Biomedicine & Pharmacotherapy 153 (2022) 113364

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Review

The effects of thymoquinone on pancreatic cancer: Evidence from preclinical studies

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Abbreviations: Akt, protein kinase B; Bax, Bcl-2 associated X protein; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma extra-large; CI, combination index; CNS, central nervous system; COX-2, ciclooxygenase 2; DTQ, dithymoquinone; GC, gas chromatography; GEM, gemcitabine; HDACs, histone deacetylases; HPLC, high-performance liquid chromatography; IgE, immunoglobulin E; JNK, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinase; MMP-9, matrix metalloproteinase 9; MTBE, 2-methoxy-2-methylpropane; mTOR, mechanistic target of rapamycin; MUC4, mucin 4; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NICD, Notch1intracellular domain; NIPAAM, N-isopropyl acrylamide; Notch1, Notch homolog 1; PAG, p-aminophenyl-1-thio-β-D-galactopyranoside; PDAC, pancreatic ductal adenocarcinoma; PEGL, polyethylene glycol; PGE2, prostaglandin E2; PKM2, pyruvate kinase M2; PTEN, phosphatase and tensin homolog; PVP, polyvinylpyrrolidone; ROS, reactive oxygen species; SLN, solid lipid nanoparticles; TLC, thin-layer chromatography; TNF-α, tumor necrosis factor alpha; TQ, thymoquinone; XIAP, X-linked inhibitor of apoptosis protein.

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https://doi.org/10.1016/j.biopha.2022.113364
Received 11 May 2022; Received in revised form 13 June 2022; Accepted 28 June 2022
Available online 8 July 2022

ARTICLE INFO

Keywords:
Thymoquinone
Pancreatic cancer
Chemopreventive/anticancer agent
Apoptosis
Molecular mechanisms
Signaling pathways

ABSTRACT

Thymoquinone (TQ) is a secondary metabolite found in abundance in very few plant species including *Nigella sativa* L., *Monarda fistulosa* L., *Thymus vulgaris* L. and *Satureja montana* L. Preclinical pharmacological studies have shown that TQ has many biological activities, such as anti-inflammatory, antioxidant and anticancer. Both *in vivo* and *in vitro* experiments have shown that TQ acts as an antitumor agent by altering cell cycle progression, inhibiting cell proliferation, stimulating apoptosis, inhibiting angiogenesis, reducing metastasis and affecting autophagy. In this comprehensive study, the evidence on the pharmacological potential of TQ on pancreatic cancer is reviewed. The positive results of preclinical studies support the view that TQ can be considered as an additional therapeutic agent against pancreatic cancer. The possibilities of success for this compound in human medicine should be further explored through clinical trials.

1. Introduction

Pancreatic cancer is a malignant disease that develops due to excessive proliferation of the cells of which the pancreas is composed, and it develops due to mutational changes in pancreatic DNA. These mutations cause the pancreatic cells to grow uncontrollably, leading to the appearance of tumor masses and causing normal cells to die or no longer function normally [1]. The causes of pancreatic cancer are not fully understood, but certain risk factors have been identified: age is one of the most important because the disease affects people between the ages of 50 and 70. Other risk factors that may cause cancer have been identified: smoking, diabetes, chronic inflammation of the pancreas-pancreatitis, family history of pancreatic cancer, obesity, lifestyle and some environmental factors [2,3].

Thymoquinone (TQ; 2-methyl-5-isopropyl-1,4-benzoquinone, (Fig. 1) is the most abundant and important bioactive constituent of several plant species, such as *Nigella sativa* L. (black-caraway, black cumin, also known as nigella or *kalonji*). In the Middle East, many diets include plants containing TQ and are considered health-promoting herbs. *N. sativa* (an annual herb) is cultivated around the Mediterranean, Syria, Egypt and India at a larger scale for TQ extraction. Black cumin seeds have traditionally been used for more than 2000 years as food but also as a medicinal plant due to the presence of fatty acids, carbohydrates, fiber, proteins and secondary metabolites such as thymoquinone, dithymoquinone, thymohydroquinone and thymol [4]. The safe uses of *N. sativa* oil and its most important constituent TQ have been confirmed by acute and chronic toxicity studies. TQ is also a bioactive element of the volatile oil of *Monarda fistulosa* L. (wild bergamot) [5] and it is present in the Lamiaceae family in species such as *Satureja montana* L.[6] and *Thymus vulgaris* L. [7].

Over the last 20 years about one-quarter of drugs have been directly isolated from plants, while in another quarter, natural compounds are chemically modified [8]. The anti-inflammatory and anti-oxidative properties of TQ have been demonstrated in different disease models such as gastric ulcer, carcinogenesis, diabetes, asthma and encephalomyelitis. TQ also acts as superoxide radical scavenger and free radical [9].

TQ has shown considerable anti-neoplastic activity against human cancer by specifically inhibiting the growth of many kinds of tumor cells without any harmful effects on normal cells [10]. As an anticancer agent, TQ operates through diverse modes of action: cell cycle arrest, reactive oxygen species (ROS) production, anti-proliferation activity, anti-metastasis activity and apoptosis induction [11–13]. TQ inhibited cell proliferation and induced apoptosis in several human cancer cell lines such as colon, breast, brain, pancreatic, and ovarian [11]. Several reports suggest an adjuvant role of TQ which may improve the quality of cancer patients.

The most common type of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC) but the benefits of gemcitabine and gemcitabine-based chemotherapy is limited. In fact, it has recently been hypothesized that combining gemcitabine with certain phytochemicals such as thymoquinone may improve the benefits of the pharmacological treatment [14].

This review aimed to provide the updated evidence of TQ, in pancreatic cancer and its modes of action demonstrated in preclinical pharmacological studies.

2. Methodology

This review used PubMed as a search engine to retrieve the most updated articles about biological activities and anticancer therapeutic potential of TQ up to 2021. The strategy of the search included the use of the following keywords: “thymoquinone” and “biological activity” or “pancreatic cancer” or “anticancer” or “cytotoxic” or “apoptosis” or “chemopreventive”. The strengths and weaknesses of articles were identified [15], and the authors selected the more useful ones for the review purpose considering full text articles in English.

3. TQ: a brief overview

3.1. Chemical characterization, isolation and synthesis

Around the year 1963, the study of the active principles of *N. sativa* was reported, being detected within the volatile compounds TQ dimer (dithymoquinone, DTQ) [16,17] and many monoterpenes such as *p*-cymene and α-pinene [18]. Obtaining a yield of 18.4 w/w TQ was

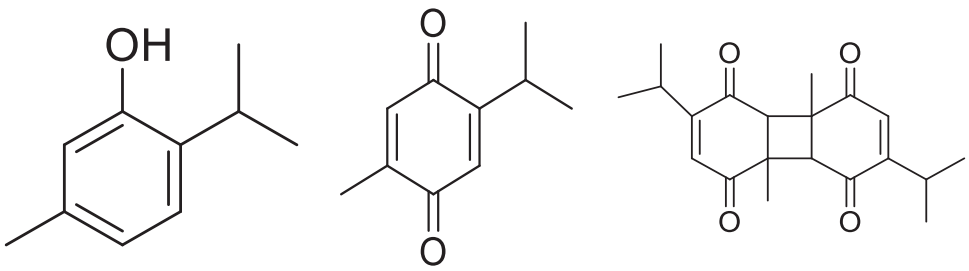

Fig. 1. Chemical structure of thymol, thymoquinone and dithymoquinone.

obtained from the volatile oils. El-Dakhakhny in 1960 also isolated TQ from the essential oil of the seed and determined that the "nigellone" isolated earlier was a dimer of TQ, which they named DTQ (Fig. 1) [19].

Chemically, thymoquinone (PubChem CID: 10281) has the molecular formula $C_{10}H_{12}O_2$ and a molecular weight of 164.204 g/mol. CAS Number: 490–91–5 [20]. The yellow crystalline molecule TQ was isolated using thin layer chromatography on silica gel. TQ has a basic quinone structure consisting of a para substituted dione conjugated to a benzene ring to which a methyl and an isopropyl side chain groups are added in positions 2 and 5, respectively (Fig. 1) [21].

When comparing two methods of extraction of the TQ, from the oil, the percentage obtained was 3 % when carrying out the extraction by hydrodistillation (using Clevinger's Apparatus), as opposed to 48 % when it is extracted by extraction with Soxhlet [22]. Obtaining a fraction rich in TQ has been achieved by extraction with supercritical fluid [23].

TQ was also obtained from *N. sativa* seeds from Abarkooh, combining maceration with 2-methoxy-2-methylpropane (MTBE) and liquid-liquid extraction with methanol as a new and efficient extraction method, accompanied by preparative high-performance liquid chromatography (HPLC) for large-scale production of TQ. With this new extraction method, they managed to obtain a purity of around 97 % of TQ [24].

The extraction with organic solvents, supercritical CO_2, or subcritical water is a better method for the separation of TQ from plant materials than hydro- or steam distillation. It has been reported within the different studies carried out that extraction with organic solvents, supercritical CO_2 or subcritical water is a better method for the separation of TQ from plant materials, compared to hydro- or steam distillation. The extraction of volatile compounds from *Monarda didyma* and *M. fistulosa*, using supercritical CO_2 extraction, was much richer in TQ [25]. However, in other research work, it was found that the best solvent for the extraction of TQ from *N. sativa* was benzene [26].

