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Efficacy and Safety of Thymoquinone on Suppression of Tumor Growth in N-butyl-N-(4-hydroxybutyl)-Nitrosamine -Induced Bladder Cancer in Rats

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Abstract

Bladder cancer is one of the most common genitourinary malignancies worldwide, the therapeutic approaches for suppression of urothelial tumor and progression toward distant metastasis are not convincing. The present study was conducted to explore the curative effect and safety of THQ in N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) induced bladder cancer in rats.

Twenty-two male Wister albino rats were allocated into three groups **G1**; served as control (n=6) and received only the vehicle. **G2**: carcinogen group (n=6); animals had 0.05% BBN in drinking water for nine weeks. **G3**: Treatment group (n=10); THQ was administered at a dose of 50mg/Kg/day orally one week prior to the last exposure of BBN and continued till week 20. The bladder wall of all the animals was examined macroscopically for assessment of the number and the surface area of the lesions using Image J software program. Histopathological evaluation was also done for abnormal morphological alterations. Serum was analyzed for measurement of total oxidant status (TOS) and nuclear factor kappa- κB (NF-κB), transforming growth factor beta-1 (TGF-β1) as well as vascular endothelial growth factor (VEGF). Safety of THQ in respect of hematological, liver, and renal function was also investigated. Bladder lesions in the THQ-treated group was reduced versus the carcinogen group. Histopathological findings in THQ-treated group demonstrated a significant improvement in the abnormal morphological growth in bladder, the TOS, NF-kβ, TGF-β, VEGF were mitigated non-significantly in the treatment group. The safety profile of THQ showed no significant deleterious effect on hematological, liver and renal function parameters with no signs of toxicity. In conclusion, THQ exerted an ameliorative effect on abnormal morphological growth in the urothelium in a rat model of bladder cancer. This might be due to antioxidant and anti-inflammatory properties of THQ. These data suggested that THQ may be effective and safe in ameliorating urinary bladder carcinogenesis.

Key words: **Urothelial cancer, Therapeutic trial, BBN, Thymoquinone, Adverse effects**

فعالية وسلامة الثيموكوينون في قمع نمو الورم في سرطان المثانة المستحدث بواسطة ن-بيوتل –ن-٤-هيدروكسي بيوتل النيتروزامين (BBN) في الجرذان

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الخلاصة

سرطان المثانة هو واحد من الأورام الخبيثة للجهاز البولي التناسلي الأكثر شيوعا في جميع أنحاء العالم، والأساليب العلاجية الموجودة لقمع الورم البولي التناسلي والتقدم نحو ورم خبيث ليست على مستوى المطلوب. وقد أجريت هذه الدراسة لاستكشاف التأثير العلاجي وسلامة الثيموكوينون في قمع نمو الورم في سرطان المثانة المستحدث بواسطة ن-بيوتل –ن-٤-هيدروكسي بيوتل النيتروزامين (BBN) في الجرذان. تم تقسيم اثنان وعشرين جرذ الويستار الى ثلاث مجموعات: مجموعة السيطرة السلبية (العدد=٦): تلقت الماء يوميا طوال فترة الدراسة. المجموعة السرطانية (العدد=٦): تلقت مادة ن-بيوتل –ن-٤-هيدروكسي بيوتل النيتروزامين (BBN) بنسبة ٠,٠٥٪ في مياه الشرب لمدة تسع أسابيع. المجموعة المعالجة بالثيموكوينون (العدد=١٠)، تم علاجهم بالثيموكوينون ٥٠ملغ/كجم/يوم عن طريق الفم ابتدأ من أسبوع ما قبل الأخير من تناول مادة BBN و استمرت حتى الأسبوع العشرين. تم فحص جدار المثانة لجميع الحيوانات لتقييم عدد و مساحة الاصابات (Lesions) الموجودة في الجدار باستخدام برنامج Image J ، كما تم إجراء التقييم النسيجي للتغيرات المورفولوجية غير الطبيعية . تم تحليل مصل الدم لقياس حالة الأكسدة الكلية (TOS) و NF-KB و TGF-B1 و VEGF . كما تم التحقيق في سلامة الثيموكوينون من أي آثار جانبية سلبية على المؤشرات الدموية ووظيفة الكبد والكلي مع عدم وجود علامات السمية.

كشفت الدراسة بان عدد الإصابات الموجودة في جدار المثانة أصبحت أقل عددا في المجموعة المعالجة مقارنة بالمجموعة السرطانية. و كذلك لوحظ تحسن ملحوظ في النتائج النسيجية المرضية و TOS و NF-KB و TGF-B1 و VEGF في المجموعة العلاجية . و اشارت نتائج بيانات السلامة لاستخدام الثيموكوينون بانه لا يوجد آثار جانبية سلبية على الدم و على وظيفة و الكبد و الكلى. نجح الثيموكوينون في تثبيط الخلايا السرطانية بشكل ملحوظ. يمكن أن تكون الاليات الأساسية لفعالية المادة هي تأثيرها القيم كمضاد للالتهابات والأكسدة. تشير هذه النتائج الى ان الثيموكوينون يمكن ان يكون مادة فعالة وآمنة في تحسن سرطان المثانة.

الكلمات المفتاحية: سرطان المثانة، تجربة علاجية، BBN ، الثيموكوينون، آثار جانبية سلبية

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Introduction

Bladder cancer is one of the most common genitourinary malignancies worldwide, of which the majority (about 90%) of bladder cancer cases are confirmed to be urothelial cell carcinoma [(1)]. About 70% of patients with bladder cancer have non-muscle-invasive bladder cancer and the rest have muscle-invasive bladder cancer; approximately one-third of patients with non-muscle invasive bladder cancer will progress to muscular-invasive bladder cancer. Urothelial carcinoma is characterized by high recurrence and distant metastasis, which are the main reasons for its poor prognosis [(2)]. Various therapeutic approaches including immunotherapy, chemotherapy, radiotherapy and surgical operations for treating bladder cancer have been attempted in the last decades and no one is without limitation, and recently no ideal treatment options are available [(3, 4)]. Therefore, it is necessary to further explore a novel therapeutic option with safer profile for the treatment of urothelial carcinoma.

Furthermore, many previous work have investigated the potential chemopreventive effects of various phytochemicals in ameliorating the initiation and progression of urothelial cancer [(5,6)]. However, the therapeutic and curative effect of a promising pharmacological option such as thymoquinone (THQ) for suppression of tumor lesions and progression toward distant metastasis need to be elucidated. Therefore, attempts to find a novel strategy for attenuation and suppression of tumor growth is of advance need.

