CHAPTER 3

RESULTS

3.1 COMPARATIVE ANALYSIS OF DEXAMETHASONE, CYCLOSPORIN A AND WORTMANNIN

3.1.1 Effect of Dexamethasone, Cyclosporin A and Wortmannin on Mast Cell Degranulation

In order to determine the effect of Dexamethasone, Cyclosporin A and Wortmannin on inhibition of mast cell degranulation, the three commercial molecules were tested in a range of concentrations in 10 µM A23187 induced RBL-2H3 allergy model. The concentrations, chosen for Dexamethasone spanned logarithmic doses of 100 µg/ml (255 µM), 10 µg/ml (25.5 µM) and 1 µg/ml (2.55 µM). An additional dosage of 50 µg/ml (127.5 µM) was included for the identification of the IC50 value. The concentrations of 100 µg/ml (84 µM), 50 µg/ml (42 µM), 10 µg/ml (8.4 µM) and 1 µg/ml (0.84 µM) were tested for Cyclosporin A for the determination of its IC50 value. Wortmannin was studied at 43 ng/ml (100 nM), 4.3 ng/ml (10 nM), 2.15 ng/ml (5 nM) and 0.43 ng/ml (1 nM).

At the end of 20min incubation with the three test molecules, the supernatant from the stimulated and control RBL-2H3 cells were assayed for hexoseaminidase activity, measured in terms of free PNP released and quantified spectrophotometrically at O.D. 405nm. The results indicated that all the 3 molecules managed to reach 50% inhibition at their highest tested
concentrations, (48%, 66% and 58% respectively for Dexamethasone, cyclosporin A and Wortmannin). At 50µg/ml, Dexamethasone exhibited 31.8% inhibition, while Cyclosporin A exhibited 57.9% inhibition. Wortmannin exhibited 31.2% inhibition at 10nM and 20.5% inhibition at 5nM concentrations. (Figure 3.1).

Figure 3.1 Study of release of Beta hexoseaminidase as a marker of mast cell degranulation

RBL-2H3 cells were incubated with various concentrations of Dexamethasone, Cyclosporin A and Wortmannin. The results have been expressed as % inhibition over A23187 stimulated control. Variance was determined using one way ANOVA. The following annotations have been designated for the p values (*: P ≤ 0.1, **: P ≤ 0.07, ***: P ≤ 0.05).

3.1.2 Effect of Dexamethasone, Cyclosporin A and Wortmannin on Cellular Cytotoxicity

Freshly isolated human PBMCs were subjected to treatment with the test molecules in their varying concentrations. At the end of a 24 h incubation period, 100 µg/ml (255 µM), 50 µg/ml (127.5 µM), 10 µg/ml (25.5 µM) and 1 µg/ml (2.55 µM) of Dexamethasone, 100 µg/ml (84 µM), 50 µg/ml (42 µM), 10 µg/ml (8.4 µM) and 1 µg/ml (0.84 µM) of Cyclosporin A and 43 ng/ml (100 nM), 4.3 ng/ml (10 nM), 2.15 ng/ml (5 nM) and
0.43 ng/ml (1 nM) of Wortmannin were tested for their effects on cellular cytotoxicity. Dexamethasone and Wortmannin exhibited a maximum toxicity of 65%, at the highest tested concentration of 255 µM and 100 nM, respectively; while Cyclosporin A exhibited a maximum of 47% toxicity. However at lower doses, no significant cytotoxicity (toxicity < 20%), was observed for either Dexamethasone or Cyclosporin A; whereas, Wortmannin exhibited a borderline toxicity of 30% at 10nM concentration, which reduced to 1% at 1 nM (Figure 3.2). Triton-X-100 served as the positive control yielding 100% cell death.

Figure 3.2 Cytotoxicity profiling of the test molecules on human PBMCs

PBMCs were incubated with the indicated concentrations of Dexamethasone, Cyclosporin A and Wortmannin for 24h. At the end of the incubation period, cell death was assessed with lactate dehydrogenase release. Results are expressed as % cell death over vehicle treated controls.

Based on the dose response analysis and the cytotoxicity data, a single concentration was chosen for each of the three analytes as follows: 50 µg/ml (125 µM) for Dexamethasone, 50 µg/ml (42 µM) for Cyclosporin A and 2.15 ng/ml (5 nM) for Wortmannin. The analytes were tested at the above single concentration, in the rest of the assays, performed under objective 1.
3.1.3 Assessment of the Test Molecules on Capacitative Calcium Entry (CCE) and Store Operated Calcium Entry (SOCE)

Based on the cytotoxicity and degranulation inhibition studies, a single concentration, namely 127.5µM for Dexamethasone, 42 µM for Cyclosporin A and 4.6 nM for Wortmannin were chosen for assessment for all further analyses of the test molecules. RBL-2H3 cells were incubated with the test molecules for 20min, and loaded with FURA 2A/M, and induced with 10 µM A23187.

![CCE and SOCE graphs](image)

**Figure 3.3 Effect on extracellular and mitochondrial calcium influx**

RBL-2H3 cells were subjected to induction with 10µM A23187 and the resultant calcium flux was measured fluorimetrically for up to 5min, at 30s intervals. The extracellular (CCE) and mitochondrial (SOCE) calcium fluxes were measured either in the presence (treated) or absence (induced) of the test molecules at the indicated concentrations: Dexamethasone (125 µM), Cyclosporin A (40 µM) and Wortmannin (4.6 nM) for 20min. Significance was tested by one way ANOVA and student’s t-test and the mean variance for both the induction model as well as test inhibition has been provided. All data points were found to be significant.
The calcium released into the supernatant upon induction was assayed at 30s intervals, either in the presence (CCE) or absence (SOCE) of external calcium. The results indicated that both Dexamethasone and Wortmannin showed a significant inhibition of the CCE, starting from 30s, that was sustained till 5min. Cyclosporin A exhibited a relatively lower level of inhibition of the calcium flux, starting from 90s. However, none of the 3 molecules showed any inhibition of the SOCE (Figure 3.3). All the data points were found to be significant (P≤0.05).

3.1.4 Time Course Analysis of the Actin Content on Activated RBL-2H3 Cell Membrane

Induction of FcεRI with 10 μM A23187 for 30s, 1, 3, 5, 20, 40 and 60min, was followed by the isolation of detergent insoluble membrane matrices from the activated RBL-2H3 cells. The lipid raft fractions were loaded with 6 U/ml Phalloidin conjugated Alexa Fluor 488 and the resultant fluorescence intensity was measured as Relative Fluorescence Units (RFUs). Results from the time course analysis indicate that, upon FcεRI induction, the RBL-2H3 cell membrane exhibits an actin dynamics, characterized by a 0.3 RFU fall in actin content at 30s, compared to non-stimulated control cells. By 20min however, the actin content rose to 0.15 RFUs over non-stimulated control (Figure 3.4). These results were reflected in the immunoblot analysis performed on activated RBL-2H3 cell membrane. The data are presented in section 3.1.8.
Figure 3.4 Time course analysis of membrane actin content upon FcεRI activation

RBL-2H3 cells were subjected to membrane activation with 10 µM A23187 at 30s, 1min, 3min, 5min, 20min, 40min and 60min. Ethanol treated, non-stimulated cells served as experimental controls. The isolated membrane fraction from the stimulated and non-stimulated cells were loaded with Phalloidin conjugated Alexa Fluor-488 for 5-10min. The expressed fluorescence has been recorded as Relative Fluorescence Units (RFUs).

3.1.5 Comparative Analysis of Dexamethasone, Cyclosporin A and Wortmannin on Membrane Actin Content

The effect of the three test molecules on the fall in actin content at 30s of FcεRI activation in RBL-2H3 cell membrane were comparatively analysed. After 30s of induction, the control cell membranes showed a 0.4 RFU fall in actin content, reflecting the results presented in section 3.1.4. Treatment with the test molecules failed to restore the membrane actin to basal levels. However, upon treatment with Cyclosporin A or Wortmannin rise in actin levels were observed beyond that of the stimulated control cell membranes. Cyclosporin A showed the maximum induction of 0.12 RFU, compared to 0.08 RFU increase exhibited by Wortmannin. Dexamethasone did not exhibit significant effects on the actin dynamics of activated RBL-2H3 cell membranes (Figure 3.5).
Figure 3.5 Quantitation of membrane actin in cells treated with the molecules at 30 sec of receptor stimulation

Treated and non-treated RBL-2H3 cells were stimulated with A23187 (10 μM) for 30s. Membrane fractions were isolated from stimulated cells and Dexamethasone (125 μM), Cyclosporin A (40 μM) and Wortmannin (4.6 nM) treated and stimulated cells. Membrane fractions from vehicle treated cells served as experimental controls. The isolated membrane fractions were loaded with phalloidin conjugated Alexa-Fluor 488 for 5-10min; the expressed fluorescence has been recorded as Relative Fluorescence Units (RFUs). * represents P≤ 0.05.

