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Bioactivities and Iridoid Determination of a Beverage Containing Noni, Cornelian Cherries and Olive Leaf Extract

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Abstract: The aim of the current study was to evaluate the iridoid content, as well as the *in vitro* and *in vivo* bioactivities, of a beverage containing noni fruit, Cornelian cherries, and olive leaf extract (Thrive Adaptogenics Max). The average total iridoid content of the beverage was 2.09 mg/mL. The major iridoids present were identified as asperulosidic acid, deacetylasperulosidic acid, oleuropein, morroniside, loganic acid, and loganin. In the 2, 2-Diphenylpicrylhydrazyl (DPPH) radical scavenging assay, remarkably high *in vitro* antioxidant activity was observed, with an IC_{50} of 3.8 μL/mL. *In vivo* bioactivities were evaluated in type 2 diabetic Sprague Dawley rats. In a dose-dependent manner, Thrive Adaptogenics Max reduced abnormal weight gain, blood glucose levels, and serum Advanced Glycation End products (AGEs), as well as improved immunity via increased T cell counts and $CD4^+/CD8^+$ratios. These results suggest that this blend of ingredients is beneficial for improving and maintaining health in the general population, as well as among those with metabolic imbalance.

Key words: Advanced glycation end products, antioxidant, cornelian cherries, iridoids, noni, olive leaf extract

INTRODUCTION

Noni (*Morinda citrifolia*), Asian Cornelian cherry (*Cornus officinalis*), European Cornelian cherry (*Cornus mas*), and olive (*Olea eruopaea*) have been used for centuries as foods and to improve health (Morton, 1992; Seeram *et al*., 2002; Rop *et al*., 2010; Erbay and Icier, 2010). Significant antioxidant activity has been found in products derived from each of these plants (Tural and Koca, 2008; Lee *et al*., 2012; Wang *et al*., 2009; Kontogianni and Gerothanassis, 2012). However, other notable bioactivities have also been discovered. Reduction of Advanced Glycation End products (AGEs) *in vitro* and *in vivo* is a reported activity of noni and the Cornelian cherries, as well as olive leaf extract (Kusirisin *et al*., 2009; Yamabe *et al*., 2007; Sasaki *et al*., 2007). Such benefits indicate that consumption of these foods is not only useful for maintaining health among the general population but also for improving health in those with impaired or imbalanced metabolism.

In addition to a number of other biologically active phytochemicals, each of these plants produces iridoids (Japon-Lujan *et al*., 2006; Deng *et al*., 2011; Gu *et al*., 1996). Iridoids found in noni fruits exhibit DNA protective activities, as well as anti-inflammatory and antioxidant activities (Choi *et al*., 2005; Kim *et al*., 2005; West *et al*., 2011). Oleuropein, a secoiridoid found in olive leaves, possesses antioxidant activity (Visioli and Galli, 1994). But it has also been shown to improve blood pressure and lipid levels (Perrinjaquet-Moccetti *et al*., 2008). The Cornelian cherries produce iridoid glycosides and secoiridoids which reduce AGEs, improve immune function, lower blood glucose levels and have neuroprotective and renal protection properties (Xu *et al*., 2006; Wang *et al*., 2010; Yokozawa *et al*., 2008). As different iridoids vary in their specific activities, combinations of these from safe food sources may provide synergistic health benefits. The aim of the current study was to evaluate the iridoid content, as well as the *in vitro* and *in vivo* bioactivities, of a pasteurized beverage containing noni fruit, Cornelian cherries and olive leaf extract, thereby gaining a better understanding of the utility of such a blend for the promotion of health.

MATERIALS AND METHODS

This study was conducted by the research and development department of Morinda, Inc. (American Fork, Utah, USA) and the pharmacology department of Tianjin Medical University (Tianjin, China) from June 2011 to January 2012.

