Cancer is one of the causes of death in many high-income countries. Among all kinds, Breast cancer is a common and fatal disease and is by far the most prevalent cancer diagnosed in women, 25.2% of all new cases in women[1]. Despite the remarkable efforts and advances in treatment, still significant number of patients dying from breast cancer. This indicates that novel and developed therapies are needed[2]. one of these new and attractive therapies is herbal medicine[3].

Artemisinin (also known as arteannuin or as qinghaosu in Chinese) is a sesquiterpenoid lactone agent, extracted from sweet wormwood (Artemisia annua) plant which has a history of more than 2000 years in Chinese traditional medicine and is also well known for the treatment of malaria[4]. The structure of Artemisinin was understood in 1977 and then modifications were done to improve its solubility in oil or water[5]. Over the past two decades, numerous studies have identified antitumor activities of malaria drugs. Nearly all these studies focused on Artemisinin derivatives. Artemisinin and its two
widely used derivatives, Artemether and Artesunate, beyond their significant anti-malarial activity also show interesting anti-cancer properties such as induction of apoptosis, inhibition of tumor growth, metastasis and angiogenesis[6]. Although the exact mechanism of action of Artemisinin is not completely comprehend but both antimalarial and anti-cancer activities of Artemisinin derivatives are assumed to be linked to iron-induced activation of their endoperoxide group and generation of toxic radical species in the cells[7]. As cancer cells are highly replicative, they have more transferrin receptors compared to normal cells and thus higher iron uptake and they become more sensitive to cytotoxic effects of Artemisinins[6]. Therefore Artemisinin is good candidates in cancer treatment because they have high potency and specificity in killing cancer cells and not normal cells.

Artesunate is a water-soluble semi-synthetic derivative of Artemisinin, and its cytotoxic effect was tested on 70 cell lines from different tumor types[8], while Artemether is a lipid-soluble methyl ether of Artemisinin that can shift the overall immune response towards the Th1 pattern[9]. So Artemisinin, Artesunate and Artemether, each have specific anti-tumor and pharmacokinetic properties; therefore we assumed that utilization these drugs as a combination may have synergistic effects in cancer treatment and increases their anti-tumor activities.

The rationale for using drugs in combination is well established in the treatment of tuberculosis[10] and infection with human immunodeficiency virus[11]. In malaria using Artemisinin based combination the rapiest effect rapid and sustained parasitological cure in patients with Plasmodium falciparum malaria[12]. Combination of agents in treatment of cancer have been used since the 1960, when Greenspan published his work describing the potential of drug combinations to increase cell kill and possibly improve response in breast cancer patients. More research also indicates that combination therapy in breast cancer offers a survival advantage[13].

**Materials and methods**

**Chemicals and Reagents**

Artemisinin, Artesunate, Artemether were obtained from Exim-Pharm International Co, India. And were dissolved in Dimethylsulphoxide (DMSO) (Merck company, Darmstadt, Germany) and Polysorbate 80 (Tween 80) (Merck company, Hohenbrunn, Germany) and stored in −20°C and further diluted in PBS for administration. Cyclophosphamide (endoxan®) was purchased from Baxter Oncology GmbH Co. (India) and was diluted in PBS for administration. 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

**In Vitro Studies**

**Cell Culture**

The mouse breast cancer cell line 4T1 was purchased from Pasteur Institute of Iran. Cells were cultured in RPMI 1640 medium (Gibco, UK) containing 2mM L-glutamine, 10% heat-Inactivated FBS (Gibco, UK), 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Gibco, USA) and maintained at 37 in a humidified atmosphere of 5% CO2. Medium was changed every two days and All experiments were performed with cells in the logarithmic growth phase.

**Isolation of Peripheral Blood Mononuclear Cell (PBMC)**

PBMCs were obtained from human whole blood using Ficoll density gradient method. 15ml of human blood was diluted with equal amount of cold PBS. The diluted blood was carefully added over 10 ml of Ficoll (Baharafshan, Iran) in a 50ml conical tube. The tube was centrifuged in 350 g at 22°C for 20 min (without brake). The layer between plasma and ficoll was collected and washed with 5 mL PBS and centrifuged in 350g at 4°C for 10 min.

