ASSESSMENT OF ANTI-INFLAMMATORY ACTIVITY AND CYTOTOXICITY OF FREEZE DRIED HYDROALCOHOLIC EXTRACT OF BIDENS ANDICOLA ON ISOLATED NEUTROPHILS

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INTRODUCTION

Bidens is a genus from which some species have been studied. Among them, Bidens pilosa and Bidens andicola are the main representatives of South America. B. pilosa Linn. var. radiata Sch. Bip., belonging to Asteraceae family is one variety of the B. pilosa species originated from South America and transmitted to China. Pharmacognostic and phytochemical studies showed the presence of phenylpropanoid glucosides, diterpenes, polycyclitlenes, flavonoids, and flavone glycosides identified as bioactive components from this plant possessing a wide range of biological/pharmaceutical activities such as antioxidant, antimicrobial, anti-inflammatory, antiseptic, liver protective, blood pressure lowering, and hypoglycemic effects. B. pilosa is used in Traditional Chinese Medicine for the treatment of periappendicular abscess, which is usually due to primary appendicitis or probably caused by various pathological conditions: Inflammatory, infective, neoplastic, immunological, ischemic, and occlusive. This plant is also employed as a folk medicine and a major ingredient of herbal tea in Taiwan believed to be able to prevent inflammation and cancer. Recently, the protein extract from it was demonstrated to have an anticancer property against colorectal cancer and induce cell apoptosis of SW480 cells via the reactive oxidative species production and glutathione depletion [1].

The study of the traditional indigenous medical practices in Amazonia and Southern Brazil brought to light ethnomedicinal use of B. pilosa Linne (Asteraceae) to treat certain tumors. This plant is also known as antitumor agent in Cuba and the Bahamas. B. pilosa is a widely occurring plant species in tropical regions and it has several popular names according to the place where it is found. In Brazil, it is generally known as Picão-preto and it is widely used as folk medicine by indigenous people to treat a variety of illnesses including pain, fever, angina, diabetes, edema (water retention), infections, and inflammation. In addition, in the Amazon and South Brazil regions, infusion and hydroalcoholic solutions of B. pilosa are also regarded as useful in the treatment of malaria and even tumors. Furthermore, B. pilosa is a popular ingredient in herb teas in China. Phytochemical analyses of B. pilosa revealed a broad chemical constitution, and it was previously believed that this fact could explain its wide use. The main compounds already isolated from the plant are polycyclitlenic and flavonoid substances. Polycyclitlenes are hydrocarbons that strongly absorb long-wave ultraviolet radiation, and their medicinal activity is altered on exposure to light (photo activation). They have been reported to possess cytotoxic effects on parasites and modified cells in culture. The specific flavonoids present in Bidens have not been fully elucidated, although luteolin, quercetin and some others have been isolated so far. Quercetin in hem has been shown to suppress tumors in mice [2].

On the other hand, B. andicola is a native and an vegetal species, named am or seco, chilipugio, pante amarillo, saitilla in Ecuador, Perú and Argentina [3,4]. This plant belongs to the Asteraceae family. B. andicola is a perennial herb [5] that grows spontaneously around crop fields in a high quantity. A peruvian ethnobotanical survey research determined that B. andicola can to be used as anti-infectious, anti-inflammatory, diuretic, antipyretic, astringent, hepatic protector, renal and bladder calculi, cystitis, prostatitis, and laryngitis [4]. In addition, the traditional use of B. andicola against mouthful is referred in Argentina [3]. Research about B. andicola chemical composition identified compounds derived from quercetin 3-O-methyl ether and quercetin, specially glycoside type compounds [6]. Quercetin 3-O-methyl ether is a flavonoid reported such as antioxidant, antitumor and anti-inflammatory on several cellular models [7,8].

