ANTIOXIDANT AND LIPID PEROXIDANTION INHIBITORY OF COMPOUNDS IN COMMON BUCKWHEAT

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ABSTRACT

Common Buckwheat (*Fagopyrum esculentum* Moench) is recognized as a healthy food in many countries because it is nutrient-rich crop. The objectives of this study was to investigate the succesful farming for buckwheat plants in the Iraqi environmental conditions, extraction and estimation of the most important nutritional and medicinal compounds from seeds plant and it's effectiveness in the inhibition of lipids peroxidation which considered to be the causative agent of the free radicals formation *in vivo*.The hexane and methanolic extraction gave many compounds from buckwheat seeds. NMR were used to determine many of the natural compounds for buckwheat plants seeds after many steps of the purification processes, using many solutions and mobile phases including: Linoleic acid, Triglyceride, Stearic acid, Ferulic acid, Flavonoid glycoside and Flavon glycoside. Compounds Linolieic acid, Ferulic acid, Flavonoid glycoside and Flavon glycoside showed activity values of 0.285, 0.362, 0.356 and 0.442 respectively at 250 µg/mL concentration in the MTT assay and inhibited LPO by 58, 73, 93 and 76% respectively at 250 µg/mL concentration. It is clear that buckwheat have strong antioxidant and lipid Peroxidation inhibitory.

Keywords: Common Buckwheat, Antioxidant, Lipid Peroxidation inhibitory.

تحديد قدرة المركبات النقية المستخلصة من نبات الحنطة السوداء في تثبيط تكون الجذور الحرة وبيروكسدة الدهون

شامل إسماعيل نعمة

مدرس

مركز دراسات الصحراء/ جامعة الأنبار

المستخلص

يُعد نبات الحنطة السوداء من المحاصيل المنتشرة زراعته في العديد من بلدان العالم لأهميته في مجالي التغذية والصناعة الدوائية على حدٍ سواء. هدفت الدراسة على تحديد إمكانية إنجاح زراعة نباتات الحنطة السوداء في الظروف البيئية العراقية واستخلاص أهم المركبات الطبيعية من بذور النبات لتحديد محتوى تلك المستخلصات من مضادات الأكسدة النباتية ومدى فعاليتها في تثبيط تكون بيروكسيد الدهون أحد العوامل المسببة لتكوين الجذورالحرة داخل الجسم الحي، أستعمل محلولي الهكسان والميثانول كمحاليل لإستخلاص المركبات النقية من بذور نبات الحنطة السوداء. إستعمل جهاز الرنين المغناطيسي النووي في تشخيص العديد من المركبات النقية المعزولة من بذور نبات الحنطة السوداء والمستخدم فيها الهكسان والميثانول كمحاليل إستخلاص واشتملت تلك المركبات على حامض اللينوليك والحامض الدهني الثلاثي وحامض الستريك وحامض الفيريوليك وكلايكوسيد الفلافونيد وكلايكوسيد الفلافون. أظهرت النتائج وجود فعالية عالية لمركبات حامض اللينوليك وحامض الفيريوليك وكلايكوسيد الفلافونيد وكلايكوسيد الفلافون في تثبيط تكون الجذور الحرة، إذ أعطى كلاً منها قيمة بلغت 0.285 و 0.362 و 0.356 و 0.442 بالتتابع في حين أعطت ذات المركبات قيمً لتثبيط تكون بيروكسيد الدهون بلغت 58 و 73 و 93 و 76% بالتتابع. يتضح لنا بأن الحنطة السوداء تمتلك مضادات أكسدة قوية ومثبطات لبيروكسدة الدهون.

الكلمات المفتاحية: الحنطة السوداء العادية، مضادات الأكسدة، تثبيط بيروكسيد الدهون.

INTERODUCTION

Common buckwheat (*Fagopyrum esculentum* Moench) is a major healthcare food reso - urce, which is rich in protein, fat, starch, mineral elements (6) as well as phenolic compounds (1), flavonoids, vitamin E (17) and amino acids (3). The clinical researches showed that buckwheat could reduce the contents of fat and sugar as well as cholesterol in blood, therefore could benefit the diabetes patients etc. Nowadays is considerable interest in the consumption of alternative crops as potential recipes for gluten-free products production too. Therefore, the use of buck - wheat for production of gluten-free pasta can have great interest for food industry.

