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Research article

Combined treatment with *Alhagi maurorum* and docetaxel inhibits breast cancer progression via targeting HIF-1α/VEGF mediated tumor angiogenesis in vivo

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ABSTRACT

Breast cancer is a challenging disease and leading cause of cancer death in women. There is no effective agent for metastatic breast cancer after surgery and chemotherapy. *Alhagi maurorum* (A.m) has been reported to exhibit an anticancer effect on various types of cancer cells in vitro. This study aimed to examine the suppressive effect of A.m alone and combined with docetaxel (DTX) on the breast cancer growth in mice models and the possible underlying mechanisms. In the present study, the mice were inoculated subcutaneously with the injections of 4T1 cells. Then, A.m, DTX, and their combination were administered intraperitoneally. The expressions of β-catenin (β-cat), FZD7, MMP2, HIF1-α, and VEGF A (vascular endothelial growth factor A) were investigated using RT-PCR method. Also, plasma alkaline phosphatase (ALP), alanine aminotransferase (GPT or ALT), aspartate transaminase (GOT or AST), serum creatinine, and urea were examined, and histological analyses of the tissues were conducted. The results demonstrated that A.m (500 mg/kg) combined with DTX significantly decreased the expression of β-cat, MMP2, and FZD7 as compared with the negative control group and monotherapies. Also, the mRNA levels of HIF1-α and VEGF A were suppressed significantly by DTX + A.m (500 mg/kg). Tumor weights and sizes were significantly lower and tumor inhibition rate was significantly higher in the DTX + A.m group. The A.m 500 mg/kg + DTX also suppressed the serum GPT level in tumor-bearing mice and decreased the serum urea level. Taken together, our findings suggest that DTX combined with A.m at an optimal dose of 500 mg/kg as the optimal dose can inhibit β-cat, FZD7, MMP2, and breast cancer growth via interrupting HIF-1α/VEGF signaling and might be used as a promising antiangiogenic agent for breast cancer treatment.

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1. Introduction

Breast cancer is the most common disease and the leading cause of cancer death in women worldwide due to the distant metastasis [1]. Identifying molecular signaling pathways involved in breast cancer and metastasis may provide new directions for cancer therapies. The growing evidence indicated that aberrant activation of Wnt/β-cat signaling is involved in cancer processes and different types of malignancies [2,3]. Wnt signaling initiates the β-cat signaling, the main molecule in the Wnt/β-cat signaling pathway. Frizzled (FZD) family receptors affect cell proliferation, migration, and differentiation [4]. After translocation to the nucleus, β-cat and activates the expression of Wnt target genes, such as VEGF [5], cyclin D1 [6], c-myc [7], and matrix metalloproteinases (MMPs) stimulating angiogenesis and tumor growth [8]. Moreover, the Wnt/β-cat signaling pathway has been found to be involved in the regulation of angiogenic factors such as VEGF. Tumor expansion gradually causes the formation of a hypoxic environment inside the tumor, stimulates hypoxia-inducible factors (HIFs), and regulates the expression of angiogenic genes. The aberrant activation of HIF-1α, a key indicator of hypoxia, is largely responsible for the overexpression of VEGF and subsequent angiogenesis [9].

The first-line treatment of metastatic breast cancer is DTX, a type of chemotherapy, i.e., a taxane found in the bark of the European yew tree [10]. It inhibits cell growth through interference with microtubule assembly and disassembly and induction of apoptosis in cells by altering the expression of the Bcl-2 family [11,12]. Treatment with DTX or other chemotherapy drugs is associated with severe side effects. Therefore, efforts are focused on finding new drugs with lower side effects, including natural products to prevent the progression of various tumor types [13]. Many plant extracts have been found to exert inhibitory effects on the growth and/or metastasis of breast cancer cells, such as curcumin, sauchinone, lycopene, denbinobin, genipin, capsaicin, and ursolic acid that act through various mechanisms [14].

Alhagi maurorum Boiss (A.m) known as camel thorn is native to the Mediterranean and Central Asia. Studies have revealed the medicinal properties of A.m in folk medicines to be used as a diaphoretic, diuretic, expectorant, and anti-ulcerogenic drug [15]. A.m has been reported to be rich in flavonoid and phenolic compounds [16]. One study showed the antitumor effect of A.m extracts on the inhibition of cell proliferation in human cancer cell lines i.e. lung large cell, renal cell adenocarcinoma, as well as some breast cancer cell lines evaluated in comparison with a normal cell line [17]. Some studies have investigated the inhibitory effect of A.m on different types of cancer, which are listed in Table 1 [18,19]. In our previous study, several medicinal plants were screened based on their background in inhibiting the growth of 4T1 breast cancer cells. Among them the one (A.m) with the best inhibitory concentration (IC50) in inhibiting the growth of 4T1 cells was then selected for molecular tests [20]. As no animal studies have been done in this field, in the present research we reported the inhibitory effect of A.m and A.m combined with DTX on breast cancer cells in vivo for the first time to show whether A.m and A.m + DTX prevent tumor growth, inhibit migration and invasion of breast cancer cells.

2. Material and method

2.1. A.m extract preparation

A.m was purchased from an authentic local store and confirmed by a botanist at the Medical Plants Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran. Ethanol (Merck, Hohenbrunn, Germany) was used for plant extraction. Hydro-alcoholic extract (ethanol 70%) was prepared and the maceration method was used for plant extraction. For this purpose, the dried powdered plant was soaked in a hydro-alcoholic solution for 48 h, and after passing through the filter paper, it was concentrated with a rotary machine and the solvent was removed. The concentrated extract was dried in a freeze dryer and the resulting powder was kept at 2–8 °C for future assays.