3.1.6 Time-course Analysis of the Phosphorylation Status of Wiskott Aldrich Syndrome Protein (WASP): A Comparative Analysis of the Effects of Dexamethasone, Cyclosporin A and Wortmannin

RBL-2H3 cells were incubated with Dexamethasone (127.5 μM), Cyclosporin A (42 μM), Wortmannin (4.6 nM) and ethanol, as the vehicle control, for 20min. The cells were then stimulated with 10μM A23187 for various time points of 30s, 5min and 10min selected based on literature review. Non-stimulated cells served as experimental controls. Upon immunoblotting, the time-course analysis performed with the non-stimulated and stimulated control cells revealed that the Ser\textsuperscript{483/484} residues of WASP are constitutively phosphorylated in RBL-2H3 cells. Taking the phosphorylation levels in vehicle treated control cells as 1 fold, no significant difference in the
levels of WASP phosphorylation at 30s and 5min of induction was observed. However, at 10min the levels of WASP phosphorylation fell 0.2 fold below control (P value: 0.005), (Figure 3.6A). Upon treatment with Cyclosporin A, the levels of constitutive phosphorylation increased to 0.16 and 0.19 folds over their stimulated controls at 30s and 10min respectively. Treatment with Dexamethasone and Wortmannin induced the phosphorylation levels to a maximum of 0.23 and 0.32 folds respectively, at 10min. However, no significant difference was observed at 30s or 5min in the phosphorylation levels of WASP, compared to stimulated control cells (Figure 3.6B).

![Figure 3.6 Protein levels study to determine the phosphorylation/activation status of WASP](image)

Figure 3.6 Protein levels study to determine the phosphorylation/activation status of WASP

Figure A represents the time course analysis of WASP phosphorylation levels after 30s, 5min and 10min of induction, as compared to vehicle treated control. In Figure B, Lane 1 represents cells induced with 10μM A23187. Lanes 2 and 3 represent cells treated with 50μg/ml concentration of Dexamethasone and Cyclosporin A, respectively. Lane 4 represents cells treated with 2.15ng/ml Wortmannin. The blots represent the phosphorylation/activation status of WASP at 30s, 5min and 10min post induction.
3.1.7 Docking analysis of Cyclosporin A against WASP-GBD

Docking study analysis of Cyclosporin A with the compact, folded, auto-inhibitory domain structure formed by the folding of the WASP GTPase Binding Domain (GBD) over its Verprolin Homology Domain (VCA) (PDB ID: 1T84) was performed. Docking performed for medium iterative steps with default parameters, showed very high binding energies, for the docking of Cyclosporin A to the auto-inhibitory loop.

Figure 3.7 Docking analysis of Cyclosporin A to WASP-GBD

Figure A is the surface view of Cyclosporin A-WASP conformation, with the best fit and least binding energy (BE). The MS representation is of the WASP-GBD, while the small ball and stick representation is that of the ligand Cyclosporin A. Figure B is the representation of the predicted H-bond formed by Cyclosporin A at the site.

Upon docking of Cyclosporin A to the WASP-GBD in the open conformation (PDB ID: 1CEE) in place of cdc42, 10 Cyclosporin A-GBD binding conformations with reasonably low binding energies (B.E) were
obtained. The best fit in the specific pocket was found to have B.E - 6.0 kcal/mol (Figure 3.7). A MS (or) Surface display of the interaction has been depicted in the Figureure. H-bond of 1.814Å Angstrom between H of NH in SER 13 and O of CO in Cyclosporin A has been predicted. Cyclosporin A also tends to form H-bonds with the nearby residues of Lys 16, Val 18 in conformations with a Binding energy of -6.0 kcal /mol or less.

3.1.8 Analysis of the effect of Cyclosporin A on membrane actin dynamics: A time-course analysis in activated RBL-2H3 cell membranes

The membrane actin dynamics was studied using resolution of the detergent extracted matrices on a 10% polyacrylamide gel, followed by immunoblotting with the anti-actin antibody. Results showed a significant (P value: 0.03) reduction (85%) in the F-actin content at 30s of induction. The membrane actin levels continued to rise over 1 and 5min, finally reaching a maximum of 140% at 20min (Figure 3.8A). Investigation of the ability of Cyclosporin A to inhibit the exhibited actin dynamics showed a significant inhibition (P value: 0.02) in the F actin dynamics at 30s. The level of membrane actin content increased approximately 20% over induced control at 30s (Figure 3.8B). Upon continued induction, Cyclosporin A was found to cause a significant (P value: 0.03) induction in the F-actin content (50% over induced control) at 5min, reaching a maximum of 155% over basal control, at 20min (Figure 3.8C). Representative immunoblots of the 45 kDa actin bands are depicted in the Figure (Figure 3.8D).
Figure 3.8 SDS-PAGE analysis of membrane actin content in FcɛRI activated RBL-2H3 mast cells

Figures A and B represent the SDS-PAGE resolution of proteins from the detergent-insoluble matrices of the membrane fraction, upon induction and upon Cyclosporin A (40 µM) treatment, respectively. Lanes 1-5 show the F-actin content at 0s, 30s, 1min, 5min and 20min of induction. Lane M shows the protein marker. The bands migrating along with 45kDa band in the marker lane, represents the F-actin band. Figure C represents the densitometric analysis of the actin bands. P < 0.05 (*) was taken to be significant. Figure D is the immunoblot analysis of the membrane actin content in IgE: DNP-HSA stimulated and Cyclosporin A (40 µM) treated and stimulated cells.
3.1.9 Confirmation of Membrane Actin Stabilization using Fluorescence Microscopy

The membrane integrity of non-stimulated, A23187 stimulated and Cyclosporin A treated and stimulated cells were studied using fluorescence microscopy at 30s, 1min, 3min, 5min and 20min of stimulation. The fluorescent images from the stimulated cells showed an apparent reduction in fluorescence towards the cell centre, as opposed to non-stimulated cells. Although, the F-actin depolymerization became apparent from the 30s time point, maximum loss of membrane integrity was observed at 3min of stimulation. The morphology and membrane integrity of the cells were observed to be restored by 20min. Upon treatment with Cyclosporin A however, although, an apparent disturbance in cell morphology was observed, the levels of actin fluorescence was observed to be maintained above the levels of stimulated control cells at all the given time points; this is true at even the 3min time point when the apparent F-actin depolymerization was observed to be maximum in stimulated control cells. By 5 and 20min, the cellular morphology was restored (Figure 3.9). These results are a reflection of the F-actin quantitation (Section 3.1.5) as well as of the Immunoblotting studies reported in section 3.1.8.
Figure 3.9 Analysis of F-actin dynamics using fluorescence microscopy

Figures A and B represent the status of actin polymerization in fixed RBL-2H3 cells under A23187 (10 µM) stimulated and Cyclosporin A (40 µM) treated and stimulated cells, respectively. The fluorescence was induced by staining the cells with phalloidin conjugated Alexa Fluor 488 stimulated under UV irradiation. The images have been obtained with 40x objective lens. Plate 1 represents vehicle treated control cells, while plates 2-6 represent cells at 30s, 1min, 3min, 5min and 20min of stimulation.

3.1.10 Comparative Analysis of Dexamethasone, Cyclosporin A and Wortmannin on Membrane Cholesterol Sequestration

The membrane and cytosolic fractions of vehicle treated and induced RBL-2H3 cells were separated using high speed and the amount of cholesterol in each fraction was estimated. Prior to membrane and cytosol separation, the cells were repleted or loaded with cholesterol using Methyl β-CycloDextrin (MBCD): cholesterol complexes. Upon receptor activation, no significant difference was observed in the membrane and cytosolic
cholesterol levels of repleted, stimulated cells, compared to repleted, non-stimulated, vehicle treated cells. Hence, a resting, non-stimulated RBL-2H3 model was used to estimate and analyze the levels of membrane and cytosolic cholesterol bio-chemically, after treatment with Dexamethasone, Cyclosporin A, Wortmannin or the control MBCD. While MBCD was observed to reduce the level of membrane cholesterol from 40 ng to 18 ng (P value: 0.07) by 2.2 fold, Dexamethasone reduced it to 35 ng (1.14 fold), Cyclosporin A to 31 ng (1.29 fold) and Wortmannin to 25 ng cholesterol/µg protein (1.6 fold), in each sample. Thus Wortmannin alone exhibited a marked (P value: 0.0933) sequestration of membrane cholesterol (1.6 fold decrease), comparable to the positive control MBCD (P value: 0.0726). On the contrary, the levels of cytosolic cholesterol was significantly (P value: 0.01) lessened upon treatment with Cyclosporin A, followed by Wortmannin; whereas, neither MBCD nor Dexamethasone exhibited a significant reduction in the levels of cytosolic cholesterol (Figure 3.10).

