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THE ANTITUMOR ACTIVITY OF THYMOQUINONE AND THYMOHYDROQUINONE *IN VITRO* AND *IN VIVO*

*S. Ivankovic[1], R. Stojkovic[1,] *, M. Jukic[2], M. Milos[2], M. Milos[3] M. Jurin[1]*
[1]*Division of Molecular Medicine, Rudjer Boskovic Institute, 10000 Zagreb, Croatia*
[2]*Department of Biochemistry and Food chemistry, Faculty of Chemical Technology, 21000 Split, Croatia*
[3]*Faculty of Sciences, Department of Physical Chemistry, University of Geneva, 1204 Geneva, Switzerland*

***Aim*: The aim of the study was to investigate antitumor activity of thymoquinone (TQ) and thymohydroquinone (THQ) *in vitro* and *in vivo*. *Materials and Methods:* In the *in vitro* experiments, L929 mouse fibroblasts and two tumor cell lines (squamous cell carcinoma (SCC VII) and fibrosarcoma (FsaR)) were used. The cells were cultured with 0.1 or 0.01 mg/ml TQ or THQ for 24 h, and cytotoxicity assay was performed with the use of crystal violet staining technique. For *in vivo* antitumor efficiency evaluation of new compounds two murine tumor models (fibrosarcoma (FsaR) and squamous cell carcinoma (SCC VII)) were used. The used dose was equal for both substances. Antitumor effect of 4 intratumoral injections of TQ and THQ at the dose of 5 mg/kg was evaluated by comparison of tumor growth kinetics between treated and control animals. *Results: In vitro* study showed that TQ and THQ exhibit statistically significant cytotoxic activity ($p < 0.01$). The cytotoxic activity was dose dependent and more expressed against tumor cells than against L929 fibroblasts. The result of antitumor activities of TQ and THQ *in vivo* reached TGI = 52% and it was statistically significant ($p < 0.05$). *Conclusion*: The results indicate that THQ antitumor activity may be improved with further dose increase of the investigated substance.**
***Key Words*: thymoquinone, thymohydroquinone, antitumor activity, *in vitro, in vivo,* mice.**

In recent years, the use of newly synthesised small molecules, combinations of therapeutic modalities or naturally occurring agents to prevent the development or recurrence of cancer has become widely accepted [1–5]. *Nigella sativa Linn*, commonly known as black seed or black cumin, is an annual plant belonging to the *Ranunculaceae* family. The herb has been traditionally used in the Indian subcontinent, Arabian countries and Europe for culinary and medicinal purposes [6–7]. *Nigella sativa* seeds contain diverse but well-characterized chemical components, which include essential oils, proteins, alkaloids and saponins [8–9].

The chemotherapeutic and chemoprotective effects of *N. sativa* extract may be due to quinones that include thymoquinone (TQ), dithymoquinone (DIM) and thymohydroquinone (THQ) that are present in the oil of this seed. TQ suppresses benzo(a)pyrene (BP)-induced forestomach tumor formation [10] and tumor formation in DMBA-initiated, TPA-promoted mouse skin [11], as well as a chemopreventive agent at the early stage of skin tumorigenesis. The antineoplastic activity of TQ may be attributed to its inhibitory effects on cancer cell growth and its capability of inducing apoptosis in cancer cells [12]. The TQ enhances the antitumor activity of cisplatin and ifosfamide [13], and improves their therapeutic index [14]. Among its wide-spectrum pharmacologic activities, TQ has antineoplastic activity against various tumor cells [11, 15–16]. DIM also contributes to the chemotherapeutic effects of *N. sativa*. *In vitro* studies indicate that DIM is equally as cytotoxic as TQ to several parental human tumor cell lines and their corresponding multidrug resistant (MDR) variants [16]. TQ and THQ exhibit limited specific inhibitory effect on COX-2 [17]. Although the antitumor activity of TQ is relatively well characterised *in vitro* the data about its antitumor activity *in vivo* are very limited. Till today data about *in vitro* and *in vivo* antitumor activities of thymohydroquinone are still unknown. Hence, the purpose of this study was to explore *in vitro* and *in vivo* antitumor activities of TQ and THQ.

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*Correspondence: Fax: +385-14560-992
E-mail: stojkov@irb.hr
Abbreviations used: CGI – cell growth inhibition; DIM – dithymoquinone; RTG – relative tumor growth; TGI – tumor growth inhibition; THQ – thymohydroquinone; TQ – thymoquinone.

