

ORIGINAL RESEARCH

Pre-Clinical Study: Antioxidant Levels and Immunomodulatory Effects of Wolfberry Juice and Other Juice Mixtures in Mice

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ABSTRACT

Although wolfberry juice, derived from the fruit of *Lycium barbarum*, has been purported by Chinese researchers to augment immune response, there is a paucity of information in scientific literature about its effects. This study was designed to evaluate the immunomodulatory effects of wolfberry juice, individually and in mixtures with other juices, using a mouse model. The antioxidant activity of wolfberry juice, and 1:1 mixtures of wolfberry juice:raspberry juice, wolfberry juice:blueberry juice, wolfberry juice:apricot juice, and wolfberry juice:pomegranate juice was analyzed. After intraperitoneal injection of these juices into mice, their impact on splenic weight and the number of splenic macrophages was investigated. Results showed that as levels of antioxidants increased in wolfberry juice mixtures, there was a corresponding increase in macrophage numbers in the spleen. The increase was most significant following injection of wolfberry juice:blueberry juice and wolfberry juice:raspberry juice. There was also a significant increase in spleen weight in mice exposed to the wolfberry

and juice mixtures in each instance. Wolfberry juice and its mixtures were shown to have immunomodulatory effects in mice by increasing splenic macrophages and splenic weight.

INTRODUCTION

Many diseases, including heart disease, cancer, and even aging, have been linked to excess production of free radicals, such as superoxide, singlet oxygen, and hydroxyl radicals.¹ These free radicals may be generated as a result of oxygen metabolism during the course of normal cellular activity, and may even be helpful in certain settings such as inside a phagocytic vesicle where free radicals may assist in the destruction of invading microorganisms. However, elevated levels of free radicals generated during periods of stress due to infection or exposure to toxic components may result in damage to host tissues.¹

Even though the body has developed a variety of ways to deal with damaging free radicals, antioxidants from dietary sources also play an important role in their control, thus limiting cellular damage.² Sources of dietary antioxidants have been actively researched in recent years with studies noting that vegetables, fruits and berries provide sources of high levels of antioxidants.² A variety of components from these plants — including phenolic flavonoids, carotenoids, and vitamins A, C and E contain antioxidant activity.^{3,4}

Medicinal use of wolfberries (*Lycium barbarum*), which grow in China, has been of great interest to Chinese scientists over the centuries. Wolfberry juice, derived from

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the fruit of *Lycium barbarum*, has specifically been purported by Chinese researchers as a way to improve one's health.⁵⁻⁷ There are reports describing the beneficial effects of wolfberry juice, such as protection of the eye from retinal damage⁵ and protection of the liver.⁶ In one recent Chinese study, after mice were subcutaneously injected with wolfberry juice, there was an increase in thymus and spleen size as well as an increase in the concentration of blood serum enzymes and anticoagulants. Researchers also noted an increase in the number of T lymphocytes and an enhanced rate of T cell maturation.⁷

Although these accounts are of interest, there is need for additional scientific studies to validate antioxidant and immune modulating properties of the juice. The current study was designed to evaluate the immunomodulatory effects of wolfberry juice and mixtures of wolfberry with other juices using a mouse model. We analyzed the antioxidant activity of wolfberry juice, Berry Young Juice® (a blend of wolfberry juice and other juices) and 1:1 mixtures of wolfberry juice:raspberry juice, wolfberry juice:blueberry juice, wolfberry juice: apricot juice, and wolfberry juice:pomegranate juice. After intraperitoneal injection of these juices into mice, their impact on splenic weight and the number of splenic macrophages was investigated.

MATERIALS AND METHODS

Mice

Swiss white female mice, weighing 20–23 grams, were obtained from Simonsen Laboratories (Gilroy, CA). Mice were divided randomly into eight groups of five mice each. Each mouse was weighed prior to injection of the appropriate juice. Animals were allowed access to drinking water and Purina Lab Chow *ad libitum* throughout the experiment, and their bedding was changed twice each week. Permission for this experimental protocol was obtained from Weber State University's Animal Care and Use Committee.

