Journal of Taibah University Medical Sciences (2016) 11(2), 145–151

Taibah University

Journal of Taibah University Medical Sciences

www.sciencedirect.com

Original Article

Chemical composition and anti-proliferative effect of Oman's *Ganoderma applanatum* on breast cancer and cervical cancer cells

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Received 24 November 2015; revised 31 December 2015; accepted 16 January 2016; Available online 26 February 2016

الملخص

أهداف البحث: لتقييم القدرة المضادة لجانوديرما أبلاناتوم على خلايا سرطان الثدي السلبي الثلاثي، وخلايا سرطان عنق الرحم، وخلايا الفيرو الطبيعية، والنمط الكيميائي لجانوديرما أبلاناتوم في سلطنة عمان.

طرق البحث: تم فحص التسمم الخلوي لمستخلص الميثانول من جانوديرما أبلاناتوم على خلايا سرطان الثدي السلبي الثلاثي، وخلايا سرطان عنق الرحم، وخلايا الفيرو بواسطة فحص الاستنبات الصغير لتيترازوليزم. كما تم وصف المصادر النشطة لمضاد السرطان من *الجانوديرما أبلاناتوم بواسطة الفصل اللوني للغاز بالإضافة إلى تحليل الطيف الكتلي*.

النتائج: أظهر مستخلص الميثانول من الجانوديرما أبلاناتوم موت الخلايا المعتمدة على الجرعة في كل من خلايا سرطان الثدي السلبي الثلاثي، وخلايا سرطان عنق الرحم بعد ٢٤ ساعة من العلاج بقيمة من ٨٤.٦ ميكروجرام/مل و٢،٤٣ ميكروجرام/مل، على التوالي. تحملت خلايا الفيرو الطبيعية العلاج لحد كبير. علاوة على ذلك، تم التعرف على سبعة جزيئات من الجانوديرما أبلاناتوم عن طريق التحليل بواسطة الفصل اللوني للغاز بالإضافة إلى تحليل الطيف الكتلي، وخمسة منهم كانت موجودة بكميات كبيرة.

الاستنتاجات: في هذه الدراسة، اندهشنا، أن الجانوديرما أبلاناتوم قد أعطى سمية وخاصة لخلايا سرطان الثدي السلبي الثلاثي، وخلايا سرطان عنق الرحم مقارنة بخلايا الفيرو الطبيعية. هذه الخاصية الكيميائية الحساسة للجانوديرما أبلاناتوم غير واضحة، وعملية موت الخلايا السرطانية التي يسببها الجانوديرما أبلاناتوم يجب دراستها بالتفصيل. بالإضافة إلى ذلك فإن النشاط المقاوم للسرطان من الجانوديرما أبلاناتوم قد يكون بسبب التأثير المتناغم من المصادر النشطة المحددة، الأمر الذي من شأنه أن يمهد الطريق إلى إمكانية عزل وتوصيف جزيء مكافحة السرطان من الجانوديرما أبلاناتوم في سلطنة عمان.

الكلمات المفتاحية: سرطان الثدي؛ سرطان عنق الرحم؛ جانوديرما أبلاناتوم؛ جاما- تيربنين؛ د-ليمونين؛ عمان

Abstract

Objectives: To evaluate the anti-proliferative potential of Oman's *Ganoderma applanatum* on triple negative breast cancer cells (MDA-MB-231), cervical cancer cells (HEp-2) and Vero cells (normal) and its chemical profiling of *G. applanatum*.

Methods: The cytotoxicity of the methanolic extract of *G. applanatum* was tested on MDA-MB-231 cells, HEp-2 cells and Vero cells by microculture tetrazolium (MTT) assay. The anti-cancer properties of *G. applanatum* were characterized using gas chromatography coupled with gas chromatography mass spectrometry (GCMS) analysis.

