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Bloodroot (*Sanguinaria canadensis* L., Papaveraceae) Enhances Proliferation and Cytokine Production by Human Peripheral Blood Mononuclear Cells in an *In Vitro* Model

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Abstract

Previous studies have suggested that phytomedicinal preparations from bloodroot (*Sanguinaria canadensis* L.) may harbor immunomodulatory properties. The purpose of this investigation was to determine the effects of alcohol tinctures and water infusions generated from bloodroot flowers, leaves, rhizomes, and roots on human peripheral blood mononuclear cell (PBMC) cytokine production and proliferation in vitro. PBMCs were collected from 16 healthy young adults and cultured with bloodroot extracts or respective controls for interleukins-1 β , -2, -8, -10, interferon- γ , and tumor necrosis factor. Proliferative capabilities of both PBMCs and K562 cells (an immortalized human myelogenous leukemia cell line) following extract treatment were determined. High-pressure liquid chromatography was used to quantify berberine, chelerythrine, and sanguinarine in the extracts and to correlate extract composition with observed effects. Overall, infusions demonstrated greater immunomodulatory capabilities than tinctures, and flower- and root-based extracts showed greater immunomodulatory properties than leaf- or rhizome-based extracts (some effects seen with root-based extracts may be due to endotoxin). Several extracts were able to augment PBMC proliferation and diminish K562 proliferation, suggesting a selective anti-carcinogenic activity. The rhizome alcohol tincture had a markedly stronger effect against K562 cells than other extracts. Chelerythrine, sanguinarine, and endotoxin (but not berberine) sometimes correlated with observed effects. The in vitro activities demonstrated here suggest bloodroot extracts may have potential as therapeutic immunomodulators.

Keywords

Cancer; chelerythrine; interferon; interleukin; medicinal plant; sanguinarine; *Sanguinaria canadensis*; tumor necrosis factor; Papaveraceae

Introduction

Bloodroot (*Sanguinaria canadensis* L., Papaveraceae) is a plant indigenous to the central and eastern United States. Native Americans had several uses for bloodroot, including use as dyes and medicines. Today, this plant is most widely used in dental care products as a remineralizing or antigingival agent (14,23), though some note an association between use and oral malignancies, such as leukoplakia (1).

Benzophenanthridine alkaloids are believed to be the primary bioactive components in *Sanguinaria* spp. (12), including sanguinarine, berberine, chelerythrine, and other related compounds. A diversity of chemical methods have been used in the past for profiling bloodroot extracts including high-performance liquid chromatography (HPLC) (21), capillary electrophoresis (22), and fast atom bombardment (FAB) mass spectrometry (2). In bloodroot, sanguinarine levels are highest in the rhizomes, less in roots, and lowest in flowers and leaves (5).

Almost all investigations have suggested sanguinarine is the most bioactive of the alkaloid group. Several medical uses for bloodroot appear to be substantiated by *in vitro* models. Most of these studies used sanguinarine (13-methyl[1,3]benzodioxolo[5,6-c]-1,3-dioxolo[4,5-i]phenanthridinium) in isolation, rather than whole-plant preparations. Sanguinarine has demonstrated anti-cancer activities *in vitro*, due to the ability to inhibit proliferation or activate components of apoptotic pathways (6,11,13,15,19). Sanguinarine also shows anti-angiogenic (3,9), anti-inflammatory (7,8), anti-platelet (17), and antimicrobial properties (4). Fewer studies have examined extracts made from whole plants rather than isolated compounds, and possibly that combinations of compounds may collectively exert different effects than lone compounds. Bloodroot extracts have demonstrated anti-inflammatory activity under *in vitro* conditions through their ability to inhibit leukotriene and prostaglandin production by lipoxygenases and cyclooxygenases (16). One study showed that a fluid extract of bloodroot containing 30–35% sanguinarine did not alter viability of leukocytes but did inhibit chemotactic response to fMLP (a synthetic peptide consisting of formyl-met-leu-phe) and the number of leukocytes in thioglycollate-elicited inflammatory exudate from rat peritonea (18).

