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A PARADOX FROM A NEUROTRANSMITTER: THE OPPOSITE EFFECTS OF SUBSTANCE P AND ITS PHYSIOLOGICAL FRAGMENTS SP (1-7) AND SP (9-11) ON THE PROLIFERATION OF HUMAN BREAST CANCER CELLS AND THE MIGRATION CAPABILITIES OF HUVEC CELLS

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Abstract – *Objective:* *Substance P (SP) is a neurotransmitter peptide, and it is known to play a major role in neurogenic inflammation, inducing local inflammatory responses, cancer, and angiogenesis. It has been previously demonstrated that SP possesses a wide range of pharmacological effects including induced cancer cell proliferation and triggered angiogenic response of tumors. However, there is no published work regarding the proliferative effects of its physiological fragments [N-terminal fragment SP (1-7) and C-terminal fragment SP (9-11)] not only on cancer cells but also on human umbilical vein endothelial cells (HUVECs).*

Materials and Methods: *Cells were treated with different doses of fragments and the cytotoxic effects on the proliferation of MDA-MB-231 cells were determined via MTT kit (Promega, Madison, WI, USA) and Cellular DNA Fragmentation Elisa Kit (Roche). Changes both in the pro-angiogenic and anti-angiogenic factor levels in media were evaluated by; Human VEGF ELISA kit (Thermo-Scientific, Waltham, MA, USA), Human Matrix Metallo-proteinases-9 Elisa kit (Legend Max), and Human Thrombospondin-1 Elisa kit (R&D Systems) for VEGF, MMP-9, and Thrombospondin-1 respectively. To determine the changes in the migration capabilities of HUVECs, a wound healing test was performed.*

Results: *According to our results, the fragments exhibit different effects not only on the proliferation of cancer cells and the release of pro/anti-angiogenic factors from the cells but also on the migration capabilities of HUVECs.*

Conclusions: *This is the first report showing that the SP fragments could behave differently from the main peptide itself.*

KEYWORDS: *Substance P, N-terminal Fragment SP (1-7), C-terminal Fragment SP (9-11), Proliferation, Anti-Angiogenesis.*

INTRODUCTION

Substance P (SP), the most important neuropeptide in cancer, inflammation, and angiogenesis, is released from the peripheral nerve C fibers. SP prolongs cancer progression and metastasis. SP is encoded by the pre-pro-tachykinin A (PPT-A) l gene and is a member of the tachykinin family which triggers the proliferation of cancer cells and the angiogenesis of tumors.

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That is why it is also the most important neuropeptide in cancer[1].

On the other hand, SP appears to show a biphasic effect on tumor growth and carcinogenesis; these effects might be due to the opposite effects of SP fragments and the main peptide, such that intact peptide is proliferative, whereas the fragments produced by proteolytic enzymes are anti-tumorigenic and theoretically anti-angiogenic[2]. According to our previous study, we showed that in contrast with intact peptide, the fragments of SP (4-11) and SP (6-11) exhibit inhibitory effects on 4T1 mouse breast cancer cell proliferation. SP (1-7) did not show the same effect on this cell line[3].

It has been shown that SP is expressed in 90% of metastatic neuroblastoma cells in the bone marrow, 68% of invasive malignant melanoma, 40% of metastatic melanoma, and 60% of in situ melanomas. SP has been found in many tumors, especially in the periphery of the tumor[4,5]. On the other hand, especially in breast cancer, SP has been detected in both cytoplasm and nuclei of tumor cells in keratocystic odontogenic tumors, oral squamous cell carcinoma, and laryngeal carcinoma tissues, compared to normal breast epithelial cells. SP-specific receptor Neurokinin-1 (NK-1) has also been shown to increase expression in many cancerous cells compared to normal cells, and the increase in the number of receptors is thought to correlate with malignancy. Also, NK-1 receptors were found in intra and peri-tumoral blood vessels. Besides, SP stimulates vascular growth and increases endothelial cell proliferation[6]. In recent years, 4 or more isoforms of the NK-1 receptor have been found in human tumor cell lines, but their functional roles remain unknown. Data show that the same NK-1 receptor isoforms are expressed in many types of cancer[7, 8]. In our previous studies, we showed that SP release from mouse breast cancer cells (4T1 and 4THMpc) was dramatically increased as compared with control groups. Based on our results, we suggested that the combination of thalidomide and radiotherapy, especially in the treatment of metastatic breast cancer patients, may cause an increase in the amount of substance P and thus cause tumor growth[3].

In another study [9], we showed that radiotherapy in the 4T1 cell line increases the amount of substance P when administered alone and in combination with endostatin; it decreases the amount of substance P both released into the medium and within the cell. In the same study, we have shown that the application of endostatin alone does not affect the amount of substance P released either in or within the 4THMpc cells. However, radiotherapy alone significantly reduced the amount of Substance P released to the medium whereas radiotherapy alone did not change the amount of intracellular substance P[9].

On the other hand, in one of our recent publications, it has been shown that radiotherapy affected the ADAM 10, and Neprilysin enzyme activities, which break down substance P in 4T1 and 4THMpc cell lines. In the 4T1 cell line, Thalidomide alone did not affect ADAM 10 enzyme activity, while radiotherapy alone inhibited this enzyme activity by 42%. However, with the combined treatment, the inhibition rate rises to 89%.

In the 4THMpc cell line, Thalidomide alone inhibits ADAM 10 enzyme activity by 66.6%. Radiotherapy alone inhibits enzyme activity by 33.3%, while combined therapy causes a 40% inhibition. In terms of the enzyme activity of neprilysin, we showed that thalidomide alone increases the enzyme activity of neprilysin by 40.9% in the 4T1 cell line and that radiotherapy alone or combined causes a 40.7% increase in this cell line. As a result, in that article, we have suggested that Thalidomide can break down substance P by increasing the enzyme activity of neprilysin and thereby exhibit anti-angiogenic effects[2].

Considering all these data, it has been suggested that the fragments released as a result of the breakdown of the SP may have similar or different biological effects compared to the main peptide. However, there were not any *in vitro* or *in vivo* studies investigating the role of Substance P fragments on angiogenesis in literature.

Angiogenesis, the formation of new blood vessels from the existing ones, is regarded as a significant characteristic of cancer cells, and depending on the facilitation of metastasis it is also essential for the growth of tumors[10]. It has been reported that SP exhibits its angiogenic effects directly on the migration and tube formation abilities of endothelial cells. It also promotes the proliferation of venular endothelial cells. However, there have been no prior studies about the effects of Substance P fragments on the angiogenic process both in pro/anti-angiogenic factor levels released from the cancer cells and on the migration capacities of HUVECs.