MATERIALS AND METHODS

Chemistry. TQ and THQ were prepared in our laboratory by the methods of Kremers et al. [18] and Stolow et al. [19]. NMR spectroscopy (Bruker AMX-400) confirmed the structures of compounds.

TQ: ^{1}H NMR ($CDCl_3$, 400 MHz): δ (multiplicity, number of protons, assignment) 1.13-1.23 (d, 6H, 2xCH_3), 2.18 (s, 3H, CH_3), 3.12-3.15 (m, 1H, CH), 4.37 (b, 2H, OH), 6.55 (s, 1H, iPr=CH-), 6.64 (s, 1H, Me=CH-).

^{13}C NMR ($CDCl_3$, 100 MHz): 15.38, 21.41, 26.45, 77.15, 130.37, 133.85, 145.29, 154.96, 187.45, 188.64.

THQ: ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.13-1.23 (d, 6H, 2xCH_3), 2.18 (s, 3H, CH_3), 3.12-3.15 15 (m, 1H, CH), 4.37 (b, 2H, OH), 6.55 (s, 1H, iPr=CH-), 6.64 (s, 1H, Me=CH-).

^{13}C NMR ($CDCl_3$, 100 MHz): 15.39, 22.68, 26.85, 77.15, 113.03, 117.69, 121.61, 133.10, 146.27, 147.72.

Cell lines and the evaluation of cytotoxicity. In the *in vitro* experiments L929 mouse fibroblasts and two tumor cell lines (SCC VII and FsaR) were used. Mentioned cell lines belong to the same genetic background, and their ability to produce solid tumors in syngeneic mice make them a very good model for antitumor activity investigations of substances *in vitro* and *in vivo*. All cell lines were grown in a RPMI 1640 medium supplemented with 10% foetal calf serum (FCS) in a humidified atmosphere of 5% CO_2 in air at 37 °C.

For each experiment, L929, SCC VII and FsaR cells were harvested and plated on 96-well microtiter plates

(100 µl/well) at an initial concentration of approximately 1 x 10^4 cells/100 µl. When the cells reached confluence (usually 24 h), the old cultured medium was replaced with a fresh one and than TQ or THQ were added to the final concentrations of 0.1 or 0.01 mg ml^{-1}. Control cells were incubated in a medium without addition of tested substances. The cells were incubated for 24 h, and then the treated and untreated cells were fixed by the addition of 1% glutaraldehyde solution for 15 min, washed with deionised water and dried in air. After that the cells were stained with 0.1% crystal violet for 20 min, then extensively washed with deionised water and allowed to dry overnight. The cells thus fixed were visualized using an inverted inverse microscope (Zeiss, Axiovert 35), equipped with the digital microscopy camera processed with the Viewfinder program. To obtain quantitative cytotoxicity data, the dye was extracted from the cells using a 0.2% solution of Triton X-100, and then absorbance was read at 595 nm (Anthos Microplate Reader HT3). Absorbance at the wave length of 590 nm is proportional to the number of surviving cells. Each experiment was made in quadruplicate and repeated three times. In addition to the cell growth inhibition (CGI) ratio was also calculated from the absorbance values by the formula: CGI = (C-T/C) x 100, where C is mean absorbance value of untreated (control) cells and T is mean absorbance value of treated cells.

Experimental animals. C3HHf/Bu Zgr/Hr male mice were used. Animals were 3 months old at the beginning of the experiments. Eight mice were used in each group per experiment. Each experiment was repeated twice. Mice were obtained from Rudjer Boskovic Institute's breeding colony. During experimental period 4 animals were kept per cage. Bottom of cage was covered with sawdust (Allspan®, Germany). Standard food for laboratory mice (4 RF 21 GLP Mmucedola srl, Italy) was used. All animals had access to food and water ad libitum. Animals were kept in conventional circumstances: light/dark rhythms 12/12 h, temperature 22 °C, and humidity 55%. All experiments were performed according to the ILAR Guide for the Care and Use of Laboratory Animals, Council Directive (86/609/EEC) and Croatian animal welfare law (NN 19/99).

