8. Summary and Conclusions

8.1 Summary

*Memecylon umbellatum* and *Nardostachys jatamansi* are two medicinal plants which have been used traditionally for inflammatory conditions and cancer and there is preliminary evidence of their anticancer activity. Both these plants can add to the armamentarium of plant-derived anticancer agents, hence, the plant extracts were investigated systematically for their anticancer activity and an attempt was made to establish the phytochemical basis of action. The methanol extract of *M. umbellatum* was prepared and partitioned to yield petroleum ether, diethyl ether, ethyl acetate and remaining aqueous fractions. For *N. jatamansi*, the petroleum ether extract was prepared by cold maceration in addition to the preparation of methanol extract that was partitioned to yield diethyl ether, ethyl acetate, and remaining aqueous fractions. The effect of the prepared fractions were tested in 4 different cancer cell lines viz. HCT-116, A549, MCF-7, and MDA-MB-231 cells after treatment with fractions for 48 h by MTT assay.

**Bioactivity-guided fractionation of ethyl acetate fraction of Memecylon umbellatum**

Of the fractions, the fraction MUEA was found to be most active with IC_{50} values of 75.23 µg/mL, 101.80 µg/mL, 116.60 µg/mL, and 69.49 µg/mL in HCT-116, A549, MCF-7, and MDA-MB-231 cells respectively. The fraction MUEA was found to be most active in MDA-MB-231 triple negative breast cancer cells.

HPTLC analysis revealed the presence of lupeol in the MUEA fraction. The anticancer activity of the identified compound, lupeol has been reported previously. Lupeol is a pentacyclic triterpene that exerts antitumor activity by cell cycle regulation and inducing apoptosis. It is reported to inhibit the proliferation of MCF-7, MDA-MB-231, and other breast cancer cells in a dose and time-dependent manner (Lambertini et al., 2005; Saleem, 2009).

Plant extracts are known to exert their therapeutic effect in a synergistic manner. To identify the other compounds responsible for the anticancer activity, broad fractionation was carried out using column chromatography following which the column sub-fractions (I) were tested for their cytotoxicity in cancer cells viz. HCT-116, MCF-7. The fraction MUEA/C1/F7/40/60 was found to be most active in both MCF-7 and HCT-116 cells. The IC_{50} value for MUEA/C1/F7/40/60 fraction was found to be 52.83 µg/mL in
HCT-116 cells and 30.2 µg/mL in MCF-7 cells. The active sub-fraction was further subjected to column chromatography for isolation of pure compounds responsible for the cytotoxic activity in cancer cells viz. HCT-116, MCF-7, and MDA-MB-231 cells. The fraction MUEA/C2/F15/70/30 was found to be most active in MCF-7, HCT-116, and MDA-MB-231 cells. The IC₅₀ value for MUEA/C2/F15/70/30 fraction was found to be 48.63 µg/mL in HCT-116 cells, 26.40 µg/mL in MCF-7 cells, and 20.73 µg/mL in MDA-MB-231 cells. The active fraction was purified using column chromatography and three compounds were isolated. The compounds were characterized using melting point mass, IR, ¹H NMR and ¹³C NMR spectroscopy. The isolated compounds MU2, MU3, and MU4 were characterized as pyrogallol, quercetin, and gallic acid. The compounds are phenolic in nature and exhibited significant cytotoxicity in HCT-116, MCF-7, and MDA-MB-231 cells with the IC₅₀ values in the range of 11-44 µg/mL in breast cancer cells and 15-84 µg/mL in colon cancer cells. Therefore, the anti-cancer activity of the MUEA fraction could be attributed to these phenolic compounds.

Our studies demonstrate for the first time, the extensive in vitro anticancer activity and the phytochemical basis of action of the leaves of Memecylon umbellatum.

Bioactivity-guided fractionation of diethyl ether fraction of Nardostachys jatamansi

Of the partitioned fractions, NJDE was found to be most active with IC₅₀ values of 21.33 µg/mL, 53.01 µg/mL, 69.94 µg/mL, and 25.04 µg/mL in HCT-116, A549, MCF-7, and MDA-MB-231 cells respectively.

