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Comparison of antioxidant properties of chlorophyll extracted from alfalfa (*Medicago sativa L.*) using enzymatic and ultrasonic extraction methods

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ABSTRACT

Nowadays, natural pigments are widely used in the food, cosmetics and sanitation industries. In recent years, numerous researches have been done on the methods of extraction and evaluation of the properties of natural pigments. The purpose of this study is comparison of enzymatic and ultrasonic extraction methods for the antioxidant properties of chlorophyll extracted from alfalfa (*Medicago sativa* L.). Antioxidant activity was performed by three methods including DPPH and ABTS free radicals scavenging and ferric reducing antioxidant power (FRAP). The results show that the concentration of chlorophyll a in alfalfa is higher than that of chlorophyll b and also the enzymatic method demonstrates higher yield in chlorophyll extraction. In addition, higher concentrations of alfalfa extract showed higher antioxidant activity in inhibiting DPPH free radicals and ferric reducing antioxidant power ($p < 0.05$). Also, with increasing the extract concentration, total phenolic compounds and flavonoid compounds increase ($p < 0.05$). Due to the better performance, the enzymatic extracted chlorophyll was used to evaluate the antioxidant properties, inhibition of free radicals DPPH and ATBS and ferric reducing antioxidant power and the result shows the higher chlorophyll concentration, the higher antioxidant properties. Due to the appropriate antioxidant effect of alfalfa chlorophyll extracted by enzymatic method, its application in food, pharmaceutical and health industries can be evaluated and industrial scale extraction systems can be designed using this technique.

. Introduction

Attention to human health has always been important in the food and pharmaceutical industries, and the use of healthy food is very effective in this regard. One of the dangerous factors for human health is oxidation reactions in the body due to the production of free radicals that lead to damage or death of cells [1]. Lowering the level of antioxidants in the body leads to premature aging, damaged or mutated cells, damaged tissue, activation of defective genes in DNA and increased pressure on the body's immune system [2]. Scientific findings indicate that fruits and vegetables contain different types of nutrients and non-nutritive substances such as pigments, which are called phytochemical compounds. These compounds have biologically beneficial effects on human health and prevent some chronic diseases such as types of cancer, cardiovascular diseases and diabetes. Among the most important functions of these compounds are free radical inhibition and antioxidant effects [3 and 4]. In fact, pigments are chemical compounds that are able to absorb light in the wavelength range of the visible region, and one of the most important advantages of natural pigments, besides their health-giving properties, is their color diversity [5]. Different parts of the body of a plant are used as pigments, which have different properties for different reasons [6]. There are four main groups of pigments in plants, including chlorophylls, carotenoids, flavonoids and anthocyanins, which have antioxidant effects. Chlorophylls are located in special cell units called chloroplasts, which act as photosynthesis agents. Chlorophylls, which are the most important light-absorbing pigments in the process of photosynthesis, and their main role is to absorb light energy and convert it into chemical energy, and are mainly blue-green or yellowish-green in color. As a food pigment, chlorophyll is responsible for transferring the stable green color to the leaves of spinach, lettuce, etc. Among the types of chlorophyll, two types of chlorophyll a and chlorophyll b, which exist in the tissues of green plants with different ratios according to light intensity and are mainly in the ratio of 3 to 1, are used in food [5]. These two types of chlorophyll have a similar structure and their difference is in their R group. If R is a methyl group (CH_3), the chlorophyll is of type a and if it is formyl (CHO), the chlorophyll is of type b. The four-pyrrole core with the head of the chlorophyll molecule is the hydrophilic pole and the phytol chain or the chlorophyll tail is the hydrophobic (or lipophilic) pole and therefore chlorophyll and similar molecules are called amphoteric compounds.[1] Minamand [7].

