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The Effects of *Plantago major* on the Activation of the Neutrophil Respiratory Burst

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ABSTRACT

Plantago major is a common plant that grows worldwide in temperate zones and is found in fields, lawns, and on the roadsides. Its leaves and seeds have been used in almost all parts of the world for centuries as a wound healer, analgesic, antioxidant, and antibiotic, as well as an immune system modulator, antiviral, antifungal, and anti-inflammatory agent. Baicalein and aucubin are the two most biologically active components of *P. major*, and both have been shown to have antioxidant, anti-inflammatory, and anticancer properties. Neutrophils have a pivotal role in wound healing and inflammation. Their principal mechanism of host defense is the killing of pathogens via the production of reactive oxygen species (ROS). The aim of the present study was to determine the *in vitro* effects of *P. major* extract, baicalein, and aucubin on human neutrophil respiratory burst activity. The cytotoxicity of the agents was assessed by lactate dehydrogenase (LDH) assays. A standard luminol-dependent chemiluminescence (CL) assay was utilized to monitor the respiratory burst of the neutrophils after exposure to *P. major* extract and its two active ingredients, baicalein and aucubin. Three replicates per group were included in each of the three runs of the experiments and analysis of variance (ANOVA) was used for statistical analysis. *P. major* and baicalein were not toxic to the cells at any of the concentrations examined. Aucubin was toxic to the cells only at the highest concentration tested ($P = 0.0081$). However, genistein was toxic to the cells at all of the concentrations examined except for the lowest concentration of 16.9 μg/ml ($P = 0.985$). *P. major* (-0.10 ± 0.11), aucubin (0.06 ± 0.16), baicalein (-0.10 ± 0.11), and genistein (-0.18 ± 0.07) all significantly ($P < 0.0001$) inhibited ROS production from the neutrophils. *P. major* extract inhibited neutrophil ROS production, as did aucubin and baicalein. Therefore, these components should be investigated further with relation to the regulation of destructive ROS production in conditions such as periodontal disease.

Key words: Aucubin, Baicalein, Neutrophil respiratory burst activity, *Plantago major*

INTRODUCTION

Medicinal plants occur naturally and are widely used in a large number of countries all around the world. People use them either in the form of traditional preparations or as pure active forms usually for their known therapeutic qualities and in order to avoid the harmful side effects of prescription medicine. One such plant is *Plantago major*, also known as Ribwort, which is readily available in many parts of the world; its leaves and seeds have been used for centuries as an anti-inflammatory, analgesic, antioxidant, anti-infective, immune-modulating, anti-ulcerogenic, anti-fungal, and anti-cancer agent, as well as for wound healing purposes.[1-6]

The properties of *P. major* are modulated by the different components of the plant. These include carbohydrates, lipids,

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alkaloids, caffeic acid derivatives, flavonoids, irioid glycosides, and other terpenoids. The chemical analysis of the leaves revealed the presence of aucubin, a glycoside, which has been reported in several studies to be a powerful anti-toxin. There are also some other effective ingredients in this plant such as baicalein, ascorbic acid, apigenin, benzoic acid, chlorogenic acid, citric acid, ferulic acid, oleanolic acid, salicylic acid, and ursolic acid.[7]

Neutrophil granulocytes (also termed polymorphonuclear leukocytes),[8] which are generally referred to as neutrophils, are the most abundant type of white blood cells (40-70%) in humans and form a crucial part of the host defense system. Neutrophils are short-lived cells normally found in the blood stream after their release from the bone marrow. However, during the acute phase of inflammation, particularly as a result of bacterial infection, bone marrow output of neutrophils increases and they migrate toward the site of inflammation to confront the pathogens. Neutrophils react within an hour of tissue injury and are the hallmark of acute inflammation.[9] They phagocytize microorganisms, internalizing and killing as many as possible. Each phagocytic event will result in the formation of a phagosome into which reactive oxygen species (ROS) are secreted for microbial destruction. The consumption of oxygen during the generation of ROS has been termed the "respiratory burst." This process plays a significant role in the inflammation-induced tissue damage and the antioxidant defense mechanisms associated with periodontal diseases and other inflammatory oral conditions.[9]

This study investigated the *in vitro* effects of *P. major* extract, baicalein, and aucubin on the respiratory burst activity of human neutrophils. The aim was to explore the potential medicinal properties of *P. major* in an effort to promote the use of this readily accessible resource for the benefit of oral and general health.

