4. RESULTS

Antioxidants or “oxidation inhibitors” exert their effects by preventing the generation of ROS and retarding the progress of many chronic diseases, including cancer, inflammation and cardiovascular diseases. Many natural as well as synthetic antioxidants are in the market for the treatment of various diseases (Nazarbahjat et al., 2014).

Oxidative stress, the imbalance between antioxidant defense and oxidant production in cells, is implicated in the onset and progression of many health problems. An approach to treating oxidative stress-related disorders is the use of exogenous antioxidants. Many studies have been undertaken to evaluate the efficacy of synthetic and naturally occurring antioxidants in combating the damaging effects of free radicals and reactive oxygen species (ROS).

Antioxidant therapy has gained an utmost importance in the treatment of these metabolic diseases. In these aspects, all around the world, the medicinal properties of plants have been investigated and explored for their potential antioxidant activities to counteract metabolic disorders, which are of high economic viability, with no side effects (Eshwarappa et al., 2015). Increase in the consumption of foods rich in antioxidant nutrients may decrease or prevent the risk of many diseases caused by oxidative stress (Kim et al., 2014a). Herbal antioxidants are of special interest to the public because of the perception of their lower toxicities compared to synthetic antioxidants (Tobwala et al., 2014).

In the present study, the antioxidant and anticancer properties of the bacoside fraction were determined using both in vitro and in silico approaches and the results obtained are presented in this chapter.

PHASE I

4.1. Yield of the bacoside fraction from Bacopa monnieri

The bacoside fraction was prepared from the aerial parts of Bacopa monnieri as explained earlier. The average yield obtained was 0.6%. The bacoside fraction was dried using a nano spray drier and used for further analysis.
Results

4.2. HPTLC analysis of the bacoside fraction

The bacoside fraction from *Bacopa monnieri* was subjected to HPTLC analysis for the identification of the presence of bacoside A, a saponin. The bacoside fraction from *Bacopa monnieri* was run along with the commercially-available standard bacoside A. The profile (R_f values, height and area of the peaks) of the standard bacoside A and bacoside fraction from *Bacopa monnieri* are shown in Table 4.1. In the chromatogram after derivatization, purple coloured bands in the day light mode observed in the respective standard and sample tracks (Plate 4.1) confirmed the presence of bacoside A in the bacoside fraction.

The HPTLC profile of the prepared bacoside fraction showed one major band (R_f 0.95) and four minor peaks (R_f values of 0.50, 0.62, 0.74 and 0.86). The R_f value of the major band corresponded exactly with that of the standard bacoside A, as can be seen from the peak densitograms (Figures 4.1 a and b) and the HPTLC chromatogram (Figure 4.2). This observation implies that the bacoside fraction prepared in the present study predominantly contained bacoside A. Other saponin compounds were also present in minor amounts.

Thus, the HPTLC analysis of the bacoside fraction prepared in the present study confirmed that the major bacoside present in the fraction is bacoside A. This was further confirmed by HPLC.

4.3. HPLC analysis of the bacoside fraction

The HPLC profile of the bacoside fraction was recorded using C18 RP column (Shimadzu equipped with PDA detector). The profile of the standard bacoside A was also recorded alongside. The results are presented in Figures 4.3 and 4.4 respectively.

The HPLC profiles of the bacoside fraction prepared and the standard bacoside A matched very well, with most of the peaks coinciding. This confirmed that the fraction prepared from the leaves in the present study was equivalent to the commercial preparation of bacoside A.
Plate 4.1
HPTLC profile of the bacoside fraction

Before derivatization
After derivatization

BMF- Bacoside fraction from *B. monnieri*, STD-Standard bacoside A

Figure 4.1
HPTLC peak densitogram of the standard and bacoside fraction

a) Standard
b) Bacoside Fraction

Figure 4.2
HPTLC Chromatogram

Table 4.1
HPTLC Peak table

<table>
<thead>
<tr>
<th>Track</th>
<th>Peak</th>
<th>Rf</th>
<th>Height</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>1</td>
<td>0.95</td>
<td>435.8</td>
<td>13048.6</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.50</td>
<td>144.0</td>
<td>7754.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.62</td>
<td>132.5</td>
<td>7191.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.74</td>
<td>102.7</td>
<td>4962.6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.86</td>
<td>207.9</td>
<td>8769.7</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.95</td>
<td>428.8</td>
<td>15534.1</td>
</tr>
</tbody>
</table>

*Understanding the anticancer activity of the bacoside fraction from Bacopa monnieri by in vitro and in silico approaches*
Results

Understanding the anticancer activity of the bacoside fraction from Bacopa monnieri by in vitro and in silico approaches

Figure 4.3
HPLC profile of the bacoside fraction

Figure 4.4
HPLC profile of the standard – Bacoside A
4.4. XRD analysis of the bacoside fraction

The XRD analysis showed one major peak at 72.63 °2Th and 6 minor peaks, as shown in Figure 4.5. The major peak was matched with that of the JCPDS (Joint Committee on Powder Diffraction Standards) database. It corresponded to the compound methane hydrate-clathrate with the molecular formula of C_{41}H_{68}O_{13}. The molecular formula of the methane hydrate-clathrate was almost similar to that of bacoside A (C_{41}H_{68}O_{13}). Thus, XRD analysis also confirmed the presence of bacoside A in the bacoside fraction prepared from *Bacopa monnieri*.

Figure 4.5
XRD profile of the bacoside fraction

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, the free radical scavenging activity and biomolecular protective effect of the bacoside fraction was assessed.

PHASE II

4.5. Radical scavenging effects of bacoside fraction

Previous studies in our laboratory showed that the methanolic extract of *Bacopa monnieri* exhibited higher radical scavenging effect when compared to other solvent extracts of varying polarity (Radha, 2010). In the present study, various concentrations of the bacoside fraction ranging from 10µg to 200µg (10µg, 25µg, 50µg, 75µg, 100µg and 200µg) were tested for
their radical scavenging effects against a battery of free radicals, namely DPPH, ABTS, superoxide, nitric oxide, hydroxyl radical and hydrogen peroxide.

4.5.1. DPPH and ABTS radical scavenging activities of the bacoside fraction

The per cent extent of DPPH and ABTS scavenging by the bacoside fraction were quantified spectrophotometrically and the results are presented in Figures 4.6 and 4.7 respectively.

**Figure 4.6**

DPPH scavenging activity of the bacoside fraction

![DPPH Scavenging Activity Graph](image)

The values are mean ± SD of triplicates

**Figure 4.7**

ABTS scavenging activity of the bacoside fraction

![ABTS Scavenging Activity Graph](image)

The values are mean ± SD of triplicates
It was observed that the bacoside fraction effectively reduced the stable radical DPPH to the yellow-coloured compound diphenyl picryl hydrazine. The extent of both DPPH and ABTS radical scavenging increased in a dose-dependent manner. In DPPH radical scavenging assay, the scavenging activity increased from 10µg to 100µg and then it plateaued. In ABTS radical scavenging assay, the activity was increased from 10µg to 75µg, with no further increase in scavenging activity.

4.5.2. Effect of the bacoside fraction on the *in vitro* generation of superoxide and nitric oxide radicals

The per cent inhibition of SO$^\cdot$ and NO generation by the presence of the bacoside fraction was calculated and the values are depicted in Figures 4.8 and 4.9 respectively.

