

Induction of apoptosis and reduction of MMP gene expression in the U373 cell line by polyphenolics in *Aronia melanocarpa* and by curcumin

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Received April 27, 2012; Accepted June 22, 2012

DOI: 10.3892/or.2012.1941

Abstract. Malignant brain tumours are rare but are the most challenging types of cancers to treat. Despite conventional multimodality approaches available for their management, the outlook for most patients remains dismal due to the ability of the tumour cells to invade the normal brain. Attention has now focused on novel therapeutic interventions such as the use of micronutrients. Both chokeberry extract (*Aronia melanocarpa*), which is rich in natural pigments such as anthocyanins and curcumin (diferuloylmethane) found in turmeric (*Curcuma longa*) have been reported to possess anticancer properties in other cancers. The aim of this study was to extend our previous research to evaluate the therapeutic potential of these two agents by testing their ability to induce apoptosis in an established glioblastoma cell line (U373). This was accomplished by treating the cells for 48 h with either chokeberry extract or curcumin, and using the Annexin-V assay. Gene profiles of 8 MMPs (2, 9, 14, 15, 16, 17, 24 and 25) and 4 TIMPs (1, 2, 3 and 4) were analysed for effects of mediators of invasion by quantitative real-time polymerase chain reaction (RT-PCR). The IC₅₀ values determined for curcumin and chokeberry extract were 15 and 200 µg/ml, respectively. Our results also suggest that curcumin induces apoptosis but chokeberry extract is necrotic to this cell line. It is possible that chokeberry extract kills the cells by other non-apoptotic pathways. In addition,

the RT-PCR results show downregulation of the gene expression of MMP-2, -14, -16 and -17 for both micronutrients. Taken together, the comparative data suggest that both curcumin and chokeberry extract may exhibit their anticancer potential by inducing apoptosis and inhibiting invasion by reducing MMP gene expression.

Introduction

Gliomas are intrinsic brain tumours which are rare, with an incidence of approximately 10 per 100,000 capita (1). The most common type of glioma is glioblastoma (GB) which is classified as grade IV. It is one of the most challenging types of cancer to treat and remains incurable. Of the biological features, such as proliferation, angiogenesis and invasion which are common to all cancers, perhaps the most significant one is invasion. Some matrix metalloproteinases or MMPs are zinc-dependent endopeptidases and have a role in degradation of the extracellular matrix (ECM). Originally, MMPs were associated with invasion and metastasis exclusively. Recent studies have documented the additional involvement of MMPs in several steps of cancer development which includes cell growth, apoptotic activity, angiogenesis and immune responses to cancer (2,3). MMPs also regulate cell growth through cleavage of cell surface-bound growth factors and receptors, apoptosis through release of death or survival factors and cell motility by revealing cryptic matrix signals or cleavage of adhesion molecules, releasing growth factors sequestered in the ECM or by integrin signalling (4). Also, cell-surface anchored MMPs or membrane-type MMPs (MT-MMPs) are expressed in gliomas which mediate extensive local intracerebral invasion and are high in vascularisation of the tumour (5). MMP dysregulation and the imbalance between metalloproteinases and their inhibitors (TIMPs) have been implicated as a potential predictor of ECM production and/or degradation (6,7). TIMPs are documented widely for their roles on the induction of apoptosis in different cancer cells *in vitro* (8).

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Key words: MTT, curcumin, *Aronia melanocarpa*, glioblastoma, apoptosis, matrix metalloproteinases

The prognosis of GB patients remains dismal despite conventional therapeutic approaches such as radio- and chemotherapy and various novel surgical approaches that have been introduced for their management. The current clinical practice guideline for diagnosis, treatment and follow-up of malignant gliomas has been reviewed by Stupp *et al* (9). Apart from surgery as the initial therapeutic approach, implantation of carmustine polymers into the resection cavity may be performed prior to radiotherapy. Chemotherapy, on the other hand, is the standard care for GB patients with temozolomide (TMZ) administered daily at a low dose of 75 mg/m² ~1.5 h before radiotherapy. Radiotherapy may be administered either as a standard fractionated focal radiotherapy (e.g. 60 Gy in 30-33 fractions) or in shorter hypofractionated regimens (e.g. 40 Gy in 15 fractions), depending on the patient's age and condition. Previous studies (10,11) have suggested that Gliadel wafer (Carmustine) may offer hope of long-term survival in malignant glioma patients but adverse systemic effects in some cases were reported. Nevertheless, there is still a strong need for other therapeutic interventions in the management of malignant gliomas to improve the quality of life and possibly the survival time.

Interest in studies on flavonoids has increased due to their proposed protective role in atherosclerosis and cancer (12). Moreover, one novel approach, proposed by Rooprai *et al* (13) is based on the hypothesis that micronutrients, such as citrus flavonoids (from tangerine peel) may have the potential to serve as anticancer agents in the treatment of malignant gliomas. Their efficacy is based on their ability to interfere with parameters of invasion. We have proposed the use of a combination of micronutrients, including selenium and tangeretin, known to have a variety of therapeutic properties such as anti-invasive and pro-apoptotic to combat brain tumours (14). We have included curcumin and polyphenolics from *Aronia melanocarpa* as part of a very large study on micronutrients screened *in vitro* to evaluate their therapeutic ability, particularly that of the induction of apoptosis and inhibition of invasion in GBs.

