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Botanical Extracts as Medical Countermeasures for Radiation Induced DNA Damage

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Technical Report

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Abstract

This study provides information for testing of readily available, low toxicity, long shelf life, easily administered botanicals which can be prophylactic radioprotectants. A selection of assays to test antioxidant capacity, metabolic and drug interactions, and DNA damage were performed to assess commercially available grape seed extract supplements and Labrador tea whole leaf extracts as potential radioprotectants. Three different commercial grape seed extracts were shown to have differing antioxidant capacities when compared to a known antioxidant (vitamin C) and radioprotectant (amifostine). Grape seed extract and Labrador tea did not interact with a well-studied drug metabolism pathway (CYP3A4), indicating that they may have potential for use as radioprotectants due to minimal drug and metabolism interactions. Using a cellular system as a model for identifying the DNA damage while allowing for minimal repair, no protection was provided by any extract. Under acellular conditions, assessing DNA damage with no repair potential resulted in increased DNA damage following radiation exposure. Overall, this study has been useful in identifying and validating a set of procedures to use in screening potential antioxidant radioprotectants. Further work explores the optimal concentrations of these and other botanical extracts as potential radioprotectants.

Résumé

La présente étude a fourni de l'information sur des substances végétales facilement accessibles, peu toxiques, à longue durée de conservation et faciles à administrer qui pourraient être utilisées en prévention comme agent radioprotecteur. Au moyen de divers essais, nous avons déterminé la capacité antioxydante, les interactions métaboliques et médicamenteuses ainsi que les dommages causés à l'ADN afin d'évaluer les effets radioprotecteurs potentiels des suppléments commerciaux à base d'extrait de pépins de raisin et d'extrait de feuilles entières de thé du Labrador. Nous avons constaté que la capacité oxydante de trois extraits commerciaux de pépins de raisin différait et qu'elle était comparable à celle d'un antioxydant (la vitamine C) et d'un agent radioprotecteur (l'amifostine). Les extraits de pépins de raisin et de thé du Labrador n'ont pas interagi avec la voie métabolique de médicament bien connue CYP3A4, ce qui démontre leur potentiel comme agent radioprotecteur avec des interactions médicamenteuses minimales. Dans le système cellulaire, aucun extrait n'a eu d'effet protecteur, tel que démontré en examinant les dommages réparables et non réparables à l'ADN. Dans les milieux acellulaires, à l'examen des dommages sans réparation de l'ADN, nous avons observé une augmentation des dommages causés à l'ADN après l'exposition au rayonnement. Globalement, cette étude a été utile pour la détermination d'un ensemble d'expériences à réaliser pour la recherche d'agents qui pourraient avoir des effets antioxydants et radioprotecteurs. D'autres travaux devront être effectués, notamment l'étude de la concentration optimale des extraits et la recherche d'autres substances végétales radioprotectrices.

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Executive summary

Botanical Extracts as Medical Countermeasures for Radiation Induced DNA damage

E. K. Kennedy, R. Liu, B. C. Foster, D. Wilkinson,
DRDC CSS TR 2012-05; Defence R&D Canada –CSS March 2012.

Introduction: The results of this study will provide much needed information on screening of potential medical countermeasures for their efficacy against radiation exposure induced DNA damage. In the past, much of the focus has been on therapeutic treatments of radiation exposure, of which, most treatments have an associated health risk, are difficult to administer, and availability is costly and limited. In contrast, the information gained from this project will target investigation into readily available compounds as potential prophylactics against radiation exposure. A selection of assays to test antioxidant capacity, metabolic and drug interactions, and DNA damage were performed to assess commercially available grape seed extract supplements and Labrador tea whole leaf extracts as potential radioprotectants. A novel assay was used to compare DNA damage in cellular and acellular environments for further clarification of the mechanisms of radiation protection. Funding was provided by the Center for Security Science (CRTI BIO-070 AP).

Results: Three commercial grape seed extracts were shown to have a range of differing antioxidant capacities comparable to a known antioxidant (vitamin C) and a radioprotectant (amifostine). There was no evidence that any of the extracts studied interacted with a well-known drug metabolism pathway, indicating their potential as promising radioprotectants due to minimal drug and metabolism interactions. The botanicals studied here were assayed by various means to assess their ability to protect DNA from radiation-induced damage. In the cellular system with minimal DNA repair potential, protection was not provided by grape seed or Labrador tea extract, as determined by examining DNA damage. Under conditions with no possibility of repair (acellular comet assay), increased DNA damage following radiation exposure was observed.

Significance: The International Atomic Energy Agency (IAEA) identified a need for safe, easily administered prophylactic and therapeutic products that would alleviate medical, public health, and psychosocial concerns to enable effective response to radiological/nuclear threats. These same products would also benefit cancer patients undergoing radiation therapies, trauma patients subjected to multiple CT scans and diagnostic imaging procedures, radiation industry accidental causalities, and astronauts subjected to cosmic radiations. Research focused on these products will lead to a better understanding of alternative therapeutics that would be easy to use in a mass casualty scenario, be of minimal risk in patients who may have low dose exposures or the worried-well with no exposures, and be readily available as prophylactics to First Responders in Chemical, Biological, Radiological, and Nuclear (CBRN) incidents.

Future plans: Based on encouraging antioxidant capacities and preliminary DNA damage analysis, further work includes the optimization for concentrations of commercial grape seed extract supplements and Labrador tea. In addition, this work has identified and validated a set of procedures to use in screening future potential antioxidant radioprotectants.

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Botanical Extracts as Medical Countermeasures for Radiation Induced DNA Damage:

E. K. Kennedy, R. Liu, B. C. Foster, D. Wilkinson,
DRDC CSS TR 2012-05; R & D pour la défense Canada –CSS mars 2012.

Introduction : Les résultats de la présente étude fourniront des renseignements très recherchés sur les contre-mesures médicales à adopter en cas d'exposition au rayonnement en vue de protéger l'ADN contre les dommages. Dans le passé, l'attention s'est essentiellement portée sur les traitements de l'exposition au rayonnement, dont la plupart sont associés à un risque pour la santé, sont difficiles à administrer, coûteux et peu accessibles. En revanche, les données recueillies dans le cadre de ce projet fourniront de l'information sur les composés facilement accessibles qui protégeraient de manière préventive contre l'exposition au rayonnement. Au moyen de divers essais, nous avons déterminé la capacité antioxydante, les interactions métaboliques et médicamenteuses ainsi que les dommages causés à l'ADN afin d'évaluer les effets radioprotecteurs potentiels des suppléments commerciaux à base d'extrait de pépins de raisin et d'extrait de feuilles entières de thé du Labrador. Nous avons comparé, à l'aide d'un nouvel essai, les dommages à l'ADN dans les milieux cellulaires et acellulaires afin de clarifier davantage les mécanismes de radioprotection. Le financement a été fourni par le Centre pour la science de la sécurité (CRTI BIO-070AP).

Résultats : Nous avons constaté que la capacité oxydante de trois extraits commerciaux de pépins de raisin différait et qu'elle était comparable à celle d'un antioxydant connu (la vitamine C) et d'un agent radioprotecteur (l'amifostine). Aucun des deux extraits n'a interagi avec l'une des voies métaboliques de médicament bien étudiée, ce qui démontre leur potentiel radioprotecteur. Nous avons analysé les deux extraits végétaux au moyen de différentes méthodes pour évaluer leur capacité à protéger l'ADN des dommages provoqués par le rayonnement. Dans le système cellulaire, aucun extrait n'a eu d'effet protecteur, tel que nous l'avons déterminé en examinant les dommages à l'ADN avec un potentiel pour la réparation minimale. Dans les milieux acellulaires, nous avons observé des dommages accrus à l'ADN après l'exposition au rayonnement.

Importance : L'Agence internationale de l'énergie atomique a fait ressortir le besoin en matière de produits prophylactiques et thérapeutiques sécuritaires et faciles à administrer qui apaiseraient les inquiétudes sur le plan médical, psychosocial et de la santé publique, et qui permettraient de répondre efficacement aux menaces radiologiques et nucléaires. Les connaissances acquises dans le cadre de ces travaux profiteront aussi aux patients en radiothérapie, aux patients traumatisés qui subissent de multiples tomodensitogrammes et examens d'imagerie diagnostique, aux accidentés du travail occasionnels ainsi qu'aux astronautes soumis à une exposition chronique à de faibles doses de rayonnement cosmique. Les résultats de ces travaux susciteront la mise au point de produits différents qui seraient faciles à utiliser dans les situations où il y aurait de nombreuses victimes, qui poseraient un risque minimal pour les patients exposés à des faibles doses ou pour les personnes non exposées inquiètes, mais en bonne santé, et qui seraient facilement accessibles aux premiers intervenants dans les incidents chimiques, biologiques, radiologiques et nucléaires.

Perspectives : Basé sur l'encouragement des capacités antioxydants et une analyse préliminaire dommages à l'ADN, d'autres travaux devront être effectués, notamment l'étude de la concentration optimale des suppléments commerciaux d'extrait de pépins de raisin et de thé du Labrador et la recherche d'autres substances végétales radioprotectrices.

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We wish to thank the Center for Security Science for support funding for this study (CRTI BIO-070AP). We would also like to thank DRDC Toronto Human Research Ethics Committee for providing us with Human Research Ethics Document Approvals so that we could conduct the necessary research. We are very grateful to our volunteers who willingly gave fingerprick blood samples for this study.

We also acknowledge Hillary Boulay Greene for her useful professional advice in the final stages of this project, Dr. John Arnason at the University of Ottawa for preliminary botanicals information and HPLC data, Aimee Jones for preliminary grape seed extract work and Dr. James McNamee for guidance in the development of the comet assay procedures.

1 Introduction

1.1 Radioprotectants

Production of free radicals (including reactive oxygen species (ROS)) *in vivo* is a major pathway resulting in radiation induced DNA damage (1, 2, 3). ROS are produced when there is an interaction between radiation and water (radiolysis of water) which can then produce DNA damaging radicals (4). Since DNA damage resulting from radiation is mostly attributed to the actions of ROS, molecules with scavenging properties (antioxidants) have great potential as radioprotectants. Botanical compounds also have potential as radioprotectants due to their anti-inflammatory, immunostimulant, and anticarcinogenic properties (5).

Organisms have evolved a variety of mechanisms for reducing cellular ROS. One mechanism of antioxidant defense is to increase the presence and activity of endogenous molecules (i.e. superoxide dismutase, catalase, glutathione peroxidase) capable of scavenging ROS. In addition, exogenous molecules (obtained from diet) are also able to scavenge or chelate oxidant molecules (1, 3, 4, 6). This study focused on the use of exogenous defence systems, using vitamin C as a supplement control; DMSO, glycerol and ethanol as chemical antioxidants; and Trolox® and amifostine as synthetic supplement controls. Commercial grape seed extract supplements (GSE) and ground whole Labrador tea leaves (LT) were tested to determine their antioxidant capacities and level of DNA damage protection from ionizing radiation. A variety of *in vitro* tests can be used to measure radioprotective effects but the end-point measured will greatly affect the conclusions (7). In addition to standard assays, this study used the cellular and acellular comet assays as more sensitive and faster approaches to observe potential protection from radiation-induced DNA damage (8, 9).

Many radioprotectants have limited usability due to availability and negative side effects at clinically effective doses (4, 5). An ideal radioprotectant produces no cumulative or irreversible toxicity, does not interact with the metabolism of other drugs, is easily administered, and has a 2 to 5 year shelf life (2, 5). This study analyzed commercially available grape seed extracts (GSE) and ground whole Labrador tea (LT) leaves as readily available, low side-effect radioprotectants.

1.1.1 Control ROS Scavengers

DMSO and glycerol are two ROS scavengers used as controls in this study for antioxidant capacity from exogenous sources (10, 11, 12, 13). By scavenging hydroxyl radicals produced from radiolysis of water, these exogenous compounds prevent the reactive species from damaging DNA (non-enzymatic process). Previous study has shown that 10 % glycerol and 10 % DMSO reduced the first mitosis yield of chromosomal rearrangements caused by radiation exposures (7.5 to 25 Gy dose) by 30 to 40 % (13). Controls used in the analysis presented here were 10 % DMSO and 4.3 % glycerol. These controls have been reported to have similar antioxidant capacity to each other at these concentrations when measuring DNA damage (10).

Ethanol was used in this experiment as a solvent for the GSE and LT extracts but is also a known to be a ROS scavenger (14, 15). Research has shown that grape seeds extracted in ethanol had maximum anti-radical activity compared to those extracted with methanol or water (12, 16). Ethanol has been previously shown to have protective effects against radiation-induced DNA damage (0 to 4 Gy) at 2.5 and 10 % (v/v) in culture medium as measured by the cytokinesis blocked micronucleus assay (17). The protective effect of ethanol was accounted for in this study.

1.1.2 Known antioxidant (vitamin C)

Vitamin C (ascorbic acid) is a well known *in vivo* and *in vitro* antioxidant (6, 8, 12, 18). It is also known that antioxidant inhibitors of one ROS mechanism may, at high concentrations, act as a pro-oxidants (19). This effect has been observed when high concentrations of vitamin C combine with free transition metal ions resulting in a release of DNA-damaging oxygen radicals (12, 19). At lower concentrations (8 to 300 μM), vitamin C reduced oxidative DNA damage to human lymphocytes measured by the cellular comet assay but had no effect on the micronucleus index at concentrations up to 2 mM (20, 21, 22). Micronuclei are caused by double strand breaks and the comet assay measures single and double stand breaks; thus, these studies suggest that vitamin C is protective against single strand breaks, damage primarily caused by the presence of ROS near DNA. In another study using the cellular comet assay, 0.2 mM to 1 mM Vitamin C reduced DNA damage, although DNA damage also increased at higher (5 mM)

concentrations (23). Vitamin C was used in this study as an exogenous control antioxidant and a well studied radioprotectant.