It should be noted that in this research we used the whole extract. In extraction by maceration method, almost all the main compounds are extracted. Researchers have previously isolated and reported the constituents of the extract of the A.m. Moreover, some researchers believe that Mother Nature has put all the compounds with the best effectiveness values together and with the best balance in a total crude extract.

2.2. Determination of median lethal dose (LD50)

The LD50 of the ethanolic extract of A.m was determined in mice by the Lorke method [21]. In a preliminary test, animals in three groups received 250, 500, or 1000 mg/kg of the A.m extract (in the normal saline), respectively. Animals were observed for 24 h for signs of toxicity and the number of deaths. The LD50 was calculated as the geometric mean of the dose that caused 100% mortality and no lethality at all [22].

Table 1
Antiproliferative activity (IC_{50} μg/ml) of A.m against some human tumor cell lines.

	Cell lines								
	MCF7	ACHN	C32	A375	LNCaP	HeLa	COR-L23	142BR	HL-60
A.m diethyl ether extract	114.7	44.6	2.7	41.1	43.9	66.9	59.9	>100	
A.m petroleum extract	105.7	48.2	62.3	45.8	99.8	40.1	47.1	>100	
A.m leaves									16
A.m flowers									22

2.3. Animals and experimental design

A total of 56 female BALB/c mice (6–8 weeks old, 18–22 g body weight) were purchased from the Pasteur Institute of Iran. All animals were kept in standard conditions with a 12-h dark–12-h light cycle at 25C°, feeding with a standard diet and water. The experimental protocol was approved by the Ethics Committee (Animal Care and Use Committee) of Shahrekord University of Medical Sciences guidelines (approval no. IR.SKUMS.REC.1397.5). All the mice were subcutaneously inoculated with 1×10^6 4T1 cells. When the tumors became palpable (after 10 days), the tumor-bearing mice were randomly divided into 7 groups (n = 8 per group): negative control group (injected with normal saline), DTX treatment group (5 mg/kg, i.p., on days 1, 7, 14), and 500 mg/kg A.m group (daily to 3 weeks. i.p), 1000 mg/kg A.m group (daily to 3 weeks, i.p), DTX (5 mg/kg, on days 1, 7, 14) + A.m (500 mg/kg), and DTX (5 mg/kg, on days 1, 7, 14) + A.m (1000 mg/kg) group. A normal group was also assigned, comprising mice not injected with 4T1 cells. Treatments were initiated 10 days after cancer cell inoculation and continued for 3 weeks. Following the termination of treatment, six mice from each group were euthanatized for hematology and blood chemistry tests, histology, and gene expression assays. The mice were screened for general health during the study period. The animal weights and tumor weights were measured using a laboratory scale (Kern, Germany, PFB 300-3, accuracy 0.001 g). When the evidence of complicacy or death was observed, the mice were killed and parts/organs required for subsequent experiments were stored. In fact, other organs were separated and saved to be examined if metastasis was seen.

2.4. Real-time PCR assay

In the real-time assay, apart from the tumor tissue, we also examined the liver tissue in order to examine the changes in gene expression in addition to the histology tests, especially if metastasis has occurred. For this purpose, the total RNA was extracted from the liver and tumor tissues using a total RNA extraction kit (Parstous). The cDNA was synthesized using an Easy cDNA Synthesis Kit (Parstous) according to the manufacturer's instructions. The prepared cDNA was then used for the quantitative PCR analysis using the g SYBR Green qPCR master mix (YEKTA TAJHIZ AZMA). The primers used were as follows: HIf -1α sense, 5′-CTGGATGCCGGTGGTCTA -3′ and antisense, 5′- ACCCCATGTATTTGTTCACGTT-3′; FZD7 sense, 5′-ATATCGCCTACAACCAGACCAT-3′ and antisense, 5′- AGGAACGGCACGGAGGAA-3′; VEGFa sense, 5′- GAGCAGAAGTCCCATGAAGTGA-3′ and antisense, 5′-CACAG-GACGGCTTGAAGATGT-3′ β-cat sense, 5′- ATGCGTTCTCCTCAGATGGTGTC-3′ and antisense, 5′- CAGAATCCACTGGTGAACCAAGC-3′ GAPDH sense, 5′- CAGCCTCGTCCCGTAGACAA -3′ and antisense, 5′- GCCGTGAGTGGAGTCATACTG-3′.

