

Phenolic Acids Profile and Antioxidant Activity of Lettuce Seeds (*lactuca sativa*)

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Abstract This investigation attempted to study the antioxidant potential and phenolic acids profile in lettuce seeds (LS), the possibility of applying LS in pharmaceutical and food industry was also in focus. The antioxidant potential of LS extracts was determined. The total phenol content of LS extracts was 775.47 ± 118.58 mg GAE/100g whereas; the total flavonoid content was 170.83 ± 13.56 mg QE/100g. LS extracts showed a considerable scavenging activity against 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) as compared with standard ascorbic acid. The standard ascorbic acid showed an IC_{50} of $12.32\mu g$ against DPPH, whereas the calculated IC_{50} for LS extract required to scavenge DPPH was 1.74 ± 0.11 mg. Phenolic acids in LS extracts were determined by using high performance liquid chromatography (HPLC). Gallic, chlorogenic, vanillic, caffeic, and coumaric acids were identified and quantified in LS extracts at a concentration of 20.30 ± 1.04 , 1327.45 ± 45.67 , 119.02 ± 4.88 , 272.33 ± 12.77 , and 912.15 ± 36.64 mg/100g respectively. Based on the obtained findings from the current research, lettuce seeds could have a potential for application in the pharmaceuticals and functional food industry.

Keywords: Lettuce seeds (LS), Phenolic acids, High performance liquid chromatography (HPLC), Total phenolics, Total flavonoid, Scavenging activity

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1. Introduction

The health benefits of lettuce are reported by several investigators [1,2,3]. The anti-inflammatory activity of lettuce extract has been attributed to the presence of phenolic compounds [4]. Quercetin is reported to present at high concentration in green lettuce leaf, and this could explain the anti-inflammatory and antioxidant potential of lettuce leaf extract [5]. Supplementation of high - cholesterol diet with 8% of red lettuce resulted in a significant decrease of plasma total cholesterol and low-density lipoprotein cholesterol in mice [6]. A randomized double-blind placebo controlled pilot study demonstrated that hyperlipidemic patients treated with lettuce seed extract capsule (1000mg once per day) augmented the effect of atorvastatin and showed a significant decrease total cholesterol, low-density lipoprotein cholesterol, and triglycerides [7]. Obese hyperglycemic mice supplemented with a red leaf lettuce at a dose of 100 or 300 mg/Kg body weight showed attenuation of hyperglycemia and improved sensitivity against insulin [8]. The authors attributed the hypoglycemic effect to the high polyphenol content in lettuce. Some studies reported the protective effect of lettuce against colorectal and lung cancers and referred to

an association between lettuce consumption and incidence of these cancers [9,10].

Among a 31 lettuce varieties studied, the highest reported content of total phenols was 820.28 mg chlorogenic acid equivalent /100 g dry matter [11]. Another investigation reported that the methanolic extract of *Lactuca sativa* has a total phenolic content of 253.31 mg catechin equivalent/g extract [12]. The antibacterial activity against Gram positive and Gram negative bacteria, and antiviral activity against Cox B-3 and HCMV viruses of the methanolic extract of *Lactuca sativa* has also been reported [12].

The antioxidant contents of green and red leaf lettuce cultured in hydroponic systems under greenhouse, and conventional soil culture were studied and concluded that red lettuce recorded the highest content of total phenol (680.2 ± 69.3 mg gallic acid equivalent /100g), whereas green lettuce exhibited the highest effect against DPPH [13]. Caffeic, chlorogenic acids and their derivatives are the major phenolic acids found in lettuce [14,15]. Another report also referred to the presence of caffeic acid as an active component in *Lactuca sativa* [16].

The current investigation attempts to study the antioxidant potential and phenolic acids profile in lettuce seeds. The possible use of lettuce seeds in pharmaceutical and food industries was also in focus.

2. Materials and Methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Methanol and water were of HPLC grade. Other chemicals were of analytical grade and obtained from BDH (Poole, UK).

2.2. Plant Material

Lettuce seeds were purchased from agricultural company, Riyadh. Specimen was deposited at the herbarium, department of botany and microbiology, college of science, King Saud University. Lettuce seeds were powdered by using a regular miller.

2.3.1. Extraction Protocol for Antioxidant Determination

Lettuce seeds (LS) powder, 2g were extracted with 25mL solution of methanol: water (4:1v/v) and kept on a rotary shaker with gentle agitation for 24h. After 24h, extract was centrifuged at 5000 rpm for 10 min. Supernatants were collected and residues were extracted with the same solution for 24h. Supernatants were pooled; solvent evaporation was carried out using rotary evaporator under vacuum (Buchi, Switzerland). After solvent evaporation, residue of extract was dissolved in distilled water, final volume was recorded and a serial dilutions of 100, 500, and 2000 were performed.

