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The evaluation of total flavonoids, total phenolic content and biological activity of Iraqi *Lipedium sativum* L. crude extract obtained by optimized ultrasound assisted extraction conditions

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Abstract

Lepidium sativum L. also known as garden cress belong to the family Brassicaceae. The plant species composed of various phytochemicals as well as powerful nutraceutical potential and possess several bioactivities like, hepatoprotective, antioxidant, anticancer, antimicrobial, hypoglycemic, gastrointestinal and bone healing activities. This research paper presents an investigation into the total flavonoids (TFC), total phenolic content (TPC) and biological activity of Iraqi *Lepidium sativum* L. The study aimed to optimize ultrasound-assisted extraction conditions to obtain a crude extract with enhanced bioactive components. Three variables were examined including methanol concentration, extraction time and ultrasound frequency. The optimum yields of extract, TFC and TPC were (3.22 ± 0.049 g/10 g of dry plant), (17.03 ± 0.060 mg RE/g) and (10.96±0.020 mg GAE/g) respectively. The optimal extraction conditions contributed to these values of experiment 2 and 3 were 70% methanol, 10 min and 40 KHz and 70% methanol, 15 min and 40 KHz respectively. The lowest IC_{50} values of optimized methanolic extracts of Iraqi *Lepidium sativum* aerial parts against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals were 31.84 µg/mL for TFC and 35.85 µg/mL for TPC . For the first time, the study provided data about the phenolic and flavonoid contents of the Iraqi plant and optimized conditions for extraction by UAE technique using single factor experiment. The plant can be acknowledged as a potential nutraceutical or functional food rich in antioxidants to combat many diseases.

Keywords

Lepidium sativum; garden cress; Ultrasound Assisted Extraction; single factor experiment; flavonoids; antioxidant activity

Introduction

Lepidium sativum L. is a fast growing plant that belongs to the family Brassicaceae. It is known by various names in different regions of the world (1). In Iraq, it is known as Rishad. Garden cress was first cultivated in Egypt and south west Asia, including Iraq and has since spread extensively throughout the world (2). *Lepidium sativum* an erect, branched, pubescent herb, with up to 60 cm in height. The upper leaves are typically entire, 2-3 cm long, oblanceolate and sessile with the bisexual white flower petals. Each siliquae possesses 2 seeds and is circularly compressed and pale green. The seeds are small, oval-shaped, silky and reddish-brown in color. People use the plant in various forms, such as in salads or as sprouts and the seed oil extract is used as a condiment (3).

Generally, food functions in providing the body with essential nutrients and to quench appetite while, consumable plants contain different biologically active components that take part in preventing and treating different illnesses (4). This species contains various classes of phytochemicals, a potent nutraceutical potential and multiple bioactivities (5). Garden cress has traditionally been used to treat asthma, uterine cancer, ulcers, hemorrhoids' bleeding, coughing, incisions, skin fungus infections, menstrual pain, back pain and polyps of the nose (6).

Chemically, garden cress contains flavonoids, phenols, coumarins, cardiac glycosides, alkaloids, amino acids and proteins (7). Phytochemical profile of Iraqi *L. sativum* ethanolic leaf extract revealed d-Proline, n,n-Dimethylaminoethanol, Butyrolactone, Benzyl nitrile, 2-Hydroxy-1-(1′-pyrrolidiyl)-1-buten-3-one (8).

Plants contain high concentrations of phytochemicals that fight free radicals and microbes and serve as nutrients that can be utilized to cure and avert a wide range of human diseases. (9). It has been reported in literature that antioxidants from natural source have greater benefits compared to synthetic source (10). Side effects of plant derived antioxidants are limited, while genotoxic effects may result from synthetically derived antioxidants (10).

Investigating the Iraqi edible plant *Lepidium sativum* for these phytochemicals is of interest. For the successful extraction of these bioactive components, new extraction methods such as ultrasonic-assisted extraction are recommended. In comparison to conventional extraction techniques, ultrasonic-assisted extraction (UAE) are not harmful to the environment because they require only a small quantity of extraction solvent, save time, low labor cost and permit extraction with lower temperatures maintaining high quality extracts (11). These advantages lead to a considerable interest to use this technique for extraction. Since experimental factors of UAE are distinct for similar phytochemicals from different plant varieties (12), it necessitates the optimization of these factors for particular phytochemicals intended to be extracted from specific plant.

