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Methodologies and limitations in the analysis of potential neuroprotective compounds derived from natural products

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ABSTRACT

Plant-derived polyphenols have attracted the attention of scientists, the public, and the media due to their potential use as nutraceutical products. The high quantities of polyphenols found in some berry species, *e.g. Vaccinium* species such as blueberries and lingonberries, and their reported antioxidant and anti-inflammatory properties, could be beneficial for brain aging and neurodegenerative disorders. The neuroprotective potential of various polyphenolic compounds have been validated using a variety of *in vivo* and *in vitro* techniques. Both *in vivo* and *in vitro* methodologies have their respective advantages and disadvantages, including, but not limited to, cost, time, use of resources and technical limitations. For example, *in vivo* studies can better evaluate the effects of protective compounds and/or their metabolites on various tissues, including the brain, whereas *in vitro* studies can better discern the cellular and/or mechanistic effects of compounds. This short review is meant to provide a synopsis of some of the inherent benefits and drawbacks of methods used for assessing neuroprotection and how findings may translate to the human population, particularly related to my specific area of research analyzing the potential neuroprotective effects of berries and their associated polyphenolic compounds.

Focal points:

- Benchside
  Both *in vivo* and *in vitro* experimental approaches are necessary to determine the full potential that berries and their constituents hold for treating and preventing neurological diseases and syndromes.
- Bedside
  Ingestion of compounds from berries may reduce the amount and severity of neurodegenerative diseases, thereby providing a form of translational preventative medicine.
- Industry
  Neuroprotective compounds from berries, including both the fruits and leaves, hold potential as nutraceutical products.
- Community
  The development of nutraceutical products with neuroprotective potential by industry could provide local economic benefits.
- Regulatory agencies
  As nutraceutical products are produced from the fruits and leaves of berries, care will need to be taken on labeling as well as claims made by the manufacturers.

1. Introduction

Polyphenols, such as flavonoids found in a variety of plant species, are a large class of compounds with reported protective activity against disorders such as cancer and cardiovascular disease [1,2]. Therefore, they have received a lot of attention from scientists, the public, and the media alike, largely due to their potential use as nutraceuticals, which are compounds believed to exert a positive effect on health. The high quantities of polyphenols found in some specific plant species, including berries (*e.g. Vaccinium* species such as blueberries and lingonberries), and their reported anti-oxidant and anti-inflammatory properties, could be beneficial for brain aging and neurological disorders [3,4]. Some of the research focus in this area has been on the positive effects that

Abbreviations: RNS, reactive nitrogen species; ROS, reactive oxygen species

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polyphenolic compounds could have on the brain if ingested as a normal part of the diet [3,5]. However, other studies have focused on specific plant-derived polyphenols and their ability to treat various brain disorders, such as stroke, traumatic brain injury (TBI) and neurodegenerative disorders such as Alzheimer's and Parkinson's disease [4,6–8]. Oxidative stress is believed to at least partially contribute to all of these forms of brain disorders, and since polyphenols generally have a high antioxidant and free radical scavenging capacity, it has been postulated that these compounds are potentially neuroprotective through antioxidant mechanisms.

2. Mechanisms of neuroprotection due to antioxidant and free radical scavenging capacity

The production of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and peroxyl radicals, and reactive nitrogen species (RNS), such as nitric oxide and peroxynitrite radicals, are a part of natural physiological reactions in the brain. However, an excessive production of ROS and RNS could lead to oxidative stress and nitrosative stress, respectively. These reactive compounds can damage lipids, proteins and DNA, leading to lipid peroxidation, altered signal transduction pathways, and the destruction of membranes and organelles [6]. The brain is particularly susceptible to oxidative stress pertaining to its high oxygen demand, and also because it is enriched with polyunsaturated fatty acids. Moreover, a high iron concentration and low levels of endogenous antioxidants are also factors responsible for the overproduction of ROS and RNS in brain cells [3,6]. This excessive production of ROS and RNS can occur rapidly and contribute to disorders such as TBI and stroke [6,8], or develop slowly over the course of years and contribute to neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases [3,7,9]. The balance between ROS and antioxidants in biological systems is referred to as redox homeostasis, which is essential for normal cell function [10]. In order to combat oxidative stress, there are several types of endogenous enzymatic antioxidants such as superoxide dismutase, catalase and glutathione peroxidase, as well as non-enzymatic glutathione. There are also several non-enzymatic antioxidants that can be obtained primarily in the diet, which include tocopherol, ascorbate, carotenoids and various polyphenolic compounds [6,11]. Given the idea that increased oxidative and nitrosative stress may be major contributors to several neurological diseases and brain aging, the ingestion of foods high in polyphenolic compounds, or dietary supplements containing their constituents, may have a positive effect on brain health [3,6].