2.3.2. Extraction Protocol for Phenolic Acids Determination

Lettuce seeds powder, 2g were weighed, and the lipid material was removed by adding 20 ml of hexane for 1h. Defatting was performed twice, then hexane layers were discarded, and 20 ml of 75% methanol was added to the residue and kept at 23°C with stirring for 2h. The suspension was centrifuged at 5000 rpm for 10 min. The precipitates were collected, and phenolic acids were extracted with 2M sodium hydroxide and left at 23°C with stirring for 4h. Suspension was acidified with 6 M HCl to pH 2.2. Phenolic acids were extracted twice using a diethyl ether and ethyl acetate (1:1 v/v) solution [17]. The organic layers (upper layers) were extracted using a separating funnel. The pooled extracts were collected, and the solvent was evaporated using a rotary evaporator under vacuum. After complete evaporation, the residue was dissolved in methanol: water (4:1v/v), and the final volume of the extract was recorded. The phenolic acids in LS extract were subjected to analysis by HPLC.

2.4. Determination of Antioxidants in LS Extracts

2.4.1. Determination of Total Phenol Content

Total phenolic content was determined by Folin-Ciocalteu's reagent method [18]. 0.5 mL of LS extract was mixed with 0.1 mL Folin-Ciocalteu's reagent and the mixture was incubated at room temperature for 15 min. Then 2.5 mL saturated sodium carbonate (7.5%) solution was added and further incubated for 30 min at room

temperature and the absorbance was measured at 760 nm. Gallic acid (GA) in a range of 0-50µg was used as standard. Total phenol values were expressed as GA equivalent (GAE), mg GAE/100g dry weight.

2.4.2. Determination of Total Flavonoid Content

Total flavonoid content was determined by Aluminum chloride method [19]. The reaction mixture of 3.0 mL consisted of 1.0 mL of extract, 1.0 mL of ethanol, 0.5 mL of aluminum chloride (1.2%) and 0.5 mL potassium acetate (120 mM), solutions were incubated at room temperature for 30 min. Absorbance was measured at 415 nm. in absolute ethanol and a range between 0-50µg was used as standard. Total flavonoid was expressed as quercetin equivalent (QE), mg QE/100g dry weight.

2.4.3. 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) free Radical Scavenging Activity

The activity of LS extracts against DPPH was estimated as follows, 3.0 mL reaction mixture composed of 1.0 mL of DPPH in methanol (0.3 mM), 1.0 mL of LS extract and 1.0 mL of methanol was incubated in dark for 10 min, the absorbance was read at 517 nm [20]. Ascorbic acid in a range between 2-40µg was used as standard. The percentage of inhibition and the IC_{50s} were calculated.

2.5. High-performance Liquid Chromatography

Analyses were performed using the Jasco LC-4000 high-performance liquid chromatography system, consisting of AS-4050 auto-sampler, two PU-4180 chromatographic pumps, CO-4061 column oven, and MD-4010 PDA detector. The stationary phase was a 5µm (4.6x150mm) Venusil XBP C18 column (Agela Technologies, Wilmington, USA), and the mobile phase was water: methanol: acetic acid at 90:20:5 (v/v/v). LS extract injection volume varied from 0.1-4.0 µL and isocratic runs were performed. Phenolic acid standards including gallic acid (GA), chlorogenic acid (CGA), hydroxyl benzoic acid (HBA), vanillic acid (V), caffeic acid (CF), and coumaric acid (CO) were separately prepared in the range between 50-300 ng/µL for calibration curves. UV intensity was monitored at 320 for CF and CGA; at 300 nm for CO; at 254 nm for GA, and V; at 280nm for HBA. Data processing was performed using the Jasco ChromNAV version 2.0 software.

2.6. Statistical Analysis

All experiments were performed in triplicate. The results are presented as mean and standard deviation, which were calculated using the MS Excel 2010 program.

