Results
3.1 Effects of C-PC and Celecoxib on growth of K562 cells

Recent studies have shown the potential use of selective COX-2 inhibitors in the treatment and prevention of colon cancer (Fournier and Gordon, 2000; Dempke et al., 2001). Growth inhibition and apoptosis have been observed in several other cancer cell lines by COX-2 inhibitors (Everts et al., 2000; Buttar and Wang, 2000; Fournier and Gordon 2000; Fosslien, 2000; Dempke et al., 2001). To test the effects of C-PC (a natural COX-2 inhibitor) and Celecoxib (a synthetic COX-2 inhibitor), human chronic myeloid leukemia cell line, K562, cultured in RPMI 1640 medium was incubated with different concentrations of C-PC and Celecoxib and the viability was examined by MTT assay. Cells were cultured in 10% FBS containing medium with or without C-PC or Celecoxib (10-100 μM) for 24, 48, 72, 96 h and cell proliferation was evaluated by the MTT assay. Under these experimental conditions a dose dependent decrease in K562 cell proliferation was observed until 48 hours after C-PC (Fig.11) and Celecoxib (Fig. 12) treatment with maximum decrease in cell proliferation being at 50 μM where the percent inhibition was 49% and 53% respectively. Since the maximum inhibition was observed in cells exposed to 50 μM C-PC / Celecoxib for 48 h, further experiments were carried under these conditions.

3.2 Effect of C-PC and Celecoxib on COX-1 and COX-2 protein expression levels

K562 cell line, cultured in RPMI 1640 medium was incubated with C-PC (25 & 50 uM) and Celecoxib (25 & 50 uM) for 48 h and the expression of COX-1 and COX-2 was monitored by Western blot analysis, with the whole cell lysate made from C-PC and Celecoxib treated cells. The results presented in Fig. 13
**Fig. 11:** Effect of C-PC on cell proliferation in K562 cells (MTT assay)

K562 cells were treated with 10, 25, 50, 100 μM of C-PC and the cell survival was determined after 24, 48, 72 and 96 h by MTT assay. The % viable cells were calculated in comparison to untreated cells. The number of cells in the control were taken as 100 %. Each data point represent the mean ± SD of four replicates.
Fig. 12: Effect of Celecoxib on cell proliferation in K562 cells (MTT assay)

K562 cells were treated with increasing concentrations (10, 25, 50, 100 uM) of Celecoxib and the cell survival was determined after 24, 48, 72 and 96 h by MTT assay. The % viable cells were calculated in comparison to untreated cells. The number of cells in the control were taken as 100 %. Values are the means ± SD of four replicates.
Fig. 13: Western blot analysis showing the effects of C-PC and Celecoxib on COX-1 protein expression in K562 cells

Whole cell lysates (50 ug) were separated on 10 % SDS-PAGE, the proteins on the gel were transferred to nitrocellulose membrane and probed with affinity purified goat polyclonal anti-COX-1 antibodies.

Lane 1: COX-1 protein positive control (from ram seminal vesicles)
Lane 2: K562 control cells
Lane 3: K562 cells treated with C-PC 25 μM
Lane 4: K562 cells treated with C-PC 50 μM
Lane 5: K562 cells treated with Celecoxib 25 μM
Lane 6: K562 cells treated with Celecoxib 50 μM
showed no significant changes in COX-1 (lanes 3-6) protein levels in C-PC and Celecoxib treated cells compared to control cells (lane 2). These results indicate that C-PC and Celecoxib have no effect on the protein levels of COX-1 in K562 cells. The results in western blot analysis of COX-2 in cells treated with C-PC/Celecoxib were presented in figure 14. As shown in figure, COX-2 in K562 cells showed a molecular weight of 90 kDa (lanes 1-5) as against the COX-2 positive control (lane 6). Also the results showed increase in COX-2 protein levels in C-PC (lane 2 & 3) and Celecoxib (lane 4 & 5) treated cells compared to that of untreated cells (lane 1).