Curcumin (diferuloylmethane; 1,7-bis (4-hydroxy 3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is a polyphenol derived from *Curcuma longa* (turmeric), a medicinal plant. Turmeric contains 3 main components; curcumin (77%), demethoxycurcumin (17%) and bisdemethoxycurcumin (3%). Together, these components are referred to as curcuminoids (15,16). Curcumin has also been reported to cross the rat blood-brain barrier (BBB) (17). Its pro-apoptotic potential has been reported in other types of somatic cancer such as breast, lung and colon (18-20). It also has a strong safety profile and a number of potent pleiotropic actions for neuroprotective efficacy. Extensive research has demonstrated that curcumin affects several targets including transcription factors, growth regulators, adhesion molecules, apoptotic genes, angiogenesis regulators and cellular signalling molecules (21). Moreover, other researchers have suggested its mode of action in that there could be participation of the death receptor pathway through Fas-ligand interaction, caspase-3 activation (22) caspase-8, BID cleavage, cytochrome c release and anti-apoptotic gene suppression (Bcl-2 and Bcl-xL) (22,23).

Polyphenolics from *Aronia melanocarpa* (chokeberry) obtained by solvent extraction (ethanol solution) include a range of flavonoids, such as chlorogenic acid, (-) epicatechin,

(+) catechin, rutin (flavones) and anthocyanins including cyanidin-3-galactoside (the primary anthocyanin in polyphenolics from *Aronia melanocarpa*), cyanidin-3-arabinose, cyanidin-3-xyloside and cyanidin-3-glucoside. Some of these epicatechins are also found in green tea, black tea, red wine, and cocoa, which include epigallocatechin gallate, resveratrol, and procyanidin. They have also been extensively investigated due to their possible role as chemopreventive agents based on their antioxidant capacities. In addition, catechins were found to inhibit hepatocyte growth factor receptor (MET kinase) activation in human colon cancer cells, possibly by competing for the kinase domain of the MET protein (24).

Apart from our own research on the anticancer effects of polyphenolics from *Aronia melanocarpa* on malignant brain tumours (14) the only other group who studied its effects in colorectal cancer cell lines have shown it to inhibit cell growth (25,26). The anthocyanins found in this extract are of particular interest because they are non-toxic and are believed to cross the BBB (27).

Thus, the aim of this study was to investigate if curcumin and polyphenolics from *Aronia melanocarpa* could induce apoptosis and effect mediators of invasion by looking at the gene profiles of 8 selected MMPs and 4 TIMPs in an established glioblastoma cell line (U373) *in vitro* compared to the normal astrocytic culture.

Materials and methods

Micronutrients

Curcumin. Curcumin is an orange-coloured powder which was donated by Indus Biotech, India. It was 97% natural and was obtained from ground turmeric rhizome by a standard solvent extraction method. Prior to use for treatment of cell cultures, it was dissolved in dimethyl sulfoxide (DMSO-Sigma Aldrich USA).

Polyphenolics from *Aronia melanocarpa*. The polyphenolics extract from *Aronia melanocarpa* is a dark purple-coloured powder which was donated by Artemis, USA. It is a purified extract containing a number of flavonoids, including chlorogenic acid (a phenolic acid that is dominant among the aromatic acids) (-) - epicatechin, (+) - catechin (catechins), rutin (a flavones) and a range of anthocyanins: cyanidin-3-O-galactoside, cyanidin-3-O-arabinoside and cyanidin-3-xyloside (14). Polyphenolics from *Aronia melanocarpa* was also diluted in DMSO prior to use.

Cell cultures. An established cell line, U373, from an adult patient with GB (grade IV, WHO Classification) was used in this study. It was kindly donated by Professor Rolf Bjerkvig (Norway) and has been tested for mycoplasma screening and confirmed to be of human origin by molecular genetics methods. The control cell culture for this study was normal astrocytes, (CC2565) used at passage 11. It was derived from normal human brain cells of an 18-year old male and purchased from Cambrex BioSciences. These two cell lines were routinely cultured as monolayers in complete medium, i.e. Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich USA) supplemented with 10% foetal calf serum and antibiotics (at the final concentration of 1% penicillin/streptomycin per ml). The physiological pH was maintained through

equilibration with 5% CO₂ atmosphere in a 37°C humidified incubator.

IC₅₀ determination using the MTT cytotoxicity assay. (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT is a residual viable biomass assay dependent on the conversion of MTT to insoluble formazan within living cells, which is subsequently solubilised in DMSO (28). It was used to determine the IC₅₀ value for each micronutrient. The formazan in the solution was quantified spectrophotometrically at 570 nm as an indicator of cell viability. Assays were carried out in triplicates in 96-well culture plates. Both CC2565 cell cultures and the U373 cell line were compared when treated separately with each micronutrient.

