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Original Article

Growth retardation and apoptotic death of tumor cells by *Artemisia herba-alba* oral administration in Ehrlich solid carcinoma bearing mice

Hanan R.H. Mohamed [a,*], Mahmoud Amer [a], Ahmad Salih A. El Faky [b]

[a] *Zoology Department, Faculty of Science, Cairo University, Giza, Egypt*
[b] *Faculty of Science, Cairo University, Giza, Egypt*

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ABSTRACT

At present, there is a rapidly growing interest in studying the cytotoxic effects of *Artemisia herba alba* Asso, Asteraceae, in various cancer cell lines. However, its antitumor effectiveness has not been investigated. Therefore, the current study was conducted to study the effect of *A. herba alba* extract on the proliferation and growth of solid tumor cells in Ehrlich Solid Carcinoma bearing mice. Oral administration of *A. herba alba* extract resulted in significant reductions in tumor size, tumor weight and mice body weight, as well as caused concurrent significant increases in the DNA breakages and apoptotic DNA damage induction in a time-dependent manner. *A. herba alba* extract also raised the expression level of p53 gene and reduced of K-ras expression in a time-dependent manner. Minor histological lesions were observed in the liver and kidney tissues sections of mice administered *A. herba alba* extract compared with the high histological lesions observed in the liver and kidney tissues of artesunate and cisplatin treated groups. Thus, we concluded that *A. herba alba* extract exhibited promising potential antitumor efficacy with greater safety than artesunate and the commercially used anticancer drug cisplatin in mice.

Introduction

Although there are many commercially available drugs to treat cancer, almost all of these drugs threaten human life through their side effects. Almost all anti-cancer drugs cause immunosuppression, anemia, fatigue, hair loss, infertility, digestive disorders such as nausea, vomiting and diarrhea as well as organ damage such as renal toxicity caused by cisplatin and heart toxicity caused by doxorubicin and idarubicin (Groopman and Itri, 1999; King and Perry, 2001; de Jonge and Verweij, 2006; Gibson and Keefe, 2006; Brydøy et al., 2007; Elad et al., 2010; Cramp and Byron-Daniel, 2012; Shaikh and Shih, 2012; Can et al., 2013).

Artesunate, a water-soluble semisynthetic derivative of artemisinin (the main active ingredient in *Artemisia annua*), is widely used for the management and treatment of complex malaria and also is highly effective against multi-drug resistant strains of *Plasmodium falciparum* (Ittarat et al., 1999; Van Agtmael et al., 1999). Moreover, artesunate has been shown to have anti-inflammatory effects in rheumatoid arthritis, allergic anaphylaxis and sepsis as well as antiviral activity against Herpes Viruses, Hepatitis B and C viruses (Romero et al., 2005; Efferth et al., 2008; Ho et al., 2014).

The cytotoxic effect of artesunate against different human cancer cell lines such as colon cancer, leukemia and melanoma cells has been proven as well as artesunate has exerted promising therapeutic efficacy against ovarian cancer, hepatoma, pancreatic cancer and esophageal cancer both *in vitro* and *in vivo* experimental models (Efferth et al., 2001; Hou et al., 2008; Chen et al., 2009; Du et al., 2010; Liu et al., 2015). However, several studies have been reported numerous toxic side effects of artesunate on various organs including brain (Genovese et al., 2000; Nontprasert et al., 2002), stomach (Eweka and Adjene, 2008) and testis (Izunya et al., 2010).

Therefore, there is ongoing research on new anticancer drugs with no or less side effects compared to the used anticancer drugs. Nowadays, there is growing research interest in assessing the antitumor potential of natural extracts and their derivatives due to lower toxicity and higher safety compared to the currently used anticancer drugs (Greenwell and Rahman, 2015). The Asteraceae family includes many aromatic and medicinal plants such as *Artemisia, Santolina, Centuria, Chrysanthemum* etc. The *Artemisia* genus belongs to the *Anthemidae* tribe that is one of the Asteraceae family and contains more than three hundred species of grasses and shrubs (Dob and Benabdelkader, 2006).

* Corresponding author.
E-mail: hananeey@sci.cu.edu.eg (H.R. Mohamed).

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Artemisia herba alba Asso, Asteraceae, has a common Arabic name Sheeh or desert wormwood. It grows in deserts and steppes in the Middle East, Spain and North Africa as well as in the Northwestern Himalayas and has been used by many cultures in folk medicine many years ago for the treatment of diabetes, hair loss, fever, diarrhea, vomiting, bronchitis, gastrointestinal tract disturbances, and muscular pains (Vernin et al., 1995; Iriadam et al., 2006; Almasad et al., 2007; Tahraoui et al., 2007; Boudjelal et al., 2013). Moreover, *A. herba alba* has exerted potent antibacterial, antispasmodic, antifungal and vaso-relaxant activities due to its chemical constituents including essential oils (largely sesquiterpenes and monoterpenoids), flavonoids, phenolic compounds and waxes (Kim et al., 2004; Saleh et al., 2006; Abou El-Hamd et al., 2010; Skiker et al., 2010).

Recently, *A. herba alba* has shown a strong cytotoxic activity against various human cancer cell lines including human colon cancer cell, laryngeal carcinoma, bladder carcinoma and myelogenous leukemia by reducing cell viability, DNA destruction and apoptosis induction in cancer cells. On the contrary, no cytotoxicity was achieved by the extract in the normal peripheral blood mononuclear cells that confirming the safety of *A. herba alba* extract to the normal cells (Lupidi et al., 2011; Khlifi et al., 2013; Tilaoui et al., 2015).

However, limited data are available on the antitumor activity of *A. herba alba* extract in *in vivo* animal models that forced us to evaluate the antitumor activity and safety of *A. herba alba* crude extract compared the semisynthetic drug artesunate and the commercially used drug *Cisplatin* in Ehrlich solid carcinoma bearing mice.

