Chemical and Physical Characterization of the Hackberry (Celtis australis) Seed Oil: Analysis of Tocopherols, Sterols, ECN and Fatty Acid Methyl Esters

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Abstract: For the very first time, the nutritional and physicochemical properties of the oil extracted from hackberry Celtis australis fruit were investigated with the aim of possible applications of such wild fruit oil. The physicochemical properties such as peroxide value, acidity, saponification, iodine value and total fat content of the extracted oil were examined extensively. The obtained results showed that peroxide value, acidity, saponification, iodine value and total fat content of the extracted oil were found to be 4.9 meq O₂/kg fat, 0.9 mg KOH/g fat, 193.6 mg KOH/g fat, 141.52 mg I₂/g fat and ~5%, respectively. The predominant fatty acid found in this wild fruit is linoleic acid which was calculated to be 73.38% ± 1.24. In addition, gamma-tocopherol (87%) and β-sitosterol (81.2±1.08)% were the major tocopherol and sterol compositions found in Celtis australis seed oil. Moreover, equivalent carbon number (ECN) analysis has indicated that the three linoleic acids are the main composition of the triglycerides extracted from Celtis australis. Also, the high value of omega 6 and β-sitosterol make this oil applicable in cosmetics and pharmaceutical applications.

Key words: Celtis australis seed oil, physical and chemical properties, fatty acid, phytosterols, tocopherols

1 Introduction

Recently, due to the increasing demand for fast foods and fatty diets in modern countries, it has become important to explore more nutritional and unsaturated edible oils from natural resources. Exploring and finding the edible oils containing high polyphenols, natural antioxidants, omega 3, 6 & 9, with less saturated fatty acid, are worth to health care. These needs have driven the governments to intensify their efforts in evaluating the nutritional values of available plants and the natural fortune of their territories⁵. Hence, various plant seeds/fruits have been studied in terms of its oil content, composition, nutritional values, oxidative stabilities, unsaturated factors, and edible or non-edible criteria. The well-known seeds/fruits oil resources that has been widely investigated includes sesame, olive, rapeseed, sunflower, soybean, corn, palm, and nuts and are widely used in preparation of food and other edible products worldwide. Some seeds/fruits oils and its butter such as Shea, Jatropha, Argan and Olive are suggested as a nutraceutical for medical applications due to their valuable antioxidants and anti-inflammatory properties⁴⁻⁶. It has been well established that the seed oils are important class of oils which can be used in industry for lubrication, soap manufacturing, fuel blending, animal food and paint manufacturing. Besides being stored as energy in living beings, oils and fats are found useful in different pharmaceutical applications as well⁶. Hence, the quest for valuable seed or fruit oils is ongoing to cater to the needs of producing food sources, cosmetic reagent and medical substances.

Celtis (Hackberries) is wildly grown and mostly linked to the tropical and warm temperate areas such as Southern Center North America, East/Southern Asia, Southern Europe and Africa⁷, that are known for their valued ornamental qualities and wood. The Hackberry tree is distinct,
possessing the gray-green colored leaves, lance-shaped and the large edible fruits. It is well known fact that the fruits of Hackberry are considered of having medicinal values. Hitherto, the nutritional, physical and chemical properties of hackberry oil have not been investigated. This study was carried out to report the above-mentioned properties intending to popularize the utilization of this wild Celtis australis seed oil all around the world. The proposed oil has high levels of linoleic acid (Omega 6), gamma-tocopherols and β-sitosterol. The distinctive high level of linoleic acid is the outstanding property of this fruit oil and the literature indicates that such oil can be used in the preparation of edible oil, cosmetics and in inducing cancer cell apoptosis. The high unsaturated fatty acid concentration indicates that this fruit can have the desired effects on the brain. In comparison to olive oil (contains high oleic acid or Omega 312,13), C. australis showed poor content of oleic acid but rich in linoleic acid, thus stating the newly reported oil as an economically viable alternative for the expensive oils. High β-sitosterol makes the proposed oil as a promising anti-inflammatory agent14,15. The gamma-tocopherol is another valuable composition of Hackberry oil that is useful in the treatment against prostate cancer16,17. This study aims to determine the nutritional and physicochemical properties of Hackberry seed oil to expand its usage in the food, cosmetics and pharmaceutical industries in the future.

