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Anticancer Activity of Fermented Solenostemm Aargel Extract and/or Low Dose Gamma Radiation Against Hepatocellular Carcinoma

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ABSTRACT

For many decades the terrestrial plants are represent an infinitive source of bioactive substances as well as pharmaceutical components which play a main role in the discovering ,developing and manufacturing of anticancer and antibiotic drugs.Solenostemmaargel is a desert plant widely used in Arabic countries as antispasmodic, anti-inflammatory and anti-rheumatic agent. It also has anti-tumor activity. Aim: the present study was oriented to investigatethe hepato-protective effect of fermentedSolenostemmaargelleave extract (100amg/kg b.wt) and/or low dose γ-radiation (0.5Gy) againsthepatocellular carcinoma (HCC)developedin rats by injection of diethylnitrosamine. Results: the data obtained revealed that thepro-apoptotic agents (caspase 3 and cytochrome c) undergoes significant elevation in HCC rats received plant extract and/or γ-radiation compared to HCC rats.While,the vascular endothelial growth factor (VEGF), hypoxia inducible factor-α (HIF-α)and monocytichemotactic protein-1(MCP-1) were significantly decreased. Also, the activity of cyclooxygenase-2 (COX-2) was significantly decreased. Moreover, the histopathological analysis revealed that a mild improvement .In conclusion, the improvement emerged in HCC rats could be attributed to the potency of extract and/or γ- radiation by induce apoptosis and reduce angiogenesis.

Keywords: Fermented Solenostemmaargel Extract/ Low Dose γ-Radiation/HCC/ Diethylnitrosamine.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of cancer that originate in the liver, it is accounting for approximately 80% of all primary liver cancers. Hepatocarcinogenesis is a complex process associated with accumulation of genetic and epigenetic changes that occur during initiation, promotion, and progression of the disease[1]. Treatment of hepatocellular carcinoma is clinically difficult, as HCC expresses consider multidrug resistance genes and is highly linked with multi-gene, multi-factor, and multi-step processes[2].The current trend of cancer research is the investigation of medicines of plant origin because of their affordability and availability with minimal side effects.

Hypoxia-inducible factor -1α (HIF-1α) is a master controller of essential adaptive responses to hypoxia, whose expression and transcriptional activity increasing exponentially with decreases in levels of cellular oxygen. In tumors, HIF-1α controls proliferation, apoptosis, metastatic spread, and glucose metabolism by performing as a transcription factor for crucial proteins. HIF-1α can also regulate the expression of several relevant factors, including pro-inflammatory cytokines, chemokines, and adhesion molecules [3].

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In hypoxia, the stabilized HIF-1α is able to translocate to the nucleus, where it induces the expression of several genes such as vascular endothelial growth factor (VEGF)[4]. Vascular endothelial growth factor (VEGF), one of the best characterized proangiogenic factors, plays a critical role during angiogenesis which is essential for tumor growth [5]. It is well known that VEGF is secreted by most solid tumors, thus activating transduction pathways which promote migration, proliferation and prolong cell survival endothelial cells [6].

Otherwise, many researchers found that the importance of HIF-1α in HCC is contributed to other markers related to inflammation. Among these markers , is cyclooxygenase-2(COX-2) which involved in chronic inflammation.Cox-2 is also associated with carcinogenesis, speed of tumor growth, and tumor aggressiveness [7].Cyclooxygenases are important enzymes that regulate the synthesis of prostaglandins . Its two isoforms (COX-1 and COX-2) have different expression patterns, with COX-1 being expressed in a broad variety of tissues. COX-2 and its main product, prostaglandin E2 (PGE2), are inducible by growth factors and inflammatory stimuli. Additionally, COX-2 has been shown to contribute in tumor development and progression. Over expression of COX-2 has been reported in many human malignancies. These findings suggest that COX-2 may be involved in carcinogenesis and/or progression of certain types of human malignancies [8].

