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Research Article

Anti-inflammatory Effect of Seeds and Callus of *Nigella sativa* L. Extracts on Mix Glial Cells with Regard to Their Thymoquinone Content

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Abstract. Anti-inflammatory effect of the alcoholic extracts of *N. sativa* seeds and its callus on mix glial cells of rat with regard to their thymoquinone (TQ) content was investigated. Callus induction was achieved for explants of young leaf, stem, petiole, and root of *N. sativa* on solid Murashige and Skoog (MS) medium containing 2,4-D (1 mg/l) and kinetin (2.15 mg/l). TQ content of the alcoholic extracts was measured by HPLC. Total phenols were determined using Folin–Ciocalteu method and antioxidant power was estimated using FRAP tests. The mix glial cells, inflamed by lipopolysaccharide, were subjected to anti-inflammatory studies in the presence of various amounts of TQ and the alcoholic extracts. Viability of the cells and nitric oxide production were measured by MTT and Griess reagent, respectively. The leaf callus obtained the highest growth rate (115.4 mg/day) on MS medium containing 2,4-D (0.22 mg/l) and kinetin (2.15 mg/l). Analyses confirmed that TQ content of the callus of leaf was 12 times higher than that measured in the seeds extract. However, it decreased as the calli aged. Decrease in the TQ content of the callus was accompanied with an increase in its phenolic content and antioxidant ability. Studies on the inflamed rat mix glial cells revealed significant reduction in the nitric oxide production in the presence of 0.2 to 1.6 mg/ml of callus extract and 1.25 to 20 μl/ml of the seed extracts. However, the extent of the effects is modified assumingly due to the presence of the other existing substances in the extracts.

KEY WORDS: callus; glial cells; inflammation; *Nigella sativa* L.; thymoquinone.

INTRODUCTION

Inflammation is observed in most diseases and assumed to be a defense reaction designed to remove noxious agents (1). Neuro-inflammation is a significant feature of brain injuries and nervous system disorders such as multiple sclerosis and Alzheimer's disease. Glial cells including astrocyte and microglia are among the brain's important immune cells. Inflamed glial cells can exacerbate neurodegenerative diseases by producing and releasing neurotoxins such as reactive oxygen species and nitric oxide (NO) (2). It has been shown that NO participates in degeneration of oligodendrocytes in multiple sclerosis and neuronal death in Alzheimer's and Parkinson's diseases (3).

Recognition of medicines which can reduce inflammation in glial cells is one of the important current issues. Considering the side effects of synthetic medicines, the World Health Organization estimates that about 80% of people, at least in Asian and African countries, trust in traditional remedies more than chemical medicines. This is true especially for herbal drugs which are found in many modern medicinal formulations such as aspirin, quinine, and triphala (4).

Black-seed with scientific name of *Nigella sativa* L. is a member of *Ranunculaceae* family native to Southwest of Asia. *N. sativa* seed has been regarded as a blessing and healing food additive in the Islamic culture and has been introduced as a medicinal herb in Avicenna's famous book, Qanun (5). Black-seed has attracted the attention of contemporary scientists too. Chemical analyses have disclosed the existence of different vitamins (6), phospholipids (7), fatty acids (8), and ascorbic acid (9) in *N. sativa* seeds. But compounds such as dithymoquinone, thymoquinone (TQ), thymol, and carvacrol (Fig. 1) which are found in black-seed have come to special attention due to the anticipated pharmaceutical properties including analgesic (10), antioxidant (11,12), and anticancer properties (13). Anti-inflammatory effects of these compounds either pure or in the black-seed extracts have also been examined in different studies including rheumatoid arthritis in rat models (14), eicosanoid generation in leukocytes (15), allergic lung inflammation in a mouse model (16), carrageenan-induced paw edema (17), ulcerative colitis (18), mouse dendritic cells (19), allergic encephalomyelitis as an animal model for the human multiple sclerosis (20), nitric oxide production by murine macrophages (21), *etc.*

Since modern plant biotechnology provides scientists with plant cells and tissue cultures which can be employed for production of active natural compounds, there is increasing interest in studying the biochemical properties of proliferated plant cells under artificial conditions and comparing the results with those of native species. Literature review shows

