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Research Article
Cytotoxic and Anti-proliferative Activities of the *Tetrapleura tetraptera* Fruit Extract on Ehrlich Ascites Tumor Cells

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Abstract

Background: *Tetrapleura tetraptera* fruit is widely used in Southern Nigeria for a range of pharmacological actions. **Methodology/Principal Findings:** In this study, the cytotoxic and antiproliferative effects of the ethanolic extract of fruit were studied *in vitro* and *in vivo* by using ehrlich ascites carcinoma cells and tumor modelling, respectively. Cytotoxic activity was determined by incubating the ehrlich ascites carcinoma cells with 0.1, 1, 10 and 100 μg mL^{-1} of the extract. *In vivo* study, 20, 40 and 80 mg kg^{-1} b.wt., doses of the extract was inoculated by intraperitoneal administration to mice following to carcinoma cells inoculation by same way. The extract was cytotoxic to carcinoma cells as assessed by both *in vitro* and *in vivo* experiments. **Conclusion/Significance:** The IC_{50} of the extract was found to be 250 μg mL^{-1}. Results showed that the *Tetrapleura tetraptera* increased the life span of mice via reducing the number of the viable EAC cells, decreasing in ascites fluid volume and tumor burden. The DNA fragmentation assay also showed that it has a possible pro-apoptotic effect.

Key words: *Tetrapleura tetraptera*, ehrlich ascites tumour, antiproliferation, cytotoxicity, phytotherapy

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Plants have been the major source of pharmaceutical agents used to treat ailments since old times and most of them are being used in the prevention and/or treatment of various diseases. On the other hand, plants used in medicinal treatment have needed to be screened for their toxicity level to detect its safety. Chemotherapeutic substances used in the treatment of cancer have been severally developed from plants like vinca alkaloids, vincristine and the isolation of the cytotoxic podophyllotoxins[1,2]. Chemotherapy has limited as it has been reported to cause serious side effects[3-5]. Therefore, there is still a continuous search and development of drugs that will decipher between normal and cancerous cells. There is also still a vast spectrum of untested tropical plants for potential chemotherapeutic drugs. The best approach in the search of antitumoral activity of natural products is to prefer natural product based on its traditional and ethnomedicinal usage. In this perspective, the traditional indigenous general and medical usage in West Africa brought to light ethnomedicinal properties of *Tetrapleurate traptera*.

Tetrapleurate tetraptera locally called "Arindan" in Yoruba, a flowering plant from the Mimosaceae family is found abundantly in Southern Nigeria. It is a flowering plant belonging to pea family and grows in West Africa natively. The fruit is a fleshy pulp with small and brownish-black. The dry fruit is used as a spice in the Southern part of Nigeria because of its pleasant aroma[6,7]. Previous investigations showed that the plant has various pharmacological effects including analgesic and anticonvulsant[8,9], anti-inflammatory and hypoglycemic[10], strong molluscicidal[11], anti-rheumatic[12], anti-malarial[13], anti-ulcerative[14] activities due to a variety of active constituents[7,12,15-23]. Pharmacological examination of this plant also showed that it significantly depressed the blood pressure in anesthetised rats in beside of its little or no hypotensive effect on anesthetised cats, dogs and rabbits[24]. However, there is not any study on the cytotoxic and antiproliferative properties of this plant extract. Therefore, the aim of this study is to determine the antiproliferative and anti-tumor potential of *T. tetraptera in vitro* and *in vivo* using by Ehrlich ascites tumor cells and tumor modelling, respectively.

MATERIALS AND METHODS

Plant collection: *Tetrapleurate tetraptera* (Taub.) fruits, a member of Mimosaceae family were collected from Osogbo, South-West, Nigeria. Plant material was identified by a botanical taxonomist, Mr. Odewo, in Lagos University. Voucher specimens were deposited in the Lagos University Herbarium, Nigeria (Voucher Number: LUH 4197).

