

Available online at [www.sciencedirect.com](http://www.sciencedirect.com)

ScienceDirect

journal homepage: [www.jfda-online.com](http://www.jfda-online.com)

## Original Article

# Effects of extracts from *Gynura bicolor* (Roxb. & Willd.) DC. on iron bioavailability in rats



Chih-Chung Wu<sup>a</sup>, Wei-Lin Chang<sup>b</sup>, Chih-Huei Lu<sup>c</sup>, Yueh-Ping Chang<sup>d</sup>,  
Jyh-Jye Wang<sup>d</sup>, Shu-Ling Hsieh<sup>b,\*</sup>

<sup>a</sup> Department of Nutrition and Health Science, Chang Jung Christian University, Tainan, Taiwan

<sup>b</sup> Department of Seafood Science, National Kaohsiung Marine University, Kaohsiung, Taiwan

<sup>c</sup> Department of Physical Medicine and Rehabilitation, Fooyin University Hospital, Pingtung, Taiwan

<sup>d</sup> Department of Nutrition and Health Science, Fooyin University, Kaohsiung, Taiwan

## ARTICLE INFO

## Article history:

Received 31 March 2014

Received in revised form

19 August 2014

Accepted 24 November 2014

Available online 24 February 2015

## Keywords:

divalent metal transporter

ferroportin

*Gynura bicolor*

iron bioavailability

## ABSTRACT

*Gynura bicolor* (Roxb. & Willd.) DC. is widely distributed in certain areas of Asia and is very popular in vegetarian cuisine in Taiwan. This study investigates the effects of *G. bicolor* extracts with different polarities of 80 mg/kg body weight (BW) *G. bicolor* alcohol extract, 80 mg/kg BW *G. bicolor* water extract, and 80 mg/kg BW *G. bicolor* ether extract on Fe bioavailability using the hemoglobin repletion efficiency assay. Wistar rats were assigned to five groups: a group receiving an iron-deficient (ID) diet; a group receiving an ID diet supplemented with ferrous sulfate (20 mg Fe/kg BW); and three groups receiving ID diets supplemented with ferrous sulfate and one of *G. bicolor* alcohol extract, *G. bicolor* water extract, or *G. bicolor* ether extract. The results indicated that the levels of hemoglobin, serum iron, serum ferritin, liver ferritin, hemoglobin regeneration efficiency, relative biological value, and hepcidin all were significantly higher than those of the ID diet group. Besides, the iron transporter divalent metal transporter-1 was significantly reduced, but iron release protein expression of ferroportin was significantly increased. It was concluded that *G. bicolor* extracts may promote iron bioavailability and regulate the expressions of divalent metal transporter-1 and ferroportin.

Copyright © 2015, Food and Drug Administration, Taiwan. Published by Elsevier Taiwan LLC. Open access under [CC BY-NC-ND license](https://creativecommons.org/licenses/by-nc-nd/4.0/).

## 1. Introduction

According to the World Health Organization, anemia is a global public health problem that primarily affects the health and social development of populations in developing and

developed countries. Iron deficiency is considered to be the most important cause of anemia [1].

Duodenum contains regulatory proteins that, via their role in iron metabolism, facilitate the balance between iron absorption and release. Dietary nonheme iron enters the intestine as Fe<sup>3+</sup>. It is converted to Fe<sup>2+</sup> by duodenal apical

\* Corresponding author. Department of Seafood Science, National Kaohsiung Marine University, 142 Haijhuang Road, Nanzih District, Kaohsiung City 81143, Taiwan.

E-mail addresses: [slhsieh@ntu.edu.tw](mailto:slhsieh@ntu.edu.tw), [slhsieh@mail.nkmu.edu.tw](mailto:slhsieh@mail.nkmu.edu.tw) (S.-L. Hsieh).

<http://dx.doi.org/10.1016/j.jfda.2014.11.001>

1021-9498/Copyright © 2015, Food and Drug Administration, Taiwan. Published by Elsevier Taiwan LLC. Open access under [CC BY-NC-ND license](https://creativecommons.org/licenses/by-nc-nd/4.0/).

cytochrome b, which exhibits ferrireductase activity and enters the intestinal cells through apical divalent metal transporter protein-1 (DMT-1). The iron entering intestinal cells is oxidized to  $\text{Fe}^{3+}$  by ferritin, and is stored as ferritin and hemosiderin [2]. When tissues need iron,  $\text{Fe}^{3+}$  is reduced to  $\text{Fe}^{2+}$  by ferritin and released into the blood circulation through the ferroportin (FPN) located on the basolateral side of intestinal cells. In addition, one of the functions of hepcidin, a hepatogenous peptide hormone, is to facilitate iron regulation and metabolism in animals. A previous study showed that hepcidin can bind to FPNs located on the basolateral side of intestinal cells and induce FPN phosphorylation and degradation, thereby increasing iron storage by intestinal epithelial cells, macrophages, and hepatocytes [3].

The main factor affecting nutrition with regard to iron is its low dietary availability. In recent years, there have been many studies regarding food iron fortification and phytomaterials. For example, a study using the Caco-2 cell model showed that, when consumed with iron, garlic can improve iron absorption through increased FPN expression [4]. A different study showed that, when fed to rats, yacon powder, which contains inulin-type fructans, acidified the intestinal mucosa by increasing cecal fermentation to produce short-chain fatty acids, thereby improving the solubility and bioavailability of iron pyrophosphate [5]. Another study showed that, when consumed by mice, *Telfairia occidentalis* affected iron absorption by decreasing the expression of duodenal DMT-1 [6]. The results of cell and animal models both evidenced that fortification of foods with iron is an effective way to improve iron nutritional status, suggesting that this approach is worth further investigation.

