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## *In vitro* cytotoxicity study of leaf, stem and bark of *Pterocarpus santalinus* Linn. F.

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#### Abstract

The leaf, stem and bark of *Pterocarpus santalinus* belonging to the family Fabaceae were evaluated for their *in vitro* cytotoxicity potential by MTT assay. The plant parts were extracted by individual and successive cold percolation method using a number of solvents of varied polarity. The extractive yield varied with different solvents. The cytotoxic study of methanol extract of all the three parts extracted by both the methods was done against HeLa cancer cell line, breast cancer cell line and normal cell line. Mitomycin C was used as positive control and DMSO as negative control.

All the three extracts showed dose dependent cytotoxic effect. When all the three parts were compared, ME extract of stem showed minimum % cell viability and both methods were compared, successive extraction methods was better than individual extraction method. Though there was not much difference in % cell viability by either method or by different parts. So it can be concluded that all the three parts showed cytotoxic effect against HeLa and breast cancer cell line.

**Keywords:** *Pterocarpus santalinus*, cancer, cytotoxicity study, MTT assay, HeLa cancer cell line, breast cancer cell line, extractive yield

#### 1. Introduction

Cancer is a major health problem worldwide. The number one cause of maximum deaths that occur in the world are cardiovascular disorders and cancer is second disease (Parkin *et al.*, 2005; Jemal *et al.*, 2007) <sup>[1]</sup> <sup>[2]</sup> There were about 14.1 million new cases of cancer, 8.2 million deaths in 2012 worldwide (Ferlay *et al.*, 2015) <sup>[3]</sup>. According to Bray and Soerjomataram, (2015) <sup>[3, 4]</sup>, there will be 23 % increase in cancer death by 2030. Cancer is a disease when abnormal cells divide in an uncontrolled way and are able to invade other tissues. Some cancers may eventually spread into other tissues. There are more than 200 different types of cancer. Most cancers start due to gene changes that happen over a person's lifetime. Cancers are divided into groups according to the type of cell they start from. They include Carcinomas, Sarcomas, Leukaemias, Lymphomas, brain tumours. Carcinoma is a type of cancer that begins in the skin or in tissues that line or cover internal organs. There are several subtypes, including adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma. Sarcoma is a type of cancer that begins in the connective or supportive tissues such as bone, cartilage, fat, muscle, or blood vessels. Leukaemia is a type of cancer that starts in blood forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and go into the blood. Lymphoma and myeloma are cancers that begin in the cells of the immune system and lastly brain and spinal cord cancers are known as central nervous system cancers.

The common cancer therapies are radiation therapy, chemotherapy, surgery, immunotherapy including utilization of monoclonal antibody and other methods (Skeel, 2003; Xu *et al.*, 2009) <sup>[5]</sup> <sup>[6]</sup>; and synthetic medication is the only option but they have many side effects, costly and not affordable by all the people. Many cancers initially respond to chemotherapy but later on they develop resistance (Johnston, 1997) <sup>[7]</sup>. There are many latest approaches to drug discovery against deadly diseases like cancer, such as combinatorial chemistry and computer based molecular modeling design, but none can replace the natural products. Plants are the best substitutes and the need of the hour is to find out new novel potential anticancer compounds with lesser side effects and cost effective (Kuetze *et al.*, 2016) <sup>[8]</sup>. Many anticancer compounds of plant origin are successfully employed with less toxicity to fight against cancer (Mondal *et al.*, 2012; Shah *et al.*, 2013; Fankam *et al.*, 2017) <sup>[9]</sup> <sup>[10]</sup> <sup>[11]</sup>, <sup>[22]</sup>. There are already some very well known anticancer compounds which are effectively being used as anticancer agents. For eg. Vinblastine and Vincristine from *Catharanthus roseus* (Cragg and Newman, 2005) <sup>[11]</sup>, <sup>[15]</sup>; Taxol from *Taxus brevifolia* (Luduen, 1998) <sup>[13]</sup>; Camptothecin from *Camptotheca acuminata* (Potmeisel and Pinedo, 1995); homoharringtonine from *Cephalotaxus harringtonia*

(Itokawa *et al.*, 2005) <sup>[15]</sup>; Combretastatins from *Combretum caffrum* (Cirila and Mann, 2003) <sup>[16]</sup>, etc *Pterocarpus santalinus* Linn.f. belongs to the family Fabaceae. It is commonly known as Red sandal and the red wood yield a natural red dye called santalin. Traditionally it has many medicinal uses and there are many reported biological activities of this endangered plant as described earlier (Donga *et al.*, 2017) <sup>[17]</sup>. In the present work, leaf, stem and bark of *P. santalinus* was extracted both by individual and successive cold percolation method using a number of solvents of varied polarity. The methanol extract of all the three parts extracted by both the methods were evaluated for their *in vitro* cytotoxicity potential, perhaps for the first time.

## Materials and methods

### Plant collection

The leaves, stem and bark of *P. santalinus* (Voucher Specimen No - Su/Bio/521/Thakrar) were collected from Surat in August, 2016 Gujarat, India. All the three parts were separated, washed thoroughly with tap water, shade dried and homogenized to fine powder and stored in closed container for further studies.

