4. Results and discussion

Ayurveda and traditional Indian system of medicines suggest many plant species which should be explored for novel leads through modern scientific evaluation. The development of leads from traditional system of medicine with the perspective of safety, efficacy and quality will help in preserving this traditional heritage and rationalize the use of natural products. Immunomodulation using medicinal plants provides an alternative to conventional chemotherapy for several diseases and overstimulated immune system. The suppression of immune system is required in autoimmune disorders. The immunostimulants are needed to overcome the immunosuppression induced by environmental factors or drugs. Further the immune system is suppressed in chronic diseases like tuberculosis, stress, and in AIDS etc. (Wagner, 1999). Historically, a number of mineral or plant based drugs have been used for immunomodulation in human diseases (Ganju, et al., 2003). The allopathic medicines with their high cost and side effects have enforced the need for alternative medicines with minimum or no side effects with low cost, particularly those belonging to the traditional system of medicine like Ayurveda. The immunomodulatory so far reported from plant origin have main effect especially on macrophage function i.e. on non-specific immunity (Mahamat et al., 2014). Although leaves of B. suaveolens and N. physalodes have been investigated for different pharmacological activities, the immunomodulatory and anticancer potential of both the plant still remains unexplored. The current study reports the extraction, fractionation and isolation from these plants for immunomodulatory and immunomodulation mediated anticancer activity.

4.1 Determination of extractable matter

The leaves of both the plant were collected from the Physic Garden of Shoolini University and the botanical identity was confirmed by Department of Forest Products, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) with UHF- Herbarium Field book no. 2916, Receipt No. 051 & 061. The leaves were shade dried and defatted using petroleum ether and then ethanolic extract was prepared by continuous hot extraction using soxhlet apparatus. The yield of petroleum ether and ethanolic extract was found out to be 7.24% & 12.3% in B. suaveolens and 8.1% & 14.6% in N. physalods respectively (Table 4.1).
Table 4.1 Extractable matter isolated from *Brugmansia suaveolens* and *Nicandra physalodes*

<table>
<thead>
<tr>
<th>Plant</th>
<th>Extractive values</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Petroleum ether extract</td>
<td>Ethanol extract</td>
<td></td>
</tr>
<tr>
<td><em>Brugmansia suaveolens</em></td>
<td>5.24% w/w</td>
<td>12.3% w/w</td>
<td></td>
</tr>
<tr>
<td><em>Nicandra physalodes</em></td>
<td>6.1% w/w</td>
<td>14.6% w/w</td>
<td></td>
</tr>
</tbody>
</table>

4.2 Successive solvent fractionation

The ethanolic extract was fractioned into alkaloidal and non-alkaloidal fractions using successive solvents, di-chloromethane, ethyl acetate, and n-butanol. The yield of alkaloidal (BS1 & NP1), di-chloromethane (BS2 & NP2), ethylacetate (BS3 & NP3) and n-butanol (BS4 & NP4) fraction is given in Table 4.2.

Table 4.2 Successive solvent fractionation

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Yield (% w/w)</th>
<th>Fractions</th>
<th>Yield (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS1</td>
<td>3.1</td>
<td>NP1</td>
<td>3.68</td>
</tr>
<tr>
<td>BS2</td>
<td>15.2</td>
<td>NP2</td>
<td>17.3</td>
</tr>
<tr>
<td>BS3</td>
<td>17.4</td>
<td>NP3</td>
<td>18.7</td>
</tr>
<tr>
<td>BS4</td>
<td>14.2</td>
<td>NP4</td>
<td>16.2</td>
</tr>
</tbody>
</table>

4.3 Phytochemical screening

All the four fractions from both the plants were subjected for preliminary phytochemical screening to access the presence of different plant secondary metabolites using qualitative tests summarized in Table 4.3.

