

Isolation and evaluation of biological efficacy of quercetol in human hepatic carcinoma cells

Huma Ali¹
Savita Dixit¹
Daoud Ali²
Abdullah A Alkahtane²
Saud Alarifi²
Bahy A Ali^{2,3}
Saad Alkahtani²

¹Department of Chemistry, Maulana Azad National Institute of Technology, Bhopal, Madhya Pradesh, India;

²Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia; ³Department of Nucleic Acids Research, Genetic Engineering and Biotechnology Research Institute, City Science Research and Technology Application, Alexandria, Egypt

Abstract: Quercetol is a polyphenolic molecule present in vegetables and fruits, and is beneficial to human and animal health. The current work aimed to test cytotoxic and apoptotic effects of quercetol on HepG2 cells. Quercetol was isolated from *Ocimum sanctum* and characterized by gas chromatography–tandem mass spectrometry (GC-MS/MS), nuclear magnetic resonance spectroscopy, and Fourier transform infrared spectroscopy. Quercetol (50–600 µg/mL) was examined for cytotoxic activity by tetrazolium salt and neutral red uptake tests and comet assay for genotoxicity, using HepG2 cells, over 24 hours. Data from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and neutral red uptake tests demonstrated quercetol-induced cytotoxicity in HepG2 cells in a concentration-dependent manner. With 4',6-diamidino-2-phenylindole staining, a significant induction of chromosomal condensation was observed at 300 µg/mL of quercetol. DNA fragmentation analysis showed that quercetol produced cell death in HepG2 cells in a concentration-dependent manner. Thus, our study suggests that an environmentally relevant concentration of quercetol, which was a chemically standardized extract from *O. sanctum*, induced cell death and DNA damage in HepG2 cells.

Keywords: NMR, FTIR, quercetol, HepG2 cells, MTT assay, apoptosis, comet assay

Introduction

Ocimum sanctum Linn commonly known as Tulsi belongs to Labiateae family. A variety of constituents including flavonoids, tannins, eugenol, dimethyl benzene, ethyl benzene, saponin, and phosphorous were detected in this plant species.¹ *O. sanctum* is a plant used in the preparation of several Ayurvedic pharmacological products.² Chanda and Nagani³ reported a wide range of beneficial effects such as anticancer, antibacterial, antimicrobial, hepato-protective, antispasmodic, anti-inflammatory, and diaphoretic actions attributed to this plant. Some investigators quantitated quercetol content in fruits (0.002–0.25 g/kg), 0.1 g/kg in vegetables, 0.004–0.016 g/L in wine (red), 0.010–0.025 g/L in tea.^{4–6} Sethi et al⁷ reported a significant decrease in sperm count, follicle-stimulating hormone, luteinizing hormone and an increase in serum testosterone levels in *O. sanctum*-treated rabbits. Oridonin produced DNA damage in HepG2 cells through reactive oxygen species (ROS) generation.⁸ Flavonoids produce a wide range of biological effects on blood vessels and heart due to antioxidant properties.⁹

Quercetol exerts antioxidant properties and shows an important role in inhibiting cancer.¹⁰ Some researchers reported that quercetol inhibits lipid peroxidation and acts as an inhibitor of xanthine oxidase, oxygen radicals scavengers in vitro.^{11–13} Skaper et al¹⁴ reported that quercetol without ascorbic acid or with ascorbic acid inhibited oxidative damage and neurons loss in skin. Scambia et al¹⁵ reported anti-proliferative effects of quercetol in cancer cells.

Correspondence: Huma Ali
Department of Chemistry, Maulana Azad National Institute of Technology, Link Road Number 3, Bhopal, Madhya Pradesh 462003, India
Email humali.manit@yahoo.com

The current study is an attempt to understand the anti-proliferative and apoptotic potential of quercetol isolated from *O. sanctum* in HepG2 cell lines.

Materials and methods

Chemicals and plants

Neutral red (NR) dye and ethidium bromide were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Antibiotics, fetal bovine serum, and DMEM/F-12 media were procured from Thermo Fisher Scientific (Waltham, MA, USA). Other reagents were high quality and purchased from local markets.