Juices

Wolfberry, blueberry, apricot, pomegranate, apricot juice and Berry Young Juice (BYJ) were obtained from the Young Living Oil Company (Provo, UT). BYJ is a patented combination of wolfberry, blueberry, pomegranate, apricot, and raspberry juices along with essential oils of citrus. Mixtures were prepared by adding equal volumes of the juices together, resulting in 1:1 v/v mixtures. Each juice or juice mixture was filter sterilized by passing the juice through a 0.45 µm membrane filter (Millipore Co., Billerica, Mass.) into a sterile 10 ml tube. The juices were refrigerated between inoculations and kept sterile. Saline was prepared by adding 0.87 gm NaCl (Fisher Scientific, Hanover Park, IL) to 100 ml of distilled water. This saline was autoclaved to insure sterility. The saline was used to inoculate one group of mice as a control.

Inoculations

After weighing each mouse, mice were randomly divided into treatment groups and inoculated intraperitoneally with 0.5 ml of filter-sterilized juice or juice mixture. Injections were repeated for four consecutive days, then each animal was rested for two days. On the eighth-day, inoculations were continued in the same manner for five more days. Intraperitoneal injection (an acceptable, alternative method to introduce substances into an animal) was chosen instead of oral administration to ensure that each animal received an accurate, repeatable dose in a manner less likely to cause injury. This method allows for quick absorption into the blood.

Specimen Collection

On the tenth day after the start of the inoculations, the animals were weighed and sacrificed by exposure to ether vapors (Halocarbon Laboratories, Riveredge, NJ). The spleen was aseptically removed from the peritoneal cavity, weighed, and processed to enumerate macrophages as noted below.

Splenic Macrophages Isolation

After being weighed, each spleen was placed in a 70 micron cell strainer (Becton Dickinson Bioscience, San Jose, CA) inside a sterile petri dish containing 10 ml of RPMI 1640 (Cellgro, Mediatech, Herndon, VA) with 10% FBS (fetal bovine serum) (Hyclone, Logan, UT). The spleen was minced with scissors and then mashed with the flat end of the syringe plunger to push out all cells. Care was taken to collect all possible cells. Cells were placed into a 50 ml centrifuge tube and spun at 1,500 rpm for five minutes at 4°C. The supernatant was discarded and 5 ml of TRIS NH₄Cl, (Fisher, Hanover Park, Il), pH 7.2, for lysing the red blood cells (RBC), was added to the pellet. The centrifuge tube was vortexed and left at room temperature for 10 minutes to allow adequate lysing of RBCs. Ten ml of 10% FBS RPMI was then added to the tube and mixed. This mixture was centrifuged for five minutes at 1,500 rpm at 4°C. Then, the supernatant was discarded. The splenic cells were resuspended in 10 ml of 10% FBS RPMI, washed a second time as previously described, and re-centrifuged. Pelleted splenic cells were suspended in 10 ml of 10% FBS RPMI and resuspended. Ten µl of this cell suspension and 90 µl of trypan blue were added to the well of a 96 well microtiter plate and mixed by pipetting. Ten µl of this was added to the counting chamber of a hemacytometer to obtain cell counts. Based on the estimate of these cell counts, the appropriate amount of biotin-conjugated rat anti-mouse CD11b monoclonal antibody (Becton Dickinson Bioscience, San Jose, CA) was added to the centrifuged splenic cells that had been resuspended in 1 ml of 10% FBS RPMI. These cells were then incubated on ice for 30 minutes. It should be noted that this antibody from BD Bioscience reacts to the α chain of Mac-1, known as CR3 (complement receptor 3), which is found on macrophages

and other immune cells in the mouse.^{8,9} Treated cells were centrifuged for 5 minutes at 1,500 rpm at 4°C and the supernatant discarded. The cells were resuspended in 1 ml of 10% FBS RPMI.

Addition of Dynabeads to the Splenic Cells

M-280 Dynabeads with streptavidin (DynaL Biotech, Lake Success, NY) were washed, centrifuged and resuspended in the calculated volume of 10% FBS RPMI. An appropriate volume of these washed Dynabeads was added to the centrifuged, resuspended splenic cells and mixed by pipetting. This cell mixture was incubated on a rotator rack at 4°C for 10 minutes. The tubes containing the cells were placed on a magnet for 5 minutes at 4°C. While the tube was still on the rack, the supernatant was removed. A second incubation of the supernatant with additional Dynabeads was repeated. One ml of 10% FBS RPMI was added to the washed cell/Dynabead mixture and mixed. Another 10 µl of this cell mixture was added to 90 µl of trypan blue to one well of a 96-well plate and mixed. Ten µl of this mixture was added to the counting chamber of a hemacytometer and the number of cells (macrophages) present was determined.