1.1.3 Known radioprotectant (WR-1065)

WR-1065 is the active metabolite of WR-2721 (phosphorothioate 5-2-(3-aminopropylamino) ethylphosphorothioic acid, also called amifostine), an FDA approved cytoprotective agent against radiation induced damage (24, 25, 26). WR-1065 (4 mM) protects mammalian cells from the direct killing of cells following 4 Gy x-ray irradiation and reduces chromosomal damage measured by the cytokinesis blocked micronucleus assay (24, 27). Lower concentrations (40 μM) also protected cells from DNA damage caused by 2 Gy x-ray irradiation, but this concentration did not reduce radiation associated cell death (27). In other studies, WR-1065 (1 mM) did not decrease intracellular oxidative stress but did decrease DNA fragmentation following hydrogen peroxide (ROS) exposure (28). WR-1065 was used in this analysis as a synthetic antioxidant radioprotectant with known cellular effects (26).

1.1.4 Grape Seed Extract

In 2010 and 2011 there were over 500 peer-reviewed papers published on the topic of grape antioxidants, indicating both an interest and a need for botanical extracts as prophylactic protectants to ROS DNA damage. Grape phenolic compounds reduce cellular oxidative stress by scavenging of ROS, inhibiting of lipid oxidation, reducing hydroperoxide formation, and affecting cell signalling pathways and gene expression (19, 29, 30). The main phenolic compounds in grapes are: anthocyanins (pigments), flavanols, flavonols, stilbenes (resveratrol) and phenolic acids (31, 32). Previous studies have shown that proanthocyanidin (oligomer chain of flavonoid) from grape seed extract has greater antioxidant capacity than vitamins C or E (33). Phenolic compounds from grapes have high bioavailability, effectively entering the human digestion system and blood within two to three hours after ingestion (30, 33, 34, 35).

The above properties, combined with a reduced chromosomal damage in blood cells exposed to grape phenolic compounds, indicate the potential for commercial grape seed extracts as prophylactic and therapeutic radioprotectants (3, 8, 36, 37). Many studies use pure extracts or mixtures of pure extracts to determine the antioxidant

properties of grape seeds (8, 19); this study aims to examine the use of pre-prepared commercial grape seed extract supplements as radioprotectants measured by antioxidant capacity, metabolic interactions, and DNA damage assays.

1.1.5 Labrador Tea

Labrador tea (*Rhododendron groenlandicum*) is an evergreen wetland plant used for centuries by Aboriginal peoples of Canada to treat weak blood, colds, dizziness, pain, heartburn, and kidney problems (38). Labrador tea leaf extracts contain high levels of phenolic compounds (20 %), have *in vitro* and *ex vivo* antioxidant activity, and demonstrate no significant cell toxicity up to 100 µg/ml concentrations (39, 40). To date, this antioxidant activity has not been measured relative to protection from radiation-induced DNA damage. This study aims to examine the use of Labrador tea leaves as a radioprotectant by measuring antioxidant capacity, metabolic interactions, and DNA damage assays.

1.2 DNA Damage Analysis – The Comet Assays

Previous work has described the use of comparing the acellular and cellular comet assays (9). The comet assay (single-cell gel electrophoresis) is a method used to measure the amount of DNA damage in a single cell based on the migration of DNA during electrophoresis (42, 43, 44). Whole cells are embedded in agarose and lysed, which results in the supercoiled structure of genomic DNA being maintained within the agarose. Single and double DNA strand breaks relax the supercoiled structure and allow loops and/or strands of DNA to migrate out of the nuclear region producing a comet-like tail. DNA damage can then be quantified by assessing the extent of DNA that migrates into the tail region of the comet and this information contributes to the calculation of percent tail intensity (the ratio of DNA in the tail to the total DNA).

The cellular comet assay detects double and single strand breaks in an intact cell thus maintaining potential for minimal DNA repair processes. Using the cellular comet assay, endogenous and exogenous radioprotective processes can be assessed (4, 6). The acellular comet assay is similar to the cellular comet assay but only damage to naked DNA already in agarose in a supercoiled structure is measured, disallowing for any chance for repair. The acellular comet assay does not maintain any cellular functions; therefore, only radioprotection by exogenous processes can be measured (4, 6). Use of these two assays will help to elucidate possible

mechanisms by which radioprotection occurs, allowing differentiation between acellular and cellular environments and their impact on DNA damage; this can include differences in DNA repair and the effects of the surrounding environment (cellular environment versus controlled maintenance buffer).

1.3 Rational, Roles, and Gaps Addressed

The International Atomic Energy Agency (IAEA) identified a need for safe, easily administered prophylactic and therapeutic products that would alleviate medical, public health, and psychosocial concerns to enable effective response to radiological/nuclear threats (45). This includes addressing the need for the development of countermeasures strategies (including broad spectrum prophylactics), management of the worried-well by providing innocuous treatment alternatives, and development of large-scale casualty management strategies. The knowledge gained through this research will also benefit medical patients undergoing radiation therapies, trauma patients subjected to multiple CT scans and diagnostic imaging procedures, radiation industry accidental patients, and astronauts subjected to cosmic radiations. The outcome of these studies will lead to the establishment of procedures to be used in the identification and testing of alternative products that would be easy to use in a mass casualty scenario (ingestible and requiring minimal medical intervention), be of minimal risk in patients who may have low dose exposures or the worried-well with no exposures, and be readily available to first responders in CBRN incidents.

2 Materials and Methods

2.1 Blood Samples

All procedures for collection and handling of fingerprick blood samples were performed according to a protocol approved by DRDC Human Research Ethics Committee. Volunteers gave informed consent and provided information regarding age, gender, and therapeutic history including recent known exposures to ionizing radiation. Fingerprick samples (50 to 200 µl) from healthy volunteers were collected into lithium heparinized collection vials (Becton Dickinson and Company, Franklin Lakes, NJ). Whole blood was then diluted to the appropriate concentration with sterile phosphate-buffered saline (PBS, pH 7.4; Sigma-Aldrich, St. Louis, MO).

2.2 Preparation of Extracts

2.2.1 Commercial Grape Seed Extracts

Three GSEs in the form of 50 to 100 mg tablets or gelatin capsules were purchased as over-the-counter natural-health products from local stores in Ottawa, Ontario, Canada (Table 1). Extracts were chosen based on unpublished HPLC data which analyzed eight commercial extracts for concentrations of potential antioxidant components (gallic acid, procyanidin B1, (+)-catechin, procyanidin B4, procyanidin B2, and (+)-epicatachin; personal communication, Dr. John Arnason, University of Ottawa). Two extracts (GSE4 and GSE6) with relatively high concentrations and one extract (GSE5) with relatively low concentration of potential antioxidant compounds were chosen for this study.

GSE4 was prepared at room temperature by grinding 30 tablets (50 mg) to a fine powder using a mortar and pestle. Powders from within approximately 30 gelatin capsules of GSE5 (100 mg gelatin capsule) and GSE6 (50 mg gelatin capsule) were respectively emptied and further ground to a fine power using a mortar and pestle. The resulting powders were stored in glass vials at 4^{o}C in the dark. All steps were performed in glass vessels at room temperature and protected from light unless otherwise specified.

Mixtures of 12.5 mg/ml GSEs were prepared by suspending powders in 80 % ethanol and mixing for 2 hr. Suspensions were transferred into 15 ml polypropylene tubes (to allow for centrifugation) with an additional two 1.0 ml rinses with 80 % ethanol

(final concentration 10 mg/ml). Suspensions were sonicated in a water bath (Branson B-3, 117 V, 50/60 Hz, 5 Amps, Branson Cleaning Equipment Company, Shelton, CONN, USA) for 30 min then centrifuged for 15 min at 1800 rcf (Thermo IEC Centra GP8R, Thermo Fisher Scientific, Ottawa, ON). The supernatants were filtered through 0.2 µm nylon filters and the remaining pellets were dried and weighed to determine stock solution concentrations (Table 1). Several isolates were prepared for each GSE and used throughout the experiments as described below. Comet assays were conducted using GSE4 as it was the first GSE purchased; future work could include analysis of GSE5 and GSE6.

2.2.2 Labrador Tea

Whole LT leaves were provided by Dr. Foster from the University of Ottawa and the Health Products Food Branch of Health Canada. Leaves were ground at room temperature into a fine powder using a mortar and pestle and stored at 4^{o}C. Stock solutions were made as described for GSE (Table 1); solutions made at different times may have slightly different concentrations and are labelled in accordance with the date made.

Table 1: Botanical extracts information.

Grape Seed extract 4 (GSE4) Tablet, assessed to have high antioxidant capacity potential (personal communication, Dr. John Arnason, University of Ottawa)		
Expiry date	June 2013	
Listed medicinal ingredients	- Grape seed extract standardized to 85 % polyphenols - Grape skin extract standardized to 15 % polyphenols	
Non-medicinal ingredients	- Magnesium stearate - Microcrystalline cellulose - Silicon dioxide - Stearic acid	
Isolate	Stock Concentration	Date Made
GSE4-1	3.60 mg / ml	15-Dec-10
GSE4-2	3.44 mg / ml	21-Feb-11
GSE4-3	3.34 mg / ml	28-Feb-11
GSE4-4	3.35 mg / ml	20-July-11
GSE4-5	3.01 mg / ml	3-Aug-11
GSE4-6	3.47 mg / ml	17-Aug-11

Grape Seed Extract 5 (GSE5) Gelatin capsule, assessed to have low antioxidant capacity potential (personal communication, Dr. John Arnason, University of Ottawa)		
Expiry date	May 2013	
Listed medicinal ingredients	- Grape seed (95 % proanthocyanidins) - Grape powder (whole fruit) - Lecithin (40 % phosphatidylcholine)	
Non-medicinal ingredients	- Rice flour - Silicone dioxide - Magnesium stearate - Gelatin	
Isolate	Stock Concentration	Date Made
GSE5-1	2.67 mg / ml	21-Feb-11
GSE5-2	2.54 mg / ml	3-Aug-11
GSE5-3	2.74 mg / ml	17-Aug-11
Grape Seed Extract 6 (GSE6) Gelatin capsule, assessed to have high antioxidant capacity potential (personal communication, Dr. John Arnason, University of Ottawa)		
Expiry date	January 2014	
Listed medicinal ingredients	- Grape seed extract (95 % polyphenols, 80 % proanthocyanidins)	
Non-medicinal ingredients	- Gelatin - Rice starch	
Isolate	Stock Concentration	Date Made
GSE6-1	1.83 mg / ml	21-Feb-11
GSE6-2	1.71 mg / ml	3-Aug-11
GSE6-3	1.33 mg / ml	17-Aug-11
Labrador Tea Extract (LT) Whole dried leaves from Dr. B. C. Foster, University of Ottawa and Health Products Food Branch of Health Canada		
Isolate	Stock Concentration	Date Made
LT	5.57 mg / ml	15-Dec-10

2.3 Toxicity Screens

Toxicity was assessed by measuring the viability of white blood cells in human whole blood after 2 hr *ex vivo* incubation at 37^{o}C in 5 % CO_2 in the experimental or control solutions using a Guava PCA™ cytometer (Guava Technologies, Inc., Hayward, CA, USA). Whole blood was diluted to 5.0 x 10^5 cells/ml with PBS then mixed 1:1 with experimental or control solutions (final concentration 2.5 x 10^5 cells/ml). Preliminary discussions involving concentrations to be tested and extraction protocols involved Aimee Jones, Diana Wilkinson, Brian Foster, and John

Arnason. All experiments were repeated at least three times and statistical analyses were performed at an alpha of 0.05.

To determine the ideal solvent for the extracts, DMSO, methanol, and ethanol were tested for toxicity between 0.08 and 10 % in PBS; 2% ethanol was chosen for GSE and LT experimental solutions based on cell toxicity results. Vitamin C (0.25 mM, L-Ascorbic acid, cell culture reagent, MP Biomedicals, Solon, OH) and WR-1065 (0.25 mM, >98 % HPLC, Sigma-Aldrich) were resuspended in dH_2O. The comet assays control solutions PBS (Sigma-Aldrich), 2 % ethanol (Fisher Scientific, Ottawa, ON), 10 % DMSO (Fisher), and 4.3 % glycerol (Fisher) were also tested for cell toxicity under comet assay conditions.

2.4 Assessment of Antioxidant Capacity

Antioxidant capacity was assessed using the Sigma Antioxidant Kit (CS0790; Sigma-Aldrich) according to manufacturer's instructions. This assay measures the antioxidant activity of hydrogen-donating compounds, and chain-breaking antioxidants compared to Trolox, and compares it to Trolox, a water-soluble vitamin E derivative. A Trolox® standard curve was prepared fresh each day and included on each plate; thus, results were calculated on a plate basis. All samples were prepared in triplicate and each experiment was repeated at least three times and statistical analyses were performed at an alpha of 0.05.

Briefly, ABTS solution (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), phosphate-citrate, and 3 % hydrogen peroxide) was added to each well with a myoglobin solution and the test sample. Test samples were: 100 µg/ml GSE4, GSE5, and GSE6; 84 µg/ml LT; 0.25 mM vitamin C; and 0.25 mM WR-1065. Samples were incubated at room temperature for 5 min and then stop solution was added to each well. Endpoint absorbances were read at 405 nm using a Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek, Winooski, VT, USA). Relative µmol TE/mg concentrations were determined using a Trolox® equivalents standard curve for each 96 well plate.

GSEs were also tested for effects from stock storage, varying from 1 to 180 days. Vitamin C was made within 1 day of use and WR-1065 was made in dH_2O within 30 min of use due to instability in solution (data not shown) and supplier instructions; as these were prepared fresh, they did not need to be assessed for storage effects.

2.5 Assessment of Metabolic Interactions using CYP3A4 Assays

Protocols and reagents were provided by Dr. Foster with the assistance of graduate student Rui Liu and all analyses were completed in his laboratory (41). All steps were performed under gold fluorescent lighting (Industrial Lighting, Ottawa, ON). Samples were prepared in triplicate, each experiment repeated at least three times, and statistical analyses were performed at an alpha of 0.05. Percent inhibition calculations were based on the differences in fluorescence between the test / test-blank wells and the mean difference between each control and test sample; controls were run with each assay.

