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Nano-Formulations of Hesperidin and Essential Oil Extracted from Sweet Orange Peel: Chemical Properties and Biological Activities

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Abstract

Citrus EOs and Flavonoids are widely recognized for their beneficial effects in possessing many biological activities, such as antioxidant, antimicrobial and cytotoxic properties, also using in food additives and in the cosmetic industry. The aim of this work is to produce nanoparticles of hesperidin and citrus EO extracted from waste orange peels, and study its biological impact compared to native products (hesperidin and citrus EO) as antioxidant, antimicrobial and cytotoxic. Obtained products (nano-formulation) are characterized by zeta potential, electron scanning microscopy, spectrophotometric method and color analysis. The final products (nano-formulation) were examined as antioxidant agent by two assays (DPPH and ABTS analysis). The antioxidant activity of nano hesperidin and citrus EO was evaluated and compared with none formulated. Nanoparticles of hesperidin and citrus EO have a great potential in DNA damage prevention compared to their respective controls which were examined by DNA damage assay induced by oxidative stress protection analysis. The in vitro cytotoxic activity of nano formulated hesperidin and citrus EO, towards (three cancerous cell lines, including: breast (MCF-7), colorectal (HCT-116) and liver (HepG2), and two non-cancerous control cell lines (fibroblast BJ-1and MCF-12F) compared with native product (citrus EO and hesperidin) were examined using MTT assay. The hesperidin and citrus EO NPs showed a positive effect on the inhibition of the proliferation of all tested cancer cell line. The antimicrobial activity of hesperidin, citrus EO and their nano formulation form was evaluated using well diffusion assay. The nano formulation form of hesperidin and citrus EO showed to improve the antimicrobial activity. These findings showed that nano-hesperidin and nano - citrus EOs play an important role as antioxidant, antimicrobial and cytotoxic agent's effects. These effects might be used for clinical trials and can represent driving formulation for novel chemotherapeutic agents and in food industry. Further studies about the therapeutic effects of nano-hesperidin and citrus EO extracted from Egyptian orange peel are requested

Key words: Orange peels, hesperidin, citrus EO, antioxidant, DNA damage, antimicrobial and cytotoxic.

1. Introduction

Citrus plants are belonging to Rutaceas family, which had several sub-types of plants, such as oranges, mandarins, limes, lemons, and grapefruits, citron and bergamot plants. According to Food and Agriculture Organization, global orange production reached 68 million tons representing 8.5% of the total fruit production [1]. The citrus fruits and its industrial products are one of the most consumed fruits worldwide [2]. In this context, 18% of citrus fruits farm products are industrially processed as juices. However, an environmental issue is associated with industrialized citrus products, which generate large amounts of wastes and by-products including peel,

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pulp, and seed residues. Due to citrus industrial processes, the derived wastes are accumulated and therefore, causing pollution problems. The accumulated citrus waste can be determined by around 15–25 thousand tons of citrus waste annually. To deal with such an environmental problem is time consuming and economically expensive [3]. In fact, the nature of citrus wastes which contain high chemical oxygen demand, low pH (3–4), high water content (80–90%) and 95% of organic matter facilitate their fermentation [5,6]. In another hand, citrus waste contains high-quality fiber, pectin in addition its composed of many valuable bioactive compounds such as hesperidin, polyphenols, flavonoids, carotenoids and citrus EOs (EOs). They have been used in a wide variety of industrial products such as food and beverage, cosmetics and pharmaceuticals [7,8]. Hesperidin (3',5,7-trihydroxy-4'-methoxy flavanone-7-6-O-α-Lrhamnosyl- D-glucose) is a flavonoid byproduct found abundantly in citrus production, mostly in sweet orange and lemon. Several reports are indicated that, hesperidin display many pharmaceutical effects such as anti-allergic, antioxidant and anti-inflammatory [3, 9]. Besides, hesperidin exhibited cytotoxic activity against different rat model carcinogenesis, including tongue, esophagus, colon and urinary bladder [2]. Citrus citrus EOs were extensively studied for their potential uses in the food industry. The composition of citrus citrus EO indicated that it contains mixtures of hydrocarbons, oxygenated compounds and nonvolatile residues, including terpenes, sesquiterpenes, aldehydes, alcohols, esters and sterols [10, 11].

In the last two decades, the nano-formulated materials have been extensively investigated for their pharmaceutical activity. Because of the production of nano-size, the physical and chemical characteristics of substances are altered, which may lead to change in the quantum size, mesoscopic, solubility, surface, charge and therefore the pharmaceutical activities [12]. The nano-materials have implicated in a wide range of applications in the field of science and technology, for instance, biomedical and electronic IT applications, as well as environmental remediation [13]. Moreover, nano-formulated drugs are considered as one of the most important and successful application. In this context, drug coupled nanocarrier has ameliorate drug delivery and their biological activity [14]. In fact, the production of nano materials may alter different physical characteristics of the original substances including, surface modification, size, shape and adsorption [15]. Among the Nano formulated strategy, polyethylene glycol (PEG) coating agent is highly recommended and effective for nanoparticles production, because of their safety and "stealth" properties, which accelerate their clinical transformations [16]. The "stealth" properties of PEG nanoparticles have long been considered to be determined the surface PEG length and density, both of which are hard to finely control [17]. The aim of this work is to produce nano-formulated hesperidin (hesperidin NPs) and citrus EO (citrus EO NPs) extracted from orange peels waste, and study its biological impact as antioxidant, antimicrobial and cytotoxic materials.

2. Materials And Methods

All chemicals used in this study were purchased from Sigma (USA) and Fluka (Switzerland) analytical grade. The peels and citrus EO (Citrus EO extraction at cold pressing technique) were obtained from El Marwa Food Industries, 6th of October Giza-Egypt. The peels were dried at room temperature then oven at 40 °C and grinded into powder.

