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Cucumis metuliferus L. Fruits Extract with Antioxidant, Anti-Inflammatory, and Antidiabetic Properties as Source of Ursolic Acid

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Abstract: To identify healthy, nutritious, and sustainable plant-based products rich in biologically active compounds, this present study was conducted, and the phytochemical composition and biological properties of the hydroethanolic ultrasound-assisted extract of the fruits of *Cucumis metuliferus* were investigated. *Cucumis metuliferus* is an unexplored fruit of a climbing plant in the Cucurbitaceae family, widely distributed in the tropical and subtropical regions of sub-Saharan Africa and whose nutritional and medicinal benefits are well known in African countries, especially. Therefore, its cultivation in other regions could influence chemical composition. The structural identification of the compounds from the hydroethanolic extract from *Cucumis metuliferus* fruits grown in Romania was carried out by chromatographic techniques (HPLC). The main compounds identified were catechin, oleanolic acid, ursolic acid, *p*-coumaric acid, and epicatechin. Subsequently, a method was proposed to isolate and characterize ursolic acid, one of the major compounds. The obtained results show that the hydroethanolic extract is rich in antioxidant compounds evaluated using the DPPH radical inhibition method (IC_{50} = 32.74 ± 0.02 µg/mL) and ABTS cation radical inhibition method (IC_{50} = 11.37 ± 0.07 µg/mL). It also demonstrate in vitro anti-inflammatory activities, such as anti-lipoxygenase (IC_{50} = 32.90 ± 0.05 µg/mL) and anti-proteinase (IC_{50} = 16.34 ± 0.07 µg/mL), and antidiabetic properties by inhibiting α-amylase (IC_{50} = 429.541 ± 0.25 µg/mL) and β-glucosidase activity (IC_{50} = 385.685 ± 0.76 µg/mL). Therefore, *C. metuliferus* fruits could be effectively used in the development of various health-promoting products, being not only appetizing, with spectacular appearance and with extended storage life, but also curative and healthy.

Keywords: *C. metuliferus*; ursolic acid; separation; antioxidant; anti-inflammatory activities

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Citation: Busuioc, A.C.; Costea, G.V.; Botezatu, A.V.D.; Furdui, B.; Dinica, R.M. *Cucumis metuliferus* L. Fruits Extract with Antioxidant, Anti-Inflammatory, and Antidiabetic Properties as Source of Ursolic Acid. *Separations* **2023**, *10*, 274. https://doi.org/10.3390/separations10050274

Academic Editors: Nguyen Van Quan and Tran Dang Xuan

Received: 31 March 2023
Revised: 20 April 2023
Accepted: 21 April 2023
Published: 23 April 2023

1. Introduction

In recent years, healthy food and the high prevalence of malnutrition have become a topic of interest as diet affects human health and the sustainability of the planet [1,2]. Increasingly, people around the world are recognizing the importance of promoting healthy and sustainable food systems in order to ensure food security for the current global population as well as for generations to come [3,4]. Today's society's high interest in swapping some foods for healthier versions and also improving the nutritional profile has led to major changes in the food industry [5,6]. In this context, due to a rich composition in a variety of essential nutrients, vitamins, minerals, antioxidant compounds, and good adaptation to climate changes, *Cucumis metuliferus* can be an important ingredient for improving different food products for healthy diets with high nutritional potential, essential for human health and sustainable food for the future. This aspect is especially important for people with special dietary restrictions due to certain medical conditions [7–11]. Different parts

of this plant (the fruit's pulp and seeds) can be used for their nutritional and medicinal properties [12,13], rapid growth, and long shelf life (over 6–7 months without cold storage) [14], alongside other nutritious vegetables and fruits, to achieve the most benefit from a healthy diet.

Cucumis metuliferus is a species of the cucurbit (Cucurbitaceae) family with ellipsoid fruits of green color that change to orange color during ripening [15]. The rind is covered with cone-shaped protuberances with pointed tips. Inside, the fruit contains a juicy, green, slimy mass with many white seeds [16]. The fruits come in two forms, the bitter form containing mainly cucurbitacins (triterpenoids), and the non-bitter form, which is more cultivated [17]. This plant is native to Africa and has been mentioned in other studies as jelly melon, horned cucumber, spiked melon, horned melon, or kiwano. It is internationally spread due to its rapid growth and adaptation to various climate conditions. Its history began approximately in 1982 with a family from New Zealand who started to cultivate it for commercial purposes. In just two years, they managed to export the "phenomenal" fruit to Japan, where it was received with curiosity and interest. Later, it became a fruit of interest among American farmers and then easily entered other markets. Under the name of horned melon, this plant soon reached Europe [12,18]. Currently, this plant is cultivated in many countries, and opinions about its taste and appearance are divided.

