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Chemical screening and identification of secondary metabolites by HPLC-MS-UV and antimicrobial activity of *Bidens pilosa* (Asteraceae) extracts

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Abstract

Our study focused on the identification of secondary metabolites by HPLC coupled with mass spectrometry and UV and the evaluation of the antibacterial and antifungal activities of the Hydroethanolic and water extracts of the whole plant of *Bidens pilosa*. Extractions were done by maceration in distilled water and in the ethanol-water mixture (70:30 v/v). Phytochemical screening was carried out following N' Guessan *et al.* The chemical composition of the crude extracts of the whole plant was determined by HPLC-MS-UV. The antibacterial and antifungal activities were evaluated according to the micro dilution method on the reference strains of *Staphylococcus aureus* ATCC43300, *Shigella flexneri* NR518, *Salmonella typhi* ATCC 6539, *Salmonella enterica* NR 13555, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* NR 592 and *Candida albicans* NR29451) as described in the Clinical Laboratory Standard Institute guidelines. Extraction yields were 16.8% and 15.5% for water and Hydroethanol extracts, respectively. Phytochemical screening revealed the presence of polyphenols, flavonoids, catechin tannins, coumarins, sterols, terpenes, Saponoside and alkaloids. The HPLC-MS-UV profile identified the following compounds: taxifolin, Brassicasterol, acetyl-vitexin-rhamnoside, paulitin, moschamin and apigenin 7-sulfate. Antibacterial activity showed that the hydroethanol extract of *Bidens pilosa* was active against all the tested microorganisms with MICs ranging from 2.5 to 10 mg / mL; MBC and MFC between 10 and 20 mg / mL. The aqueous extract was active only on *Candida albicans* and *Escherichia coli* with MICs of 10 and 20 mg / mL respectively. These antimicrobial properties may partly justify the use of *Bidens pilosa* in traditional pharmacopoeia for the treatment of infectious diseases.

Keywords: *Bidens pilosa*, chemical screening, antimicrobial activity

Introduction

Infectious diseases are responsible for 17 million deaths worldwide each year, and remain thereby the leading cause of death^[1]. More than half of deaths due to infectious diseases occur in developing countries^[2]. The consequences of infectious diseases are notably: an increase in morbidity and mortality; an increase in health care costs caused by longer hospitalizations, and the need to use more expensive and often more toxic drugs^[3]. Thus, an interesting pathway for the search for new active compounds could be the traditional medicine. Population growth and limited access to pharmaceutical drugs in developing countries are contributing factors to increased demand for traditional medicines^[4-5]. In Africa, this demand is due to limited access to adequate care facilities; on the other hand, conventional medicine, which, is very often considered as the appropriate method of treatment, is very expensive to the common people. Given this therapeutic orientation, our interest fell on *Bidens pilosa* (Asteraceae). This plant has long been used in traditional African medicine for its healing properties and ability to treat diseases such as dysentery, diarrhea, typhoid fever and dermatitis^[6]. The present study was aimed at identifying the major chemical groups of the aqueous and hydroethanol extracts of *Bidens pilosa* and at determining the concentrations capable of inhibiting tested fungi and bacteria (Gram-positive and Gram-negative).

Materials and Methods

The whole plant of *Bidens pilosa* was harvested on January 18, 2018 in Batela, a locality within Bagangté, the head quarter of the NDE division in the West region of Cameroon. Samples were identified at the Cameroon national herbarium by Mr. Ngansop as *Bidens Pilosa*. L (Asteraceae) in comparison with those of Letouzey (No. 3417) and Malzy (No. 302)

registered at the National Herbarium respectively under No.487/SRFk and No.14921/SRF/Cam. The antibacterial and antifungal activities were evaluated according to the micro dilution method as described in the Clinical Laboratory Standard Institute guidelines [7].

