International Journal of Phytomedicine 3 (2011) 182-191

http://www.arjournals.org/index.php/ijpm/index

Original Research Article

ISSN: 0975-0185

In vitro evaluation of the antimicrobial and antioxidant properties of extracts from whole plant of *Alternanthera pungens* H.B. & K. and leaves of *Combretum sericeum* G. Don

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Abstract

Resistance to current antimicrobial agents continues to increase and render difficult the fight against microbial diseases. There is an urgent need to find new disposable and affordable remedies to face this problem. Plants have been used for centuries for health cure and constitute an important source of drugs.

In this study, the aqueous, water-ethanol, and water-acetone extracts of *Alternanthera pungens* H. B. & K. and *Combretum sericeum* G. Don were evaluated for antimicrobial and antioxidant activities. The antimicrobial activity was evaluated by disc diffusion and microdilution assays. Antioxidant Activity Index (AAI) was determined for antioxidant activity evaluation.

The extracts from the leaves of *C. sericeum* exhibited potent antimicrobial activity. The highest inhibition zone diameters (IZD) were obtained with these extracts. The lowest minimum inhibitory concentration (MIC) (0.625 mg/ml) was recorded against Enterococcus faecalis for all the extracts from this plant and against Lysteria Monocytogenes for the water-acetone extract only. *C. Sericeum* showed also strong antioxidant activity with AAI comparable to standard antioxidant compounds such as Vitamin C and BHA. A. Pungens showed weak antimicrobial and antioxidant activities, comparatively to *C. Sericeum*. Phytochemical analyses showed also high total phenolic and total flavonoid contents in *C. Sericeum* extracts than in A. pungens. The use of these plants in traditional medicine is justified and they constitute a source for further investigations for traditional enhanced drug and active molecules discovery.

Keywords: *Combretum sericeum*, *Alternanthera pungens*, antimicrobial activity, antioxidant activity index.

Introduction

Antimicrobial resistance is one of the world's most serious public health problems. Many of the microbes (bacteria, viruses, protozoa) that cause infectious disease no longer respond to common antimicrobial drugs [1]. There is an urgent need to find new efficient antimicrobial drugs [2]. This situation has increased the interest for medicinal plants from traditional medicine. These plants have been used for centuries as remedies for human diseases and continue to do important services at human being. The widespread use of

traditional medicine particularly by the poorest patients in the world can have several explanations: i) the high cost of pharmaceutical drugs for many people in the developing countries; ii) the emergence of multiple drug resistant pathogens with major disease outbreaks which highlighted mortality and morbidity particularly in developing countries [3]; iii) natural plant products have served as a very important replacement for the resistant strains [4]; iv) socio-cultural effect of traditional medicine over many people in the world; v) in many countries, less than half of the population has access to public health services [5]. Thus, in some Asian and African countries, 80% of the population depends on traditional medicine for primary health care [6].

Meanwhile, there is a lack of scientific data on the efficiency, the toxicity and the harmlessness of most of plants used traditionally as medicines. The evaluation of the rich heritage of traditional medicine is essential [7] and can lead to the discovery of new active molecules against pathogens or lead to ameliorated traditional drug which is cheaper, efficient and available for the population.

Combretum sericeum G. Don (Rubiaceae) and *Alternanthera pungens* H. B. & K. (Amaranthaceae) are two plants used in traditional medicine in Burkina Faso and many countries in the world. *C. sericeum* is used to combat diarrhea, cough, dysentery, pneumonia and fever [8]. As to, A. pungens is used traditionally against dysentery, venereal diseases, cholera, and many parasitic diseases [9, 10]. It is interesting and important to verify whether their traditional uses are supported by scientific data or merely based on folklore.

In the present work, the main goal was to investigate the antimicrobial activity and the antioxidant properties of some extracts from these two medicinal plants.

Material and Methods

Plant Material

The leaves of *Combretum sericeum* and whole plant of *Alternanthera pungens* were collected near Sapone, a village located 45 km south of Ouagadougou in March 2009. Their selection was based on their use by traditional healers and ethnobotanical survey data given by the literature. The plants were first identified by a local traditional healer and taxonomically authenticated at the Department of Plant Biology and Ecology of the University of Ouagadougou, Burkina Faso.

The collected plant parts were carefully dried until total dryness at ambient temperature in the laboratory under continuous ventilation, away from sun light and dust. The samples were then powdered with a mechanical crusher and the powder was hermetically sealed in polythene bags and stored away from light and moisture at room temperature until use.