Materials and methods

Chemicals

Artesunate drug was purchased from the Shanghai-Sudan Pharmaceutical Company, Ltd. Khatoum North, Sudan while Cisplatin was purchased in the form of colorless, clear solution in amber vials under the trade name of Unistin (KUP-United Douglas Pharm., USA, Pharmaceutical CO.) and each vial (50 ml) contains 50 mg cisplatin.

Plant material and preparation of Artemisia herba alba *extract*

Artemisia herba alba Asso, Asteraceae, recently known as *Seriphidium herba-alba*, was collected from South Sinia (Saint Catherine), Egypt in June 2014 and a voucher specimen has been deposited in the herbarium of St. Katherine protectorate, Egypt with voucher specimen number SH-1101.

To prepare the *A. herba alba* crude extract, about 200 g of the dried *Artemisia* material was soaked in a flask containing one liter of 70% ethanol for two days with frequent agitation at room temperature. The mixture was then, filtered to discard any solid material using filter papers and the filtrate extract were then concentrated using a water path at 70 °C for 3 h, let to cool down and the crude extract was finally collected and saved at −4 °C temperature for later treatment.

Animals

Six to eight-week-old female BALB/c mice were used and were obtained from the National Cancer Institute (NCI) animal house Unit. The mice were left for one week before the experiments began to adapt with the animal house conditions under normal dark/light cycle and were supplied diets and water *ad libitum*. Animals handling and experiments conducted in this study were approved by the Institutional Animal Care and Use Committee at Faculty of Science, Cairo University (CU-IACUC) Egypt with approval number CU/I/S/11/16.

Ehrlich solid carcinoma induction

Ehrlich Solid Carcinoma (ESC) was induced into forty female mice using Ehrlich Ascites Carcinoma (EAC) cells that purchased from the NCI (Cairo, Egypt) and obtained from the ascitic fluid of female mice bearing EAC. On day zero about 2.5×10^6 viable EAC cells present in 0.2 ml of diluted ascitic fluid (1:10) with saline was injected intramuscularly in the left thigh of each mouse to induce solid tumors (Perry, 2008).

Treatment schedule

Once the solid tumor formed and appeared on the 14 day of EAC cells injection; mice were randomly divided into four groups, ten mice per group: the first one is the negative control group (Group 1) that was orally administered deionized distilled, and the remaining three groups (Groups 2-4) were treated with cisplatin (5 mg/kg b.w *i.p.*), artesunate (300 mg/kg b.w *p.o.*) or *A. herba-alba* crude extract (300 mg/kg b.w *p.o.*), respectively, daily for five of ten consecutive days.

Body weight and tumor size and weight

After tumor implantation, tumor size and mice weights were measured three times at the 15, 20 and 25 day of EAC inoculation. Tumor size was measured using Vernier caliper while tumor weight was estimated using the formula:

$$\text{Tumor weight(mg)} = \text{Length(mm)} \times (\text{width(mm)})^2/2.$$

Collection of organs

Mice were euthanized using isoflurane (1–4%), sacrificed by decapitation and dissected after either 5 or 10 days of the first treatment. Liver, kidney, and tumor tissues were collected for subsequent analyses. One-half of the tumors and the vital organs were fixed in 10% formalin for histopathological analysis while the other half were stored in sterilized Eppendorfs at −80 °C for later molecular assays.

Detection of DNA damage induction

The level of DNA damage was measured in tumor tissues only using alkaline comet assay (Tice et al., 2000). Briefly: a 10 μl of the clear cell suspension was mixed with low melting agarose (0.5%) and spread on a slide coated with normal melting agarose (1%). Slides were left for gel solidification and then incubated in cold lysis buffer for 24 h in darkness. The denaturized un-winded DNA was electrophoresed in a fresh alkaline buffer for 30 min at 25 V and 300 mA, neutralized, fixed in 100% cold ethanol and stored at room temperature until they were scored. Before imaging, slides were stained with ethidium bromide and 50 comet cells per animal were analyzed using a TriTek CometScore™ Freeware v1.5 scoring software. Tail length in px, %DNA in tail and tail moment were used as indicators for DNA damage.

Mutation screening using SSCP-PCR analysis

Single strand conformational polymorphism-polymerase chain reaction (SSCP-PCR) analysis was conducted in the tumor tissue to screen mutation in the p53 (exon 5) and K-ras (exon 1) genes. Whole genomic DNA was first extracted from tumor tissues using

Zymo Research Genomic DNA™ –Tissue Mini-prep kits and the desired exons were amplified using the primer sequences listed in Table S1 in Supplementary material previously designed by Gutierrez et al. (1992) and Watanabe et al. (1999). Finally the denaturated PCR products were electrophoresed in 20% polyacrylamide gel at 90 V, stained with ethidium bromide and photographed using UV trans-illuminator (Stratagene, USA).

Measuring the expression of p53 and K-ras genes

The expression levels of p53 and K-ras genes were measured in tumor tissues using Real Time Polymerase Chain Reaction (RT-PCR). Whole RNA was extracted according to the instructions of Gene JET RNA Purification Kit (Thermo scientific, USA) and then converted into complementary DNA (cDNA) using Revert Aid First Strand cDNA Synthesis Kit (Thermo scientific, USA). Quantitative SYBR green-based real time polymerase chain reaction (RT-PCR) was conducted using the 7500 Fast system (Applied Biosystem 7500, Clini lab, Egypt) using the same primers sequences listed in Table S1. Expression levels of the tested genes were normalized using the housekeeping gene β actin gene and quantified using the comparative *Ct* (DD*Ct*) method.

Histological examination

Portions of tumor, kidney and liver tissues were collected and fixed in 10% neutral buffered Formalin, processed and embedded in Paraffin wax, sectioned at 4 μm and stained with Hematoxylin and Eosin. Sections were photographed and examined by specialized Histopathologist.

