



# Phytochemical screening and properties of botanical crude extracts and ethyl acetate fractions isolated by deep eutectic solvent

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## Abstract

Botanicals, being safe because of their natural origin, are associated with potential health benefits due to their isoflavones-rich content. Isoflavones as plant-generated secondary metabolites are formed to defend against environmental stress such as UV radiation, mechanical damage, or any other attack from the surroundings. Kudzu (*Pueraria* roots and flowers), red clover (*Trifolium pratense*), and soy (*Glycine max*) are of significant importance in the medical field. Isoflavones were reported to exhibit a variety of biological functions, e.g., anti-aging, anticancer, and microbiome modulations. This study focuses attention on the extraction and quantification of isoflavones from kudzu roots (KR), kudzu flowers (KF), red clover (RC), and soy molasses (SM), and determining their total polyphenols (TPC), flavonoids (TFC), and antioxidant activity (AOA). Novel green ultrasound-assisted extraction (UAE) technology was developed to extract and quantify the isoflavones. The results showed the quantity of isoflavones in KR, KF, RC, and SM crude extracts to be 1.01%, 2.9%, 0.70% and 0.67%, respectively. The fractionation using ethyl acetate (EA) improved the yield that became 1.48%, 2.5%, 1.3% and 0.89% in the KR, RC and SM extract fractions, respectively. This investigation eventually showed that our green UAE methodology is a safe and efficient method for recovering isoflavones. The total isoflavone contents were recorded as follows: KF>KR>RC>SM. We concluded that these botanicals are a crucial source of isoflavones that exhibit strong antioxidant properties.

## Keywords

*Pueraria* species  
*Glycine Max*  
*Trifolium pratense*  
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## 1. Introduction

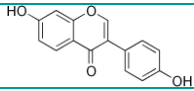
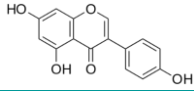
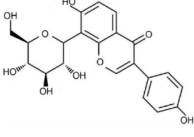
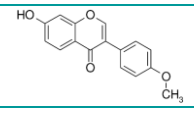
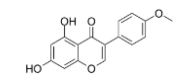
Isoflavones are secondary metabolites that represent the most abundant category of plant polyphenols. Dietary soy, red clover and kudzu contain primarily genistein, daidzein, glycitein, biochanin A [1]. The similarity of these compounds to  $\beta$ -estradiol is responsible for the protection against age-related and hormone-dependent diseases. Substantial shreds of evidence confirmed the fundamental health benefits of the consumption of these isoflavones. Therefore, Japan has declared a recommended daily dietary dose of isoflavones as 15–22 mg supplementation/day for a 60-kg man [2].

Soy (*Glycine max*), red clover (*trifolium pretense*) and kudzu (*Pueraria montana*) are crucial plants in the medical field. Each of these botanicals contains a unique profile of

isoflavones in a considerable quantity [2]. The evaluation of the safety of isoflavone-containing botanicals depends primarily on their ability to induce effects on the metabolism of estrogen, but this evaluation can only be carried out accurately with a proper characterization of the isoflavones involved. These isoflavones have been reported to exhibit a variety of biological functions, e.g. antiaging, anticancer, and microbiome modulations [3]. The abundant representative aglycone chemotypes are presented in Table 1 below. The data are provided as described by U.S. Department of Agriculture Natural Resources Conservation Service [4].

Kudzu (*Pueraria* species) is a *Fabaceae* family that is primarily found in temperate climates worldwide, including Japan and China, and is used as a herbal medicine to treat the common cold, headaches, diarrhea, and hypertension.

**Table 1** Chemical structure, names and formula of isoflavones in soybean, red clover and kudzu [4].

Isoflavone	Chemical structure	Chemical name
Daidzein		7-Hydroxy-3-(4-hydroxyphenyl)-4-benzopyrone
Genistein		5,7-Dihydroxy-3-(4-hydroxyphenyl)-4-benzopyrone
Puerarin		4H-1-Benzopyran-4-one, 8-β-D-glucopyranosyloxy-7-hydroxy-3-(4-hydroxyphenyl)-(daidzein 8-C-glucoside)
Formononetin		7-hydroxy-3-(4-methoxyphenyl)-4-benzopyrone (biochanin B)
Biochanin A		5,7-Dihydroxy-3-p-methoxyphenyl-4H-chromen-4-one

For centuries, particularly in Asia, Kudzu roots and flowers have been commonly used as a food and medicine and also have been documented to control diabetes, prevent cardiovascular diseases, and exhibit antioxidants, antihypertensives, anti-inflammatory, antiestrogenic, and cholesterol-lowering activities [5].

Red clover is a legume forage abundant in phenolic compounds which have been used fresh (in salads) or thermally processed for teas since ancient times [6, 7]. It was found that phytoestrogens isoflavones present in the biomass of the above-ground part of red clover can prevent various types of diseases and hormonal-related disorders [8, 9]. The most abundant isoflavones therein are biochanin A and formononetin, with genistein and daidzein occurring at 5 to 10% of the concentrations of biochanin A and formononetin [10]. These isoflavones are effective antioxidants and may have free radical scavenging activity [11].