Monocyte chemotactic protein-1 (MCP-1) is a member of the chemokine family composed of 76 amino acids, and it is 13 kDa in size, it is a multifunctional factor involved in various aspects of liver pathogenesis, including acute liver injury, chronic HBV/HCV infection, cirrhosis and tumorigenesis[9]. It is secreted by hepatic stellate cells, hepato- cytes, Kupffer cells and biliary epithelial cells [10] .Monocyte chemotactic protein-1 is primarily identified as a potent chemotactic factor for attracting monocytes, macrophages and other inflammatory cells to the site of inflammation during tissue injury and infection [13]. Studies in recent years reveal that functions of MCP-1 are far beyond tissue repair; it participates in pathophysiological development of various diseases including cancer and obesity [11].

Solenostemmaargel is a desert plant, belongs to Asclepiadaceae family. It is widely extent in, Egypt, Sudan Libya, Chad, Algeria, Saudi Arabia and Palestine[3] . This family is a gorgeous source of many important groups; indoline, alkaloids, steroids, steroidal alkaloids, pregnanes and their glycosides which possess antitumor and anticancer activities [12]. The plant is widely used in traditional folkloric medicine as antispasmodic, anti-inflammatory and anti-rheumatic agent .Smoke inhalation and infusion of the whole plant is used in treatment of diabetes mellitus, hypercholesterolemia, jaundice, cough, cold and measles. It was described to alleviate gastrointestinal cramp, urinary tract infection and menstrual disturbance. Also, this plant has anti-bacterial, antifungal and antioxidant activity [13].

The induction of apoptosis is considered as one of promising mechanisms of cancer therapy. Caspase activation plays a central role in the triggering of apoptosis. The activated caspases cleave a variety of target proteins, thereby disabling important cellular processes and breaking down structural components of the cell [14]. The release of cytochrome-c from the mitochondrial inter-membrane space into the cytosol is the precondition of caspase-dependent intrinsic apoptosis pathway. Caspase-3 is the main caspase involved in both extrinsic and intrinsic apoptosis pathway [15]. The present work is an endeavor to examine the anti-proliferative mechanisms of fermented leave extract of argel and/or γ-radiation against HCC in rats.

MATERIALS AND METHOD

Microorganisms and Culture Conditions the acticacid bacteria (LAB) strains used in this study were: Lactobacillus rhamnosus, ATCC 7469 and Lactobacillus acidophilus, ATCC 4356. All cultures were stored at −25°C in liquid MRS)de Man, Rogosa and SharpeLactobacilli media)(Oxoid, Basingstoke, Hampshire, UK) with 20% sterile glycerol[16].

Probiotic Fermentation of Solenostemmaargel Leaves Solenostemmaargel leaves were purchased from the local market and allowed to shade dried at room temperature and grinded. The S. argel leaves powder was fermented by 2% mixed culture of L. rhamnosus and L. acidophilus for 24 hrs at 37Oc[12].

Ethanolic Extract Preparation

30 gram of fermented plant materials were extracted by immersing them in 150 ml ethanol for 48 hrs at room temperature under dark conditions, then filtered through clean muslin cloth followed by a filter paper. The process was repeated again for another 24 hrs. The extracts were concentrated under vacuum by rotary evaporator at 40 °C. The dry extracts were stored at -80°C [12].

Chemicals

All chemicals used in the present study purchased from Sigma Chemical Company, St. Louis, U.S.A investigation were of analytical grade.

Animals

Male outbreed albinorats originally obtained from National Cancer Institute (NCI) (120-140g) were used as experimental animals throughout the experiment period. The animals were housed under standard laboratory condition and fed a balanced diet with free access to water ad libitum. Animal experimentations were consistent with the guidelines of ethics by Public Health Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) in accordance with the recommendations for the proper care and use of laboratory animals approved by Animal Care Committee of the National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt.

