Inhibitory effects of hydroethanolic extracts from three Cameroonian medicinal plants on proteins inflammation and growth of multi-resistant strains of Mycobacterium tuberculosis

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1. INTRODUCTION

The research of medicinal plants through their secondary metabolites possessing anti-inflammatory and antimicrobial properties have been on the rise due to their potential use in the therapy of various chronic and infectious diseases like tuberculosis. Epidemiology and experimental studies have implicated pro-inflammatory damage arising from the denaturation process of functional and structural proteins and membrane lipids as the primary cause of chronic and infectious diseases. Due to risk of adverse effects encountered with the use of synthetic antibiotics, medicinal plants may offer an alternative source for antimicrobial agent with significant activity against various pathogens1. In addition, a number of antibiotics have lost their effectiveness due to the development of resistant strains, mostly through the expression of resistance genes1.

Inflammation is a complex process, which is considered as a primary physiologic defence mechanism that helps body to protect itself against burn, toxic chemicals, allergens, other noxious stimuli or infectious diseases. Uncontrolled and persistent inflammation can play an etiological role in many of these chronic diseases and can be a source of worsening
infectious diseases. Although it is a defence mechanism, the complex events and mediators involved in the inflammatory reaction can contribute or aggravate many respiratory diseases like pneumonia or tuberculosis. Tuberculosis (TB) is an infectious and inflammatory disease caused by Mycobacterium tuberculosis complex bacteria. It generally affects the lungs and is transmitted from a patient through exhaled droplets that diffuse into the air to a healthy person. In 2009, the World Health Organization (WHO) estimated that one third of the world’s population has been infected by the bacillus of Koch and 10% of infected individuals have developed the disease. In 2016, 8.5 and 8.3 million new TB cases were estimated by WHO at for the years 2013 and 2014 respectively. In 2017, 10 million new cases of tuberculosis were recorded with about 1.3 million dead. Thus, these incidence rates show that Mycobacterium tuberculosis remains the pathogen responsible for the leading cause of death in humans compared to other microbial species. This resistance is due to the inadequate use of drugs which provokes the emergence of multidrug resistant strains like isoniazid or Rifampicin resistant strains, Multi-Drug resistant strains (MDR) and Ultra-Drug resistant strains (XDR). To solve this public health problem, the WHO suggested to look for an alternative and effective therapies. Therefore, the development of potent antimycobacterial drugs that also possesses anti-inflammatory effects with fewer side effects is necessary from medicinal plants origin.

The used of Cameroonian medicinal plants as TB treatment is recurrent as well as ethnobotanical survey has shown that several medicinal plants like Allium sativum L. (Amaryllidaceae), Drypetes gossweilleri S. MOORE (Putranjivaceae) and Pentadactyla brazzeana Baill. (Pentadactylaceae) could be an approach of choice. These plants possess diverse biological activities including anti-carcinogenic, anti-atherosclerotic, antithrombotic, antimicrobial, anti-inflammatory and antioxidant effects of their essential oils. In the Nkam sub-division (Littoral region of Cameroon), people often used their crude extracts deriving from maceration, decoction and infusion to treat tuberculosis. Even if the antimycobacterial potential of the essential oil extracted from these plants have been shown against resistant strains, the present study was undertaken to evaluate the phytochemical components. In vitro anti-inflammatory and anti-mycobacterial efficiency of hydroethanolic extracts from three cameroonian medicinal plants against multi-resistant strains of Mycobacterium tuberculosis.

2. MATERIAL AND METHODS

2.1. Chemical reagents and solvents

Glycerol and Tween 80 were purchased from Sigma-Aldrich (France). The Boivine Serum Albumin (1 mM), Phosphate Buffer Saline (0.2 M; pH 7.4), Middlebrook 7H9 OADC (oleic acid, albumin, dextrose, catalase) supplement, Alamar Blue solution and PANTA (Polymixin, Amphotericin B, Naladixic acid, Trimethoprim and Azlocillin) antibiotic supplement were purchased from Becton Dickinson (USA). All other reagents and solvents were of analytical grade.

