

# Anti-Sports Anaemia Effects of Verbascoside and Martynoside in Mice

## Authors

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## Key words

- verbascoside
- martynoside
- anti-sports anaemia

## Abstract

This paper aims to investigate the effects of verbascoside and martynoside isolated from *Pedicularis dolichocymba* on sports anaemia. Forty mice were divided into four groups: Group R (control group, nonsupplemented and maintained at rest), Group E (nonsupplemented and undergoing exercise), Group VE (supplemented with verbascoside 10 mg/kg per day and undergoing exercise), and Group ME (supplemented with martynoside 10 mg/kg per day and undergoing exercise). After 5 weeks intensive swimming exercises, we measured the RBC count, the hemoglobin concentration, the hematocrit (Hct), the mean corpuscular hemoglobin concentration

(MCHC) and the mean corpuscular hemoglobin (MCH). We studied the shapes of RBC and measured the plasma malonyldialdehyde (MDA). We found Group E showed lower RBC, hemoglobin and Hct levels, higher MCHC, MCH, plasma MDA levels and the abnormally shaped RBCs percentage than Groups R, VE and ME. Group ME showed lower RBC and Hct levels, higher MCH, plasma MDA levels and the abnormally shaped RBCs percentage than Group VE. The results indicated that verbascoside and martynoside have the potential of antagonizing sports anaemia, the mechanism of this effect might be related to preventing RBC from free radical damage. Moreover, verbascoside was found to be more active than martynoside.

## Introduction

There have been reports on sports anaemia associated with intensive physical exercise [3,23]. Sports anaemia not only reduces exercise capacity, but also does harm to athletes' health. Many reasons have been found responsible for sports anaemia, such as gastrointestinal and urinary tract bleeding, iron deficiency and hemolysis, etc. [6,19,24,27]. Among them red blood cell (RBC) destruction caused by intensive physical exercise is the most emphasized one [2,8,28], and oxidant stress is a well-documented cause of RBC mechanical destruction [5,11,22].

Natural products are important sources in drug development. *Pedicularis* species are herbs widely used in traditional Chinese remedies for treatment of collapse, exhaustion and senility [17]. Phenylpropanoid glycosides (PPGs) are characteristic compounds of *Pedicularis* species [7], and have been reported to possess antioxidative properties, inhibiting linoleic acid peroxidation in micelles [29], scavenging superoxide and hydroxyl radicals [12,26]. Verbascoside and mar-

tynoside are two PPGs we isolated from *P. dolichocymba* (○ Fig. 1) [4], which are reported to have the effect of retarding skeletal muscle fatigue [14]. Verbascoside also has the effect of reducing oxidative stress in muscle caused by exhaustive exercise by decreasing the concentration of free radicals and the level of lipid peroxidation [13]. Furthermore, verbascoside might have the effect of moderating oxidative stress and erythrocyte membrane fluidity during immobilization [15]. There are no reports, however, about antagonizing sports anaemia effects of verbascoside and martynoside. In our study their effects on hematological parameters, RBC shapes and oxidant stress in exercised mice were investigated.

## Materials and Methods

### Animals

Male mice (Kun-ming strain by origin, grade SPF), weighing  $29.00 \pm 1.65$  g and aged 5 weeks were used for the study. They were obtained from the

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Experimental Animal Center of Guangdong Medical College, Zhanjian, Guangdong, China. The certificate number of the animal breeder is 2004A029. Ten animals were housed per cage under the controlled conditions of temperature (18–24 °C), humidity (40%), and a light/dark cycle (12 h/12 h) with access to food and water ad libitum. Animals were allowed to acclimatize to the laboratory before the commencement of the experiment. Procedures were approved by the Guangdong Province Laboratory Animal Care and Use Committee. Our study also meets the ethical standards of the IJSM [9].

### Experimental design

Forty mice were randomly divided into four groups (n=10): nonsupplemented and maintained at rest (Group R), serving as control; nonsupplemented and undergoing exercise (Group E); supplemented with verbascoside 10 mg/kg per day and undergoing exercise (Group VE); and supplemented with martynoside 10 mg/kg per day and undergoing exercise (Group ME). The training groups underwent swimming exercises in a water container measuring 60 cm in height, 19 cm in diameter, and 40 cm in depth at the temperature of 29 °C for five weeks. The swimming duration was progressively increased: 30 min/day for the first week, 60 min/day for the second week, 90 min/day for the third week, 120 min/day for the fourth week, and 150 min/day for the fifth week. The mice in the training groups swam once a day for 5 days per week. All the mice were weighed twice a week during the experiment. One mouse in Group E died of a disease on the fifteenth day of the experiment.

