Science Letters ISSN 2345-5463 – An International Triannually Journal

Review Article
April 2017 | Volume 5 | Issue 1 | Pages 93-104

ARTICLE INFO

Received
December 15, 2016
Accepted
February 12, 2017
Published
April 15, 2017

***Corresponding Author**
Sana Javaid Awan
E-mail
sana.javaidawan@yahoo.com

Keywords
Angiogenesis and apoptosis
Cassia fistula
Cancer
Plant extract

How to Cite
Awan SJ, Tariq K, Nasir GA, Batool W, Sheraz MA, Ahmed S, Maqbol T, Ali U, Ansari F. Cassia fistula seed extract enhances apoptosis in HeLa cell line via activating annexin V and p53. Sci Lett 2017; 5(1):93-104

Scan QR code to see this publication on your mobile device.

Open Access

Cassia fistula Seed Extract Enhances Apoptosis in HeLa Cell Line via Activating Annexin V and p53

Sana Javaid Awan*, Kanza Tariq, Ghazanfer Ali Nasir, Wajjiha Batool, Muneeb Ahmed Sheraz, Sajjad Ahmed, Tahir Maqbool, Usman Ali, Farheen Ansari

Institute of Molecular Biology and Biotechnology, The University of Lahore, Lahore, Punjab, Pakistan

Abstract

Cancer is one of the top causes of death in the prosperous countries. Conventional plants are a precious source of novel cytotoxic agents and are still performing superior role in health concerns. The study was aimed to evaluate the effect of *in vitro* anticancer activity of *Cassia fistula* (CF) seed extracts against the HeLa cell line and umbilical cord-derived mesenchymal stem cells (UC-MSCs). CF seeds were extracted using three solvents (ethanol, ethyl acetate and petroleum ether). For the estimation of anti-proliferation in HeLa cells and UC-MSCs, MTT assay and for cell viability, trypan blue and crystal violet assays were done. Angiogenic potential was checked via immunocytochemistry and ELISA of vascular endothelial growth factor (VEGF). Immunocytochemistry of annexin-V and p53 was performed for the estimation of apoptosis in HeLa cells and UC-MSCs. Furthermore, ELISA for annexin-V, lactate dehydrogenase (LDH) assay and antioxidant enzymes activity assays were also performed. The seed extract showed reduced viability, angiogenesis and proliferation of HeLa cells with increased apoptosis. Whereas, anti-oxidative enzymes showed higher activity in seed extract treated cancer cells as compared to untreated cells. It was observed that the CF seed extract could induce apoptosis and improve the antioxidant status of HeLa cells along with the inhibition of proliferation and angiogenesis, especially, when extraction was done with ethyl acetate.

Introduction

In spite of exceptional advances in the discovery and development of novel cancer therapeutics, cancer still the second leading cause of death worldwide whose uncontrolled progression, invasiveness makes it resistant to conventional treatment [1, 2]. The main reason behind the cancer cell formation and progression is an imbalance between proliferation and apoptosis [3, 4]. Cervical cancer is a very common form of cancer and a leading cause of subsequent death occurring in women [5, 6]. HeLa cells are used widely in all kinds of cervical cancer research and play an important role in the research of cervical cancer cell biology, diagnosis, and treatment of cervical cancer [1].

For a long time, plants derived compounds have been at the leading edge as important components of anticancer therapies and have assumed an indispensable part in the prevention and treatment of cancer, because of easy availability, and relatively low toxicity compared to chemotherapy [1, 7-9]. The compounds of plants can be readily administered orally as part of the patient's dietary intake. Naturally derived compounds from plants also generally more tolerated and non-toxic to normal human cells. There are more than 3000 plant species to treat cancer have been reported and about thirty plant-derived compounds have been isolated and tested in clinical trials for cancer [1]. *Cassia fistula* is a pharmaceutical plant that has a huge association with medicine and thought to be functioning as an antioxidant, antibacterial and anti-inflammatory agent [10]. Several findings related to *Cassia fistula* plant based on the animal model have confirmed the pharmacological safety and efficacy and have opened a new window for human health management.

Thus, the present study was designed for the demonstration of targeted therapy via, *Cassia fistula* seed extracts for the treatment of cancer cell line through the inhibition of proliferation and inhibition of angiogenesis in HeLa cells along with the induced apoptosis and oxidative stress.

Materials and Methods

Sampling of umbilical cord and HeLa cell lines

Umbilical cord-derived mesenchymal stem cells (UC-MSCs) were taken from the hospital after cesarean unit in a sterile container having normal saline added with 100U penicillin (GIBCO, USA) and 100µg/ml streptomycin (GIBCO, USA). The HeLa cell line was acquired from cell culture laboratory established at The University of Lahore. The HeLa cell line was preserved in the liquid nitrogen and restored for further use.

Isolation and culturing of UC-MSCs and HeLa cell line

The cord was taken and washed directly with normal saline to remove contamination. After washing, it was cut down into small pieces (explants). Further, the pieces were placed on the culture plate with the help of forceps. Dulbecco's modified eagle's medium high glucose (DMEM-HG) (Caisson's Lab, USA) containing 100U penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS) (GIBCO, USA) was added to explants and HeLa cells in a humidified atmosphere of 5% CO_2 at 37°C. The medium was changed after every two days until cells reached the stage of sub-culturing. When cultivated HeLa cells and UC-MSCs achieved 70-80% confluence, the cells were subjected to sub-culturing. Trypsinization with trypsin-EDTA (Caisson's Lab, USA) was done to make these cells detached from the surface. After sub-culturing, these cells were re-plated and propagated up to 2nd passage.

***Cassia fistula* seed extract**

The seed extract of *Cassia fistula* (CF) was acquired from Microbiology lab arranged in The University of Lahore. The CF seeds were extracted using three solvents separately: ethanol (CFS-E), ethyl acetate (CFS-EA) and petroleum ether (CFS-PE). Lyophilized types of these extracts were taken and 20 mg/ml stock arrangements were made by dissolving them in Dimethyl sulfoxide.

Treatment of HeLa cell line and UC-MSCs with plant extract

HeLa cell lines and UC-MSCs at 2nd passage were cultured in 6-well (~2x10^6 per well) and 96-well plates (~2x10^4 per well) for immunocytochemistry

and for cell proliferation and biochemical assays. The treatments were given as 1000 µg and 500 µg of the CF seed extract in DMEM (pre-treatment medium) according to the previously described method [11]. Both, HeLa cell lines and UC-MSCs were divided into four groups. One group for untreated UC-MSCs (UT-UCMSCs) and untreated HeLa cells (UT-HE) served as controls. Remaining three groups were UCMSCs and HeLa treated with CFS-E (T-UCMSCs-CFS-E and T-HE-CFS-E), UCMSCs and HeLa treated with CFS-EA (T-UCMSCs-CFS-EA and T-HE-CFS-EA) and UCMSCs or HeLa treated with CFS-PE (T-UCMSCs-CFS-PE and T-HE-CFS-PE). After 24 hours, 6 well plates were used for immunocytochemistry and 96 well plates were used for cell proliferation and viability assays, while after treatment, medium collected from 96 well plates and 6 well plates were used for biochemical analysis.