**Figure 4.8**
Superoxide inhibition by the bacoside fraction

![Graph showing superoxide inhibition](image)

The values are mean ± SD of triplicates

**Figure 4.9**
Nitric oxide inhibition by the bacoside fraction

![Graph showing nitric oxide inhibition](image)

The values are mean ± SD of triplicates
Results

The various concentrations of the bacoside fraction exhibited considerable superoxide and nitric oxide scavenging activities in a dose-dependent manner. The superoxide and nitric oxide scavenging activity increased from 10µg to 50µg of the bacoside fraction, after which, a plateau in the activity was observed.

4.5.3. Hydrogen peroxide scavenging activity of the bacoside fraction

The ability of the bacoside fraction to scavenge H$_2$O$_2$ in an *in vitro* system was studied and the results are expressed in Figure 4.10.

![Figure 4.10](image)

**Figure 4.10**

H$_2$O$_2$ Scavenging activity of the bacoside fraction

The values are mean ± SD of triplicates.

All the different concentrations of the bacoside fraction exhibited good H$_2$O$_2$-scavenging effects. The scavenging activity increased with increase in concentration till 50µg. There was no further marked increase in H$_2$O$_2$ scavenging activity with higher concentrations of the fraction.

4.5.4. Hydroxyl radical scavenging activity of the bacoside fraction

The hydroxyl radical has high reactivity and is short-lived. The extent of TBARS produced in the reaction is taken as a measure of hydroxyl radical production. The inhibition of TBARS production is, thus, considered as a measure of hydroxyl radical scavenging efficiency. The exposure to H$_2$O$_2$ caused the maximum damage, which was very effectively counteracted by the bacoside fraction from *Bacopa monnieri*. The percent TBARS formation decreased with increase in concentration from 5µg to 50µg, after which, there was no further decrease in TBARS formation (Figure 4.11).
Understanding the anticancer activity of the bacoside fraction from *Bacopa monnieri* by *in vitro* and *in silico* approaches

The values are mean ± SD of triplicates. The H$_2$O$_2$ treated group was fixed as 100 % and the per cent TBARS formed in the other groups were calculated relative to this.

From the free radical scavenging assays, it was clear that the extent of scavenging increased up to a dose of 50µg, and thereafter exhibited a plateau in superoxide, nitric oxide, H$_2$O$_2$ and hydroxyl assays. In order to further optimize the dose, biomolecular protective effect of the bacoside fraction from *Bacopa monnieri* was also assessed.

### 4.6. Effect of the bacoside fraction on oxidative damage to biomolecules

ROS are generated by redox reactions and the Fenton reaction of H$_2$O$_2$, as well as iron that generates the hydroxyl radical, which causes severe damage to DNA, proteins and lipids (Nicolaou *et al*., 2013). Thus, it was felt imperative to study the effects of the bacoside fraction on oxidant-induced damage to lipids, DNA and proteins. As the first step, different concentrations (10, 25, 50, 75, 100 and 200µg) of the bacoside fraction powder were used to assess the protective effect against oxidant (H$_2$O$_2$) induced damage to lipids *in vitro*.

#### 4.6.1. Effect of the bacoside fraction against *in vitro* lipid peroxidation

The damage to lipids and the extent to which the bacoside fraction inhibited this process was quantified by measuring the extent of lipid peroxidation (LPO). To ascertain the damage to lipids, three different
Results

Membrane models were studied. They were RBC ghosts (plasma membrane devoid of intracellular membranes), liver homogenate (a mixture of plasma membrane and internal membranes) and precision-cut liver slices (intact cells). The extent of inhibition of LPO was studied in the presence of the bacoside fraction.

Figure 4.12

Inhibition of lipid peroxidation in different membrane preparations by the bacoside fraction

The values are mean ± SD of triplicates

The per cent inhibition of in vitro LPO by the bacoside fraction in the three membrane systems (Figure 4.12) showed that the maximum inhibition was observed in the liver slices, followed by the liver homogenate and then the RBC ghosts. A statistical comparison was not made as the protocols for each were distinctly different from each other.

The extent of in vitro LPO was inhibited in a dose-dependent manner by the bacoside fraction from 10µg to 50µg. Thereafter, a near-linear activity was observed with higher concentrations. Our results showed that the bacoside fraction rendered protection to lipid molecules against oxidant-induced in vitro lipid peroxidation.
Results

Thus, from the results obtained with the free radical scavenging assays and the extent of LPO, it was inferable that 50µg of bacoside fraction was the minimum concentration at which a maximum response was elicited. Further increase in concentration did not result in a further increase in the response. Therefore, this concentration (50µg) was chosen as the optimal dose for further analysis.

4.6.2. Protective effects of the bacoside fraction from *Bacopa monnieri* on oxidative damage to DNA

The ultimate biomolecular target of the oxidative damage is DNA. In the present study, the extent of protection rendered by the bacoside fraction to DNA exposed to oxidants was studied. Different sources of DNA, belonging to various evolutionary hierarchical levels, were used for the analysis. Both the commercially available DNA preparations and DNA from intact cells were used. They were,

- Lambda DNA (linear, viral phage)
- pUC18 DNA (plasmid, circular, bacterial)
- Herring sperm DNA (genomic, haploid, fish)
- Calf thymus DNA (genomic, diploid, mammal)
- Human peripheral blood lymphocytes (intact human cells)

4.6.2.1. Protective effects of the bacoside fraction to λ DNA and pUC18 DNA

The extent of damage induced by H$_2$O$_2$ to DNA from these sources and the protective effects of the bacoside fraction were studied by viewing the migration pattern of the DNA in agarose gels. The results are presented in Plate 4.2.

In both the DNA types, H$_2$O$_2$ caused a significant extent of damage. This was evident by a decrease in the intensity of specific bands in lane 2, wherein the DNA was treated with oxidant alone. Bacoside fraction reversed this damage, which could be seen in lane 4, as indicated by the intact bands. The bacoside fraction, by itself, did not cause any DNA damage. This
observation was reiterated by the Integrated Density Values (IDV) of the bands, recorded using a digital gel documentation software (Alpha Ease FC of Alpha Digidoc 1201), the values of which are presented in Table 4.2.

Plate 4.2
Migration patterns of λ DNA and pUC18 DNA treated with H₂O₂ with and without bacoside fraction

<table>
<thead>
<tr>
<th>Sample</th>
<th>IDV of the bands</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λ DNA</td>
<td>pUC18 DNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Without H₂O₂</td>
<td>With H₂O₂</td>
<td>Without H₂O₂</td>
<td>With H₂O₂</td>
<td></td>
</tr>
<tr>
<td>No extract</td>
<td>909039</td>
<td>595733</td>
<td>43731</td>
<td>5932</td>
<td></td>
</tr>
<tr>
<td>Bacoside fraction</td>
<td>906337</td>
<td>563141</td>
<td>41583</td>
<td>39823</td>
<td></td>
</tr>
</tbody>
</table>

Among the two DNA preparations from the lower organisms, the bacterial plasmid DNA was more susceptible to oxidative damage and was also more receptive to the protective effect by the bacoside fraction. The extent of damage by H₂O₂ in the DNA from the viral source was lower; the extent of protection was also lower in λ DNA. The IDV of the bands clearly proved this observation.
4.6.2.2. Protective effects of the bacoside fraction on H₂O₂ induced damage to herring sperm and calf thymus DNA

The results of the quantification of oxidative damage to herring sperm DNA is schematically presented in Figure 4.10.

