Chapter 3
Objective III

To check the key findings in imatinib resistant cells from objective I and objective II in imatinib sensitive cells.

The followings are the key findings from object I and II.

1. Imatinib resistant K562 cells shows TRAIL resistant. But Piper betel leaves derived phenolic compound Hydroxychavivol sensitizes Imatinib resistant K562 cells to TRAIL mediated apoptosis.

2. In previous study, overexpression of antiapoptotic proteins, FLIP, XIAP is responsible for TRAIL resistance property in imatinib resistant K562 cells. Hydroxychavicol targeted and degrades XIAP and FLIP in imatinib resistant K562 cells. In this way, hydroxychavicol sensitizes imatinib resistant K562 cells to TRAIL mediated apoptosis.

3. Proteosomal mediated degradation pathway is involved in Hydroxychavicol mediated XIAP and FLIP degradation.

4. ROS played a major role in Hydroxychavicol -mediated TRAIL sensitization.

5. Hydroxychavicol downregulates XIAP and FLIP in ROS dependant manner.

Next, the above key results from imatinib resistant CML cell line K562 have now been checked in Imatinib-sensitive CML cell lines K562.

Materials:

Hydrogen peroxide used in this study was purchased from Merck Life Science Pvt. Ltd (India). The antibodies against XIAP, c-FLIP, anti-mouse IgG HRP-linked antibody and anti-Rabbit IgG HRP-linked antibody were bought from Cell Signaling Technology (Denver, Massachusetts, USA). Anti-γ-actin antibody used as a control was bought from Biobharati LifeScience (India). rhTRAIL was bought from R&D Systems Inc. (Minneapolis, Minnesota, USA). iScript Reverse Transcription Supermix and Sso-Fast Evagreen Supermix were

purchased from BIORAD. FITC Annexin V was taken from BD Pharmingen. Propidium iodide was brought from Sigma-Aldrich.JNK inhibitor SP600125, ERK inhibitor PD98059 were purchased from Abcam (Cambridge, MA, USA). NAC (N-acetyl-L-cysteine) were bought from Calbiochem. Fetal Bovine Serum (FBS) Standard (origin Brazil) was purchased from Gibco®.Life Technologies, USA

Methods:

Cell lines and culture condition: K562 cell line was a kind gift from Dr Santu Bandyopadhyay of Indian Institute of Chemical Biology. Cells were maintained in RPMI medium supplemented with 10% fetal bovine serum and 0.1% ampicillin and 1% penicillin-streptomycin at 37°C in a humidified atmosphere with continuous flow of 5% CO2.

Western blot analysis: Cells were washed in ice-cold PBS and extracted for 15 min with a buffer containing 50 mmol/l Tris/HCl (pH: 7.5), 140 mmol/l NaCl, 5 mmol/l EDTA, 5 mmol/l NaN3, 1% (v/v) Triton X-100, 1% (v/v) Nonidet P40, 1 mmol/l EGTA and protease inhibitor cocktail. Lysates were cleared by centrifugation at 15 000 rpm for 15 min in an Eppendorf centrifuge, and protein concentrations were determined using Lowry protein assay. Proteins were denatured in 1%SDS-containing reducing sample buffer and the same total protein amount was transferred on to an Immun-Blot PVDF Membrane (BIORAD). The membranes were probed with specific antibodies. Immunocomplexes were detected using horseradish peroxidase-conjugated secondary antibodies followed by Clarity Western ECL Substrate detection (BIORAD) using BIORAD ChemiDoc MP Imaging System

Annexin V/PI binding assay: 1.0 × 105 cells were seeded in 24 well plates and the treatments were done. Cellswere washed with 1X PBS two times. Cells were washed with 1X Annexin V/ PI binding buffer and annexinV/FITC was added according to the manufacturer's protocol. Next 50 µg/ ml PI was added and flow cytometrywas done mixing the cells with400µl of 1X Annexin V binding buffer. The instrument, used was BD Accurieand analysis was done with BD C6 software.

Real time PCR:

Using Trizol reagent the total RNA was isolated from appropriately treated cells according to the manufacturer's instructions. RNA quality was checked by agarose gel electrophoresis and concentration was determined using Synergy H1 Microplate Reader. cDNA was prepared from isolated total RNA using iScriptTM ReverseTranscription Supermix for RT-qPCR (BIO RAD). Real-time PCR was done with SsoFastTM Evagreen Supermix (BIO RAD), in CFX96 Touch™ Real-Time PCR Detection System (BIO RAD). 40 cycles were run for eachround of PCR for total saturation and the Cq values were considered for further analysis. All RT-qPCR setupwere done in duplicate sets and the complete experiments were independently repeated at least three times. For graphical representation of the gene expression data, Actin was used as house-keeping gene and the mRNA levels were normalized accordingly.

The primers used were:

CFLIP

Forward: 5'-GGC CGA GGC AAG ATA AGC AA-3'

Reverse: 5'-TTG TCC CTG CTC CTT GAA CA-3'

XIAP

Forward :5'-GGA CCC TCC CCT TGG AC-3'

Reverse: 5'-TGA TGT CTG CAG GTA CAC AAG TT-3'

Statistical analysis: Data were analyzed as mean ± SD of at least three independent experiments and statistically significant differences between mean values (from three independent experiment at least) were determined using Student's t-test.

Results:

Imatinib-sensitive K562 cells showed resistance against TRAIL-induced apoptosis:

To check the cytotoxicity of TRAIL to K562 CML cell lines (imatinib sensitive), K562 cells were incubated with TRAIL for 24 hrs. Annexin V/PI binding assay indicated that no cell death as compare with control in K562 cells by TRAIL (Fig 1A). So, Imatinib sensitive K562 cells show resistance against TRAIL induced apoptosis.