HPTLC analysis revealed that the NJM extract, NJPE and NJDE fractions were found to be enriched with lupeol and β-sitosterol which might have contributed to the anticancer activity of the extract/fractions. Lupeol causes G₂/M arrest in prostate cancer cells which is mediated through cyclin-B-regulated signalling pathway (Prasad et al., 2008). Further, it is reported that lupeol induced G₂/M phase arrest and apoptosis in DMBA-induced carcinogenesis with upregulation of Bax and caspase-3 and downregulation of bcl-2 and survivin genes (Nigam et al., 2007). In addition, recent studies have confirmed the cytotoxic effect of β-sitosterol in MDA-MB-231 cells at concentration of 16 µmol/L which is mediated through induction of apoptosis by upregulation of bax/bcl-2 ratio, downregualtion of IAP family and caspase activation (Park et al., 2008). Moreover, another group confirmed the cytotoxic and pro-apoptotic effect of β-sitosterol in MDA-MB-231 cells providing valuable insight into the chemopreventive and therapeutic...
efficacy of β-sitosterol (Vundru et al., 2013). Overall, lupeol and β-sitosterol are reported to have multi-target action with immense anticancer potential modulating key signalling pathways that are implicated in various types of cancer, modulation of antioxidant enzyme levels in disease states, and reducing free radical generation (Saleem, 2009, Baskar et al., 2010). Hence, these compounds can serve as excellent chemopreventive as well as therapeutic agents. NJDE exhibited the highest anticancer activity in MDA-MB-231 cells which could be attributed to the presence of lupeol and β-sitosterol along with the presence of other sesquiterpenes since the rhizomes are considered to be rich in jatamanshic acid, jatamansone, patchouli alcohol, nor-seychelanone, seychellen, alpha and beta patchoulene, valeranone, valeranal, nardol, calarenol, and nardostachone. Sesquiterpenes are a promising class of natural compounds in cancer drug discovery with 3 compounds; artemisinin, thapsigargin and parthenolide currently being evaluated in clinical trials (Ghantous et al., 2010). Other anticancer terpenoids from N. jatamansi include ursolic acid and 3-O-arabinosyl ursolic acid isolated from the chlorform:methanol fraction that showed cytotoxicity in lung (A-549), prostate (DU-145), breast cancer (MCF-7), and neuroblastoma (SK-N-SH) cells (IC\textsubscript{50}: 18-32 mM) (Rekha et al., 2013). Hence, the anticancer activity of the whole extract NJM and NJDE fraction against breast cancer cells could be attributed to the presence of a variety of compounds.

To identify the other compounds responsible for the anticancer activity, broad fractionation was carried out using column chromatography following which the column sub-fractions (I) were tested for their cytotoxicity in cancer cells viz. HCT-116, MCF-7. The fractions NJDE/C1/F2/95/5 and NJDE/C1/F4/85/15 were found to be most active in HCT-116 cells with an IC\textsubscript{50} value of 15.48 µg/mL and 17.72 µg/mL respectively. In MCF-7 and MDA-MB-231 cells, NJDE/C1/F2/95/5 fraction exhibited IC\textsubscript{50} values of 85.82 µg/mL and 18.13 µg/mL respectively and NJDE/C1/F4/85/15 fraction demonstrated IC\textsubscript{50} values of 42.77 µg/mL and 19.58 µg/mL respectively.

The active fraction NJDE/C1/F2/95/5 was purified using column chromatography and white crystalline compound was isolated from the NJDE/C2/F1/100PE fraction and the compound NJ1 was isolated. The compound was characterized as nardin using melting point mass, IR, \textsuperscript{1}HNMR and \textsuperscript{13}CNMR spectroscopy. Nardin is a sesquiterpene acid and its activity was further evaluated in various cancer cells. It was found to exhibit significant cytotoxicity in HCT-116, MCF-7, and MDA-MB-231 cells with the IC\textsubscript{50}
values, 78.39 µg/mL in MCF-7 and 30.58 µg/mL in MDA-MB-231 breast cancer cells and 67.47 µg/mL in HCT-116 cells. From the active sub-fraction NJDE/C2/F2/99/1, a yellow colored oil was obtained and GC-MS analysis was carried out to determine the chemical constituents of the oil. The fraction was found to be rich in patchouli alcohol (27.45%), Globulol (7.17%), Spathulenol (11.77%), Juniper camphor (7.80%), and valeric acid, 2,6-dimethylnon-1-en-3-yn-5-yl ester (10.85%). The patchouli alcohol rich oil also demonstrated significant cytotoxicity to cancer cells with IC$_{50}$ values of 30.89 µg/mL in MCF-7 cells, 18.37 µg/mL in MDA-MB-231 cells and 17.79 µg/mL in HCT-116 cells. Therefore, the anti-cancer activity of the NJDE fraction could be attributed to these compounds.

