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Potential Anti-Cancer Activity and Protection from Hyperlipidemia by *Citrus limon* Fruit

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Abstract

The present research aimed at studying different bioactive constituents and the potential hypolipidemic effect of the roasted lemon powder along with the anticancer activity of its methanol extract. Volatile compounds and fatty acids′ profile in roasted lemon were analyzed. Individual phenolic compounds and total phenolic and flavonoidal contents and DPPH• scavenging capacity of the methanol extract were determined. The anticancer activity of the extract was evaluated in human cell line from hepatocellular carcinoma (Hep-G2) and breast adenocarcinoma (MCF-7). The hypolipidemic effect of roasted lemon was assayed in Triton X-100 induced hyperlipidemic rat. Results demonstrated D-Limonene to be the major volatile compound while hisperdin was the main phenolic compound. Total phenolics, flavonoids and DPPH• scavenging activity were 8.59± 0.098 mg gallic acid equivalent, 8.10± 0.17 mg catechin equivalent and 5.05± 0.07 mg trolox equivalent/g, respectively. Linoleic acid was the major fatty acid while linolenic acid was present as 10.04%. The anticancer activity of the extract showed IC50 to be 240 and 148µg/ml towards Hep-G2 and MCF-7, respectively. *In-vivo* experiment demonstrated that intake of roasted lemon improved hyperlipidemia and reduced cardiovascular risk factor, malondialdehyde, transaminases, creatinine and body weight gain. It could be concluded that roasted lemon methanol extract showed anticancer activity towards Hep-G2 and MCF-7, with superiority to MCF-7. The roasted lemon possessed hypolipidemic and antioxidant activities along with cardio-, hepato- and reno-protective effects with body weight reducing ability. Such effects might be related to the presence of volatile compounds specially D- Limonene, the phenolic and flavonoidal compounds and linolenic acid.

Keywords: Roasted lemon; Volatile compounds; Methanol extract; Phenolic compounds; Fatty acids; Cancer cell line; Hyperlipidemic rats.

Introduction

Citrus limon fruit (L.) is considered as a rich source of bioactive constituents like phenolic acids, flavonoids, terpenes, tannins, fibers, vitamin C and essential oil represented mainly by limonene [1,2]. Lemon fruit was demonstrated previously to possess different biological activities including antioxidant, anti-inflammatory, antimutagenic and anti-proliferated effects [3,4]. Therefore, as a whole, lemon fruit may be considered as a functional food that may have protective effect towards chronic diseases. Due to the aforementioned bioactivities, lemon fruit may have cardiovascular protective effect and might possess anti-cancer effect. The daily use of lemon in culinary purpose in both fresh and dry roasted forms stimulates the research team to study its anti-cancer effect along with the protective effect towards hyperlipidemia which has a strong correlation to cardiovascular diseases (CVDs).

Inflammation and oxidative stress are among the considered main causes of chronic diseases like cancer and CVDs. Hypercholesterolemia (TC) and elevated low density lipoprotein-cholesterol (LDL-C) together with reduced high-density lipoprotein (HDL-C) and elevated TC/HDL-C are considered risk factors for CVDs. Elevated oxidative stress

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represented by high malondialdehyde (MDA) is an another risk factor for CVDs [5] which might be due to the possible production of oxidized LDL.

Triton X-100 is a nonionic surfactant which is used to induce experimental hyperlipidemia in rats for testing new hypolipidemic remedies [6-8]. In addition to hyperlipidemia, Triton X-100 was reported to produce elevation in liver fat and inflammation that may have a hand in inducing hypercholesterolemia and CVDs in such rat model [7]. Drug used to reduce cholesterol like atorvastatin, has a low bioavailability [9] therefore it must be prescribed in large dose which could subsequently increase the drug side effects on liver and kidney along with induction of myopathy [10]. Therefore, nutraceuticals from lemon fruit and the whole lemon fruit that may have no side effects might be an alternative or complementary remedy to atorvastatin.

Breast and liver cancer are among the common cancers worldwide. Liver cancer is one of the most resistant types of cancer to chemotherapy therefore searching new remedy is crucial, lemon alcohol extract might be beneficial. Also, more than 2.3 million new cases and 685,000 deaths from breast cancer were reported in 2020. It has been predicted that by 2040 the burden from breast cancer would increase to more than 3 million new cases and 1 million deaths per year [11]. The presence of phenolic compounds and a part of the volatile bioactive constituents in lemon alcohol extract might have anticancer effect [4,12].

The aim of the present research was to study the essential oils, fatty acids′ composition, total phenolic, total flavonoids and individual phenolic acids and flavonoids in roasted dry lemon. The *in-vitro* antioxidant activity of roasted lemon was also assessed. *In-vivo* hypolipidemic effect of the whole roasted lemon powder was studied in triton X-100 induced hyperlipidemic rats. The anticancer effect of the methanol extract was assayed using liver and breast human cancer cell line. Roasted lemon was used in the present study instead of fresh lemon, to investigate whether the preserved roasted lemon still possesses functional activity or not.

Materials and Methods

Materials:

The lemon fruits (*Citrus limon* L.), Family Rutaceae, Egyptian variety, were obtained from local market, Cairo, Egypt and washed thoroughly to remove dust and other foreign materials. Reference standard materials and reagents as gallic acid, catachin, chlorogenic acid, caffeic acid, cinnamic acid, ferulic acid, p-coumaric, caffeine, Hesperidin, fatty acids, Folin–Ciocalteu, DPPH, sodium carbonate were purchased from Sigma-Aldrich Chemical Co. Inc. (Louis, USA). HPLC-grade acetonitrile and methanol were obtained from thermo fisher chemicals, USA). Triton X-100 was purchased from Loba Chemie Pvt Ltd, a manufacturer of laboratory reagents and fine chemicals (Mumbai, India).