2.5.1 CYP3A4 Fluorescent Microwell Plate Assay

Extracts were screened for their ability to inhibit cytochrome P450 isoform 3A4 (CYP3A4) marker substrate dibenzylfluorescein (DBF) (BD Gentest™, Mississauga, ON) in an *in vitro* fluorescent microwell plate assay (42). Test and control solutions were added to clear-bottom 96 well plates. Control wells contained dH_2O or ethanol (concentration dependent on test substance solvent), NADPH (beta-nicotinamide adenine dinucleotide phosphate, reduced form, Sigma-Aldrich) solution, and marker substrate DBF. Blank wells contained dH_2O or ethanol and buffer solution. Test wells contained extract or ketoconazole (positive control, Calbiochem Merck, Darmstadt, Germany) and NADPH solution. Test-blank wells consisted of corresponding extract or ketoconazole, and buffer solution. CYP3A4 enzyme was then added to all wells. Initial fluorescence was measured at 485nm emission and 530nm excitation; microwell plates were then incubated at 37^oC for 20 min before final fluorescence was measured.

2.5.2 CYP3A4 Liquid Chromatography Assay

Extracts were screened for their ability to decrease the metabolism of testosterone (Steraloids Inc., Newport, RI, USA), a CYP3A4 marker substrate. Test solutions were incubated then stopped by an equal volume of methanol. Samples were then filtered prior to HPLC analysis to remove particulates and eliminate the strong fluorescence interference. Methanol extraction and filtration leaves only organic material (testosterone and metabolites) for HPLC analysis, thus eliminating the effect of quenching from the extracts as observed in the microwell plate assay. Control vials contained dH_2O or

ethanol (concentrations dependant on test substance), NADPH solution, and marker substrate (testosterone). Blank tubes contained dH_2O or ethanol and buffer solution. Test tubes contained extract or ketoconazole positive control, and NADPH solution. Test-blank tubes consisted of corresponding extract or ketoconazole, and buffer solution. CYP3A4 enzyme was then added to all samples. Samples were incubated at 37^{o}C for 30 min before the liquid-to-liquid extraction by 500 µl ethyl acetate (twice) and resuspended in methanol. Samples were filtered through 0.45 um PTFE filters (Fisher) and sonicated for 5 min.

As described in Foster *et al.* (2004) (41), extracts were analyzed on a Luna-C18 (150 x 2 mm, 3 um particle size, S/N 563913-12; Phenomenex Inc, Torrance, CA, USA) Agilent 1100 series HPLC (Agilent Technologies, Waldbronn, Germany) with a diode array detector. The flow rate was 1 ml/min and oven temperature was set at 40 °C using a linear gradient starting with an elution system containing acetonitrile:water (20:80 v/v) for 2 min to a final system containing acetonitrile:water (80:20 v/v) at 8 min and maintained for 12 min. The peaks were detected at 254 nm wavelength.

2.6 Comet Assay

2.6.1 Acellular Comet Assay

This protocol was adapted from the alkaline comet assay (9, 42, 43, 44). All steps were performed under minimal light at room temperature unless otherwise stated. Whole blood was diluted to 2.5 x 10^6 cells/ml with PBS and mixed 1:10 v/v with 0.55 % low-melting point agarose in PBS (pH 7.4) at 42^{o}C (BioRad Laboratories, Mississauga, ON). A 50 µl aliquot of this suspension was pipetted and spread evenly across a 4 cm^2 area on GelBond film (Mandel Scientific, Guelph, ON) then allowed to solidify at room temperature for 5 min. Samples were placed in lysis buffer (2.5 M NaCl, 100 mM tetra-sodium EDTA, 10 mM Tris base, 1 % N-lauryl sarcosine, 10 % DMSO, 1 % Triton-X 100, pH 10.0) for 2 hr. The samples were then placed into separate 50 ml conical centrifuge tubes (Fisher) with 40 ml of maintenance buffer (2.5 M NaCl, 100 mM tetra-sodium EDTA, 10 mM Tris base, pH 10.0) with the following sample concentrations: GSE4 (90 µg/ml in 2 % ethanol), LT (84 µg/ml in 2% ethanol) , 2 % ethanol, 4.3 % glycerol, 10 % DMSO, and PBS. GSE4 was the first purchased commercial grape seed

extract, followed later by the purchase of GSE5 and GSE6; thus, experiments were initiated using GSE4. The tubes were wrapped in aluminum foil and maintained at room temperature. Samples were then irradiated as described below. Following irradiation, samples were maintained in the foil wrapped tubes overnight and electrophoresed the following day.

2.6.2 Cellular Comet Assay

This protocol was adapted from the alkaline comet assay (9, 42, 43, 44). All steps were performed under minimal light at room temperature, unless otherwise stated. Briefly, whole blood was diluted to 5 x 10^5 cells/ml with PBS and mixed 1:1 (v/v) with experimental solutions (GSE or LT) or controls in 1.5 ml centrifuge tubes at a volume of 50 µl. Samples include GSE4 (90 µg/ml in 2 % ethanol), LT (84 µg/ml in 2% ethanol), 2 % ethanol, 4.3 % glycerol, 10 % DMSO, and PBS. Samples were incubated for 2 hr at 37^{o}C in 5 % CO_2.

Samples (20 µl) were then irradiated on ice to minimize DNA repair (for 0 to 5 min, exposure times were dose dependent) and mixed 1:10 v/v with 0.55 % low melting point agarose in PBS (pH7.4) at 42^{o}C (BioRad). A 50 µl aliquot of this suspension was pipetted and spread evenly across a 4 cm^2 area on GelBond film (Mandel Scientific) and allowed to solidify for 5 min. Samples were placed in lysis buffer (2.5 M NaCl, 100 mM tetra-sodium EDTA, 10 mM Tris base, 1 % N-lauryl sarcosine, 10 % DMSO, 1 % Triton-X 100, at pH 10.0) for 2 hr.

2.6.3 Electrophoresis

Following irradiation (acellular comet assay) or lysis (cellular comet assay), samples on GelBond films were rinsed in distilled water and immersed in electrophoresis buffer (0.3 M NaOH, 10 mM tetra-sodium EDTA, 0.1 % (w/v) 8-hydroxyquinoline, 2 % DMSO, pH 13.1) for 30 min to allow DNA to unwind. Samples were then transferred to electrophoresis tanks containing 700 ml of electrophoresis buffer and electrophoresed for 17 min at approximately 200 mA, resulting in 1.135 V/cm. Samples were then transferred to neutralization buffer (0.4 M Trizma, pH 7.15) for 30 min and placed in 99 % ethanol for 2 hr and dried overnight.

2.6.4 Scoring and Statistics

Dried slides were stained with 1/10,000 dilution SYBR Gold (Molecular Probes, Invitrogen Canada Inc, Burlington, ON) for 20 min then rinsed in distilled water and analyzed using Comet IV image analysis software (Perceptive Instruments Ltd, Suffolk, UK); sixty comets were scored per sample. The comets chosen for scoring had to satisfy three criteria: 1) could not be in contact other comets; 2) were more than two fields of view away from the edge of the agarose; and 3) contained a head with a predominantly circular shape. Parameters used by the Comet Assay IV software were head length, tail length, head intensity, tail intensity, tail moment, total area, mean grey, total intensity, and width. Outliers for all parameters assessed were identified as values greater than three times the 75^{th} quartile or less than one-third of the 25^{th} quartile.

Each experiment was repeated at least three times and statistical analyses were performed at an alpha of 0.05. The geometric mean tail intensity values were used to assess the level of DNA damage. As discussed by Lovell *et al.* and Duez *et al.* (46, 47), the comparison of data was performed using the geometric mean sample values.

2.7 Irradiations

Acellular comet assay (DNA samples in maintenance buffer) or cellular comet assay (whole blood) samples were exposed to a dose of 2 Gy using DRDC's ^{60}Co gamma beam-150C irradiator. Whole blood was irradiated in 25 µl volumes in 1.5 ml centrifuge tubes (Fisher) in a 1 mm thick ice tray behind 2.2 mm of ice. Acellular comet assay samples were irradiated in 50 ml conical centrifuge tubes (1 mm thick, Fisher) in 40 ml transfer buffer, resulting in 2.5 mm transfer buffer in front of the agarose-embedded sample. The dose rate was periodically checked and verified using a calibrated electrometer (Farmer model #2670A; Nuclear Enterprise Technology Ltd., Beenham, UK) and an ionization chamber (NE model #2581; Nuclear Enterprise Technology Ltd.). The dose rate in air was determined to be approximately 11.0 Gy / h at 1 m from the source with an uncertainty of 1.2 % (confidence level 95 %). Irradiations were performed in accordance with IAEA recommendations to ensure homogenous exposure to the samples (46).

3 Results

3.1 Toxicity Screens

3.1.1 Solvent and Controls Toxicity

Concentrations from 0.08 to 10 % of DMSO, ethanol, and methanol were tested for their effect on cell viability under the comet assay conditions to determine the maximum concentration and most ideal solvent to use for the GSEs and LT extractions (Figure 1). While methanol had the least toxic effect at 10 %, none of the solvents tested were significantly toxic to whole blood at 5 % (v/v) under the conditions tested (Annex A.1). Based on Dr. Foster's recommendations (personal communication) for ideal extraction conditions, and consideration of solvents concentrations, less than 5 % ethanol was chosen as the optimal solvent and maximum concentration.

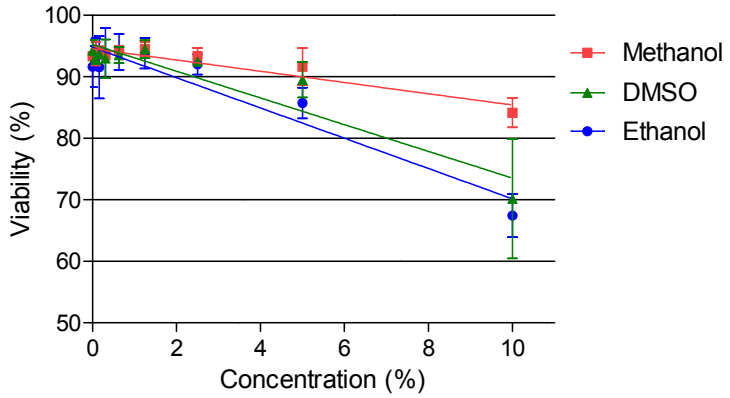

Figure 1: Toxicity assay to determine maximum concentrations of solvents for extracts.

Fresh whole red blood was incubated in the solvents at 2.5 x 10^5 cells / ml for 2 hr at 37°C, 5 % CO_2. Viability was assessed using a Guava PCA cytometer. Points represent the mean ± SEM (n = 3); samples were analyzed by one-way ANOVA followed by Dunnett's post-hoc tests for specific comparisons, $\alpha = 0.05$ (Annex A.1).

Glycerol and DMSO were chosen as ROS scavenger controls, thus, were tested for toxicity (Figure 2) (Appendix A.2). 10 % DMSO, although toxic to the cells under these conditions (Figure 2), was studied previously and shown to have protective effects against radiation-induced DNA damage (13). 4.3 % glycerol was shown to be non-toxic under the tested conditions (Figure 2) and have similar scavenger ability (10).

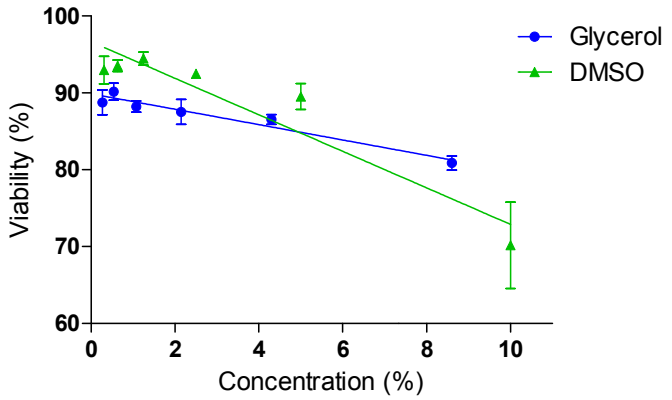

Figure 2: Toxicity assay for controls DMSO and glycerol.

Fresh whole red blood was incubated in the solvents at 2.5 x 10^5 cells / ml for 2 hr at 37°C, 5 % CO_2. Viability was assessed using a Guava PCA cytometer. Points represent the mean ± SEM (n = 3); samples were analyzed by one-way ANOVA followed by Dunnett's post-hoc tests for specific comparisons, $\alpha = 0.05$ (Annex A.2).

3.1.2 Extract Toxicity

Extracts tested for toxicity include GSE4, GSE5, GSE6, LT, vitamin C, and WR-1065. GSE4 at 90 µg/ml in 2 % ethanol and LT at 180 µg/ml in 4 % ethanol did not have a significant effect on cell viability under the comet assay conditions (Figure 3); these extracts were tested further using the comet assays. The 90 µg/ml concentration of GSE4 was chosen as a maximum concentration for further experiments as it did not result in a significant change of viability from controls (Annex A.3). The 84 µg/ml concentration of LT was chosen as the maximum concentration as it did not result in a change in viability from the controls; greater concentrations had increased variability in viability assessment, and it was below the 100 µg/ml toxicity limit as previously published data indicated (40). Both vitamin C and WR-1065 were resuspended in dH_2O and did not have toxic effects under the conditions tested up to and including 1 mM (Figure 4, Annex A.4).

GSE5 can be used at a maximum concentration of 45 µg/ml and GSE6 can be used at a maximum concentration of 22.5 µg/ml without affecting cell viability under the comet assay conditions. To date, only GSE4 and LT have been tested using the comet and CYP3A4 assays due to time constraints. GSE5 and GSE6 were tested for antioxidant capacity at a concentration of 100 µg/ml to be consistent with the GSE4.

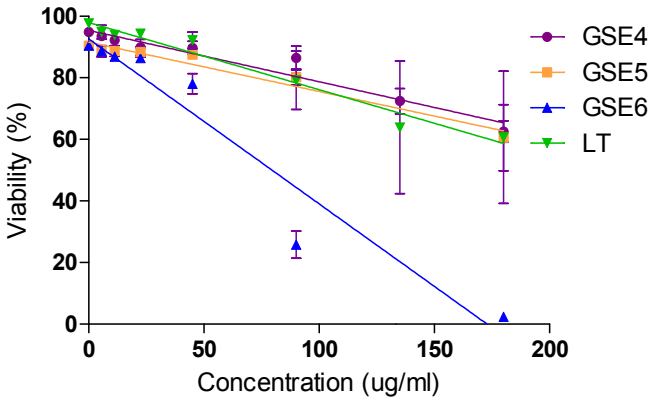

Figure 3: Toxicity assessment versus concentration for GSE4, GSE5, GSE6, and LT.