2 Experimental

2.1 Materials and chemicals

The analytical standard reference material (C4-C24) of fatty acid methyl esters mixture, triglycerides (including glyceryl tripalmitoleate (tripalmitolein), glyceryl tristearate, etc.), sterols (including campesterol, beta-sitosterol, stigmasterol, stigmastanol, etc.), 5-α-cholestan and 1-eicosanol were obtained from Sigma-Aldrich (America). Chloroform, acetone, n-hexane, methanol and acetonitrile as HPLC grade solvents were ordered from Samchun Pure Chemical Co., Ltd (Gyeonggi, South Korea). Potassium iodide (KI), glacial acetic acid and n-propanol were purchased from Merck Company (Darmstadt, Germany).

Hackberry fruits were collected from wild trees from village areas (Nohdeh) in the northwest of Iran (Nowdeh, Khalkhal, Ardebil, Iran). Khalkhal is located at 37.3481 latitude and 48.4453 longitude and it is situated at 1790 meters above sea level. Nowdeh have four season with, spring, cool summer (+20°C), autumn and cold winter/snowing (−10°C + 8°C). 

2.2 Instruments

Gas chromatography series 6000 from Young Lin Instrument Co., Ltd (Gyeonggi, Korea) was used for the analysis of free fatty acid methyl esters and sterols profile. Conditions for fatty acid methyl esters analysis were as follow: RESTEK capillary column (L 100 m, ID 0.2), injection port temperature: 280°C, FID detector temperature: 320°C, carrier gas: H2 (1 mL/min), flame: air (300 mL) and H2 (30 mL), sample volume: 1 μL. Conditions for determination of sterols were as follow: Teknokroma TRB-5 capillary column (L 30 m, ID 0.35), injection port temperature: 280°C, FID detector temperature: 320°C, carrier gas: H2 (1 mL/min), flame: air (300 mL) and H2 (30 mL), sample volume: 1 μL. For determination of tocopherols and triglycerides (ECN), respectively.

2.3 Extraction of Hackberry (C. australis) seed oil

In order to extract the oil content of the Hackberry seed, a simple solvent extraction method was applied as follows: a flask was placed into the oven operating at 103°C for 1 h, then in a desiccator for 10 min and accurately weighed. In another flask, 100 g of dried and powdered Hackberry fruit seeds were weighed and 150 mL n-hexane was added to it. This flask was shaken (WiseCube shaker, Germany) at 150 rpm for 30 h at 25-35°C in dark and then the content of it was filtered by ashes Whatman filter paper grade 5, 125 mm. The residue on the filter paper was washed off with 100 mL of n-hexane. The filtrate was then transferred into the pre-weighed flask and the solvent was evaporated using a rotary evaporator (Heidolph, type: Heizbad WB, Germany) operating at 35°C under vacuum for 45-60 min. Finally, the flask and its content was desiccated for 1 hour in an oven at 35-40°C and weighed again to calculate the weight of the extracted oil (ISO 659: 2009). Finally, the oil content obtained was calculated to be 5% for the proposed C. australis oil (Fig. 1).

2.4 Physicochemical properties of oil

2.4.1 Determination of the saponification and iodine value

Saponification value is a measure of the mass (in milligrams) of potassium hydroxide required to saponify 1 gram of the fat to be produced with the experimental conditions specified6,18. The desired calculations and the titration based experimental test needed were carried out using the same protocols as that reported previously16. Hence, ISO 3657:2013 method was used for determining the saponification value of hackberry seed oil. Iodine value corresponds to the consumption of iodine (mg) to saturate 100 g of seed oil. The measurement of iodine value in Hackberry oil extract was conducted using a titration process corresponding to ISO 3961:2009 method.
2.4.2 Determination of peroxide value

Peroxide value \( (PV) \) was obtained as described in ISO 3960:2017.

