breast cancer patients (Wang et al., 2015).

Boswellia serrata is a medical plant that primarily grows in India and has been shown to exhibit immense potential in combating cancer (Roy et al., 2016). Boswellia species are widely used in traditional treatments for a wide range of diseases including inflammation, rheumatoid arthritis, Crohn's disease and leukocyte infiltration (Ammon, 2002; Etzel, 1996). Frankincense, an aromatic resin hardened from exuded gums obtained from trees of the genus Boswellia (Burseraceae family) (Frank et al., 2009), is used as an effective remedy for treating cancer, dysentery, ringworm, boils and blood diseases (Siddiqui, 2011). In traditional treatment, frankincense is used for respiratory distress, digestive diseases, joint pain and inflammatory conditions, as well as cancerous diseases and shows antimicrobial activity against various microorganisms (Siddiqui, 2011). The frankincense oil and boswellic acid (a major chemical component in *Boswellia*) can significantly suppress the survival of the J82 bladder cancer cells (Frank et al., 2009). As reported by Huang et al. (2008) *B. serrata* extract that contains β-boswellic acids and related compounds inhibits TPA (tumor promoter 12-O-tetradecanoylphorbol-13-acetate)-induced tumor promotion in mice by suppressing up-expression of pro-inflammatory cytokine proteins (Huang et al., 2008). Glaser et al. (1999) have also found that boswellic acids can induce apoptotic pathways and have cytotoxic effects on malignant glioma cells at low micromolar concentrations. In breast cancer patients with brain metastases, *B. serrata* reduces breast cancer metastasis and brain tumor formation by inhibiting lipoxygenase-2 enzyme (Flavin, 2007). It has also been shown acetyl-11-keto-beta-boswellic acid (AKBA) at 10mg/kg/day has been associated with inhibition of prostate tumor growth in mice. In this type of cancer, it was observed that AKBA inhibits tumor growth by inhibiting vascular endothelial growth factor (VEGF) and subsequently inhibiting angiogenesis (Pang et al., 2009).

Previous studies have not reported any serious, long-term, or irreversible adverse effects for *B. serrata* (Ernst, 2008). Therefore, it can be used as a low-risk product in the treatment of various diseases, including cancer. On the other hand, the 4T1 tumor is a suitable experimental animal model for human mammary cancer (Pulaski and Ostrand-Rosenberg, 2000). In addition, more than 50% cancer therapy compounds have been recognized to have the ability to induce apoptosis and also can be found in natural products (Dholwani et al., 2008; Yadav et al., 2012). Therefore, we investigated the inhibitory effect of *B. serrata* alcoholic extract (BSE) on tumor growth, metastasis and angiogenesis in 4T1 breast cancer mouse model.

Materials and methods

Reagents

Methanol (MeOH) was purchased from Merck in Darmstadt, Germany. Thiazolyl blue tetrazolium bromide, dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), Dulbecco's Modified Eagle's Media (DMEM), trypsin, penicillin, and streptomycin were purchased from Sigma-Aldrich (St. Louis, Missouri). Whatman filter paper was purchased from Millipore. 4T1 cells were purchased from the Pasteur Institute of Iran (National Cell Bank of Iran). Ki-67 antibody (mouse monoclonal, dilution 1:500) and CD31 antibody (mouse monoclonal, dilution 1:500) were from Dako (Carpinteria, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. All other tissue culture reagents and additives were obtained from Sigma (St. Louis, Missouri).

Preparation of extract

Several publications have appeared in recent years documenting polar (such as methanol) extracts of plants have stronger anti-cancer effects than nonpolar extracts (Arpornsuwan and Punjanon, 2006; Betancur-Galvis et al., 1999; Uddin et al., 2011), the decision was made to use methanol extract in the present research. The dried powder of *B. serrata* gum resin (50g) (Collected from Kurdistan, Iran) was extracted in 500 ml of methanol in a mechanical shaker at room temperature for 48h. After cooling down, the mixture was then filtered with Whatman No. 1 filter paper. The filtrates obtained from methanol extraction was evaporated to dryness at 40°C in a rotary evaporator. The dried sample of extract allowed to cool stored at 4 °C in a sterile bottle for further analysis. Sterile distilled water was used to dissolve dry powder.

Ethics statement

The experiments were performed based on the

Principles of Laboratory Animal Care by the US National Institute of Health (NIH publication No. 85-23, revised 1985).

Animals

Female BALB/c mice (body weight, 20–22g) were obtained from the Pasteur Institute, Karaj, Iran. The mice were maintained in isolation under specified pathogen-free conditions and under controlled temperature (22±0.5°C) and light (12h light/ dark cycle), and food and water were supplied *ad libitum*.