Results: The methanolic extract of *G. applanatum* elicited dose-dependent cell death on both MDA-MB-231 and HEp-2 cells after 24 h of treatment, with an IC_{50} value of 84.6 μg/ml and 43.2 μg/ml, respectively. Normal vero cells were able to significantly withstand the treatment. Furthermore, seven molecules were identified from *G. applanatum* by GCMS analysis, and five of them were present in considerable amounts, namely, γ-terpinene (30.3%), D-limonene (23.6%), cis-2-methyl-4-pentylthiane-s,s-dioxide (15.3%), β-cymene (12.7%) and α-terpinolene (8.1%).

Conclusion: In this study, surprisingly, *G. applanatum* exhibited toxicity particularly towards tumour cells (MDA-MB-231 and HEp-2) compared to normal vero cells. This tumour-specific chemo-sensitivity of *G. applanatum* is unclear, and the mechanism of cancer cell death induced by *G. applanatum* should be studied in detail. In addition, the observed anti-cancer activity of *G. applanatum* might be due to the synergistic effects of the identified properties. This

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Peer review under responsibility of Taibah University.

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 http://dx.doi.org/10.1016/j.jtumed.2016.01.007

would pave the way for isolation and characterization of the potential anti-cancer molecule from Oman's *G. applanatum*.

Keywords: Breast cancer; Cervical cancer; *Ganoderma applanatum*; D-Limonene; Oman; γ-Terpinene

Introduction

Cancer is the leading and second leading cause of death in developed and developing countries, respectively.[1] Despite technological and social development, cancer has become one of the most common diseases of concern and a leading cause of human suffering and death. One in four deaths in the United States is due to cancer. Of the different cancer types, breast cancer is the most common malignancy in women throughout the world, and it accounts for 18% of all female cancers; there are approximately 600,000 annual deaths worldwide from breast cancer.[2,3] Noticeably, breast cancer is the most common cancer in the Sultanate of Oman, and it accounts for one in every five new cases of cancer detected among females.[4] After breast cancer, cervical cancer is the second most frequent cancer and the leading cause of cancer death in women worldwide, with approximately 470,000 new cases and 233,000 deaths per year.[5]

Chemotherapy is the principal and feasible cancer treatment strategy in comparison with other therapeutic formats. However, the effective dose of chemotherapy is often accompanied by post-treatment clinical consequences such as drug resistance and tumour relapse, along with severe side effects. A number of natural and synthetic molecules have been reported as cancer chemotherapeutic agents; however, no single compound has emerged as a drug with null toxicity. Tamoxifen and cisplatin are standard care of chemotherapy for breast cancer and cervical cancer, respectively, but their use elicits significant post-treatment toxicity. In this scenario, discovering molecules from natural sources remains an active area of cancer drug discovery research. Natural compounds represent an invaluable source for the development of powerful therapeutics. Given their high structural diversity, substances with a biological origin are used as lead structures for drug discovery. Moreover, natural compounds show high effectiveness by addressing targets that exert central functions because nature has used targeting structures that are of the utmost importance for most organisms.[6–8]

Our group is mainly involved in finding natural product-based drugs 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.[9] In this study, we explored the anti-cancer potential of Oma's *Ganoderma applanatum* (Mushroom). Mushrooms have been used widely as flavourful foodstuffs and for medicinal purposes. In recent years, mushrooms have emerged as an important class of bioactive actions, i.e., antitumour,[10] immunological[11] and hypoglycaemic activities.[12] The most significant medicinal effect of mushrooms is their antitumour activity, which has attracted the attention of the public around the globe. *G. applanatum*, a species of basidiomycete, is called '*Elfvingia applanata*'.[13] This mushroom has been used in folk medicine for the treatment of various ailments, including cancer. Components derived from *G. applanatum* can modulate the humoral immune response.[14–16] Further, antibacterial and antiviral activities of the methanol and aqueous extracts of *G. applanatum* have been reported[17–19] without any toxicity.[20] *G. applanatum* is reported to induce apoptosis in gastric cancer cells (SGC-7901),[21] and polysaccharides derived from *G. applanatum* have been shown to be effective anti-tumour agents.[22] However the cytotoxic effects of Oman's *G. applanatum* on triple negative breast cancer cells (MDA-MB-231) and cervical cancer cells (HEp-2) remain unexplored. To address this issue, in this study, we report the anti-cancer potential of Oman's *G. applanatum* on MDA-MB-231, HEp-2 and Vero (normal) cells along with their chemical profiling.