### Extraction

The dried powder of (leaf, stem and bark) of *Pterocarpus santalinus* was extracted by individual cold percolation method (Parekh and Chanda, 2007) <sup>[18]</sup> and by successively cold percolation method (Kaneria and Chanda, 2013) <sup>[19]</sup> using different solvents like petroleum ether (PE), toluene (TO), ethyl acetate (EA), acetone (AC), methanol (ME) and water (AQ) of varied polarity. The procedure of extraction followed is as described earlier (Parekh and Chanda, 2007; Kaneria and Chanda, 2013) <sup>[18]</sup> <sup>[19]</sup> <sup>[21]</sup>.

### *In vitro* cytotoxicity study

#### MTT assay

Cell viability of the methanol extract of leaf, stem and bark was evaluated by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Labieniec and Gabryelak, 2003) <sup>[25]</sup>. 150  $\mu$ l of cells were seeded in 96-well plates (Becton Dickinson (BD), USA) at the density of  $1.1 \times 10^4$  viable cells/well and incubated for 24 h to allow cell attachment. Following attachment, the medium was replaced with complete medium (150  $\mu$ l/well) containing methanol extract of leaf, stem and bark (concentrations ranging from 2 to 200  $\mu$ g/ml) for 72 h. Following treatment, the cells were washed with PBS and incubated with 100  $\mu$ l/well fresh medium containing 0.5 mg/ml MTT. The MTT-containing medium was removed after 3 h incubation in dark condition. The MTT formazan was dissolved in 100  $\mu$ l/well DMSO and optical density was determined at 570 nm using an ELISA plate reader (Bio-Tek, USA). Standard anticancer drug Mitomycin was used as positive control. Cell viability was calculated by the following equation:

Cell viability (%) =  $(A_S/A_{Control}) \times 100$  where,  $A_S$  is the absorbance of the cells incubated with the methanol extract  $A_{Control}$  is the absorbance of the cells incubated with the culture medium only.

## Results and discussion

### Extractive yield

Extraction method directly affects the extraction efficiency of polyphenols, flavonoids and other phytoconstituents from different plant parts. Extraction yield refers to the %age of crude extract obtained from a dried plant sample through a

solvent extraction procedure for further analysis. In the present work, extraction was done by individual cold percolation method and successive cold percolation method using a number of solvents with different polarities. In individual cold percolation method, the crude plant extract is first defatted with non-polar solvent petroleum ether and then individually they are extracted with different solvents. In successive cold percolation method, the crude plant extract is first defatted with non-polar solvent petroleum ether and then it is extracted successively in solvents with increasing polarity and finally in water (aqueous).

The extractive yield of leaf, stem and bark of *P. santalinus* in different solvent extracts (PE, TO, EA, AC, ME, AQ) is given in Fig.1. In individual cold percolation method, the extractive yield in leaf ranged from 0.89% to 8.63%; in stem it ranged from 0.42% to 7.02% and in bark it ranged from 0.28% to 15.86% (Fig.1 A-C). Maximum extractive yield was in bark ME extract. In successive cold percolation method the extractive yield in leaf ranged from 0.43% to 4.77%; in stem it ranged from 0.44% to 2.51%. And in bark ranged from 0.32% to 2.80% (Fig.1 D-F). Maximum extractive yield was in leaf AQ extract.

When the two extraction methods were compared, maximum extractive yield was found in individual cold percolation method; and when all the three parts (leaf, stem and bark) and six solvent extracts (PE, TO, EA, AC, ME, AQ) were compared, maximum extractive yield was in bark-ME extract.

### *In vitro* cytotoxicity study

The cytotoxicity study is done only in ME extract of all the three parts leaf, stem and bark of *P. santalinus* extracted by both the methods. There are a number of methods to evaluate the efficacy of natural anticancer plant extracts or compounds. The most common *in vitro* models include MTT assay, XTT assay, Trypan blue dye exclusion assay, Sulforhodamine B assay and Lactic dehydrogenase assay; while the most popular *in vivo* model is Induction of Ehrlich ascites carcinoma (Nagani and Chanda, 2013) <sup>[21]</sup>. MTT assay is the most common assay which is generally employed to evaluate the cytotoxicity of plant extracts, or essential oils of metal nanoparticles like AgNPs, AuNPs or ZnONPs against cancer cell line and normal cell line. Doxorubicin (Fankam *et al.*, (2017) <sup>[11]</sup>, <sup>[22]</sup>, berberine (Pendota *et al.*, 2017) <sup>[23]</sup>, cyclophosphamide (Mercy Ranjitham *et al.*, 2013) <sup>[24]</sup> cell5, Mitomycin C (Labieniec and Gabryelak, 2003) <sup>[20]</sup>, <sup>[25]</sup> are generally used as standard or reference drugs.

