JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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J. Agric. Food Chem., **Just Accepted Manuscript** • Publication Date (Web): 17 May 2017

ACS Publications

Journal of Agricultural and Food Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Consumption of watermelon juice enriched in L-citrulline and pomegranate ellagitannins enhanced metabolism during physical exercise

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ABSTRACT

L-citrulline is a non-essential amino acid precursor of arginine and indirectly a precursor of nitric oxide (NO), which is a vasodilator and increases mitochondrial respiration. On the other hand, the antioxidant pomegranate ellagitannins are precursors of urolithin A, which has been associated with mitophagy and increased muscle function. To elucidate if a single dose of watermelon enrichment with these compounds have a positive effect after a high intensity exercise (8 sets of 8 repetitions of half squat exercise), a double-blind randomized crossover in vivo study was performed in healthy male subjects (n=19). Enrichment juices maintained basal levels of blood markers of muscle damage, such as lactate dehydrogenase and myoglobin, and showed a significant maintenance of force during the exercise and a significant decrease in the rating of perceived exertion and muscle soreness after exercise. A positive effect was observed between L-citrulline and ellagitannins improving the ergogenic effect of watermelon juice.

Keywords*: arginine, ergogenic aid, myoglobin, lactate-dehydrogenase, urea, creatinine, L-citrulline, ellagitannins, watermelon, pomegranate*

INTRODUCTION

Developing the most effective and efficient method to maximize performance is the focus of scientists and coaches.[1] High intensity exercise causes an accumulation of ammonia in the blood. Ammonia is produced in skeletal muscle when AMP (adenosine monophosphate) is de-aminated to IMP (inosine monophosphate) during the resynthesis of ATP, which increases the rate of glycolysis and accumulation of blood lactate and finally increases fatigue.[2] In addition, eccentric exercise produces delayed-onset muscle soreness which is usually extended for several days. The acute muscle damage from eccentric exercise can cause local inflammation,[3] oxidative stress,[4] and release of Ca^{2+}-activated proteases.[5] This muscle damage produces muscular fatigue which limits performance, decreasing force, peak power and/or speed.[6] The serum level of skeletal muscle enzymes is a marker of the functional status of muscle tissue, and varies widely in both pathological and physiological conditions.[7] As a result of the damage to the sarcolemma, several myocellular proteins are released into the blood stream and the increase of plasma concentrations of myoglobin, creatin kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are typically used as indirect markers of muscle fiber damage.[8] Therefore, athletes commonly use legal ergogenic aids as a method to increase exercise performance especially by eliminating fatigue symptoms.[9]

As result, the beverage industry is researching natural juices without added sugars and rich in bioactive compounds with healthy properties or positive effects in sportsmen, which could be considered as functional foods and could be substitutes for pharmacological products or energetic drinks with high sugar content. Interestingly, watermelon juice is a rich source of lycopene and L-citrulline.[10] Lycopene is an important antioxidant with anticancer properties [11] and L-citrulline, is a non-essential amino acid which reduces lactic acid accumulation, allowing a higher resistance exercise performance to exhaustion.[6] Furthermore, L-citrulline is an essential component of the urea cycle in the liver, being responsible for detoxification of ammonia via conversion to urea.[6,12] Additionally, L-citrulline is precursor of arginine with positive effects after a high intensity exercise.[13] About 80% of citrulline is metabolized by the kidneys into arginine,[14] and finally arginine is converted to citrulline and nitric oxide (NO) by nitric oxide synthase.[15] NO is a potent vasodilator, which helps increase blood flow and mitochondrial respiration, particularly during exercise [14,15] and increases muscle contractility, muscle repair, muscle blood flow, glucose uptake and resistance exercise performance. [16,17] For this reason, in sports physiology, NO has also received much interest, and supplements of NO are thought to be an ergogenic aid.[14] However, the reactive oxygen species (ROS) generated during intense exercise inactivate the NO in mammalian tissues, while antioxidants would enhance the biological actions of NO by protecting the NO against oxidative destruction.[18] Pomegranate juice has been reported to have a higher antioxidant effect than grape juice, blueberry juice, red wine, ascorbic acid and α-tocopherol in protecting NO against inactivation by reactive oxygen species. [19] The antioxidant effect is due to pomegranate juice being a rich source of potent polyphenolic antioxidants[20] Therefore, ellagitannins can protect against exhaustive exercise induced oxidative injury in sportsmen.[21] Moreover, supplementation with polyphenols (ellagitanins) from pomegranate extract significantly improves isometric strength 2-3 days after eccentric exercise.[22, 23] Additionally, Trexler et al. reported the ergogenic effect of pomegranate extract in runners, showing a higher vitality scale, blood flow and vessel diameter with the consumption of pomegranate extract in comparison to placebo. [24] On the other hand, the ellagitannin metabolite urolithin A (50 mg/kg/day in mice) induce mitophagy, improving the mitochondrial respiratory capacity and enhancing muscle strength.[25]

Several previous studies have used citrulline malate (CM) (pharmaceutical drug used as popular sport supplement) or L-citrulline during a supplementation period previous to exercise to test the effect of this bioactive compound. However, the bioavailability of L-citrulline is greater when it is contained in a matrix of watermelon.[26] Not many previous studies have investigated the effect of a unique dose of L-citrulline or L-citrulline and ellagitannins in watermelon juice on resistance exercise and blood biochemistry of sportsmen. Therefore, the aim of this study was to analyze the ergogenic effects of two different doses (0.5 and 3.3 g per 200 mL) of L-citrulline in watermelon juice matrix and the positive effect of ellagitannins (22.0 mg per 200 mL) on submaximal resistance exercise performance to exhaustion in trained resistance athletes.

MATERIALS AND METHODS

Subjects' characteristics. Nineteen healthy male subjects (age: 23.9 ± 3.7 years; stretch stature: 177.4 ± 5.3 cm; body mass: 75.2 ± 7.6 kg) participated in this study. The inclusion criteria for this study were the following: 18-30 years of age, the subjects had at least four years resistance training experience and performed exercise three times per week, none of the subjects had any musculoskeletal disorder within six months before the study, no lifestyle factors or diseases that could decrease NO production and no consumption of supplements within the last years (branched-chain amino acids, protein, L-arginine, L-citrulline). Moreover, subjects were also asked to refrain from caffeine and alcohol 24 hours before each test and avoid exhaustive training in the 48 hours preceding each testing session. Prior to their participation, all subjects provided signed informed consent, which was approved by the University's Institutional Review Board and in accordance with the Declaration of Helsinki.

Beverage tested. Three different watermelon juices from Fashion watermelon cultivar and a placebo beverage (elaborated with a sugars solution in water and colorant to seem like the watermelon juice colour, without L-citrulline and ellagitannins) were produced. The watermelon juices were manufactured with a specially designed process in order to maintain the maximum level of citrulline. Watermelon juice (WJ), watermelon juice enriched in L-citrulline (3.3 g per serving) (CWJ) and a mix of watermelon juice and a concentrate of pomegranate from whole fruit enriched in L-citrulline (3.3 g per serving,) and ellagitannins (22.0 mg per serving) (CWPJ). The external L-citrulline added to the juice, to supplement the watermelon juice and increase the total citrulline content, was from Acofarma® (Barcelona, Spain) and the ellagitannins (Pomegranate Fruit Concentrate) produced by AMC Innova (AMC Juice & Drink S.A company, Espinardo, Murcia, Spain) with a Proprietary Process under patent. The characteristics of different beverages are shown in **Table 1**. L-citrulline, pH, tritatable acidity (TA) and total soluble solid (TSS) were determined according to Tarazona-Díaz et al. [10] and ellagitannins according to Peña et al.[27] procedures.

