



An investigation of anti-proliferative and apoptotic potential of ethanolic leaf and stem extracts of *Andrographis megamalayana* Gnanasek *et al.*, (Acanthaceae) against hepatocarcinoma cell line (Hep G2)

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Abstract: Cancer is an insidious disease characterized by mortality in humans. Medicinal plants offer a holistic approach for the development of novel drug to combat awful diseases which manifest viz., cancer, liver disorders, herpes, fever, hepatitis and other chronic disorders etc. The aim of the present study was to elucidate and analyze the potentiality of ethanolic leaf and stem extracts of *Andrographis megamalayana* a species of high ethnobotanical importance on anti-proliferative, apoptotic gene expression by caspases 3 and DNA fragmentation against human liver carcinoma (Hep G2) cell line. Among various concentrations ranging from 50 - 250 µg/ml of the extracts evaluated, ethanolic leaf extract revealed the highest anti-proliferative effect on Hep G2 cell line with IC₅₀ value of 121.8 µg/ml. Evidently, 100 µg / ml concentration of ethanolic leaf extract had a significant up regulatory effect on the expression of caspase3 enzyme. Whereas in the loss of mitochondrial membrane potential, DNA fragmentation strongly support the ability that 100 µl concentration of ethanolic leaf extract of *A. megamalayan* was found to induce cancer cell apoptosis. According to the results pertaining to the present study the ethanolic leaf extract of *A. megamalayan* harbor a lead source of novel metabolite and a natural cytotoxic agent which inhibited the proliferation of cancer cell by inducing cell apoptosis. Elucidation of phytochemical characterization is further recommended to fully understand the underlying mechanism of action on cancer cell death and in identification of responsible phytocompound.

Keywords: *Andrographis megamalayana*, Anti-proliferative, apoptosis, Caspase 3, DNA fragmentation.

I. INTRODUCTION

As majority of cancer therapies severely affects the host's normal cells (Mascarenhas, 1994), the use of natural products has now been contemplated unique value in the control of disease (Suffness and Pezuto 1990). Hepatocarcinoma is the sixth most common disease both in developed and developing countries with a substantial life loss. Owing to its tremendous aggressive nature and poor survival rate, there is a constant demand for new therapies to treat and prevent the onset of this life threatening disease. Apoptosis is a process of programmed cell death that occurs in multicellular organisms through caspases, a key agent of cell death, which includes apoptosis (Susan Elmore, 2007). Caspases are grouped as initiators and effectors according to their specific function. It acts as cascades which activate one another (Ge *et al.*, 2016). They are crucial in cell death and apoptosis takes place in all the cells to maintain homeostasis and cellular integrity. Cancer treatment therapies target this apoptotic pathway by increasing apoptosis in cells and thus prevents cancer. The advent of novel synthetic treatment has major drawback of expensiveness and carries a bound list of adverse effects viz. loss of appetite, bleeding, thrombocytopenia, hypertension edema, fatigue, lung disorders and hair loss (Aggarwal *et al.*, 2009). According to World Health Organization (WHO) about 80 per cent of the global populations rely on medicinal formulations obtained from plant sources. Many studies revealed that chemo protective properties of plants that are used in traditional medicines are thought to be safe and potential in targeted diseases. Plants naturally synthesis secondary metabolites which were now being investigated for their anticancer efficacy which leads to the development of novel clinical drug targets.

Andrographis is a member of the family Acanthaceae. They are widely distributed throughout the tropical Asian countries. It is used extensively as an anticancer herb in traditional systems of medicine and also as a home remedy for various diseases in India. Native population of this genus are wide spread throughout the southern parts of India. They are used widely by the traditional healers for the treatment of liver disorders, herpes, fever, hepatitis and other chronic disorders (Okhuarobo *et al.*, 2014). They were found to exert cytotoxic effects on

almost all the cell line tested with the underlying mechanism involving cell cycle arrest, anti-proliferation, apoptosis etc., (Islam et al., 2018). The present study species, *A. megamalayana* is reported recently to be endemic to southern Western Ghats of India (Gnanasek et al., 2016). As it belongs to the genus, *Andrographis*, a potential cluster of species with phylogenetic sources (Gnanasek et al., 2015) reveal the capacity of *A. megamalayana* in curing the diseases like cancer, liver disorders, hepatitis etc., (Saeed et al., 2015). In this context, it is known that there is no scientific validation in this species for its therapeutic property. Therefore, the outcome of the research in this species may be the first report and expecting to have more value in pharmaceutical industries.

