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Antioxidant activity of Northern Ontario medicinal plants and their protective effect on the H9c2 cardiovascular cells against hydrogen peroxide mediated oxidative stress

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Abbreviations:
AAPH: 2,2'-Azobis (2-methylpropionamidine) dihydrochloride, ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt), CVD: Cardiovascular diseases, DMEM: Dulbecco's Modified Eagle's Medium, DPPH: 2,2-diphenyl-1-picrylhydrazyl, EC_{50}: half maximal effective concentration, H9c2 cells: rat cardiomyoblast cell line, H2DCFDA: 2',7'-dichlorodihydrofluorescein diacetate, H_2O_2: Hydrogen peroxide, MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, ORAC: Oxygen Radical Absorption Capacity, ROS: Reactive Oxygen Species, µM TE/ gdw: µM Trolox Equivalence/ gram dry weight, mg GAE/ 10 gdw: mg Gallic Acid Equivalence/10 gram dry weight

Keywords:
Medicinal plants; antioxidant activity; H9c2 cardiovascular cells; Northern Ontario; oxidative stress

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Abstract
Increased intracellular oxidative stress generated by the propagation of reactive oxygen species (ROS) has been associated with cardiovascular diseases. In the present investigation, the cardio-protective effect of *Cornus canadensis* L., *Petasites frigidus* (L.) Fr., *Ledum palustre* L. and *Prunella vulgaris* L. northern Ontario medicinal plants on the H9c2 cardiomyocytes against hydrogen peroxide (H_2O_2) mediated oxidative stress was evaluated (*in vitro*). The total phenolic contents and antioxidant activity were also determined. The antioxidant activity is observed in the following hierarchy: *C. canadensis* leaf>*P. vulgaris* leaf>*L. palustre* leaf>*C. canadensis* stem>*P. vulgaris* stem>*L. palustre* stem >*P. frigidus* leaf. A high correlation ($R^2 \geq 0.91$) was observed between the total phenolic contents and antioxidant capacity. Pre-treatment of the H9c2 cells with 100 µg/ml of *P. vulgaris* and *P. frigidus* leaf extracts significantly reduces intracellular ROS levels and prevents cell death from the H_2O_2 treatment. However, the *C. canadensis* and *L. palustre* extract pre-treatment proved lethal to the H9c2 cells. These results indicate that *P. vulgaris* and *P. frigidus* extract pretreatments are biologically tolerant and could protect against cell damage from oxidative stress with potential implications in the food and pharmaceutical industry.

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1. Introduction

1.1 Oxidative stress

Oxidative stress is generated by the propagation of reactive oxygen species (ROS) that exist in various forms, including the superoxide ion ($O_2^{\cdot-}$), hydroxyl radical ($HO^{\cdot}$), hydroperoxyl radical ($HO_2^{\cdot}$), peroxyl radical ($ROO^{\cdot}$), singlet oxygen species ($^1O_2$), alkoxyl radical ($RO^{\cdot}$) and peroxynitrite ion ($ONOO^{-}$) (Teow et al., 2007; Ou et al., 2002). These radical species are the initiators of radical chain reactions known to augment lipid peroxidation, DNA oxidation and protein damage in cells with detrimental effects on the biological system. Accordingly, an extravagance in the intracellular ROS levels has been correlated with the onset of aging, carcinogenesis, cardiovascular diseases, and other chronic disease pathogenesis (Markesbery, 1997; Ou et al., 2012; Schumacker, 2006; Sohal & Weindruch, *1996).*

1.2. Oxidative stress is a mediator of cardiovascular diseases

Cardiovascular disease (CVD) is the leading cause of mortality and disability worldwide (Mensah & Brown, 2007). In the United States, CVD accounted for approximately 800,000 deaths in 2003 and over $400 billion in lost revenue from the associated health care costs and lost productivity (Mensah & Brown, 2007). Over the past few decades, many endeavours in biomedical research have established ROS toxicity as one of the major culprits of cardiovascular dysfunction and abnormality (Kukreja & Hess, 1992). In addition, Zern and Fernandez (2005) reported that the heart tissue is more susceptible to oxidative stress due to the relatively lower expression of the antioxidant enzymes (i.e. glutathione peroxidase and superoxide dismutase). Subsequently, strategic supplementation of the diet with antioxidant rich foods or nutraceuticals has potential in CVD preventative regiments.

1.3 Role of medicinal plants in CVD prevention the rapeutics

Considerable quantities of epidemiologic studies have demonstrated the beneficial effect of phenolic phytochemicals in the prevention and treatment of CVD and coronary diseases (Bouayed et al., 2009; Hertog et al., 1993; Kaur & Kapoor, 2008). Medicinal plants are an excellent resource of phenolic compounds that are predominantly less toxic than their synthetic alternatives (Kahl & Kappus, 1993). Natural products also garner tremendous appeal in the pharmaceutical industry from their environmentally friendly marketable reception. However, despite the abundance of plant resources utilized in traditional medicinal practices in northern Ontario, we found an absence of ethnopharmacological research in this region. Thus, the objective of this study is to evaluate the antioxidant activity and protective effect of northern Ontario medicinal plants against hydrogen peroxide (H_2O_2) induced intracellular oxidative stress in the H9c2 cardiovascular cells.