Cell proliferation and viability assays

MTT assay

For the evaluation of the proliferative potential of HeLa cells after treatment, 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT) (Invitrogen Inc., USA) assay was performed using a 96-well plate. A monolayer of cells was washed with phosphate buffer saline (PBS) and incubated in 100µl serum free DMEM medium and 25µl MTT solution (5mg/ml) for 2 hours. The purple color formazan crystals appeared were then solubilized with 10% sodium dodecyl sulfate (SDS) and absorbance was taken at 570 nm. Percentage viability was calculated by previous method [12].

$$\%\ \text{Cell viability} = \frac{\text{Experimental } (OD_{570})}{\text{Control } (OD_{570})} \times 100$$

Cell viability assays

Trypan blue was used for the evaluation of cell viability as an exclusion agent for alive and dead cells. The cells were washed with PBS three times leading to incubation in trypan blue (Invitrogen Inc., USA) for 15 minutes. After 15 minutes, cells were washed again with PBS three times and observed under a microscope. Cells stained with trypan blue were considered as dead. The cell viability percentage was calculated by dividing the number of cells excluding trypan blue with a total number of cells multiplying by 100.

For the crystal violet assay, the medium was discarded from 96-well plate and the plate was washed with PBS. 0.1% crystal violet dye mixed with 2% ethanol was added and incubated at room temperature for 15 minutes. The dye was discarded and washed carefully with water. After washing, 200µl of 1% SDS was added to solubilize the color absorbed by viable cells and left for 5-10 minutes. Absorbance was taken at 540 nm or 595 nm on a microtiter plate.

Lactate dehydrogenase (LDH) assay

LDH activity was estimated using pre and post treatment media by using LDH assay kit (AMP Diagnostics, Austria). The procedure adopted was, according to manufacturer's instructions. Briefly, 5µl medium was mixed with 100µl of working reagent in a 96 well plate and incubate for 5 minutes. Absorbance was taken by spectrophotometer at 340 nm.

Immunocytochemistry

HeLa cells and UC-MSCs plated onto 6 well plates were subjected to immunocytochemistry after 24 hours treatment. Treatment medium was removed and washing was given to each well 5 times with Tris buffer saline containing 0.05% tween 20 (TBS-T). Cells were fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature and washed to remove traces of PFA. Bovine serum albumin (BSA; 5%) was used to block nonspecific binding for 30 minutes. Another washing was given with TBS-T and now the cells were incubated with vascular endothelial growth factor (VEGF) (Santa Cruz Biotechnology, USA), annexin V (Santa Cruz Biotechnology, USA) and p53 (Santa Cruz Biotechnology, USA) rabbit polyclonal primary antibodies for 90 minutes at 37°C. Further, the cells were washed again and then incubate with FITC conjugated donkey anti-rabbit secondary antibody (Santa Cruz Biotechnology, USA) for 90 minutes at 37°C. Once more cells were washed and stained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich, USA) and incubated at room temperature for 15 minutes. Finally, cells were rinsed

with TBS-T and preserved in mounting medium. Later, the cells were observed under the FLoid® cell imaging station (Life Technologies, USA).

Enzyme-linked immunosorbent assay (ELISA)

Solid phase sandwich ELISA was performed for VEGF and annexin V (Santa Cruz Biotechnology, USA) in 96-well plate (Corning, USA). The plate was coated with VEGF and annexin V antibody and incubated for 120 minutes. After washing three times with TBS-T added 1% BSA for 30 minutes meant for blocking. After blocking, 200 µl sample (for VEGF, the post-treated medium was used while for annexin V, post-treated cell lysate was used) was loaded to each well and incubated for 60 minutes. The sample was removed and washed well three times, added horse reddish peroxidase (HRP) conjugated donkey anti-rabbit secondary antibody (Santa Cruz biotechnology, USA) and incubated for 120 minutes at 37°C. After washing, 100 µl of chromogenic solution 3, 3, 5, 5-tetramethylbenzidine (TMB) (Invitrogen Inc., USA) and 0.18 M (H_2SO_4) were added after 15 minutes to stop reaction then absorbance was taken at 450 nm.

Evaluation of antioxidant enzymes

Catalase (CAT) assay

Catalase assay was performed in 96-well plate. One well contained 50mM KH_2PO_4 (pH 7.0) and medium from experimental groups served as blank while other wells contained 12.5mM hydrogen peroxide (H_2O_2) in 50mM KH_2PO_4 (pH 7.0) and medium from different experimental groups. After keeping in dark for 45 to 60 seconds, optical density was measured at 240 nm against blank.

Superoxide dismutase (SOD) assay

The SOD assay was performed in 96 wells plate and reaction mixture contained 100mM KH_2PO_4 buffer (pH 7.8), 0.1mM EDTA, 13mM methionine, 2.25mM nitro-blue tetrazolium chloride (NBT), 60µM riboflavin and medium from pre-treatment and post-treatment groups. Optical density was measured at 560nm against the blank via a spectrophotometer.

Ascorbate peroxidase (APOX) assay

APOX assay was performed in 96-well plate. The reaction mixture contained 100mM KH_2PO_4 buffer (pH 7.0), 0.5mM ascorbate and 0.3mM H_2O_2 and remaining was a medium from a different group of HeLa cell line. After 3 minutes optical density was measured at 290 nm.

Statistical analysis

All data of experimental groups were expressed as mean ± SEM in triplicate experiments. Quantitative data obtained from different experimental groups were statistically analyzed via graph pad software by using one-way ANOVA for cell viability assays and immunocytochemistry graphs. Two-way ANOVA was used for MTT assay, ELISA, LDH, and antioxidants. A P-value less than 0.05 was considered as significant.

Results

Decreased proliferation and viability in CF seed treated HeLa cells

After treatment of HeLa cells with CF seed extracts, cell viability was assessed by trypan blue to detect dead and live cells. HeLa cells and UC-MSCs both treated with ethanol, ethyl acetate and petroleum ether extract, were stained with trypan blue. A significantly large number of blue colored cells were observed in T-HE-CFS-E, T-HE-CFS-EA and T-HE-CFS-PE groups, indicating more dead cells as compared to normal HeLa cells. Whereas, less numbers of blue color cells were observed in T-UCMSCs-CFS-E, T-UCMSCs-CFS-EA and T-UCMSCs-CFS-PE groups (Fig. 1).