Buckwheat seeds and hulls contain compo - nends with healing properties and biological activity, such as flavonoids and flavon, phen - olic acid, condensed tannins, phytosterols and fagopyrins. Phenolic compounds comprise important secondary metabolites with many physiological functions including antioxidant, antitumor, anti-inflammatory and free radical scavenging activities (15, 16). Flavonoids are phytonutrients with chelating properties, acting as antioxidants inhibiting lipid peroxi - dation, chelate redox-active metals and atten - uate reactive oxygen species (ROS) damages (4). Buckwheat's flavonoid compounds decr - ease blood cholesterol, helping the reduction of a high blood pressure. In addition, buckwheat reduces cellular proliferation and therefore it protects the colon against carcinog -enesis (9). The antioxidant activity in buckw - heat exhibited a statistically significant relation -nship with its total phenolics, as well as rutin content (5).In this study, antioxidant and anti-inflam matory activities of pure isolates when used the hexane and methanolic the solvent of extracting from buckwheat seeds were determined using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide MTT (10, 11) lipid peroxidation LPO (2, 12). Also, to show the purification, structure elucidation and bioactivity studies of extracts isolates as a measure to determine their functional food quality.

MATERIALS AND METHODS

General Experimental Procedures: All solvents used for isolation and purification were of ACS reagent grade (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Silica gel plates (250 and 500 µm; Analtech, Inc., Newark, DE, USA). TLC plates were viewed under UV light at 254 and 366 nm in a Spectroline CX-20 ultraviolet fluorescence analysis cabinet (Spectroline Corp., Westbury, NY, USA) and sprayed with 10% sulphuric acid solution. NMR spectra were recorded on a 500 MH_Z (Varian Unity ±500, ^1H NMR). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphe - nyltetrazolium bromide], (TBHQ) *tert*-butyl - hydroquinone, (BHA) butylated hydrox - yanisole, (BHT) butylated hydroxytoluene. The fluorescent probe 3-[p-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid was purch - ased from Molecular Probes (Eugene, OR, USA). All reagents were stored in Laboratory at Michigan State University-USA. MTT antioxidant activity was tested on a Bio-Tek Elx800 universal microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). LPO assay was tested on a Turner model 450 fluorometer (Barnstead/ Thermolyne Corp., Dubuque, IA, USA).

Extraction and Isolation: The dried weight (170 g) from seeds of Common buckwheat *Fagopyrum esculentum* Moench grown in Baghdad- Iraq. From this, samples were packed in cardboard boxes and sent to Baghdad for onward shipment to Michigan State University. The dry weight (170 g) from seeds sample was packed in a glass column and eluted sequentially with hexane (1 L) and methanolic (1 L). The evaporation of organic solvent under vacuum at 35 °C afforded seeds by hexane (3.79 g) and methanolic extracts (5.55 g).An aliquot of seeds extract by hexane (250 mg) was fractionated by silica gel vacuum liquid chromatography (VLC) and eluted under gradient conditions using hexane/acetone (10:1, 5:1, 1:1, v/v). the fractions collected were A, 29 mg; B, 114.8 mg and C, 106.2 mg.An aliquot of fraction A (25 mg) was purified by preparative TLC (hexane/acetone, 20:1, v/v; and $CHCl_3$/MeOH, 200:1, v/v) to yield 1 (7.2 mg). An aliquot of fraction B (100 mg) was purified by preparative TLC (hexane/ acetone, 15:1, v/v; and $CHCl_3$/MeOH, 200:1, v/v) to yield compounds 2 (5.3 mg). An aliquot of fraction C (100 mg) was purified by preparative TLC

