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Research Article

CHEMOTHERAPEUTIC POTENTIAL OF NOVEL XANTHONE SOURCED FROM *SWERTIA CHIRATA* AGAINST SKIN CARCINOGENESIS

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ABSTRACT

Objective: *Swertia chirata* forms a rich source of bio-active compounds, among which xanthones form an important part. Among the xanthones present in it, 1,5,8 Tri-hydroxy-3-methoxy xanthone (TMX) was found to be the most active. The present study aims to evaluate the chemotherapeutic potential of it against metastatic skin cancer cell lines.

Methods: In this study, the antitumor activity of TMX (the active component of chirata plant) was evaluated in A431, SKMEL-5, and A375 cell line by using *in-vitro* assays such as cell viability assay, cell cycle analysis, caspase 3 activity assay, intracellular reactive oxygen species (ROS) level determination by dichlorofluorescein diacetate, and quantitative real-time polymerase chain reaction (qRT-PCR).

Results: *In vitro* studies showed that TMX from *S. chirata* exhibited significant antitumor activity by inducing apoptosis and restricting proliferation in both melanoma and non-melanoma skin cancer cell lines, but no such activity was seen in normal skin cancer cell line WS1. The qRT-PCR analysis revealed that in both the melanoma ad non-melanoma cell lines, TMX could exert its antitumor activity by downregulating c-Myc, cyclin-D1, and β-catenin and up-regulating Wnt antagonist gsk-3β, thereby suppressing wnt self-renewal pathway, but such regulation was absent in normal cell line.

Conclusions: TMX from chirata could effectively inhibit the proliferation of metastatic skin cancer (both melanoma and non-melanoma) cell lines while being non-toxic to normal cell lines. The chemotherapeutic potential of TMX against metastatic skin cancer cell lines was achieved by downregulating several key regulatory genes enabling the suppression of the self-renewal pathway, the chief reason behind the invasiveness of cancer cells.

Keywords: Chirata, Metastasis, Melanoma, Squamous cell carcinoma, Xanthones.

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INTRODUCTION

Skin cancer is the most prevalent among all human cancers. In 2020, more than 100,000 people in the U.S. are expected to be diagnosed with either melanoma or non-melanoma skin cancer. Nearly 7000 are expected to die due to metastasis [1]. There are three major types of skin cancers: Basal cell carcinoma (BCC), squamous cell carcinoma (SCC), and melanoma. BCC has an excellent prognosis, but most cancer-related deaths were due to SCC's invasive nature and melanoma. Around 50% of the people affected with melanoma are likely to develop the invasive type, and people affected with SCC are more susceptible to death from any cause than the general population [2,3]. The Wnt/β-catenin signaling pathway and its regulator c-Myc, cyclin-D1, and β-catenin are important in both melanoma progression and non-melanoma type of skin tumors [4]. Aberrant activation of Wnt/β-catenin signaling leads to a rise in the nuclear accumulation of β-catenin, which leads to transcription of oncogenes such as c-Myc and cyclin D1. Together, these oncogenes play a crucial role in the maintenance of cancer stem cells (CSCs), which play a nodal role in metastasis [5]. Therefore, targeting these molecules could pave the way for a drug that could downregulate CSCs and so, targeting drug-resistant, metastatic form of cancers and providing a better prognosis for patients is the need of the hour. Phytometabolites have the eye of cancer researchers as they have lesser toxicity; some of them showed activity against CSCs and thereby was proved to be highly efficacious against drug-resistant metastatic cancers [6]. Among the endless array of medicinal plants, *Swertia chirata* deserves special mention due to its multiferous medicinal usage. *S. chirata* is rich in several xanthones, among which 1,5,8-trihydroxy-3-Methoxy xanthone (TMX) was considered to hold the most promise [7]. We found that it could inhibit breast cancer cells at a low dosage of 1 μM while being non-toxic to normal fibroblasts at a much higher concentration, which signified specificity to cancer cells (data not shown). In another study, we found that TMX could restrict DMBA/croton oil-induced carcinogenesis to mild dysplastic lesions at the low dosage of 20 μg/kg in Swiss albino mice and could also significantly increase survivability (data not shown). These observations lead us to hypothesize that TMX could be efficacious against metastatic skin cancer while being non-toxic to normal cells. Therefore, in this study, we aim to evaluate and elucidate the mechanism by which TMX could exert antitumor activity against SCC cell line A431 and melanoma cell lines SKMEL-5 and A375 while being non-toxic to normal cell line WS1.