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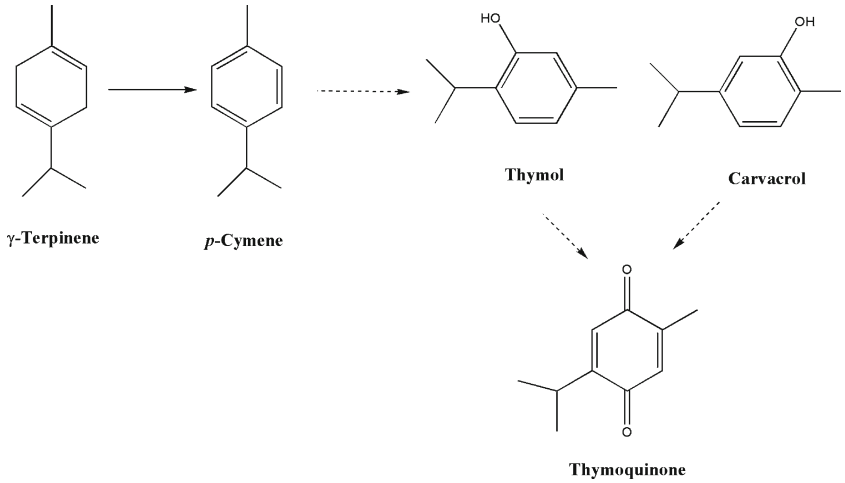

Fig. 1. Proposed pathway for TQ biosynthesis (44)

that the callus induction of black-seed was reported in 1975 by Banerjee and Gupta (22). Cytological abnormalities in the callus cells during sub-cultures (23), thymol content (24), and antimicrobial potential of the callus extracts (25) were also studied in later works. In pursuit of these studies and in view of new trends in biomedicines, the callus induction of *N. sativa* native to Iran was examined and the anti-inflammatory effect of the alcoholic extracts of black-seed and *N. sativa* calli on mix glial cells with an emphasis on the TQ content was also investigated. Results of these studies are presented and discussed in this paper.

MATERIALS AND METHODS

Seed Samples and Chemicals

Seeds of *N. sativa* were obtained from the Natural Resources Gene Bank of Institute of Forest and Rangeland (RIFR) in Tehran. Seeds had been collected from the Ardebil and Qazvin regions in the North-West of Iran. *N*-(2-furanylmethyl)-1H-Purin-6-amine (kinetin) and 2,4-dichlorophenoxyacetic acid (2,4-D) were purchased from Duchefa Biochemie (Netherlands). Folin–Ciocalteu phenol reagent, methanol (HPLC grade), and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt). Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from GIBCO. Trypsin, trypan blue dye, lipopolysaccharide (LPS) from *Escherichia coli*, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Griess reagent, thymoquinone, penicillin, and streptomycin were purchased from Sigma-Aldrich Corporation.

Seed Germination and Callus Induction

Seeds were sterilized by ethanol for 1 min under laminar air flow and rinsed three times with sterile water followed by sodium-hypocholorite (0.3%) for 5 min. consequently they were washed three times with sterile water. The sterilized seeds sprouted on a half-strength hormone-free Murashige and Skoog medium (MS). To make 1/2MS medium, all the required ingredients were used at halved concentrations (26). To induce callus, explants excised from different parts (roots, stems, petioles, and leaves) of young plantlets were put on solid MS medium containing agar (0.8%), sucrose (5%), 2,4-D (1 mg/l), and kinetin (2.15 mg/l) (27). The resulting callus cells were primarily proliferated on the same medium containing 2,4-D (0.22 mg/l) and kinetin (2.15 mg/l). The fast growing cells were subcultured every 3 weeks. Weights of the fresh and dried calli were measured for biomass calculation.

Extracts of *N. sativa* Seed and Callus

Seeds (50 g) were powdered and extracted by ethanol (96%) using a Soxhlet (250 ml). The solvent was evaporated in a Rotary Evaporator (Buchi Rotavapor R-200, Switzerland) at 20°C until an oily substance was obtained. Callus samples (1 g) were smashed in a mortar containing alcohol (4 ml) at room temperature and successively subjected to filtration and centrifugation. The callus extraction resulted in a sticky solid substance upon evaporation at room temperature. The results of extraction were stored in the dark at 4°C.

Total Phenolic Substances and Antioxidant Measurements

Total phenols were determined using Folin–Ciocalteu method and values were expressed as gallic acid equivalent (28). Antioxidant power was estimated using FRAP, ferric reducing/antioxidant power, assay and values were expressed as ascorbic acid equivalent (29).