Thymoquinone (THQ) has been demonstrated to have antioxidant, anti-inflammatory and immunomodulatory activities [(7-9)], it is regarded as a promising agent for treating cancer. The previous researches have demonstrated that THQ has antitumor effects on various kinds of cancers, such as breast cancer, osteosarcoma, glioblastoma and bladder cancer [(10-14)]. Furthermore, an *in vivo* study has reported that THQ has a potential to inhibit the growth of tumors [(15)]. Previous studies have also confirmed that THQ can inhibit the invasion and metastasis in various types of cancers [(16, 14)]. However, few studies have examined the potential effect of THQ on tumor growth and progression in bladder carcinogenesis and no study has been carried out on the suppressive and curative efficacy of THQ in bladder cancer. Therefore, the aim of the study was to investigate the potential suppressive and curative ability of THQ on N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)-induced urinary bladder carcinogenesis in albino rats and to assess the safety profile of this bioactive substance in the study.

Material and Methods

Animals and induction of bladder cancer

The experiment was carried out on 22 six to eight weeks -old male Wistar albino rats of 160-180 g weight, they were obtained from the animal house of University of Sulaimani. The induction of bladder cancer was performed by using 0.05% N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN); BBN was from TCI Tokyo Chemical Industry Co., Ltd., Japan with a concentration of 97.4% it was diluted to 0.05% in water and administered *ad libitum*. The animals had also free access to food. The induction period has been decided based on the previous studies with few modification [(5,17)]. The drinking water has been changed every day and water bottles were covered with aluminum foil to prevent light exposure [(18)]. Ultrasound sonography for the bladders was used to detect bladder wall thickness and urothelial changes in order to confirm the tumor induction. This procedure was carried out under light anesthesia in horizontal and sagittal sections using a 12-MHz linear transducer [(19)].

Experimental design and ethical consideration

The rats were acclimatized in plastic cages at temperature 25 ± 1 °C and relative humidity 50 to 70%. with a 12/12-hour circadian cycle for one week. The rats were randomly classified into three groups:

G1: Control Group (n=6) the animals were received only tap water during the study.

G2: BBN Carcinogen Group (n=6) animals had 0.05% BBN in drinking water for 9 weeks for tumor induction. Then this group was left solely with tap water till the end of the experiment (week 20) for cancer progression and tumor growth.

G3: Treatment Group (n=10) BBN-Thymoquinone Group, BBN 0.05% was given in the drinking water for 9 weeks for tumor induction. Thymoquinone 50mg/Kg (from Glentham Life Sciences- United Kingdom) was given orally one week prior to the last dose of BBN (i.e week 8) and continue to be given till the end of the experimental period which was 20 weeks. All the animals in this period consumed their normal diet and water plus their treatment. All the procedures and animals handling were according to the guidelines approved by the University of Sulaimani. The study protocol was approved by the College of Pharmacy Ethics and Registration Committee. The protocol of this study was obtained from a previous work with few modifications [(17)] and the dose of THQ was selected based on the previous studies [(20,21)]. The experimental protocol is summarized in Figure 1.

At the end of 20th weeks, the rats were sacrificed to investigate the therapeutic approach of THQ on macroscopical, microscopical and biochemical parameters.

Blood collection and harvesting of bladder

Blood: At the end of week 20, blood was collected from all the rats. At first, the animals were hypnotized with chloroform inhalation, then the blood immediately obtained from the heart and then collected in a tube without anticoagulant for serum collection and EDTA tube for complete blood count (CBC) test.

Organs: after animal scarification and before removal of the bladder, the bladder inflated with 10% formaldehyde through urethral catheterization, then a ligature (suturing silk) tied around the bladder neck to keep appropriate distention and to ensure clear photographing after fixation (Figure 2), then the bladder was removed and kept in 10% formaldehyde. After 24 hours, it was cut off and photographed for gross assessment.

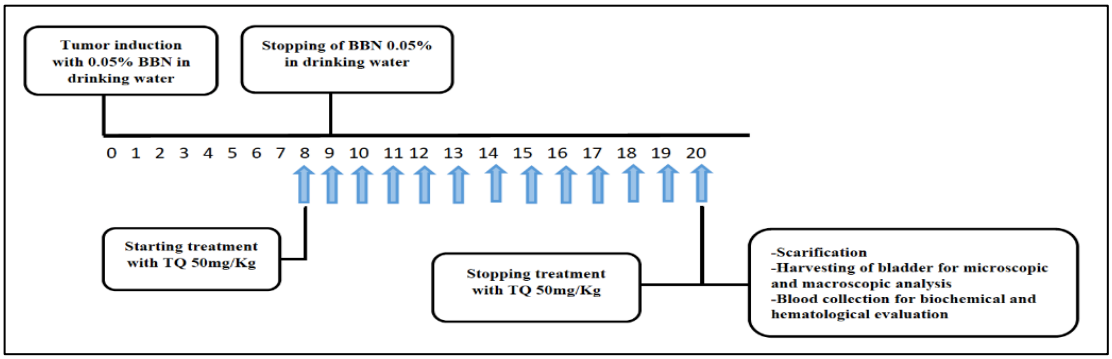

Figure 1. Schematic diagram of the study design

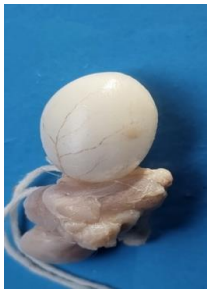

Figure 2. Filling the bladder with 10% of formaldehyde via catheterization for pre-fixation of tumor lesion followed by ligation of bladder neck with a thread to prevent emptying of bladder.

Outcome measures

Quantitative macroscopic evaluation

For macroscopic quantitative analysis, the number of lesions per each rat and the total number of the lesions per each group were recorded and the surface area of the lesions were calculated by using Image J software program.

Microscopic evaluation

Following macroscopic assessment, histopathological evaluation was also done for abnormal morphological alterations. Pre-fixed tissues were gently dehydrated and embedded in melted paraffin blocks. With the aid of semi-automated rotary microtome, paraffinized tissues were sectioned to 5 µm. Then after, the tissue sections were fixed on glass slides, dried on hot plate then deparaffinized and cleaned with two steps xylene changes, later on placed in a hot oven at 50°C for 30 minutes. Finally, bladder sections were stained with Harris's hematoxylin and eosin solution, cleaned with xylene and cover slipped, then examined and evaluated quantitatively under light microscope.

