

Antioxidant properties of 6-methoxyflavonol glycosides from the aerial parts of *Chenopodium bonus-henricus* L.

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Dedicated to Acad. Ivan Juchnovski on the occasion of his 80th birthday

Radical scavenging and antioxidant activities of nine flavonoids previously isolated from the aerial parts of *Chenopodium bonus-henricus* L. were established using DPPH and ABTS free radicals as well as inhibition of lipid peroxidation in a linoleic acid system by the ferric thiocyanate method. Patuletin glycosides, compounds **1**, **2** and **7** showed the highest DPPH (85.78%, 85.59%, 86.07%) and ABTS activity (90.64%, 90.27%, 90.76%) compared to Vitamin C. Spinacetin and 6-methoxykaempferol glycosides compounds **4**, **6** and **3**, **5** respectively possessed a significantly low DPPH activity and demonstrated a high ABTS radical-scavenging activity. Spinacetin and 6-methoxykaempferol glycosides, compounds **8** and **9**, containing esterified ferulic acid in their moiety, demonstrated a moderate DPPH (53.46%, 45.52%) and a high ABTS (83.41%, 74.51%) activity. All flavonoids inhibited significantly the lipid peroxidation of linoleic acid. The results suggest that the aerial parts of *C. bonus-henricus* L. could be a potential source of flavonoids with radical-scavenging and antioxidant activities..

Key words: *Chenopodium bonus-henricus*; flavonoids; DPPH; ABTS; FTC assays

INTRODUCTION

The human body has a complex system of natural enzymatic and non-enzymatic antioxidant defenses which counteract the harmful effects of free radicals and other oxidants. Free radicals are responsible for causing a large number of diseases including cancer [1], cardiovascular disease [2], neural disorders [3], Alzheimer's disease [4], mild cognitive impairment [5], Parkinson's disease, alcohol induced liver disease [6], ulcerative colitis [7] and atherosclerosis [8]. Protection against free radicals can be enhanced by ample intake of dietary antioxidants. Foods containing antioxidants and the antioxidant nutrients may be of major importance in disease prevention. On the other hand the most widely used synthetic antioxidants in food (butylated hydroxytoluene BHT, butylated hydroxyanisole BHA, propyl galate PG and tertiary butylhydroquinone TBHQ) have been suspected to cause or promote negative health effects. For this reason there is a growing interest in utilization of natural sources as antioxidants. *Chenopodium bonus-henricus* L. (Amaranthaceae) is widespread in Europe, western Asia and North America. The leaves and flowering tops of Good King Henry are used as a vegetable in the same manner as spinach in some European traditional cuisines. In Italy,

Spain and England it is used in soups or stews and roughly in salads. In Turkey it is known as "yabanı ispanak" (wild spinach). Canadians have also cultivated the plant as a daily vegetable. The shoots and flower clusters are eaten respectively like asparagus and broccoli [9].

In Bulgarian folk medicine the roots of *C. bonus-henricus* are known as "chuyen" and have been applied externally to treat skin inflammations, wounds and boils. The infusion of the drug has been used as a mild laxative. In Bulgarian food industry the aqueous extract of the roots has been employed in production of halva [10].

Nine flavonol glycosides of patuletin, 6-methoxykaempferol and spinacetin were recently isolated from the aerial part of *C. bonus-henricus*. All flavonoids (100 μ M), compared to silybin, significantly reduced the cellular damage caused by CCl_4 in rat hepatocytes, preserved cell viability and GSH level, decreased LDH leakage and reduced lipid damage. High concentrations of compounds showed marginal or no cytotoxicity on HepG2 cell line. The experiment data suggest that the glycosides of 6-methoxykaempferol, spinacetin and patuletin are a promising and safe class of hepatoprotective agents [11]. These results stimulated us also to investigate the radical-scavenging and antioxidant activities of these flavonoids using DPPH and ABTS free radicals and

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inhibition of lipid peroxidation in a linoleic acid system by the ferric thiocyanate method (FTC assay).

EXPERIMENTAL

General experimental procedures, chemicals and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma-Aldrich (Steinheim, Germany). Linoleic acid (99%) was obtained from Acros organics (Geel, Belgium). Potassium persulphate, Vitamin C (200 mg/2 ml) and ammonium thiocyanate were purchased respectively from Valerus (Sofia, Bulgaria), Sopharma (Sofia, Bulgaria) and Fisher Chemicals (Loughborough, United Kingdom). All chemicals, including the solvents were of analytical grade. UV-VIS, Biochrom Libra S70 (Cambourne, United Kingdom) was used to measure the absorbance.

