Conclusion and key finding

CONCLUSION AND

KEY FINDING
6 Conclusion and key finding

Cancer remains as the one of the leading causes of death of millions of people all over the world, despite the advancement in modern techniques for diagnosis and treatment. Lung adenocarcinomas account for the second leading cause of cancer deaths. The incidences are on the rise globally, especially in the Asian countries. Among the FDA approved drugs for cancer and infectious diseases 60% and 75% respectively are from natural sources (Newman et al., 2003). Currently, lots of interest exists among researchers in drug discovery from natural sources for cancer treatment and prevention (Balunas & Kinghorn, 2005). A large number of plant species have been screened and also bio assayed worldwide by scientist for this purpose (Edeoga et al., 2005, Mehta & Pezzuto, 2002).

The main purpose of this study was to evaluate the anti-proliferative properties of some medicinal plants. Eight plants [Vitex negundo (Leaves), Lantana camara (Leaves), Bauhinia variegate (Leaves), Bauhinia racemosa (Leaves), Bauhinia purpurea (Bark, leaves), Argyreia nervosa (Leaves, root), Butea monosperma (Leaves), Kigelia pinnata (Leaves)] were selected and their methanolic extracts were screened for anticancer activity in vitro using the MTT assay. Lung adenocarcinoma cell line (A-549) and chicken embryo fibroblast (CFC) were chosen for the study. The main results of screening were as follows:

1. *V. negundo*, *B. racemosa* and *B. monosperma* plant’s methanolic extract were found to have promising activity in vitro. These plant’s methanolic extract were more active on cancerous cells than the non-cancerous cells. *V. negundo* have low LD$_{50}$ value (213±1.34 µg/ml) against cancerous cells (A-549) but high LD$_{50}$ value (443.31±2.45 µg/ml) against primary culture of chick embryo fibroblast (CFC) cells. Similarly *B. racemosa* and *B. monosperma* plant’s methanolic extracts also shows low LD$_{50}$ values respectively (263.6±1.94 and 205±1.98 µg/ml) against cancerous cells (A-549) but high LD$_{50}$ values against primary culture of chick embryo fibroblast (CFC) cells (376±2.14 and 390.82±4.5 µg/ml) respectively. From the above result *V. negundo*, *B. racemosa* and *B. monosperma* were selected for further study.

2. Partially purified fractions of these three plants were again screened for their cytotoxic activity against cancerous cells (A-549) and primary culture of chick embryo fibroblast (CFC) cells used as normal cells. From all screened extract ethylacetate extract of *V. negundo* was found to possess selective toxicity on the lung cancer (A-549) cell lines, whilst exhibiting minimal toxicity on the normal primary culture of chick embryo fibroblast (CFC) cells.
3. The methanolic and ethylacetate extract of *V. negundo* was investigated for its preliminary phytochemical analysis. Phytochemical analysis showed the presence of steroid, alkaloids, flavanoids, tannins, saponin, terpanoids and phenolic secondary metabolites. Total phenolic and flavanoid content in ethylacetate extract of *V. negundo* was \((211 \pm 1.53\, \text{mg GAE/g dw})\) and \((211 \pm 1.53\, \text{mg GAE/g dw})\) respectively. For ABTS activity, \(\text{IC}_{50}\) value for methanolic \((72.15\, \mu\text{g/ml})\) and ethylacetate fraction \((61.25\, \mu\text{g/ml})\) of *V. negundo* was compared with standard BHT \((8.66\, \mu\text{g/ml})\).

4. The methanolic and ethylacetate extract of *V. negundo* was studied for its antioxidative activity using DPPH and ABTS assay. The \(\text{IC}_{50}\) value for ethylacetate extract of *V. negundo* was \(245.52 \pm 2.78\, (\mu\text{g/ml})\) which exhibited somewhat higher scavenging activity than methanolic extract of *V. negundo* which was \((309.17 \pm 1.68\, \mu\text{g/ml})\). Thus ethylacetate extract has a good DPPH scavenging activity.

5. ROS generation in A-549 cells treated with Ethylacetate fraction of *V. negundo* can be monitored using fluorescence methods. 2', 7'-dichlorofluorescein diacetate fluorescence probe was used for qualitative and quantitative analysis. Oxidative stress was observed in the cells treated with ethyl acetate fraction of *V. negundo* compare to the control cells. As ROS level was increased intensity of fluorescence was also increased. At lower concentration, the ROS production was lower but as concentration increased, the significant increase in ROS was observed.

6. The ethylacetate extract of *V. negundo* was further investigated for its mechanism of anticancer activity through apoptotic pathway.

- VnEAE caused death of A-549 cells through the apoptotic pathway which was confirmed by the appearance of DNA ladder- the hallmark of apoptotic death).
- After treatment of A-549 cells with \(\text{LD}_{50}\) value of VnEAE, several morphological changes were observed. These changes include rounding up of the cells, detaching from the substrate, membrane blebbing, presence of apoptotic body and finally lesser number of cells in the culture flask of A-549 cells.
- In DAPI staining morphological examination revealed cell shrinkage, chromatin condensation and loss of cell-to-cell contact when VnEAE treated A-549 cells. This indicated that A-549 cells undergo apoptosis.
- qRT-PCR assays revealed that the mRNA levels of P53, Bax, caspase-3 and caspase-9 genes increased significantly with increasing time in the cells treated with \(\text{LD}_{50}\) value of VnEAE compared with the control group gene. Bcl-2 gene expression level was decreased with increasing time. These results indicated that the increased
expression of caspase-3 and caspase-9 may contribute to apoptosis induced by VnEAE.

7. Using column chromatography technique ethylacetate fraction of *V. negundo* was subjected for separation of compounds using TLC, HPLC, LC-MS and $^1$H NMR technique. Using above technique, two compounds were identified: 1) luteolin and 2) p-hydroxybenzoic acid.

8. *In-vitro* cytotoxicity study of luteolin and p-hydroxy benzoic acid was checked against A-549 cells using MTT assay. LD$_{50}$ value for luteolin and P-hydroxy benzoic acid were 43.65 ± 2.3 and 53.82 ± 1.4 µM respectively.

9. Caspase-3 is key player in apoptosis mechanism because both intrinsic and extrinsic pathway activate caspase 3 which ending in a death of the cells. Using the EnzChek Caspase-3 Assay Kit #1 with Z-DEVD–AMC substrate the caspase-3 activity in the luteolin and p-hydroxy benzoic acid treated A-549 cells was assessed and activity was increased with time over that of untreated cells. The activation of caspase-3 activity suggests that its cytotoxicity was caused by the induction of apoptosis.

It was concluded from the current study that *V. negundo* capable of inducing growth-suppressive and apoptosis effects in A-549 cells, suggesting that *V. negundo* possesses selective anti lung cancer activity against cancerous cells compare to the normal cells, might be a novel and attractive remedial contender for tumor treatment in clinical practice.