

***In vitro* cytotoxicity screening of wild plant extracts from Saudi Arabia on human breast adenocarcinoma cells**

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ABSTRACT. This study investigated the *in vitro* anticancer activities of a total of 14 wild angiosperms collected in Saudi Arabia. The cytotoxic activity of each extract was assessed against human breast adenocarcinoma (MCF-7) cell lines by using the MTT assay. Among the plants screened, the potential cytotoxic activity exhibited by the extract of *Lavandula dentata* (Lamiaceae) was identified, and we analyzed its anticancer potential by testing antiproliferative and apoptotic activity. Our results clearly show that ethanolic extract of *L. dentata* exhibits promising cytotoxic activity with an IC₅₀ value of 39 µg/mL. Analysis of cell morphological changes, DNA fragmentation and apoptosis (using an Annexin V assay) also confirmed the apoptotic effect of *L. dentata* extract, and thus, our data call for further investigations to determine the active chemical constituent(s) and their mechanisms of inducing apoptosis.

Key words: Cytotoxicity; Apoptosis; Cancer; MCF-7; Saudi Arabia

INTRODUCTION

Breast cancer is a leading cause of death in women worldwide (American Cancer Society, 2012). There has long been standing interest in the identification of natural products for the treatment of various diseases for thousands of years. Natural products possess immense pharmacological significance in the development of drugs (Dixon et al., 2007; Baker et al., 2007; Harvey, 2008) including cancer (Graham et al., 2000; Figueroa-Hernández et al., 2005; Madhuri and Pandey, 2009; Tan et al., 2011; Newman and Cragg, 2012; Kuno et al., 2012), and were discovered through plant bioprospecting (Mann, 2002). The majority of drug candidates, such as paclitaxel, etoposide, camptothecin, vinca alkaloids, indole alkaloids, podophylotoxin derivatives, etoposide and teniposide, currently used in clinical cancer chemotherapy, were originally derived from plants. The efficacy of chemotherapy, radiotherapy, hormonal therapy, or surgery, which are mainly used for the treatment of cancer, are well-known for side effects (Stopeck and Thompson, 2012); hence, the identification of novel natural products that possess better effectiveness against cancer, but less harmful effects have become desirable (Lachenmayer et al., 2010), and therefore, natural products are continuously being explored worldwide. The floral elements of unique arid plant biodiversity of Saudi Arabia are being practiced in folk medicine since ancient times (Rahman et al., 2004). Plants that grow under harsh desert stress conditions produced a high concentration of secondary metabolites that impart a wide range of pharmacological effects including anticancer activities (Harley et al., 2012). As part of our efforts to study wild plants from desert regions for pharmacological activities, our study provides data on the cytotoxic potential from a total number of 14 extracts from wild flowering plants of Saudi Arabia.

MATERIAL AND METHODS

Plant materials and preparation of crude extracts

A total of 14 flowering plants growing wild in nature were collected along with voucher specimens (Table 1) from different geographical regions of Saudi Arabia. The plants were identified through consultation of the flora of Saudi Arabia (Chaudhary, 2001), and specimens were housed at the Herbarium of the King Saud University (KSUH) in Riyadh, Saudi Arabia. The collected plant materials were rinsed thoroughly with tap water to remove extraneous contaminants and then cut into small pieces, oven-dried at 50°C until the dry weight stabilized, and grounded into a powder with an electric-grinder. A crude extract was prepared by macerating the powdered plant materials (1000 g) in 95% ethanol at room temperature for 1 week. Extracts were filtered and concentrated using a rotary evaporator at low temperature and pressure. The crude extracts were weighed and stored at -20°C until use.

Dilution of test materials and reference drugs

The crude extract from each plant was initially dissolved in 50% ethanol. Concentrated stock solution (100 mg/mL) of each extract was prepared, diluted to 1.0 mg/mL by adding complete cell culture media, and then serially diluted with the same media to obtain working solutions of 6 concentrations: 1.0, 0.50, 0.25, 0.12, 0.06 and 0.03 mg/mL. Doxorubicin

(Sigma Aldrich, St. Louis, MO, USA) was dissolved in complete cell culture medium at a concentration of 100 μ M for use as a positive control.

Table 1. Plant extracts screened in this study.

