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Vigna unguiculata L. Walp. Leaves as a Source of Phytochemicals of Dietary Interest: Optimization of Ultrasound-Assisted Extraction and Assessment of Traditional Consumer Habits

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Vigna unguiculata L. Walp. is an African crop spread worldwide mainly for pulses production. Despite being a neglected and under-utilized food, cowpea leaves are a rich source of phytochemicals and micronutrients. The aim of the work is to characterize the phytochemical composition of cowpea leaves by an optimized ultrasound-assisted extraction (USAE) and to compare raw and boiled leaves. A three-level factorial design (Box-Behnken) was employed for the optimization of the USAE considering three different parameters (% ethanol, drug-to-solvent ratio, and number of cycles). The optimized extracts were characterized by LC/MS/MS. Finally, leaves were boiled at 100 °C for 30 min to simulate traditional cooking procedures and compared to raw leaves. The best extraction condition was EtOH/H_2O 1:2 v/v, drug to solvent ratio 1:47 w/v, and 3 extraction cycles. The phytochemicals identified mainly belong to the family of phenolic acids, flavonoids, terpenoids, and alkaloids. Boiled leaves revealed a significant loss of most phytochemicals and a net decrease of their antioxidant activity compared to the raw ones. The results highlight the potential nutraceutical value of cowpea leaves whilst the impoverishment triggered by traditional consumer habits pushes the need to evaluate alternative cooking procedures helpful in the maintenance of their phytochemical properties.

Introduction

Vigna unguiculata L. Walp., also known as cowpea, is an African pulse of high nutritional interest for its seeds that are consumed in many regions of the world, and are a source of micronutrients, proteins, amino acids, and phytochemicals important for the human diet.[1] Furthermore, they are characterized by low-fat content whilst they have been recognized as a source of minerals such as potassium, calcium, phosphorus, and vitamins such as niacin, folates, and tocopherols.[1] *V. unguiculata* output has significantly increased in the last decades, reaching 8.9 million metric tons in 2019.[2] The spread of this crop worldwide is nowadays a matter of fact so that cowpea is cultivated in many continents (e.g., Asia, Europe, and South America), particularly in the tropical regions.[1] Additionally, pulses are crops of particular interest also from an environmental point of view, as they are highly adaptable to harsh environmental conditions (e.g. drought, reduced soil tillage, and low agrochemicals input) standing out as a promising crop for counteracting the hindrances posed by climate change.[3,4] Furthermore, cowpea can provide ground cover, control weeds, and fix up to 80% of nitrogen (N_2), thus representing a key element for the long-term viability of agricultural systems.[5]

Although the main interest in cowpea cultivation and consumption deals with seeds, in Africa and some Asian countries leaves are also consumed boiled or fried as a side dish.[6,7] Cowpea leaves are also used for the prevention and treatment of several human disorders such as burns, adenitis, and measles, and are known sources of micronutrients, such as zinc, iron, and beta-carotene.[8,9] However, raw leaves display minor traces of some anti-nutrients, such as oxalates and alkaloids, whose occurrence is limited by the cooking procedure.[1] Due to their low exploitation cowpea leaves are nowadays considered a "neglected and underutilized" food despite their nutritional value. Some African food campaigns (e.g., slow food) were launched in the last years in order to allow their commercialization and supply in the market but, for the moment, their usage is still limited to African countries (https://www.growfurther.org/) and their uptake is still very low. Moreover, relatively little is known about their phytochemical composition in terms of secondary compounds such as polyphenols, terpenes, and alkaloids.[10] Therefore, the novelty of the present study lies in the valorization of cowpea leaves as a source of antioxidant compounds to promote their consumption. Moreover, we aim at adding a piece to their chemical composition that - to date - reports them as one of the indigenous African vegetables richest in micronutrients, such as

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iron and vitamin A.[11] In the end, besides being an interesting matrix at the micronutrient level, the exploitation of *V. unguiculata* leaves deals also with the context of a circular economy approach, since the valorization of waste matrixes is a matter of concern in terms of environmental sustainability, as highlighted in the Sustainable Development Goals of the Agenda 2030 (i.e., SDG 12).

The growing attention towards environmental safeguarding highlights the need to reduce as much as possible the use of organic solvents.[12] Nowadays, mixtures of hydro-alcoholic solvents are among the most exploited to recover compounds of nutraceutical interest. Initially, policies connected with the reduction of the environmental impact in the context of solvent-to-liquid extraction processes aimed at the optimization of extraction procedures by favoring the exploitation of GRAS solvents (e.g., ethanol instead of methanol).[13] The recent advances in terms of solvent management foresee the evaporation of the organic solvent exploited within a process, thus guaranteeing the possibility of its reusage. However, such tools (such as the rotary evaporator) require other issues to be considered in terms of economic and environmental impacts (e.g., costs related to energy waste). Therefore, an emerging frontier in phytochemical studies is to develop extraction techniques aimed at optimizing yields by exploiting a solvent as eco-friendly as possible, such as water.[14] Furthermore, technologies such as ultrasound-assisted extraction (USAE) significantly increase the extraction efficiency in an inexpensive way compared to traditional methods, while at the same time increasing the stability of phytochemicals, as it allows the reduction of the temperature.[15] The advantages of USAE have already been highlighted in different studies, such as the one from[16] in which the cavitation procedure enhanced the extraction yield by 24% compared to traditional extraction while also reducing the time by 90%.