Cell culture and MTT assay

The 4T1 triple negative breast cancer cells were purchased from the Pasteur Institute of Iran. The cells were cultured in 75cm^2 culture flasks in DMEM containing 10% FBS, penicillin (100IU/ml) and streptomycin (100mg/ml) with 5% (v/v) CO_2 at 37°C for 24h. Cells were separated by trypsin and seeded at a density of 1×10^4 cells/ml in 96-well polystyrene culture plates. Cells were cultured in different concentrations of extract (0, 5, 20, 40, 80, 100, 150, 200 and 250µg/ml). After 24 hours of treatment, the cells were employed to test the cell viability by the MTT method. The 200µl of MTT solution (5mg/ml) was added to each well and the plate was incubated for 3 hours. After removing the supernatant, 100 microliters of DMSO were added to each well, then the plate was incubated for 20 minutes. DMSO was used for dissolving the formazan crystals and cells were incubated at 37°C. Absorbance was measured in each well, including the samples (treated cells), control (untreated cells) and blank (wells containing medium only) at 570nm by ELISA Plate Reader. Cell viability (%) was calculated for all groups compared to the control sample. All experimental samples were performed in triplicate.

Tumor inoculation and *in vivo* anti-breast tumor study

Animals were randomly divided into Five groups (n=5) and received 1×10^5 4T1 cells/0.1 ml of phosphate buffer solution subcutaneously. Following the injection of 4T1 cells, treatment groups (BSE50, BSE150 and BSE250) were orally administered a daily dose of BSE at doses of 50, 150 and 250mg/kg, respectively. The control group was administered only distilled water. On day 22, the animals were weighed and then anesthetized with ethyl ether, after which they were sacrificed and their tumor tissues were collected. In addition, the liver and lung tissues were collected and then visually examined for metastasis. The following formula was used to calculate the tumor inhibition rate: Tumor inhibitory rate= [(tumor weight of the control group− tumor weight of experimental group)/tumor weight of the control group]×100.

Histological Analysis

The mice were sacrificed by suffocating with diethyl ether in a glass desiccator. Tumors and organs including lung and liver were harvested and fixed immediately in 10% buffered formalin phosphate and embedded in paraffin. For histopathological assessment, 10 sections of each block were randomly selected, deparaffinized with xylene, stained with hematoxylin-eosin (H&E), and each slide was examined under a light microscope with the assistance of a pathologist. Percentage of tumor metastatic and necrotic area was assessed microscopically by ImageJ software as a percentage of total area. An average of 5 fields in each tissue section was evaluated.

Immunohistochemical (IHC) analysis of 4T1 tumors

Paraffin embedded specimens were deparaffinized followed by antigen retrieval using citric acid buffer (pH 6.0, 95°C for 20min). Slides were treated with 1% hydrogen peroxide in methanol to block endogenous peroxidase activity. After 20 min blocking in 5% horse serum, slides were incubated overnight at 4°C with the following primary Ki-67 and CD31 antibodies. Next, the slides were incubated with 2µg/ml of biotinylated secondary antibody for 30min at room temperature. Subsequently, the sections were stained using Standard Ultra-Sensitive ABC Peroxidase Staining kit (Pierce/Thermo Fisher Scientific, San Jose, CA) and 3,3'- diaminobenzidine (DAB), counterstained by hematoxylin, dehydrated and mounted with a cover slide. Slides were also counterstained with H&E. Staining was performed according to standard protocol. Each value represents the average of five different fields.

Data analysis

Level of significance between different treatment groups relative to control was determined by one-way

analysis of variance (ANOVA). The least significant difference (LSD) was used as the post-hoc test. $P<0.05$ was considered statistically significant. All data were presented as mean ± standard error of the mean (SEM) and was analyzed using the statistical package "SPSS 16.0"..

Results

The effect of *B. serrata* hydroalcoholic extract on 4T1 cell viability

B. serrata hydroalcoholic extract demonstrated a substantial anti-cancer effect against the 4T1 cell line. The percentage of cell viability at doses of 5, 20, 40 and 80µg/ml of extract was not significantly different with the control group (100±5%, $P>0.05$). However, concentrations of 100, 150, 200 and 250mg/ml of extract significantly reduced the survival of 4T1 cells ($P<0.05$, Fig. 1). The IC_{50} value was considered as the concentration of the extract that caused a 50% decrease in cell viability relative to the negative control which was constituted by cell culture and DMSO without the extract. IC_{50} value for *B. serrata* hydroalcoholic extract was found to be 92.3µg/ml by MTT assay.

Body weight

Body weight was measured on days 1, 6, 12, 18 and 22 using digital scales. There was no significant difference in body weight on different days between treatment groups with the control group (P>0.05, Fig. 2).