It is well known that the chemical compounds of each species may vary due to various factors such as variety, maturity, growing conditions, and soil [16]. Moreover, the variation in the chemical composition can lead to differences in therapeutic properties [19]. Therefore, different parts of medicinal plants can potentially be excellent sources of phytochemical compounds with biological activity. In general, fruits contain a combination of important nutrients, such as proteins, carbohydrates, fats, vitamins, and minerals. The seeds are rich in linoleic and oleic acids, which are important for human health. In particular, some studies show that oleic acid can help lower blood pressure [17,20,21]. γ-tocopherol and α-tocopherol identified in considerable quantities in kiwano fruits are two types of vitamin E known for their antioxidant capacity [22]. The fruit of *C. metuliferus* has a wide list of health benefits due to its varied phytochemical composition. The pulp contains considerable amounts of β-carotene, vitamin A, and vitamin C, which are very important for strengthening the immune system, night vision, or the curative effect of the skin [23,24]. In addition, a wide range of other secondary metabolites has been identified, such as glycosides, alkaloids, flavonoids, saponins, steroids, tannins, and terpenoids [25]. The fruits are also characterized by a high content of minerals such as iron, potassium, phosphorus, zinc, magnesium, copper, calcium, and sodium [15]. Among the numerous secondary metabolites contained by *C. metuliferus* species, ursolic acid (UA) (3β-3-hydroxy-urs-12-en-28-oic-acid) is a compound of great interest. This pentacyclic triterpenoid compound has low toxicity and is known for its beneficial therapeutic properties, including antioxidant, antimicrobial, hepatoprotective, immunomodulatory, antiviral, antitumor, chemopreventive, anti-inflammatory, cardioprotective, antihyperlipidemic, and hypoglycemic properties [26,27]. Thus, some studies have shown that it can help prevent and treat obesity by modulating fat and glucose metabolism and reducing fat storage in cells [28]. In addition, ursolic acid can help to relieve diabetes symptoms by lowering blood sugar levels and stimulating insulin secretion. In the case of the inflammatory process, UA can help to reduce it by modulating the activity of inflammatory enzymes and blocking molecules that trigger inflammation [26,27,29]. Various technologies are known for UA extraction and purification, both traditional (maceration, Soxhlet, heat reflux) and modern (microwaves, ultrasound, and supercritical fluid) [27,30]. In recent years, researchers have explored structural modifications of ursolic acid intending to produce novel derivatives that possess enhanced biological activities and increased bioavailability [31–34].

For the purification of various compounds from extracts of various plants, different methods and different auxiliary substances were used [35,36]. Other methods for the purification of UA are based on chromatographic separation of crude extracts, on the column with macroporous adsorbent resins or silica gel [37], by thin-layer chromatography [38],

or by fractioned extraction with solvents. Ursolic acid is almost non-toxic and is used in various cosmetics and health products either as an active ingredient or in a mixture with other active substances. In addition, it can be used as a natural compound in the semi-synthesis of numerous new bioactive molecules [39].

Our study aimed to assess the phytochemical profile and the antioxidant, anti-inflammatory, and antidiabetic activities of ultrasound-assisted extracts from *C. metuliferus* "Tempus" fruits grown in Romania (Southeastern Europe). Furthermore, the study proposes a new method for the separation and purification of one of the major compounds, ursolic acid, from *C. metuliferus* "Tempus" fruit extracts, which will be further explored in future studies as a lead compound for the development of new derivatives with therapeutic properties.

2. Materials and Methods

2.1. Reagents, Plant Materials, and Apparatus

All reagents and solvents used for isolation, purification, and analysis were purchased from Sigma-Aldrich and Merck (Darmstadt, Germany). The ethanol used for extraction was of analytical grade. The standard compound ursolic acid (≥95%, HPLC grade) was also purchased from Merck. All optical density measurements for antioxidant, anti-inflammatory, and antidiabetic activities were performed with a multiplate reader (Tecan Trading AG, Tecan Pro 200, Männedorf, Switzerland). The fruits used in this study were collected at the ripening stage from the Station of Vegetable Research and Development (SCDL), Buzau, Muntenia Region, Romania (Southeastern Europe, 45°09′30.1″ N 26°49′39.2″ E). An ultrasonic bath was used for the extraction with solvents (Bandelin Sonorex Ultrasonic, Berlin, Germany, with a digital timer, temperature control, and a frequency of 35 kHz).

2.2. Preparation of Crude Exact of C. metuliferus

Fruits of *C. metuliferus* "Tempus" were examined for integrity, absence of dust, and insect contamination, then cleaned in the first stage with ultrapure water as a first step. Then, the fresh fruits were washed with deionized water, cut into small pieces, and dried at 50 °C in a hot air oven for 48 h. The dried fruits were ground in an electronic mill, and the resulting powder was passed through a 60-mesh sieve. The obtained powdered and dried material was stored in a dark and dry place until use.

The dry material was successively extracted three times, with hexane, under reflux, filtered, and dried, followed by a repetitive extraction with 70% ethanol in an ultrasonic bath. The optimal time and temperature conditions for ultrasonic extraction were selected from the literature [40]: 40 ± 5 °C and 60 min. The final step consisted of the evaporation of the solvent with a rotary evaporator. The obtained extract was kept at 2–4 °C and further used in the following analyses and for the isolation and purification of the ursolic acid [41,42].

2.3. HPLC-DAD Analysis of the Extract

High-performance liquid chromatography studies using photodiode arrays (HPLC-DAD) were performed with equipment (Rigol Technologies Inc., Beijing, China), with an autosampler, binary pump, vacuum degasser, and photodiode array detector. A Synergi Polar-RP C18 (250 × 4.6 mm, 4 µm) analytical column (Phenomenex, Chesire, UK), preceded by a security cartridge, was used for chromatographic separation. Quantification of the different analytes was performed according to the validated method as described in other studies [43]. Quantification curves were generated by injecting 1 to 50 mg/L of the standard compound solutions at five different concentrations. Thus, correlation coefficients (R^2) greater than 0.99 were obtained, indicating a good linearity of the HPLC-DAD method. The mobile phase was a mixture of 0.1% formic acid in water (*v*/*v*) and 0.1% formic acid in methanol (*v*/*v*) [13]. UV-visible spectra (210–520 nm) for the analyzed compounds were recorded so that each compound was analyzed at the corresponding wavelength; the data are presented in Table 1. The organic compounds were identified by combining the

following data: comparison with standard compounds, retention time, and elution order, and quantified by using quantification curves of the analyzed compounds.