Today, there is a growing desire to replace artificial colors in the food and health industries with colors of natural origin. Several side effects such as allergic effects, neurological and behavioral complications (such as hyperactivity) as well as toxicity caused by the consumption of artificial pigments have been observed in the long term [8]. This is despite the fact that the colors derived from natural sources, in addition to having good quality, efficiency and sensory characteristics, have many medicinal effects such as anti-cancer, anti-inflammatory, anti-cholesterol and diabetes effects and therefore play an important role in improving health. Humans are in charge [9]. Antioxidants are compounds that delay oxidative processes and exist in both synthetic and natural forms. The side effects of synthetic antioxidants include carcinogenesis and mutagenesis [10]; Therefore, it is necessary to use natural antioxidants such as tocopherols, flavonoids, chlorophylls and plant pigments. Antioxidants can reduce oxidative stress in cells. Therefore, their prescription is useful and effective for the treatment of many diseases [11].

One of the plants for extracting chlorophyll is alfalfa. Alfalfa (.*Medicago sativa* L) herbaceous and perennial plant from the legume family*Fabaceae*), with English names*alfalfa* And*Lucerne* Is. It contains various plant chemicals such as alkaloids, amino acids and essential enzymes, phytosterols, phytoestrogens, flavons, flavonoids, minerals, saponin, vitamins [12]. Due to its richness in vitamins and phytoestrogens, this plant is used as a food additive in several developed countries [13]. In Iran, alfalfa is the most common crop grown. The different parts of this plant, such as aerial parts and seeds, have been investigated from

[1] .Amphiphilic compounds

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different aspects, including the ability to withstand environmental stresses, especially salinity and drought, for agricultural and livestock applications, as well as the effect in the treatment of some diseases caused by oxidative stress. However, according to the reports that there is a wide range of effective chemical compounds in the alfalfa plant and since the alfalfa plant grows in a wide geographical area and its cultivation is easy and inexpensive, it is necessary to identify the maximum potential of this plant and to develop new agents that are active have good biological properties, should be used [14].

Considering that the use of different properties of plants in different industries requires extraction, it is necessary to evaluate different extraction methods in order to find the most optimal and efficient method. Because the quality and quantity of the extract, in addition to depending on the plant organ, also depends on the choice of the appropriate extraction method [15]. In recent years, the use of enzymes in the extraction industries of plant bioactive compounds has received more attention due to their biological benefits; Because from the environmental point of view, the enzyme extraction process is also a green and environmentally friendly method. Enzyme extraction as an effective and new way to release bound compounds and increase total performance, by adding specific enzymes such as cellulase, hemicellulase and pectinase during the extraction process through breaking the cell wall and hydrolysis of polysaccharide can increase the recovery [10]. . In this study, the extraction of chlorophyll using enzyme and ultrasound methods from alfalfa plant is discussed.

2- Materials and methods

2- 1- Plant preparation

alfalfa plant (.*Medicago sativa L*) with the variety (Hamadani alfalfa) from Sari city located in Mazandaran province (Iran) in February 2018 and after confirming the scientific name, alfalfa plant leaves were dried and then powdered using a mill.

2- 2- Extraction method

2-2-1- Enzyme method

In enzymatic extraction, first one gram of alfalfa sample was mixed with 10 ml of aqueous buffer solution or acid buffer (pH=3.5) and homogenized for one minute. Then from the enzyme solution*PectineX Ultra SP L* (Novozyme, Denmark) containing a mixture of pectinase, cellulase and hemicellulase enzymes, with a concentration of 5%, was used to extract from the tampon solution prepared with alfalfa powder in water, for 120 minutes and at a temperature of 40 degrees Celsius [16].

2-2-2- Ultrasound method

For ultrasonic extraction, 100 grams of alfalfa was added to one liter of 96% ethanol (Merck, Germany) and at a frequency of 25 kHz and a power of 120 watts, a temperature of 35 °C and a time of 1 hour for extraction using an ultrasonic method (model S60 H (made by Elma, Germany) was used. The samples were centrifuged (Hettick, Germany) at a temperature of 4 degrees and around 8000 rpm for 5 minutes and the supernatant was collected [17].