Therefore, in this study, we aimed to investigate the possible roles of SP fragments on angiogenesis *in vitro*. We planned to carry out this research in 2 different cell types since there is no study on the angiogenic and/or anti-angiogenic properties of SP fragments. In this context, our first target was to detect non-cytotoxic doses of SP fragments in the human breast cancer cell line that we selected as a model. After determining

the doses, the possible effects of the fragments on VEGF (Vascular Endothelial Growth Factor), MMP-9 (Matrix Metallopeptidase 9), and Thrombospondin-1, which are the most important factors in the angiogenesis process, were evaluated. These selected factors are the first to be studied in all studies investigating the anti-angiogenic properties of any active substance.

However, the angiogenesis process is based on the proliferation, migration, and tube-forming properties of blood vessels. Therefore, we also aimed to determine the possible effects of fragments on vascular endothelial cells. In this context, the migration test showed whether the fragments affected the migration ability of the vascular endothelial cells directly.

As mentioned above, this study aims to investigate the possible anti-angiogenic effects of SP fragments on the levels of two proangiogenic factors (VEGF and MMP-9) and one anti-angiogenic factor (Thrombospondin-1) released from human breast cancer cell line MDA-MB-231 and the direct effects of the fragments on the migration capabilities of Human Umbilical Vein Endothelial Cell Line HUVEC.

MATERIALS AND METHODS

Cell culture conditions

MDA-MB-231 (ATCC® HTB-26™, Manassas, VA, USA) and HUVEC-C (ATCC® CRL-1730™, Manassas, VA, USA) cells were cultured in RPMI (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 10 µg/mL gentamicin (Genta, İ.E. Ulagay İlaç Sanayi Türk A.Ş. Turkey) and 5% sodium pyruvate (Gibco, Carlsbad, CA, USA). The cells were incubated in 5% CO_2 with 95% humidity at 37°C.

Cell proliferation

Cell proliferation was estimated using a CellTiter 96 aqueous nonradioactive cell proliferation assay (Promega, Madison, WI, USA), which is based on the cleavage of 3-(4,5 dimethylthiazol-2-yl)- 5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium (MTS) into a formazan product which is soluble in tissue culture medium. Briefly, cells were seeded at 5×10^3 cells per well in 200 µl complete medium onto 96-well plates and allowed to attach for 24 h. After the cells reached 80–90% confluence, the media was removed, and cells were washed with phosphate-buffered saline (PBS). Subsequently, cells were treated with different concentrations of SP fragments (10 µg; 1 µg; 100 ng; 10 ng and 1 ng) and were prepared in 1% FBS containing a complete medium. Each treatment was performed in eight-well replicates. The cells were grown at 37°C for 24 h, 48 h, and 72 h. The medium was gently aspirated to terminate the experiment, 180 µl serum-free complete medium and 20 µl of MTT were added to each well, and cells were incubated for 4 h. The absorbances at 450 nm were measured in a microplate reader (Thermo Labsystems Multiskan Spectrum, Thermolabsystem, Chantilly, VA, USA) using wells without cells as background. Sample readings were calculated by subtracting the average of background absorbances. All experiments were performed at least four times.

DNA fragmentation assay

To correct the proliferation test results and to figure out the primer reason for the cell death, Human Cellular DNA Fragmentation ELISA kit (Roche, Thomas Scientific, Swedesboro, NJ USA), assay was used. $1x10^6$ cells were seeded to six-well plates and incubated for 24 hours. After the incubation period, cells were treated with three doses (100 ng; 10 ng and 1ng) of both fragments, and experiments were terminated at the end of the 24 h incubation period. Samples were examined according to manufacturers' protocol. Absorbances were read at 370 nm with a microplate reader (Thermo Labsystems Multiskan Spectrum, Thermo Lab System, Chantilly, VA, USA).

Determination of VEGF, MMP-9, and Thrombospondine-1 levels by ELISA

To determine the possible effects of SP fragments on VEGF (Quantikine Human VEGF ELISA kit; R&D Systems Inc., Minneapolis, MN, USA), MMP-9 (SensoLyte Human MMP-9 ELISA kit; Anaspec, Fremont, CA, USA), and Thrombospondine-1(Human Thrombospondine-1 Elisa Kit R&D Systems Inc., Minneapolis, MN, USA) levels released from MDA-MB-231 cells were used following the manufacturer's protocols. Human VEGF, MMP-9, and Thrombospondine-1 standards were diluted and used as standards. Serial dilutions (starting from 1000 pg/mL to 15. 6 pg/mL) were used to establish the standard curve. Average results from four independent experiments were used to compare non-treated and treated cells.

Migration assay

Whether SP fragments have a direct effect on the migration properties of vascular endothelial cells was demonstrated by a modified scratch wound healing assay based on the literature. HUVECs ($25x10^5$ per mL) were seeded in a 35 mm- plate until a monolayer formed for 24 hours. A 5 mL syringe without a needle was used to draw a smooth scratch through the cells. Then, the cells were cultured in medium with 1% FBS containing 10 ng of SP, SP (1-7), and SP (9-11). The cells were then incubated for 24 h. Photos were taken at the beginning (Time 0) 12 and 24 hours after the treatment via a Euromex Microscope (Arnhem, The Netherlands) (with 10x Objective) and compared with non-treated (Control) and SP-treated (Positive control) groups. The photos were analyzed using ZEN 3.1 Blue edition software (Göttingen, Germany). The gap closure was calculated as below:

$$\% \text{ of gap healing} = (W_0 - W_h)/ W_0 \times 100$$

In this equilibrium, W_0 shows the gap width at 0 hours (Time 0), Wh shows the gap width at n hour. Data were acquired from three independent experiments.

RESULTS

Cell proliferation results

In this study, the effects of SP fragments on cell viability were evaluated by MTT test at the end of three different incubation times (24, 48, and 72 hours). Both fragments with a concentration of 100 ng showed a cytotoxic effect after 24 hours incubation period. SP exhibited the expected proliferative effect. As we have suggested in our hypothesis, both of the fragments showed cytotoxic effects in different doses, especially during the incubation periods of 24 and 48 hours.