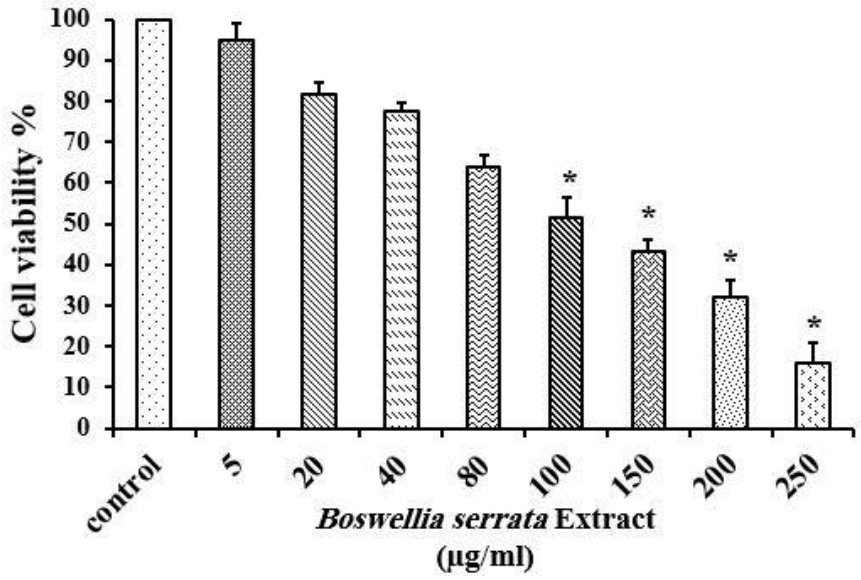

Fig.1. The effect of *B. serrata* gum resin alcoholic extract on 4T1 cell viability (MTT assay) (n=3).

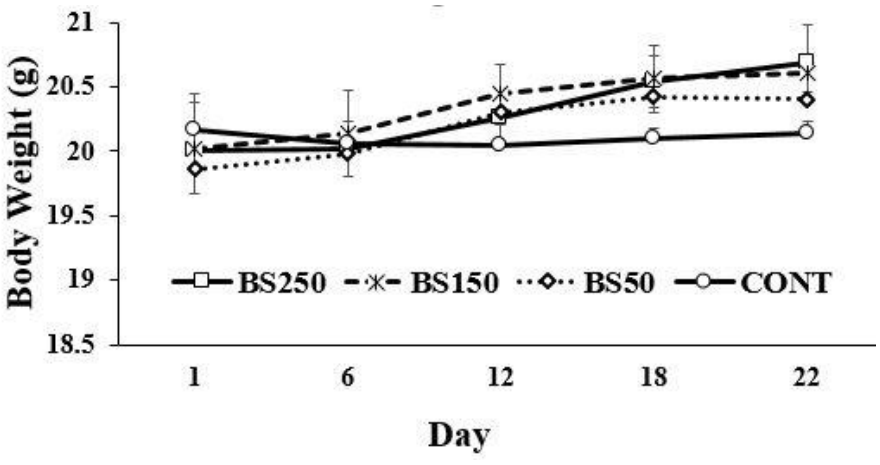

Fig.2. Body weight in BALB/c mice treated with *B. serrata* gum resin extract. Body weight was measured at days 1, 6, 12, 18 and 22. BSE250, 150 and 50: *B. serrata* extract in different concentration (250, 150 and 50mg/kg); Cont: control group (n= 5).

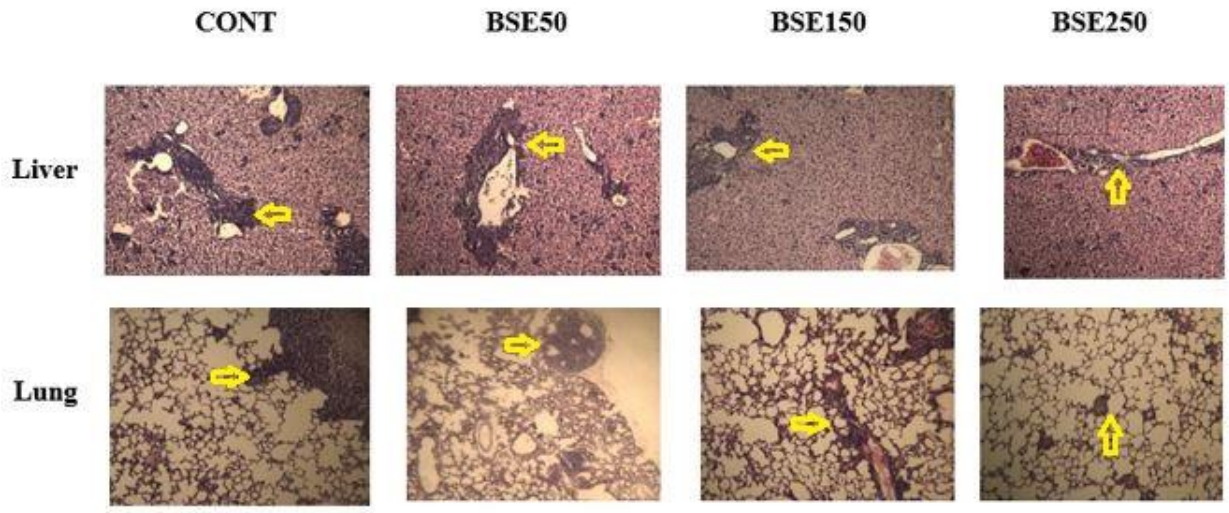

Fig.3. Hematoxylin eosin staining of paraffin sections from the liver and lung tissues (H&E stain; 400x magnification). BSE250, 150 and 50: *B. serrata* gum resin extract in different concentration (250, 150 and 50mg/kg); Cont: control group. Arrows indicate metastatic cell (n = 5).

