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Phytochemical screening, antioxidant, antimicrobial, and cytotoxicity evaluation of the leaf extracts of *Bidens pilosa* by the T'boli tribe in South Cotabato, Philippines

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ABSTRACT: This study determined the bioactive compounds and evaluated the antioxidant, antimicrobial, and cytotoxicity activities of *Bidens pilosa* aqueous and ethanolic extracts against bacterial and fungal pathogens and various human cancer cell lines. The *B. pilosa* leaves were extracted using ethanol and water as solvents. The aqueous (BA) and ethanolic (BE) extracts of *B. pilosa* were subjected to qualitative phytochemical screening. The phosphomolybdate method was used to determine the total antioxidant activity of the extracts. The antimicrobial activity of the extracts against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa*, and *Candida tropicalis* was determined using the Kirby-Bauer method. Lastly, the brine shrimp lethality test (BSLT) and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay in the human breast, colon, and liver cancer cell lines were used to evaluate the cytotoxicity activities of the more potent extract. Steroids, flavonoids, tannins, saponins, alkaloids, and cyanogenic glycosides were present in *B. pilosa* leaf extracts. BE showed higher antioxidant and antimicrobial activities than BA. However, BSLT results showed BA was more toxic than BE based on their lethal concentration 50 (LC_{50}) values after 24-hour exposure. MTT assay also showed that BA was more potent against the colon cancer (HCT116) cell line, followed by breast (MCF-7) and liver (Hep G2) cancer cell lines. The *B. pilosa* extracts possess potent antioxidant, antimicrobial, and anticancer activities. These biological activities are due to the presence of various natural products present in its leaves. Further investigations are needed to understand the pharmacological properties of *B. pilosa* fully.

KEYWORDS: Free radicals; HCT 116; human cancer; MTT assay; traditional medicine.

1. INTRODUCTION

Of the 13,500 plant species in the Philippines, more than 3,500 are considered indigenous, and almost 1,500 are known as medicinal plants. However, only about 120 medicinal plants were scientifically validated for safety and efficacy [1]. The Philippines is also a rich melting pot of different cultures, which includes the T'boli tribe. The tribe mainly settles in southwestern Mindanao, particularly in South Cotabato, around Lake Sebu [2]. The T'boli has traditional herbal medicine for specific illnesses. The "*m'wanga*" (tribal therapist) or "*m'tonbu*" (herbal healer) leads the healing rituals to cure conditions ranging from "*sentengeb*" (minor) to "*nasal be tonok*" (most serious) such as "*b'latu*" (tumor or myoma), "*tenbalung*" or "*henayam*" (hemorrhage) using various herbal plants, such as *B. pilosa* [3,4].

B. pilosa Linn. (Asteraceae), commonly known as "*Sl'ot*" by the T'bolis, is an annual plant widely distributed in waste places, mainly at medium altitudes, rising to 2,200 meters from Batanes and the Babuyan Islands and Northern Luzon to Mindanao [5]. As a leafy vegetable, it is a rich source of food and medicine with an excellent amount of fiber and certain mineral elements good for humans and animals [6-8]. T'boli tribe

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uses *B. pilosa* to treat toothaches, headaches, vomiting, diarrhea, and inflammations. Flowers are directly smelled and dabbed on the forehead and neck three times a day for three days to treat headaches and vomiting. When chewed thrice daily, the roots, flowers, or balled-up leaves can also treat toothache. Pounded leaves are applied to sore eyes and snake and insect bites. Leaves of *B. pilosa* are prepared as a tea to treat malaria, flatulence, diarrhea, and ulcers [5].

Over the centuries, the T'bolis' knowledge of traditional herbal medicine was passed from generation to generation. Plants have always been the major reservoir of novel bioactive compounds since herbal medicines have helped develop and improve human health care [9-11]. Some plants possess medicinal properties due to different complex bioactive substances in various compositions, which occur as secondary metabolites. These secondary metabolites can affect the physiology of the human body [12-15]. Secondary metabolites, which include tannins, saponins, flavonoids, steroids, alkaloids, cyanogenic glycosides, and anthraquinones, can boost the immune system, prevent oxidative stress caused by free radicals, inhibit the growth of pathogenic microorganisms, and have possible use in treating cancer [9-16]. Therefore, this present study evaluated the antioxidant, antimicrobial, and anticancer activities of *B. pilosa* and determined its phytochemical constituents.

2. RESULTS & DISCUSSION

Medicinal plants are assuming greater interest in the fundamental health care of individuals and communities in many developing countries. Treating various illnesses using medicinal plants has been widely accepted among indigenous people worldwide [17-21]. The information and folk knowledge regarding the medicinal and therapeutic uses of these native plant materials have been handed down from generation to generation through verbal communication [22]. In this study, we have demonstrated the antioxidant, antimicrobial, and anti-cancer activities of *B. pilosa* to validate the use of this common medicinal plant by the T'boli tribe in South Cotabato, Philippines.