This vegetal resource is widely used by the indigenous communities of Ecuador as dye, anti-flu, tonic and refreshing and it represents a good alternative to economy to the indigenous communities if its effectiveness and security are guaranteed. The aim of this research was to evaluate anti-inflammatory activity and cytotoxicity in vitro of hydroalcoholic extract of B. andicola, as a way of rescuing and validate the ancestral wisdom of indigenous people who use this natural resource to treat their illnesses.
METHODS

Plant material
B. andicola sample was collected in Ecuador, Chimborazo Province, San Andrés sector, S 01° 34’ 28.065’’ W 079° 42’ 59.681’’; average height: 3124 m. The plant material was taxonomically identified by a botanist Jorge Caranqui at Escuela Superior Politécnica de Chimborazo, and a specimen was deposited at Herbarium. The B. andicola aerial parts were collected, and the hydroalcoholic extract was obtained for use in the study.

Reagents
Ficoll-paque, modified Hank’s solution, Zimo saa D and Triton X-100 was purchased from Sigma-Aldrich, S.L. (USA), water-soluble tetrazolium salt (WST-1) from Roche (USA), aspirin from J.T. Baker (USA), dimethyl sulfoxide (DMSO) and ammonium chloride from Merck (Germany), deionized water was used in all experimental procedures. All other reagents were of analytical or high-performance liquid chromatography grade as appropriate.

Equipment
Heidolph Hei-VAP Advantage Rotary Evaporator (Germany), Micro Modulyo Freeze dryer - 1.5 L Thermo (USA), Multiskan™ GO Microplate Spectrophotometer Thermo Scientific™ (USA), Branson 2510DTH Sonicator, Vortex Gemmy VM300 (Taiwan).

Hydroalcoholic extract obtention
Ultrasound-assisted extraction is a rapid technique that can also be used with mixtures of insoluble substances. The hexane phase concentrated less polar compounds, while the aqueous phase concentrated more polar flavonoids [9].

B. andicola hydroalcoholic extract was obtained from aerial parts (leaves, flowers, and stems) following a standardized methodology. From 10 g of aerial parts of B. andicola were extracted with ethanol 70% v/v, sonicated for 15 minutes, followed by vortex for 15 minutes and finally defatted with n-hexane. Hydroalcoholic fraction was concentrated under controlled conditions (50°C, -0.5 bar) in a rotary evaporator, and finally, the aqueous residue was freeze-drying to obtain the hydroalcoholic extract of B. andicola.

Preliminary phytochemical screening test for B. andicola
Phytochemical screening of the plant extract was carried out to investigate the presence of secondary metabolites such as flavonoids, terpenoids, saponins, tannins, and phenols using standard procedure [10].

Total flavonoids determination
The total flavonoids were measured by a colorimetric assay modified by Boukrhis et al. (2012). 1 mL aliquot of the appropriately diluted sample or standard solution of quercetin (20, 40, 60, 80, and 100 mg/L) was added to a 10 mL volumetric flask containing 4 mL of H₂O. At zero time, 0.3 mL of NaNO₂ (5%, w/w) was added to the flask. After 5 minutes, 0.3 mL AlCl₃ (10%, w/w) was added. At 6 minutes, 2 mL of NaOH (1 M) was added to the mixture. Immediately, the reaction flask was diluted to volume with the addition of 2.4 mL of H₂O and thoroughly mixed. The absorbance of the mixture, characterized by a pink color, was determined at 510 nm compared to a water control. The total flavonoids were measured by a colorimetric assay modified by Boukrhis et al. (2012). The total flavonoids were expressed as dry weight mg/g quercetin equivalents (QE) [11]. For quercetin, the curve absorbance against concentration was described by the equation: $y = 0.0012x - 0.0345 \ (R^2 = 0.9997)$.

Total phenolics determination
The total phenol content of extracts was determined using the phenol reagent (Boukrhis et al., 2010) with few modifications in the sample volume. Briefly, 2 mL aliquot of the extract was assayed with 500 µL of phenol reagent and 500 µL of aqueous sodium carbonate (20%, w/v). The mixture was vortexed and diluted with water to a final volume of 5 mL. After incubation for 30 minutes at room temperature, the absorbance was measured at 765 nm. The total phenols were expressed as gallic acid equivalents (GAE/g of dry weight), using a calibration curve of a freshly prepared gallic acid solution [11]. For the gallic acid, the curve absorbance versus concentration is described by the equation: $y = 0.0012x - 0.0345 \ (R^2 = 0.9997)$.

