Kafkas Univ Vet Fak Derg
23 (5): 817-823, 2017
DOI: 10.9775/kvfd.2017.17971

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
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Research Article

Antitumorigenic Activity of the Herbal Mixture-AK27 on Ehrlich Ascites Carcinoma in Mice

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Article Code: KVFD-2017-17963 Received: 01.05.2017 Accepted: 07.07.2017 Published Online: 09.07.2017

Citation of This Article

Demirkan İ, Ertekin T, Korkmaz M, Kılıç İ, Çevik-Demirkan A, Bozkurt F, Konuktürk A: Antitumorigenic activity of the herbal mixture-AK27 on ehrlich ascites carcinoma in mice. *Kafkas Univ Vet Fak Derg,* 23 (5): 817-823, 2017. DOI: 10.9775/kvfd.2017.17971

Abstract

The treatment of cancer is highly challenging and contains surgery, chemotherapy and radiotherapy alone or combinations which have various side effects on the patient health. This study aimed at observing the possible antineoplastic activity of AK27-herbal mixture, a combination of pistachio resin, rhus resin, pollen, *Nigella sativa* seed, pomegranate skin and olive oil, on experimentally induced Ehrlich ascites carcinoma (EAC) in a mouse model. EAC-bearing mice were evaluated by tumor cell count (viable and non-viable), median survival time, percentage increase in the life span and live body weight changes up to 30 days of EAC inoculation. Volume of EAC cells and viable cell count were found to be significantly decreased in AK27 treated groups when compared to EAC control group ($P<0.01$). The highest viable cell count (mean $15.5x10^7$/mL/mouse) and EAC volume (average 12 mL/mouse) was measured in the cancer group. Administration of AK27 mixture before tumor challenge prevented the EAC development whereas simultaneous administration or after tumor initiation, AK27 significantly reduced the number of viable EAC cells with respect to cancer control group. After the 4th day onwards until the 12th day significant differences were observed between groups in terms of live body weight ($P<0.001$). All animals in cancer control group died within 12 days as expected. Mean life span in AK27 treated groups were varied from 24 to 26 days with percentage increase in life span of from 100 to 150. The present study demonstrated that AK27-compound was exhibited promising antitumor efficacy in EAC bearing mice.

Keywords: *Ehrlich ascites carcinoma, Pistachio, Rhus, Nigella sativa, Pollen, Olive oil*

Farelerde AK27 Bitkisel Karışımının Ehrlich Ascites Karsinoma Üzerine Antitümorijenik Etkisi

Özet

Kanser sağaltımı oldukça zor ve hastanın sağlığı üzerine olumsuz etkileri olan cerrahi, kemoterapi ve radyoterapinin tek başına veya kombinasyonlarını kapsar. Bu çalışmanın amacı antep fıstığı reçinesi, sumak reçinesi, polen, çörek otu tohumu, nar kabuğu ve zeytinyağı kombinasyonundan oluşan AK27-bitkisel karışımın muhtemel antineoplaistik etkinliğini fare modelinde deneysel oluşturulan Ehrlich asites karsinomunda (EAK) göstermektir. EAK'lı fareler tümör hücre sayısı (canlı ve ölü), ortalama yaşam süresi, yaşam süresinde yüzde artış ve canlı ağırlık değişimi yönünden değerlendirildi. En yüksek canlı hücre sayısı (ortalama $15.5x10^7$/mL/fare) ve EAK hacmi (ortalama 12 mL/fare) kanser grubunda ölçüldü. AK27 karışımının tümör oluşturulmasından önce verilmesi EAK gelişimini engellerken aynı anda veya tümör oluştuktan sonra verilmesi kanser kontrol grubu ile karşılaştırıldığında canlı EAK hücre sayısını anlamlı düzeyde azalttığı gözlendi. Dördüncü günden itibaren 12. güne kadar canlı ağırlık artışı bakımında gruplararasında anlamlı farklar tespit edildi ($P<0.001$). Beklenildiği gibi kanser kontrol grubundaki hayvanların tümü 12. günde öldü. AK27 uygulanan gruplarda ortalama yaşam süresi 24 ile 26 gün arasında artarken yaşam süresinde yüzde artış 100 ile 150 arasında değişti. Bu çalışma AK27 bileşiminin EAK'lı farelerde dikkate değer antitümör etkisinin olduğunu gösterdi.

