

Adjustment of Prescription by ORAC assay to Enhance the Effect of Medicinal Liqueur

Yuan-Cai Liu¹, Yue-Jun Yang², Mo-Si Chen^{2*}, Lei Wang¹, Shi-Guang Lu², Sheng-Hua Liu¹,
Tao Yin², Yi-Min Shan³

1. Jing Brand Co., Ltd., Daye, China

2. Hubei Provincial Key Lab for Quality and Safety of Traditional Chinese Medicine Health Food, Daye, China

3. Jing Brand Research Institute, Daye, China

* E-mail: ccmms@sina.com.cn

Abstract: Objective The ingredient herbs of JING liqueur were studied, and prescription was adjusted by oxygen radical absorbance capacity (ORAC) assay to enhance the effect of this medicinal liqueur. **Method** Values of very herb's ORAC were measured, the proportions of high ORAC value herbs were increased, while the proportions of low ORAC value herbs were decreased. Adjusted JING liqueur was compared with primary product by the test of anti-fatigue and enhance immunity experiment. **Result** Compared with primary product, the anti-fatigue effect of adjusted JING liqueur was significantly increased, and the enhance immunity effect of adjusted JING liqueur was obviously improved. **Conclusion** The effect of JING liqueur could be enhanced by adjusting proportions of every ingredients across their ORAC, increased proportions of high ORAC value herb could obviously improve the anti-fatigue and enhance immunity efficacy.

Key words: medicinal liqueur, ORAC value, anti-fatigue, enhance immunity

1. Introduction

Traditional Chinese Medicine select various herbs to treat different diseases follow the theory of Chinese medical recipe, but many prescriptions have not been verified by experiment, and the adjustment of prescription is always by empiricism, hence searching for appropriate methods to verify and adjust traditional Chinese medicine prescription has significant value. Vinum is considered to be an effective dosage form, JING liqueur is a kind of medicinal liqueur produced by Jing Brand Co., LTD. which alcohol concentration is 35% vol, ingredients including rhizoma dioscoreae, angelica sinensis, cistanche, wolfberry, astragalus membranaceus, epimedium, cortex cinnamomi and syzygium aromaticum. The prescription of above herbs in JING liqueur is considered has the function of anti-fatigue and enhancing immunity. The finished product of JING liqueur is stable in composition, fingerprintings of JING liqueur was shown in Figure 1, and could be discriminated by electronic nose [1].

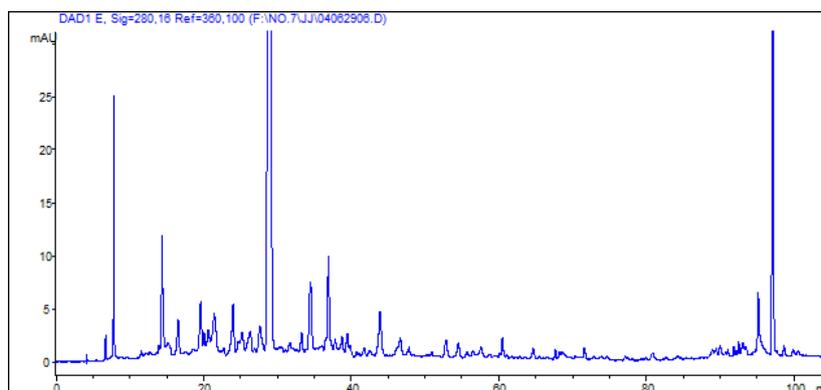


FIGURE 1. Fingerprintings of JING liqueur.

It was reported that physiological fatigue and decline in immunity related to oxygen radical. Oxidative stress and exhaustion play an important role in physical fatigue [2]. Hard work or intense exercise can lead to the production and accumulation of excess reactive free radicals, which results in oxidation stress injury to the body. Exhaustion theory suggests that excess metabolite accumulation in body is one of the most important reasons

lead to fatigue besides energy source depletion [3]. Several studies have shown that exogenous antioxidants could reduce exercise-induced oxidative stress and produce anti-fatigue effect [4]. Oxidative stress could cause damage in organs, induce varieties of cytokines and enzymes dysfunctional, plant extracts with antioxidant activity have the effect of anti-inflammatory and enhance immunity [5]. It was found that exogenous antioxidant led to significant suppression of serum IL-6, TNF- α levels, and significant enhancement of serum IL-2, IL-4, IL-10 levels in immunity-enhancing model [6].