Irradiation procedures

Whole body γ-irradiation was performed with a Canadian Cs137 GammaCell-40 at the NCRRT. Cairo, Egypt, at a dose rate 0.61 Gy/min. Rats were exposed to a single dose of 0.5Gy according to experimental design.

Experimental design

Rats were divided into 8 equal groups(15 rats/group) as follows: (1)control group (C): animals were received intrapretoneal 0.2 ml saline along with experimental time course ,(2)Diethylnitrose amine DEN(D):rats were received oral administration 20mg/kg b.wt./day(5days/week) for six weeks(16),(3)Fermented Solenostemmaargel (argel) group(F): rats of this group received oral administration of 100mg/kg b.wt./day of the extract for 7 weeks,(4) Irradiated group (R):rats were exposed 0.5 Gy gamma radiation on the 7th day from the beginning of experiment (5) Fermented Solenostemmaargel (argel) + R (FR): rats werereceived oral administration of extract as group (3) and exposed to gamma radiation as group (4).(6) Fermented Solenostemmaargel (argel) -Diethylnitrose amine(FD): rats of this group were received extract as group (3)and received with diethylnitrosamine as group (2)(7)Diethylnitrose amine –irradiated(DR) : rats were injected with diethylnitrosamine like group(2) and exposed to gamma radiation like group (4)(8)Fermented Solenostemmaargel (argel) -Diethylnitrose amine -irradiated (FDR): animals were received extract as group (3) and treated with diethylnitrosamine as group(2) and exposed to gamma radiation as group (4).

Biochemical Assays

Serum HIF was estimated using rat HIF-1α ELISA kit(sandwich ELISA Catalog No : E-EL-R0513 96T.Elabscience USA),serum VEGF level was measured using a rats-VEGF ELISA kit (quantikine R&D system, USA),MCP-1 concentration in serum was quantified using rat ELISA kit (R&D Systems, Minneapolis, MN) and the level of Cox-2 was assessed using Sandwich ELISA kit(LSBio-LS-F5730).

RNA extraction and real time quantitative PCR determination (RT-PCR)

RNA was extracted from the tumor tissue homogenate using the RN easy plus mini kit (Qiagen, Venlo, Netherlands),according to the manufacturer's instructions. The RNA concentration was determined spectropmjhotometrically at 260 nm using the Nano Drop ND-1000 spectrophotometer (Thermo Fisher scientific, Waltham,USA) and RNA purity was checked by means of the absorbance ratio at 260/280 nm. RNA integrity was assessed by electrophoresis on. The Relative expression levels of genes for caspase-3 and cytochrome c were determined by qRT-PCR. (1 µg) of RNA were used in the cDNA synthesis reaction, which was performed using the Reverse Transcription System(Promega, Leiden, The Netherlands). RNA was incubated at 70°C for 10 min to prevent secondary structures. The RNA was supplemented with MgCl2 (25mM), RTase buffer (10X), dNTP mixture (10mM), oligo d (t) primers, RNAse inhibitor (20 U) and AMV reverse transcriptase (20 U/µl). This mixture was incubated at 42°C for 1 h.

Quantitative Real Time PCR

qPCR was performed in an optical 96-well plate with an ABI PRISM 7500 fast sequence detection system (Applied Biosystems, Carlsbad, California) and universal cycling conditions min

95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C). Each 10 µl reaction contained 5 µl SYBR Green Master Mix (Applied Biosystems), 0.3 µl gene-specific forward and reverse primers (10 µM), 2.5µl cDEN and 1.9 µl nuclease-free water. The sequences of PCR primer pairs used for each gene are shown in Table I.