Methods:

Dehydration technique.

Pretreatment of the whole lemon fruits:

Lye treatment of whole lemon fruits with 1% sodium hydroxide solution was applied to remove outer waxy layer to improve moisture diffusion through outer peel as recommended by Nasr et al. [13].

Drying process

Whole lemon fruit was dried at 60 °C in a hot air oven (Fisher Bioblock Scientific, Model Do 315H) until the moisture content was below 20% according to Özcan et al.[14]. Then the dried whole lemon fruit was roasted at 180 ºC for 1 hour to get the final product as illustrated in Fig. 1. Roasted lemon was crushed into fine powder for the subsequent experiments.

Fig 1. Roasted lemon

Determination of total phenolics, total flavonids, and antioxidant activity

Extraction

One gram of roasted lemon powder was extracted twice with 10 mL of methanol and homogenized using the Ultra-Turrax homogenizer for 20 min. the extract was centrifuged at 10,000 rpm for 20 min. The supernatant was collected prior to analysis.

Determination of total phenolics content:

The total phenolic content was determined by Folin-Ciocalteu (FC) reagent according to Zilic et al. [15]. Briefly, the extract (50 µL) was reacted with 100 µL of FC reagent. After 5 min, the mixture was neutralized with 500 µL of 20% aqueous sodium carbonate solution and the total volume was adjusted to 4 mL with distilled water. After 40 min, the absorbance was measured at 725 nm against the solvent blank. The total phenolic content was obtained from the calibration curve of gallic acid. The result was expressed as mg gallic acid equivalent (GAE) per g of sample.

Determination of total flavonids content:

Colorimetric assay of total flavonid content using aluminum chloride was implemented according to Zilic et al. [15]. Briefly, 100 µL of extract was mixed with 300 µL of a 10% aluminum chloride solution. After 5 min, 300 µL of 5% sodium nitrite was added and the volume adjusted to 2.5ml. The solution was left 5 min and 1.5 mL of 1 M sodium hydroxide was added. The absorbance was measured at 510 nm against the solvent blank. The total flavonid content was obtained from catechin calibration curve, and the result was expressed as mg catechin equivalent (CE) per g of sample.

Determination of DPPH radical scavenging activity:

Free radical scavenging capacity of the roasted lemon extract was determined using the stable DPPH$^{\bullet}$ according to Hwang and Do Thi [16]. The absorbance was measured at 517 nm against a pure methanol after 60 min of incubation in a dark condition at ambient temperature. Percent inhibition of the DPPH free radical was calculated by the following equation:

Inhibition (%) = 100 × [(*A control-A sample)/A control*]

Where:

A control is the absorbance of the control reaction (containing all reagents except the test compound).

A sample is the absorbance with the test compound.

The calibration curve was prepared using Trolox. Results were expressed as mg Trolox equivalents (TE)/g sample).

Assessment of phenolic compounds profile by High Performance Liquid Chromatography (HPLC)

Roasted lemon powder (1g) was pre-extracted by alkaline hydrolysis (20 ml of 2M sodium hydroxide) in a quick fit flask and flushed with nitrogen. The sample was shacked for 4 h at room temperature. After hydrolysis the pH was adjusted to 2 with 6 M hydrochloric acid. Then, the phenolic compounds were extracted twice with 50 ml ethyl ether and ethyl acetate 1:1. The organic phase was separated, collected and evaporated at 45°C to dryness and the remaining was reconstituted in 1ml methanol.

Chromatographic analysis of phenolic compounds was performed using HPLC series 1100 (Agilent Technologies, CA, USA) according to Kim et al. [17] with some modifications. HPLC system equipped with a quaternary pump, an autosampler injector, a diode array detector, and Agilent Eclipse XDB C18 (150 - 4.6 µm; 5 µm) column. Ten microliters of samples were injected. the gradient separation of phenolic compounds was done using acetonitrile (solvent A) and 2 % acetic acid in water (solvent B) at a flow rate of 1 mL/min. Elution was initiated at 0% solvent A; the percentage of solvent A was increased from zero to 15% in 30 min, and to 50% in 20 min, then to 100% for 5 min. finally back to initial point (0 % of A) in 5 min. The benzoic and cinnamic acid derivatives, and flavonid compound were monitored at 280, 320, and360 nm respectively. Identification and quantification of detected compounds were performed by comparing the retention times and peak areas with those of reference standards.

Determination of volatile compounds by Headspace – Solid Phase Microextraction Gas Chromatography – Mass Spectrometry (HS-SPME-GC–MS) analysis:

Volatile compounds in the roasted lemon were extracted and detected using the HS-SPME-GC–MS methods published by Zhang et al. [18]. The extraction method was as follows: 5 g whole dried lemon powder was put in the 20 mL headspace vial then, the sample vial was heated for 20 min at 50°C to accelerate extraction equilibrium of the headspace for volatile compounds. Then volatile compounds extraction was carried out by inserting a 65 µm divinylbenzene/ Polydimethylsiloxane SPME fiber (Supelco, Bllefonte, PA, USA) into the head space of the vial. At the end of the extraction, the fiber was desorbed into the injection port of the GC at 250°C for 5 min.