The cytotoxicity of ME extract of leaf, stem and bark of *P. santalinus* by individual cold percolation method and successive cold percolation method was evaluated on HeLa cancer cell line, breast cancer cell line and normal cell line by the MTT assay with Mitomycin C as positive control and DMSO as negative control. The results are given in Figs. 2– 7. The cytotoxicity of ME extract of leaf, stem and bark by individual cold percolation method against HeLa cancer cell line is given in Fig. 2. All the three extracts showed dose dependent cytotoxic effect on HeLa cancer cell line i.e. as the concentration of extract increased, % cell viability decreased indicating the cytotoxic effect of ME extract of leaf, stem and bark of *P. santalinus*. When the concentration of ME extract was in the range 2-200  $\mu$ g/ml, the % cell viability of leaf was in the range 100% to 47.06%; of stem was in the range 100% to 52.94% and of bark was in the range 100% to 47.06%. At a concentration of 50  $\mu$ g/ml, leaf ME extract treated HeLa cells showed 76.47% cell viability, stem ME extract treated HeLa cells showed 73.53% cell viability and bark ME extract

treated HeLa cells showed 67.65 % cell viability. The % cell viability of positive control at the same concentration was 35.29 %. At highest concentration i.e. at 200 µg/ml, the cell viability of leaf, stem and bark was 47.06%, 52.94% and 47.06% respectively. Amongst the three parts, minimum cell viability or maximum cytotoxic effect was shown by leaf and bark ME extract by individual cold percolation method.

The cytotoxicity of ME extract of leaf, stem and bark by successive cold percolation method against HeLa cancer cell line is given in Fig. 3. All the three extracts showed dose dependent cytotoxic effect on HeLa cancer cell line i.e. as the concentration of extract increased, % cell viability decreased indicating the cytotoxic effect of ME extract of leaf, stem and bark. When the concentration of ME extract was in the range 2-200 µg/ml, the % cell viability of leaf was in the range 100% to 58.82%; of stem was in the range 100% to 41.18% and of bark was in the range 100% to 44.12%. At a concentration of 50 µg/ml, leaf ME extract treated HeLa cells showed 82.35% cell viability, stem ME extract treated HeLa cells showed 73.53% cell viability and bark ME extract treated HeLa cells showed 58.82 % cell viability. At highest concentration i.e. at 200 µg/ml, the cell viability of leaf, stem and bark was 58.82%, 41.18% and 44.12% respectively. Amongst the three parts, minimum cell viability or maximum cytotoxic effect was shown by stem ME extract by successive cold percolation method.

The cytotoxicity of ME extract of leaf, stem and bark by individual cold percolation method against breast cancer cell line is given in Fig. 4. All the three extracts showed dose dependent cytotoxic effect on breast cancer cell line i.e. as the concentration of extract increased, %cell viability decreased indicating the cytotoxic effect of ME extract of leaf, stem and bark. When the concentration of ME extract was in the range 2-200 µg/ml, the % cell viability of leaf was in the range 100% to 48.39%; of stem was in the range 100% to 61.29% and of bark was in the range 100% to 51.61%. At a concentration of 50 µg/ml, leaf ME extract treated breast cells showed 77.42% cell viability, stem ME extract treated breast cells showed 83.87% cell viability and bark ME extract treated breast cells showed 83.87% cell viability. At highest concentration i.e. at 200 µg/ml, the cell viability of leaf, stem and bark was 48.39%, 61.29% and 51.61% respectively. Amongst the three parts, minimum cell viability or maximum cytotoxic effect was shown by leaf ME extract by individual cold percolation method.

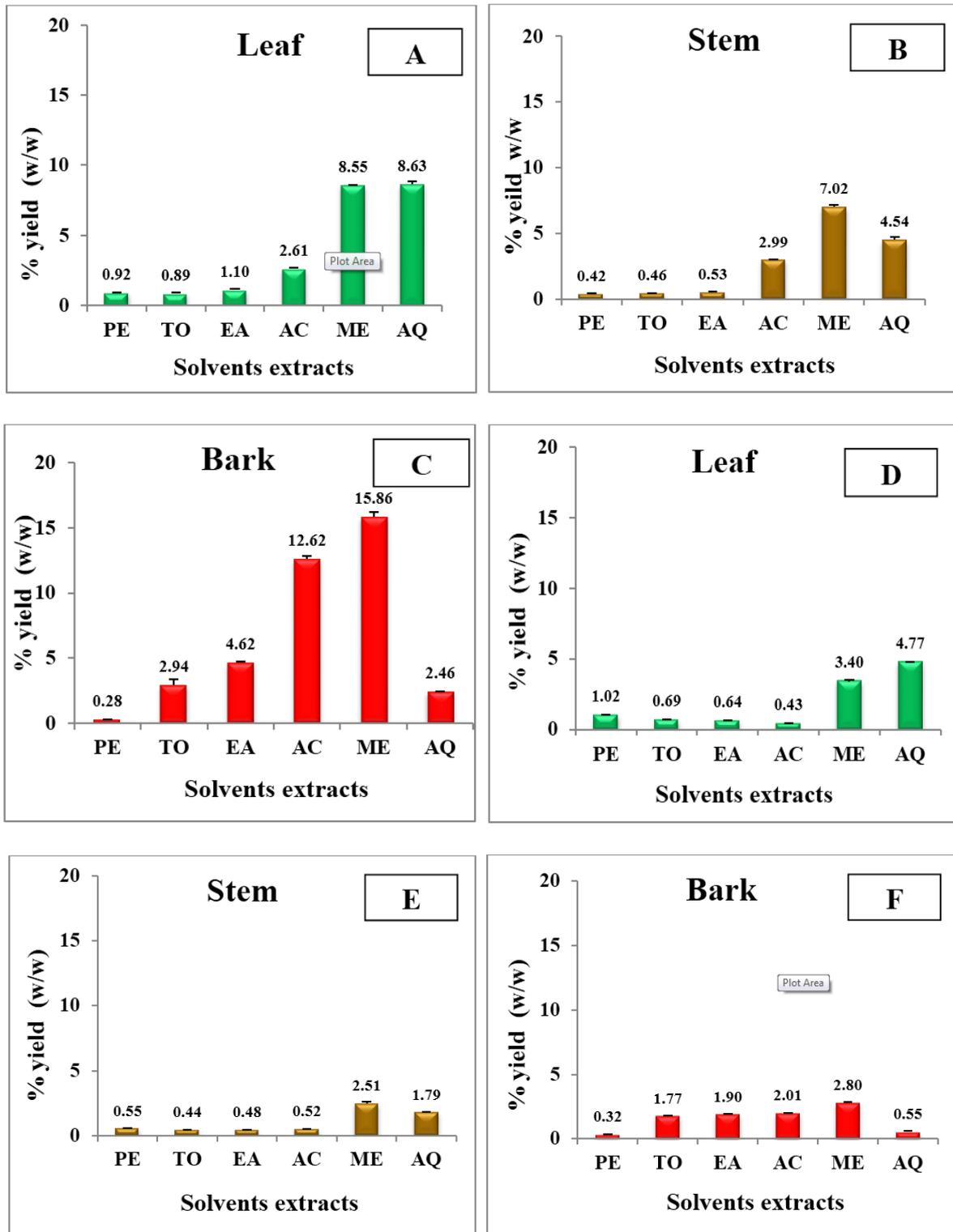
The cytotoxicity of ME extract of leaf, stem and bark by successive cold percolation method against breast cancer cell line is given in Fig. 5. All the three extracts showed dose dependent cytotoxic effect on breast cancer cell line i.e. as the concentration of extract increased, %cell viability decreased indicating the cytotoxic effect of ME extract of leaf, stem and bark. When the concentration of ME extract was in the range 2-200 µg/ml, the % cell viability of leaf was in the range 100% to 67.74%; of stem was in the range 100% to 54.84% and of bark was in the range 100% to 48.39%. At a concentration of 50 µg/ml, leaf ME extract treated breast cells showed 87.10% cell viability, stem ME extract treated breast cells showed 77.42% cell viability and bark ME extract treated breast cells showed 77.42% cell viability. At highest

