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Original Article

In Vitro Antiradical and Antioxidant Activity of Flavonoid Glycosides and Their Aglycones: Diosmin and Hesperidin Case Study

Lamyae Yachi[1]*, Madiha Alami chentoufi[2], Hajar Benhaddou[1], Brahim Mojemmi[1], Mustapha Bouatia[1]

ABSTRACT

In recent years, several works have been carried out on the beneficial effect of flavonoids which act as natural antioxidants and help to neutralize free radicals. We analysed the antioxidant activity of two flavonoids, diosmin, and hesperidin, known for their anti-haemorrhoidal effect, before and after acid hydrolysis, for comparison with their corresponding aglycones, namely diosmetin and hesperetin. Ascorbic acid was used as a positive control. For antioxidant activity evaluations, three methods including 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH•) scavenging assay, phosphomolybdate method, and Ferric Reducing Antioxidant Power method (FRAP) were used. The results were expressed as a percentage of inhibition of DPPH• radical and half-maximal inhibitory concentration values (IC50), for the first assay. And for the others, as mg of ascorbic acid equivalents per 100 g of powder: the quantity of ascorbic acid required to produce the same antioxidant activity as 100 g of sample (noted ''Ascorbic Acid Equivalent Antioxidant Capacity'': AEAC mg/100g). Aglycones exhibited significant in vitro antioxidant activity compared to glycosides ($p<0.05$ DPPH assay; $p<0.001$ Phosphomolybdate and FRAP assays), with antioxidant effect dose-dependent at the concentration levels used.

Keywords: flavonoid glycoside, flavonoid aglycone, antioxidant activity, diosmin, hesperidin.

Lamyae Yachi[1]*, Madiha Alami chentoufi[2], Hajar Benhaddou[1], Brahim Mojemmi[1], Mustapha Bouatia[1]

[1]Laboratory of Analytical Chemistry and Bromatology, Faculty of Medicine and Pharmacy, Mohammed V University Imp. Souissi, 10100 Rabat, MOROCCO.

[2]Laboratory of Life and Health Science, Faculty of Medicine and Pharmacy, Abdelmalek Essaidi University, Tanger, MOROCCO.

Correspondence

Y. Lamyae

Laboratory of Analytical Chemistry and Bromatology, Faculty of Medicine and Pharmacy, Mohammed V University Imp. Souissi, 10100 Rabat, MOROCCO.

E-mail: lamyae.yachi@fmp.um5.ac.ma

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INTRODUCTION

Flavonoids are polyphenolic secondary metabolites that are ubiquitously found in plants, which have recently been studied extensively for their vast antioxidant properties. Antioxidants are substances that mop up free radicals and prevent them from causing cell damage. Thus, the interest in the potential health benefits of flavonoids has increased owing most of all to their potent antioxidant and free radical scavenging activities observed *in vitro*[1]. They can prevent injury caused by free radicals through various mechanisms, as direct scavenging of reactive oxygen species, metal chelating activity[2], inhibition of oxidases[3] and others ways. In the last decade, there has been an emerging opinion that flavonoids may exert antioxidant actions at enzymatic targets involved in multiple signalling pathways such as those involving protein kinases and redox-sensitive cysteines[3]. In the human diet, they are most concentrated in fruits, vegetables, teas and cocoa[4].

Used as phlebotonic to prevent and treat cardiovascular diseases[5-7], nowadays, numerous studies discuss the prospects of therapeutic potential of flavonoids as antibacterial[8], antifungal[9], antidiabetic[7,8,10], acetylcholinesterase inhibitor[11] and anti-inflammatory in neurodegenerative and autoimmune disorders[12,13], anti-mutagenic[14], etc. The differences in the structure and state of oxidation are the basis of flavonoids classification. The skeleton structure of flavonoids, diphenylpropane (C6C3C6), contains two aromatic rings designated as A- and B-rings that are bridged by another C-ring[15]. They are classified into various groups which include flavonols, flavan-3-ols, flavones, flavanones, isoflavones, and anthocyanins[16] (Figure 1).

