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PHYTOCHEMICAL STUDY AND ANTIBACTERIAL, ANTIFUNGAL AND ANTIOXIDANT PROPERTIES OF *BRIDELIA FERRUGINEA* AND *PTELEOPSIS SUBEROSA*

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ABSTRACT

The antifungal, antimicrobial, antioxidant and cytotoxic activities of methylene chloride, methanol and hydroethanolic extracts of the leaves of *Bridelia ferruginea* and sterm bark of *Pteleopsis suberosa* were investigated against six Gram Positive and Gram negative strains bacteria and six species of *Aspergilus.* The antimicrobial activity was investigated by the microtest method using p-iodonitrotetrazolium and the antifungal activity by measuring the mycelial and sporulation inhibition. The phytochemical study was performed on Thin Layer Chromatography and antioxidant activity was assayed using qualitative and quantitative 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging test. The methanolic extract of *Pteleopsis suberosa* showed better Minimum Inhibitory Concentration against all the tested bacteria. The strongest activity was observed with the methanolic extract of *Pteleopsis suberosa against S. aureus meticillin resistant* with Minimum Inhibitory Concentration value of 78µg/ml. All extracts showed an interesting sporulation inhibitory percentage up to 60% whereas little to moderate inhibition was obtained against mycelial development (9.18 to 57.53%). The antioxidant activity of extracts increased in dose dependent manner. The methanolic and hydroethanolic extracts were the most active with inhibitory percentage values varying from 86.09 to 100%. In the brine shrimp lethality bioassay, the LC_{50} values of tested extracts ranged between 3.74 to 45.38 mg/ml. The results obtained indicated that the plant have interesting bioactive principles and support the use of these plants in the treatment of infectious diseases.

INTRODUCTION: The emergence of pathogens resistant to antibiotics represents a serious problem for public health. The resistance of pathogens such as bacteria and fungi to drugs is increasingly high. These pathogens caused major problems throughout the world. Unfortunately, a number of antibiotics produced by the pharmacological industries do not allow fighting the diseases caused by these pathogens.

Plants have been used for centuries as remedies for human diseases. As a result of increasing need for new and better antimicrobial and antifungal drugs, research work was carried out to study scientifically medicinal plants. Various antifungal and antimicrobial agents have been explored, but the control of fungal diseases has not yet been achieved [1-2].

Single or combination of plants is used by traditional healers to treat various diseases. These traditional healers possess a good knowledge of the use of medicinal plants. They have also a number of superstitious beliefs related to different types of diseases and possess a good knowledge in using herbal drugs. Many Ethnobotanists all over the world stressed the importance of investigating plants for new antimicrobial and antifungal agents [3-5].

The role of free radical reactions in disease pathology is also well established, suggesting that these reactions are necessary for normal metabolism but can be detriment to health as well [6]. It is know that free radical play a fundamental role in several diseases such as arteriosclerosis, cancer, diabetes mellitus, hypertension, renal failure, liver disease, AIDS etc [7-10]. Further to the remarkable toxicity and the mutagenic effects of synthetic antioxidants, the attentions turned to the natural antioxidants. Numerous medicinal plants extracts or constituents have proven to show free radical scavenging activity [5, 11-12].

According to World Health Organization, 80% of the world population still relies mainly on plant drugs [13].

Bridelia ferruginea (Euphorbiaceae) traditionnaly used by traditional healers is a shrub from 1 to 8 cm. The decoction of stem bark is used per os to treat epilepsy, oedemas, irritability of the infant. It was also used to treat gastralgias, anaemia, dysenteria and rheumatisms. The roots were also used with *Euphorbia hirta* to treat gonorrhea and blennorragia [14].

Pteleopsis suberosa (Combretaceae) is a small tree use by traditional healers in Bénin. It is used traditionally to treat various diseases. The decoction of roots is used to treat dystocy and the stem bark to treat dysentery, eruptive fever, and epilepsy [14].

