**International Journal of Botany Studies**
**ISSN: 2455-541X; Impact Factor: RJIF 5.12**
**Received: 07-01-2020; Accepted: 08-02-2020; Published: 11-03-2020**
**www.botanyjournals.com**
**Volume 5; Issue 2; 2020; Page No. 37-44**

# Anti: Proliferative efficacy of the leaf extract of *Aristolochia tagala* cham, on hep G2 cell line

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**Abstract**
Aristolochia tagala Cham. is an important medicinal plant used to treat tumors in ethno botanical Practices. During the present investigation, dried leaf powder of A. tagala was extracted with three different solvents by decoction method and were subjected to MTT cytotoxicity assay against HepG2 – human liver carcinoma cell line. Methanolic leaf extract showed best anti-proliferative activity against HWepG2 cell line with an $IC_{50}$ value of 80 µg/ml than other two extracts. Cell cycle arrest was studied by PI staining and the treated cells were arrested at G2/M phase. Apoptosis was assessed by annexin-V and PI staining, the results revealed high annexin-V and low PI staining representing early apoptotic stages and finally Caspase-3 expression in the treated cells were analyzed by using flow cytometry having negligible caspase-3 activity hinting for a caspase-3 independent pathway leading to cell death. In the present study methanolic leaf extract of A. tagala showed a competitive anti-proliferative and apoptotic activity against HepG2 cell line giving a partial scientific validation for its usage in traditional system of medicine. Further the affirmative results call for advanced studies on this medicinally important plant is needed.

**Keywords:** *Aristolochia Tagal*, mtt, hepg2, $ic_{50}$, annexin-v and anti-proliferative

## 1. Introduction
An anomaly in the cell cycle results in the uncontrolled cell division lead to the most dreaded disease – the Cancer, which is the major cause of deaths all over the world accounting for a massive 23% of death in USA and 7% in India [1]. Among the different types of cancer, hepatocellular carcinoma is the 6th most prevalent malignancy worldwide [16]. The majority of the hepatocellular carcinoma are caused due to cirrhosis which is majorly triggered by alcohol consumption, or by infection of hepatitis B or C viruses. The diagnosis of the disease is done by noninvasive and invasive techniques such as liver function test and AFP test (α-fetoprotein in the serum is tested) by taking blood samples, ultrasound, CT or MRI scanning or by liver biopsy. The treatment is determined based on the stage and severity of disease and are categorized into three groups as curative treatments (for very early and early stages of disease) which include surgical resection, radiofrequency ablation therapy and liver transplantation; palliative treatments (for intermediate and advanced stages of disease) and symptomatic therapy (for terminal stage of disease) which include chemotherapy [4]. However even after curative surgical therapies 50% of the cases may show recurrence of the disease and similarly chemotherapies also fail to completely cure the disease in many cases due to the resistance shown by the disease towards medication or they may have severe side effects [9]. Due to these reasons there is an increased usage of complementary and alternative medicines including traditional herbal medicines all over the world to prevent and treat cancer [18]. Among the vast range drugs confirmed by FDA to treat cancer only few are obtained from plant sources like vinca alkaloids, taxanes, podophyllotoxins, etc [13]. However plenty of medicinal plants are used in different traditional systems to treat cancer which require scientific validation and many more plants are yet to be discovered for their medicinal properties.

*Aristolochia tagala* is one such important plant which is used to treat tumour, as an antidote against poisonous snake and scorpion bites, to cure abdominal pain, bilious disorders, fever and headache by several tribal settlements in India and Bangladesh [12, 5, 3]. Hence scientific validation of anti-proliferative efficacy of this plant through *in vitro* experiments was chosen for the present work, the results of which can boost up further in-depth studies leading to novel drug formulation to cure cancer.

## 2. Materials and Methods
### 2.1 Collection of plant material
Frequent field trips were undertaken to Bisle reserve forest, Sakleshpur taluk, Hassan district, Karnataka to collect *Aristolochia tagala* for the current research work and identified using flora. Herbarium was prepared and submitted to Botanical Survey of India, Pune for authentication (Accession No. 136269). Fresh and healthy leaves were washed in running tap water to remove soil and dirt. Excess of water was blotted out and dried under shade at room temperature.

### 2.2 Preparation of crude extract
The shade dried leaves were powdered and extracted with three different solvents separately by hot decoction method. 50 grams of leaf powder was treated with 250 ml each of three different solvents *viz*., methanol, chloroform and petroleum ether in a tightly closed conical flask at 50°C in hot water bath for 4 hours after which it was filtered with Whatman No.1 filter paper to collect the extract. The collected extracts were further kept in hot air oven (at 50°C) for the evaporation of excess solvent to obtain paste like crude extract (yield: methanolic extract – 3.41g, chloroform extract – 5.54g and petroleum ether extract – 1.47g). These

extracts were stored in eppendorf tubes (sealed with paraffin tape) which were stored in refrigerator at 4°C until further use.