The cell viability was also estimated using crystal violet staining of HeLa cell line and UC-MSCs. When these (T-HE-CFS-E, T-HE-CFS-EA, and T-HE-CFS-PE) groups were treated with CF seed extract, those showed fewer numbers of living cells as compared to untreated HeLa cells. Both normal UMSCs and treated groups (T-UCMSCs-CFS-E, T-UCMSCs-CFS-EA, and T-UCMSCs-CFS-PE) showed similar results. Cancer cell (HeLa cells) death was observed in all groups, treated with ethanol, ethyl acetate, and petroleum ether CF seed extracts. MTT reagent was used for the analysis of cell proliferation and significantly lower proliferation in T-HE-CFS-E, T-HE-CFS-EA and T-HE-CFS-PE groups were observed in treated cells compared to untreated HeLa cells (Fig. 2).

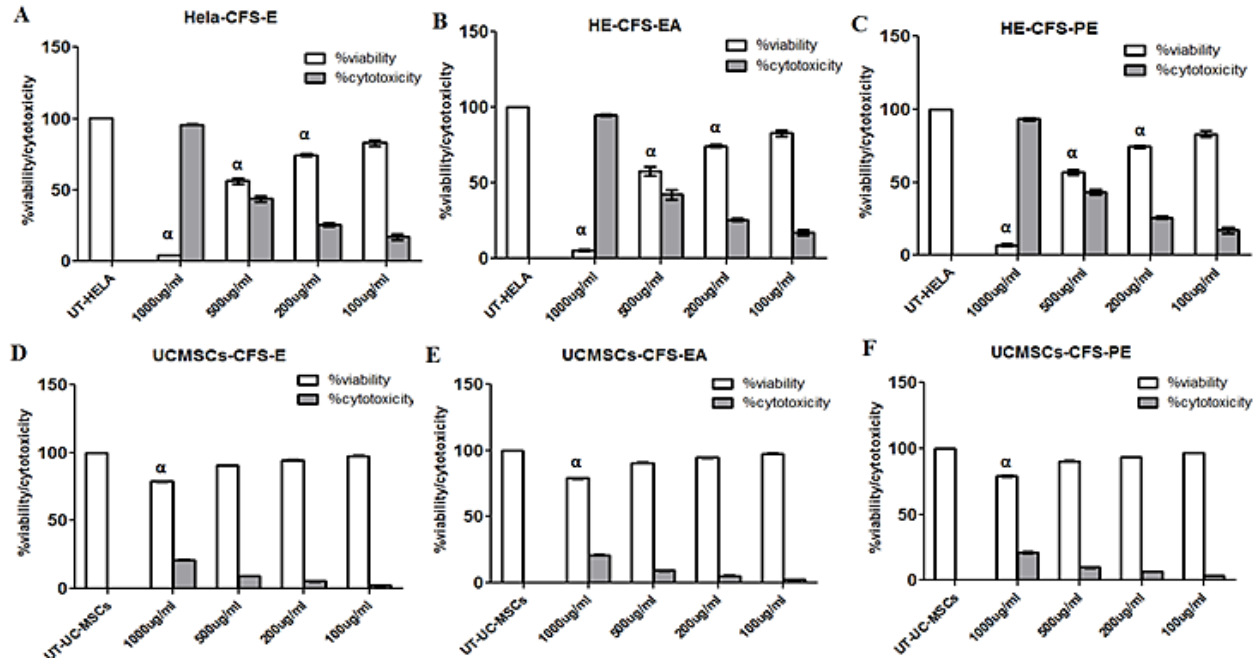

Fig. 1 Cytotoxicity analysis of different doses (100ug/ml-1000ug/ml) of *Cassia fistula* seed extracts. **(A)** Cytotoxicity analysis of CFS-E on HeLa cells; **(B)** cytotoxicity analysis of CFS-EA on HeLa cells; **(C)** cytotoxicity analysis of CFS-PE on HeLa cells; **(D)** cytotoxicity analysis of CFS-E on UC-MSCs **(E)** cytotoxicity analysis of CFS-EA on UC-MSCs; **(F)** cytotoxicity analysis of CFS-PE on UC-MSCs. Values were taken as mean ± SEM and α shows the significant difference between viabilities of treated groups and untreated controls ($p<0.05$).

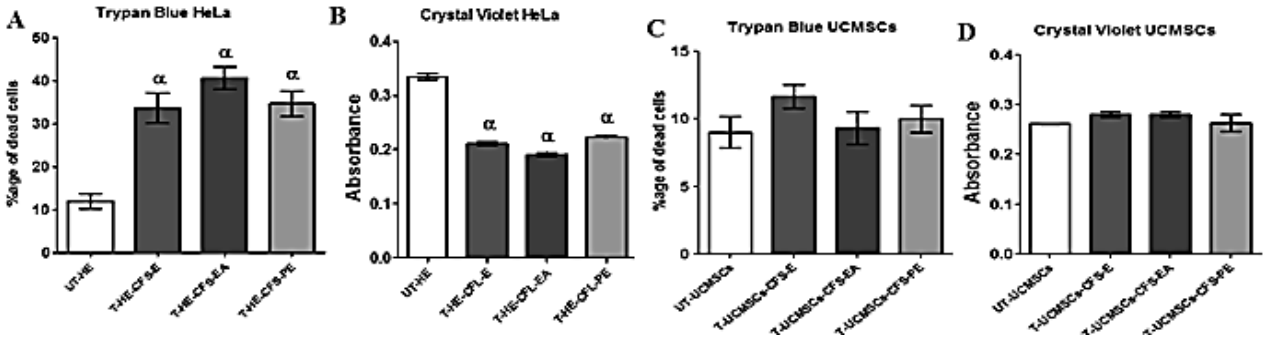

Fig. 2 Cell viability analysis of HeLa cell line and UC-MSCs after treatment with *Cassia fistula* seed extracts. **(A)** Percentage of dead cells in both untreated and treated HeLa cell lines; **(B)** numbers of living cells in both untreated and treated HeLa cell line; **(C)** Percentage of dead cells in both untreated and treated UC-MSCs; **(D)** number of living cells in both untreated and treated UC-MSCs. Values were taken as mean ± SEM and α shows the significant difference between viabilities of treated groups and untreated controls ($p<0.05$).

Reduced angiogenic potential of HeLa cells after treatment with *Cassia fistula* seed extract

After treatment, reduced angiogenesis was observed in HeLa cells with CF seed extracts via estimating the level of vascular endothelial growth factor (angiogenesis protein) in post-treated HeLa cells. According to our results, treatment of cell line with seed extracts lowered the angiogenesis level in cancer cells. The VEGF levels were estimated via immunocytochemistry and ELISA. Immunocytochemistry showed decreased levels of VEGF in T-HE-CFS-E, T-HE-CFS-EA, and T-HE-CFS-PE groups compared to non-treated HeLa cells; whereas, in T-UCMSCs-CFS-E, T-UCMSCs-CFS-EA and T-UCMSCs-CFS-PE groups, the levels of VEGF remained same as that of non-treated UC-MSCs (Fig. 3).