The purpose of the present investigation was to explore the effects of bloodroot extracts on peripheral blood mononuclear cells (PBMCs) collected from young human adult donors. PBMCs were stimulated *in vitro* with extracts and observed for effects on proliferation and cytokine production compared to solvent vehicle controls. Additionally, tumor-killing capacity of the extracts was assessed. HPLC was performed on all extracts to determine concentrations of three bioactive alkaloids: berberine, chelerythrine, and sanguinarine. Results from the immunomodulatory assays were then compared to the phytochemical data to examine potential correlations between extract composition and observed bioactivity.

Materials and Methods

Plant material

Specimens of bloodroot, *Sanguinaria canadensis* L. (Papaveraceae), were harvested with root bundles intact from East River Valley Access, Ames, Iowa, in late April, 2006, as the plants were flowering. Recent research has suggested that bioactive molecules, such as alkaloids, are at their highest concentrations mid-spring (10). The whole plants were transferred to the laboratory within 15 min after harvest and refrigerated briefly before separation of the plants into the four organ parts, flower, leaf, rhizome, and root, and were then extracted. The plant material was diced with a surgical scalpel and then incubated with the solvent at a ratio of 1:4 (plant:solvent, weight:volume) for 1 h under agitation on a horizontal shaker, followed by filtering through sterilized tulle to remove particulate matter. Three different types of extract

were prepared from each organ. The solvent for the alcohol tincture was 50% ethanol and 50% Nanopure water (Gemini System; Aries Filter Works; West Berlin, NJ). Room temperature Nanopure water was used as the solvent for the cold water infusion, whereas near-boiling Nanopure water was used as the solvent for the hot water infusion. Following filtering, extracts were stored at -80°C for 3 d prior to use in immune assays.

HPLC analysis of alkaloid content

Phosphoric acid, triethylamine, sanguinarine, berberine, and chelerythrine were purchased from Sigma Chemical Company (St. Louis, MO). HPLC grade acetonitrile and water were obtained from Fisher. The analysis and quantification of sanguinarine, berberine, and chelerythrine from extracts was performed using a Hewlett Packard 1090 HP liquid chromatograph using an internal diode array detector with detection at a wavelength of 280 nm. The column used was a Phenomenex C-18 ProdigyTM-RP (5 μ , 250 $\times$ 4.6mm ID). The solvents used during chromatography were A-10mM triethylamine in 25% acetonitrile (v:v) in HPLC water pH 2.5 and B-10 mM triethylamine in 90% acetonitrile (v:v) in HPLC water pH 2.5. The elution profile was as follows: 5% B for 5 min, 5–25% B for 15 min, 25–100% B for 5 min, 100% B for 5 min, 100–5% B for 5 min. The flow rate was 1 mL min⁻¹ and the injection volume was 50 μ L. Each sample was diluted 1:9 in solvent A. To identify peaks, standards were run before every other sample. Quantification of each peak was determined from a 5-point calibration curve based on standard solutions of alkaloids containing 0.015–5.0mg/mL, with a detection limit of 0.010 mg/mL, prepared in solvent A.

Human subjects and PBMC isolation

All protocols were approved by the Iowa State University Institutional Review Board prior to the experiment and all subjects gave informed consent prior to participation. A total of 16 subjects between the ages of 18 and 34 participated in the study (10 males, 6 females). All of the subjects were healthy (no illnesses within the past two weeks, no chronic illnesses that would influence immune parameters) and none of the subjects reported taking any medications or over-the-counter products that would influence immune parameters in our study. Phlebotomy was performed at the antecubital vein and blood collected in heparinized tubes. Ficoll-Paque plus (Amersham Pharmacia Biotech, Piscataway, NJ) gradient centrifugation was used to isolate PBMCs. Cells were stained with Trypan blue and counted manually using a hemacytometer. All cultures were adjusted to 1.0×10^6 cells mL⁻¹ in AIM-V media (GIBCO/Invitrogen, Carlsbad, CA).

Cell culture and immune assays

Stock bloodroot extracts were diluted in a two-step procedure in all immune assays and K562 anti-proliferation assay. Immediately prior to use, extracts were diluted 1:12.5 in AIM-V media and kept on ice. Extracts were further diluted 1:21 when added to the wells containing cells and medium (50 μ L of extract into 1 mL of cell culture in the case of all cytokine assays, 5 μ L into 100 mL in the case of proliferation assay). In this manner extracts were standardized against each other by final dilution. All cultures were kept under 37 $^{\circ}\text{C}$, 5.0% carbon dioxide in a humidified atmosphere.