We worked with seven microbial strains including Gram-positive and Gram-negative bacteria and yeasts (*Staphylococcus aureus* ATCC43300, *Shigella flexneri* NR518, *Salmonella typhi* ATCC 6539, *Salmonella enterica* NR 13555, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* NR 592 and *Candida albicans* NR29451) some of which have known resistance to conventional antibiotics.

These strains were provided by the laboratory of microbiology and phytochemistry of the University of Yaoundé I. The media used were prepared following the procedure prescribed by the manufacturer: Muller Hinton Agar Agar (MHA) for the activation of bacterial strains and Nutrient Broth for the determination of Minimal Inhibitory, Bactericidal and Fungicidal Concentrations. The whole plant of *Bidens pilosa* was harvested, cleaned and dried under the shade at room temperature for two weeks, then pulverized with a mechanical grinder. Three hundred grams of powder were macerated in 1600 ml of the ethanol / distilled water mixture (70/30, V / V) and in 3 L of distilled water for 72 hours, the solvent being renewed every 24 hours. For the hydroethanol extract, the filtrate obtained was evaporated at 70 °C in a rotary evaporator in order to remove the ethanol whereas the aqueous phase was dried in the oven at 40 °C to obtain the dry extract.

Phytochemical screening was carried out following the method of N' Guessan *et al* (2009) [8]. We prepared an aqueous solution of our extracts at 1%. One gram (1 g) each extract was weighed into a beaker and the water was gradually being added up to 100 milliliters while stirring; the whole was homogenized under magnetic stirrer until complete dissolution of the extracts in distilled water.

Polyphenols identification test

The test was carried out with ferric chloride (FeCl₃). In two test tubes containing respectively 2 mL of extract and 2 mL of distilled water, we added four drops of a solution of 10% diluted iron per chloride. The development of a greenish color indicated the presence of polyphenols.

Tannin identification using Stiasny reagent (chloridic acid / Formalin) at 30%

Two milliliters of extract were mixed with 15 ml of Stiasny reagent, the whole mixture was warmed in a water bath at 80 °C for 30 minutes. The development of a precipitate in the form of a flake showed the presence of catechin tannins.

Identification of Gallic tannins

The previous solution was filtered and the filtrate was saturated with sodium acetate and then 3 drops of ferric chloride were added. The appearance of an intense black blue color indicated the presence of Gallic tannins

