Anti-Cancer Effect of Plum Extract on U-87 Glioblastoma Multiforme Brain Cancer Cells

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Abstract

Brain cancer is a very devastating disease which is difficult to treat and the survival rate of brain cancer patients is low. Present treatments have limited success; therefore, there is a constant search for new brain cancer treatments and strategies. Oxidative stress plays a role in the initiation and progression of brain cancer. Fruits and vegetables are rich in phenolic compounds that have superior antioxidant properties. Among fruits, plum extract contains high levels of phenolic compounds and has been shown to exhibit anticancer properties. The objective of this study was to determine effects of plum extract on proliferation of brain cancer cells. A standardized preparation of plum extract, PE60, was used to determine its total polyphenols content, total flavonoids content, and anti-oxidation activity. Aqueous, methanolic, and DMSO solutions of PE60 were used to determine their effects on cell viability of U-87 cells by MTT assay. Apoptosis was assayed by analyzing nuclear morphology and caspase activation. PE60 was found to be rich in polyphenols (575-600mg/g of dry weight). The antioxidant activity in PE60 solutions ranged from 3250-3500mM equivalent of Trolox/g dry weight. When cells were treated with aqueous PE60 solution, the cells’ viability was significantly decreased by 20% (p<0.05) at 25μg/ml, and about 80% (p<0.05) of cells died at 250μg/ml PE60 solution. When cells were treated with PE60 dissolved in methanol or DMSO, the cells’ viability was decreased significantly by 30-40% at 250μg/ml. Our data show that the effect of plum extract was mediated through an apoptotic pathway involving caspase-3 activation. In conclusion, this study showed that aqueous PE60 solution was better than that of methanolic or DMSO solutions for inhibiting U-87 brain cancer cell growth. Our data suggest that plums have nutritional compounds that can potentially be effective for preventing or inhibiting brain cancer.

Keywords: Antioxidants; Brain cancer; Plum extract; Polyphenols

Introduction

Cancer is considered to be a major public health problem worldwide and is the second leading cause of death globally, causing an estimated 9.6 million deaths in 2018 [1]. According to the National Cancer Institute (2016), the most common causes of cancer deaths are lung, bronchus, colorectal, and prostate cancers in men and lung, bronchus, breast, and colorectal cancers in women [2]. Brain and other nervous system cancers are relatively rare compared to other cancers [3], and are most frequently diagnosed among people aged 55-64 years though they can develop in any age group [4]. According to the World Health Organization (WHO), there are 120 different types of brain cancers [5]. However, some of the most common brain tumor types include oligodendrogliaoma, astrocytoma, and meningioma [6]. Gliomas make up about 30% of all brain tumors and about 80% of all malignant brain tumors [7]. The brain tumors arise from glial cells which are normally found in brain tissues [8]. Glial cells support nerve cells with energy and nutrients and maintain the blood-brain barrier [9]. About 5% of brain tumors are caused by hereditary genetic conditions such as neurofibromatosis, tuberous sclerosis, Li-Fraumeni syndrome, and Turcot’s syndrome [10].

The hereditary and somatic mutations in oncogenes and tumor suppressor genes play an important role in tumor initiation and progression [11]. Tissues with decreased concentrations of antioxidant enzymes and enhanced concentrations of Reactive Oxygen Species (ROS) initiate mutagenic and carcinogenic conditions that have potential for genomic alteration [12]. The brain, because of its high metabolic activity and relatively decreased capacity for cellular regeneration, is particularly susceptible to the damaging effects of ROS [13]. Reactive species, such as hydroxyl radicals, can react with nucleotide bases. Additionally, they can damage chromatin proteins and cause modifications and genomic instability in chromosomes, therefore, resulting in gene expression alterations [14]. The carcinogenic potential induced by oxidative stress is related to a balance between ROS generation and its neutralization by anti-oxidation processes. An excess ROS accumulation may induce DNA damage and then interfere with crucial cellular processes [14].

