

HYDROXYSAFFLOR YELLOW A REVERSE CHEMOTHERAPY RESISTANCE VIA JNK AND P38 MAPK SIGNAL PATHWAY IN OVARIAN CANCER CELLS

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Abstract

Cisplatin based combination chemotherapy has become a conventional treatment for ovarian cancer. However, the appearance of drug resistance during the ovarian cancer treatment is one of the main obstacles for improving the therapeutic perspective of ovarian cancer. The aim of this study was to confirm the hypothesis that Hydroxysafflor yellow A (HYSYA) can reverse chemotherapy resistance in ovarian cancer cells. *In vitro* study on A2780/DDP ovarian cell line was used to monitor cell proliferation by cytotoxicity assay and real time cellular analysis associated with flow cytometry that intend to determine if HYSYA enhance cisplatin sensitivity through apoptosis. *In vivo* experiments on mice was used to identify the HYSYA effects on cisplatin sensitivity. The *in vitro* studies showed that MAPK signal pathways were suppressed in cisplatin resistant ovarian cells A2780/DDP. Also, HYSYA enhanced cisplatin sensitivity in cisplatin resistant organisms, *in vivo*. Mechanistically, HYSYA increase P-JNK and P-38 levels, but has no effect on P-ERK, indicating that HYSYA enhanced cisplatin sensitivity via JNK and P38 MAPK signalling pathways.

Rezumat

Chimioterapia bazată pe combinații cu cispaltin a devenit un tratament convențional pentru cancerul ovarian. Cu toate acestea, prevalența rezistenței medicamentoase în timpul tratamentului cancerului ovarian este unul dintre principalele obstacole în calea îmbunătățirii perspectivelor terapeutice. Scopul acestui studiu a fost confirmarea ipotezei că *safflorin A* (HYSYA) poate inversa rezistența la chimioterapie în celulele cancerigene ovariene. Studiul *in vitro* asupra liniei celulare A2780/DDP a fost utilizat pentru a monitoriza proliferarea celulară prin analiza citotoxicității și analiza celulară în timp real asociată cu citometria în flux, pentru a determina dacă HYSYA crește sensibilitatea la cisplatin. S-au realizat experimente *in vivo*, pe șoareci, pentru a identifica efectele HYSYA asupra sensibilității la cisplatină. Studiile *in vitro* au arătat că semnalele celulare au fost suprimate în celulele ovariene A2780/DDP rezistente la cisplatină. De asemenea, HYSYA a îmbunătățit *in vivo* sensibilitatea la cisplatină în organismele rezistente. HYSYA a crescut nivelurile P-JNK și P-38, dar nu a avut niciun efect asupra P-ERK, indicând faptul că HYSYA acționează prin intermediul căilor de semnalizare JNK și P38 MAPK.

Keywords: Hydroxysafflor yellow A; chemotherapy resistance; MAPK; ovarian cancer; A2780/DDP cell line

Introduction

Ovarian cancer is the most common gynaecologic cancer, having the highest mortality rate from the gynaecological tumours. Due to lack of a complete early diagnosis, about 70% ~ 80% cancer patients are diagnosed in the advanced stage. Cisplatin based combination chemotherapy has become a conventional treatment of ovarian cancer. Most ovarian cancer patients have high initial response to cisplatin. However, the appearance of drug resistance during the treatment of ovarian cancer has become the main obstacle in improving the therapeutic perspective of ovarian cancer. Studies have found that about 75% ~ 80% of epithelial ovarian cancer began to respond

to chemotherapy, but at least 80% of all patients end up with drug resistance [1, 2].