2-3- Measurement of chlorophyll

To measure the amount of chlorophyll, the supernatant obtained after centrifugation in the previous steps and in any of the enzymatic or ultrasonic methods was diluted using 80% acetone (Merck, Germany). Then, the diluted samples were placed in one centimeter plastic cells for examination with a visible spectrophotometer (Hitachi, Japan). After that, it was placed in the visible spectrophotometer with ethanol solvent as a control and the investigated wavelengths were 663 and 645 nm. At the end, the absorption numbers in equations 1 and 2 were replaced and the amount of chlorophyll was calculated in terms of milligrams per gram of fresh weight of the sample [17].

Chlorophyll a = (19.3 × A663 -0.86 × A645) V/100W

Chlorophyll b = (19.3 × A645 -3.6 × A663) V/100W

A: Absorption of light at wavelengths of 663, 645

V: the volume of the filtered solution (the upper solution from the centrifuge)

W: fresh weight of the sample in grams

At this stage, a calibration curve was drawn by the device using different concentrations from the target sample. The optimal sample in terms of the amount of chlorophyll extracted with the desired solvent was prepared in different concentrations and was checked at the maximum wavelength where the samples showed a peak. The optimized sample and solvent were selected and entered the next phase of the experiment. The stable complex of

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prepared chlorophyllin samples was checked by phosphate buffer control at 405 nm wavelength, which is the absorption intensity of visible light of chlorophyllins.

2-4- Measurement of total phenolic compounds

In alfalfa extract, the total amount of phenolic compounds present was measured by spectroscopic method with Folin-Siocaltio reagent (Merck, Germany) and the results were expressed based on milliequivalents of gallic acid per gram [19].

5-2- Measurement of flavonoid compounds

Amounts of flavonoid compounds of alfalfa extract using the method of Devanto et al[2] done and the results were expressed based on milligrams per hundred grams of dry weight [20].

6-2- Antioxidant tests

2- 6- 1- Investigating DPPH free radical inhibition[3]

For this purpose, 1 ml of different concentrations of extract (ppm 500, 1000, 1500 and 2000) was added with 1 ml of 0.1 mM DPPH solution (Merck, Germany) and the resulting mixture was shaken well and kept in the room for 15 minutes. It was placed in the dark and then the optical absorption of the samples was read at a wavelength of 517 nm against a control. It should be mentioned that all these steps about[4]BHA was used as standard antioxidant [21].

= DPPH free radical inhibition percentage

100x (witness absorption/(sample absorption - control absorption))

2- 6- 2- Investigation of radical inhibition[5]ABTS

Evaluation of ABTS radical scavenging according to the study of Hay et al[6] It was done with a slight change. At first, 7 mM ABTS solution was mixed with 2.45 mM potassium persulfate and placed at room temperature and in a dark place for 16 hours. The working solution was prepared by diluting the initial solution in 80% ethanol so that the absorbance at 734 nm wavelength was read as 0.7. The solution prepared from chlorophyll was mixed with the final solution of ABTS and the absorbance was read at a wavelength of 734 nm [22].

2-6-3- Investigating the reductive power of iron (FRAP[7])

To check the reductive power of iron, 0.1 g of alfalfa extract was homogenized with 5 ml of distilled water in a cold porcelain mortar in an ice bath. The resulting homogenate was filtered using Whatman No. 1 filter paper, and then 50 microliters of the obtained extract was added to 1.5 milliliters of FRAP reagent (300 mM sodium acetate buffer with pH = 3.6, ferric-tripyridyl-s-triazine and ferric chloride). be added The resulting mixture was vortexed and incubated for 4 minutes at 30°C. The absorbance of the solutions was read at 593 nm compared to the control (containing 50 µl of distilled water with 1.5 ml of FRAP reagent). It should be noted that ammonium ferrous sulfate was used as a control for comparison [23].

2-7- Data analysis

Statistical analysis of data was done using SPSS version 22 software. One-way analysis of variance was used to evaluate the presence or absence of a significant difference at the 5% level between the measured values. It should be mentioned that in all stages of analysis and analysis, the permissible error for rejecting Ho was considered 5%.