Hence, the aim of the present work is to evaluate the phytochemical composition of cowpea leaves to promote their exploitation as a food or food supplement, by identifying the best USAE to recover the secondary compounds occurring in cowpea leaves. Finally, the stability of the above-mentioned phytochemicals is evaluated by comparing the yield and the antioxidant composition between raw and boiled samples to address the role of traditional processing on the nutraceutical value of cowpea leaves.

Experimental Section

Samples collection

V. unguiculata plants (accession n. TVU11733) were cultivated in Sanremo, Italy at the CREA Research Center for Vegetable and Ornamental Crops of Sanremo (IM, Italy, GPS: 43.816887, 7.758900) between July and November 2021. The plot included two rows (12 m long) with 50 plants per row, the distance between the rows was 1 m, and between the two rows was a central irrigation line (40 cm wide and 1.5 cm deep). All the plants were watered once a week by filling the central line that separated the two rows with tap water. No fertilization or fertigation was applied. At the end of the growing season, leaves were collected and washed to remove grounds and impurities and then stored at −20 °C.

Chemicals

Ultrapure H_2O (18 MΩ) was obtained using a Milli-Q purification system (Millipore, Bedford, USA). Solvents for samples extraction and characterization (ethanol, methanol, gallic acid, Trolox, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Folin-Ciocalteu reagent) were purchased from Sigma Aldrich®, Germany. Mass-grade solvents (methanol, acetonitrile, and formic acid) were obtained from Romil®, Italy.

Design of Experiment (DoE)

Leaves were freeze-dried (SP scientific, Pennsylvania) for 24 h to obtain a dry matrix that was processed to a fine powder through a mill (IKA®, Staufen, Germany) for laboratory purposes.

The fine powder was extracted by exploiting an ultrasound bath (ARGO LAB, Italy) that was maintained in the following conditions throughout the extraction processes: bath temperature of 30 °C, frequency of 37, and sonication time of 10 min. For each extraction, 300 mg of dry powder was used. For optimization of the USAE parameters, a three-level factorial design in 27 runs (Box-Behnken) was used in order to investigate the role of three fixed effects that were i) % ethanol in the extraction solvent (20-50-80 % *v/v*), ii) drug to solvent ratio (1 : 10-1 : 40-1 : 80 *w/v*), iii) the number of cycles (2-3-4) on the recovery of antioxidant compounds evaluated as the total phenolic content (mg gallic acid equivalents, GAE/g dry leaves), antioxidant capacity (TEAC, mg of Trolox equivalents/g dry leaves), and DPPH radical scavenging activity (TEAC, mg of Trolox equivalents/g dry leaves). Response surface methodology (R Studio version 1.4.1, package *rsm*) was used for experimental design and data analysis. In the extraction process, the influence of the fixed parameters on the response variables was assessed using a confidence level of 95% for all the variables.

The quadratic model proposed for each response variable (Yi) was:

$$Yi = a + bA + cB + dC + eA^2 + fB^2 + gC^2 + hAB + iAC + jBC + \varepsilon;$$

where "A" is the solvent composition; "B" is the drug/solvent ratio "C" is the number of cycles; "a" is the intercept; "b", "c", and "d" are the linear coefficients; "e", "f", and "g" are quadratic coefficients; "h", "i", and "j" are the interaction coefficients; and "ε" is the error variable.

This quadratic model was estimated considering the R^2 value (% variation explained by the model). Additionally, a lack-of-fit test was done for the models from the analysis of variance to estimate the significance of the amount of variability not explained by the regression model. When the lack of fit *p*-value was incalculable, the model residual distribution was evaluated as reported in *Supporting Information* (Figure S1, Figure S2). Graphically, a response surface for each variable was obtained and significant differences were considered with $p < 0.05$. The models were built by using the average values coming from three biological extractions for each tested condition (see Table 1 for the results).

Cooking procedure

Leaves were boiled for 30 min to mimic the conditions of consumption by following some traditional African receipts.[6] After boiling, the excess water was removed from the leaves which were then freeze-dried. Dry leaves were ground and extracted following

Table 1. Mean and SD values of the analyzed conditions (expressed as the total phenol content (TPC) and Trolox Equivalent Antioxidant Capacity based on the ABTS ($TEAC_{ABTS}$) and DPPH ($TEAC_{DPPH}$) scavenging activity) included in the response surface statistical models.