Chemicals, Reagents and Media

Quercetin and 1,1-Diphenyl-2-picryl hydrazyl (DPPH) and Folin Ciocalteu reagent were purchased from Fluka Chemika (Switzerland). Gallic acid, Butylated Hydroxyanisole (BHA) and the other chemicals were from Sigma-Aldrich (St Louis, MO,USA). All solvents were analytical grade. Plate Count Agar (PCA), Mueller-Hinton Agar and Broth (MHA and MHB), Potato Dextrose Agar and Broth (PDA and PDB) were purchased from Liofilchem, Italy

Microbial Strains

Nine referenced bacterial strains including Gram-negative and Gram-positive and one fungal strain were used in this study. These microorganisms were Enterococcus faecalis CIP 10907, Shigella dysenteriae 5451 CIP, Proteus mirabilis ATCC 35659, Salmonella enterica CIP 105150, Enterobacter aerogenes CIP 104725, Bacillus subtilus ATCC 21332, Listeria monocytogenes CRBIB 13134, Pseudomonas aeruginosa ATCC 19249, Staphylococcus aureus ATCC 9144 and

Candida albicans. Most of these microorganisms are implicated in infectious diseases in Burkina Faso.

Crude Extracts Preparation

Water-ethanol (30/70, v/v) extract, water-acetone (30/70, v/v) extract and water (100%) extract were prepared from dry powder. 25 g of powder from each sample were soaked with 250 ml of the appropriate solvent mixture and left under shaking conditions at room temperature (25 to 30 °C) for 24 h. Water extract was prepared by decoction mixing 25 g of powder to 250 ml of distilled water. The mixture was boiled for 1 hour. Each extract was filtered using Whatman N°1 filter paper and solvents were completely removed at low pressure with a rotary evaporator (BÜCHI, Labortechnik, Switzerland). The extracts were then concentrated, freeze-dried and stored at 4°C until analysis.

Antibacterial Susceptibility Testing

The antibacterial susceptibility of the extracts was evaluated by agar disc diffusion method [11, 12]. Tested bacterial strains were grown in MHB for 18 to 24 h at 37°C and adjusted to 10^8 cells/ml with reference to the 0.5 Mac Farland standard turbidometry [13, 14].These inocula were used to inundate sterile Petri dishes (90 mm diameter) containing 15 ml MHA in order to prepare lawn cultures. The sterile filter paper discs (6 mm diameter) were impregnated with 10 µl corresponding to 1000 µg of each extract dissolved in DMSO at 100 mg/ml and filtered with a Millipore filter (0.22µm) for sterilization. Then, the discs were placed on lawn cultures and the Petri dishes were subsequently incubated at 37°C for 24 h. Gentamicin (10µg) and Ciprofloxacin (5µg) (Liofilchem, Italy) were used as the positive control. Discs impregnated with DMSO were also included to the assay as control. The results were recorded by measuring the inhibition zone around each disc. All tests were performed in duplicate and the antibacterial activity was expressed as the mean of diameters of inhibition zone produced.

Minimum Inhibitory Concentration (MIC)

MIC of the extracts was determined using the broth microdilution method [15]. The inocula of the bacterial strains were prepared as described above. The extracts were first dissolved in 10% DMSO and then sterilized as described previously. 100 µl of this stock extract solution were transferred into the first well of a 96-well sterile plate (Cellstar, Greiner Labortechnik, Germany) previously filed with 100 µl of nutrient broth. Serial twofold dilutions were made in to 11 consecutive wells. Into each well 95 µl of nutrient broth and 5 µl of the bacterial inocula were added to achieve concentrations of extracts ranging from 5 to 0.0049 mg/ml. The final volume in each well was 200 µl. Growth control wells and sterility control wells were included in the plate. Each plate was mixed on a plate shaker at 300 rpm for 20 seconds and then incubated at 37°C for 24 h. Bacterial growth was indicated by the presence of turbidity and a pellet on the well bottom. The MIC is the lowest extract concentration demonstrating no visible growth in the broth.

Minimum Bactericidal Concentration (MBC)

The MBC is the lowest concentration of the extract at which 99.99% or more of the initial inoculum was killed. The MBC was determined using results from the MIC test as described previously [12, 16]. 100 µl from each well demonstrating no visible growth were removed to spread onto Petri dishes filed with sterilized PCA medium. Petri dishes were incubated at 37°C for a total period of 48 h. The lowest concentration of the extract which showed no bacterial growth was noted and recorded as the MBC.