3. Results and Discussion

3- 1- Chlorophyll values

The concentration of chlorophylls a and b extracted from alfalfa plant using ultrasonic and enzymatic methods in terms of milligrams per gram of wet weight can be seen in Figure 1, and if it can be seen, the concentration of chlorophyll a is higher than the concentration of chlorophyll b, and in the enzymatic method, more chlorophylls are extracted. became Also, according to the results of analysis of variance, no significant difference was observed between the values of chlorophylls a and b in enzymatic and ultrasonic extraction methods at the 5% probability level ($p < 0.05$).

Sabrian et al [24] showed in their studies on alfalfa that the amount of chlorophyll a is higher than chlorophyll b. Also, the research

2. Dewanto et al., 2002

3. 2,2-diphenyl-1-picrylhydrazyl

4. Butylated hydroxyanisole

5. 2, 2'-Azinobis-3-ethylbenzthiazoline-6-sulphonic acid

6. He et al., 2012

7. Ferric Reducing Antioxidant Power

results of Dias et al[8] [25] and Kong et al[9] [26] that on lettuce leaves and microalgae*Chlorella vulgaris* were carried out, showed that the amounts of chlorophyll a were higher than chlorophyll b.

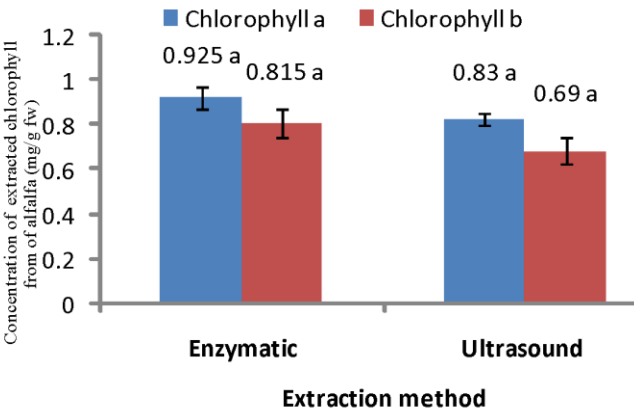

Fig 1 Concentration of extracted chlorophylls from alfalfa in two extraction methods.

2-3- Amounts of total phenol

In Figure (2), the results showed that with the increase in the concentration of the extract, the amount of total phenol in the alfalfa plant extract increases. Although in low concentration of the extract i.e. 300 µM, the ultrasound method had a better performance, but the amount of total phenol with the increase in the concentration of the extract indicated the improvement of the performance of the enzyme method. The results of the analysis of variance also showed a significant difference at the 5% level between different concentrations of the extract to evaluate the amounts of total phenol in each of the mentioned extraction methods ($p < 0.05$).

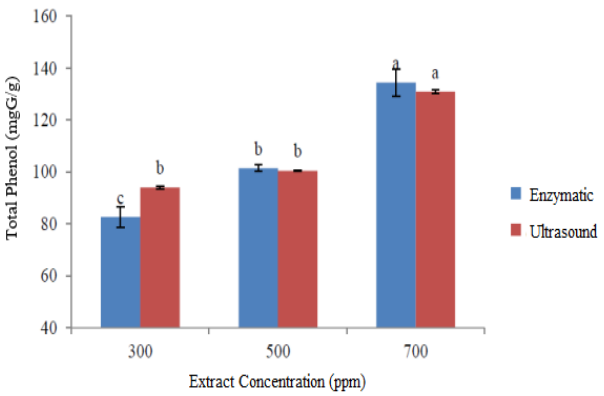

Fig 2 Total phenol of total extracted chlorophyll from alfalfa by different methods.

3- 4- Quantities of flavonoid compounds

In Figure (3), the amount of flavonoid compounds in alfalfa plant extract increases with the increase of different concentrations of enzyme extracts and ultrasound of this plant, and its amount is higher in enzyme method compared to ultrasound method. In each of the extraction methods, the analysis of variance evaluation results showed a significant difference at the 5% level between different concentrations of the extract to evaluate the amounts of flavonoid compounds ($p < 0.05$).