Gas Chromatography – Mass Spectrometry (GC – MS) analysis:

The analysis was performed on an Agilent 8890 GC System, coupled to a mass spectrometer (Agilent 5977B GC/MSD). Volatile compounds were separated on an HP-5ms fused silica capillary column (30 m × 0.25 mm × 0.25 µm), and the oven temperature program was set as follows: the initial temperature was 50°C, held for 2 min, ramped at 5°C/min to 200°C, then held for 3 min and increased at 10°C/min to 280°C, held for 5 min at final

temperature. The volatiles were injected in the GC with a split less mode. Helium carrier gas was used at a flow rate of 1 mL/min. The injection port temperature was 250°C, and the injection mode was manual. Mass spectrometric detection parameters were set as follows: ionization method EI, electron energy 70 eV, ion source temperature 230°C, MS quadrupole temperature 150°C, mass scanning range mass-to-charge ratio (m/z) 39–500 amu, The isolated peaks were identified by matching them with data from the library of mass spectra (National Institute of Standard and Technology, NIST).

Determination of fatty acid methyl esters by gas-chromatography:

A dried sample of roasted lemon was extracted by petroleum ether (60-80 ºC) using soxhlet and the oil was obtained after evaporation of the solvent under vacuum. The fatty acid composition was determined by the conversion of oil to fatty acid methyl esters prepared by adding 1.0 mL of n-hexane to 15 mg of oil followed by 1.0 mL of sodium methoxide (0.4 mol), according to the modified method of Zahran and Tawfeuk [19]. The mixtures were vortexed for 30 seconds and were allowed to settle for 15 minutes. The upper phase containing the FAMEs was recovered and analyzed by gas chromatography (GC-FID). FAMEs were identified by comparing their relative and absolute retention times to those authentic standards of FAMEs. The fatty acid composition was reported as a relative percentage of the total peak area.

Type and Condition of GC Analysis: Perkin Elmer Auto System XL Equipped with flame ionization detector (FID) was used. Fused silica capillary column ZB-Wax (60 m x 0.32 mm i.d) was used. Oven temperature was maintained initially at 150°C, then programmed from 150 to 220 °C at a rate of 3 °C/ min, then held at 220 °C for 20 min. Injector temperature was 240°C. Detector temperature was 250°C. Carrier gas was Helium with flow rate of 1ml/ min.

Anti-cancer (Cytotoxic) activity of roasted lemon methanol extract

Two cancer cell lines HepG-2 (liver cancer cells) and MCF-7 (breast cancer cells) were supplied from National Cancer Institute, Cairo University, Egypt. The anti-cancer activity of crude methanol extract of roasted lemon was tested using cell line technique [20]. The methanol extract was prepared as the aforementioned method under determination of total phenolic; the pure extract was obtained after evaporation of the solvent under vacuum. The cells of HepG-2 and MCF-7 were separately plated in 96-multiwell plate for 24 hours to be attached to the wall plate before treatment with the extract. Different concentrations from the extract were prepared (0, 62.5, 125, 250 and 500 µg/ ml) and applied to the cell monolayer, 6 well were used for each individual dose. The plate was incubated for 48 hours at 37ºC in 5% CO2 atmosphere, then cells were fixed and stained with sulfo-rhodamin-B stain, then excess stain was washed with acetic acid while the attached stain was recovered with Tris EDTA buffer. Color intensity was then measured in an ELISA reader. Survival fraction from the cancer cells were plotted against the extract concentrations. IC50 (the concentration of the extract which reduces the survival of the exposed cancer cells to 50%) was obtained from the curve.

***In-vivo* hyperlipidemic experiment**

Animals

Male Wistar rats with body weight of 170-180 g were purchased from the Animal House Unit of the National Research Centre, Egypt. The rats were kept in stainless steel cages with 12 hour light/dark cycle and given food and water *ad libitum*. Animals were treated in accordance with the guidelines and regulations of the National Institute of Health Guide for Care and Use of Laboratory Animals (NIH No. 85: 23 revised 1985) and the ethics committee of the National Research Centre, Cairo, Egypt.

Preparation of Triton X-100

Exactly one gram triton was dissolved in saline up to 15 ml. Rats were given 100 mg triton/ kg rat body weight through intra-peritoneal injection according to Gundamaraju et al. [6]. Meanwhile normal control rats were given the same amount of saline intra-peritoneal as being the vehicle

Diets′ composition

Balanced diet was fed to rats of the normal control (NC) and dyslipidemic control (HC) groups. The balanced diet composed of 12% casein, 8% corn oil, 70.5% starch, 5% wheat bran, 3.5% mineral mixture and 1% vitamin mixture. The test group was given a balanced diet containing 5% roasted lemon powder on the expense of starch.

Design of the animal experiment

Three groups were assigned, each of eight rats. Rats of group 1 and 2 were fed on balanced diet while rats of group 3 consumed balanced diet containing 5% roasted lemon powder. In the day 18, rats were fasted overnight and in the subsequent day (day 19) rats of the second and third groups were given 100 mg of triton X-100/kg rat body weight intra-peritoneal while that of the first group were

treated intraperitoneal with saline as the vehicle. The aforementioned diets were continued fed to rats with the same previous sequence. The normal control (NC) group was represented by group 1, group 2 served as the hyperlipidemic control (HC) while the third group was the test group. In the day 21, rats were weighed and fasted. Rats were anesthetized by pentobarbital and blood samples were received in heparinized tubes, and centrifuged for separation of the plasma. Plasma triglycerides (TGs), total cholesterol (TC), HDL-C and LDL-C were determined according to the previously described colorimetric methods [21-24]. Plasma malondialdehyde (MDA) was assessed as biomarker of lipid peroxidation and oxidative stress by applying the method of Satoh [25]. Liver function was assessed by determination of alanine transaminase (ALT) and aspartate transaminase (AST) activities [26]. Plasma creatinine and urea were assessed as indicator of kidney function [27,28]. The ratio of TC/HDL-C was calculated to indicate the risk to cardiovascular diseases [29].

