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Changes in gene expression of *CXCR4*, *CCR7* and *BCL2* after treatment of breast cancer cells with saponin extract from Tribulus terrestris

T. E. GORANOVA[1,*], S. S. BOZHANOV[2], V. S. LOZANOV[3], V. I. MITEV[1,3], R. P. KANEVA[1,3], E. I. GEORGIEVA[2]

[1]*Molecular Medicine Center, Medical University – Sofia, 2 Zdrave Street, 1431 Sofia, Bulgaria;* [2]*Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Akad. G. Bonchev Street, Bldg. 21, 1113 Sofia, Bulgaria;* [3]*Department of Medical Chemistry and Biochemistry, Medical University – Sofia, 2 Zdrave Street, 1431 Sofia, Bulgaria*

**Correspondence: goranova@mmcbg.org*

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Saponins are natural substances produced by a large number of plants, one of which is Tribulus terrestris L. (TT). They have been reported to possess an antitumor activity exerted by regulating various signaling pathways in the cell. Although the mechanisms of action of saponin extracts from various plants have been widely studied, limited data are available about TT. The present study aimed to analyze the impact of saponin extract from TT on cell processes in breast carcinoma cell lines. The variations in expression of a group of 32 selected genes were examined by real-time PCR after saponin treatment of MCF7 and MCF10A cell lines. Only three genes – *CXCR4*, *CCR7* and *BCL2*, showed changes in their mRNA levels after the application of the herb extract. While *CXCR4* expression was reduced in both cell lines, *CCR7* and *BCL2* levels decreased only in tumorigenic MCF7 cells, implying cell-specificity of the saponin action. Our results suggested that TT extract containing saponins was likely to affect the processes of apoptosis and metastasizing of cancer cells. Further in vivo studies will show its applicability as an anticancer therapeutic agent.

Key words: saponins, Tribulus terrestris, breast cancer, CXCR4, CCR7, BCL2

Tribulus terrestris L. (TT) is a herb, widely spread in Mediterranean region, Southern Asia and Africa [1]. It has been used as an aphrodisiac, diuretic, antihypertension, antimicrobial, antiacetylcholine, hemolytic and anthelmintic substance as well as to treat coughs, kidney failure and cancer [1-3]. The biologically active compounds in TT are considered to be the steroidal saponins [4]. Saponins are a group of naturally occurring plant glycosides with strong foam-forming properties in aqueous solution [5]. They have been found not only in TT, but also in a variety of other higher plants. However, among Bulgarian herbs TT is the one that is known to contain a big fraction of saponins, mainly protodioscin [4]. A lot of studies have been performed on plant extracts and have reported that saponins exert an antitumor effect on various cancer types [5-7]. Saponins from TT have been found to suppress proliferation and induce apoptosis in fibroblast cells [8] and to activate apoptotic processes in breast cancer cells [9]. However, there are limited data about the mechanisms involved. Studies on saponins from other plants have shown that they exert a cytotoxic effect by regulating proliferation mediators (cyclins and CDKs), modulating signaling pathways (mTOR, ERK1/2, etc.) elevating proapoptotic molecules levels, etc. [10-13].

In the present study we analyzed the effect of saponin extract from TT on the expression of 32 genes involved in the breast cancer formation and development. The selected group included genes from various pathways responsible for cell migration and metastasis formation, apoptosis, DNA repair, DNA demethylation, regulation of the cell cycle, transcription and angiogenesis.

Materials and methods

Purification of saponins from Tribulus terrestris. The purification was done as previously described [8, 9]. Briefly, the powder from aerial parts of the herb was extracted three times with 70% ethanol and the extract was evaporated to dryness under vacuum at temperature below 50°C. The residue was dissolved in water and extracted by aqueous butanol three times. The butanol extract was evaporated to dryness under vacuum and then was subjected to chromatography

on silica gel. Saponins were eluted from the column using mixture of CHCl3-MeOH-H2O (50:10:1, v/v/v/). The contents of saponins in the fraction selected for the experiments were determined to be more than 99% by photometric analysis described previously [14]. In addition, the fraction was standardized on base of protodioscin contents by RP-HPLC [15] using a commercially available standard (ChromaDex, Inc., Santa Ana, CA). The used fraction was found to contain no less than 94 % protodioscin.

