CHAPTER 6

SUMMARY

...and to make an end is to make a beginning, the end is where we start from.
-T.S. Eliot
Environmental mutagens and carcinogens are instrumental in initiation, promotion and progression of several kinds of cancer. The exposure to these xenobiotics is often unavoidable and create a great risk to human health. A complimentary approach is to render the host organism more resistant to the attack of mutagens and carcinogens by supplementing the diet with chemopreventive agents. Intake of sufficient amounts of antimitagens and/or anticarcinogens is believed to confer a preventive effect on the initiation and development of human cancers. Phytochemicals are secondary metabolic products produced by plants in response to environmental stresses. Thousands of these phytochemicals have been identified and when consumed in human diet may affect chronic disease risk. Evidences from epidemiological and laboratory studies have demonstrated that some plants eaten whole or their active constituents taken in isolation show adequate protective effects against human carcinogenesis and mutagenesis. Several plant extracts or their constituents have proved to contain variety of antimitagenic/antigenotoxic substances.

Amongst the number of ways by which phytochemicals can protect against cell proliferation, one of the ways is by destroying reactive oxidative species (ROS) that initiate carcinogenesis through oxidative damage of DNA. Oxidative stress can damage DNA, lipids, proteins, and carbohydrates leading to impaired cellular function and enhanced inflammatory reactions. Several studies have revealed link between cancer and cyclooxygenase-2 because prostaglandins (PGs) are mediators of inflammation. The association of chronic inflammation with development of human cancer is well recognized. There are number of reports of involvement of inflammatory process in the initiation and progress of cancer. COX-1 and COX-2 are involved in the control of inflammatory reactions and catalyze the conversion of arachidonic acid to PGH₂, the precursor of PGs. Inhibition of COX-1, the constitutive form may result in gastric ulceration whereas COX-2 is inducible and is thought to be causative factor of cellular
injury and may ultimately lead to carcinogenesis. There is great demand for natural COX-2 inhibitors with fewer side effects.

Medicinal plants are the potential candidates for cancer chemoprevention because they may harbor chemopreventive agents with inhibitory effects on the initiation, promotion and progression of carcinogenesis. *Rubia cordifolia* L. commonly known as ‘majeeth’ is one such medicinal plant whose roots are used in ayurvedic system of medicine to treat various skin disorders, menstrual disorders and urinary disorders. Apart from its medicinal value, this plant has also been used as natural food colorant and as natural hair dyes. *Glycyrrhiza glabra* L. is also an important medicinal plant, the dried rhizomes of which are commonly known as ‘licorice’, and find an important place in Ayurvedic system of medicine as a ‘rasayana’ with implicated use in treatment of respiratory and digestive disorders. Keeping in view the importance of the above medicinal plants in Ayurvedic system of medicine, the present study was planned to isolate and purify antigenotoxic factor(s) present in these important medicinal plants. The isolated phytochemicals were also checked for their potential to inhibit COX-2 in *in vitro* studies.

The roots of *R. cordifolia* were powdered and percolated with 80% methanol to obtain the methanol extract (MeOH-RC). The solvent was evaporated on rotary vacuum evaporator and lyophilized to remove the moisture from the extracts. The MeOH-RC was made aqueous by dissolving in distilled water and further fractionated with a series of organic solvents viz. hexane, chloroform, ethyl acetate and *n*-butanol in order to obtain the Hexane fraction (Hex-RC), Chloroform fraction (CHCl₃-RC), Ethyl acetate fraction (EtOAc-RC), *n*-Butanol fraction (But-RC) respectively. The solvents were evaporated and dried fractions were lyophilized. Hex-RC was subjected to column chromatography (aluminium oxide) and eluted with a gradient of hex/EtOAc and then with EtOAc/MeOH. The fraction eluting in pure methanol resulted in isolation of ‘RUC-1’. CHCl₃-RC was subjected to column chromatography (aluminium oxide) and eluted with a gradient of hex/EtOAc and then with EtOAc/MeOH. The fraction eluting in hex/EtOAc (98:2) and another fraction eluting in EtOAc/MeOH (90:10) led to
the isolation of ‘RUC 2’ and ‘RUC-1’ respectively. Column chromatography (silica gel) of \textit{EtOAc-RC} resulted in the separation of ‘RUC-3’.