Statistical analysis

Data from *in-vivo* experiment were expressed as mean± SE. Data were statistically analyzed by applying one-way analysis of variance followed by the Tukey multiple comparison test using the SPSS statistical program. Differences were considered significant at P value less than 0.05. Data from anticancer test, *in-vitro* antioxidant activity, total phenolic and flavonoids were expressed as means± SD.

Results

The values of total phenolics, flavonoids and DPPH scavenging activity were 8.59± 0.098 mg GAE/g, 8.10 ± 0.17 mg CE/g and 5.05 ±0.07 mg TE/g roasted lemon, respectively (Table 1). Different phenolic compounds of roasted lemon fruits that analyzed by HPLC were shown in table 2.The major flavonoid was hisperdin followed by catechin, then apegnin-7-glycoside, naringin, rutin and quercetin. Gallic acid, protocatechuic acid, *p*-coumaric acid and cinnamic acid were the prominent phenolic acids followed by caffeic, syringic acid, ferulic and *p*-hydroxybenzoic acid while vanillic acid showed the least level.

The relative percentages of volatile compounds of the roasted lemon are present in table 3. D-Limonene was the major essential oil followed by β-Pinene, γ-Terpinene, α-Pinene and α-Bergamotene (38.94, 22.45, 8.54, 4.44 and 3.60, respectively), the relative % of all other volatile compounds were less than 3.

Table 1. Antioxidant activity as DPPH scavenging activity, total phenolics and total flavonoids contents of roasted lemon fruits.

Parameters	Values
Total phenolic (mg GAE/g)	8.59 ± 0.098
Total flavonids (mg CE/g)	8.10 ± 0.170
Antioxidant activity DPPH (mg TE/g)	5.05 ±0.070

GAE= gallic acid equivalent
CE = catechin equivalent
TE= Trolox equivalent

The fatty acids′ composition of the petroleum ether extract of roasted lemon fruit is demonstrated in table 4. It could be noticed that Linoleic acid (ω-6) was the major fatty acid (34.05%) followed by palmitic which is saturated fatty acid (26.35%) and oleic (21.22%) as representative of ω-9 fatty acid while linolenic acid (ω-3) was present as 10.04%. The percentage stearic, palmitoleic and arachidic were 5.57, 0.57 and 0.55, respectively. The total unsaturated fatty acids represented by 65.88% which were higher than total saturated fatty acids (32.47). The ratio ω-6/ω-3 was calculated to be 3.4/1.

The anticancer results are shown in Fig. 2. It could be noticed that the methanol extract of roasted lemon produced marked anticancer effect towards HepG-2 and MCF-7 human cell line with IC50 value of 240 and 148 µg/ ml, respectively.

The results of the *in-vivo* hyperlipidemia experiment are outlined in table 6 and 6. The injection of rats by triton X-100 produced significant increase in plasma TGs, TC, LDL-C and TC/HDL-C along with reduction of HDL-C compared to the NC group. Feeding rats a diet containing 5% roasted lemon powder produced significant reduction in TG, TC, LDL-C and TC/HDL-C with increased HDL-C compared to the HC group. Only plasma TC and TC/HDL-C of the group given 5% lemon diet showed insignificant change from the NC group while surprisingly the TGs showed significant reduction. Plasma MDA, creatinine, AST and ALT were significantly elevated in the HC group compared to the NC group while urea showed insignificant effect. The test group fed on 5% lemon diet produced significant reduction on such parameters that matched the normal level in case of the MDA. Plasma creatinine and urea of the rats fed on lemon diet demonstrated significant reduction compared to the NC group while ALT and AST activity showed significant higher level. Final body weight and body weight gain of the HC group showed insignificant reduction compared to the NC group. Such parameters demonstrated significant decreases in the

group fed on lemon diet compared to the NC group and insignificant changes compared to the HC group.

Table 2. Different phenolic compounds (µg/g) of roasted lemon fruits by HPLC.

Compound	Concentration (ug/g)
Gallic acid	29.431
Protocatechuic acid	23.303
p-hydroxybenzoic acid	7.005
Catechin	669.927
Chlorogenic acid	ND*
Caffeic acid	14.205
Syringic acid	11.836
Vanillic acid	1.479
Ferulic acid	7.678
p-coumaric acid	21.829
Rutin	69.077
hisperdin	2556.851
naringin	86.390
apegnin-7-glycoside	173.223
Cinnamic acid	20.694
quercetin	11.355
Kaempferol	76.341
Chrysin	ND*

ND= not detected*

Discussion

Citrus limon methanol extract showed marked anticancer effect against Hep-G2 and MCF-7 in the present study which might be due to its total phenolic and flavonoidal contents seen in the current results. Such contents were demonstrated previously to possess anticancer effect which might be related to their anti-inflammatory activity [30]. As a matter of fact the methanol extract in the present study most probably contains volatile constituents due to its extraction on cold. *Citrus limon* and other citrus fruit peel was reported to possess anticancer effect due to presence of essential oil specially limonene [31]. Li et al though reported cytotoxicity effect of *Citrus limon* essential oil however it was of the least effect among 4 citrus fruit, the mechanism include G0/G1 cell cycle arrest in lung cancer [32]. Reduction of the expression of inflammatory cytokine IL-6, cyclooxygenase- 2 and nuclear transcription factor kappa B p65 by *C-limon* in mice ear edema was reported in the aforementioned study together with radical scavenging activity which might be involved in the mechanism of action as anticancer. The flavonoids hesperidin and rutin which are present in the methanol extract as shown from the present study were reported to have anticancer activity [33]. Catechin which is present in appreciable amount in the lemon methanol extract was reported previously to inhibit A549 cells thereby suppress cancer cell proliferation [34].