Our studies demonstrate for the first time, the extensive in vitro anti-cancer activity of PA-rich oil and nardin establishing the phytochemical basis of action of the roots and rhizomes of Nardostachys jatamansi.

The isolated compounds were tested for their underlying mechanistic basis of activity. It is a well known fact that apoptosis is the preferred mode of action for anticancer compounds. Thus, modulation of apoptosis is considered extremely useful in the management and therapy or cancer chemoprevention. Additionally, extensive research is underway to focus on the elucidation and analysis of the cell cycle machinery and signalling pathways that modulate cell cycle. Therefore, targeting the higher proliferation rate of cancer cells can be used to inhibit tumor growth by preferential killing of rapidly proliferating cells. Compounds that simultaneously modulate these two cornerstones of effective anticancer therapeutics are highly desirable.

Our results demonstrated that the NJDE fraction of N. jatamansi acts through the activation of apoptotic pathway evident through increased fold expression of Caspase 3/7 in MDA-MB-231Br cells. The effect of NJDE fraction could be attributed to the synergistic activities of PA-rich oil and nardin. In cell cycle analysis, NJDE fraction caused cell cycle arrest in MDA-MB-231 cells in the G$_0$/G$_1$ phase. In colon cancer cells, NJDE fraction caused G$_0$/G$_1$ arrest that could be due to the cumulative effect of PA-rich oil and nardin. Both PA-rich oil and nardin exhibited G$_0$/G$_1$ arrest in HCT-116 cells and G$_2$/M arrest in MCF-7 cells.

In addition, NJM and fractions, NJPE, NJDE, and NJEA significantly (p < 0.001) reduced the colony formation of MDA-MB-231 cells over a period of 12 days thereby
suggesting the long-term antiproliferative effect of NJM/fractions. Clonogenic assay is considered as the gold standard to determine the anticancer activity of drugs. Owing to their ability to inhibit colony formation of MDA-MB-231 cells, we speculate that NJM/fractions could significantly contribute to the reduction of metastases.

The MUEA fraction of Memecylon umbellatum acts through the activation of apoptotic pathway through increased fold expression of Caspase 3/7 in MDA-MB-231Br cells. The effect of MUEA fraction could be attributed to the phenolic compounds pyrogallol, quercetin, and gallic acid. All compounds exhibited significant induction of apoptosis as evident through the nuclear staining methods. In cell cycle analysis of MCF-7 cells, the active fraction MUEA/C2/F15/70/30 increased the proportion of cells in the S phase. MCF-7 cells treated with pyrogallol showed an increase in the percentage of cells in the G2/M phase while treatment with quercetin increased the proportion of MCF-7 cells in the S phase and G2/M phase as compared to negative control cells. MUEA fraction demonstrated G0/G1 arrest in HCT-116 cells while quercetin exhibited G2/M arrest in HCT-116 cells. Quercetin is reported to cause G2/M arrest in U937 cells (Lee et al., 2006). Moreover, the active column sub-fractions of both plants demonstrated significant selectivity towards breast cancer cells as compared to normal MCF-10A cells, a highly desirable feature of anticancer therapeutics.

Overall, MUEA fraction and its active components: pyrogallol, quercetin, and gallic acid act by the preferred mode of cell death i.e. induction of apoptosis and cell cycle arrest in colon and breast cancer cells (both ER+ and triple negative). NJDE fraction and its active components: PA-rich oil and sesquiterpene acid, nardin act by the preferred mode of cell death i.e. induction of apoptosis and cell cycle arrest in colon and breast cancer cells.