Tumor models. For *in vivo* antitumor efficiency evaluation of new compounds two standard murine tumor models (fibrosarcoma (FsaR) and squamous cell carcinoma (SCC VII)) were used. Both tumor models belong to the same genetic background (originated from the C3H mice strain). SCC VII cells belong to the epithelial tumor of ectodermal origin, whereas FsaR cell line is of mesodermal origin, so that we can compare the tumors with the same genetic background and different histogenesis. Fibrosarcoma (FsaR), and squamous cell carcinoma (SCC VII) had been maintained in C3HHf/Bu Zgr/Hr mice. For the experimental purpose tumor cells (5 x 10^5 in 100 µl of RPMI) were transplanted subcutaneously into the thigh of the right leg using a tuberculin syringe and a 25-gauge needle. The viability of the cells was determined by Trypan blue dye exclusion test and it was over 95%.

Test substances and dosage. All substances were stored at +4 °C and freshly dissolved in distilled water immediately prior to injection (for each application). Three days after tumor cells inoculation TQ or THQ (5 mg/kg in a volume of 100 µl) were injected intratumourally for the first time. The application was repeated on days 4, 5 and 11 following tumor cells inoculation, so that the total received dose was 20 mg/kg. The chosen dose is one half of LD_{50} of more toxic substance (thymoquinone) applied i.p. as a single dose therapy [20]. The control group received on same days 100 µl of distilled water.

Tumor response. Tumor response (endpoint) was studied as a tumor growth delay study [21]. In order to get a tumor volume, three orthogonal diameters (A, B and C) of a growing tumor were measured with calliper on days 8, 11, 14, 17 and 21 after the tumor cell injection. The tumor volume (V) was calculated by the formula V = ABCπ/6. Antitumor effect was evaluated by a comparison of a tumor growth between treated and untreated (control) groups. The relative tumor growth (given in % of control) was calculated by the formula: RTG = (T/C) x 100, where C is mean tumor volume of control (untreated) group and T is mean tumor volume of treated groups. Tumor growth inhibition (TGI) was calculated from the RGI by formula 100-RGI %.

Statistical analysis. Statistical analyses were conducted by "Statistica for Windows 4.0" — Stats soft Inc. USA 1993. Obtained data were tested firstly by Shapiro-Wilks W-test, Levene's test for normality and homogeneity of variance, respectively. After that a one-way ANOVA test was applied to assess the overall difference among experimental groups and if ANOVA was significant than differences among the means were assessed by the Tukey multiple comparison test. Statistical significance was defined as $p < 0.05$.

RESULTS

Cytotoxic assay. The effect of thymoquinone and thymohydroquinone was tested on normal fibroblasts cell line (L929) and two tumor cell lines (SCC VII and FsaR). The effects of therapy with TQ and THQ on cells growth are shown on photomicrographs (Fig. 1). Data show that control cells were of high density with well-defined morphological characteristics of certain cultures. 24 h after addition of TQ or THQ noticeable changes were visible in the morphology and density of treated cells. Almost all treated cells became rounded and their number was reduced in comparison with the control cell culture. In order to quantify the toxicity of TQ and THQ a crystal violet assay was performed. The cytotoxic effects of 0.01 and 0.1 mg/ml of TQ and THQ on cells growth are shown in Fig. 2. In the higher concentration (0.1 mg/ml) both tested substances exhibited statistically significant cytotoxic activity against tumor cells (~ 87 and 92% for SCC VII and FsaR cells, respectively, $p < 0.001$). The cytotoxic activity was dose dependent and it was up to 44% for lower dose (0.01 mg/ml) and up to 92% for higher dose (0.1 mg/ml) of TQ against tumor cell lines . The antiproliferative ability of THQ was up to 17% for lower dose (0.01 mg/ml) and up to 92% for higher dose (0.1 mg/ml) against tumor cell lines. The results obtained with the use of "lower" dose of TQ were statistically significantly better