The isolation of TQ has also been achieved using thin-layer chromatography (TLC) to later be identified by gas chromatography (GC) and HPLC, according to the work carried out by Abou Basha [16,27]. The volatile components of *N. sativa* seeds were isolated using microwave-assisted extraction obtained 38.23 % of TQ and other compounds identified using gas chromatography *p*-cymene (28.61 %), 4-isopropyl-9-methoxy-1-methyl-1-cyclohexene (5.74 %), longifolene (5.33 %), α-thujene (3.88) and carvacrol (2.31 %) [28].

Several HPLC methods have been described for the quantification of TQ [29,30]. TQ easily reacts with the amino or thio groups of amino acids, undergoing a series of oxidation-reduction reactions, leading to the formation of semiquinone and thymohydroquinone. In the case of semiquinones, they can undergo a redox cycle that leads to the generation of ROS [21].

To improve the biological activity of the TQ molecule, various groups of study have studied the synthesis and structural characterization of new TQ analogues, with potent anti-pro-life activities against pancreatic cancer cell lines. In 2010, Banerjee, Padhye, Azmi, Wang, Philip, Kucuk, Sarkar and Mohammad [31]m synthesized TQ analogues, modifying the carbonyl and benzenoid sites, to be subsequently evaluated against pancreatic cancer cell lines. Of all the synthesized analogues, three molecules showed greater activity than TQ based on the inhibition of cellulite growth, induction of apoptosis and modulation of the transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [31]. In 2013, Yusufi, Banerjee, Mohammad, Khatal, Venkateswara Swamy, Khan, Aboukameel, Sarkar and Padhye [32] found a greater sensitization to pancreatic cancer in the i*n vitro* system, by one of the synthesized analogues from TQ.

TQ studies have continued in recent years, given the constant search to understand the mechanism that TQ presents in the damage of cancer cells, but without affecting normal cells. Therefore, it is necessary to know the chemistry, optimal methods of identification, isolation and synthesis of TQ. Since 1999, it had been possible to obtain TQ from the oxidation of carvacrol and thymol with hydrogen peroxide catalyzed by Mn III porphyrins [33].

There are studies where it indicates the obtaining of TQ from thymol by a biotransformation process, using *Synechococcus* sp. In this study, 1.5 % efficiency was obtained in obtaining TQ, possibly due to the toxicity of thymol [34].

There are also studies where TQ is used as a starting molecule for the synthesis of benzoxazole compounds with biological activity [35]. TQ, has also served as the starting compound for the elaboration of other derivatives, the synthesis of DTQ has been reported by photoirradiation of TQ in a single step [36].

3.2. Bioavailability of TQ

The beneficial criteria of *N. sativa* seeds and TQ are mainly related to the existence of the lipophilic constituent of quinine in its structure. This lipophilic nature facilitates efficient and easy access of the molecule to cellular and subcellular structures [37]. TQ has been reported to be responsible for killing cancer cells through a process that involves apoptosis and cell cycle arrest [38].

These anticancer activities have limited its clinical translation by its poor bioavailability and hydrophobicity. For this reason, there are studies that encapsulate TQ in nanoparticles to improve its supply and limit undesirable cytotoxicity [39].

The solubility of TQ in aqueous medium has been reported to be $<$1.0 mg/mL at room temperature. Along with its high lipophilicity, TQ has slow absorption, rapid metabolism, rapid elimination, low bioavailability, and low physicochemical stability. All these characteristics have led to various studies to improve its bioavailability in recent years.

These limitations can be ameliorated using nano-formulations of TQ, which can eliminate its bad pharmaceutical qualities to achieve better therapeutic efficacy [40]. Some groups have proposed the use of diverse nano-formulations, which can protect the TQ.

TQ encapsulation passively directs the drug to the liver and releases the drug in a controlled and effective manner, improving the oral bioavailability of this hydrophobic molecule. In general, this method of encapsulating drugs aims to reduce the biodegradability of unstable substances, control or sustained release. Nanoformulations advantages are small size and biocompatibility with tissues and cells, stable in the blood and relatively non-toxic. [41]. Examples of nanoformulations: solid lipid nanoparticles loaded with TQ (TQ-SLN) [42], or TQ loaded in a colloidal drug carrier known as nanostructured lipid carrier [43,44]. Encapsulated TQ nanoparticles have also been synthesized using biodegradable hydrophilic polymers such as polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG) to overcome the poor aqueous solubility, thermal sensitivity, and light effect to the TQ [45]. Consequently, these changes improve the minimal systemic bioavailability of TQ, helping greatly with the effective treatment of cancer [46]. TQ-SLN has been used for the effective treatment of liver cirrhosis [47]. As well as the formulation of a nanostructured lipid carrier loaded with TQ, with potential characteristics of becoming a potent drug for the treatment of breast cancer [48].

To enhance the antioxidant properties of TQ without any toxicity, NIPAAM (N-isopropyl acrylamide) nanoparticles coated with PAG (p-aminophenyl-1-thio-β-D-galactopyranoside) have been synthesized followed by encapsulation of TQ in its hydrophobic core [49]. Nanoemulsion-based administration can be used efficiently in the encapsulation of bioactive TQ since it is stable for 6 months [50].

Liposomes have also been used to act as solubilizing agents and drug carriers [51]. TQ increases its bioavailability and absorption by cells [52] and the combined use of TQ and cyclodextrins in pharmaceutical preparations [53], forming aggregates in some cases nanometric [54]. The combination with conventional chemotherapeutic drugs could produce a greater therapeutic effect as well as reduce the toxicity of the latter[55].

4. Preclinical studies related to TQ effects in pancreatic cancer: potential mechanisms of action

4.1. In vitro studies

Studies of cytotoxicity on cancer cells are the basis of its potential antineoplastic effect on cancer [56,57]. In that sense, the cytotoxic effect on these cells and induction on various pathways involved in apoptosis have been demonstrated [58]. TQ exert an antiproliferative effect of MIA PaCa-2 cells at concentrations of 25–150 μM of TQ dose-dependent [59]. Furthermore, dose-dependent TQ exposure (0–50 μM) for 48 h in PANC-1, AsPC-1, and BxPC-3 cells shows a significant decrease in cell viability and has exhibited down-regulation of anti-apoptotic protein Bcl-2 (B-cell lymphoma 2) and Bcl-xL (B-cell lymphoma-extra large), and an increase in Bax (Bcl-2 associated X protein) apoptotic proteins. Also, an increase in cytochrome C which activates caspase-3 and −9 to execute apoptosis, and an accumulation of cells in the G_0/G_1 phases were observed [60]. Similar results have been evidenced on MIA PaCa-2 and AsPC-1 cells at concentrations of 10–50 μM of TQ associated with up-regulation of p53 and down-regulation of Bcl-2 [61]. On the other hand, TQ can decrease the expression of mucin 4 glycoprotein (MUC4), expressed in an exacerbated way in pancreatic cancer cells, contributing to the regulation of differentiation, proliferation, metastasis, and chemoresistance. By incubating MUC4-expressing FG/COLO357 and CD18/HPAF cells with TQ (50–100 μM) for 24 h, decrease the MUC4 expression through the proteasomal pathway and mediated apoptosis by c-Jun NH2-terminal kinases and Mitogen-activated p38 kinases [62].

Regarding the treatment of pancreatic cancer, there is commonly resistance to drugs as Gemcitabine (GEM), but it has been shown that pre-treatment of pancreatic cancer cells with TQ can increase the sensitivity of cells to this drug. The synergistic combination of GEM with TQ was evaluated on PANC-1 cells resistant to GEM and MIA PaCa-2. The combination index (CI) revealed that pre-treatment of TQ together with GEM synergistically inhibited the proliferation of cancer cells. The pyruvate kinase M2 isoform (PKM2), involved in the metabolism of cancer cells, showed a negative regulation in the presence of a TQ + GEM CI of 36 ± 0.66 and 25 ± 5.25 on the MIA PaCa-2 and PANC-1 cells, respectively. Therefore, the effectiveness of TQ in combination with GEM in inhibiting cell proliferation, inducing apoptosis, and downregulating PKM2 expression, reflects promise in the treatment of pancreatic cancer [63]. Additionally, when exposing PANC-1, AsPC-1 and BxPC-3 cells to TQ concentrations of 25, 21 and 10 μM, respectively, for 48 h, followed by 24 h from GEM incubation (0–50 μM), a cytotoxic effect of TQ was observed. A dose of 100 nM GEM has been shown to cause significant cell death [60]. A recent study showed that TQ can exert a synergistic effect with juglone, another cytotoxic dietary molecule for pancreatic cancer cells [64]. Cell viability of MIA PaCa-2 cells was evaluated by exposing them to TQ, juglone, and a combination of both. The IC_{50} (24.75 μM) value for juglone and TQ in combination was significantly higher than juglone or TQ alone. Juglone only killed Mia-Paca-2 cells by ferroptosis. This additive effect occurred at concentrations of 75 % and 90 % of the cells. Moderate synergy was observed at concentrations of 40.90 μM juglone and 511.19 μM TQ [64].

