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# The Effect of Gnidilatimonoein from *Daphne mucronata*, on the Adhesive Property of Human Platelets

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## ABSTRACT

**The adhesive interaction between tumor cells and the host cells or the extracellular matrix plays a crucial role in tumor metastasis. To evaluate the mediation of cell adhesion by *Daphne mucronata*, an anti-cancer medicinal plant in Iranian folk medicine, the adhesion of thrombin activated human platelets to the cultured monocytes or HL-60 cells was investigated under the effect of the plant extract (0.54 mg/ml) or one of its purified components (gnidilatimonoein, 0.94 µM). Treatment of the platelets with the plant extract or the active component, for various time intervals, followed by their activation by thrombin resulted in 80-90% reduction in the number of monocytes with more than 10 attached platelets. Similarly, under almost all identical conditions, the adhesion of the activated platelets to HL-60 cells was decreased by 90%. The adhesion of thrombin activated human platelets to the plant treated HL-60 cells was also reduced significantly (by 95%). These data clearly indicate that *Daphne mucronata* is capable of mediating tumor metastasis through effecting cell adhesion properties of the cells.** *Iran. Biomed. J. 8 (3): 143-147, 2004*

*Keywords*: Cell adhesion, *Daphne mucronata*, Cancer, Metastasis

## INTRODUCTION

One of the major causes of mortality in cancer is metastasis, which is considered a multi-step cellular event. The pathogenesis of metastasis includes release of the tumor cells from the original site, entry into circulation, formation of homotypic (tumor cell to tumor cell) or heterotypic (tumor cell to platelets) aggregates (emboli), arrest of the emboli in the capillary bed of the target tissue, extroversion from the circulation and finally aggressive and selective growth in the target tissue [1-4]. It is believed that the formation of emboli, which results in protection of tumor cells from the invading immune system, is an important step in the metastatic cascade. It has been shown that the adhesive property among cells in emboli or the adhesion of tumor cells to the capillary beds is mediated by some plasma membrane glycoproteins, which serve either as receptors or ligands [5-8]. These surface molecules bind complementary molecules on adjacent normal or tumor cells or within the extracellular matrix (ECM). Thus, these molecules play an important role in tumor invasion and metastasis.

Various cytokines such as TNF, IL-1, IL-6 and GM-CSF, produced by tumor cells or the surrounding tissues, stimulate the expression of cell surface adhesion molecules [9, 10]. These surface molecules such as endothelial leukocyte adhesion molecule (ELAM-1), intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) usually mediate the adhesion or recruitment of neutrophils and lymphocytes to the stimulated vascular endothelium in the processes of tissue damage or inflammation [11, 12]. It is suggested that tumor metastasis takes place under the effect of cytokines, with a similar analogy to neutrophil recruitment to the damaged sites [12].

In our previous investigation [13], we found that treatment of breast-tumor bearing rats with the plant extract prevented the growth of the tumor compared to the untreated control rats. In this report, we investigated the effect of *Daphne mucronata* extract and its active component on tumor metastasis by studying the adhesiveness between activated human platelets and the cultured human monocytes or HL-60 cells.

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## MATERIALS AND METHODS

***Materials.*** The cell culture medium (RPMI 1640), FBS and penicillin-streptomycin were purchased from Gibco BRL (Life Technologies, Paisley, Scotland). The cell culture Petri dishes were obtained from Nunc. (Denmark). Cell lines were purchased from the Pasteur Institute of Iran (Tehran). Chloroform, diethylether and silica gel 60 were obtained from Merck (Germany), Silica gel G for TLC from Fluka (Sweden), The blood samples from the Iranian Blood Bank (Tehran, Iran), theophylline from Sigma (USA) and all the other chemicals used in this investigation have been obtained from Aldrich Chemical Co. (UK).

***Plant material and the purification of gnidilatimonoein.*** The collection and processing of the plant material, extraction and purification of gnidilatimonoein have been achieved as described in detail previously [14]. The molecular structure of gnidilatimonoein is shown in Figure 1.