![Figure 3.10 Quantification of membrane and cytosolic cholesterol contents in RBL-2H3 cells](image)

Figure 3.10 Quantification of membrane and cytosolic cholesterol contents in RBL-2H3 cells

Figure represents the comparative membrane and cytosolic cholesterol levels in resting, Dexamethasone, Cyclosporin A, Wortmannin and MBCD treated cells. MBCD (10mM) served as the positive control. The results have been expressed as cholesterol in ng/µg of protein. • and * represents P<0.1, P < 0.05 respectively.
3.1.11 Comparative Analysis of Dexamethasone, Cyclosporin A and Wortmannin on Phosphorylation/Activation of Cellular Signaling Mediators

In order to study the status of cellular activation, the whole cell lysates of non-stimulated, stimulated or Dexamethasone, Cyclosporin A or Wortmannin treated and stimulated RBL-2H3 cells were probed with anti-P Tyr antibody. The results showed a significant (P value: 0.001) stimulation in the levels of protein phosphorylation, 30s after FcεRI activation. Upon treatment with Dexamethasone, Cyclosporin A or Wortmannin, the cellular phosphorylation was observed to be reduced 0.3 fold, 0.2 fold and 0.2 fold, respectively, below stimulated control. The positive control, Methyl-β-Cyclodextrin (MBCD), showed a slightly higher inhibition (P value: 0.002), at 30s. However, 5min after FcεRI activation, a marginal increase was observed in the levels of protein phosphorylation (0.04 fold over non-stimulated control), with no significant change in the phosphorylation levels upon treatment with Cyclosporin A. Dexamethasone exhibited a continued reduction in the levels of cellular phosphorylation (0.05 fold), followed by Wortmannin (0.2 fold). MBCD exhibited a marginal increase (P value 0.01), (Figure 3.11).
Figure 3.11 Western blot analysis of the activation status of events down-stream to FcεRI activation

Figures A and B represents the phosphorylation levels of cellular proteins, at 30s and 5min of induction respectively. Lane 1 is the vehicle treated control cells, Lane 2 represents A23187 induced cells. Lanes 3, 4, 5 and 6 represent Dexamethasone, Cyclosporin A, Wortmannin and MBCD treated cells, respectively. Figures C and D are the densitometric representation of the blots, expressed as fold phosphorylation, over vehicle control.

3.1.12 Analysis of the Phosphorylation Status of Protein Kinase B (PKB/AKT)

Immunoblotting studies with stimulated and non-stimulated RBL-2H3 whole cell lysates with the phospho-detect anti-AKT antibody, showed that, upon stimulation for 30s, 1min, 5min and 30min, the levels of AKT phosphorylation increased 2.4, 1.8, 4.2, 2.4 folds over non-stimulated control cells, respectively. Treatment of the test molecules, Dexamethasone, Cyclosporin A and Wortmannin with RBL-2H3 cells at the time points of least and maximum AKT phosphorylation/activation, namely 1 and 5min, it was observed that only cyclosporin A exhibited inhibition in the levels of
AKT phosphorylation at both the tested time points with a reduction of 0.3 fold over control cells being observed. Dexamethasone and Wortmannin, conversely, exhibited stimulation in the levels of AKT-phosphorylation to 0.2 fold over control cells at 1min. The phosphorylation levels rose to 0.6 fold and 1 fold over control cells, respectively, at the 5min time point (Figure 3.12).

Figure 3.12 Protein levels study to determine the phosphorylation/activation status of AKT

Figure A represents the time course analysis of AKT phosphorylation levels at 30s, 1min, 5min and 30min of induction, as compared to vehicle treated control. The corresponding densitometry values have been represented as fold increase over control. In Figure B, Lane 1 represents vehicle treated control cells. Lanes 3 and 4 represent cells treated with 50µg/ml of Dexamethasone and Cyclosporin A respectively. Lane 5 represents cells treated with 2ng/ml Wortmannin. The blots represent the phosphorylation/activation status of AKT at 1min and 5min post induction. The corresponding densitometry values have been represented as fold increase over control.
3.1.13 A Time Course Analysis of the Effect of Dexamethasone, Cyclosporin A and Wortmannin on NFκB Translocation and IκB Degradation

Upon probing the nuclear extract with the anti-p65 antibody, Wortmannin showed the maximum inhibition (1.2 fold reduction under stimulated control) of the p65 nuclear translocation at 1h of stimulation. This was followed by Dexamethasone (0.7 fold) and Cyclosporin A (0.5 fold). At 2h post stimulation, Wortmannin showed the maximum inhibition (0.6 fold reduction), followed by Cyclosporin A (0.4 fold reduction) and Dexamethasone (0.2 fold reduction) below stimulated control. By 3h of stimulation, all three molecules exhibited a significant inhibition of the p65 nuclear translocation, with Wortmannin showing the maximum effect (0.9 fold reduction), followed by Cyclosporin A (0.7 fold) and Dexamethasone (0.6 fold). Conversely, the probing of the cytosolic fraction with the anti-IκB antibody revealed that, after 1h of induction, Wortmannin inhibited the degradation of IκB to 1 fold over stimulated control. However, only Dexamethasone sustained the effect upon continued induction (2h) showing a 0.35 fold increase in IκB levels. By 3h post stimulation, all three molecules fail to inhibit IκB degradation (Figure 3.13).
Figure 3.13 Western blot analysis of p65 nuclear translocation and IκB degradation

Lane 1 represents uninduced, vehicle treated control cells. Lane 2 represents cells induced with 10µM A23187. Lanes 3 and 4 represent cells treated with 50µg/ml concentration of Dexamethasone and Cyclosporin A, respectively. Lane 5 represents cells treated with 5nM Wortmannin. The blots represent the protein levels of NFκB (Figure A) and IκB (Figure B) in the nuclear and cytosolic extracts, respectively, at 1h, 2h and 3h post induction.

3.1.14 Analysis of the mRNA Expression Levels of TNF-α

PCR analysis for the expression of TNF-α mRNA expression revealed that dexamethasone completely abrogated (0.8 fold under stimulated control) TNF-α expression at 30min post stimulation and sustained the effect until 1h (0.3 fold). Cyclosporin A showed a moderate inhibition (0.6 fold and 0.3 fold) at 30min and 1hour respectively. Wortmannin, on the other hand, showed a partial inhibition at 30min (0.6 fold), but the inhibition was observed to be lost upon further stimulation, up to 2h (Figure 3.14).
Figure 3.14 RT-PCR analysis of TNF- alpha mRNA expression levels

Lane 1 represents uninduced, vehicle treated control cells. Lane 2 represents cells induced with 10μM A23187. Lanes 3 and 4 represent cells treated with 50μg/ml concentration of Dexamethasone and Cyclosporin A, respectively. Lane 5 represents cells treated with 5nM Wortmannin. M represents the 100bp ladder and N is the negative control reaction for the PCR analysis. The gels represent the TNF-alpha mRNA expression levels 30min (A) and 1h (B) post induction, in the presence or absence of the inhibitor molecules. GAPDH served as the internal control to normalize the PCR reaction.
3.2 ISOLATION OF SOLASODINE

3.2.1 Selection and Screening of Medicinal Plants with Anti-allergic potential: A Preliminary Analysis against Hexoseaminidase Release from Activated RBL-2H3 Cells

Two medicinal plants, Solanum xanthocarpum and Clerodendron serratum were selected for screening for their anti-allergic potential. The plant materials were authenticated at Centre for Advanced Studies in Botany, University of Madras, India and deposited at the herbarium of Tissue culture and Drug discovery lab, Anna University Chennai. The dried plant materials were sequentially extracted with Dichloromethane and Methanol. Extraction with Dichloromethane was performed to sequester the non-polar constituent compounds in Solanum xanthocarpum, following which Methanol extraction was performed to elute the polar compounds. This was done to prevent any masking effect of the activity of polar compounds by the non-polar compounds. Extraction of 100 g of the dried aerial parts of S. xanthocarpum, consisting mainly of berries and stem, yielded approximately 40 g (d.w) of the Dichloromethane (SXD) and 38 g (d.w) of the Methanol (SXM) extract. Extraction of C. serratum bark yielded approximately 17.0 g (d.w) of the Dichloromethane (CSD) and 30 g (d.w) of the Methanol (CSM) extracts. The extracts were tested for their % degranulation inhibition against the IgE: DNP-HSA stimulated RBL-2H3 cells in an in vitro allergy model. Cyclosporin A (40 µM) served as positive control and IgE: DNP-HSA treated cells served as stimulated control. Vehicle treated, non-stimulated cells served as experimental controls. Results indicate that SXD, SXM, CSD and CSM inhibit degranulation to 44.3%, 77.1%, 57.5% and 38.5% respectively, at the highest tested concentration of 100µg/ml (Figure 3.15). SXM exhibited maximum activity and was selected for further studies.
Figure 3.15  Screening of plant extracts for their anti-allergic potential

Dichloromethane and Methanol extracts from S. xanthocarpum and C. serratum, were tested for their potential of degranulation inhibition as a measure of $\beta$-hexoseaminidase release. The results have been expressed as % inhibition over stimulated control.

3.2.2  Dose Response Analysis of the Anti-allergic Potency of SXM: A Function of Hexoseaminidase Release

In order to determine the extent of anti-allergic activity of S. xanthocarpum methanol extract (SXM), and its IC50 dose, its effect on mast cell degranulation was studied. The anti-allergic activity was measured as a
function of hexoseaminidase release, in the IgE: DNP-HSA induced RBL-2H3 cells. The results obtained as optical density have been expressed as % inhibition over induced control. The concentrations were chosen as the four logarithmic doses, 100, 10, 1 and 0.1µg/ml. At these concentrations, SXM exhibited 77%, 42%, 32% and 16% inhibitions respectively. In order to narrow down the IC50 value empirically, two additional doses of 50 and 20 µg/ml were tested and they were observed to inhibit degranulation to 61% and 40% respectively. Therefore further studies were carried out at 50µg/ml. (Figure 3.16). The positive control Cyclosporin A (40 µM) exhibited 50% inhibition of degranulation.. IgE: DNP-HSA treated cells served as stimulated control. Vehicle treated, non-stimulated cells served as experimental controls.