Table 1. Chemical composition of the hydroethanolic extract of the fruit of *C. metuliferus* from HPLC-DAD analysis.

Compound	RT *	HPLC-DAD λ, nm	HPLC-DAD µg/g of Extract	SD **	RSD %	UHPLC-MS Exact Mass	UHPLC-MS $[M-H]^-$ Ion (m/z)
Hydroxybenzoic acids							
Gallic acid	5.9	272.00	401.39	1.46	0.36	170.02152	169.01302
Catechin	17.6	280.00	6576.76	3.86	0.06	290.07904	289.071
Epicatechin	23.9	280.00	2016.71	3.59	0.18	302.04265	289.071
Procianidin B2	24.3	230.00	35.88	0.22	0.61	578.14242	577.1558
Procianidin A2	29.9	230.00	756.10	1.86	0.25	578.14242	577.1558
Flavonols							
Rutin	31.4	265.00	39.42	0.23	0.60	610.15338	609.14613
Quercetin	35.8	365.00	5.98	0.56	9.43	302.04265	301.23813
Quercetin-3-D-galactoside	32.0	265.00	11.62	0.28	2.45	464.09548	463.0876
Kampferol-3-glucoside	33.6	265.00	21.90	0.70	3.20	448.10056	447.09331
Kampferol	37.8	365.00	5.14	1.49	28.88	286.04774	285.13422
Hydrocinnamic acids							
Neochlorogenic acid	10.5	325.00	22.89	0.53	2.31		
Chlorogenic acid	22.3	325.00	77.39	0.15	0.19	354.09508	354.09508
Caffeic acid	22.9	325.00	58.65	0.57	0.96	180.04226	179.03501
p-Coumaric acid	28.9	325.00	116.11	1.46	1.26	164.04734	163.03954
trans-Ferulic acid	30.5	325.00	34.27	1.86	5.42	194.05791	193.05066
Triterpenes							
Oleanolic acid	45.8	210.00	551.83	3.25	0.59	456.36034	455.35309
Ursolic acid	45.9	210.00	577.31	3.19	0.55	456.36034	455.35309

* RT—retention time; ** SD—standard deviation.

2.4. Isolation and Purification of Ursolic Acid from C. metuliferus Extract

The isolation and purification of ursolic acid were carried out from the hydroethanolic extract of *C. metuliferus* species, obtained as previously described, using silica gel column chromatography, then analyzed by HPLC-DAD. The ethanolic extract was subjected to column chromatography using a silica gel column (30 × 2.0 cm) preconditioned with chloroform. For elution, a solution of 2 to 4% methanol in chloroform was used. The eluent was collected at every 3–5 mL fraction. After, all the fractions that were collected during the elution process were subjected to TLC testing on silica gel 60 F254 plates (Merck, Darmstadt, Germany). A mixture of chloroform: methanol (95:5, v/v) was used as the mobile phase, and UV light was used for revealing (254 nm, 365 nm, and white light). The fractions with the same Rf were combined and subjected to solvent evaporation [41,44].

2.5. Structure Elucidation and Identification of the Isolated Compound

2.5.1. Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (FTIR-ATR) Analysis

The obtained fractions were analyzed using a Nicolet iS50 FT-IR spectrometer (Thermo Scientific, Waltham, MA, USA) assembled with an ATR accessory. The analysis was performed with a resolution of 4 cm^{-1} by coaddition of 32 scans in the range 4000 cm^{-1}–400 cm^{-1}. Air was carried out as a reference for the background spectrum before each sample analysis, and the diamond crystal was cleaned with alcohol after each sample. Analyses were performed in a temperature-controlled room (21 ± 2 °C) [45].

2.5.2. Analysis of Fraction by UHPLC-MS

In order to certify the presence of ursolic acid in the obtained fraction, the analysis using ultrahigh-performance liquid chromatography coupled with mass spectrometry

(UHPLC-MS) was performed. A method similar in certain parameters was used, with a mobile phase similar to that previously described, while maintaining the parameters for MS. For ionization, a HESI (Heated ElectroSpray Ionization) ion source in negative mode was used. The calibration solution was used for external calibration in both negative and positive modes. Full scan HRMS (high-resolution mass spectrometry) analysis of the compounds was accomplished using a mass spectrometer from Q-Exactive. Full scan data in negative mode were recorded at 70,000 FWHM resolving power, at m/z 200. A scan range of m/z 100–1000 Da was chosen. The temperature of the heat source and capillary was set at 300 °C [46,47].

2.5.3. NMR Analysis

Samples for NMR (nuclear magnetic resonance) experiments were performed in deuterated solvent, CD_3OD, and analyzed using a Varian mercury-plus spectrometer, 400 MHz for ^1H. ^1H chemical shifts were reported in parts per million (ppm), while the coupling constant (J) was Hertz (Hz) (Figures S1–S3, Supplementary Materials) [48].

2.6. Antioxidant Assay

2.6.1. DPPH Scavenging Activity

The antioxidant activity of the extract was analyzed by the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging test [49]. This method is based on the change of purple color to yellow, resulting in a reduced structure of the DPPH radical. The method was performed in a 96-well plate, and the absorbance was recorded at 517 nm after 30 min using a microplate reader. Calculations were made according to the literature [46,50]. The standard compound Trolox was used as a positive control due to its known antioxidant properties [51].

2.6.2. ABTS Radical Cation Decolorization Assay

For this antioxidant activity, a previously prepared solution of $ABTS^{\bullet+}$ (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) was used [52,53]. The assay was performed according to the descriptions in the literature, with minor modifications [54,55]. The absorbance of the solution was measured at 734 nm in a 96-well plate using the microplate reader. Samples were prepared by mixing 100 µL of the kiwano extract with 100 µL of $ABTS^{\bullet+}$ radical cation solution, and after 30 min, the absorbance was measured. Results were conveyed using a Trolox calibration curve.