The active fractions were also tested for their in vivo antitumor activity using two animal models: DMBA-induced breast cancer in Sprague-Dawley rats and MDA-MB-231Br xenograft model in nude mice. In the DMBA model, the MUEA fraction demonstrated significant (p < 0.05) reduction in tumor growth volume and tumor weight at 200 mg/kg in the DMBA-induced breast cancer in SD rats. Elemental analysis revealed significant hypercalcemia in DMBA treated animals while MUEA fraction treated animals showed significant (p < 0.05) reduction in serum calcium levels. Antioxidant parameters in mammary gland showed diminished lipid peroxidation (p < 0.05) in MUEA treated groups at both doses. MUEA was more effective at the higher dose i.e. 200 mg/kg. In
MDA-MB-231 xenograft model, MUEA at 150 mg/kg showed significant (p<0.05) reduction of tumor growth.

NJDE fraction at both doses was found to exhibit significant (p < 0.05) reduction in tumor growth volume and tumor weight in the DMBA-induced breast cancer in SD rats. In combination with doxorubicin, NJDE was found to demonstrate cardioprotective activity and better overall health of the animals as shown by maintenance of body weight, lesser reduction in WBC count and serum AST levels as compared to doxorubicin treated animals. Oxidative stress and trace elements have been implicated in the development of breast cancer. Elemental analysis revealed significant hypercalcemia in DMBA treated animals while treatment groups showed significant reduction (p < 0.05) in serum calcium levels. Of note, antioxidant parameters in mammary gland showed diminished lipid peroxidation (p < 0.05) in NJDE treated groups. Overall, NJDE was more effective at the higher dose i.e. 200 mg/kg. In MDA-MB-231 xenograft model, NJDE/C1/F4/85/15 fraction at 25 mg/kg exhibited significant (p<0.05) inhibition of MDA-MB-231Br tumor in vivo from Day 5 onwards. The column sub-fractions PA-rich oil and NJDE/C2/F2/91/9 also demonstrated significant (p<0.05) tumor growth inhibition of MDA-MB-231Br xenograft at 50 mg/kg dose.

To summarize, NJDE and MUEA exhibit significant antitumor potential in DMBA-induced breast cancer in SD rats. NJDE is cardioprotective in nature as it was found to alleviate the symptoms of myocardial damage/cardio-myopathy associated with doxorubicin treatment. Overall, fractions MUEA and NJDE have immense anticancer potential by modulating key signalling pathways that are implicated in various types of cancer, modulation of antioxidant enzyme levels in disease states, and reducing free radical generation thereby combating oxidative stress.

8.2 Conclusion

Our results suggest that *Memecylon umbellatum* and *Nardostachys jatamansi* may serve as an excellent lead for the development of anticancer agents for colon and breast cancer. The anticancer activity of the MUEA could be attributed to the phenolic compounds; pyrogallol, gallic acid and quercetin that exhibit significant cytotoxicity to colon and breast cancer cells cancer cells and act via the preferred mode of action of anticancer therapeutics viz. induction of apoptosis and cell cycle arrest.
The anticancer activity of *Nardostachys jatamansi* could be attributed to nardin and PA-rich oil that exhibit significant cytotoxicity to colon and breast cancer cells and act via induction of apoptosis and G0/G1 arrest in cancer cells. *In vivo*, the fractions MUEA and NJDE exhibit significant anticancer activity in animal models of breast cancer in both DMBA-induced breast cancer and MDA-MB-231Br xenograft breast cancer model.

### 8.3 Future research initiatives

- Further testing of the isolated compound, nardin and PA-rich oil in different cancer cells and understanding their effect on the expression of cell cycle checkpoint inhibitors
- Mechanistic evaluation of the sub-fraction/isolated compounds on important signalling pathways affected in breast cancer
- Mechanistic evaluation of active sub-fractions/compounds in xenograft models of breast cancer in particular triple negative breast cancer
- Development of formulation for the active sub-fractions and testing for their *in vitro* and *in vivo* anticancer activity

### 8.4 References