Caspase-3	Sense: 5-CAAACCACCAAGTGGAGGAG-3 Antisense: 3'-GTGGGTGAGGAGCACGTAGT-5'
Cytochrome C	Sense: 5′-TTTGGATCCAATGGGTGATGTTGAG-3 Antisense: 5′TTTGAATTCCTCATTAGT AGCTTTTT TGAG-3′
GAPDH	Sense:5'-CTCCCATTCTTCCACCTTTG-3' Antisense:5'-CTTGCTCTCAGTATCCTTGC-3'

Data were analyzed with the ABI Prism sequence detection system software and quantified using the v1·7 Sequence Detection Software from PE Biosystems (Foster City, CA). For each target gene, relative expression was calculated using the comparative threshold cycle method. All values were normalized to the Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) gene signal as a housekeeping gene, generating Δ cycle threshold (C t) value (ΔC t = C t target gene – C t reference gene[17]. The relative gene expression was calculated according to the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt = ΔCt experimental setting − ΔCt control setting [18].

Histopathological Study

Autopsy samples were taken from the liver of rats in different groups and fixed in 10% formol saline for 24 hrs . Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for 24hrs. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by slidge microtome. The obtained tissue sections were collected on glass slides, deparaffinized and stained by hematoxylin and eosin stain for routine examination through the light microscopy [19].

Statistical analysis

Statistical analyses of all data were presented as the mean ± Standard Error (SE). One-way ANOVA test followed by Tukey post hoc test for multiple comparisons were performed.Statistical analyses were performed by Differences were considered statistically significant for values of P<0.05. All data were analyzed by SPSS PC-software version 20for Microsoft Windows (SPSS Inc., Chicago, IL, USA).

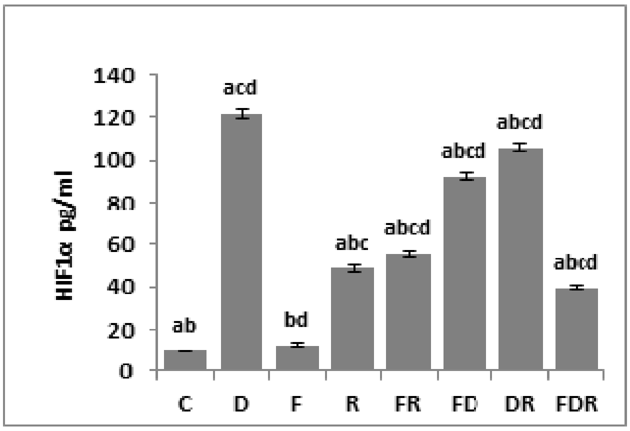

Fig (1):Effect of fermented argel and/or γ-radiation on HIF-α serum level in rats. Each value represents the mean ± SE (n=6). Significance level at p< 0.05, where (a) significant vs control (C), (b) significant vs DEN (c) significant vs fermented argel (F) and (d) significant vs irradiated rats group (R).

The effect of argel and/or gamma radiation on angiogenic factor VEGF was shown in figure 4.The data revealed significant elevation in D group in compared to control.There was significant amelioration occurred in animal injected with DEN treated with argel alone or in combined with radiation in compared to C and D groups.(Fig 2)

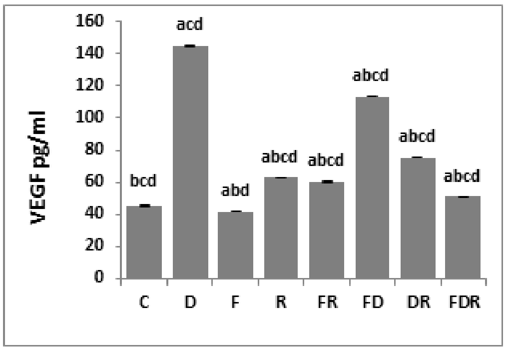

Fig (2): Effect of fermented argel and/or γ-radiation on VEGF serum level in rats. Each value represents the mean ± SE (n=6). Significance level at p< 0.05, where (a) significant vs. control (C), (b) significant vs. DEN (c) significant vs. fermented argel (F) and (d) significant vs. irradiated rats group (R).

Figure 3 represnts the effect of fermented argel and /or low dose radiation on monocyte chemoattractant protein -1 in rats injected with DEN.The results performed significant increase in D group in compared with control group.Otherwise,the ameliorative influence of argel in groups treated with argel alone or in combination with R and/or DEN as compared to control group were appeared.