Anahtar sözcükler: *Ehrlich ascites karsinomu, Antep fıstığı, Sumak, Nigella sativa, Polen, Zeytin yağı*

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INTRODUCTION

Cancer is one of the most aggressive disease and closely associated to the causes of morbidity and mortality in humans with approximately 14 million new cases in 2012 [1]. By 2020, the number of new cases is expected to rise as high as 70%. It is the second leading cause of death globally, and was responsible for 8.8 million deaths (approximately 1 in 6 mortality) in 2015 [2].

Cancer is the disease of all vertebrated animals and can be defined as uncontrolled cellular growth, invasion and spreading of local cells from the primary site to other sites in the body to generate new colonies of cancer cells [3].

The nature of cancer can be evaluated by experimental tumor models especially transferable tumor cells from one individual to the other [4]. Under *in vivo* conditions transplanting tumor tissues subcutaneously from mouse to mouse was pioneered by Ehrlich and Apolant [5]. Then the liquid form of carcinoma "Ehrlich ascites carcinoma" (EAC) was deposited into the peritoneum of the mouse and achieved successful passage thus making next studies possible [6]. EAC is known as undifferentiated carcinoma, and is originally hyperdiploid, has high transplantable capability, no-regression, rapid proliferation, shorter life span, 100% malignancy and also has no tumor specific transplantation antigen [7].

The management of cancer is challanging and usually treated by one of or combinations of surgery, chemotherapy and/or radiotherapy. In metastasized cases, the treatment becomes more drastic and patients suffer various side effects during and/or after therapy. Several anti-tumor agents have cytotoxic effects and been designed to stop tumor growth [8]. Long term use of chemotherapeutics is closely associated with significant negative outcomes on patients' health status. Therefore several natural compounds have been investigated without any detectable side effects.

In folkloric and traditional medicine several plants, vegetables and herbs have been frequently used for the prevention or treatment of malignant masses as nutraceuticals and they served as the main source of cancer chemoprevention drug discovery and development [9-11].

Plant derived natural products (remedies) such as pistachio, rhus, *Nigella sativa* seed, olive oil, pommegrande skin have received significant attention due to their ignorable side effects on comsumers/patients. It was shown that pistachio or its resin possessed apoptotic activity through cytotoxic and apoptosis-inducing effects on human hepatoma cell line [12]. *Rhus coriaria* exhibits anticancer activities (suppressing angiogenesis, metastasis and tumor growth) by promoting cell cycle arrest and autophagic cell death of the breast cancer cells [13,14]. *N. sativa* has long been recognised as antitumorigenic effects especially on hard splenic masses [15]. It has been exclusively used for a wide range of tumors. Intraperitoneal administration of *N. sativa* extract dramatically restricted soft tissue sarcomas chemically induced in albino mice [16,17]. Several studies have shown that bee pollen has greater or lesser antimutagenic properties in certain types of cancer [18,19]. It has been also shown the growth of breast, prostate, colon and lung cancer cells in culture were successfully and selectively inhibited by pomegranate (*Punica granatum)* extracts [20]. Olive oil phenols as chemopreventive and therapeutic effects against cancer has been reviewed [21,22] and shown that olive oil prevented experimentally induced colon cancer in a mouse model [23].

We believe that due to many facets of cancer malignancy using single plant derived substance may have only limited effect however a combination of these matters may generate synergistic impact on the tumor cells. Thus, here, we evaluated possible effect of the combination (AK27 herbal mixture) of pistachio resin, rhus resin, pollen, *N. sativa* seed, pomegranate skin and olive oil on experimentally induced EAC in a mouse model.