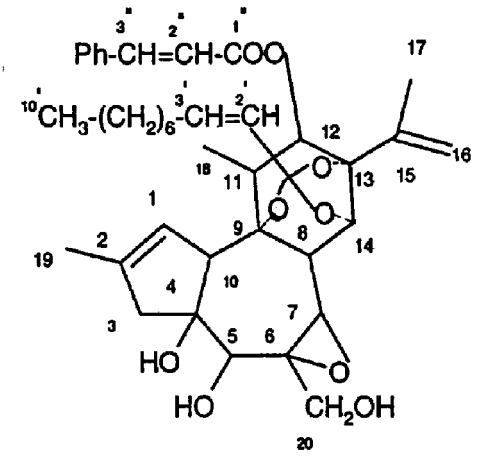



**Fig. 1.** The molecular structure of gnidilatimonoein.

***Platelet isolation.*** The human blood sample was centrifuged at 200 ×g for 10 min. The supernatant was separated and mixed in 1:1(v/v) portion with citrate-citric-dextrose (CCD) buffer (sodium citrate, 93 mM; citric acid, 7 mM; dextrose, 140 mM; adenosine, 5 mM and theophylline, 3 mM, pH 6.5). The platelet suspension was centrifuged at 18,000 ×g for 20 min. The pellet was resuspended again in CCD and centrifuged two times to remove the remaining red blood cells. The final platelet pellet was suspended in Hank's balanced salt solution (pH 7.2) [9].

***Monocyte isolation.*** The monocytes were isolated from human blood sample by the adherence technique [15]. The RPMI-1640 containing 10% FCS and heparin (10-15 IU/ml) was added to the blood sample in 1:1 ratio (v/v) in a Petri dish and kept at 37°C in an incubator with 5% $CO_2$. After 2 h of incubation, the medium was decanted out and the Petri dish was rinsed four times with heparin/PBS solution (containing per liter: NaCl, 8 g; KCl, 0.2 g; $Na_2HPO_4$, 1.15 g; $KH_2PO_4$, 0.2 g and heparin, 10 IU/ml; pH 7.2). The attached monocytes, devoid of red blood cells, were detached from the Petri dish using a rubber policeman, centrifuged and resuspended in RPMI-1640 in a final concentration of $2 \times 10^6$ monocyte/µl. The monocyte suspension, kept at 37°C, was used within 24 h after preparation.

***Treatment of platelets and HL-60 cells with the plant extract and gnidilatimonoein.*** The HL-60 cells ($1 \times 10^6$) were cultured in RPMI-1640 containing 10% FBS, streptomycin (100 µg/ml) and penicillin (100 U/ml) [16] and then were incubated at 37°C in an incubator with 5% $CO_2$ atmosphere for 24 h before plant treatments. Twenty-seven microliter of the diluted plant extract (1 to 50 equivalent to 0.54 mg plant leave powder) or the purified component (0.94 µM) were given to the cells once a day for two consecutive days. The non-toxin doses of the plant extract and gnidilatimonoein have been worked out separately (data not shown). The cell viability was accessed by trypan blue exclusion test.

***Cell adhesion assay.*** This assay was performed according to the Larsen's method [17]. The treated or untreated platelet ($2 \times 10^8$ cells.ml$^{-1}$) was activated by thrombin (0.25 U.ml$^{-1}$) at 22°C for 20 min. Twenty microliter of this activated platelet suspension was mixed with an equal volume of the cell suspension (monocytes or HL-60 cells at $2 \times 10^6$ cells.ml$^{-1}$). The cell mixture was incubated at 22°C for 20 min. An aliquot of the cell suspension was counted and the percentage of the cells with various numbers of platelets attached to them was evaluated.

## RESULTS AND DISCUSSION

The effect of *D. mucronata* crude extract and one of its purified active components (gnidilatimonoein) on the adhesive property of the activated platelets to human monocytes are shown in Figure 2.