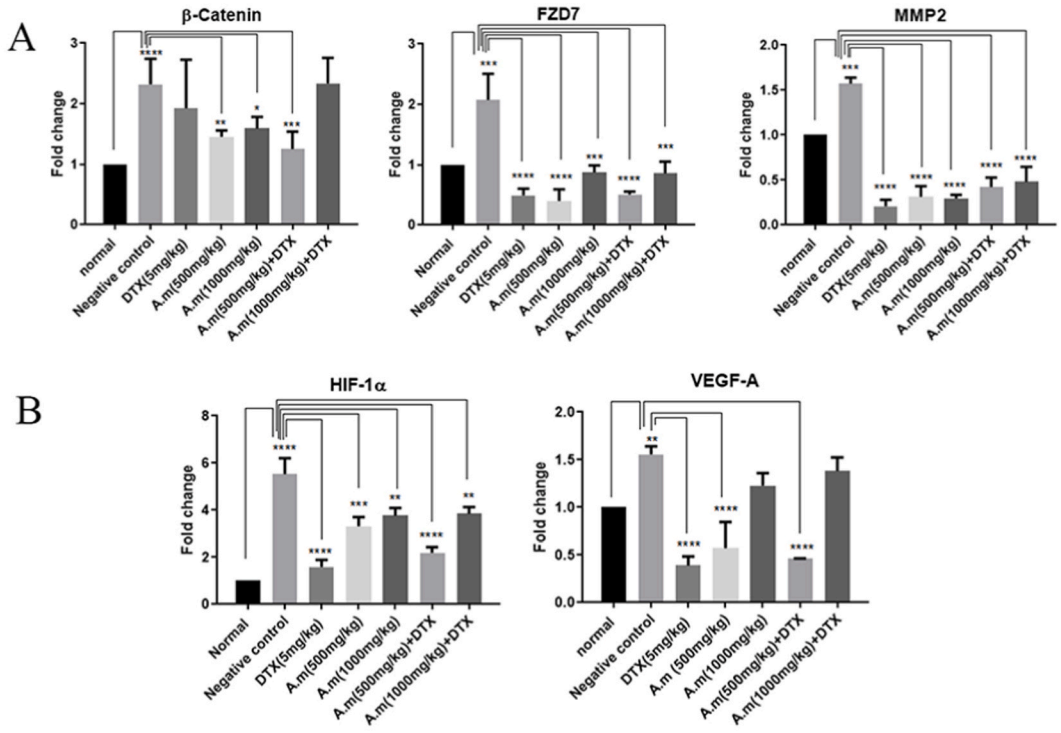

Fig. 1. Effect of DTX and/or A.m on the gene expression involved in Wnt signaling path. Quantitative real-time PCR was used to assay the effects of treatment with A.m and DTX, used as a single drug or in combination, on (A): β-cat and FZD7 and MMP2 expression and (B): HIF1-α and VEGFA expression in tumor tissue in 4T1 mouse breast cancer model. Data represent the mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

2.5. Assessment of serum biochemical parameters

At the end of the experiment, all mice were sacrificed, blood samples were collected in centrifuge tubes, allowed to clot, and then centrifuged at 3000 rpm for 20 min to separate the sera, which were preserved at −20 °C until used for biochemical assays. The stored sera were used to evaluate liver function tests: Plasma alkaline phosphatase (ALP), alanine aminotransferase (GPT or ALT), aspartate transaminase (GOT or AST), serum creatinine, serum urea, and serum uric acid using BT2000 system.

2.6. Histological assay

Following blood collection, the mice were sacrificed with ketamine (80 mg/kg IP) -xylazine (10 mg/kg IP). Tumors and organs, including lungs, kidneys, spleen, and liver, were removed and fixed in 10% formaldehyde and then embedded in paraffin. These organs were dissected and stored to be histologically examined to see if metastasis had occurred and to be further investigated if so. The 3–5-mm-thick sections were cut from the paraffin block and stained with Hematoxylin and Eosin (H&E). With the assistance of a pathologist, each slide was examined using a light microscope. At least 3 fields from each slide of each group were examined to evaluate the histological changes.

2.7. Statistical analysis

All experiments were performed in triplicate. The results were expressed as Mean ± Standard deviation (SD). Two-way Analysis of Variance (ANOVA) was used to analyze significant differences. The significance level was defined as $P < 0.05$.

3. Results

3.1. Effect of DTX and A.m on FZD7/β-cat signaling and MMP2 expression in tumor

We investigated the effect of DTX and A.m on the expression of FZD7/β-cat signaling, a major factor in breast cancer [23], and on MMP2 signaling, which plays a critical role in the progression of the disease [24]. The expression levels of β-cat and FZD7 genes in tumors were determined by the real-time PCR assay. As shown in Fig. 1, β-cat expression in mice was significantly inhibited in 500 mg/kg A.m ($p < 0.01$), and 500 mg/kg A.m + DTX ($p < 0.001$) groups. DTX + A.m (500 mg/kg) was more effective. Moreover, A.m