According to our results, the high doses of fragments have an anti-proliferative effect, unlike the main peptide SP. At its lowest doses (1 ng), SP (1-7) also showed a proliferative effect, like SP, while the C-terminal end SP (9-11) showed an anti-proliferative effect for all three incubation times. At this point, the hypothesis "Fragments have the opposite effect of SP" has been confirmed at least in terms of proliferation.

The opposite effects of fragments on the proliferation of MDA-MB-231 cells for 24, 48, and 72 hour incubation periods were shown in Figures 1, 2, and 3, respectively.

According to this result, both of the N and C –terminal fragments had cytotoxic effects on MDA-MB-231 cells. As it is seen from Figure 1, SP (9-11) had a strong cytotoxic effect in all doses except the lowest one. However, these cytotoxic effects were lower than Doxorubicin-HCl. Doxorubicin-HCl results which merged these data were obtained from our previous study[11].

After the 48-hour incubation period, both fragments showed cytotoxic effects on MDA-MB-231 cells. However, this cytotoxic effect was in contrast to the two fragments. For the N-terminal fragment SP (1-7), the cytotoxicity decreased towards its higher concentration. In opposite, for

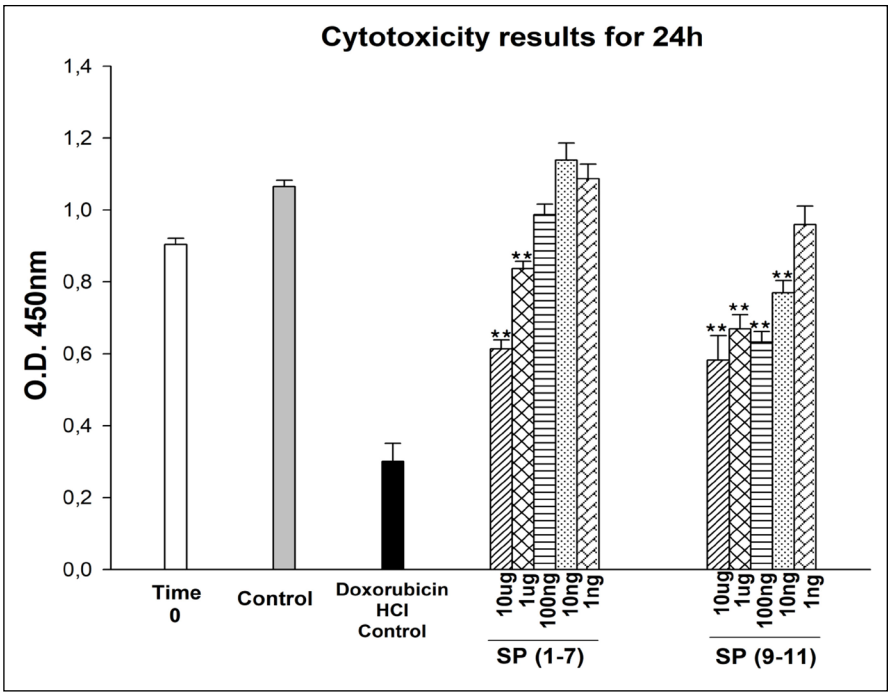

Fig. 1. Cytotoxic effects of fragments on cell proliferation for 24-hour incubation period were shown. The time 0 bar showed the initial cell amount at the beginning of the experiment. The control bar reflects the non-treated group. Doxorubicin-HCl was used as a positive control for this experiment (p <0.001).

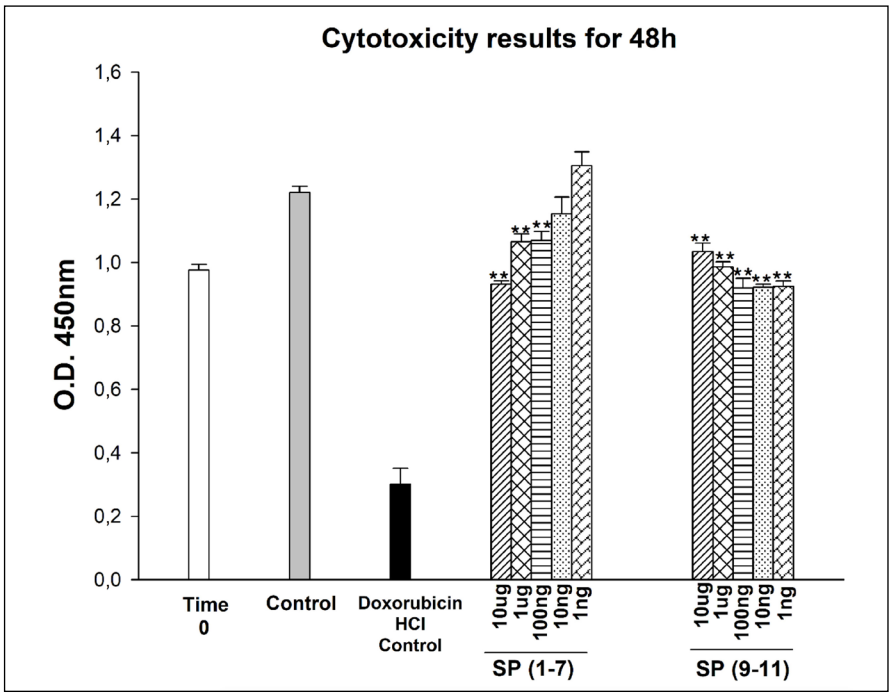

Fig. 2. Cytotoxic effects of fragments on cell proliferation for 48-hour incubation period were shown. The time 0 bar showed the initial cell amount at the beginning of the experiment. The control bar reflects the non-treated group. Doxorubicin-HCl was used as a positive control for this experiment (p <0.001).

the C-terminal fragment SP (9-11), the cytotoxicity increased towards its higher concentration.

As seen in Figure 3, similar cytotoxic effects were observed for both fragments. Interestingly, for 72-hour incubation period results, N-terminal fragment SP (1-7) exhibited a biphasic effect. It caused a strong cytotoxic result up to its higher concentration (10 ng); however, surprisingly it also showed a proliferative effect on MDA-MB-231 cells. The other puzzling result was that the minimal concentration caused the most significant cytotoxicity on this cell line.

In sum, in contrast to the proliferative effect of SP, both fragments have cytotoxic effects.

