doi.org/10.36721/PJPS.2020.33.3.SUP.1233-1238.1

Resistomycin, a pentacyclic polyketide, inhibits the growth of triple negative breast cancer cells through induction of apoptosis and mitochondrial dysfunction

Ammara Riaz[1], Azhar Rasul[1]*, Ghulam Hussain[2], Malik Saadullah[3], Bilal Rasool[1], Iqra Sarfraz[1], Muqaddas Masood[4], Muhammad Asrar[1], Farhat Jabeen[1] and Tayyaba Sultana[1]

[1]Department of Zoology, Government College University, Faisalabad, Pakistan
[2]Department of Physiology, Government College University, Faisalabad, Pakistan
[3]Department of Pharmaceutical Chemistry, Government College University, Faisalabad, Pakistan
[4]The Key Laboratory of Molecular Epigenetics of MOE, Institute of Genetics and Cytology, Northeast Normal University (NENU), Changchun, China

Abstract: Triple negative breast cancer (TNBC) stands among the most fatal and aggressive malignancies of women all over the world. This study was based on the screening of natural compounds library to identify potentially active entities against TNBC, which has been reported to be resistant towards currently available chemotherapeutics. A library of 100 compounds was screened against TNBC and non-TNBC breast cancer cell lines. Compounds that exhibited cytotoxicity and inhibited 80% growth of cancer cells at the concentration of 10µg/ml were considered as hits. 4 out of 100 screened compounds exhibited anti-proliferative potential against breast cancer while 2 out of 4 showed significant anti-cancer potential only against TNBC. In this article, we report the anti-cancer potential of resistomycin, a pentacyclic polyketide metabolite, which is isolated from bacterial strain *Streptomyces* spp. Resistomycin inhibited the growth of TNBC cells (MDA-MB231) more efficiently than non-TNBC (MCF-7) cells. Further, the molecular mechanism lying behind the anticancer potential of resistomycin was found to be induction of apoptosis and mitochondrial dysfunction. Resistomycin has significant potential to dissipate MMP to induce apoptosis in TNBC cells. The results of this research provide evidence to support this bioactive natural entity as an anti-TNBC drug candidate in future.

Keywords: Triple negative breast cancer, natural compounds, resistomycin, anticancer.

INTRODUCTION

Triple negative breast cancer (TNBC) is enlisted among the most aggressive malignancies of breast in women all over the world with 5-year survival rate of 60% after diagnosis (Jitariu *et al*., 2017). TNBC lacks the expression of estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2) (Gadi and Davidson, 2017). Due to the lack of expression of drug targets in TNBC, it is resistant towards the currently available chemotherapeutics. Moreover, its poor prognosis and high proliferative potential than any other type of breast cancer further aggravates the situation (Cleator *et al*., 2007; Han *et al*., 2019). Hence, there is dire need to figure out the effective agents to improve therapeutics against this fatal malignancy (Riaz *et al*., 2019).

Natural compounds have proved their worth as an irreplaceable tool in drug discovery during recent years (Atanasov *et al*., 2015). Several *in vivo*, pre-clinical as well as clinical studies have affirmed the potential of natural compounds against cancer (Choudhari *et al*., 2019). In order to find the potent cytotoxic compounds against TNBC, we have screened the natural compounds library. MDA-MB231 cells were selected as TNBC model cells to figure out the anti-TNBC compounds (Chavez *et al*., 2010). After screening, we obtained four cytotoxic compounds, out of which, eriocalyxin B and resistomycin were found to be more effective inhibitors of TNBC cells. We have recently reported the anti-cancer potential of EriB (Riaz *et al*., 2019), while this research article reports the potential of another compound, resistomycin, against TNBC.

Resistomycin is a pentacyclic polyketide metabolite isolated from the bacterial *Streptomyces* spp. (Jakobi and Hertweck, 2004; Jones and Elliot, 2017). To date, resistomycin has been known to exert anticancer effects against few cancers such as colon (Nadysev *et al*., 2018), hepatic, cervical (Vijayabharathi *et al*., 2011) and gastric carcinoma (Adinarayanaa *et al*., 2006), however, the exact mechanism lying behind its cytotoxic effects still needs to be investigated. Our research on this compound is an effort to determine anti-proliferative potential of resistomycin towards MDA-MB231 and the mechanism of resistomycin-induced apoptosis in MDA-MB231 cells.