2.7. In Vitro Anti-Inflammatory Activities

2.7.1. Anti-Lipoxygenase Activity

To perform the test, a reaction mixture (200 µL) was obtained by adding 160 µL of sodium phosphate buffer (pH 8.0) to 10 µL of the sample dissolved in Tris buffer (pH 7.4) at various concentrations, and also 20 µL of lipoxygenase. The reaction mixture was incubated at 25 °C for 10 min. To initiate the reaction, 10 µL of the linoleic acid solution was added as substrate and incubated at 25 °C for 6 min, and the absorbance at 234 nm was read in the microplate reader [46,56,57]. Indomethacin (250–255 µg/mL) was used as a positive control due to its known anti-lipoxygenase properties. The percentage of inhibition was calculated using the following formula:

$$\% \, Inhibition = (A_{Control} - A_{Sample})/(A_{Control}) \times 100 \quad (1)$$

2.7.2. Anti-Proteinase Action

In this method, samples dissolved in 20 mM Tris-HCl buffer (pH 7.4)) were mixed with trypsin solution. The reaction mixture was incubated for 5 min at 37 °C and then 0.3 mL of 1.5% casein (*w*/*v*) was added. Then, the obtained mixture was again incubated for 20 min. The final step to complete the reaction was performed by adding 200 µL of 70% perchloric acid. Prior to spectrophotometric analysis of the samples, consisting of absorbance mea-

surement at 210 nm, the mixture was centrifuged for 5 min at 5000 rpm [58,59]. Aspirin (10–500 µg/mL), a known inhibitor of proteinase activity, was used as a positive control. The percentage of inhibition was calculated according to the following equation:

$$\% \, Inhibition = (A_{Control} - A_{Sample})/(A_{Control}) \times 100 \quad (2)$$

2.8. In Vitro Evaluation of the Antidiabetic Activity

The α-amylase inhibition test was assessed with the 3,5-dinitrosalicylic acid (DNS) method [60], with minor modifications. Briefly, 80 µL of each sample or positive control was added to 40 µL of α-amylase solution (2 U/mL, α-Amylase from *Aspergillus oryzae*, EC Number: 232-588-1, Sigma-Aldrich®) and incubated for 20 min at 37 °C. After incubation, 140 µL of 1% starch solution was added to each tube and incubated again for 30 min. The reaction was terminated by adding 0.40 mL of DNS 1% and heating it in a boiling water bath for 10 min. The reaction mixture was cooled to room temperature and then diluted with 0.80 mL phosphate buffer when the absorbance at 540 nm was measured. The blank sample was prepared by replacing the plant extract samples with 0.20 mL of phosphate buffer. A positive control sample was prepared using acarbose (0.025–1 mg/mL), a known amylase inhibitor [61]. The α-amylase inhibitory activity was expressed as a percentage of inhibition and calculated according to the following formula:

$$\% \, \alpha\text{-}amylase \, Inhibition = (A_{Control} - A_{Sample})/(A_{Control}) \times 100 \quad (3)$$

The β-glucosidase inhibition test was assessed as previously described [62]. Briefly, a reaction mixture was obtained from 15 µL phosphate buffer (pH 5.0), 20 µL p-nitrophenyl-β-D-glucopyranoside (1mg/mL, Sigma-Aldrich®) and 20 µL sample or positive control at different concentrations. The 96-well plates were incubated in a microplate reader for 10 min at 37 °C. Then, 5 µL β-glucosidase (from almonds, Sigma-Aldrich®) solution was added (2.5 mg/mL) to each well and incubated again for 30 min at 37 °C. The reaction was terminated by addition of 0.140 mL buffer (pH = 10). Absorbance was measured at 410 nm using a microplate reader. As positive control, acarbose was used, being a known inhibitor of glucosidase [62]. The β-glucosidase inhibitory activity was expressed as a percentage of inhibition and calculated according to the Formula (4).

$$\% \, \beta\text{-}glucosidase \, Inhibition = (A_{Control} - A_{Sample})/(A_{Control}) \times 100 \quad (4)$$

2.9. Statistical Analysis

The carried experiments were achieved in triplicate, and the presented data represent the average of the three individual determinations ± standard deviation (SD). Statistical evaluation of the obtained data was estimated by one-way analysis of variance (ANOVA) to find out significant differences ($p \leq 0.05$). The inhibitory concentration IC_{50} values were calculated from linear regression analysis of the percent activity depending on concentration by using the GraphPad Prism software (vs. 5.0).

3. Results and Discussion

3.1. Preparation of Crude Exact of C. metuliferus

The current study presented an adapted method for obtaining a hydroethanolic ultrasound-assisted extract of *C. metuliferus*. Further, for the purification and characterization of ursolic acid, one of the major terpenoid compounds, two steps were proposed, using two solvents with different polarities. The first step was to remove the other compounds. The combined filtrate obtained after extraction was concentrated under reduced pressure to obtain, from 1 kg of plant material, 183 g of crude extract, which has been used for the following analyses and also for the isolation and purification of the major terpenoid compound, ursolic acid. Figure 1 shows the scheme for obtaining the crude extract starting from the fresh fruits of *C. metuliferus*.