MATERIALS AND METHODS

The study was approved by the Institutional Review Board (IRB) of Indiana University Purdue University under the application for research not subject to FDA or common rule definitions of human subjects research, prior to commencement of the study (e.g., IRB study number: 0309-56). No human subjects were recruited specifically for this study. The buffy coat samples were purchased from Indiana Regional Blood Center and were de-identified. *P. major* (30×) extract was purchased from Washington Homeopathic Products (Berkeley Springs, WV, USA). Baicalein, aucubin, and genistein were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Neutrophil isolation

Six buffy coats, separated from healthy adult human donor blood, were purchased from the Central Indiana Regional Blood Center in Indianapolis, Indiana. To obtain the buffy coats, de-identified healthy human donor blood was collected by the blood center in citrate phosphate dextrose solution anticoagulant bags and centrifuged at 2000 × *g* at 4°C for 4 min. Buffy coat layers were then drawn off by the blood center and provided for this study. In the laboratory, the buffy coats were diluted in a 1:1 ratio with Rosewell Park Memorial Institute Medium (RPMI) (Sigma Chemicals, St. Louis, MO, USA) to maximize the efficiency of separation. The neutrophils were isolated from the buffy layer by the double dextran gradient method as described previously.[10] Briefly, 3 ml aliquots of HISTOPAQUE-1119 (Sigma Chemicals) were placed in test tubes and then 3 ml of HISTOPAQUE-1077 (Sigma Chemicals) was added to each tube. Then, 6 ml of buffy coat/RPMI mixture was layered on the top, centrifuged, and washed three times with media, followed by centrifugation after each wash. The cells were then re-suspended, stained with Trypan Blue (Sigma Chemicals) to verify viability, and counted to determine the number of neutrophils.

Lactate dehydrogenase cytotoxicity assays

Cellular membrane integrity was monitored by the permeability assay based on the release of lactate dehydrogenase (LDH) into the media. The Cytotoxicity Detection Kit-PLUS (Roche Applied Science, Mannheim, Germany) measures the conversion of tetrazolium salt to a red formazan product. The amount of formazan produced is proportional to the amount of LDH released into the culture medium as a result of cytotoxicity.

Neutrophils were added to six-well plates (100,000 cells per well). After 3 h, the neutrophils were incubated at 37ºC for 2 h with *P. major* extract diluted in media, as well as with aucubin, baicalein, or genistein at various concentrations. Total cell death (high control) was generated by the addition of 1.9 ml of serum-free RPMI and 0.1 ml of lysis solution to control cells as described by the manufacturer. Untreated cells served as the low control and represented no cytotoxicity. Samples (100 µl) were transferred to 96-well plates. An aliquot (100 µl) of the reconstituted mix as per the manufacturer was added to each well. The plates were then incubated for 30 min at room temperature. The absorbance was recorded at 490 nm in a microplate reader (Titertek, Multiskan MCC, Flow Laboratories, McLean, VA, USA). The experiments were repeated six times and the mean values calculated. The percentage release of LDH was calculated from the treated cells by comparing it with the maximum release of LDH as follows:

cytotoxicity (%) = (experimental value – low control)/(high control – low control) × 100%.

Chemiluminescence assays

These assays were performed as per the previously established protocols using the Bio-Orbit 1251 Luminometer (Bio-Orbit, Turku, Finland).[10] For each of the three runs of the experiments, there were 16 reaction cuvettes. To each reaction cuvette, the following were added: 500 µl of neutrophil suspension of 500,000 cells in RPMI-1640 media, 300 µl of phosphate-buffered saline (PBS), and 100 µl luminol that served as a chemiluminescence (CL) probe for signal augmentation. It was dispensed at baseline and then after 30 min, 3% of *P. major* extract, 0.01 µg/ml of aucubin, 0.085 µg/ml of baicalein, or 16.9 µg/ml of genistein was added. A negative control that contained no neutrophils and a positive control of *N*-formyl-methionyl-leucyl-phenylalanine (fMLP, 10^{-5} M) treated neutrophil sample were included. The reaction was followed for 90 min and this represented the neutrophil activation phase. Neutrophil activation was recorded in millivoltage and the intervals were calculated. Data analyses were performed on the mean values of the triplicate experiments.

High-performance liquid chromatography

Twenty microliters of the *P. major* extract (30×) were processed through a high-pressure liquid chromatography (HPLC) instrument equipped with a series 200 pump, a 785A tunable UV-VIS detector, and a Brownlee C-18 reverse phase column (25 cm × 4.6 mm, 5 µm particle size), all from Perkin-Elmer (Shelton, CT, USA). The chromatography was performed with a flow rate of 1 ml/min and with acetonitrile/water mobile phase at 210 nm.