2.1. Qualitative Phytochemical Screenings

The qualitative phytochemical analyses of *B. pilosa* aqueous extract (Table 1) revealed the presence of major bioactive compounds, such as steroids, flavonoids, tannins, saponins, alkaloids, and cyanogenic glycosides. The *B. pilosa* ethanolic extract has only revealed the presence of steroids, flavonoids, saponins, and alkaloids. Anthraquinones were not detected in both extracts.

Table 1. Qualitative phytochemical screening results of the *B. pilosa* aqueous and ethanolic extracts.

Phytochemicals screened	*B. pilosa* ethanolic extract	*B. pilosa* aqueous extract
Steroids	+++	+++
Flavonoids	+++	+++
Tannins	-	+++
Saponins	+	+
Alkaloids	++	++
Cyanogenic glycosides	-	+++
Anthraquinones	-	-

Level of Detection: (+) turbid, (++) moderate, (+++) heavy, (-) not detected

2.2. Total Antioxidant Activities

This study used phosphomolybdate to analyze the antioxidant activities of *B. pilosa* aqueous and ethanolic extracts. Presented in Table 2 are the antioxidant activities of *B. pilosa* aqueous and ethanolic extracts expressed in their ascorbic acid (AA) and butylated hydroxytoluene (BHT) equivalents. Results have shown that *B. pilosa* ethanolic extract exhibits higher antioxidant activities than *B. pilosa* aqueous extract.

Ascorbic acid and butylated hydroxytoluene equivalents showed that *B. pilosa* extracts exhibited potent antioxidant properties essential to combat free radicals and oxidative stress. A previous study revealed that ethanolic and ethyl acetate extracts of *B. pilosa* whole plant exhibit protection from oxidative damage in normal human erythrocytes [23]. Several antioxidant compounds are present in the crude extract. These compounds could synergistically enhance the antioxidant activity of the plant. These results imply that *B.*

pilosa can be a good source of antioxidants. The efficiency and efficacy of its antioxidant activities depend on the availability and capacity to donate hydrogen or electron atoms [24,25].

Table 2. Antioxidant activities of *B. pilosa* aqueous and ethanolic extracts.

Crude Plant Extracts	AA Equivalents (ppm)	BHT Equivalents (ppm)
B. pilosa aqueous extract	7.60	38.88
B. pilosa ethanolic extract	13.67	49.85

2.3. Antimicrobial Properties

Both the *B. pilosa* aqueous (BA) and *B. pilosa* ethanolic (BE) extracts exhibit varying antimicrobial activities against *S. aureus, B. subtilis, E. coli, P. aeruginosa*, and *C. tropicalis* based on the results of the disc diffusion assay (Table 3). In an antimicrobial study on *B. pilosa*, collected from Baja California Sur, Mexico, its ethanolic extract did not exhibit antimicrobial activity against *B. subtilis, S. aureus, S. faecalis, E. coli,* and *C. albicans* [26]. However, following the antimicrobial results of this present study, it is interesting that the aqueous and ethanolic leaf extracts of *B. pilosa* inhibited the growth of *S. aureus, B. subtilis, E. coli, P. aeruginosa*, and *C. tropicalis*. This divergence in the plant's biological activity may be due to different compositions of phytochemical profiles across geographical regions [27,28]. Reports have shown that various environmental factors exogenously influence the secondary metabolite levels present in plants [29].

Table 3. Mean zone of inhibition for *B. pilosa* aqueous and ethanolic extracts against bacterial and fungal strains.

Microorganisms	Mean Zone of Inhibition (mm)		
	BA	BE	Tetracycline/Ketoconzale
Staphylococcus aureus	8.3	9.0	11.0
Bacillus subtilis	8.0	9.0	10.7
Escherichia coli	6.7	8.0	12.3
Pseudomonas aeruginosa	7.0	7.3	12.7
Candida tropicalis	7.0	8.0	10.7

2.4. Toxicity against Brine Shimp nauplii

BSLT and MTT assay revealed the cytotoxicity and anti-cancer properties of *B. pilosa*, especially its aqueous extract. Table 4 shows the mortality percentage of *A. salina* nauplii after 6 h and 24 h exposure to different concentrations of *B. pilosa* extracts. The nauplii mortality occurred in a dose-dependent manner.

