Chapter-V

SUMMARY & CONCLUSION
The present study focused on isolation of an immunomodulatory compound from *Prunus cerasus* L. the sour cherry (from Kashmir) and its therapeutic applications particularly against cancer. The review of literature revealed that some parts of the plant predominantly, the fruit extracts have a chemopreventive effect against various ailments e.g. inflammation, stroke, gout etc. However there was hardly any comprehensive evidence based studies reported where different parts of the plant have been demarcated on the basis of their immunological bioactivity profile. Keeping in view the importance of immune response of body to cure immunological or immune related diseases like diabetes, cholestremia, cancer etc., in the present study attempts have been made to provide an in-depth studies regarding immunomodulatory and anticancer activities conferred to the methanolic fruit extract and find out the active constituent(s) principally responsible for the bioactivity.

The whole study was completed in a sequence of three phases using a detailed panel of in vitro and in vivo experimental systems. In the phase-I, three types of solvent extracts i.e. methanolic, hydro-methanolic and aqueous were prepared from five different Parts of *Prunus cerasus* L. Total 15 extracts were prepared from the selected parts i.e. seed, fruit, leaves, root and shoot-bark and were screened in vitro for their immunological activity, employing various tests: Nitroblue Tetrazolium (NBT) reduction potential, inducible Nitric Oxide Synthase (iNOS) and bactericidal activities as well as mitogenic potential. After preliminary screening, PcMFE (Pc-methanolic fruit extract) was selected, because of its maximum immunological bioactivity in vitro.

The selected extract ‘PcMFE’ was subjected to in vivo immunomodulatory studies using assays for cell mediated and humoral immune response as well as macrophage function, cytokine expression profile. Results showed that under in vivo experimental conditions also, the PcMFE reproduced promising and convincing immunostimulatory effects on all the tested immune parameters. Hence on the basis of in vitro as well as in vivo immunological experimental studies it was found that PcMFE is the potent immunomodulatory agent/extract.

In order to explore the chemical constituents responsible for the immunological activity of the selected PcMFE, the extract was subjected to bioactivity guided fractionation by partitioning PcMFE into aqueous and ethyl acetate fractions. The in vitro lymphocyte proliferation assay was used to select the more bioactive fraction. The
ethyl acetate fraction was more immunopotent than the aqueous fraction. Hence ethyl acetate fraction (EAFR) was taken up for detailed in vivo immunological studies as well as for isolation of chemical signatures. The in vivo immune-modulation studies of the selected EAFR was conducted in BALB/c mice employing a panel of assays and the results indicated that EAFR efficiently augmented the functions of T and B cells and macrophages. This is evidenced from our findings that EAFR increased T and B cell proliferation, supported Th1 as well as Th2 immunity, enhanced macrophage function and immunoglobulin secretion thereby suggesting that EAFR stimulated cell mediated and humoral immunity. Further the results also revealed that the percentage of CD4+ and CD8+ T lymphocytes in SRBC-immunized mice were greatly augmented by EAFR. The enhanced percentages of both CD4+ and CD8+ T lymphocytes in spleen indicated that both Th and CTLs were activated greatly by EAFR.

In the next phase, chemical constituents were isolated from the bioactive EAFR of PcMFE by Column chromatography. Four compounds were isolated as quercetin, daidzin, rutin and chlorogenic acid. The HPLC analysis data showed that both the PcMFE and its EAFR are rich in daidzin followed by quercetin, chlorogenic acid and rutin. The in vitro immunocharacterization of four isolated molecules showed that daidzin and chlorogenic acid were immunostimulators whereas quercetin and rutin were immunosuppressive. Overall daidzin showed the maximum immunomodulatory potential and hence was selected for further in depth immunological studies in BALB/c mice. The results of in vivo immunological study reproduced that daidzin treatment produced significant immunostimulatory effects on cell mediated and humoral immunity, besides the expression level of selected cytokines i.e. IL-4 as well as IFN-γ and TNF-α also increased.

In the phase–III, the selected extract PcMFE and its isolated chemical constituents were assessed for therapeutic efficacy against cancer using five different cancer cell lines (A-549, THP-1, MCF-7, PC-3 and NCI-H322) by MTT assay. The results indicated that PcMFE showed significant inhibitory activity against almost all tested cell lines with maximum effect being observed against Lung cancer cell line NCI-H322. The cytotoxicity profile of four marker molecules against above mentioned cancer cell lines revealed that quercetin exhibited maximum cytotoxicity potential followed by daidzin whereas chlorogenic acid and rutin didn’t show any significant
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cytotoxicity. The in vivo anticancer activity of PcMFE and two selected bioactive molecules i.e. quercetin and daidzin was tested in EAC/EAT tumor mouse models. PcMFE treatment as well as quercetin showed maximum tumor growth inhibition in EAC bearing mice as compared to Daidzin. Hence quercetin was selected for further in depth comprehensive studies to find out the molecular mechanism of cytotoxicity in NCI-H322.

The basis of cell death by the PcMFE or marker molecules happened to be due to apoptosis. It may again be pointed that cancer cells always evade apoptosis and the agents that induce apoptosis might be the promising anticancer therapeutics. Therefore the relative potential for the induction of cell death due to apoptosis by quercetin was evaluated by employing various biological end points of apoptosis in human lung NCIH322 cells. The results of Cell cycle Arrest demonstrate that exposure of NCI-H322 cells to quercetin enabled apoptotic cell death as evidenced by apoptotic bodies formation and increase in sub-Go hypo-diploid DNA fraction. The impairment of cell migration by quercetin against NCIH322 cell line in wound healing assay showed that quercetin inhibited wound clouser in a concentration dependent manner. Loss in mitochondrial transmembrane potential ($\Delta\Psi_m$) as a result of mitochondrial perturbation was measured by flowcytometer and results showed that quercetin induced a remarkable increase in $\Delta\Psi_m$.

Overall the present study contains research findings that contribute to our understanding of the usefulness of sour cherry fruit and its isolated bioactive components for therapeutic application based on their different biological activities specifically immunomodulation and ant-cancerous properties. In addition, these findings provide basic mechanism of actions of the methanolic fruit extract of Prunus cerasus (PcMFE) that laid down the basis for the future in depth mechanistic studies for many other therapeutic applications.
Conclusion:

From this study it is concluded that PcMFE played an important role in the modulation of the immune response as well as exhibited significant anticancer activity and thus may have applications as an effective immunotherapeutic agent. These findings will assist in identifying potential therapeutic targets of different diseases that can be manipulated for the therapeutic benefit.

This study also showed the activity profile of the extract vis-a-vis its chemical signature, leading to the identification of the component(s) that play major part in the respective activities. The isolated compounds: Daidzin can be employed as an immunomodulator whereas quercetin though immunosuppressive can be employed to cure various cancers.

Further studies regarding the interactions between different chemical constituents isolated from the PcMFE for modulation of immune related disorders are recommended.