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In vitro Screening of the Leaf Extracts from *Gardenia ternifolia* (Forest Gardenia) for their Anticancer Activity

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Authors' contributions

This work was carried out in collaboration between all authors. Authors DST, SD and MR designed the study, performed the experimental process and wrote the first draft of the manuscript. Author GGS wrote the protocol. Authors KNN, VM, DDT and PTM managed the literature searches. Author BZG identified the plant species. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of the study is to evaluate the cytotoxicity of the crude extracts of *Gardenia ternifolia* (Oliv.) in human prostate cancer (PC-3) and breast cancer (MCF-7) cell lines.
Study Design: Successive extractions of *Gardenia ternifolia* leaves were performed, using petroleum ether 60-80ºC, chloroform, ethyl acetate, methanol and methanol 80%. The cytotoxicity of these extracts on human breast cancer (MCF-7), prostate cancer (PC-3), and non-cancerous rat skeletal muscle (L6) cell lines were analyzed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay.
Place and Duration of Study: This work was performed at PSG College of Pharmacy, Coimbatore, India, from 01 September 2014 to 30 December 2014.

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Methodology: The powdered leaves of *Gardenia ternifolia* were dried and kept at room temperature (27ºC) and then extracted by maceration. Successive extractions were followed starting with petroleum ether 60-80ºC, chloroform, ethyl acetate, methanol and methanol 80%. Furthermore, the extracts were concentrated under reduced pressure and dried at room temperature. Anti-cancer activities of the various extracts were assayed by MTT assay on MCF-7, PC-3, and L6 cell lines.

Results: For MCF-7 cell lines, the total extracts showed moderate CC_{50} (50% cytotoxic concentration) of respectively 21.62 µg/mL and 45.44 µg/mL for chloroform and ethyl acetate extracts. The CC_{50} of petroleum ether 60-80ºC, methanol and methanol 80% crude extracts were found to be more than 100 µg/ml. For PC-3 cell lines, the CC_{50} of the extracts were of 9.66 µg/ml, 24.47 µg/ml and 92.10 µg/ml, respectively for chloroform, ethyl acetate and methanol extracts. The CC_{50} of the crude extracts of petroleum ether 60-80ºC and methanol 80% were more than 100 µg/ml.

Conclusion: The chloroform extract of *Gardenia ternifolia* showed better cytotoxicity effect in PC-3 than MCF-7 cell lines, comparatively to the other extracts. This could be due to the different secondary metabolites extracted with the chloroform solvent. Therefore, it could be suggested that *Gardenia ternifolia* could be developed as a possible therapeutic agent against human prostate cancer.

Keywords: Gardenia ternifolia; prostate cancer; breast cancer; MCF-7; PC-3; L6.

1. INTRODUCTION

Over the past decade, herbal medicines have become a topic of global importance, making an impact on world health and international trade. Medicinal plants continue to play a central role in the healthcare system of large proportions of the world's population [1]. This is particularly true in developing countries, where herbal medicine has a long and uninterrupted history of use [2]. Moreover, the continuous usage of herbal medicine by a large proportion of the population in the developing countries is largely due to the high cost of pharmaceuticals and healthcare. In addition, herbal medicines are more acceptable in these countries from their cultural and spiritual points of view [3].

Cancer is one of the diseases which are considered as a major public health burden in both developed and developing countries. Every year, millions of people are diagnosed with cancer, leading to death in a majority of the cases. There are more than hundred different known cancers that affect humans, notably prostate cancer, breast cancer, lung cancer, colon cancer, etc [4-6]. In 2002, Parkin and collaborators estimated that there were 10.9 million new cases, 6.7 million deaths and 24.6 million persons living with cancer around the world [7]. In 2012, about 14.1 million new cases of cancer had been reported globally (excluding skin cancer other than melanoma). It caused about 8.2 million deaths or 14.6% of all human deaths. Several authors recognize that cancer is the second leading cause of death after cardiovascular diseases in the United States of America, where one in four deaths are due to cancer [8].