Recent research reports that certain plant chemicals, such as terpenes, polyphenols, and thiols have much more powerful antioxidant
properties than well-known antioxidant vitamins [15]. Plants and vegetables are rich in flavonoids, and their beneficial functions as antioxidant agents and cancer-preventers are well known [16]. In the last decade, bioactive phytochemicals have been regarded as mostly non-toxic compounds for the treatment of many forms of cancers, including brain tumors and gliomas [17]. Several authors reported inhibition of cell proliferation of glioma by compounds such as Curcumin (Cur), isothiocyanate derivatives, resveratrol, and catechins, such as Epigallocatechin-3-Gallate (EGCG) [18-21].

The plum is considered a super food that is rich in phytonutrients [22]. It is a rich source of antioxidant compounds like flavonoids, phenolic acids, and other phenolic compounds, all of which are effective as natural antioxidants and protect the body from unwanted radicals and toxins. Most of its antioxidant power is due to the high levels of neochlorogenic and chlorogenic acids, phenolic acids, and anthocyanins [23, 24]. The objective of the present study was to characterize a standard preparation of plum extract for its anti-oxidation activity and to determine its effects on cell proliferation of glioblastoma cells.

Materials and Methods

Cells and reagents

U-87 cells were purchased from ATCC (Manassas, VA). DMEM media, penicillin, and streptomycin were purchased from Gibco-Fisher (Grand Island, NY). Fetal Bovine Serum (FBS) was purchased from RAMBIO (Missoula, MT). The PE60 powder, a standardized preparation of plum extract containing 60% phenolic compounds, (P. L. Thomson, Morristown, MA) was kindly donated by Dr. Suzette Pereira from Abbott Nutrition (Columbus, OH). Folin-Ciocalteu, aluminum chloride, Diphenyl-1-Picrylhydrazyl (DPPH), quercetin, gallic acid, trolox, 3-(4,5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT), paraformaldehyde, and 2, 2’-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma Chemical Co. (St Louis, MO). The caspase inhibitors z-VAD-FMK and z-DEVD-FMK were purchased from Biovision (Atlanta, GA).

Preparation of PE60 extracts

PE60 powder was dissolved either in water, 80% methanol, or DMSO (25% or 50%) at 250mg/ml (stock solution). The solutions were centrifuged at 2000g for 10 minutes to remove trace amounts of undissolved material. The clear solution was stored at -20°C until used. The stock solution was diluted with serum-free media to 0-250mg/ml each time when used for cell treatment. The final concentration of methanol and DMSO did not exceed 0.1%. A control containing appropriate amounts of methanol and DMSO was included along with the test samples.

Determination of Total Phenolic Content (TPC)

The total phenolic content of PE60 solutions was determined by using the Folin-Ciocalteu method as previously described [25]. The total phenolic content of the PE60 extract was calculated as gallic acid equivalents (mg GAE/g of dry extract).

Determination of Total Flavonoid Content (TFC)

An aluminum chloride complex-forming assay was used to determine the total flavonoid content of the PE60 solutions [26]. Quercetin was used as a standard and flavonoid content was determined as quercetin equivalents (mg QE/g of dry extract).

Anti-oxidation activity

The anti-oxidation activity of PE60 solutions was determined by using the 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) method [27]. The data is reported as trolox equivalent (mM TE/g of dry extract).

Oxygen scavenging capacity

The oxygen scavenging capacity of PE60 solutions was evaluated according to the previously reported protocol [28, 29]. Trolox equivalents were calculated using a standard curve and expressed in mM tolux equivalents per gram of dried plum extract (mM TE/g of dry extract).

