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Original Article

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Anti-Breast Cancer Effects of Thymoquinone-Chemotherapeutic Combinations: A Systematic Review of the Latest *In Vitro* and *In Vivo* Studies

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Abstract

Background: Breast cancer is a leading malignancy among women globally, with chemotherapy as a cornerstone of treatment. However, the side effects and toxicity associated with chemotherapy necessitate the exploration of adjunctive therapies to improve efficacy and reduce adverse effects. Thymoquinone (TQ) has shown potential anti-cancer properties. This systematic review aimed to evaluate the effectiveness of TQ in combination with chemotherapeutic agents in treating breast cancer.

Methods: This study thoroughly reviewed and synthesized existing research following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 guidelines. The selected databases, including PubMed, ProQuest, ScienceDirect, Epistemonikos, and Google Scholar, were searched over the past 10 years. Eligibility criteria were based on the PICOS framework, focusing on experimental studies involving TQ-chemotherapy combinations. Data extraction and quality assessment were performed using SYRCLE and SCIRAP tools. This review included 18 *in vitro* and six *in vivo* studies.

Results: Findings revealed that TQ enhances the efficacy of chemotherapeutic agents by inducing apoptosis, enhancing autophagy, inhibiting tumor growth, and regulating cancer cell signaling pathways as well as multiple phases of the cell cycle. Additionally, TQ reduced chemotherapy-related toxicity, such as heart, blood, liver, and kidney damage, and also improved patient tolerance. Nanoparticle-based delivery systems further amplified these synergistic effects.

Conclusions: The TQ-chemotherapy combination shows significant potential as a therapy for breast cancer, enhancing treatment efficacy while mitigating side effects. Future clinical studies are needed to establish its safety and therapeutic applicability.

Keywords: Anti-cancer effect; Breast cancer; Thymoquinone; *Nigella sativa*; Systematic review

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Introduction

Breast cancer is a leading malignancy among women globally, significantly impacting public health and socioeconomic structures [1]. According to the World Health Organization (WHO), it constitutes about 24.5% of all cancers diagnosed in women, with annual case numbers rising [2]. Currently, chemotherapy remains a vital part of breast cancer treatment, often combined with surgery and radiation therapy [3]. Several drugs have also been developed for breast cancer management, such as antibody-drug conjugates (ADCs) and immune checkpoint inhibitors (ICIs) [4-6]. Chemotherapy, while effective in targeting tumor tissues, is associated with significant side effects that cause discomfort and burden for patients. These drugs also affect normal cells, leading to adverse effects that can hinder patient adherence to cancer therapy [3, 7]. Therefore, there is a critical need for alternative or adjunctive therapies that can provide practical anti-cancer effects with minimal toxicity [8].

One potential solution is integrating natural compounds with established chemotherapeutic drugs. Thymoquinone (TQ) is recognized for its medicinal properties and potential anti-cancer effect [9, 10]. The ability of TQ to induce apoptosis, inhibit cell proliferation, enhance immune response, and reduce cell viability in cancer cells makes it a candidate for combination therapy in breast cancer treatment [11]. It is supported by evidence that cancer is a multifactorial disease influenced by metabolic dysregulation and inflammation, with markers such as the neutrophil-to-lymphocyte ratio underscoring the pivotal role of immune modulation in treatment [12, 13]. Leveraging TQ may lead to more effective and less toxic breast cancer treatments, with studies exploring its synergistic effects with

chemotherapeutic agents to enhance efficacy and reduce side effects [14]. TQ, a primary compound in black cumin (*Nigella sativa*), can regulate redox systems and inhibit cell proliferation, migration, and tumor growth through various signaling pathways [15, 16]. Additionally, TQ may alleviate chemotherapy-induced complications, such as kidney damage, enhancing its therapeutic benefits [15, 17].

The extensive research on TQ has laid a robust groundwork for its anti-cancer properties, particularly in breast cancer therapy. Despite the valuable insights from these studies, there are still gaps in understanding TQ's full therapeutic potential and mechanisms of action of TQ when used in combination with standard chemotherapeutic agents [18, 19]. This systematic review analyzed the recent effects of TQ-chemotherapeutic combinations in breast cancer treatment. It combines the latest *in vitro* and *in vivo* findings to provide a comprehensive understanding of TQ's efficacy and mechanism of action in treating breast cancer. This study is also intended to be a reference for advancing to clinical trials.

Materials and Methods

Source of data and search strategy

The PRISMA 2020 guidelines were used to structure this systematic review [20]. The data source comprised accessible studies from five databases: PubMed, ProQuest, ScienceDirect, Epistemonikos, and Google Scholar, utilizing a combination of search terms: "((("Thymoquinone") OR ("TQ") OR ("Nigella sativa") OR ("Cuminum") OR ("Black cumin") OR ("Black caraway") OR ("Kalonji") OR ("Black seed")) AND ("Breast cancer") AND ("In vivo") AND ("In vitro")". The approval of the Institutional Review Board and adherence to ethical guidelines concerning human or animal subjects are irrelevant to this study.

Inclusion and exclusion criteria

The studies included in this systematic review must meet specific criteria based on the PICOS framework: P (population): *in vitro and in vivo* studies; I (intervention): treatment with TQ-chemotherapeutic combinations; C (comparison): negative (saline or untreated cell) and positive (chemotherapeutic alone); O (outcome): anti-cancer effects; S (study): experimental studies.

Clinical trials, protocols, conference proceedings, news articles, editorials, posters, review articles, presentations, and studies without a control group were excluded. Moreover, studies without full-text access, non-English publications, and those published before 2015 were excluded.

Data extraction and quality assessment

Basic data were extracted, including 1) the corresponding author of the selected study, 2) the year of publication, and 3) the country where the study was conducted. Subsequently, the table of characteristic results was divided into two tables: one for *in vitro* studies and another for *in vivo* studies. Data extraction and quality assessment were performed independently by four investigators (MBI, RSD, FS, and PMA) using the Systematic Review Center for Laboratory Animal Experimentation (SYRCLE) risk of bias tool for *in vivo* studies [21] and Science in Risk Assessment and Policy (SCIRAP) tool to evaluate the methodological quality of *in vitro* toxicity studies [22-25]. Furthermore, the Grades of Recommendation, Assessment, Development, and Evaluation (GRADE) Working Group's Guideline Development Tool (GRADEpro GDT) was utilized [26, 27].

