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# Thymoquinone inhibits cell proliferation through regulation of G1/S phase cell cycle transition in N-nitrosodiethylamine-induced experimental rat hepatocellular carcinoma

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## HIGHLIGHTS

- TQ inhibits cell proliferation.
- TQ down regulates the expression of proteins controlling the G1/S phase of cell cycle.
- TQ treatment greatly reduced liver injury markers and tumor markers.
- Treatment with TQ was greatly reduced the hyper AgNORs expression in NDEA induced hepatocellular carcinoma.

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## ABSTRACT

Dysregulated cell proliferation and tumorigenesis is frequently encountered in several cancers including hepatocellular carcinogenesis (HCC). Thus, agents that inhibit cell proliferation and restrain hepatic tumorigenesis through cell cycle regulation have a beneficial effect in the treatment of hepatocellular carcinogenesis. The present study was aimed to investigate the efficacy of thymoquinone (TQ), an active compound derived from the medicinal plant *Nigella sativa*, on N-nitrosodiethylamine (NDEA) [0.01% in drinking water for 16 weeks]-induced hepatocarcinogenesis in experimental rats. After experimental period, the hepatic nodules, liver injury markers and tumor markers levels were substantially increased in NDEA induced liver tumors in rats. However, TQ (20 mg/kg body weight) treatment greatly reduced liver injury markers and decreased tumor markers and prevented hepatic nodule formation and reduced tumor multiplicity in NDEA induced hepatic cancer bearing rats and this was evident from argyrophilic nucleolar organizer region (AgNORs) staining. Moreover, the uncontrolled cell proliferation was assessed by specific cell proliferative markers [proliferating cell nuclear antigen (PCNA) and Ki67] by immunofluorescence, immunoblot and analysis of mRNA expression. Simultaneously, we assessed the activity of TQ on G1/S phase cell cycle regulation with specific cell cycle proteins ($p21^{WAF1/CIP1}$, CDK4, Cyclin D1 and Cyclin E) by immunoprecipitation in experimental rats. Treatment with TQ significantly reduced the detrimental alterations by abrogating cell proliferation, which strongly induced G1/S arrest in cell cycle transition. In conclusion, our results suggest that TQ has a potent anti proliferative activity by regulating the G1/S phase cell cycle transition and exhibit a beneficial role in the treatment of HCC.



## 1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancies of the liver and the third leading cause of cancer death worldwide (Tsai and Chung, 2010). More than 80% of the new cases are detected mostly in developing countries of Asia and Africa. However, the incidence rate is 2–3 times higher in developing countries, and globally it has become a fastest growing cause of

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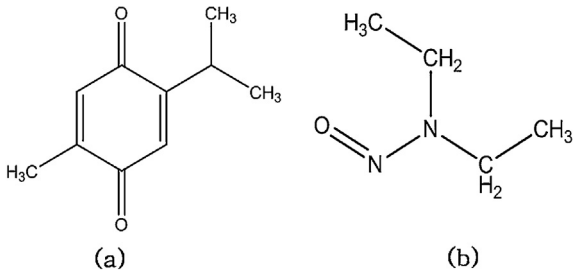


**Fig. 1.** Chemical structure of thymoquinone (a) and N-nitrosodiethylamine (b).

cancer death (Jemal et al., 2010; Sohal and Sun, 2011; Thomas et al., 2011). The vast majority of HCC cases are attributed to cirrhotic liver associated with viral hepatitis (B and C), alcohol, obesity, iron overload, and mainly due to dietary carcinogens, including aflatoxins and nitrosamines (Perz et al., 2006).

N-nitrosodiethylamine (NDEA) (Fig. 1b) is widespread in the environment, and it is present in foods, beverages, tobacco smoke, agricultural chemicals, cosmetics, and industrial pollution. These are the major risk factors of liver diseases, and their endogenous formations causes a wide range of tumors, and are hazardous to human health NDEA is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animal models (Perz et al., 2006; Brown, 1999; Reh and Fajen, 1996; Smith et al., 1997, 2001). It is known to cause perturbations in the nuclear enzymes involved in DNA repair/replication (Ramakrishnan et al., 2006). The reactive oxygen species (ROS) formation occurs during the metabolic biotransformation of NDEA and leads to carcinogenesis by upregulation of biochemical, intracellular signaling pathways, and gene expression. One of the most promising applications in the emerging field of cancer treatment is to discover the adjuvant or palliative therapy for solid tumors with negligible cytotoxicity to the normal cells. Hence, the identification of novel compound preferentially from plant origin have been shown to be promising chemotherapeutic agent. Thus bioactive compound provide a novel opportunity to improve the existing standard of care for HCC and other cancers (Newman, 2008).