Many phytochemicals have antioxidant activity and may help protect cells against the oxidative damage caused by free radicals. The Oxygen radical absorbance capacity (ORAC) assay has become a popular method for measuring the antioxidant or radical scavenging capacity of biological samples, and was found valuable in researching the characteristics of herbs [7]. This research is to enhance the effect of JING liqueur by adjust proportions of every ingredients across their ORAC value.

2. Material preparation

Dried herbs were purchased and identified by Hubei University of Chinese Medicine, 300g herb was weight and dissolved in 1000 ml 50% alcohol. After be vibrated for 3h and 4000 r/min centrifuged for 10 min, supernatant was collected for ORAC assays.

The JING liqueur was provided by Jing Brand Co., LTD., the low/middle/high doses of treated groups were converted from 10/20/30 fold of adult daily intake (100ml/60kg.bw), and concentrated to the gavage doses for animal (0.2ml/10g.bw, 15% vol).

3. Experimental method

3.1 ORAC assays

The ORAC measurement was conducted based on the method described by Ou et al [8]. This method was modified so it can be used for a 96-well microplate. All the ORAC assays were conducted on a microplate reader (synergy2, Bio-Tek, USA). AAPH was used as a peroxy radical generator. Trolox was used as a standard, and fluorescein (FL) was used as a fluorescent probe with an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

Herbs were powdered and dissolved in alcohol as previously mentioned, supernatants were diluted to proper concentrations for analysis. FL and AAPH were dissolved in 50 mM potassium phosphate buffer (pH=7.4). Then 25 μ L of diluted flavonoid standards were mixed with 75 μ L of 48 nM FL-disodium. The solution was incubated for 15 min at 37°C. Then 100 μ L of AAPH solution (170 mM) was injected into each well. The fluorescence was taken immediately every 2 min for 3 h at 37°C. The reaction mixture without flavonoids was used as a blank. All measurements were taken in triplicate. The final ORAC values were calculated using the differences of area under the decay curves between a blank and a sample (i.e., net area). The ORAC units were expressed as trolox equivalents (μ mol Trolox equivalent/100g). Various concentrations of Trolox were used as standards for the calibration curve. After values of very herb's ORAC were measured, the proportions of high ORAC value herbs were increased, while the proportions of low ORAC value herbs were decreased. Adjusted JING liqueur was compared with primary product by the test of anti-fatigue and enhance immunity experiment.

3.2 Anti-fatigue experiment

3.2.1 Forced Swimming Test

Sixty SPF male KM mice (18-22g) were randomized into six groups: control group (were perfused with distilled water), alcohol group (were perfused with 15% ethanol), primary product group (converted from 30 fold of primary product for adult daily intake), and adjusted product low/middle/high dose groups (converted from 10/20/30 fold of adjusted product for adult daily intake). All groups received intragastric administration 0.2ml/10g.bw once a day for 30 days.

The protocol was adapted from a previous study with some modifications [2]. 30 min after the last treatment administration, mice were underwent an exhaustive swimming test. The mice were placed individually in the swimming pool with water depth more than 40 cm, maintained at 25 \pm 1°C. A weight equivalent to 5% of body weight was attached to the root of mouse tail, and endurance for each mice was measured as swimming times recorded from the beginning of the time in the pool to exhaustion. The swimming period was considered the time spent floating, struggling, and making necessary movements until exhaustion and possible drowning. When the mice were unable to remain on the water surface, they were considered exhausted.

3.2.2 Determination of Blood Lactate

Mice were grouped and treated as above, plasma lactate levels were determined before and after exercise. A 10 min swimming test was performed 30 min after the last treatment administration. Blood samples were collected from the angular vein plexus of pretreated mice when swimming test terminated, and collected again after a 20

min rest. The serum samples were collected after centrifugated, lactate levels were determining by diagnostic kit (Biosino, China).

3.2.3 Hepatic Glycogen Determination

Mice were grouped and treated as above, 30 min after the last treatment administration the liver was excised and weighed for tissue glycogen level analysis. The hepatic glycogen levels were measured as described [2]. For each mouse, 75 mg of liver was finely cut, and glycogen levels were determining by diagnostic kit (Biosino, China).

