

Citrus Limetta Extracts Inhibit Oxidative Stress in Human Skin Organ Culture

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ABSTRACT

The current study is part of the development of medicinal plants known for their therapeutic virtues. We were then interested in studying sweet lime (*Citrus limetta*) known for its richness in different compounds characterized by their important biological properties. The present work focused on extracting the peel of sweet lime by the maceration technique. The determination of phenolic compounds (hexane: 2.57 ± 0.0198 ; ethanol 150 ± 3.4 ; aqueous: 164.64 ± 2.37 mg equivalent in mg GAE/g DM), and in flavonoids (hexane: 1.544 ± 0.045 ; ethanol 64.991 ± 1.61 ; aqueous: 75.915 ± 0.65 mg equivalent mg QE/ g DM). Antioxidant activities were studied by direct, reactive oxygen species assay using chemical systems (TAA, DPPH, ABTS+, FRAP). The antioxidant effect on lipid peroxidation was studied in the biological system (cell culture), by measuring the levels of MDA and DC in HeLa cell line after H₂O₂ treatment. Our results showed protection against lipid peroxidation, highlighted by a significant decrease in MDA and DC levels ($p < 0.5$ and $p < 0.01$, respectively). The results showed a cytotoxic effect of the hexane extract on HeLa cell lines. In addition, the histological study on the skin fragments showed dose-dependent protection of our extracts against lesions caused by hydrogen peroxide.

Introduction

Herbal medicines have formed the basis of traditional medical systems for centuries and occupy an interesting place in modern pharmacology. The World Health Organization (WHO) estimates that about 80% of the world's population still depends on herbal medicines to treat various diseases because of their high availability and economic reasons [1]. Citrus, belonging to the Rutaceae family, is one of the most widely grown crops globally, especially in tropical climates and temperate regions [2]. The health benefits

of citrus have been mainly associated with its high contents on antioxidant molecules such as Vitamin C and Polyphenols known for their benefic impact on human health [3]. Citrus belonged to the family Rutaceae and several commercial citrus varieties such as sweet orange, grapefruit, lime, and lemon and were considered to possess natural compounds with several health benefits [4]. The beneficial effects of citrus fruits can be attributed to their chemical constituents, including vitamins, dietary fiber, carotenoids, flavonoids, lipids, and essential oils [5,6].

Citrus limetta belongs to a type of citrus commonly known as sweet lemon, which can be cultivated in South and Southeast Asia [7]. It is small, between oval and spherical, with a greenish-yellow peel, rich in polyphenols, flavonoids, flavanones, and flavones [8]. *Citrus limetta* Risso (Rutaceae) is known to be an antihyperglycemic plant in Mexican folklore [9]. *C. limetta* fruits are used for the common cold, decreasing cholesterol level, fever regulation, regulating inflammation, digestive disorders, and so forth as well as blood pressure modulator [10,11]. Flavonoids hesperidin and naringin are found to be present in the peel part of the fruit of *Citrus limetta* [12]. *C. limetta* comprised of 8–10% peel, which is perishable waste material creates a challenge in processing industries and pollution monitoring agencies [13]. Interestingly, *C. limetta* fruit peels possess various pharmacological activities due to flavonoids in a significant quantity [14,15].

In this context that we proposed to study in a first part the extraction of fruit peels from the *Citrus limetta* plant by maceration using three solvents (hexane, water and ethanol). In the second part, we studied the content of total polyphenols and flavonoids and the antioxidant effect. First, the antioxidant power was studied in a chemical system (TAA, DPPH, ABTS, and FRAP). Then, the biological system used HeLa cell lines by measuring MDA and CD levels. In addition, to study the cytotoxic effect, MTT test was realized using HeLa cells lines. Finally, the antioxidant activity of the three extracts of *Citrus limetta* was evaluated on skin explants.

Materials and Methods

Plant Materials and Extraction Procedure

The *Citrus limetta* fruits were purchased from the local market / Sfax, south of Tunisia, in March 2019. The fruits were carefully washed, cut into small pieces, and dried in an oven at 60 °C. 100 g of plant material were treated overnight with different solvents (ethanol, hexane, and water) under gentle stirring. The different extracts were filtered through a cellulose filter, lyophilized, and frozen at -80 °C until use.

