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Research Article

Fermented Pomegranate (*Punica granatum*) Peel Extract as a Novel Anticancer Agent Targeting Angiogenesis and Metastasis

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ABSTRACT

Inhibitors of tumor angiogenesis and metastasis are rapidly emerging as important new drug candidates for cancer therapy. Our previous work has revealed that solid state fermentation improved the antioxidant activity of pomegranate. In the present study, the ability of the fermented and unfermented pomegranate in inhibiting tumor growth, metastasis and angiogenesis were investigated. The cytotoxicity and *in vitro* anticancer evaluation of the fermented and unfermented pomegranate has been assessed against five human cancer cell lines (liver HepG2, breast MCF-7, prostate PC3, colon HCT116 and lung A549). The results revealed that the fermented and unfermented pomegranate exert their actions in HepG2, MCF-7 and HCT116 through inhibition of the activity of both urokinase (uPA) and histone deacetylase (HDAC) which implicated in the development of cancers. fermented pomegranate extract revealed promising anticancer activity compared to the activity of the commonly used anticancer drug, doxorubicin with decreasing the level of vascular endothelial growth factor (VEGF) as a marker of angiogenesis and inhibition in a metastatic as measured by reduction in the activity of elastase enzyme. In conclusion, the results suggested that the fermented and unfermented pomegranate (especially fermented pomegranate**)** methanolic extracts can be used as good candidate for novel therapeutic strategies for cancer possessed significant anticancer activity through regulation angiogenesis and metastasis of cancer.

Keywords: Pomegranate, fermentation, anticancer, metastasis, angiogenesis.

INTRODUCTION

Epidemiological studies have suggested that herbal medicines and fruit extracts play a major role in the prevention and treatment of many types of cancer [1,2].

Punica granatum Linn. (Punicaceae), commonly known as pomegranate, is a small tree native to the Mediterranean region. The plant possesses an immense therapeutic value. A number of biological activities such as antitumor [3]; antibacterial [4]; antidiarrheal [5]; antifungal [6]; antiulcer [7] have been reported with various extracts/constituents of different parts of this plant. Pomegranate, especially flower of pomegranate, has extensively been used in Unani and Ayurvedic systems of medicine [8]. Peels are often the waste part of various fruits. These wastes have not generally received much attention with a view to being used or recycled rather than discharged. This might be due to their lack of commercial application. Interestingly, the peel and seed fractions of some fruits have higher antioxidant activity than the pulp fractions [9,10].

Solid-state fermentation (SSF) of an edible plant is a biotechnological strategy that may induce health beneficial naturally occurring antioxidant components including polyphenols, tocopherols and ascorbic acid during microbial fermentation [10, 11].

Urokinase plasminogen activator (uPA) is a serine protease that functions in the conversion of the circulating plasminogen to the active, broad-spectrum, serine protease plasmin. uPA is secreted as an inactive single-chain proenzyme by many different cell types and exists in a soluble or cell-associated form by binding to a specific membrane uPA receptor (uPAR) [12,13]. The uPA is involved in many physiological functions and, along with members of the matrix metalloproteinases family; it has been implicated in cancer invasion and metastatization [14,15]. Besides the proteolytic function, upon binding to uPAR, uPA is involved in initiating versatile intracellular signal pathways that regulate cell proliferation, adhesion and migration [16]. Urokinase is implicated in a large number of malignancies, e.g. cancers of breast, lung, bladder, cervix, kidney, stomach and brain [17,18]. The role of uPA in human cancer progression is further supported by clinical evidences demonstrating that high tissue levels of its components correlate with a poor prognosis in different types of cancer as breast, gastrointestinal cancers [19,20].

Histone deacetylases (HDACs) are a class of enzymes playing an important role in gene expression [21]. Because it has been reported that its inhibition brought about cell-cycle arrest and induced differentiation [22], HDAC is considered a target for new types of pharmaceuticals. A

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Table 1: Cytotoxicity (IC_{50}, µg/ml) of unfermented and fermented pomegranate against five types of human malignant cell lines as measured with SRB assay method.