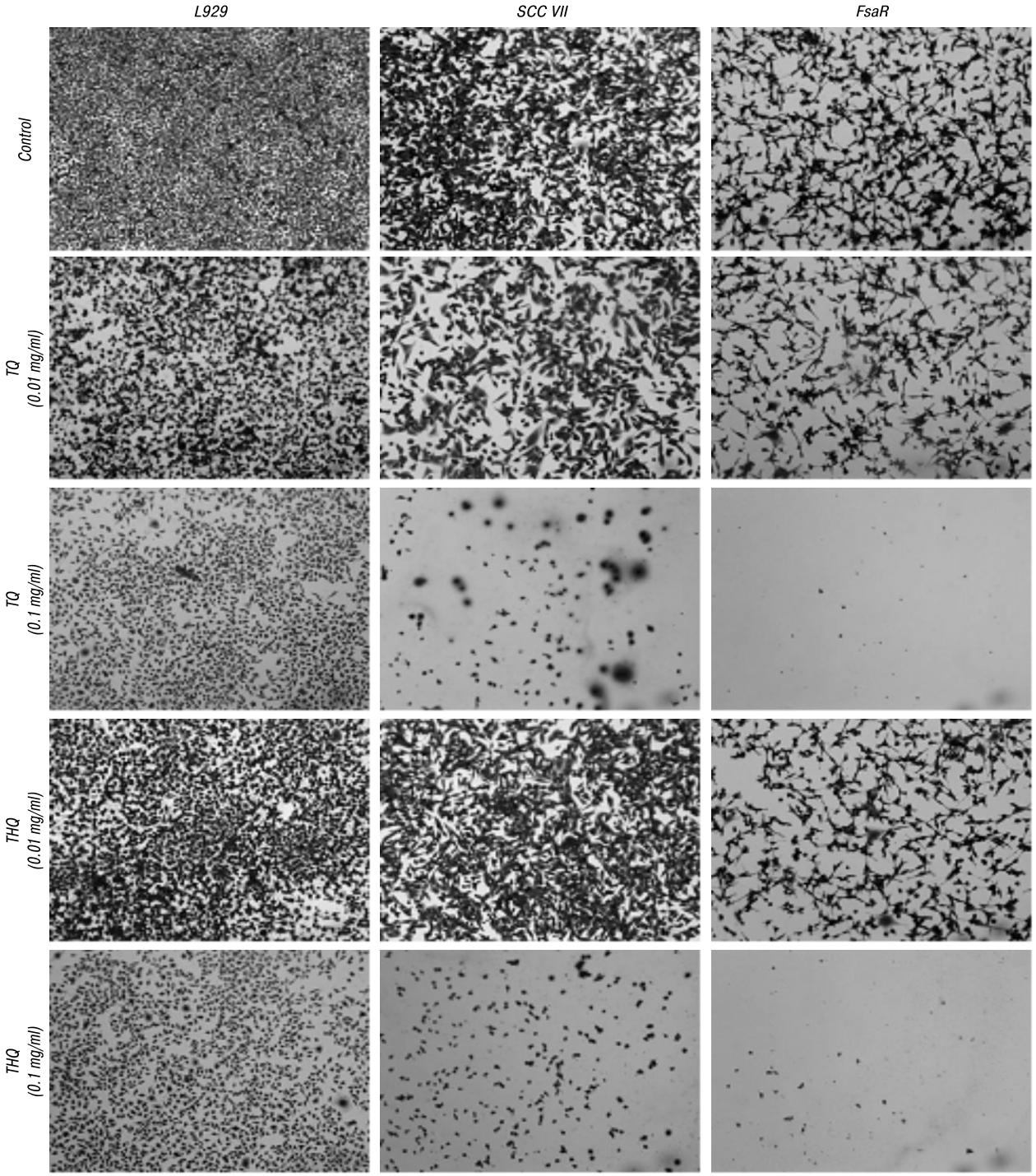

Fig. 1. Photomicrographs of fixed and crystal violet stained control and treated cells 24 h after the addition of 0.1 and 0.01 mg ml^{-1} of thymoquinone (TQ) or thymohydroquinone (THQ)

than those obtained with the use of "lower" dose of THQ. The effect of "lower" doses of TQ or THQ against normal mice fibroblasts (L929) was very similar (21–57%) to the antiproliferative effects against tumor cell lines. On the other hand the antitumor effect of "higher" doses of TQ or THQ was cell specific and it was statistically much more expressed against tumor cell (between 86% and 92%) than against normal fibroblasts (62–63%).

Antitumor activity evaluation in vivo. The goal of the *in vivo* experiments was to determine antitumor activity of TQ and THQ on two different murine tumor models (squamous cell carcinoma (SCC VII) and fibrosarcoma (FsaR)). From the Fig. 2, in which results of therapy with TQ and THQ are shown, it could be seen that both used substances exert very good antitumor activity against SCC VII and FsaR. The TGI value reached with the use of TQ against SCC VII was up to 52% and up to 43% against FsaR. Therapeutic effect (TGI value) achieved with the use of THQ was up to 49% against SCC VII and up to 33% against FsaR. Statistically significant difference in antitumor activity *in vivo* between TQ and THQ has not been found in this study.