Spectrophotometeric and HPLC Analyses

Spectrophotometric analyses were carried out in the conventional quartz cells (1 ml) using a Jena (Specord 210, Analytik) spectrophotometer. The reversed phase HPLC analyses of the different extracts were carried out on a KNAUER (Germany) HPLC equipped with a C18 (Beckman, USA) analytical column. An isocratic method using a mixture of water, methanol, and 2-propanol (50:45:5% *v*/*v*) as mobile phase at a flow rate of 0.8 ml/min was employed and detector was set at 254 nm. The injection volume was 20 µl. Commercial TQ (1 mg/ml) was used as the standard sample in these experiments.

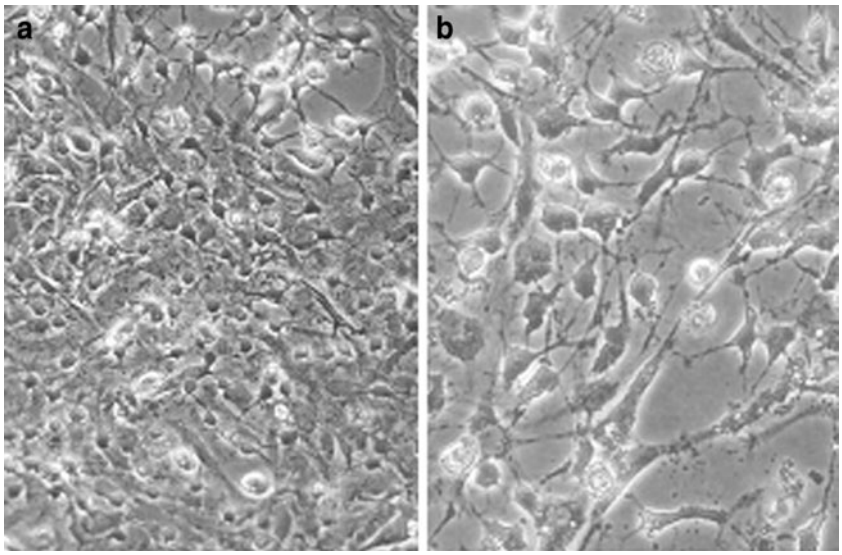

Fig. 2. Primary mix glial cell culture after 14 days observed by an inverted microscope **a** 32×, and **b** 40×

Primary Mix Glial Cell Culture and Treatment

Primary glial cell cultures were prepared from Wistar rats (1 to 3 days old) with the approval of Bioethic Committee of the Health Ministry. In brief, brain was removed aseptically and separated from cerebellum. Blood vessels and membranes were discarded. The brain tissues were dissected and fragmented mechanically then incubated in 25-cm^2 culture flasks

