Screening Togolese medicinal plants for few pharmacological properties

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ABSTRACT

Background: *Terminalia macroptera* Guill. et Perr. (Combretaceae), *Sida alba* L. (Malvaceae), *Prosopis africana* Guill et Perr. Taub. (Mimosaceae), *Bridelia ferruginea* Benth. (Euphorbiaceae), and *Vetiveria nigritana* Stapf. (Asteraceae) are traditionally used in Togolese folk medicine to treat several diseases including microbial infections. **Objective:** This study aimed to investigate the antimicrobial, antioxidant, and hemolytic properties of the crude extracts of the above-mentioned plants. **Materials and Methods:** The antimicrobial and the antioxidant activities were assayed using the NCCLS microdilution method and the DPPH free radical scavenging, respectively. Human A+ red blood cells were used to perform the hemolytic assay. Phenolics were further quantified in the extracts using spectrophotometric methods. **Results:** Minimal inhibitory concentrations in the range of 230–1800 μg/ml were recorded in the NCCLS broth microdilution for both bacterial and fungal strains with methanol extracts. The DPPH radical scavenging assay yielded interesting antioxidant activities of the extracts of *P. africana* and *T. macroptera* (IC_{50} values of 0.003 ± 0.00 μg/ml and 0.05 ± 0.03 μg/ml, respectively). These activities were positively correlated with the total phenolic contents and negatively correlated with the proanthocyanidin content of the extracts. The hemolytic assay revealed that great hemolysis occurred with the methanol extracts of *T. macroptera, S. longepedunculata,* and *B. ferruginea.* **Conclusion:** These results support in part the use of the selected plants in the treatment of microbial infections. In addition, the plant showed an interesting antioxidant activity that could be useful in the management of oxidative stress.

Key words: Antimicrobial, antioxidant, hemolytic, phenolics

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INTRODUCTION

Infectious diseases are world's leading cause of deaths, killing millions of people annually.[1] An important factor is that the drug resistance to human microbial pathogens has been increasing both in the developing countries and the developed countries due to the inappropriate use of antibiotics. The multidrug resistant bacterial and fungal strains have further complicated the treatment of infectious diseases in immunocompromised AIDS and cancer patients. This has highlighted the need for new antimicrobial substances.[2-4]

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Plants have been by far used as medicines for treating a variety of diseases and complaints. Phytotherapy in Africa is particularly widespread and up to 80% of the population still relies on plants for primary healthcare. The plant preparations and medications continue to be used in the treatment of numerous disorders, including eczema, malaria, respiratory disorders, and infectious diseases.[5,6] In fact, plants have an almost limitless ability to synthesize aromatic substances, which are phenols or their oxygen substituted derivatives. Some of them are secondary metabolites and are involved in the plant defense against micro-organisms. For some of these plant concoctions, the antimicrobial activity has been proven;[7-9] however, for many of them, the evidence is anecdotal. Consequently, many traditionally used antiseptic agents have yet to be subjected to thorough scientific investigation.[4,10]

Togo is a country located in western Africa in the subequatorial zone. The country benefits from an excellent floristic biodiversity and the indigenous people have old knowledge of the medicinal plant uses.[11,12] Thus, the majority of the Togolese people living in rural areas traditionally use plants for nutrition and medical purposes. Therefore, the Togolese government recognizes the importance of traditional medicine as a key provider of primary healthcare and is promoting the integration of the traditional healing into the official healthcare system.[13] In recent years, the plants used traditionally for therapeutic purposes have attracted the attention of the researchers and some plants have been screened with success.[14,15] However, the information on the pharmacological properties of Togolese medicinal plants is scanty. The present study was aimed at documenting few pharmacological properties of six medicinal plants namely *Bridelia ferruginea* Benth. (Euphorbiaceae), *Prosopis africana* Taub. (Fabaceae), *Securidaca longepedunculata* Fresen. (Polygalaceae), *Sida alba* L. (Malvaceae), *Terminalia macroptera* Guill. and Perr. (Combretaceae), and *Vetiveria nigritana* Stapf (Poaceae) used in the Togolese traditional medicine to treat several infectious diseases. The screened pharmacological properties included the antimicrobial, hemolytic, and antioxidant activities.