Compound	IC_{50} (µg/ml)				
	HepG2	MCF-7	PC3	HCT116	A549
Doxorubicin	20.10 ± 2.00	24.00±2.50	18.00±2.00	19.25±2.00	25.50±2.70
Unfermented	28.40 ± 2.60	27.60 ± 3.00	78.00±9.00	24.60 ± 2.60	82.60±6.40
Fermented	19.20±1.90	23.80 ±2.40	58.00±3.90	21.10 ± 1.96	70.40±4.80

Data are expressed as means ± S.E. of three separate experiments.

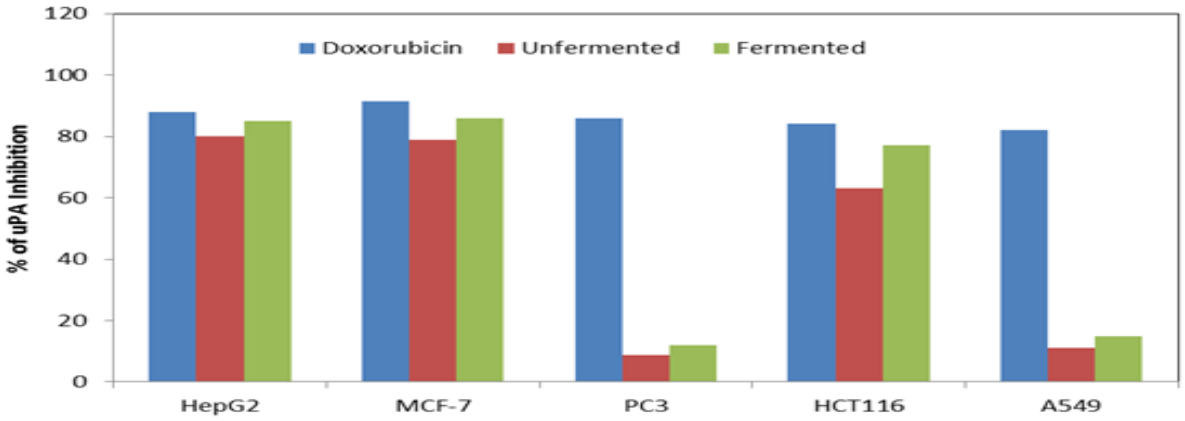

Fig. 1: The percent of uPA inhibition of the unfermented and fermented pomegranate on different cell lines. Data were expressed as percentage changes as compared with control cancer cells (DMSO treated)

possible application of HDAC inhibitors would be treatment of cancers [23].

Angiogenesis is essential for the initiation, progression and metastasis of solid tumor. Overexpression of angiogenic factors can direct the endothelial cell proliferation and sprouting in tumor mass as well as maintain vascular state of the tumor for the growth [24]. Vascular endothelial growth factor (VEGF) has been identified as the most important angiogenic factor for tumor progression because it is released by a variety of tumor cells and overexpresses in different human cancers. Drugs that can inhibit the production of VEGF or block its receptor signaling show significant inhibition of tumor growth [25,26]. In addition, metastasis of cancer cells to distant sites is one of the major deciding factors in cancer outcome. In fact, prognosis of cancer is mainly determined by the invasiveness of the tumors and its ability to metastasize. There is a cascade of events leading to the metastasis of tumors. These include separation from the primary site, circulation through blood or lymph, adhesive to the basement membrane (composed mainly of heparan sulfate, elastin, and collagen), invasion and proliferation at distant sites. Any compound which can inhibit one of the steps in the cascade will be useful in the inhibition of tumor metastasis and tumor growth [27]. Elastase is a broad-range proteolytic enzyme thought to be a tumor promoter involved in increasing tumor cell invasiveness by facilitating cell motility and transendothelial migration as it has the ability to degrade basement membrane and ECM glycoproteins such as elastin, fibronectin, as well as adhesive molecules and junctional cadherins [28]. Furthermore, Taniguchi et al [29] postulated that increased elastase activity destroy the barrier between tumor and the local circulatory system, either lymphatic or hematogenous, and result in at least loco-regional metastases.