Redox Status, inflammation, proliferation and angiogenesis assessment

Serum was separated after blood collection by centrifugation at 5000 r.p.m. for 10 minutes, then stored at -70ºC. Serum levels of the following biomarkers were measured: Total Oxidant Status (TOS), Nuclear factor kappa-B (NF-KB), Transforming growth factor beta-1 (TGF- β1) and Vascular endothelial growth factor (VEGF), using Rat ELISA kit from Bioassay Technology laboratory, Harborne Road, Birmingham, England respectively.

Safety profile for thymoquinone

The hematological indices, renal and liver function test were measured to evaluate the safety of THQ in the studied dose.

Statistical analysis

The analyses were performed with the help of GraphPad prism 9.4.0 software. Ordinal quantitative variables were expressed as means and standard of error of mean (SEM). The differences between the groups were determined by One way ANOVA test. In all cases, the level of statistical significance was set at $p<0.05$.

Results

Confirmation of bladder urothelial changes

The bladder urothelial changes and formation of the lesions were confirmed by ultrasound imaging at week 20. Figure 1 demonstrates a representative rat with changes in the bladder wall.

A. Ultrasound view

B. Macroscopic view

Figure 3. Ultrasound imaging (Sonography) of the bladder of a representative rat with cancer induction at week 20. A. Ultrasound view shows the projection of the lesions from the bladder wall. B. Macroscopic view shows large visible lesions.

Macroscopic evaluation of the urothelial lesions

The formaldehyde pre-fixed bladders were opened and photographed then the images were analyzed by Image J software program (Figure 4). The percentage of rats with tumor in each group, a number of lesions per each rat, and the surface area of the lesions per rat were then evaluated (Table 1). The incidence of lesions formation in THQ-treated group was lower (50%) in comparison with carcinogen group (83%). The mean number of lesions per rat in THQ-treated group (1.5± 0.54) was also lower than in carcinogen group (4±1.16). The surface area of lesions per rat was remarkably smaller in THQ-treated group (2.19±0.8) than in carcinogen group (16.28±11.39) but the difference was statistically not significant.

Table 1. Macroscopic evaluation of the urothelial lesions

	Control (n=6)	Carcinogen (n=6)	Treatment (n=10)
Rats with lesions (%) in each group	0	83% (5 in 6)	50% (5 in 10)
Number of Lesions per rat (mean±SEM)	0	4±1.16*	1.5± 0.54*
Area of the lesions (mm^2) per rat (mean±SEM)	0	16.28±11.39*	2.19±0.8*

Values are expressed as percentage and mean±SEM (Standard error of mean). Mann Whitney test was used to determine the differences between different groups. * indicates statistically significant difference with control group (p-value <0.05)

Macroscopic appearance of the urothelial wall in the representative animals of the three group is shown in figure 4. The control group which received only tap water shows a normal appearance of the bladder, however the bladders of the carcinogen group show multiple large visible lesions with clear hypervascularization an indicator of neoangiogenesis. In THQ-treated group there is a remarkable reduction in the number of lesions

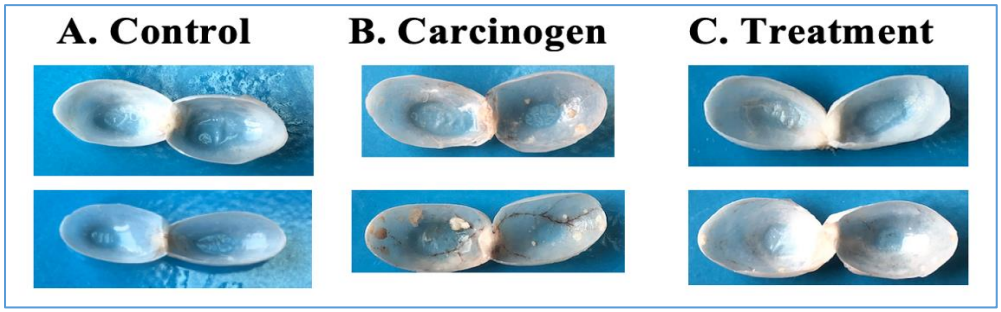

Figure 4. Macroscopic evaluation of the bladders at the end of week 20. Represented by two bladders in each group. A. Control group; received only tap water. B. Carcinogen group; had 0.05% BBN for 9 weeks. C. Treatment; received 50mg/kg thymoquinone orally for 12 weeks starting from week 8 to 20.

Microscopic histomorphology analysis

For microscopic examination, morphometric changes such as hyperplasia, dysplasia, papillary or nodular hyperplasia and infiltrative growth together with atypia and neoplastic transformation of urothelium was observed. Table 2 shows the lesion scoring and grading system of bladder sections. Control group shows a normal urothelial pattern in comparison with carcinogen group. At week 20 data analysis in the THQ-Treated group that received 50mg/Kg of THQ for 12 weeks starting from week 8 to the end of trial demonstrate significant improvement in the abnormal morphological growth in the urothelium, evident by noticeable reduction in the hyperplasic area, high grade dysplasia, suppression of the papillary growth as well as infiltrative and carcinoma in situ growth in comparison with N-Butyl-N-(4-hydroxybutyl) nitrosamine (BBN) for 9 weeks, and show highly significant preneoplastic glandular-like growth (NG), manifested by large acinar-like proliferation, together with infiltration growth (IG) toward the lamina propria (Figure 5).

Table 2. Histological quantitative evaluation of urothelium neoplastic changes at week 20

Experimental Groups N=10	Hyperplasia# (Mean%)**	Dysplasia# (Mean%)**	Papillary growth* (Mean %)**	Infiltrative growth* (Mean %)**	Carcinoma in situ (Cis)* (Mean %)**	Lesion Scoring (0 -100%)	Lesion Grading	Neoplastic Grading
G1†-Control	2.56 % ^{A}	0.38 % ^{A}	0.59 % ^{A}	0.08 % ^{A}	0.04 % ^{A}	10-50 %	normal	No atypia
G2-Carcinogen	75.48 % ^{E}	81.43 % ^{E}	77.81 % ^{E}	84.25 % ^{E}	92.46 % ^{E}	75-100 %	critical grade	Invasive
G3-Treatment	44.92 % ^{C}	47.33 % ^{C}	38.69 % ^{C}	32.51 % ^{C}	46.72 % ^{C}	25-50 %	medium grade	Dysplasia

Notes: *Area of papillary like growth, infiltrative growth, and carcinoma in situ were estimated in (µm). #Hyperplasia and dysplasia were estimated in calculated counted cell number. **Each value represents mean percentage of (n=10). Statistical comparison among groups: Mean values with different capital letters have significant differences at ($P < 0.05$). †: **G1:** Control group received only the vehicle **G2:** Carcinogen group received N-Butyl-N-(4-hydroxybutyl) nitrosamine (BBN) for 9 weeks. **G3:** Treatment group received BBN for 9 weeks and thymoquinone 50mg/Kg for 12 weeks staring from week 8 to 20.