Study design. The present study used a double-blind randomized crossover within subjects design and included a separate test for each of four beverages. Three different watermelon juices from Fashion watermelon cultivar were evaluated (WJ, CWJ and CWPJ) compared to a placebo beverage (without L-citrulline and ellagitannins) in each subject in a randomized order.

Training protocol. Three hours after consuming a standardized breakfast, training load was determined by 1RM for the half squat exercise. Before testing 1RM, a warm-up with 5-min of cycling on a cycle ergometer (Ergoline GmbH, Bitz, Germany) at 75 W followed by 10 repetitions at 50% of the perceived 1RM and active stretching exercises were performed. After, 1RM loads were determined according to standard.[28] This load was used to calculate exercise intensity for the four subsequent session trials. In every session, the subjects lifted loads that allowed only 8 sets of 8 repetitions (8RM) to be performed with 2 min rest between sets of half squat. The 8RM load was established by 1RM testing and was adjusted by approximately ± 2.5% if subjects performed ±1 repetitions or by approximately ± 5% if subjects performed ± 2 repetitions[29] every session. The eccentric phase of each exercise was performed in 3 s (controlled by digital metronome), whereas the concentric phase was performed at maximum velocity. The subjects were supervised by an experienced lifter to ensure that volitional fatigue was achieved safely, and the control of the rest was strict. Mean and peak force (N) and power (w) variables were monitored during each set of half squat exercises via a linear position transducer (Chronojump, Barcelona, Spain) that was attached to the bar.

Tests. Every 7 days to allow subjects' recovery between the tests, four different beverages were tested by each subject at different days in randomized order. For each test, 1 h after the beverage intake (200 mL), subjects performed the warm-up described previously in 1RM testing and subsequently, the isokinetic dynamometer test was carried out, followed by the training protocol and finally the isokinetic dynamometer test. All tests were performed at the same time of day and were also separated 7 days. For each subject, the food and total amount of water intake for 24 h prior to each trial was accounted for in an individualized food log book used for the nutrition recall and the first trial's dietary intake was followed for the subsequent trial.

Experimental and analytical determinations. Anthropometric, one-repetition maximum (1RM) load for the half squat exercise and blood variables were determined.

Stretch stature and body mass, were measured using a Seca720 scale (Seca Ltd., Germany). Heart rate (HR) was recorded (Polar RS800; Polar Electro Oy; Kempele, Finland) during all the training sessions. After the completion of each session, rating of perceived exertion (RPE) was analyzed using a 6-20 RPE scale.[30] Furthermore, muscle soreness for lower limbs was measured using a 1-5 muscle soreness scale 1 h, 24-h and 48-h after the completion of each test.

Isokinetic dynamometer test. Peak torque values and torque-angle of both legs during knee flexion and extension were measured by an isokinetic dynamometer (Biodex 3, Biodex Corporation, Shirley, NY, USA). The subjects were seated and stabilized by straps so that only the knee to be tested was moving with a single degree of freedom. The hip extensors and flexors in the dominant leg were tested concentrically. The motor axis was visually aligned with the axis of the knee. Both the 'dynamic ramping' (limb acceleration and deceleration) and 'gravity correction' features were used in all tests to avoid previously documented problems, such as torque overshoot and gravity effects. The dynamometer was calibrated at the beginning of each session. Before the trial set, a specific warm-up consisting of two series at 50 and 80% of the subject perceived maximum effort were carried out. The test started 5 min after the warm-up trials had been completed to prevent fatigue. All subjects performed five continuous maximum effort concentric contractions of the knee flexors and extensors at the angular velocity of $60° \cdot s^{-1}$. The first and last repetitions were excluded from the data analysis. Only the highest peak torque values of the fitted curve of the flexors and extensors of each leg were used in the analysis. Later, the resistance training session started. Immediately after the training session the subjects performed an isokinetic test as described previously.

Plasma analyses. Hematological tests were conducted on the subjects to analyze serum blood markers of muscle damage and biochemical parameters such as arginine, myoglobin, ferritine, C-reactive protein, potassium, uric acid, urea, cholesterol, tryglicerides, fasting glucose, creatinine, CK, LDH, AST and ALT. Five hematological tests (6.5 mL of blood samples) were carried out for each subject, one previous to the first test (basal) and the rest immediately after the completion of each test. Venous blood samples were collected from each subject by antecubital venipuncture with a vacutainer system to determine the basic biochemistry, arginine content and muscle damage related enzymes. After making withdrawals, samples were kept at 2 °C. It was expected to take at least 30 min until complete blood coagulation. Samples were centrifuged for 10 min at 3,800 rpm to separate formed elements and fibrin clot and supernatants were recovered for further analyses following the sanitary procedures.

L-arginine was determined as described.[31] An aliquot (40 µL) of plasma was mixed with 40 µL of 1.5 M $HClO_4$ to precipitate proteins. To this solution, 900 µL of HPLC-grade water and 20 µL of 2 M K_2CO_3 were added. The mixture was centrifuged at 10.000 *g* for 1 min and 100 µL of the supernatant was injected into a liquid chromatograph (HPLC, Waters, Milford, MA, USA) with fluorescent detector (Agileserie 1200). Arginine was quantified by comparison with an external standard of arginine (Sigma Chemicals, Madrid, Missouri, USA) and results are expressed in mg per dL. The potassium ion was determined by ion selective electrode using an Easy Electrolites analyser (Medica Corporation, Berford, USA) and results are expressed as mEq per L.

The rest of the serum biochemical analytes were measured using an autoanalizador Spinteach 640 (Spinreact, Girona, Spain), reagents and chemicals were supplied with the purchased commercial kits (Spinreact, Girona, Spain), different methods used for analysis of biochemical analytes were:1) The determination of blood enzymes was conducted using AST by the International Federation of Clinical Chemistry (IFCC) enzymatic-UV method, ALT by the IFCC enzymatic-UV method, LDH by the German Society of Clinical Chemistry (*Deutsch Gesellschaftfür Klinische Chemie*, DGKC) kinetic-UV method and CK by the N-acetylcysteine (NAC) kinetic-UV method and the results are expressed in U per L, 2) glucose by glucose oxidase-peroxidase enzymatic colorimetric method, 3) creatinine by Jaffé colorimetric kinetic method, 4) urea by urease-glutamate dehydrogenase kinetic method, 5) uric acid by uricase-peroxidase enzymatic colorimetric method, 6) myoglobin by turbilatex myoglobin latex turbidimetry, 7) ferritin by turbilatex ferritin latex turbidimetry. Glucose, creatinine, urea and uric acid are quantified in mg per dL, while myoglobin and ferritin are quantified in ng per mL.

Statistical analysis. Statistical analysis was performed using the statistical program SPSS (SPSS 22 for Windows, SPSS Inc. Chicago IL.). The distribution of data was initially verified by the Shapiro-Wilktest. Repeated measures ANOVA (isokinetic dynamometer data, multipower data and parameters blood test: glucose, uric acid, creatinine, ferritin, potassium, creatine kinase) with pairwise comparisons post hoc test using the Bonferroni corrections or Friedman (parameters blood test: total cholesterol, triglycerides, urea, AST, ALT, LDH, arginine, myoglobin and reactive protein C) with Wilcoxon post hoc test performed with the Bonferroni corrections was used depending on data normality. $P < 0.05$ was considered statistically significant. Data are presented as mean ± standard error (SD).