concentration i.e. at 200 µg/ml, the cell viability of leaf, stem and bark was 67.74%, 54.84% and 48.39% respectively. Amongst the three parts, minimum cell viability or maximum cytotoxic effect was shown by bark ME extract by successive cold percolation method.

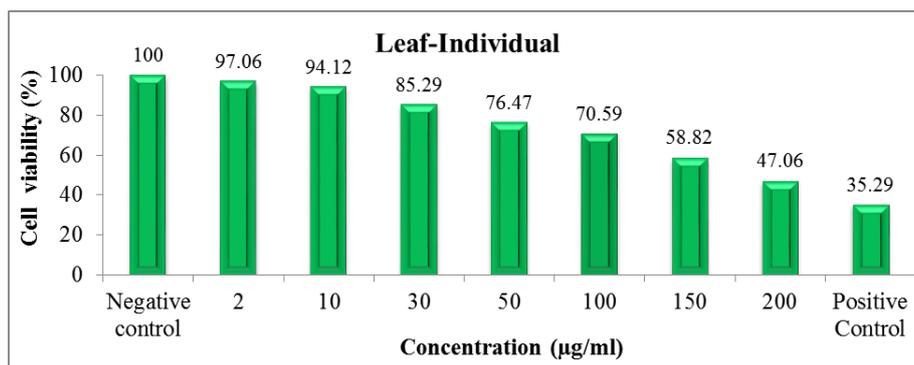
The cytotoxicity of ME extract of leaf, stem and bark by individual cold percolation method against Normal fibroblast cancer cell line is given in Fig. 6. All the three extracts showed dose dependent cytotoxic effect on Normal fibroblast cancer cell line i.e. as the concentration of extract increased, %cell viability decreased indicating the cytotoxic effect of ME extract of leaf, stem and bark. When the concentration of ME extract was in the range 2-200 µg/ml, the % cell viability of leaf was in the range 100% to 44.44%; of stem was in the range 100% to 44.44% and of bark was in the range 100% to 47.22%. At a concentration of 50 µg/ml, leaf ME extract treated Normal fibroblast cells showed 72.22% cell viability, stem ME extract treated Normal fibroblast cells showed 66.67% cell viability and bark ME extract treated Normal fibroblast cells showed 69.44% cell viability; while that of standard drug showed 30.56% cell viability, indicating that the studied extracts were better than the standard positive control.

