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Safety and whole-body antioxidant potential of a novel anthocyanin-rich formulation of edible berries

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Abstract

Edible berry extracts rich in anthocyanins possess a broad spectrum of therapeutic, pharmacologic and anti-carcinogenic properties. Six berry extracts (wild blueberry, bilberry, cranberry, elderberry, raspberry seeds and strawberry), singly and in combination, were studied in our laboratories for antioxidant efficacy, cytotoxic potential, cellular uptake and anti-angiogenic properties. Combinations of edible berry extracts were evaluated to develop a synergistic formula, OptiBerry, which exhibited high oxygen radical absorbance capacity (ORAC) value, low cytotoxicity and superior anti-angiogenic properties compared to the other combinations tested. The current study sought to determine the broad spectrum safety and antioxidant potential of OptiBerry *in vivo*. Acute oral LD_{50} of OptiBerry was greater than 5 g/kg in rats. Acute dermal LD_{50} of OptiBerry was greater than 2 g/kg. No changes in the body weight or adverse effects were observed following necropsy. Primary skin and eye irritation studies were conducted in New Zealand albino rabbits. OptiBerry was classified as slightly irritating to the skin (primary skin irritation index 0.3) and minimally irritating to the eye (maximum mean total score 6.0). The antioxidant potential of OptiBerry was investigated in rats and mice by assessing GSH redox status in tissues as well as by a unique state-of-the-art electron paramagnetic resonance (EPR) imaging of whole-body redox status. A clinically relevant hyperbaric oxygen (HBO) exposure system (2 atm, 2 h) was employed to study the antioxidant properties of OptiBerry. OptiBerry feeding (8 weeks) significantly prevented HBO-induced GSH oxidation in the lung and liver of vitamin E-deficient Sprague Dawley rats. Furthermore, OptiBerry-fed mice, when exposed to HBO, demonstrated significant protection in whole-body HBO-induced oxidation compared to the unfed controls by EPR imaging. Taken together, these results indicate that OptiBerry is reasonably safe and possess antioxidant properties. (Mol Cell Biochem **281:** 197–209, 2006)

Key words: berry anthocyanins, glutathione, hyperbaric oxygen, *in vivo*, OptiBerry, oxidative stress, safety studies

Introduction

Nutrition is a major tool in health preservation and disease prevention. The therapeutic property of edible berries has been long known [1]. More recently, it has been observed that edible berries may have potent chemopreventive properties [2–6]. Berries are rich in anthocyanins, flavonoid glycosides, responsible for the red, violet, purple and blue color

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of the fruit. Dietary consumption of anthocyanin improve the overall antioxidant defense status of human plasma [7]. On one hand, the search is on for specific medical drugs that would efficiently limit angiogenesis [8, 9]. On the other hand, diet-based approaches to limit angiogenesis are being actively explored [2, 3, 10–15]. Recently, we studied the anti-angiogenic properties of several edible berries and developed a mixture (OptiBerry) of such extracts that exhibit potent effects *in vitro* [16]. Next, we developed a novel *in vivo* model to study the mechanisms regulating angiogenic outcomes [17, 18]. OptiBerry proved to be clearly effective in limiting angiogenesis *in vivo* by regulating the expression of inducible MCP-1 [17–19]. These favourable developments with OptiBerry led to the current study aimed at testing the safety and antioxidant efficacy of edible berries *in vivo*.

Safety is a key concern for dietary supplements [20]. Although OptiBerry represents a novel formulation wholly derived from edible berries [16], we undertook to systematically test OptiBerry for safety *in vivo*. Edible berries are known to possess potent antioxidant properties [21]. Beyond testing the safety of OptiBerry, we tested whether the berry mix was able to protect against oxidant insult *in vivo*. Hyperbaric oxygen (HBO) therapy poses risk of oxygen toxicity [22]. We employed a clinically relevant HBO exposure system to investigate the antioxidant properties of OptiBerry. The effect of OptiBerry feeding for 8 weeks on HBO-induced GSH oxidation in the lung and liver of vitamin E deficient Sprague Dawley rats was examined. We also employed a state-of-the-art electron paramagnetic resonance (EPR) imaging approach to test the effect of HBO and OptiBerry feeding on the whole body redox status of mice immediately after exposure to HBO.

Materials and methods

Chemicals

The anthocyanin-rich combination of berry extract, OptiBerry BX-600 (Lot no. 302019, InterHealth Nutraceuticals, Benicia, CA), used in this study is a standardised blend of wild blueberry, (*Vaccinium angustifolium* (fruit) extract), strawberry *Fragaria chiloensis* (fruit) powder, cranberry *Vaccinium macrocarpon* (fruit) powder, wild bilberry *Vaccinium myrtillus* (fruit) extract, elderberry *Sambucus nigra* (fruit) extract and raspberry *Rubus idaeus* (seed) powder (patent-pending). Unless otherwise stated, all the chemicals and biochemicals used in this study were obtained from Sigma Chemical Company (St. Louis, MO). 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl (carbamoyl-PROXYL) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI).

Animals and treatment

All safety testing were conducted at the Product Safety Laboratories (East Brunswick, NJ, USA) in compliance with the Good Laboratory Practices (GLP) as defined in 21 CFR 58, 1987: US Food and Drug Administration (FDA) GLP Standards and in accordance with Organization for Economic Co-operation and Development (OECD) Guidelines for Testing of Chemicals, Procedures 402 (February, 1987), 404 (April, 2002), 405 (April, 2002), 425 (December, 2001). Detailed animal protocols are provided in individual toxicological assessment.

