CHAPTER 3

RESULTS

3.1 EXTRACTION OF PHYLLANTHUS URINARIA AND AEGLE MARMELOS WITH DIFFERENT ORGANIC SOLVENTS

Dried plant powder of *P. urinaria* was extracted with different solvents sequentially from non-polar to polar namely hexane, ethylene glycol, ethyl acetate, methanol and water. The dried plant powder of *A. marmelos* was extracted with different solvents from non-polar to polar namely hexane, dichloromethane, ethyl acetate, methanol and water (Figure 3.1). Each of these extracted compounds was dried in rotatory evaporator, under reduced pressure. 10 mg of the dried powder from each of the solvent extract were reconstituted to 1 ml with the respective solvents. The working preparations of the extracts were expressed as dilution of 1:1, 1:50 and 1:100 from the original stock preparation.

3.1.1 Thin Layer Chromatography (TLC)

Thin Layer Chromatography was carried out to find out the various compounds present in *P. urinaria* and *A. marmelos*. Four types of solvent system were used a) 25% ethyl acetate in hexane b) 50% ethyl acetate in hexane c) 100% ethyl acetate and d) 5% methanol in ethyl acetate. In case of *P. urinaria* 25% ethyl acetate in hexane was found to be the best solvent system because in that solvent system maximum number of compounds separated from
Figure 3.1 Extraction of *Phyllanthus urinaria* and *Aegle marmelos* with different organic solvents

Sequential extraction of both the plants with different organic solvents starting from non-polar to polar, after that these extracts were dried under reduced pressure in a rota evaporator and reconstituted in the respective solvent and used for bioassay.
the origin of the TLC plate (Figure 3.2A). For *A. marmelos* 50% ethyl acetate in hexane was found to be an ideal solvent system as shown in Figure 3.2B.

### 3.1.2 \[^{3}\text{H} \] Thymidine Incorporation on HEp-2 cells in the presence of different solvent extracts of *P. urinaria* and *A. marmelos*

Incorporation of radioactive thymidine is an index of the extent of a proliferation exhibited by a proliferating cell. Growing and dividing cells take up thymidine for incorporation into DNA during active DNA synthesis. Reduction in the incorporation of thymidine in the presence of plant extracts indicates that the plant is cytotoxic or anti-proliferative.

The different solvent extracts of *P. urinaria* were added to HEp-2 cells at a concentration of 50 µg / ml. Thymidine incorporation studies showed marked inhibition of proliferation in ethyl acetate extract, whereas the untreated control cells and the solvent controls of all the respective solvents showed no inhibition of proliferation (Figure 3.3A). The extracts of the other solvents namely hexane, ethylene glycol, acetone, methanol and water extracts were also tested, and it was found that since the inhibition of proliferation was maximum with *P. urinaria* ethyl acetate fractions, this was taken up for further purification.

The different solvent extracts of *A. marmelos* were added to HEp-2 cells at a concentration of 50 µg / ml. The antiproliferative effects of different solvent extract were analysed by \[^{3}\text{H} \]-Thymidine incorporation experiments at day 1, day 2 and day 3 time points, whereas the untreated control cells and solvent controls of all the respective solvents also showed no inhibition of proliferation. Maximum inhibition of proliferation was found in ethyl acetate extract (Figure 3.3B), therefore studied further ethyl acetate extract.
In order to find out various compounds present in *P. urinaria* and *A. marmelos* four types of solvent systems were used and the separated chromatogram was viewed under UV and Iodine chamber.

3.2 A) Thin layer chromatography profile of *P. urinaria* with each of the solvent extracts. 1 Hexane extract, 2 Ethylene glycol extract, 3 Ethyl acetate extract, 4 Methanol extract, 5 Water extract. Products are resolved in 50% ethyl acetate and 50% methanol and visualised in UV and Iodine chamber.

3.2 B) Thin layer chromatography profile of *A. marmelos* with each of the solvent extracts. 1 Hexane extract, 2 DCM extract, 3 Ethyl acetate extract, 4 Methanol extract, 5 Water extract. Products are resolved in 50% ethyl acetate and 50% methanol and visualised in UV and Iodine chamber.
Figure 3.3  $[^3]$H Thymidine Incorporation on HEp-2 cells in the presence of different solvent extracts of 	extit{P.urinaria} and 	extit{A.marmelos}

3.3 A) Anti-proliferative effect of 	extit{P.urinaria} with different solvent extracts on HEp-2 cells monitored by $[^3]$H-thymidine incorporation for 24 hours, 48 hours and 72 hours. Control is untreated cells. Ethyl acetate extract showed maximum inhibition of proliferation.

3.3 B) Anti-proliferative effect of 	extit{A.marmelos} with different solvent extracts on HEp-2 cells monitored by $[^3]$H-thymidine incorporation for 24 hours, 48 hours and 72 hours. Control is untreated cells. Ethyl acetate extract showed maximum inhibition of proliferation.

Hex : Hexane; Ethegly : Ethylene glycol, EtoAc : Ethyl acetate;
DCM : Dichloromethane; Met.: Methanol.
3.1.3 Dose response study of *P. urinaria* and *A. marmelos* ethyl acetate extract

To facilitate the difference between toxicity and optimum dose, which is required to elicit 50% of inhibition, the dose response studies were carried out. The doses used were 100 μg, 50 μg, 10 μg and 1 μg of the plant extracts in the respective solvents. In order to determine the toxicity of the solvents alone, a solvent control was included in the studies. Representative histograms of counts per minute/mg protein (cpm/mg protein) are shown in Figure 3.4 A for *P. urinaria* crude ethyl acetate extract. For a dose of 10 μg of the extract an optimum inhibition of 55% was observed as compared to the untreated control cells. Dose response study of *A. marmelos* ethyl acetate extract at different concentrations of 100 μg, 50 μg, 10 μg and 1μg were added to the cells. The optimum dose was found to be 10 μg/ml (Figure 3.4 B).

3.1.4 Purification of active fraction by column chromatography

In order to elute the active fraction from *P. urinaria* various concentrations of hydrocarbons like hexane - ethyl acetate (3:1), hexane -ethyl acetate (1:1), ethylene glycol (100%) and methanol - ethyl acetate (1:19) were tested on TLC. The best resolution was obtained with hexane - ethyl acetate (3:1). In normal phase silica gel column the above mentioned solvent system was used to elute the active compound. The fraction was concentrated under reduced pressure and the homogeneity of the compounds was determined by TLC by using different solvent system. In the C-18 HPLC column 6 peaks were obtained and purity was checked by TLC (Figure 3.5A) and compound 3 was subjected to structural characterisation studies and the molecular weight and structure of the lead compound was determined using MASS Spectroscopy and NMR studies.
Figure 3.4  Dose response study of *P. urinaria* and *A. marmelos* ethyl acetate extract on HEp-2 cells

3.4 A) Dose response study of *P. urinaria* ethyl acetate extract at different concentrations of 100 µg, 50 µg, 10 µg and 1 µg added to the cells. The optimum dose was found to be 10µg/ml. Control is untreated cells and S.cont is solvent control.