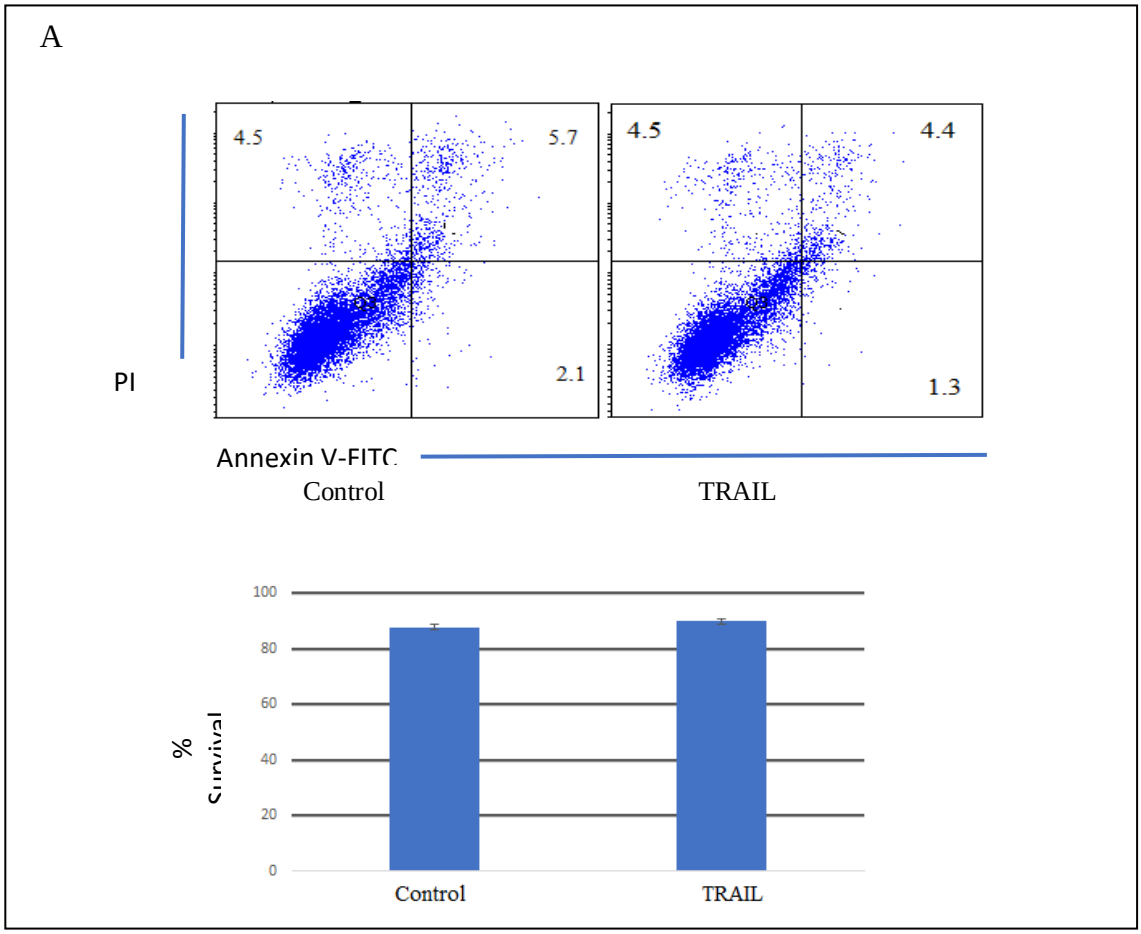


Figure 1

Fig1: CML cell lines had showed resistance against TRAIL-induced apoptosis: Imatinib sensitive K562 cells were treated with 200 ng/ml of TRAIL, and annexin V/PI binding assay was performed in flow cytometer. Data represents mean ±SD of three independent experiments (*P<0.05).

CML cell lines were sensitized by Hydroxychavicol to TRAIL-induced apoptosis

To check the effect of Hydroxychavicol on TRAILinduced cytotoxicity in Imatinib sensitive K562 cells (K562(S)), K562(S) cells were treated with either 8 µM of Hydroxychavicolalone or in combination with 200 ng/ml of TRAIL. Apoptosis assay (annexin V/PI binding assay) indicated that Hydroxychavicol alone induced apoptosis of around 60% but increased up to 88% when TRAILwas applied along with Hydroxychavicol [Fig 2A]. These data indicated that Hydroxychavicol facilitates TRAIL-induced apoptosis in K562(S) cells.