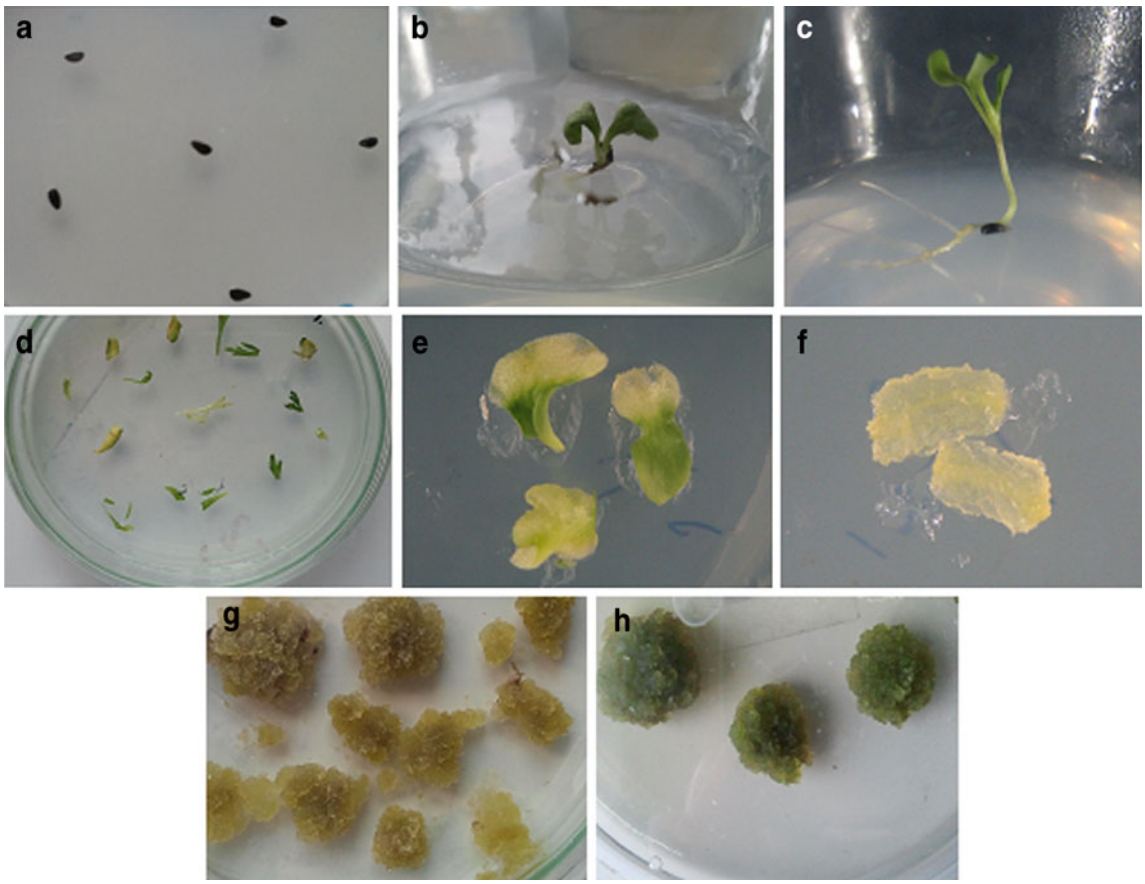

Fig. 3. **a** *N. sativa* seeds, **b** sprouted seeds, and **c** plantlet growing on hormnon-free 1/2MS medium at 25°C applying a photoperiod of 16:8 h (light–dark cycle). **d** Excised explants from *N. sativa* young plantlet on MS medium supplemented with 2,4-D (1 mg/l) and kinetin (2.15 mg/l). **e**, **f** Induced calli to grow on MS medium supplemented with 2,4-D (0.22 mg/l) and kinetin (2.15 mg/l). Calli grown **g** in the absence of light and **h** in the presence of light at 25°C

Table I. Callus Induction Time, Average Weight of Wet Callus and Biomass of Different Explants of *N. sativa*

Sample	Leaf	Petiole	Stem	Root
Callus induction (days)	6–7	7–8	7–9	9–10
Growth rate of wet callus (mg/day)	115.4±2.8	62±3.4	67.5±5.9	33.57±5.3
Biomass (mg/g of wet callus)	152	368	327	750

The calli were proliferated on a MS medium supplemented with 2,4-D (0.22 mg/l) and kinetin (2.15 mg/l) in dark at 25°C and were subcultured every 3 weeks

containing 5 ml of growth medium [DMEM containing FBS (10%), penicillin (100 U/ml), and streptomycin (100 mg/ml)]. Mixed glial cultures were maintained at 37°C in a 5% CO_2 incubator and medium renewed twice a week. Confluence (70–80%) was achieved in about 14 days (Fig. 2). Then, glial cells were dissociated by trypsin and centrifuged (1,000 rpm) at 4°C for 5 min. Supernatant was discarded and samples were counted with trypan blue stain and the hemocytometer. Glial cells were seeded in 96 well plates (2×10^4 cells/well) for control, treatments of LPS (15 μg/ml), DMSO and different doses (0.5, 1.25, 2.5, 5, 10, and 20 μl/ml) of seeds extract (NSO) or callus extracts (NSC) (0.2, 0.4, 0.8, 1.6, and 3.2 mg/ml) in the presence or absence of LPS (all in triplets). FBS concentration was decreased to 1% to minimize the possible protective effects on the extracts. The maximum dose of DMSO used as solvent in the treatments (0.1%) was tested and showed to be nontoxic. Glial cells were also examined for morphological changes by an inverted microscope (Axiovert 25, Germany) before and after treatments.

Cell Viability and NO Measurement

Conversion of yellow MTT to blue formazan indicates mitochondrial activity and as a result, cell viability (30). MTT (10 μl, 0.5 mg/ml) was added to 100 μl of the medium of each treatment after 24 and 48 h and incubated at 37°C for 4 h. Then, cells were lysed in DMSO (100 μl) and subjected to the absorbance reading at 580 nm on a Multiskan RC (Labsystems, Finland) microplate reader.