Thanks to its various health-promoting properties, soy isoflavones are currently important targets in food and nutritional diets, such as those directed at reducing cholesterol levels, cancer prevention, and alleviation of osteoporosis and menopause syndrome. Because of the environmental impacts associated with the generation of by-products during the processing of soy-based food, there is a desperate need to recycle this waste in order to reduce the production costs and maximize the overall use of food [12]. In general, soy molasses, a dark brown syrup rich in carbohydrates and isoflavones, is produced concomitantly with the concentrate of soy protein [13].

Food and Agriculture Organization (FAO) of the United Nations estimated the annual needs of kudzu, and soybeans to be 50 and 96.5 million tons, respectively. Thus, it is technologically and economically valuable to recover isoflavones from these sources [14, 15]. Since it contains the highest concentration of isoflavones and biological activity in its biowaste, KR, RC and SM were chosen for their antioxidant activities to be extracted and examined.

To extract the main individual flavonoid and phenolic compounds of the botanical sources, various solvents with different polarities were previously used to extract antioxidant components and reversed-phase high-performance liquid chromatography (HPLC) analysis was performed. For the extraction of isoflavones, traditional methods, including maceration, percolation, decoction, infusion, Soxhlet extraction, and hot reflux extraction have all been used [11]. These methods, however, appear to have some disadvantages, such as long extraction times and low performance. In addition, the large-scale use of organic solvents can affect the environment and leave some traces in the extract [16]. Therefore, rapid, efficient, environmentally friendly and effective methods for the isolation and quantification of isoflavones were necessary to be developed.

Ultrasound-assisted extraction (UAE) has been used for the isolation of isoflavones in conjunction with green solvents such as water and ionic liquids to address these issues. In terms of less organic solvent, less time and less labor, this approach is more favorable and effective for obtaining high yield and quality [17].

Natural deep eutectic solvents (NADESs) are a new green solvent with certain properties that are shared with ionic liquids. A range of beneficial properties is also exhibited, including simple preparation procedures from cheap and sustainable starting materials. In addition, they are environmentally friendly and have outstanding physicochemical and biodegradable characteristics. In the current study, NADESs were produced by mixing a quaternary ammonium salt (choline chloride) with a citric acid (a hydrogen-bond donor) (HBD). Both components were sufficiently able to form a tight bond that helps to extract and crack KR, KF, RC and SM cell walls to obtain isoflavones [18].

## 2. Materials and Method

### 2.1. Chemicals, Reagents, and Equipment

Reference standards of daidzein, genistein, puerarin, formononetin and biochanin A standards were purchased from Sigma Aldrich (Missouri, USA), with a purity of each ≥98% and used without further purification. Methanol 95% (HPLC grade), formic acid, and acetonitrile (HPLC grade) were obtained from Sigma Aldrich. Choline chloride (99%) pharmaceutical grade was purchased from Acros Organics, Belgium. Citric acid (99%, food-grade) was bought from Sigma Aldrich. Quercetin, Gallic acid and 2, 2-diphenyl-1-picrylhydrazyl (DPPH), were all purchased from Sigma Aldrich, Missouri, USA. Ethyl acetate and ethanol were purchased from “Chemreaktivsnab” company, Ufa, Russia. All other reagents and chemicals used in this study were of analytical grade.

Elma ultrasonic cleaner was used for the extraction process. Also, a laboratory centrifuge (PE-6926) with a rotor of 10x5 ml was utilized for separating the extraction mixtures. Spectrophotometer UV 1800 Shimadzu was also used

for the analysis of total polyphenols and antioxidant activity. A hot oven (dry Oven UN55, Memmert, Germany) was utilized for our experiment. Also, high-performance liquid chromatography (HPLC) was used for quantifying isoflavone content.

## 2.2. Chemicals, Reagents, and Equipment

Dried kudzu (*Pueraria lobata*) roots (KR) and kudzu flowers (KF) were purchased from Xi'an Sgonek Biological Technology (Shaanxi Sheng, China) and red clover flowers (*Trifolium pratense*) were purchased from Parapharm company (Russian Federation). The roots and flowers were then dried to constant weight at 40 °C and pulverized to a homogeneous powder using an electric blender. The resulting powder was stored at room temperature until subsequent quantitative analysis. Similarly, soy molasses (SM) (*Glycine max*), a by-product of soy protein concentrate, was purchased from Agroproduct CJSC, Kaliningrad, Russia and stored at 20 °C until further usage.

## 2.3. Preparation of NADESs

The powder was extracted using NADESs at the material ratio of NADESs to kudzu root or red clover in volume (mL)/weight (g) 20:1. NADESs used in the current study comprise a two-component mixture of choline chloride and citric acid at 1:2 molar ratio. For extraction of SM, NADESs were prepared by mixing equimolar quantities of choline chloride and citric acid, 1:1 (Table 2). Briefly, the two-component mixture was transferred into a glass seal and distilled water (20%) was added, the final mixture was heated at 60–80 °C under constant stirring until a transparent solution was observed [19]. The prepared NADESs were stored in dark until subsequent utilization.