Total Phenol Determination

Total phenols were determined using the Folin-Ciocalteu reagent according to the method of Singleton and Rossi [16]. Briefly, 50 µl aliquot of the extract was mixed with 250 µl of Folin reagent and 500 µl of sodium carbonate (20%, w/v). The mixture was vortexed and diluted with water to a final volume of 5 ml. After incubation for 30 min at room temperature, the absorbance was read at 765 nm. Total phenols were expressed as gallic acid equivalents (GAE), using a freshly prepared gallic acid solution calibration curve.

Total Flavonoid Determination

Total flavonoids were measured by the colorimetric assay described by Zhishen, et al. [17]. Total flavonoids were expressed on a dry weight basis as quercetin equivalents, using a freshly prepared quercetin solution calibration curve.

Antioxidant Capacity Assays

a. Phosphomolybdenum Assay

The total antioxidant activity of the extracts was evaluated using the Prieto, et al. methodology [18]. Briefly, 100 µl of the extract was mixed with 1 ml of the phosphomolybdenum reagent (600 mM sulfuric acid, 28 mM sodium phosphate, 4 mM ammonium molybdate). After incubation at 95 °C for 90 min, the absorbance was measured at 695 nm. The total antioxidant activity was expected to be the effective concentration (EC50) compared to Vit C as a positive control.

b. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Activity Assay

The radical scavenging activity of extracts against DPPH free radicals was measured using the method of Cao, et al. [19] slightly modified as follows: 20 µl of appropriately diluted samples or Vitamin C solutions was added to 190 µl of DPPH solution (100 µM). The mixture was shaken vigorously and allowed to reach a steady-state at room temperature for 30 min. Then, the absorbance was measured at 517 nm with a Beckman spectrophotometer.

c. ABTS Radical Scavenging Activity Assay

The antiradical activity was performed by the ABTS+ free radical decolorization assay as developed by Re, et al. [20]. The reaction mixtures containing 100 µl of the sample at different concentrations and 900 µl of reagent were incubated at 30 °C for 6 min. The absorbance was measured at 734 nm.

d. Ferric-Reducing Antioxidant Power (FRAP)

The principle of this method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous, colored form in the presence of antioxidants [21]. Briefly, the FRAP reagent contained 2.5 ml of a 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine, Sigma) solution in 40 mmol/L HCl plus 2.5 ml of 20 mmol/L FeCl₃ and 25 ml of 0.3 mol/L acetate buffer, pH 3.6 and was prepared freshly and warmed at 37°C. Aliquots of 40 µl sample supernatant were mixed with 0.2 ml distilled water and 1.8 ml FRAP reagent. The absorbance of the reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37°C for 10 min. The 1 mmol/L FeSO₄ was used as the standard solution. A spectrophotometer performs the

absorbance reading against a blank at 700 nm. Ascorbic acid is used as a positive control. The increase in the absorption capacity of the components indicates an increase in iron reduction.

e. MTT Cell Proliferation Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay measures the cell proliferation rate and, conversely, reduces cell proliferation viability when metabolic events lead to apoptosis or necrosis. The yellow compound MTT (Sigma) is reduced by mitochondrial succinate dehydrogenase to the blue formazan, depending on the cell viability. Cells were grown on microtiter plates (200 μ l of cell suspension/well) in 96 well microplates with serial extract dilutions. After 72 h, 20 μ l of MTT solution (5 mg/ml) was added to each well. The plate was incubated for 4 h at 37 °C in a CO₂ incubator. After that, 180 μ l of the medium was removed from each well and replaced by 180 μ l of (DMSO/ methanol) (V:V). When all the crystals were dissolved, absorbance was measured at 570 nm with a microplate reader (Elx 800 microplate reader) [22].

f. Malondialdehyde (MDA) Determination

The MDA production was evaluated using thiobarbituric acid-reactive species (TBARS) assay. Adherent cells were detached using trypsin/EDTA solution and centrifuged at 3000 rpm for 10 min. The pellet was resuspended in 500 μ l of deionized water and lysed by five cycles of sonication during 20 s at 35% (Sonisc, vibracell). One millilitre of TBA solution (15% trichloroacetic acid, 0.8% thiobarbituric acid, 0.25 N HCl) was added. The mixture was heated at 95 °C for 15 min to form MDA-(TBA)₂ adducts. Optical density (OD) was measured by a spectrophotometer (Biochrom, Libra S32) at 532 nm. Values were reported to a calibration curve of 1,1,3,3-tetraethoxypropane (1.1.3.3 TEP) [23].

