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TumoricidalEffect of Trigonella foenum-graceum Extract and Selenium Nanoparticleson Ehrlich Carcinoma Bearing Mice٤

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ABSTRACT	۱۱

The present study was designed to examine the antitumor effect of Trigonella foenum-graceum either alone or combined with selenium nanoparticles. Ehrlich ascite carcinoma (EAC) cells and 4 groups of female mice were used. Solid Ehrlich carcinoma (EC) was induced by inoculation of 2.5×10^6 cells in the left thighs of each animal. Mice were gavage orally by 2.5 µg/0.1 ml of *Trigonella foenum*graceum extract either alone or combined with selenium nanoparticles daily for one month. Tumor size, serum tumor markers (TNF- α , IFN- γ and Granzyme-B and Caspase-3) were evaluated. Oxidative $\frac{1}{2}$ and antioxidant markers, Histopathological, apoptotic and necrotic examination were determined in tumor tissues. Significant inhibition in tumor size. Caspase-3 and Granzyme-B activity Were significantly elevated along with diminished tumor size while, TNF- α and IFN- γ levels were decreased in serum. Meanwhile, oxidative stress marker (MDA) was significantly decreased in tumor tissue? In addition, tumor GSH content and CAT activity were increased. Histopathological, apoptotic and nečrotic examinations were context with the previous results. It could be concluded that Gg extract either alone or combined with SeNPs exhibited antitumor activity and this is reflected by reduction in tumor size, decrease of serum TNF- α and IFN- γ , increase in serum caspase-3 and Granzyme-B, reduction in turnor 22 MDA and increase in tumor GSH and CAT which cause regulate tumor regression.

INTRODUCTION

Fenugreek (*Trigonella foenum-graceum*) is a leguminous herb belonging to fabaceae, cultivated throughout the world especially in the Asia and North African countries. It is best known for prešence of pungent aromatic compounds in their seeds that gives color, flavor and aroma to food. It has been used as a medicinal plant since more than 4000 years in various parts of world. It has wide therapëutic applications including carminative, aphrodisiac and lactation stimulant in women after childbirth in traditional Chinese medicines. Its ability to treat wounds and sore muscles had made its use with in science (**Bano** *et al.*, **2016**). Fenugreek has antidiabetic activity, antiplasmodic activity, hypolipidefinic activity, immunological activity, antibacterial activity, anthelmintic activity, anti-inflammatory, analgesic activity and antioxidant activity (**Yadav** *et al.*, **2011**). It also has gastro and hepatoprotective properties (**Pandian** *et al.*, **2002**). It is also used in cardiovascular health issues (**Hannan** *et al.*, **2003**).

The main chemical components of *Trigonella foenum-graceum* are fibers, flavoñoids, polysaccharides, saponins, fixed oils and some identified alkaloids (**Mohammed** *et al.*, 2006). Mature seeds mainly contain amino acid, fatty acid, vitamins, saponins and a large quantity of folic^{ξ} acid (84mg/100g) (**Mohammed** *et al.*, 2006). The chemical constituents of fenugreek possessing anticancer activity are phytoestrogens and saponins. Saponins selectively inhibit cell division in tumor cells and can activate apoptotic programs that can lead to programmed cell death (Shivangi *et al.*, 2016). Flavonoids and catechins were first shown to be apoptotic in human carcinoma cells (Ahmed *ét^{{z}al., 2016*).

2000).

The trace element selenium (Se) is important for health of humans and animals. The role of seleno-compounds as chemopreventive and chemotherapeutic agents has been \mathbf{t}^{\vee} supported by a large number of epidemiological, preclinical and clinical studies (El-Batal \wedge et al., 2012). Selenium nanoparticle (Nano-Se) is a novel Se species with novel biological ⁹ activities and low toxicity compared with other Se compounds like sodium selenite, selenomethinine, and methyl selenocysteine this makes Nano-Se a good candidate in clinical 07 cancer treatment (El-Batal et al., 2012).

AIM OF THE WORK

In the current study, we sought to achieve the emerging nano-based approaches suitable to be used as imaging techniques for cancer treatment by fenugreek extract either alone or combined with SeNPs.

MATERIALS AND METHODS

Animals

Female outbreed Swiss albino mice originally obtained from National Cancer Institute (NCI) (20-25g) were used as experimental animals. All the studied animals were conducted in accordance with criteria of the investigation and ethics committee of the community laws governing the use of 21 experimental animals.

Ehrlich Ascites Carcinoma Cell Line (EAC).

Ehrlich Ascites Carcinoma, were obtained from National Cancer Institute (NCI), Cairo university .The cells were propagated as ascites in female Swiss albino mice by weekly intraperitoneal inoculation 20 of 2.5 x 10⁶ cells/ mouse (Salem *et al.*, 2011).

77 Preparation of Fenugreek extract Fenugreek extract is produced by putting flask contains 10 g of Fenugreek seeds ٦٧ with 20 ml D.W in autoclave at 120° C for 20 min. then filtration to get Fenugreek ٦٨ 79 extract. ٧.

Preparation of nanoparticle :

Selenium dioxide 1mM solution was mixed with aqueous extract of fenugreek powder 1.11 v/v . The mixture was stirred at room temperature and exposed to gamma ray at 40 kGy. This led to the immediate formation of SeNPs visualized as pink color solution. Then SeNPs were immediately characterized by Transmission electron microscopy (TEM), Dynamic light scattering measurement 10 (DLS) and Fourier transform infrared spectroscopy (FTIR).

Transmission electron microscopy (TEM)

SeNPs suspension were loaded on carbon-coated copper grids and solvent was allowed to evaporate by incubation at 37°C for 30min in an incubator. The size and morphology of the SeNPs Were estimated by TEM (JEOL electron microscope JEM-100 CX) operating at 80 kV accelerating volvage. ٨.