NO production in the culture supernatants was assessed by Griess reagent. Accordingly, after 24 and 48 h of treatment, 100 μl of each sample was mixed with Griess reagent (100 μl). After 15 min, absorbance was measured at 540 nm (31). In statistical analyses, *P* values less than 0.05 were regarded significant.

RESULT AND DISCUSSION

Seedlings, Callus Induction, and Proliferation

All seeds sprouted on 1/2MS solid medium in a range of 7 to 15 days (Fig. 3a, b). Seeds which had been collected from the Ardebil region sprouted 3 to 5 days sooner than those collected from the Qazvin region. Qazvin and Ardebil are located on the hilly altitudes of Alborz and Sabalan mountains, respectively, in North-West of Iran. Ardebil is closer to Caspian Sea and has an obvious colder climate than Qazvin. Employing a similar procedure, Elhag *et al.* had observed 50% seedling of black-seeds collected from some parts of Ethiopia and Saudi Arabia after 2 weeks (32). This difference could be an age-related phenomenon as young, a few-months-old, seeds were used in this study. Nevertheless, the data indicate that seedling of black-seed does not seem to be a limiting factor for *in vitro* studies and applications.

Young plantlets of black-seed grew on the same medium of seedling (Fig. 3c). Interestingly, all the explants excised from roots, stems, petioles, and leaves (Fig. 3d) produced callus on solid MS medium containing 2,4-D (1 mg/l) and kinetin (2.15 mg/l) in the dark (Fig. 3e, f). However, the time of induction was different. The shortest time was obtained for the leaf explants, about 6 days, and the longest time was observed for the root explants, beyond 9 days. These results are comparable to those reported by Chand and Roy (23) and suggest that callus induction, similar to the seedling step, is not a difficult and time-consuming stage during the *in vitro* studies on *N. sativa*.

Considering the summarized results in Table I, the following order is concluded for the growth rate of the wet calli: leaf>stem>petiole>root. But in regard to biomass, the order of root>petiol>stem>leaf is obtained. In other words, the leaf callus was more friable and watery (Fig. 3g and h) as compared with the calli of root, stem, and petiole. Results of successive subculture of the same calli of different tissues are illustrated in Fig. 4. It is understood that all the examined calli achieved the highest rate of proliferation in the second subculture and showed aging in the third subculture. Data published by Chand and Roy (23) and Al-Ani (24) indicated that the calli obtained from the leaf explants had the highest growth rates. However, the

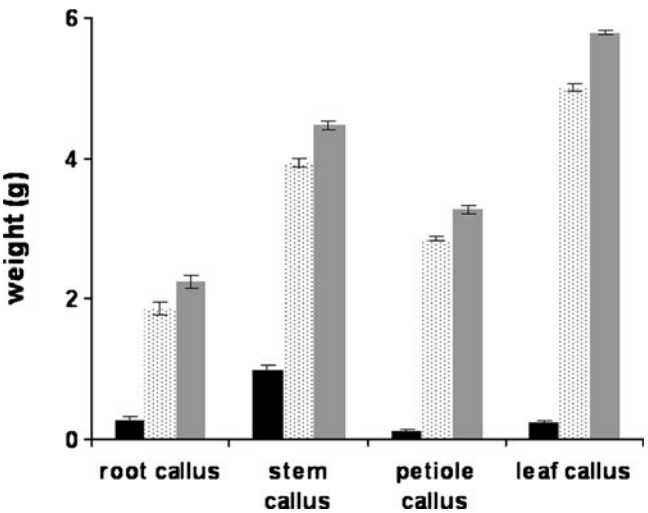

Fig. 4. Weights of the wet calli at the end of first subculture (*dark bars*), second subculture (*dotted bars*), and third subculture (*gray bars*). Calli were subcultured every 3 weeks on solid MS medium supplemented with 2,4-D (0.22 mg/l) and kinetin (2.15 mg/l). See the "MATERIALS AND METHODS" section for details

Table II. Subpeak Area of TQ in HPLC Chromatograms of the Standard and the Extracts Samples

