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Phytochemistry and Antioxidant Activities of the Methanolic Extract Obtained from the Leaves of *Citrus limon* (L.) Osbeck

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Abstract: This study was aimed to identify the phytochemistry and antioxidant activities of the methanol extract obtained from the leaves of *Citrus limon* (L.) Osbeck (CLM). HPLC analyses revealed the presence of various phenolics (gallic acid, catechin, vanillic acid, coumaric, resveratrol) and flavonoids (rutin and apigenin).Qualitative analyses showed phenolics (302.91 µg EAG/mg), tannins (36.86 ± 0.71µg ECT/mg) and flavonoids (19.77 ± 0.06 µg EQ/mg) and coumarins. CLM exhibited a significant concentration-dependent *in vitro* antioxidant activity against DPPH radical and reducing power (FRAP test). This study concluded that the methanol extract obtained from the leaves of *C. limon* possess an antioxidant potential.

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1. INTRODUCTION

The medicinal plants were used for their particular beneficial properties for human health [1]. Indeed, they were used in different ways including decoction, maceration, and infusion. One or more of their parts can be used as root, leaf and flower. According to Solecki [2], medicinal plants have been used by humans for nearly 60,000 years for their therapeutic purposes. About 35,000 plants species were used globally for medicinal purposes. Despite the growing influence of the modern health system, medicinal plants continue to meet a significant need [3]. They were used or in the form of oils, extracts, aqueous or organic solutions. Preparations contain one or more active ingredients that can be used for therapeutic purposes [4]. Indeed, they can use to help the human body in scavenging free radicals and active oxygen which leads to cell injury and apoptosis. Therefore, they may protect the cells from the damage of the stress oxidative and various diseases.

In this context, the main purpose of this work was to study the phytochemical and antioxidant activities of the methanolic extract of the leaves of *C. limon.* Indeed, *C. limon* is known under the name "Lemon tree", is a member of the family Rutaceae, known for its wide

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therapeutic use, for example, the infusion of the leaves is used for the treatment of obesity, diabetes, diseases cardiovascular, brain disorders and certain types of cancer [5, 6]. Then, the essential oil has antimicrobial activity [7], antifungal [8]. Then, in our work has been revealed, found that the essential oil of the leaves of *C. limon* had a protective effect against a high dose of aspirin induced toxicity in different organs in Wistar albino rats and *in vitro* induced damage in intestine epithelial cells (IEC)-6 cells [9, 10, 11].

Consistently, the present work is aimed to: i) analyse the composition of the methanolic extract of *C. limon*, ii) analyse qualitative and quantitative of the extract and iii) study the antioxidant activity of the extract using two methods (DPPH test and reducing power).

2. MATERIAL and METHODS

2.1. Standards Chemicals

Solvents, reagents and standards were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Plant Material

The leaves of *C. limon* were collected in the region of Sidi Aich (Gafsa) in January 2018. It identified with the botanist in the Faculty of Science of Gafsa (Dr. Lefi El Kadri). Then, the leaves were cleaned and then dried at room temperature (approximately 20% relative humidity and 24°C temperature) for 48 h. The dry leaves were then finally ground with a mortar.

2.3. Sample Extraction

The powder of *C. limon* (1 g) was immersed into 10 mL of methanol and macerated for 24 h in an erlenmeyer flask with stirring. Then, the content was filtered with Whatman No. 1 filter paper. The resulting filtrate was evaporated by a rotary evaporator at 35 °C under reduced pressure. The dry extract was stored in the dark bottles and refrigerated at 4°C.

2.4. HPLC Analysis Condition

The HPLC separation of the active compounds was carried out using variant prostar HPLC equipped with a C-18 reverse phase column (Varian,250 mm*4.6 mm, particle size 5 µm), a ternary pump (model Prostar 230) and a Prostar 330 diode array detector at a gradient elution. Eluant A was methanol (100%); eluant B was 0.05% acetic acid aqueous solution. Gradient conditions was initial=35% A and 65% B, 30 min=50% A and 50% B, 40 min= 90% A and 10% B. the flow rate was 1 mL $min^{-1}$and the injection volume was 20 µL at 25°C. The identification was performed at 290 nm for phenolic acids and at 365nm for flavonoids, based on the comparison with the retention times of standards.

2.5. Phytochemical Screening

According to the tests of Ravishankara et al [12], the presences of various phytoconstituents were determined by performing different qualitative tests on methanol extract of the leaves of *C. limon* (CLM).