![Figure 3.16](image)

<table>
<thead>
<tr>
<th>Log value</th>
<th>Conc. (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-7</td>
<td>0.1</td>
</tr>
<tr>
<td>-6</td>
<td>1</td>
</tr>
<tr>
<td>-5</td>
<td>10</td>
</tr>
<tr>
<td>-4.7</td>
<td>20</td>
</tr>
<tr>
<td>-4.3</td>
<td>50</td>
</tr>
<tr>
<td>-4</td>
<td>100</td>
</tr>
</tbody>
</table>

Figure 3.16 A Dose response analysis of SXM against degranulation inhibition as a function of hexoseaminidase release

RBL-2H3 cells were incubated with different concentrations of SXM. The β-hexoseaminidase released from SXM treated RBL-2H3 cells have been expressed as % inhibition over induced control. Variance was determined using one way ANOVA. ***, ** and * represent P< 0.0001, P< 0.005 and P< 0.05 respectively.

3.2.3 Cytotoxicity Profiling of SXM in RBL-2H3 and PBMCs

To determine the toxicity potential of Solanum xanthocarpum, SXM at the highest tested dose in the study, 100µg/ml, was tested against the
normal human Peripheral Blood Mononuclear Cells (PBMCs) and the RBL-2H3 cells. At the end of 24h incubation, cell death was measured as a function of lactate dehydrogenase release. SXM exhibited 27% cell death (P value: non-significant); in PBMCs, whereas in RBL-2H3, SXM exhibited negative toxicity (Figure 3.17). Triton-X-100 served as the positive control yielding 100% cell death.

![Figure 3.17 Cytotoxicity profiling of SXM](image)

**Figure 3.17 Cytotoxicity profiling of SXM**

RBL-2H3 cells and PBMCs were incubated with 100 µg/ml SXM for 24h. At the end of the incubation period, cell death was assessed with lactate dehydrogenase release. Results are expressed as % cell death over vehicle treated controls. No significant cytotoxicity was observed. P<0.05 (*) was taken to be significant.

### 3.2.4 Isolation of the Anti-Allergic Principles of Solanum xanthocarpum: the Steroidal Alkaloids (SAs)

In order to select for the steroidal alkaloids documented to be rich in the berries of the plant S. xanthocarpum, saponification and fractionation of SXM, followed by column chromatography in a silica gel 200-400 mesh size column was performed (Breinhölder et al 2002). The protocol yielded 14 pooled fractions; the profile has been shown in the TLC plate (Figure 3.18). The TLC plates were developed using the ammonium molybdate spray
reagent at a temperature of 45º - 50º C. The fractions were then further tested for the bio-activity using β-hexoseaminidase release assay. Fractions 4 and 5 exhibited 44 and 43% inhibition respectively; fractions 7, 8 and 9 exhibited 47%, 17% and 43% inhibition respectively. Fraction 13 exhibited the highest % inhibition of 78% followed by fraction 14, which was observed to exhibit 64% inhibition (Figure 3.19A). Further chloroform-methanol based washes of the fractions yielded 4 isolates, Molecule 1 (SXM 1) from fraction 4, Molecule 2 (SXM 2) from fraction 7, Molecule 3 (SXM 3) from fraction 9 and Molecule 4 (SXM 4) from fraction 11-14 with maximum percentage being found in fraction 13 (Figure 3.19B).

Figure 3.18  TLC analysis of pooled fractions 1-14

TLC analysis of the fractions 1-14 resolved using 70% chloroform in Methanol. The TLC plates were developed on an Ammonium Molybdate spray reagent at 45º-50º C.
Figure 3.19  Analysis of the anti-allergic potential of pooled fractions 1-14

Figure A represents the β-hexoseaminidase release of RBL-2H3 cells treated with SXM and pooled column fractions 1-14. The anti-allergic potential of the column fractions has been represented as % inhibition over stimulated control. Figure B represents the TLC profile of the SXM and the 4 steroidal alkaloids isolated from it, resolved on Ethyl acetate/ Formic Acid/ Acetic / Water (10: 2: 2: 1). Lane 1 represents, SXM, lane 2 is SXM 1, lane 3 is SXM 2, lane 4 represents SXM 3 and lane 5 represents SXM 4.

3.2.5  Dose Response Analysis of SXM 1, 2, 3 and 4: Anti-allergic Activity as a Measure of % Degranulation Inhibition

SXM 1, 2, 3 and 4 were assayed for their inhibition of β-hexoseaminidase release at 100, 50, 20, 10, 1, 0.1 and 0.01 µg/ml concentrations. SXM 1 exhibited a maximum inhibition of 72% at the highest tested concentration of 100µg/ml and the IC50 dose was calculated to be 37.3ng/ml (Figure 3.20). SXM 2 exhibited a maximum inhibition of 72%,
with a IC50 dose calculated to be 3.618µg/ml (Figure 3.21). SXM 3 exhibited a maximum inhibition of 70% at the highest tested concentration of 100µg/ml and the IC50 dose was calculated to be 3.36µg/ml (Figure 3.22). SXM 4 exhibited a maximum inhibition of 66% at the highest tested concentration of 100µg/ml and the IC50 dose was determined to be 32.87µg/ml (Figure 3.23). Cyclosporin A (40 µM) served as positive control and IgE: DNP-HSA treated cells served as stimulated control. Vehicle treated, non-stimulated cells served as experimental controls.

<table>
<thead>
<tr>
<th>Log value</th>
<th>Conc. (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-7</td>
<td>0.1</td>
</tr>
<tr>
<td>-6</td>
<td>1</td>
</tr>
<tr>
<td>-5</td>
<td>10</td>
</tr>
<tr>
<td>-4.7</td>
<td>20</td>
</tr>
<tr>
<td>-4.3</td>
<td>50</td>
</tr>
<tr>
<td>-4</td>
<td>100</td>
</tr>
</tbody>
</table>

Figure 3.20  Dose response analysis of SXM 1 against degranulation inhibition as a function of hexoseaminidase release

RBL-2H3 cells were incubated with different concentrations of SXM 1. The β-hexoseaminidase released from SXM treated RBL-2H3 cells have been expressed as % inhibition over induced control. Variance was determined using one way ANOVA. ** and * represent P< 0.005 and P< 0.05 respectively.
Figure 3.21 Dose response analysis of SXM 2 against degranulation inhibition as a function of hexoseaminidase release
RBL-2H3 cells were incubated with different concentrations of SXM 2. The β-hexoseaminidase released from SXM treated RBL-2H3 cells have been expressed as % inhibition over induced control. Variance was determined using one way ANOVA. P< 0.05 (*) was taken to be significant.

Figure 3.22 Dose response analysis of SXM 3 against degranulation inhibition as a function of hexoseaminidase release
RBL-2H3 cells were incubated with different concentrations of SXM 3. The β-hexoseaminidase released from SXM treated RBL-2H3 cells have been expressed as % inhibition over induced control. Variance was determined using one way ANOVA. ** and * represent P< 0.005 and P< 0.05 respectively.
Figure 3.23 Dose response analysis of SXM 4 against degranulation inhibition as a function of hexoseaminidase release

RBL-2H3 cells were incubated with different concentrations of SXM 4. The β-hexoseaminidase released from SXM treated RBL-2H3 cells have been expressed as % inhibition over induced control. Variance was determined using one way ANOVA. P< 0.05 (*) was taken to be significant.

3.2.6 Cytotoxicity Profiling of SXM 1, 2, 3 and 4

The isolates were profiled for their cytotoxic effects on normal human PBMCs and the RBL-2H3 allergy model. At the end of 24h incubation, SXM 1, 2 and 3 exhibited almost 0% toxicity in the RBL-2H3 allergy model and negative toxicity in the normal cells (PBMCs). SXM 4 induced toxicity of 9% (Figure 3.24). Triton-X-100 served as the positive control.
Figure 3.24  Cytotoxicity profiling of SXM and SXM 1, 2, 3 and 4

Figure A represents the cytotoxicity profiling of cells treated with 100 µg/ml SXM, SXM 1, 2, 3 and 4 on RBL-2H3 cells. Figure B represents the cytotoxicity profiling of cells treated with 100 µg/ml SXM, SXM 1, 2, 3 and 4 on PBMCs. Results are expressed as % cell death over vehicle treated controls. No significant cytotoxicity was observed. P< 0.05 (*) was taken to be significant.

From the dose response analysis, it was observed that SXM 2 exhibited the maximum % inhibition of hexoseaminidase release (72%), at 100 µg/ml, while also, exhibiting an IC50 value of 3.6µg/mL. Additionally, SXM1 also appears to induce the proliferation of PBMC’s (50%) as observed
in the Figure 3.24. Hence SXM 2 was chosen for further structural elucidation and in vitro studies.