2.5 x 10^5 cells/ml were incubated for 2 hr at 37°C, 5 % CO_2 and viability was assessed using a Guava PCA cytometer. Points represent the mean ± SEM (n = 3); samples were analyzed by one-way ANOVA followed by Dunnett's post-hoc tests for specific comparisons, $\alpha = 0.05$ (Annex A.3).

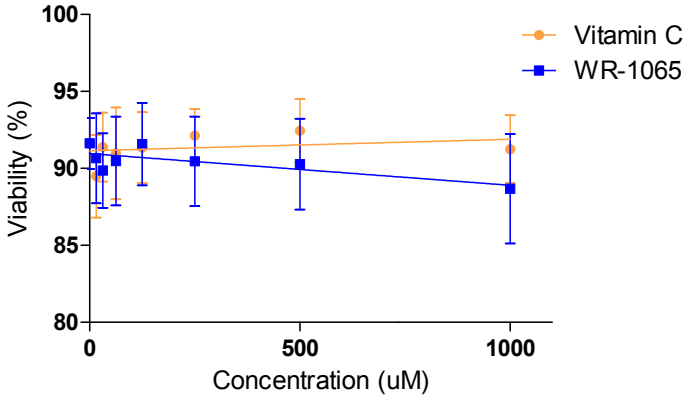

Figure 4: Toxicity assessment versus concentration for vitamin C and WR-1065.

2.5 x 10^5 cells/ml were incubated for 2 hr at 37°C, 5 % CO_2 and viability was assessed using a Guava PCA cytometer. Points represent the mean ± SEM (n = 3); samples were analyzed by one-way ANOVA followed by Dunnett's post-hoc tests for specific comparisons, $\alpha = 0.05$ (Annex A.4).

3.1.3 Extract Incubation Time Effect on Toxicity

Incubation time of 2 to 6 hr was assessed for affect on viability for PBS, 4.3 % glycerol, 2 % ethanol, and 90 µg/ml GSE4 (Figure 5). A 2 hr incubation time was chosen as it resulted in no significant change in viability for all samples (Annex A.5) except DMSO (Figure 1).

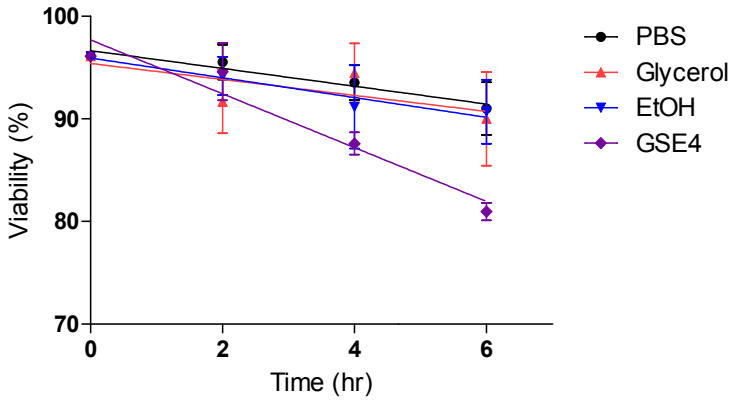

Figure 5: Effect of incubation time on cell viability for GSE4 and controls.

2.5 x 10^5 cells/ml were incubated for 2, 4, and 6 hr at 37°C, 5 % CO_2 and viability was assessed using a Guava PCA cytometer. Points represent the mean ± SEM (n = 3); samples were analyzed by two-way ANOVA comparing solvent and incubation time followed by Bonferroni post-hoc tests for specific comparisons, $\alpha = 0.05$ (Annex A.5).

3.2 Assessment of Antioxidant Capacity

Antioxidant capacity was measured as Trolox®-equivalents (TE), a water-soluble vitamin E derivative using the Trolox®-equivalents antioxidant assay (TEAC). When oxidized, metmyoglobin forms ferryl myoglobin which subsequently oxidizes ABTS to ABTS*, a soluble chromogen. Antioxidants prevent the production of ABTS* in a concentration-dependent manner.

3.2.1 Antioxidant Capacity of Extracts and Controls

The Trolox®-equivalents (TE) of the measured GSEs were: GSE4, 37.4 µmol TE /mg; GSE5, 7.8 µmol TE /mg; and GSE6, 44.2 µmol TE /mg; LT, 6.1 µmol TE /mg; vitamin C, 40.1 µmol TE /mg; WR-1065, 38.2 µmol TE /mg (Figure 6). Controls PBS, 10 % DMSO, 4.3 % glycerol, and 2 % ethanol had no effect (Annex B.1).

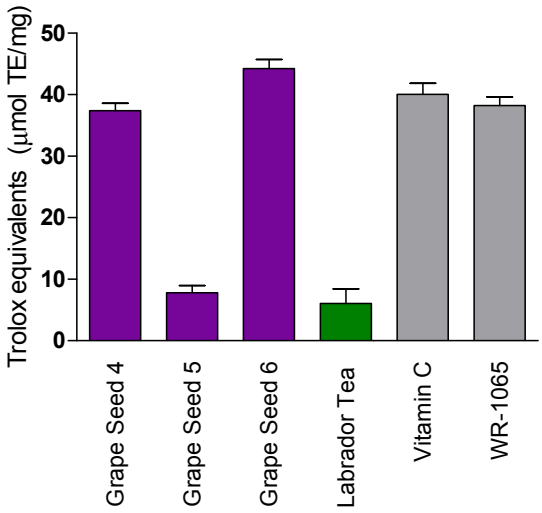

Figure 6: Antioxidant capacity expressed as Trolox ® equivalents (µmol TE /mg).

Assessed using antioxidant capacity for GSE4, GSE5, GSE6, LT, vitamin C, and WR-1065. Vitamin C solutions were used within 1 day of being made and WR-1065 samples were used within 30 min of being made. GSEs TE activities were not shown to degrade over time (Annex A.5) and represent a selection of preparations. Each bar represents the mean ± SEM (n > 3). Controls (PBS, 10 % DMSO, 4.3 % glycerol, and 2 % ethanol) did not have any statistically relevant antioxidant capacity (Annex B.1).

3.2.2 Effect of Extract Stock Solution Age

Three separate stock solutions of each GSE4, GSE5, and GSE6 (Annex A.6) were compared to ensure antioxidant capacity was not affected by stock solution age; extract ages compared were 1, 15, and 180 days (Figure 7). None of the GSEs significantly changed in antioxidant capacity as measured in Trolox®-equivalents (Annex A.6). Vitamin C and WR1065 were made fresh, therefore antioxidant capacity over time was not determined; changes in antioxidant capacity of LT was not assessed due to time limitations.

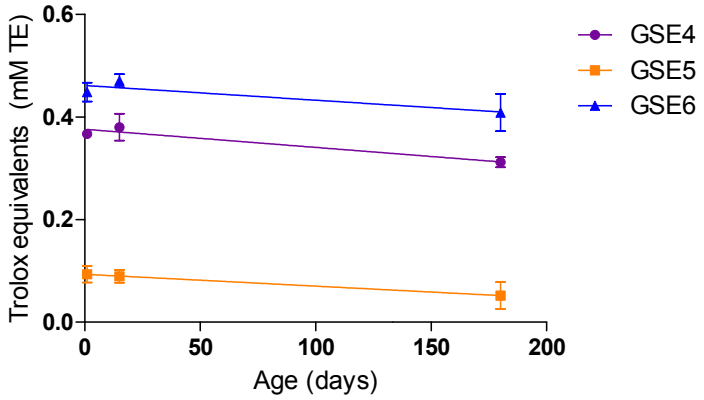

Figure 7: GSE antioxidant capacity over time.

Comparison of single GSE 4, 5, and 6 extract isolates analyzed at different ages (180, 15, and 1 day). Points represent the mean ± SEM (n = 3); samples were analyzed by one-way ANOVA followed by Dunnett's post-hoc tests for specific comparisons, $\alpha = 0.05$ (Annex A.6).

3.3 Assessment of Metabolic Interactions using CYP3A4 Assays

Natural health products (including antioxidants) can decrease CYP3A4-mediated metabolism of substrates, indicating a potential for drug metabolism interactions if they are taken together with other therapeutic health products. GSE4 (90 µg/ml), LT (84 µg/ml), vitamin C (1 mM), and WR-1065 (1 mM) were tested for their effect on CYP3A4-mediated metabolism of marker substrates. Future work could include analysis of GSE5 and GSE6 using these assays, due to time constraints they were not completed in this analysis.

3.3.1 CYP3A4 Fluorescent Microwell Plate Assay

DBF is de-alkylated by CYP3A4 to form a fluorescent complex which can be quantified. GSE4 and LT inhibited DBF metabolism 95.9 % and 62.0 % respectively according to the fluorescent microwell plate assay (Figure 8). Vitamin C and WR-1065 inhibited DBF metabolism -0.1 % and 5.5 % respectively. Ketoconazole is a known inhibitor of CYP3A4-mediated metabolism and is included as a positive control. The unexpectedly high inhibition observed in this assay by GSE4 and LT could be caused by quenching of the fluorescent signal, a limitation of the assay. Therefore, and HPLC version of the assay was performed, where only final metabolites are measured thus avoiding the potentially quenching effects of the extracts (Figure 9).

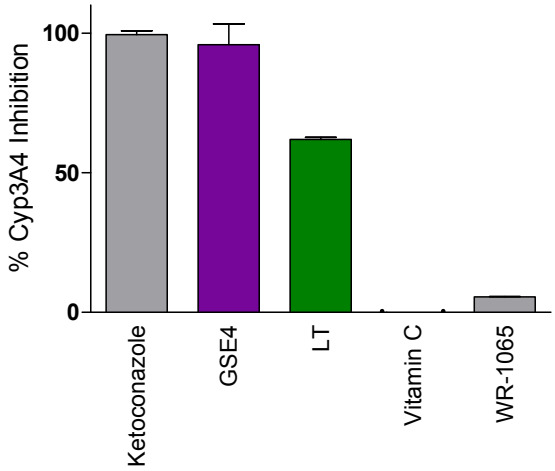

Figure 8: CYP3A4 inhibition plate reader assay.

DBF (dibenzylfluorescein) is de-alkylated by CYP3A4 to form a fluorescent fluorescein which can be quantified. Ketoconazole was included as a positive control. Change in fluorescence is measured using a plate reader. CYP3A4 inhibition of LT (84 μg/ml), GSE4 (90 μg/ml), 1 mM vitamin C (176.12 μg/ml), and 1 mM WR-1065 (207.16 μg/ml) compared to the control ketoconazole. Each bar represents the mean ± SEM (n = 3).

3.3.2 CYP3A4 Liquid Chromatography Assay

Testosterone is metabolized to 6-b-hydroxytestosterone by CYP3A4, a process which can be quantified by liquid chromatography. CYP3A4 inhibitors, such as ketoconazole, can inhibit this reaction close to 100 % and can be used as a control to assess other potential CYP3A4 inhibitors. In this study, GSE4 and LT inhibited testosterone metabolism 1.5 % and 2.9 % respectively (Figure 9). This method is not affected by quenching from reagents because direct levels of 6-beta-OH-testosterone (metabolite) produced is measured. This is in contrast to the plate reader assay where the fluorescence of extracts, substrates, and metabolites are all measured together. We show here that quenching in the plate reader assay resulted in false high CYP3A4 inhibition values while HPLC values are considered correct due to the increased accuracy of the assay because of direct metabolite measurement.

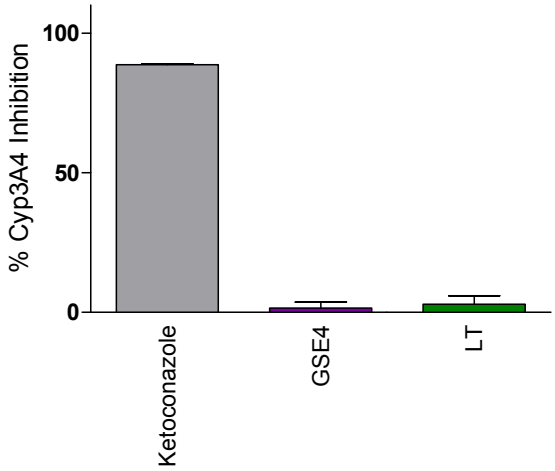

Figure 9: CYP3A4 HPLC assay for GSE4 and LT.

CYP3A4 inhibition of LT (84 μg/ml), and GSE4 (90 μg/ml), compared to the control ketoconazole. Testosterone is metabolized by CYP3A4 to 6-beta-OH-testosterone which was quantified by HPLC. Each bar represents the mean ± SEM (n = 3).

3.4 Comet Assay

3.4.1 Acellular versus Cellular Comet Assay

DNA damage can be quantified using the comet assays by assessing the extent of DNA that migrates into the tail region of the comet; this information contributes to the calculation of percent tail intensity (the portion of DNA in the tail). The cellular comet assay detects DNA damage which has occurred in a live cell with the capacity for repair versus the acellular comet assay which detects DNA damage occurring to naked DNA outside of the cellular environment with no possibility for repair.

In this report, ^{60}Co gamma-ray irradiated samples of naked DNA and whole blood (0, 1, 2, and 4 Gy) were analyzed using both assays (acellular and cellular comet assays) (Figure 10). As observed previously, the acellular comet assay protocol demonstrated greater tail intensity (indicator of DNA damage) compared to the standard cellular comet assay ($p < 0.0001$, Annex A.6), thus requiring separate dose response curves.

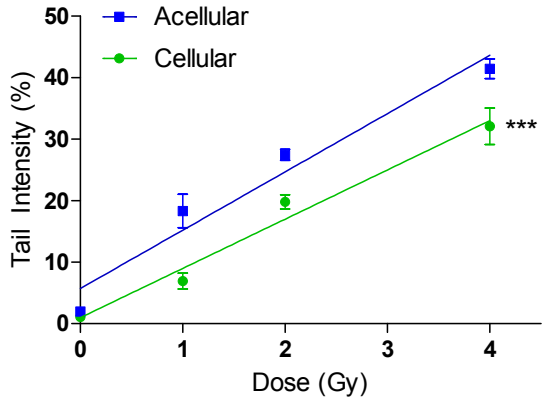

Figure10: Differences between the acellular and cellular comet assays.