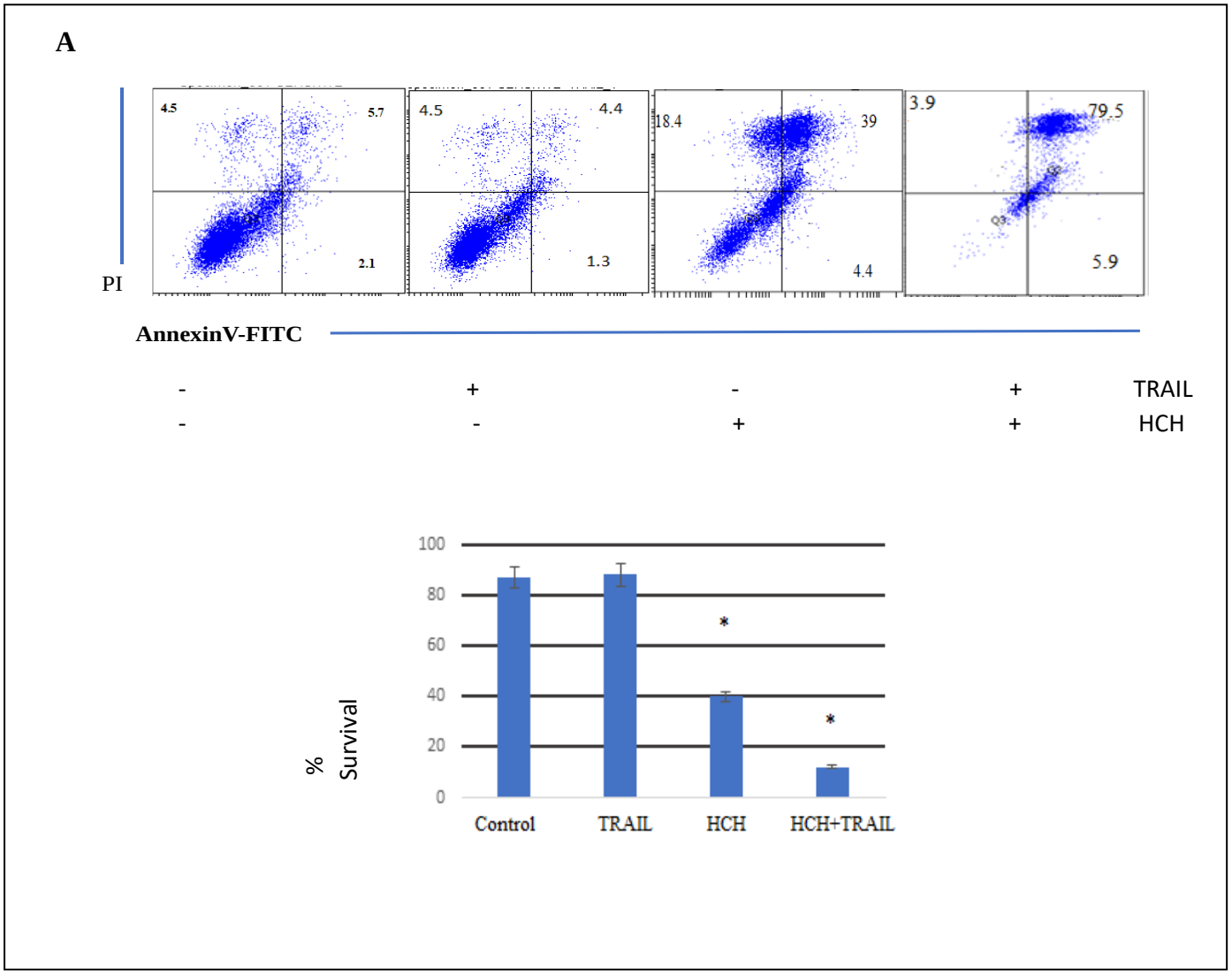


Figure 2

Figure 2: CML cell lines were sensitized by Hydroxychavicol to TRAIL-induced apoptosis : K562(R) cells were treated with indicated doses of Hydroxychavicol (HCH) and 200 ng/ml of TRAIL. Annexin V/PIbinding assay was performed in flowcytometer. Data represents mean ±SD of three independent experiments (*P<0.05).

Hydroxychavicol-mediated TRAIL sensitization of K562(S) cell was ROS dependent:

In our previous study, we have shown that Hydroxychavicol increased intracellular ROS level [159]. Therefore, It might be possible that here ROS has some role to play insensitizing K562(S) cells to TRAIL-mediated apoptosis. Therefore, we preincubated cells with ROS scavenger NAC and then treated with TRAIL and Hydroxychavicol (HCH). AnnexinV/PI binding assay showed that NAC significantly reversed TRAIL-mediated enhancement of apoptosis indicating the involvement of ROS (Fig. 3A).

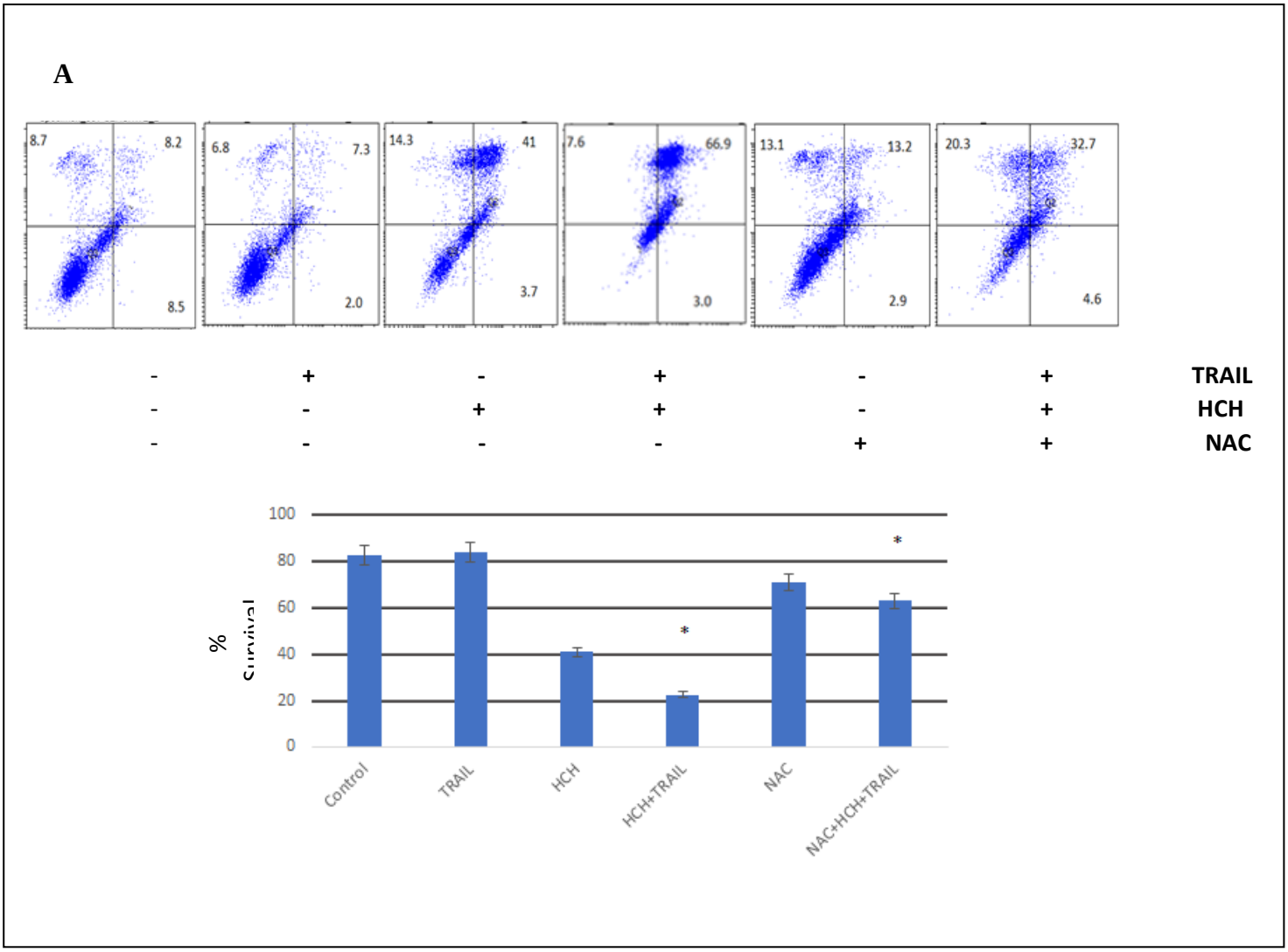


Figure 3

Figure 3: Hydroxychavicol-mediated TRAIL sensitization of K562(R) cell was ROS dependent: K562(S) cells were treated with HCH and TRAIL in presence and absenceof 1 mmol/l NAC for 24 h and Annexin V/PI binding assay was performed in flowcytometer. Results were representative of three independentexperiments.

So, it is concluded that ROS was involved in Hydroxychavicol-mediated TRAIL sensitization of K562(S) cell.

The protein level of XIAP and FLIP was decreased by Hydroxychavicol

The overexpression of antiapoptotic genes is most common mechanism of apoptosis inhibition. There are three antiapoptotic family of proteins, including Bcl2, inhibitors of apoptosis proteins (IAPs) and FLICE-inhibitory proteins (FLIPs). They regulate caspase-dependent apoptotic pathways. So, we wanted to check whether any change of these anti apoptotic proteins could be responsible here. Imatinib sensitive K562 cells were treated with Hydroxychavicol at different doses for 20 hrs. Whole cell lysates were subjected to western blot analysis. Western Blot analysis data indicated that FLIP and XIAP were decreased significantly when treated with increasing concentration of Hydroxychavicol [fig 4].