MATERIALS AND METHODS

Cell culture

TNBC and non-TNBC (MDA-MB231 and MCF-7

*Corresponding author: e-mail: drazharrasul@gmail.com

respectively) cells were cultured in DMEM media supplemented with 10 percent fetal bovine serum (FBS) and 1 percent antibiotics. The incubation of cancerous cells was done in atmosphere of 5 percent CO_2 at 37°C and cells were allowed to grow till they attained 70-80% confluency (Di *et al*., 2014).

Cell proliferation assay

TNBC and non-TNBC cells were seeded in 96 well plate (5×10^3 cells/well) in DMEM comprising 10 percent FBS along with 1 percent antibiotics for 12 h. Stock solution of 2mg/ml of each compound was prepared in DMSO. Cells were treated with test compounds for 48 h. MTT solution (10µl) was added to each well after which the cells were re-incubated. After 4 hours, the media was discarded from each well and replaced with DMSO (150 µl/well). At Multimode Reader (Thermo Scientific, USA), the absorbance was taken at 490 nm (Rasul *et al*., 2011). The experiment was done in triplicate and the inhibition ratio was calculated by the following formula:

$$1\%\frac{[\text{A490(control)} - \text{A490(treated)}]}{\text{A490(control)}}\times100$$

Where, "I" denotes the inhibition rate and "A" represents the absorbance at 490 nm.

Measurement of cell apoptosis

Annexin V/PI assay along with FACS (flow cytometric analysis) was performed to measure the cellular apoptosis (Rasul *et al*., 2012b). Cells were grown in in 6 wells plates (8×10^4 cells/well) and treated with the various concentrations (0.2, 0.4, 0.8µM) of resistomycin for 48 hours. Followed by that, the cells were washed two times with PBS and staining was done with Annexin V-FITC (Beyotime PR. China) and PI (10µl) in 500µl binding buffer for 15 min in dark. The percentage of cells in early and late phases of apoptosis was measured by FACs analysis.

Mitochondrial Δψm assay

After the seeding of TNBC cells in 6 well plate (8×10^4 cells/ml), incubation was done for 12 h in CO_2 incubator. Next, the treatment of various concentrations (0, 0.2, 0.4, 0.8µM) of resistomycin was given to cells. Cells were stained by JC-1 solution (10µl/well). Mitochondrial transmembrane potential was probed by FACs analysis (Rasul *et al*., 2012a).

STATISTICAL ANALYSIS

The data obtained after experiments was statistically analysed by using Origin lab 8. The pattern of data representation was mean ± standard deviation. Statistical significance was recorded by paired *t*-test and the differences were considered as statistically significant at $P<0.05$.

RESULTS

Screening of natural compounds library

During this study, natural compounds library was screened to find out potent and selective compounds against TNBC by using human MDA-MB231 and MCF-7 cells. Out of 100 natural compounds screened, 4 hit compounds (eriocalyxin B, resistomycin, brevicarine, emetine) inhibited the proliferative potential of TNBC as well as non-TNBC cancer cells while two natural compounds (eriocalyxin B, resistomycin) have selective and significant inhibitory potential against TNBC cells as compared to non-TNBC cells (table 1).

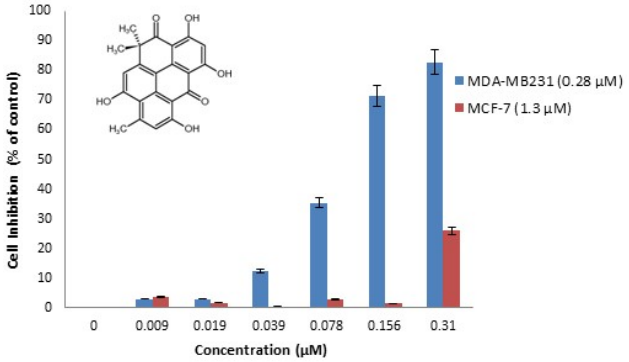


Fig. 1: Resistomycin caused inhibition of growth in MCF-7 and MDA-MB231 breast cancer cells. Cancer cells were given treatment of 0.31, 0.156, 0.078, 0.039, 0.019, 0.009, 0 µM of resistomycin for 48 h. Values are expressed as means ± SD. $P<0.05$ compared to the control group.