Determined by measuring the comet tail intensity induced from doses of ^{60}Co gamma-ray irradiation 0 to 4 Gy using the comet assay. Points represent the mean ± SEM (n = 3); samples were analyzed by two-way ANOVA comparing radiation dose and comet assay type followed by Bonferroni post-hoc tests for specific comparisons, $\alpha = 0.05$ (Annex A.7) (*** $p < 0.0001$).

3.4.2 Effect of Ethanol on Radiation-Induced DNA Damage

The effect of 2 % ethanol was assessed using the acellular and cellular comet assays for changes in radiation-induced DNA damage as measured by percent tail intensity (Figure 11). Ethanol (2 %) did not significantly reduce the background amount of DNA damage (at 0 Gy) in either the acellular or cellular comet assay ($p > 0.05$) (Annex A.8). In the acellular comet assay, ethanol did significantly reduce the amount of radiation-induced DNA damage ($p < 0.05$) at 1 Gy dose and above ($p < 0.01$). In the cellular comet assay, 2 % ethanol significantly reduced the amount of radiation-induced DNA damage at 2 Gy dose and above ($p < 0.01$). GSE4 and LT values have been normalized within each experimental repeat to account for this protective effect from ethanol.

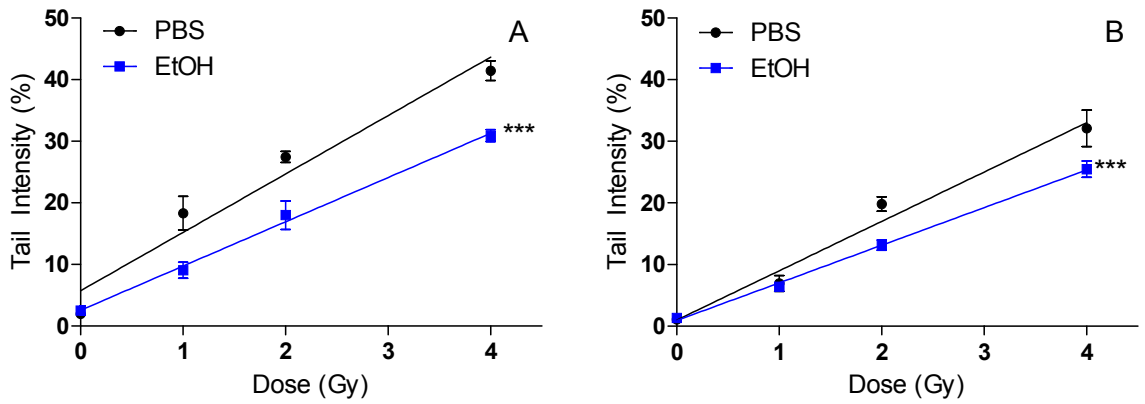

Figure 11: Effect of ethanol compared to PBS control on radiation-induced DNA damage

Determined by measuring the comet tail intensity induced from doses of ^{60}Co gamma-ray irradiation 0 to 4 Gy using the comet assay. (A) Dose response curve for acellular and (B) cellular comet assay. Each data point represents the geometric mean ± SEM (n = 3); samples were analyzed by two-way ANOVA comparing radiation dose and solvent (ethanol) followed by Bonferroni post-hoc tests for specific comparisons, α = 0.05 (Annex A.8).

3.4.3 Effect of Control ROS Scavengers on Radiation-Induced DNA Damage

The effect of DMSO and glycerol were assessed using the acellular and cellular comet assays for changes in percent tail intensity (Figure 12). Controls of 10 % DMSO and 4.3 % glycerol were chosen as they had been reported to have similar antioxidant capacity at these concentrations (10). Neither control had a significant protective effect on the background percent tail intensity (estimate of endogenous DNA damage) when cells were not exposed to radiation ($p > 0.05$) (Annex A.9). At doses of 2 to 4 Gy, glycerol did not have a significant protective effect when measured by the acellular comet assay (Figure 12 A; $p > 0.05$), but did have a protective effect ($p < 0.05$) when measured using the cellular comet assay (Figure 12 B). DMSO was protective of DNA in the acellular comet assay at 1, 2, and 4 Gy ($p < 0.001$), but only demonstrated a significant difference at 2 and 4 Gy in the cellular comet assay ($p < 0.001$).

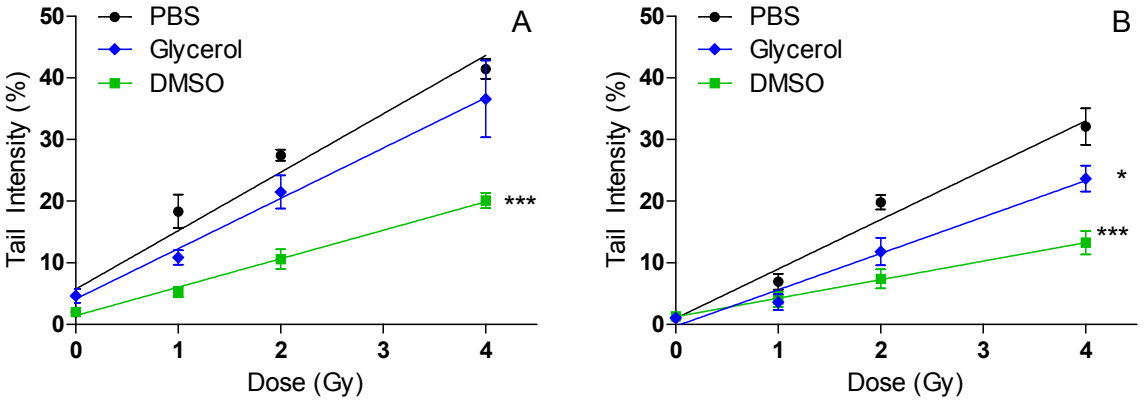

Figure 12: Effect of DMSO and glycerol controls on radiation-induced DNA damage

Determined by measuring the comet tail intensity induced from doses of ^{60}Co gamma-ray irradiation 0 to 4 Gy using the comet assay. (A) Dose response curve for acellular and (B) cellular comet assay. Each data point represents the geometric mean ± SEM (n = 3); samples were analyzed by two-way ANOVA comparing radiation dose and solvent (DMSO or glycerol) followed by Bonferroni post-hoc tests for specific comparisons, $\alpha = 0.05$ (Annex A.9).

3.4.4 Effect of Aging (or long term storage) on Extracted Stock Solutions

Stock solutions of grape seed extract made fresh (< 3 days) or stored (> 1 month) were compared to determine if age of extracted solutions had an effect on the amount of DNA damage (Figure 13). For both the acellular and cellular comet assays, the age of extract solution did not significantly affect the levels of radiation-induced DNA damage as measured by percent tail intensity ($p = 0.1923$) (Annex A.10).

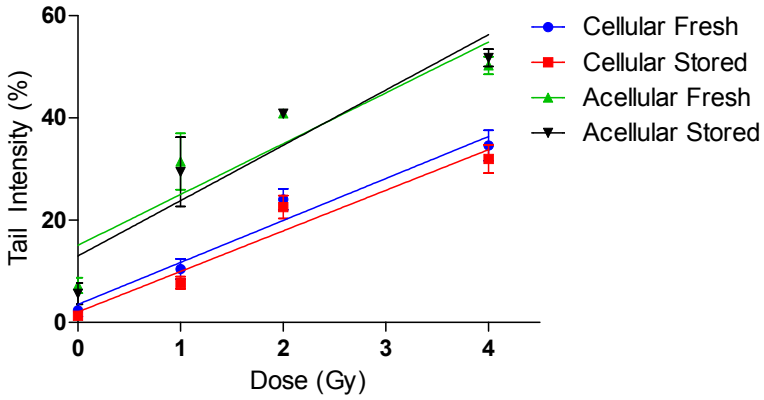

Figure 13: Comparison of fresh or stored GSE4 to determine extract stability.

Determined by measuring the comet tail intensity induced from doses of ^{60}Co gamma-ray irradiation 0 to 4 Gy using the acellular and cellular comet assays. Age of GSE4 did not significantly affect percent tail intensity (estimate of DNA damage) as measured by the comet assays ($p = 0.1923$). Points represent the mean ± SEM ($n = 3$); samples were analyzed by two-way ANOVA comparing dose and extract age followed by Bonferroni post-hoc tests for specific comparisons, $\alpha = 0.05$ (Annex A.10).

3.4.5 Effect of Grape Seed and Labrador Tea Extracts on Radiation-Induced DNA Damage

The effect of GSE4 and LT ROS scavengering ability was assessed using the acellular and cellular comet assays by measuring changes in percent tail intensity (estimate of DNA damage) (Figure 14). Neither GSE4 nor LT caused a significant effect to the levels of background DNA damage (percent tail intensity at 0 Gy). In the acellular comet assay (A), 90 µg/ml GSE4 had a significant DNA damaging effect (increased percent tail intensity) at 2 and 4 Gy ($p < 0.01$) (Annex A.11); LT (84 µg/ml) had a significant damaging effect at 1, 2, and 4 Gy ($p < 0.01$). Neither GSE4 nor LT had any effect on comet tail intensity in the cellular comet assay (B, $p > 0.05$).

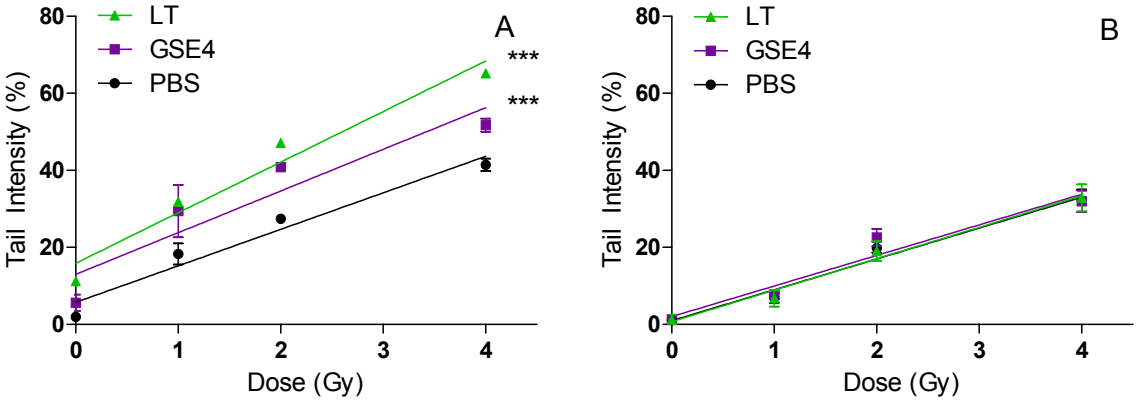

Figure 14: Effect of GSE4 and LT on radiation-induced DNA damage

Determined by measuring the comet tail intensity induced from doses of ^{60}Co gamma-ray irradiation 0 to 4 Gy using the comet assay. (A) Dose response curve for acellular and (B) cellular comet assay (*** $p < 0.0001$). Each data point represents the geometric mean ± SEM ($n = 3$); samples were analyzed by two-way ANOVA comparing radiation dose and treatment (GSE4 or LT) followed by Bonferroni post-hoc tests for specific comparisons, $\alpha = 0.05$ (Annex A.11).

4 Discussion

This study was undertaken to provide information on readily available botanicals which may have the potential to be used as prophylactic radioprotectants. Previous studies of pure compounds from grape seed extract have shown a high antioxidant activity using free-radical scavenging tests and DNA-damage protection assays (19). This study analyzed commercially available grape seed extract supplements (GSEs) and whole leaves of Labrador tea (LT) as promising radioprotectants. These compounds were specifically selected because they were expected to demonstrate low side effects, be easily administered, have a long shelf life, and have radioprotectant potential. This work sets a platform for studying additional botanicals by identifying and validating a set of procedures to be used for screening potential radioprotectants with antioxidant properties.

4.1 Antioxidant Capacity

Previous studies have shown that proanthocyanidin from grape seed extract has greater antioxidant capacity than vitamin C or E (33). In this study, we show that two of the three GSEs (GSE 4 and GSE6) had similar antioxidant capacity as vitamin C and WR-1065 (GSE4 = 37.4 μmol TE /mg , GSE5 = 7.8 μmol TE /mg, GSE6 = 44.2 μmol TE /mg , vitamin C = 40.1 μmol TE /mg, WR-1065 = 38.2 μmol TE /mg). Kedage *et al*. reported similar antioxidant capacities, as reported in this study, using the TEAC assay for varieties of whole grape extracts (49). The compounds tested in this study were not pure extracts; therefore results may have differed by: 1) containing less antioxidant compounds per weight due to the presence of filler-material; 2) different extraction processes; 3) loss of material during filtering; 4) solubility in ethanol versus other solvents used in previous studies; and 5) confounding effects from other components (filler material) in these commercial products. However, it is encouraging to have observed comparable antioxidant capacities in the commercial extracts as found in pure extracts and fresh grapes.

Previous studies have shown that Labrador tea leaf extract had 16 ± 2 μmol TE/mg antioxidant capacity, approximately the same capacity as the known standard gallic acid (40). The LT in this study had 6.1 μmol TE/mg antioxidant capacity. The difference in the observed TE capacity between previous studies and that observed here is possibly caused by the use of different substrates to measure Trolox® equivalents, extraction processes, or different solvents.

LT has a lower antioxidant capacity than GSEs, vitamin C, and WR-1065, but, by having some level of antioxidant capacity indicates it still has potential as an antioxidant botanical radioprotectant.

4.2 Drug and Metabolic Interactions

The ideal radioprotectant produces no cumulative or irreversible toxicity, does not interact with the metabolism of other drugs, is easily administered, and has a 2 to 5 year shelf life (2, 5). Drug interactions occur when the efficacy or toxicity of a drug is changed by the presence of another substance. In this case, we studied a drug metabolic interaction of botanical extracts administered for radioprotection. Although drug metabolism can be affected by a variety of factors, a drug disposition process commonly investigated for assessing botanical drug interactions is the metabolism of substrates by the Phase I drug metabolizing enzyme CYP3A4 (50, 51). CYP3A4 is involved in a variety of drug and metabolism interactions, including approximately 50 % of orally administered drugs (51). It is therefore imperative that the botanical extracts being proposed as radioprotectants do not significantly alter CYP3A4 mediated metabolism (52). In this study, the HPLC CYP3A4 assay showed that GSE4 and LT inhibited CYP3A4 metabolism of testosterone by -0.1 and 5.5 % respectively. Therefore, both GSE4 and LT showed little to no interaction with CYP3A4 mediated metabolism. These results indicate that they both may have a potential as radioprotectants due to their low risk for drug interactions, although further in-depth drug interaction testing is still required.