Laddered DNA fragmentation assay

Apoptotic DNA fragmentation was estimated in the tumor, liver and kidney tissues based on the Sriram et al. (2010) protocol. Briefly, small piece of tissues was gently homogenized and lysed in Tris EDTA buffer. Genomic DNA was extracted and electrophoresed in 1% agarose gel at 70 V, visualized using a UV trans-illuminator and photographed.

Statistical analysis

Results were analyzed using Statistical Package of the Social Sciences (SPSS 23). Two-way analysis of variance (ANOVA) was choesn to study the effect of the treatment type, experimental time and their interaction on all the studied parameters. Duncan'stest was utilized to examine the similarities among all the experimental groups. Independent t-test was executed to compare between the experimental times. Regression analysis and correlation coefficient analysis were applied to study the relationships between the studied variables. Data were expressed as a mean ± standard error (SEM).

Results

Body weight and tumor size and weight

The results summarized in Table 1 showed that the oral administration of *A. herba alba* extract at the dose level of 300 mg/kg resulted in statistically significant reductions in the body and tumor weight ($p < 0.001$) after 5 and 10 days of the first administration as was the case with cisplatin and artesunate treatments compared with the negative control levels. On the other hand, a statistically significant decrease in tumor size was observed only after 10 days of *A. herba alba* extract administration similar to those observed in artesunate and cisplatin treated groups compared to the negative control value (Table 1). Moreover, two way analysis of variance (ANOVA) revealed that body weight as well as tumor size and weight are highly affected by the experimental time (5 and 10 days) and treatment type.

DNA damage in tumor tissue

Results of the comet assay showed that oral administration of *A. herba alba* extract caused dramatic damage to the genomic DNA of the tumor tissue as revealed by the observed statistical significant elevations in tail length, %DNA in tail and tail moment ($p < 0.001$) at the two time intervals even higher than the DNA damage caused by cisplatin or artesunate treatment compared with the negative control group (Table 2). Examples for the observed comet nuclei in negative control and different treated groups were shown in Fig. 1. Furthermore, tail length, %DNA in tail and tail moment are significantly affected by the experimental time and type of treatment as revealed by results of two way ANOVA analysis. This was further confirmed by the results of regression analysis that confirmed the moderate negative correlations between tail moment and tumor size ($r = -0.51$) at the two sampling times but the moderate negative correlation ($r = -0.45$) was shown between tail moment and tumor weight only after 10 days of the first treatment as shown in Fig. S1 provided in the Supplementary material.

SSCP-PCR analysis

The results of SSCP-PCR analysis showed that oral administration of *A. herba alba* extract (300 mg/kg b.w) for five and ten consecutive days did not result in any mutation in the p53 and K-ras genes of the tumor tissue where the patterns of the exons 5 and 1 of p53 and K-ras genes, respectively, were identical to that of the negative control group (Fig. S2 in the supplementary material). Similarly, the pattern of p53 exon 5 and K-ras exon 1 of artesunate and cisplatin groups at the two time intervals five and ten days was identical to that of the negative control group as shown in Fig. S2 .

P53 and K-ras expression in tumor tissue

The expression level of p53 gene was highly elevated ($p < 0.001$) in tumor tissue after oral administration of *A. herba alba* extract (300 mg/kg b.w) for five and ten consecutive days compared with the negative control value and even higher than the elevations observed in cisplatin and artesunate groups as shown in Fig. 2. Strong negative correlations between the p53 expression level and tumor size ($r = -0.81$) after 5 days and ($r = -0.90$) after 10 days were shown by regression analysis curves as well as strong negative correlations between tumor weight and expression level of p53 gene was indicated only after 10 days of the first treatment as shown in Fig. S3 in the Supplementary material.

On contrary, oral administration of *A. herba alba* extract for five and ten consecutive days resulted in significant decreases in the expression level of K-ras gene compared with its level in the negative control group and even lower than the reduced K-ras expression level in artesunate and cisplatin treated groups (Fig. 2). Strong positive correlations were found between the expression level of K-ras gene and tumor size ($r = +0.99$) at the two intervals and tumor weight ($r = +0.86$) after 5 days and ($r = +0.98$) after 10 days as shown in regression analysis curve (Fig. S4 in the Supplementary material). Moreover, variations in the expression level of either p53 or K-ras genes were highly dependent on the experimental time and treatment types as shown from two way ANOVA analysis.

Table 1
Effect of treatment and experimental time as well as their interaction on the body weight, tumor size and tumor weight. Data is represented as mean ± standard error of mean (SEM).

Parameter	Group	Experimental time (days)		Two-ways (ANOVA)		
		5	10	Treatment	Time	Interaction
Body weight (g)	Negative control	27.40 ± 1.52^{A}	26.20 ± 2.49^{A}	F = 20.21, $p < 0.001$	F = 67.25, $p < 0.001$	F = 3.97, $p < 0.05$
	Cisplatin	24.96 ± 0.11^{B}	20.00 ± 2.12^{aB}			
	Artusunate	24.92 ± 0.22^{B}	19.40 ± 1.34^{aB}			
	Artemisia	24.94 ± 0.22^{B}	20.80 ± 1.92^{aB}			
Tumor size (mm^2)	Negative control	0.15 ± 0.04^{A}	0.97 ± 0.30^{aA}	F =23.57, $p < 0.001$	F ± 96.66, $p < 0.001$	F ± 20.88, $p < 0.001$
	Cisplatin	0.13 ± 0.05^{A}	0.36 ± 0.07^{B}			
	Artusunate	0.11 ± 0.02^{A}	0.27 ± 0.09^{aB}			
	Artemisia	0.11 ± 0.03^{A}	0.31 ± 0.11^{aB}			
Tumor weight (μg)	Negative control	10.16 ± 2.89^{A}	283.48 ± 33.34^{aA}	F = 215.28, $p < 0.001$	F ± 740.61, $p < 0.001$	F ± 202.52, $p < 0.001$
	Cisplatin	6.02 ± 1.82^{B}	54.55 ± 4.72^{aB}			
	Artusunate	5.94 ± 1.73^{B}	52.20 ± 8.62^{aB}			
	Artemisia	6.75 ± 2.97^{B}	62.92 ± 14.79^{aB}			