Acute oral toxicity

The acute oral toxicity evaluation (up and down procedure) was conducted with rats to determine the potential of OptiBerry to produce acute oral toxicity from a single dose *via* the oral route in compliance with the good laboratory practices (GLP) as defined in 21 CFR 58, 1987: US FDA GLP Standards and in accordance with OECD guidelines for testing of chemicals, procedure 425, adopted December, 2001. Three healthy young adult female, nulliparous and non-pregnant albino Sprague Dawley rats (aged 9–10 weeks old, initial body weight 188–197 g) were obtained from Ace Animals, Inc. (Boyertown, PA), and singly housed in suspended stainless steel cages with mesh floors which conform to the size recommendations in the most recent Guide for the Care and Use of Laboratory Animals DHEW (NIH). Litter paper was placed beneath the cage and was changed at least three times per week. The rats had free access to standard rat chow (Purina Rodent Chow# 5012) and filtered tap water ad libitum, and maintained at a controlled temperature (20–24 °C) and light cycle (12 h light/12 h dark). The animals were acclimated to the laboratory conditions for at least 10–14 days prior to initiation of dosing.

Before each dosing, rats were fasted overnight and examined through the fasting period for health and weight (initial). Individual doses were calculated based on initial body weights at a dose level of 5000 mg/kg. The test substance was administered as a 25% w/w suspension in distilled water using a stainless steel ball-tipped gavage needle. Following administration, each animal was returned to its designated cage and the feed was replaced 3–4 h after dosing. Individual body weights were recorded again on days 7 and 14 (termination) after dosing. The animals were observed for mortality, signs of gross toxicity and behavioural changes at least once daily for 14 days after dosing. Observations included gross evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavioral pattern. Particular attention was directed to observations of tremors, convulsions,

salivation, diarrhea and coma. All rats were euthanised by CO_2 inhalation at the end of the 14-day observation period and gross necropsies were performed on all animals. Tissues and organs of the thoracic and abdominal cavities were examined.

Acute dermal toxicity

The acute dermal toxicity evaluation was conducted in rats to determine the potential for OptiBerry to produce toxicity from a single topical application in compliance with 21 CFR 58, 1987: US FDA GLP Standards and in accordance with OECD guidelines for testing of chemicals, procedure 402, adopted February, 1987. Five healthy young adult albino Sprague Dawley male rats (aged 10–11 weeks old, initial body weight 290–307 g) and five young adult female, nulliparous and non-pregnant albino Sprague Dawley rats (aged 10–11 weeks old, initial body weight 200–215 g) were obtained from Ace Animals, Inc. (Boyertown, PA), and singly housed in suspended stainless steel cages with mesh floors which conform to the size recommendations in the most recent Guide for the Care and Use of Laboratory Animals DHEW (NIH). Litter paper was placed beneath the cage and was changed at least three times per week. The rats had free access to standard rat chow (Purina Rodent Chow no. 5012) and filtered tap water ad libitum, and maintained at controlled temperature (19–23 °C) and light cycle (12 h light/12 h dark). The animals were acclimated to the laboratory conditions for 21 days.

On the day prior to application, the five male and five female animals were prepared by clipping (Oster model no. A5-small) the dorsal area and the trunk. After clipping and prior to application, the animals were examined for health and weight (initial) and the skin checked for any abnormalities. Individual doses were calculated based on the initial body weights, taking into account the concentration of the test mixture. Prior to application, OptiBerry was moistened with distilled water to achieve a dry paste by preparing a 75% w/w mixture. OptiBerry (2 g/kg of body weight) was then applied to a 2 in. × 3 in., 4-ply gauze pad and placed on a dose area of approximately 2 in. × 3 in. (approximately 10% of the body surface). The gauze pad and entire trunk of each animal were then wrapped with 3 in. Durapore tape to avoid dislocation of the pad and to minimize loss of the test substance (OptiBerry). The rats were then returned to their designated cages. The day of application was considered as day 0 of the study. After 24 h of exposure of the test substance (OptiBerry), the pads were removed and the test sites were gently cleansed of any residual test substance. Individual body weights of the animals were recorded prior to OptiBerry application (initial) and again on days 7 and 14 (termination). The animals were observed for mortality, signs of gross toxicity and behavioral changes during the first several hours after application and at least once daily thereafter for 14 days. Observations included gross evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavioral pattern. Particular attention was directed to observations of tremors, convulsions, salivation, diarrhea and coma. All the rats were euthanised *via* CO_2 inhalation on day 14. Gross necropsies were performed on all animals at terminal sacrifice. Tissues and organs of the thoracic and abdominal cavities were examined.

Primary skin irritation

The primary skin irritation test was conducted on two young adult male New Zealand albino rabbits and one young nulliparous non-pregnant female New Zealand albino rabbits (aged 13 weeks old, initial body weight range at receipt 2.3–2.4 kg, estimated body weight at the start of the experiment was 2.8–3.2 kg) to determine the potential for OptiBerry to cause irritation after a single topical application in compliance with 21 CFR 58, 1987: US FDA GLP Standards and in accordance with OECD guidelines for testing of chemicals, procedure 404, adopted in April of 2002. The rabbits were obtained from Robinson Services, Inc. (Clemmons, NC), and singly housed in suspended stainless steel cages with mesh floors which conform to the size recommendations in the most recent Guide for the Care and Use of Laboratory Animals DHEW (NIH). Litter paper was placed beneath the cage and was changed at least three times per week. The rabbits were allowed free access to lab chow (Purina Rabbit Chow no. 5326, St. Louis, MO) and filtered tap water ad libitum, and maintained at controlled temperature (20–22 °C) and light cycle (12 h light/12 h dark). Animals were acclimated to the laboratory conditions for a period of 28 days prior to initiation of dosing.