ORAC (Oxygen Radical Absorption Capacity) Assay to Determine Antioxidant Activity of Juice Mixtures

Juice samples were sent on dried ice to Brunswick Laboratory (Wareham, MA) which performed the hydro-ORAC-fl assay. This assay was used to measure the antioxidant activity of the juice mixtures.^{10,11,12}

Determination of Brix Values

A Brix refractometer (Atago Co. Ltd., Kirkland, WA) was used to determine the Brix value of juices. Brix is a value given to wines and juices indicating % total solids by weight. These dissolved solids are mostly related to the con-

centration of sugars, but some dissolved solids could be due to other components such as acids, minerals, and phenols.¹³

Statistical analysis

The ANOVA test (analysis of variance) was used to determine if treatments were significant, and Tukey's Studentized Range (HSD) Test for variables was used to determine the significance of individual treatments (Tables 2, 4, and 6). The student t test for comparing means was used to generate Table 7.¹⁴ SAS Software and Users Guide version 8.2 (1999, SAS Institute Inc., Cary, NC) was used to perform this analysis.

RESULTS

Antioxidant Levels of the Juice Mixtures

Before being injected into the mice, the level of antioxidant activity in the juices was determined by Brunswick Labs using the ORAC procedure (Table 1). Note that the 1:1 mixture of wolfberry juice with raspberry juice had the highest antioxidant level, followed by wolfberry juice with blueberry juice, wolfberry juice with pomegranate juice, wolfberry juice alone, and, finally, wolfberry juice mixed with apricot juice. Thus, prior to injection, each of these juices had high levels of antioxidant activity.

Comparison of Health, Body Weight, and Spleen Weight between Experimental and Control Groups

Animals in both experimental and control groups tolerated the inoculation procedure well and appeared healthy throughout this experiment. There was no significant change in average body weight between groups of mice injected with wolfberry juice and its mixtures as compared with control mice injected with saline alone (Table 2). Yet, the average

Table 1: Antioxidant levels of wolfberry (WB) juice and 1:1 mixtures of WB juice with other juices as measured using the ORAC analysis¹

Juice	ORAC _{hydro FL}	ORAC _{lipo FL}	ORAC _{total FL}
	µmole TE/L ¹	µmole TE/L ¹	µmole TE/L ¹
WB: raspberry juice 1:1	170,668	2,799	173,467
WB: blueberry juice 1:1	143,966	2,292	146,258
WB: pomegranate juice 1:1	60,198	1,087	61,285
WB juice alone	33,868	1,252	35,120
WB: apricot juice 1:1	31,683	944	32,627

1. The ORAC analysis is an assay of the scavenging capacity of antioxidants against the peroxy radical in which fluorescein is the probe. This assay was performed by Brunswick Labs of Wareham, MA.

ORAC_{hydro FL} is a measure of the water-soluble antioxidant capacity.

ORAC_{lipo FL} is a measure of the lipid soluble antioxidant capacity.

ORAC_{total FL} is the sum of ORAC_{hydro FL} and ORAC_{lipo FL}.

TE/L is the Trolox equivalent unit expressed as micromole TE per liter (µmole TE/L).

Trolox is a water-soluble vitamin E analog used as the calibration standard in this assay.

Table 2: Tukey's Studentized Range (HSD) Test for variable: Average weight of mice

Treatment group IP injection with	Average body weight of 5 mice (gm)	Significance of the treated group from saline group p (0.05)
Saline	24.50	—
wolfberry juice	24.93	NS ¹
wolfberry juice: blueberry juice (50:50 w/w)	22.90	NS ²
wolfberry juice: raspberry juice (50:50 w/w)	24.60	NS
wolfberry juice: pomegranate juice (50:50 w/w)	24.30	NS
wolfberry juice: apricot juice (50:50 w/w)	26.63	NS ²

1. NS = not significant

2. Significant difference between these two groups.

Table 3: ANOVA for spleen weight from different treatment groups

Source	DF	MS	F value	P
treatments (6)	5	0.0040	15.93	0.001
error	29	0.0002		
correction	34			