2.2. Collection and authentication of plant materials

Three aromatics and/or medicinal plants namely D. gossweilleri stem-barks, P. brazzeana roots and A. sativum bulbs were collected at 6 a.m from Littoral, West and North Regions of Cameroon respectively in January 2016. The botanical identification and authentication of the plants was carried out at the National Herbarium of Cameroon (Yaoundé) where voucher specimens are kept under the 25749/SR/Cam, 42918/SR/Cam and 25742/SR/Cam respectively.

2.3. Extraction procedure

The D. gossweilleri stem-barks, P. brazzeana roots and A. sativum bulbs were air-dried at room temperature and weighed. 200 g of aliquots of each pulverized and dried plant material were extracted by maceration in hydroethanolic mixture (20:80; v/v), twice for 48 hours at room temperature (27 ± 2°C) in enclosed flasks. Each sample was filtered through a Whatman N° 1 filter paper, and evaporated under reduced pressure with rotary evaporate to obtain hydro-ethanolic extracts. These extracts were stored at 4°C until use. The extraction yields were calculated as the ratio of the mass of hydroethanolic extract to the mass of the starting plant material. It expressed as a percentage.

2.4. Phytochemical screening of hydroethanolic extracts

Preliminary phytochemical screening of secondary metabolites were carried out according to the methods described by Harborne, and Tress and Evans.

2.5. In vitro anti-inflammatory assay

2.5.1. Bovine Serum Albumin (BSA) denaturation assay

Anti-denaturation assay was conducted as described by Betote et al., with slight modifications. The reaction mixture was consisting of test hydroethanolic extracts or sodium diclofenac and 5% aqueous solution of bovine serum albumin. The mixture were incubated at 37°C for 20 min and then heated to 70°C for 15 min; after cooling the samples, the turbidity was measured at 660 nm using a spectrophotometer of the brand Thermo Fisher Scientific: Evolution 300 UV-VIS. The experiment was performed in triplicate. The percent inhibition of bovine serum albumin denaturation was calculated as follows, where Absocontrol is the absorbance without sample, Abocontrol is the absorbance of sample (hydroethanolic extracts/standard).

\[
\text{I} (\%) = \frac{\text{Abs control} - \text{Abs extracts/sodium diclofenac}}{\text{Abs control}} \times 100
\]

2.5.2. Proteinase inhibitory action

The test was performed according to the modified method of Oyedepo et al. The reaction mixture (2 mL) was containing 0.06 mg trypsin, 1 mL of 20 mM Tris-HCl buffer (pH 7.4) and 1 mL test hydro-ethanol extracts or sodium diclofenac of different concentrations. The reaction mixture was incubated at 37°C for 5 minutes and then 1 mL of 0.8% (W/V) casein was added. The mixture was incubated for an additional 20 min, 2 mL of 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage of inhibition of proteinase inhibitory activity was calculated.

\[
\text{I} (\%) = \frac{\text{Abs control} - \text{Abs extracts/diclofenac sodium}}{\text{Abs control}} \times 100
\]

Where, Abocontrol is the absorbance of control tube and Aboextract/diclofenac sodium is the absorbance of sample tube.

2.6. Anti-mycobacterial activity assay

2.6.1. Mycobacterial cultures of Mycobacterium tuberculosis.
Three *Mycobacterium tuberculosis* strains isolated from patients at the tuberculosis control centre in the South Region of Cameroon and characterized at the Laboratory for Tuberculosis Research and Pharmacology (the Biotechnology Centre of the University of Yaoundé I) where they have been screened to drug resistant using Line probe assay. The tested strains included an Isoniazid resistant strain codify as AC6, Rifampicin resistant strain (AC96) and *M. tuberculosis* extensively drug resistant strain (XDR). These bacterial strains are maintained on slant of Middlebrook 7H11 Agar medium supplemented with 10 % Middlebrook 7H9 OADC supplement and PANTA an antibiotic supplement.