3. Results and Discussion

The calibration curves of gallic acid and quercetin for the quantification of total phenol and total flavonoid contents are shown in Figure 1. The total phenol content of LS extracts was 775.47 ± 118.58 mg GAE/100g (Figure 2). Six lettuce cultivars were studied for their antioxidants

and reported to have a total phenolics ranged from 8.4 to 12.9 mg catechin equivalents /g [21]. The antioxidant contents of green and red leaf lettuce cultured in hydroponic systems under greenhouse, and conventional soil culture was studied and concluded that red lettuce recorded the highest content of total phenol of 680.2 ± 69.3 mg GAE /100g [13]. The reported total phenolic content of baby red leaf lettuce was 1.4-1.7 mg/g [1]. The total flavonoid content of LS extract was 170.83 ± 13.56 mg QE/100g (Figure 2). Lettuce seeds under salt stress with 100 mM NaCl resulted in a significant increase of total flavonoid content in lettuce leaves that reached 300mg catechin equivalents/100g [22].

Lettuce seed extract showed a considerable scavenging activity against DPPH as compared with standard ascorbic acid. The standard ascorbic acid showed an IC_{50} of $12.32 \mu\text{g}$ against DPPH, whereas the calculated IC_{50} for LS extract required to scavenge DPPH was 1.74 ± 0.11 mg (Figure 3). Extracts from five lettuce varieties were studied for their anti-scavenging activity against DPPH; the IC_{50} s were ranged between 303.56 to $4485.41 \mu\text{g/ml}$ [23] which is comparable to our obtained findings.

Correlation coefficients, retention times, and the slopes of the calibration curves of gallic, chlorogenic, vanillic, caffeic, and coumaric acids are shown in Table 1. The chromatographic patterns of LS extracts at different wavelengths for quantification of, chlorogenic, caffeic, vanillic, coumaric, and gallic acids are shown in Figure 4- Figure 8 respectively.

Our findings showed that LS extract has a very high content of chlorogenic acid that reached 1327.45 ± 45.67 mg/100g (Figure 4). Chlorogenic acid reported to inhibit metastasis, induce apoptosis, and improve antitumor immunity in breast cancer [24]. Two reports concluded the effectiveness of chlorogenic acid in the treatment of colon cancer, one report concluded that chlorogenic acid decreases the expression of the miR.31 oncogene [25], while the other report stated that chlorogenic acid suppressed the progression of colon cancer via the RTK-MEK-ERK and NF- κ B pathways [26]. A dose composed of

chlorogenic acid at a concentration of 5mg/kg body weight and tetrahydrocurcumin at a concentration of 80 mg/kg body weight exhibited potential antidiabetic effect in streptozotocin- nicotinamide induced diabetic rats [27,28].

Caffeic acid is also detected in LS extracts at a high concentration of 272.33 ± 12.77 mg/100g (Figure 5). Italian head lettuce has chlorogenic acid content ranges from 15.93 ± 1.59 to 22.24 ± 7.74 mg/100 g, and caffeic acid from 3.87 ± 0.43 to 7.59 ± 2.66 mg/100 g [29]. Caffeic acid derivatives are associated with the reduction of plasma cholesterol and the inhibition of LDL oxidation, also caffeic acid is reported to inhibit the growth of cancer cells and to induce tumor cell apoptosis [30]. 3,5-dicaffeoylquinic acid, a caffeic acid derivative has been reported to attenuate diabetes by inhibiting α -glucosidase and increase of plasma insulin level [31].

LS extracts also showed a vanillic acid concentration of 119.02 ± 4.88 mg/100g (Figure 6, Table 2). The antibacterial effect of vanillic acid has been reported [32,33]. Injection of vanillic acid at concentration of 10mg/kg reported to suppress concanavalin A-induced liver injury in mice by decreasing serum cytokines levels [34].

Surprisingly and interestingly coumaric acid was found at a high concentration of 912.15mg/100g (Figure 7) in the methanol extract which is usually liberates free phenolic compounds. Coumaric acid has been reported to have a significant effect on glucose and lipid metabolism, and improve metabolic disorders through GLUT2 activation [35]. The methanolic extract of LS as a rich source of coumaric acid could have a potential use in pharmaceutical and food industry [35]. A randomized double-blind placebo controlled pilot study attributed the hypolipidemic effect of lettuce seed extract on hyperlipidemic patients to desoxylactucin, lactucin-15-oxalate, lactucopicrotin, lactucopicrotin-15-oxalate, and lactucin as major antioxidants in LS extract [7], however, the findings of our study could explain the hypolipidemic effect exerted by lettuce seed (LS) due to the presence of chlorogenic, coumaric, caffeic, and vanillic acids in LS extract in considerable concentrations.