Table 4.3 Preliminary phytochemical screening of *B. suaveolens*

<table>
<thead>
<tr>
<th>Test</th>
<th>BS1</th>
<th>BS2</th>
<th>BS3</th>
<th>BS4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Alkaloid</td>
<td>+</td>
<td></td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Saponins</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Proteins</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
</tbody>
</table>
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Table 4.3 continued

<table>
<thead>
<tr>
<th></th>
<th>NP1</th>
<th>NP2</th>
<th>NP3</th>
<th>NP4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tannins</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavanoids</td>
<td>_</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycoside</td>
<td>_</td>
<td>+</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Sterols</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Triterpenoids</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>_</td>
</tr>
</tbody>
</table>

Table 4.4 Preliminary phytochemical screening of *N. physalodes*

<table>
<thead>
<tr>
<th>Test</th>
<th>NP1</th>
<th>NP2</th>
<th>NP3</th>
<th>NP4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Alkaloid</td>
<td>+</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Saponins</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Proteins</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Tannins</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavanoids</td>
<td>_</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycoside</td>
<td>_</td>
<td>+</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Sterols</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Triterpenoids</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>_</td>
</tr>
</tbody>
</table>

4.4 Bioactivity guided fractionation based on cell viability assay

The fractions were analyzed at selected concentrations (25, 50, 100 and 200 µg/ml) for PBMC proliferation using MTT assay. The non-alkaloidal ethyl acetate fractions of both the plant (i.e. BS3 and NP3) activates human PBMC significantly in a dose dependent manner (Figure 4.1 and 4.2). DMSO and Con A (a known T cell activator) were used as negative and positive controls respectively. Further, bioactive fractions were subject to column chromatography on silica gel 60-120 eluting with hexane-ethyl acetate. Elutes were divided into and four broad sub-fractions based on TLC (i.e. BS3a, BS3b, BS3c, BS3d from BS3 and NP3a, NP3b, NP3c, NP3d from NP3). All the four broad sub-fractions were again subjected to immunomodulatory evaluation.
Figure 4.1 Effect of BS1-BS4 fractions of *B. suaveolens* on proliferation of human PBMC 48 h after incubation in MTT assay. The values are mean±SEM of two independent observations done in triplicates. Data are expressed as mean±S.E. *p<0.05, **p<0.01 and ***p<0.001 compared with control group determined by one-way ANOVA (Dunnet’s test).

Figure 4.2 Effect of NP1-NP4 fractions of *N. physalodes* on proliferation of human PBMC 48 h after incubation in MTT assay. The values are mean±SEM of two independent observations done in triplicates. Data are expressed as mean±S.E. *p<0.05, **p<0.01 and ***p<0.001 compared with control group determined by one-way ANOVA (Dunnet’s test).

Fractions BS3b and NP3a showed significant immunostimulating activity in human PBMC in a dose dependent manner with the maximum effect at 40 µg/mL (Figure 4.3 and 4.4). To isolate the active compounds responsible for the immunostimulatory activity, the active sub-fractions from both the plants (i.e. BS3b and NP3a) were again
subjected for re-column. Based on the TLC pattern, elutes were divided into for sub fractions (BS3A, BS3B, BS3C, BS3D from BS3b and NP3A, NP3B, NP3C, NP3D from NP3a). All the fractions were again subjected for \textit{in vitro} immunostimulatory activity.

**Figure 4.3** Effect of sub fractions BS3a-BS3d on proliferation of human PBMC 48 h after incubation in MTT assay. The values are mean±SEM of two independent observations done in triplicates. Data are expressed as mean±S.E. *p<0.05, **p<0.01 and ***p<0.001 compared with control group determined by one-way ANOVA (Dunnet’s test).

**Figure 4.4** Effect of sub fractions NP3a-NP3d proliferation of human PBMC 48 h after incubation in MTT assay. The values are mean±SEM of two independent observations done in triplicates. Data are expressed as mean±S.E. *p<0.05, **p<0.01 and ***p<0.001 compared with control group determined by one-way ANOVA (Dunnet’s test).
BS3A, BS3B, NP3A and NP3B showed significant immunostimulating property and the maximum effect was obtained at 10 µg/mL as compared to control group (Figure 4.5 and 4.6). Since BS3A, BS3B, NP3A and NP3B showed significant immunostimulatory activity, therefore, all the four fractions were subjected for a panel of in vivo immunomodulatory assays.

**Figure 4.5** Effect of sub fractions BS3A-BS3D proliferation of human PBMC 48 h after incubation in MTT assay. The values are mean±SEM of two independent observations done in triplicates. Data are expressed as mean±S.E. *p<0.05, **p<0.01 and ***p<0.001 compared with control group determined by one-way ANOVA (Dunnet’s test).