\[
PV\ = \frac{(V_s - V_b) \times (C_s - C_0) \times 1000}{m}
\]  

Where \( PV \) is peroxide value \( \text{meq O}_2 \text{ per kg} \), \( m \) is the weight of oil sample \( \text{g} \), \( V_s \) is the volume of thiosulfate 0.01 N which is used for titration of the sample solution, \( \text{mL} \), \( V_b \): the volume of thiosulfate 0.01 N which is used for titration of the blank solution, \( \text{mL} \), \( C_s \) is the exact concentration of thiosulfate 0.01 N, \( \text{mole/lit} \) and \( C_0 \) is the approximate concentration of thiosulfate 0.01 N, \( \text{mole/lit} \)

2.4.3 Acidity value or free fatty acid value

Acidity value was calculated due to the high levels and concentrations of the Linoleic acid based on titration method Eq. 2.19, 20.

\[
\text{Free fatty acid as linoleic acid} = \frac{28.04 \times V \times N}{W}
\]

Where \( V \) is the volume in \text{mL} of standard potassium hydroxide, \( N \) is the normality of potassium hydroxide \( 0.01 \text{ N} \), \( W \) is the weight of sample in gram.

2.4.4 Fatty acid methyl esters profile

The fatty acid methyl esters profile was studied using the method described in the ISO 12966-2: 2017 and different obtained peaks were identified and interpreted in accordance with the ISO 12966-4: 2015. Briefly, 0.1 g oil was hydrolyzed and esterified with 200 \( \mu \text{L} \) of 2 molar KOH in methanol. Then, methyl ester fatty acids were extracted with 2 \( \text{mL} \) \text{n-hexane} and 1 \( \mu \text{L} \) of the upper phase was injected into GC-FID.

2.4.5 Determination of phytosterols

Sterols content of Hackberry oil was determined based on the described method in the ISO12228:2014. Briefly, 5 gram of oil was saponified with 50 \( \text{mL} \) of 2 M KOH in ethanol (5-alpha-cholestan used as internal standard). The unsaponified matter was then extracted with 100 \( \text{mL} \) diethyl ether and continuously was washed with excess distilled water to remove the alkaline contents. The organic phase was collected and evaporated with a vacuum rotary set-up at \( 40 ^\circ \text{C} \). Then reconstituted with 1 \( \text{mL} \) chloroform and introduced on TLC (silica gel F254) followed by eluting with a mixture of 65 \( \text{mL} \) \text{n-hexane} and 35 \( \text{mL} \) of diethyl ether. The first line from the bottom was separated and washed with chloroform, dried and derived before GC-FID analysis.

2.4.6 Triacylglycerol analysis

Triacylglycerols (TAGs) with equivalent carbon number (ECN) of 42 to 50 (ECN42, ECN44, ECN46, ECN48 and ECN50), were analyzed by HPLC-RF (refractive index detector) relative to the described method in COI/T.20/DOC. 20 – 2010. Briefly, 0.5 gram oil was added in the silica gel column (1 g), then eluted with 20 \( \text{mL} \) \text{n-hexane}. This was then evaporated and reconstituted with 1 \( \text{mL} \) of acetone and 20 \( \mu \text{L} \) was injected into HPLC-RF.

2.4.7 Tocopherol analysis

A stock standard solution of each reference material of alpha, beta, delta and gamma-tocopherol was prepared in ethanol and stored at \( -20 ^\circ \text{C} \) in dark for 1 month. The
working solutions were prepared from the stock solution and then injected to the HPLC. The standard calibration curves of each tocopherol were obtained by the dilution method. The working solutions were prepared per two weeks.