DISCUSSION

Thymoquinone is a promising compound with significant *in vitro* and *in vivo* antitumor activities against different tumor

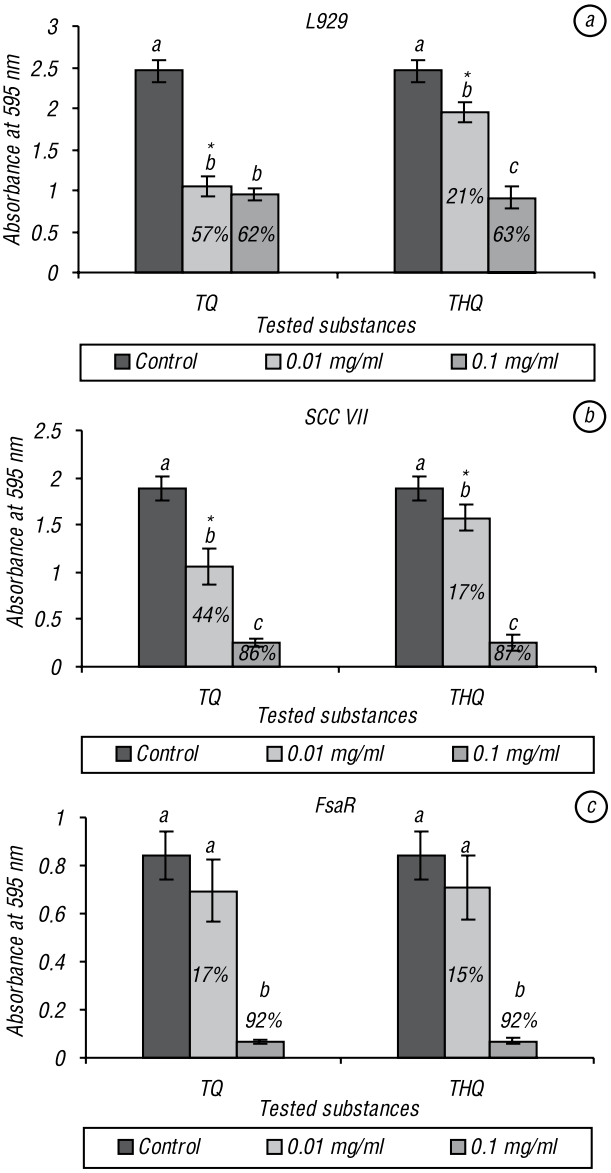

Fig. 2. Cytotoxic effects of 0.01 and 0.1 mg/ml of thymoquinone (TQ) and thymohydroquinone (THQ) on mouse fibroblast L929 (*a*), squamous cell carcinoma SCC VII (*b*) and fibrosarcoma FsaR (*c*) cell lines as evaluated by the crystal violet cytotoxic assay. Inside of each of the treated group bars the values of the cell growth inhibition (CGI %) was also presented. The asterisk (*) indicates statistically significant difference ($p < 0.01$) in activity between same concentrations of TQ and THQ on particular cell lines. Small letters a, b, c (for each tested compound on particular cell line) — bars that do not have common letters are significantly different, $p < 0.01$

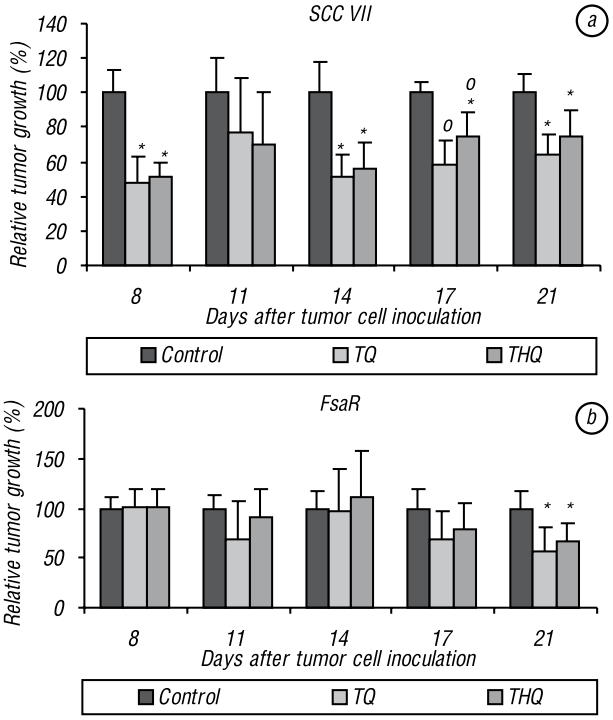

Fig. 3. Effects of application of TQ or THQ on the growth of mouse carcinoma SCC VII (*a*) and fibrosarcoma Fsar (*b*). The asterisk (*) indicates statistically significant difference ($p < 0.05$) in activity between treated and control group. The open circle (o) indicate statistically significant difference ($p < 0.05$) between tested substances