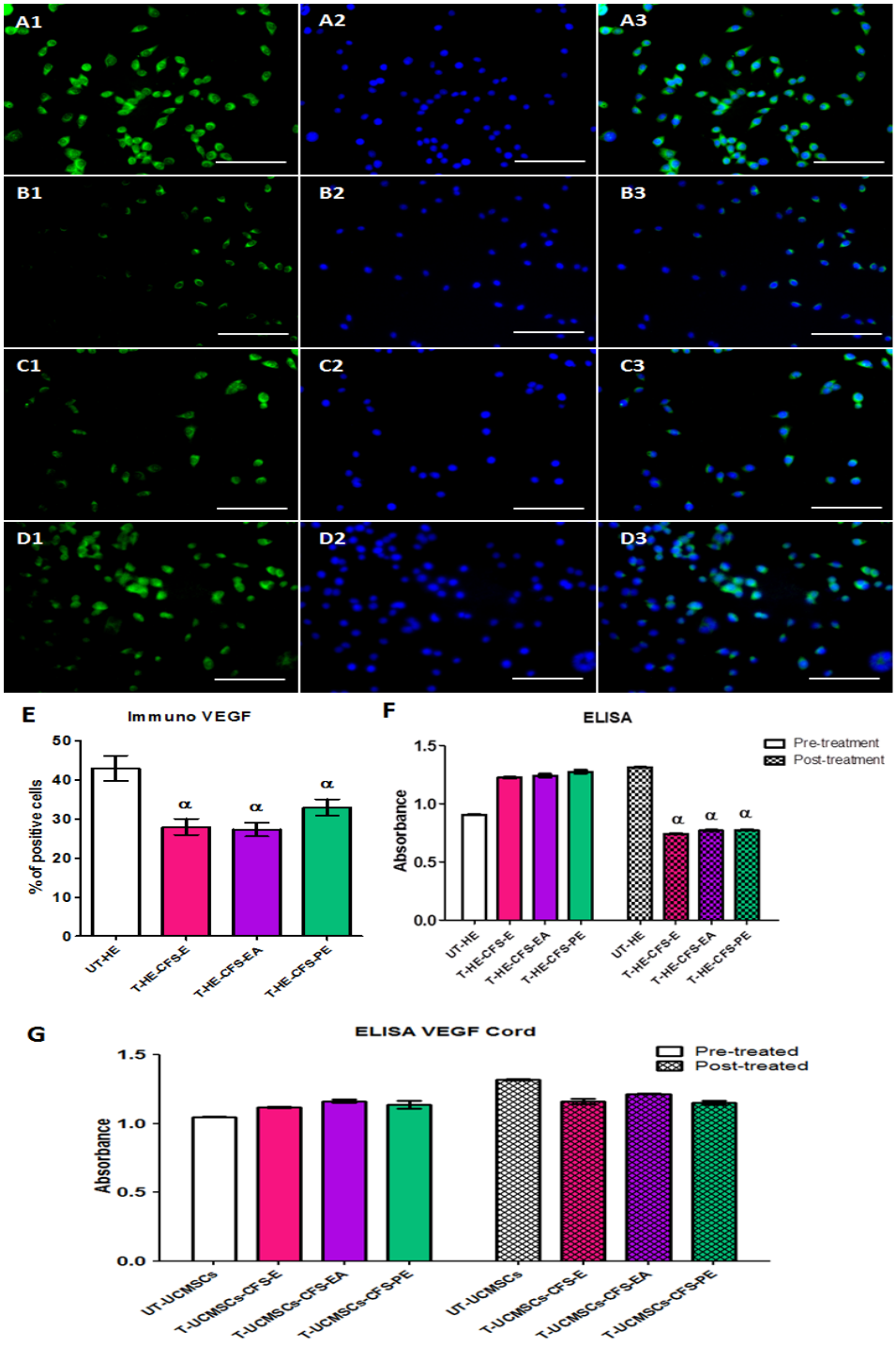

Fig. 3 Expression analysis of VEGF in untreated and treated groups. (**A1-A3**) Untreated HeLa cells (A1: anti-VEGF, A2: DAPI, A3: merge anti-VEGF/DAPI); (**B1-B3)** HeLa cells treated with ethanol *Cassia fistula* seed extract (B1: anti-VEGF, B2: DAPI, B3: merge anti-VEGF/DAPI); (**C1-C3)** HeLa cells treated with ethyl acetate *C. fistula* seed extract (C1: anti-VEGF, C2: DAPI, C3: merge anti-VEGF/DAPI); (**D1-D3)** HeLa treated with petroleum ether *C. fistula* seed extract (D1: anti-VEGF, D2: DAPI, D3: merge anti-VEGF/DAPI) **(**antibody stained in green, nuclei stained with DAPI were in blue color) (X200). **(E)** The graphical representation shows low percentage level of VEGF in HeLa cells treated with ethanol, ethyl acetate and ether extract of *C. fistula* seed as compared to untreated cells. **(F)** The graphical demonstration shows a significantly reduced level of VEGF in HeLa cells in different treatment groups measured via ELISA. **(G)** The graph shows no significant change in VEGF level in UC-MSCs treated with ethanol, ethyl acetate and ether extract of *C. fistula* seed in comparison to untreated UC-MSCs cells and shows that *C. fistula* seed treatment does not affect the untreated cells. Values were taken as mean ± SEM and α shows the significant difference between viabilities of treated groups and untreated controls ($p<0.05$).

Enhanced apoptosis in HeLa cells treated with *Cassia fistula* seed extract

ELISA and Immunocytochemistry were also done to evaluate the apoptosis level by using annexin-V antibody. As Annexin-V is a principal factor of apoptosis, untreated HeLa cells showed low apoptosis level, whereas T-HE-CFS-E, T-HE-CFS-EA, and T-HE-CFS-PE groups showed high apoptosis level. While in T-UCMSCs-CFS-E, T-UCMSCs-CFS-EA and T-UCMSCs-CFS-PE groups, there was the non-significant difference in VEGF level (Fig. 4). To evaluate the apoptosis level using p53 antibody, immunocytochemistry was done. In our findings, untreated HeLa cells showed low apoptosis level, whereas, in T-HE-CFS-E, T-HE-CFS-EA and T-HE-CFS-PE groups, the level of apoptosis was high. However, the ethyl acetate extract showed high p53 level compared to other two extracts. Our results showed high LDH released in post-treatment groups of HeLa cell line. The LDH release in T-HE-CFS-E, T-HE-CFS-EA, and T-HE-CFS-PE groups was higher compared to untreated group (Fig. 5)

Increased antioxidant potential of HeLa cells after treatment with *Cassia fistula* seed extract

After treating HeLa cells with CF seed extracts using ethanol, ethyl acetate, and petroleum ether, and then co-culturing these cancer cells separately, the medium was collected from each of the treated cells and analyzed for ascorbate peroxide (APOX), catalase (CAT) and superoxide dismutase (SOD) activity. It was observed that APOX, CAT, and SOD activities were significantly increased compared to untreated one (Fig. 6)