Taxon	Family	Voucher specimen	Percentage survival					
			1.0	0.5	0.25	0.12	0.06	0.03
<i>Aizoon canariense</i>	Aizoaceae	FMA22 (KSUH)	75	78	80	85	91	98
<i>Alhagi maurorum</i>	Fabaceae	FMA34 (KSUH)	60	64	70	73	78	88
<i>Anastatica hierochuntica</i>	Brassicaceae	FMA20 (KSUH)	70	72	76	84	88	90
<i>Capparis spinosa</i>	Capparaceae	FMA27 (KSUH)	72	76	81	86	91	95
<i>Centaurothamnus maximus</i>	Asteraceae	FMA29 (KSUH)	62	68	71	76	80	89
<i>Clutia lanceolata</i>	Euphorbiaceae	FMA12 (KSUH)	52	58	65	72	81	84
<i>Echinops sheilae</i>	Asteraceae	FMA28 (KSUH)	60	65	72	75	82	87
<i>Fagonia indica</i>	Zygophyllaceae	FMA14 (KSUH)	50	68	62	68	75	78
<i>Hyphaene thebaica</i>	Arecaceae	FMA26 (KSUH)	72	80	84	91	96	98
<i>Lavandula dentata</i>	Lamiaceae	FMA8 (KSUH)	25	31	37	42	46	59
<i>Malva parviflora</i>	Malvaceae	FMA19 (KSUH)	72	75	81	85	93	97
<i>Neurada procumbens</i>	Neuradaceae	FMA9 (KSUH)	56	65	83	88	90	96
<i>Teucrium oliverianum</i>	Lamiaceae	FMA3 (KSUH)	66	69	73	76	89	91
<i>Tribulus macropterus</i>	Zygophyllaceae	FMA30 (KSUH)	56	64	79	83	89	96

The extracts were subjected to cytotoxicity testing against MCF-7 cells by MTT assay using two-fold dilutions of six concentrations (1.0 to 0.03 mg/mL).

Cell culture methods

The human breast adenocarcinoma cell line (MCF-7) was procured from ATCC (Rockville, MD, USA). The cells were cultured in a humid environment at 37°C and 5% CO₂ in minimum essential medium (Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen). At 85-90% confluence, cells were harvested using 0.25% trypsin/ EDTA solution and sub-cultured onto 6-well or 96-well plates according to the experimental requirements.

Cytotoxicity assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay developed by Mosmann (1983) with modification was used to screen the cytotoxic activity of plant extracts. Briefly, the MCF-7 cells (1 x 10⁴ cells/well) were grown overnight on 96-well flat bottom cell culture plates, and were then exposed to 6 different concentrations (1.00, 0.5, 0.25, 0.12, 0.06, and 0.03 mg/mL) of each ethanolic extract of plants for 24 h. In addition, negative/vehicle controls, and a positive control (Doxorubicin) were also used for comparison. After the completion of desired treatment, 10 μ L MTT reagent (Invitrogen) prepared in 5.0 mg/mL phosphate buffered saline (PBS) was added to each well and further incubated for 3 h at 37°C. Finally, the medium with MTT solution was removed, and 200 μ L of DMSO (Sigma Aldrich) were added to each well and further incubated for 20 min. The optical density (OD) of each well was measured at 550 nm by using a Synergy microplate reader (BioTek, Winooski, VA, USA). Results were generated from 3 independent experiments and each experiment was performed in triplicate. The percentage of cytotoxicity compared to the untreated cells was determined. The potential cytotoxic activity exhibited by the extract of *Lavandula dentata* was chosen, and further MTT assay was performed with 6

lower ranges of concentrations (300, 150, 75, 37.5, 18.75, and 9.37 $\mu\text{g}/\text{mL}$) to determine the IC_{50} value (the concentration at which 50% cell proliferation is inhibited).

Morphological changes analysis

MCF-7 cells were treated with IC_{50} concentration of crude ethanolic extract of *L. dentata* for 24 h. After the end of incubation period, cells were observed under phase contrast inverted microscope equipped with a digital camera (Olympus IX51, Tokyo, Japan) at 400X magnification.