On the day before application, rabbits were prepared by clipping (Oster model no. A5-small) the dorsal area and the trunk. On the day of dosing, but prior to application, the rabbits were critically examined for health and the skin checked for any abnormalities, and three healthy rabbits without preexisting skin irritation were selected for the test. Individual doses were calculated based on the initial body weights, taking into account the concentration of the test mixture. On the day of application (day 0), OptiBerry was moistened with distilled water to achieve a dry paste by preparing a 75% w/w mixture. Five-tenths of a gram of OptiBerry (0.67 g of test mixture) was placed on a 1 in. × 1 in., 4-ply gauze pad and applied to a 6 cm^2 intact dose site on each rabbit. The pad and the entire trunk of each rabbit were then wrapped with semi-occlusive 3 in. Micropore tape to avoid dislocation of the pad. Elizabethan collars were placed on each rabbit and they were

Table 1. Primary dermal irritation study of albino rabbits: Scoring criteria for dermal reactions[a] [24]

	Evaluation of dermal reactions	
Value	Erythema and eschar formation	Edema formation
0	No erythema	No edema
1	Very slight erythema (barely perceptible), edges of area not well defined	Very slight edema (barely perceptible) edges of area not well defined
2	Slight erythema (pale red in color and edges definable)	Slight edema (edges of area well defined by definite raising)
3	Moderate to severe erythema (defined in color and area well defined)	Moderate edema (raised approximately 1 mm)
4	Severe erythema (beet to crimson red) to slight eschar formation (injuries in depth)	Severe edema (raised more than 1 mm and extending beyond area of exposure)
4	Total possible erythema score	Total possible edema score

[a]Eight total possible primary irritation score.

returned to their designated cages. After 4 h of exposure to OptiBerry, the pads and collars were removed and the test sites were gently cleansed of any residual test substance.

Individual dose sites were scored according to the Draize scoring system (Table 1) [23, 24] at approximately 1, 24, 48, and 72 h after patch removal. The classification of irritancy was obtained by adding the average erythema and edema scores for the 1, 24, 48, and 72 h scoring intervals and dividing by the number of evaluation intervals (4). The resulting primary dermal irritation index (PDII) was classified as shown in Table 2.

The animals were also observed for signs of gross toxicity and behavioural changes at least once daily during the test period. Observations included gross evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern. Particular attention was directed to observation of tremors, convulsions, salivation, diarrhea and coma.

Primary eye irritation

The primary eye irritation test was conducted with rabbits to determine the potential for OptiBerry to produce irritation from a single installation *via* the ocular route in compliance with 21 CFR 58, 1987: US FDA GLP Standards and in accordance with OECD guidelines for testing of chemicals, procedure 405, adopted April, 2002. Three female, nulliparous and non-pregnant New Zealand albino rabbits (aged 13 weeks old, initial body weight range at receipt 2.0–2.3 kg, estimated body weight at the start of the experiment 2.6–3.0 kg) were obtained from Robinson Services, Inc. (Clemmons, NC) and singly housed in suspended stainless steel cages with mesh floors which conform to the size recommendations in the most recent Guide for the Care and Use of Laboratory Animals DHEW (NIH). Litter paper was placed beneath the cage and was changed at least three times per week. The rabbits were allowed free access to lab chow (Purina Rabbit Chow no. 5326, St. Louis, MO) and filtered tap water ad libitum, and maintained at controlled temperature (17–24 °C) and light cycle (12 h light/12 h dark). Animals were acclimated to laboratory conditions for a period of 22 days prior to initiation of dosing.

Table 2. Descriptive rating for mean primary dermal irritation index[a]

Primary Dermal Irritation Index (PDII)	Classification
0	No irritation
>0–2.0	Slight irritation
2.1–5.0	Moderate irritation
>5.0	Severe irritation

[a]US EPA Addenum 3 on data reporting to pesticide assessment guidelines; dermal irritation, January 1988.

Prior to instillation, both the eyes of rabbits were examined using a fluorescein dye procedure. One drop of 2% ophthalmic fluorescein sodium was instilled into both the eyes of each rabbit. The eyes were rinsed with physiological saline (0.9% NaCl) approximately 30 s after installation of the fluorescein. Using an ultraviolet light source, the eyes were checked for gross abnormalities according to the "Scale for Scoring Ocular Lesions" (Table 3) [24]. Three healthy animals without pre-existing ocular irritation were selected for the test. One-tenth of a milliliter (0.04 g) of OptiBerry was instilled into the conjunctival sac of the right eye of each rabbit by gently pulling the lower lid away from the eyeball. The upper and lower lids were then gently held together for about 1 s before releasing, to minimise the loss of the test substance. The left (control) eye of each animal remained untreated and served as a control. The rabbits were then returned to their designated cages. Ocular irritation was evaluated macroscopically using a high-intensity white light

Table 3. Scale for scoring ocular irritation [23]

I. Cornea	
(A) Opacity-degree of density (area most dense taken for reading)	
No ulceration or opacity	0
Dulling of normal luster, details of iris clearly visible	1[b]
Easily discernible translucent areas, details of iris slightly obscured	2[b]
Nacreous areas, no details of iris visible, size of pupil rarely discernible	3[b]
Opaque cornea, iris not discernible through the opacity	4[b]
(B) Area of cornea involved	
No ulceration or opacity	0
One quarter or less but not zero	1
Greater than one quarter, but less than half	2
Greater than half, but less than three quarters	3
Greater than three quarters, up to whole area	4
Total maximum	80[a]
II. Iris	
(A) Values	
Normal	0
Markedly deepened rugae, congestion, swelling, circumcorneal injection (any or all of these or combination thereof), iris still reacting to light (sluggish reaction is positive)	1[b]
No reaction to light, hemorrhage, gross destruction (any or all of these)	2[b]
Total maximum	10[c]
III. Conjuctivae	
(A) Redness (refers to palpebral and bulbar conjunctivae excluding cornea and iris)	
Blood vessels normal	0
Some blood vessels definitely hyperemic (injected above) normal	1
Diffuse, deeper crimson color, individual vessels not easily discernible	2[b]
Diffuse beefy red	3[b]
(B) Chemosis: lids and/or nictitating membranes	
No swelling	0
Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of lids	2[b]
Swelling with lids about half closed	3[b]
Swelling with lids more than half closed	4[b]
(C) Discharge	
No discharge	0
Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to the lids	2
Discharge with moistening of the lids and hair, and considerable area around the eye	3
Total maximum	20[d]
Total Maximum Score Possible	110[e]

[a]Score equals A × B × 5.
[b]Figures indicate positive effect.
[c]Score equals A × 5.
[d]Score equals (A + B + C) × 2.
[e]Total Maximum Score Possible (110) represents the sum of all scores obtained for the cornea, iris and conjunctivae.