Thymoquinone (TQ) (Fig. 1a) is a major bioactive ingredient isolated from *Nigella sativa* and has been reported for its anti-inflammatory, antioxidant, and anti-neoplastic effects both *in vitro* and *in vivo* (Gurung et al., 2010; Gali-Muhtasib et al., 2006; Li et al., 2010). Moreover, TQ could act as a free radical, superoxide radical scavenger and conserving the activity of various antioxidant enzymes. In recent studies, anti-proliferative and therapeutic effect of TQ has been reported against a wide variety of cancer, including breast cancer, ovarian cancer (Shoieb et al., 2003), colorectal cancer (Gali-Muhtasib et al., 2004), pancreatic cancer (Worthen et al., 1998), osteosarcoma (Roepke et al., 2007), leukemia (El-Mahdy et al., 2005), fibrosarcoma and lung cancer (Kaseb et al., 2007), and squamous cell carcinoma (Das et al., 2012). Recently, Li et al. have reported that TQ inhibits cell proliferation and induces apoptosis in multiple myeloma cells (Li et al., 2010). We have previously reported that anticancer activity of TQ in mouse neuroblastoma (Neuro-2a) cells through caspase-3 by down regulation of XIAP (Paramasivam et al., 2012). In animal models, TQ inhibited the forestomach tumors and enhanced the anti-tumor activity in pancreatic cancer (Salem, 2005). The exact mechanisms of TQ in inhibiting cell proliferation and how it restrained the tumor growth are yet to be studied.

Cell cycle is regulated by protein complexes composed of cyclins and cyclin-dependent kinases (CDKs). Aberrant activation of cyclins and CDKs has been observed in numerous primary tumors which correlate with failure of cell cycle control which resulting in uncontrolled proliferation (King and Cidlowski, 1998). Several studies

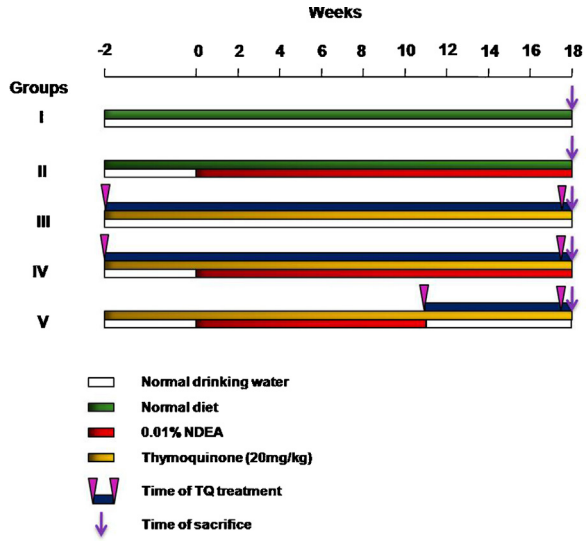


**Fig. 2.** Schematic representation of the experimental protocol involving NDEA exposed experimental rat hepatocellular carcinogenesis.

documented that dysregulation of cell cycle causes uncontrolled proliferation and contribute to the tumor growth and neoplastic transformation in various malignancies, including breast, osteosarcoma, colon cancer and hepatocellular carcinoma (Woo et al., 2012). Therefore, inhibition of G1/S phase cell cycle transition by suppressing the Cyclin D1/CDK4 protein may block the development of carcinogenesis. This present study was attempted to explore the antiproliferative efficacy of TQ through cell cycle arrest at G1/S phase against NDEA induced HCC.

## 2. Materials and methods

### 2.1. Experimental animals and diet

Pathogen-free adult male Wistar strain albino rats (*Rattus norvegicus*) weighing about 150–180 g were obtained from The King Institute, Chennai, India. The animals were acclimatized to standard laboratory conditions including a controlled environment at 24 ± 1 °C and 50 ± 10% relative humidity with the alternating 12:12-h dark–light cycle for 1 week before the beginning of the study. The animals were fed a commercial pelletted diet (M/s Hindustan foods Ltd., Bangalore, India) and provided drinking water *ad libitum*. All the experiments were designed and conducted according to the ethical norms approved by Institutional animal ethics committee guidelines (IAEC No. 01/086/09) regulated by the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

### 2.2. Chemicals and their sources

NDEA and TQ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies for PCNA, Ki67, $p21^{WAF1/CIP1}$, CDK4, Cyclin D1 and Cyclin E were purchased from Santa Cruz biotech, USA. All other chemicals used were purchased from Bio Basic (USA), Genei (Bangalore) and Sisco Research Laboratories (Mumbai) India.

### 2.3. Experimental design

A schematic representation of the experimental protocol is given in Fig. 2. Following an acclimatization period of 1 week with standard basal diet, rats were randomly divided into five groups (10 animals per group) based on a power analysis. Group I is normal control; Group II is NDEA alone (Induced); Group III is TQ alone; Group IV is Preventive treatment (Pre treatment = TQ + NDEA); Group V is Curative/Post treatment (NDEA + TQ); Groups II and IV were given 0.01% NDEA in drinking water for 16 weeks and Group V for 11 weeks to induce HCC. TQ at a concentration of 20 mg/kg body weight was administered orally for weekly 3 alternative days to rats of Group IV, for two weeks before the experiment, and to rats of Group V for last 5 weeks of the experiment as per duration of treatment schedule. Food and water intake and behavioral changes were monitored every 2 weeks. At the end of the experimental period rats were fasted overnight and the body weight of each rat was monitored. Then the rats were anaesthetized and sacrificed by decapitation. The relative liver weight was calculated as the percentage ratio of liver weight to the body weight and stored at −20 °C for further analysis.