Materials and Methods

Chemicals

Dulbecco's modified eagle medium (DMEM), minimum essential medium (MEM), RPMI 1640 medium and foetal bovine serum were purchased from Sigma Aldrich Chemical Co (St. Louis, USA). All other chemicals used in this study were of pure analytical grade.

Collection

G. applanatum was collected from a different area of Ibra, Oman, and it was identified by Jackson Anchakunju, Botanist, A'Sharqiyah University (ASU). A voucher specimen is deposited in our herbarium collection.

Extraction

Freshly collected *G. applanatum* was shade dried for seven days and pulverized using a mechanical grinder. Powdered material was extracted with methanol using a Soxhlet apparatus. Then, a dried extract was obtained by evaporating the methanol at room temperature. The dried extract was stored at −20 °C until used for the experiment.

Gas chromatography mass spectrometry (GCMS) analysis

GCMS analysis was performed on a Perkin Elmer Clarus 680 GC System fitted with an Rtx®-5MScapillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; maximum temperature, 250 °C) and 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/min. The injection, transfer line and ion source temperatures were 270, 240 and 240 °C, respectively. The ionizing energy was 70 eV. The electron multiplier (EM) voltage was obtained by auto-tuning. All data were obtained by collecting the full-scan mass

spectra within the scan range of 40–550 amu. The sample was prepared in hexane, and 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

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% foetal bovine serum and 1% antibiotics (Penicillin/streptomycin) and maintained in a humidified cell incubator at 37 °C and 5% CO_2.

Drug preparation

A stock solution (10 mg/ml) of *G. applanatum* extract was prepared in dimethylsulfoxide (DMSO). Different concentrations (25, 50, 75 and 100 µg/ml) of extract were 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 (1×10^5/well) were seeded in a 96-well plate (100 µl/well) and allowed to adhere firmly overnight in DMEM, MEM and RPMI 1640 medium, respectively, with 10% FBS. Then, the cells were treated with different concentrations of freshly prepared extracts for 24 h. Then, the medium was removed, and the cells were incubated with MTT reagent (5 mg/ml) for 4 h and violet crystals dissolved in DMSO, and the absorbance was read at 540/690 nm. The absorbance of the control (without treatment) was considered to be 100% cell survival. Doxorubicin was used as a positive control. The morphology of the cells was photographed after the treatment period using an Olympus microscope at 100× magnification.

Statistical evaluation

Data are presented as the mean ± SD of four duplicates of three independent experiments. Experimental data were evaluated using Student's 't' test and one- or two-way analysis of variance (ANOVA). Significant differences between each set of data were considered at the confidence levels of $p < 0.05$ and $p < 0.001$.

Results

We tested the anti-proliferative potential of the methanolic extract of *G. applanatum* on MDA-MB-231, HEp-2 and vero cells (normal). We found that *G. applanatum*-derived active principles elicited dose-dependent cell death on both MDA-MB-231 and HEp-2 cells after 24 h of treatment (Figure 1) with an IC_{50} value of 84.6 µg/ml and 43.2 µg/ml, respectively (Table 1). Interestingly, *G. applanatum* did not exhibit significant cytotoxicity on normal vero cells (Figure 1). Noticeably, HEp-2 cells were more sensitive

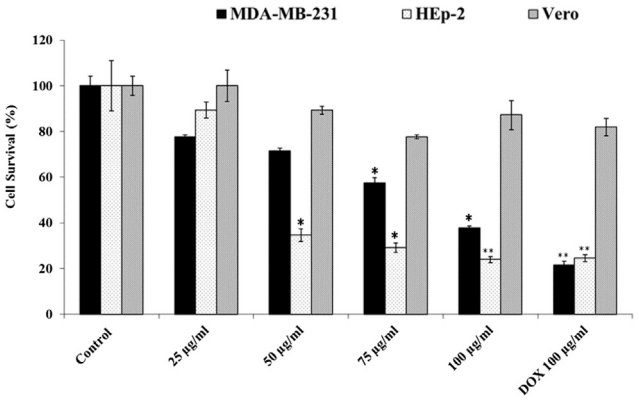

Figure 1: Cytotoxicity of *Ganoderma applanatum* on breast cancer (MDA-MB-231), cervical cancer (HEp-2) and Vero (normal) cells.