The cytotoxic properties in pancreatic ductal adenocarcinoma (PDA) cells have been mechanistically proved because TQ increased p21 WAF1 expression, inhibited histone deacetylase (HDAC) activity, inducing hyperacetylation of histones, inhibiting the proliferation of cells and inducing apoptosis. In relation with these cytotoxic properties, TQ has also demonstrated to act as an anti-inflammatory agent in pancreatic cancer cells (HS766T PDA cells), significantly reducing the synthesis of MCP-1, TNF-α, IL-1 and Cox-2 after 24 h treatment, with a better profile than the control substance trichostatin A. Considering that cancer patients reveal a general pro-inflammatory state, these results are promising as TNF-α blockers are being studied in patients with certain solid cancers [65].

4.2. In vivo studies

Regarding studies *in vivo* models, TQ has been exerting effects in decreasing the size of the tumor and anti-metastasis.

A study conducted by Wu, Chen, Shen, Huang and Jiang [66] analyzed the effects of TQ on the model of human pancreatic carcinoma by surgical orthotopic implantation in nude mice. After 3-week implantation, mice were administered a low-dose (L-TQ 5 mg/kg) and high-dose (H-TQ 20 mg/kg) by intragastric (i.g.) via. GEM in doses of 100 mg/kg by intraperitoneal (i.p.) via was used control. At week 8 post-implantation, tumour weight, inhibition rate, and the presence of metastasis were evaluated. The tumour growth-inhibiting rates of L-TQ treatment, H-TQ treatment, and GEM treatment were 42.57 %, 64.11 %, and 54.77 %, respectively. The rates of metastasis in the L-TQ group and H-TQ group were 60 % and 50 % versus GEM treatment (100 %). Immunohistochemistry showed inhibition of Matrix Metalloproteinase 9 (MMP-9), X-linked inhibitor of apoptosis protein (XIAP), and Ki-67 protein in mice with TQ treatment [66].

Another study by Mu et al. [60], analyzed the TQ effect on orthotopic pancreatic cancer PANC-1 cells xenograph mice model. Six-week-old female BALB/c nude was used into four treatment groups: control, GEM (50 mg/kg, i.p. three times per week), TQ (1 mg/mouse, i.g. daily), and combination of GEM and TQ treatment. After 35 days, the mice were sacrificed and pancreatic tumours were isolated. The rate average weight of pancreatic tumor tissues in mice treated with TQ and GEM alone was significantly lighter, but the combination treatment of TQ and GEM resulted in 81.7 % and 85 % reduction in tumour weight. Furthermore, NF-κB was moderately down-regulated by TQ treatment alone, but mice treated with TQ and GEM exert a considerable reduction of NF-κB DNA binding activity with up-regulation of caspase-3 activity and down-regulation of Bcl-2, Bcl-xL and surviving proteins. On the other hand, the expression of Notch1, NICD, PTEN, Akt, mTOR, and S6 was analyzed. The authors propose that Notch1/PTEN and Akt/mTOR/S6 regulatory pathways play important roles in the mechanism by TQ induced apoptosis and prevented GEM insensitivity on pancreatic cancer [60].

Another study by Relles et al. [61] used a xenograft model for pancreatic ductal adenocarcinoma (PDAC) generated in 4-week-old male nude mice with AsPC-1 or Hs766T PDAC cells were injected subcutaneously. The mouse was treated with TQ (30 mg/kg, i.p.). At week 5 animals were sacrificed for tumour analysis and evaluation of acetylation expression levels. The treatment with TQ significantly reduced by 67 % of the tumour size of the animals. Furthermore, the TQ modifies the H4 acetylation by decreased histone deacetylases (HDACs) expression inducing the pro-apoptotic signalling pathway [61].

The most important mechanism of action of TQ as adjunctive in pancreatic cancer are summarized in Fig. 2 and Table 1.

5. Toxicology Investigations

Safety and toxicology consist in identifying and analyzing the effects of TQ on living organisms, but also in evaluating the symptoms and their mechanisms in TQ's intoxications. Adverse reactions can be subtle or severe and occur locally at different levels of the body, affecting a tissue or organ [68].

In a recent study, Abukhader [69] claimed that the maximum tolerated dose for i.p. and oral TQ was determined in Wistar rats (males and females). There were different signs of toxicity in rats that received i.p. injection from those who received the PO. Rats given the i.p. injection of TQ showed signs of toxicity related to acute pancreatitis, and rats that received oral TQ ingestion showed signs of transient toxicity. Deaths have been reported at a dose of 500 mg/kg due to complications of intestinal obstruction. Signs of weight loss, diarrhoea, mild abdominal distension, and shortness of breath (indicators of generalized peritonitis) were observed in 34 % of rats receiving 300 and 500 mg/kg within 48 h post-dose. After that, the rats regained their weight, and the signs of

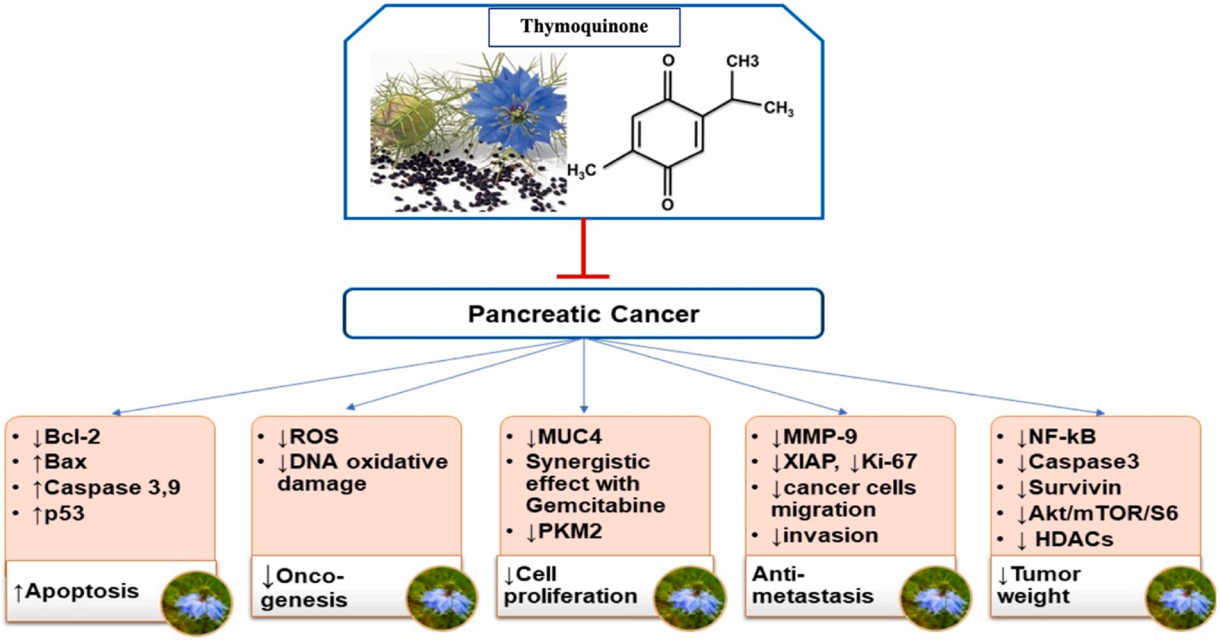

Fig. 2. Major mechanisms of TQ as chemopreventive/anticancer agent in pancreatic cancer. Abbreviations: ↑ increase, ↓ decrease, Akt protein kinase B, Bax Bcl-2 associated X protein, Bcl-2 B-cell lymphoma 2, HDACs histone deacetylases, MMP-9 matrix metalloproteinase, mTOR mammalian target of the rapamycin signaling pathway, MUC4 mucin 4, NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells, PKM2 pyruvate kinase M2, ROS reactive oxygen species, XIAP X-linked inhibitor of apoptosis protein.

toxicity began to disappear by the 5th day of the experiment. There was a difference in the incidence of these signs of TQ-related toxicity between male and female rats [69].

5.1. Side effects/ adverse reactions in the gastrointestinal tract

TQs are generally well tolerated but can cause gastric intolerance with the following manifestations: epigastric pain, abdominal cramps, flatulence, nausea, vomiting, gastralgia, anorexia. Diarrhoea or constipation may rarely occur. Pseudomembranous enterocolitis can occur extremely rarely. Even if they are the most common side effects, they are also the best tolerated and probiotics seemed to have beneficial effects [70,71]. Compared to other antibiotics they occur with a moderate frequency [72].

5.2. Side effects/ adverse reactions at the renal level

Very rarely, crystalluria, haematuria and even acute renal failure, interstitial nephritis and reversible nephritis on discontinuation of treatment may occur during renal TQ treatment. The occurrence of crystalluria during treatment with TQ (clinical investigations) has been extensively studied, but there is no evidence that TQ leads to urolithiasis or adversely affects renal function [73].