RESULTS AND DISCUSSION

Effect on half-squat and isokinetic dynamometer performance. The different juices did not show any effect on mean average force (Figure 1A). However, the peak average force was higher in the subjects with intake of watermelon juice enriched in L-citrulline and significant differences were detected between CWPJ (1820.6 ± 369.8 N) respect to placebo (1662.7 ± 353.0 N) and WJ (1650.9 ± 409.5 N) (Figure 1A). On the other hand, no significant differences were found in mean and peak of average power among beverages (Figure 1B). Previous works had shown a positive effect of citrulline-malate (8 g) beverage enhancing the athletic anaerobic performance to increase the numbers of repetitions respect to placebo beverage.[16,32] However, Cutrufello et al.[33] did not observe an ergogenic effect when a single dose of L-citrulline (6 g) was taken 1 or 2 h before exercise testing in 22 subjects (11 males and 11 females), suggesting higher doses and for longer supplementation periods.

The differences between pre and post 8RM exercise in isokinetic peak torque at $60^{\circ}\cdot s^{-1}$ are shown in Figure 2. A reduction in knee extension peak torque was observed with increased citrulline content in watermelon juices, and a significant reduction in the decrease in extension peak torque was observed in the juice with citrulline (3.3 g) and ellagitannins (22.0 mg) (CWPJ) respect to placebo (-10.4 ± 26.6 *vs* -52.0 ± 29.3N·m, respectively). On the other hand, no significant differences were observed between beverages in knee flexion isokinetic peak torque (data not shown). Nevertheless, Bailey et al.[34] observed a significant effect to enhance endurance exercise performance after 6 g of citrulline supplementation for 7 days, but no significant effect was detected after 6 g of arginine supplementation for 7 days. In addition, several studies have shown that CM supplementation before resistance exercise attenuates fatigue occurring to the working muscle.[32,35] Furthermore, the use of CM might be useful to increase athletic performance in high intensity anaerobic exercises with short rest times.[32] A possible explanation for this might be that CM stimulates hepatic ureogenesis and promotes the renal reabsorption of bicarbonates. These metabolic actions had a protective effect against acidosis and ammonia poisoning and explain the anti-fatigue properties of CM in humans.[6] On the other hand, a supplemented pomegranate juice (650 mg of gallic acid equivalents per day) during 8 days improved strength recovery in leg and arm muscles following eccentric exercise, with no dose response effect.[23] In our study, citrulline and ellagitannins have shown a positive effect, probably because of the antioxidant effect of ellagitannins, increasing antioxidant enzyme activities before and after exhaustive exercise and thus protecting against exhaustive exercise induced oxidative injury in sportsmen[21] and protecting NO against oxidative destruction, resulting in augmentation of the biological actions of NO.[19] On the other hand, recently Ryu et al.[25] observed an improvement of exercise capacity in rodents after ingestion of urolithin A (a type of microflora human metabolite of dietary ellagic acid derivatives or ellagitannins), with a dose of 50 mg/kg/d in mice which is equivalent to 4 mg/kg/d in humans, because of mitophagy induced by urolithin A. Therefore, ellagitannins from pomegranate could have an additional effect on antioxidant power and the mitophagy in skeletal muscle, removing the dysfunctional mitochondria and improving the mitochondrial respiratory capacity. Urolithin A has been described as enhancing muscle strength and robustly augmenting running endurance without increasing lean muscle mass.[25] Therefore, ellagitannins as urolithin A could improve muscle cell quality rather than quantity.

Effect on physical activity intensity perception and muscle soreness perception. The subjects that took juices showed a lower heart rate (WJ: 156.7 ± 17.4 bpm; CWJ: 156.5 ± 19.9 bpm; CWPJ: 156.6 ± 19.5 bpm) respect to placebo (164.1 ± 17.1 bpm), although no significant differences were detected between the beverages tested (Figure 3A). According to previous results the beverage designed to increase NO production did not induce a stimulant response in the heart rate during exercise.[16,36] Moreover, Bailey et al.[34] observed a significant decrease in blood pressure after citrulline supplementation (6 g for 7 days). The reduction in blood pressure through reduction of intracellular calcium level,[37] might be due to NO-cyclic guanosine monophosphate (cGMP)-related smooth muscle relaxation.[34]

The 8RM exercise was felt to be hard and highly stressful by subjects, principally when juices were not administered (Figure 3B). After the 8RM exercise, the RPE was significantly lower when the CWJ and CWPJ were administered respect to placebo. These results are very important as they show a relationship between RPE and 8RM and knee extension isokinetic test. The ability to demonstrate lower perceived exertion for a greater work output has attractive implications for performance. These findings are like those presented by Glenn et al.[16] who observed lower overall feelings of exertion (8%) with resistance-trained females consuming CM (8 g citrulline malate + 8 g dextrose) respect to placebo (8 g dextrose) 1 hour before exercise.

On the other hand, muscle soreness perception decreased with the time after the test in all treatments, except for placebo and WJ where maximum values were observed 24 h after 8RM exercise (Figure 4). Subjects that took the CWPJ reported the lowest muscle soreness values 1 h after 8RM exercise (placebo and WJ showed around 31.2% and 22.9% higher score than CPWJ), without significant differences with CWJ. 24 h after

8RM exercise, subjects that took CWPJ and CWJ showed around 60% and 44% of muscle soreness reduction respect to placebo, without significant differences between the different juices. Finally, 48 h after exercise subjects who had taken the enrichment juices before exercise were completely recovered from exercise (muscle soreness values 1.1 ± 0.2 in CWJ and 1.0 ± 0.0 in CWPJ), while the subjects who had taken the placebo showed a similar muscle soreness value (2.1 ± 1.3) (Figure 4). Furthermore, subjects who took CWPJ (1.9 ± 0.7) 1h after exercise showed a similar muscle soreness compared with placebo at 48 h after exercise. These results are consistent with those of Pérez-Guisado & Jakeman[32] who reported a detrimental percentage value of 40% with a CM supplementation compared to placebo 24 and 48 h after exercise in the same muscle soreness scale. However, Tarazona-Díaz et al.[26] observed a significant muscle soreness reduction either in enriched watermelon juice (6 g of L-citrulline per 500 mL) or in watermelon juices (1.17 g of L-citrulline per 500 mL) 24 h after a maximum exercise test on a cycle ergometer. These differences between both studies could be attributed to the different nature of the exercises used in each test. Furthermore, a previous study reported that pomegranate juice supplementation attenuated muscle soreness of elbow flexor muscles after eccentric exercise, but did not attenuate muscle soreness in knee extensor muscles.[38] These authors described this fact as resulting from the daily use of legs for standing and ambulation, offering added protection from soreness. However, in this study, the 8RM exercise promoted the appearance of different degrees of muscle soreness regardless of the supplementation used. Regarding the acute effect on the attenuation of muscle soreness 1 hour after the exercise in CWPJ trails, Trombold et al.[22] found a lower level of muscle soreness perception 2 hours after eccentric exercise in subjects who drunk a beverage supplemented with ellagitannins from pomegranate extract compared to placebo. However, these differences were not observed from 24 to

96 hours after the eccentric exercise, although the isometric strength was significantly improving 2-3 d after eccentric exercise.[22] The improvement of mitochondrial activity promoted by ellagitannin metabolites,[25] could contribute to ATP production through the induction of mitochondrial content, like coumestrol which is a natural organic compound.[39]

Effect on blood biomarkers. Plasma levels of arginine were significantly increased with the consumption of L-citrulline (Figure 5A). Arginine content in the volunteers who took CWJ was 2.23 ± 0.68 mg per dL and CWPJ provided 2.32 ± 0.47 mg per dL in comparison to placebo (1.68 ±0.30 mg per dL) and WJ (1.67 ± 0.27 mg per dL), indicating that citrulline was effectively converted into arginine. Our results are in agreement with those of previous studies showing that L-citrulline supplementation increases levels of L-arginine. Mandel et al.[40] observed the highest plasma citrulline and arginine concentrations 1-2 h after only a dose of watermelon ingestion (3.3 kg wet weight of ripe watermelon). In addition, a lower quantity of watermelon juice (similar to 0.26 kg) intake for three weeks of daily ingestion increased plasma arginine concentrations too.[31] Bailey et al.[41] also demonstrated that watermelon juice supplementation (16 days taking 300 mL day^{-1}) increased plasma L-citrulline, L-arginine and nitrite.