Non-repairable cisplatin-induced DNA damage leads to the activation of a multi-branched signalling cascade with proapoptotic outcomes. Starting with the technological development and modernization of daily living, people have been exposed to a growing number of toxins and other harmful substances, and this has led to an explosion of cancers. Activation of signalling pathways that initiate tumorigenic changes in the target cell may be triggered by some external factors such as bisphenol [3]. Genetic and epigenetic alterations in the components of this complex signalling network have been associated with variable levels of resistance to cisplatin [4]. In order to avoid this

resistance, new pharmaceutical nanotechnology forms have been tried for transport to the target of the cytostatics [5, 6]. Studies suggest that proapoptotic signal transducers such as mitogen-activated protein kinases (MAPK) family may contribute to the cisplatin-resistant phenotype [7, 8]. The MAPKs in mammals include extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and P38 MAPK. These enzymes are serine-threonine protein kinases that regulate various cellular activities including proliferation, differentiation, apoptosis or survival, inflammation, and innate immunity [9]. These findings lead to the development of compound targeted MAPK signal pathway to reverse drug resistance.

Cancer is not a modern disease, it has always existed, and people have fought against it with vegetal extracts, the only products existing at the time. Hydroxysafflor yellow A (HSYA) is a compound with a single chalcone glycoside structure, with the highest efficacy from water soluble pharmacological active compounds of safflower. HSYA showed anti-inflammatory properties, antioxidant effects, cell survival modulation, apoptosis and inhibition of tumour angiogenesis [10]. However, little is known about the role of HSYA in anti-drug resistance in cancer treatment. This study intended to identify if HSYA can influence the cisplatin resistance and its mechanism of action in order to provide a solution for advanced ovarian cancer resistance.

Materials and Methods

Materials

Cell cultures and reagents

The antibodies against Erk1/2, P-Erk1/2, JNK, P-JNK, P38, P-P38 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were purchased from Sigma Chemical (St. Louis, MO, USA). Hydroxysafflor yellow A (HSYA) was purchased from Jiangyin Tian Jiang Pharmaceutical Co. Ltd. (Jiangyin, China). The human ovarian cancer cell line resistant to cisplatin A2780/DDP and A2780 were purchased from Shanghai cell bank of Chinese Academy of Sciences (Zhejiang, China). Human A2780/DDP ovarian cancer cells were routinely cultivated in RPMI-1640 medium containing 10% FBS and 2 µg/mL cisplatin, at 37°C with 5% CO₂. Cells were passaged at 80% confluency, using 1 mmol/L ethylene diamine tetra acetic acid (EDTA) - 0.025% trypsin for 3 - 5 min, and sub-cultured at a ratio of 1:3 - 1:5. Cells at the logarithmic growth phase were collected for experiments.

Cytotoxicity assay

One day before treatment, A2780, A2780/DDP cells were plated at a density of 5000 cells *per* well in 96-well plates. The cells were treated with cisplatin and HSYA. After 24-hours treatment, the cell viability was determined using Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions (Dojindo,

Kumamoto, Japan). The cell survival rates were expressed as mean ± S.D. from at least three independent experiments.

Real time cellular analysis (RTCA) for monitoring cell proliferation

First, A2780 and A2780/DDP cells at the logarithmic growth phase were obtained and the cell concentration was adjusted to 5×10^4 /mL. E-Plate16 test board with 50 µL medium was used to determine the background resistance value. Then, 100 µL of the above-mentioned cell suspension was added into each well of E-Plate16. The E-Plate 16 was then placed on the RTCA station in the incubator, with cell index detected at intervals of 15 min. In the second day, according to the experimental design, the 5 µL working fluid was added to the cell wall along the whole wall. The cells were divided in 3 groups as follows: the first one was treated with cisplatin (25 µg/mL), the second one received HSYA (1 mg/mL) + cisplatin (25 µg/mL) and a control group. For each group the RTCA test was done every 1 minute for two hours to observe the short-term effect of the drugs and then, every 15 min for a total of 72 hours to observe the long-term effect of the drug.