Exp. Run	A	B	C	TPC (mg GAE/g)	$TEAC_{ABTS}$ (mg TE/g)	$TEAC_{DPPH}$ (mg TE/g)
1	1:4	1:10	2	5.62 ± 0.18	8.10 ± 0.26	1.30 ± 0.62
2	1:1	1:10	2	10.53 ± 0.88	6.54 ± 1.47	2.45 ± 0.53
3	4:1	1:10	2	5.56 ± 0.76	2.64 ± 1.09	0.95 ± 0.25
4	1:4	1:10	3	8.64 ± 0.05	9.83 ± 0.35	2.43 ± 0.33
5	1:1	1:10	3	11.47 ± 2.14	6.87 ± 1.66	4.09 ± 0.86
6	4:1	1:10	3	9.12 ± 0.77	4.84 ± 0.34	1.80 ± 0.09
7	1:4	1:10	4	7.58 ± 0.35	6.41 ± 0.33	2.44 ± 0.18
8	1:1	1:10	4	6.72 ± 0.37	5.40 ± 0.18	2.22 ± 0.20
9	4:1	1:10	4	3.89 ± 0.66	3.00 ± 0.52	1.81 ± 0.31
10	1:4	1:40	2	10.99 ± 2.07	11.92 ± 2.58	4.32 ± 1.23
11	1:1	1:40	2	13.12 ± 0.48	13.63 ± 0.72	4.74 ± 0.53
12	4:1	1:40	2	9.33 ± 0.39	6.26 ± 0.60	4.14 ± 1.66
13	1:4	1:40	3	11.88 ± 2.70	14.32 ± 0.90	4.99 ± 0.60
14	1:1	1:40	3	13.39 ± 0.23	14.54 ± 0.58	4.73 ± 1.20
15	4:1	1:40	3	9.88 ± 0.37	7.14 ± 0.58	4.31 ± 1.39
16	1:4	1:40	4	10.21 ± 0.58	8.04 ± 0.48	3.74 ± 0.13
17	1:1	1:40	4	9.56 ± 0.28	7.37 ± 0.54	3.46 ± 0.11
18	4:1	1:40	4	7.43 ± 0.27	4.71 ± 0.11	3.00 ± 0.16
19	1:4	1:80	2	11.3 ± 1.08	10.0 ± 0.21	5.11 ± 0.13
20	1:1	1:80	2	9.27 ± 0.35	8.80 ± 0.15	3.30 ± 0.14
21	4:1	1:80	2	6.38 ± 0.30	4.67 ± 0.05	3.33 ± 0.06
22	1:4	1:80	3	12.3 ± 0.36	10.65 ± 0.35	5.82 ± 0.21
23	1:1	1:80	3	9.95 ± 0.11	9.00 ± 0.14	4.16 ± 0.27
24	4:1	1:80	3	7.00 ± 0.03	4.70 ± 0.07	3.40 ± 0.01
25	1:4	1:80	4	11.5 ± 0.47	9.88 ± 0.25	5.30 ± 0.36
26	1:1	1:80	4	8.84 ± 0.08	7.41 ± 0.03	2.86 ± 0.13
27	4:1	1:80	4	7.86 ± 0.33	4.31 ± 0.03	3.41 ± 0.03

the best combination of variables based on the DoE results to address the impact of the cooking treatment on the antioxidant composition of leaves ($n = 10$, 5 per treatment).

Evaluation of the total phenol content and total antioxidant activity

The response variables (Y_i) considered in the regression models were obtained by analyzing the extracts for their TPC based on the Folin-Ciocalteu assay and the TEAC based on the ABTS and DPPH assays.

The analysis was carried out following the protocols suggested by Amigoni et al..[17] The same analysis was performed to compare raw and boiled leaves.

Untargeted metabolomic analysis

Both raw and boiled leaves were chemically characterized by High Resolution Mass Spectrometry (HR-MS) by using the ACQUITY UPLC H-class system coupled with the Xevo G2-XS QTof Mass Spectrometer (Waters Corp., Milford, MA, USA) through an ESI source. All the analytes were separated on a Zorbax SB-C18 column (100 mm×2.1 mm, 3.5 μm). The mobile phases were MS grade H_2O (A) and MeCN (B), both containing 0.1% formic acid (HCOOH) and analyte elution was performed according to the following gradient: 0–2 min, 5–10% B linear gradient; 2–10 min, 10–45% B linear gradient; 10–11 min isocratic 45% B,11-13 min 45–80% B linear gradient, 13–15 min 80–95% B linear gradient, 15–20 min isocratic 95% B. After each run, the column was washed for 5 mins with 95% B and then equilibrated for further 5 mins at the initial conditions (5% B) before the next sample injection. Elution was performed at a flow rate of 0.4 mL/min, and the injection volume was 10 μL. The column temperature was set at 30 °C. Full-scan MS data were acquired both in positive and negative ionization modes and the spectra were recorded in the range of *m/z* 100–1200. The source parameters were as follows: electrospray capillary voltage 2.0 kV, source temperature 150 °C, and desolvation temperature 500 °C. The cone and desolvation gas flows were 20 and 1000 L/h, respectively. A scan time of 0.5 s was employed. The mass spectrometer was calibrated with 0.5 M sodium formate and leucine-enkephalin (100 pg/μl) was used as LockMass (*m/z* 554.2615, 0.8 kV ionization voltage for negative mode; *m/z* 556.2677, 0.8 kV ionization voltage for positive mode), which was infused at 8 μl/min and acquired for 1 s each 30 s. A quality control (QC) sample was repeatedly analyzed at the beginning of the sample list and every 3 samples. The ionizing compounds occurring in samples were characterized by Data Dependent Acquisition (DDA) setting as ion intensity threshold a value of 5×10^4 and

additional targeted MS/MS experiments were performed for precursors not fragmented in the DDA experiments. The MassLynx software (version 4.2) was used for instrument control, data acquisition, and data processing. Metabolite identification was performed based on the experimental accurate mass measurement and fragmentation profile, taking into consideration the mass error, isotopic pattern, and the overlapping of fragmentation profiles with data reported in the literature, in public databases and by using the UNIFI Software 1.9.4 EN (Waters, USA) with the library "Waters Traditional Medicine Library" provided by Waters, USA.