Discussion

Efforts to work with new anti-tumorigenic compounds, which extremely effect the cancer cell proliferation, are extending to address the problematic issue of chemotherapy resistance of many tumors. Though there are some drugs that have low toxicity but still inhibit the angiogenic potential of the cancer cell. Nowadays, naturally occurring agents, especially from plant sources are considered to be the key players in the development of potential drugs for life-threatening diseases [14, 15]. Studies suggested that the cause of many cancers is dietary imbalance and diet changes can eliminate the risk of these cancers [13, 16]. Further, studies also proved that food with high antioxidant activities like fruits and vegetables could affect the prevalence of cancer [16, 17]. Thus, there is a dire need to discover the new anticancer agents from plants that have high antioxidant activities. The major aim of the present study was to investigate the anti-cancer activity of CF seed extracts against the HeLa cell line. For this purpose, seeds were extracted using different solvents, i.e., ethyl acetate, ethanol, and ether. Four main compounds are reported to be present in CF seed including rhein which is most active anti-cancer compound; other three are thymol, oleic acid and palmitic acid [11]. Many studies have revealed the anticancer properties of these compounds as they have the potency to inhibit tumor cell growth, anti-proliferative and induce apoptosis [18, 19]. Further, in this study evaluation of effects of seed extracts on normal cells were also studied.

It is assumed that the extent of cancer invasion, growth and metastasis is determined by its angiogenesis [13, 20]. Therefore, it can also be assumed that by targeting the angiogenesis one can design potential anticancer strategy which inhibits the new vessel formation, thus, reduces the oxygen and nutrient supply to actively proliferating cancer cells [21, 22]. Most of the present day anti-cancer agents extensively target all rapidly proliferating cells that resulted in adverse effects, including the digestive problems immunosuppression and loss of body hair [23]. On the other hand, therapies involving anti-angiogenic strategies are reported to have fewer side effects because neo-angiogenesis is a rare phenomenon in the healthy adult body except in the endometrium of the uterus where neo-angiogenesis is a frequent event. Therefore, the blockage of VEGF pathway is considered to be the most appropriate approach for limiting the angiogenesis of tumor. The VEGF blockers have been reported to study in many patients with progressive cancers along with the preclinical studies [24-27]. In the present study, significantly low angiogenesis in HeLa cell line was observed after treatment with CF seed extracts as evidenced by both ELISA and immunocytochemistry. In a previous research, plant extracts have been used to target the VEGF for the suppression of

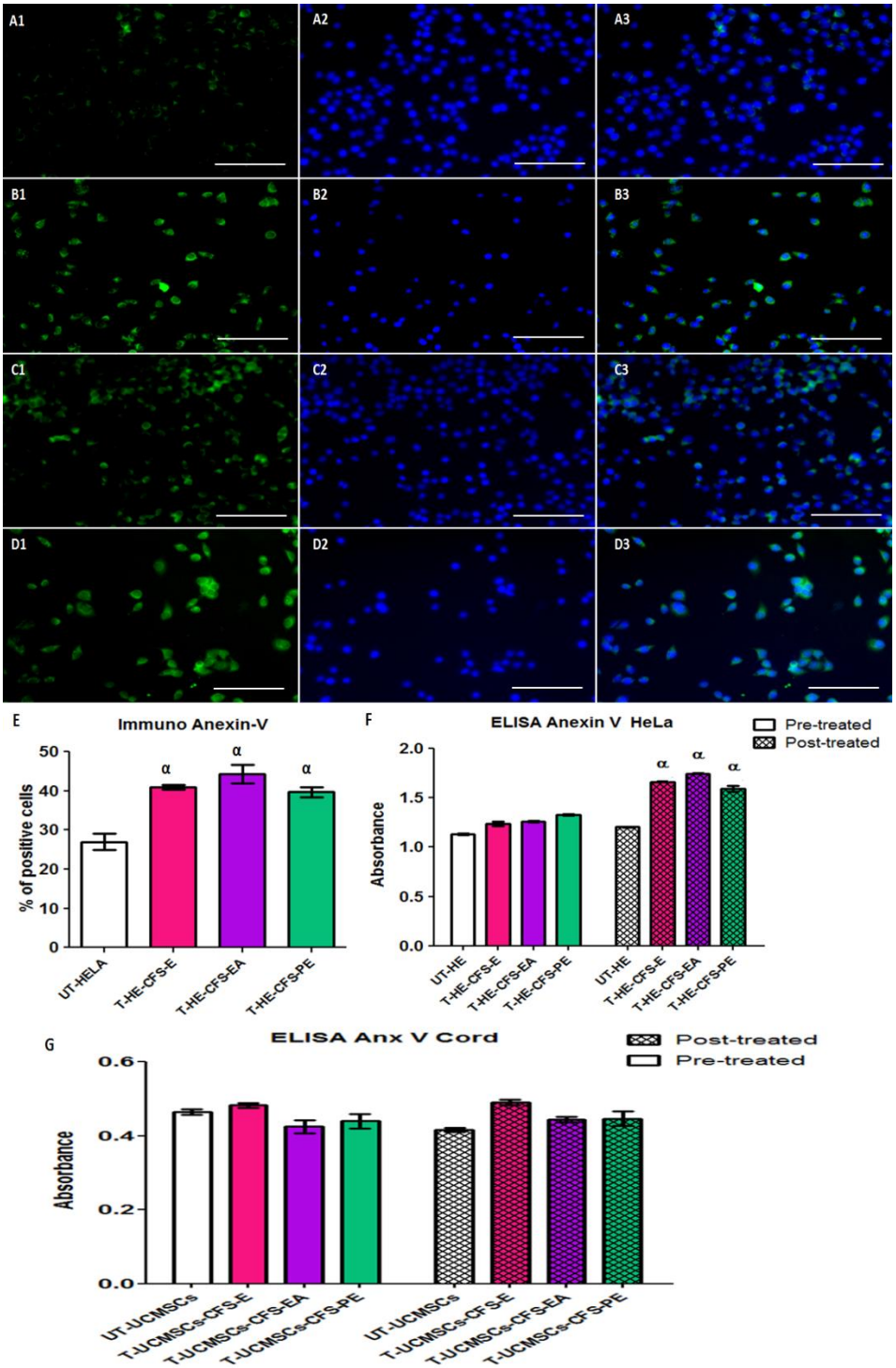

Fig. 4 Expression analysis of annexin-V in untreated and treated groups. **(A1-A3)** Untreated HeLa cells (A1: anti-annexin-V, A2: DAPI, A3: merge anti-annexin-V /DAPI); (**B1-B3)** HeLa cells treated with *Cassia fistula* seed extract in ethanol (B1: anti-annexin-V, B2: DAPI, B3: merge anti-annexin-V /DAPI); (**C1-C3)** HeLa cells treated with *C. fistula* seed extract in ethyl acetate (C1: anti-annexin-V, C2: DAPI, C3: merge anti-annexin-V /DAPI); (**D1-D3)** HeLa cells treated with *C. fistula* seed extract in petroleum ether (D1: anti-annexin-V, D2: DAPI, D3: merge anti-annexin-V/DAPI) (antibody stained in green, nuclei stained with DAPI are in blue color) (X200). **(E)** The graphical representation shows the high percentage level of annexin-V in HeLa cells treated with ethanol, ethyl acetate and petroleum ether extract of CF seeds compared to untreated cells. **(F)** The graphical demonstration shows a significantly increased annexin-V level in different treatment groups measured via ELISA. **(G)** The graph shows no significant change of annexin-V in untreated and treated UC-MSCs. Values were taken as mean ± SEM and α shows the significant difference between viabilities of treated groups and untreated controls (p<0.05).