In this study, the ethanolic leaf and stem extracts of *A. megamalayana* were evaluated for anti-proliferative, apoptotic gene expression by Caspase 3 and DNA fragmentation studies using human hepatocarcinoma (HepG2) cell line.

II. MATERIALS AND METHODS

Procurement and preparation of plant materials

The leaf and stem parts of *A. megamalyana* were collected from the Megamalai hills, Theni district, Tamilnadu, India. The authenticity of the selected plant materials were duly identified and confirmed by comparison with the reference specimens preserved at Botanical Survey of India, Southern Circle, Coimbatore, India. The voucher specimens [Vide no: BSI/SRC/5/23/2017/Tech/2900] were lodged in the department herbarium for further reference. The plant materials were cleaned, washed with copious amounts of distilled water, shade dried, chopped into bits, and coarsely powdered in a Willy mill [Nippon electrical, Chennai, India] to 60-mesh size for extraction.

Preparation of crude plant extracts

Fifty grams of coarsely powdered plant samples were exhaustively extracted with ethanol using Soxhlet apparatus. The extracts were filtered and concentrated to dryness under reduced pressure using rotary vacuum evaporator (RE300; Yamato, Japan) and the lyophilized powders were stored at 20°C until used directly for the assessment of anti-proliferative activities.

Determination of *in vitro* anti-proliferative screening by MTT assay

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0×10^5 cells/ml using DMEM medium containing 10% FBS. To each well of a 96 well microtitre plate, 100 µl of the diluted cell suspension (approximately 10,000 cells/well) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, the monolayer was washed once with medium and 100 µl of different test sample concentrations prepared in maintenance media were added per well to the partial monolayer in microtitre plates. The plates were then incubated at 37°C for 48 hrs in 5% CO₂ atmosphere, and microscopic examination was carried out and observations were recorded every 24 hours. After 48 hours, the sample solutions in the wells were discarded and 20 µl of MTT (2mg/ml) in MEM-PR (MEM without phenol red) was added to each well. The plates were gently shaken and incubated for 3 hours at 37°C in 5% CO₂ atmosphere. The supernatant was removed and 50 µl of iso-propanol was added and the plates were shaken gently to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540nm. The percentage of growth inhibition was calculated and concentration of drug or test samples needed to inhibit cell growth by 50% values were generated from the dose-response curves for each cell line.

Induction of Apoptosis

Gene Expression assay by Caspase 3

HEP-G2 cell was plated in 6 well plates at a density of 5×10^5 cells/well and grown for 24 hours. Various concentrations of samples with solely added DMSO (solvent) for the control regimen were grown at 37° C in a humidified 5% CO₂ for 12 h. About 5×10^5 cells were lysed in lysis buffer (1% Triton X-100, 0.32 M sucrose, 5mM EDTA, 10 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 1 mM PMSF, 1 lg/ml aprotinin, 1 mg/ml leupeptin) for 30 min at 4°C followed by centrifugation at 10, 000g for 20 min. For caspase-3 activity determination, 50 ul reaction mixtures with SYBRgreen substrate peptides was specifically used for caspase-3. The substrate peptide (200 uM) was incubated at 37°C with cytosolic extracts in reaction buffer (100 mM HEPES, 10% sucrose, 10 mM DTT, 0.1% 3-[3-chloamidopropyl] dimethylammonio) 1-propanesulfonate. The results were determined in fluorescence colour which was observed after 2 h (excitation wavelength, 400 nm; emission wavelength, 505 nm) with a fluorescence plate reader.

