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Comparison of the contents of total polyphenol, total flavonoid, and flavonoid derivatives in unfermented and fermented barley sprouts

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Abstract Barley (*Hordeum vulgare*) belongs to the Poaceae family. This study compared the polyphenol and flavonoid levels of unfermented and fermented barley sprouts using spectrophotometric assays. The findings indicated that fermentation greatly boosted the flavonoid content but caused only a slight increase in the polyphenol content. However, this does not imply that fermentation has no effects whatsoever on the polyphenol content of barley sprouts. This was due to the fact that some flavonoids cannot be detected by the wavelength used to calculate the overall polyphenol concentration. Both samples were subjected to high-performance liquid chromatography analysis and detected the flavonoids lutonarin, saponarin, isoorientin, isovitexin, and tricin—all of which have bioactive properties—most notably known for their antioxidant activity. These results augment the ongoing phytochemical profiling research and can possibly valorize the already thriving barley industry.

Keywords Fermentation · Flavonoid · *Hordeum vulgare* · High-performance liquid chromatography analysis · Polyphenol

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Introduction

Polyphenols are a well-studied class of natural compounds found in the majority of plants [1]. These compounds have various applications in the health, cosmetics, and food industry [1,2] owing to their anticancer [1], antihyperlipidemic [3], anti-inflammatory [4], and antioxidant activities [5]. The degree of its effects varies with the kind of polyphenol used [1]. The presence of these bioactive properties is not surprising because polyphenols are secondary metabolites—compounds that are utilized for plant survival, contrary to primary metabolites, which are needed for the planted basic developmental functions [4,6]. Because plants are non-motile organisms, they need to synthesize these compounds to fend for themselves in the wild [7]. These compounds usually function as ultraviolet (UV) screens and antioxidant agents that protect plants from UV radiation and other stressors [4,6].

Flavonoids are a type of polyphenols. They are by far the most prevalent class of polyphenolic compounds that are present in all plants [8]. Flavonoids are generally composed of a 15-carbon skeleton that is made up of two phenyl rings and one heterocyclic ring [9]. Based on the various substituents on the rings, the level of benzo-pyrone saturation, and the connecting location of ring B, flavonoids can be divided into multiple subclasses and contain approximately 9,000 different derivatives [10]. This group of compounds has been proven to have anti-inflammatory [11], anticancer [12], and antibacterial [13] properties.

Barley (*Hordeum vulgare*) is a plant that is well known for its high content and variety of flavonoids [14,15]. It is the fourth most popular grain crop worldwide [16]. It has high concentrations of dietary fibers, minerals, flavonoids, and phenolic acids [16]. Proanthocyanidins, catechins, saponarins, and lutonarins are some of the known phenolic acids and flavonoids found in barley, respectively [15,16].

This plant is one of the ancient crops still being grown today. There are records of barley being grown in ancient Egypt as long as 17,000 years ago [17,18]. Indeed, ancient civilizations like that

of Egypt must have known the health benefits of barley [17]. It was consumed even then due to its high dietary fiber content, which promotes digestion and overall gut health [19].

Fermentation is a process by which microorganisms improve the nutritional value of a food by converting the conjugated form of phenolics into its free form [20,21]. Humans have been fermenting their food since time immemorial [22]. Fermentation of foods is popular worldwide because of its amazing health benefits [23]. Fermentation has also been thought to increase the phytochemical content of a plant, which, in turn, also increases its antioxidant activity [21]. With the emergence of many oxidative diseases, a lot of the so-called 'superfoods' have also been emerging [24]. Although there is no clear scientific basis, superfoods are foods that are considered superior owing to their bioactive properties [25]. One such superfood is barley. Fermentation of barley, which in itself has antioxidant properties, will increase its capacity for antioxidant activities [26]. The poor bioavailability of flavonoids decreases their benefits [27]. However, fermentation has been observed to increase the bioavailability of these compounds [27].

In this study, the polyphenol and flavonoid contents of unfermented and fermented barley sprouts (UBS and FBS, respectively) were compared to elucidate the effects of fermentation on the polyphenol and flavonoid contents of barley. The results of this study could potentially help in developing supplements to augment the polyphenol and flavonoid intake of people and, consequently, improve their health. Ultimately, the results of this study can contribute to the valorization of the already thriving barley industry.

Materials and Methods

Plant materials

Barley (*H. vulgare*) seeds were obtained from Sang Byeok Co. Ltd., Pocheon, Republic of Korea.

