

# *In vitro* effects of *Yunnan Baiyao* on canine hemangiosarcoma cell lines

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## Abstract

*Yunnan Baiyao* is a Chinese herbal medicine that has been utilized for its anti-inflammatory, haemostatic, wound healing and pain relieving properties in people. It has been utilized in the veterinary profession to control bleeding in dogs with hemangiosarcoma (HSA) and has been anecdotally reported to prolong survival times in dogs with this neoplasm. This study evaluated the *in vitro* activity of *Yunnan Baiyao* against three canine HSA cell lines after treatment with increasing concentrations of *Yunnan Baiyao* (50, 100, 200, 400, 600 and 800  $\mu\text{g mL}^{-1}$ ) at 24, 48 and 72 h. Mean half maximum inhibitory concentration ( $\text{IC}_{50}$ ) at 72 h for DEN, Fitz, SB was 369.9, 275.9 and 325.3  $\mu\text{g mL}^{-1}$ , respectively. Caspase-3/7 activity increased in correlation with the  $\text{IC}_{50}$  in each cell line which was confirmed by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, APO-BRDU Kit; BD Biosciences, San Jose, CA, USA) assay. VEGF in cell supernatant was also quantified. Overall, the study found that *Yunnan Baiyao* causes dose and time dependent HSA cell death through initiation of caspase-mediated apoptosis, which supports future studies involving *Yunnan Baiyao*.

## Keywords

canine hemangiosarcoma,  
Chinese herbal medicine,  
*Yunnan Baiyao*

## Introduction

Hemangiosarcoma (HSA) is a highly malignant neoplasm of vascular endothelial cell origin. HSA is a relatively common neoplasm in the dog, accounting for up to 21% of all soft tissue sarcomas and 0.3–2% of all malignant tumours in this species.<sup>1–4</sup> The incidence of disease is significantly higher in large breed dogs such as German Shepherds, Golden and Labrador Retrievers.<sup>4–7</sup> HSA can affect any tissue in the body; however, the spleen is the most common site of tumour development, accounting for 50–65% of all canine HSAs.<sup>2</sup> HSA is also the most common primary cardiac tumour and tumours of the right atrium account for 3–25% of all HSAs in the dog.<sup>8</sup> Other common sites include the subcutaneous tissues (13–17%) and the liver (5–6%).<sup>9</sup> Canine HSA is an aggressive malignancy, characterized by pathologic angiogenesis and early, aggressive metastasis that is poorly chemo-sensitive.<sup>3,9–19</sup> Previously reported prognostic factors for canine HSA include location

(cutaneous versus viscera), histological grading and stage.<sup>5,9–19</sup> Despite available multi-modal therapies to address local and systemic disease, few patients survive beyond 6 months with most succumbing to symptoms associated with metastatic disease.

Malignant tumours of the vascular endothelium are rare in humans; however, this type of cancer is extremely aggressive when it does occur. HSA, also called angiosarcoma, accounts for approximately 2% of soft tissue sarcomas in humans and most commonly occurs in liver, spleen, breast and scalp. As in dogs, this tumour frequently metastasizes and despite multimodal treatment, 5-year survival rates remain between 10 and 35%.<sup>20–24</sup>

The lack of effective adjuvant therapies warrants the investigation of novel treatment options and in recent years, traditional Chinese medicine (TCM) has been receiving increased attention for the treatment of malignant neoplasia. *Yunnan Baiyao* is an herbal TCM that has been used frequently by veterinarians and their clients as an adjunctive treatment

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for canine HSA. It has been anecdotally reported to prolong survival times and control bleeding in dogs with this aggressive neoplasm.

*Yunnan Baiyao* is a well-known Chinese herbal patent formula that has been utilized for its anti-inflammatory, haemostatic, wound healing and pain relieving properties in people for over 100 years. It was developed in the Yunnan Province of China around 1902 and gained popularity among Chinese soldiers during World War II for use as a haemostatic agent on the battlefield.<sup>25,26</sup> *Yunnan Baiyao* has been shown to improve clotting and enhance platelet function.<sup>26–30</sup> This may benefit canine patients with HSA due to the frequency of clotting abnormalities and potential for fatal haemorrhage although this was not evaluated in this study.

*Yunnan Baiyao* is a class-1 protected TCM and the exact herbal formula is a trade secret. Due to this protected status, component analysis and quality control measures for *Yunnan Baiyao* have been slow to develop; however, due to international demand for quality assurance and the development of Good Manufacturing Practice (GMP), the product is now labelled to identify its major components per 0.5 g serving.<sup>31</sup> The following ingredients are listed based on 2011 manufacturer's label: 200 mg Tienchi ginseng root (*Panax notoginseng*), 85 mg *Ajuga forrestii* Diels plant, 66.5 mg Chinese yam root, 57.5 mg *Dioscoreae nipponica* Makino root, 36 mg *Erodium stephanianum* and *Geranium wilfordii* plant, 30 mg *Dioscoreae parvilora* ting root and 25 mg *Inula cappa* plant (*Yunnan Baiyao*; Yunnan Baiyao Group, Kunming, China).

There is a vast body of scientific literature showing that components of *Yunnan Baiyao* have various anti-cancer properties; however, studies on *Yunnan Baiyao* itself as an anti-cancer therapy have not been previously performed.<sup>32–37</sup>

*Panax notoginseng* root extract (NGRE), which is a major component of *Yunnan Baiyao*, showed significant growth inhibition and increased apoptosis of SW480 human colorectal cancer cells *in vitro*. NGRE also enhanced cell growth inhibition when combined with either 5-fluorouracil or irinotecan.<sup>32</sup> The saponin ginsenoside Rd, isolated from *P. notoginseng*, was shown to inhibit proliferation of human cervical cancer (HeLa) cells

*in vitro* and induce apoptosis by upregulation of Bax, downregulation of Bcl-2 and activation of the caspase-3 pathway.<sup>33</sup> Additionally, *P. notoginseng* has been documented to inhibit DNA synthesis and cell proliferation in human umbilical vein endothelial cells (HUVEC) *in vitro*.<sup>34,35</sup>