**Preparing Drugs**

Artemisinin, Artesunate, Artemether and cocktail form of drugs were dissolved in lowest amount of DMSO and tween 80 to prepare a stock solution. The stock solution was filtered through a 0.22 μm micro pore filter and stored at 4°C. The stock was diluted with PBS to prepare other doses. The maximal dilution of DMSO in the wells of the plate did not exceed .45%. Drugs were freshly prepared for each test or administration.

**MTT Cytotoxicity Assay**

The cytotoxic effect of our drugs was determined by MTT (Merck, Germany) assay. To perform this test 1 × 104 4T1 cells were seeded into each well of a 96 well-plate in 200 μl of RPMI 1640 medium, after a
24-h incubation the culture medium was removed and 200 µl of fresh medium was added to each well, and then the cells were treated with 20 µl of indicated concentrations of ART, ARTs, ARTm and the cocktail of these drugs and incubated for 24 and 48 h in 37°C (the final concentration of drugs in the wells was between 15 to 135 µg). For control cells, equal volumes of DMSO and tween 80 were added. After 24 and 48 h the medium was removed and 200 µl of fresh medium and 20 µl of MTT solution (5 mg/ml in PBS) was added to each well. After 4 h of incubation in 37°C, the formazan crystals were dissolved in 100 µl of DMSO and the absorbance was measured at background wavelength of 540 nm using a micro plate reader (Lab Systems Multiskan). Three independent experiments were done in triplicate. The same process was performed on human PBMCs at a density of 1×105 cell per well.

The viability of cells was presented as the percentage of control as follows:

Viability(%): ODsamples+ (ODControl – ODDMSO) / ODcontrol×100

**In Vivo Studies**

**Animal Experiments**

Six-to-eight-week-old female BALB/c mice with weight of 16-20 g were obtained from Pasteur Institute of Iran. The animals were housed and fed for one week in a specific pathogen-free conditions; Animal care and treatment were conducted in conformity with the guideline of Animal Care and Research Committee of Tarbi at Modaress University.

**Plan of Study**

The Tumors were established by subcutaneous injection of 8 × 105 4T1 cells, suspended in 100 µl of Phosphate-buffered saline (PBS), into the posterior flank region of each mice(day 0). The mice were inspected for tumor formation every two days. After 10 days, the tumor size was recorded and the treatment was started. The length (L) and width (W) of the tumors were measured daily by a single person using digital Caliper. The volume (V) of each tumor was estimated according to the formula:

Volume =[(π×L×W)^2/6).

When the average size of tumor achieved 90 mm3, 30 tumor-bearing mice were randomized into six groups, each consisting of five mice. Treating mice with drugs started and continue for 12 days after the first drug administration,

The first group was treated with 20mg/100 µl of Artemisinin daily.

The second group was treated 20mg/ 100 µl of Artesunate daily.

The third group was treated 20mg/ 100 µl of Artemether daily.

The 4th group was treated with the combination of the drugs at the same dose.

The 5th group was treated with Cyclophosphamide as positive control.

The last group received the drugs solvent.

**Antigen preparation**

Tumor at the size approximately 3000 mm3 was extracted from a breast cancer-bearing BALB/c mouse. The tumor tissue was cut into small pieces in PBS and passed through a 150μm Mesh filter. The suspension was then underwent the freeze-thaw process for five times. To inactivate serine proteases, 1 mM of phenyl methyl sulfonylfluoride (PMSF) (Gibco, USA) was added to the cell lysate. The cell lysate was centrifuged in 3000 g for 15 min at 4°C and the supernatant was dialyzed against 1 L of PBS buffer with stirring for 24 h at 4 °C. The PBS buffer was changed after 12 h of stirring. The extract was then filtered through a 0.22μm filter and its concentration was determined using the Bradford method and stored at −20°C for further use. The concentration of antigen was 1mg/ml.

**Separation of Splenic Mononuclear Cells (MNCs)**

Mice were sacrificed by cervical dislocation on the 20th day; spleens was resected under sterile conditions and were suspended in cold PBS containing 2% FBS. The splenic cell suspension was RBC-lysed with a solution of 0.75% NH4Cl and Tris buffer (0.02%) (PH=7.4). The cells were washed and the single-cell suspension was prepared in RPMI 1640 containing 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, and 100 IU/ ml penicillin and 100µg/ml streptomycin. To define the viability and density of cells in the suspension, Trypan blue dye exclusion method was used and the viability of splenocytes was >95%.
**Splenocyte Cytokine Production**

The isolated spleen MNCs was cultured in 24-well plates (Nunc, Denmark) in a final concentration of $2 \times 10^6$ cells/mL and 20 μg/mL of purified tumor lysate was added to stimulate the cells. After 72h of incubation at 37°C and 5% CO2, the supernatants of spleen cells were collected and the level of IFN-γ and IL-4 cytokines was measured using enzyme-linked immunosorbent assay (ELISA) technique. Mouse IL-4 and IFN-γ kits (R&D, USA) were purchased, and all the procedures were carried out according to the manufacturer’s guidelines. Each sample was analyzed in triplicate.