Chemicals and apparatus

High-performance liquid chromatography (HPLC) was performed using a Perkin Elmer Flexar, Quat with pump, autosampler, and photodiode array detector (Perkin Elmer, Shelton, CT, USA). HPLC-grade solvents such as methanol (MeOH), water, acetic acid, and acetonitrile (ACN) were purchased from J. T. Baker (Radnor, PA, USA). Gallic acid, quercetin, lutonarin, saponarin, isoorientin, isovitexin, and tricin (Fig. 1) were provided by Natural Product Institute of Science and Technology (www.nist.re.kr), Anseong, Korea.

BS cultivation and fermentation

Barley seeds were soaked in water for three days and then placed on a shelf and kept moist for eight days. The germinated barley seeds were planted in a rice nursery tray until the shoots were 7 cm long. Subsequently, the grown BS were plucked, oven dried, and eventually powdered with a grinder to obtain the UBS samples. To obtain the FBS samples, first, glucose and *Lactococcus lactis* were dissolved in purified water. Subsequently, the solution was used to knead the UBS powder, which was then placed in a fermentation container for two weeks at 25-30 °C. Finally, the FBS samples were dried and powdered.

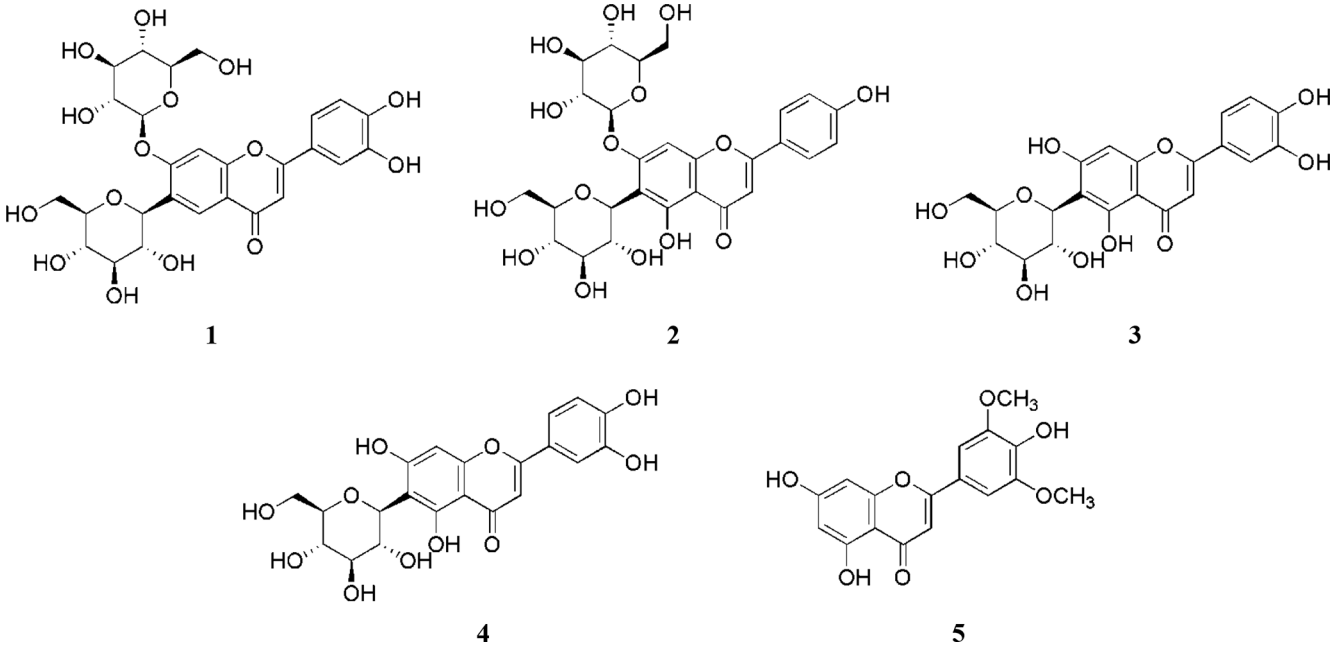

Fig. 1 Chemical structures of lutonarin (**1**), saponarin (**2**), isoorientin (**3**), isovitexin (**4**), and tricin (**5**)

Sample extraction methods

Each 10 g of UBS and FBS powder was extracted thrice with 200 mL ethanol for 3 h using a reflux extractor. Subsequently, the mixture was filtered and vacuum evaporated to obtain an extract. The extraction yields of UBS and FBS were 39 and 33%, respectively.