(MagLite) in accordance with Draize *et al.* at 1, 24, 48, and 72 h post-instillation (Table 3). The fluorescein eye evaluation was used at 24 h to verify the absence of corneal damage. Individual irritation scores were recorded for each animal. In addition to the observations of the cornea, iris and conjunctivae, any other lesions were noted. The average score for all rabbits at each scoring period was calculated to aid in data interpretation. Time intervals with the highest mean score

Table 4. Descriptive rating of maximum mean total primary eye irritation scores [25]

Maximum mean total score	Classification	Requirement for maintenance of classification
0.0–0.5	No irritation	Up to 0.5 at 1 h with zeros at 24 h; otherwise increase one level
0.6–2.5	Practically no irritation	With zeros at 24 h; otherwise increase one level
2.6–15.0	Minimal irritation	With zeros at 48 h; otherwise increase one level
15.1–25.0	Mild irritation	With zeros at 96 h; otherwise increase one level
25.1–50.0	Moderate irritation	With 7 days mean $\leq$20 and individual total scores $\leq$10 in at least 60% of rabbits with no total score $>$ 30; otherwise, increase one level
50.1–80.0	Severe irritation	With 7 days mean $\leq$40 and individual total scores $\leq$30 in at least 60% of rabbits with no total score $>$ 60; otherwise increase one level
80.1–100.0	Extreme irritation	With 7 days mean $\leq$80 and individual total scores $\leq$60 in at least 60% of rabbits with no total score $>$ 100; otherwise increase one level
100.1–110	Maximal irritation	With 7 days mean $>$80 and individual total scores $>$60 in at least 60% of rabbits; otherwise decrease one level

(maximum mean total score – MMTS) for all rabbits were used to classify the test substance (OptiBerry) by the system of Kay and Calandra (Table 4) [25].

The animals were also observed for signs of gross toxicity and behavioural changes at least once daily during the test period. Observations included gross evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern. Particular attention was directed to the observation of tremors, convulsions, salivation, diarrhea and coma.

Evaluation of antioxidant properties

Animals and dietary supplementation protocol for testing antioxidant properties. Male and female rats (aged 6 weeks) (Sprague-Dawley Harlan, Indianapolis, IN, USA) were divided into the following three groups: (i) E^-/HBO (vitamin E deficient, placebo fed, exposed to HBO insult; HBO, 24 h prior to tissue harvest exposed to pure O_2 at 2 atm for 2 h) group; male ($n = 5$) and female ($n = 3$) rats). IACUC (Institutional Animal Care and Use Committees) approval for this experiment was obtained from the Ohio State University Medical Center (approved protocol no. 00A0173 and 2005A0006). Rats were fed on a vitamin E deficient diet (TD 88163, Harlan; α-tocopherol levels below detection limits). (ii) E^-/OptiBerry/HBO group (vitamin E deficient, OptiBerry fed, exposed to HBO insult; male ($n = 5$) and female ($n = 3$) rats) (iii) E^+/control rats (vitamin E sufficient standards laboratory diet, not exposed to HBO or not fed with OptiBerry; male ($n = 3$) and female ($n = 3$) rats). The OptiBerry-fed rats were supplemented with an OptiBerry suspension (40 mg/ml in H_2O, aliquoted and stored in $-20\,^{\circ}C$). Body weights were taken weekly and the animals gavaged accordingly at 20 mg/kg dose for 8 weeks. Placebo rats were gavaged with H_2O for 8 weeks as well. At 8 weeks of treatment (14 weeks of age) the OptiBerry and placebo treated rats were exposed to HBO (hyperbaric oxygen). Tissue harvest followed 24 h later.

Glutathione assay

GSH was detected using an HPLC coulometric electrode array detector (CouloArray Detector Model 5600 with 12 channels, ESA Inc., Chelmsford, MA). Sample preparation, mobile phase and column used for HPLC glutathione assay were as previously described [26]. As an improvement to the previously reported methods, the current method implemented a coulometric electrode array detector for the detection of glutathione. This system uses multiple channels with different redox potentials [27]. Glutathione was detected at channels set at the following potential: (i) 600, (ii) 700 and (iii) 800 mV. Signals from the channel set at 800 mV were used for quantification.

Vitamin E extraction and analysis

Vitamin E extraction and analysis from the liver was performed as described previously using a HPLC-coulometric electrode array detector (CouloArray Detector model 5600 with 12 channels; ESA Inc., Chelmsford, MA, USA) [27, 28]. This system uses multiple channels with different redox-potentials. α-Tocopherol was detected on a channel set at 200 mV.

EPR imaging of whole-body redox status in mice

A unique, state-of-the-art technique was developed to evaluate the antioxidant potential of OptiBerry against HBO-induced oxidative stress in mice in a whole-body scenario. Vitamin E deficient mice (aged 8 weeks) (Harlan, Indianapolis, IN, USA) were kept on an E-deficient diet as above for 7 days before EPR imaging was performed. IACUC approval for this experiment was obtained from the Ohio State University Medical Center. HBO treated group was subjected to hyperbaric oxygen at 2 atm pressure for 2 h in a chamber before administration of anesthesia, whereas the control group was kept in the room air all the time. Mice in each group were anesthetised by intra-peritoneal injection of ketamine (90 mg/kg body weight) and xylazine (20 mg/kg). 3-Carbamoyl PROXYL solution (150 mg/kg of body weight) was injected into the tail vein and the mouse was placed in prone position in a quartz tube of 3.5 cm diameter. EPR spectra were immediately taken on the body of the mouse at regular intervals of 4 min and 16 projections were taken for each time point for 2D imaging of redox status. The measurements were done on a custom-built EPR spectrometer [29] with 750 MHz microwave bridge unit and a 40 mm diameter loop gap re-entrant resonator. The microwave power was set at 32 mW, and the modulation frequency was 100 kHz. Spectral acquisitions were performed using custom-developed data acquisition software (SPEX) that was capable of automated data acquisition and recording. For construction of 2D images, 16 projections were taken and the spectral data was deconvoluted using the line shape of the zero-gradient spectrum. Images were reconstructed from the deconvoluted data by filtered back projection [30].