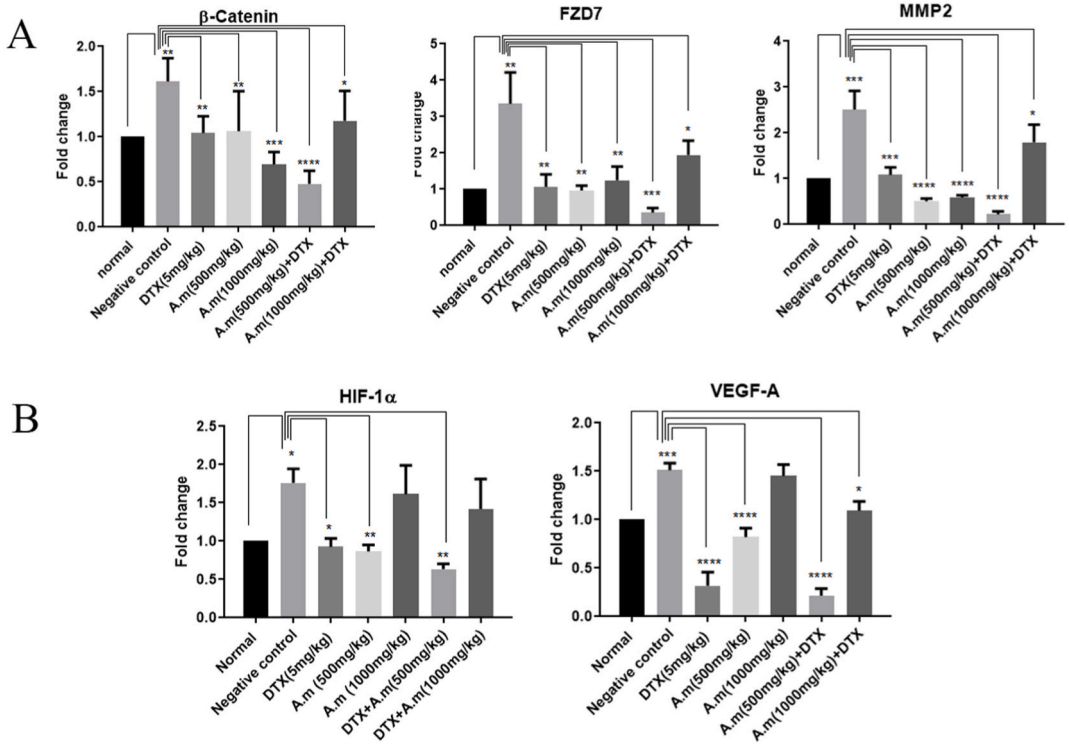

Fig. 2. Effect of DTX and/or A.m on the gene expression involved in Wnt signaling path. Quantitative real-time (PCR) assay showing the effects of A.m and DTX, used as a single drug or in combination, on (A): β-cat and FZD7 and MMP2 expression and (B): HIF1-α and VEGFA expression in liver tissue in 4T1 mouse breast cancer model. Data represent the mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

decreased the FZD7 expression compared to the negative control group, at dosages of 500 mg/kg ($p < 0.0001$), and 1000 mg/kg ($p < 0.001$) alone, as well as combined with DTX, respectively ($p < 0.0001$ and $p < 0.001$). The expression of MMP-2 was detected by RT-PCR in our study. As depicted in Fig. 1 MMP-2 was down-regulated in DTX, A.m 500 and 1000 mg/kg, DTX + A.m 500 mg/kg and DTX + A.m 1000 mg/kg groups in tumor tissue ($p < 0.0001$) (Fig. 1 A).

3.2. Effect of DTX and A.m on HIF1-α/VEGF A signaling in tumor

To find out the possible mechanism of A.m in the inhibition of angiogenesis we investigated VEGF and HIF-1α expression. A.m could modulate the level of HIF-1α and VEGF A expression. The HIF-1α and VEGF are commonly overexpressed in some kinds of cancers, including prostate, breast, and ovarian cancer cells [25]. DTX and A.m (500, and 1000 mg/kg) inhibited the hypoxia-induced HIF-1α/VEGF signaling axis. HIF-1α protein is oxygen-sensitive and gets degraded under normoxic conditions. Fig. 1B shows that the expression level of HIF1-α ($p < 0.0001$) and VEGF A ($p < 0.01$), increased in the negative control group in tumor tissue, treatment with DTX ($p < 0.0001$), A.m 500 mg/kg ($p < 0.001$), and A.m 1000 mg/kg ($p < 0.01$), alone and combined with DTX ($p < 0.0001$, $p < 0.01$), down-regulated the HIF-1α level in tumor compared to the control group, and DTX was more effective ($p < 0.0001$). DTX + A.m 1000 mg/kg and A.m 1000 mg/kg had no significant effect on the expression of VEGF A in tumor, but other treatment groups, DTX ($p < 0.0001$), A.m 500 mg/kg ($p < 0.0001$), and A.m 500 mg/kg + DTX ($p < 0.0001$) significantly down-regulated the level of VEGF A.

3.3. Effects of DTX and A.M on the expression of b-cat, FZD7, and MMP2 genes in liver

The ability of combined treatment with DTX and A.m on β-cat/FZD7 and MMP2 in liver tissue was assessed by RT-PCR in mice. RT-PCR analysis (Fig. 2) showed a significant down-regulation in the expression of β-cat by DTX ($p < 0.01$), A.m 500 mg/kg ($p < 0.01$), A.m 1000 mg/kg ($p < 0.001$), DTX + A.m 500 mg/kg ($p < 0.0001$) and DTX + A.m 1000 mg/kg ($p < 0.05$) groups in liver tissue compared with the negative control group. Significant reductions in the expression of FZD7 in DTX, A.m 500 mg/kg, and A.m 1000 mg/kg ($p < 0.01$), also DTX + A.m 500 mg/kg ($p < 0.001$) and DTX + A.m 1000 mg/kg ($p < 0.05$) were observed. The expression of MMP2 in all groups was observed (Fig. 2A).

3.4. Effect of DTX and A.m on the expression of HIF1-α and VEGF A genes in liver

The results showed that DTX, A.m 500 mg/kg, and DTX + A.m 500 mg/kg significantly decreased the expression of HIF1-α and VEGF A compared to the negative control group, and DTX + A.m 500 mg/kg was more effective. However, A.m 1000 mg/kg had no significant effect on the HIF1– α and VEGF A expression, but DTX + A.m 1000 mg/kg significantly decreased the level of VEGF A in liver tissue compared to the negative control group ($p < 0.05$) (Fig. 2B).