Preparation of test product: Noni fruits were harvested in French Polynesia and allowed to fully ripen. The fruit was then processed into a puree by mechanical removal of the seeds and skin at a good manufacturing certified fruit processing facility in Mataiea, Tahiti. Olive leaf extract was prepared by extraction of dried *O. europaea* leaves

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with ethanol, followed by removal of the solvent and residual water by evaporation. The resulting extract was reduced to a powder with a particle size <0.420 mm and total moisture <6%. Ripe *C. mas* and *C. officinalis* fruits were harvested during the autumn season. *C. mas* fruit was harvested in the Anatolia region of Turkey, whereas *C. officinalis* fruit was harvested in the Shaanxi and Hunan provinces of China. The seed pits were removed from fruit with automatic fruit pitters. Batches of *C. mas* fruit were processed into puree in an auger press micro-mesh (4 mm) screen fruit pulper. Depitted *C. officinalis* fruits were dried at low heat and then shipped to the U.S. where the reconstituted juice was extracted by pumping/filtration through a stainless steel mesh screen. All ingredients were blended and pasteurized at a good manufacturing certified fruit processing facility in American Fork, Utah to produce the finished product used in this study, Thrive Adaptogenics Max (Morinda, Inc. Utah, USA). The total solids content (mean±standard deviation) of the final test product was 20.24±0.48%.

Iridoid analysis: Samples from separate batches were analyzed. Analysis for iridoids was performed by High Performance Liquid Chromatography (HPLC), according to a previously reported method (Deng *et al*., 2011). Prior to analysis, one g of sample was dissolved in 10 mL 1:1 (v/v) water: methanol and then filtered through a 0.45 μm PTFE filter. Iridoid standards were dissolved in methanol (MeOH) to a concentration of 1 mg/mL and further diluted to produce standard curves for iridoids identified in the samples. Chromatographic separations were performed on a Waters 2690 separations module coupled with 996 PDA detectors (Waters Corporation, Milford, MA, USA), equipped with a C18 column. Elution was accomplished with two mobile phases, MeCN and 0.1% formic acid in H_2O (v/v), with a flow rate of 0.8 mL/min. A linear gradient of 100% aqueous formic acid (0.1%) for 0-5 min, followed by 70% aqueous formic acid and 30% MeCN for 40 min, was used. The PDA detector was monitored in the range of 210-400 nm. The injection volume was 10 μL for each of the sample solutions. The column temperature was maintained at 25ºC. Iridoids were identified in the samples by comparison of retention times and UV absorbance of compounds in the samples and the standards.

2, 2-Diphenylpicrylhydrazyl (DPPH) radical scavenging assay: In the DPPH test, the beverage was diluted serially with deionized water. The diluted samples, and a water blank, were combined 1:1 (v/v) with 0.4 mM DPPH in ethanol. The absorbance of each sample, in triplicate, and blank was read at 515 nm with a Synergy™ HT microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) after incubation at 25±2ºC for 60 min. Percent radical scavenging activity was calculated by dividing the difference in absorbance of the blank and the sample by the absorbance of the blank alone. The concentration of Thrive Adaptogenics Max which scavenged 50% of the DPPH radicals (IC_{50}) was calculated with the linear equation that best fit the plot of concentration versus scavenging activity (R^2>0.99).

Efficacy in type 2 diabetic sprague dawley (SD) rats: Fifty healthy male SD rats, 200-220 g, were acquired from the Tianjin Drug Research Center (Tianjin, China). These were acclimated for one week while receiving standard rodent feed, composed of 23% protein, 53% carbohydrates, and 5% fat, respectively. Following acclimation, the rats were randomly divided into five groups of 10 animals each. One group, the normal control, continued to be fed the standard diet. The four other groups were fed a high fat and sugar diet composed of 20% protein, 48% carbohydrates, and 22% fat. Water and feed were provided *ad libitum*. The normal control group and one of the groups receiving the high fat diet, the diabetic controls, were fed 2 mL saline twice per day by gavage. The remaining three groups received, twice daily by gavage, 2 mL of solutions containing 25% (low dose), 50% (mid dose), or 100% (high dose) Thrive Adaptogenics Max. All animals were fed saline or test product solutions at the same time every morning and evening. After four weeks of the various treatments and feeding of the assigned diets, the animals were fasted for 12 h and administered a single abdominal injection, at 30 mg/kg body weight (b.w.), of 0.5% streptozotocin in citrate buffer (pH 4.2). The normal control animals did not receive the streptozotocin solution, but were administered citrate buffer only. All groups were provided water and the standard diet for another 7 days then fasted over night.