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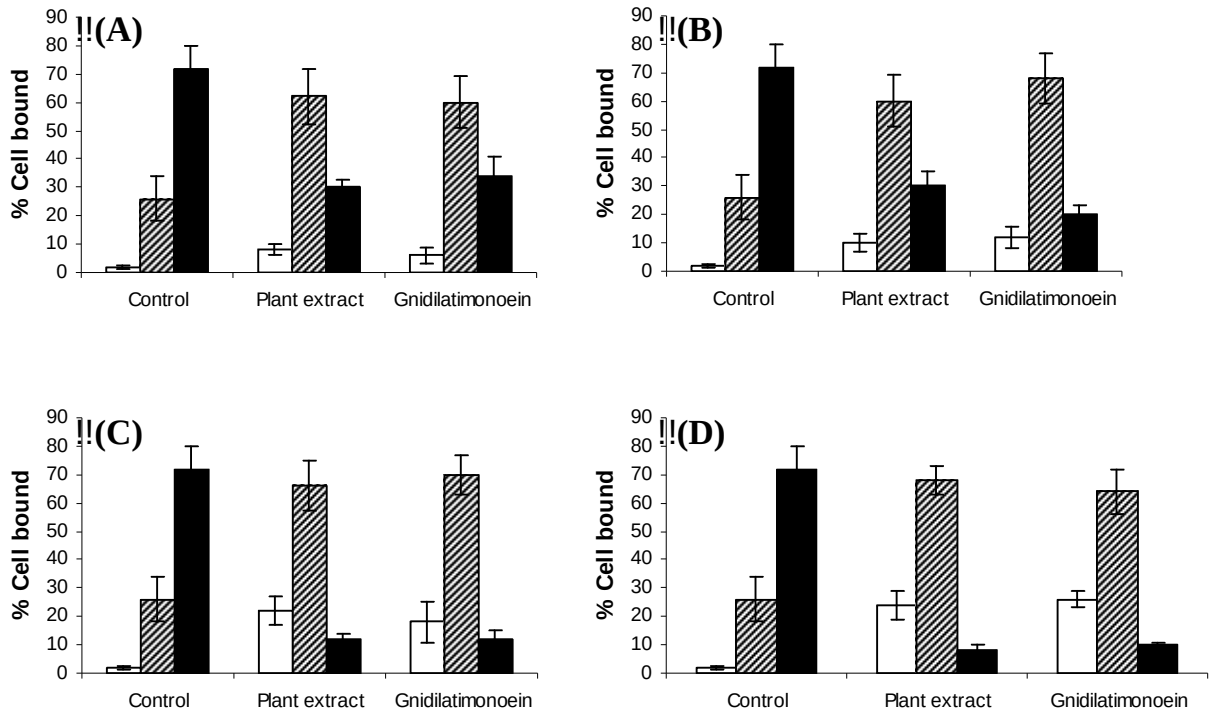



**Fig. 2.** Time dependant effects of the diluted plant extract (27 µl.ml$^{-1}$ equivalent to 0.54 mg of plant leaf powder) or gnidilatimonoein (0.94 µM) on the adhesive property of thrombin activated platelets to human monocytes. The platelets were incubated with a single non-toxic dose of the stimuli for half **(A)**, one **(B)**, two **(C)** and twenty-four **(D)** h and then stimulated with thrombin (0.25 U/ml) for twenty min. The activated platelets ($2 \times 10^8$ cells/ml) were added to $2 \times 10^6$ cells/ml human monocytes and incubated at 22°C for twenty min followed by inspection and scoring of the monocytes with various numbers of attached platelets: monocytes with less than five ( □ ), between 5 to 10 ( ▨ ) and more than 10 ( ■ ) attached platelets.

The biological activities of gnidilatimonoein and the crude extract have been previously measured by the brim shrimp test [18]. In scoring the monocytes with attached platelets, we counted 100 monocytes in three categories: (a) monocytes with more than 10 attached platelets; (b) monocytes having 5 to 10 attached platelets and (c) monocytes with less than 5 attached platelets.