Evaluation of apoptosis by flow cytometry

For detection of apoptosis by FACS, A2780/DDP cells were treated with cisplatin (25 µg/mL) and HSYA (1 mg/mL) for 24 hours. Then the cells were collected for annexin V-phycoerythrin and 7-amino-actinomycin D (7-AAD) double staining. In short, the cells were washed with phosphate buffered saline (PBS) for three times and stained according to the manufacturer's instructions (Guava Nexin® Reagent kit, Millipore, USA). Samples were then analysed using Guava EasyCyte Plus (Millipore, USA) within 30 mins after the staining.

Animal experiment on nude mice model of transplanted tumour

Twenty female BALB/c nude mice, 4 weeks old were obtained from Shanghai Sippr-BK laboratory animal Co. Ltd. The animal care committee of Zhejiang Chinese Medical University approved the animal experiment and the code number of the animals was SCXK (HU) 2013-0016. All of the experimental nude mice were kept under specific pathogen-free (SPF) conditions in the Laboratory Animal Centre of Zhejiang at a relative humidity of 45% - 60% and a temperature of $26 \pm 2^\circ\text{C}$. The code number of the laboratory animal center was SYXK (ZHE) 2014-0008. A2780/DDP cells concentration were adjusted to 5×10^7 /mL. Then 200 µL of the above-mentioned cell suspension was injected subcutaneously into the right flank of each nude mouse. After two weeks, every nude mouse has grown a tumour at least a volume of approximately 60 mm³. Then, 20 nude mice were randomly divided into 4 experimental groups as follows: cisplatin group treated with 3

mg/kg body weight cisplatin, HSYA group treated with 1.1 g/kg body weight HSYA, cisplatin plus HSYA group that received 3 mg/kg bw cisplatin and 1.1 g/kg bw HSYA and the control group that received physiological saline solution. The treatment was done for each group once every three days for five weeks. Health status and tumour growth of mice in each group were checked daily, with an electronic digital calliper and weekly measurement of tumour size as well as tumour volume was calculated according to the formula:

$$\text{tumour volume (mm}^3\text{)} V = (W^2 \times L)/2,$$

where, W is tumour width and L is tumour length. At the end of the experiment, the animals were euthanized and the organs were collected.

Western blot analysis

For biochemical analysis, cells and tissues were washed with ice cold PBS (Beyotime Institute of Biotechnology, Jiangsu, China) and lysed in radio-immunoprecipitation assay lysis buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1% Triton X100; 1% sodium deoxycholate; 0.1% sodium dodecyl sulphate (SDS); Beyotime Institute of Biotechnology). The lysates were kept in ice for 30 mins and clarified by centrifugation at 12,000 x g for 25 mins at 4°C. The clarified lysate was then collected and the Erk1/2, P-Erk1/2, JNK, P-JNK, P38, P-38 and GAPDH proteins were separated by 12% SD Spolyacrylamide gel electrophoresis (30100 µg protein/lane) which was later transferred to a polyvinylidene fluoride membrane (all from Beyotime Institute of Biotechnology). After the transfer, the membranes were incubated in 5% milk for an hour and then with antibodies diluted in fat-free milk. The membranes were then washed with Tris buffered saline and Tween 20 (Beyotime Institute of Biotechnology) and incubated with the HRP (horseradish peroxidase) conjugated secondary antibodies. Immunoreactive proteins were visualized using a BeyoECL Plus kit (Beyotime Institute of Biotechnology).

Statistical analysis

Statistical analysis was conducted using SPSS, version 18.0 (SPSS, Inc., Chicago, IL, USA). Each experiment was performed at least 3 times. Data were indicated as mean values ± standard deviation and differences were evaluated using Student's t-test. A probability value < 0.05 was considered statistically significant.