In each column, the mean values marked with the same superscript letter are similar (insignificant, $p > 0.05$) whereas those with different ones are significantly differed ($p < 0.05$).
$p > 0.05$: insignificant, $p < 0.05$, $p < 0.01$ and $p < 0.001$: significant effect at $\alpha = 0.05$, 0.01 and 0.001, respectively.
[a] Represent significant differences ($p < 0.05$) in comparison to the corresponding group after 5 days.

Table 2
Effect of treatment and experimental time as well as their interaction on the studied comet parameters. Data is represented as mean ± standard error of mean (SEM).

Comet parameter	Group	Experimental time (days)		Two-ways (ANOVA)		
		5	10	Treatment	Time	Interaction
Tail length (μm)	Negative control	4.39 ± 0.55^{A}	3.53 ± 1.97^{aA}	F = 17.20, $p < 0.001$	F ± 83.20, $p < 0.001$	F ± 13.48, $p < 0.001$
	Cisplatin	2.40 ± 0.39^{B}	6.44 ± 2.31^{aB}			
	Artusunate	2.28 ± 0.99^{B}	8.68 ± 2.90^{aB}			
	Artemisia	6.77 ± 1.80^{C}	7.92 ± 1.56^{B}			
%DNA in tail	Negative control	3.38 ± 0.72^{A}	5.34 ± 2.22^{aA}	F = 479.28, $p < 0.001$	F = 44.43, $p < 0.001$	F = 8.00, $p < 0.001$
	Cisplatin	7.37 ± 1.61^{B}	8.64 ± 2.82^{B}			
	Artusunate	6.02 ± 0.67^{AB}	7.79 ± 2.61^{B}			
	Artemisia	38.84 ± 5.18^{C}	49.28 ± 1.33^{aC}			
Tail moment	Negative control	0.17 ± 0.06^{A}	0.59 ± 0.50^{aA}	F = 62.85, $p < 0.001$	F = 75.94, $p < 0.001$	F = 18.83, $p < 0.001$
	Cis-platin	0.27 ± 0.10^{A}	1.25 ± 0.30^{aB}			
	Artusunate	0.13 ± 0.05^{A}	1.24 ± 0.36^{aB}			
	Artemisia	3.74 ± 1.07^{B}	4.28 ± 0.89^{C}			

In each column, the mean values marked with the same superscript letter are similar (insignificant, $p > 0.05$) whereas those with different ones are significantly differed ($p < 0.05$).
$p > 0.05$: insignificant, $p < 0.05$, $p < 0.01$ and $p < 0.001$: significant effect at $\alpha = 0.05$, 0.01 and 0.001, respectively.
[a] Represent significant differences ($p < 0.05$) in comparison to the corresponding group after 5 days.

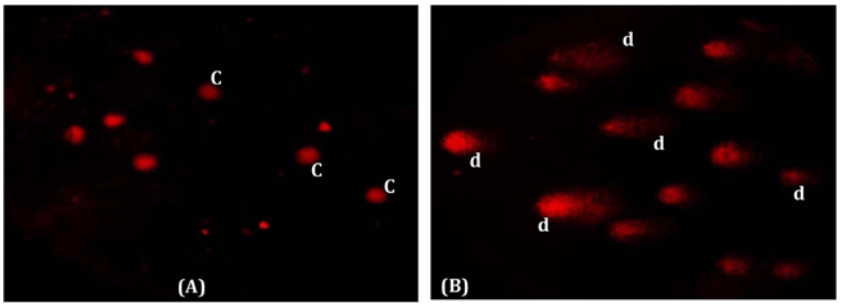

Fig. 1. Representative photos for the observed comet nuclei in the negative control and the three treated groups showing undamaged (A) and damaged (B) comet nuclei with various DNA damage degree. C: control undamaged nuclei; d: damaged nuclei.

Histological examination

Tumor tissues

Histological examination of the tumor tissue sections of the untreated control mice have shown EAC cells infiltration into the dermal layer of the thigh and well developed solid tumors with criteria of malignancy including pleomorphism, anaplasia, hyperchromasia and frequent atypical mitotic figures with muscle necrosis at the two experimental intervals 5 and 10 days (Fig. 3a,b). However, oral administration of *A. herba alba* at the dose level 300 mg/kg resulted in apoptosis and necrosis of tumor cells after 5 as well as massive mononuclear cells infiltration with higher apoptotic and necrotic tumor areas were observed after 10 days of the first administration as shown in Fig. 3g,h.

Likewise apoptotic tumor cells and necrotic tumor area with mononuclear cells infiltrations were observed in the sections of tumor tissues obtained from cisplatin (5 mg/kg) (Fig. 3c,d) and artesunate (300 mg/kg) (Fig. 3e,f) treated mice at the two time intervals 5 and 10 days of the first treatment.