3.4 B) Dose response study of *A. marmelos* ethyl acetate extract at different concentrations of 100 µg, 50 µg, 10 µg and 1 µg added to the cells. The optimum dose was found to be 10µg/ml. Control is untreated cells and S.cont is solvent control.
In order to find out the active fraction in *A. marmelos* different concentrations of hydrocarbons were tested on TLC, for the best resolution. The best resolution was obtained with Hexane-ethyl acetate (5:5). The above-mentioned solvent system was used to elute the active compound. The fraction was concentrated under reduced pressure and the homogeneity of the compounds was determined by TLC. According to TLC profile they were pooled and 6 fractions were obtained and purity was checked by TLC, Fraction 5 showed maximum purity and also demonstrated single spot (Figure 3.5B). This fraction was subjected to structural characterisation studies and the molecular weight and structure of the lead compound was determined using MASS Spectroscopy and NMR studies.

3.1.5 \[^3\text{H}\] Thymidine Incorporation on HEp-2 cells in the presence of different column fractionated ethyl acetate extracts of *P. urinaria* and *A. marmelos*

The effect of different column fractionated extracts of *P. urinaria* and *A. marmelos* were checked on HEp-2 cells at a concentration of 10 µg/ml. The inhibition of proliferation was represented graphically in terms of cpm/mg protein. As shown in Figure 3.6 *P. urinaria* fraction 3 showed maximum inhibition of thymidine uptake by day 1 and day 2. In case of *A. marmelos* fraction 5, showed maximum inhibition of proliferation by day 1, day 2 (Figure 3.6B).
Figure 3.5 Thin Layer Chromatography (TLC)

In order to find out the various compounds present in the column fractionated *P. urinaria* and *A. marmelos* four types of solvent system were used and the separated chromatogram was viewed under UV and Iodine chamber.

3.5 A) Thin layer chromatography profile of *P. urinaria* ethyl acetate extract column fractionated fractions. C, Crude ethyl acetate extract and Lane 1 to 6 showed different fractions obtained from HPLC. Products are resolved in 50% ethyl acetate and 50% methanol and visualised in UV and Iodine chamber.

3.5 B) Thin layer chromatography profile of *A. marmelos* ethyl acetate extract column fractionated fractions. C, Crude ethyl acetate extract and Lane 1 to 6 showed different fractions obtained from column chromatography. Products are resolved in 50% ethyl acetate and 50% methanol and visualised in UV and Iodine chamber.
Figure 3.6 [³H] Thymidine Incorporation on HEp-2 cells in the presence of different column fractionated ethyl acetate extracts of *P. urinaria* and *A. marmelos*.

3.6 A) Anti-proliferative effect of column fractionated ethyl acetate extracts of *P. urinaria* on HEp-2 cells monitored by [³H]-thymidine incorporation for day1 and day2. Different fractions of the same ethyl acetate fractions were tested. Control is untreated cells. SC is solvent control. Fraction 3 showed maximum inhibition of proliferation.

3.6 B) Anti-proliferative effect of column fractionated ethyl acetate extracts of *A. marmelos* on HEp-2 cells monitored by [³H]-thymidine incorporation for 12 hours, 24 hours and 36 hours. Different fractions of the same ethyl acetate fractions were tested. Control is untreated cells. S.cont is solvent control. Fraction 5 showed maximum inhibition of proliferation.
3.1.6 Dose dependent analysis of *P. urinaria* and *A. marmelos* Pure compounds in HEp2 cells

The pure compounds thus isolated were studied for dose response on HEp-2 cells. Cells were treated with different doses of the pure compounds over a period of day1, day2 and day3 and proliferative potential was measured using $[^3]H$-Thymidine incorporation. The optimum inhibition of thymidine uptake was observed only with 2 μg/ml of the *P. urinaria* pure compound (Figure 3.7 A). In case of *A. marmelos* pure compound optimum activity was at a concentration of 5 μg/ml (Figure 3.7 B). These optimum concentrations of both pure compounds were used in all other bioassays.

3.1.7 Structural elucidation of active molecule by NMR spectroscopy and Mass spectrometry for purified compounds of *P. urinaria* and *A. marmelos*

The structure of the active compound of both *P. urinaria* and *A. marmelos* was determined by $^1$H, $^{13}$C and $^1$H - $^{13}$C COSY NMR spectroscopy and electrospray ionisation mass spectrometry. The NMR experiments were done in CDCl$_3$ solution and MASS Spectrometry using acetonitrile-water spray condition.

As shown in Figure 3.7 A for *P. urinaria* the $^1$H and $^{13}$C NMR data showed characteristic peaks for lignan skeletons and confirmed presence of 5 OMe groups. The mass spectra confirmed elemental composition C$_{24}$H$_{32}$O$_8$ (448). The structure of the active compound was elucidated as 7'-hydroxy-3',4',5,9,9'-pentamethoxy-3,4-methylene dioxy lignan (Figure 3.8 A).
Figure 3.7 Dose response study of *P. urinaria* and *A. marmelos* pure compound on HEp2-cells

3.7 A) Dose response study of *P. urinaria* pure compound at different concentrations of 100 µg, 50 µg, 10 µg, 2 µg and 1 µg added to the cells. The optimum dose was found to be 2 µg/ml. Control is untreated cells and S.cont is solvent control.

3.7 B) Dose response study of *A. marmelos* pure compound at different concentrations of 100 µg, 50 µg, 10 µg, 5 µg and 1 µg added to the cells. The optimum dose was found to be 2 µg/ml. Control is untreated cells and S.cont is solvent control.
Figure 3.8 Structural elucidation of active molecule by NMR spectroscopy and Mass spectrometry for purified compounds of both *P. urinaria* and *A. marmelos*

3.8 A) The structure of the active compound from *P. urinaria* was determined as 7'-hydroxy-3',4',5,9,9'-pentamethoxy-3,4-methylene dioxy lignan

3.8 B) The structure of the active compound from *A. marmelos* was determined as 9-hydroxy-decahydro-furo[3,2-g]chromen-7-one
In case of A. marmelos the $^1$H and $^{13}$C NMR data showed characteristic peaks for furo-chromen-7-one skeletons. The mass spectra confirmed elemental composition C$_{11}$H$_6$O$_4$ (202). The structure of the active compound was elucidated as 9-hydroxy-decahydro-furo [3,2-g]chromen-7-one (Figure 3.8B).