2.1. Extraction of crude hesperidin.

About 200g of Sweet orange peels powder were extracted with 600 ml of petroleum ether (40-60°C) under reflux condenser at 50°C. After 1.5h the mixture was filtrated through Buchner funnel, the filtrate was discarded and the residue was dried at room temperature. The dry powder was extracted with 600 mL of methanol and heated under reflux for another 2h at 65°C. The mixture was filtrated through Whatman filter paper No.1, then the filtrate was concentrated by rotary evaporator. The yielded syrup residue was converted to crystallized from by dilute acetic acid (6%), and yielding orange needles (crude Hesperidin) melting point was 268°C according to Lahmer *et al*., (2015) [18].

2.2. Nanoparticle preparation

Nano-hesperidin and Nano-citrus EO were prepared as previously described by Werdin González *et al* (2014) [19] with some modifications. In brief, 50 g of PEG 8,000 were melted at 65 °C on a hotplate stirrer in a clean flask. While stirring a 16.5 g of hesperidin or citrus EO (previously dissolved into 3 ml of Tween 80) were added in a drop wise to the melted PEG (drop/5sec), then the mixture was sonicated using a DAIGGER ULTRA-SONIC Model GEX

750, USA for 15 min. The mixture was then cooled at −4 °C for 2h and completely ground in a refrigerated mortar. Finally, the product was sieved using a stainless steel sieve (230 mesh), stored at 25 ± 0.5 °C in an airtight container, and used for the bioassay within the following 48 h.

*2.3. **Nanoform characterization***

Particle size of the produced NPs were analyzed using Dynamic Light Scattering (DLS) analyzer (Nano- ZS, Malvern instruments Ltd.,UK) as well as to evaluate the surface charge at 25 °C, zeta potential values, and the NP dimension, expressed in terms of Z-average size (d), and polydispersity index (PDI). The morphology of the hesperidin-NPs was visualized using transmission electron microscope TEM (JEM 2100 HRT, HIGH RESOLUION MADE IN JAPAN). In brief, after 24 h, aliquots of each hesperidin-NP or citrus EO-NP were suspended in 10 mL of distilled water and then the suspension was filtered using Whatman NO1 filter paper. Fourier transform infrared spectroscopy FTIR was used to evaluate the association level between materials during nanoparticles production which was evaluated using (VERTEX 80v, BRUKER, Germany) at 4 cm^{-1} resolution and measurement scale range of 4000-400 cm^{-1} [19].

2.3.1. DPPH radical scavenging activity

The antioxidants activity of hesperidin, hesperidin–NPs, citrus EO and citrus EO–NPs as well as Nano-control were tested following a protocol as described by Shin, (2012) [20] with slightly modifications. Briefly, 50 µL containing different concentration (0.05, 0.01, 0.15, 0.2, 0.5, 1 mg/ml) of each sample were mixed with 2.95 mL of DPPH 0.04% dissolved in methanol. The mixture was then vigorously shacked and set to react at 37°C in a dark place for 30 min. The absorbance was measured at 517 nm against vitamin C, and the scavenging activity % was calculated using the following equation:

DPPH• scavenging activity (Inhibition %) = [(Ac – As)/Ac] X 100

(Ac: absorbance of the DPPH solution and A_s: absorbance of the sample).

2.3.2. ABTS radical scavenging activity

The potential of ABTS radical scavenging of the mentioned samples was measured using a modified method of Floegel *et al*. (2011) [21]. Briefly, 980 µL of ABTS solution previously adjusted to 0.7 absorbance at 734 nm were added to a mixture of 20 µL containing different concentration (0.05, 0.01, 0.15, 0.2, 0.5, 1 mg/ml) of each sample. The mixture was then set to react at 37 °C for 10 min in dark. The absorbance was measured at 734 nm and the ABTS radical scavenging % was calculated using the equation:

% Inhibition = $[(A_0 – A_1) /A_0]$ X 100

(A_0 is the $ABTS^{\bullet+}$ absorbance of the control reaction and A_1: is the $ABTS^{\bullet+}$absorbance of the sample).

2.3.3. Fenton's reagent induced DNA damage protection.

The potential prevention of DNA damage induced by oxidative stress Type-Fenton's reagent was evaluated according to previous study reported by Leba *et al*., (2014) [22]. In brief, 3µl of (60µg/µl) DNA Ribonuclease Inhibitor (RNH1) plasmid was mixed with various concentration of hesperidin, hesperidin–NPs, citrus EO and citrus EO–NPs *and Nano-control* (0.5, 0.75 and 1.0 mg/ml) with or without Fenton's reagent (5mM of H_2O_2 and 0.35mM of $FeSO_4$ and 0.60mM of EDTA) and the final volume was completed to 25µl phosphate buffer (H_2PO_4, 8.3mM, pH 7.4). The mixture was incubated for 20 min at 37°C. Also, 3µl of RNH1 plasmid DNA (25µg/µl) was used as DNA protection control. After 20 min samples were loaded into agarose gel 1.5% and the separated bands were analyzed.

2.4. Evaluation of antimicrobial activity

The antimicrobial activity of hesperidin, hesperidin–NPs, citrus EO and citrus EO–NPs were tested against different strains of bacteria, including gram positive (*Bacillus cereus* EMCC 1080 and *Staphylococcus aureuse* ATCC 13565), and gram negative (*Escherichia coli* O157-H7 ATCC 51659, *Salmonella typhi* ATCC 15566 and *Pseudomonas aeruginosa* NRRL B-272). Also antifungal activity was tested against six fungal species (*Aspergillus flavus* NRRL 3357, *A. parasiticus* SSWT 2999, *A. niger* ITEM 10027, *A. ochracious* ITAL 14, *F. proliferitum* MPVP 328 *and Penicillium verrucosum* BFE 500). Antibacterial activity was conducted using well diffusion assay on nutrient agar medium. The tested bacterial plates were incubated at 37 Ċ for 24 h. After incubation the inhibition zones were measured by ruler in mm. Potato dextrose agar (PDA) was used for antifungal activity using well diffusion technique.