Table 4: Tukey's Studentized Range (HSD) Test for variable: Average weight of spleen

Treatment groups IP injection with	Average spleen weight of 5 mice (gm) after treatment	Significance from the saline control p (0.05)
saline	0.0885	
wolfberry juice	0.1489	S ¹
wolfberry juice: blueberry juice (1:1 v/v)	0.1427	S
wolfberry juice: raspberry juice (1:1 v/v)	0.1694	S
wolfberry juice: pomegranate juice (1:1 v/v)	0.1409	S
wolfberry juice: apricot juice (1:1 v/v)	0.1259	S

1. S= significant

weight of the mice injected with the wolfberry juice:blueberry juice (1:1 v/v) combination was significantly different from the average weight obtained from those injected with wolfberry juice: apricot juice (1:1 v/v) at a (p<0.05).

An ANOVA test for spleen weight (Table 3) revealed a significant difference between the different treatment groups (p<0.001). Further analysis using Tukey's Studentized Range Test showed a significant increase (p<0.05) in the splenic weight of mice treated with each juice combination as compared to the splenic weight of the mice treated with saline (Table 4).

Splenic Phagocytic Cell Counts

ANOVA, comparing splenic phagocyte numbers (Table 5), indicated a significant difference between the different treatment groups (p<0.0001). Although Table 6 shows

increases in splenic phagocytic cell counts for all groups injected with wolfberry juice and its mixtures as compared to cell counts in the control mice, Tukey's Studentized Range Test, used to compare individual treatment groups against each other, indicated that treatment with wolfberry:blueberry (1:1) and wolfberry:raspberry (1:1) juices significantly increased splenic phagocytic counts (p<0.05). Based on the experimental protocol, most of these splenic cells would be macrophages.^{8,9}

Immunomodulating Effects of Berry Young Juice (BYJ)

Studies comparing the effect of three different lots of Berry Young Juices® (BYJ) on spleen weight and splenic phagocytic counts were also done. The lots of BYJ studied varied in terms of the Brix value for each lot, being either 16°, 18°, or 22°. Table 7 shows that for all three lots of BYJ

Table 5: ANOVA of average splenic phagocyte number in each treatment group

Source	DF	MS	F value	P
treatments (6)	5	332.9890	8.66	0.0001
error	29	38.4705		
correction	34			

Table 6: Tukey's Studentized Range (HSD) Test for variable: Splenic phagocytes in mice

Treatment groups IP injection with	Average # of splenic phag. of 5 mice (x 10 ⁶)	Significance from the saline control p (0.05)
saline	13.55	—
wolfberry juice	22.32	NS ¹
wolfberry juice: blueberry juice (1:1 v/v)	26.70	S ²
wolfberry juice: raspberry juice (1:1 v/v)	36.02	S
wolfberry juice: pomegranate juice (1:1 v/v)	19.88	NS
wolfberry juice: apricot juice (1:1 v/v)	18.50	NS

1. NS = not significant 2. S= significant

there was a statistically significant difference in spleen weights and phagocytic counts between the mice injected with BYJ compared with control mice.

Impact of ORAC Total Levels of the Juices on Average Splenic Phagocytes

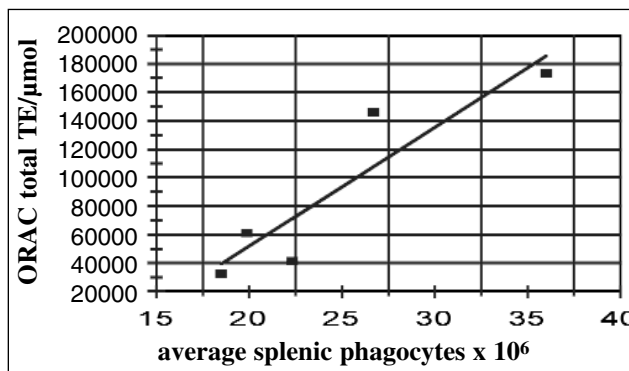
Figure 1 shows that as the total oxygen radical absorption capacity (ORAC) levels of the juices increase, there is a corresponding increase in splenic phagocytes among treated mice. This indicates that antioxidant levels in the juices were enhancing the production and/or survival of splenic phagocytes. Either way, the higher the antioxidant levels of the juices, the more phagocytes become available to counter offending infectious agents. Although factors other than antioxidant level probably influence the number of splenic phagocytes, the R² value for this linear plot is 0.839223, indicating a significant correlation.