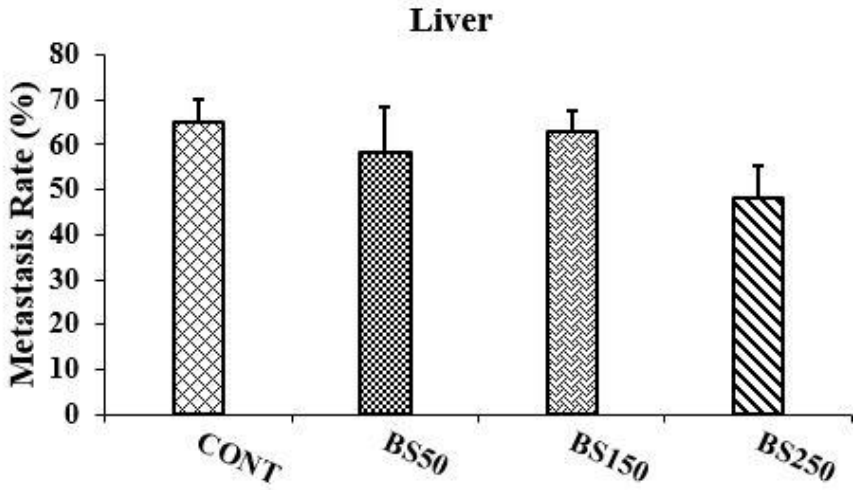

Fig.4. The levels of liver metastasis (%) cells in 4T1 breast cancer mouse model (H&E stain; 400x magnification). BSE250, 150 and 50: *B. serrata* gum resin extract in different concentration (250, 150 and 50mg/kg); Cont: control group (n = 5).

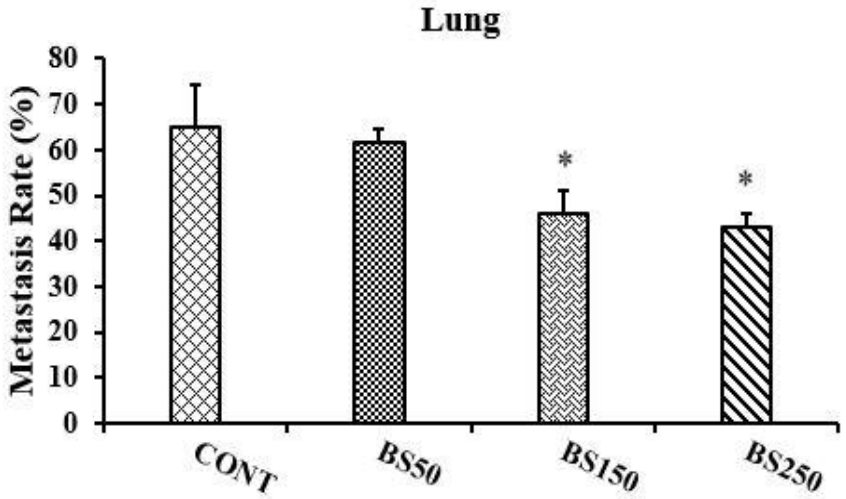

Fig.5. The levels of lung metastasis (%) cells in 4T1 breast cancer mouse model. (H&E stain; 400x magnification). BSE250, 150 and 50: *B. serrata* gum resin extract in different concentration (250, 150 and 50mg/kg); Cont: control group (n = 5).