4.3 DNA damage

4.3.1 Control ROS Scavengers

DMSO is a ROS scavenger of hydroxyl radicals produced from radiolysis of water thereby preventing ROS from damaging DNA (10, 11, 12, 13). In this study, 10 % DMSO significantly reduced the radiation-induced DNA damage in the cellular and acellular environments, indicating uptake and localization of DMSO near the DNA prior to radiation exposure. Because DMSO provided both acellular and cellular DNA protection, it is likely independent of cellular activity, offering a passive scavenger process for DNA protection. The results in this study are consistent with other studies,

indicating that the chosen assays are capable of detecting ROS scavengers by measuring DNA damage (10, 12, 13).

Glycerol is also a ROS scavenger of hydroxyl radicals produced from radiolysis of water thereby limiting ROS from damaging DNA (10, 12, 13). The comet assay used 4.3 % glycerol as it was predicted to have similar scavenging abilities as 10 % DMSO but was not toxic to cells under these conditions (10). Glycerol provided protection from radiation-induced DNA damage in the cellular environment (minimal repair) but not in the acellular environment (no repair). It is possible that 4.3 % glycerol, under cellular assay conditions, optimized the minimal DNA repair processes occurring at the low temperature in the comet assay. More replicates of the acellular comet assay will increase the power of the statistical analyses, potentially increasing assay resolution.

4.3.2 Grape Seed Extract

The acellular comet assay detects DNA damage without the potential for DNA repair processes, increasing the sensitivity and specificity of the assay (9). At the maximum concentration where there is no significant effect on cell toxicity, GSE4 increased the amount of DNA damage at all radiation exposure doses examined but did not cause a statistical increase in background DNA damage at no exposure. It is possible that GSE4 is being affected by the radiation and becomes more damaging to the DNA in the acellular environment where molecular transport was not needed for components to be in proximity of the DNA. Research using pure extracts has shown that 75 to 300 μg / ml grape extracts increased non-repaired damage (double strand DNA breaks) in lymphocytes, suggesting that high concentrations of some components of grape seed extracts can increase DNA damage (19, 48). This increase in DNA damage from high concentrations of antioxidants has also been observed in vitamin C, WR-1065, β-carotene, and lycopene (19, 23, 28, 53). The resulting increase in DNA damage when exposed to radiation may be caused by an antioxidant's interaction with free metal ions generating DNA-damaging oxygen radicals, an effect observed with high concentrations of flavonoids and phenolic compounds (12, 19). It was hoped that the high concentration of EDTA (100 mM, metal chelator) in the maintenance buffer would have avoided this effect. It is also possible that other ingredients in the commercial grape seed extract (magnesium stearate, microcrystalline cellulose, silicon dioxide, stearic acid) were

affected by the radiation and become more damaging to the DNA. Future work will involve testing lower concentrations of GSE4 to determine if an optimal concentration exists in this system.

Cellular DNA damage was assessed under conditions of minimal DNA repair (cellular comet assay). The data presented here show no protection of DNA in the cellular environment and the increase in radiation-induced DNA damage observed in the acellular environment was not observed. Future work could include testing GSE4 and LT for rates of transport into the cell to determine if this lack of effect is due to lack of molecular uptake. Repeating experiment by irradiating cells at 37^{o}C could help elucidate this hypothesis. It is not recommended to investigate higher concentrations of GSEs as these assays were performed at the maximum concentration which did not reduce cell viability.

4.3.3 Labrador Tea

The LT was also assessed in both the acellular and cellular environment. As observed with GSE4, no DNA protection was observed in the cellular environment and the extract resulted in increased radiation-induced DNA damage in the acellular environment at all doses above background. As hypothesized with GSE4, it is possible that interactions with free metal ions generate DNA-damaging oxygen radicals as observed in other phenolic antioxidants (12, 19). The LT was prepared from dried whole leaves and therefore did not contain filler components thought to be involved in the increased damage observed in the GSE4.

Cellular DNA damage was assessed under conditions of minimal DNA repair (cellular comet assay). The data presented here show no protection of DNA in the cellular environment; although the increase in DNA damage observed in the acellular environment was not observed. Future work could include testing LT for rates of transport into the cell to determine if this lack of effect is due to lack of molecular uptake, as recommended for GSE4. As suggested for GSE4, repeating experiment by irradiating cells at 37^{o}C could help elucidate this hypothesis. It is not recommended to investigate concentrations of LT greater than 84 μg/ml, but to determine if a lower optimal dose exists. Additional types of antioxidant assay could also be performed to determine if non-phenolic antioxidant compounds are present in the LT.

4.4 Conclusions

This work sets a platform for studying additional botanicals by identifying and validating a set of procedures to use in screening potential antioxidant radioprotectants. Although neither GSE nor LT leaves provided protection from radiation-induced DNA damage at the concentrations tested, further work could be done to optimize the concentrations or explore other potential radioprotectants using this collection of assays. Two additional GSEs (GSE5 and GSE6) with differing antioxidant capacities have been prepared and are ready to be tested using the comet assays. Additional compounds which satisfy the needs for a radioprotectant could include commercial supplements of blueberry, cranberry, mint, or ginger extracts.

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Annex A Statistics

A.1 *Maximum solvent concentration determination.*

Determined by measuring cell viability. 2.5 x 10^5 cells were incubated for 2 hr at 37°C, 5 % CO_2 and viability was assessed using a Guava PCA cytometer. Data (n = 3) were analyzed by one-way ANOVA followed by Dunnett's post-hoc tests for specific comparisons, $\alpha = 0.05$.

Ethanol		
P value	< 0.0001	
P value summary	***	
Are means signif. different? (P < 0.05)	Yes	
Number of groups	9	
F	20.96	
R square	0.9031	
Dunnett's Multiple Comparison Test	**95% CI of diff**	**Significant? P < 0.05?**
0.00 vs 0.08	-9.976 to 5.642	No
0.00 vs 0.16	-7.709 to 7.909	No
0.00 vs 0.31	-10.01 to 5.609	No
0.00 vs 0.63	-10.21 to 5.409	No
0.00 vs 1.25	-9.976 to 5.642	No
0.00 vs 2.50	-8.142 to 7.476	No
0.00 vs 5.00	-1.875 to 13.74	No
0.00 vs 10.00	16.42 to 32.04	Yes

Methanol		
P value	< 0.0001	
P value summary	***	
Are means signif. different? (P < 0.05)	Yes	
Number of groups	9	
F	12.65	
R square	0.8490	
Dunnett's Multiple Comparison Test	**95% CI of diff**	**Significant? P < 0.05?**
0.00 vs 0.08	-4.189 to 3.389	No
0.00 vs 0.16	-4.623 to 2.956	No
0.00 vs 0.31	-3.989 to 3.589	No
0.00 vs 0.63	-4.789 to 2.789	No
0.00 vs 1.25	-4.956 to 2.623	No
0.00 vs 2.50	-3.856 to 3.723	No
0.00 vs 5.00	-2.123 to 5.456	No
0.00 vs 10.00	5.377 to 12.96	Yes

DMSO		
P value	< 0.0001	
P value summary	***	
Are means signif. different? (P < 0.05)	Yes	
Number of groups	9	
F	13.47	
R square	0.8569	
Dunnett's Multiple Comparison Test	**95% CI of diff**	**Significant? P < 0.05?**
0.00 vs 0.08	-7.356 to 10.16	No
0.00 vs 0.16	-8.223 to 9.289	No
0.00 vs 0.31	-7.456 to 10.06	No
0.00 vs 0.63	-8.023 to 9.489	No
0.00 vs 1.25	-8.956 to 8.556	No
0.00 vs 2.50	-6.956 to 10.56	No
0.00 vs 5.00	-4.023 to 13.49	No
0.00 vs 10.00	15.34 to 32.86	Yes

A.2 *Viability assessment versus concentrations of glycerol and DMSO.*

Determined by measuring cell viability. 2.5 x 10^5 cells were incubated for 2 hr at 37°C, 5 % CO_2 and viability was assessed using a Guava PCA cytometer. Data (n = 3) were analyzed by one-way ANOVA followed by Dunnett's post-hoc tests for specific comparisons, $\alpha = 0.05$.

Glycerol		
P value	< 0.0001	
P value summary	***	
Are means signif. different? (P < 0.05)	Yes	
Number of groups	8	
F	109.1	
R square	0.9708	
Dunnett's Multiple Comparison Test	**95% CI of diff**	**Significant? P < 0.05?**
0 vs 0.270	-6.288 to 9.654	No
0 vs 0.540	-7.080 to 7.680	No
0 vs 1.080	-5.155 to 9.605	No
0 vs 2.150	-4.455 to 10.30	No
0 vs 4.300	-3.480 to 11.28	No
0 vs 8.600	2.195 to 16.95	Yes
0 vs 17.200	49.65 to 64.40	Yes

DMSO		
P value	< 0.0001	
P value summary	***	
Are means signif. different? (P < 0.05)	Yes	
Number of groups	9	
F	13.47	
R square	0.8569	
Dunnett's Multiple Comparison Test	**95% CI of diff**	**Significant? P < 0.05?**
0.00 vs 0.08	-7.356 to 10.16	No
0.00 vs 0.16	-8.223 to 9.289	No
0.00 vs 0.31	-7.456 to 10.06	No
0.00 vs 0.63	-8.023 to 9.489	No
0.00 vs 1.25	-8.956 to 8.556	No
0.00 vs 2.50	-6.956 to 10.56	No
0.00 vs 5.00	-4.023 to 13.49	No
0.00 vs 10.00	15.34 to 32.86	Yes

A.3 Viability assessment versus concentration for GSE4 and LT.

Determined by measuring cell viability. 2.5 x 10^5 cells were incubated for 2 hr at 37°C, 5 % CO_2 and viability was assessed using a Guava PCA cytometer. Data (n = 3) were analyzed by one-way ANOVA followed byDunnett's post-hoc tests for specific comparisons, $\alpha = 0.05$.

GSE4		
P value	< 0.0001	
P value summary	***	
Are means signif. different? (P < 0.05)	Yes	
Number of groups	8	
F	18.39	
R square	0.8009	
Dunnett's Multiple Comparison Test	**95% CI of diff**	**Significant? P < 0.05?**
0 vs 5.625	-9.198 to 11.85	No
0 vs 11.25	-7.876 to 13.17	No
0 vs 22.5	-5.514 to 15.53	No
0 vs 45	-5.390 to 15.65	No
0 vs 90	-2.146 to 18.90	No
0 vs 135	11.93 to 32.97	Yes
0 vs 180	21.80 to 42.85	Yes

GSE5		
P value	< 0.0001	
P value summary	***	
Are means signif. different? (P < 0.05)	Yes	
Number of groups	7	
F	12.62	
R square	0.7829	
Dunnett's Multiple Comparison Test	**95% CI of diff**	**Significant? P < 0.05?**
0 vs 5.6200	-6.794 to 10.95	No
0 vs 11.2500	-7.159 to 10.58	No
0 vs 22.5000	-6.796 to 10.95	No
0 vs 45.0000	-5.996 to 11.75	No
0 vs 90.0000	1.346 to 19.09	Yes
0 vs 180.0000	13.56 to 31.30	Yes

GSE6		
P value	< 0.0001	
P value summary	***	
Are means signif. different? (P < 0.05)	Yes	
Number of groups	7	
F	242.9	
R square	0.9858	
Dunnett's Multiple Comparison Test	**95% CI of diff**	**Significant? P < 0.05?**
0 vs 5.6200	-7.291 to 10.94	No
0 vs 11.2500	-5.515 to 12.71	No
0 vs 22.5000	-5.063 to 13.16	No
0 vs 45.0000	3.270 to 21.50	Yes
0 vs 90.0000	55.55 to 73.78	Yes
0 vs 180.0000	78.94 to 97.16	Yes

LT		
P value	0.1748	
P value summary	ns	
Are means signif. different? (P < 0.05)	No	
Number of groups	8	
F	1.719	
R square	0.4292	
Dunnett's Multiple Comparison Test	**95% CI of diff**	**Significant? P < 0.05?**
0 vs 5.625	-44.02 to 49.77	No
0 vs 11.25	-43.12 to 50.67	No
0 vs 22.5	-43.52 to 50.27	No
0 vs 45	-41.33 to 52.46	No
0 vs 90	-28.37 to 65.41	No
0 vs 135	-13.06 to 80.72	No
0 vs 180	-9.841 to 83.95	No

A.4 Viability assessment versus concentration for vitamin C and WR-1065.

Determined by measuring cell viability. 2.5 x 10^5 cells were incubated for 2 hr at 37°C, 5 % CO_2 and viability was assessed using a Guava PCA cytometer. Data were analyzed by one-way ANOVA followed byDunnett's post-hoc tests for specific comparisons, $\alpha = 0.05$.

Vitamin C		
P value	0.9813	
P value summary	ns	
Are means signif. different? (P < 0.05)	No	
Number of groups	8	
F	0.2041	
R square	0.05619	
Dunnett's Multiple Comparison Test	**95% CI of diff**	**Significant? P < 0.05?**
0 vs 15.624	-9.517 to 9.517	No
0 vs 31.25	-11.41 to 7.627	No
0 vs 62.5	-10.99 to 8.040	No
0 vs 12.5	-11.36 to 7.677	No
0 vs 250	-12.14 to 6.892	No
0 vs 500	-12.47 to 6.565	No
0 vs 1000	-11.26 to 7.770	No

WR-1065		
P value	0.9967	
P value summary	ns	
Are means signif. different? (P < 0.05)	No	
Number of groups	8	
F	0.1139	
R square	0.03215	
Dunnett's Multiple Comparison Test	**95% CI of diff**	**Significant? P < 0.05?**
0 vs 15.624	-10.14 to 12.08	No
0 vs 31.25	-9.346 to 12.87	No
0 vs 62.5	-9.961 to 12.26	No
0 vs 12.5	-11.06 to 11.16	No
0 vs 250	-9.936 to 12.28	No
0 vs 500	-9.761 to 12.46	No
0 vs 1000	-8.161 to 14.06	No

A.5 Effect of incubation time on cell viability for GSE4 and controls.