Results

Study selection

Through the use of several databases, a total of 2,138 articles were obtained. After removing 436 duplicate entries, 1,702 articles were filtered by examining their titles and abstracts, excluding 1,594 articles. Additionally, specific inclusion and exclusion criteria were applied, leading to the elimination of 87 articles. Consequently, this systematic review focused its analysis on 21 original articles [28-48]. The process and results of the literature screening are shown in Figure 1.

Characteristics of the studies

All included studies used an *in vivo* and *in vitro* study, published in English, and occurred between 2015 and 2023. The characteristics and primary outcomes are presented in Table 1 [28-37, 40, 42-47] for *in vitro* studies and Table 2 [29-31, 38, 39, 41] for *in vivo* studies. The *in vitro* studies examined data concerning cell viability, the apoptosis/DNA fragmentation rate, autophagy rate, necrotic rate, gene expression, tumor stem cell detection, cell cycle distribution, wound healing rate/cancer cell migration, cell invasion, and side effects of TQ and chemotherapeutic agent treatments. Meanwhile, the *in vivo* studies examined data concerning inhibitory effect, tumor gene expression, $CD4^+$ and $CD8^+$ expression, myeloid cell count, apoptotic effect, and antiangiogenic effect.

Quality evidence and risk of bias

This review uses the SCIRAP tool to evaluate the quality of *in vitro* study reports analyzed via Microsoft Excel™. The review focused on five main areas: test compounds and controls, test systems, administration of test compounds, data collection and analysis, and funding/competing interests, covering 23 topics. Of the 18 studies reviewed, two did not meet solubility and control vehicle criteria [28, 46], while 14 exhibited a high risk of bias related to contamination screening [28, 29, 32-37, 40, 43-47]. Additionally, seven studies lacked detail on metabolic competence [28, 32, 34, 37, 45, 46, 48], one did not specify cell passages [28], two had a high risk of bias associated with

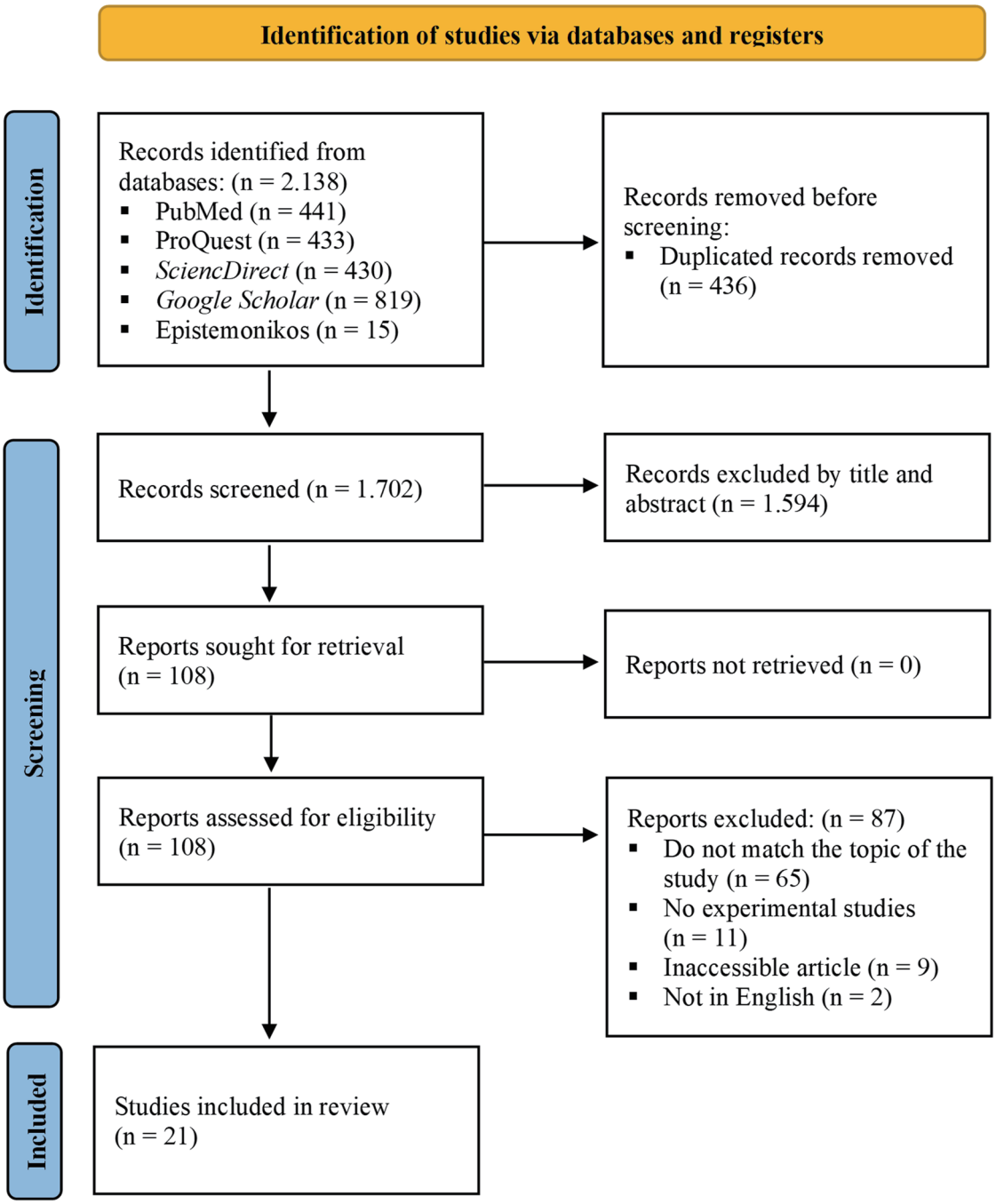

Figure 1. Flowchart illustrating the article selection procedure in accordance with the PRISMA 2020 guidelines for performing a systematic review.

compound administration [28, 37], and one study presented a high risk of bias related to funding and competing interests [48]. Nonetheless, over 60% of the studies demonstrated a low bias risk, particularly in data collection and analysis (Supplementary Material 1, jocmr.elmerjournals.com).