Recently, a greater emphasis has been given towards the researches on alternative and complementary medicine that deals with cancer management [9]. Phytochemicals have been proposed to offer protection against a variety of chronic ailments including cardiovascular diseases, obesity, diabetes and cancer [7,10]. The research on medicinal plants could be encouraged to find an upcoming solution against new form of cancer diseases.

An attempt has been made to review some medicinal plants used for the prevention and treatment of cancer. Various anticancer herbs have been identified, which execute their therapeutic effect by inhibiting cancer-activating enzymes and hormones. Several anticancer agents including taxol, vinblastine, vincristine and etoposide derived from plants are in clinical use worldwide. Promising agents such as flavopiridol, roscovitine, combretastatin A-4, betulinic acid, and silvestrol are in clinical or preclinical development [1,7,9]. Pharmaceutical research done in technologically advanced countries has considerably improved the quality of the herbal medicines used in the treatment of cancer. Some herbs are used, on one hand, to reduce the toxic side effects of radiotherapy and chemotherapy and on the other hand to protect the body from cancer by enhancing detoxification of the body's

functions. Scientists worldwide are concentrating on the herbal medicines to boost immune cells of the body against cancer [7]. Vinca alkaloids, vinblastine and vincristine, are phytoconstituents obtained from Madagascar periwinkle, *Catharanthus roseus* (Apocynaceae) and introduced a new area of the use of plant material as anticancer agents [7,10].

This work was focused on the study of the leaves' extracts of *Gardenia ternifolia* subsp. *Jovis-tonantis*, which is known under the vernacular name of Lembanzau (Kikongo name in DR Congo). This plant is used in traditional medicine to treat malaria (bark decoction), worms (leaves and roots decoction), coughs and respiratory ailments (bark decoction) [11]. Five extracts of *Gardenia ternifolia* were prepared and their cytotoxicity was performed against human prostate cancer cells (PC-3) and on human breast cancer cell lines (MCF-7).The toxicity of the extracts was evaluated in non-cancerous rat skeletal muscle (L6) cell lines. The PC-3 is an androgen independent prostate cancer cell lines. It is negative for androgen receptor (AR) expression and hence it doesn't require androgen for proliferation. The null expression of AR means that it doesn't respond to AR antagonist conferring resistance for the hormonal therapy [12]. The PC-3 expresses the high levels of estrogen receptor (ER) – β. The MCF-7 was negative to ER-β and expresses ER-α and wild type AR. The MCF-7 responds to hormonal therapy because the ER-α mediated proliferation of MCF-7 cell lines could be controlled by using ER-α antagonist like tamoxifen [13].

2. MATERIALS AND METHODS

2.1 Plant Material Collection and Identification

The leaves of *Gardenia ternifolia*, the plant material under the study, were collected in Kisantu (Province of Central Congo, Democratic Republic of Congo, in June 2014 and was authenticated by Mr. B.L. Nlandu of the INERA (Institut National d'Etudes et Recherches Agronomiques).

2.2 Preparation of the Extracts and Isolation

The powdered leaves of *Gardenia ternifolia* were dried and kept at room temperature. 30 g of the powdered leaves were extracted by maceration, using successive extractions starting with petroleum ether 60-80℃, chloroform, ethyl acetate, methanol and methanol 80% (Hi-media, India). The extraction was repeated twice for each used solvent, in order to optimize the extraction. Then the extract was concentrated under reduced pressure, dried at room temperature and weighed to give solvent solubles. All extracts were dissolved in dimethylsulfoxide (DMSO) to give a stock concentration of 100 µg/µl. The stock solution was then serially diluted with the culture medium. The test concentrations for the extracts were of 0.5, 1, 5, 10, 50, 100, and 500 µg/mL. The concentration of DMSO never exceeded 1% in any of the 96 wells.