g. Conjugated Dienes

After sonication, cell lysates were extracted with 3 ml (chloroform: methanol) (2:1 v/v). After centrifugation at 3000 rpm for 15 min, 2 ml of the organic phase was transferred into another tube and dried at 45 °C. The dried lipids were dissolved in 2 ml of methanol, which was determined by absorbance at 233 nm. This corresponds to the maximum absorbance of the extracted compounds [24].

Evaluation of the Antioxidant Activity of *Citrus Limetta* Extract on Skin Explants

a. Stress Induction

After removal, the fragments are washed three times with PBS (1 \times) and then incubated for 15 min at room temperature in

the presence of an antibiotic and an antifungal. The fragments are then sterile cut into small pieces and cultured in a 24-well plate at the air / liquid interface, in the presence and absence of the 3 extracts at different concentrations (0.5 mg / ml; 1 mg / ml; 2 mg / ml) for 2 hours at 37 °C. After incubation, the culture medium is eliminated, and then two successive washes with PBS (500 μ l) are carried out. Hydrogen peroxide is then added at a concentration of 1.5 mM in RPMI to induce oxidative stress on the skin explants. After 2 h of incubation at 37 °C, all the fragments are recovered in tubes containing formalin and stored at 4 °C until the histological sections are made.

b. Histopathological Examination

For light microscopic examination, samples were fixed in 10% neutral buffered formalin, dehydrated in an ascending ethanol series (65%, 75%, and 95%), cleared in xylene, and embedded in paraffin. Paraffin sections (5 μ m) were stained with hematoxylin and eosin using a routine method. The assembly step consists of bonding the blades containing the sections.

c. Analysis data

Statistical studies are carried out using the SPSS program (19.0). The t-Student test carried out the comparison between the means. The results are represented in mean \pm standard deviation (DS).

Results

Total Phenolic and Flavonoid Compounds

First, we were interested in evaluating the levels of anthocyanins, total phenolic, and flavonoid compounds in the different *Citrus limetta* extracts using Follin-Ciocalteu colorimetric and AlCl₃ methods, separately. The results show that the aqueous and ethanolic extract contains (166.4 \pm 0.81 mg GAE / g DM; 152.29 \pm 1.8 mg GAE / g DM) respectively. On the other hand, the hexane extract has a low polyphenol content (2.57 \pm 0.02 EAG / g DM) (Table 1). Regarding flavonoids contents, our results indicate 75.9 \pm 0.65 mg QE/g DM; 64.9 \pm 1.64 mg QE/g DM for aqueous and ethanolic extract, respectively. In addition, a small number of flavonoids for hexane extract was detected (1.54 \pm 0.045 EC / g DM) (Table 1).

Antioxidant Activity

a. Phosphomolybdenum Assay

The power of ammonium phosphomolybdate in the present study is expressed in mg equivalent of ascorbic acid (vitamin C) / g of dry matter. Our results indicate a total antioxidant activity for the *Citrus limetta* peels extracts (Table 1).

Table 1: Total antioxidant activity of *Citrus limetta* extracts.

Sample	Aqueous	Ethanolic	Hexane
Total phenol content (mg GAE/g DM)	166.4 ± 0.81	152.29 ± 1.8	2.57 ± 0.02
Flavonoides (mg QE/ g DM)	75.9 ± 0.65	64.9 ± 1.6	1.54 ± 0.04
Total antioxidant activity (mg AA/g DM)	738.86 ± 0.005	417.84 ± 0.001	429.57 ± 0.004

b. The Scavenging Activity for DPPH Radicals

Table 2: ABTS and DPPH IC₅₀ values for the extract of *Citrus limetta* (HEOA), Vit C and TROLOX.