The tested plates were incubated at 25 Ċ for 48 h (EUCAST, 2015) [23].

2.5. Cytotoxic activity

All cell lines used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Three human, cancerous cell lines were used in this study including colorectal adenocarcinoma (HCT-116, ATCC® CCL-247™), mammary adenocarcinoma (MCF-7, ATCC® HTB-22™) and hepatocellular carcinoma (HepG2, ATCC® HB-8065™), and noncancerous skin fibroblast BJ-1 (ATCC® CRL-2522™). Cell lines were cultured in DMEM/high glucose supplemented with 2 mM L-glutamine, 10% FBS and 1% penicillin/streptomycin kept in Corning® 75cm-1² U-Shaped canted neck cell culture flask with vent cap (Corning, New York, USA). The sub-confluent cultures (70–80%) were there trypsinized (Trypsin 0.05%/0.53 mM EDTA) and spilt depending on the seeding ratio [24, 25].

2.6. MTT assay

Cells (1×10^5/well) were plated into 100 µl of medium/well in 96-well plates (Hi media). After 48 hours incubation, the cells reach the confluence. The media was then replaced with RPMI-1640 media containing different concentration of hesperidin, hesperidin–NPs, citrus EO and citrus EO–NPs. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 20µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-- tetrazolium bromide cells (MTT) phosphate- buffered saline solution were added. After 4h incubation, 130 µl of 0.04M HCl/ isopropanol were added. Viable cells were determined by the absorbance at 570nm with reference at 655nm. Measurements were performed in 3 times, and the concentration required for a 50% inhibition of viability (IC50) was determined graphically. The absorbance at 570 nm was measured with a microplate reader (Bio-Rad, Richmond, CA), using wells without sample containing cells as blanks. The effect of the samples on the proliferation of human breast cancer cells was expressed as the % cell viability, using the following formula:

% cell viability = A570 of treated cells / A570 of control cells × 100% [26].

2.7. Statistical analysis

Data were statistically analyzed using Co-stat statistical package data according to Anonymous, (1989) [27].

3. Results And Discussion

3.1. Nanoparticles characterization

3.1.1. Transmission Electron Microscope TEM

TEM analysis is a technique that uses electron beam to directly imaging and measure nanoparticle size. TEM images of prepared hesperidin–NPs and citrus EO–NPs showed that the particle size was in the range of approximately 4–70 and 53–126 nm, respectively (Figure 1 A (b) and B (b)) compared to PEG-NPs as control (Figure 1 A (a) and B (a)). In this context, it has been reported that spherical shaped AgNPs synthesized using citrus plant extract was 50 nm sized [28]. While, heterogeneously shaped AgNPs synthesized using lemon leaves extract has been reported with size range between 15 and 30 nm [29]. Interestingly, these nanoparticles have not displayed any adhesion or agglomeration. Likewise, decreasing of particle size will improve dissolution velocity. Increasing dissolution velocity can conduct to improve bioavailability especially to biopharmaceutical class system (BCS) II which dissolution velocity is the rate limiting step [30]. In fact, nanoformulations can solve problems related to EO volatility, poor water solubility and the tendency to oxidize [21]. In addition, nanoparticles are able to release the active compounds at the site of action gradually [31], and also minimize the toxic effects on non-target organisms [32].

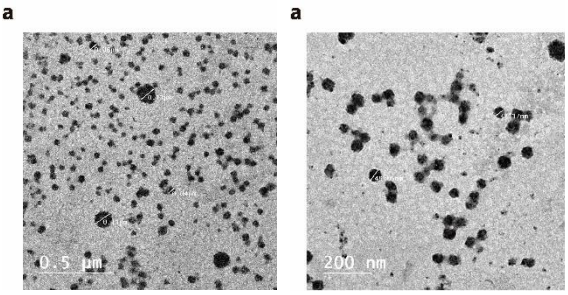

Figure 1. Transmission electron microscope micrographs of Hes-NPs and Citrus EO-NPs. A) Represents TEM of PGE-NPs in the left panel (a) and Hes-NPs in the right panel (b). B) represents TEM of PGE-NPs in the left panel (a) and Citrus EO-NPs in the right panel (b).