3.1.8 **Assessment of anti-proliferative activity of both P. urinaria and A. marmelos crude ethyl acetate extract and pure extract on other cancer cell lines like HEp2, MCF-7, EL-1 monocytes and HeLa cells**

Assessment of anti-proliferative activity was measured by [3H]-Thymidine incorporation assay in HEp-2 cells and three different cell lines. The crude ethyl acetate extracts of both the plants were added to the cells at a concentration of 10 μg/ml and pure compounds were added at a concentration of 2 μg/ml (P. urinaria) and 5μg/ml (A. marmelos) and the effect was monitored for 12 hours and 24 hours. The respective histograms of P. urinaria (Figure 3.9A) and A. marmelos (Figure 3.9B) inhibited upto 45% to 65% of cell proliferation with different cell lines tested.

3.1.9 **Assessment of cytotoxicity of both crude and pure extracts on HEp-2, MCF-7, EL-1 monocytes and HeLa cells, by LDH (Lactate Dehydrogenase) release MTT Assay**

Apart from anti-proliferative activity the cytotoxicity of the both crude ethyl acetate extract and pure compound in different tumour derived cell lines was also determined. The Cytotoxicity was measured by total amount of LDH released from the cells at the desired time points.
Figure 3.9  Assessment of cytotoxicity of both crude and pure extracts of *P. urinaria* and *A. marmelos* on HEp-2, MCF-7, EL-1 monocytes and HeLa cells, by [³H] Thymidine Incorporation assay

3.9 A) Anti-proliferative effect of *P. urinaria* crude ethyl acetate fraction (10μg/ml) and pure compound (2μg/ml) on HEp-2, MCF-7, EL-1 monocytes and HeLa cells showing 45% to 65% of death in different tumor derived cells. Control is untreated cells. S.cont is solvent control.

3.9 B) Anti-proliferative effect of *A. marmelos* crude ethyl acetate fraction (10 μg/ml) and pure compound (5 μg/ml) on HEp-2, MCF-7, EL-1 monocytes and HeLa cells showing 45% to 50% of death in different tumor derived cells. Control is untreated cells. S.cont is solvent control.
The crude ethyl acetate extracts of both the plants were added to the cells at a concentration of 10 µg/ml and pure compounds were added at a concentration of 2 µg/ml (P. urinaria) and 5 µg/ml (A. marmelos) and the effect was seen for 12 hour and 24 hours and the respective histograms of P. urinaria (Figure 3.10A) and A. marmelos (Figure 3.10B). These results suggest that in the doses tested, with the plants extract, both crude and pure showed 45% to 65% cytotoxicity on the different tumour cells.

3.1.10 Morphological assessment and cell viability by trypan blue assay on HEp-2 cells after treatment with the plant-extract

Morphological observation of HEp-2 cells on treatment with the crude ethyl acetate extract and pure compounds of both P. urinaria and A. marmelos showed cell shrinking and display bubbles in the cytoplasm and ruffling of the plasma membrane. Towards the third day, tiny vesicles were observed around the dying cells, which were later identified as apoptotic bodies. The solvent controls did not show any changes in morphology.

Cell viability at the end of 3 days was checked by trypan blue exclusion assay, the exclusion of the dye by the viable cells and the dye uptake by the dead cells. It was found that the cells treated with crude ethyl acetate extracts and pure compounds of both the plants showed dye uptake indicating a 50% to 60% dead population (Figure. 3.11).

3.2 CYTOSKELETAL REARRANGEMENTS INDUCED BY BOTH P. URINARIA AND A. MARMELOS CRUDE EXTRACT AND PURE COMPOUND ON HEp-2-CELLS

A great variety of natural products are used as anticancer agents, their anti-mitotic activity being due to their interaction with microtubular protein.
Figure 3.10 Assessment of cytotoxicity of both crude and pure extracts of *P. urinaria* and *A. marmelos* on HEp-2, MCF-7, EL-1 monocytes and HeLa cells, by LDH (Lactate Dehydrogenase) release MTT Assay

3.10 A) Anti-proliferative effect of *P. urinaria* crude ethyl acetate fraction (10 µg/ml) and pure compound (2 µg/ml) on HEp-2, MCF-7, EL-1 monocytes and HeLa cells showing 45% to 65% of death in different tumor derived cells. Control is untreated cells. S.cont is solvent control.

3.10 B) Anti-proliferative effect of *A. marmelos* crude ethyl acetate fraction (10 µg/ml) and pure compound (5 µg/ml) on HEp-2, MCF-7, EL-1 monocytes and HeLa cells showing 45% to 50% of death in different tumor derived cells. Control is untreated cells. S.cont is solvent control.
The action of *P. urinaria* and *A. marmelos* both crude ethyl acetate extracts and pure compounds on microtubule dynamics in HEp-2 cells was examined. The morphological changes observed under the phase contrast microscope such as cell rounding, blebbing and loss of cell anchorage prompted the study of integrity of cytoskeletal elements such as tubulin.

### 3.2.1 Disruption of tubulin network in HEp-2 cells

Microtubules are polymers that form a network framing the cytoskeleton in the cells. The changes in the tubulin network of cytoskeleton in HEp-2 cells following the addition of the crude ethyl acetate extracts and pure compounds of both the plant were analysed. HEp-2 cells were treated with the plant extracts for 12 hours and changes in the tubulin pattern were observed. After the addition of plant extracts HEp-2 cells became vacuolated with the tubulin network forming a peripheral accumulation of tubulin inside the cells. The spatial organisation of the tubulin cytoskeletal network showed disorganised microtubule pattern with subsequent peripheral accumulation and condensation at the centre of the cells forming microtubule centers (Figure 3.12), which were viewed staining with yellow colour.

