The Science of Monavie

This compilation is the result of a collaborative effort of our team & focuses on scientific publications related to Monavie juice blends.

1 – University of Florida study shows: Brazilian berry destroys cancer cells in lab. January 2006.

2 – Monavie & Diabetes: Q&A - Facts and Testimonial

3 – Richard Bird's Collection on Articles on Acai berry and Monavie

4 – Antioxidant Capacity and other Bioactivities of the Freeze-Dried Amazonian Palm Berry, Acai
Journal of Agricultural & Food Chemistry: 10/7/2006 American Chemical Society

5 – Increased Antioxidant Capacity & Inhibition of Lipid Peroxidation in Healthy Adults Consuming and Acai Fruit-Based Juice.
USDA Arkansas Children's Nutrition's Center & A.G. Schauss Natural Products Research AIBMR Life Sciences, Puyallup, WA – ISHS 2009

6 - In Vitro and In Vivo Antioxidant and Anti-inflammatory Capacities of Antioxidant-Rich Fruit and Berry Juice Blend.
Results of a Pilot and Randomized, Double-Blinded, Placebo-Controlled, Crosseover Study. Journal of Agricultural & Food Chemistry: 8/22/2008

7 – Anti-Oxidant capacities of flavonoid compounds isolated from acai pulp. Food Chemistry, October 1st 2010

8 - What is Monavie Mmun: Product information page

9 - What is Monavie Active: Product information page

10 – What is Monavie Pulse: Product information page
11 - Pain reduction and Improvement of range of motion after consumption of Monavie Active

12 - What is Monavie Pulse: Product information page

13 – What is EMV Energy drink; Product information page

University of Florida News [http://news.ufl.edu]

Brazilian berry destroys cancer cells in lab, UF study shows

Filed under Agriculture [http://news.ufl.edu/research/agriculture/] , Health [http://news.ufl.edu/research/health/] , Research [http://news.ufl.edu/research/] , Sciences [http://news.ufl.edu/research/sciences/] on Thursday, January 12, 2006.

GAINESVILLE, Fla. — A Brazilian berry popular in health food contains antioxidants that destroyed cultured human cancer cells in a recent University of Florida [http://www.ufl.edu] study, one of the first to investigate the fruit's purported benefits.

Published today in the Journal of Agricultural and Food Chemistry [<a href=] , the study showed extracts from acai (ah-SAH'-ee) berries triggered a self-destruct response in up to 86 percent of leukemia cells tested, said Stephen Talcott [http://fshn.ifas.ufl.edu/faculty/STTalcott/] , an assistant professor with UF's Institute of Food and Agricultural Sciences [http://www.ifas.ufl.edu] .

"Acai berries are already considered one of the richest fruit sources of antioxidants," Talcott said. "This study was an important step toward learning what people may gain from using beverages, dietary supplements or other products made with the berries."

He cautioned that the study, funded by UF sources, was not intended to show whether compounds found in acai berries could prevent leukemia in people.

"This was only a cell-culture model and we don't want to give anyone false hope," Talcott said. "We are encouraged by the findings, however. Compounds that show good activity against cancer cells in a model system are most likely to have beneficial effects in our bodies."

Other fruits, including grapes, guavas and mangoes, contain antioxidants shown to kill cancer cells in similar studies, he said. Experts are uncertain how much effect antioxidants have on cancer cells in the human body, because factors such as nutrient absorption, metabolism and the influence of other biochemical processes may influence the antioxidants' chemical activity.

Another UF study, slated to conclude in 2006, will investigate the effects of acai's antioxidants on healthy human subjects, Talcott said. The study will determine how well the compounds are absorbed into the blood, and how they may affect blood pressure, cholesterol levels and related health indicators. So far, only fundamental research has been done on acai berries, which contain at least 50 to 75 as-yet unidentified compounds.

"One reason so little is known about acai berries is that they're perishable and are traditionally used immediately after picking," he said. "Products made with processed acai berries have only been available for about five years, so researchers in many parts of the world have had little or no opportunity to study them."

Talcott said UF is one of the first institutions outside Brazil with personnel studying acai berries. Besides Talcott, UF's acai research team includes Susan Percival [http://fshn.ifas.ufl.edu/faculty/SSPercival/WWW/DrPercival.htm] , a professor with the food science and human nutrition department [http://fshn.ifas.ufl.edu/index.html] , David Del Pozo-Insfran, a doctoral student with the department and Susanne Mertens-Talcott, a postdoctoral associate with the pharmaceutics department [http://www.cop.ufl.edu/departments/PC/index.htm] of UF's College of Pharmacy [http://www.cop.ufl.edu/root4/index.htm] .

Acai berries are produced by a palm tree known scientifically as Euterpe oleracea, common in floodplain areas of the Amazon River, Talcott said. When ripe, the berries are dark purple and about the size of a blueberry. They contain a thin layer of edible pulp surrounding a large seed.

Historically, Brazilians have used acai berries to treat digestive disorders and skin conditions, he said. Current marketing efforts by retail merchants and Internet businesses suggest acai products can help consumers lose weight, lower cholesterol and gain energy.

"A lot of claims are being made, but most of them haven't been tested scientifically," Talcott said. "We are just beginning to understand the complexity of the acai berry and its health-promoting effects."

In the current UF study, six different chemical extracts were made from acai fruit pulp, and each extract was prepared in

seven concentrations.

Four of the extracts were shown to kill significant numbers of leukemia cells when applied for 24 hours. Depending on the extract and concentration, anywhere from about 35 percent to 86 percent of the cells died.

The UF study demonstrates that research on foods not commonly consumed in the United States is important, because it may lead to unexpected discoveries, said Joshua Bomser [http://hec.osu.edu/hn/facstaff/display.php?name=Joshua%20Bomser] , an assistant professor of molecular nutrition and functional foods at The Ohio State University [http://www.osu.edu/] in Columbus, Ohio.

But familiar produce items have plenty of health-giving qualities, he said.

"Increased consumption of fruits and vegetables is associated with decreased risk for many diseases, including heart disease and cancer," said Bomser, who researches the effects of diet on chronic diseases. "Getting at least five servings a day of these items is still a good recommendation for promoting optimal health."

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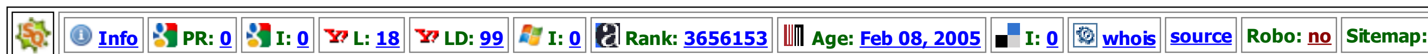


Here is a great helper for people with diabetes!

Monavie & diabetes

It is said that Monavie is a great healer for a lot of illnesses and affections, but can Monavie heal or at least help against diabetes? Can diabetics really drink Monavie?

In the following, we will analyze some monavie & diabetes facts which, hopefully will lead to whether or not Monavie does mend diabetes. First, let's see what monavie and diabetes have in common. Well, the connection between the two is that monavie has a couple of ingredients that really hate diabetes.

Monavie & diabetes Fact No.1

Acerola Cherries, one of Monavie's ingredients, besides being very rich in minerals, vitamins and a powerful antioxidant, acerola cherries fight against diabetes for ages.

It is a well known fact that native people have long used acerola cherries for treating diabetes, among other affections. So, we could say Monavie - Diabetes 1-0 :)

Cure diabetes

Click here for a FREE bottle of aca berry for people w want to lose weigh

Monavie & diabetes Fact No.2

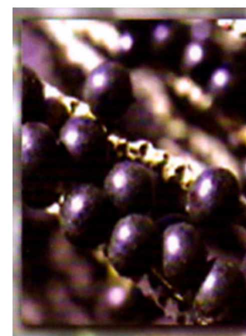
The black chokeberry, or aronia as it is mostly known as, is so powerful that it can fight against cardiac diseases and even cancer. But does it help monavie against diabetes? Yes, it does. Aronia has in its components, important trace minerals and it has been used for a long time as an anti-diabetic food.

Get help in the battle against diabetes! Click here!

Monavie & diabetes Fact No.3

A close relative of the blueberries, the bilberry is known for its capacity to

strengthen the eye capillaries. Also, the bilberry is used in the process of slowing down the age related macular degeneration, night blindness and most importantly, it slows down diabetes related eye disorders.



Monavie & diabetes Fact No.4

Blueberries act as a great antioxidant, and are partially responsible for keeping us smart, young and healthy. Also, blueberries have a long history of being used against diabetes.

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Monavie Fruit
Monavie Drink
Monavie Blog
Monavie Japan

Monavie & diabetes Fact No.5

AdsSpy: 4 sites by this AdSense ID

Type 2 Diabetes Diet
Type 2 Diabetes Diet - Foods To Eat & Foods To Avoid!
Nibbledish.com/Type-2-Diabetes-Diet

Buy Mona-v-i-e Now
Become a Preferred Customer Buy Direct Best Prices!
www.thegreatproduct.com/greatjuice

Diabetes Type 1 Diabetes
Answers to your questions about type 1 diabetes and more!
www.ivillage.com

Free Diabetes Meal Plan
Eat Right & Lose Weight to Control Diabetes. Free from Everyday Health
www.EverydayHealth.com/DiabetesMeal

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Pomegranate, another Monavie ingredient has more antioxidants than red wine, orange juice, green tea or cranberry juice.

It was a known fact that pomegranate is very effective in clearing plaque from inner arterial walls and recent studies show that pomegranate can be used with good results in combating diabetes (among many diseases).

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Type 2 Diabetes
Monavie Price
Monavie Story
Monavie Online
Acai Drink

Monavie & diabetes Fact No.6

Prunes are rich in minerals, phenols, and have an very high ORAC value. Their drying process increases their antioxidant powers by over 6 times!

It has been shown that prunes can be used to can prevent/treat diseases related to aging. Prunes can also be used for treating diabetes.

Monavie & diabetes Fact No.7

Know as the "herb of longevity", the wolfberry contains a powerful combination of polysaccharides and antioxidants, which enhance the activity of the immune system.

Among the many health conditions that medicine has found wolfberries to help we will find diabetes.

Well, it looks like we have a clear winner in this Monavie & diabetes match, but we're not done yet. Maybe your still wondering if diabetics can drink monavie and feel better. Maybe all that was said before still dindn't convince you. This is why we will explore some more.

Monavie & diabetes Fact No.8

Because of its low Glycemic value which is 4 grams of naturally occurring sugar and 2 carbs per 1 oz., monavie won't spike your blood sugar levels and is safe for diabetics.

The next two facts are two questions that people just like you have asked. And the answers are in...

Monavie & diabetes Fact No.9

QUESTION:
Are diabetics able to take the MonaVie Active?

ANSWER:
Technically glucosamine is a carbohydrate. The body is not able to convert it into glucose so it does not provide additional sources of glucose. If you are a diabetic, check first with your doctor and always monitor your blood sugar. In diabetes, many factors can lead to changing blood sugar levels. Studies show glycosamine did not affect insulin sensitivity in humans. (Pouwels 2001)

Monavie & diabetes Fact No.10

QUESTION:
Are the Monarch products safe for Type I Diabetics?

ANSWER:
It is very rare for a Diabetic Type I to be overweight, and in this case, it is not infrequent for Diabetic Type I to be misdiagnosed as such and really be Diabetic Type II. Typically the shakes, bars, and vitamins can be used as snacks, although, as always, we recommend that you consult with your health care provider. With diabetes, it is always advisable to check sugar blood levels regularly, especially when you make any changes in diet.

And here is a testimonial of a diabetes & monavie case:

Monavie & diabetes testimonial

I am 27 years old, and my work includes having been a Bounty Hunter in Colorado
for about 8 years. About 4 years ago, I noticed I was losing the feeling in my feet. Walking became increasingly difficult because the numbness was making it hard to balance myself. The doctor told me I had Type II diabetes and had probably had it for 5-6 years. I started drinking 'Acai Active Blend' on January 8th, 2005 and by March I had gained back almost all the feeling in my feet. Walking is natural and easy again, and my knees don't crack the way they used to.

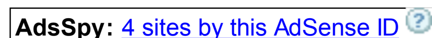
I'm drinking a total of around 3 ounces a day. I'm also giving my 5-year-old daughter two ounces a day of the 'Acai Original Blend'. After about 6-8 weeks, I began noticing that she was much more calm and relaxed. She seemed to have a new sense of well being and serenity about her and was thinking things through before just acting on them as she'd done in the past. Even her teacher at school has commented and says Laree is now getting her work done on time, has much better concentration and is less aggressive on the playground. This product has made a dramatic difference in our lives, and I'll greatly appreciate it forever.

---Shaun Lawrence - Colorado

A little monavie & diabetes conclusion

In conclusion, can diabetics drink Monavie (acai juice)? Evidence shows that monavie does help against diabetes. Nonetheless, before taking monavie to treat diabetes and any other illness for that matter, see at least one doctor and ask him if its the case for monavie or not. Each diabetes case is different from another and although monavie usually defeats diabetes, you have to get a second opinion from your doctor.

I wish you all the luck and a great life from now on! Thank you for reading about monavie & diabetes!



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Richard Bird's collection of articles on Acai

1 The product and the company 2005-2010

Condensed and updated version of talk by Jeff Graham on MonaVie and Acai, originally given in 2005. [Updated by myself 2010].

In 2000-2001, Jeff Graham, Dr. Alexander Schauss and Ken Murdock started **a company called K2A** for the purpose of searching the world for unique natural products and ingredients with a potential for impact the wellness of humanity -- and then bringing those products to the marketplace.

Decades ago, Ken Murdock became the founder of herbal giant **Nature's Way,** which he sold to a large, German pharmaceutical company in 2001. It remains the world's largest herbal company. Ken is highly regarded throughout the industry for his insistence upon quality and science behind every product with which he has worked. In early 2001, these men became aware of Acai fruit during a trip to Brazil. There ensued a great deal of **scientific research** by K2A, deemed necessary because very little documented science on Acai existed at that time.

They learned that the natives of northern Brazil had eaten this wild grown fruit for **hundreds of years** as a mainstay of their diets. Interestingly, these natives, despite much poverty in the area, have an intense amount of energy, excellent health and a very low incidence of skin cancer -- in spite of great exposure to the relentless rays of the equatorial sun.

The K2A scientific work is ongoing, and in 2005, the **K2A principals crossed paths with Dallin Larsen and the MonaVie research team.** Although K2A had entertained thoughts of bringing an Acai formula of their own to market, they found a great synergy with MonaVie's administration, integrity, product quality and purpose -- and made a decision to **align themselves** with MonaVie. With the vast majority of the world's body of science behind the Acai berry having been done by K2A, much of it now in conjunction with the **United States Department of Agriculture,** K2A filed and has received grant of two separate patent applications related to Acai -- the exact Acai that is only available through MonaVie **1) a specific CLAIMS patent 2) a PROCESS patent.**

There is now a variety of Acai-based products sold in assorted retail stores (and already by various and sundry MLM companies trying to capitalize on MonaVie's success) ... but the **superior quality** Acai that distinguishes MonaVie is exclusively available to MonaVie and **NO** other company.

As their own Acai production plans had earlier evolved, K2A built a **state-of-the-art Acai processing plant** at the edge of the rain forest near Belem, Brazil. Their studies revealed that precious phytonutrients in the Acai berries began **degrading and breaking down** very shortly after harvest, so it was **crucial to begin preserving and processing them** immediately after the berries were harvested. The K2A freeze-drying process (now patented) is **exclusively** for MonaVie's products.

The modern school built by K2A to provide quality education for the many children of the plant workers was recently renamed the MonaVie School.

From the rain forest of northern Brazil, Acai fruit is harvested in "clusters" on stems that grow at the top of three different varieties of palm trees. Only ONE Acai palm (Euterpe Oleracea) produces fruit with a level of **nutritional value** that makes it highly commercial. Only the **ripest and peak quality** Acai berries from each cluster are selected and used for MonaVie. Berries that are less ripe and of lesser quality are sold to **other** companies who use conventional processing methods.

It was MonaVie's **scientifically-formulated blend of 19 powerful and healing fruits of the world** that originally captured the attention of K2A. Other Acai formulas that surfaced in 2005 seemed hastily blended with little more than ACCEPTABLE TASTE guiding them. (Acai alone is bitter and must be blended with other ingredients for palatability.)

To further distinguish MonaVie from other products, **Dr. Alexander Schauss** (now the world's #1 scientific expert on Acai and recipient of the coveted, **2005 Linus Pauling Lecture Award**) has been working tirelessly with the USDA in documenting the efficacy of FREEZE DRIED Acai that is covered under the recent patent applications and used in MonaVie.

Dr. Schauss is the **lead author** of numerous scientific paper s dealing with nutrition, health and behavior. His works on acai (along with 11-12 other authors, including Dr. Wui, one of the USDA's own top specialists on antioxidants) are freely available online at www.aibmr.com and in the **Journal of Agricultural and Food Chemistry**--the Creme de la Creme of peer reviewed journals in the nutritional

Especially in winter months when colds and flus are widespread, many people take additional Vitamin C for its protective and immediate, antioxidant effects. These effects are known to **dissipate** quite rapidly, thus people must continue taking more Vitamin C daily or even hourly in some cases to keep replenishing it as it is used up. (There is much anecdotal data showing that Vitamin C can fight free radicals that cause cellular damage and bring down our immune systems, leaving us more vulnerable to contracting illness.) Quite unlike Vitamin C, the freeze dried Acai berry's antioxidant effect is **both immediate AND long lasting,** thus providing an ever more powerful impact, a **cumulative** effect as additional quantities are consumed. Our specific type of Acai has been found to be **biologically active within SECONDS after ingestion, as well as hours and DAYS later.** And ONLY MonaVie now contains the Acai protected by the patent pending, freeze-drying technology. All "competitors" that list Acai as an ingredient are thus using fruit prepared by other processing methods known to be inferior to our freeze drying.

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From the begining, our MonaVie formula has been backed by **extensive science.** The 19 different fruits were scientifically selected because of their known benefits and spectrum of pigmentation and phytochemicals. It had been frequently demonstrated that MonaVie delivers health benefits like no other product in the marketplace. Now, with the change to freeze-dried Acai as well as adding an **additional quantity of Acai powder as well as a greater amount of the pulp,** the results are even more dramatic than before! (The change to our new, GREEN bottle was a decision of our marketing department, and as the clear bottle inventory is used up, the change to green will be complete. The bottle COLOR is not related to the quality of the contents!) **The Significance of Flash Pasteurization and Freeze Drying** Pasteurization of products such as MonaVie is essential -- to assure there will be **no microbes** that could later cause problems. Although standard, "kettle" pasteurization was originally used for MonaVie (a 4-5 hour process), we now use the increasingly popular **"flash" pasteurization,** which takes only 30-45 seconds as the formula moves continuously through the bottling pipelines. This results in a **more consistent color and flavor** ... a "crisper" product, although there will always be SOME differences because our 19 fruits are harvested during different growing seasons in different locales. (The Acai is harvested 10-11 months of the year from the regions where Acai palm trees thrive.)

Much of Dr. Schauss' research with the USDA has been related to the **antioxidant** potential of **freeze dried** Acai powder vs. the more conventional, **spray-dried** Acai powder. The USDA has long considered the **cranberry** the world's most powerful antioxidant fruit, boasting a score of **94 ORAC units** per gram of dry weight. (ORAC means the ability to control and destroy one particular type of free radical.) Astonishingly, the **"Opti Acai"** (A2K's trade name for the exclusive, freeze-dried powder now used in MonaVie) from their Brazilian plant near the rain forest, compared on a gram to gram basis, gives us a **thunderous advantage** in the marketplace. Look at the comparison, dry weight, gram to gram basis ...

PRODUCT . . . . . ORAC SCORE PER GRAM

Cranberry . . . . 94 units

Spray-dried Acai . . . . . 61-74 units

Freeze-dried Acai . . . . **1027 units ! ! !**

Jeff Graham also noted the manipulation of published ORAC scores by many companies who **"spike"** their formulas with Vitamin C or Vitamin E (and are not legally required to disclose such spiking!) in order to artificially inflate their scores.

Company growth has been staggering since our premier MonaVie formula was unveiled on January 9, 2005. Without question, the BEST is yet to come!

2. Sustainable Harvesting and Rainforest Preservation
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MonaVie is committed to sustainable harvesting and preserving the Brazilian Rain Forest. It uses skilled local harvesters to gather the berries by hand. This helps to create legitimate jobs for the local people which enables them to better provide for their families and also helps to reduce poaching which destroys the rain-forest

The economy being created through the sustainable harvesting of the açai berry and production of MonaVie is stretching all the way up the Amazon river, and is helping to create education, build schools and change people's lives.

Rainforest Preservation

Prior to the emergence of the açai harvesting industry the 'hearts of palm' or 'palmito' would have been harvested from the trees by poachers. This small section of the tree would then be sold for a small sum. Whilst helping the local people to survive the harvesting of the heart of palm also kills the whole tree and contributes significantly to the destruction of the rain-forest. MonaVie are committed to reducing this practice and helping the preserve the rain forest through both sustainable harvesting of the açai berry and education.

In May 2009, Randy Larsen, Executive Vice President and Cofounder of MonaVie, visited the city of Brasilia to execute the Sustainable Harvesting and Rainforest Preservation Education Agreement between MonaVie and the Ministry of the Environment, Chico Mendes Institute (a branch of the Brazilian government). The unique partnership will further help preserve the Amazon Rainforest and promote sustainable harvesting of the açai berry.

3. The M.O.R.E. Project

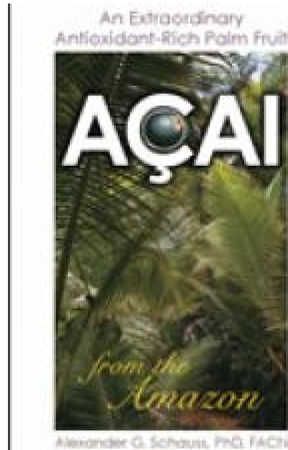
In addition to their sustainable harvesting and rain-forest preservation initiatives, MonaVie's primary charitable cause is the M.O.R.E. Project, which stands for**M**onaVie **O**peration **RE**scue.

The M.O.R.E. Project has a number of aspects to it's work. These are focused primarily in into 4 areas:

- **Hope and Dignity** – helping to restore the hope and belief of the people living in the Favelas, or slums, so that they can make more of their lives.
- **Education** – providing education and building schools so that both children and adults can learn trades and skills so they can get a good job and provide for their families.
- **Sheltered Homes** – these are safe havens for children who would otherwise be on the streets, most likely involved in drug trafficking or prostitution. Instead they are cared for by trained foster parents and receive love, care, education and have access to medical care.
- **Home Repair** – helping to repair the homes of the people living in the Favelas, or slums, so that they they have basic amenities and a more habitable living environment.

4. General literature

Acai (Euterpe oleracea): An Extraordinary Antioxidant-Rich Palm Fruit from the Amazon



By: Alexander G. Schauss, PhD, FACN

Dr. Alex Schauss' discovery of a previously little known palm fruit's remarkable antioxidant activity, led to publication in the *Journal of Agricultural and Food Chemistry* that confirmed it had the highest peroxyl radical scavenging activity of any food in the world. Since his discovery numerous authors have referred to it as a "super food."

In the new edition of this book, the author details the pathway to discovery and recounts the findings of numerous laboratories that collaborated in studying this fruit, now known worldwide as "Acai."

The impact of the author's discovery has led to government protection of millions of acres of palm trees in the delicate rain forest of Amazonia. Today over 400,000 liters of acai is consumed a day by nearly 1.2 million inhabitants that live in the floodplains of the Amazon River near Belem, owing to the growing body of information about its nutritional content and antioxidant activity. In addition, it has become a major export food of Brazil and resulted in one of the fastest growing network marketing companies in the world. However, not all "acai" products are the same; many contain a fraction of the antioxidant activity of a proprietary freeze-dried acai that has been the subject of extensive research by numerous scientists and laboratories.

ISBN: 978-0-9814906-4-9,
Published by BioSocial Publications, Tacoma, WA. Published September, 2009.

(NaturalNews Nov 2006) New research appearing in the Journal of Agricultural and Food Chemistry has found that although the Amazonian acai berry has the highest antioxidant content of any food, certain commercial processing methods may strip the fruit of much of its nutrition.

Two studies led by Alex Schauss from AIBMR Life Sciences -- along with colleagues from the U.S. Department of Agriculture, the University of California and other institutions -- examined the antioxidant properties of OptiAcai-brand freeze-dried acai fruit pulp and skin powder, as measured by the powder's oxygen radical absorbance capacity (ORAC).

Schauss and his team found that the OptiAcai product had a 1026.9 ORAC score, compared to an ORAC score of 155 for other commercially available freeze-dried acai samples.

"What mystifies me is that the acai products we tested in the commercial marketplace had a fraction of the antioxidant activity reported in our paper for OptiAcai," Schauss said.

The OptiAcai product also demonstrated "extraordinarily high" peroxyl scavenging activity -- the highest of any food researched so far -- as well as high superoxide scavenging activity. According to Schauss, the ability of acai berries to halt the formation of damaging free radicals could have profound implications for treatment of a wide range of diseases.

Schauss says that although the OptiAcai product was impressive in its antioxidant content, not all acai products available on the market are as effective.

"We believe there are many reasons for the lower ORAC values of various acais that have been on the market for some time," Schauss said. "First, freeze-drying is superior to spray drying or air drying in retaining phytochemicals and nutrients, but more expensive. We believe that other suppliers have not considered the issue of enzymatic degradation of the fruit."

Much of the acai shipped from Brazil to the United States or Europe is sent in large frozen blocks that fail to prevent nutritional degradation, Schauss said. For that reason, many commercially available acai products have low ORAC levels.

Consumer advocate Mike Adams, author of "The Seven Laws of Nutrition," said acai berries have a pleasant taste and provide high-density natural medicine to consumers.