Dynamic light scattering measurement (DLS)

Average particle size and size distribution were determined by the dynamic light scattering (DLS). Technique (PSS-NICOMP 380-ZLS, USA); 250µl of suspension were transferred to a disposable low volume cuvette. After equilibration to a temperature of 25°C for 2 min., five measurements After ٨٤ performed using 12runs of 10s each. 10

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of the samples were recorded in KBr pellets using an FTIR spectrophotometer (JASCO FT-IR -3600) and spectrum was collected at a resolution of 4cm⁻¹ in wave number region of 400 to 4000cm⁻¹ to identify the possible molecules responsible for the reduction of selenium ions and to confirm ٨٩ FPP capped SeNPs.

In vitro study \geq

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Chemosensitivity of nanoparticles (Cell viability):

Antitumor effect of *Trigonella foenum-graceum* extract and/or SeNPs was assessed? by observation of changes with respect to viable and nonviable tumor cell count. Cytotoxicity effects of the nanoparticles on tumor cells were determined according to the method of **El- Merzabani** *et al.* (1979). In order to detect the cytotoxicity of nanoparticles, EACs were treated with nanoparticles at the concentrations of 1,2,3,4,5,6,7,8,9,10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml. The EACs were obtained by needle aspiration of ascites fluid from the preinoculated mice under aseptic condition and ultra violet laminar airflow system. The percentages of non-viable cells were determined by counding dead and viable EACs. To differentiate between dead and viable EAC cells, trypan blue stain was used. Then the percentages of non-viable cells (NVC) were calculated according to the following equations $NVC = C/T \times 100$, where (C) is number of non-viable cells and (T) is total number of viable cells.

Experimental Design

The animals were allowed 7 days for adaptation. 60 mice were then randomly distributed into 4 equal groups, 15 mice for each. The animal groups were recognized as follows: G1: Normal control group. Normal mice neither injected nor treated. G2: Ehrlich carcinoma (EC) bearing group. Mice were intramuscularly injected with 0.2ml of

 2.5×10^{6} /ml/mouse viable Ehrlich ascite carcinoma cells in the left thigh.

G3: EC bearing fenugreek extract group. Mice were injected intramuscularly with 0.2ml of 2.5×10^6 Ehrlich ascite carcinoma cells in the left thigh then after one day of tumor inoculation fenugreek extract gavage $2.5 \mu g/0.1$ ml orally every day for one month.

G4: EC bearing fenugreek extract combined with SeNPs group. Mice were injected intramuscullarly

with 0.2ml of 2.5×10^6 Ehrlich ascite carcinoma cells in the left thigh then after one day of tubior inoculation fenugreek extract combined with SeNPs gavage $2.5 \mu g/0.1$ ml orally every day for one mohth. Monitoring the tumor size

Tumor size was monitored twice or thrice weekly throughout the experiment. The tumor size being measured regularly using Vernier calipers and represented in terms of tumor size. The tumor size was estimated using the following formula: Tumor size $(mm^3) = 4 (A/2) (B/2)^2 = 0.25 A.B^2$, where AV is the major axis and B is the minor axis (Ghoneum *et al.*, 2008). The mean tumor size with the corresponding standard error was calculated in each experimental group. One month after treatments, experiment was terminated and all animals were sacrificed.

Sample preparation:

After one month of treatments, mice were anaesthetized using diethyl ether and sacrificed. Blood and tumor from animals of each group were collected and used for the proposed studies.

Preparation of serum:

Animals were sacrificed and the blood was collected from heart puncher using disposable plassic syringes, drained in tube, and left for coagulation. The blood was centrifuged and the upper layer (sertin) was taken. TNF- α , IFN- γ , Granzyme-B and Caspase-3 were measured in serum of each group.

Tissue samples:

The EC tumor tissue of experimental animals were dissected out, washed and divided into two parts, one part was kept in 10% formalin for histopathological studies, apoptosis detection and the other part was prepared in ice-cold saline (0.9%) using a potter-Elvehjem homogenizer to give a 10% homogenates which were used for determination of biochemical parameters.

Biochemical analysis:

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In serum, the levels of tumor necrosis factor-alpha, Interferon-gamma, Granzyme-B) Tand Caspase-3 activities were assayed by the standard sandwich enzyme-linked immune-sorbent (ELISA) assay technique using ELISA kit (K0331186, KOMABIOTECH, Seoul, Korea) following "the manufacturer's instructions, In Ehrlich carcinoma tumor tissues, lipid peroxidation, Reduced glutathione and Catalase were measured colorimetrically as described by Yoshioka et al. (1979), Beutler et al. 189 (1963) and Sinha (1972) respectively.

Statistical Analysis

The obtained data was expressed as mean \pm standard error (SE). All data were analyzed statistically using one-way analysis of variance (ANOVA) followed by Student's t-test. Statistical significance was considered at P < 0.05. Statistical Package for Social Sciences (SPSS) for Windows version 17.0 software was used for this analysis (Harnett and Horrell, 1998). 122 120

Histopathological Examination:

Following mice sacrificing tumor tissues were rapidly dissected and excised, rinsed in satine solution and cut into suitable pieces, then fixed in neutral buffered formalin (10%) for 24 hours, following fixation, the specimens were dehydrated in ascending series of alcohol, then tissue specifichs were cleared in xylene and embedded in paraffin at 60°C. Section of 5 microns thickness was cut by slidge microtome. The obtained tissue sections were collected on the glass slides and stained by haematoxylin and eosin stain for histopathological examination by the light microscope (Banchroft)et al., 1996). Another tissue sections (2-4 µm thick) were cut from paraffin embedded blocks by microtome and mounted from warm water (40 $^{\circ}$ C) onto charged adhesive slides. By using a mixture of 100 μ g/ml acridine orange and 100 µg/ml ethidium bromide prepared in PBS, the apoptosis and necrosis stalifing were analyzed (Ribble et al., 2005). The tissue uptake of the stain was monitored under a fluores defice microscope. 107

RESULTS

Morphology of nanoparticles:

The distribution of the particles size, DLS was performed, and its outcomes were linked 109 17. to the TEM results. The average particle size was defined by DLS technique and was 171 determined as 117 nm in SeNPs as noted in Fig. 1.