**Figure 4.6** Effect of sub fractions of BS3A-BS3D proliferation of human PBMC 48 h after incubation in MTT assay. The values are mean±SEM of two independent observations done in triplicates. Data are expressed as mean±S.E. *p<0.05, **p<0.01 and ***p<0.001 compared with control group determined by one-way ANOVA (Dunnet’s test)
4.5 Evaluation of immunomodulatory activity by hemagglutination

The fractions active in PBMC proliferation assay were further evaluated for activity to stimulate B and T cells in relation to serum immunoglobulins IgM and IgG in presence of T dependent antigen (SRBCs). The antibody response was observed by hemagglutination titre. IgM titers were measured in mice serum of different groups, collected 7 days after immunization and treatment. The agglutinated lattice maintains the RBC’s in a suspended distribution, typically viewed as a diffuse reddish solution. The formation of the lattice depends on the concentrations of the antibody and RBC’s, and when the relative antibody concentration is too low, the RBC’s are not constrained by the lattice and settle to the bottom of the well. The results showed significant increase titres in mice treated with BS3A, BS3B, NP3A and NP3B at 50 and 100 mg/kg after 7 days when compared with negative control. A similar profile was observed after 14 days, with IgG predominating over IgM (Figure 4.7). BS-3B showed the better activity at 100 mg/Kg among all four fractions. The augmentation of humoral immunity to T-dependent antigen (SRBC) shows the increased responsiveness of macrophages since the antibody production is closely related to the co-operation of macrophages, T and B lymphocyte response (Benacerraf, 1978).

![Figure 4.7](https://example.com/figure4.7.png)

**Figure 4.7** Effect of BS3A, BS3B, NP3A and NP3B on hemagglutination antibody titres in mice. The animals were immunized on day 0. Primary antibody titres were determined on day 7 and secondary 14 days after challenge. Values are means±SE (n=5); *p<0.05, **p<0.01, and ***p<0.001 (control vs. BS3A, BS3B, NP3A and NP3B-treated groups; one-way ANOVA followed by Dunnet’s test).
4.6 Evaluation of type IV hypersensitivity

DTH response is a type IV hypersensitivity reaction and progresses when antigen sensitizes T\textsubscript{DTH} cells. It is an expression of cell-mediated immunity. It plays an important role in various inflammatory disorders (Abid et al., 2012). These reactions are characterized by invasions of large number of non-specific inflammatory cells. These inflammatory cells are Th1 subpopulation while sometimes T\textsubscript{C} cells are also involved. The antigen presentation through appropriate APCs leads to the activation of T\textsubscript{DTH} cells that results secretion of various cytokines includes IFN-\gamma. These cytokines recruit and stimulate macrophages, thus promoting phagocytic activity. It has been reported that DTH reactions play a vital role in host defense against parasites and bacteria that can live and proliferate intracellularly (Gongora et al., 2000). Treatment with fractions BS-3A, BS-3B, NP-3A and NP-3B enhanced the DTH reaction. DTH reaction to SRBC is given in Figure 4.8, in which data are expressed in terms of the footpad thickness. After administration of the BS3A, BS3B, NP3A and NP3B (50 and 100 mg/Kg, p.o.), a significant increase in footpad thickness was observed at 100 mg/Kg after 24 and 48 h as compared with the control group, suggesting heightened infiltration of macrophages to the inflammatory site.

![Figure 4.8](image)

**Figure 4.8** DTH response was determined in SRBC immunized, BS3A, BS3B, NP3A and NP3B treated mice at 24 and 48 h after antigen challenge. Data are mean±S.E. of six animals. *p<0.05, **p<0.01 and ***p<0.001 compared with control group determined by one-way ANOVA (followed by Dunnet’s test)
4.7 Carbon clearance assay
In view of the crucial role played by macrophages in synchronizing the processing and presentation of antigen to B-cells, fractions BS3A, BS3B, NP3A and NP3B were evaluated for its influence on macrophage phagocytic function. Oral administration of BS3A, BS3B, NP3A and NP3B (50 and 100 mg/Kg) for 7 days and 30 min prior to injection of colloidal carbon increased the clearance rate of carbon particles from mouse reticulo-endothelial system. A significant increase in phagocytic index was obtained at all dose levels and the maximum effect was observed at 100 mg/Kg whereas BS-3B showed better response among all fractions (Figure 4.9).