2.3 Cytotoxicity Assay

Anticancer activity was assayed by the standard 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) (obtained from HiMedia Leading Biosciences Company: India) assay colorimetric procedure. While MCF-7, PC-3 and L6 cell lines were obtained from National Centre for Cell Science, Pune in India. It was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum 2mM glutamine, amphotericin (3 µg/ml), gentamycin (400 µg/ml), streptomycin (250 µg/ml), penicillin (250 units/ml) and 1 µg/ml (for MCF-7 alone) of insulin in an incubator (thermo scientific) at 5% CO_2, 37℃ (obtained from Gibco Bio Resource Biotech., Pune, Maharashtra, India).

The cells were trypsinized at sub-confluence and $2x10^3$ cells/well were seeded in each of the 96 wells using culture medium. The viability was tested using trypan blue dye with the help of a hemocytometer and 95% of viability was confirmed. After 24 hrs, the fresh medium with the extracts were added to the respective wells and incubated for 72 hrs. After incubation, the following assays were performed. The old medium was removed and a fresh medium was added. The medium used for incubation of the extracts had phenol red, which could interfere with the colorimetric assay. The Phenol red is an indicator used to detect the changes in the pH of the medium. The fresh medium would be without phenol red and contains 10 µl of MTT (5 mg/ml stock solution). The plates were then incubated for 4 hrs. The medium containing the extracts were discarded and the purple formazan crystals, which were formed in the cells, were dissolved with 100 µl of DMSO. The optical density was measured at 570 nm and the percentage of toxicity was calculated as following.

Percentage of cytotoxicity = {1 – [(At-Ab)/ (Ac-Ab)]} x100

Where:

At = Absorbance value of test compound,

Ab = Absorbance value of blank and

Ac=Absorbance value of control (DMSO treated).

The graph pad prism software was used to calculate the CC_{50} [14], which was defined as the concentration required for reducing the cell number by 50% compared to that of control cells.

3. RESULTS AND DISCUSSION

The different extracts were concentrated under reduced pressure and yielded (0.16 g, 0.53%), (3.2 g, 10.67%), (2.70 g, 9.00%), (0.64 g, 2.13%) and (0.32 g, 1.07%) for petroleum-ether 60-80℃, chloroform, ethyl acetate, methanol and methanol 80%, respectively. The Table 1 shows the CC_{50} of the five leaf extracts of *G. ternifolia*. The CC_{50} of the extracts were derived after plotting a dose response curve (DRC) for log concentration vs % cytotoxicity. The Fig. 1 shows the DRC of chloroform extract and standard drug paclitaxel. On one hand, from the analysis as shown in the Table 1, the chloroform and ethyl acetate extracts of *G. ternifolia* showed the CC_{50} values of 21.62 µg/ml and 45.44 µg/ml, respectively against human breast cancer cell lines (MCF-7). On the other hand, against human prostate cancer cells (PC-3), the CC_{50} of the extracts were of 9.66 µg/ml, 24.47 µg/ml, and 92.10 µg/ml for chloroform, ethyl acetate and methanol extracts, respectively. The petroleum ether 60-80ºC and methanol 80% extracts were inactive. As reported by Wall and collaborators [15], any plant extracts/compound with a CC_{50} value below than 20 µg/ml could be accepted as potent cytotoxic extracts/compounds. This is conformed for the chloroform extract of this plant, with a CC_{50} value of 9.66 µg/ml. The chloroform extract also has a better safety ratio with PC-3 cell lines because it is 7.02 times selective in killing the cancer cells (PC-3) than the non-cancerous L6 cell lines. The cell proliferation control is considered to be a potentially effective strategy for the control of tumor growth [15,16]. The present study interestingly revealed that the treatment of PC-3 cell lines with the chloroform extract resulted inhibition of the cell proliferation.

Fig. 2A represents the control cells (solvent treated). Figs. 2B and 2C represent the chloroform extract and paclitaxel treated cells, respectively (20 µg/ml for 72 hrs). One can also see that the test and standard treated cells were morphologically deteriorated and the cell density was also decreased in comparison to the solvent control.