Preparation of samples and standard solutions

The UBS and FBS extracts and lutonarin, saponarin, isoorientin, isovitexin, and tricin were dissolved in MeOH to obtain a concentration of 30 mg/mL for the extracts and 0.5 mg/mL for the five standards. Subsequently, they were sonicated for 20 min and filtered using a polyvinylidene fluoride membrane filter (pore size: 0.45 μm). Then, the standard solutions were diluted to obtain concentrations that were appropriate for calculations in the quantitative analysis.

Total polyphenol content

The total polyphenol content of the UBS and FBS extracts was measured as described in a previous study, with slight modifications [28]. Briefly, 60 μL of each sample was mixed with 40 μL, Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA). Next, 100 μL of 7.5% Na_2CO_3 was added and the samples were allowed to react at room temperature in the dark for 30 min. The absorbance of the samples was measured at 760 nm using a microplate reader (Epoch; BioTek, Winooski, VT, USA). The total polyphenol content was calculated based on a standard curve constructed using different concentrations of gallic acid.

Total flavonoid content

The total flavonoid content of the samples was measured using a modified version of the method described in a previous study [29]. Briefly, 100 μL of 2% aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$) was added to 100 μL of the extract and incubated for 10 min. Subsequently, the absorbance of the samples was measured at 430 nm using a microplate reader (Epoch; BioTek, Winooski, VT, USA). The total flavonoid content was calculated based on a standard curve constructed using different concentrations of quercetin.

HPLC conditions

The UBS and FBS samples were quantitatively analyzed using a reverse phase HPLC system with a YMC Pack-Pro C18 column (250 mm × 4.6 mm, 5 μm). The column temperature was maintained at 30 °C. The mobile phase was composed of 0.2% acetic acid in water (A) and ACN (B). The elution was performed using a gradient system. The gradient elution conditions were 90% A from 0 to 5 min, 85% A at 10 min, 65% A at 25 min, 50% A at 30 min, and 0% A from 35 to 40 min. The sample injection volume was 10 μL, the mobile phase flow rate was 1.0 mL/min, and the detector wavelength was set at 330 nm.

Calibration curve

The lutonarin, saponarin, isoorientin, isovitexin, and tricin standard solutions were serially diluted to a minimum of five concentrations to construct the respective calibration curves. The linearity of the calibration curve was determined based on the correlation coefficient (r^2), and the content of the target compound was calculated using the equation of the calibration curve. In the calibration correction function of the five compounds, the X-axis (μg/mL) represents the concentration, the Y-axis represents the peak area, and the value to be substituted is the mean value (n =3) ± standard deviation.

Results and Discussion

The polyphenol and flavonoid contents of UBS and FBS were analyzed in this study. Previous studies have reported an increase in polyphenol and flavonoid contents of BS after fermentation [9,30,31]. This is because most polyphenolic compounds (flavonoids being the most common) exist in a bound form and fermentation converts these compounds into their free form, increasing their content [27]. Table 1 summarizes the total polyphenol and flavonoid contents of UBS and FBS. The total polyphenol content of UBS (2.512 mg GAE/g extract) was only slightly lower than that of FBS (4.395 mg GAE/g extract). However, the total flavonoid content of UBS (3.505 mg QE/g extract) was considerably lower than that of FBS (19.695 mg QE/g extract). These results might look inconclusive because the increase in flavonoid content was quite profound, whereas the increase in polyphenol content was not of a great magnitude. However, this is because the wavelength (760 nm) used for determining the total polyphenol content cannot detect certain flavonoids [32]. The flavonoids reported in this study were detected at a wavelength of 410 nm. Flavonoids are generally detected at shorter wavelengths [32]. Furthermore, the increase in the polyphenol and flavonoid contents after fermentation with a *Lactobacillus* bacteria can be attributed to the hydrolysis of these compounds to release their free forms [30].