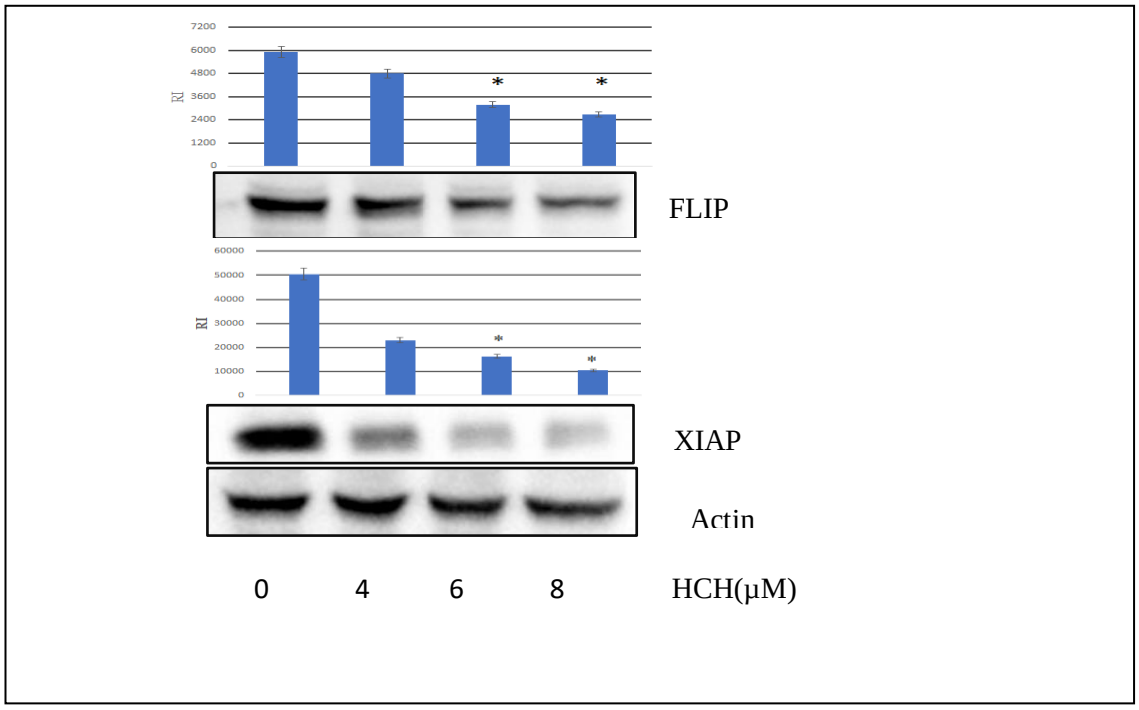
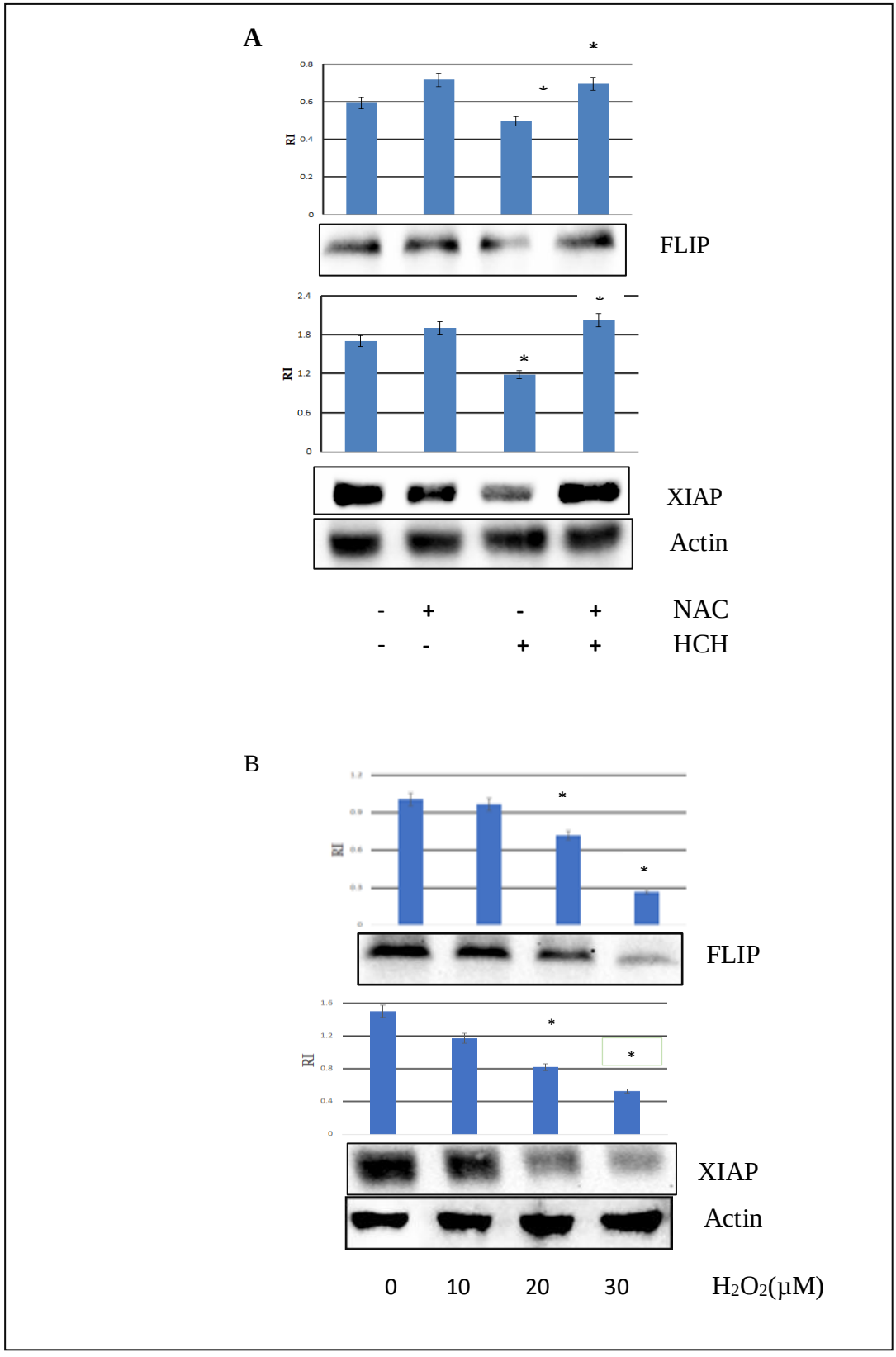


Figure 4

Figure 4: The protein level of XIAP and FLIP was decreased by Hydroxychavicol: K562(S) cells were treated with different doses of Hydroxychavicol for 20 hrs. Western blot analysis was done for checking the level of XIAP and FLIP. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments (*P<0.05).