Glycosylation occurs mostly in positions 5 and 7, while methylation and acylation, on the hydroxyl groups of B ring. In plants, flavonoids do not occur as aglycones, but the most frequent forms are the glycoside derivatives[12].

The flavonoids are limited by their low bioavailability[17]. To a certain extent, the glycosides are like pro-drugs, which improve the solubility of aglycones by linking with sugar moieties. Indeed, the glycosylation may improve the bioavailability of flavonoids; their glycosides maintain higher levels in plasma and have longer mean residence time in the blood than those of aglycones. However, to produce effects *in vivo*, the flavonoid glycosides are commonly hydrolyzed to their aglycones[18].

Glycosidic bonds can be hydrolysed assisted by glycoside hydrolases, like rhamnosidases[19]. Alternatively, acidic hydrolysis of a glycoside represents a common chemical method to obtain its aglycones[20]. Thus, the present study aims to evaluate the antiradical and antioxidant activities of two flavonoids, diosmin and hesperidin, before and after acid hydrolysis, in comparison with their aglycones, namely, diosmetin and hesperetin.

MATERIALS AND METHODS

Acid hydrolysis of flavonoid glycosides

The preparation of the aglycones diosmetin and hesperetin was carried out according to the method described by Zhang et al. (2014)[20], based on the acid hydrolysis of flavonoid glycosides. Sulfuric acid (20 mL) was added to a 1 L beaker containing 500 mg of

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the flavonoid glycoside and the mixture was agitated using ultrasound to ensure complete dissolution at room temperature. Distilled water (20 mL) was then carefully added, as the reaction is highly exothermic. After cooling, the reaction mixture was poured into distilled water (150 mL) in a single portion to induce crystallization. The resulting yellow-brown crystals were collected by filtration, washed with distilled water (100 mL), and further purified using 70% (v/v) aqueous methanol.

Characterization of glycoside aglycones

Infrared (IR) spectral characterization was performed using a JASCO FT-IR 460 Plus Fourier transform spectrophotometer in the range of 4000–400 cm^{-1}. Samples were triturated with dried potassium bromide (KBr) and compressed into transparent pellets approximately 1 mm thick using an evacuable KBr die and a hydraulic press. The characteristic absorption bands of the aglycones were recorded and compared with reference data.

DPPH• radical scavenging activity assay

The antiradical activity of the samples was evaluated using the method described by Popovici et al. (2009)[21], with minor modifications. An aliquot of 0.1 mL of each sample solution, at concentrations ranging from 0.5 to 5 mg/mL, was added to 3.9 mL of a methanolic solution of DPPH• (25 µg/mL). The mixture was incubated for 30 min in the dark at room temperature, and absorbance was measured at 517 nm. The percentage of DPPH• inhibition (I%) was calculated using the following equation:

$$I\,\% = \left(1 - \frac{Ae}{Ao}\right) \times 100$$

where Ao is the absorbance of the control and Ae is the absorbance in the presence of the sample

Phosphomolybdate assay

Total antioxidant capacity was determined using the phosphomolybdate method described by Saeed et al. (2012)[22] An aliquot of 300 µL of sample solution was mixed with 3 mL of reagent solution containing 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. The mixture was vortexed and incubated in a water bath at 95°C for 90 min. After cooling, absorbance was measured at 695 nm against a blank. Results were expressed as milligrams of ascorbic acid equivalent antioxidant capacity per 100 g of powder (AEAC mg/100 g).