METHODS

Plant material

S. chirata (whole plant) was collected from a local plant supplier of Kolkata and was authenticated by Dr. S.R. Das, Ex-Survey Officer, Central Research Institute (Ayurveda), Kolkata. The plant specimen has been preserved in the Herbarium of the Central Research Institute (Ayurveda), Kolkata.

Isolation and purification of TMX from *S. chirata*

Purified TMX was isolated from the plant of *S. chirata* Buch. Ham (4 kg). The crude extract was prepared by normal hexane in the Soxhlet apparatus for 72 h. The extract was concentrated to a thick yellowish liquid (250 g). This liquid was kept in the refrigerator for 24 h. A pale yellow amorphous solid (2.4 g) was separated out from the chilled liquid. The solid was filtered and purified by repeated crystallization from ethanol. Finally, shining yellow needle-shaped crystals (TMX) was obtained (yield 0.52%), having a melting point 270–271°C. All the compounds were identified by detailed spectral analyses such as U.V., I.R., NMR, and mass spectrometry. Extraction and purification of this

secoiridoid glycoside were done at the National Research Institute for Ayurvedic Drug Development, Kolkata, India. The process of isolation is patented, Indian Patent no -191129, dated March 26, 2002.

Cell line and cell culture condition

Cell lines A431, A375, SKMEL-005, and WS1 were obtained from National Centre for Cell Sciences, Pune, India. Cell lines SKMEL-005 and WS1 were maintained in Eagle's Minimum Essential Medium, with 10% fetal bovine serum, 2 mM glutamine, 100 U penicillin, and 100 µg/ml of streptomycin. Cells were grown at 37°C in a humidified CO_2 incubator with 5% CO_2. Refeeding with fresh growth medium and subculturing (using 0.05% trypsin-ethylenediaminetetraacetic acid [EDTA]) of cells was done as per requirement. A431 and A375 were maintained in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum, 2 mM glutamine, 100 U penicillin, and 100 µg/ml of streptomycin. Cells were grown at 37°C in a humidified CO_2 incubator with 5% CO_2. Refeeding with fresh growth medium and subculturing (using 0.05% trypsin-EDTA) of cells was done as per requirement [8].

Cytotoxicity analysis

Cytotoxic effects of TMX on A431, A375, SKMEL-005, and WS1 cells were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test as described in the manufacturer's protocol (HiMedia, India). Briefly, the cells (5×10^3) were seeded into 96-well plates. After 24 h, the cells were treated with different concentrations of TMX (1–20 µM) for 48 h, then cell viability test was performed, and the dose-response curve was drawn [9].

Cell cycle analysis

Programmed cell death or apoptosis is an ordered and orchestrated cellular event that encompasses a series of biochemical and morphological changes. The pattern of cell cycle phase distribution of all the cells was analyzed by fluorescence-activated cell sorting (Calibur, Becton Dickinson, USA) assay by 50 µg/ml of propidium iodide staining, and data were analyzed by cell quest pro software [10].

Caspase 3 activity assay

Caspase 3 enzyme activity assay was performed with the caspase-3 colorimetric assay kit (Abcam) to detect the caspase-3 enzyme activity. Briefly, after 24 h of different treatments, the supernatant was removed, and the cells were trypsinized. Then, the cells were collected and centrifuged at 14,000 rpm for 5 min. Cell lysis buffer was then added and the cells were retained on ice and centrifuged. Protein concentration was measured with Bradford assay [11]. Proteins (50 µg) diluted by the addition of cell lysis buffer. Then, 50 µL 2× reaction buffer and 5 µL DEVD-pNA 4 mM substrate were added. The plates were then incubated at 37°C for 2 h, and reading was taken at 405 nm. The changes in caspase 3 activities were assessed by comparing these data with the level of the untreated control.

Evaluation of cellular damage by quantitating intracellular reactive oxygen species (ROS) generation

On the verge of maintaining intracellular levels of free radicals, a number of cellular defense mechanisms evolved to meet the need to maintain a balance between generation and removal of this oxidative stress from the cell. The relative level of ROS was measured by spectrofluorimetric (Cary Varian) analysis of both cancer and normal cells following the method previously performed in our laboratory described by Yamamoto *et al.* [12].

Quantitation of mRNA of different genes

The real-time RT-PCR method has been extensively used as a fast and appropriate method for analyzing mRNA expression. The technique is a combination of canonical RT-PCR along with fluorescence resonance energy transfer as it requires the help of fluorogenic dye like SYBR Green for gene quantification [13].

