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RESEARCH ARTICLE

Study for Murine Mammary Adenocarcinoma Implanted in Female Albino Mice for Evaluation of Antitumor Activity of *Trigonella foenum graecum* (Fenugreek) Seed Alkaloid Extract against Breast Cancer

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Abstract

The current study was designed to prepare the alkaloid extract of the *Trigonella foenum graecum* and studying the therapeutic potential of cancer in laboratory mice with a study of the physiological effect of the alkaloid extract, 48mice were used for studying, in the following groups (8 mice in each group) : The first group infused normal group injection with normal saline for 2 weeks and was considered a negative control group. The second group was its untreated tumor-bearing mice group for two weeks and was considered a positive control group. The third group was tumor-bearing mice treated using the 80 g / kg for 2 weeks. The fourth group was treated using alkaloids extract and 120 g / kg for 2 weeks. The fifth group was not infected with the tumor and was injected with the 80 g / kg alkaloids extract for 2 weeks. The sixth group was not infected with the tumor and was injected with the 120 g / kg alkaloids extract for 2 weeks. The results of the study showed that the tumor inhibition rate in the group rats treated with the plant extract 80 g / kg for two weeks was higher by significant difference ($P < 0.05$) than the group treated with 120 g/kg alkaloids. The results of the CEA study and TP53 production showed a significant difference ($P < 0.0001$) between the control groups and the treated and untreated tumor groups (positive control). No significant difference was found between positive control groups and tumor groups. The results of the physiological effect on the study groups showed higher levels of AST and ALT in tumor aggregates compared with control group but did not show significant differences between the groups injected with alkaloid extract only compared to the control group.

Keywords: *Fenugreek; Seed alkaloid extract; Trigonella foenum graecum; and female Albino mice.*

Introduction

Cancer is a most dangerous disease that threatens human life in various parts of the world, considered the second causes of death in the world after heart disease [1].Breast cancer is the second cancer effect on people worldwide and the most common cancer among females [2]. In Iraq, breast cancer accounting for approximately one-third of the registered female cancers, according to the latest Iraqi Cancer Registry [3].

Currently, many studies have been carried out to isolate the active novel compounds from medical plants that used as raw materials for extraction of active ingredients which used in the synthesis of different drugs. Like in case of laxatives, blood thinners, antibiotics, antimalaria ana anticancer [4] that are proven to have potent effects against many varieties of maladies, including cancer [5]. Various active compounds derived from medicinal plants have been assessed for their efficacy and tolerability in the treatment of human breast cancer.Some of these plant species, including Taxus brevifolia ,Taxus baccata, Podophyllum peltatum, Camptotheca accuminata, and Vinca rose a have well-recognized anticancer activity in breast and other human malignancies[6]. Fenugreek is one of the oldest plants which have been identified as medicinal plant by the researchers around the world.

It is therapeutic properties have also which include the treatment of number of diseases such as diabetes, hypercholesterolemia, inflammation, antioxidant, antimicrobial and several kinds of cancers. [7].The Trigonella seeds or Fenugreek are rich source of calcium, iron, b-carotene ,alkaloid, yellow coloring matter, tannic acid, diosgenin, fixed and volatile oils, vitamin A and other vitamins [8]

This compound has multiple medicinal properties such as Anticancer, Anti-Inflammatory, Antiseptic, Aphrodisiac, Astringent, Bitter, Demulcent, Emollient, Expectorant, Anthelmintic, Wound healing and Gastro protective[9].Alkaloids, one of the secondary metabolites which are produced by plants as toxic substances. Out of the 27,000 different alkaloids, more than 17,000 have different pharmacological properties including anticancer activities [10].

Tumor Markers are biochemical substances elaborate by tumor cells either due to the cause or effect of malignant process, The first tumor marker used for diagnostic purposes of different cancer (colorectal, pancreatic, breast, ovary, head and neck, bladder, kidney, and prostate cancers) was the CEA antigen, found over expressed in serum of oncological patients compared to healthy individuals [11].

There is many factor es which was observed to play a crucial role in cancer development; it is the apoptosis (programmed death of cells) appears as the critical functioning of p53 in tumor suppression [12]. Protein 53 (p53) or Tumor protein 53(Tp 53) was originally defined as an oncogenic Protein could allow abnormal cells to proliferate, resulting in cancer. As many as 50 % of all human tumors contain p53 mutants [13]. Biochemical analyses are useful in chronic toxicity studies because they serve as indicators of cell damage when enzymes are leaked into the blood [14].