Detection of apoptosis with Annexin-FITC using flow cytometry. The binding of the calcium-dependent phosphatidylserine binding protein Annexin V to externalised residues is a hallmark of apoptosis in many cells. A fluorochrome-labelled Annexin V can be used in combination with a DNA-binding dye such as propidium iodide to differentiate between live cells (negative for both dyes), cells in early apoptosis (Annexin positive but propidium iodide negative) and dead cells (positive for both dyes). Cells were treated with a range of concentrations of curcumin and chokeberry and the percentage of live, dead and apoptotic cells were assessed. Cells were harvested and washed before being re-suspended in 500 µl of Annexin-V binding buffer (Pharmingen, USA) with 2.5 µl Annexin-V-FITC (Pharmingen) and incubated at room temperature for 15 min. Propidium iodide (50 µl) (50 µg/ml, Sigma) was added just before samples were measured. Samples were analysed on a FACSCalibur (Becton-Dickinson, USA) with FITC fluorescence being measured using a 530/30 bandpass filter and propidium iodide fluorescence being measured using a 670 nm longpass filter. At least 10,000 events were acquired and data were analysed using the CellQuest software (Becton-Dickinson).

RNA extraction. After 48-h treatment with selected concentrations of either curcumin or polyphenolics from *Aronia melanocarpa*, cells were harvested using Cell Dissociation Solution (Sigma) before 2 ml of RNAzol reagent (Biogenesis) was added to the pellet. The next steps were carried out at 4°C. Chloroform (0.2 ml) was added to the solubilised RNA and vortexed for 15 sec followed by centrifugation at 4,000 rpm for 30 min. Equal volumes of both the supernatant (800 µl) taken from the upper phase and isopropanol were mixed. The sample was incubated for 15 min and centrifuged at 4,000 rpm for 30 min before 2 ml of 70% ethanol was added to the pellet. This centrifugation was repeated twice for 15 min, each. The pellet was then resuspended in 100 µl of double autoclaved water and incubated in a water bath for 15 min at 60°C as to dissolve the RNA. The RNA (100 µl) was then diluted in 300 µl of double distilled water for measuring the optical density at 260 nm in order to determine the RNA concentration. Each sample (3 µl) was diluted (x100) in double autoclaved water prior to spectrophotometric analysis, in triplicate. The control used was double autoclaved distilled water. RNA concentration was calculated as follows: Mean value x 100 (dilution factor) x 40 µg/ml (conversion factor for RNA).

Reverse transcription (RT) reactions. An aliquot (1 µg) of total RNA was reverse transcribed using 2 µg of random hexamers (Amersham) and 200 units of Superscript II reverse transcriptase (Invitrogen). The sequences for primers and probes have been previously described (5) whereas 18S RNA was used as an endogenous control to account for differences in the extraction and reverse transcription of the total RNA. PCR was performed using the manufacturer's protocol for ABI Prism 7700 Sequence Detection System (Applied Biosystems). This cDNA synthesis reaction mixture was pre-incubated for 10 min at 21°C. Reverse transcription reaction was carried out for 50 min at 42°C and then heated to 95°C for 5 min to stop the reaction.

Quantitative PCR (polymerase chain reaction). PCRs were performed in 25-µl reaction volumes. Each reaction volume contained the equivalent of 5 ng of the RT reaction product, 50% TaqMan 2X PCR Master mix (Applied Biosystem) 100 nM of each forward and reverse primer and 200 nM probe. Each PCR cycle consisted of a heat denaturation step for 94°C for 1 min, a primer-annealing step at 50°C for 30 sec and a polymerization step for 1 min at 72°C. To determine the relative RNA levels within the samples, standard curves for the PCR reaction were prepared by using the cDNA from a standard sample and making 2-fold serial dilutions covering the range equivalent to 20-0.625 ng of RNA; for 18S analyses, the range was 4-0.125 ng. Messenger RNA levels were normalized to 18S rRNA levels and are presented with the levels in the control sample set to 1 (29).

Statistical analysis. The absorbance values obtained from the MTT assay were analysed using the StatsDirect and Microcal (Origin) programmes. The average of 6-wells was taken for each concentration studied and compared with the control cells treated with a medium (100% viable) in order to obtain the percentage of residual viable cells. The dose response curve formed (of each micronutrient) conferred the IC₅₀ values.