### 2.6.2. In vitro anti-mycobacterial assessment of extracts.

The geometric serial broth microdilution method was carried out according the Microplate Alamar Blue Assay (MABA) described previously by Collins and Franzblau and modified by Jimenez-Arellanes *et al.*. A stock solution was prepared by diluting the respective sample in 10 % tween 80. Stock solution was then added to Middlebrook 7H9 broth to reach final sample concentrations ranging from 5000 µg/mL to 9.765 µg/mL Serial dilutions were inoculated with mycobacteria inocula (1.5 × 10⁶ cells/mL prepared from the Middlebrook 7H9 broth medium supplemented with glycerol, tween 80 and Middlebrook 7H9 OADC and PANTA supplements). Each 96 wells microtiter plates was mixed and incubated at 37 °C for 7 days. Positive controls consisted of Rifampicin and Isoniazid at 250 µg/mL and negative controls was contained no drugs and blank contained no inoculum and/or drug. The concentration of tween 80 in the assay was kept at a concentration to ensure that the effect on bacterial growth was minimal. Upon incubation periods, 40 µL of 0.02 % resazurin were added to individual wells and the plates reincubated for additional 1 day and checked daily for color change. Change in resazurin color from blue to pink indicated reduction of the indicator and thus bacterial growth. The Minimal Inhibitory Concentration (MIC) was defined as the lowest concentration of samples at which the microorganism did not demonstrate growth.

### 2.6.3. Determination of mycobactericidal effect.

Mycobactericidal effect of hydroethanolic extracts and standard drugs on pathogens was determined by microdilution method. The Minimal Bactericidal Concentrations (MBC) of promising hydroethanolic extracts was assessed by sub-culturing MIC test microtiter plates on Middlebrook broth 7H9 + 10 % (AADC+ PANTA). The MBC was considered as highest dilution or lowest concentration at which no growth occurred in the medium. All the experiments were done in duplicate and repeated three times. The anti-mycobacterial effect was deemed bactericidal or bacteriostatic depending on the ratio: MBC/MIC. If CMB/CMI is lower than four, the anti-mycobacterial effect of plants extracts is bactericidal and bacteriostatic when CMB/CMI higher than four.

### 2.7. Statistical analysis.

Each data was expressed as mean ± SD (n = 3). The analysis was done by ANOVA followed by test Dunnett’s. The difference between standard and hydroethanolic extracts concentrations was considered significant at p<0.05. The graphical representation of the data and IC₅₀ was performed using the Graph Pad Prism 8.0.1 software (Microsoft, USA).

### 3. RESULTS

#### 3.1. Phytochemical Screening of hydroethanolic extracts.

The qualitative analysis of phytochemical compounds of *A. sativum*, *D. gossweilleri* and *P. brazzeana* were carried out and hydroethanolic extracts showed the presence of various chemical constituents such as phenols, polyphenols, flavonoids, alkaloids, catechic tannins, triterpenes, steroids, anthocyanins and leucoanthocyanins. These results show high level of its possible medicinal value. The results are shown in (Table 1).

### Table 1: Preliminary phytochemical screening

<table>
<thead>
<tr>
<th>Nº</th>
<th>Secondary metabolites</th>
<th>Hydroethanolic extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phenols</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Polyphenols</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Catechic tannins</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Triterpenes</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Steroids</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Anthocyanins</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Leucoanthocyanins</td>
<td>+</td>
</tr>
</tbody>
</table>

**Legend:** (+) indicate the presence of active constituents; (−) indicates the absence of active constituents.

Unlike other classes of secondary metabolites that are ubiquitous to all three plant extracts, phenols were found exclusively in *D. gossweilleri* and steroids in *A. sativum*.