*Gynura bicolor* (Roxb. & Willd.) DC., a perennial plant belonging to the Asteraceae family, originates from tropical areas in Asia. Leaves of *G. bicolor* distinctively show a reddish purple color on their abaxial sides, contrasting with the typical green color seen on the adaxial side [7]. Previous studies have shown that *G. bicolor* possesses high antioxidant activity and performs many biological functions with no toxic effects [8]. The pigment sources and physiological effects of *G. bicolor* are thought to be related to its rich flavonoid content, including anthocyanins, quercetin, kaempferol, quercitrin, isoquercitrin, and rutin [9,10]. In addition, according to the Nutritional Composition Database of Taiwan, *G. bicolor* is rich in iron, having iron levels as high as 41  $\mu\text{g/g}$ , and 100 g of *G. bicolor* contains 15 mg of vitamin C (ascorbic acid), which can promote iron absorption. *G. bicolor* is consumed as a cooked vegetable and is believed to have a wide range of benefits that include anticancer, anti-inflammatory, and possibly antihypertensive effects [11,12]. However, promotion of iron absorption by *G. bicolor* has not been reported.

The purpose of this study was to determine whether the administration of *G. bicolor* extracts in Wistar rats could promote iron absorption and bioavailability. The blood biochemical indicators, including hemoglobin (Hb), serum iron, serum ferritin, liver ferritin, serum iron, total iron-binding capacity (TIBC), transferrin saturation (TS), Hb regeneration efficiency (HRE), relative biological value (RBV), hepcidin, and duodenal mucosa expressions of iron metabolism proteins (DMT-1 and FPN), were examined using an Hb repletion efficiency assay.

## 2. Methods

### 2.1. Preparation of the *G. bicolor* alcohol extract, *G. bicolor* water extract, and *G. bicolor* ether extract

*G. bicolor* (Roxb. & Willd.) DC. was purchased from Yuanshan Village (Ilan, Taiwan). Leaves of *G. bicolor* were removed, cleaned, and blended in 95% alcohol or cold water (4°C, w/w: 1/1). After standing for 1 hour, the suspension was centrifuged at 5000g for 10 minutes at 4°C, after which the filtrate was concentrated under reduced pressure (30–40°C). Finally, the extracts were dried in a rotary vacuum dryer. The percent yields of the alcohol and water extracts were 0.9% and 4.2% (w/w), respectively. In addition, the leaves of *G. bicolor* homogenates were extracted with ether (v/v: 1/1) for 6 hours at 4°C, after which the extracts were stirred on a stirring plate for 4 hours and then dried in a rotary vacuum dryer. The percent yield of the ether extract was 0.3% (w/w).

### 2.2. *G. bicolor* composition analysis

The total flavonoid and phenolic content of *G. bicolor* extracts was determined using the colorimetric method of Jia et al [13]. The rutin content was determined using high-performance liquid chromatography, as described by Krizman et al [14]. The  $\beta$ -carotene and chlorophyll contents were analyzed in accordance with Xu et al [15] and Witham et al [16], respectively. The total phenolic content was determined using the Folin–Ciocalteu procedure described by Singleton and Rossi [17] with gallic acid as the standard and was expressed ( $\mu\text{g}$ ) as gallic-acid equivalent (GAE)/g of extract. The anthocyanin content was determined according to the method of Padmavati et al [18] with quercetin as the standard and was expressed ( $\mu\text{g}$ ) as quercetin equivalent/g of extract.

### 2.3. Experimental design

Weanling male Wistar rats (Lasco, Taipei, Nangang, Taiwan), weighing  $50 \pm 5$  g, were housed individually in stainless-steel cages. The experiments were carried out in accordance to internationally accepted guidelines on laboratory animal use and the protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of National Kaohsiung Marine University of Taiwan.

Weanling male Wistar rats (Lasco, Taipei, Nangang, Taiwan), weighing  $50 \pm 5$  g, were housed individually in stainless-steel cages. The temperature was maintained at 25°C with 12-hour light/dark periods. Food and deionized water were freely available [19]. Each rat was fed a Fe-deficient powder diet (9G0T, test diet, 5 mg Fe/kg) [20] for 14 days (depletion period), and the Hb concentration was monitored via blood drawn from the tail. When the Hb concentration was sufficiently low (6 g/dL), the rats were organized into five groups, based on the average body weight (g).

In the regeneration period, rats were fed a Fe-powder diet containing 20 mg Fe/kg body weight (BW) as ferrous sulfate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ) (Panreac, Barcelona, Spain) and were supplemented with different polarities of the *G. bicolor* extracts

[80 mg/kg BW *G. bicolor* alcohol extract (GBAE), *G. bicolor* water extract (GBWE) or *G. bicolor* water extract (GBEE)] for 14 days.

#### 2.4. Iron bioavailability and hematological parameters

Following overnight fasting, Wistar rats were sacrificed by carbon dioxide euthanasia. Blood was withdrawn using the intracardiac method, and serum was prepared for the analysis of hematological parameters by the automated hematology analyzer (Sysmex, Kobe, Japan). The Hb concentrations and Fe consumption results were used to estimate the following parameters [21]:

- (1) Hb Fe gain (Fe mg/rat), assuming that the total blood volume was 6.7% of body weight and Fe content in Hb was 0.335%:

$$\text{Hb Fe gain} = [\text{body weight (g)} \times \text{Hb (g/dL)} \times 6.7 \times 0.335]/1000$$

- (2) HRE:

$$\% \text{HRE} = [\text{Hb Fe (final)} - \text{Hb Fe (initial)} \times 100]/\text{Fe intake (mg/rat)}$$

- (3) RBV:

$$\% \text{RBV} = 100 \times (\% \text{HRE test group}/\% \text{HRE ferrous sulfate group})$$

Serum iron and TIBC (Randox Laboratories, Crumlin, Co., Antrim, UK) concentrations were determined using commercial kits for colorimetric iron determination at 590 nm. The protocol described by the manufacturer was used.