Antifungal Sensitivity Test

The extracts were screened for antifungal activities against Candida albicans. This test was performed by disc diffusion method and microdilution technique [17] with minor modifications. The discs (6 mm in diameter) were saturated with 10 µl (1000 µg) of the extracts separately, allowed to dry and were placed on Petri dishes containing PDA and inoculated with

the fungal strain. The Petri dishes were incubated at 28°C for 48 to 96 h. Standard disc of Nystatin (100IU) (Liofilchem, Italy) was used as positive control. MIC and MFC (minimal fungicidal concentration) were assessed as described above.

Antioxidant Activity Index

The Antioxidant Activity Index (AAI) was assessed according to the method described by Scherer and Godoy [18]. This method is based on the DPPH radical test. Briefly, the working reagent was prepared by dissolving 10 mg of DPPH in 100 ml ethanol. Graded concentrations of extracts ranging from 0.781 to 100 µg/ml obtained by two-fold dilutions were prepared and 100 µl of each dilution were mixed with 100µl of the working solution of DPPH in a 96-well plate. Absorbancies were measured at 517 nm after 15 min incubation at room temperature in the dark. Ascorbic acid (Vitamin C) and Butylated Hydroxyanisole (BHA) were used as references. The ability to scavenge DPPH radical was calculated by the following equation:

$$\%RSA = \frac{Acontrol - Asample}{Acontrol} X100$$

A = Absorbance at 517 nm

The IC_{50} (concentration providing 50% inhibition) of extracts and standards was determinate using regression curves in the linear range of concentrations. The AAI was then calculated as follows:

$$AAI = \frac{[DPPH](\mu g/mL)}{IC_{50}(\mu g/mL)}$$

[DPPH] is the final concentration of DPPH.

We considered criteria of Scherer and Godoy [18] according to which plant extracts show poor antioxidant activity when AAI < 0.5, moderate antioxidant activity when AAI between 0.5 and 1.0, strong antioxidant activity when AAI between 1.0 and 2.0, and very strong when AAI > 2.0.

Phytochemical screening

Crude extracts were screened for, Alkaloids, Coumarins, Flavonoids, Tannins, Anthracenosides, Anthocyanosides, cardiac glycosides, Saponosides, terpenes and steroids detection using standard procedures of analysis [19, 20, 21]

Total Phenolic Content

The total phenolic contents of the different extracts were determined according to the Folin-Ciocalteu Method [22] adapted to a 96 well-plate [23] with minor modifications as described by [14] using gallic acid as standard. The absorbance was measured at 735 nm using a multiwell plate reader (µQuant Bio-Tek Instrument, Inc, USA). All analyses were done in triplicate and results (average of triplicate analysis) were expressed as gallic acid equivalent per gram of lyophilized sample.

Total Flavonoid Content

Total flavonoid contents were determined by the aluminium chloride ($AlCl_3$) colorimetric assay method [24] adapted to 96 well-plate, using quercetin as a standard [14]. The total flavonoid contents (average of the triplicate analysis) were expressed as Quercetin equivalents in milligrams per gram sample.

Results

The inhibition zone diameters (IZD) obtained in the antimicrobial susceptibility assays for the plants extracts and standard antimicrobial drug discs are presented in **Table 1**. The highest IZD was recorded with the water-ethanol extract of *C. sericeum* (CsWEE) against E. faecalis (20 mm) followed by water extract (CsWE) against the same microorganism (18 mm). With the exception of Pseudomonas aeruginosa, all tested bacteria showed sensitivity to both plants extracts but the efficiency of the extracts in inhibition was varied from one microorganism to another and also depends on type of extract and plant. A. pungens extracts exhibited lesser inhibitory effect comparatively to those of C. sericeum. Contrary to *C. sericeum* extracts, A. pungens extracts did not show antifungal activity.

Table 1: Inhibition zone diameters (mm) produced by the extracts from *C. Sericeum* and *A. Pungens* in disc diffusion method.