### 2.3 Cell culture

The experiments were carried out at Stellixir Biotech Pvt. Ltd. Bangalore. HepG2 cell line was procured from National Center for Cell Science, Pune, India. The obtained cell line was cultured and maintained on Dulbecco's Modified Eagle's Medium (DMEM) with low glucose, supplemented with Fetal Bovine Serum (FBS) and Streptomycin. The culture vials were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide and when the cells reached 70% – 80% confluency they were used for the following *in vitro* assays. For all the following experiments the sample was prepared by reconstituting the crude leaf extracts of *A. tagala* in less than 0.1% Dimethyl sulfoxide (DMSO) and making it up to the required quantity with DMEM. In each of the following experiment the activity of the sample was compared with that of the standard drug i.e., Camptothecin.

### 2.4 MTT Cytotoxicity Assay

Cytotoxicity assay on HepG2 cells was conducted using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric method (Mosmann, 1983) [11]. 300 µl of medium containing 50,000 cells per well was seeded on to 96 well plate and was incubated for 24 hours. After incubation, the spent medium was removed and treated with 300 µl of samples of various concentrations ranging from 0 - 320 µg per well. These treated cells were further incubated for another 24 hours. The extract was removed and treated with 100 µl of MTT (5mg/10ml of 1X Phosphate Buffered Saline) per well. Later it was incubated in dark for 4 hours. The MTT salt reduced to purple coloured compound called formazan by dehydrogenase enzyme present in the living cells, which was solubilized using DMSO and the colour intensity was measured by reading the absorbance at 590 nm using a spectrophotometer. Percentage of inhibition was calculated using MS Excel and $IC_{50}$ value was calculated using Graphpad Prism Version 6.0 software. Among the three extracts tested, the one which showed best result was further subjected for its activity through the following three experiments at the concentration equivalent to its $IC_{50}$ value obtained in MTT assay.

### 2.5 Cell Cycle Arrest Analysis

2ml of DMEM with $2\times10^5$ cells per well were seeded onto 6 well plate and incubated for 24 hours for growth of the cells; following that the spent medium was removed, treated with 2ml plant extract and incubated for another 24 hours. After the incubation period, methanolic leaf (better $IC_{50}$ value was selected) extract was removed, washed with PBS, trypsinized and cells were harvested with 2ml of fresh medium which was centrifuged to get the cell pellet. This cell pellet was washed with PBS and fixed with 70% ethanol. These fixed cells were treated with Ribo nuclease-A (R Nase A) to remove RNA content, stained with pro pidium iodide (PI) and analyzed using flow cytometry to know the effect of extract on cell cycle and at what stage the cells were arrested.

### 2.6 Annexin V: PI Staining Assay

Methanolic leaf extract-induced apoptosis in HepG2 cell line was determined by flow cytometry using the Annexin V-FITC conjugated apoptosis detection kit (BD Biosciences).

The HepG2 cells were seeded at a density of $2\times10^5$cells in 2ml of DMEM per well in a 6 well plate and was incubated for 24 hours. Subsequently the spent medium was removed and treated with 2ml of extract and incubated for another 24 hours. After incubation the extract was removed, washed with PBS, trypsinized and cells were harvested with 2ml of fresh medium which was centrifuged to get the cell pellet. The cell pellet was further washed with PBS twice and re-suspended in 1X annexin-V binding buffer (by BD Pharmingen). It was then stained with FITC Annexin-V, vortexed and incubated in dark at room temperature for 15 min; after which PI stain in 1X binding buffer was added to it and analyzed by flowcytometry to examine the percentage of necrotic and apoptotic cells.

### 2.7 Caspase-3 expression assay

HepG2 cells at $2\times10^5$cells in 2ml of DMEM was seeded per well in a 6 well plate and was incubated for 24 hours. After that the spent medium was removed, 2ml of methanolic leaf extract was added and incubated for another 24 hours. Following this the extract was removed and cells were harvested and pelleted using the standard procedure. The cell pellet was washed with PBS and incubated with 0.5 ml of BD Cytofix solution for 10 minutes. Later it was washed with 0.5% bovine serum albumin (BSA) in 1X PBS and 0.1% sodium azide. The cell pellet was now treated with Caspase-3 antibody, mixed well and incubated in dark at room temperature for 30 minutes. Later it was washed with 1X PBS combined with 0.1% sodium azide, and cell pellet was re-suspended in 0.5 ml of PBS and analyzed by flowcytometry to record the percentage of cells showing caspase-3 expression.

## 3. Results and Discussions

Cytotoxicity of *A. tagala* extracts on HepG2 cell line by MTT assay: Abnormal cell proliferation and differentiation leads to the formation of benign and malignant tumours. Chemo and radiotherapies advised for treatment of cancer may have severe side effects [6]. Hence herbal based alternative medicines are gaining more prominance. *Aristolochia tagala* is one such ethno-botanical medicinal plant used by several tribes to treat tumours. In the current research study anti-proliferative efficacy of leaves of *A. tagala* on HepG2 cell line was focused. Very few reports are available regarding the cyto-toxic effect of A. *tagala*.