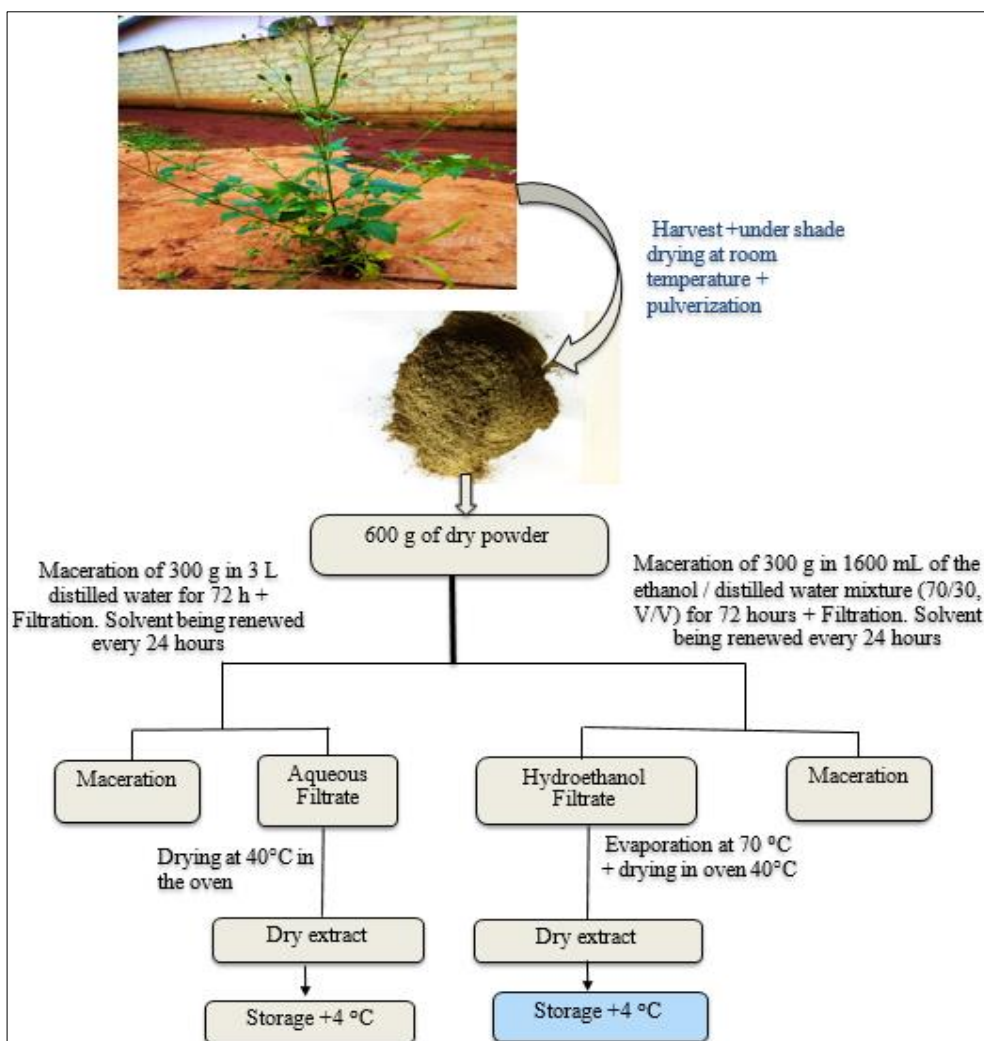


Fig 1: Diagram showing the extraction process of *Bidens pilos*

Flavonoid identification using Shinoda test

Two mL of the extract were mixed with 1 mL of 50% methanol. Magnesium chips were added, followed by a few drops of concentrated hydrochloric acid. An effervescent exothermic reaction had to occur. The appearance of an intense pink-orange or purplish coloration indicated the presence of flavonoids.

Alkaloid Identification using Mayer Test

We put 2 mL of the extract solution in the test tube and 2 mL of distilled water in the control tube. Subsequently we added 3 drops of Mayer reagent to each tube. The development of a creamy white or yellow-white precipitate indicated the presence of alkaloids.

Anthocyanin identification test

In a test tube containing 0.1 g of extract, dissolved in 2 ml of distilled water, the introduction of 2 ml of concentrated sulfuric acid caused the appearance of a red-orange color, which then turned to purple blue upon the addition of ammonia, indicating the presence of anthocyanins.

Saponoside identification using foam test.

We put 2 mL of the extract solution in the test tube and 2 mL of distilled water in the control tube. Then the test tube was vigorously shaken for 15 minutes lengthwise. After shaking, it was allowed to settle for 15 min. The persistence of the foam of a height greater than 1 cm indicated the presence of saponins.

Identification of anthraquinones using Bornträger test

In a test tube, 1 mL of extract was introduced to which 1 mL of 1/5 diluted HCl was added; we then heated the mixture in a water bath for 20 minutes, after cooling, it was extracted with 20 mL of chloroform. To the organic phase, 0.5 mL of 25% NH₄OH was added. The appearance of a red or purple color indicated the presence of anthraquinones.

Identification of sterols and triterpenes using Liebermann Burchard test

A quantity of extract (5 mg) was dissolved in a test tube containing 1 ml of methanol, and 0.2 ml of each of the following reagents: chloroform, glacial acetic acid, concentrated H₂SO₄; the appearance of an intense red color which turned to purple and then to green or blue indicated the presence of triterpenes and sterols respectively.