3.3 Morphological and ultrastructural changes

3.3.1 Phase contrast microscopy

Phase contrast microscopy pictures of K562 cells treated with C-PC and Celecoxib (50 uM) for 48 h were taken to observe the altered morphological features. Cells grown in complete medium in the absence of C-PC or Celecoxib were round in shape with characteristic features of lymphoid cells (Fig. 15A). However, after 48 h of incubation with C-PC (Fig.15B) Celecoxib (Fig. 15C) showed decrease in cell number with cytoplasmic shrinkage and marked convolution of cellular surfaces. Many cells displayed protuberances of the plasmamembrane that would eventually separate into membrane-bound apoptotic bodies.

3.3.2 Fluorescence microscopic studies

A distinguishing feature of apoptosis is the condensation and fragmentation of nuclear chromatin, which can be monitored by fluorescence
**Fig. 14:** Western blot analysis showing the effects of C-PC and Celecoxib on COX-2 protein expression in K562 cells

Whole cell lysates (50 ug) were separated on a 10 % SDS-PAGE. After electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with affinity purified goat polyclonal anti-COX-2 antibodies.

- Lane 1: K562 control cells
- Lane 2: K562 cells treated with C-PC 25 uM
- Lane 3: K562 cells treated with C-PC 50 uM
- Lane 4: K562 cells treated with Celecoxib 25 uM
- Lane 5: K562 cells treated with Celecoxib 50 uM
- Lane 6: COX-2 positive control (rh COX-2)
Fig. 15: Phase contrast photomicrographs showing the effect of C-PC and Celecoxib in K562 cells

K562 cells were treated with C-PC (50 μM) and Celecoxib (50 μM) for 48 h and cells were photographed under phase contrast microscopy (Magnification 400 X). Arrows indicate a typical apoptotic cell with apoptotic bodies.
microscope. K562 cells were exposed to various concentrations of C-PC and Celecoxib (25 & 50 uM) for 48 h, and then assessed for morphological signs of apoptosis by staining with DAPI. Nuclear condensation and apoptotic bodies, a hallmark of apoptosis, were observed in cells treated with C-PC (Fig 16 B & C) and Celecoxib (Fig. 16 D &E). Also the number of apoptotic cells increased with the increasing concentration of C-PC/ Celecoxib. Chromatin of apoptotic cells was segregated and compacted into sharply delineated masses, very close to the nuclear envelope, as indicated by the arrows.

### 3.3.3 Ultrastructural changes - SEM & TEM

In the light of changes observed under phase contrast and fluorescence microscopes, further studies were undertaken for detailed analysis of morphological and ultrastructural changes on SEM and TEM. To determine whether the antiproliferative effects of C-PC were associated with apoptosis, we examined the ultrastructural changes of K562 cells treated with 50 uM C-PC / Celecoxib for 48h. Apoptotic cell death was confirmed by scanning and transmission electron microscopy, which revealed characteristic ultrastructural features of apoptosis. SEM studies of C-PC and Celecoxib treated cells revealed the presence of membrane blebbing, which might be due to a deep cytoskeleton rearrangement, causing progressive changes in cell shape, organelle distribution, cell shrinkage and severing junctions with its neighbours and loss of microvilli (Fig. 17B). TEM gives the qualitative bidimensional image of the sectioned samples. The cell volume of the C-PC/Celecoxib treated cells was reduced, which indicated shrinkage of cytoplasm, while the plasma membrane remained well defined. The cells showed typical nuclear fragmentation and condensed
**Fig. 16:** Fluorescence microscopic studies showing C-PC and Ceiecoxib induced nuclear DNA fragmentation in K562 cells stained with DAPI.

Nuclear morphology of K562 cells was observed under a fluorescence microscope (Olympus BH2RFC) after treatment with C-PC & Ceiecoxib for 48 h. The arrows are pointed to the apoptotic cells. (Magnification 400X).