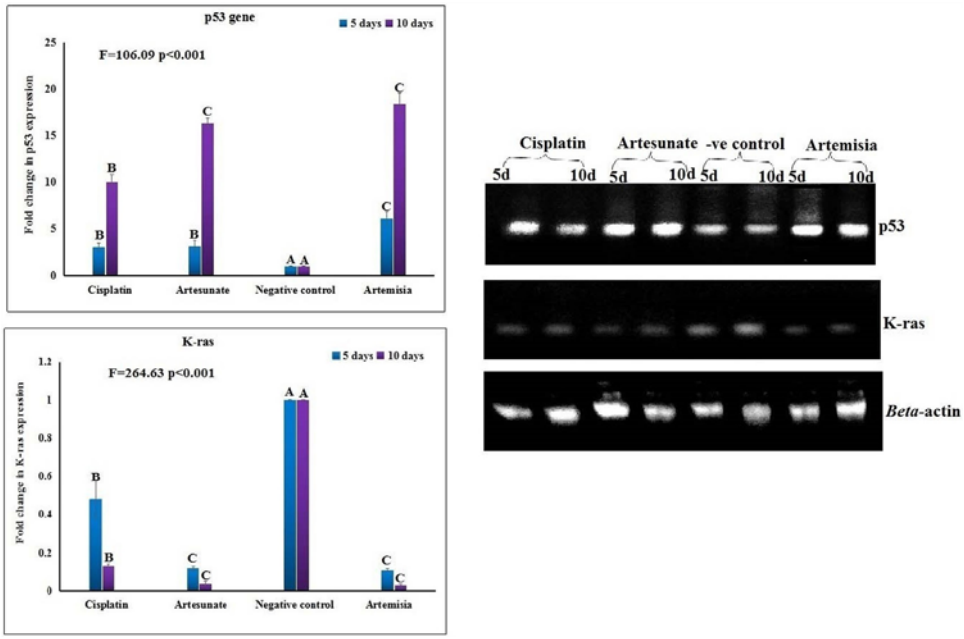

Fig. 2. The expression levels of p53 and K-ras genes in tumor tissues of the negative control and the three treated groups. Left: Charts showing the fold change in the expression of p53 and K-ras genes β-actin gene for standardization. Results are expressed as mean ± Standard Error (S.E). Two ways ANOVA was used to compare between the four groups and different letters indicated statistical significant difference. D: days. Right: Pattern of the RT-PCR products of p53, k-ras and *Beta*-actin genes electrophoresed on agarose gel stained with ethidium bromide.

Liver tissues

Histological examinations of the liver tissues sections have shown necrosis of most of hepatocytes of the liver obtained from the negative control mice with evidence of metastasis as shown in Fig. 4a. On contrary, no evidence of metastasis was observed in the liver tissue sections of mice treated with cisplatin, artesunate or *A. herba alba* but heavy infiltration of mononuclear inflammatory cells was found between the swelled and degenerated hepatocytes of liver tissues of cisplatin injected mice (Fig. 4b). Similarly, degenerative changes of some hepatocytes with vacuolation and necrosis of others were observed in the liver sections of artesunate treated mice (Fig. 4c) as well as diffusely neuroses hepatocytes with pyknotic nuclei and faint cytoplasm was seen in the liver sections of *A. herba alba* treated mice (Fig. 4d).

Kidney tissues

As shown in Fig. 4a kidney section of mice from negative control group showing vacuolation of glomerular epithelium and renal epithelium with evidence of metastasis. On the other hand, no evidence of metastasis was seen in the kidney sections of cisplatin, artesunate and *A. herba alba* groups. However, vacuolar degeneration of glomerular epithelium and renal epithelium were observed in the kidney of cisplatin group (Fig. 4b) and massive necrosis and atrophy of renal tubules were seen in the kidney section of artesunate group (Fig. 4c) but only congestion of the interstitial blood vessel was reported in the kidney section of *A. herba alba* group (Fig. 4d).

Laddered DNA fragmentation

As seen in Fig. 5 the oral administration of *A. herba alba* extract resulted in a dramatic degradation of the tumor genomic DNA after 5 and 10 days of the first administration, as demonstrated by the observed smeared pattern of DNA electrophoresed on agarose as in the case of artesunate and cisplatin groups. On the other hand, oral administration of *A. herba alba* caused slight fragmentation of the genomic DNA of liver and kidney tissues as shown from the less fragmentized DNA compared to the highly degraded DNA in cisplatin and artesunate treated groups (Fig. 5).

Discussion

Although chemotherapy is a successful treatment against diverse forms of cancer either alone or in combination with radiotherapy or/and surgery, all these therapeutic drugs almost cause many side effects that prompt scientists to seek new anticancer drugs that selectively kill cancer cells without affecting normal cells. As a result, in recent years, the interest in the use of natural extracts and their derivatives has increased in the prevention and treatment of cancer. Many natural products have been found to have potential anticancer effects both *in vitro* and *in vivo*. Therefore, this study was conducted to evaluate the safety and antitumor activity of the *A. herba alba* crude extract compared to the semisynthetic drug artesunate as an attempt to be used as an anticancer drug with few or no side effects.

In the current study Ehrlich Solid Carcinoma bearing mice was used as *in vivo* experimental animal model for tumor because Ehrlich ascites carcinoma (EAC) cells, the most commonly used tumors cells, are highly undifferentiated and have a rapid growth rate that making them extremely sensitive to chemotherapy likewise human tumors (Ozaslan et al., 2011).

The results of physical monitoring revealed the potential efficacy of orally administered *A. herba alba* extract in the treatment of solid carcinoma, as manifested by the observed significant decreases in the body weight, tumor size and tumor weight during the experimental period as in the case of artesunate administration even reached their values in cisplatin treated group (positive control) (Table 2). Consequently, our results confirmed the previously demonstrated cytotoxic effects of *A. herba alba* extract in different human cancer cell lines (Lupidi et al., 2011; Khlifi et al., 2013; Tilaoui et al., 2015).