Leaves of *O. sanctum* were accumulated from Sanjevani; Bhopal, Madhya Pradesh, India. The current study was approved by the ethical committee of Maulana Azad National Institute of Technology.

Extraction and isolation of quercetol

Quercetol was isolated from *O. sanctum* using the method shown in Figure 1.

Quercetol

Isolated compound was subjected to ultra violet (UV) spectrophotometer, FTIR gas chromatography–tandem mass spectrometry (GC-MS/MS), and NMR spectroscopy. Figure 2A shows UV spectra analysis of isolated compound. Analysis of infrared (IR) spectra appeared a broad peak for O-H stretch may be hydrogen bonded from 3,412 to 2,712/cm. Stretch 1,664, 1,649, 1,560, and 1,450/cm showed C=C stretch of

benzene and 1,242/cm for C-O stretch. 941, 864, 823, 794, and 702/cm showed peaks for substituted benzene (Figure 2B). ¹H NMR spectra of the compound showed proton signals at δ 7.23 (1H, H-6), δ 7.28 (1H, H-8), δ 7.02 (1H, H-2'), δ 7.33 (1H, H-5'), δ 7.54 (1H, H-6'); δ 3.56 (1, OH-5), δ 3.51 (1, OH-7), δ 2.19 (1, OH-3), δ 2.03 (1, OH-3'), and δ 1.56 (1, OH-4') (Figure 2C). The (–ve) electro spray ionization mass spectra of the compound exhibited ion [M-H][–] at *m/e* 303, demonstrating a relative weight of 302 (Figure 2D). The result showed high content of the flavonoid 3,3',4',5,7-pentahydroxyflavone (quercetol). The compound was purified by re-crystallization with CH₃OH to produce 99% pure quercetol.

HepG2 cells and treatments

Human hepatic carcinoma (HepG2) cells were procured from National Centre For Cell Science, Pune, India. HepG2 cells were cultivated in DMEM/F-12 media with 10% fetal bovine serum and penicillin-streptomycin (100 unit/mL) in a CO₂ incubator (5%, 37°C). After growth, HepG2 cells were divided into other culture plates and flasks. A stock solution of quercetol (10 mg/mL) was prepared in DMSO and diluted in cell culture media to doses (50, 100, 300, and 600 μ g/mL). HepG2 cells unexposed to quercetol act as a control in each assay.

Cell shape

Shape of HepG2 cells was seen after treatment of various dosages of quercetol for 48 hours by an inverted microscope (DM IL; Leica, Wetzlar, Germany).

MTT assay

MTT test was done as described by Mossman.¹⁶ HepG2 cells were treated with quercetol (0, 50, 100, 300, and 600 μ g/mL) for 24 hours.

NRU test

NRU test assay was performed according to Borenfreund and Puerner method.¹⁷

MMP

Measurement of mitochondrial membrane potential (MMP) in HepG2 cell line due to quercetol (0, 50, 100, 300, and 600 μ g/mL) for 24 hours was done according to JC-1 mitochondrial membrane potential kit (Item no 10009172) from Cayman Chemical (Ann Arbor, MI, USA).

Assay for condensing of chromosome

Condensed chromosome in HepG2 cells due to quercetol exposure was observed by 2-(4-amidinophenyl)-1H-indole-6-carboxamide (DAPI) staining.