Generation of dose-response curves and calculation of IC_{50} values

The dose-response curves were generated to calculate the inhibitory concentrations (IC_{50}) of four hit compounds obtained after the screening of natural compounds against the TNBC and non-TNBC cancer cell lines (MDA-MB231 and MCF-7 respectively). EriB and resistomycin have potential to inhibit the growth of TNBC cells significantly with IC_{50} values of 3±1.7µM and 0.28±0.2 µM respectively while their cytotoxic potential against non-TNBC cells was found to be relatively lower with IC_{50} values of 67±10.7µM and 1.3±0.5µM respectively. Interestingly, resistomycin anti-proliferative potential against MDA-MB231 cells is four times greater as compared to MCF-7 cells. Therefore, we were interested to find the mechanism of resistomycin-induced apoptosis in MDA-MB231 cells. Details of compounds, their molecular weight and IC_{50} values against TNBC and non-TNBC cell lines are provided in table 2.

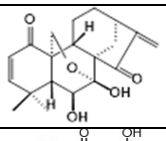
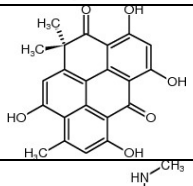
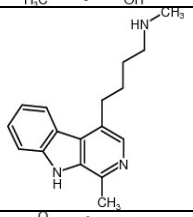
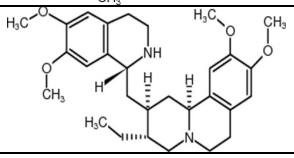
Resistomycin significantly inhibits the proliferation of MDA-MB-231 cells

The inhibition in the growth of MDA-MB231 and MCF-7 cells was determined by MTT assay. Our results show that resistomycin possesses significant antiproliferative

Table 1: Summary of hit rate obtained after screening of natural compound library against TNBC and non-TNBC cells by MTT assay

Library name	No. of compounds screened	Final dose	Potent Hits	Hit rate	Hits with Selective Inhibitory Potential against MDA-MB231 cells
Natural Compound Library	100	10µg/ml (10-40 µM)	4	4 %	2

Table 2: IC_{50} values of hit compounds against MDA-MB231 and MCF-7 cells along with their molecular weight and chemical structure.

	Natural Compounds (Potent Hits)	Chemical Structure	Molecular Weight	IC_{50}	
				MDA-MB-231	MCF-7
1	Eriocalyxin B		344.4 g/mol	3 µM ± 1.7	67 µM ± 10.7
2	Resistomycin		376.4 g/mol	0.28 µM ± 0.2	1.3 µM ± 0.5
3	Brevicarine		480.6 g/mol	0.97 µM ± 0.3	0.74 µM ± 0.2
4	Emetine		267.37 g/mol	16.6 nM ± 2.5	14.9 nM ± 2.5

potential against TNBC (MDA-MB231) cells. Treatment of cells with various concentrations of resistomycin (0.31, 0.156, 0.078, 0.039, 0.019, 0.009, 0µM) caused a dose-mediated inhibition of TNBC cells as represented in fig. At the dose of 0.31µM, resistomycin inhibited the growth of 80% TNBC cells while at the same dose, resistomycin inhibited the growth of more than 20% non-TNBC cells which clearly represents that resistomycin's effective growth inhibitory potential towards TNBC cells.

Resistomycin inhibits proliferation via induction of apoptosis in MDA-MB231 cells

The results of FACs analysis show the dose-dependent apoptosis induction in TNBC cells after the exposure of cells to different concentrations of resistomycin (0, 0.2, 0.4, 0.8µM). Fig. 2 represents AV/PI double staining of MDA-MB231 cells after fourteen hours treatment with resistomycin. The percentage of cells undergoing early apoptosis (AV+/PI-) after the exposure of cells to 0, 0.2, 0.4, 0.8µM of resistomycin was 8.09±0.38%, 24.37± 2.5%, 37.96±2.7% and 44.20±3.1% respectively while cells in late apoptotic phase (AV+/PI+) were 1.04± 0.06%, 2.82±0.09%, 3.76±1.23% and 4.5±1.29% respectively, however, necrotic cell percentage (AV-/PI+) was found to be 0.61±0.02%, 2.70±0.98%, 3.34±0.93%, and 6.71±1.31% respectively.