2. Objective of Research

In the present investigation *Cornus canadensis L., Petasites frigidus* (L.) Fr., *Ledum palustre* L. and *Prunella vulgaris* L. plants were selected from the original list of 48 northern Ontario medicinal plants (Hassan et al., 2012) based on their prescribed anticancer and/ or anti-inflammatory applications in indigenous communities. We report on the total phenolic contents and antioxidant activity of the leaf and/or stemextractsof these plants through the Folin-Ciocalteu, DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt), ORAC (Oxygen radical absorption capacity), and EC_{50} (half maximal effective concentration) assays. Their cytotoxicity to the H9c2 cardiovascular cells upon 24 hours pre-treatment, and protective effect against the H_2O_2 treatment were also evaluated through the *in vitro* MTT (3-(4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) and cell-permeant H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) assays, respectively. Flow cytometer was utilized to count the fluorescently active H9c2 cells in the H_2DCFDA assay. The antioxidant activity of *C. canadensis* and *P. frigidus* plantshas never

been studied. Although the antioxidant activity of *P. vulgaris* (Zhang et al., 2011) and *L. palustre* (Nam, 2006) has been previously reported, geographical variation in the northern Ontario region may be present. Overall, this is the first reporting of the protective effect of *P. vulgaris*, *L. palustre*, *C. canadensis* and *P. frigidus* leaf extracts on H9c2 cardiovascular cells against the H_2O_2 mediated oxidative injury (*in vitro*). The findings in this study could provide natural alternatives to the treatment or prevention of CVD with implications in the pharmaceutical and food industries.

3. Materials and Methods

Increased intracellular oxidative stress has been associated with higher incidence of CVD. In the present investigation, the protective effect of northern Ontario medicinal plant pre-treatment on H9c2 cardiovascular cells against H_2O_2 mediated oxidative injury was evaluated. The levels of oxidative stress and cell death upon pre-treatment with plant extracts were determined through the H_2DCFDA and MTT assays, respectively. In addition, the antioxidant activity and total phenolic contents of plant extracts were determined through the Folin-Ciocalteu, ABTS, DPPH, EC_{50} and ORAC assays.

3.1 Instruments and chemicals

The absorbance was measured using Bio-Rad Smart Spec Plus spectrophotometer. Synergy HT Biotek using GEN 5 software was utilized for the measurement of fluorescence. Beckman coulter ViCell XR using the Vicell software (ViCELL203) was used to calculate cell count, and BD FACS caliber flow cytometer using the BD Cell quest pro software (Version 5.2.1) was utilized in the H_2DCFDA assay. In the MTT assay, BioTek Powerwave X5 (BioTek, Winooski, VT, USA), using the KC4 software was used to read absorbance of the 96 well flat bottom polystyrene microplate (Corning). The chemicals MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), potassium persulfate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, Folin-Ciocalteau phenol reagent and anhydrous sodium carbonate were purchased from Sigma-Aldrich (Canada). ACS grade methanol was purchased from Fisher Scientific (Canada). The cardiac (H9c2) cells were obtained from American Type Culture Collection (Manassas, VA) and the H_2DCFDA solution was purchased from Molecular Probes, Eugene, OR, USA.

3.2. Collection of plant material

The *Cornus canadensis L., Petasites frigidus* (L.) Fr., *Ledum palustre* L. and *Prunella vulgaris* L. plant materials in this study were collectedin Thunder Bay, Ontario,Canada (North America) between July 22nd - August 10th, 2011. The specimens were identified by Erika North, herbarium curator and contract lecturer at Lakehead University, Thunder Bay, Ontario, Canada. All voucher specimens were deposited in -80° C fridge at the Department of Biology lab (CB3037), Lakehead University, Canada.

3.3 Extraction

The leaf and/or stem tissues of the collected plant samples were dried in dark over a period of 24-48 hours at 30° C. The dried plant samples (2 g) were ground up, milled through No. 40 mesh and extracted twice with 50ml of ethanol (95% v/v) over a period of 48 hours at room temperature. All extracts were filtered through Whatman No.1 filter paper, evaporated to dryness under rotary evaporator at 34° C in vacuum and further dried under high vacuum overnight. The dried extracts were stored in parafilmed glass vials at -80° C until required for analysis.

3.4 Cell culture methods

The cardiac (H9c2) cells were cultured in 25 cm^3, 75 cm^3 and 150 cm^3 cell culture flasks as per the recommended protocol: Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, MO) with 10% fetal calf serum (Hyclone, Pittsburgh, PA), 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA); 37° C, 5% CO_2 and 100% humidity. Cells were seeded in an appropriate amount of medium and allowed to settle 24 hours prior to treatment exposure. By convention, experimental cultures were grown in the absence of serum and antibiotic to prevent their potential effects upon the data.

3.5 MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is used as a quantitative index of activity of mitochondrial and cytosolic dehydrogenases, which, in living cells, reduce the yellow tetrazolium salt to produce a purple formazan dye that can be measured spectrophotometrically. Cells were seeded onto sterile flatbottom 96-well plates

and incubated overnight to achieve the desired confluence. Plated cells (~ 8 x 10^5 cells/ well) were subjected to the plant extracts (100 µg/ml) pre-treatment, 1 µL of 99.85% methanol pretreatmentor no treatment for 24 hours and then analyzed for cell viability. In another set, the plated cells were pre-treated with the plant extracts (100 µg/ml) and Trolox (50 µM) for 24 hours followed by a 30 minute 200 µM H_2O_2 treatment in serum- and antibiotic-free media. In the H_2O_2 control, the seeded cells were grown overnight and then treated with 200 µM H_2O_2 for 30 minutes. Prior to the addition of MTT reagent or H_2O_2, the medium in wells was aseptically removed via vacuum suction. The MTT reagent was added to wells to achieve a final concentration of 10 % (v/v), and cells were incubated at 37° C for an additional 4 hours, during which time the MTT reagent was converted to purple formazan crystals in living cells according to their metabolic activity. Following this, the incubation media was aspirated and 50 µL of dimethylsulfoxide was added to each well to solubilize the formazan crystals. Following 10 minutes of agitation on a Belly Dancer shaker (Stovall, Greensboro, NC, USA) at its highest setting, absorbance was measured spectrophotometrically at a wavelength of 490 nm (650 nm correction wavelength). Viability of the H9c2 cells in treated wells was assessed relative to control wells, which were considered to represent 100 % viability.