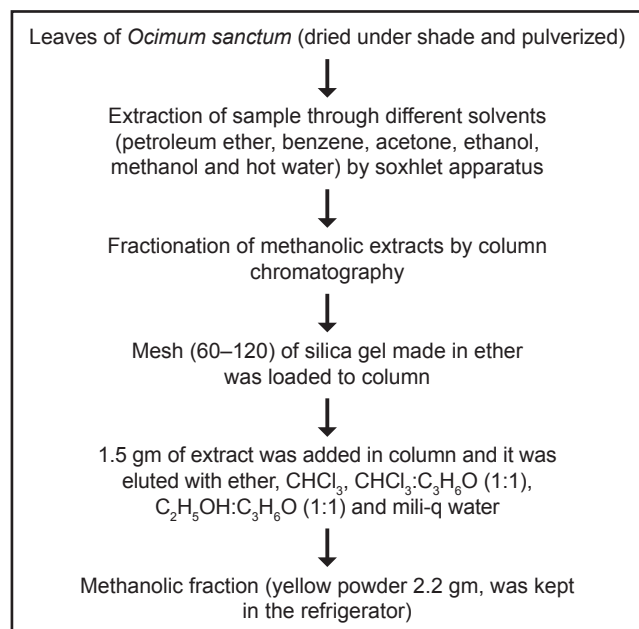


Figure 1 Method for isolating quercetol from *O. sanctum*.



Figure 2 Characterization of isolated compound of *Ocimum sanctum* (quercetol) by (A) UV spectra, (B) FTIR spectra, (C) (a) NMR spectra, (b) structure of quercetol, and (D) mass spectra.

Abbreviations: FTIR, Fourier transform infrared spectroscopy; NMR, nuclear magnetic resonance; UV, ultra violet; ms, mass spectra.

Caspase-3 activity

Twenty four hours later, HepG2 cell culture with or without quercetol were cleaned thrice and reseeded in culture media. Caspase-3 activity was determined by caspase-3 (Red-DEVD-FMK) detection kits and Glomax[®] multi detection system. The method was used as described by the manufacturers.

DNA strand breakage

DNA strand breakage was done by Comet test method.¹⁸

Analysis of results

The result was presented as average, and statistical analysis was done by ANOVA. $P < 0.05$ was used as significant.

Results

Alteration in HepG2 cells

Untreated HepG2 cells are represented in Figure 3A. HepG2 cells are detached from culture plate surface and changed into round shape at 300 and 600 $\mu\text{g}/\text{mL}$ quercetol exposure (Figure 3B–C).

Viability and MMP of HepG2 cells

We studied the role of mitochondria (reduction of MTT) and lysosome activity (NRU) as end points of cell toxicity. MTT results confirmed a dose-related cell death after exposure

to quercetol in HepG2 cells (Figure 4A). NRU test data is presented in Figure 4B. Data indicated a dose-based decayed in HepG2 cell lines treated to quercetol for 24 hours. MMP declined after 24 hours exposure of quercetol to HepG2 cells using the JC-1 fluorescent probe (Figure 5). Decline in MMP was observed as dose related and highly significant ($P < 0.05$) at 600 $\mu\text{g}/\text{mL}$ of quercetol as compared with control.

Condensing of chromosome and caspase-3 enzyme

Condensed chromosome was observed by DAPI staining. HepG2 cells treated with quercetol (50, 100, and 300 $\mu\text{g}/\text{mL}$) for 24 hours induced chromatin condensation (Figure 6). Caspase-3 was induced in cells with quercetol treatment (Figure 6C). HepG2 cells were treated with quercetol (50, 100, and 300 $\mu\text{g}/\text{mL}$) over 24 hours; the activity of caspase-3 was upgraded in a dose-based manner.

DNA fragmentation

Fragmentation of DNA was quantified as percent DNA in the untreated and quercetol (50, 100, and 300 $\mu\text{g}/\text{mL}$) treated cells. The cells treated with quercetol revealed more fragmented DNA than untreated HepG2 cells. Maximum DNA fragmentation was seen at 300 $\mu\text{g}/\text{mL}$ quercetol in HepG2 cells (Figure 7).