3.3 Enhance immunity experiment

3.3.1. Carbon Clearance Test in Vivo

Mice were grouped and treated as above, at the end of the experiment, mice were injected with 100ml/kg of Indian ink via the tail vein. Blood samples were got from angular vein at 2 and 10 min after injection. An aliquot (20 μ L) of blood was mixed with 2 mL 0.1% sodium carbonate solution, and the absorbance of this solution was determined at 600 nm. The rate of carbon clearance (K) was calculated from the following equation [9]:

$$K = (\log A_{10} - \log A_2) / (t_{10} - t_2) \quad (1)$$

where A = absorbance at blood collected at the respective time point,

t = time of blood collection (min).

The phagocytic index (α) was calculated from the following equation:

$$\alpha = [\text{body weigh}/(\text{liver weight} + \text{spleen weight})] \times K^{1/3}$$

Liver, spleen and thymus were exteriorized and weighed after mice were sacrificed.

3.3.2 Hemolysis of Sheep Red Blood Cell (SRBC) at 50% (HC₅₀)

Mice were intragastrically treated with drug for 30d, Aliquots (0.2 mL) of 2% sheep red blood cells (SRBC) were injected into the abdominal cavity of mice. After 5 days, blood samples were drawn and serum samples were obtained.

Serum samples were diluted at 1:300 (v/v) with saline, 1mL of diluted serum samples were added into glass tubes, and then 0.5 mL of 10% SRBC suspension and 1 mL of 10-fold diluted guinea pig complement were added into the tubes. After incubation at 37°C for 30 min, the reaction was stopped by putting the tubes in an ice bath, and the reaction mixtures were centrifuged at 2000r/min for 10 min. An aliquot (1mL) of the resultant supernatant was mixed with 3mL of Doshi reagent (0.2 g potassium ferricyanide, 0.05 g potassium cyanide, 1.0 g sodium hydrogen carbonate in 1 L distilled water). In parallel, absorbance for 100% hemolysis was obtained from the following preparation: 100 μ L 20% SRBC mixed with 400 μ L red blood cell lysis buffer, and then 0.25 mL of the mixture was added Doshi reagent to 4 ml to develop the color for the measurement of absorbance. The absorbance at 540 nm for assay mixtures was measured and the 50% hemolytic concentration (HC₅₀) of the serum was estimated by the following equation: Sample HC₅₀ = (sample absorbance/50% hemolytic absorbance) \times dilution factor [9].

3.4 Statistical analysis

The results were presented as the mean \pm SEM, statistical comparison of data was performed by one-way analysis of variance (ANOVA) when the data conformed to normal distribution, and the Wilcoxon rank sum test was used when data did not comply with the normal distribution, $p < 0.05$ was considered significant.

4 Result

4.1 ORAC Value Comparison of Herbs

As showed in Table 1, the ORAC values of every herb in the JING liqueur were different.

TABLE 1. ORAC Value Comparison of Herbs

Herb	ORAC (μ mol TE/100g)
rhizoma dioscoreae	25949 \pm 1978
angelica sinensis	18721 \pm 1272
cistanche	303670 \pm 42883
wolfberry	14155 \pm 1294
astragalus membranaceus	87101 \pm 13419
epimedium	106428 \pm 8412
cortex cinnamomi	80247 \pm 10972
syzygium aromaticum	183169 \pm 8914

The prescription of JING liqueur was adjusted based on the ORAC value of herbs, and new product was generated: total content of drugs remain the same, but the proportions of high ORAC value herbs (desertliving cistanche, syzygium aromaticum and herba epimedii) were relative increased, while the proportions of low

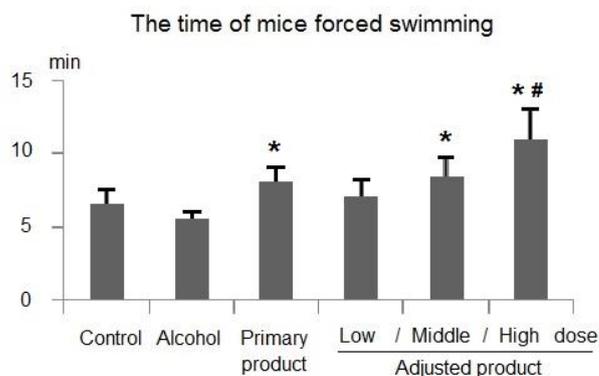
ORAC value herbs (*lycium barbarum* and *angelica sinensis*) were relative decreased. The effect of adjusted product was compared with the primary product by functional tests of antifatigue and enhancing immunity.

