Austin Journal of Medical Oncology

Open Access

Austin Publishing Group

Special Article - Breast Cancer

Growth Inhibitory Effect of *Cymbopogan Schoenanthus* on Triple Negative Breast Cancer (MDA-MB-231) and Cervical Cancer (HEp-2) Cells: Piperitone and Elemol as an Active Principle

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Received: July 11, 2016; **Accepted:** August 09, 2016; **Published:** August 11, 2016

Abstract

Cancer is the leading causes of mortality among human population of all ages. Noticeably breast and cervical cancer are the most common malignancy in women throughout the world. Chemotherapy is feasible format to treat the patients but emerging issues such as tumor relapse, drug resistance, and metastasis remain as unresolvable challenges. Although thousands of chemotherapeutic agents are reported for cancer cell killing effect, due to its extreme toxicity and accumulation of new mutation in response to therapy raises the question on their use in clinic. Finding a new molecule from natural sources and through synthetic approach to arrest cancer cell proliferation with low toxicity on normal counterpart remains an active area in cancer drug research discovery. In this study we tested the cytotoxic effect of Oman's *Cymbopogan schoenanthus* on breast cancer cells (MDA-MB-231), cervical cancer cells (HEp-2) and Vero cells (normal) and we analyzed their chemical profile by Gas Chromatography Coupled Mass Spectrometry (GCMS).

Materials and Methods: MDA-MB-231, HEp-2 and Vero cells were treated with different concentration (25, 50, 75 and 100 µg/ml) of methanolic extract of *C. schoenanthus* for 24 hrs and cell viability measured by MTT assay. Doxorubicin was used as positive control. Morphology of treated cells was photographed after treatment. Further GC MS chemical profiling of extract was done.

Results: The data reveals that extract can induce the considerable dose dependent cell death at each concentration tested after 24hr treatment whereas, vero cells sustained survival despite of treatment. MDA-MB-231 and HEp-2 cells attained necrotic state after 24h of treatment. Further chemical profiling data indicates that piperitone is major component about 38.6% followed by elemol (27.9%), alpha eudesmol (14.5%) and beta eudesmol (4.6%).

Conclusion: It is clearly evidenced from the results that *C. schoenanthus* derives active principles specifically targeting tumor cells with very low toxicity on normal cells. Anti-cancer activity of *C. schoenanthus* might be due to synergistic effect of active ingredients. Mechanism of cancer cell death induced by *C. schoenanthus* and *in vivo* xenograft studies is ongoing on our laboratory.

Keywords: Breast cancer; Cervical cancer; *Cymbopogan schoenanthus*; Piperitone; Oman

Introduction

Cancer is the leading causes of mortality throughout the world. In fact, it is responsible for 7.6 million deaths in 2008 [1]. It has been projected that the cancer mortality rate will extend to about 30.1 million by 2030 [1]. Among the different cancer types, Breast Cancer (BC) is the most common malignancy in women throughout the world, and it accounts for about 18% of all female cancers and there are approximately 600,000 annual deaths worldwide [2,3]. Followed by breast cancer, cervical cancer is the second most frequent cancer and the second leading cause of cancer death in women worldwide, with approximately 470,000 new cases and 233,000 deaths per year [4].

The high mortality rate is largely due to lack of effective therapies for eliminating disease in women with high-grade breast cancer and cervical cancer and the lack of response to chemotherapy of inoperable disease. Chemotherapy is the feasible way of comparing other therapeutic formats and it is often chosen by clinician to treat the patients. Molecules used in chemotherapy are only effective when applied to patients with small tumors detected in an early stage, so chemotherapy is not effective in patients with terminal cancer or metastasis. Furthermore, the compounds used generates severe side effects associated with their necrotic activity [5,6], a fact that indicates the need to seek new compounds, ones with high anti-proliferative activity on cancer cells but low necrotic effects on normal cells.

Citation: Hakkim FL, Al-Buloshi M and Achankunju J. Growth Inhibitory Effect of *Cymbopogan Schoenanthus* on Triple Negative Breast Cancer (MDA-MB-231) and Cervical Cancer (HEp-2) Cells: Piperitone and Elemol as an Active Principle. Austin J Med Oncol. 2016; 3(1): 1027.

Searching for new effective anticancer drugs includes broad spectrum of biological and biochemical testing. Besides cytotoxicity, a new drug candidate should have other properties such as good selectivity and low system toxicity.