5.3. Side effects/ adverse reactions at the eye level

TQs are generally used in eye treatments without side effects. Ocular toxicity appears to be dose-dependent and structurally dependent on quinolone. Phototoxicity and neurotoxicity, a toxic effect on ocular collagen (which may be associated with Achilles tendon damage), have been reported. Corneal precipitates are advantageous forms of storage but can delay healing and even lead to corneal perforation in about 10 % of cases [74].

5.4. Side effects/ adverse reactions at the level of the joints and muscles

TQ presents a risk of chondrotoxicity in humans under therapeutic conditions. This risk in some TQs may even be increased because cartilage lesions are not always associated with clinical symptoms. Several cases of acute arthralgia have been reported in TQ in the first generations in which the causal relationship remained unclear. The highest incidence of arthropathy was reported after treatment with pefloxacin (8 cases out of 63). A similar group treated with ofloxacin did not have such complications. TQ can cause tendonitis and Achilles tendon rupture even after a short period of treatment. Because TQ chelates divalent and trivalent metal ions such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, and Al^{3+} it seems that an electrolyte balance disorder is possible which increases the risk of these side effects [75].

5.5. Side effects/ adverse reactions at the cardiovascular level

After treatment with TQ, hypotension and tachycardia have been reported as side effects. However, the cardiotoxic potential differs from one patient to another. The information available from the studies indicated indicates that TQ has a risk factor to increase the QT interval (QT interval measures a portion of the heartbeat recorded on the electrocardiogram, an important electrocardiogram parameter) and to cause arrhythmia. After the withdrawal of TQ from therapy (due to cardiovascular incidents), more attention is paid to prolonging the QT interval. However, the relationship between cardiotoxic side effects and TQ therapy is still uncertain, with numerous studies being conducted [76].

5.6. Side effects/ adverse reactions at the central nervous system (CNS) level

TQ can also cause neurotoxic side effects. Dizziness has been reported in half of the patients treated with TQ. First of all, a clear distinction must be made between mild neurotoxic signs (headache, dizziness, fatigue, drowsiness, abnormal vision, restlessness, nightmares) of the CNS and severe, extremely rare, < 0.5 %, (psychotic reactions, hallucinations, depression, convulsions) in which therapy should be discontinued. These reactions appear only a few days after the start of therapy and disappear when the medication is stopped. From all the existing studies, it appears that neurotoxic reactions are not strictly

Table 1
The most relevant preclinical studies and mechanisms of action of thymoquinone in different pancreatic cancer cell lines and animal models.

Tested compound	Dose	Experimental Model	Effects/ mechanisms	References
***In vitro* studies**				
TQ	25–150 μM	MIA PaCa-2	↑ cytotoxicity, ↓ cells proliferation	[59]
TQ	10 μM	MiaPaCa-2, BxPC-3, AsPC-1, HPAC	↓cell growth, ↑apoptosis, ↑NF-κB, ↓Bcl-2, ↓Bcl-xL, ↓survivin, ↓XIAP, ↓COX-2, ↓PGE2	[9]
TQ	50–73 μM	FG/COLO357, CD18/HPAF	↓MUC4 expression through the proteasomal pathway ↑apoptosis, ↑ JNK, ↑ p38 MAPK	[62]
TQ	10–50 μM	MIA PaCa-2 AsPC-1	↑ apoptosis, ↓ anti-apoptotic protein: ↓Bcl-2 y ↓Bcl-xL ↑ apoptotic proteins: ↑Bax, ↑caspase-3, ↑caspase-9 accumulation of cells in the G_0 /G_1 phases	[61]
TQ	2.5 μM	MiaPaCa-2 BxPC-3	↓DNA binding capacity of NF-kB, ↓cell viability, ↑apoptosis	[32]
TQ	25, 21, 10 μM	PANC-1, BxPC-3, AsPC-1	↓Notch1, ↓NICD, ↑PTEN, ↓Akt/mTOR/S6 ↓phosphorylation, nuclear translocation of p65, ↓TNF-α, ↓Bcl-2, ↓Bcl-xL, ↓XIAP, ↑ pro-apoptotic molecules, ↑caspase-3, ↑caspase-9, ↑Bax, ↑cytochrome c	[67]
TQ	25–75 μM	PDA	↓MCP-1, TNF-α, interleukin (IL)−1β and Cox-2	[65]
TQ/(GEM) combination	2,36 μM 25 μM	MIA PaCa-2 PANC-1	Chemosensitization. ↓PKM2 involved in cancer cells metabolism	[63]
TQ and TQ/GEM combination	25, 21, 10 μM 100 nM	PANC-1, AsPC-1 BxPC-3	↑ cytotoxicity ↑ significant death cell.	[60]
TQ	50 – 100 μM	FG/COLO357 CD18/HPAF	↑ apoptosis, ↓expression of MUC4, ↑c-Jun NH2, ↑p38	[62]
TQ/juglone combination	40.90 μM 511.19 μM	MIA PaCa-2	↑cytotoxicity/ Synergic effect	[64]
***In vivo* studies**				
TQ	5 and 20 mg/kg for 2 weeks	Human pancreatic carcinoma by surgical implantation in nude mice	↓MMP-9, ↓ XIAP, ↓Ki-67 proteins ↓ tumor growth ↓ metastasis	[66]
TQ GEM/TQ combination.	TQ 1 mg/kg i.g. for 5 weeks GEM 50 mg/kg i.p. 3 times per week	PANC-1 cells Xenograph mice model	↓ tumor weight ↓ NF-κB,↓ion of caspase-3 activity Notch1/PTEN, Akt/mTOR/S6 regulatory pathways modulation.	[60]
TQ	30 mg/kg i.p. 5 weeks	Xenograft PDAC mice model	↓ tumor size Pro-apoptotic signaling pathway modulation by H4 acetylation.	[61]

Abbreviations: ↑increase, ↓decrease, Akt protein kinase B, Bax Bcl-2 associated X protein, Bcl-2 B-cell lymphoma 2, Bcl-xL B-cell lymphoma-extra large, COX-2 ciclooxygenase 2, GEM gemcitabine, JNK c-Jun N-terminal kinases, MAPK mitogen-activated protein kinase, MMP-9 matrix metalloproteinase, mTOR mechanistic target of rapamycin, MUC4 mucin 4, NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells, NICD Notch1intracellular domain, Notch1 Notch homolog 1, PDAC pancreatic ductal adenocarcinoma, PGE2 prostaglandin E2, PKM2 pyruvate kinase M2, PTEN phosphatase and tensin homolog, TNF-α tumor necrosis factor alpha, TQ thymoquinone, XIAP X-linked inhibitor of apoptosis protein.

related to very high plasma concentrations of TQ. There may be an explanation for the concentrations of TQ that reach the brain tissue. There are few data on TQ concentrations in brain tissue. TQ concentrations were measured in a small group of brain-operated patients. Their variability was found to be 2–20 times the corresponding concentration in the cerebrospinal fluid. The stimulant action in the CNS appears to be due to inhibition of the binding of gamma-aminobutyric acid to its central receptors, a dose-dependent effect [77].

5.7. Side effects/adverse reactions to the teguments

Frequency of side effects teguments it is estimated to be between 0.4 % and 2.1 %. The most frequently reported manifestations are erythema, pruritus, urticaria and other skin manifestations. Phototoxicity is one of the clearest examples of the effect of chemical structure on biological capacity. Phototoxic reactions have been described for treatment with the majority of TQ. In general, it is recommended that patients undergoing treatment for TQ avoid sun exposure [78].

5.8. Side effects/adverse reactions - various

Adverse reactions such as haematological disorders (thrombocytopenia, leukopenia, anaemia) have been reported in some cases. Transient increases in plasma liver enzymes have also been observed. Occasional TQs have been reported: fever, hot flashes, chest pressure, sweating, palpitations, rhinitis, vaginal irritation [79].

5.9. Allergy to TQ

TQ can cause immediate and late hypersensitivity reactions. Hypersensitivity reactions to TQ (especially anaphylactic reactions) have become more common in the last decade, given their increasing use in medical practice. The most common reactions described in the literature are generalized pruritus, maculopapular rash, urticaria, angioedema and anaphylaxis. Rarely, fixed drug reactions or toxic epidermal necrolysis may also occur. These reactions are mostly immunoglobulin (Ig) E-mediated and there appears to be cross-recapacity within this class of drugs [80].