Effect of resistomycin on mitochondrial membrane potential (MMP)

In order to investigate that whether resistomycin induced apoptosis through loss of MMP, we have measured ΔΨm via MMP sensor, JC-1 and FACS analysis. Our study demonstrated that resistomycin has potential to dissipate MMP actively in MDA-MB231 cells dose-dependently. Fig. 3A displays the level of MMP in the resistomycin-treated cells (0, 0.2, 0.4, 0.8µM). The results clearly indicate that resistomycin-induced apoptosis is linked to MMP dissipation.

DISCUSSION

TNBC comprises 15% of invasive breast cancers which are characterized by lack of ER, PR and HER2 (Portha *et al*., 2016). Due to the absence of diagnostic receptors and frequently developed resistance against chemotherapeutics (Kim *et al*., 2018), there is an ultimate

need to screen compound libraries against TNBC for the identification and characterization of potent drug candidates to provide better treatment for these aggressive tumors. In this context, natural products with medicinal properties provide rationale towards the discovery of drugs against cancer since times (Zupko *et al*., 2015).

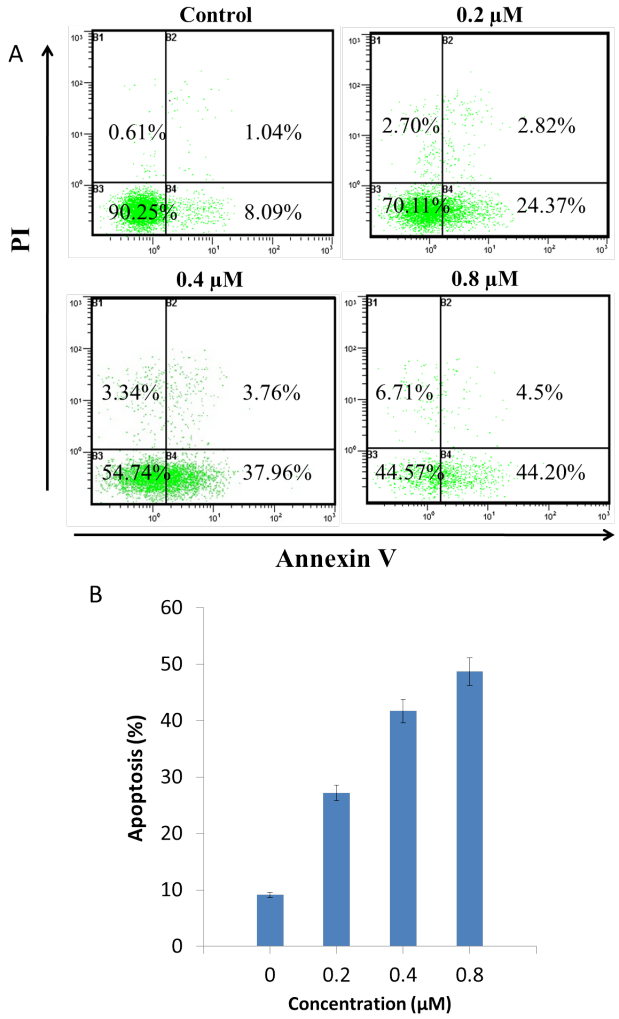


Fig. 2: Analysis of apoptosis in resistomycin treated-MDA-MB231 cells by flow cytometry. (A) Dot plot for FACs analysis, B1: AV-/PI+ (Necrotic cells), B2: AV+/PI+ (late apoptotic cells), B3: AV-/PI- (viable cells), B4: AV+/PI- (early apoptotic cells). (B) Bar graph illustrating the percentage of cells in early and late apoptotic phases after treatment with resistomycin. Values are expressed as means ± SD. P<0.05 compared to the control group.

Resistomycin, a pentacyclic polyketide metabolite from bacterial strain *Streptomyces* spp., has been known to possess antifungal (Zhang *et al*., 2013), antiviral (Roggo *et al*., 1994) and anti-proliferative properties. The results of our study demonstrate significant anticancer potential of resistomycin against the proliferation of TNBC cell line, MDA-MB231, by the induction of apoptosis. The results of this study are found to be consistent with the previous studies which report the anti-cancer potential of resistomycin against colon (Nadysev *et al*., 2018) and gastric cancer (Adinarayanaa *et al*., 2006). Our results demonstrate the dose-dependent cytotoxic effects of resistomycin against TNBC as previously reported for liver and cervical cancer cells (Vijayabharathi *et al*., 2011). The IC_{50} values reported for resistomycin against liver (HepG2) and cervical (HeLa) cancer are 0.005µg/ml 0.006µg/ml respectively. Moreover, authors also demonstrated the greater cytotoxic potential of resistomycin as compared to 5-FU in HeLa and HepG2 cells treated with similar doses of resistomycin and 5-FU, indicating the significant cytotoxic potency of resistomycin against cancer (Vijayabharathi *et al*., 2011).