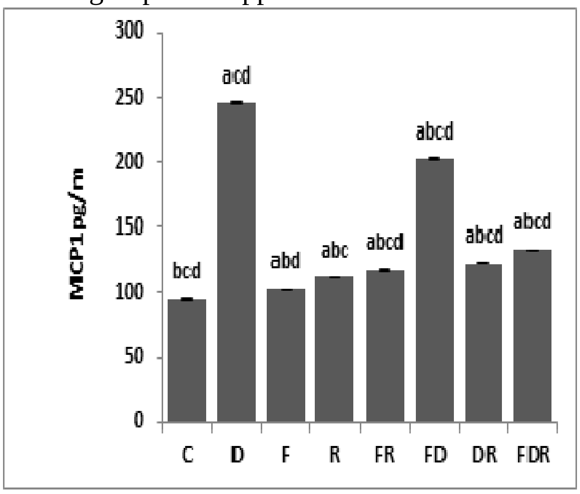

Fig (3): Effect of fermented argel and/or γ-radiation on MCPI serum level in rats. Each value represents the mean ± SE (n=6). Significance level at p< 0.05, where (a) significant vs. control (C), (b) significant vs. DEN (c) significant vs. fermented argel (F) and (d) significant vs. irradiated rats group (R).

In the present work we aimed to evaluate the anti-inflammatory effect of fermented argel by investigate Cox2 inflammatory marker .The control group was 0.99±0.009.There was no significant change in F group (1.09±0.02) in compared with control group. Likewise, there was significant elevation in D and DR groups 11±0.13 and 3.8±0.06 respectively. However, there was significant improvement in Cox2 serum level in FR group 4.2±0.14 as compared to D (Fig. 4) (P < 0.05). Similarly, in FDR group there was significant increase in compared to control group (1.76±0.06).

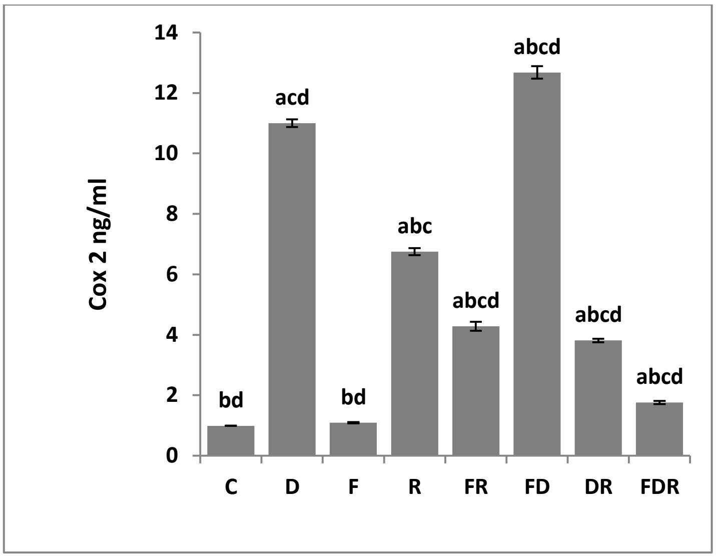

Fig (4): Effect of fermented argel and/or γ-radiation on Cox-2 serum level in rats. Each value represents the mean ± SE (n=6). Significance level at p< 0.05, where (a) significant vs control (C), (b) significant vs DEN (c) significant vs fermented argel (F) and (d) significant vs irradiated rats group (R).

The expression profile of caspase-3 is presented in Fig 5a.The results revealed that in groups of F,R and D the expression of caspase3 gene was significantly elevated with fold change values 1.2,2.6 and 1.6 in compared to control group (P < 0.05)respectively .Likewise, significant increasewas revealed in group injected with DEN and then administered with fermented argel and low dose γ-radiationin compared to C and D groups with fold 3.3 and 1.9 (P < 0.05) respectively. Similarly, cytochrome c gene expression in these groups (F,R,D)documented a fold of change values 1.6,4 and 2.5 respectively in compared to control. The elevation was also remarked in rats injected with DENand treated with fermented argel and radiation with 5.7 and 2.3 as compared with C and D respectively (Fig5b).

