CHAPTER-6
CONCLUSION

An effective strategy for identifying potential anti-cancer molecules, should be based upon validation of those plants whose ethnobotanical and ethnopharmacological use has shown promise rather than mass screening of plants in general. The use of herbs, plants, homeopathic, ayurvedic and traditional medicines have been outlined as a part of CAM therapies from ancient times, however the effectiveness of such therapies against cancer management and prevention is still uncertain either due to lack of scientific data or safety related issues. An understanding of the use of CAM therapies in mainstream cancer treatment therefore is the need of the hour.

To facilitate an evaluation of the anticancer potential of certain CAM sources [Triticum aestivum or wheatgrass (IIWE, CWE, MWE and AWE), Achyranthes aspera roots (EAA and AAA), mushroom species- Pleurotus ostreatus (PAE), Macroliopia procera (MAE) and Auricularia polytricha (AAE) and homeopathic drug- Ruta graveolens or Ruta (10M, 1M, 200C, 30C and MT)] various assays were carried out on COLO-205 cells which served as the in vitro colon cancer model. As conventional cancer therapy is associated with a number of side effects which basically kill or disrupt the normal cells, therefore the cytotoxic effects on normal cells using NRK-52E cell line was also assessed.

Since free radicals have been known to play a significant role in the initiation and the progression of cancer, therefore the presence of free radical scavengers in the botanical extracts would help in the management of cancer. The results of studies pertaining to the evaluation of the antioxidant potential of the various plant extracts revealed that the methanolic extracts of wheatgrass, ethanolic of Achyranthes aspera and the aqueous extract of Auricularia polytricha possessed the highest free radical scavenging activity. These observations support the theory behind the anticancer activity of plant extracts as reported by a number of workers [270--273].
In contrast, the aqueous extracts of wheatgrass (AWE), *Achyranthes aspera* (AAA) and *Pleurotus ostreatus* (PAE), showed greater cytotoxicity against COLO-205 cells, although they possessed weaker antioxidant activity thereby indicating factors other than anti-oxidants maybe playing a role. Similar observations were also reported wherein cell death was not directly related to the anti-oxidant potential present in a plant. Studies pertaining to the hexane, methanol, ethyl-acetate and aqueous extracts of *Alpinia pahangensis*, revealed that the antioxidant and cytotoxic potential of each extract was not directly proportional [274]. Cytotoxicity testing of various potencies of homeopathic drug *Ruta* revealed that 30C and MT showed maximum cytotoxic actions when compared to other potencies i.e. 10M, 1M and 200C.

Colon cancer cells over time develop a number of mutations which play a major role in their survival and pathogenesis [45]. The ability of various plant extracts to either induce cell death or to stall proliferative capabilities is well documented in various plants such as *Polygonum avicula*, *Origanum vulgare L* and *Philadelphus coronaries* which exhibited cytotoxicity and apoptotic activities against various *in vitro* cancer models [275-277]. In the development of colon cancer, the ability of the cells to proliferate is an important factor right from the initiation stage. An added advantage to the cancer cells is this continuous division leads to the acquisition of more defects in key genes which allow their growth to carry on unchecked [229]. Various *in vitro* assays were performed to evaluate anti-proliferative properties of CAM sources other than cytotoxicity evaluation. Amongst all CAM sources, AWE, AAA, PAE, 30C and MT of *Ruta* showed enhanced capabilities to slow down the proliferation of COLO-205 cells.

Cell death can be classified as either necrotic or apoptotic. Since evasion of apoptosis is a key factor by which cancer cells proliferate therefore it was necessary to confirm the manner by which cytotoxicity by the CAM sources was seen in the COLO-205 cells. Cell death in the presence of the CAM sources was evaluated by various qualitative and quantitative methods. The results indicated that the cell death was indeed via the apoptosis pathway.

From the knowledge gathered from the above investigations, the most potent extract and potency from each CAM source i.e. AWE from wheatgrass extracts, AAA from *Achyranthes aspera* root extracts, PAE from mushrooms and 30C and MT from *Ruta* was further selected to understand the underlying mechanism behind the cellular cytotoxicity in COLO-205 cells. Alterations in the levels of key molecular players related to apoptosis (caspase-9, caspase-8, caspase-3, Bax and Bcl-2) were evaluated. Up-regulation of caspase-9, caspase-8, caspase-3,
Bax and downregulation of Bcl-2 expression in the CAM treated cells indicated that CAM treatment had the ability to induce apoptosis. The results are in agreement with published work on the ability of plants to induce apoptosis in cancer cells. Cytotoxic and apoptosis inducing effects of four different fractions of *Amorphophallus campanulatus* tuber i.e. petroleum ether fraction (PEF), chloroform fraction (CHF), ethyl acetate fraction (EAF) and methanolic fraction (MEF) were investigated on the colon cancer cell line, HCT-15. The results suggested that, amongst all fractions, CHF had potent cytotoxic and apoptotic activity [278]. The anti-cancer effects of the crude extract of *Solanum lyratum* (SLE) on human colon cancer COLO-205 cells indicated SLE led to increased levels of Bax, caspase-9, caspase-3, p53 and p21 proteins along with decreased levels of cyclin B1, suggesting cell cycle arrest leading to apoptosis in COLO-205 cells upon treatment [279]. The leaf extracts of *Achyranthes aspera* displayed a dose and time dependent cytotoxicity on various *in vitro* models of colon, breast, lung, prostate and pancreatic cancer [185] *Pleurotus tuber-regium* extract exhibited cytotoxicity and anti-proliferative activity against human acute promyelocytic leukemia cells (HL-60) [245].