RNA isolation

Total RNA was extracted from HepG2 cells that are treated with ethanolic leaf and stem extracts of *A. megamalayana* and vehicle (0.1 % DMSO) for 24 h using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. RNA samples were properly dissolved by incubating at 55°C for 10 min. DNA contamination was removed by treatment with DNase I (Invitrogen #18047019) for 30 min at 37°C followed by heat inactivation of DNase I prior to quantification using spectrophotometer. The purified RNA was stored at -80°C until further use.

cDNA synthesis and real-time PCR

First strand cDNA synthesis was done using iScript™ cDNA synthesis kit (Biorad#170-8897) by following manufacturer's protocols. The cDNA obtained was used to perform real-time qRT-PCR. Real-time PCR was performed using SYBR Green qPCR Supermix as per the manufacturer's recommendations. Amplification was carried out in a Rotor-Gene200 with SYBR Green detection and melt curve analysis. Primers used Sequences Caspase-3-F: 5' AATTCAAGGGACGGGTCATG-3'; Caspase-3-R: 5'- GACACAATACACGGGATCTG3' are specific for caspase-3. Reaction mixtures contained 10 ng cDNA, SYBR Green qPCR Supermix UDG, 300 nM each of forward and reverse primer and final volume of 15 µL. All Samples were investigated and the melting Curves obtained after each PCR amplification confirmed the specificity of the SYBR green assay.

DNA fragmentation analysis

The monolayer cell culture was trypsinized and cell count was adjusted to 1×10^5 cells/mL using DMEM containing 10% Fetal bovine serum (FBS). In each well of 6-well plate, 2 mL of cell suspension was added and incubated at 37°C for 24 hours in an atmosphere of 5% CO₂. After 24 hours, when a partial monolayer was formed, the supernatant was discarded and 2 mL of drug dilution prepared in maintenance medium and having concentration below its average, IC₅₀ was added to each well in duplicate. The plates were kept for incubation at 37°C in an atmosphere of 5% CO₂. After 24 hours of exposure, cells were harvested by trypsinization and centrifuged for 5 minutes at 3000 rpm. Supernatant was discarded; the cell pellet was dissolved in 1 mL of digestion buffer and 50 µL of proteinase K. The mixture was incubated at 45°C overnight for digestion. DNA was extracted with an equal volume of phenol/chloroform using the standard phenol/chloroform extraction procedure. The aqueous phase was separated and treated with 40 µL of RNase at room temperature for 2 hours. The impurities were removed with equal volume of chloroform. After gentle shaking, aqueous phase was separated and the chloroform layer was discarded. DNA was precipitated by addition of 1 to 1.5 mL of ice-cold ethanol. The DNA was pelleted by centrifugation at 10,000 rpm for 5 minutes at 4°C and the pellet was re-suspended in 100 µL of TE buffer. The DNA was dissolved in 100 µL of TE buffer (10 mM Tris HCl and 1 mM EDTA, pH 10.0) and electrophoresis of the DNA (at 100 V for 2 hours) was carried out on 1.5% agarose gels at 65 V for 45 minutes or until tracking dye moved to the bottom. The bands were visualized by staining with ethidium bromide.

Statistical analysis

For *in vitro* anti-proliferative activity of the extracts, the results were recorded as mean $\pm$ standard deviation (mean $\pm$ S.E.M.) (n = 3) and subjected to one way analysis of variance (ANOVA) followed by post hoc Duncan's multiple range test using SPSS latest version. $p < 0.05$ was chosen as the criterion for statistical significance.