Aspartate transaminase (AST) is a marker enzyme found in many tissues including the liver, kidney, muscle, heart, brain and lung. The amount of AST in the blood relates to the extent of tissue damage [15]. Alanine transaminase (ALT) is another parameter used to assess liver ill health. It is a marker enzyme most common in the liver but can also be found in the plasma. An elevated level of ALT is an indicator of medical conditions such as hepatitis, liver damage, diabetes, congestive heart disease or bile duct problems [16].

Materials and Methods

Plants Collection

The Trigonella seeds were bought fresh from local market of Kufa, Najaf, Iraq, during May 2016. The plants were classified by specialists in the Botanical. University of kufa.

The plant seeds dried under shade for 10 days at room temperature and dried, then the seed was ground and stored at room temperature until further use.

Total Alkaloid Extraction

Total alkaloids were extracted according to Harborne [17]. Briefly, 10 g of plant dry powder was extracted with 80% ethanol for 24 h in a continuous extraction by soxhlet apparatus 250 ml volume. The extract was filtered by Whatman No. 1 filter paper and then, the filtrate was concentrated by a rotary evaporator under vacuum at 45°C until the solution reached to 10 ml. The concentrated extract was transferred to a biker and 2 N HCl was added gradually to adjust the pH value up to 2. Then, the pH value of the extract was adjusted to 10 using NH OH, and washed with 10 ml chloroform 3 times. The chloroform portion was dried to obtain the total alkaloid extract. The dried extract was weighed and preserved in a clean container at 4°C for use.

Qualitative Detection of Alkaloids

To detect the presence of alkaloids in plant extracts some qualitative tests were performed using Mayer's, Hager's and Dragendorff 's reagents. Mayer's reagent used to screen all types of alkaloids, that prepared by Harborne [18]. Hager's test appeared a yellow color precipitate that indicates to the presence of alkaloids [19].Furthermore, Dragendorff's reagent was used to investigate alkaloids in plant extract. The formation of an orange color indicated the test was positive [20].

Experimental Design

Transplantation of Tumour Cells in Mice

Ahmed Majeed 2003 (AM3) also named (AN3) mammary adenocarcinoma transplantable tumor line [21] was supplied from ICCMGR, Experimental Therapy Department. Transplantation of tumor cell in mice, Single tumor (mammary adenocarcinoma) bearing mouse, was used to obtain tumor cells which were later transplanted into adult female albino mice. The following protocol was followed to perform the transplantation process [22].

Experimental Animal

Fourth eight Albino Swiss females mice, aged

6-8 weeks,supplied and housed at Iraqi Center for Cancer and Medical Genetic Research (ICCMGR) animal house unite under controlled conditions (room temperature 25 ± 2°C, and photoperiod of 12 h Light: Dark cycle)[23]. The animals were divided into five groups, (n= 8) as follows:

Group1: Control group. Mice of this group maintained on a standard pellet diet and water applied were used as a normal or negative control group and injected intraperitoneally with normal slain that sacrificed after 2weeks.

Group 2: Tumor group. Mice of this group were inoculated in abdominal region with AM3 cells. Each mouse was injected subcutaneously with 2 x 10^6 AM3/mL as tumor or positive control group and injected intraperitoneally with normal slain that sacrificed after 2weeks.

Group 3: Tumor, alkaloid extract treated group. Mice of this group were inoculated with 2 x 10^6 AM3/mL in abdominal region and injected intraperitoneally with alkaloid extract (80mg/kg b. wt) that sacrificed after 2weeks.

Group 4: Alkaloid extract injected group. Mice of this group were inoculated with 2 x 10^6 AM3/mL in abdominal region and injected intraperitoneally with alkaloid extract (120mg/kg b. wt) that sacrificed after 2weeks.

Group 5: Alkaloid extract injected group. Mice of this group were inoculated with 2 x 10^6 AM3/mL in abdominal region and injected intraperitoneally with alkaloid extract (80mg/kg b. wt) that sacrificed after 2 weeks.

Group 6: Alkaloid extract injected group. Mice of this group were inoculated with 2 x 10^6 AM3/mL in abdominal region and injected intraperitoneally with alkaloid extract (120mg/kg b. wt) that sacrificed after 2weeks.

