6. Summary and Conclusion

In the recent years, research has focused on the discovery of novel anticancer compounds, including natural compounds that can specifically target cancer cells and minimize the possible side effects on normal cells. The plants are the potential source of antioxidants, as well as other phytocompounds, which possess therapeutic and curative properties.

India is one of the major biodiversity hotspots with several medicinal plants. However, many of these plants are underexploited for their medicinal properties. The traditional knowledge of the usage of some plants can serve as an innovative and powerful discovery engine for newer, safer and affordable medicines.

With this background, the plant chosen for the present study was *Bacopa monnieri*. *Bacopa monnieri*, is a well-known herb for its memory enhancing properties. In the present study, the bacoside fraction was prepared from *Bacopa monnieri* and its antioxidant and anticancer properties were determined using both *in vitro* and *in silico* approaches. There are no systematic studies reported on the anticancer effect of the bacoside fraction. Hence, the bacoside fraction from *Bacopa monnieri* was chosen for this study.

The work was carried out in four distinct phases. In the first phase, the bacoside fraction was prepared from *Bacopa monnieri* and characterized by HPLC, HPTLC and XRD. The results of Phase I showed that the active component bacoside A, a saponin, was present in the bacoside fraction isolated from *Bacopa monnieri*.

In phase II, in order to understand the antioxidant activity of the bacoside fraction, the free radical scavenging activities and biomolecule-protective effects of the bacoside fraction was assessed. The bacoside fraction was tested for the free radical scavenging activity in cell-free systems against a battery of radicals, namely DPPH, ABTS, hydrogen peroxide, SO\(^*\), NO and hydroxyl radicals.
A dose-dependent increase in the free radical scavenging activity was observed up to 50µg, after which, no further increase in activity was recorded.

The bacoside fraction was then tested for its ability to protect biomolecules exposed to an oxidant. The biomolecules analyzed were lipids, DNA and proteins. Lipids are the most susceptible targets of oxidative attack, followed by DNA as the ultimate target of oxidative damage. The proteins, which carry out all the cellular functions, are also highly susceptible to oxidative damage under physiological conditions.

Three different membrane preparations were exposed to oxidants and the effect of the bacoside fraction to inhibit LPO in each system was analyzed. The membrane lipid preparations used were goat RBC ghosts, goat liver homogenate and goat liver slices. The maximum inhibition of LPO was observed in the liver slices, followed by liver homogenate and then RBC ghosts up to 50µg, after which, a plateau in activity was observed. Hence, 50µg dose was selected for the further studies.

DNA from sources of varying hierarchy of evolution were used to study the protective effect of bacoside fraction against oxidative damage. The protein damage was analyzed by the formation of protein carbonyls and 1D SDS-PAGE. Bacoside fraction exhibited a significant protective effect against various hierarchical levels of DNA and protein oxidation. Thus, it was clear that bacosides protected all the major biomolecules against oxidative damage. There was a slightly higher degree of protection of lipids in the intact cells, while the other lipid preparations as well as the DNA and protein preparations showed more or less similar extent of protection.

From phase II, it was clear that the bacoside fraction possessed good free radical-scavenging activity and offered significant biomolecular protection against oxidative stress, both in cell-free systems and in intact cells.

In Phase III, oxidative stress induced-apoptosis in non-transformed (buccal) and transformed (KB oral carcinoma) cells were studied. The influence
Summary and Conclusion

of etoposide, a standard chemotherapeutic drug, in the presence and the absence of the bacoside fraction was evaluated in both primary buccal cell culture and KB (oral carcinoma) cells. The parameters used to determine the apoptotic features of the cells were the morphological (Giemsa staining) and nuclear changes (PI, EtBr, DAPI and AO/EtBr staining). The cytotoxicity assays (MTT and SRB assays) were also carried out to evaluate the cell viability. The cell cycle analysis was analysed using ADDCP (Apoptosis, DNA Damage, Cell Proliferation and Cell Cycle Analysis) kit, using flow cytometry. The effect of bacoside fraction on the genomic profile of cancer was also studied.

The results obtained from the cytotoxicity assays and various staining techniques showed a strong protective effect of bacoside fraction in the primary buccal cells and cytotoxic effect in the KB cells when treated with or without etoposide. Thus, the present study showed the differential effect of the bacoside fraction, which selectively kills the cancer cells and protects the normal cells. The bacoside fraction, in combination with the etoposide, further augmented the cytotoxicity towards the cancer cells and protected the normal cells.

This is a very significant observation, as the bacoside fraction discriminates between the non-cancerous and cancerous cells, and targets its action on the cancer cells. It is inferable that the fraction is able to selectively recognize a specific component expressed by the cancer cells alone. More in-depth mechanistic studies are needed to confirm this influence and to identify the signal process involved.