3.2.7 Structure Elucidation of SXM 2

The UV absorption ($\lambda_{\text{max}}$), for SXM 2 was found to be 203nm. In proton $^1$H (Figure 3.25) and $^{13}$C NMR (Figure 3.26) studies, SXM 2 gave the following shifts in resonance, indicated as peak values: $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 5.31 (br, S, 1H), 4.95 (d, $J$ = 5.2 Hz, 1H), 4.68 (m, 6H), 4.48 (d, $J$ = 5.6, 1H), 4.26 (d, $J$ = 7.6 Hz 1H), 4.19 (m, 1H), 1.09 (m, 4H), 0.96 (br, S m, 4H), 0.86 (m, 4H). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 140.4, 121.0, 100.4, 97.4, 77.7, 77.0, 76.6, 75.3, 75.1, 73.6, 71.8, 70.6, 70.5, 68.6, 62.3, 55.9, 49.5, 47.1, 36.7, 36.3, 34.0, 31.6, 31.5, 31.0, 30.5, 19.4, 19.1, 17.7, 16.1, 15.2.

$^1$H NMR (400 MHz) and $^{13}$C NMR (100 MHz) spectral data of solasodine in DMSO-$d_6$. Chemical shifts are in $\delta$ ppm and coupling constants ($J = \text{Hz}$) are given in parenthesis. The molecule was found to have a molecular weight of 413m/z [M+2] (Figure 3.27). Based on the number of Protons and Carbon atoms and the exhibited chemical coupling represented by the peak shifts, SXM 2 was predicted to have a molecular formula of C$_{27}$H$_{43}$O$_2$N, with the corresponding chemical structure similar to that of Solasodine (Figure 3.28A). The Steroidal Alkaloid was thus identified as Solasodine (Bhat et al 2008; Usabillaga et al 1997). The identity of the molecule was also confirmed by TLC analysis using the Chloroform/Methanol (8: 2) solvent system which showed an Rf of 0.275 (Figure 3.28B). Further HPLC analysis of the molecule was performed using acetonitrile: water solvent system, in the ratio 80: 20. A single peak was observed in the 3$^{rd}$min (Figure 3.29), (Trivedi and Pundarikakshudu 2007).
Figure 3.25  $^1$H NMR analysis of SXM 2
Figure represents the proton NMR analysis of SXM 2.

Figure 3.26  $^{13}$C NMR analysis of SXM 2
Figure represents the $^{13}$C NMR analysis of SXM 2.
Figure 3.27  Mass fragmentation pattern of SXM 2

Figure represents the mass fragmentation pattern of SXM 2

Figure 3.28  Chemical structure and Rf of Solasodine

Figure A represents the chemical structure of Solasodine, of the molecular formula C_{27}H_{43}O_{2}N. Figure B represents the TLC analysis of Solasodine performed using the solvent system 80% chloroform in Methanol. Solasodine yielded a Rf of 0.275.
Figure 3.29  HPLC analysis of Solasodine

Figure represents the HPLC analysis of Solasodine in the acetonitrile: water (80: 20) solvent system, eluted under isocratic conditions. Run time was 15min.

The structurally characterized Solasodine was investigated for its anti-allergic activity against the induced RBL-2H3 cells as an in vitro allergy model. All furthur studies were carried out at the IC50 concentration of SXM (50µg/ml) and Solasodine (9µM).

3.3  BIOLOGICAL POTENTIAL OF SXM AND SOLASODINE

3.3.1  Immunoblot Analysis of the Effect of SXM and Solasodine on Membrane Actin Dynamics: A Time-Course Analysis in Activated RBL-2H3 Cell Membranes

The effect of SXM and solasodine on FceRI induced cytoskeletal rearrangement was studied by determining the membrane actin content at 0s, 30s, 1, 5 and 20min of allergic induction (Figure 3.30). Immunoblotting with the anti-actin antibody was performed of the detergent extracted matrices, from cells treated with SXM (50µg/ml) and Solasodine (9µM) Densitometric
analysis of the resolved actin bands indicated that SXM caused a marked 10% increase in the membrane actin levels at the 30s time point (92%). At 1 and 5min time points the membrane actin levels in SXM treated cells stood at 97% and 124% respectively. At 20min, the actin content remained close to basal levels at 115% and did not reach the 140% induction, seen in stimulated control cells. Treatment with Solasodine exhibited a significant stimulation of 15% in the membrane actin levels (96%) over stimulated control at 30s. The actin levels then started to decrease in the ensuing time points (88% at 1min; 94% at 5min) and a maximum down regulation of 1.6 fold was observed at 20min (90%) of stimulation. The actin levels in all the treated samples were analyzed in comparison to DNP: HSA stimulated control cells.

Figure 3.30 Effect of SXM and Solasodine on membrane actin dynamics

Figures A, B and C shows the 45 kDa actin bands from an immunoblotted, SDS-PAGE resolution of proteins, from the detergent-insoluble matrices of the membrane fraction from DNP:HSA stimulated, SXM and Solasodine treated cells respectively. Lane 1 shows the membrane actin content in vehicle treated control RBL-2H3 cells, lanes 2-6 show the membrane actin content in DNP: HSA stimulated, SXM treated or Solasodine treated and stimulated RBL-2H3 cells at 0s, 30s, 1min, 5min and 20min of induction, respectively. Figures D and E represent the densitometric analysis of the actin bands.
3.3.2 Confirmation of the Effect of SXM and Solasodine on Membrane Actin Dynamics in Activated RBL-2H3 Cells using Fluorescence Microscopy

The membrane integrity of non-stimulated, A23187 stimulated cell controls (Figure 3.31 A) and cells treated with SXM (Figure 3.31 B) and Solasodine (Figure 3.31 C) were studied using fluorescence microscopy at 1, 3, 5 and 20min of stimulation.

Upon treatment with SXM and solasodine the cellular morphology was observed to be maintained at all the time points tested. An apparent loss in fluorescence towards the cells centre was observed by the 3 and 5min time points on treatment with SXM. This loss in fluorescence was however restored by 20min, and the cells resembled non-stimulated control cells. On treatment with Solasodine, the fall in fluorescence intensity became apparent at 5min of induction. At 20min of stimulation, uneven and patchy fluorescence in the cell membrane was observed. This is reflective of the observed loss in membrane actin content reported in section 3.3.1.
Figure 3.31 (Continued)

A

Uninduced Control Cells

A23187 stimulated cells

1 min 3 min

5 min 20 min
Figure 3.31  Analysis of F-actin dynamics using fluorescence microscopy: SXM

Figures A, B and C represent the status of actin polymerization in fixed RBL-2H3 cells under stimulated (A), SXM (50 µg/ml) treated and stimulated (B) and Solasodine (9µM) treated and stimulated (C) conditions at various time points respectively. The fluorescence was induced by staining the cells with phallloidin conjugated Alexa Fluor 488 stimulated under 488 nm excitation wavelength. The images have been obtained with 40x objective lens, with an emission wavelength of 568 nm.

3.3.3 Effect of SXM and Solasodine on WASP Ser\textsuperscript{483/484} phosphorylation

RBL-2H3 cells were incubated with SXM, Solasodine or with Methanol as the vehicle control, for 20min. Non-stimulated cells served as experimental controls. Similar to the results reported in section 3.1.6, no significant stimulation was observed in the levels of WASP residue Ser\textsuperscript{483/484} phosphorylation upon induction with 10µM A23187 for 30s, 5 and 10min. A
fall of 0.2 fold below control was observed in the phosphorylation levels at the 10min time point (Figure 3.32 A). The time course analysis performed with SXM treated cells revealed that, SXM inhibited WASP Ser^{483/484} phosphorylation at 30s of stimulation to 0.4 fold below control, but the phosphorylation returned to basal levels at 5min of stimulation. Upon treatment with Solasodine, a fall of 0.3 fold and 0.4 fold below control, in the levels of WASP Ser^{483/484} phosphorylation was observed at 30s and 5min of stimulation, respectively; at 10min of stimulation, a fall in the phosphorylation levels to 0.1 fold below control was observed (Figure 3.32B).

Figure 3.32 Determination of the phosphorylation/activation status of WASP

Figures A, B, C represents the time course analysis of WASP phosphorylation levels after 30s, 5 and 10min of induction, respectively. This is in comparison to vehicle treated control, in SXM (50 µg/ml) and Solasodine (9 µM) treated RBL-2H3 cells. Lane 1 represents vehicle treated control cells; lane 2 represents cells stimulated with 10µM A23187; lanes 3 and 4 represent cells treated with SXM (50 µg/ml) and Solasodine (9 µM) respectively. Figures D, E, F represents the densitometric analysis of the blots expressed as fold increase or decrease over stimulated control.
3.3.4 Effect of Solasodine on Actin “Comet-Tail” Motor Apparatus Formation for the Propulsion of Granules in Exocytosis

The cytosol of RBL-2H3 is filled with granules containing the preformed mediators, released on degranulation signals. The cell-free granules were isolated from RBL-2H3 treated with Solasodine, as well as from untreated control cells, using the method described in section 2.15. The cell-free granules from each of the samples were then stimulated with PMA and stained with acridine orange for 1h. The fluorescent dye acridine orange has been shown to accumulate in granules, revealing the accumulation of actin as a “comet-tail” like structure, that motors the propulsion of granules towards their destination (Taunton et al 2000), in this case, the mast cell membrane. Interestingly, when the cell-free granules from the cells treated with Solasodine were stimulated for 1h with PMA and stained with acridine orange, it exhibited an inhibition of comet tail formation on the granules indicating that solasodine could be inhibiting the granule movement towards the cell membrane, as part of the exocytosis process. The appearance of the granules resembled granules from non-stimulated, control cells (Figure 3.33).

