***In vitro* assessment of apoptosis induction and cell cycle arrest in colon cancer cell line of a chemically analyzed *Hypericum triquetrifolium* extract**

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**Abstract**

Natural derived drugs are desired for anticancer treatment and some plant products are being used to treat cancer. This *in vitro* study aimed to investigate the role of apoptosis, cell cycle modulation and cell cycle arrest in the observed *Hypericum triquetrifolium* extract (HTE)-induced cytostatic effects in human colon cancer cell line, HCT-116. We observed that 50% ethanol (in water) HTEinduced cell death via an apoptotic process as assayed by Annexin V-Cy3 assay. Exposing HCT-116 to 0.064, 0.125, 0.25 and 0.5 mg/ml for 24 hours led to 50%, 71.6%, 85% and 96% apoptotic cells respectively. HCT-116 cells treated with 0.25 and 0.5 mg/ml HTE, for 3 hours resulted in 38.9% and 57.2% cleavage of caspase-3-specific substrate respectively. RT-PCR analysis revealed that HTEextract had no effect on mRNA levels of Apaf-1 and NOXA. Moreover, we clearly demonstrated that 0.125 mg/ml and 0.25 mg/ml THE for 24 hoursattenuated the cell cycle progression machinery in HCT-116 cells. GC/MS analysis of the extract identified 51 phytochemicals, some are reported as apoptosis inducers and cell cycle arrest agents. These results suggest that HTEinduced apoptosis of human colon cells is mediated primarily through the caspase-dependent pathway Taken together, HTEseems to be a potent therapeutic agent for colon cancer growth.

**Introduction**

Cancer is the second leading cause of mortality in humans worldwide. It is estimated that one in 3 women and one in 2 men in the United States will develop cancer in their lifetime. Increases in the number of individuals diagnosed with cancer each year, due in large part to aging and growth of the population, as well as improving survival rates, have led to an ever-increasing number of cancer survivors {Mullan, 1985 #2;Wang, 2012 #1}. Colorectal cancer (CRC) is the second leading cause of cancer death. The development of colorectal cancer proceeds through a series of genetic alteration involving the activation of oncogenes and loss of tumor suppressor genes. The first step in colon carcinogenesis involves loss of functional APC (Adenomatous Polyposis Coli) gene, which is a tumor suppressor gene followed by inactivation of the p53 gene (a sensor essential for the checkpoint control that arrests cells with damaged DNA in the G1 phase), resulting in formation of polyps on the inside of colon wall. Although much has yet to be learned about why some individuals develop colon cancer and others do not, certain genetic and environmental factors are known to increase a person’s chance of developing the disease {Mullan, 1985 #2} {Bedi, 1995 #3}.

Surgery, chemotherapy, and radiotherapy, either alone or in combination, have been considered conventional strategies for cancer treatment in the last century. With the rapid development of molecular medicine, novel therapeutic approaches, such as immunotherapy, molecular targeted therapy, and hormonal therapy, have been proposed to improve clinical outcome for cancer patients. However, those therapeutic approaches are not always effective and clinical outcome in survival rates is still poor {Mullan, 1985 #2;Wang, 2012 #1}

There has been a substantial increase in the use of complementary and alternative medicines, including dietary supplements and medicinal plants for cancer treatment. Several *in vitro*, cellular, and animal studies have evaluated the effects of herbal and other specialty products on the development and progression of colorectal cancer {Satia, 2009 #4}

The use of agents targeting the cell cycle machinery has long been considered as an ideal strategy for cancer therapy. These drugs target the abnormal expression of CDKs, mitotic kinases/kinesins, or affect the cellular checkpoints, resulting in cell-cycle arrest and subsequently induction of apoptosis in cancer cells. Cell cycle-based agents can be grouped into categories that reflect their molecular targets such as CDK inhibitors: inhibition of CDKs would selectively block tumor growth without compromising normal cells, checkpoint inhibitors: Targeting the S and G2 checkpoints and mitotic inhibitors {Chen, 1999 #6;Mahadevan, 2011 #7;Schoffski, 2009 #5}

Apoptosis induction is a useful mechanism to modulate cancer progression especially when there are mutations that alter the ability of the cell to undergo apoptosis and allow transformed cells to keep proliferating rather than dying. It would be therapeutically advantageous to tip the balance in favor of apoptosis over mitosis in tumors, if possible. The progressive accumulation of genetic alterations (APC, p53 and ras) governs the transition of normal colorectal epithelium to adenocarcinomas {Fearon, 1990 #8}