These side effects of TQ, summarized in Fig. 3, must be considered used for potential therapeutic uses. However, as previously mentioned,

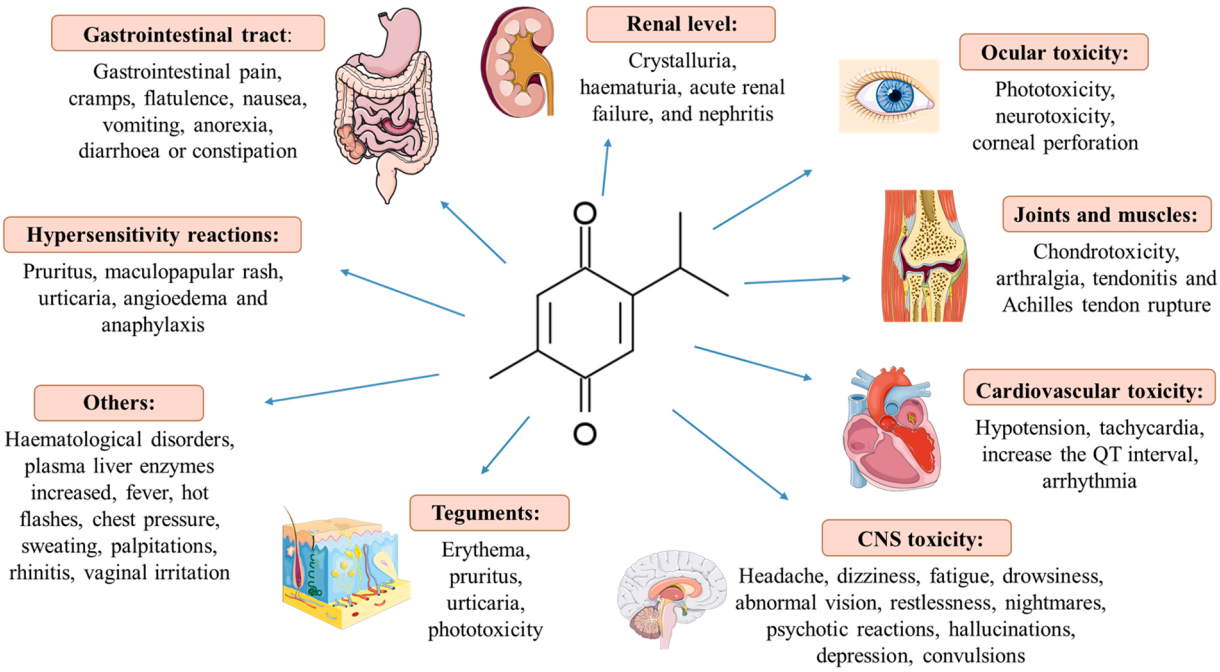

Fig. 3. TQ potential adverse effects collected in the scientific literature. It should be noted that many of these side effects are very rare.

most of them are rare and at high doses of TQ.

6. Discussion

Despite the invention of numerous strategies to treat cancer (such as surgery, chemotherapy, radiotherapy, targeted therapy, immunotherapy, etc.), the search continues for new additional ones to discover secondary metabolites, which allow fighting cancer using natural plants and its bioactive components [81,82].

TQ is the main bioactive component of the oils extracted from *N. sativa* (commonly known as black cumin or black seed) and *Nigella sativa* var. *hispidula* Boiss., various studies have reported anti-cancer effects through various mechanisms of action together with numerous preclinical studies [83,84]. TQ can also be found in other plants such as the fresh cones of *Cupressus sempervirens* L. [85], in *M. fistulosa* [86], in *Origanum vulgare* L. [87], *Juniperus communis* L. [88], *Tetraclinis articulata* (Vahl) Mast. [89].

As a result of extensive structure-capacity research, high-potency TQ, extended capacity spectrum, improved absorption and distribution properties have been achieved. TQ has evolved to the point where they are useful for treating a variety of serious systemic infections [90]. Its pharmacological properties are related to its anti-inflammatory, antidiabetic, antihypertensive, antimicrobial, analgesic, immunomodulatory, spasmolytic, hepatoprotective, renal protective, gastroprotective, a bronchodilator, antioxidant and antineoplastic agents *in vitro* and *in vivo* [91,92]. Although TQ is a potential candidate for the treatment of different diseases, its highly hydrophobic nature limits its oral bioavailability.

The anticancer potential is achieved through several aspects: including apoptosis promotion, cell cycle arrest and ROS generation [93]. Additionally, it strengthens the immune system and the side effects associated with traditional cancer therapy.

An important aspect to mention is related to the stability of the TQ. It has been reported that TQ was not affected by the action of acids, basic and oxidative forced conditions, but the action of light and heat was important, observing degradation products due to these actions [94], criterion related to the properties and chemical characteristics of TQ. The anticancer activities of TQ are limited by its poor bioavailability and hydrophobicity. For this reason, some studies encapsulate TQ in nanoparticles to improve its supply and limit undesirable side effects. When free TQ is compared with TQ nanoparticle formulations, it is observed that the latter have much better anti-cancer and anti-inflammatory activities than free TQ, enhancing clinical translation.

TQ has a cancer therapeutic potential with low toxicity, however adverse reactions can be severe and occur locally at different levels of the body. To be noted that TQ inhibit phosphorylation of MAPK pathway which is also essential fact for bone and teeth formation. For this reason, the development of clinical trials with TQ during developmental phases is a major concern. Obviously, clinical trials cannot be carried out in pregnant or lactating women, and patients with hypersensitivity to TQ or plants rich on this phytochemical. In addition, the toxicity and pharmacokinetic behavior not only dependent on its concentration, but also of its route administration. These details need to be considered to avoid severe effects in future clinical trials.

7. Conclusion and final remarks

Herbal medicines as complementary medicines have played an important role in ancient medicine and it is still widespread in modern times, both in rural and urban areas. TQ is the principal constituent of *N. sativa* and has a wide spectrum of medicinal effects. Also, several medicinal plants rich in TQ have beneficial biological effects. Preclinical studies, *in vitro* and *in vivo,* show a therapeutic potential of TQ against pancreatic cancer but the data still scarce. The knowledge from the animal model systems makes known that TQ has several therapeutic possibilities as a low toxicity, probable antioxidant effects, and consequently cytoprotection, with a synergistically capacity with currently used chemotherapeutic agents, capacity against compound resistance (especially antibiotics) as well as antagonistic effects with well-known toxic compounds making TQ. Further *in vitro* and *in vivo* research is required to better understand the safety of TQ and mechanisms involved of TQ against pancreatic cancer to posterior conduction of clinical trials to confirm the benefits of this phytochemical in humans.

Conflict of interest statement

All authors have made a significant contribution to the study and

paper and declare no conflicts of interest.

Data availability

Data will be made available on request.

Acknowledgement

This work was supported by ANID CENTROS BASALES ACE 210012. and Medical University of Lublin, grant number 07/2022.