The objective of the present study was to investigate the phytochemical constituents of *Pteleopsis suberosa* and *Bridelia ferruginea* and to evaluate the antibacterial, antifungal, antioxidant activities against seven bacteria strains, six species of *Aspergilus*.

The inhibitory effect of extracts against free DPPH radical and cytotocixity on *Artemia salina* were also evaluated.

MATERIAL AND METHODS

Plant material: In the present study, the leaves of *Bridelia ferruginea* and bark of *Pteleopsis suberosa* were used. Plants were collected from Ouidah, in Atlantic Department, the southern commune of Bénin, in January 2010. The collection and identification were carried out by botanists from university of Abomey-Calavi. The parts collected for each plant were dried for two weeks in laboratory (22°C).

Preparation of Plants Extracts: The dried plants were ground to a fine powder using an electric grinder (EXCELLA Mixer Grinder with 3 S.S.JARS. Model: excella QTY:1PC). Air dried plant of each species (100 g) was extracted three times with methylene chloride (400 ml) at room temperature. The plant extracts were filtered through Whatman N°1 filter paper and pooled together. The filtrates were concentrated to dryness in a rotary evaporator at 40-50°C. The residue was dried under extractor hood and then extracted three times with methanol (400 ml) using same procedure. 50 g of each plant material were also extracted two times with 500 ml of 20% ethanol in water for 2 h. The filtrates of each extraction were taken to dryness under vacuum and the residues were stored in a freezer until used for biological assay.

Microorganisms: Seven bacteria strains: *Escherichia coli* CIP 53126, *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* CIP82118, *Salmonella aboni* CIP 8039, *Staphylococcus aureus* Meticillin Resistant (SARM), *Staphylococcus epidermidis* obtained from Laboratoire de Biophotonique et Pharmacologie, University of Strasbourg, France and Six fungal species, *Aspegillus flavus* CMBB75, *A. parasiticus* CMBB20, *A. ochraceus* CMBB91, *A. nidulans* CMBB90, *A. terreus* CMBB94 and *A. fumigatus* CMBB89 obtained from the Laboratory of Biochemistry and Molecular Biology of University of Abomey-calavi were used in the present study.

Phytochemical study: Phytochemical analysis for major constituents was achieved on Thin Layer Chromatography plate (Alugram, Silicagel 60 F_{254}) using a standard procedures [15-16]. The tests for alkaloids, coumarins, anthracenes derivatives, flavonoïds, essential oils, lignans, naphtoquinones, pigments, saponins, terpenoids and tannins.

Minimum Inhibitory Concentration (MIC): The Minimum inhibitory concentrations were determined by serial dilution microplate bioassay using specific dye p-iodonitrotetrazolium violet as an indicator of growth [17]. All extracts were reconstituted to 20 mg/ml with a mixture of acetone/Muller Hinton (v/v). MIC was determined by two fold serial dilutions of extracts beyond the level where no inhibition of growth of microorganisms was observed. 100 µl of Muller Hinton (DIFCO: Becton Dickinson France S.A) broth culture of bacteria (10^6 CFU/ml) was added to each well contains extract. The microplates were incubated at 37°C for 18 h, after which 40 µl of *p*-iodonitrotetrazolium (0.2 mg/ml) solution in water were added to each well and the microplates were incubated at 37°C. After 1 h of incubation, the MIC values were recorded. The total activities (TA) of extracts were determined by dividing the MICs with the quantity extracted from 1 g of the plant material [18].

Antifungal Assay: Antifungal activity of extracts was evaluated against six species of *Aspergilus* as described by Saadabi [19]. The mixture of sterilized Potato Dextrose Agar-extract (PDA: 39 g of powder of PDA in 1 liter of distilled water) at 1 mg/ml was poured in sterile disposable petri dishes. After solidified, 100 spores prepared in tween 25% were dropping in the center on the petri dishes which were left at 25°C. After 5 days the diameter of mycelia was measured and the number of spores was counted microscopically using malassez cell. Each assay was run in triplicate. Two petri dishes without extract were used as control. The inhibitory percentage (***PI***) of extracts was determined according to the formula below [20]:

$$PI\ (\%) = \frac{A - B}{A} \times 100$$

In which PI means: Inhibitory Percentage; A = Average diameter of the mycelia or estimated number of spores of control; B = Average diameter of the mycelia or estimated number of spores of tested dishes (with extract).