In vivo studies' bias risk was assessed using the SYRCLE tool, supported by RevMan 5.4. All studies showed a high risk of bias related to blinding (performance bias) and an unclear risk regarding allocation concealment. Among the six studies, three exhibited a high risk of bias in random housing and blinding (detection bias) [29-31]. Three studies also showed an unclear risk of bias concerning blinding (detection bias) [38, 39, 41] and sequence generation [29-31]. However, all studies showed a low risk of bias regarding baseline characteristics, incomplete outcome data, selective reporting, and other biases (Supplementary Material 2 and 3, jocmr.elmerjournals.com).

Table 1. Characteristics and Main Outcomes of *In Vitro* Studies Included

Author (years)	Country	Intervention group	Control group	Cellular type	Carrier	Duration	Outcomes
Soni et al, 2015 [28]	India	TQ + PTX	PTX alone	MCF-7 breast cancer cell	PLGA nanoparticle	24 h	1) TQ + PTX significantly showed lower cell viability compared to PTX alone. $P < 0.001$. 2) TQ lowers the effective concentration (IC_{50}) of PTX. 3) TQ + PTX showed 0.688 (synergistic effect).
Sakalar et al, 2016 [29]	Turkey	TQ + PTX	PTX alone, untreated embryonic fibroblasts cell (PMEFCFL-P1)	4T1 (ATCC CRL-2539) breast cancer cells	-	48 h	1) TQ + PTX significantly showed lower cell viability compared to PTX alone. $P < 0.0001$. 2) Lower dose TQ showed higher apoptosis compared to untreated cells. $P < 0.05$. 3) TQ modulated apoptosis-related genes, cytokine, and p53 signaling pathway genes.
Bashmail et al, 2018 [40]	Saudi Arabia	TQ + GCB	GCB alone, untreated cell	MCF-7 and T47D breast cancer cells	-	24, 48, and 72 h	1) TQ lowers the effective concentration (IC_{50}) of GCB. 2) TQ + GCB showed 0.15 (strong synergistic effect). 3) TQ + GCB showed significantly higher apoptosis, increased cell death in the pre-G phase, and cell cycle arrest at S phase compared to GCB alone. $P < 0.05$. 4) TQ + GCB significantly increased autophagic cell death compared to control untreated cells. $P < 0.05$.
Zidan et al, 2018 [42]	Egypt	DOX + TQ + F2 gel, DOX + F2 gel, TQ + F2 gel	Free DOX, free TQ, untreated cell	MCF-7 breast cancer cells	F2 gel nanofibers	24 h	1) DOX + TQ + F2 gel showed lower cell viability compared to other treatments. $P < 0.05$. 2) DOX + TQ + F2 gel induced the highest apoptosis, 88.6%, compared to the control group.
Ibiyeye et al, 2019 [43]	Malaysia	DOX + TQ-ACNP, DOX-ACNP, TQ-ACNP	Free DOX + TQ, free TQ, free TQ	MDA-MB-231 breast cancer cell, MCF10A normal breast cell, 3T3 normal fibroblast cell	ACNP	24, 48, and 72 h	1) TQ + DOX showed lower cell viability than other treatments. $P < 0.05$. 2) Free TQ + DOX showed 0.8 at 48 h (slight synergism), while TQ + DOX-ACNP showed 0.5 at 48 h (synergism). 3) Free TQ increased the late apoptosis in TQ + DOX compared to DOX alone. 4) DOX + TQ-ACNP has the highest percentage in SubG0 (dead cells) and S phase at 48 h. 5) TQ has been shown to significantly inhibit cancer cell invasion and migration in TQ + DOX compared to other treatments. $P < 0.05$.
Khan et al, 2019 [44]	Saudi Arabia	TQ + cyclo	Cyclo alone, untreated cell	SKBR-3 and MDA-MB-231 breast cancer cells	-	48 h	1) TQ enhanced the growth inhibition of $Her2^+$ and $Her2^-$ breast cancer cells in combination with cyclo, inhibited p-5473-Akt, increased the expression of its inhibitor PTEN, and decreased cyclin D1 compared to cyclo alone. $P < 0.001$. 2) TQ + cyclo increased cells to arrest in sub-G1 and G1 compared to untreated cells. $P < 0.05$.
Odeh et al, 2019 [45]	Jordan	TQ + DTX	DTX alone	MCF-7 breast cancer cell	-	72 h	1) TQ lowers the effective concentration (IC_{50}) of DTX. 2) TQ + DTX showed 0.552 - 0.803 (synergistic effect).

Table 1. Characteristics and Main Outcomes of *In Vitro* Studies Included - *(continued)*