	Diameters of Inhibition zone (mm)								
	Extracts						Standards		
bacteria	**CsWAE**	**CsWEE**	**CsWE**	**ApWAE**	**ApWEE**	**ApWE**	**Cipro**	**Genta**	**Nyst**
Enterococcus faecalis 10907 CIP	14	20	18	10	8	8	34	30	Nd
Shigella dysenteriae 5451 CIP	8	13	10	8	7	7	26	24	Nd
Proteus mirabilis ATCC 35659	12	11	10	9	7	7	28	Nd	Nd
Salmonella enterica CIP 105 150	12	10	9	10	8	7	34	28	Nd
Enterobacter aerogenes CIP 104 725	10	11	12	8	8	8	8	20	Nd
Bacillus subtilus ATCC 21 332	12	12	10	10	11	8	32	28	Nd
Listeria monocytogenes CRBIB 13 134	14	14	10	8	8	0	30	26	Nd
Pseudomonas aeruginosa ATCC 19249	8	8	8	0	0	0	30	20	Nd
Staphylococcus aureus ATCC 9144	12	10	8	8	7	7	29	29	Nd
Fungi									
Candida albicans	10	10	10	0	0	0	Nd	Nd	20

Nd = not determinated; Cipro = Ciprofloxacin (5µg); Genta = Gentamicin (10 µg); Nyst = Nystatin (100IU); Cs = C. Sericeum; Ap = A. Pungens; WAE = water-acetone extract; WEE = water-ethanol extract; WE = water extract.

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) or fungicidal concentration (MFC) in mg/ml obtained by microdilution method.

Bacteria	**CMI and CMB (mg/ml)**											
	CsWAE		**CsWEE**		**CsWE**		**ApWAE**		**ApWEE**		**ApWE**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Enterococcus faecalis 10907 CIP	0.625	2.5	0.625	2.5	0.625	2.5	5	>5	>5	>5	>5	>5
Shigella dysenteriae 5451 CIP	2.5	5	2.5	5	5	>5	>5	>5	>5	>5	>5	>5
***Proteus mirabilis* ATCC 35659**	2.5	5	5	>5	>5	>5	>5	>5	>5	>5	>5	>5
Salmonella enterica CIP 105 150	2.5	5	5	nd	5	>5	>5	>5	>5	>5	>5	>5
Enterobacter aerogenes CIP 104 725	5	>5	5	>5	>5	>5	5	>5	>5	>5	>5	>5
***Bacillus subtilus* ATCC 21 332**	1.25	1.25	2.5	5	5	>5	5	>5	5	>5	>5	>5
Listeria monocytogenes CRBIB 13 134	0.625	1.25	2.5	5	5	>5		>5	>5	>5	>5	Nd
Pseudomonas aeruginosa ATCC 19249	>5	>5	>5	>5	>5	>5	Nd	Nd	Nd	Nd	Nd	Nd
***Staphylococcus aureus* ATCC 9144**	2.5	5	5	>5	>5	>5	>5	>5	>5	>5	>5	>5
Fungi	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Candida albicans	5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5

Nd = not determinated; Cs = C. Sericeum; Ap = A. Pungens; WAE = water-acetone extract; WEE=water-ethanol extract; WE= water extract.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values summarized in **Table 2** confirmed the antibacterial and antifungal activity of the plants. The lowest MIC (0.625 mg/ml) was recorded with all the extracts of *C. sericeum* on E. faecalis and only the water-acetone extract (CsWAE) on L. monocytogenes. The lowest MBC (1.25 mg/ml) was observed with the CsWAE on B. subtilus and L. monocytogenes.

The antioxidant activities of the extracts are pointed out in **Table 3**. As it can be seen in this table, the AAI of the extracts from *C. sericeum* ranged from 4.59 to 8.26 and can be compared to AAI of Vit C and BHA (AAI values of 10.66 and 7.30 respectively) while those of A. pungens ranged from 0.20 to 0.47. In addition to that, **Figure 1** and **Figure 2** show the DPPH radical inhibition effect of respectively *C. sericeum* extracts and A. pungens extracts in comparison with vitamin C and DHA.

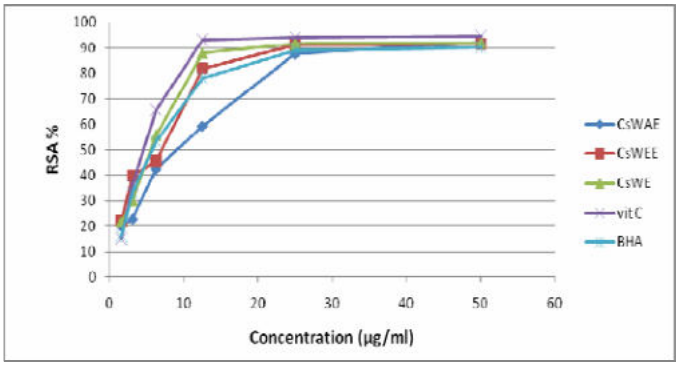

Figure 1: DPPH radical scavenging activity of *C. sericeum* extracts compared to vitamin C and BHA. Cs = C. sericeum; WAE = water-acetone extract; WEE = water-ethanol extract; WE= water extract.