Isolation of human neutrophils
Heparinized fresh venous blood sample was drawn from healthy volunteers and neutrophils were isolated [12]. Whole blood was added to Ficoll-paque, it was centrifuged for 30 minutes at 1500 rpm. After discarding the supernatant, red blood cells traces were lysed by mixing it with hypotonic ammonium chloride solution (0.83%). It was centrifuged again, and the neutrophils were washed with modified Hank’s solution (MHS), pH 7.4 and resuspended at concentration of 10⁷ cells/mL in a MHS appropriate volume [13].

Cell viability assay
Metabolically active cells reduce tetrazolium salts into colored formazan compounds. Therefore, tetrazolium salt-based colorimetric assays detect viable cells only. These sensitive assays can readily be performed in a microtiter plate with relatively few cells using modified method [14]. In this study, the human isolated neutrophils (10⁷ cells/mL) were incubated with test compounds for 30 minutes then WST-1 (250 µM) was added and incubated in shaking water bath at 37°C for 3 hr. The absorbance was measured at 450 nm. Triton X-100 (0.1%) was used as negative control showed 0.00% cell viability [13]. DMSO 5% was used as positive control exhibited 100.00% cell viability. The OD is the mean of five replicates. Cell viability percent was calculated using the following formula and results are shown in Table 1.

$\% \text{Cell viability} = \frac{OD - OD_{DMSO}}{OD_{DMSO}} \times 100$

Anti-inflammatory assay
Anti-inflammatory activity was determined using modified assay [15]. This in vitro assay is based on the reduction of WST-1 in the presence of activated neutrophils. Anti-inflammatory assay was determined in a total volume of 250 µL MHS (pH 7.4) containing 10⁵ neutrophils/µL, 500 µM WST-1 and various concentrations of test compounds. Control contained buffer, neutrophils, and WST-1. All compounds were equilibrated at 37°C and the reaction was initiated by adding opsonized Symosan A (15 mg/mL), which was prepared by mixing it with human pooled serum, followed by centrifugation at 3000 rpm and pellet was resuspended in phosphate buffer solution. Absorbance was measured at 450 nm [13]. Aspirin was used as a positive control that is widely used as nonsteroidal anti-inflammatory drugs for treatment of several inflammatory diseases [16,17]. IC₅₀ values were calculated by comparing to the DMSO used as blank and expressed as produced superoxide anions inhibition percent.

Statistical analysis
All values are expressed as mean±SD. A value of p<0.05 was considered to indicate a significant difference. ANOVA test and Tukey’s were performed to establish significant differences for multiple comparisons. Student’s t-test was used to compare control group and the individual better performance treatment.

Table 1: Cell viability percent at different concentrations of extract

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. andicola hydroalcoholic extract</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>27.679±0.329</td>
</tr>
<tr>
<td>100</td>
<td>30.399±0.372</td>
</tr>
<tr>
<td>50</td>
<td>32.959±0.474</td>
</tr>
<tr>
<td>25</td>
<td>36.639±0.262</td>
</tr>
</tbody>
</table>

B. andicola: Bidens andicola, SD was standard deviation of five replicates, SD: Standard deviation.
RESULTS AND DISCUSSION

9.65 g of freeze-dried extract was obtained from 100 g of aerial parts of B. andicola. The isolation of fraction reveals a high yield of extraction approximately 10%. The preliminary phytochemical screening of hydroalcoholic extract revealed the presence of secondary metabolites such as flavonoids, phenols, and saponins; in addition, the presence of sugars; and in a little amount coumarins and catechins. These results are related with the previous research [6] that shown the presence of flavonol 7-O-glycosides with quercetin or quercetin 3-methyl ether as their aglycons and chalcone ester glycoside. The total flavonoid content of B. andicola expressed like QE/100 g of the plant was 9.987±0.123, and total phenolics content of B. andicola expressed like GAE/100 g of the plant was 12.058±0.274. These results suggest that B. andicola possesses a high quantity of flavonoids and phenolic compounds in its composition. It is clearly established when B. andicola is compared with another Bidens species like Bidens biterminata, in which the flavonoid content is in the range of 0.004-0.016% at different parts of the plant [18]. Nevertheless, B. pilosa total flavonoid content in the extracts measured using a colorimetric assay developed by Zhishen et al. (1999), was 66.2% (w/w) [19], in another research in which B. andicola was fractionated by the use of solvents of increasing polarity, ethyl acetate extract of B. pilosa herb had the highest total flavonoid content 14.66% (w/w), and methanolic extract had the highest phenolic content 7.61% (w/w) [20]. This meaning that B. andicola possesses an appreciable amount of flavonoids, without becoming as large as the case of B. pilosa. However, the phenol content of B. andicola in relation to B. pilosa is almost double.