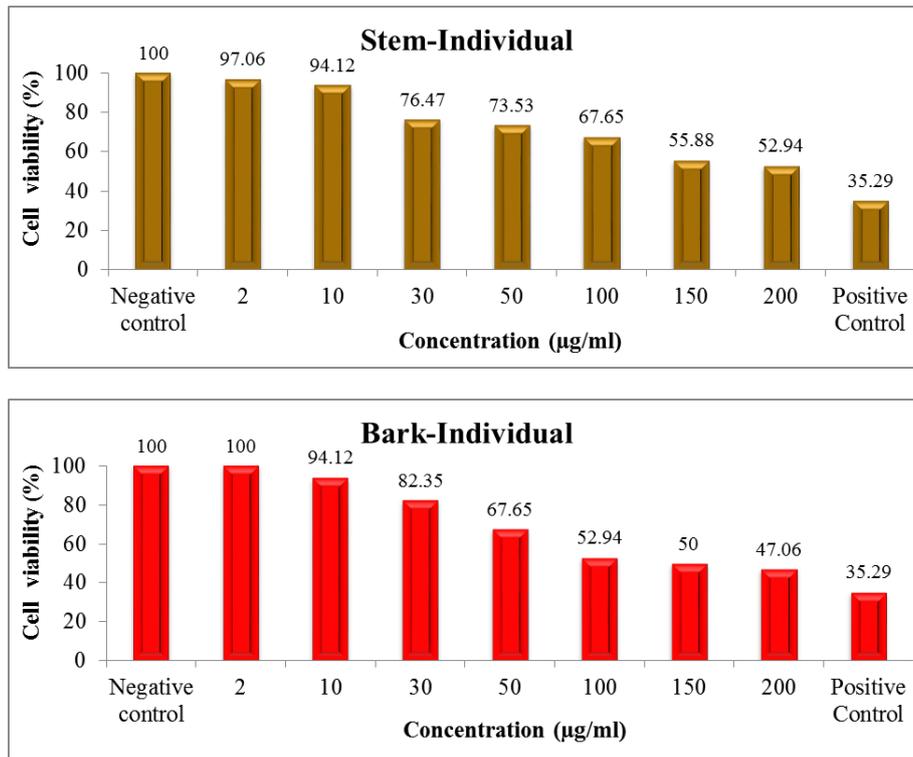
The cytotoxicity of ME extract of leaf, stem and bark by successive cold percolation method against Normal fibroblast cancer cell line is given in Fig. 7. All the three extracts showed dose dependent cytotoxic effect on Normal fibroblast cancer cell line i.e. as the concentration of extract increased, %cell viability decreased indicating the cytotoxic effect of ME extract of leaf, stem and bark. When the concentration of ME extract was in the range 2-200 µg/ml, the % cell viability of leaf was in the range 100% to 55.56%; of stem was in the range 100% to 41.67% and of bark was in the range 100% to 44.44%. At a concentration of 50 µg/ml, leaf ME extract treated Normal fibroblast cells showed 77.78% cell viability, stem ME extract treated Normal fibroblast cells showed 75.00% cell viability and bark ME extract treated Normal fibroblast cells showed 61.11% cell viability; while that of standard drug showed 30.56% cell viability, indicating that the studied extracts were better than the standard positive control.

When all the three parts were compared, ME extract of stem showed minimum % cell viability and both methods were compared, successive extraction methods was better than individual extraction method. Though there was not much difference in % cell viability by either method or by different parts. So it can be concluded that all the three parts showed cytotoxic effect against HeLa and breast cancer cell line. Some of the examples of plant extracts on different cell lines is reported by other researchers for eg. *K. odoratissima* leaf extract showed dose-dependent cytotoxicity against MDA-MB468, K562, SKOV3, Y79, and A549 cancer cell lines as reported by Momtazi *et al.*, (2017) [26]. Little to no cytotoxicity effects of extracts and isolated compounds from leaves of *Pappea capensis* is reported by Pendota *et al.*, (2017) [23]. *A. graveolens* seed extracts were found to be more cytotoxic to cancer cells as compared to the normal cells (Rakad and Jumaily, 2010) [27].

