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Extracting and studying the antioxidant capacity of polyphenols in dry linden leaves (*Tilia cordata*)

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Abstract
Medicinal potentials of the flowers and leaves of *Tilia* species (Linden) make them valuable plants in pharmacognosy researches. The present study is an attempt to investigate the optimal conditions for the extraction of phenolic compounds from *Tilia cordata* leaves harvested from the coastal mountains located at the west of Syria. The optimal extraction conditions of phenolic compounds were determined using the conventional liquid–solid method using different ethanol–water ratio, temperature and extraction time. Total phenolic content and the radical scavenging activity of a number of the dried leaves extracts were determined by Folin-Ciocalteu reagent and scavenging of DPPH (2,2- diphenyl-1-picrylhydrazyl) radical respectively. The best phenolic yield (1.37±0.0021 g/100 g DW) was obtained using 40% ethanol at 75 °C for 30 min. Whereas the best IC_{50} (0.3303±0.0896 mg/ml) was obtained using ethanol 60% at 75 °C for 30 min and was correlated with a phenolic yield of 0.49±0.0014 g/100g DW. The high content of phenolic compounds and antioxidant activity found in the dried leaves extract of linden leaves indicated that it might be considered as a potential source of nutraceuticals in the future.

Keywords: Linden leaves, polyphenols, antioxidant, extraction

Introduction
Oxidative stress is the state of imbalance between antioxidant activity and free radicals [1]. Since natural defense may not be efficient, excessive exposure or production of free radicals could harm cellular macromolecules [2-4] leading to several degenerative diseases including arthritis, cancer, cataract, brain dysfunction, cardiovascular diseases and aging [4, 5]. However, free radicals could be controlled naturally by antioxidants that act as free radicals scavengers [5]. Antioxidants consumption plays an important role in reducing the risk of several chronic diseases including cardiovascular diseases, cancer and diabetes [1, 2].

Phenolic compounds are plant secondary metabolites closely responsible for the sensory properties of food [6]. They are believed to be the dominant antioxidants in most plants [7] by acting as reducing agents as well as hydrogen donors. There is growing evidence that consumption of plant foods is inversely related with the incidence of degenerative diseases [5]. However, consumption of fruits, vegetables, teas, etc. may not be sufficient which led researchers to look for alternative sources of antioxidants. Medicinal herbs are one of these sources [2]. Consumers' interest in medicinal herbs and nutritional supplements has increased since they have no side effects compared to synthetic drugs and due to their low cost [8].

The genus *Tilia L.* (common names; linden and lime) from Tiliaceae family consists of about 44 species [9]. *Tilia cordata* is used in folk medicine as sedative in anxiety and sleeping disorders. It also has diuretic, demulcent and diaphoretic properties [9, 10]. It is also useful for the treatment of hypertension caused by arteriosclerosis and for the treatment of cold-related coughs [9]. These plants contain a number of derivatives such as hydrocarbons, esters, terpenoids, quercetin, kampferol, phenolic compounds, condensed tannins and scopoletin [10]. Linden flowers have been used to treat several illnesses like bacterial infections as well as their effects in reducing tension. Alcoholic extracts have antibacterial properties while flower infusion is used to treat diseases of respiratory tract [11]. These effects could be attributed to the presence of flavonoids and tiliroside. Duda-Chodak *et al*. reported that the phenolic content of Linden flowers was 38.6 mg catechin/g [12] and according to Buřičová and Réblová the antioxidant activity according to DPPH method was 58.8 ± 6.3 mg ascorbic acid/ g dry flowers [2]. Linden leaves are also used in folk medicine for their diaphoretic properties [9]. Thus, it was important to determine the antioxidant activity of linden leaves in order to support its traditional use with scientific evidence. It is also important to compare linden leaves properties with those of flowers as a potential more abundant substitute.

In this present research, we concentrated on finding the efficient method for extracting polyphenols from dry small-leaved linden (*T. cordata*) collected from coastal mountains of the west of Syria and studying their polyphenolic content and antioxidant properties. The lack of researches on the capacity of the phenolic content of the leaves of linden encouraged us to study this field.

