Phytochemical and genotoxicity studies of *Citrus reticulata* aerial part in mice

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Background and objectives
The genus *Citrus* (family Rutaceae) is known to contain many bioactive compounds like flavonoids that protect mice against genotoxicity because of their antioxidant and free radical scavenging properties. The aim of this study was to investigate the phytochemical constituents and the protective effect of the ethanolic extract of the aerial part of *Citrus reticulata* cultivated in Saudi Arabia against genotoxicity induced by benzo(a)pyrene (BaP) in mice.
Materials and methods
The major constituents from of the aerial part of *C. reticulata* were isolated using different chromatographic techniques. Identification of compounds was realized through R_f values, shift reagents, and spectroscopic tools such as ultraviolet and nuclear magnetic resonance. The constituents of both unsaponifiable and methylated fatty acids were identified using Gas Liquid Chromatography (GLC) analysis. Essential oil constituents of peels and aerial part of *C. reticulata* were obtained by hydrodistillation and analyzed by gas chromatography–mass spectrometry. PBS, 1% LMPA, and ethidium bromide were used for the comet assay and quantitative analysis of DNA fragmentation in liver tissue in male rats was determined. About 50 male mice were used in this study, which were allocated in five groups (10 animals each) and treated with BaP and *C. reticulata* [total ethanolic extract (TE) and petroleum ether fraction].
Results and conclusion
Phytochemical investigation of the ethanolic extract from the aerial part of *C. reticulata* revealed five flavonoids (1–5). GLC analysis of the unsaponifiable fraction showed the presence of α-tocotrienol and α-tocopherol, which belong to the group of vitamin E. A total of 22 compounds were identified in the essential oils of *C. reticulata* blanco, 12 compounds were found in the aerial part, and 12 compounds were found in the fruit peels. The ethanolic extract was tested for the first time against genotoxicity induced by BaP in mice using the comet assay. TE significantly reduced the damage of DNA caused by BaP in mice. There was a statistically significant increase ($P \leq 0.05$) in the DNA fragmentation in the liver tissues of male mice and an increased rate of DNA damage in mice blood cells in the BaP group. Treatment with TE has a significant liver and blood cell protection by inhibiting the rate of DNA damage. These findings led us to conclude that the aerial part of *C. reticulata* is useful to reduce the genotoxicity induced by hazardous chemical agents.

Keywords:
Citrus reticulata, comet assay, flavonoids, genotoxicity testing, phytochemical constituents

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Introduction

Many environmental and industrial chemicals are capable of leading to genotoxic effects in exposed organisms. Even though, mutagenic and carcinogenic events are caused by the boosted subjection to hazardous chemicals. It has been proved through epidemiological studies that cancer and several other diseases can be avoided through the intake of fruits and vegetables [1]. However, there is a possibility that antimutagenic or anticarcinogenic effects could be caused by such consumption of fruits and vegetables [2]. The genus *Citrus* is known to contain many bioactive compounds such as phenolics, flavonoids, essential oil, coumarins, carotenoids, and vitamins [3]. Flavonoids are the most common in *Citrus* spp. [4] and have many biological properties, including hepatoprotective, antithrombotic, antibacterial, antiviral, and anticancer activity, due to their antioxidant and free radical scavenging properties [1]. Previous studies using the micronucleus test have

reported that *Citrus* extract protects mice against genotoxicity induced by cyclophosphamide in the bone marrow cells [5,6]. Hesperidin was the major flavonoid in *Citrus reticulata* extract according to high-performance liquid chromatography results [7]. It was found that *Citrus* fruits include vitamin C, which is one of the multiple cancer chemopreventive agents, and also vitamin E [3,8]. The diversity and genetic relatedness in *Citrus* spp. have been evaluated by isozymes technique since 1980 [9]. The most influential carcinogen is benzo(a)pyrene (BaP), which is embryo toxic and teratogenic in animals [10]. In environmental samples, the level of BaP could be a fine sign for carcinogenic potential contamination [11]. BaP is metabolized by the liver microsomal mixed function oxidase system by highly reactive compounds that can be bounded with specific targeted sites of DNA, which is of critical importance in creating the BaP-induced carcinogenesis [12]. One of the most salient events in the development of a number of human malfunctions that are caused by overwhelming the biological defense system against carcinogens, stress, and drugs is the oxidative damage of biological molecules. Some carcinogenic events and oxidative-related disorders can be restrained or even treated by the human consumption of antioxidant compounds, or compounds that ameliorate or empower the biological antioxidant mechanisms [13].

The present study was conducted to investigate the phytochemical composition and the protective effect of the ethanolic extract of the aerial part of *C. reticulata* against genotoxicity induced by BaP in mice. These investigations have not been previously reported from of the aerial part of *C. reticulata* cultivated in Saudi Arabia.

Materials and methods

Phytochemical investigations

Plant material

The aerial part of *C. reticulata* blanco (mandarin) was collected from Najran, Kingdom of Saudi Arabia, during December 2015 and identified by Dr Ali Al-Jaleel, National Centre for Horticultural Research and Development, King Abdul-Aziz University, Jeddah, Saudi Arabia.

Apparatus

Electrothermal 9100 instrument melting point apparatus was used. GLC conditions of the unsaponifiable matter and fatty acids were performed on Hewlett Packard-HP 6890 series (United States), GC system, equipped with flame ionization detector. Nitrogen gas was used as a carrier gas. The analysis of the unsaponifiable matter was carried out using capillary column (HP-1 methyl siloxane) and at an oven temperature of 50°C/11.7 min from 80 to 325°C; injection and detector temperature was 300°C. The operating conditions for fatty acid methyl ester analysis were capillary column polyethylene glycol (60 m×320 mm), and the column temperature was 260°C. Ultraviolet (UV) data were measured on a Shimadzu (Japan) UV-240 spectrophotometer. The nuclear magnetic resonance (NMR) spectra were recorded at 300 (^{1}H) and 75 (^{13}C) MHz on a Varian Mercury 300 (Varian, United Kingdom). NMR spectra were run in a dimethyl sulphoxide (DMSO-d_6), and chemical shifts were given in δ (ppm) relative to tetramethylsilane (TMS) as the internal standard.