than MDA-MB-231 cells upon *G. applanatum* treatment. Further, both MDA-MB-231 and HEp-2 cells lost their adherence and morphology and were found to be necrotic based on their size and shape after 24 h of treatment with different concentrations of extract (Figure 2 A, B). Conversely, the normal vero cells withstood *G. applanatum* treatment (Figure 2C), indicating that *G. applanatum* has a specific chemo-sensitization property towards tumour cells.

To identify the molecules responsible for the observed cytotoxicity on both MDA-MB-231 and HEp-2 cells, we analysed the chemical profiling of *G. applanatum*-derived extract by GC MS. In total, seven compounds were identified by GCMS. Of the seven compounds, five were present in significant quantities, namely, γ-terpinene (30.3%), D-limonene (23.6%), cis-2-methyl-4-pentylthiane-s,s-dioxide (15.3%), β-cymene (12.7%), and α-terpinolene (8.1%) (Table 2; Figure 3). The identified components were terpenoids, and the observed anti-cancer effect might be due to their synergistic effect.

Discussion

Breast cancer and cervical cancer are the most deadly diseases among women. Targeting agents such as monoclonal antibodies have failed to abort cancer progression due to variations in the receptors' expression in both breast and cervical cancer. To overcome these hurdles and to achieve high therapeutic response, finding new drug molecules with the least toxicity is highly warranted. In this study, we explored the antiproliferative efficacy of Oman's *G. applanatum* on MDA-MB-231 cells and HEp-2 cells along with vero cells (normal). In this context, *gonaderma* is well enumerated for anti-cancer activity, and in particular, *Gonaderma lucidium* is well regarded for cytotoxicity in

Table 1: IC_{50} values of *Ganoderma applanatum*-derived extracts on different cell lines.