Sample	ABTS values	DPPH IC ₅₀
Aqueous	0.56	0.88
Ethanolic	0.6	1.38
Hexane		0.57
Vit C	-	0.41
TROLOX	0.4	-

DPPH molecules that contain a stable free radical have widely been used to evaluate the radical scavenging ability of antioxidants. Therefore, the *Citrus limetta* free radical scavenging activities were assayed using DPPH (Table 2). At all the tested concentrations,

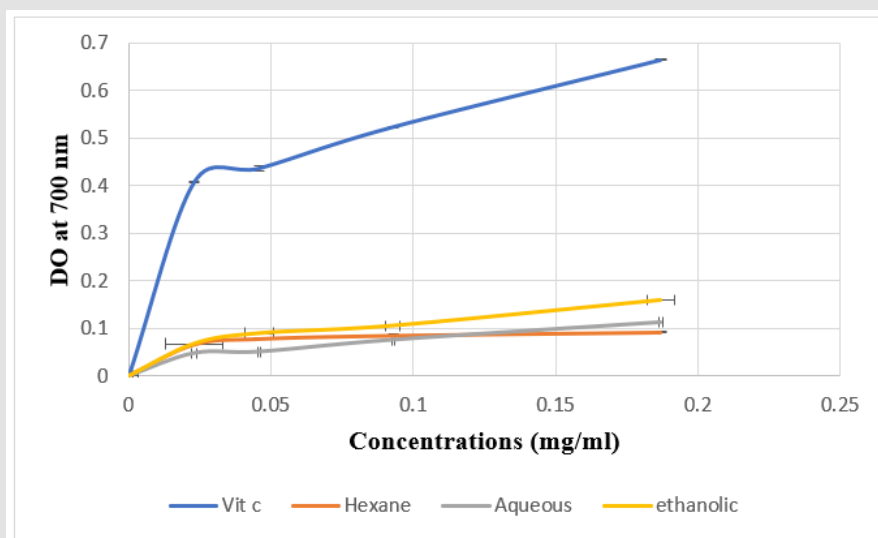
Citrus limetta aqueous, ethanolic, and hexane extracts showed a significant anti-radicular effect attending 84%, 83%, and 87%, respectively, compared to their synthetic antioxidant positive control (BHT).

c. The Scavenging Activity for ABTS

The antioxidant activity test results of the ABTS+ radical by ethanolic, aqueous, and hexane extracts attend 89,9%; 90,10 and 8,24%, respectively (Table 2).

d. Ferric-Reducing Antioxidant Power (FRAP)

The FRAP assay is usually used to measure the capacity of the sample to reduce the ferric complex to the ferrous form. However, our results showed that the three *Citrus limetta* peel extracts have a weak antioxidant activity compared to vitamin C (Figure 1).

**Figure 1:** Absorbance of standard Vit C and extracts of *Citrus limetta* peel by Ferric Reducing Antioxidant Power (FRAP) assay.

Note: Various concentrations of extracts (0 to 0.25 mg/mL) and acid ascorbic were mixed with FRAP reagents. The reduction of ferric ion (Fe³⁺) to ferrous form (Fe²⁺) by extracts produces an intense blue light revealed as a change in absorption at 700 nm. Results were expressed as mean inhibition percentage (%) ± standard deviations (n = 3). Ascorbic acid at various concentrations (0 to 0.25 mg/mL) was used as standard.

e. Cytotoxicity Effect of *Citrus limetta* Peel Extract

To investigate the cytotoxic effect of *Citrus limetta* peel extracts on HeLa human cell line, cells were treated with various concentrations of extracts (Hexane, ethanolic and aqueous) ranging

from 4 to 0.0625 mg/ml for 48 hours, and then submitted to the MTT test. Our results showed a cytotoxic effect of the hexane extract on the HeLa line depending on the concentrations used. The other two extracts (Ethanol and Aqueous) have less than 50% cytotoxicity for the concentrations used (Figure 2).