3.1.2. Fourier-transform infrared spectroscopy (FTIR)

The FTIR is a technique used in nanoparticle studies, where the resulted spectrum is expressing the interference level between the compounds and nanomaterial. FTIR spectra of native hesperidin and nano-hesperidin are presented in Figure 2. The absorption band at 3393 cm^{-1}corresponding to hydroxyl group O–H stretching vibration. The band at 2875 cm^{-1} is due to alkane C–H stretching vibration. The FTIR spectrum peak of carbonyl C=O stretch appeared at 1645 cm^{-1}. The bands at 1455–1348 cm^{-1} are attributed to aromatic C=C stretch, and the aromatic C–O stretches at 1298 and 1047 cm^{-1}. The FTIR spectra of native hesperidin showed characteristic bands because of existence of different functional groups like, 3329, 2918, 1645, 1519, 1063 cm^{-1} which could be attributed to O–H stretching vibration, C–H stretching, C=O stretching, C=C stretching and C–O stretching, respectively. Both spectra of hesperidin and Nano hesperidin have the same characteristic peaks but with some difference which may due to the minor chemical interaction of native hesperidin and PEG matrix [33, 34]. Main peaks of native citrus EO were observed at 2964, 2917, 2855, 1676, 1644, 1435, 1375, 1241, 1198, 1147, 1050, 1015, 956, 885, 797, 541 and 427 cm^{-1}. The vibrational bands around 2900 cm^{-1}, 1700 cm^{-1}, and 1100 cm^{-1} may include spectral features arising from C-H, C=O, and C-O stretching vibrations of terpenoid components, respectively [35]. The peak around 2964 cm^{-1} corresponds to the –CH3 asymmetric and symmetric stretching vibrations. The peaks at 2917 cm^{-1}, 1676 cm^{-1}, 1644 cm^{-1}, 1435 cm^{-1}, 1375 cm^{-1}, 1147 cm^{-1}, 885 cm^{-1}, and 797cm^{-1} correspond to the C-H stretching vibrations of alkanes, C=O stretching vibrations, C=C stretching vibrations of alkanes, C-H bending vibrations of alkanes, O.H. bending vibrations of phenols, C-O stretching vibrations of tertiary alcohols, C-H stretching vibrations of aromatics and C=C bending vibrations of alkanes, respectively [36]. The results indicated that, the most spectrum of PEG nano-carrier bands were loaded in Citrus EO-NPs. In addition, some spectrum of native citrus EO was loaded in Citrus EO-NPs at 427, 1644, and 2859 cm^{-1}, with new bands at 668, 1960, 2163 and 3478 cm^{-1}. The presence of new beaks may be due to a chemical reaction of Citrus EO with matrix (PEG).

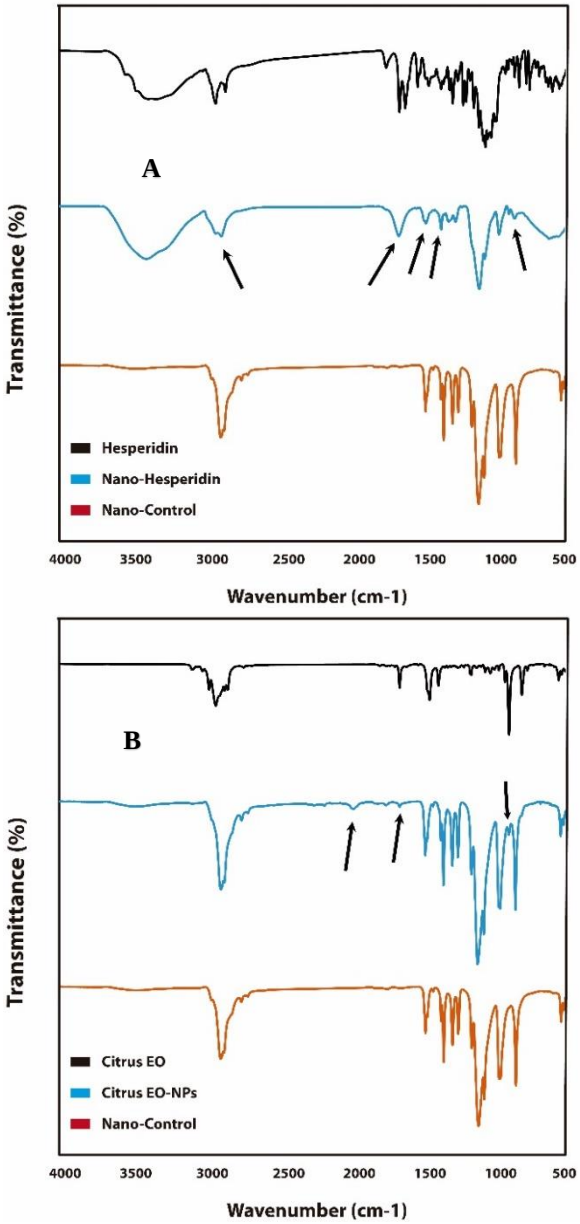

Figure 2. Fourier-transform infrared spectroscopy (FTIR) spectra of Hes–NPs and Citrus EO–NPs. A) Represents FTIR Spectrum of hesperidin (Black), Hes–NPs (blue) and PEG-NPs (Red). B) represents FTIR Spectrum of Citrus EO (Black), Citrus EO–NPs (blue) and PEG-NPs (Red).

3.1.3. Particle size distribution

Particle size and Zeta potential are a limiting factor of nanomedicine effectiveness at drug delivery and stability levels. The Zeta Potential gives an indication about particles surface charge [37]. The results of particle size (nm), Polydispersity index (PDI), and Zeta potential (mV) analysis revealed that the diameter of produced hesperidin–NPs, Citrus EO–NPs and PEG-NPs were 337.2 432.5 and 360.9 nm respectively (Table 1). Thus, the larger particle size formation may

be due to greater viscosity of the dispersed phase during ultra-sonication [38]. In another hand, PDI value of hesperidin–NPs and Citrus EO–NPs were 0.243 and 0.560 respectively points to a narrow size distribution [39]. Hesperidin and Citrus EO loaded NPs exhibited a positive surface charge (+4.89 and +2.93mV respectively), while the unloaded nanoparticles were + 4.50 mV (Table 1). However, it has been reported that ZP ranged from 0 - 5 mV indicated a rapid coagulation or flocculation of produced nanoparticles [40].

Table. 1. Particle size, Polydispersity index (PDI) and Zeta potential of hesperidin–NPs, citrus EO–NPs and PEG-NPs.

