ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

The apoptotic properties of *Salvia aegyptiaca* and *Trigonella foenum-graecum* extracts on Ehrlich ascites carcinoma cells: the effectiveness of combined treatment.

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ABSTRACT

Cancer still remains the most aggressive killer worldwide. We suppose that plants are the best alternative cancer treatment, as they provide efficacious agents for modern medicine. Hence, we investigated the antitumor activities of sage (*Salvia aegyptiaca*) leaves aqueous extract and fenugreek (*Trigonella foenum-graecum*) seeds methanolic extracts against Ehrlich ascites carcinoma (EAC). The antioxidant properties of both extracts were investigated. The anticancer efficacy of treatments was evaluated by investigating the apoptosis by flow ctometry, DNA fragmentation, chromosomal aberrations and SDS-PAGE. Additionally, anti-angiogenic effects as well as the main oxidative stress markers were also evaluated. The results of sage and fenugreek in vivo treatments inhibited EAC cells proliferation and ascites formation. Additionally, attenuated micro vessel density count in the histological sections of treated mice peritoneum was noted. Consequently, the life span of EAC bearing mice was enhanced noticeably. The cell cycle analysis revealed a prominent decrease in aneuploidy DNA population. Agarose gel electrophoresis also confirmed DNA fragmentation. The antiproliferative potential was proved also as cell death occurrence among treatment groups and they were proved by SDS-PAGE protein analysis and cytogenetically by chromosomal aberrations elevation and all results showed significant antitumor activities by cell death enhancement when compared to control group. The enhanced antioxidant status in extracts-treated EAC bearing mice was evident from decline in lipid peroxidation level and increased level of glutathione. In conclusions, the present study revealed that sage leaves and fenugreek seeds may have a great potential to be exploited for the discovery and development of new anticancer drugs.

Keywords: *Salvia aegyptiaca*; *Trigonella foenum-graecum*; Ehrlich ascites carcinoma; Apoptosis; Antitumor activities.

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ISSN: 0975-8585

INTRODUCTION

Ehrlich ascites carcinoma (EAC) model has been used as a transplantable tumor model to investigate the antineoplastic activities of new anticancer therapies [1,2]. EAC derives from a mouse adenocarcinoma with poorly differentiated malignant tumor and grows in ascites fluid as well as solid tumor and it has the ability to grow in almost all mice strains [3]. Its preference as an experimental malignancy depends on its high chance of tumor development even when subsequent passages are done.

Cancer chemoprevention can be defined as the preventing, slowing down or reversing the tumorigenesis process by administration of one or more natural and/or chemical compounds. The ideal anticancer agent should minimally effective for normal cells. One of the major limitations in the currently available chemical treatments for cancer is their side effects [4]. Hence, usage of natural sources as an alternative cancer therapy is thought to have a great value for cancer control due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive properties [5,6].

Salvia aegyptiaca L. (Egyptian sage) is belonging to family of Lamiaceae. Traditionally sage has been used to treat nervous and mental conditions with anti-inflammatory and anti-bacterial properties [7,8]. There are some chemical compounds like flavonoids, terpenoids and essential oils present in different species of *Salvia* [9]. In this regard, *Salvia officinalis* contains 1, 8-cineole, camphor, borneol, bornyl acetate, camphene, α-and β-thujone, linalool, α-and β-caryophyllene, α-humulene, α- and β-pinene, viridiflorol, pimaradiene, salvianolic acid, rosmarinic acid, carnosolic acid, ursolic acid and etc. [10,11].

Trigonella foenum-graecum L. (fenugreek) is a member of the legume family. The aromatic fenugreek seeds can be roasted, ground to a powder and used as an ingredient in curries, chutneys and teas [12]. Traditionally, fenugreek seeds are used as a remedy for the treatment of diabetes and hypercholesterolemia, moreover, fenugreek have also been reported to exhibit pharmacological properties such as antitumor, antiviral, antimicrobial, anti-inflammatory, hypotensive and antioxidant activities [13,14,15]. The chemical composition of fenugreek includes alkaloids (trigonelline, choline, gentianine and carpaine), saponins, steroid sapogenins (such as fenugreekine), glycoside (such as 4, 5-dimethyl-3-hydroxy-2 [5H]-furanone), coumarin, flavonoids and tannins, including quercetin [16]. Seeds also contain amino acid 4-hydroxyisoleucine [17]. The plant has a wide variety of therapeutic properties and is used as a traditional functional food. However, some toxic effects have been noted with its use [12].