Results

Acute oral toxicity

In the present study, a single oral administration of OptiBerry was provided to female Sprague Dawley rats to assess its acute toxicity following Up and Down procedure in accordance with the OECD guidelines for testing of chemicals, procedure 425 (adopted December 2001). OptiBerry, at the limit dose of level of 5000 mg/kg body weight, did not cause any mortality and did not demonstrate any signs of gross toxicity, adverse pharmacologic effects or abnormal behaviour in the treated female rats following dosing and during the observation period of 14 days thereafter. All the animals survived, gained normal body weight and appeared active and healthy during the study. No gross abnormalities or pathological alterations were noted for any of the organs of the rats when necropsied at the conclusion of the 14-day observation period. Based on these results and under the conditions of this study, the acute oral LD_{50} of OptiBerry is greater than 5000 mg/kg of body weight in female rats.

Acute dermal toxicity

Acute dermal toxicity of OptiBerry was conducted in male and female Sprague Dawley rats to determine the potential for OptiBerry to cause toxicity from a single topical application in accordance with the OECD guidelines for testing of chemicals, procedure 402 (adopted February 1987). All animals survived, gained normal body weight, and appeared active and healthy during the study. There were no signs of dermal irritation, gross toxicity, adverse pharmacologic effects or abnormal behaviour. No gross abnormalities were noted for any of the animals when necropsied at the conclusion of the 14-day observation period. Under the conditions of this study, the single dose acute dermal LD_{50} of OptiBerry is greater than 2000 mg/kg of body weight in both male and female rats.

Primary skin irritation

Primary dermal irritation potential of OptiBerry was evaluated within male and female New Zealand albino rabbits to evaluate the potential of OptiBerry to produce irritation after a single topical application in compliance with 21 CFR 58, 1987: US FDA GLP Standards and in accordance with OECD guidelines for testing of chemicals, procedure 404 (adopted April, 2002). Following application of OptiBerry, all the animals appeared active and healthy. Apart from the dermal irritation noted below, there were no signs of gross toxicity, adverse pharmacologic effects or abnormal behaviour. One hour after patch removal, very slight erythema was observed at all the three treated sites. The overall incidence and severity of irritation decreased with time. All animals were free from dermal irritation within 24 h. A summary of Draize primary dermal irritation scoring criteria for dermal reactions is presented in Table 1, while descriptive rating for mean primary dermal irritation index (PDII) is presented in Table 2.

Under the conditions of this study, the PDII for OptiBerry was calculated to be 0.3, thus classifying OptiBerry to be slightly irritating to the skin (Table 5).

Primary eye irritation

A primary eye irritation test was conducted with New Zealand albino rabbits to determine the potential for OptiBerry to cause irritation from a single instillation via the ocular route in compliance with 21 CFR 58, 1987: US FDA GLP Standards and in accordance with OECD guidelines for testing of

Table 5. Primary dermal irritation scores in male and female New Zealand albino rabbits after exposure to OptiBerry[a]

Time post instillation (h)	Incidence of dermal irritation		Total PDI[b]	Total PDII[c]
	Erythema	Edema	Mean score	Overall Index
1	1.0	0.0	1.0	0.3
24	0.0	0.0	0.0	
48	0.0	0.0	0.0	
72	0.0	0.0	0.0	

[a]Average values ($n = 3$).
[b]Primary dermal irritation (PDI) = average erythema + average edema.
[c]Primary dermal irritation index (PDII) = PDI for 1, 24, 48, and 72 h/4.

chemicals, procedure 405, adopted April, 2002. The Draize Scale for scoring eye lesions is presented in Table 3. The Kay and Calandra Scheme for classifying eye irritants are presented in Table 4. All animals appeared active and healthy. There were no signs of gross toxicity, adverse pharmacologic effects or abnormal behaviour. No corneal opacity or iritis were observed in any of the treated eye during the study. One hour following OptiBerry instillation, all treated eyes exhibited conjunctivitis (Table 6). Individual eye irritation scores are presented in Table 6. The overall severity of irritation decreased with time. All animals were free of ocular irritation within 48 h. Under the conditions of this study, the maximum mean total score (MMTS) of OptiBerry powder is 6.0, classifying OptiBerry to be minimally irritating to the eye.

Evaluation of antioxidant properties

Hyperbaric oxygen therapy (HBOT) delivers 100% O_2 at 2–3 atm pressure and patients typically receive 10–30 treatments, depending upon the diagnosis. These treatments are usually 60–120 min long, may be given several days a week and performed in specialised chambers at facilities with physician supervision. HBOT is capable of elevating arterial pO_2 as high as 1200 mmHg. While HBOT may improve the oxygenation status of several tissues, it poses a clear risk of oxygen toxicity [31, 32]. Like many other risk factors including cigarette smoking, HBOT may not result in immediate manifestation of clinical abnormalities in most cases. However, it is general knowledge that exposure of biological cells and tissues to pure O_2 may result in oxidative stress and genotoxicity [22]. We utilized this *in vivo* model system to test the antioxidant efficacy of OptiBerry. The standard laboratory feed for rodents is heavily enriched with the antioxidant vitamin E. Typical levels of vitamin E in the chow exceed the human recommended dietary allowance for vitamin E by over 15-folds. Excessive tissue vitamin E in the tissues of laboratory rodents may pose a problem to study oxygen toxicity. As a result, we generated vitamin E deficient rats by feeding the rodents with vitamin E deficient diet (Fig. 1). HBO treatment resulted in substantial increase in GSSG levels in the rat lung. Such increase in lung GSSG level was significantly lower in OptiBerry-fed male rats compared to

Table 6. Incidence, severity and reversibility of ocular irritation in New Zealand albino rabbits after exposure to OptiBerry

Time post instillation (h)	Incidence of ocular irritation		Severity of irritation	
	Corneal opacity	Iritis	Conjunctivitis	MMT[a] score
1	0/3	0/3	3/3	6.0
24	0/3	0/3	3/3	2.0
48	0/3	0/3	0/3	0.0
72	0/3	0/3	0/3	0.0

[a]Maximum mean total score (MMTS).