Statistical analyses

We used MS Dial software version 4.9 for the peak picking, deconvolution, noise level setting, and identification of metabolites. The identified peaks were aligned by exploiting the QC sample, also used to allow the monitoring of the response of the instrument. Deconvoluted chromatograms were normalized on the Total Ion Current (TIC) and analyzed through a Principal Component Analysis (PCA) to account for the effect of the condition of consumption followed by PERMANOVA to account for statistical significance ($\alpha = 5\,\%$) in R by exploiting the *vegan* package.

Results

Definition of the best USAE condition

By the response surface methodology, for each of the tested variables, a second-order polynomial model was interpolated. The results of the models are reported in Table 2.

Results show that both the percentage of ethanol in the extraction solvent, the number of extraction cycles, and the drug/solvent ratio significantly affected the recovery of phenolic compounds (Figure 1).

The stationary point with the highest yield was EtOH 40 % v/v, 1 to 45 (w/v) drug/solvent ratio, and 3 extraction cycles. Considering the antioxidant compounds recovery based on the ABTS scavenging activity, the RSM model showed that each independent variable has an impact on the recovery of antioxidant compounds with a stationary point of response surface at EtOH 25 % v/v, 1 to 45 (w/v) drug/solvent ratio, with 3 extraction cycles (Figure 2).

Finally, the model based on the DPPH scavenging activity showed that the significant parameters were the drug/solvent

Table 2. Response Surface Methodology models output. Letters indicate the coefficients expressed in Equation (1). *: maintained as a blocking factor; n.s.: not significant; ** Not Available (see model validation in Figure S1 and Figure S2).

	TPC (mg/g GAE)		$TEAC_{ABTS}$ (mg/g TE)		$TEAC_{DPPH}$ (mg/g TE)	
Coefficient	Value	*p*-value	Value	*p*-value	Value	*p*-value
a	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
b	n.s.	n.s.	n.s.	n.s.	n.s.*	n.s.*
c	0.64	<0.001	0.85	<0.001	0.384	<0.001
d	10.04	0.012	10.66	0.005	4.46	0.019
e	−0.002	0.014	−0.002	0.012	n.s.	n.s.
f	−0.02	0.001	−0.03	<0.001	−0.01	<0.001
g	−1.75	0.009	−1.92	0.003	−0.75	0.019
h	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
i	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
j	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Adj. R^2	62.6 %	<0.001	82.4 %	<0.001	68.5 %	<0.001
Lack of fit	N.A.**	N.A.**	N.A.**	N.A.**	1.002	0.433

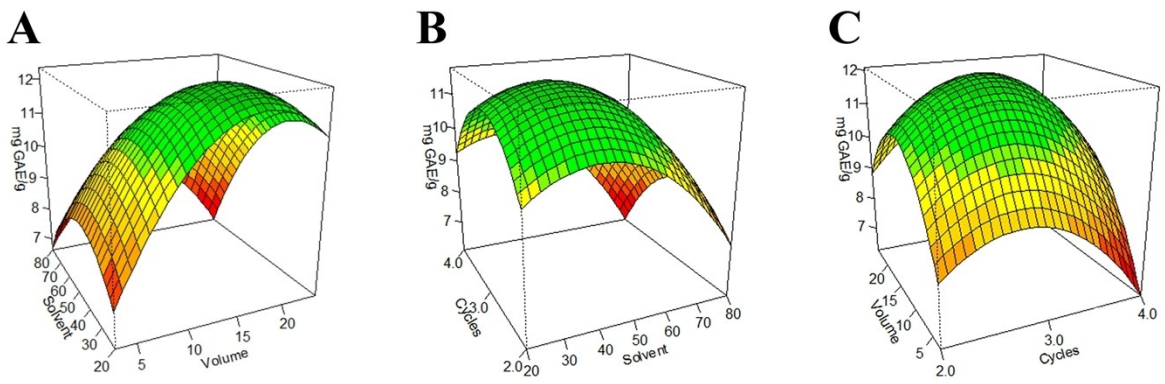

Figure 1. Response surface methodology plots representing the relationship between the yield variable (TPC) as a function of the drug-to-solvent ratio, solvent composition, and the number of extraction cycles.