Extraction

The dried aerial part of *C. reticulata* (500 g) was grounded and defatted by treatment with 2 l petroleum ether (PE) (60–80°C). The PE was distilled in-vacuo at 50°C giving a residue of 10 g. The defatted powdered was extracted by percolation with 80% aqueous ethanol (5 l) at room temperature. The collected ethanol was evaporated in-vacuo at 50°C under reduced pressure, affording 62 g of total ethanolic extract (TE).

Preliminary phytochemical screening

The ethanolic extract of *C. reticulata* was analyzed for the presence of various phytochemical constituents like carbohydrates, tannins, saponnins, flavonoids, leucoanthocyanins, terpenes/steroids, alkaloids, anthraquinones, cardiac glucosides, which were identified using standard phytochemical procedures according to the previous experiences [14–16].

Investigation of the lipoidal matter

Two grams of PE obtained from *C. reticulata* were saponified by refluxing with 50 ml of alcoholic KOH (10%) for 3 h to give the unsaponifiable matter and fatty acids. Determination the hydrocarbon and sterol contents from the unsaponifiable matter [17] was carried out by using the GLC apparatus and the identification was achieved by comparing the retention time of their peaks with those of authentic (Table 1). The fatty acids fraction was obtained by acidification of the aqueous alkaline solution by 2 N HCl. The isolated fatty acids were methylated by refluxing with absolute methanol containing 5% H_2SO_4 for about 1 h according to the method described by Iverson and Sheppard [18]. Identification of the fatty acid methyl esters was achieved by comparing the retention time of their peaks with those of authentic by using GLC analysis (Table 2).

Isolation of the essential oil

The essential oil isolation was carried out by water distillation using a Clevenger-type apparatus of air-dried *C. reticulata* (150 g of each aerial part and peels) for 3 h according to the method recommended by the European Pharmacopeia to produce oils [19]. The oil was dried over anhydrous sodium sulfate, filtered, and stored at −4°C until analysis. The chemical composition of *C. reticulata* essential oil is shown in Table 3.

Table 1 GLC analysis of the unsaponifiable fraction present in the petroleum ether extract of *Citrus reticulata*

Names of compound	RT	Percentage
Tridecane	10.78	0.16
Pentadecane	11.54	0.47
Hexadecane	12.21	1.12
Heptadecane	13.80	0.21
Octadecane	14.79	2.27
Nonadecane	15.80	0.47
Eicosane	16.96	7.13
Heneicosane	18.31	1.62
Docosane	19.49	6.87
Tricosane	20.61	3.57
Tetracosane	21.57	8.98
Pentacosane	22.90	1.64
Hexacosane	23.11	14.68
Heptacosane	24.45	3.25
Octacosane	25.05	1.96
α-Tocotrienol	25.28	9.97
Nonacosane	26.94	5.54
Triacontane	27.48	1.62
α-Tocopherol	27.76	2.40
Cholesterol	28.50	4.94
Campesterol	29.32	2.94
Stigmasterol	30.25	2.50
β-Sitosterol	31.32	0.86
β-Amyrin	33.14	14.83

Gas chromatography–mass spectrometry analysis

The analysis of the essential oil was performed using gas chromatography–mass spectrometry (GC–MS) Finnigan mat SSQ 7000. The model of GC is Trace GC 2000, produced by Thermo Finnigian Company, Italy, injection volume 1 µl, injection

Table 2 GLC analysis results of saponified fraction present in the petroleum ether of *Citrus reticulata*

Names of compound	RT	Percentage
Butyric acid (4 : 0)	4.50	27.85
Caproic acid (6 : 0)	8.41	16.94
Caprylic acid (8 : 0)	11.86	4.33
Capric acid (10 : 0)	15.03	1.61
Henedecanoic acid (11 : 0)	17.77	0.69
Pentadecanoic acid (15 : 0)	23.60	2.42
Margaric acid (17 : 0)	30.25	4.32
Palmitoleic acid (16 : 1)	31.17	5.26
Linoleic acid (18 : 2)	36.30	2.09
Linolenic acid (18 : 3)	37.28	6.12
Arachidonic acid (20 : 4)	42.56	1.70
5,8,11,14,17-Eicosapentaenoic acid (20 : 5)	43.37	9.32
Heneicosanoic acid (21 : 0)	44.19	5.61
Behenic acid (22 : 0)	46.45	3.81
cis-13-Docosenoic acid (22 : 1)	47.50	7.93

Table 3 GC/MS analysis of the essential oil of peels and aerial part of *C. reticulata*

No.	RT	Compound Name	Peels aerial part %		M. Wt.	M. F.
1	7.86	3-Hexen-1-ol	–	0.70	100	$C_6H_{12}O$
2	8.28	1-Hexanol	–	0.04	102	$C_6H_{14}O$
3	11.18	Sabinene	–	0.08	136	$C_{10}H_{16}$
4	11.24	2-Thujene	–	0.14	136	$C_{10}H_{16}$
5	11.95	*a*-Myrcene	0.05	–	136	$C_{10}H_{16}$
6	13.05	Limonene	67.29	0.17	136	$C_{10}H_{16}$
7	13.61	3-Methylene-1,6-heptadiene	–	0.16	108	C_8H_{12}
8	13.96	1,2-Cyclononadiene	32.26	–	122	C_9H_{14}
9	15.77	*a*-Terpinolene	–	4.48	136	$C_{10}H_{16}$
10	16.00	Linalool	0.04	91.02	154	$C_{10}H_{18}O$
11	16.48	*Trans*-Pinene hydrate	–	0.11	154	$C_{10}H_{18}O$
12	18.04	4-Terpineol	–	2.68	154	$C_{10}H_{18}O$
13	18.07	*a*-Terpineol	0.10	–	154	$C_{10}H_{18}O$
14	18.25	Linalyl propionate	–	0.25	210	$C_{13}H_{22}O_2$
15	20.31	carvone	0.05	–	150	$C_{10}H_{14}O$
16	20.81	Perilla alcohol	0.02	–	152	$C_{10}H_{16}O$
17	21.47	4-Hydroxy-2-methyl-acetophenone	0.03	–	150	$C_9H_{10}O_2$
18	23.44	*a*-Cubebene	0.03	–	204	$C_{15}H_{24}$
19	23.55	*b*-elemene	–	0.19	204	$C_{15}H_{24}$
20	25.77	Germacrene D	0.01	–	204	$C_{15}H_{24}$
21	26.81	*d*-Cadinene	0.02	–	204	$C_{15}H_{24}$
22	27.43	Hedycaryol	0.05	–	222	$C_{15}H_{26}O$