In this investigation, the variation in the number of monocytes with more than 10 attached platelets, under the effect of the plant extract or gnidilatimonoein, has been the basis for evaluating the adhesive properties of the cells. According to Figure 2A, normally about 72% of monocytes are usually saturated with more than 10 activated platelets and about 26% of them contain between 5 to 10 attached platelets and the remaining 2% contained only 5 or less attached platelets. However, treatment of platelets for various time intervals changed this distribution so that the majority of monocytes (60-62%) had less than 10 attached platelets and the number of monocytes with more than 10 attached platelets dropped to almost 8%, after platelets were treated with the plant extract or gnidilatimonoein for two hours. Based on these data, we may conclude that in 1-2, h the plant extract or its active component will exert their full effects on the platelets, probably through the molecular cell surface modifications which is reflected as changes in the cell adhesive properties.

Similarly, Figure 3 indicates that treatment of platelets, for various time intervals, with the plant extract or gnidilatimonoein followed by 20 min of activation with thrombin, changed the pattern of the platelet adhesions to HL-60 cells. In the absence of treatment of the platelets, only 20% of HL-60 cells were covered with more than 10 platelets and the majority of HL-60 cells had between 5 to 10 attached platelets. However, after treatments of platelets with the crude extract or gnidilatimonoein, only 2-4% of HL-60 cells contained more than 10 platelets per cell. Treatment of platelets with the plant extract or gnidilatimonoein for 24 h also resulted in drastic changes in platelet distribution pattern on HL-60 cells. As it is evident from Figure 3A, before platelet treatments, the majority of HL-60 cells (60%) contained 5-10 attached platelets.



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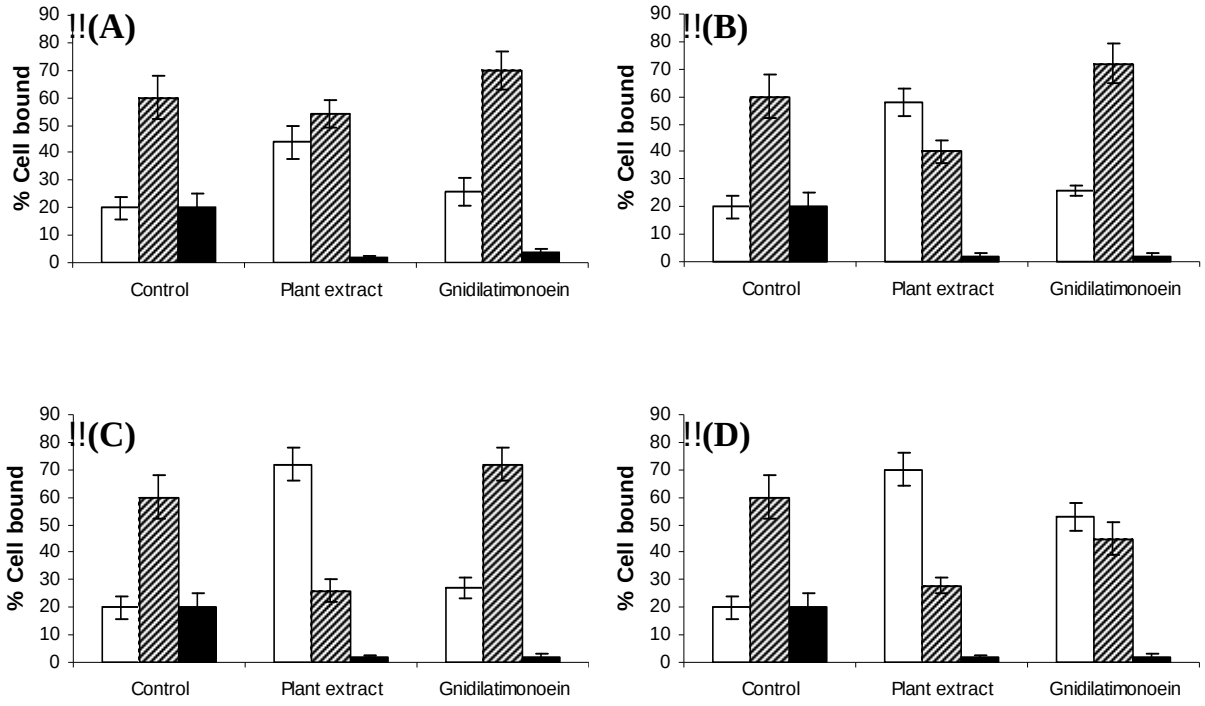