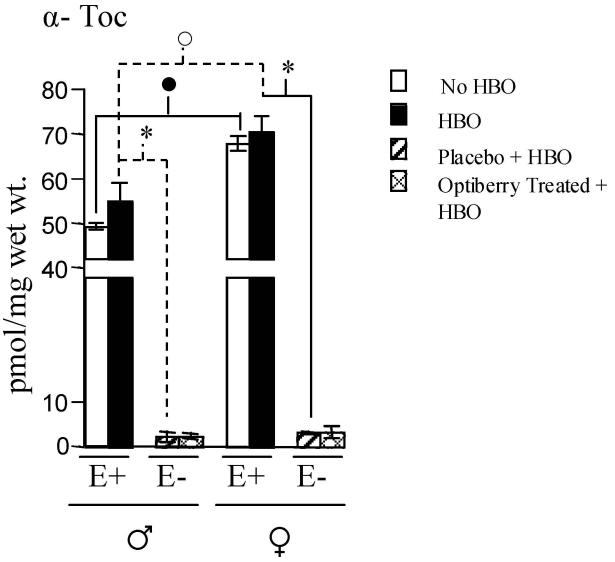

Fig. 1. α-Tocopherol levels in rat liver. Rats were randomly divided into the following four groups: (i) E^+ group – fed on a standard rat chow that is enriched with α-tocopherol (~200 nmol/g), (ii) E^+ group – fed on a standard rat chow that is enriched with α-tocopherol (~200 nmol/g) and exposed to pure O_2 at 2 atm for 2 h, (iii) E^-; group – fed on a vitamin E deficient diet treated with placebo and exposed to pure O_2 at 2 atm for 2 h and (iv) E^-; group – fed on a vitamin E deficient diet treated with OptiBerry and exposed to pure O_2 at 2 atm for 2 h. Vitamin E analysis was performed using HPLC. $^*P < 0.05$ significantly different compared between E^+ HBO treated and E^-; HBO treated placebo. $^\bullet P < 0.05$ significantly different compared between male E^+ control and female E^+ control. $^\circ P < 0.05$ significantly different compared between male E^+ HBO treated and female E^+ HBO treated.

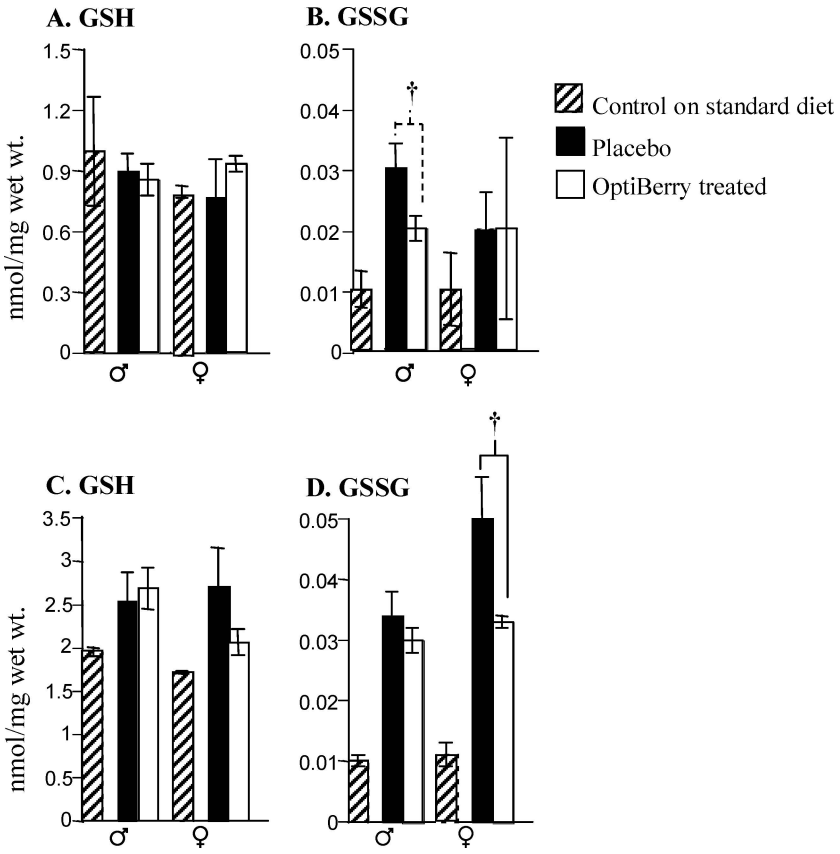

Fig. 2. (A) GSH levels in rat lung. (B) GSSG levels in rat lung. (C) GSH levels in rat liver. (D) GSSG levels on rat liver. Rats were randomly divided into the following three groups: (i) control group – fed a standard rat chow that is enriched with (-tocopherol (∼200 nmol/g) (ii) E^- group – fed a vitamin E deficient diet, exposed to pure O_2 at 2 atm for 2 h and treated with a placebo, (iii) E^- group – fed a vitamin E deficient diet, exposed to pure O_2 at 2 atm for 2 h and treated with OptiBerry. Glutathione analysis was performed using HPLC. $^\dagger p < 0.05$ significantly different compared between placebo and OptiBerry treated groups.

that of placebo-fed controls (Fig. 2B). HBO exposure also resulted in substantial oxidation of hepatic GSH to GSSG. In the female rats, OptiBerry feeding significantly prevented HBO-induced GSH oxidation (Fig. 2D). These effects of HBO on GSSG formation were studied 24 h after HBO exposure.