Identification of coumarins

In a test tube, 2 mL of extract and 6 mL of chloroform were successively introduced; then the tube was sealed and allowed to stand for 15 minutes. After filtration and evaporation in a water bath, we added 1 ml of 10% NH₄OH. Then we observed the mixture under UV at 365 nm. Intense fluorescence in the tube indicates the presence of coumarins.

Mucilage identification test

Two millilitres of extract and 2 mL of distilled water were respectively introduced into the test and the control tubes. A volume of absolute ethanol (5mL) was added to each tube. Development of a fluffy precipitate by mixing indicated the presence of mucilage.

The High Performance Liquid Chromatography analysis

The samples were prepared in acetonitrile at a concentration of 250 µg/mL. A volume (5 µL) was injected into the system. The separations were performed using a Synergi MAX-RP 100A column (50 x 2 mm, particle size 2.5 µ) with a gradient H₂O (+ 0.1% HCOOH) (A) / acetonitrile (+0.1 % HCOOH) (B) (flow rate 500 µL / min). The samples were analyzed using a gradient program as follows: 95% isocratic for 1.5 min, linear gradient at 100% B for 6 min, after 100% B isocratic for 2 min, the system returned to its initial state (95% A) 1 min, and was equilibrated during 1 min. The Mass Spectrometer coupled to the apparatus made it possible to obtain the mass spectra of the various compounds. The spectrometer was connected to an Ultimate 3000 HPLC system (Thermo Fisher, USA) consisting of an LC pump, a diode array detector (DAD) (λ: 215, 254, 280, 330 nm), auto sampler and the column oven (50 °C). High resolution mass spectra were obtained with a QTOF spectrometer (Broker, Germany) equipped with a high resolution ESI source. The spectrometer operated in positive mode (mass range: 100-1500, with a scanning frequency of 1.00 Hz) providing high precision mass measurements with a deviation of 2 ppm using Na Formate as a standard. The following parameters were used for the experiments: 4.5 kV sputtering voltage, 200 °C capillary temperature. Nitrogen was used as cladding gas (4 L / min).

Antimicrobial assay

The MHA medium was used for the activation of the strains and Nutrient Broth for the different antibacterial tests. These media were prepared according to the instructions prescribed by the manufacturer by dissolving 1.3 g of powder in 100 mL of distilled water. Nutrient Broth medium was then sterilized by autoclaving at 121 ° C. ± 1 ° C. for 15 minutes. Extracts were prepared at a concentration of 100 mg / mL in sterile distilled water. As a result, 300 mg of extracts were weighed and dissolved in 3 ml of distilled water and stirred until completely dissolved. The standard drugs were ciprofloxacin and fluconazole for bacteria and yeasts respectively. These were prepared at a concentration of 2 mg / mL in 0.1 N HCl for ciprofloxacin and distilled water for fluconazole. The strains were activated by subculture on Mueller Hinton Agar agar then incubated for 24 h to obtain fresh colonies. Few previously activated bacterial and fungal colonies were removed with a wire loop and suspended in sterile physiological water until a turbidity visually matched with 0.5 Mac Farland standards (bacterial concentration of 1.5x10⁸ CFU/mL and yeast concentration of 2.5 x10⁶ CFU/mL). The suspension afterwards was adjusted to the final density required for the antimicrobial tests according to the specifications of the Committee of the antibiogram of the French Society of Microbiology. The concentrations of inoculum used were 5x10⁶ bacteria/mL and 5x10⁴ yeasts/mL.

Resultants and Discussion

Phytochemical screening and identification of some compounds

The extraction yields obtained by maceration of 600 g of whole plant powder were 16.8% and 15.5% for water and hydroethanol (70: 30; V / V), respectively.

The results of phytochemical screening are recorded in the table 1. It revealed the presence of polyphenols, flavonoids, coumarins, saponosides, triterpenes, sterols, alkaloids and mucilages in both water and hydroethanol extracts.