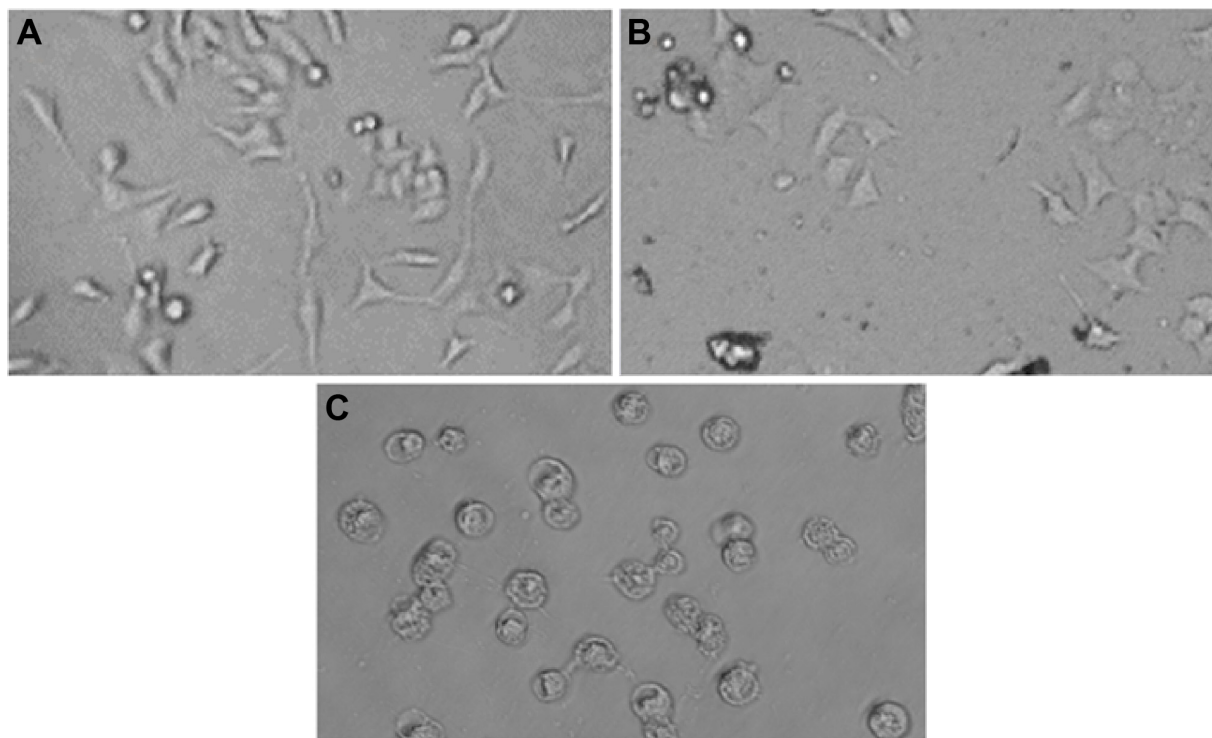


Figure 3 Morphology of HepG2 cells.

Notes: (A) Control, (B) at 300 $\mu\text{g}/\text{mL}$ of quercetol, and (C) at 600 $\mu\text{g}/\text{mL}$ of quercetol for 24 hours.