DPPH radical-scavenging assay

Scavenging activity of flavonoids against DPPH radical was assessed according to the method of Blois with some modifications [12]. Briefly, 0.3 mL of each compound in MeOH (0.1 mM) was mixed with 0.3 mL of DPPH methanol solution (0.1 mM). The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm. The ability to scavenge DPPH radical was calculated by the following equation:

$$\% \text{DPPH radical scavenging activity} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100,$$

where $\text{Abs}_{\text{control}}$ is the absorbance of DPPH radical in MeOH, $\text{Abs}_{\text{sample}}$ is the absorbance of DPPH radical solution mixed with a sample. Vitamin C (0.1 mM) was used as a positive control. All determinations were performed in triplicate (n=3).

ABTS radical-scavenging assay

For ABTS assay, the procedure followed the method of Kokanova-Nedialkova *et al.* [13] with some modifications. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulphate solution. The working solution was then prepared by mixing the two stock solutions in

equal quantities and allowing them to react for 12-14 h at room temperature in the dark. The solution was then diluted by mixing 1 mL ABTS solution with 60 mL methanol. A fresh ABTS solution was prepared for each assay. 0.2 mL of each compound in MeOH (0.1 mM) was allowed to react with 1 mL of the ABTS solution and the absorbance was taken at 734 nm after 7 min using a spectrophotometer. The ABTS scavenging capacity of the compound was calculated as:

$$\% \text{ABTS radical scavenging activity} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100,$$

where $\text{Abs}_{\text{control}}$ is the absorbance of ABTS radical in methanol; $\text{Abs}_{\text{sample}}$ is the absorbance of an ABTS radical solution mixed with a sample. Vitamin C (0.1 mM) was used as a positive control. All determinations were performed in triplicate (n=3).

Determination of antioxidant activity in linoleic acid system by the FTC method

The antioxidant activity of studied compound (0.1 mM) against lipid peroxidation was measured through ferric thiocyanate assay, as described by Takao *et al.* [14] with some modifications. The reaction solution, containing 0.2 mL of each compound in MeOH (0.1 mM), 0.2 mL of linoleic acid emulsions (25 mg/mL in 99% ethanol), and 0.4 mL of 50 mM phosphate buffer (pH 7.4), was incubated in the dark at 40°C. A 0.05 mL aliquot of the reaction solution was then added to 1.5 mL of 70% (v/v) ethanol and 0.1 mL of 30% (w/v) ammonium thiocyanate. Precisely 3 min after the addition of 0.1 mL of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance of the resulting red color was measured at 500 nm. Aliquots were assayed every 24 h until the day after the absorbance of the control solution (without compound) reached maximum value. Vitamin C (0.1 mM) was used as a positive control. All determinations were performed in triplicate (n=3).

Statistical analysis

Statistical program "MEDCALC" was used for analysis of the data. The results were expressed as mean (\pm standard deviation, SD) of three independent experiments, each performed in triplicate.