Sample	Area%	mg/ml	Phenolic content[a]	Antioxidant power[b]
Standard thymoquinone (1 mg/ml)	90	1	–	–
Seed extract	67	0.74	–	–
Leaf callus grown in dark (1 week old)	3.47	3.17	–	–
Leaf callus grown in dark (2 week old)	9.61	**8.78**	–	–
Leaf callus grown in dark (3 week old)	2.93	2.68	–	–
Root callus grown in dark (2 week old)	4.42	0.82	–	–
Root callus grown in dark (3 week old)	0.91	0.17	–	–
Leaf callus grown in light (4 week old)	1.76	1.61	–	–
Leaf callus grown in dark (4 week old)	1.17	1.07	–	–
Bright parts of leaf and root calli grown in dark (4 week old)	1.23	0.68	686	2.88
Dark parts of leaf and root calli grown in dark (4 week old)	0.19	0.1	1250	6

The bold data shows one of the important results of this reseach concerning with the production of Thymoquinone
[a] Extracted data from Folin–Ciocalteu method showing the amount of total phenolic compounds equivalent to gallic acid (millligrams per liter)
[b] Data obtained from FRAP tests expressing the antioxidant ability equivalent to ascorbic acid (micromolar)

reported growth rates are varied from 18.7 mg/day by Al-Ani (24) to 142.8 by Al-Said *et al.* (33). Although the obtained growth rate for the leaf callus in this study, 115.4 mg/day, is similar to what Al-Said *et al.* reported, it is important to note they had used a different hormonal treatment (33).

Thymoquinone Analysis

Several chemical analyses have confirmed TQ as one of the main ingredients (8 to 27%) of the *N. sativa* seed extracts (10,34). Al-Said *et al.* had worked on the effect of different precursors such as thymol and menthol on the biosynthesis of TQ (33). Results reported by Al-Ani also confirmed thymol production in the callus obtained from the *N. sativa* leaf explants (24). Given these results, production of TQ in the selected calli of leaf and root which had been grown in the dark was examined. Experiments showed that the calli proliferated in light became green after 2 weeks while those grown in the dark had a pale yellow appearance (Fig. 3g and h). However, the advent of browning, assumingly due to an increase in the phenolic content, in both groups of calli was evident.

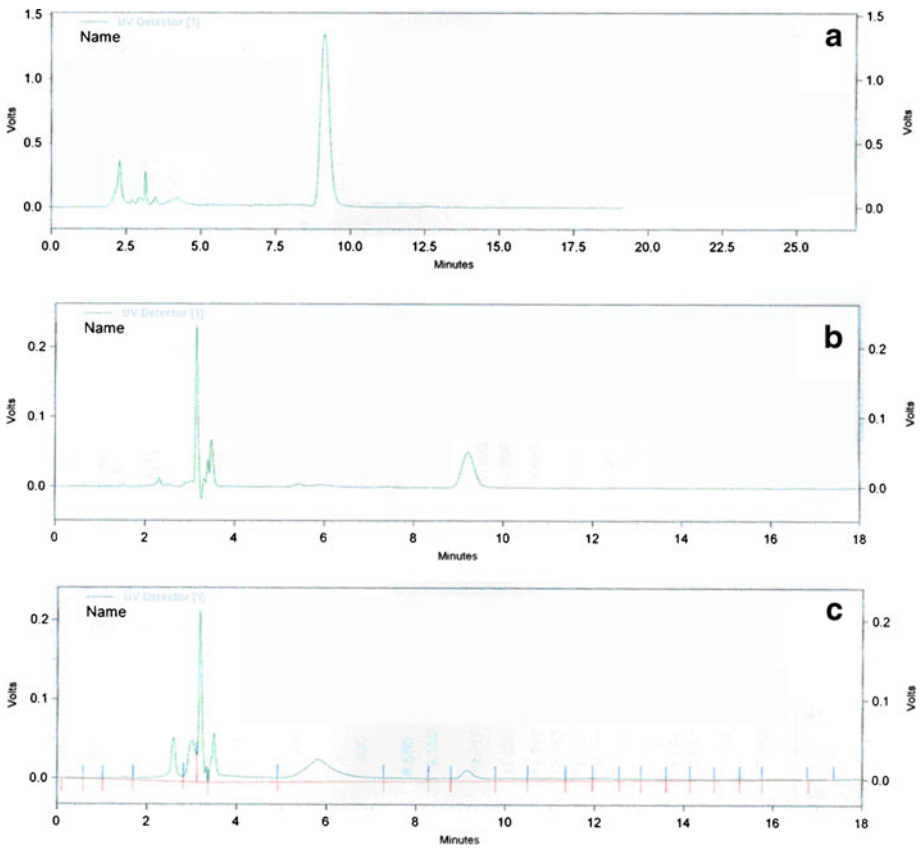

Fig. 5. HPLC chromatograms of the extracts of **a** the seeds, **b** bright parts of the leaf callus at the second week and **c** fourth week of the second subculture. See the "MATERIALS AND METHODS" section for the experimental details