Qualitative Antioxidant Activity: The aim of this method was to evaluate the preliminary antioxidant activity before quantitative evaluation [21]. Each extracts were spotted on the starting point on silica gel sheet (Kieselgel 60 $F_{254,}$ Merk) which was developed in the mixture of Ethyl acetate/Methanol/water (100:2:1). After dry, the silica gel plate was sprayed with a solution of 2% DPPH in methanol. Any bleaching of the purple color background of DPPH (purple color) reagent within 10 min was taken as positive result.

Quantitative Antioxidant Activity: The quantitative antioxidant activity was determined according to the method described previously by Velazquez [22]. Three stock solutions of extracts (300; 30 and 3µg/ml) were prepared and tested in the final concentrations of 100, 10 and 1µg/ml. 750 µl of stock solution of each extract and 1500 µl of a 2% solution of DPPH in methanol were introduced into dry and sterile tubes. For each concentration a blank and a negative control are prepared. The Blank consists of 750 µl of extract and 1500 µl of methanol. The negative control consists of 1500 µl of the solution of DPPH (2%) and 750 µl of methanol. Each test was done in triplicate and quercetol was used as positive control. The test tubes were incubated in dark at room temperature. After 20 mn, the optical density of each mixture was measured at 517 nm using spectrophotometer (Jenway Jenova). The inhibitory Percentage of DPPH radical which means the antioxidant activity of extracts and quercetol was calculated as follow [23].

$$\%I = [1 - (DO_S - DO_B)/DO_C] \times 100$$

In which %I = Inhibitory Percentage of DPPH radical; DO_S = Absorbance of sample; DO_B = Absorbance of Blank; DO_C = Absorbance of control.

Brine Shrimp Lethality Bioassay: The assay was performed as described by Keymanesh et *al*. (2009). The brine shrimp eggs were hatched in normal seawater for 72 h to obtained nauplii larva. The stock solution of each extracts (3 mg/ml) was obtained by dissolving 15 mg in 200 µl ethanol and 4.80 ml of seawater. For the assay, 1 ml of seawater containing 15 living naupli to 1 ml of extracts at 3 mg/ml was added. Six concentrations ranging from 1.5 to 0.075 mg/ml; obtained by a twofold serial dilution of stock solution were tested. Each experiment was done in triplicate and control was prepared using seawater and 15 naupli. After 24 h incubation at room temperature, survivor's nauplii were counted. The Lethal concentration (LC_{50}) was determined graphically.

RESULTS AND DISCUSSION:

Phytochemical Results: Phytochemical analysis of *Bridelia ferruginea and Pteleopsis suberosa* are presented in **Table 1**.

TABLE 1: PHYTOCHEMICAL ANALYSIS OF *BRIDELIA FERRUGINEA AND PTELEOPSIS SUBEROSA*

	Bridelia ferruginea	*Pteleopsis suberosa*
Alkaloids	--	--
Coumarins	+	+
Anthracenic derivatives	++	--
Flavonoids	++	--
Essential oil	+++	++
Lignans	--	--
Naphtoquinones	++	--
Pigments	++	+++
Saponins	--	--
Triterpenoids	++	+++
Tannins	+	++

(--): Absence; (+): trace; (++): medium; (+++): presence

The phytochemical screening of *B. ferruginea* showed the presence of coumarins, anthracenic derivates, flavonoids, essential oil, naphtoquinons, pigments, triterpens and tannins *whereas P. suberosa* showed the presence of coumarins, essential oil, pigments, triterpens and tannins. These results are comparable to those obtained by Owoseni and Baba-moussa respectively on ethanolic extract of leaves and barks of *Bridelia ferruginea and Pteleopsis suberosa* [24-25].