MATERIALS AND METHODS

Chemicals and biochemicals

Catechin, gallic acid, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), phosphate buffer saline (PBS), and folin reagent were obtained from Sigma Chemical Company (St. Louis, France). All media for microbial growth were from Bio-Rad (France). Ascorbic acid and sodium carbonate were from Merck (Germany). All the other chemicals were of analytical grade.

Preparation of extracts

The plant materials were harvested in June 2010 at Sokodé, in the Central Region of Togo, according to their traditional uses.[16] The collected samples were roots of *V. nigritana* Stapf and the leaves of *B. ferruginea* Benth., *P. africana* Taub., *S. longepedunculata* Fresen., *S. alba* L., and *T. macroptera* Guill. and Perr. These samples were botanically authenticated at the Department of Botany of University of Lomé where voucher specimens were deposited. The fresh plant materials were then air-dried at ambient temperature in a laboratory and pulverized using a mechanical grinder. The powdered materials (500 g) were extracted with 1.5 l of 70% methanol by cold maceration with constant agitation for 24 h, then filtered through Whatman no. 1 filter paper, and freeze dried.

Phytochemical analysis

The total phenolic compounds from lyophilized methanolic extracts were quantified using the Folin–Ciocalteu method.[17] The assay was performed in 96-well plates as follows. The Folin–Ciocalteu reagent (25 µl; 50%, v/v) was added to 10 µl of 20 mg/ml (w/v) of each lyophilized plant extract dissolved in water. After 5-min incubation at room temperature, 25 µl of 20% (w/v) sodium carbonate and 140 µl of water were added to a final volume of 200 µl per well. After 30-min incubation at ambient temperature, the absorbance was read at 725 nm using a multiwell plate reader (µQuant Bio-Tek Instrument, Inc., USA). Raw absorbance was automatically recorded using KC junior software, version, 1.31.5 (Bio-Tek instrument, Inc. USA). All the assays were carried out in duplicate. Gallic acid was used as standard and the results were expressed as milligram of gallic acid equivalent (GAE) per gram of the lyophilized sample.

The proanthocyanidin content was determined using the butanol/HCl method.[18] For each sample, 2% (w/v) of $NH_4Fe(SO4)_2 \times 12\ H_20$ was prepared by dissolving the reagent in 2 M HCl. Then, 0.2 ml of this solution was added to 7 ml of *n*-butanol. HCl (95:5, v/v) to constitute the working solution. Each lyophilized extracts (100 mg) were dissolved in the working solution and the mixture was incubated at 95°C for 40 min. Afterward, the mixture was distributed in 96-well plates in duplicate (200 µl/well) and the absorbance at 550 nm was measured. The proanthocyanin content was calculated using catechin as standard ($A^{1\%}$ at 550 nm = 280). The results were expressed as milligram of catechin equivalent (CE) per gram of the lyophilized sample.

DPPH radical scavenging activity

The antioxidant activity of the plant extracts was assayed using the stable 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging with ascorbic acid as standard.[19] For each test, 50 µl of the extract or standard (0.01–625µg/ml) dissolved in methanol was added to 150 µl of 1 mM methanolic DPPH in 96-well plates. After incubation at 37°C for 30 min, the absorbance was determined at 517 nm. The assay was conducted in duplicate. The free radical scavenging activity (RSA) was calculated as follows:
RSA (%) = 100 × (1 − As/Ac). Where As was the absorbance of the sample at 517 nm and Ac the absorbance of the control at 517 nm. The EC_{50} value was the concentration of the sample required to scavenge 50% DPPH free radicals. This was determined using linear regression analysis.