RT, retention time.

mode: split less, carrier gas was helium at the flow rate of 1.0 ml/min, main column (DB-5) and 5% phenyl methyl polysiloxan. Temperature program was 40°C for the first 1 min, which was then raised up to 250°C by 2°C/min for 45 min. MS model used was Finnigan SSQ 7000; mass range was 40–300, and scan time was 5 s. For GC–MS detection, an electron ionization system was used with ionization energy of 70 eV.

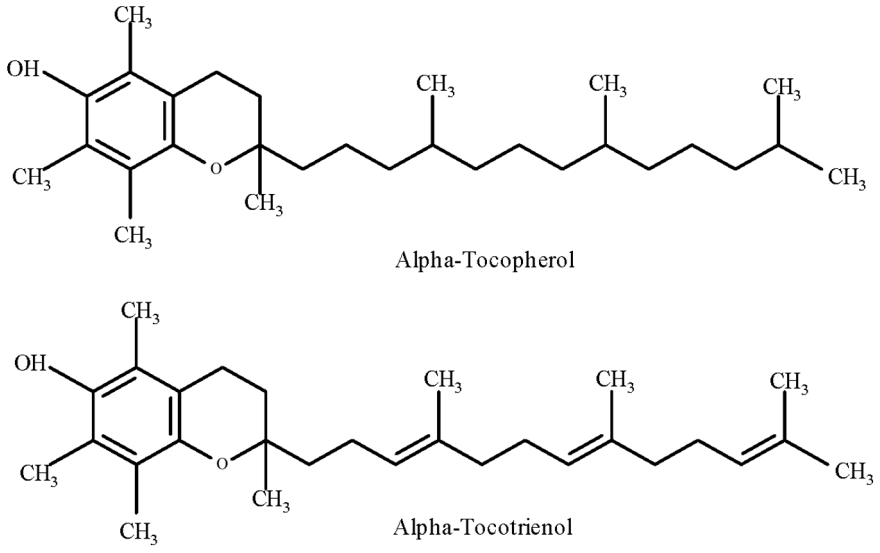
Materials used for chromatographic analysis

Column chromatography (CC) was carried using silica gel 70–230 mesh (Fluka, Switzerland) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Thin-layer chromatography (TLC) was performed on silica gel GF_{254} precoated plates (Fluka). The chromatograms were visualized under UV light at 365 nm before and after exposure to ammonia vapour and $AlCl_3$ reagent (1 g powder of $AlCl_3$ in 100 ml of ethanol, R_1). *Solvent systems*: S_1, dichloromethane/methanol (9 : 1); and S_2, benzene/ethyl acetate (8 : 2), were used for TLC, whereas S_3, 15% aqueous acetic acid, and S_4, *n*-butanol/acetic acid/water and BAW (4 : 1 : 5, top layer), were used for the paper chromatography. The nature of the sugar was confirmed by TLC using cellulose F_{254}, *n*-butanol/pyridine/acetic acid/ethyl acetate/H_2O (50 : 20 : 10 : 25 : 20) (S_5), and aniline hydrogen phthalate spray reagent (R_2).The reagents and chemicals used were of analytical grade. After spraying with R_1, dry chromatogram was heated at 105°C for 10 min and visualized under UV light.

Isolation and identification of compounds

The ethanolic extract (TE, 60.0 g) was subjected to silica gel CC, eluted with dichloromethane (DCM) and a dichloromethane and methanol gradient with increasing polarity. Two main fractions were obtained, fraction I (6.4 g, DCM/MeOH, 8 : 2) and fraction II (17.0 g, DCM/MeOH, 6 : 4). The first fraction, Fr-I, was subjected to repeated column chromatography using silica gel (250 g) and eluted with DCM/MeOH (8 : 2) as an eluent to give compound **1**. A semi-pure compound was obtained, followed by an application on Sephadex LH-20 (methanol) to give pure compound **1** (30 mg). Fr-II was chromatographed on Si CC and eluted with increasing polarities of DCM/MeOH mixtures, giving four semi-pure compounds, followed by application, separately, on Sephadex LH-20 (methanol) to give pure compounds, **2** (22 mg), **3** (18 mg), **4** (25 mg), and **5** (15 mg). The purity of compounds **1–5** was checked by TLC using a solvent system S_1 for compounds **1** and **2**, whereas S_2 was used for compounds **3–5**. While PC using the solvent systems S_3 and S_4 were used for checking the purity of compounds **1–5**, spray reagent R_1 was used for all compounds **1–5**. Structure elucidation of five compounds was realized through R_f values, color reactions, chemical investigations (acid hydrolysis for compound **5**), and spectral investigations as UV and NMR [20,21]. The acid hydrolysis was carried out with 5% HCl for 3 h under reflux followed by extraction with ethyl acetate to get aglycones where the aqueous liquor was analyzed for sugar [22], as well as comparison with the available reference samples. All compounds were characterized mainly by spectroscopic methods, UV, MS, ^1H, ^{13}C NMR, and through comparison of physical and spectral data with that in the literature. They were identified as quercetin **1**, kaempferol **2**, naringenin **3**, apigenin **4**, and

Figure 1



α-Tocopherol and α-tocotrienol structures

hesperidin **5** (Fig. 2). Their spectral data of isolated compounds were illustrated as the following:

Quercetin **1**: Yellow needle crystals, m.p. 310–313°C, R_f: 0.26 (S_3), 0.76 (S_4), UV: λ_{max} (nm) MeOH: 257, 273 sh, 306 sh, 369; +NaOMe: 262, 328 sh, 440; +$AlCl_3$: 271, 313sh, 445; +$AlCl_3$/HCl: 267, 300 sh, 358 sh, 425; +NaOAc: 274, 325 sh, 395; +NaOAc/H_3BO_3: 264, 295 sh, 386; ^{1}H NMR (DMSO-d_6): δ (ppm) 12.49 (1H, s, OH-5), 7.67 (1H, d, *J*=2.2 Hz, H-2′), 7.54 (1H, dd, *J*=2.2 and 8.4 Hz, H-6′), 6.89 (1H, d, *J*=8.4 Hz, H-5′), 6.40 (1H, d, *J*=2.0 Hz, H-8), 6.19 (1H, d, *J*=2.0 Hz, H-6); ^{13}C NMR: δ (ppm) 176.6 (C-4), 164.0 (C-7), 160.9 (C-5), 156.4 (C-9), 147.6 (C-2), 147.2 (C-4′), 145.2 (C-3′), 135.8 (C-3), 122.1 (C-1′), 120.3 (C-6′), 115.6 (C-5′), 115.2 (C-2′), 103.2 (C-10), 98.4 (C-6), 93.5 (C-8).

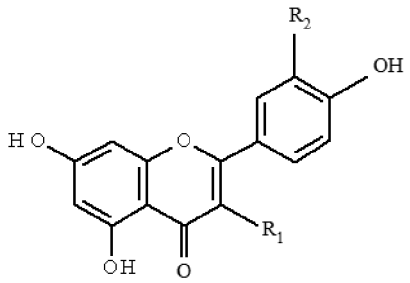
Kaempferol **2**: Yellow crystals, m.p. 275–278°C, R_f: 0.40 (S_3), 0.83 (S_4), UV: λ_{max} (nm) MeOH: 267, 325 sh, 367; +NaOMe: 285, 322, 428; +$AlCl_3$: 265, 305 sh, 350, 420; +$AlCl_3$/HCl: 265, 305 sh, 350, 420, +NaOAc: 275, 300 sh, 380; +NaOAc/H_3BO_3: 270, 295 sh, 370; ^{1}H NMR (DMSO-d_6): δ (ppm): 12.52 (1H,s,OH-5), 8.05 (2H, d, *J*=8.0 Hz, H-2′/6′), 6.91 (2H, d, *J*=8.0 Hz, H-3′/5′), 6.41 (^{1}H, d, *J*=1.8 Hz, H-8), 6.18 (^{1}H, d, *J*=1.8 Hz, H-6); ^{13}C NMR: δ (ppm) 176.0 (C-4), 164.1 (C-7), 161.1 (C-5), 159.4 (C-4′), 156.3 (C-9), 146.8 (C-2), 133.6 (C-3), 129.5 (C-2′/6′), 122.7 (C-1′), 113.4 (C-3′/5′), 103.1 (C-10), 98.3 (C-6), 93.6 (C-8).

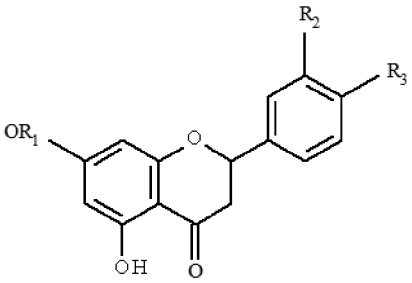
Naringenin **3**: Yellow needles, m.p. 250–252°C, R_f: 0.32 (S_3), 0.87 (S_4), UV: λ_{max} (nm) MeOH: 289, 327; +NaOMe: 250, 273 sh, 323; +$AlCl_3$: 305, 373; +$AlCl_3$/HCl: 305, 371; +NaOAc: 290, 330 sh; +NaOAc/H_3BO_3: 293, 330 sh; ^{1}H NMR (DMSO-d_6): δ (ppm): 12.16 (1H, s, OH-5), 7.37 (^{2}H, d, *J*=8.4 Hz, H-2′/6′), 6.86 (2H, d, *J*=8.4 Hz, H-3′/5′), 5.89 (^{2}H, s, H-6/8), 5.46 (1H, d, *J*=2.8 Hz, H-2), 3.40, 2.69 (2H, dd, *J*=13.0, 17.1 Hz, H-3), ^{13}C NMR: δ (ppm) 196.4 (C-4), 166.7 (C-7), 163.6 (C-5), 163.0 (C-9), 157.9 (C-4′), 128.7 (C-1′), 128.1 (C-2′/6′), 115.4 (C-3′/5′), 101.8 (C-10), 96.1 (C-6), 95.2 (C-8), 78.4 (C-2), 42.0 (C-3).

Apigenin **4**: Light yellow needles, m.p. 348–350°C, R_f: 0.13 (S_3), 0.88 (S_4), UV: λ_{max} (nm) MeOH: 267, 298 sh, 335; +NaOMe: 275, 325 sh, 392; +$AlCl_3$: 275, 301, 347, 382; +$AlCl_3$/HCl: 275, 299, 344, 381; +NaOAc: 274, 300 sh, 380; +NaOAc/H_3BO_3: 267, 301 sh, 337; ^{1}H NMR (DMSO-d_6): δ (ppm): 12.94 (1H,s,OH-5), 7.94 (2H, d, *J*=9 Hz, H-2′/6′), 6.94 (2H, d, *J*=9 Hz, H-3′/5′), 6.68 (^{1}H, s, H-3), 6.52 (1H, d, *J*=2.1 Hz, H-8), 6.25 (1H, d, *J*=2.1 Hz, H-6); ^{13}C NMR: δ (ppm) 182.0 (C-4), 164.6 (C-7), 164.2 (C-2), 162.0 (C-4′), 161.9 (C-5), 157.8 (C-9), 129.0 (C-2′/6′), 121.8 (C-1′), 116.5 (C-3′/5′), 104.2 (C-10), 103.4 (C-3), 99.2 (C-6), 94.2 (C-8).