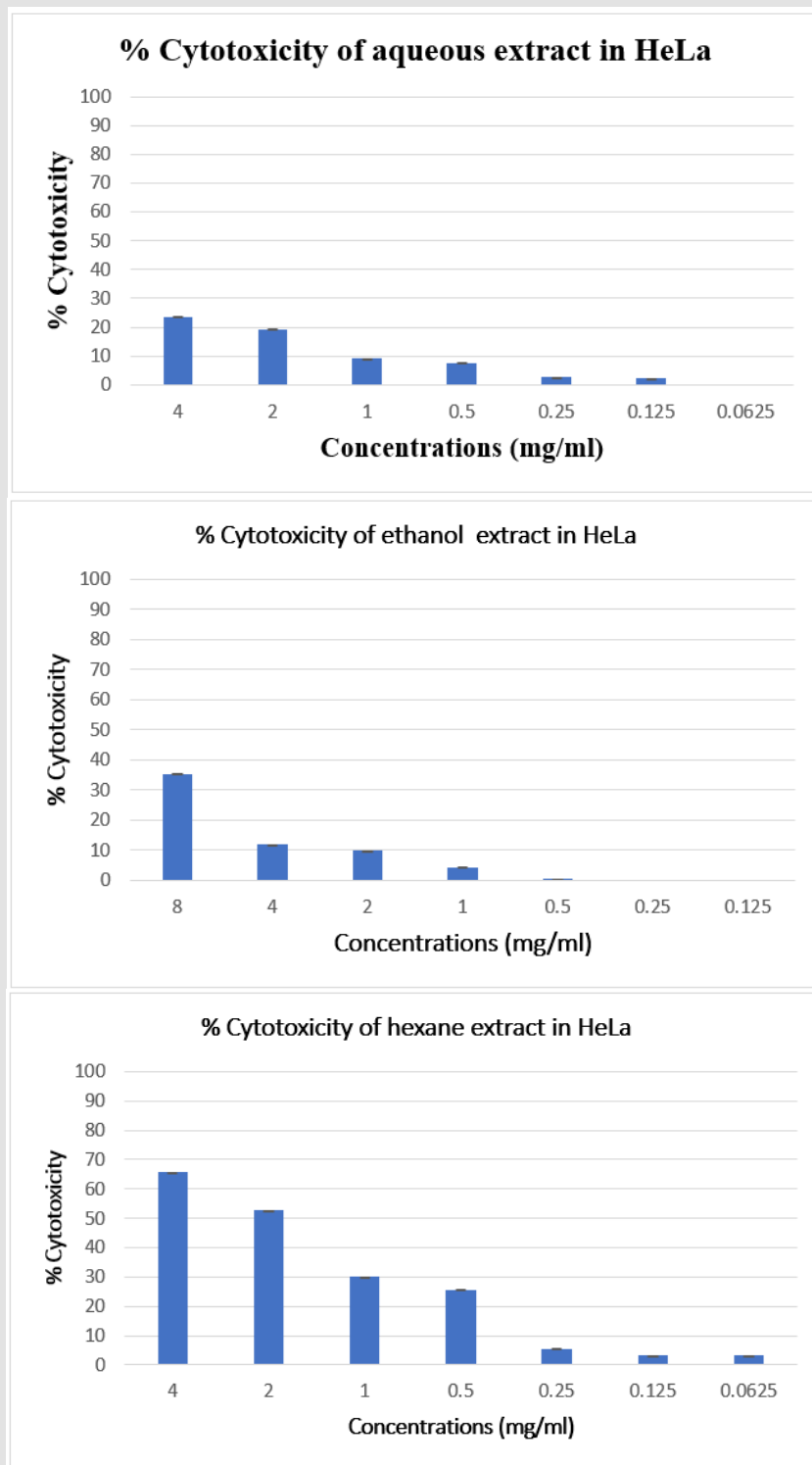


Figure 2: Cytotoxic effect of Citrus limetta peel extracts on HeLa cell line.

Note: The inhibitory effect of different doses on cell growth was determined by MTT assay. Cells were treated with extracts at a concentration ranging from 4 to 0.0625 mg/ml. The percent of growth reduction was calculated from the extinction difference between treated cell culture and the control. Results are the means of two repetitions.

f. Effect of *Citrus Limetta* Peel Extracts on Lipid Peroxidation Activity

The investigation of the *Citrus limetta* extracts biological antioxidant activity was carried in HeLa human cell line. Cells were cultured with or without the addition of extracts for 48 hours. Oxidative stress was induced by adding 0,2 mM H_2O_2 . Hydrogen peroxide and *Citrus limetta* extracts with a 0.25 mg/ml concentration were added to the HeLa human cell line simultaneously for 30 minutes.

g. Malondialdehyde (MDA)