($CHCl_3$/MeOH, 10:1, v/v) to yield compounds 3 (8.4 mg). An aliquot of the methanolic extract D (250 mg) was fractionated by MPLC (C18 column) eluted under gradient conditions using MeOH/H_2O (gradient elution, 1:9, 3:7, 5:5, 7:3, 9:1, v/v) and finally with MeOH (100%). the fractions collected were E, 33.1 mg; F, 104.4 mg and G, 112.5 mg. Compound 4 (3.5 mg) was isolated from fraction E by preparative TLC ($CHCl_3$/MeOH, 5:1, v/v). An aliquot of fraction F (12.7 mg) was purified by preparative TLC ($CHCl_3$/MeOH/H_2O, 4:1:0.1 v/v) to afford compound 5. An aliquot of fraction G (3.0 mg) was purified by preparative TLC ($CHCl_3$/MeOH/ H_2O, 5:4:0.1 v/v) to yield compound 6 (Figure 1).

Compound 1:colorless oil (13)
(9*Z*, 12*Z*)-octadeca-9,12-dienoic acid
^{1}H-NMR spectral data identical to those of Linoleic acid (13).

Compound 2: Colorless oil (14)
^{1}H-NMR spectral data identical to those of Triglyceride (14).

Compound 3: Colorless oil (13)
^{1}H-NMR spectral data identical to those of Stearic acid (13).

Compound 4: Amorphous powder (17)
(*E*)-3-(4-hydroxy-3-methoxyphenyl) acrylic acid
^{1}H-NMR spectral data identical to those of Ferulic acid (17).

Compound 5: Yellow powder (7, 18)
2-(3,4-dihydroxyphenyl)-7-hydroxy-5-((3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yloxy)-4*H*-chromen-4-one
^{1}H-NMR spectral data identical to those of Flavonoid glycoside (7, 18).

MTT Antioxidant Assay: MTT assay was performed according to our previous report (10, 11). Stock solutions of test extracts and positive controls (TBHQ) were prepared in DMSO (10 mg/mL for extracts and 1 mg/mL for controls). An aliquot of 10 µL of test samples, 190 µL of MTT water solution (1 mg/mL) and 800 µL of DMSO were vortexed in a capped glass vial (2 mL) for 24 h.

Compound 6: Yellow powder (8) (4-oxo-3-phenyl-4*H*-chro -men-6-yl) holmium ^{1}H-NMR spectral data identical to those of Flavon glycoside (8).