3. Screening of potential neuroprotective compounds using *in vitro* approaches

Potential neuroprotective compounds, including those from natural sources, are often screened for effects utilizing sterile cell culture techniques. In these approaches, compounds are added to brain derived cells and challenged with toxic substances known to produce cell damage and death, such as glutamate or hydrogen peroxide, which will generate excessive ROS and/or RNS [12,13]. This approach can include immortalized cell lines [14], or primary cells, which are generally derived from embryonic or neonatal brains of mice or rats [15,16]. One of the major advantages of this approach is that compounds can be screened for protective activity over the course of a few weeks, compared to *in vivo* approaches, which can take months. Also, these studies can aid in determining a concentration of a compound at which it is efficacious, or the level at which the potential protective compound itself becomes toxic. These initial findings could produce leads as to which specific compounds or extracts from plant sources merit further studies in whole animals (Table 1).

My laboratory has used primary cells to test the effects of various berry extracts for neuroprotection. Using biochemical assays, we found that extracts of the fruits and leaves of blueberry and lingonberry plants had high levels of polyphenols, such as anthocyanins, tannins, and flavonoids [16]. Overall, the levels of these compounds were significantly higher in the leaves of these plants *versus* the fruits. Total antioxidant capacity, in terms of radical scavenging activity and reducing power, was much higher in the leaves of both plants as compared to their fruits. We next tested the effects of the extracts against glutamate-mediated excitotoxicity, a pathological process partially involving overproduction of ROS and RNS. Cortical cell cultures were exposed to glutamate (100 μM) for 24 h. Glutamate-exposed cells displayed morphological alterations such as disrupted cell bodies, and increased dark punctae, which is often indicative of condensed nuclei and delayed cell death [16]. Glutamate also caused significant cell loss after 24 h. While lingonberry fruit extract did not provide protection from glutamate toxicity, blueberry fruit extracts were extremely protective. Cultures treated with leaf extracts of lingonberry and blueberry showed no cell loss in the presence of glutamate, indicating a strong protective effect of both the leaf extracts. We have also investigated protective effects of extracts using an *in vitro* model of traumatic injury [17], which causes significant cell loss after 24 h. Injury in the presence of extracts from bilberries, blueberries and lingonberries caused significantly less cell loss and damage. We found similar effects when cells were injured in the presence of oxyresveratrol, a potent antioxidant derived from mulberry wood [15].

An advantage of our approach is that we can fairly quickly analyze specific compounds at various concentrations for protective effects as well as test various extracts, which include several compounds, as it may be necessary to have more than one compound present in order to produce optimal efficacy. Also, cell cultures from mice can often be derived from genetic models of disease, such as Alzheimer's disease [18], against which extracts

Table 1
Advantages and disadvantages to *in vitro* and *in vivo* experimental approaches to studying potential neuroprotective agents.

Types of model(s)	Advantages	Disadvantages
In vitro	– Are often less expensive than *in vivo* Approaches – Can quickly screen compounds for neuroprotective potential – Good for discerning cellular effects of compounds and mechanism of action	– Exact cytoarchitecture of the brain is not maintained – Compounds are often tested at concentrations that are not necessarily achieved in nervous system tissue – Cell lines have been genetically modified in some way, so may not represent the true characteristics of cells in the brain
In vivo	– Can more adequately evaluate the potential protective effects of compounds or their metabolites in specific brain areas – Can determine effects of compounds using behavioural experiments – Can determine the extent to which compounds in the diet can enter the brain	– Are often more expensive than *in vitro* studies due to animal maintenance and other costs – Often take much longer to screen protective effects than *in vitro* studies (*i.e.* months *versus* weeks)