MTT cytotoxicity assay is colorimetric assay based on the breaking down of tetrazolium salt to formazan by the hydro genase enzyme present within the living cells. By knowing the quantitiy of formazan produced, number of living cells can be determined. Hence this method can be used to measure cytotoxicity, proliferation and activation of cells [11].

Cytotoxicity of the methanolic, chloroform and petroleum ether leaf extracts (coded as AT1, AT2 and AT3 respectively) of *A. tagala* on HepG2 cell line was analysed by MTT colorimetric assay. Among the three extracts tested, methanolic extract (AT1) showed the best results

With lowest $IC_{50}$ value (Figure 1). AT1 showed the highest percentage of inhibition of 78% at the highest concentration tested (320 µg/ml), whereas at the same concentration AT2 showed 71.95% and AT3 showed 77.20% of inhibition. And the results were depicted in the form of a graph to calculate $IC_{50}$ value using Graphpad prism version 6.0 software, AT1 proved to have the best cytotoxicity by showing lowest $IC_{50}$ value of 76.99 µg/ml (Figure 2) where as AT2 and AT3 were 87.24 µg/ml and 98.73 µg/ml. Hence for the further studies only AT1 was selected.

These results are in complete agreement with the findings of Garg *et al*., (2007) that alcoholic extract of stem and leaves of *A. tagala* showed $IC_{50}$ value of 70 µg/ml against COLO (human colon cancer cell line), 40 µg/ml against MCF-7 (human breast cancer cell line), 9 µg/ml against KB (human oral cancer cell line), 13 µg/ml against PA-I (human ovary cancer cell line) and 20 µg/ml against WRL-68 (human liver cancer cell line) [18]. The high cytotoxicity effect in their study may be due to the fact that they used both stem and leaf for extraction. Hadem *et al*., (2014) [7]. conducted *in vivo*

Experiments on mice using methanolic root extract of *A. tagala*, which attenuated the increased activities of cancer marker enzyme acetylcholine esterase (AChE) when exposed to carcinogen diethyl nitrosamine (DEN). They have also reported the activities of antioxidant enzymes which decreased following DEN administration, were significantly increased in mice treated with *A. tagala* extract. The results obtained in the present study and the reports of previous workers suggest that alcoholic extract of *A. tagala* plant parts have phyto compounds with antioxidant potential which can scavenge the free radicals that can trigger oncogenesis and tumour proliferation; thereby preventing and curing the cancer.

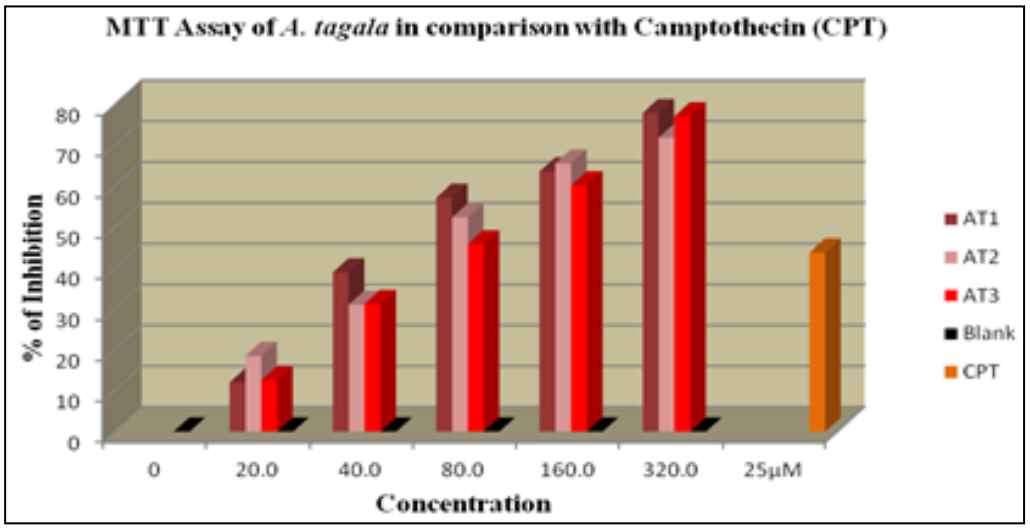


**Fig 1:** Cytotoxicity of AT1, AT2 and AT3 at various concentrations in comparison with that of Blank and Camptothecin (CPT) a standard drug in MTT assay. Calculation and Graph were prepared using MS Excel software.