Antibacterial test

The minimal inhibitory concentrations (MICs) were determined using the broth microdilution in 96-well plates according to National Committee for Clinical Laboratory recommendations.[20] The following strains were tested: *Escherichia coli* CIP 105182, *Proteus mirabilis* CIP 588104, *Serratia marcescens* CIP 6755, *Shigella flexneri* CIP 8248, *Salmonella paratyphi* A. CIP 5539, *Salmonella enteritidis* CIP 8297, *Klebsiella pneumoniae* CIP 52144, *Staphylococcus aureus* ATCC 25923, *Mucor rouxi* ATCC 24905, *Fusarium oxysporum* CIP 525 72, and *Rhizopus nigricans* ATCC 622713. The microbial suspensions, corresponding to 0.5 McFarland standards, were prepared by suspending overnight colonies in 0.85% NaCl. These suspensions were diluted with the Mueller–Hinton broth to inoculate 96-well plates containing twofold serial dilutions of extracts. For the fungi, the Mueller–Hinton broth was replaced by the Sabouraud broth. The drug concentrations ranged from 10 to 2500 μg/ml and the final volume in wells was 200 μl. The final inocula as determined by the colony counts from the growth control wells were approximately 10^5 cfu per well. The plates were incubated in air at 37°C for 24 h. The MIC was recorded as the lowest extract concentration demonstrating no visible growth in the broth.

Hemolytic assay

The hemolytic assay was carried out by adopting the method recommended by the World Health Organisation[21] with slight modifications. Four milliliters of venous blood (group A rhesus positive) was collected from a healthy volunteer in a citrate tube. The blood was centrifuged at 2500 rpm for 10 min to discard the plasma. Afterward, the red blood cells were washed three times with PBS ×1 by centrifugation at 2500 rpm for 10 min and then suspended in the same PBS at 2% hematocrit. Fifty microliters of serial dilution of extracts was added to 150 μl of this cell suspension in 96-well plates resulting in extract concentrations of 10–4000 μg/ml. The extracts were dissolved in DMSO and diluted with PBS to have a final concentration below 1% in the first wells. A control experiment was conducted separately by replacing the extracts by DMSO to ensure that DMSO had no significant influence on cells' hemolysis. Another control experiment was performed with extracts diluted in PBS without the red blood cells to correct for interfering compounds' absorbance at 450 nm. The plates were incubated at 37°C under permanent agitation for 2 h. Afterward, the absorbance was measured at 450 nm. Saponin R was used as standard and the results were expressed as saponin equivalent. The experiment was carried out in duplicate.

Statistical analysis

The results were expressed as means followed by standard deviations. Statistical significance was determined by one-way analysis of variance and Fisher's test, with the level of significance at $P < 0.05$. Pearson's linear regression analysis was used to observe the correlation between the phenolic contents and antioxidant activities.

RESULTS

Phenolic content and free radical scavenging

The results of chemical analysis are summarized in Table 1. According to the table, the highest concentration of total phenolic compounds was detected in the leaves of *S. longepedunculata, T. macroptera*, and *B. ferruginea* (27.25 ± 0.66 mg GAE/g, 11.34 ± 0.47 mg GAE/g, and 10.29 ± 1.57 mg GAE/g, respectively). However, *T. macroptera* followed by *P. africana* and *B. ferruginea* had a high amount of proanthocyanidins (93.55 ± 1.90 mg CE/g, 56.51 ± 4.64 mg CE/g, and 20.23 ± 0.36 mg CE/g, respectively). There was a low correlation between the total phenolic content and the proanthocyanidin content ($r^2 = -0.2$). Indeed, the extract with the highest total phenolic content, *S. longepedunculata*, did not contain systematically the largest amount of proanthocyanidins.