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Fig. (2): Transmission Electron Microscopy (TEM)

11. The samples were recorded in KBr pellets using an FTIR spectrophotometer and spectrum was collected at a resolution of 4cm⁻¹ in wave number region of 400 to 4000cm⁻¹ 111 to identify the possible molecules responsible for the reduction of selenium ions and to 171 confirm FPP capped SeNPs as in Fig. 3. 177



□ In vitro studies:

- Chemosensitivity of fenugreek extract either alone or combined with SeNPs on Ehrlich Ascite 119 carcinoma cells

The tumoricidal effect of different concentrations of fenugreek extract either alone or combined with SeNPs on Ehrlich cells viability is shown in Table (1). The low concentration (10 µg/mll) of ۱۸۲ fenugreek extract decreases the tumor cells viability by 15%.

The cytotoxicity of fenugreek extract led to the death of Ehrlich carcinoma cells. The median lethal concentration of fenugreek extract was 70 µg/ml for Ehrlich carcinoma cells. At a concentration

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of 20 μ g/ml fenugreek extract led to the death of 20% of Ehrlich carcinoma cells and at a concentration of 90 μ g/ml fenugreek extract led to the death of 65% of Ehrlich carcinoma cells.

The low concentration (10 μ g/ml) of fenugreek extract combined with SeNPs decreases the tumor cells viability by 20%. The median lethal concentration of fenugreek extract was 60 μ g/ml/for Ehrlich carcinoma cells. For concentration of 20 μ g/ml led to the death of 25% of Ehrlich carcinoma cells and at a concentration of 90 μ g/ml fenugreek extract combined with SeNPs led to the death of 80% of Ehrlich carcinoma cells.

 Table (1): The effect of Trigonella foenum-graceum extract and Trigonella foenum-graceum extract

 combined with SeNPs on the viability of Ehrlich ascites carcinoma cells.

Concentration (µg/ml)	<mark>Trigonella foenum-</mark> graceum extract		Trigonella foenum-graceum extra combined with SeNPs	
	% of viable cells	% of dead cells	%of viable cells	% of dead cells
0	99	1	99	1
9	90	10	90	10
10	85	15	80	20
20	80	20	75	25
30	75	25	70	30
40	70	30	65	35
50	65	35	60	40
60	60	40	50	50
70	50	50	40	60
80	40	60	30	70
90	35	65	20	80

In vivo studies

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Ehrlich Carcinoma Tumor Size Monitoring:

The size of solid Ehrlich carcinoma (EC) in the left thigh of mice was measured eight times along one month starting from EC tumor cells inoculation and beginning of tumor formation in control \mathcal{PC} bearing mice. The delay of inhibition in tumor size in mice treated by fenugreek extract either alone or combined with SeNPs comparing with EC group is illustrated in Fig (4). It is clear that the mean size of the left thigh of healthy normal mice is 17.55 mm³ and the inoculation of 2.5 million of EC cells $\mathbf{in} \cdot \mathbf{0.2}$ ml physiological saline in the left thigh of healthy normal mice produced a solid tumor with a mean size of 95.67±3.83 mm³ on the 7th day after tumor inoculation after tumor inoculation. EC tumor size

exceeds 400 mm³ on the 10^{th} day after tumor inoculation. The increase of EC tumor size proceeds by days reaching $2583.33\pm35.7 \text{ mm}^3$ on the 30^{th} days after tumor inoculation.

The data obtained revealed lesser tumor size through the observation period in groups of experimental animals daily treated with fenugreek extract at the next day after tumor inoculation for one month. At the 7th, 10th and 30th days after tumor inoculation tumor size were 60.5 ± 4.42 , 74 ± 4.75 and 1682.5 ± 48.36 mm³ respectively. The tumor size of mice treated with fenugreek extract combined with selenium nanoparticles at the next day after tumor inoculation for one month every day showed $(86.17\pm5.31, 136.33\pm5.07 \text{ and } 1694.33\pm13.94 \text{ mm}^3 \text{ on } 7^{th}, 10^{th} \text{ and } 30^{th} \text{ days respectively})$.



Fig. (4): Effect of fenugreek extract either alone or combined with SeNPs on EC tumor size.

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Tumor markers responses

Caspase-3, Granzyme-B, Serum tumor necrosis factor-alpha (TNF- α) and Serum Interferon gamma^Y ¹ ^o (IFN- γ) detection

Data revealed that female mice inoculated with EC and treated with fenugreek extract daily for one month recorded a significant increase in caspase-3 activity, a significant decrease in GranzymbAB activity, a significant decrease in TNF- α Level and a significant decrease in IFN- γ Level in compared to EC group. On the other hand, daily treatment of female mice inoculated with EC and treated with Licorice extract combined with SeNPs for one month predicts an increase in caspase-3 activity has increase in Granzyme-B activity, a decrease in TNF- α level and a decrease in IFN- γ level compared to EC group.

Table (2): Effect of fenugreek extract either alone or combined with SeNPs on Caspas	e-3, Granzyme-
B, TNF- α and IFN- γ levels of mice bearing EC.	220

Groups Parameter	G1	G2	G3	G4
Caspase-3 (µmol pNA/min/ml)	2.2±0.03	2.83±0.07	3.1±0.23	7.69±0.06 ab
Granzyme-B	78.63±2.16	14.1±0.62	13.63±1.01	46±1.89
(pg/ml)	D	a	а	ab
TNF-α	30.89±0.88	113.47±4.02	39.5±1.85	53.81±2.42
(pg/ml)	b	а	ab	ab

IFN-γ	17.47±0.48	85.96±2.35	18.59±0.67	35.4±0.95
(pg/ml)	0	u	0	ao
All data are the means of 10 records.				

222 222 a: significant against N at $P \le 0.05$ b: significant against EC at $P \le 0.05$ 227 229 ۲۳. 777

> Oxidative stress and antioxidant markers in tumor tissues

Tumor tissue MDA, CAT and GSH activity are represented in Table (3) The data revealed that female mice bearing EC represents a highly significant increase in tumor MDA and a highly significant 170 decrease in tumor GSH and CAT in compared to N group.