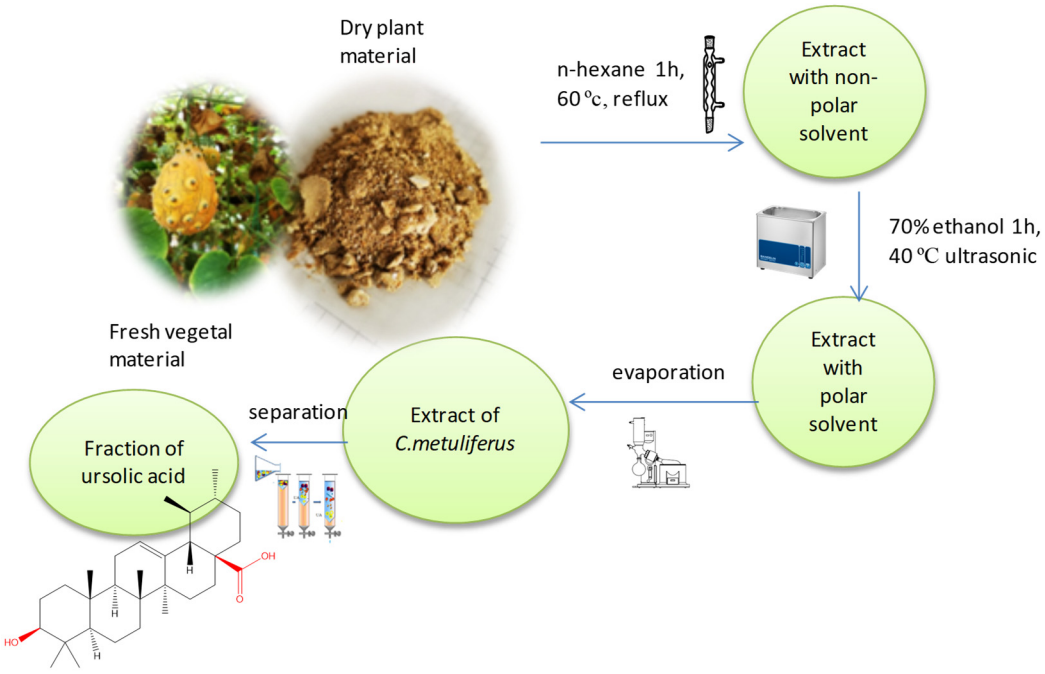

Figure 1. Separation scheme starting from the fruits of *C. metuliferus*.

3.2. Identification of Bioactive Compounds by HPLC

The results of this study concerning the chemical composition of the hydro-ethanolic extract from the fruit of *C. metuliferus*, obtained by HPLC-DAD analysis, are presented in Table 1. Thus, for the compound class of flavan-3-ols, the highest amount of catechin (6576.76 ± 3.89 µg/g dry extract) and epicatechin (2016.71 ± 3.59 µg/g dry extract) and the lowest amount of procyanidin B2 (35.88 µg/g dry extract) were determined.

In the case of hydroxycinnamic acids compounds, *p*-coumaric acid (116.11 ± 1.46 µg/g dry extract) was identified in the highest concentration, followed by chlorogenic acid (77.39 ± 0.15 µg/g dry extract). Several representative flavonoids, such as rutin (39.42 ± 0.23 µg/g dry extract) and kaempferol-3-glucoside (21.90 ± 0.70 µg/g dry extract), were identified in much smaller amounts compared to other compounds. Additionally, the pentacyclic triterpenoids, ursolic acid (577.31 ± 3.19 µg/g dry extract), and oleanolic acid (551.831 ± 3.25 µg/g dry extract) were identified in high concentrations. A composition rich in the biologically active triterpenoid ursolic acid of the extract was highlighted, which was further investigated by isolation and characterization in the next step of our study.

According to other studies from the literature, fruits of *C. metuliferus* grown in an ecological farm on the Fruška gora Mountain (Pannonia plain, Novi Sad, Republic of Serbia) showed considerable amounts of polyphenols, expressed as gallic acid (GAE), in different parts of the plant, such as 58.22–74.90 mg GAE/100 g in pulp, between 1728.94 and 1923.52 mg GAE/100 g in peel extracts samples, and 141.25 mg GAE/100 g in seed extracts [23]. Another study that demonstrated the neuroprotective action of this plant presented the chemical composition of the fruits from Hby local farm located at Cinfães, Douro, Portugal, with smaller concentrations of gallic acid (11.7 ± 0.6 mg/100 g), *p*-coumaric acid (2.86 ± 0.14 mg/100 g), protocatechuic acid (7.69 ± 0.38 mg/100 g), (+)-catechin (4.86 ± 0.24 mg/100 g), and (-)-epicatechin (4.58 ± 0.23 mg/100 g) [63]. Several studies on the African species of *C. metuliferus* also highlighted the presence of a wide range of vitamins (vitamin A 198.51 mg/mL, B6 835.00 mg/mL, B12 0.10 mg/mL, C 682.00 mg/mL, D 5.28 mg/mL, E 0.42 mg/mL) [64,65] and minerals (Ca, Al, Mg, Zn, K) [22,64,66].

3.3. Analysis and Identification of Ursolic Acid

All the obtained fractions after the separation of the phytochemical compounds from the hydroethanolic extract of *C. metuliferus* fruits by using column chromatography were

characterized through FT-IR, and the corresponding fraction of ursolic acid was identified. Ursolic acid was isolated from the ultrasound-assisted extract from kiwano fruit (Table 2) and further characterized by IR spectroscopy, mass spectroscopy, and ^{1}H NMR, and the results were confirmed by comparing them with other studies [67–69].

Table 2. Characterization of the isolated compound.