Statistical methods

One-sided tests were used to compare each group in the LDH assays with the negative control (untreated cells). CL data from each experimental run were divided by the positive control mean to standardize the data from the different experimental runs. CL values for each of the groups were summarized (mean, standard deviation, standard error, minimum and maximum). Comparisons between the groups for differences in CL values were performed using mixed-model analysis of variance (ANOVA). The ANOVA included a term for group, as well as a random effect for experimental run. The analyses were performed using the ranks of the CL values to satisfy the distributional assumptions required for ANOVA.

RESULTS

LDH cytotoxicity assays

The cytotoxicity was examined at different concentrations of *P. major*, aucubin, baicalein, and genistein. *P. major* was prepared in ethanol, whereas aucubin, baicalein, and genistein were prepared in dimethylsulfoxide (DMSO). The alcohol and DMSO at the final concentrations utilized for the agents were not toxic to the cells (data not shown). *P. major* and baicalein were not toxic to the cells at any of the concentrations examined [Table 1]. Aucubin was toxic to the cells only at the highest concentration tested (100 µg/ml, $P = 0.0081$) [Table 1]. However, genistein was toxic to the cells at all of the concentrations examined except for the lowest concentration of 16.9 µg/ml ($P = 0.985$) [Table 1].

Active CL

P. major (−0.10 ± 0.11), aucubin (0.06 ± 0.16), baicalein (−0.10 ± 0.11), and genistein (−0.18 ± 0.07) all significantly (all with $P < 0.0001$) inhibited ROS production from the neutrophils [Figure 1].

HPLC for *P. major* extract

The data were analyzed with Turbochrom software from Perkin-Elmer. Standards of the components of the materials tested were run on the HPLC at known concentrations. Linear calibration curves were used to calculate the concentrations of aucubin and baicalein based on the area of the chromatographic peaks at the corresponding retention times. Detection limits for each are defined as 5 times the signal to noise ratio. *P. major* extract (30×) contained 0.05 µg/ml of aucubin and 0.0425 µg/ml of baicalein.

Table 1. Cytotoxicity of *plantago major*, aucubin, baicalein, and genistein

	Mean±Std. Dev	*P* value
Plantago major, %		
0.011	−8.41±0.09	0.98
0.023	−10.07±0.08	0.99
0.046	−7.93±0.11	0.97
0.187	−7.79±0.12	0.97
0.375	−7.08±0.14	0.96
0.75	−6.51±0.15	0.94
1.5	−6.68±0.13	0.95
3	−2.02±0.04	0.68
Aucubin, µg/ml		
0.78	−9.79±0.10	0.99
1.5	−9.07±0.11	0.98
3.15	−8.59±0.13	0.98
6.25	−8.58±0.13	0.98
12.5	−8.87±0.13	0.98
25	−8.45±0.14	0.98
50	−8.62±0.13	0.98
100	11.49±0.17	0.0081*
Baicalein, µg/ml		
0.25	−0.59±0.01	0.75
0.5	−0.79±0.01	0.82
1	−0.91±0.00	0.85
2	−0.64±0.01	0.78
Genistein, µg/ml		
16.89	−4.15±0.09	0.985
33.78	14.22±0.06*	0.0001*
67.56	45.99±0.09*	0.0001*
108.09	53.53±0.08*	0.0001*

*Significant at $P<0.05$ compared to untreated cells

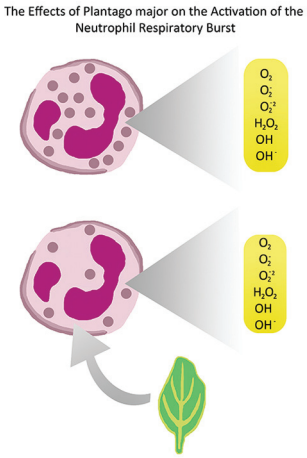

Figure 1. Chemiluminescence results showing the effects of *Plantago major*, aucubin, baicalein, and genistein on ROS production from the neutrophils

DISCUSSION

Neutrophils play a key role in the host response against invading pathogenic microorganisms in periodontal disease and other oral conditions. They represent the frontline of defense in acute infections. A major mechanism is through phagocytosis and killing of the microorganism either through oxidative or

non-oxidative mechanisms. However, although their major effects are protective, neutrophils release toxic products that are thought to be partly responsible for the associated tissue destruction, such as ROS.[9] Some investigators had thus described the relationship of neutrophils and the tissue destruction as a proverbial double-edged sword. There is cumulative evidence now to implicate ROS in the pathogenesis of several diseases including AIDS, cancer, atherosclerosis, chronic inflammatory conditions such as periodontal and oral diseases, and in the aging process.[11,12]

For this reason, there is a growing interest in natural antioxidants found in herbs and medicinal plants that may act as scavengers by reducing the action of ROS and free radicals to support their use in the treatment of chronic inflammatory diseases. The present study investigated the effects of *P. major* and two of its main components, baicalein and aucubin, on the human neutrophils' respiratory burst activity and ROS production *in vitro*. A standard CL method was used to measure the generation of ROS by human neutrophils. This technique was chosen because of its accuracy, high sensitivity, and simplicity.