Results

Curcumin and polyphenolics from *Aronia melanocarpa* reduce glioma cell viability. The MTT viability assay was used to assess the cytotoxicity of curcumin and polyphenolics from *Aronia melanocarpa* on normal astrocytic cultures, CC2565 at passage number 11 by treating them with the micronutrients compared to untreated cells (with DMEM only). The data obtained (Fig. 1A) show that generally, curcumin and polyphenolics from *Aronia melanocarpa*, at all the concentrations selected for the study, were not toxic (>90% viability) to them. In contrast, the U373 established brain tumour cell line, showed a decrease in cell viability in a dose-responsive manner (Fig. 1B and C). The IC₅₀ values for U373, determined for curcumin and polyphenolics from *Aronia melanocarpa* were 15 and 200 µg/ml, respectively. These results indicate that curcumin and polyphenolics from *Aronia melanocarpa*, at the concentrations analysed, are not toxic to normal astrocytic cells but induce cell death in the U373 cell line.

Curcumin and polyphenolics from *Aronia melanocarpa* cause cell death by different mechanisms. To evaluate the

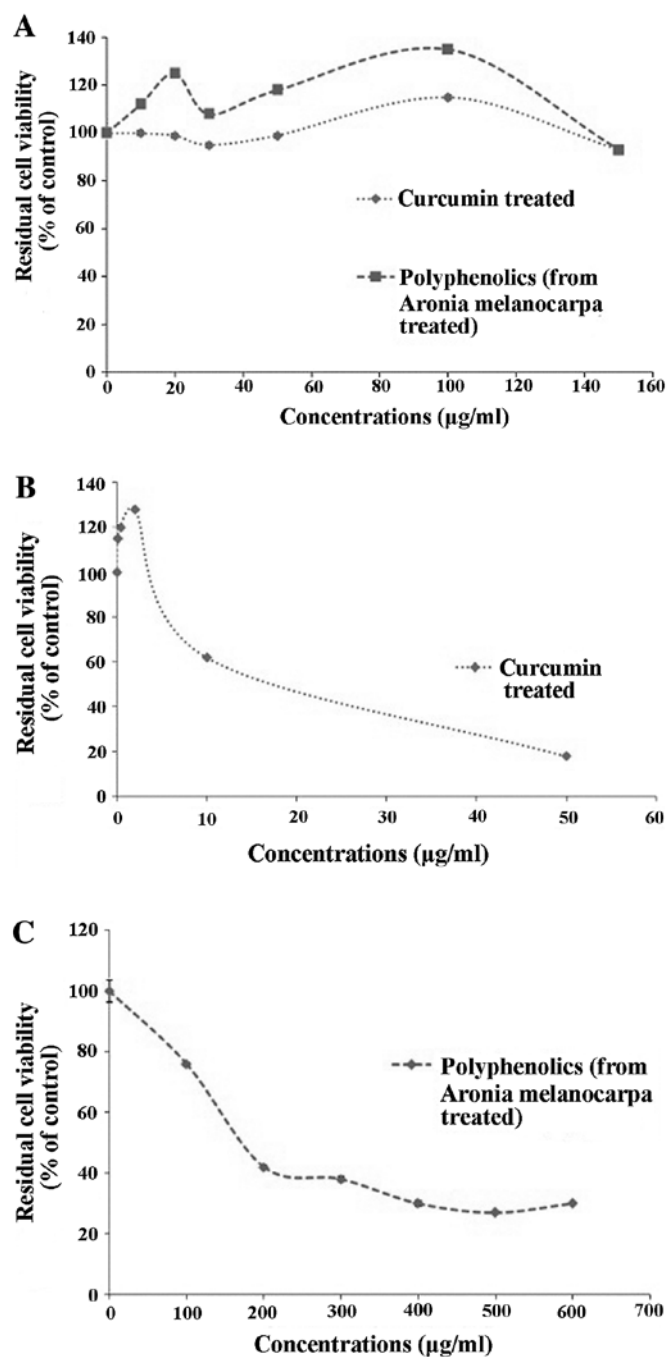


Figure 1. Effect of curcumin or polyphenolics from *Aronia melanocarpa* on cell viability of CC2565 (normal brain cells) and U373 cells determined by the MTT assay. (A) CC2565 cells were treated with the indicated concentrations of curcumin or polyphenolics from *Aronia melanocarpa* E for 48 h. Dose dependent effect of (B) curcumin or (C) polyphenolics from *Aronia melanocarpa* E treatment for 48 h on U373 cells. The experiments were repeated at least 3 times.

effects of curcumin and polyphenolics from *Aronia melanocarpa*, flow cytometric assessment of apoptosis and cell death by the Annexin V/propidium iodide assay was carried out. The advantage of the flow cytometry is that it is possible to measure not just dead cells but also early apoptotic cells (30). The ability to induce cell death by polyphenolics from *Aronia melanocarpa* within a range of different concentrations, 30–60 µg/ml (Fig. 2B) and curcumin, 5–20 µg/ml (Fig. 2A),