Physical Properties	Ursolic Acid Fraction
Color	white
Melting Point	291–293 °C
Solubility	soluble in ethanol, DMSO
Rf Value Solvent system: chloroform: methanol (95:5, *v*/*v*)	0.41

3.3.1. FT-IR Analysis

All the fractions obtained after the separation of the phytochemical compounds from *C. metuliferus* fruit's ethanolic extract using column chromatography were characterized through FT-IR analysis, and the functional groups were identified based on data in the literature. The fraction corresponding to ursolic acid was of interest, and in this study, the ATR-FTIR spectrum identified as ursolic acid was analyzed in a range of 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1}. Table 3 shows the vibration bands of ursolic acid and their characteristic functional groups. Interpretation and assignment of IR bands (Figure 2) were made based on data in the literature concerning ursolic acid [67–70].

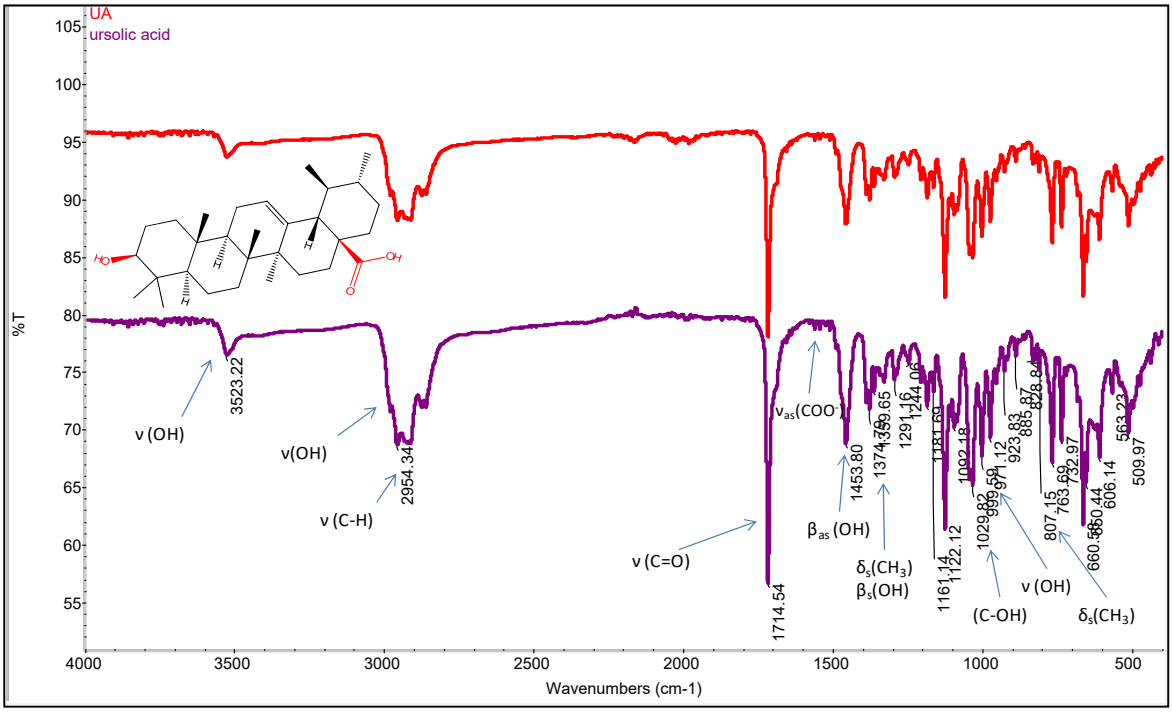

Figure 2. The FTIR-ATR spectra of pure ursolic acid (purple spectra) and isolated ursolic acid (UA, red spectra).

Table 3. The main characteristic experimental FTIR-ATR bands (cm^{-1}) of standard commercial UA and isolated UA from *C. metuliferus* fruits extract and their assignments.

Assignments	ATR-IR Bands (cm^{-1})	
	Standard Commercial UA	**Isolated UA from *C. metuliferus***
ν_{ass} (OH) w	3523.33	3523.22
ν (O–H) w	2965.41	2966.61
ν (CH) m	2953.90; 2918.75	2954.34; 2918.32
ν_{ass} (C=O) s	1714.66	1715.54
ν_{as} (COO^-) w	1553.56	1543.80
β_{as} (OH) m	1453.78	1454.54
ν_s (COO^-; C=O) w	1405.15	1406.03
δ_s (CH_3) w	1385.72	1386.45
β_s (OH) m	1374.72	1375.79
ν(C–OH) m	1029.87	1030.96
γ (−C=C−, CH) m	999.56	999.59
ν (C–C, C–O, C–H) w	971.08	972.12
δ_s (CH_3) w	807.15	808.14

and intensities: s—strong, w—weak, m—medium; ν_{ass}—asymmetric stretch; ν_s—symmetric stretch.

3.3.2. Sample Analysis by UPLC-MS

The UHPLC-MS chromatographic data demonstrated that the isolated compound was the ursolic acid (supplementary material) by using the mass spectra.

3.3.3. NMR Analysis

The ursolic acid was identified using ^{1}H-NMR spectroscopy by comparing the UA assignments with data in the literature. The ^{1}H-NMR spectra of the isolated UA revealed the presence in the higher field region of five methyl groups by a corresponding singlet (0.77, 0.88, 0.95. 0.97, and 1.10 ppm), two methyl groups by a doublet (0.87 and 0.99 ppm), and the peaks were characteristic for the skeleton of ursane-type triterpenes [71,72]. Two singlet peaks at δH 1.10 ppm, characteristic for the methyl group of oleanane triterpene skeleton, and at δH 1.29 ppm for the methylene protons –CH_2- were also observed in the isolated UA. The peak at δH 3.3 ppm from the lower field region, representing H, may be due to the hydroxyl group, and at δH 4.94 ppm, an olefinic proton peak was assigned to the H of a triterpene.