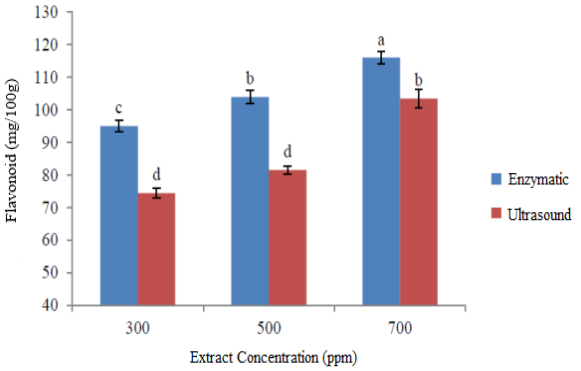

Fig 3 Total flavonoid of total extracted chlorophyll from alfalfa by different methods.

5-3- The results of antioxidant tests

The results of the antioxidant effects of alfalfa plant extract with DPPH free radical inhibition tests and iron reduction power are described in figures (4) and (5) and show a direct relationship between the concentration of extracts extracted from alfalfa by enzymatic and ultrasound methods and their antioxidant activity, and of course, the method Enzyme has been associated with better performance. The

8. Dias et al., 2014

9. Kong et al., 2012

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results of analysis of variance for both mentioned methods indicated the rejection of Ho and indicated a significant difference at the 5% level between different concentrations of the extract in the evaluation of antioxidant activity ($p < 0.05$). In the research of Bora and Sharma [27] and Al-Dassari[10] [28] Also, with the increase in concentration of alfalfa extract, the DPPH free radical inhibition percentage increases. Rana et al[11] [29] also investigated the antioxidant properties of alfalfa plant and the results showed that with increasing concentration of alfalfa extract, the level of inhibition of DPPH and ABTS free radicals and the power of reducing iron increases. Kamali et al. [30] also in their research on the medicinal plant Zarin (*Dracocephalum kotschyi*) showed that increasing the concentration of the extract has a significant effect on free radical inhibition, and with increasing concentration, an increase in free radical inhibition was observed, and synthetic antioxidants showed higher antioxidant activity. These results were similar to the achievement of Shariatifar [31] on Hayza grass plant.

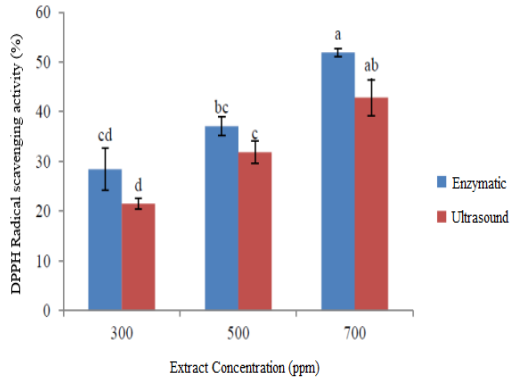


Fig 4 DPPH Radical scavenging activity of different concentration of extracted chlorophyll from alfalfa by different methods.

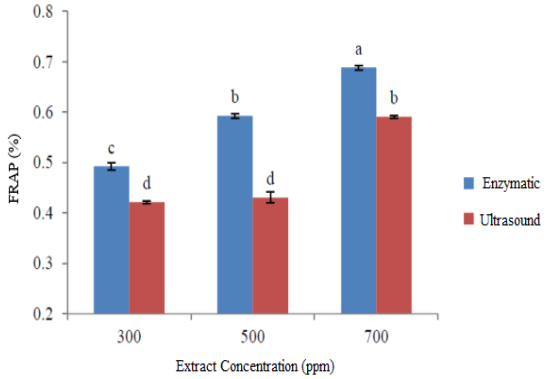


Fig 5 FRAP of different concentration of extracted chlorophyll from alfalfa by different methods.

According to the previous graphs and comparing the performance of enzymatic and ultrasonic extraction methods, it has been concluded that the enzymatic method has a better performance; Therefore, chlorophylls extracted by enzymatic method have been used in the evaluation of antioxidant properties. The results of evaluating the antioxidant effect of different concentrations of total chlorophyll extracted from alfalfa plant in the form of measuring DPPH free radical inhibition, ABTS radical inhibition and iron reduction power can be seen in figures (6) to (8) respectively. According to these graphs, the antioxidant activity was higher in higher concentrations of extracted chlorophyll. The results of the analysis of variance also indicated the rejection of Ho and indicated a significant difference at the 5% level between different concentrations of total chlorophyll to evaluate the antioxidant activity ($p < 0.05$) and showed that all groups differed with each other at a significant level of 5%.