DNA fragmentation assay results

DNA fragmentation analysis is one of the most important markers that reveal whether cell death occurs by the apoptotic pathway or not. Since the anti-proliferative effects can be observed during the 24-hour incubation period, DNA fragmentation was also evaluated at the end of the 24-hour

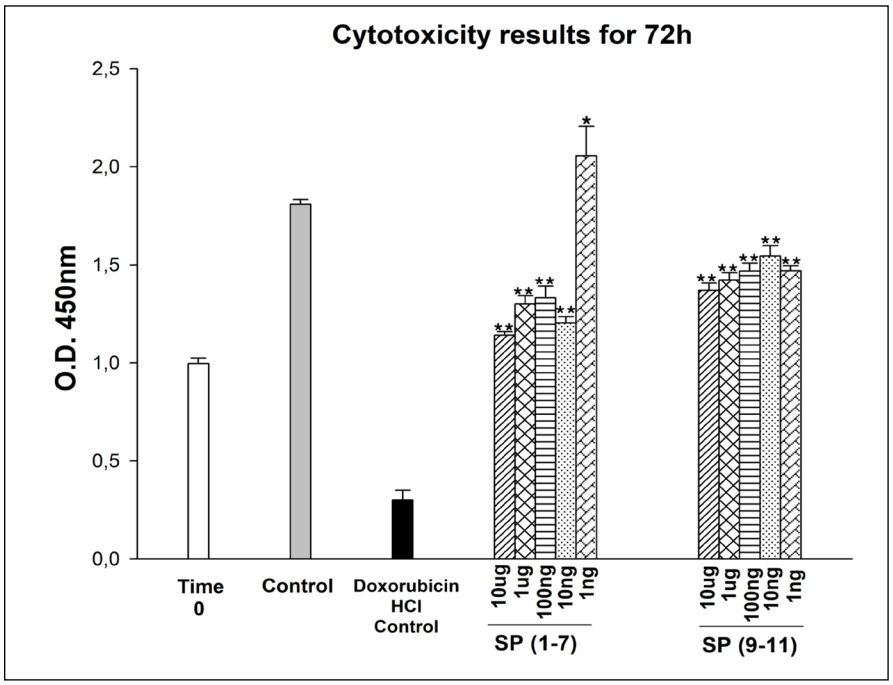

Fig. 3. Cytotoxic effects of fragments on cell proliferation for 72-hour incubation period were shown. The time 0 bar showed the initial cell amount at the beginning of the experiment. The control bar reflects the non-treated group. Doxorubicin-HCl was used as a positive control for this experiment (p <0.01; p <0.001).

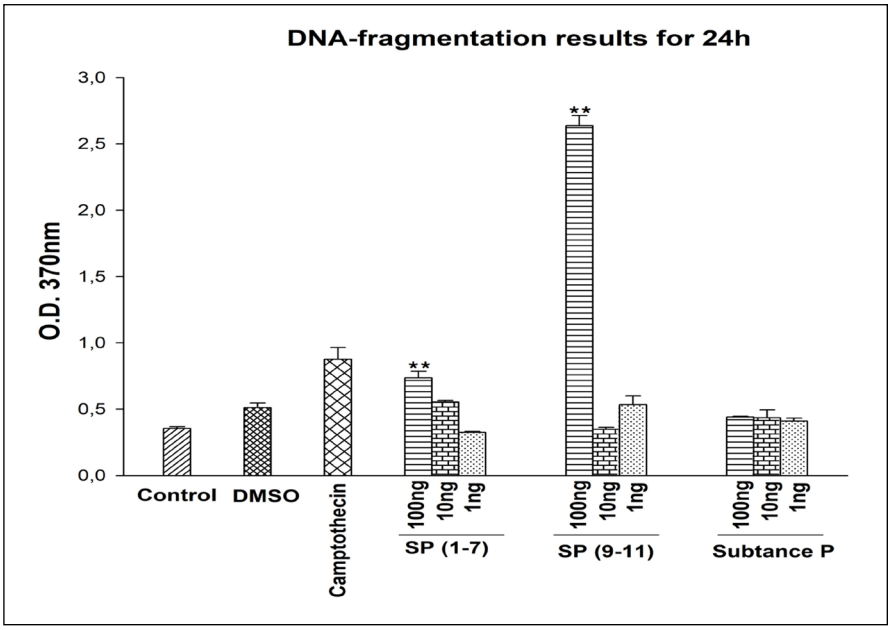

Fig. 4. The effects of fragments on DNA breaks depending on their cytotoxicity for a 24-hour incubation period were shown. The control bar showed the amount of DNA fragmentation in the non-treated control group. Camptothecin and its solvent DMSO are used as positive controls. SP was used for another control to compare its effect to the fragments (p <0.001).

incubation period. Camptothecin, which is known to kill cells by creating fractures in DNA in experiments, was used as a positive control. According to the MTT test results, DNA fragmentation experiments were performed in three doses (1, 10, and 100 ng / mL), in which we had an anti-proliferative effect. The results were given in Figure 4.

According to results shown in Figure 4, while SP did not cause significant DNA fragmentation, SP (1-7) and SP (9-11) at all three doses caused a statistically significant increase in the amount of DNA fragmentation, unlike the main peptide itself. Similar to the cytotoxic effect, SP (9-11), which contains the C terminal end of the SP, had an opposite effect on DNA fragmentation. Especially, both highest doses of fragments caused DNA breaks. According to these results, we suggest that SP fragments show their cytotoxic effects by creating fractures in DNA.

VEGF, MMP-9, and Thrombospondin-1 Elisa Assay Results

The effects of fragments at three doses which were determined according to the cytotoxicity test results, on the amount of VEGF (48-hour period), MMP-9 (72-hour period), and Thrombospondine-1