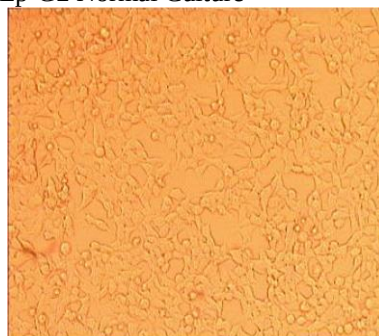
III. RESULTS

Anti-proliferative screening

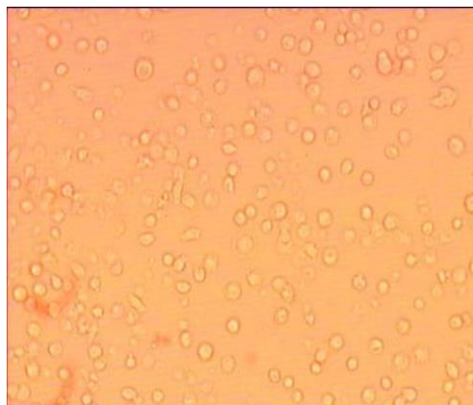
The cell viability and *in vitro* cytotoxic effect was performed to elucidate the efficacy of crude ethanolic extract of *A. megamalayana* leaf and stem against human liver cancer (HEp G2) cell line. The ability of the cells to survive a toxic insult has been the basis of the anti-proliferative assay. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The cleavage of MTT to a blue formazan derivative by living cells is the basis of this assay. In this study, the efficacy of the studied plant extract on proliferation in Hep G2 cells were depicted and presented in represented in Figure 1, 2 & 3. Results revealed that after 48 hours of incubation, the cell viability of ethanolic leaf extract of *A. megamalayana* evidenced 30% of activity, while their stem extract contributed 43% of activity. Apparently, the cell viability of the cancer cell line reduced nearly to 70% after incubation with the ethanolic extracts of *A. megamalayana* leaf and stem for 48 hours. However, the activity was lower for ethanolic leaf extract compared to that of the stem extract. After 48 hours of incubation and at the concentration range of 50 - 250 µg / ml the IC₅₀ values for the leaf and stem extracts (Table 1) were 121.88 µg / ml and 165.85 µg / ml, respectively.

Fig 1: Anti-proliferative effect of the ethanolic leaf and stem extracts of *A. megamalayana* against Hep G2 cell line.

HEp-G2 Normal Culture



Ethanolic Leaf extract > 200 µg/ml



Ethanolic Stem extract > 200 µg/ml

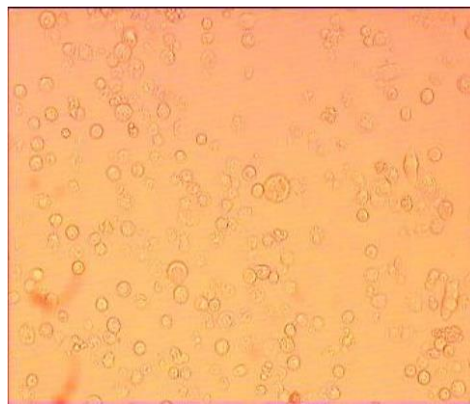


Table 1: Anti-proliferative studies for the ethanolic leaf and stem extracts of *A. megamalayana*.

S.No.	Sample	Hep-G2 IC ₅₀ (µg/ml)
1	LEAF	121.88
2	STEM	165.85

Fig 2: Percent cell viability for the ethanolic leaf extract of *A.megamalayana* at 250µg/ml concentration against Hep G2 cancer cell line.