Wild yam root (*Dioscoreae spp.*), another major component of *Yunnan Baiyao*, was shown to have the most potent effects on cell viability and induction of apoptosis in a murine malignant neuroblastoma cell line when compared with 373 other naturally derived herb, seed, root, plankton and fungi extracts.<sup>36</sup> Wild yam root has also been shown to induce anti-proliferative and pro-apoptotic effects in a range of tumour cells by G2/M arrest, downregulation of NF- $\kappa$ B, Akt, cyclin D, c-myc and initiating PARP cleavage/DNA fragmentation.<sup>36</sup> *Dioscoreae nipponica* extract exerted dose dependent inhibition on the invasion, motility, secretion of MMPs and u-PA in murine melanoma (B16F10) and human melanoma (A2058) cells *in vitro*.<sup>37</sup> It was also shown to inhibit activation of NF- $\kappa$ B and increase expression of I- $\kappa$ B in the B16F10 cells *in vitro*. Additionally, lung metastasis formation was significantly reduced in mice treated with the extract versus the control group *in vivo* in the same study.<sup>37</sup>

Novel therapeutic options are needed if we hope to improve outcomes associated with canine HSA. Studies on the anti-cancer properties of *Yunnan Baiyao* components combined with anecdotal evidence to its efficacy suggest that it may enhance the traditional medical approach to treatment of canine HSA. This study aims to take the first step in evaluating the biological activity of *Yunnan Baiyao* against canine HSA cells *in vitro*. We studied *Yunnan Baiyao*'s ability to inhibit growth of canine HSA cells and to induce apoptosis. Cell survival assays were performed for HSA cell lines exposed to *Yunnan Baiyao*. Apoptosis was investigated by measuring caspase-3/7 activity and the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). Changes in cell cycle kinetics were evaluated using flow cytometry. Due to the association of increased VEGF levels in dogs with HSA,<sup>38</sup> we also investigated levels of VEGF found in supernatant from untreated and *Yunnan Baiyao* treated HSA cells. The information gained from this

study will be used to establish a proof-of-concept for clinical use as well as support for further *in vitro* investigation of the use of *Yunnan Baiyao* as a novel anti-cancer agent.

## Materials and methods

### Cell cultures

Three established canine HSA cell lines were evaluated: DEN-HSA, Fitz-HSA (provided by Dr Ilene Kurzman, University of Wisconsin, Madison, WI, USA) and SB-HSA (provided by Dr Stuart Helfand, Oregon State University, Corvallis, OR, USA). DEN was established from a renal HSA from a Golden Retriever, Fitz was from a splenic HSA of a Golden Retriever and SB was obtained from a subcutaneous HSA of a German Shepherd dog.<sup>39,40</sup> It has recently been shown that DEN and Fitz were derived from the same source.<sup>40</sup> This does not mean that DEN and Fitz might not show differences in drug sensitivity as they have been cultured as separate cell lines for several years now and may have 'drifted' apart. All cell lines were cultured under standard conditions (37 °C, 5% CO<sub>2</sub>, humidified air). DEN and Fitz were maintained in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (Cellgro, Mediatech, Manassas, VA, USA). SB was maintained in Roswell Park Memorial Institute (RPMI, Buffalo, NY, USA) medium supplemented with 10% heat-inactivated FBS, sodium pyruvate, L-glutamine, HEPES, penicillin AND streptomycin.

### *Yunnan Baiyao* preparation

*Yunnan Baiyao* (Yunnan Baiyao Group) was generously provided as a stock powder (4 g per vial) by Dr Shen Huisheng Xie (University of Florida, Gainesville, FL, USA). A 200 mg mL<sup>-1</sup> stock solution was prepared in 0.1% dimethylsulfoxide (DMSO) at room temperature, vortexed for 5 min and filtered with a 0.22 µm filter. Aliquots of the stock solution were stored at -20 °C and protected from light. Dilutions of the stock solution were prepared immediately prior to use in cell culture medium such that the DMSO concentration did not exceed 1%.

### Evaluation of cell viability

The DEN and Fitz cells were plated at 5000 per well and SB cells were plated at 10 000 per well in 100 µL media in 96-well flat-bottom plates (Falcon, Becton Dickinson Bedford, MA, USA). The plates were incubated under standard conditions for 24 h. After 24 h, *Yunnan Baiyao* was added to the wells at increasing concentrations (50, 100, 200, 400, 600 and 800 µg mL<sup>-1</sup>) in 100 µL media solution. Control wells were prepared for each assay containing media with 1% DMSO only or 800 µg mL<sup>-1</sup> *Yunnan Baiyao* in 100 µL media solution. After incubation times of 24, 48 or 72 h, the relative viable cell number was assessed using a one-step tetrazolium-based (MTS) colorimetric assay (CellTiter-Blue® Cell Viability Assay, Promega, Madison, WI, USA) in accordance with the manufacturer's specifications. Fluorescence was quantified with a fluorescence plate reader at an excitation wavelength of 530 nm and emission wavelength of 590 nm. Relative viable cell number was assessed by means of triplicate wells for each drug concentration and triplicate wells for each control, and each experiment was repeated three times.

### Effect of *Yunnan Baiyao* on apoptosis

To measure and characterize cell death, the effects on caspase-3/7 activity were assessed as an important signalling and effector step in the apoptotic cascade. The DEN and Fitz cells were plated at 5000 per well and SB cells were plated at 10 000 per well in 100 µL media in 96-well flat-bottom plates (Falcon, Becton Dickinson). The plates were incubated under standard conditions for 24 h. After 24 h, *Yunnan Baiyao* was added to the wells at increasing concentrations (50, 100, 200, 400, 600 and 800 µg mL<sup>-1</sup>) in 100 µL media solution. Control wells were prepared for each assay containing cells and media with 1% DMSO only or 800 µg mL<sup>-1</sup> *Yunnan Baiyao* in 100 µL media solution. After incubation for 24, 48 or 72 h, caspase-3/7 activity was measured using a commercial assay (Apo-ONE® Homogeneous Caspase-3/7 Assay; Promega) performed in accordance with the manufacturer's specifications. Fluorescence was quantified with a fluorescence plate reader at an excitation wavelength of 485 nm and emission

wavelength of 528 nm. All samples were analysed in triplicate, and each experiment was repeated three times with each of the cell lines.