Cell viability was determined using WST-1 metabolizing activity, a reliable method to this purpose and with multiple usefulness as the case of the determination of decrease cytotoxicity induced by H2O2 of some subfractions of the red ginseng extract [21]. Table 1 shows the cell viability expressed by percentage. Cell viability decreases with increase the B. andicola hydroalcoholic extract concentration. There is a statistically significant difference between the B. andicola concentrations tested (ANOVA p<0.001) and results differ from each other (Tukey p<0.001).

Table 2 shows the B. andicola hydroalcoholic extract anti-inflammatory activity percents. It demonstrated proportional relation with B. andicola hydroalcoholic extract concentrations, there is a statistically significant difference between the treatments (ANOVA p<0.001) and results differ from each other (Tukey p<0.001) with the exception of aspirin that differs compared to the hydroalcoholic extract of B. andicola at 200 ppm (Tukey p=0.009). Genus Bidens is widely studied for its biological activity and phytochemicals [22]. Medicinal properties of members of genus Bidens are due to polyacetylenic glycosides, aurones, auron glycosides, p-coumaric acid derivatives, flavonoids and flavonoid glycosides, sesquiterpenes, phenylpropanoid glucosides, and diterpenes [23,24]. These components would be responsible for its anti-inflammatory activity. Flavonoids have various biological activities, which are mainly related to its ability to inhibit enzymes and its effects on immune responses [25]. The pharmacological effects of quercetin, such as its antioxidant, anti-inflammatory, antiallergic, anti-aging, and anticancer activities, as well as its regulating effect on interleukin (IL)-6, IL-8, tumor necrosis factor, histamine, and tryptase release in mast cells, are well acknowledged [26].

Table 3 shows both responses anti-inflammatory activity and cell viability calculated at 200 µg/mL of B. andicola hydroalcoholic extract and aspirin. Results indicate that B. andicola hydroalcoholic extract has an appreciable anti-inflammatory activity. However, the extract does not achieve the same level of anti-inflammatory activity than aspirin (p<0.003 Student’s t-test). Nevertheless, the potential of B. andicola hydroalcoholic extract to its possible use like anti-inflammatory is evident. In the same way, cell viability comparison between B. andicola hydroalcoholic extract and aspirin differ from each other (p<0.001 Student’s t-test). At a concentration of 200 µg/mL, although the anti-inflammatory activity of this is easy to deduce that B. andicola has a great potential as an ant-inflammatory.

## Table 2: Anti-inflammatory percent at different concentrations of extract

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Anti-inflammatory activity (%)</th>
<th>B. andicola hydroalcoholic extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>80.138±0.729</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>76.993±0.695</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>71.947±0.531</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>66.827±1.225</td>
<td></td>
</tr>
</tbody>
</table>

## Table 3: Anti-inflammatory percent and cell viability values of extract and positive control at 200 µg/mL.

<table>
<thead>
<tr>
<th>Components</th>
<th>Anti-inflammatory percent</th>
<th>Cell viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. andicola hydroalcoholic extract</td>
<td>80.138±0.729</td>
<td>27.679±0.329</td>
</tr>
<tr>
<td>Aspirin</td>
<td>82.117±0.762</td>
<td>23.359±0.504</td>
</tr>
</tbody>
</table>

| B. andicola: Bidens andicola. SD was standard deviation of five replicates, SD: Standard deviation |

**CONCLUSION**

Especially, the indigenous populations of the Sierra region of Ecuador have used B. andicola to treat their inflammatory diseases. The research carried out is a contribution so that the traditional use of this resource that is currently underutilized can be an opportunity for development of communities so that they maintain the ancestral use of the plant and can use it rationally to conserve it and in the future to take advantage of it to improve their economy.

**ACKNOWLEDGMENT**

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**REFERENCES**

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