## 2.4. NADESs-based ultrasound-assisted extraction of kudzu roots

We used a NADES-based ultrasound-assisted extraction (UAE) procedure for the extraction of isoflavones from KR, KF, RC and SM [20]. Approximately 1 g of the dry powder was accurately weighed into a 50 mL beaker, to which 20 mL of NADES solution was immediately added. The mixture was processed by ultrasonic extraction at a frequency of 37 kHz and a power of 580 W at 60 °C for 3 hours using an ultrasonic extraction instrument. Isoflavones were gradually extracted into the NADESs phase, and a viscous suspension was obtained. The suspension was centrifuged at 6000 rpm for 10 min to separate the solid and liquid phases. The liquid extract was fractionated in a separatory funnel containing ethyl acetate and the resulting organic layer was concentrated using a rotatory evaporator until dry. The final extract was then stored at –20 °C until further use.

A certain amount of the resulting dry powder was diluted with methanol. Then, the concentrations of isoflavones in the final solution were determined by liquid chromatography (LC) using calibration curves. The same procedure was applied in triplicate for all experimental treatments.

Extraction yields ( $E_y$ ) were calculated as follows:

$$E_y = \frac{C_f \cdot V_s}{m_s}, \quad (1)$$

where  $C_f$  is the concentration of isoflavones found in NADESs based on LC analysis,  $V_s$  is the diluted suspension volume, and  $m_s$  is the mass of the test sample.

## 2.5. Evaluation of extraction efficiency

### 2.5.1. Quantification of isoflavones

Quantification of isoflavone content in the extract was performed on an HPLC system (Agilent Technologies 1100, version 2.3) consisting of a quaternary pump (Model G7111B), a UV/Vis detector (Model G7117C) coupled with DAD analysis software, an autosampler (Model G7129A), a column oven (Model G7130A) and a vacuum degasser module. A column (250×4.6 mm) was used for the separation of active compounds. The elution solvents consisted of (solvent A, 95%) containing water and 0.1% acetic acid and (solvent B, 5%) containing methanol and 0.1% acetic acid. Linear gradient elution of solvent B was applied up to 100% over 21.5 min, at a flow rate of 0.7 mL/min, as a mobile phase. The temperature of the column was kept at 30 °C and the injection volume was 5 µL. A quantitative analysis of the five most characteristic peaks of isoflavone content in the extracts was applied. The isoflavones were separated on a reversed stationary phase and detected with UV/Vis at 254 nm.

The individual isoflavones were quantified using their corresponding standard calibration curves with a concentration of approximately 1.6 µg/mL. The level of isoflavones was expressed in g/100 g of KR, KF, RC, or SM for both crude extract and ethyl acetate fraction.

### 2.5.2. Assessment of Scavenging activity of DPPH free radical using EPR method

The DPPH radical scavenging effect was estimated as previously described by Chen et al. with little modification [21]. 0.5 mM DPPH solution in methanol was prepared and 200 µL of the DPPH solution was then added to 25 µL of each test sample.

**Table 1** Natural deep eutectic solvents (NADESs) used in extraction experiments.

Materials	Components		Molar ratio	Solid/solvent ratio	Appearance
	HBA <sup>a</sup>	HDB <sup>b</sup>			
–	HBA <sup>a</sup>	HDB <sup>b</sup>	–	–	–
Pueraria lobata roots	Choline chloride	Citric acid	1:2	1:20	Transparent colourless liquid
Pueraria lobata flowers					
Trifolium pratense					
Soy molasses			1:1	1:3	

<sup>a</sup> Hydrogen bond acceptor.

<sup>b</sup> Hydrogen bond donor.

The absorbance was then immediately measured using an electron paramagnetic resonance (EPR) spectrometer (Bruker, Germany).

Ascorbic acid, a well-known antioxidant, was used as a positive control for the assay. The antioxidant activity was calculated for plotting the standard curve. The antioxidant activity of extracts was determined in mM of Ascorbic acid equivalency. Ascorbic acid at the levels of (1, 0.5 and 0.25 mM) was used as standard and blank, respectively. The intensity of the EPR spectrum decreases with an increasing concentration of Ascorbic acid, which increases the percentage inhibition (%); the percentage inhibition of the EPR spectrum was calculated using the following equation:

$$I(\%) = \frac{I_0 - I}{I_0} \cdot 100, \quad (2)$$

where  $I_0$  is the double integral of the EPR signal of the water-ethanol mixture radical (225  $\mu$ l of DPPH in methanol),  $I$  - the double integral of the EPR signal of a mixture of DPPH in methanol (200  $\mu$ l) and (25  $\mu$ l) of the sample. The EPR spectrum of DPPH changing in time in the process of reaction with an antioxidant is recorded over five minutes time scale.