Ursolic acid (3β-hydroxy-urs-12-en-28-oic acid): powder, white, ^{1}H-NMR (CD_3OD) (supplementary material); ^{1}H-NMR (CD_3OD): 1.63 & 1.69 (H-1,2H, m), 1.65 (H-2, 2H, m), 3.13 (H-3, 2H), 0.74 (H-5, 1H, d), 1.43 & 1.29 (H-6, 2H, m), 1.29 (H-7, 2H, m), 1.56 (H-9, 1H, m), 1.91 (H-11, 2H, q), 5.21 (H-12, 1H, t), 1.62 (H-15, 2H, m), 1.58 & 1.63 (H-16, 2H, m), 2.20 (H-18, 1H), 1.38 (H-19, 1H, m), 1.36 (H-20, 1H, m), 1.32 (H-21, 2H, m), 1.53(H-22, 2H, m), 0.95 (H-23, 3H, s), 0.77 (H-24, 3H, s), 0.97 (H-25, 3H, s), 0.84 (H-26, 3H, s), 1.10 (H-27, 3H, s), 0.87 (H-29, 3H, d), 0.95 (H-30, 3H, d).

3.4. Evaluation of the Antioxidant Activity

It is known that oxidative stress leads to the production of free radicals, and this can affect various cellular events, some of which are cancer proliferation, inflammation, and aging [73,74]. Various in vitro and in vivo studies have been performed to demonstrate the antioxidant of *C. metuliferus* extracts and ursolic acid, but only a few investigated the anti-inflammatory properties [65,75,76]. Table 4 shows the results of the two methods applied by us for the evaluation of the antioxidant activity data compared with Trolox and ascorbic acid, known for their antioxidant properties.

The purpose of this present research was to analyze the antioxidant activity of the hydroethanolic extract and also of the purified fraction of ursolic acid obtained from *C. metuliferus.* The obtained fraction of ursolic acid presented IC_{50} values (IC_{50} DPPH = 4.27 ± 0.009 μg/mL and IC_{50} ABTS = 6.96 ± 0.014 μg/mL) close to those reported by data in the literature

for the pure compound [77–79], greater than that of ascorbic acid and Trolox. The extract demonstrated good antioxidant activity, greater than Trolox and ascorbic acid in an ABTS assay (IC_{50} = 11.37 ± 0.071 µg/mL). The antioxidant activity of *C. metuliferus* extract can be due to its rich composition in various active compounds, such as catechin, epicatechin, oleanolic acid, ursolic acid, and gallic acid.

Table 4. Evaluation of the antioxidant activity.

Samples	IC_{50} DPPH (µg/mL)	IC_{50} ABTS (µg/mL)
Fruit extract	32.74 ± 0.022 ^{d}	11.37 ± 0.071 ^{b}
Isolated ursolic acid	4.27 ± 0.009 ^{a}	6.96 ± 0.014 ^{a}
Trolox	10.57 ± 0.002 ^{b}	32.56 ± 0.002 ^{c}
Ascorbic acid	20.34 ± 0.034 ^{c}	13.76 ± 0.044 ^{b}

The values succeeded by the same letters (a, b, c, d) in the same column show no statistically significant differences ($p < 0.05$) according to the analysis of variance (ANOVA).

Various studies on the extracts from *C. metuliferus* fruits cultivated in different geographic areas and also extracts from various parts of the plant showed different results [22]. Thus, other studies presented that the antioxidant activity of the extracts from the pulp by the DPPH method was 77.98 ± 2.13 µmol TE/100 g, by the ABTS method was 2508.69 ± 23.14 µmol TE/100 g, and that the peel extracts recorded higher activity for DPPH, 211.53 ± 7.95 µmol TE/100 g and 7845.91 ± 57.13 µmol TE/100 g for ABTS, respectively [22]. Seeds have weak antioxidant activity, as evaluated by the DPPH method (49.21 ± 1.15 µmol TE/100 g) and by the ABTS method (1425.196 ± 21.96 µmol TE/100 g) [22]. Another study showed that the DPPH and ABTS radical scavenging capacity of the methanolic extract from kiwano whole fruit was 20.88 µmol Trolox, equivalent per g, and, 185.36 µmol Trolox equivalent per g, respectively [23]. According to some studies, ursonic acid, which can be semi-synthesized by the oxidation of UA, has therapeutic potentials that are similar to or stronger than UA in some aspects. In particular, ursonic acid showed better anticancer, antiproliferative, and antiprotozoal effects [77]. Therefore, UA can be a good lead compound for the development of new derivatives with various therapeutic properties.

3.5. Evaluation of the in Vitro Anti-Inflammatory Activities

It is known that inflammation is liable for many diseases, such as diabetes, multiple sclerosis, arthritis, Parkinson's, and Alzheimer's among others, and is also associated with the progression of cancer cells [19,80].

Table 5 shows the results of this study, from which it appears that the proteinase inhibition activity of the *C. metuliferus* extract is mostly due to the presence of ursolic acid in its composition. The obtained results demonstrate that the isolated compound has a good anti-lipoxygenase activity, even if the values are lower compared to the known standards used, i.e., indomethacin and aspirin, so the *C. metuliferus* extract can be used as an alternative or as an adjuvant in suppressing inflammation.

Table 5. Evaluation of the anti-inflammatory and antidiabetic activities.