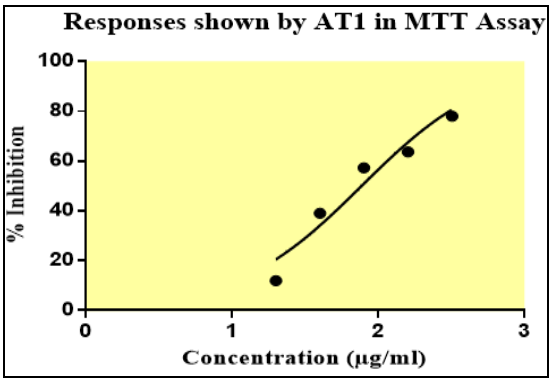


**Fig 2:** A graph of AT1 showing percentage of inhibition in MTT assay. Calculation and Graph were prepared using Graphpad prism version 6.0.

No previous reports are available on the effect of *A. tagala* leaf extract against cancer cell lines on cell cycle arrest, apoptosis and caspase-3 activity analysis hence the present study.

### 3.1 Effect of AT1 on Cell Cycle

To analyze the effect of AT1 on cell cycle of HepG2 cancer cells, they were treated with AT1 at a concentration of 80 µg/ml (a value near to its $IC_{50}$ value in MTT assay). The treated cells were fixed, stained with PI and analyzed with flowcytometry. The effect of AT1 was similar to that of the standard (Camptothecin) (Figures 3, 4 & 5). In both the cases majority of the cells were arrested at G2/M phase. Cells treated with CPT showed 74.56% of them to be arrested at G2/M phase whereas in case of AT1 treatment 71.43% were arrested at G2/M phase proving that AT1 triggered cell death at G2/M phase of the cell cycle. In case of untreated cells, the cells were found to be present in various stages (G0/G1, S and G2/M phases) showing that cell cycle was unaffected (Figure 6).

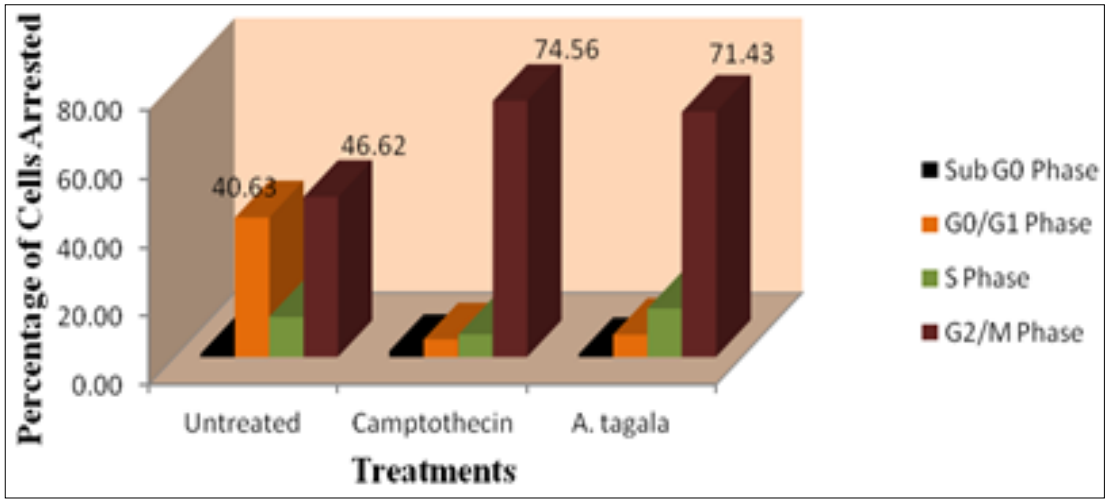


**Fig 3:** Bar graph representation of the percentage HepG2 cells arrested at various stages of cell cycle due to AT1 and CPT treatments