MATERIAL and METHODS

Formulation of AK27 Mixture

A total of 100 g preparation of AK27 mixture contained followings and prepared by one of the author (AK). Resin of pistachio was collected from branches of pistachio trees approximately 5 year-old and 10 cm in diameter after pruning. Similarly rhus resin was obtained after pruning of the young branches (1-2 year-old) of the rhus plants. Freshly collected resins were melted at 50-55°C for 15 min and kept at room temperature overnight and then overlayed as a thin layer on a marble block to solidify. Thin layer of resins were removed from the marble and finely crushed into powder form. Ten g of each resin powder was mixed. Shadow-dried skin of unrippen pomegranates (local name: Delieksi) were crushed into fine particles. From this, 15 g was added to the formulation. *N. sativa* seeds and bee-pollen were purchased from a local herbalist and both were further triturated by a fine grinder. Twenty-five g of *n. sativa* seed and 20 g of pollen were added to the mixture. The formulation was filtered through a fine particular sized drain. Finally 20 g of natural olive oil (produced locally in a traditional manner) was added to the formulation and mixed until the end-product was viscous.

Animals and Handling

Handling animals carried out in accordance with the ethical guidelines for the care of laboratory animals of Afyon Kocatepe University, Turkey (Ethical approval no: 49533702-110, 14.06.2016). All effort made to control the experimental pain in conscious animals. A total of 84 eight-week-old, weighing 24-25 g male BALB-c mice were used and allocated into six groups each having 14 mice. Animals

were climatised and housed before the experiment for 10 days at the university's experimental animal research center and kept in separate polyacrylic cages without contacts between groups i.e. four animals per cage. All animals received 3 g standard pellet feed per day with *ad libitum* access to water. Room temperature was kept at 22-24ºC and humidity 50%±2. The light cycle was 12 h dark then 12 h light. The air in the room was cleaned by a bio-filter system (Airsopure S980, Airsopure International, USA).

EAC and Stock Animals

EAC cells were obtained from Department of Anatomy, Faculty of Medicine, Erciyes University, Kayseri, Turkey [24]. The tumor cells were maintained in our laboratory by serial intraperitoneal passage in male BALB-c mice for 7-10 days. EAC cells were tested for viability and contamination using trypan blue dye exclusion technique. Cell viability was usually found to be 95% or more. Tumor cell suspensions were prepared in phosphate buffered saline (PBS). Finally EAC cells were implanted into the peritoneal cavity of experimental groups' to establish the animal model for ascites carcinoma.

Experimental Study

Animals in group I (n=14) were given freshly prepared 2 g/mouse of AK27 mixture (0.2 g of pistachio resin, 0.2 g of rhus resin, 0.3 g pommegranate skin, 0.5 g of *N. sativa* seeds, 0.4 g of pollen, and 0.4 g of olive oil per mouse) orally for 7 days then $3x10^6$ EAC cells/mouse (0.2 mL/mouse) was administered intraperitoneally. AK27 mixture feeding was continued until the end of the experiment (to observe preventive effect of AK27 mixture). In group II (n=14) immediately after intraperitoneal administration of $3x10^6$ EAC cells/mouse, oral AK27 mixture (2 g/mouse) was given and continued until the end of the experiment (to observe effect of tumor initiation) whereas in grup III (n=14) after 5 days of single intraperitoneal administration of $3x10^6$ EAC cells/mouse, oral AK27 mixture (2 g/mouse) was given and continued until the end of the experiment (to observe effect on tumor development). Group IV (n=14) served as cancer control group where mice received intraperitoneal $3x10^6$ EAC cells/mouse and then daily oral 0.9% NaCl (0.2 mL/mouse) was administered. For the AK27 control, group V (n=14) mice had single injection of intraperitoneal normal saline solution (0.2 mL/mouse) followed by oral AK27 mixture (2 g/mouse) until the end of the experiment (AK27 mixture control). Group VI serves as sham control in which routine feeding was practiced. The experiment was terminated at the 30th day of the study.