On the other hand, after exercise the highest myoglobin levels were observed in placebo (149.54 ± 96.50 ng per mL) respect to basal and CWPJ juice (68.35 ± 6.84 ng per mL and 70.96 ± 15.96 ng per mL, respectively) and no significant differences were observed with WJ and CWJ juices (99.50 ± 21.68 and 98.81 ± 23.11 ng per mL, respectively) (Figure 5B**)**. Myoglobin is a marker of muscle damage, which can be auto-oxidate during exercise.[42] Lippi et al.[43] reported that the major increment over the pre-half-marathon value was recorded for myoglobin, the concentration of which increased nearly 3-fold. The increased plasma myoglobin concentration represents secondary symptoms of damaged muscle after plasma membrane damage.[8, 44] Additionally, the antioxidant and anti-inflammatory polyphenols from pomegranate fruit could aid in exercise recovery by enhancing nutrient delivery to skeletal muscle and neutralizing the ROS, at least in part. Thus, Trexler et al.[24] showed that the ingestion of pomegranate extract in an exercise bout led to enhanced vessel diameter, blood flow, and delayed fatigue in highly active participants. Additionally, the optimization of mitochondrial energy production by ellagitannins[25] could improve the aerobic metabolism and reduce the muscle damage. Therefore, these compounds could have a synergic effect reducing the oxidative stress and inflammation at the site of muscle damage immediately following a bout of eccentric exercise.[23]

Regarding plasma skeletal muscle enzymes concentration as markers of the functional status of muscle tissue, significant differences were observed in AST, ALT and LDH, but no significant differences were observed in CK (Figure 6). The placebo showed a significantly higher plasma AST and CWPJ showed a significantly higher plasma ALT concentration (33.60 ± 10.07 U per L and 24.20 ± 9.51 U per L, respectively) compared to AST and ALT basal concentrations (24.93 ± 9.91 U per L and 22.13 ± 8.56 U per L) (Figures 6A and 6B). AST and ALT are indices of cellular necrosis and tissue damage in skeletal muscle. These are also released from activated muscles, and levels can increase after acute physical exercise. The increase is linked to performance intensity and duration. In American football players, AST and ALT values measured before and after a game showed a significant increase in AST due to muscular damage; increased AST was also correlated with muscle cramps during twice-a day practices in training camp.[45] Córdova et al.[46] studied volleyball players through one season, and AST and ALT values were found to be higher than in non-sportsmen after the training.

In our experiment, exercise induced a significant increase of LDH in placebo consumption (467.29 ± 77.02 U per L) compared to basal levels (390.64 ± 33.00 U per L). However, no significant differences were observed between LDH basal levels and the levels with any drink containing citrulline (Figure 6C). Given the potential ergogenic mechanisms of citrulline involving oxygen delivery and mitochondrial efficiency, it is possible that citrulline, and ellagitannins supplementation preferentially enhances aerobic exercise capacity compared to higher-intensity anaerobic activities. These mechanisms activated aerobic glycolysis and therefore the reaction of pyruvate to lactate is reduced thereby decreasing LDH compared to the placebo.[2, 6, 14, 25, 39]

Finally, the plasma CK levels showed high variations between drinks, although no significant differences were reported among beverages (Figure 6D). The plasma CK levels range from basal level around 167.05 ± 99.92 U per L to placebo level around 239.67 ± 138.69 U per L. These results may be due to our blood samples being collected immediately after exercise. After prolonged exercise, total serum CK activity is markedly elevated for 24 hours after the exercise bout when participants rest, and may remain so for 48-72 hours.[7] For example, Goodman et al. [47] observed that serum myoglobin levels increased significantly immediately after a 21-km run, while CK levels increased significantly only 24 h thereafter.

Results regarding the plasma substrates concentration (uric acid, urea, creatinine and fasting glucose) are shown in Figure 7. No significant differences were observed between basal uric acid concentration with respect to the levels for the rest of beverages (Figure 7A). However, WJ showed significantly higher plasma uric acid concentration (15%) than CWPJ. Uric acid is the final product of purine catabolism. Thus, during an intense exercise an additional source of energy was from ADP, by producing 1 ATP and 1 AMP from 2 ADP. While the ATP is used for energy, the AMP is degraded to IMP, which is catabolized finally to uric acid.[2] High-intensity exercise results in a decrease in muscle adenine nucleotide pool ([ATP], [ADP], [AMP]) and an increase in IMP and ammonia. It could be possible that citrulline enhances the aerobic energy, by producing ATP and AMP from 2ADM, decreasing lactate production via the anaerobic pathway,[34, 48] and the synergic effect of ellagitannins, enhanced the mitochondrial activity and promoted the aerobic energy [25, 39] and neutralized the oxidative stress during exercise, as in intensive exercise the xanthine oxidase (XOD) enzyme utilizes hypoxanthine or xanthine as a substrate and O_2 as a cofactor to produce superoxide ($\cdot O_2-$) and uric acid.[49]

A similar trend was observed in plasma urea concentrations, where no significant differences were observed between basal concentration with respect to the levels for the different juices (Figure 7B). However, in this case, the placebo showed significantly higher plasma urea concentration (21%) than CWPJ (Figure 7B). Decreases in the plasma urea concentrations after exercise with citrulline and ellagitannins supplementation indicated that citrulline supplementation could decrease proteolysis (in this case, independently of citrulline dose) and that ellagitannins have a positive effect with citrulline.

During physical exercises of high intensity and short duration, phosphocreatine is the energy substrate, by rapid depletion of ATP converted into creatinine. Plasma levels of creatinine were significantly increased with the consumption of juices respect to placebo and basal levels: Placebo 102%, WJ 112%, CWJ 112% and CWPJ 113% (Figure 7C). L-arginine is known to actively participate in the synthesis of creatine (a rate of about 1-2 g per day). Diets supplemented with L-arginine increase intramuscular creatine phosphate concentrations between 1% and 2% in laboratory animals; thus, this may enhance the response to anaerobic exercise.[14] Moreover, L-arginine has been suggested to increase creatine delivery to skeletal muscle based on the ability to increase muscle blood flow.[50] Previous studies have described that supplementation of citrulline reduces fatigue, stimulates hepatic ureogenesis and promotes the renal absorption of bicarbonates.[6] These metabolic actions could explain the antifatigue properties of citrulline because of the protective effect against acidosis and ammonia poisoning. In fact, the citrulline malate supplementation (6 g per day during 15 days) increases around 34% the rate of oxidative ATP production during exercise and around 20% the rate of phosphocreatine recovery after exercise, indicating an important contribution of oxidative ATP synthesis to the energy production.[48] Additionally, L-citrulline malate supplementation can enhance the production of arginine derived metabolites as creatinine and nitrite, creatinine, ornithine and urea.[13]

The levels of fasting glucose obtained with CWPJ consumption were similar to those obtained before exercise (82.17 ± 8.56 mg per dL and 72.29 ± 14.53 mg per dL, respectively), while placebo (124%), WJ (120%) and CWJ (120%) showed the highest levels respect to placebo (Figure 7D). Glucose is the primary energy source of ATP production in skeletal muscle, by glycolysis or aerobic oxidation. These results may be due to a synergistic effect between citrulline and ellagitannins since they both increase blood flow and improve muscle glucose uptake because of the increased NO production and the optimization of energy methabolism, maintaining lower LDH concentrations than placebo or WJ. In this sense, another natural organic compound, coumestrol, showed an increase in mitochondrial content in myocytes with an elevation of cellular ATP levels and an increase of glucose uptake.[39] On the other hand, the intake of 63% of functional watermelon pomace juice for 4 weeks in Zucker Diabetic Fatty Rats increased arginine availability and improved the glycemic control, reducing the glucose levels probably by increased NO synthesis and insulin sensitivity with the decrease of serum concentrations of glucose.[51]

Considerations and limitations. Test were done every 7 days, although with a separation of 72 h is enough time to allow subject´s recovery between the tests. On the other hand, the time between the intake of different beverages (7 days) is also enough washout period to allow the elimination of pomegranate juice ellagitannin metabolites are present in human plasma and urine, which are disappear around 48 hours[52].The results of current research study are consistent with previous data reporting that L-citrulline and ellagitannins have an ergogenic effect in resistance exercise performance to exhaustion [15-16, 22].