On the final day of the study (day 35), all animals received an abdominal injection of glucose solution at 2 g/kg b.w. Glucose levels in blood from the tail vein of each animal were measured with a glucometer (Changsha Sannuo Biologic and Sensing Technology, Inc., Changsha, Hunan, China) at 0 and 2 h after glucose injection. Following the glucose test, the rats were anesthetized with ether, and 1.5 mL blood was removed from the orbital sinus. For the detection of T-cell subsets, 1 mL blood was mixed with heparin. For detection of AGEs, 0.5 mL blood was centrifuged at 3000 rpm for 10 min, followed by collection of the serum.

Heparinized blood samples were prepared for flow cytometry analysis with a T cell subset detection kit (MultiSciences Biotech Co., Ltd., Hangzhou, Zejiang, China). This involved labeling of specific lymphocytes with fluorescently-labeled antibodies for $CD3^+$, $CD4^+$ and $CD8^+$ proteins, according to the kit manufacturer's instructions. Labeled cells were detected with a flow cytometer (Beckman Coulter, Brea, CA, USA) and the percentages of $CD3^+$, $CD4^+$ and $CD8^+$ T-cell subsets were determined. The $CD4^+/CD8^+$ ratios were also calculated.

Centrifuged blood samples were prepared for AGE detection with an enzyme linked immunosorbent assay (ELISA) kit (Shanghai YanHui Biotechnical, Inc., Shanghai, China), according to the kit manufacturer's instructions. Fluorescent detection of labeled AGE's was performed with a Bio-Rad 550 microplate ELISA reader (Bio-Rad, Hercules, CA, USA). Animal weights were also recorded throughout the study.

The means and standard deviations of each measurement were calculated for each group. Statistical differences between control and test groups were evaluated with Student's t-test, using SPSS® software version 16.0 (IBM Corporation, Armonk, NY, USA).

RESULTS AND DISCUSSION

Iridoid analysis: The phytochemical analysis revealed that Thrive Adaptogenics Max is an iridoid rich beverage, with total iridoid content (mean±standard deviation) of 2.09±0.08 mg/mL. The major iridoids present were identified as asperulosidic acid deacetylasperulosidic acid, oleuropein, morroniside, loganic acid and loganin. Noni fruit puree is the source of asperulosidic acid and deacetylasperulosidic acid (West *et al*., 2011). Oleuropein is a well known and well researched iridoid in olive leaf extract (Japon-Lujan *et al*., 2006). Morroniside and loganin are contributed primarily by *C. officinalis* fruit (Gu *et al*., 1996; Du *et al*., 2008). Our analysis resulted in the first discovery of loganic acid in *C. mas* fruit. The concentrations found in the beverage indicate that processing conditions are suitable for the preservation of the iridoids.

DPPH radical scavenging assay: In the DPPH radical scavenging assay, remarkably high *in vitro* antioxidant activity was observed, with an IC_{50} of 3.8 μL/mL. The antioxidant activity of this beverage is much greater than those reported for several fruits and fruit juices. The IC_{50}'s reported for mangosteen, orange, pomelo, grapes and papaya ranged from 11.18-32.80 mg/mL, while those of orange juice, grape, rose apple, and jackfruit were from 50.62-110.46 mg/mL (Surinut *et al*., 2005). Different varieties of pomegranate juice are also reported to have a higher IC_{50}'s, ranging from 15.98-23.98 μL/mL (Elfalleh *et al*., 2009). The DPPH radical scavenging activity of Thrive Adaptogenics Max is at least three times greater than that reported for any of these other fruit products.

Efficacy in type 2 diabetic rats: Average weights throughout the study are summarized in Table 1. There were no differences in the initial weights of the groups. However, significant differences from normal controls were evident by week 2 in the diabetic control and low dose groups. At the same time point, mid and high dose group weights were no different than those of normal controls. At week 4, high dose group weights were not significantly different from those of normal controls. During the entire study, weight gain was greatest in the diabetic control group, which received no test product. Significant reductions in weight gain of the high and mid dose groups, as compared to the diabetic control group, were also apparent throughout the entire study. A trend of lower weight was also present in the low dose group, reaching statistical significance at week 4. Overall, weight differences were dose-dependent and demonstrated that supplementation with the test product reduced weight gain in rats fed a high fat and high sugar diet.