![Figure 4.9](image)

**Figure 4.9** Effect of BS3A, BS3B, NP3A and NP3B on carbon clearance in mice. Rate of carbon clearance, termed hereafter in the text as phagocytic index (K). Values are means±SE (n=5); *p<0.05, **p<0.01, and ***p<0.001 (control vs. BS3A, BS3B, NP3A and NP3B treated groups; one-way ANOVA followed by Dunnet’s test).

4.8 Th1 and Th2 cytokines
It has been reported that Cytokines play an important role in the regulation of the immune response against antigens and infectious agents. Th1 and Th2 cytokine are secreted during antigenic response. The Th1 cells produce IL-2 and IFN-γ while Th2 cells produce IL-4. The proliferation of T and B cells with BS3A, BS3B, NP3A and NP3B may be because of cytokine-mediated mechanism, since IFN-γ and IL-2 are up-regulated in treated groups. Th1 cytokines (IFN-γ and IL-2) are important immunomodulatory molecules that protects against intracellular pathogens for example viral infections. They activate macrophages, induce the generation of T cells, and regulate Th1 and Th2 cells. The Th2 cells or mast cells produces IL-4 that activate monocytes or macrophages. The balance between Th1 and Th2 cytokine production is
helpful in understanding the consequences of different immune reactions (Fang et al., 2005). BS3A, BS3B, NP3A and NP3B up-regulated the production of IL-4 with a significant release of IFN-γ and IL-2 thus regulating the Th1 and Th2 balance. The maximum response was observed at 100 mg/Kg dose compared with the control group and BS-3B was found most active among all the fractions (Figure 4.10, 4.11 and 4.12).

**Figure 4.10** Effect of BS3A, BS3B, NP3A and NP3B on IFN-γ production in mice sera. Serum was collected 4 h after the final oral administration of BS3A, BS3B, NP3A and NP3B (50 and 100 mg/Kg). IFN-γ concentration was measured by enzyme-linked immunosorbent assay (ELISA kit, BD Opt EIA set) according to the instructions of the manufacturer. Data are mean±S.E. of six animals. *p<0.05, **p<0.01 and ***p<0.001 compared with control group determined by one-way ANOVA (Dunnet’s test).

**Figure 4.11** Effect of BS3A, BS3B, NP3A and NP3B on IL-2 production in mice sera. Serum was collected 4 h after the final oral administration of BS3A, BS3B, NP3A and NP3B (50 and 100 mg/Kg). IL-2 concentration was measured by enzyme-linked immunosorbent assay (ELISA kit, BD Opt EIA set) according to the instructions of the manufacturer. Data are mean±S.E. of six animals. *p<0.05, **p<0.01 and ***p<0.001 compared with control group determined by one-way ANOVA (Dunnet’s test).
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Figure 4.12 Effect of BS3A, BS3B, NP3A and NP3B on IL-4 production in mice sera. Serum was collected 4 h after the final oral administration of BS3A, BS3B, NP3A and NP3B (50 and 100 mg/Kg). IL-4 concentration was measured by enzyme-linked immunosorbent assay (ELISA kit, BD Opt EIA set) according to the instructions of the manufacturer. Data are mean±S.E. of six animals. *p<0.05, **p<0.01 and ***p<0.001 compared with control group determined by one-way ANOVA (Dunnet’s test).

4.9 Macrophage function assay

The effect of BS3A, BS3B, NP3A and NP3B on macrophage function was assessed by measuring the amount of nitric oxide produced from peritoneal macrophages of treated mice. Griess reagent was used to measure the nitrite levels, the stable end-product of NO metabolism. The nitrite concentration was determined by extrapolation from a sodium nitrite standard curve and the results are expressed in μM. The effect of BS3A, BS3B, NP3A and NP3B (50 and 100 mg/Kg) on nitric oxide production is shown in Figure 4.13. A significant increase in nitric oxide was found at 100 mg/Kg in all the fractions as compared with the control group. Macrophages are important cells for the immune system which play an important role in host defense mechanisms for protection from microbial invaders. When macrophages are stimulated with foreign substances, a variety of cytokines and chemicals are released to induce fundamental defense systems. Among them, TNF-α is a representative cytokine secreted by macrophages that plays a key role in the cytokine network, e.g. T cell and NK cell activation (Tanaka et al., 1999). Also, TNF-α is a major pro-inflammatory cytokine characteristically produced at sites of inflammation by macrophages and is considered to help in eliminating certain invaders. Its levels in plasma are directly correlated with the ability of phagocytes to generate superoxide and to increment the activity of iNOS and, thus, NO levels (Miesel et al., 1996). Furthermore, TNF-α produced by activated macrophages, enhances the
cytotoxic action of macrophages. We observed that BS3A, BS3B, NP3A and NP3B strongly augmented the release of nitric oxide from macrophages. Our results thus suggested that BS3A, BS3B, NP3A and NP3B is capable of stimulating immune functions of macrophages through increase in TNF-α synthesis and NO production. The exact mechanism by which BS3A, BS3B, NP3A and NP3B stimulates the macrophages and their subsequent NO production could be due to macrophage specific TNF-α release, which plays a dominant role in the augmentation of macrophage function.