Preparation of ethanolic extract: The collected fruits of the plant material were thoroughly washed with tap water and then with distilled water to remove any dirt. The fruits were stayed in dark room until all parts dried. Then the dried parts were put in hot air oven (Nuve, France) at 50°C for 4-6 h to remove excess moisture of fruits. They were separately crushed gently by using a mixer grinder to make a powder form. The crushed powder (500 g) was extracted with ethanol (2.5 L) using by Soxhlet apparatus (Gerhardt, Germany). Extraction was run until to get clear solvents. Then the extract was concentrated using by Rotary evaporator (Heidolph, Germany) under low pressure and temperature to remove the solvents[25] and 14 g extract was obtained (2.8% yield).

Phytochemical characterization of *Tetrapleurate tetraptera* extract: In order to determine which type of the compounds contains the extract, the extract was subjected to silica gel Thin Layer Chromatography (TLC) using chloroform-ethyl acetate (8:2), chloroform-methanol (8:2), ethyl acetate and ethyl acetate-ethanol mobile phases. The TLC was carried out on silica gel 60 precoated plates, F-254 (Merck). The spots on the TLC plates were visualized by UV_{254}, UV_{365} and spraying with 1% vanillin-H_2SO_4 followed by heating (at 105°C)[26].

Animals: Six weeks, male, 25-30 g weighting Swiss albino mice were provided from Department of Physiology, Faculty of Medicine, Gaziantep University, Turkey. They were housed in polycarbonate cages in a room temperature (25±2°C) and 12 h light/dark cycle with humidity and fed with standard diet and water ad libitum. The study was conducted after approval from local animal ethic committee of the Gaziantep University (Ethical Number: 05/2012-13) and then the animal experiments were carried out in accordance with the protocols of local animal care.

EAC cells: The EAC cells were obtained from Professor Zekiye S. Altun, Istanbul University, Turkey. They were maintained by intraperitoneal injection of 1×10^6 cells/mice in a regular period[41]. The EAC cells were counted by a cell counter (Cedex, Roche) according to the trypan blue dye exclusion method. The EAC cell viability was >90%. The EAC cell suspension was prepared in Phosphate Balanced Salt (PBS) solution at pH 7.4

to final concentrations of 1×10^6 viable cells mL^{-1}. Mice were inoculated intraperitoneally (i.p.) with 1×10^6 viable EAC cells in 0.2 mL[27-31].

***In vitro* cytotoxicity:** *In vitro* cytotoxic activity was performed according to the trypan blue cytotoxic assay[32]. Briefly, the stock solution from *T. tetraptera* fruit ethanolic extract was used prepared PBS to a 1000 μg mL^{-1} final concentration. Serial dilutions (100, 10, 1 and 0.1 μg mL^{-1}) were prepared in PBS. Two hundred microliter sample solution was placed in tubes and 600 μL PBS was added on it. 1×10^6 EAC in 100 μL volume was added to the tubes. Only PBS was also prepared as a control. They were incubated at 37°C for 3 h and 100 μL trypan blue was added to all test tubes. The EAC cells were counted in a cell counter (Cedex, Roche) according to the trypan blue dye exclusion method. Results were expressed as percentage cell viability[33].

Tumor growth response: The animals were divided into extract-treated "test" and physiological saline (0.9%) and 5-fluorouracil (5-FU, 1000 mg/20 mL, Kocak Farma, Turkey)-treated "control" groups of 10 animals per group. 1×10^6 cells/mouse were inoculated i.p. on day '0' and *T. tetraptera* Extract (TTE) was given per orally (p.o.) at a dose of 20, 40 and 80 mg kg^{-1} b.wt./day 24 h after inoculation. Control groups were treated with same volume of 0.9% physiological saline. All treatments were continued for 14 days. The ascitic fluid from EAC-bearing mice was isolated by peritoneal lavage after death. The Mean Survival Time (MST) of each group was noted. The anticancer efficacy of TTE was compared with that of positive control group (5-FU, 20 mg kg^{-1} b.wt./day). The MST and Increased Life Span (ILS%) was calculated using the following equations[34-35]:

$$MST = \frac{\text{First death day+last death day}}{2}$$

$$ILS\ (\%) = \left(\frac{\text{MST of treated group}}{\text{MST of control group}} - 1\right)\times 100$$

Anticancer activity: Animals were divided into 6 groups (n: 10). All groups were inoculated with EAC cells[28-31,36] (1×10^6 cells/mouse in 0.2 mL volume) i.p. except group 1. This was taken as day zero. After 24 h, TTE at different concentrations were received i.p. in daily administration.