### 3.3 ASSESSMENT OF APOPTOSIS IN HEp-2 CELLS

Genetic instability contributes to the origin of cancer as well as to the ability of cancer cells to become resistant to various therapies. Because of this, cytotoxic rather than cytostatic therapies might be most effective against this disease. Many oncogenes and tumour suppressors mediate their effects by interfering with or inducing apoptotic signalling. Thus, apoptotic pathways
HEp-2 cells treated with *P. urinaria* and *A. marmelos* crude ethyl acetate extracts and pure compounds for 12 hours enhanced the tubulin polymerisation and caused the accumulation of tubulin in the periphery of the cells, which are stained in yellow colour while untreated control cells did not show any tubulin polymerisation.
might be significantly altered in cancer cells relative to untransformed cells, and these differences might present a therapeutic window that can be exploited for development of cancer drugs. Studies on dying cells reveal two distinct types of cell death, distinct in their nature and biological significance - apoptosis and necrosis. Necrosis or accidental cell death, is the non-physiological or passive type of cell death and it is usually caused by extreme trauma or injury to the cell. Apoptotic cell death has been shown to occur in diverse cell types and is triggered by a number of extracellular and intracellular signals. Apoptosis is associated with typical morphological changes in the cells, such as plasma membrane blebbing, chromatin condensation and fragmentation of the cell into apoptotic bodies that are rapidly phagocytosed by neighbouring cells.

3.3.1 Propidium Iodide staining of HEp-2 cells to study nuclear morphology

Apoptotic nuclei can be visualised under a fluorescent microscope using DNA-binding dyes such as Propidium Iodide, as fragmented packets of intense fluorescence, and can be distinguished from viable nuclei, which show a uniform, diffused staining.

Monolayer of HEp-2 cells was treated with both crude ethyl acetate extract and pure compounds at respective concentration and the extent of apoptosis was assessed by propidium iodide staining. HEp-2 cells were treated with both the P. urinaria and A. marmelos crude ethyl acetate extract and pure compound for 72 hours. The treated monolayer of HEp-2 cells contained more apoptotic cells than untreated monolayer. Figure 3.13 showed characteristic nuclear fragmentation of nuclei in treated HEp-2 cells whereas the untreated control cells did not show any nuclear fragmentation.
HEp-2 cells were treated with crude extract and pure compound of *P. urinaria* and *A. marmelos* for three days. Both the crude extracts and pure compounds exhibited 50 to 60% of characteristic nuclear fragmentation when compared to control. Control Untreated cells did not show any significant nuclear fragmentation.

Figure 3.13 Nuclear localisation of HEp-2 cells on treatment with crude extracts and pure compounds by propidium iodide staining
The apoptotic cells displayed the characteristic features of reduced size, intense fluorescence of condensed nuclear chromatin and formation of membrane blebs. These data further quantitatively supported by FACS analysis.

3.3.2 AnnexinV staining of HEp-2 cells

The externalisation of Phosphatidyl Serine in cells which undergo apoptosis is one of the characteristic features of apoptotic cells. Annexin V staining bind specifically to phosphotidyl serine and can be used to monitor apoptosis.

HEp-2 cells on exposure to both *P. urinaria* and *A. marmelos* crude ethyl acetate extract and pure compound for three days showed the presence of the externalisation of Phosphotidyl Serine which were viewed green staining in colour (Figure 3.14) when compared untreated control cells, which did not show any staining.

3.3.3 Fluorescence Activated Cell Sorting (FACS) Analysis

Further confirmation of apoptosis came from the analysis of DNA content of HEp-2 cells by flow cytometry, which revealed reduced DNA, sub G1 peaks in HEp-2 cells exposed to the crude ethyl acetate extracts of both the plants. The crude ethyl acetate extracts of both plants were added to the cells at a concentration of 10 µg/ml and pure compounds were added at a concentration of 2 µg/ml (*P. urinaria*) and 5 µg/ml at the end 72 hours, DNA content in
**Figure 3.14** AnnexinV staining of HEp-2 cells on treatment with crude extracts and pure compounds

HEp-2 cells were treated with crude extract and pure compound of *P. urinaria* and *A. marmelos* for three days. Both the crude extracts and pure compounds showed the presence of externalisation of Phosphatidyl Serine when compared to control. Control-Untreated cells did not show any staining.
subG1 peaks were analysed. A gradual increase was observed in the percentage of total cells showing apoptotic DNA content on prolonged exposure; HEp-2 cells treated for 72 hours with *P. urinaria* crude ethyl acetate extract showed 64.7% of apoptotic cells and pure compound showed 70.3% of cells turning apoptotic. In case of HEp-2 cells treated with *A. marmelos* crude ethyl acetate extract and pure compound showed respectively 45.9% and 85.1% of the total cell population became apoptotic. (Figure 3.15) A leftward shift of the cycling population is obvious in the HEp-2 cells indicating that as the exposure to these plants extract increases the number of cells exiting the cell cycle and undergoing apoptosis.

### 3.4 ASSESSMENT OF TELOMERASE ACTIVITY BY TELOMERIC REPEAT AMPLIFICATION PROTOCOL ASSAY (TRAP)

A major goal in developing new molecular therapeutics is to identify targets that are differentially expressed between normal and cancer cells. Most immortal cell lines derived from human cancers or transformed *in vitro* maintain telomeres by endogenous expression of telomerase. Telomerase is a ribonucleoprotein whose activity has been detected in germ line cells, immortal cells, and most cancer cells. Except in stem cells, which have a low level of telomerase activity, its activity is absent from normal somatic tissues. Understanding the regulation of telomerase activity is critical for the development of potential tools for the diagnosis and treatment of cancer. In addition, telomerase inhibitors could also be used as chemopreventive agents in high cancer-susceptibility individuals or in early stage cancer to prevent overgrowth of metastatic cells. The increased level and frequency of telomerase
Figure 3.15 Flow cytometry analysis of HEp-2 cells on treatment with the crude extracts and pure compounds at 72 hours

HEp-2 cells were treated with crude extract and pure compound of *P. urinaria* and *A. marmelos* for three days. Percentage of apoptosis was analysed against PI fluorescence showing distinct subG₀ peaks and which is expressed as A₀. The extent of apoptosis was high in both crude extracts and pure compounds when compared to control. Control-Untrated cells did not show any peak in subG₀.
activity in cancers when compared with normal cells makes telomerase an
extremely attractive target for anti-cancer strategies, particularly as it should be
possible to use telomerase-based therapeutics over a broad range of
cancers. Telomerase activity was monitored in HEp-2 and EL-1
monocyte cell lines by TRAP assay, which is very sensitive assay.