3.5. Effect of DTX and A.m on liver function markers

The serum ALP showed no significant effect among groups. The GPT and GOT were found to be significantly higher in negative control mice when compared to the normal animals. The elevated activities of the serum GPT ($p < 0.0001$) and GOT ($p < 0.001$) observed in the negative control mice may be due to tumor-induced hepatic damage and the subsequent leakage of these enzymes into the circulation. Administration of A.m 500 mg/kg ($p < 0.01$), A.m 1000 mg/kg ($p < 0.05$), and combined treatment with DTX and A.m 500 mg/kg ($p < 0.0001$) caused a significant reduction in the serum level of GPT as compared to the negative control group but no significant effect was found on the ALP and GOT level. In addition, the results showed that DTX had no effect on the ALP, GPT, and GOT in all groups (Fig. 3). The data in Fig. 4 showed that mice with breast cancer had a significant increase in the serum levels of urea compared to the normal group ($p < 0.0001$). DTX, A.m 500 mg/kg ($p < 0.001$), A.m 1000 mg/kg, and DTX + A.m 500 mg/kg ($p < 0.0001$) significantly reduced total urea when compared to the negative control group, although they showed no significant effect on the serum creatinine levels among groups (Fig. 4).

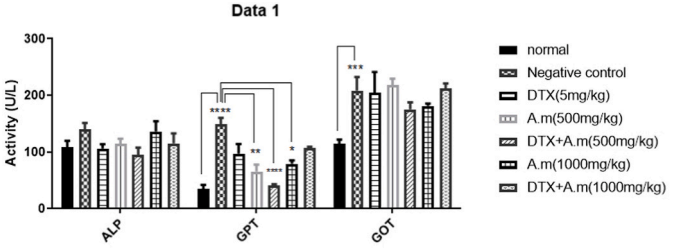

Fig. 3. Effect of DTX and/or A.m on serum liver enzymes in 4T1 mouse breast cancer model. The results of DTX and/or A.m (500, 1000 mg/kg) didn't show the effect on the ALP and GOT level in serum, but decreased the level of GPT in serum. The DTX + A.m 500 mg/kg was more effective. Data represent the mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3.6. Measurement of body weight and tumor weight

At the end of the experiment, the animals were weighted and tumors were excised from each animal for the examination of tumor weight and assessment of the antitumor effect of DTX and A.m. The body weight of the control group animals (normal and without any treatment) remained the same throughout the study period (Fig. 5); however, a significant body weight loss was found in the negative control group. There was a weight loss recorded after treatment with A.m and DTX. After DTX, A.m 500 mg/kg, and DTX + A.m500 mg/kg administration, the body weight started to increase at day 15 suggesting that DTX and A.m treatment ameliorated the damage to overall body metabolism. Fig. 6 shows the tumor weight that was not the same for all animals. The tumor weight decreased significantly compared to the negative control group after treatment with A.m 500 mg/kg ($p < 0.05$) and A.m 500 mg/kg + DTX ($p < 0.01$). However, the injection of DTX, DTX + A.m 1000 mg/kg, and A.m 1000 mg/kg had no significant effect on the tumor weight. For the group treated with A.m 500 mg/kg + DTX, there was a more significant decrease in tumor weight after treatment compared to the negative control group ($p < 0.01$). Photographs of tumors are presented in Fig. 7 for viewing and comparison. There was no significant difference in the spleen or kidney weight between the treated groups and the negative control group, even the histological examination showed no obvious lesion. The effect of A.m and DTX on the weight of mice, tumors, spleens and livers are listed in Table 2.

3.7. Histopathological examination results

The effects of DTX and A.m on the lung, kidney, spleen, and liver metastasis in mice were also investigated. The examination of liver, spleen, lung, and kidney H&E sections revealed normal morphological features in the normal group (Fig. 8). Concordantly, the histological results showed no metastasis in any of the groups. Also, microcytic hypochromic cells with mitotic divisions were seen in the breasts of the negative control group and treated groups (Fig. 8). Treatment with DTX and A.m and combined treatment reduced the number of tumor cells. In more analysis, hemorrhage was found in the liver and lung in all groups compared to the control group. Treatment with DTX and A.m decreased hemorrhage in tissue and combined treatments were found to be more effective.

4. Discussion

This study was conducted to compare the anti-tumor effects of A.m extract alone and in combination with DTX on the 4T1-induced breast cancer mouse model cell-line for the first time. Chemotherapy with DTX is the first-line therapy among cancer patients but induces toxicity and resistance in cancer cells, and treating with a single drug is rarely effective [13]. Combination therapy is now considered to be a treatment for many types of cancers, based on agents targeting cell signaling pathways that interact with the cell cycle, proliferation, or angiogenesis. Previous studies have reported that A.m possesses anti-inflammatory, antiproliferation, and antitumor properties on different cancer cell lines [18,26]. Moreover, the results of our previous study showed that combined treatment with DTX + A.m possesses synergistic antitumor effects on 4T1 cells [20]. In the present study, we focused on the combined efficacy of A.m and DTX in vivo.

The results of this study confirmed that co-treatment of mice with DTX and A.m 500 mg/kg reduced the tumor weight compared to a single treatment, thus reducing the toxicity and side effects of DTX. Also, our results showed that tumor weight was significantly affected by A.m 500 mg/kg and DTX + A.m 500 mg/kg. Though the tumor weight in A.m 1000 mg/kg + DTX group was lower than that in the negative control group, this difference was not significant. Additionally, the mice weight loss was more noticeable in the DTX + A.m 1000 mg/kg group than that in other groups, but the weight loss of mice in the combined DTX + A.m 500 mg/kg group was near to the DTX and A.m 500 groups suggesting that A.m 500 mg/kg in combination with DTX was better than the dose of 1000 mg/kg. In fact, it is possible that the dose of A.m 1000 mg/kg alone or in combination with DTX caused a greater weight loss in mice due to the