### Plant and chemistry

*Pedicularis dolichocymba* was collected in Zhongdian, Yunnan, China in August 2003 and identified by Prof. Hong Wang, Kunming Institute of Botany, Chinese Academy of Sciences. The dried whole plants of *P. dolichocymba* were extracted by 95% ethanol and then concentrated under reduced pressure. The residue was dissolved in hot water and successively extracted by EtOAc. The EtOAc portion was eluted by CHCl<sub>3</sub>-MeOH (20: 1) over silica gel column to give verbascoside, and was further separated over silica gel and Sephadex LH-20 column to give martynoside (○ Fig. 1). Detailed purification and identification had been described in our previous publication [4].

### Drug administration

The mice in Groups VE and ME were given 10 mg/kg of verbascoside and martynoside (dissolved in 0.86% NaCl) respectively for

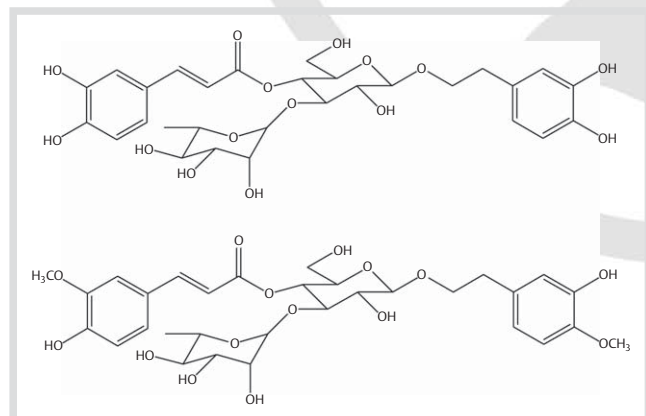


Fig. 1 Chemical structures of verbascoside and martynoside.

five weeks through an intragastric gavage (ig) once per day. The mice in Groups R and E were administrated 0.86% NaCl solution in the same volume and using the same method.

### Collection and preparation for blood samples

On the 36<sup>th</sup> day of the experiment, the exhaustion time during forced swimming was determined for Groups VE, ME, R and E. After exhaustive swimming, mice took a break of 24 h. The blood of mice was obtained by cardiac puncturing into an anticoagulated heparin solution, which was used for the basic hematological study, the RBC was visualized using scanning electron microscopy and analysis of plasma malonyldialdehyde.

### Basic hematological study

RBC count, hemoglobin concentration, hematocrit (Hct), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), and mean corpuscular volume (MCV) were calculated using the pocH-100i hematology analyzer (Sysmex Corporation, Kobe, Japan) for the heparinized blood samples.

### Preparation for Red Blood Cell (RBC) by Scanning Electron Microscopy (SEM)

Five mice were selected randomly from each group for RBC visualization by SEM. After washing RBC with 0.86% NaCl solution (pH 7.4), blood samples were centrifuged at 1 000 rpm for 10 min to obtain the pellet containing the RBCs. The process was repeated 3 times to obtain a pure preparation of RBCs. The RBCs were then fixed in 2.5% glutaraldehyde (2.5% in 0.1 M sodium phosphate buffer, pH 6.0) at 4 °C.

### SEM

After being fixed for 3 days at 4 °C, the RBCs were washed with decreasing concentration of phosphate buffer following dehydration in graded ethanol (from 50%) and finally in absolute ethanol. The dehydrated RBCs were mounted on a brass stub, dried at room temperature and sputteringly coated with gold for 10 min at 1.2 kV. The cell surface architecture was visualized by the SEM (Philips XL30; Philips Co., Eindhoven, Netherlands) operated at 25 kV accelerating potential. Morphological changes of erythrocytes were observed, classified and counted by SEM [1, 18].

### Analysis of Plasma Malonyldialdehyde (MDA)

Plasma MDA level was measured by thiobarbituric acid assay described by the MDA kit. The kit was provided by the Nanjing Jiancheng Bioengineering Institute, Nanjing, China.

### Statistics

All measurements were expressed as mean ± standard deviation. The SPSS 10.0 package was used for the statistical analysis. The changes in body weight, hematological parameters and plasma MDA level were assessed by the one-way analysis of variance (ANOVA). The percentage of abnormally shaped RBC was compared by the chi-squared test. Statistical significance was accepted at values of  $p < 0.05$ .