Figure 4.13
Inhibition of oxidant-induced damage to herring sperm and calf thymus DNA by the bacoside fraction

It was found that H₂O₂ caused an increased extent of damage to herring sperm DNA. The extent of damage decreased markedly in the presence of the bacoside fraction. This indicated the protective effect rendered by the bacoside fraction against the oxidant. Similar results were also observed with calf thymus DNA (Figure 4.13). This proved that the bacoside fraction possesses good protective effect against oxidative damage to DNA.
4.6.2.3. Effect of the bacoside fraction on the damage induced by H$_2$O$_2$ to DNA in intact cells

The DNA damaging effect of H$_2$O$_2$ in intact cells was studied by following the formation of comets in human peripheral blood cells exposed to the oxidant in vitro. The effect of the bacoside fraction is presented in Table 4.3. The photographic record of the comets in each of the treatment groups is depicted in Plate 4.3.

### Table 4.3

Effect of the bacoside fraction on DNA damage induced by H$_2$O$_2$ in human peripheral blood cells

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>No. of cells with comet/100 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without H$_2$O$_2$</td>
</tr>
<tr>
<td>Control</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Bacoside fraction</td>
<td>7 ± 2 a</td>
</tr>
</tbody>
</table>

The values are mean ± SD of triplicates

a – Statistically significant (P<0.05) compared to untreated control  
b – Statistically significant (P<0.05) compared to H$_2$O$_2$ alone treated group  
c – Statistically significant (P<0.05) compared to the respective bacoside fraction treated group

### Plate 4.3

Comet bearing peripheral blood lymphocytes

- Untreated control
- H$_2$O$_2$ treated
- Bacoside fraction
- Bacoside fraction + H$_2$O$_2$
H₂O₂ exposure caused a steep increase in the number of cells with comets. In the positive control (cells treated only with H₂O₂), the DNA was severely damaged. The co-treatment with the bacoside fraction and H₂O₂ significantly decreased the number of cells expressing the DNA damage. Thus, the results indicated that the bacoside fraction was able to protect the DNA in peripheral blood cells against oxidative damage (Plate 4.3). These observations suggested that the bacoside fraction is effective in counteracting the DNA damage.

4.6.3. Protective effect of the bacoside fraction on oxidative damage to proteins

The oxidative damage to proteins and the effect of bacoside fraction on the extent of this damage was studied by quantifying the protein carbonyl levels spectrophotometrically and by following the migration pattern of an oxidant-exposed protein in 1D gel electrophoresis. The results obtained are presented below.

4.6.3.1. Effect of the bacoside fraction on protein carbonyl formation

The effect of bacoside fraction on protein oxidation is depicted in Table 4.4. The levels of protein carbonyl significantly increased in the presence of the oxidant. On co-treatment with the bacoside fraction, a significant decrease in the oxidation of proteins was observed when compared to that of the group treated with the oxidant alone. This showed the protective effect of the bacoside fraction against protein oxidation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein carbonyl (nmol/mg protein)</th>
<th>Without H₂O₂</th>
<th>With H₂O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Extract</td>
<td>8.44 ± 1.11</td>
<td>35.97 ± 3.17²</td>
<td></td>
</tr>
<tr>
<td>Bacoside fraction</td>
<td>5.25 ± 0.57²</td>
<td>18.30 ± 0.91³</td>
<td></td>
</tr>
</tbody>
</table>

The values are mean ± S.D. of triplicates

a – statistically significant (p<0.05) compared to untreated control
b – statistically significant (p<0.05) compared to H₂O₂ control
c – statistically significant (p<0.05) compared to the respective bacoside fraction control
4.6.3.2. Effect of the bacoside fraction on protein migration on 1D Gel

The effect of the bacoside fraction on protein oxidation \textit{in vitro} was evaluated by 1D gel electrophoresis. It is evident from the results of the SDS-PAGE depicted in Plate 4.4, that the intensity of the bands in the H$_2$O$_2$-treated group (lane 2) showed a significant decrease when compared to that of the untreated control (lane 1). This effect was counteracted by the co-treatment with the bacoside fraction (lane 4). The integrated density values of the bands obtained are shown in Table 4.5.

Plate 4.4

Effect of the bacoside fraction on the migration of proteins subjected to oxidative stress

![Image of 1D gel electrophoresis]

Lane 1: Untreated control
Lane 2: BSA + H$_2$O$_2$
Lane 3: BSA + Bacoside fraction
Lane 4: BSA + Bacoside fraction + H$_2$O$_2$

Table 4.5

<table>
<thead>
<tr>
<th>Sample</th>
<th>IDV of the bands</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Band 1</td>
</tr>
<tr>
<td>Control</td>
<td>115920</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>56355</td>
</tr>
<tr>
<td>Bacoside fraction</td>
<td>80730</td>
</tr>
<tr>
<td>Bacoside fraction + H$_2$O$_2$</td>
<td>74562</td>
</tr>
</tbody>
</table>
The results of the second phase of this study, thus, showed that 50µg of the bacoside fraction exhibited marked free radical scavenging activity and significant biomolecular protection against oxidative stress, both in cell-free systems and in intact cells.

**PHASE III**

4.7. **Effect of the bacoside fraction on etoposide induced stress in primary buccal cells and KB cells**

It is evident from the results obtained from phase II that the bacoside fraction can render protection against oxidant induced stress. Therefore, it is necessary to study the effect of the bacoside fraction on oxidative stress induced-apoptosis in non-transformed (buccal) (Plate 4.5a) and transformed (KB oral carcinoma) (Plate 4.5b) cells.

**Plate 4.5**

a) Human buccal cells

![Human buccal cells](image1)

b) Human oral carcinoma (KB) cells

![Human oral carcinoma (KB) cells](image2)
Apoptosis is one of the many cell death motifs, which is not only crucial for embryogenesis, development and homeostasis of the body, but also plays an important role in the occurrence and development of tumours (Zu et al., 2014). Cytotoxic drugs kill cancer cells by increasing ROS to cause apoptosis or necrosis (Zhao et al., 2014). Oxidative stress was induced by etoposide in both non-transformed (buccal) and transformed (KB) cells. Etoposide, an anticancer drug, was used as the standard oxidant in both the cell types in this phase.

In the present study, the influence of etoposide in the presence and the absence of the bacoside fraction in both primary buccal cell culture and KB cells were evaluated by cytotoxicity assays (MTT and SRB) and various (membrane and nuclear) staining techniques. Non-cancerous primary buccal cell culture was used as a control for the cancerous cell line (KB cells).

4.7.1. Effect of bacoside fraction on the viability of primary buccal cells and KB cells

The effect of the bacoside fraction on the viability of primary cultured buccal cells and KB oral carcinoma cells was quantified using MTT and SRB assays. The per cent viability obtained in the various treatment groups in buccal cells and KB cells are represented schematically in Figures 4.14 and 4.15 respectively for the MTT assay, and Figures 4.16 and 4.17 respectively for the SRB assay.