2.6. Determination of Polyphenol Contents

2.6.1. Determination of Total Phenolic Contents

The polyphenols were determined using the method of Singleton and Rossi. [13]. 50 µL of diluted plant extract were mixed with 400 µL of folin ciocalteu reagent. After 8 min, 500 µL of sodium carbonate (Na_2CO_3) at a concentration of 7.5 g/L were added. After incubation the reaction mixture for 1 h at room temperature and in the dark, the absorbance was measured at 725 nm. The total phenolic content was expressed as gallic acid equivalents per milligrams dry extract (µg GAE/mg) by calibration curve was performed by gallic acid at different

concentrations (15.625 to 500 µg/L), under the same conditions and the same steps of the assay. All measurements are repeated three times.

2.6.2. Determination of Total Flavonoid Contents

Total flavonoid content was determined as described by Dewanto et al. [14]. Briefly, 1 mL of the sample was mixed with 75 µL of 5 % sodium nitrite solution. After incubation for 5 min, 150 µL of 10 % aluminum chloride solution was added, and the mixture was left standing for 5 min, and then 0.5 mL of 1 M sodium hydroxide was added to the solution. Then, the volume of the reaction mixture was made with 2.5 mL of distilled water and then mixed well.

The absorbance was determined at 510 nm using quercetin as a standard. Results were expressed in micrograms of quercetin equivalent per milligram of extract (µg EQ/ mg) [14].

2.6.3. Determination of Total Flavonoid Contents

Total tannin contents were determined using the method of Heimler et al. [15]. The sample (400 µL) was added to 3 mL of vanillin solution (4%) and 150 µL of concentrated H_2SO_4 (sulfuric acid). Then the mixture was incubated for 15 min in the dark. Finally, the absorbance was read at 500 nm. The total content was determined from calibration ranges used with catechin (0-300 µg/mL) and expressed as in micrograms of catechin equivalent per milligram of extract (µg ECT/mg).

2.7. Antioxidant Activities

2.7.1. DPPH Radical Scavenging Assay

The effects of the methanol extract of *C. limon* on DPPH radical was determined following the method reported by Blois [16]. Briefly, 25 µL of diluted extract at different concentrations (50 – 200 µg/mL) were mixed with 975 µL methanolic of solution of DPPH (0.5 mM). In parallel, 25 µL of methanol was mixed with 975 µL of solution methanolic of DPPH. After 30 min of incubation in the dark, the absorbance was measured at 515 nm. BHT was used as standards of antioxidants. All the measurements were done three times. The results were expressed by the percent of inhibition (I %) according to the formula:

$$\text{I \%} = \left[\frac{(\text{Absorbance control} - \text{Absorbance sample})}{\text{Absobance control}}\right] \times 100$$

2.7.2. The Ferric Reducing Antioxidant Power (FRAP)

The evaluation of the ferric reducing antioxidant power (FRAP) of the sample was determined by the method of potassium ferricyanide-ferric chloride as described by the method described by Chu et al. [17]. The absorbance was measured at 700 nm, plotted against extract concentration (µg/mL) and compared with BHT used as a standard solution.

2.8. Statistical Analysis

All experimental measurements were performed in triplicate and are expressed as mean of ± three standard deviation analyzes [mean (SE) ± standard deviation]. Statistical analysis was executed using one-way analysis of variance (ANOVA). A value of $p< 0.05$ was considered statistically significant.

3. RESULTS and DISCUSSION

3.1. Chemical Analysis by High Performance Liquid Chromatography (HPLC)

The HPLC analysis of CLM revealed presence of phenolic acids and flavonoids (Table 1). There were five phenolic acids identified in the extract including gallic acid, catechin, vanillic acid, coumarin acid, and resveratrol with the retention times respectively, 6.28 min,

9.387 min, 20.58 min, 30.45 min and 32.48 min (Figure 1-A) and there were two flavonoids identified including rutin and apigenin with respectively retention times 22.040 min and 32.2 min (Figure 1-B). The HPLC elution profile phenolic acids also showed seventeen peaks of unknown compounds and fourteen peaks for flavonoids. This analysis was confirmed with the phytochemical investigation which revealed significant levels of polyphenols, tannins and flavonoids. Our obtained values were different from those reported in the peels of *Citrus limon* [18] which found that the analysis by HPLC-DAD-MS/MS of the peel of the extract methanolic of *C. limon* identified hesperidin, rutin, eriocitrin, diosmin, and hyperoside, as the main compounds.