**Table 1** Effects of martynoside and verbascoside on body weights (g).

	<sup>b</sup> Group R	<sup>a</sup> Group E	<sup>b</sup> Group VE	<sup>b</sup> Group ME
before the experiment	28.91 ± 1.11	29.36 ± 1.30	29.47 ± 1.67	29.24 ± 1.23
the end of first week	31.49 ± 0.94	31.85 ± 1.17	32.29 ± 1.55	32.82 ± 1.05
the end of second week	34.11 ± 1.64	30.88 ± 2.61 <sup>††</sup>	31.45 ± 1.18 <sup>††</sup>	31.53 ± 0.83 <sup>††</sup>
the end of third week	35.90 ± 1.57	31.12 ± 2.19 <sup>††</sup>	31.98 ± 1.73 <sup>††</sup>	32.31 ± 1.03 <sup>††</sup>
the end of fourth week	36.03 ± 1.54	31.17 ± 2.41 <sup>††</sup>	32.43 ± 2.33 <sup>††</sup>	32.47 ± 1.32 <sup>††</sup>
the end of fifth week	36.81 ± 2.02	30.18 ± 2.91 <sup>††</sup>	33.06 ± 1.51 <sup>††§§</sup>	32.61 ± 1.33 <sup>††§§</sup>

<sup>a</sup> Before the experiment and the end of first week of the experiment N=10, From the end of second week to the end of fifth week of the experiment N=9; <sup>b</sup>N=10. <sup>§</sup>p<0.05, <sup>§§</sup>p<0.01, compared with the E Group; <sup>††</sup>p<0.01, compared with the Group R

**Table 2** Effects of martynoside and verbascoside on hematological parameters.

	Group R <sup>b</sup>	Group E <sup>a</sup>	Group VE <sup>b</sup>	Group ME <sup>b</sup>
RBC (× 10 <sup>12</sup> /L)	8.30 ± 0.50	5.26 ± 0.89 <sup>††</sup>	8.05 ± 0.36 <sup>§§, ††</sup>	7.21 ± 0.31 <sup>††, §§</sup>
Hb (g/L)	134.10 ± 10.50	118.78 ± 22.43 <sup>†</sup>	134.10 ± 8.65 <sup>§</sup>	132.00 ± 5.43 <sup>§</sup>
Hct (%)	38.05 ± 3.14	25.99 ± 4.38 <sup>††</sup>	37.20 ± 1.81 <sup>§§, †</sup>	33.91 ± 2.34 <sup>††, §§</sup>
MCV (fl)	47.36 ± 1.68	49.98 ± 6.04	46.10 ± 1.52	47.60 ± 2.28
MCH (pg)	16.87 ± 2.05	24.90 ± 4.41 <sup>††</sup>	16.36 ± 1.43 <sup>§§, †</sup>	19.15 ± 2.76 <sup>§§</sup>
MCHC (g/L)	356.89 ± 49.30	430.67 ± 49.19 <sup>††</sup>	355.30 ± 30.34 <sup>§§</sup>	383.44 ± 33.41 <sup>§</sup>

<sup>a</sup>N=9, <sup>b</sup>N=10. <sup>†</sup>p<0.05, <sup>††</sup>p<0.01, compared with Group R; <sup>§</sup>p<0.05, <sup>§§</sup>p<0.01, compared with Group E; <sup>†</sup>p<0.05, <sup>††</sup>p<0.01, compared with Group ME. Abbreviations: red blood cell (RBC), hemoglobin concentration (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC)

**Table 3** Percentages of abnormally shaped RBCs of Groups R, E, VE and ME.

	Abnormally shaped RBCs (%)
<sup>a</sup> group R	11.80 ± 3.03
<sup>a</sup> group E	61.60 ± 7.40 <sup>††</sup>
<sup>a</sup> group VE	31.20 ± 7.69 <sup>††, §§, ††</sup>
<sup>a</sup> group ME	54.80 ± 9.12 <sup>††, §</sup>

<sup>a</sup>N=5. <sup>††</sup>p<0.01, compared with Group R; <sup>§</sup>p<0.05, <sup>§§</sup>p<0.01, compared with Group E; <sup>††</sup>p<0.01, compared with Group ME. Abbreviations: red blood cells (RBCs)

## Results

### Effect of verbascoside and martynoside on general state of health of mice in groups

The mice in all groups were active and well with shiny fur, obviously increased appetites and body weights. There were no significant differences in the first week ( $P>0.05$ ). The mice in Groups E, VE and ME had decreased appetites and body weights which were lower than those of Group R ( $P<0.01$ ) in the second week. Body weights of the mice in Groups E, VE and ME were significantly lower than those of Group R from the third week to the fifth week ( $P<0.01$ ). The mice in Group E showed reduced activity, thinned fur, decreased appetite, and obviously lost body weight in the fifth week, while those symptoms significantly lessened in the mice of Groups VE and ME with increased appetites and body weights, which were higher than those of Group E ( $P<0.01$ ,  $P<0.05$ ) (○ **Table 1**).