Despite of few investigational studies concerning the Iraqi plant species, literature search has not documented the evaluation of phenolic contents and antioxidant potential of the plant's aerial parts. The objective of the study is the evaluation of flavonoid and phenolic contents, antioxidant potential and the optimization of the extraction conditions of phenolic compounds using ultrasound-assisted extraction (UAE) (12).

Materials and Methods

Chemicals

Methanol (Scharlan, Spain), petroleum ether (Alpha Chemika, India), sodium hydroxide (Applichem- Gmbh, Germany) and sodium carbonate (Thomas Baker, India), Folin-Ciocalteu's (Sigma, Germany) and AlCl3 (LaboratoryReagent/India).

Plant material

Iraqi Garden cress was purchased in March, 2023 from Baghdad's local vegetable shops and authenticated at the College of Science, University of Baghdad. The plant was washed to eliminate impurities and dirt. Subsequently, the aerial parts were dried indoors for period of 2 weeks then grinded, weighted and stored until extraction.

Extraction of plant constituents

One hundred and fifty grams of powdered plant material macerated in 750 mL of petroleum ether for 2 days at room temperature with intermittent agitation for defatting. This step was repeated thrice. The extract was filtered and plant left to dry to remove solvent residue. Thereafter the dried plant subjected to Ultrasonic-assisted extraction (UAE) using a probe ultrasonicator (Q sonicator LLC/USA) and methanol as extraction solvent. The UAE was conducted with different experimental conditions (13).

Single Factor Experimental Design

Using a single-factor experiment (13), the effect of each variable on the extraction of garden cress was evaluated. In each experiment, one variable is altered while the remaining variables kept constant. Three variables were used including extraction solvent concentrations (methanol 50%, 70% and 90%) (14), extraction durations (5, 10 and 15 min) and ultrasound frequencies (20, 40 and 60 kHz) while the solvent/plant material ratio (10:1) and temperature (25 °C) remained constant during all experiments. Using a probe ultrasonicator, plant material exposed directly to ultrasonic waves and the crude methanolic extracts were strained and dried at 40 ˚C in a rotary evaporator under vacuum. The dried filtrates kept in refrigerator at 4 °C until further investigation.

Preliminary phytochemical evaluation of crude extracts

Using the following chemical assays (15, 16), phenolics and flavonoids were identified in dried methanolic crude extracts.

Test for Flavonoids: Addition of few drops of NaOH solution to 1 mL of crude methanolic extract, appearance of yellow color refers to the presence of flavonoids.

Test for Phenolics: After dissolving 1 mL of crude methanolic extract in 1 mL of 5% ferric chloride, appearance of dark green to black color indicate the presence of phenolics.

Estimation of the Total Flavonoid Content (TFC) of each experimental condition

The total flavonoids content (TFC) was assessed in accordance with a protocol (15). Rutin standard solutions with the concentrations (0.156, 0.312, 0.625 and 1.25 mg/mL) were prepared to establish the Rutin standard curve. To prepare the samples, 1 mg of every extract dissolved in 10 mL of D.W. In separate tubes, 4 mL of D.W and 0.3 mL of 5% $NaNO_2$ were added to 1 mL of each extract and each standard solution. After 5 min, 0.3 mL of the 10% of aluminum chloride was incorporated into all tubes and left to stand for 5 min. Finally, 2 mL of 1M sodium hydroxide added and left to stand for 30 min at room temperature. The absorbance was measured at 510 nm against blank solution using UV-VIS spectrophotometer. The standard curve for Rutin was generated by plotting each concentration versus its corresponding absorption. The TFC was demonstrated as (mg RE/g) of plant material.