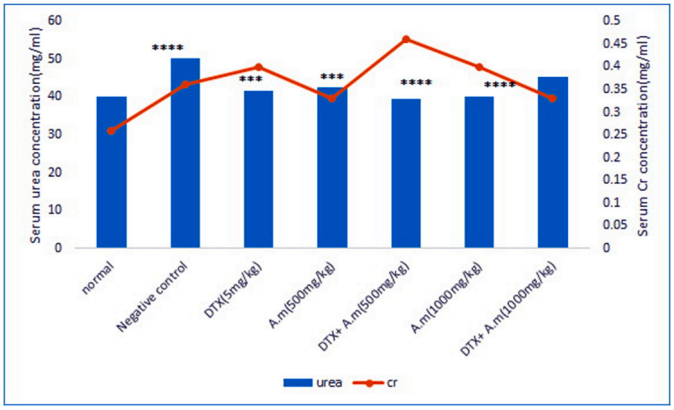

Fig. 4. Effect of DTX and/or A.m on serum urea and creatinine in 4T1 mouse breast cancer model. The results showed that treatment with DTX, A.m (500, 1000 mg/kg), and combined DTX with A.m 500 mg/kg decreased the serum urea level significantly ($p < 0.0001$), but showed no significant effect on the serum creatinine level. Data represent the mean ± SD, ****$p < 0.0001$, ***$p < 0.001$.

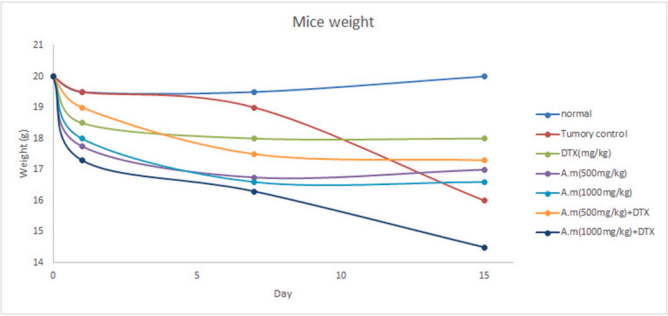

Fig. 5. Effect of DTX and/or A.m on the body weight of mice in 4T1 mouse breast cancer model. Graph shows the body weight from groups, DTX, A.m (500, 1000 mg/kg), and DTX + A.m (500, 1000 mg/kg). DTX was more effective compared to the ones in other groups. Data represent the mean ± SD.

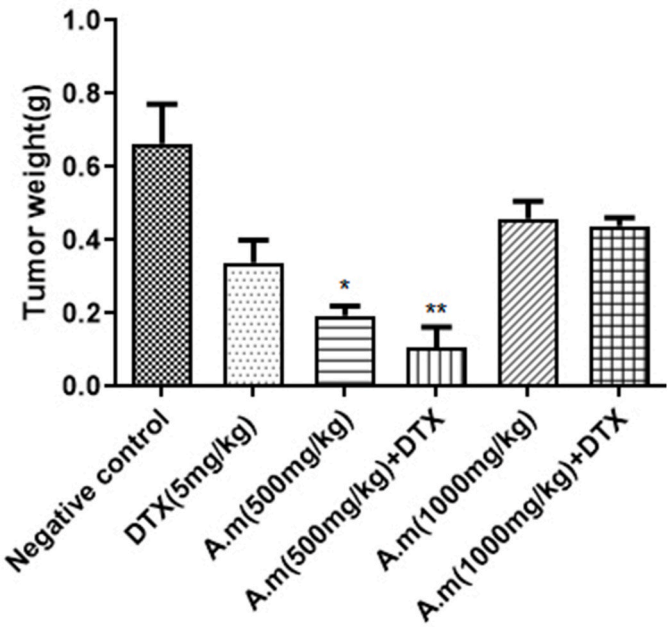

Fig. 6. Effect of DTX and/or A.m on tumor weight tissue in 4T1 mouse breast cancer model. The graph shows tumor weight from groups DTX, A.m (500, 1000 mg/kg), and DTX + A.m (500, 1000 mg/kg). DTX + A.m 500 mg/kg was more effective than other groups. Data represent the mean ± SD. *$p < 0.05$, **$p < 0.01$.

toxicity of the higher dose and did not have much effect in reducing the tumor size.

We also examined the effect of combined treatment with DTX and A.m on the Wnt/β-cat and FZD7 pathway. Experimental evidence suggests that A.m extract exerts multiple different suppressive effects on Wnt/β-cat pathway components. Activation of the Wnt/β-cat pathway plays an essential role in cell proliferation, differentiation, invasion, and metastasis in many types of human cancers [27,28]. Canonical Wnt/β-catenin signaling cascade was reported to be triggered through the interaction of Wnt ligands with the seven-pass transmembrane Frizzled (FZD) receptor and the lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptor. This leads to the activation of disheveled (Dsh) and translocation of cytosolic β-cat into the nucleus, resulting in controlling target oncogenes that are involved in cancer cell proliferation, tumor invasion and metastasis, such as VEGF and MMPs [29,30]. In this research we showed that β-cat and FZD7 expression were down-regulated in tumor and liver tissues by A.m and DTX when mice were treated either alone or in combination mode. The results showed that DTX combined with a lower dose of A.m (500 mg/kg) was more effective. Among MMPs, a component in the Wnt/β-cat pathway that may be affected, we focused on MMP-2 because several groups have shown that MMP-2 is the major extracellular matrix degradation enzyme that is highly expressed in breast cancer and its increased expression is associated with tumor aggressiveness, metastasis, and poor prognosis [31]. The results indicated that combined treatment with DTX and A.m 500 mg/kg significantly reduced MMP2 expression in liver tissue rather than in DTX group alone. Currently, there is no reported study regarding the molecular mechanisms of the action of A.m on cancer. Considering the factors playing pivotal roles in the metastasis of breast cancer and angiogenesis, this study indicates for the first time that A.m at a low dose of 500 mg/kg (lower dose) can