DISCUSSION

Although berries are considered to be an important dietary source of antioxidants,^{2,15,16} few scientific studies exist of the wolfberry juice or its mixtures with other juices⁵⁻⁷ that study their impact on the immune system. Thus, this current study was undertaken primarily to study the level of antioxidants in the juices, and their effect on splenic weight and macrophage numbers.

Determination of the ORAC levels of the mixtures of wolfberry:raspberry juice and wolfberry juice:blueberry juice (Table 1) revealed that their antioxidant capacity was about three times higher than ORAC fl values of juices

Figure 1. Antioxidant levels of the juices injected intraperitoneally¹ and the average splenic phagocytes after the treatment.



¹ 9 times over 12 days

Formula for the line: y = 8357.8 x - 115295 R squared = 0.839223

determined in a study by Ou et al.¹⁰ In contrast, the ORAC values of wolfberry juice, wolfberry:pomegranate juice (1:1), and wolfberry: apricot juice (1:1) were in a range similar to the ORAC of fruit juices from that study.¹⁰ It can be concluded that since there was a constant concentration of wolfberry juice in each juice mixture, the added juice had a significant impact on the antioxidant level. It should also be noted that the water-soluble component contained the highest concentration of antioxidants and thus had the most impact on the total ORAC value.

Our results support the view that increased levels of antioxidants in wolfberry juice mixtures increase the number of macrophages in the spleen, as illustrated by our

Table 7: Immunomodulatory effects of BY juice with different Brix values on mice

Treatments Average (° Brix value)	Average # of spleen weight (Gm)	Splenic macrophages (10⁶)
saline	0.0969	12.54
BY juice (22°)	0.1377 (p<0.05)	25.98 (p<0.001)
saline	0.0967	11.07
BY juice (18°)	0.1213 (p<0.0001)	22.37 (p<0.05)
saline	0.0853	12.43
BY juice (16°)	0.1727 (p<0.0001)	25.90 (p<0.05)

mouse model. Depending on the circumstances, this increase in macrophages may not always be a good prognostic indicator.¹⁷ However, an increase of splenic phagocytes to this degree in a healthy animal would indicate increased ability to fight disease since macrophages are the main phagocytic cells in the spleen, providing an essential defense by filtering infectious agents from the blood.¹⁷

Although other components of the juices may be involved, our data indicate that the antioxidants in wolfberry juice or its mixtures have a strong influence on the number of splenic phagocytes in an otherwise healthy animal as shown by the R² value of the linear plot (0.839223). This result also fits with data in which the level of antioxidants in the blood of treated animals was higher than in those animals receiving only saline (data not shown).

Even though an increase in macrophage numbers is important, subsequent studies should be done to determine whether the macrophages are more able to kill phagocytized bacteria or not. Since macrophages can kill through the production of free radicals such as reactive oxygen intermediates and reactive nitrogen intermediates, it would be interesting to determine if this system is affected by the presence of increased antioxidants.

Previous research in humans has shown that ingestion of cranberry juice elevated plasma antioxidant levels in healthy females.¹⁸ Another study noted an increase in antioxidant levels in the plasma of HIV-positive individuals after ingestion of fruits and vegetables.¹⁹ These findings,¹⁸⁻¹⁹ as well as those of this current study, suggest that further studies in humans be conducted to document the antioxidant and immunological capacity of wolfberry juice and its mixture with other juices. We would expect increases in plasma antioxidant levels after ingestion of the juices used in this study as well, especially since they had high initial antioxidant levels as determined by the ORAC assay. It is important to emphasize that although this and other studies have focused on the impact of the antioxidant components

in the berries/fruits, there are a variety of other compounds present which may also exert an effect on the immune system. Therefore, the improvements/changes noted could be due in part to the antioxidants, but may also be due to other components of the juices.

In summary, wolfberry juice and its mixtures, which contained high levels of antioxidants, when introduced into an animal were used to increased spleen weight and the number of splenic macrophages. A relationship was noted that as antioxidant levels of the juice increased, the number of splenic phagocytes increased correspondingly. Thus, wolfberry juice and its mixtures were shown to have beneficial immunomodulatory effects in mice.

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