A: K562 control cells
B: K562 cells treated with C-PC 25 uM
C: K562 cells treated with C-PC 50 uM
D: K562 cells treated with Ceiecoxib 25 uM
E: K562 cells treated with Ceiecoxib 50 uM
Fig. 17: Scanning electron micrographs showing C-PC treated K562 cells

A. Control

B. C-PC 50 μM

Ultra structural morphology in K562 cells treated with C-PC (50 μM) for 48 h. A. Control cells with occasional microvilli B. C-PC treated cells showing clumping and shortening of microvilli, cell shrinkage and membrane blebbing, holes and cytoplasmic extrusions.
chromatin with the formation of apoptotic bodies (Fig. 18 B & C). However in the control cells, the nuclei are intact with high nucleus/cytoplasm ratio (Fig. 18A).

3.3.4 C-PC and Celecoxib induced DNA fragmentation in K562 cells

In addition to morphological evaluation, apoptosis induction by C-PC and Celecoxib was ascertained by using an assay developed to measure DNA fragmentation, a biochemical hallmark of apoptosis. During later stages of apoptosis internucleosomal cleavage of cellular DNA by endonucleases to 180 bp or oligomers of 180 bp fragments could be detected by extraction of nuclear DNA and agarose gel electrophoresis. As illustrated in (Fig. 19), agarose gel electrophoresis of DNA extracted from K562 cells treated with C-PC and Celecoxib at concentrations of 10, 25 and 50 μM for 48 h revealed a progressive increase in the non-random fragmentation into a ladder of 180–200 bp (lanes 2-7). The degree of nuclear DNA fragmentation was directly proportional to the concentration of C-PC/Celecoxib. Such a pattern corresponds to internucleosomal cleavage, reflecting the endonuclease activity characteristic of apoptosis. Control cells did not show any internucleosomal DNA fragmentation (lane 1).

3.4 DNA content assay by fluorescence activated cell sorter (FACS)

The induction of apoptosis in C-PC and Celecoxib treated cells was further verified by flow cytometric analysis of DNA content. Loss of DNA is a typical feature of apoptotic cells. Propidium iodide (PI) staining of DNA, which is taken up into the nucleus of apoptotic and necrotic cells, was used to measure the relative numbers of dead cells (Pullen et al., 1981). Furthermore, since
Ultrastructural changes induced by C-PC & Celecoxib (50 μM) in K562 cells. Cells were harvested, fixed in 2.5 % glutaraldehyde and analyzed by transmission electron microscopy. Chromatin condensation and nuclear fragmentation is clearly seen in C-PC and Celecoxib treated cells. Control cells showed distinguishable diffused interchromatin.
Fig. 19: Agarose gel electrophoresis showing internucleosomal DNA fragmentation induced by C-PC and Celecoxib in K562 cells

Agarose gel electrophoresis of DNA extracted from K562 cells treated with C-PC and Celecoxib for 48 h. After treatment cells were lysed and total cellular DNA was extracted and electrophoresed on a 1% agarose gel containing 0.05 mg/ml ethidium bromide at 5 V/cm. The gels were then photographed under UV illumination.