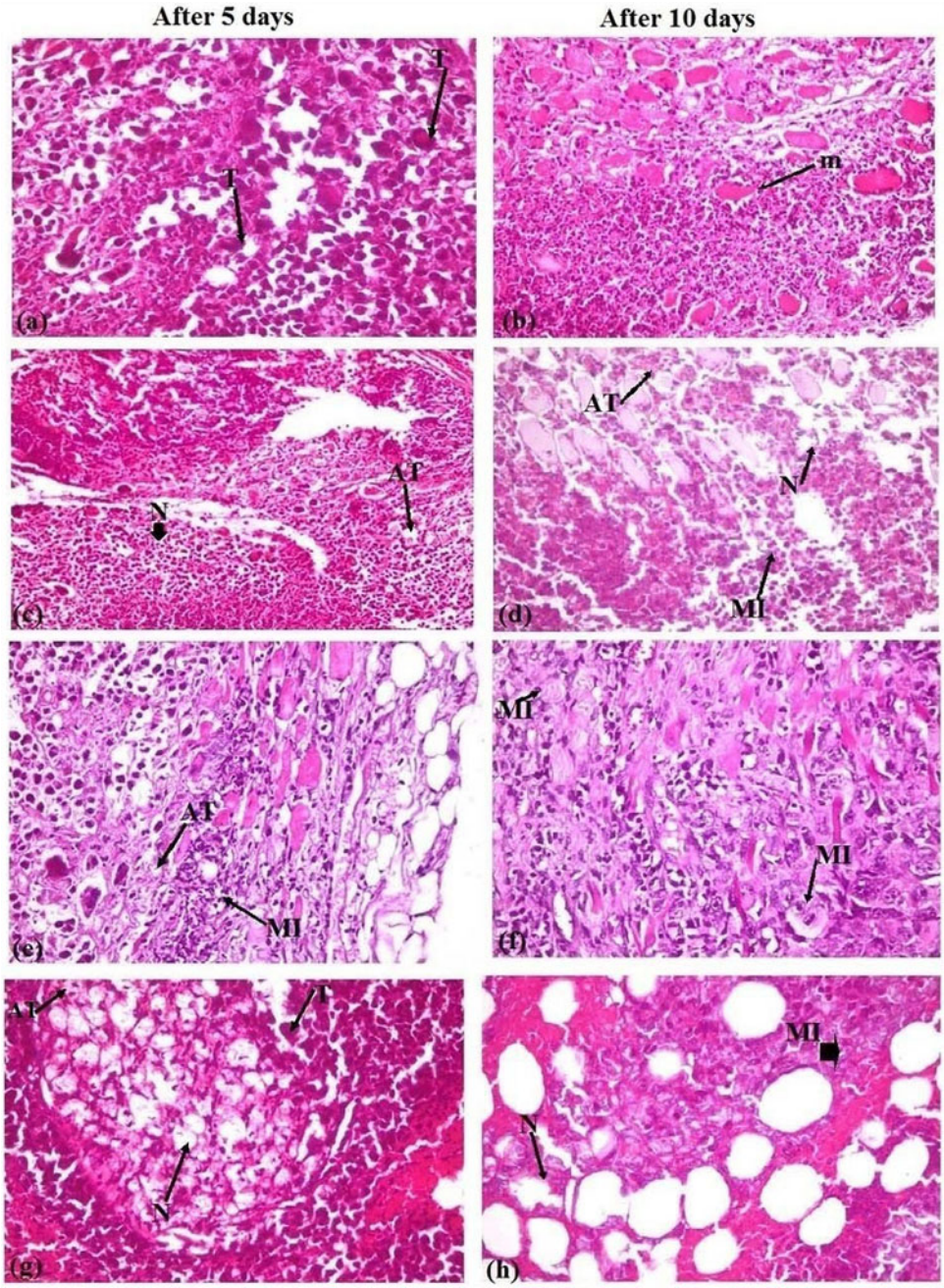

Fig. 3. Histopathological examination of solid tumors from negative control tumor bearing mice (a-b); cisplatin (5 mg/kg) treated mice (c-d); artesunate (300 mg/kg) treated mice (e-f) and *Artemisia herba-alba* (300 mg/kg) treated mice (g-h) sacrificed after 5 and 10 days of the first treatment (H&E X400). T: tumor; m: muscle; AT: apoptotic tumor cell; N: necrosis and MI: mononuclear cells infiltration.

Various mechanisms have been proposed for the antitumor efficacy of *A. herba alba* extract including reduced cell viability and induction of DNA damage and apoptosis (Lupidi et al., 2011; Khlifi et al., 2013). Our finding of significant increases in tail length, %DNA in tail and tail moment shown in Table 2 confirmed the damage of tumor cell DNA by *A. herba alba* extract administration even higher than those resulting from artesunate administration through the accumulative induction of single and double DNA stranded breaks during the experimental period as the alkaline comet assay detected both single and double stranded DNA breaks (Tice et al., 2000).

This accumulative induction of single and double stranded DNA breaks represent a stimulus for apoptotic DNA damage induction and thus triggered apoptosis of tumor cells (Lips and Kaina, 2001; Tounekti et al., 2001). Double stranded DNA breaks are one of the most serious and lethal DNA damage types as a single double stranded DNA breaks is sufficient to disturb cell genomic integrity or kill it (Jackson and Bartek, 2009) as well as these breaks act as signals for activation of the tumor suppressor p53 gene thereby mediate apoptosis (Bartkova et al., 2006; Di Micco et al., 2006). Therefore, the induction of apoptotic DNA damage in tumor tissue by *A. herba alba* extract was manifested by the smeared pattern of tumor genomic DNA on agarose gel and can be attributed to the induced concurrent significant elevations in the p53 expression levels and significant decreases in the expression level of proto-oncogene K-ras by oral administration of *A. herba alba* and artesunate even better those induced by cisplatin in consistence with the study of Lupidi et al. (2011).