Minimum Inhibitory Concentration (MIC): The Minimum Inhibitory Concentration (MIC) and Total Activity values are recorded in **Table 2**. The results showed that the MIC values ranging from 0.078 to 2.5 mg/ml. All extracts inhibited one or more microorganisms. The antibacterial activity of *P. suberosa* extracts was higher than *B. ferruginea* extracts. The methanolic extract of *P. suberosa* was the most active with MIC value of 78µg/ml against *Staphylococus aureus* meticillin resistant (SARM) which was the most sensible among the tested bacteria while *E. faecalis* the most resistant. The results previously obtained with aqueous and ethanolic extracts of *B. ferruginea* are similar to those obtained in our study with the hydro-ethanolic extract against *E. coli, S. aureus and P. aeruginosa* (CMI > 470 µg/ml) [26-27]. The interesting antimicrobial activity obtained here could be due to the presence of flavonoids and tannins in the extracts [28-30].

TABLE 2: MINIMUM INHIBITORY CONCENTRATION VALUES IN mg/ml AND TOTAL ACTIVITY IN ml OF EXTRACTS: DM: METHYLENE CHLORIDE, ME: METHANOL, ETOH/H_2O: ETHANOL/WATER

	Minimum Inhibitory Concentration (mg/ml)					
Species	***Bridelia ferruginea***			***Pteleopsis suberosa***		
Extracts / µorganismes	DM	Me	EtOH/H_2O	DM	Me	EtOH/H_2O
E.coli	Nd	1.25	2,5	-	0.63	-
S.aureus	>2.5	1.25	2,5	-	0.15	-
S.epidermidis	>2.5	0.63	2.5	>2.5	0.31	0.63
S.a Met Resistant	1.25	0.31	0.63	0.16	0.078	0.16
E.faecalis	5	5	2.5	-	0.63	1.25
P.aeruginosa	5	2.5	2.5	-	1.25	-
S.aboni	2.5	5	-	-	2.5	0.63
Total quantity in mg extracted from 1 g						
	26.80	153.24	158.94	11.54	8.44	42.2
Total activity in ml/g						
E.coli	Nd	122.59	63.57	-	13.5	-
S.aureus	<10.72	122.59	63.57	-	54.01	-
S.epidermidis	<10.72	245.18	63,57	<4.61	27.01	67.52
S.a Met Resistant	21.44	490.36	254.3	73.85	108.20	270.08
E.faecalis	5.36	30.65	63.57	-	13.5	33.76
P.aeruginosa	5.36	61.29	63.57	-	6.75	-
S.aboni	10.72	30.65	-	-	3.37	67.52

Antifungal Assay: The antifungal activity was evaluated against six *Aspergillus* species. Six extracts obtained from *Bridelia ferruginea and Pteleopsis suberosa* were tested for their effect against mycelial growth and sporulation of fungi. Results are compiled in **Table 3 and 4**. All tested extracts were active against fungi by inhibiting sporulation. The Inhibitory Percentage (IP) of sporulation ranging from 53.98% to 99.09 %. The most interesting activity was obtained with methanol extract of *B. ferruginea* against *Aspergillus flavus* with an IP value of 99.09%. *Aspergillus flavus* and *Aspergillus fumigatus* were the most sensitive for extracts (94.91% ≥ IP ≥ 99.09%).

Table 3: Antifungal activity of extracts on sporulation stage of *Aspergillus* species

	Inhibitory Percentage of sporulation (%)					
Extracts	***A. flavus***	***A. parasiticus***	***A. terreus***	***A.ochraceus***	***A. nudilans***	***A. fumigatus***
***Bf* DM**	96,06±0,00	53,98±0,12	82,41±0,07	83,11±0,12	85,11±0,06	97,70±0,00
***Bf* Me**	97,47±0,01	77,34±0,04	89,28±0,08	78,22±0,05	92,31±0,01	99,09±0,00
***Bf* H_2O**	95,87±0,01	94,13±0,18	55,80±0,11	66,66±0,26	89,91±0,01	96,74±0,00
***Ps* DM**	95,87±0,01	82,34±0,06	80,53±0,02	92,27±0,02	83,01±0,01	93,32±0,00
***Ps* Me**	96,92±0,01	89,69±0,03	80,53±0,01	86,30±0,07	86,37±0,03	70,14±0,01
***Ps* H_2O**	94,91±0,01	62,01±0,12	86,16±0,02	86,30±0,04	58,05±0,04	69,57±0,06