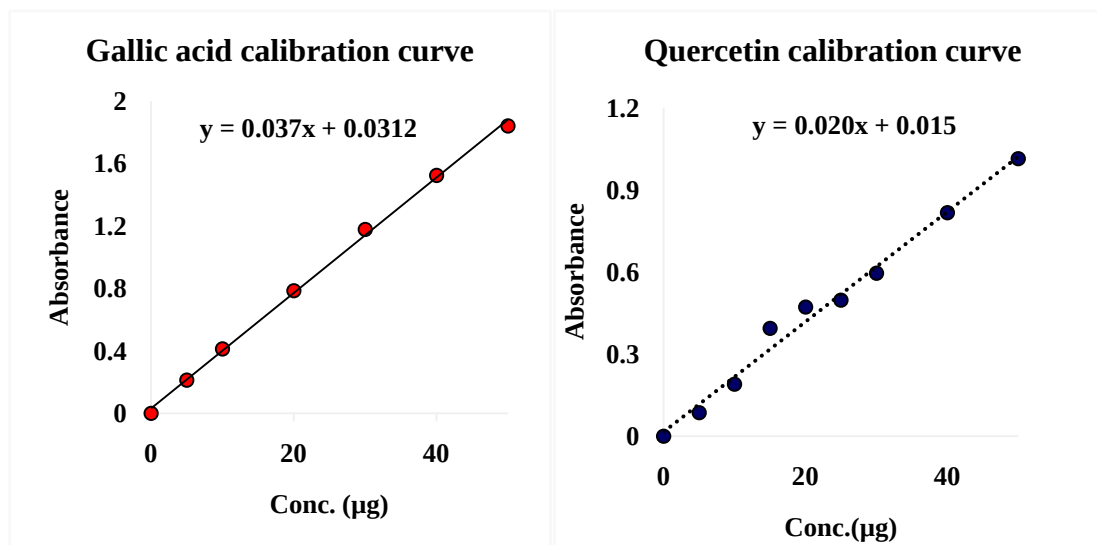


Figure 1. Calibration curves of Gallic acid and Quercetin

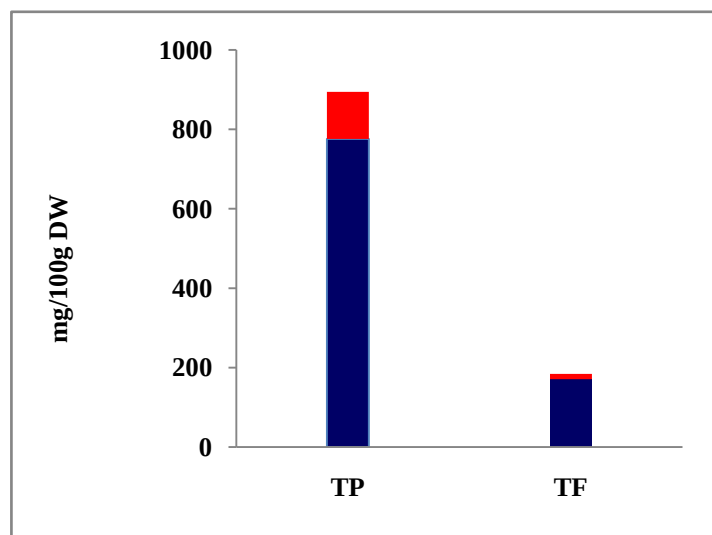


Figure 2. Total phenolics, TP (mg GAE/100g DW), and total flavonoid, TF (mg QE/100g DW) in lettuce seeds (LS) extracts. Data are expressed as mean $\pm$ SD for three samples

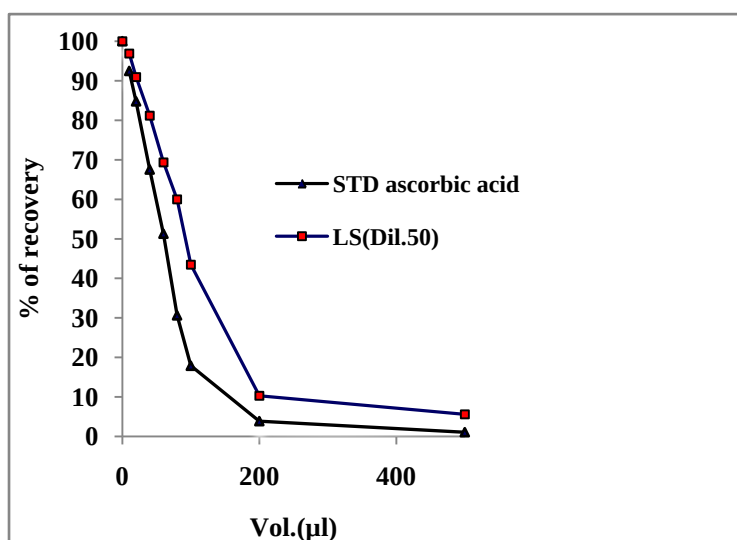


Figure 3. Lettuce seeds (LS) extract scavenging activity against DPPH. Data shown are means of three different extract preparations