The rhizomes of \textit{Glycyrrhiza glabra} were powdered and percolated with 80\% methanol to obtain the methanol extract (\textit{MeOH-GG}). The solvent was evaporated on rotary vacuum evaporator and lyophilized to remove the moisture from the extract. The methanol extract was made aqueous by dissolving in distilled water and further fractionated with a series of organic solvents viz. hexane, chloroform, ethyl acetate and \textit{n}-butanol in order to obtain the \textbf{Hexane fraction (Hex-GG)}, \textbf{Chloroform fraction (CHCl$_3$-GG)}, \textbf{Ethyl acetate fraction (EtOAc-GG)}, \textbf{\textit{n}-Butanol fraction (But-GG)} respectively. \textit{EtOAc-GG} was subjected to medium pressure liquid chromatography by using Borosilikutat 3.3, Code No. 17982 (Buchi Switzerland) column and packed with silica gel for flash chromatography (230-400 mesh) at a flow rate of 10\% and 30mBar pressure and eluted with a gradient of hex/EtOAc and then with EtOAc/MeOH. The fractions eluting in hex/EtOAc (70:30) resulted in the isolation of ‘\textbf{Gly-1}’ and fractions eluting in EtOAc/MeOH (75:25) yielded yellow coloured compound ‘\textbf{Gly-2}’. \textit{MeOH-GG} was subjected to dry column chromatography, and seven fractions (250 ml each) were collected with a gradients of chloroform: methanol. The fraction collected from chloroform: methanol (75:25) yielded the phytochemical ‘\textbf{Gly-3}’. The last two fractions collected from CHCl$_3$: MeOH (25:75) and MeOH (100\%) were dried, pooled and subjected to reverse phase column chromatography using silica gel RP-18, with a running solvent CHCl$_3$: EtOAc: MeOH (40:40:20). Ten fractions were collected (100 ml) each. The fifth fraction yielded the compound ‘\textbf{Gly-4}’.

Various spectroscopic techniques viz. $^1$H-NMR, $^{13}$C-NMR, DEPT-90, DEPT-135, and mass spectroscopy were used to analyse the chemical characteristics of various isolated fractions. RUC-1, RUC-2, and RUC-3 from \textit{Rubia cordifolia} were elucidated as Alizarin, Mollugin, and Lucidin primveroside respectively. Gly-1, Gly-2, Gly-3 and Gly-4 from \textit{Glycyrrhiza glabra} were elucidated as Umbelliferone, Isoliquiritin apioside, Kaempferol and Glycyrrhizic acid respectively. For structure elucidation of the fractions showing single spots, NMR spectra were recorded on Brucker Avance-300. To
find the molecular weight mass spectra were recorded on QTOS-Micro, Waters Micromass.

The antigenotoxic potential of various extract/fractions was evaluated by SOS chromotest in E.coli PQ37 tester strain and in human blood lymphocytes by Comet assay against the genotoxicity induced by hydrogen peroxide and 4-nitroquinoline-1-oxide. The extract/fractions were also tested for their radical scavenging activity by 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay. Pure molecules RUC-1, RUC-2, RUC-3, Gly-1, Gly-2, Gly-3, Gly-4 were evaluated for COX-2 inhibitory activity in vitro.

The different fractions of Rubia cordifolia modulated the genotoxic activity of both H₂O₂ and 4NQO to varying amounts. In SOS chromotest with E.coli PQ37, The potent antigenotoxic activity against both H₂O₂ and 4NQO was exhibited by mollugin (RUC-2), with IC₅₀ of 3.90 µg/ml and 4.26 µg/ml respectively. In case of Alizarin (RUC-1) respective IC₅₀ of 5.92 µg/ml and 6.11 µg/ml against H₂O₂ and 4NQO were observed. Hex-RC, CHCl₃-RC and EtOAc-RC also significantly inhibited the genotoxicity of H₂O₂ and 4NQO. In human blood lymphocytes using the Comet assay, the DNA damage induced by hydrogen peroxide was reduced by 80.49 % by the highest non-toxic dose (10 µg/ml) of mollugin. It also inhibited the 4NQO induced DNA damage by 73.06 %. Alizarin (RUC-1), Hex-RC, CHCl₃ –RC and EtOAc-RC also significantly reduced the tail moment induced by H₂O₂ and 4NQO. In the DPPH radical scavenging assay, Mollugin, Alizarin, Hex-RC, CHCl₃-RC and EtOAc-RC showed significant DPPH radical scavenging activity. Mollugin showed good activity as COX-2 inhibitors (88.53% inhibition at 10⁻⁵ M).

From G.glabra, the phytochemical Isoliquiritin apioside (Gly-2), Glycyrrhizic acid (Gly-4), Umbelliferone (Gly-1), Kaempferol (Gly-3), along with EtOAc-GG, MeOH-GG showed significant antigenotoxic activity against the SOS inducing potency of H₂O₂ and 4NQO respectively in SOS chromotest. The DNA damaging effect of H₂O₂ and 4NQO in human blood lymphocytes was inhibited (≥ 70%) by Isoliquiritin apioside, Glycyrrhizic acid, Kaempferol, EtOAc-GG and MeOH-GG as assessed using comet assay. Isoliquiritin apioside and Glycyrrhizic acid have also been found to
possess selective COX-2 inhibitory activity. Isoliquiritin apioside and Glycyrrhizic acid showed significant DPPH radical scavenging activity.

The chemopreventive effects of various phytochemicals have often been associated with their antioxidative and anti-inflammatory activities. Some fractions from both *R. cordifolia* and *G. glabra* have been found to possess significant antigenotoxic activity against oxidative mutagens. The fractions with potent antigenotoxic activity also show potential anti-inflammatory activity as assessed by COX-2 inhibition. This leads us to suggest that the phytochemicals from both *R. cordifolia* and *G. glabra* possess significant chemopreventive potential.