Apoptosis induction assay

Apoptosis was measured using flow cytometry to quantify the levels of detectable phosphatidylserine on the outer membrane of apoptotic cells (Evens et al., 2004). An annexin-V FITC apoptosis detection Kit (BD Biosciences, San Jose, CA, USA) was used for the differentiation of apoptotic and necrotic cells. Briefly, MCF-7 cells at a density of 1×10^5 cells/mL were incubated with extract (IC_{50} concentration) of *L. dentata* for 24 h. All adhering cells were harvested using trypsin/EDTA solution and washed twice with PBS before being transferred to a sterile centrifuge tube (1×10^6 cells/mL). Samples were prepared following manufacturer instructions. Annexin-V/propidium iodide (PI) fluorescence was analyzed for each sample using a FACSCalibur flow cytometer (BD Biosciences). A total of 10,000 events were acquired for each sample and data were analyzed using the Cell Quest Pro software (BD Biosciences).

Apoptotic DNA ladder assay

MCF-7 cells were treated with various concentrations of crude ethanolic extract of *L. dentata* (30, 20 and 10 $\mu\text{g}/\text{mL}$) for 24 h. After incubation, DNA from treated and untreated cells was extracted using an apoptotic DNA ladder kit (Roche, Mannheim, Germany), following manufacturer instructions. The quantity and purity of extracted DNA was estimated by measuring OD at A_{260} nm and A_{280} nm using a GeneQuant UV spectrophotometer (Amersham Biosciences, Amersham, UK). DNA samples were separated using 1% agarose gel electrophoresis and stained with 10 $\mu\text{g}/\text{mL}$ ethidium bromide. Finally, the DNA was visualized under UV light and images were captured using a gel documentation system (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All experiments were carried out with 3 replicates and values are reported as means \pm standard error (SE). Microsoft Office Excel was used for calculations and plotting the estimated means and standard deviations in the graphs. Data were statistically analyzed using the Student *t*-test and applying a significance level of $P < 0.05$.

RESULTS

In this study, we carried out an initial screen of ethanolic extracts from 14 species (belonging to twelve families) of flowering plants that grow wildly in Saudi Arabia: *Aizoon*

canariense L. (Aizoaceae), *Alhagi maurorum* Medik. (Fabaceae), *Anastatica hierochuntica* L. (Brassicaceae), *Capparis spinosa* L. (Capparaceae), *Centaurothamnus maximus* (Forssk.) Wagenitz & Dittrich (Asteraceae), *Clutia lanceolata* Forssk. (Euphorbiaceae), *Echinops sheilae* Kit Tan (Asteraceae), *Fagonia indica* Burm. f. (Zygophyllaceae), *Hyphaene thebaica* (L.) Mart. (Arecaceae), *L. dentata* L. (Lamiaceae), *Malva parviflora* L. (Malvaceae), *Neurada procumbens* L. (Neuradaceae), *Teucrium oliverianum* Ging. Ex Benth. (Lamiaceae), and *Tribulus macropterus* Boiss. (Zygophyllaceae). Screening was performed against human breast adenocarcinoma cells. To date, the identification of candidates having anticancer potential from the arid floristic biodiversity of the Arabian gulf region in general is concerned; an exhaustive literature survey reveals that there are such limited documentation (Amin and Mousa, 2007; Mothana et al., 2009), and wild plants of Saudi Arabia in particular have been previously poorly explored. Apart from some recent reports (Almehdar et al., 2012; Elkady, 2013) on medicinal plants, a perusal of literature also reveals that the plants screened in this study have not been previously included in a plant bioprospecting program for anticancer activity. The percentage viability of cells after treatment of the extract indicates that out of the above-mentioned 14 plants extracts, only *L. dentata* induced over 40% cell death at a minimum treatment concentration 0.3 mg/mL (Table 1). Dose response studies of all the extracts are summarized in Figure 1.