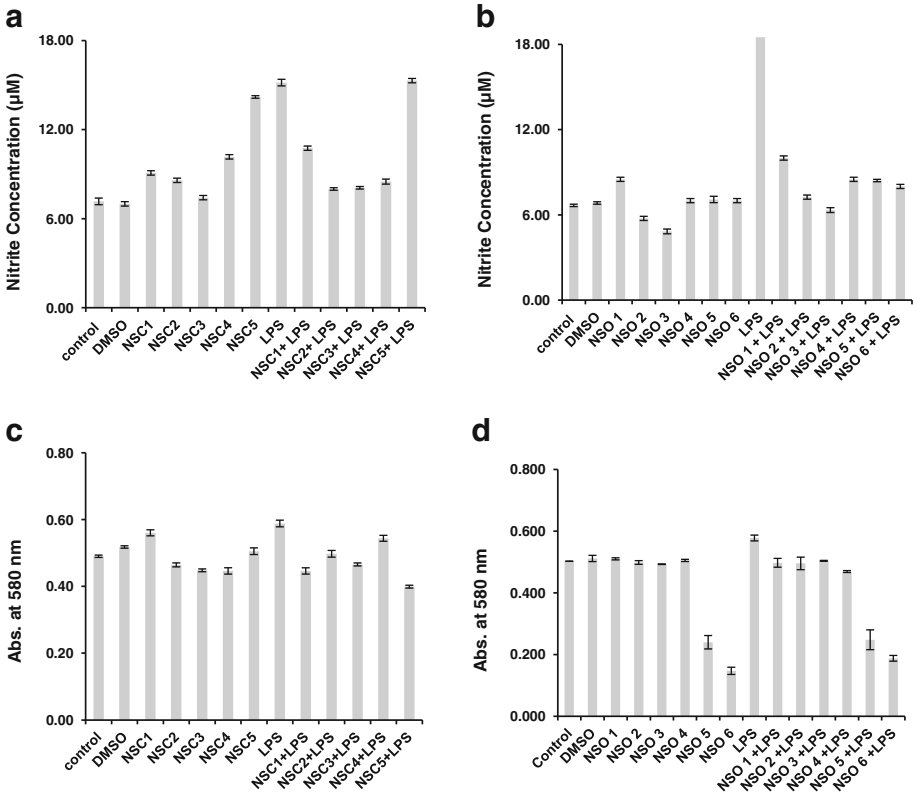

Fig. 6. Nitrite concentration in the supernatant of the mix glial cell cultures after 48 h of treatment with different concentrations **a** of NSC (0.2, 0.4, 0.8, 1.6, and 3.2 mg/ml) designated as NSC1 to NSC5, **b** of NSO (0.5, 1.25, 2.5, 5, 10, and 20 μl/ml) designated as NSO1 to NSO6. Results of MTT test for the mix glial cells treated with **c** five different concentrations of NSC, and **d** six different concentrations of NSO

The HPLC analysis of the standard TQ sample by the method introduced in the experimental section showed a retention time of 9.2 min for TQ with high reproducibility. Data provided in Table II indicates that the extract of the leaf callus contained the highest amount of TQ (8.78 mg/ml). These results also reveal that TQ production in the leaf callus reached its peak in the second week of the second subculture and then decreased as the callus aged (data of the weekly analyses is not shown here). Impressively calculation, based on the employed biomass of the samples for extraction, showed that the TQ content of the leaf callus was about 12 times higher than the TQ content of the seeds extract. As mentioned, some callus cells gradually darkened. Similar observation on the black-seed callus was reported by Elhag (32). To observe the effect of the age-related browning and light conditions on the TQ content, the leaf calli were divided into two groups and proliferated in the presence and absence of light. Both groups were allowed to age for 4 weeks. HPLC analysis disclosed that the TQ content of the dark cells was much lower than that in the bright cells. Interestingly, the callus which had been grown in light showed higher amounts of TQ after 4 weeks (Table II). This observation suggests that light does not disrupt TQ biosynthesis.