Group 1: Normal control
Group 2: Disease control, EAC (1×10^6 cells/mice)
Group 3: EAC (1×10^6 cells/mice)+20 mg kg^{-1} TTE i.p.
Group 4: EAC (1×10^6 cells/mice)+40 mg kg^{-1} TTE i.p.
Group 5: EAC (1×10^6 cells/mice)+80 mg kg^{-1} TTE i.p.
Group 6: EAC (1×10^6 cells/mice)+5-FU (20 mg kg^{-1}, i.p.)

Carcinoma assessment was performed by observing the changes with respect to body weight, viable and nonviable EAC cell count and DNA fragmentation assay.

After 14 days of treatment, all the animals were fasted for 12 h but still allowed to access to water. The animals from each group were euthanized by ether anaesthesia. The total number of EAC cells was counted by the trypan blue[28-31,37] using by the Cedex counting machine[38].

DNA fragmentation assay: The DNA fragmentation assay was performed using by the modified method of Ueda *et al.*[39] as described. In brief, the EAC cells were collected from animals and washed twice with PBS. Pelleted cells were lysed in lysis buffer (10 mM tris-HCl buffer, 10 mM EDTA and 0.2% triton X-100, pH 8.0) on ice for 10 min. The lysate was centrifuged at 6000 rpm for 20 min. And then the supernatant was extracted with PCIAA (phenol-chloroform-isoamyl alcohol solution, 25:24:1). The mixture was centrifuged at 6000 rpm for 20 min and the upper layer was decanted off and precipitated with 3M NaCl and cold ethanol (1:20) at -20°C overnight. After drying, the isolated DNA was dissolved in TE (tris EDTA) buffer. The RNA contamination was eliminated by incubation with RNa seat 37 °C for 30 min. Loading buffer (Bromophenol blue) was added and DNA electrophoresed on 1.5% agarose gel in TBE (40 mM tris, 20 mM Boric acid, 1 mM EDTA) at 100 V for 45 min and visualized by EtBr staining under UV light.

Statistical analysis: Results are expressed as percentages or Mean±Standard Deviation. The SPSS 11.0 package statistic program was used for data analysis and student t-test was used for determining the significance between mean values within a group. Values of $p<0.05$ was expressed as a significant.

RESULTS

This results showed that the death rate of EAC cells *in vitro* increased with elevation of TTE concentration (Fig. 1). Ethanolic extract of TTE showed cytotoxic effect against to EAC cells. The IC_{50} was calculated as a 250 μg mL^{-1} (Fig. 2).

The effect of TTE on the survival time of EAC-bearing mice was shown in Table 1. The MSTs for the control groups including diseased group and 20 mg kg^{-1} 5-FU group were

Table 1: Survival time of EAC-bearing mice

Group No.	Treatment	Day of first death (DFD)	Day of last death (DLD)	MST (days) (DFD+DLD/2)	ILS (%) ((T/C)-1)
1	EAC only	7	11	9.0	-
2	EAC+20 mg kg^{-1} 5-FU	11	12	11.5	27.8
3	EAC+20 mg kg^{-1} TTE	8	11	9.5	5.6
4	EAC+40 mg kg^{-1} TTE	9	11	10.0	11.1
5	EAC+80 mg kg^{-1} TTE	10	12	11.0	22.2