The non-radioactive detection of telomerase activity was followed
after the cells were exposed to both *P. urinaria* and *A. marmelos* crude ethyl
acetate extract and pure compound for 12 hours. Inhibition of telomerase
activity in both HEp-2 and EL-1 monocyte cells which was directly assessed by
number TRAP products formed, shows the capability of the isolated telomerase
which is present in total protein. In case of pure compounds, telomerase
inhibition was higher than crude ethyl acetate extract in both the plants.
Significant decrease in the activity of telomerase was observed in case of cells
treated with *P. urinaria* (Figure 3.16A), and *A. marmelos* (Figure 3.16 B). 36 bp
TS primer served as internal control for the TRAP reaction. Lane 7 and Lane 8
are served as negative controls. Lane 7 is pre-incubated sample with the RNAse
and Lane 8 pre-heated sample at 95°C.

3.5 APOPTOTIC SIGNALLING IN HEp-2 CELLS

3.5.1 Analysis of Proto-oncogene by Northern blot

In order to ascertain the specific mechanistic pathways play a role in
apoptotic signalling was analysed in detail.

HEp-2 cells showed elevated levels of c-myc mRNA expression on
treatment with both crude ethyl acetate extract and pure compound of
*P. urinaria* and *A. marmelos* at an early time points of 12 hours (Figure 3.17).
HEp-2 untreated control cells and solvent control did not show any basal levels
of c-myc expression at 12 hours.
Figure 3.16 Assessment of telomerase activity by Telomeric Repeat Amplification Protocol (TRAP) assay

3.16 A
Lane 1. HEp-2 Untreated control, Lane 2. Treated with crude ethyl acetate extract of *P. urinaria*, Lane 3. Treated with pure compound of *P. urinaria*, Lane 4. Control untreated EL-1 monocytes, Lane 5. Treated with crude ethyl acetate extract of *P. urinaria*, Lane 6. Treated with pure compound of *P. urinaria*, Lane 7. RNAse treated negative control, Lane 8. Pre incubated at 95°C and Lane 9. Marker.

3.16 B
Lane 1. HEp-2 Untreated control, Lane 2. Treated with crude ethyl acetate extract of *A. marmelos*, Lane 3. Treated with pure compound of *A. marmelos*, Lane 4. Control untreated EL-1 monocytes, Lane 5. Treated with crude ethyl acetate extract of *A. marmelos*, Lane 6. Treated with pure compound of *A. marmelos*, Lane 7. RNAse treated negative control, Lane 8. Pre incubated at 95°C and Lane 9. Marker.

Both *P. urinaria* and *A. marmelos* crude ethyl acetate extracts and pure compounds showed significant decrease in the activity of telomerase, which was assessed by the TRAP products formed.
Figure 3.17 Northern blot analysis of c-myc and c-H-ras in HEp-2 cells at 12 hours on treatment with plant extracts

HEp-2 Untreated control (Lane 1), Treated with crude ethyl acetate extract of P.urinaria (Lane 2), Treated with pure compound of P.urinaria (Lane 3), Treated with crude ethyl acetate extract of A.marmelos (Lane 4), Treated with pure compound of A.marmelos (Lane 5), Solvent control (Lane 6).

c-myc, a proto-oncogene gets elevated in HEp-2 cells on treatment with both the crude plant extracts and pure compounds while untreated control cells and solvent control did not show any activation.

c-H-ras, an oncoprotein showed significant expression in untreated control HEp-2 cells whereas in cells treated with P.urinaria crude ethyl acetate extract and pure compound for 12 hours, down regulation of c-H-ras was observed. But A.marmelos did not show any significant activity.
Suppression of \(c-H\)-ras mRNA expression was seen in HEp-2 cells treated with both crude ethyl acetate extract and pure compound of \(P.\ urinaria\) for 12 hours when compared to that of control. HEp-2 untreated cells showed prominent levels of \(c-H\)-ras mRNA expression at 12 hours. In case of cells treated with \(A.\ marmelos\), c-H-ras mRNA expression was not significant. When compared to their respective untreated control HEp-2 cells (Figure 3.17).

3.5.2 Analysis of proinflammatory cytokine TNF \(\alpha\) mRNA level in HEp-2 cells

Tumour necrosis factor (TNF) is a cytokine that is mainly produced by activated macrophages; also lymphoid cells, natural killer cells, neutrophils, keratinocytes, and fibroblasts can produce this cytokine in response to various challenges. The first interest in TNF arose from its potential antitumour properties because of a specific cytotoxic effect of TNF-\(\alpha\) on several transformed cell lines. Also \textit{in vivo}, TNF-\(\alpha\) showed considerable antitumour activity in a number of murine tumour models. The antitumour effect of TNF-\(\alpha\) is mediated by direct cytotoxic effects on the tumour cells as well as by several effects on the host cells. Because TNF receptors (TNF-R) are ubiquitously expressed, it is not surprising that almost all cell types respond to TNF-\(\alpha\).

TNF-\(\alpha\) mRNA expression was analysed in HEp-2 cells after 12 hours of treatment of both the crude extract and pure compounds of the plants. As shown in Figure 3.18 A RT-PCR analysis of \(P.\ urinaria\) with crude ethyl acetate extract and pure compound did not show any significant increase in the expression level, whereas in \(A.\ marmelos\) the crude ethyl acetate extract and pure compound were capable of elevating the levels of TNF-\(\alpha\) expressing a product of 501 bp. The untreated control cells did not show any expression and HPRT, a housekeeping gene was used as an internal control (Figure 3.18 B).
Figure 3.18 RT-PCR analysis of TNF-α expression in HEp-2 cells at 12 hours on treatment with crude extract and pure compound of *P. urinaria* and *A. marmelos*

Molecular weight marker (Lane 1), HEp-2 Untreated control (Lane 2), Treated with crude ethyl acetate extract of *P. urinaria* (Lane 3), Treated with pure compound of *P. urinaria* (Lane 4), Treated with crude ethyl acetate extract of *A. marmelos* (Lane 5), Treated with pure compound of *A. marmelos* (Lane 6), negative control (Lane 7).

3.18 A Analysis of TNF-α mRNA (501bp) expression in HEp-2 cells after 12 hours of treatment showed elevated levels of expression when compared to untreated control cells only in case of *A. marmelos* while *P. urinaria* did not show any activation.

3.18 B Equal amounts of RNA were analysed for HPRT (410bp) expression as an internal control.
3.5.3 Analysis of cytokine IFN-γ mRNA level in HEp-2 cells

The interferon's (IFN's) are a group of cytokines, which in addition to their antiviral activity are capable of modulating a variety of cellular responses. One such prominent effect of IFN's is their potent anti-mitogenic action, which can be observed both on malignant and non-malignant cells of many different origins. IFN’s are also used in the clinic, mainly in malignant and viral diseases, and their cell growth -inhibitory effect has been suggested to be of major importance in their anti-tumour and anti-viral action. The aim of the present study is to provide insight into the molecular mechanisms by which IFN’s modulate cell cycle progression in various cell types.