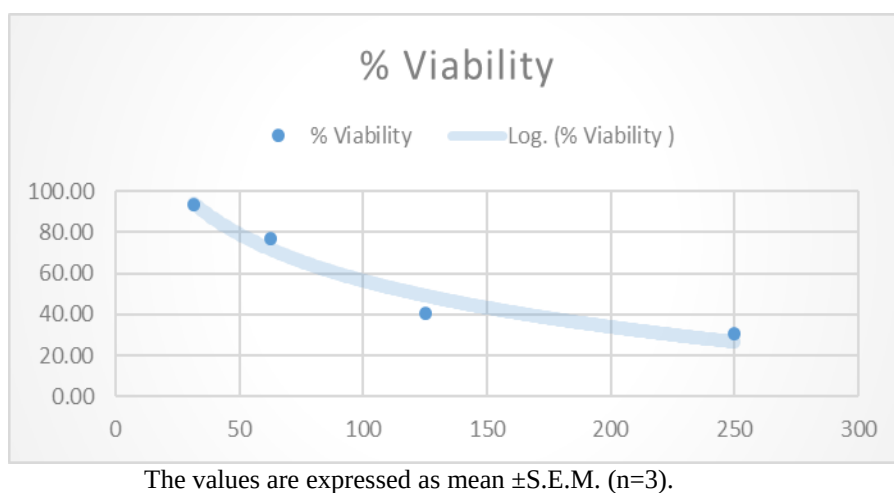
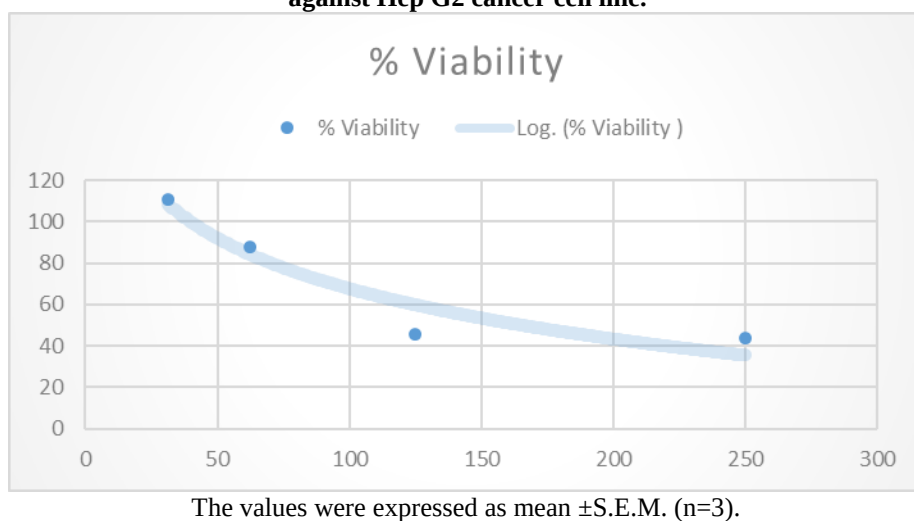


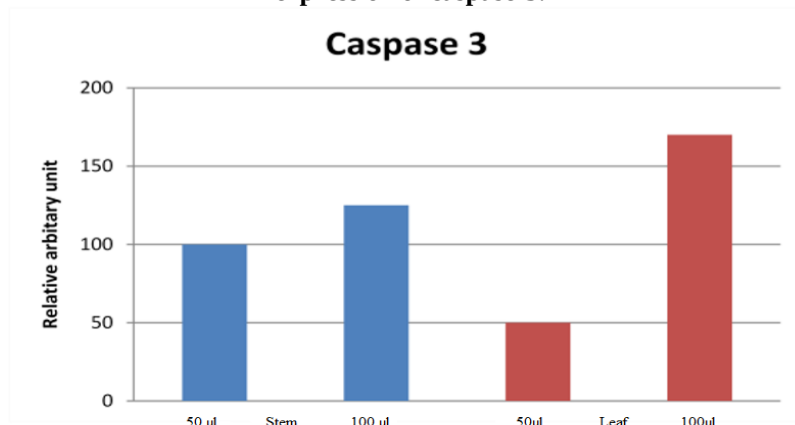
Fig 3: Percent cell viability for the ethanolic stem extract of *A.megamalayana* at 250µg/ml concentration against Hep G2 cancer cell line.