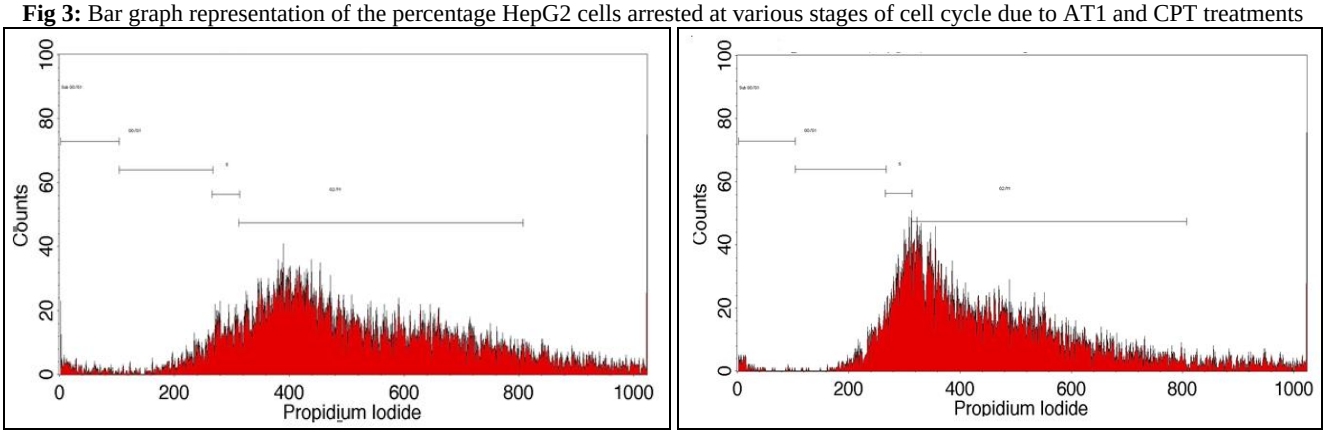


**Fig 4 and 5:** Histogram representation of HepG2 cells found in various stages due to CPT and AT1 treatments respectively

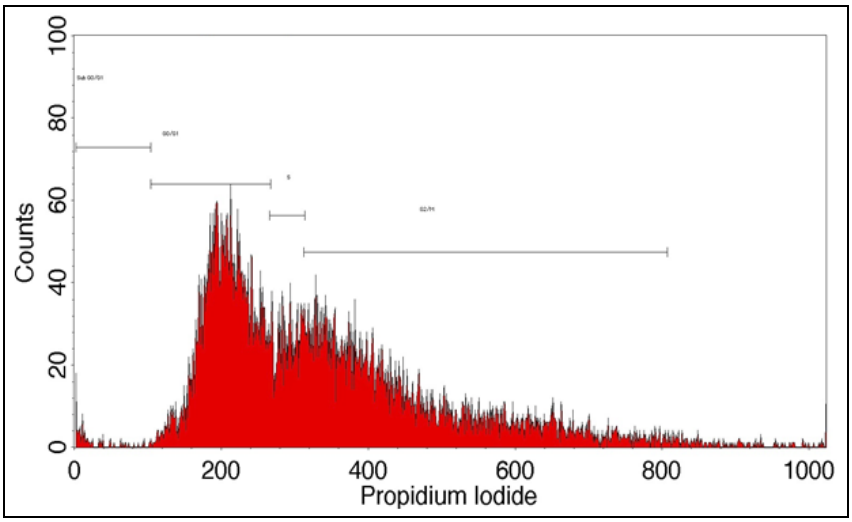


**Fig 6:** Histogram representation of untreated HepG2 cells found in various stages

Capranico *et al.*, (2012) [2] recorded that, when human colon cancer cells were treated with camptothecin, the cells showed an increase in the p53 responsive genes which caused an interruption of the mitosis - related gene expression of the cells leading to the cell cycle arrest at G2 phase. The results of the present study tallies with the findings of Capranico *et al*., that camptothecin (standard drug) used as positive control, arrested the cell cycle of HepG2 cells at G2/M phase. The methanolic leaf extract of *A. tagala* showed 71.43% of cells arrest at G2/M phase compared to standard.

**Apoptosis Assay**

Annexin-V and PI staining is common method used to detect the apoptosis or necrosis in a dying cell. One of the early changes that occur during apoptosis is the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer. Annexin-V is a phospholipid-binding protein with high affinity for PS. Hence this protein can be used as a sensitive probe for PS exposure upon the cell membrane. Release of PS can be seen in both the cases of apoptosis and necrosis. However in case of apoptosis the cell does not lose its integrity unlike necrosis; hence does not release genetic material and other

cellular contents to the exterior in the early stages. In case of necrosis the cell breaks down simultaneously along with the release of PS. The genetic material thus released can be detected by staining with PI. Even before the annexin-V binds with PS, large quantity of released genetic material will get stained with PI. Hence the low annexin-V and high PI stained cells can be considered as necrotic cells; the cells that bind with both annexin-V and PI can be regarded as late apoptototic or secondary necrotic cells and those that bind only with annexin-V can be considered as cells in early apoptotic stages [15]. Apoptosis caused in HepG2 cells due to the treatment of cells with AT1 (80 µg/ml) and CPT (25 µM) was detected by Annexin-V & PI Staining technique. The treated cells were fixed, analyzed with flowcytometry and quadrant plot graphs were drawn. In the quadrant plots the horizontal axis represented the annexin-V stained cells and vertical axis represented the PI stained cells. In case of CPT treatment, 61.65% of cells were found to live cells, 26.67% were in early apoptotic stages and 10.36% were in late apoptototic stages (Figure 7 & 8). 48.37% of the cells treated with AT1 did not get stained with PI or Annexin-V representing the live cells and 48.65% of the cells were recorded with only Annexin-V staining representing cells in early apoptototic stages (Figure 9). Out of all the untreated cells 85.77% of them were live cells and 13.87% of them were showing necrotic stages (Figure 10). Cell death can occur in many different ways, major of them being apoptosis, necrosis and stress induced cell death. Stress induced cell death and Necrosis are pathological events which kill only damaged cells, may trigger regeneration and scar formation. Induction of necrosis of cancer cells can release cellular debris and components of the dying cell that can trigger immune reactions which can also have adverse effects on host tissue; whereas induction of apoptosis to cancer cells can kill the cells with less side effects and immune reactions and hence apoptosis is the preferred mode to kill cancer cells.[19, 14] In the present study 48.65% of HepG2 cells treated with 80 µg/ml of *A. tagala* methanolic leaf extract exhibited high annexin-V and low PI staining indicating early apoptosis, 10.36% showed late apoptotic (or secondary necrotic) stage and only 1.32% of them displayed necrosis making this extract an ideal candidate for apoptosis in tumour cells.