The oral gavages of female mice bearing EC by fenugreek extract daily for one month recorded decrease in tumor MDA and GSH activity and a significant increase in CAT in compared to EC bearing group. Treatment of female mice bearing EC with fenugreek extract combined with SeNPs daily for one month predicts decrease in tumor MDA, increase in tumor GSH and CAT in compared to EC group.⁹

Table (3): Effect of fenugreek extract either alone or combined with SeNPs on MDA, CAT and 251 ۲٤٢ GSH levels of mice bearing EC.

Groups Parameter	G1	G2	G3	G4
MDA	64.67±2.33	117.83±6.29	112.83±4.55	91.67±1.69
(μM/gm tissue)	b	^a	a	ab
Catalase (µM Catalase/gm tissue)	1.5±0.1	0.2±0.01	0.5±0.01 ab	1.5±0.5 ab
GSH	2.33±0.09	1.6±0.14	1.36±0.11	1.75±0.09
(mg GSH/gm tissue)	b	a	a	a

All legends as in table (2)

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- Histopathological examination of Ehrlich carcinoma (EC):

Histopathological examination possessed normal muscle histology (Fig. 5 A) of non-infice bearing Ehrlich carcinoma. Ehrlich carcinoma (EC) tissue section under light microscope showed compact and aggregation of the tumor tissue cells spread within the muscular tissues. EC showed groups of large, round and polygonal cells, with pleomorphic shapes, hyperchromatic nuclei and binucleătion. Several degrees of cellular and nuclear pleomorphic were seen in (Fig. 5 B&C). EC of mice gavage orally by fenugreek extract daily for one month after 1 day of tumor inoculation represents extensive areas of necrotic EC cells and other areas contain of remnants, apoptotic and some pyknotic nuclei (Fig. 6 A, B&C). Photomicrographs in sections of Ehrlich carcinoma of mice gavage orally by fenugreek extract combined with SeNPs daily for one month represents extensive areas contain of remfants, 100 apoptotic and some pyknotic nuclei after 1 day of tumor inoculation (Fig. 7 A&B).



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- Fig. (5): Photomicrograph in sections of EC. A: Normal control muscle section in Albino mike
represents normal muscular fiber. B & C: Control EC. Note: EC cells invaded muscular
tissue; (↔) tumor cells encircled the muscles cěllsť.
(H and E stain, A&B X100- C X 400)



Fig. (6): Photomicrographs in sections of Ehrlich carcinoma of mice gavage orally by fenugreek extract daily for one month. A, B& C: gavage after 1 day of tumor inoculation represents extensive areas of necrotic EC cells (star) and other areas contain of remnants, apoptotic and some pyknotic nuclei (▲). (H and E stain, A X100- B& C X 400)



Fig. (7): Photomicrographs in sections of Ehrlich carcinoma of mice gavage orally by fenugreek extřáct combined with SeNPs daily for one month. A& B: gavage after 1 day of tumor inoculařiôň represents extensive areas contain of remnants, apoptotic and some pyknotic nuclei (▲)." (H and E stain, A X100- B X 400)

Apoptotic and necrotic examination of Ehrlich carcinoma (EC):

Apoptotic and necrotic stained by Acridine orange / propidium iodide stain and examined under a fluorescent microscope. Normal muscle tissue section represents vital tissue regions stained in green color (Fig. 8 A). Control section of EC represents vital tissue stained in green stain with no zones of necrosis (orange cells) or apoptosis (yellow cells) in addition to the presence of vital green regions and some vacuolated areas (Fig. 8 B&C).

Treatment of mice orally by fenugreek extract daily for one month represents extensive areas of necrotic EC cells and other areas contain of remnants of apoptotic nuclei and some vacuolated areas for gavage treatment after 1 day of tumor inoculation (Fig 9 A&B). Combined treatment of fenugreek extract with SeNPs daily for one month represents extensive areas of necrotic EC cells and other areas contain of remnants of apoptotic nuclei and some vacuolated areas for gavage after 1 day of tumor inoculation (Fig. 10 A&B).





Fig. (8): Fluorescent imaging of sections in Ehrlich carcinoma stained by Acridine orange / propidium iodide stain. **A:** Normal muscle represents vital tissue regions stained in green (red blocked arrows). **B&C:** Control Ehrlich carcinoma represents vital green regions (1) and some vacuolated areas (▲). (A&C X250, BX100)



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Fig. (9): Photomicrographs in sections of Ehrlich carcinoma Fluorescent imaging of sections in Ehrlich carcinoma stained by Acridine orange / propidium iodide stain of mice gavage orally by fenugreek extract daily for one month. A& B: gavage after 1 day of tumor inoculation represents extensive areas of necrotic EC cells (1)
 and other areas contain of remnants of apoptotic nuclei (●) and some vacuolated areas (▲). (A& B x 250)

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۳0. Fig. (10): Photomicrographs in sections of Ehrlich carcinoma Fluorescent imaging of 501 sections in Ehrlich carcinoma stained by Acridine orange / propidium iodide stain 507 of mice gavage orally by fenugreek extract combined with SeNPs daily for one 505 month. A& B: gavage after 1 day of tumor inoculation represents extensive areas 305 of necrotic EC cells (•) and other areas contain of remnants of apoptotic nuclei 000 (star) and some vacuolated areas (▲). 807 (A& B x 250) 30V