Gene expression by Caspase 3

Gene expression by Caspase 3 was performed in order to delineate the mechanism of cell death mediated by ethanolic leaf and stem extracts of *A. megamalayana*, which was characterized further for apoptosis. HepG2 cells were exposed to extracts for various periods of time and RT-PCR was performed to determine the expression of several apoptotic related gene expression. The evaluation of caspase 3 activity was carried out with two varied concentration of the test drug viz., 50 μ l and 100 μ l (Fig 4) which elevated the mRNA expression of Caspase 3. With the dose induced apoptosis and activation of caspase 3 gene, the results revealed that the ethanolic leaf extract at 100 μ g / ml concentration unveiled significant up regulatory effect on the expression of caspase3 gene than the ethanolic stem extract. However, ethanolic leaf extract at higher concentrations revealed positive response against the expression of caspase-3 gene.

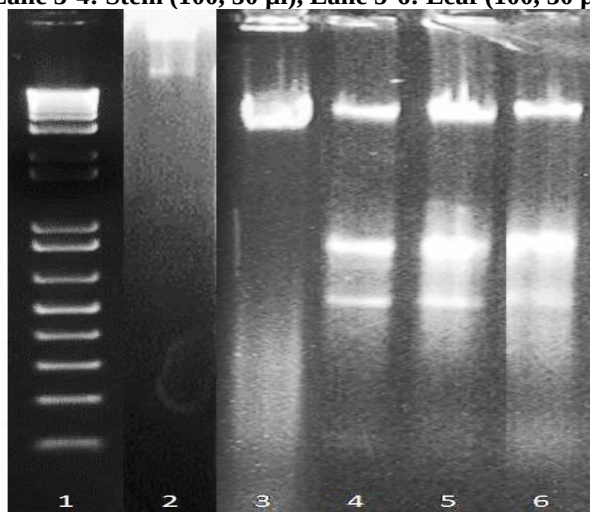
Fig 4: The effect of ethanolic leaf and stem extracts of *A. megamalayana* (50 μ l and 100 μ l) on protein expression of caspase-3.



DNA Fragmentation

DNA fragmentation is a hallmark feature of apoptosis. A DNA fragmentation assay was used to determine whether the action of the extract was associated with apoptosis or not. The DNA fragmentation was analysed using typical DNA ladder formation. The cells were treated with various concentration of extracts at various time intervals (24, 48 and 72 hours). The DNA was extracted from the apoptotic cells and were separated in an agarose gel. The results revealed that the extracts have great potential to degrade the DNA. Whereas, the ethanolic leaf extracts at 50 μ l and 100 μ l concentration clearly visualize degradation of chromosomal DNA into small internucleosomal fragments which is the clear evidence that the cells underwent apoptosis (Fig 5). DNA analysis also revealed typical ladders early at 48 and 72 hours after treatment, which indicates the apoptosis.

Fig 5: Lane 1: DNA Ladder (1kb), Lane 2: Negative control, Lane 3-4: Stem (100, 50 μ l), Lane 5-6: Leaf (100, 50 μ l).



IV. DISCUSSION

Anti-proliferative screening

Anti-proliferative screening is a technique used for the preliminary assessment of the plant extract for anticancer property. It is based on one or more mammalian cell lines being grown under controlled conditions where they actively undergo mitotic division. In our experiment, *A. megamalayana* was found to substantially decrease the MTT uptake of the studied human liver cancer cell line. The results obtained from this study would be useful in understanding the inhibitory mode of *A. megamalayana* which can induce cell death of HepG2 cells via apoptosis.