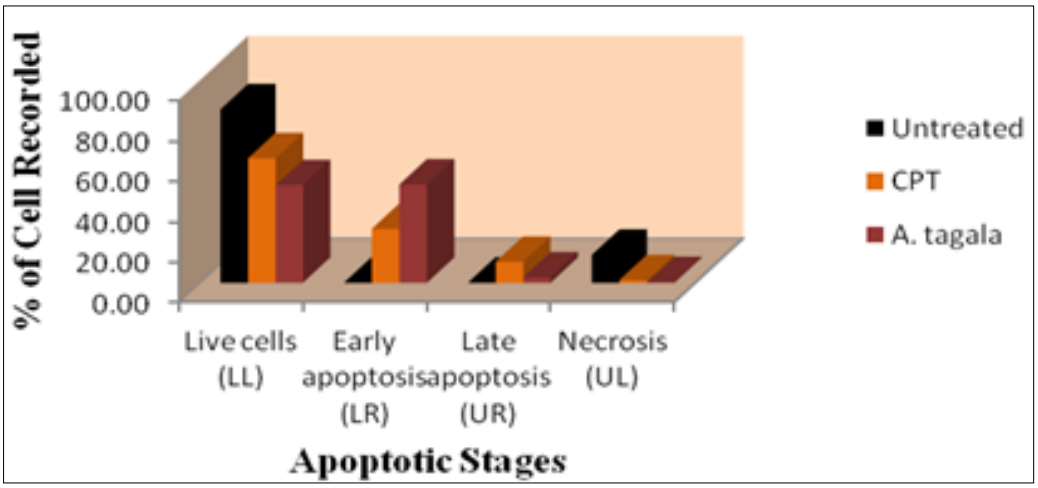


**Fig 7:** Bar graph representation of the percentage HepG2 cells showing various stages of apoptosis due to AT1 and CPT treatments