3.6 H_2DCFDA (Oxidative Stress) assay

Oxidative stress in the form of intracellular ROS was determined via the H_2DCFDA assay. The cells were seeded at 8 x 10^5 cells/ well onto sterile flatbottom 6-well plates (Costar 3516) and cultured overnight. There were four groups in this study: (1) Control group, cells were untreated; (2) H_2O_2 group, cells were exposed to freshly prepared 200 µM H_2O_2 for 30 minutes; (3) Positive control group, cells were exposed to freshly prepared 50 µM Trolox for 24 hours and then treated with 200 µM H_2O_2 for 30 minutes; (4) Plant extract group, cells were pre-treated with *P. frigidus*, *C. canadensis*, *P. vulgaris* or *L. palustre* leaf extracts (100 µg/ml) for 24 hours and then treated with 200 µM H_2O_2 for 30 minutes. Prior to the H_2O_2 treatment, the medium in wells was aseptically aspirated. After treatments, the cells were washed with PBS and incubated for 30 minutes with H_2DCFDA according to the manufacturer's instructions. The H_2DCFDA was then aspirated and the cells were washed with PBS. The cells were then carefully removed from the culture dish by trypsinization and the relative fluorescence of samples was measured via flow cytometer as per the manufacturer's instructions. A minimum of 10, 000 gated events were acquired per trial, unless stated otherwise. Mean fluorescence (specifically: geometric mean fluorescence) was understood to be directly proportional to levels of intracellular ROS.

3.7 ABTS antioxidant assay

For ABTS assay, the procedure by Thaipong et al., (2006) was used with modifications. The stock solution of 7.4 mM ABTS and 2.6 mM potassium thiosulfate was mixed in equal quantities and allowed to react in darkness at room temperature for 12-16 hrs. Then, 1 ml of this working solution was mixed with 20 ml methanol to obtain an absorbance reading of 1.16 ± 0.05 units at 734 nm. Plant extracts (150 µl) were allowed to react with 2850 µl of the diluted ABTS*+ for 2 hrs in dark. Then the absorbance was taken at 734 nM. This was done in triplicates and the results expressed as µM Trolox Equivalent/gram dry weight (µM TE/gdw). The Trolox standard curve was linear between 25 µM and 625 µM (R^2 = 0.9927).

3.8 DPPH antioxidant assay

The DPPH assay was performed according to the method established by Brand-Williams et al., (1995). The stock solution was prepared by dissolving 24 mg DPPH in 100 ml methanol. The working solution was prepared by mixing 10 ml of stock solution in 45 ml methanol to obtain a reading of 1.13 ± 0.05 at 515 nm. Plant extracts (150 µl) were allowed to react with 2850 µl of DPPH working solution at room temperature for 24 hrs in dark. The absorbance was read in triplicates at 515 nm and the results expressed as µM Trolox Equivalent/gram dry weight (µM TE/gdw). The Trolox standard curve was linear between 25 µM and 825 µM (R^2 = 0.9907).

3.9 Determination of EC_{50}

This assay was performed according to Mensor et al., (2001). Briefly, 1 ml of freshly prepared 0.3 mM DPPH methanol solution was added to 2.5 ml of sample solution in triplicate, and allowed to react at room temperature for 30 minutes. The absorbance was measured at 518 nm using methanol as blank. The EC_{50} value, extract concentration that can reduce 50 % of the initial DPPH radical concentration, was calculated through a dose-response curve expressed either as a linear or logarithmic relationship. DPPH solution (1.0 ml at 0.3 mM) plus methanol (2.5 ml) was used as a negative control and Trolox as positive control.

3.10 Oxygen radical absorption capacity assay
The ORAC procedure was performed according to Teow et al., (2007). Briefly, 100 µl of 12 nm fluorescein, 20 µl of methanol plant extract, and 80 µl of 24 mM AAPH (2,2'-Azobis (2-methylpropionamidine) dihydrochloride) were loaded and mixed in flat bottom, polystyrene 96-well microplate. The plate was agitated at slow speed for 15 seconds prior to first reading (Excitation/emission 484 nm/520 nm), in 1 min interval for 120 minutes at ambient conditions (pH 7.4, 37° C) chamber. Area under the kinetic curve (AUC) was calculated using PRISM 5 software and the ORAC value was expressed as µM TE/gdw. AAPH and fluorescein was used as blank, AAPH in exclusion was used as negative control and fluorescein in absence of AAPH and extract was positive control. The Trolox standard curve was linear between 5 µM TE to 50 µM TE (R^2 = 0.9882).

3.11 Total phenolic content assay
The total phenolic content assay was performed according to procedure established by Ou et al., (2012). Briefly, at 0 min, 200 µl Folin-Ciocalteau phenol reagents was added to a reaction mixture of 200 µl plant extract and 2.6 ml dd H_2O and mixed rigorously at room temperature. After 6 minutes, 2 ml of 7 % anhydrous sodium carbonate solution was added and mixed rigorously. The mixture was incubated in dark for 90 minutes and absorbance was read at 750 nm. The total phenolic content was expressed as mg Gallic Acid Equivalent/10 gdw (mg GAE/10 gdw). The standard curve was linear between 30 µg GA/ml to 300 µg GA/ml (R^2 = 0.9982).