RNA was isolated from both normal and cancer cells using Roche high pure RNA isolation kit according to the manufacturer's protocol. cDNA synthesis was done and real-time analysis was performed by cDNA Synthesis Kit and FastStart Essential DNA Green Master from Roche Life Science, respectively, according to the manufacturer's protocol. All the data were normalized by using GAPDH as an internal standard. The list of Primers is provided in Table 1.

Statistical analysis

Statistical analysis was performed to find out any significance in our studies. A t-test was performed for the analysis where *p<0.005 was considered to be statistically significant. Data were represented as mean with a standard deviation of at least three different experiments.

RESULTS

The anti-skin cancer activities of TMX were confirmed by *in-vitro* assay like MTT assay, as shown in Fig. 1. Exposure of cells to different concentrations of the TMX (1 µM–20 µM) caused significant toxicity to cancer cells at low dosage, whereas no toxicity was observed in the case of normal cells at higher dosages. IC_{50} for cancer cells was seen at a low concentration of 5 µM for SKMEL-5, 4 µM for A375, and 2 µM for A431 at 48 h, whereas normal cells showed 98% viability at the highest concentration of 20 µM for 48 h.

In Fig. 2, a significant (p<0.005) downregulation of G0G1 followed by up-regulation (p<0.005) of Sub G0 of cancer cells was observed upon treatment with TMX at the IC_{50} concentrations for each respective cancer cell lines. No significant modulation (p>0.005) was observed in normal cell line WS1 upon treatment with 20 µM of TMX. Data were acquired after 48 h of treatment. No change in the phase distribution of control cells was observed for normal cells after treatment with the highest dosage of 20 µM.

As shown in Fig. 3, induction of apoptosis was validated by caspase-3 activity assay, where a significant (p<0.005) (almost 5 fold) increase in caspase-3 activity was observed for all the cancer cells at their respective IC_{50} dosage compared to their respective untreated control, and in line with our previous results, no change in caspase -3- activity was observed between control and treated in case of normal cells at the highest dosage (p>0.005).

Table 1: List of primers used for qRT-PCR

c-Myc	F.P. CGTCCTCGGATTCTCTGCTC
	R.P. CTTCGCTTACCAGAGTCGCT
Cyclin D1	F.P.GTGCTGTCTGGGAAGATGTCC
	R.P. ACCCATTCTGGAATACCGGC
βcatenin	F.P. GCGCCATTTTAAGCCTCTCG
	R.P. GTAAGCCATCACCTTCGCCT
Gsk-3β	F.P.CAGGCAATGGAAAAGGGCAA
	R.P.AGGTTAATGAGACCGGGGGA

qRT-PCR: Quantitative real time polymerase chain reaction

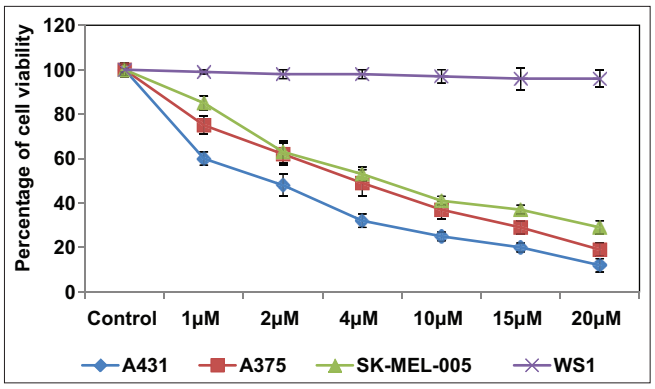

Fig. 1: Cellular viability assay by MTT analysis (cytotoxic effects) of 1,5,8 Tri-hydroxy-3-methoxy xanthone after 48 h of treatment against A431, A375, SK-MEL-005, and WS1. Data presented as mean±SD. Each experiment was done in triplicate and was repeated at least 3 times

In Fig. 4, findings of caspase-3 activity assay were validated by evaluating intracellular ROS level in cancer and normal cells signifying cellular cytotoxicity. Significant ($p<0.005$) up-regulation in intracellular ROS level was observed upon treatment with IC_{50} dosage in cancer cells, whereas no significant ($p>0.005$) change was observed in normal cells after treatment with the highest dosage of 20 μM.