The effect of both *P. urinaria* and *A. marmelos* on IFN-γ mRNA expression in HEp-2 cells were analysed after 12 hours of treatment of both the crude extract and pure compounds of the plants. As shown in Figure 3.19 A RT-PCR analysis of both *P. urinaria* and *A. marmelos* with crude ethyl acetate extract and pure compound shown major augmentation in the expression level of IFN-γ expressing a product of 510 bp. The untreated control cells did not show any expression of IFN-γ and HPRT, a housekeeping gene was used as an internal control (Figure 3.19 B).

3.5.4 Analysis of cytokine iNOS mRNA level in HEp-2 cells

Inducible NOS (iNOS) is a Ca2+-independent isoform that is expressed, upon induction by cytokines, in several cell types including macrophages, endothelial cells, and hepatocytes. Expression of the inducible nitric oxide synthase (iNOS) gene results in profound physiologic consequences. Therefore, the regulation of iNOS expression is
RT-PCR analysis of IFN-γ expression in HEp-2 cells at 12 hours on treatment with crude extract and pure compound of *P. urinaria* and *A. marmelos*

Molecular weight marker (Lane 1), HEp-2 Untreated control (Lane 2), Treated with crude ethyl acetate extract of *P. urinaria* (Lane 3), Treated with pure compound of *P. urinaria* (Lane 4), Treated with crude ethyl acetate extract of *A. marmelos* (Lane 5), Treated with pure compound of *A. marmelos* (Lane 6), negative control (Lane 7).

**3.19 A** Analysis of IFN-γ mRNA (510bp) expression in HEp-2 cells after 12 hours of treatment showed elevated levels of expression in both crude extracts and pure compounds of *P. urinaria* and *A. marmelos* when compared to untreated control cells.

**3.19 B** Equal amounts of RNA were analysed for HPRT (410bp) expression as an internal control.
tightly controlled. In humans, synergistic combinations of cytokines (IL-1β+ TNFα + INF-γ) are important for the transcriptional regulation of iNOS gene expression.

In order to find out the effect of both *P. urinaria* and *A. marmelos* crude ethyl acetate extract and pure compound on iNOS mRNA expression in HEp-2 cells result reveals that the iNOS mRNA expression was greatly augmented in cells treated with both *P. urinaria* and *A. marmelos* crude ethyl acetate extract and pure compound. At the same time point, the untreated control HEp-2 cells does not show any expression of iNOS (Figure 3.20 A) and HPRT, a housekeeping gene was used as an internal control (Figure 3.20 B).

### 3.6 NF-κB REGULATION OF CELLULAR APOPTOSIS AND PROLIFERATION

Nuclear Factor-κB is transcription factor that induces the immunoglobulin κ chain, cytokines such as IL-1, IL-2, IL-6, IFN-γ and cell adhesion proteins. It also induces anti-apoptotic proteins and inhibits TNF-α and anti-cancer drug induced apoptosis. Therefore NF-κB function inhibitors may be useful as anti-inflammatory and anti-cancer agents.

#### 3.6.1 Assessment of NF-κB activation in HEp-2 cells

To analyse whether NF-κB is activated in HEp-2 cells on exposure to these plant extracts. A dose response analysis was carried out using different doses ranging from 5-40 μg/ml of both crude and pure compounds. The nuclear extracts of cells exposed to different doses of plant extracts was further analysed by Electro mobility shift assay. The analysis of nuclear extracts
Figure 3.20 RT-PCR analysis of iNOS expression in HEp-2 cells at 12 hours on treatment with crude extracts and pure compounds of *P. urinaria* and *A. marmelos*

Molecular weight marker (Lane 1), HEp-2 Untreated control (Lane 2), Treated with crude ethyl acetate extract of *P. urinaria* (Lane 3), Treated with pure compound of *P. urinaria* (Lane 4), Treated with crude ethyl acetate extract of *A. marmelos* (Lane 5), Treated with pure compound of *A. marmelos* (Lane 6), negative control (Lane 7).

3.20 A Analysis of iNOS mRNA(210 bp) expression in HEp-2 cells after 12 hours of treatment showed elevated levels of expression in both crude extracts and pure compounds of *P. urinaria* and *A. marmelos* when compared to untreated control cells.

3.20 B Equal amounts of RNA were analysed for HPRT (410bp) expression as an internal control.
isolated from HEp-2 cells, demonstrated that there is no activation of NF-κB in HEp-2-cells at different time intervals ranging from 0-24 hours (data not shown).

3.6.2 Suppression of NF-κB activation in Lipopolysaccharide (LPS) induced HEp-2 cells

NF-κB pathway is also a key mediator of genes involved in the control of the cellular proliferation and apoptosis. Anti-apoptotic genes that are directly activated by NF-κB include the cellular inhibitors of apoptosis (c-IAP1, c-IAP2, and IXAP), the TNF receptor–associated factors (TRAF1 and TRAF2), the Bcl-2 homologue A1/Bfl-1, and IEX-IL.

Activation of NF-κB was analysed in LPS stimulated HEp-2 cells, A. marmelos crude ethyl acetate extract and pure compound was capable of suppressing the NF-κB activity but P. urinaria does not showed any significant activity when compared to LPS treated control HEp-2 cells (Figure 3.21). The inhibition of the NF-κB pathway by these plant extracts may be associated with suppression of Iκ -B phosphorylation and its subsequent degradation.

3.7 ANALYSIS OF DIFFERENT TARGET GENES IN HEp-2 CELLS BY RNAse PROTECTION ASSAY [RPA]

The RPA system is a highly sensitive and specific method for simultaneous detection of up to 12 different mRNA species in a single sample of total RNA. The method utilizes specific riboprobes, which are generated using DNA-dependent RNA polymerases from the bacteriophages T7
Figure 3.21 Analysis of NF-κB activation in HEp-2 cells on treatment with *P. urinaria* and *A. marmelos* crude ethyl acetate extracts and pure compounds by Electro mobility shift assay

Lane 1: Untreated HEp2 cells, Lane 2: Positive control with LPS, Lane 3: LPS+*P. urinaria* Crude ethyl acetate extract, Lane 4: LPS+*P. urinaria* pure compound, Lane 5: LPS+*A. marmelos* Crude ethyl acetate extract, Lane 6: LPS+*A. marmelos* pure compound