The radical scavenging activity was expressed as a percentage decrease in absorbance at 517 nm. All the extracts showed a significant percentage radical scavenging activity in a dose-dependent manner. The assay yielded very low IC_{50} values with *P. africana* (0.003 ± 0.00 μg/ml) and *T. macroptera* (0.05 ± 0.03 μg/ml). These two extracts displayed a very high antioxidant activity compared with

Table 1: Phenol contents and DPPH scavenging of extracts

Extract	Total phenolic compounds, GAE (mg/g)	Proanthocyanidins content, CE (mg/g)	DPPH inhibition, IC_{50} (μg/ml)
T. macroptera	11.34 ± 0.47	93.55 ± 1.90	0.05 ± 0.03
S. alba	1.56 ± 0.53	14.065 ± 0.18	8.95 ± 0.52
P. africana	3.03 ± 0.22	56.51 ± 4.64	0.003 ± 0.00
S. longepedunculata	27.25 ± 0.66	3.845 ± 0.28	79.35 ± 0.00
V. nigritana	5.70 ± 0.47	6.415 ± 0.02	5.70 ± 3.34
B. ferruginea	10.29 ± 1.57	20.23 ± 0.36	3.83 ± 2.02
Ascorbic acid	–	–	4.79 ± 0.02

–: nonapplicable.

the standard antioxidant, ascorbic acid. This activity was highly correlated with the total phenolic content ($r^2 = 0.9$); however, a negative correlation was recorded with the proanthocyanidin content ($r^2 = -0.5$).

Hemolytic activity

In the present study, the hemolytic assay revealed that total hemolysis occurred with concentrations above 6 µg/ml saponin R and the measured optical density was higher than 210 at 450 nm. The results of the assay are presented in Figure 1a and b. According to the figure, the hemolytic effect was dose dependent and the extracts of *T. macroptera, S. longepedunculata*, and *B. ferruginea* had the highest hemolytic activity. At 4000 µg/ml, these extracts yielded hemolysis equivalent to 7.66, 6.90, and 5.34 µg/ml saponin R, respectively. *B. ferruginea, S. alba* and *P. africana* were less hemolytic. Hemolysis yielded by 4000 µg/ml extracts were 5.26, 3.27, and 3.02 µg/ml saponin R, respectively.

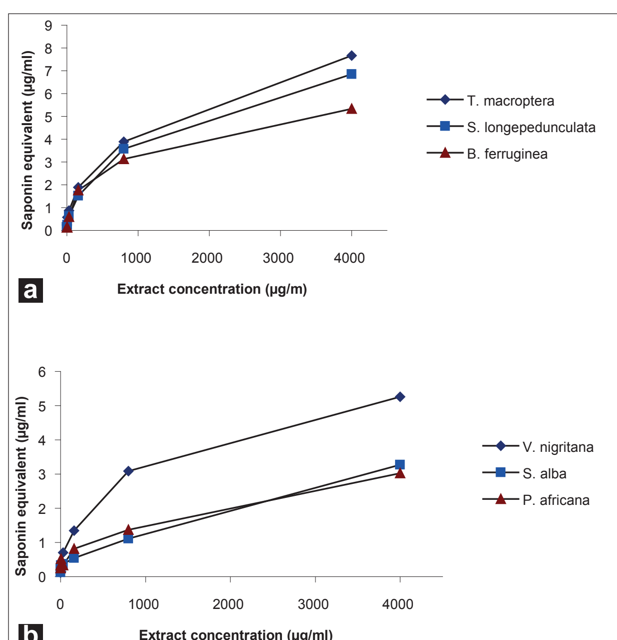

Figure 1: Hemolytic activity of methanolic extracts: (a) *Terminalia macroptera, Securidaca longepedunculata,* and *Bridelia ferruginea;* (b) *Vetiveria nigritana, Sida alba,* and *Prosopis africana*