Author (years)	Country	Intervention group	Control group	Cellular type	Carrier	Duration	Outcomes
Alkhatib et al, 2020 [46]	Saudi Arabia	TQ + DTX + B-NE	Free TQ and DTX	MCF-7 and MDA-MB-231 breast cancer cells	B-NE	48 h	1) TQ + DTX in B-NE exhibited a lower IC_{50} than free DTX and other treatments. 2) TQ + DTX in B-NE showed 0.6-0.9 (synergistic effect). 3) TQ + DTX in B-NE significantly showed higher apoptosis compared to DTX alone. $P < 0.05$. 4) TQ + DTX and free TQ or DTX significantly showed higher autophagy than untreated cell. $P < 0.05$.
Bashmail et al, 2020 [47]	Saudi Arabia	TQ + PTX	PTX alone, TQ alone	MCF-7 and T47D breast cancer cells	-	24 and 48 h	1) TQ did not enhance PTX potency but significantly eliminated tumor-associated resistant cell clones to PTX. $P < 0.05$. 2) TQ + PTX showed 1.6 - 4.6 (antagonistic effect). 3) TQ + PTX significantly increased apoptosis, pre-G phase population, autophagic cell death fluorescence, and reduced $CD44^+/CD24^-$ compared to PTX alone. $P < 0.05$.
Bawadud et al, 2020 [48]	Saudi Arabia	NE-DTX + TQ, free DTX + TQ	Free DTX alone, free TQ alone, drug-free NEs	MCF-7 and MDA-MB-231 breast cancer cells	NE	48 h	1) DTX + TQ-NE and free DTX alone significantly increased DNA fragmentation compared to free TQ alone and drug-free NEs. $P < 0.05$. 2) DTX + TQ-NE significantly reduced $CD44^+/CD24^-$, SNAIL-1, and TWIST-1 compared to other treatments. $P < 0.05$. 3) DTX + TQ-NE caused significant arrest at the G2/M phase ($P < 0.0001$) and the S phase ($P < 0.0021$) compared to untreated cells and free TQ alone.
Zafar et al, 2020 [30]	India	DTX + TQ in ULNCs and CLNCs	Free DTX alone, free TQ alone, DTX-LNCs	MCF-7 and MDA-MB-231 breast cancer cells	CLNCs and ULNCs	24 and 48 h	1) TQ + DTX in CLNCs and ULNCs significantly showed lower cell viability and higher cell inhibition than free DTX, free TQ, and DTX-LNCs. $P < 0.05$.
Zafar et al, 2020 [31]	India	DxTq-LNCs	DTX-LNCs, free DTX alone, free TQ alone	MCF-7 and MDA-MB-231 breast cancer cells	Long circulating sub-100 nm mPEG-DSPE- Vitamin E TPGS-LNCs	24 and 48 h	1) DxTq-LNCs significantly showed lower cell viability than free DTX alone and DTX-LNCs. $P < 0.05$. 2) TQ lowers the effective concentration (IC_{50}) of DTX in LNCs. 3) DxTq-LNCs showed 0.53-0.79 (synergistic effect).
El-Far et al, 2021 [32]	Egypt	TQ + DOX	DOX alone, untreated cell	MCF-7 breast cancer cell	-	5 days after DOX treatment and 24 h after TQ treatment	1) TQ + DOX and TQ alone significantly increased apoptosis compared to untreated cell compared to untreated cells ($P < 0.001$ and $P < 0.05$, respectively). 2) TQ significantly increased the Bax/Bcl2 ratio and caspase-3 activity compared to DOX alone and untreated cells. $P < 0.01$.

Table 1. Characteristics and Main Outcomes of *In Vitro* Studies Included - *(continued)*

Author (years)	Country	Intervention group	Control group	Cellular type	Carrier	Duration	Outcomes
Zheng et al, 2022 [33]	China	TQ + 5-FU	5-FU alone	BT-549 and MDA-MD-231 breast cancer cells	-	24 and 48 h	1) 5-FU + TQ significantly showed lower cell viability, higher cell inhibition, and increased apoptosis than 5-FU or TQ alone. $P < 0.01$. 2) 5-FU + TQ was more effective in prolonging the S phase of the cell cycle than other treatments.
Anandan et al, 2023 [34]	India	TQ + PTX	PTX alone, TQ alone	MCF-7 breast cancer cells	-	24 h	1) TQ + PTX significantly showed lower cell viability than PTX and TQ alone. $P < 0.05$. 2) TQ lowers the effective concentration of PTX.
Bawadud et al, 2023 [35]	Saudi Arabia	NE-DTX + TQ, free DTX + TQ	Free DTX alone, free TQ alone, drug-free NEs	Human ductal carcinoma cells T47D	NEs	48 h	1) NE-DTX + TQ had a lower IC_{50} than free DTX alone and other treatments. 2) Free TQ + DTX showed 7.9 (antagonistic effect), while NE-DTX + TQ showed 0.75 (synergistic effect). 3) NE-DTX + TQ significantly increased apoptosis ($P < 0.0001$) and reduced $CD44^{+}/CD24^{-}$ ($P < 0.0002$) compared to free DTX alone. 4) NE-DTX + TQ had the highest rate of autophagic vacuole formation compared to other treatments. $P < 0.0001$.
Loo et al, 2023 [36]	Malaysia	TQ + GCB, in LLCNs, free TQ + GCB	Free TQ alone, free GCB alone, drug-free LLCNs, untreated cell	MCF10A non-malignant breast epithelial and MCF-7 breast cancer cell	LLCNs	24, 48, and 72 h	1) TQ + GCB in LLCNs had a lower IC_{50} compared to drug-free LLCNs. 2) Free TQ + GCB and TQ + GCB in LLCNs significantly showed the lowest cell viability compared to other treatments. $P < 0.05$. 3) TQ + GCB in LLCNs showed 0.87 (synergistic effect), while free TQ + GCB showed 0.92 to 1.40 (additive to antagonistic effect).
Mousavinasab et al, 2023 [37]	Iran	TQ + Cis	TQ alone, Cis alone	MCF-7 breast cancer cell	-	24, 48, and 72 h	1) TQ + Cis significantly showed lower cell viability compared to Cis alone. $P < 0.05$. 2) TQ lowers the effective concentration of Cis. 3) TQ + Cis significantly induced the highest caspase-9, caspase-3, PPAR, p53, Bax, and the lowest Bcl-2 compared to other treatments. $P < 0.00001$.

ACNP: aragonite calcium carbonate ($CaCO_3$) nanoparticle; Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; B-NE: borage nanoemulsion; Cis: cisplatin; CLNCs: chitosan-coated lipid nanocapsule; Cyclo: cyclophosphamide; DOX: doxorubicin; DTX: docetaxel; GCB: gemcitabine; 5-FU: 5-fluorouracil; LLCNs: lyotropic liquid crystalline nanoassemblies; LNCs: lipid nanocapsules; NE: nanoemulsion; p-5473-Akt: phosphorylated Akt; PLGA: poly lactic-co-glycolic acid; PPAR: peroxisome proliferator-activated receptor; PTEN: phosphatase and TENsin homolog; PTX: paclitaxel; TQ: thymoquinone; ULNCs: drug-loaded uncoated lipid nanocapsule.