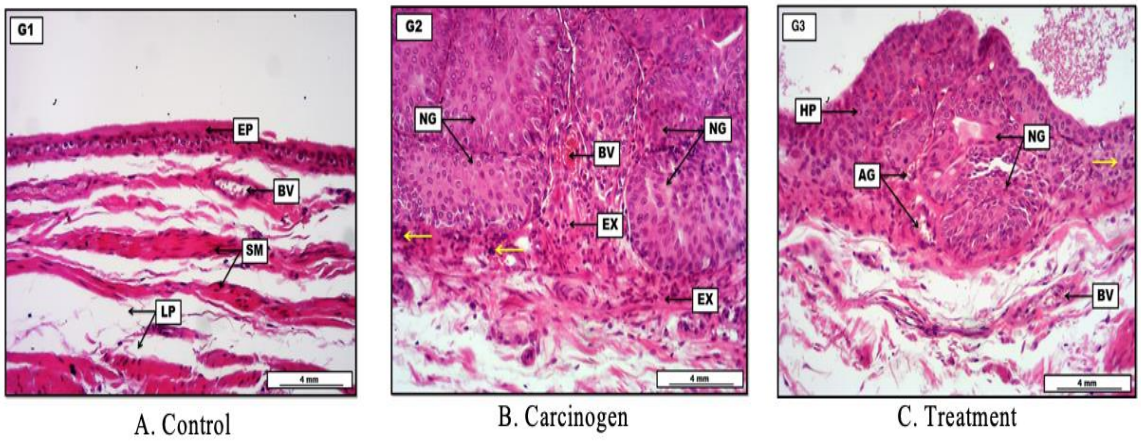

Figure 5. Photomicrograph of urinary bladder at week 20 from groups; G1: Control group received tap water for 20 weeks, reveal no obvious morphological changes, apparent by typically aligned urothelial epithelium (EP), together with classically appeared lamina propria (LP) with the presence of mildly congested blood vessel (BV) with normally appeared smooth muscle fibers (SM). G2: Carcinogen group received N-Butyl-N-(4-hydroxybutyl) nitrosamine (BBN) for 9 weeks, display extremely significant preneoplastic glandular-like pattern in growth (NG), demonstrated by large acinar-like proliferation, with many areas of inflammatory exudates (EX) in between, the section also shows infiltration of mononuclear inflammatory cells (yellow arrows). Moreover, there is moderate significant vascular congestion within the submucosa (BV). G3: Treatment group received BBN for 9 weeks with thymoquinone 50mg/kg for 12 weeks staring from week 8 to 20, shows significant low-grade epithelial hyperplasia (HP) together with clear and significant preneoplastic glandular pattern of growth (NG), with evidence of new angiogenesis (AG) and low-grade dysplasia (yellow arrow). Presence of some congested blood vessels (BV). H&E. Scale bar: 4 mm.

Serum level of total oxidant status (TOS)

The TOS was significantly increased in carcinogen group in comparison with the control group. TOS was mitigated in treatment group (50mg/Kg of THQ for 12 weeks) in non-significant manner in comparison with carcinogen group (Figure 6).

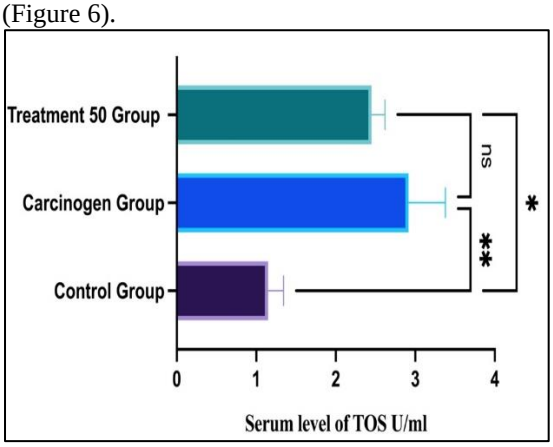

Figure 6. Serum level of Total oxidant status (TOS) in different groups, Nuclear Factor Kappa B (NF-κβ) in different groups at week 12. Values are presented as mean ± SEM. * P-value <0.05, ** P-value <0.01 are statistically significant.

Serum level of nuclear factor kappa β (NF-kβ)

At week 20, serum level of NF-kβ significantly elevated in carcinogen group and treatment group in compare to the control group (Figure 7).

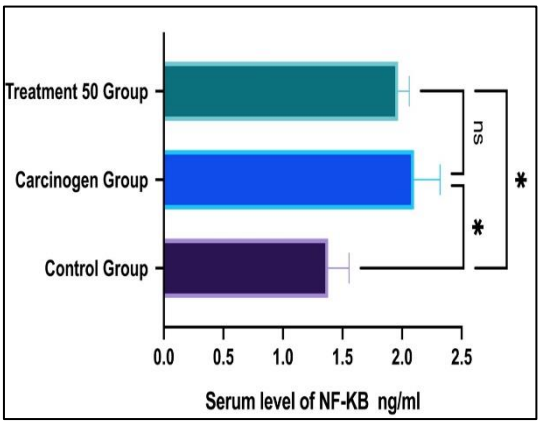

Figure 7 Serum level of NF-kβ in different groups, Nuclear Factor Kappa B (NF-κβ) in different groups at week 20. Values are presented as mean ± SEM. * P-value <0.05, are statistically significant.

Serum level of transforming growth factor beta (TGF-β)

The elevation of the serum level of TGF-β and VEGF in carcinogen group was significant compared to the control group. We observed that THQ as a treatment approach (starting from week 8 to week 20) mitigated TGF-β and VEGF in non-significant manner compare to BBN treated group.