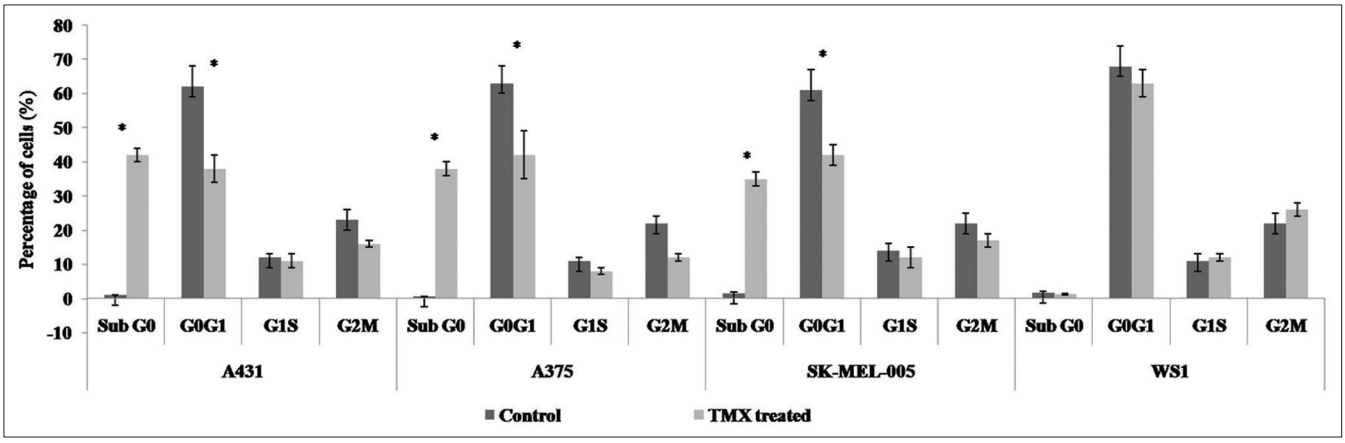

Fig. 2: Cell cycle phase distribution was determined by propidium iodide staining in fluorescence-activated cell sorting. Data were represented as ±SD *indicates a significant difference versus untreated control $p<0.005$. Each experiment was done in triplicate and was repeated at least 3 times

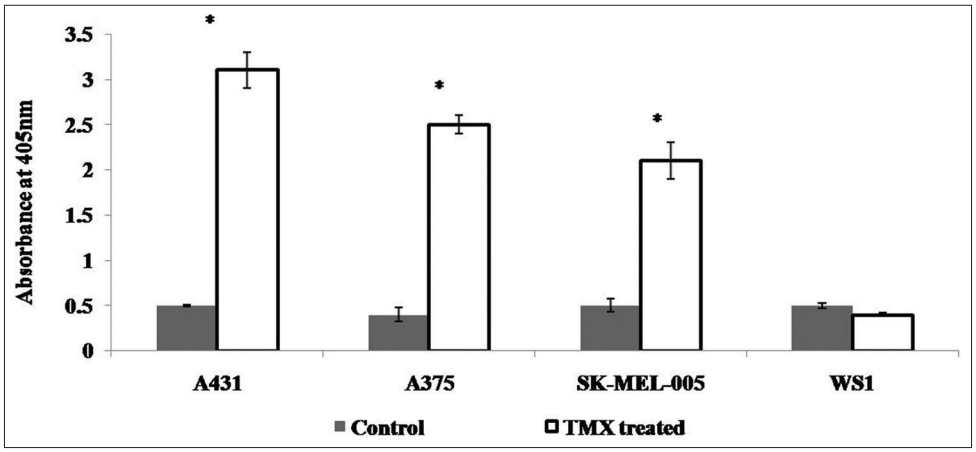

Fig. 3: Activities of caspase 3 in the A431, A375, SK-MEL-005, and WS1 cells after treatment with 1,5,8 Tri-hydroxy-3-methoxy xanthone (TMX) at IC_{50} dosage for cancer cell lines and the highest dosage of 20 μM for the normal cell line. Data were taken 48 h after TMX treatment. Each value shows that the mean±standard deviation *indicates a significant difference versus untreated control $p<0.005$. Each experiment was done in triplicate and was repeated at least 3 times