After H_2O_2 treatment, a very significant increase in MDA levels

in the HeLa line was observed, in comparison with the untreated cells, indicating the presence of a state of oxidative stress following the treatment of these cells by H_2O_2 ($p < 0.01$). HeLa cells treated with the three extracts: ethanol, hexane, and aqueous, induce a very significant decrease in MDA levels ($p < 0.01$ respectively) (Figure 3).

h. Conjugated Dienes (CD)

HeLa cell line treated with H_2O_2 induces a significant increase in CD levels compared to untreated cells ($p < 0.001$) (Figure 3). Conversely, treating cells with *Citrus limetta* extracts causes a significant decrease in CD levels. This decrease is highly significant for hexane and ethanol ($p < 0.001$) and significant for the aqueous extract ($p < 0.05$).

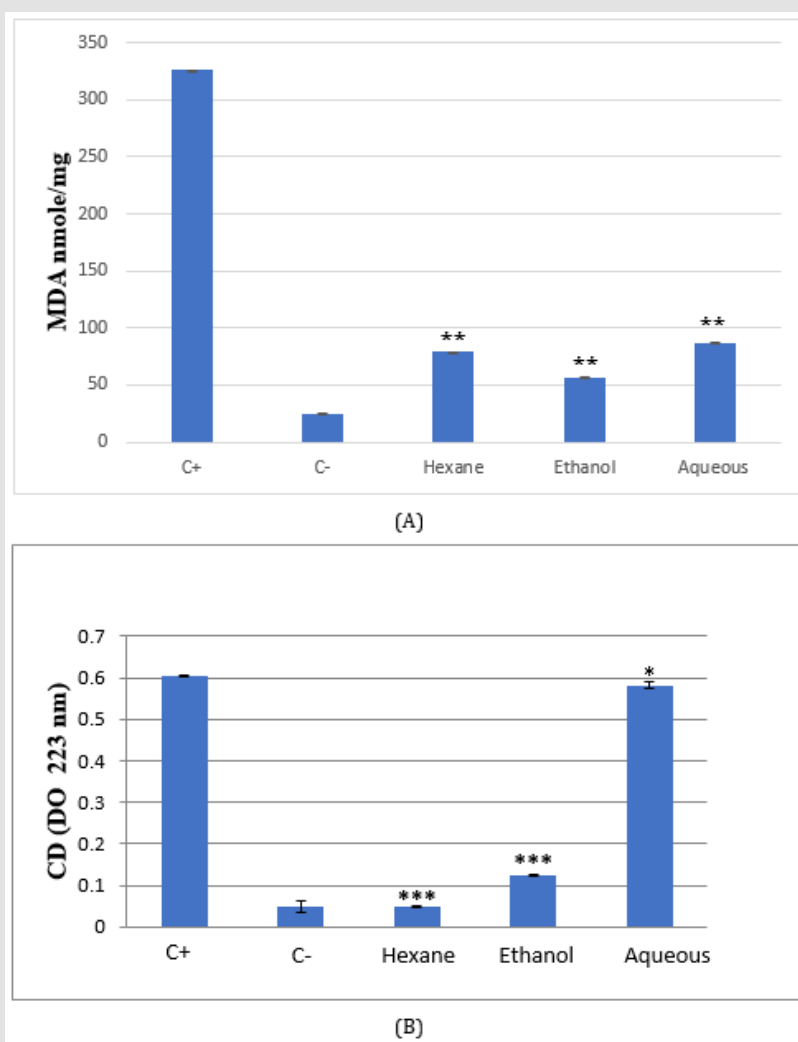


Figure 3: MDA (A) and conjugated diene (B) levels in *Citrus limetta* peel extracts supplemented HeLa cell line.