Hesperidin **5**: Pale yellow powder, m.p. 259–262°C, R_f: 0.79 (S_3), 0.60 (S_4), UV: λ_{max} (nm) MeOH: 285, 330; +NaOMe: 287, 355; +$AlCl_3$: 308, 377; +$AlCl_3$/HCl: 308, 377, +NaOAc: 285, 330; +NaOAc/H_3BO_3: 285, 332; ^{1}H NMR (DMSO-d_6): δ (ppm): 12.03 (1H, s, OH-5), 6.95 (1H, d, *J*=2.0, H-2′), 6.88 (1H, *J*=8.0, H-5′), 6.83 (1H, dd, *J*=8.0, 2.0, H-6′), 6.14 (1H, d,

Figure 2





	R_1	R_2
Apigenin	H	H
Kaempferol	OH	H
Quercetin	OH	OH

	R_1	R_2	R_3
Naringenin	H	H	OH
Hesperidin	Rut	OH	OCH_3

Rut =rutinose (6-*O*-α-rhamnosyl-D-glucose)

Structures of isolated compounds

J=2.0, H-8), 6.13 (1H, d, *J*=2.0, H-6), 5.50 (1H, dd, *J*=11.1, 5.3, H-2), 4.97 (1H, d, *J*=7.2, H-1″), 4.54 (^{1}H, br s, H-1‴), 3.78 (3H, s, OCH_3-4), 3.29–3.63 (9H, m, sugar protons), 3.11 (1H, dd, *J*=17.3, 11.1 Hz, H-3α), 2.76 (1H, dd, *J*=17.1, 5.2 Hz, H-3β), 1.07 (3H, d, *J*=6.2 Hz, H-6); ^{13}C NMR: δ (ppm) 197.2 (C-4), 165.5 (C-7), 163.3 (C-5), 162.8 (C-9), 148.2 (C-4′), 146.7 (C-3′), 131.1 (C-1′), 118.2 (C-6′), 114.3 (C-2′), 112.3 (C-5′), 103.6 (C-10), 100.9 (C-1‴), 99.7 (C-1″), 96.7 (C-6), 95.9 (C-8), 78.7 (C-2), 76.6 (C-3″), 75.8 (C-5″), 73.3 (C-2″), 72.4 (C-4‴), 71.0 (C-4″), 70.6 (C-2‴), 69.7 (C-3‴), 68.5 (C-5‴), 66.4 (C-6″), 56.0 (OCH_3), 42.0 (C-3), 18.2 (C-6‴).

Biological investigation

Animals

Adult albino male mice (*n*=50) weighing 20–25 g were obtained from the Animal House Colony of the National Research Center, Dokki, Egypt. The animals were kept individually in a wire-bottomed cages at room temperature (25±2°C) under 12 h dark/light cycles. They were maintained on a standard laboratory diet and water *ad libitum*. The animals were allowed to acclimatize their new conditions for 1 week before commencing experiment, and then they were allocated into five groups (10 mice/group).

Experimental design

Selection of the doses of BaP of the aerial part of *C. reticulata* (TE and PE). The animals were acclimatized for a period of 1 week and were classified into the following groups: group 1, which included untreated control animals for 4 weeks treatment period; group 2, including animals treated with DMSO for 4 weeks; group 3, including animals treated with a single dose of 50 mg/kg body weight of BaP dissolved in DMSO; and groups 4 and 5, in which animals were orally treated (20 mg/kg body weight) with TE and PE for 4 weeks. At the end of the experimental period, five animals were killed and fasting blood samples were withdrawn from the retro-orbital venous plexus under diethyl ether anesthesia. Blood samples were received in EDTA containing tubes for the comet assay. Afterwards, the other five animals were killed and the liver samples were removed and stored at −80°C for DNA fragmentation.

Quantitative analysis of DNA fragmentation

DNA fragmentation in liver tissue was determined according to the method described by Perandones *et al*. [23]. In brief, 10–20 mg of liver or kidney tissues were ground in 400 µl hypotonic lysis buffer (10 mmol/l Tris base, 1 mmol/l EDTA and 0.2% Triton X-100), centrifuged at 3000*g* for 15 min at 4°C and the supernatant containing small DNA fragments was separated. One-half of the volume was used for gel electrophoresis and the other half together with the pellet containing large pieces of DNA were used for quantification of fragmented DNA by the diphenylamine. The samples were treated with equal volumes of absolute isopropyl alcohol and 0.5 mol/l NaCl to precipitate the DNA, stored at 20°C overnight and centrifuged at 2000*g* for 15 min. The proportion of fragmented DNA was expressed as a percentage of total DNA appearing in the supernatant fractions to the total amount of DNA in the pellet and supernatant.

Comet assay (single-cell gel electrophoresis)

The procedure used for measuring the degree of DNA damage was the comet assay, which was performed as described by Singh *et al*. [24]. The whole blood was diluted with PBS (Sigma Chemicals, Germany). Add equal volumes of diluted blood and 1% low melting point agarose (LMPA; Sigma Chemicals) to the coated slide. Place the cover slip and put the slide on a slide tray resting on ice packs until the agarose layer hardens (~5–10 min). Gently slide off the cover slip and add a third agarose layer (80 µl LMPA) to the slide. Replace the cover slip and return to the slide tray to allow the agarose layer to solidify (~5–10 min). The slides were made to allow DNA unwinding and expression of alkali-labile sites and electrophoresis was conducted by applying 300 V and 30 mA in the dark for 40 min. Each slide was stained with ethidium bromide (Sigma Chemicals). A total of 100 randomly captured comets from each slide were examined at ×400 using an automated fluorescence microscope (Nikon, Tokyo, Japan) with an excitation filter of 520–590 nm and the images were captured on a computer equipped with the comet score software (Komet IV). To quantify the DNA damage, tail length and tail moment were evaluated. Tail length (length of DNA migration) is related directly to the DNA fragment size and presented in micrometers. It was calculated from the center of the cell. The tail moment was calculated as the product of the tail length and the fraction of DNA in the comet tail.