Table 3 Effect of thymoquinone on serum level of Transforming growth factor-Beta 1 (TGF-β1) and vascular endothelial growth factor (VEGF) in different groups at week 20

Biomarkers	Control Group	Carcinogen Group	Treatment 50 Group
Serum Transforming growth factor-Beta 1 (TGF-β1) mean±SEM	95.12±13.97	165±10.79*	126.6±9.896
Serum vascular endothelial growth factor (VEGF) mean±SEM	189.2±23.46	296.9±42.28*	254.7±15.09

Values are presented as mean ± SEM. * P-value <0.05 are statistically significant with the control.

Effects of thymoquinone on hematological parameter

Different hematological indices have been measured at the end of the experiment to investigate the safety profile of THQ in the chemically induced-bladder cancer model. Our finding reveals non-significant differences between all groups (Table 4).

Table 4. Effects of thymoquinone on hematological parameter

Parameters	Control	Carcinogen	Treatment
WBC	11.77±2.708	9.278±2.201	12.16±1.479
Neutrpohil %	27.3±0.6083	24±1.058	25.51±1.844
Neutrpohil	3.047±1.005	2.203±0.4826	3.163+0.4464
Lymphcoyte	5.743±1.754	6.205±1.409	6.67±0.4687
Lymphcoyte %	59.38±4.844	67.48±0.9911	65.55±2.124
Monocyte	0.9125±0.2844	0.7025±0.2593	1.031±0.213
Monocyte %	7.2±1.97	7.05±0.9836	8.033±0.7219
Eosinophil	0.0975±0.02955	0.1375±0.07181	0.05667±0.0115
Eosinophil %	0.4667±0.08819	1.15±0.5204	0.45±0.06798
RBC	7.44±0.316	7.44±0.1377	7.458±0.1539
HGB	14.7±0.5583	14.85±0.1658	14.96±0.3755
HCT	40.58±1.409	39.6±0.5958	40.43±1.042
PLT	595.3±48.45	645±21.84	594.6±58.04
PCT	0.4497±0.03824	0.4555±0.01867	0.4639±0.01307
MPV	8.075±0.5437	7.05±0.06455	7.542±0.1323
PDW	9.533±0.1202	8.2±0.2082	9.775±0.3852
P-LCR	20.93±1.135	17.53±0.5603	20.9±1.036

Values are presented as mean ± SEM.

Effects of thymoquinone on renal and liver function

After measuring different renal and hepatic parameters of the animals at week 20 of the study, the data revealed that the effect of THQ on renal and liver function parameters compare to the negative group and BBN group was not significant except serum creatinine was significantly elevated in THQ-treated group in compare to control group. (Table 5).

Table 5. Effects of thymoquinone on renal and liver function

Parameters	Control	Carcinogen	Treatment
Albumin	3.563±0.06957	4.09±0.1449	3.968±0.06859
ALP	160.7±14.33	134.4±7.264	151.9±9.557
ALT	37.4±3.762	36.98±4.14	42.16±2.825
AST	150.1±11.77	158.4±15.78	170±8.235
Bilirubin	0.075±0.00497	0.0702±0.007749	0.0959±0.00869
BUN	16.33±0.8819	14±0.5477	15.22±0.7027
Serum Creatinine	0.29±0.01581	0.382±0.0102	0.405±0.01863***
Uric acid	1.4±0.1826	1.7±0.1958	1.478±0.1128
Urea	35.43±1.964	29.76±1.096	33.92±1.63

Values are presented as mean ± SEM. *** highly significant with the control group

Discussion

Bladder cancer remains one of the most frequent malignancies that endanger people's health worldwide. The progression and metastasis after tumor initiation sometime is inevitable. Various preclinical; *in vitro* and *in vivo* studies have been conducted to mitigate the development and progression of BC. Additionally, many preventive strategies have been investigated to reduce morbidity and the recurrence rate especially in high risk people. Various phytochemicals have been then investigated during the recent years as potential chemopreventive agents for BC [22]. Thymoquinone has emerged as a highly promising option in the recent years for the prevention of numerous cancers [16,23,24]. To our knowledge this is the first study that investigates the curative efficacy of THQ in a chemically-induced bladder cancer. The principal findings of this study were 1) BBN was successfully induced the morphometric changes in rat's bladder. 2) THQ exerted a potential curative efficacy in amelioration of abnormal morphological growth in the urothelial wall in compare to BBN-carcinogen group. 3) Total oxidative status of the BBN-carcinogen group was significantly enhanced, this was attenuated in THQ-treatment group, but the difference was not significant. 4) Non-significant reduction has been observed in the serum level of inflammation, proliferation and angiogenesis biomarkers in THQ-treatment group. 5) A favorable safety profile for THQ has been adopted based on biochemical and hematological parameters.

To be mentioned BBN is an organ specific BC inducer. Exposure to BBN may occur due to inhalation of N-nitroso compound found in tobacco smoking. Furthermore, environmental and infectious metabolite partly accounts for BBN toxicity [25]. BBN has been used in animal studies to induce bladder cancer, several factors interfere with the urothelial lesion induced by BBN, such as: time of exposure, concentration of BBN, strain and species [26].

Various models have been developed for induction of BC in experimental animals [27]. The present study used BBN model because it resembles the human disease in histological characteristics, BBN can be given orally in drinking water and it is metabolized into an urothelium carcinogenic agent, namely N-butyl-N-(3-carboxypropyl)-nitrosamine, which has direct carcinogenic effect on urinary bladder epithelium [25]. In the present study, all rats exposed to BBN in carcinogen group developed urothelial lesions with many pathological conditions which is characterized by hyperplasia, dysplasia, papillary or nodular hyperplasia and infiltrative growth together with atypia and neoplastic transformation of urothelium. In the quantitative evaluation of urothelial wall, there was a significant reduction in the mean percentage of the above mentioned abnormal morphological changes in THQ-treated animals in which 50mg/Kg/BW of THQ was given for 12 weeks starting one week prior to BBN administration. The improvement in the histopathological pattern of urinary bladder with the intake of THQ are in line with other experimental studies in which THQ was tried for suppression and treatment of neoplastic changes as evidence in colon [28] , renal [29] and other cancer models [30].