In our previous work we studied the antioxidant properties of unfermented and fermented methanolic extracts of pomengrate peel with investigating their protection effect against cardiotoxicity and nephrotoxicity induced by adrymicin and the results revealed that the fermentation process appears to improve the antioxidants properties of the extract [10]. In the same direction and in continuing effort to find more potent and selective anticancer compounds, herein, we examined the effect of fermented and unfermented of methanolic extracts of pomengrate peel for their anticancer activity against five different human cancer cell lines including liver HepG2, breast MCF-7, prostate PC3, colon HCT116 and lung A549 with studying their mechanism of action that may act through inhibition the activity of both urokinase and histone deacetylase with studying their antiangiogenesis and antimetastasis activities.

MATERIALS AND METHODS

Chemicals: Fetal bovine serum (FBS) and L-glutamine, were obtained from Gibco Invitrogen Company (Scotland, UK). Dulbecco's modified Eagle's (DMEM) medium was provided from Cambrex (New Jersey, USA). Dimethyl sulfoxide (DMSO), doxorubicin, penicillin, streptomycin and Sulfo-Rhodamine-B stain (SRB) (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals and reagents used in this study were of analytical grade and purchased from Sigma-Aldrich chemical Co. (St. Louis, MO, USA).

Table 2: The effect of unfermented and fermented pomegranate on the level of VEGF in different cell lines.

Compound	VEGF (pg/mg protein)				
	HepG2	MCF-7	PC3	HCT116	A549
Doxorubicin	133.42±15.70	110.50±12.80	109.60±15.80	135.00±17.80	120.70±19.90
DMSO	925.46±93.90[a]	750.60±76.60[a]	600.00±62.12[a]	610.20±64.30[a]	850.76±77.70[a]
Unfermented	220.67±24.10 [a,b]	240.22±25.00 [a,b]	420.37±45.00 [a,b]	255.88±28.40 [a,b]	610.33±64.60 [a,b]
Fermented	150.30±17.50 [b]	190.60±21.60 [a,b]	360.77±40.00 [a,b]	210.00±23.50 [a,b]	420.17±44.30 [a,b]

Data were expressed as Mean ± Standard error (S.E.) of three independent experiments. [a] and [b] is significant difference from doxorubicin and DMSO – cancer treated cells respectively at ($p < 0.05$).

Cell lines and culturing*:* Anticancer activity screening for the tested extracts utilizing 5 different human tumor cell lines including liver HepG2, breast MCF-7, prostate PC3, colon HCT116 and lung A549 cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). The tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (GIBCO), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37 ºC in humidified atmosphere containing 5% CO_2. Cells at a concentration of 0.50 x 10^6 were grown in a 25 cm^2 flask in 5 ml of complete culture medium.

In vitro antiproliferative assay: The antiproliferative activity was measured *in vitro* using the Sulfo-Rhodamine-B stain (SRB) assay according to the previous reported standard procedure [30]. Cells were inoculated in 96-well microtiter plate (10^4 cells/ well) for 24 h before treatment with the tested extracts to allow attachment of cell to the wall of the plate. Test extracts were dissolved in DMSO and diluted with saline to the appropriate volume. Different concentrations of the extracts under test (0 - 100 µg/ml) were added to the cells. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the extracts for 48 h. at 37°C and in atmosphere of 5% CO_2. After 48 h cells were fixed, washed, and stained for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and attached stain was recovered with Tris-EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration is plotted to get the survival curve for each cell line after the specified time. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated and the results are given in Table 1.