Samples	Anti-Lipoxygenase Activity IC_{50} (µg/mL) *	Anti-Proteinase Action IC_{50} (µg/mL) *	α-amylase Inhibition IC_{50} (µg/mL) *	β-glucosidase Inhibition IC_{50} (µg/mL) *
Hydroethanolic extract	32.90 ± 0.045 ^{b}	16.34 ± 0.067 ^{b}	429.541 ± 0.252 ^{c}	385.685 ± 0.758 ^{c}
Ursolic acid fraction	18.61 ± 0.086 ^{a}	12.53 ± 0.044 ^{a}	394.264 ± 0.143 ^{b}	322.412 ± 0.517 ^{b}
Aspirin		160.20 ± 0.020^{c}		
Indomethacin	45.12 ± 0.014 ^{c}			
Acarbose			341.577 ± 0.398 ^{a}	308.474 ± 0.296 ^{a}

* The values succeeded by the same letters (a, b, c) in the same column show no statistically signifcant differences ($p < 0.05$) according to the analysis of variance (ANOVA).

Terpenoids are one of the classes of compounds present in plants that have demonstrated anti-inflammatory potential, ursolic acid being one of the representatives of this class [81]. The ability of ursolic acid to effectively inhibit aggregation of human platelet induced by arachidonic acid (IC_{50} = 0.26 mM) was also demonstrated through in vivo studies, compared to the activity of the known compounds quercetin (IC_{50} = 4.41 μM) and aspirin (28–71% inhibition at 150–300 mg/kg) [76]. Ursolic acid has been shown to exhibit weaker inhibitory activity (IC_{50} = 10 μM) than patuletin, but stronger activity than quercetin [75].

3.6. In Vitro Evaluation of the Antidiabetic Activity

The present research was designed to also evaluate the possible antidiabetic activity of *C. metuliferus* hydroethanolic extract by using in vitro enzyme assays. To investigate the pharmacological potential of hydroethanolic extract and isolated UA as amylase and glucosidase inhibitors in the development of new drugs to keep off and treat diabetes, they underwent specific tests. The results of the in vitro inhibition tests of α-amylase and α-glucosidase (Table 5) displayed that *C. metuliferus* extract had a similar inhibitory effect ($IC_{50\ \alpha\text{-amylase}}$ = 429.541 ± 0.252 μg/mL and $IC_{50\ \beta\text{glucosidase}}$ = 385.685 ± 0.758 μg/mL) with that of the standard drug acarbose ($IC_{50\ \alpha\text{-amylase}}$ = 341.577 ± 0.398 μg/mL and $IC_{50\ \beta\text{glucosidase}}$ = 308.474 ± 0.296 μg/mL). Several other studies demonstrated that triterpenoids and their derivatives could efficaciously inhibit the activity of glucosidase and amylase to diminish the absorption of carbohydrates [34,82]. These findings suggest that *C. metuliferus* extract could decrease the postprandial glucose level by inhibiting the activities of β-glucosidase or α-amylase, which are key enzymes in the digestion process of complex carbohydrates from food into adsorbable monosaccharides.

4. Conclusions

In this present work, the HPLC analysis was used to identify the chemical composition of the hydroethanolic extract of *Cucumis metuliferus*. The obtained results highlight the presence of 17 known phytochemical compounds in different amounts, including ursolic acid. A method of identification and isolation of ursolic acid, which presents antioxidant and anti-inflammatory activity, from the fruits of *Cucumis metuliferus* was proposed. The structure of the isolated compound was determined by IR spectroscopy, mass spectroscopy, and ^{1}H NMR, and the data were confirmed by comparison with previously reported data. The obtained fraction showed antioxidant, anti-inflammatory, and antidiabetic activities, as well as the hydro-ethanolic extract. *C. metuliferus* exhibited considerable enzymatic inhibitory activity on key enzymes involved in diabetes and encourages the use of its extracts and phytochemical constituents, such as ursolic acid, in the prevention and therapy of diabetes mellitus. The results demonstrate the good activity of the hydro-ethanolic extracts obtained from fruits of *Cucumis metuliferus* "Tempus" grown in Romania due to the bioactive compounds identified in the extract, with major potential for various uses in disease management, as an alternative medicine to improve the overall health and wellness.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10050274/s1, Figure S1: MS Spectra of fraction of *C. metuliferus* spicies with ursolic acid (negative 16 mode) performed on mass spectrometry; Figure S2: Proposed fragmentation pattern of ursolic acid, Figure S3. The ^{1}H NMR spectrum of the ursolic acid fraction.

Author Contributions: Conceptualization, R.M.D. and A.C.B.; Data curation, A.V.D.B., A.C.B. and B.F.; Funding acquisition, R.M.D. and B.F.; Investigation A.C.B., G.V.C. and A.V.D.B.; Methodology, R.M.D., A.V.D.B., A.C.B. and B.F.; Project administration R.M.D. and A.C.B.; Resources R.M.D. and B.F. Software A.V.D.B.; Supervision, R.M.D. and B.F.; Visualization A.C.B., A.V.D.B. and G.V.C.; Writing—original draft, A.C.B., A.V.D.B. and R.M.D.; Writing—review and editing, R.M.D., A.V.D.B., A.C.B. and B.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The data presented in this study are available in the figures and supplementary materials.

Acknowledgments: The authors are grateful for the technical support provided by the MoRAS and ECEE Centers, and by "Dunarea de Jos" University, to Costel Vînătoru (Plant Genetic Resources Bank for Vegetables, Floriculture, Aromatic, and Medicinal Plants Buzău, 56 Nicolae Bălcescu Street, 120187, Buzau, Romania), and the Research-Development Station for Vegetable Growing Buzău for the plant material that was used in this study.

Conflicts of Interest: The authors declare no conflict of interest.

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