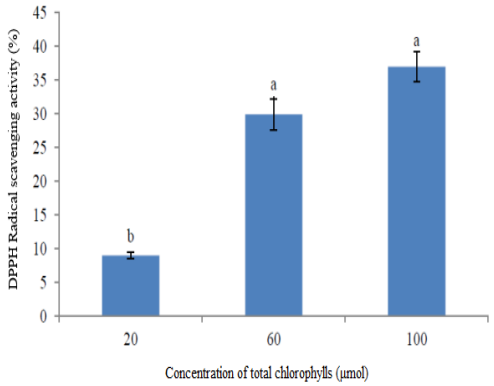
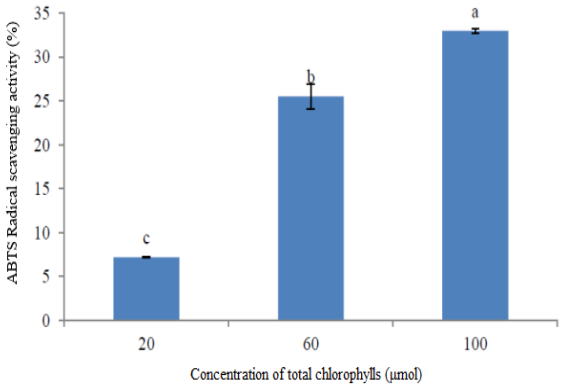


Fig 6 DPPH Radical scavenging activity of different concentration of total chlorophyll concentration.



[10] Al-Dosari, 2012

[11] Rana et al., 2010

Fig 7 ABTS Radical scavenging activity of different concentration of total chlorophyll concentration.

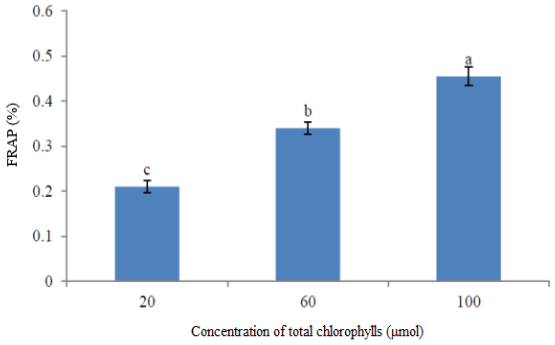


Fig 8 FRAP of different concentration of total chlorophyll concentration.

4- Discussion

The results of this research based on enzymatic extraction and ultrasonic extraction from alfalfa showed that the concentration of chlorophyll a was higher than the concentration of chlorophyll b. Enzyme method has more advantages and in higher concentrations of this plant extract and chlorophyll extracted from it, the antioxidant activity, i.e. DPPH free radical inhibition and iron reduction power and ABTS free radical inhibition are also higher. The results of this experiment showed that the antioxidant activity of the samples has a direct relationship with the amount of total phenolic and flavonoid compounds. Some studies also showed that the high content of phenolic compounds is the main reason for the high antioxidant activity of some extracts, including polar extracts [31]. Phenolic compounds act effectively as hydrogen donors, so they are considered as an effective antioxidant [32].

Alfalfa plant extract also contains phenolic, anthocyanin and flavonoid compounds, which have antioxidant properties, and according to the results of this research, chlorophyll extracted by an enzymatic method from this plant showed a more significant antioxidant effect, and the enzyme extraction method can be used as a stimulus to increase the amount of chlorophyll. did Phenolic compounds with high molecular weight have a great ability to scavenge free radicals, and the different antioxidant activity and free radical inhibition may be partially related to the wide variety of antioxidant substances such as phenols, ascorbate and carotenoids.