Despite the great progress in modern medicine, traditional medicine has always been practiced{Zaid, 2012 #9}. Herbal medicine such as Garlic, onion, black seeds and olive oil and leaf as well as *H. triquetrifolium* are prescribed for cancer treatment and prevention {Chandra, 2010 #11;Volanis, 2010 #10} The safety and effectiveness of alternative medicine is not always scientifically proven. Based on knowledge of traditional herbal medicine and on our preliminary studies, this *in vitro* study aims to investigate the role of apoptosis, cell cycle modulation and cell cycle arrest in the observed *H. triquetrifolium* extracts- induced cytostatic effects in colon cancer cell line HCT-116.

**Materials and Methods**

**Materials**

Cells of the human colorectal cell line HCT-116 was purchased from (ATCC, CCL-247), and all tissue culture reagents including fetal bovine serum and standard culture medium RPMI-1640 were purchased from Biological Industries (Beit Haemek, Israel). MTT reagent (sigma Aldrich), LDH kit (Promega, WI, USA), cell cycle kit (Thermo Fisher, USA), RNA isolation kit (QIAGEN), all other materials were purchased from Sigma Aldrich.

**Preparation of plant extracts**

*H. triquetrifolium* (aerial parts) were purchased from (Al Alim- Medicinal Herb Center, Zippori, Israel). One hundred grams of air-dried plant (aerial part) material was added to 1L of 50% EtOH (in water) and boiled for 30 min then stirred for 24 hours at room temperature. The extract thus obtained was filtered using filter paper, aliquot and frozen at -800C until use {Kadan, 2018 #13;Saad, 2005 #12}

**Silylation Derivatization**

Sample of the dried extract was re-dissolved and derivatized for 90 min at 3700C (in 40 μl of 20 mg/ml methoxyamine hydrochloride in pyridine) followed by a 30 min treatment with 70 μl MSTFA [N-methyl- N(trimethylsilyl)trifluoroacetamide] at 370C and centrifugation. One microliter of the derivatized sample was injected into the gas chromatograph coupled with mass selective detector (GC/MS). {Mintz-Oron, 2008 #54}

**Gas Chromatography-Mass Spectrometry Analysis**

The GC/MS system was comprised of a COMBI PAL autosampler (CTC analytics), a Trace GC Ultra gas chromatograph equipped with a PTV injector, and a DSQ quadrupole mass spectrometer (ThermoElectron Cooperation, Austin, USA). GC was performed on a 30 m x 0.25 mm x 0.25 μm Zebron ZB-5ms MS column (Phenomenex, USA). The PTV split technique was carried out as follows: sugars were analyzed with the split 1:100, and the lower-abundance compounds were analyzed with the split 1:10. PTV inlet temperature was set at 45°C, followed with a temperature program: hold at 45°C for 0.05 min, followed by raising to 70°C with ramp rate of 10°C/sec, hold at this temperature for 0.25 min, then followed by transfer-to-column stage (raising to 270°C with ramp rate of 14.5°C/sec; hold at 270°C for 0.8 min), and followed by a cleaning stage (raising to 330°C with a ramp rate of 10°C/sec; hold at 330°C for 10 min). The interface was heated to 300°C, and the ion source was adjusted to 250°C. Helium was used as the carrier gas at a flow rate of 1.2 ml/min. The analysis was performed under the following temperature program: 1 min isothermal at 40°C, followed by a 15°C/min ramp to 320°C, and then holding this temperature for 4.5 min. Mass spectra were recorded at 9 scans/sec with an m/z 40–450 scanning range from 5 till 10 min, and with an m/z 50–600 scanning range from 10 till 24 min. For the analysis of the lowabundance compounds the filament was switched off from 12.95 till 13.60 min. in order to prevent damage to the MS detector from the high concentration sugar compounds. {Mintz-Oron, 2008 #54}.

**Identification of Components**

Phytochemical compounds were putatively identified by correlation of their RI and mass spectrum to those present in the mass spectra library of Max-Planck-Institute for Plant Physiology, Golm, Germany (Q\_MSRI\_ID, http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd\_msri.html) and the commercial mass spectra library NIST05 (http://www.nist.gov/). The response values for metabolites resulting from the Xcalibur processing method were normalized to the ribitol internal standard {Kadan, 2016 #14;Mintz-Oron, 2008 #54}.