For the TNF, IFN- γ , IL-2, and IL-10 cytokine assays, cells were plated in 1mL aliquots in 24-well Costar plates. Cells were challenged with 50 μ L of ethanol solvent control (control for tinctures), media solvent control (control for infusions), or one of the 12 bloodroot extracts (described above), providing 14 wells plated per subject and challenged with each of the 14 conditions. Aliquots of 250 μ L were harvested for assay of cytokine production at different time points, TNF (24 h), IFN- γ (48 h), and IL-2 and IL-10 (72 h). All aliquots were kept frozen at -20°C until testing via ELISA (BD Biosciences Pharmingen, San Diego, CA).

For the IL-1 β and IL-8 cytokine assays, cells were plated as above and challenged with ethanol solvent control or one of the four bloodroot tinctures (described above). Only tinctures were tested due to low cell count. Aliquots of 250 μ L were collected for both IL-1 β and IL-8 at 24 h. The aliquots were frozen until tested via ELISA.

For the PBMC proliferation assay, 100 μ L of cells were plated per well in 96-well flat-bottom Costar plates. Cells were stimulated with 5 μ L of ethanol solvent control, media solvent control, or one of the 12 bloodroot extracts, with all conditions replicated in triplicate. Cultures were grown undisturbed for 4 d and then proliferation was assessed via CellTiter (Promega, Madison, WI) and read in a Bio-Rad plate reader (Hercules, CA).

For the K562 anti-proliferation assay, K562 cells (an immortalized human myelogenous leukemia line) were courteously provided by Dr. Joan E. Cunnick (Iowa State University, Ames, IA) and cultured for 1.5 weeks in CRPMI (500 mL RPMI [Gibco] + 10% FBS [HyClone] + 10 mL penicillin/streptomycin [Sigma]). Cells were washed, counted, and adjusted to 1.0×10^5 in the same media. Aliquots (5 mL) of cells were placed into 25 cm² culture flasks and stimulated with 250 μ L of ethanol solvent control, media solvent control, or one of the 12 bloodroot extracts. Each condition was performed in triplicate, generating a total of 42 flasks. After 48 h in culture, flasks were agitated gently to resuspend cells and a 10 μ L aliquot was withdrawn from each flask. Each aliquot was combined with 40 μ L Trypan blue and 50 μ L of media to generate a diluted sample of which 10 μ L was placed under a hemacytometer for viability counting.

Endotoxin determination

Sterile water (Hospira, Inc., Lake Forest, IL) was used for all extracts and all glassware used in the extraction process was baked at 180°C for 20 h prior to use to minimize endotoxin contamination. Endotoxin levels (endotoxin units, EU mL⁻¹) were determined for stock extracts and sterile water (0 EU mL⁻¹) using Bio-Whittaker QCL 1000 kits (Cambridge, MA).

Statistics

For the TNF, IFN- γ , IL-2, IL-10, PBMC proliferation, and anti-K562 proliferation assays, univariate ANOVA was used to test for main effects due to organ, extract type, or organ $\times$ extract type interactions using SPSS (Chicago, IL). Because only ethanol tinctures were tested in the IL-1 β and IL-8 assays, one-way ANOVA was used for this statistical analysis. When analyses reported significant effects, follow-up posthoc tests (LSD) were done using Bonferroni corrections for multiple comparisons.

To determine correlations between extract phytochemical composition and observed immunomodulatory properties, Pearson correlation coefficients were determined between the phytochemical constituents and the immune variables. If a significant correlation was observed, a linear regression model was used to test the extent to which the immune variable may be affected by endotoxin level and/or by phytochemical constituent. In this stepwise regression, the phytochemical constituent was added in the first step, whereas endotoxin level was added in the second step. If endotoxin accounted for the primary effect on immune variable, the plant constituent would no longer be a significant predictor of the immune variable and instead endotoxin would be statistically significant.

Results

Endotoxin values

Endotoxin levels differed among the stock extracts with the higher levels of endotoxin extracted from roots, especially the cold water extracts (Table 1). For all of the tissues except the leaf,

the water (cold or hot) extract contained more endotoxin than the ethanol extract. The ethanol extract of the leaf contained more endotoxin than the leaf cold water extract. The reported endotoxin values, however, only represent endotoxin levels from stock extracts and are the not final diluted endotoxin levels added to cell cultures.