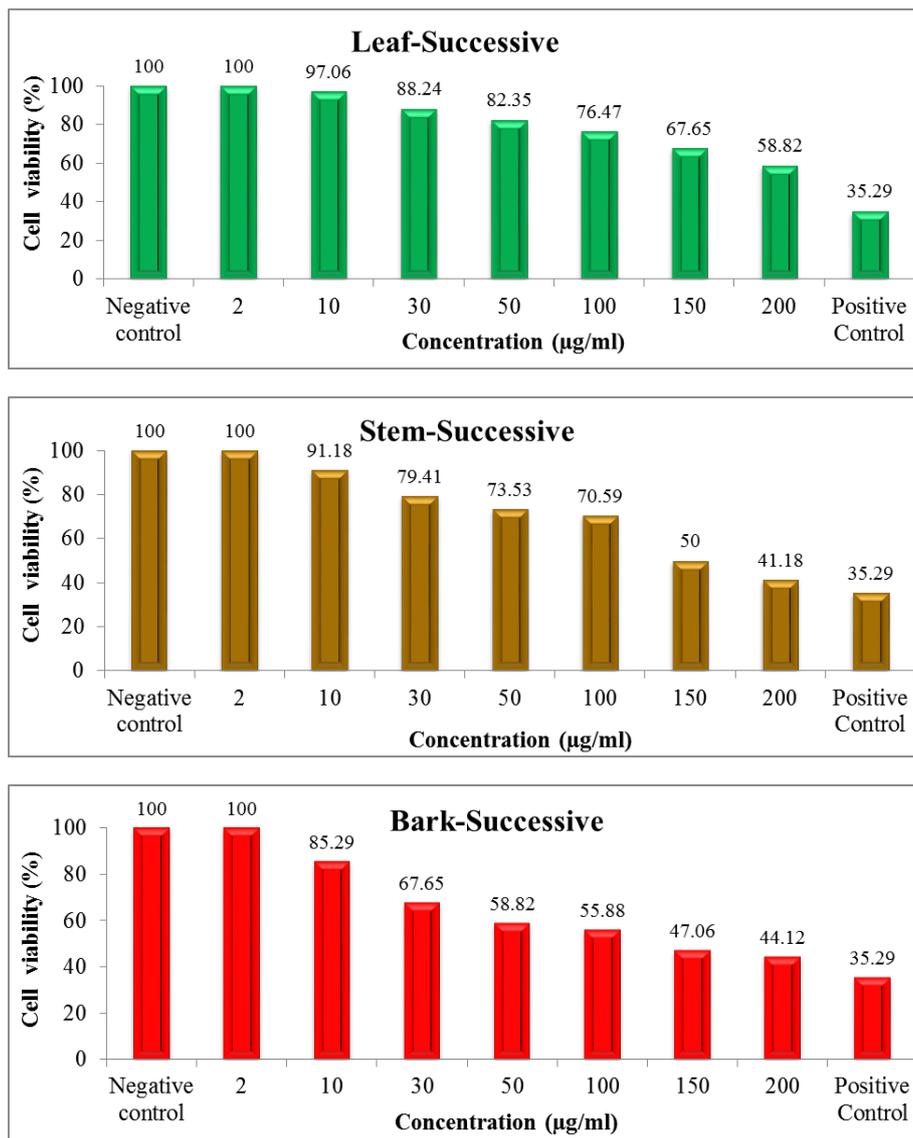


**Fig 1:** The extractive yield of different solvent extracts of *P. santalinus*; A, B and C. (Individual Cold Percolation Method) and D, E and F (Successive Cold Percolation Method)