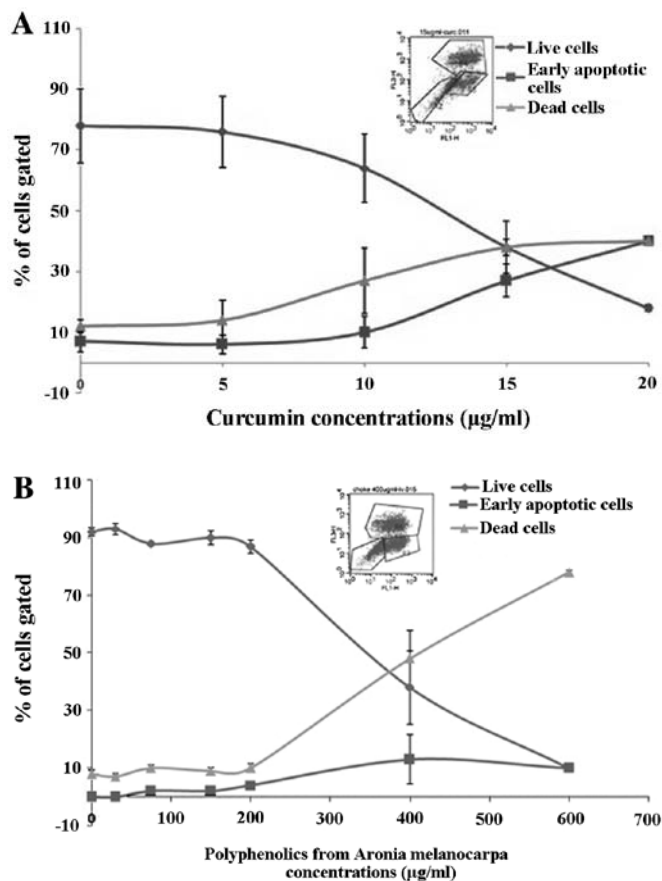


Figure 2. Population of live, apoptotic and dead cells gated via flow cytometry (Annexin V assay) after 48-h treatment with (A) curcumin or (B) polyphenolics from *Aronia melanocarpa*. At least 10,000 events were acquired by FACSCalibur and analysed using the CellQuest software.

was then assessed, closer to their IC_{50} values determined by the MTT viability assay.

For the polyphenolics from *Aronia melanocarpa*, all concentrations studied induced more necrosis (82%) than apoptosis (11%) in the cells particularly at the highest concentration of 600 µg/ml (Fig. 2B). This population included the late apoptotic cells (as seen with both propidium iodide and Annexin V positivity). Percentages of live, early apoptotic and dead cells were calculated in comparison to the untreated cells and a representative scatter plot was included next to each graph plotted to illustrate each cell population gated. Low percentages of the early apoptotic cells were gated almost at the same extent at all concentrations studied (1–15%). The highest two concentrations caused the apoptotic population of cells to increase but only up to 14 and 11% for cells treated with 400 and 600 µg/ml of polyphenolics from *Aronia melanocarpa*, respectively.

In contrast, higher percentages of the early apoptotic cell population were seen following treatment with curcumin, particularly at concentrations of 15 and 20 µg/ml when the apoptotic cells gated were 26 and 41%, respectively (Fig. 2A). When comparing early apoptotic and dead cell populations gated, it was seen that as curcumin concentrations increased, the dead cell population increased more than the apoptotic one. Interestingly, at the highest concentration of curcumin (20 µg/ml) used, both early apoptotic and dead cells appeared