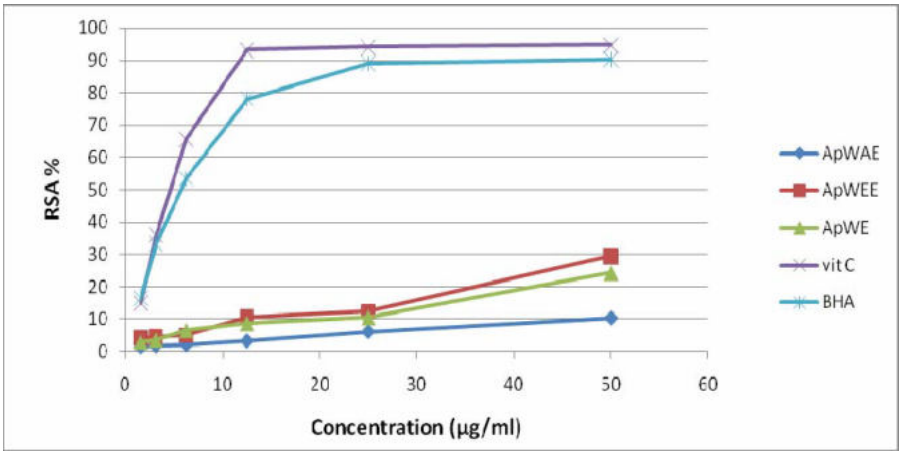

Figure 2: DPPH radical scavenging activity of A. pungens extracts compared to vitamin C and BHA. Ap = A. pungens; WAE = water-acetone extract; WEE = water-ethanol extract; WE=water extract.

Table 3: antioxidant activity of *C. sericeum* and A. Pungens extracts by DPPH free radical scavenging method.

Extracts	Regression curve's equations	R^2	CI_{50} (µg.mL^{-1})	AAI
CsWAE	y = 6.120x + 13.00	R² = 0.989	6.05	8.26
CsWEE	y = 5.351x + 15.05	R² = 0.968	6.53	7.65
CsWE	y = 2.883x + 18.65	R² = 0.969	10.87	4.59
ApWAE	y = 0.401x + 1.214	R² = 0.972	121.66	0.41
ApWEE	y = 0.436x + 3.453	R² = 0.981	106.76	0.47
ApWE	y = 0.201x + 0.942	R² = 0.997	244.07	0.20
Vit C	y = 10.360x + 1.313	R² = 0.993	4.69	10.66
BHA	y = 5.615x + 11.53	R² = 0.966	6.85	7.30

Cs = C. Sericeum; Ap = A. Pungens; WAE = water-acetone extract; WEE=water-ethanol extract; WE= water extract.

Table 4 presents the results of the Preliminary phytochemical screening. This screening attested the presence of several secondary metabolites in the extracts. Alkaloids, an important group of active compounds were not detected in the extracts of both plants. Saponosids were also not detected in all the extracts of C. sericeum. Absence of Flavonoids, anthracenosides and Anthocyanosides were also observed in the water extract of A. pungens.

The content of total phenolics and total flavonoids are presented in **Table 5**. The contents of total phenolic in terms of gallic acid equivalent (standard curve equation: $y = 0.061x + 0.0034$, $R^2 = 0.9982$) ranged from 18.595 to 407.429 mg GAE/100g of lyophilized extract. The high contents were recorded with water-acetone extracts (407.429 mg GAE/100g for CsWAE and 337.362 mg GAE/100g for ApWAE). Total flavonoids (standard curve equation: $y = 0.035x + 0.026$, $R^2 = 0.999$) were also abundant in water-acetone extracts than Water-ethanol and water extracts (**table 5**).

Table 4: Results of the preliminary phytochemical screening.