The extent of DNA damage (using anti-γH2AX antibodies), the extent of cell proliferation (using anti-BrdU antibodies), the cell cycle events (using DAPI) and apoptosis (using anti-cleaved PARP antibodies) were studied using flow cytometry. The results of flow cytometry added strong support to our earlier results, wherein increased apoptotic death was observed upon treatment with bacoside and / or etoposide. This was mediated by cell cycle arrest at sub-G₀ phase. The flow cytometric analysis using specific antibodies revealed that the bacoside fraction increases the extent of apoptosis and the extent of DNA damage, while decreasing cell proliferation and cell cycle operation in KB cells.
These results unequivocally prove that the bacoside fraction exerts a pro-apoptotic effect on the cancerous KB cells, by influencing cell cycle and proliferation.

The effect of bacoside fraction on the gene expression profile in KB cell line was studied using RT-PCR cancer pathway finder array. The array profiles the expression of 84 genes representative of six biological pathways involved in transformation and tumourigenesis. The etoposide treatment influenced adhesion, angiogenesis, cell cycle control and DNA damage repair, and signal transduction pathways of the cancer genome.

The bacoside fraction upregulated many genes involved in the adhesion, cell cycle control, DNA damage and its repair, apoptosis and cell senescence, and signal transduction. Among the genes involved in the adhesion pathway, marked upregulation was observed in many genes. This implies that the bacosides aid in the adhesion of the cancer cells to their site of origin, thereby preventing their migration (or metastasis) to other locations. In the group of genes involved in cell cycle control and DNA repair, many genes showed a high magnitude of expression compared to the control. This observation implies that the bacoside fraction can influence the production of gene products that can control the cell cycle and repair damaged DNA. Additionally, among the genes of the cell cycle control, CDKN1A, the gene coding for cyclin-dependent kinase inhibitor 1A, was markedly downregulated in bacoside treated KB cells. From this pattern, it can be inferred that the cdk, which is involved in controlling the cell cycle, remains active, as the inhibitor synthesis is downregulated.

The maximum fold change of upregulation was observed with the genes associated with apoptotic and cell senescence pathways. These changes indicate that the apoptotic pathway is induced at the gene level by the bacosides. Notable increase in the fold-change values were observed with the genes involved in signal transduction. As signal transduction processes and transcription factors are involved in the regulation of several functions, both in normal and cancer cells, the change in expression may be taken as a supportive process to the molecular action of the bacosides.
The genes involved in angiogenesis showed an increase expression level upon exposure to bacosides. IFNA1 codes for Interferon A1, which has a negative effect on cancer. Similarly, TGFB1 is a ced gene that induces apoptosis. Thus, the induction of the expression of these genes support the protective action of bacosides. The genes associated with the pathways of invasion and metastasis were upregulated, while TIMP1 was severely downregulated. The reason for these changes are also evading at present, especially as both TIMP1 and TIMP3 are metalloprotease inhibitors, and exhibit contradicting expression pattern.

The results, thus, showed that the bacoside fraction exerted a very profound influence on the cancer-related pathway genes, much higher in magnitude than etoposide.

In phase IV, the compound bacoside A, was subjected to in silico studies in order to analyze the interaction of the compound with various apoptotic (Bcl2, Bax, Bak, MDM2 and TRAIL-R) and cancer (p53, LOX, PARP, protein kinase C and tubulin) targets. The ADME profile implied very poor oral absorption of the compound from the gastrointestinal tract. This was a surprising observation, as reports have already proven the memory-enhancing property of bacoside A. The structure of bacoside A has a glucose and an arabinose moiety associated with it. This opened up the possibility of the compound being co-transported with the sugars. To affirm this, the structure of bacoside A was docked to the Glucose Transporter Protein (GlcTP), which is involved in transport in glucose across the cell membranes. The results showed that bacoside A exhibited strong interaction with the GlcTP, which was mediated by an induced-fit mechanism. Our study is the first to report the possible mechanism by which bacoside A may be absorbed across the membrane barrier.

The in silico studies performed with bacoside A on various targets supports the interaction pattern of bacoside A to the selected apoptotic and cancer targets. The compound bacoside A showed an effective docking to all the selected targets, except tubulin. The docking pattern re-iterated that bacoside A can induce apoptosis in the cancer cells.
From the present study, it can be concluded that the bacoside fraction has strong antioxidant and anticancer properties. The anticancer properties are mediated in the KB oral carcinoma cells by a multi-faceted and multi-angled approach. Thus, this study provides insights into the molecular mechanisms of the anticancer effect of bacoside fraction, and provides a deeper understanding into the possibilities of its use in combination therapy with chemotherapeutic agents like etoposide.

**SUGGESTIONS FOR FUTURE RESEARCH**

The outcome of the present study has opened up a number of avenues for future research. Some of them that can be suggested for active research are given below.

- The anticancer activity of the bacoside fraction can be tested in various cancer cell lines of different tissue origin to know whether the activity is tissue-specific.
- The apoptosis-inducing activity of the bacoside fraction can be analyzed further for the mechanism involved using other molecular markers of apoptosis.
- The effects of the bacoside fraction can be evaluated *in vivo* using experimental animals.
- The proteomic profile of the cancer cells treated with the bacoside fraction may be characterized.
- The nanoparticles of the bacoside fraction can be synthesized and their effect on the various cancer cell lines can be studied.