Results and Discussion

HSYA enhanced cisplatin sensitivity in cisplatin resistant cells

First, we confirmed the cisplatin resistance character of A2780/DDP cells. As shown in Figure 1A, the survival of A2780/DDP cells is increased in cisplatin treatment compared with A2780 cells. After the treatment with 50 µg/mL cisplatin the viability of

since the cell viability of A2780/DDP cells was 63% compared with only 19% viability for A2780 cells. Then, we investigated the potential role of HSYA in A2780/DDP cells. These cells were treated with cisplatin (25 µg/mL) alone or combined with HSYA (1 mg/mL). As a result, the viability of A2780/DDP cells was significantly decreased by HSYA and cisplatin-combined treatment (Figure 1B). After 12 hours of treatment the A2780/DDP cell viability in cisplatin + HSYA group was 89% compared with 94% in cisplatin group and after 60 hours of treatment the A2780/DDP cell viability was only 23% compared with 58% in cisplatin group. RTCA analysis showed that although the proliferation of A2780/DDP cells was inhibited by cisplatin treatment, the cells still survived (Figure 1C). In contrast, HSYA and cisplatin combined treatment led to significant cell death (Figure 1C). These results suggested that HSYA enhanced cisplatin-sensitivity in cisplatin resistant cells.

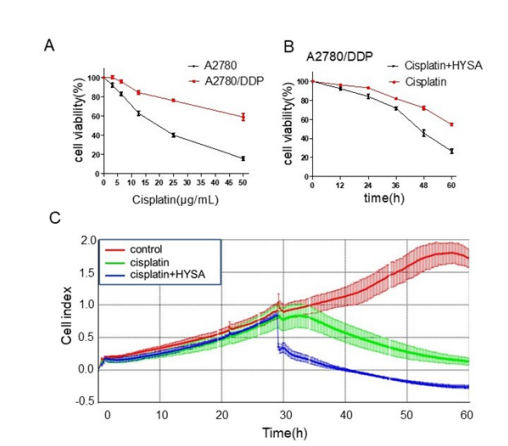


Figure 1.

HSYA enhanced cisplatin sensitivity in cisplatin resistant cells. (A) The viability of A2780 and A2780/DDP cells treated with cisplatin. (B) The viability of A2780/DDP cells treated with cisplatin and HSYA. (C) Cell proliferation of A2780/DDP cells treated with cisplatin and HSYA

HSYA enhanced cisplatin sensitivity through apoptosis

Apoptosis was the major cell death type induced by cisplatin. To determine whether HSYA enhanced cisplatin induced apoptosis in A2780/DDP cells, the cells were treated with cisplatin (25 µg/mL) alone or combined with HSYA (1 mg/mL) for 24 hours (Figure 2A and 2B). The proportion of AV(Annexin-V)+PI (propidium iodide) + (late stage of apoptosis) was increased from 0.09% in control group cells to 6.33% of the cisplatin group, and to 15.66% of the cisplatin + HSYA group. The number of AV+PI- (early stage of apoptosis) increased from 0.36% to 9.36%. These results suggested that HSYA enhanced cisplatin sensitivity through induced apoptosis.

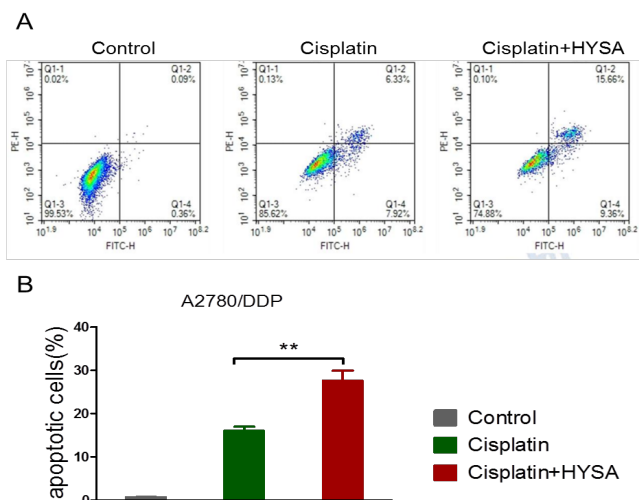


Figure 2.