3.2. Phytochemical Screening

As seen in Table 2, the preliminary phytochemical screening of methanol extract revealed the presence of phenolic compounds, flavonoids, tannins and coumarins. As reported in Table 3, the total phenolic level, the flavonoids and the tannins of the methanol extract of *C. limon* were found to be between 302.91 µg GAE/mg, 19.77µg EQ/mg and 36.86 µg ECT/mg of extract, respectively. These results were different with reported by Bocco et al. [19] which found that the main compounds of the seeds of *C. limon* were caffeic acid, p-coumaric acid, ferulic acid and sinapinic acid for the phenolic compounds and among the flavonoids, eriocitrin and neoeriocitrin were found.These differences were due to the conditions of drying and preservation of the sample. The difference can also be attributed to environmental factors or genotypes of the Citrus plant used [20]. It is owing to what parts of the plant used. Indeed, many researchers have shown that the peels and seeds contained more biologically active compounds and they are an interesting source of phenolic compounds. Also, the season of the collect of the plants, the method of extraction, the solvent used for extraction are the main factors of variation.

3.3. DPPH Radical Scavenging Activity

The antioxidant activity *in vitro* was evaluated using the reducing power, 2,2-diphenyl-1-picrylhydrazyl radical and phosphomolybdenum assay. Figure 2 showed a decrease in the concentration of DPPH radicals due to the scavenging ability of methanol extract and standard. At 45 and 185 µg/mL of methanol extract and BHT respectively exhibited 50 % inhibition.

Table 1. Main compounds identified in the methanolic extract of *Citrus limon* by HPLC

Compounds	Concentration (µg/mL)	Area
Gallic acid	4,64	4585430
Catechin	5,90	408880
Vanillic acid	2,71	1664397
Coumaric acid	1,21	746569
Resveratrol	32,41	4741123
Rutin	30,84	3191654
Apigenin	19,63	904947

3.4. Ferric Reducing Antioxidant Power Assay

The reducing capacity was based on the reduction of Fe^{3+} to Fe^{2+} by transfer of an electron and the hydrogen donors, which results in a green color. Figure 3 showed the reductive capability of the methanol extract of the leaves of *C. limon* and the standard BHT. It is found that the reducing power increased with increasing concentration. Therefore, the reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The antioxidant activity depends on the content of phenolic compounds of the sample and the position and number of grouping hydroxylated [21].

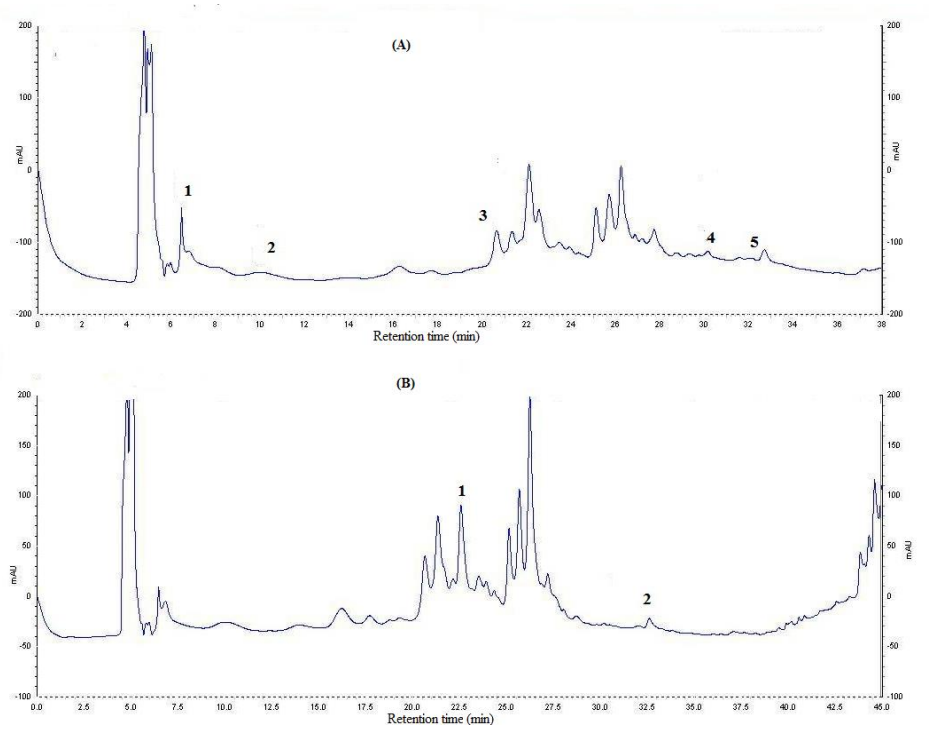

Figure 1. High-performance liquid chromatography (HPLC) of the methanolic extract of the leaves of *Citrus limon*, (A) HPLC profile of phenolic acids at 280 nm (1: Gallic acid, 2: Catechin, 3 : Vanillic acid, 4 : Coumarin acid, 5 : Resveratrol); (B) HPLC profile of flavonoids at 360 nm (1 : Rutin; 2: Apigenin).