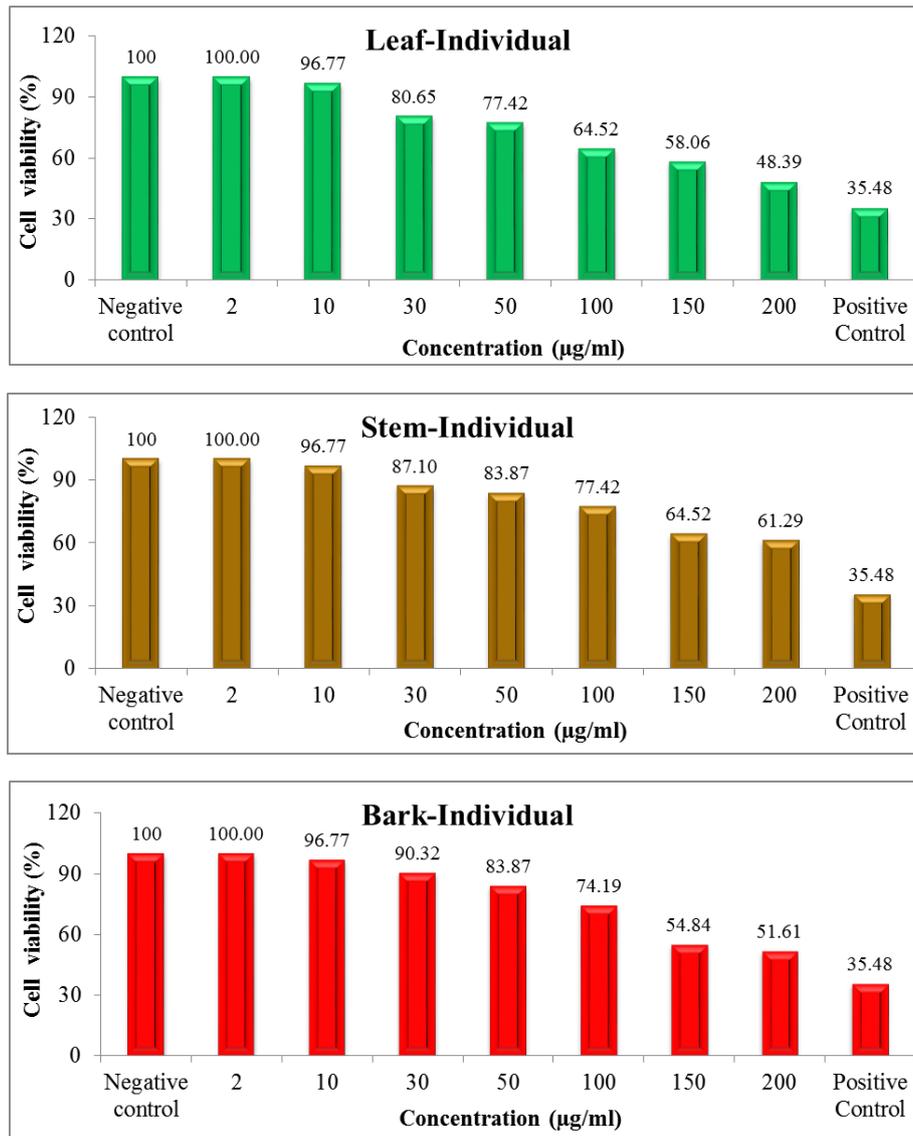




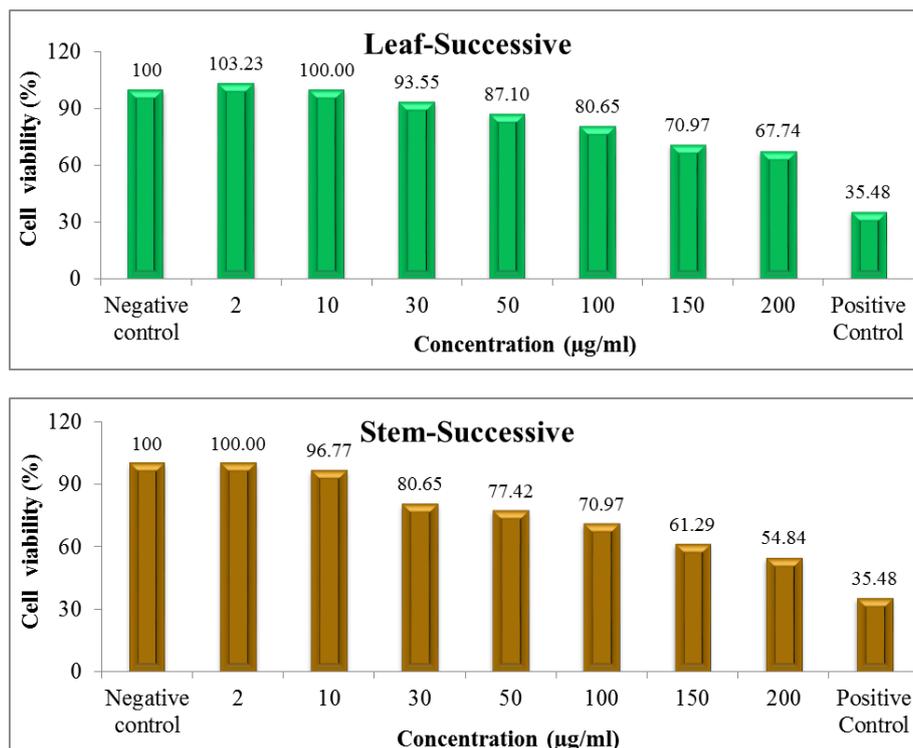
**Fig 2:** In vitro cytotoxicity of ME extract of *P. santalinus* against HeLa cell line by individual cold percolation method.



**Fig 3:** In vitro cytotoxicity of ME extract of *P. santalinus* against HeLa cell line by successive cold percolation method.



**Fig 4:** *In vitro* cytotoxicity of ME extract of *P. santalinus* against breast cancer cell line by individual cold percolation method.



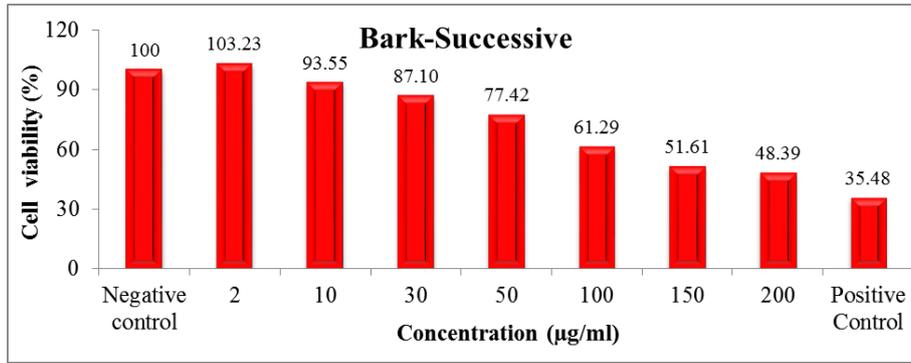


Fig 5: *In vitro* cytotoxicity of ME extract of *P. santalinus* against breast cancer cell line by successive cold percolation method.