Cell lines	IC_{50}(µg/ml)
MDA-MB-231	84.6
Hep-2	43.2
Vero	>100

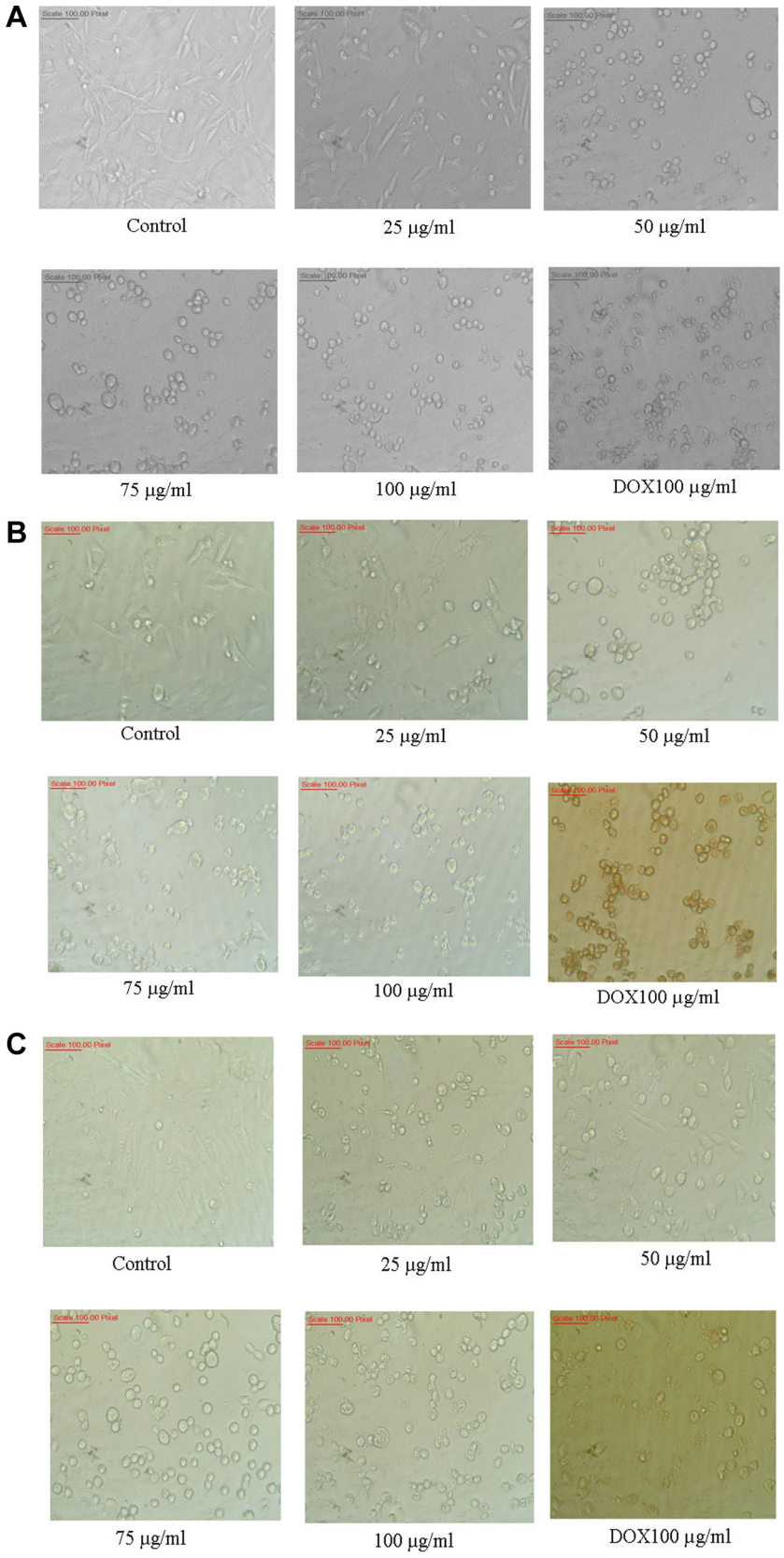

Figure 2: A, Effect of *Ganoderma applanatum* on MDA-MB-231 cell morphology. B, Effect of *Ganoderma applanatum* on HEp-2 cell morphology. C, Effect of *Ganoderma applanatum* on Vero cell morphology.

Table 2: Chemical composition of Oman's *Ganoderma applanatum*.

S. No.	Compound name	Retention time	Area	%
1	D-Limenone	19.31	4,615,044	23.676,394
2	γ-Terpinene	20.75	5,917,792	30.359,836
3	β-Cymene	19.04	2,477,768	12.711,604
4	α-Terpinolene	22.19	1,596,407	8.1,899,895
5	3N-Hexylthiane-s, s-dioxide	25.59	1,357,745	6.9,655,904
6	Cis-2-methyl-4-pentylthiane-s, s-dioxide	27.24	3,001,685	15.399,437
7	3-Carene	18.28	525,733	2.6,971,491

numerous cancer types such as breast,[23,24] cervical,[25] gastric,[26] prostate,[27] hepatocarcinoma,[28] and lung cancers.[29] Our group is involved in finding potential drug candidates from natural resources; gonaderma species attracted us due to their anti-cancer potential. However, limited reports are available on the anti-cancer potential of gonaderma species other than *G. lucidium*. In the current study, we found that Oman's *G. applanatum* showed cancer cell-specific toxicity compared to normal cells after 24 h (Figure 1).