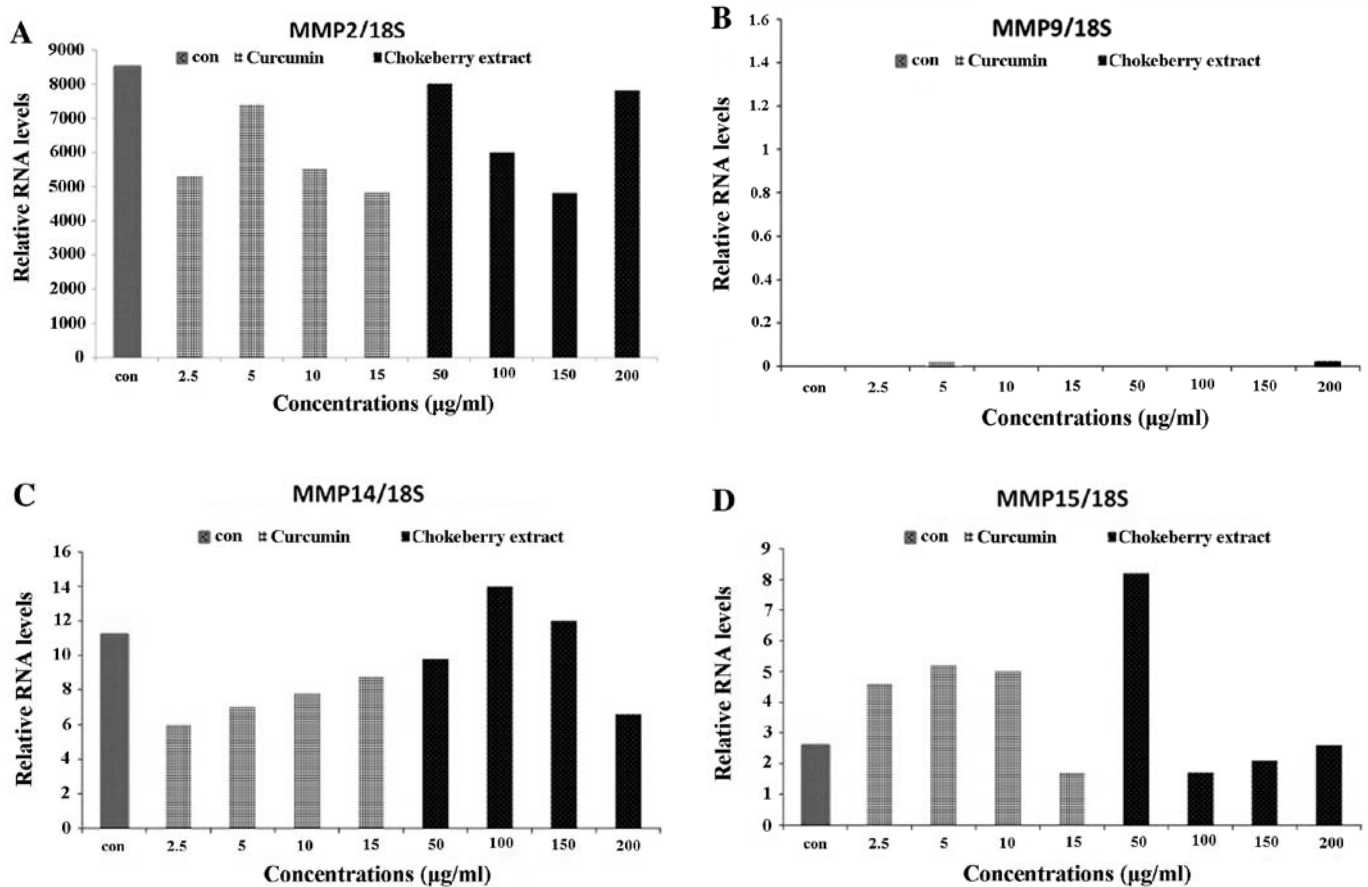


Figure 3. Relative RNA expression of the MMP-2, -9, -14 and -15 (A-D) in U373 cell line following curcumin and polyphenolics from *Aronia melanocarpa* treatment separately. RNA levels measured are presented as ratios of target gene levels to 18S rRNA levels (ratios normalized such that sample 1 was equal to 1). The experiment was repeated at least twice.

to share the same effect due to similar percentage of cells (~40% for each population) gated. The representative scatter plots (Fig. 2) show that both curcumin and polyphenolics from *Aronia melanocarpa* cause cell death in the glioma cell line, while polyphenolics from *Aronia melanocarpa* mainly induce necrosis. The effect of curcumin is mediated equally by necrosis and early apoptosis.

Reduction in MMP gene expression with polyphenolics from *Aronia melanocarpa*. Relative RNA expression levels of 8 MMPs (MMP-2, 9, 14, 15, 16, 17, 24 and 25) in the U373 cell line following treatment with ranges of curcumin 10-50 µg/ml or polyphenolics 50-400 µg/ml from *Aronia melanocarpa* as shown in Figs. 3 and 4. RNA expression was normalized to 18S rRNA levels and is expressed relative to the levels in untreated cells. Generally, the results in Figs. 3 and 4 show that RNA levels of all the MMPs and TIMPs studied were reduced compared to the untreated cells at the different concentrations of the micronutrients. However, the extent of reduction in each case was different as seen in the raw data. In some cases such as MMP-9, a minimum amount was expressed either in the control or curcumin/polyphenolics from *Aronia melanocarpa*-treated cells. The most prominent reduction was seen in MMP-2 (Fig. 3A) whereas MMP-14, -15 (Fig. 3C-D) and -16 (Fig. 4A) showed slight reduction of the RNA levels at both IC₅₀ values of each micronutrient. A similar reduction was seen with

MMP-17 (Fig. 4B) but to a greater extent in cells treated with 200 µg/ml polyphenolics from *Aronia melanocarpa*. The level of MMP-24 (Fig. 4C) remained unchanged at the IC₅₀ value for curcumin but was slightly increased for the IC₅₀ value of polyphenolics from *Aronia melanocarpa*. With MMP-25 (Fig. 4D), negligible changes were observed with either polyphenolics from *Aronia melanocarpa* or curcumin at all concentrations studied.

Discussion

The present study showed that both curcumin and polyphenolics from *Aronia melanocarpa* were non-toxic to the control human brain cell culture, (CC2565) as illustrated in Fig. 1A. This confirms earlier reports that curcumin is safe to be consumed even at higher concentrations because it does not cause harmful effects on normal cells. Moreover, extensive clinical and metabolic studies including those of Aggarwal *et al* (15) have reported that 8-12 g/day is safe to be consumed (31). Similarly, the non-toxic effects of anthocyanins and polyphenolics from *Aronia melanocarpa* are consistent with other reports in malignant colon cancer cells studies (32).