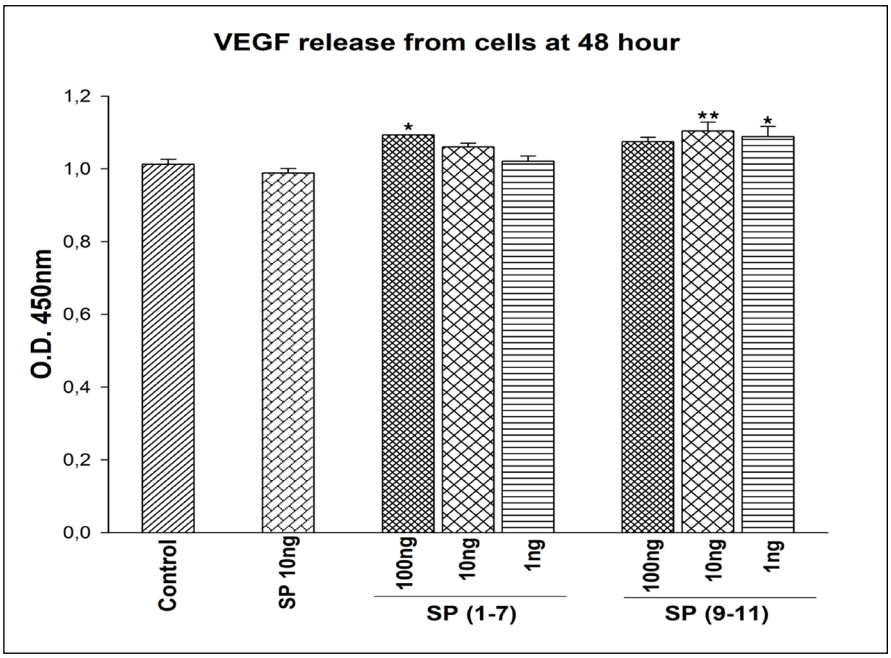

Fig. 5. The effects of fragments on VEGF release from MDA-MB-231 cells after 48-hour incubation period were shown. The control bar showed basal VEGF release in the non-treated control group. SP (10 ng) was used as a positive control to compare to the fragments (p <0.01; p <0.001).

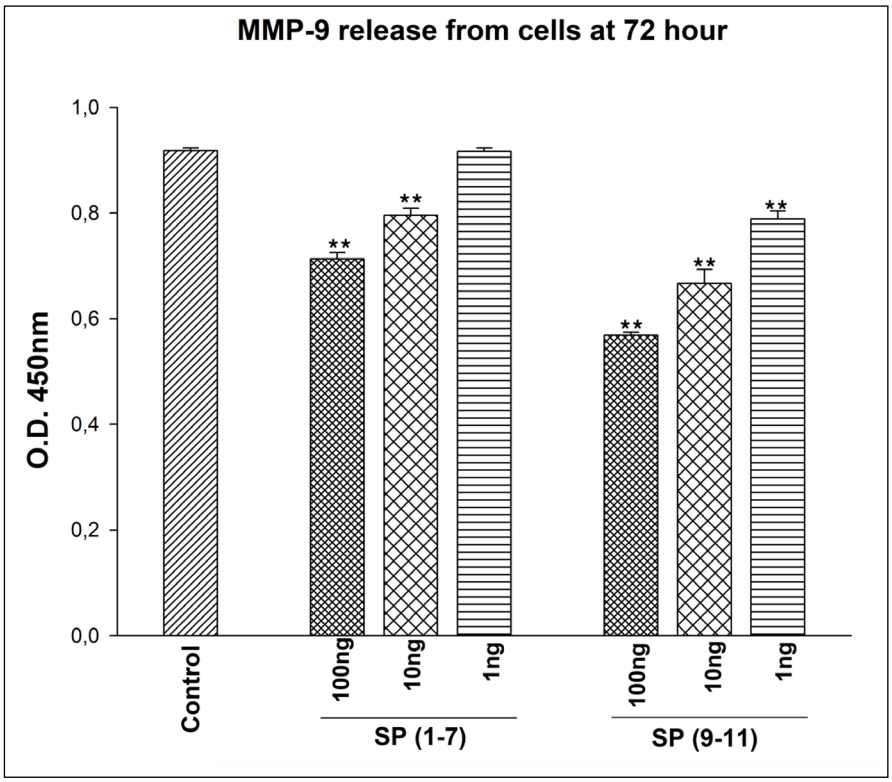

Fig. 6. The effects of fragments on MMP-9 release from MDA-MB-231 cells after a 72-hour incubation period were shown. The control bar showed basal MMP-9 release in the non-treated control group (p <0.001).

(48-hour period) released from MDA-MB-231 cells into the medium, were determined and given in Figure 5, Figure 6, and Figure 7, respectively.

As can be observed from Figure 5, the amount of VEGF released from the MDA-MB-231 cells was increased as compared to the control group. Interestingly, this increase was found statistically significant at the highest concentration in the application of the 1-7 fragment of the substance P, which is the N-terminal end of the substance P but was also significant at the low doses of the C-terminal end. Substance P itself also increases the amount of VEGF alone. In this context, both substance P fragment administration in the MDA-MB-231 human breast cancer cell line has a similar effect to the main peptide.

The amount of MMP-9 released from MDA-MB-231 cells instead of fattening was measured at the end of the 72-hour incubation period and the results are given in Figure 6. Both fragments cause a decrease in the amount of MMP-9 compared to the control group. Therefore, for both fragments, they exhibit anti-angiogenic behavior as they cause a decrease in the amount of MMP-9 released from cells.

Another protein that we investigated based on the possibility that the fragments might exert their anti-angiogenic effects by increasing the amount of an anti-angiogenic factor was Thrombospondin-1 (TSP-1). Amounts of thrombospondin-1 released from MDA-MB-231 cells instead of fattening were measured at the end of the 48-hour incubation period and the results are presented in Figure 7.

According to our results, the amount of thrombospondin-1 released from the MDA-MB-231 cells to the medium significantly decreased in Substance P (10 ng / mL) and fragment 9-11 (1 and 100 ng / mL) treated groups compared to the control group. A significant increase was found in the highest dose of fragment 1-7 as compared to control.

Considering all these results, Substance P and its N-terminal and C-terminal fragments showed different effects depending on their concentration and application times in terms of both cytotoxic and anti-angiogenic effects.

Migration Test Results

To figure out the migratory effects of SP fragments due to the induction of endothelial cell motility was assessed by the migration test. The results are given in Figure 8.