Hence, the present study was conducted to evaluate the antitumor potential of sage leaves aqueous extract and fenugreek seeds methanolic extract and their combination on EAC grown in mice.

MATERIALS AND METHODS

Plant materials

Preparation of *Salvia aegyptiaca* extract

Sage was obtained from open markets and was shade dried at room temperature (30±2°C) and the dried leaves were ground into fine powder using pulverizer. The powdered part was sieved and kept in deep freezer until the time of use. One hundred gram of dry fine powder was extracted with 20x (w/v) of hot water (85°C) for 3 h. The extract was filtered with Whatman No. 1 filter paper to remove insoluble particles. The filtrate was lyophilized with a freeze-dryer-cryodo. The dried extract was then stored in -20°C until used.

Preparation of *Trigonella foenum-graecum* extract

Fenugreek seeds were obtained from open markets and were ground into fine powder using pulverizer. One hundred gram of dry fine powder was extracted with 20x (w/v) of 80% methanol, and kept at room temperature. After 5 days the mixture was filtered and the solvent was evaporated by rotary evaporator (at 40-50 °C and 150 rpm). The dried extract was then stored in -20°C until used.

Measurement of total polyphenols, flavonoids and *in vitro* free radical scavenging assay

Determination of total phenols

The total polyphenolic contents (TPC) were measured using Folin-Ciocalteau reagent based on the oxidation of polyphenols to a blue colored complex with an absorbance maximum of 750 nm. Calibration curve was prepared using gallic acid as standard for TPC which was measured as mg gallic acid equivalents (GAE) per milliliter of the sample (µg/ ml).

Determination of flavonoid content

For the assessment of flavonoids a colorimetric method was used. Briefly, 1.50 ml of the deionized water was added to 0.25 ml of the sample and then 90 µl of 5% Sodium nitrite ($NaNO_2$). Six min later, after addition of 180 µl of 10% $AlCl_3$, mixture was allowed to stand for another 5 min before mixing 0.6 ml of 1M NaOH. By adding deionized water and mixing well, final volume was made up to 3 ml. The absorbance was measured at a fixed wavelength 510 nm. Calibration curve was prepared using quercetin as standard for total flavonoids which was measured as mg quercetin equivalents (QE) per milliliter of the sample (µg/ml).

Determination of DPPH radical scavenging activity

The free radical scavenging capacity was evaluated by the 2, 2-Diphenyl -1- picrylhydrazyl (DPPH) assay. In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical species, the absorption decreases. Briefly, 1 ml of 0.25 mM solution of DPPH in methanol was added to 50, 100, 150 and 200 µl of sample in 950, 900, 850 and 800 µl methanol, respectively. After 20 min, the absorbance was measured at 517 nm. Ascorbic acid was used as a positive control. The percentage DPPH decolorisation of the sample was calculated by the equation, % DPPH scavenging = $[(A_{control} - A_{sample})/A_{control}] -100$, where A is the absorbance.

Experimental Animals:

Experiments were performed on female albino mice, 6–8 weeks old, weighing 25 ± 5 obtained from the Holding Company for Biological Products and Vaccines (**VACSERA, Cairo, Egypt)**. After an acclimatization period of one week, the animals were divided into four groups (6 mice per group) and housed in wire bottomed cages in a room under standard condition of illumination with a 12-hrs light-dark cycle at 25±2ºC. The mice were provided water and a balanced diet *ad libitum*. We followed the European Community Directive (86/609/EEC) and national rules on animal care that was carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals 8^{th} edition.

Tumor cells:

The parent EAC cell line was supplied by the National Cancer Institute, Cairo University, Egypt. EAC was maintained in BALB/c mice in the ascites form. EAC counts were done by a Neubauer hemocytometer using the trypan blue dye (0.4%) exclusion method.