![Graph](image)

**Figure 4.13** Influence of BS3A, BS3B, NP3A and NP3B on the formation of nitrite contents in mouse peritoneal macrophage ex vivo. Values are means±SE (n=5); *p<0.05, **p<0.01, and ***p<0.001 (control vs. BS3A, BS3B, NP3A and NP3B-treated groups; one-way ANOVA followed by Dunnet’s test).

### 4.10 Cellular lysosomal phosphatase activity in macrophages

Lysosomal phosphatase activity was measured by the optical density of p-nitrophenol, a colored hydrolytic product of p-nitrophenyl phosphate (p-NPP). Stimulation of lysosomal phosphatase activity increased at both the concentration of BS3A, BS3B, NP3A and NP3B and the maximum response was measured at 100 mg/Kg (Figure 4.14). It has been shown that lysosomal enzyme are involved in the degradation of killed bacteria (Armstrong and Hart, 1975). The activated macrophages with enhanced microbicidal capacity contain significantly increased quantities of lysosomal hydrolases (Cummings et al., 1980) suggesting role of lysosomal enzymes in microbial destruction. BS3A, BS3B, NP3A and NP3B enhanced the cellular lysosomal phosphatase activity in macrophages, suggesting its role in amelioration of infection.
following microbial invasion. Comparatively BS-3B showed maximum activity among all the four fractions.

![Graph showing the effect of BS3A, BS3B, NP3A and NP3B on lysosomal enzyme activity in Balb/c macrophages. Values are means±SE; *p<0.05, **p<0.01, and ***p<0.001 (control vs. BS3A, BS3B, NP3A and NP3B-treated groups; one-way ANOVA followed by Dunnet’s test).]

**Figure 4.14** Effect of BS3A, BS3B, NP3A and NP3B on lysosomal enzyme activity in Balb/c macrophages. Values are means±SE; *p<0.05, **p<0.01, and ***p<0.001 (control vs. BS3A, BS3B, NP3A and NP3B-treated groups; one-way ANOVA followed by Dunnet’s test).

### 4.11 Splenocyte proliferation in mice

To confirm the immunostimulatory effect of the bioactive fractions BS3A, BS3B, NP3A and NP3B on the cellular immune response, the proliferation of splenocytes in response to Con A (5 μg/mL) and LPS (10 μg/mL) was evaluated. The results, shown in Figure 4.15 indicate that the proliferation in bioactive fractions BS3A, BS3B, NP3A and NP3B treated 50 and 100 mg/Kg was stimulated significantly as compared with the control group with the maximum effect at 100 mg/kg dose.
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Figure 4.15 Influence of BS3A, BS3B, NP3A and NP3B (50 and 100 mg/Kg) on proliferation of T and B lymphocytes ex-vivo. Mice were exposed to both the doses of BS3A, BS3B, NP3A and NP3B p.o. daily for 14 days. Splenocytes were isolated and stimulated with sub-optimal amounts of mitogens; Con A (5 μg/mL), LPS (10 μg/mL) for T and B cell proliferation, respectively. Cells were incubated for 48 h and proliferation was measured by MTT reduction assay. Splenocyte proliferation is expressed as stimulation index. Values are means±SE; *p<0.05, **p<0.01, and ***p<0.001 (control vs. BS3A, BS3B, NP3A and NP3B-treated groups; one-way ANOVA followed by Dunnet’s test).