	Hesperidin - NPs	Citrus EO-NPs	PEG-NPs
Partial size (nm)	337.2	432.5	360.9
Pdi	0.243	0.560	0.588
Zeta potential (mV)	+4.89	+2.93	+4.50

3.1.4. Radical-scavenging activity on DPPH and ABTS

The antioxidants activity of hesperidin–NPs and citrus oil-NPs were evaluated by ABTS and DPPH assays. Different concentrations were tested and the concentration that correspond to 50% antioxidant (IC_{50}) was calculated (Table 2). The native hesperidin and citrus EO showed the highest antioxidant activity, with IC_{50} 0.061 and 0.099 µg/ml in DPPH assay, while IC_{50} values were 0.114 and 0.082 using ABTS assay, respectively. Whereas, the antioxidant of hesperidin–NPs and citrus EO–NPs were 0.053 and 0.085 µg/ml in DPPH assay and 0.055 and 0.11 µg/ml in ABTS assay, respectively. The results showed that no significant differences between Nano-form and native form of hesperidin and citrus EO were noticed using DPPH assay. While, there is significant differences between them by using ABTS assay. The results indicated that, hesperidin-NPs enhanced the potential antioxidant activity may due to their nano-formulation. Thus, nano-formulations may be altering several physical properties, therefore increasing the antioxidants potency [21, 41]. Previous studies have been reported that hesperidin plays a direct role in reactive oxygen species (ROS) scavenging including activation of antioxidant enzymes, metal chelating activity, reduction of α-tocopheryl radicals, inhibition of oxidases. Additionally, hesperidin was able to reduce superoxide ions *in vitro*. Moreover, hesperidin exhibited a potential protective effect of liposomes from peroxidation induced by UV-irradiation [42-43].

In other hand, citrus citrus EO is enriched in monoterpenes that explain the high antioxidant activity of the citrus EO. However, citrus EOs which contain monoterpene hydrocarbons, oxygenated monoterpenes and/or sesquiterpenes have greater antioxidative properties [44]. In addition, the chemical composition of citrus citrus EO declared the presence of 1,8-cineol, α-pinene, β- pinene that attributed to the antioxidants activities thus, many reports contributed to antioxidant potential of the citrus EOs often refer to the synergism, antagonism and additively [45].

3.2. DNA damage protection

The DNA damage protection activity of Hes-NPs and citrus EO-NPs were evaluated using DNA of *RHN1* plasmid treated with Fenton's reagent. In general, plasmid DNA has the supercoild form (scDNA) as a major form and many accompaed with traces of the relaxed from open circular, ocDNA). The supercoiled DNA (scDNA) migrates faster on agarose gel than ocDNA. Therefore, the charge in the intensity of those forms can indicate the damage effect on DNA (single strand beark) which lead to decrease of scDNA and increase of ocDNA. Extream stress on plasmid DNA enhance the damage of DNA (double strand break) then enhance the previous changes in addition to form linear DNA which appear as clear band scDNA and ocDNA bands. Fenton's reagent containing hydrogen peroxide (H_2O_2) that react with ferrous iron ($FeSO_4$) releases $OH^{\bullet}$ radical induced DNA damage. As a result, Hes-NPs and citrus EO-NPs are able to protect DNA damage of *RHN1* plasmid. The obtained results also indicated that by increasing the concentration of hesperidin and citrus EO; the antioxidant status could be changed to prooxidant which induced oxidative stress. These results showed that hesperidin at 2.5 µg/ml was able to protect DNA damage. While, at higher concentration 5 µg/ml exhibited pro-oxidant activity when the supercoiled band was almost disappeared (Figure 3A lane 5-6). However, Hes-NPs increases the protection potential of DNA from damage protection at all tested concentrations (2.5 and 5 µg/ml) compared to their respective controls (Figure 3 lane 1-4). In addition, no significant differences were observed post NPs control treatment compared to negative control one (lane 7-8). Mostly, human DNA damage induced in response to oxidative stress (OS). Plants and plant derivatives act as an alternative medicinal therapeutic modality.

Table 2. Antioxidant activity (IC50) of hesperidin, hesperidin–NPs, citrus EO, citrus EO–NPs and PEG-NPs.

IC50 (µg/ml)	Hesperidin	Hesperidin-NPs	LSD	Citrus EO	Citrus EO-NPs	PEG-NPs	LSD
DPPH	$0.061^{b} \pm 0.001$	$0.053^{b} \pm 0.002$	0.147	$0.099^{b} \pm 0.003$	$0.085^{b} \pm 0.004$	$2.50^{a} \pm 0.128$	0.144
ABTS	$0.114^{b} \pm 0.005$	$0.055^{c} \pm 0.007$	0.013	$0.082^{c} \pm 0.005$	$0.11^{b} \pm 0.02$	$4.59^{a} \pm 0.007$	0.025

All experiments were performed in triplicate; all data are expressed as the mean ± SD.
Means with different letters (at the same row) are significantly different at $p \leq 0.05$.

They are rich in the secondary metabolites such as vitamins, flavonoids, pigments, sterols and minerals [46]. Bioactive plant derived components exhibited cytotoxic, antimicrobial, antioxidant and DNA damage protection properties [47]. Naringenin is one of these bioactive compounds derived from citrus fruits which has displayed cytotoxic activities against human colon cancer [48]. It has been reported that, Naringenin protected plasmid DNA from damage induced by UVB. Fenton's reagent-mediated hydroxyl radical releases in the presence of hydrogen peroxide (H_2O_2) and Fe^{3+}, therefore, single or double strands DNA break causes various chronic diseases [49]. The results indicated that hesperidin loaded NPs enhanced the protection capacity of DNA damage induced by Fenton reagent compared to hesperidin alone which may be due to nano-formulations which altering the free drug delivery inside the cells at the site of action, therefore accumulate more hesperidin and citrus EO inside the cells [50]. Similar to our findings that, Hes-NPs protects DNA damage, citrus EO and citrus EO-NPs displayed DNA damage protection activity. However, the nano-formulation of Citrus EO-NPs showed an improvement in DNA damage protection was observed due to Nano-formulation of citrus EO, indicating that CEO has high cytotoxic effect. In this context, there are few studies on cytotoxic activity of Citrus EO on DNA damage protection were carried out [51, 52].