References

[1] J.D. Mizrahi, R. Surana, J.W. Valle, R.T. Shroff, Pancreatic cancer, Lancet 395 (10242) (2020) 2008–2020.
[2] M. Sharifi-Rad, N.V.A. Kumar, P. Zucca, E.M. Varoni, L. Dini, E. Panzarini, J. Rajkovic, P.V.T. Fokou, E. Azzini, I. Peluso, A.P. Mishra, M. Nigam, Y. El Rayess, M. El Beyrouthy, L. Polito, M. Iriti, N. Martins, M. Martorell, A.O. Docea, W. N. Setzer, D. Calina, W.C. Cho, J. Sharifi-Rad, Lifestyle, oxidative stress, and antioxidants: back and forth in the pathophysiology of chronic diseases, Front. Physiol. 11 (2020) 21.
[3] J. Sharifi-Rad, C.F. Rodrigues, F. Sharopov, A.O. Docea, A. Can Karaca, M. Sharifi-Rad, D. Kahveci Karıncaoglu, G. Gülseren, E. Şenol, E. Demircan, Y. Taheri, H.A. R. Suleria, B. Özçelik, K. Nur Kasapoğlu, M. Gültekin-Özgüven, C. Daşkaya-Dikmen, W.C. Cho, N. Martins, D. Calina, Diet, lifestyle and cardiovascular diseases: linking pathophysiology to cardioprotective effects of natural bioactive compounds, Int. J. Environ. Res. Public Health 17 (7) (2020).
[4] J. Ansary, F. Giampieri, T.Y. Forbes-Hernandez, L. Regolo, D. Quinzi, S. Gracia Villar, E. Garcia Villena, K. Tutusaus Pifarre, J.M. Alvarez-Suarez, M. Battino, Nutritional value and preventive role of *Nigella sativa* l. and its main component thymoquinone in cancer: an evidenced-based review of preclinical and clinical studies, Molecules 26 (8) (2021) 2108.
[5] H. Gali-Muhtasib, A. Roessner, R. Schneider-Stock, Thymoquinone: a promising anti-cancer drug from natural sources, Int. J. Biochem. Cell Biol. 38 (8) (2006) 1249–1253.
[6] G. Kreibich, M. Czakó-Graham, R. Grebenau, W. Mok, E. Rodriguez-Boulan, D. D. Sabatini, Characterization of the ribosomal binding site in rat liver rough microsomes: ribophorins I and II, two integral membrane proteins related to ribosome binding, J. Supramol. Struct. 8 (3) (1978) 279–302.
[7] A.S. Butt, N. Nisar, T.A. Mughal, N. Ghani, I. Altaf, Anti-oxidative and anti-proliferative activities of extracted phytochemical compound thymoquinone, J. Pak. Med. Assoc. 69 (2019) 1479–1485.
[8] P. Vuorelaa, M. Leinonenb, P. Saikkuc, P. Tammelaa, J.P. Rauhad, T. Wennberge, H. Vuorela, Natural products in the process of finding new drug candidates, Curr. Med. Chem. 11 (11) (2004) 1375–1389.
[9] S. Banerjee, A.S. Azmi, S. Padhye, M.W. Singh, J.B. Baruah, P.A. Philip, F. H. Sarkar, R.M. Mohammad, Structure-activity studies on therapeutic potential of Thymoquinone analogs in pancreatic cancer, Pharm. Res. 27 (6) (2010) 1146–1158.
[10] B. Almajali, H.A.N. Al-Jamal, W.R.W. Taib, I. Ismail, M.F. Johan, A.A. Doolaanea, W.N. Ibrahim, Thymoquinone, as a novel therapeutic candidate of cancers, Pharmaceuticals 14 (4) (2021) 369.
[11] R.L. Gurung, S.N. Lim, A.K. Khaw, J.F. Soon, K. Shenoy, S. Mohamed Ali, M. Jayapal, S. Sethu, R. Baskar, M.P. Hande, Thymoquinone induces telomere shortening, DNA damage and apoptosis in human glioblastoma cells, PLoS One 5 (8) (2010), e12124.
[12] C.C. Woo, A.P. Kumar, G. Sethi, K.H. Tan, Thymoquinone: potential cure for inflammatory disorders and cancer, Biochem. Pharmacol. 83 (4) (2012) 443–451.
[13] J. Pang, N. Shen, F. Yan, N. Zhao, L. Dou, L.C. Wu, C.L. Seiler, L. Yu, K. Yang, V. Bachanova, Thymoquinone exerts potent growth-suppressive activity on leukemia through DNA hypermethylation reversal in leukemia cells, Oncotarget 8 (2017) 34453–34467.
[14] M.B. Djamgoz, V. Jentzsch, Integrative management of pancreatic cancer (PDAC): emerging complementary agents and modalities, Nutr. Cancer (2021) 1–24.
[15] S. West, V. King, T.S. Carey, K.N. Lohr, N. McKoy, S.F. Sutton, L. Lux, Systems to rate the strength of scientific evidence, Evid. Rep. Technol. Assess. (47) (2002) 1–11.
[16] L.I.A. Basha, M.S. Rashed, H.Y. Aboul-Enein, TLC assay of thymoquinone in black seed oil (*Nigella sativa* Linn) and Identification of dithymoquinone and thymol, J. Liq. Chromatogr. 18 (1) (1995) 105–115.
[17] L. Canonica, G. Jommi, C. Scolastico, A. Bonati, The pharmacologically active principle in *Nigella sativa*, Gazz. Chim. Ital. 93 (11) (1963) 1404–1407.
[18] O.A. Badary, O.A. Al-Shabanah, M.N. Nagi, A.M. Al-Bekairi, M.M.A. Elmazar, Acute and subchronic toxicity of thymoquinone in mice, Drug Dev. Res. 44 (2–3) (1998) 56–61.
[19] H. Gali-Muhtasib, N. El-Najjar, R. Schhneider-stock, The medicinal potential of black seed (*Nigella sativa*) and its components, Advances in, Phytomedicine 2 (2005) 133–153.
[20] PubChem-Database, Thymoquinone, National Center for Biotechnology Information Updated. ⟨https://pubchem.ncbi.nlm.nih.gov/compound/10281⟩. (Accessed 16 August 2018). (2018).
[21] R. Schneider-Stock, I.H. Fakhoury, A.M. Zaki, C.O. El-Baba, H.U. Gali-Muhtasib, Thymoquinone: fifty years of success in the battle against cancer models, Drug Discov. Today 19 (1) (2014) 18–30.
[22] M. Burits, F. Bucar, Antioxidant activity of *Nigella sativa* essential oil, Phytother. Res. PTR 14 (5) (2000) 323–328.
[23] Z. Solati, B.S. Baharin, H. Bagheri, Supercritical carbon dioxide (SC-CO_2) extraction of *Nigella sativa* L. oil using full factorial design, Ind. Crops Prod. 36 (1) (2012) 519–523.
[24] Z. Ghanavi, A.A. Velayati, P. Farnia, A.M. Naji, S. Kalatehjari, Extraction and purification of anticancer thymoquinone from seeds of Nigella sativa by preparative high-performance liquid chromatography, J. Med. Plants Prod. 9 (Special) (2020) 73–79.
[25] H. Sovova, M. Sajfrtova, M. Topiar, Supercritical CO_2 extraction of volatile thymoquinone from *Monarda didyma* and *M. fistulosa* herbs, The, J. Supercrit. Fluids 105 (2015) 29–34.
[26] M.S. Iqbal, A. Ahmad, B. Pandey, Solvent based optimization for extraction and stability of thymoquinone from *Nigella sativa* Linn. and its quantification using RP-HPLC, Physiol. Mol. Biol. Plant Int. J. Funct. Plant.Biol. 24 (6) (2018) 1209–1219.
[27] H.Y. Aboul-Enein, L.I. Abou-Basha, Simple HPLC method for the determination of thymoquinone in black seed oil (*Nigella sativa* Linn), J. Liq. Chromatogr. 18 (5) (1995) 895–902.
[28] X. Liu, J.H. Park, A.M. Abd El-Aty, M.E. Assayed, M. Shimoda, J.H. Shim, Isolation of volatiles from *Nigella sativa* seeds using microwave-assisted extraction: effect of whole extracts on canine and murine CYP1A, Biomed. Chromatogr. BMC 27 (7) (2013) 938–945.
[29] O.A. Ghosheh, A.A. Houdi, P.A. Crooks, High performance liquid chromatographic analysis of the pharmacologically active quinones and related compounds in the oil of the black seed (*Nigella sativa* L.), J. Pharm. Biomed. Anal. 19 (5) (1999) 757–762.
[30] G.M. Hadad, R.A. Abdel Salam, R.M. Soliman, M.K. Mesbah, High-performance liquid chromatography quantification of principal antioxidants in black seed (*Nigella sativa* L.) phytopharmaceuticals, J. AOAC Int. 95 (4) (2019) 1043–1047.
[31] S. Banerjee, S. Padhye, A. Azmi, Z. Wang, P.A. Philip, O. Kucuk, F.H. Sarkar, R. M. Mohammad, Review on molecular and therapeutic potential of thymoquinone in cancer, Nutr. Cancer 62 (7) (2010) 938–946.
[32] M. Yusufi, S. Banerjee, M. Mohammad, S. Khatal, K. Venkateswara Swamy, E. M. Khan, A. Aboukameel, F.H. Sarkar, S. Padhye, Synthesis, characterization and anti-tumor activity of novel thymoquinone analogs against pancreatic cancer, Bioorg. Med. Chem. Lett. 23 (10) (2013) 3101–3104.
[33] R.R.L. Martins, M.G.P.M.S. Neves, A.J.D. Silvestre, A.M.S. Silva, J.A.S. Cavaleiro, Oxidation of aromatic monoterpenes with hydrogen peroxide catalysed by Mn(III) porphyrin complexes, J. Mol. Catal. A Chem. 137 (1) (1999) 41–47.
[34] S. Rasoul-Amini, E. Fotooh-Abadi, Y. Ghasemi, Biotransformation of monoterpenes by immobilized microalgae, J. Appl. Phycol. 23 (6) (2011) 975–981.
[35] U. Glamoclija, S. Padhye, S. Spirtovic-Halilovic, A. Osmanovic, E. Veljovic, S. Roca, I. Novakovic, B. Mandic, I. Turel, J. Kljun, S. Trifunovic, E. Kahrovic, S. Kraljevic Pavelic, A. Harej, M. Klobucar, D. Zavrsnik, Synthesis, biological evaluation and docking studies of benzoxazoles derived from thymoquinone, Molecules 23 (12) (2018).
[36] A.L. Myers, Y.-P. Zhang, M.A. Kramer, W.G. Bornmann, A. Kaseb, P. Yang, H. T. Tran, A practical synthesis and X-ray crystallographic analysis of dithymoquinone, a photodimer of thymoquinone, Lett. Org. Chem. 9 (10) (2012) 762–766.
[37] Y.K. Mahmoud, H.M.A. Abdelrazek, Cancer: thymoquinone antioxidant/pro-oxidant effect as potential anticancer remedy, Biomed. Pharmacother. Biomed Pharmacother. 115 (2019), 108783.
[38] A.M. Shoieb, M. Elgayyar, P.S. Dudrick, J.L. Bell, P.K. Tithof, In vitro inhibition of growth and induction of apoptosis in cancer cell lines by thymoquinone, Int. J. Oncol. 22 (1) (2003) 107–113.
[39] F. Ballout, Z. Habli, O.N. Rahal, M. Fatfat, H. Gali-Muhtasib, Thymoquinone-based nanotechnology for cancer therapy: promises and challenges, Drug Discov. Today 23 (5) (2018) 1089–1098.
[40] S. Dewanjee, P. Chakraborty, B. Mukherjee, V. De Feo, Plant-based antidiabetic nanoformulations: the emerging paradigm for effective therapy, Int J. Mol. Sci. 21 (6) (2020) 2217.
[41] C. Rathore, N. Upadhyay, R. Kaundal, R.P. Dwivedi, S. Rahatekar, A. John, K. Dua, M.M. Tambuwala, S. Jain, D. Chaudari, P. Negi, Enhanced oral bioavailability and hepatoprotective activity of thymoquinone in the form of phospholipidic nano-constructs, Expert Opin. Drug Deliv. 17 (2) (2020) 237–253.
[42] S. Ramachandran, S. Thangarajan, Thymoquinone loaded solid lipid nanoparticles counteracts 3-Nitropropionic acid induced motor impairments and neuroinflammation in rat model of Huntington's disease, Metab. Brain Dis. 33 (5) (2018) 1459–1470.
[43] S.I. Abdelwahab, B.Y. Sheikh, M.M. Taha, C.W. How, R. Abdullah, U. Yagoub, R. El-Sunousi, E.E. Eid, Thymoquinone-loaded nanostructured lipid carriers: preparation, gastroprotection, in vitro toxicity, and pharmacokinetic properties after extravascular administration, Int. J. Nanomed. 8 (2013) 2163–2172.
[44] A.S. Haron, S.S. Syed Alwi, L. Saiful Yazan, R. Abd Razak, Y.S. Ong, F.H. Zakarial Ansar, H. Roshini Alexander, Cytotoxic effect of thymoquinone-loaded nanostructured lipid carrier (TQ-NLC) on liver cancer cell integrated with hepatitis B genome, Hep3B, Evid. Based Complement. Altern. Med. 2018 (2018), 1549805.
[45] I. Pinzaru, D. Coricovac, C. Dehelean, E.A. Moacă, M. Mioc, F. Baderca, I. Sizemore, S. Brittle, D. Marti, C.D. Calina, A.M. Tsatsakis, C. Şoica, Stable PEG-coated silver nanoparticles - a comprehensive toxicological profile, Food Chem. Toxicol. Int. J. Publ. Br. Ind. Biol. Res. Assoc. 111 (2018) 546–556.