Cells were cultured in 25 cm² flasks with 0.25 mg/ml of extracts for 48 hours. Oxidative stress was induced by the addition of cells for 30 min. TBARS and conjugated diene (CD) were compared to untreated cells (C-), cells treated with H_2O_2 (C+), and cells treated with extracts.

i. The Protective Effect on Skin Explants in Culture

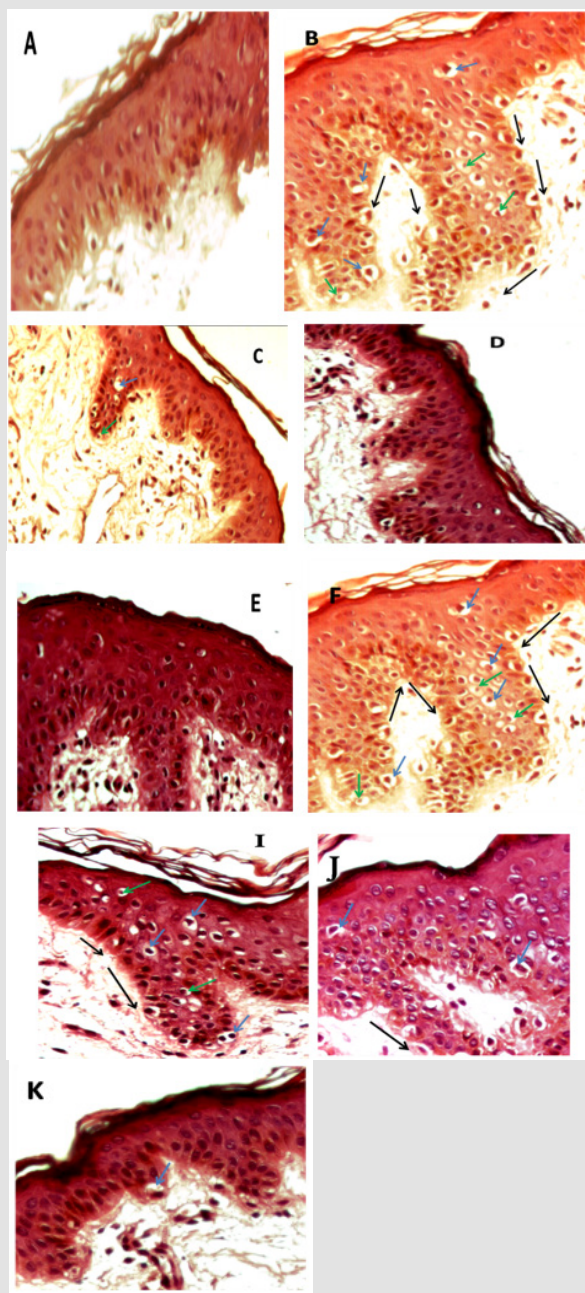


Figure 4: Micrograph of skin sections treated with the three extracts of *Citrus limetta*.

Note: A: control B: treated with H_2O_2 (1.5 mM) C: traitée with hexane extract (0.5 mg/ml) then with H_2O_2 (1.5 mM) D: treated with hexane extract (1 mg/ml) then H_2O_2 (1.5 mM) E: treated with hexane extract (2 mg/ml) then H_2O_2 (1.5 mM) F: treated with aqueous extract (0.5 mg/ml) then H_2O_2 (1.5 mM) G: treated with aqueous extract (1 mg/ml) then H_2O_2 (1.5 mM) H: treated with aqueous extract (2 mg/ml) then H_2O_2 (1.5 mM) I: treated with ethanolic extract (0,5 mg/ml) then H_2O_2 (1.5 mM) J : treated with ethanolic extract (1 mg/ml) then H_2O_2 (1.5 mM) K : treated with ethanolic extract (2 mg/ml) then H_2O_2 (1.5 mM).

- Detachment of the basal lamina
- Pycnotic nucleus
- Vacuolar degeneration

The evaluation of the antioxidant activity of the three *Citrus limetta* extracts was carried out on skin explants maintained in culture in the presence of different concentrations of each extract (0.5 mg/ml; 1 mg/ml; 2 mg/ml). The results obtained are compared with those found with explants cultured in the presence and absence of hydrogen peroxide (Figure 4). Our results have shown that culturing of skin explants in the complete medium does not affect the histo-architecture of the skin (Figure 4A). However, treatment with hydrogen peroxide at a concentration of 1.5 mM leads to structural disorganization of the skin, which manifests itself in the detachment of the membrane which separates the dermis and the epidermis. In addition, in the granular layer of the epidermis, the cells presented a necrotic appearance which resulted in retraction of the nuclei (pycnotic nuclei) and vacuolar degeneration (Figure 4B).