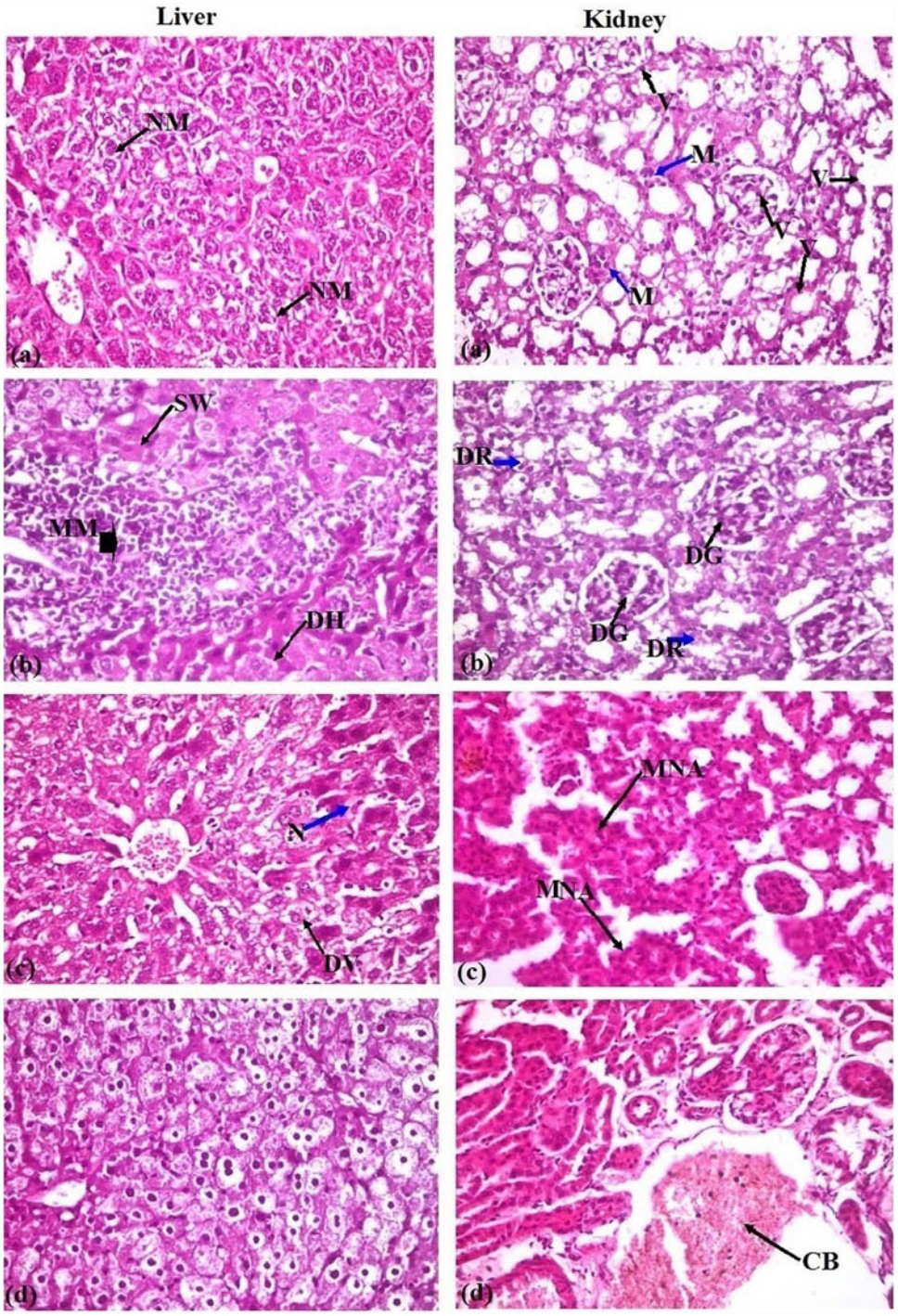

Fig. 4. Histological examination of liver and kidney tissues of negative control tumor bearing mice (a); cisplatin (5 mg/kg) treated mice (b); artesunate (300 mg/kg) treated mice (c) and *Artemisia herba-alba* (300 mg/kg) treated mice (d) sacrificed after 10 days of the first treatment (H&E X400). NM: Necrotic hepatocyte with evidence of metastasis; V: Vacuolation; M: Mestastasis; SW: Swollen hepatocyte; DH: Degenerated hepatocyte; MM: Massive mononuclear cells infiltration; DG: Degenerated glomerulus; DR: Degenerated renal epithelium; N: Necrosis; DV: Degenerated vacuolated hepatocyte; MNA: Massive necrosis and atrophy and CB: Congested blood vessel.

The demonstrated upregulation in the expression of p53 gene inhibited the expression of proto-oncogene K-ras gene as well as 1,8-cineole, an essential oil of *A. herba alba* has been shown to highly decrease the tumor promoter Transforming growth factor-beta (TGF-β1) thereby suppressed the proliferation of tumor cells (Moding et al., 2016; Abdallah et al., 2019).

Mutation screening using SSCP-PCR analysis revealed the absence of mutation in both p53 and K-ras genes thus the forementioned variations in the expression of both p53 and K-ras genes did not depend on sequence variation rather depended on signals such as the induced DNA strand breaks by oral administration of *Artemisia* extract or artesunate likewise cisplatin.