Phytochemical profiling

HPLC analysis of the stock extracts indicated the presence of the known bioactive compounds (berberine, chelerythrine, and sanguinarine) in extracts from bloodroot flowers, leaves, rhizomes, and roots (Figures 1–4). The flower, leaf, and root extracts collectively contained all three bioactive compounds, but only chelerythrine and sanguinarine were detected in rhizome extracts. Differences among the extraction methods were also apparent with the ethanol extractions, in general, exhibiting the highest levels of the bioactive compounds and the cold water extractions exhibiting the lowest levels. The ethanol extract of the rhizome exhibited the highest concentrations of both chelerythrine and sanguinarine, but was devoid of berberine (Table 2). In the current study, berberine was the least abundant of the three alkaloids, with the highest concentrations being observed in flower-alcohol and root-alcohol extracts.

PBMC cytokine production and proliferation

In vitro cytokine production by human PBMCs cultured individually with each of the bloodroot extracts indicated significant organ, extract type, and organ $\times$ extract type interactions for all four cytokines (all $p < 0.001$). Cells cultured with the alcohol extract from roots demonstrated a statistically significant increase in IL-2 production compared with control wells after 48 h (Figure 5a). Cells cultured with hot-water flower extract, cold-water root extract, and hot-water root extract exhibited statistically significant increases in IL-10 production after 72 h as compared with control ($p \leq 0.002$ for extracts) (Figure 5b), whereas cells cultured with alcohol flower extract and cold-water flower extract showed a trend ($p = 0.01$ and 0.015 , respectively) (Figure 5b). IFN- γ production after 72 h was significantly higher in cells cultured with cold-water flower extract, hot-water flower extract, and alcohol root extract ($p < 0.001$ for all extracts) (Figure 6a). Several cell cultures demonstrated significantly higher production of TNF after 24 h when cultured with various bloodroot extracts (Figure 6b): alcohol, cold-water, and hot-water flower extracts, alcohol leaf extract, cold-water rhizome extract, and cold-water and hot-water root extracts ($p < 0.001$ for all extracts).

In vitro IL-1 β and IL-8 production by human PBMCs cultured with the ethanol extracts only was observed (Figure 7). A significant effect of organ was present for both cytokines (both $p < 0.001$). Cells cultured with extracts from flower or leaf exhibited significantly greater IL-1 β production after 24 h compared to control wells (both $p < 0.001$; Figure 7a). Interleukin-8 production was significantly lower in wells receiving rhizome extract compared to all other conditions ($p < 0.001$; Figure 7b).

In vitro proliferation of human PBMCs cultured for 4 d with each of the bloodroot extracts individually indicated significant organ and organ $\times$ extract interactions ($p < 0.001$) existed, but no significant effects by extract type ($p = 0.152$) (Figure 8). Most of the extracts (all flower extracts, leaf alcohol and hot-water extracts, rhizome cold-water extract, and root cold-water and hot-water extracts) significantly increased proliferation of human PBMCs (all $p \leq 0.001$).

K562 anti-proliferative activities

Proliferation of K562 cells cultured for 48 h with each of the bloodroot extracts individually exhibited significant organ, extract type, and organ $\times$ extract type interactions ($p \leq 0.003$ for all observations) (Figure 9). The alcohol extract of the rhizome significantly diminished K562 cell counts ($p = 0.003$), in fact leaving no visible viable cells in any of the cultures. The flower alcohol and the root alcohol extracts demonstrated a trend towards reducing K562 counts

($p=0.006$ and 0.005 , respectively). Any effects due to leaf alcohol extract were not significant ($p=0.018$).

Correlations and stepwise regression

Pearson correlation coefficients were calculated to determine if a relationship existed between content of the extracts and observed immune outcomes. When a significant correlation was discerned, a linear stepwise regression model was used to gauge the contribution of the various constituents. Chelerythrine and sanguinarine content in the extracts were highly correlated (Pearson correlation coefficient=0.998; $p<0.001$), such that high levels were found together in the same plant part. IL-8 levels appeared to be influenced by chelerythrine and sanguinarine, such that higher levels of either phytochemical were associated with reduced IL-8 production (Figure 7b and Table 3).