[46] S. Bhattacharya, M. Ahir, P. Patra, S. Mukherjee, S. Ghosh, M. Mazumdar, S. Chattopadhyay, T. Das, D. Chattopadhyay, A. Adhikary, PEGylated-thymoquinone-nanoparticle mediated retardation of breast cancer cell migration by deregulation of cytoskeletal actin polymerization through miR-34a, Biomaterials 51 (2015) 91–107.
[47] A. Singh, I. Ahmad, S. Akhter, G.K. Jain, Z. Iqbal, S. Talegaonkar, F.J. Ahmad, Nanocarrier based formulation of Thymoquinone improves oral delivery: stability assessment, in vitro and in vivo studies, Colloids Surf. B Biointerfaces 102 (2013) 822–832.
[48] W.K. Ng, L. Saiful Yazan, L.H. Yap, W.A.G. Wan Nor Hafiza, C.W. How, R. Abdullah, Thymoquinone-Loaded Nanostructured Lipid Carrier Exhibited Cytotoxicity towards Breast Cancer Cell Lines (MDA-MB-231 and MCF-7) and Cervical Cancer Cell Lines (HeLa and SiHa), BioMed. Res. Int. 2015 (2015), 263131.
[49] S.K. Verma, S. Rastogi, K. Javed, M. Akhtar, I. Arora, M. Samim, Nanothymoquinone, a novel hepatotargeted delivery system for treating CCl4 mediated hepatotoxicity in rats, J. Mater. Chem. B 1 (23) (2013) 2956–2966.
[50] Z. Tubesha, Z. Abu Bakar, M. Ismail, Characterization and stability evaluation of thymoquinone nanoemulsions prepared by high-pressure homogenization, J. Nanomater. 2013 (2013), 453290.
[51] B. Salehi, D. Calina, A.O. Docea, N. Koirala, S. Aryal, D. Lombardo, L. Pasqua, Y. Taheri, C. Marina Salgado Castillo, M. Martorell, N. Martins, M. Iriti, H.A. R. Suleria, J. Sharifi-Rad, Curcumin's nanomedicine formulations for therapeutic application in neurological diseases, J. Clin. Med. 9 (2) (2020).
[52] F. Odeh, S.I. Ismail, R. Abu-Dahab, I.S. Mahmoud, A. Al Bawab, Thymoquinone in liposomes: a study of loading efficiency and biological activity towards breast cancer, Drug Deliv. 19 (8) (2012) 371–377.
[53] T. Cardoso, C.I.C. Galhano, M.F. Ferreira Marques, A. Moreira da Silva, Thymoquinone β-cyclodextrin nanoparticles system: a preliminary study, Spectrosc.: Int. J. 27 (2012), 902486.
[54] R. Abu-Dahab, F. Odeh, S.I. Ismail, H. Azzam, A. Al Bawab, Preparation, characterization and antiproliferative activity of thymoquinone-beta-cyclodextrin self assembling nanoparticles, Die Pharm. 68 (12) (2013) 939–944.
[55] B. Salehi, P. Lopez-Jornet, E. Pons-Fuster López, D. Calina, M. Sharifi-Rad, K. Ramírez-Alarcón, K. Forman, M. Fernández, M. Martorell, W.N. Setzer, N. Martins, C.F. Rodrigues, J. Sharifi-Rad, Plant-derived bioactives in oral mucosal lesions: a key emphasis to curcumin, lycopene, chamomile, *Aloe vera*, green tea and coffee properties, Biomolecules 9 (3) (2019).
[56] B. Salehi, J. Sharifi-Rad, E. Capanoglu, N. Adrar, G. Catalkaya, S. Shaheen, M. Jaffer, L. Giri, R. Suyal, A.K. Jugran, D. Calina, A.O. Docea, S. Kamiloglu, D. Kregiel, H. Antolak, E. Pawlikowska, S. Sen, K. Acharya, M. Bashiry, Z. Selamoglu, M. Martorell, F. Sharopov, N. Martins, J. Namiesnik, W.C. Cho, *Cucurbita* plants: from farm to industry, Appl. Sci. 9 (16) (2019) 21.
[57] B. Salehi, M. Shivaprasad Shetty, V.A.K. N, J. Živković, D. Calina, A. Oana Docea, S. Emamzadeh-Yazdi, C. Sibel Kılıç, T. Goloshvili, S. Nicola, G. Pignata, F. Sharopov, M. Del Mar Contreras, W.C. Cho, N. Martins, J. Sharifi-Rad, *Veronica* plants-drifting from farm to traditional healing, food application, and phytopharmacology, Molecules 24 (13) (2019).
[58] B. Salehi, J. Sharifi-Rad, F. Cappellini, A. Reiner, D. Zorzan, M. Imran, B. Sener, M. Kilic, M. El-Shazly, N.M. Fahmy, E. Al-Sayed, M. Martorell, C. Tonelli, K. Petroni, A.O. Docea, D. Calina, A. Maroyi, The therapeutic potential of anthocyanins: current approaches based on their molecular mechanism of action, Front. Pharmacol. 11 (2020) 20.
[59] S. Rooney, M.F. Ryan, Effects of alpha-hederin and thymoquinone, constituents of *Nigella sativa*, on human cancer cell lines, Anticancer Res. 25 (3b) (2005) 2199–2204.
[60] G.G. Mu, L.L. Zhang, H.Y. Li, Y. Liao, H.G. Yu, Thymoquinone pretreatment overcomes the insensitivity and potentiates the antitumor effect of gemcitabine through abrogation of Notch1, PI3K/Akt/mTOR regulated signaling pathways in pancreatic cancer, Dig. Dis. Sci. 60 (4) (2015) 1067–1080.
[61] D. Relles, G.I. Chipitsyna, Q. Gong, C.J. Yeo, H.A. Arafat, Thymoquinone promotes pancreatic cancer cell death and reduction of tumor size through combined inhibition of histone deacetylation and induction of histone acetylation, Adv. Prev. Med. 2016 (2016), 1407840.
[62] M.P. Torres, M.P. Ponnusamy, S. Chakraborty, L.M. Smith, S. Das, H.A. Arafat, S. K. Batra, Effects of thymoquinone in the expression of mucin 4 in pancreatic cancer cells: implications for the development of novel cancer therapies, Mol. Cancer Ther. 9 (5) (2010) 1419–1431.
[63] A. Pandita, B. Kumar, S. Manvati, S. Vaishnavi, S.K. Singh, R.N. Bamezai, Synergistic combination of gemcitabine and dietary molecule induces apoptosis in pancreatic cancer cells and down regulates PKM2 expression, PLoS One 9 (9) (2014), e107154.
[64] N. Karki, S. Aggarwal, R.A. Laine, F. Greenway, J.N. Losso, Cytotoxicity of juglone and thymoquinone against pancreatic cancer cells, Chem. -Biol. Interact. 327 (2020), 109142.
[65] N. Chehl, G. Chipitsyna, Q. Gong, C.J. Yeo, H.A. Arafat, Anti-inflammatory effects of the *Nigella sativa* seed extract, thymoquinone, in pancreatic cancer cells, HPB 11 (5) (2009) 373–381.
[66] Z.H. Wu, Z. Chen, Y. Shen, L.L. Huang, P. Jiang, [Anti-metastasis effect of thymoquinone on human pancreatic cancer], Yao Xue Xue Bao 46 (8) (2011) 910–914.
[67] C. Rathore, N. Upadhyay, R. Kaundal, R. Dwivedi, S. Rahatekar, A. John, K. Dua, M.M. Tambuwala, S. Jain, D. Chaudari, Enhanced oral bioavailability and hepatoprotective activity of thymoquinone in the form of phospholipidic nano-constructs, Expert Opin. Drug Deliv. 17 (2) (2020) 237–253.
[68] B. Salehi, A. Prakash Mishra, M. Nigam, N. Karazhan, I. Shukla, A. Kiełtyka-Dadasiewicz, B. Sawicka, A. Głowacka, M.S. Abu-Darwish, A. Hussein Tarawneh, A.V. Gadetskaya, C. Cabral, L. Salgueiro, M. Victoriano, M. Martorell, A.O. Docea, A. Abdolshahi, D. Calina, J. Sharifi-Rad, *Ficus* plants: state of the art from a phytochemical, pharmacological, and toxicological perspective, Phytother. Res. PTR (2020).