Table 1: Qualitative phytochemical composition of whole plant extracts of *Bidens pilosa*

	Test	Water extract	Hydroethanol extract
Polyphenols	Ferric chloride 10 %	+	+
Flavonoids	Shinoda	+	+
Catechic tannins	Stiany	-	+
Gallic tannins	Fe Cl ₃ 2%	-	-
Coumarins	NH ₄ OH 10%	+	+
Saponoside	Foam	+	+
Anthocyanins	H ₂ SO ₄ et NH ₄ OH	-	+
Triterpenes and sterols	Liebermann –Burchard	+	+
Quinones	NaOH 10%	+	-
Alcaloids	Mayer	+	+
Mucilage		+	+

Legend: + (presence), - (absence)

Analysis of MS and UV spectral data in comparison with what is documented in the literature led to the identification of three compounds notably Ts1, Ts2 and Ts3. Indeed, the mass spectrum of Ts1 shows the molecular ion at m/z 305.24 $[M + H]^+$ which corresponds to the taxifolin with the molecular formula, C₁₅H₁₂O₇ and molar masse of 304.24 g/mol (Figure 1); This compound has previously been isolated by Chen *et al* (2016) [9]. On the other hand, the mass spectrum of Ts2 shows a molecular peak at m/z 421.34 $[M + Na]^+$, a base peak at m/z = 399.35 $[M + H]^+$ and corresponds to Brassicasterol (Figure 2); The compound, Brassicasterol with a molar mass $M = 398,35$ g/mol was recently isolated from two species of Asteraceae by Babota *et al* (2018) [10]. The mass spectrum of Ts3 shows the molecular ion at m/z 621.30 $[M + H]^+$ which corresponds to acetyl-vitexin-rhamnoside with empirical formula, C₃₅H₅₆O₉ (Figure. 3). This compound with $M = 620, 30$ g/mol shared the same characteristics with the one isolated from another Asteraceae family in 2007 by Bilia *et al* [11].

Antibacterial and antifungal properties of *Bidens pilosa* extracts

The Minimal Inhibitory Concentrations of *Bidens pilosa* extracts

The antimicrobial activities of the plant extracts, ciprofloxacin and fluconazole were tested on seven reference strains (six bacteria and one yeast) as described in the methodology section.

At the tested concentration of 20 mg/mL, the aqueous extract of *Bidens pilosa* showed no activity against *Staphylococcus aureus* ATCC43300, *Salmonella typhi* ATCC 6539, *Salmonella enterica* NR 13555, and *Pseudomonas aeruginosa*

NR 592 but was active against *Escherichia coli* ATCC 25922, and *Candida albicans* NR29451 with a MIC of 20 mg / mL and 10 mg / mL respectively. This result differs from the work of Owoyemi *et al* (2017) and those of Deshnee *et al* (2013) [12-13] who worked on the same microorganisms and obtained minimal inhibitory concentrations between 50 and 100 mg / mL respectively. In contrast, the work of Yaouba *et al* (2017) and those of Owoyemi *et al* (2017) [14-12] show a sensitivity of the aqueous extract against *Staphylococcus aureus*, *Shigella flexneri*, *Salmonella enterica*, *Salmonella typhi* and *Pseudomonas aeruginosa* with concentrations ranging from 50 mg / ml to 512mg / mL. These differences could be explained by the use in their work of the 90% ethanol recognized for its bacteriostatic properties, during the extract of the secondary metabolites.

On the other hand, the hydroethanolic extract was active against all the tested microorganisms with MIC ranging from 2.5 mg / mL to 20 mg / mL. These results are in agreement with those of Owoyemi *et al* (2017); Lawal *et al* (2015) then Deshnee *et al* (2013) [12-15-13] who worked on a methanol extract of dry leaves and same microbial strains and obtained minimal inhibitory concentrations ranging from 0.6 to 50 mg / mL. In addition, it is interesting to notice that the hydroethanolic extracts also exhibited activities both against Gram positive and Gram negative organisms, revealing thereby the broad spectrum action of *Bidens pilosa* extracts. Generally, the antibacterial and antifungal activities are justified by the presence of terpenes, sterols and especially the abundance of flavonoids in the crude extracts of *Bidens pilosa*.