As observed from the figures, etoposide treatment caused a steep decrease in the survival of both buccal and KB cells, indicating that if does not discriminate between the two types of cells. The viability of the buccal cells improved markedly in the presence of the bacoside fraction in the buccal cells. The fraction, by itself, showed mild toxicity to these (normal) cells.

On the other hand, in KB cells, the fraction, by itself, caused a steep decrease in the viability, which was comparable to that of etoposide treatment. This observation implies the anticancer effect of the bacoside fraction. It was interesting to note that the bacoside fraction decreased the survival of only the KB cells to such an extent, and not the buccal cells.
Results

**Figure 4.14**
Effect of the bacoside fraction on the viability of buccal cells subjected to oxidative stress as determined by MTT assay

![Bar chart showing the effect of bacoside fraction on cell viability](image)

The values are mean ± SD of triplicates.
The viability of the untreated (negative) control group was fixed as 100% and the per cent viabilities in the other groups were calculated relative to this.

**Figure 4.15**
Effect of the bacoside fraction on the viability of KB cells subjected to oxidative stress as determined by MTT assay

![Bar chart showing the effect of bacoside fraction on cell viability](image)

The values are mean ± SD of triplicates.
The viability of the untreated (negative) control group was fixed as 100% and the per cent viabilities in the other groups were calculated relative to this.
Results

Understanding the anticancer activity of the bacoside fraction from *Bacopa monnieri* by *in vitro* and *in silico* approaches

**Figure 4.16**
Effect of the bacoside fraction on the viability of buccal cells subjected to oxidative stress as determined by SRB assay

![Graph showing effect of bacoside fraction on viability of buccal cells](image1)

The values are mean ± SD of triplicates.

The viability of the untreated (negative) control group was fixed as 100% and the per cent viabilities in the other groups were calculated relative to this.

**Figure 4.17**
Effect of the bacoside fraction on the viability of KB cells subjected to oxidative stress as determined by SRB assay

![Graph showing effect of bacoside fraction on viability of KB cells](image2)

The values are mean ± SD of triplicates.

The viability of the untreated (negative) control group was fixed as 100% and the per cent viabilities in the other groups were calculated relative to this.
Results

When etoposide and the bacoside fraction were co-administered to the KB cells, there was a further reduction in the survival of the cells, which was more evident in the SRB assay. This observation suggests that the bacoside fraction exhibits a differential effect between the non-cancerous and cancerous cells, in that, it protects the non-cancerous buccal cells from etoposide-induced cell death, while rendering the cancerous KB cells more susceptible to the chemotherapeutic agent.

4.7.2. Effect of the bacoside fraction on the morphological changes in etoposide induced stress in primary buccal cells and KB cells

The morphological changes observed in primary buccal cell culture and KB cells stained with Giemsa are depicted in Table 4.6 and Figure 4.18 respectively. There is a steep increase in the number of cells (cancerous) showing apoptotic morphology in both primary buccal cell culture and KB cells (Plates 4.6a and 4.7a). A differential response was observed in the two types of cells, when treated with the bacoside fraction. The bacoside fraction did not cause any toxic effects to non-transformed buccal cells, whereas they caused a steep increase in the number of apoptotic cells in KB cells.

In the primary buccal cell culture, the presence of the bacoside fraction, along with the oxidant showed a decrease in the apoptotic cells when compared with that of the etoposide alone treated group (Plate 4.6a). On the other hand, in the case of KB cells, bacoside fraction alone showed an increase in the number of apoptotic cells. On co-treatment with the etoposide and the bacoside fraction in KB cells, there was a further increase in the number of apoptotic cells. This clearly showed that the bacoside fraction augmented the cytotoxicity of the chemotherapeutic agent (etoposide) only in the cancer cells. Our results show that the bacoside fraction protected the non-transformed buccal cells from oxidant induced cytotoxicity.
### Table 4.6

Effect of the bacoside fraction on the morphological changes in buccal and KB cells subjected to oxidative stress as determined by Giemsa staining

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Number of Apoptotic cells / 100 cells</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buccal cells</td>
<td>KB cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Without Etoposide</td>
<td>With Etoposide</td>
<td>Without Etoposide</td>
<td>With Etoposide</td>
</tr>
<tr>
<td>Without bacoside fraction</td>
<td>7 ± 2</td>
<td>46 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11 ± 2</td>
<td>81 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>With bacoside fraction</td>
<td>12 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29 ± 2&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>71 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85 ± 2&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The values are mean ± S.D of triplicates

- a – Statistically significant (P<0.05) compared to untreated control
- b – Statistically significant (P<0.05) compared to etoposide alone treated group
- c – Statistically significant (P<0.05) compared to the respective bacoside fraction treated group

### Figure 4.18

Effect of the bacoside fraction on the apoptotic ratio in buccal and KB cells subjected to oxidative stress (Giemsa staining)
4.7.3. Effect of the bacoside fraction on the nuclear changes in primary buccal cells and KB cells

The nuclear changes that occur during apoptosis in the transformed and untransformed cells exposed to etoposide in the presence / absence of the bacoside fraction were analysed using the various nuclear stains, namely EtBr, PI, DAPI and AO/EtBr. The number of apoptotic cells was counted and the apoptotic ratio was calculated for each treatment group. The results are shown in the Tables 4.7 – 4.10 and Figures 4.19 - 4.22 respectively.

In both primary buccal cells and KB cells, there was a drastic increase in the number of apoptotic cells with apoptotic nuclear morphology in the etoposide treated groups compared to that of untreated control. In the buccal cells, the co-administration of the bacoside fraction with etoposide, caused a decrease in the number of apoptotic cells when compared to that of the etoposide treated group.

However, in KB cells, this co-treatment increased the proportion of apoptotic cells. This showed the differential response of the bacoside fraction exhibited in untransformed and transformed cells. The nuclear morphology of both primary buccal cells and KB cells exposed to etoposide in the presence/absence of the bacoside fraction that were viewed after various nuclear staining methods namely EtBr, PI, DAPI and AO/EtBr are presented in Plates 4.6 (b to e) and 4.7 (b to e) respectively.

It is clear from the results obtained with the viability assays and the staining techniques that the bacoside fraction exhibits an anticancer effect on KB oral carcinoma cells, but was non-toxic in normal buccal cells at the same dose. The fraction also rescued the buccal cells from the toxic effects of etoposide, the standard chemotherapeutic agent, which did not discriminate between normal and cancer cells, killing both alike. However, in the KB cells, the bacoside fraction not only caused increased death but itself, but also augmented the cytotoxic effect of etoposide. This trend was confirmed by the spectrophotometric viability assays (MTT and SRB), as well as the morphological (Giemsa) and nuclear (EtBr, PI, DAPI and AO/EtBr) staining techniques. All the staining techniques clearly showed apoptotic changes, thus confirming that the type of cell death occurring was apoptosis.
Results

Having confirmed this, the anticancer effect of the bacoside fraction was analyzed at the molecular level to understand the possible mechanisms involved.