In migration experiments, we tested the effect of SP fragments on the migration abilities of HUVEC cells, and we have illustrated the size of the wound after the wound was closed at certain hour intervals (time 0, 6,12, and 24 hours) and calculated the area size in mm. The lengths of the opened spaces (wounds) were marked on the pic-

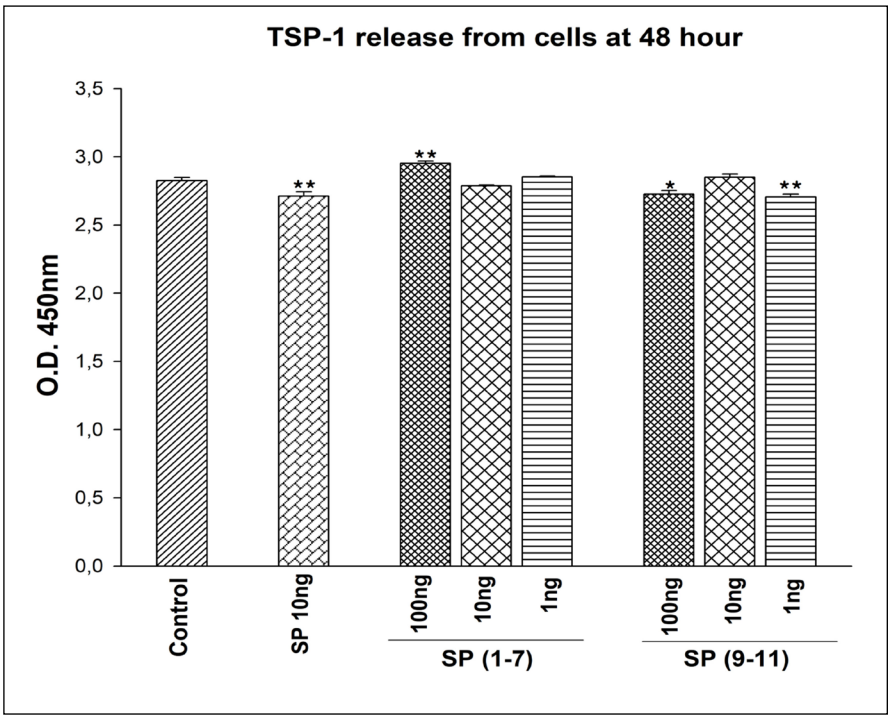

Fig. 7. The effects of fragments on TSP-1 release from MDA-MB-231 cells after 48-hour incubation period were shown. The control bar showed basal MMP-9 release in the non-treated control group. SP (10 ng) was used as a positive control (p <0.01; p <0.001).

tures at the level of mm. The starting time was set to "time O" and these measurements were done immediately after opening the wound at the beginning of the experiments.

Using the distances at 0 hours, 6 hours, 12 hours, and 24 hours in total, how much the wound closed within 24 hours was calculated as "% closure" by proportioning the control group for each dose of each fragment (Table 1).

According to the experimental results, there was a significant reduction in wound closure for all doses of both fragments as compared to the control group. We showed that both of the fragments suppress the migration abilities of HUVEC cells and cause a significant reduction in the wound healing rate.

DISCUSSION

The main point that we wanted to investigate in this study was to figure out any anti-angiogenic properties of SP fragments opposite of the intact peptide, itself. Two opposite fragments of SP were chosen for this study. These are Fragment 1-7 (SP (1-7)) and Fragment 9-11 (SP (9-11)).

SP is released from the sensory nerve fibers and it is one of the most important cancer-associated neurotransmitters. While studies on SP itself and NK-1 receptor antagonists continue intensively, several synthetic SP fragments have been shown to inhibit cancer growth, and these fragments are in the clinical trial phase[12]. Similarly, in our previous study, we tested the potential cytotoxic properties of SP (1-7), SP (1-6), SP (8-11), and SP (9-11) fragments on the mouse breast cancer cell line. We have shown that all fragments, except the fragment (1-7), are effectively cytotoxic. In another study of us, conducted in 2011, we applied SP (4-11), SP (6-11), SP (1-7) fragments (100-0.001 nM) to 4T1 cells in combination with radiotherapy (45 gray cobalt60) and SP. We have shown that all fragments except (1-7) show a cytotoxic effect on this cell line. These fragments also increased the cytotoxic effect of radiotherapy. However, substance P does not affect the cytotoxicity of these fragments[3].

Physiological fragments that are released as a result of the breakdown of SP with ADAM-10 and Neprilysin enzymes are almost identical in structure, so that physiological fragments, like their synthetics, can suppress the physiological effects of SP. The most studied physiological fragments are SP (1-7). SP (1-7) has been shown to have pain-relieving properties and increase dopamine release. All of the SP fragment-based studies are related to the immune responsiveness of them and their effects on interleukins are generally well-investigated[13]. Besides, since there are no new studies in the literature, the references are dated to the past.

It has been suggested that the administration of the SP (1-7) fragment may be a potential antagonist against SP-induced responses. The idea that SP (1-7) fragment may be an internal modulator of SP functions in this study was suggested by Herrera-Marschit et al[14] that suggested SP (1-7) fragment has a painful effect while SP triggers pain formation.