Cell culturing and anti-proliferation assay

The U-87 brain tumor cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen; Carlsbad, CA) and supplemented with penicillin (100units/ml), streptomycin (100µg/ml) and 10% FBS (complete media). The cells were incubated in a humidified incubator at 37°C with 5% CO2. Medium was changed every 3 days and cells were subcultured when they became confluent. The effect of PE60 on cell proliferation was determined using a MTT assay as per manufacturer instructions. Briefly, U-87 brain cancer cells (10,000/well) were cultured in a 96 well plate. After 24 hours of incubation in a complete medium, cells were treated with a final concentration of 0 (0.1% methanol or DMSO as control), 10, 25, 50, 100, 150, 200, or 250mg/ml of PE60 plum solution for another 24 hours in serum-free medium. Cells were then incubated with 20µl of MTT solution (5mg/ml in DMSO) for 3 hours and formation of purple crystals were quantified (after dissolving the crystal in 200ml of DMSO) in a 96 well plate reader (Molecular Devices, San Jose, CA) at 570 nm. The results are expressed as % change from control.

Effect of PE60 on nuclear morphology

U-87 brain cancer cells were cultured in a 96 well plate for 24 hours in 100ml of a complete medium. Cells were then incubated with a final concentration of 0, 10, 25, 50, 100, 150, 200, or 250mg/ml of PE60 plum solution for 24 hours. After treatment, the cells were removed and washed once with Phosphate Buffered Saline (PBS) and then fixed with cold 3% paraformaldehyde for 15 minutes on ice. The cells were washed 3 times with PBS and then 50ml of 1:1000 dilution of Hoechst 33342 (10mg/ml) dye was added as per manufacturer’s instructions (Thermo Scientific, Rockford, IL). The plate was kept at room temperature for 10 minutes before the cells were washed three times with 100ml PBS. Microscopic images were taken in the dark at different magnifications using a Nikon Fluorescence Microscope.

Role of caspases in PE60-induced cell death

Cells were cultured in a 96 well plate as described above, and treated with a final concentration of either 0.1% DMSO (control), 10µM of z-VAD-FMK (generic caspase inhibitor) or 10µM of z-DEVD-FMK (specific caspase-3 inhibitor) for 1 hour prior to treatment with PE60. After caspase inhibitor pretreatment, cells were incubated with or without PE60 (100µg/ml) for 24 hours in a CO2 incubator as described above. The effect of caspase inhibitors on PE60-induced cell death was determined by assaying total viability of U-87 cells using a MTT assay as described above.
Results

Characterization of PE60 preparations for anti-oxidation activity

The different preparations of PE60 were characterized for anti-oxidation potential by assessing their total phenolic content, total flavonoid content, anti-oxidation activity, and total oxygen scavenging activity. The data shown in figure 1 indicate that water- and DMSO-extracted PE60 contained TPC of 575-600mg Gallic Acid Equivalents (GAE)/g of dry weight. However, the methanol extract had a significantly lower TPC concentration of around 450mg/g of dry extract.

![Figure 1: Total phenolic content in PE60 preparations.](image1)

The amount of TFC in different PE60 preparations is shown in figure 2. Similarly, the water and DMSO preparations contained TFC of 525-550mg Quercetin Equivalents (QE)/g of dry weight, whereas the methanol extract contained a significantly lower amount of TFC (425mg QE/dry weight) compared to that of other preparations.

![Figure 2: Total flavonoid content in PE60 preparations.](image2)

The anti-oxidation activity in different preparations of PE60 is shown in figure 3. The data indicate that all preparations have a similar extent of anti-oxidation activity and represented about 3250-3500mM equivalence of Trolox activity/g of dry weight.

![Figure 3: Total anti-oxidation activity in PE60 preparations.](image3)

The oxygen scavenging activity of different preparations of PE60 extract is shown in figure 4. It is evident from the data that all preparations contained a similar capacity for scavenging free oxygen. The total oxygen scavenging activity in these preparations varied from 4000-5000mM Trolox equivalent/g of dry weight.