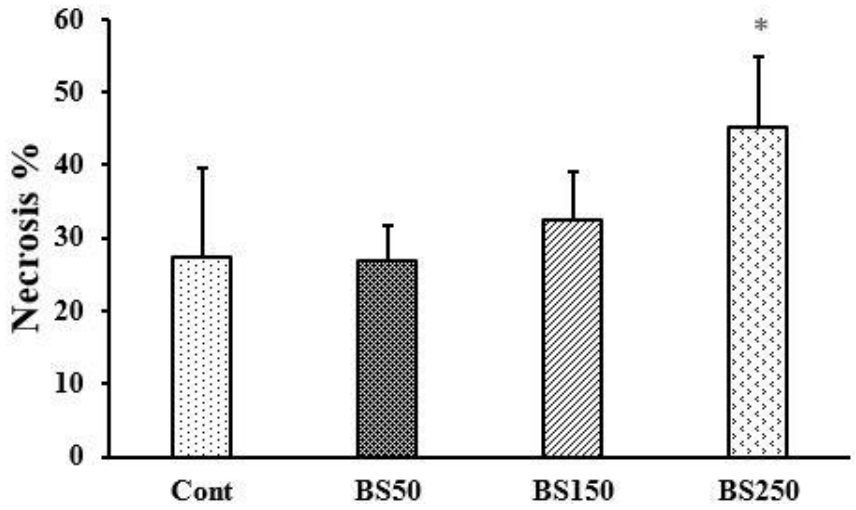

Fig.6. The levels of tumor necrosis (%) in 4T1 breast cancer mouse model. (H&E stain; 400x magnification). BSE250, 150 and 50: *B. serrata* gum resin extract in different concentration (250, 150 and 50mg/kg); Cont: control group (n = 5).

Table 1: Effect of *B. serrata* gum resin extract on tumor inhibition rate in 4T1 breast cancer model (n = 5). $^{*}P<0.05$ compare to control group.

Groups	Tumor Weight (g)	Inhibition Rate (%)
Cont	2.64±0.34	-
BSE50	2.51±0.81	0.04
BSE150	1.96±0.57	25.7
BSE250	1.37±0.43	41.8*

Tumor inhibition rate

We used an implanted 4T1 mouse model to investigate the effects of BSE on tumor growth. Table 1 shows the tumor weight and percentage of tumor growth inhibition in the treatment and control groups. Statistical analysis showed that BSE250 suppressed tumor growth by 41.8%, which was a significant inhibitory effect compared to the control group ($P<0.05$). In the treated groups with BSE50 and BSE150, tumor growth was inhibited by 0.04% and 25.7%, respectively, which was not significantly different with the control group.

Histological analysis and necrosis rate

To observe metastatic cells in the liver and lung tissues of all experimental groups, H&E staining was used in the present study. Histopathological study with H&E staining has shown metastatic cells in small and large groups between liver and lung cells (Fig. 3). In the macroscopic examination, the elevation of the size of the liver and the lung tissues was detectable.

In this study, liver metastasis in the treatment groups did not show any significant difference with the control group ($P>0.05$, Fig. 4); however, the rate of lung metastasis in the BSE150 and BSE250 treatment groups showed a significant decrease compared to the control group ($P<0.05$, Fig. 5).

The level of the necrotic area in the group receiving the highest dose of BSE (250mg/kg) was significantly higher than the control group; however, the percentage of the necrotic area in other treatment groups (BSE50 and BSE150) did not show any significant difference with the control group (Fig. 6).

Immunostaining changes for cell proliferation (Ki-67) and neovascularization (CD31) of tumor tissues

To determine how BSE decreases tumor growth and metastasis, immunohistochemical studies were used to examine markers of proliferation and angiogenesis, Ki-67 and CD31, respectively (Figs 7 and 8). Ten pathologic slides were randomly selected and the mean number of positive cells in each group were used for statistical analysis. The number of Ki-67 cells in BSE250, BSE150 and BSE50 groups is about 38%, 17% and 10% lower than the control group. The results of immunohistochemistry studies showed that a dose of 250mg/kg of BSE could significantly