4.12 Isolation and characterization

Isolation: Two compounds SUPH036 022A and SUPH036 022B were isolated using semi preparative HPLC. The yield of both the compounds was found out to be 0.074% and 0.069% of the crude drug respectively. Out of SUPH036 022A and SUPH036 022B only SUPH036-022A is fully characterized.

Characterization:

NMR spectra of SUPH036-022A

The compounds was solubilize in methanol and had limited solubility in chloroform, so spectra was recorded in CD$_3$OD.

$^1$H-NMR (400MHz, δ, CD$_3$OD, TMS=0): δ=1.77 (H, m, Ha at C4), 1.97 (H, m, Hb at C4), 2.36 (2H, m, H at C3), 3.56 (3H, s, OCH$_3$ at C7), 3.64 (3H, s, OCH$_3$ at C10), 4.09 (H, q, H at C5), 6.71 (H, d, H at C9, $J=8.1$ Hz), 7.76 (H, d, H at C8, $J=8.1$ Hz).

$^{13}$C-NMR (100MHz, δ, CD$_3$OD, TMS=0): 175.94 (C6), 175.26 (C2), 163.37 (C7), 133.02 (C9), 122.76 (C10), 116.06 (C8), 53.19 (OCH$_3$ at C7), 52.55 (C5), 52.17 (OCH$_3$ at C10) and 30.36 (C3 and C4).
The $^1$H NMR showed two multiplets centered at 1.77 and 1.97 ppm accounting for two protons each. There were two singlet accounting for three protons each at 3.56 and 3.64 ppm respectively, for methoxy groups in the aromatic ring. A quartet appeared at 4.09 ppm ($J = 3.52$ Hz) indicating an α-hydrogen at position 5. A doublet appeared at 6.71 ppm with $J = 8.1$ Hz which appears to be proton at carbon 9 in ring B which couples with the proton at carbon 8 in ring B appearing at 7.76 ppm has a doublet with $J = 8.1$ Hz. The compound does not show 2,4-dinitrophenylhydrazine (2,4-DNP) test possibly because DNP is a nucleophile and was unable to approach carbonium ion from the side of hydroxyl away from the oxygen of carbonyl. To further confirm the position of hydroxyl group, the dehydration reaction was carried out, thus eliminating the hydroxyl group. The dehydrated compound showed positive test for 2,4-DNP. Thus the tentative structure was proposed as (5S)-5-Hydroxy-7,10-dimethoxy-4,5-dihydro-2H-1-benzooxocine-2,6(3H)-dione (Figure 4.16).

The tentative structure was further supported by $^{13}$C NMR data. $^{13}$C NMR shows signal at 30.45 ppm which tentatively represent both (carbon 3 and 4). Two peaks appears at 52.19 and 52.17 ppm showing presence of methoxy group at carbon 7 and 10. Two carbonyl in the ring A appears at C2 and C6, which is further supported by FTIR peak at 1722 nm. The structure was further supported by the Dept experiment. Dept showed only one CH$_2$ signal at 30.45 ppm for both (carbon 3 and 4) the carbon. Further 2D heteronuclear cosy showed that two methylene carbon have merged at 30.45 ppm while in $^1$H NMR, the methylene appear on Ha and Hb with different chemical shifts. The tentatively assigned structure matches with the mass spectra which showed M+1 peak at 266 accounting for C$_{13}$H$_{14}$O$_6$. It showed a base peak at 196 due to M-(C-OCH$_3$,
43+28=71, M 195+H=196. The compound showed $\lambda_{\text{max}}$ at 310 and 361 nm, on hydrolysis besides shift of two chromophores to benzonoid signals, an extra chromophore appears with $\lambda_{\text{max}}$ 322 nm due to ion conjugation of hydrolyzed product at $\alpha$-$\beta$ unsaturated carbonyl.

### 4.12 Cell viability assay

The preliminary *in vitro* immunomodulatory of SUPH036 022A and SUPH036 022B were evaluated (1, 2.5, 5, and 10 $\mu$g/mL). SUPH036 022A and SUPH036 022B showed significant immunostimulating activity at 2.5 $\mu$g/mL as compared to control group (Figure 4.16). DMSO and Con A (a known T cell activator) treated cells were used as negative and positive control respectively. The activity platued at 2.5 $\mu$g/mL followed by decrease in proliferation at higher doses though effect was equipotent to Con A at 10 $\mu$g/mL of all these compounds.