"As with many of the smaller berries such as blueberries, sea buckthorn and goji berries, acai berries pack a potent nutritional punch in a tiny package," Adams said. "I recommend them to anyone interested in improving their health."

5. Videos:
Sustainable Harvesting and Rainforest Preservation

(Try to ignore the Chariots of Fire style music!)

http://www.youtube.com/v/550NTmi0xM0&rel=0&border=1&color1=0x3a3a3a&color2=0x999999&hl=en_US&feature=player_embedded&fs=1

8604 *J. Agric. Food Chem.* **2006,** *54,* 8604–8610

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antioxidant Capacity and Other Bioactivities of the Freeze-Dried Amazonian Palm Berry, *Euterpe oleraceae* Mart. (Acai)

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The fruit of *Euterpe oleraceae*, commonly known as acai, has been demonstrated to exhibit significantly high antioxidant capacity *in vitro*, especially for superoxide and peroxyl scavenging, and, therefore, may have possible health benefits. In this study, the antioxidant capacities of freeze-dried acai fruit pulp/skin powder (OptiAcai) were evaluated by different assays with various free radical sources. It was found to have exceptional activity against superoxide in the superoxide scavenging (SOD) assay, the highest of any food reported to date against the peroxyl radical as measured by the oxygen radical absorbance capacity assay with fluorescein as the fluorescent probe ($ORAC_{FL}$), and mild activity against both the peroxynitrite and hydroxyl radical by the peroxynitrite averting capacity (NORAC) and hydroxyl radical averting capacity (HORAC) assays, respectively. The SOD of acai was 1614 units/g, an extremely high scavenging capacity for $O_2^{\bullet-}$, by far the highest of any fruit or vegetable tested to date. Total phenolics were also tested as comparison. In the total antioxidant (TAO) assay, antioxidants in acai were differentiated into "slow-acting" and "fast-acting" components. An assay measuring inhibition of reactive oxygen species (ROS) formation in freshly purified human neutrophils showed that antioxidants in acai are able to enter human cells in a fully functional form and to perform an oxygen quenching function at very low doses. Furthermore, other bioactivities related to anti-inflammation and immune functions were also investigated. Acai was found to be a potential cyclooxygenase (COX)-1 and COX-2 inhibitor. It also showed a weak effect on lipopolysaccharide (LPS)-induced nitric oxide but no effect on either lymphocyte proliferation and phagocytic capacity.

KEYWORDS: ***Euterpe oleraceae*; acai; reactive oxygen species (ROS); antioxidant; $ORAC_{FL}$; NORAC; HORAC; superoxide; SOD; TAO; cyclooxygenase (COX); macrophage phagocytosis assay; nitric oxide assay; lymphocyte proliferation assay**

INTRODUCTION

High intake of fruits and vegetables was found to positively associate with lower chance of many diseases by epidemiological studies and clinical trials. Antioxidant capacity was believed to be one of the possible mechanisms, though others are also involved. Acai, fruits of *Euterpe oleraceae* Martius, is consumed in a variety of beverages and food preparations in the native land in Brazil, Colombia, and Suriname and used medicinally as an antidiarrheal agent (*1*, *2*). Recently, much attention has been paid to its antioxidant capacity and possible role as a "functional food" or food ingredient (*3*−*6*). *Euterpe oleraceae* fruit pulp has been reported to quench peroxyl radicals, peroxynitrite, and *in vitro* hydroxyl radicals by the TOSC assay (*4*). In another study, the antioxidant activity of acai frozen pulp was determined on the basis of the inhibition of copper-induced

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peroxidation of liposome and the inhibition of the co-oxidation of the linoleic acid and β-carotene system (*5*). Phytochemical composition and nutrient analysis of acai have been presented in our former paper (*7*). Here, we focus on its antioxidant capacities evaluated by different assays with various free radical sources to further enhance our knowledge of this fruit's health potential.

Free radicals are consistently formed as byproducts of aerobic metabolism in the human body (*8*). They are generally reactive oxygen or nitrogen species (ROS or RNS). The most common ROS and RNS *in vivo* are superoxide ($O_2^{\bullet-}$), hydroxyl radical ($OH_2^{\bullet}$), peroxyl radical ($RO_2^{\bullet}$), nitric oxide ($^{\bullet}NO$), and peroxynitrite ($ONOO^-$). These ROS have been associated with many chronic and degenerative diseases including vascular diseases, diabetes, cancer, and overall aging (*9*−*11*). Dietary antioxidants are believed to be good external sources to counteract free radicals in the body (*12*). A large number of methods have been developed to evaluate total antioxidant capacity (TAC) of food samples. Nevertheless, few of them have been used widely due to the difficulty of measuring TAC owing to limitations associated with methodological issues and free radical sources (*13*). In this study, the TAC of acai was evaluated by a series of oxygen radical absorbance capacity assay with fluorescein as the fluorescent probe ($ORAC_{FL}$) based assays, including hydrophilic $ORAC_{FL}$ (H-$ORAC_{FL}$), lipophilic $ORAC_{FL}$ (L-$ORAC_{FL}$), peroxynitrite radical averting capacity (NORAC), and hydroxyl radical averting capacity (HORAC). As a comparison, total phenolics was also measured by the Folin−Ciocalteu method. Moreover, several novel antioxidant capacity assays including the superoxide scavenging (SOD) assay, total antioxidant (TAO) assay, and inhibition of ROS formation in a functional, cell-based assay using freshly purified human neutrophils from healthy donors were also performed. Results from these assays are expected to provide additional information to help us better understand the antioxidant capacity of acai.

Bioactivities based on mechanisms other than antioxidant activities may also contribute to the overall health benefits of acai. In this study, we conducted several assays related to anti-inflammation and immune functions, including the cyclooxygenase (COX-1 and COX-2) inhibitor assay, macrophage phagocytosis assay, nitric oxide assay, and lymphocyte proliferation assay.

MATERIALS AND METHODS

Plant Material. Freeze-dried acai (*Euterpe oleracea*) fruit pulp/skin powder (OptiAcai) was obtained from K2A LLC (Provo, UT). The berries were collected in Belem, Brazil. Within hours of harvesting, acai berries were frozen and stored at −20 °C until transferred for freeze drying. The freeze-dried acai powder was kept at −20 °C until analyzed.

Chemicals and Standards. *ORAC-Based Assays and Total Phenolics.* 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein (sodium salt) (Fl), cobalt(II) fluoride tetrahydrate, and picolinic acid were obtained from Aldrich (Milwaukee, WI). Randomly methylated β-cyclodextrin (Trappsol, Pharm Grade, RMCD) was obtained from Cyclodextrin Technologies Development Inc. (High Springs, FL). Folin−Ciocalteu's phenol reagent, sodium carbonate, sodium hydrogen carbonate ($NaHCO_3$), dihydrorhodamine-123 (DHR-123), caffeic acid, and gallic acid were all purchased from Sigma (St. Louis, MO). Potassium phosphate dibasic (K_2HPO), potassium phosphate monobasic (KH_2PO_4), and hydrogen peroxide were obtained from VWR (West Chester, PA). 3-Morpholinosydnonimine hydrochloride (SIN-1) was provided by Toronto Research Chemicals (North York, Ontario, Canada). Other solvents were purchased from Fisher (Fair Lawn, NJ).

SOD Assay. Hydroethidine was from Polysciences, Inc. (Warrington, PA). Xanthine oxidase (from butter milk, catalog number X4875), xanthine, and superoxide dismutase (from bovine erythrocytes, catalog number S 2515) were purchased from Sigma-Aldrich (St. Louis, MO). Manganese(III) 5,10,15,20-tetra(4-pyridyl)-21*H*,23*H*-porphine chloride tetrakis(methochloride) was obtained from Aldrich (Milwaukee, WI,).

TAO Assay. TAO iodine reagent was provided by Shanbrom Technologies, LLC (Ojai, CA). A 710A+ basic ion selective meter was obtained from Thermo-Electron Corp (Waltham, MA).

Inhibition of ROS Formation in Human PMN Cells. Histopaque 1119 and 1077 are both from Sigma-Aldrich (St. Louis, MO). DCF-DA is from Molecular Probes (Eugene, OR).

COX-1 and COX-2 Inhibitor Assay. Arachidonic acid and COX-1 and COX-2 enzymes are all purchased from Cayman Chemical (Ann Arbor, MI).

Macrophage Phagocytosis Assay, Nitric Oxide Assay, and Lymphocyte Proliferation Assay. RPMI-1640 media was purchased from Invitrogen (Carlsbad, CA) and phosphate-buffered saline (PBS) from Hyclone (Logan, UT). Lipopolysaccharide (LPS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) and naphthylethylene diamine dihydrochloride (NEDD) were obtained from Sigma (St. Louis, MO). Other reagents were of analytical grade (Bangalore, India).

Total Phenolic Analysis. The acetone/water/acetic acid (AWA) extracts were subjected to total phenolics measurement by Folin−Ciocalteu reagent according to the method of Wu et al. (*14*). The results were expressed as milligrams of gallic acid equivalents per 100 g of fresh weight (mg of GAE/100 g of FW).

$ORAC_{FL}$-Based Assays. Freeze-dried acai (0.035 g) was extracted with 20 mL of acetone/water (50:50 v/v) for 1 h at room temperature on an orbital shaker. The extracts were centrifuged at 5900 rpm, and the supernatant was ready for H-$ORAC_{FL}$, HORAC, and NORAC analysis. An acai sample (1 g) was extracted with hexane/dichloromethane two times (10 mL × 2). The supernatants were combined for L-$ORAC_{FL}$ analysis (*15*).

The H-$ORAC_{FL}$ assay was conducted on the basis of a report by Ou and co-workers (*16*), modified for the FL600 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT). The FL600 microplate fluorescence reader was used with fluorescence filters for an excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm. The plate reader was controlled by software KC4 3.0. For L-$ORAC_{FL}$, a sample solution was prepared according to a previous paper (*17*). Then the L-$ORAC_{FL}$ was also measured in the same plate reader based on a published procedure (*17*).

The HORAC assay is based on a report by Ou and co-workers and modified for the FL600 fluorescence microplate plate reader (Bio-TeK Instruments, Inc., Winooski, VT) (*18*).

$ONOO^-$ scavenging was measured by monitoring the oxidation of DHR-123 according to a modification of the method of Chung et al (*19*). Briefly, a stock solution of DHR-123 (5 mM) in dimethylformamide was purged with nitrogen and stored at −80 °C. A working solution with DHR-123 (final concentration, fc, 5 M) diluted from the stock solution was placed on ice in the dark immediately prior to the study. The buffer of 90 mM sodium chloride, 50 mM sodium phosphate (pH 7.4), and 5 mM potassium chloride with 100 M (fc) diethylenetriaminepentaacetic acid (DTPA) was purged with nitrogen and placed on ice before use. $ONOO^-$ scavenging by the oxidation of DHR-123 was measured with a microplate fluorescence reader FL600 with excitation and emission wavelengths of 485 and 530 nm, respectively, at room temperature. The background and final fluorescent intensities were measured 5 min after treatment with or without SIN-1 (fc 10 M) or authentic $ONOO^-$ (fc 10 M) in 0.3 N sodium hydroxide. Oxidation of DHR-123 by decomposition of SIN-1 gradually increased, whereas authentic $ONOO^-$ rapidly oxidized DHR-123 with its final fluorescent intensity being stable over time.

SOD Assay. An acai sample (0.02 g) was extracted with 20 mL of an acetone/water mixture on a shaker for 1 h. The mixture was centrifuged at 5900 rpm and 20 °C for 10 min. The supernatant was used for SOD assay. SOD assay was carried out on the basis of an in-house protocol (Brunswick Labs, Wareham, MA) on a Precision 2000 eight channel liquid handling system and Synergy HT microplate UV−vis and fluorescence reader, both from Bio-Tek Inc. (Winooski, VT).

TAO Assay. Acai powder (4 g) was placed in 40 mL of 10% (w/v) soluble polyvinyl pyrrolidone (PVP; BASF, Kollidon 17PF) and

incubated for 2 h at 37 °C. The extract was centrifuged at 4000 rpm for 10 min, and the supernatant was decanted from the tube and serially diluted 2-fold to 1:8. Iodine reagent (0.1 mL) was mixed well with diluted supernatant. The iodide level (μg/mL) in the sample was determined by an Orion Iodide Sure-Flow solid-state combination electrode (Waltham, MA) at 30 s and 30 min after addition of iodine reagent. The TAO value is equivalent to ppm (μg/mL) of iodide formed.

Inhibition of ROS Formation in Human PMN Cells. Heparinized blood samples were obtained from healthy volunteers upon informed consent. The blood was immediately layered on top of a double gradient of Histopaque 1119 and 1077 (both from Sigma-Aldrich, St. Louis, MO), and centrifuged for 25 min at room temperature. The top layer of mononuclear cells was removed. The second layer of cells between the two gradients, which represents almost 100% neutrophils, was harvested and used for the evaluation of ROS formation. Cells were washed twice in phosphate-buffered saline without calcium or magnesium.

An extract of the test product was prepared by adding acai powder (0.5 g) to 5 mL of phosphate-buffered saline, pH 7.4. This mixture was vortexed repeatedly and allowed to sit at room temperature for 1 h. Prior to use, insoluble particles were removed by centrifugation and subsequent filtration using a 0.22 μm cellulose−acetate syringe filter. This liquid was used to prepare a series of 100-fold dilutions in phosphate-buffered saline without calcium or magnesium. Freshly purified human neutrophils were preincubated with acai extracts over a wide range of dilutions and then incubated at 37 °C for 90 min. Following a wash to remove compounds within the extracts that could interfere with the oxidation marker, cells were loaded with 0.5 μM DCF-DA (Molecular Probes, Eugene, OR) for 1 h at 37 °C. All samples, except for the untreated control samples, were then exposed to 167 mM H_2O_2 for a period of 45 min to induce oxidative stress. Samples were washed to remove the peroxide, transferred to cold RPMI, and stored on ice in preparation for immediate acquisition by flow cytometry, using a FACSCalibur cytometer (Becton-Dickinson, San Jose, CA). Untreated samples were run before and after running all other samples, to verify that spontaneous oxidation was minimal.

Intracellular levels of DFC-DA fluorescence intensity in untreated versus challenged cells in the presence versus absence of the test product were analyzed by flow cytometry. A standard curve of DCF-DA fluorescence intensity as a result of treatment with known amounts of hydrogen peroxide was used to produce an estimation of the effectiveness of a given natural product in terms of quenched hydrogen peroxide molecules. Data was collected in triplicate for controls and duplicate for each sample concentration. Dose levels are reported in volumetric parts per billion. Statistical significance was determined using Student's t-test.

COX Assay. Acai powder (2 g) was extracted with 50% acetone and tested directly without further dilution. The sample was incubated at 37 °C with Tris buffer (0.5 mL) in the reaction chamber followed by 5 μL of 100 μM heme in DMSO. To the solution, 5 μL of COX-1 (or 10 μL of COX-2) enzyme solution was added (used as received from supplier). The mixture was incubated for 1 min. A 5 μL sample (in DMSO or ethanol) was added and incubated for 1 min. Arachidonic acid (5 μL) was added, and the reaction rate was monitored. The oxygen concentration was monitored in real time by an Oxytherm (Hansatech Instrumental, Norfolk, England). The initial oxygen consumption rate is obtained from the kinetic curve. In the presence of inhibitors, the initial rate decreased. The IC_{50}, the concentration at which the initial oxygen consumption rate decreased by 50%, was used to express the COX-1 and -2 inhibition activity.

Nitric Oxide Assay, Macrophage Phagocytosis Assay, and Lymphocyte Proliferation Assay. Acai was diluted in media containing 5% DMSO to a concentration of 10 mg/mL. It was subjected to sonication at 35 kHz for 10 min in a Bandelin Sonorex sonicator. The supernatant was collected, sterilized though a 0.22 μm filter, and used immediately for the assay.

Mouse splenocytes were isolated according to previously published procedures with minor modifications (*20*).

The lymphocyte proliferation assay was performed per previously established protocols (*20*, *21*). Splenocytes were plated in 96-well plates on day 0 (0 h) at 5×10^5 cells/well (200 μL per well) in RPMI-1640 media containing 10% fetal bovine serum (FBS). The plates were incubated for 2 h at 37 °C in a CO_2 incubator (5% CO_2) to allow the cells to recover. A Trypan blue dye exclusion test was performed at the time lymphocytes were seeded in the well. Cell viability was >95%. Acai extracts were added to the wells at the requisite concentrations, and the plates were incubated again for 24 h at 37 °C in a CO_2 incubator (5% CO_2). LPS (5 μg/mL) was also added to some wells as a positive control for the assay. Cell proliferation was checked at 24 h using the MTT assay.

The nitric oxide assay was performed as per previously established protocols (*22*−*24*). Briefly, J774A.1 cells were plated in 96 well plates on day 0 (0 h) at 5×10^5 cells/mL (200 μL per well) in RPMI-1640 medium containing 10% FBS. The plates were incubated overnight (for about 16 h) at 37 °C in a CO_2 incubator (5% CO_2). The old media was removed from the wells after 16 h, and fresh media was added onto the cells. LPS or herbal extract at different concentrations was added to the wells. The cells were incubated for 48 h at 37 °C in a CO_2 incubator (5% CO_2). At the end of 48 h, the supernatants were collected and used for the nitric oxide (NO) assays.

In order to perform the NO assay, 100 μL of the sample supernatant was added in a 96-well plate. Greiss reagent (100 μL) was added to each well, and the samples were incubated at room temperature for 10 min. After 10 min incubation, the absorbance was measured at 540 nm. A standard curve was made with different concentrations of $NaNO_2$, and the data was expressed in terms of micromoles of $NaNO_2$. The experiment was run three times with six replicates per data point.

The macrophage phagocytosis assay was performed per previously established protocols (*25*). Briefly, J774A.1 cells were plated in 35 mm Petri dishes on day 0 (0 h) at 4×10^5 cells/dish (2 mL per 35 mm Petri dish) in RPMI-1640 medium containing 10% FBS. The plates were incubated for 6 h at 37 °C in a CO_2 incubator (5% CO_2) to allow the cells to adhere to the plates. The herbal extracts were added at different concentrations and the plates were incubated overnight (for about 16 h) at 37 °C in a CO_2 incubator (5% CO_2). The old media was removed from the plates after 16 h, and fresh media was added onto the cells. Yeast cells were added onto the plates at a 1:8 (macrophage/yeast) ratio, and the cells were incubated again at 37 °C in a CO_2 incubator (5% CO_2) for 1 h. At the end of 1 h, the supernatant was discarded, and the cells were washed twice with phosphate-buffered saline to remove unattached yeast cells. The cells were then fixed with methanol, stained with Geimsa stain, and observed under the oil immersion lens of the microscope for calculating the phagocytotic index.

Cells were counted in different fields, and a minimum of 100 macrophages were observed per sample. The phagocytotic index was expressed in two sets of parameters: percentage infected macrophages and average number of yeast per 100 infected macrophages. Attached but noninternalized yeast were not counted. The experiment was run three times with three replicates per data point.

The statistical analysis was performed using the GraphPad Prism program. A one-way analysis of variance (ANOVA) was performed on the data to analyze for significance, followed by a Neuman−Keuls test to compare multiple samples. A value of $P < 0.05$ was considered to be significant.

RESULTS

$ORAC_{FL}$, HORAC, NORAC, SOD, and Total Phenolic (TP) Content of Acai. H-$ORAC_{FL}$, L-$ORAC_{FL}$, HORAC, NORAC, SOD, and total phenolics (TP) content of freeze-dried acai are reported in **Table 1**. Total antioxidant capacity (TAC) was calculated as sum of H-$ORAC_{FL}$ and L-$ORAC_{FL}$.

Antioxidant Capacity from TAO. Antioxidant capacity of freeze-dried acai from the TAO assay is shown in **Figure 1**. In this assay, the antioxidant values of "slow-acting" (measured at 30 min) and "fast-acting" (measured at 30 s) were differentiated.

Inhibition of ROS Formation. Pretreatment of human neutrophils with freeze-dried acai extracts prior to induction of ROS by H_2O_2 treatment resulted in a significant reduction in

Table 1. Antioxidant Capacity from ORAC Assay with Different Free Radicals, SOD Assay, and Total Phenolics (TP) of Freeze-Dried Acai and Other Acai Products[a]

sample	H-$ORAC_{FL}$[b] (μmol TE/g)	L-$ORAC_{FL}$[c] (μmol TE/g)	TAC[d] (μmol TE/g)	NORAC (μmol TE/g)	HORAC (μmol GAE/g)	SOD (unit/g)	TP[e] (mg GAE/g)
freeze-dried acai	997	30	1027	34	52	1614	13.9

[a] Data was expressed as mean of duplicate measurements. [b] Hydrophilic $ORAC_{FL}$. [c] Lipophilic $ORAC_{FL}$. [d] Total antioxidant capacity, calculated as the sum of H-$ORAC_{FL}$ and L-$ORAC_{FL}$. [e] Total phenolics.

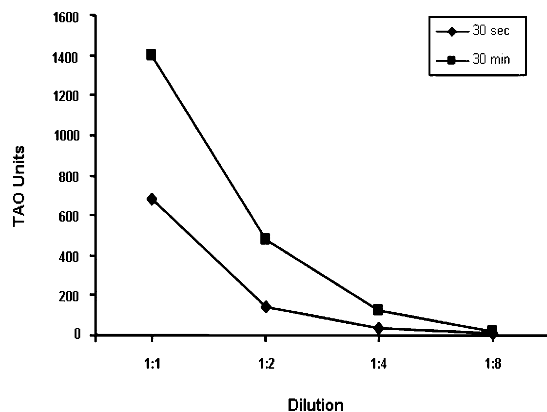

Figure 1. Total antioxidant (TAO) activity of freeze-dried acai, in which TAO assay differentiates antioxidant into "slow-acting" (30 min) and "fast-acting" (30 s) components.

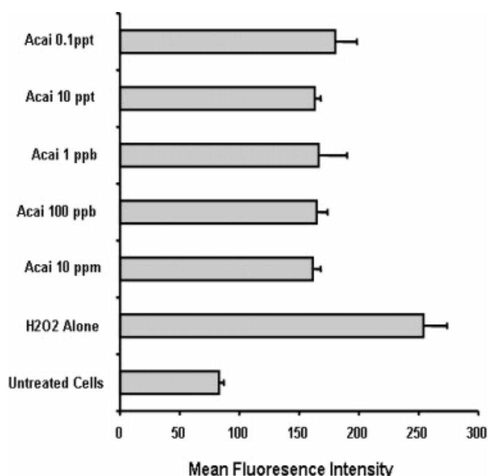

Figure 2. Freeze-dried acai reduced the H_2O_2-induced formation of reactive oxygen species (ROS) in freshly purified human neutrophils.

Table 2. Results from COX Assay of Freeze-Dried Acai

sample	IC_{50} of COX-1[a] (mg/mL)	IC_{50} of COX-2[a] (mg/mL)	IC_{50} ratio (COX-1 vs COX-2)
freeze-dried acai	6.96	12.50	0.56

[a] The result is reported as the IC_{50} (50% enzyme activity inhibition concentration).

ROS production. The formation of ROS was significantly inhibited, even at extremely low doses of freeze-dried acai. (**Figure 2**).

COX Inhibition Effects. Inhibition of COX-1 and COX-2 by freeze-dried acai is shown in **Table 2**. The IC_{50} ratio of COX-1 vs COX-2, which indicates the selectivity of the sample in inhibition of COX enzymes, is also presented (**Table 2**).

Lymphocyte Proliferation Activity. Freeze-dried acai did not show any effect on lymphocyte proliferation at the concentrations tested (5−1000 μg/mL) at a 24 h assay point. LPS (5 μg/mL), the positive control for the assay, demonstrated a 1.55-fold increase in lymphocyte proliferation over cell controls. The latter result is in keeping with the data usually obtained with LPS in this assay (**Figure 3**).

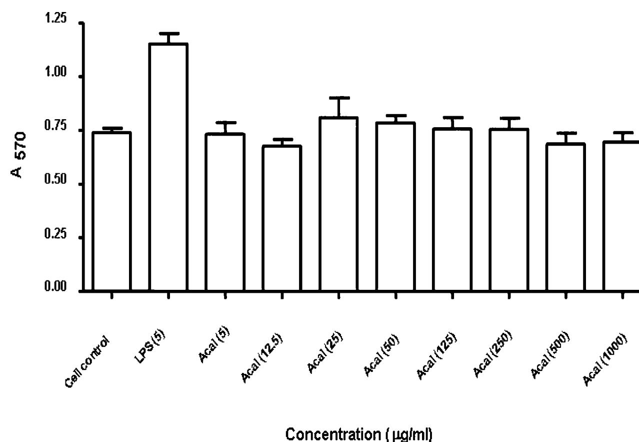

Figure 3. Effect of freeze-dried acai on lymphocyte proliferation.

Nitric Oxide Assay. The freeze-dried acai did not show any effect on NO release by J774A.1 macrophages at the above concentrations (250−2500 μg/mL) at a 48 h assay point. LPS (5 μg/mL), the positive control for the assay, demonstrated a 15.66-fold increase in NO release over cell controls. Freeze-dried acai at the above concentrations demonstrated a significant dose-dependent inhibition of LPS-induced nitric oxide in this assay (**Figure 4**).

Macrophage Phagocytosis Activity. Freeze-dried acai at 5−250 μg/mL increased macrophage infection by about 1.4−1.5-fold over control values. However, this effect did not appear to be dose dependent, and infection levels came down to control values with 500 μg/mL acai. There was a significant increase in the number of yeast engulfed per macrophage at 5 μg/mL acai, but the effect was not evident at higher concentrations (**Figure 5**).