3.12 Statistical analysis
The results were expressed as means ± standard deviation (SD) of three replicate measurements. Statistical significance analysis was determined through the two-way ANOVA test in PRISM 5 software.

Table 1: The antioxidant capacities and total phenolic content of the northern Ontario medicinal plants

Medicinal Plant	Plant Part	ABTS [a](µM TE/ gdw)	DPPH [b](µM TE/ gdw)	EC_{50} [c] (µg/ ml)	ORAC [d] (µM TE/ gdw)	Total Phenolic Content (mg GAE/ 10 gdw)
C. canadensis L.	Leaf	172.67 ± 2.80	177.95 ± 3.95	14.00 ± 0.45	261.67 ± 4.85	359.80 ± 0.71
	Stem	127.11 ± 0.48	112.69 ± 0.95	27.50 ± 0.96	220.64 ± 5.54	256.35 ± 0.13
L. palustre	Leaf	157.33 ± 2.13	161.54 ± 3.05	15.00 ± 0.06	376.76 ± 8.92	349.80 ± 1.90
	Stem	96.44 ± 0.57	97.18 ± 0.60	38.29 ± 0.17	259.35 ± 8.70	179.82 ± 1.37
P. frigidus	Leaf	57.56 ± 0.37	44.62 ± 0.80	35.00 ± 1.80	226.26 ± 3.02	128.80 ± 1.80
P. vulgaris	Leaf	160.00 ± 1.57	169.23 ± 4.70	17.23 ± 0.97	466.99 ± 5.80	350.60 ± 3.30
	Stem	98.89 ± 5.91	97.95 ± 0 .30	21.99 ± 0.68	305.66 ± 7.51	249.13 ± 3.25

Note: all results are expressed as mean ± SD of triplicate measurements [a]ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt; [b]DPPH: 2,2-diphenyl-1-picrylhydrazyl; c EC_{50}: concentration necessary to neutralize 50% of the DPPH radical at a concentration of 82µM; [d]ORAC: oxygen radical absorption capacity assay µM TE/ gdw: µM Trolox Equivalence/ gram dry weight; mg GAE/ 10 gdw: mg Gallic Acid Equivalence/ 10 gram dry weight.

Table 2: Correlation between antioxidant capacities and total phenolic contents

	DPPH	TPC[a]	EC_{50}	ORAC
ABTS	0.98	0.91	0.89	0.38
ORAC	0.30	0.32	0.40	
EC_{50}	0.84	0.93		
TPCa	0.94			

[a]TPC: total phenolic contents

4. Results

4.1 Antioxidant Capacities of Plant Extracts
In this study, the leaf and / or stem tissues of *P. frigidus*, *P. vulgaris*, *C. canadensis* and *L. palustre* plants were analyzed for the total phenolic contents and *in vitro* antioxidant activity through the *in vitro* DPPH, ABTS, ORAC and EC_{50} assays (Table 1).

In the DPPH, ABTS and EC_{50} assays, a range in activity of 44.62 - 172.67 µM TE/ gdw, 57.56 -177.95 µM TE/ gdw and 14.00 - 38.29 µg/ml was observed, representing a variation of approximately 4, 3 and 3-fold, respectively. Furthermore, in the DPPH and ABTS assays, the following hierarchy in activity was observed: *C. canadensis* leaf >*P. vulgaris* leaf >*L. palustre* leaf >*C. canadensis* stem >*P. vulgaris* stem >*L. palustre* stem >*P. frigidus* leaf. The EC_{50} values of *C. canadensis*, *P. vulgaris* and *L. palustre* leaf extracts were comparable (between 14.00 - 17.23 µg/ml), however, *P. frigidus* leaf extract demonstrates relatively low activity with an EC_{50} of 35.00 ± 1.80 µg/ml. In the ORAC assay, antioxidant capacityin the range of 226.26 - 466.99 µM TE/ gdw was observed, representing a variation of 2-fold. In contrast with the DPPH

and ABTS assays, the highest activity is observed for the *P. vulgaris* leaf extract, followed by the extracts of *L. palustre* leaf, *P. vulgaris* stem, *C. Canadensis* leaf, *L. palustre* stem, *P. frigidus* leaf and *C. canadensis* stem. Overall, the leaf tissues of medicinal plants display higher antioxidant activity relative to the stem extracts.

The total phenolic content of medicinal plants was determined through the Folin-ciocalteu method. The phenolic contents in the northern Ontario medicinal plants range from 128.80 - 359.80 mg GAE/10 gdw, representing a variation of 3-fold. The trend in phenolic contents was comparable to the EC_{50}, ABTS and DPPH assays: *C. canadensis* leaf >*P. vulgaris* leaf >*L. palustre* leaf >*C. canadensis* stem >*P. vulgaris* stem >*L. palustre* stem >*P. frigidus* leaf.

The correlation between different assays was also analyzed in this study (Table 2). A high correlation ($R^2 \geq 0.84$) was observed between the EC_{50}, DPPH and ABTS antioxidant assays. Also, a strong relationship ($R^2 \geq 0.91$) was observed between the antioxidant capacity and total phenolic contents. However, the ORAC assay failed to show a good correlation with the antioxidant assays or the total phenolic contents ($0.40 \geq R^2 \geq 0.30$).