Biochemical assays: The cells in culture medium were treated with 20 µl of 1/10 of IC_{50} values of fermented and unfermented pomegranate or the standard reference drug, doxorubicin, then incubated for 48 h at 37 ºC, in a humidified 5% CO_2 atmosphere. The cells were harvested and homogenates were prepared in saline using a tight pestle homogenizer until complete cell disruption for further biochemical analysis. The supernatants obtained after centrifugation of cell homogenates from HepG2, MCF-7 and HCT116 cell lines was used for further investigations and total cellular protein was assayed by the method of Lowry et al [31].

Determination the level of uPA protein expression: The urokinase (uPA) protein expression was determined using AssayMax human uPA ELISA kit (Assaypro, USA) according to manufacturer's instructions. The concentration of uPA in the samples was determined and the percentage of uPA inhibition was calculated as compared with control cancer cells (DMSO treated).

The effect of unfermented and fermented pomegranate on histone deacetylase (HDAC) activity: Histone deacetylase inhibitors represent a promising new class of compounds for the treatment of cancer. The activity of HDAC in the lysate of five cancer cells treated with fermented and unfermented pomegranate was measured using a colorimetric assay kit (BioVision, Mountain View, kit no. K331-100). The procedure involves the use of the HDAC colorimetric substrate (Boc-Lys (Ac)-pNA) and a trichostatin A (TSA), as a known inhibitor of HDAC activity. The activity was recorded as µM deacetylated substrate/mg protein from standard curve and was expressed as percentage of control untreated cells.

The effect of unfermented and fermented pomegranate on VEGF expression: The effect of fermented and unfermented pomegranate on the level of the vascular endothelial growth factor (VEGF) as a marker for angiogenesis was determined in five cell lines using a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit obtained from Glory Science Co., Ltd (TX 78840, USA). The level of VEGF in samples was calculated (pg/mg protein) as triplicate determinations from the standard curve.

The effect of unfermented and fermented pomegranate on elastase activity: The elastase activity is determined in cell homogenates by its catalytic effect on the *N*-succinyl-trialanyl-*p*-nitroanilide substrate releasing *p*-nitroaniline (*p*NA) which is measured photometrically at 405 nm [32].

Table 3: The effect of unfermented and fermented pomegranate on the elastase activity in different cell lines.

Compound	Elastase (U/mg protein)				
	HepG2	MCF-7	PC3	HCT116	A549
Doxorubicin	0.21±0.03	0.31±0.04	0.60±0.07	0.40±0.05	0.50±0.06
DMSO	1.98±0.08[a]	2.19±0.09 [a]	3.00±0.32	2.00±0.23	3.30±0.40[a]
Unfermented	0.42±0.05[b]	0.52±0.06 [b]	2.40±0.25 [a]	0.88±0.09[a, b]	2.70±0.30 [a]
Fermented	0.33±0.03[b]	0.46±0.05 [b]	2.20±0.21 [a]	0.80±0.07 [a,b]	2.33±0.26 [a]

Data were expressed as mean ± S.E. (n=3), [a] and [b] is significant difference from doxorubicin and DMSO - treated cells respectively at ($p < 0.05$).