Lane 1: K562 control cells
Lane 2: K562 cells treated with C-PC 10 uM
Lane 3: K562 cells treated with C-PC 25 uM
Lane 4: K562 cells treated with C-PC 50 uM
Lane 5: K562 cells treated with Celecoxib 10 uM
Lane 6: K562 cells treated with Celecoxib 25 uM
Lane 7: K562 cells treated with Celecoxib 50 uM
Lane 8: 100 bp ladder
apoptosis, but not necrosis, involves degradation of DNA, the staining pattern obtained with PI was used to establish whether cell death was due to apoptosis or necrosis. Two different ways of staining with PI are generally observed: PI in the presence of a permeabilising and fixing agent results in the staining of DNA in living, apoptotic and necrotic cells; and PI in a physiological buffer, stain cells that are dead due to apoptosis or necrosis. In the present study K562 cells treated with C-PC/ Celecoxib (25 & 50 \( \mu \text{M} \)) for 48 h were taken for FACS analysis. Fig. 20 illustrates the DNA content histograms obtained after PI staining of permeabilized cells that had been treated with C-PC and Celecoxib (25 & 50 \( \mu \text{M} \)) for 48 h. In agreement with DNA fragmentation results, a typical sub-diploid apoptotic peaks were observed in K562 cells treated with 25 \( \mu \text{M} \) and 50 \( \mu \text{M} \) C-PC (Fig. 20 B & C) and Celecoxib (Fig 20 D & E) for 48 h. The FACS analysis of control cells, on the other hand, showed prominent G1, followed by S and G2/M phases (Fig. 20 A). Only 2.97 % of these cells showed hypodiploid DNA (sub G0/G1 peaks). This value of 2.97 % hypodiploid DNA in control cells increased to 14.11 % and 20.93 % cells in C-PC (25 & 50 \( \mu \text{M} \)) treated cells (Fig.20 B& C) and 15.86% and 20.95 % in Celecoxib (25 & 50 \( \mu \text{M} \)) treated cells (Fig 20 D &E). These studies thus reveal increase of hypodiploid apoptotic cells in response to C-PC and Celecoxib treatment in a concentration-dependent manner and the decrease of the cells at S and G2 phase of cell cycle. This result suggested a possibility that C-PC induced apoptosis occurs at S and G2 phase of the cell cycle.
**Fig. 20:** Quantification of apoptosis by flow cytometric analysis (FACS)

Celecoxib for 48 h were determined using propidium iodide staining by flow cytometry.

A. Control
B. K562 cells treated with C-PC (25 uM)
C. K-562 cells treated with C-PC (50 uM)
D. K-562 cells treated with Celecoxib (25 uM)
E. K-562 cells treated with Celecoxib (50 uM)
3.5 Signal transduction pathways

3.5.1 C-PC and Celecoxib treatment evokes cytochrome c release

One of the major apoptotic pathways is activated by the release of apoptogenic protein, cytochrome c, from mitochondria into the cytosol. The release of cytochrome c, one of the most important respiratory-chain proteins, from the mitochondria into the cytosol is the hallmark of cells undergoing apoptosis (Liu et al., 1996; Martinou et al., 2000). To specify the molecular basis of apoptosis the release of cytochrome c into the cytosol was measured in K562 cells treated with C-PC and Celecoxib, by Western blot analysis employing mouse monoclonal cytochrome c antibodies. As shown in Fig. 21 untreated cells cytochrome c (lane 1) was not detectable in the cytoplasm, whereas the levels of cytosolic cytochrome c significantly increased after C-PC (lanes 2 & 3) and Celecoxib treatment (lanes 4 & 5). As shown in Fig. 22 the levels of cytochrome c in the cytosol were elevated within 6 hours after treatment with C-PC (lanes 2 & 3) and the levels were further increased by 12 hours (lanes 4 & 5) with later stabilization (lanes 6 & 7).

3.5.2 PARP cleavage in response to C-PC and Celecoxib treatment

PARP, poly (ADP-ribose) polymerase, has been implicated in many cellular processes including apoptosis and DNA repair. PARP is primarily found in the nucleus and is activated by DNA strand breaks. PARP is a 116-kDa protein, which converts nicotinamide adenine dinucleotide (NAD) to nicotinamide and protein-linked ADP-ribose polymers. The DNA repair enzyme, PARP has been recognized as a representative death substrate that is cleaved and
K562 cells were treated with C-PC and Celecoxib for 48 h, cytosolic proteins (50 ug) were separated on a 15 % SDS-PAGE, and after electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with mouse monoclonal cytochrome-c antibodies.