4.2 Cell viability of the H9c2 Cells

In this study, viability of the H9c2 cardiomyocytes after the plant extracts pretreatments (100 µg/ml), with and without the H_2O_2 (200 µM) induced oxidative stress was determined. Only the leaf extracts of medicinal plants were tested for cell cytotoxicity due to their higher activity in the *in vitro* antioxidant assays. Trolox (50 µM) was used as the positive control. The survival of cardiomyocytes decreased significantly to 76.94 ± 10.61 % after a 30 min exposure to H_2O_2 (Figure 1a). However, pre-treatment with the Trolox solution, *P. vulgaris* or *P. frigidus* leaf extracts increasedthe survival rate of the H9c2 cells to over 90 %.

Survival rate of the H9c2 cells in the *C. canadensis* and *L. palustre* leaf extract pretreatments was 59.34 ± 9.22 % and 52.19 ± 9.82 %, respectively (Figure 2a). After the H_2O_2 treatment, *L. palustre* leaf extract pre-treatment maintains viability near 52%. However, viability of the H9c2 cells pretreated with the *C. canadensis* leaf extract further decreases to 43.67 ± 9.00 % ($p < 0.05$).

4.3 H_2DCFDA oxidative stress assay

Hydrogen peroxide is a major propagator of oxidative stress in cells, and has been utilized as the model radical source in many studies on cardiovascular diseases. Following the cell cytotoxicity assays, the H_2DCFDA method was utilized to evaluate the level of total intracellular ROS production in response to the H_2O_2 treatment. The H9c2 cells treated with H_2O_2 (200 µM, 30 min) show a significant increase in the total ROS production (376.02 ± 29.34 a.u) compared to the non-stress control (259.67 ± 11.49 a.u) (Figure 1b). However, pre-treatment of the cardiac cells with Trolox solution, *P. frigidus*, or *P. vulgaris* leaf extracts significantly reduces the total ROS productionto 212.15 ± 5.05, 190.14 ± 4.03 and 168.48 ± 8.79 a.u, respectively. Also, the mean logarithmic fluorescence intensity of H9c2 cells, indicative of the intracellular ROS level, in thesepretreatments was lower than the non-stress control (Figure 1c). Overall, the *P. vulgaris* leaf extract pre-treatment displays the most significant reduction in total ROS levels among all the different pre-treatment.

The total intracellular ROS production in the *C. canadensis* and *L. palustre* leaf extract pre-treatment could not be determinedsince only 609 ± 49 and 2632 ± 100 gated events were captured in the flow cytometric analysis, respectively (Figure 2b, c). As can be seen in the logarithmic distribution depicted in Figure 2b and c, the peak at the control borderline (between 10^3- 10^4 fluorescence intensity units) for *C. canadensis* and *L. palustre* leaf extracts pre-treatment could not be generated.

5. Discussion

Many studies in biomedical research have emphasized the role of oxidative stress in the pathogenesis of CVD (Dhalla et al., 2000). The intake of phenol rich dietary or nutraceutical supplements may balance the cellular redox homeostasis in CVD at-risk patients by delaying or inhibiting the autoxidation of susceptible biomolecules. As such, the discovery of novel phenolic compounds from medicinal plants, fruits, vegetables or synthetic sources is a lucrative area of research. In the present investigation, the antioxidant activity and the beneficial effect of *P. frigidus*, *P. vulgaris*, *C. canadensis* and *L. palustre* northern Ontario medicinal plants on the H9c2 cardiomyocytes were evaluated.

Figure 1: The cytotoxic and protective effect of *P. vulgaris* and *P. frigidus* leaf extract pretreatment on the H9c2 cardiovascular cells against H_2O_2 mediated oxidative stress. Trolox (50 µM) was used as the positive control. **(a.)**

Cell viability of the H9c2 cells upon pretreatment with Trolox and the extracts (24 hours), with and without the H_2O_2 treatment (30 min, 200 µM). **(b.)** Total intracellular ROS production in the H9c2 cells measured with the H_2DCFDA assay. **(c.)** ROS level distribution in the H9c2 cells, expressed as logarithm of fluorescence intensity versus the cell count (~10, 000 gated events/ graph). Three independent experiments were performed for the two-way ANOVA analysis: NS, not significant; *, p<0.01; **, p< 0.05 as compared to the control (non stress)