### 2.5.3. Assessment of Scavenging activity of DPPH free radical using spectrophotometric method

The capacity to scavenge the "stable" free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay was established based on a method described by Tian et al. with some modifications [22]. Briefly, 100  $\mu$ l of KR, KF, RC or SM extract was made up to a volume of 3 ml using methanol and 1 ml of 0.8 mM DPPH was added to the diluted extract. The reaction mixture was shaken vigorously and incubated at room temperature for 30 min in dark. The DPPH radical reaction was monitored by recording absorbance (Abs.) at 514 nm. The decreased absorbance value of reaction mixture indicated an increased percentage of free radical scavenging activity. The measurements were investigated using spectrophotometric analysis. The percentage inhibition or free radical scavenging activity was calculated by using the following formula:

$$\text{Inhibition (\%)} = \frac{\text{control abs.} - \text{sample abs.}}{\text{control abs.}} \cdot 100 \quad (3)$$

where the control contained only methanol and DPPH solution. The inhibition % was calculated from the plot of the inhibition curve. All the reactions were monitored in duplicates and the value was expressed as the mean  $\pm$  standard deviation (S.D.).

### 2.5.4. Total polyphenol content

Total phenolic content of KR, KF, RC and SM extracts was purified [23] and subsequently determined according to the previously described method [24] with slight modifications. Briefly, samples (0.25 mL) or standard gallic acid (0, 50, 100, 150, 250, and 500 mg/L) solution were pipetted into the assay tubes. Then, Folin Ciocalteu solution (0.5 mL) and distilled water (5.5 mL) were mixed and homogenized.

20% Na<sub>2</sub>CO<sub>3</sub> (1 mL) was added after 5 min of incubation. The assay tubes were incubated at 20 °C for 2 h, and the absorbance was measured at 765 nm for 30 min against a blank (distilled water) by a spectrophotometer (UV-1800, Shimadzu USA,). Total phenol content was calculated from the standard curve of gallic acid ( $y=0.0038x+0.0487$ ,  $R^2=0.9982$ ) and the result was expressed as mg of gallic acid equivalents per mMol/L of extract.

### 2.5.5. Total flavonoids -The NaNO<sub>2</sub>-Al (NO<sub>3</sub>)<sub>3</sub>-NaOH colorimetric method

The NaNO<sub>2</sub>-Al (NO<sub>3</sub>)<sub>3</sub>-NaOH colorimetric method was conducted as described by Huang et al. with few modifications. Briefly, 0.5 mL of extract was mixed with 2 mL of 30% ethanol and 0.15 mL of NaNO<sub>2</sub> (5%, w/v). After 5 min the mixture was reacted with 0.15 mL of Al(NO<sub>3</sub>)<sub>3</sub> (10%, w/v) for 6 min. Then, 2 mL of 1 M NaOH was added, and the mixture was adjusted to 5 mL with 0.2 mL of 30% ethanol. After incubation at room temperature for 10 min, the absorbance was read at 510 nm. The amounts of Al(NO<sub>3</sub>)<sub>3</sub> and NaOH solutions were substituted with the same amount of 30% ethanol in the blank. Similarly, 0.5 mL aliquots of KR, KF, RC and SM extracts were reacted in the same way. The total flavonoid content of samples was expressed as quercetin equivalent, and the calibration curve ranged from 0 to 500  $\mu$ g mL<sup>-1</sup>.

### 2.6. Statistical Analysis

The extractions were repeated twice, and all determinations were carried out in triplicate. The values are presented as means  $\pm$  standard deviation. All the parameters were analyzed at least at a 95% significance level ( $p < 0.05$ ) using GraphPad Prism analysis 08.0.2.

## 3. Results

### 3.1. Quantification of isoflavones in kudzu roots, kudzu flowers red clover and soy molasses

In order to quantify the bioactive components in extracts, a calibration curve of five different isoflavones was plotted. The experimental data showed coefficient of determination ( $R^2$ ) values for all standards of more than 0.9996 as shown in Table 3. This indicates that this model is optimized and applicable to describe the response of the experiment to the five isoflavones. Representative standard samples including puerarin (A), daidzein (B), genistein (C), formononetin (D) and biochanin (E) were used for quantification purposes.

**Table 3** Regression equation,  $R^2$  and extraction yield of isoflavone.

Isoflavones	Regression equation	$R^2$
Puerarin	$y=156.3464x+287792$	0.99966
Daidzein	$y=236.9975x+9.2923$	0.99995
Genistein	$y=276.2041x+19.7115$	0.99979
Formononetin	$y=202.7583x+21.7709$	0.99980
Biochanin A	$y=256.2404x+13.2775$	0.99983

### 3.2. Detection of isoflavones bioactive compounds in kudzu root by HPLC-DAD

The crude extract and its sub-fraction were analyzed by HPLC-DAD system as shown in Table 4. The five main isoflavones were efficiently extracted by HPLC and were identified as daidzein, genistein, puerarin, formononetin and biochanin A according to the retention times of the respective isoflavone standards. The results show that crude extract of KR consists mainly of daidzein, genistein and puerarin, while the components identified in the extract after ethyl acetate fractionation were daidzein, genistein, puerarin and formononetin. Daidzein concentration in the fraction was significantly higher in comparison to the crude extract, as shown in Figure 1. The amounts of formononetin and biochanin A are too small or neglected in both extracts. These isoflavones are distributed in a fraction of different polarities. The contents determined are consistent with those published by Zhang et al. [25].