Lane 1: K562 control cells
Lane 2: K562 cells treated with C-PC 25 uM
Lane 3: K562 cells treated with C-PC 50 uM
Lane 4: K562 cells treated with Cele 25 uM
Lane 5: K562 cells treated with Cele 50 uM
**Fig. 22:** Western blot analysis showing release of cytochrome c into the cytosol in K562 cells treated with C-PC for different time periods

K562 cells were treated with CPC (0-24 h), cytosolic proteins (50 ug) were separated on a 15 % SDS-PAGE, and after electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with mouse monoclonal cytochrome-c antibodies.

Lane 1: K562 control cells (0 h)
Lane 2: K562 cells treated with C-PC 25 μM (6 h)
Lane 3: K562 cells treated with C-PC 50 μM (6 h)
Lane 4: K562 cells treated with C-PC 25 μM (12 h)
Lane 5: K562 cells treated with C-PC 50 μM (12 h)
Lane 6: K562 cells treated with C-PC 25 μM (24 h)
Lane 7: K562 cells treated with C-PC 50 μM (24 h)
Paragraph 1:

inactivated by down-stream caspases. In response to growth factor withdrawal or exposure to a variety of chemotherapeutic compounds (Shah et al., 1996), PARP is cleaved to generate 85 and 23 kDa fragments. To determine whether PARP is cleaved in C-PC and Celecoxib induced cell death, we treated K562 cells with 25 & 50 uM C-PC and Celecoxib for 48 h and PARP cleavage was monitored with PARP antibodies, which specifically recognizes the 23 kDa fragment of the cleaved PARP and uncleaved 116 kDa PARP. Fig. 23 illustrates the gradual increase in the proportion of the Mr 23,000 cleavage product and decrease in the proportion of 116 kDa uncleaved PARP with increasing concentrations of C-PC (lanes 2 & 3) and Celecoxib (lanes 4 & 5). In the control cells, however, no fragment of PARP was observed, except the uncleaved 116 kDa protein (lane 1). The extent of PARP Cleavage in C-PC treated cells (lanes 3 & 4), however, was much higher than the same in Celecoxib treated cells (lanes 4 & 5).

3.5.3 Bcl-2/ Bax ratio modulation

Different proteins of the Bcl-2 family have been implicated in triggering or preventing apoptosis. Bax and Bcl-2 are the proteins associated with the mitochondrial membrane and their ratio is crucial for cell survival. In light of the recent reports that attributed COX-2 inhibitor-induced apoptosis to bcl-2 downregulation (Liu et al., 1998; Sheng et al., 1998), studies were undertaken to test whether Bcl-2 expression is affected after C-PC/Celecoxib treatment in K562 cells. Changes in the expression of cellular anti-apoptotic proteins, Bcl-2 and of the pro-apoptotic protein Bax, following C-PC and Celecoxib treatment (25 & 50 uM) for 48 h were examined by Western blotting. As shown in Fig. 24 untreated
Fig. 23: Western blot analysis showing the cleavage of PARP in cell extracts of C-PC and Celecoxib treated cells.

Whole cell lysates from K562 cells treated with C-PC and Celecoxib for 48 h, were fractionated on a 12 % SDS-PAGE. After electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and the proteins were probed with anti-PARP antibodies. This antibody recognizes both uncleaved PARP (116 kDa) and the cleaved fragment (23 kDa).

Lane 1: K562 control cells
Lane 2: K562 cells treated with C-PC 25 uM
Lane 3: K562 cells treated with C-PC 50 uM
Lane 4: K562 cells treated with Celecoxib 25 uM
Lane 5: K562 cells treated with Celecoxib 50 uM
Fig. 24: Immunoblot analysis of Bcl-2 expression in K562 cells treated with C-PC and Celecoxib

K562 cells were treated with C-PC and Celecoxib (25 & 50 uM) for 48 h, and the whole cell lysates (50 ug) were separated on a 15 % SDS-PAGE and the proteins on the gel were transferred to nitrocellulose membrane and probed with affinity purified goat polyclonal anti-Bcl-2 antibodies.