A treatment trial with THQ 50mg/Kg mitigated oxidative stress but the difference was statistically not significant in comparison with BBN only treated group. This finding is in tune with the several previous studies in which THQ displayed antioxidant potential against carcinogens-induced oxidative injury via various mechanism including up-regulation of anti-oxidant and cytoprotective enzyme levels such as superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase that can combat the oxidative stress-induced tumorigenesis [31]. The present study further explored the other contributing factors associated with BBN-induced changes in the urothelial wall including inflammation, proliferation and formation of new blood vessels, therefore the effect of THQ on the most relevant biomarkers such as NF-kβ and TGF-β and VEGF

were evaluated. Thymoquinone as a suppressive approach in post- tumor induction exerted a non-significant reduction in all these biomarkers. This finding is inconsistent with other previous studies that documented an obvious anti-inflammatory property of THQ [32,33] as THQ was effective in suppressing NF-KB-mediated activity and preventing cancer progression in colon cancer [34,35]. Additionally, THQ exerted antitumor effect on bladder cancer in other experimental *in vitro* and *in vivo* models via down regulation of NF-Kβ and its regulated pathway such as X-linked inhibitor of apoptosis protein (XIAP) [36]. However, the non-significant effect in this study might be related to the dose and duration of THQ administration which might be not sufficient to exert anti-inflammatory action in this model.

The effect of THQ on TGF-β was also showed a statistically non-significant changes in this biomarker which inconsistent with the efficacy of THQ in downregulating TGF-β/Smad2/3 as an essential intracellular signaling in prostate cancer cell line [16]. Furthermore, another study on hepatocellular carcinoma *in vivo* model, confirmed the ability of THQ in inducing apoptosis via downregulating Bcl2 and transforming growth factor-beta 1 (TGF- β1) alongside with upregulating TNF-related apoptosis-inducing ligand (TRAIL) and caspase-3 [37]. Transforming growth factor-β1 (TGF-β1) is a multifunctional cytokine that regulates cell proliferation, growth, differentiation as well as cell movement [38]. In the present study, a non-significant change has been observed in the serum level of TGF- β1 after 12 weeks of the administration of 50mg/Kg TQ, it seems that TQ has a modulatory effect on TGF-β1 signaling pathway for suppression of tumor growth and progression of cancer.

To investigate the inhibitory effect of THQ on tumor-angiogenesis, the present study evaluated VEGF as a biomarker of formation of new blood vessels. The results showed that THQ exerted a non-significant alteration in the serum level of this biomarker, this was not comparable with the previous *in vivo* study which was conducted by Al-Trad *et al*. to investigate the role of 50mg/Kg orally administered THQ against the development of benign prostatic hyperplasia in six Wistar rats for two weeks. The finding of that study emphasized on the THQ's ability in the amelioration of VEGF level in the treated group as well as prostate weight/body weight ratio, epithelial hyperplasia, serum interleukin 6 (IL-6) levels, and the expressions of TGF-1[39].

Additionally, another study conducted on osteosarcoma to investigate the antitumor and anti-angiogenic effects of THQ *in vitro* and *in vivo*, stated that THQ treatment significantly suppresses angiogenesis via downregulating the expression of VEGF in these models of osteosarcoma [40].

The safety profile of anticancer agents is one area that needs attention since it frequently puts some efficacious anticancer medications in jeopardy. As per current understanding, THQ is recognized as a harmless phytochemical compound that potentially have many beneficial effects including cardioprotective [41], nephroprotective [42], hepatoprotective [43], neuroprotective [44], gastroprotective [45] effects in various animals and human models. As anticipated for this class of medications, THQ therapy in our trial was associated with a safe haematological and biochemical data, including a safe value for the functions of kidney and liver which is in agreement with what would be expected for this compound. The most recent clinical study has reported a favorable safety profile for THQ in a phase I clinical trial on healthy individuals [46].

In the present study, THQ shows no significant effect on hematological profile. Our finding has been supported by Ong *et al* [47] who reported that THQ administration for 28 days to male BALB/c mice resulted in no significant changes in hematological parameters.

Our study reveals that the effect of THQ as a treatment approach on liver enzymes (ALT, AST and ALP) was not significant in compare to control and carcinogen group. Several *in vivo* studies demonstrated hepatoprotective effects of THQ via attenuation of reactive oxygen species and mitigation of inflammation [43,48].

The nephroprotective potential of THQ against kidney damage was confirmed in several animal based experimental model of kidney injury [42,49]. It has been found that oxidative stress is a leading cause of renal damage, for this reason anti-oxidant effect of THQ is considered the main mechanism by which THQ protect kidney against nephrotoxic chemicals such as sodium nitrite [50] At week 20 no significant elevation has been noted in all renal function parameters THQ-treated group compared to the other groups, except serum creatinine level in the treatment group with 50mg/Kg for 12 weeks was significantly higher than the control group in which the rats received only the vehicle, this result is not in agreement with the findings of Guo *et al*. in which THQ significantly reduced the serum creatinine and blood urea nitrogen levels and alleviating the sepsis-induced acute kidney injury [51]. The finding of the current study might be related to inability of THQ in the studied dose and duration in reversing the abnormal alteration in renal cells which has been happened by BBN-carcinogen agent. Further studies are required to elucidate the effect of THQ in different doses and different models of carcinogenesis.

Conclusion

In conclusion, THQ exerted an ameliorative effect on abnormal morphological growth in the urothelium in a rat model of bladder

cancer. This might be due to antioxidant and anti-inflammatory properties of THQ. This finding is promising in implementation of THQ alone or as a safe adjuvant therapy for the treatment of the development of bladder cancer.

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Ethics Statements

All the experimental procedures on the animals were conducted in accordance with the institutional animal care guidelines.

Author Contribution

Karmand S. Hamaamin Qadir, conducted literature review, performed the experiment, assisted in data analysis, contributed to the manuscript writing, reviewed and edited the manuscript. Bushra H. Marouf, conceived and designed the study, supervised the research, wrote the first draft of the manuscript, statistically analyzed the data, reviewed, revised and approved the final manuscript. All authors discussed the results and contributed to the final manuscript.