### 3.3. Detection of isoflavones bioactive compounds in kudzu flowers by HPLC-DAD

The crude extract and its sub-fraction were analyzed by HPLC-DAD system as shown in Table 5. The main isoflavone was efficiently extracted by HPLC and was identified as genistein. The results show that crude extract of KF consists mainly of genistein and a low quantity of puerarin, daidzein and biochanin A, while the components identified in the extract after ethyl acetate fractionation were mainly genistein. Genistein concentration in the fraction was significantly higher in comparison to the crude extract, as shown in Figure 2. The amounts of other compounds are too small or neglected in both extracts. The contents determined are consistent with those previously published [26].

### 3.4. Detection of isoflavones bioactive compounds in red clover by HPLC-DAD

In our study, the five standards were also quantified in both crude extracts and the EA fraction of red clover by HPLC method. It was found that formononetin and biochanin A as the main compounds in all studied samples (approximately 0.28 and 0.20%, respectively), while only small amounts of daidzein and genistein were detected (approximately 0.1% and 0.12%, respectively). The fractioning using ethyl acetate significantly improved the yield of both daidzein and genistein. It was also observed that puerarin was completely absent in red clover except for a negligible quantity in the crude extract.

Typical HPLC chromatograms of extracts of red clover are shown in Figure 3. In the EA fraction case, the HPLC-UV chromatogram showed a rise to sharper peaks particularly formononetin and biochanin A [10]. The concentrations on isoflavone profile of both crude extract and EA fraction are shown in Table 6. Interestingly, the isoflavone composition of red clover showed Biochanin A slightly predominant over formononetin in the crude extract, while showed the opposite approach when fractionated with EA. The ethyl acetate

fraction represented the total amount of isoflavones (1.3%) which was 3.6 times higher than the quantity in the crude extract (0.36%), as shown in Table 6.

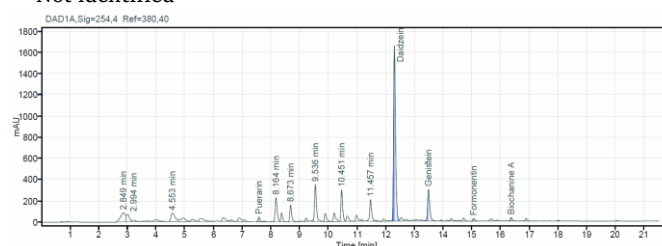
### 3.5. Detection of isoflavones bioactive compounds in soy molasses by HPLC-DAD

The crude extract of SM and its sub-fraction were also analyzed by HPLC-DAD system. Daidzein and genistein were only identified among the five standards in both crude extract and its subfraction. The results in Table 7 show that ethyl acetate fraction of SM recorded its concentration as significantly higher than in crude extract. The contents determined are corresponding to those published by Gu et al. [27]. The chromatogram below indicated the peaks of both daidzein and genistein as recognized by HPLC in SM fractions as elucidated in Figure 4.

**Table 4** Content of isoflavone compounds in kudzu roots as determined by HPLC-DAD.

Isoflavones	Isoflavone content in kudzu roots		
	Crude extract	Ethyl acetate fraction	Crude extract
Parameters	Concentration (g/100 g extract)	Concentration (g/100 g extract)	Concentration (g/100 g DM)
Puerarin	0.52	0.05	0.94
Daidzein	0.02	1.2	0.036
Genistein	0.002	0.2	0.036
Formononetin	NI <sup>a</sup>	0.02	NI <sup>a</sup>
Biochanin A	Negligible	0.02	Negligible
Sum	0.542	1.48	1.012

<sup>a</sup> Not identified



**Figure 1** Representative HPLC-UV chromatographic profile for ethyl acetate fraction of kudzu roots.

**Table 5** Content of isoflavone compounds in kudzu flowers as determined by HPLC-DAD.

Isoflavones	Isoflavone content in kudzu roots		
	Crude extract	Ethyl acetate fraction	Crude extract
Parameters	Concentration (g/100 g extract)	Concentration (g/100 g extract)	Concentration (g/100 g DM)
Puerarin	0.1	NI <sup>a</sup>	0.94
Daidzein	0.1	0.02	0.1
Genistein	0.94	2.47	2.7
Formononetin	NI <sup>a</sup>	NI <sup>a</sup>	NI <sup>a</sup>
Biochanin A	0.1	0.01	0.1
Sum	0.97	2.5	2.9

<sup>a</sup> Not identified

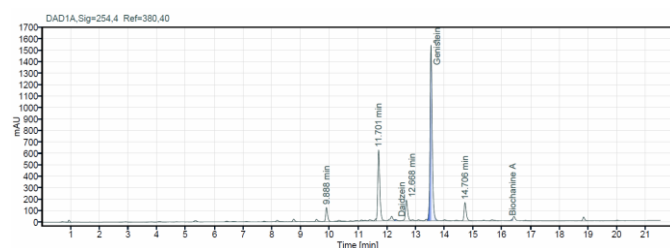


Figure 2 Representative HPLC-UV chromatographic profile for ethyl acetate fraction of kudzu flowers.