Table 1: Mean weight (g), ±standard deviation, of treatment groups by week

Group	Initial	Week 2	Week 4	Week 5
Normal control	214±10.9	268±10.1	330±13.0	363±13.1
Diabetic control	214±10.2	291±11.8**	368±13.8**	408±14.0**
Low dose	214±11.2	283±11.6**	353±12.1$^{**\#}$	398±14.0**
Mid dose	214±10.3	278±11.5$^{\#}$	343±11.6$^{*\#\#}$	396±12.4$^{**\#\#}$
High dose	214±10.7	275±12.1$^{\#}$	340±13.2$^{\#\#}$	381±12.2$^{**\#\#}$

*: p<0.05; **: p<0.01 (compared to normal controls); #: p<0.05; ##: p<0.01 (compared to diabetic controls)

Blood glucose levels at 0 and 2 h after glucose injection, or challenge, are presented in Table 2. Glucose levels in all groups fed the high fat and sugar diet were significantly greater than the normal control group. But significant dose-dependent reductions in blood glucose, as compared to the diabetic controls, were seen in the low, mid and high dose groups. Animals with 0 and 2 h glucose levels >7.0 and >11.0 mmol/L, respectively, were categorized as diabetic. The percentage of animals categorized as diabetic in each group is also presented in Table 2. All animals in the diabetic control group were above these limits, demonstrating the successful induction of diabetes. As with mean blood glucose, a dose-dependent decrease in the percentage of diabetic rats occurred in the test product groups. It is remarkable that no animals met the diabetic threshold in the high dose group.

T cell subsets in each group are summarized and compared in Table 3. The percentage of T cells among total lymphocytes is expressed by $CD3^+$ values. $CD4^+$ cells are T helper cells that play a central role in regulating the immune system, whereas $CD8^+$ cells (cytotoxic or suppressor cells T cells) down regulate immune function (Hung *et al*., 1998; Jiang and Chess, 2006; WHO, 2007). The $CD4^+/CD8^+$ ratio, also referred to as the T-lymphocyte helper/suppressor profile, is an indicator of immune system function. In a properly functioning immune system, this ratio is greater than one, as $CD4^+$ cell count should be greater than $CD8^+$. Reductions in $CD4^+/CD8^+$ ratios have been associated with immune deficiency (Reinherz and Schlossman, 1980;Kiecolt-Glaser *et al*., 1986). Human studies have demonstrated an inverse association between T helper cell

Table 2: Mean blood glucose, ±standard deviation, and percentage of animals categorized as diabetic (%DM) by glucose level, at 0 and 2 h after glucose challenge

	0 (h)		2 (h)	
Group	Glucose (mmol/L)	%DM	Glucose (mmol/L)	%DM
Normal control	4.32±0.39	0	6.28±0.53	0
Diabetic control	7.93±0.57*	100	12.1±0.58*	100
Low dose	6.90±0.57$^{* \#\#}$	50	11.3±0.91$^{* \#}$	60
Mid dose	6.48±0.46$^{* \#\#}$	20	10.7±0.68$^{* \#\#}$	30
High dose	6.16±0.47$^{* \#\#}$	0	10.2±0.55$^{* \#\#}$	0

*: p<0.01 (compared to normal controls); $^{\#}$: p<0.05; $^{\#\#}$: p<0.01 (compared to diabetic controls);

Table 3: Mean percentage, ±standard deviation, of T cells subsets and $CD4^+/CD8^+$ ratio by treatment group

Group	$CD3^+$%	$CD4^+$%	$CD8^+$%	$CD4^+/CD8^+$
Normal control	54.5±5.49	49.6±8.22	29.9±5.38	1.71±0.45
Diabetic control	37.9±6.50**	30.3±6.70**	39.1±5.19**	0.75±0.254**
Low dose	40.4±7.72**	32.5±7.07**	37.1±4.42**	0.91±0.301**
Mid dose	43.5±6.87**	39.2±5.08$^{** \#\#}$	35.2±3.11*	1.13±0.229$^{** \#\#}$
High dose	46.7±8.75$^{* \#}$	44.0±8.77$^{\#\#}$	32.8±6.27$^{\#}$	1.43±0.512$^{\#\#}$