**Cell culture**

Cells of the human colorectal cell line HCT-116 (ATCC, CCL-247) were grown in RPMI-1640 medium (Roswell Park Memorial Institute) with high glucose content (4.5 g/l), supplemented with 10% vol/vol fetal calf serum, 1% nonessential amino acid, 1% glutamine, 100 U/ml penicillin. Cells were maintained in humidified atmosphere of 5% CO2 at 370C.

**MTT (cell viability)**

The tetrazolium dye, MTT, is a colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to the purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells. 20,000 cells were seeded per well of 96-microtiter plates. Twenty-four hours after cell seeding, cells were incubated with increasing concentrations of plant extracts(0-1000 g/mL) for 24 at 37°C. Cells were then washed in phosphate buffered saline and incubated in serum-free RPMI to which 0.5 mg/mL MTT was added to each well (100 µL), and incubated for four hours. Then the medium was removed and the cells were incubated for 15 minutes with 100 µL of acidic isopropanol (0.08 N HCl**)** to dissolve the formazan crystals. The absorbance of the MTT formazan was determined at 570 nm in an Elisa reader. Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells {Kadan, 2013 #17}

**Lactate Dehydrogenase**

The Lactate dehydrogenase (LDH) assay measures the leakage of the cytoplasm located enzyme LDH into the extracellular medium as an indicator for the plasma membrane rupture. LDH activity was measured in both the supernatants and the cell lysate fractions using CytoTox 96, a non- radioactive cytotoxicity assay kit (Promega, WI, USA) in accordance with the manufacturer’s instruction. The absorbance was determined at 490 nm with 96-well plate ELISA reader {Kadan, 2013 #17}

**Apoptosis detection**

HCT-116 cells were seeded in 24 well plate and incubated with *H. triquetrifolium* (0-500g/ml) extract for 24 h followed by apoptosis detection by Acridine Orange (green) and Annexin-V CY3 (red) according to the manufacture protocol. Annexin V-Cy3 Apoptosis Detection Kit (abcam) is based on the observation that soon after initiating apoptosis, cells translocate the membrane phosphatidyl-serine (PS) from the inner face of the plasma membrane to the cell surface. Once on the cell surface, PS can be easily detected by staining with a fluorescent conjugate of Annexin V, a protein that has a high affinity for PS. The detection was determined by fluorescence microscopy {Saydam, 2005 #18}

**Caspase-3 activity assay**

HCT-116 treated as described in the apoptosis detection method and caspase 3 activity was detected according to the manufacture protocol. Caspase 3 assay, Colorimetric (Sigma-Aldrich), is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase 3, resulting in the release of the p-nitroaniline (pNA) moiety. The concentration of pNA is calculated from the absorbance values at 405 nm with 96-well plate ELISA reader {Masters, 2001 #19}

**Cell cycle assay**

HCT-116 treated as described in the apoptosis detection method. The cells were treated with FUCCI staining (Premo FUCCI Cell Cycle Sensor, BacMam 2.0) according to manufacturer’s instructions in order to detect the cell cycle stage. This indicator employs a red (RFP) and a green (GFP) fluorescent proteins fused to different regulators of the cell cycle, Cdt1 and geminin. In the G1 phase of the cell cycle, only Cdt1 tagged with RFP may be visualized, thus identifying cells in the G1 phase with red fluorescent nuclei. In the S, G2 and M phases, Cdt1 is degraded and only geminin-tagged with GFP remains, thus identifying cells in these phases with green fluorescent nuclei. During the G1/S transition both proteins are present in the cells, allowing GFP and RFP fluorescence to be observed as yellow fluorescence {Napoli, 2013 #20}.

**FACS analysis**

HCT-117 cells were seeded in 24 well plate and a day after they were treated with the plant extract (0-250g/ml) for 24 h, trypsinized and fixed with 70% ethanol. After fixation, cells were centrifuged and incubated for 4 min at 1500 rpm at 4°C. Then, the cells were suspended with 5 mg/ml propidium iodide and 50 μg/mL RNase A. After incubation for 20 min at room temperature, fluorescence intensity was analyzed using Bacton Dickinson flow cytometer {Hershko, 2004 #21}.