Table 4. Mortality percentage of *A. salina* nauplii after 6-hour and 24-hour post-treatment at different concentrations of *B. pilosa* aqueous and ethanolic extracts

Crude Plant Extracts	Mortality Percentage (%)							
	6-hour Exposure				24-hour Exposure			
	1000 ppm	500 ppm	100 ppm	10 ppm	1000 ppm	500 ppm	100 ppm	10 ppm
B. pilosa aqueous extract	76.67	63.33	20.00	20.00	100.00	100.00	96.67	26.67
B. pilosa ethanolic extract	63.33	63.33	23.33	6.67	70.00	80.00	20.00	13.33

Moreover, after 6 h and 24 h exposure, BA has shown higher mortality of *A. salina* nauplii (LC_{50}=330.45 ppm; LC_{50}=11.93 ppm) than BE (LC_{50}=380.32 ppm; LC_{50}=284.38 ppm) as shown in Table 5. The LC_{50} values and the cell viability percentage of *B. pilosa* aqueous extract provide valuable information regarding its anti-cancer potential.

Table 5. Acute and chronic toxicity LC_{50} values of *B. pilosa* aqueous and ethanolic extracts.

Crude Plant Extracts	LC_{50} values (ppm)	
	6-hour Exposure	24-hour Exposure
B. pilosa aqueous extract	330.45	17.93
B. pilosa ethanolic extract	380.32	284.38

2.5. Cytotoxicity against Human Cancer Cell Lines

The cytotoxic and antiproliferative effects of *B. pilosa* aqueous and ethanolic extracts against HCT 116, MCF-7, and Hep G2 are shown in Table 6. The viability percentage of the cancer cell lines after exposure to BA and the positive control, digitonin, revealed that BA is highly potent to HCT 116, followed by MCF-7 and Hep G2. These results indicate that BA can effectively inhibit the proliferation of HCT 116 cancer cells.

Table 6. Cell viability percentage of *B. pilosa* aqueous and ethanolic extracts.

Cancer Cell Lines	Cell Viability Percentage (%)	
	BA (100 µg/mL)	Digitonin (100 µg/mL)
Colon cancer (HCT-116)	11.65	22.94
Breast cancer (MCF-7)	36.19	0.60
Liver cancer (HepG2)	78.48	7.82

Studies have shown that *B. pilosa* extracts exhibit antiangiogenic and antiproliferative activities [30,31]. Isolated bioactive compounds from *B. pilosa* were potent against the proliferation of human umbilical vein endothelial cells (HUVEC) [31]. Meanwhile, hot water extracts of *B. pilosa* whole plant suppressed the proliferation of five human leukemic cell lines, such as L1210, K562, U937, Raji, and P3HR1 [30].

3. CONCLUSION

In conclusion, traditional herbal medicine provides essential baseline information for drug discovery and development. *B. pilosa* extracts possess potent antioxidant, antimicrobial, and anti-cancer activities, which confirm their use in the traditional herbal medicine of the T'boli tribe. These pharmacological activities are due to various natural products present in its leaves. Further investigations are needed to fully understand the pharmacological and toxicological properties of *B. pilosa*. The aqueous extract (BA) was prepared using 800 g of fresh leaves that were cut into pieces, boiled in 1000 mL of distilled water for 5 mins, filtered, cooled, and subjected to lyophilization.

4. MATERIALS AND METHODS

4.1. Preparation of Plant Extracts

Fresh leaves of *B. pilosa* were collected from Lake Sebu, South Cotabato, Philippines. The leaves were washed, cleaned, and air-dried at a temperature of 35-40°C for three weeks. Dried leaves were chopped and pulverized. Ethanolic extraction was done by macerating 100 g of *B. pilosa* leaf powder in 95% ethanol at a 1:10 (w/v) ratio for seven days. The mixture was filtered to collect the filtrate. The filtrate was concentrated under reduced pressure using a rotary evaporator. The crude ethanolic extract (BE) was stored in an amber vial and kept at -20 °C until further use.

4.2. Qualitative Phytochemical Screening

The phytochemical screening of the crude ethanolic and aqueous extracts of *B. pilosa* was carried out using qualitative analyses for tannins, saponins, flavonoids, steroids, alkaloids, cyanogenic glycosides, and anthraquinones following the stand phytochemical screening procedures compiled and as described by [32] with slight modifications. Data were recorded using the 3-point scale scoring based on the *Handbook of Philippine Medicinal Plants* [33].

4.3. Total Antioxidant Activity Screening

The total antioxidant activities of the extracts were evaluated using the phosphomolybdate radical scavenging activity [33]. The method is based on the reduction of Mo (VI) to Mo (V) by the extracts and subsequent formation of a green phosphate -Mo (V) complex. The plant extract (0.1 mL) was mixed with a 3.0 mL reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The solution was incubated at 95°C for 90 min, cooled to room temperature, and the absorbance was measured at

695 nm against a blank. Ascorbic acid and butylated hydroxytoluene were used as the standard references to draw the standard curves. The total antioxidant activities were expressed as micrograms of ascorbic acid (AA) and butylated hydroxytoluene (BHT) equivalents and were done in triplicates.