Figure 3.33 Acridine Orange staining of cell-free granules

The cell free granules subject to stimulation with PMA and loaded with 10 µl acridine orange (4 mg/ml) for 1h. 10 µl of the cell-free sample was loaded on to glass slides and viewed under an excitation wavelength of 505 nm and emission wavelength of 520 nm, in a fluorescence microscope. Plates 1-3 represents cell-free granules from vehicle treated, stimulated (PMA) or Solasodine (9 µM) treated cells, respectively.
3.3.5 Quantitation of the Total and Membrane F-actin Content in Activated RBL-2H3 cells: A Comparative Analysis

Fluorimetric quantitation of the actin content in the activated RBL-2H3 cells as a whole, as well as its membrane fractions revealed that the pattern of actin dynamics in the total F-actin and membrane actin fractions closely resembled each other, over all the time points tested. At any given time, 25% of the cellular actin was found to exist in the cytosol and 75% in the membrane fraction. At 30s of stimulation, a characteristic fall (0.3 RFUs below control) in the actin content was observed in the membrane fraction, the total F-actin content exhibiting a fall of 0.2 RFUs below control. The membrane F-actin content was found to reach its peak at 20min, (0.106 RFUs over stimulated control). This was reflected in the total F-actin content also, (0.04 fold over stimulated control); the increase however, was not statistically significant. By 40min, both the membrane actin and the total F-actin contents were observed to recover to basal levels. The basal levels of actin content were stably maintained from 40min for up to 1h of stimulation (Figure 3.34).
Figure 3.34 Time Course analysis of total and membrane F-actin content in stimulated RBL-2H3 cells

Figure A and Figure B represents the quantitation of total and membrane F-actin content in stimulated RBL-2H3 cells (10 µM A23187), after 30s, 1min, 3min, 5min, 20min, 40min and 60min of FceRI activation. The fixed whole cells were loaded with Phalloidin conjugated Alexa Fluor 488 and the fluorescence measured using the fluorimeter. The actin contents have been expressed as Relative Fluorescence Units.

3.3.6 Quantitation of the Total F-actin Content in RBL-2H3 at 40min of A23187 Stimulation, upon Treatment with SXM and Solasodine

The effect of SXM and Solasodine were probed against the total F-actin content at 40min of stimulation. Results from the study, indicated that
SXM exhibits a significant inhibition (P value: 0.0001) of total actin content at 40min. Interestingly, Solasodine exhibited minimum inhibition of the total F-actin content at 40min of stimulation (Figure 3.35). This study was conducted to determine if the inhibition exhibited by Solasodine on cytosolic actin (“comet-tail” formation), was a global cellular phenomenon, causing a consequent disruption in RBL-2H3 membrane stability, at 40min of stimulation.

![Figure 3.35](image)

**Figure 3.35  Quantitation of membrane actin in treated cells at 40min of receptor stimulation**

Treated and non-treated RBL-2H3 cells were stimulated with A23187 (10 µM) for 40min. Membrane fractions were isolated from vehicle treated control cells, cells stimulated with 10 µM A23187, SXM (50 µg/ml) or Solasodine (9 µM) treated cells. The isolated membrane fractions were loaded with phalloidin conjugated Alexa-Fluor 488 for 5-10min; the expressed fluorescence has been recorded as Relative Fluorescence Units (RFUs). *** and * represent P< 0.0001 and P< 0.05 respectively.

3.3.7  Fluorescence Microscopy based Confirmation of the Membrane Integrity upon Treatment with SXM and Solasodine against A23187 Stimulation

The fluorescence microscopic images indicated that the cellular morphology and membrane integrity were restored in A23187 stimulated cells at 40min of stimulation. Upon comparison of treated cells, it was found that,
SXM treated cells showed the maximum restoration of cellular morphology and membrane integrity. Conversely, cells treated with Solasodine showed patchy fluorescence, with a disruption of cellular morphology being observed (Figure 3.36).

**Figure 3.36** Fluorescence microscopic view of membrane actin after 40min of FceRI activation

Fluorescence microscopic view of the cellular morphology and membrane integrity in A23187 (10 μM) stimulated cells; the time period of stimulation and receptor activation was 40min. Magnification was 400x.

### 3.3.8 Assessment of SXM and Solasodine on Cytosolic Calcium Influx

The effect of SXM on mitochondrial (SOCE), (Figure 3.37A) and extracellular calcium influx (CCE), (Figure 3.37B) was measured
fluorimetrically. 50µg/ml SXM reduced the magnitude of CCE, from a maximum of 2.2 RFU at 30s of allergic induction to around 2 RFU. This effect was sustained for up to 5min post stimulation. However, after an allergic stimulation, an induction of 0.6 RFU was observed in the levels of SOCE. Treatment with Solasodine exhibited the maximum inhibition of SOCE (1 RFU) at 30s of stimulation. The effect was sustained for up to 5min, till when the experiment was continued. Solasodine also exhibited a maximum of 0.3 RFU inhibition of CCE, with no significant changes in the calcium flux over the time period, under study.

![Figure 3.37](image)

**Figure 3.37  Effect of SXM and Solasodine on calcium flux**

RBL-2H3 cells were incubated with SXM (50µg/ml), Solasodine (9 µM) or vehicle (methanol), induced with 10µM Aβ1-42 in the presence of 1mM EGTA (A) or 1mM CaCl2 (B). The resultant calcium flux was measured fluorimetrically for up to 5min, at 30s intervals, represented as Relative Fluorescence Units. Significance was tested by one way ANOVA and student’s t-test and the mean variance for both the induction model as well as test inhibition has been provided. *** and ** represent P< 0.001 and P< 0.05 respectively.
3.3.9 Assessment of the Effect of SXM and Solasodine on PLA2 Activity Profile

The cytosolic PLA2 (cPLA2) activity was measured as a function of radio-labeled C14 arachidonic acid release. cPLA2 activity inhibition levels of 35% and 31% over A23187 stimulated controls, were observed upon treatment with SXM (50 µg/ml) and Solasodine (0.009 mM) respectively (Figure 3.38). The commercial cPLA2 inhibitor used as the experimental positive control exhibited a maximum of 79% inhibition of cPLA2 activity.

![Graph showing inhibition of PLA2 activity](image)

Figure 3.38 Effect of SXM on prostaglandin bio-synthetic enzyme, PLA2

Inhibition of PLA2 activity by SXM (50µg/ml) or Solasodine (9µM). Commercial cPLA2 inhibitor served as the positive control. PLA2 activity was measured as C14 labeled Arachidonic acid release. The results have been given as % inhibition of activity over A23187 (10 µM) stimulated control. P value of <0.05 was taken to be significant.

3.3.10 Assessment of the Effect of SXM and Solasodine on COX Activity Profile

The commercial COX enzymes in two different dilutions of 1:1000 and 1:10000 were incubated with the IC50 concentrations of SXM and Solasodine. Incubating the treated enzymes with their commercial substrates, revealed that neither SXM nor Solasodine significantly affected the activity.
profile of COX-1 enzyme. Upon incubation with COX-2 enzyme, both SXM and Solasodine exhibited a 9% inhibition of enzyme activity (Figure 3.39).

![Figure 3.39](image)

**Figure 3.39** Effect of SXM on prostaglandin bio-synthetic enzyme, COX

Figure represents the COX 1 and COX 2 activity profiles upon treatment with SXM (50ug/ml) or Solasodine (9 µM) treatment, in commercial COX enzymes. Results have been represented as % inhibition over purified enzyme control. Commercial inhibitors were used as positive controls in the enzyme assays. P value of <0.05 was taken to be significant.

### 3.3.11 A Comparative Analysis of the Effects of SXM and Solasodine on the Phosphorylation Levels of PKB/AKT Ser\textsuperscript{473} in Activated RBL-2H3 Cells

Upon probing the whole cell lysates of vehicle treated and A23187 stimulated RBL-2H3 cells, with Ser\textsuperscript{473} specific p-AKT antibody for 30s, 1, 5 and 30min, phosphorylation of Ser\textsuperscript{473} PKB/AKT was observed at 1 and 5min. Vehicle treated, non-stimulated cells served as experimental controls (Figure 3.40 A). Upon treatment with SXM and Solasodine, SXM exhibited 0.15 and 0.2 folds of inhibition at 1min and 5min of stimulation, respectively. The activity was reflected by Solasodine which exhibited 0.4 and 0.2 folds of inhibition at the two tested time points (Figure 3.40 B, C).
**Figure 3.40 Effect of SXM and Solasodine on AKT phosphorylation**

Figure A represents the time course analysis of Akt phosphorylation at 30s, 1min, 5min and 30min of A23187 (10 µM) stimulation. Figure B represents the levels of AKT phosphorylation in the whole cell lysate of RBL-2H3 at 1min and Figure C represents Akt phosphorylation levels at 5min of stimulation. Lane 1 represents cells stimulated with 10ȝε Aβγ1κ7. Lanes 2-3 represent cells treated with SXε (50 ȝg/ml) and Solasodine (λ µε). The bar graphs represent the corresponding densitometric analysis of the p-AKT bands.