Animal grouping:

Mice were inoculated intraperitoneally (i.p.) on day 0 with (2.5×10^6) viable tumor cells per mouse in a volume of 0.1 ml of cell suspension in physiological saline (0.9%). The groups and the design of the experiment were as follows:

Group 1: EAC (served as –ve control).
Group 2: EAC + SE (i.p 100 mg/ kg/day).
Group 3: EAC + FGE (i.p 100 mg/ kg/day).
Group 4: EAC + SE+FGE (i.p 50 mg/ kg/day of each).
Group 5: EAC + 5-FU (i.p 20 mg/kg/day).

The treatments were continued for 4 days started at the 6th day after tumor transplantation. The dose of SE and FGE selected on the basis of previous study **[18]**.

Ascites volume

Twenty-four hours following the last administration, on the 11th day, either untreated or treated EAC bearing mice were killed under mild ether anesthesia. The ascitic fluid was collected from the peritoneal cavity, and volume was measured.

Percentage increase of life span (ILS)

Mean survival time (MST) of each group containing four mice was monitored by recording the mortality daily of the experimental mice and % ILS was calculated using the following equatio **[19]**.

MST = (day of first death + day of last death)/2

%ILS = (MST of treated group/MST of control group) x 100

Peritoneal angiogenesis and microvessel density (MVD)

After harvesting the EAC cells, the peritoneum was cut open and the inner lining of the peritoneal cavity was examined for extent of neovasculature and photographed. Formaldehyde fixed and paraffin embedded tissues of peritoneum from EAC bearing mice either treated or untreated groups was taken and 5 micron sections were prepared and stained with Hematoxylin and Eosin. The blood vessels were counted according to **[20]**.

Cell cycle analysis by flow cytometry

For the determination of cell cycle phase distribution, EAC cells harvested from tumor-bearing mice were permeabilized and nuclear DNA was labeled with propidium iodide (PI) using Cycle TEST PLUS DNA reagent kit. Cell cycle phase distribution of nuclear DNA was determined on FACS, fluorescence detector equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale) using CellQuest software (Becton Dickinson). A total of 10.000 events were acquired and the results as percent of cells in G_0/G_1, S or G_2/M phases have been displayed.

DNA extraction and agarose gel electrophoresis

DNA extraction was done according to salting out extraction method **[21]** with some modifications **[22]**. Gels were prepared using 1.8% normal melting point agarose in 1X Tris borate EDTA buffer. The apoptotic bands of DNA fragmentation appeared and located at 180 bp and its multiples 360, 540 and 720 bp against thirteen bands of DNA marker (100–3000 bp).

Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) method for determining the total proteins

This technique was done using 12% continuous polyacrylamide gel preparation **[23]**. Gels were digitally photographed then the intensity of bands were analyzed using (Biogene software, France)

Chromosomal aberrations (CAs) assay

For the analysis of EAC chromosomes, colchicine (1mg/ml) was added to Ehrlich ascites tumor fluid and left 1hr, then the cells were resuspended in hypotonic solution (0.075 M KCl) at 37 °C. The cells were centrifuged, fixed by freshly-prepared 3:1 (v/v) methanol:glacial acetic acid. The fixed cells were resuspended and they were dropped onto glass slides and left to air-dry and then the slides were stained with 3% (w/v) Giemsa in phosphate buffer **[24]**. The frequency of chromosomal abnormalities was estimated in 300 metaphases for each animal.

ISSN: 0975-8585

Oxidative stress markers

Ascites was used to determine malondialdehyde (MDA) as indicator of lipid peroxidation by reaction of thiobarbituric acid according to the method of **[25]**. Nitrite/nitrate (nitric oxide; NO) **[26]** and glutathione (GSH) **[27]** were also chemically measured.

Statistical analysis

Results were expressed as the mean ± standard error of the mean (SEM). Data for multiple variable comparisons were analyzed by one-way analysis of variance (ANOVA). For the comparison of significance between groups, Duncan's test was used as a post hoc test according to the Statistical Package for the Social Sciences (SPSS version 20.0).

RESULTS

Total phenolic and flavoniod contents in plant's extract:

The high content of phenolic compounds in SE and FGE are 205.7 mg GAE/g extract and 211.3 mg GAE/g extract, respectively were observed. Whereas, flavonoids are 136.2 mg QE/g extract and 98.6 mg QE/g extract, respectively.