The principal limitation of the present study was that an additional test with a beverage without L-citrulline and the same dose of ellagitannins tested, to analyze the only effects of ellagitannins on strength performance, was not included. Thus, we could discriminate if ellagitannins plus L-citrulline could have an additive or synergic effect in sportsmen. Although, the positive effect of both compounds has been demonstrated. Additionally, all subjects were men and the results could variate in other type of populations as women. On the other hand, in our study the subjects were not classified according to their urolithin metabotypes.[53] Future research studies with a stratification of volunteers, according to their urolithin metabotypes, could provide and additional tool to diminish the variability in the effects, and probably would show a higher effect in metabotype A or B than in metabotype 0. [25, 54]

In conclusion, a unique dose of 200 mL watermelon juice enrichment with citrulline (3.3 g 200 mL^{-1}) showed an ergogenic effect, which was improved with ellagitannins supplementation (22.0 mg 200 mL^{-1}) from pomegranate fruit concentrate. These functional juices have shown a benefit in sportsmen increasing the average peak force around 3% and reducing around 5 times the decrease in peak torque. Moreover, the subjective RPE and muscle soreness were lower than placebo in enrichment juices. At the same time, levels of some biochemical markers associated with muscle damage such as LDH, myoglobin, uric acid and urea were maintained. These kinds of beverages could be useful also in workers that need an extra physical effort. The promising results should take into account the synergic effect of the natural fruit drinks matrix. If using another fruits matrix, results should be confirmed by similar human studies. Moreover, the decrease in plasma glucose levels could be an interesting subject for study in future works due to the impact in diabetes illness.

ACKNOWLEDGMENT

The authors are grateful to AMC INNOVA JUICE AND DRINKS, S.L. for providing the beverages with the functional ingredients extracted in their facilities and Oriol Abellán for the help in recruiting subjects. The authors would like to thank all the individuals who participated in the study. Martínez-Sánchez is holder of a postdoctotal grant ("Juan de la Cierva") from Spanish Ministry (MINECO).

Notes

The authors declare no conflicts of interest associated with the current study.

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Funding

This work was supported by the Spanish National Centre for the Development of Industrial Technology (CDTI, Economy and Competitiveness Ministry).

FIGURE CAPTIONS

Figure 1. Effect of different beverages on the average of media and maximum force (A) and power (B) in squat exercise. Different letters in the same parameter show significant differences between beverages.

Figure 2. Changes in isokinetic test at 60º s^{-1} of angular velocity after the exercise. Different letters show significant differences between beverages.

Figure 3. Effect of different beverages on cardiac frequency (A) and the rating of perceived exertion (RPE) (B) immediately after squat exercise. Different letters show significant differences between beverages.

Figure 4. Effect of different beverages on muscle soreness 1 h, 24 h and 28 h after squat exercise. Different capital letters for the same beverage show significant differences between the time and different lower case letters for the same time show significant differences between beverages.

Figure 5. Effect of different beverages in plasma markers such as arginine (A) and myoglobin (B) after squat exercise. Different letters show significant differences between beverages.

Figure 6. Effect of different beverages in plasma marker enzymes such as (A) aspartate aminotransaminase (AST), (B) alanine aminotransferase (ALT), (C) lactate dehydrogenase (LDH), and (D) creatine kinase (CK) after of squat exercise. Different letters show significant differences between beverages.

Figure 7. Effect of different beverages in plasma substrates such as (A) uric acid, (B) urea, (C) creatinine and (D) fasting glucose after squat exercise. Different letters show significant differences between beverages.

Table 1. Physicochemical Characteristics and Content of Bioactive Compounds in the Different Beverages

	Placebo	WJ[z]	CWJ[z]	CWPJ[z]
Sugars content (g L^{-1})	51.45 ± 2.57 ns	47.18 ± 1.66 ns	48.74 ± 1.34 ns	52.56 ± 3.87 ns
Luminosity (L*)	23.94 ± 0.76 c	30.29 ± 0.43 b	31.37 ± 0.14 a	30.68 ± 0.40 ab
Hue angle[y]	14.51 ± 3.27 c	42.90 ± 1.31 a	39.93 ± 0.16 b	45.21 ± 1.69 a
Chroma[x]	9.13 ± 0.97 c	15.33 ± 0.52 b	22.51 ± 0.12 a	15.78 ± 0.71 b
pH	3.12 ± 0.14 b	4.70 ± 0.04 a	4.65 ± 0.13 a	4.70 ± 0.04 a
Total acidity (g 100 mL^{-1})	0.19 ± 0.02 a	0.13 ± 0.01 c	0.15 ± 0.01 bc	0.16 ± 0.00 b
Total solids soluble (ºBrix)	11.01 ± 0.14 a	8.67 ± 1.13 b	9.23 ± 0.12 b	9.53 ± 0.16 b
L- Citrulline (g 200 mL^{-1})	ND	0.5 ± 0.1 b	3.3 ± 0.3 a	3.3 ± 0.5 a
Ellagitannins (mg 200 mL^{-1})	ND	ND	ND	22.0 ± 0.8

[z]WJ (watermelon juice), CWJ (watermelon juice enriched with L-citrulline), CWPJ (mix of watermelon and pomegranate juice enriched with L-citrulline). Sugars contents = sum of glucose, fructose, and sucrose. [y]Hue angle (°h = $\tan^{-1}$ (b*/a*)). [x]Chroma = $[(a^*)^2 + (b^*)^2]^{1/2}$. ND, no detected. Values are means (n = 3) ± SD. Different letters in the same row show significant differences between beverages.

Figure 1.