#### 2.5. Ferritin contents and TS

Blood samples were centrifuged at 3000g for 10 minutes. The liver samples were scraped (in a buffer containing 8mM  $\text{KH}_2\text{PO}_4$ , 12mM  $\text{K}_2\text{HPO}_4$ , and 1.5% KCl; pH 7.4) with a homogenizer. The homogenate was centrifuged at 3000g for 10 minutes at 4°C. Ferritin was assayed using an enzyme-linked immunosorbent assay kit (R&D System, Minneapolis, MN, USA). Serum and liver ferritin concentrations were extrapolated from standard curves, as described by the manufacturer's protocol. TS was calculated from serum iron and TIBC using the following equation:

$$\text{TS (\%)} = \text{serum iron}/\text{TIBC} \times 100\%.$$

#### 2.6. Serum hepcidin contents

Serum hepcidin concentrations were determined using commercial enzyme-linked immunosorbent assay kits (Sunred, Shanghai, Baoshan, China). Serum hepcidin concentrations were extrapolated from standard curves, as described in the manufacturer's protocol.

#### 2.7. Protein expression

Each duodenal mucosa sample was scraped with a glass slide and homogenized [in RIPA (radio-immunoprecipitation assay) buffer containing 150mM NaCl, 50mM Tris-HCl (pH 8.0), 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate), 1mM PMSF (phenylmethylsulfonyl fluoride), 2  $\mu\text{g}/\text{mL}$  aprotinin, 1  $\mu\text{g}/\text{mL}$  leupeptin, and 1mM sodium orthovanadate] with a homogenizer on ice [6]. The homogenate was centrifuged at 12,000g for 20 minutes at 4°C. Protein concentrations were determined using the Lowry et al's [22] method. Samples in sample buffer (v/v = 1/1) were heated at 95°C for 5 minutes and then loaded on a 10% gel. The proteins separated were then transferred to polyvinylidene fluoride membranes using a semidry transfer apparatus (Bio-Rad, Philadelphia, PA, USA). Each membrane was blocked with 5% nonfat milk for 2 hours and probed with DMT-1, FPN, or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Santa Cruz Bioscience, CA, USA) antibodies diluted in Tris-buffered saline. Cross-reactivity was observed with peroxidase-linked anti-IgG. Colorimetric protein detection used 3,3'-0-diaminobenzidine (Sigma Chemical, St. Louis, MO, USA). Band intensities were measured with an AlphaImager 2000 (Alpha Innotech, San Leandro, CA, USA).

#### 2.8. Statistical analysis

Data are expressed as the means  $\pm$  standard deviation from at least three independent experiments. Differences among treatments were analyzed by one-way analysis of variance and Duncan's test using the SPSS version 12.0 statistical software (SPSS Inc., Chicago, IL, USA). Statistically significant differences were defined as  $p < 0.05$ .

### 3. Results

#### 3.1. *G. bicolor* extract composition

The composition analysis result of *G. bicolor* extracts was shown in Table 1. The main phytoactive ingredients contained in GBAE were 3.08  $\mu\text{g}/\text{g}$  of quercetin, 506  $\mu\text{g}/\text{g}$  of rutin, 29.8  $\mu\text{g}/\text{g}$  of gallic acid, 102  $\mu\text{g}/\text{g}$  of chlorophyll, 2.39  $\mu\text{g}/\text{g}$  of  $\beta$ -carotene, 22.5 mg of GAE/g of total phenolics, 51.4 mg of cyanidin chloride equivalents (CyE)/g of anthocyanin, and 3.26  $\mu\text{g}/\text{g}$  of iron; in GBWE were 2.84  $\mu\text{g}/\text{g}$  of gallic acid, 22.5  $\mu\text{g}/\text{g}$  of chlorophyll, 1.63  $\mu\text{g}/\text{g}$  of  $\beta$ -carotene, 2.11 mg of GAE/g of total phenolics, 47.6 mg of CyE/g of anthocyanin, and 10.1  $\mu\text{g}/\text{g}$  of iron; and in GBEE were 25.3  $\mu\text{g}/\text{g}$  of quercetin, 11500  $\mu\text{g}/\text{g}$  of chlorophyll, 7460  $\mu\text{g}/\text{g}$  of  $\beta$ -carotene, 2.40 mg of GAE/g of total phenolics, 49.1 mg of CyE/g of anthocyanin, and 1.44  $\mu\text{g}/\text{g}$  of iron.

#### 3.2. Effect of *G. bicolor* extracts on iron bioavailability and hematological parameters in rats

Changes in Hb content in experimental rats prior to and after iron repletion are shown in Table 2. The statistical results show that, after iron repletion, the ferrous sulfate group and GBAE + ferrous sulfate, GBWE + ferrous sulfate, and

**Table 1 – Chemical composition analysis of *Gynura bicolor* and its extracts.**

Ingredients	<i>G. bicolor</i>	GBAE	GBWE	GBEE
Quercetin ( $\mu\text{g/g}$ )	0.11	3.08	ND	25.3
Rutin ( $\mu\text{g/g}$ )	6.12	506	ND	ND
Gallic acid ( $\mu\text{g/g}$ )	7.50	29.8	2.84	ND
Chlorophyll ( $\mu\text{g/g}$ )	629	102	22.5	11500
$\beta$ -Carotene ( $\mu\text{g/g}$ )	27.3	2.39	1.63	7460
Total phenolic (mg of GAE/g)	0.13	22.5	2.11	2.40
Anthocyanin (mg of CyE/g)	4.65	51.4	47.6	49.1
Iron ( $\mu\text{g/g}$ )	3.94	3.26	10.1	1.44

CyE = cyanidin chloride equivalent; GAE = gallic acid equivalent; GBAE = *G. bicolor* alcohol extract; GBEE = *G. bicolor* ether extract; GBWE = *G. bicolor* water extract; ND = not detected.