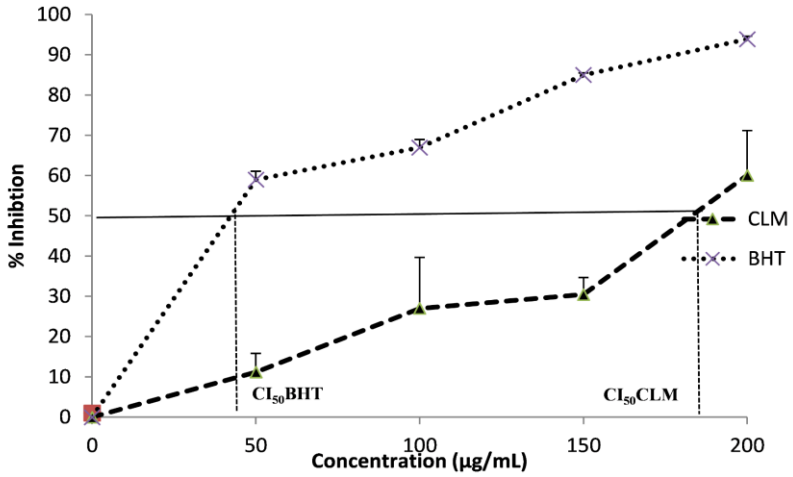

Figure 2. Free radical scavenging activity of the methanolic extract of the leaves of *Citrus limon* at different concentrations. Values are represented as mean ± standard deviation (n = 3).

Table 2. Phytochemical composition of extract methanolic of *Citrus limon*

Phytochemicals	Phenols	Flavonoids	Tanins	Coumarins
CLM	+ + +	+	+ +	+ +

+ : presence

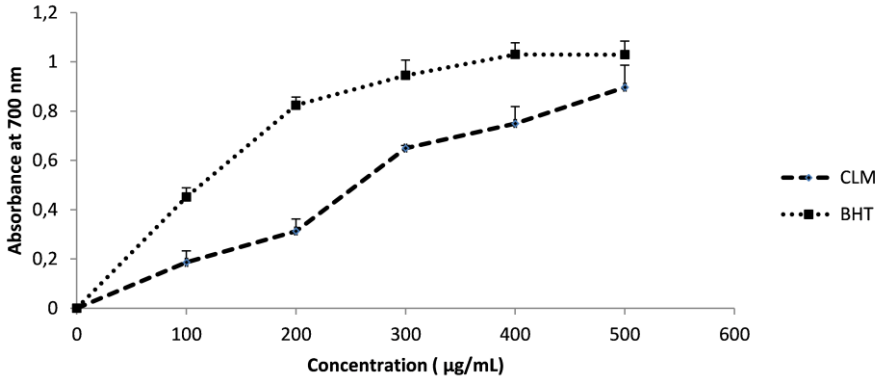

Figure 3. FRAP assay at various concentrations of the methanolic extract of the leaves of *Citrus limon* and the synthetic antioxidant BHT. Each value represented as mean ± standard deviation (n = 3).

Table 3. Polyphenols and total tannin contents were measured by colorimetric methods (The values are expressed by means SEM (n = 3)

	Total phenol content	Total Flavonoid content	The content tannin
CLM	302.91 (a)	19.77 ±0.06 (b)	36.86±0.71 (c)

CLM: methanol extract of the leaves of *Citrus limoni* (a) microgram of gallic acid equivalents per milligrams of extract (µg GAE/mg), (b) micrograms of quercetin equivalent per milligrams of extract (µg EQ/mg), (c) micrograms of catechin equivalent per milligram of extract (µg ECT/mg)

4. CONCLUSION

The metabolites secondary remained the subject of much research *in vivo* and *in vitro*, in particular the search for new natural constituents such as phenolic compounds. This study reveals that *Citrus limon* is rich in bioactive phytochemicals, including phenolic acids, tannins and flavonoids and has antioxidant activities. These results give scientific evidence of the benefits of the traditional plants and provide a promising composition of natural antioxidant phytochemicals.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

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