MST: Mean survival time and ILS: Increase in life span

Table 2: Body weight changes

Group No.	Treatment	Before inoculation	After inoculation	After treatment	Change in body weight (%)
1	EAC only	30.7+6.7	32.6+7.2	39.3+10.7	27.9
2	EAC+20 mg kg^{-1} 5-FU	36.2+5.6	36.7+6.2	32.6+11.3	-9.0
3	EAC+20 mg kg^{-1} TTE	28.4+2.0	30.3+2.5	30.1+4.9	5.0
4	EAC+40 mg kg^{-1} TTE	33.2±3.2*	33.5±2.8*	32.6±3.8*	-1.8
5	EAC+80 mg kg^{-1} TTE	32.3±3.0*	32.7±3.5*	31.0±2.9*	-4.1

*p<0.05, EAC: Ehrlich ascites carcinoma, TTE: *T. tetraptera* extract and 5-FU: 5-fluorouracil

Table 3: Tumor volume of mice with EAC

	EAC only	EAC+20 mg kg^{-1} TTE	EAC+40 mg kg^{-1} TTE	EAC+80 mg kg^{-1} TTE	EAC+5-FU
Ascites tumor volume (mL)	4.2+1.1	1.4+1.1	3.2±0.4*	1.9±1.7*	0.2+0.0
EAC packed cell volume (%)	43.7+5.8	37.5+0.7	44.6±9.5*	36.3±8.1*	20.5+0.7
Blood PCV (%)	32.0+8.5	40.0+12.2	41.4±10.0	43.6±9.1	30.8+0.8
Tumor weight (g)	4.4+1.1	1.5+1.1	3.1±0.4*	2.0±1.7*	0.2+0.0

*p<0.05 EAC: Ehrlich ascites aarcinoma, PCV: Packed cell volume, TTE: *Tetrapleura tetraptera* extract and 5-FU: 5

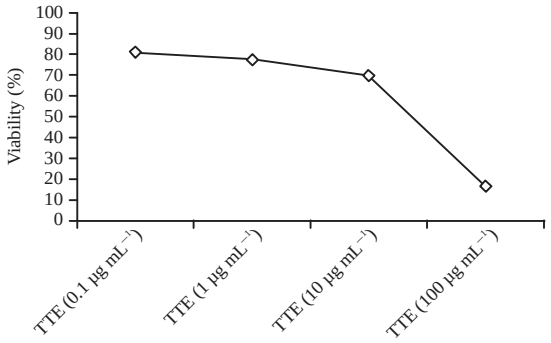

Fig. 1: *In vitro* cytotoxic activity of the *Tetrapleura tetraptera* fruit

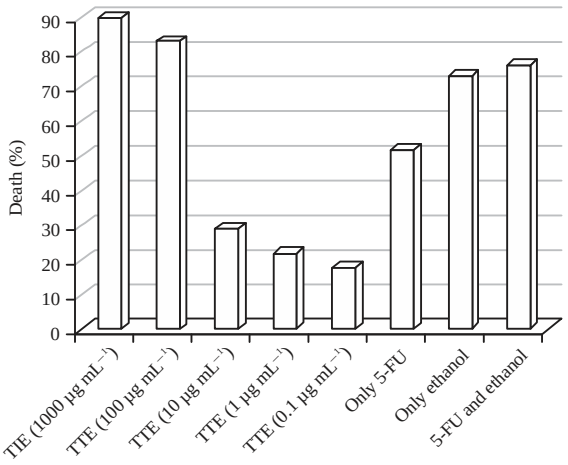

Fig. 2: Death rate of the *Tetrapleura tetraptera* fruit in EAC cells, TTE: *Tetrapleura tetraptera* extract, 5-FU: 5-fluorouracil

9 and 11.5, respectively whereas they were 9.5, 10 and 11 days, respectively for the groups treated with 20, 40 and 80 mg kg^{-1} TTE. The ILS% of EAC-bearing mice treated with 20, 40 and 80 mg kg^{-1} TTE and 20 mg kg^{-1} 5-FU were found to be 5.55, 11.11, 22.22 and 27.77%, respectively as compared to the diseased control (Table 1). At all doses of TTE, there was a significant reduction in increased body weight as compared to EAC-bearing mice ($p<0.05$) (Table 2). Treatment with TTE also caused a significant reduction in tumor volume and viable tumor cell count at all doses as compared to the EAC control group (Table 3-4).