Cell lines and saponin treatment. As a model system in our study two adherent human cell lines – the non-tumorigenic breast epithelial cell line MCF-10A and the breast carcinoma cell line MCF-7, were used. Both cell lines were purchased from the American Type Culture Collection (ATCC). MCF-7 breast tumor cell line was derived from the pleural effusion of a patient with invasive breast ductal carcinoma [16]. MCF-10A cell line was established from normal breast tissue of a patient with fibrocystic breast disease [17]. Cultures were maintained in monolayers at 37°C in a humidified atmosphere containing 5% CO2. MCF-7 was grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate and 1% non-essential amino acids. MCF-10A cells were grown in the same medium, supplemented with 10 μg/ml insulin, 20 ng/ml human epidermal growth factor (hEGF) and 0.5 μg/ml hydrocortisone.

The cells were split at 80-90% confluence using trypsin/EDTA and sub-cultured at a dilution of 1:3-1:6. The culture medium was changed twice a week, and passages 6–40 were used for the experiments.

MCF-10A and MCF-7 cells were seeded into six-well plates at a concentration of 250 000 cells per well. The control cells were seeded at the same time as the cells that would be treated with saponins later. After 24 hours both cell lines were incubated with the saponin fraction from TT at a final concentration of 90 μg/ml for 3, 7, 24, 48, and 72 hours. The selected concentration was shown to have the strongest antitumor ef-

Table 1. A list of the selected genes and primers for gene-expression analysis

Gene Symbol	Name	Reference	QuantiTect Primer Assay
AKT1	v-akt murine thymoma viral oncogene homolog 1	OMIM: 114480	Hs_AKT1_1_SG
ATM	ataxia telangiectasia mutated	OMIM: 114480	Hs_ATM_1_SG
BAX	BCL2-associated X protein	[9, 18]	Hs_BAX_1_SG
BCL2	B-cell CLL/lymphoma 2	[9, 18]	Hs_BCL2_1_SG
BRCA1	breast cancer 1, early onset	OMIM: 114480	Hs_BRCA1_1_SG
BRCA2	breast cancer 2, early onset	OMIM: 114480	Hs_BRCA2_1_SG
BRIP1	BRCA1 interacting protein C-terminal helicase 1	OMIM: 114480	Hs_BRIP1_1_SG
CASP8	caspase 8, apoptosis-related cysteine peptidase	OMIM: 114480	Hs_CASP8_1_SG
CCR7	chemokine (C-C motif) receptor 7	OMIM: 114480	Hs_CCR7_1_SG
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	OMIM: 114480	Hs_CDH1_1_SG
CHEK2	checkpoint kinase 2	OMIM: 114480	Hs_CHEK2_1_SG
CXCR4	chemokine (C-X-C motif) receptor 4	OMIM: 114480	Hs_CXCR4_1_SG
EZH2	enhancer of zeste homolog 2 (Drosophila)	[19]	Hs_EZH2_1_SG
HMMR	hyaluronan-mediated motility receptor (RHAMM)	OMIM: 114480	Hs_HMMR_1_SG
KDM1A	lysine (K)-specific demethylase 1A (LSD1)	[20]	Hs_KDM1A_1_SG
KDM5B	lysine (K)-specific demethylase 5B (JARID1B)	[20]	Hs_KDM5B_1_SG
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	OMIM: 114480	Hs_KRAS_1_SG
KRIT1	KRIT1, ankyrin repeat containing	[21]	Hs_KRIT1_1_SG
MAP3K11	mitogen-activated protein kinase kinase kinase 11	OMIM: 114480	Hs_MAP3K11_1_SG
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	OMIM: 114480	Hs_MYC_1_SG
NQO2	NAD(P)H dehydrogenase, quinone 2	OMIM: 114480	Hs_NQO2_1_SG
ORAI1	ORAI calcium release-activated calcium modulator 1	[22]	Hs_ORAI1_1_SG
PALB2	partner and localizer of BRCA2	OMIM: 114480	Hs_PALB2_1_SG
PHB	prohibitin	OMIM: 114480	Hs_PHB_1_SG
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha	OMIM: 114480	Hs_PIK3CA_1_SG
PPM1D	protein phosphatase, Mg2+/Mn2+ dependent, 1D	OMIM: 114480	Hs_PPM1D_1_SG
RAD54L	RAD54-like (S. cerevisiae)	OMIM: 114480	Hs_RAD54L_1_SG
RB1CC1	RB1-inducible coiled-coil 1	OMIM: 114480	Hs_RB1CC1_1_SG
RHOC	ras homolog family member C	[23]	Hs_RHOC_1_SG
SLC22A18	solute carrier family 22, member 18	OMIM: 114480	Hs_SLC22A18_1_SG
TP53	tumor protein p53	OMIM: 114480	Hs_TP53_1_SG
XRCC3	X-ray repair complementing defective repair in Chinese hamster cells 3	OMIM: 114480	Hs_XRCC3_1_SG