Determined by measuring cell viability. 2.5 x 10^5 cells were incubated for 2, 4, and 6 hr at 37^{o}C, 5 % CO_2 and viability was assessed using a Guava PCA cytometer. Data (n = 3) were analyzed by two-way ANOVA comparing solvent and incubation time followed by Bonferroni post-hoc tests for specific comparisons, α = 0.05.

Source of Variation	**% of total variation**	**P value**	
Interaction	15.07	0.0212	
Treatment	9.40	0.5011	
Time	31.97	< 0.0001	
Subjects (matching)	29.2166	0.0003	
Bonferroni posttests			
0 vs 2			
Treatment	**95% CI of diff.**	**P value**	**Summary**
PBS	-7.772 to 6.639	P > 0.05	ns
Glycerol	-11.64 to 2.772	P > 0.05	ns
EtOH	-9.139 to 5.272	P > 0.05	ns
GSE4	-8.705 to 5.705	P > 0.05	ns
0 vs 4			
Treatment	**95% CI of diff.**	**P value**	**Summary**
PBS	-9.772 to 4.639	P > 0.05	ns
Glycerol	-8.839 to 5.572	P > 0.05	ns
EtOH	-12.14 to 2.272	P > 0.05	ns
GSE4	-15.71 to -1.295	P<0.01	**
0 vs 6			
Treatment	**95% CI of diff.**	**P value**	**Summary**
PBS	-12.31 to 2.105	P > 0.05	ns
Glycerol	-13.31 to 1.105	P > 0.05	ns
EtOH	-12.61 to 1.805	P > 0.05	ns
GSE4	-22.34 to -7.928	P<0.001	***

A.6 GSE age comparing GSE4, GSE5 and GSE6 isolates (1 to 180 days).

Determined by measuring antioxidant capacity. Data (n = 3) were analyzed by one-way ANOVA followed by Dunnett's post-hoc tests for specific comparisons, $\alpha = 0.05$.

GSE4		
P value	0.0398	
P value summary	*	
Are means signif. different? (P < 0.05)	Yes	
Number of groups	3	
F	4.713	
R square	0.5115	
Dunnett's Multiple Comparison Test	**95% CI of diff**	**Significant? P < 0.05?**
1 day vs 180 days	-0.006618 to 0.1171	No
1 day vs 15 days	-0.07512 to 0.04862	No

GSE5		
P value	0.2845	
P value summary	ns	
Are means signif. different? (P < 0.05)	No	
Number of groups	3	
F	1.450	
R square	0.2437	
Dunnett's Multiple Comparison Test	**95% CI of diff**	**Significant? P < 0.05?**
1 day vs 180 days	-0.02878 to 0.1123	No
1 day vs 15 days	-0.06628 to 0.07478	No

GSE6		
P value	0.2484	
P value summary	ns	
Are means signif. different? (P < 0.05)	No	
Number of groups	3	
F	1.632	
R square	0.2662	
Dunnett's Multiple Comparison Test	**95% CI of diff**	**Significant? P < 0.05?**
1 day vs 180 days	-0.05113 to 0.1306	No
1 day vs 15 days	-0.1131 to 0.06863	No

A.7 Differences between the acellular and cellular comet assays.

Determined by measuring the comet tail intensity induced from doses of ^{60}Co gamma-ray irradiation 0 to 4 Gy using the comet assay. Data (n = 3) was analyzed by two-way ANOVA comparing both dose of radiation and assay type, $\alpha = 0.05$.

Source of Variation	% of total variation	P value	P value summary
Interaction	2.01	0.0356	*
Assay	6.91	< 0.0001	***
Dose	88.14	< 0.0001	***

A.8 Effect of ethanol compared to PBS control on Radiation-induced DNA damage.

Determined by measuring the comet tail intensity induced from doses of ^{60}Co gamma-ray irradiation 0 to 4 Gy using the comet assay. Data (n = 3) was analyzed by two-way ANOVA comparing both dose of radiation and solvent (PBS versus EtOH) followed by Bonferroni post-hoc tests for specific comparisons, $\alpha = 0.05$.

Acellular			
Source of Variation	**% of total variation**	**P value**	**P value summary**
Interaction	2.05	0.0200	*
Dose	5.14	< 0.0001	***
Solvent	88.68	< 0.0001	***
Bonferroni posttests	**95% CI of diff.**	**P value**	**Summary**
0	-5.553 to 6.543	P > 0.05	ns
1	-13.46 to -1.359	P < 0.05	*
2	-14.55 to -2.452	P<0.01	**
4	-15.08 to -2.987	P<0.01	**

Cellular			
Source of Variation	**% of total variation**	**P value**	**P value summary**
Interaction	2.16	0.0207	*
Dose	4.63	< 0.0001	***
Solvent	88.81	< 0.0001	***
Bonferroni posttests	**95% CI of diff.**	**P value**	**Summary**
0	-5.500 to 4.690	P > 0.05	ns
1	-7.948 to 2.243	P > 0.05	ns
2	-12.72 to -2.530	P<0.01	**
4	-13.18 to -2.992	P<0.01	**

A.9 Effect of DMSO and glycerol controls on Radiation-induced DNA damage.

Determined by measuring the comet tail intensity induced from doses of ^{60}Co gamma-ray irradiation 0 to 4 Gy using the comet assay. Data (n = 3) were analyzed by two-way ANOVA comparing both dose of radiation and compound (DMSO and glycerol) followed by Bonferroni post-hoc tests for specific comparisons, $\alpha = 0.05$.

Acellular PBS vs. DMSO			
Source of Variation	**% of total variation**	**P value**	**P value summary**
Interaction	9.29	< 0.0001	***
Compound	23.94	< 0.0001	***
Dose	64.47	< 0.0001	***
Bonferroni posttests			
Dose	**95% CI of diff.**	**P value**	**Summary**
0	-5.549 to 5.636	P > 0.05	ns
1	-18.66 to -7.474	P<0.001	***
2	-22.43 to -11.24	P<0.001	***
4	-26.94 to -15.76	P<0.001	***

Acellular PBS vs Glycerol			
Source of Variation	**% of total variation**	**P value**	**P value summary**
Interaction	1.96	0.2873	ns
Compound	1.96	0.0595	ns
Dose	88.47	< 0.0001	***
Bonferroni posttests			
Dose	**95% CI of diff.**	**P value**	**Summary**
0	-8.165 to 13.50	P > 0.05	ns
1	-18.30 to 3.365	P > 0.05	ns
2	-16.78 to 4.878	P > 0.05	ns
4	-15.70 to 5.962	P > 0.05	ns

Cellular PBS vs DMSO			
Source of Variation	**% of total variation**	**P value**	**P value summary**
Interaction	12.13	< 0.0001	***
Compound	18.45	< 0.0001	***
Dose	63.01	< 0.0001	***
Bonferroni posttests			
Dose	**95% CI of diff.**	**P value**	**Summary**
0	-6.098 to 5.548	P > 0.05	ns
1	-9.873 to 1.773	P > 0.05	ns
2	-18.50 to -6.857	P<0.001	***
4	-24.68 to -13.03	P<0.001	***

Cellular PBS vs Glycerol			
Source of Variation	**% of total variation**	**P value**	**P value summary**
Interaction	2.46	0.0737	ns
Compound	5.00	0.0008	***
Dose	87.86	< 0.0001	***
Bonferroni posttests			
Dose	**95% CI of diff.**	**P value**	**Summary**
0	-6.767 to 6.707	P > 0.05	ns
1	-10.08 to 3.390	P > 0.05	ns
2	-14.71 to -1.240	P < 0.05	*
4	-15.18 to -1.703	P < 0.05	*

A.10 Comparison of fresh or stored GSE to determine extract stability at 4°C.

Determined by measuring the comet tail intensity induced from doses of ^{60}Co gamma-ray irradiation 0 to 4 Gy using the comet assay. Data (n = 3) were analyzed by two-way ANOVA comparing both dose of radiation and followed by Bonferroni post-hoc tests for specific comparisons, $\alpha = 0.05$.

Source of Variation	% of total variation	P value	P value summary
Interaction	0.08	0.9715	ns
Stored/Fresh	0.61	0.1923	ns
Dose	94.06	< 0.0001	***

A.11 Effect of GSE 4 and LT on Radiation-induced DNA damage.

Determined by measuring the comet tail intensity induced from doses of ^{60}Co gamma-ray irradiation 0 to 4 Gy using the comet assay. Data (n = 3) were analyzed by two-way ANOVA comparing both dose of radiation and compound (GSE and LT) followed by Bonferroni post-hoc tests for specific comparisons, $\alpha = 0.05$.

Acellular PBS vs GSE4			
Source of Variation	**% of total variation**	**P value**	**P value summary**
Interaction	1.47	0.0267	*
Compound	5.67	< 0.0001	***
Dose	90.90	< 0.0001	***
Bonferroni posttests			
Dose	**95% CI of diff.**	**P value**	**Summary**
0	-2.899 to 10.25	P > 0.05	ns
1	-2.113 to 11.03	P > 0.05	ns
2	6.804 to 19.95	P<0.001	***
4	3.714 to 16.86	P<0.01	**

Acellular PBS vs LT			
Source of Variation	**% of total variation**	**P value**	**P value summary**
Interaction	2.02	0.0579	ns
Compound	17.92	< 0.0001	***
Dose	76.55	< 0.0001	***
Bonferroni posttests			
Dose	**95% CI of diff.**	**P value**	**Summary**
0	-1.025 to 19.56	P > 0.05	ns
1	3.198 to 23.78	P<0.01	**
2	9.335 to 29.92	P<0.001	***
4	13.43 to 34.01	P<0.001	***

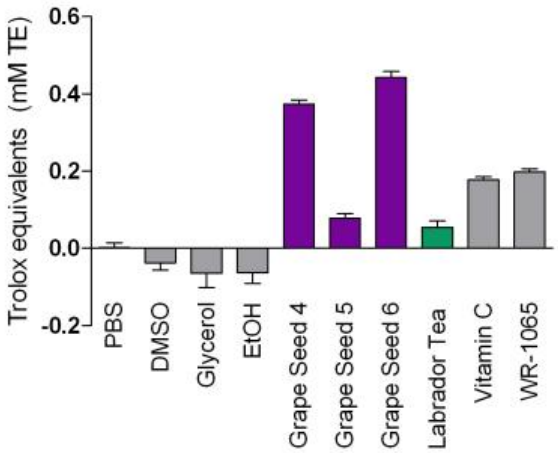
Cellular PBS vs GSE4			
Source of Variation	**% of total variation**	**P value**	**P value summary**
Interaction	0.20	0.8627	ns
Compound	0.14	0.4826	ns
Dose	95.24	< 0.0001	***
Bonferroni posttests			
Dose	**95% CI of diff.**	**P value**	**Summary**
0	-7.045 to 7.498	P > 0.05	ns
1	-6.418 to 8.125	P > 0.05	ns
2	-4.515 to 10.03	P > 0.05	ns
4	-7.392 to 7.152	P > 0.05	ns

Cellular PBS vs LT			
Source of Variation	**% of total variation**	**P value**	**P value summary**
Interaction	0.05	0.9863	ns
Compound	0.00	0.9927	ns
Dose	93.99	< 0.0001	***
Bonferroni posttests			
Dose	**95% CI of diff.**	**P value**	**Summary**
0	-8.338 to 8.745	P > 0.05	ns
1	-8.655 to 8.428	P > 0.05	ns
2	-9.341 to 7.741	P > 0.05	ns
4	-7.775 to 9.308	P > 0.05	ns

Annex B Extra Figures

B.1 Antioxidant potential expressed as Trolox ® equivalents

Samples tested include 10 % DMSO, 4.3 % glycerol, 2 % EtOH (solvent), 100 ug/ml GSE, and 90 ug/ml LT, 0.25 mM vitamin C (HPLC grade ascorbic acid), and 0.25 mM WR-1065 (metabolically active amifostine). Each bar represents the mean ± SEM (n > 3). Vitamin C solutions were made within 1 day of use and WR-1065 samples were made within 30 min of use. GSE and LT extracts were not shown to degrade over time (see corresponding tables) and represent a selection of preparations and ages.



List of symbols/abbreviations/acronyms/initialisms

α	alpha
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid, phosphate-citrate
ANOVA	analysis of variance
CBRN	chemical, biological, radiological, and nuclear
CRTI	CBRNe Research Technology Initiative
CT	computed tomography scan
cm	centimeter (10^{-2})
^{60}Co	Cobalt 60
CO_2	carbon dioxide
CYP3A4	cytochrome P450 isoform 3A4
DBF	dibenzylfluorescein
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DND	Department of National Defence
DRDC	Defence Research and Development Canada
DRDKIM	Director Research and Development Knowledge and Information Management
EDTA	ethylenediamine tetra-acetic acid
et al	et alia, and others
EtOH	ethanol
GSE	grape seed extract
HPLC	high-performance liquid chromatography
hr	hour
Hz	Hertz
IAEA	International Atomic Energy Agency
KCl	potassium chloride
LT	Labrador tea
mM	millimolar (10^{-3})
ml	milliliter (10^{-3})
μg	microgram (10^{-6})

µl	microliter (10^{-6})
µM	micromolar (10^{-6})
µmol	micromoles (10^{-6})
M	molar
min	minute
n	statistical number of repeats
NaCl	sodium chloride
NADPH	beta-nicotinamide adenine dinucleotide phosphate
nm	nanometer (10^{-9})
PBS	phosphate buffered saline
PHA	phytohaemagglutinin
PTFE	polytetrafluoroethylene
R&D	Research and Development
rcf	relative centrifugal force
ROS	reactive oxygen species
SEM	standard error of the mean
TEAC	Trolox® equivalents antioxidant assay
V	Volt
v/v	volume to volume
WR-1065	metabolically active amifostine

Glossary

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid, phosphate-citrate)

A chemical compound used to observe the reaction kinetics of specific enzymes acellular DNA damage; used to indirectly follow the reaction kinetics of any hydrogen peroxide-producing enzyme, or to simply quantify the amount of hydrogen peroxide in a sample

acellular DNA damage

Not occurring in whole cells, acellular DNA damage occurs following cell lysis.

acellular comet assay

A method used to measure the amount of DNA damage in a single cell based on the migration of DNA during electrophoresis. DNA damage is done following cell lysis, thus no DNA repair mechanisms are available.

amifostine

A cytoprotective adjuvant used in cancer chemotherapy and radiotherapy involving DNA-binding chemotherapeutic agents.