GBEE + ferrous sulfate groups showed significant differences compared to the ID group ( $p < 0.05$ ). Moreover, GBAE + ferrous sulfate and GBEE + ferrous sulfate groups were significantly higher than the other groups.

Table 3 shows the changes in serum iron, TIBC, and TS of the GBAE + ferrous sulfate, GBWE + ferrous sulfate, and GBEE + ferrous sulfate groups after iron repletion. The serum iron concentrations of the ferrous sulfate, GBAE + ferrous sulfate, GBWE + ferrous sulfate, and GBEE + ferrous sulfate groups were  $148 \pm 11 \mu\text{g/dL}$ ,  $254 \pm 30 \mu\text{g/dL}$ ,  $178 \pm 23 \mu\text{g/dL}$ , and  $225 \pm 14 \mu\text{g/dL}$ , respectively, wherein the GBAE + ferrous sulfate group had the most significant change ( $p < 0.05$ ). The TS value of the ferrous sulfate group after iron repletion was  $35.0 \pm 5.3\%$ , and those of the groups that were given GBAE or GBEE, together with ferrous sulfate, were significantly increased to  $47.4 \pm 1.6\%$  and  $47.0 \pm 5.5\%$ , respectively. Changes in serum ferritin, liver ferritin, and hepcidin are shown in Table 3. The results show that after supplementing iron, the liver ferritin concentrations of the ferrous sulfate, GBAE + ferrous sulfate, GBWE + ferrous sulfate, and GBEE + ferrous sulfate groups were  $161 \pm 5 \mu\text{g/dL}$ ,  $220 \pm 5 \mu\text{g/dL}$ ,  $198 \pm 7 \mu\text{g/dL}$ , and  $184 \pm 1 \mu\text{g/dL}$ , respectively, wherein the liver ferritin concentration of the GBAE + ferrous sulfate group was significantly higher than that of the ferrous sulfate, GBWE + ferrous sulfate, and GBEE + ferrous sulfate groups (Table 3) ( $p < 0.05$ ).

The effects of iron repletion on HREs and RBVs of the GBAE + ferrous sulfate, GBWE + ferrous sulfate, and

GBEE + ferrous sulfate groups are shown in Table 4. The results show that after iron repletion, RBVs of the ferrous sulfate, GBAE + ferrous sulfate, GBWE + ferrous sulfate, and GBEE + ferrous sulfate groups were  $100 \pm 0$ ,  $150 \pm 8$ ,  $135 \pm 7$ , and  $132 \pm 4$ , respectively, wherein the GBAE + ferrous sulfate group showed the most significant change in RBV ( $p < 0.05$ ).

### 3.3. Effect of *G. bicolor* extracts on DMT-1 and FPN protein expressions in rat duodenum

The effects of *G. bicolor* extracts of different polarities on the expressions of DMT-1 and FPN, which regulate iron metabolism of the rat duodenum, are shown in Fig. 1. The results show that, compared with the ID group, DMT-1 expression levels significantly decreased in various groups that were given *G. bicolor* extracts of different polarities after iron repletion. Likewise, DMT-1 expression levels of the groups that were given GBAE or GBWE were significantly lower compared to those of the ferrous sulfate group ( $p < 0.05$ ). In addition, the results showed that after iron repletion, FPN expression significantly increased in the groups that were given *G. bicolor* extracts of different polarities compared with the ID group, and the increasing trend in the GBAE group was significant ( $p < 0.05$ ).

## 4. Discussion

Previous studies have shown that phytoactive ingredients can considerably affect iron absorption [23]. Organic acids present in vegetables, such as citric acid, malic acid, and lactic acid, and trace nutrients, such as vitamin A and  $\beta$ -carotene, increase iron absorption [24,25]. The  $\beta$ -carotene in foods may form a complex with iron, maintaining the solubility of iron in the intestine and, at the same time, reducing the inhibition of iron absorption caused by phytic acid, tannic acid, and polyphenols [26]. In addition, some studies have demonstrated that vitamin A and  $\beta$ -carotene also increase the absorption of nonheme iron, thereby increasing Hb content [25], and that  $\beta$ -carotene-rich vegetables (carrots or amaranth) can increase iron availability in grains. Supplementation with  $\beta$ -carotene products can increase the iron bioavailability of grains by 19.6–102%, indicating that  $\beta$ -carotene can promote iron absorption [27].

**Table 2 – Effects of *Gynura bicolor* extracts with different polarities on the 14-day repletion hemoglobin in rats.<sup>a,b</sup>**

Groups	Hemoglobin (g/dL)		
	Initial	14 d repletion	14 d gain
ID	$5.05 \pm 0.56^a$	$5.34 \pm 0.18^b$	$0.29 \pm 0.12^c$
ID + ferrous sulfate	$5.11 \pm 0.78^a$	$9.93 \pm 0.58^a$	$4.82 \pm 0.46^b$
ID + GBAE + ferrous sulfate	$4.47 \pm 1.08^a$	$9.80 \pm 0.53^a$	$5.33 \pm 1.11^a$
ID + GBWE + ferrous sulfate	$4.90 \pm 1.04^a$	$9.63 \pm 0.15^a$	$4.73 \pm 1.16^b$
ID + GBEE + ferrous sulfate	$4.70 \pm 1.84^a$	$10.20 \pm 0.20^a$	$5.50 \pm 1.65^a$

Data are presented as mean  $\pm$  SD. Significance of different groups was evaluated by Duncan's test. Mean values followed by the same superscript letter are not significantly different ( $p < 0.05$ ).