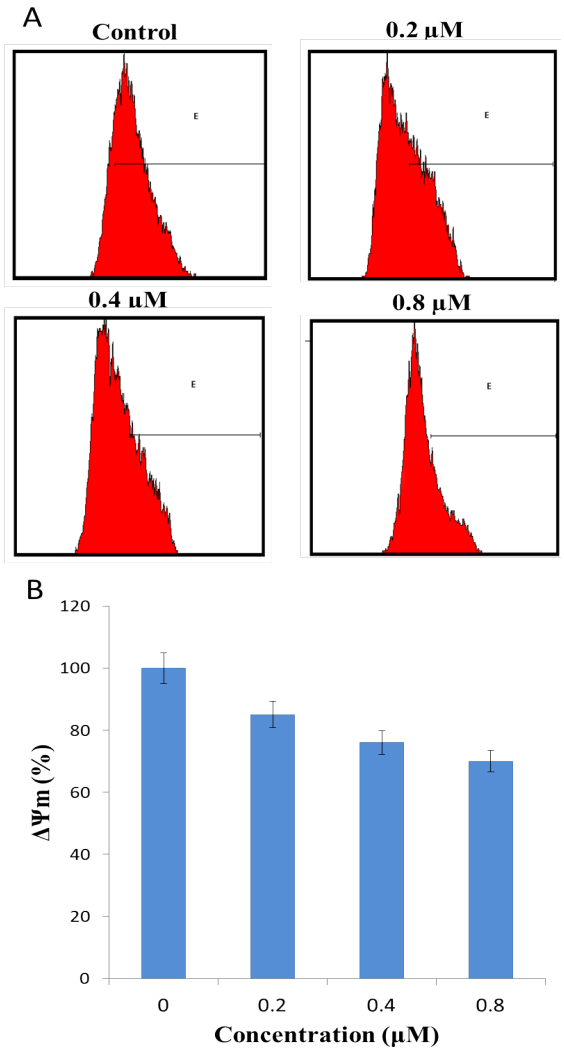


Fig. 3: Analysis of ΔΨm in resistomycin treated MDA-MB231 cells relative to control cells (A) Flow cytometry analysis showing the dissipation of MMP in resistomycin treated (0, 0.2, 0.4, 0.8µM) cells. (B) Graph presenting the percentage loss of MMP in resistomycin treated (0, 0.2, 0.4, 0.8µM) MDA-MB-231 cells. Values are expressed as means ± SD. P<0.05 compared to the control group.

Anticancer activity of natural compounds is governed by the induction of apoptosis in cancerous cells (Safarzadeh *et al*., 2014). Programmed cell death proceeds via intrinsic and extrinsic pathways (Xu *et al*., 2019). The intrinsic cell-death pathway is activated via modulation of Bcl-2 family proteins, MMP dissipation, release of cytochrome c, and these factors ultimately cause activation of caspase-3 which induce apoptosis (Plati *et al*., 2011). As resistomycin has been previously known to induce apoptosis in colon (Nadysev *et al*., 2018), gastric carcinoma (Adinarayanaa *et al*., 2006) hepatic, and cervical cancer cells (Vijayabharathi *et al*., 2011), however, there is very limited data available on targets of resistomycin in cancer cells. In this study, we have demonstrated that resistomycin dissipates MMP to induce apoptosis in TNBC cells. These results are found to be consistent with literature citing the potential role of mitochondrial trans-membrane depolarization in natural compounds-induced apoptosis in cancer cells (Khan *et al*., 2012). In the light of the results from this study, it is suggested that *in vivo* efficacy and toxicity profile of resistomycin should be explored to pave a way for this bioactive compound as a promising anti-TNBC drug candidate in the near future.