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power of the samples was determined according to the method of Oyaizu (1986)[23]. One milliliter of each sample solution (0.5–5 mg/mL) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixtures were incubated at 50°C for 20 min. After incubation, 2.5 mL of 10% trichloroacetic acid was added, followed by dilution with distilled water (50:50, v/v). Finally, 0.5 mL of 0.1% ferric chloride was added to 5 mL of each prepared sample. Absorbance was measured at

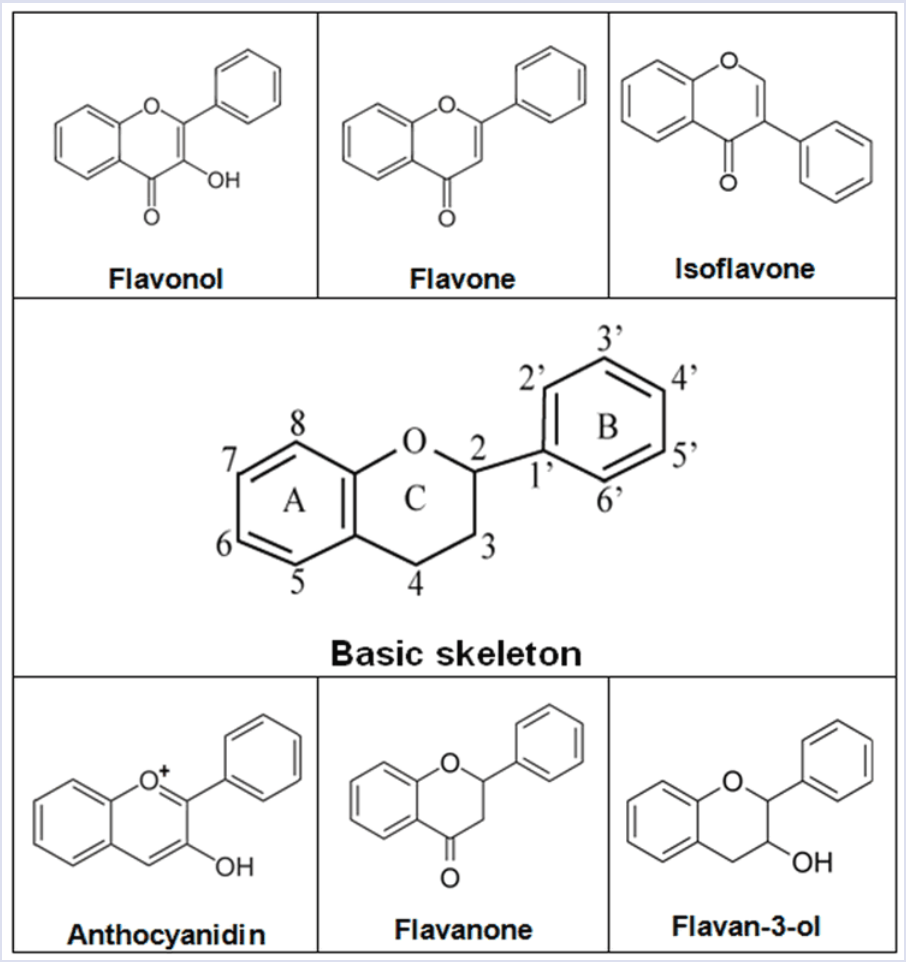

Figure 1. Basic skeleton structure of flavonoids and their classes

700 nm. Results were expressed as AEAC mg/100 g of powder.

Statistical analysis

All experiments were performed in triplicate, and results were expressed as mean ± standard deviation (SD). Statistical analysis was conducted using one-way analysis of variance (ANOVA) to determine significant differences between means, with $p < 0.05$ considered statistically significant.

RESULTS AND DISCUSSION

The Diosmin and Hesperidin flavonoids

Diosmin and hesperidin are flavonoids belonging to the flavone and flavanone classes, respectively. Both compounds are O-glycosylated at position 7 by a rutinoside moiety and contain methoxy and hydroxyl groups on the B ring. They are commonly found in plants of the Rutaceae family[24]. Diosmin, also known as diosmetin-7-O-rutinoside[25], differs from hesperidin by the presence of a C2=C3 double bond in the C ring (Figure 2). Diosmin can be obtained by extraction of hesperidin from citrus peels followed by chemical dehydrogenation[25,26].