Aborus et al. (2016) investigated the phenolic and flavonoid contents of two different varieties of BS—a hybrid variety, 'NS565' (BSNS), and a nonhybrid variety, 'Golozrni' (BSG). The results of their study were profoundly different from those observed during the present study. The total polyphenol contents

Table 1 The total polyphenol and flavonoid contents in UBS and FBS

Sample	Total polyphenol content (mg GAE/g extract)	Total flavonoid content (mg QE/g extract)
UBS	2.512±0.141	3.505±0.360
FBS	4.395±0.053	19.695±0.218

Note: GAE, gallic acid equivalent; QE, quercetin equivalent

Table 2 The calibration curves for lutonarin (**1**), saponarin (**2**), isoorientin (**3**), isovitexin (**4**), and tricin (**5**)

Compound	t_R	Calibration equation[a]	Correlation factor, r^2 [b]
1	16.99	Y = 4415X + 2162.2	0.9999
2	18.96	Y = 16261X + 6712.7	1.0000
3	19.50	Y = 5267.8X - 1234.7	0.9999
4	20.89	Y = 15006X + 8017.5	0.9999
5	33.17	Y = 7358.3X + 2669.1	1.0000

[a]Y = peak area, X = concentration of standards (μg/mL)
[b]r^2 = correlation coefficient of five calibration data points (n =3)

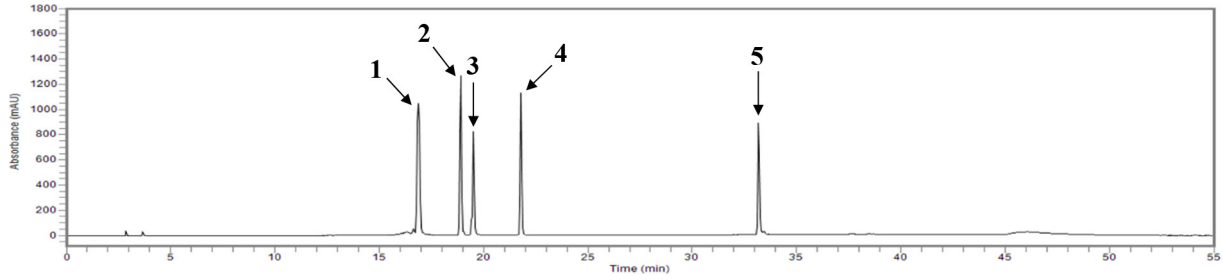

Fig. 2 HPLC chromatogram of lutonarin (**1**), saponarin (**2**), isoorientin (**3**), isovitexin (**4**), and tricin (**5**)

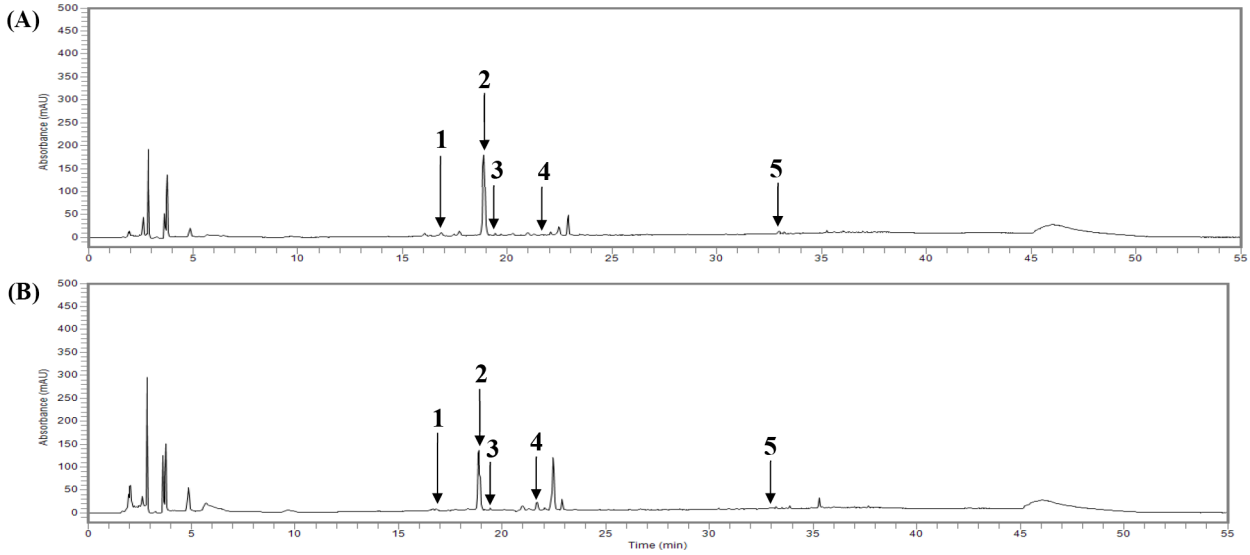

Fig. 3 HPLC chromatograms of UBS (A) and FBS (B) extracts. **1**, **2**, **3**, **4**, and **5** are the peaks representing lutonarin, saponarin, isoorientin, isovitexin, and tricin, respectively