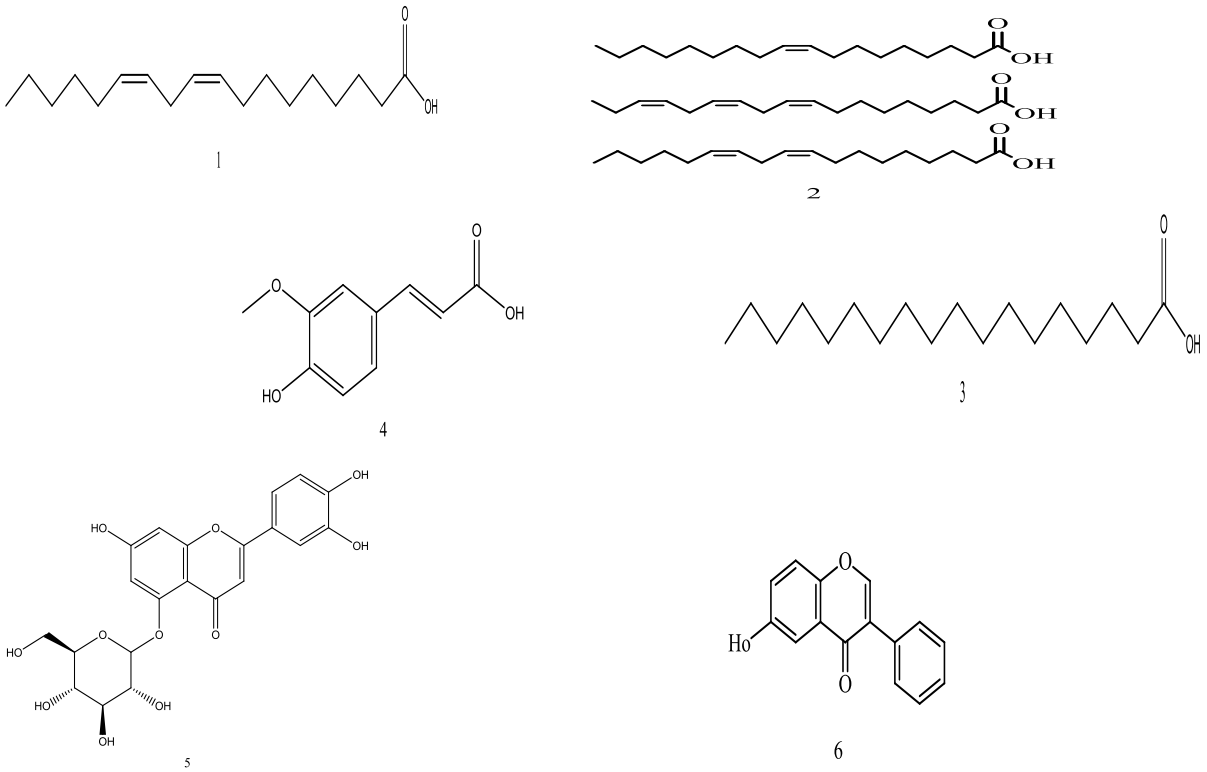

Figure 1. Structures of pure isolates compounds from Common buckwheat: Linoleic acid (1), Triglyceride (2), Stearic acid (3), Ferulic acid (4), Flavonoid glycoside (5) and Flavon glycoside (6).

for 1 min, which was then incubated at 37°C An aliquot (200 µL) of the reaction mixture was pipetted to a 96-well cell culture plate and the absorbance was at 570 nm. in duplicate on a Bio-Tek Elx800 universal microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

Lipid Peroxidation inhibitory Assay:

The extract 250 µg/mL and positive controls (TBHQ at 10 µM) were tested for lipid peroxidation (LPO) inhibitory activities by using fluorescence spectroscopy on a Turner model 450 fluorometer (Barnstead/ Therm - olyne Corp.) according to the reported proc - edure (2,12). The liposome, unilamellar vesi - cles (ULV), was prepared according to the published procedure. The peroxidation was initiated by the addition of 20 µL of $FeCl_2.4H_2O$ (0.5 mM) to the assay mixture [HEPES (100 µL), 1 M NaCl (200 µL), N_2-sparged Millipore water (1.64 mL), DMSO or test sample (20 µL) and 20 µL of liposome suspension. The fluorescence was monitored at 0, 1 and 3 min. and every 3 min. thereafter up to 21 min. The decrease in fluorescence intensity over time (21 min.) indicated the rate of peroxidation. Each sample was assayed in duplicate, and the percent inhibition was calculated with respect to DMSO control.

RESULTS AND DISCUSSION

Buckwheat seeds used in this study was brown in color. To determine the accurate dry weight of plant materials used for extraction. The yield of extract was minute in quantity and combined on the basis of the TLC profiles prior to purification. The methanolic extract of seeds contained trace amounts of components by TLC. Before purification extracts were evaluated for their antioxidant activities (Figure 1). We routinely use MTT and LPO assay to determine the antioxidant activity of natural extracts and hence detects most antioxidant compounds that are reducing agents. On the other hand, inhibition of LPO detects free radical scavenging capacity of extracts and test compounds. Biochemical reactions *in vivo* generate free radicals. The reaction of free radicals with lipids, protein and nucleic acids result in oxidative damage and leads to a number of diseases including cancer, cardiovascular disease and arthritis (2, 11). Antioxidants scavenge these free radicals generated *in vivo* and prevent such unwanted biochemical reactions. Similarly inflammation signaling pathways produse intermediates or inflammation causing hormones.

The pure isolates were tested for antioxidant activity. The MTT assay results of compounds 1-6 showed absorbance values of 0.285, 0.157, 0.133, 0.362, 0.356 and 0.442 at 570nm, respectively at 250 µg/mL concentration. While compound 3, steric acid showed little activity as indicated by the poor absorbance value of 0.133 at 250 µg/mL concentration. Vitamine C was the most active and showed an absorbance value of 0.48 at 25 µg/mL similar to the activity of fraction K and TBHQ (Figure 2). The LPO inhibition allowed us to evaluate the radical scavenging capacity of test samples. Compound 5 showed the strongest LPO inhibition by 93% and 4,6 showed 73, 76% at 250 µg/mL, concentration. Linolieic acid 1, also showed moderate LPO inhibition by 58% at 250 µg/mL concentration. TBHQ was the most active and showed inhibition value of 90% at 10 µg/mL similar to the inhibition of BHA and BHT (Figure 3).