Diosmin was first isolated in 1925 from *Scrophularia nodosa* L. and introduced as a therapeutic agent in 1969[27]. It is widely used for its phlebotonic and vasoprotective effects in the treatment of chronic venous insufficiency, lymphedema, and varicose veins. Several clinical trials have demonstrated its efficacy in the management of acute and chronic hemorrhoidal symptoms[28]. Its therapeutic effectiveness is enhanced when administered in combination with hesperidin. A randomized, triple-blind, controlled clinical trial involving 134 patients showed that a combination of diosmin, hesperidin, and troxerutin provided faster symptom control and reduced edema and thrombosis in acute hemorrhoidal disease[29].

Hesperidin is a naturally occurring flavanone glycoside abundant in *Citrus aurantium* and has been reported to exhibit antidiabetic, antioxidant, and anti-inflammatory activities[30]. Owing to their wide range of biological properties, diosmin and hesperidin are considered promising agents for the treatment of oxidative stress-related disorders[31].

Effect of acid hydrolysis on antioxidant activity

Acid hydrolysis of diosmin and hesperidin yielded their corresponding aglycones, diosmetin and hesperetin. Structural confirmation of the obtained aglycones was achieved by IR spectroscopy, and the characteristic absorption bands were consistent with previously reported reference data[20], confirming successful deglycosylation.

In vitro antiradical and antioxidant activity evaluation

The antioxidant properties of diosmin, hesperidin, and their aglycones were evaluated using three complementary in vitro assays. In all cases, the aglycone forms exhibited markedly higher antioxidant activity than their corresponding glycosides.

In the DPPH radical scavenging assay, diosmetin and hesperetin showed significantly stronger antiradical activity than diosmin and hesperidin, with a clear concentration-dependent effect.

The IC_{50} values revealed the following order of activity:

Diosmin > Hesperidin > Diosmetin > Hesperetin (Figure 3, Table 1)

Lower IC_{50} values indicate higher radical scavenging efficiency. Consistent results were obtained with the phosphomolybdate and FRAP assays. The antioxidant capacity, expressed as ascorbic acid equivalent antioxidant capacity (AEAC), showed that aglycones possessed significantly higher reducing and total antioxidant capacity than glycosylated forms. Hesperetin exhibited the highest antioxidant activity, followed by diosmetin, whereas diosmin and hesperidin showed comparatively weak activity (Figure 4, Table 2).

Statistical analysis confirmed that the differences between aglycones and their corresponding glycosides were significant ($p < 0.05$) for all assays (Table 3).

Structure–activity relationship

These findings are in agreement with previous studies demonstrating that glycosylation reduces the in vitro antioxidant activity of flavonoids compared to their aglycone counterparts[5,7,33]. The removal of sugar moieties increases the availability of free hydroxyl groups, which play