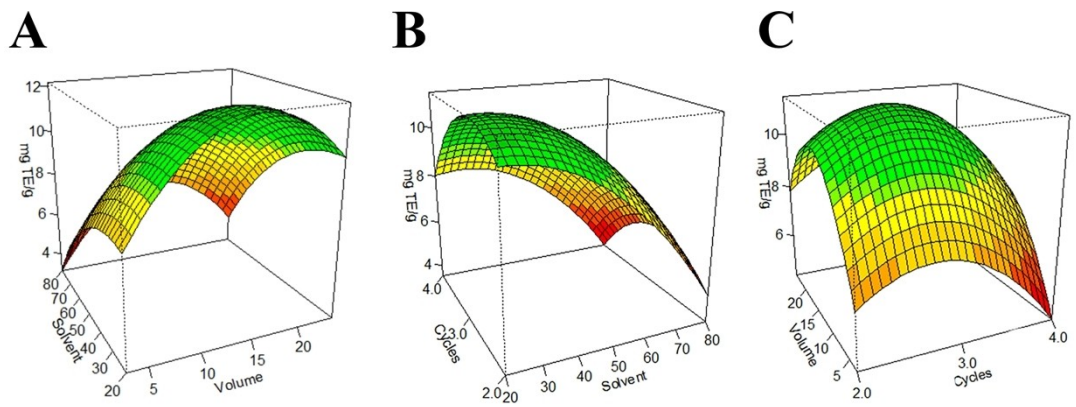

Figure 2. Response surface methodology plots representing the relationship between the yield variables ($TEAC_{ABTS}$) as a function of the drug-to-solvent ratio, solvent composition, and the number of extraction cycles.

ratio and the number of cycles with a stationary point at 1:52 (w/v) drug/solvent ratio and 3 extraction cycles (Figure 3).

Regarding this model, the solvent was not found to be a relevant parameter in the response surface, but it was maintained as a blocking factor to improve the goodness of fit of the model itself. For each analysis, the R^2 value, the model significance, and – when calculable – the lack of fit significance is reported in Table 2.

Overall, based on the results obtained and considering the output of all the analyses, we defined the combination EtOH/H_2O 1:2 v/v; 1:47 (w/v) drug/solvent ratio and 3 extraction cycles as the best conditions for the recovery of phytochemicals endowed with antioxidant activity by exploiting the USAE.

Characterization of the phytochemical profile

For samples extracted with the optimal USAE condition defined, the phytochemical profile was investigated by HR-MS analysis. The chromatographic profile is reported in Figure 4.

Their relative identification is reported in Table 3.

The results of the metabolomic analysis underline that the phenolic composition is not the only responsible for oxidative radical scavenging. Indeed, most of the compounds identified belong to the family of phenolic acids, flavonoids, and terpenes besides a putative alkaloid and a fatty acid. Most of the compounds detected occurred in the form of glycosides or glycoside derivatives. For instance, peak 13 showing a *m/z* value of 903.2172 $[M–H]^-$ was supposed to be a quercetin derivative due to the presence of a MS/MS fragment at *m/z* 300 with the main MS/MS fragment at *m/z* 757 indicating a loss of a rhamnoside unit (146 Da). Therefore, we identified it as the same compound of peak 8 with the addition of a rhamnose moiety. The same pattern explains the identification of peak 14 exhibiting a *m/z* of 933.2299 $[M–H]^-$. In this case, however, the difference between the pseudomolecular ion and the fragment at *m/z* 757 suggests the loss of a glucuronate unit (176 Da). Peak 16 shows a pseudomolecular ion at 771.1729 with a MS/MS fragmentation pattern of 625 and 300 that is representative of a quercetin. Similarly, peak 19 identity with a *m/z* 815.2013 $[M–H]^-$ is explainable considering that in the fragmentation pattern the MS/MS fragment *m/z* 639 exhibits a difference of 176 Da, suggesting the loss of a glucuronate unit.

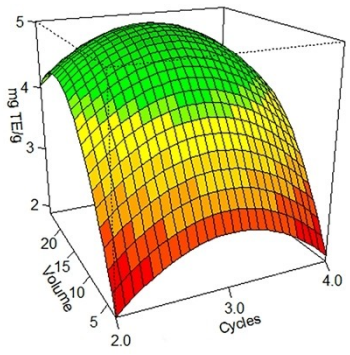

Figure 3. Response surface methodology plots representing the relationship between the yield variables ($TEAC_{DPPH}$) as a function of the drug-to-solvent ratio and the number of extraction cycles.

Procedure of consumption

The results obtained by the untargeted metabolomic analysis on raw and boiled leaves, obtained with the best USAE conditions, are displayed in Figure 5.

The principal component analysis identifies clear differences between raw and boiled samples ($p = 0.009$) driven by the loss of most of the compounds during the cooking process, due to the dispersion in the boiling water and to the heat degradation of many compounds. The separation between the two conditions occurs mainly on the PC1 accounting for 77.9% of the variability. The PC2 explains 6.7% of the variability and PC3 only 4.6%. These results are confirmed also by the evaluation of the antioxidant activity of raw and boiled leaves showing that the amount of antioxidant compounds is from about 8 ($TEAC_{ABTS}$) to 32 ($TEAC_{DPPH}$) fold lower in boiled samples compared to the raw ones as shown in Table 4.