RESULTS AND DISCUSSION

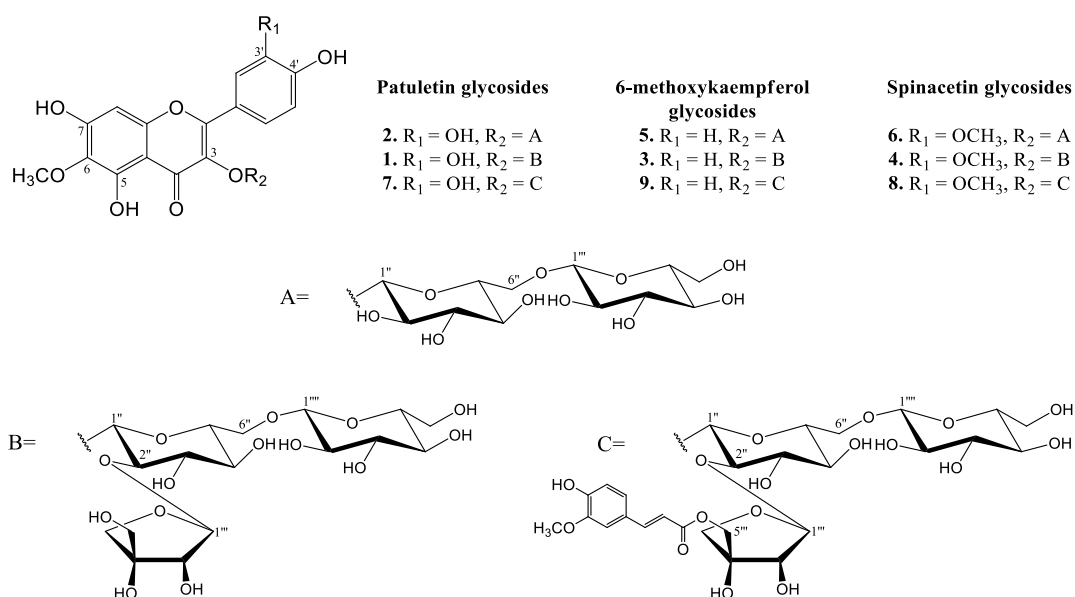
In the present study nine flavonoids, respectively patuletin 3-O- $[\beta$ -apiofuranosyl(1 \rightarrow 2)]- β -glucopyranosyl(1 \rightarrow 6)- β -glucopyranoside **1**, patuletin 3-O-gentiobioside **2**, 6-methoxykaempferol 3-O- $[\beta$ -apiofuranosyl(1 \rightarrow 2)]- β -glucopyranosyl(1 \rightarrow 6)- β -glucopyranoside **3**, spinacetin 3-O- $[\beta$ -apiofuranosyl(1 \rightarrow 2)]- β -glucopyranosyl(1 \rightarrow 6)- β -glucopyranoside **4**, 6-methoxykaempferol 3-O-gentiobioside **5**, spinacetin 3-O-gentiobioside **6**, patuletin-3-O-(5'''-O-E-feruloyl)- β -D-apiofuranosyl(1 \rightarrow 2)[β -D-glucopyranosyl (1 \rightarrow 6)]- β -D-glucopyranoside **7**, spinacetin-3-O-(5'''-O-E-feruloyl)- β -D-apiofuranosyl (1 \rightarrow 2)[β -D-glucopyranosyl(1 \rightarrow 6)]- β -D-glucopyranoside **8** and 6-methoxykaempferol-3-O-(5'''-O-E-feruloyl)- β -D-apiofuranosyl(1 \rightarrow 2)[β -D-glucopyranosyl (1 \rightarrow 6)]- β -D-glucopyranoside **9** (Fig. 1) were tested for their radical-scavenging and antioxidant activities. The radical-scavenging activities of compounds **1-9** (0.1 mM in MeOH) were compared with those of ascorbic acid at the same concentration (0.1 mM in MeOH) and expressed as % of inhibition against DPPH and ABTS (Table 1). There are three criteria for effective radical scavenging activity of flavonoids. The first important criteria is the presence of o-dihydroxy structure in the B ring, which confers higher stability to the radical form and participates in electron delocalization. The 2,3 double bond in conjugation with a 4-oxo function in the C ring is responsible for electron delocalization from the B ring.

Table 1. DPPH and ABTS radical-scavenging activities of flavonoids **1-9**.

Compound	DPPH %	ABTS %
1	85.78 \pm 0.05	90.64 \pm 0.05
2	85.59 \pm 0.36	90.27 \pm 0.09
3	7.16 \pm 0.44	70.18 \pm 0.67
4	13.56 \pm 0.05	71.81 \pm 0.38
5	4.13 \pm 0.54	70.94 \pm 0.47
6	14.62 \pm 0.13	74.41 \pm 0.01
7	86.07 \pm 0.40	90.76 \pm 0.16
8	53.46 \pm 0.53	83.41 \pm 0.65
9	45.52 \pm 0.52	74.51 \pm 0.30
Ascorbic acid	86.46 \pm 0.07	90.79 \pm 0.19

The antioxidant potency is related to structure in terms of electron delocalization of the aromatic nucleus. Where these compounds react with free radicals, the phenoxyl radicals produced are stabilized by the resonance effect of the aromatic nucleus. The third important criteria for a maximum radical scavenging potential is the presence of the 3- and 5-OH groups with 4-oxo function in A and C rings. Rice-Evans *et al.* also reported that the glycosylation of flavonoids reduces their activity when compared to the corresponding aglycones [15].