3.3. Antimicrobial activity

The data obtained in Tables (3 and 4) show the antimicrobial activity of native hesperidin and citrus EO, and their nanoformulation form. Citrus EO and its nanformulaion forms showed antibacterial activity against: *B. cereus* and *S. aureuse*, only (gram positive) while there is no antimicrobial activity against gram negative: (*E. coli*, *S. typhi* and *P. aeruginosa*), which data of gram negative bacteria is not showed. Results revealed that the inhibition zone is from 10.0 to 11.33 mm against *B. cereus* and *S. aureus* respectively for native citrus EO. While for the same bacterial the citrus EO–NPS was ranged from 8.33 to 8.75 mm respectively (Table 3). The inhibition zone developed by Citrus EO-NPs was lower than that obtained by citrus EO because one gram of Citrus EO-NPs contains 33.3% native EO. No activity was obtained against all tested bacteria in presence of hesperidin and its nanoparticles. Antifungal activity: The data presented in Table 4 show that the antifungal activity of hesperidin, citrus EO, and their nanoparticles against six fungal species.

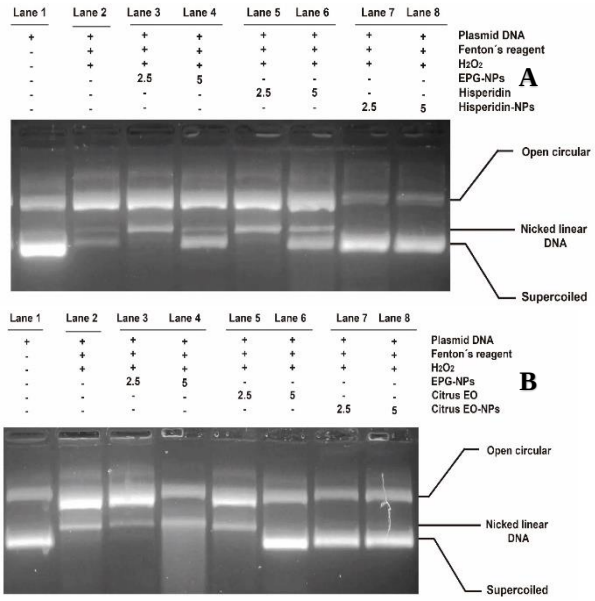

Figure 3. In vitro analysis of DNA damage protection capacity. A) Represent DNA damage of hesperidin and Hes–NPs. B) Represent DNA damage of Citrus Eo and Citrus EO-NPs. Lane 1: *RHN1* DNA Plasmid, Lane 2: *RHN1* DNA Plasmid treated with Fenton's reagent, Lane 3-4: PEG-NPs control PEG-NPs control, Lane 5-6: Hesperidin in A and Citrus EO in B, and Lane 7-8: Hes-NPs in A and Citrus EO-NPs in B. Three concentration were used in this assay (2.5 and 5 µg/ml), respectively. All the reaction mixtures were incubated for 20 min at 37°.

The results of hesperidin and its nanoparticles showed significant difference with miconazole as standard against all tested fungi. While, no significant differences were noticed between hesperidin and its nanoparticles against the three tested fungi (*A. parasiticus*, *F. proliferitum* and *P. verrucosu*). Halo zone was 13.6, 13.5 and 11.3 mm respectively for hesperidin and were 14.0, 11.6 ad 12.8 mm respectively for hesperidin-NPs. Also, citrus EO and its nanoparticles showed significant difference with miconazole as standard against all tested fungi with no significant difference between them except *A. niger* which was 15.0 and 11.5 mm for citrus EO and its nanoparticles respectively. The obtained halo zones developed by their nanoformulaion form against all tested fungi, can be explained by the effect of nano-formulations technology which have involved in physical properties alteration, including particles size, solubility and shapes [51]. The mechanism of the antimicrobial effects may be attributed to the combined effects of adsorption of phenolic compounds to bacterial membranes that disrupt the membrane, followed by leakage of cellular contents, formulation of hydroperoxide and metal chelation by phenolics [53]. The major flavonoid compounds in citrus fruits which are effective antimicrobial agents are hesperidin and naringin. In a previous study [54] the authors found that the acid hydrolysis of Citrus peel extract enhanced the antimicrobial activity by increasing the free phenolic forms of hesperidin and naringin. Most studies investigating the action of citrus EOs against food spoilage organisms and food borne pathogens agree with the present study. Generally, citrus EOs are slightly more active against Gram-positive than Gram negative bacteria. The Gram-negative organisms are less susceptible to the action of anti-bacterial is perhaps to be expected, since they possess an outer membrane surrounding the cell wall, which restricts diffusion of hydrophobic compounds through its covering lipopolysaccharide [55, 56].

Table 3. Antibacterial activity of Citrus EO and Citrus EO–NPs at 100 mg/ml.

Zone Inhibition (mm)					
Bacterial strain	Citrus EO	Citrus EO -NPs	PEG -NPs	Ceftriaxone 1mg/ml	LSD
B. cereus	11.33^{b} ±0.58	8.75^{c}±1	0.0^{d}	16.0^{a} ±1.00	1.43
S. aureus	10.0^{b}± 1	8.33^{c}±0.58	0.0^{d}	17.6^{a}±1.15	1.53

All experiments were performed in triplicate; all data are expressed as the mean ± SD.

Means with different letters (at the same row) are significantly different at $p \leq 0.05$

3.4. Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration of hesperidin, citrus EO and their nano-formulation forms were presented in figure (4 a, b). MIC of citrus EO against *B. cereus* and *S. aureus* was 0.33 and 0.25 mg/ml respectively, and was 3 and 1.3 mg/ml respectively for citrus EO –NPs (figure 4a). The MIC of samples against the six species of fungi showed that the hesperidin had a MIC from 1.0 to 4.0 mg/ml against *A. niger* and *A. parasiticus* respectively, and ranged from 2.0 to 8.3 mg/mL for *F. proliferitum* and *A. parasiticus* respectively for hesperidin-NPs. Also, the MIC of citrus EO recorded 0.5 to 1.3 mg/ ml for *P. verrucosum* and *A. parasiticus* respectively. While, the MIC of citrus EO –NPs was ranged from 2.3 to 4.3 mg/ ml for *A. parasiticus* and *A. niger* respectively.