#### TUNEL assay

Detection of fragmented DNA, one of the later steps in apoptosis, was performed using a TUNEL assay (APO-BRDU Kit; BD Biosciences, San Jose, CA, USA). Cells were plated into six-well plates (50 000 per well DEN, 75 000 per well Fitz and 100 000 per well SB) and placed in the incubator under standard conditions for 24 h. After 24 h, *Yunnan Baiyao* was then added to the wells (50, 100, 200, 400, 600 and 800  $\mu\text{g mL}^{-1}$ ). Control wells were prepared for each assay containing cells and media with 1% DMSO. After incubation, the cells were fixed with 1% (w/v) paraformaldehyde in phosphate buffered saline (PBS) and kept at  $-20^{\circ}\text{C}$  until assayed. The commercial assay was performed in accordance with the manufacturer's specifications. The APO-BRDU kit is a two-colour staining method for labelling DNA breaks and total cellular DNA in order to detect apoptotic cells by flow cytometry. Apoptotic cells with exposed 3'-hydroxyl DNA ends were labelled with brominated deoxyuridine triphosphate nucleotides (BR-dUTP). FITC labelled anti-BrdU mAb provided by the commercial kit was then used to stain apoptotic cells. Propidium iodide (PI) was used as a counterstain to label total cellular DNA for cell cycle analysis. Flow cytometry was performed using a flow cytometer (FACSort; BD Biosciences) with a green fluorescence (520 nm) and a red fluorescence (623 nm) detection. Data were processed by use of Cell Quest software (Cell Quest software, version 3.3; BD Biosciences). The samples were pooled and this assay was performed as a single run for all three cell lines at 24, 48 and 72 h. The percentage of apoptotic cells and cell cycle kinetics were evaluated.

#### VEGF enzyme linked-immunosorbent assay

A commercial enzyme linked-immunosorbent assay (ELISA) kit (Quantikine Canine VEGF ELISA Kit; R&D systems, Minneapolis, MN, USA) was used to measure VEGF levels in the cell culture supernatants before and after treatment with

*Yunnan Baiyao*. The kit contains Sf21-expressed, recombinant VEGF and antibodies raised against the recombinant protein. Results obtained for naturally occurring canine VEGF show linear curves that are parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural canine VEGF.<sup>39</sup> In brief, HSA cell lines were plated into six-well plates (50 000 per well DEN, 75 000 per well Fitz and 100 000 per well SB) and placed into the incubator under standard conditions for 24 h. Then *Yunnan Baiyao* (50, 100, 200, 400, 600 and 800  $\mu\text{g mL}^{-1}$ ) was added to the wells. Untreated (control) wells containing cells only were also plated. After incubation for an additional 24, 48 or 72 h the supernatant was removed, centrifuged and stored at  $-20^{\circ}\text{C}$  until assayed. The samples were added in duplicate to a 96-well plate and the VEGF immunoassay was performed in accordance with the manufacturer's specifications. All samples were run in duplicate and calibration on the microtitre plate included a standard series of dilutions of recombinant human VEGF. The optical density of the standard solutions was plotted against their corresponding concentrations to generate a standard curve and allow determination of all VEGF concentrations. All samples were analysed at the same time. This assay has been previously validated for measurement of canine VEGF.<sup>39</sup>

#### Statistical analysis

Statistical analyses were performed with Sigma-Plot software (SigmaPlot for Windows, version 12.5; Systat Software, Erkrath, Germany). Cell survival data were fitted to a four-equation regression model to determine the mean half maximum inhibitory concentration ( $\text{IC}_{50}$ ) for each cell line. The  $\text{IC}_{50}$  was defined as the drug concentration that caused 50% cell death compared with the control. For the cell viability assay and caspase-3/7 assay, a two-way analysis of variance (ANOVA, two-factor repetition) was used to determine whether time and concentration had an effect on cell viability and caspase-3/7 activity, and pair-wise multiple comparisons procedures (Hom-Sidak method) were performed for *post hoc* analysis. To account for changes in cell number which may influence

levels of apoptosis, the reading was normalized to the cell viability of non-untreated cells at the same time-point under investigation. For the VEGF assay, a one-way ANOVA was used to determine if time had an effect on median VEGF concentrations of controls incubated for 24, 48 and 72 h. A two-way ANOVA was then used to analyse if *Yunnan Baiyao* concentration had an effect on mean VEGF levels for all three cell lines treated at 72 h. To account for changes in cell number which may influence VEGF levels, the reading was normalized to the cell viability of non-treated cells at the same time-point under investigation. Overall significance was set at  $P = 0.05$ .

## Results

### Effects of *Yunnan Baiyao* on cell viability

For all three canine HSA cell lines, cell viability decreased after incubation with higher concentrations of *Yunnan Baiyao* at 24, 48 and 72 h. (see Fig. 1A–C). For the DEN cell line, a significant decrease in cell viability was found at  $\geq 400 \mu\text{g mL}^{-1}$  concentrations at 24 h, and at  $\geq 200 \mu\text{g mL}^{-1}$  concentrations at 48 and 72 h ( $P < 0.001$ ). For the Fitz cell line, a significant decrease in cell viability was found at  $\geq 400 \mu\text{g mL}^{-1}$  concentrations at 24 and 48 h, and at  $\geq 200 \mu\text{g mL}^{-1}$  concentrations at 72 h ( $P < 0.001$ ). For the SB cell line, a significant decrease in cell viability was found at  $\geq 400 \mu\text{g mL}^{-1}$  concentrations at 24 and 48 h, and at  $\geq 200 \mu\text{g mL}^{-1}$  concentrations at 72 h ( $P < 0.001$ ).

Cell viability data were fitted to a four-equation regression model in order to determine the  $\text{IC}_{50}$  for each cell line (see Table 1). The  $\text{IC}_{50}$  values at 72 h were 275.9 and 325.3  $\mu\text{g mL}^{-1}$  for the Fitz and SB cell lines, respectively. The  $\text{IC}_{50}$  was slightly higher at 369.3  $\mu\text{g mL}^{-1}$  for the DEN cell line at 72 h. The correlation coefficient or  $R^2$  value was evaluated to determine the goodness of fit of the derived values for each dose response curve. The mean  $R^2$  value for DEN, Fitz and SB was 0.98 at 72 h where unity is considered a perfect correlation.

The duration of *Yunnan Baiyao* incubation time (24, 48 and 72 h) was found to be a significant factor ( $P < 0.001$ ) in mean cell viability for all three cell lines, with the proportion of cell viability of *Yunnan Baiyao* treated cells to cell viability of the

control samples decreasing with time. Time was found to be a significant factor for concentrations  $\leq 200 \mu\text{g mL}^{-1}$  for the DEN and Fitz cell lines and at  $\leq 100 \mu\text{g mL}^{-1}$  for the SB cell line. Time was no longer a factor at concentrations  $\geq 400 \mu\text{g mL}^{-1}$  for all three cell lines.