fect – MCF7 tumor cells had the lowest viability (34%), while the normal cells viability was 88% [9]. The cells were then incubated for 3, 7, 24, 48, and 72 hours and their viability was checked to confirm that there were enough alive cells for RNA extraction. PBS (solvent of the saponin fraction) was added to each of the respective controls. All the experiments were done in triplicates.

Total RNA extraction and real-time PCR. Total RNA was isolated from saponin-treated and control cells of both MCF-7 and MCF-10A cell lines at 0, 3, 7, 24, 48 and 72h after treatment/ cultivation using RNAspin mini (GE Healthcare) according to the manufacturer's protocol. The quality of RNA was checked by denaturing electrophoresis on a formaldehyde gel. The amount of RNA was determined spectrophotometrically.

Thirty-two genes were selected on the basis of published information (OMIM: 114480 and PubMed search) so that they represent various cell processes in breast cancer – tumor cell migration and metastasizing, DNA repair and demethylation, regulation of the cell cycle, transcription, apoptosis and angiogenesis – Table 1. Gene expression was analyzed by real-time PCR on Rotor-Gene Q (Qiagen). First, 1 μg RNA of each sample underwent reverse transcription using High-capacity cDNA reverse transcription kit (Applied Biosystems) according to manufacturer's recommendations. Real-time PCR reaction was performed in 25 μl volume and the mixture included: 1x RotorGene SYBR Green PCR Mix, 1x QuantiTect Primer Assay for the respective gene (Table 1) and 100 ng cDNA. The conditions were: initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 15 sec, primer annealing at 55°C for 30 sec and synthesis with data acquisition at 72°C for 30 sec. Each sample was examined in triplicates; negative and no template controls were evaluated as well. Beta-actin expression was used for normalization. To determine the relative quantity (RQ) for each gene, the ddCt method was applied [24]. Expression levels were determined at 3, 7, 24, 48 and 72h with/ without saponin treatment compared to the levels at 0h. T-test was used to calculate the statistical significance of the results. P values below 0.05 were accepted as statistically significant.

Results

Thirty-two selected genes were analyzed for variations in their expression after saponin treatment of MCF-7 and MCF-10A cell lines by real-time PCR. In order to reduce the bias of in vitro cultivation, which might affect changes in mRNA levels with time, the expression of each gene in treated cells was compared to the expression of the same gene in control cells at the same time point. When data were analyzed, altered expression was found in only three genes – *CXCR4, CCR7* and *BCL2*.

The time-dependent profile of expression in the non-treated cells was different for the two cell lines. While *CXCR4* expression slowly increased with time in MCF7 – Fig. 1a; in MCF10A cell line more than 50 times higher levels of *CXCR4*