Table 4. Antifungal activity of hesperidin, hesperidin–NPs, Citrus EO, Citrus EO–NPs and PEG-NPs at 100 mg/ml.

fungi	*A. flavus*	*A. parasiticus*	*A. niger*	*A. ochracious*	*F. proliferitum*	*P. verrucosum*
Hesperidin	12.00^{c} ± 0.5	13.67^{b} ± 2.75	11.67^{c} ±0.76	14.17^{b} ±1.26	13.50^{b} ±1.32	11.33^{b} ±0.76
Hesperidin - NPs	15.5^{b} ± 0.87	14.0^{b} ± 1.0	13.83^{b} ±0.76	12.17^{c} ±0.76	11.67^{b} ±2.93	12.83^{b} ±1.61
PEG-NPs	0.0^{d}	0.0^{c}	0.0^{d}	0.0^{d}	0.0^{c}	0.0^{c}
Miconazole 1mg/ml	20.3^{a} ±1.03	20.6^{a} ±2.36	20.3^{a} ±0.76	18.5^{a} ±0.50	17.3^{a} ±1.04	17.2^{a} ±2.57
LSD	1.35	3.54	1.24	1.46	3.18	2.93
Citrus EO	13.67^{b} ± 1.53	13.17^{b} ± 1.15	15.0^{b}±1.0	10.50^{b}±0.0	15.67^{ab}±0.76	12.33^{b}±1.76
Citrus EO - NPs	14.17^{b} ± 1.26	12.0^{b} ± 1.1.80	11.50^{c}±1.32	11.0^{b}±0.87	12.67^{b}±3.06	12.50^{b}±0.50
LSD	2.1	3	1.71	0.94	3.12	2.96

All experiments were performed in triplicate; all data are expressed as the mean ± SD.

Means with different letters (at the same Colum) are significantly different at $p \leq 0.05$

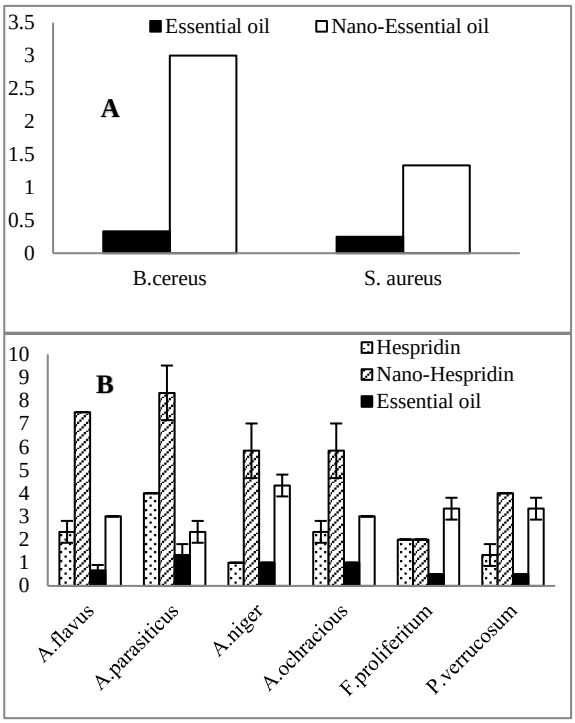

Figure 4. The minimum inhibitory concentration (MIC) of hesperidin, hesperidin-NPs, Citrus EO and Citrus EO -NPs against some fungi (b) and Citrus EO and Citrus EO-NPs against some bacteria (a).