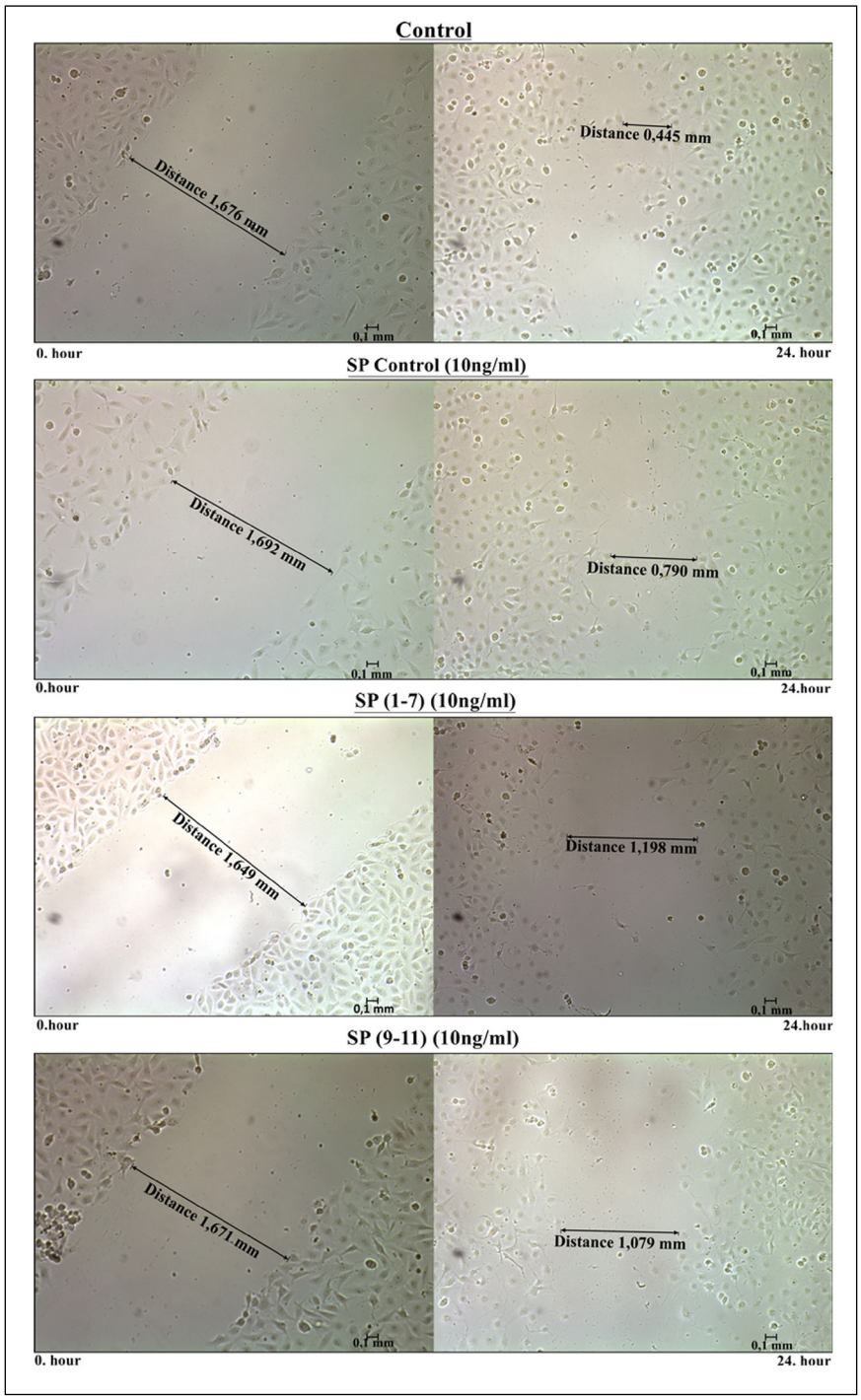

Fig. 8. Scratches were created using a 5 mL syringe without a needle. Photos were taken 12 and 24 hours after the treatment via a Euromex Microscope with 10x Objective. The photos were analyzed using ZEN 3.1 Blue edition software.

TABLE 1. Closure Rates of Gaps.

Fragment Doses	*% Closure Rates of Gaps as Compared to Control Group*	
	Fragman SP (1-7)	*Fragman SP (9-11)*
1 (ng/mL)	45.7%	47.6%
10 (ng/mL)	49%	60.9%
100 (ng/mL)	49.2%	44.4%

It has also been shown that SP (1-9) and SP (6-11) have similar modulating effects as the main peptide, but the presence of fragments does not have a signal-reproducing effect generated by the SP. SP's carboxy-terminal analogs [(Pro-4, Glu (OBzl) 11) -SP 4-11, Hyp4, Glu (OBzl) 11) SP 4-11, (c Hyp4, Glu (OBzl) 11) SP (4 -11) and (kPro4, Glu (OBzl) 11) SP 4-11] have been shown to increase TNF-α expression in human monocytes and macrophages similarly to SP itself, and NK-1 receptor antagonists inhibit this effect, andIt has been suggested that its effect originates from the carboxy-terminal end[15].

The synthetic version of the SP (4-11) fragment and NK-1 antagonist (D-Pro4, D-Trp7,9, Phe11) SP (4-11), and NK-2 receptor antagonists GR71251 and L 668,169 (at concentrations of 10-5 M) were tested *in vitro* on human skin fibroblasts and these NK-2 antagonists changed the dose-response curve triggered by SP[16]. However, it is also suggested that when the SP is disintegrated as a result of *in vitro* treatment with rat, mouse, and human microsomes, the released fragments are different for all three organisms, therefore SP fragments are broken into different fragments in different organisms, and these fragments may play important roles on different biomolecular mechanisms in these organisms[17].

CONCLUSIONS

Therefore, within the scope of the study, we wanted to determine the effects and results of N-terminal SP (1-7) and C terminal SP (9-11) as follows:

1. Substance P has a proliferative effect on cancer cells. We determined the anti-proliferative or cytotoxic effects of fragments with MTT and DNA fragmentation assays. According to our results, we showed that the application of SP (1-7) at the doses of 1 and 10 µg/mL in the first 24 hours significantly killed MDA-MB-231 cells and had a cytotoxic effect. SP (9-11) fragment did not show a significant cytotoxic effect only at the lowest dose (1 ng), while it showed a cytotoxic effect at all other doses (1 µg/mL, 10 µg, 100 ng, 10 ng). Unlike SP, both fragments are cytotoxic at the end of the 24-hour incubation period and have an adverse effect in contrast to the main peptide. At the end of the 48 hours incubation period, SP (9-11) showed a cytotoxic effect in all doses, while SP (1-7) showed the significant cytotoxic effect in the highest three doses. According to these results, for both fragments, at the end of this incubation period, they showed the opposite effect of the main peptide. At the end of the 72-hour incubation period, only the lowest dose (1 ng / mL) of SP (1-7) showed a significant proliferative effect, while in all other doses, were cytotoxic in contrast to the main peptide. Whether this cytotoxic effect seen in cells is due to apoptosis was also determined with a DNA fragmentation assay. According to these results, the amount of DNA fragmentation at the highest dose increased significantly in both fragment applications.
2. Substance P has an angiogenic effect. According to our hypothesis, we expected at least one of these fragments may have anti-proliferative or cytotoxic effects. In this context, to determine the possible anti-angiogenic effects of the fragments, the amounts of VEGF, MMP-9, and thrombospondin-1 from pro and anti-angiogenic factors released from the cancer cells were measured. According to our results, the amount of VEGF released from MDA-MB-231 cells instead of fattening caused only a significant decrease in the 100 ng / mL dose of the SP (1-7) fragment at the end of the 24 hours incubation period. There was an increase in the amount of VEGF release in all other doses of SP (1-7) and all doses of SP (9-11). Especially at doses of 1 ng/mL, VEGF amounts significantly increased compared to control. In this context, it has been suggested that after the first 24 hours incubation period, fragments exhibit similar effects to the main peptide on VEGF release. At the end of the 48-hour incubation period, there was an increase in the amount of VEGF released at all doses of both fragments, similar to 10 ng of SP administration. Statistically, this increase was found significant in some doses. It has been assumed that the amount of VEGF released shows both peptide-like behavior in both fragments. When the amounts of MMP-9 released from MDA-MB-231 cells are compared to the control group, it could be said that they cause a significant decrease in both fragment treatments. From this point of view, after the first 24 hours, the fragments reduce the amount of MMP-9 released and show anti-angiogenic effects in contrast to the main peptide. At the end of the 48 hours incubation period, we can say that the effects of the first 24 hours disappeared. While only 100 ng / mL dose of SP (1-7) fragment caused a significant increase, in almost all other doses MMP-9 was nearly the same as the control group. At the end of the 72-hour incubation period, there is a surprising reduction in all doses of both fragments (except the 1 ng/mL dose of SP (1-7)) compared to the control group. MMP-9 amounts were decreased with the application of fragments depending