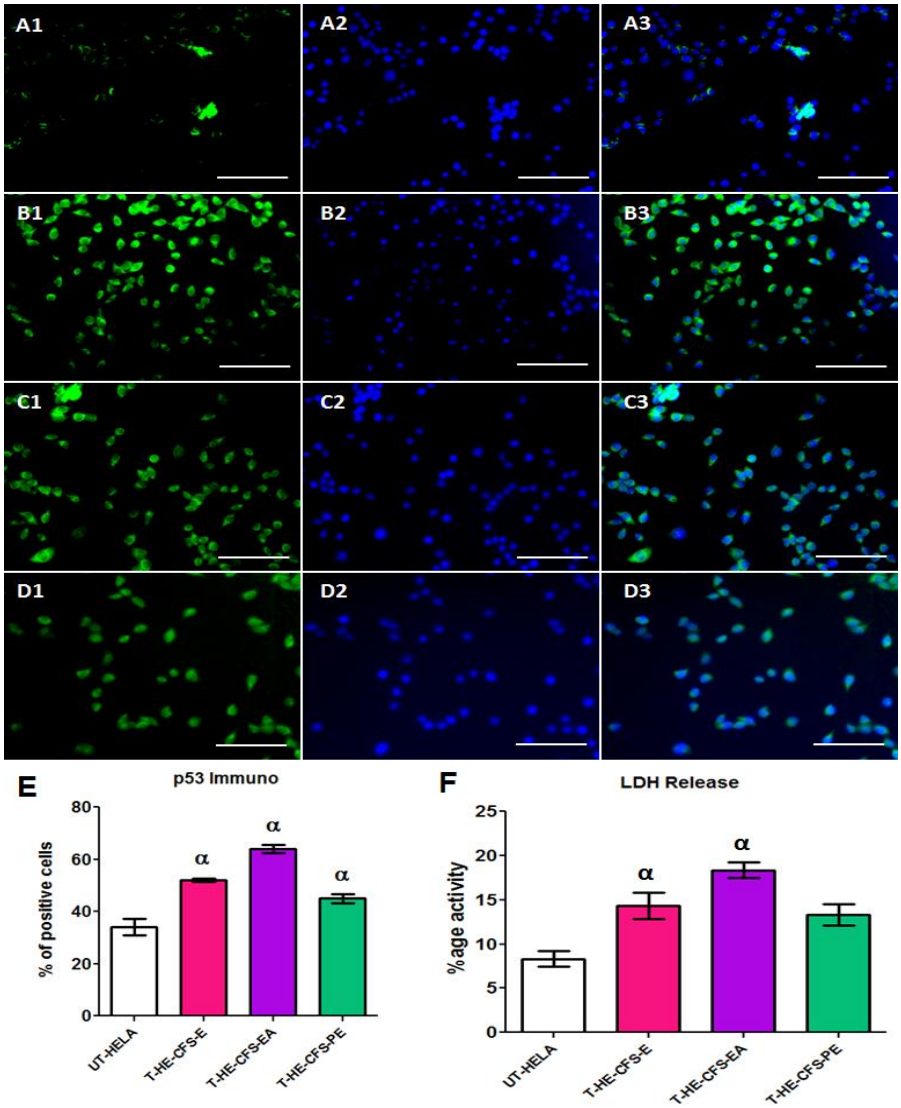

Fig. 5 Expression of p53 in untreated and treated groups. **(A1-A3)** untreated HeLa cells (A1: anti-p53, A2: DAPI, A3: merge anti-p53/DAPI); (**B1-B3)** HeLa cells treated with *Cassia fistula* seed extract with ethanol (B1: anti-p53, B2: DAPI, B3: merge anti-p53/DAPI); (**C1-C3)** HeLa treated with *C. fistula* seed extract in ethyl acetate (C1: anti-p53, C2: DAPI, C3: merge anti-p53/DAPI); (**D1-D3)** HeLa cells treated with *C. fistula* seed extract in petroleum ether (D1: anti-p53, D2: DAPI, D3: merge anti-p53/DAPI) **(**Antibody-stained in green, nuclei stained with DAPI are in blue color) (X200). **(E)** The graphical representation shows the high percentage level of p53 in HeLa cells treated with ethanol, ethyl acetate, and petroleum ether extracts of *C. fistula* seed compared to untreated cells. **(F)** The graphical demonstration shows the LDH release in untreated and in treatment groups. Values were taken as mean ± SEM, where $^{\alpha}$ shows the significant difference between untreated and in treatment groups ($p< 0.05$).

angiogenesis [28]. Similarly, extracts used in this study must contain some inhibitory factors of VEGF pathway that lowered the VEGF level and suppressed the angiogenesis of HeLa cells. On the other hand, when UC-MSCs were subjected to treatment with the same extracts those showed slight but nonsignificant lower levels of VEGF pre and post treatment. A slight reduction of VEGF was observed even in UC-MSCs group which showed that this treatment could prove to be an effective antiangiogenic therapy for cancer cells with the least effects on normal cells.

The MTT is a positively charged tetrazolium salt, which is reduced by viable cells with active metabolism, in which it freely enters and forms purple colored formazan product. MTT assay measures the cell viability due to its reductive activity as it can convert the tetrazolium compound to

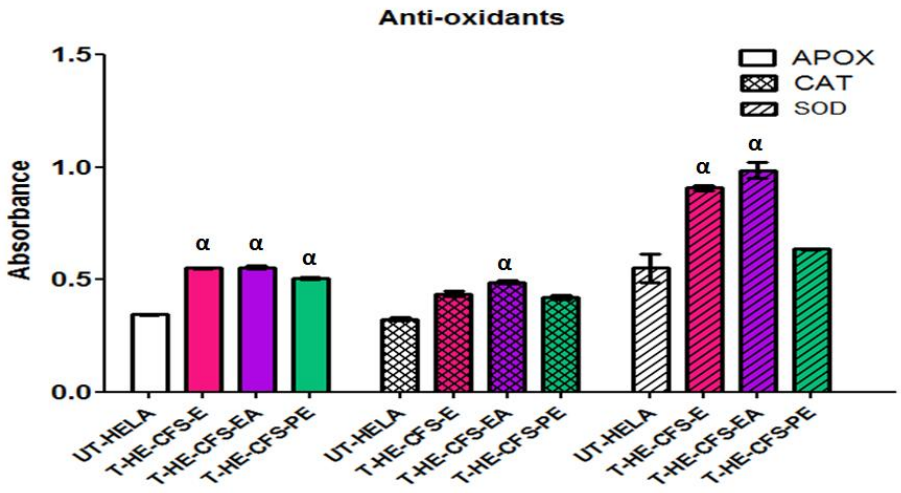

Fig. 6 Evaluation of antioxidant activity of HeLa cells treated with the *Cassia fistula* seed extracts in ethanol, ethyl acetate, and petroleum ether. Values were taken as mean ± SEM and α shows the significant difference between viabilities of treated groups and untreated controls ($p<0.05$).