Lane 1: K562 control cells
Lane 2: K562 cells treated with C-PC 25 uM
Lane 3: K562 cells treated with C-PC 50 uM
Lane 4: K562 cells treated with Celecoxib 25 uM
Lane 5: K562 cells treated with Celecoxib 50 uM
cells expressed high levels of Bcl-2 protein (lane 1) where as in cells treated with C-PC (lane 2 & 3) and Celecoxib (lane 4 & 5) the expression of Bcl-2 protein is down regulated in a dose dependent manner. Bax protein levels, however, were not altered on C-PC and Celecoxib treatment (Fig. 25). As a result of decreased Bcl-2 with no change in Bax, the ratio of $\text{Bcl-2}/\text{Bax}$ reduced significantly during C-PC and Celecoxib treatment.

3.5.4 c-Myc expression and modulation

Myc proteins are known to be critical regulators of apoptotic mechanisms. To examine the possible contribution of c-Myc to the C-PC and Celecoxib induced cell death, Western blot analysis was performed in K562 cells with specific antibodies. Under conditions of exponential growth, continuous exposure to C-PC (lanes 2 & 3) and Celecoxib (lanes 4 & 5) produced a marked increase in c-Myc expression in a dose dependent manner (Fig. 26).

3.5.5 Effect of C-PC and Celecoxib on PKC activity

Protein kinase C (PKC) is a $\text{Ca}^{2+}$ and phospholipid-dependent serine/threonine protein kinase with fundamental importance in cellular growth control. Alterations in PKC have been linked to the increased cell proliferation in response to tumor promotion. Therefore measurements were carried out as function of time of exposure in order to assess the effect of C-PC and Celecoxib on PKC activity. After treatment with C-PC and Celecoxib PKC activity decreased in a dose dependent manner (Fig. 27), indicating desensitization of PKC upon treatment. This study suggests that antiproliferative effects of C-PC and Celecoxib might be mediated through PKC pathway.
Fig. 25: Immunoblot analysis of Bax expression in C-PC and Celecoxib treated K562 cells

K562 cells were treated with C-PC and Celecoxib for 48 h, and the whole cell lysates (50 ug) were separated on a 12 % SDS-PAGE and the proteins on the gel were transferred to nitrocellulose membrane and probed with mouse monoclonal anti-Bax.

Lane 1: K562 control cells
Lane 2: K562 cells treated with C-PC 25 uM
Lane 3: K562 cells treated with C-PC 50 uM
Lane 4: K562 cells treated with Celecoxib 25 uM
Lane 5: K562 cells treated with Celecoxib 50 uM
**Fig. 26:** Immunoblot analysis of c-Myc expression in C-PC and Celecoxib treated K562 cells

K562 cells were treated with C-PC and Celecoxib for 48 h, and the whole cell lysates (50 μg) were separated on a 10 % SDS-PAGE and the proteins on the gel were transferred to nitrocellulose membrane and probed with affinity purified rabbit polyclonal anti c-Myc.

Lane 1: K562 control cells
Lane 2: K562 cells treated with C-PC 25 μM
Lane 3: K562 cells treated with C-PC 50 μM
Lane 4: K562 cells treated with Celecoxib 25 μM
Lane 5: K562 cells treated with Celecoxib 50 μM
Fig. 27 A: Effect of C-PC and Celecoxib on PKC activity (at 15 min)

K562 cells were treated with C-PC and Celecoxib (25 uM & 50 uM) for 15 min. PKC activity was determined using MBP (4-14) as described in materials and methods and percent of total PKC activity (bars) from three experiments performed in duplicate were calculated. Results are the mean ± SD of three experiments performed in duplicate.
K562 cells were treated with C-PC and Celecoxib (25 uM & 50 uM) for 30 min. PKC activity was determined using MBP(4-14) as described in materials and methods and percent of total PKC activity (bars) from three experiments performed in duplicate were calculated. Results are the mean ± SD of three experiments performed in duplicate.
3.6. Purification of C-PC from *Spirulina platensis*