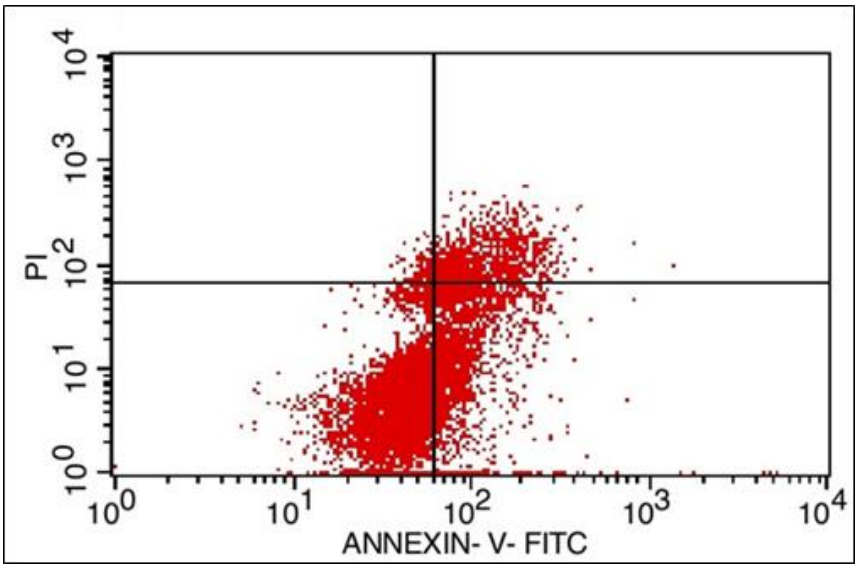


**Fig 8:** Quadrant plot representing cells in various stages of apoptosis due treatment with CPT

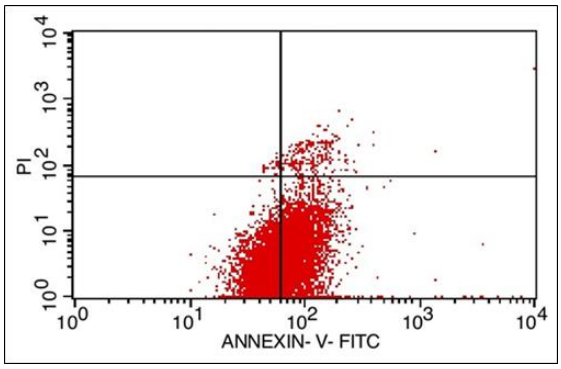


**Fig 9:** Quadrant plot representing cells in various stages of apoptosis due treatment with AT1

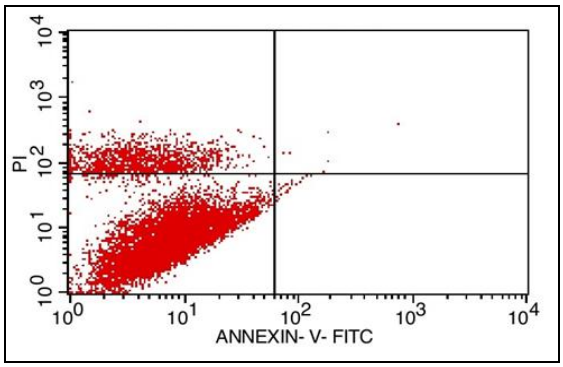


**Fig 10:** Quadrant plot representing untreated live cells (treatment with blank i.e., DMEM)

**Analysis for the presence of active caspase-3**

Apoptosis can be triggered due to numerous factors. Based on these factors apoptosis can occur in two different pathways, *viz*., extrinsic and intrinsic pathways. In extrinsic pathway, the death ligands (ex. TNF-α, FASL, TRAIL etc) bind to trans membrane death receptors (ex. TNFR1, FAS, DRS etc) present on cells preordained to die. These ligand bound receptors cluster together and recruit adaptor proteins (like FADD, TRADD etc) and form death inducing signaling complex (DISC) on cytoplasmic side which activates caspase cascade by inducing autocleavage of initiator caspase (caspase-8) which in turn activates executioner caspases (caspase-3, 6 or 7). In case of intrinsic pathway the stress on the cell causes activation and binding of the cytoplasmic proteins like BAX and BID to the outer membrane of the mitochondria and interact with a mitochondrial protein (BAK) causing the release of the Cytochrome c into the cytosol. Thus released cytochrome c interacts with Apoptotic Protease Activating Factor -1 (APAF-1) which forms apoptosome and triggers the activation of another intiating caspase (caspase-9) that further activates the executionary caspases (caspase-3, 6 or 7). Bax can also be activated by caspase 8 of extrinsic pathway thereby linking both the pathways [17]. P53 is another tumor suppressor protein that

gets activated due to stress on cell and can activate both intrinsic and extrinsic pathways of apoptosis [10]. In both pathways the effectors *viz*., caspase-3, 6 or 7 are activated which promote further apoptotic processes by cleaving cellular substances. Among them, capase-3 is mentioned to be a major effector caspase.[18, 19]

In the past Hadem *et al*., in 2015 have showed that Diethyl nitrosamine (DEN) induced hepatocellular carcinoma in mice, occurs though increased levels of TNF-α which mediates inflammation and cancer through activation of NF-kB in mice which were reduced by methanolic root extract of *A. tagala*, nonetheless, occurrence of apoptosis and the its pathway in the treated mice tumour cells were not mentioned by them [8]. Hence in the current research work the HepG2 cells were treated with methanolic leaf extract of *A. tagala* (AT1) and Camptothecin (CPT) to cause apoptosis and were tested for the presence of active caspase-3 in them following a standard protocol as mentioned before. However outcome of the experiment illustrated that only 4.07% cells treated with AT1 & 12.94% of cells treated with that of CPT showed the presence of active caspase-3. These results may indicate that either the Hepg2 cells treated with AT1 and CPT utilize the caspase-3 at a faster rate to undergo apoptosis or they undergo caspase-3 independent pathway of apoptosis or parapoptosis. Further research on these aspects and on isolation of specific active metabolites from methanolic leaf extract of *A. tagala* can through light on the exact pathway of cell death caused by the treatment of HepG2 cells with this plant extract which can further help in drug designing to combat hepatocellular carcinoma or (in general) cancer efficiently.

To check if the cell death caused by AT1 and CPT treatment was by capase-3 dependent pathway of apoptosis, the AT1 and CPT treated cells along with the untreated cells were treated with caspase-3 antibody that would bind with the active caspase-3 produced within the cells and were analyzed with flowcytometry. The results demonstrated that both in the CPT and AT1 treated cells very few cells showed the presence of active caspase-3 i.e,, 12.94% of CPT treated cells and 4.07% of AT1 treated cells signaled for the presence of active caspase-3 within them (Figure 11, 12, 13 & 14).