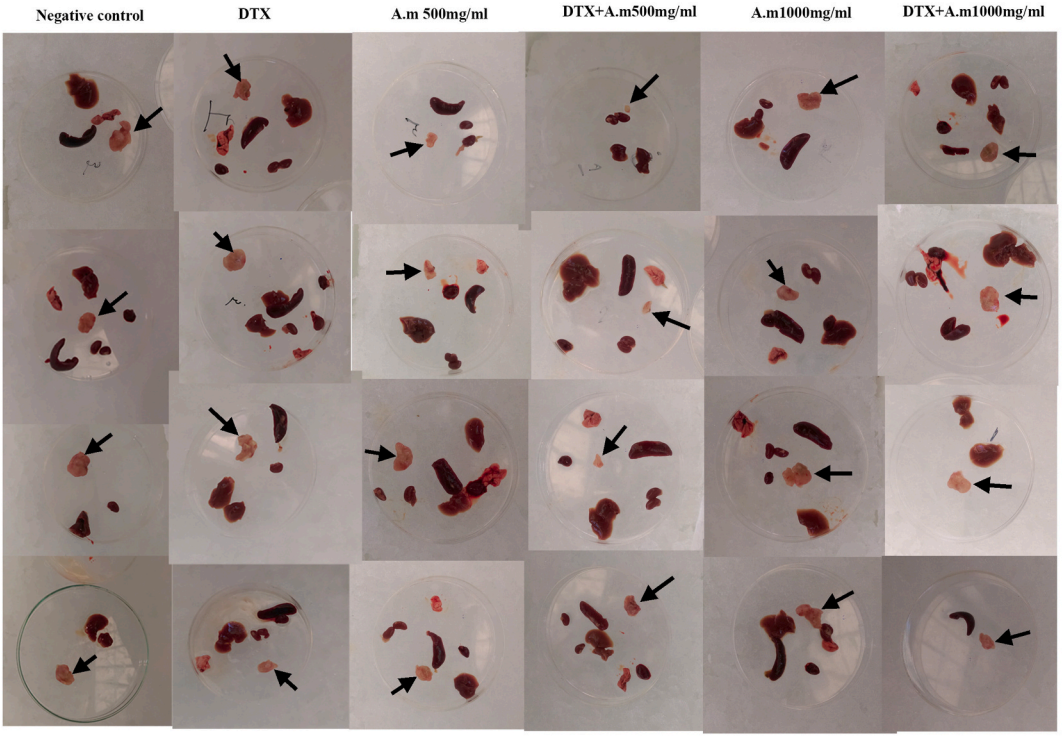

Fig. 7. In this figure, tumors removed from the body of the mice can be seen and compared. After sacrificing the mice, the tumors were removed and their sizes were measured with a caliper. As shown in the figure, the size of the tumors in the DTX + A.m 500 mg/kg group showed a significant reduction.

Table 2
Effect of DTX and/or A.m on the tumor weight, bodyweight, spleen, and kidney weight.

Groups	Mice weight (day 1) (gr)	Mice weight (day 7) (gr)	Mice weight (day 15) (gr)	Tumor weight (gr)	Tumor size (cm)	Spleen weight (gr)	Kidney weight
Normal	20	19.5	20	0	0	0.115	0.207
Negative control	20	19	16	0.578	1.7*0.89	0.674	0.265
DTX	20	18	18	0.408	1.02*1.5	0.469	0.228
A.m(500 mg/kg)	20	16.75	17	0.290	0.87*0.85	0.331	0.188
DTX + A.m(500 mg/kg)	20	16.6	17.3	0.104	0.63*0.55	0.293	0.198
A.m(1000 mg/kg)	20	17.5	16.6	0.459	1.41*0.92	0.377	0.212
DTX + A.m(1000 mg/kg)	20	16.3	14.5	0.459	1.21*0.92	0.312	0.207

down-regulate MMP2, β-cat, FZD7 (Figs. 1A & 2A), HIF1-α, and VEGF-A expression (Figs. 1B and 2B) as compared with the negative control group, the factors that play pivotal roles in the metastasis of breast cancer and angiogenesis. Indeed, we found the suppression of the 4T1 cell invasion by A.m and DTX to be correlated with the inhibition of MMP-2 activity and FZD7.

In this study, we also investigated the A.m effect on the angiogenesis factors to better understand the mechanism by which A.m inhibits tumor progression and angiogenesis. We showed the effect of A.m on the expression of VEGF, one of the most important factors in angiogenesis, and found that A.m 500 mg/kg and DTX + A.m 500 mg/kg could significantly reduce VEGF A expression in tumor and liver tissues, but A.m 1000 mg/kg had no significant effect, although DTX + A.m1000 mg/kg significantly reduced the expression of VEGF A in liver. Previous studies have shown that HIF-1α, as the main factor in low oxygen conditions regulates the activation of angiogenesis and triggers gene expression, such as VEGF [32,33]. Therefore, HIF-1α itself is an upstream factor that activates the β-Cat pathway contributing to the activation of the HIF-1α/VEGF axis in angiogenesis [34]. Consequently, we suggest that the modulation of HIF1-α/VEGF signaling may be one of the mechanisms implicated in the suppression of cell proliferation in breast cancer by A.m extract. Our present findings showed that A.m 500 mg/kg and DTX alone significantly inhibited the HIF1-α and VEGF A expression, and the combined effect of A.m 500 mg/kg and DTX on the expression of HIF-1α was superior to that of the DTX in liver (Figs. 1B and 2B).