of BSG and BSNS were 479.02 mg GAE/100 g and 713.25 mg GAE/100 g DW, respectively. The total flavonoid contents of both varieties were also different (BSG: 274.18 mg GAE/100 g DW; BSNS: 278.07 mg GAE/100 g DW). The differences in the results can be attributed to the differences in the varieties and the fermentation processes used.

In a similar study, Ding et al. (2020) fermented a black barley variety to investigate whether fermentation affected the total polyphenol and total flavonoid contents of the barley. The total polyphenol and total flavonoid contents of the unfermented barley samples were 43.22 mg/100 g and 2743.69 mg/100 g, respectively. However, after fermentation, the total polyphenol and total flavonoid contents increased to 75.49 mg/100 g and 3093.08 mg/100 g, respectively.

The HPLC analysis detected five flavonoid derivatives of great importance—two flavonoid glycosides (lutonarin and saponarin) and three flavones (isoorientin, isovitexin, and tricin) (Fig. 1). These five flavonoid derivatives showed good retention times (Table 2, Fig. 2). The chromatograms of both UBS and FBS showed the presence of peaks corresponding to these flavonoid

Table 3 Contents of the different flavonoid derivatives in UBS and FBS

Sample	Contents (mg/g ext.)				
	1	**2**	**3**	**4**	**5**
BS	0.32±0.00	2.11±0.01	0.19±0.00	tr	0.15±0.00
FBS	0.20±0.00	1.62±0.01	0.18±0.00	0.18±0.00	0.09±0.00

tr: trace; **1**, **2**, **3**, **4**, and **5** indicate lutonarin, saponarin, isoorientin, isovitexin, and tricin, respectively

derivatives (Fig. 3). As mentioned previously, these flavonoid derivatives are most notably known for their antioxidant properties [11-13]. They also exhibit other bioactive properties; lutonarin, saponarin, isoorientin, isovitexin, and tricin have anti-neuraminidase [2] antidiabetic [15] activities, anti-depressant [33], anticancer [34], and anti-inflammatory [35] activities, respectively.

The contents of the flavonoid derivatives in the UBS and FBS samples were considerably different. In the UBS samples, among the five flavonoid derivatives detected (Table 3), saponarin (2.11 mg/g extract) content was the highest, followed by lutonarin (0.32 mg/g extract), isoorientin (0.19 mg/g extract), and tricin (0.15 mg/g extract). Meanwhile, only trace amounts of isovitexin were detected in the UBS samples. In the FBS samples, fermentation decreased the contents of the flavonoid derivatives except for isovitexin. Saponarin content (1.62 mg/g extract) was still the highest, followed by lutonarin (0.20 mg/g extract), isoorientin and isovitexin (0.18 mg/g extract), and tricin (0.09 mg/g extract). Interestingly, fermentation increased the content of isovitexin.

The results of this study are compelling because they go against what has been previously reported in the literature [30,31,36-38]. As mentioned above, fermentation should increase the flavonoid content of a plant because the process per se converts bound flavonoids into free form [27]. A patented study conducted by Kim et al. (2019) revealed that the levels of isovitexin and other flavonoid derivatives, such as saponarin and luteolin, in BS of a similar length as used in the present study, increased when fermented. However, Kim et al. (2019) performed fermentation using *Lactobacillus fermentum* augmented with an enzyme complex. In addition, their method of fermentation was quite different from the one used in the present study. The differences in the results obtained by Kim et al. (2019) and those obtained in the present study can be attributed to these differences. However, this is only a postulation because the mechanisms behind the fermentation are not within the scope of the present study.

Much research is needed to be done to create a complete profile of the total polyphenol and total flavonoid contents of different plants, let alone the same plant (the same plant can have different varieties with different phytochemical profiles). Hence, comparison with literature can be challenging for studies like this because the sample and methods used may vary among studies. Nevertheless, the results of this study add to the literature and contribute to the existing total polyphenol and total flavonoid profiling studies in the realm of natural products science.

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