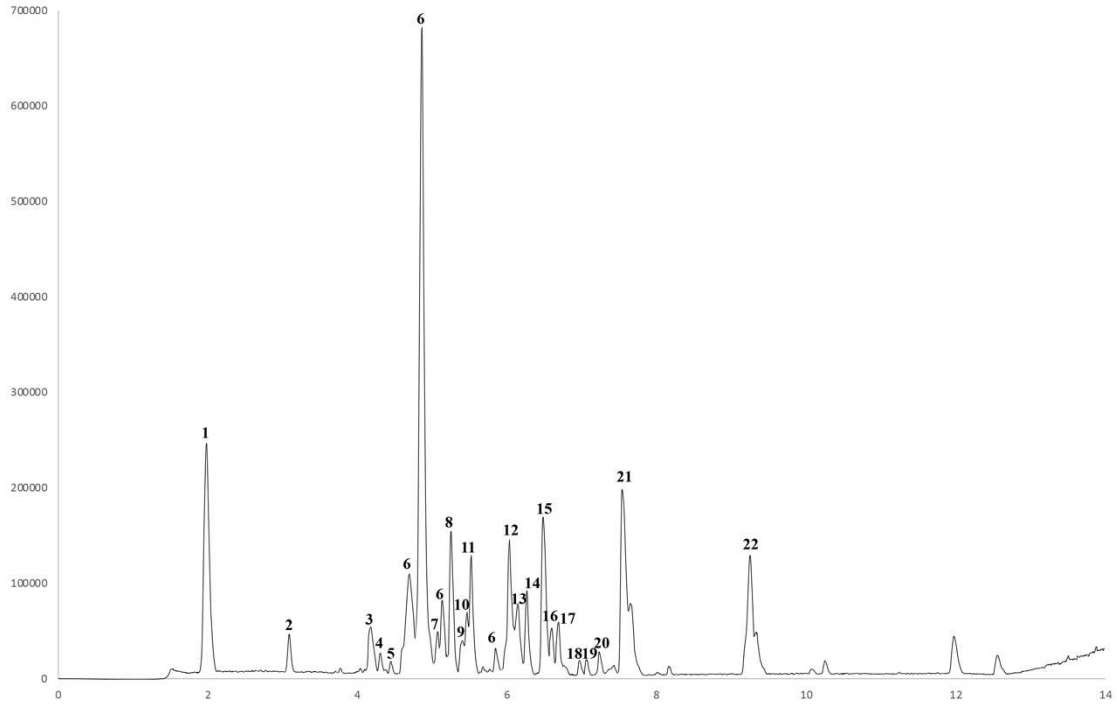

Figure 4. Representative base peak intensity chromatogram of *Vigna unguiculata* leaves extracts acquired in negative ionizing MS mode.

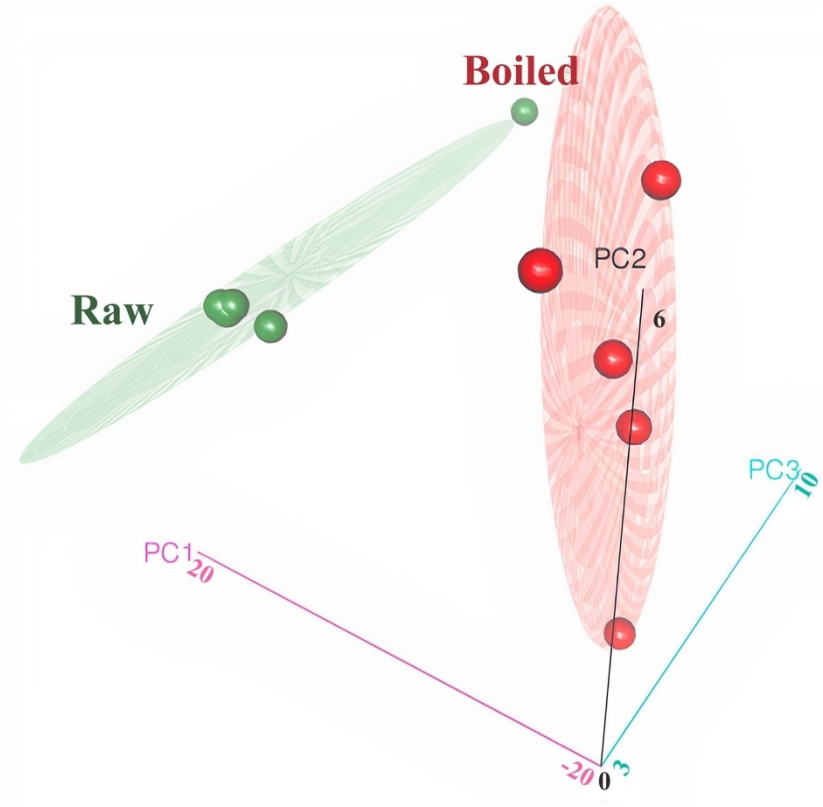

Figure 5. Principal Component Analysis (PCA) performed on the metabolomic profile of raw and boiled cowpea leaves in negative ion current.

Table 3. Identification of the phytochemicals occurring in cowpea leaves by HR-MS analysis.