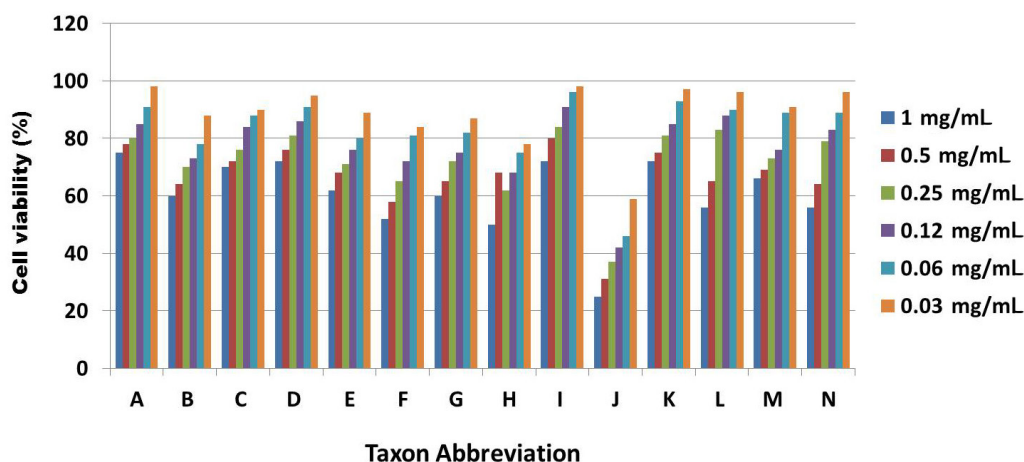


Figure 1. A total of 14 plant extracts were screened for their ability to inhibit MCF-7 cell proliferation using MTT assay after 24 h of exposure to the extracts. Most of the extract showed mild cytotoxicity at higher concentrations. However, ethanolic extract of *Lavandula dentata* showed high toxicity at lower concentration. Data are reported as mean values of three independent experiments. Taxon abbreviations: **A.** *Aizoon canariense*; **B.** *Alhagi maurorum*; **C.** *Anastatica hierochuntica*; **D.** *Capparis spinosa*; **E.** *Centaurothamnus maximus*; **F.** *Clutia lanceolata*; **G.** *Echinops sheilae*; **H.** *Fagonia indica*; **I.** *Hyphaene thebaica*; **J.** *L. dentata*; **K.** *Malva parviflora*; **L.** *Neurada procumbens*; **M.** *Teucrium oliverianum*; **N.** *Tribulus macropterus*.

Based on these screening results (Table 1), the extract of *L. dentata* was selected, and subjected to IC_{50} determination (Figure 2). The relative number of viable cells as a percentage of control was calculated, defining the absorbance at 550 nm for the control as 100%. The IC_{50} value was graphically obtained by plotting the percentage growth inhibition against the corresponding different concentrations of the test compound used. The extracts of *L. dentata*

showed cytotoxicity at an IC_{50} value of approximately 39 $\mu\text{g}/\text{mL}$, while doxorubicin showed 63% growth inhibition at 100 μM (data not shown). The anticancer potential was analyzed according to proliferation inhibition and apoptotic activity. No significant change in morphology was observed in control cells. The cells appeared to have a normal shape, were attached to the surface, and reached about 95-100% confluence (Figure 3A). Characteristics of apoptotic cell death were evident in *L. dentata* treated samples. Cell shrinkage, loss of cell adhesion, reduced cell density, and cell debris were clearly observed (Figure 3B).

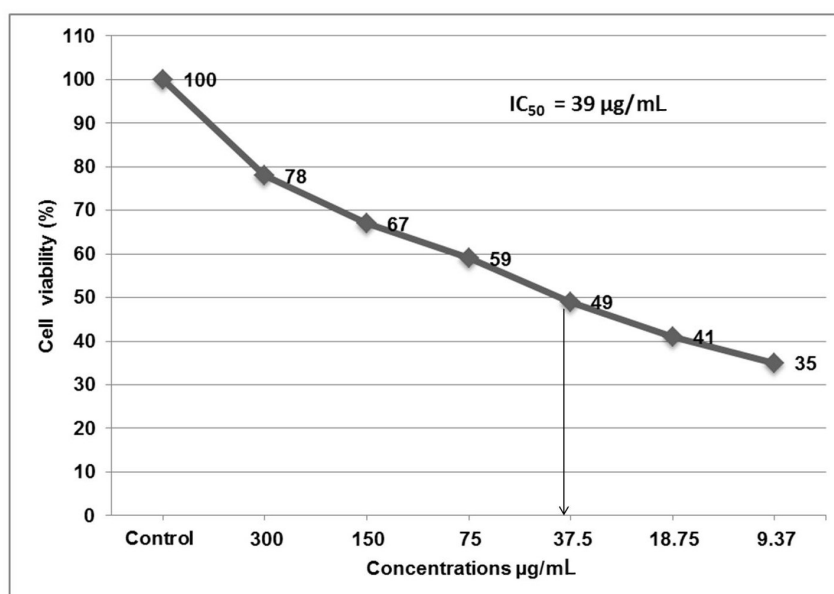


Figure 2. Inhibition of MCF-7 cell proliferation by crude ethanolic extract of *Lavandula dentata*. Cells were treated with indicated concentrations of extract for 24 h, and cell viability was determined by the MTT assay. The IC_{50} value was estimated at 39 $\mu\text{g}/\text{mL}$ (indicated by arrow).