The results on the antimicrobial properties of *Bidens pilosa* extracts are summarized in Table 2.

Table 2: MIC (mg/mL) of crude extracts from *Bidens pilosa*

Strains	Water Extract	Hydroethanolic extract	Ciprofloxacin and Fluconazole
<i>Staphylococcus aureus</i> ATCC43300	>20	5	0.064
<i>Shigella flexneri</i> NR 518	>20	10	0.064
<i>Salmonella enterica</i> NR13555	>20	10	0.064
<i>Salmonella typhi</i> ATCC 6539	>20	10	0.064
<i>Escherichia coli</i> ATCC 25922	20	10	0.064
<i>Pseudomonas aeruginosa</i> NR 592	>20	10	0.064
<i>Candida albicans</i> NR29451	10	2,5	0.064

MIC: Minimal Inhibitory Concentration

Minimal Bactericidal Concentration (MBC) and Minimal Fungicidal Concentration (MFC) of *Bidens pilosa* extracts

The MFC of the water extract on *Candida albicans* is greater than 20 mg/mL whereas the MBCs were not determined as the MICs were greater than 20 mg / mL. The hydroethanol

extract registered a MBC of 10 mg/mL for *Staphylococcus aureus*; 20 mg/mL for *Shigella flexneri* and *Candida albicans* but greater than 20 mg/mL for *Salmonella enterica*, *Salmonella typhi*, *Escherichia coli* and *Pseudomonas aeruginosa*.

Table 3: MBC (mg/mL) and MFC (mg/mL) of crude *Bidens pilosa* extracts

Strains	Water extract	Hydroethanolic extract	Ciprofloxacin and Fluconazole (mg/mL)
<i>Staphylococcus aureus</i> ATCC43300	nd	10	0.064
<i>Shigella flexneri</i> NR 518	nd	20	0.064
<i>Salmonella enterica</i> NR13555	nd	>20	0.064
<i>Salmonella typhi</i> ATCC 6539	nd	>20	0.064
<i>Escherichia coli</i> ATCC 25922	>20	>20	0.064
<i>Pseudomonas aeruginosa</i> NR 592	nd	>20	0.064
<i>Candida albicans</i> NR29451	>20	20	0.064

CMB: Minimal Bactericidal Concentration; CMF: Minimal Fungicidal concentration, nd: not determined

Determination of the MBC/MIC and MFC/MIC ratios of *Bidens pilosa* extracts

The MBC/MIC and MFC/MIC ratios are 2 and 8 respectively (Table 4), thereby indicating the bactericidal and fungistatic property of hydroethanolic extract.

Table 4: MBC/MIC and MFC/MIC ratios of *Bidens pilosa* extracts

Strains	Water extract	Hydroethanolic extract	Ciprofloxacin and Fluconazole
<i>Staphylococcus aureus</i> ATCC43300	/	2	1
<i>Shigella flexneri</i> NR 518	/	2	1
<i>Salmonella enterica</i> NR13555	/	/	1
<i>Salmonella typhi</i> ATCC 6539	/	/	1
<i>Escherichia coli</i> ATCC 25922	/	/	1
<i>Pseudomonas aeruginosa</i> NR 592	/	/	1
<i>Candida albicans</i> NR29451	/	8	1