Effect of PE60 preparations on U-87 glioblastoma cell viability

Different solvent preparations (water, methanol, and DMSO) were used to determine the effect of PE60 on U-87 glioblastoma brain cancer cells. When cells were treated with water extract of PE60, cell viability was decreased significantly to 80% (p<0.05) at 25µg/ml. Cell viability was decreased further in a concentration-dependent manner by increasing the PE60 concentration over 25µg/ml (Figure 5). A decrease in cell viability was observed and only 20% (p<0.05) survived at 250µg/ml.
was observed up to 200mg/ml. However, when the concentration of PE60 was increased to 250mg/ml, a significant reduction in cell viability of 30% was observed (Figure 5). However, when cells were treated with PE60 extract in 50% DMSO, a non-significant effect on cell viability was observed at all tested concentrations (Figure 5).

**Effect of PE60 on cell death through apoptosis**

The effect of PE60 on programmed cell death (apoptosis) was determined by using caspase inhibitors (apoptosis inhibitors). One of the hallmarks of apoptosis is the blebbing of cellular nuclei, as examined by microscopy. The data shown in figure 6 indicate that upon increasing the concentration of water soluble PE60, the number of intact nuclei was reduced in a dose-dependent manner. The effect of PE60 on cell viability, as determined by MTT assay, in the presence of caspase inhibitors is shown in figure 7. The PE60 treatment at 100mg/ml caused a significant reduction in cell viability to 40% (p<0.05). However, PE60 treatment (100mg/ml) in the presence of a specific caspase-3 inhibitor (10µM) improved cell viability significantly to 55% (p<0.05), whereas a generic caspase inhibitor did not cause any significant improvement in PE60-induced reduction in cell viability (Figure 7).
Discussion

Plant extracts are typically reconstituted in water or DMSO for studies. During our preliminary studies, we observed variable results when PE60 extract powder was reconstituted in water or DMSO, suggesting that different solvents may have dissolved different compounds from the extract. During this study, we therefore investigated the effect of PE60 extract dissolved in three different solvents. Water, 80% methanol, 25% DMSO and 50% DMSO were used to dissolve components from PE60 for the purpose of testing their effects on U-87 glioblastoma multiforme brain cancer cells.

Our data indicate that water as a solvent was more effective against brain cancer cells than methanol or DMSO. The solubility of polyphenol highly depends on the polarity of the solvent. These solvents were chosen because of their capabilities for dissolving compounds with variable polarity. Methanol is less polar than water because of a nonpolar carbon being attached to a hydroxyl group. The more polar alcohol has a shorter nonpolar carbon chain attached to a polar hydroxyl group. Therefore, dimethyl sulfoxide, DMSO, is less polar than methanol and the data indicate that water soluble polyphenols or other water soluble compounds may be more active than that of less-polar polyphenols dissolved in methanol or DMSO. The solvents commonly used in polyphenol extractions are water, ethanol, methanol, ethyl acetate, and acetone [30]. However, the most widely used solvents are ethanol and water because they are only mildly toxic and have relatively high polarities resulting in the highest yields [31]. The use of ethanol is restricted because of local regulatory laws. Other non-polar solvents such as acetone or chloroform were not used during the present investigation because of their toxic solvent residues, and the safety issues regarding worker exposure, disposal of waste, and environmental pollution.

When examining Total Polyphenol Content (TPC), we validated that the PE60 has 575-600mg/g and about 60% polyphenols, which is the same as what is reported by the manufacturer [32]. The water and DMSO solutions have a similar amount of TPC while the methanol solution has less than 60% polyphenols. It appears that some polyphenols may be less soluble in methanol than in water. Similar to TPC, we found that the methanol solution has less flavonoids than water or DMSO solutions. There is no difference in anti-oxidation activity between any of our PE60 preparations. In methanol, we found that there is less TPC and TFC but the anti-oxidation activity is similar to water and DMSO. It is possible that some flavonoids in the methanol solution may have been more active than in water or DMSO solutions as different polyphenols contain variable hydroxyl groups. For oxygen scavenging capacity, all preparations were in the same range. Flavonoids and polyphenols exert vital effects on the central nervous system by shielding neurons against stress-induced damage, suppressing neuroinflammation, and improving cerebral functions [33]. They potentially act as neuromodulators by protecting the brain from several pathological conditions that include neuro-degenerative disorders such as Alzheimer’s and Parkinson’s disease, diseases of the cardiovascular system, infections, brain cancer, and many others [34]. Conclusively, flavonoids and polyphenols provide anti-oxidant and pro-oxidant functions to the human body and have an extensive range of benefits, such as to destroy brain cancer and improve other cerebral disorders [35]. Polyphenols have various beneficial health effects such as antioxidant activities and may neutralize the destructive reactivity of undesired reactive oxygen/nitrogen species produced as a byproduct during metabolic processes in the body. These molecules can be found naturally in fruits, vegetables, cereals and beverages.