DPPH radical-scavenging activity for plant's extract:

The antioxidant activities of SE and FGE were evaluated by measuring DPPH radical scavenging activity. Figure 1 depicts the dose-response curves for the DPPH radical-scavenging activities of the SE and FGE. The inhibitory concentration (IC_{50}) at which there is 50% reduction of free radicals by SE or FGE was found to be 5.29 µg/ml or 11.18 µg/ml, respectively, which showed increase in its value when compared to the standard ascorbic acid (4.22 µg/ml).

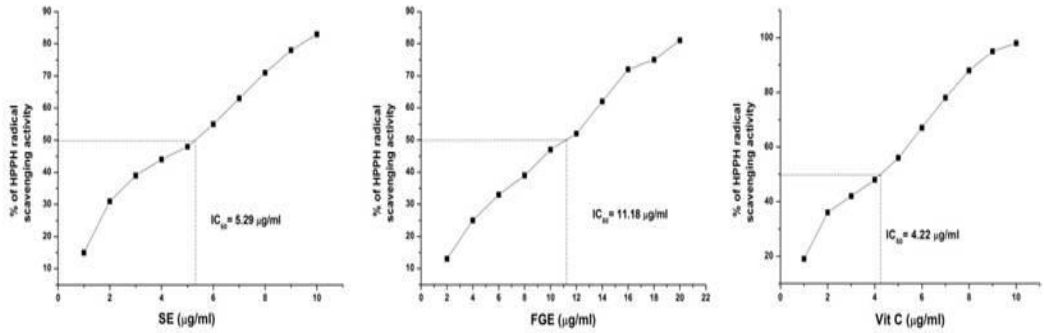

Figure 1. DPPH radical scavenging activity of sage extract (SE) and fenugreek extract (FGE) using vitamin C (Vit C) as standard. Data are represented as mean ± SEM of two independent experiments each performed in duplicate.

Survival time

The effect of the SE and FGE and their combination treatments on survival time of EAC bearing mice is shown in Table 1. The median survival time of animals in the control group was 18 days. This number significantly ($p < 0.05$) increased to 29 when animals were treated with SE. This increase in life span is 61.1%. The median survival time of animals treated with FGE, decreased to 15 days, i.e. the 16.6% decrease in life span. Whereas, the median survival time of animals treated with 5-FU, significantly ($p < 0.05$) increased to 27 days, i.e. the 50% increase in life span. The effect of the combination of these plants on the median survival time of animals was significantly ($p < 0.05$) increased to 30 days and the life span increase to 66.6%.

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Table 1. Mean survival time of mice treated with sage extract (SE), fenugreek extract (FGE) and 5-FU.

Group	Control	SE	FGE	SE+FGE	5-FU
Median survival days	18.0 ± 1.0	29 ± 0.4[a]	15 ± 0.4[a]	30± 0.4[a]	27 ± 0.5[a]
Life span (%)	100	161.1	83.3	166.6	150
ILS (%)*	----	61.1	-16.7	66.6	50

* ILS, increase of life span; ILS = (T − C)/C × 100. T = mean survival time of treated mice, C = mean survival time of control group.
Values are the mean ±SEM (n = 7).
[a]$p < 0.05$: significant change with respect to the control group.
[b]$p < 0.05$: significant change with respect to 5-FU group.

Ascites secretion

The volume of ascites secretion of control and the different treated groups were measured. About 37%, 28% and 36% of ascites secretion were reduced upon SE, FGE or SE+FGE-treatment, respectively. Moreover, there was also no significant difference regarding ascites secretion between SE or SE+FGE and 5-FU.