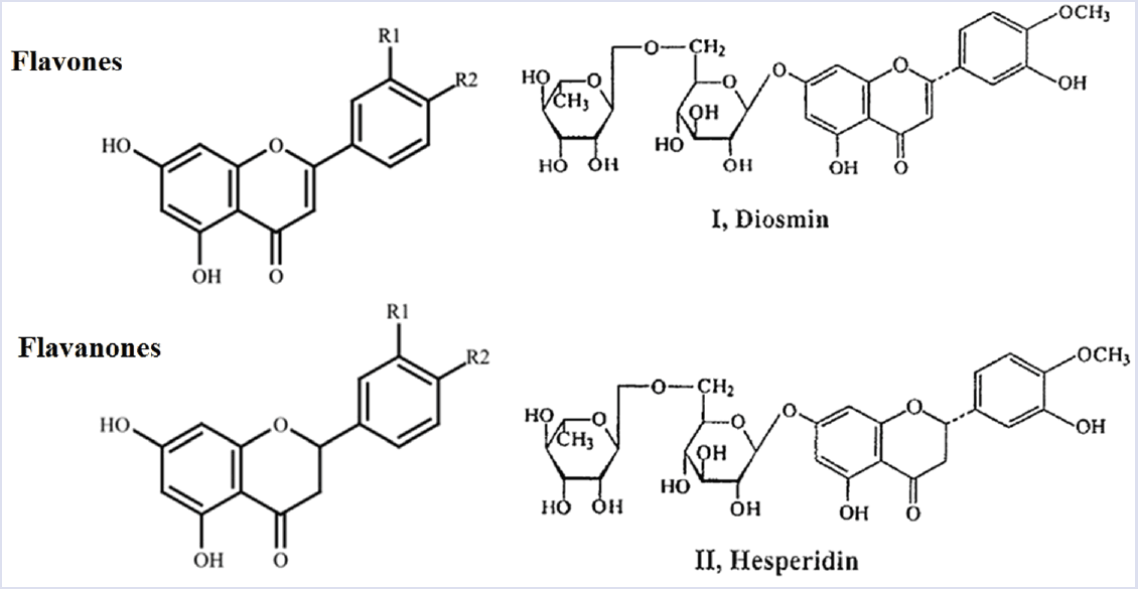

Figure 2. Chemical structure of diosmin I. and hesperidin II[16,23]

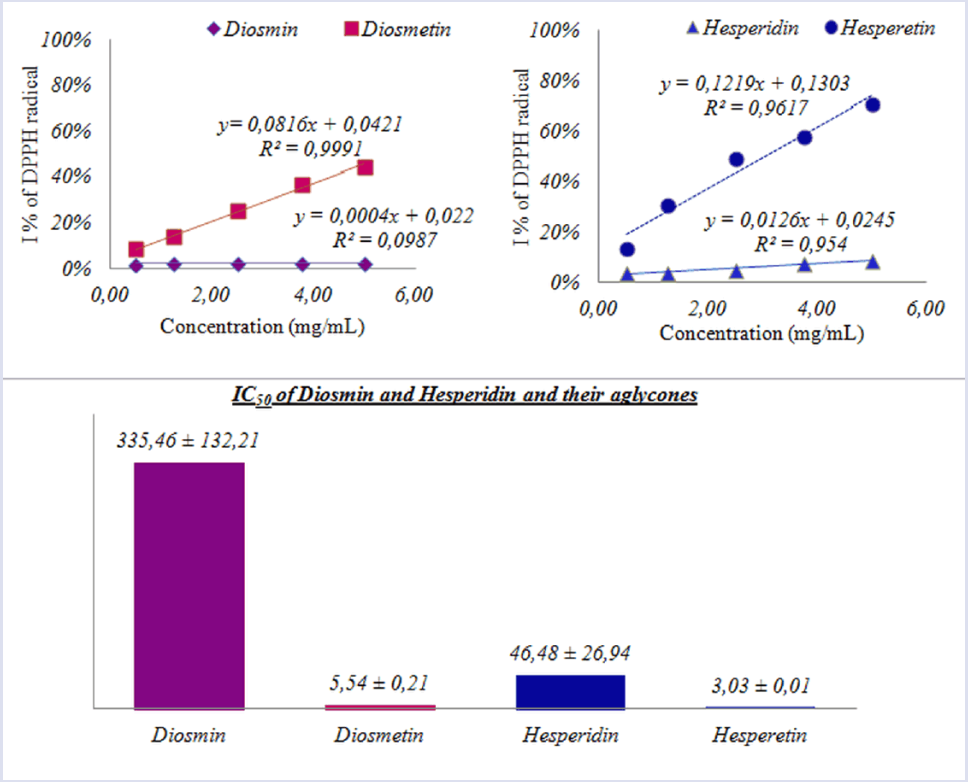

Figure 3. DPPH radical scavenging activitiy of diosmin, hesperidin and their aglycones