GBAE = *G. bicolor* alcohol extract; GBEE = *G. bicolor* ether extract; GBWE = *G. bicolor* water extract; ID = iron-deficient diet; SD = standard deviation.

<sup>a</sup> Iron concentration was 20 mg/kg BW rat.

<sup>b</sup> GBAE, GBWE, and GBEE concentration was 80 mg/kg BW rat.

**Table 3 – Effects of *Gynura bicolor* extracts with different polarities on serum iron, TIBC, TS, serum ferritin, liver ferritin, and hepcidin in rats.<sup>a,b</sup>**

Groups	Serum iron ( $\mu\text{g/dL}$ )	TIBC ( $\mu\text{g/dL}$ )	TS (%)	Serum ferritin ( $\mu\text{g/dL}$ )	Liver ferritin ( $\mu\text{g/dL}$ )	Hepcidin (ng/L)
ID	52 $\pm$ 4 <sup>e</sup>	596 $\pm$ 12 <sup>a</sup>	11.7 $\pm$ 3.1 <sup>c</sup>	100 $\pm$ 6 <sup>c</sup>	155 $\pm$ 2 <sup>d</sup>	98 $\pm$ 5 <sup>c</sup>
ID + ferrous sulfate	148 $\pm$ 11 <sup>d</sup>	485 $\pm$ 20 <sup>b</sup>	35.0 $\pm$ 5.3 <sup>b</sup>	133 $\pm$ 3 <sup>b</sup>	161 $\pm$ 5 <sup>d</sup>	133 $\pm$ 1 <sup>a</sup>
ID + GBAE + ferrous sulfate	254 $\pm$ 30 <sup>a</sup>	506 $\pm$ 19 <sup>b</sup>	47.4 $\pm$ 1.6 <sup>a</sup>	136 $\pm$ 1 <sup>ab</sup>	220 $\pm$ 5 <sup>a</sup>	129 $\pm$ 3 <sup>a</sup>
ID + GBWE + ferrous sulfate	178 $\pm$ 23 <sup>c</sup>	469 $\pm$ 29 <sup>b</sup>	38.2 $\pm$ 7.4 <sup>b</sup>	147 $\pm$ 7 <sup>a</sup>	198 $\pm$ 7 <sup>b</sup>	124 $\pm$ 3 <sup>ab</sup>
ID + GBEE + ferrous sulfate	225 $\pm$ 14 <sup>b</sup>	480 $\pm$ 28 <sup>b</sup>	47.0 $\pm$ 5.5 <sup>a</sup>	143 $\pm$ 7 <sup>ab</sup>	184 $\pm$ 1 <sup>c</sup>	117 $\pm$ 3 <sup>b</sup>

Data are presented as mean  $\pm$  SD. Significance of different groups was evaluated by Duncan's test. Mean values followed by the same super-script letter are not significantly different ( $p < 0.05$ ).

GBAE = *G. bicolor* alcohol extract; GBEE = *G. bicolor* ether extract; GBWE = *G. bicolor* water extract; ID = iron-deficient diet; SD = standard deviation; TIBC = total iron-binding capacity; TS = transferrin saturation.

<sup>a</sup> Iron concentration was 20 mg/kg BW rat.

<sup>b</sup> GBAE, GBWE, and GBEE concentration was 80 mg/kg BW rat.

Quercetin and rutin are phytochemicals with great potential for promoting iron absorption. Previous studies have shown that rutin and quercetin can reduce the oxidation–reduction activity, labile plasma iron, and serum iron overload, and can effectively facilitate the loading of metal ions onto iron-transport proteins [23,28]. Quercetin can promote iron absorption and bioavailability by facilitating the reduction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  in foods [23]. Animal model studies have shown that an increase in the prenatal quercetin intake of adult mice increases the expression of cytokines [hepcidin, interleukin (IL)-1, IL-6, and IL-10] associated with hepatic iron metabolism, thereby significantly increasing iron storage [29]. Bacchan et al [28] have proposed that the antioxidant activity of quercetin may be dependent on its ability to pass labile plasma iron through the extracellular matrix and transfer it onto ferritin. The present study results indicate that *G. bicolor* extracts are rich in active ingredients that promote iron absorption, such as  $\beta$ -carotene, quercetin, and rutin. The main phytoactive ingredients contained in GBAE were 3.08  $\mu\text{g/g}$  of quercetin, 506  $\mu\text{g/g}$  of rutin, and 2.39  $\mu\text{g/g}$  of  $\beta$ -carotene; the main phytoactive ingredients contained in GBWE were 1.63  $\mu\text{g/g}$  of  $\beta$ -carotene; and the main phytoactive ingredients

contained in GBEE were 25.3  $\mu\text{g/g}$  of quercetin and 7460  $\mu\text{g/g}$  of  $\beta$ -carotene.