Cell cycle progression in EAC cells

To find out the mechanism of tumor cell killing by SE, FGE and SE+FGE we analyzed tumor cell cycle phase distribution. The flow cytometric data described the effect of SE, FGE and SE+FGE on cell cycle phase distribution of EAC DNA. On day 10 after EAC inoculation, the content of aneuploidy DNA (82.92% before treatment, Figure 2a) was decreased markedly (0%, Figure 2b) in SE, FGE and SE+FGE treated mice. Moreover, DNA content in G_0/G_1 phases was decreased in SE treated mice to 66.65%, in FGE to 55.01% and in SE+FGE to 53.45% versus the untreated mice (38.49%) as well as DNA content in S and G_2/M phase are also decreased in the treated groups. These results suggested the breakdown of EAC DNA resulting in tumor killing. The obvious ramifications were the growth arrest of EAC.

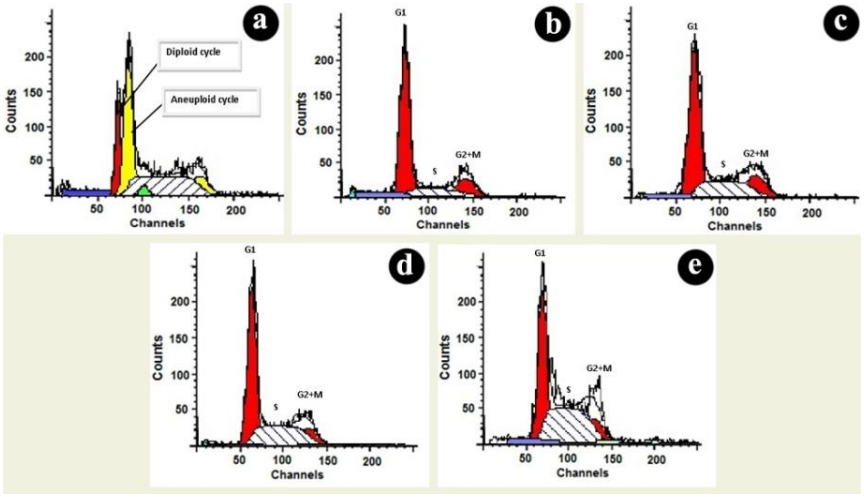

Figure 2. Flowcytometric analysis of EAC cell cycle phase distribution. EAC cells from tumor-bearing mice treated with vehicle (a), sage extract (b), fenugreek extract (c), SE+FGE (d) or 5-FU (e). Histogram display of DNA content (*x*-axis, PI-fluorescence) versus counts (*y*-axis) has been shown.

DNA fragmentation in EAC cells

An attempt was made to find out whether the inhibition of proliferation of EAC cells by SE, FGE and SE+FGE was due to induction of apoptosis in EAC cells. Apoptosis is characterized by fragmentation of DNA. We further verified the effect of SE, FGE or SE+FGE on DNA damage of EAC cells. As expected, SE, FGE or SE+FGE treatments caused DNA fragmentation which then leads to the formation of DNA ladder in EAC cells. The electrophoretic pattern of DNA was shown in (Figure 3). The analysis revealed that DNA from control EAC cells (lane: 1) was found to be intact (undamaged), while the administration of ES (lane: 2) showed moderated

ISSN: 0975-8585

DNA apoptotic fragmentation with laddering pattern in EAC cells and FGE administration (lane: 3) showed necrotic DNA fragmentation. The effect of SE+FGE treatment (lane: 4) showed moderated damage in DNA. Further, we evaluated this fragmentation as optical densities of 180 bp band and its multiples. As shown in Table 2, high intensity of fragmented DNA was detected at 360, 540 and 720 bp in case of FGE treatment.

Table 2. Maximum intensity of fragmented DNA in treated and untreated EAC cells.

Group	Control	SE	FGE	SE+FGE	5-FU
720 bp	-	22 ± 7.3	119 ± 10.9	119 ± 11.7	127 ± 9.5
540 bp	-	20 ± 5.6	90 ± 12.3	150 ±5.3	150 ± 8.3
360 bp	-	10 ± 3.0	43 ± 10.5	154 ± 6.7	220 ± 11.0
180 bp	-	6 ± 2.2	8 ± 6.9	34 ±11.6	190 ± 10.1

Data are represented as mean ± SEM of two independent experiments each performed in duplicate.