EAC Cell Count

Six of the mice in each group were sacrificed at 10th day and abdominal ascites were removed to observe viability of the cells by microscopy. One ml of EAC ascites was added 1 mL of PBS making 2 mL total. A 100 microliter of diluted EAC was mixed with 100 microliter of trypan blue solution (Sigma, T8154, product of UK). Then approximately 50 microliter was subjected to cell count using a counting chamber (Thoma, Iso Lab, Germany). Five large squares of each counting area of the chamber was considered for cell count. The cells that did not stained by trypan blue were considered as viable and those stained were non-viable. All viable and nonviable cells were counted.

Volume of EAC

Ascites fluid were withdrawn from the abdomen of sacrificed mice and centrifuged at 3000 rpm for 10 min at 4°C. Subtracting the volume of the supernatant gave the volume of ascites fluid.

Mean Survival Time and Percentage Increase in Life Span

Mice that were not sacrificed (n=8 in each group) were observed for the mean survival time (MST) and percentage increased in life span (PILS) according to quotations given below [25]. The live body weight of animals were measured every other day up to 30 days.

$$MST = \frac{\text{Total survival time of each mouse in group}}{\text{Total number of mice in group}}$$

$$PILS = \frac{\text{MST of treated group}}{\text{MST of control group}} - 1 \times 100$$

Statistics

Data were analysed using SSPS for windows. Differences between groups were evaluated by ANOVA and Duncan tests. Significant level was set at P<0.05.

RESULTS

Antitumor activity of AK27 compound against EAC-bearing mice was evaluated by tumor cell count (viable and non-viable), median survival time, percentage increase in the life span and live body weight changes. The findings of the study indicated that the AK27 compound produced significant antitumor effects on mice with EAC.

Live Body Weight Increase

When the live body weight (LBW) increases of the mice were considered, the differences between groups were not significant on days 0 and 2 (P>0.05). After the 4th day onwards until the 12th day significant differences were observed between groups (P<0.001). The highest mean LBW was noted in group IV (cancer control group) then followed by groups III and II. On day 4, no significant difference was seen in groups III and IV however the difference was significant between 6 and 12 days of the study *(Table 1)*.

After 14 days, the highest LBW increase was observed in group III (mean 28.36 g) followed by groups II (27.44 g) and I (27.11 g). The lowest LBW increase was in groups

V (26.19 g) and VI (25.39 g). AK27 significantly ($P<0.05$) maintained the body weight of mice in groups I, II and III toward normal with respect to EAC control animals (group IV) *(Fig. 1)*. Increase in the LBW was 44.9% for group IV however it was 8.6%, 11.4% and 15.1% for groups I, II and III, respectively. In control groups LBW was increased 6.1% and 5.3% *(Table 2)*.

Cell Count and EAC Volume

Volume of EAC cells and viable cell count were found to be significantly decreased in AK27 treated group when compared to EAC control group ($P<0.01$). No viable or non-viable CEA cells or abdominal fluid was recovered from groups I, V and VI however the highest viable cell