Total Phenolic Substances and Antioxidant Measurements

Analysis of the dark and bright parts of the leaf calli of *N. sativa* by Folin–Ciocalteu method disclosed that the phenolic content of the dark cells was about 1.8 times higher than that of the bright cells (Table II). Measuring the antioxidant ability of these cells by FRAP test also revealed about twofold antioxidant power for the dark parts of the calli as compared with the bright parts (Table II). These results are worthwhile from a biosynthetic point of view as they suggest that TQ formation does not guarantee TQ accumulation in the black-seed callus cells. Presumably, TQ participates in further chemical/enzymatic reactions which increase the phenolic content of the cells. Although the details of TQ biosynthesis pathway is not known, it is assumed that hydroxylation of compounds such as *p*-cymene, thymol, and carvacrol (Fig. 1) with further oxidation, most probably by cytochrome p450, form a major part of this pathway (34). Considering the high chemical activity of quinones, increasing the TQ concentration in cytosol may trigger chemical reactions such as conjugation and ring fusing reactions which ultimately increases the polyphenolic content of the cells. Finally, it is worthwhile to mention that the TQ content of the frozen callus remains constant. In contrast, drying the calli at 37°C aggressively decreases TQ. Gradual decrease in the TQ content of the leaf callus which was maintained at 25°C accompanied by formation of some other compounds is observed in the corresponding HPLC chromatograms illustrated in Fig. 5b, c.

Study of Anti-Inflammatory Effects

As mentioned in the "INTRODUCTION", several studies have been carried out to examine the anti-inflammatory

effects of the *N. sativa* seeds or its ingredients such as thymol and TQ in various disorders (35,36). In view of these results, the anti-inflammatory effect of TQ and the alcoholic extracts of both black-seed and its callus on mix glial cells were studied. LPS, a bacterial product, stimulates glial cells and make them activated. During inflammation, the activated glial cells produce NO (37) which is assayed by Griess method. Cell viability tests also help to estimate cytotoxicity of the employed materials. Mix glial cells were selected because they are regarded as complete immune cells of the central nervous system (38,39) and the astrocyte–microglia interactions play an important role in the biological behavior of these cells (40–42).

The mix glial cells of rat were treated first with various concentrations of LPS. Results (not shown here) indicated that LPS was effectively inflammatory at a concentration of 15 μg/ml after 48 h of treatment. Treating the mix glial cells with the leaf callus extract (NSC) showed a pro-inflammatory effect at concentrations above 1.6 mg/ml. But it had anti-inflammatory effect on the inflamed mix glial cells at concentrations between 0.2 and 1.6 mg/ml (Fig. 6a, c). In contrast, the seeds extract (NSO) was not pro-inflammatory at the range of concentration examined in this study (0.5 to 20 μl/ml). However, its toxic effect at concentrations above 10 μl/ml was evident after 1 h of treatment (Fig. 6d). Treating the inflamed mix glial cells with NSO proved anti-inflammatory effect of the extract at a range of 1.25 to 5 μl/ml (Fig. 6b). Considering the results of the HPLC analyses, an average TQ content of 10 to 80 μM was estimated for the effective concentration range of NSC and 4.5 to 22 μM for NSO. In a parallel experiment on TQ, it was observed that TQ had anti-inflammatory effect on the inflamed mix glial cells at concentrations 1.25 to 2.5 μM and pro-inflammatory effects at above 10 μM (data not shown).

These results indicate that the extracts of both seeds and the callus of *N. sativa* have moderate anti-inflammatory potential as compared with pure TQ. From a concentration point of view, they are comparable with what has been reported for acetaminophen. Acetaminophen (50 μM) increases neuronal cell survival and inhibits inflammation (43). But the observed differences in the cytotoxicity and anti-inflammatory effects of NSO and NSC may be ascribed to the different compositions of these extracts observed in the corresponding HPLC chromatograms (Fig. 5a, b).

CONCLUSION

Seedling, callus induction, and cell proliferation of *N. sativa* can be achieved in a short time period under cost-effective conditions. The callus obtained from the young leaf explant is friable with acceptable growth rate. This callus has also a great potential for TQ production. Biochemical studies on the anti-inflammatory effect revealed the moderate potential of the alcoholic extract of this callus for controlling inflammation in the mix glial cells of rat. However, the extent of the observed anti-inflammatory power cannot be directly ascribed to the TQ content of the callus.

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