### Effect of verbascoside and martynoside on hematological parameters

RBC count, Hb concentration and Hct of Group E were significantly lower than those of Groups R, VE and ME ( $p<0.01$ ,  $p<0.05$ ), while MCH and MCHC of Group E were significantly higher than those of Groups R, VE and ME ( $p<0.01$ ,  $p<0.05$ ). RBC count and Hct of Group VE were significantly higher than those of Group ME ( $p<0.01$ ,  $p<0.05$ ), while MCH values of Group VE

were significantly lower than those of Group ME ( $p<0.05$ ) (○ **Table 2**).

### Effect of verbascoside and martynoside on RBC shape

Results from SEM showed that most RBCs exhibited normal discoid structures in Group R. There were many abnormally shaped RBCs such as torocytes in Group E, whose percentage was significantly higher than that of Groups R, VE and ME ( $p<0.01$ ,  $p<0.05$ ). The percentage of abnormally shaped RBCs of Group VE was significantly lower than that of Group ME ( $p<0.01$ ) (○ **Table 3** and ○ **Fig. 2**).

### Effect of verbascoside and martynoside on Plasma MDA

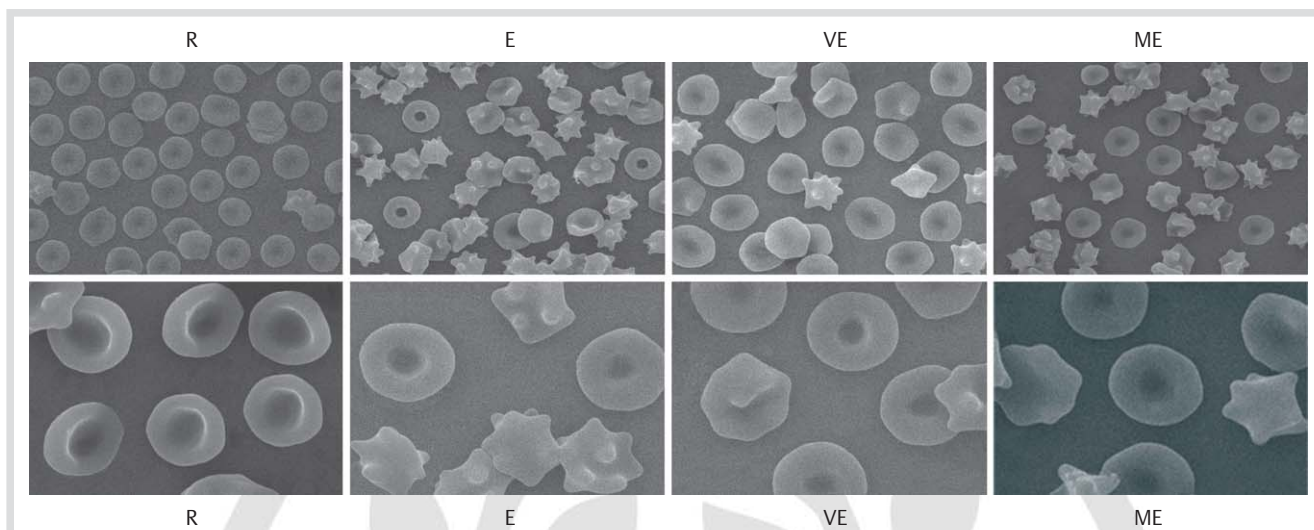
The plasma MDA values of Group E were significantly higher when compared with those of Groups R, VE and ME ( $p<0.01$ ), and the plasma MDA values of Group VE were significantly lower than that of Group ME ( $p<0.01$ ) (○ **Table 4**).

## Discussion

This study examined the effects of verbascoside and martynoside on hematological parameters, RBC shapes and oxidant stress. The doses of verbascoside and martynoside used were based on the literature [13] and on the preliminary experiment results.