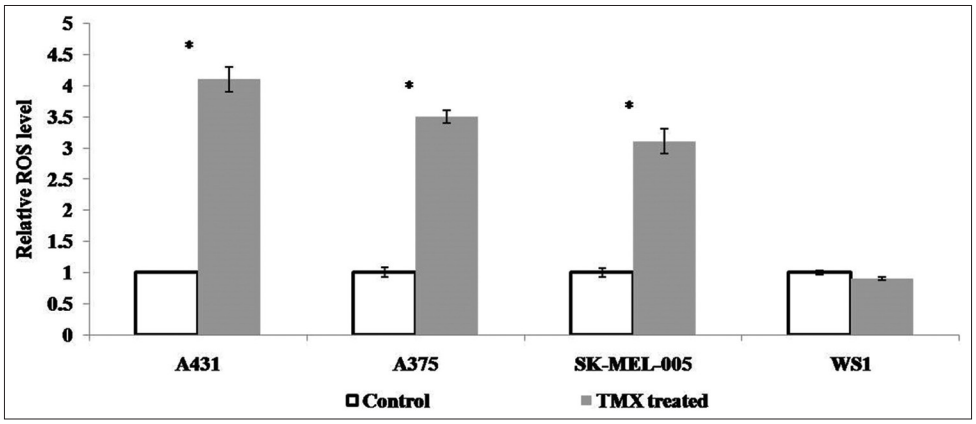

Fig. 4: Intracellular reactive oxygen species generation determination in the A431, A375, SK-MEL-005, and WS1 cells after treatment with 1,5,8 Tri-hydroxy-3-methoxy xanthone (TMX) at IC_{50} dosage for cancer cell lines and highest dosage of 20 μM for the normal cell line. Data were taken 48 h after TMX treatment. Each value shows that the mean±SD *indicates a significant difference versus untreated control $p<0.005$. Each experiment was done in triplicate and was repeated at least 3 times

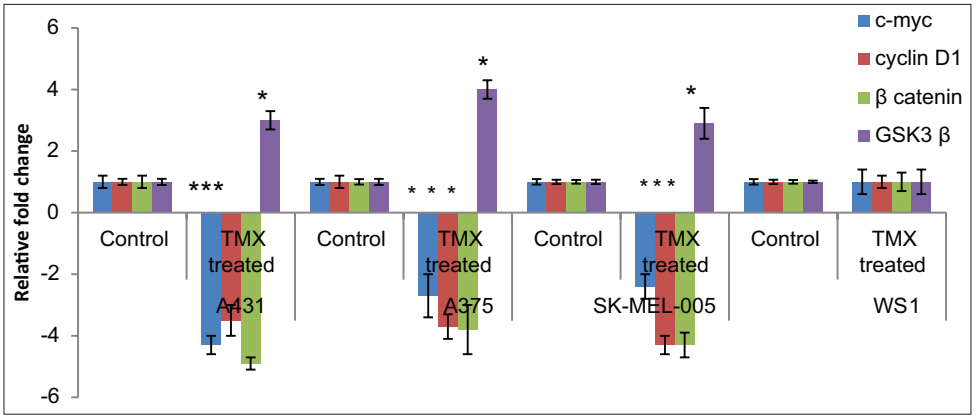

Fig. 5: Real time-PCR analysis to determine the effects of 1,5,8 Tri-hydroxy-3-methoxy xanthone on c-Myc, cyclin D1, β-catenin, and GSK3β. mRNA levels. Data were normalized for each respective cell line taking GAPDH as an internal control. Data were represented as mean fold changes±standard deviation *significant difference to control P<0.005. Each experiment was done in triplicate and was repeated at least 3 times

In Fig. 5, molecular mechanism of antitumor efficacy of TMX against both melanoma and non-melanoma cancer cell lines was investigated by monitoring the modulation of some key regulatory genes such as β-catenin, c-Myc, and cyclin-D1, which controls wntβ catenin pathway, which plays an important role in regulating expression and maintenance of CSCs [14]. CSCs, as we know, play a nodal role in controlling all the complexities related to cancer [15]. Here we see that in the case of cancer cells treated with IC_{50} dosage of TMX significantly ($p>0.05$) almost (3-5 fold) down-regulated β-catenin, c-Myc, and cyclin-D1 and up-regulated (almost 3 fold) wnt antagonist gsk-3β, leading to the suppression of wnt beta-catenin self-renewal pathway which forms the basis of its antitumor efficacy. No modulations in these key regulatory genes were observed in the case of control and TMX treated WS1 cell line, signifying that the antitumor efficacy of TMX is specific for melanoma and non-melanoma skin cancer while being non-toxic to normal cells.