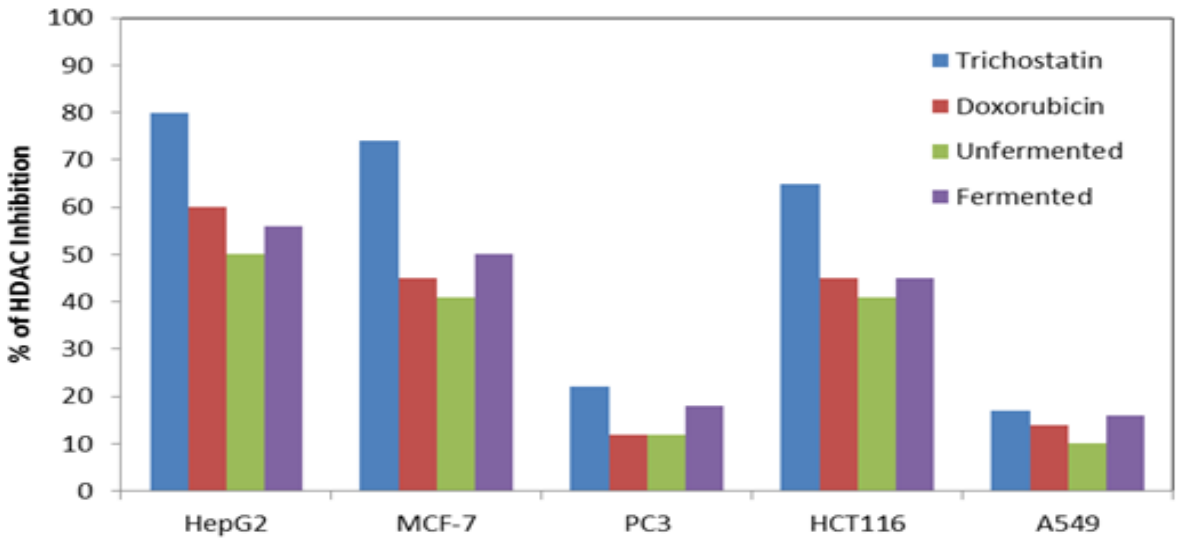

Fig. 2: The percent of HDAC inhibition of the unfermented and fermented pomegranate on different cell lines. Data were expressed as percentage changes as compared with control cancer cells (DMSO treated).

The elastase activity was determined as U/mg protein.

Statistical analysis: The results are reported as Mean ± Standard error (S.E.) for at least three experiments. Statistical differences were analyzed by one way ANOVA test followed by student's *t* test wherein the differences were considered to be significant at $p < 0.05$.

RESULTS

In vitro antiproliferative activity: The antiproliferative activity of unfermented and fermented pomegranate was evaluated against 5 different human cancer cell lines including liver HepG2, breast MCF-7, prostate PC3, colon HCT116 and lung A549 using SRB colorimetric assay, in comparison with doxorubicin as reference drug.

The antiproliferative activities are expressed by median growth inhibitory concentration (IC_{50}) and provided in Table 1. From the results it is evident that although fermented and unfermented pomegranate displayed potent growth inhibitory activity against HepG2, MCF-7 and HCT116, they had poor activity against PC3 and A549 cell lines.

In case of HepG2, fermented and unfermented pomegranate exerted antiproliferative activity with IC_{50} values of 28.40 ± 2.60 and 19.20 ± 1.90 µg/ml near to the IC_{50} of the reference drug, doxorubicin (IC_{50}: 20.10 ± 2.00 µg/ml). For MCF-7 the IC_{50} was 27.60 ± 3.00 and 23.80 ± 2.40 µg/ml (doxorubicin IC_{50}: 24.00±2.50 µg/ml). For HCT116 the IC_{50} was 24.60 ± 2.60 and 21.10 ± 1.96 µg/ml (doxorubicin IC_{50}: 19.25±2.00 µg/ml). It is clear that, while IC_{50} of both unfermented and fermented pomegranate was closed to the value of the doxorubicin in the three cell lines (HepG2, MCF-7 and HCT116), fermented pomegranate was more potent than the doxorubicin in HepG2 and MCF-7 and closed to doxorubicin in the HCT116 cell line.

Inhibitory effect of unfermented and fermented pomegranate on uPA: To identify the mechanism of action responsible for the cytotoxicity of unfermented and fermented pomegranate, the level of uPA protein expressed in the five cell lines were estimated quantitatively. The result revealed that the data of uPA expression were in consistent with the cytotoxicity activity.