RBC count, Hb concentration and Hct are recognized as indexes of anaemia diagnosis. Results in ○ **Table 2** show that RBC count, Hb concentration and Hct of Group E were significantly lower than those of Group R, which suggests that sports anaemia occurred after five weeks of intensive swimming exercise. Furthermore, significant differences of these three hematological parameters in Groups E, VE, and ME suggest that verbascoside and martynoside show potential effects on antagonizing anaemia during intensive exercise, and verbascoside was more active than martynoside. There are many reasons reported to explain





**Fig. 2** Morphological changes in RBCs of Groups R, E, VE and ME. The microscope magnified the object 3000 diameters in the first line. The microscope magnified the object 8000 diameters in the second line.

**Table 4** Plasma MDA levels of Groups R, E, VE and ME.

	<sup>b</sup> Group R	<sup>a</sup> Group E	<sup>b</sup> Group VE	<sup>b</sup> Group ME
MDA (μmol/L)	4.49 ± 0.26	23.29 ± 2.21 <sup>††</sup>	10.33 ± 1.04 <sup>††,§§,‡‡</sup>	13.32 ± 7.09 <sup>††,§§</sup>

<sup>a</sup>N=9, <sup>b</sup>N=10, <sup>††</sup>p<0.01, compared with Group R; <sup>§§</sup>p<0.01, compared with Group E; <sup>‡‡</sup>p<0.01, compared with Group ME. Abbreviations: malonyldialdehyde (MDA)

sports anaemia [6,19,24,27], among which RBC destruction caused by intensive physical exercise is the most emphasized one [2,8,28]. Results in **Table 2** also show that MCH and MCHC of Group E were significantly higher than those of Group R, which suggests that sports anaemia is partly caused by RBC destruction. SEM results in **Table 3** and **Fig. 2** not only support this viewpoint, but also indicate that verbascoside and martynoside exhibited a significant protective effect on RBC, and verbascoside was more active than martynoside. This suggests that the more noticeable anti-sports anemia effect of verbascoside might be related to the more potent protective role of verbascoside on RBC.

Oxidant stress is a well-documented cause of RBC mechanical destruction [11,22,25]. The increase in plasma MDA seems to serve as an indicator of oxidative stress related to damage of lipids and proteins of RBC, which not only leads cell to membrane-lipid peroxidation and subsequent RBC destruction, but also modifies RBC membrane proteins such as spectrins and band 3, and causes RBC destruction [2,20,21,28]. Strenuous exercise may lead to a remarkable increase of free radical production [5]. Results in **Table 4** show that plasma MDA values in Group E were significantly higher when compared with those of Group R, which is consistent with the literature [10,16]. Moreover, the significant differences of plasma MDA level in Groups E, VE, and ME suggest that verbascoside and martynoside have the effect of rapidly removing free radicals, and verbascoside is more active than martynoside. This suggests that verbascoside and martynoside exhibited a significant protective effect on RBC which would prevent RBC from free radical damage.

Verbascoside and martynoside are PPGs with two similar aromatic rings. There are two hydroxyl groups at meta and para

positions in each aromatic ring in verbascoside, while the meta and para hydroxyls are substituted by two methoxyl groups in martynoside (**Fig. 1**). It has been reported that verbascoside and martynoside have the ability of scavenging the reactive oxygen species (ROS) and the antioxidant properties of these PPGs are dependent on the number of phenol hydroxyl groups at conjugating positions. The higher the number of these groups, the stronger are their antioxidant activities [12,14]. Results from this study also demonstrated that the free radical removal effect of verbascoside was better than that of martynoside, which is consistent with the literature [26].

Verbascoside and martynoside have also shown to be effective in lessening symptoms of fatigue caused through intensive physical exercise such as reduced activity, thinned fur, decreased appetite, lost body weight, and so on. We suggest that this is related to their antagonizing sports anaemia. In addition, this result also suggests that verbascoside and martynoside might be active compounds of *Pedicularis* species' strong effect in promoting physical performance in exercised rats.

This research shows that verbascoside and martynoside have the potential on antagonizing sports anaemia, whose mechanism might be related to their preventing RBC from free radical damage. Verbascoside was however found to be more active than martynoside. Results from this study not only suggest that verbascoside and martynoside may be active compounds of *Pedicularis* species' strong effect of antagonizing sports anaemia, but also could provide partial experimental basis for verbascoside used in sports medicine.

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