4.2 The anti-fatigue effect of adjusted JING liqueur

At the end of experiments, there was not significant difference in body weight between groups. Compared with primary product, the anti-fatigue effect of adjusted JING liqueur was significantly enhanced. The time of mice forced swimming in primary product group was increased to 8.1 ± 0.9 min compared with control (6.6 ± 1.0 min) and alcohol groups (5.5 ± 0.6 min), further more the adjusted product in high dose group could obviously increased the time to 11.0 ± 2.0 min (Figure 2a). The AUC of plasma lactate after exercise in the high dose adjusted product groups was decreased from 15.2 ± 2.6 to 11.8 ± 1.9 compared with primary product group (Figure 2b). As Figure 2c showed, in the low and middle dose adjusted product groups, the hepatic glycogen level of mice was increased from 13.5 ± 0.6 mg/g to 27.5 ± 1.4 and 23.4 ± 1.2 mg/g compared with control, but the efficacy could not be observed in high dose adjusted and primary product groups.

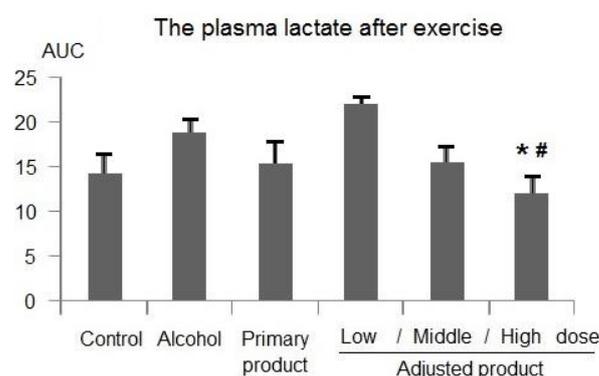
4.3 The enhance immunity effect of adjusted JING liqueur

Compared with primary product and control group, the enhance immunity effect of adjusted JING liqueur was obviously improved. The humoral immune function experiment demonstrate that, HC_{50} was increased from 49.0 ± 1.8 to 56.2 ± 2.1 in the low dose group, and 56.1 ± 1.7 in the middle dose group, while primary product group was 50.8 ± 2.8 (Figure 3 a). The monocyte-macrophage function experiment demonstrate that, the rate of carbon clearance was increased from 3.3 ± 0.1 to 4.4 ± 0.2 in the middle dose group, and 4.1 ± 0.2 in the high dose group, while primary product group was 3.4 ± 0.2 (Figure 3 b).



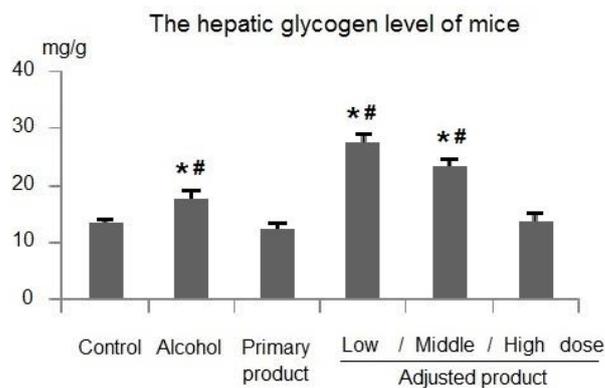
* $P < 0.05$ compare with control, # $P < 0.05$ compare with primary product.

(A)



* $P < 0.05$ compare with control, # $P < 0.05$ compare with primary product.

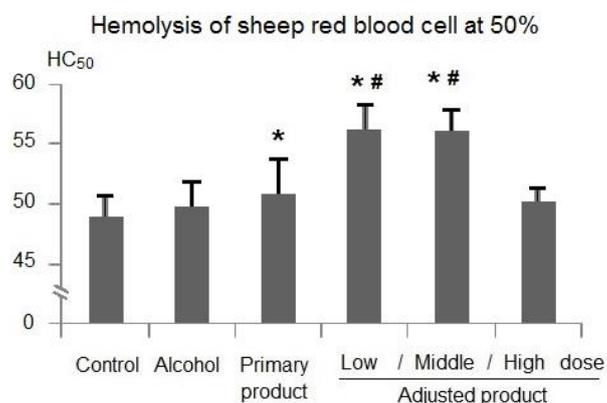
(B)



* P<0.05 compare with control, # P<0.05 compare with primary product.