Although the fermented and unfermented pomegranate did not showed activity against uPA in case of PC3 and A549 cell lines, they exhibited a good activity in HepG2 (80 and 85%); MCF-7 (79, 86%); HCT116 (63 and 77%) which reached near to the effect of the doxorubicin on HepG2, MCF-7 and HCT116 (88%, 91.50% and 84% respectively) (Figure 1). It is clear from the data that fermented pomegranate exerted good result comparing to unfermented.

The effect of unfermented and fermented pomegranate on VEGF level: To further investigate the involvement of VEGF in the unfermented and fermented pomegranate-induced anti-angiogenic effects against liver, breast, prostate, colon and lung cancer cell lines. Cells were treated with fermented and unfermented pomegranate. Results showed that the protein expression of VEGF was reduced after treatment with fermented and unfermented pomegranate in the five cell lines as shown in Table 2. The results revealed that fermented and unfermented pomegranate were found to be potent and selectively similar to doxorubicin against human VEGF as compared with control cancer cells. These results were consistent with cell cytotoxicity activity against the five cell lines. This reduction in VEGF level after protector administration could be related to the anti-angiogenic actions.

The effect of unfermented and fermented pomegranate on HDAC activity: The activity of HDAC in the lysate of five cancer cell lines treated with unfermented and fermented pomegranate, doxorubicin as well as Trichostatin, as a known inhibitor was measured and the data were calculated as percentage of inhibition as compared to the control cancer cells. While, treatment of hepatic HepG2, breast MCF-7, prostate PC3, colon HCT116 and lung A549 cancer cells with Trichostatin resulted in 80, 74, 22, 65 and 17% inhibition respectively as compared with control DMSO-treated cancer cells,

while the treatment with unfermented and fermented pomegranate resulted in significant inhibition in the activity of HDAC in the five cell lines (HepG2, MCF-7, PC3, HCT116 and A549) by 50, 41, 12, 41 and 10% in case of unfermented while decreased by 56, 50, 18, 45 and 16% in case of fermented (Figure 2).

Taken together, these findings suggested that there are correlation between the cytotoxicity of the both extracts and inhibition of the urokinase and histone deacetylase activities.

The effect of unfermented and fermented pomegranate on elastase activity: Several studies suggested that targeting the activity of elastase might be a beneficial antitumor therapy. In the present study, results showed that the activity of elastase enzyme was significantly increased in the five cancer cells. While treatment with unfermented and fermented pomegranate resulted in decrease in the activity of elastase activity in all cell lines especially HepG2, MCF-7 and HCT116 while the effect of fermented on the treated cell lines was more potent than the unfermented reached to the effect of doxorubicin (Table 3).

DISCUSSION

The investigation of tumor growth inhibitors is a major obstacle in the medical field [33]. For these reasons, the development of novel anticancer drugs is still necessary and has very much demand.

Most pomegranate (*Punica granatum* Linn., Punicaceae) fruit parts are known to possess enormous antioxidant activity. In our previous study we used the yeast candidate *Kluyveromyces marxianus* NRRL Y-8281, to enhance the antioxidant activities of pomegranate peel by modulating polyphenolic substances during solid state fermentation, in an attempt to increase the antioxidant properties of methanolic extract of pomegranate peels [10] and examined both unfermented and fermented pomegranate extracts for their cytotoxicity activity against five human cancer cell lines including liver HepG2, breast MCF-7, prostate PC3, colon HCT116 and lung A549.

The results of cytotoxicity test revealed that, although both unfermented and fermented pomegranate extracts exert cytotoxicity activity against HepG2, MCF-7 and HCT116, they had poor activity against PC3 and A549 cell lines. Moreover, fermented pomegranate was more potent than the doxorubicin in HepG2 and MCF-7 and closed to doxorubicin in the HCT116 cell line. The fermentation process appears to improve the anticancer activity of the pomegranate extract by enhancement the antioxidants properties of the extracts [10].