MFC: Minimal Fungicidal concentration; MBC: Minimal Bactericidal Concentration

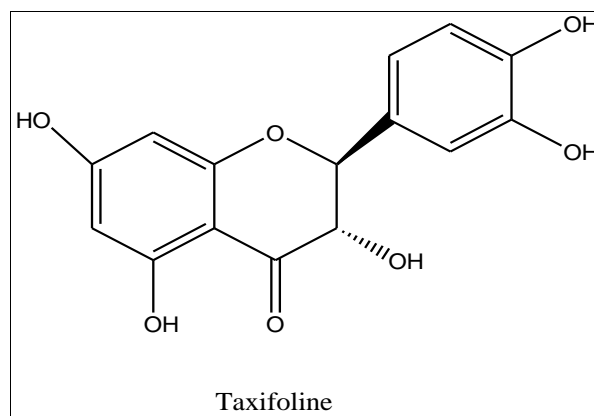
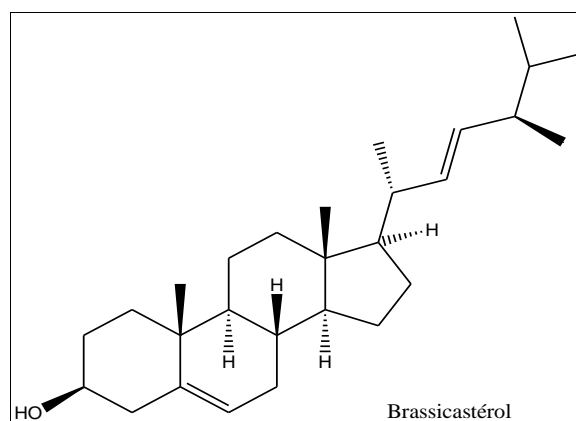
Conclusions

The objectives of the present study were to phytochemically screen the water and hydroethanol crude extracts of *Bidens pilosa* and to evaluate its antibacterial and antifungal properties. The chemical screening of the plant revealed the presence of polyphenols, flavonoids, coumarins, triterpenes, sterols, alkaloids and saponosides in both extracts. However, the absence of tannins and anthocyanins in the aqueous extract and Gallic tannins, and quinones in the hydroethanolic extract was observed. HPLC-MS analysis coupled with the comparison of the molecular weights of each peak as documented in the literature, revealed the presence of three flavonoids (taxifolin, acetyl-vitexin-rhamnoside and apigenin 7-sulfate) and a sterol (brassicasterol).

The aqueous extract was active against *Escherichia coli* and *Candida albicans*. However, the hydroethanolic extract was active against all the tested microbial strains at different concentrations. The MBC/MIC and MFC/MIC ratios attest that the hydroethanolic extract was bactericidal against *Staphylococcus aureus* ATCC43300 and *Shigella flexneri* NR 518 but fungistatic against *Candida albicans*.

The current evidence indicates that the presence flavonoids, sterols, and sesquiterpenes as revealed by HPLC-MS may be responsible for the antibacterial and antifungal properties of *Bidens pilosa*.

The identified moschamin is an indole alkaloid. Alkaloids are generally known to exhibit analgesic, tranquilizing and healing properties. All these properties could justify the use of *Bidens pilosa* in traditional medicine.