DISCUSSION

An Internet search using the words "acai and antioxidant" entered into Google.com resulted in over 200 000 hits. Other than its antioxidant capacity, it was interesting to note how many health benefits were reported for acai. Yet, little research has been reported in the literature, while even less existed to support its claimed health benefits. In this study, antioxidant capacities of acai were investigated by different assays in an effort to fully understand the scope of its antioxidant capacities. Moreover, other possible bioactivities of acai related to inflammatory processes and its effect on markers related to immune function were also performed. Due to the complexity of the antioxidant defense system and involvement of many different types of free radicals in the body, a single antioxidant assay cannot provide us a complete picture of the antioxidant capacity of a given food *in vitro*, much less *in vivo*. Thus, several different antioxidant assays were used to study the antioxidant capacity of acai *in vitro*.

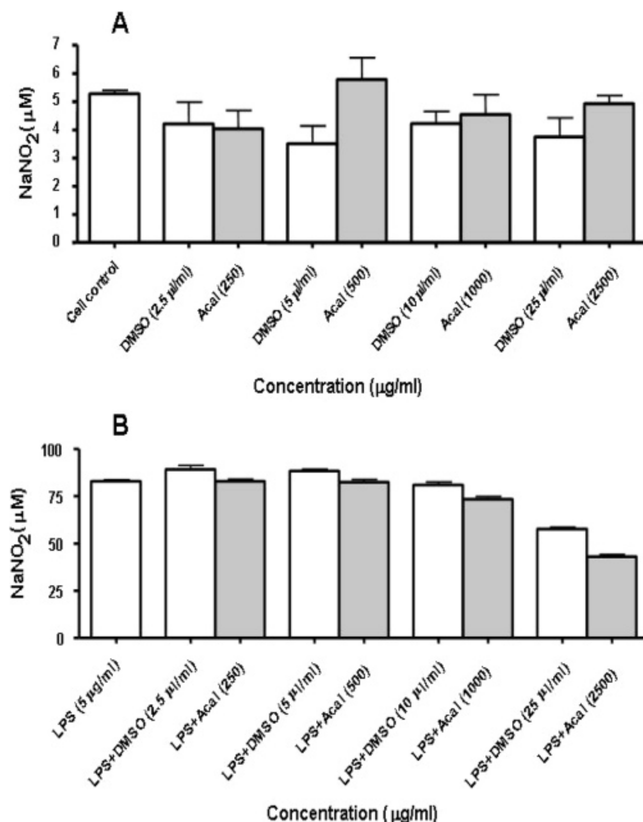

Figure 4. Effects of freeze-dried acai on nitric oxide release by J774 cells (**A**) and on LPS-stimulated nitric oxide release by J774 cells (**B**).

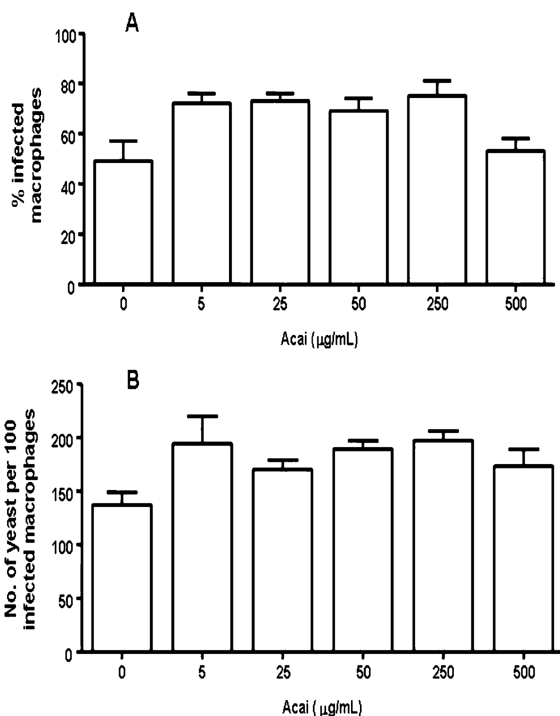

Figure 5. Effects of freeze-dried acai on J774 macrophage infection by yeast cells (**A**) and number of yeast engulfed by J774 macrophage (**B**).

The original $ORAC_{FL}$ assay was designed to measure the antioxidant capacity of foods toward peroxyl radicals, and it can be conducted to measure both hydrophilic and lipophilic antioxidants (*16*, *17*). From our results, the H-$ORAC_{FL}$ of freeze-dried acai was 996.9 μmol TE/g, which is significantly higher than that of most dark colored berry or any fruit or vegetable tested to date when appropriately converting fresh weight to dry weights (*14*). The L-$ORAC_{FL}$ of freeze-dried acai was 30 μmol TE/g, thereby yielding a total ORAC of 1026.9 μmol TE/g. Contradictorily and surprisingly, the contents of anthocyanins, proanthocyanidins, and other polyphenol compounds in this freeze-dried product were found to be much lower than those found in blueberry or any other berries with elevated H-$ORAC_{FL}$ values. To make things even more confusing, the total phenolics in acai was found to be only 13.9 mg/g GAE. In a recent paper, the ratio between hydrophilic $ORAC_{FL}$ and total phenolics was found to vary dramatically from less than 2 to more than 100 for different groups of foods (*14*). For most fruits and vegetables, this ratio is about 10. However, the ratio in acai is 50, five times greater than that found for any other fruit. This "unusual" ratio raises questions whether acai contains much stronger antioxidants than those found in other berries on an equal weight basis. Determining which antioxidants contributed to this unusual ratio warrants further work.

Freeze-dried acai has an oily feel when rubbed between the fingers, suggesting that acai contains fairly large amounts of lipophilic compounds. The L-$ORAC_{FL}$ is 29.6 μmol TE/g, which is higher than any berry samples tested to date (*14*).

HORAC and NORAC, two assays developed from ORAC, were adopted to measure antioxidant capacity of acai toward $OH^{\bullet}$ and $ONOO^{-}$, two of the major cell-killing ROS in the human body (*26*). The HORAC value of freeze-dried acai was 52 μmol GAE/g, which is similar to that of grapes but lower than that of dark colored berries (*18*). From our limited data (unpublished data), the NORAC value of freeze-dried acai is among the average of other fruits.

Superoxide ($O_2^{\bullet -}$) is believed to be the cause of other ROS formations such as hydrogen peroxide, peroxynitrite, and hydroxyl radicals. Therefore, $O_2^{\bullet -}$ scavenging capacity in the human body is the first line of defense against oxidative stress. It has been reported that overexpression of superoxide dismutase and catalase in transgenic flies extended life-span by as much as one-third, perhaps, due to decreased oxidative stress reflected by lower protein carbonyl contents (*27*). Superoxide scavenging capacity in blood is considered very important in maintaining antioxidant status. The most studied SOD from any natural source is wheat sprout SOD, ranging from 160 to 500 units/g for different samples (unpublished data). The SOD of acai was 1614 units/g, meaning acai has extremely high scavenging capacity to $O_2^{\bullet -}$, by far the highest of any fruit or vegetable tested to date.

The total antioxidant (TAO) assay was developed to permit rapid and simple determination of a sample's antioxidant capacity. The TAO assay is based on the iodine−iodide oxidation−reduction (redox) reaction, with the formation of iodide in the sample proportional to the antioxidant (or reducing) capacity of the sample. The TAO assay also differentiates antioxidants into a "slow-acting" component, which includes complex organic antioxidants (e.g., phenolics) and a "fast-acting" or "vitamin-C-like" component. The "fast-acting" antioxidants were measured at 30 s, whereas the "slow-acting" antioxidants were measured at 30 min. The combination of these values is the total antioxidant capacity (*28*). The TAO assay results for freeze-dried acai clearly showed that the antioxidant capacity of "slow-acting" antioxidants was stronger than that of "fast-acting" antioxidants (**Figure 1**).

Freeze-dried acai was also assayed for inhibition of ROS formation in freshly purified human neutrophils. Freeze-dried acai demonstrated a substantial inhibitory effect on the ROS formation in human neutrophil cells (**Figure 2**). Freeze-dried

acai displayed a maximum effect at a concentration of 1−10 parts per trillion (ppt, v/v). A nonmonotonic dose response was observed, which is typical in this type of *in vitro* cell-based assay, where a complex blend of active ingredients results in multiple factors contributing to intracellular oxidative stress. The ROS inhibition by the freeze-dried acai extract was effective at extremely low doses. The level of ROS formation was not brought back to the level of the positive control at any of the acai dilutions tested, including 0.1 ppt. This data indicates that the active antioxidant compounds in the freeze-dried acai are able to enter human cells in a fully functional form and perform oxygen quenching at extremely low doses.

Other Bioactivities of Acai. Antioxidants are surely not the only reason that we should eat fruits and vegetables. There are hundreds and even sometimes thousands of compounds in foods. Many of them contribute to health benefits through mechanisms other than antioxidant activity. Proteins in acai pulps have been found to show high antitryptic activity and considerable inhibition activity toward human salivary α-amylase (*29*). The effects of acai polyphenolics on the antiproliferation and induction of apoptosis in HL-60 human leukemia cells have also been investigated (*30*). Hence, we primarily focused on the possible effects of freeze-dried acai on immune parameters and some anti-inflammatory markers.

The lymphocyte proliferation assay (LPA) is a measure of immune activation/stimulation. It measures the ability of lymphocytes placed in short-term tissue culture to undergo clonal proliferation when exposed to a foreign substance/mitogen. This assay helps evaluate the immunostimulatory/immunosuppressive activity of a mitogen. Freeze-dried acai fruit demonstrated no significant effect on lymphocyte proliferation across a very wide concentration range (5−1000 μg/mL) in this assay (**Figure 3**) (*31*).

Nitric oxide (NO) is an inorganic free radical that functions as an intracellular messenger and effector molecule. It is produced during the conversion of arginine to citrulline and its production is catalyzed by the enzyme nitric oxide synthase (NOS) (*32*). NOS has three isoforms: NOS I, II, and III. Out of these three isoforms, only NOS II is inducible and is produced during macrophage activation (*33*). Macrophage activation is thus accompanied by the induction of inducible nitric oxide synthase and sustained release of NO (*34*). Synthesis of NO endows macrophages with cytostatic or cytotoxic activity against viruses, bacteria, fungi, protozoa, helminths, and tumor cells (*35*, *36*). Freeze-dried acai fruit did not affect nitric oxide release by J774A.1 macrophages at a concentration of 250−2500 μg/mL (**Figure 4**).

Neutrophils/macrophages play a major role in phagocytosis of microorganisms and other foreign entities that enter the body. Compounds that increase the phagocytic capacity of these cells are potent immunostimulators. Thus, this assay can be used to gauge the potential immunostimulatory effect of a substance. Freeze-dried acai was found to increase macrophage activity slightly (1.4−1.5-fold over control values) at concentrations of 5−250 μg/mL (**Figure 5**). However, this effect did not appear to be dose dependent, and activity levels came down to control values at 500 μg/mL. There was also a significant increase in the number of yeast engulfed per macrophage at 5 μg/mL, but the effect was not statistically significant at higher concentrations. This suggests that lower concentrations of freeze-dried acai may be activators of macrophage phagocytosis but possibly not at higher concentrations. Thus on the whole, it appears that freeze-dried acai probably possesses minimal immunostimulatory properties at concentrations higher than 5 μg/mL. Lower concentrations (less than 5 μg/mL) might be immunostimulatory, but additional study for this is needed. We also observed an inhibition in nitric oxide levels within J774A.1 macrophages with acai treatment. Since increased nitric oxide is associated with increased killing of microorganisms, and we have examined only initial macrophage phagocytosis and not yeast killing at later time points, it is probable that acai does not significantly enhance immunity *in vitro*.

Interestingly, the freeze-dried acai at 250−2500 μg/mL demonstrated a significant dose-dependent inhibition of LPS-induced nitric oxide (**Figure 4**). Since inhibition of LPS-induced nitric oxide has been correlated with anti-inflammatory activity (*37*), this result suggests that the freeze-dried acai may be used as a potent anti-inflammatory substance and thus may find applications in allergic and autoimmune disorders.

Only recently has the mechanism of botanicals been investigated at the molecular biology level by using COX-1 and COX-2 inhibitory assays to measure the pain-relieving and anti-inflammatory potential of herbal supplements (*38*). Freeze-dried acai showed mild inhibition capacity *in vitro* based on the COX-1 and COX-2 assays (**Table 2**). The IC_{50} ratio of COX-1 vs COX-2 indicates the selectivity of the sample in inhibition of COX enzymes. When the ratio is one, there is no selectivity. If the ratio is smaller than one, the sample inhibits COX-1 better than COX-2. If the ratio is larger than one, the sample inhibits COX-2 better. Therefore, freeze-dried acai inhibits the COX-1 enzyme more efficiently than the COX-2 enzyme.

Conclusion. In this study, freeze-dried acai fruit pulp/skin powder has been shown to be extremely powerful in its antioxidant properties against superoxide ($O_2^{\bullet -}$) by SOD assay. The freeze-dried acai fruit pulp/skin powder was also shown to be excellent against the peroxyl radical ($RO_2^{\bullet}$), with the highest reported total ORAC (1026.9 μmol TE/g) of any fruit or vegetable, and mild against both peroxynitrite ($ONOO^-$) and hydroxyl radical ($OH^{\bullet}$) by $ORAC_{FL}$-based assays. In addition, this freeze-dried acai was found to be a potential COX-1 and COX-2 inhibitor. These findings may have significant value as to this fruit's antioxidant role in aging and disease. Although this study proved that antioxidants in freeze-dried acai are able to enter human cells in a fully functional form *in vitro*, more studies are warranted to determine safety and efficacy of acai *in vivo*.

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Increased Antioxidant Capacity and Inhibition of Lipid Peroxidation in Healthy Adults Consuming an Açai (*Euterpe oleracea*) Fruit-Based Juice

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Abstract

The in vitro and in vivo properties of an açai based juice blend (MonaVie Active®, Salt Lake City, UT) were evaluated. Initially, a phenolic profile was determined for the beverage, and a cell-based antioxidant protection (CAP-e) assay (Honzel et al., 2008) was performed, which showed that the antioxidants in the beverage could penetrate erythrocytes and significantly protect the cells from oxidative damage in vitro ($p < 0.001$). Polymorphonuclear (PMN) cells exposed to the beverage showed significantly reduced formation of reactive oxygen species (ROS) and also had significantly reduced migration toward three pro-inflammatory chemoattractants.

A randomized, double-blind, placebo-controlled, cross-over study was performed on the beverage using 12 healthy subjects, 19-52 years of age (Jensen et al., 2008). At both one and two hours post consumption, a statistically significant increase in antioxidant capacity within subjects was noted based upon the CAP-e assay performed on serum ($p < 0.03$ and $p < 0.015$). Additionally, a statistically significant decrease in serum lipid peroxidation was noted using the thiobarbituric acid reactive substances assay (TBARS) at two hours after consumption ($p < 0.01$), together suggesting an in vivo antioxidant effect and, hence, bioavailability of the beverage.

INTRODUCTION

The palm tree *Euterpe oleracea* Mart. grows primarily in the rain forest of the Amazon. British, Portuguese and American botanists of the 18th and 19th century documented the use of the fruit of *E. oleracea,* known as the açai berry, as one of the primary foods consumed by natives living in the Amazon (Schauss, 2008). The berry has a favorable nutritional composition and a long harvest season (May to December). Up to 7,000 *E. oleracea* palms have been found to grow per acre in the Amazonian flood plains, making the availability of the fruit viable for commercial consumption worldwide. The palm has extraordinary resilience to flooding and wet soil conditions and has the ability to withstand equatorial solar radiation when it serves as the canopy of the rain forest. The palm can grow to a height of 32 meters and fruit for nearly 100 years (Schauss, 2008), although to maximize commercial production of the fruit, the palm stands are thinned at eight to ten years of age.

Each berry is about 2.5 cm in size, appearing almost black at maturity. The berries are not sweet nor do they have a taste familiar to most palates. Yet, in large cities along the Amazon, particularly in Amapa and Para states, Brazil, daily consumption of açai averages up to 2.0 liters of fresh juice a day. The pulp is also added to a wide range of staple foods, such as manioc (*Manihot esculenta*), served as a thick soup, or to fish or meat dishes (Schauss, 2008).

Anthocyanins, proanthocyanidin polymers, and other flavonoids are the predominant phytochemicals in açai (Schauss et al., 2006b). Besides a complement of vitamins, minerals, trace elements, soluble and insoluble fiber, phytosterols (beta-sitosterol, campesterol, and sigmasterol), and a low sugar content (1.3%), the total polyunsaturated, monounsaturated, and saturated fatty acids in açai contribute 13%, 60%, and 26%, respectively, to its total fatty acids content. Oleic acid (56%), palmitic acid (24%) and linoleic acid (13%) are the dominant fatty acids. Nineteen amino acids, including all of the essential amino acids, are found in açai (Schauss et al., 2006b).

Unlike spray-dried açai, which has a comparatively unimpressive ORAC score, freeze-dried (FD) açai has a Total ORAC of 1,027 µmol TE/g (Schauss, 2008; Schauss et al., 2006a). The ORAC-lipophilic scavenging activity of açai, at 29.6 µmol TE/g, is the highest of any known fruit. Açai's superoxide ($O_2^{\cdot-}$) scavenging capacity, based on the superoxide radical absorbance capacity (SOD) assay, is 1,614 units/g, the highest reported to date for any fruit or vegetable by over 1,000 units/g. FD açai also has high hydroxyl and peroxynitrite radical scavenging activity based on the hydroxyl radical averting capacity (HORAC) and peroxynitrite radical averting capacity (NORAC) assays (Schauss et al., 2006a). The Trolox Equivalent Antioxidant Capacity (TEAC) assay and Ferric Reducing Antioxidant Power (FRAP) assay show similar high antioxidant capacity values for FD acai of 744 µmol TE/g and 249 µmol TE/g, respectively (Schauss, in press).

The total phenolics of açai were calculated as 13.9 mg gallic acid equivalents/g. The ratio between ORAC-hydrophilic scores and total phenolics has been found to vary dramatically in foods. However, the ratio for FD açai is five times greater than that found in other fruits. A possible explanation for this could be that the antioxidants found in açai are unusually strong and not just reliant on its polyphenolics, but rather on higher molecular weight oligomers (Schauss et al., 2006a).

It has been demonstrated that açai's antioxidants can enter human cells and protect these from oxidative damage in the CAP-e cell based antioxidant protection assay using erythrocytes (Honzel et al., 2008). Furthermore, açai-treated human neutrophil cells show a dose-dependent inhibition of H_2O_2-induced ROS formation, at doses down to 0.1 ppt (Schauss et al., 2006a). When comparing the doses at which an antioxidant protection and doses at which an anti-inflammatory effect are seen, the data suggest that anti-inflammatory cellular signaling is responsible for the reduced ROS formation. Lastly, açai has shown mild cyclooxygenase COX-1 and COX-2 inhibitor action and has been found to have an slight inhibitory effect on production of lipopolysaccharide (LPS)-induced nitric oxide (Schauss et al., 2006a).

MonaVie Active is a proprietary juice blend made with açai berries as its primary ingredient. Because of the encouraging anti-inflammatory and antioxidant findings described previously for the açai berry, we were inspired to examine for similar in vivo properties of this beverage in humans in a step-wise fashion.

MATERIALS AND METHODS

A detailed description of all materials and methods can be found in the recent publication by G.S. Jensen and colleagues (Jensen et al., 2008). The methods are briefly described here.

Phenolic Analysis

Phenolic acid analysis was performed on the beverage using the techniques of HPLC (Agilent 1100HPLC system, Agilent Technologies, Palo Alto, CA) and mass spectrometry (4000 Q TRAP mass spectrometer, Applied Biosystems, Foster City, CA).

Cell-Based Antioxidant Protection of Erythrocytes Assay (CAPE-e), Polymorpho-Nuclear (PMN) Cell Migration and Reactive Oxygen Species Production In Vitro

Peripheral human venous blood was obtained for the in vitro studies from healthy volunteers, and PMN and erythrocytes were isolated from the blood. The erythrocytes were utilized in the CAP-e assay, where the cells were treated with the beverage, washed,

and then stained with a fluorescent probe that detects free radical damage. Cells were then treated with the free radical producer hydrogen peroxide, and fluorescence in the cells was measured using flow cytometry.

The PMN cells were treated with the beverage and used to measure ROS formation and migration toward three different pro-inflammatory chemoattractants: bacterial peptide f-Met-Leu-Phe (fmlp), leukotriene B4 (LTB4), or interleukin-8 (IL-8). Fluorescence intensity was also utilized to determine these measurements.

Human Clinical Study

The randomized, double-blind, placebo-controlled, cross-over human clinical study was approved by the Sky Lakes Medical Center Institutional Review Board. Seven healthy subjects participated in a pilot study, and 12 in the succeeding clinical trial. Due to the difficulty in developing a liquid placebo that could not be discerned, an encapsulated placebo was made using potato flakes and purple food coloring. Subjects were purposefully left unsure as to whether the capsules were the placebo or a solid version of the product. Two methods of serum antioxidant evaluation were performed on the subjects' blood and compared in the pilot study, including the ORAC and the CAP-e assays. Because ORAC testing did not result in a trend toward increased antioxidant activity in the subjects, it was not utilized in the following clinical trial. Subjects in the clinical trial arrived for testing after an overnight fast. A baseline blood sample was collected, and then the subjects consumed 120 ml of the juice blend. Blood was drawn again for analysis at one and two hours after consumption. CAP-e assay (as described above) and TBARS assay (utilizing a kit from Cayman Chemical Co, Ann Arbor, MI) were performed on serum samples. Subjects were tested in a randomized fashion after consumption of the beverage or placebo on different days at least one week apart, and data was compared at baseline and after treatment in a within subject design.

Statistical Analysis

Analysis of the data obtained in these studies was performed using the student's t test, two-tailed t test, and analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Phenolic Analysis

Major phenolics found in the juice blend included anthocyanins, proanthocyanidinds, and phenolic acids. The overall profile was expectedly somewhat different than that of previously described freeze dried açai itself, since the beverage contains other fruits besides its main ingredient açai. However, several key phenolics found previously in açai, such as cyaniding 3- glucoside and cyaniding 3-rutinoside (Schauss et al., 2006b) were also major anothocyanins found in the beverage blend.

Cell-Based Antioxidant Protection of Erythrocytes Assay (CAPE-e), Polymorpho-Nuclear Cell Migration and Reactive Oxygen Species Production In Vitro

In vitro, the beverage showed a highly significant dose-dependent antioxidant protection effect in the CAP-e assay (p values ranged from < 0.001 at highest beverage concentration of 10 g/L and < 0.045 for lowest concentration of 0.016 g/L). PMN cells which produce ROS very quickly upon stimulation, showed dose-dependently significantly less ROS formation after treatment with the beverage ($p < 0.003$ at the highest dose). Complex dose effects were seen when looking at migration toward the bacterial peptide fmlp; attraction was enhanced or inhibited depending upon the whether the dose was low or high respectively. However, a more clear dose-dependent inhibitory effect on migration of PMN cells was seen toward LTB4 ($p < 0.05$) and IL8 ($p < 0.03$).

Human Clinical Study

In the clinical study, intake of the beverage rendered a statistically significant increase in serum antioxidant capacity in the subjects blood at both the one hour and two

hour post consumption time points ($p < 0.03$ and $p < 0.015$, respectively) as measured by the CAP-e assay. Consumption of the beverage also resulted in a statistically significant decrease in lipid peroxidation in the blood after two hours ($p < 0.01$), as measured by the TBARS assay. A 45% correlation was calculated at the two-hour time point between the increased antioxidant capacity and the reduction in lipid peroxidation.

It remains unclear whether these statistically significant results found after intake of the juice blend were wholly due to the açai ingredient, as a combination of açai and the other fruit components added to the juice could also be responsible for the results. Further in vivo studies are being carried out to determine possible mechanisms of action. A more detailed description and discussion of all results can be found in the recent publication by G. S. Jensen and colleagues (Jensen et al., 2008).

CONCLUSIONS

The highly statistically significant antioxidant effect of treatment with MonaVie Active açai-based juice on cells in the CAP-e assay both in vitro and in vivo in human subjects suggests that the antioxidant components from the beverage are able to enter living cells and protect the cells against oxidative insult and are also able to enter serum after consumption and assist in protection in vivo. The significant reduction of lipid peroxidation as measured using the TBARS assay further suggests that consumption of this beverage results in the protection of lipids from oxidative damage. While it is unclear if these results are due solely to the high açai content of the juice, the results correlate well to previous data performed on the freeze-dried açai berry alone.

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In Vitro and in Vivo Antioxidant and Anti-inflammatory Capacities of an Antioxidant-Rich Fruit and Berry Juice Blend. Results of a Pilot and Randomized, Double-Blinded, Placebo-Controlled, Crossover Study

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This study investigated the in vitro and in vivo antioxidant and anti-inflammatory properties of a juice blend (JB), MonaVie Active, containing a mixture of fruits and berries with known antioxidant activity, including açai, a palm fruit, as the predominant ingredient. The phytochemical antioxidants in the JB are primarily in the form of anthocyanins, predominantly cyanidin 3-rutoside, cyanidin 3-diglycoside, and cyanidin 3-glucoside. The cell-based antioxidant protection of erythrocytes (CAP-e) assay demonstrated that antioxidants in the JB penetrated and protected cells from oxidative damage ($p < 0.001$), whereas polymorphonuclear cells showed reduced formation of reactive oxygen species ($p < 0.003$) and reduced migration toward three different pro-inflammatory chemoattractants: fmlp ($p < 0.001$), leukotriene B4 ($p < 0.05$), and IL-8 ($p < 0.03$). A randomized, double-blinded, placebo-controlled, crossover trial with 12 healthy subjects examined the JB's antioxidant activity in vivo. Blood samples at baseline, 1 h, and 2 h following consumption of the JB or placebo were tested for antioxidant capacity using several antioxidant assays and the TBARS assay, a measure of lipid peroxidation. A *within subject* comparison showed an increase in serum antioxidants at 1 h ($p < 0.03$) and 2 h ($p < 0.015$), as well as inhibition of lipid peroxidation at 2 h ($p < 0.01$) postconsumption.