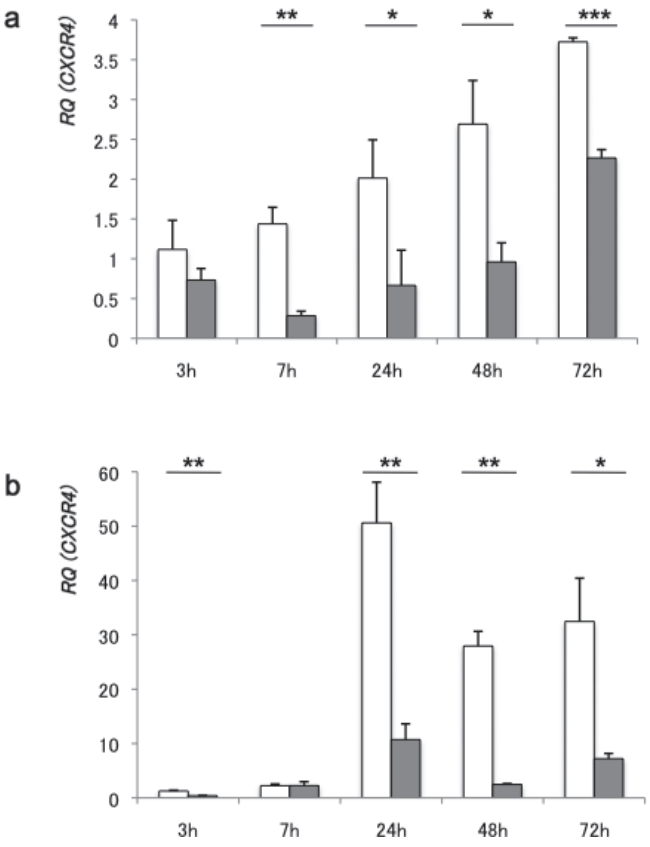

Figure 1. Fold expression change of *CXCR4* gene at 3h, 7h, 24h, 48h and 72h after cultivation/ treatment of MCF7 (a) and MCF10A (b) cells. White columns refer to non-treated, and grey columns – to treated with saponin cells. Standard deviation is shown with bars. RQ – relative quantity. * - p<0.05; ** - p<0.01; * - p<0.001.**

were observed at 24h, then lower expression was detected – Fig. 1b. All three replicates of the experiment showed high levels of CXCR4 in MCF10A cell line at 24h. However, when the cells were treated with saponin fraction from TT, the expression of *CXCR4* decreased in both cell lines. Significant down-regulation of *CXCR4* was observed starting from 7h and 24h after treatment of MCF7 and MCF10A cells, respectively. Decrease in *CXCR4* levels was also detected 3h after the application of saponin extract to MCF10A cells; however at 7h there was almost no difference between the treated and the non-treated MCF10A cells.

CCR7 levels varied also between the cell lines – in MCF7 non-treated cells *CCR7* first increased reaching a peak at 24h after which there was a decrease – Fig. 2a; in MCF10A slow rise with a peak at 72h was observed – Fig. 2b. Even though MCF7 saponin-treated cells showed lower expression of *CCR7* at all times except for 3h after treatment, statistically significant difference between treated and control cells was found only at 24h ($p=4.5\times10^{-5}$). In MCF10A cell line significant increase of *CCR7* was detected 7h post-treatment (p=0.026).

Gene expression of *BCL2* in MCF7 changed slightly with time – Fig. 3a; while in MCF10A it was more unstable – Fig. 3b. When compared with the one in saponin-treated cells a decrease was found at 7h after application and the trend was kept up to the 72h. The only exception was at 3h post-treatment of MCF7 when a higher level of *BCL2* was detected in treated cells. In MCF10A significant rising of *BCL2* was observed at 24h and 72h after saponin application.

Other examined genes showed no statistically significant change of expression after treatment with plant extract, although there were small differences in the time-dependent expression of some genes between the two non-treated cell lines (data not shown).

Discussion

Saponins are promising candidates for cancer therapy found in many herbal plants. However, they differ in structure and thus, in the way they exert antitumor effects. In the present study we analyzed the impact of the saponin fraction from TT on the expression of a group of selected genes in MCF7 and MCF10A cell lines. We found that expression of three genes – *CXCR4, CCR7* and *BCL2,* was significantly influenced by the saponin application.

CXCR4 gene encodes a chemokine G-protein coupled receptor, which is highly expressed in epithelial cells [25]. Our results on non-treated MCF10A cells are in agreement with the previous reports as this cell line, which is of epithelial origin, showed increase in *CXCR4* levels, especially 24h after cultivation when the mRNA levels were more than 50 times higher than at the beginning – Fig. 1b. Even though overexpression of *CXCR4* in MCF10A cell line has been reported before [26], we could not find in the literature any particular data explaining why the levels of *CXCR4* were higher in non-cancerous than in cancerous cells. Moreover, there are no published data about the expression change of *CXCR4* in those cell lines with time. It might be a feature of the selected cell line, which enables its in vitro cultivation.