.**Table 3.** Volatile compounds in the roasted lemon

RT	Compound name	Relative %
4.196	Furfural	1.14
6.142	α-Phellandrene	0.56
6.325	α-Pinene	4.44
6.685	Camphene	0.84
7.292	Sabinene	0.33
7.395	β-Pinene	22.45
7.71	β-Myrcene	1.24
8.414	α-Terpinene	1.03
8.631	p-Cymene	2.83
8.763	D-Limonene	38.94
9.249	β-Ocimene	0.28
9.564	γ-Terpinene	8.54
10.393	Terpinolene	1.33
12.911	Terpinen-4-ol	0.85
13.272	α-Terpineol	1.27
14.754	Carvone	0.57
17.288	δ-EIemene	0.83
18.41	Geranyl acetate	0.25
18.719	β-Elemene	0.96
19.463	β-Caryophyllene	2.4
19.806	α-Bergamotene	3.60
20.315	Aromandendrene	0.41
20.996	α-Gurjunene	0.27

In the study of Xi et al. caffeic acid (9.31-741 µg/g fruit) and chlorogenic acid (2.7-527.5 µg/g fruit) were the dominant phenolic acids in five tested lemon fruit cultivars; gallic was 1.64-9.05 µg/g fruit while ferulic acid was traces [35]. In the aforementioned study, flavanones were the major flavonoid in lemon while hesperidin was the predominant flavanone (10-3315 µg/g fruit) with the presence of rutin as 2.29-30.57 µg/g fruit; however the fresh fruit was

grounded by a freezer mill which is different from the present technique.

Table 4. Fatty acid composition of dried lemon as percentage of total fatty acids.

No.	Fatty acids	Relative concentration (%)
1	Palmitic acid (C16:0)	26.35
2	Palmitoleic acid (C16:1)	0.57
3	Stearic acid (C18:0)	5.57
4	Oleic acid (C18:1)	21.22
5	Linoleic acid (C18:2)	34.05
6	Linolenic acid (C18:3)	10.04
7	Arachidic acid (C20:0)	0.55
Total saturated fatty acids		32.47
Total unsaturated fatty acids		65.88
Omega-6/Omega-3		3.4/1

The reduction in MDA in the *in-vivo* dyslipidemic rats fed on 5% roasted lemon and the *in-vitro* DPPH$^{\bullet}$ scavenging activity of roasted lemon methanol extract might be attributed to the antioxidant property of total phenolic and flavonoid that present in lemon [36]. According to Xi et al. the DPPH scavenging value of the lemon fruit was 3.10 to 7.96 % among 5 lemon cultivars which agreed with the present results [35]. This was supported by a study carried out by Tounsi et al. that showed *in-vitro* antioxidant effect of *C. limon* from four Tunisian varieties [37]. Restoration of reduced glutathione, superoxide dismutase and liver function indicated by ALT and AST was reported on administration of *citrus limon* fruit in rats with liver toxicity [38, 39]. Therefore the improved liver function in the present study on feeding lemon diet might be ascribed to the antioxidant and anti-inflammatory activity of lemon. Elwan et al. also demonstrated the antioxidant effect of the dry lemon fruit [40]. Bouzenna et al. reported that the essential oil of *Citrus limon* has protective effect on hepatotoxicity and nephrotoxicity induced by aspirin in rats which was ascribed to its MDA reducing effect and antioxidant enzymes elevating effect in both liver and kidney [41].

The improved lipid profile of rats after feeding lemon diet in the current study agreed with the study of Trovato et al. and Elwan et al. [40, 42]. The reduction in total cholesterol in rats might be due to presence of flavonoids specially hesperidin in addition to dietary fibers in lemon fruit. Cholesterol reduction by hesperidin might be due to amelioration of HMG-CoA [42]. Dry lemon was reported to contain dietary fibers like cellulose, hemicellulose and lignine [40] which may participate in its hypolipidemic effect. It was also reported [43]that the essential oil of lemon showed, hypocholesterolemic and antiatherogenic effect in rabbit along with *in-vitro* antioxidant activity preventing oxidation of LDL which agreed with the present study that showed improved dyslipidemia, reduced MDA and reduced CVDs risk factor represented by TC/HDL-C. The flavonoids analyzed in the methanol extract can function as direct antioxidant, free radical scavengers and have the capacity to modulate enzymatic activities and protect membranes. Peel and whole fruit of lemon were reported to have significantly higher level of phenolics than other fruit part [35]. The presence of omega-3 fatty acid represented by linolenic acid in the roasted lemon might participate in the hypolipidemic activity and MDA reducing effect as reported previously in rat [44, 45]. The ratio omega-6/omega-3 in roasted lemon (3.4/1) which is lower than 4/1 indicated its cardio-protective effect as demonstrated in the study of Djuricic and Calder [46]

The major volatile compound in lemon in the study of Lee et al was limonene followed by γ-terpinene, α-pinene and β- pinene [43] which was similar to the present study however the relative% was different than the current results which might be due to the used drying technique for drying lemon. Also the study of Li et al. [32] showed the major essential oil to be D- limonene; in addition to different volatile compounds that similar to the present study.