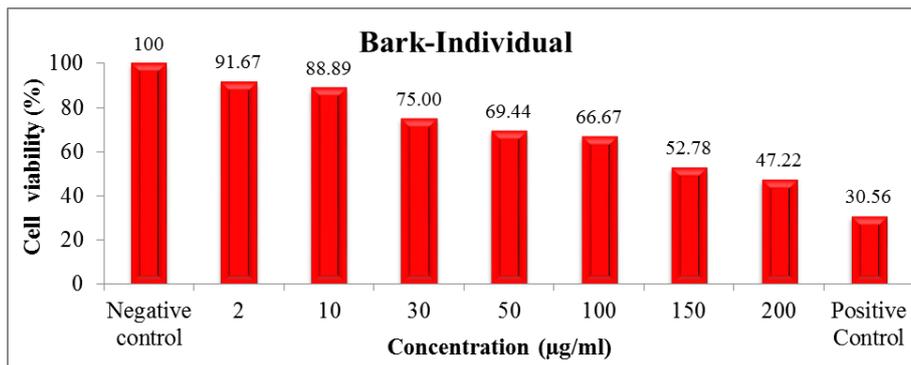
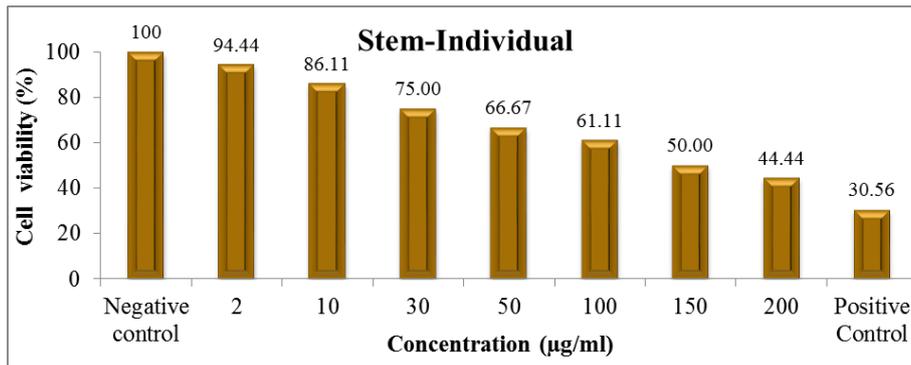
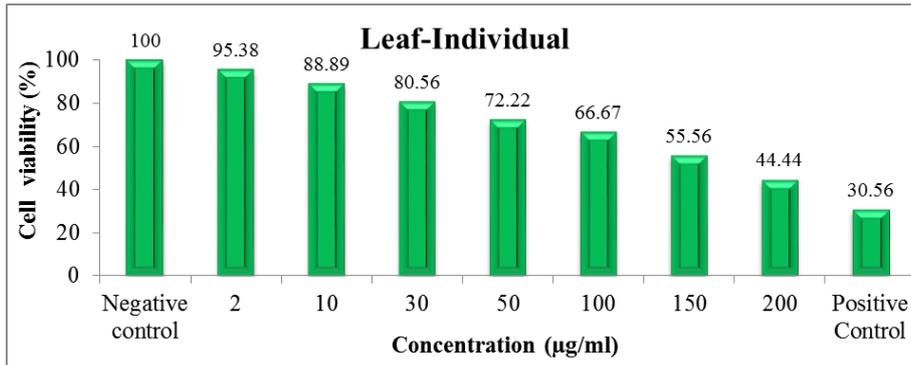
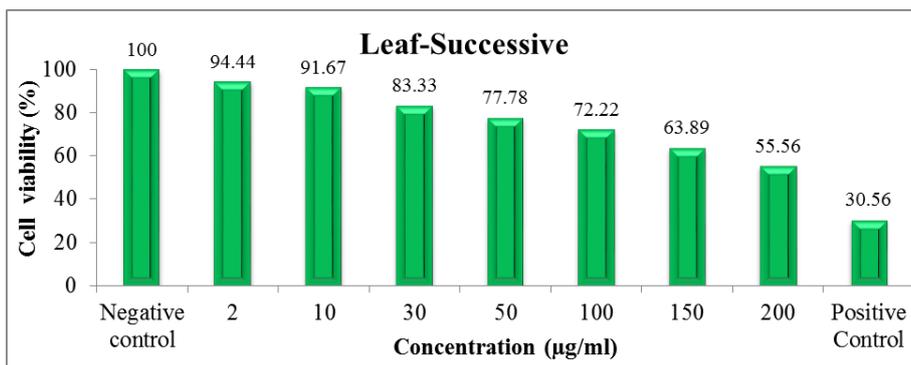
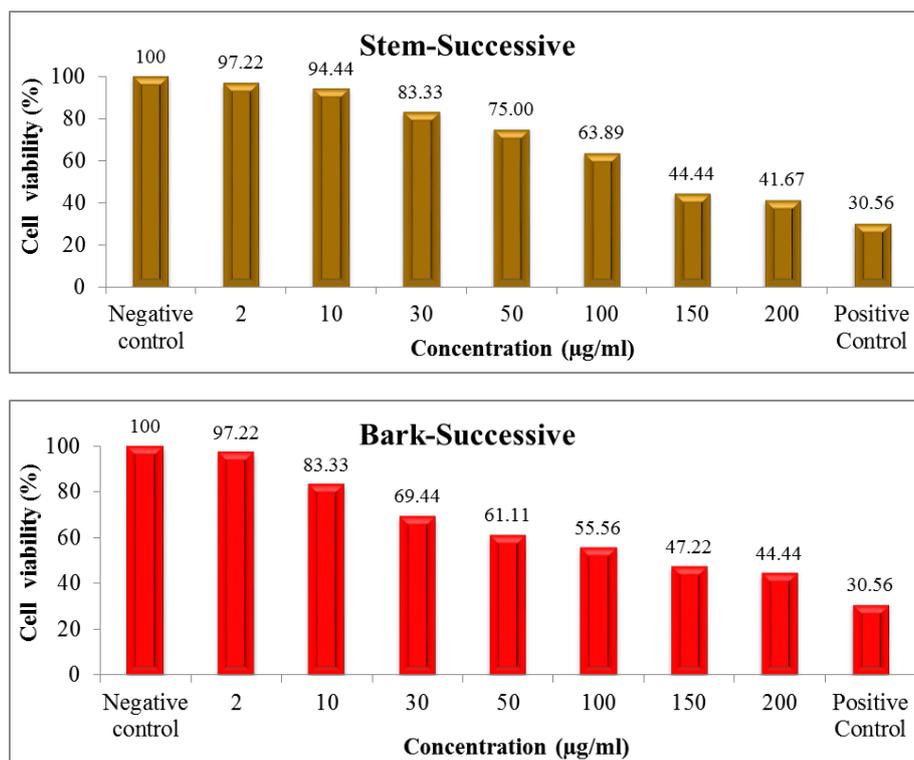


Fig 6: *In vitro* cytotoxicity of ME extract of *P. santalinus* against Normal fibroblast cell line by individual cold percolation method.





**Fig 7:** *In vitro* cytotoxicity of ME extract of *P. santalinus* against Normal fibroblast cell line by successive cold percolation method.

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