Summary

Leaf extracts of *Glycosmis pentaphylla* (Retz.) DC inhibited the cell growth of cancer cells, compared to the bark extracts.

Bioactivity guided fractionation and *in vitro* screening by SRB assay in a panel of cancer and normal cells resulted in shortlisting four active fractions. The shortlisted fractions viz. PEG2, PEG5, EF1 and DCM2 showed higher cytotoxicity to breast cancer cells. The toxicity was selective to the cancer cells. Fractions showed two to three-fold increase in IC$_{50}$ values in normal breast (MCF-10A) and kidney (Vero) cells.

The fractions were standardized by HPLC and HPTLC. Lupeol and β-sitosterol were detected by HPTLC. Fraction PEG2 showed presence of lupeol (2.508 ± 0.34%) and β-sitosterol was detected in PEG5 (14.14 ± 0.93%) and DCM2 (0.79 ± 0.07%). HPLC detected fumaric acid (9.91 ± 0.005 µg/mg) and quercetin (23.77 ± 0.01 µg/mg) in DCM2 fraction. Quercetin (2.195 ± 0.004 µg/mg) and kaempferol (35.278 ± 0.002 µg/mg) were present in EF1 fraction.

Two isolates were obtained from the DCM2 fraction. Isolate 1 was identified as cycloartenol with molecular formula C$_{30}$H$_{50}$O. Isolate 2 was a simple aliphatic alcohol.

Lupeol, Chrysin and Cycloartenol were reported for their presence in this plant for the first time through this study.

Selected fractions were able to inhibit the colony formation of MCF-7 and MDA-MB-231 breast cancer cells.

Cell cycle analysis by flow cytometry showed G$_0$/G$_1$ arrest in MCF-7 and G$_2$/M arrest in MDA-MB-231 by the fractions. The cell lines being different in the ER (estrogen receptor) status, the results might be indicating that in MCF-7 cells (ER$^+$) the fractions inhibited CDK4/6 through the estrogen receptors. The activity might be also correlated with the increased expression of p-53 and decreased Bcl-2 expression. In MDA-MB-231 cells (ER$^-$), the CDK inhibition might be through ER independent mechanism.

When a cell is arrested in cell cycle and if the damage is not repairable it is directed to the apoptotic pathway. The ability of the studied fractions viz. PEG2, PEG5, DCM2 and EF1 to induce apoptosis in breast cancer cells was evident from the morphological changes like condensed nuclei and formation of apoptotic bodies found after Hoechst staining and AO/EB dual staining.

DNA fragmentation pattern seen in agarose gel electrophoresis after the treatment with fractions supported the apoptosis inducing ability of the fractions.
Apoptosis was confirmed by Annexin V staining by flow cytometry. The fractions were inducing apoptosis, and not necrosis in breast cancer cells.

The ability of the fractions to induce apoptosis through the intrinsic/mitochondrial apoptotic pathway was confirmed by caspase 3/7 assay in MCF-7 and MDA-MB-231 breast cancer cells. The fractions were able to activate the executioner caspases 3 and 7 which is confirmatory that the apoptosis occurred through the mitochondrial pathway.

Western blot studies of the key apoptotic regulators like p53, Bax, Bcl-2, cytochrome c and caspase-9 in MCF-7 cells was done. Results showed that the tested fractions were able to induce p53 (tumor suppressor gene), Bax, cytochrome c and caspase-9 which are the pro-apoptotic markers, and inhibit Bcl-2 expression which is an anti-apoptotic marker. These studies further confirmed the activation of intrinsic apoptotic pathway by the fractions.

In MDA-MB-231 cells, the levels of p-53, cleaved PARP, Bad and cleaved caspase-3 were increased, evidenced from the ELISA confirming the activation of mitochondrial apoptotic pathway.

Anti-metastatic potential was initially tested by scratch wound assay. All fractions were able to significantly prevent cell migration at 48 h compared to the control MCF-7 cells. In MDA-MB-231 cells, all fractions except EF1 were able to significantly inhibit migration at 24 h and 48 h.

Effect on MMP-9 expression studied by western blot presented that all the fractions except EF1 significantly reduced the levels in MCF-7 cells. Hence, the fractions have anti-metastatic potential, which might be further confirmed in suitable in vivo models.