Flavonoids are secondary metabolites that protect plants against ultraviolet rays, pathogens and herbivores due to the creation of a defense mechanism. Flavonoids have antioxidant properties and play a role in regulating enzyme activities and producing primary metabolites [33]. Considering the importance and many uses of secondary metabolites in modern human life, investigating the sources of secondary metabolites in plants as organic sources can be very useful [34]. The increase of phenolic compounds is also directly related to the increase of antioxidant properties, and therefore, alfalfa plant can be used as a source of secondary metabolites in the pharmaceutical and food industries due to its antioxidant properties and due to the presence of anthosanins, flavonoids and phenolic compounds. take It can also be used as a source of natural antioxidants in food and pharmaceutical industries.

5- General conclusion

In this research, chlorophyll pigment and alfalfa extract were used to perform antioxidant and antimicrobial tests. Enzymatic and ultrasound methods were used for extraction. The results showed that in the enzymatic method, the measured values for total phenol and flavonoid compounds of the extract were higher and also the antioxidant performance of the extract was more significant. For this purpose, the enzyme method was used as the basis of the work and this method was used to evaluate the stability and measure the antioxidant and antibacterial power of alfalfa chlorophyll. In measuring total phenol compounds and flavonoids in the extract, the results of the enzyme method were more favorable and the increase of all these variables were in line with the increase in the concentration of the extract. The enzyme method in evaluating the antioxidant properties of the extract, i.e., DPPH free radical inhibition and iron reduction power, is more favorable than the ultrasound method, and with the increase in the concentration of the extract, the antioxidant property also increases.

6- Resources

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مقایسه ویژگی‌های آنتی‌اکسیدانی کلروفیل استخراج‌شده از گیاه یونجه (*Medicago sativa* L.) با بهره‌گیری از روش‌های استخراج آنزیمی و فراصوت

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چکیده

امروزه از رنگ‌دانه‌های طبیعی به‌صورت گسترده‌ای در صنایع غذایی، آرایشی و بهداشتی استفاده می‌گردد و طی سال‌های اخیر تحقیقات متعددی پیرامون روش‌های استخراج و بررسی خواص رنگ‌دانه‌های طبیعی انجام یافت. هدف از این تحقیق نیز مقایسه ویژگی‌های آنتی‌اکسیدانی کلروفیل استخراج‌شده از گیاه یونجه (*Medicago sativa* L.) با بهره‌گیری از روش‌های استخراج آنزیمی و فراصوت می‌باشد. فعالیت آنتی‌اکسیدانی با سه روش مهار رادیکال‌های آزاد DPPH و ABTS و قدرت احیاکنندگی آهن (FRAP) انجام گرفت. نتایج نشان داد که غلظت کلروفیل a در گیاه یونجه بیشتر از غلظت کلروفیل b بوده است و همچنین روش آنزیمی نیز عملکرد بالاتری در استخراج کلروفیل نشان داده است. علاوه براین غلظت‌های بالاتر عصاره یونجه فعالیت آنتی‌اکسیدانی بیشتری در مهار رادیکال آزاد DPPH و نیز قدرت احیاکنندگی آهن نشان داد (p.<۰/۰۵). همچنین، با افزایش غلظت عصاره، مقدار ترکیبات فنلی کل و ترکیبات فلاونوئیدی افزایش یافت (p.<۰/۰۵). به دلیل عملکرد مطلوب‌تر، از کلروفیل استخراج‌شده به روش آنزیمی در ارزیابی خواص آنتی‌اکسیدانی یعنی مهار رادیکال‌های آزاد DPPH و ATBS و نیز قدرت احیاکنندگی آهن استفاده گردید و با افزایش غلظت کلروفیل نیز خواص آنتی-اکسیدانی افزایش یافت. با توجه به اثر آنتی‌اکسیدانی مطلوب کلروفیل استخراج‌شده به روش آنزیمی از گیاه یونجه می‌توان کاربرد آن را در صنایع غذایی، دارویی و بهداشتی مورد ارزیابی قرار داد و با استفاده از این تکنیک سامانه‌های استخراجی در مقیاس صنعتی طراحی نمود.