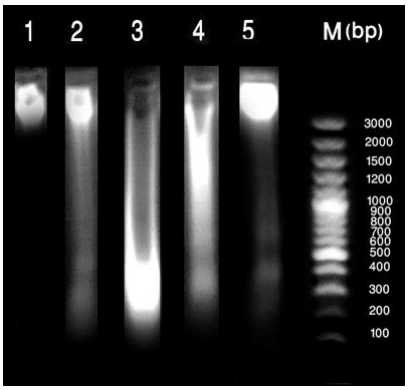

Figure 3. In vivo effects of sage extract, fenugreek extract and 5-FU on DNA fragmentation of Ehrlich ascites carcinoma cells. DNA run and detected on 1.8% agarose gel electrophoresis. Lane 1: DNA from control EAC cells, lane 2: DNA from SE treated EAC cells and lane 3: DNA from FGE treated EAC cells, lane 4: DNA from SE+FGE treated EAC cells, lane 5: DNA from 5-FU treated EAC cells. M: 100 bp DNA ladder marker.

Micro vessel density (MDV) count

We examined the micro vessel density in the peritoneal wall tissue of control as well as treated groups. Figure 4 shows the hematoxylin and eosin staining of peritoneal wall tissue from EAC bearing mice either untreated or treated with SE, FGE, SE+FGE or 5-FU. It is evident from the present results that significantly larger number of blood vessels was seen in the peritoneum of control mice as compared to the decreased blood vessels seen in different treated mice. Peritoneum section from EAC-bearing mice showed 4.3 ± 1.0 blood vessels whereas in case of SE treated mice 1.7 ± 0.4 blood vessels, 1.9 ± 0.4 from FGE treated mice, 2.4 ± 0.7 from SE+FGE treated mice and 2.0 ± 0.5 from mice treated with the standard drug were observed (Table 3).

Table 3. Micro vessel density in peritoneum section from mice treated with sage extract (SE), fenugreek extract (FGE) and 5-FU.

Group	Control	SE	FGE	SE+FGE	5-FU
Micro vessel density (MDV)	4.3±1.0	1.7±0.4[a]	1.9±0.4[a]	2.4±0.7[a]	2.0±0.5[a]

Values are the mean ±SEM (n = 7).
[a] $p < 0.05$: significant change with respect to the control group.
[b] $p < 0.05$: significant change with respect to 5-FU group.

ISSN: 0975-8585

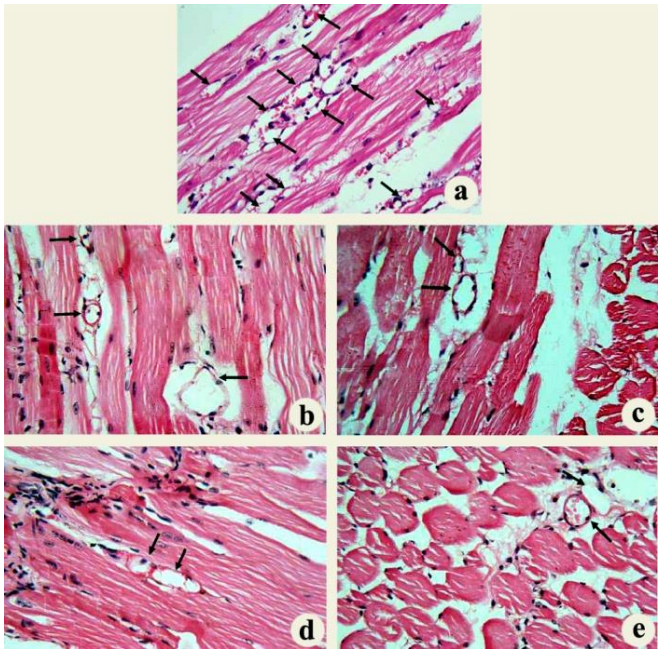

Figure 4. Histopathological H&E studies of the peritoneal wall of EAT tumor-bearing mice treated with vehicle (a), sage extract (b), fenugreek extract (c), SE+FGE combination (d) or 5-FU (e). H&E × 400.

Determination of the total proteins profile of EAT cells (SDS-PAGE):

The protein profile of electrophoresed EAT cells pattern on 12% SDS-PAGE revealed that protein from control EAT cells was found to be intact (undamaged), while the protein pattern of treated groups indicated the presence of proteins degradation (smear shape) which reflect the evidence of cell death (Figure 5). Moreover, three bands were detected in the proteins obtained at 34 KDa (a), 37 KDa (b) and 39 KDa (c) in SE, FE, SE+FGE and 5-FU treated groups.