[69] M.M. Abukhader, The effect of route of administration in thymoquinone toxicity in male and female rats, Indian J. Pharm. Sci. 74 (3) (2012) 195–200.
[70] J. Sharifi-Rad, C.F. Rodrigues, Z. Stojanović-Radić, M. Dimitrijević, A. Aleksić, K. Neffe-Skocińska, D. Zielińska, D. Kołożyn-Krajewska, B. Salehi, S. Milton Prabu, F. Schutz, A.O. Docea, N. Martins, D. Calina, Probiotics: versatile bioactive components in promoting human health, Medicina 56 (9) (2020).
[71] P. Mitrut, A.O. Docea, A.M. Kamal, R. Mitrut, D. Calina, E. Gofita, V. Padureanu, C. Gruia, L. Streba, Colorectal cancer and inflammatory bowel disease, Intech (2016).
[72] F. Shakeri, Z. Gholamnezhad, B. Mégarbane, R. Rezaee, M.H. Boskabady, Gastrointestinal effects of *Nigella sativa* and its main constituent, thymoquinone: a review, Avicenna J. Phytomed. 6 (1) (2016) 9–20.
[73] Y.-F. Liou, Y.-S. Hsieh, T.-W. Hung, P.-N. Chen, Y.-Z. Chang, S.-H. Kao, S.-W. Lin, H.-R. Chang, Thymoquinone inhibits metastasis of renal cell carcinoma cell 786-O-SI3 associating with downregulation of MMP-2 and u-PA and suppression of PI3K/Src signaling, Int J. Med. Sci. 16 (5) (2019) 686–695.
[74] T. Kocatürk, E. Erkan, İ. Meteoğlu, M. Ekici, A. Karul Büyüköztürk, İ. Yavaşoğlu, H. Çakmak, V. Dayanır, M. Balkaya, Effects of topical thymoquinone in an experimental dry eye model, Turk. J. Ophthalmol. 48 (6) (2018) 281–287.
[75] N. Zhu, Y. Xiang, X. Zhao, C. Cai, H. Chen, W.-b Jiang, Y. Wang, C. Zeng, Thymoquinone suppresses platelet derived growth facto' Bb induced vascular smooth muscle cell proliferation, migration and neointimal formation, J. Cell. Mol. Med. 23 (2019) 8482–8492.
[76] H. Liu, H.Y. Liu, Y.N. Jiang, N. Li, Protective effect of thymoquinone improves cardiovascular function, and attenuates oxidative stress, inflammation and apoptosis by mediating the PI3K/Akt pathway in diabetic rats, Mol. Med Rep. 13 (3) (2016) 2836–2842.
[77] T. Farkhondeh, S. Samarghandian, S. Hozeifi, M. Azimi-Nezhad, Therapeutic effects of thymoquinone for the treatment of central nervous system tumors: a review, Biomed. Pharmacother. 96 (2017) 1440–1444.
[78] H. Aslam, M. Shahzad, A. Shabbir, S. Irshad, Immunomodulatory effect of thymoquinone on atopic dermatitis, Mol. Immunol. 101 (2018) 276–283.
[79] S.A. Qadi, M.A. Hassan, R.A. Sheikh, O.A. Baothman, M.A. Zamzami, H. Choudhry, A.L. Al-Malki, A. Albukhari, M. Alhosin, Thymoquinone-Induced reactivation of tumor suppressor genes in cancer cells involves epigenetic mechanisms, Epigenet. Insights 12 (2019), 2516865719839011-2516865719839011.
[80] M.S. Al-Qubaisi, A. Rasedee, M.H. Flaifel, E.E.M. Eid, S. Hussein-Al-Ali, F. H. Alhassan, A.M. Salih, M.Z. Hussein, Z. Zainal, D. Sani, A.H. Aljumaily, M. I. Saeed, Characterization of thymoquinone/hydroxypropyl-β-cyclodextrin inclusion complex: application to anti-allergy properties, Eur. J. Pharm. Sci. 133 (2019) 167–182.
[81] T.A. Sani, E. Mohammadpour, A. Mohammadi, T. Memariani, M.V. Yazdi, R. Rezaee, D. Calina, A.O. Docea, M. Goumenou, L. Etemad, S. Shahsavand, Cytotoxic and apoptogenic properties of dracocephalum kotschyi aerial part different fractions on CALU-6 and MEHR-80 lung cancer cell lines, Farmacia 65 (2) (2017) 189–199.
[82] B. Salehi, E. Capanoglu, N. Adrar, G. Catalkaya, S. Shaheen, M. Jaffer, L. Giri, R. Suyal, A.K. Jugran, D. Calina, A.O. Docea, S. Kamiloglu, D. Kregiel, H. Antolak, E. Pawlikowska, S. Sen, K. Acharya, Z. Selamoglu, J. Sharifi-Rad, M. Martorell, C. F. Rodrigues, F. Sharopov, N. Martins, R. Capasso, *Cucurbits* plants: a key emphasis to its pharmacological potential, Molecules 24 (10) (2019).
[83] Y. Zheng, Q. Zhang, X. Hu, A comprehensive review of ethnopharmacological uses, phytochemistry, biological activities, and future prospects of *Nigella glandulifera*, Med. Chem. Res. 29 (7) (2020) 1168–1186.
[84] S.N. Goyal, C.P. Prajapati, P.R. Gore, C.R. Patil, U.B. Mahajan, C. Sharma, S. P. Talla, S.K. Ojha, Therapeutic potential and pharmaceutical development of thymoquinone: a multitargeted molecule of natural origin, Front. Pharmacol. 8 (2017) 656.
[85] M. Milos, J. Masteli, A. Radoni, Free and glycosidically bound volatile compounds from cypress cones (*Cupressus sempervirens* L.), Croat. Chem. Acta 71 (1) (1998) 139–145.
[86] H.A. Johnson, L.L. Rogers, M.L. Alkire, T.G. McCloudand, J.L. McLaughlin, Bioactive monoterpenes from *Monarda fistulosa* (Lamiaceae), Nat. Prod. Lett. 11 (4) (1998) 241–250.
[87] M. Milos, J. Mastelic, I. Jerkovic, Chemical composition and antioxidant effect of glycosidically bound volatile compounds from oregano (*Origanum vulgare* L. ssp. hirtum), Food Chem. 71 (1) (2000) 79–83.
[88] J. Mastelic, M. Milos, D. Kustrak, A. Radonić, Essential Oil and Glycosidically Bound Volatile Compounds from the Needles of Common Juniper (*Juniperus communis*, L.) (2000).
[89] S. Pagola, A. Benavente, A. Raschi, E. Romano, M.A.A. Molina, P.W. Stephens, Crystal structure determination of thymoquinone by high-resolution X-ray powder diffraction, AAPS PharmSciTech 5 (2) (2004) e28-e28.
[90] A.F. Majdalawieh, M.W. Fayyad, G.K. Nasrallah, Anti-cancer properties and mechanisms of action of thymoquinone, the major active ingredient of *Nigella sativa*, Crit. Rev. Food Sci. Nutr. 57 (18) (2017) 3911–3928.
[91] M.A. Khan, H.C. Chen, M. Tania, D.Z. Zhang, Anticancer activities of *Nigella sativa* (black cumin), Afr. J. Tradit. Complement. Altern. Med. AJTCAM 8 (5 Suppl) (2011) 226–232.

[92] E.Z. Dajani, T.G. Shahwan, N.E. Dajani, Overview of the preclinical pharmacological properties of *Nigella sativa* (black seeds): a complementary drug with historical and clinical significance, J. Physiol. Pharmacol. Off. J. Pol. Physiol. Soc. 67 (6) (2016) 801–817.

[93] O.M. Zlatian, M.V. Comănescu, A.F. Roşu, L. Roşu, M. Cruce, A.E. Găman, C. D. Călina, V. Sfredel, Histochemical and immunohistochemical evidence of tumor heterogeneity in colorectal cancer, Rom. J. Morphol. Embryol. = Rev. Roum. De. Morphol. Et. Embryol. 56 (1) (2015) 175–181.

[94] R.M. Soliman, R.A. Abdel Salam, B.G. Eid, A. Khayyat, T. Neamatallah, M. K. Mesbah, G.M. Hadad, Stability study of thymoquinone, carvacrol and thymol using HPLC-UV and LC-ESI-MS, Acta Pharm. 70 (3) (2020) 325–342.