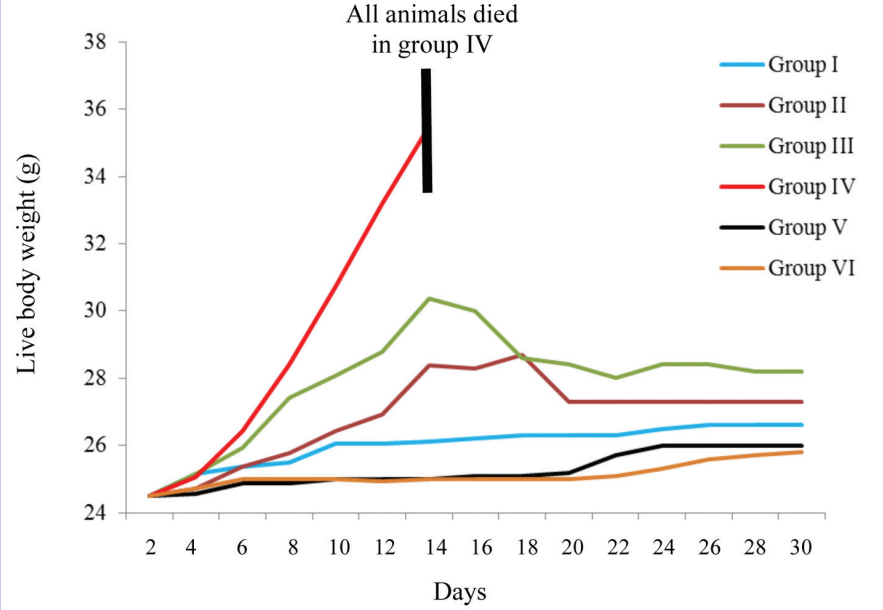


Fig 1. Representation of mean life span and body weight of normal and EAC bearing mice. Note: Animals in cancer control group (IV) were died at the 12th day of the experiment

Table 1. *Live body weight changes in normal and EAC bearing mice (first 12 days of the experiment)*

Groups	Days													
	0		2		4		6		8		10		12	
	Live body weight (g)													
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
I	24.50	0.68	25.14	0.53	25.36^{bc}	0.63	25.50^{cd}	0.65	26.07^{c}	0.47	26.07^{bc}	0.47	26.13^{d}	0.35
II	24.50	0.51	24.71	0.83	25.36^{bc}	0.74	25.79^{c}	0.89	26.43^{c}	0.85	26.93^{b}	1.27	28.38^{c}	2.62
III	24.50	0.52	25.14	1,29	25.93^{ab}	1.21	$27,43^{b}$	1,55	28.07^{b}	1.94	28.79^{b}	2.08	30.38^{b}	2.62
IV	24.50	0.52	25.07	0,83	26.43^{a}	1.16	28.43^{a}	1.60	$30,71^{a}$	2.13	33.21^{a}	2.67	35.50^{a}	2.78
V	24.50	0.52	24.57	0.51	24.86^{c}	0.36	24.86^{d}	0.36	25.00^{d}	0.00	25.00^{c}	0.00	25.00^{d}	0.00
VI	24.50	0.51	24.71	0.47	25.00^{c}	0.00	25.00^{cd}	0.00	25.00^{d}	0.00	24.93^{c}	0.27	25.00^{d}	0.00
Total	24.50	0.56	24.89	0.81	25.49	0.95	26.17	1.66	26.88	2.35	27.49	3.23	28.40	4.16
p	NS		NS		<0.001		<0.001		<0.001		<0.001		<0.001	

$^{a,b\,c,d}$ *Different letters respresent significant differecences between groups ($P<0.05$)*

Table 2. *Initial, final, average and increase in live body weight of mice*

Groups	Initial Body Weight (g)	Final Body Weight (g)	Average Body Weight (g)	Increase in Body Weight (%)
I	24.5	26.6	25.9	8,6
II	24.5	27.3	26.7	11.4
III	24.5	28.2	27.7	15.1
IV	24.5	35.5	28.1	44.9
V	24.5	26.0	25.6	6.1
VI	24.5	25.8	25.2	5.3

Table 3. *Viable and non-viable cell count and volume of EAC in mice*

Groups	Viable Cell Count (cell x 10^7/mL)			Non-viable Cel Count (cell x 10^7/mL)			EAC Volume (mL)
	Lowest	Highest	Mean	Lowest	Highest	Mean	
I	-	-	-	-	-	-	-
II	8.1	29.5	15.5	0.5	2	1.4	6
III	21.5	30.2	25.5	0.3	0.9	0.6	8
IV	30.1	44.3	33.1	0.2	0.5	0.4	12
V	-	-	-	-	-	-	-
VI	-	-	-	-	-	-	-