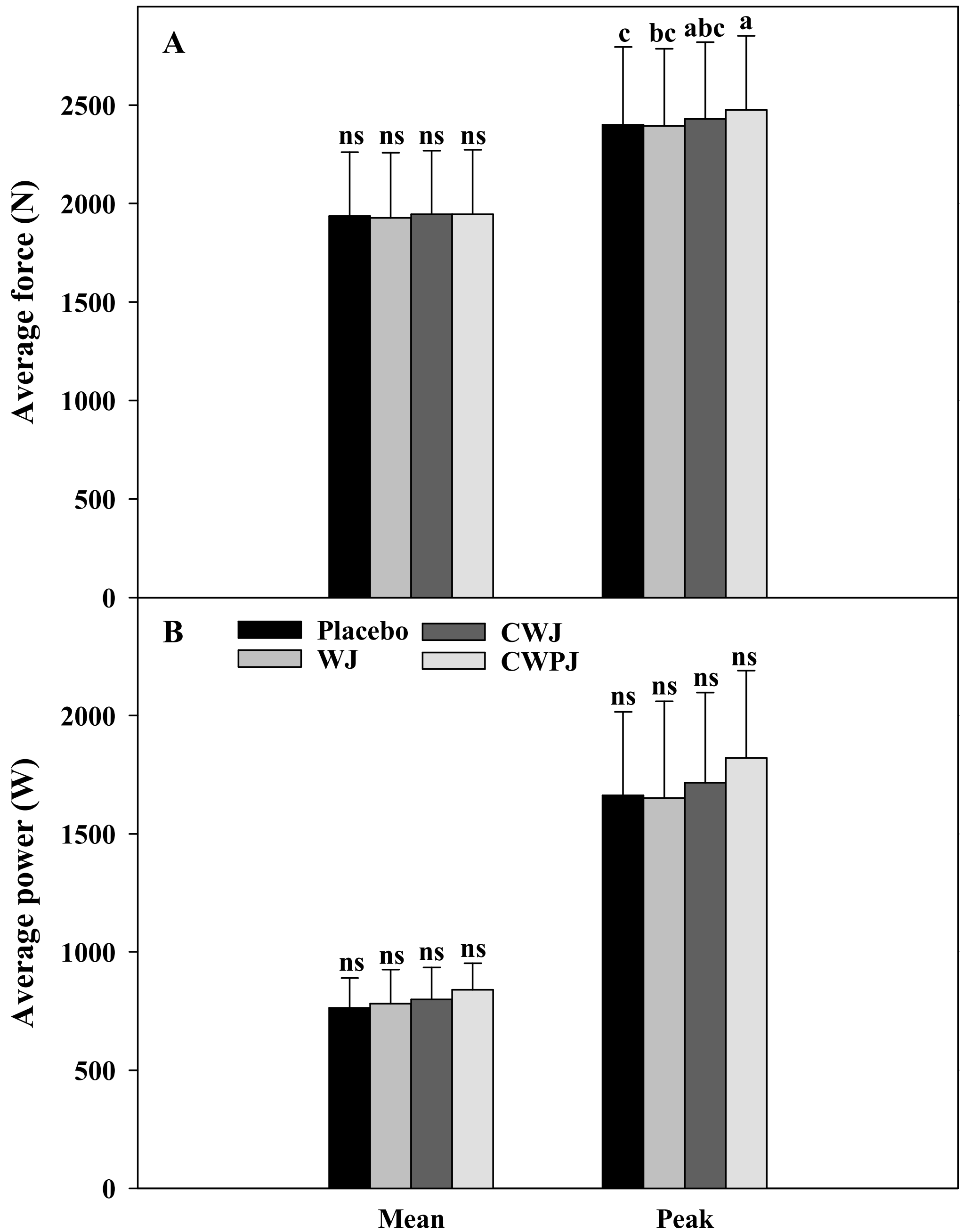

A
B
Average force (N)
Average power (W)
0
500
1000
1500
2000
2500
ns ns ns ns
c bc abc a
Placebo
WJ
CWJ
CWPJ
ns ns ns ns
Mean
Peak

Figure 2.

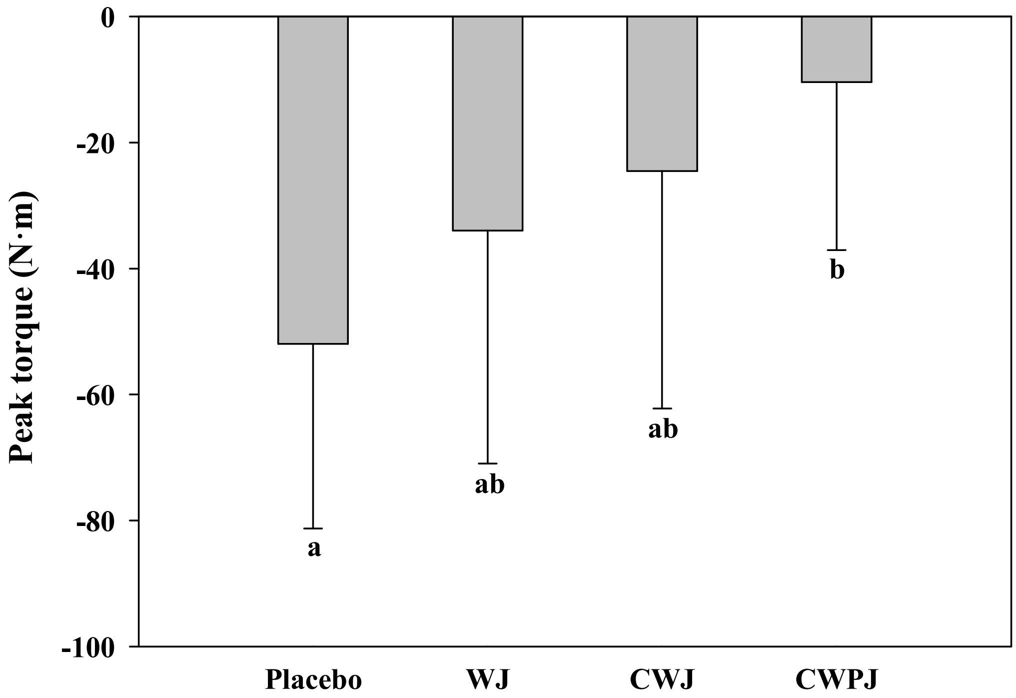


Figure 3.