In this scenario bioactive phytochemicals attracted much attention due to their enormous chemical variability from the high diversity of plants and since they are exhibiting the ability to inhibit cancer cytogenesis by suppressing the tumor initiation, promotion, and progression are being considered as potential biocompatible anticancer agents. In this regard, the anti-proliferative activity of several phytochemical extracts was reported [7-9]. But emerging drug resistance and relapse due to accumulation of mutation in response to therapy is always problematic. These hurdles always induce the researchers to search for an alternative. In the quest for new therapeutics, plants were and still considered as one of the main sources of biologically active materials. It has been estimated that about 50% of the prescription products in Europe and USA were originated from natural products, including plants or their derivatives [10]. Our group involved in finding the natural product based drug for cancer treatment from Oman's medicinal plants. Recently, we reported that heavy terpenes derived from Oman's frankincense resin can induce significant breast cancer cell death [11]. In this study we tested anti proliferative efficacy of active principles derived from Oman's *Cymbopogan schoenanthus*. Essential oil extracted from *C. schoenanthus* reported for Anthelmintic [12] and insecticidal activity [13]. Ethanol and aqueous extracts of *C. schoenanthus* elicit significant anti-stress [14] and anti-nephrotoxic effect [15] respectively. Anti-cancer activity of *cymbopogan* species has been well enumerated in the literature [16- 19]. However anti-proliferative potential of *C. schoenanthus* not been reported yet. In the current study we report the chemical profiling and *in vitro* cytotoxic efficacy of *C. schoenanthus* on breast cancer cells (MDA-MB-231), cervical cancer cells (HEp-2), and vero cells (normal cells).

Materials and Methods

Plant collection

C. schoenanthus was collected from different area of Ibra, Oman. Plant was identified by Jackson Anchakunju, Botanist, A'Sharqiyah University (ASU). Voucher specimen is deposited in our herbarium collections.

Extraction

Freshly collected *C. schoenanthus* was shade dried for seven days and pulverized using mechanical grinder. Powdered material was extracted with methanol using soxhelet apparatus. Later the dried extract was obtained by evaporating methanol at room temperature. The dried extract was stored in -20°C until use for experiment.

Gas Chromatography Mass Spectrometry (GCMS) analysis

GCMS analysis was performed on a Perkin Elmer Clarus 680 GC System, fitted with a Rtx®-5MScapillary column (30 m×0.25 mm i.d. × 0.25 µm film thickness; maximum temperature, 250°C), coupled to a Perkin Elmer Clarus SQ8S MS. Ultra-high purity helium (99.9999%) was used as carrier gas at a constant flow of 1 ml/minute. The injection, transfer line and ion source temperatures were 270, 240 and 240°C, respectively. The ionizing energy was 70 eV. Electron multiplier (EM) voltage was obtained from autotune. All data were obtained by collecting the full-scan mass spectra within the scan range 40-550 amu. The injected sample volume was 1 µl with a split ratio of 50:1. The oven temperature program was 60°C and accelerated at a rate of 3°C /minute-240°C. The unknown compounds were identified by comparing the spectra obtained with mass spectrum libraries (NIST MS20 2011).

Cell lines and culture method

Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium (MEM), RPMI 1640 medium, fetal bovine serum were purchased from Sigma Aldrich chemical Co (St. Louis, USA). All the other chemicals used in this study were of pure analytical grade. MDA-MB-231, HEp-2 and Vero cells were purchased from ATCC, USA. MDA-MB-231, HEp-2 and Vero cells were cultured in DMEM, MEM and RPMI 1640 medium respectively with 10% fetal bovine serum and 1% antibiotics (Penicillin/streptomycin) and maintained in humidified cell incubator at 37°C and 5% CO_2.

Drug preparation

Stock solution of *C. schoenanthus* extract was prepared in Dimethylsulfoxide (DMSO). Different concentration of (25, 50, 75 and 100 µg/ml) extract was prepared in cell culture medium before use.

3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) cell proliferation assay

MDA-MB-231, HEp-2 and Vero cells (1X105/well) were seeded in 96 well plate (100 µl/well) and allowed to adhere firmly overnight in DMEM, MEM and RPMI 1640 medium respectively with 10% FBS. Then cells were treated with different concentration of freshly prepared extracts for 24h. Then medium was removed and cells were incubated with MTT reagent (5 mg/ml) for 4h and violet crystals dissolved in DMSO and absorbance was read at 540/690 nm. Absorbance of control (without treatment) was considered as 100% cell survival. Doxorubicin was used as positive control. Morphology of cells was photographed after treatment period by Olympus microscope at 100X magnification.