CXCR4 is also upregulated in many cancer types including breast cancer [27]. The same was observed in the studied tumorigenic cell line, MCF7 – Fig. 1a. The high level of *CXCR4*

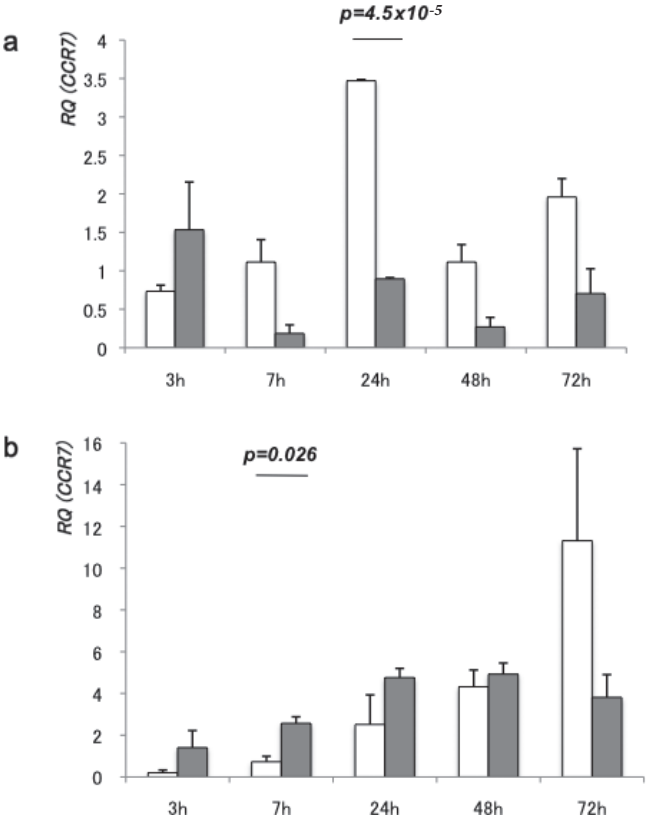

Figure 2. Fold expression change of *CCR7* gene at 3h, 7h, 24h, 48h and 72h after cultivation/ treatment of MCF7 (a) and MCF10A (b) cells. White columns refer to non-treated, and grey columns – to treated with saponin cells. Standard deviation is presented with bars. RQ – relative quantity. Only p values below 0.05 are shown.

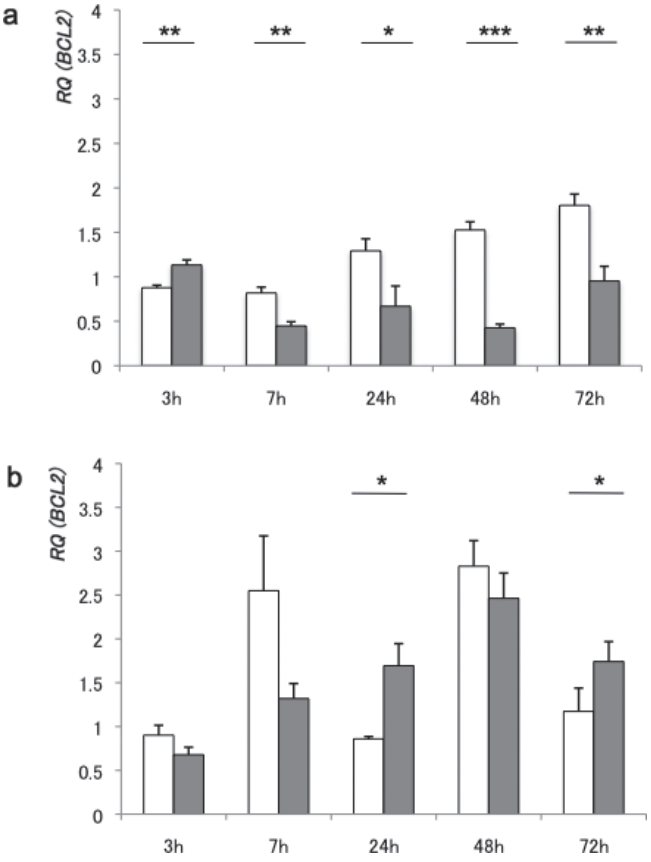

Figure 3. Fold expression change of *BCL2* gene at 3h, 7h, 24h, 48h and 72h after cultivation/ treatment of MCF7 (a) and MCF10A (b) cells. White columns refer to non-treated, and grey columns – to treated with saponin cells. Standard deviation is shown with bars. RQ – relative quantity. * – p<0.05; ** – p<0.01; * – p<0.001.**

mRNA in tumor cells is believed to be involved in their survival under hypoxia [28]. Further, overexpression of *CXCR4* has been associated with metastases formation and poor prognosis of patients with breast and other types of cancer [29-31], which explains the significant interest towards CXCR4 inhibitors [32, 33].