**Fig 1:** Tax if oline**Fig 2:** Brassi casterol

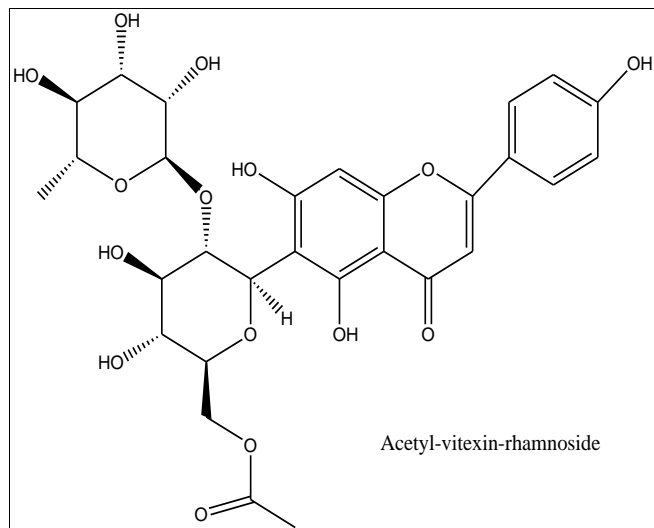


Fig 3: Acetyl-vitexin-rhamnoside

12. Owoyemi OO, Muftau KO. Phytochemical Screening and Antibacterial Activities of *Bidens pilosa* L. and *Tridax procumbens* L. on Skin Pathogens. *International Journal of Modern Biology and Medicine*. 2017; 8(1):24-46.
13. Deshnee N. Safety and efficacy of traditional medicinal plant combinations for the treatment of sexually transmitted infections in Northern Maputaland, South Africa [Thèse]. Master of Pharmacy: University of the Witwatersrand, 2013, 21.
14. Yaouba A, Tchikoua R, Tatsadjieu NL. Antibacterial effect of plant extracts against some pathogenic bacteria. *International Journal of Natural Products Research*. 2012; 4(1):83-7.
15. Lawal OA, Amisu KO, Akinyemi SK, Sanni AA, Simelane BC, Mosa RA, *et al.* *In vitro* Antibacterial Activity of Aqueous Extracts of *Bidens pilosa* L. (Asteraceae) from Nigeria. *British Microbiology Research Journal*. 2015; 8(4):525-31.

References

1. Desenclos J-C, De Valk H. Les maladies Infectieuses émergentes: importance en santé publique, aspects épidémiologiques, déterminants et prévention. *Médecine ET maladies infectieuses*. 2005; 35(2):49-61.
2. World Health Organization. Meeting of the Strategic Advisory Group of Experts on Immunization. *Weekly Epidemiological Record*. 2012; 87(21):201-16.
3. Carle S. La résistance aux antibiotiques: UN enjeu de santé publique important *Pharmactuel*. 2010; 42(1):6-20.
4. Ladoh-Yemeda CF, Vandi D, Dibong SD, Mpondo M pondo E, Wansi JD, Betti JL, *et al.* Étude ethno botanique des plantes médicinales commercialisées dans les marchés de la ville de Douala, Cameroun. *Journal of Applied Biosciences*. 2016, 9450-68.
5. Mammeri H, Van De Loo M, Poirel L, Martinez-Martinez L, Norman P. Emergence of Plasmid-Mediated Quinolone Resistance in *Escherichia coli* in Europe. *Antimicrobial Agents and Chemotherapy*. 2005; 49(1):71-6.
6. Kouakou LK, Traore F, Abo JC, Ehouan EE Effets pharmacology d'un extrait aqueux de *Bidens pilosa* L. (Asteraceae) sur le système cardiovasculaire de mummifiés *Afrique Science*. 2007; 3(2):284- 304.
7. CLSI, Performance standards for antimicrobial susceptibility testing; Fifteenth informational supplement. NLSI document M100-S25. 2015; 35(3):1-16.
8. N' Guessan K, Kadja B, Zirih G, Traoré D, Aké-Assi L. Screening phytochimique de quelques plantes médicinales ivoiriennes utilisées en pays Krobou (Agboville, Côte-d'Ivoire). *Sciences & Nature*. 2009; 6(1):189-220.
9. Chen G, Li X, Saleri F, Guo M. Analysis of Flavonoids in *Rhamnus davurica* and Its Ant proliferative Activities. *Molecules*. 2016; 1275(21):2-14.
10. Babota M, Mocan A, Vlase L, Cris O, Ielciu I, Gheldiu AM, *et al.* Phytochemical Analysis, Antioxidant and Antimicrobial Activities of *Helichrysum arenarium* (L.) Moench. And *Antennaria dioica* (L.) Gaertn. Flowers. *Molecules*. 2018; 409(23):2-15.
11. Bilia AR, Eterno F, Bergonzi MC, Giovanni M, Vincieri FF. Evaluation of the content and stability of the constituents of mother tinctures and tinctures: The case of *Crataegus oxyacantha* L. and *Hieracium pilosella* L. *Journal of Pharmaceutical and Biomedical Analysis*. 2007; 44:70