The extraction of tocopherols was performed based on the method described by E. Gemino et al., with some modifications. Briefly, the oil sample was dissolved in n-hexane and the supernatant was separated. Then 600 μL of methanol was added to 200 μL of the supernatant of the sample. The mixture was vortexed and then centrifuged at 3000 × g for 5 min. The supernatant was separated and filtered by a 0.45 μm filter. The sample was stored at −20°C for 1 week. Tocopherols analysis was carried out using a HPLC (Yung Lin 9100, Korea) equipped with the UV detector and RP-ODS-C18 analytical column (25 cm × Id. 0.46 mm, 5 μm). A mobile Phase was the water/acetonitrile/methanol: 5/45/50 and eluted at a flow rate of 2 mL/min. UV detection was performed at 292 nm and the analytical column temperature was set at 45°C. Detection of tocopherol peaks was carried out by comparison of the each detected tocopherol peak area of the sample with the standard curves.

3 Results and Discussion

3.1 Saponification and iodine values

The saponification value is equal to the hydrolysis degree of triglycerides that describe the average molecular weight of fatty acids and the nature of fatty acid chains (long or short chain). The fatty acids with long chain possess low saponification value, which is good for soap industry. In this study, the results showed that the saponification value of the C. australis oil was found to be 193.6 mg KOH/g, which is considered as high. Hence, among the common cold press oils, the C. australis provided higher soap value as compared to sesame (186 mg KOH/g), sunflower (190 mg KOH/g), walnut (189 mg KOH/g) and less value than almond (207 mg KOH/g) and pumpkin seeds oil (285 mg KOH/g).

Iodine value is used to describe the degree of unsaturated fat and oils. In the current study, iodine value was found to be 141.52 mg I2/g fat which shows high unsaturated fatty acid (-C=C-) content in C. australis oil, which is comparable with walnut (132 I2/g), sunflower (118 I2/g), sesame (120 I2/g), peanut (107 I2/g) and grape seed oil (124 I2/g).

3.2 Fatty acids composition

According to the GC-FID chromatogram illustrated in Fig. 2, the fatty acids composition of the C. australis seed oil was obtained as follows (Table 1): myristic acid (C14, (0.02±0.007) %), palmitic acid (C16, (6.16±0.9) %), stearic acid (C18, (3.04±0.2) %), oleic acid (C18:1 cis, (14.94±0.79) %), linoleic acid (C18:2 cis, (73.38±1.24) %), arachidic acid (C20, (0.31±0.06) %), linolenic acid (C18:3 cis, (0.51±0.02) %), eicosanoic acid (C20:1, (0.29±0.033) %) and other methyl asters ~0.6% (C17, C17:1, C22 and C24). As shown, the main fatty acid in Iranian Celtis australis oil was linoleic acid (Omega 6). The linoleic acid which is a very minor lipid in human bodies and one of the two exceptional fatty acids which cannot be synthesized in the human body. Besides, Omega 6 has recently received great attention due to its anticancer and atherosclerosis properties. It is a dietary compound that can lower the risk of heart diseases, besides its various benefits including immunomodulatory, osteosynthetic, anti-obesitic, apoptotic effects and women’s health. Its molecular action may be linked to the activation and binding to peroxisome proliferator-activated receptors which regulate DNA expression and balance the number of different DNA products in the cell.

3.3 Phytosterols

Phytosterols which are natural compounds resembling cholesterol which dominates in the fields relevant to food nutrition, pharmacology and cosmetics. Down to the molecular level and regarding the biological linked function, these compounds play a main role in stabilizing and building the bilayers of cell membranes, along with suppressing heart diseases linked to high cholesterol levels by its cholesterol reducing action. Figure 3 displays the phytoster-
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The amounts of the various sterol compounds were quantified as follow (Table 1): cholesterol (0.18 ± 0.02 %), brassicasterol (0.11 ± 0.01 %), campesterol (7.45 ± 0.30 %), stigmasterol (0.43 ± 0.09 %), α-tocopherol (5.1% ± 0.22%)

Table 1 The results for fatty acid, sterols and tocopherols composition of C. australis oil.