4.4. Antimicrobial Activity Screening

4.4.1. Microorganisms

The antimicrobial activities of the extracts were screened against two gram-positive bacteria, *S. aureus* (BIOTECH 1582) and *B. subtilis* (BIOTECH 1679), two gram-negative bacteria, *E. coli* (BIOTECH 1634) and *P. aeruginosa* (BIOTECH 1335), and the yeast strain *C. tropicalis* (BIOTECH 2085). Test microorganisms were purchased from the University of the Philippines Los Baños-Philippine National Collection of Microorganism (PNCM) of the National Institute of Molecular Biology and Biotechnology (NIMBB) (Los Baños City, Philippines) and maintained on appropriate agar plates by the manufacturer's instructions. Subsequently, colonies of each strain were resuspended in 10% glycerol, frozen, and stored at -80°C.

4.4.2. Kirby-Bauer disk diffusion susceptibility method

The antimicrobial activities of the extracts were determined using the Kirby-Bauer disk diffusion susceptibility method [34, 35]. The bacterial strains were cultured overnight on Müller–Hinton agar at 37°C, while the yeast strain was cultured on Sabouraud dextrose agar at 25°C. Suspension of each microorganism was prepared in physiological saline solution (0.9%) using the McFarland optical density of 0.5, and 200 µL of each microorganism was evenly spread on the appropriate medium using sterile cotton swabs. Disks of Whatman filter paper grade 3 were prepared using a regular paper puncher (disk size: 6 µm) and were sterilized in an autoclave. Six dried filter paper disks were evenly distributed on the agar plates. Stock or diluents (20 µL) of the plant extracts were dropped in the center of each filter paper disk. An equal amount of 100% ethanol and distilled water were used as blank controls. The plates were left uncovered for 20 min inside a sterilized biosafety cabinet to dry the solutions. The plates were then covered and incubated at 37°C for 24 hours and 27°C for 48 hours for bacteria and yeast, respectively. After incubation, the plates were observed for the formation of a clear inhibition zone around the disk, which would be indicative of the presence of antimicrobial activity. The diameter of the zone of inhibition was measured and recorded. Microbial spectrum and susceptibility of the *B. pilosa* leaf extracts were compared with commercially available antibiotic and antifungal, Tetracycline (30 µg/disc) and Ketoconazole (10 µg). All experiments were done in triplicate.

4.5. Brine Shrimp Lethality Test

The bioassay was carried out according to the method [36] with slight modifications. After hatching, nauplii were collected using a dropping pipette. Ten milligrams (10 mg) of each extract (crude ethanol and aqueous) were dissolved in 10 mL of filtered seawater. The stock solutions for the crude ethanolic (1,000 ppm) and aqueous (1,000 ppm) extracts were further serially diluted to produce 500, 100, and 10 ppm. Test tubes with 5.0 mL of the diluted test solutions in different concentrations were prepared in triplicates, using seawater as a control. Active brine shrimps (n=10) were transferred into each test tube using a Pasteur pipette. The mortality rates and the acute and chronic effects were determined after 6 and 24 hours. The mortality percentage was calculated using the formula,

Mortality percentage = (Number of dead nauplii)/(Initial number of live nauplii)× 100 %

The plant extracts' 50% lethal concentrations (LC_{50}) were calculated using Probit analysis at 95% confidence intervals. LC_{50} values less than 1,000 ppm are considered toxic, while LC_{50} values more than 1,000 ppm are considered non-toxic [36].

4.6. Cytotoxicity Activity Screening

4.6.1. Cell lines and reagents

The human colon (HCT 116), liver (Hep G2), and breast (MCF-7) cancer cell lines (ATTC, Manassas, VA, USA) were purchased from ATTC, Manassas, VA, USA. Cells were kept in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL) in a humidified atmosphere of 5% CO2 at 37°C. Media were changed every 3 days, and cells were passaged at 80% confluency.

4.6.2. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) Assay

Two hundred microliters (200 µL) of Dulbecco's Modified Eagle Medium (DMEM) containing semi-confluent cells (1 × 105 cells) were added to 96-well plates in triplicate and were incubated for 24 h. After removing the old cell culture medium, the cells were exposed to 200 µL of leaf extracts prepared in DMEM (10 and 100 µg/mL). The cells were then incubated for 48 hours, and cytotoxicity was assessed using the tetrazolium salt reduction (MTT assay) [37,38]. The DMEM with plant extracts was removed from each well, and 110 µL of MTT reagent (0.5 mg/mL) prepared in phenol red-free DMEM was added and incubated for another 4 hours. Then, the MTT reagent (85 µL) was removed and replaced with 50 µL of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals. The absorbance of the resulting purple solution was measured using an ELISA microplate reader at 570 nm. Non-treated cells and digitonin were used as negative and positive controls, respectively.

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