models [16, 22, 23]. However its mechanism(s) of action is still unknown. The present knowledge about antitumor activity of thymohydroquinone is very limited and till today there is no data about antitumor activity of THQ *in vivo*. This study was undertaken to demonstrate the antitumor effects of TQ and THQ *in vitro* and *in vivo* and revealed that both substances (TQ and THQ) exhibited good antiproliferative activity against tumor cells *in vitro* which is in agreement with literature findings [11, 15, 16, 22, 23]. Furthermore it shows that antiproliferative activity of TQ and THQ is dose dependent and more pronounced towards tumor cell lines *in vitro*. Similar observation of TQ inhibiting activity against human colon cancer cells (HTC-116) was observed pointing to the involvement of *p*53 dependent mechanisms [24]. These results as well as our presented results indicate that TQ triggers apoptosis in a dose dependent manner. Apoptosis induction by TQ was associated with several fold increase in mRNA expression of *p*53 and the downstream *p*53 target gene, *p*21WAF1 (oedema). A marked increase in *p*53 and *p*21WAF1 protein level and a significant inhibition of anti-apoptotic Bcdl-2 protein were found in TQ treated cell cultures. However, *p*53-null HCT-116 cells were less sensitive to TQ-induced growth arrest and apoptosis [24] what correspond to the reaction of FsaR cells (*p*53 wild type is less expressed) [25]. The role of *p*53 in TQ apoptotic action is supported by the data from literature pointing that co-cultivation of TQ and pifithrin-alpha, a specific inhibitor of *p*53, resulted in Bcl-2, *p*53 and *p*21WAF1 restored to untreated control levels. Further, THQ in a lower dose used was less effective than TQ against fibroblast and carcinoma cells what might correspond to its less pronounced toxicity *in vivo*, and there are no reports in literature about that compound's antitumor action. Possible action of thymoquinones on tumor angiogenesis is of further interest, too. Antitumor activity of TQ and THQ against used murine tumors *in vivo* was very promising, especially for THQ. Optimistic expectations are based on the fact that antitumor activity of THQ was as good as antitumor activity of TQ regardless of fact that used dose of THQ was two times less toxic than used dose of TQ (less than $\frac{1}{4}LD_{50}$ of THQ versus $\frac{1}{2}LD_{50}$ of TQ) [20]. The present study shows that the antiproliferative activity of tested substances is dose dependent and the future expectations were put on even better therapeutic results for THQ with further dose elevation.

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ПРОТИВООПУХОЛЕВЫЙ ЭФФЕКТ ТИМОХИНОНА И ТИМОГИДРОХИНОНА *IN VITRO* AND *IN VIVO*

***Цель*: исследовать противоопухолевую активность тимохинона (ТХ) и тимогидрохинона (ТГХ) *in vitro* и *in vivo*. *Материалы и методы:* в экспериментах *in vitro* использовали линию клеток фибробластов мыши L929 и две линии опухолевых клеток мыши (плоскоклеточной карциномы SCC VII и фибросаркомы FsaR). По достижении монослоя, клетки инкубировали с 0,1 или 0,01 мг/мл ТХ или ТГХ, после чего цитотоксический эффект определяли по включению кристаллического фиолетового. Противоопухолевый эффект 4-кратного внутриопухолевого введения ТХ или ТГХ в дозе 5 мг/кг *in vivo* в экспериментальных моделях FsaR и SCC VII определяли по кинетике опухолевого роста. *Результаты:* исследования *in vitro* показали, что ТХ и ТГХ обладают статистически значимой цитотоксической активностью ($p < 0,01$), причем таковая носит дозозависимый характер и в большей степени проявляется по отношению к линиям опухолевых клеток, чем фибробластов. Противоопухолевый эффект соединений статистически достоверный, ингибирование опухолевого роста достигало 52% ($p < 0,05$). *Выводы*: противоопухолевая активность ТГХ может быть повышена путем дальнейшего повышения дозы вещества.**

***Ключевые слова*: тимохинон, тимогидрохинон, противоопухолевая активность, *in vitro, in vivo,* мыши.**