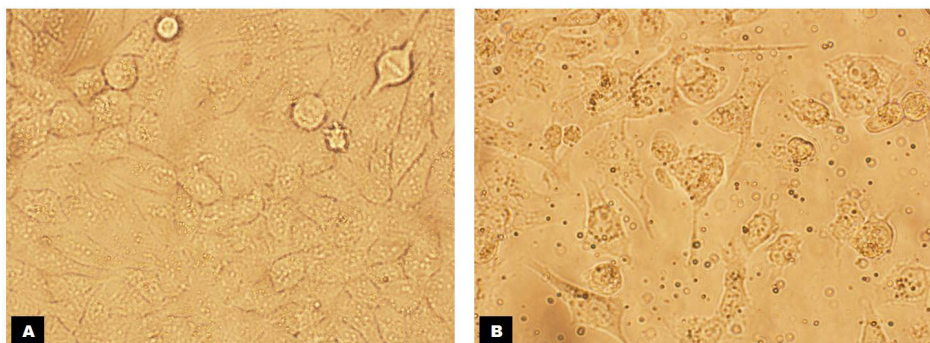


Figure 3. Morphological changes in MCF-7 cells after the cells were exposed to IC_{50} concentration of ethanolic extract of *Lavandula dentata* for 24 h. **A.** Untreated cells appeared in normal shape with about 95-100% confluence. **B.** Cell shrinkage, loss of cell adhesion, reduced cell density along with cell debris were observed in treated cells. Images were captured in inverted microscope (Olympus, Japan) at 400X magnification.

DNA fragmentation resulting from significant DNA damage was observed as a ladder pattern by agarose gel electrophoresis (Figure 4). The DNA from all 3 treated-group DNA showed 180-200 base pair internucleosomal DNA fragments, whereas untreated DNA appeared as single band. Furthermore, we quantified the extent of apoptosis in cells labeled with Annexin-V/PI staining using flow cytometry. For control MCF-7 cells, no significant difference in the proportion of normal cells and those in early or late apoptosis were observed (Figure 5A). Only 2-3% cells were dead or undergoing apoptosis which is a normal event for cells growing in cultures. However, after exposure to crude ethanolic extract of *L. dentata* (30 $\mu\text{g}/\text{mL}$ for 24 h), the proportion of early and late apoptotic cells increased significantly ($P < 0.05$) as compared with control cells with values of about 25 and 10%, respectively (Figure 5B).

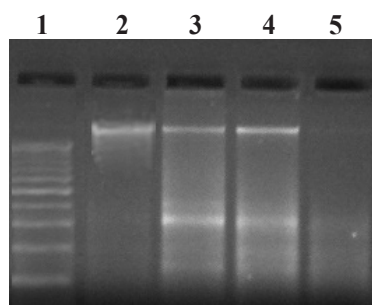


Figure 4. Analysis of genomic DNA fragmentation in MCF-7 cells after treatment for 24 h with crude ethanolic extract of *Lavandula dentata* (30, 20, 10 $\mu\text{g}/\text{mL}$). DNA Fragmentation was assessed on agarose gel electrophoresis. Lane 1 = 100 bp DNA ladder used as marker; lane 2 = control; lane 3 = 30 $\mu\text{g}/\text{mL}$; lane 4 = 20 $\mu\text{g}/\text{mL}$; lane 5 = 10 $\mu\text{g}/\text{mL}$.