Table 2. Characteristics and Main Outcomes of *In Vivo* Studies Included

Author (years)	Country	Sample	Route	Intervention/dose	Control group	Animal (age)	Weight (g)	Duration	Outcomes
Şakalar et al, 2016 [29]	Turkey	Total 20 (control = 6, low dose TQ = 6, high dose TQ = 8)	Intraperitoneal injection	0.64 mg/kg TQ + 1.25 mg/kg PTX, 2.4 mg/kg TQ + 1.25 mg/kg PTX	PTX alone, DMSO and/or 50% ethanol in ddH_2O	Female balb/c mice (8 - 12 weeks)	26 - 28	8 days (low dose group), 11 days (high dose group)	1) TQ significantly decreased tumor growth (weight) in PTX + TQ compared to PTX alone. $P < 0.007$ in lower dose and $P < 0.001$ in higher dose.
El-Ashmawy et al, 2017 [38]	Egypt	Ten/group (normal control, tumor control, F2 gel, free DOX, DOX + F2 gel, free TQ, TQ + F2 gel, and DOX + TQ + F2 gel)	Subcutaneous injection	DOX + F2 gel (200 µL with 100 µg of DOX), TQ + F2 gel (200 µL with 3 mg of TQ), and DOX + TQ + F2 gel (300 µL with 100 µg of DOX and 3 mg of TQ), free DOX (5 mg/kg), free TQ (3 mg/mouse)	Normal control, tumor control, F2 gel (100 µL/mouse)	Female albino mice (6 - 8 weeks)	18 - 22	28 days	1) TQ significantly decreased tumor growth (volume) and increased inhibition rate in DOX + TQ and TQ alone compared to DOX alone. $P < 0.05$. 2) DOX + TQ + F2 gel decreased Bcl-2 and P53 expression compared to other treatments. $P < 0.05$. 3) TQ reduces the side effects of DOX on the heart as evidenced by decreased cardiac markers when combined with DOX. $P < 0.05$.
Gomaa et al, 2018 [39]	Egypt	Five/group (PB, gold nanoparticles (AuNPs), AuNPs/TQ conjugate, AuNPs/TQ + Cisplatin 10, and AuNPs/TQ + Cisplatin 40)	Intraperitoneal injection	AuNPs (21.4 µg/mouse) (AuNPs group), AuNPs/TQ conjugate (1 mg/mouse) (AuNPs/TQ group), AuNPs/TQ (1 mg/mouse) plus cisplatin (10 µg/mouse) (AuNPs/TQ + Cis10 group) or AuNPs/TQ (1 mg/mouse) plus cisplatin (40 µg/mouse) (AuNPs/TQ + Cis40 group)	PBS	Female Swiss albino mice (6 - 8 weeks)	25 ± 2	6 days	1) TQ significantly decreased tumor growth (weight) and increased $CD4^+$, $CD8^+$, granulocytic, and monocytic cell populations in AuNPs/TQ + Cis compared to untreated cells. $P < 0.05$.

Table 2. Characteristics and Main Outcomes of *In Vivo* Studies Included - *(continued)*

Author (years)	Country	Sample	Route	Intervention/dose	Control group	Animal (age)	Weight (g)	Duration	Outcomes
Mosalam et al, 2020 [41]	Egypt	Ten/group (ECis, ECis + ETQ, ECis + ETQ + EPTX, LCis, LCis + LTQ, and LCis + LTQ + LPTX, tumor control)	Cis and TQ was given intraperitoneal injection, PTX was given subcutaneous injection	7.5 mg/kg Cis (on 12th and 18th days for early groups, while on 19th and 25th days for late groups), 20 mg/kg TQ, 15 mg/kg PTX (TQ and PTX injected daily for 10 days)	ECis alone, LCis alone, tumor control PEG 200 µL/mouse/day	Female albino mice (6 - 8 weeks)	20 - 22	28 days	1) TQ significantly decreased tumor growth (weight), increased inhibition rate, $CD4^+$, $CD8^+$, and apoptosis rate in ECis + ETQ + EPTX and LCis + LTQ + LPTX compared to ECis or LCis alone and untreated cells. $P < 0.05$.
Zafar et al, 2020 [30]	India	Ten/group (control and TQ groups)	Direct contact	3.3 µM DTX + 6.6 µM TQ in CLNCs carrier	DTX alone, TQ alone, NS	Chick Embryo Chorioallantoic Membran (CAM) (9 days)	-	1 and 2 days	1) TQ increased vascular inhibition (antiangiogenic effect) in TQ + DTX-CLNCs compared to DTX alone and untreated cells.
Zafar et al, 2020 [31]	India	Total 9 (NS = 3, 2 DTX = 3, DTX + TQ co-encapsulated lipid nanocapsules (DxTq-LNCs) = 3)	Intravenous tail vein injection	DTX + TQ - LNCs (equivalent to 0.3 mg DTX and 0.6 mg TQ) containing 2 mg/kg DTX	DTX alone, NS	Female balb/c mice	20 - 25	14 days	1) TQ significantly increased inhibition rate (volume) in DTX + TQ - LNCs compared to DTX alone. $P < 0.05$. 2) TQ significantly reduces liver, kidney, and blood toxicity of DTX as indicated by elevated SOD and GSH levels, reduced MDA, AST, and ALT, along with decreased BUN and creatinine, and increased WBC and RBC counts in DTX + TQ - LNCs. $P < 0.05$.

ALT: alanine transaminase; AST: aspartate transaminase; BUN: blood urea nitrogen; CK-MB: creatine kinase-MB; CLNCs: chitosan-coated lipid nanocapsule; DOX: doxorubicin; DTX: docetaxel; ECis: early cisplatin; GCB: gemcitabine; GSH: glutathione; LCis: late cisplatin; LDH: lactate dehydrogenase; MDA: malondialdehyde; NS: normal saline; PBS: phosphate buffer saline; PTX: paclitaxel; RBC: red blood cell; SOD: superoxide dismutase; TQ: thymoquinone; WBC: white blood cell.

The certainty of evidence was analyzed using GRADE criteria with the GRADEpro GDT tool, focusing on five aspects: risk of bias, inconsistency, indirectness, imprecision, and other considerations presented descriptively [26, 27]. Four results were rated high certainty [29, 38, 39, 41], while two were rated moderate certainty [30, 31]. Further details are available in Supplementary Material 4 (jocmr.elmerjournals.com).