Materials and methods

Chemicals

Folin-Ciocalteau (2N) reagent (Sigma-Aldrich, Switzerland), 2,2-diphenyl-1-picryl-hydrazyl (DPPH) (Sigma-Aldrich, USA), Sodium carbonate (Himedia, India), Gallic acid (Sigma-Aldrich, China).

Sample Preparation

Linden leaves were harvested from the coastal mountains located at the west of Syria. The leaves were dried in air for several days. The moisture content of the dried leaves was determined and they were then milled using a home blender until they formed a fine powder.

Extracts Preparation

Four different concentrations of ethanol were used for extraction: 20%, 40%, 60% and 80%. An aliquot of 0.5 g of powdered linden leaves was added to 10 ml of one of the four different concentrations of ethanol. The extraction for each concentration was then conducted at three different temperatures: maceration at room temperature and water bath at 45 °C and 75 °C. All previous samples were extracted for 30 minutes or 60 minutes.

Total Phenolic Content

Total phenolic content in each of the 24 extracts was determined according to the Folin-Ciocalteu method described by Skerget *et al*. with little modifications [13].

To 1 ml of each 10 fold diluted extract, 0.5 ml of Folin-Ciocalteu reagent (2N) was added and then diluted with 4.5 ml of distilled water. After a time interval of 5 min, 4 ml of Na2CO3 (7.5%) were added. The samples were mixed and incubated for 30 min at 40 °C. The absorbance of the resulting blue-colored solutions was measured at 734 nm.

Measurements were performed, based on a standard calibration curve of gallic acid in ethanol. The mean (±SD) results of duplicate analyses were expressed as gallic acid equivalents (GAE) in milligrams per gram of dry-material.

DPPH Assay

A spectrophotometric test based on an electron-transfer reaction monitoring absorbance, DPPH, was used as described by Brand-Williams *et al*. [14]. Two hundred µL of each extract were added to 2 mL DPPH (100 µM/L methanol), the mixture was manually shacked. After 1h of incubation in the dark and at room temperature, the absorbance of the samples was measured at 520 nm.

The percentage of scavenging activity was calculated according to the following equation:

$$Scavenging\ activity\ (\%) = \frac{A_C - A_S}{A_C} \times 100$$

Where, A_C is the absorbance of the control and A_s is the absorbance of the sample.

Statistical Analysis

The experiments were conducted in triplicates. All results were presented as means ± standard deviations. The differences between treatments were determined by applying the Student's t-test.

Results and discussion

Different studies have showed that the conventional temperature for polyphenol extraction ranged between 25 °C and 80 °C [15] as temperatures above 80 °C have shown to decrease the extraction yields due to possible degradation of phenolic compounds that is caused by hydrolysis, internal redox reactions and polymerization [16]. Other studies had assumed that conventional range of temperature for polyphenol extraction yield should not be less than 55 °C and not more than 60 °C [17]. It's also convenient that increasing extraction time will decrease the phenolic yield. In our study, the highest yield of phenolic compounds was reached after only 30 minutes of extraction at 75 °C as shown in Fig. 1.

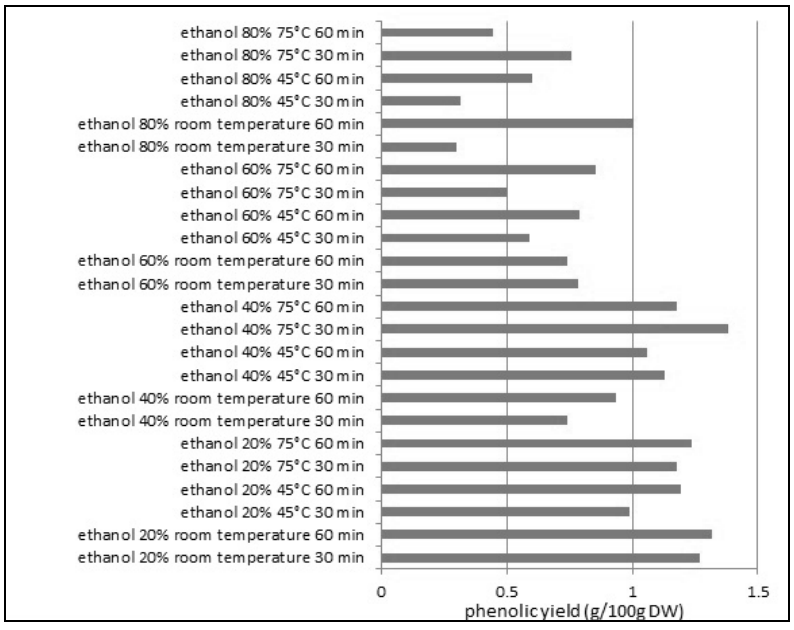

Fig 1: Influence of different extraction parameters on the phenolic yield of *T. cordata* leaves extract.