**Statistical Analyses**

Data were analyzed using GraphPad software version 6. At first, normality of the data and homogeneity of variances were tested with K–S and Levene’s statistical tests, respectively. P values less than 0.05 were considered as statistically significant. The data are presented here as mean ± SD of three independent experiments.

**RESULTS**

**Evaluation The cytotoxicity Effect Of Drugs In Vitro**

To examine the cytotoxic effects of the drugs and their combination form on 4T1 cell line and normal cells we used MTT assay to evaluate the cytotoxic effect. The results in figure 1 showed that cocktail drug none significantly increase the cytotoxic effect during 24 and 48 hrs of incubation. However there were no significant differences between the combination and Artesunate or Artemetheralone. No significant cytotoxic effect was noticed on the normal cells.

![Figure 1(a)](image1a.png)
![Figure 1(b)](image1b.png)
![Figure 1(c)](image1c.png)
![Figure 1(d)](image1d.png)

**Figure 1(a).** Cytotoxic effects of drugs on 4T1 cells in 24 h. **(b).** Cytotoxic effects of drugs on 4T1 cells in 48 h. **(C).** Comparison between cytotoxic effects of Cocktail form on 4T1 cells in 24 & 48 h. **(d) Cytotoxic effects of drugs on normal blood cells (PBMC)**
Comparative Studies on the Antitumor Effect of Artemisinin, Artesunate and Artemether Against StageII Breast Cancer in Animal Model

Evaluation the Tumor Volume of Mice Following Treatment with Drugs

In order to figure out the effect of drugs on in vivo tumor size, 30 mice were used. After the tumor volume reached to the average size of 90 mm$^3$, animals were divided and injected with drugs according to material and methods. The results in figure 2 showed that Artemisinin and cocktail groups significantly reduced the rate of tumor growth compared to control group. No significant difference was observed between tumor volume of Artesunate or Artemether group and control.

![Figure 2](chart showing the mean ±S.E. tumor volume in 6 groups of mice for 12 days. 20 mg/kg of drugs and the same volume of PBS were intraperitoneally injected to the groups. The treatments were administered on days 8 to 20. The results were analyzed with ANOVA statistical test. Significant difference (p<0.05) was seen in Artemisinin and cocktail groups.)

Evaluation the Weight of Mice Following Treatment with Drugs

Weight of mice was measured daily as an indicator of their general health. The figure 3 no significant difference was observed between mice weight in any groups.

![Figure 3](The weight(g) of animals treated with Artemisinin, Artesunate, Artemether and Cocktail drugs)

Evaluation the level of Cytokine after Treatment with Drugs

Splenocytes from treated mice were isolated and cultured in vitro. Cells were stimulated by lysate antigens for 72 h and supernatant was used to measure the concentration of IL-4 and IFN-γ by ELISA technique. The results in figure 4 indicated a significant increase (p < 0.05) in IFN-γ concentration in all groups including cocktail. But only production of IL-4 was decreased in the Splenocytes of Artemisinin and cyclophosphamide groups. No significant difference was observed in cocktail group.

![Figure 4](chart showing the mean ±S.E. tumor volume in 6 groups of mice for 12 days. 20 mg/kg of drugs and the same volume of PBS were intraperitoneally injected to the groups. The treatments were administered on days 8 to 20. The results were analyzed with ANOVA statistical test. Significant difference (p<0.05) was seen in Artemisinin and cocktail groups.)
Discussions

Today there are different ways to treat cancer, such as chemotherapy, Radiotherapy, surgery. The main goal in Cancer Therapy is achieve desired concentration of drug to the tumor site and destroying tumor cells with Minimal damage of normal cells[14]. Artemisininand its derivatives have this important anti-cancer feature. In this study we tried to provide an evidence to show that the Artemisinin in the combination with its derivatives has the ability in restricting tumor growth in mouse model of breast cancer[15, 16].