ANOVA (analysis of variance)

A statistical method for making simultaneous comparisons between two or more means; a statistical method that yields values that can be tested to determine whether a significant relation exists between variables.

One-way

An analysis technique for determining whether any mean is significantly different from other means in single factor experiments.

Two-way

A statistical test to study the effect of two categorical independent variables on a continuous outcome variable. Two-way ANOVAs analyze the direct effect of the independent variables on the outcome, as well as the interaction of the independent variables on the outcome.

anthocyanins

A type of phytochemical and are the pigments responsible for the red and blue colors of fruits and vegetables, which may have anticancer properties based on their antioxidant activities that defend cells against harmful carcinogens.

anticarcinogenic

Any chemical which reduces the occurrence of cancers, reduces the severity of cancers that do occur, or acts against cancers that do occur, based on evidence from in vitro studies, animal models, epidemiological studies and/or clinical studies.

anti-inflammatory

The property of a substance or treatment that reduces inflammation.

antioxidant

A molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent.

antioxidant capacity

A measure of how effectively antioxidants work against free radicals.

β-carotene

An organic compound and classified as a terpenoid. It is a strongly-coloured red-orange pigment abundant in plants and fruits. As a carotene with beta-rings at both ends, it is the most common form of carotene. It is a precursor (inactive form) of vitamin A.

bioavailability

The ability of a drug or other substance to be absorbed and used by the body. Orally bioavailable means that a drug or other substance that is taken by mouth can be absorbed and used by the body.

Bonferroni multiple comparison test

A correction is based on the idea that if an experimenter is testing *n* dependent or independent hypotheses on a set of data, then one way of maintaining the familywise error rate is to test each individual hypothesis at a statistical significance level of *1/n* times what it would be if only one hypothesis were tested. Used in two-way ANOVA to test if specific pairs of means differ.

Botanical compound

A product containing plants or ingredients made from plants.

cellular DNA damage

DNA damage occurring while still contained within a life, functioning cell; can include various levels of DNA repair, cytotoxic effects, enzymatic modifications, etc.

chelate

Chelation is the formation or presence of two or more separate bindings between a polydentate (multiple bonded) ligand and a single central atom. Usually these ligands are organic compounds, and are called chelants, chelators, chelating agents, or sequestering agents.

chromosome

An organized building of DNA and protein that is found in cells. It is a single piece of coiled DNA containing many genes, regulatory elements and other nucleotide sequences.

^{60}Co (Cobalt 60)

A radioactive isotope of cobalt with mass number 60; a source of exceptionally intense gamma rays.

comet tail intensity

The percentage of DNA found in the comet tail versus the head; a measurement of DNA damage. It is the recommended endpoint for the alkaline comet assay as it is the most consistent measurement endpoint, behaves in a dose-responsive manner, and has defined and comparable units.

CT (computed tomography) scan

A method of body imaging where the x-ray beam rotates around you. Small detectors measure the amount of x-rays that make it through the body area of interest.

CYP3A4 (cytochrome P450 isoform 3A4)

A member of the cytochrome P450 mixed-function oxidase system, is one of the most important enzymes involved in the metabolism of xenobiotics in the body. CYP3A4 is involved in the oxidation of the largest range of substrates of all the CYPs.

CYP3A4 marker substrate

A substrate that is metabolized by CYP3A4 or another substance metabolized CYP3A4 such that changes in the marker substrate's reaction kinetics indicates a change (increase or decrease) in CYP3A4 function.

cytokinesis blocked micronucleus assay

Measurements of micronuclei which are small nuclei separated from the main nucleus and contain chromosomes or chromosome fragments, derived from mitotic spindle dysfunction or acentric fragments as an indication of radiation-induced DNA damage.

DNA

Deoxyribonucleic acid, a self-replicating material present in nearly all living organisms as the main constituent of chromosomes. It is the carrier of genetic information.

DNA repair

Enzymic correction of errors in DNA structure and sequence that protects genetic information against environmental damage and replication errors.

Dunnett's multiple comparison test

Dunnett's test compares group means. It is specifically designed for situations where all groups are to be pitted against control group. Its goal is to identify groups whose means are significantly different from the mean of the control group.

endogenous

Having an internal cause or origin; Growing or originating from within an organism.

ex vivo

The testing of a substance by exposing it to (excised) living cells (but not to the whole, multicelled organism) in order to ascertain the effect of the substance (for example, pharmaceutical) on the biochemistry of the cell.

exogenous

Of, relating to, or developing from external factors; Growing or originating from outside an organism.

mitosis

A type of cell division that results in two daughter cells each having the same number and kind of chromosomes as the parent nucleus, typical of ordinary tissue growth.

flavanols

Flavan-3-ols (sometimes referred to as flavanols) are a class of flavonoids that use the 2-phenyl-3,4-dihydro-2H-chromen-3-ol skeleton. These compounds include the catechins and the catechin gallates; are building blocks for proanthocyanidins.

flavonols

Flavonols are a class of flavonoids that have the 3-hydroxyflavone backbone (IUPAC name : 3-hydroxy-2-phenylchromen-4-one). Their diversity stems from the different positions the phenolic -OH groups; are present in a wide variety of fruits and vegetables.

free metal ions

A metal is a chemical element that is a good conductor of both electricity and heat and forms cations and ionic bonds with non-metals. An ion is an atom or molecule with a net electric charge due to the loss or gain of one or more electrons.

free radical

An atom or group of atoms with at least one unpaired electron; in the body it is usually an oxygen molecule that has lost an electron and will stabilize itself by stealing an electron from a nearby molecule.

gene expression

The process by which a gene’s coded information is converted into the structures present and operating in the cell.

geometric mean

A statistical average of a set of transformed numbers often used to represent a central tendency in highly variable data, such as water quality. It is calculated from data transformed using powers or logarithms and then transformed back to original scale after averaging.

Gray

The SI unit of absorbed radiation dose of ionizing radiation (for example, X-rays), and is defined as the absorption of one joule of ionizing radiation by one kilogram of matter (usually human tissue).

hydroperoxide

A compound containing an O_2H group.

IAEA (International Atomic Energy Agency)

An international organization that seeks to promote the peaceful use of nuclear energy, and to inhibit its use for any military purpose, including nuclear weapons. The IAEA was established as an autonomous organization on 29 July 1957.

immunostimulant

Substances (drugs and nutrients) that stimulate the immune system by inducing activation or increasing activity of any of its components.

in vitro

A procedure performed in vitro (within the glass) is performed not in a living organism but in a controlled environment, such as in a test tube or Petri dish.

in vivo

With in a living organism; a laboratory experiment performed in which the substance under study is inserted into a living organism.

ionizing radiation

Ionizing radiation consists of subatomic particles or electromagnetic waves that are energetic enough to detach electrons from atoms or molecules, thus ionizing them. The occurrence of ionization depends on the energy of the individual particles or waves, and not on their number.

irradiation

The condition of being exposed to radiation.

ketoconazole

A strong CYP3A4 inhibitor; synthetic antifungal drug used to prevent and treat fungal skin infections.

Labrador tea

A low-growing northern shrub of the heath family, with fragrant leathery evergreen leaves that are sometimes used as a tea substitute.

lycopene

A bright red carotene and carotenoid pigment and phytochemical found in tomatoes and other red fruits & vegetables, such as red carrots, watermelons and papayas (but not strawberries or cherries). Although lycopene is chemically a carotene, it has no vitamin A activity.

lipid oxidation

A chemical reaction involving unsaturated lipids with oxygen to yield hydro peroxides; degradation of the hydro peroxides yields a variety of products including alkanols, alkenols, hydroxyalkenols, ketones, alkenes, etc.

lymphocyte

A form of small leukocyte (white blood cell) with a single round nucleus, occurring especialy in the lymphatic system.

magnesium stearate

Magnesium salt, derived most commonly from plants, used as a binder in foods.

microcrystalline cellulose

A plant-derived cellulose powder that binds other ingredients in a formula.

myoglobin

A red protein containing heme that carries and stores oxygen in muscle cells. It is structurally similar to a subunit of haemoglobin.

NADPH (beta-nicotinamide adenine dinucleotide phosphate)

Used in anabolic reactions, such as lipid and nucleic acid synthesis, which require NADPH as a reducing agent.

natural health products

Natural Health Products are defined (in Canada) as: vitamins and minerals, herbal remedies homeopathic medicines, traditional medicines such as traditional Chinese medicines, probiotics, and other products like amino acids and essential fatty acids.

phenolic compounds

A class of chemical compounds consisting of a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group. The simplest of the class is phenol.

proanthocyanidin

Phytonutrients found in some foods and herbs, particularly in certain types of grape seeds and pine bark, that have powerful antioxidant activity; a class of flavanols.

prophylactic

A medicine or course of action used to prevent disease.

quenching

Quenching refers to any process which decreases the fluorescence intensity of a given substance. A variety of processes can result in quenching, such as excited state reactions, energy transfer, complex-formation and collisional quenching.

radiolysis

The molecular decomposition of a substance by ionizing radiation.

radioprotectant

Substance that prevents or lessens the effects of radiation.

reactive oxygen species

Highly reactive oxygen–containing free radicals that are generated during oxidative metabolism. ROS can react with and damage lipids, proteins, and DNA in cells, causing oxidative stress. Common ROS include hydrogen peroxide, superoxide radicals, and hydroxyl radicals.

scavenger

A scavenger in chemistry is a chemical substance added to a mixture in order to remove or inactivate impurities or unwanted reaction products.

silicon dioxide

A common additive in the production of foods, where it is used primarily as a flow agent in powdered foods, or to absorb water in hygroscopic applications.

solvent

The liquid in which a solute is dissolved to form a solution.

standard (cellular) comet assay

A method used to measure the amount of DNA damage in a single cell based on the migration of DNA during electrophoresis. DNA damage is done prior to cell lysis.

stearic acid

A naturally-derived, plant-based fatty acid used as a thickener and co-emulsifier.

stilbenes

Either of two isomeric hydrocarbons, diphenylethylene, but especially the trans isomer, used in the manufacture of dyes and many other compounds.

synthetic supplement

A substance taken to remedy the deficiencies in a person's diet made artificially by chemical reactions.

therapeutic

Administered or applied for reasons of health; applied post problem / infection.

toxicity

Toxicity is the degree to which a substance can damage an organism.

Trolox ®

Hoffman-LaRoche's trade name for 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble derivative of vitamin E. It is an antioxidant, like vitamin E, and is used in biological or biochemical applications to reduce oxidative stress or damage.

Vitamin C

Vitamin C or L-ascorbic acid or L-ascorbate is a naturally occuring essential nutrient for humans (meaning that it must be provided by the diet because the body cannot manufacture it), in which it functions as a vitamin. In living organisms, ascorbate is an anti-oxidant, since it protects the body against oxidative stress.

Vitamin E

Vitamin E is a naturally occurring essential nutrient (meaning that it must be provided by the diet because the body cannot manufacture it), in which it functions as a vitamin. It is an antioxidant that protects cell membranes and other fat-soluble parts of the body.

worried-well

Individuals free from illness who are nonetheless concerned about their physical state and frequently and inappropriately use medical services.

WR-1065

The active cytoprotective thiol metabolite of amifostine.

Index

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This study provides information for testing of readily available, low toxicity, long shelf life, easily administered botanicals which can be prophylactic radioprotectants. A selection of assays to test antioxidant capacity, metabolic and drug interactions, and DNA damage were performed to assess commercially available grape seed extract supplements and Labrador tea whole leaf extracts as potential radioprotectants. Three different commercial grape seed extracts were shown to have differing antioxidant capacities when compared to a known antioxidant (vitamin C) and radioprotectant (amifostine). Grape seed extract and Labrador tea did not interact with a well-studied drug metabolism pathway (CYP3A4), indicating that they may have potential for use as radioprotectants due to minimal drug and metabolism interactions. Using a cellular system as a model for identifying the DNA damage while allowing for minimal repair, no protection was provided by any extract. Under acellular conditions, assessing DNA damage with no repair potential resulted in increased DNA damage following radiation exposure. Overall, this study has been useful in identifying and validating a set of procedures to use in screening potential antioxidant radioprotectants. Further work explores the optimal concentrations of these and other botanical extracts as potential radioprotectants.

La présente étude a fourni de l'information sur des substances végétales facilement accessibles, peu toxiques, à longue durée de conservation et faciles à administrer qui pourraient être utilisées en prévention comme agent radioprotecteur. Au moyen de divers essais, nous avons déterminé la capacité antioxydante, les interactions métaboliques et médicamenteuses ainsi que les dommages causés à l'ADN afin d'évaluer les effets radioprotecteurs potentiels des suppléments commerciaux à base d'extrait de pépins de raisin et d'extrait de feuilles entières de thé du Labrador. Nous avons constaté que la capacité oxydante de trois extraits commerciaux de pépins de raisin différait et qu'elle était comparable à celle d'un antioxydant (la vitamine C) et d'un agent radioprotecteur (l'amifostine). Les extraits de pépins de raisin et de thé du Labrador n'ont pas interagi avec la voie métabolique de médicament bien connue CYP3A4, ce qui démontre leur potentiel comme agent radioprotecteur avec des interactions médicamenteuses minimales. Dans le système cellulaire, aucun extrait n'a eu d'effet protecteur, tel que démontré en examinant les dommages réparables et non réparables à l'ADN. Dans les milieux acellulaires, à l'examen des dommages sans réparation de l'ADN, nous avons observé une augmentation des dommages causés à l'ADN après l'exposition au rayonnement. Globalement, cette étude a été utile pour la détermination d'un ensemble d'expériences à réaliser pour la recherche d'agents qui pourraient avoir des effets antioxydants et radioprotecteurs. D'autres travaux devront être effectués, notamment l'étude de la concentration optimale des extraits et la recherche d'autres substances végétales radioprotectrices.

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Antioxidant; Botanical; Commercial Grape Seed Extract; Labrador Tea; Radioprotectant; Comet Assay; CYP3A4; DNA damage; Prophylactic; Reactive Oxygen Species; Medical Countermeasures