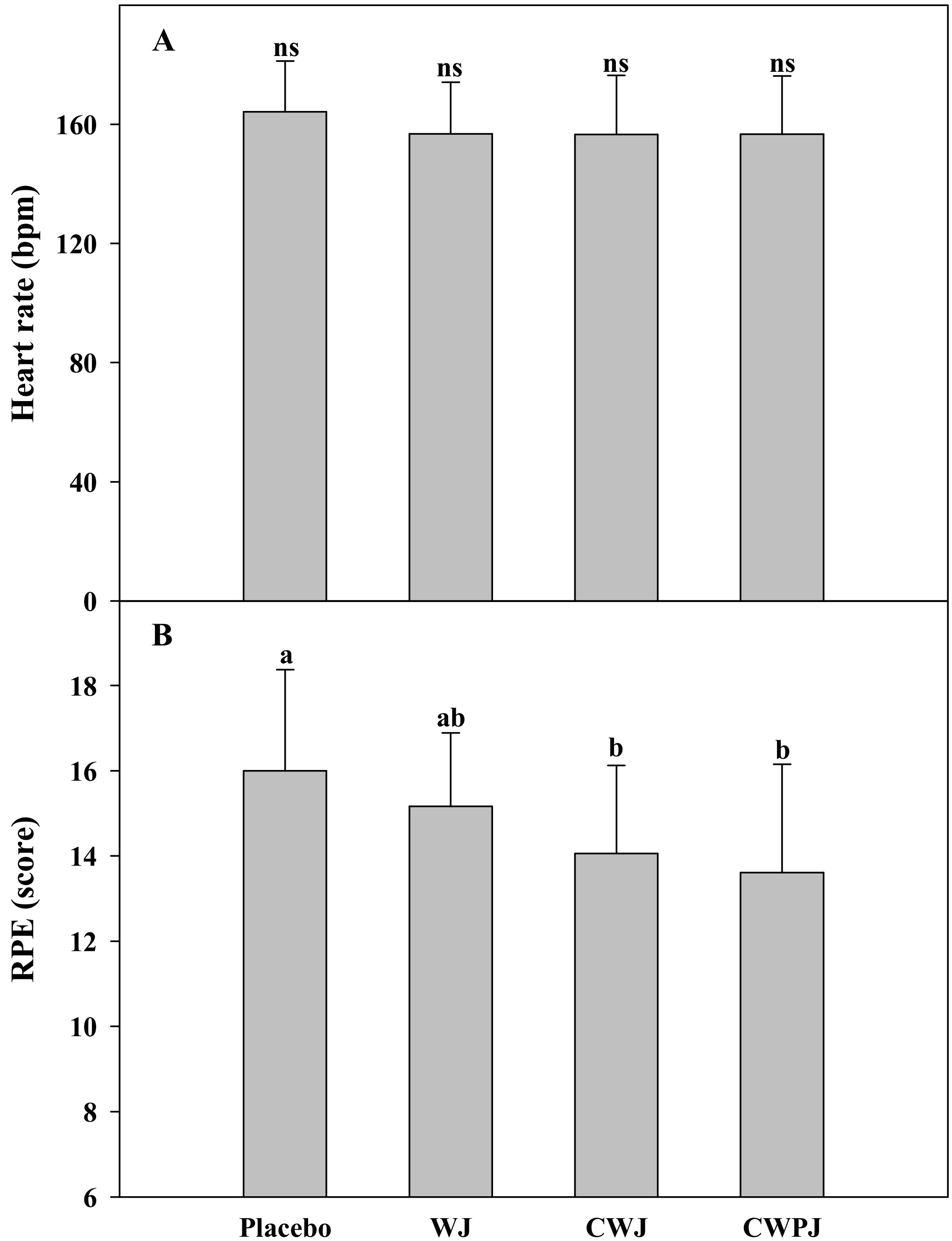

A
ns
ns
ns
ns
Heart rate (bpm)
0
40
80
120
160
B
a
ab
b
b
RPE (score)
6
8
10
12
14
16
18
Placebo
WJ
CWJ
CWPJ

Figure 4.

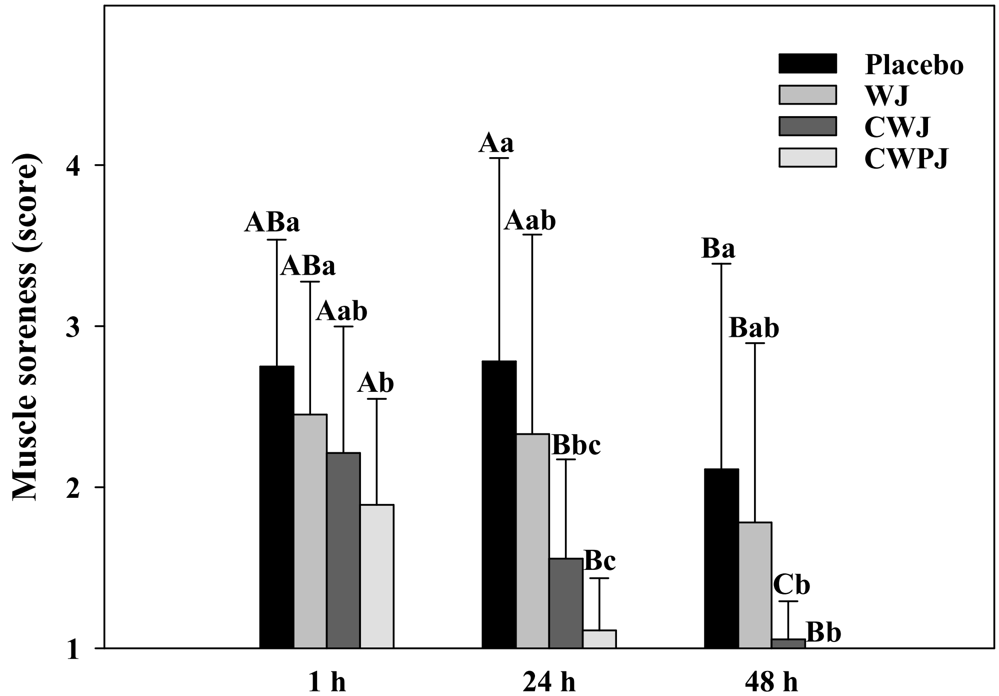

Placebo
WJ
CWJ
CWPJ
Muscle soreness (score)
1
2
3
4
ABa
ABa
Aab
Ab
Aa
Aab
Bbc
Bc
Ba
Bab
Cb
Bb
1 h
24 h
48 h

Figure 5.

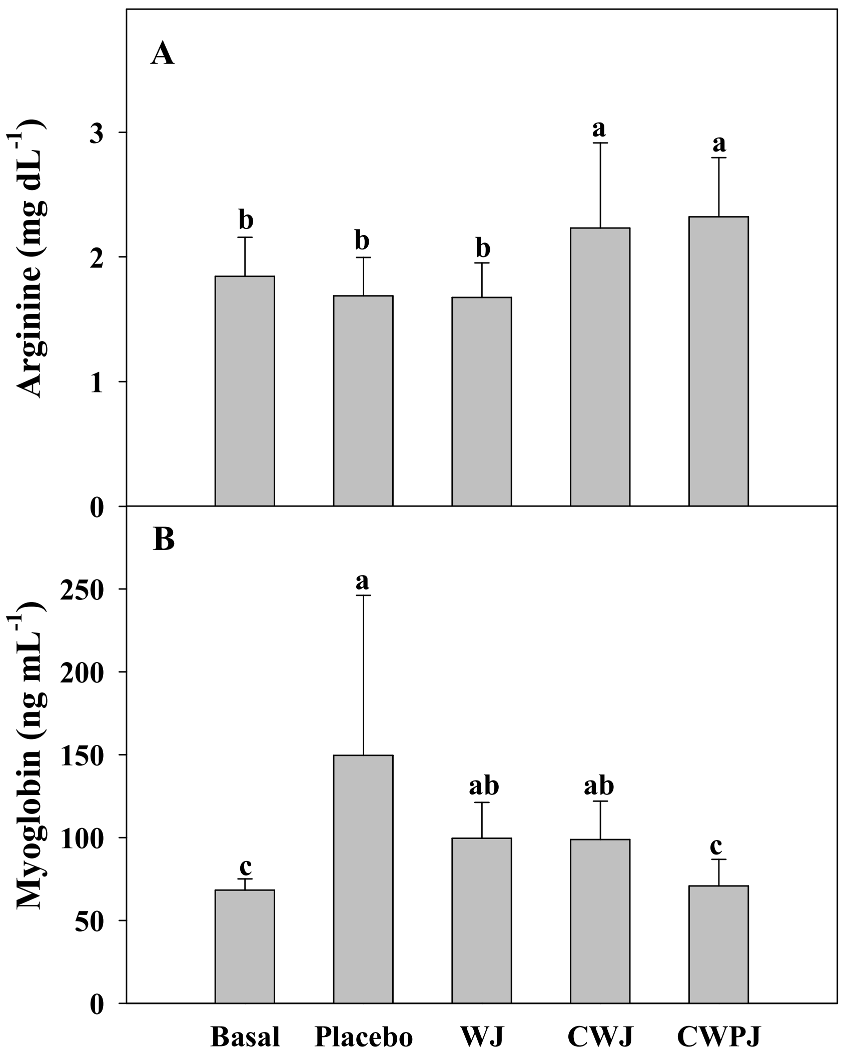

A
Arginine (mg dL-1)
0
1
2
3
b
b
b
a
a
B
Myoglobin (ng mL-1)
0
50
100
150
200
250
c
a
ab
ab
c
Basal
Placebo
WJ
CWJ
CWPJ

Figure 6.