Overall outcomes of the studies

The *in vitro* studies summarized in Table 1 reveal important interactions between TQ and conventional chemotherapeutic agents. Eleven studies on cell viability demonstrated that TQ combined with chemotherapeutics consistently resulted in the lowest cell viability [28-31, 33, 34, 36, 37, 42-44]. Among 10 studies assessing half-maximal inhibitory concentration (IC_{50}), six indicated that TQ effectively reduced the required concentration of chemotherapeutics, enhancing their anticancer efficacy [28, 31, 34, 37, 40, 45]. However, four studies found no significant enhancement in potency, although the TQ-chemotherapy combinations still yielded the lowest IC_{50} values [35, 36, 46, 47]. One study noted that TQ could overcome resistance in paclitaxel (PTX)-resistant cancer cell lines [47]. Furthermore, nine studies evaluated the combination index (CI), with five showing a synergistic effect between TQ and chemotherapeutics [28, 31, 40, 45, 46], particularly when TQ was in carrier form [35, 36, 43]. In contrast, one study observed an antagonistic effect in its free-drug form [47].

In a review of nine studies on apoptosis rates, eight showed that TQ combined with chemotherapeutic agents significantly increased apoptosis compared to either treatment alone or untreated cells [32, 33, 35, 40, 42, 43, 46, 47]. At the same time, one study noted that TQ alone also heightened apoptosis [29]. The TQ-chemotherapeutic combination further enhanced DNA fragmentation compared to chemotherapeutics alone [48] and showed notable improvements in wound healing and cell invasion [43]. Among four studies on autophagy, three indicated that chemotherapeutics enhanced autophagy when combined with TQ [35, 46, 47]. In contrast, one study reported that TQ significantly increased autophagy compared to chemotherapy alone and untreated cells [40].

Six studies analyzing the cell cycle found that TQ combined with conventional chemotherapeutic agents significantly increased cell death across several phases, including pre-G, sub-G0, S, sub-G1, and G1 [33, 40, 43, 44, 47, 48]. Additionally, four studies on tumor stem cells and breast cancer stem cells (BCSCs) showed that TQ significantly decreased $CD44^+$/$CD24^-$ when combined with chemotherapeutics compared to either treatment alone [35, 40, 47, 48].

Five studies on gene expression demonstrated that TQ significantly regulates genes in breast cancer treatment when combined with chemotherapeutic agents. TQ upregulated apoptosis-related genes, cytokines, p53 signaling pathway [29, 37], and tumor suppressor genes such as p21 and BRCA [29]. It inhibited phosphorylated Akt (p-5473-Akt), increased phosphatase and TENsin homolog (PTEN) expression [44], and decreased SNAIL-1, TWIST-1, and cyclin D1 levels [44, 48]. Additionally, TQ enhanced the B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax)/Bcl-2 ratio [32, 37] and boosted the activities of caspases 3, 7, 9, 12, and peroxisome proliferator-activated receptor (PPAR) [29, 32, 37].

The *in vivo* studies summarized in Table 2 indicate that TQ significantly enhances the efficacy of conventional chemotherapeutic agents. Five studies found that the combination of TQ and chemotherapy notably inhibited tumor growth compared to other treatments [29, 31, 38, 39, 41]. Two studies revealed TQ's crucial role in regulating breast cancer-related genes by reducing Bcl-2 levels, increasing P53 levels, and enhancing IL-2 expression while suppressing Notch 1 and vascular endothelial growth factor (VEGF) [38, 41]. Safety analyses showed that TQ mitigated chemotherapy-related side effects, reducing heart toxicity [38] and increasing splenocyte counts [39]. One study noted liver and kidney minimal toxicity with TQ-lipid nanocapsules (LNCs) compared to docetaxel (DTX) [31]. Additionally, the TQ-chemotherapy combination increased $CD4^+$ T cells compared to chemotherapy alone and showed varying effects on $CD8^+$ T cells [39, 41]. The combination also enhanced the presence of granulocytic and monocytic cells [39], and improved apoptosis rates [41] and antiangiogenic effects compared to either treatment alone [30].

Discussion

Breast cancer has overtaken lung cancer as the most frequently diagnosed cancer and is the fifth leading cause of cancer-related deaths globally [2]. To date, research into effective therapies to reduce mortality from this disease continues to grow and remains a significant concern. One of the challenges in its management is that each chemotherapy drug has its limitations and potential side effects. This systematic review analyzed the recent effects of TQ-chemotherapeutic combinations in breast cancer treatment. The findings from the conducted identification and analysis reveal that combining TQ with chemotherapeutic agents consistently led to the lowest levels of cell viability. In addition, this study found that most studies showed that TQ effectively reduced the concentration of chemotherapy required and showed a synergistic effect between TQ and chemotherapy agents.

Multiple studies across various cancer cell lines have documented a synergistic effect of TQ, indicating its potential to enhance the efficacy of conventional chemotherapy [49, 50]. For instance, the TQ-cisplatin combination significantly increased cytotoxicity against oral squamous cell carcinoma, helping to address cisplatin's limitations, such as drug resistance and toxicity [51]. Mahmood and Hamaamin (2022) highlighted TQ's ability to induce apoptosis and inhibit cell proliferation, supporting its combined use with other chemotherapeutic agents [52]. Additionally, research by Celebioglu et al (2022) demonstrated that combining TQ with etoposide reduced cell viability and exhibited a potentially synergistic effect, improving therapeutic outcomes [53].

However, some studies indicate that TQ may not enhance the potency of chemotherapeutic agents and can even exhibit antagonistic effects in breast cancer cell lines. For instance, Bashmail et al (2020) found that TQ increased PTX's anti-breast cancer activity despite mathematical antagonism, poten-

tially due to its reduction of BCSCs ($CD44^+/CD24^-$), associated with drug resistance. TQ increased apoptotic and necrotic cell death in T47D cells with PTX and induce autophagy in MCF-7 cells [47]. Moreover, Motaghed et al (2014) reported that TQ's combination with certain chemotherapies could diminish efficacy, especially in estrogen receptor-positive breast cancer, likely by downregulating the epidermal growth factor receptor (EGFR) pathway [54]. These findings underscore the complexity of integrating natural compounds like TQ with conventional chemotherapy in breast cancer treatment.