Determination of Biochemical Parameters

On the 14th day, from each mouse (0.75 to 1) mL of blood were collected from the heart directly using disposable syringe. The blood sample was placed in serum gel tube and left for 30 minutes. The serum was prepared via centrifugation at 3000 rpm for 10 minutes and kept frozen at -20°C [24] for estimation of AST and ALT by using an auto analyzer mind ray chemistry analyzer (BS-230 China), mouse Carcino Embryonic Antigen (CEA) Kit and mouse Tumor protein p53(TP53) Kit (Elabscience, chine) were used in the study.

Statistical Analysis

Data were expressed as means ± S.E. Statistical analyses were performed using graghpresem program followed by LSD test. The $p \leq 0.05$ were considered a significant for all data of the results.

Results

The soxhlet extraction procedure using the soxhlet showed that the total alkaloid getting 4.4 gram of dry alkaloid extract from 252 gram of DW of plant. Table 1 showed the qualitative detection of alkaloids present in the plant extracts using different reagent. The qualitative analysis of extracts appears the presence of alkaloids by changing color in reagents .

Table 1: Qualitative detection of alkaloids in plant extracts using different reagents

Reagent	Resulted	color
Mayer's reagent	+	Creamy precipitat
Hager's reagent	+	Yellow color
Dragendorff's reagent	+	Orange color
+ : Indicate the positive results		

Treatment of Mammary Adenocarcinoma by Alkaloid Extract

Two doses(80 and 120 g/kg) are choses to study anti-tumor effect of extract in 2 weeks of treatment and determent tumor value Table (2) and growth inhibition of tumor table (3) . Animals treated with plant extract showed a significant regression of the tumor during the experiment period, where the result showed significant decrease in tumor volume size of treated group with 80 g/kg extract comparing with the control group at level ($p<0.05$) but showed non-significant decrease in the 120 g/kg extract treated group . Tumor growth inhibition occurred at day 3 from starting of the treatment for 80g/kg up to 86.8 with a maximum inhibition reached up to 90.1 at day 6, then decrease to reach up

to 75.9% at day 14. Treated group with 120 g/kg recorded varying rates of inhibition growth from 79.2 until it reaches the final growth inhibition rate reached up to 57.5 % occurred at the end of experimental period.

Table 2: Comparison of relative tumor volume among groups (2 weeks)

Groups	Days							LSD
	0 day Mean± SE	3th Mean± SE	6th Mean± SE	8th Mean± SE	10th Mean± SE	12th Mean± SE	14th Mean± SE	
Control	0.520 ± 0.3	0.622 ± 0.3	1.152 ± 0.6	1.39 ± 0.6	1.73 ± 0.7	2.07 ± 0.7	2.43 ± 0.7 A	1.66
80g/kg	0.151±0.04	0.075±0.02a	0.109±0.04a	0.133±0.05a	0.207±0.09a	0.32 ± 0.2a	0.57 ± 0.4a	0.49
120g/kg	0.133± 0.04	0.124±0.04	0.504 ± 0.2	0.577 ± 0.2	0.93 ± 0.4	1.009±0.4A,B,	1.019±0.4 A ,B	0.81
LSD	0.43	0.43	0.8	0.83	1.19	1.24	1.45	

Table 3: Comparison of Growth inhibition among groups (2 weeks)

Groups	Days					
	3th Mean± SE	6th Mean± SE	8th Mean± SE	10th Mean± SE	12th Mean± SE	14th Mean± SE
80g/kg (2w)	86.8%	90.1%	89.7%	87.7%	84%	75.9%
120g/kg(2w)	79.2%	54.1%	55.5%	56.9%	49.5%	57.5%

Tumor Marker Parameters of Mice

CEA and TP53 Tumor Marker

The study showed a significant increase ($P < 0.001$) in the marker levels(CEA) and (TP35) in the treated with 80 and 120 g / kg concentrations and untreated tumor groups compared with the control at a treatment period of 2 weeks (table 4).There was no significant ($P > 0.05$) difference between other groups.

Table 4: The levels of CEA and TP53 in mice treated with extract

Group	CEA ng/mL Mean ± SE 2week	TP53 ng/mL Mean ± SE 2week
Control	0.076 ± 0.004	0.21 ± 0.02
Tumor only	1.79 ± 0.16 a	0.61 ± 0.05 a
Tumor + 80 g\k extract	1.48 ± 0.11 a	0.50 ± 0.05 a
Tumor + 120g\k extract	1.51 ± 0.08 a	0.59 ± 0.07 a
LSD value	0.32	0.13

The study showed that there were significant increase ($P < 0.05$) in the level of AST ana ALT concentration in the tumor only group of animals compare with the control group, also a significant increase ($P < 0.05$) in the level of AST concentration in the animals group (tumor + 80 and 120 g/kg extract) when compared with control, the results showed a significant increase ($P < 0.05$) in the Tumor + 120g\k extract group when compared with tumor group only. The result showed at the concentration level of ALT were significant increase ($P < 0.05$) in the tumor only group of animals compare with the control group but not significant in comparison with all experimental groups and with different concentration (80 and 120 g / kg).