water enzymatically [29, 30]. Our results indicate that the HeLa cells, when treated with seed extracts of CF, shows the significantly low level of proliferation, whereas when UC-MSCs treated with the extracts, they show the non-significant effect on proliferation. Moreover, other cell viability assays, including trypan blue and crystal violet also showed the similar results. In trypan blue assay, more dead cells were found in HeLa cells after treatment with plant extracts as compared to the percentage of dead cells in UC-MSCs after the same treatment. Similarly, the amount of crystal violet absorbed by the live cells was more in UC-MSCs group compared to HeLa cell treatment group. Maybe this is due to the lowering of angiogenesis that inhibits the growth of cancer cells.

Anticancer drugs, along with plant-derived ones, generally exert their cell death effects by inducing apoptosis in tumor cells [31]. The mechanisms of action of plant-derived anticancer drugs are numerous and most of them induce apoptotic cell death that may be intrinsic or extrinsic, and caspase and/or p53-dependent or independent mechanisms [1]. In many studies, annexin V staining shows a high percentage of apoptosis after treatment with plant extracts [32]. In our findings, it was observed that the seed extracts of CF induced apoptosis in HeLa cells via both p53 and annexin V-dependent mechanism in an increasing apoptosis pattern when treated with petroleum ether, ethanol, and ethyl acetate. While the ethyl acetate extract was found to be the most effective in the induction of apoptosis as evidenced by analysis of p53 and annexin V via ELISA and immunocytochemistry. On the other hand, UC-MSCs showed non-significant results in this assay too, showing that these extracts do not enhance the apoptosis in normal cells thus proving to be the best candidate for anticancer therapy.

The LDH is an enzyme released when damage happens to cell membranes and is estimated in supernatants of cell cultures [33]. In many studies, control cells showed lesser LDH release and cells treated with plant extracts showed higher LDH release [34]. Our results are also in agreement with the previous studies as we found more LDH release in post-treated HeLa cell lines compared to the UC-MSCs. Moreover, in post-treated HeLa cell line, ethyl acetate group showed the highest LDH release compared to the ethanol group. While, petroleum ether showed the low LDH release compared to the other two extracts, but still higher levels than untreated HeLa cells. Oxidative stress is considered to be the primary cause of cancer and occur as a result of an imbalance between the increasing demand for oxygen and nutrients by rapidly proliferating tumor cells and an inadequate, dysfunctional blood supply resulting from tumor angiogenesis [35-37]. Cancer cells demonstrate the Warburg effect, according to which even in the presence of sufficient oxygen cell prefer glycolysis for their energy production [38-40]. Oxidative stress

results in increased level of reactive oxygen species (ROS), but the cancer cells can adjust against ROS levels by using this metabolic shift that can save them from apoptosis due to oxidative damage thus rapid proliferation is there [41, 42]. Antioxidative enzymes (APOX, CAT, and SOD) effect the proliferation of a cell in a positive way, but when these antioxidants are given in compliance with some anti-proliferative therapy, it enhances the efficacy of the therapy by reducing the levels of ROS. In the present study, APOX, CAT, and SOD activities were increased when the HeLa cell line was treated with CF seed extracts, thereby, oxidative stress was decreased, which resulted in increased CF seed extract's anti-proliferative efficiency. Thus, from the present study, it is concluded that CF seed extracts exhibit the property of inducing apoptosis and reducing the proliferation of cancer cell with augmentation of their anti-oxidant ability. Moreover, when seed extract was prepared in ethyl acetate its efficiency was significantly increased.

Conflict of interests

The authors declare that they have no competing interest.

Acknowledgements

We would like to acknowledge Prof. Dr. MH Qazi, Vice Chancellor, The University of Lahore to provide us with the facilities for this research. We would also like to say thanks to our entire lab staff for being there to help us when needed.

References

[1] Gali-Muhtasib H, Hmadi R, Kareh M, Tohme R, Darwiche N. Cell death mechanisms of plant-derived anticancer drugs: beyond apoptosis. Apoptosis 2015; 20(12):1531-62.

[2] Ji S, Zhang B, Liu J, Qin Y, Liang C, Shi S, et al. ALDOA functions as an oncogene in the highly metastatic pancreatic cancer. Cancer lett 2016; 374(1):127-35.

[3] Shroff GK, Kuna R, Dirsipam K, Yalagala R, Mitra P. Harnessing Impaired Energy Metabolism in Cancer Cell: Small Molecule-Mediated Ways to Regulate Tumorigenesis. Anti-cancer Agents in Med Chem 2011; 11(3):272-9.

[4] Meier P, Finch A, Evan G. Apoptosis in development. Nature 2000; 407(6805):796-801.

[5] Tian Q, Zang YH. Antiproliferative and apoptotic effects of the ethanolic herbal extract of *Achillea falcata* in human cervical cancer cells are mediated via cell cycle arrest and mitochondrial membrane potential loss. J Buon 2015; 20(6):1487-96.

[6] Nayak A, Satapathy SR, Das D, Siddharth S, Tripathi N, Bharatam PV, et al. Nanoquinacrine induced apoptosis in cervical cancer stem cells through the inhibition of hedgehog-GLI1 cascade: Role of GLI-1. Sci Rep 2016; 6:20600.

[7] Bhalodia N, Nariya P, Acharya R, Shukla V. In vitro antibacterial and antifungal activities of *Cassia fistula* Linn. fruit pulp extracts. Ayu 2012; 33(1):123.

[8] Paul J, Gnanam R, Jayadeepa RM, Arul L. Anti cancer activity on Graviola, an exciting medicinal plant extract vs various cancer cell lines and a detailed computational study on its potent anti-cancerous leads. Curr Top Med Chem 2013; 13(14):1666-73.

[9] Lindamulage IK, Soysa P. Evaluation of anticancer properties of a decoction containing *Adenanthera pavonina* L. and *Thespesia populnea* L. BMC Complement Altern Med 2016; 16(1):70.

[10] Jarald E, Joshi S, Jain D, Edwin S. Biochemical evaluation of the hypoglycemic effects of extract and fraction of *Cassia fistula* linn. in alloxan-induced diabetic rats. Indian J Pharm Sci 2013; 75(4):427.

[11] Irshad M, Mehdi SJ, Al-Fatlawi AA, Zafaryab M, Ali A, Ahmad I, et al. Phytochemical composition of Cassia fistula fruit extracts and its anticancer activity against human cancer cell lines. J of Bio Active Prod Nat 2014; 4(3):158-70.