Table 4.7
Effect of the bacoside fraction on the nuclear changes in buccal and KB cells subjected to oxidative stress as determined by EtBr staining

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Number of Apoptotic cells / 100 cells</th>
<th>Buccal Cells</th>
<th>KB Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without</td>
<td>With</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Etoposide</td>
<td>Etoposide</td>
</tr>
<tr>
<td>Without bacoside</td>
<td></td>
<td>6 ± 1</td>
<td>43 ± 3</td>
</tr>
<tr>
<td>fraction</td>
<td></td>
<td></td>
<td>a</td>
</tr>
<tr>
<td>With bacoside</td>
<td></td>
<td>13 ± 2</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>fraction</td>
<td></td>
<td>a</td>
<td>a,b,c</td>
</tr>
</tbody>
</table>

The values are mean ± S.D of triplicates

a – Statistically significant (P<0.05) compared to untreated control
b – Statistically significant (P<0.05) compared to etoposide alone treated group
c – Statistically significant (P<0.05) compared to the respective bacoside fraction treated group

Figure 4.19
Effect of the bacoside fraction on the apoptotic ratio in buccal and KB cells subjected to oxidative stress (EtBr staining)
Results

Table 4.8

Effect of the bacoside fraction on the nuclear changes in buccal and KB cells subjected to oxidative stress as determined by PI staining

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Number of Apoptotic cells / 100 cells</th>
<th>Buccal Cells</th>
<th>KB Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without Etoposide</td>
<td>With Etoposide</td>
</tr>
<tr>
<td>Without bacoside fraction</td>
<td></td>
<td>5 ± 3</td>
<td>45 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>With bacoside fraction</td>
<td></td>
<td>14 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30 ± 1&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The values are mean ± S.D of triplicates

- a – Statistically significant (P<0.05) compared to untreated control
- b – Statistically significant (P<0.05) compared to etoposide alone treated group
- c – Statistically significant (P<0.05) compared to the respective bacoside fraction treated group

Figure 4.20

Effect of the bacoside fraction on the apoptotic ratio in buccal and KB cells subjected to oxidative stress (PI staining)
Table 4.9

Effect of the bacoside fraction on the nuclear changes in buccal and KB cells subjected to oxidative stress as determined by DAPI staining

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Number of Apoptotic cells / 100 cells</th>
<th>Buccal Cells</th>
<th>KB Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without Etoposide</td>
<td>With Etoposide</td>
<td>Without Etoposide</td>
</tr>
<tr>
<td>Without bacoside fraction</td>
<td>4 ± 3</td>
<td>48 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5 ± 4</td>
</tr>
<tr>
<td>With bacoside fraction</td>
<td>12 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29 ± 3&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>65 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The values are mean ± S.D of triplicates

<sup>a</sup> – Statistically significant (P<0.05) compared to untreated control
<sup>b</sup> – Statistically significant (P<0.05) compared to etoposide alone treated group
<sup>c</sup> – Statistically significant (P<0.05) compared to the respective bacoside fraction treated group

Figure 4.21

Effect of the bacoside fraction on the apoptotic ratio in buccal and KB cells subjected to oxidative stress (DAPI staining)
Table 4.10
Effect of the bacoside fraction on the live and dead population in buccal and KB cells subjected to oxidative stress as determined by AO/EtBr staining

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Number of Apoptotic cells / 100 cells</th>
<th>Buccal Cells</th>
<th>KB Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without Etoposide</td>
<td>With Etoposide</td>
</tr>
<tr>
<td>Without bacoside fraction</td>
<td></td>
<td>7±1</td>
<td>45±3 a</td>
</tr>
<tr>
<td>With bacoside fraction</td>
<td></td>
<td>15±2 a</td>
<td>30±1 a,b,c</td>
</tr>
</tbody>
</table>

The values are mean ± S.D of triplicates

a – Statistically significant (P<0.05) compared to untreated control
b – Statistically significant (P<0.05) compared to etoposide alone treated group
c – Statistically significant (P<0.05) compared to the respective bacoside fraction treated group

Figure 4.22
Effect of the bacoside fraction on the apoptotic ratio in buccal and KB cells subjected to oxidative stress (AO/EtBr staining)
Plate 4.6
Effect of bacoside fraction on the morphological and nuclear changes in buccal cell culture

<table>
<thead>
<tr>
<th>Untreated control</th>
<th>Etoposide treated</th>
<th>Bacoside Fraction</th>
<th>Bacoside + Etoposide</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Giemsa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b) EtBr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c) PI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d) DAPI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e) AO/EtBr</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Plate 4.7
Effect of bacoside fraction on the morphological and nuclear changes in KB cells

a) Giemsa
b) EBr
c) PI
d) DAPI
e) AO/EtBr
4.7.4. Effect of bacoside fraction on apoptosis, DNA damage, cell proliferation and cell cycle analysis in oral cancer cell line (KB)

The important cellular events that occur during the manifestation of anticancer effect of an agent will encompass a profound influence on the extent of DNA damage and cell proliferation. This will, in turn, influence the cell cycle operation and, ultimately, result in cell death – either by apoptosis or by other death types. Our results with the staining techniques to visualize morphological and nuclear changes revealed that the type of cell death occurring in KB cells, exposed to bacoside fraction, was apoptosis.

Following this, 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 also followed using flow cytometry. The scattergrams of the flow of cells and their corresponding peak histograms are shown in Figure 4.23. From these, it can be clearly observed that a major proportion of cells in the control group were in $G_0$-$G_1$ phase, indicating that they were not apoptotic. Additionally, in this group, cells were observed in all phases of the cell cycle, suggesting that, they were dividing and proliferating.

**Figure 4.23**
Flow cytometric analysis of the oral carcinoma (KB) cells

<table>
<thead>
<tr>
<th>Control</th>
<th>Etoposide</th>
<th>Bacoside fraction</th>
<th>Bacoside + Etoposide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scattergram</td>
<td><img src="image1.png" alt="scattergram" /></td>
<td><img src="image2.png" alt="scattergram" /></td>
<td><img src="image3.png" alt="scattergram" /></td>
</tr>
<tr>
<td>Cell cycle</td>
<td><img src="image4.png" alt="cell cycle" /></td>
<td><img src="image5.png" alt="cell cycle" /></td>
<td><img src="image6.png" alt="cell cycle" /></td>
</tr>
</tbody>
</table>
In the groups treated with etoposide and/or bacoside fraction, most of the cell population was locked in the sub-G0 phase. This corresponds to the late apoptotic phase, suggesting the cytotoxicity of etoposide/bacoside.

The 2D-scattergrams obtained with the intensities of the fluorescent tags on the specific antibodies that indicate DNA damage, cell proliferation, progress of the cell cycle and apoptosis, are presented in Figure 4.24. The inferences that can be made from the patterns obtained in the 2D-scattergrams are presented below.

**Figure 4.24**

**Effect of bacoside fraction on the apoptosis, DNA damage, cell proliferation and cell cycle analysis in KB cells using flow cytometry**

Control 1  Etoposide Treated 2  Bacoside fraction 3  Bacoside + Etoposide 4
The extent of cell cycle operation was measured using DAPI, the results of which are presented as A1, A2, A3 and A4, corresponding to the control, etoposide treatment, bacoside treatment and combined treatment respectively. The control group showed active cell cycle, as seen by the number of cells in the lower right (LR) quadrant of A1. Treatment with etoposide and bacoside fraction, both alone or in combination, decreased this intensity drastically, indicating that KB cells were arrested in division. This was also reflected in the extent of proliferation (upper left quadrants of A1-A4), which was increased in the control group, but drastically decreased in the other groups.

