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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.

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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)

can be tested. However, there are many disadvantages to this approach as well. For example, the exact cellular architecture of cells in the brain is not achieved in culture, which can alter the response of cells to traumatic insults. In addition, it is difficult to know if the concentration range of various compounds we test *in vitro* is a realistic level that can be achieved in the brain without conducting studies in whole animals. Also, cell lines have in some way been genetically modified so that they can become immortalized, which is most often not a realistic situation to which data can be extrapolated to the intact animal.

4. *In vivo* studies of potential neuroprotective compounds

Several studies using whole animals, which are usually rodents, have been conducted recently in order to evaluate the effects of berries on the nervous system. In particular, the potential of berries and their constituents to protect the brain from aging and neurodegenerative disease has gained increased attention in recent years. For example, dietary supplementation with polyphenol-containing fruits, including blueberries, can decrease age-related behavioral deficits in rats [5]. In a recent study conducted with a mouse model of Alzheimer's disease, treatment with berries rich in polyphenols (*e.g.* bilberries) decreased the extent of behavioral abnormalities associated with the disease [18]. Other experimental studies have shown that rats fed a diet enriched with blueberries can protect the brain against oxidative stress and associated learning deficits [19]. Perhaps most surprisingly, diets enriched with blueberries have been demonstrated to later protect animals from the damage induced by insults as severe as ischemic stroke [20,21].

These types of approaches offer several advantages over cell models. The models are more realistic in the sense that animals must ingest a diet containing berries or their extracts, as would humans. Animals can also be fed for various periods of time, and with different percentages of berries constituting the diet. Several behavioral tests can be administered in animals to measure motor function and/or cognitive functions and the results can be compared to animals treated with a non-berry enriched diet. Also, at the end of behavioral testing several other analyses can be conducted, such as histological analysis of various brain areas, and the effects of berry-enriched diets can be measured in other parts of the body as well, including the heart, liver and kidneys. Also, as mentioned above, the effects of various berries can be tested in genetic mouse models for various diseases, such as Alzheimer's disease [18]. Despite these many advantages of *in vivo* approaches, there are also some drawbacks. The experiments can be expensive, especially when considering the cost of maintenance of many animals over the cost of several weeks. As with *in vitro* approaches, animals often receive a high amount of berries or extracts in their diets that may not be realistically achieved with humans. Also, many studies do not analyze the extent to which berry-derived polyphenols entered the brain.

5. Issues regarding bioavailability

There is now substantial evidence suggesting that the ingestion of diets high in berries can have positive effects on the brain [22,23]; however it is still not conclusive whether this is due to direct or indirect effects on nervous system tissue. Some research has demonstrated that dietary polyphenols can cross the blood–brain-barrier [22], and anthocyanins specifically have been detected in brain tissue after oral administration to rodents [24–26] as well as pigs [27,28]. Some estimates of specific anthocyanins in brain tissue are in the sub-nanomolar range (~0.2–0.25 nmol/g tissue) [25,26], whereas some others are as low as the femtomolar range [28]. It is difficult for us to make direct comparisons to such *in vivo* studies with our recent *in vitro* work [16], as we added whole extract and not specific polyphenols. The final concentration of blueberry and lingonberry extracts that we tested was 0.833 μg/ml of fruit extract and 0.083 μg/ml of leaf extract. In other previous work we conducted chemical analysis of commercially available lingonberry extracts and have found that these extracts contain an estimated 63.7 mg of cyanidin-3-galactoside per 100 mg of fresh extract weight (unpublished data). If our fresh lingonberry extracts contained a similar amount of this compound, this would translate to the cultured cells being exposed to approximately a 10 nM concentration of fruit extract and 1 nM in leaf extract. Talavera et al. [25] detected a level of another cyanidin compound (cyanidin-3-glucoside) of 0.25 nmol equivalent per gram of tissue. Therefore, the amount of extract that we tested for neuroprotective effects in cultures is likely slightly higher than what might be achieved in the brain after oral administration. Also, the amount we added to cultures is much higher than femtomolar estimates in pigs that had ingested polyphenols orally [28]. However, polyphenol measurements occurred 18 h postprandial in these studies, so it is possible that polyphenol levels in the brain may have been higher if measured earlier. Most *in vivo* studies also feed animals berry-rich diets for several weeks. However, the extent to which berry-derived polyphenols enter the brain from short periods of ingestion (*e.g.* a day or a week), or how long these constituents stay present in the brain is not known (Fig. 1).

It is also possible that polyphenolic compounds contained in extracts that are tested *in vitro* may not be the predominate forms that would actually enter the brain, as some recent studies have found that although anthocyanins have a fairly high bioavailability, they also undergo significant metabolism, producing diverse metabolites [29,30]. Some evidence suggests that certain polyphenolic compounds are maintained in their natural glycosylated form [24,25]. Xenobiotic metabolism also likely contributes to the amounts and forms of polyphenols that cross the blood–brain barrier, as additional evidence has demonstrated that glucuronide forms of anthocyanins can be detected in the brain [28]. Lastly, a greater amount of polyphenols may enter the brain when the blood–brain-barrier is compromised, which can occur following traumatic brain injury or stroke. For example, the compound oxyresveratrol can get into the brain to a larger extent once it is compromised after a stroke [31]. Much more research is needed in order to determine the types of polyphenolic compounds that can enter the brain, and to what extent.