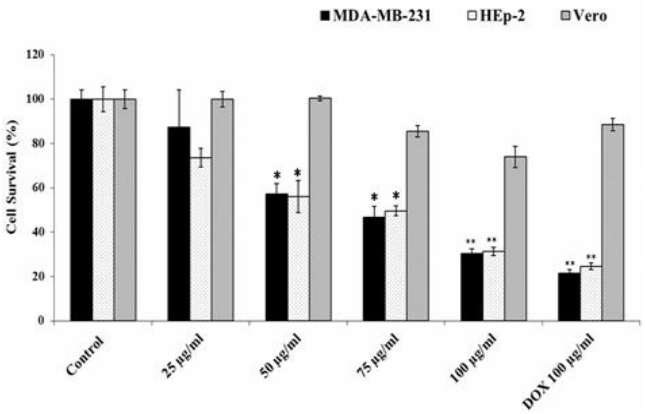

Figure 1: Anti proliferative effect of *Cymbopogan schoenanthus* on breast cancer (MDA-MB-231), cervical cancer (HEp-2) and Vero (normal) cells. Values are presented as mean ± SE of four duplicates of three independent experiments. Asterisks indicates the significant difference compare to control (*: P < 0.05, **: P < 0.001).

Table 1: IC50 value of *Cymbopogan schoenanthus* derived extracts on different cell lines.

Cell lines	IC_{50} (µg/ml)
MDA-MB-231	67.4
Hep-2	74.3
Vero	>100

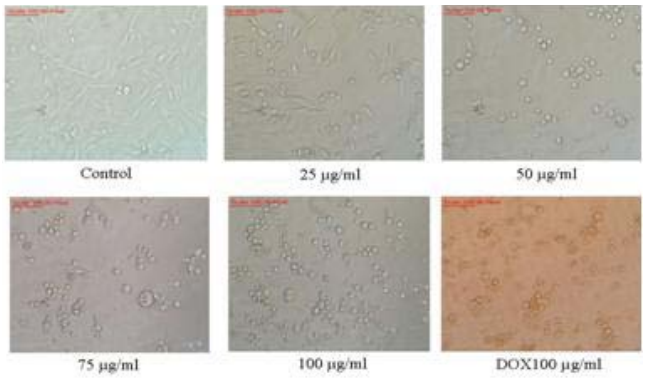

Figure 2A: Effect of *Cymbopogan schoenanthus* on MDA-MB-231cells morphology.

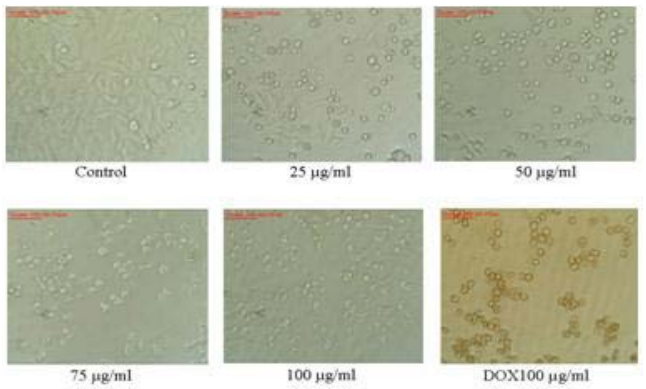

Figure 2B: Effect of *Cymbopogan schoenanthus* on HEp2 cells morphology.

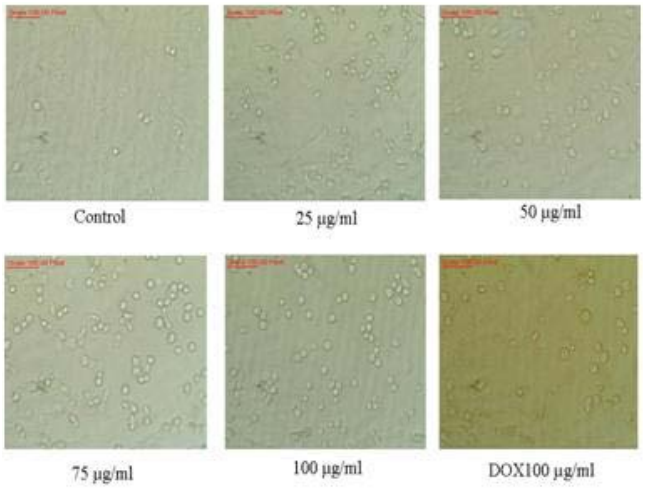

Figure 2C: Effect of *Cymbopogan schoenanthus* on Vero cells morphology.