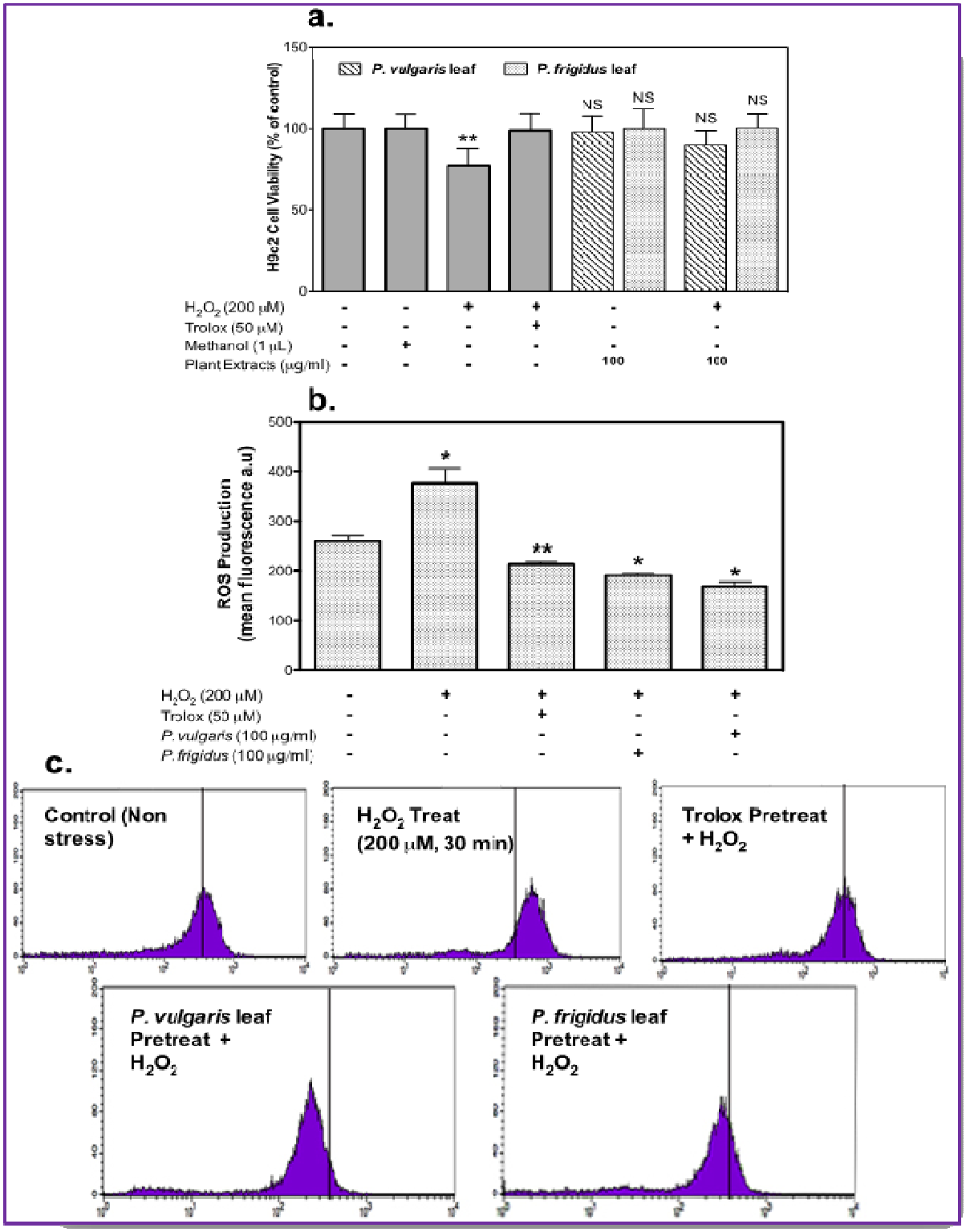
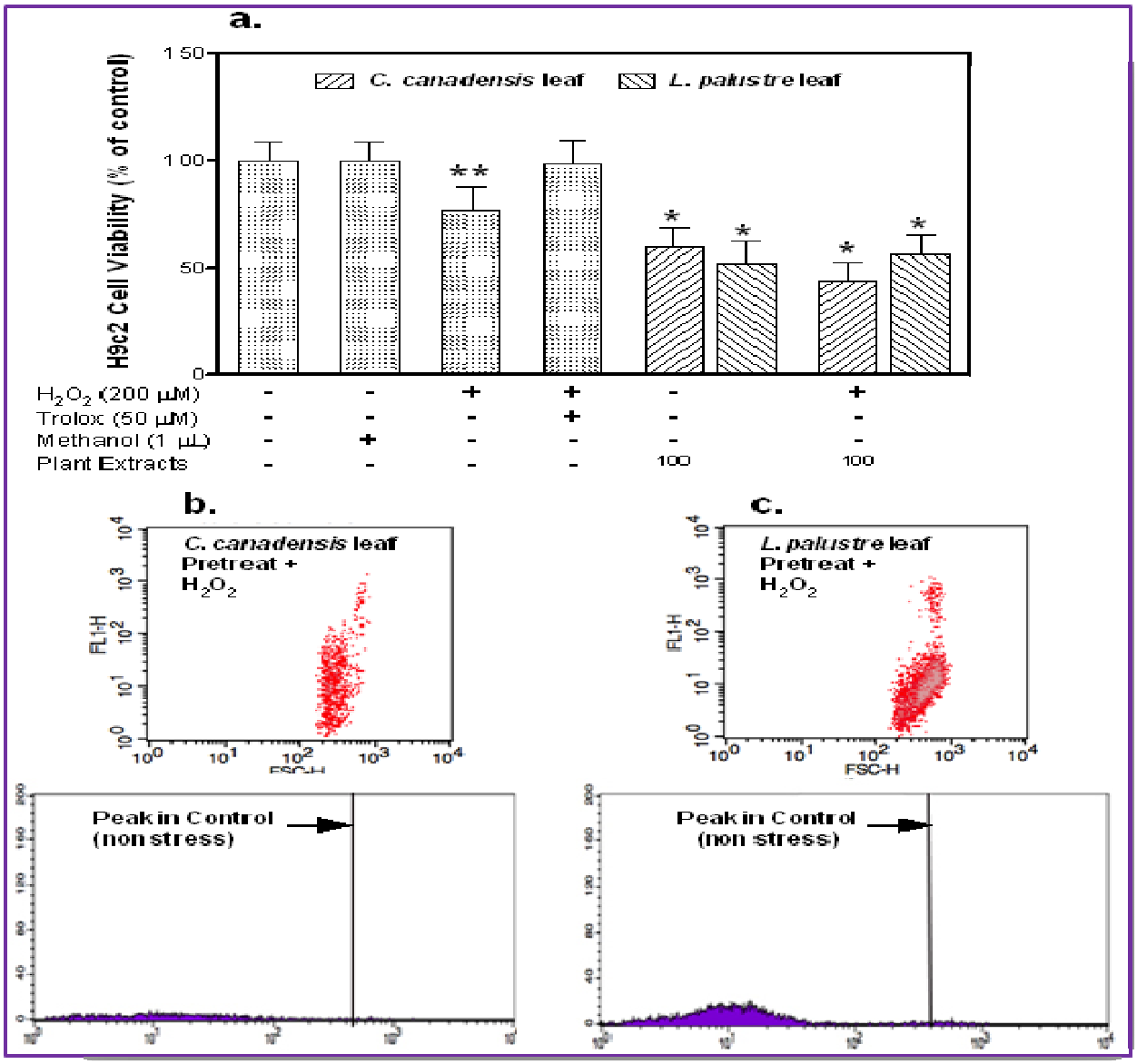