The reduced body weight due to intake of lemon diet in the present study reflects the anti-obesity effect of roasted lemon. In contrast to the present results a previous study feeding diet containing 1% and 2% of dried lemon (drying was at 40ºC without roasting) to rabbit produced significant increase in body weight gain [40] which might be due to difference in drying techniques and /or the percentage of lemon powder. On the other hand herbal mixture containing lemon juice was demonstrated to have anti-obesity effect [47].

It is worthy to mention that the combination of bioactive constituents either in the roasted lemon or in the methanol extract might have synergistic effect in elucidating the different health benefits seen in the present study

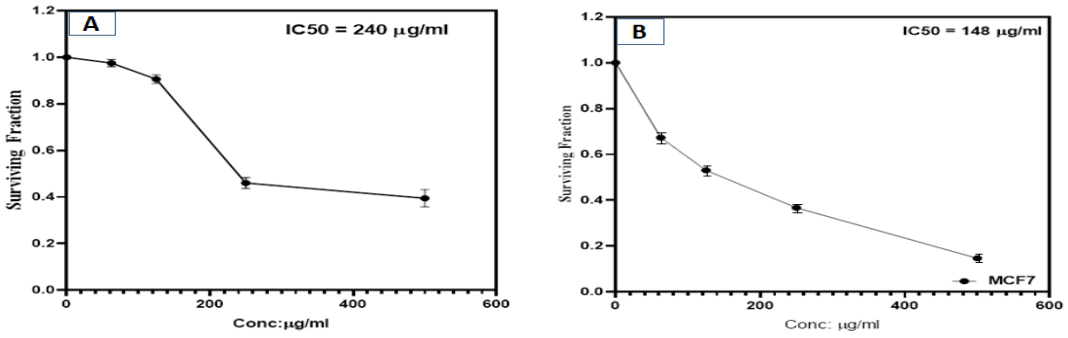

Fig 2. Survival fractions of cancer HepG2 cells (A) and MCF7 (B) against lemon extract concentration.

Table 5. Plasma parameters of different experimental groups± SE

Plasma parameters	Groups		
	Normal control	Hyperlipidemic control	Lemon treated group
TC (mg/dL)	75.2^{c}±1.72	119.9^{a}±2.26	85.4^{b}±2.13
Triglycerides (mg/dL)	58.0^{b}±2.59	111.9^{a}±1.65	29.0^{c}±1.3
HDL-C (mg/dL)	43.9^{b}±1.03	39.1^{c}±1.72	49.5^{a}±1.55
Low density lipoprotein-cholesterol (mg/dL)	19.7^{c}±0.86	58.4^{a}±1.51	30.1^{b}±1.38
TC/HDL-C	1.7^{b}±0.02	3.1^{a}±0.1	1.7^{b}±0.04
Malondialdehyde (nmol/ml)	2.5^{b}±0.24	4.3^{a}±0.27	2.2^{b}±0.24
Creatinine (mg/dL)	1.15^{b}±0.11	1.48^{a}±0.14	0.82^{c}±0.06
Urea (mg/dL)	64.2^{a}±0.95	68.7^{a}±1.75	52.1^{b}±1.68
Aspartate transaminase activity (U/L)	28.3^{c}±3.17	64.5^{a}±2.5	37.7^{b}±1.05
Alanine transaminase activity (U/L)	40.1^{c}±2.11	82.1^{a}±1.99	61.3^{b}±2.64

Different superscript letters in the same row means significant difference
TC: Total cholesterol, HDL-C: High density lipoprotein-cholesterol.

Table 6. Body weight parameters (mean± SE)

Groups	Body weight parameters		
	Initial body weight (g)	Final body weight (g)	Body weight gain (g)
Normal control	175.7±1.91	243.5^{a}±8.42	67.8^{a}±8.185
Hyperlipidemic control	175.0±1.77	235.0^{ab}±8.01	60.0^{ab}±9.09
Lemon fed group	175.5±3.170	222.0^{b}±12.84	46.5^{b}±10.03

Different superscript letters in the same column means significant difference

Conclusion

The methanol extract of roasted lemon showed anticancer activity towards Hep-G2 and MCF-7, which was superior in the second case. Roasted lemon demonstrated hypolipidemic effect with reduction of oxidative stress and improvement of liver and kidney functions along with reduction of cardiovascular risk and body weight gain. Such effects might be related to the presence of volatile compounds specially D- Limonene, the phenolic and flavonoidal compounds and linolenic acid demonstrated in the present study together with the DPPH• scavenging activity shown by the methanol extract.