When given synthetic hepcidin-25 (10 $\mu\text{M}$ ) and cultivated for 24 hours, Caco-2 cells exhibited decreased apical iron absorption via the reduction of mRNA and protein expression of DMT-1 in iron-responsive elements (DMT1 + iron-responsive element). However, the expression of iron-regulated gene-1 (the gene encoding FPN) was not affected by hepcidin, indicating that hepcidin affects mainly the mechanism of apical iron absorption and, thus, reduces absorption of dietary iron [30]. For Caco-2 and J774 cells (rat macrophage cells) that were cultured in synthesized hepcidin-25 for 24 hours, hepcidin-25 can inhibit iron release from macrophages by inducing FPN degradation and can inhibit iron absorption of intestinal cells by inhibiting DMT-1 transcription [31]. When Caco-2 cells were cultured for 24 hours with the active ingredient of garlic (diallyl disulfide) and  $^{55}\text{FeCl}_3$ , the results showed that the mRNA and protein expression of FPN increased [4], indicating that garlic can promote iron absorption by increasing FPN expression. Mice that received synthesized hepcidin (hepcidin-25) by intraperitoneal injection showed that the injection of hepcidin-25 did not affect liver iron storage or Hb levels, but inhibited the iron absorption of duodenal apical membranes

**Table 4 – Effects of *Gynura bicolor* extracts with different polarities on the 14-day repletion hemoglobin Fe gain, HRE, and RBV in rats.<sup>a,b</sup>**

Groups	Hemoglobin Fe gain <sup>c</sup> (mg Fe/rat)	HRE <sup>d</sup> (%)	RBV <sup>e</sup> (%)
ID + ferrous sulfate	3.44 $\pm$ 0.23	5.6 $\pm$ 0.3 <sup>b</sup>	100 $\pm$ 0 <sup>c</sup>
ID + GBAE + ferrous sulfate	4.03 $\pm$ 0.23	8.5 $\pm$ 0.4 <sup>a</sup>	150 $\pm$ 8 <sup>a</sup>
ID + GBWE + ferrous sulfate	3.52 $\pm$ 0.10	7.6 $\pm$ 0.3 <sup>a</sup>	135 $\pm$ 7 <sup>b</sup>
ID + GBEE + ferrous sulfate	3.27 $\pm$ 0.12	7.5 $\pm$ 0.5 <sup>a</sup>	132 $\pm$ 4 <sup>b</sup>

Data are presented as mean  $\pm$  SD. Significance of different groups was evaluated by Duncan's test. Mean values followed by the same super-script letter are not significantly different ( $p < 0.05$ ).

GBAE = *G. bicolor* alcohol extract; GBEE = *G. bicolor* ether extract; GBWE = *G. bicolor* water extract; HRE = hemoglobin regeneration efficiency; ID = iron-deficient diet; RBV = relative biological value; SD = standard deviation.

<sup>a</sup> Iron concentration was 20 mg/kg BW rat.

<sup>b</sup> GBAE, GBWE, and GBEE concentration was 80 mg/kg BW rat.

<sup>c</sup> Hemoglobin Fe gain = [body weight (g)  $\times$  Hb (g/dL)  $\times$  6.7  $\times$  0.335]/1000.

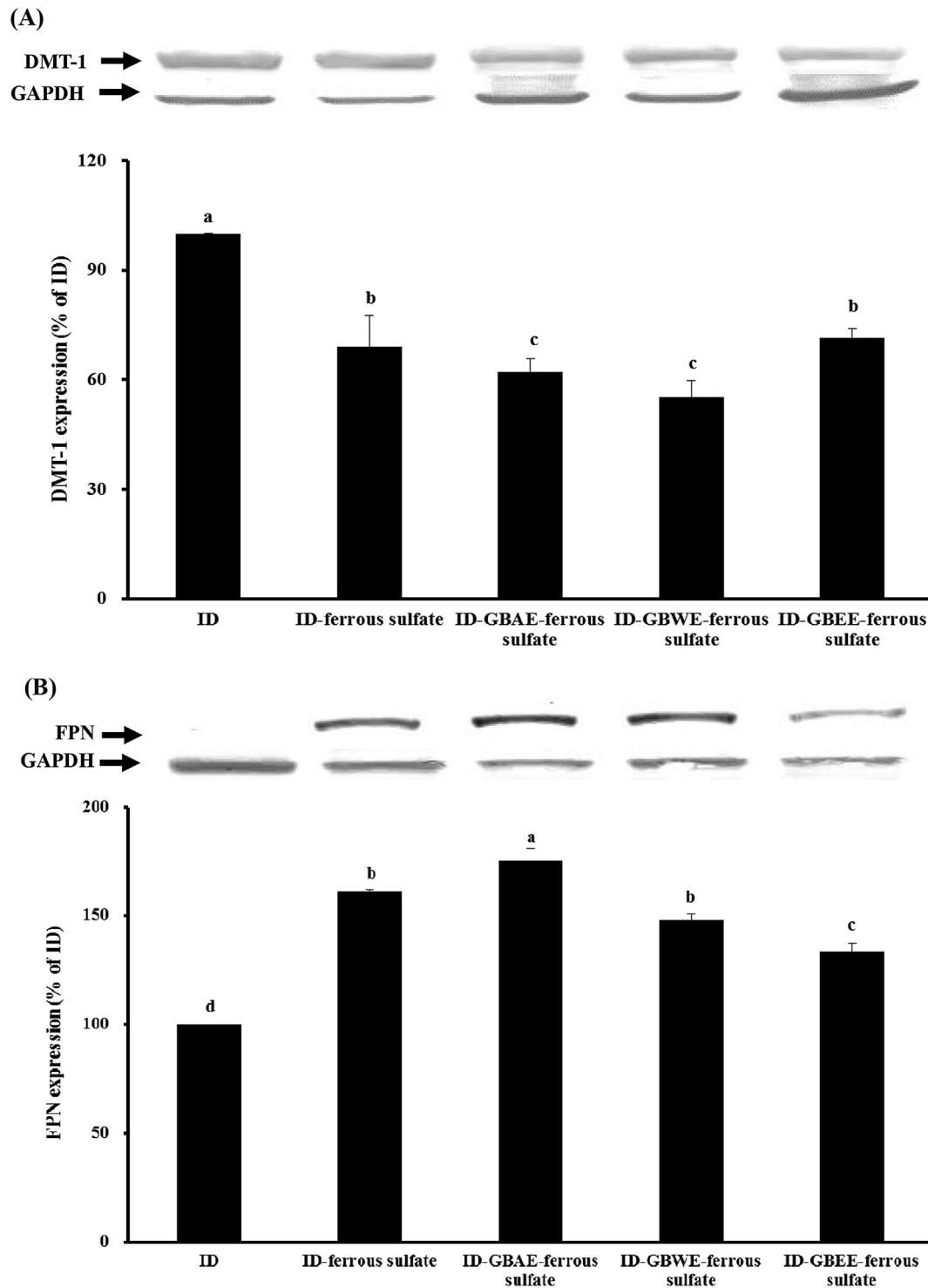
<sup>d</sup> HRE% = [Hb Fe (final) – Hb Fe (initial)  $\times$  100]/Fe intake (mg/rat/13 days).