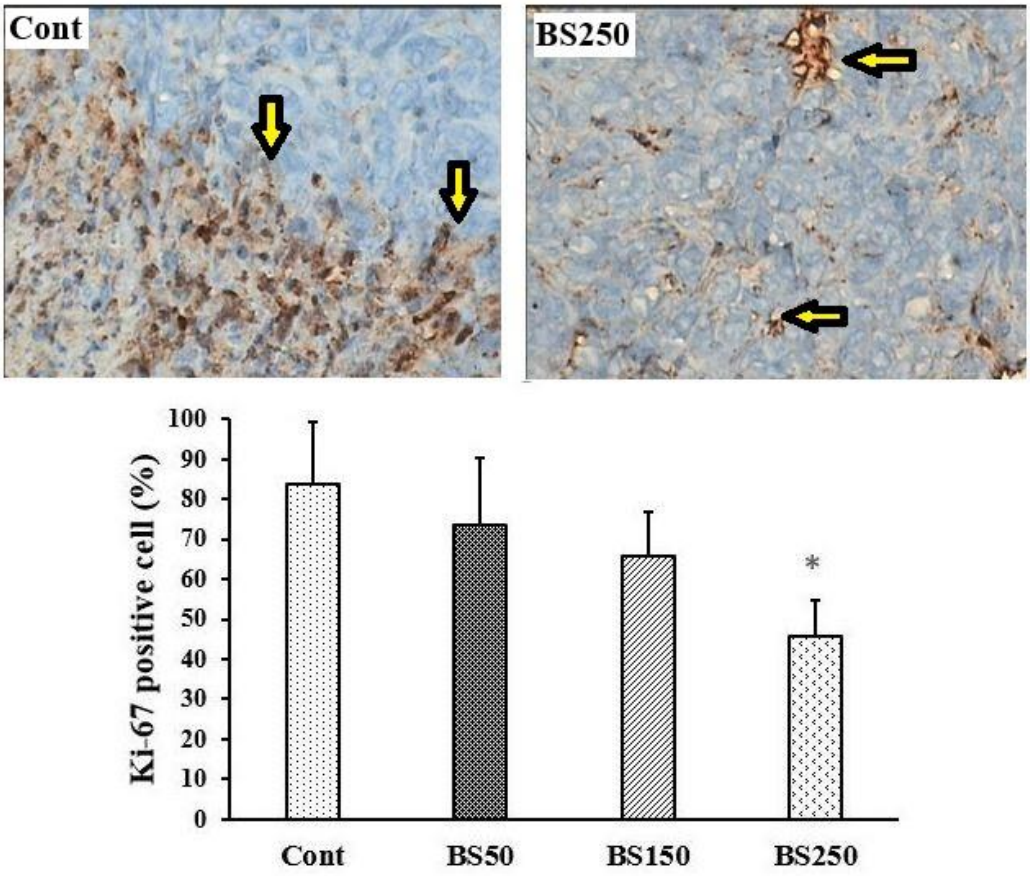

Fig.7. The levels of Ki-67 (%) in 4T1 breast cancer mouse model. (IHC stain; 400x magnification). BSE250, 150 and 50: *B. serrata* gum resin extract in different concentration (250, 150 and 50mg/kg); Cont: control group (n = 5).

decrease the number of Ki-67 positive cells compared to the control group (Fig. 7). However, in BSE50 and BSE150 groups, the rate of cell proliferation did not differ significantly with the control group. The expression of CD31 in the treatment groups with BSE150 and BSE50 was not significantly different with the control group. However, the level of angiogenesis in BSE250 group was significantly lower than the control group (Fig. 8).

Discussion

In our paper, the focus of attention was on anti-cancer effects of *B. serrata* in the 4T1 breast cancer model. Our results showed that BSE reduced the cell survival of 4T1 cells *in vitro*. There was also a decrease in tumor growth and metastasis rate in the *in vivo* environment. On the other hand, BSE showed a significant effect on the reduction of angiogenesis (CD31) and cell proliferation (Ki-67).

In this study, the effect of the alcoholic extract of *B. serrata* on 4T1 cells was investigated by MTT assay. The results of the MTT assay technique showed that *B. serrata* alcoholic extract could significantly reduce the proliferation of 4T1 cancer cells and also had a dose-dependent cytotoxic effect on the survival of 4T1 cells. The inhibitory effect of 250µg/ml concentration of extract was significantly higher than other concentrations. For several years, great effort has been devoted to the study of anti-cancer activity of *Boswellia* species (Chashoo et al., 2011; Hoernlein et al., 1999; Jing et al., 1999; Liu et al., 2002a; Xia et al., 2005). As reported by Shao et al. (1998) boswellic acids from the gum resin of *Boswellia serrata* indicate anti-tumor activity in human leukemia HL-60 cells by reducing DNA, RNA and protein synthesis with IC_{50} values ranging from 0.6 to 7.1µM. In addition, it has also been shown that *B. serrata* extract and boswellic acids induce intrinsic and extrinsic apoptotic pathways (Bhushan et al., 2007; Hoernlein et al., 1999). The ability of boswellic acids to induce apoptosis has been investigated on a variety of cell lines such as myeloid leukemia cells, metastatic melanoma, fibrosarcoma, brain tumor cells, HCT-116 human colon cancer cells, Hep G2 cell line, liver cancer and glioma cells (Liu et al., 2002a; Streffer