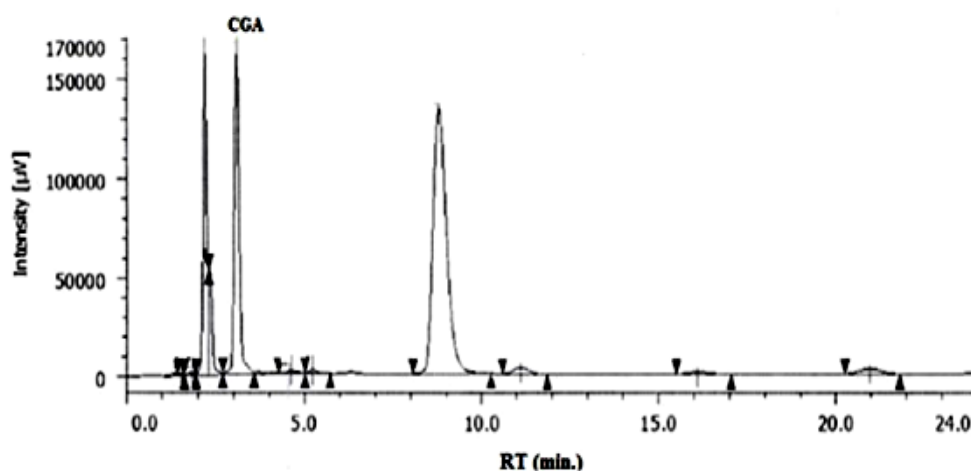


Figure 4. Chromatogram of 0.1 μ l of LS extract measured at 320nm for chlorogenic acid (CGA) determination

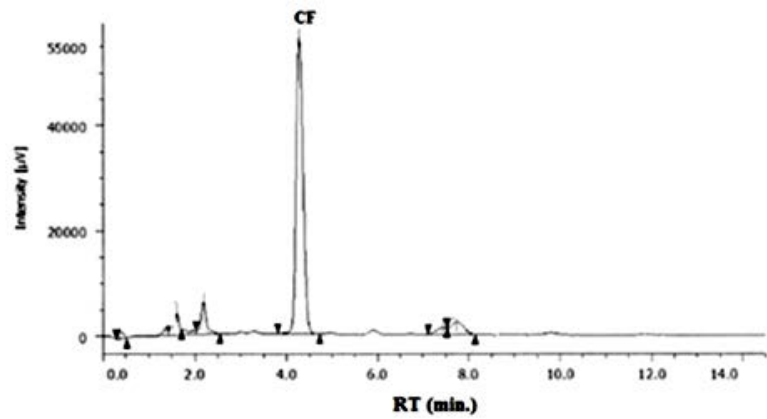


Figure 5. Chromatogram of 0.1µl of LS extract measured at 320nm for caffeic acid (CF) determination

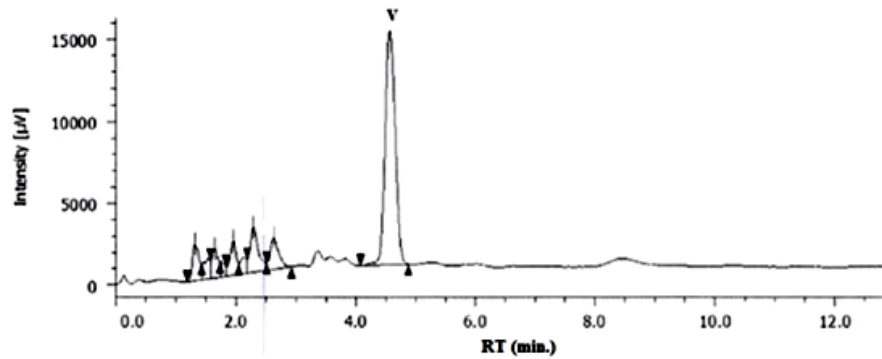


Figure 6. Chromatogram of 0.1µl LS extract measured at 254nm for vanillic acid (V) determination