Artemisinin is used in treating malaria for years and has little side effects. Beside direct cytotoxic effect on cancer cells, Artemisinin induces apoptosis, inhibits angiogenesis and also reduces regulatory T cells[17, 18]. Artesunate is a water soluble derivative of Artemisinin and applies its anti-cancer effect by inducing nitric oxide in cancerous cells and also reducinganti-apoptotic proteins like Bcl-2. Artesunate decreases the level of VEGF and therefore prevents metastases and invasion of tumor[19, 20]. Artemether, the oil soluble derivative of Artemisinin, induces oxidative damage in DNA [21]. and also decreases the level of drug resistant mRNAs. Artemether’s oil solubility feature increases its penetration into cancer cells’ membrane[22, 23].

In this study we compared the effect of Artemisinin with its derivatives as sole and combination in killing cancer cells in-vitro, reducing tumor growth and also cytokine production. As Artemisinin has less cytotoxic effects compared to conventional chemotherapy drugs. Artemisinin half life is short in plasma[24] and because Artemisinins each has a specific anti-tumor feature therefore using them as a combination may intensify their anti-tumor properties.

For checking the cytotoxicity rate of drugs, 4T1 murine cell line and human PBMCs were considered as breast cancer and normal cell respectively. MTT results indicatethat Artemisinin alone and in combination form have significant cytotoxic and inhibitory effects on tumor cell growth on Breast cancer cell line 4T1. Artemisinin in the concentration of 105 microgram, Artesunate and Artemether in 90 microgram and the combination form in 75 microgram killed 50% of 4T1 cells. So this can be concluded that using these drugs as a combination increases their cytotoxic activity in vitro. In accordance wihtother similar studies that was performed on noroblstomacells[25] we also found that Artesunate and Artemether are more cytotoxic in killing cancerous cellsin comparison to Artemisinin.Also there were no significant differences in the cytotoxic activity between the Artesunate and Artemether within 24 and 48 hour treatment had no significant difference.These incidents was observed in other studiesand it is probably because of the short half life of drugs[26-28].
Since there is high demand of Fe2⁺ iron in the cancerous cells as compared to normal cells, it is expected to see more cytotoxicity on tumor cells than in normal ones. Here we observed that even in higher concentrations of drugs their cytotoxic effect was not changed on normal cells and this shows that combining these drugs dose not intensify their cytotoxic effects against normal cells.

Intraperitoneal injection (IP) of drugs led to the following results;

Artemisinin could induce significant inhibition to tumor growth comparing to control group.

Cocktail drugs induce significant decrease in tumor growth comparing to control group.

Artesunate and Artemether also induce significant decrease in tumor growth comparing to control group.

The pharmacokinetic of Artemisinin is more than Artesunate and Artesunate is more than Artemether, while the pharmacokinetic of Cocktail drugs was more than Artesunate and Artemether and is nearly equal to Cyclophosphamide.

Th1 polarization in tumor surrounding provides a suitable condition for anti-tumor responses. It is of great importance that the generation of IFN-γ by Th1 is a help for the cytotoxic T cells to be activated and to be the effector cells in tumor cells killing and finally makes the immune response stronger against cancerous cells. Our results clarify that IP injection of all drugs including the cocktail form increases the level of IFN-γ and leads to significant anti-tumor activity in BALB/c model of breast cancer. Previous studies have shown that Artemisinin and its derivatives have the ability to reduce the number of regulatory T cells, control tumor growth, immune modulatory properties and shift the immune system to cell immunity. Previous studies show that Artemisinin and Artemether solely could change the level of IFN-γ and IL-4. Based upon previous data our results, combination drug could be hopeful for therapeutic effect, at least as supplement drug to decrease in tumor growth and increase in shifting toward Th1.

Numerous studies have been carried out on the antitumor properties of Artemisinin and its derivative. In recent years many efforts have been made to increase the therapeutic properties of the conventional drugs, our results showed that combining these drugs can improve the efficiency of their cytotoxicity in vitro and their tumor growth inhabitation in tumor bearing mice. However we suggest that some modifications like changing the dose of treatment or schedule of injections can help us gain a better understanding of the mechanism of the cocktail form of drugs.

References


Comparative Studies on the Antitumor Effect of Artemisinin, Artesunate and Artemether Against Stage II Breast Cancer in Animal Model


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