Purification of C-PC from *Spirulina platensis* was achieved by a combination of salt precipitation and ion-exchange chromatography. C-PC was precipitated from Spirulina extract by ammonium sulfate (0-30% initially and 30-50% later). The 30-50% ammonium sulfate precipitate was used for further purification on DE-52 column. The dialyzed ammonium sulfate extract was loaded on to DE-52 column. The bound C-PC was eluted with a linear increasing salt concentration (0-0.5 M NaCl, 100 ml) at 1 ml/min (Fig. 28). The sample is considered pure, if the ratio of A620/A280 is above 4.0 (Boussiba & Richmond, 1979). In the ion-exchange column chromatography only fractions having A620/A280 ratio above 4.0 were pooled and scanning spectrum of the pooled samples were taken. Fig. 29 presents the typical visible scanning spectrum of highly purified C-PC, showing absorbance maximum at 620 nm. Details of the purification steps are given in Table 3. As shown in Table 3, the absorbance ratios for the C-PC increased at each step of purification. From one gram of dry Spirulina cells, 60-70 mg pure C-PC was isolated. The yield was sacrificed for the sake of purity.

The homogeneity of the purified protein was checked by electrophoresis under non-denaturing (Native-PAGE) and denaturing (SDS-PAGE) conditions. Native PAGE revealed the presence of a single band at 39 kDa (Fig. 30 A) and SDS-PAGE performed under denaturing conditions showed the presence of two bands corresponding to α (19 kDa) and β subunits (20 kDa) (Fig. 30 B). In order to study the mode of action of C-PC, polyclonal antibodies were raised against
Anion exchange chromatography of C-PC on DEAE-Cellulose (DE-52) column: 30-50 % ammonium sulfate fractionated proteins were loaded on to pre equilibrated DE-52 column and after washing the column bound proteins were eluted with linear salt gradient (0-0.5 M NaCl). The eluted fractions were checked for absorbance at 280 and 620 nm.
Fig. 29: Visible scanning spectrum of C-PC purified from *Spirulina platensis*
Table 3: Purification profile of C-PC from *Spirulina platensis*

<table>
<thead>
<tr>
<th>Step of purification</th>
<th>Absorbance ratios (A_{620}/A_{280})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1.08</td>
</tr>
<tr>
<td>Precipitation with (NH₄)₂SO₄ (0-15%)</td>
<td>0.45</td>
</tr>
<tr>
<td>Precipitation with (NH₄)₂SO₄ (30-50%)</td>
<td>2.98</td>
</tr>
<tr>
<td>DE-52</td>
<td>4.8</td>
</tr>
</tbody>
</table>
Fig. 30: Native PAGE and SDS-PAGE of C-PC
purified C-PC. Western blotting with increasing concentration of C-PC confirmed that the antibody is specific to protein of interest (Fig.31).

3.7 Effects of C-PC -mechanistic studies

3.7.1 Immunolocalization

The subcellular distribution of C-PC was determined in K562 cells using laser scanning confocal microscopy. Polyclonal antibodies of C-PC were used to determine its in situ localization in C-PC treated cells. This was done by using fluorescence labeled anti-rabbit antisera and monitoring the cells exposed to anti-Phycocyanin antiserum on confocal microscopy. These studies showed a strong immunofluorescence signal in the cytosol with no signal in the nuclear compartment (Fig.32) of C-PC treated cells. Native-PAGE analysis confirmed the intactness of the protein in C-PC treated cells (Fig.33). As shown in figure, there is time dependent increase in the uptake of C-PC into the cells, reaching maximum by 24 h exposure. Since the size of the protein is equal to that of the positive control (lane 6), it is assumed that the whole C-PC is entering the cells. These studies thus demonstrate the entry of intact C-PC into the cells and then probably showing effects on K562 cells.

