed, obtaining a MIC value ≤ 1 mg/mL against five of the eight tested pathogens followed by GD and GH, which both exhibited good antimicrobial activity against half (four) of the tested pathogens. ZH was the least active, not obtaining a MIC value of ≤ 1 mg/mL against any of the tested pathogens (Table 11).

Table 10. Summary of antimicrobial activity of plant extracts against wound pathogens.

Pathogen	Number of extracts with MIC value ≤ 1 mg/mL	Average MIC value (mg/mL)
M. terrae	4	3.40
E. coli	0	8.75
P. aeruginosa	5	1.88
Streptococcus A	4	0.99
C. albicans	0	2.29
MRSA	7	1.10
C. tetani	5	0.96
S. epidermidis	7	0.91

Table 11. Lowest MIC values obtained per plant extracts and pathogens against which MIC values of ≤ 1 mg/mL were obtained.

Plant extract	Lowest MIC value (mg/mL)	Pathogen against which the lowest MIC value was obtained	Pathogens against which the extract obtained an MIC value of ≤ 1 mg/mL
ZD	0.078	MRSA	*M. terrae*, *P. aeruginosa*, *Streptococcus* A, *C. tetani*, *S. epidermidis*, MRSA
ZE	0.3125	*M. terrae*	*M. terrae*, *C. tetani*, MRSA
ZH	1.25	*P. aeruginosa*, *Streptococcus* A, *C. tetani*, *S. epidermidis*, MRSA, *C. albicans*	None
ZM	0.625	*C. tetani*, MRSA	*C. tetani*, MRSA
GD	0.3125	*P. aeruginosa*, *Streptococcus* A, *S. epidermidis*, MRSA	MRSA, *P. aeruginosa*, *Streptococcus* A, *S. epidermidis*
GE	0.3125	*P. aeruginosa*, *Streptococcus* A, MRSA	MRSA, *P. aeruginosa*, *Streptococcus* A, *C. tetani*, *S. epidermidis*
GH	0.625	*P. aeruginosa*, *Streptococcus* A, MRSA, *C. tetani*	*C. tetani*, *P. aeruginosa*, *Streptococcus* A, MRSA
GM	0.625	*S. epidermidis*	*S. epidermidis*
HD	<0.156	*M. terrae*	*S. epidermidis*, *M. terrae*
HE	0.625	MRSA, *S. epidermidis*	MRSA, *S. epidermidis*
HH	<0.156	*M. terrae*	*M. terrae*
HM	0.625	*P. aeruginosa*, *S. epidermidis*	*P. aeruginosa*, *S. epidermidis*

ZD, ZE, ZM, GD, GE, GH, GM, HD, HE, HH and HM all exhibited good antimicrobial activities against some of the pathogens tested. ZD, ZE, and ZM showed excellent antimicrobial activity against *M. terrae*, *P. aeruginosa*, *Streptococcus* A, *S. epidermidis*, *C. tetani* or MRSA. Antimicrobial activity showed by extracts of *Z. mucronata* was as expected since the antimicrobial activity of different extracts of *Z. mucronata* against different pathogens has been published. Good antimicrobial activity for methanol and acetone extract of both the leaves and bark of *Z. mucronata* against *S. aureus, Bacillus subtillis, Klebsiella pneumoniae* have been reported (MIC ≤ 6.25 mg/mL) (Mthethwa 2009). Antimicrobial activity of ethanol extracts of *Z. mucronata* against *S. aureus* and *E. coli* has also been reported (Adamu et al. 2004). Olajuyigbe and Afolayan (2011) said antimicrobial activity of methanolic extracts of the bark of *Z. mucronata* against *E. coli* and *S. aureus.*

The findings of the potent antimicrobial activity of some plant extracts in this study support the use of these plant extracts in traditional medicine. The medicinal plants studied here are all used in the treatment of wounds, and the antimicrobial activity reported for these plants supports the traditional use of these plants. The prevention of microbial wound infections is one of the critical factors in wound healing and wound management (Ayyanar and Ignacimuthu 2009; Davis and Perez 2009). However, more studies need to be carried out to confirm the role of the different natural products in the observed biological activity.

Conclusion

The yields per extraction of the two extraction methods were not significantly different, which indicates the preference of the cold extraction method to lower the risk of destroying valuable compounds. *Z. mucronata* showed high antioxidant activity that could be attributed to flavonoids, alkaloids, and terpenes, as reported earlier. High apoptotic activity was reported for *H. ciliatum.* Heliotropium species were said to possess toxic pyrrolizidine alkaloids and indicine-n-oxide. Antitumor activity has also been recorded in some Heliotropium species. Diterpene was found in Gnidia species, which could explain the toxicity observed for *G. polycephala*, though detail analysis is required to confirm this. Low toxicity was published for the water extracts of the three plants. Water extracts are commonly used in conventional medicine and the low toxicity observed here is of significance to their application in the treatment of other diseases and ailments other than cancer. The low toxicity could also indicate a low concentration of the active compound since crude extracts were analyzed. Antimicrobial activity of extracts against different wound pathogens is not surprising as these plants are also used in the treatment of wounds.

Nevertheless, many aspects of this research need to be further investigated to confirm the observed activity. The simple assays employed in this study gave good results about the potential of these plant extracts as antioxidant, anticancer and antimicrobial agents. More in-depth studies are necessary to confirm the activity of the promising extracts. These include the employment of other antioxidant assays such as the total antioxidant assay, whole phenolic assay, and the superoxide assay to mention a few and compare the antioxidant potential of the extracts through different methods. For the anticancer assay, numerous cell lines were used, some of which were only used against some of the extracts and not others. Also, for some tests such as the APOPercentage™ assay, the test concentrations were high, and as a result, it was not possible to determine the IC50 concentrations. In future, the use of fewer cell lines and instead the test of all plant extracts at different levels against the cell lines is suggested as this will enable the comparison of the activity of various extracts against a particular cell line and give their IC50 concentrations. An assay-guided fractionation and purification of the crude extracts are also recommended because this will provide detail information as to which

fraction of the crude extract is active and the percentage activity as compared to the crude extract.

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