#### 3.2. In vitro Anti-inflammatory Activity.

##### 3.2.1. Inhibition of Protein (BSA) denaturation.

The hydroethanolic extracts of *A. sativum*, *D. gossweilleri* and *P. brazzeana* displayed significant activity (Figure 1). The hydroethanolic extracts of *P. brazzeana* at 250 and 500 µg/mL showed highest inhibitions 96.28 % and 98.03 % of Bovine Serum Albumin denaturation (p<0.05) respectively. While, *A. sativum* and *D. gossweilleri* showed 97.60 % and 93.81 % of inhibition of protein denaturation at 500 µg/mL respectively. The hydroethanolic extracts of *P. brazzeana* has also showed a higher inhibition concentration 50 of 91.67 µg/mL than *A. sativum* (113.10 µg/mL) and *D. gossweilleri* (159.50 µg/mL) extracts. These extracts are effective in inhibiting heat induced albumin denaturation compared with that of the standard Sodium diclofenac (IC₅₀ of 6.97 ± 0.98 µg/mL) (Figure 2).
3.2.2. Proteinase Inhibitory Action.

The hydroethanolic extracts of *A. sativum*, *D. gossweileri* and *P. brazzeana* exhibited significant anti-proteinase activity (Figure 3). The best inhibition concentration 50 was observed from stem-barks extract of *D. gossweileri* (31.92 μg/mL) and *P. brazzeana* roots extract (33.62 μg/mL) in decreasing order was *A. sativum* hydroethanolic extract (56.93 μg/mL). The standard Sodium diclofenac drug (8.39 μg/mL) showed the best proteinase inhibitory action (Figure 4).

Figure 3: Effect of hydroethanolic extracts of *A. sativum*, *D. gossweileri* and *P. brazzeana* on the inhibition of proteinase inhibitory action (p<0.05).
3.3. Antimycobacterial Assay.

The hydroethanolic extracts of *P. brazzeana*, *D. gossweileri* and *A. sativum* were evaluated for their anti-mycobacterial effects against three resistant strains of *M. tuberculosis*. The results are presented in Table 2. The strains used possess a drug-resistant profile representing the majority of clinical isolates of tuberculosis patient’s drug control in the south region of Cameroon. These results showed that the hydroethanolic extracts of *A. sativum*, *D. gossweileri* and *P. brazzeana*, exhibited moderate anti-mycobacterial activity against the resistant strains *M. tuberculosis* AC15, AC25 and U with MICs ranging from 312.5-2500 µg/mL. Out of these extracts, *A. sativum* has exhibited very high anti-tuberculosis activities on both the isoniazid resistant strain and Extensively drug resistant strain U2 with same MIC value of 312.5 µg/mL. While the MDR *M. tuberculosis* AC25 has been shown less susceptibility to the plant extracts. A bactericidal effect (I < MBC/MIC ≤ 2) has been observed with all hydroethanolic extracts against Isoniazid (AC15) and Rifampicin (AC25) resistant strains, and XDR strain (U2) respectively; with the exception of the hydroethanolic extract of *A. sativum* that showed a bacteriostatic effect (MBC/MIC ≤ 4) on *M. tuberculosis* U2.

Table 2: Plant materials used for anti-mycobacterial activity, MICs and MBCs values of their hydroethanolic extracts, against resistant strains of *Mycobacterium tuberculosis* AC15, AC25 and XDR.