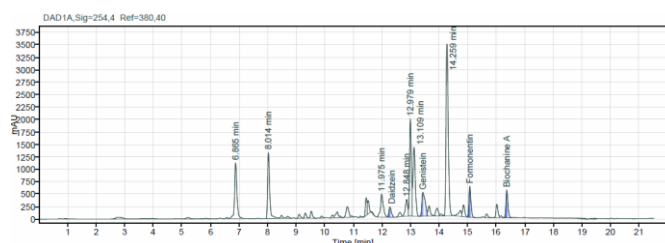


Figure 3 Representative HPLC-UV chromatographic profile for ethyl acetate fraction of red clover sample.

Table 6 Content of isoflavone compounds in red clover as determined by HPLC-DAD.

Isoflavones Parameters	Isoflavone content in red clover		
	Crude extract	Ethyl acetate fraction	Red clover flowers
	Concentration (g/100 g extract)	Concentration (g/100 g extract)	Concentration (g/100 g DM)
Puerarin	0.004	NI	NI
Daidzein	0.02	0.11	0.1
Genistein	0.03	0.21	0.12
Formononetin	0.15	0.56	0.28
Biochanin A	0.16	0.42	0.2
Sum	0.36	1.30	0.70

Table 7 Content of isoflavone compounds in soy molasses as determined by HPLC-DAD.

Isoflavones Parameters	Isoflavone content in soy molasses		
	Crude extract	Ethyl acetate fraction	Soy molasses bio-waste
	Concentration (g/100 g extract)	Concentration (g/100 g extract)	Concentration (g/100 g DM)
Puerarin	NI <sup>a</sup>	NI <sup>a</sup>	NI <sup>a</sup>
Daidzein	0.03	0.36	0.31
Genistein	0.06	0.53	0.36
Formononetin	NI <sup>a</sup>	NI <sup>a</sup>	NI <sup>a</sup>
Biochanin A	NI <sup>a</sup>	NI <sup>a</sup>	NI <sup>a</sup>
Sum	0.09	0.89	0.67

<sup>a</sup> Not identified

Table 8 Total polyphenols, total flavonoids, and antioxidant activities in the extracts of kudzu roots and soy molasses.

Parameters	Kudzu roots	Kudzu flowers	Red clover	Soy molasses
TPC GA equ. (mmol/L)	223.1±1.07	618.4±2.207	438±8.46	330.5±8.45
TFC Quercetin equ. (mmol/L)	201.2±10.35	181.5 ±11.6	366.5±13.58	133.1±11.3
AOA-Inhibition spectrophotometer (%)	94.14±0.85	98.79±0.33	90.4±0.06	91.37±0.27
AOA (mM Asc. Acid) (mmol/L)	2.205±0.05	4.24±0.0012	2.49±0.004	2.03±0.02

### 3.6. DPPH free radical scavenging activity

The percentage scavenging effect of DPPH radicals vs the concentrations of extract fractions and standards (mM Asc. Acid) were plotted. It was found that the percentage of DPPH radical scavenging activity of all the extracts was found to be isoflavone-concentration-dependent. The inhibition percentage of KR, KF, RC and SM was recorded as 94%, 98.79%, 90.4% and 87%, respectively, using spectrophotometric analysis, as shown in Table 8. Similarly, KF fraction showed a higher inhibition % over five minutes scale. This explains the KF showed the best results while SM exhibited the lowest. At the same time, the equivalency of antioxidant activity was recorded in the following order: KF>KR>RC>SM using EPR technique, 2.205, 4.24, 2.49, and 2.03, respectively. The EPR spectra and inhibition % explained the antioxidant activity of all extracts and its equivalency to ascorbic acid in both Figures 5 and 6.

### 3.7. Total polyphenols and total flavonoids

The total polyphenols and total flavonoids were calculated according to the equivalency of gallic acid and quercetin concentration, respectively (Figure 7a, 7b).

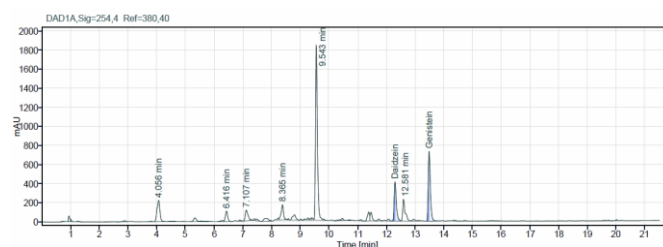


Figure 4 Representative HPLC-UV chromatographic profile for ethyl acetate fraction of soy molasses.