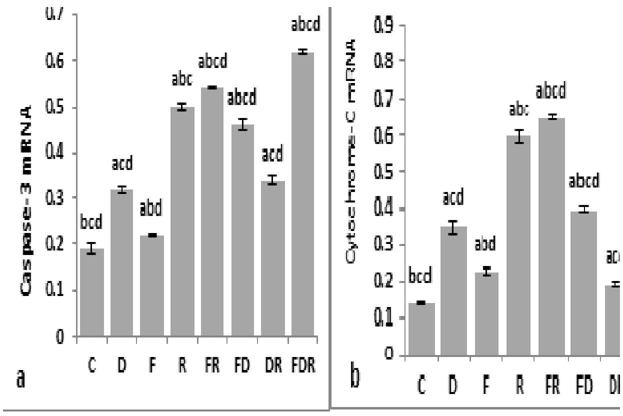

Fig (5):Effect of fermented argel and/or γ-radiation on Caspas3 and cytochrome c serum level in rats. Each value represents the mean ± SE (n=6). Significance level at $p< 0.05$, where (a) significant vs control (C), (b) significant vsDEN(D) (c)fermented argel (F) and(d) significant vs irradiated rats group (R).

Histological Examination of the Liver

In group of rats kept as control, histopathological examination to liver section showed that there was no histopathological alteration and the normal histological structure of central vein and portal area with the surrounding hepatocytes were recorded in(C).Meanwhile,group injected with DEN degenerated dysplastic hepatocytes were divided into lobules by proliferated fibroblasts and inflammatory cells infiltration(D) . Group of rats administrated extract only showed no histopathological alteration as recorded(F).In group exposed to low dose whole body radiation, there was congestion and dilatation were detected in both central and portal veins associated with degeneration in the hepatocytes in diffuse manner(R). Whereas, there was no histopathological alteration recorded in group of rats exposed to radiation and administrated the extract (FR).

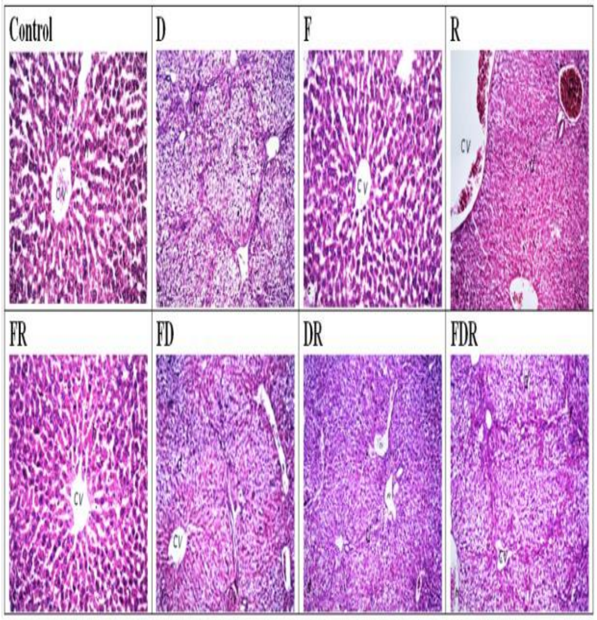

Fig (6):Photomicrograph represents the effect of fermented argel and/or γ-radiation on liver tissue of different tested groups

Mild degenerative change was detected in the hepatocytes with dilatation in the central and portal veins in group treated with argel and injected with DEN(FD).The hepatocytes showed degenerative change associated with dilatation in the central and portal veins in DR group. There was fibrosis observed in mild form and divided the degenerated hepatocytes into lobules in association with dilatation and congestion in the central and portal veins (FDR) (Fig 6)