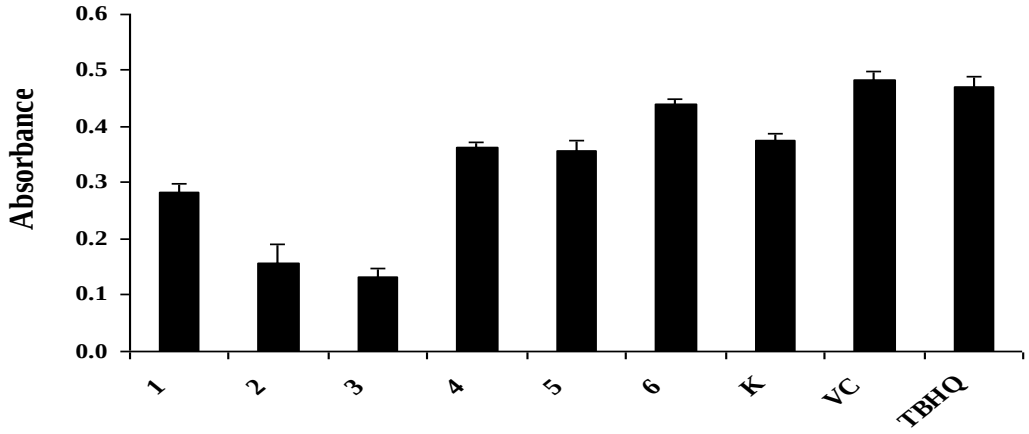

Figure 2. Absorbance values at 570 nm of compounds at 250 µg/mL obtained after reaction with MTT at 37 °C. Vitamin C and TBHQ were used as positive controls at 25 µg/mL.

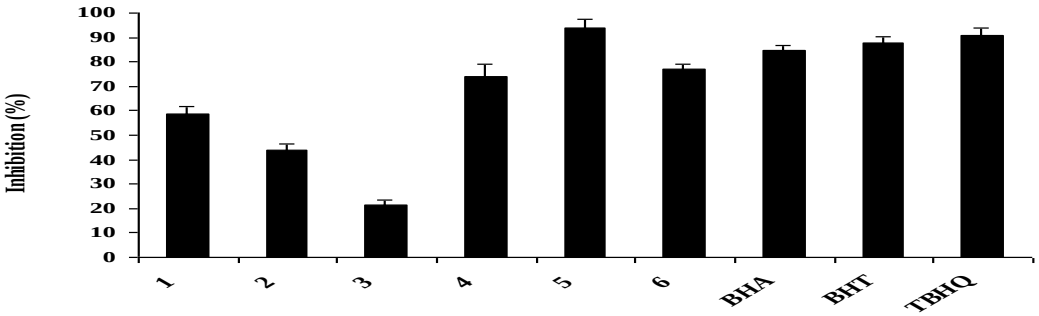

Figure 3. Inhibition of LPO by compounds at 250 µg/mL. Commercial antioxidants BHA, BHT and TBHQ were tested at 10 µM. The oxidation of lipid was initiated by the addition of Fe^{2+} ions. The varying concentrations of positive controls used in these assays were to yield comparable activity profiles between 0 and 100% by test extracts and positive controls alike. Vertical bars represent the standard deviation of each data point (*n*= 2).

ABBREVIATIONS USED

MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diph - enyltetrazolium bromide; LPO, lipid perox - idation; TLC, thin-layer chromatography; UV, ultraviolet; NMR, nuclear magnetic resonance; TBHQ, *tert*- butylhydroquinone; BHA, butylated hydroxyanisolene; BHT, butylated hydroxytoluene; DMSO, dimethylsulfoxide.

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