In the current study we found lower *CXCR4* levels in both cell lines after saponin treatment. It is important to mention that the observed decrease in expression of *CXCR4* was stable in time for MCF7. The equal levels of *CXCR4* in treated and control MCF10A cells at 7h implied that the reduction in expression of *CXCR4* in these cells required more time. Even though there are no data about saponins from TT, *CXCR4* downregulation at protein level has been detected after treatment of cells with saponins extracted from various other plants like ginseng [32].

The gene *CCR7* encodes a chemokine receptor that binds its ligand *CCL21*, which is widely found in lymph nodes. Thus, upregulation of *CCR7* has been associated with lymph node metastasizing [34]. Increased levels of *CCR7* have been found in a number of tumor types, one of which is breast cancer [35-37]. As expression of *CCR7* is reportedly correlated with lymphatic metastasis and poor prognosis in breast cancers, it could be a good therapeutic target in breast cancer therapy [38].

Our control cells also showed a peak in the levels of *CCR7* mRNA – at 24h for MCF7 and at 72h for MCF10A – Fig. 2a/2b. Although MCF10A is not a cancer cell line, its *CCR7* increased with time, which might be a result of the cell response to long cultivation. Notably, saponin treatment of MCF7 cells resulted in a decrease of *CCR7*, suggesting an anti-metastatic activity. On the other hand, MCF10A cells showed an increase of *CCR7* after treatment, which should be further analyzed functionally. Moreover, while the lower level of *CCR7* might be associated with apoptosis, it has also a vast effect on lymphocyte localization and could cause their accumulation in epithelial tissues [39]. This two-sided effect of *CCR7* expression should be taken into consideration in future in vivo studies on antitumor activity of TT extract.

The product of *BCL2* gene is a mitochondrial membrane protein that blocks apoptosis. It is overexpressed in many types of cancer [40, 41]. It should be mentioned that in our study the non-tumorigenic cell line MCF10A showed higher levels of *BCL2* than MCF7, even though they were not stable in time, probably as a result of cell cycle fluctuations [42]. High expression of *BCL2* has been reported to slow growth by increasing the time cells spend in G1, thus lengthening the cell cycle [43].

MCF7 cells that were treated with saponin extract from TT showed decrease in *BCL2* levels, which is in agreement with the previously reported potential antitumor activity of the herb [9]. Downregulation of *BCL2* in a hepatoma cell line after its treatment with saponins has been reported not for TT, but for the asparagus extract [44]. However, application of saponins to MCF10A cells caused increase in *BCL2* levels. Thus, the effect of saponins on *BCL2* expression is likely to be cell-specific.

It has been recently shown that CXCR4 and CCR7 interact to form functional dimeric receptor and to promote cancer metastasis [45]. Activation of the chemokine receptors by their respective ligands, CXCL12 and CCL21 specifically reduces the sensitivity of metastatic breast cancer cells to anoikis by a distinct mechanism of selective regulation of pro-apoptotic Bmf and anti-apoptotic Bcl-xL proteins [46]. However in benign or non-invasive cancer cells, while individual receptors are still expressed on the cell surface, the CXCR4/ CCR7 heterodimer is not present and both receptors are silent. The CXCR4 and CCR7 functional inter-dependency in metastatic cells was also shown, as inactivation of either receptor is sufficient to almost completely abrogate the activation of the other and to alter the cells' metastatic phenotype both in vitro and in vivo [45].

In summary, our results showed that saponins from TT exert their antitumor role by lowering the expression of *CXCR4* and *CCR7*, which is likely to result in reducing the metastatic potential of the cells. This coupled with a decrease of the *BCL2* levels in the tumorigenic cell line, points to a mechanism of action related to activation of apoptosis. However, further analysis of the impact of saponins at in vitro and in vivo models should be performed to elucidate this issue.

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