Baicalein and aucubin are believed to be among the most important chemicals found in *P. major*. Baicalein, a flavonoid, and aucubin, an irioid glycoside, are considered the most biologically active components of the plant.[1] Baicalein is known for its potential antioxidant properties and as a free radical scavenger. It is also known for its anti-inflammatory activities, specifically its ability to inhibit inflammatory cytokines in human mast cells.[3,4] Aucubin is the major irioid glycoside isolated from the leaves and has been determined to be a specific inhibitor of nuclear factor-kappa B (NF-κB) activation in mast cells, which explains its anti-inflammatory properties. Aucubin has also been shown to have anticancer properties, since it enhances human lymphocyte proliferation and the secretion of interferon γ.[9] The concentrations of aucubin and baicalein selected for the CL assays were below the toxic levels as determined in the LDH assays. Aucubin at 0.01 μg/ml and baicalein at 0.085 μg/ml were chosen based on the LDH assays and the literature.[13-15] The data from the HPLC experiment detected 0.05 μg/ml of aucubin and 0.0425 μg/ml of baicalein in the *P. major* 30× extract. Therefore, the concentrations of aucubin and baicalein used in the CL assays were close to the concentrations detected in *P. major*. However, these concentrations of aucubin and baicalein, as well as the *P. major*, inhibited ROS production almost completely, so future studies examining dose responses of these agents are needed to determine if the crude *P. major* extract works better than the individual components (aucubin or baicalein) in inhibiting ROS production.

Genistein was used as a control. Genistein is the major natural phytoestrogen polyphenolic nonsteroidal isoflavonoid found in soybeans.[15,16] Genistein serves as a control for inhibition due to its antioxidant and anti-inflammatory properties through its down regulation of cytokine-induced signal transduction events in the cells of the immune system.[16,17] Genistein suppresses the NF-κB activation induced by ROS and subsequently inhibits the transcription of a variety of inflammatory genes.[16]

Non-toxic levels of *P. major*, baicalein, aucubin, and genistein significantly inhibited the ROS production from the activated neutrophils. This indicates the potential antioxidant and anti-inflammatory effect of *P. major* and the agents. These results are supported by the findings of studies and reports on the wound healing activity of *P. major*.[18-20] A clinical trial has also determined *P. major* to be effective for the treatment of chronic bronchitis.[21]

Chiang *et al*.,[22] studied the cytotoxic, antiviral, and immunomodulatory effects of *P. major* on various human leukemia, lymphoma, and carcinoma cells and concluded that extracts of *P. major* possess a broad spectrum of antiviral activities, as well as the activities that modulate cell-mediated immunity.[22] Stef *et al*.,[23] analyzed the total antioxidant and scavenging capacity of *P. major*, among many other herbal medicines, and concluded that it should be considered as an antioxidant compound, as it presented both reducing power and radical scavenging capacity.[23] Similarly, Kumarasamy *et al*.,[24] reported that the *P. major* seed extracts exhibited significant free radical scavenging activity. They concluded that these natural antioxidant compounds from plant extracts may help to develop new drug candidates for antioxidant therapy.

Since there is currently sufficient evidence to implicate the contribution of ROS in the pathogenesis of a many diseases including periodontal and oral infections, and since neutrophils are recognized as a primary source for ROS, agents that suppress ROS production from neutrophils present promising therapeutic approaches for these diseases. Oxidative stress occurs when the production of ROS exceeds the body's natural antioxidant defense mechanisms, causing damage to biomolecules such as lipids, proteins, and DNA. This oxidative stress is responsible for a major part of the general drop in cellular functions associated with many human diseases.

In conclusion, the results of the present study showed that the *P. major*, baicalein, and aucubin inhibited ROS production by human neutrophils. This makes them promising candidates for future extensive studies to provide evidence for their therapeutic potential and prove their benefits in the prevention and treatment of periodontal and oral conditions.

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