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References

1. Bruno L., Spadafora N.D., Iaria D., Chiappetta A., Bitonti M.B. Developmental stimuli and stress factors affect expression of ClGLP1, an emerging allergen-related gene in *Citrus limon*. *Plant Physiol Biochem* **79**, 31–40 (2014).
2. Rauf A., Uddin G., Ali J. Phytochemical analysis and radical scavenging profile of juices of *Citrus sinensis, Citrus anrantifolia, and Citrus limonum*. *Org Med Chem Lett* **4**, 5 (2014). doi:10.1186/2191-2858-4-5.
3. Campelo L.M., Goncalves F.C., Feitosa C.M., Freitas R.M. Antioxidant activity of Citrus limon essential oil in mouse hippocampus. *Pharm Biol* **49**, 709–715 (2011).
4. Matsumoto T., Takahashi K., Kanayama S., Nakano Y., Imai H., Kibi M., Imahori D., Hasei T., Watanabe T. Structures of antimutagenic constituents in the peels of *Citrus limon*. *J Nat Med* **71**(4), 735-744 (2017). doi: 10.1007/s11418-017-1108-3.
5. Romuk E., Wojciechowska C., Jacheć W., Zemła-Woszek A., Momot A., Buczkowska M., Rozentryt P. Malondialdehyde and uric acid as predictors of adverse outcome in patients with chronic heart failure. *Oxid Med Cell Longev* **9**, 9246138 (2019). doi: 10.1155/2019/9246138.
6. Gundamaraju R., Hwi K.K., Singla R.K., Vemuri R.C., Mulapalli S.B. Antihyperlipidemic potential of *Albizia amara* (Roxb) Boiv. bark against Triton X-100 induced hyperlipidemic condition in rats. *Pharmacogn Res* **6**, 267–273 (2014).
7. Al-Okbi S.Y., Al-Siedy E.S.K. Potential anti-dyslipidemia and hepatoprotection of functional food components represented by tetracosanol and mixture of policosanol in Triton X-100 induced dyslipidemic rats. *Egyptian Journal of Chemistry* **65**(11), 313-321 (2022).
8. Al-Okbi S.Y., Mohammad A.A., Hamed T.E., Elewa T.A., Desoukey M.A. Quinoa and quinoa food products as nutritious and functional foods for protection from dyslipidemia. *Egyptian Journal of Chemistry.* Accepted in 2023, doi: 10.21608/EJCHEM.2023.206437.7881
9. Lennernas H. Clinical pharmacokinetics of atorvastatin. *J Clin Pharmacokinet* **42**, 1141–1160 (2003).
10. Hermann M., Bogsrud M.P., Molden E., Asberg A., Mohebi B.U., Ose L., Retterstøl K. Exposure of atorvastatin is unchanged but lactone and acid metabolites are increased several-fold in patients with atorvastatin induced myopathy. *J Clin Pharmacol Ther* 79, 532–539 (2006).
11. Arnold M., Morgan E., Rumgay H., Mafra A., Singh D., Laversanne M., Vignat J., Gralow J.R., Cardoso F., Siesling S., Soerjomataram I. Current and future burden of breast cancer: global statistics for 2020 and 2040. *Breast* **66**, 15-23 (2022). doi: 10.1016/j.breast.2022.08.010
12. Tinh N.T.T., Sitolo G.C., Yamamoto Y., Suzuki T. (2021). Citrus limon peel powder reduces intestinal barrier defects and inflammation in a colitic murine experimental model. *Foods* **10**, 240 (2021). https://doi.org/ 10.3390/foods10020240
13. Nasr S.E., Hassan M.M., Ahmed T.H. Engineering studies on lemon fruit drying with electric oven. *Zagazig Journal of Agricultural Research* **48**(3), 747-759 (2021).
14. Özcan M.M., Ghafoor K., Al Juhaimi F., Uslu N., Babiker E.E., Mohamed Ahmed I.A., Almusallam I.A. Influence of drying techniques on bioactive properties, phenolic compounds and fatty acid compositions of dried lemon and orange peel powders. *Journal of food science and technology* **58**(1),147–158 (2021).
15. Zilic S., Serpen A., Akillioglu G., Jankovic M., Gokmen V. Distributions of phenolic compounds, yellow pigments and oxidative enzymes in wheat grains and their relation to antioxidant capacity of bran and debranned flour. *Journal of Cereal Science* **56**, 652-658 (2012).
16. Hwang E.S., Do Thi N. Effects of extraction and processing methods on antioxidant compound contents and radical scavenging activities of Laver (*Porphyra tenera*). *Preventive Nutrition and Food Science* **19**, 40-48 (2014).
17. Kim K.H., Tsao R., Yang R., Cui S.W. Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. *Food Chemistry* **95**, 466-473 (2006).
18. Zhang P., Zhou Z. Postharvest Ethephon degreening improves fruit color, flavor quality and increases antioxidant capacity in 'Eureka' Lemon (Citrus Limon (L.) Burm. F.). *Scientia Horticulturae* 248, 70–80 (2019).
19. Zahran H.A., Tawfeuk H.Z. Physicochemical properties of new peanut (Arachis hypogaea L.) varieties. *OCL* **26**, 19 (2019). https://doi.org/10.1051/ocl/2019018
20. Cordero C.P., Morantes S.J., Páez A., Rincón J., Aristizábal F.A. *Fitoterapia* **80**, 364-368 (2009).
21. Richmond W. Preparation and properties of a cholesterol oxidase from Nocardia sp. and its application to the enzymatic assay of total cholesterol in serum. *Clin Chem* **19**, 1350–1356 (1973).
22. Lopes-Virella M.F., Stone P., Ellis S., Colwell J.A. Cholesterol determination in high-density lipoproteins separated by three different methods. *Clin Chem* **23**, 882–884 (1977).
23. Bucolo G., David H. Quantitative determination of serum triglycerides by the use of enzymes. *Clin Chem* **19**, 476–482 (1973).
24. Schriewer H., Kohnert U., Assmann G. Determination of LDL cholesterol and LDL apolipoprotein B following precipitation of VLDL in blood serum with phosphotungstic acid/MgCl2. *J Clin Chem Clin Biochem* 22, 35–40 (1984).
25. Satoh K. Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. *Clin Chim Acta* **90**, 37–43 (1978).
26. Reitman S., Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Am J Clin Pathol* **28**, 56–63 (1957).
27. Bartels H., Böhmer M., Heierli C. Serum creatinine determination without protein precipitation. *Clin Chim Acta* **37**, 193-7 (1972). https://doi.org/10.1016/0009-8981(72)90432-9 PMid:5022083