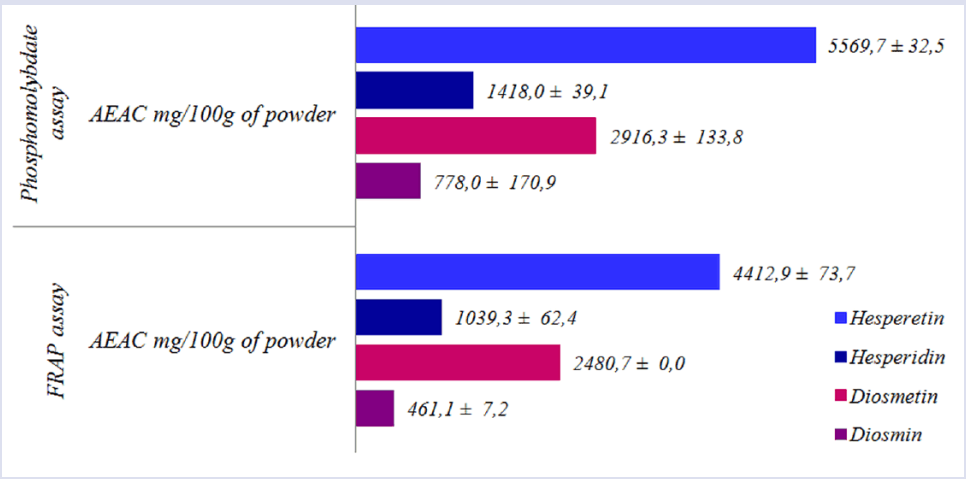

Figure 4. Antioxidant activity of diosmin, hesperidin, and their aglycones determined by phosphomolybdate and FRAP assays.

a key role in hydrogen donation and radical stabilization. Enzymatic or chemical conversion of flavonoid glycosides into aglycones has been reported to enhance antioxidant and anti-inflammatory activities in various systems[19,34-36].

The antioxidant activity of flavonoids is strongly influenced by structural features. Free hydroxyl groups are the primary contributors to radical scavenging activity, while methoxy substitutions can modulate activity depending on their position[3,37]. The higher antioxidant activity observed for hesperidin and hesperetin compared with diosmin and diosmetin may be related to the absence of the C2=C3 double bond. This observation is consistent with the findings of Zheng et al. (2019), who reported that, in polar environments, flavanones exhibit stronger antiradical activity than flavones or flavonols differing only by the presence of this double bond[38].

CONCLUSION

Flavonoids, a class of secondary plant phenolics, are well known for their antioxidant activity and are widely found in several plants and plant products. In our work, the evaluation of the antioxidant activity of diosmin and his flavanone, hesperidin, in comparison with their corresponding aglycones has shown that the diosmetin and hesperetin have a much higher *in vitro* antioxidant potential than the glycoside forms. The antioxidant effect was dose-dependent at the concentration levels used.

Table 1. IC_{50} values obtained for flavonoid glycosides and aglycones

	IC_{50}
Diosmin	**335,46 ± 132,21**
Diosmetin	5,54 ± 0,21
Hesperidin	46,48 ± 26,94
Hespertin	3,03 ± 0,01

Table 2. Antioxidant capacity (AEAC) of flavonoids determined by phosphomolybdate and FRAP assays

	Phosphomolybdate assay AEAC mg/100mg of power	FRAP assay AEAC mg/100mg of power
Hesperetin	5569,7 ± 32,5	4412,9 ± 73,7
Hesperidin	1418,0 ± 39,1	1039,3 ± 62,4
Diosmetin	291$,3 ± 133,8	248,07 ± 0,0
Diosmin	778,0 ± 170,9	461,1 ± 7,2

Table 3. One-way ANOVA (p-values) comparing flavonoid glycosides and aglycones.

	Diosmin VS Diosmetin	Hesperidin VS Hesperetin
DPPH assay	(0.012)	(0.049)
Phosphomolybdate assay	(< 0.001)	(< 0.001)
FRAP assay	(< 0.001)	(< 0.001)

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