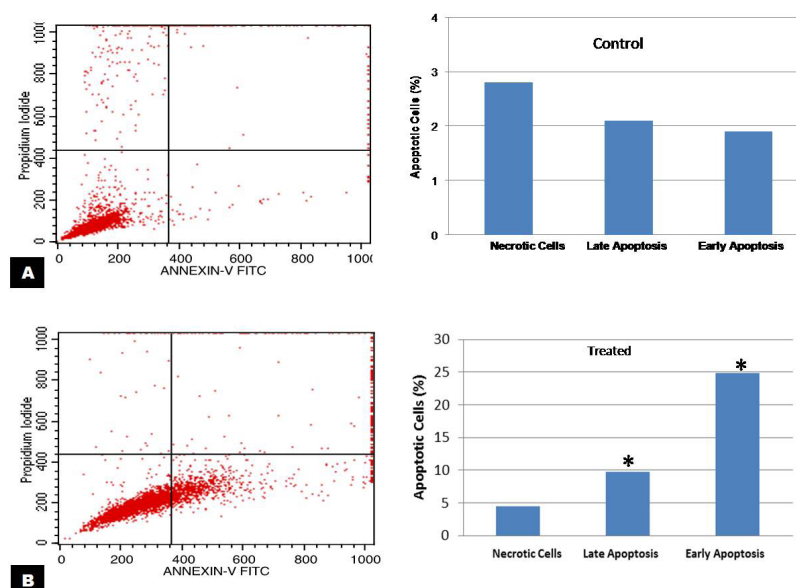


Figure 5. Apoptosis analysis of MCF-7 cells treated with IC_{50} concentration of *Lavandula dentata* extract for 24 h using annexin-V FITC and propidium iodide staining. **A.** Flow cytometric scans of untreated cells showed only 2-3% of cells in early or late apoptosis stage. **B.** In treated cells, significant increase ($P < 0.05$) marked by asterisks in percentage distribution of cells in early (25%) and late (10%) apoptosis were observed.

DISCUSSION

The genus *Lavandula* (commonly known as lavender) belongs to the family Lamiaceae and comprises 28 species. Many members of the family Lamiaceae are well-known for their pharmacological effects such as anticonvulsant, sedative, antispasmodic, analgesic, antioxidant, or local anaesthetic activity (Ghelardini et al., 1999; Lis-Balchin and Hart, 1999; Hosseinzadeh et al., 2000; Kovatcheva et al., 2001). Phytochemical analyses of lavender have demonstrated the presence of many monoterpenes (especially linalyl acetate, linalool), which are responsible for its pharmacologic activity (Lis-Balchin and Hart, 1999; Gilani et al., 2000). Owing to its delightful odor, lavender has been widely used in perfumes and cosmetics (Gilani et al., 2000), and oils from a variety of lavender species have been demonstrated to have neurological, antimicrobial, pesticidal, and dermatological activities (Cavanagh and Wilkinson, 2002). The oil of *L. angustifolia* is chiefly composed of linalyl acetate and linalool and is considered to be one of the mildest of known plant essential oils with known effects on wound healing, cytotoxicity to human skin cells (Prashar et al., 2004), and inflammation and analgesia (Hajhashemi et al., 2003). An aqueous extract of *L. angustifolia* protected the neurons against glutamate toxicity (Buyukokuroglu et al., 2003). Moon et al. (2006) demonstrated that low ($\leq 1\%$) concentrations of *Lavandula angustifolia* and *Lavandula intermedia* oil can completely eliminate *Trichomonas vaginalis*, *Giardia duodenalis* and *Hexamita inflata*. An aqueous extract of *Lavandula stoechas* possess cytotoxic and genotoxic effects (Çelik and Aslantürk, 2007). Berrington and Lall (2012) evaluated *L. spica* for anticancer activity on the cervical epithelial carcinoma (HeLa) cell line. *L. dentata* has previously been reported to have range of biological activities, such as anticonvulsant, sedative, antispasmodic (Gilani et al., 2000), wound healing, rheumatic, urine retention, kidney stones, antiseptic (Rahman et al., 2004), and antiprotozoal (Al-Musayeib et al., 2012).

By screening 14 ethanolic extracts of wild plants from Saudi Arabia for their anti-proliferative properties against human adenocarcinoma breast cancer (MCF-7) cell lines, we found that most extracts showed mild or no toxicity, whereas the crude ethanolic extract of *L. dentata* exhibited promising cytotoxic activity. The identification of novel bioactive compounds with anti-cancer properties, and the elucidation of the mechanisms by which the anti-cancer properties derived from the natural products are of immense importance. The results of this study provide the basis for further investigation of *L. dentata* for potential identification of novel bioactive compounds with therapeutic and anti-cancer properties.

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