### Effects of *Yunnan Baiyao* on apoptosis

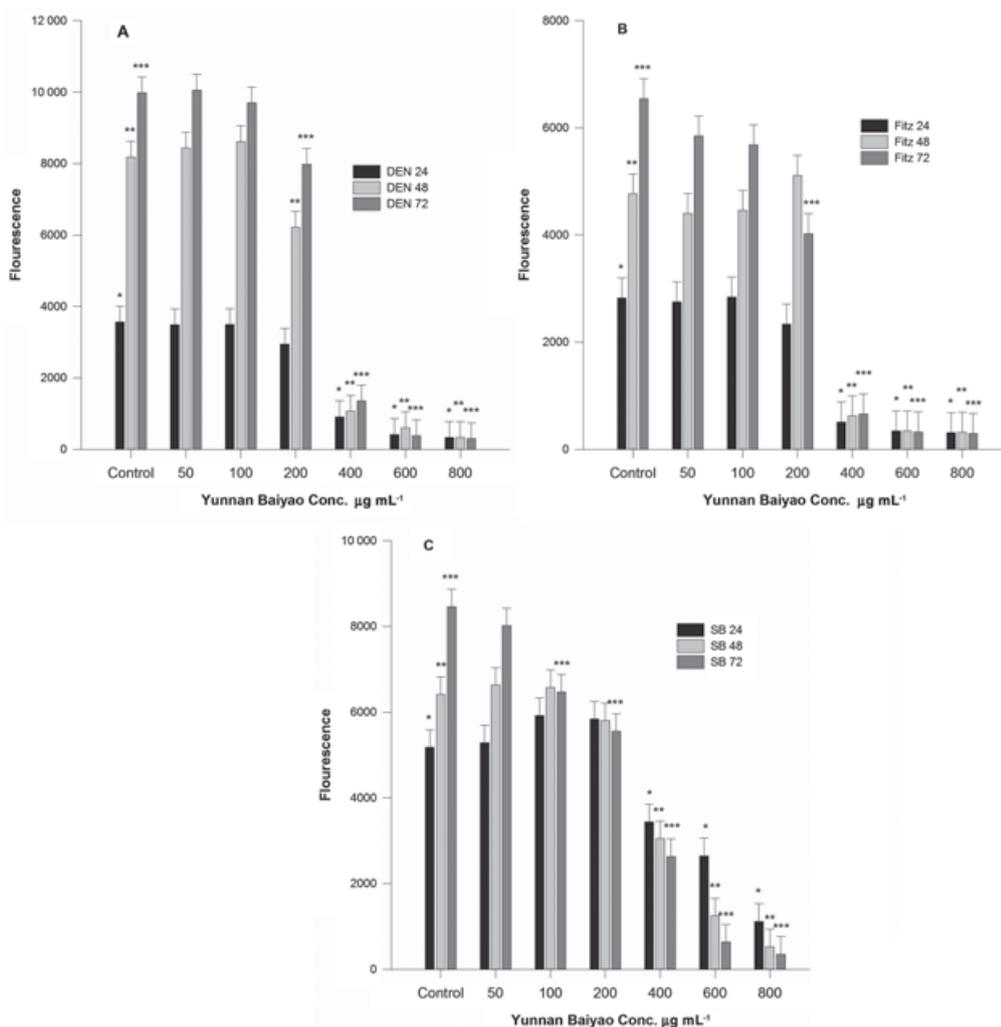
#### Caspase-3/7

Overall, the duration of *Yunnan Baiyao* incubation time and concentration were significant ( $P < 0.001$ ) factors in the mean caspase-3/7 activity (apoptosis) for all cell lines (see Fig. 2A–C). For the DEN cell line, significant increases in caspase-3/7 were found at  $\geq 400 \mu\text{g mL}^{-1}$  concentrations ( $P < 0.001$ ) at 24, 48 and 72 h. For the Fitz cell line, significant increases in caspase-3/7 activity were found at  $\geq 400 \mu\text{g mL}^{-1}$  for 24 and 48 h, and at  $\geq 200 \mu\text{g mL}^{-1}$  at 72 h ( $P < 0.001$ ). For the SB cell line, significant increases in caspase-3/7 activity were found at  $\geq 600 \mu\text{g mL}^{-1}$  for 24 h,  $\geq 400 \mu\text{g mL}^{-1}$  at 48 h, and at  $\geq 200 \mu\text{g mL}^{-1}$  at 72 h ( $P < 0.001$ ). This suggests that the SB cell line may be more sensitive to the effects of *Yunnan Baiyao* than the other two cells lines.

Of note is that the caspase-3/7 activity relative to the number of viable cells increased significantly compared with the control sample at close approximation with the  $\text{IC}_{50}$  of each cell line (see Table 1 and Fig. 2A–C). The duration of incubation time with *Yunnan Baiyao* was also a significant factor in caspase-3/7 activity in all three cell lines ( $P < 0.001$ ).

#### TUNEL assay

Flow cytometry was used to detect the number of TUNEL-positive cells as a measure of percentage of apoptotic cells in the population (see Table 2 and Fig. 3). Samples for all three cell lines at 24, 48 and 72 h were pooled and the TUNEL assay was performed as a single experiment in order to minimize cost and sample processing time. No appreciable levels of apoptosis were noted in the control samples or in cells treated at *Yunnan Baiyao* concentrations  $\leq 100 \mu\text{g mL}^{-1}$ . However, there was a statistically significant ( $P < 0.001$ ) increase in the percentage of apoptotic cells at concentrations  $\geq 200 \mu\text{g mL}^{-1}$  for all cell lines incubated for 72 h (see Table 2). Specifically, at the 200  $\mu\text{g mL}^{-1}$



**Figure 1.** *Yunnan Baiyao* causes a concentration dependant decrease in HSA cell viability over time as measured by the CellTiter-Blue Cell Viability Assay. An increase in fluorescent signal is correlated with an increase in viable cells. Control samples are designated as 24 h (\*), 48 h (\*\*) and 72 h (\*\*\*). Error bars represent standard deviation (SD). A statistically significant decrease in cell viability compared with untreated control sample at the corresponding time point is represented on the graph by \*, \*\* and \*\*\* for 24, 48 and 72 h, respectively. (A) DEN cell line treated with increasing concentrations of *Yunnan Baiyao*. (B) Fitz cell line treated with increasing concentrations of *Yunnan Baiyao*. (C) SB cell line treated with increasing concentrations of *Yunnan Baiyao*.

concentration the percentage of apoptotic cells (TUNEL-positive) was 19.63, 56.34 and 86.47% for DEN, Fitz and SB, respectively. The percentage increased to 90.63, 99.69 and 98.98% for DEN, Fitz, and SB (respectively) at the 400  $\mu\text{g mL}^{-1}$  concentration; then remained high at 600 and 800  $\mu\text{g mL}^{-1}$  (see Table 2). Curiously, there was also a statistically significant ( $P < 0.001$ ) increase in percentage of apoptotic cells at the 50 and 100  $\mu\text{g mL}^{-1}$  concentrations at 24 h for the SB cell line which was not noted in the other cell lines (data not shown).