Patuletin glycosides, compounds **1**, **2** and **7** showed the highest DPPH (85.78, 85.59 and 86.07 %) activity respectively, compared to ascorbic acid (86.46 %) (Table 1). The compounds **1**, **2**, **7** possess 3', 4'-ortho-dihydroxy configuration in B ring, C2-C3 double bond configured with a 4-keto arrangement and a presence of free 5-OH and 7-OH groups, which determine their radical-scavenging activities.

**Fig. 1.** 6-Methoxyflavonol glycosides from the aerial parts of *Chenopodium bonus-henricus* L.

Spinacetin and 6-methoxykaempferol glycosides, respectively compounds **4** (13.56 %), **6** (14.62 %) and **3** (7.16 %), **5** (4.13 %) possessed significantly low DPPH activity and probably it is due to the absence of ortho-dihydroxy configuration. In spinacetin glycosides (compounds **4** and **6**) the 3'-OH group is methylated and the O-methylation of the hydroxyls of the catechol B-ring resulted in a decrease of the antioxidant activity with regard to the patuletin glycosides. In 6-methoxykaempferol glycosides (compounds **3** and **5**) 3'-OH group is missing and these compromise the antioxidant capacity. On the other hand spinacetin and 6-methoxykaempferol glycosides, compounds **8** and **9**, containing esterified ferulic acid in their moiety, demonstrated moderate DPPH (53.46 % and 45.52 %) activity. Ferulic acid possesses three distinctive structural motifs that can possibly contribute to its free radical scavenging capability. The presence of electron donating groups on the benzene ring (3-methoxy and more importantly 4-hydroxyl) of ferulic acid gives the additional property of terminating free radical chain reactions. The next functionality - the carboxylic acid group in ferulic acid with an adjacent unsaturated C-C double bond - can provide additional attack sites for free radicals and thus prevent them from attacking the membrane. In addition, this carboxylic acid group also acts as an anchor of ferulic acid, by which it binds to the lipid bilayer, providing some protection against lipid peroxidation [16].

Patuletin glycosides, compounds **1** (90.64 %), **2** (90.27 %) and **7** (90.76 %) showed the highest ABTS radical-scavenging activity. Activity of these compounds were similar to those of a classical antioxidant, ascorbic acid (90.79 %) (Table 1). Spinacetin and 6-methoxykaempferol glycosides, respectively compounds **4** (71.81 %), **6** (74.41 %), **8** (83.41 %) and **3** (70.18 %), **5** (70.94 %), **9** (74.51 %) also possessed a high ABTS radical-scavenging activity. Their lower activity is probably due to the absence of ortho-dihydroxy configuration in ring B. The results show that compounds **8** and **9** which contain esterified ferulic acid again demonstrate higher activity than the other spinacetin and 6-methoxykaempferol glycosides.

In the present study, the inhibition of lipid peroxidation of flavonoids **1-9** (0.1 mM) was determined in linoleic acid system using the FTC method. During linoleic acid peroxidation, peroxides were formed and these compounds oxidized Fe²⁺ to Fe³⁺. The Fe³⁺ ion formed a complex with SCN⁻, which had a maximum absorbance at 500 nm. Thus, a high absorbance value was an indication of high peroxide formation during the emulsion incubation. The presence of antioxidants in the mixture minimizes the oxidation of linoleic acid and reduce the absorption, respectively.

The absorbance of the control showed a steady increase and reached a maximal value of 1.20 on the 7th day (Fig. 2).