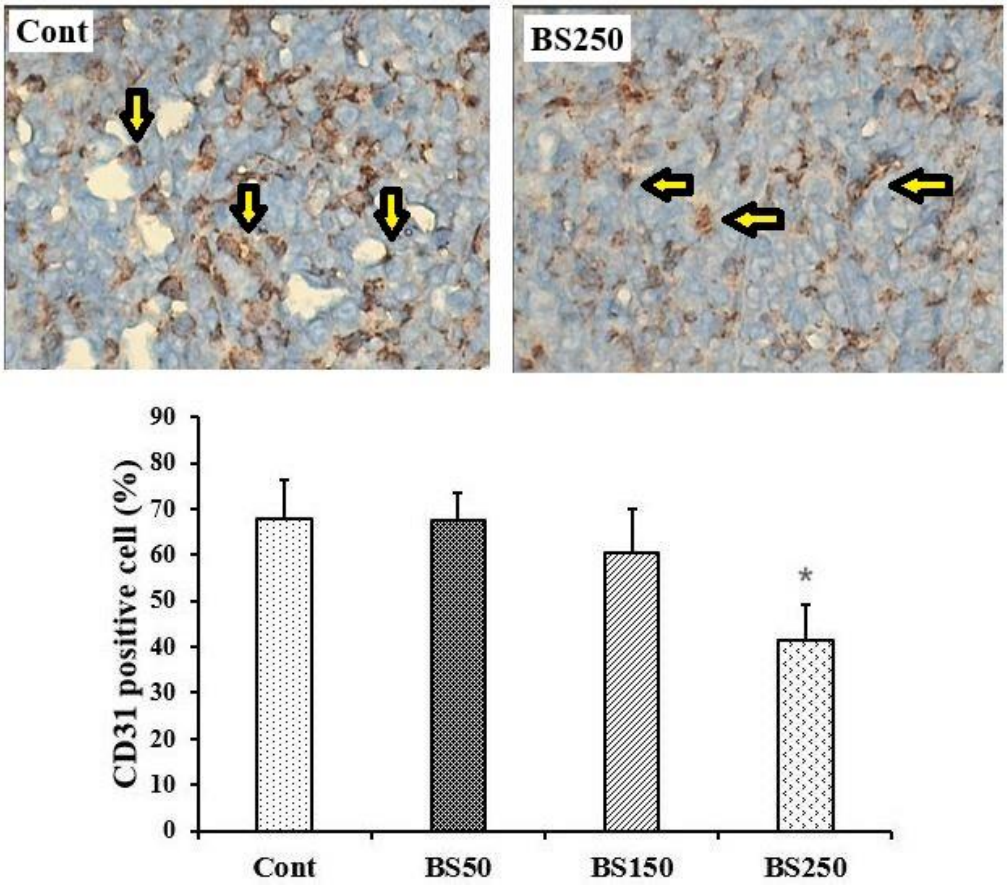

Fig.8. The levels of CD31 (%) in 4T1 breast cancer mouse model. (IHC stain; 400x magnification). BSE250, 150 and 50: *B. serrata* gum resin extract in different concentration (250, 150 and 50mg/kg); Cont: control group (n = 5).

et al., 2001; Wang et al., 2018; Xia et al., 2005; Zhao et al., 2003). It has also been shown that AKBA, has a cytotoxic effect on meningioma cells *in vitro* (Park et al., 2002). Conti et al. (2018) also reported that combination of AKBA with radiotherapy inhibits factors which involved in cell death regulation and tumor progression. Suhail et al. (2011) studied inhibitory effect of *Boswellia sacra* essential oil on various human breast cancer cells, such as T47D, MCF-7 and MDA-MB-231 and normal MCF10-2A cells and showed that *B. sacra* essential oil induces tumor cell-specific apoptosis and suppresses tumor aggressiveness, but normal cells of the breast tissue were very resistant to *B. sacra* oil. In addition, the anticancer activity of boswellic acid has been indicated against human prostate cancer cell line PC-3 (Gandhi et al., 2016). Anticancer activity of boswellic acid against human colon cancer cells has also been shown in a recent paper by Wang et al. (2018) in which boswellic acid has a dose-dependent effect against the HCT-116 colon cancer cells via induction of apoptosis, cell cycle arrest and inhibition of cell migration and PI3K/AKT signaling pathway. The results obtained by Huang et al. (2018) also showed that 11-carbonyl-β-boswellic acid inhibits the growth of non-small cell lung cancer H446 cells *in vitro*. According to previous studies, it could be argued that the *B. serrata* alcoholic extract reduces 4T1 cell viability by stimulating apoptosis and disturbing DNA, RNA and protein synthesis.

Our anti-breast cancer study showed that percentage changes in tumor weight in BSE250 group were significantly lower than the control group, while the BSE150 and BSE50 groups did not differ with the control group. In addition, immunohistochemical studies with light microscopy confirmed decreasing in cell proliferation (Ki-67) and the inhibition effect of BSE250 on tumor growth. The results obtained by Syrovets et al. (2004) show that AKBA triggers apoptosis in the chemo-resistant and androgen-independent human PC-3 prostate cancer cells, both *in vitro* and *in vivo*. Agrawal et al. (2011) also studied the possible role of boswellic acid isolated from *B. serrata* on ascites and solid Ehrlich tumor and showed that boswellic acid induces tumor cell apoptosis through activating the pro-apoptotic bcl-2 family and caspase-3. Increase in cytoplasmic DNA-histones complex, pre-G1 peak in cell cycle, activity