To confirm these results, we performed histopathology of tissues. Pathological data also demonstrated that the lung, spleen, and

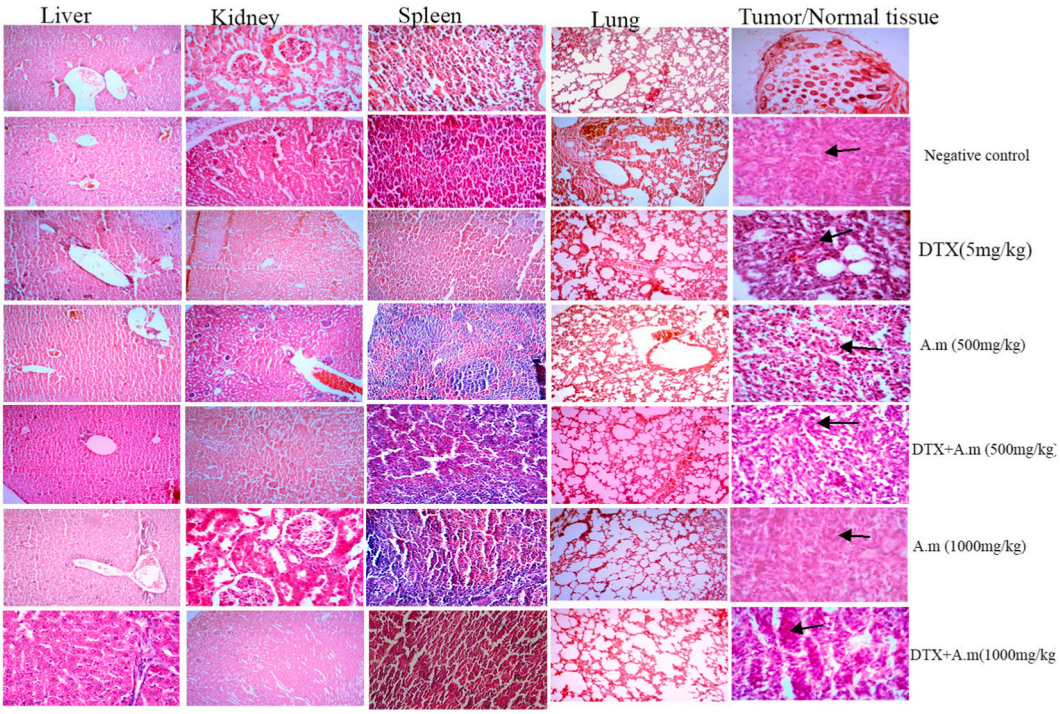

Fig. 8. Representative sections of tumor, kidney, spleen, lung, and liver tissues from mice untreated and treated with different doses of A.m and/or DTX following H&E staining as observed under a light microscope (200 ×). The pathology results showed that tumor cells were observed in breast tissues in all groups compared with the normal group. Treatment with DTX, A.m (500 and 1000 mg/kg), and combined treatment decreases the number of tumor cells in tumors. No metastasis was seen in lung, kidney, liver, and spleen tissues.

liver were not significantly affected by the metastasis of tumor cells in all groups. Interestingly, renal damage was alleviated when DTX was combined with A.m. We further tested the renal function by measuring serum creatinine and urea level. There was a significant difference in urea serum in the negative control group indicating toxicity to the kidney that was reduced when treated with A.m and DTX. Reinforcing these data, the renal histopathological findings revealed abnormality of the kidneys. Based on both biochemical analysis and histopathological examination, A.m and DTX affect kidney function. In agreement with our results, previous studies have shown that some plants have hepatoprotective properties against the side effects of chemical drugs [35]. Treatment with different doses of A.m and DTX did not change the plasma level of ALP and GOT liver enzymes. A.m 500 mg/kg combined with DTX significantly reduced the GPT level in serum compared with the negative control group. Nevertheless, the liver of mice treated with A.m and DTX exhibited areas with less hemorrhage. It is suggested that the observable adverse effects of A.m in this study are associated with a dose of 500 mg/kg. These results could be in line with other findings in RT-PCR, which showed that in mice treated with DTX + A.m 500 mg/kg the expression of angiogenic and progression genes was down-regulated compared to those treated with DTX and A.m alone.

In conclusion, our results demonstrated that DTX combined with A.m elicits an additive anticancer effect in the breast cancer model via the suppression of Wnt/β-cat and HIF1-α/VEGF pathway. We also found the inhibitory effects of DTX and A.m on FZD7 and MMP2 were stronger than those on β-cat and VEGF. The present study suggests that DTX combined with A.m 500 mg/kg is a promising novel anticancer treatment strategy for breast cancer.

Author contribution statement

Nayereh Bahamin: Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Mahmoud Rafieian-Kopaei: Shahin Ahmadian: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data. Iraj Karimi: Gholamreza Mobini: Analyzed and interpreted the data. Amir Hossein Doustimotlagh: Wrote the paper. Elham Bijad: Mahshid Shafiezadeh: Contributed reagents, materials, analysis tools or data.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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