**Total RNA isolation and cDNA synthesis**

HCT-116 treated as described in the apoptosis detection method. Total RNA was isolated from cells using Rneasy Plus Mini Kit (QIAGEN) according to manufacturer’s instructions, and immediately frozen at -800C until use. DNase-treated RNAs was used to synthesize cDNA with the Transcriptor First Strand cDNA Synthesis Kit using random hexamers as specified by the manufacturer (Maxima First Strand cDNA Synthesis Kit for RT-PCR by Thermo).

Real time PCR amplification (RT-PCR) and advanced relative quantification analysis was achieved using a Light Cycler 480 instrument (Roche Applied Science) with software version LCS480 1.5.039. All reactions were performed in duplicates with the light Cycler Fast Start DNA Master SYBER Green I Kit (Roche Applied Science) in a final 20 µl volume with 2.5 mM MgCl2, 0.2 µM of each primer and 2 µL cDNA. Amplification conditions consisted of an initial pre-incubation at 95°C for10 min (polymerase activation) followed by amplification of the target cDNA for 45 cycles (95°C for 15 s, 60°C for 20 s and extension time at 72°C for 30 s) {Hershko, 2004 #21}.

**Statistical Analysis**

Error limits were cited and error bars were plotted and represent simple standard deviations of the mean. When comparing different samples, results were considered to be statistically different when P < 0.05. Statistical calculations were conducted using SPSS version 21.0.

**Results**

**Cell viability measurements**

The effects of HTE on cell viability were evaluated here in HCT-116 colon cancer cell line by MTT and LDH assays. Cells seeded in 96 well plates (20,000 cell/well) were exposed to increasing concentrations (0-1 mg/ml) of HTE for 24h. Concentrations that caused less than 10% cell death were considered as a non-toxic. HTE were non-toxicup to 0.5 mg/ml, the IC50 was about 1mg/ml (Fig.1). Concentrations higher than 0.5 mg/ml caused a significant reduction in the cell viability.

Membrane integrity can be evaluated by measuring LDH activity. Lactated dehydrogenase, an enzyme located in the cytoplasm, catalysis the conversion of lactate and pyruvate. When LDH is found in the media of the cells, there are two possible causes: the first is cellular death and the second is a leak in a cell membrane. HCT-116 cells seeded in 96 well plate (20,000 cell/well) were exposed to increasing concentrations (0-1 mg/ml) *HTE* for 24h. Concomitant with the MTT results, no significant changes in LDH levels were detected in the culture medium after exposure to extracts of *HTE* at concentrations up to 0.25 mg/ml (Fig.2).

Based on MTT and LDH results, *H.triquetrifolium* was used up to0.5 mg/ml in the following experiments.

**Apoptosis detection**

Annexin-V is capable of detecting cells in early apoptosis stages via membrane-associated processes, by binding to the phosphatidylserine (PS) head groups. Annexin test was performed on HCT-116 cell line after exposure to 0.064, 0.125, 0.25, 0.5 mg/ml of *HTE*. Characteristically, Annexin-V binds only to the surface of the HCT-116 membrane in apoptotic cells, where it binds to the exposed Phosphatidylserine head groups. As shown in Fig.3B, when compared with control treated cells, treatment of HCT-116 cells with *THE*  (0.125 and 0.25 mg/ml for 24 hours) resulted in a significant increase of apoptosis (red membrane). Indeed, *HTE* induced 50 ± 9 %, 71.6±8%, 85 ± 5 % and 96 ± 1.5 % apoptosis at 0.064, 0.125, 0.25 and 0.5 mg/ml (Fig.3A and 3B). The putative apoptosis inducers compounds structure and names are shown in Fig. 3C. Most of the compounds are fatty acids and phenols.

To further assess *HTE* competency to induce apoptosis, its effect on caspase-3 activation in HCT-116 cells was analyzed. As shown in Figure 4, treatment of HCT-116 cells with 0.25 and 0.5 mg/ml *HTE*, for 3 hours resulted in 38.9 ±1.5% and 57.2 ± 3% cleavage of caspase-3-specific substrate respectively. Staurosporine (1 M) led to 100% apoptosis. These results are in line with the previous Annexin V-Cy3 apoptosis detection assay. Taken together, the results indicate that *HTE* capacity to induced apoptosis in HCT-116 cells is mediated at least in part through the activation of caspase-3.

**mRNA Levels of Apaf-1 and NOXA**

Next, we examined the effect of *HTE* on the expression of Apaf-1 and NOXA, two proteins involved to the intrinsic apoptosis pathway. Their mRNA levels were detected by real time PCR (RT-PCR) and normalized to GAPDH levels. RT-PCR analysis point out no effect for *HTE* on Apaf-1 and NOXA mRNA levels (data not shown).