DISCUSSION

Induction of angiogenesis and inhibition of apoptosis are common phenomena in cancer proliferation and progression[20]. During cancer development, cancer cells are suffering from hypoxia as a result of insufficient oxygen supply. The oxygen level in cancer cell is 1-2% less than normal, so tumor cell tends to undergo angiogenesis. HIF-1 is a transcription factor [21], which is the major regulator of O2 tension[22].In the present work, HIF level displayed significant increase (Fig 1). The elevation of HIF-1 is associated with induction of VEGF, which then enhance the development of new blood vessels to supply tumor with oxygen for their growth [3],[23] which documented that the concentration of circulating VEGF was found to tightly associated with advanced HCC and tumor metastasis. In group injected with DEN both enzymes expression

(Caspase-3 and cytochrome c) were elevated in less extend this might be due to the mechanism has been related to substantial hepatocyte apoptosis induced by DEN injection [24]. In the same line, the current data revealed this controversial role which may explain the significant increase in caspase-3 and cytochrome c in D group and the significant elevation of HIF-1α and VEGF levels in the same group. Otherwise, the amelioration of HIF-1 and VEGF were significantly noticed in rats developed HCC and treated with argel and γ-radiation might be regarded to the pro-apoptotic effect of argel and the immune enhancement of low dose radiation [25], stated the enhancement activity of low dose γ-radiation on lymphocyte. In cancer progression, MCP-1 has involved as a major chemokine which can mediate many types of tumor-promoting cross-talks between tumor cells and tumor microenvironment [26].It play a role in metastasis and angiogenesis by HIF-1 induction [26].Furthermore, many investigation have revealed that MCP-1 gene have many binding sites for HIF-1 in the promotor ,that might be the reasons for increase MCP-1 expression in respond to HIF stabilization[27].In this study, the significant elevation of MCP level in D group could be as a result of HIF. This elevation was improved in FDR group. Occurrence of hypoxia is coupled with inflammation[28].VEGF over expression can be mediated by the activation of NF-kβ signaling by expressing COX-2 gene[29].This might explain the increase of COX-2 level in HCC induced rats. However, the anti- inflammatory effect of argel is responsible for the significant improvement found in rats treated with argel and exposed to γ-radiation[30].

Induction of apoptosis is one of the recent goals of all cancer treatment methods; the disturbance of apoptosis is key event in cancer.Caspase-3and cytochrome c (the main extrinsic and intrinsic apoptotic factors) were estimated. The present data indicate the significant elevation of the expression of both casepase-3 and cytochrome c in groups treated with argel and / or low dose γ-radiation as compared to control group might explain the pro-apoptotic activity of argel in both apoptotic pathways (extrinsic and intrinsic).The significant increase in the expression in FDR is might be due to the synergistic apoptotic effect of both argel and low dose radiation[31].Revealed that both argel and radiation have a synergistic effect against tumor by induction of TNF-α which enhance capase3 and cytochrome c[32] . Furthermore, The biochemical finding in our results was confirmed by results of histopathological examinations. The histopathological observations of the liver of DEN-treated rats revealed well-differentiated HCC hepatocytes with disorganized hepatic lobular architecture and obvious cellular damage. These finding are in agreement with the histological investigation of liver tissues in the study of Seydi*et al* 2015 [33]**.**The administration of argel and /or γ-radiation resulted in significant ameliorations ranged from mild degenerative changes and mild fibrosis in hepatocytes.

In conclusion, among many regulatory factors in tumor angiogenesis, hypoxia-inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF)and monocyte chemotactic protein 1(MCP1) play vital roles in this process .Hypoxia is a potent stimulus for inflammation and remodeling, and HIF-1 can activate transcription of several inflammatory cytokines, chemokines and growth factors. Argel and low dose γ-radiation may have a synergistic effect against HCC by enhancement of apoptosis and downregulate angiogenesis.

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