The phenolic yield is affected by the solubility of phenolic compounds and the solvent's polarity [15, 16]. Ethanol, methanol and acetone at different concentrations with distilled water have commonly been used as solvents for the extraction of polyphenols [18, 19]. In this study different ethanol fractions with distilled water (20%, 40%, 60%, 80%) were used.

As shown in Fig. 1, 20% and 40% ethanol samples recorded higher phenolic yield than 60% and 80% ethanol samples. This can be explained by the high solubility of oxygen in 60% and 80% ethanol samples which play an important role in facilitating degradation reactions of polyphenols [17]. Also, the low solubility of polyphenols in 60% and 80% ethanol samples is due to the hydrogen bonds formed between proteins and polyphenols, which are normally present in plant cells, in high concentrated organic samples [20].

In addition, extraction using 20% ethanol resulted in a decrease of phenolic yield along increasing temperature and time of extraction. This concludes that an amount of ethanol has been lost as a gas due to the rising temperature (room temperature, 45 ^{0}C, 75 ^{0}C) which will affect the phenolic yield. In contrast, extraction using 40% ethanol resulted in an increase in phenolic yield from 0.74±0.0014 g/100 g DW to 1.37±0.0021 g/100 g DW along increasing temperature from 20 °C (room temperature) to 75 °C, respectively.

The differences between treatments were determined by applying the Student's t-test. We have noticed that the T calculated values were higher than the tabular one with three repetitions and freedom degree of 2 (4.303) [21] which means that there were statistical differences between treatments. On the other hand, we compared the quantities of phenolic compounds which we obtained and the relative value 0.75 g/100 g DW obtained by Lugemwa *et al*. [4] during the extraction of linden leaves collected from stores in USA at room temperature for 96 h using a combination of ethyl acetate, ethyl alcohol and water (4.5:4.5:1). Statistical results indicated that all of the values obtained during our experiment were higher than the relative one which means that our protocol used for the extraction of phenolic compounds form Linden leaves is better than other protocols.

Negri *et al*. [10] analyzed the hydro-ethanolic extract by using liquid chromatography with mass spectrometry and found that the major flavonoids were: quercetin-3,7-di-O-rhamnoside, kaempferol-3,7-di-O-rhamnoside and kaempferol 3-O-(6"-p-coumaroyl glucoside) or tiliroside. These flavonoids and phenolic compounds suppress or scavenge radical oxygen species either by enzyme inhibition or by chelating trace elements in free radical generation; or up-regulating and protecting the antioxidant enzymes involved in the defenses [22]. A previous study conducted by Lugemwa *et al*. [4] had showed that the phenolic content of linden leaves was 375.6 ± 7.8 mg/l (equivalent to 0.75 mg/100 g DW). This value was correlated with an IC50 of 3.76 ± 0.10 mg/ml. Our results indicated that the radical scavenging activity of the extract was not related to its phenolic content as the maximum yield of phenolic compounds gave an IC50 of about 0.6611 mg/ml as shown in figure 2, whereas the best IC_{50} (0.3303±0.0896 mg/ml) was obtained using ethanol 60% at 75 °C for 30 min and was correlated with a phenolic yield of 0.49±0.0014 g/100g DW. Several studies in the literature had reported that there is no positive correlation between antioxidant activity and the quantity of phenolic compounds [23, 24].