The patterns in B1-B4 represent the extent of DNA damage in correlation with cell proliferation. The control KB cells exhibited a lower intensity corresponding to DNA damage (UL quadrant) and higher proliferation (LR quadrant). In the treated groups, the extent of DNA damage increased markedly, while the extent of proliferation decreased. This observation reiterates the anticancer effect of both etoposide and bacoside fraction in that they cause increased DNA-damage, which may reflect the DNA fragmentation during cell death.

C1-C4 depict the extent of proliferation in relation to the extent of apoptosis. The scatter patterns once again prove that there is decreased proliferation, which is a consequence of increased apoptosis.

The 2D-scattergrams represented as D1-D4 present the extent of DNA damage versus apoptotic death. Both these processes showed high correlation, reflecting that the increased apoptosis had resulted in a high level of DNA fragmentation.

Thus, the results of the flow cytometry studies, added strong support to our earlier results, wherein increased apoptotic death was observed upon treatment with bacoside and / or etoposide. 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.
4.7.5. Effect of bacoside fraction on the gene expression profile in oral cancer cell line (KB)

The effect of bacoside fraction on the gene expression profile in KB cell line was studied using RT-PCR cancer pathway finder array. The Human Cancer PathwayFinder™ RT² Profiler™ PCR Array profiles the expression of 84 genes representative of six biological pathways involved in transformation and tumourigenesis. The details of the array and its contents are presented as an Annexure to this dissertation. The six pathways analyzed in the cancer pathfinder microarray are

- cell cycle control and DNA repair,
- apoptosis and cell senescence,
- signal transduction molecules and transcription factors,
- adhesion,
- angiogenesis,
- invasion and metastasis.

The intensities of the fluorogen reflecting the extent of gene expression was quantified and compared between untreated, etoposide treated and bacoside fraction treated KB cells, using qPCR assay. The intensities were normalized and compared, as per manufacturer’s instructions, using the online software. From the resultant values, heat maps of gene expression were constructed. For this, the fold changes in the intensities were compared between control and etoposide as one group, and control and bacoside as the other, in two independent experiments. The assays were also conducted in triplicates. The results are given below.

Figure 4.25 (a) shows the heat map in the control versus etoposide treated KB cells. As can be inferred from the heat map, etoposide treatment influenced a few genes involved in adhesion, angiogenesis, cell cycle control and signal transduction pathways of the cancer genome. These observations suggest that etoposide executes its anticancer activity by influencing these pathways at the gene level.
Results

Figure 4.25
Heat map of differential gene expression in etoposide and bacoside treated KB cells

a) Control Vs Etoposide  b) Control Vs Bacoside Fraction

The effect of the bacoside fraction on the levels of the gene expression in comparison with the control is presented in Figure 4.25 (b). It is evident from the pattern obtained that bacoside influenced all the pathways of the cancer genome. The fold change in the intensities was also very high in the bacoside treated group, when compared to the etoposide treated group.

The genes that were clearly upregulated in the cells treated with etoposide, were also upregulated in the bacoside-treated group. However, in addition to those, many other genes showed upregulation upon bacoside treatment. It is evident from these results that the bacoside fraction exerts a multi-faceted attack on cancer (KB) cells, influencing the expression of several cancer pathway genes.

From the fold change, the scatter plots (Figure 4.26 a, b) and volcano plots (Figure 4.27 a, b) were also generated. These very clearly show that the pattern of gene expression in KB cells is more profoundly influenced by the bacoside fraction, compared to etoposide.
In order to compare the effects of etoposide and bacoside fraction on individual pathways, the expression levels corresponding to the genes of individual pathways were constructed from the heat maps generated. These are presented in Figure 4.28.
Figure 4.28

Effect of bacoside fraction on the gene expression profile in individual pathways in KB oral carcinoma cells
Results

It was inferable from the results obtained that 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 with ITGA2, ITGA4, ITGB1, MCAM, SYK and EPDR1. These results suggested that the bacoside fraction enhances adhesion, thus preventing metastasis.

In the group of genes involved in cell cycle control and DNA repair, ATM, BRCA1, CDKN2A, CHEK2, MDM2 and S100A4 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. As cancer is a disorder resulting from loss of cell cycle control (Fu et al., 2015) and DNA damage (Montagner et al., 2015), this is probably a mechanism by which the anticancer effect of bacoside fraction is evoked.

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. This observation added strength to our results presented above.

Many of the genes associated with apoptotic and cell senescence pathways showed a tendency of upregulation in the bacoside-treated KB cells, among which, the maximum fold change of upregulation was observed with APAF1, BAD, FAS, HTAT1P2 and TNFRSF25. These changes indicate that the apoptotic pathway is induced at the gene level by the bacosides. This observance is also supported by our results in the earlier experiments, which showed increased apoptotic death in KB cells by the treatment with bacoside fraction. The gene expression studies also indicated that the process of cell senescence may also be upregulated at the gene level. This also bears much significance, as cancer progresses by dedifferentiation (Ostrakhovitch et al., 2015), and thus, any agent that induces cell senescence will revert the cancerous condition.
Genes involved in signal transduction were also upregulated to a large extent by the bacoside fraction. Notable increase in the fold-change values were observed with AKT1, ERBB2, NFKB1 and SNCG. 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.

Among the genes involved in angiogenesis, ANGPT2, IFNA1, IGF1, TEK and TGFB1, showed an increased expression level upon exposure to bacosides. IFNA1 codes for Interferon A1, which has a negative effect on cancer. Similarly, TGFB1 is a ced gene (Annexure) that induces apoptosis. Thus, the induction of the expression of these genes support the protective action of bacosides.

The possible mechanism behind the induction of ANGPT2 (coding for angiopoietin 2), IGF1 (insulin-like growth factor 1) and TEK (tyrosine kinase) is unclear. More studies involving the pathway of angiogenesis are needed to unravel the mechanism involved.

Similarly, of the genes associated with the pathways of invasion and metastasis, MET, MTA2, PLAUR, S100A4, SERPINE1 and TIMP3 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.

Thus, the results of the expression of the genes involved in cancer-related pathways, show that the bacoside fraction can influence the process of cancer from several angles, right at the genetic level. This observation opens up exciting possibilities for future therapeutic avenues.

The patterns also clearly show that the bacoside fraction exerts a much stronger influence on the cancer genome, compared to etoposide. This is possibly the reason for the strong differential effect observed with the staining protocols presented earlier in this phase of the study.
Thus, from Phase III, it was observed that the bacoside fraction showed very good anti-apoptotic activity in non-cancerous cells and pro-apoptotic activity in cancer cells. It also exhibited a marked differential effect at the gene expression level. Following this, *in silico* studies were carried out to determine the interaction of bacoside A to the apoptotic and cancer targets.

**PHASE IV**

4.8. *In silico* docking of bacoside A with apoptotic and cancer targets

Docking is the computational routine to determine the probable binding manners of a ligand to the dynamic site of a receptor. The docking process fits the ligand in the binding site and makes available an image of the dynamic site, with interaction points known as grid (Bala *et al*., 2014). *In silico* studies have several advantages like cost factor and time consumption over the experimental determination of compound-protein interaction. Bacoside A was subjected to *in silico* studies for their efficacy against the target proteins involved in apoptosis and cancer using the commercially available Schrödinger software. The 2D structure of the compound bacoside A (Figure 4.29) was retrieved from PubChem and it was converted into 3D format using 2D sketcher. The target proteins selected for the present study are listed in Table 4.11.