Statistical analysis

The statistical evaluation was performed using two-tailed Student's *t*-test. A difference at *P* value of less than 0.05 was considered statistically significant.

Results and discussion

Phytochemical analyses

The preliminary phytochemical investigation of the ethanolic extract of the aerial part of *C. reticulata* revealed the presence of carbohydrates, phytosterols, flavonoids, leucoanthocyanins, and tannins, whereas triterpenoids, cardiac glycosides, anthraquinones, alkaloids, and saponins were absent. These results are in agreement with earlier studies conducted by other researchers in India and Sudan [25–27].

GLC analysis of the unsaponifiable fraction revealed the presence of 24 compounds (Table 1) that are classified into hydrocarbon compounds, which were the major content (61.56%) followed by triterpene compound, β-amyrin, (14.83%), tocochromanols (12.30%), and phytosterols (11.24%). Hexacosane (14.68%) was the higher hydrocarbon content.

Tocochromanols are composed mainly of α-tocotrienol (9.97%) and α-tocopherol (2.40%), which belong to the group of vitamin E compounds (Fig. 1). These compounds were detected for the first time in the unsaponifiable fraction of the aerial part of *C. reticulata*.

Cholesterol (4.94%) was the major phytosterol, followed by campesterol (2.94%), stigmasterol (2.50%), and β-sitosterol (0.86%). The fruit peel oil furnishes a number of constituents; cholesterol, campesterol, stigmasterol, and β-sitosterol have been reported [28]. Furthermore, β-sitosterol (34.4%), campesterol (24.2%), stigmasterol (18.1%), and cholesterol (3.1%) were determined by GC–MS in roots [29].

GLC analysis of fatty acid methyl esters enabled the identification of 15 fatty acids, nine saturated fatty acids together with six unsaturated fatty acids (Table 2). In the present study, we showed that the saturated fatty acid methyl esters represent 67.58% and the major acids were butyric acid (27.85%), caproic acid (16.94%). On the other hand, the unsaturated fatty acids represented 32.42% of the total fatty acids fraction, in which 5,8,11,14,17-eicosapentaenoic acid (9.32%) and *cis*-13-docosenoic acid (7.93%) were the most abundant identified unsaturated fatty acids.

Many fatty acids were isolated and identified from the fruit peels of *C. reticulata* [30]. Different varieties of *C. reticulata* in the seed oil of Nigerian plant revealed the presence of fatty acids such as palmitic (12.10–28.00%), oleic (26.10–45.30%), linoleic (29.00–38.00%), and other fatty acids including stearic and linolenic acids [31]. The seeds of *C. reticulata* are reported to have linoleic acid, palmitic acid, oleic acid, linolenic acid, and stearic acid, whereas linoleic acid is the major fatty acid present in seed oil [32,33].

The components of essential oils from the aerial part and fruit peel of *C. reticulata* blanco (mandarin) cultivated in Saudi Arabia are shown in Table 3. Using GC–MS, 22 components were identified. In each oil 12 compounds were determined in the aerial part oil linalool (91.02%); the oxygenated monoterpene hydrocarbon was detected as a major component; however, limonene (67.29%) was the most abundant in the fruit peel oil.

Similar to our findings, limonene (69.90%), the monoterpene hydrocarbon, was the main constituents of essential oil from fresh peels of *C. reticulata*, cultivated in Pakistan [34]. Fanciullino *et al.* [35] investigated the chemical variability of peel and leaf oils of 35 mandarin cultivars, belonging to five different species. All the investigated samples exhibited limonene (95.70–62.50%) as the major component in the peels oil and linalool (≤59.60%) as the principal component in the aerial part oil [35]. Recently, limonene was reported as the major component (91.70–95.10%) of the peel oil of nine cultivars of mandarins belonging to the species *C. reticulata* blanco [36].

Isolation and purification of the two chromatographic fractions I and II delivered five compounds. Structure elucidation of all isolated compounds was realized through R_f values, color reactions, chemical investigations, and spectral investigations as UV and NMR [20,21]. The two chromatographic fractions I and II were subjected to column chromatography to get quercetin **1** from Fr-I and four compounds, kaempferol **2**, naringenin **3**, apigenin **4**, and hesperidin **5** were isolated from Fr-II. Characterization of the isolated compounds (Fig. 2) was achieved through their physical, chemical, chromatographic, and spectral analyses (UV, MS, ^{1}H NMR, and ^{13}C NMR). The analytical data were in agreement with those reported in the other studies in the literature [20,21,37].

Biological analysis

Single-cell gel electrophoresis called 'comet assay' after the shape of the images (comets) seen under the microscope. To study DNA damage and repair, comet assay is a basic, rapid, and sensitive technique. It is a micro gel electrophoresis procedure that allows the measurement of DNA damage in each cell. The alkaline version, introduced by Singh *et al.* [24], diagnoses DNA breakage, alkali-labile sites, open repair sites, and cross links.

For this technique, cells are mixed with agarose gel, which is spread onto a microscope slide. High salt concentrations and detergents are then lysed with the cells. In the same alkali buffer, the remaining nuclear DNA is afterwards denaturated and electrophoresed. The DNA fragments voyage out of the nucleus and head to the positive pole. The slides are dyed with a fluorochrome-like ethidium bromide after electrophoresis. A number of damage parameters are measured by using an image analysis procedure [24,38].