**3.3.12 A Time Course Analysis of the Effect of SXM and Solasodine on NFκB Translocation and IκB Degradation**

The effect of SXM and Solasodine on NFκB p65 nuclear translocation (Figure 3.41 A, B) and IκB (35 kDa) degradation (Figure 3.41 C, D) was studied by western blotting analysis, at 1h, 2h and 3h post allergic stimulation. Upon stimulation, the levels of NFκB in the nuclear extract was
seen to rise 1.8, 0.8 and 0.02 folds over basal control; simultaneously, the levels of IκB was seen to fall 0.4, 0.4 and 0.5 fold under basal controls. Upon treatment with SXM, it was observed that the nuclear translocation of NFκB fell 0.3, 0.3 and 0.4 folds below A23187 stimulated control cells at 1h, 2h and 3h respectively. The inhibition was found to be significant at the 2h and 3h time points. On the contrary, SXM failed to inhibit IκB degradation at the 1h time point; but the levels rose to stabilize by the 2h and 3h time points. Solasodine exhibited significant inhibition of p65 nuclear translocation at 1 and 2h of stimulation (1.8, 1 and 0.15 folds below stimulated control). Solasodine also exhibited a sustained abrogation of IκB degradation to 0.03, 0.6 and 0.1 folds over stimulated control cells at 1h, 2h and 3h of stimulation, respectively.

Figure 3.41 (Continued)
**Figure 3.41 Effect on p65 nuclear translocation**

The blots represent the levels of p65 subunit in the nuclear fraction of RBL-2H3 at 1h (A), 2h (B) and 3h (C) post induction. Lane 1 represents non-stimulated, vehicle treated control cells. Lane 2 represents cells stimulated with 10μM A23187. Lanes 3 and 4 represent cells treated with SXε (50 μg/ml) and Solasodine (0.009 mM), respectively.

**Densitometric analysis**
Graph represents the corresponding densitometric analysis of the p65 bands.

**Effect on IκB degradation**

The blots represent the levels IκB in the cytosolic fraction of RBL-2H3 at 1h (D), 2h (E) and 3h (F) post induction. Lane 1 represents non-stimulated, vehicle treated control cells. Lane 2 represents cells stimulated with 10μM A23187. Lanes 3 and 4 represent cells treated with SXε (50 μg/ml) and Solasodine (0.009 mM), respectively.

**Densitometric analysis**
Graphs represent the corresponding densitometric analysis of the IκB bands.

### 3.3.13 Assessment of the Effect of SXM and Solasodine on Membrane Cholesterol Sequestration

Using the non-stimulated RBL-2H3 cells, the membrane and cytosolic cholesterol levels were estimated bio-chemically using the Liebermann-Burchard reaction. Methyl-β-Cyclodextrin was used as the positive control and exhibited a 55% decrease in membrane cholesterol, without significantly affecting the cytosolic cholesterol levels (Figure 3.42 A). Upon treatment with SXM and Solasodine, SXM did not exhibit
significant effects on the sequestration of RBL-2H3 membrane cholesterol (32 ng cholesterol/ µg protein), while stimulating the cytosolic cholesterol levels 2.5 fold and 2.3 fold over control cells, whereas, Solasodine exhibited the most significant sequestration of membrane cholesterol, reducing the levels from 39 ng cholesterol/ µg protein in control cells to 22 ng cholesterol/ µg protein. (1.8 fold decrease). However, Solasodine stimulated the levels of cytosolic cholesterol to 3.8 fold over control cells.

Figure 3.42 Quantification of membrane and cytosolic cholesterol contents in RBL-2H3 cells

Figure A represents the cholesterol contents in resting and A23187 activated RBL-2H3 cells. Figure B represents the comparative membrane and cytosolic cholesterol levels in resting cells treated with SXM (50 µg/ml) and Solasodine (9 µM), respectively. The results have been expressed as cholesterol in ng/ µg of protein. ** and * represents P < 0.005 and P < 0.05 respectively.
3.3.14 Time Course Analysis of SXM and Solasodine on Phosphorylation/Activation of Cellular Signaling Mediators

In order to study the status of cellular activation, the whole cell lysates of non-stimulated, stimulated, SXM or Solasodine treated and stimulated RBL-2H3 cells were probed with anti-P Tyr antibody. At 30s and 5min of cellular stimulation, the time points being chosen based on literature review, 0.6 fold (P value: 0.001) and 0.2 fold (P value: 0.001) increase in the levels of protein phosphorylation were observed. Treatment with SXM (50 µg/ml) produced a resultant fall in the cellular phosphorylation levels to 0.6 fold and 0.2 fold below stimulated control cells, at 30s and 5min respectively. Although Solasodine (0.009 mM) inhibited protein phosphorylation levels to 0.2 fold below stimulated control at 30s, it was unable to sustain its potency for up to 5min of stimulation (Figure 3.43).
Figure 3.43 Western blot analysis of the activation status of events down-stream to FceRI activation

Figures A and B represent the phosphorylation levels of cellular proteins, at 30s and 5min of stimulation respectively. Lane 1 represents non-stimulated, vehicle treated control cells. Lane 2 represents cells stimulated with 10μM A23187. Lanes 3 and 4 represent cells treated with SXM (50 μg/ml) and Solasodine (0.009 mM) respectively. Graphs represent the corresponding densitometric analysis of the tyrosine phosphorylation status, expressed as fold phosphorylation over vehicle control.

3.4 COMBINATORIAL CHEMISTRY

To investigate combination therapy of solasodine and the other isolates of Solanum xanthocarpum in the inhibition of mast cell degranulation.
3.4.1 Preliminary Combinatorial Analysis at the IC50 Concentrations of Solasodine with SXM1, SXM3 and SXM4

Preliminary analyses to assess for the possible existence of synergy between solasodine and the other three isolates SXM 1, SXM 3 and SXM 4 were performed. The molecules were tested in all their different combinations of two, at their IC50 concentrations. The IC50 concentration for SXM was observed to be 1 µg/ml, and for Solasodine, SXM 3 and SXM 4 it was observed to be 10 µg/ml as reported in section 3.2.5. IC50 combinations of Solasodine/SXM 1 exhibited 31% inhibition of hexoseaminidase release. A combination of Solasodine/SXM 3 exhibited 30% inhibition. And a combination of solasodine/SXM4 showed 46% inhibition of hexoseaminidase release (Figure 3.44). Cyclosporin A (40 µM) served as positive control and A23187 (10 µM) treated cells served as stimulated controls. Vehicle treated, non-stimulated cells served as experimental controls.

Figure 3.44 Analysis of synergistic interactions between IC50 doses of Solasodine, SXM 1, SXM 3 and SXM 4

Solasodine (0.009 mM), SXM 1 (98.6 mM), SXM 3 (0.004 mM) and SXM 4 (0.07 mM) were combined at their IC50 doses in all of their combinations of two. The resulting degranulation inhibition potential was measured as β-hexoseaminidase release. The results are expressed as % inhibition over stimulated control.
3.4.2 Dose Response Analysis of Combination of Solasodine with SXM 1, SXM 3 and SXM 4

Solasodine with the other three isolates SXM 1, SXM 3 and SXM 4 in their combinations of two, as stated in section 3.41, were assessed for their potency on degranulation inhibition using the preliminary hexoseaminidase release assay. Three logarithmic doses of 0.1µg/ml, 1 µg/ml and 10 µg/ml were selected. Solasodine was studied as a combination of two at each of the selected concentrations. Of the 27 possible combinations tested, Solasodine / SXM 4 (Table 3.3) at a combination of each 10 µg/ml, exhibited 76 (± 9) % inhibition. The results are tabulated below. Cyclosporin A (40 µM) served as positive control and A23187 (10 µM) treated cells served as stimulated controls. Vehicle treated, non-stimulated cells served as experimental controls.

Table 3.1 Determination of synergism/antagonism between Solasodine and SXM 1

<table>
<thead>
<tr>
<th>Solasodine</th>
<th>SXM 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1µg/ml</td>
</tr>
<tr>
<td>0.1µg/ml</td>
<td>39.52± 0.44</td>
</tr>
<tr>
<td>1µg/ml</td>
<td>38.58± 4.10</td>
</tr>
<tr>
<td>10µg/ml</td>
<td>41.07±0.58</td>
</tr>
</tbody>
</table>

Combination study between Solasodine and SXM 1 over three logarithmic doses of 10, 1 and 0.1 µg/ml. The values represent the % inhibition of degranulation inhibition over stimulated control, by each of the combinations, at the specified doses.
Table 3.2 Determination of synergism/antagonism between Solasodine and SXM 3

|        | SXM 3  
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1µg/ml</td>
</tr>
<tr>
<td>0.1µg/ml</td>
<td>33.03±1.32</td>
</tr>
<tr>
<td>1µg/ml</td>
<td>27.31±3.2</td>
</tr>
<tr>
<td>10µg/ml</td>
<td>28.91±3.29</td>
</tr>
</tbody>
</table>

Combination study between Solasodine and SXM 3 over three logarithmic doses of 10, 1 and 0.1 µg/ml. The values represent the % inhibition of degranulation inhibition over stimulated control, by each of the combinations, at the specified doses.