HSYA enhanced cisplatin sensitivity through apoptosis. (A) Apoptosis of A2780/DDP cells treated with cisplatin and HSYA was determined by flow cytometry. (B) Graphic apoptotic rate of A2780/DDP cells

HSYA enhanced cisplatin sensitivity in vivo

We employed the xenograft model to investigate the *in vivo* role of HSYA in cisplatin resistance. After 5 weeks treatment, the HSYA group showed a decrease of the tumour volume, followed by the cisplatin group and then cisplatin + HSYA with the smallest tumour volume (Figure 3A and 3B). The tumour tissues were analysed by HE staining and the results showed that there were more apoptotic cells in cisplatin combined

with HSYA group compared with the cisplatin group (Figure 3C and 3D). The average tumour volumes of the control, HSYA, cisplatin and cisplatin+ HSYA groups were 1984.29, 1566.81, 1104.23 and respectively 695.48 mm³. The average tumour weights of the control, HSYA, cisplatin and cisplatin+ HSYA groups were 4.71, 4.54, 3.53 and respectively 2.76 g. These results demonstrated that HSYA enhanced cisplatin sensitivity *in vivo*.

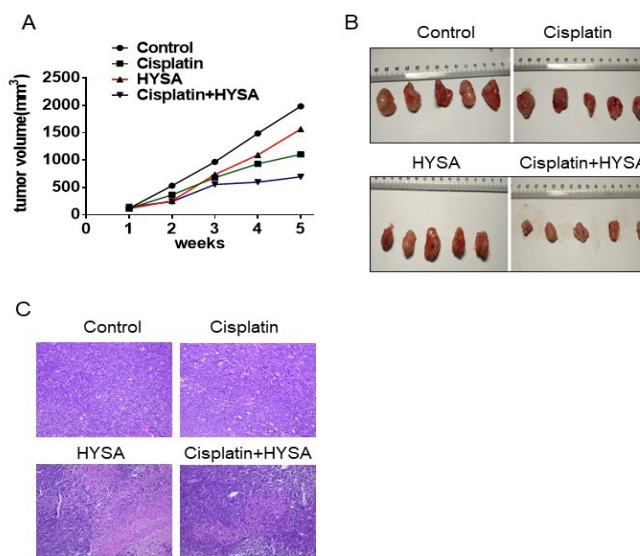


Figure 3.

HSYA enhanced cisplatin sensitivity *in vivo*. (A) The growth of tumour treated with cisplatin and HSYA. (B) The tumours after 5 weeks by cisplatin and HSYA treatment. (C) HE staining of the tumours

HSYA enhanced cisplatin sensitivity via JNK and P38 MAPK signal pathways

Apoptosis maybe controlled by various signal pathways, including MAPK signal pathways [10-13].

ERK, JNK, P38 are three sub-pathways of MAPK. We found that the levels of P-ERK, P-JNK, P-P38 were down regulated in A2780/DDP cells compared with A2780 cells (Figure 4A), indicating

that MAPK signal pathways were suppressed in cisplatin resistant cells. When A2780/DDP cells were treated with HSYA, P-JNK and P-38 levels but not P-ERK were significantly activated (Figure

4B) compared with GAPDH, indicating that HSYA enhanced cisplatin sensitivity *via* JNK and P38 MAPK signalling pathways.

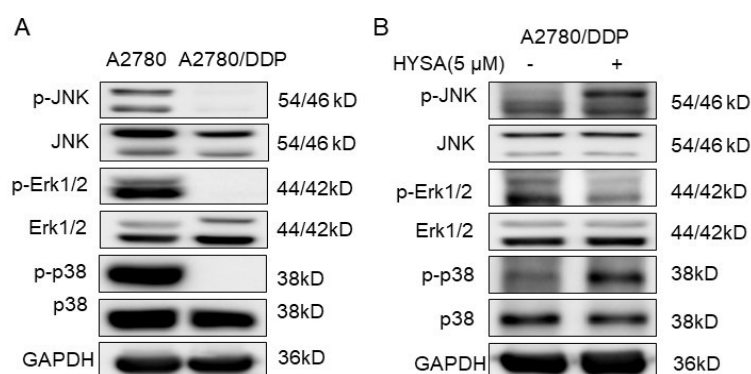


Figure 4.