**Figure 4.29**

**Structure of bacoside A**
Table 4.11

Target proteins selected from apoptosis and carcinogenesis

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Apoptotic Targets</td>
</tr>
<tr>
<td>1.</td>
<td>Bcl2</td>
</tr>
<tr>
<td>2.</td>
<td>MDM2</td>
</tr>
<tr>
<td>3.</td>
<td>Bak</td>
</tr>
<tr>
<td>4.</td>
<td>Bax</td>
</tr>
<tr>
<td>5.</td>
<td>TRAIL-R</td>
</tr>
<tr>
<td></td>
<td>Cancer Targets</td>
</tr>
<tr>
<td>6.</td>
<td>p53</td>
</tr>
<tr>
<td>7.</td>
<td>LOX</td>
</tr>
<tr>
<td>8.</td>
<td>PARP</td>
</tr>
<tr>
<td>9.</td>
<td>Tubulin</td>
</tr>
<tr>
<td>10.</td>
<td>Protein kinase C</td>
</tr>
</tbody>
</table>

The 3D structures of the target proteins were obtained from the Protein Data Bank (PDB) and were refined using the protein preparation wizard module. The ADME and molecular docking studies were performed to characterize the compound, bacoside A.

4.8.1 ADME studies of bacoside A

In the present study, bacoside A was first subjected to ADME profiling, using QikProp module of the Schrödinger Drug Design Suite. The results obtained are presented in Table 4.12.

From the results of QikProp, it was evident that the druggability of bacoside A was good, as reflected by the H-bond donors and acceptors, octanol-water partition co-efficient as well as the compliance to Lipinski’s rule of five. However, the ADME profile showed that bacoside A has very poor permeability and shows poor oral absorption from the GI tract. This observation was quite surprising and intriguing, because the compound is a known active component, exhibiting memory-enhancing activities (Verma et al., 2014). This effect, which is
also exploited in the traditional medicinal practices of India, would not be possible without the compound being absorbed from the GI tract, as the preparations from *Bacopa monnieri* are all administered orally.

**Table 4.12**

**ADME results of bacoside A using QikProp 3.6**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Descriptors</th>
<th>Standard values</th>
<th>Bacoside A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Molecular Weight (Da)</td>
<td>130-725</td>
<td>768.98</td>
</tr>
<tr>
<td>2.</td>
<td>Number of H bond donors</td>
<td>0.0/6.0</td>
<td>7</td>
</tr>
<tr>
<td>3.</td>
<td>Number of H bond acceptors</td>
<td>2.0-20.0</td>
<td>20.45</td>
</tr>
<tr>
<td>4.</td>
<td>Qp log P for octanol/water</td>
<td>-2.0/6.5</td>
<td>1.82</td>
</tr>
<tr>
<td>5.</td>
<td>Apparent CaCO2 permeability (nm/sec)</td>
<td>&lt;25-poor, &gt;500-great</td>
<td>31.541</td>
</tr>
<tr>
<td>6.</td>
<td>Apparent MDCK permeability (nm/sec)</td>
<td>&lt;25-poor, &gt;500-great</td>
<td>11.798</td>
</tr>
<tr>
<td>7.</td>
<td>Lipinski’s rule of 5 violations</td>
<td>Maximum 4</td>
<td>3</td>
</tr>
<tr>
<td>8.</td>
<td>% human oral absorption in GI tract (±20%)</td>
<td>&lt;25% Poor</td>
<td>25.551</td>
</tr>
</tbody>
</table>

Therefore, in the present study, we probed the molecule further using *in silico* methods. A closer analysis of the structure of bacoside A revealed that it is always associated with two sugar moieties, namely glucose and arabinose. Thus, it is possible that the compound, bacoside A, is co-transported with glucose, by the glucose transporter protein (GlcTP). In order to confirm this possibility, docking of bacoside A was attempted with GlcTP (PDB ID - 4PYP). The docking was not very efficient when the target protein (GlcTP) was used as such. Therefore, an induced fit docking was attempted between the compound and the target, and the result showed that there is excellent interaction between GlcTP and bacoside A, as presented below.

Table 4.13 shows the docking score of the two structures, while Plate 4.8 shows the pictorial representation of the docking and the interactions. It is evident from the high Glide score (-13.504) and induced-fit score (-900.476) that bacoside A
is co-transported into the cells, by the GlcTP, after an induced-fit interaction. Our study is the first to report this possibility, as elaborated in the Discussion chapter.

**Table 4.13**

<table>
<thead>
<tr>
<th>Target</th>
<th>IFD Score</th>
<th>Glide Score</th>
<th>Glide H bond</th>
<th>Glide VDW</th>
<th>Glide E Model</th>
<th>Glide energy</th>
<th>Glide Conformer index</th>
<th>Glide pose index</th>
<th>H bond</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP</td>
<td>-900.476</td>
<td>-13.504</td>
<td>-0.708</td>
<td>-49.851</td>
<td>-105.757</td>
<td>-75.475</td>
<td>138</td>
<td>47</td>
<td>9</td>
</tr>
</tbody>
</table>

**Plate 4.8**

Interaction of the bacoside A with glucose transporter protein

Following this, the compound bacoside A was docked to the prepared proteins with Glide in standard precision mode. A correlation was calculated by the Glide score. For the prediction of the results, parameters like Glide score, Glide energy, H-bonds and good van der Waals interactions, were analysed. These indicated the binding affinity of ligand towards the target molecules.

The docking results showed that bacoside A effectively interacted with the target proteins involved in apoptosis and carcinogenesis, as seen from Table 4.14 and Table 4.15 respectively. The docking and hydrogen bond interactions of bacoside A with the apoptotic and cancer targets are shown in Plates 4.9 and 4.10 respectively. The amino acid interactions of bacoside A with the apoptotic and cancer targets are shown in Table 4.16, Table 4.17, Plate 4.9 and Plate 4.10 respectively.
All the apoptotic and cancer target proteins exhibited good docking efficiency. Bacoside A showed better interaction with Bax among the apoptotic targets, whereas among the cancer targets, better docking efficiency was observed with p53. The ligand bacoside A failed to dock with the cancer target protein tubulin.