Generally, the residual cell viability decreased as the concentrations increased, suggesting that very high concentrations could have been toxic to the cells. The IC₅₀ value for curcumin was 15-20 µg/ml (Fig. 1B). A similar pattern was seen

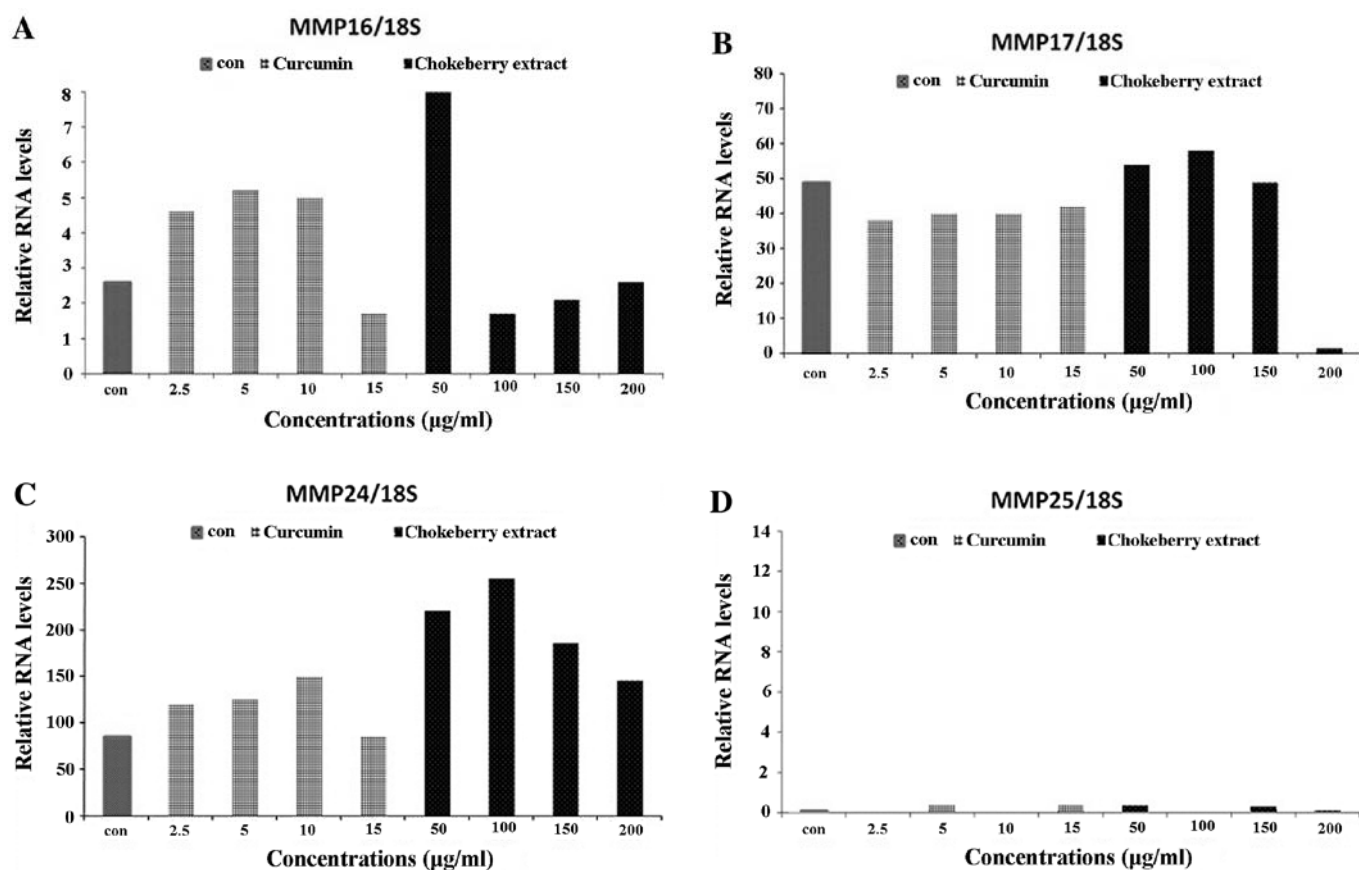


Figure 4. Relative RNA expression of the MMP-16, -17, -24 and -25 (A-D) in the U373 cell line following curcumin and chokeberry extract treatment separately. RNA levels measured are presented as ratios of target gene levels to 18S rRNA levels (ratios normalized such that sample 1 equals 1). The experiment was repeated at least twice.

for cells treated with polyphenolics from *Aronia melanocarpa* giving an IC_{50} of ~ 200 $\mu\text{g/ml}$ (Fig. 1C). These values probably could not be achieved physiologically as both curcumin and polyphenolics from *Aronia melanocarpa* have rapid and extensive metabolisms which explains their poor bioavailability. However, as U373 is an established GB cell line, which has homogenous cell populations of cells, probably stem cells, unlike the low passage biopsy-derived primary cultures which are heterogenous. It is therefore not surprising that it requires a high IC_{50} value for either micronutrient compared to primary cultures. The IC_{50} value was much lower (30 $\mu\text{g/ml}$) when biopsy-derived cell cultures were treated with polyphenolics from *Aronia melanocarpa* (unpublished data). A brain tumour stem cell study by Inagaki *et al* (33) demonstrated a small population of cancer stem cells (CSCs) with preserved stem cell properties even after many serial passages under non-adherent/adherent culture conditions. Other studies have suggested that cells experiencing *in vitro* propagation are likely to be different from the original tumour's signature, possibly being responsible for the attenuated resistance observed (34-36).