Table 5: The levels of AST and ALT in mice treated with extract during 2 week

Group	AST UI/mL Mean ± SE	ALT UI/mL Mean ± SE
Control	25.6 ± 1.6	26.8 ± 0.7
Tumor only	36.7 ± 1.7a	31.6 ± 1.8a
Tumor + 80 g\k extract	32.3 ± 1a	29.5 ± 1.3
Mice + 80 g\kg extract	23.5 ± 1.8	28.5 ± 1.3
Tumor + 120 g\k extract	30.6 ± 1.2a,b	28.2 ± 1.3
Mice + 120 g\kg extract	23.7 ± 0.6	27.6 ± 0.9
LSD value	4.6	3.7

Discussion

Medicinal plants contain some organic compounds which provide definite physiological action on the human body, and these bioactive substances include alkaloids [25]. Total alkaloids were detected by changing the color of specialized reagents. Treated animals with two concentration extract showed significant decrease in tumor volume size of treated group with 80 g/kg

extract comparing with the control group. The results of this study indicate that the alkaloid extract of the *Trigonella* plant has cytotoxity on tumor cancer, this alkaloids may be similar to the alkaloids used in the manufacture of anticancer drugs because it have many mechanism that Alkaloids are capable of modulating key signaling pathways involved in proliferation, cell cycle, and metastasis making them the chief components of several clinical anticancer agents [26].

PI3k/Akt signal transduction cascade is one of the several cellular proliferative pathways which promote a normal cell cycle progression by modulating cyclins and pro-apoptotic proteins. Many cytotoxic agents target DNA and the Akt pathway to block cell proliferation and induce apoptosis [27].

Nuclear Factor-Kappa B (NF-kB) pathway is an inducible nuclear transcription factor that activates genes involved in cell survival and proliferation [28]. Alkaloids have been found to suppress tumorigenesis by targeting the NF-kB pathway such as Taxol that targeting NF-kB pathway [29]. Some alkaloids, derived from plants, activate caspases, are important agents in the programmed cell death pathways. Such as scutebarbatine A, a major alkaloid in Scutellaria barbata. Was found to exhibit its anti-proliferative activity against human lung carcinoma cells through the cleavage of caspases-3 and -9 [30].

The importance mechanism of several of alkaloids resides in their ability to act on cell cycle checkpoints to induce cell cycle arrest. This allows to directs the cell towards apoptosis [31] , for instance, noscapine) isolated from Papaver somniferum induced G2/M arrest in various types of cancers such as breast cancer [32].Most of these medically exploited alkaloids function as therapeutics by mainly provoking DNA damage, inducing apoptosis, and acting as anti-proliferative agents; however, their associated toxicity urges the finding of new natural compounds that have the potential to selectively target cancerous cells [33].

In their study of plant extracts, the inhibitory effect was based on the lower concentrations than the high concentrations, it showed degrease in tumor value in group that treatment with 80 g/kg during 2 weeks. Also the result showed non-significant decrease in the 120 g/kg extract treated group. Tumor growth inhibition occurred at day 3 from starting of the treatment for 80g/kg up to 86.8 with a maximum inhibition reached up to 90.1 at day 6, then decrease to reach up to 75.9% at day 14.

Treated group with 120 g/kg recorded varying rates of inhibition growth from 79.2 until it reaches the final growth inhibition rate reached up to 57.5 % occurred at the end of experimental period. This depends on several factors, such as the concentration of the material and the molecular weight. The low concentrations of the material allow it is to penetrate the outer membrane of the cell wall. [34].

This study showed increase in ALT and AST level in tumor peering group, ALT and AST are the major critical enzymes in the biological processes. The liver could easily be exposed to internal stimuli which produce reactive oxygen species. The oxidative stress could damage the liver cells [35]. Oxidative stress and inflammation are related to cancer development; at the same time, oxidative stress and inflammation could also lead to damaged liver cells [36].

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