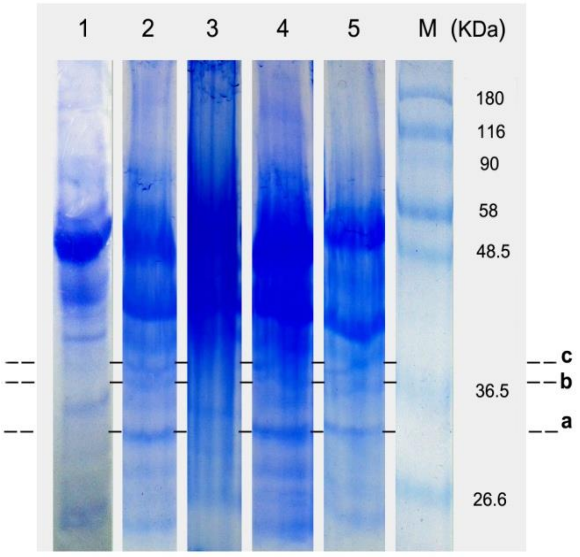

Figure 5. Digital photograph of SDS-PAGE protein electrophoresis separations of EAT cells shows the effect of plants' extract (sage 100 mg/kg/day & fenugreek 100 mg/kg/day) and 5-FU drug (20 mg/kg/day) after 4 days of treatment, where, 1-4 lanes resembles EAT control group, EAT+SE group, EAT +FGE group, EAT SE+FGE group and EAT +5-FU group respectively; and M: (26.6-180 KDa) Marker. a, b and c: detected differences in protein bands profile among treatment groups.

Chromosomal aberrations (CAs) assay:

The results showed that SE, FGE and SE+FGE treatments caused total chromosomal aberrations (TCA: 3.106, 2.533 and 2.666 respectively) with a significant increase when compared to the EAT control (TCA: 1.553), while nearly the same effect at the case of FE when compared to the standard drug (5-FU) (TCA: 2.613).

Oxidative stress markers

Effect of SE, FGE and SE+FGE on lipid peroxidation, nitric oxide and glutathione levels in tumor bearing mice are presented in Table 4. MDA levels in EAC cells were markedly ($p < 0.05$) decreased in EAC treated groups as compared to corresponding values of the untreated control group. However, NO levels were significantly ($p < 0.05$) increased in EAC treated groups. Moreover, GSH contents in EAC cells were increased significantly ($p < 0.05$) compared to the untreated controls.

Table 4. Oxidative stress markers in Ehrlich ascites tumor from mice treated with sage extract (SE), fenugreek extract (FGE) and 5-FU.

Group	Control	SE	FGE	SE+FGE	5-FU
MDA (mmol/mg protein)	35.24 ± 1.44	27.82 ± 1.28[a]	29.78 ± 1.44[a]	24.660 ± 1.33[a]	26.18 ± 1.93[a]
NO (µmol/mg protein)	22.07 ± 4.41	99.11 ± 7.47[ab]	64.21 ± 4.87[ab]	54.35 ± 6.39[ab]	79.87 ± 6.38[a]
GSH (nmol/mg protein)	17.40 ± 0.99	24.78± 1.11[ab]	66.46± 3.27[ab]	33.64± 2.83[ab]	44.98± 4.35[a]

Values are the mean ±SEM (n = 7).
[a] $p < 0.05$: significant change with respect to the control group.
[b] $p < 0.05$: significant change with respect to 5-FU group.

DISCUSSION

Cancer chemoprevention utilizes chemical or natural substances to revert or inhibit malignancy and metastasis. The use of natural medicinal sources or dietary agents is being increasingly utilized as an effective way for the management of cancer **[28,3]**. The Ehrlich ascitic tumor implantation induced local inflammatory responce, with increasing vascular permeability, which results in an intense edema formation, cellular migration and a progressive ascitic fluid formation **[29]**. Ascitic fluid is the essential nutritional source for proliferation of tumor cells and a rapid increase in ascitic fluid with tumor growth would provide the essential nutritional requirement of tumor cells **[30]**. In the present study, regular and rapid increases in ascitic tumor volume were observed in EAC tumor bearing mice, while in groups were taken the treatments, a decreased in tumor volume was observed supporting the beneficial anticarcinogenic effect of sage and fenugreek.