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References

1. Klotz L, Brausi MA. World Urologic Oncology Federation Bladder Cancer Prevention Program: A global initiative. Urol Oncol Semin Orig Investig. 2015;33(1):25–9.
2. Alfred Witjes J, Lebret T, Compérat EM, Cowan NC, De Santis M, Bruins HM, et al. Updated 2016 EAU Guidelines on Muscle-invasive and Metastatic Bladder Cancer. Eur Urol. 2017;71(3):462–75.
3. Williams SK, Hoenig DM, Ghavamian R, Soloway M. Intravesical therapy for bladder cancer. Expert Opin Pharmacother. 2010;11(6):947–58.
4. Lou K, Feng S, Zhang G, Zou J, Zou X. Prevention and Treatment of Side Effects of Immunotherapy for Bladder Cancer. Front Oncol. 2022;12:879391.
5. Parada B, Reis F, Cerejo R, Garrido P, Sereno J, Xavier-Cunha M, et al. Omega-3 Fatty Acids Inhibit Tumor Growth in a Rat Model of Bladder Cancer. BioMed Res Int. 2013;2013:1–11.
6. Grubbs CJ, Lubet RA, Koki AT, Leahy KM, Masferrer JL, Steele VE, et al. Celecoxib inhibits N-butyl-N-(4-hydroxybutyl)-nitrosamine-induced urinary bladder cancers in male B6D2F1 mice and female Fischer-344 rats. Cancer Res. 2000;60(20):5599–602.
7. Mahmoud YK, Abdelrazek HMA. Cancer: Thymoquinone antioxidant/pro-oxidant effect as potential anticancer remedy. Biomed Pharmacother . 2019; 115:108783.
8. Kohandel Z, Farkhondeh T, Aschner M, Samarghandian S. Anti-inflammatory effects of thymoquinone and its protective effects against several diseases. Biomed Pharmacother. 2021; 138:111492.
9. Majdalawieh AF, Fayyad MW. Immunomodulatory and anti-inflammatory action of Nigella sativa and thymoquinone: A comprehensive review. Int Immunopharmacol. 2015;28(1):295–304.
10. Rajput S, Kumar BNP, Sarkar S, Das S, Azab B, Santhekadur PK, et al. Targeted Apoptotic Effects of Thymoquinone and Tamoxifen on XIAP Mediated Akt Regulation in Breast Cancer. Bauer JA, editor. PLoS ONE. 2013;8(4):e61342.
11. Gurung RL, Lim SN, Khaw AK, Soon JFF, Shenoy K, Mohamed Ali S, et al. Thymoquinone Induces Telomere Shortening, DNA Damage and Apoptosis in Human Glioblastoma Cells. Santos J, editor. PLoS ONE. 2010;5(8):e12124.
12. Ahmad A, Mishra RK, Vyawahare A, Kumar A, Rehman MU, Qamar W, et al. Thymoquinone (2-Isopropyl-5-methyl-1, 4-benzoquinone) as a chemopreventive/anticancer agent: Chemistry and biological effects. Saudi Pharm J. 2019;27(8):1113–26.
13. Almajali B, Al-Jamal HAN, Taib WRW, Ismail I, Johan MF, Doolaanea AA, et al. Thymoquinone, as a Novel Therapeutic Candidate of Cancers. Pharm Basel Switz. 2021 ;14(4):369.
14. Zhang M, Du H, Wang L, Yue Y, Zhang P, Huang Z, et al. Thymoquinone suppresses invasion and metastasis in bladder cancer cells by reversing EMT through the Wnt/β-catenin signaling pathway. Chem Biol Interact. 2020;320:109022.
15. Woo CC, Hsu A, Kumar AP, Sethi G, Tan KHB. Thymoquinone Inhibits Tumor Growth and Induces Apoptosis in a Breast Cancer Xenograft Mouse Model: The Role of p38 MAPK and ROS. Cheng JQ, editor. PLoS ONE. 2013;8(10):e75356.
16. Kou B, Liu W, Zhao W, Duan P, Yang Y, Yi Q, et al. Thymoquinone inhibits epithelial-mesenchymal transition in prostate cancer cells by negatively regulating the TGF-β/Smad2/3 signaling pathway. Oncol Rep. 2017; Available from: http://www.spandidos-publications.com/10 .3892 /or.2017.6012
17. Fernandes J, Sereno J, Garrido P, Parada B, Cunha M, Reis F, et al. Inhibition of Bladder

Tumor Growth by Chitooligosaccharides in an Experimental Carcinogenesis Model. Mar Drugs. 2012;10(12):2661–75.

18. Amponsa VO, Shuman L, Ellis J, Wang E, Walter V, Owens RG, et al. Carcinogen-induced bladder cancer in the FVB mouse strain is associated with glandular differentiation and increased Cd274/Pdl-1 expression. Am J Clin Exp Urol. 2019;7(3):139–52.

19. Dornelas CA, Fechine-Jamacaru FV, Albuquerque IL, Magalhães HIF, Souza AJS de, Alves LA, et al. Chemoprevention with green propolis green propolis extracted in L-lysine versus carcinogenesis promotion with L-lysine in N-Butyl-N-[4-hydroxybutyl] nitrosamine (BBN) induced rat bladder cancer. Acta Cir Bras. 2012;27(2):185–92.

20. Gali-Muhtasib H, Ocker M, Kuester D, Krueger S, El-Hajj Z, Diestel A, et al. Thymoquinone reduces mouse colon tumor cell invasion and inhibits tumor growth in murine colon cancer models. J Cell Mol Med. 2007;12(1):330–42.

21. Asaduzzaman Khan Md, Tania M, Fu S, Fu J. Thymoquinone, as an anticancer molecule: from basic research to clinical investigation. Oncotarget. 2017;8(31):51907–19.

22. Koh YC, Ho CT, Pan MH. Recent advances in cancer chemoprevention with phytochemicals. J Food Drug Anal. 2020 ;28(1):14–37.

23. Relles D, Chipitsyna GI, Gong Q, Yeo CJ, Arafat HA. 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.

24. Imran M, Rauf A, Khan IA, Shahbaz M, Qaisrani TB, Fatmawati S, et al. Thymoquinone: A novel strategy to combat cancer: A review. Biomed Pharmacother. 2018;106:390–402.

25. Vasconcelos-Nóbrega C, Colaço A, Lopes C, Oliveira PA. Review: BBN as an urothelial carcinogen. Vivo Athens Greece. 2012(4):727–39.

26. Arentsen HC, Hendricksen K, Oosterwijk E, Witjes JA. Experimental rat bladder urothelial cell carcinoma models. World J Urol. 2009;27(3):313–7.

27. John BA, Said N. Insights from animal models of bladder cancer: recent advances, challenges, and opportunities. Oncotarget. 2017;8(34):57766–81.

28. Jrah-Harzallah H, Ben-Hadj-Khalifa S, Almawi WY, Maaloul A, Houas Z, Mahjoub T. Effect of thymoquinone on 1,2-dimethyl-hydrazine-induced oxidative stress during initiation and promotion of colon carcinogenesis. Eur J Cancer. 2013;49(5):1127–35.