Overall, the greatest percentage change in apoptosis occurred between 200 and 400  $\mu\text{g mL}^{-1}$  for DEN, Fitz and SB which correlates with the calculated  $\text{IC}_{50}$  for all three cell lines (see Table 1 and Fig. 3).

### Cell cycle analysis

Cell cycle analysis was performed on data recorded for all three cell lines at 24, 48 and 72 h (see Fig. 4A–C). The DEN cell line (see Fig. 4A) when

**Table 1.** Cell viability data were fitted to a four-equation regression model in order to determine the IC<sub>50</sub> for each cell line

Cell line	24 h	48 h	72 h
DEN ( $\mu\text{g mL}^{-1}$ )	313.4	313.4	369.3
Fitz ( $\mu\text{g mL}^{-1}$ )	356.5	285.8	275.9
SB ( $\mu\text{g mL}^{-1}$ )	497.6	414.4	325.3

The IC<sub>50</sub> for the Fitz and SB cell lines decreased with increasing exposure time to *Yunnan Baiyao*. The IC<sub>50</sub> for the DEN cell line slightly increased at 72 h when compared with the 24 and 48 h time points.

compared with control cells at 24 and 48 h, showed a moderate increase in G1- and G2-phases and a corresponding decrease in S-phase at concentrations ranging from 50 to 400  $\mu\text{g mL}^{-1}$ . In contrast, at 72 h the DEN cell line showed moderate decreases in G1- and G2-phases with a corresponding increase in S-phase at concentrations ranging from 50 to 400  $\mu\text{g mL}^{-1}$ . Both Fitz and SB cell lines showed moderate increases in G2-phase compared with control cells at all time points at concentrations ranging from 50 to 400  $\mu\text{g mL}^{-1}$ , with G1-phase being the predominant cell phase.

Remarkably, all cell lines at concentrations >200–400  $\mu\text{g mL}^{-1}$  and at all time points the cell phases disappeared and were replaced by DNA debris (see Fig. 4A–C). This DNA debris, found to the left of the G1 peak in the cell cycle histogram, is considered a sign of late apoptosis due to endonuclease cleaving of DNA. This is an unusual finding, but consistent with the TUNEL assay's recording of virtually 100% (see Fig. 3) apoptotic cells at concentrations >200–400  $\mu\text{g mL}^{-1}$ .

### Effects of *Yunnan Baiyao* on VEGF concentrations

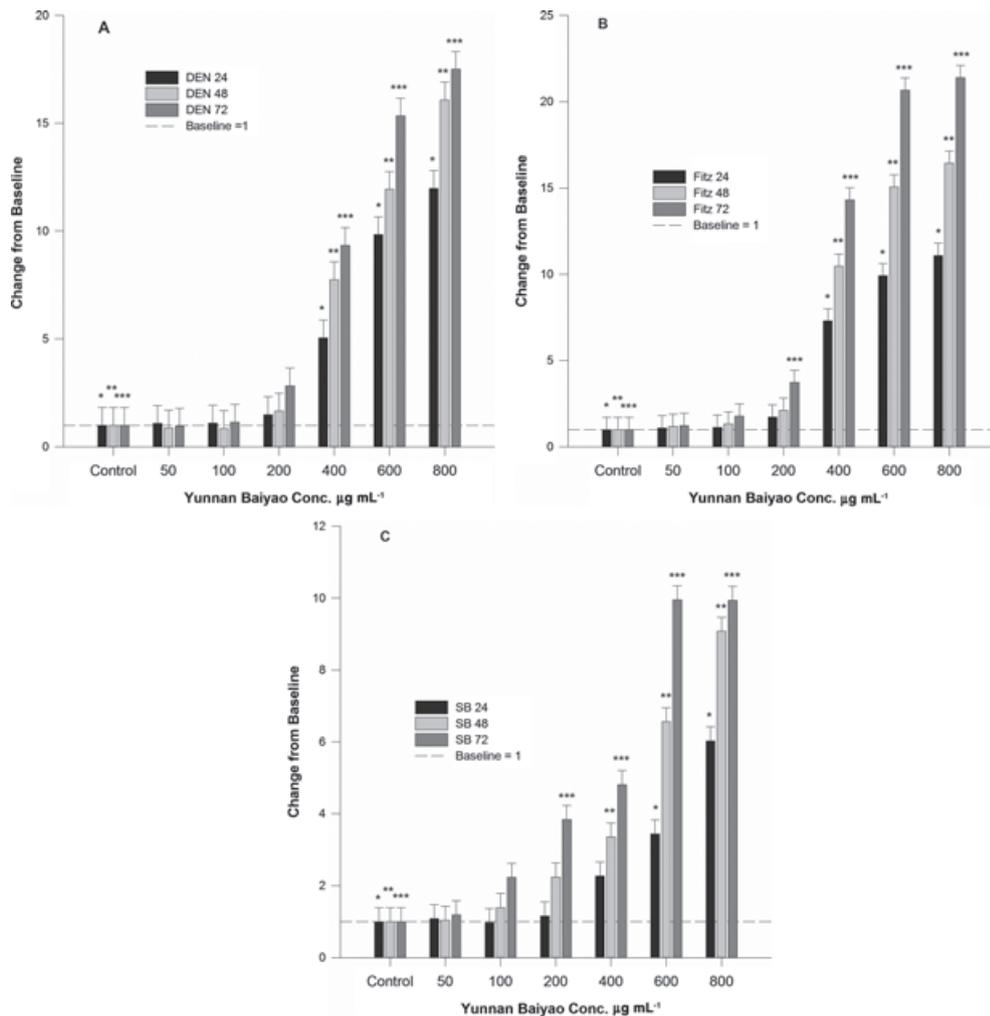
In the DEN, Fitz and SB cell lines, the untreated (control) cells expressed increasing levels of VEGF over time (see Table 3). The DEN cell line expressed the highest levels of VEGF followed by Fitz and then SB at all time points. Of note is that the untreated SB cells expressed a VEGF level that remained below the detectable threshold of the standard curve (<19.5  $\text{pg mL}^{-1}$ ) at 24 h.

VEGF levels were only measured in cells that were treated with increasing concentrations of *Yunnan Baiyao* at 72 h because of the cost of test kits.