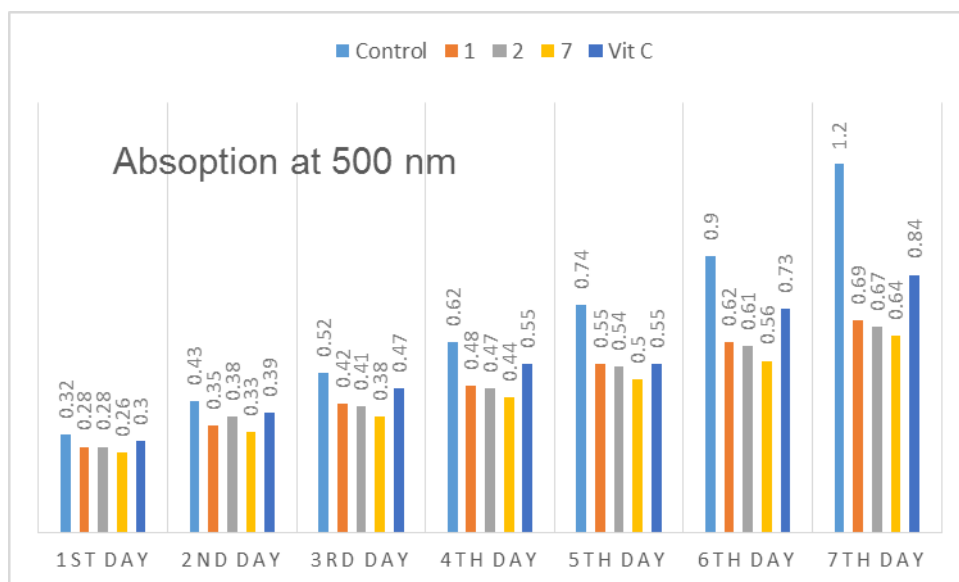


Fig. 2. Antioxidant activity of patuletin glycosides **1**, **2**, **7** in linoleic acid system.

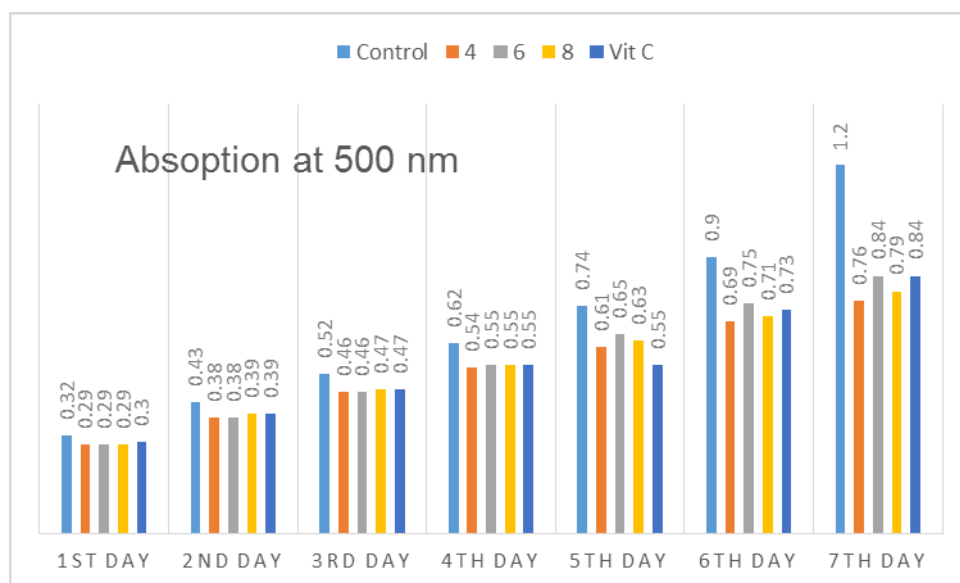


Fig. 3. Antioxidant activity of spinacetin glycosides 4, 6, 8 in linoleic acid system.