6. Other potential mechanisms of protection of natural compounds

Due to the plethora of information suggesting that oxidative stress can contribute to a variety of disease states, including neurological disorders, arguably the ability of polyphenolic compounds to act as antioxidants has received the most attention. Although anthocyanins and flavonols may exert some of their neuroprotective effects by acting as antioxidants, they likely have other important effects, such as scavenging reactive nitrogen species, activating protective cell signaling pathways, or altering the expression of various proteins [22,23,32,33]. This idea is strengthened by evidence that the bioavailability of these compounds in the brain is much lower than the levels of endogenous antioxidant compounds [33]. Also, the compound resveratrol, which is found in high levels in grape skins and seeds, was initially believed to exert positive health benefits by acting as an antioxidant compound. However, recently it has been found that resveratrol exhibits its potential anti-aging effects by acting primarily as a phosphodiesterase inhibitor [34].

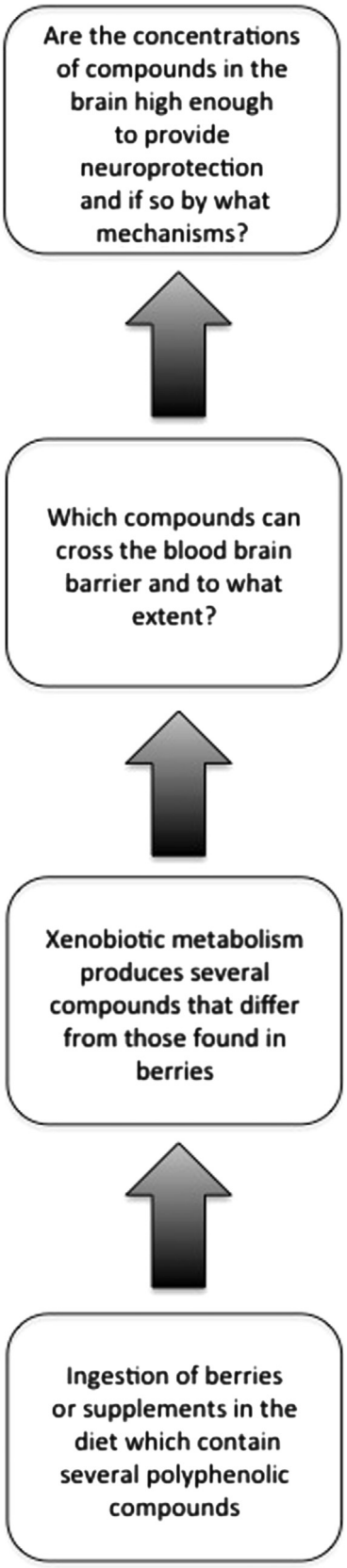

Fig. 1. Issues related to bioavailability and neuroprotective potential of polyphenols in berries.

It is possible that polyphenols exert beneficial properties through hormetic effects. For example, low levels of these compounds may activate the transcription factor Nrf2, which can induce the production of endogenous antioxidant enzymes and other protective compounds [22,33]. Polyphenols can also decrease the level of pro-inflammatory mediators in the brain, such as interleukins and tumor necrosis factor-$\propto$ [23]. The anti-nitrosative properties may also play a significant role in protecting the brain from damage compared to antioxidant effects, by scavenging excessive damaging compounds such as peroxynitrite. It is also possible that polyphenols have an indirect effect on the brain that leads to positive effects. For example, antioxidant effects could be positive for the cardiovascular system, which may lead to an increase in blood flow to the brain. This effect could certainly improve cognitive function both in experimental animals and in humans. These latter biological effects of polyphenols in the brain are poorly understood and warrant further investigation.

7. Conclusions

A large body of evidence suggests that plant-derived polyphenolic compounds can be beneficial for the nervous system. Many of these compounds have strong antioxidant capacity, which could potentially be used to treat debilitating disorders such as stroke or trauma. However, it is unlikely that these compounds on their own could be significant treatments for such disorders due to their complexity and the ability to deliver a substantial level of these compounds to the brain. Findings from *in vitro* and *in vivo* studies have shown overall positive results that set the basis for translational preventative medicine. For example, ingestion of various species of berries could result in increased cognitive function and prevention, or a delay in onset, of various neurodegenerative diseases. It is currently unknown if a specific polyphenolic compound, such as in the form of a supplement, or a combination of several compounds derived directly from fruits for example, would provide greater protection. More studies are needed to determine the bioavailability of polyphenols or similar substances, when ingested in different amounts or various periods of time. Although these types of studies primarily need to be conducted using *in vivo* experimentation, i*n vitro* studies could significantly aid in deciphering the cellular mechanisms by which polyphenols provide protection.

Executive summary

- Some naturally occurring compounds hold promise as possible neuroprotective agents. These agents include polyphenolic compounds from berries, which have high antioxidant capacity and demonstrate neuroprotection in many studies.
- Determining the potential of natural products to protect the brain should be based on well-designed *in vitro* and *in vivo* experiments. More work also needs to be done in order to determine the degree to which specific compounds can enter the brain.
- Polyphenols may be neuroprotective by acting as antioxidants or through other diverse cellular mechanisms, and more research is necessary to decipher these mechanisms of action.
- Both the fruits and leaves of certain types of berries demonstrate neuroprotective qualities. Therefore, compounds from these sources hold potential for the further development of nutraceutical products.

Conflict of Interest

None Declared.

Ethical approval

None Declared.

Role of the funding source

None Declared.

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