KEYWORDS: Antioxidant; anti-inflammatory; lipid peroxidation; cell-based antioxidant protection assay (CAP-e); oxygen radical absorbance capacity (ORAC) assay; açai, *Euterpe oleracea*; fruit juice; thiobarbituric acid reactive substances assay (TBARS)

INTRODUCTION

Reactive oxygen and nitrogen species play key roles in normal physiological processes, including cellular life/death processes, protection from pathogens, various cellular signaling pathways, and regulation of vascular tone (*1*). Oxidative stress is caused by an insufficient capacity of biological systems to neutralize excessive free radical production, which can contribute to human diseases and aging (*2*), including cardiovascular disease (*3*), neurodegenerative disease and age-related cognitive decline (*4*), obesity and insulin resistance (*5*), as well as immune system dysfunction (*6*). Oxidative stress also contributes to the accumulation of damaged macromolecules and organelles, including mitochondria (*4, 7*).

The antioxidant capacity of foods, juices, and teas has been linked to in vivo protection from oxidative stress in numerous studies. A recent study assessed the increase in plasma antioxidant capacity after the consumption of either an antioxidant-poor meal or the same antioxidant-poor meal with the addition of a known quantity of whole fruits added (*8*). This study

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showed that the consumption of an antioxidant-poor meal results in a decrease in plasma antioxidant capacity and that adding fruits to the same meal not only prevented this decrease but also led to an increase in antioxidant capacity. Furthermore, the study is important because it addresses fruits and juices consumed in normal amounts, in contrast to studies of highly enriched extracts or purified compounds.

There is a diverse array of methods currently employed for testing the antioxidant capacity of various foods and natural products. Novel research is currently directed toward possible therapies aimed at reversing the decline in mitochondrial ATP production and increased production of reactive oxygen species (ROS) (*3, 4*). For the chemical estimation of antioxidants in foods, the oxygen radical absorbance capacity (ORAC) test has been proposed as a preferred assay with possible relevance to human biology (*9*). The assay has been used for the assessment of total antioxidant capacity in human serum (*10*) and antioxidant uptake after the consumption of fruits and berries (*8*). We have developed a validated cell-based antioxidant protection assay using human erythrocytes (CAP-e), which we have used to document antioxidant capacity both in antioxidant-rich natural products and in serum obtained after the consumption of such products (*11, 12*).

The proprietary fruit and berry juice blend (JB) MonaVie Active contains the Amazonian palm fruit, açai (*Euterpe oleracea* Mart.) as the predominant ingredient (*13*), along with lesser amounts of the following fruits and berries in descending order of dominance: white grape (*Vitis* L.), Nashi pear (*Pyrus pyrifolia*), acerola (*Malpighia glabra*), aronia (*Aronia melanocarpa*), purple grape (*Vitis* L.), cranberry (*Vaccinium macrocarpon*), passion fruit (*Passiflora edulis*), apricot (*Prunus armeniaca*), prune (*Prunus* L.), kiwifruit (*Actinidia deliciosa*), blueberry (*Vaccinium* L.), wolfberry (*Lycium barbarum*), pomegranate (*Punica granatum*), lychee (*Litchi chinensis*), camu camu (*Myrciaria dubia*), pear (*Pyrus* L.), banana (*Musa acuminata*), and bilberry (*Vaccinium myrtillus*).

This study was undertaken to examine whether the antioxidants and anti-inflammatory compounds known to be present in the individual components of the unprocessed ingredients present in the JB are in a form able to enter into and protect human cells in vitro. Furthermore, the study also investigated the bioavailability of these compounds following ingestion of the JB and its effect on serum indicators of oxidative damage.

MATERIALS AND METHODS

Chemicals and Apparatus. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and fluorescein (sodium salt) (Fl) were obtained from Aldrich (Milwaukee, WI). Randomly methylated β-cyclodextrin (Trappsol) (Pharm grade) (RMCD) was obtained from Cyclodextrin Technologies Development Inc. (High Springs, FL). Folin−Ciocalteu's phenol reagent, sodium carbonate, sodium hydrogencarbonate ($NaHCO_3$), Sephadex LH-20, formic acid, gallic acid, and all other phenolic acids were purchased from Sigma (St. Louis, MO). Potassium phosphate dibasic (K_2HPO_4) and potassium phosphate monobasic (KH_2PO_4) were obtained from VWR (West Chester, PA). Standards of 3-*O*-β-glucosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (six mixed anthocyanin standards, HPLC grade) were obtained from Polyphenols Laboratories (Sandnes, Norway). Methanol, acetonitrile, methylene chloride, and acetic acid (HPLC grade) were from Fisher (Fair Lawn, NJ). Phosphate-buffered saline, hydrogen peroxide, and RPMI-1640 cell culture medium were purchased from Sigma-Aldrich (St. Louis, MO). DCF-DA was from InVitrogen (Carlsbad, CA). Flow cytometry was performed using a FacsCalibur flow cytometer (Becton-Dickinson, San Jose, CA), and fluorescence reading of 96-well microtiter plates was performed using a TECAN SpectraFluor plate reader (TECAN US, Durham NC).

Study Overview. This study was conducted in three stages. Initial testing was performed to evaluate the phytochemical contents of the JB, as well as the antioxidant capacity of the JB in cell-based assays in vitro. The second stage was a pilot study with five healthy adults who were tested on a single study day, to identify the time course for antioxidant absorption and bioactivity of the JB in vivo. Blood samples were collected immediately prior to consumption of 120 mL (4 oz) of the JB and at 30, 60, and 120 min following consumption. The selection of these time points was based on previous studies using pomegranate juice (*14*). The third stage was a randomized, double-blinded, placebo-controlled, crossover study of 12 healthy adults ranging from 19 to 52 years of age, in which all 12 study participants were tested on both the placebo and JB on different days. Serum antioxidant and lipid peroxidation levels were compared at baseline and in response to the placebo or JB in a *within subjects* study design.

Polyphenols and Antioxidant Capacities of JB. *Sample Preparation.* Ten milliliters of the JB was centrifuged at 4000*g* for 10 min. Supernatant was harvested and extracted by hexane twice (5 mL × 2) to separate lipophilic components. The hexane layer was used to measure lipophilic ORAC, whereas the aqueous layer was used for phytochemical analysis, hydrophilic ORAC, and total phenolic measurements.

Phytochemical Analysis. One milliliter of aqueous solution was filtered using a 0.22 μm Teflon syringe filter (Cameo, MN) for qualitative and quantitative analysis of ACN analysis following the method reported previously (*15*). Five milliliters of aqueous solution was fractioned by Sephadex LH-20 for proanthocyanidin analysis following the published method for proanthocyanidin analysis and quantization (*16*). Phenolic acids analysis was carried out using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) coupled to the 4000 Q TRAP mass spectrometer (Applied Biosystems, Foster City, CA) according to a recently published method (*17*).

ORAC Assay. The hydrophilic and lipophilic $ORAC_{FL}$ assays were carried out on a FLUOstar Galaxy plate reader (BMG Labtechnologies, Durham, NC), based on published procedures (*18, 19*). The results are expressed as micromoles of Trolox equivalents per milliliter of juice (μmol TE/mL).

Total Phenolic Assay. The aqueous layer was subjected to total phenolics measurement by Folin−Ciocalteu reagent according to the method of Wu et al. (*18*) The results are expressed as milligrams of gallic acid equivalents per milliliter of juice (mg GAE/mL).

Consumable Test Products: Preparation for in Vitro and in Vivo Testing. Five 750 mL bottles from the same batch of JB were received from the manufacturer. Because many antioxidants are degraded by exposure to light and air, a new bottle was opened on each clinical study day to ensure a consistent antioxidant concentration. The in vivo pilot study was performed to examine any possible effect of juice consumption. Therefore, a dose of 120 mL was chosen. This dose is at the high end of the manufacturer's recommended daily dose of 60−120 mL and has been mentioned in testimonials of consumers as supporting a generalized sense of well-being and relieving symptoms of an inflammatory nature.

For the in vitro cell-based studies, one bottle of JB was opened under sterile conditions and aliquoted into a series of vials. Care was taken to fill each vial to a maximum, to minimize exposure to oxygen and thus to prevent potential oxidation of the compounds during the storage period. The vials were stored at 4 °C and protected from light until required for in vitro testing. On each in vitro test day a vial of JB was prepared for addition to cell cultures by removal of solids and nonaqueous compounds by standard laboratory procedures, including centrifugation and filtration using a cellulose acetate syringe filter. Serial dilutions were then prepared in phosphate-buffered saline (PBS) and were used only on the day of preparation.

The in vitro antioxidant assays were performed using the sterile filtrate, whereas the clinical study evaluated ingestion of the whole juice. The filtration removes solids and lipid agglomerates, which removes the antioxidants responsible for the $ORAC_{lipophilic}$ value from the test product while retaining water-soluble compounds that are responsible for the $ORAC_{hydrophilic}$. Dry weight assessment of the amount of dissolved material to be added to cell cultures found that 100 mL of the JB contains 40% solids, which were removed by centrifugation

and filtration prior to addition to the cell cultures. The filtrate contained 100 g/L of dissolved material. Taking the 40% solids into account, unaltered JB contains 60 g/L of dissolved material. Thus, consumption of 120 mL of the whole juice is equivalent to 7.2 g of dissolved material.

The JB is a complex, robustly flavorful, dark purplish liquid, with a substantial amount of pulp, giving the juice a characteristic appearance that is very difficult to reproduce for placebo purposes. Therefore, we decided against developing a liquid placebo. An encapsulated placebo product was created, and study participants were purposefully left uncertain about whether they were being fed a new test product or a placebo. The placebo was prepared by mixing white potato flakes, which in contrast to some other types of potato flakes do not increase serum antioxidant levels (*20*), with a purplish food-coloring blend. The mix was redried, ground, and put into vegetable-based capsules. Two capsules containing 0.5 g each were consumed by the study participants.

Purification of Peripheral Blood Cell Subsets for in Vitro Testing. Peripheral venous blood for the in vitro work was obtained from healthy human volunteers between 19 and 52 years of age after informed consent was obtained and upon approval by the Sky Lakes Medical Center Institutional Review Board (FWA 2603). Blood samples were obtained using a sterile technique and processed immediately. The whole blood was layered onto a Histopaque 1119 density gradient. Following centrifugation, the polymorphonuclear (PMN) cell fraction and the erythrocyte fraction were harvested and washed. The PMN cells were used for testing of ROS formation and migration, and the erythrocytes were used for the CAP-e assay (see below).

Cell-Based Antioxidant Protection of Erythrocytes (CAP-e) Assay. The CAP-e assay (*11, 12*) utilizes human red blood cells as a model for antioxidant uptake and protection of a simplistic, noninflammatory cellular model that is intimately involved in redox processes in vivo. The CAP-e assay was used to evaluate the antioxidant capacity of JB in vitro and in vivo. For the in vitro assay, serial dilutions of JB were added to the assay. For testing antioxidant uptake in vivo, serum samples obtained before and after consumption of either the JB or placebo were added to the cells.

Purified red blood cells from a type O+ donor were washed extensively in physiological saline and stored at 4 °C. Red blood cells were distributed in triplicate in 96-well V-bottom microplates (NUNC, Roskilde, Denmark) and pretreated with either the JB or serum from the subjects receiving either the placebo or JB. Repeated washing ensured that any antioxidant compounds not absorbed by the cells were removed prior to analysis. The cells were stained with DCFDA—a probe that becomes fluorescent with free radical damage. One triplicate set of wells was left untreated as a control to record the background level of oxidation in the red blood cells. All other wells were treated with hydrogen peroxide, which penetrates the cells and triggers severe intracellular oxidative damage. The fluorescence intensity of the cells was analyzed by flow cytometry, and the subsequent data analysis was performed using FlowJo software (TreeStar Inc., Ashland, OR).

Evaluation of Reactive Oxygen Species (ROS) Formation by PMN Cells. Evaluation of the inhibition by the JB of ROS production was done using freshly purified human PMN cells, as previously described (*21, 22*). In brief, PMN cells were incubated at 37 °C and 5% CO_2 for 90 min, either untreated or treated with serial dilutions of JB. The PMN cells were washed twice in PBS and resuspended in the precursor dye DCF-DA (Molecular Probes, Eugene, OR) and incubated for 1 h at 37 °C. All samples, with the exception of the negative controls, were then exposed to hydrogen peroxide for 45 min to induce ROS formation. Samples were washed twice in PBS to remove the peroxide, transferred to cold RPMI 1640, and stored on ice. The intensity of ROS-induced DCF-DA fluorescence was analyzed immediately by flow cytometry, as described above for the CAP-e assay. The mean fluorescence intensity (MFI) of PMN cells was compared between untreated, hydrogen peroxide-treated, and JB-pretreated cells. A reduction in MFI in samples pretreated with the JB prior to challenge with hydrogen peroxide would indicate that the JB mediated a reduction in ROS production.

PMN Migration in Vitro. The evaluation of random and directed PMN migration in vitro was performed using Millipore double-chamber migration 96-well culture plates; the top and bottom chambers are separated by a 3 μm porous filter. PMN cells were plated at 10^6/mL in the top chambers, with or without the addition of serial dilutions of JB. The bottom chambers contained either culture medium alone to assess random migration or one of the following three different pro-inflammatory chemoattractants: bacterial peptide f-Met-Leu-Phe (fmlp), leukotriene B4 (LTB4), or interleukin-8 (IL-8) to assess directed migration. All culture conditions were performed in triplicate. Plates were incubated for 18 h at 37 °C and 5% CO_2 to allow for PMN migration from the top into the bottom chambers. The following day the top chambers were removed, and the cells in the bottom chambers were transferred to V-bottom 96-well plates and stained with CyQuant (Molecular Probes). The fluorescence intensity, proportional to the numbers of cells that had migrated from the top to the bottom wells, was measured using a TECAN SpectraFluor plate reader.

In Vivo Testing of Antioxidant Uptake and Lipid Peroxidation. *General.* The study was approved by the Sky Lakes Medical Center Institutional Review Board (FWA 2603). Recruitment of study participants included an initial interview by a nurse to evaluate whether each volunteer met the inclusion/exclusion criteria. Volunteers who met these criteria were scheduled for a medical examination. Subjects who met the requirements of the medical examination were enrolled in the study. Exclusion criteria were as follows: under 18 or over 55 years of age; pregnancy; severe stress; asthma and/or allergies requiring daily medication; any known chronic illness; obesity, smoking; recreational drug use; impaired digestive function (including previous major gastrointestinal surgery); regular consumption of the JB or other products with a high concentration of known antioxidants. A total of 19 study subjects were interviewed. Two subjects were excluded due to obesity, and one was excluded on the basis of a high daily antioxidant intake. Two subjects were unable to participate due to their work schedule. Of the remaining 14, 7 participated in the pilot study and 12 participated in the clinical trial.

Pilot Study. The pilot study involved seven study participants, of which two were excluded from analysis due to incomplete blood sampling. Thus, the analysis of the pilot study was based on five study participants from whom repeat blood draws were performed. Two methods for the evaluation of the antioxidant capacity of the serum samples were employed: (1) the ORAC assay and (2) the CAP-e assay. Samples from the initial pilot study of five subjects were shipped to Brunswick Laboratories (Norton, MA) for serum ORAC analysis. Samples were tested in serial dilutions and plotted onto a standard curve to acquire the data expressed as Trolox equivalents. An average was made based on the data from each serial dilution that was within the range of the standard curve. Because the ORAC testing did not result in a trend toward increased antioxidant activity, it was not used in the subsequent randomized controlled trial. Instead, the thiobarbituric acid reactive substances (TBARS) test was utilized for the reasons discussed below. Testing of the serum samples in the CAP-e assay showed an increase in serum antioxidants following JB consumption. Therefore, the CAP-e assay was utilized as well for the randomized controlled trial.

Randomized, Placebo-Controlled Crossover Clinical Trial. Twelve volunteers were scheduled for two study days at least one week apart. On each study day, the participants spent approximately 3 h at the clinic, including filling in initial daily intake questionnaires, baseline blood draws, consumption of the test product, and subsequent blood sample collection. The study participants were instructed to avoid vigorous exercise for a period of 24 h prior to arriving at the clinic. They were also instructed to eat a light meal and to avoid meat, fruits, or greens, as well as to abstain from consuming alcohol, coffee, or melatonin the night before. Subjects were scheduled to arrive at the clinic following an overnight fast and were instructed to consume no food, vitamins, or other nutritional supplements the morning of each study day. A daily intake questionnaire was used to track last meal, last snack, and amount of exercise within the past 24 h, to ensure that volunteers were in compliance. Sample collection was performed at the same time of the day (7:00−10:00 a.m.) to minimize the confounding effect circadian fluctuations may present on metabolic function. Upon arrival to the clinic, the study participants were allowed to consume water as needed. On each study day, volunteers were instructed to complete a questionnaire aimed at determining any exceptional stress-related circumstances

that might affect them, as well as to provide for the tracking of any adverse events or sickness during the past week. After completion of the questionnaire, a baseline blood sample was drawn. Immediately after collection of the baseline sample, 120 mL of the JB was provided for consumption. Blood samples were subsequently drawn at 1 and 2 h after ingestion of the test item. At each time point, 6 mL of blood was drawn into serum separator vials. The vials were placed at room temperature for 30 min to allow for complete coagulation. The vials were centrifuged at 400*g* for 10 min, and the serum was pipetted into multiple aliquots, which were frozen for later testing of antioxidant status. The serum antioxidant status was assessed using the CAP-e assay as described above. The extent of lipid peroxidation in the serum was determined using the TBARS assay. The TBARS assay is a well-established method for screening and monitoring lipid peroxidation (*23*). A commercially available kit for this assay was used according to the instruction manual from the manufacturer (Cayman Chemical Co., Ann Arbor, MI).

Statistical Analysis. For the in vitro antioxidant testing, a simple comparison of arithmetic means was performed using Student's *t* test, using Microsoft Excel (Microsoft, Redmond, WA). Probabilities (p) based on the statistics were computed as the tail area of the *t* distribution using the two-tailed *t* test, at a 5% level of significance. For the in vivo study of antioxidant uptake and the effect on lipid peroxidation, the normalized data for each person and each time point after placebo consumption were subtracted from the same data set after JB consumption. Statistical significance was performed using Student's *t* test. Statements regarding whether a given mean value differed from a reference mean value were evaluated by two-tailed independent as well as paired *t* tests and were supported if $p < 0.05$. To evaluate whether there was greater antioxidant uptake after JB consumption compared to placebo, a two-within factor and repeated measures analysis of variance (ANOVA) was used, where treatment (JB or placebo) was one within-group factor and time of blood draw was the other. The α levels were set at 0.05 with the two-tailed tests. Analysis was performed with SPSS version 12.5 (SPSS, Chicago, IL).

RESULTS

Polyphenols and Antioxidant Capacities of JB. Major polyphenol compounds including anthocyanins, proanthocyanidins, and phenolic acids, along with antioxidant capacities measured by ORAC and total phenolics, are presented in **Table 1**. Compared to freeze-dried açai (*13*), the polyphenol profile of the JB is different. However, certain key polyphenols, such as the anthocyanins cyanidin 3-glucoside and cyanidin 3-rutinoside, appeared as major anthocyanins in the JB as they are in the freeze-dried berry. The total antioxidant capacity of the JB measured by ORAC is 22.8 μmol TE/mL, which is higher than the range of ORAC values for most common juices (*18, 24*).

In Vitro Cell-Based Antioxidant Protection of Erythrocytes (CAP-e) Assay. The JB showed a clear dose-dependent antioxidant protection effect in the CAP-e assay, indicating that compounds in th JB are able to cross the plasma membrane of living cells and, subsequently, are able to provide significant protection from oxidative damage within the cells (**Figure 1**). The data were highly significant. At a concentration of 10 g/L, the p value was <0.001. Even at the lowest dose of JB (0.016 g/L) the antioxidant protection of the cells was statistically significant ($p < 0.045$).

Inhibition of Reactive Oxygen Species (ROS) Formation by Polymorphonuclear (PMN) Cells. PMN cells are highly reactive immune cells capable of producing ROS immediately upon stimuli. ROS production can be inhibited by antioxidants available to enter live cells and can also be further inhibited by anti-inflammatory compounds in a complex product. The pretreatment of PMN cells by the JB resulted in inhibition of th formation of ROS by the PMN cells (**Figure 2**). Untreated

Table 1. Phytochemical Analysis of the Juice Blend

		μg/mL of juice
proanthocyanidins	total	0.472005
	1-mer	0.0118
	2-mer	0.001255
	3-mer	0.0108
	4-mer	0.00565
	polymer	0.4425
anthocyanins	cyanidin 3-diglycoside	57.3
	cyanidin 3-sambubioside	10.2
	cyanidin 3-glucoside	26.5
	cyanidin 3-arabinoside	13.3
	cyanidin 3-rutinoside	69.9
phenolic acids	total	1.50
	protocatechuic acid (3,4-OH BA)	0.58
	caffeic acid (3,4-OH CA)	0.20
	3-(4-hydroxyphenyl)propionic acid (4-OH PPA)	0.41
	p-coumaric acid (3-OH CA)	0.31

	mg GAE[a]/mL of juice
total phenolics	1.48

		μmol TE[b]/mL of juice
ORAC	H-ORAC	22.2
	L-ORAC	0.61

[a] GAE, gallic acid equivalents. [b] TE, Trolox equivalents.

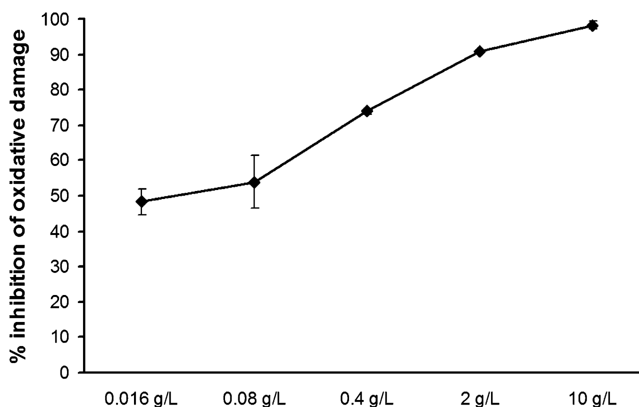

Figure 1. Antioxidant capacity of the JB evaluated in vitro, using the CAP-e assay. Red blood cells were used as a cellular model for evaluating the ability of antioxidants to cross the plasma membrane into living cells. Red blood cells were exposed to serial dilutions of the JB and subsequently exposed to an oxidative challenge. The graph shows the percent inhibition of oxidative damage. The treatment of cells with the JB resulted in statistically significant protection from oxidative damage over a broad range of dilutions, from the highest concentration of 10 g/L in cell culture ($p < 0.001$) to the lowest concentration tested ($p < 0.045$).

PMN cells (no JB, no H_2O_2) served as a baseline, and PMN cells treated with H_2O_2 in the absence of JB served to show maximum ROS production. PMN cells treated with the JB produced fewer ROS than cells treated with H_2O_2 in the absence of JB. The inhibition of ROS formation was dose-dependent, and at the highest dose of JB tested (1 g/L), the inhibition was highly significant ($p < 0.003$).

Effects on PMN Migration toward Inflammatory Stimuli. JB treatment of PMN cells showed differential effects under different culture conditions. The PMN response to JB treatment resulted in nonlinear, complex dose-responses for random

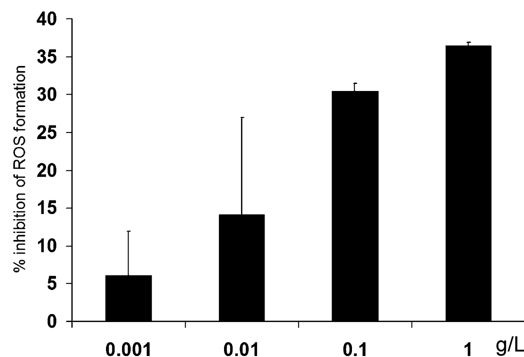

Figure 2. Capacity of the JB to reduce formation of ROS by PMN cells tested in vitro. PMN cells were exposed to serial dilutions of the JB and then challenged by H_2O_2 to produce ROS. Untreated cells (no JB, no H_2O_2) served as negative controls. The graph shows the percent inhibition of ROS formation as calculated means ± standard deviations of triplicate samples, compared to the negative and positive controls. A clear dose-dependent inhibition of ROS formation was caused by the JB. The effect was statistically significant with $p < 0.003$ at the highest dose of JB. All tests were performed in triplicate and repeated three times using cells from different donors.

migration (**Figure 3a**) as well as migration toward the bacterial peptide fmlp (**Figure 3b**). At the dose of 0.1 g/L, the JB significantly enhanced PMN random migration ($p < 0.05$). At the dose of 1 g/L, the PMN migration toward fmlp was significantly inhibited ($p < 0.001$). In contrast, the JB treatment of PMN cells showed a less complex, more linear, dose-response when PMN cells migrated toward two pro-inflammatory compounds made during inflammation, leukotriene B4 (LTB4, **Figure 3c**) and interleukin-8 (IL-8, **Figure 3d**). At the highest JB dose tested (1 g/L), significant inhibition of PMN migration was observed toward LTB4 ($p < 0.05$) and IL-8 ($p < 0.03$).

Pilot Study in Vivo. During an initial exploratory phase prior to the full study, the total antioxidant status in serum was assayed using both the ORAC and CAP-e assays. Whereas the CAP-e assay showed an increase in the antioxidant capacity in all five of the subjects, the ORAC test did not ($p < 0.2$, at 2 h postconsumption). On the basis of these findings the ORAC assay was not used in the full study and instead the TBARS test was employed.