The EAC cells stained with trypan blue for exclusion of viability or non viablity and giemsa for morphological characteristics were shown in figures (Fig. 3 and 4, respectively). Fragmented DNA which extracted from TTE-treated cells was seen in agarose gel electrophoresis (Fig. 5). The DNA of the control group was found to be intact while of 5-fluorouracil-treated cells was more intense. Phytochemical screening of the extract on TLC showed that it contains mainly terpenic compounds. In particular, the TLC chromatogram of the extract using polar mobile phases, ethyl acetate-ethanol (1:1) and methanol indicated that there were two polar terpenic or steroidal compounds.

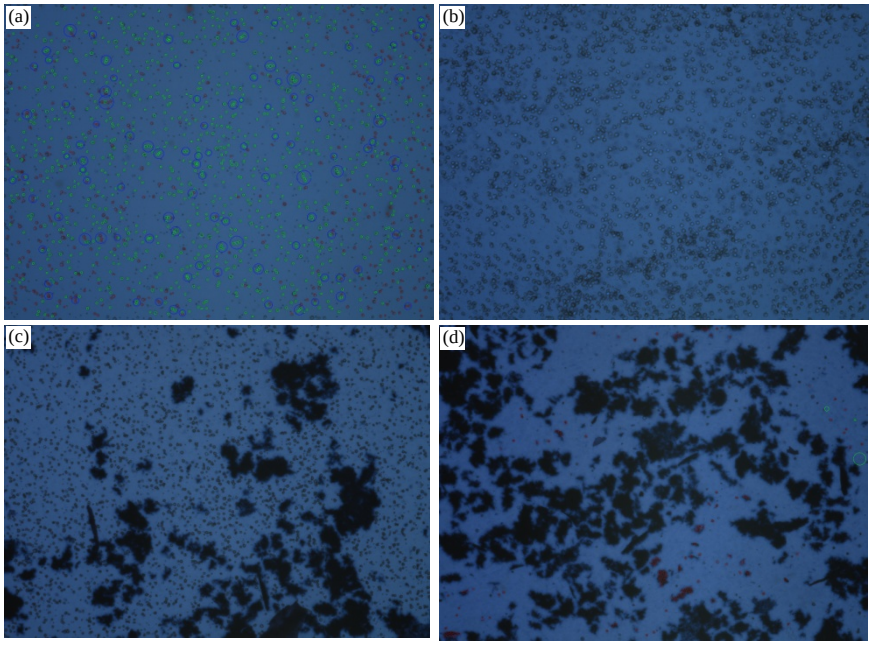

Fig. 3(a-d): EAC cells stained with trypan blue, (a) EAC only, (b) EAC+5-FU, (c) EAC+20 mg kg^{-1} TTE and (d) EAC+80 mg kg^{-1} TTE