Activation of NF-κB was analysed in LPS stimulated HEp-2 cells. *A. marmelos* crude ethyl acetate extract and Pure compound is capable of suppressing NF-κB activation and *P. urinaria* did not show any significant suppression when compared to control HEp-2 cells+LPS (lane 2).
or T3. The polymerases drive the synthesis of antisense RNA probes from DNA templates, each of distinct length and each representing a unique sequence in a specific mRNA species. The resulting linear probes are hybridised with target mRNA. After free probes and other single-stranded RNA molecules are digested with RNase, the hybridized probe/target RNA duplexes are resolved according to size on polyacrylamide gels and transferred to a nylon membrane. Quantification of the RNA fixed to the membrane by UV crosslinking follows, based on the 32P labeled antisense RNA probes incorporated into the sample mRNA. Experiments were designed to study levels of mRNA transcripts of numerous apoptosis-regulating genes by RPA. Three commercially available gene templates were obtained from BD Pharmingen: hCK-3 (different cytokines), hAPO-2c (Bcl-2 family), and hAPO-3c (TNF-receptor family).

3.7.1 RNAse Protection assay for different cytokines in HEp2 cells

Experiments were designed to study levels of mRNA transcripts of different types of cytokines by RPA. Commercially available gene templates were obtained from BD Pharmingen: hCK-3 (different cytokines).

Treatment of HEp-2 cells with both *P. urinaria* and *A. marmelos* crude ethyl acetate extracts and pure compounds for 12 hours upregulated the mRNA levels of IFN-γ when compared to the untreated control cells. TNF-α mRNA was elevated only by *A. marmelos* crude ethyl acetate extract and *A.marmelos* pure compound (Figure 3.22). In these experiments, L32 and GAPDH served as loading controls.
Figure 3.22 Analysis of different cytokines in HEp-2 cells at 12 hours on treatment with crude extracts and pure compounds by RNAse Protection Assay [RPA]

Unprotected probe (Lane 1), Yeast RNA control (Lane 2), Untreated control cells (Lane 3). Treated with crude ethyl acetate extract of *Purinaria* (Lane 4). Treated with pure compound of *Purinaria* (Lane 5). Treated with crude ethyl acetate extract of *A.marmelos* (Lane 6). Treated with pure compound of *A.marmelos* (Lane 7).

HEp-2 cells on treatment with *Purinaria* crude ethyl acetate extract and pure compound showed elevated levels of IFN-γ whereas *A.marmelos* crude ethyl acetate extract and pure compound showed elevated levels of both IFN-γ and TNF-α at 12 hours when compared to untreated control cells.
3.7.2 RNAse Protection assay for Bcl-2 family proteins in HEp2 cells

The role of Bcl-2 family proteins in apoptosis experiments were understood by studying the levels of mRNA transcripts of numerous apoptosis-regulating genes by RPA. We have used commercially available gene templates were obtained from BD Pharmingen: hAPO-2 c (Bcl-2 family genes).

Treatment of HEp-2 cells with *P. urinaria* crude ethyl acetate extract and pure compound for 24 hours showed down regulation of Bcl-2 and upregulation of BAX (Figure 3.23) when compared to untreated control cells. In the same time point cells treated with *A. marmelos* crude ethyl acetate extract and pure compound did not show any significant activity when compared to untreated control cells (Data not shown). In these experiments, L32 and GAPDH served as loading controls.

3.7.3 RNAse Protection assay for death receptors and caspases in HEp-2 cells

In order to study the role of TNF-α, TNFRI and TRADD family proteins in apoptosis, experiments were designed to measure levels of mRNA transcripts of several apoptosis-regulating genes by RPA. Commercially available gene templates were obtained from BD Pharmingen: hAPO-3 c (TNF-receptor family).

HEp-2 cells on exposure to *P. urinaria* crude ethyl acetate extract and pure compound for 24 hours showed upregulation of Fas and caspase 8 at 24 hours when compared to untreated control cells. In the same time point HEp-2 cells on exposure to *A. marmelos* crude ethyl acetate extract and pure
Unprotected probe (Lane 1), Yeast RNA control (Lane 2), Untreated control cells (Lane 3) Treated with crude ethyl acetate extract of \textit{P. urinana} (Lane 4), Treated with pure compound of \textit{P. urinana} (Lane 5), Treated with crude ethyl acetate extract of \textit{A. marmelos} (Lane 6), Treated with pure compound of \textit{A. marmelos} (Lane 7).

\textbf{HEp-2} cells on treatment with \textit{P. urinana} crude ethyl acetate extract and pure compound showed up-regulation of \textit{bax} and down regulation of \textit{Bcl-2} at 24 hours when compared to untreated cells.

\textbf{HEp-2} cells on treatment with \textit{A. marmelos} crude ethyl acetate extract and pure compound did not show any significant activity when compared to the control.
compound showing elevated levels of TNFRp55 (TNFRI) and TRADD, Fas and caspase 8 (Figure 3.24). These results indicate that *A. marmelos* was capable of inducing apoptosis by elevating TNF-α expression, which in turn activate TNFRI, and recruit TRADD and activate the Caspase 8. In these experiments, L32 and GAPDH served as loading controls.

3.8 WESTERN BLOT ANALYSIS OF DIFFERENT APOPTOTIC SIGNAL TARGETS LIKE \textit{bcl2}, p53, TRADD, CASPASE 8 AND CASPASE 3

Although apoptosis is a conserved and highly regulated mechanism of cell death, its inducers are diverse. They include proteins of the tumour necrosis factor (TNF) family, genotoxic and cytotoxic agents such as anticancer drugs and g-radiation, and anti-inflammatory drugs such as aspirin and menadione. The intracellular mediators of apoptosis have been relatively well characterised. Regardless of the nature of the stimulus, the commitment to apoptosis occurs as a result of the activation of members of the caspase family of cysteine proteases. These enzymes are present in cells as inactive precursors that are proteolytically activated on induction of apoptosis.

3.8.1 Analysis of \textit{bcl-2} protein expression in HEP-2 cells

Mitochondria are central to the apoptosis activation pathway in many physiological and pathological conditions. Members of the Bcl-2 family of proteins are known to affect mitochondrial function and regulate the release of apoptosis-activating factors. Anti-apoptotic members of Bcl-2 family (\textit{e.g.} Bcl-2 and Bcl-xL) act primarily to preserve mitochondrial integrity by suppressing the release of cytochrome \textit{c}. In contrast, pro-apoptotic members (Bax, Bid, etc.) induce the release of cytochrome \textit{c} and cause mitochondrial dysfunction.
Figure 3.24 Analysis of death receptor and caspases in HEp-2 cells at 24 hours on treatment with crude extracts and pure compounds by RNase Protection Assay [RPA]

Unprotected probe (Lane 1), Yeast RNA control (Lane 2), Untreated control cells (Lane 3). Treated with crude ethyl acetate extract of *P.urinaria* (Lane 4), Treated with pure compound of *P.urinaria* (Lane 5), Treated with crude ethyl acetate extract of *A.marmelos* (Lane 6), Treated with pure compound of *A.marmelos* (Lane 7).