DISCUSSION

Α

Classical very potent chemotherapeutic agents have been used against several tumor types for several decades. However, they have the disadvantage of affecting both tumor cells and normal tells, with the concomitant secondary effects including cardiotoxicity, cytotoxicity, neurotoxicity, nephrotoxicity, and ototoxicity (**Wang** *et al.*, **2012**). Some of these chemotherapeutic-associated problems have been solved by the use of nanoparticle formulations of these drugs. The most important advantage of these novel formulations is that they preferentially target tumor cells by the enhanced permeability and retention (EPR) phenomenon exhibited by solid tumors compared with normal tissues. In addition, nanoparticles as therapeutics carriers have other unique properties of higher therapetic efficacy, lower toxicity and the ability to encapsulate and deliver poorly soluble drugs (**Wang** *et al.*, **2012**). The elaboration of nanoparticles of uniform shape, size and composition is a dynamic growing research field in cancer medicine. Novel improved biodegradable and biocompatible nanoparticle formulation with increasing bioavailability, in vivo stability, intestinal absorption, solubility, sustained and targeted delivery to site of action combined with therapeutic effectiveness, are being developed (**Ochekpe** *et al.*, **2009**). Since the size of the nanoparticles is significantly smaller than a cell, they'can deliver a large payload of drugs, contrast agents or fluorescent probe onto the surface or interior of the

cell, without disrupting its function (Matteo *et al.*, 2006). These particles are able to deep penetrate tissues, going through the fenestration of the small blood-vessel epithelial tissue. They can enterview systemic blood circulation without forming blood platelet aggregates. Their reduced particle size entails high surface area and hence a strategy for faster drug release (Matteo *et al.*, 2006).

Selenium (Se) is an essential trace element required by many organisms. It is a crucial cofactor of antioxidant enzymes such as glutathione peroxidases and thioredoxin reductases (Srivastava and Mukhopadhyay, 2013). As the selenium nanoparticles (SeNPs) possess antimicrobial and anticancer properties, they can be used as nanomedicines (Wadhwani *et al.*, 2016). In addition, they exhibit tess toxicity as compared to their inorganic and organic counterparts (Shakibaie *et al.*, 2010).

On the other hand, many anticancer drugs exert adverse side effects, which can be severe for deadly. Thus, identification of novel anticancer compounds from natural products was proposed as a safer alternative and a promising strategy for cancer prevention or treatment. Many traditional herbal medicines and certain food constituents exhibit anti-inflammatory and antioxidant effects, suggesting "their potential as chemopreventive or therapeutic agents (Jasim, 2014).

In a number of studies, extract of fenugreek seeds and some of their constituents have shown anticarcinogenic potency. Consumption of fenugreek was accompanied with decreased polyamines (spermine, spermidine, and putrescine) content in tumor tissue (**Jasim**, **2014**). The effect of biologically active constituent of fenugreek seeds on breast cancer cell lines caused G1 cell cycle arrest by down regulating cyclin D1, cdk-2 and cdk-4 expression in both estrogen receptor positive ER (+) and estrogen receptor negative ER (-) breast cancer cells resulting in the inhibition of cell proliferation and induction of apoptosis (**Jasim**, **2014**).

In the present study, the cytotoxicity of either fenugreek seeds extracts either alone or combined with selenium nanoparticles on Ehrlich carcinoma cell line was carried out.

The present study demonstrated that fenugreek extract could exert a high cytotoxicity against Ehrlich ascite carcinoma cell line. The median lethal concentration of fenugreek extract was 70 μ g/ml and the median lethal concentration of fenugreek extract combined with SeNPs was 60 μ g/ml. ^{mqq}</sup>

Fenugreek extract were cytotoxic in vitro to a panel of cancers but not normal cells. Also, fenugreek extract have an inhibitory growth to breast, pancreatic and prostate cancer cell lines (Shi \Rightarrow angi et al., 2016). $\xi \cdot \chi$

Fenugreek extract selectively inhibit cell division in tumor cells and can activate apostotic programs, which can lead to programmed cell death (Shivangi *et al.*, 2016). Meanwile, the cytotoxickty effect of nanoparticles is due to their adherence to the cell membrane, particle internalization and degradation of products in the cell culture medium or inside the cells (Abbasalipourkabir *et al.*, 2011).

Our experimental data revealed that the positive control mice develop Ehrlich tumor bulb exceeded 1cm³ (500 mm³)14 days after tumor inoculation (ATI) of viable EAC cells. Also, microscopic investigations showed compact and aggregation of the tumor tissue cells spread within the muscular tissues with pleomorphic shapes, hyperchromatic nuclei and binucleation without necrosis or apoptosis. The effect of ROS production as a result of tumor growth, on other organs in the body can be explained as follows: ROS cause activation in nuclear factor κ B (NF- κ B) and phosphorylation of its inhibitor (I κ B). Thus, they enable NF- κ B to translocation in the cell nucleus and binds to DNA and regulates the transcription of various target genes (i.e. inducible nitric oxide synthase, cyclooxygenase II, cytokines, etc.), which contribute to cell damage. Interestingly, in tumor cells cytokines activate NF- κ B, which protects the tumor cells from TNF- α induced apoptotic cell death. NF- κ B activation in cancer

cells regulates transcription of genes involved in cell proliferation, antiapoptosis and invasion. Thus, activation of NF- κ B induces tumor growth and metastasis and reduces cytokines-induced apostosis (Hanafi and Asmaa, 2015).

In the present study, regular and rapid increases in tumor volume were observed in EC tumor bearing mice, while in groups were taken the treatments, a decreased in tumor volume was observed supporting the beneficial anticarcinogenic effect of fenugreek. On the other hand EC of mice taily gavage orally by fenugreek extract either alone or combined with selenium nanoparticles represents histopathologically extensive areas contain of remnants, apoptotic and pyknotic EC cells.

Context with the findings of **Thippeswamy and Salimath** (2006) the tested extracts of fenugreek have potent proapoptotic effects on EC cells in vivo and the inhibitory effect of fenugreek on EC cells growth may be due to induction of apoptosis. The reduction in tumor volume was due to the treat $\hbar \xi$ cell induced inhibition in cell cycle progression (Meikrantz and Schlegel, 1995).

In an in vivo study the effect of fenugreek seed powder along with its bioactive compound were able to inhibit the formation preneoplastic lesion (Shivangi *et al.*, 2016). Suppressed the expression of proapoptotic protein bcl-2 and there was an increase in the expression of caspase-3, an antiapostosis protein (Shivangi *et al.*, 2016). Several studies on anticancer properties of chemical constituents of fenugreek have been done and have shown positive results. Some constituent of alkaloids, called "trigonelline," has revealed potential forusein cancer therapy (Shivangi *et al.*, 2016).