Downregulation of antiapoptotic proteins XIAP and FLIP was ROS-dependent

Now, we wanted to check Hydroxychavicol (HCH)-mediated XIAP and FLIP downregulation is ROS mediated or not. We preincubated K562(S) cells with NAC and treated with HCH. Western blot analysis with the whole cell lysates for XIAP and FLIP showed that NAC treatment significantly reversed HCH mediated XIAP and FLIP downregulation (Fig. 5A). This indicated that ROS might be involved in the downregulation of XIAP and FLIP.If ROS played a key role in the downregulation of FLIP and XIAP, then adding ROS externally should show similar effect. When, imatinib sensitive K562 cells were treated with various doses of H_2O_2, western blot analysis showed that H_2O_2 dose dependently decreased both XIAP and FLIP (Fig. 5B). K562(S) cells were treated with 30 μM H_2O_2 for various time points. The whole cell lysate was subjected to western blot analysis with XIAP and FLIP antibody. Result indicated that FLIP level decreased after 18 hours while XIAP level decreased after 16 hours of H_2O_2 treatment [Fig. 5C].


A
RI
FLIP
XIAP
Actin
- + - +
- - + +
NAC
HCH
B
RI
FLIP
XIAP
Actin
0 10 20 30
H2O2(μM)

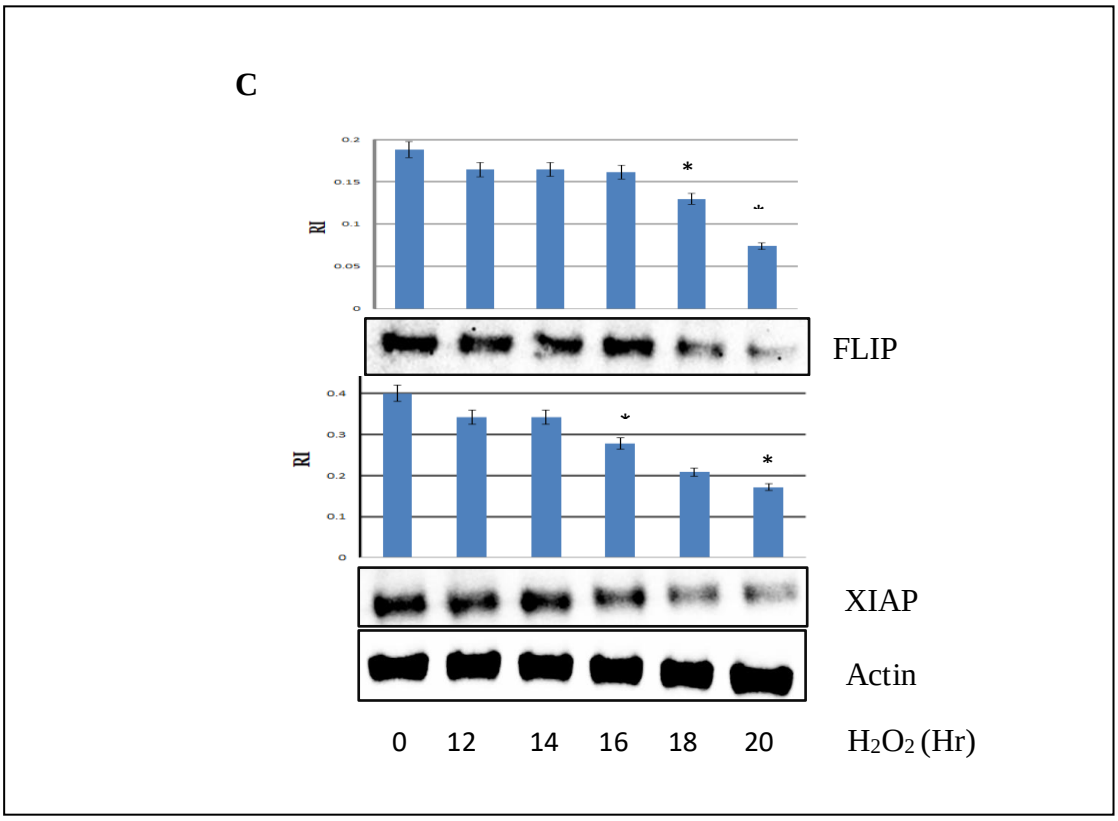


Figure 5

Figure 5: Downregulation of antiapoptotic proteins XIAP and FLIP was happened in a ROS-dependent manner: **A)** K562 (S) cells were pre-treated with 1mM NAC for 1 hr and then HCH was added and incubated for overnight. Whole cell lysates were extracted and Immunoblot analysis of FLIP and XIAP was performed. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *p < 0.05. **B)** Imatinib sensitive K562 cells were treated with indicated doses of H_2O_2 for overnight. Western blot analysis of FLIP and XIAP was performed from whole cells. Actin was usedas loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean± SD of three independent experiments. *p < 0.05 **C)** Immunoblot analysis of FLIP and XIAP was performed from imatinib sensitive K562 cells after treating the cells with 30µM H2O2 for the indicated time points. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis andindicate mean ± SD of three independent experiments. *p < 0.05.

XIAP and FLIP at mRNA level was not altrered by Externally treated H_2O_2 in imatinib sensitive K562 cells:

We checked whether there is any alteration of XIAP, FLIP proteins at mRNA level, cells were treated with different doses of H_2O_2. Real time RT qPCR with the total RNA isolated from H_2O_2 treated K562(S) cell showed that there is no any alternation of either XIAP or FLIP at RNA level. [Fig 6]. So, it is concluded that ROS mediated XIAP and FLIP modification is post translational mediated.