3.5. Cytotoxic activity

After 24 h of incubation, the viable number of MCF-7 and HepG-2 cells was significantly decreased in response to hesperidin-NPs and citrus EO-NPs compared to BJ-1 and MCF-12F normal cells (Table 5). Results showed that, hesperidin–NPs and Citrus EO–NPs at 100 µg/ml displayed growth inhibition of MCF-7 and HepG-2 with death % of 59.5 and 69.3%, in comparison hesperidin–NPs and 64.6 and 85.4% for Citrus EO–NPs treatments, respectively. However, hesperidin and Citrus EO showed the highest potential cytotoxic activity against HCT-116 which 88.1 and 57.3%, respectively. The results also showed an increase the cytotoxic effect of hesperidin and Citrus EO at normal cells Bj-1 and MCF-12F. Whereas the nanoform of hesperidin and Citrus EO reduced the cytotoxic effect against normal cells (MCF12F and Bj-1) with death% 7.4 and 14.8 in MCF-12F and 1.4 and 3.05% at BJ-1, respectively. We cannot exclude that each one gram of hesperidin-NPs and Citrus EO–NPs contains 33.3% of native hesperidin and Citrus EO. The results indicated that hesperidin-NPs and Citrus EO-NPs have improved the cytotoxic activity, and reduced the cytotoxic effects of the hesperidin and citrus EO against normal cells. In fact, nano-formulations have different physical properties, including shape, size and charge which might influenced as rapid drug delivery inside the cells at the site of action, therefor accumulate more hesperidin or citrus oil inside the cells [50]. In addition, PEG-NPs showed very low cytotoxic activity against tested cell lines. The concentration causing 50% inhibition of growth of cells (IC_{50}) was calculated as follows for hesperidin was 112.3, 77.5 and 56.1 µg/ml against MCF-7, HepG-2 and HCT-116, respectively. While the IC_{50} values of hesperidin against MCF12F and BJ-1 normal cell lines were 751 and 393 µg/ml respectively. In other hand, the IC_{50} values of hesperidin-NPs was 82.9, 72.4 and 75.6 µg/ml against MCF-7, HepG-2 and HCT-116 and 1522 and 2202 µg/ml against MCF12F and Bj-1, respectively. The IC_{50} of citrus EO were 93.8, 67.6 and 88.2 µg/ml against MCF-7, HepG-2 and HCT-116, respectively and 510 and 310 µg/ml against MCF12F and Bj-1 normal cell lines, respectively. Whereas, the IC_{50} values of citrus EO -NPs were 76.4, 58.3 and 105.2 µg/ml MCF-7, HepG-2 and HCT-116 and 2316 and 3437 µg/ml on MCF12F and Bj-1 respectively. The cytotoxic activity of hesperidin could be a result of its antioxidant capacity [57] which inhibit cell proliferation [58], and display antimutagenic effect [59]. Among dietary flavonoids hesperidin and its derivatives, (such hesperidin) were highly potent cytotoxic agents and inhibiting neoplastic transformation in murine fibroblasts [60]. Citrus EOs are among of the most valuable plant products used in medicine [61]. D-Limonene, the most abundant constituent of orange citrus EO, has been shown to have anti-proliferative and apoptosis-inducing effects [62, 63]. Thus it has been used as a chemo preventive and chemotherapeutic agent against multiple types of tumors [64]. Other components of the citrus EO, such as -pinene, have been shown to inhibit growth of non-small-cell lung carcinoma cells [65]. Citral could reduce the proliferation of MDA-MB-231 cells by inhibiting the cancer stem cell marker ALDH1A3 (Aldehyde dehydrogenase 1 family, member A3) [66]. The results revealed that, nano-formulation of hesperidin and citrus EO have pharmaceutical properties, including the antioxidants, antibacterial and cytotoxic activities and this result may be caused by nano-formulation forms which have been changed in physical properties, including particles size, solubility and shapes [67].

Table 5. Cytotoxic activity and IC_{50} of hesperidin-NPs, hesperidin, citrus EO-NPs and citrus EO at 100µg/ml.

	MCF-7	MCF12F	HepG2	HCT-116	BJ-1
	Cytotoxicity % (at 100 µg/ml)				
Hesperidin	45.66^{b}± 1.07	7.40^{a}±0.92	66.33^{b}±1.70	88.13^{a}±1.96	11.28^{a}±1.31
Hesperidin -NPs	59.52^{a}±1.66	3.05^{b}±0.24	69.37^{a}±1.54	66.32^{b}±1.05	2.76^{b}±0.43
PEG-NPs	8.08^{c}±1.09	2.42^{b}±0.44	8.13^{c}±0.74	5.61^{c}±0.64	1.66^{b}±0.18
LSD	2.59	1.2	2.77	2.66	1.6
Citrus EO	54.46^{b}± 1.35	9.53^{a}±0.44	74.57^{b}±1.27	57.31^{a}±1.61	14.85^{a}±0.95
Citrus EO -NPs	64.61^{a}±1.33	2.23^{b}±0.20	85.46^{a}±1.48	49.47^{b}±1.38	1.40^{b}±0.07
PEG-NPs	9.23^{c}±0.65	2.07^{b}±0.10	8.41^{c}±0.48	6.39^{c}±0.47	1.79^{b}±0.09
LSD	2.31	0.57	2.3	2.5	1.1
	IC_{50} µg/ml				
Hesperidin	112^{b}.33±2.53	751.76^{c}±5.44	77.59^{b}±1.43	56.12^{c}±1.07	393.01^{c}±3.70
Hesperidin -NPs	82.90^{c}±1.63	1522.32^{b}±9.47	72.45^{b}±1.35	75.66^{b}±1.07	2202.37^{b}±11.62
PEG-NPs	1621.11^{a}±3.17	2438.35^{a}±7.93	623.68^{a}±4.70	825.14^{a}±5.32	2669.33^{a}±9.46
LSD	5.04	15.56	5.87	6.37	17.8
Citrus EO	93.84^{b}±1.49	510.80^{c}±5.60	67.69^{b}±1.51	88.20^{c}±1.60	310.68^{c}±7.28
Citrus EO -NPs	76.43^{c}±1.46	2316.81^{a}±11.45	58.33^{c}±1.94	105.22^{b}±3.30	3437.17^{b}±13.44
PEG-NPs	1661^{a}.24±5.28	2397.73^{b}±7.89	623.34^{a}±7.72	819.85^{a}±6.38	5120.07^{a}±17.02
LSD	6.54	17.29	9.34	8.49	26.38

All experiments were performed in triplicate; all data are expressed as the mean ± SD.
Means with different letters (at the same Column) are significantly different at $p \leq 0.05$.

4. Conclusion

hesperidin and citrus EO is a naturally occurring substances found in many fruits and vegetables including orange peels. Flavonoids and terpenoids have undergone a number of research studies as to their possible benefits against diseases, among other health issues. The current work aimed to study the biological properties of native hesperidin and citrus EO xtracted from Egyptian sweet orange peel and their nanoparticles forms. The obtained data revealed that the small size of nanoparticles is hopeful to improve tremendous biological activities including antioxidant, DNA damage prevention, antimicrobial and cytotoxic activity. The obtained results showed that the tested nanoparticles have strong cytotoxicity against three cancer cell line (HepG-2, MCF-7 and HCT-116) more than native forms. However, their tiny effect on normal cell line opens the door to its use as a drug delivery system to enhance the selectivity against only cancer cells. In the future these nanoparticles could be used as natural antioxidants, antimicrobial agents and nature preservatives in food products. In addition, they have cytotoxic activity against cancer cell lines, and protect DNA from damage which magnify its nutritional impact on our health and protection from cancer.

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