The chemopreventive activity of the methanolic extract of fenugreek seeds may be due to the rich chemical constituents (such as, saponins, flavonoids, alkaloids, galactomannans) that are present in the seed working synergistically at various stages of angiogenesis (Shivangi *et al.*, 2016). $\xi \gamma \gamma$

Fenugreek was reported to have an ability to inhibit further growth of cancer cells without harming the healthy cells of the body. $\xi \gamma \gamma$

The mechanism of selenium nanoparticles in reducing the tumor size may be through the tongcirculating nanoparticulate carriers. They are able to efficiently deliver the chemotherapeutics to \$6 hd tumors by exploiting the enhanced permeability and retention effect and thus can significantly enhanced the therapeutic index of the drug or improve reducing undesirable side effects. Studies recorded that ultra-low size particles can efficiently be targeted to the tumor tissue through the combined effects of extravagation and long circulation in blood (Savita and Amarnath, 2009).

Our results demonstrated apoptosis suppression in solid EC tumors as evidenced by ξ the significant reduction in the level of apoptotic molecules (caspase-3 and granzyme B), compared to hon EC-bearing mice. Apoptosis is a programmed cell death that maintains the stability of the internal environment through removing genetic mutations and unstable cells. However, this process is inhibited in cancer, which leads to the accumulation of various genetically unstable cells. The disturbance in the apoptotic regulators leads to tumor proliferation and growth (Medhat *et al.*, 2017).

Caspases (C: cysteine protease mechanism, **aspase**: ability to cleave after aspartic acid) are aspartate-directed cysteine proteases that play a key role in the initiation and execution of apoptosis for PCD, necrosis and inflammation, failure of which may cause tumor development and several autoimmune diseases. Once activated, they cleave cellular substrates, leading to morphological hallmarks of apoptosis (Hanafi and Asmaa, 2015).

In our study, treatment of experimental animals bearing EC with fenugreek extract either $\hat{a}\hat{b}\hat{h}\hat{h}$ or combined with SeNPs represents significant increase in tumor caspase-3 levels when compared $\hat{a}\hat{h}\hat{h}$ their corresponding activity in EC bearing mice. $\hat{z} \circ \hat{q}$

This increase in caspase-3 activities postulated the effect of apoptosis on MCF-7 cell line in Caspase 3, 8, 9, p53, Fas, FADD, Bax and Bak activation. There are various mechanisms through which apoptosis can be induced in cells such as the expression of pro and anti-apoptotic proteins.² The

mitochondrial apoptotic pathways and death receptor pathways are the two major pathways.⁵ The mitochondria have a central role in regulating the caspase cascade and apoptosis (**Shafi** *et al.*, **2009**). Caspases have a central role in the apoptotic process in that they trigger a cascade of apoptotic pathways (**Shah** *et al.*, **2003**).

Also, the activity of caspase-3 is increased in tumor cells due to the inactivation of P53 (tumor suppressor protein), which is responsible for protecting cells from tumorigenic alterations (Hanafi and Asmaa, 2015).

Caspase activation leads to apoptosis through two main pathways. One pathway involves a turnor necrosis factor (TNF) receptor at the cell surface, which recruits caspase-8 through the adaptor protein FAS-associated death domain (FADD) leading to the activation of caspase-8. The intrinsic pathway involves the release of cytochrome c from mitochondria, a key intermediate step in the apoptotic proteess that leads to the activation of caspase 9 (Hanafi and Asmaa, 2015). Cytosolic cytochrome c binkly to Apoptotic protease-activating factor-1(Apaf-1) forming complex containing Apaf-1and cytochrome c $\chi\gamma\gamma$

In the same direction, SeNPs inhibited cancer cell growth partially by caspase-mediated apoptosis, which was through the downregulation of androgen receptor (AR) phosphory at expression at both transcriptional and translational levels. SeNPs treatment activated the Akt/Mdm2 pathway, and initiated AR phosphorylation, ubiquitination and degradation. The cancer suppression function of SeNPs consisted of at least two mechanisms, regulation of AR transcription and promotion of AR protein degradation (Kong et al., 2011).

Granzyme is a family of serine proteases is contained within the cytoplasmic granules of cytotoxic lymphocytes (CLs), and the pore-forming protein, perforin. According to the model of granulemediated apoptosis, killing involves degranulation and subsequent transfer of these proteases into the cytoplasm of the target cell, where they rapidly induce apoptosis (Birkedal and Taylor, 1982). This process is inhibited in cancer, which leads to the accumulation of various genetically unstable cells (Medhat et al., 2017).

Our results demonstrated apoptosis suppression in solid EC tumors as evidenced by the significant reduction in the level of apoptotic molecules (caspase-3 and granzyme B), compared to non EC-bearing mice. Apoptosis is a programmed cell death that maintains the stability of the internal environment through removing genetic mutations and unstable cells. $\xi q \gamma$

Treatment of experimental animals bearing EC with fenugreek extract either alone or combined with SeNPs represents significant increase in granzyme B level when compared with their corresponding activity in EC bearing mice.

This increase in caspase-3 activities postulated that Granzyme B has a similar preferented as caspases for cleaving protein peptide bonds C-terminal to Aspartate residue. Granzyme B is capable of direct proteolytic processing and activation of the executioner procaspase-3 and -7. On the other hand, there are contradictory reports on the direct Granzyme B-mediated procaspase-6 proteolytic activation (**Ilona and Evzen, 2010**). Moreover, the apoptotic procaspases including procaspase-8, -10, -9, and -2 were reported to serve as substrates for the active Granzyme B. However, it should be emphasized that Granzyme B can proteolytically cleave these initiator procaspases but it cannot activate them. The initiator procaspases are activated exclusively by homodimerization in specific multiprotein activation platforms such as apoptosome, DISC and PIDDosome (**Ilona and Evzen, 2010**).