3.7.2 Expression of stress proteins

Hsp60, the eukaryotic homolog of the bacterial chaperonin, is the mitochondrial matrix protein induced by stress and form within the mitochondria. This chaperonin complex that is important for mitochondrial protein folding and function. Apoptosis is closely linked to mitochondrial dysfunction such as reduction in mitochondrial transmembrane potential or release of cytochrome c
Fig. 31: Western blot analysis of C-PC

Various fractions collected during C-PC purification were separated on 15 % SDS-PAGE and after electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with anti-C-PC antibodies raised against pure CPC in rabbit.

Lane 1: Crude
Lane 2: Pellet (8000 rpm)
Lane 3: Supernatant (8000 rpm)
Lane 4: 0-30 % (NH₄)₂SO₄ supernatant
Lane 5: 30-50 % (NH₄)₂SO₄ pellet
Lane 6: DE-52 fraction
Confocal image showing the localization of C-PC in K562 cells fixed in paraformaldehyde using C-PC polyclonal antibodies. After treatment cells were permeabilized, washed with PBS, incubated with rabbit polyclonal anti C-PC antibodies and then with incubated with anti rabbit FITC conjugated secondary antibody. Cells were washed and were observed under Meridian ULPIMA laser scanning confocal microscope with the use of mounting medium for fluorescence.

A: Control K-562 cells

B: K-562 cells treated with CPC (50 uM)
Whole cell lysates of K562 cells treated with 50 uM C-PC for different time intervals were separated on 15 % native-PAGE, transferred to nitrocellulose membrane and probed with rabbit polyclonal anti-C-PC.

Lane 1: K562 control cells
Lane 2: K562 cells treated with CPC 50 uM (0 h)
Lane 3: K562 cells treated with CPC 50 uM (12 h)
Lane 4: K562 cells treated with CPC 50 uM (24 h)
Lane 5: K562 cells treated with CPC 50 uM (48 h)
Lane 6: +ve control C-PC
into the cytosol. Therefore in the present study it is evaluated whether mitochondrial Hsp60 is involved in the apoptosis induced by C-PC. As shown in the Fig 34 a dose-dependent and time dependent increase in the levels of mitochondrial stress protein, Hsp60, was observed in the cytosolic extracts of C-PC (lanes 2-7) treated cells, when compared to the untreated control samples (Lane 1).

3.7.3 C-PC suppressing telomerase activity of K562 cells

The disruption of the telomeric structure and/or function by drugs interacting with the telomerase enzyme has evolved into a promising way of anticancer drug development. To assess the effects of C-PC on telomerase activity of K562 cells, TRAP (Telomerase repeat amplification protocol) assay was employed. After 5-day incubation of cells with different concentrations of C-PC (1-10 uM), telomerase activity of K562 cells was measured. As shown in Fig.35, the telomerase activity of K562 cells decreased in a concentration-dependent manner.
**Fig. 34:** Effect of C-PC on Hsp60 protein expression by immunoblot analysis

K562 cells were treated with C-PC and cytosolic extracts were separated on 10 % SDS-PAGE. After electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with mouse monoclonal anti-hsp60 antibodies.

Lane 1: K562 control cells  
Lane 2: K562 cells treated with C-PC 25 μM (6 h)  
Lane 3: K562 cells treated with C-PC 50 μM (6 h)  
Lane 4: K562 cells treated with C-PC 25 μM (12 h)  
Lane 5: K562 cells treated with C-PC 50 μM (12 h)  
Lane 6: K562 cells treated with C-PC 25 μM (24 h)  
Lane 7: K562 cells treated with C-PC 50 μM (24 h)
Detection of telomerase activity in K562 cells treated with C-PC for 5 days. Telomerase activity was determined using the TRAP-ELISA method as described in materials and methods. Values are the mean ± SD of data from at least three independent experiments.