Although not statistically significant, TNF production followed a similar pattern such that higher levels of chelerythrine or sanguinarine were associated with reduced TNF. TNF, however, was influenced by endotoxin level in the plant part and therefore a better predictive model of TNF production included both endotoxin and chelerythrine or sanguinarine. In a regression model, inclusion of endotoxin and either phytochemical met statistical significance (chelerythrine: adjusted $r^2=0.338$, $p=0.041$; sanguinarine: adjusted $r^2=0.412$, $p=0.037$). IL-10 production appeared to be influenced by endotoxin level alone and not by chelerythrine or sanguinarine.

Finally, no significant correlation between extract constituents and IL-1 β , IL-2, or IFN- γ was present, suggesting that unidentified constituents in the plant may influence these cytokines. Proliferation of human PBMCs was negatively correlated with chelerythrine (Table 3) but not sanguinarine content. Proliferation of K562 cells was negatively correlated with both chelerythrine and sanguinarine (Table 3).

Discussion

In this study, the cytokine- and proliferation-modulating capabilities of bloodroot extracts in human PBMC and K562 cell cultures was investigated. The endotoxin concentrations in all of the extracts were tested as any contaminating endotoxin could influence *in vitro* immune results. In a previous publication (20), the effects of various concentrations of pure endotoxin in our cell model were demonstrated. A 50 μL aliquot of a solution containing 10 EU/mL added to 1 mL of cells (at 1.0×10^6 cells mL^{-1}) did not influence production of IL-1 β , IL-2, IL-10, or TNF as compared with a control solution containing no endotoxin (a dilution regimen identical to that used in this study). When 50 μL of a solution containing 100 EU/mL was added to 1 mL of cells, however, production of all of the cytokines, except IL-2, increased. Endotoxin concentrations in the flower, leaf, and rhizome bloodroot extracts were at levels too low to influence immune outcomes (for comparison, divide each endotoxin level reported for the stock extracts by 12.5).

In contrast, endotoxin concentrations in the root extracts were much higher. Endotoxin concentrations in the root-alcohol extract were at a quantity (52 EU/mL) where they may or may not be influential (20), whereas levels in the root-cold water and root-hot water extracts (440 and 200 EU mL^{-1} , respectively) were sufficiently great enough to influence the immune parameters. Both roots and rhizomes are belowground parts and the larger concentrations of endotoxin in the root extracts may be due to surface area:volume differences between the roots and rhizomes.

While the rhizomes have a greater circumference than roots, the roots have a greater surface area. Thus, the surface area of the roots potentially correlates with more sites for bacterial

attachment and hence potentially more endotoxin. Data from root-stimulated cultures in this investigation should be considered with caution due to endotoxin concentrations. The correlation tests suggest that the higher levels of endotoxin in root influenced IL-10 production and may also explain, in part, the observed effect on TNF.

TNF and IL-1 β are cytokines associated with the inflammatory cascade and produced principally by macrophages. The strongest effects from the bloodroot extracts were seen with TNF and IL-1 β , but endotoxin may contribute to some of the observed effects. At least one extract from each of the four plant organs stimulated TNF production (Figure 6b), whereas both alcohol flower extract and alcohol leaf extract upregulated IL-1 β production, suggesting that bloodroot extracts stimulate macrophage cytokine production. The quantified phytochemical constituents were inversely related to TNF and IL-1 β , indicating that another constituent not yet identified may contribute to cytokine production by macrophages.

Interleukin-8, associated with inflammation, is produced during the early-phase response, primarily by neutrophils. Bloodroot extracts either had no effect on IL-8 production or, in the case of alcohol rhizome extract, decreased IL-8 production significantly (Figure 7b). The alcohol rhizome extract had the highest level of sanguinarine and chelerythrine, raising the possibility that these constituents act to suppress IL-8 production.

Interleukin-10 inhibits pro-inflammatory cytokine synthesis, primarily by down-regulating macrophage cytokine production, suggesting IL-10 may be produced by T-regulatory cells in an effort to quell immune responses. The IL-10 may also be produced by monocytes and macrophages under certain conditions. In this investigation, both the cold-water and hot-water root extracts greatly augmented IL-10 production (Figure 5b). All three flower extracts also increased IL-10 production, but to a much lesser extent than the cold- and hot-water extracts. The observed correlation between root extracts and IL-10 production suggested that IL-10 is influenced to a great extent by endotoxin level, which was highest in root extracts. Endotoxin is thought to primarily influence IL-10 production in monocytes/macrophages and therefore monocytes/macrophages could be the source of IL-10 in our experiments. The results suggest that if a bloodroot constituent affected IL-10 production in this model, the effect of alkaloids was minimal in comparison to endotoxin. Alkaloids may better account for the IL-10 effects in flower extracts, however.