Table 3.3 Determination of synergism/antagonism between Solasodine and SXM 4

|        | SXM 4  
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1µg/ml</td>
</tr>
<tr>
<td>0.1µg/ml</td>
<td>33.53616±4.74832</td>
</tr>
<tr>
<td>1µg/ml</td>
<td>28.63352±5.28</td>
</tr>
<tr>
<td>10µg/ml</td>
<td>16.04659±1.46702</td>
</tr>
</tbody>
</table>

Combination study between Solasodine and SXM 4 over three logarithmic doses of 10, 1 and 0.1 µg/ml. The values represent the % inhibition of degranulation inhibition over stimulated control, by each of the combinations, at the specified doses.

3.4.3 Dose Response Analysis of Combination of Solasodine/ SXM 4 with SXM 1 and SXM 3

To further check the best combination from the previous study Solasodine/ SXM4 with the other isolates as a combination of three, at each of their three logarithmic concentrations were investigated. Of the possible combinations tested with Solasodine/SXM4/ SXM 1 the combination of 0.1µg/ml: 0.1µg/ml: 0.1 µg/ml: exhibited 50 % (± 0.2) inhibition in the degranulation inhibition assay.
Table 3.4 Determination of synergism/antagonism between Solasodine/ SXM 4/ SXM 1

<table>
<thead>
<tr>
<th>Drug Combinations of Solasodine/SXM 4/ SXM 1</th>
<th>% inhibition of hexoseaminidase release ±S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1/ 0.1/ 0.1</td>
<td>49.54167 ± 0.176777</td>
</tr>
<tr>
<td>0.1/ 1/ 0.1</td>
<td>37.70833 ± 3.49395</td>
</tr>
<tr>
<td>0.1/ 10/ 0.1</td>
<td>39.875 ± 4.22887</td>
</tr>
<tr>
<td>0.1/ 0.1/ 1</td>
<td>43.95833 ± 2.062395</td>
</tr>
<tr>
<td>0.1/ 1/ 1</td>
<td>33.04167 ± 4.20106</td>
</tr>
<tr>
<td>0.1/ 10/ 1</td>
<td>20.375 ± 5</td>
</tr>
<tr>
<td>0.1/ 0.1/ 10</td>
<td>46.08333 ± 4.896026</td>
</tr>
<tr>
<td>0.1/ 1/ 10</td>
<td>39.54167 ± 0.058926</td>
</tr>
<tr>
<td>0.1/ 10/ 10</td>
<td>43.875 ± 3.719249</td>
</tr>
<tr>
<td>1/ 0.1/ 0.1</td>
<td>41.66667 ± 2.592725</td>
</tr>
<tr>
<td>1/ 1/ 0.1</td>
<td>33.95833 ± 3.719249</td>
</tr>
<tr>
<td>1/ 10/ 0.1</td>
<td>37.91667 ± 4.32412</td>
</tr>
<tr>
<td>1/ 0.1/ 1</td>
<td>43.25 ± 2.239171</td>
</tr>
<tr>
<td>1/ 1/ 1</td>
<td>40.29167 ± 1.001735</td>
</tr>
<tr>
<td>1/ 10/ 1</td>
<td>33.45833 ± 4.015611</td>
</tr>
<tr>
<td>1/ 0.1/ 10</td>
<td>30.08333 ± 5</td>
</tr>
<tr>
<td>1/ 1/ 10</td>
<td>29.41667 ± 5</td>
</tr>
<tr>
<td>1/ 10/ 10</td>
<td>33.20833 ± 3.712311</td>
</tr>
<tr>
<td>10/ 0.1/ 0.1</td>
<td>39.83333 ± 1.178511</td>
</tr>
<tr>
<td>10/ 1/ 0.1</td>
<td>44.125 ± 3.830162</td>
</tr>
<tr>
<td>10/ 10/ 0.1</td>
<td>38.16667 ± 1.178511</td>
</tr>
<tr>
<td>10/ 0.1/ 1</td>
<td>39.91667 ± 4.660323</td>
</tr>
<tr>
<td>10/ 1/ 1</td>
<td>39.58333 ± 4.01735</td>
</tr>
<tr>
<td>10/ 10/ 1</td>
<td>37.25 ± 4.838835</td>
</tr>
<tr>
<td>10/ 0.1/ 10</td>
<td>40.79167 ± 1.237437</td>
</tr>
<tr>
<td>10/ 1/ 10</td>
<td>47.54167 ± 4.426356</td>
</tr>
<tr>
<td>10/ 10/ 10</td>
<td>34.29167 ± 3.948013</td>
</tr>
</tbody>
</table>

Combination study between Solasodine/ SXM 4 with SXM1 over three logarithmic doses of 10, 1 and 0.1 µg/ml. The values represent the % inhibition of degranulation inhibition over stimulated control, by each of the combinations, at the specified doses.
The isolates Solasodine / SXM 4/ SXM 3 (Table 3.5) in combinations of 0.1 µg/ml: 1 µg/ml: 0.1 µg/ml exhibited 54 (± 15) % inhibition. None of the other combinations tested exhibited any significant increase in their anti-allergic potency. The results are tabulated below. Cyclosporin A (40 µM) served as positive control and A23187 (10 µM) treated cells served as stimulated controls. Vehicle treated, non-stimulated cells served as experimental controls.

**Table 3.5 Determination of synergism/antagonism between Solasodine/ SXM4 with SXM 3**

<table>
<thead>
<tr>
<th>Drug Combinations of Solasodine/SXM4/ SXM 3</th>
<th>% inhibition of hexoseaminidase release±S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1/ 0.1/ 0.1</td>
<td>48.99464 ± 4.278632</td>
</tr>
<tr>
<td>0.1/ 0.1/ 1</td>
<td>41.01485 ± 4.006507</td>
</tr>
<tr>
<td>0.1/ 0.1/ 10</td>
<td>41.66938 ±0.07526</td>
</tr>
<tr>
<td>1/ 0.1/ 0.1</td>
<td>38.15741 ± 4.250982</td>
</tr>
<tr>
<td>1/ 0.1/ 1</td>
<td>35.21948 ±1.524611</td>
</tr>
<tr>
<td>1/ 0.1/ 10</td>
<td>33.16792 ± 4.194264</td>
</tr>
<tr>
<td>10/ 0.1/ 0.1</td>
<td>33.0625 ± 4.47307</td>
</tr>
<tr>
<td>10/ 0.1/ 1</td>
<td>41.67463 ± 4.79648</td>
</tr>
<tr>
<td>10/ 0.1/ 10</td>
<td>33.38943 ± 4.07862</td>
</tr>
<tr>
<td>0.1/ 1/ 0.1</td>
<td>53.6845 ±5</td>
</tr>
<tr>
<td>0.1/ 1/ 1</td>
<td>34.49495 ±3.214122</td>
</tr>
<tr>
<td>0.1/ 1/ 10</td>
<td>27.67677 ±5</td>
</tr>
<tr>
<td>1/ 1/ 0.1</td>
<td>20.75758 ± 4.64226</td>
</tr>
<tr>
<td>1/ 1/ 1</td>
<td>19.24242 ± 4.78547</td>
</tr>
<tr>
<td>1/ 1/ 10</td>
<td>21.11111 ± 5.285445</td>
</tr>
<tr>
<td>10/ 1/ 0.1</td>
<td>29.89899 ±5</td>
</tr>
<tr>
<td>10/ 1/ 1</td>
<td>17.47475 ± 0.999949</td>
</tr>
<tr>
<td>10/ 1/ 10</td>
<td>6.464646 ±4.856691</td>
</tr>
<tr>
<td>0.1/ 10/ 0.1</td>
<td>33.73737 ±0</td>
</tr>
<tr>
<td>0.1/ 10/ 1</td>
<td>29.24242 ±4.785368</td>
</tr>
<tr>
<td>0.1/ 10/ 10</td>
<td>28.78788 ±3.856946</td>
</tr>
<tr>
<td>1/ 10/ 0.1</td>
<td>35.30303 ± 4.78501</td>
</tr>
<tr>
<td>1/ 10/ 1</td>
<td>28.43434 ±4.64262</td>
</tr>
<tr>
<td>1/ 10/ 10</td>
<td>26.81818 ± 2.785572</td>
</tr>
<tr>
<td>10/ 10/ 0.1</td>
<td>25.40404 ±5.785419</td>
</tr>
<tr>
<td>10/ 10/ 1</td>
<td>27.22222 ± 1.928473</td>
</tr>
<tr>
<td>10/ 10/ 10</td>
<td>33.68687 ±5</td>
</tr>
</tbody>
</table>

Combination study between Solasodine/ SXM 4 with SXM3 over three logarithmic doses of 10, 1 and 0.1 µg/ml. The values represent the % inhibition of degranulation inhibition over stimulated control, by each of the combinations, at the specified doses.