In groups I, V and VI no ascites fluid was obtained

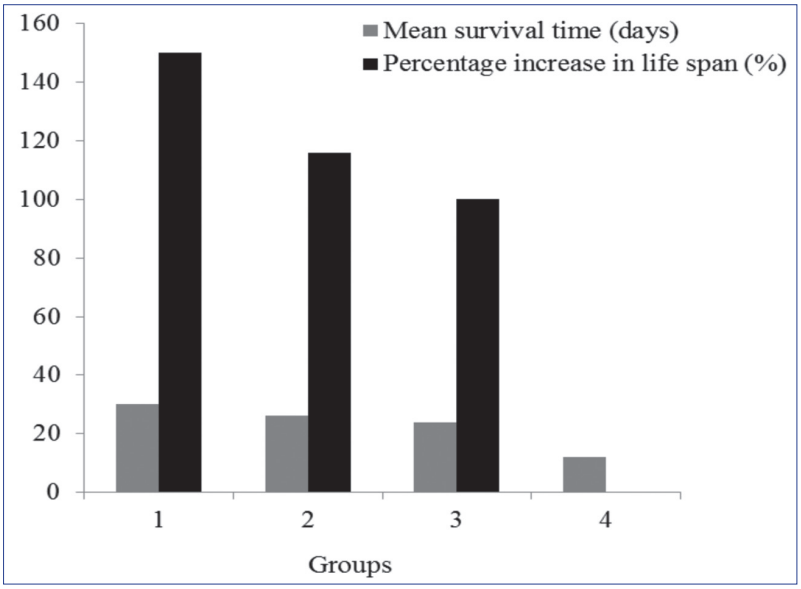


Fig 2. Chart showing mean survival time and percentage increase in life span in normal and tumor bearing mice

count (lowest 8.1x10^7/mL, highest 29.5x10^7/mL and mean 15.5x10^7/mL) and EAC volume (12 mL) was measured in group IV *(Table 3)*. Administration of AK27 before tumor challenge (group I) prevented the EAC development whereas simultaneous administration (group II) or after tumor initiation (group III), AK27 significantly reduced the number of viable CEA cells with respect to cancer control group (P<0.05) displaying negative effects on initiation and development of the tumor.

Longevity of Animals

All animals in group IV (cancer control) were died within 12 days as expected. No dead was observed in group I where AK27 was given 1 week before the EAC challenge. However in groups 2 and 3, two and three dead out of 8 animals were noted, respectively. In the cancer control group, the median survival time was 12 days whereas it significantly increased in AK27 treated groups to 30 days in group I, 26 days in group II and 24 days in group III with PILS of 150, 116 and 100, respectively *(Fig. 2)*.

Effect of AK27 on Normal Mice

After oral administration of AK27 compound at a dose of 2 g/day/mouse for 30 days, none of the mice exhibited either clinical side effects or abnormal behavioral responses (such as aggressiveness, inactiveness, loss of appetite, slow movement, dullness, dizziness, erection of hairs, and hypothermia; data not shown) in group V. Moreover repeated daily oral doses of 2 g/mouse for 30 days also did not have any effect on the live body weight of the mormal mice.

DISCUSSION

Nature was the main source of food, protection, clothing, transportation and remedies for humans since ancient times for survival in this planet [26]. This is also true today's modern world. Natural products are also considered as one of the major contributor that can be used for the design and development of potential chemotherapeutic agents [27]. A number of plant extracts have been used for major health problems for instance the management and combat of cancer and cancer related diseases in traditional medicine however, only a few of them have been scientifically explored [28].

The treatment of cancer is not promising in all cases. Several side effects on the patient's health is still significant

obstacle in modern medicine today. These effects may be short term or long term [29].

Using plant derived extracts remedy principles indicate cytotoxicity towards tumor cells [30] and antitumor activity in experimental animal models [31].