In Vivo Protection from Oxidative Damage—Serum Antioxidant Status. The randomized, placebo-controlled trial involving 12 people in a within-subject design was performed as outlined in **Figure 4**. The description of the study population is shown in **Table 2**. Consumption of the JB resulted in an increase in the serum antioxidant capacity within 2 h of consumption in 11 of 12 study participants. The antioxidant capacity was tested using the CAP-e assay for serum samples obtained at baseline and at 1 and 2 h postconsumption (**Figure 5**). The increase in serum antioxidant capacity was statistically significant both at 1 h ($p < 0.027$) and at 2 h ($p < 0.015$) postconsumption. When a paired t test was performed on the normalized data from each person's response to placebo versus JB, the significance at 2 h postconsumption was even stronger ($p < 0.01$).

In Vivo Protection from Oxidative Damage—Serum Lipid Peroxidation Status. Consumption of the JB resulted in a decrease in serum lipid peroxidation within 2 h of consumption in 10 of the 12 study participants (**Figure 6**). The level of lipid peroxidation in the sera was evaluated using the TBARS test. The paired analysis, that is, the individual changes for each study participant consuming either JB or placebo, showed that the decrease in lipid peroxidation had not reached statistical

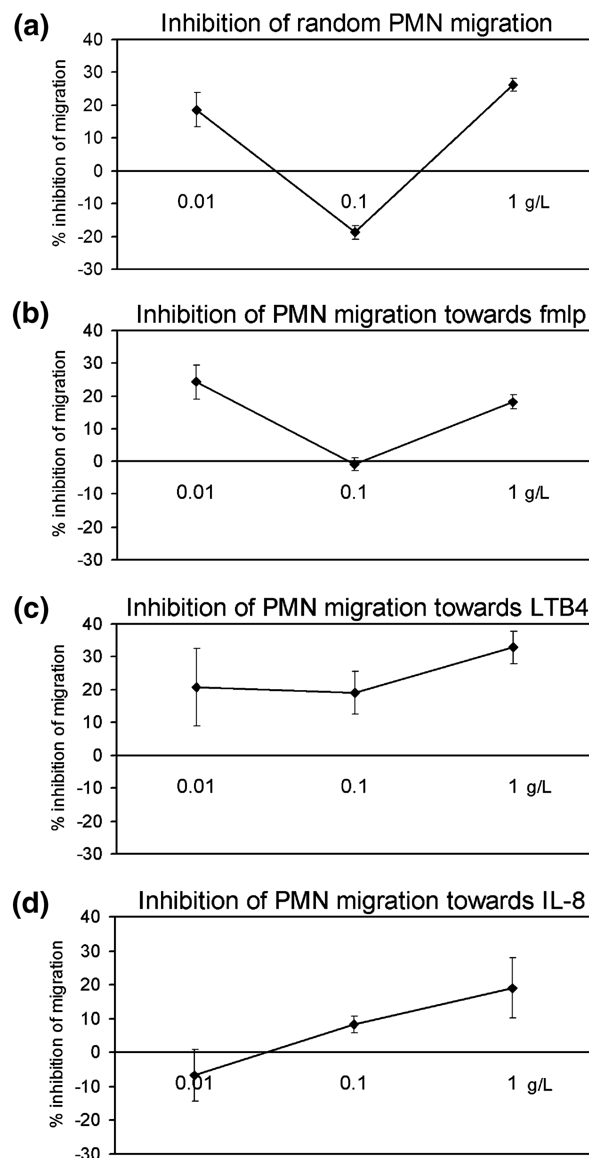

Figure 3. Treatment of PMN cells with the JB in vitro altered PMN migration under four different test conditions: random migration (**a**); and directed migration toward three chemoattractants bacterial peptide fmlp (**b**), LTB4 (**c**), and IL-8 (**d**). The graphs show percent inhibition of PMN migration as the mean ± standard deviations. At a dose of 1 g/L JB, random PMN migration was inhibited ($p < 0.05$), whereas at 0.1 g/L JB, random PMN migration was increased ($p < 0.05$). At the dose of 1 g/L JB, the PMN migration toward fmlp was significantly inhibited ($p < 0.001$). In contrast, pro-inflammatory, directed chemotaxis was inhibited for both inflammatory markers LTB4 ($p < 0.05$) and IL-8 ($p < 0.03$). All test conditions, serial dilutions, and positive and negative controls were performed in triplicates. The testing was repeated three times with cells from different donors.

significance at 1 h postconsumption ($p < 0.15$), but did achieve statistical significance at 2 h ($p < 0.01$). Analysis of the variance (ANOVA) of the repeated measures showed statistical significance for the treatment effect ($p < 0.02$).

The antioxidant uptake was correlated with the decrease in lipid peroxidation in vivo. A 45% correlation was observed between the increased antioxidant capacity in the serum and the reduction in lipid peroxidation at 2 h postconsumption (**Figure 7**).

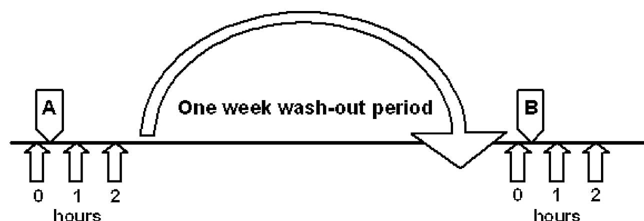

Figure 4. Schematic diagram of the randomized, placebo-controlled crossover study design for the in vivo study. Each of the 12 volunteers participated on two different clinic days, one week apart to allow for wash-out of active constituents. On each clinic day, a baseline blood draw was performed (0 h), and a consumable “A” or “B” was ingested. Two more blood samples were drawn at 1 and 2 h postconsumption. Volunteers were randomized to receive either placebo or JB on the first versus second clinic day (see also **Table 2**).

Table 2. Description of the Study Population

	males	females	total study population
average age (years)	25.5 ± 11.1	42.3 ± 13.8	33.9 ± 5.5
age (range)	19−48	20−52	19−52
height (cm)	177.8 ± 14.5	164.3 ± 7.3	171.0 ± 13.0
weight (kg)	79.5 ± 4.8	72.0 ± 13.5	75.7 ± 10.3
body mass index	25.6 ± 6.2	26.8 ± 5.2	26.3 ± 5.5
randomization: JB then PL	3	3	6
randomization: PL then JB	3	3	6

DISCUSSION

The study reported here was performed to evaluate whether the antioxidants present in the antioxidant-rich fruit and berry juice blend (JB) are capable of protecting living cells from oxidative damage in vitro, as well as to evaluate their bioavailability and biological effect in vivo. Using the CAP-e assay (*11*) to evaluate the activity of antioxidants in a noninflammatory cell-based system, it was determined that a significant amount of the antioxidants found in the JB are available to living cells, are capable of penetrating the plasma membrane of the cells, and able to protect the cells from intracellular oxidative damage in vitro.

The JB showed anti-inflammatory effects in several in vitro assays using the inflammatory PMN cell. JB treatment of PMN cells showed both a reduction in ROS formation and altered migratory behavior. In particular, the effect on PMN migration indicated altered cellular behavior beyond a simple contribution of antioxidants. PMN migration involves both random and directed migration behavior. Random migration reflects PMN cell scavenging for invaders as part of normal immune surveillance. In contrast, directed migration toward a chemoattractant involves migration toward and into sites of inflammation. It is biologically relevant to distinguish the effects of natural products on our innate defense mechanisms, such as random migration and migration in response to bacterial invaders, as opposed to inflammatory mediators made by the host. Therefore, it is of

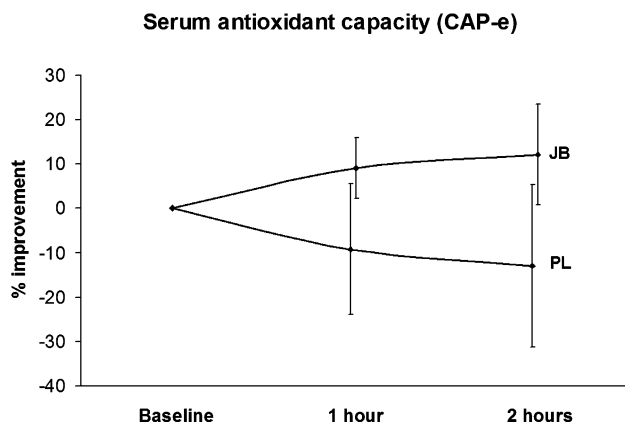

Figure 5. Consumption of JB resulted in an increase in antioxidants available to living cells. Serum samples obtained at baseline and 1 and 2 h after consumption of either JB or placebo (PL) were tested in the CAP-e assay. Data from each person per test day were normalized to examine changes in antioxidant capacity for each person over time after consumption of either JB or PL. The average and standard deviations for each group of data are shown for JB and PL. The difference in serum antioxidant capacity between JB and PL was statistically significant both at 1 h ($p < 0.03$) and 2 h ($p < 0.015$) postconsumption.

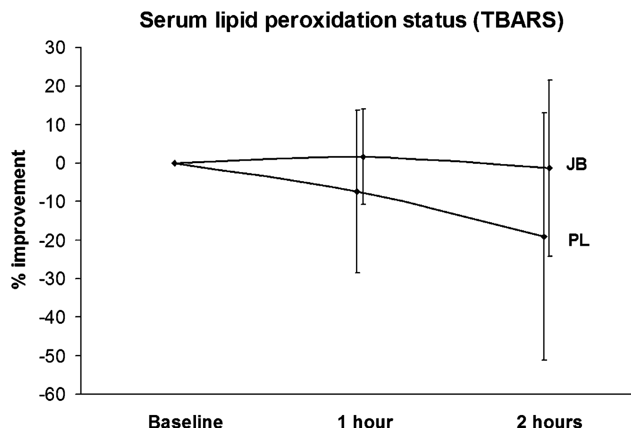

Figure 6. Consumption of JB resulted in a decrease in lipid peroxidation in vivo. Serum samples obtained at baseline and 1 and 2 h after consumption of either the JB or placebo (PL) were tested using the TBARS assay. Data from each person per test day were normalized to examine changes in antioxidant capacity for each person over time after consumption of either JB or PL. The average and standard deviations for each group of data are shown. The difference between JB and PL did not reach statistical significance at 1 h ($p < 0.11$) or 2 h ($p < 0.22$). However, when paired analysis was performed, by which each person’s response to JB was compared to the same person’s response to PL, a significant reduction of lipid peroxidation in the serum was observed at 2 h postconsumption ($p < 0.01$).

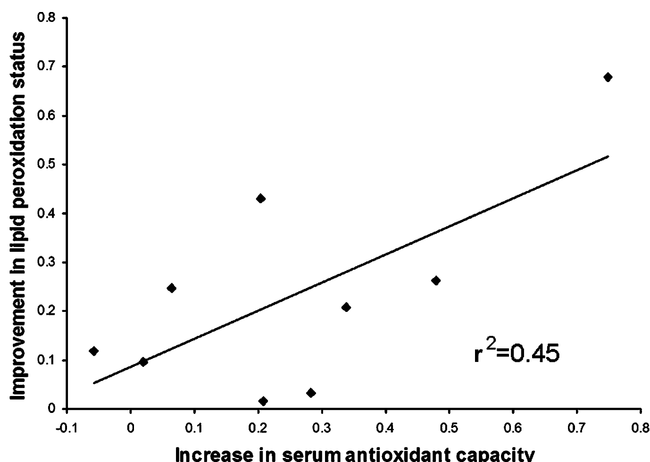

Figure 7. Comparison of the postconsumption reduction in lipid peroxidation and the increase in antioxidants capable of crossing the plasma membrane of living cells. The individual difference between a subject’s levels of lipid peroxidation after consumption of JB versus placebo was plotted against the same individual’s differences in serum antioxidant status. A linear trend line showed a 45% correlation coefficient between the improved serum antioxidant levels as measured by the CAP-e assay and the improvement in lipid peroxidation status as measured by the TBARS assay on serum samples collected 2 h postconsumption.

interest that JB treatment of freshly purified human PMN cells in vitro affected random and fmlp-directed migratory behavior differently from PMN migration toward the inflammatory mediators LTB4 and IL-8. We speculate that the anti-inflammatory properties of JB in vivo may allow normal immune surveillance while at the same time reducing inflammatory conditions.

On the basis of the preliminary in vitro data, the in vivo investigation of JB was performed. Consumption of the JB resulted in an overall increase in the antioxidant capacity of serum collected from the study subjects when compared to the placebo group, as measured by the CAP-e assay, but the trend was not as clear for the serum ORAC test. Because the CAP-e assay is cell-based, it is possible that some antioxidants from JB were able to accumulate and be retained in the cells, thus providing a more sensitive testing system, in contrast to the chemical ORAC assay. Therefore, the CAP-e assay was chosen for the subsequent randomized placebo-controlled crossover study.

The increase in serum antioxidant status upon consumption of JB was more clearly observed when the data from consumption of the JB were compared with the same study subject's data from placebo consumption in a paired statistical analysis. There was no evidence of a placebo effect in this study.

In only a few of the study subjects did the antioxidant capacity remain consistent with baseline values after consumption of the placebo. A slight increase in oxidative damage was observed in approximately 50% of the participants (i.e., a gradual decrease in antioxidant capacity) over the 2 h following consumption of the placebo. ROS are produced as a consequence of normal aerobic metabolism. We speculate that the observed increase in the potential for inducing oxidative damage in some of the study participants receiving the placebo may reflect a natural decline in their antioxidant status due to the overnight fast and increased depletion of food-derived antioxidants prior to the morning blood draws. Thus, by performing paired analysis, the decrease in antioxidant capacity as a result of fasting was taken into account when the same participant's response to JB consumption was analyzed.

Consumption of the JB resulted in an increase in serum antioxidant compounds that are able to enter living cells and protect them from oxidative damage in 11 of 12 study subjects, as evaluated by applying the serum samples to the CAP-e assay.

A decrease in lipid peroxidation in the sera of 11 of the 12 participants was observed 2 h after consumption of the JB, as demonstrated by comparing the normalized differences between the results of the TBARS data on sera collected 2 h following consumption of either the JB or placebo. This suggests that a rapid reduction in lipid peroxidation may occur in vivo within a 2 h period after consumption of the JB.

In conclusion, ingestion of the JB demonstrated a substantial antioxidant capacity for protecting cells from oxidative damage in vitro in this study. Furthermore, JB consumption led to a rapid increase in serum antioxidants, as measured by the cell-based assay for protection from oxidative damage. JB consumption also resulted in a statistically significant decrease in serum lipid peroxidation within 2 h of consumption. This effect is likely due to increased serum antioxidant capacity.

Antioxidant consumption, along with anti-inflammatory treatment, is being critically evaluated as a potential strategy for reversal of disease progression (*25*). It has been suggested that the apparent failure of multiple larger clinical trials to document reversal of disease processes may be linked to the choice of antioxidants. In particular, the frequent use of vitamins C and E in such studies may be due to availability and low cost, but may not have been the best choice, as both vitamins have pro-oxidant capacity as well (*26*). In contrast, studies on polyphenols may be much more promising, and may be more relevant, as these types of antioxidants are the most abundant in a health-conscious diet (*27−29*). Given the high content of certain specific polyphenols in the JB, the increased antioxidant protection in vivo after consumption of the JB, and the anti-inflammatory capacity in vitro, further research is warranted to evaluate whether JB consumption may provide reversal of risk markers in subjects with conditions such as arthritis, obesity, chronic viral diseases, cardiovascular disease, and compromised cognitive function as well as other conditions associated with chronic inflammation.

ABBREVIATIONS USED

CAP-e, cell-based antioxidant protection of erythrocytes; fmlp, bacterial peptide f-Met-Leu-Phe; GA, gallic acid; IL-8, interleukin-8; LTB4, leukotriene B4; ORAC, oxygen radical absorbance capacity assay; PMN, polymorphonuclear cells; RBC, red blood cell; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances assay; TE, Trolox equivalents.

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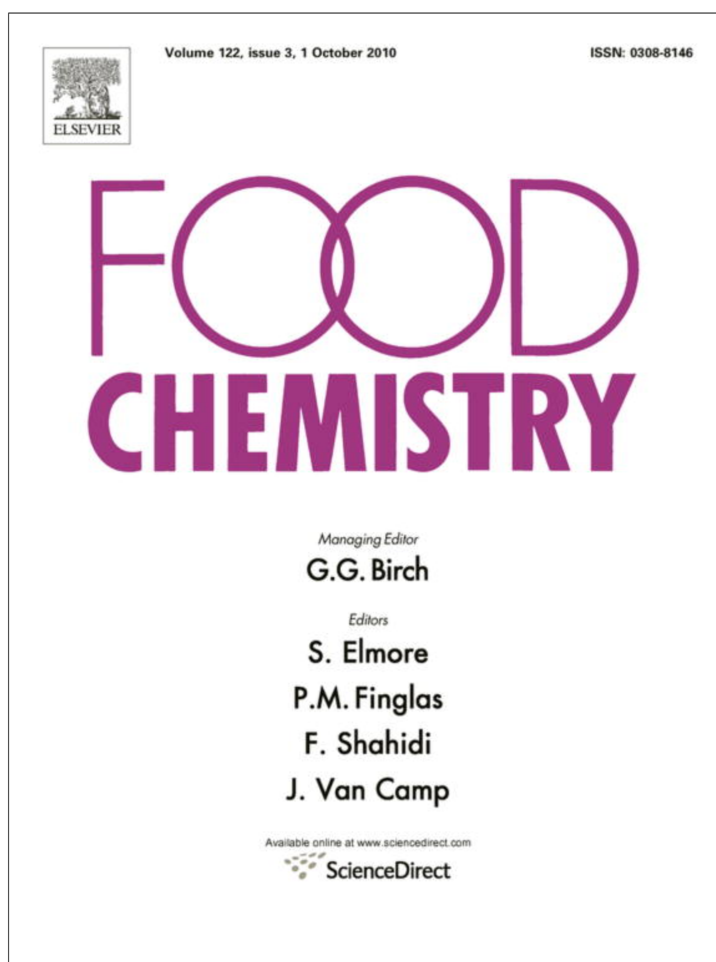

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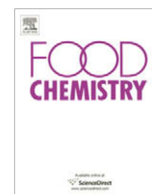

Anti-oxidant capacities of flavonoid compounds isolated from acai pulp (*Euterpe oleracea* Mart.)

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ABSTRACT

Acai fruit (*Euterpe oleracea* Mart.) has been demonstrated to exhibit extremely high anti-oxidant capacity. Seven major flavonoids were isolated from freeze-dried acai pulp by various chromatographic methods. Their structures were elucidated as orientin (**1**), homoorientin (**2**), vitexin (**3**), luteolin (**4**), chrysoeriol (**5**), quercetin (**6**), and dihydrokaempferol (**7**) by NMR, MS and compared with the reported literature. Compounds **3** and **6** were reported from acai pulp for the first time. Anti-oxidant capacities of these flavonoids were evaluated by oxygen radical absorbance capacity (ORAC) assay, cell-based anti-oxidant protection (CAP-e) assay and reactive oxygen species (ROS) formation in polymorphonuclear (PMN) cells (ROS PMN assay). ORAC values varied distinctly (1420–14,800 μmol TE/g) among the seven compounds based on numbers and positions of hydroxyl groups and/or other substitute groups. The ORAC values of aglycones are generally higher than that of glycosides. CAP-e results indicated that only three compounds (**4**, **6** and **7**) could enter the cytosol and contribute to the reduction of oxidative damage within the cell. The ROS PMN assay showed that five compounds (**2**–**3** and **5**–**7**) demonstrated exceptional effects by reducing ROS formation in PMN cells, which produced high amounts of ROS under oxidative stress. In evaluating the anti-oxidant capacity of natural products, combining both chemical and cell-based assays will provide more comprehensive understanding of anti-oxidant effects and potential biological relevance.

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1. Introduction

Acai (*Euterpe oleracea*) belongs to the family Arecaceae (palm tree). It is indigenous to South America. The palm trees grow at a density of 2500–3500 trees per hectare, serving as the predominant canopy plant inside the Amazon River delta in Para and Amapa states, Brazil, covering a range of over three million hectares (Brondizio, Safar, & Siqueira, 2002). The nutrient-dense and polyphenol-rich pulp of acai fruit is consumed as a fruit juice (Rodriques et al., 2006). The single seed, which accounts for 87% of the fruit, is expelled, while the skin is removed during pulping. A popular juice in Brazil prepared from the pulp of acai is consumed in a variety of beverages and food preparations (Schauss, 2010; Schauss et al., 2006a). Acai pulp has received much attention in recent years due to its extremely high anti-oxidant capacity and its role as a "functional food" or food ingredient. Freeze-dried acai pulp had dramatically high anti-oxidant value measured by ORAC (1027 μmol TE/g) (Schauss et al., 2006a), which is indeed higher than that of any fruit or vegetable that have been analysed and reported by USDA (Wu et al., 2004). Anti-oxidant capacities and other bioactivities of acai were studied in human, animal and cell culture models (Del, Percival, & Talcott, 2006; Jensen, Schauss, Beaman, & Ager, 2009; Jensen et al., 2008; Spada et al., 2009).

Since its high anti-oxidant capacity was revealed, numerous research investigations have focused on analyses and assessments associated with the anti-oxidant capacities of the acai pulp, juice or extracts (Honzel et al., 2008; Rufino, Fernandes, Alves, & Brito, 2009; Schauss, Jensen, Wu, & Scherwitz, 2009; Schauss et al., 2006a; Wu et al., 2004). Polyphenols have been associated with the anti-oxidant activity in fruits and vegetables. Major polyphenolic components found in acai include anthocyanins, proanthocyanidins, other flavonoids and lignans, etc. (Gallori, Bilia, Bergonzi, Barbosa, & Vincieri, 2004; Schauss et al., 2006b). Anthocyanins and proanthocyanidins are considered major anti-oxidants in fruits, but their concentrations are relatively low in acai (Schauss et al., 2006b). The contributions of the anthocyanins to the overall anti-oxidant capacities of acai were estimated to be only approximately 10% (Lichtenthäler et al., 2005). A recent paper also suggested that the components other than anthocyanins in acai contributed to antiproliferative activity against C-6 rat brain glioma cells (Hogan et al., 2010). The flavonoids were found to be

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the major polyphenols in acai (Gallori et al., 2004; Schauss et al., 2006b). Many studies have demonstrated that flavonoids have strong anti-oxidant activities and anti-inflammatory properties (Beara et al., 2009; Leong et al., 2010; Li et al., 2009). However, very few studies have determined the anti-oxidant capacity of individual polyphenol compounds found in acai pulp. There is only one study that has primarily studied lignans (Chin, Chai, Keller, & Kinghorn, 2008).

On the other hand, due to given limitations in analytical procedures, flavonoids have only been tentatively identified by HPLC or mass spectrometric methods, resulting in many flavonoids in the pulp remaining unidentified, much less determined for possible bioactivity. Therefore, the first objective of this study was to identify the major flavonoid compounds in acai pulp. Systemic isolation and fractionation in freeze-dried acai pulp is being reported in this paper. Seven flavones and their C-glycosides were obtained from freeze-dried acai pulp and their structures were elucidated by NMR, MS and the results compared to the literature. Among them, two compounds were identified in acai pulp for the first time.

The vast majority of studies that assess anti-oxidant capacities of acai and other anti-oxidant rich fruits and berries have utilised chemical-based assays. Unfortunately, such assays do not reflect or correlate with the test item's cellular response. The second objective of this study was to evaluate the anti-oxidant capacities of these flavonoids with one chemical-based assay and two cell-based assays: ORAC, CAP-e assay and ROS PMN assay. By doing these additional assays, we hope to understand not only their *in vitro* anti-oxidant capacities, but also their behaviours in living cells to reduce oxidative stress.

2. Materials and methods

2.1. Plant material

Freeze-dried acai (*Euterpe oleracea*) fruit pulp was obtained (Earth Fruits LLC, Belem, Brazil). The fruit was collected in Para state, Brazil, and processed within hours of harvesting to pure pulp and stored at −20 °C until transferred for freeze drying.

2.2. Chemicals and reagents

2.2.1. Extraction and isolation

Ninety five percentage EtOH, MeOH, petroleum ether and $CHCl_3$ were purchased from Shanghai Zhengxing Chemical Plant (Shanghai, China). EtOAc and acetone were obtained from Sinopharm Chemical Reagent Corporation (Shanghai, China). Silica gel (100–200 mesh) and Sephadex LH-20 were supplied by the Branch of Qingdao Marine Chemical Corporation (Qingdao, China) and Shanghai Juyuan Biotechology Corporation (Shanghai, China), respectively. Diatomite was obtained from Sinopharm Chemical Reagent Corporation (Shanghai, China).

2.2.2. ORAC-based assay

2,2′-Azobis(2-amidinopropane)dihydrochloride (AAPH) was purchased from Wako Chemicals, USA (Richmond, VA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and fluorescein (sodium salt) (FL) were obtained from Sigma–Aldrich (Milwaukee, WI). Potassium phosphate dibasic (K_2HPO_4) and potassium phosphate monobasic (KH_2PO_4) were obtained from VWR (West Chester, PA). Microplates (48-well, Falcon 3230) were purchased from VWR (West Chester, PA).

2.2.3. CAP-e assay and inhibition of ROS formation by PMN cells

The following buffers and reagents were obtained from Sigma–Aldrich (St. Louis, MO): phosphate-buffered saline (PBS), RPMI-1640 culture medium, hydrogen peroxide 30% solution (H_2O_2), dimethyl sulfoxide (DMSO), Histopaque 1077, and Histopaque 1119. Dichloroflurescein diacetate (DCF-DA) was obtained from Molecular Probes (Eugene, OR), a subdivision of Invitrogen (Carlsbad, CA).