The pretreatment of the skin explants at different concentrations of each *Citrus limetta* peel extract made it possible to minimize the oxidative damage induced by hydrogen peroxide depending on the concentration used (Figures 4C-4N). It is observed that the protective effect is most important at the level of the fragments treated with the hexane extract (Figures 4C-4E), so the total disappearance of the oxidative damage by the treatment with the high concentration of hexane extract at 2 mg/ml.

Discussion

Plants contain different phenolic compounds, including simple phenolics, phenolic acids, hydroxycinnamic acid derivatives and flavonoids [25]. The content of total polyphenols and flavonoids was determined. Our results agree with those of Mohanty, et al. [15] who showed the content of phenolic and flavonoid compounds in the aqueous and ethanolic extract of *Citrus limetta* [15]. Similarly, Safdar et al showed that the 4 peel extracts of *Citrus reticulata* L (methanols, ethanol, acetone, and ethyl acetone) presented a polyphenol content varying according to the polarity of the solvents used in the extraction [26]. Our results agree with the work of Hegazy et al. [27] who found that the polyphenol content varies between the different extracts of orange peel [27]. According to Chan et al. the choice of extraction solvent is essential because it allows the type and quantity of polyphenols to be estimated [28]. The variations in the polyphenol content depend on the polarities of the solvents used and their concentration level.

Citrus limetta extracts analyzed in this work showed potent radical scavenging activity. We have demonstrated that G_{EO} has an interesting antioxidant activity which is highlighted by the TAC, DPPH, ABTS+, FRAP. Our results agree with the literature that showed that citrus possesses an anti-free radical activity DPPH, according to Padilla, et al. [29]. The *Citrus limetta* aqueous extract can trap the DPPH radical with a percentage inhibition of

42.5%. Other *Citrus reticulata* L methanolic extract studies have shown a high antioxidant potential (72.83 ± 1.22%) [26]. Other work studied three extracts of the peel orange (*Citrus aurantium*) that showed significant antioxidant power with low IC₅₀ values (acetone 781.9 µg / ml; ethanol 1,137.9 µg / ml; methanol 1,402.9 µg / ml) [30]. The same extract showed comparable antioxidant activity to TROLOX using the ABTS radical. Our results agree with Oboh et al., who showed the presence of an antioxidant effect of the peel of *Citrus sinensis*, *Citrus paradisi*, and *Citrus maxima* extracts against the radicals ABTS [31].

The *Citrus limetta* extracts had a weak FRAP value, which was in disaccord with the results obtained by Farha et al. that confirmed that the *Citrus limetta* methanolic extract has moderate antioxidant (iron-reducing capacity) activity [32]. The variation in antioxidant activity depending on the tests can be explained by the fact that this antioxidant activity depends not only on the concentration but also on the structure and antioxidants nature [33]. Our results showed a cytotoxic effect-dependent concentration of *Citrus limetta* extracts with Hexane on HeLa cell lines. Other studies on the aqueous extract of *Citrus unshiu* (a species of the same family as the *Citrus limetta*) showed the presence of a cytotoxic power of the latter on the line MDA-MB-231 with a concentration of 1,5 mg/ml. This cytotoxic effect is attributed to the disruption of the mitochondrial transport system [34]. On the other hand, Diab et al. showed that the ethanolic peel extracts of *Citrus sinensis* L reduced the HL-60 cell line viability [35].

According to the results, to investigate the antioxidant activity of our extract on the cells model, we choose a concentration that induces less than 20% of toxicity. The concentration used is 0.25 mg/ml in all the experiences. HeLa cells are treated simultaneously with H₂O₂ and extracts. Our results induce a very significant decrease in MDA levels, which agrees with the work, which showed that the methanolic extract of *Citrus limetta* peel exhibits antioxidant activity demonstrated by a reduction of MDA levels [36]. To the best of our knowledge, our study is the first to determine the antioxidant potentials of *Citrus limetta* peel on the skin by analyzing histopathological findings.

Conclusion

Citrus limetta is a promising source of natural antioxidants, as indicated by its high contents of polyphenols, flavonoids and by its considerable DPPH and ABTS free radical scavenging activities and FRAP value. In addition, the histological study of skin fragments has shown dose-dependent protection of our extracts against lesions.

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This work is dedicated to the memory of Pr. Saloua Lassoued Elamri. We will never forget her, and she is always in our hearts.

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