DISCUSSION

The toxic nature of present anticancer drugs has led to a search for novel drugs that are selectively toxic to cancer cells [16]. There is a marked increase in the number of anticancer drugs of plant origin as they are found to be potentially non-toxic [17,18], which are tested on cells (including various cancer cell lines) [19] and experimental animals after purification and then sent to clinical trials [20]. From 2006 to 2014, there was a significant increase in the number of newly discovered anticancer molecules from 50,000 to 3,26,000, among which 1,76,000 failed to show selective toxicity [21]. Therefore, the search for a molecule that is selectively toxic to cancer cells while being non-toxic to normal cells continues to be of prime interest to cancer researchers. The wide spectrum of biological activities of *S. chirata* is attributed to the pharmacologically bioactive compounds belonging to different classes such as xanthones and their derivatives [7]. Among the most abundant xanthones found in *S. chirata*, TMX was found to be the most bio-active against breast cancer cell lines, both *in-vivo* (mouse) and *in-vitro* (human). TMX elicited antitumor efficacy at a dose that is significantly lower compared to established chemotherapeutic xanthone α-mangosteen (data not shown). TMX holds great potential for being developed into chemotherapeutic drugs due to their selective toxicity to cancer cells. The skin is the largest organ of our body and holds the greatest reserve of CSCs [22]. It is these CSCs that are responsible for the metastatic phenotype of cancer cells [22]. Deaths from skin cancer occur due to the metastatic transformation of both SCC and melanoma. The upregulation of the wnt/β-catenin pathway leads to the metastatic transformation of skin cancers melanoma and SCC [23]. β-catenin, one of the central molecules in the regulation of CSC and CSC related self-renewal pathway acts as a co-activator of oncogenes such as c-Myc and cyclin D1. The β-catenin level is tightly regulated by GSK3β dependent phosphorylation of exon3, which controls proteasomal degradation of β-catenin [24]. In this study, in line with our previous data, TMX elicited selective toxicity to both melanoma and SCC cell lines while being non-toxic to skin fibroblast cell lines. TMX elicited cytotoxicity to melanoma cell lines SK-MEL-005 and A375 at a low dose of 5 μM and 4 μM. Similarly, TMX elicited cytotoxicity to SCC cell line A431 at a low dose of 2 μM. Interestingly, TMX was non-toxic to skin fibroblast cell line WS1 at a much higher dose of 20 μM. Cytotoxicity to cancer cell lines was achieved by ROS mediated apoptosis, but no such apoptotic effect was seen in the normal cell line. This finding validated our hypothesis that TMX elicited selective cytotoxicity to cancer cell lines. The chief reason behind the metastatic phenotype of cancer cells is the CSC self-renewal pathway. Wnt/β-catenin self-renewal pathway plays a key role in the metastatic transformation of both SCC and melanoma, which ultimately leads to mortality. In this study, TMX treatment successfully downregulated the wnt/β-catenin self-renewal pathway by downregulating CSC modulators β-catenin, c-Myc, and cyclin D1 and up-regulating wnt antagonist GSK-3β. This modulation of the wnt/β-catenin self-renewal pathway by TMX treatment plays a key role in the restriction of metastatic melanoma and SCC.

CONCLUSION

TMX from *S. chirata* was previously seen to counteract the metastatic breast cancer cells both *in-vitro* and mouse model [25]. In the present study, we examined the effect of TMX on metastatic SCC and melanoma cell lines as well as normal cell lines, and it was found that TMX exhibits selective toxicity to cancer cells by inducing apoptosis to cancer cells at lower dosage while being non-toxic to normal cells. On investigating the molecular mechanism behind the antitumor efficacy of TMX against metastatic SCC and melanoma cell lines, we found that in all the cancer cell lines, there was a significant upregulation of the agonist (β-catenin, c-Myc, and cyclin D1) of wnt/β-catenin pathway and suppression of antagonist (GSK-3β) of the same pathway, this scenario was reversed upon treatment with TMX which lead to suppression wnt/β-catenin pathway. Therefore, suppression of the wnt/β-catenin pathway by TMX acts as a key event behind its antitumor potential against metastatic SCC and melanoma cell lines.

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AUTHORS' CONTRIBUTIONS

Atish Barua, Prosenjit Saha, and Dr. Chinmay Kumar Panda designed the research work, Pritha Choudhury assisted in drafting the manuscript.

CONFLICTS OF INTEREST

The authors have declared that they have no conflicts of interest with respect to current research.

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