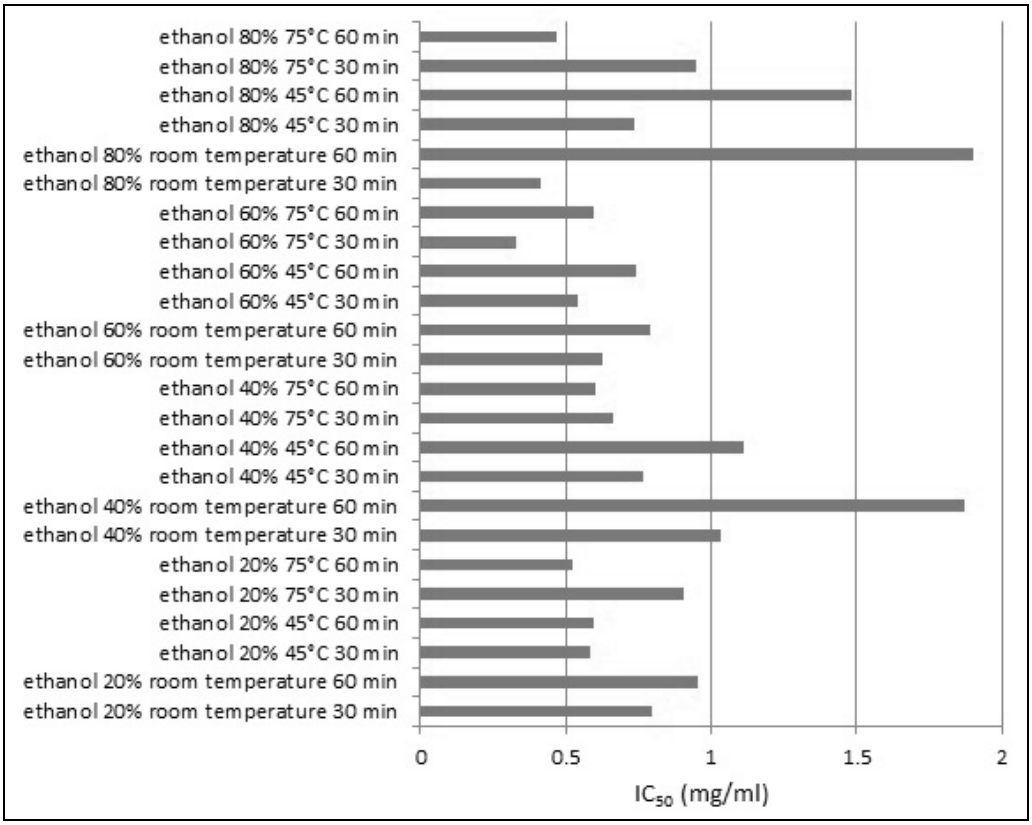

Fig 2: Influence of different extraction parameters on the IC_{50} of T. cordata leaves extract.

Finally, we noticed that the content of phenolic compounds in the ethanolic extract of *Tilia cordata* flowers was about 28.74±4.03 mg/100g which is significantly lower than the relative value of those found in the leaves [25] The lower phenolic content was not correlated with lower antioxidant capacity as the IC50 of *Tilia cordata* flowers' methanolic

extract was 0.116±0.012 mg/ml which is significantly better than the IC50 of leaves' extract found in our research [26].

These results can show that even if phenolic compounds play an important role as antioxidants, there are other compounds in plants which have antioxidant activity which must be considered. Another point is the influence of non-phenolic compounds on Folin-Ciocalteu reagent. Some other compounds such as ascorbic acid, which is one of the main constituents of linden leaves, might also react with Folin-Ciocalteu reagent and affect color measurement [27]. Even more, simple phenols, although they are not effective antioxidants, react with Folin–Ciocalteu reagent [28]. Also, it should be taken into consideration that different phenolic compounds may show different antioxidant activities, depending on their structure, as well as synergistic or antagonistic effect of other compounds, which are present in the crude extract [29].

Conclusion

The ethanolic extracts of *Tilia cordata* leaves is a rich source of polyphenols (1.37±0.0021 g/100 g DW) and exhibit high antioxidant activity (IC_{50}=0.3303±0.0896 mg/ml calculated as DPPH scavenging activity). The prepared extract could be used for the preparation of natural supplements or functional foods in order to supply human organisms with bioactive compounds of antioxidant activity. However, purification of polyphenol extracts and in vivo evaluation should be further studied.

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