Antimicrobial activity

The methanolic extracts of the screened plants in the present study yielded MIC values in the range of 450–1800 µg/ml for *Proteus mirabilis* CIP 588104, which was susceptible to all the extracts [Table 2]. The other bacteria were selectively inhibited by one or more extracts. Indeed, *E. coli* CIP 105182, which was found to be the most resistant bacterial strain of the study, was only inhibited by *S. longepedunculata* (MIC = 450 µg/ml). *S. paratyphi* A. CIP 5539, *S. enteritidis* CIP 8297, *K. pneumoniae* CIP 52144, and *S. aureus* ATCC 25923 were inhibited by two extracts with MIC values ranging from 600 to 1800 µg/ml. The lowest MIC value (MIC = 230 µg/ml) was recorded with *S. longepedunculata* on *S. flexneri* CIP 8248, which was also inhibited by *S. alba* and *P. africana* (MIC = 1800 µg/ml, for these extracts). *S. marcescens* CIP 6755 was inhibited by four extracts, namely, *P. africana, S. longepedunculata, V. nigritana*, and *B. ferruginea* with MIC values in the range of 450–1800 µg/ml.

The fungal strains tested also showed selective susceptibility to extracts. Thus, *Fusarium oxyporum* CIP 525 72 was only inhibited by *S. longepedunculata* (MIC = 1200 µg/ml), while *M. rouxi* ATCC 24905 and *Rhizopus nigricans* ATCC 622713 were susceptible to *T. macroptera* and *S. longepedunculata* (MCI = 1200 µg/ml).

S. longepedunculata seemed to be the most active plant. Despite the fact that this plant yielded the lowest MIC value, it also inhibited the greatest number of microbial strains. In fact, all fungal strains were inhibited by this extract; in addition 6/8 bacterial strains were inhibited. *S. alba* seemed to be less active. Its extract failed in the inhibition of fungal strains and only inhibited 2/8 bacterial strains. *V. nigritana,*

Table 2: Minimal inhibitory concentration of extracts

Micro-organisms	*T. macroptera*	*S. alba*	*P. africana*	*S. longepedunculata*	*V. nigritana*	*B. ferruginea*
Escherichia coli CIP 105182	–	–	–	450	–	–
Proteus mirabilis CIP 588104	450	1800	1800	1800	1800	450
Serratia marcescens CIP 6755	–	–	1800	450	1800	1800
Shigella flexneri CIP 8248	–	1800	1800	230	–	–
Salmonella paratyphi A. CIP 5539	–	–	–	–	1800	1800
Salmonella enteritidis CIP 8297	–	–	–	–	–	–
Klebsiella pneumoniae CIP 52144	–	–	1800	900	–	–
Staphylococcus aureus ATCC 25923	1200	–	–	600	–	–
Mucor rouxi ATCC 24905	1200	–	–	1200	–	–
Fusarium oxysporum CIP 525 72	–	–	–	1200	–	–
Rhizopus nigricans ATCC 622713	1200	–	–	1200	–	–

All values are expressed in µg/ml. –: For MIC value > 2500 µg/ml.

B. ferruginea and *P. africana* also failed in the inhibition of fungal strains.

DISCUSSION

The present study aimed to assess the phenolic content, free radical scavenging, and antimicrobial properties of the methanol extracts of the screened plants. Phenolics are a group of highly hydroxylated compounds present in the extractive fraction of several plant materials. Polyphenols in plants include hydroxycoumarins, hydroxycinnamate derivatives, flavanols, flavonols, flavanones, flavones, anthocyanins, and proanthocyanidins often called condensed tannins. Our results revealed that *T. macroptera* had the highest content in proanthocyanidins. This plant was previously known to possess a large amount of phenolic compounds of which some were isolated and identified. Thus, two glucoside gallates, namely, vanillic acid 4-*O*-beta-D-(6'-*O*-galloyl) glucopyranoside and 3,3',4'-tri-*O*-methylellagic acid, and two hydrolyzable tannins, isoterchebulin and 4,6-*O*-isoterchebuloyl-D-glucose, were isolated from the bark of the plant.[22,23] *S. longepedunculata* was also found to possess a high amount of phenolic compounds, with quercetin as the major compound being isolated and identified using reverse-phase, high-pressure liquid chromatography.[24]