There is mounting evidence that Granzyme B can kill cells via a caspase-independent pathway (Lord *et al.*, 2003). The serine protease and the caspases appear to cleave some of the same cellular substrates, resulting in the demise of the cells (Walker *et al.*, 1994). The Granzyme B not only activates

pro-death functions within a target, but also has a previously unidentified role in inactivating pro-growth signals to cause cell death (**Thomas** *et al.*, **2000**).

TNF-alpha is a cytokine produced by the innate immune cells and implicated in the promotion or inhibition of tumor development. Tumor cells or inflammatory cells in the tumor microenvironment produce it. The role of TNF-alpha in chronic inflammatory diseases and tumor-promoting effects is well recognized as well as the role in promoting tumor cell survival through the induction of genes encoding NFkB- dependent antiapoptotic molecules. Other actions, which may enhance tumor progression, include; promotion of angiogenesis, metastasis and impairment of immune surveillance by suppressing T cell responses as well as cytotoxic activity of activated macrophages (Hanafi and Asmaa, 2015).

Genetic poly morphisms, which enhance TNF-alpha production, are associated with increased risk of hepatocellular carcinoma (HCC) as well as other tumors such as multiple myeloma, bladder carcinoma, gastric carcinoma and breast carcinoma. Overall, TNF-alpha is an important factor invôlvêd in initiation, proliferation, angiogenesis, and metastasis of various types of cancers (Hanafi and Asmaa, 2015).

The experimental data reveals that female mice bearing EC represents a significant increase in serum TNF- α level of tumor bearing mice in compared to normal control group.

The elevation in the TNF- α level in EC mice may be attributed to the increase in the production of ROS by macrophages which stimulate lipid peroxidation or initiating a potentially harmful immune response and stimulate neutrophil chemotaxis or activates transcriptional factor NF- κ B which in turn increases the production of proInflammatory cytokines (Hanafi and Asmaa, 2015).

Our data reveals that treatment of experimental animals bearing EC with fenugreek extract either alone or combined with SeNPs represents a significant decrease in serum TNF- α level in compared to EC group and a significant increase in compared to normal control level. Fenugreek extract inhibited TNF-induced invasion by inhibiting the proliferation of tumor cells and stopping the cells ffom progressing to G1 (Liu *et al.*, 2005), downregulated the expression of antiapoptotic, proliferative, fand angiogenic gene products (Yin *et al.*, 2004). Also, Fenugreek extract suppressed TNF-induced invasion by tumor cells, and this inhibition correlated with the downregulation of MMP-9 and e0X-2 (Esteve *et al.*, 2002).

Mansour et al. (2010) postulated that use of selenium nanoparticle significantly decrease \mathfrak{TNF} - α concentration in the plasma of mice bearing EC.

Interferon gamma (IFN-\gamma) is a dimerized soluble cytokine that is the only member of the type II class of interferons (**Gray and Goeddel, 1982**). IFN- γ is produced predominantly by natural Killer (NK) and natural killer T (NKT) cells as part of the innate immune response, and by CD4 Th1 and CD8 cytotoxic T lymphocyte (CTL) effector T cells once antigen-specific immunity develops (**Schoenborn and Wilson, 2007**). IFN- γ is also produced by non-cytotoxic innate lymphoid cells ($\mathbf{fl} \leq \mathbf{C}$), a family of immune cells first discovered in the early 2010s (**Artis et al., 2015**).

EC bearing mice showed high increases in the activity of IFN- γ due to its role in systemie 4 fd local immunity and in almost all inflammatory responses (**Ikeda** *et al.*, 2002).

Treatment of experimental animals bearing EC with fenugreek extract either alone or combined with SeNPs represents significant decrease in IFN- γ level when compared with their corresponding activity in EC bearing mice.

In the last years, many researches demonstrated the immunoregulatory activity of fenugréek extract. Among the compounds of them is believed to play an important role in stimulating the body's immune ability. It affects the body's nonspecific and specific immune functions and activates immune cells. In addition, it also showed immunoregulatory activity (Fontes *et al.*, 2014).

Oxidative stress is occurred due to a disturbance in the balance between the production of \mathbb{ROS} and the efficiency of the antioxidant defense. In other words, oxidative stress results if excessive production of ROS overwhelms the antioxidant defense system or when there is a significant decrease or lack of antioxidant defense (Kang, 2002). Moreover, severe oxidative stress is not only known to cause DNA damage and mutations of tumor suppressor genes, which are initial events in carcinogenesis (Kang, 2002), but can also play an important role in the promotion of multistep carcinogenesis (Alimed *et al.*, 1999).

The end product of lipid peroxidation, malondialdehyde, due to its high cytotoxicity and inhibitory action on protective enzymes, is suggested to act on tumor development (Kang, 2002). Lipid peroxidation plays an important role in the control of cell division (Diplock *et al.*, 1994) associated with pathological conditions of a cell. Moreover, it has been claimed that MDA acts as a tumor promoter and co-carcinogenic agent because of its high cytotoxicity and inhibitory action on protective enzymes. Also, the tumor development might be responsible for the antioxidant depletion and also the increased concentration of lipid peroxidation products (Hanafi and Asmaa, 2015).

The increase in levels of lipid peroxidation in tumor tissue might be attributed to the deficiency of antioxidant defense mechanisms or probably due to the generation of reactive oxygen species (ROS) and altered redox statuses, which are common biochemical aspects in tumor cells. ROS can react with the polyunsaturated fatty acids of lipid membranes and induce lipid peroxidation. In addition, eaMier studies observed increased lipid peroxidation and decreased antioxidant levels in the cancer parients (Hanafi and Asmaa, 2015).

Our data revealed that experimental revealed that animals bearing EC represents a significant decrease in tumor GSH content in compared to EC group. $\circ V \mathfrak{t}$

The depletion in GSH level in tumor tissue may be attributed to the enhanced utilization $\delta f'$ the antioxidant system as an attempt to detoxify the free radicals generated by Ehrlich solid cells or δV the diminished activity of glutathione reductase due to the deficiency or inactivation of glucose-6-phosp Mate dehydrogenase, the main supplier for NADPH which is necessary to change oxidized glutathione δV the reduced form (Hanafi and Asmaa, 2015).