In the present study, *in vitro* anti-proliferative potential of crude ethanolic leaf and stem extracts of *A. megamalayana* against human liver cancer cell line HepG2 has been evaluated and summarized (Table 1 and Fig.1, 2&3). As judged in the microculture tetrazolium test (MTT), out of the two extracts examined, ethanolic leaf extract strongly and specifically inhibited the proliferation of the cell line tested. The anti-proliferative effect may be due to the release of cytochrome c from mitochondria, prominent to apoptosis. In a similar fashion notable observations were reported for the ethanolic extract of *A. paniculata* leaf extract treated for 24h on HEp2 cell line resulted in a dose and time dependent inhibition of growth (Padmalochana, 2017), whereas in another report the ethanolic leaf extract of *Garcinia cowa* inhibited the growth of cancer cell (Wahyuni et al., 2015). Likewise, Aqueous leaf extract of *Pandanus odoratissimus* also exhibited anti-proliferative effect against calu-6 cell line (Raj et al., 2014). Naveen et al, 2011 reported that leaf extract of *C. punctatum* served as a potential source for proliferation of PBMCs cell line. The plant extracts of *Bidens pilosa*, *Centella asiatica*, *Cnicus benedictus*, *Dicoma capensis* were reported to possess inhibitory activity against MDA-MB-231, MCF-7, and MCF-12A cell line (Steenkamp and Gouws, 2006). Subsequently it is evident that the observed anti-proliferative activity of the study plant might be due to the fact that the presence of phytochemicals could therefore have directly inhibited the proliferation of cancer cells *in vitro*, via cell cycle arrest by synergistically.

Induction of Apoptosis

Gene expression by Caspase 3

Caspase-3 is a vital initiator of apoptosis and so it was surprising that Caspase-3 deletion upsurges apoptosis. Caspase-3 is the biomarker which is used as a parameter for the study. Caspase-3 is a protease that is most often associated with cell death which catalyzes many cellular proteins and alter the morphology of nucleus and the process of apoptosis. The result of this study indicates that the ethanolic leaf extract at higher concentration express higher caspases 3 activity (Fig4) in comparison with that of the stem extracts. This fact evidences the existence of caspase-like proteases in plants. The similar research conducted by Abdullah et al., (2017) also demonstrated that *Annona muricata* leaf extract at 10 µg/ml concentration depicted higher caspase-3 activity than that of the tested standard. In another study also the methanolic leaf extracts of the plant species, *Holarrhena floribunda* induced concentration-dependent increase in caspase-3 activity against HeLa cell lines Badmus et al. (2015). And Liang et al. (2018) suggested that the occurrence of apoptosis by the expression of cleaved caspase-3 was significantly increased in *Pinnigorgia* sp at 6 µg/mL concentration when treated with HSC-T6 cell line. The studies manifest that the gene expression elevates with the increase in concentration of plant extracts in cancer cell lines.

DNA Fragmentation analysis

The DNA laddering technique is used to visualize the endonuclease cleavage products of apoptosis (Wyllie, 1980). This assay involves abstraction of DNA from a lysed cell homogenate followed by agarose gel electrophoresis. This results in a distinctive "DNA ladder" with every band in the ladder detached in size by approximately 180 base pairs. Hence, DNA gel electrophoresis method was used to regulate the conceivable mode of cell death caused by 50 µl dose of ethanolic leaf extract of *Andrographis megamalayana* as represented in Fig 5. Similarly crude leaf extracts of *Andrographis paniculata* also exhibited potent apoptogenic activity in DNA fragmentation (Saengkhae, 2008). Also Fieber et al.(2013) also stated that greater DNA ladder suggested greater apoptosis in detached cells. The notable activity was determined for the studied plant species in a dose-dependent increase of apoptotic fragments at the tested doses of *C. sativus* (Samarghandian et al., 2013). Based on the fact it can be presumed that *Andrographis megamalayana* expressed significant apoptotic effects against HepG2 cancer cell lines.

Conclusion

Andrographis megamalayana was found to possess anticancer properties against human liver carcinoma HepG2 cell line and it could be used as potent drug candidate to be used in pharmaceutical preparations. It is recommended that the extracts can be used as a supportive therapy for the treatment of cancer which selectively exterminates the cancer cells rendering protection to normal cells. The ethanolic leaf extract enhanced profound caspase-3 and DNA fragmentation analysis activity than its stem counterpart. However, further research is needed to determine the active fraction of *A. megamalayana* leaves on anti-proliferative, caspase-3 and DNA fragmentation. It will enrich the scientific validation of *A. megamalayana* leaf as a potential apoptotic agent for liver cancers.

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