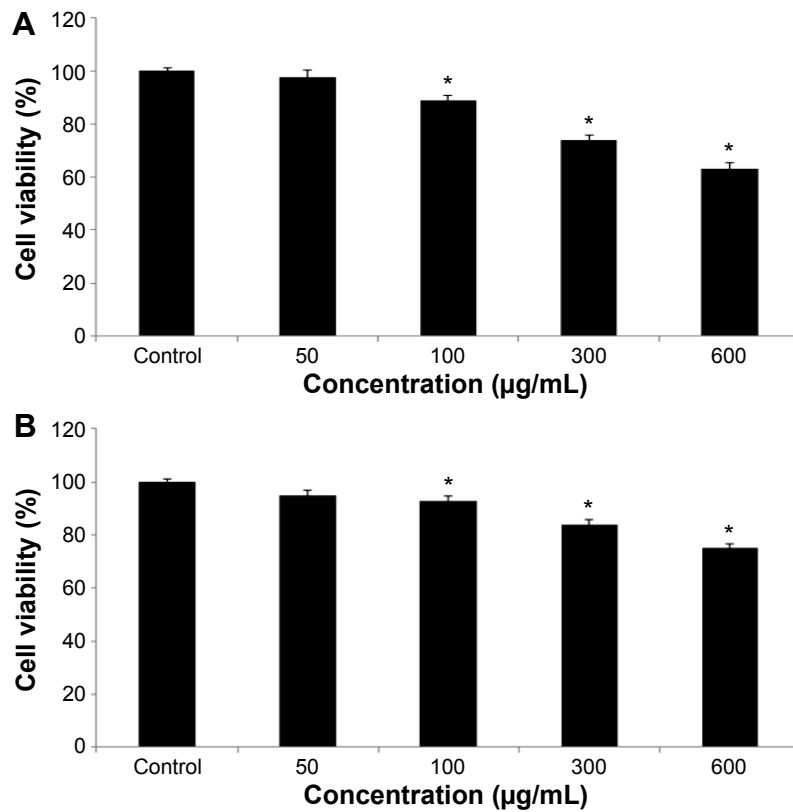


Figure 4 Toxic effect of quercetol in HepG2 cells as determined by (A) MTT and (B) NRU tests.

Notes: Data are expressed as percentage viability of cells exposed to quercetol relative to control cells and mean \pm SE of three experiments. * $P < 0.05$ vs control, using one-way ANOVA.

Abbreviations: ANOVA, analysis of variance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NRU, neutral red uptake; SE, standard error.

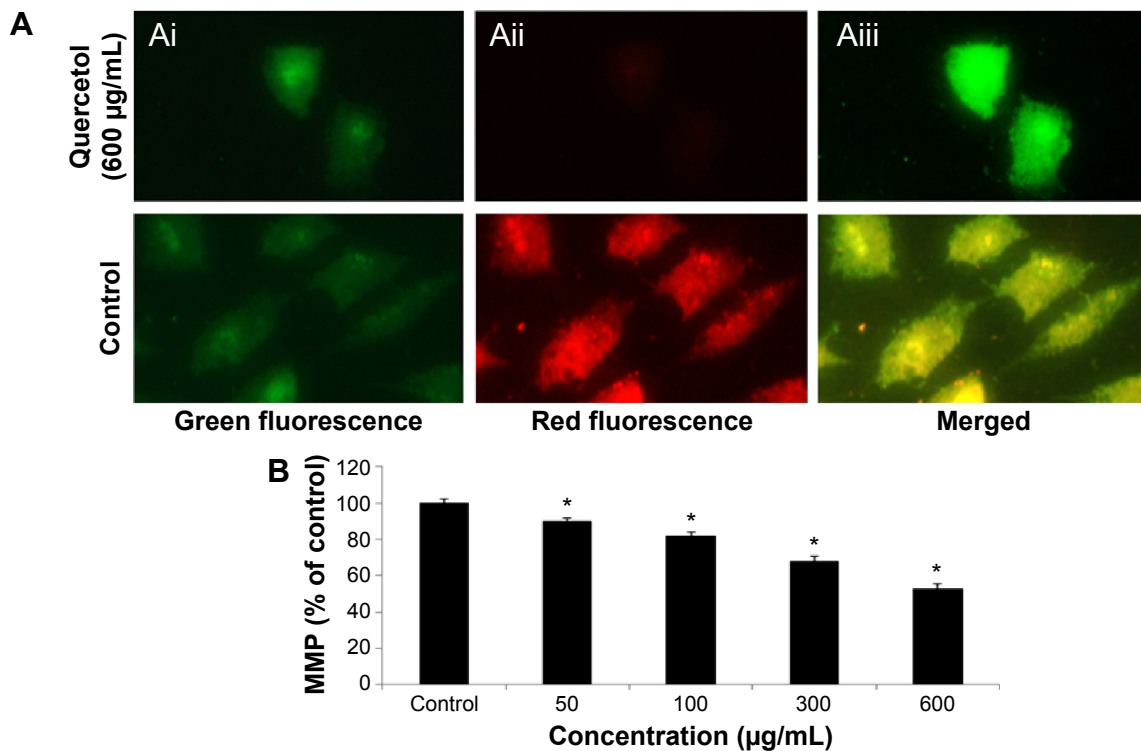


Figure 5 Impact of quercetol on the MMP of HepG2 cells.

Notes: Panel (Ai) shows green fluorescence (JC-1 monomer) only, panel (Aii) shows red fluorescence (JC-1 aggregate) only, and the panel (Aiii) shows merged (green–red fluorescence). (B) % MMP ratio. Data are expressed as the mean \pm SE of three experiments. * $P < 0.05$ vs control, using one-way ANOVA.