<sup>e</sup> RBV% = HRE% test group/HRE% ferrous sulfate group.

without affecting the proportion of iron transferred into blood circulation [32].

Compared with the ID + ferrous sulfate group, the iron parameters for the groups given *G. bicolor* extracts combined

with ferrous sulfate showed that GBAE and GBEE significantly improved Hb levels, TS, serum iron levels, liver ferritin levels, HRE, and RBVs; and GBWE significantly improved serum iron levels, serum ferritin levels, liver ferritin levels, HRE, and



**Fig. 1** – Effects of *Gynura bicolor* extracts with different polarities on (A) DMT-1 and (B) FPN protein expression in rat duodenum. Results expressed as mean  $\pm$  SD. Significance of different groups was evaluated by Duncan's test. Different alphabet letters indicate that the DMT-1 and FPN protein expressions are statistically different from each other ( $p < 0.05$ ). Iron concentration was 20 mg/kg BW rat. GBAE, GBWE, and GBEE concentration was 80 mg/kg BW rat. DMT-1 = divalent metal transporter-1; FPN = ferroportin; GBAE = *G. bicolor* alcohol extract; GBEE = *G. bicolor* ether extract; GBWE = *G. bicolor* water extract; ID = iron-deficient diet (5 mg/kg).

RBVs. In addition, GBAE significantly reduced the expression of iron transporter DMT-1, but iron release protein expression of FPN was significantly increased.

DMT-1 and FPN play important roles in the iron absorption of duodenum [2,3]. DMT-1 not only accounts for dietary-iron absorption in the intestine, but also constitutes a crucial component of the transferrin cycle by mediating Fe<sup>2+</sup> transport across the endosomal membrane. DMT-1 expression is increased under iron deficiency, and it is decreased to regulate the iron amount when iron is supplemented [33]. FPN is expressed in many iron-exporting cells, including placental syncytiotrophoblasts, and plays a fundamental role in the release of iron from tissues into the bloodstream [34]. On the contrary, FPN expression is decreased under iron deficiency, and it is increased to enhance the release of iron from enterocytes when iron is supplemented [35]. In this study, GBAE affected ferrous sulfate absorption by decreasing DMT-1 expression and increasing FPN expression. Previous studies have shown that phytoactive ingredients can considerably affect iron absorption. Thus, the significant effects of GBAE on enhancing iron absorption probably result from its richness in various phytoactive ingredients. The results indicated that all *G. bicolor* extracts with different polarities (GBAE, GBWE, and GBEE) significantly increase ferrous sulfate absorption, and the most significant effect is observed in the GBAE group. The optimal concentration of the GBAE group is worth further investigation.

In summary, the results presented that *G. bicolor* extracts can improve the levels of iron absorption and storage proteins (liver ferritin) by regulating DMT-1 and FPN expressions and, therefore, promoting iron absorption. It is speculated that the effect of *G. bicolor* in promoting iron absorption may be related to its rich phytoactive ingredients, such as  $\beta$ -carotene, quercetin, rutin, and other related polyphenolic compounds. As *G. bicolor* can promote iron absorption, use of *G. bicolor* as iron supplements to treat iron deficiency anemia seems very promising.

## Acknowledgments

This study was supported by a grant (FH-HR-101-02) from the Fooyin University Hospital, Pingtung, Taiwan.