Estimation of the Total Phenolic Content (TPC) of each experimental condition

Folin – Ciocalteu assay with slight modification was used to quantify Total Phenolic Content (TPC) (16). To construct a standard curve, serial dilutions of gallic acid standard were prepared with concentrations of 30, 40, 60 and 80 g/mL. For preparing the samples, 1 mg of each extract was mixed in 10 mL of D.W. In separate tubes, 1 mL of the Folin-Ciocalteu reagent was added to 1 mL of each standard solution and each extract. After 5 min, 5 mL of water was added and mixed, then 1 mL of 10% Na_2CO_3 added and incubated in a dark place for 60 min at ambient temperature. Then, 760 nm was used to measure the absorbance against a blank. Total Phenolic Content was calculated as (mg GA / g) of dried plant.

Antioxidant Activity Evaluation using (DPPH) Reagents

To study the power of obtained extracts to scavenge DPPH free radicals, a standard method with min modification was used (17). The concentration of the stock solution of extract was 1 mg/mL prepared in methanol. Serial dilutions were prepared 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.99 and 0.97 g/mL. One mL of DPPH methanolic solution (1 mg/mL) added to one mL of each diluted solution. The absorbance was then measured at 517 nm after incubation period of 30 min in dark place at 25 °C. The control contains all reagents excluding the extract. The percent inhibition calculated applying (Eqn.1) and the half maximal inhibitory concentrations (IC_{50}) were calculated by plotting the inhibition (%) against Log (concentrations). All values were compared to vitamin C (Ascorbic acid) standard.

$$\text{Inhibition } (\%) = (A_0 - A_1/A_0) \times 100 \quad \text{……………………....(Eqn.1)}$$

A_0: DPPH solution absorbance, A_1: sample absorbance

Analytical Statistics

All experiments were done in triplicates and presented as mean± standard deviation. The Statistical Analysis System - SAS (Statistical. Version 9.6th ed., 2018) program was used to detect the effect of different factors on study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significantly compare between means in this study. $p \leq 0.01$ considered significant. IC_{50} values were calculated by non-linear curve fitting. One way ANOVA and Tukey's multiple comparisons test used to determine the extraction efficiency on TFC, TPC and radical scavenging activity (RSA). A p- value less than (0.05) regarded as statistically significant. Calculations were performed by Graph Pad prism 8.

Results and Discussion

Analysis of a single factor experiment

Using single factor experimental analysis, the parameters of UAE of the current study were optimized.

Impact of Extraction Time

Time of extraction is a crucial variable that can impact the extraction efficiency. Evaluating the effect of time on extraction efficiency of studied plant parts, experiments were conducted over periods of 5, 10 and 15 min with constant parameters of 70% methanol, 40 kHz ultrasound frequency and 10:1 solvent/solid ratio. The optimal time that showed the highest extract yield (3.22± 0.03 g /10 g) was 10 min, but further increase in extraction time to 15 min, the yield decreased to (2.63± 0.03 g / 10 g) of dry plant (Table 1 and Fig. 1). The yield was higher in comparison to a study (18) that showed methanolic extract yield (20.50%) from *L. sativum* leaves obtained by soxhlet extraction method. Additionally, the highest levels of TFC and TPC were obtained by optimal conditions of experiments 2 and 3 respectively. Determination of TFC was based on the linearity of the Rutin calibration curve ($y = 0.7907x + 0.052$) and the regression coefficient ($R^2 = 0.9854$) (Fig. 2). The highest level of TFC was (17.03 ± 0.06 mg/g) as Rutin equivalent of dry plant material (Table 1). On the other hand, the TPC was increased to (10.96± 0.02 mg GAE/g) when extraction time extended to 15 min (Table 1). The TPC of the methanolic extracts were calculated depending on linearity of Gallic acid calibration curve ($y = 0.0052x + 0.02$) and regression coefficient ($R^2 = 0.9812$) (Fig. 3). The selected parameters significantly enhanced TFC whereas no significant effect was shown on TPC except for experiment 3. This

Table 1. Experimental conditions of UAE, Yield, TFC and TPC in extracts obtained by UAE