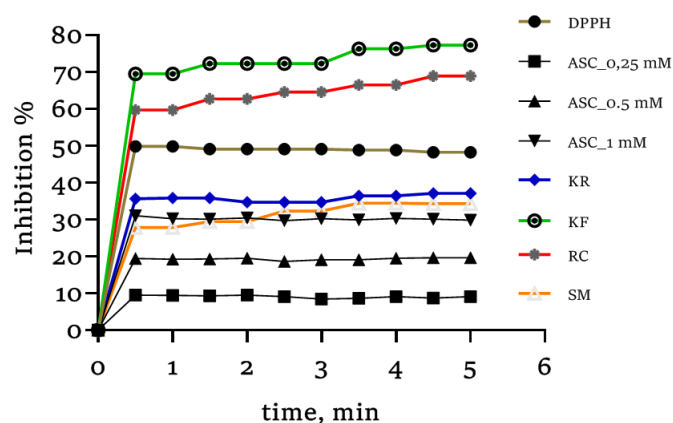
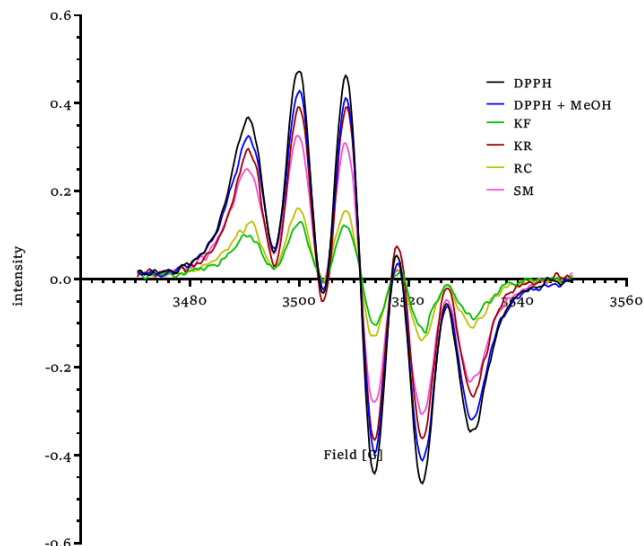
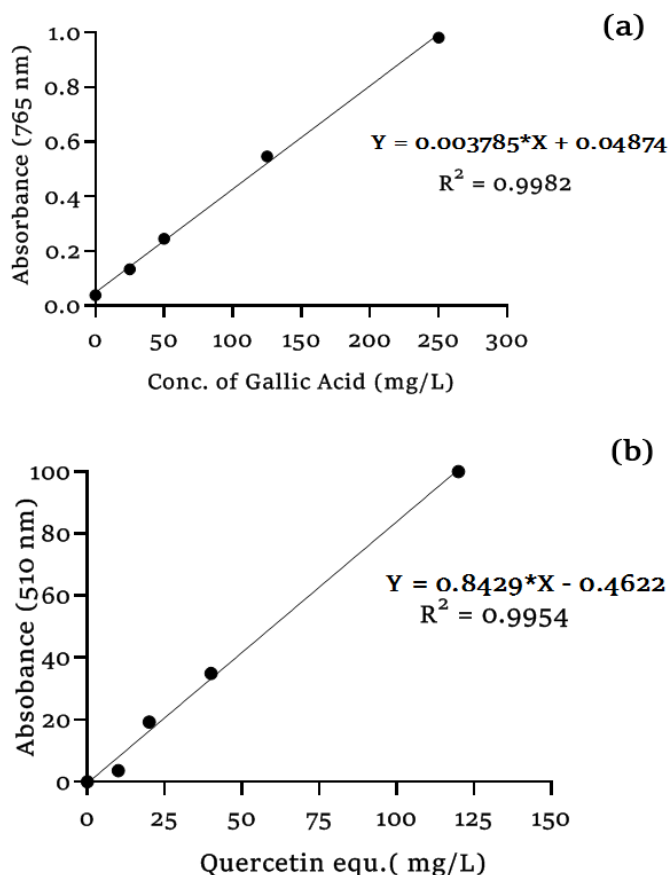


Figure 5 EPR spectra and inhibition percent for quantification of intensity and ascorbic acid equivalency, respectively.

As shown in Table 8, total phenolic content and total flavonoids were recorded in the following order: KF>KR>RC>SM. The values in Table 8 indicated that TPC was 223.1, 618.4, 438 and 330.5 GA equivalent (mmol/L) in KR, KF, RC and SM respectively, while TFC was recorded as 201.2, 181.5, 366.5, and 133.1 quercetin equ. (mmol/L) for KR, KF, RC and SM respectively.



**Figure 6** EPR spectra and inhibition percent for quantification of intensity and ascorbic acid equivalency, respectively.



**Figure 7** Gallic acid and Quercetin standard calibration curves for the quantification of total polyphenol content and total flavonoids, respectively.

#### 4. Discussion

Many botanicals contain phytoestrogens, which are steroid-like plant compounds that have beneficial effects on humans. Research has shown that many of these benefits may be linked to isoflavones [28]. Therefore, the previous researchers attempted to isolate these isoflavones from botanical sources. Kudzu roots, red clover and soy molasses are crucial sources of isoflavones. These botanical materials (soy, red clover, and kudzu) are characterized by the content of a five isoflavones (puerarin, daidzein, genistein, formononetin, and biochanin A).

Soy molasses, red clover, and parts of kudzu (roots and flowers) are regarded as waste in the manufacturing process. In the previous studies, it was concluded that these biowastes exhibited potential activities, especially puerarin, daidzein and genistein that showed stronger antioxidant activity [25]. Additionally, it was recorded to have high quantities of polyphenols and flavonoids. Extraction of isoflavones in KR, KF, RC and SM using NADESs has not been conducted before, according to the best of our knowledge. The results showed the efficacy of using natural solvents for extracting isoflavones compared to other organic or toxic solvents. Green extraction and subsequent organic solvent partitioning have not yet been applied to isolate the abundant bioactive compounds in the above-mentioned botanicals. The current research was carried out to isolate, quantify and fractionate the isoflavones present in these plants using HPLC DAD system. The HPLC is a sufficiently reliable method for the determination of compounds based on internal standards. Therefore, it was used in our study to analyze our extracts.