(*Bf*): *Bridelia ferruginea*; (*Ps*): *Pteleopsis suberosa*; (DM): Dichlorométhane, (Me): Méthanol; EtOH/H_2O: Ethanol/Eau

TABLE 4: ANTIFUNGAL ACTIVITY OF EXTRACTS AGAINST MYCELIAL DEVELOPMENT STAGE OF A*SPERGILLUS SPECIES.*

Extracts	Inhibitory Percentage of mycelia development (%)					
	A. flavus	***A. parasiticus***	***A. terreus***	***A. ochraceus***	***A. nudulans***	***A. fumigatus***
Bf DM	18,36±0,1	27±0,01	41,1 ±0,06	34,48 ±0,08	7,46 ±0,02	11,36±0,22
Bf Me	20,41±0,0	41±0,12	46,57±0,0	48,27±0,02	32,83±0,03	34,10±0,10
Bf H_2O	12,24±0,0	27±0,09	57,53±0,0	43,1 ±0,00	43,28±0,15	36,36±0,13
Ps DM	24,44±0,0	29±0,01	46,57±0,0	50,00±0,04	35,82±0,03	11,36±0,14
Ps Me	19,38±0,1	28±0,02	45,2 ±0,01	43,10±0,05	31,34±0,09	27,27±0,22
Ps H_2O	9,18 ±0,01	27±0,04	36,98±0,0	39,65±0,05	37,31±0,01	15,90±0,21

(*Bf)*: *Bridelia ferruginea*; *(Ps*): *Pteleopsis suberosa*; (DM): Dichlorométhane ; (Me): Méthanol; (EtOH/H_2O): Ethanol/Eau

Contrary to sporulation, the Inhibitory Percentage (IP) of extracts against mycelial development was weak (2.98% to 53.42%). Only 11 tests out of 36 showed an IP value up to 40%. The hydroethanolic extract of *B. ferruginea* and methylene chloride extract of *P. suberosa* showed interesting activity with IP values of 57.53% and 50.00% respectively. The other extracts showed weak results (7.46% - 48.27%).

Antioxidant activity: Antioxidant activity of extracts was determined for their scavenging potential of the stable DPPH free radical in both qualitative and quantitative tests.

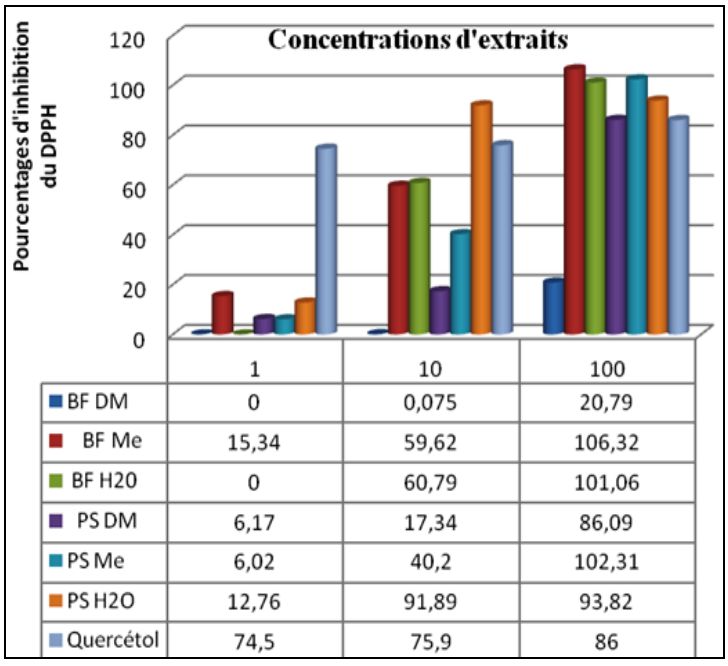
- **Qualitative antioxidant activity of extracts: Figure 1** showed the qualitative inhibition of DPPH radical by extracts**.** The results obtained showed that methanolic and hydroethanolic extracts of both species presented the most interesting activities. Methylene chloride extracts showed respectively weak to low inhibition of DPPH radical. In the works of Dramane,the methanolic extract of bark of *B. ferruginea* gave similar positive results [27].