CONCLUSION

To the best of author's knowledge, this is first report on resistomycin anti-cancer potential towards MDA-MB231 cells. Our study illustrated that resistomycin inhibits the proliferative capability of MDA-MB231 cells more efficiently than non-triple negative breast cancer (MCF-7) cells. Resistomycin halts the proliferation of TNBC cells by inducing apoptosis and dissipating the MMP. Moreover, the low inhibitory concentrations of this compound against TNBC cell line support its effective cytotoxic potential. MDA-MB231 cells are not only model for TNBC but are also glycolytic dependent cancer cells (Li *et al*., 2014), thus, it is recommended that further studies should also investigate the role of resistomycin against metabolic enzymes especially PKM2 (Pyruvate kinase M2). Our results provide a rationale for resistomycin to be potentiated as a lead candidate against TNBC but *in vivo* efficacy and biosafety profile of resistomycin needs to be further investigated**.**

ACKNOWLEDGEMENTS

This study was supported by The Nagai Foundation Tokyo, Japan (NFT-R-2018) and NRPU Research Grants, (8381/Punjab/NRPU/R&D/HEC/2017;8382/Punjab/NRPU /R&D/HEC /2017).

REFERENCES

Adinarayanaa G, Venkateshana MR, Bapirajub VVSNK, Sujathab P, Premkumarb J, Ellaiahb P and Zeeckc A (2006). Cytotoxic Compounds from the Marine Actinobacterium *Streptomyces corchorusii* AUBN1/71. *Russian. J. Bioorg. Chem.*, **32**(3): 295-300.

Atanasov AG, Waltenberger B, Pferschy-Wenzig EM, Linder T, Wawrosch C, Uhrin P, Temml V, Wang L, Schwaiger S, Heiss EH, Rollinger JM, Schuster D, Breuss JM, Bochkov V, Mihovilovic MD, Kopp B, Bauer R, Dirsch VM and Stuppner H (2015). Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnol Adv.*, **33**(8): 1582-1614.

Chavez KJ, Garimella SV and Lipkowitz S (2010). Triple negative breast cancer cell lines: One tool in the search for better treatment of triple negative breast cancer. *Breast Dis.*, **32**(1-2): 35-48.

Choudhari AS, Mandave PC, Deshpande M, Ranjekar P and Prakash O (2019). Phytochemicals in Cancer Treatment: From Preclinical Studies to Clinical Practice. *Front Pharmacol.*, **2019**: 10.3389/ fphar.2019.01614.

Cleator S, Heller W and Coombes RC (2007). Triple-negative breast cancer: therapeutic options. *Lancet Oncol*, **8**(3): 235-244.

Di W, Khan M, Rasul A, Sun M, Sui Y, Zhong L, Yang L, Zhu Q, Feng L and Ma T (2014). Isoalantolactone inhibits constitutive NF-kappaB activation and induces reactive oxygen species-mediated apoptosis in osteosarcoma U2OS cells through mitochondrial dysfunction. *Oncol Rep.*, **32**(4): 1585-1593.

Gadi VK and Davidson NE (2017). Practical Approach to Triple-Negative Breast Cancer. *J. Oncol. Pract.*, **13**(5): 293-300.

Han J, Lim W, You D, Jeong Y, Kim S, Lee JE, Shin TH, Lee G and Park S (2019). Chemoresistance in the Human Triple-Negative Breast Cancer Cell Line MDA-MB-231 Induced by Doxorubicin Gradient Is Associated with Epigenetic Alterations in Histone Deacetylase. *J Oncol.*, **2019**: 10.1155/2019/1345026.

Jakobi K and Hertweck C (2004). A gene cluster encoding resistomycin biosynthesis in Streptomyces resistomycificus; exploring polyketide cyclization beyond linear and angucyclic patterns. *J. Am. Chem. Soc.*, **126**(8): 2298-2299.

Jitariu AA, Cimpean AM, Ribatti D and Raica M (2017). Triple negative breast cancer: The kiss of death. *Oncotarget*, **8**(28): 46652-46662.

Jones SE and Elliot MA (2017). Streptomyces Exploration: Competition, Volatile Communication and New Bacterial Behaviours. *Trends Microbiol.*, **25**(7): 522-531.

Khan M, Yi F, Rasul A, Li T, Wang N, Gao H, Gao R and Ma T (2012). Alantolactone induces apoptosis in glioblastoma cells via GSH depletion, ROS generation, and mitochondrial dysfunction. *IUBMB Life*, **64**(9): 783-794.