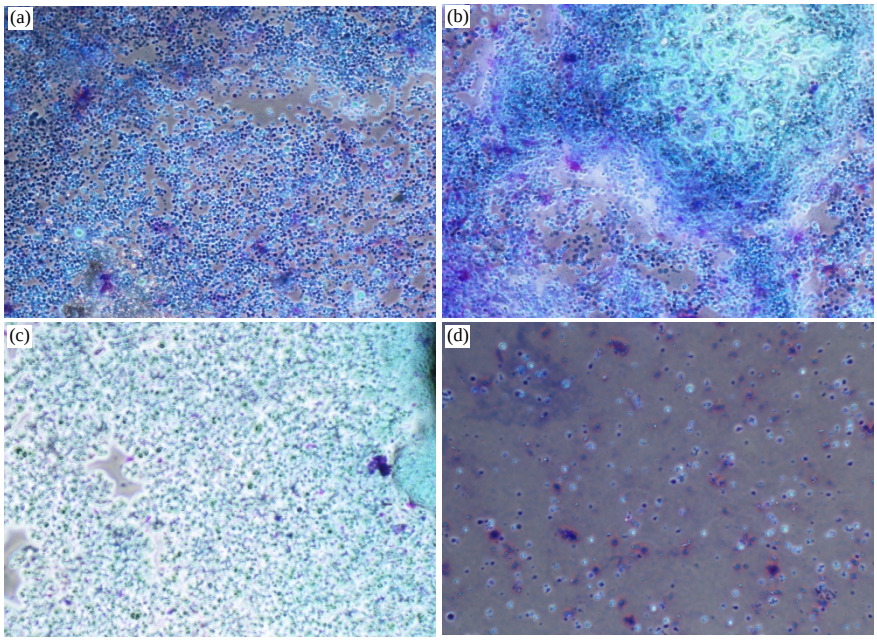

Fig. 4(a-d): EAC cells stained with giemsa, (a) EAC only, (b) EAC+5-FU, (c) EAC+20 mg kg^{-1} TTE and (d) EAC+80 mg kg^{-1} TTE

Table 4: Tumor viability of mice with EAC

Group No.	Treatment	Viable cell count	Dead cell count	Total cell count	Total cell concentration (cells mL^{-1})	Viability (%)	Mortality (%)
1	EAC only	1086	420	1506	418.51×10^5	72.1	27.9
2	EAC+20 mg kg^{-1} 5-FU	190	462	652	181.19×10^5	29.1	70.9
3	EAC+20 mg kg^{-1} TTE	12	381	393	109.21×10^5	3.1	96.9
4	EAC+40 mg kg^{-1} TTE	31	1137	1168	324.58×10^5	2.7	97.3
5	EAC+80 mg kg^{-1} TTE	3	120	123	34.18×10^5	2.4	97.6

EAC: Ehrlich ascites carcinoma, TTE: *Tetrapleura tetraptera* extract and 5-FU: 5