FIGURE 1: QUALITATIVE ANTIOXIDANT EFFECT OF EXTRACTS FROM *B. FERRUGINEA* AND *P. SUBEROSA*

- **Quantitative Antioxidant Activity: Figure 2** shows the antioxidant potential of six extracts from *B. ferruginea* and *P. suberosa* expressed as Inhibitory percentage of DPPH radical in comparison to the positive control (quercetol). The results obtained showed that the antioxidant activity of extracts is concentration dependent. It has been shown that the scavenging effects on the DPPH radical increase with increasing concentration of samples and standards to a certain extent [28].

  The methanol and hydroethanol extracts of both species showed the most interesting antioxidant activity. At 1µg/ml, the antioxidant activity turns around 10% whereas quercetol showed an inhibitory percentage of 74.5%. Methylene chloride extract of *B. ferruginea* possesses no activity which result was in accordance with that obtained by the qualitative method. At 10µg/ml, four extracts out of six presented an antioxidant activity up to 40%. The hydroethanolic extract was the most active with an IP value of 91.89%.

  At 100 µg/ml, except methylene chloride extract of *B. ferruginea* (20, 79%), the other five extracts showed inhibitory percentages ranging from 86 to 100%, similar to quercetol (86%). Our results suggested that *B. ferruginea* and *P. suberosa* contain compounds with strong radical scavenging activity.



	1	10	100
BF DM	0	0,075	20,79
BF Me	15,34	59,62	106,32
BF H20	0	60,79	101,06
PS DM	6,17	17,34	86,09
PS Me	6,02	40,2	102,31
PS H2O	12,76	91,89	93,82
Quercétol	74,5	75,9	86

FIGURE 2: QUANTITATIVE ANTIOXIDANT EFFECT OF EXTRACTS FROM *B. FERRUGINEA* AND *P. SUBEROSA*

Brine Shrimp Lethality Bioassay: Brine shrimp lethality test results are showed in **Table 5**. The LC_{50} values of tested extracts ranged between 3.74 to 45.38 mg/ml. The hydroethanolic extract was the most toxic on the shrimps with LC_{50} value of 3.74 mg/ml. The methylene chloride extracts of both species showed similarly LC_{50} values of 22.37 and 20.42 mg/ml respectively for *B. ferruginea* and *P. suberosa*. The higher LC_{50} value was exhibited by methanol extract of *P. suberosa* (45.38 mg/ml). According to the results previously obtained by Zakaria [29] we concluded that all extracts tested in his study exhibited no toxicity, giving LC_{50} values higher than 100µg/ml.

TABLE 5: BRINE SHRIMP LETHALITY ASSAY OF EXTRACTS FROM *B. FERRUGINEA* AND *P. SUBEROSA*

Species	Extracts	LC_{50}	Coefficient co-relation
Bridelia ferruginea	DM	22,37	0,68
	Me	13,63	0,58
	$EtOH/H_2O$	3,74	0,88
Pteleopsis suberosa	DM	20.42	0,68
	Me	45,38	0,38
	$EtOH/H_2O$	Nt	0

CONCLUSION: In the present study, biological activity of six extracts from *B. ferruginea* and *P. suberosa* was investigated. We can conclude that both species may have antibiotic and antifungal activity as they exhibited potent antibacterial, antifungal, antioxidant activities. Chance to find constituents with antifungal, antimicrobial activities were apparent in the two species. These findings give a scientific basis to the traditional uses of *B. ferruginea* and P. suberosa.

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