**Cell cycle modulation**

The effect of *HTE* on HCT-116 cell cycle was tested using FACS analysis as described in the methods. Cells were exposed to 0.125 and 0.25 mg/ml at 4, 8, and 24 hours. Treatment of HCT-116 cells with *HTE* at concentrations of 0.125 and 0.25 mg/ml for 24 hours resulted in significant higher level of subG1 phase (22.2 and 22.6% respectively), compared with non-treated cells (0.39%) (Fig. 5). No significant changes in the percentage of cells in S and G2-M phases were recorded. Treatments for 4 and 8 hours with *HTE* did not have a significant effect on cell cycle distribution and cell viability. For further examination of the role *HTE* on cell cycle modulation, FUCCI Cell Cycle Sensor assay was applied. HCT-116 cells challenged with 0.125 and 0.25 mg/ml *HTE* for 24 hours were arrested at G1 phase (red cells) (Fig. 5B). The putative cell cycle arresting compounds are mainly Terpenoids, phenols and fatty acids (Fig. 5C). These compounds are also known as apoptotic inducers except for Dihydroxyacetone (Table 1).

**GC-MS analysis of the *H.triquetrifolium* extract**

The phytochemicals profile of *HTE* was complemented by GC/MS metabolite profiling of derivatized extracts as described in the methods. A total of 51 identified metabolites were monitored (Table 1). Most of the compounds are terpenoids, organic acids, alcohols and sugars. Interestingly, 21 compounds detected here are associated with either anti-cancer, apoptosis induction and cell cycle arrest activity (Table 1). All together these potential active compounds consist 23.2% out of the total amount of the detected chemicals.

**Discussion**

The evaluation of herbal medicines may indicate novel strategies for the treatment of colorectal cancer, which remains the second leading cause of cancer death. Herbal medicine such as Garlic, onion, black seeds, olive oil and leaf and *H. triquetrifolium* are prescribed for cancer treatment and prevention {Chandra, 2010 #11;Volanis, 2010 #10}

In this *in vitro* study, *HTE* was non-toxicup to 0.5 mg/ml. Our study indicated that treatment of colon cancer cell line (HCT-116) with *H.triquetrifolium* extracts resulted in significant induction of apoptosis as detected by Annexin-v staining. The apoptotic effects of *H.triquetrifolium* were further confirmed by measuring caspase-3 activity. Caspases are a family of proteases that mediate cell death and are important for the process of apoptosis. Activated caspase-3 is the key executioner of apoptosis progression. Treatment of HCT-116 cells with *H.triquetrifolium* extract resulted in a dose-dependent activation of caspase-3. Taken together, *H.triquetrifolium-* induction of apoptosis is mediated at least in part by caspase-3 activation.

The release of cytochrome c from the mitochondria serves trigger for the formation of the apoptosome, an oligomeric protein complex consisting of apoptotic protease-activating factor-1 (Apaf1), procaspase-9, and cytochrome c {Fadeel, 2008 #22}. This leads, in turn, to the activation of caspase-3. NOXA is a member of bcl-2 family and is described as a p53 target gene, serving as a candidate mediator of p53-induced apoptosis {Oda, 2000 #23}. We had selected both NOXA and Apaf-1 genes for further analysis of the mechanisms underlying extracts-induced apoptosis. However, RT-PCR analysis revealed that *H.triquetrifolium* extracts had no effect on mRNA levels of Apaf-1 and NOXA, suggesting that apoptosis induction was not mediated via the transcriptional regulation of these genes. Apoptosis is tightly regulated by anti-apoptotic and pro-apoptotic effectors molecules, and can be mediated by several distinct pathways. Gene expression is often interpreted in terms of protein levels. Production and maintenance of cellular protein requires a remarkable series of linked processes, transcription, processing and degradation of mRNAs to the translation, localization, modification and programmed destruction of the proteins themselves{Vogel, 2012 #24}. The induction of apoptosis by *H.triquetrifolium* extracts is, most probably, at a post-transcriptional level.

We also determined the effect of *H.triquetrifolium* extracts on the cell cycle progression. Our finding of a significant increase in the percentage of HCT-116 cells with a subG1 DNA content suggests that this extract induced apoptosis and thereby disrupted the uncontrolled cell cycle progression.