2.3. Instrumentation

Electrospray ionisation mass spectrometry (ESIMS) data were measured on Micromass Q-TOF spectrometer (Milford, MA). ^1H NMR spectra were recorded on Varian Inova 400 or 500 MHz NMR spectrometer (Palo Alto, CA) using tetramethylsilane (TMS) as an internal standard in dimethyl sulfoxide-d_6 (DMSO-d_6). ORAC analyses were carried out on a FLUOstar Galaxy plate reader (BMG Labtech, Duraham, NC). A fluorescence filter with an excitation wavelength of 485 nm and an emission wavelength of 520 nm was used.

2.4. Extraction and isolation

The freeze-dried acai pulp powder (1800 g) was mixed with diatomite, percolated with 95% alcohol for 2 weeks. After evaporation of solvents under the vacuum, the residue (244 g) was then mixed with diatomite, extracted with petroleum ether, chloroform, ethyl acetate, acetone, and methanol, successively. The $CHCl_3$ and EtOAc fractions were then chromatographed over silica gel and Sephadex LH-20 columns and purified by preparative thin layer chromatography (PTLC) (Fig. 1).

$CHCl_3$ extract (9 g) was loaded into an open silica gel column ($CHCl_3$:MeOH = 90:1). Fraction 27–34 was subjected to Sephadex LH-20 column, eluted with MeOH. Sub-fraction 11–18 was then purified by PTLC (petroleum ether:EtOAc = 1:3) to get compound **5** (R_f = 0.34, 8 mg).

EtOAC extract (19 g) was loaded into an open silica gel column, using a gradient of increasing polarity with $CHCl_3$ and MeOH mixture as solvent.

Fraction 71–86 ($CHCl_3$:MeOH = 50:1) was subjected to Sephadex LH-20 column, eluted with MeOH. Sub-fraction 16–24 was then purified using PTLC ($CHCl_3$:MeOH = 5:1) to yield compound **7** (R_f = 0.61, 7 mg).

Fraction 87–99 ($CHCl_3$:MeOH = 30:1) was subjected to Sephadex LH-20 column, eluted with MeOH. Compound **4** (3 mg) came from sub-fraction 21–23 by recrystallisation in MeOH.

Fraction 100–112 ($CHCl_3$:MeOH = 10:1) was subjected to Sephadex LH-20 column, eluted with MeOH. Sub-fraction 33–40 was then purified by PTLC ($CHCl_3$:MeOH = 5:1) to yield compound **6** (R_f = 0.29, 9 mg).

Fraction 118–136 ($CHCl_3$:MeOH = 10:1) was subjected to Sephadex LH-20 column, eluted with MeOH. Compound **3** (2 mg) was obtained from sub-fraction 11–12 by recrystallisation in MeOH.

Fraction 137–159 ($CHCl_3$:MeOH = 10:1) was subjected to Sephadex LH-20 column, eluted with MeOH. Sub-fraction 14–26 was then purified using PTLC (EtOAc:MeOH:H_2O = 8:2:1) to afford compounds **1** (R_f = 0.55, 21 mg) and **2** (R_f = 0.49, 11 mg).

2.5. Oxygen radical absorbance capacity (ORAC) assay

Seven pure flavonoids (>95%) were weighed exactly, dissolved in MeOH and diluted properly with phosphate buffer (0.75 M, pH 7.0). The dilution factors were in the range of 50–800 folds depending on the compounds. The ORAC assay was conducted based on the method reported by our group (Wu et al., 2004). Briefly, the assay was carried out on a FLUOstar Galaxy plate reader used with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The temperature of the incubator was set to 37 °C. The micro plate loaded with samples and

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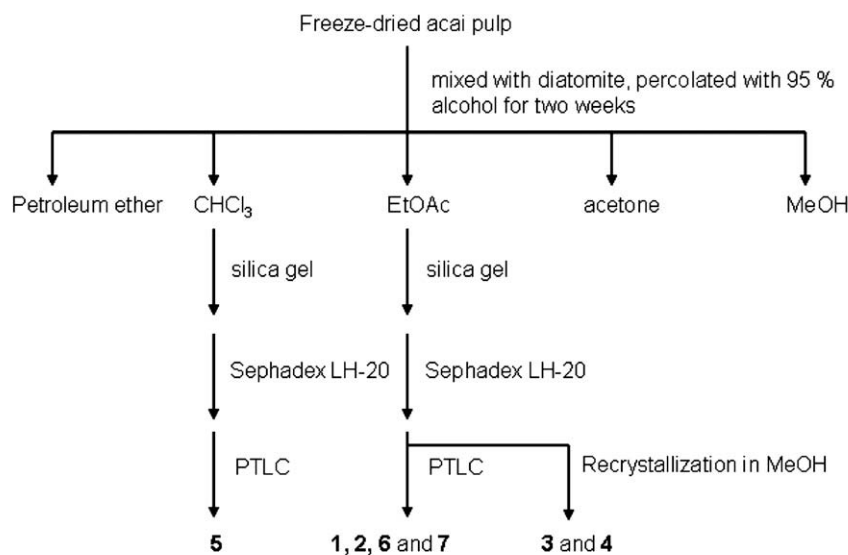

Fig. 1. The process of extraction, isolation and fractionation of flavonoids from acai.

standards were incubated for 5 min prior to run. Fluorescein was used as fluorescence probe; AAPH was used as peroxyl generator; Trolox as standard. The results were expressed as μmol Trolox equivalent (TE) per gramme.

2.6. Purification of red blood cells (RBC) and polymorphonuclear (PMN) cells

Healthy human volunteers between the ages of 20 and 50 years served as blood donors after informed consent, as approved by the Sky Lakes Medical Center Institutional Review Board (Klamath Falls, OR). Peripheral venous blood samples were drawn into sodium heparin and layered onto a double-gradient of Histopaque 1119 and 1077. The vials were centrifuged at 2400 rpm for 25 min. The PMN-rich lower interface was harvested using sterile transfer pipettes. PMN cells and the RBC fraction were washed twice in PBS without calcium or magnesium at 2400 rpm for 10 min. Then the core of the packed RBC was transferred into new vials and again washed twice in PBS without calcium or magnesium at 2400 rpm for 10 min. RBC aliquots were stored at 4 °C until use in the CAP-e assay. PMN cells were used immediately for the ROS assay.

2.7. Cell-based anti-oxidant protection of erythrocytes (CAP-e) assay

CAP-e assay was conducted following the method published by Honzel et al. (2008), but using an accelerated and more sensitive microplate-based protocol.

RBC cell suspension was prepared for the CAP-e assay by adding packed RBC (0.1 ml) into PBS (10 ml). The cell suspension was distributed in a V-bottom 96-well microtiter plate. Twelve wells were not treated with any source of anti-oxidants, and served as negative controls (six wells) and positive controls (six wells) for minimum versus maximum oxidative damage. Twelve wells were treated with a standard source of a known anti-oxidant (gallic acid) across six different serial dilutions, where each dilution was tested in duplicate. The remaining wells were treated with the isolated compounds from acai, where each compound was tested at 6 serial dilutions, and each dilution was tested in duplicate. RBC were incubated with anti-oxidants for 20 min, which was chosen based on testing of various incubation times on RBC anti-oxidant uptake of standard anti-oxidant compounds. Anti-oxidants not absorbed by the cells were removed by washing twice in PBS at 2400 rpm for 2.5 min. The cells were lysed and the precursor dye was added to the wells. Incubation was performed at room temperature for 15 min, followed by two washes. Oxidation was carried out using the peroxyl free radical generator AAPH for 1 h. The green fluorescence intensity, as a measure of oxidative damage, was measured at 488 nm using a Tecan Spectrafluor plate reader (40 flashes, optimal gain). The inhibition of oxidative damage was calculated as the reduced fluorescence intensity of product-treated cells, compared to cells treated only with the oxidising agent. The CAP-e value reflects the IC_{50} dose of the test product. This is then compared to the IC_{50} dose of the known anti-oxidant gallic acid. The CAP-e value was expressed as gallic acid equivalent (GAE) per gramme.

2.8. Inhibition of reactive oxygen species (ROS) formation by polymorphonuclear (PMN) cells

Evaluation of inhibition by seven flavonoids of ROS production was performed by using primary human polymorphonuclear (PMN) cells (ROS PMN assay) as previously described (Honzel et al., 2008). Freshly purified human PMN were exposed to the test products. During the incubation with a test product, any anti-oxidant compounds able to cross the cell membrane can enter the interior of the PMN cells. Compounds capable of transducing a signal across the cell membrane can do so. Then the cells were washed, loaded with the DCF-DA dye, which turns fluorescent upon exposure to reactive oxygen species. Formation of ROS was triggered by addition of H_2O_2. The fluorescence intensity of the PMN cells was evaluated by flow cytometry. The low fluorescence intensity of untreated control cells served as a baseline and PMN cells treated with H_2O_2 alone served as a positive control. If the fluorescence intensity of PMN cells exposed to an extract, and subsequently exposed to H_2O_2, was reduced compared to H_2O_2 alone, this indicates that a test product has anti-oxidant and/or anti-inflammatory effects. In contrast, if the fluorescence intensity of PMN cells exposed to test product was increased compared to H_2O_2 alone, this indicates that a test product has pro-inflammatory effects.

2.9. Statistical analysis

Statistical analysis was performed using Sigma Stat 3.5 for Windows (San Jose, CA). Statistical significance was tested using

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Student's *t*-test with a *P* value of less than 0.05 indicating a significant difference between data sets.

3. Results and discussion

3.1. Characterisation of compounds

According to the initial screening, flavonoid compounds were mainly present in $CHCl_3$ and EtOAc fractions (Fig. 1). Therefore, we separated and purified compounds from these two fractions. After repeated separation by various chromatographic methods, seven known compounds were obtained (Fig. 2). Their structures were elucidated by ESIMS, ^{1}H NMR spectra and compared with literature.

Orientin (**1**), yellowish amorphous powder; ESIMS: m/z = 447 $[M-H]^-$; ^{1}H NMR (DMSO-d_6) ppm: δ 13.12 (1H, s, 5-OH), 7.32 (1H, d, J = 8.4 Hz, H-6′), 7.23 (1H, s, H-2′), 6.58 (1H, d, J = 8.4 Hz, H-5′), 6.19 (1H, s, H-3), 5.71 (1H, s, H-6), 4.72 (1H, d, J = 9.6 Hz, glc-H-1″), 3.92–3.26 (5H, m, glc-H-2″-6″). The data were consistent with the known compound (Leong et al., 2010).

Homoorientin (**2**), yellowish amorphous powder; ESIMS: m/z = 447 $[M-H]^-$; ^{1}H NMR (DMSO-d_6) ppm: δ 13.62 (1H, s, 5-OH), 7.19 (1H, d, J = 8.4 Hz, H-6′), 7.15 (1H, s, H-2′), 6.59 (1H, d, J = 8.4 Hz, H-5′), 6.19 (1H, s, H-3), 5.92 (1H, s, H-8), 4.56 (1H, d, J = 9.6 Hz, glc-H-1″), 4.16–3.15 (5H, m, glc-H-2″-6″). The data were consistent with the known compound (Leong et al., 2010).

Vitexin (**3**), yellowish amorphous powder; ESIMS: m/z = 431 $[M-H]^-$; ^{1}H NMR (DMSO-d_6) ppm: δ 10.27 (1H, s, 5-OH), 8.01 (2H, d, J = 8.4 Hz, H-2′, 6′), 6.92 (2H, d, J = 8.4 Hz, H-3′, 5′), 6.77 (1H, s, H-3), 6.28 (1H, s, H-6), 4.92 (1H, d, J = 9.6 Hz, glc-H-1″), 4.88–3.76 (5H, m, glc-H-2″-6″). The data were consistent with the known compound (Leong et al., 2010).

Luteolin (**4**), yellowish amorphous powder; ESIMS: m/z = 285 $[M-H]^-$; ^{1}H NMR (DMSO-d_6) ppm: δ 12.94 (1H, s, 5-OH), 7.40 (1H, d, J = 8.4 Hz, H-6′), 7.40 (1H, s, H-2′), 6.89 (1H, d, J = 8.4 Hz, H-5′), 6.63 (1H, s, H-3), 6.44 (1H, s, H-8), 6.18 (1H, s, H-6). The data were consistent with the known compound (Hartwig, Maxwell, Joseph, & Phillips, 1990).

Chrysoeriol (**5**), yellowish amorphous powder; ESIMS: m/z = 299 $[M-H]^-$; ^{1}H NMR (DMSO-d_6) ppm: δ 12.94 (1H, s, 5-OH), 7.36 (1H, d, J = 8.4 Hz, H-6′), 7.32 (1H, s, H-2′), 6.74 (1H, d, J = 8.4 Hz, H-5′), 6.34 (1H, s, H-3), 5.78 (1H, s, H-8), 5.54 (1H, s, H-6), 3.71 (3 H, s, OCH_3-3′). The data were consistent with the known compound (Awaad, Maitland, & Soliman, 2006).

Quercetin (**6**), yellowish amorphous powder; ESIMS: m/z = 301 $[M-H]^-$; ^{1}H NMR (DMSO-d_6) ppm: δ 12.42 (1H, s, 5-OH), 9.26 (1H, s, 3-OH), 7.66 (1H, d, J = 2.0 Hz, H-2′), 7.54 (1H, dd, J = 8.0, 2.0 Hz, H-6′), 6.88 (1H, d, J = 8.0 Hz, H-5′), 6.40 (1H, d, J = 2.0 Hz, H-6), 6.18 (1H, d, J = 2.0 Hz, H-8). The data were consistent with the known compound (Awaad et al., 2006).

Dihydrokaempferol (**7**), yellowish amorphous powder; ESIMS: m/z = 287 $[M-H]^-$; ^{1}H NMR (DMSO-d_6) ppm: δ 11.96 (1H, s, 5-OH), 7.30 (2H, d, J = 8.4 Hz, H-2′, 6′), 6.78 (2H, d, J = 8.4 Hz, H-3′, 5′), 5.79 (1H, d, J = 1.6 Hz, H-8), 5.74 (1H, d, J = 1.6 Hz, H-6), 4.99 (1H, d, J = 11.2 Hz, H-2), 4.50 (1H, d, J = 11.2 Hz, H-3). The data were consistent with the known compound (Nafady et al., 2003).

After a literature search, orientin (**1**), homoorientin (**2**), vitexin (**3**), luteolin (**4**) and quercetin (**6**) were isolated and identified as pure compounds from acai for the first time. Two of them, vitexin and quercetin (**3** and **6**), were reported from acai for the first time. Though some previous studies identified some flavonoids from acai, only a few of them (Chin et al., 2008) were separated and obtained. Most flavonoids were tentatively identified by HPLC-UV or HPLC-MS analysis (Gallori et al., 2004; Pacheco-Palencia, Duncan, & Talcote, 2009), therefore the bioactivities of pure compounds from acai could not be studied. It is very important to obtain the pure compounds from acai for studying responsible compounds having anti-oxidant capacities in acai.

3.2. Anti-oxidant capacity from ORAC

The ORAC values obtained for the seven flavonoids from ORAC assay is shown in Table 1. ORAC values varied distinctly among these seven compounds (1420–14800 μmol TE/g) based on the numbers and positions of hydroxyl groups and other substitute groups. A previous study reported that with compounds having the same basic chemical structure, the ORAC value of flavonoid was proportional to the number of hydroxyl substitutions on the structure (Cao, Sofic, & Prior, 1997). However, according to our study, the ORAC value of each flavonoid was not only determined by the number of hydroxyl groups, but also depended on the positions of hydroxyl groups in the structures. The structure of vitexin (**3**) is very similar to that of orientin (**1**). The difference is a lack of one hydroxyl group at C-3′ of vitexin (**3**). ORAC values of these two

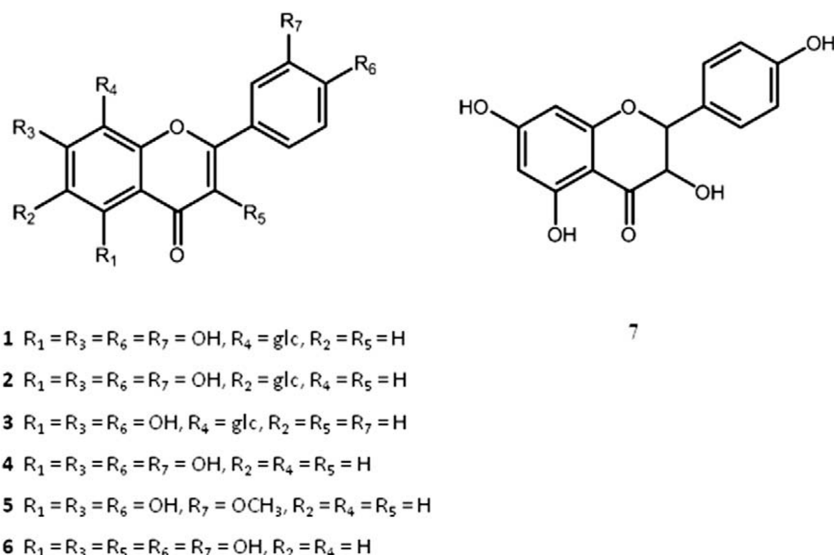

Fig. 2. The structures of seven flavonoid compounds (1–7) isolated from acai. The corresponding compound names are shown in Table 1.

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Table 1
Antioxidant capacities from ORAC assay and CAP-e assay[a].

No.	Compound name	ORAC (μmol TE/g)	CAP-e (GAE/g)
1	Orientin	1700 ± 78.9	N/A[b]
2	Homoorientin	1420 ± 63.3	N/A
3	Vitexin	14,800 ± 451	N/A
4	Luteolin	7870 ± 350	5040 ± 260
5	Chrysoeriol	4400 ± 189	N/A
6	Quercetin	12,300 ± 1070	5510 ± 443
7	Dihydrokaempferol	8390 ± 93.8	3980 ± 126

[a] Data was expressed as mean ± SD ($n = 3$).
[b] No value was obtained.

compounds were very different. Vitexin (**3**) has the highest ORAC (14800) among the seven compounds, whereas ORAC of orientin (**1**) was only 1700. The results also showed that ORAC values of aglycones are generally higher than that of C-glycosides. For instance, the ORAC value of the aglycone luteolin (**4**) was four times higher than that of glycosides, orientin and homoorientin (**1** and **2**). The *O*-methylation of the hydroxyl groups reduced the ORAC values (about two times), such as chrysoeriol (**5**) vs. luteolin (**4**). Likely the conjugation between 4′-OH and 4-C=O distinctly affects the anti-oxidant activity (e.g. vitexin and dihydrokaempferol, **3** and **7**), while mono-OH substitution at 4′ (vitexin, **3**) has stronger anti-oxidant activity (approximate 8–10 times) than di-OH substitution at 3′ and 4′ (e.g. orientin and homoorientin, **1** and **2**).

3.3. CAP-e anti-oxidant capacity

Anti-oxidant capacities of the seven flavonoids were also measured by the recently developed cell-based anti-oxidant protection (CAP-e) assay. Data from the CAP-e assay reflects whether anti-oxidants can enter into and protect live cells from oxidative damage. The results of CAP-e values of seven flavonoids are shown in Table 1. Remarkably, orientin, homoorientin, vitexin and chrysoeriol (**1**–**3** and **5**) did not have any CAP-e values at any tested concentrations (Fig. 3), which meant that they were not able to penetrate into live cells and provided protection against oxidative stress. As these compounds were not able to enter into and protect RBC in the CAP-e assay, no inhibition of oxidative damage was seen, and no IC_{50} were calculated. The other three compounds, luteolin, quercetin, and dihydrokaempferol (**4**, **6** and **7**) could enter into the live cells with quercetin (**6**) displaying the highest CAP-e value. The IC_{50} for compounds **4**, **6**, and **7** were 0.00635 ± 0.00033, 0.00581 ± 0.00047, and 0.00803 ± 0.00025 mg/ml, respectively.

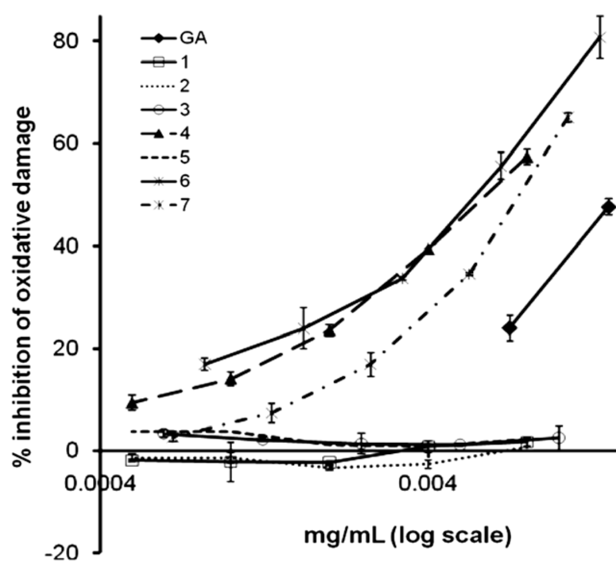

Fig. 3. CAP-e values of seven flavonoid compounds. The corresponding compound names are shown in Table 1. GA represents gallic acid, which was used as standard.

The results showed that the compounds (**4**, **6** and **7**) being able to get into living cells were all aglycones, and their C-glycosides (**1**–**3**) cannot penetrate into living cells. Surprisingly, chrysoeriol (**5**), as an aglycone, was not able to enter living cells at all. It is the only aglycone that bears a methoxy group. So we speculate that this structural property may increase its hydrophobicity thus prevent this compound from entering living cells.

3.4. Inhibition of ROS formation by ROS PMN assay

Inhibition effects of the seven flavonoids against ROS formation were evaluated by the ROS PMN assay are shown in Fig. 4. The ROS PMN assay monitors the combined effect of a test product on an inflammatory cell type, and the data reflects a combination of at least three different mechanisms: (1) anti-oxidants penetrate into the cell and neutralise free radicals, similar to the CAP-e assay; (2) anti-inflammatory compounds mediate cell signalling at the cell surface, reprogramming the PMN cell to a less inflammatory behaviour, resulting in a reduction in formation of ROS; and (3) pro-inflammatory compounds capable of supporting innate immune functions mediate a signal at the cell surface, resulting in an increase in the PMN cell function, thus increasing the production of ROS (Honzel et al., 2008). All compounds tested were shown to reduce ROS formation at various doses. However, their behaviours were very different. Orientin (**1**) and luteolin (**4**) showed dose responses in which only higher doses displayed inhibition effects (Fig. 4). Four compounds, homoorientin, vitexin, quercetin, and dihydrokaempferol (**2**, **3**, **6** and **7**), were shown to reduce ROS formation best at the lowest concentrated dilution of 8×10^{-7} mg/ml (Fig. 4). Interestingly, chrysoeriol (**5**) exhibited inhibition effects indistinguishably at doses varied from 8×10^{-7} to 8×10^{-3} mg/ml (Fig. 4). One apparent reason for these dramatic behavioural differences is the chemical structure. Chrysoerial (**5**), for example, is the only one among these seven compounds that bears a methoxy group. This structural characteristic might be responsible for the unique inhibitory effects of this compound, which inhibited ROS formation at both low and high doses ranging from 8×10^{-7} to 8×10^{-3} mg/ml (Fig. 4). But exactly how the chemical structure affects ROS production and the potent anti-inflammatory effect seen at the lowest dose tested for these compounds warrant further investigation.