*: p<0.05; **: p<0.01 (compared to normal controls); $^{\#}$: p<0.05; $^{\#\#}$: p<0.01 (compared to diabetic controls)

percentage and the severity (in HbA1C levels) and duration of type 2 diabetes (Sumida, 1991). A similar reduction in relative $CD4^+$ count was also observed in patients progressing towards onset of type 1 diabetes (Al-Sakkaf *et al*., 1989). As revealed in Table 3, $CD3^+$, $CD4^+$, and $CD4^+/CD8^+$ values are significantly lower in all the diabetic groups than in the normal control group, with the exception of the high dose group. $CD4^+$ and $CD8^+$ percentages in the high dose group, as well as the $CD4^+/CD8^+$ ratio, were not significantly different than those in the normal control group. Further, there was a trend of increased total $CD3^+$ cells with increased test product dose, with high dose values being significantly greater than those in the diabetic control group. A similar trend was observed with $CD4^+$ values and $CD4^+/CD8^+$ ratios, with statistically significant increases occurring in the mid and high dose groups. $CD8^+$ percentages were greatest in the diabetic control group. However, these decreased as test product dose increased. As $CD8^+$ percentage is inversely associated with $CD4^+$ percentage, the changes are expected. The $CD8^+$ percentage in the high dose group is significantly less than that of the diabetic control group. These changes demonstrate improvements in immune system function that occur with increased ingestion of the test product.

Serum AGE levels were highest in the diabetic control group, Table 4. As expected, AGEs were lowest in normal controls. Interestingly, there was no significant difference between the level in this group and that of the high dose group. Further, the mid dose also had significantly lower AGEs than the diabetic controls. As such, a dose dependent trend of reduced AGEs was evident in the groups receiving the test product. AGEs, products of nonenzymatic glycation of proteins (Maillard reaction), accumulate during aging and in a number of diseased states, such as diabetes, chronic inflammation, and kidney failure (Ramasamy *et al*., 2005). AGE formation occurs over extended periods of time and is associated with elevated glucose levels (Singh *et al*., 2001). Therefore, the reduction in blood glucose levels by the test product may be a major reason for lower AGEs. AGE formation is also accelerated by oxidative stress (Miyata *et al*., 1997). It is likely, therefore, that the antioxidant activity of the test product helps control AGE levels. Cross-links formed between intra and extracellular molecules and AGEs lead to a wide variety of health complications, due to altered cell structure and function and the induction of inflammation (Goldin *et al*., 2006; Naidoo *et al*., 2011). Therefore, controlling AGE formation may slow the progression of pathologies associated with aging and deviations from normal metabolism, such as metabolic syndrome and diabetes (Lee and Cerami, 1994; Kitano *et al*., 2004; Peppa and Vlassara, 2005).

Table 4: Mean AGE level, ±standard deviation, by treatment group

Group	AGE (pg/mL)
Normal control	26.4±3.74
Diabetic control	36.9±4.67 **
Low dose	32.7±4.26 **
Mid dose	31.4±4.32 $^{* \#}$
High dose	30.1±5.19 $^{\#\#}$

*: p<0.05; **: p<0.01 (compared to normal controls); $^{\#}$: p<0.05; $^{\#\#}$: p<0.01 (compared to diabetic controls)

CONCLUSION

The combination of noni, Cornelian cherries, and olive leaf extract appears to have significant potential for improving and maintaining health. Such a blend provides a useful dietary source of iridoids, which are known to have a wide variety of bioactivities. The results of this study suggest that the iridoid rich beverage may help mitigate adverse health effects associated with the aging process, something that confronts all people. Those with metabolic imbalances, such as diabetes and metabolic syndrome, may also benefit from supplementation with the beverage. People with metabolic syndrome constitute a large segment of the population. Its prevalence among developed nations, such as the US and the European

Union, is ever increasing (Ford *et al*., 2004). In 2006, 34% of the US adult population met the criteria for metabolic syndrome (Ervin, 2009). Surprisingly, similar percentages were reported for adult males in the Okayama Prefecture of Japan (Miyatake *et al*., 2006). Metabolic syndrome is also an increasing public health problem in emerging economies, such as Malaysia and Russia (Tan *et al*., 2011; Metelskaya *et al*., 2011) and effective interventions may have a major impact on these populations.

Previous studies of the ingredients in the test product have revealed a wide range of possible positive health effects. Therefore, the benefits of Thrive Adaptogenics Max are not likely to be limited to what has been demonstrated in the current study. Rather, our data may represent just a fraction of a larger set of biological activities.

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