Fig. 3A represents the control cells treated with DMSO. Figs. 3B and 3C represent the chloroform extract and paclitaxel treated cells (10 µg/ml for 72 hrs). One can also see that, most of the cells were morphologically deteriorated with the chloroform extract treatment. The cell density was decreased in chloroform extract and paclitaxel treatment when compared to the solvent control.

Table 1. Bioactivity

Extract/Isolate	CC_{50} (µg/ml)*			Safety ratio	
	MCF-7 (Breast cancer)	PC-3 (Prostate cancer)	L6 (Non-cancer)	L6/MCF-7	L6/PC3
Petroleum-ether 60-80ºC	>100	>100	>100	×	×
Chloroform	21.62 ± 1.6	9.66± 2.6	67.89± 1.2	3.14	7.02
Ethyl acetate	45.44 ± 2.2	24.47± 1.1	63.21± 4.8	1.39	2.58
Methanol	>100	92.10± 6.5	>100	×	×
Methanol 80%	> 100	> 100	>100	×	×
Paclitaxel	27.73± 3.5	22.34± 0.86	37.86± 1.8	1.36	1.69

**The CC_{50} value represents mean ± S.D of three individual experiments*

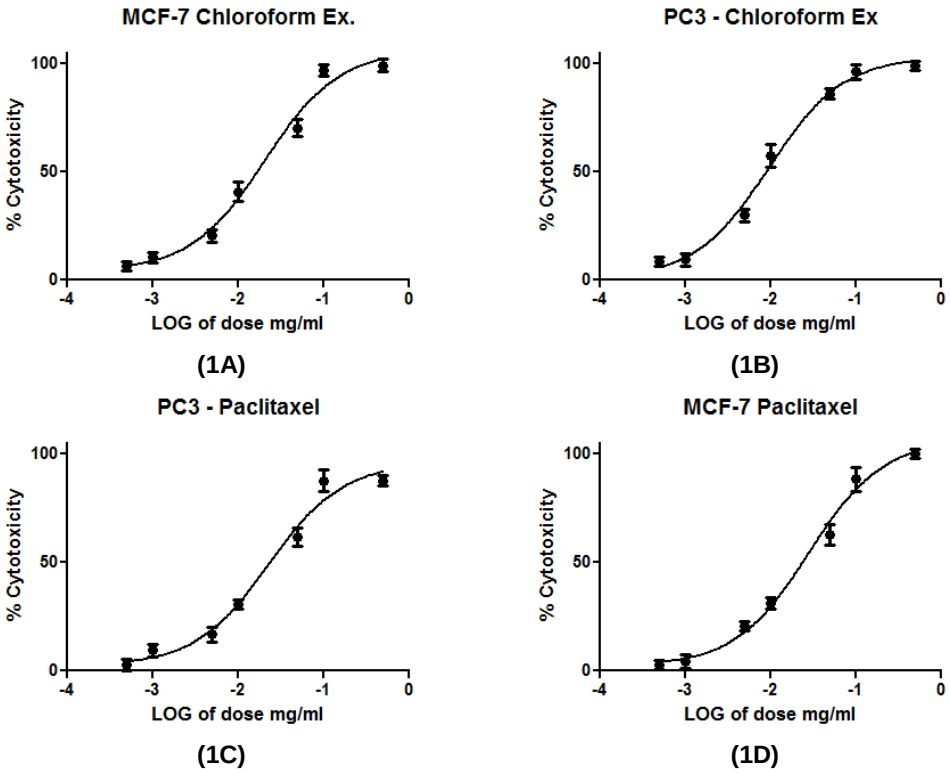


Fig. 1. Dose response curve of chloroform and paclitaxel