Apoptosis of tumor tissues was further confirmed by the appearance of apoptotic tumor cells during the experimental period in the tumor sections of mice administered *A. herba alba* or artesunate. Necrosis of tumor cells observed after 5 and 10 days of *A. herba alba* indicated the death of tumor cells since apoptotic bodies in the absence of phagocytes lose their integrity and undergo secondary

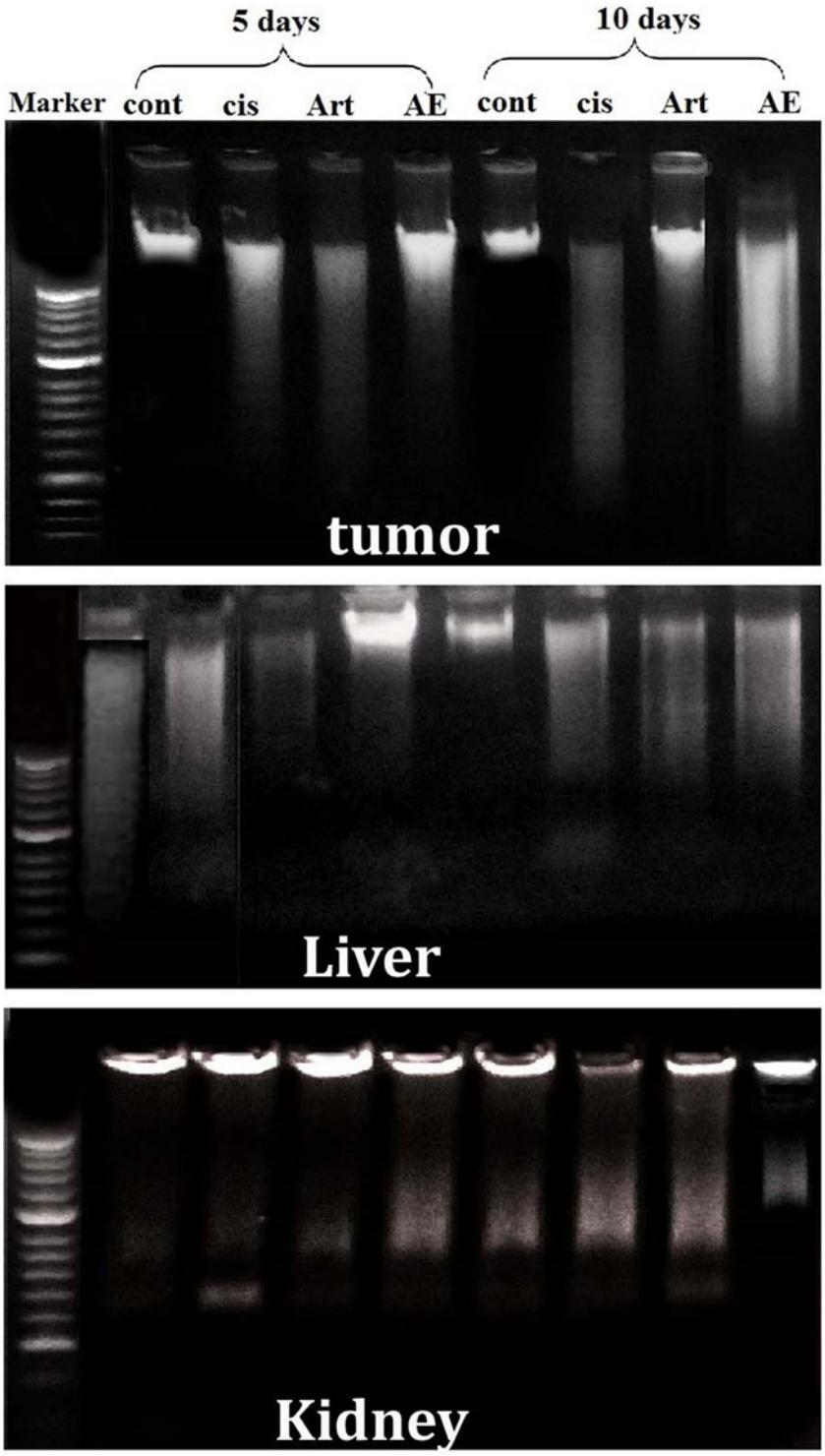

Fig. 5. Pattern of the genomic DNA extracted from the tumor, liver and kidney tissues at the two time intervals: 5 and 10 days on agarose gel (1%) stained with ethidium bromide. cont: negative control; Cis: Cisplatin: Art: artesunate and AE: *Aremisia* extract groups.

or apoptotic necrosis releasing inflammatory contents into the surrounding tissues (Schwartz and Bennett, 1995; Fink and Cookson, 2005).

The inflammatory mononuclear cells infiltrations seen in the tumor sections of mice administered *A. herba alba* or artesunate confirmed the previously reported inflammations and reactive oxygen species (ROS) generations by *Artemisia* crude extract and artesunate (Du et al., 2010; Lupidi et al., 2011). Increased the ROS production causes oxidative damage to cellular macromolecules including proteins, lipids and even DNA resulting in diverse kinds of oxidative damage *e.g.* single base and sugar phosphate damage, as well as strand breaks (Bjelland and Seeberg, 2003; Cadet et al., 2003; Dizdaroglu, 2003).

Regarding the safety of *A. herba alba* no evidence of metastasis was observed in the liver and kidney tissue sections of mice bearing tumor after 10 days of *Artemisia* extract first administration likewise artesunate and cisplatin as well as slight histological injuries including congested renal blood vessel and necrotic hepatocytes

only were observed in *Artemisia* group compared with the high histological injuries observed in artesunate and cisplatin group. These results are in consistence with previous studies that reported the safety of *A. herba abla* extract on normal cells *in vitro* (Lupidi et al., 2011; Khlifi et al., 2013).

Conclusion

Artemisia herba alba has a potential antitumor activity against induced solid tumors in mice by induction of single and double stranded DNA breaks that act as signals for p53 mediated apoptosis in tumor tissues likewise artesunate. However, *A. herba alba* has shown a greater safety to liver and kidney tissues compared with artesunate and cisplatin. Therefore, further studies are required to test possible uses of *A. herba alba* as anticancer drug.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

HM and A-EF designed, executed experimental work, performed data analyses and wrote the manuscript. HM revised the final manuscript.

Conflict of interest

All authors declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bjp.2019.06.007.

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