Figure 2: The effects of *L. palustre* and *C. canadensis* leaf extract pretreatment on the viability and ROS accumulation in the H9c2 cells. **(a.)** MTT cell viability assay. Flow cytometer scatter plot and logarithmic distribution of intracellular ROS levels in the H9c2 cells upon H_2O_2 (30 min, 200 µM) treatment after the 24 hour

pretreatment with *C. canadensis* **(b)** and *L. palustre* **(c)** leaf extracts. Three independent experiments were performed for the two-way ANOVA statistical analysis: *, p<0.01; **, p< 0.05 as compared to the non stress control



5.1 Northern Ontario medicinal plants exhibit high antioxidant capacities

In order to determine the antioxidant activity of crude extracts from medicinal plants, it is crucial to implement a variety of methods with varying reaction mechanisms. In this study, both the *in vitro* hydrogen atom transfer (HAT) and single electron transfer (ET) methods were used. The HAT based methods (ORAC assay) utilize a competition based reaction scheme, which reflect on the kinetics between the antioxidant and radical species; whereas, the ET based methods (DPPH and ABTS assays) utilize the end-point of redox reactions to quantify antioxidant activity (Huang et al., 2005). Our investigation reveals that C. canadensis, *L. palustre* and *P. vulgaris* extracts possess strong antioxidant activity, whereas, *P. frigidus* leaf extract consistently ranked low. There was a high correlation (R^2≥0.91) between the antioxidant capacity and total phenolic contents, which conforms to prior studies (Kontogianni & Gerothanassis, 2012; Maisuthisakul et al., 2008). Particularly, the EC_{50} of *C. canadensis* (14.00 µg/ml) was comparable to rutin (14.16 µg/ml) (Mensor et al., 2001), a dietary polyphenolic compound, which has reported protective effects against cancer (Alía et al., 2006) and rheumatoid arthritis (Ostrakhovitch & Afanas'ev, 2001)

In the ORAC assay, *P. vulgaris* and *L. palustre* extracts display the highest activity, whereas, *C. canadensis* and *P. frigidus* extractsrank poor. This discrepancy between HAT and ET methods can escalate from incorporation of an alternate reaction mechanism intrinsic to HAT-based method (Dudonne et al., 2009). Overall, the leaf extracts of *P. vulgaris* and *L. palustre* displaya high total phenolic content and antioxidant activity in both HAT and ET-based methods.

In this study, the leaf extracts of medicinal plants exhibit higher antioxidant activity and phenolic contents relative to the stem extracts. This may be due to a higher surface area to volume ratio in leaf tissues, which precedes greater exposure to UV-B radiation (Cen & Bornman, 1993). In a study by Price et al. (1995), the concentration of quercetin glycoside (a natural flavonol) in grapes tissue increased 7.5-fold (4.5 mg/L to 33.7 mg/L) as a direct outcome of increased exposure to sunlight. However, resistance to herbivory or competition against other plants in the habitat may be alternative hypothesis to explain the higheractivity in these extracts. Overall based on the greater radical scavenging capacity and phenolic contents, only the leaf extracts of medicinal plants were further investigated for protective effect on the H9c2 cells against H_2O_2 induced oxidative stress.

5.2 P. vulgaris and P. frigidus leaf extract pretreatments protect H9c2 cells against H_2O_2 mediated oxidative stress and cell death

Hydrogen peroxide is a major source of ROS in cells, capable of crossing the cellular membrane, causing DNA damage and lipid per oxidation (Xing & Jian, 2011). In the H_2DCFDA assay, the H_2DCFDA dye passively diffuses into cells and isoxidized by intracellular ROS to the fluorescent DCF. Hence, it is a direct measure of the total intracellular ROS level, expressed as mean fluorescent arbitrary units (a.u.). In this study, treatment of the H9c2 cells with hydrogen peroxide (200 µM) lead to a significant accumulation of intracellular ROS. However, pre-treatment with the leaf extract of *P. vulgaris* before exposure to the H_2O_2 treatment, decreased the total intracellular ROS levels by an astonishing 180.43 a.u. compared to the H_2O_2 control (figure 1b, c). In addition, the total intracellular ROS level in *P. vulgaris* leaf extract pre-treatment was statistically lower than the non-stress control by 78.84 a.u., indicating that it protects the H9c2 cardiomyocytes against both H_2O_2 and aerobic metabolism induced oxidative insults. Although similar findings were observed for the *P. frigidus* leaf extract pre-treatment, the *P. vulgaris* extract was considerably more effective in protecting cardiovascular cells against oxidative stress.