DNA-strand breaks and alkali-labile sites become obvious, and the extent of DNA migration correlates with the amount of DNA damage in the cell in its alkaline version, which is mainly manipulated. To detect DNA single-strand breaks and/or alkali-labile sites with the single-cell approach typical of cytogenetic assays, the comet assay merges the simplicity of biochemical methods. The comet assay has a number of benefits like its basic and quick performance, its accuracy for detecting DNA damage, the examination of data at the level of the individual cell, the manipulation of severely tiny cell samples, and the usability of virtually any eukaryote cell population. On the economical level, the cost of performing the assay is extremely low, which greatly facilitates and enhances the possibilities of comet measurements [38,39]. The comet assay has manifold applications in fundamental research for DNA damage and repair, and in genotoxicity testing of novel chemicals and pharmaceuticals [40]. Here, we describe the method of alkaline comet assay to detect and measure DNA damage. Generation of DNA damage is considered to be an important initial event in carcinogenesis [41].

The results of DNA fragmentation in liver of mice are presented in Table 4 (Figs 3 and 4). The BaP significantly increased the DNA fragmentation in the liver by 19.5% as compared with the control group (7.50%). Treatments with PE and TE of *C. reticulata* significantly brought down the levels of DNA damage to 11.80 and 9.30%, respectively. In addition, the percent of DNA inhibition against BaP using TE was in particular higher (85.00%) compared with the PE fraction (64.17%).

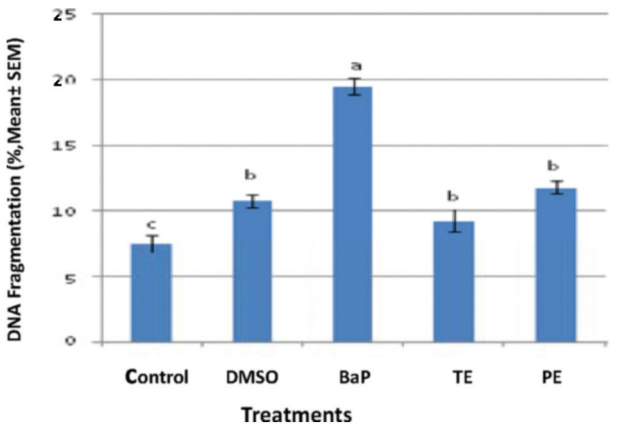
Table 4 DNA fragmentation detected in mice from the experimental group treated with different *Citrus reticulata* fractions

Treatments	DNA fragmentation (%) (mean±SEM)	Change	Inhibition (%)
Control	7.50 ± 0.65^{c}	0	0
DMSO	10.80 ± 0.48^{b}	3.30	72.50
BaP	19.50 ± 0.66^{a}	12.00	0
TE	$9.30\pm0.85^{b,c}$	1.80	85.00
PE	$11.80\pm0.44^{b,c}$	4.30	64.17

BaP, benzo(a)pyrene; DMSO, dimethyl sulfoxide; PE, petroleum ether fraction; TE, total ethanol extract. Means with different superscripts (a,b and c) between groups in the same column are significantly different at $P<0.05$.

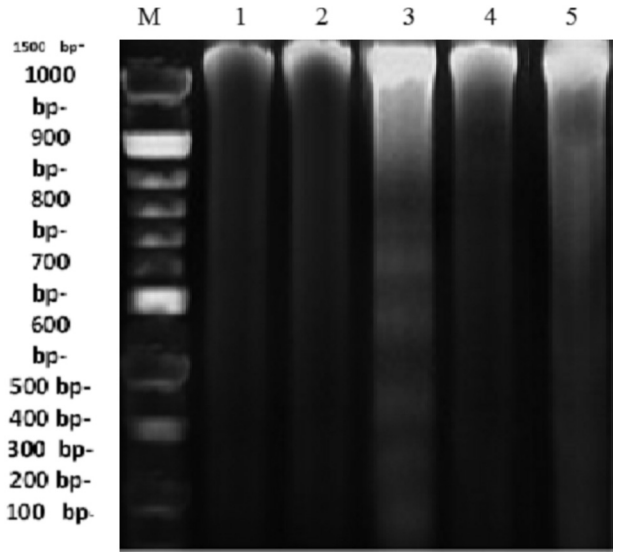
In the present study, the amount of DNA damage in the cell was estimated from tail length as the extent of the migration of the genetic material in the direction of the anode. During electrophoresis, cell DNA was seen to more rapidly migrate toward the anode at higher concentrations than at the lowest concentration. Even the comet tail tended to increase when exposed with

Figure 3



DNA damage in mice from the experimental group treated with TE and PE of *C. reticulata*. BaP, benzo(a)pyrene; DMSO, dimethyl sulfoxide; PE, petroleum ether; TE, total ethanolic extract

Figure 4



DNA fragmentation detected with agarose gel of DNA extracted from mice samples by using the DNA gel electrophoresis laddering assay. M represents Ladder DNA (100 bp). Lane 1 represents the control group. Lane 2 represents the DMSO group. Lane 3 represents the BaP group. Lane 4 represents TE. Lane 5 represents PE. BaP, benzo (a)pyrene; DMSO, dimethyl sulfoxide; PE, petroleum ether; TE, total ethanolic extract