3.5. Results of anti-oxidant capacities from different assays

In this study, anti-oxidant capacities of seven flavonoids isolated from acai were investigated by three assays. Of these three assays, ORAC is one of most widely used chemical-based anti-oxidant assays, which measures anti-oxidant inhibition of peroxyl radical induced oxidation (Prior, Wu, & Schaich, 2005). However, a chemical-based assay is solely based on chemical reaction and do not reflect the cellular physiological conditions, including cellular uptake versus signal transduction. Mechanisms of anti-oxidants going beyond direct scavenging of free radicals may be involved in disease prevention and health promotion. Therefore, there is need for cell-based anti-oxidant assays (Liu & Finley, 2005). The CAP-e assay is a newly developed cell-based assay using erythrocytes to address the question of whether anti-oxidants in complex natural products enter the cytosol and contribute to the reduction of oxidative damage within the cell. The assay allows for semiquantification specifically of those anti-oxidants that are capable of penetrating into live cells (Honzel et al., 2008). Based on the CAP-e data, another cell-based as-

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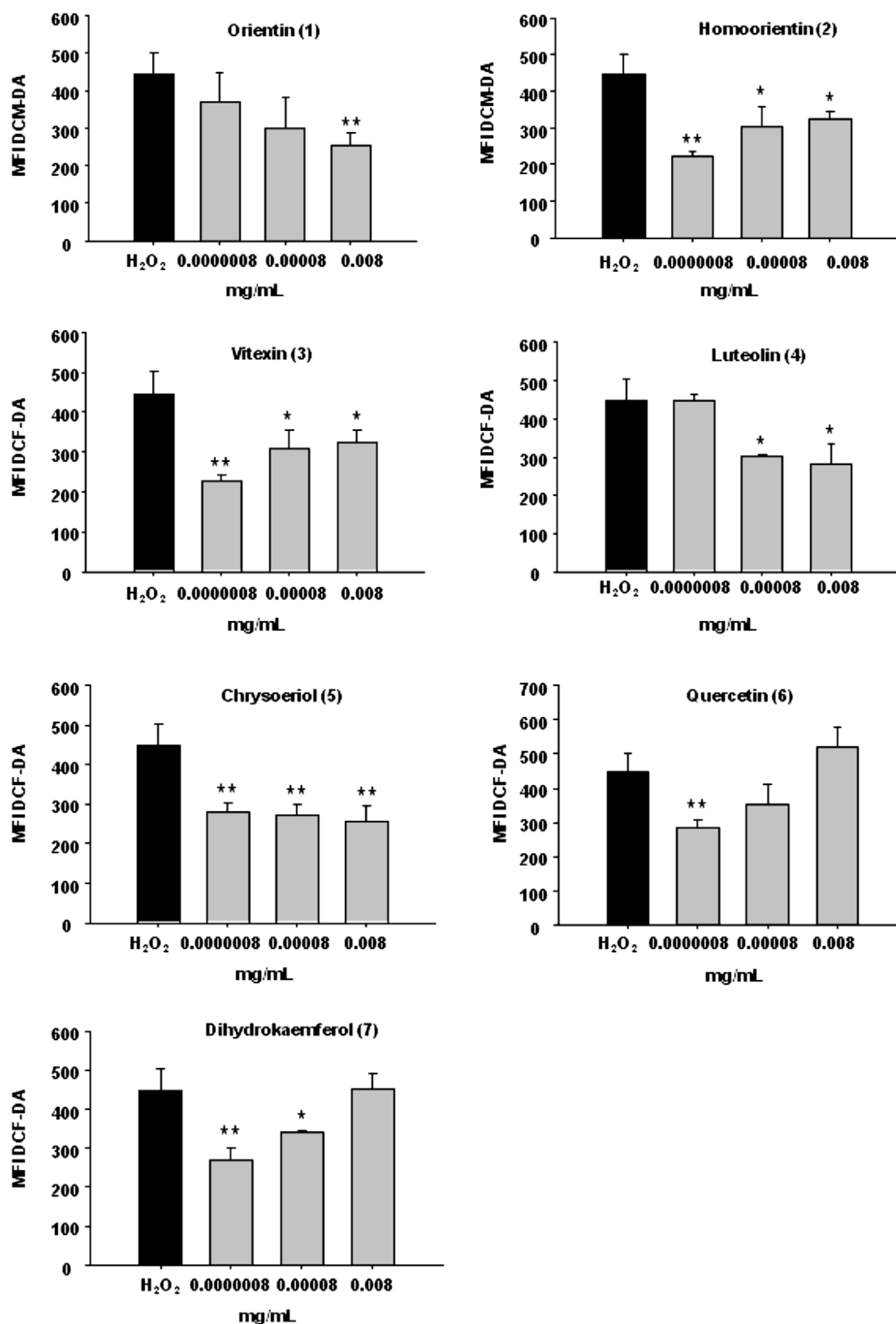

Fig. 4. Results from the ROS PMN assay for seven flavonoid compounds. The mean fluorescence intensity (MFI) for the reporter dye DCF-DA is plotted. It is proportional to ROS formation under each assay condition. Black bars indicate H_2O_2 treatment and grey bars indicate different doses of each flavonoid ($^{*}P < 0.05$ and $^{**}P < 0.01$).

say using primary pro-inflammatory PMN cell was performed, which allows for a more complex assessment of the properties of natural products *in vitro*, were conducted. PMN cells are an important part of our innate immune defense and are capable of rapid production of ROS in response to both oxidative damage and pro-inflammatory stimuli such as microbial invaders. The PMN cell can respond to compounds in natural products extracts in the three aforementioned mechanisms. Thus, data obtained from a PMN-based assay may be interpreted better in the context of data from the CAP-e assay (Honzel et al., 2008).

The combination of ORAC to CAP-e data and ROS PMN data give a good foundation for making further decisions and fully

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understand the scope of acai compounds' anti-oxidant capacities. For instance, vitexin (**3**) responsible for the highest ORAC value may not match the three compounds, luteolin, quercetin and dihydrokaempferol (**4**, **6** and **7**) that performed best in the CAP-e assay. The four compounds, orientin, homoorientin, vitexin and chrysoeriol (**1**–**3** and **5**) that showed no bioactivity in the CAP-e bioassay, may be responsible for the signalling at the cell membrane level, where entry into the cell is not necessary for the biological activity involved in the ROS PMN assay. Basically, the seven compounds were divided into four groups according to the results from three assays. Group 1, orientin (**1**), showed relatively low ORAC, and does not penetrate into live cells. Group 2, homoorientin, vitexin and chrysoeriol (**2**, **3**, **5**), had exceptional anti-inflammatory effect and compound **3** had the highest ORAC, but neither of these compounds were able to penetrate into live cells. Group 3, luteolin (**4**), had some anti-inflammatory effect, relatively high ORAC, and can penetrate into live cells. Group 4, quercetin and dihydrokaempferol (**6**–**7**), both of them have demonstrated exceptional anti-inflammatory effects and the ability to penetrate into live cells. Remarkably, quercetin (**6**) in group 4 displayed the best anti-oxidant capacities in all three assays, which included high ORAC and CAP-e value at the lowest dose of 8×10^{-7} mg/ml inhibiting ROS formation.

4. Conclusions

In this study, seven flavonoids (flavones and their C-glycosides) were isolated and structurally identified from freeze-dried acai pulp. Vitexin and quercetin (**3** and **6**) were reported from acai pulp for the first time. Our data confirmed that flavonoids are major polyphenols in acai pulp. In order to determine the major anti-oxidants in acai pulp, the anti-oxidant capacities of these flavonoids were evaluated with one chemical-based assay and two cell-based assays: ORAC, CAP-e assay and ROS PMN assay. However, anti-oxidant capacities of these compounds varied significantly based on their chemical structures and the assays used. In general, flavonoid aglycones showed not only higher anti-oxidant capacities than their C-glycosides, but also the ability to penetrate into living cells. Except for sugar moieties, the methoxy group appears to be another important structural factor that could lead to dramatic changes of flavonoids in terms of their anti-oxidant capacity and the ability to penetrate into living cells.

Due to the complexity of the anti-oxidant defense system in the body, a single anti-oxidant assay cannot reflect all aspects of activities of a given compound. In evaluating the anti-oxidant capacities of natural products, combining both chemical and cell-based assays will provide a useful approach towards understanding the anti-oxidant effects of natural compounds and their biological relevance to any health benefits observed *in vivo*. Such combined use of assays may prevent premature conclusions being made about which compounds are responsible for a foods biological effects, based on reliance of chemical assays.

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MONAVIE (M)MŪN™

PRODUCT INFORMATION PAGE

MonaVie (M)mūn™ is a beneficial blend of 19 fruits and Wellmune®—clinically shown to promote proper immune function. Formulated with antioxidant rich MVāo²™, a proprietary complex featuring the superfruits açai and maqui, this delicious juice helps protect your body year round. It's your daily defense for a healthier life.

THE PREMIER AÇAI BLEND®

MonaVie (M)mūn is a delightful fusion of the following19 fruits, which were specifically chosen for their ability to nutritionally support your immune health and overall well being:

Açai, maqui, grape, apple, acerola, aronia, black currant, elderberry, cranberry, blood orange, sea buckthorn, pear, blueberry, cupuaçu, strawberry, baobab, lingonberry, bilberry, and camu camu.

KEY BENEFITS

- Supports your immune system, which helps safeguard your body against potentially harmful microorganisms.
- Helps optimize proper immune function.
- Promotes and maintains an overall sense of good health and well being.
- Benefits young and old alike—ages four and up.

ESSENTIAL FACTS

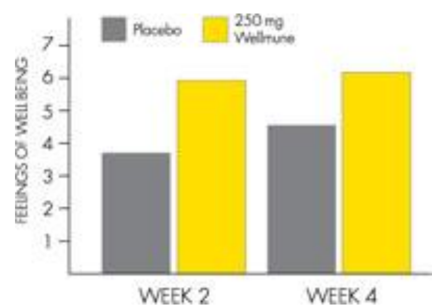
Safeguard. Optimize. Shield. The SOS approach of MonaVie (M)mūn arms your body against everyday challenges.

- *Safeguards your health.* Just like the daily practices of wearing a seatbelt or brushing your teeth help protect your body, drinking four ounces of MonaVie (M)mūn every day helps support your overall health.
- *Optimizes your natural defenses.* MonaVie (M)mūn supports your immune system with a unique complex carbohydrate that's supported by seven clinical studies and more than $250 million in research and development.
- *Shields your body.* Everyday challenges such as poor eating habits, heavy workloads, and lack of sleep and exercise can take a toll on your health. MonaVie (M)mūn supports your immune system, a complex network of specialized cells that helps shield against potentially harmful microorganisms.

WELLMUNE AND IMMUNE FUNCTION

Elite athletes, such as marathon runners, place a significant amount of physical stress on their bodies. Similar to other burdens on the body (e.g., lack of sleep, poor diet, emotional strain), exercise stress can compromise your immune system.

In a four week study* examining the effects of Wellmune in marathon athletes, participants reported an improvement in their feelings of well being and in the maintenance of their overall health.



* Journal of Sports Science and Medicine (2009) 8, 509-515

WHO SHOULD USE MONAVIE (M)MŪN™?

MonaVie (M)mūn is for people ages 4 and up who want to supplement their daily diets with beneficial antioxidants and who are interested in promoting vitality, well being, and immune health.

NUTRITION FACTS

MONAVIE (M)MŪN

Nutrition Facts	Amount, %DV*
Serving Size	2 fl. oz. (60 ml)
Calories	40
Calories from Fat	5
Total Fat	0.5 g, 1%
Sodium	10 mg, 0%
Potassium	80 mg, 2%
Total Carbohydrate	9 g, 3%
Dietary Fiber	2 g, 8%
Sugars	6 g
Protein	<1 g, 0%
Vitamin A	2500 IU, 50%
Vitamin C	30 mg, 50%
Vitamin E	15 IU, 50%
Niacin	5 mg, 25%
Vitamin B6	0.5 mg, 25%
Vitamin B12	1.5 mcg, 25%
Pantothenic Acid	2.5 mg, 25%
Zinc	1.5 mg, 10%

Not a significant source of saturated fat, trans fat, cholesterol, calcium, or iron.
* Percent Daily Value based on a 2,000 calorie diet.

Other Ingredients: Proprietary MVāo^{2}™ Complex [açai blend (açai, juçara–freeze-dried powder and puree), maqui–reconstituted fruit juice], reconstituted fruit juice blend (grape, apple, acerola, aronia, black currant, elderberry, cranberry, blood orange, sea buckthorn, pear, blueberry, cupuaçu, strawberry, baobab, lingonberry), fruit puree blend (pear, bilberry, cranberry, camu camu), maltodextrin (soluble dietary fiber), Wellmune® (baker's yeast beta-glucan), citric acid, natural flavor, sodium benzoate, strawberry (freeze-dried powder), ascorbic acid, dl-alpha-tocopherol acetate, vitamin A palmitate, calcium d-pantothenate, pyridoxine hydrochloride, zinc oxide, niacinamide, cyanocobalamin.

RECOMMENDED USE

Drink 2 ounces twice daily. Shake well before using. Refrigerate after opening.

Go ahead, drink to your health!

MONA·VIE®

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What is MonaVie Active?

Enhances your body's joint health. MonaVie Active features the additional benefits of plant-derived glucosamine, which has been scientifically shown to promote healthy joint function by targeting mobility and flexibility. Designed to support joint performance and recovery, this vital formula delivers the resources your body needs to get moving.

· Individuals who regularly consume MonaVie Active have reported an increase in vitality, better health, an overall sense of well being, and an improvement in joint health.

· Initial research* suggests that drinking four ounces of MonaVie Active on a daily basis can enhance your body's antioxidant activity.

· Gives your body the resources it needs to help fight free radicals that target joints.

· Helps you maintain a healthy and active lifestyle.

* For more information about this study, please visit www.monavie.com/science.

Is MonaVie Active juice safe for children and pregnant or nursing women?

MonaVie Active contains glucosamine hydrochloride, which has been extensively tested and found to be safe and effective; however, tests involving pregnant and nursing women have been limited. Thus, MonaVie recommends that pregnant and nursing women consult their health care practitioners prior to use.

What is glucosamine hydrochloride?

Glucosamine hydrochloride is a natural compound found in the body that is used to help make and form cartilage. Glucosamine has also been shown to help lubricate, cushion, and protect healthy joints.

Is one form of glucosamine more effective than another?

There are three types of glucosamine: glucosamine hydrochloride (HCl), N-acetyl glucosamine, and glucosamine sulfate. Both glucosamine HCl and glucosamine sulfate are rapidly converted into "free" glucosamine in the stomach. So, from a medicinal standpoint, they are equally effective. However, glucosamine HCl is 79 percent glucosamine, while glucosamine sulfate is only between 47 and 52 percent glucosamine. We formulated MonaVie Active juice with glucosamine HCl because this form of glucosamine contains the highest level of free glucosamine.

What are esterified fatty acids?

Esterified fatty acids promote joint health, improving flexibility and mobility. Esterification is the general name for a chemical reaction in which two chemicals (typically an alcohol and an acid) form an ester as the reaction product.

Why does the MonaVie Active label list "tree nuts (palm nut)"?

MonaVie Active's esterified fatty acids are derived from a 100 percent vegetable source (palm nut oil). The (US) FDA has made it mandatory to list all sources of potential allergens found in a product on its label. Eight major foods or food groups have been identified as common allergy causing foods in some individuals. Of these foods tree nuts are included. Since palm nuts are a tree nut and the esterified fatty acids come from palm nut oil, it is necessary to list it on the product label.

How might esterified fatty acids cause an allergic reaction?

Allergic reactions are caused by proteins. The esterified fatty acids in MonaVie Active may contain an extremely low level—an almost undetectable level—of protein from palm nuts. Because the amount of protein is so minute, it is unlikely that people with tree nut allergies will have an allergic reaction; however, in extremely sensitive persons it is best to avoid it all together. If you have an allergy to palm nuts, it is best to consult with your physician prior to drinking MonaVie Active.

If I have an allergy to peanuts, will I also be allergic to tree nuts?

No, not necessarily. Individuals with peanut allergies only should not have an allergic reaction to MonaVie Active because the product is not produced in a facility that manufactures peanuts or peanut oil.

What is the nutritional content in 4 oz. of Active juice?

Calories: 120

Calories from Fat: 20

Total Fat: 2 g, 3%*

Cholesterol: 0 mg, 0%*

Potassium: 220 mg, 6%*

Sodium: 20 mg, 1%

Total Carbohydrate: 20 g, 8%*

Dietary Fiber: 1 g, 4%*

Sugars: 12 g

Protein: 1 g, 2%*

Calcium: 25 mg, 3%*

Vitamin A: 125 IU, 3%*

Vitamin C: 60 mg, 100%*

Iron: 1.5 mg, 8%*

Vitamin K: 32 mcg, 40%*

*Percent Daily Value based on a 2,000 calorie diet.

Not a significant source of saturated fat or trans fat.

Pain reduction and improvement of range of motion after consumption of MonaVie Active™, an Açai-rich fruit/berry juice blend.

Gitte S. Jensen, Alexander G. Schauss, Marcie A. Mitzner, Kimberlee A. Redman, David M. Ager.
NIS Labs, Klamath Falls Oregon; AIBMR Life Sciences, Puyallup Washington; Cascade Chiropractic and Rehabilitation, Klamath Falls Oregon.

Abstract

The objective was to evaluate the impact of consumption of an Açai-rich fruit/berry juice blend on pain and range of motion. An open-label clinical pilot study was performed by recruiting 14 study participants with some limitations in range of motion associated with pain and affecting their activities of daily living.

The study participants were supplied with juice for 12 weeks, and instructed to consume four ounces (120 mL) daily for the duration of the study. The study participants went through a medical exam at study entry and exit, and were assessed by a nurse (structured interview, questionnaires, blood samples), and a chiropractor (range of motion assessment). The pain levels were scored using a visual analogue scale (VAS). The ROM assessment was performed using dual digital inclinometry, using the J-Tech wireless system as recommended by the American Medical Association guidelines.

Serum testing showed a significant increase in antioxidant activity already after 2 weeks of consumption, using the CAP-e cell-based antioxidant protection assay. A reduction of inflammation was seen as a reduction of C-Reactive Protein (CRP).

Introduction

The test product for this study was MonaVie Active™ (MVA), a fruit- and berry-based juice blend, with a very high level of anti-oxidants. It contains the following eight exotic fruits and berries that have been studied separately for their high anti-oxidant content: Açai, Pomegranate, Wolfberry (Goji berry), Camu Camu, Passion fruit, Aronia, Acerola, and Bilberry. It also contains 10 more ordinary fruits, including: Apricot, Purple grape, White grape, Lychee, Banana, Kiwi, Pear, Cranberry, Blueberry, and Prune. In addition, MVA also contains glucosamine.

Testimonials claim many types of health improvements after MVA consumption. These include recovery from chronic pain syndromes and improved cardiovascular health. We have previously conducted an *in vivo* study documenting that consumption of four ounces of MV results in an increase in antioxidant activity in the serum, as measured by the CAP-e assay [1, 2] and that this increased serum antioxidant activity translates into positive consequences for oxidative stress, as measured by reduction in lipid peroxidation within two hours after consumption of the juice blend [2].

Based on these data it was of interest to examine whether the increased antioxidant activity may have a positive effect on known inflammatory conditions. For this study, study participants with some degree of joint pain and generalized muscle pain were chosen.

Study design

Fourteen human subjects were enrolled in the 12-week study, to identify the time course of changes in symptoms of pain and reduced ROM. Subjects were monitored at baseline, and after 2, 4, 8, and 12 weeks. Blood was taken at each visit, and a structured nurse interview specifically addressed any subjective changes.

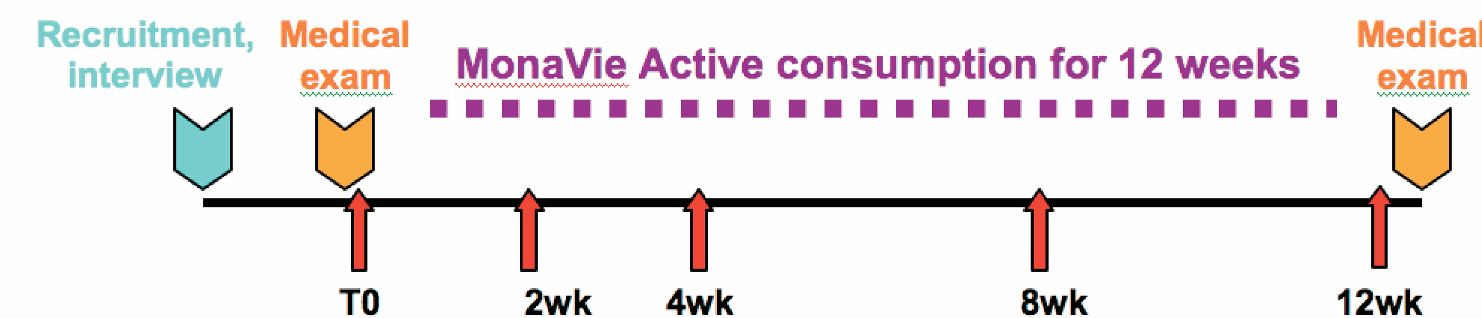

Figure 1. Study design for the 12 week study. Red arrows indicate visits to the clinic, assessment of pain and range of motion, and blood draws.

Vol. #	Gender	Age	Primary Complaint	Other Pain
01	M	84	Joint pain	Agent Orange Rash
02	F	68	Joint pain	Right leg muscular pain
03	F	47	Joint pain - knees	–
04	F	59	Rheumatoid Arthritis	Right knee & hip pain
05	M	51	Chronic inflammation of the spine and lower back	Joint pain - multiple
06	F	44	Joint pain	–
07	F	54	Chronic muscle pain	Knee pain
08	F	59	Joint pain - knees	–
09	F	54	Chronic muscle pain, joint pain	Lupus
10	F	70	Joint pain	Lower back pain right hip/leg pain
11	M	47	Joint pain	Knee & hip pain
12	F	60	Joint pain - knees	Lower back pain
13	M	58	Joint pain - knees	Shoulder, elbow, wrist pain
14	F	47	Joint pain - knees	Hand & finger pain

Results

Range of Motion (ROM)

In all study participants, the ROM at baseline was primarily reduced in the lumbar and lower extremity areas. Consumption of MVA resulted in an increase in lumbar and lower extremity ROM as well as an improvement in overall generalized pain for the study group as a whole. Comparison of the means of the grouped data was performed using a two-tailed un-paired (independent) *t*-test. For the lumbar ROM, there was improvement over the entire 12 weeks ($p < 0.02$). Improvement for the lower extremity ROM was observed by 12 weeks ($p < 0.05$).

Method for assessing ROM using J-TECH Tracker Freedom® Wireless

The J-Tech Tracker Freedom® system uses dual inclinometry protocols from the American Medical Association (AMA) and wireless instruments to measure composite range of motion and strength evaluation. This system is controlled with a foot switch; therefore, permitting all instrumented tests to be accurately recorded without returning to the computer. The following instrumented tests were performed for this study:

- Dual Inclinometry (range of motion = ROM) for spine and extremities;
- Dynamometer Muscle Testing (strength);
- Algometry (tenderness; pain threshold and tolerance);
- Grip Testing (grip strength).

The assessment was performed by a chiropractor with experience in detailed ROM assessment. Thus, one operator performed all ROM assessments throughout the study.

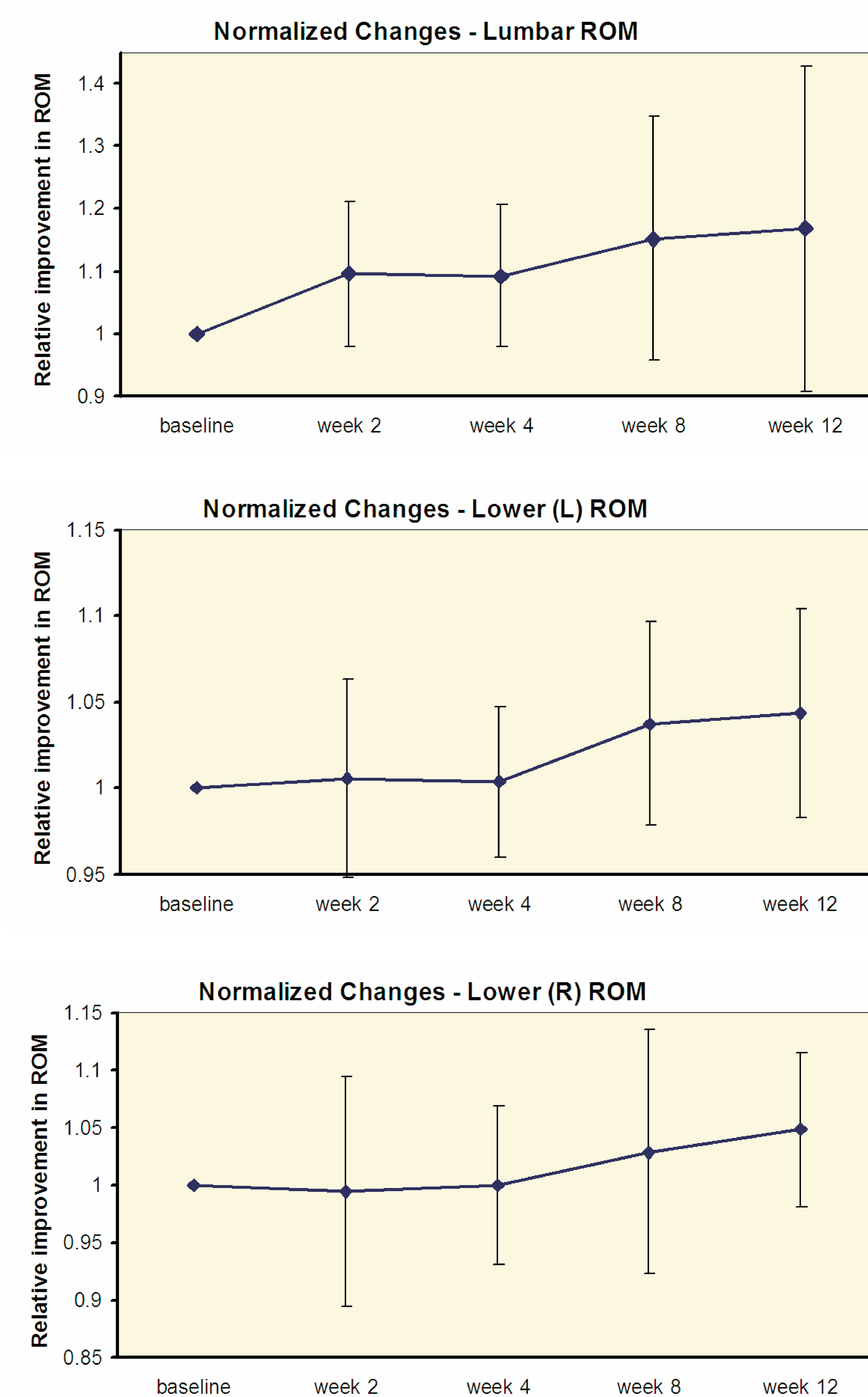

Figure 2. The normalized change in ROM is shown. The improvements are shown for lumbar ROM (top), lower left extremity (mid), and lower right extremity (bottom). Each person's cumulative ROM at study start was set to "1", and improvements in ROM are indicated by higher relative ROM score. The averages of the normalized values are shown for each visit. The vertical bars indicate the standard deviation. The improvement in lumbar ROM reached statistical significance already at 2 weeks ($p < 0.02$), and the improvement in ROM for lower extremities reached significance at 12 weeks ($p < 0.05$).

Pain

The self-reported pain level, as scored on the visual analogue scale, was significantly reduced by 12 weeks of MVA consumption ($p < 0.01$).