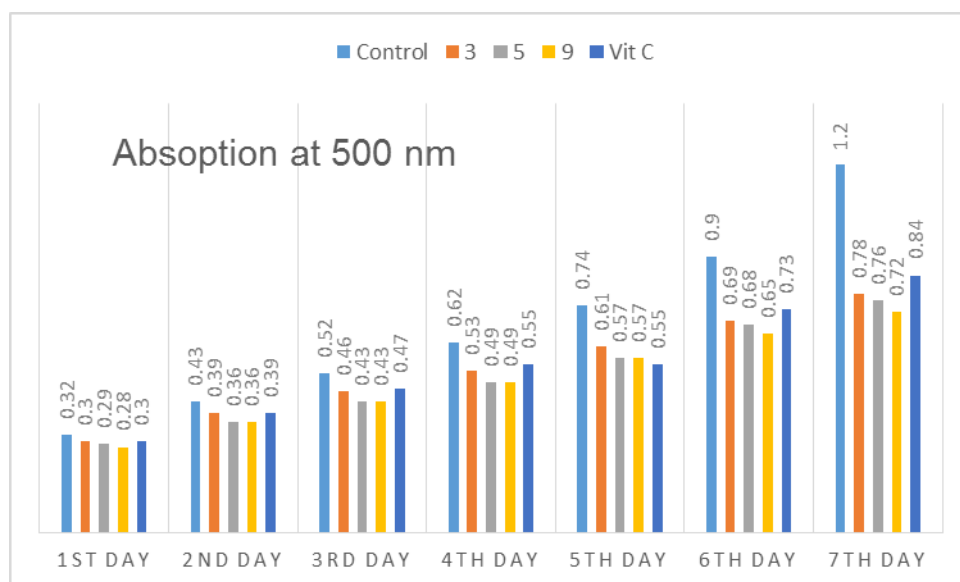


Fig. 4. Antioxidant activity of 6-methoxykaempferol glycosides 3, 5, 9 in linoleic acid system.

The patuletin glycosides compounds 1, 2 and 7 showed the highest capacity to inhibit linoleic acid peroxidation, with absorbance values always under 0.69 during the 7 days of testing (Fig. 2). Their activity was higher than the activity of Vitamin C, a widely used commercial antioxidant and may be it was due to the presence of ortho-dihydroxyl configuration in ring B.

On the other hand spinacetin and 6-methoxykaempferol glycosides, respectively compounds 4, 6, 8 (Fig. 3) and 3, 5, 9 (Fig. 4) also hindered the oxidation of linoleic acid and their activity is similar to that of Vitamin C.

These results indicate that patuletin, spinacetin and 6-methoxykaempferol glycosides, compounds

1-9, can significantly inhibit the peroxidation of linoleic acid and reduce the formation of hydroperoxide, thus implying that these flavonoids are powerful natural antioxidants.

CONCLUSION

Patuletin glycosides (1, 2 and 7) showed the highest DPPH and ABTS radical-scavenging activity. Spinacetin (4 and 6) and 6-methoxykaempferol (3 and 5) glycosides possessed significantly low DPPH activity and demonstrated a high ABTS radical-scavenging activity, compared to Vitamin C. On the other hand spinacetin and 6-methoxykaempferol glycosides (compounds 8 and 9), containing esterified ferulic acid in their moiety,

demonstrated a moderate DPPH and a high ABTS activity. All flavonoids inhibited significantly the lipid peroxidation of linoleic acid. The highest diminution was demonstrated again by patuletin glycosides and they are much more effective than Vitamin C. The results suggests that the wild spinach (*C. bonus-henricus* L.) could be a potential source of flavonoids with radical-scavenging and antioxidant activities.

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АНТИОКСИДАНТНИ СВОЙСТВА НА 6-МЕТОКСИФЛАВОНОЛОВИ ГЛИКОЗИДИ ОТ НАДЗЕМНИТЕ ЧАСТИ НА *Chenopodium bonus-henricus* L.

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(Резюме)

Радикал-свързващата и антиоксидантната активност на девет по-рано изолирани от надземната част на *Chenopodium bonus-henricus* L. флавоноиди е установена чрез DPPH и ABTS свободни радикали и чрез инхибиране на липидна пероксидация в система на линоленова киселина чрез фери-тиоцианатен метод. Гликозидите на патuletин, съединения **1**, **2** и **7** показват най-висока DPPH (85.78%, 85.59%, 86.07%) и ABTS активност (90.64%, 90.27%, 90.76%) сравнена с активността на Vitamin C. Гликозидите на спинацетин и 6-метоксикемпферол, съответно съединения **4**, **6** и **3**, **5** притежават значително ниска DPPH активност и проявяват висока ABTS радикал-свързваща активност. Гликозидите на спинацетин и 6-метоксикемпферол, съединения **8** и **9**, съдържащи в молекулата си естерифицирана ферулова киселина проявяват умерена DPPH (53.46%, 45.52%) и висока ABTS (83.41%, 74.51%) активност. Всички флавоноиди значително възпрепятстват окислението на линоленовата киселина. Въз основа на получените резултати надземните части на *C. bonus-henricus* L. могат да бъдат потенциален източник на флавоноиди с радикал-свързваща и антиоксидантна активност.