Our results also showed that there was a variation in the proportion of apoptotic and dead cells which could be due to the cells' response depending on the state of the cells at the time. In addition, it also suggested that at high concentration, the method of cell death may have changed. As seen in Fig. 2B, polyphenolics from *Aronia melanocarpa* show a rapid killing from 200 $\mu\text{g/ml}$ upwards whereas curcumin (Fig. 2A) is more

subtle from 5 $\mu\text{g/ml}$. Hence, it is implied that unlike curcumin, polyphenolics from *Aronia melanocarpa* may not be apoptotic. In addition, the polyphenolics from *Aronia melanocarpa* may appear to kill the cells by a non-apoptotic (or at least a non-Annexin positive) pathway whereas curcumin appears to target Annexin V positive populations. The percentage of dead cells as indicated by propidium iodide staining shows that this did not change significantly over the range of concentrations used, but the percentage of apoptotic cells did. This may account for the discrepancy seen with the MTT assay which generally measures a final end point rather than early apoptosis. The MTT viability assay detects metabolically active cells which then do not necessarily correlate with the flow cytometry data. Recently, other workers have reported the induction of apoptosis by curcumin in somatic cancers where the degree of response may not be comparable to our study on brain tumours. Saha *et al* showed that at 50 μM (18.4 $\mu\text{g/ml}$) induced apoptosis in 60% of human lung cancer cells (37). Moreover, a similar effect was seen at 10 $\mu\text{mol/l}$ (3.7 $\mu\text{g/ml}$) in melanoma cells (38) and 20-75 μM (7-28 $\mu\text{g/ml}$) in breast and colon cancer (18,20). Perhaps in these different types of cancer, low concentrations may cause apoptosis while higher ones may lead to other forms of cell death, such as necrosis and autophagy.

However, to the best of our knowledge, there are no other reports in the literature for *Aronia melanocarpa*'s potential for induction of apoptosis except for our own recent findings

in another study on its powerful anti-invasive potential in GB cells (unpublished data). Nevertheless, polyphenolics from *Aronia melanocarpa* have been reported to have inhibitory effects on cell growth and cell cycle at a range of 50-75 $\mu\text{g/ml}$ (25,26) but non-cytotoxic on healthy human tissue.

Generally, both curcumin and polyphenolics from *Aronia melanocarpa* were found to downregulate the RNA levels of all the MMP and TIMP RNA studied at their IC_{50} values. High levels of MMP-2 which has been implicated in invasion were detected in the untreated cells. Both micronutrients downregulated MMP2 levels in this study, suggesting their anti-invasive potential. Whereas, in the U373 cells, hardly any MMP-9 (Fig. 3B) and MMP-25 (Fig. 4D) expression could be detected, either in the untreated or curcumin/polyphenolics from *Aronia melanocarpa*-treated cells.

It is well documented that high levels of MT-MMPs, such as MMP-14 are expressed by gliomas and it facilitates MMP-2 activation thus playing a role in the regulation of invasiveness (39). Although our results show that there were some increases at the concentrations below the IC value of either curcumin or polyphenolics from *Aronia melanocarpa* in MMP-14 (Fig. 3C) and -15 (Fig. 3D) the expression of both MMPs was reduced to a similar extent by 15 $\mu\text{g/ml}$ curcumin when compared with the control. MMP-16 expression was also reduced at all concentrations studied (Fig. 4A). Thus, the reduction of the expression of MMP-14, -15 and -16 may suggest inhibition of invasion. MMP-24 is a cell membrane-anchored MMP which is brain specific and mainly expressed in the cerebellum (40). Surprisingly, its levels elevated generally with both micronutrients (Fig. 4C), similar to the effect seen on MMP-25 with the treatment of selenium (41).

In conclusion, it is possible that curcumin and polyphenolics from *Aronia melanocarpa* with its complex and ranged phenolic compounds could selectively induce apoptosis or inhibit invasion in the established malignant brain tumour cells, U373. Further studies include comparison of the therapeutic potential of these and other micronutrients in primary cultures, particularly with the use of spheroids in 3D invasion assays and *in vitro* angiogenesis assays.

Acknowledgements

The authors are grateful to the Have a Chance Inc., RoseTrees Trust, CALM and Ministry of Higher Education, Malaysia for their financial support.

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