of caspase 3 and PARP complex have been reported by Liu et al. (2002b) as another apoptosis inducer effect of boswellic acids. Investigation of *Boswellia ovalifoliolata* anti-apoptotic effects with western blot technique has shown that *B. ovalifoliolata* extract significantly reduces phosphorylated nuclear factor-κB (NF-kB, ser536), PCNA and anti-apoptotic protein (Bcl-2) and increases Bax protein in MDA-MB-231 and MDA-MB-453 breast cancer cells (Thummuri et al., 2014). It has been reported that triterpenoid extracted from *B. serrata* inhibits Bcl-2 protein and transfers Bax to the mitochondrial membrane and also induces release of cytochrome C, AIF and Smac/DIABL into the cytosol and initiates the process of apoptosis (Bhushan et al., 2007). As reported by Zhang et al. (2013), inhibition of NF-kB, cyclooxygenase-2 (COX-2) and 5-lipoxygenase can be another apoptotic mechanism of triterpenoid resinous metabolites from the genus *Boswellia*. Recently it has been shown that *Boswellia serrata* extract and 3-O-Acetyl-β-boswellic acid down-regulate multiple targets counter to the cell survival, proliferation and metastasis of triple-negative breast cancer cell (MDA-MB-231)(Mazzio et al., 2017). Summing up the previous studies and our results, it can be concluded that *B. serrata* reduces tumor growth in the 4T1 breast cancer model by inhibition of cell survival pathways and induction of apoptosis.

Our data, not showing any significant reduction in liver metastasis as compared to the control group, but lung metastasis significantly reduced in BSE150 and BSE250 groups. Angiogenesis also significantly reduced in BSE250 group in comparison with the control group. Angiogenesis is an important process involved in tumor growth and is essential for cell proliferation and metastasis of cancer cells (Folkman, 1992). Therefore, suppressing the angiogenesis pathway is a promising strategy for the treatment of metastatic cancer (Folkman, 1992). It has been shown that boswellic acid analog BA145 inhibits VEGF induced expression of VEGFR-1/R-2 and HIF-1α/1β in PC-3 cells in a dose dependent manner and suppresses activation of PI3K/Akt pathway (Pathania et al., 2015). In the pancreatic cancer cell lines, AKBA inhibits NF-kB activity and transcription of VEGF, MMP-9 and COX-2 genes (Park et al., 2011). Also, it has been reported that treatment of prostate cancer cells with AKBA suppresses the expression of different receptors, including androgen receptors, death receptor 5 and VEGF-R2 receptors (Pang et al., 2009). This evidences suggest that AKBA reduces the growth of prostate cancer cells by inhibiting angiogenesis and inducing apoptosis (Pang et al., 2009). In a paper by Kunnumakkara et al. (2009), it was shown that AKBA inhibits IL-6-induced STAT3 activation in melanoma cells. Inhibition of STAT-3 signal pathway leads to inhibition of genes involved in cell proliferation, cell survival and angiogenesis (Kunnumakkara et al., 2009). In addition, Yadav et al. in 2012 indicated that AKBA (50-200 mg/kg) in orthotopic models of colorectal cancer reduces metastasis in liver, spleen and lung tissues and also reduces vascular density (CD31), cell proliferation (Ki-67). The results obtained by Flavin in 2007 suggest that, in breast cancer patients with brain metastases, *B. serrata* reduces breast cancer metastasis and brain tumor formation by inhibiting the lipoxygenase-2 enzyme. Recent data reported by Ranjbarnejad et al. in 2017 indicate that *B. serrata* extract inhibits proliferation, angiogenesis and migration and induces apoptosis in HT-29 cells by inhibiting of mPGES-1 and decreasing the PGE2 level and its downstream targets. Xia et al. (2017) has also found that *Boswellia sacra* gum resin hydro-distillates may have cancer chemo-preventive effects on urothelial cell carcinoma of the urinary bladder in transurethral resection of bladder tumor patients (Xia et al., 2017). Inhibition of genes involved in cell proliferation, cell survival and angiogenesis can be considered as a possible mechanism for the *B. serrata* alcoholic extract in reducing angiogenesis and metastatic rate in the 4T1 breast cancer mouse model. In our future research, we intend to concentrate on anti-cancer mechanisms in *B. serrata*.

Morioka et al. (2003) reported that tumor necrosis has not linear correlation with tumor volume in breast cancer xenograft models. However, there are reports that there is a direct relationship between tumor volume and tumor necrosis and angiogenesis (Leek et al,. 1999). It seems that in our study, angiogenesis rate inhibition in treated groups led to a difference in the incidence of tissue necrosis in BSE250 group than with the control group.

Conclusion

In this study, we used *in vivo* and *in vitro* techniques to investigate the anti-tumor effects of *B. serrata*

alcoholic extract on the 4T1 breast cancer mouse model. From the research that has been carried out, it is possible to conclude that *B. serrata* alcoholic extract can reduce tumor growth in 4T1 breast cancer model. This suppressing effect of *B. serrata* gum resin was confirmed by immunohistopathology studies, which showed a decrease in cell proliferation (Ki-67) and angiogenesis (CD31). This finding suggests that anti-cancer properties of BSE could also be useful for developing a novel medical herb for the treatment of breast cancer. The next stage of our research will be to investigate the anti-cancer molecular mechanisms of *B. serrata*.

Acknowledgments

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Conflict of interest

The authors declare that they have no competing interests.

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