Exp. No	Experimental conditions			Yield (g/10g)	TFC (mg RE/g)	TPC (mg GAE/g)
	Time (min)	Solvent (%)	Frequency (%)			
1 - 5		70	40	2.55 ± 0.02	8.80 ± 0.104	6.67 ± 0.072
2	10	70	40	3.22 ± 0.03	17.03 ± 0.06	8.13 ± 0.06
3	15	70	40	2.63 ± 0.03	9.61 ± 0.01	10.96±0.02
4	10	50	40	2.91 ± 0.01	11.19 ± 0.04	8.27 ± 0.04
5	10	90	40	2.88 ± 0.02	16.36 ± 0.01	7.96 ± 0.07
6	10	70	20	2.72 ± 0.06	9.23 ± 0.01	6.65 ± 0.07
7	10	70	60	2.66 ± 0.03	9.80 ± 0.05	6.33 ± 0.03

may suggest that only the time factor had an effect on TPC yield.

The increase in extraction time prolonged the contact of bioactive compounds with the extraction solvent and hence, increases their solubility. Regarding the phe-

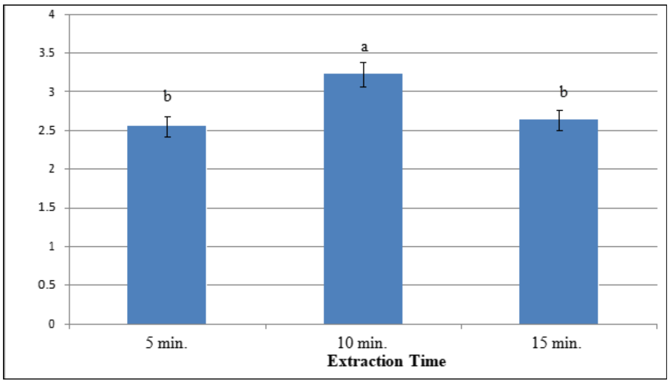

Fig. 1. The impact of extraction time on the extraction efficiency (yield) of the Iraqi garden cress aerial parts extracts.

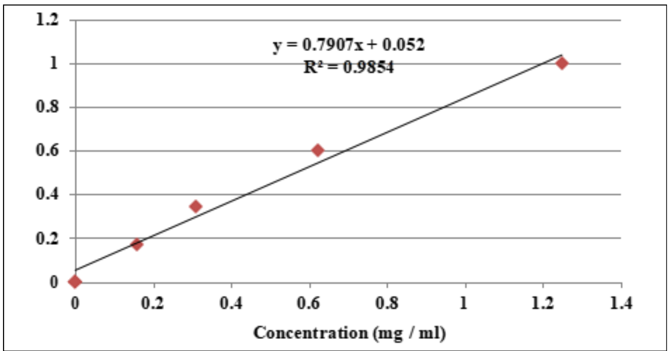

Fig. 2. Calibration curve of Rutin standard to determine TFC of aerial parts of Iraqi garden cress extract obtained by different experiments of UAE.

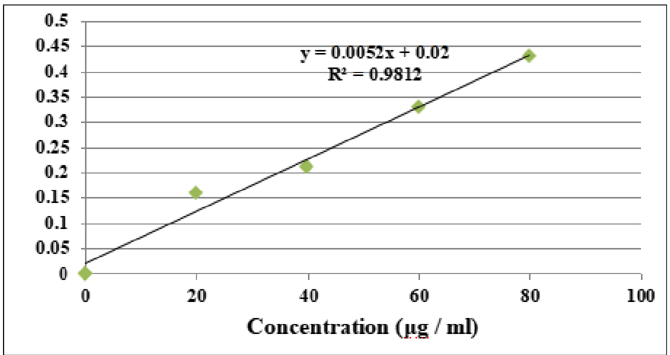

Fig. 3. Calibration curve of Gallic acid standard for determination of TPC of aerial parts of Iraqi garden cress extracts obtained by different experiments using UAE.