To account for changes in cell number that may influence VEGF levels, the reading was normalized to the cell viability of non-treated cells at the same time-point under investigation (see Fig. 5). No significant VEGF changes from baseline were found for the DEN cell line at 72 h for any concentration of *Yunnan Baiyao*. The SB cell line showed a statistically ( $P < 0.001$ ) significant fold ( $\times$ ) increase from baseline in VEGF levels at 50 ( $\times 2.9 \pm 1.1$ ), 100 ( $\times 64.5 \pm 3.7$ ), 200 ( $\times 19.1 \pm 1.9$ ) and 600  $\mu\text{g mL}^{-1}$  ( $\times 2.9 \pm 0.4$ ) of *Yunnan Baiyao*. The maximum increase in VEGF concentration by the SB cell line at 100  $\mu\text{g mL}^{-1}$ , was  $3170 \pm 0$   $\text{pg mL}^{-1}$ , which was significant considering the control cell concentration was only  $58.1 \pm 2.2$   $\text{pg mL}^{-1}$  (see Table 3). The Fitz cell line also showed a statistically ( $P < 0.001$ ) significant fold increase from baseline in VEGF levels when compared with control cells at 100 ( $\times 3.7 \pm 0.1$ ), 200 ( $\times 4.5 \pm 0.1$ ), 600 ( $\times 3.8 \pm 0.8$ ) and 800  $\mu\text{g mL}^{-1}$  ( $\times 4.0 \pm 0.3$ ) of *Yunnan Baiyao*. Nevertheless, the fold increase for Fitz was lower when compared with the SB cell line. Both SB and the Fitz cell line lacked statistical significance at 400  $\mu\text{g mL}^{-1}$  of *Yunnan Baiyao*. The SB cell line also lacked significance at 800  $\mu\text{g mL}^{-1}$  which was due to the wide standard deviation from the mean (see Fig. 5).

### Discussion

This study showed that *Yunnan Baiyao* causes time and concentration dependant death of canine HSA cells. The Cell Titer Blue results showed a decrease in cell viability with increasing concentrations of *Yunnan Baiyao*. Results from the APO-ONE caspase-3/7 and TUNEL assays suggested that this decrease in cell viability occurred due to apoptosis. Caspase-3 activation occurs downstream of both the extrinsic and intrinsic apoptotic pathways; thus, should reflect the amount of apoptosis occurring regardless of the pathway. In this study, caspase-3/7 activity was shown to increase in correlation with the IC<sub>50</sub> consistently in each cell line which was confirmed by the TUNEL assay. These results suggest that caspase-mediated apoptosis is a mechanism of cell death in all three cell lines. The TUNEL assay showed an increase in the percentage of cells undergoing apoptosis as



**Figure 2.** *Yunnan Baiyao* causes a concentration dependant increase in caspase-3/7 activity in HSA cells over time as measured by the Apo-ONE Homogenous Caspase-3/7 Assay. An increase in fluorescent signal is correlated with an increase in caspase-3/7 activity which is an important signalling and effector step in the apoptotic cascade. The results are expressed as a ratio of change compared with the baseline apoptosis measured in the control at 24, 48 and 72 h (dotted line represents baseline of 1). Control samples are designated as 24 h (\*), 48 h (\*\*) and 72 h (\*\*\*). Error bars represent standard deviation (SD). A statistically significant increase in caspase-3/7 activity compared with the untreated control sample at the corresponding time point is represented on the graph by \*, \*\* and \*\*\* for 24, 48 and 72 h, respectively. (A) Level of apoptosis measured in the DEN cells treated with increasing concentrations of *Yunnan Baiyao*. (B) Level of apoptosis measured in the Fitz cells treated with increasing concentrations of *Yunnan Baiyao*. (C) Level of apoptosis measured in the SB cells treated with increasing concentrations of *Yunnan Baiyao*.

the concentration increases in correlation with the APO-ONE caspase-3/7 results. This suggests that later mechanisms in the apoptotic cascade, such as DNA fragmentation are also involved in inhibition of HSA cell growth by *Yunnan Baiyao*. The mechanism by which *Yunnan Baiyao* causes apoptosis has not been elucidated. It is possible that *Yunnan Baiyao* could cause blockage of a receptor that triggers initiation of apoptotic pathways

through downregulation of anti-apoptotic factors or upregulation of apoptotic factors which has been previously shown with *P. notoginseng*.<sup>33</sup> Another possibility is that it may directly alter downstream signalling proteins in the apoptotic pathway.

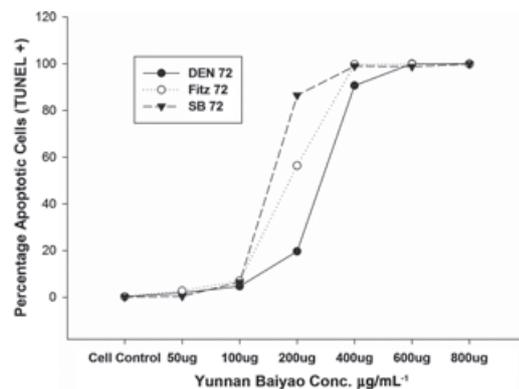
Cell cycle analysis did show some minor change in cell cycle kinetics. The changes were not inconsistent with normal cell cycling. No evidence was found to indicate cell cycle arrest was present in

**Table 2.** This table demonstrates the percentage of apoptotic cells as detected by the APO-BRDU Kit for cells incubated for 72 h at increasing *Yunnan Baiyao* concentrations

<i>Yunnan Baiyao</i> concentration (mg mL <sup>-1</sup> )	Den (%)	Fitz (%)	SB (%)
0	0.29	0.20	0.08
50	1.91	2.76	0.41
100	4.68	7.01	6.47
200	19.63 <sup>a</sup>	56.34 <sup>a</sup>	86.47 <sup>a</sup>
400	90.63 <sup>a</sup>	99.69 <sup>a</sup>	98.98 <sup>a</sup>
600	100.00 <sup>a</sup>	99.81 <sup>a</sup>	98.78 <sup>a</sup>
800	100.00 <sup>a</sup>	99.78 <sup>a</sup>	99.84 <sup>a</sup>

A significant increase in apoptosis occurred at *Yunnan Baiyao* concentrations  $\geq 200 \mu\text{g mL}^{-1}$  in each cell line (DEN, Fitz and SB).

<sup>a</sup>Represents a statistically significant increase in apoptotic cells compared with the untreated control sample.