on the incubation period and dose-dependent manner. The amount of Thrombospondin-1 released from the MDA-MB-231 cells into the medium causes a significant increase in the application of SP (1-7) fragments at the end of the 48 hours incubation period compared to the control, while 100 ng and 1ng/mL SP (9-11) fragment applications caused a significant decrease in the amount of released Thrombospondin amounts. According to these results, unlike SP main peptide, a 100 ng/mL dose of SP (1-7) showed an anti-angiogenic effect. 100 and 1 ng/mL doses of SP (9-11) showed an SP-like enhancing effect. Therefore, the clearest result we can say about SP fragments is that the fragments show bilateral effects similar to the SP parent peptide or vice versa depending on the factors examined, their concentration, and incubation time, and this is a paradox.

3. In our experiments to investigate the effect of SP fragments on the migration abilities of HUVEC cells, we have illustrated the size of the wound after the fixed wound formation after the wound was closed at certain time intervals and calculated the area size in mm using a computer. The lengths of the wounds are marked on the pictures at the level of a millimeter. The start time was set to "O. Hour" and measurements were done immediately after opening the wound. Using the distances at 0,6,12 and 24 hours. Wound lengths were calculated as percent (%) closure by proportioning the control group for each dose of each fragment. According to our results, there is a significant reduction in wound closure for all doses of both fragments compared to the control group. In sum, both fragments kill cancer cells by acting in reverse to the parent peptide and suppress the migration capabilities of vascular cells in contrast to the intact peptide.

In conclusion, this is the first report indicating that SP fragments may have anti-angiogenic effects on HUVECs *in vitro*. Fragments also showed a cytotoxic effect on the breast cancer cell lines.

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REFERENCES

1. Munoz M, Rosso M, Pérez A, Coveñas R, Rosso R, Zamarriego C, Piruat JI. The NK1 receptor is involved in the antitumoural action of L-733,060 and in the mitogenic action of substance P on neuroblastoma and glioma cell line. Neuropeptides 2005; 39: 427-432.
2. Simsek E, Aydemir E, Korcum AF, Fişkin K. Thalidomide combined with irradiation alters the activity of two proteases. Mol Med Rep 2015; 11: 1535-1541.
3. Simsek Oz E, Aydemir E, Korcum AF, Fiskin K. 2011, Thalidomide and irradiation combination therapy increases substance P levels in vitro. Exp Ther Med 2011; 2: 529-535.
4. Palma C, Bigioni M, Irrissuto C, Nardelli F, Maggi CA, Manzini S. Anti-tumour activity of tachykinin NK1 receptor antagonists on human glioma U373 MG xenograft. Br J Cancer 2000; 82: 480-487.
5. Munoz M, Rosso M, Covenas R, Montero I, Gonzales-Moles MA, Robles MJ. Neurokinin-1 receptors located in human retinoblastoma cell lines: antitumor action of its antagonist, L-732,138. Investig Ophthalmol Vis Sci 2007; 48: 2775-2781.
6. Singh D, Joshi DD, Hameed M, Qian J, Gascon P, Maloof PB, Mosenthal A, Rameshwar P. Increased expression of preprotachykinin-I and neurokinin receptors in human breast cancer cells: implications for bone marrow metastasis. Proc Natl Acad Sci U S A 2000; 97: 388-393.
7. Brener S, Gonzales-Moles MA, Tostes D, Esteban F, Gil-Montoya JA, Ruiz-Avila I, Bravo M, Munoz M. A role for the substance P/NK-1 receptor complex in cell proliferation in oral squamous cell carcinoma. Anticancer Res 2009; 29: 2323-2329.
8. Estaban F, Gonzales-Moles MA, Castro D, Martin-Jean MM, Redondo M, Ruiz-Avila I, Rosso M, Munoz M. Expression of substance P and neurokinin-1 receptor in laryngeal cancer: linking chronic inflammation to cancer promotion and progression. Histopathology 2009; 54: 258-260.
9. Aydemir E, Şimşek E, Korcum AF, Fişkin K. Endostatin enhances radioresponse in breast cancer cells via alteration of substance P levels. Oncol Lett 2011; 2: 879-886.
10. Aydemir E, Şimşek E, Korcum AF, Fişkin K. Endostatin and irradiation modifies the activity of ADAM10 and neprilysin in breast cancer cells. Mol Med Rep 2016; 14: 2343-2351.
11. Imir N, Aydemir E, Simsek E, Gokturk RS, Yesilada E, Fiskin K. Cytotoxic and immunomodulatory effects of Ebenus boissieri Barbey on breast cancer cells. Genet Mol Res 2016; 18: 15.
12. Heasley LE. Autocrine and paracrine signaling thorough neuropeptide receptors in human cancer. Oncogene 2001; 20: 1563-1569.
13. Tomoe S, Iwamoto I, Yoshida S, Tomioka H. The potencies of substance P, substance P fragments, and compound 48/80 for granulocyte infiltration in mouse skin. Allergy 1992; 42: 119-124.
14. Herrera-Marschitz M, Terenius L, Sakurada T, Reid MS, Ungerstedt U. The substance P(1-7) fragment is a potent modulator of substance P actions in the brain. Brain Res 1990; 521: 316-320.

15. Ho WZ, Stravropoulos G, Lai JP, Hu BF, Magafa V, Anagnostides S, Douglas SD. Substance P C-terminal octapeptide analogues augment tumor necrosis factor-alpha release by human blood monocytes and macrophages. J Neuroimmunol 1998; 82: 126-132.
16. Morbidelli L, Maggi CA, Ziche M. Effect of selective tachykinin receptor antagonists on the growth of human skin fibroblasts. Neuropeptides 1993; 24: 335-341.
17. Pailleux F, Vachon P, Lemoine J, Beaudry F. Targeted liquid chromatography quadrupole ion trap mass spectrometry analysis of tachykinin related peptides reveals significant expression differences in a rat model of neuropathic pain. Neuropeptides 2013; 47: 261-271.