To evaluate whether the apoptotic changes seen upon treatment with the CAM sources correlated with arrest of the COLO-205 cells at various phases of the cell cycle, the cells were treated with PI, a DNA specific dye. Accumulation of cells at different phases of cell cycle was observed at each treatment such as AWE at Go/G1, AAA at S-phase, PAE at Go/G1 and 30C and MT at G2/M phase. Cell proliferation in normal cells is regulated by cell cycle checkpoints at the G1/S and G2/M phases to check for accurate replication of DNA as well as their segregation, respectively.

Several studies over the years have shown a direct correlation between the cell cycle and cancer and moreover mutations in the cell cycle regulators have been reported for various cancers, including that of the colon. As a result of this cancerous COLO-205 cells have the ability to overcome these checkpoints and allow the mutated cells to progress through the cell cycle [254]. If CAM treatment can overcome this mutated property of the cancer cells then it would go a long way in controlling the progress of colon cancer.
In order to throw further light on the cell cycle arrest by the CAM sources gene expression studies of CKIs was carried out and an explanation was sought on the role of CKIs in the undergoing cell cycle arrest. Up-regulation of phase specific CKIs indicated selective cell cycle arrest. It is known that increased expression of p16, p21 and p27 are associated with G0/G1 arrest, while over-expression of p21 and p27 are linked to S or G2/M phase arrest. The results showed that p21 gene expression was found to be up regulated in all CAM (AWE, AAA, PAE, 30C and MT of *Ruta*) treated COLO-205 cells suggesting arrest of the cell cycle. However, the levels of p16 were seen to be constant in the cells treated with 30C and MT of *Ruta*, thereby indicating G2/M arrest by *Ruta*. Although the levels of p27 were increased upon treatment with AWE, AAA, 30C and MT of *Ruta*, in the case of PAE treatment no drastic variation was seen. Since the arrest in the cell cycle by PAE was at the Go/G1 phase these results were in agreement. The cytotoxic effect of these CAM treatments on COLO-205 cancer cells was through up-regulation of CKI gene expression and blockage of cell cycle at specific phases of the cell cycle, finally leading to apoptosis.

The cytotoxicity as well as the effect on proliferation and apoptosis observed after treating normal NRK-52E cells with CAM sources was non-significant as compared to the cancer cells. Not only does this resolve the safety concerns related to CAM therapies it also provides an exciting window of therapeutic opportunity for preferentially eliminating colon cancer cells with minimal damage to the surrounding normal tissue and might encourage the use of CAM therapies as an alternative treatment option.

Synergism is a theory that is quite popular nowadays and is based upon the hypothesis that a mixture of extracts/potencies is more powerful source of anti-cancer agents than a single extract [266]. Based on such this hypothesis, a cocktail mix was prepared by combining the IC_{50} of AWE, AAA, PAE and MT of *Ruta*. The effect was tested on both cancer and non-cancerous cell lines. The cocktail mix showed enhanced cytotoxicity and apoptosis induction in COLO-205 cells along with increased levels of apoptosis and cell cycle arrest related genes as compared to individual extract treatment to COLO-205 cells. In addition, when the results were compared to those of the normal NRK-52E cells the level of toxicity was non-significant.
In summary, this study demonstrates for the first time that aqueous extract of wheatgrass, *Achyranthes aspera* roots and *Pleurotus ostreatus* and MT of *Ruta* either individually or as a cocktail mix lead to growth inhibition of COLO-205 cells. The natural compounds present in AWE, AAA, PAE and MT modulate apoptosis and cell cycle pathways that are frequently blocked in human cancers. Therefore, they may provide novel opportunities for cancer drug development. A proposed mechanism of action by the CAM sources acting as a cocktail mix is proposed in Figure 6.1.

![Diagram of proposed mechanism of action](image)

**Figure 6.1** The proposed mechanism of action by the CAM sources acting as a cocktail mix on COLO-205 cells