[12] Irshad M, Ahmad A, Zafaryab M, Ahmad F, Manzoor N, Singh M, et al. Composition of *Cassia fistula* oil and its antifungal activity by disrupting ergosterol biosynthesis. Nat Prod Commun 2013; 8(2):261-4.

[13] Hampannavar GA, Karpoormath R, Palkar MB, Shaikh MS. An appraisal on recent medicinal perspective of curcumin degradant: Dehydrozingerone (DZG). Bioorg Med Chem Lett 2016; 24(4):501-20.

[14] Teiten MH, Gaascht F, Dicato M, Diederich M. Anticancer bioactivity of compounds from medicinal plants used in European medieval traditions. Biochem Pharmacol 2013; 86(9):1239-47.

[15] Wang Z, Dabrosin C, Yin X, Fuster MM, Arreola A, Rathmell WK, et al. Broad targeting of angiogenesis for cancer prevention and therapy. Sem Cancer Biol 2015; 35 Suppl:S224-43.

[16] Nasri H, Rafieian-Kopaei M. Medicinal Plants And Antioxidants: Why They Are Not Always Beneficial? Iran J Public Health 2014; 43(2):255-7.

[17] Esakkirajan M, Prabhu N, Arulvasu C, Beulaja M, Manikandan R, Thiagarajan R, et al. Anti-proliferative effect of a compound isolated from Cassia auriculata against human colon cancer cell line HCT 15. Spectrochim Acta A Mol Biomol Spectrosc 2014; 120:462-6.

[18] Sadeghnia HR, Ghorbani Hesari T, Mortazavian SM, Mousavi SH, Tayarani-Najaran Z, Ghorbani A. Viola tricolor induces apoptosis in cancer cells and exhibits antiangiogenic activity on chicken chorioallantoic membrane. Biomed Res Int 2014; 2014:625792.

[19] Medda R, Lyros O, Schmidt JL, Jovanovic N, Nie L, Link BJ, et al. Anti inflammatory and anti angiogenic effect of black raspberry extract on human esophageal and intestinal microvascular endothelial cells. Microvasc Res 2015; 97:167-80.

[20] Griggs J, Metcalfe JC, Hesketh R. Targeting tumour vasculature: the development of combretastatin A4. Lancet Oncol 2001; 2(2):82-7.

[21] Ng KW, Salhimi SM, Majid AM, Chan KL. Anti-angiogenic and cytotoxicity studies of some medicinal plants. Planta Med 2010; 76(9):935-40.

[22] Nussbaumer S, Bonnabry P, Veuthey JL, Fleury-Souverain S. Analysis of anticancer drugs: a review. Talanta 2011; 85(5):2265-89.
[23] Hosseini A, Ghorbani A. Cancer therapy with phytochemicals: evidence from clinical studies. Avicenna J Phytomed 2015; 5(2):84-97.
[24] Huijbers EJ, van Beijnum JR, Thijssen VL, Sabrkhany S, Nowak-Sliwinska P, Griffioen AW. Role of the tumor stroma in resistance to anti-angiogenic therapy. Drug Resist Updat 2016; 25:26-37.
[25] Lei X, Chen M, Nie Q, Hu J, Zhuo Z, Yiu A, et al. In vitro and in vivo antiangiogenic activity of desacetylvinblastine monohydrazide through inhibition of VEGFR2 and Axl pathways. Am. J. Cancer Res 2016; 6(4):843-58.
[26] Kubota Y. Tumor angiogenesis and anti-angiogenic therapy. Keio J Med 2012; 61(2):47-56.
[27] Choedon T, Dolma D, Mathan G, Kumar V. Molecular insights into the anti-cancer properties of Traditional Tibetan medicine Yukyung Karne. BMC Complement Altern Med 2014; 14(1):380.
[28] Lu L, Zhang L, Wai MS, Yew DT, Xu J. Exocytosis of MTT formazan could exacerbate cell injury. Toxicol In Vitro 2012; 26(4):636-44.
[29] Stockert JC, Blazquez-Castro A, Canete M, Horobin RW, Villanueva A. MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. Acta Histochem 2012; 114(8):785-96.
[30] Tait SW, Ichim G, Green DR. Die another way--non-apoptotic mechanisms of cell death. J Cell Sci 2014; 127:2135-44.
[31] Ovadje P, Ma D, Tremblay P, Roma A, Steckle M, Guerrero J-A, et al. Evaluation of the efficacy & biochemical mechanism of cell death induction by piper longum extract selectively in in-vitro and in-vivo models of human cancer cells. PLoS One 2014; 9(11):e113250.
[32] Plackal Adimuriyil George B, Tynga IM, Abrahamse H. In Vitro Antiproliferative Effect of the acetone extract of *Rubus fairholmianus* gard. Root on human colorectal cancer cells. BioMed Res Int 2015; 2015:165037.
[33] Saad B, Dakwar S, Said O, Abu-Hijleh G, Battah FA, Kmeel A, et al. Evaluation of medicinal plant hepatotoxicity in co-cultures of hepatocytes and monocytes. Evid Based Complement Alternat Med 2006; 3(1):93-8.
[34] Lendahl U, Lee KL, Yang H, Poellinger L. Generating specificity and diversity in the transcriptional response to hypoxia. Nature Rev Genet 2009; 10(12):821-32.
[35] Bailey KM, Wojtkowiak JW, Hashim AI, Gillies RJ. Targeting the metabolic microenvironment of tumors. Adv Pharmacol 2012; 65:63-107.
[36] Toyokuni S. Molecular mechanisms of oxidative stress-induced carcinogenesis: from epidemiology to oxygenomics. IUBMB life 2008; 60(7):441-7.
[37] Queiroz EA, Puukila S, Eichler R, Sampaio SC, Forsyth HL, Lees SJ, et al. Metformin induces apoptosis and cell cycle arrest mediated by oxidative stress, AMPK and FOXO3a in MCF-7 breast cancer cells. PLoS One 2014; 9(5):e98207.
[38] Grüning N-M, Ralser M. Cancer: sacrifice for survival. Nature 2011; 480(7376):190-1.
[39] Warburg O. On the origin of cancer cells. Science 1956; 123(3191):309-14.
[40] Fuchs-Tarlovsky V. Role of antioxidants in cancer therapy. Nutrition 2013; 29(1):15-21.
[41] Anastasiou D, Poulogiannis G, Asara JM, Boxer MB, Jiang J-k, Shen M, et al. Inhibition of pyruvate kinase M2 by reactive oxygen species contributes to cellular antioxidant responses. Science 2011; 334(6060):1278-83.
[42] Greenwell, M., & Rahman, P. K. Medicinal Plants: Their Use in Anticancer Treatment. Int J Pharm Sci Res 2015; 6(10):4103-4112.
[43] Rahmani, A. H. (2015). Cassia fistula Linn: Potential candidate in the health management. Pharmacog Res 2015; 7(3):217-24.