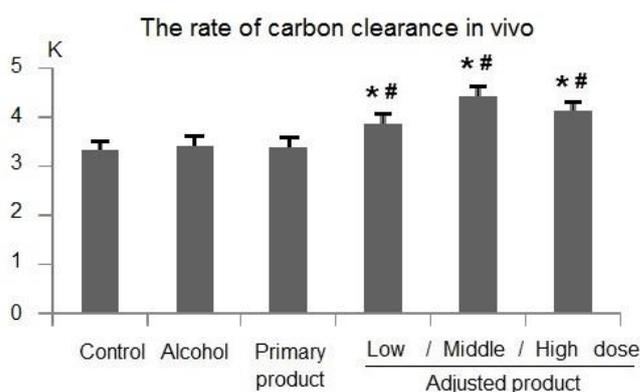
(C)

FIGURE 2. The anti-fatigue effect of JING liqueur. (A) The time of mice forced swimming. (B) The plasma lactate after exercise. (C) The hepatic glycogen level of mice.



* P<0.05 compare with control, # P<0.05 compare with primary product.

(A)



* P<0.05 compare with control, # P<0.05 compare with primary product.

(B)

FIGURE 3. The enhance immunity effect of JING liqueur. (A) Hemolysis of sheep red blood cell at 50%. (B) The rate of carbon clearance in vivo.

5. Discussion

Exhaustion theory suggests that energy source depletion and excess metabolite accumulation lead to fatigue [3]. In order to maintain physical functioning, the body primarily performs glucose metabolism, which is

dependent on the glycolytic pathway and results in significant consumption of glycogen and accumulation of fatigue-inducing metabolites, including lactic acid (LC). It mainly manifests as a physical decrease in muscle tone and exercise tolerance due to an accumulation of LC and other metabolites [10]. The LC concentrations of the adjusted JING liqueur treatment groups were significantly decreased compared with those of the control group and primary product. Glycogen serves as a form of energy storage in animals and is stored primarily in the cells of the liver and the muscles, and energy is supplied via hepatic glycogen degradation when blood glucose is depleted. The adjusted JING liqueur treatment groups could also increase hepatic glycogen compared with control group and primary product. The results of this study suggested that JING liqueur may improve the energy efficiency and decrease the accumulation of LC in body. Besides above, hard work or intense exercise will lead to the production and accumulation of excess reactive free radicals, which results in oxidation stress injury to the body. The blood LC removal function of adjusted JING liqueur enhanced with dose increase, but hepatic glycogen add was not increase with dose, that demonstrated the anti-fatigue effect of adjusted JING liqueur was closely related to eliminate oxydic and metabolic products.

Oxidative injury may induce weakened immunity, and it has been proved that plants extract nutrients which contain exogenous antioxidants could improve immunologic function [6]. Related research shows that, herb extracts with high ORAC value have neutrophil-modulating activity [11]. Although fermentation may affect aromatic compositions of the wine [12], but ORAC is not affected. Oxidative stress associated with increased levels of lipid peroxidation (LPO) and other thiobarbituric acid reactive intermediates are linked to immune injury such as cancer progression. The decline in SOD and catalase activities in body might be because of the increase in circulating LPO, which reportedly results in the accumulation of superoxide anions that are capable of traversing membranes causing deleterious effects [5]. It was reported that antioxidant substance such as *Ganoderma lucidum* polysaccharides (GLP) could improve the levels of serum and gastric tissue SOD, CAT and GSH-Px toward the control values in a dose-dependent manner, and significantly reduced the levels of serum IL-6 and TNF- α levels and increased the levels of serum IL-2, IL-4 and IL-10 in GLP-treated rats compared to gastric cancer model rats, demonstrated the effect of enhance immunity was from antioxidant activities [5].

Several studies have shown that exogenous antioxidants can reduce exercise-induced oxidative stress [4]. Specific nutrients or herbal supplements could reduce metabolite production and/or improve energy utilization. A trend has been to investigate a variety of plants as new potential sources of antioxidants. Many phytochemicals have antioxidant activity and may help protect cells against the oxidative damage caused by free radicals. The ORAC assay has become a valuable and popular method for measuring the antioxidant or radical scavenging capacity of biological samples. Research into the antioxidant properties of herbs may provide information on the mechanism of action of the plant extracts, the utility of the medicines in mitigating against oxidative damage and help to identify the presence of specific antioxidant constituents [7].

Our study provides evidence to support traditional medicinal liqueur has the activity of antifatigue and enhance immunity, and the effect of JING liqueur could be enhanced by adjusting proportions of every ingredient across their ORAC, increased the proportions of high ORAC value herb could obviously improve the anti-fatigue and enhance immunity efficacy.

Conflict of Interest

The authors declare no conflict of interest.

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