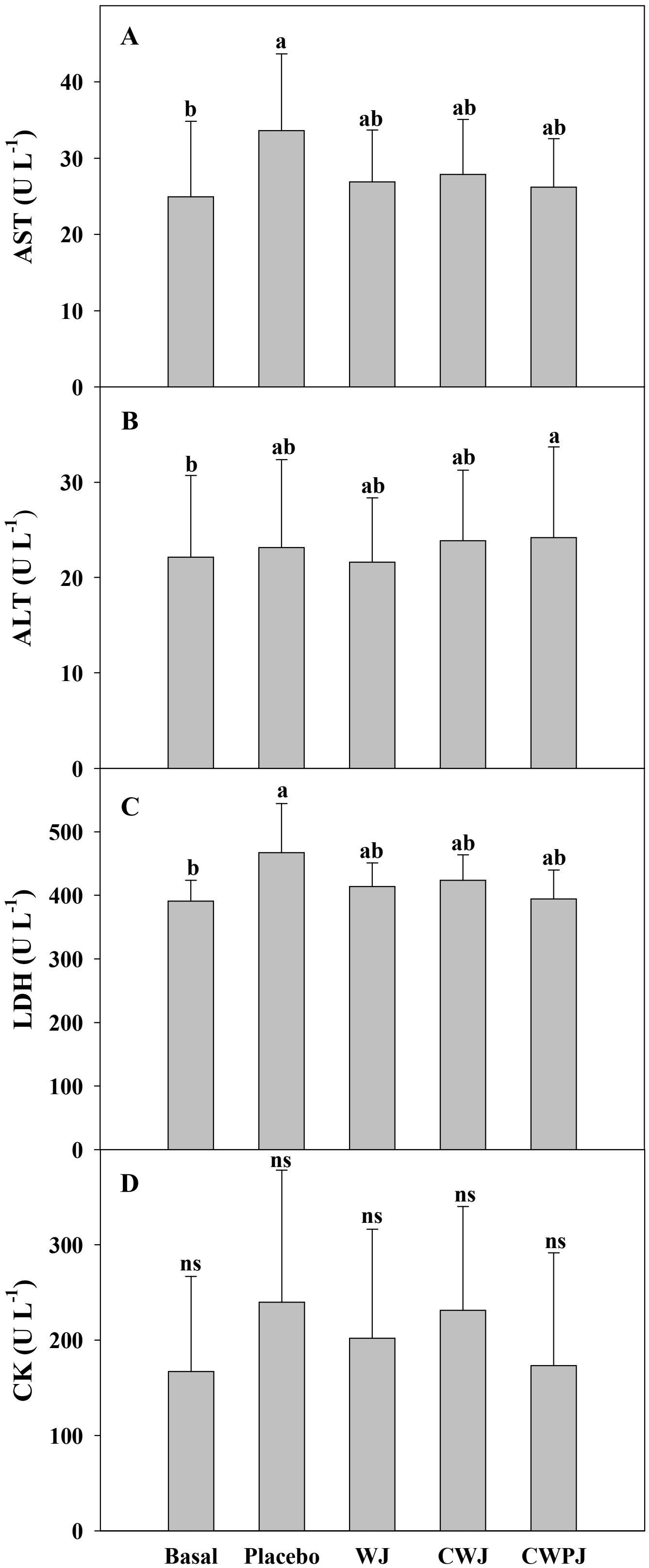
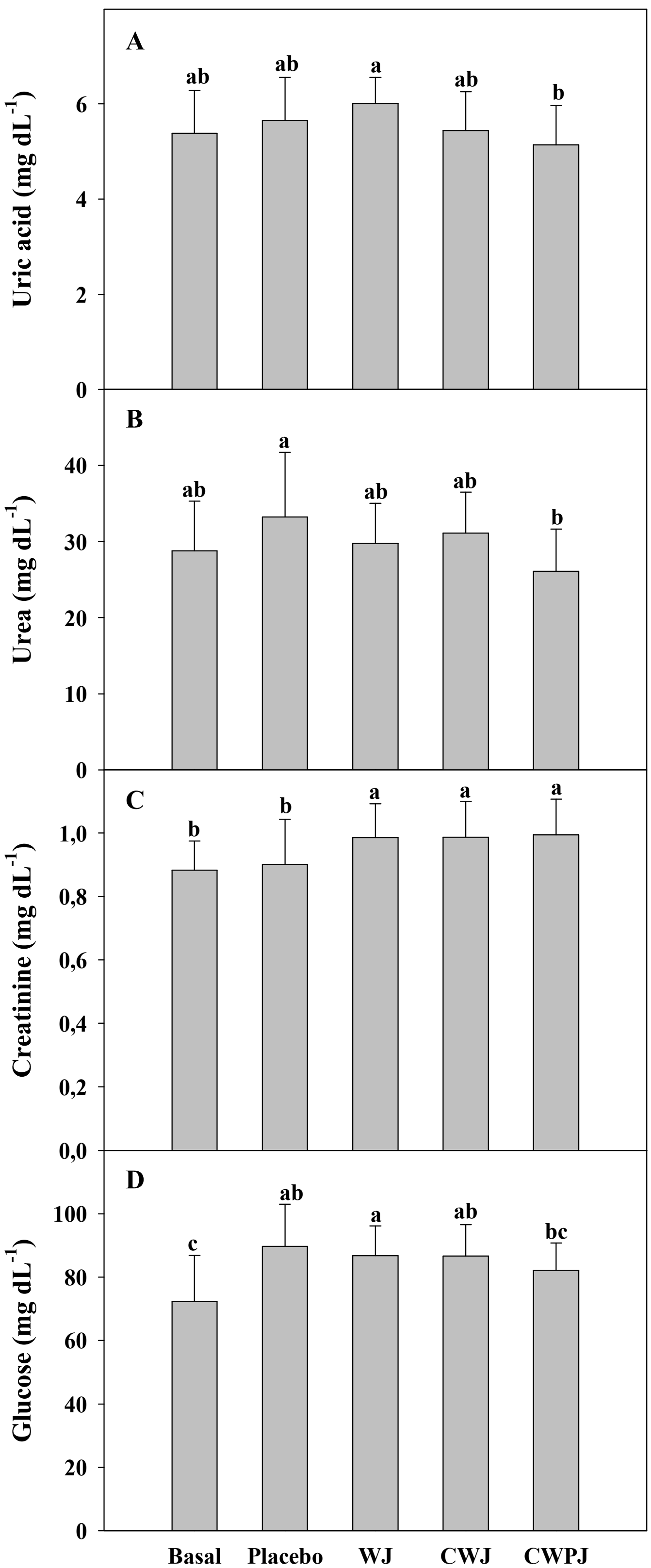
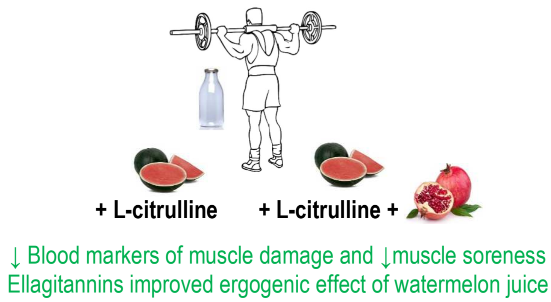

A
B
C
D
AST (U L-1)
ALT (U L-1)
LDH (U L-1)
CK (U L-1)
a
b
ab
ns
Basal
Placebo
WJ
CWJ
CWPJ

Figure 7.


A
B
C
D
Uric acid (mg dL^{-1})
Urea (mg dL^{-1})
Creatinine (mg dL^{-1})
Glucose (mg dL^{-1})
ab
ab
a
ab
b
ab
a
ab
ab
b
b
b
a
a
a
c
ab
a
ab
bc
Basal
Placebo
WJ
CWJ
CWPJ


+ L-citrulline
+ L-citrulline +
↓ Blood markers of muscle damage and ↓muscle soreness
Ellagitannins improved ergogenic effect of watermelon juice