Pistachio consumption reduced cancer mortality and may protect prostate cancer, colorectal and colon cancers. *In vitro* studies and those studies carried out on animals suggested that the health properties of pistachios can be attributed partially to the content of the nut's dietary antioxidants activity [32]. Anti-cancer properties of *N. sativa* has been exclusively reviewed by Randhawa and Alghamdi [13]. The thymoquinone main constituent of the volatile oil of *n. sativa* seed enhanced the anti-cancer effect in rats and mice due to its antioxidant action [33] that interferes with DNA synthesis coupled with enhancement of detoxification processes and apoptosis and cell cycle [34]. Sumac is the generic name for genus Rhus that cover over 250 plant species and has a long historical background of use by indigenous people for medicinal and other uses including antimutagenic and antitumorigenic activities [35]. Anti-tumorigenic activities can be attributed to its promotion of cell cycle arrest [14]. Pollen possesses a wide range of primary and secondary metabolites that exhibit various properties and bioactivities i.e. anticarcinogenic. This effect is associated with the development by cytotoxic activity [19]. Elsewhere, the efficacy of pomegranate was evaluated and concluded that it has a very high antioxidant activity associated with anti-proliferative, anti-invasive and pro-apoptotic entities in various cancer cell lines and animal models [36] for example, pomegranate significantly suppresses TNFα-induced COX-2 protein expression and NF-κB binding suggesting anti-proliferative activity [37].

In our study the combination of aforementioned plants was formulated and successfully used for the prevention and treatment of CEA in the mouse model. The rationale behind combination approach may be explained that many tumors arise from a single malignant cell, by the time they are clinically detectable (1 cm^3 or 10^9 cells), they contain a heterogeneous population of cells. When tumor mass contains 10^6 cells, inherent drug resistance may develop [38]. This genetic instability may further be associated with a tumor that initially responds to treatment but later relapses due to resistance clones grow predominantly. Sometimes single agent therapy is not curative therefore induction regime should contain multiple drugs [39] and combination protocols must maximize cell kill and maintain acceptable toxicities, broaden the range of efficacy against a heterogeneous tumor population and prevent or slow the development of resistant tumor cells [40].

EAC is highly aggressive and causing mortality within 12 days in mice. Nascimento et al.[41] reported that the longevity of EAC bearing mice were 12 days maximum similar to the current study in which all animals in EAC treated group were died within 12 days.

To observe antineoplastic effect of agents on the EAC model the live body weight (LBW) of animals are compared between groups [42]. Our study showed that LBW in cancer group increased dramatically until death however it was not the case in groups I and II indicating EAC prevention effect. In group III, increase in LBW was not significant at 30th day of the tumor challenge mimicking anticancer activity.

It was suggested that studies directed to longevity after cancer cell inoculation should be monitored at least 30 days since the distribution of death occurrence with a 50% longevity of 14.3 days in mice models [43]. We observed the animals up to 30 days.

The prolongation of lifespan of animals is highly reliable and valuable criteria for judging the anticancer drugs effectiveness in experimental investigations [44]. Enhancements of life span by 25% are more over that of the control can be considered as effective antitumor response of the drug in question [45]. MST and PILS in study groups were significantly longer than control group in our study. This observation displays that increased lifespan of tumor bearing mice in experimental groups further corroborates the antitumor potential of AK27 mixture.

To conclude, the results of our study showed enhanced antitumor activity of AK27 compounds on experimentally induced EAC in mice. AK27 mixture formulation appeared to be stable at room temperature, safe to use and easy to administer by means of oral gavage. No side effects of AK27 was observed at a dose of 2g per mouse in this study. However, the maximum tolerated dose should be determined and also this formulation requires further evaluation to identify the possible antineoplastic action/mechanism (synergism or contribution) of the combinatorial fashion. Eventually, future phase studies in human cases should be considered.

Acknowledgements

The authors would like to thank Prof. Dr. Korhan Altunbaş, Afyon Kocatepe University for his valuable contribution to EAC cell count analysis.

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