Kim C, Gao R, Sei E, Brandt R, Hartman J, Hatschek T, Crosetto N, Foukakis T and Navin NE (2018).

Chemoresistance Evolution in Triple-Negative Breast Cancer Delineated by Single-Cell Sequencing. *Cell*, **173**(4): 879-893 e813.

Li W, Liu J, Jackson K, Shi R and Zhao Y (2014). Sensitizing the therapeutic efficacy of taxol with shikonin in human breast cancer cells. *PLoS One*, **9**(4): e94079.

Nadysev GY, Tikhomirov AS, Lin MH, Yang YT, Dezhenkova LG, Chen HY, Kaluzhny DN, Schols D, Shtil AA, Shchekotikhin AE and Chueh PJ (2018). Aminomethylation of heliomycin: Preparation and anticancer characterization of the first series of semi-synthetic derivatives. *Eur. J. Med. Chem.*, **143**: 1553-1562.

Plati J, Bucur O and Khosravi-Far R (2011). Apoptotic cell signaling in cancer progression and therapy. *Integr Biol. (Camb)*, **3**(4): 279-296.

Portha H, Jankowski C, Cortet M, Desmoulins I, Martin E, Lorgis V, Arnould L and Coutant C (2016). Non-metastatic triple-negative breast cancer in 2016: Definitions and management. *Gynecol Obstet Fertil*, **44**(9): 492-504.

Rasul A, Khan M, Yu B, Ma T and Yang H (2011). Xanthoxyletin, a coumarin induces S phase arrest and apoptosis in human gastric adenocarcinoma SGC-7901 cells. *Asian Pac. J. Cancer Prev.*, **12**(5): 1219-1223.

Rasul A, Yu B, Khan M, Zhang K, Iqbal F, Ma T and Yang H (2012a). Magnolol, a natural compound, induces apoptosis of SGC-7901 human gastric adenocarcinoma cells via the mitochondrial and PI3K/Akt signaling pathways. *Int. J. Oncol.*, **40**(4): 1153-1161.

Rasul A, Yu B, Zhong L, Khan M, Yang H and Ma T (2012b). Cytotoxic effect of evodiamine in SGC-7901 human gastric adenocarcinoma cells via simultaneous induction of apoptosis and autophagy. *Oncol. Rep.*, **27**(5): 1481-1487.

Riaz A, Rasul A, Hussain G, Zubair M, Samiullah K, Sarfraz I, Masood M, Jabeen F, Sultana T and Sultana S (2019). Eriocalyxin B induces apoptosis in human triple negative breast cancer cells via inhibiting STAT3 activation and mitochondrial dysfunction. *Pak J Pharm Sci.*, **32**(6): 2483-2848.

Roggo BE, Petersen F, Delmendo R, Jenny HB, Peter HH and Roesel J (1994). 3-Alkanoyl-5-hydroxymethyl tetronic acid homologues and resistomycin: new inhibitors of HIV-1 protease. I. Fermentation, isolation and biological activity. *J. Antibiot (Tokyo)*, **47**(2): 136-142.

Safarzadeh E, Sandoghchian Shotorbani S and Baradaran B (2014). Herbal medicine as inducers of apoptosis in cancer treatment. *Adv. Pharm. Bull.*, **4**(Suppl 1): 421-427.

Vijayabharathi R, Bruheim P, Andreassen T, Raja DS, Devi PB, Sathyabama S and Priyadarisini VB (2011). Assessment of resistomycin, as an anticancer compound isolated and characterized from Streptomyces aurantiacus AAA5. *J. Microbiol.*, **49**(6): 920-926.

Xu X, Lai Y and Hua ZC (2019). Apoptosis and apoptotic body: Disease message and therapeutic target potentials. *Biosci Rep.*, **39**(1): 1-17.

Zhang YL, Li S, Jiang DH, Kong LC, Zhang PH and Xu JD (2013). Antifungal activities of metabolites produced by a termite-associated Streptomyces canus BYB02. *J. Agric. Food Chem.*, **61**(7): 1521-1524.

Zupko I, Jaeger W, Topcu Z and Wu CC (2015). Anticancer properties of natural products. *Biomed. Res. Int.*, **2015**: 10.1155/2015/242070.