**Table 4.14**

Glide SP docking of bacoside A with the apoptotic target proteins

<table>
<thead>
<tr>
<th>Target</th>
<th>Glide Score</th>
<th>Glide H bond</th>
<th>Glide VDW</th>
<th>Glide E Model</th>
<th>Glide energy</th>
<th>Glide Conformer Index</th>
<th>Glide pose index</th>
<th>H bond</th>
<th>Good contacts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl2</td>
<td>-6.41769</td>
<td>-0.49734</td>
<td>-33.7994</td>
<td>-66.1133</td>
<td>-52.7532</td>
<td>189</td>
<td>157</td>
<td>7</td>
<td>415</td>
</tr>
<tr>
<td>MDM2</td>
<td>-6.70935</td>
<td>-0.9091</td>
<td>-32.252</td>
<td>-70.8092</td>
<td>-54.6921</td>
<td>155</td>
<td>88</td>
<td>6</td>
<td>293</td>
</tr>
<tr>
<td>Bak</td>
<td>-6.90696</td>
<td>-1.52012</td>
<td>-32.1623</td>
<td>-77.141</td>
<td>-54.6389</td>
<td>238</td>
<td>327</td>
<td>6</td>
<td>305</td>
</tr>
<tr>
<td>Bax</td>
<td>-7.53737</td>
<td>-0.91105</td>
<td>-30.5518</td>
<td>-75.8138</td>
<td>-54.2825</td>
<td>165</td>
<td>343</td>
<td>8</td>
<td>305</td>
</tr>
<tr>
<td>TRAIL-R</td>
<td>-6.35516</td>
<td>-0.30187</td>
<td>-30.9234</td>
<td>-72.5563</td>
<td>-54.5133</td>
<td>36</td>
<td>123</td>
<td>5</td>
<td>317</td>
</tr>
</tbody>
</table>

**Table 4.15**

Glide SP docking of bacoside A with the cancer target proteins

<table>
<thead>
<tr>
<th>Target</th>
<th>Glide Score</th>
<th>Glide H bond</th>
<th>Glide VDW</th>
<th>Glide E Model</th>
<th>Glide energy</th>
<th>Glide Conformer Index</th>
<th>Glide pose index</th>
<th>H bond</th>
<th>Good contacts</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>-7.16793</td>
<td>-0.45746</td>
<td>-40.0546</td>
<td>-86.7595</td>
<td>-61.0554</td>
<td>19</td>
<td>62</td>
<td>5</td>
<td>314</td>
</tr>
<tr>
<td>LOX</td>
<td>-5.98269</td>
<td>-0.45705</td>
<td>-28.3609</td>
<td>-72.2436</td>
<td>-53.6901</td>
<td>376</td>
<td>236</td>
<td>7</td>
<td>281</td>
</tr>
<tr>
<td>PARP</td>
<td>-6.56377</td>
<td>-1.21319</td>
<td>-27.4667</td>
<td>-75.5076</td>
<td>-56.1894</td>
<td>425</td>
<td>351</td>
<td>4</td>
<td>271</td>
</tr>
<tr>
<td>Protein kinase C</td>
<td>-5.81127</td>
<td>-0.5006</td>
<td>-20.7838</td>
<td>-58.4483</td>
<td>-46.4902</td>
<td>135</td>
<td>255</td>
<td>4</td>
<td>321</td>
</tr>
</tbody>
</table>
### Table 4.16

**Amino acid interactions of bacoside A with the apoptotic target proteins**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Target</th>
<th>Bacoside A</th>
</tr>
</thead>
</table>
| 1.    | Bcl2   | 1992:B:ASN169(O):(H)4884  
       |        | 3297:A:ASN169(H):(O)4771  
       |        | 3297:A:ASN169(H):(O)4768  
       |        | 4407:B:TRP173(H):(O)4772  
       |        | 4472:B:ARG180(H):(O)4766  
       |        | 4468:B:ARG180(H):(O)4766  
       |        | 467:A:PRO120(O):(H)4885 |
| 2.    | MDM2   | 1073:B:ASP68(O):(H)3270   
       |        | 1073:B:ASP68(O):(H)3269   
       |        | 494:A:ASP84(O):(H)3254    
       |        | 495:A:ASP84(O):(H)3241    
       |        | 2020:A:ASN79(H):(O)3158   
       |        | 1057:B:TYR67(O):(H)3262   |
| 3.    | Bak    | 1233:A:ASP158(O):(H)6040  
       |        | 1233:A:ASP158(O):(H)6043  
       |        | 1701:X:THR32(O):(H)6057   
       |        | 4692:X:THR38(H):(O)5947   
       |        | 4696:X:THR38(H):(O)5947   
       |        | 1716:X:LEU34(O):(H)6030   |
| 4.    | Bax    | 1286:B:ASP33(O):(H)8564   
       |        | 1246:B:GLN28(O):(H)8592   
       |        | 1246:B:GLN28(O):(H)8591   
       |        | 1274:B:GLN32(O):(H)8563   
       |        | 5508:B:GLN32(H):(O)8481   
       |        | 5703:B:LYS64(H):(O)8474   
       |        | 1311:B:ALA42(O):(H)8576   
       |        | 1311:B:ALA42(O):(H)8573   |
| 5.    | TRAIL-R| 1244:B:ASP269(O):(H)4469  
       |        | 1727:A:ARG118(O):(H)4476  
       |        | 1258:B:GLU271(O):(H)4448  
       |        | 1767:A:GLU123(O):(H)4477  
       |        | 1972:A:PRO150(O):(H)4461  |
### Results

Understanding the anticancer activity of the bacoside fraction from *Bacopa monnieri* by *in vitro* and *in silico* approaches

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Target</th>
<th>Bacoside A</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.</td>
<td>LOX</td>
<td>7126:A:ASP442(O):(H)21741 7123:A:ASP442(O):(H)21715 17844:B:ASP442(O):(H)21742 17818:B:LYS441(O):(H)21735 7101:A:LYS441(O):(H)21743 8414:A:SER522(H):(O)21634 4003:A:ARG246(H):(O)21624</td>
</tr>
</tbody>
</table>

From the amino acid interactions of all the target proteins with bacoside A, it was clear that the interaction was more with the polar amino acids such as Asn, Trp, Arg, Ser, Thr, Tyr, Gln, Asp, Lys and Glu. The ligand also showed interactions with specific hydrophobic amino acids such as Pro, Ala and Leu in all the protein targets studied. All the cancer target proteins exhibited interactions only with polar amino acids, while the apoptotic targets revealed interactions with more polar amino acids and a few non-polar amino acids. Thus, from the *in silico* studies, it was confirmed that the compound bacoside A has good docking efficiency with both apoptotic and cancer targets.
Plate 4.9

Interaction of bacoside A with apoptotic targets

Docking Interaction | H-bond Interactions | Amino acid interactions

Bcl2

MDM2

Bak

Bax

TRAIL-R
Results

Plate 4.10
Interaction of bacoside A with cancer targets

Docking Interaction | H-bond Interactions | Amino acid interactions

| p53 |  |  |
| LOX |  |  |
| PARP |  |  |
| Protein kinase C |  |  |
Thus, the present study demonstrated that bacoside fraction prepared from the plant, *Bacopa monnieri*, was rich in the bacoside A saponin. This compound may be responsible for the antioxidant and apoptosis-modulating effects of *Bacopa monnieri*. The study also validates the strong antioxidant potential of the bacoside fraction. It also showed protection against the biomolecules like lipids, DNA and proteins, against oxidative damage induced *in vitro*.

The study showed the differential effect exhibited by the bacoside fraction, which rendered anti-apoptotic effect in primary buccal culture and pro-apoptotic activity in KB cells. Flow cytometric analysis showed that the bacoside fraction can arrest the KB cells in sub-G₀ phase, resulting in increased apoptosis, combined with decreased proliferation and increased DNA damage. It also influences the gene expression in all the pathways involved in the pathogenesis of cancer. Novel *in silico* studies using induced-fit docking enabled the unraveling of the transport mechanism of bacoside across the membrane. Further *in silico* docking with apoptotic and cancer target proteins reaffirmed the anticancer effect of bacoside A.

Thus, the results of the present study proved the anticancer properties of the bacoside fraction and unfolded the possible mechanisms involved in the process. The outcome of the research work is discussed in the next chapter with the support of relevant published articles.