29. Zhang Y, Fan Y, Huang S, Wang G, Han R, Lei F, et al. Thymoquinone inhibits the metastasis of renal cell cancer cells by inducing autophagy via AMPK/mTOR signaling pathway. Cancer Sci. 2018;109(12):3865–73.

30. Majdalawieh AF, Fayyad MW, Nasrallah GK. Anti-cancer properties and mechanisms of action of thymoquinone, the major active ingredient of Nigella sativa. Crit Rev Food Sci Nutr. 2017;57(18):3911–28.

31. Kassab RB, El-Hennamy RE. The role of thymoquinone as a potent antioxidant in ameliorating the neurotoxic effect of sodium arsenate in female rat. Egypt J Basic Appl Sci. 2017;4(3):160–7.

32. Woo CC, Kumar AP, Sethi G, Tan KHB. Thymoquinone: Potential cure for inflammatory disorders and cancer. Biochem Pharmacol. 2012;83(4):443–51.

33. Hossen MJ, Yang WS, Kim D, Aravinthan A, Kim JH, Cho JY. Thymoquinone: An IRAK1 inhibitor with in vivo and in vitro anti-inflammatory activities. Sci Rep. 2017;7:42995.

34. Chen MC, Lee NH, Hsu HH, Ho TJ, Tu CC, Chen RJ, et al. Inhibition of NF-κB and metastasis in irinotecan (CPT-11)-resistant LoVo colon cancer cells by thymoquinone via JNK and p38: TQ Inhibits NF-κB and Metastasis in CPT-11-R Cells Via JNK and p38. Environ Toxicol. 2017;32(2):669–78.

35. Alshyarba M, Otifi H, Al Fayi M, A Dera A, Rajagopalan P. Thymoquinone inhibits IL-7-induced tumor progression and metastatic invasion in prostate cancer cells by attenuating matrix metalloproteinase activity and Akt/NF-κB signaling. Biotechnol Appl Biochem. 2020;bab.2062.

36. Mu H qi, Yang S, Wang Y jun, Chen Y he. [Role of NF-κB in the anti-tumor effect of thymoquinone on bladder cancer]. Zhonghua Yi Xue Za Zhi. 2012;92(6):392–6.

37. Helmy SA, El-Mesery M, El-Karef A, Eissa LA, El Gayar AM. Thymoquinone upregulates TRAIL/TRAILR2 expression and attenuates hepatocellular carcinoma in vivo model. Life Sci. 2019;233:116673.

38. Kajdaniuk D, Marek B, Borgiel-Marek H, Kos-Kudła B. Transforming growth factor β1 (TGFβ1) in physiology and pathology. Endokrynol Pol. 2013;64(5):384–96.

39. Al-Trad B, Al-Zoubi M, Qar J, Al-Batayneh K, Hussien E, Muhaidat R, et al. Inhibitory Effect of Thymoquinone on Testosterone-Induced Benign Prostatic Hyperplasia in Wistar Rats: Amelioration of benign prostatic hyperplasia by thymoquinone. Phytother Res. 2017;31(12):1910–5.

40. Peng L, Liu A, Shen Y, Xu HZ, Yang SZ, Ying XZ, et al. Antitumor and anti-angiogenesis effects of thymoquinone on osteosarcoma

through the NF-κB pathway. Oncol Rep. 2013 ;29(2):571–8.

41. Bocsan IC, Pop RM, Sabin O, Sarkandy E, Boarescu PM, Roșian ȘH, et al. Comparative Protective Effect of Nigella sativa Oil and Vitis vinifera Seed Oil in an Experimental Model of Isoproterenol-Induced Acute Myocardial Ischemia in Rats. Molecules. 2021;26(11):3221.

42. Saleem U, Ahmad B, Rehman K, Mahmood S, Alam M, Erum A. Nephro-protective effect of vitamin C and Nigella sativa oil on gentamicin associated nephrotoxicity in rabbits. Pak J Pharm Sci. 2012;25(4):727–30.

43. El-Far AH, Korshom MA, Mandour AA, El-Bessoumy AA, El-Sayed YS. Hepatoprotective efficacy of Nigella sativa seeds dietary supplementation against lead acetate-induced oxidative damage in rabbit – Purification and characterization of glutathione peroxidase. Biomed Pharmacother. 2017;89:711–8.

44. Farkhondeh T, Samarghandian S, Shahri AMP, Samini F. The Neuroprotective Effects of Thymoquinone: A Review. Dose-Response. 2018;16(2):155932581876145.

45. Ahmad S, Najmi A, Kaundal M, Akhtar M. Gastroprotective Effect of Thymoquinone on Water Immersion Restraint Stress Induced Ulceration in Rats. Drug Res. 2017;67(06):366–72.

46. Thomas JV, Mohan ME, Prabhakaran P, Das S S, Maliakel B, I.M. K. A phase I clinical trial to evaluate the safety of thymoquinone-rich black cumin oil (BlaQmax®) on healthy subjects: Randomized, double-blinded, placebo-controlled prospective study. Toxicol Rep. 2022;9:999–1007.

47. Ong YS, Saiful Yazan L, Ng WK, Noordin MM, Sapuan S, Foo JB, et al. Acute and subacute toxicity profiles of thymoquinone-loaded nanostructured lipid carrier in BALB/c mice. Int J Nanomedicine. 2016;11:5905–15.

48. Nili-Ahmadabadi A, Tavakoli F, Hasanzadeh G, Rahimi H, Sabzevari O. Protective effect of pretreatment with thymoquinone against Aflatoxin B(1) induced liver toxicity in mice. Daru J Fac Pharm Tehran Univ Med Sci. 2011;19(4):282–7.

49. Samarghandian S, Azimi-Nezhad M, Mehrad-Majd H, Mirhafez SR. Thymoquinone Ameliorates Acute Renal Failure in Gentamicin-Treated Adult Male Rats. Pharmacology. 2015;96(3–4):112–7.

50. Elsherbiny NM, Maysarah NM, El-Sherbiny M, Al-Gayyar MM. Renal protective effects of thymoquinone against sodium nitrite-induced chronic toxicity in rats: Impact on inflammation and apoptosis. Life Sci. 2017;180:1–8.

51. Guo LP, Liu SX, Yang Q, Liu HY, Xu LL, Hao YH, et al. Effect of Thymoquinone on Acute Kidney Injury Induced by Sepsis in BALB/c Mice. BioMed Res Int. 2020;2020:1–7.

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