Abbreviations: ANOVA, analysis of variance; MPP, mitochondrial membrane potential; SE, standard error.

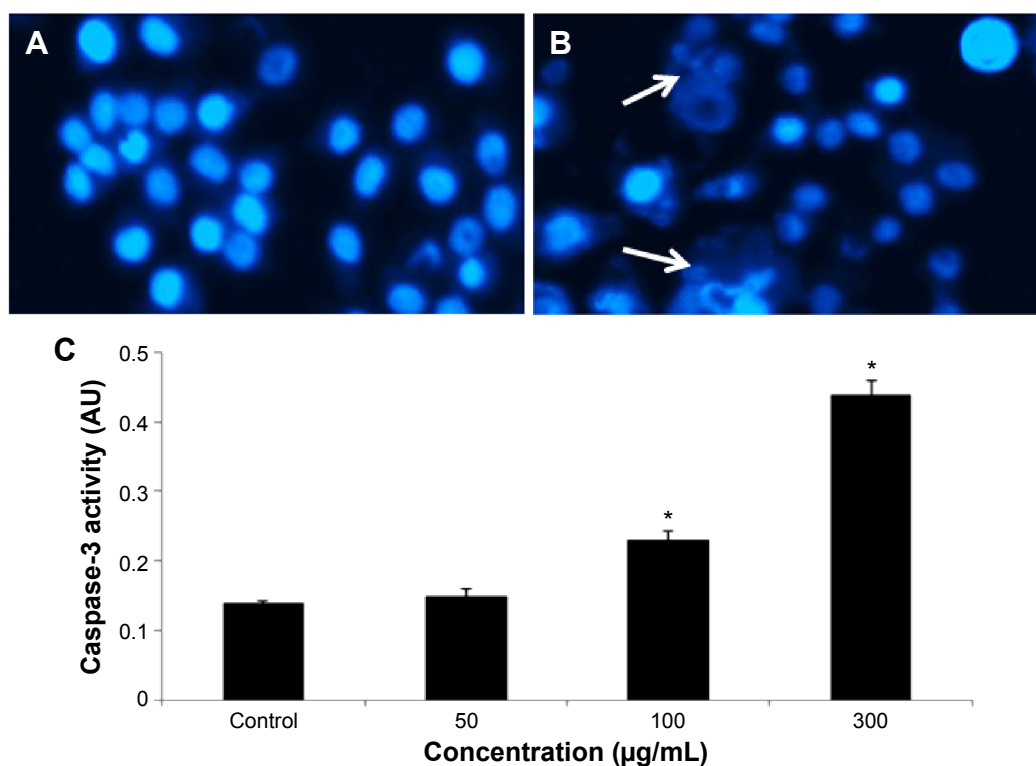


Figure 6 Induction of condensed chromosome and caspase-3 enzyme activity in HepG2 cells after exposure to quercetol.

Notes: (A) Control, (B) at 300 µg/mL of quercetol for 24 hours (arrows indicate condensed chromosome), and (C) Caspase-3 activity. Each value represents the mean \pm SE of three experiments. * $P < 0.05$ vs control, using one-way ANOVA.

Abbreviations: ANOVA, analysis of variance; SE, standard error.

Discussion

Nutritional foods are important sources for the treatment of some types of cancers, leading to the development of potential novel agents. Several of the molecules available from foods have been shown to exert anticancer activities on cancer cells. Lee et al¹⁹ have reported these effects in animal and cells. This study exposes the quercetol effects on HepG2 cells and explores important possible mechanisms by which quercetol induces toxicity on HepG2 cell lines. This data indicates that quercetol has genotoxic and apoptotic effects on HepG2 cells. Before observing cytotoxic and genotoxic effect of quercetol, we had characterized and purified through re-crystallization with methanol to produce quercetol (99% purity).

Confiction of a few plant extracts which normally used cell toxicity assessment system has been well reported by earlier researchers. Therefore, we have used two assays, namely MTT and NRU, to find out the toxicity of quercetol in HepG2 cells because it increases validity of results. In this study, quercetol produced cell toxicity in concentration-related matter as determined by NRU and MTT tests.