nolic and flavonoid contents, literature search showed few studies of *L. sativum* aerial parts. The current study showed higher recovery of TFC than TPC in contrast to studies by (18, 19) that revealed higher levels of TPC rather than TFC acquired by conventional extraction methods. The TPC and TFC yields of the present study were 4.6 and 10.6 times higher than (19) that revealed TPC (2.36±0.10 mg GAE/g and TFC (1.61± 0.007 mg QE/g) respectively and 1.1 and 11.1 times higher than (19) that showed (9.93 mg GAE/g and 1.53 mg QE/g) respectively. However, TPC level was lower than (20) that showed (184.14 ± 2.5 µg GAE/mg whereas TFC of present study was 1.3 times higher than one report (20) that showed TFC of (12.63±1.5 µg QE/mg). The differences in phenolic and flavonoid contents of *L. sativum* between the aforementioned studies and the current study may be influenced by factors like method of extraction, variation in genotype of *L. sativum*, environmental conditions for cultivation and partly of its sporophytic self-incompatibility system (21). Moreover, the TPC yield of the present study was compared to Ratananikom (13), that showed significant increase in yields of TPC and TFC of Dill extract when UAE time was extended to 30 min without oxidizing phenolic and flavonoid content, whereas the current study showed an increment in TPC and a decline in TFC when time extended to 15 min. Other relevant studies were consistent with present study that showed decrease in TFC when UAE time extended from 40 to 60 min that lead to oxidation and destruction of active compounds (22, 23). However, a study by (24) showed an increase in period of extraction did not significantly affected the extraction of balsam content, TPC and TFC of propolis that is inconsistent with the current study regarding TPC. Other studies revealed longer time of UAE was required for extraction of phenolics from different matrices compared to shorter optimal time of the current study. For instance, investigations are on UAE of antioxidants from grape pomace to study the influence of different parameters (25). Concerning the time of extraction, the investigators observed that the optimal conditions suggested a 25 min sonication. Reports are on the optimized the extraction parameters of Ultrasound Assisted Extraction of phenolic compounds and antioxidants obtained from grape seeds and results showed time of extraction was 29.03 min and 30.58 min respectively (26). It was recognized that extraction of phenolic constituents from various matrices manifested extended extraction time compared to the studied plant aerial parts.

Impact of extraction solvent concentrations

In this experiment, flavonoids and phenolic compounds were extracted from plant material using a solvent mixture of methanol and water. To examine the impact of methanol/water concentrations on extraction yield, different concentrations of aqueous methanol solutions were used with other parameters held constant. Table 1 and Fig. 4 illustrates the solvent effect on extraction efficacy. The 70% methanol was deemed to be the optimal solvent for efficient extraction with highest phenolic and flavonoid content. It was notable that the extract yield decreased to 2.88± 0.02 g when the concentration was increased to 90% methanol.

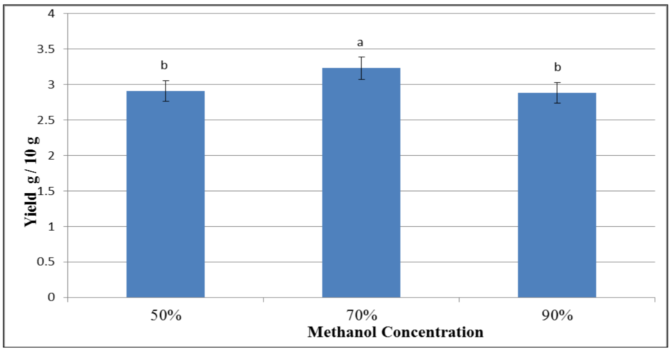

Fig. 4. The impact of extraction solvent on the extraction efficiency (yield) of the Iraqi Garden cress aerial parts extracts.