28. Fawcett J.K., Scott J.E. A rapid and precise method for the determination of urea. *J Clin Pathol* **13**(2), 156-9 (1960). doi: 10.1136/jcp.13.2.156
29. Al-Okbi S.Y., Abd El Ghani S., Elbakry H.F.H., Mabrok H.B., Nasr S.M., Desouky H.M., Mahmoud K.F. Kishk Sa′eedi as a potential functional food for management of metabolic syndrome: A study of the possible interaction with pomegranate seed oil and/ or gum Arabic. *J Herbmed Pharmacol* **10**(3), 319-330 (2021). doi: 10.34172/jhp.2021.37.
30. Jonathan H. Crane: Lemon growing in the Florida Home Landscape. *US Florida* 1153, 1095-1100 (2013).
31. Nair S.A., Sr R.K., Nair A.S., Baby S. Citrus peels prevent cancer. *Phytomedicine* **50**, 231-237 (2018). doi: 10.1016/j.phymed.2017.08.011.
32. Li C., Cai Q., Wu X., Tan Z., Huang S., Wei C., Zhang W., Chen Z., Zhang L., Xiang H. Variation in compositions and biological activities of essential oils from four citrus species: *Citrus limon*, *Citrus sinensis*, *Citrus paradisi*, and *Citrus reticulata*. *Chem Biodivers* **19**(4), e202100910 (2022). doi: 10.1002/cbdv.202100910
33. Mohammed F.Z., Gurigis A.A., Gamal A. Assessment of antiproliferative activity of *Capparis spinosa* L extract against Ehrlich ascites carcinoma in Swiss albino mice. *Biochemistry Letters* **13**(5), 54-78 (2018).
34. Sun H., Yin M., Hao D., Shen Y. Anti-cancer activity of catechin against a549 lung carcinoma cells by induction of cyclin kinase inhibitor p21 and suppression of cyclin E1 and P-AKT. *Appl Sci.* 10, 2065 (2020). 10.3390/app10062065
35. Xi W., Lu J., Qun J., Jiao B. Characterization of phenolic profile and antioxidant capacity of different fruit part from lemon (*Citrus limon* Burm.) cultivars. *J. Food Sci. Technol*. **54**(5), 1108-1118 (2017). doi: 10.1007/s13197-017-2544-5.

36. Panche A.N., Diwan A.D., Chandra S.R. Flavonoids: an overview. *Journal of Nutritional Science* **5**(47), 1-15 (2017).
37.Tounsi M.S., Wannes W.A., Ouerghemmi I., Jegham S., Njima Y.B., Hamdaoui G., Zemni H., Marzouk B. Juice components and antioxidant capacity of four Tunisian Citrus varieties. *Journal of the Science of Food and Agriculture* **91** (1), 142–151 (2011).
38.Kaur M., Sandhir R. Comparative effects of acute and chronic carbofuran exposure on oxidative stress and drug metabolizing enzymes in liver. *Drug and Chemical Toxicology* **29** (4), 415–421(2006).
39. Jaiswal S.K., Gupta V.K., Siddiqi N.J., Pandey R.S., Sharma B. Hepatoprotective effect of *Citrus limon* fruit extract against carbofuran induced toxicity in Wistar rats. *Chinese Journal of Biology* Volume **2015**, Article ID 686071, 10 pages (2015) http://dx.doi.org/10.1155/2015/686071
40. Elwan H.A.M., Dawood D.H., El-Shafei S.M.A., Abd El-Rahman A.A., Abdel-Latif S.A., Mohany M., Alqahtani F., Alqahtani S., Al-Rejaie S.S. The potential role of *Citrus limon* powder as a natural feed supplement to boost the productive performance, antioxidant status, and blood biochemistry of growing rabbits. *Animals* **9**, 426 (2019). doi:10.3390/ani9070426
41. Bouzenna H., Dhibi S., Samout N., Rjeibi I., Talarmin H., Elfeki A., Hfaiedh N. The protective effect of *Citrus limon* essential oil on hepatotoxicity and nephrotoxicity induced by aspirin in rats. *Biomed Pharmacother* **83**, 1327-1334. (2016) doi: 10.1016/j.biopha.2016.08.037
42.Trovato A., Monforte M.T., Barbera R., Rossitto A., Galati E.M., Forestieri A.M. Effects of fruit juices of *Citrus sinensis* L. and *Citrus limon* L. on experimental hypercholesterolemia in the rat. *Phytomedicine* **2** (3), 221–227 (1996).
43. Lee H., Woo M., Kim M., Noh J.S., Song Y.O. Antioxidative and cholesterol-lowering effects of lemon essential oil in hypercholesterolemia-induced rabbits. *Prev Nutr Food Sci* **23**(1), 8-14 (2018).
44. Al-Okbi S.Y., El-qousy S.M., El-Ghlban S , Moawad H.F. Role of borage seed oil and fish oil with or without turmeric and alpha-tocopherol in prevention of cardiovascular disease and fatty liver in rats. *J. Oleo Sci*. **67** (12), 1551-1562 (2018).
45. Al-Okbi S.Y., Mohammed S.E., Al-Siedy E.S.K., Ali N.A. Fish oil and primrose oil suppress the progression of Alzheimer's like disease induced by aluminum in rats. *J. Oleo Sci*. **69** (7), 771-782 (2020)
46. Djuricic, I., Calder, P.C. Beneficial outcomes of omega-6 and omega-3 polyunsaturated fatty acids on human health: An update for 2021. *Nutrients* **13**, 2421 (2021). https://doi.org/10.3390/nu13072421
47. Pandeya P.R., Lamichhane R., Lamichhane G., Lee K-H., Lee H.K., Rhee S-j., Jung H-J. 18KHT01, a potent anti-obesity polyherbal formulation. *Front Pharmacol* **12**, 807081 (2021). doi: 10.3389/fphar.2021.807081