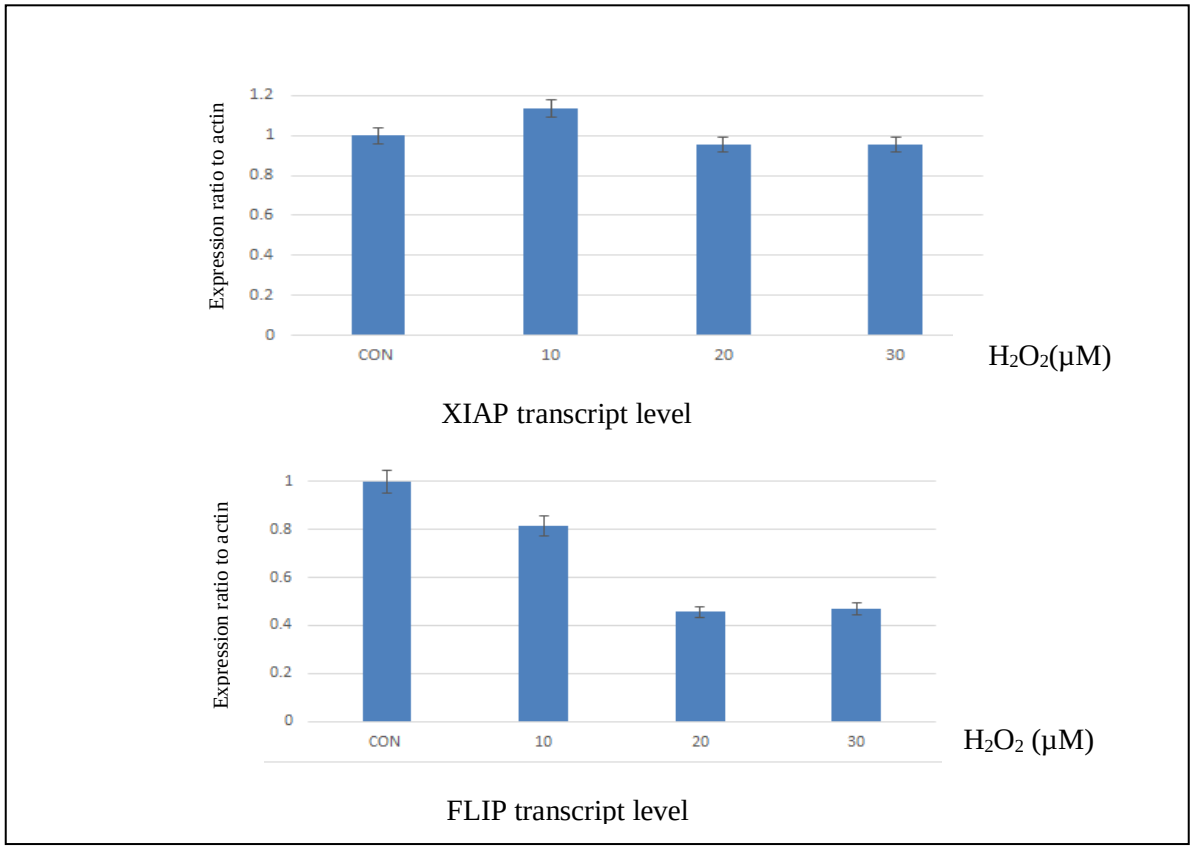


Figure 6

Fig 6: XIAP and FLIP at mRNA level was not altrered by Externally treated H_2O_2 in imatinib sensitive K562 cells: Imatinib sensitive K562 were treated with increasing concentration of H_2O_2 for 14 hrs and then total RNA was extracted from cells. Relative levels of endogenous FLIP and XIAP mRNA were assessed by Real time PCR and graphically represented as fold change. Data represents mean ± SD of three independent experiments.

FLIP and XIAP were downregulated by H_2O_2 via Lysosomal dependant pathway but not proteosomal pathway

Cellular proteins level was reduced by Lysosomal or Proteosomal degradation pathway. Therefore, we wanted to check involvement of any of these two pathways was responsible for H_2O_2 mediated XIAP and FLIP downregulation.

Now, K562(S) cells were pretreated with 1.5 µM of Proteasome inhibitor MG132 and then treated with 30 µM H_2O_2.Whole cell lysates were taken after 18 hours then subjected to western blot analysis. The result indicated that proteasome inhibitor MG132 did not reverse H_2O_2 mediated FLIP and XIAP downregulation [Fig 7A]. So, proteosomal pathway is not involved in H_2O_2 mediated XIAP and FLIP degradation. To check the involvement of Lysosomal pathway, K562(S) cells were pre-treated with 20 µM of Chloroquine, an inhibitor of autophagy pathway by impairing autophagosome fusion with lysosomes then cells were incubated with 30 µM H_2O_2 for 18 hours. Whole cell lysates were then subjected to western blot analysis with anti-XIAP and anti-FLIP antibody. Result indicated that Chloroquine reverses H_2O_2 mediated XIAP and FLIP downregulation. So, it is confirmed that H_2O_2 mediated XIAP and FLIP downregulation is occurred via lysosomal dependant [Fig 7B].