The phenolic and flavonoid content were higher in the present study compared to that used 80% methanol to extract phenolics from aerial parts of *L. sativum* by conventional method with highest TPC and TFC values of (2.36 mg GAE/g extract) and (1.61 mg QE/g extract) respectively (19). While, the effect of solvent concentration used in UAE of the present study agreed with (13) in which both studies showed an increase in phenolic and flavonoid contents when alcohol concentration increased to 70%. The result of the current study was also consistent with other studies that revealed the effect of methanol of various concentrations on the extraction of phenolics and flavonoids (27-30). Also, Corona-Jiménez (31) demonstrated the impact of solvents' polarities in UAE technique and found higher TPC obtained from chia seeds using methanol. While, the present study was inconsistent that demonstrated the optimal conditions for UAE using lower concentration of ethanol (50%), a 60% amplitude and 20 min sonication time contributed to greater phenolics and flavonoid contents and antioxidant capacities from mango residue (32). The solvent polarity is directly affected by the methanol concentration, while penetration of ultrasound relies on solvent's dielectric constant and improved with the amount of water in aqueous methanol as reported by (33).

Impact of Ultrasound Frequencies

The ultrasound frequency may be a crucial factor that influences the extraction efficiency and it is absent in conventional methods. The investigation revealed that an increase in the ultrasonic frequency from 20 to 40 kHz, the extract yield increased from 2.72± 0.06 g to 3.22± 0.03 g, indicating an increase in power of extraction. Further increase in ultrasonic frequency to 60 kHz, decreased extract yield to 2.66± 0.03 g (Table 1) and (Fig. 5). The probable cause for this phenomenon is that the intensity of cavitation in solution becomes stronger when frequency increases, and hence the time of cavitation is prolonged leading to release of target constituents. However, further increase in ultrasound frequency, the cavitation intensity would become weaker due to the reduction in cavitation bubble size, resulting in lower yield of target constituents (34). The influence of ultrasonic frequency on extraction of the present study is consistent with a study that showed an increase in ultrasound frequency from 18 to 54 kHz, increased the extraction yield of anthocyanin (34). It was noted that ultrasonic frequency significantly elevated the yield of the amount of proteins and polyphenols acquired from shoots of vine (35) and a like observation was noticed in polyphenols and anthocyanins obtained from grape pomace (36) and eggplant peel (37). Whereas, the results were inconsistent with (24) that showed an increase in amplitude from 20-100%, the TPC elevated with 17.5%, the content of balsam elevated with 23.3% and TFC elevated with 29.1% respectively.

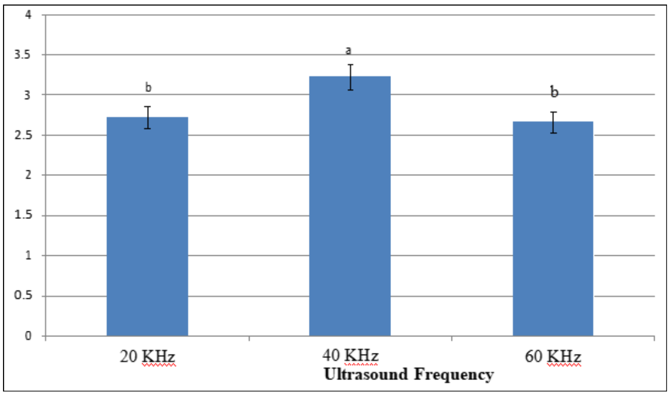

Fig. 5. The effect of ultrasound frequency on the extraction efficiency (yield) of the Iraqi garden crest aerial parts extracts.

Preliminary phytochemical evaluation of crude extract

The aqueous methanolic extracts obtained from various experiments were subjected to simple, quick and economical phytochemical screening assays before the evaluation of TFC and TPC. The appearance of yellow and dark green to black color indicated the presence of flavonoids and phenolics respectively.