Statistical evaluation

Data presented as mean ± SE of four duplicates of three independent experiments. Experimental data were evaluated by students 't' test and one or two way Analysis Of Variance (ANOVA). Significant difference between each set of data were considered at the confidence level of $p<0.05$ & $p<0.001$.

Results and Discussion

Breast cancer is among the most widespread diseases with a fatal outcome. A distinct breast cancer subtypes characterized by lack of estrogen and progesterone receptors expression (ER negative and PR negative, respectively) and no overexpression of Human Epidermal Growth Factor Receptor 2 (HER2) are known as Triple-Negative Breast Cancers (TNBC) [20]. Due to deficiency of the appropriate receptors, the aggressive subgroup of breast cancer is resistant to existing targeted treatments (trastuzumab or hormonal treatments) [21]. In addition cervical cancer induced by Estrogen (ER) through its nuclear receptors ERα and ERβ [22]. Over 99% of human cervical cancers are positive for sexually transmitted Human Papilloma viruses (HPVs) [23]. Currently available prophylactic vaccines that inhibit infection by a subset of high-risk HPVs hold promise for reducing cervical cancer incidence in future generations for women [24]. However, these vaccines do not protect women who are already infected or afflicted by the cancer.These hurdles led the researchers to develop a drug for TNBC and cervical cancer with low systemic toxicity. The use of tumor cell lines allows investigators to test compounds under highly controlled and reproducible conditions. The availability of well-characterized cell lines enables parallel screening assays in which the effect of a drug candidate on proliferation of multiple tumour cell lines can be determined [25]. In this study, we tested the anti-proliferative potential of *C. schoenanthus* on TNBC cells (MDA-MB-231), cervical cancer (HEp-2), vero cells (normal). We found that *C. schoenanthus* elicited dose dependent cell death on both MDA-MB-231 and HEp-2 cells after 24h of treatment (Figure 1) with an IC50 value of 67.42 µg/ml and 74.3 µg/ml respectively (Table 1). Whereas, interestingly *C. schoenanthus* does not exhibited cytotoxicity on normal vero cells (Figure 1). This results indicates that *C. schoenanthus* has specific chemo-sensitization property towards tumor cells. Further both MDA-MB-231 and HEp-2 cells lost their adherence and found to be necrotic based on their size and shape after 24h of treatment (Figure 2A,2B). However, mechanism of cell death induced by *C. schoenanthus* remains unclear. Vero cells treated with *C. schoenanthus* withstand for 24h remain attached with firm morphology and we found that there was no significant cell death (Figure 2C). In support of our results, essential oil extracted from *cymbopogan* species shown to be an effective arresting agent of MCF-7 cell proliferation [16]. Similarly Cymbopogon citratus extract reported to be an anti-proliferative agent against breast cancer cells (MDA-MB 231 and MCF-7) [17]. Cymbopogon flexuosus derived essential oil found to be an anti-cancer agent in wide array of tumor cell lines such as colon (HT-29, HCT-15, SW-620, 502713), lung (A549, HOP-62, H-226), cervix (SiHa), oral (KB), prostate (DU-145) and promyelocytic leukemia (HL-60) cells [18]. Further Cymbopogon citratus derived polysaccharides elicited significant cell death in different tumor cells (Siha and LNCap) by activating intrinsic apoptotic signaling pathways [19].

Table 2: Chemical composition of Oman's *Cymbopogan schoenanthus*.