EPR imaging of whole-body redox status in mice

We employed an EPR approach to test the effect of HBO and OptiBerry feeding on whole-body redox status of mice immediately after the exposure to HBO. Here, mice were chosen because our EPR cavity is suited for the size of mice. Anesthetized mice were injected with a nitroxyl radical through tail vein, placed in EPR resonator, and images were taken every 4 min. Antioxidants reduce the nitroxyl radical to hydroxyamine, and thus the signal decays with time. A higher intensity signal from an area indicates lower reduction capability. Data was obtained from a custom-built 750 MHz EPR spectrometer [30]. In the control group, high signal intensity was observed in the peritoneum at 4 and 8 min and in the bladder at 12 and 16 min. In the placebo-fed HBO group, higher signal intensity is observed at all time points as compared to the control group, and the nitroxyl radical was retained in the body for a longer time. This indicates a clear shift of the whole-body redox status towards oxidation in response to HBO treatment. When OptiBerry-fed mice were exposed to HBO, based on the intensity and retention of signal, it was clear that OptiBerry feeding prevented HBO-induced oxidation response in a whole-body mouse model (Fig. 3).

Discussion

The pharmacologic, medicinal and therapeutic benefits of edible berries are well established. We have studied the

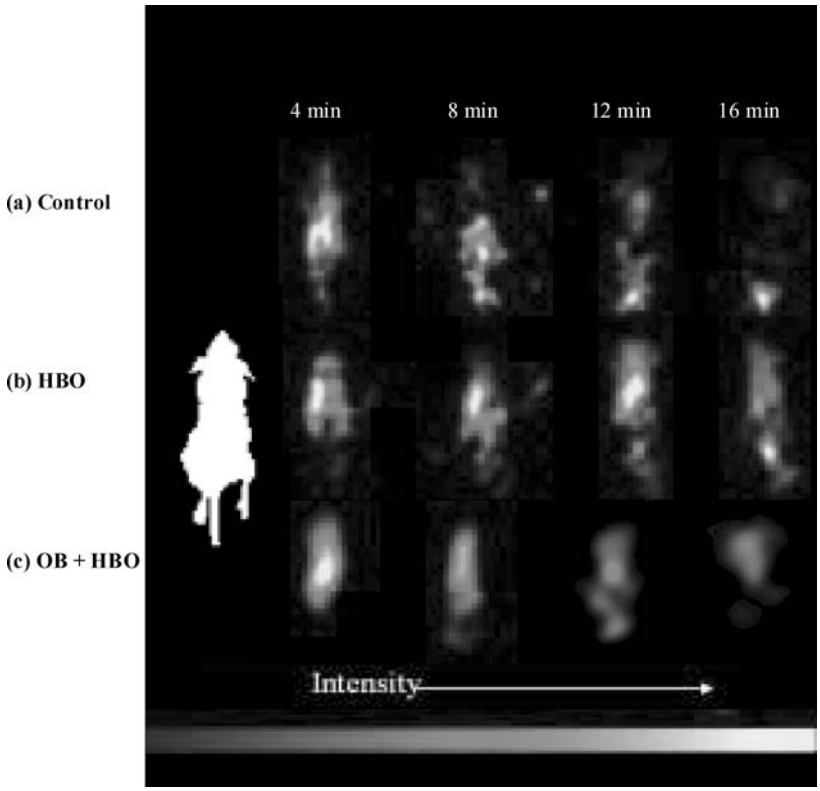

Fig. 3. Comparison of redox status in mice *in vivo*. Anesthetised mice were injected with a nitroxyl radical through tail vein, placed in EPR resonator, and images were taken every 4 min. Antioxidants reduce the nitroxyl radical to hydroxyamine, and thus the signal decays with time. A higher intensity signal from an area indicates lower reduction capability. Data is obtained from a custom-built 750 MHz EPR spectrometer with the following parameters: 32 mW microwave power, 0.7 G modulation amplitude, 100 kHz modulation frequency, 80 ms time constant, 35G scan range and 750 MHz microwave frequency. (A) Control group: A high signal intensity is observed in the peritoneum at 4 and 8 min, and in the bladder at 12 and 16 min (B) HBO group: On treatment with hyperbaric O_2 at 2 atm for 120 min, a higher signal intensity is observed at all time points as compared to the control group, and the nitroxyl radical stays in the body for a longer time. (C) Treatment with Optiberry-fed diet for 2 weeks and hyperbaric oxygen at 2 atm for 120 min: Optiberry, a novel antioxidant, reduces the signal intensity obtained. The radical is retained in the body for a much shorter time period than the other two groups.

anti-angiogenic properties of several extracts of edible berries including blueberry, bilberry, elderberry, cranberry, strawberry and raspberry seeds. Based on such investigations, we have developed a novel synergistic combination, OptiBerry, of these six berry extracts that exhibit potent effects *in vitro* and *in vivo* [16, 19, 27]. These favourable developments with OptiBerry led to the current study aimed at testing the broad spectrum safety and antioxidant potential of OptiBerry in *in vivo* settings. We used a unique, state-of-the-art electron paramagnetic resonance (EPR) imaging technique to demonstrate the antioxidant potential of OptiBerry against hyperbaric oxygen (HBO)-induced oxidative stress in mice in a whole-body scenario.

Safety of nutraceuticals is a major issue with respect to public health. Thus, in compliance with the FDA guidelines, we were led to conduct a broad spectrum of safety evaluation on OptiBerry to provide information on safety/toxicity/health hazards likely to arise from an exposure to OptiBerry. An acute oral toxicity test was conducted with rats to evaluate the potential for OptiBerry to produce toxicity from a single dose *via* the oral route. The median lethal dose (LD_{50}) of OptiBerry after single oral administration in female Sprague Dawley rats was found to be more than 5000 mg/kg body weight. In a second set, an acute dermal toxicity test was conducted with rats to evaluate the potential for OptiBerry to produce toxicity from a single topical application. The acute dermal LD_{50} of OptiBerry was found to be greater than 2000 mg/kg of body weight in both male and female rats. Furthermore, primary skin and eye irritation potentials of OptiBerry were evaluated in New Zealand albino rabbits. The primary skin irritation index was determined to be 0.3, thus classifying OptiBerry to be slightly irritating to the skin. OptiBerry was also found to be minimally irritating to the rabbit eye. Thus, these results demonstrate the safety of OptiBerry.