Kudzu roots and flowers were shown to contain large amounts of isoflavones (an average of 1.8 to 12% dry matter), including puerarin, daidzein, gallic acid and quercetin [26, 29, 30]. The concentration of puerarin was recorded 1.01% in dry kudzu roots. Similarly, our extraction technique showed that the concentration of isoflavones was three times higher than that obtained by Tong-Rong et al. (0.33%) where methanol was used for the recovery of isoflavones [21]. Nevertheless, KF showed the highest concentration of isoflavones content (2.9%), particularly after the fractionation using ethyl acetate. The most abundant compound after fractionation was genistein (2.7%). These outcomes are in agreement with the previously published results [26].

At the same time, Yang L. et al. extracted and quantified total isoflavones in KR conventionally using ethanol with a 1.86%, which is slightly higher than in our extract despite the use of ethanolic extraction technique for 24 h three times [25]. This show that our technology was highly efficient and more time-consuming despite the slightly lower concentration obtained. The fractionation using ethyl acetate improved the yield of daidzein in case of KR.

Also, our chromatographic conditions described enabled us to quantify the isoflavone profile of RC of red clover before and after partitioning using EA rapidly and simply. It was

recorded in literature that there are factors that may affect the concentration of isoflavones in RC, such as growing period, genetic factors, cultivar, temperature, light, nutrition, etc. [31]. Since the predominant compounds in RC are formononetin and biochanin A, it was necessary to compare our outcome with the previously published results. Formononetin and biochanin A were recorded as 0.28% and 0.2%, respectively. These results were obtained by Nurgiin et al. (0.025 to 0.3%) from different origins, conditions and extraction techniques (methanol and acid hydrolysis) [32]. Meanwhile, the total isoflavones (0.70%) in our extracts exhibited slightly higher results than RC clover extracts analyzed by Burdette et al. after 3 h extraction using ethanol as extracting solvent [33]. However, total isoflavones in our ethyl acetate fraction were 2 times higher, as shown in Table 6.

The concentration of daidzein and genistein in soy molasses was quantified to be 0.67% in SM bio-waste. These results are 16 times higher than those obtained by Gu et al. [27]. This observation highlights the potential role that NADESs played in extracting these components from SM. Also, the ethyl acetate fractionation maximized the yield of isoflavones significantly more than that in the crude extract.

In this study, a novel green extraction method based on deep eutectic solvents (NADESs) was applied to the simultaneous extraction of bioactive compounds from KR, KF, RC and SM. The NADESs proved to be efficient solvents for the extraction of isoflavones using ultrasonic-assisted extraction as in comparison to the conventional extraction methods previously utilized. Hence, citric acid/ChCl combination was proved to be the efficient extraction solvent of NADESs for obtaining the three main compounds under optimized conditions.

Phenolic compounds, including isoflavones, are known for their efficient radical scavenging activity resulting from the hydroxyl groups at various positions and the ortho-dihydroxy structure in their B ring [34]. Recent investigations have shown that phenolic compounds contribute significantly to antioxidant activities [35]. Since EA fraction exhibited the highest concentration of isoflavones, it was selected for the subsequent AOA, TPC and TFC. KR, SM and SM showed total phenolic contents of 223.1, 618.4, 438, and 330.5 GA equivalent (mmol/L), respectively. These results are higher than that was obtained by Filipiak-Szok et al. (6.3%) and by Batista et al. (0.15%) [5]. Similarly, it was found that EA fraction showed the highest antioxidant and free radical scavenging activity in comparison to the other fractions [11]. This result suggests that KR, RC and SM extracts are rich sources of phenolic compounds.

Since the concentration of isoflavone in KF extract was recorded as higher than that of KR, RC and SM, antioxidant activity was similarly determined the highest in kudzu root fraction. In summary, KR, KF, RC and SM showed high antioxidant activities and these activities were well matched with the total phenolic contents and total flavonoids determined in this study. The results suggested that KR, KF, RC and SM could also be used as a source of antioxidants and antiaging compounds.

## 5. Conclusion

This research shows that KR, KF, RC and SM are good botanical sources of isoflavones and may be useful for the functional or therapeutic treatment of inflammation, bone loss, metabolic disorders, and aging caused by oxidative stress. The extraction technique and fractionation of the extracts allowed obtaining the bioactive components in higher concentrations compared to the reported conventional methods. A green and rapid NADESs-UAE method was developed for efficient extraction of three bioactive compounds, puerarin, daidzein, and genistein, from KR, KF and SM. On the other side, formononetin and biochanin A were predominant in RC. The antioxidant activities were shown to change in a following series: KF>KR>RC>SM, and were significantly correlated with total polyphenols and flavonoids in the extracts. Higher quantities of chemically interesting and biologically active natural antioxidants could be provided by further investigation of various extraction conditions. Overall, it seems reasonable to consider these botanical waste extracts as a new valuable source for various pharmaceuticals.

## Supplementary materials

No supplementary materials are available.

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## Conflict of interest

The authors declare no conflict of interest.

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