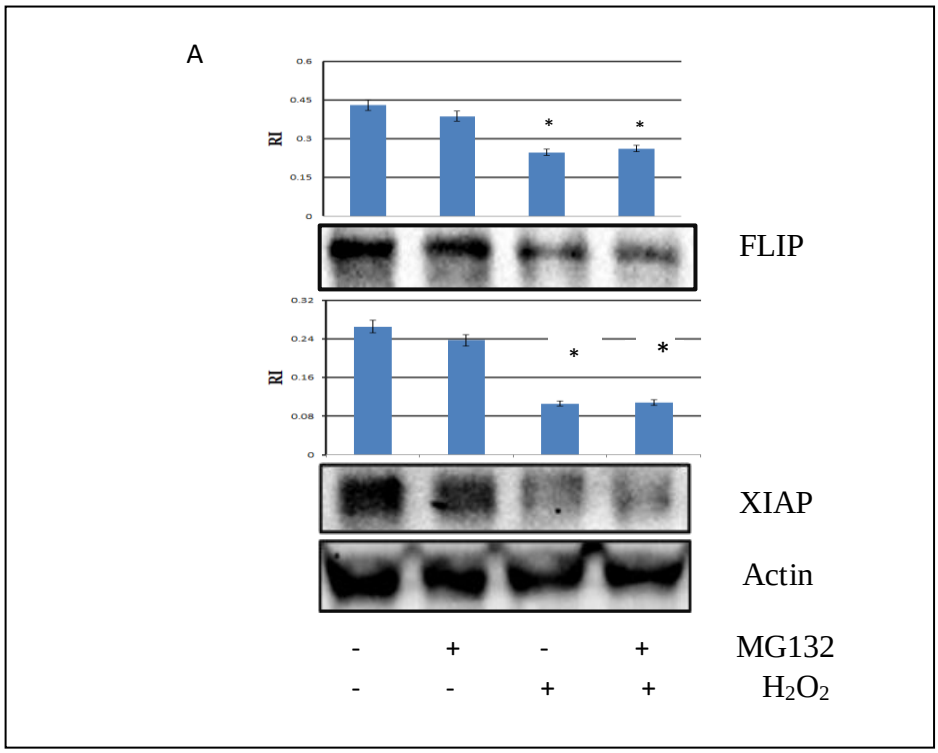


Figure 7A

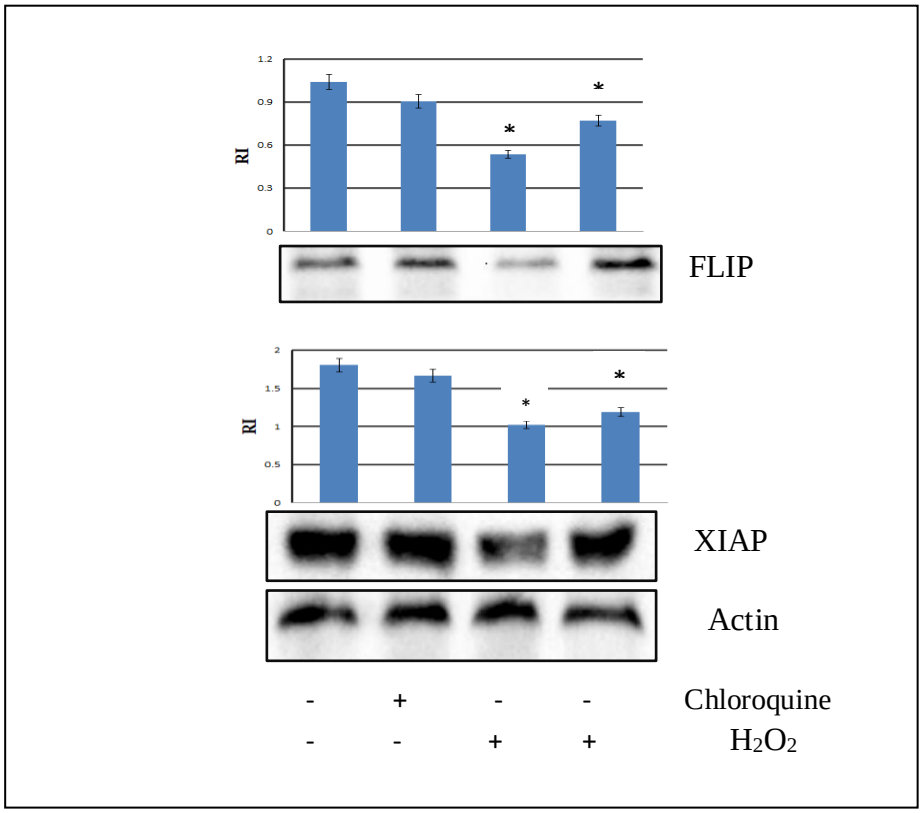


Figure 7B

Fig 7: FLIP and XIAP were downregulated by H_2O_2 via Lysosomal dependant pathway not proteosomal pathway. **A)** K562 (S) cells were preincubated with 2 µM of MG132 and then 30µM H_2O_2. Whole cell lysates were subjected to western blot analysis with anti-FLIP and anti-XIAP antibodies. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$. **B)** K562(S) cells were preincubated with 20 µM Chloroquine and then treated with 30 µM H_2O_2. Whole cell lysates were subjected to western blot analysis with anti-FLIP and anti-XIAP antibodies. Actin was used as loading control. Data were presented graphically as ratio to actin after densitometry analysis and indicate mean ± SD of three independent experiments. *$p < 0.05$.

So, it is concluded that H_2O_2 mediated XIAP and FLIP downregulation was lysosomal dependant not proteosomal in K562(S) cells. From objective II, we have seen that H_2O_2 mediated XIAP and FLIP downregulation is proteosomal dependant not lysosomal in K562(R) cells.

H_2O_2 did not significantly induce apoptosis in imatinib sensitive K562 cells:

K562(R) cells were treated with different doses of H_2O_2 for 24 hours. Annexin V-PI binding assay indicated that highest concentration of H_2O_2 causes little bit cell death as compare to control untreated K562 cells. [Fig8].