To identify the mechanism of action responsible for the cytotoxicity of unfermented and fermented pomegranate, the level of uPA protein expression and HDAC activity in the five cell lines were estimated. The result revealed that although the unfermented and fermented pomegranate did not showed activity against uPA and HDAC in case of PC3 and A549 cell lines, they exhibited a good inhibitory activity in HepG2, MCF-7 and HCT116 (Figure 1,2).

Taken together, these findings suggested that there are correlation between the cytotoxicity of the unfermented and fermented pomegranate and inhibition of the uPA and HDAC activities. The unfermented and fermented pomegranate exert anti-carcinogenic activity in HepG2, MCF-7 and HCT116 cancer cell lines through down-regulation the activity of these enzymes that may reduce the cell proliferation and resulted in significant growth inhibitory.

Angiogenesis is essential for tumor progression because tumor mass cannot grow bigger than 2 mm^3 without the nourishment of blood vessels. Moreover, vascularization is required for the process of extravasation in metastasis [24,34]. Hence, establishment of chemotherapeutic strategy by blocking angiogenesis attracts much attention in recent years. Treatment of cancer cells with unfermented and fermented pomegranate markedly decrease VEGF expression in the five cancer cell lines which correlated with the down regulation of elastase activity, which are consistent with the in vitro cytotoxicity of both extract especially fermented one. VEGFR-1 and VEGFR-2 are receptors distinctively present on vascular endothelial cells, which have strong association with cell proliferation, migration and induction of vascular permeability suggests that unfermented and fermented pomegranate could attenuate tumor progression via inhibition of angiogenesis and metastasis [35,36]. As a matter of fact, there is a close relationship between matrix metalloproteinases (MMPs) and VEGF in tumor progression, of which MMP-9 induces the release of biologically active VEGF in the culture of ovarian tumor cells and in ascites of ovarian tumor-bearing mice [37]. MMPs are secreted by recruited macrcophages, mast cells and fibroblasts, of which one important role of MMPs is to degrade and remodel the surrounding extracellular matrix (ECM) by proteolysis. When ECM is degraded, angiogenic factors being sequestered there in addition to cytokines and tumor cells will be released together, further facilitating the growth and infiltration of new blood vessels into the tumor [38]. The correlated interactions between VEGF, MMPs and other ECM-related proteases such as urokinase plasminogen activator (uPA) as well as their potential regulation by unfermented and fermented pomegranate during control of cancer progression were elucidated in this study.

Elastase is a broad-range proteolytic enzyme thought to be a tumor promoter involved in increasing tumor cell invasiveness by facilitating cell motility and transendothelial migration as it has the ability to degrade basement membrane and ECM glycoproteins such as elastin, fibronectin, as well as adhesive molecules and junctional cadherins [28]. Moreover, elastase considered to be protease that is able to degrade insoluble elastin, a structural component of elastic tissues such as blood vessel, skin, lung, liver and breast tissues [39]. Furthermore, Taniguchi et al [30] postulated that increased elastase destroy the barrier between tumor and the local circulatory system, either lymphatic or hematogenous, and result in at least loco-regional metastases. In the present study, results showed that the activity of elastase

enzyme was significantly increased in the five cancer cells. The treatment of the cancer cells with unfermented and fermented pomegranate, resulted in decrease in the activity of elastase activity of especially in fermented pomegranate in HepG2, MCF-7 and HCT116 treated cells. This finding confirmed that unfermented and fermented pomegranate are effective antimetastatic agents and there being a reasonably good correlation between the antimetastatic activity of these agents and their elastase inhibitory activity.

CONCLUSIONS

To summarize, we conclude that unfermented and fermented pomegranate is able to attenuate tumor growth by down regulation uPA and HDAC activities which may reduce the cell proliferation and resulted in significant growth inhibitory accompanied by direct effect on VEGF production and metastasis by inhibition elastase activity. In the present study, unfermented and fermented pomegranate were found to have multiple molecular targets. All these evidences suggest that unfermented and fermented pomegranate is a potential candidate for further development as an adjuvant in modern chemotherapeutic treatment of different types of cancers.

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