

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

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ABSTRACT

Objective: The aim of this study was to evaluate anti-inflammatory activity and cytotoxicity *in vitro* of hydroalcoholic extract of *Bidens andicola*.

Methods: *B. andicola* hydroalcoholic extract was obtained from aerial parts of *B. andicola*, following a standardized methodology. Briefly, aerial parts of *B. andicola* were extracted with ethanol 70% v/v and defatted with n-hexane, hydroalcoholic fraction was concentrated under controlled conditions in a rotary evaporator, and finally the residue was freeze-drying to obtain the hydroalcoholic extract of *B. andicola*. Anti-inflammatory activity and cytotoxicity assays were carried out using *in vitro* isolated neutrophils model using stable water-soluble tetrazolium salts.

Results and Conclusions: The *in vitro* anti-inflammatory assay on isolated neutrophils demonstrated that the hydroalcoholic extract showed anti-inflammatory activity compared to aspirin, with inflammatory inhibition percent values of 80.138 ± 0.729 to hydroalcoholic extract of *B. andicola* and 82.117 ± 0.762 to aspirin, each tested in five replicates at the concentration of 200 ppm of hydroalcoholic extract or reference.

Keywords: Anti-inflammatory, Cytotoxicity, *Bidens andicola*, Cell proliferation reagent, Water-soluble tetrazolium salts.

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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, polyacetylenes, 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 an 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 polyacetylenic and flavonoid substances. Polyacetylenes 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. Interestingly, quercetin has been shown to suppress tumors in mice [2].

On the other hand, *B. andicola* is a native and an vegetal specie, named amorseco, chilipiga, planetaamarillo, 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 of crop fields in a high quantity. A peruvian ethnobotanical survey research determined that *B. andicola* can 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, anticancer, and anti-inflammatory on several cellular models [7,8]. This vegetal resource is widely used by the indigenous communities of Ecuador 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 town, sector S 01° 34' 28.065'' W 078° 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, Zimosan A 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 2510DTHSonicator; Vortex GemmyVM300 (Taiwan).

Hydroalcoholic extract obtention

Ultrasound-assisted extraction is a rapid technique that can also be used with mixtures of in miscible solvents. The hexane phase concentrated less polar compounds, while the aqueous alcohol phase concentrated 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, follow 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 developed by Zhishen et al. (1999). 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 expressed as dry weight mg/g quercetin equivalents (QE). For quercetin, the curve absorbance against concentration was described by the equation $y = 0.0012x + 0.0218$ ($R^2 = 0.9991$) [11].

Total phenolics determination

The total phenol content of extracts was determined using the phenol reagent (Boukhris 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. For the gallic acid, the curve absorbance versus concentration is described by the equation $y = 0.0012x - 0.0345$ ($R^2 = 0.9997$) [11].

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.

$$\%Cellviability = \frac{OD}{OD_{DMSO}} \times 100$$

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 10⁴ 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 Zymosan 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

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

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

AO2

RESULTS AND DISCUSSION

9.65 g of freeze-dried extract was obtained from 100 g of aerial parts of *B. andicola*. There is a relative 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* possess a high quantity of flavonoids and phenolic compounds in its composition. This is clearly established when *B. andicola* is compared with another *Bidens* species like *Bidens biternata*, 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 in 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, aurons, 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). However, 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-

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

Concentration (µg/mL)	Anti-inflammatory activity (%) <i>B. andicola</i> hydroalcoholic extract
200	80.138±0.729
100	76.999±0.695
50	71.947±0.538
25	66.827±1.225

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

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

Components	Responses at 200 µg/mL (%)	
	Anti-inflammatory percent	Cell viability
<i>B. andicola</i> hydroalcoholic extract	80.138±0.729	27.679±0.329
Aspirin	82.117±0.762	23.359±0.504

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

inflammatory percentage of aspirin differs from that of the extract, it is easy to deduce that *B. andicola* has a great potential as an anti-inflammatory.

Around the world, genus *Bidens* has been used in traditional medicine as anti-inflammatory, antimalarial, antiulcer, antiallergic, anticancer, antidiabetic, and antibacterial agents [18]; in this sense, it can be determined that the ethnobotanical use from which we started have been validated at the level of the isolated neutrophils anti-inflammatory model.

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 become 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.

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