| FAMEs            | Value %     | Sterols         | Value %     | Tocopherols | Value %     |
|------------------|-------------|-----------------|-------------|-------------|-------------|
| Myristic acid (C14:0) | (0.02 ± 0.007%) | cholesterol     | (0.18 ± 0.02%) | delta-tocopherol | 5.1% ± 0.22% |
| Palmitic acid (C16:0) | (6.16 ± 0.9%) | brassicasterol  | (0.11 ± 0.01%) | gamma-tocopherol | 87% ± 1.91% |
| Stearic acid (C18:0) | (3.04 ± 0.2%) | campesterol    | (7.45 ± 0.30%) | alpha-tocopherol | 3.8% ± 0.19% |
| Oleic acid (C18:1 cis) | (14.94 ± 0.79%) | stigmasterol  | (0.43 ± 0.09%) | –            | –            |
| Linoleic acid (C18:2 cis) | (73.38 ± 1.24%) | delta-7-campesterol | (0.45 ± 0.06%) | –            | –            |
| Arachidic acid (C20:0) | (0.31 ± 0.06%) | clerosterol    | (1.04 ± 0.09%) | –            | –            |
| Linolenic acid (C18:3 cis) | (0.51 ± 0.02%) | β-sitosterol  | (81.2 ± 1.08%) | –            | –            |
| Eicosenoic acid (C20:1) | (0.29 ± 0.033%) | delta-5-avenasterol | (6.49 ± 0.88%) | –            | –            |
| Other methyl asters ~ (C17:0, C17:1, C22:0 and C24:0) | 0.6% | – | – | – |

This suggests the potential applications of the current oil extract in preventing a variety of diseases, mainly cancer, as it has been reported that γ-tocopherol suppresses tumor and arrests the growth and spread of breast and prostate cancer cells.

3.4 Tocopherols

Tocopherols are the major form of Vitamin E and are fat-soluble that can be found in high levels in vegetable oils. Tocopherols with high antioxidant activity can improve oil oxidative stability and also can reduce cancer risk. Due to the position of the methyl groups, the chemical structure of the tocopherols is named alpha, beta, gamma, and delta. Hence, in this study, the tocopherol of the C. australis oil was extracted and analyzed with HPLC-UV as shown in Fig. 4. As can be seen, that γ-tocopherol was no doubt the most dominant among the identified tocopherols (Table 1); delta-tocopherol 5.1% ± 0.22%, gamma-tocopherol 87% ± 1.91%, alpha-tocopherol 3.8% ± 0.19%. This suggests the potential applications of the current oil extract in preventing a variety of diseases, mainly cancer, as it has been reported that γ-tocopherol suppresses tumor and arrests the growth and spread of breast and prostate cancer cells.

3.5 Triglycerides

Equivalent carbon number (ECN) is used to describe the nature of triglycerides in terms of the fatty acids positions of 1, 2 and 3 on glycerol. The fatty acid position is impor.
tant to validate the quality and purity of the vegetable oil that is important in oil marketing. ECN test method is a strong test to determine the presence of external vegetable oil in olive oils. Triglyceride composition analysis is a suitable test for the detection of external oil in the edible oils. Since, nutrition and physical/chemical properties of oils are highly dependant on triglycerides and location of fatty acids. Fatty acid positions at 1, 2 and 3 for \textit{C. australis} oil is calculated based on ECN analysis and listed in Table 2. Results show that trilinoleic acid (41.13) is higher than other fatty acids such as oleic-dilinoleic (24.87) and palmitic-oleic-palmitic (5.01). Hence, according to the performed analysis, the main composition of the tryglycerids in \textit{C. australis} oil was found to be comprised of three linoleic acid on the three positions of glycerol.

### 4 Conclusion

In this report, the physicochemical and nutritional value of wild tree oil (Hackberry) was studied and reported for the first time. The extracted oil held great benefits and promising applications mainly in food, cosmetics and medical industries, especially, as a treatment for cancer owing to its high percentage of valuable nutrition and composition such as unsaturated fatty acids of linoleic acid or Omega 6 (73.38\% ± 1.24), gamma-tocopherol (87\% ± 1.91) and \(\beta\)-sitosterol (81.2\% ± 1.08). The obtained results suggested the possibilities of using \textit{C. australis} as a cheaper and even better alternative for the application in the cosmetic and pharmaceutical industry, subjected to further analysis.

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