Similarly, IFN- γ production (associated with activation of CD8-mediated cytotoxic function) was augmented by flower and root extracts, but the flower extracts were more effective than root extracts in augmenting IFN- γ production (Figure 6a). These results suggest that bloodroot flower and root extracts have the ability to modulate T cell cytokine production. IFN- γ production was not correlated with levels of sanguinarine, chelerythrine, berberine, or endotoxin, suggesting that some other phytochemical constituent influences IFN- γ .

Interleukin-2, a cytokine also produced by T cells, did not follow this pattern. Only cultures stimulated with alcohol root extract demonstrated a significant increase in IL-2 production, and this change in IL-2 would be considered very modest (Figure 5a). IL-2 is produced exclusively by T cells in an effort to enhance T cell proliferation. Curiously, whereas only alcohol extract of root stimulated IL-2, several bloodroot extracts demonstrated an ability to enhance PBMC proliferation (Figure 8). Cells cultured in the flower-alcohol, flower-cold water, flower-hot water, leaf-alcohol, leaf-hot water, rhizome-cold water, root-cold water, and root-hot water extracts demonstrated significantly greater proliferation as compared with controls.

Note that alcohol root extract did not significantly increase PBMC proliferation, whereas the cold- and hot-water extracts of root did. This difference in extract effects on PBMC proliferation is interesting since the extracts with the highest concentrations of sanguinarine

and chelerythrine (alcohol and hot water extracts of rhizomes and alcohol extract of root) demonstrated the lowest levels of proliferation, suggesting that other phytochemical constituents may promote proliferation. Given the different responses of IL-2 and proliferation, the findings suggest that the bloodroot extracts that upregulate proliferation may be working through a different proliferative pathway than that mediated by IL-2. Endotoxin did not influence IL-2 or proliferation, a finding that is not surprising given that T-lymphocytes do not respond directly to endotoxin to the same degree as monocytes/macrophages.

Several of the extracts diminished K562 proliferation following 48 h incubation and none of the extracts promoted proliferation (Figure 9). These findings, in comparison with PBMC proliferation, suggest that bloodroot extracts do not influence normal human PBMCs and cancerous cells in a similar manner. Second, these results suggest anti-carcinogenic properties in the bloodroot extracts, consistent with results published by other investigators using isolated compounds (6,11,13,15,19). Thus, whole-organ bloodroot extracts may represent a potential source of future herbal therapies for cancer.

Apparently both the flower and root extracts of bloodroot contained more effective immunomodulatory activity than other extracts. Unfortunately, most previously published reports related to this species use sanguinarine alone, and others do not report from which organ their preparations were gleaned. Thus, comparing results of the current study with those from other laboratories for immunomodulatory activity is difficult. Interestingly, some immunomodulatory effects, such as IFN- γ modulation, appear to be unrelated to levels of sanguinarine, suggesting other plant phytochemical constituents may be important. Other immune responses appear to be decreased by incubation with extracts that contain higher levels of sanguinarine (TNF- α and IL-8), signifying a possible immunosuppressive or anti-inflammatory role for sanguinarine.

Water extracts appeared to be stronger immunomodulators than ethanol-water extracts. No single preparation performed consistently better than others, although flower hot water and root cold water modulated the greatest number of parameters. Cold water extract of leaf and hot water extract of rhizome did not modulate any of the parameters investigated. An alcohol tincture has been reported to demonstrate anti-inflammatory properties by others (16). Considering all the information, difficulty arises in any attempt to conclude that any one extract was more efficacious than others in promoting PBMC cytokine production or proliferation compared with other extracts. The results, however, do suggest that both solvent and organ selection are important in determining outcomes under experimental conditions.

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Figure 1.
HPLC chromatogram for flower extracts.
Chart: A = cold water, B = hot water, C = ethanol.
Abbreviations: Ber = berberine, Che = chelerythrine, San = sanguinarine.

Figure 2.
HPLC chromatogram for leaf extracts.
Chart: A = cold water, B = hot water, C = ethanol.
Abbreviations: Ber = berberine, Che = chelerythrine, San = sanguinarine.