S.No	Compound Name	Retention Time	Area	%
1	Piperitone	30.6	2.54+E	38.667599
2	alpha terpineol	27.69	3795043	0.5785442
3	beta elemene	36.88	11369853	1.7333037
4	elemol	43.55	1.83E+08	27.900315
5	gamma Selinene	47.04	23709976	3.6145225
6	Alpha Eudesmol	48.01	95522440	14.562141
7	beta gurjuene(calarene)	38.98	4964950	0.7568934
8	beta eudesmol	53.92	30598892	4.6647194
9	Lavandulyl acetate	35.84	14692161	2.2397807
10	Agaruspirol	47.21	10093076	1.5386625
11	3,7,11 trimethyl-3-hydroxy-6,10 dodecadien-1-yl-acetate	30.07	6488145	0.9891004
12	D-gemma crene	41.01	5042674	0.7687422
13	beta cadinene	42.37	7258985	1.1066129
14	3-Carene	20.01	1041932	0.1588398
15	beta-Phellandrene	19.48	1873695	0.2856398
16	alpha-Phellandrene	18.16	774027	0.1179983
17	beta-himachalene	47.24	2076686	0.3165852

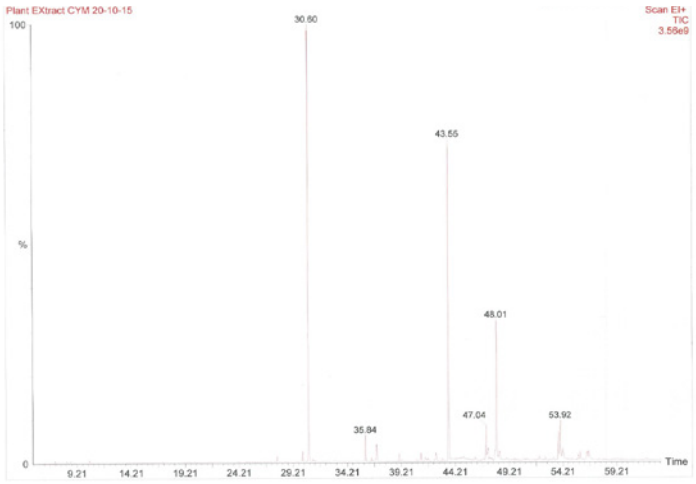

Figure 3: GC chromatogram of Oman's *Cymbopogan schoenanthus* extract.

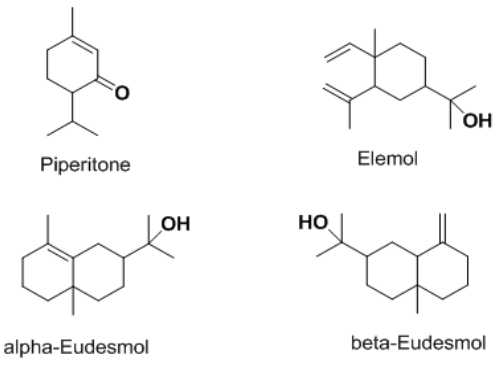

Figure 4: Structures of major compounds identified from *Cymbopogan schoenanthus*.

To identify anti-cancer active principle of C. schoenanthus, we analyzed their chemical profiling by GCMS method. Chemical profiling data reveals that piperitone is major component about 38.6% followed by elemol (27.9%), alpha eudesmol (14.5%) and beta eudesmol (4.6%) (Table 2; Figure 3,4). This is not surprising since numerous studies reported the presence of piperitone in *cymbopogan* species derived essential oil [16,17]. In particular, essential oil of *C. schoenanthus* contains 61 % of piperitone [26]. However, our data suggests that considerable amount of piperitone can be obtained by simple soxhelet extraction rather than extracting piperitone following complicated essential oil extraction methods such as hydro-distillation and microwave assisted hydro-distillation, it requires sophisticated set up and noticeably it is not cost effective. Also, as the essential oil is volatile, long term stability of active constituents of oil is remaining questionable. In our study, observed anticancer

effect of *C. schoenanthus* might be due to synergistic effect of active ingredients in particular due to high content of piperitone. Piperitone shown to be an effective active principle of *Cymbopogon citratus, Mentha citrata* and *Mentha longifolia* [17,27] and it exhibited anti-cancer activity in variety of tumor cells and it goes in agreement with our study.

Conclusion

In conclusion, to the best our knowledge this is the first report on breast and cervical cancer cell killing effect of Oman's *C. schoenanthus* and piperitone was found to be a major active principle. However, the *in vitro* mechanism of cancer cell death induced by *C. schoenanthus* should be studied in detail before commencing the *in vivo* xenograft model experiment.

Acknowledgement

We thank Mr. Manoharan and Mr. Gibin, Lonestar Alpha laboratories, Muscat, Oman for GCMS analysis.

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