This review emphasizes that TQ synergizes with chemotherapeutic agents to enhance therapeutic outcomes by improving DNA fragmentation, inducing autophagy, inhibiting tumor growth, and promoting apoptosis. TQ supports apoptotic body formation and increases histone H2A.X phosphorylation, an early indicator of DNA damage [55, 56]. It can induce necrosis by activating reactive oxygen species (ROS) and modulating key signaling pathways like p38 MAPK, which are vital in mediating cell death responses [57-59]. In breast cancer models, TQ depletes tumor-associated stem cells through autophagy, sensitizing them to apoptosis and improving treatment efficacy [46, 60]. Furthermore, TQ downregulates anti-apoptotic proteins like Bcl-2, promotes apoptosis across various cancer cell lines, and affects their migratory and invasive abilities, underscoring its therapeutic potential [46, 60-62].

This study demonstrated that TQ combined with chemotherapeutics significantly enhanced the inhibitory effects on wound healing and cell invasion in *in vitro* models. Consistent with previous research, TQ effectively inhibits breast cancer cell migration and invasion by downregulating epithelial-to-mesenchymal transition (EMT) markers such as Twist1 and Zeb1 while upregulating E-cadherin, which is essential for maintaining epithelial integrity [63, 64]. TQ also inhibits EMT in prostate cancer by negatively regulating the transforming growth factor-beta (TGF-β)/Smad2/3 signaling pathway [65] and reduces renal cancer cell migration and invasion by downregulating matrix metalloproteinase-2 (MMP-2) and urokinase-type plasminogen activator (u-PA) [66]. Additionally, TQ increased antiangiogenic effects by inhibiting key signaling pathways, such as VEGF, reducing pro-inflammatory cytokines, and directly affecting endothelial cells [67, 68].

Current evidence indicates that TQ effectively inhibits tumor cells during various phases of development. The cell cycle's progression through the G1, S, G2, and M phases is regulated by cyclin-dependent kinases (CDKs) and cyclins [69, 70]. Recent studies show that TQ induces cell cycle arrest in breast cancer cell lines, particularly in triple-negative breast cancer, affecting G0/G1, G1/S, or G2/M phases depending on the cell type [16, 71]. Our findings support previous research that TQ induces G2/M arrest in doxorubicin-resistant breast cancer and spindle carcinoma cells, which is linked to decreased levels of cyclin B1 and cell division cycle 25/Cdc25 phosphatase, along with increased p53 expression [72].

Our review highlights that the combination of TQ with chemotherapeutic agents significantly decreases the population of $CD44^+/CD24^-$ BCSCs, a phenotype linked to chemoresistance and poor prognosis. This reduction is vital since $CD44^+/CD24^-$ cells contribute to tumor initiation and recurrence due to their stem-like properties [73]. By targeting this population, TQ may help overcome the challenges posed by cancer stem cells, potentially leading to improved patient prognostic outcomes [74].

This study found that TQ can overcome resistance in PTX-resistant cancer cell lines, addressing a significant challenge in breast cancer treatment. TQ sensitizes resistant cells by modulating key signaling pathways related to cell survival and apoptosis. For instance, Khan et al (2023) reported that TQ inhibits the Akt signaling pathway, promoting pro-apoptotic protein expression while downregulating anti-apoptotic proteins like Bcl-2 and X-linked inhibitor of apoptosis protein (XIAP) [75]. Additionally, Woo et al (2013) emphasized TQ's role in inducing ROS production, which contributes to cancer cell apoptosis, suggesting a mechanism to counteract resistance [55]. Furthermore, Abdelfadil et al (2013) demonstrated that TQ induces apoptosis in various cancer cell lines via the p38 mitogen-activated protein kinase (MAPK) pathway [76]. Collectively, these findings indicate that TQ enhances the efficacy of existing chemotherapeutics and offers a promising strategy to overcome drug resistance, potentially improving patient outcomes.

The *in vivo* results of this study indicate that TQ plays a vital role in regulating breast cancer-related genes by reducing Bcl-2 levels, increasing P53 levels, and suppressing genes like Notch1 and VEGF while enhancing interleukin 2 (IL-2) expression. Consistent with *in vitro* findings, TQ influences gene expression and cell behavior in breast cancer by inducing apoptosis and modulating the p53 signaling pathway and tumor suppressor genes like p21 and BRCA. TQ inhibits p-5473-Akt and increases PTEN expression while decreasing SNAIL-1 and TWIST-1 levels, thereby enhancing the Bax/Bcl-2 ratio and increasing caspase-3 activity. By downregulating Bcl-2, TQ promotes apoptosis and restores normal apoptotic pathways disrupted in cancer [77, 78], while its upregulation of P53 further supports tumor suppression [60, 79]. Additionally, the suppression of Notch1 and VEGF expression highlights TQ's ability to inhibit tumor growth and angiogenesis [52], and the enhancement of IL-2 suggests a potential boost in immune responses against tumors [43]. Collectively, these findings underscore TQ's potential as a therapeutic agent in breast cancer treatment by modulating key regulatory pathways involved in tumor survival and progression.

This study found that the combination of TQ and conventional chemotherapy enhances anti-breast cancer effects by increasing helper T cells and myeloid cells. The elevation of $CD4^+$ and $CD8^+$ T cells indicates a strong adaptive immune response vital for effectively targeting and eliminating tumor cells [80, 81]. TQ likely contributes to this immune enhancement by promoting T-cell activation and proliferation, strengthening the body's defenses against breast cancer [82]. Additionally, the increase in myeloid cells suggests that TQ facilitates the recruitment of these immune cells to the tumor site, amplifying the anti-tumor response [83, 84]. Granulocytic and monocytic cells are crucial for inflammation and immune surveillance, supporting tumor suppression [85]. These findings highlight TQ's potential as an immunomodulatory agent that targets cancer cells directly and boosts the immune system, presenting a promising strategy for combination therapies that integrate cytotoxic effects and immune activation [86].