![Figure 4.17](image_url)  
*Figure 4.17* Effect of SUPH036-022A and SUPH036-022B on proliferation of human PBMC. PBMC treated for 48 h with SUPH036-022A and SUPH036-022B different at different concentrations (1, 2.5, 5 and 10 $\mu$g/mL) and assayed for cellular proliferation by MTT assay. The values are mean±SEM of two independent observations done in triplicates.

### 4.13 Co-culture MTT micro-cytotoxicity assay

The anti-proliferative activity of co-cultured PBMCs with test compound activated immune response on cancer cell lines was evaluated. Cancer cell proliferation was inhibited immune response of PBMCs cells that mimics *in vivo* conditions where immune cells interact with cancer cells leading to their apoptosis (Yeap et al., 2007). As breast cancer is the most common cancer worldwide in females (Ferlay et al., 2015), MCF-7 was chosen for the assay. Results revealed that SUPH036 022A and SUPH036
022B treated PBMC showed significantly better anticancer effect as compare to untreated cells (Figure 4.17). The target cell line was incubated at (1:2.5, 1:5, 1:10 and 1:20) target:effector (T:E) ratios respectively. SUPH036 022A and SUPH036 022B (2.5 µg/mL) treated PBMC showed 59±2.1% (p<0.05) cell cytotoxicity against MCF-7 cells at 1:10 ratio after 24 h whereas control group. The anticancer effects shown by SUPH036 022A and SUPH036 022B (2.5 µg/mL) stimulated PBMC were even better than the PBMC treated with Con A (10 µg/mL), a known T cell stimulator (Berger, 1979). Thus SUPH036 022A and SUPH036 022B activated immune cells inhibit proliferation of co-cultured cancer cells.

![Figure 4.18](image_url)

Figure 4.18 Effect of SUPH036-022A and SUPH036-022B treated PBMC (Effector-E) on the cell death of human cancer cell line (Target-T). SUPH036-022A and SUPH036-022B (2.5µg/mL ), control and Con A (10µg/ml) treated PBMC were co-cultured with MCF-7 cell line for 24 h at different E:T ratios and assayed for cellular death by MTT assay. The values are mean ± SD of two independent experiments done in triplicate.

### 4.14 Cytokine production

Cytokines are the biochemical transmitters that induce immune response. They activate the immune response during antigenic attack. Therefore, SUPH036 022A and SUPH036 022B were further evaluated for ability to induce the IL-2 and IFN-γ cytokine production. SUPH036 022A and SUPH036 022B (2.5 µg/mL) showed significantly increased IL-2 and IFN-γ cytokine level in human PBMCs compared to the negative control treated PBMCs 48h h post treatment (Figure 4.18 and Figure 4.19).
**Chapter 4**

**Results and discussion**

**Figure 4.19** Induction of IFN-γ was measured after 48 h treatment of human PBMCs with control, Con A, SUPH036-022A and SUPH036-022B. IFN-γ concentration was measured by enzyme-linked immunosorbent assay (ELISA kit, BD Opt EIA set) according to the instructions of the manufacturer. Data are expressed as mean±S.E. *p<0.05, **p<0.01 and ***p<0.001 compared with control group determined by one-way ANOVA (Dunnet’s test).

**Figure 4.20** Induction of IL-2 was measured after 48 h treatment of human PBMCs with different control, Con A, SUPH036-022A and SUPH036-022B. IL-2 concentration was measured by enzyme-linked immunosorbent assay (ELISA kit, BD Opt EIA set) according to the instructions of the manufacturer. Data are expressed as mean±S.E. *p<0.05, **p<0.01 and ***p<0.001 compared with control group determined by one-way ANOVA (Dunnet’s test).
4.15 Effect on ROS generation
Reactive Oxygen Species play an important role in induction of apoptosis and depolarizing mitochondria. Target cells were incubated with stimulated effector cells at different ratios for 6 h and analyzed by fluorometry after staining with DCFH-DA (Borutaite and Brown, 2003). There was very low DCF fluorescence in the control group. However, cells treated with SUPH036 022A and SUPH036 022B stimulated PBMCs (2.5 µg/mL) at ratio of 1:10 showed a significant increase in DCF positive MCF-7 cell population (Figure 4.20). The level of ROS generation in MCF-7 cells was 79.09%, and 73.99%, respectively after 6 h exposure to SUPH036 022A and SUPH036 022B (2.5 µg/mL) stimulated PBMCs in 1:10 (T:E) ratio. Treatment with Con A was found to enhance ROS generation by 77.39% as compared to control DCF fluorescence of vehicle (DMSO) treated cells. The ROS production results in can produce deleterious effects on mitochondrial membrane potential ($\Delta \Psi_{\text{mt}}$) (Sun et al., 1999; Richter and Vollgraf, 1998; Wink and Mitchell 1998).