Figure 3.
HPLC chromatogram for rhizome extracts.
Chart: A = cold water, B = hot water, C = ethanol.
Abbreviations: Ber = berberine, Che = chelerythrine, San = sanguinarine.

Figure 4.
HPLC chromatogram for root extracts.
Chart: A = cold water, B = hot water, C = ethanol.
Abbreviations: Ber = berberine, Che = chelerythrine, San = sanguinarine.

Figure 5.

Peripheral blood mononuclear cell production of (a) interleukin-2 and (b) interleukin-10.

Asterisks (*) represent statistical significance ($P \leq 0.05$) as compared with controls; daggers indicate a trend towards significance ($0.05 < P < 0.10$); bars represent standard errors.

Abbreviations: OH = alcohol extract, CW = cold water extract, HW = hot water extract, W = water control.

Figure 6.

Peripheral blood mononuclear cell production of (a) interferon- γ and (b) tumor necrosis factor. Asterisks (*) represent statistical significance ($P \leq 0.05$) as compared with controls; bars represent standard errors.

Abbreviations: OH = alcohol extract, CW = cold water extract, HW = hot water extract, W = water control.

Figure 7. Peripheral blood mononuclear cell production of (a) interleukin-1 β [IL-1 β] and (b) interleukin-8 [IL-8] by alcohol extracts. Asterisks (*) represent statistical significance ($P \leq 0.05$) as compared with controls; bars represent standard errors.

Figure 8.

Peripheral blood mononuclear cell proliferation in an optical impedance assay.

Asterisks represent statistical significance ($P \leq 0.05$) as compared with appropriate controls, bars represent standard errors.

Abbreviations: OH = alcohol extract, CW = cold water extract, HW = hot water extract, W = water control.

Figure 9. K562 anti-proliferative activity as represented by cell counts following treatment. Asterisks represent statistical significance ($P \leq 0.05$) as compared with appropriate controls, bars represent standard errors.

Table 1

Endotoxin levels in bloodroot extracts.

Stock extract	Endotoxin level (EU mL ⁻¹)
Control - ethanol	Below detection limit
Control - water	Below detection limit
Flower - ethanol	6.9
Flower - cold water	13.2
Flower - hot water	26.5
Leaf - ethanol	2.9
Leaf - cold water	2.4
Leaf - hot water	20.1
Rhizome - ethanol	48.3
Rhizome - cold water	200.5
Rhizome - hot water	168.3
Root - ethanol	645.7
Root - cold water	≅5500
Root - hot water	≅2500

Table 2

Concentrations of berberine, chelerythrine, and sanguinarine in bloodroot extracts.

Extract	Berberine	Chelerythrine	Sanguinarine
	-----($\mu\text{g}/5 \mu\text{L}$)-----		
Flower, Cold Water	ND ¹	0.029	ND
Flower, Hot Water	BL ²	0.091	BL
Flower, Ethanol	0.025	0.119	0.054
Leaf, Cold Water	0.023	0.052	0.021
Leaf, Hot Water	BL	0.070	0.076
Leaf, Ethanol	0.037	0.149	0.100
Rhizome, Cold Water	ND	0.136	0.223
Rhizome, Hot Water	ND	0.765	1.431
Rhizome, Ethanol	ND	2.065	3.852
Root, Cold Water	BL	0.050	0.136
Root, Hot Water	BL	0.047	0.061
Root, Ethanol	0.036	0.343	0.601

¹ ND = not detected² BL = below limits of quantification (<0.010 mg/mL)

Table 3

Significant correlations among phytochemical constituents and observed immune effects.

Variables	Pearson correlation coefficient	P-value
Chelerythrine and sanguinarine	−0.998	<0.001
Interleukin-8 and chelerythrine	−0.985	0.015
Interleukin-8 and sanguinarine	−0.990	0.010
Interleukin-10 and endotoxin	0.748	0.005
Tumor necrosis factor and chelerythrine+endotoxin	0.338	0.041
Tumor necrosis factor and sanguinarine+endotoxin	0.412	0.037
PBMC proliferation and chelerythrine	−0.671	0.017
Anti-K562 proliferation and chelerythrine	−0.578	0.049
Anti-K562 proliferation and sanguinarine	−0.580	0.048