GC/MS of the *H.triquetrifolium* extract was examined to detect bioactive compounds. 21 compounds detected here (out of 51) are associated with either anti-cancer, apoptosis induction and cell cycle arrest activity. Most of the cell cycle arrest and apoptosis inducers detected compounds her are phenolic, terpenoids, fatty and organic acids and alcohols. Indeed, plants extract contains phenolic compounds or long chain fatty acids {Adebayo, 2019 #67;Intisar, 2012 #66} are reported as anticancer potential agents {Cevik, 2019 #69;Owen, 2000 #68}. The specific reported activity of each compound detected her is detailed in Table 1. Further studies are required to separate the detected compounds and asses their anticancer activity as a pure compound or either few separated compounds.

Further studies are needed to map the different genes and the specific cellular pathway that influence induction of apoptosis by *H.triquetrifolium*. Identification and isolation of agents that can induce apoptosis and cell cycle arrest in cancer cells is of high priority. *H.triquetrifolium* extract seem to possess a potent therapeutic activity for colon cancer via cell cycle arrest and apoptosis induction. Clearly, their effects of untransformed cells need to be further studied before they can be used as anti-cancer agents.

**Acknowledgments**

The authors would like to acknowledge The Association of Arab Universities Research Fund, Al-Qasemi and AAUP Research Foundations for providing their financial support. SK appreciates the “Ministry of Science Technology and Space” for the scholarship.

**Legends:**

**Fig.1:** MTT assay in HCT-116 cells after 24 hours treatment with varying concentration of HTE. The absorbance of the MTT formazan was determined at 620 nm using ELISA reader. Cell viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells. Values represent mean ±SD of three independent experiments carried out in triplicate. T-test of statistical calculations were conducted using SPSS version 21.0. \*p < 0.05 , \*\* p < 0.01, significant as compared with controls.

**Fig.2**: LDH leakage from HCT-116 cells after 24 hr incubation with varying concentration of HTE*.* The leakage of the cytoplasm-located LDH into the extracellular medium is measured. The absorbance was determined at 492 nm using ELISA reader. Values represent mean ±SD of three independent experiments carried out in triplicate. T-test of statistical calculations were conducted using SPSS version 21.0. \*p < 0.05, significant as compared with controls.

**Fig 3:** Determination of apoptosis induced by *HTE* in HCT-116 cells. Cells were exposed to the plant extract (up to 0.25 mg/ml) for 24 hours. Apoptosis was determined using Acridine Orange (green) and Annexin-V CY3 (red) staining assay and was monitored by fluorescent microscope. Apoptosis expressed as a percentage of treated cells to untreated cells. (A) Values represent mean ±SD of three independent experiments carried out in triplicate. T-test of statistical calculations were conducted using SPSS version 21.0. \*p < 0.05 , \*\* p < 0.01, significant as compared with controls. (B) Representative Fluorescent microscope images showing co-staining with Acridine Orange and Annexin-V CY3. (C) Chemical structure of the putative apoptotic inducers detected compounds.

**Fig.4**: Analysis of intracellular caspase-3 activity in HCT-116 cells post 3 hours post treatment with Staurosporine (1 M), *H.triquetrifolium* extracts. Cell lysates were combined with the caspase-3-specific substrate in a standard reaction buffer. Cleavage of caspase-3-specific substrate compared to the Staurosporine (STS, 1 M) treated cells. The absorbance was determined at 405 nm using ELISA reader. Values represent mean ±SD of three independent experiments carried out in triplicate. T-test of statistical calculations were conducted using SPSS version 21.0. \*\* p < 0.01 significant as compared with control.

**Fig 5:** Effect of *H.triquetrifolium* extracts on cell cycle distribution of HCT-116 cells. (A) Cellular DNA was stained with propidium iodide and flow cytometric analysis was done to determine the cell cycle distribution post treatment with *H.triquetrifolium* extracts for 4, 8, and 24 hours as described in the Methods. (B) Fluorescence images of HCT-116 cells using FUCCI Cell Cycle Sensor .Cells were treated with 0.125 and 0.25 mg/ml *H.triquetrifolium* extracts for 24 hours. FUCCI is designed for live- cell imaging of cell cycle progression through three critical phases; G0/G1(Red), G1/S (yellow), and G2/M (Green). (C) Chemical structure of the putative cell cycle arresters detected compounds.