Evaluation of Antioxidant Activity using DPPH Assay

The potential of each extract to get rid of free radicals was evaluated using the DPPH assay. The antioxidant activities of extracts were demonstrated as percentage of inhibition (%) and IC_{50} values (µg/mL) (Table 2) and (Fig. 6). The study showed highly significant ($p \leq 0.0001$) enhancement of RSA by extraction conditions. Experiment 2 exhibited the greatest RSA (91.2%) with half-maximal concentration of (31.84 µg/mL) whereas, extract obtained by experiment 1 possessed the least neutralizing capacity (59%) with concentration (52.93 µg/mL) compared to vitamin C standard (29.97 µg/mL). The extract of experiment 2 manifested stronger antioxidant activity (lower IC_{50}) than that showed IC_{50} values of (126.43 ± 0.14 µg/mL) and (149.541µg/mL) for methanolic and ethanolic leaves extract of Iraqi *L. sativum* respectively (9, 18). Whereas, the studies revealed that the IC_{50} value of methanolic extract of *L. sativum* seeds was (62 µg/mL) (38) and showed IC_{50} (318.91 ppm) for the Turkish *L. sativum* seed extract (39). The study noted highly significant effects ($p < 0.0001$) of both TFC and TPC on antioxidant activities. The enhanced antioxidant activities of the present study may be due to the high quality of extracts obtained by optimized UAE conditions compared to seed extracts of the aforementioned studies obtained by conventional extraction methods.

Table 2. Log (IC_{50}), IC_{50}, RSA (%) and R^2 of Ascorbic acid (Vitamin C) and extracts obtained by different UAE experimental conditions

	Vit C	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Exp 6	Exp 7
Log IC_{50}	1.477	1.724	1.503	1.554	1.638	1.577	1.752	1.640
IC_{50}(µg/ mL)	29.97	52.93	31.84	35.85	43.48	37.76	56.47	43.61
RSA (%)	96.3	59.1	91.2	80.4	76.5	85.6	67.2	70.3
R^2	0.9701	0.9959	0.9949	0.9970	0.9977	0.9958	0.9866	0.9854

Exp.= Experiment; Radical Scavenging Activity= RSA; Regression Coefficient =R^2

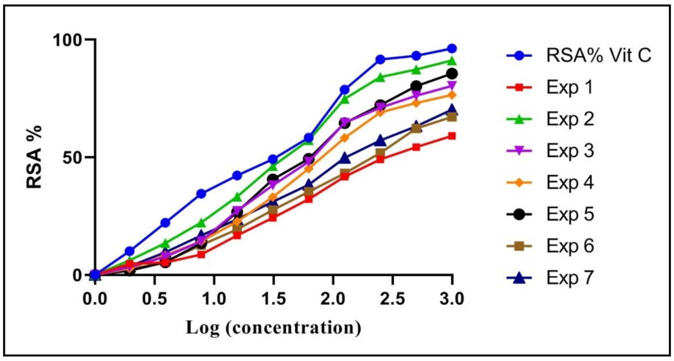

Fig. 6. Percentage of Radical scavenging activity of investigated extracts obtained from different UAE conditions against Log (concentration) values.

It has been reported that aerial parts of Egyptian *L. sativum* contained flavonols (Quercetin and Kaempferol) and flavones (Apigenin and Luteolin) (41). Although, further analysis of the Iraqi *L. sativum* extracts needed to reveal their constituents, the maximum antioxidant activity of optimized extract may correlate to the presence of these compounds.

Conclusion

For the first time, the study provided data about the phenolic and flavonoid contents of the Iraqi *L. sativum* aerial parts and optimized conditions for extraction by UAE technique using single factor experiment. The optimal extraction conditions by UAE were determined for TFC and TPC. The conditions of all experiments represented by extract yield significantly influenced the TFC yield but not TPC except for experiment 3. Moreover, the study found that the optimal yields were higher than those obtained by conventional extraction methods of previous studies, indicating the efficiency of UAE method. It was realized that the TFC was higher than TPC by UAE method, whereas contrary results were seen with conventional methods, designating different extraction behaviour by UAE toward phenolic compounds and matrices. The IC_{50} was determined and optimal extract was the strongest antioxidant. The Iraqi plant has demonstrated a strong antioxidant activity with a good source of phenolic and flavonoid contents and can be acknowledged as a potential nutraceutical or functional food rich in antioxidants to combat many diseases and recommended to be included regularly in daily meals.

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Authors contributions

The author contributed to all the work, starting from experimental design, analysis, interpretation of data and writing the whole manuscript.

Compliance with ethical standards

Conflict of interest: The author declares that they have no competing interests.

Ethical issues: None.

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