The water solution of PE60 was more effective for reducing cell viability of U-87 glioblastoma cells than that of methanol or DMSO solutions. This may be due to the fact that there are some specific components of polyphenols or flavonoids in water preparations that have better anticancer effects against brain cancer cells. It is also possible that water soluble PE60 solution may contain other polar anti-oxidants such as vitamin C, which is also known for its anti-oxidation and anti-cancer effects [36]. During our investigations, we did not identify the individual components of water, methanol, 25% and 50% DMSO preparations as it was beyond the scope of the present study. Future additional studies are required to identify the components in PE60 preparations using analytical approaches such as HPLC and/or HPLC-MS.

Many anticancer drugs cause cancer cell death due to induction of apoptosis which involves a cascade of caspase activation from proteolytic enzymes present in an inactive state as “pro-caspases” [37]. The initiator caspases (caspase-8 or caspase-9) cause the activation of executory caspase-3. We found that PE60 causes changes to nuclear morphology, and indicator of apoptosis resulting from caspase activity. In order to validate the role of PE60 in inducing apoptosis, we used caspase-3 inhibitor. We found that the PE60 treatment in the presence of a specific caspase-3 inhibitor improved cell viability significantly by 15% while the generic caspase inhibitor did not cause any significant improvement. The inhibition of cell death by caspase-3 inhibitor validated the involvement of caspase 3 activation and supports the theory of induction of apoptosis by PE60 in U-87 cells. Apoptosis is a vital biological process that aids organisms in disposing of unwanted cells from bodily development, homeostasis and disease [38]. Caspase-3 inhibitor is a protein under the Inhibitor of Apoptosis (IAP) family that suppresses apoptosis by providing a mechanism to control the unwanted death of cells and regulate cell division [39]. Conversely, caspase-3 is significant for cell death in crucial tissues and cell types, and is vital for several characteristic changes in the morphology of cells as well as various biochemical processes connected with apoptosis [40]. Caspase-3 is processed by autoproteolytic cleavage that assembles active heterotetrameric enzymes. Consequently, caspase-3 selectively acts on tissues, and this can be explained by two mechanisms: A shortage of the vital caspase-3 protease that supplements for caspase-3 at a crucial stage in the neural process of apoptosis or caspase-3 may position itself at the center of a vital neural death pathway. Therefore, caspase-3 brings about essential effects in organisms that include cell deaths and crucial nuclear and morphological processes that ensure the well-being of the life of organisms [41]. We found that caspase-3 inhibitor improved cell viability while generic caspase inhibitor had no effect, suggesting that PE60 induced its effects through apoptosis. Once caveat to this study is that the inhibitor amounts used were not at a high enough concentration to block the activation of all caspases. Further studies are required to determine the actual chemical nature of the PE60 solutions. The present study has identified that water extract of PE60 containing water soluble compounds including polyphenols may have the potential to inhibit U-87 glioblastoma brain cancer cells.

Authors’ Contribution

Concept, design, and manuscript preparation, editing, and review: Rafat A Siddiqui.
Review of results, and preparation, editing, and review of the manuscript: Andrea R. Beyer and Jason Younkin.

Experimental studies, data acquisition, data analysis, statistical analysis: Haiwen Li and Ashwaq Alsufyani

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Conflicts of Interest

The authors have no conflicts of interest to declare.

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