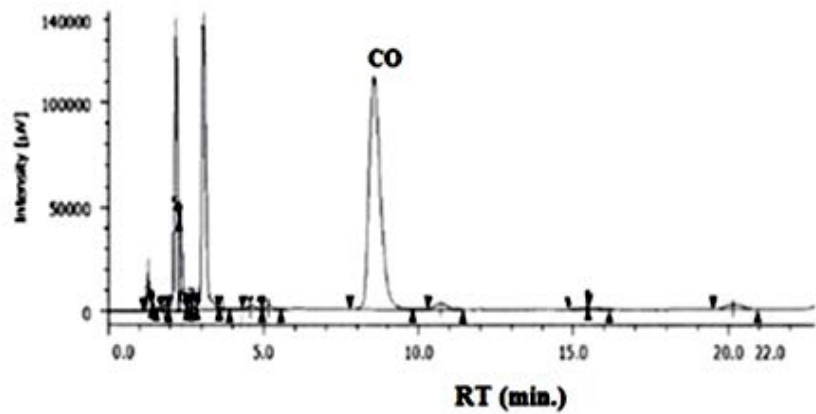


Figure 7. Chromatogram of 0.1µl of LS extract (methanol step) measured at 300nm for coumaric acid (CO) determination

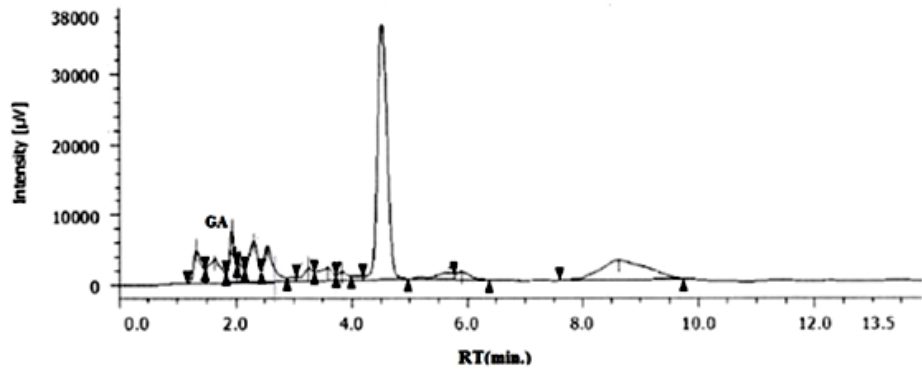


Figure 8. Chromatogram of 0.5µl LS extract measured at 254nm for gallic acid (GA) determination

Table 1. Correlation coefficients, retention times, slopes and detection channels of the calibration curves of standard gallic, chlorogenic, vanillic, caffeic, and coumaric acids

Phenolic acid	Slope	Correlation coefficient	Retention time (min.)	Detection channel(nm)
Gallic (GA)	$y=27531.3+763.881*x$	0.984581	1.65	254
Chorogenic (CGA)	$y=20160.5+1145.78*x$	0.987564	3.04	320
Vanillic (V)	$y=20787.6+1229.75*x$	0.993677	4.563	254
Caffeic (CF)	$y=89158.9+1910.72*x$	0.986130	4.30	320
Coumaric (CO)	$y=31907.6+3933.40*x$	0.994905	8.55	300

Table 2. Gallic, chlorogenic, vanillic, caffeic, and coumaric acid content in lettuce seed (LS) extracts. Data are expressed as mean $\pm$ SD for three samples

Phenolic acid	(mg/100g DW)
Gallic (GA)	20.30 $\pm$ 1.04
Chorogenic (CGA)	1327.45 $\pm$ 45.67
Vanillic (V)	119.02 $\pm$ 4.88
Caffeic (CF)	272.33 $\pm$ 12.77
Coumaric (CO)	912.15 $\pm$ 36.64

Gallic acid showed the lowest concentration (20.30 $\pm$ 1.04 mg/100g DW) among other phenolic acids in LS extracts (Figure 8). A report conducted by Brazaityté et al., [36] who studied the changes of phenolic content in lettuce cultivation under different light conditions and seasons in greenhouses concluded that gallic acid content ranges between 2.0 – 4.9 mg/100g DW.

4. Conclusion

The results of the present study showed that lettuce seeds contained considerable concentrations of chlorogenic, vanillic, caffeic, and coumaric acids. To the best of our knowledge, no report in the literature studied the phenolic acids profile in LS extracts. Based on these findings, lettuce seeds could have a potential for application in the pharmaceuticals and functional food industry.

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Disclosure Statement

The author reports there are no competing interests to declare.

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