Angioinhibition and induction of apoptosis in tumor cells are the recent strategies for the treatment of cancer. Thus the discovery of angiogenic inhibitors with proapoptotic potential would provide a valuable therapeutic importance. In present study, we observed angiogenesis in ascites tumor-bearing peritoneal walls. Moreover, the tested extracts of sage and fenugreek have potent antiangiogenic and proapoptotic effects on EAC cells *in vivo*. Paraffin sections of the peritoneum stained with H & E showed reduction in microvesssel density count suggesting that sage and fenugreek have an antiangiogenic activity. These findings reinforce evidence that the anti-inflammatory actions of sage and fenugreek modify the peritoneal microenvironment and reduce other factors related to the formation and development of cancers, for example, reduction of neovascularization and ascites accumulation **[31]**.

Following the treatments with sage or fenugreek, we observed a significant induction of apoptosis and increased chromosomal aberration index and the extracted DNA showed a ladder pattern when analyzed by gel electrophoresis and this degradation is through the activation of endonuclease suggesting an association of the antineoplastic effect of the extracts on EAC cells with the induction of apoptosis. The inhibitory effect of sage or fenugreek on EAC cell growth may be due to induction of apoptosis. The results of SDS-PAGE of EAT cells supported the presence of cell death and degradation in addition to the appearance of new bands which could be for proteins that associated with apoptosis like caspase-3 (34 KDa), caspase-9 (37 KDa) and vascular endothelial growth factor VEGF (39 KDa).

The cell cycle phase distribution of the EAC cells obtained from EAC treated groups was studied by flow cytometry to investigate whether the reduction in tumor volume was due to the treatment induced inhibition in cell cycle progression. Cell cycle and apoptosis may be linked, and they provided an argument to support that. (1) apoptosis is almost present in proliferating cells. (2) molecules acting on cells in late G_1 phase are also required for apoptosis. (3) passaging of the of a cell from late G_1 to the S phase is controlled by p53. Finally, artificial manipulation of the cell cycle could either abolish or potentiate apoptosis **[32]**.

Oxidative stress and lipid peroxidation may play important roles in carcinogenesis. Tumor cells are sensitive to the LPO products which are also mediators of oxidative stress **[33]**. A depleted endogenous antioxidant molecules such as GSH with enhanced free radicals generation and LPO is documented in carcinogenesis **[34]**. Many cancer cells with pro-oxidant status promote oxidative stress, which increases the survival potential of the tumor cells by mutations induction, redox signaling activation and pro-survival factors stimulation **[35]**. GSH is a tripeptide and considered as the major endogenous antioxidant produced by cells and helps to protect cells from ROS **[36]**. In the current study, the GSH levels in the experimental treated mice were found to be significantly higher than those in the EAC untreated control mice. These results revealed that the antitumor activities of sage and fenugreek were accompanied by the enhancement of non-enzymatic antioxidant protection and inhibiting EAC-induced intracellular oxidative stress.

NO may also play a prominent role in cytotoxicity induced by the different extracts. Our results showed that sage and fenugreek significantly increased the production of NO in the ascites fluid of treated animals. The elevation of NO levels may be due to the activation of macrophages present in the ascites fluid. It has been found that macrophages play an important role against neoplastic cells to initiate cell death programs **[37]**. Further, NO can cause growth arrest and apoptosis because of its high reactivity with iron-and thiol-containing macromolecules, thereby inhibiting enzymes of DNA synthesis and repair **[38]**.

In conclusion, we demonstrated that sage and fenugreek extracts may act as antioxidant, antiangiogenesis and pro-apoptotic agents. Further studies are necessary to identify the active principle responsible for pro-apoptotic and antiangiogenic actions of both sage and fenugreek.

Conflict of interests

Authors declare that there is no conflict of interests regarding the publication of this article.

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