![Figure 4.21](image)

Figure 4.21 Effect of SUPH036-022A and SUPH036-022B stimulated effector cells induced on the generation of ROS in MCF-7 cells. MCF-7 cells were incubated with effector cells at different T:E ratio (1:5, 1:10 and 1:20) for 6 h. Cells were stained with DCFH-DA and analyzed using fluorometer. Data are Mean ± S.E. from two similar experiments. *p<0.05, **p<0.01 and ***p<0.001 compared to untreated control determined by one-way ANOVA (Dunnet’s test)

4.16 Mitochondrial membrane potential assay
The loss of mitochondrial membrane potential is facilitated through opening of mitochondrial permeability transition pores (PTP) largely due to Bax translocation,
which in turn, conduct the leakage of cytochrome c and pro-apoptotic proteins from mitochondria to the cytosol (Bhushan et al., 2006). Decrease in $\Delta \Psi_{mt}$ is a critical step in cells undergoing apoptosis. Rhodamine fluorescence in target cells (MCF-7 cells) exposed to SUPH036 022A and SUPH036 022B (2.5$\mu$g/mL) stimulated effector cells in ratio (1:5, 1:10 and 1:20) after 6 h exposure. The fluorometric analysis revealed that SUPH036 022A and SUPH036 022B stimulated effector cell causes 65.76% and 63.04% decrease in mitochondrial potential in MCF-7 cells as compared to control group (Figure 4.21). Treatment with Con A was found to cause 55.07% decrease in $\Delta \Psi_{mt}$ compared to control.

The loss of mitochondrial membrane potential is association with increased production of ROS activating caspases. The SUPH036 022A and SUPH036 022B stimulated effector cells treated MCF-7 cells showed activation of mitochondria-mediated apoptotic pathway as indicated by shift in mitochondrial membrane potential across the membrane. The results matched with generation of ROS intermediates, thus indicating that activation of redox sensitive transcription as mechanism for activation of apoptotic pathway.

Figure 4.22 SUPH036-022A and SUPH036-022B stimulated effector cells induced loss of mitochondrial membrane potential ($\Delta \Psi_{mt}$). MCF-7 cells were incubated with effector cells at different T:E ratio (1:5, 1:10 and 1:20) for 24 h. Cells were stained with Rhodamine-123 (1 $\mu$M/mL) for 1 h and analyzed in fluorometer. Data are Mean ± S.E. from two similar experiments. *p<0.05, **p<0.01 and ***p<0.001 compared to untreated control determined by one-way ANOVA (Dunnet’s test)
4.17 Cell cycle analysis

MCF-7 cells co-cultured with stimulated effector cells at 1:10 ratio for 24 h exhibited increase in sub-G1 DNA fraction (Figure 4.22). The sub-G1 fraction was <5% in control cells, while it increased to ~28% and 23% in MCF-7 treated with SUPH036 022A and SUPH036 022B stimulated effector cells, respectively. Treatment with Con A stimulated effector cell was found to cause 26% increase in sub-G1 phase after treatment with effector cell at 1:10 ratio. These results suggested that co-cultured SUPH036 022A and SUPH036 022B stimulated effector cells lead to the induction of apoptosis. Involvement of both reactive oxygen species and loss of mitochondrial potential might be the probable mechanism for the induction of apoptosis. Further, the G2/M phase of cell cycle remained unaffected indicating that effector cells does not produce any mitotic inhibition or caused cell cycle delay.
Figure 4.23 DNA cell cycle analyses in SUPH036-022A and SUPH036-022B treated effector cell with target MCF-7 cells. MCF-7 cells were exposed to stimulated effector cells in 1:10 ratio for 24 h and stained with PI to determine DNA fluorescence and cell cycle phase distribution. Fraction of cells from SUB G1, G0G1, S and G2M phases analyzed from FL2-A vs. cell counts is shown in percentage.