HSYA enhanced cisplatin sensitivity *via* JNK and P38 MAPK signalling pathways. (A) The expression of p-Erk1/2, Erk1/2, p-JNK, JNK, p-p38, p38 and GAPDH in A2780 and A2780/DDP cells (B) The expression of p-Erk1/2, Erk1/2, p-JNK, JNK, p-p38, p38 and GAPDH in A2780/DDP cells after HSYA treatment

Ovarian cancer is the most malignant tumour in gynaecological tumours, and cisplatin is widely used as a basic drug for ovarian cancer chemotherapy. In patients with advanced ovarian cancer, the rate of recurrence was 70% after chemotherapy, and the rate of chemotherapy resistance was 70%, which directly affects the 5 years survival rate of advanced ovarian cancer patients [14]. Classical chemotherapy produces severe side effects, of which severe immunosuppression favours severe bacterial infections with treatment resistant germs such as *Staphylococcus aureus* [15, 16, 17] or fungus from *Aspergillus*, *Fusarium* [18]. Therefore, screening resistant reversal drugs from traditional Chinese medicine and developing a specific resistance reversal agent will help to improve the 5 years survival rate of advanced ovarian cancer patients. This process is triggered by autocrine and paracrine signals. Thus, agents that may block or reverse this process may offer a promising therapeutic strategy to limit cancer diffusion.

In the current study, cytotoxicity assay and real time cellular analysis used to monitor cell proliferation showed that HSYA enhanced cisplatin sensitivity in cisplatin resistant cells. Flow cytometry suggested that HSYA enhanced cisplatin sensitivity through apoptosis. Animal experiments showed that HSYA enhanced cisplatin sensitivity *in vivo*. HSYA blocks the expression of apoptosis markers in the Western blotting assays. We further showed that HSYA can enhance cisplatin sensitivity *via* JNK and P38 MAPK, which is associated with apoptosis. These results provide new mechanistic bases for the therapeutic application of HSYA in the patients with ovarian cancer.

Conclusions

HSYA, has shown to have anti-apoptotic, inflammatory effects, antioxidant, cell survival modulating potential and it inhibits the tumour angiogenesis. Most importantly, to the best of our knowledge, our study is the first to demonstrate that the anti-apoptotic effect of HSYA was associated with reverse chemotherapy resistance in cultured ovarian cancer cells. Our results offer a new perspective on the role of HSYA in preventing the progression of ovarian cancer.

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References

1. Coleman RL, Monk BJ, Sood AK, Herzog TJ, Latest research and treatment of advanced-stage epithelial ovarian cancer. *Nat Rev Clin Oncol.*, 2013; 10: 211-224.
2. Leitao MM Jr, Chi DS, Surgical management of recurrent ovarian cancer. *Semin Oncol.*, 2009; 36: 106-111.
3. Jalal N, Surendranath AR, Pathak JL, Yu S, Chung CY, Bisphenol A (BPA) the mighty and the mutagenic. *Toxicol Rep.*, 2018; 5: 76-84.
4. Galluzzi L, Senovilla L, Vitale I, Michels J, Martins I, Kepp O, Castedo M, Kroemer G, Molecular mechanisms of cisplatin resistance. *Oncogene*, 2012; 31:1869-1883.
5. Kadry MO, Abdel-Megeed RM, El-Meliegy E, Abdel-Hamid AZ, Crosstalk between GSK-3, c-Fos, NFκB and TNF-α signaling pathways play an ambitious role in Chitosan Nanoparticles Cancer Therapy. *Toxicol Rep.*, 2018; 5: 723-727.