## REFERENCES

- [1] World Health Organization. World health report 2001: iron deficiency anemia assessment, prevention, and control. Geneva, Switzerland: World Health Organization; 2001.
- [2] Friedman A, Arosio P, Finazzi D, Koziorowski D, Galazka-Friedman J. Ferritin as an important player in neurodegeneration. *Parkinsonism Relat Disord* 2011;17:423–30.
- [3] Ganz T. Molecular control of iron transport. *J Am Soc Nephrol* 2007;18:394–400.
- [4] Nahdi A, Hammamia I, Brasse-Lagnel C, Pilard N, Hamdaoui MH, Beaumont C, El May M. Influence of garlic or its main active component diallyl disulfide on iron bioavailability and toxicity. *Nutr Res* 2010;30:85–95.
- [5] Lobo AR, Cocato ML, Borelli P, Gaievski EHS, Crisma AR, Nakajima K, Nakano EY, Colli C. Iron bioavailability from ferric pyrophosphate in rats fed with fructan-containing yacon (*Smallanthus sonchifolius*) flour. *Food Chem* 2011;126:885–91.
- [6] Hamlin F, Latunde-Dada GO. Iron bioavailability from a tropical leafy vegetable in anaemic mice. *Nutr Metab* 2011;8:1–7.
- [7] Shimizu Y, Imada T, Zhang H, Tanaka R, Ohno T, Shimomura K. Identification of novel poly-acylated anthocyanins from *Gynura bicolor* leaves and their antioxidative activity. *Food Sci Technol Res* 2010;16:479–86.
- [8] Rosidah, Yam MF, Sadikun A, Ahmad M, Akowuah GA, Asmawi MZ. Toxicology evaluation of standardized methanol extract of *Gynura procumbens*. *J Ethnopharmacol* 2009;123:244–9.
- [9] Yoshitama K, Kaneshige M, Ishikura N, Araki F, Yahara S, Abe K. A stable reddish purple anthocyanin in the leaf of *Gynura aurantiaca* cv. 'Purple Passion'. *J Plant Res* 1994;107:209–14.
- [10] Lu H, Pei Y, Li W. Studies on flavonoids from *Gynura bicolor* DC. *Chin J Modern Appl Pharm* 2010;27:613–4 [In Chinese].
- [11] Lin JY, Liu YL, Lin BF, Hsiao CC, Tsai SJ. Antioxidant, antimutagenic and immunomodulatory potentials of *Gynura bicolor* and *Amaranthus gangetcaue*. *Taiwanese J Agric Chem Food Sci* 2004;42:231–41 [In Chinese].
- [12] Li WL, Ren BR, Min-Zhuo, Hu Y, Lu CG, Wu JL, Chen J, Sun S. The anti-hyperglycemic effect of plants in genus *Gynura* cass. *Am J Chin Med* 2009;37:961–6.
- [13] Jia Z, Tang M, Wu J. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem* 1999;64:555–9.
- [14] Krizman M, Baricevic D, Prosek M. Determination of phenolic compounds in fennel by HPLC and HPLC-MS using a monolithic reversed-phase column. *J Pharm Biomed Anal* 2007;43:481–5.
- [15] Xu F, Yuan QP, Dong HR. Determination of lycopene and  $\beta$ -carotene by high-performance liquid chromatography using Sudan I as internal standard. *J Chromatogr B* 2006;838:44–9.
- [16] Witham FA, Blaydes DF, Devlin RM. Chlorophyll absorption spectrum and quantitative determination. In: Exercise in plant physiology. Boston: PWS Publishers; 1986. p. 128–31.
- [17] Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Viticult* 1965;16:144–58.
- [18] Padmavati M, Sakthivel N, Tahara KV. Differential sensitivity of rice pathogens to growth inhibition by flavonoids. *Phytochemistry* 1997;46:499–502.
- [19] AOAC. Official methods. Bioavailability of iron, rat hemoglobin regeneration bioassay. In: Williams S, editor. Official methods of analysis vol. 45. Arlington: Association of Official Analytical Chemists; 1995. p. 62–3.
- [20] American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies. *J Nutr* 1977;107:1340–8.
- [21] Mahoney AW, Van Orden CC, Hendricks DG. Efficiency of converting food iron into haemoglobin by the anaemic rat. *Nutr Metab* 1974;17:223–30.
- [22] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–75.
- [23] Vlachodimitropoulou E, Naftalin RJ, Sharp PA. Quercetin is a substrate for the transmembrane oxidoreductase Dcytb. *Free Radic Biol Med* 2010;48:1366–9.
- [24] Gillooly M, Bothwell TH, Torrance JD, MacPhail AP, Derman DP, Bezwoda WR, Mills W, Charlton RW. The effects of organic acids, phytates and polyphenols on the absorption of iron from vegetables. *Br J Nutr* 1983;49:331–42.

- [25] Garcia-Casal MN, Layrisse M, Solano L, Barón MA, Arguello F, Llovera D, Ramírez J, Leets I, Tropper E. Vitamin A and betacarotene can improve nonheme iron absorption from rice, wheat and corn by humans. *J Nutr* 1998;128:646–50.
- [26] Garcia-Casal MN, Irene L, Miguel L.  $\beta$ -Carotene and inhibitors of iron absorption modify iron uptake by Caco-2 cells. *J Nutr* 2000;130:5–9.
- [27] Gautam S, Platel K, Srinivasan K. Influence of  $\beta$ -carotene-rich vegetables on the bioaccessibility of zinc and iron from food grains. *Food Chem* 2010;122:668–72.
- [28] Baccan MM, Chiarelli-Neto O, Pereira RMS, Esposito BP. Quercetin as a shuttle for labile iron. *J Inorg Biochem* 2012;107:34–9.
- [29] Vanhees K, Godschalk RW, Sanders A, van Waalwijk van Doorn-Khosrovani SB, van Schooten FJ. Maternal quercetin intake during pregnancy results in an adapted iron homeostasis at adulthood. *Toxicology* 2011;290:350–8.
- [30] Yamaji S, Sharp P, Ramesh B, Srail SK. Inhibition of iron transport across human intestinal epithelial cells by hepcidin. *Blood* 2004;104:2178–80.
- [31] Mena NP, Esparza A, Tapia V, Valdés P, Núñez MT. Hepcidin inhibits apical iron uptake in intestinal cells. *Am J Physiol Gastrointest Liver Physiol* 2008;294:192–8.
- [32] Laftah AH, Ramesh B, Simpson RJ, Solanky N, Bahram S, Schumann K, Debnam ES, Srail SKS. Effect of hepcidin on intestinal iron absorption in mice. *Blood* 2004;103:3940–4.
- [33] Wang J, Pantopoulos K. Regulation of cellular iron metabolism. *Biochem J* 2011;434:365–81.
- [34] Donovan A, Lima CA, Pinkus JL, Pinkus GS, Zon LI, Robine S, Andrews NC. The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. *Cell Metab* 2005;1:191–200.
- [35] Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, Kaplan J. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* 2004;306:2090–3.