Peak number	Retention time (min)	Name	Molecular Formula	*m/z* observed	MS/MS ions	Reference
1	1.98	Gentisic acid 5-O-glucoside	$C_{13}H_{16}O_9$	315.0696 $[M-H]^-$	152, **108**	[18]
2	3.09	Disaccharide	$C_{13}H_{24}O_9$	323.1353 $[M-H]^-$	119, 113, **101**, 89	[19]
3	4.18	Coumaric acid-O-glucoside	$C_{15}H_{17}O_8$	325.093 $[M-H]^-$	163, **119**	[18]
4	4.26	Saccharide (formate)		451.2552 $[M-HCOO]^-$	405	[20]
5	4.45	Citrusin C	$C_{17}H_{26}O_7$	387.1658 $[M-H]^-$	**163**	Traditional Medicine Library
6	4.86, 5.40	Roseoside (formate)	$C_{19}H_{30}O_8$	431.1901 $[M-HCOO]^-$	385, 223, **153**	[20]
7	5.13	Saccharide (formate)		435.2221 $[M-HCOO]^-$	**389**, 225	[20]
8	5.25	Quercetin-arabinosyl-diglucoside	$C_{32}H_{38}O_{21}$	757.1796 $[M-H]^-$	300	[21]
9	5.46	Coumaroyl-glucarate isomer	$C_{15}H_{16}O_{10}$	355.1045 $[M-H]^-$	209, 163, **119**	[22]
10	5.52	Quercetin di-hexoside	$C_{27}H_{30}O_{17}$	625.1429 $[M-H]^-$	**300**, 151	[21]
11	5.85	Dihydro-roseoside (formate)	$C_{19}H_{32}O_8$	433.2081 $[M-HCOO]^-$	**387**, 223	[20]
12	6.03	Myricetin-O-pentoside	$C_{20}H_{18}O_{12}$	449.2039 $[M-H]^-$	269, **209**	[23]
13	6.15	Quercetin-arabinosyl-glucoside-rhamnoside	$C_{37}H_{44}O_{26}$	903.2172 $[M-H]^-$	**757**, 300	see the text
14	6.26	Quercetin-arabinosyl-diglucoside-glucuronide	$C_{38}H_{46}O_{27}$	933.2299 $[M-H]^-$	757, **300**	see the text
15	6.48	Caffeoyl-quinic acid derivative		415.1957 $[M-H]^-$	179	[24]
16	6.60	6-Hydroxyluteolin7-O-(6‴-O-*p*-coumaroyl)-sophoroside	$C_{36}H_{43}O_{16}$	771.1729 $[M-H]^-$	**625**, 300	[25]
17	6.69	Quercetin-feruloyl-diglycoside	$C_{31}H_{28}O_{15}$	801.1832 $[M-H]^-$	**625**, 300	[26]
18	6.97	Isorhamnetin-sophoroside-rhamnoside	$C_{34}H_{42}O_{21}$	785.1909 $[M-H]^-$	639, **315**, 300	[27]
19	7.06	Isorhamnetin-sophoroside-glucuronide	$C_{34}H_{40}O_{23}$	815.2013 $[M-H]^-$	639, **315**, 300	see the text
20	7.23	Pinoresinol-acetyl-hexoside	$C_{28}H_{34}O_{12}$	561.1985 $[M-H]^-$	**357**, 151	[28]
21	7.53, 7.65	Alkaloid	$C_{21}H_{35}N_3O_2$	362.2804 $[M-H]^+$	344, 273, 139, **112**	Traditional medicine library
22	9.25, 9.33	Oxo-dihydroxy-octadecanoic acid	$C_{18}H_{32}O_5$	327.2157 $[M-H]^-$	229, 211, **171**	[20]

Table 4. Comparison of the TPC and TEAC between raw and boiled leaves. Different letters indicate differences occurring at the statistical level ($p < 0.05$).

Condition	TPC (mg GAE/g)	$TEAC_{ABTS}$ (mg TE/g)	$TEAC_{DPPH}$ (mg TE/g)
Raw leaves	8.09 ± 0.34^a	8.12 ± 0.49^c	3.94 ± 0.22^e
Boiled leaves	0.77 ± 0.07^b	0.93 ± 0.08^d	0.12 ± 0.07^f