HEp-2 cells on treatment with *P.urinaria* crude ethyl acetate extract and pure compound showed elevated levels of Fas and caspase 8 at 24 hours when compared to untreated control cells.

HEp-2 cells on treatment with *A.marmelos* crude ethyl acetate extract and pure compound showed elevated levels of TNFRp55(TNFRI), TRADD, Fas and caspase 8 at 24 hours when compared to untreated control cells.
Immunoblot analysis of Bcl-2 in HEp-2 cells on exposure to both *P. urinaria* and *A. marmelos* crude ethyl acetate extracts and pure compounds for 36 hours showed down regulation of bcl-2 in cells treated with *P. urinaria* crude ethyl acetate extract and pure compound (Figure 3.25 A) when compared to the untreated control cells while *A. marmelos* extracts did not down regulate bcl2 activity. These data correlate with the down regulation of bcl-2 at message (mRNA) level.

### 3.8.2 Analysis of p53 protein expression in HEp-2 cells

The p53 protein is a transcription factor and tumour suppressor that is lost during tumourigenesis in 30–70% of clinical tumour samples. The presence of the wild-type p53 gene in a tumour correlates with a favorable response to chemotherapy, and, in experimental systems, elevated p53 signalling contributes to the induction of apoptosis by DNA damaging agents.

Immunoblot analysis of p53 in HEp-2 cells on exposure to both *P. urinaria* and *A. marmelos* crude ethyl acetate extracts and pure compounds for 24 hours showed up-regulation of p53 in cells treated with *A. marmelos* crude ethyl acetate extract and pure compound (Figure 3.25 B) when compared to the untreated control cells while *P. urinaria* does not shows any significant activity.

### 3.8.3 Analysis of TRADD protein expression in HEp-2 cells

Death Receptors - i.e. cell surface receptors that transmit apoptotic signals initiated by specific ligands - play a central role in instructive apoptosis. These receptors can activate death caspases within seconds of ligand binding,
Figure 3.25 Immunoblot analysis of Bcl2, p53 and TRADD in HEp-2 cells at 36 hours on treatment with crude extracts and pure compounds

Molecular weight marker (Lane 1), HEp-2 Untreated control (Lane 2), Treated with crude ethyl acetate extract of *P. urinaria* (Lane 3), Treated with pure compound of *P. urinaria* (Lane 4), Treated with crude ethyl acetate extract of *A. marmelos* (Lane 5), Treated with pure compound of *A. marmelos* (Lane 6).

3.25 A) Immunoblot analysis showed down regulation of Bcl2 in HEp-2 cells when treated with *P. urinaria* crude ethyl acetate extract and pure compound while *A. marmelos* did not show any significant activity in Bcl-2 expression when compared to the untreated cells.

3.25 B) Immunoblot analysis of p53 (3.25 B) and TRADD (3.25 C) showed activation of both p53 and TRADD in cells treated with *A. marmelos* crude ethyl acetate extract and pure compound while *P. urinaria* did not show any activation of p53 and TRADD when compared to untreated cells.
causing an apoptotic demise of the cell within hours. TNFR1-Associated Protein with Death Domain (TRADD): TRADD is a protein which specifically binds to oligomerised TNFR1, but not to CD 95, by homotypic interactions between the death domains. The function of TRADD probably is to recruit RIP. The cleavage of RIP results in the blockage of TNF-induced NF-kappaB activation. RIPc, one of the cleavage products, enhances interaction between TRADD and FADD/MORT1 and increases cells' sensitivity to TNF.

Immunoblot analysis of TRADD in HEp-2 cells on exposure to both *P. urinaria* and *A. marmelos* crude ethyl acetate extracts and pure compounds for 36 hours showed up-regulation of TRADD in cells treated with *A. marmelos* crude ethyl acetate extract and pure compound (Figure 3.25 C) when compared to the untreated control cells whereas *P. urinaria* does not shows any significant activity.

### 3.8.4 Analysis of Caspase 8 and Caspase 3 protein expression in HEp-2 cells

Caspases are synthesised as zymogens or proenzymes that contain a prodomain in addition to one large subunit and one small subunit. Caspases can be subdivided by phylogenetic analysis, according to their substrate specificity or the length of their prodomain. Initiator caspases, like caspase-8, contain long prodomains, which facilitate their interaction with specific ‘adapter’ proteins. Exposure to apoptotic stimuli results in clustering of these adapter proteins, bringing initiator caspases in close proximity to one another to promote trans-catalytic activation. Initiator caspases then activate various short prodomain, ‘effector caspases’, including caspases-3, 6 and 7.
Immunoblot analysis of Caspase-8 and Caspase-3 in HEp-2 cells on exposure to both *P. urinaria* and *A. marmelos* crude ethyl acetate extracts and pure compounds for 36 hours showed activation of Caspase-8 in cells treated with both *P. urinaria* and *A. marmelos* crude ethyl acetate extracts and pure compounds (Figure 3.26 A) when compared to the untreated control cells which in turn cleave the pro caspase-3 (32 kDa) into active Caspase-3 (17 kDa) (Figure 3.26 B). These data also further supported by our RPA analysis were activation of Caspase-8 was noted at message (mRNA) level.
Figure 3.26 Immunoblot analysis of Caspase 8 and Caspase 3 in HEp-2 cells at 36 hours on treatment with crude extracts and pure compounds

Molecular weight marker (Lane 1), HEp-2 Untreated control (Lane 2), Treated with crude ethyl acetate extract of *P. urinaria* (Lane 3), Treated with pure compound of *P. urinaria* (Lane 4), Treated with crude ethyl acetate extract of *A. marmelos* (Lane 5), Treated with pure compound of *A. marmelos* (Lane 6).

Immunoblot analysis of both caspase 8 and caspase 3 in HEp-2 cells showed activation of caspase 8 (3.26 A) and caspase 3 (3.26 B) on treatment with crude ethyl acetate extracts and pure compounds of *P. urinaria* and *A. marmelos* for 36 hours when compared to untreated control cells.