We used 24 h as the time point for our *in vitro* dose-dependent study as per the guidelines of the American National Cancer Institute (NCI). NCI recommends 24 h of exposure time to evaluate the efficacy of crude extracts on cell lines.[30] However, a time-dependent study is required to alleviate the limitations of our cytotoxicity results. Further, we use less than 1% DMSO as a vehicle to treat the cells. It is well reported that less than 1% DMSO is permissible in *in vitro* assays because at this concentration, DMSO does not elicit significant damage to cells.[31] Therefore, we conclude that the observed cytotoxicity on HEp-2 and MDA-MB-231 cells is due to *G. applanatum*-derived properties and not because of DMSO. A limitation of our study is that we did not include respective normal cells of tumour tissue origin; instead, we used vero cells as a normal control for the *in vitro* MTT assay. Vero cells are fibroblasts derived from the adult African monkey kidney[32]; fibroblastic cells are known to be associated with every tissue. In addition, fibroblast cells support the tissue architecture and play a pivotal role in drug response. A number of *in vitro* anti-cancer studies used vero cells as normal controls to screen crude extracts[33–36] and their results are in agreement with our results. Further, our data reveal that *G. applanatum* extract sensitizes the HEp-2 cells more prominently than MDA-MB-231 cells. This indicates that the molecules present in the *G. applanatum* extract may target the receptors of HEp-2 cells. Because MDA-MB-231 cells lack receptors, *G. applanatum*-derived active principles might not be able to elicit considerable cell death when compared to the effect of HEp-2 cells.

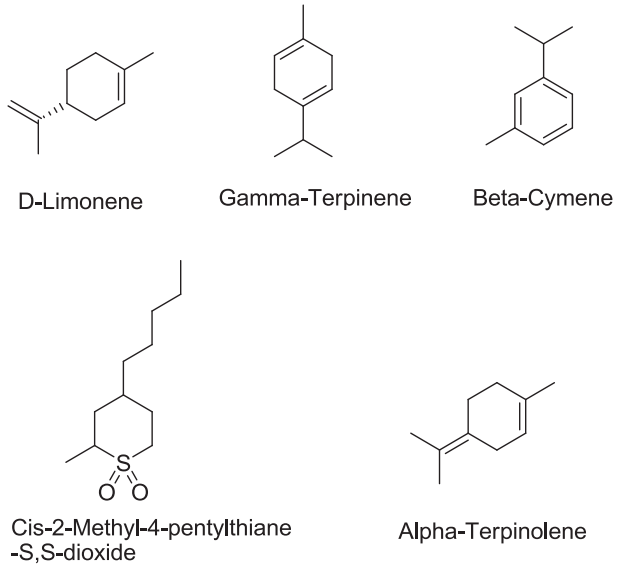

Figure 3: Structures of principal compounds identified from *Ganoderma applanatum*.

This observation motivated us to identify active molecules from *G. applanatum*, and we analysed them using GCMS. We found that γ-terpinene, D-limonene, cis-2-methyl-4-pentylthiane-s,s-dioxide, β-cymene and α-terpinolene are the principal components of *G. applanatum*. In support of our cytotoxicity results, the identified compounds in Oman's *G. applanatum,* γ-terpinene and D-limonene are well reported for their presence in numerous medicinal plants and their cytotoxic potential on different tumour cells.[37–42] Further abundant presence of these anti-cancer terpenes is observed in medicinal plant-derived essential oils.[43,44,40] However, our study suggests that the unique quantity of γ-terpinene and D-limonene can be obtained from Oman's *G. applanatum* by simple Soxhlet extraction method, rather than by following complicated essential oil extraction methods such as hydro-distillation and microwave-assisted hydro-distillation, which require sophisticated set-up and are not cost-effective. In addition, because essential oil is volatile, the long-term stability of the active constituents of oil remain questionable.

In conclusion, the observed anti-proliferative potential of Oman's *G. applanatum* on MDA-MB-231 and HEp-2 cells may be due to the synergistic action of the identified components. However, the mechanism of cancer cell death induced by *G. applanatum* remains unclear. Further isolation and characterization of anti-cancer active molecules from *G. applanatum* and studies of their tumour remission efficacy in an in vivo xenograft model are mandatory to translate this formulation to clinic application.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contribution

FLH designed the study, conducted research, provided research materials, organized data and written the manuscript, MA interpreted the data. JA collected the plant material and extracted the organic molecules. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

Acknowledgements

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

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How to cite this article: Hakkim FL, Al-Buloshi M, Achankunju J, Chemical composition and anti-proliferative effect of Oman's *Ganoderma applanatum* on breast cancer and cervical cancer cells. Journal of Taibah University Medical Sciences 2016;11:145–151.