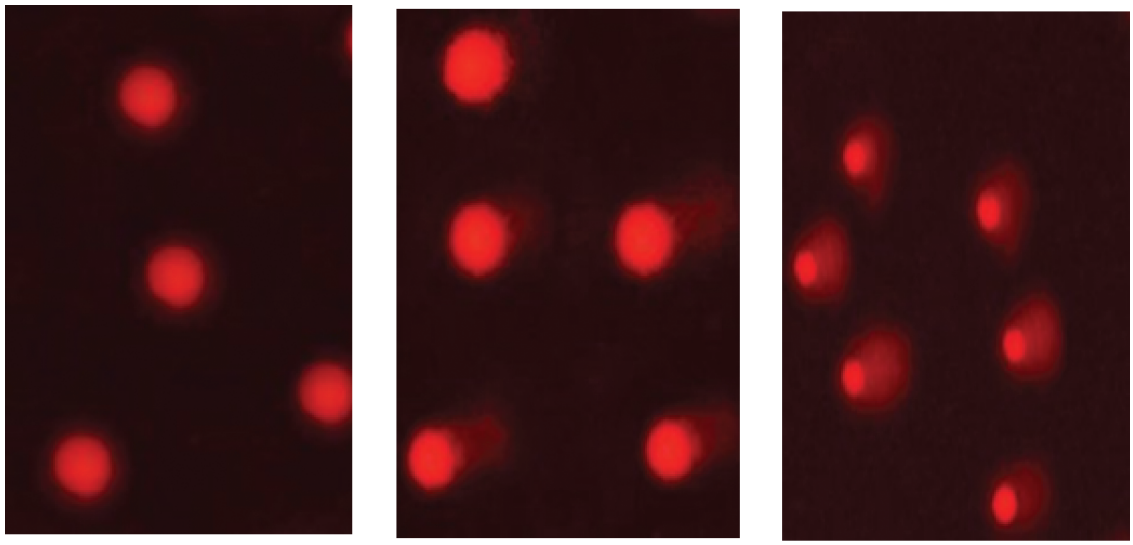
BaP, TE, and PE (Fig. 5). It shows DNA damage determination, as evident from Table 4, corresponds to DNA from control, DMSO, BaP, TE, and PE fraction groups of mice, respectively. It was observed that the treatment with BaP drug resulted in significantly ($P<0.01$) higher DNA damage as compared with controls. However, DNA damage was significantly ($P<0.05$) reduced in mice treated with TE. In addition, treatment of mice with PE decreased significantly the DNA damage induced by BaP treatment; however, TE was more effective than was PE in the reduction of the DNA damage (Table 5).

Evaluation of the role of plant extracts and their phytochemical antigenotoxic and antimutagenic compounds is essential to prevent cancer and other disorders [42]. No antigenotoxic activity of *C. reticulata* has been demonstrated till now. In the present study, antimutagenic and antigenotoxic effects of *C. reticulata* against DNA damage induced by BaP in mice using the comet assay as trying to get new potential antigenotoxic agents for possible use in protection. Therefore, the effect of TE and PE of *C. reticulata* was tested for DNA damage induced by BaP in mice.

Our investigation showed that TE gives higher activity against genotoxicity. This may be attributed to the presence of various secondary metabolites that may be responsible for the pharmacological and therapeutic properties of the plant [43,44]. Moreover, the presence of flavonoid compounds in the crude ethanolic extract is responsible for its protective effect [45].

Carvalho *et al.* [42] stated that flavonoids have antimutagenic activity, whereas Silva *et al.* [46] reported some flavonoids are potentially harmful chemicals because they show mutagenic activity in bacteria and mammalian test. The mutagenic property of flavonoids can be explained by the fact that they can act as pro-oxidants, which may result in oxidative DNA

Figure 5



Visual score of normal DNA (class 0), DNA damage (classes 1 and 2), and DNA damage (class 3) using the comet assay in mice samples exposed to DMSO, BaP, TE, and PE. BaP, benzo(a)pyrene; DMSO, dimethyl sulfoxide; PE, petroleum ether; TE, total ethanolic extract

Table 5 Visual score of DNA damage in mice liver tissues from different treated groups

Treatments	Number of animals	Number of cells		Class**				DNA damaged cells (%) (mean±SEM)
		Analyzed*	Comets	0	1	2	3	
Control	4	400	33	367	25	8	0	8.30 ± 0.65^{c}
DMSO	4	400	54	346	21	17	16	13.50 ± 0.87^{b}
BaP	4	400	103	297	24	46	33	25.80 ± 1.70^{a}
TE	4	400	47	353	21	14	12	$11.80\pm1.18^{b,c}$
PE	4	400	51	349	26	14	11	$12.80\pm0.63^{b,c}$

BaP, Benzo(a)pyrene; PE, petroleum ether fraction; TE, total ethanol extract. *Number of cells examined per a group. **Class 0=no tail; 1=tail length < diameter of the nucleus; 2=tail length between 1X and 2X the diameter of the nucleus; and 3=tail length > 2X the diameter of the nucleus. Means with different superscripts (a, b and c) between groups in the same column are significantly different at $P<0.05$.

damage, with consequent mutagenicity. Furthermore, flavonoids can act as inhibitors or inducers of a variety of cellular processes containing the inhibition of growth factor signaling pathways and enzyme activity and induction of tumor suppressor genes, apoptosis, and DNA damage [47].

Consequently, TE of *C. reticulata* was effective in preventing DNA damage induced by BaP and showed good antigenotoxic activities because of the protective effects of flavonoid compounds against DNA damage. Activity of PE fraction against genotoxicity showed no significant DNA damage ($P<0.05$) compared with BaP and the trend of reduced comet tail length. This is may be attributed to the fact that PE fraction contained fatty acids and sterols [48] and the presence of vitamin E plays an important role in human nutrition and health [49], whereas tocochromanols that belong to the group of vitamin E have good antioxidant activity due to the ability of their heterocyclic chromanol ring system to give the phenolic hydrogen to lipid-free radicals [50]. Lewinska *et al.* [51] stated that fatty acids do not show any oxidative stress-mediated cytotoxic or genotoxic effects. Moreover, there is an interrelationship between fatty acids and vitamin E that is important to many aspects of metabolism [52]. Furthermore, vitamin E and the unsaponifiable fraction have antimutagenic activity [53]. In another study, Saeidnia *et al.* [54] recorded that β-sitosterol is not genotoxic and/or cytotoxic agent.

Conclusion

The ethanolic extract TE of *C. reticulata* cultivated in Saudi Arabia was effective in preventing DNA damage induced by BaP and showed good antigenotoxic activity. GLC analysis of the PE fraction showed the presence of vitamin E for the first time. In addition, essential oils of peels and aerial part were detected. Chromatographic isolation of the ethanolic extract resulted of five flavonoids, quercetin, kaempferol, naringenin, apigenin, and hesperidin. Both flavonoid compounds and vitamin E play an important role in human nutrition and health. These results showed that TE of the aerial part of *C. reticulata* is useful to reduce the genotoxicity induced by hazardous chemical agents and in finding out the quality of the raw material for drug production.

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Conflicts of interest

There are no conflicts of interest.

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