The combination therapy of TQ and conventional chemotherapy for breast cancer has shown potential safety benefits

Table 3. Summary of Beneficial and Adverse Effects of TQ-Chemotherapy Combination in Clinical Practice

Benefit	Adverse effect
TQ itself has a direct anticancer effect and increases the immune response [29, 39-41].	Potential drug interactions and toxicities depend on the concentration of TQ, drug carrier, and combination chosen [35, 36, 47, 54].
Enhance the efficacy of various chemotherapeutic agents [28, 31, 34, 37, 40, 45].	Possible variability in patient response [88].
Reducing chemotherapy-induced toxicity [31, 38, 39].	
Improve cost-effective adjunct chemotherapy by reducing the required doses of conventional treatments [28, 31, 34-37, 40, 43, 45, 46].	

and reduced side effects in various studies. This review indicates that TQ mitigates chemotherapy-related toxicities by lowering cardiac injury markers like lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB), which are the indicators of heart toxicity. This cardioprotective effect is crucial, given the frequent cardiotoxicity associated with specific chemotherapeutic agents, especially anthracyclines [87]. Additionally, TQ may help protect immune cells during treatment, although specific evidence regarding splenocyte counts is limited. TQ also exhibits minimal toxicity to the liver and kidneys, evidenced by normal liver enzyme, blood urea nitrogen (BUN), and creatinine levels, highlighting its potential as a safe adjunct therapy. These findings underscore TQ's role in enhancing chemotherapy efficacy while improving safety profiles, making it a promising candidate for combination therapies that minimize adverse effects and maximize therapeutic benefits (Table 3) [28, 29, 31, 34-41, 43, 45-47, 54, 88].

This systematic review has certain limitations, as it includes only *in vivo* and *in vitro* (preclinical) studies, which highlights the need for clinical studies. Additionally, the data encompass a wide range of variables, complicating the ability to perform a thorough meta-analysis for all variables. Furthermore, more than 50% of the *in vivo* studies exhibit a high risk of bias related to blinding and random assessments. However, despite these limitations, this systematic review has significantly contributed to the provision of preclinical evidence regarding the efficacy of TQ in treating breast cancer.

Over the next 5 years, we anticipate progression to phase I/II clinical trials, particularly targeting triple-negative breast cancer subtypes, where therapeutic options remain inadequate. Advancements in pharmaceutical technology may address TQ's hydrophobicity and stability limitations through sophisticated liposomal or polymer-based delivery systems. TQ may set a precedent for integrating traditional medicinal compounds into contemporary oncology protocols. However, critical questions regarding optimal dosing regimens, specific chemotherapeutic combinations, and patient stratification criteria must be resolved through rigorously designed clinical trials with comprehensive safety and efficacy endpoints.

Conclusions

The systematic review suggests that based on *in vitro* and *in vivo* studies, the TQ exhibits significant anti-cancer properties, particularly in breast cancer treatment. TQ effectively induces apoptosis, enhances autophagy, inhibits tumor growth, and regulates cancer cell signaling pathways as well as multiple phases of the cell cycle. Combined with chemotherapeutic agents, TQ enhances efficacy, reduces required drug dosages, and mitigates side effects such as healthy cell toxicity. Furthermore, advanced delivery systems such as nanoparticles and lipid carriers amplify these effects, ensuring targeted action and improved therapeutic outcomes. Despite its promising role in improving breast cancer treatment outcomes and reducing chemotherapy toxicity, further clinical trials are needed to validate its safety, efficacy, and mechanisms in human applications.

Supplementary Material

Suppl 1. Risk-of-bias and applicability concerns graph of *in vitro* studies.

Suppl 2. Risk-of-bias and applicability concerns summary table of *in vivo* studies.

Suppl 3. Risk-of-bias and applicability concerns graph of *in vivo* studies.

Suppl 4. Certainty of evidence from *in vivo* studies.

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Conflict of Interest

All the authors declare that there is no conflict of interest.

Informed Consent

Not applicable.

Author Contributions

NQ was involved in developing the research concept and conducting the systematic review. MBI, FS, RSD, and PMA significantly contributed to the article's literature search, quality assessment, and language enhancement. L, ZH, and DP reviewed, edited, and approved the final manuscript, while all authors endorsed the draft and affirmed its originality.

Data Availability

The authors declare that data supporting the findings of this study are available within the article and its supplementary information files.

Abbreviations

ACNP: aragonite calcium carbonate ($CaCO_3$) nanoparticle; ADCs: antibody-drug conjugates; ALT: alanine transaminase; AST: aspartate transaminase; Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; B-NE: borage nanoemulsion; Brca 1: breast cancer susceptibility gene 1; BUN: blood urea nitrogen; CDK: cyclin-dependent kinase; Cdkn1a: cyclin-dependent kinase inhibitor 1a; Cis: cisplatin; CK-MB: creatine kinase-MB; CLNCs: chitosan-coated lipid nanocapsule; CTNNB1: cadherin-associated protein beta-1; Cyclo: cyclophosphamide; DOX: doxorubicin; DTX: docetaxel; ECis: early cisplatin; EMT: epithelial-mesenchymal transition; Fasl: Fas ligand; 5-FU: 5-fluorouracil; GCB: gemcitabine; GSH: glutathione; Gstp: glutathione S-transferase p; Hes1: hairy and enhancer-of-split 1; Hic 1: hypermethylated in cancer 1; ICIs: immune checkpoint inhibitors; IFN-γ: interferon gamma; IL-6: interleukin-6; IL-2: interleukin-2; Jag1: jagged 1; LCis: late cisplatin; LDH: lactate dehydrogenase; LLCNs: lyotropic liquid crystalline nanoassemblies; LNCs: lipid nanocapsules; MDA: malondialdehyde; NE: nanoemulsion; NF-κB: nuclear factor-kappa B; NS: normal saline; PBS: phosphate buffer saline; p-5473-Akt: phosphorylated Akt; PI3K/Akt: phosphatidylinositol 3-kinase/protein kinase B; PLGA: poly lactic-co-glycolic acid; PPAR: peroxisome proliferator-activated receptor; PTEN: phosphatase and TENsin homolog; PTX: paclitaxel; RBC: red blood cell; SOD: superoxide dismutase; TNF-α: tumor necrosis factor alpha; TQ: thymoquinone; Trail: tumor necrosis factor-related apoptosis-inducing ligand; ULNCs: drug-loaded uncoated lipid nanocapsule; VEGF: vascular endothelial growth factor; WBC: white blood cell

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