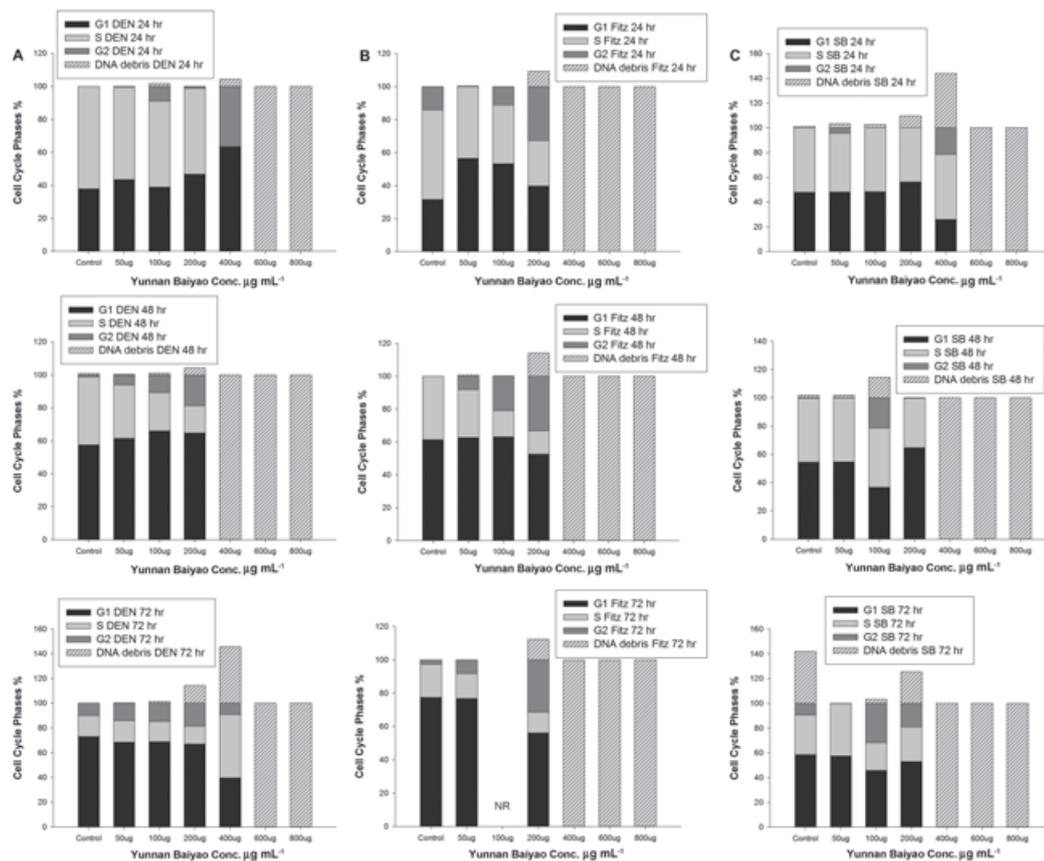


**Figure 3.** This graph demonstrates the percentage of apoptotic cells as detected by the APO-BRDU Kit for cells incubated for 72 h at increasing *Yunnan Baiyao* concentrations. A significant increase in apoptosis occurred at *Yunnan Baiyao* concentrations  $\geq 200 \mu\text{g mL}^{-1}$  in each cell line (DEN, Fitz and SB).

either G1- or G2-phases of the cell cycle. This differs from previous data which showed that wild yam root arrested cells in the G2/M phase.<sup>36</sup> This may be due to the fact that *Yunnan Baiyao* consists of a combination of herbs which has different bioactivity than the individual components. Nonetheless all three cell lines at concentrations  $>200\text{--}400 \mu\text{g mL}^{-1}$  and at all time points showed induction of apoptosis (DNA debris) to the exclusions of all cell phases (see Fig. 4A–C). This DNA debris, found to the left of the G1 peak in the cell cycle histogram, considered a sign of late

apoptosis is due to endonuclease cleaving of DNA. This unusual finding, was supported by the TUNEL assay's recording of virtually 100% (see Fig. 3) apoptotic cells at concentrations  $>200\text{--}400 \mu\text{g mL}^{-1}$ . These cells were still metabolically active since Cell Titer Blue activity was recorded for all cell lines at concentrations  $>200\text{--}400 \mu\text{g mL}^{-1}$ , although at significantly reduced levels (see Fig. 1A–C). Further investigation is required to explain the rapid induction of nuclear endonuclease activity by *Yunnan Baiyao*.

VEGF levels in cell supernatant were measured in untreated (control) cells and found to increase over time for all three cell lines. Although the VEGF levels for SB were negligible at 24 and 48 h, it was significant at 72 h (see Table 3). We have previously reported *in vitro* VEGF concentrations for these cell lines at 24, 48 and 72 h and the findings from this study are consistent with the variation found in our previous report.<sup>41</sup> We then evaluated the ability of *Yunnan Baiyao* to modulate VEGF levels in the cell supernatant for all three cell lines at 72 h. *Yunnan Baiyao* did cause some significant increases in VEGF levels in Fitz and SB cell lines, but not in the DEN cell line (see Fig. 5). The increases in VEGF occurred at concentrations of *Yunnan Baiyao* that were approaching the IC<sub>50</sub> for Fitz and SB, namely 275.9 and 325.3  $\mu\text{g mL}^{-1}$ , respectively. Moreover, these concentrations of *Yunnan Baiyao* were also consistent with the induction of apoptosis in the cell lines. These findings are not unlike our previous findings from a report that showed the induction of VEGF when mastinib concentrations approached the IC<sub>50</sub> for Fitz and SB.<sup>41</sup> However, in the report by Lyles *et al.* the DEN cell line was marginally affected, but similar to this study the SB cell showed the greatest fold increase of VEGF. This needs to be investigated further by examining the effects of these drugs on cellular pathways involved in VEGF signalling and production, e.g. hypoxia inducible factor 1 $\alpha$  (HIF1 $\alpha$ ). Interestingly, human cancer patients treated with anti-angiogenic tyrosine kinase inhibitors show increased plasma levels of VEGF and placental growth factor in the face of clinical efficacy.<sup>42</sup> The relationship between cell supernatant concentration and *in vivo* plasma concentration of VEGF is not clear. In the study by Clifford C *et al.*, median VEGF concentrations actually



**Figure 4.** Cell cycle analysis was performed using flow cytometry and propidium iodide counter staining and data were recorded for all three cell lines at 24, 48 and 72 h. Remarkably, all cell lines at concentrations >200–400 µg mL<sup>-1</sup> and at all time points the cell phases disappeared and were replaced by DNA debris. This DNA debris is considered a sign of late apoptosis due to endonuclease cleaving of DNA which correlates with the noted increase in caspase-3/7 activity. (A) Cell cycle kinetics measured in the DEN cells treated with increasing concentrations of *Yunnan Baiyao*. (B) Cell cycle kinetics measured in the Fitz cells treated with increasing concentrations of *Yunnan Baiyao*. (C) Cell cycle kinetics measured in the SB cells treated with increasing concentrations of *Yunnan Baiyao*.

decreased with increasing stage of disease and 4 of 17 dogs with HSA did not have detectable VEGF levels in the plasma.<sup>38</sup> This may be due to the fact that VEGF can differ within the tumour versus in circulation or this may not be the primary factor involved with progression of HSA in dogs.