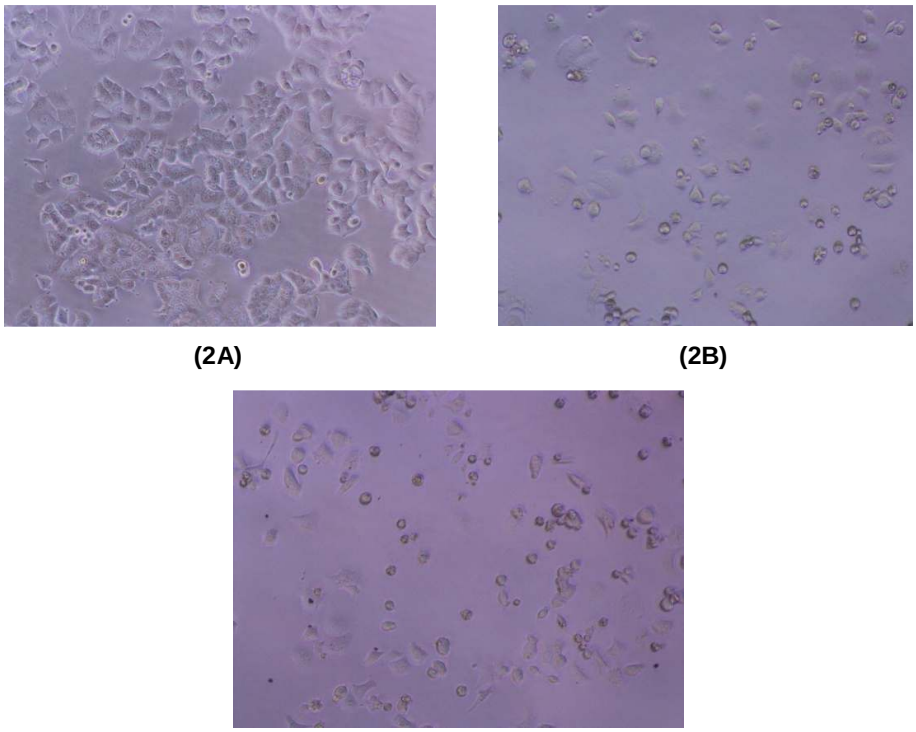
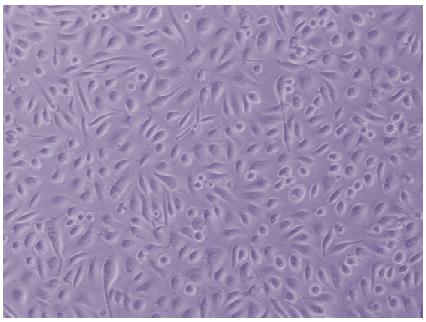
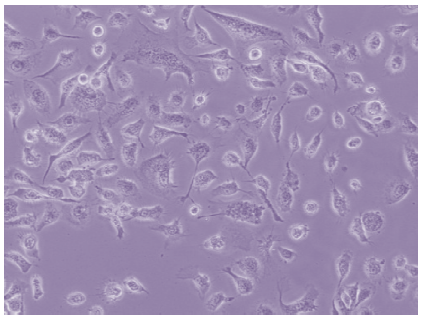


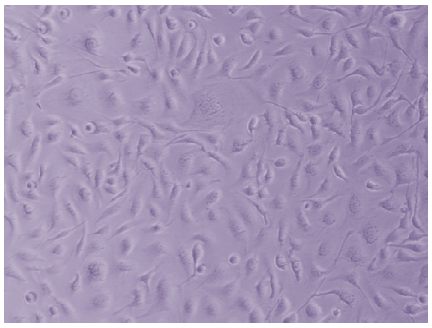
Fig. 2. Morphology of untreated (DMSO) and treated MCF-7 cells (10X)



(3A)



(3B)



(3C)

Fig. 3. Morphology of untreated (DMSO) and treated PC-3 cells (10X)

4. CONCLUSION

In this work, we aimed to evaluate the anticancer properties of five crude extracts of *Gardenia ternifolia,* using the tetrazolium salt method (MTT method). We have shown that the five extracts don't have inhibitory effect on the MCF-7 cell lines. However, on PC-3 cell lines, the chloroform extract showed better activity, comparatively to the other extracts and could be accepted as potent cytotoxic extract. In the future, we will further analyze the phytochemical constituents of the chloroform extract and possible mechanism of anti-cancer activity.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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