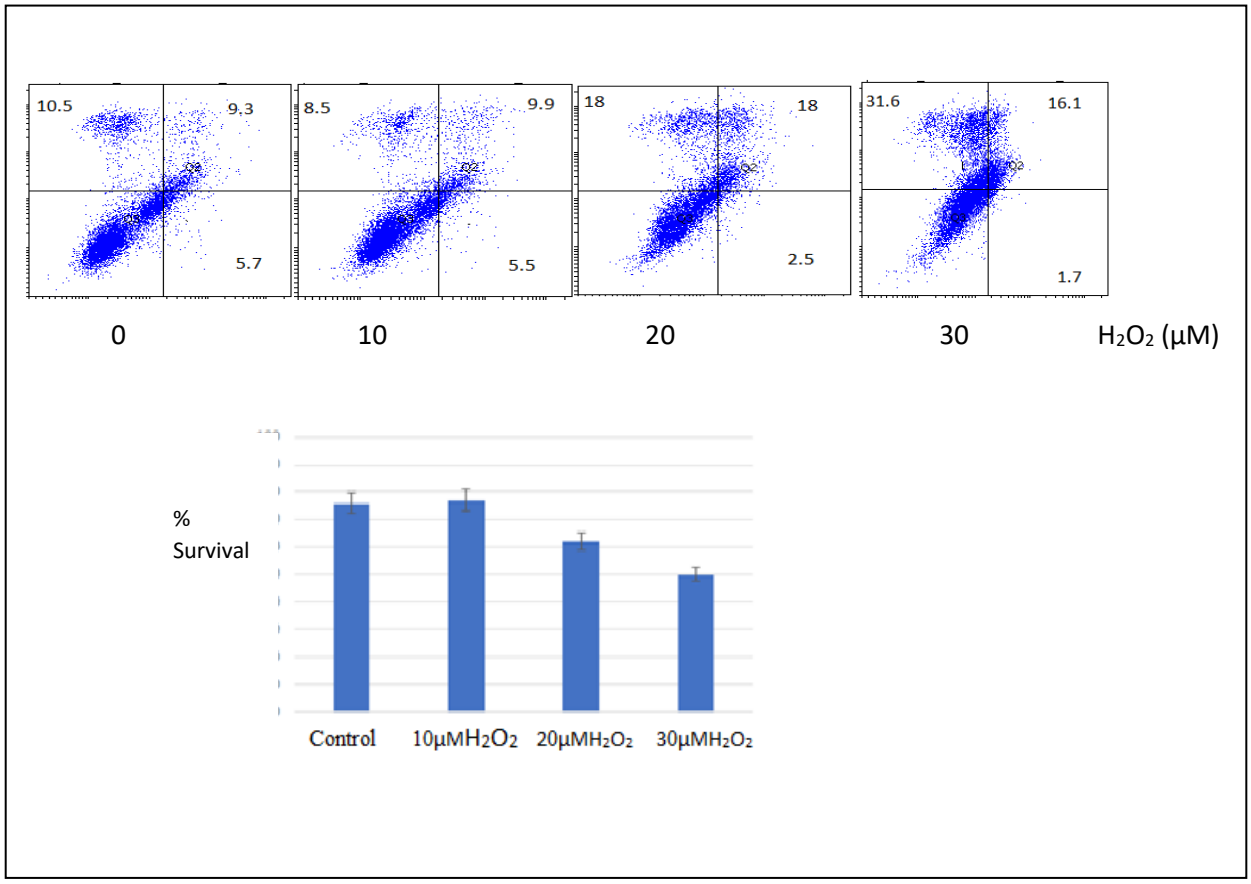


Figure 8

Figure 8: Significant apoptosis by H_2O_2 was not seen as compare to control in imatinib sensitive K562 cells: Imatinib sensitive K562 were treated with indicated doses of H_2O_2. After 24hrs, cells were subjected to Annexin V/ PI binding assay by flow cytometry. Results were representative of three independent experiments.

Hydroxychavicol mediated TRAIL induced apoptosis was regulated by MAPK signaling pathway:

MAPK pathway is a well-established downstream signaling pathway of ROS. It has been known that ROS oxidizes Trx to dissociate from ASK-1 for its activation, resulting in the activation of JNK and p38 pathways. Moreover, ROS have been shown to activate ERK by activating growth factor receptors in the absence of the growth factor receptor ligands**.** So, we have checked whether ERK/JNK signaling pathway regulates Hydroxychavicol mediated TRAIL induced apoptosis. So, imatinib sensitive K562 cell were preincubated with JNK inhibitor, SP600125 then treated with TRAIL followed by Hydroxychavicol. SP revered Hydroxychavicol and TRAIL mediated apoptosis. So, it is concluded that JNK signalling pathway promotes Hydroxychavicol mediated TRAIL induced apoptosis [Fig 9]. Imatinib sensitive K562 cell were preincubated with ERK inhibitor, PD98059then treated with TRAIL followed by Hydroxychavicol. PD promotes Hydroxychavicol mediated TRAIL induced apoptosis. So, It is concluded that ERK signaling pathway inhibited Hydroxychavicol (HCH) mediated TRAIL induced apoptosis [Fig 9].

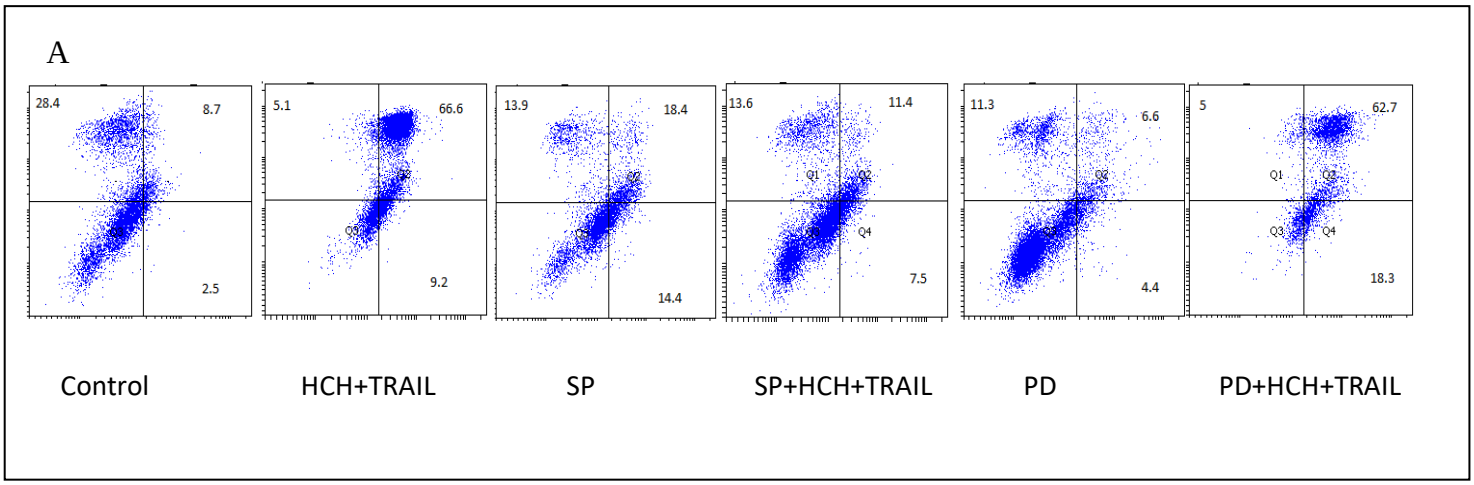


Figure 9

Figure 9: Hydroxychavicol mediated TRAIL induced apoptosis was regulated by MAPK signaling pathway: Imatinib sensitive K562 cell were preincubated with JNK inhibitor, SP600125 or ERK inhibitor PD then treated with TRAIL followed by Hydroxychavicol. Annexin V/PI binding assay was performed in flowcytometer. Results were representative of three independent experiments.

Discussion:

We have found some key findings in K562(S) cells from objective I, II in K562(R) cells.

In imatinib sensitive and Imatinib resistant K562 both cell lines, Hydroxychavicol sensitizes CML cells to TRAIL mediated apoptosis. Because K562(S) and K562(R) cells shows TRAIL resistant. In both cell lines, Hydroxychavicol-mediated TRAIL sensitization is ROS dependant because ROS scavenger NAC significantly reverses Hydroxychavicol and TRAIL mediated apoptosis. In previous objective, we have observed that ROS mediated activated ERK/JNK signaling pathway involed in antiapoptotic protein FLIP, XIAP downregulation in K562(R). We also have known that XIAP and FLIP have been responsible for TRAIL resistance. So, we have targeted antiapoptotic proteins XIAP and FLIP. Hydroxychavicol downregulated XIAP and FLIP in K562 (S) cells that was similar in case of K562(R) cells. In previous study, we knew that Hydroxychavicol induces intracellular ROS in CML cells. In this study, Hydroxychavicol degrades XIAP and FLIP in ROS dependant manner. Because externally ROS treatment degrades XIAP and FLIP in imatinib sensitive and imatinib resistant K562 cells. In imatinib sensitive K562 cells, ROS degrades XIAP and FLIP in lysosomal dependant pathway. In imatinib resistant K562 cells, ROS degrades XIAP and FLIP in proteosomal dependant pathway.