This is the first documentation of *Yunnan Baiyao*'s ability to cause a decrease in cell viability via apoptosis in canine HSA cells. It lends evidence to the anecdotally reported improvement in survival times in canine patients with HSA receiving this medication.

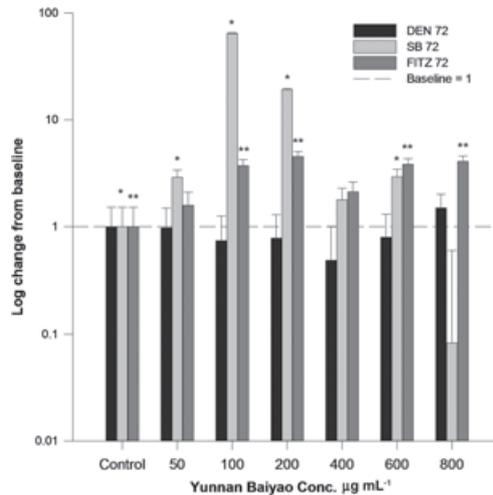
Pharmacokinetic studies on *Yunnan Baiyao* itself have not been performed; however, studies of the major component, *P. notoginseng*, have been performed. A pharmacokinetic study of intravenous

**Table 3.** The untreated (control) cells expressed increasing levels of VEGF over time

Mean VEGF (pg mL <sup>-1</sup> )	DEN	Fitz	SB
24 h	437.0 ± 24.3	107.7 ± 2.0	0 ± 0
48 h	1192.6 ± 34.9	241.7 ± 3.1	25.7 ± 16.8
72 h	2532.7 ± 86.9	392.0 ± 5.0	58.1 ± 2.2

All samples were run in duplicate and the mean VEGF values ± standard deviation are reported here. VEGF levels were only statistically increased for the 72 h time point compared with the 24 h baseline (\*P < 0.001).

panaxatrol disuccinate sodium, a ginsenoside derivative, was performed in healthy human volunteers and human patients with advanced solid tumours. The steady-state peak concentration,



**Figure 5.** VEGF levels were measured in canine HSA cell supernatant after treatment with increasing concentrations of *Yunnan Baiyao* at 72 h. A logarithmic scale has been used due to the wide variation in VEGF levels among cell lines (dotted line represents baseline of one). The DEN cell line had no significant increases or decreases ( $P < 0.001$ ) in VEGF levels. Significant increases ( $P < 0.001$ ) in VEGF levels were found in the Fitz (\*\*) cells treated with 100, 200, 600 and 800  $\mu\text{g mL}^{-1}$  *Yunnan Baiyao*, and for the SB (\*) cells with 50, 100, 200 and 600  $\mu\text{g mL}^{-1}$  *Yunnan Baiyao*.

average concentration and mean steady state AUC in plasma were  $13.96 \pm 15.48$ ,  $0.15 \pm 0.29$  and  $148.00 \pm 117.18 \text{ mg L}^{-1}$ , respectively. An intravenous injection at a dose of  $100 \text{ mg m}^{-2}$  has been suggested for further phase II clinical trials.<sup>43</sup> We can not necessarily correlate what is achievable *in vitro* to *in vivo* availability based upon our study as we are not examining an individual component of *Yunnan Baiyao*. However; the average  $\text{IC}_{50}$  for the three cell lines across the three time points (24, 48 and 72 h) was  $350.17 \mu\text{g mL}^{-1}$  (equal to  $350.17 \text{ mg L}^{-1}$ ). This value does exceed the above noted steady state peak concentration and average concentration in plasma but is in a similar range with the mean steady state concentration achieved in plasma.<sup>43</sup> On the basis of dosing of other chemotherapeutic medications in veterinary medicine and the results of this *in vitro* study, this would appear to be a clinically attainable dose in the canine patient. It should also be noted that the  $\text{IC}_{50}$  data presented here is based on the entire *Yunnan Baiyao* compound and separation of the individual ingredients is more likely to result in even more comparable data. Pharmacokinetic

studies in canine patients on the individual components as well as whole compound *Yunnan Baiyao* are needed to have a better understanding of clinically achievable levels. Ginsenosides have also been identified as pharmacokinetic markers in the serum of rats after oral administration of *P. notoginseng*.<sup>44</sup> *Panax notoginseng* may serve as a marker of *Yunnan Baiyao* plasma concentration in the future.

Novel medications for the treatment of canine HSA are needed and Chinese herbal medications are being studied at an increasing rate for the purposes of cancer treatment in people. Increased demand for herbal medications worldwide as well as voluntary use of Good Agricultural Practice (GAP) has advanced knowledge as well as safety of these medications.<sup>31</sup> A nutrient and metal analysis on various marketed herbal products showed that contaminants such as Ni, Pb and Cd were equal to or lower than previously reported. Concentrations of these minerals were also below National Research Council proposed tolerances at recommended dosing.<sup>45</sup> Another study performed HPLC specifically on different *Yunnan Baiyao* batch preparations and showed that the total content of 13 saponins varied insignificantly (<4.78%) for different batches of powder and capsule forms when purchased from the *Yunnan Baiyao* Group.<sup>46</sup> On the basis of these studies, *Yunnan Baiyao* also appears to be a safe medication for further study in the canine and human patient.

In conclusion, *Yunnan Baiyao* induces both time-dependant and concentration-dependant cell death through apoptosis in canine HSA cells *in vitro*. This is the first study to document *Yunnan Baiyao*'s ability to induce apoptosis in canine HSA cells and the associated  $\text{IC}_{50}$  values. VEGF expression was also documented in untreated (control) and treated HSA cells. The information gained from this study supports the further investigation of *Yunnan Baiyao* in treatment of canine HSA in the laboratory and clinical settings.

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### Conflicts of interest

The authors have declared no conflicting interests.

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