Tirkey *et al.* (2005) indicated that oxidative stress causes depletion of intracellular GSH, a reducing agent with its sulfhydryl group leading to serious consequences. The decrease could be due to a feedback inhibition or oxidative inactivation of enzyme protein caused by ROS generation that can in turn impair the antioxidant defense mechanism leading to increased lipid peroxidation (Ohta *et/dil.*, 2004). Excessive lipid peroxidation can cause increased glutathione consumption (Manda and Bhatta, $\circ \wedge \circ$

On the other hand, our data revealed that experimental animals bearing EC represents a significant decrease in tumor CAT content in compared to EC group. $\circ \Lambda V$

When CAT activity is reduced, the level of hydrogen peroxide increased in cancer tissue. This may correspond with the report, which showed that some human cancer lines produced a large arrivant of hydrogen peroxide (Szatrowski and Nathan, 1991). Indeed, the levels of glutathione, CAT and GSH-Px, have been shown to be significantly altered in malignant cells (Oberley and Oberley, 1997) and in primary cancer tissues homeostasis and stress adaptation in cancer cells or could also be due to exhaustion of the glutathione and antioxidant enzymes because of increased peroxidation (Manimaria and Rajneesh, 2009). Alternatively, it is possible that the antioxidant system is impaired because of an abnormality in the anti-oxidative metabolism due to the cancer processes.

Treatment of experimental animals bearing EC with fenugreek extract either alone or combined with SeNPs represents a decrease in levels of lipid peroxidation, an increase in catalase activity and the significant change in reduced glutathione in tumor tissue in compared to EC group.

The decrease in MDA level when compared with their corresponding level in EC bearing field explain the more pronounced delay in tumor size and the protective activity of fenugreek aqueous extract against tumor progression and the return of muscular tissue to its normalization. The fenugreek extract inhibited the promoter of LPO by blocking the production of thiobarbituric acid reactive substances (TBARS) (Umesh and Najma, 2014).

According to the results obtained, it could be postulated that lipids, especially polyunsaturated fatty acids (PUFA) in the membranes, are very susceptible to free radical attack that can initiate lipid peroxidation (Halliwell and Gutteridge, 1999). Lipid peroxidation plays an important role in the control of cell division (Diplock *et al.*, 1994).

Glutathione (GSH), the most abundant non-enzymatic antioxidant present in the cell, plays an important role in the defense against oxidative stress-induced cell injury. In the cells, glutathione⁴ is present mainly in its reduced form. Reduced GSH can be converted to oxidized glutathione (GSSG) which is revertible to the reduced form with the glutathione reductase (GR). Cells are also equipped with the enzymatic antioxidant mechanisms that play an important role in the elimination of free radicals (Hanafi and Asmaa, 2015).

Indeed, the level of glutathione have been shown to be significantly altered in malignant defises and in primary cancer tissues, suggesting aberrant regulation of redox homeostasis and stress adaptation in cancer cells or could also be due to exhaustion of the glutathione and antioxidant enzymes because of increased peroxidation. Alternatively, it is possible that the antioxidant system impaired as a consequence of an abnormality in the anti-oxidative metabolism due to the cancer processes (Hanafi and Asmaa, 2015).

Our data revealed that treatment of experimental animals bearing EC with fenugreek extract either alone or combined with SeNPs represents un-significant change in tumor GSH content in compared to EC group.

The depletion in glutathione level has been reported to enhance the cell death and apoptošiš of the tumor cells along with the loss of essential sulfhydryl groups that result in an alteration of the calcium homeostasis and eventually loss of cell viability (Hanafi and Asmaa, 2015).

GSH is essential for cell survival and its depletion increases the cellular susceptibility to apoptosis (Morales *et al.*, 1998). High intracellular GSH levels have been related to apoptosis resistance (Cazanave *et al.*, 2007), and GSH depletion has been shown either to induce or potentiate apoptosis (Tormos *et al.*, 2004).

Moreover, the inhibition of antioxidant enzymes activities and a reduction in glutathione level as a result of tumor growth were also reported (**Gupta** *et al.*, **2004**). This phenomenon could be attributed to the exhaustion of these antioxidants especially glutathione and glutathione-containing enzymes in the detoxification of free radicals and peroxides generated due to tumor inoculation. These free radicals conjugate with GSH and ultimately protect the cells and organs from oxidative stress.

Catalase is mainly catalyzes the dismutation of hydrogen peroxide (H_2O_2) into water and molecular oxygen and used by cells to defend against the toxic effects of hydrogen peroxide, which is generated by various reactions and/or environmental agents or by the action of superoxide dismutates while detoxifying superoxide anion (Michiels *et al.*, 1994).

When CAT activity is reduced, the level of hydrogen peroxide increased in cancer tissue. This may correspond with the report, which showed that some human cancer lines produced a large amount of hydrogen peroxide (Szatrowski and Nathan, 1991). Indeed, the level of glutathione and CAT have been shown to be significantly altered in malignant cells (Oberley and Oberley, 1997) and in printary cancer tissues (Murawaki *et al.*, 2008), suggesting aberrant regulation of redox homeostasis and stress adaptation in cancer cells or could also be due to exhaustion of the glutathione and antioxidant enzymes

because of increased peroxidation (Manimaran and Rajneesh, 2009). Alternatively, it is possible that	Ĺ
the antioxidant system is impaired as a consequence of an abnormality in the anti-oxidative metabolism	L
due to the cancer processes.	
CONCLUSION 75A	
From the results of the present study it is clear that fenugreek extract either alone of ξ^{q} combined with selenium nanoparticles exhibited significant antitumor activity reflected by \circ reduction in tumor size, Inhibition of tumor cytokine profile (TNF- α and INF- γ), increased \circ tumor apoptotic profile (caspase-3 and Granzyme-B), decreased lipid peroxidation and \circ^{γ} increased antioxidant markers activities (GSH and CAT) in tumor tissue, Histopathological, \circ^{γ} apoptotic and necrotic examinations of tumor tissue.	
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