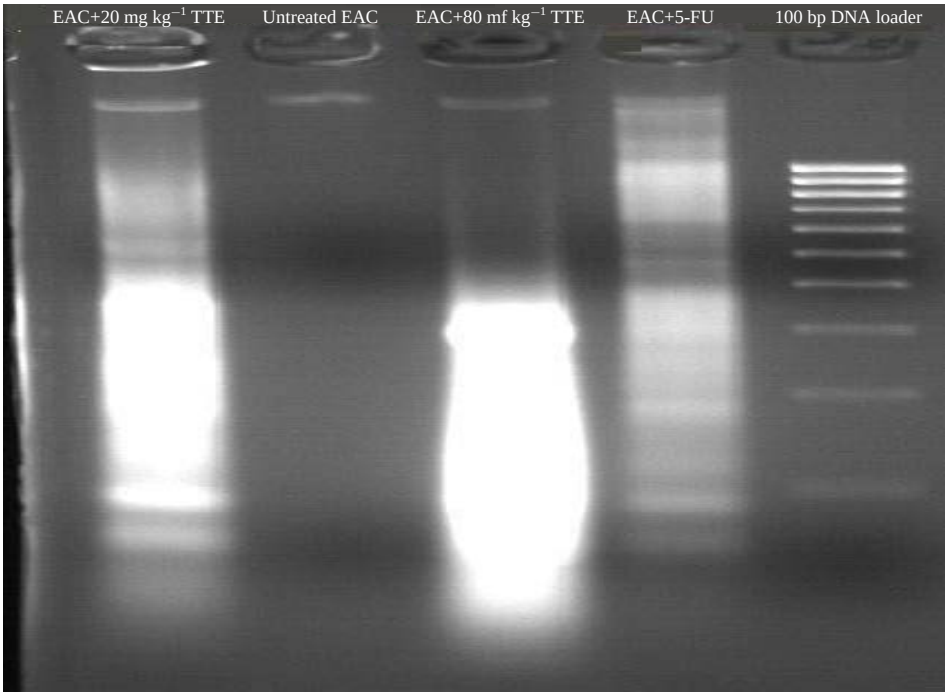

Fig. 5: DNA fragmentation in EAC cells

DISCUSSION

This study was aimed to evaluate the cytotoxic and antiproliferative potentials of *T. tetraptera* ethanolic extract in EAC-bearing mice. The prolongation of life span in experimental animals which were treated therapeutic agents is one of the most reliable criteriato assess the value of anticancer drugs[32,40]. The TTE treatment increased the life span of EAC-bearing mice via reducing the number of viable EAC cells, decreasing in tumor volume and tumor burden. It was reported that a 25% increase in the lifespan of EAC-bearing animals should be considered to be an indicator of drug activity[41]. In this study, although 5-FU had an approximately 28% increasing rate in the lifespan of EAC-bearing mice, 80 mg kg^{-1} TTE had a 22% increasing rate. This study showed that this proximate increasing in lifespan resulted by TTE treatment was significant rate for a candidate therapeutic in cancer.

In vitro cytotoxicity assay showed us TTE was toxic for the EAC cells as there was an increase in the number of nonviable cells, depends on its increasing concentration. The cytotoxic activity of TTE might be related with the mechanisms other than direct cytolytic effects including tumor cell lysis directly or the microenvironment destroying directly. Fecchio *et al.*[42] reported that local inflammatory reactions were induced by EAC cell implantation and this situation resulted in an intense edema formation, cellular migration and a progressive ascetic fluid formation. Tumor cells increase as well as ascitic fluid increasing because ascitic fluid is the source of nutrition for tumor cells. Results showed TTE treatment reduced the tumor burden as depicted by the reduction in ascitic fluid volume. Cytotoxic activity of TTE might be related with the reduction of ascitic fluid.

In order to understand the mechanism of antitumor effect by TTE, DNA fragmentation of wholly grown tumor was treated with the extract. In this regard, *T. tetraptera* prove to be a potential anti-proliferative and apoptosis inducing activity. Phytochemical screening of the extract on TLC showed that it contains mainly terpenic compounds. In particular, the TLC chromatogram of the extract using polar mobile phases, ethyl acetate-ethanol (1:1) and methanol indicated that there were two polar terpenic compounds. These results were in agreement with previous reports. According to literature survey, it is shown that *T. tetraptera* fruits are rich in the glycosides of oleanolic acid triterpenes[7,15,18,20,22,23]. Aridanin, which is an N-acetyl glycoside of oleanoic acid triterpenoidis aprincipal component of *T. tetraptera* fruits[16,17,21,43-44]. Therefore, cytotoxic activity of the ethanolic extract of the *T. tetrapteura* fruits can be attributed to this principal component of the plant. On the other hand, there can synergistic and/or antagonistic interactions between other minor compounds and aridanin. Various biological activities including depressant[22], neuropharmocological[44], molluscicidal actions[7,16,17,43] of aridanin have been previously

reported. However, there are no reports on the cytotoxic activity of the fruits of *T. tetraptera*, aridanin and its other components. Therefore, the aim of this study group was to isolate and characterize compound(s) responsible for cytotoxic activities of the extract. It has also been shown that scopoletin, a coumarin derivative is one of the other principal components of the fruits of *T. tetraptera*[15,45]. Havada kalmis gibi. However, in the current study, the TLC analysis did not allow the detection of this compound.

CONCLUSION

The results of this study revealed that the *Tetrapleura tetraptera* increased the life span of mice via reducing the number of the viable EAC cells, decreasing in ascites fluid volume and tumor burden. Moreoever, DNA fragmentation assays also confirmed that it has a possible pro-apoptotic effect. The ingradients of *Tetrapleura tetraptera* sap required to be purified for a detailed research on.

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