During chemotherapy of several cancers, anticancerous drugs are used. Some researchers reported that ROS normally comprises H_2O_2 , OH^\bullet , and O_2^- anions, so it induces

impairment in cell organelles as nucleic acid damage and finally cell death occurred.^{20,21} We have seen elevation of caspase-3 activity in HepG2 cells after treatment with quercetol. Quercetol may produce free radicals after their relations with cells. ROS may oxidize and reduce proteins, nucleic acids, and lipids molecule as a consequence of cell damage. In this study, quercetol significantly produced toxicity in HepG2 cells. In the present study, quercetol produced cell death by apoptosis that identified by biochemical and morphological features. Quercetol caused condensation and disintegration of chromosomes in HepG2 Cells.

Quercetol displays an incidental effect on nuclear materials due to its capability to produce ROS. Impairments of nuclear material can either produce carcinogenic cells or cell death, which disturb functions of normal cells. We found the DNA damaging effect of quercetol in HepG2 cells through single gel test that is adept at identifying double- and single-strand breaks in DNA fragmentation.²²

In conclusion, these data demonstrate that quercetol has the ability to induce fragmentation of DNA in HepG2 cells. However, it is obvious that further research including cell culture and animal studies are needed to obtain more information and to make concise evaluations on the subject.

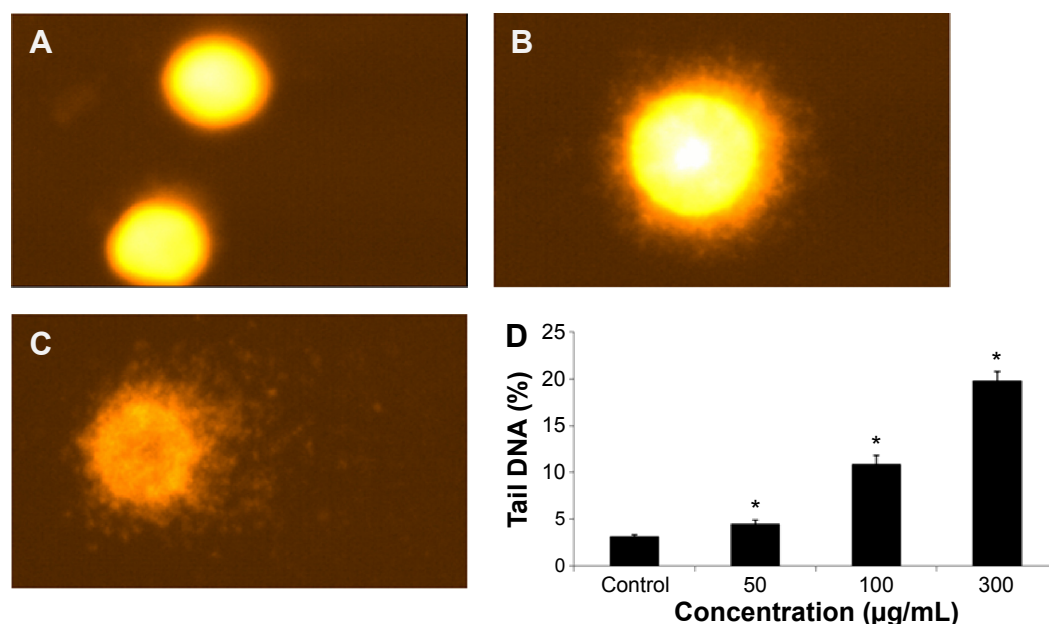


Figure 7 Fragmentation of DNA in HepG2 cells due to 24 hour quercetol exposure.

Notes: (A) Untreated cell, (B) at 100 µg/mL of quercetol, (C) at 300 µg/mL of quercetol, (D) percentage tail DNA. Each value represents the mean ± SE of triplicate tests. * $P < 0.05$ vs control, using one-way ANOVA.

Abbreviations: ANOVA, analysis of variance; SE, standard error.

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Disclosure

The authors report no conflicts of interest in this work.

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