The antioxidant activity of polyphenols is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching oxygen, or decomposing peroxides. Indeed, the antioxidant activity of some plant extracts is highly correlated with their phenolic content, both total phenolics and proanthocyanidins.[7] In this study, the antioxidant activity was more correlated with the total phenol content. The crude methanolic extracts of *P. africana* and *T. macroptera* exhibited a significant antioxidant activity which may be relevant in the treatment of oxidative stress, although it is difficult to extrapolate this directly to preparations used by the traditional healers. The antioxidant activity of *B. ferruginea* has already been investigated by Adetutu *et al.* A moderate antioxidant activity was recorded with the ethanolic extract of the leaves of the plant through DPPH free radical scavenging.[25] The investigation of *S. alba* also yielded a weak antioxidant activity correlated with the total phenolic content of the plant.[26] Our results are in accordance with these previous reports.

Most of the plants screened in this study are used for wound healing in native areas by the indigenous people.[27-29] This suggests that possible antimicrobial compounds may occur in these plants. In fact, a key factor in curing chronic wounds, gastroenteritis, and infectious diseases is the failure to combat multifactorial infections including *E. coli, Staphylococcus, Pseudomonas*, and *Proteus* species.[30,31] According to Fabry *et al*, for crude extracts of plants to be considered as potentially useful therapeutically, they must have MIC values below 8 mg/ml, while Gibbons suggests that the isolated phytochemicals should have the MIC value below 1 mg/ml. In the present study, some extracts displayed MICs in the range of 230–1800 μg/ml, showing great antimicrobial potential of the plants.[32,33] These results are in agreement with previous literature reports, since all these plants, excluding *S. alba*, have been screened for the antimicrobial activity. In the particular case of *B. ferruginea*, this plant was screened two times for the antibacterial activity by Adetutu *et al*. These screening yielded MICs in the range of 470–950 μg/ml,[25,34] while Silva *et al,* found MIC values in the range of 150–5000 μg/ml for several fractions of *T. macroptera*.[35] Adamu *et al*, found inhibition of bacterial strains by 200 mg/ml of the *V. nigritana* ethanolic extract in the agar well diffusion assay.[36] Furthermore, the phenolics were demonstrated to be responsible for the antimicrobial activity of the plant.[22,23]

As these plants are use to cure wounds by the direct application of the concoction, it was judicious to seek for the possible hemolytic effects of the extracts. The *in vitro* cytotoxity on the erythrocyte membrane of various plant extracts has been studied from time to time and correlated with their constituents, such as natural polyphenolic antioxidants from green and black tea,[37] epicactechin isomers from jasmine green tea,[38] steryl glycosides from tubers of *Momordica cochinchinensis*,[39] triterpenoid saponins from *Heteropappus altaicus, H. biennis,* and *Helianthus annuus*.[40,41]

The hemolytic activity in this study was determined by comparison with a reference compound, saponin R which has a hemolytic activity of 1000 units/g. Our results revealed a dose-dependant hemolytic activity of the extracts. *T. macroptera, S. longepedunculata*, and *B. ferruginea* were the most hemolytic plants. Many of these medicinal plant materials may contain saponins and the above-mentioned compounds. The most characteristic property of saponins is their ability to cause hemolysis. When added to a suspension of blood, saponins produce changes in erythrocyte membranes causing hemoglobin diffusion into the surrounding medium. Previous studies showed that saponin-lysed erythrocytes do not reseal, suggesting that saponin damage to the lipid bilayer is irreversible.[42]

Saponins occurring in the plants used in the traditional medicine worldwide can often account for their therapeutic action including antibacterial, antiviral, anti-inflammatory, antiprotozoal, and antitumor activities[43] Although the saponins are extremely toxic to cold-blooded animals, their

oral toxicity in mammals is low.[44] It is evidence that the main route of the administration of traditional concoctions is oral, but in the case of the screened plants, they are used to treat wound by directed application in some cases.[27,45,46] The concoctions based on these plants should be therefore managed with precaution to avoid notable adverse effects, according to our results.

The results of the present study support partially the use of the selected plants in traditional medicine notably in the treatment of microbial infections. Further screenings are need for the isolation and the identification of the active principles.

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