Discussion

Optimized extraction protocol and phytochemical characterization

By interpreting the results originating from the different assays adopted, we found that under optimal conditions the retrieval of the phytochemicals is maximum when 1 g of the dry matrix is extracted with 47 mL of solvent. This is because the extractants summon as many compounds as possible until the saturation point is reached. This is confirmed by the observation that the extractant needs to be renewed three times to gain optimal recovery. If combined with the optimal drug-to-solvent ratio and with three cycles of extraction to let total exhaustion of the matrix, it is also possible to lower significantly the amount of organic solvent that in our study was found to be optimal in a ratio 1:2 v/v in combination with water, which is a percentage significantly lower compared to that of many studies where the extractant is represented by at least 50% v/v of organic solvent.[29,30] The analysis of the phytochemical composition of cowpea leaves displayed that most of the compounds occurring within the matrix are conjugated with glycosides or sugar moieties that are likely to increase the overall polarity of the phytocomplex. This may justify the need for a low amount of ethanol to obtain the optimal recovery of antioxidant compounds, making cowpea leaves an interesting matrix as regards their usage as a food supplement. As regards cowpea leaves, few studies focused on their phytochemical composition.[18,26] Conversely, most of the documentation about the composition of cowpea leaves is related to the micronutritional composition, highlighting the occurrence of many classes of vitamins and microelements.[31,32] In the present case, we were able to detect an array of compounds belonging to the category of secondary metabolites that have not been described yet in the present matrix. Many of these compounds are endowed with documented antioxidant properties, the majority being glycosides of phenolics (such as gentisic acid, coumaric acid, and pinoresinol), flavonoids (myricetin, luteolin, quercetin, and isorhamnetin) and sesquiterpenes such as the case of isomeric forms of roseoside, besides than a compound which was assigned to the formula $C_{21}H_{35}N_3O_2$ identifiable as an alkaloid already described in leaves.[33] Most of the above-mentioned compounds are known to exhibit many nutraceutical properties, including anticarcinogenic, antiviral, antidiabetic and some related to the anti-inflammatory activities studied in different *in vitro* models.[34,35] For instance, a study by Frankish et al.[36], highlighted that roseoside exhibits antihyperglycemic effects by enhancing the action of insulin or by increasing the glucose metabolism or glucose homeostasis in diabetic animals. Likewise, the occurrence of myricetin derivatives and long chain fatty acids such as oxo-dihydroxy-octadecenoic acid is a proxy of anti-inflammatory activities.[37] Furthermore, these phytochemicals may be of interest also for the food industry. For instance, isolated quercetin is marketed as a dietary supplement for its health-beneficial effects among which its antihypertensive effects and its potential to improve endothelial function are the most relevant.[38] This may hypothesize the use of leaves in the food supplement sector, especially if coupled with a sustainable extraction method such as that described by the experimental design presented. Conversely, other chemical components may act as anti-nutrients, for instance alkaloids, that generally are associated with negative effects on human health.[39] However, the majority of them was lost or significantly reduced in concentration after culinary processes such as the common boiling process, as highlighted by the untargeted metabolomic analysis. Indeed, cowpea leaves are usually considered agricultural waste and are mainly exploited as fodder.[1] However, as highlighted in the HR-MS analysis, it is a matrix endowed with promising biologically active compounds exploitable to produce high-added value products, in line with a circular economy perspective, widely advocated to abandon the linear economy model and to promote a more sustainable renewal of wastes deriving from the agricultural and food supply chains (Agenda 2030, SDG 12).

Nutritional value of a neglected and underutilized food and impact of the consumer habits

While the value of cowpea at the nutritional and nutraceutical level has been widely investigated concerning seeds,[1,40] leaves composition has not been completely elucidated yet. Cowpea leaves belong to a long series of many African neglected and underutilized foods, mainly vegetables, that have been incorporated into the human diet thanks to their nutritional value and adaptability to climate change.[41] Furthermore, such African vegetables can be used to achieve some of the United Nations Sustainable goals like the SDG n.2 that aims at interrupting hunger and all forms of malnutrition, improving the nutritional value of food products and ensuring the full potential of these crops.[41] For example, a recent study by Tepe and Lemken[42] analyzed consumer demand for traditional porridge combined with cowpea leaf powder. The results obtained underlined the feasibility of enriching conventional foods well accepted into the diets but of low nutritional value. This alternative usage of cowpea leaves can be a practical approach to introduce nutritious vegetables into the diet not only to counteract the hindrances related to micronutrient deficiencies, but also with those related to oxidative stress in light of the results obtained in the present study.

Moreover, only a few researches have investigated the effects of the cooking method on the nutraceutical composition of cowpea leaves, frequently focusing only on specific compounds. For instance, a recent study evaluated the concentration of ascorbic acid in *V. unguiculata* leaves after different cooking procedures.[43] In detail, they compared three different methods: microwaving, steaming, and boiling, with the latter yielding the least concentration of ascorbic acid. This observation supports our results that indicated the boiling method as highly impactful on the phytochemical composition. Although boiling represents the most common cooking procedure for *V. unguiculata* leaves, as it can reduce toxicity and increase palatability,[1] it can significantly reduce their phytochemical content, thus worsening their nutraceutical value. A comparable

impact has been already documented for many other vegetables, whose phytochemical composition is known to be preserved by avoiding direct simmering and preferring more preservative preparation methods[44–46] which can support the stability of a wide array of nutrients such as proteins, sugars, vitamins, and glucosinolates. Further studies should evaluate the effects of reducing cooking time and using other cooking methods such as microwaving or steaming to identify the best consumption practices in the view of preserving as much as possible the nutritional value of such food sources.

Conclusions

In the present work we described a fast and economically feasible method for the extraction of phytochemicals from cowpea leaves. The optimized condition resulted to be obtained with a combination EtOH/H_2O 1:2 v/v, in a drug-to-solvent ratio equal to 1:47 w/v coupled with three extraction cycles. The characterization of the matrix showed the occurrence of many different secondary compounds with potential bioactive effects. However, to optimize the assumption of the antioxidants occurring in the matrix it is essential to identify alternative methods to common boiling exploited in traditional African receipts to preserve as much as possible the properties of cowpea leaves. Moreover, the occurrence of compounds of potential nutraceutical interest supports the idea to exploit the agricultural wastes of this crop for the production of plant-based foods.

Author Contributions

Conceptualization E. P., L. G., Methodology C. S., A. C., M. B., L. G., Validation E. P., C. S., M. B., L. G., Data Curation E. P., C. S., Writing – Original Draft E. P., L. G. Project administration M. L., Supervision Funding acquisition M. L.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: ultrasound-assisted extraction · *Vigna unguiculata* · experimental design optimization · HR-MS · traditional food consumption

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