6. Danciu C, Pinzaru IA, Dehelean CA, Hancianu M, Zupko I, Navolan D, Licker M, Ghiulai RM, Șoica CM, Antiproliferative and antimicrobial properties of pure and encapsulated rutin. *Farmacia*, 2018; 66(2): 302-308.
7. Mansouri A, Ridgway LD, Korapati AL, Zhang Q, Tian L, Wang Y, Siddik ZH, Mills GB, Claret FX, Sustained activation of JNK/p38 MAPK pathways in response to cisplatin leads to Fas ligand induction and cell death in ovarian carcinoma cells. *J Biol Chem.*, 2003; 278: 19245-19256.
8. Brozovic A, Osmak M, Activation of mitogen-activated protein kinases by cisplatin and their role in cisplatin-resistance. *Cancer Lett.*, 2007; 251: 1-16.
9. Kim EK, Choi EJ, Compromised MAPK signaling in human diseases: an update. *Arch Toxicol.*, 2015; 89:867-82.
10. Yang F, Li J, Zhu J, Wang D, Chen S, Bai X, Hydroxysafflor yellow A inhibits angiogenesis of hepatocellular carcinoma via blocking ERK/MAPK and NF- κ B signaling pathway in H22 tumor-bearing mice. *Eur J Pharmacol.*, 2015; 754: 105-114.
11. Sui X, Kong N, Ye L, Han W, Zhou J, Zhang Q, He C, Pan H, p38 and JNK MAPK pathways control the balance of apoptosis and autophagy in response to chemotherapeutic agents. *Cancer Lett.*, 2014; 344: 174-179.
12. Mehedintu C, Antonovici M, Brinduse L, Bratila E, Stanculescu R, Berceanu C, Bratu O, Pituru S, Onofriescu M, Matasariu DR, The influence of progesterone on immunohistochemical markers in endometriosis. *Rev Chim (Bucharest)*, 2018; 69(3): 581-584.
13. Li Q, Chen M, Liu H, Yang L, Yang T, He G, The dual role of ERK signaling in the apoptosis of neurons. *Front Biosci (Landmark Ed)*, 2014; 19: 1411-1417.
14. Pectasides D, Farmakis D, Koumariou A, The role of neoadjuvant chemotherapy in the treatment of advanced ovarian cancer. *Oncology*, 2005; 68(1): 64-70.
15. Zlatian O, Balasoiu AT, Balasoiu M, Cristea O., Docea AO, Mitrut R, Spandidos DA, Tsatsakis AM, Bancescu G, Calina D, Antimicrobial resistance in bacterial pathogens among hospitalised patients with severe invasive infections. *Exp Ther Med.*, 2018; 16: 4499-4510
16. Ungureanu A, Zlatian O, Mitroi G, Drocaș A, Țircă T, Călina D, Dehelean C, Docea AO, Izotov BN, Rakitskii V, Cioboată R, Spandidos DA, Tsatsakis AM, Găman A, *Staphylococcus aureus* colonisation in patients from a primary regional hospital. *Mol Med Rep.*, 2017; 16: 8771-8780.
17. Georgescu M, Tăpăloagă PR, Tăpăloagă D, Furnaris F, Ginghină O, Negrei C, Giuglea C, Bălălu C, Ștefănescu E, Popescu IA, Georgescu D, Evaluation of antimicrobial potential of nigella sativa oil in a model food matrix. *Farmacia*, 2018; 66(6): 1028-1036.
18. Tănase A, Coliță A, Ianoși G, Neagoe D, Brănișteanu DE, Călina D, Docea AO, Tsatsakis A, Ianoși SL, Rare case of disseminated fusariosis in a young patient with graft vs. host disease following an allogeneic transplant. *Exp Ther Med.*, 2016; 12: 2078-2082.