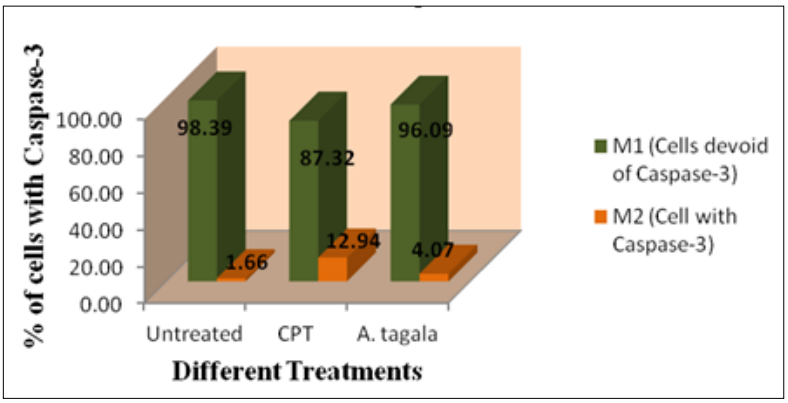


**Fig 11:** Comparative account of caspase-3 activity in untreated, CPT treated and AT1 treated cells

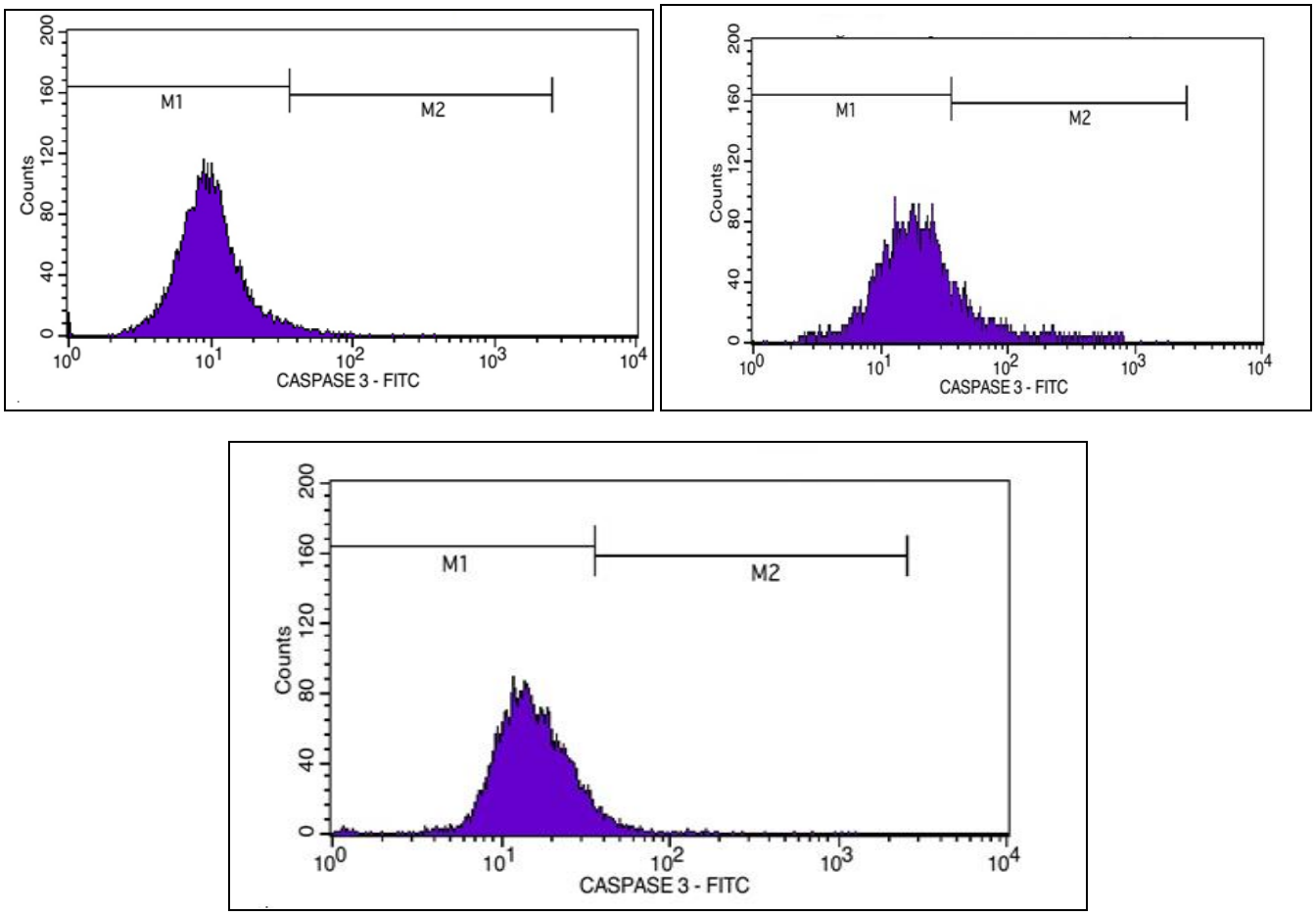


**Fig 12, 13 & 14:** Histograms representing the cells with and without caspase-3 activity in untreated, CPT treated and AT1 respectively

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