	Combretum sericeum			*Alternanthera pungens*		
Chemical Groups	**Water-acetone extract**	**Water-ethanol extract**	**Water extract**	**Water-acetone extract**	**Water-ethanol extract**	**Water extract**
Alkaloids	—	—	—	—	—	—
Coumarins	++	++	+	++	++	+
Flavonoids	++	+	+	++	++	—
Tannins	++(gallic)	++ (gallic)	++(gallic)	++(catechic)	++(catechic)	+(catechic)
Anthracenosides	++	—	+	++	++	—
Anthocyanosides	++	+	++	+	+	—
Saponosids	—	—	—	++	++	+ /-
Triterpenoids/ steroids	++	+	++	++	++	+

— = Not Detected; ++ =Abundant; + = not abundant; +/- = very scanty

Table 5: Total phenolic content (TPC) and Total flavonoid content (TFC) by respectively the Folin Ciocalteu assay and aluminium chloride assay of extracts from *C. sericeum* and A. Pungens.

Extracts	TPC (mg GAE/100g)	TFC (mg QE/100g)
CsWAE	407.429	83.4883
CsWEE	287.929	56.269
CsWE	18.595	1.843
ApWAE	337.762	79.873
ApWEE	276.926	49.582
ApWE	60.762	nd

Nd = Not determinated; Cs = C. Sericeum; Ap = A. Pungens; WAE = water-acetone extract; WEE=water-ethanol extract; WE= water extract.

Discussions

The present study was performed to test the in vitro antimicrobial and antioxidant activities of *C. sericeum* and A. pungens. These plants are used in folk medicine for the treatment of many diseases including bacterial diseases, parasitic diseases and diarrhea [25, 9, 10].

The study showed that the different extracts from these plants inhibited the growth of nearly all

microorganisms used in the essay, indicating the presence of antimicrobial compounds in these plants. Preliminary phytochemical screening showed the presence of flavonoids, tannins, anthocyanosides, anthracenosides, coumarins and steroids/triterpenoids. Most of these secondary metabolites have been reported to have multiple medicinal properties [26]. For example, flavonoids are reported to have antiviral, antibacterial, antifungal, antioxidant, anti-inflammatory, oestrogenic, Vascular, antitumor, anticarcinogenic, antimutagenic activities and other biological activities [26, 27, 28]. Likewise, anthocyanosides have a wide range of biological activities including antioxidant, anti-inflammatory, antimicrobial and anti-cancer activities and are able to protect against coronary heart diseases [29, 30]. Commonly, several studies have linked the biological activities of plants to phenolic compounds that they contain [31, 30] Our results show that the extracts with high level of phenolic compounds were also the most active against microorganisms or exhibited higher antioxidant activity. The extract from *C. sericeum* exhibited strong antioxidant activity comparable to standard antioxidant compounds used in this assay contrary to A. pungens extracts which showed poor antioxidant activity (**Figure 1&2**) It is evident that nature and class of these chemical compounds varied from one plant to another and one extract to another. Indeed, more than 8000 flavonoid and 500 anthocyanin structures were reported by the year 2000 [32].That could justify the differences in activity of the different extracts from the same plant or different plants. Indeed, it can be seen in **Table 5** that CsWE with TPC value of 18.595 mg GAE/100g gave higher antimicrobial activity and strong antioxidant activity than ApWE with TPC value of 60.762 mg GAE/100g. Flavonoids, anthracenosides and anthocyanosides which are present in CsWE and absent in ApWE could be the basis of this difference in activity. The presence of revealed compounds in the extracts can so justify their antioxidant, antibacterial and antifungal activities and also justify the use of these plants to treat many diseases in traditional medicine. Alkaloids were not detected with Dragendorff's reagent in our extracts in this study. This group of secondary metabolites is known for medicinal uses for centuries and one of their common biological properties is their toxicity against cells of foreign organisms [33]. Their absence tends to indicate that the antimicrobial and antioxidant activities of both plants could be attributed to phenolic compounds.

Conclusion

As conclusion, this study confirm the multiple uses of *A pungens* and *C. sericeum* for the treatment of many infectious diseases and place them as candidate for further investigations for Enhanced Traditional Drug utilizable as Complementary and Alternative Medicines development and new active compounds discovery.

Authors contribution

Cheikna ZONGO is the main author; **A. SAVADOGO** revised the protocols, the manuscript and provided reference bacterial strains; **M. S. SOMDA** participated to all experiments; **J. KOUDOU** Contributed to protocols elaboration and advised in manuscript preparation and **A. S. TRAORE** is Director of the CRSBAN, supervisor of this work, provided partially financial support.

Acknowledgments

The authors are very much thankful to the International Programme in the Chemical Sciences (ISP/IPICS) for the partial financial support during this work and to the UEMOA (West African Economic and Monetary Union) for supporting our research center.

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