Western blot studies on the expression of HIF-1α, a key inducer of angiogenesis in cancer cells was done in MCF-7 cells. The fractions PEG2, PEG5, EF1, but not DCM2 were able to decrease the HIF-1α expression. This implied on the anti-angiogenic potential of G. pentaphylla (Retz.)DC.

ROS and NO production in LPS-challenged RAW264.7 cells were inhibited by the fractions with an order of activity, DCM2> EF1>PEG2>PEG5. Since LPS acts through the toll-like receptor 4 (TLR-4) and further activates NFκB to induce many inflammatory mediators, the inhibitory activity showed by the fractions of G. pentaphylla indicated on its anti-inflammatory potential. Ethnopharmacologically, the plant is a well-known anti-inflammatory agent.

In vivo studies on DMBA-induced mammary tumor bearing Sprague Dawley rats showed that the fractions PEG2, EF1 and DCM2 at a dose of 400 mg/kg, p.o. were able to
significantly reduce the tumor weight and volume. The fraction PEG5 which has shown significant activity \textit{in vitro} was unable to show a significant activity \textit{in vivo} at 400 mg/kg, p.o. But this might be due to the dose selected or pharmacokinetic profile and hence maybe studied at a higher dose.

- There was no significant difference in body weight change and organ indices of liver, kidney, spleen and heart in animals treated with the fractions. The spleen index was found to be significantly lowered in doxorubicin (standard) treated animals. No significant difference in the blood parameters was found in animals treated with fractions, but doxorubicin treated animals presented a significantly lower RBC count and hemoglobin content.

- The lipid peroxidation level in mammary tissue was significantly elevated in DMBA control animals and doxorubicin treated animals. The fractions were able to significantly reduce the level when compared to DMBA control animals. Catalase levels were significantly lowered in DMBA control and doxorubicin treated animals. Animals treated with the fractions significantly elevated the levels (near to normal level) when compared to DMBA control.

- The nitrate and nitrite levels were significantly higher in DMBA control animals. The levels in animals treated with the fractions were near to normal. This might be due to the anti-oxidant property of the fractions.

- The serum AST levels were elevated in all the animals when compared to normal control. ALT levels were normal in all the animals. Serum urea levels were significantly higher in DMBA control and doxorubicin treated animals, but normal in animals treated with the fractions.

- Histopathology of mammary tissue implicated that DMBA induced infiltrating ductal carcinoma in rats. All the treatments were able to reduce the necrosis and restore the normal breast architecture to some extent. Even though the fraction PEG5 could not significantly reduce the tumor volume at 400 mg/kg, p.o. the histopathology showed that it was able to reduce necrosis. DMBA control and doxorubicin treated animals showed degeneration of the renal tubular epithelium. In liver, mild periportal inflammation characterized by infiltration of lymphocytes was found in DMBA control animals. Doxorubicin treated animals showed mild vacuolar degeneration in liver. The histopathological studies of liver and kidneys of animals treated with fractions showed normal architecture.
In EAC tumor model, the fractions PEG2, PEG5 and EF1 showed significant anti-cancer activity by increasing the mean survival time and life span of animals. The altered hematological and anti-oxidant enzyme levels were also brought near to normal levels by the fractions.

*In vivo* studies are pointing towards the advantage of natural products as being devoid of any major toxic effects.

**Conclusion**

*Glycosmis pentaphylla* (Retz.) DC has shown promising results *in vitro* and also *in vivo* in mammary tumor model. Our studies have supported the ethnopharmacological use of the plant as an anti-cancer medicine by the traditional healers. Natural products due to their variety of chemical components have the property to act in a moderate way devoid of severe toxicities. They are also able to target and act through multiple signaling pathways. *Glycosmis pentaphylla* (Retz.) DC fractions were able to act through different pathways *in vitro* and showed anti-cancer, apoptotic, anti-angiogenic, anti-metastatic and anti-inflammatory activities. *In vivo* studies implicated the low toxicity level of the plant as compared to the standards, doxorubicin and cisplatin. The plant may be a source of new lead compounds for anti-cancer activity and may be explored further.

**Future directions**

- Study of the effect of fractions on the hormonal status in animals.
- Study the fractions for anti-metastatic and anti-angiogenic activities *in vivo*.
- Study of fractions on transplantable tumor models.
- Isolation and characterization of new compounds from active fractions.
- Study the anti-inflammatory potential of the plant.
- Studies to relate the anti-cancer constituents of the plant to the identified pathophysiological cancer targets.