The tripeptide glutathione (gamma-glutamyl-cysteinyl-glycine; GSH) is the most abundant low-molecular-weight

thiol, and GSH/glutathione disulfide is the major redox couple in animal cells. The synthesis of GSH from glutamate, cysteine and glycine is catalysed sequentially by two cytosolic enzymes, gamma-glutamylcysteine synthetase and GSH synthetase. Compelling evidence shows that GSH synthesis is regulated primarily by gamma-glutamylcysteine synthetase activity, cysteine availability and GSH feedback inhibition. Animal and human studies demonstrate that adequate protein nutrition is crucial for the maintenance of GSH homeostasis. In addition, enteral or parenteral cystine, methionine, *N*-acetyl-cysteine, and L-2-oxothiazolidine-4-carboxylate are effective precursors of cysteine for tissue GSH synthesis. Glutathione plays important roles in antioxidant defense, nutrient metabolism and regulation of cellular events (including gene expression, DNA and protein synthesis, cell proliferation and apoptosis, signal transduction, cytokine production and immune response and protein glutathionylation). Oxidative stress can result from exposure to excessive amounts of endogenous and exogenous electrophiles. The concentration of GSH, the most abundant intracellular non-protein thiol and important antioxidant, declines with age and in some age-related diseases. The underlying mechanism, however, is not clear. Glutathione deficiency contributes to oxidative stress, which plays a key role in aging and the pathogenesis of many diseases (including Kwashiorkor, seizure, Alzheimer's disease, Parkinson's disease, liver disease, cystic fibrosis, sickle cell anemia, HIV, AIDS, cancer, heart attack, stroke and diabetes). New knowledge of the nutritional regulation of GSH metabolism is critical for the development of effective strategies to improve health and to treat these diseases [33].

HBO treatment is applied as a therapy for a wide variety of diseases with symptoms caused by lack of oxygen in the target tissues. However, it is known that exposure to high concentrations of oxygen may lead to oxidative stress and cause cell and tissue damage. Oxygen toxicity and possible cancer-promoting effects of HBO therapy have been a matter of serious concern. Although a cancer-inducing effect of HBO was not found to date, recent studies clearly indicated an induction of oxidative DNA damage in the blood cells of healthy subjects after HBO under therapeutic conditions. The biological significance of this finding has been investigated in a series of *in vitro* and *in vivo* tests. These studies have been recently reviewed [30]. We employed a clinically relevant HBO exposure protocol to study the antioxidant properties of OptiBerry. OptiBerry feeding prevented HBO-induced GSSG formation in the lung and liver of vitamin E-deficient Sprague Dawley rats. Accumulation of lung tissue oxidised glutathione (GSSG) is recognised as a marker of oxidant induced lung injury [34]. Oxygenation of the lung induces GSSG formation [35] and such perturbation of GSH redox status in the lung often accompanies lipid peroxidation [36]. Thus, the observed protective effects of OptiBerry against HBO-induced changes in the lung redox status is expected to have direct bearing on the general health of this vital organ.

The liver is the central detoxifying organ and one of the most widely recognised function of GSH is its defense against toxic compounds, whether exogenous, such as electrophilic xenobiotics, or endogenous, such as reactive oxygen species, generated during normal oxidative metabolism and/or stress. However, another no less significant role of GSH, namely, its function as a reservoir and vehicle for packaging and transport of cyst(e)ine has received significant attention. Because GSH is relatively more auto-oxidation resistant and stable than cyst(e)ine (CYSH), it serves as the preferred form for storage and transport of the latter, especially in the extracellular and relatively much less reduced (than intracellular) milieu, where CYSH oxidises to cystine (CYSS) rapidly. Over the past two decades, significant work has been going on to delineate the intra- and extrahepatic (interorgan) turnover, transport and disposal of GSH and define the quantitative role of these processes in interorgan homeostasis of GSH, CYSH and CYSS. These studies have identified the liver as the central organ of interorgan GSH homeostasis, with sinusoidal GSH efflux as the major determinant of plasma GSH, CYSH, CYSS and thiol-disulfide status of plasma [37]. Akin to the scenario in the lung, levels of GSSG in the liver serve as an index of stress and injury [38]. Maintenance of appropriate glutathione redox homeostasis in the liver is central to overall hepatic metabolism as well as immunity, which in turn directly influence general health of the organism [39, 40]. Thus, the observed protective effects of OptiBerry against HBO-induced perturbation of GSH redox state in the liver are likely to be of significance to overall health.

Recently, He *et al.* [41] established the ability of EPR imaging to provide non-invasive *in vivo* mapping of the redox status of the skin of living rats. The redox status was measured using a topically applied nitroxyl spin probe, (15)N-PDT. Free radicals and other paramagnetic species, play an important role in the cellular injury and pathophysiology. EPR spectroscopy and imaging has emerged as an important tool for non-invasive *in vivo* measurement and spatial mapping of free radicals in biological tissues. Extensive applications have been performed in small animals such as mice and recently applications in humans have been performed. A variety of spatial, and spectral–spatial EPR imaging applications have been performed. These techniques, along with the use of biocompatible paramagnetic probes, including particulate suspensions and soluble nitroxide radicals, enable spatial imaging of the redox state in a variety of biomedical applications [42]. We employed this novel EPR imaging approach to test the effect of HBO and OptiBerry feeding on whole-body redox status of mice, immediately after the exposure to HBO. This experimental system provided data supporting the GSH/GSSG data to substantiate the antioxidant properties of OptiBerry. Taken

together, these results indicate that OptiBerry is safe and that it may exhibit whole-body antioxidant properties when consumed orally for an extended period of time.

Acknowledgments

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