**Fig. 3.** Time dependant effects of the plant extract or gnidilatimonoein on the adhesive property of the activated platelets to HL-60 cells under all identical conditions presented in Figure 2.

However, keeping the platelets under the effect of plant extract or gnidilatimonoein for 24 h reduced this population to 28% and consequently increased the population of HL-60 cells with less than 5 attached platelets to 70%. Based on the cumulative data of Figures 2 and 3, we can clearly conclude that the plant extract or its purified active component has induced some cell surface changes in the platelets resulting in their adhesive properties to human monocytes and HL-60 cells.

In order to evaluate the same phenomenon on HL-60 cells, under the effects of the plant extract and gnidilatimonoein, we treated the HL-60 cells with a single safe dose (27 µl, equivalent to 0.54 mg of the plant leave powder) or gnidilatimonoein (0.94 µM). The cells were separated from the medium by centrifugation and resuspended in RPMI-1640 medium. The attachment of the activated platelets to these cells was than evaluated. Based on the results presented in Figure 4, it is evident that only 1% of HL-60 cells contained more than 10 attached platelets compared to the 20% in the control sample. In addition, the majority of the treated HL-60 cells (88%) contained less than 5 attached platelets compared to the 20% of the untreated HL-60 cells in the control group. The purified compound (gnidilatimonoein) exerted almost the same effect, as the crude extract on the adhesive property of HL-60 cells. Based on Figure 4, we can clearly state that the capability of HL-60 cells to get saturated with activated platelets is drastically modified.

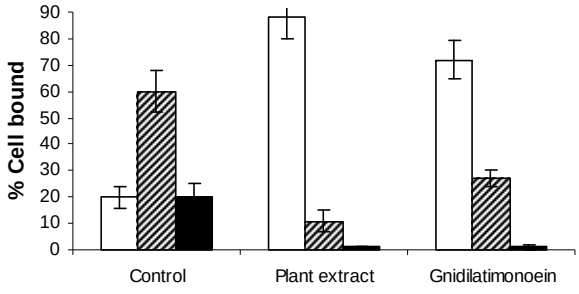



**Fig. 4.** Variation in the adhesive properties between thrombin activated platelets and the plant treated HL-60 cells. The HL-60 cells were treated with a non-toxic dose of the diluted plant extract (27 µl equivalent to 0.54 mg plant leaf powder) or gnidilatimonoein (0.94 µM) for 24 h. The cells were centrifuged and resuspended in RPMI-1640 medium in a concentration of $2 \times 10^6$ cells/ml. An aliquot of HL-60 cells (20 µl) was mixed with 20 µl ($2 \times 10^8$ cell/ml) of thrombin-activated platelets. The mixture was incubated at 22°C for 20 min followed by scoring HL-60 cells with different number of attached platelets: HL-60 cells with less than five ( □ ), between 5 to 10 ( ▨ ) and more than 10 ( ■ ) attached platelet.



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The accumulated data of Figures 2 to 4 clearly show that the adhesive affinity of platelets for monocytes and/or HL-60 cells is reduced by *D. mucronata*. These observations may suggest a mechanism of action for *D. mucronata* in reducing the rate of breast-tumor metastasis, which has been observed in our laboratory using animal models.

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