The loss of cardiomyocytes by apoptosis or cell death also contributes towards the development of CVD (Xing & Jian, 2011). A study by Xing and Jian (2011) demonstrated that excess H_2O_2 down regulates Bcl2 and up regulates the Bax gene in the H9c2 cells, which induces apoptosis. The antioxidant defense system protects against cell death and oxidative stress induced by a surge in the H_2O_2, xanthine oxidase, metals or other toxic species. It is composed of enzymatic (Glutathione peroxidase, GP; superoxide dismutase, SOD; glutathione-S-transferase, GST; catalase) and non-enzymatic elements (Vitamin C and E, glutathione, albumin)(Prior et al., 2005) that are essential for the intracellular ROS quenching and maintenance of the redox homeostasis in cells (Lillig & Holmgren, 2007). In the MTT assay, it was shown that the *P. vulgaris* and *P. frigidus* leaf extract pretreatments prevent cell death induced by H_2O_2 (figure 1a). The H9c2 cell viability in these pre-treatment, after the H_2O_2 treatment, was statistically insignificant compared to the non-stress control. It's possible that *P. vulgaris* and *P. frigidus* leaf extract, containing rosmarinic, caffeic and oleanolic acidprotected the H9c2 cells from xanthine oxidase mediated oxidative stress by lowering the intracellular ROS levels and inducing SOD, GST, catalase and glutathione in a concentration dependent manner. However, the actual regulation of biomolecules in the H9c2 cellular machinery by *P. vulgaris* pre-treatment is open to future investigations. Also, no prior studies have been performed on *P. frigidus*; thus, the chemical basis of its therapeutic action is open to discovery. Overall, the present study presents strong evidence for the beneficial effect of *P. vulgaris* and *P. frigidus* leaf extracts pre-treatment on the H9c2 cardiomyocytes.

5.3 The C. canadensis and L. palustre leaf extract pretreatments aredetrimental to the H9c2 cells

In the *in vitro* antioxidant assays, the leaf extracts of *C. canadensis* and *L. palustre* display strong antioxidant activity. However, as shown in figure 2 a, these extracts were highly cytotoxic to the H9c2 cells.

Nearly half of the cardiomyocytes were eliminated in the pre-treatment process (without the H_2O_2 treatment). Previous studies on the *C. canadensis* extract have isolated iridoid glycosides (Stermitz & Krull, 1998), which may have cytotoxic properties against the H9c2 cells. Also, the HPLC and GC-MS analysis of *L. palustre* aerial parts has isolated monoterpenes, sesquiterpenes, esculin, quercetin glycosides, acetylated flavonoids, glucoside fraxin and dihydric alcohols (Chosson et al., 1998; Fylaktakidou et al., 2004; Nam, 2006). The cytotoxic effect of two quercetin glycosides from *L. palustre* against the human mouth epidermal carcinoma has been reported before (Dampc & Luczkiewicz, 2013). However, the exact chemical basis of the cytotoxicity against H9c2 cardiac cells is still unknown. Overall, due to the lethality of these extracts to H9c2 cardiomyocytes, their protective effect against the H_2O_2 treatment could not be determined.

Research Highlights

The present investigation demonstrates that pre-treatment of the cardiomyocytes (H9c2 cell line) with *P. vulgaris* or *P. frigidus* crude extracts significantly alleviates H_2O_2 associated oxidative stress and cell death. These extracts also exhibit a relatively high antioxidant and total phenolic content profiles. The evidence suggests that these extracts, particularly the *P. vulgaris* leaf extract, produce antioxidant phytopharmaceuticals that may eliminate oxidative stress mediated CVD symptoms.

Limitations

In this study, the antioxidant capacities and total phenolic contents of medicinal plants were determined. In addition, their protective effect on the H9c2 cardiovascular cells was evaluated. However, the chemical basis of bioactivity and modulatory effect on the biological system was not researched.

Recommendations

Future studies should focus on analytical evaluation of the medicinal plant extracts via HPLC, LC, IC or GC coupled with MS and NMR. Bioactivity mediated fractionation can elucidate the chemical source of activity. In addition, the *in vitro* synergistic or antagonist action of bioactive metabolites in combination with other extracts or pharmaceutical drugs should be investigated.

Funding and Policy Aspects

Despite the tremendous success of drug discovery from natural resources, the pharmaceutical industry has retracted its investigation of plants as sources of novel phytochemicals. The government should invest in Ethnobotanical research and provide incentives for pursuing alternative medicine for the treatment of human illnesses.

Conclusion

Hydrogen peroxide causes generation of ROS and up regulation of the Bax gene leading to apoptosis, DNA oxidation, lipid peroxidation, and protein damage. In our study, we showed that a 30-minute treatment of the H9c2 cell with hydrogen peroxide (200 µM) increased the total intracellular ROS levels and induced cell death. The leaf extracts of *P. vulgaris* and *P. frigidus* pre-treatment protects the H9c2 cells against cell death and reduces intracellular ROS levels from oxidative stress induced by H_2O_2. However, the leaf extracts of *C. canadensis* and *L. palustre* were heavily cytotoxic to the H9c2 cells, since nearly 50% of the cardiomyocytes were terminated in the 24-hour pre-treatment process. As mentioned before, ROS overproduction is one of the major mechanisms of H_2O_2 induced cell injury. In this study, the *P. vulgaris* leaf crude extract was the most effective in defending the cells through scavenging the ROS. In contrast to the *P. frigidus* leaf extract, which displays a relatively low antioxidant capacity, the higher efficacy of *P. vulgaris* reflects its strong antioxidant activity in the *in vitro* DPPH, ABTS, ORAC and EC_{50} assays. Regarding the high level of phenolic contents in *P. vulgaris* leaf extract, it is possible that the antioxidant potential and modulatory effect on the intracellular ROS defense system is due to its polyphenolic content. In addition, the observed bioactivities of *P. vulgaris* and *P. frigidus* extracts elude towards the presence of potential phytopharmaceutical grade therapeutic metabolites that may have CVD preventative properties. Overall, the observed bioactivities of plants studied correlate with their medicinal uses in aboriginal communities of the northern Ontario region.

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Conflict of Interest

The authors declare no conflict of interest

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