Method for assessing pain

A visual analogue scale was used to track current pain levels at each visit. The scale was without increment marks, and was 10 centimeters long. The study participants were instructed to make a mark anywhere on the line reflecting current pain level on the day of the visit. The score was measured on the scale in centimeters.

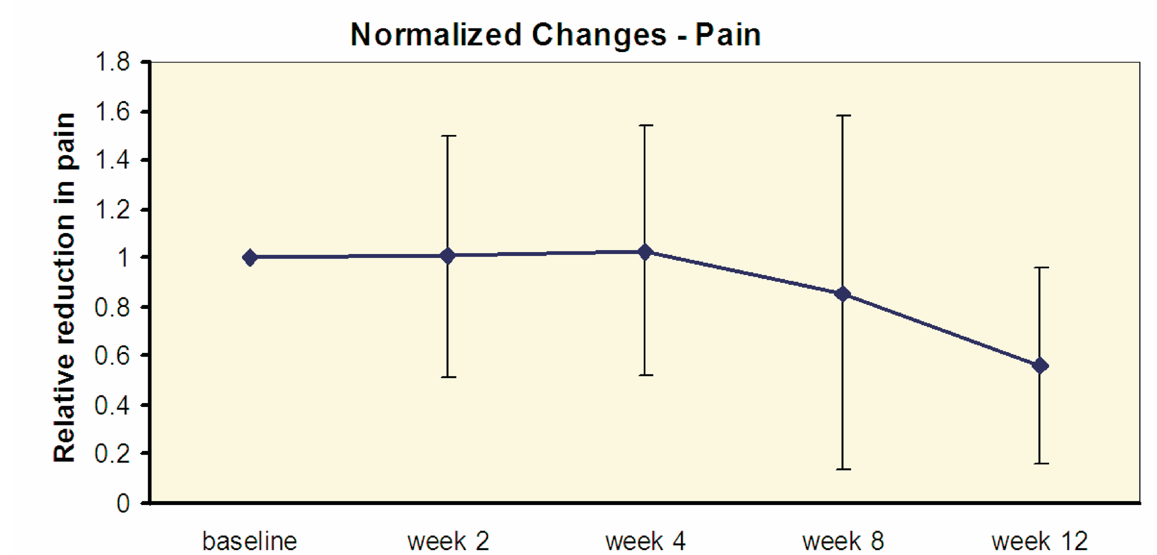

Figure 3. The relative changes in pain scores are shown. Each person's pain score at study start was set to "1", and reductions in pain levels are indicated by lower pain scores. The averages of the normalized values are shown for each visit. The vertical bars indicate the standard deviation. Of the 14 study participants, volunteers 6 and 9 were removed from the pain analysis, because other pain issues complicated the analysis of pain pertaining to the primary complaint at study start. Among the remaining 12 study participants, the pain reduction seen after 12 weeks was highly significant ($p < 0.01$).

Serum antioxidant and inflammatory status

The serum antioxidant status, as monitored by the CAP-e cell-based antioxidant protection assay, was significantly improved already after 2 weeks of consumption of MVA ($p < 0.05$), and kept improving throughout the 12 weeks of study ($p < 0.00001$) (Figure 4).

A reduction in the inflammatory marker CRP was seen after 12 weeks, but did not reach statistical significance ($p < 0.22$).

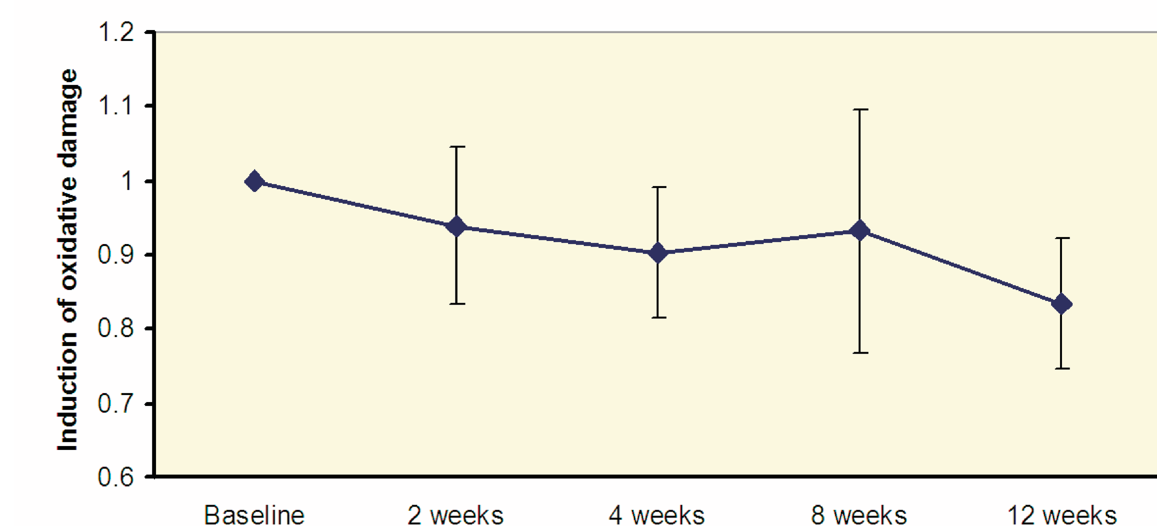

Figure 4. The relative changes in serum antioxidant activity, as measured by the CAP-e cell-based antioxidant protection assay, are shown. Each study participant's serum samples were tested in quadruplicate. The level of oxidative damage that could be induced in the presence of a person's baseline serum was set to "1". Data from later visits were normalized to the baseline. The averages of the normalized values are shown for each blood draw. The vertical bars indicate the standard deviation. The test evaluates whether the addition of serum helps protect cells from oxidative damage in vitro. Serum samples obtained after consumption of MVA contained more antioxidants able to enter into and protect cells from oxidative damage. The improvement was statistically significant ($p < 0.05$).

Conclusions

We conclude that consumption of MVA contributes to quantifiable improvements in motility and relief of pain. This is likely due to an increase in antioxidant activity against oxidative stress, leading to a decrease in inflammatory status.

Acknowledgements

The study was performed at NIS Labs, an independent contract research lab. The study was sponsored by MonaVie LLC, Utah.

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What is MonaVie Pulse?

Helps maintain existing healthy cholesterol levels. MonaVie Pulse delivers added heart health benefits derived from plant sterols* (which studies suggest play a key factor in lowering cholesterol), resveratrol, and omega 3 fatty acids. Scientifically formulated with your heart in mind, this delicious formula offers key nutritional support to those watching their cholesterol.

- Boasts heart healthy levels of plant sterols to help maintain existing healthy cholesterol levels.

- Features resveratrol, which supports your cardiovascular system by helping protect healthy blood vessels.

- Delivers powerful antioxidant polyphenols to promote a healthy cardiovascular system.

* Foods containing at least 0.4 grams per serving of plant sterols, eaten twice a day with meals for a daily total intake of at least 0.8 grams and as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease. Two servings (4 ounces) of MonaVie Pulse contain 0.8 grams of plant sterols.

What is a serving size of MonaVie Pulse juice and MonaVie Pulse Gel?

Drink two ounces of MonaVie Pulse juice twice daily. One serving (two ounces) provides 0.4 grams of plant sterols. For Pulse Gel, take one packet twice daily. One packet (one serving) also provides 0.4 grams of plant sterols.

How does the amount of acai found in Pulse compare to Original and Active? Does it contain more or less acai?

Acai remains as the crown jewel and is the primary ingredient in MonaVie Pulse. The amount of acai in MonaVie Pulse is equivalent to MonaVie Active and Original.

Why were plant sterols (phytosterols), omega-3 fatty acids, and resveratrol added? Aren't these already found in açai?

Plant sterols, often called phytosterols, are naturally occurring fats found in plants. Açai, a source of healthy fats, does contain some phytosterols. They are found in MonaVie Active and Original, but by adding more plant sterols to MonaVie Pulse, the product is able to support a healthy heart and maintain existing healthy cholesterol levels.

What new fruits have been added to MonaVie Pulse?

Concord grape, raspberry, strawberry, blackberry, elderberry, yumberry, tart cherry, prickly pear, pineapple, and cupuaçu.

Why did MonaVie choose to add these fruits to the Pulse juice blend?

Concord grape, raspberry, strawberry, blackberry, elderberry, yumberry, and tart cherry were identified for their antioxidant content, especially polyphenols. Diets rich in fruits that provide a variety of polyphenol antioxidants have been shown to benefit overall health as well as the maintenance of cardiovascular health. Cupuaçu and yumberry help keep MonaVie on the cutting-edge of nutritional trends. Native to the Amazon Rainforest, cupuaçu has been praised as one of the next great superfruits for its nutritional properties. Pineapple and prickly pear have been added to enhance MonaVie Pulse's flavor and nutritional profile.

What are plant sterols?

Many plants include some substance known as plant sterols, phytochemical compounds found in the fatty tissues of plants. Wheat germ, sesame oil, wheat bran, and Brussels sprouts are rich in plant sterols. When plant sterols are consumed, they compete with cholesterol for absorption in the body. However, it is difficult to consume enough of these foods on a daily basis to derive enough plant sterols to maintain healthy cholesterol levels.

How do plant sterols work?

From your body's point of view, plant sterols look a lot like cholesterol. Studies suggest that as you eat a meal with sufficient amounts of plant sterols, the body absorbs less cholesterol because plant sterols actually compete with cholesterol for absorption in the body. Foods containing at least 0.4 g per serving of plant sterol esters, eaten twice a day with meals for a daily total intake of at least 0.8 g, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease. Two servings of MonaVie Pulse juice (4 oz.) supply 0.8 g of plant sterols.

How long do I need to consume MonaVie Pulse before changes are seen?

Studies have been conducted on the plant sterols contained in MonaVie Pulse. In these studies, cholesterol-lowering benefits were detected after consuming 0.8 grams of plant sterols daily for 4 weeks or more. MonaVie is currently conducting a study to determine whether Pulse, which contains 0.8 grams of plant sterols in 4 oz. of juice, is able to produce the same benefits over the same amount of time.

Are plant sterols safe?

Numerous human and toxicological studies conducted with plant sterols have shown no adverse effects in most populations. However, if you have a specific concern, health condition, or question regarding the consumption of plant sterols, we recommend you consult with your physician prior to consuming MonaVie Pulse.

Is MonaVie Pulse safe for women who may be pregnant or lactating?

If you are pregnant or lactating and have a specific concern or question about consuming MonaVie Pulse, we recommend you consult with your physician prior to consuming the product.

MonaVie goes to great lengths to ensure its products are safe. This includes using ingredients with Generally Recognized As Safe (GRAS) status as required by the FDA prior to sale. MonaVie also ensures that its products are manufactured according to Current Good Manufacturing Practices as required by the FDA.

In addition to these requirements, MonaVie employs rigorous testing to ensure its products meet its pre-determined high quality standards for safety and purity. However, despite our confidence in the safety of MonaVie Pulse, MonaVie does not replace the advice of a physician.

Can I take MonaVie Pulse with my cholesterol-lowering medication?

Although MonaVie products are safe for consumption as food products, you should consult with your physician if you are concerned about ingredient contraindications.

Can I take Pulse with Active and/or Original? If I take both, how should I take them?

Yes, you can drink MonaVie Pulse with MonaVie Active or MonaVie Original. To get the full joint health benefits from MonaVie Active, you will need to drink 4 ounces of MonaVie Active as directed; to get the full benefits from the plant sterols, you will need to drink 4 ounces of MonaVie Pulse as directed.

Can children consume MonaVie Pulse?

MonaVie Pulse is targeted for health-conscious men and women who are concerned with cholesterol levels, heart health, and overall nutrition and well-being.
Monavie Pulse FAQ

Why should I be concerned about my cholesterol?

According to the American Heart Association, elevated blood cholesterol is a major risk factor in cardiovascular health. Nearly 100 million Americans have elevated cholesterol (>200 mg/dL) and, oftentimes, they don't even know it. Taking action now to maintain healthy cholesterol levels will help prevent such problems from arising.

Cholesterol levels can often be modified by lifestyle changes, such as eating a diet low in saturated fat and cholesterol, and high in whole grains, fruits, and vegetables. MonaVie Pulse contains plant sterols, which studies suggest play a key role in maintaining healthy cholesterol levels already within the normal range. Consuming plant sterols as a regular part of a healthy diet low in saturated fat and cholesterol provides another natural option in the maintenance of cardiovascular health.

Do governments or professional health associations recommend consuming plant sterols?

In the US, the National Cholesterol Education Program (NCEP) suggests the use of plant sterols in conjunction with other lifestyle changes produces positive effects on cholesterol levels achieved through dietary means. (The NCEP is a branch of the National Heart, Lung, and Blood Institute of the National Institutes of Health.)

Have any clinical trials been done with plant sterols?

Plant sterols (phytosterols) have been clinically evaluated in many different populations, under different circumstances over the past 50 years. In a number of studies plant sterols have demonstrated the ability to improve cholesterol levels in humans. The health benefits of phytosterols have been well established, and public health authorities, such as the US National Cholesterol Education Program (NCEP), are beginning to include phytosterols as part of their dietary recommendations (NCEP, 2001).

What if I am concerned about cholesterol, joint health and antioxidant nutrition, but I only want to use one product? Which product should I take?

All of the MonaVie products contain açai and a blend of 18 additional body-beneficial fruits. MonaVie Original is for individuals mainly interested in antioxidant nutrition and aging. Built on the foundation of MonaVie Original, MonaVie Active also provides the additional benefits of glucosamine for joint health. MonaVie Pulse offers a potent blend of antioxidant nutrients for cardiovascular health and enough plant sterols (.8 grams per 4 oz, or 2 packets) to help maintain existing healthy cholesterol levels.

What nutrition information is available for MonaVie Pulse Juice?

Label Information, MonaVie Pulse juice

100% Juice

Nutrition Facts

Serving Size: 2 fl. oz. (60 ml)

Calories: 45

Calories from Fat: 10

Total Fat: 1 g 1%*

Potassium: 80 mg, 2%

Sodium: 30 mg 0%*

Total Carbohydrate: 8 g 3%*

Dietary Fiber: <1 g 2%*

Sugars: 7 g

Protein: <1 g 0%*

Vitamin A: 4%*

Vitamin C: 20%*

Vitamin K: 15 mcg, 20%

Iron: 2%*

* Percent Daily Value based on a 2,000 calorie diet.

Not a significant source of saturated fat, trans fat, cholesterol, or calcium.

Ingredients: Proprietary blend of açai (freeze-dried powder, puree), reconstituted fruit juice blend (concord grape blend, pineapple, apple, prickly pear, pomegranate, elderberry, yumberry, bilberry, blackberry, blueberry, cherry, cranberry, raspberry, aronia), puree fruit blend (acerola, strawberry, cupuaçu, camu camu), plant sterols (emulsified with corn syrup solids, polyglycerol esters of fatty acids, gum acacia), Apple Phyto-Phenolics® (polyphenol blend), omega-3 (cranberry seed oil), resveratrol, natural flavors, potassium sorbate (preservative), sodium benzoate (preservative), citric acid.

MONAVIE E$^{MV™}$ and MONAVIE E$^{MV™}$ LITE

PRODUCT INFORMATION PAGE

For use in the United States only.

Recharge your body and mind with a boost of sustained energy. MonaVie E^{MV} and MonaVie E^{MV} Lite feature a proprietary blend of antioxidant rich fruits, including the superfruits açai and maqui, and natural sources of energy. Great tasting and lightly carbonated, these healthy formulas increase performance, endurance, and concentration by kicking up your energy level and keeping it there—without a subsequent crash. Finally, two healthy solutions to your body's everyday energy needs.*

KEY BENEFITS

- Scientifically formulated to provide longer lasting energy without the unhealthy "high" and "low" typically associated with energy drinks.*
- Features healthy, all natural sources of energy to increase vigor, performance, alertness, and endurance.*
- Contains no artificial flavors, colors, sweeteners, or preservatives.

ESSENTIAL FACTS

- In independent taste tests, both MonaVie E^{MV} and MonaVie E^{MV} Lite were ranked No. 1 over leading energy drinks and preferred for color, sweetness, and aftertaste.
- A market analysis of 30 energy drinks in the retail and direct sales channels, including the top five selling products, showed that 97 percent contain artificial colors, sweeteners, flavors, and/or preservatives.
- Over the next five years, the U.S. energy drink market is expected to nearly double. *(Euromonitor International: Country Sector Briefing, April 2008.)*

LONGER LASTING ENERGY

Go the distance with MonaVie E^{MV} and MonaVie E^{MV} Lite. Fortified with 100 percent natural sources of energy, these advanced formulas feature Palatinose,™ a carbohydrate energy source found naturally in honey, sugar cane, and sugar beets. Scientifically shown to promote longer lasting energy, Palatinose metabolizes more slowly than sucrose and high fructose corn syrup—typical ingredients in other energy drinks—promoting a steady stream of energy over a longer period of time.*

ON THE LIGHTER SIDE

MonaVie E^{MV} Lite offers a delicious, reduced calorie alternative to traditional energy drinks. Unlike MonaVie E^{MV}, MonaVie E^{MV} Lite contains 20 percent fruit juice and is naturally sweetened with calorie free stevia (Reb-A 99%)—the purest stevia leaf extract available. It's a healthy, lighter way to meet your body's everyday energy needs.

*These statements have not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, or prevent any disease.

FREQUENTLY ASKED QUESTIONS

WHAT IS MONAVIE E^{MV} AND MONAVIE E^{MV} LITE?

MonaVie E^{MV} and MonaVie E^{MV} Lite are healthy energy drinks made with antioxidant rich fruits, including the superfruits açai and maqui. These refreshing beverages deliver a unique blend of ingredients to keep you fully charged. Unlike most energy drinks that are loaded with synthetic stimulants, MonaVie E^{MV} provides a healthy energy alternative that enhances performance, boosts endurance, increases alertness, and reduces fatigue. MonaVie E^{MV} Lite is for the calorie-conscious consumer. With 75 calories per serving and all-natural sources of caffeine, it delivers a powerful blend that fuels the body and mind. Drink MonaVie E^{MV} and MonaVie E^{MV} Lite for a jitter-free, sustained energy boost.

HOW DOES MONAVIE E^{MV} LITE DIFFER FROM MONAVIE E^{MV}?

MonaVie E^{MV} contains 80 percent fruit juice, whereas MonaVie E^{MV} Lite contains 20 percent. In addition, MonaVie E^{MV} Lite is sweetened with stevia (Reb-A 99%), an all natural, zero-calorie sweetener, making it a great option for those who are looking for a boost of energy with fewer calories.

WHAT CLAIMS CAN I MAKE ABOUT MONAVIE E^{MV} AND MONAVIE E^{MV} LITE?

- Natural energy source.
- Enhances performance.
- Supports endurance.
- Increases energy.
- Promotes alertness, mental clarity, and focus.
- Provides longer lasting energy.
- Healthy. Sustained. Energy.™

DOES MONAVIE E^{MV} AND MONAVIE E^{MV} LITE CONTAIN CAFFEINE?

Yes, both MonaVie E^{MV} and MonaVie E^{MV} Lite contain natural sources of caffeine from guarana, green tea, and yerba mate. They are free of synthetically made caffeine.

WHAT FRUITS ARE USED IN MONAVIE E^{MV} AND MONAVIE E^{MV} LITE?

There are eight fruits in MonaVie E^{MV} and MonaVie E^{MV} Lite: açai, maqui, apple, grape, pear, acerola, cupuaçu, and camu camu.

WHAT IS STEVIA?

Stevia is an all natural, zero-calorie sweetener extracted from the leaves of a South American plant, *Stevia rebaudiana*. This plant originates from areas in Paraguay and Brazil, where it has been used for centuries as a maté tea sweetener. Stevia extracts have been widely used in many countries as a sugar substitute, but the Reb-A 99% stevia in MonaVie E^{MV} Lite is the purest form available.

FORMULATION

MONAVIE E^{MV}

Healthy. Sustained. Energy.™

80% Juice. No artificial flavors, colors, sweeteners, or preservatives.

Supplement Facts

Serving Size: 1 can, 8.4 fl. oz. (250 ml)

Amount Per Serving		% Daily Value
Calories	160	
Total Carbohydrate	40 g	13%*
Sugars (from fruit)	22 g	
Palatinose™	15 g	
Vitamin C	60 mg	100%
Niacin	40 mg	200%
Vitamin B_6	4 mg	200%
Vitamin B_{12}	12 mcg	200%
Pantothenic Acid	20 mg	200%
Sodium	20 mg	<1%
Proprietary Energy Blend	16,107 mg†	

Palatinose, D-ribose, green tea extract (leaf)§, guarana extract (seed)§, cha de burge extract (leaf), maca extract (root), yerba mate extract (leaf)§, panax ginseng extract (root), L-tyrosine.

*% Daily Values are based on a 2,000 calorie diet.
†Daily Value not established.

Other Ingredients: Reverse osmosis carbonated water, proprietary fruit blend (açai, maqui), reconstituted fruit juice blend (apple, grape, pear), fruit puree blend (acerola, cupuaçu, camu camu), acerola extract (fruit), natural flavor, citric acid, xanthan gum, pectin, niacinamide, calcium pantothenate, pyridoxine hydrochloride, cyanocobalamin.

MONAVIE E^{MV} LITE

Healthy. Sustained. Energy.™

20% Juice. No artificial flavors, colors, sweeteners, or preservatives.

Supplement Facts

Serving Size: 1 can, 8.4 fl. oz. (250 ml)

Amount Per Serving		% Daily Value
Calories	75	
Total Carbohydrate	18 g	6%*
Sugars (from fruit)	7 g	
Palatinose™	10 g	
Vitamin C	60 mg	100%
Niacin	40 mg	200%
Vitamin B_6	4 mg	200%
Vitamin B_{12}	12 mcg	200%
Pantothenic Acid	20 mg	200%
Sodium	20 mg	<1%
Proprietary Energy Blend	11,165 mg†	

Palatinose, guarana extract (seed)§, D-ribose, green tea extract (leaf)§, cha de burge extract (leaf), maca extract (root), yerba mate extract (leaf)§, panax ginseng extract (root), L-tyrosine.

* % Daily Values are based on a 2,000 calorie diet.
† Daily Value not established.

Other Ingredients: Reverse osmosis carbonated water, proprietary fruit blend (açai, maqui), reconstituted fruit juice blend (grape, apple, pear), malic acid, fruit puree blend (acerola, cupuaçu, camu camu), citric acid, natural flavor, xanthan gum, acerola extract (fruit), pectin, stevia extract (Reb-A 99%), niacinamide, calcium pantothenate, pyridoxine hydrochloride, cyanocobalamin.

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AIBMR Life Sciences, Inc.

Natural and Medicinal Products Research

Effects of MonaVie Active on Antioxidant Capacity in Humans

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Free radicals are highly reactive molecules that are associated with the degenerative aging process. Free radicals steal electrons from healthy cells causing cellular damage. They are byproducts of normal metabolic processes in the body and are produced by exposure to environmental pollutants including cigarette smoke, smog, harmful chemicals, toxins, and sunlight. Antioxidants found in fruits and vegetables help counter the effects of the damaging free radicals.

A study conducted by an independent laboratory in the United States commissioned by the Natural and Medicinal Products Research division of AIBMR Life Sciences was performed to determine whether drinking MonaVie Active affects antioxidant activity and or levels in human serum. The study began with a pilot study that determined which assays and follow-up schedule to use.

The main study used two measures of antioxidant activity: 1) the Thiobarbituric Acid Reactive Substances (TBARS) assay, a well-established method for screening and monitoring of lipid peroxidation; and, 2) the Reactive Oxygen Stress in Red Blood Cells (ROS RBC) assay that measures the ability of antioxidants to enter into a living cell and protect it from damage.

Clinical Study

Methods. We employed a crossover study with twelve participants, all of whom took both MonaVie Active and a placebo at two separate stages in the study. All participants were told they were consuming MonaVie Active. Each treatment was separated by a seven-day washout period where the participants did not consume MonaVie Active. The placebo consisted of two capsules of potato flakes dyed purple to look like MonaVie Active. Blood samples were drawn from the subjects prior to consuming MonaVie Active and placebo, and at one and two hours following consumption, to determine baseline antioxidant levels. Subjects were randomized to receive either 4 ounces of MonaVie Active or placebo first. Results were compared and contrasted on an individual basis and on a group basis.

Results. The TBARS measure of lipid peroxidation shows more antioxidant activity when the subjects were consuming MonaVie than when consuming the placebo. An inspection of individual subjects revealed that 83.3% of subjects had a decrease (relative to the placebo) in lipid peroxidation after taking MonaVie. A treatment by repeated measures analysis of variance showed this positive effect to be statistically significant at ($p = 0.04$).

Likewise, the ROS RBC cell-based assay revealed that when subjects consumed MonaVie Active they had a rise in antioxidant activity at both one and two hours after consumption. An inspection of individual results revealed that 82% of subjects had a small to moderate increase (relative to placebo) in antioxidant activity within cells. A treatment by repeated measures analysis of variance showed this effect to be statistically significant ($p = 0.03$).

In summary, MonaVie Active has not only shown experimentally to increase antioxidant activity in human serum, leading to a decrease in lipid peroxidation in the blood stream, but there is also evidence that MonaVie Active provided antioxidants that are able to penetrate cells. This study confirms that by drinking four ounces of MonaVie Active containing Brazilian acai berry, the subjects had a significant increase in their antioxidant capacity and inhibition in lipid peroxidation, which reduces the risk of cholesterol being oxidized. Oxidized cholesterol has been shown to contribute to damage to the internal lining of the arteries.

Complete and final results of this study are expected to be published in scientific literature in 2008 after presentation of the findings at an international scientific nutritional symposium in the fall of 2007.

Important Note: This study was conducted in a relatively small number of healthy adults. Further studies with larger sample sizes are needed before these results can be generalized to the population-at-large.