<table>
<thead>
<tr>
<th>No.</th>
<th>Plant species</th>
<th>Part used in this study</th>
<th>Extraction yields (%)</th>
<th>AC15</th>
<th>AC25</th>
<th>MBC</th>
<th>MIC</th>
<th>MBC/MIC</th>
<th>MIC</th>
<th>MBC</th>
<th>MIC</th>
<th>MBC/MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>D. gossweileri</em></td>
<td>Stem-barks</td>
<td>13.19%</td>
<td>1250</td>
<td>1250</td>
<td>1</td>
<td>2500</td>
<td>5000</td>
<td>2</td>
<td>1250</td>
<td>1250</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td><em>P. brazzeana</em></td>
<td>Roots</td>
<td>6.42%</td>
<td>2500</td>
<td>2500</td>
<td>1</td>
<td>2500</td>
<td>2500</td>
<td>1</td>
<td>625</td>
<td>1250</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td><em>A. sativum</em></td>
<td>Bulbs</td>
<td>8.55%</td>
<td>312.5</td>
<td>312.5</td>
<td>1</td>
<td>2500</td>
<td>2500</td>
<td>1</td>
<td>312.5</td>
<td>1250</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>Rifampicin (RIF)</td>
<td></td>
<td></td>
<td>0.98</td>
<td>3.91</td>
<td>4</td>
<td>1.95</td>
<td>n.d</td>
<td>n.d</td>
<td>15.63</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>5</td>
<td>Isoniazid (INH)</td>
<td></td>
<td></td>
<td>7.81</td>
<td>n.d</td>
<td>n.d</td>
<td>0.12</td>
<td>0.98</td>
<td>8</td>
<td>7.81</td>
<td>n.d</td>
<td>n.d</td>
</tr>
</tbody>
</table>

Legend: * The present extraction yields were calculated in percentages (w/w); MIC: minimal inhibitory concentration; MBC: minimal bactericidal concentration; n.d: not determined; AC15: Isoniazid resistant strain of *M. tuberculosis*; AC25: Rifampicin resistant strain of *M. tuberculosis* and XDR: Extremely drug resistant strain of *M. tuberculosis*.

4. DISCUSSION

The results of phytochemical screening confirmed the use of *P. brazzeana* roots, *D. gossweileri* stem-barks and *A. sativum* bulbs in traditional medicine. We found the secondary metabolites classes, anti-inflammatory, and antimycobacterial activities in these plant extracts. The general qualitative analysis of hydroethanolic extracts of *P. brazzeana*, *D. gossweileri* and *A. sativum* were carried out and extracts showed the presence of various secondary metabolites such as phenols, polyphenols, flavonoids, alkaloids, cathelic tannins, triterpenes, steroids, anthocyanins and leucoanthocyanins showing that these extracts have a high level of medicinal value. The study carry out by Montaut et al., and Nyegue et al., reported the presence of sulfur-containing compounds found in stem barks such as glucosinolin (4-hydroxybenzyl) and glutocarpein (benzyl glucosinolate) in gossweileri extracts and *P. brazzeana* roots meanwhile organosulphur compounds like allicin, an ajouene were found in *A. sativum* hydroethanolic extract[22,28,29]. The presence of these compounds might be responsible for the diverse of biological activities[51].
The extracts of *D. gossweileri*, *P. brazzeana* and *A. sativum* were assessed for its anti-inflammatory activity and the results were compared with Sodium diclofenac standard. Denaturation of proteins like Bovine Serum Albumin is a well-documented cause of inflammation. Maximum inhibition was observed from roots of *P. brazzeana* hydroethanolic extract, which has showed 98.03 % of inhibition of BSA denaturation at 500 μg/mL. Diclofenac sodium, standard anti-inflammatory drug showed 94.28 % of inhibition of protein at the concentration of 500 μg/mL. Same to the inhibition of denaturation of bovine serum albumin, *P. brazzeana* hydroethanolic extract has showed best percentage of inhibitory value (74.19 %) using the proteinase Inhibitory action assay.

The secondary metabolites present in *A. sativum* hydroethanolic extracts like allin and ajene or di-methyl sulphone and tri-methyl sulphone inhibit the complex events and mediators involved during the inflammatory reaction by reducing the level of reactive oxygened compounds and some inflammatory mediators like Tumour Necrosis Factors (TNFα)23. These extracts may also inhibit the release of lysosomal content of neutrophils at the site of inflammation. These neutrophils lysosomal constituents include bactericidal enzymes and proteinases, which upon extracellular release cause further tissue inflammation and damage. The obtained results indicated that the secondary metabolites contained in the hydroethanolic extracts of *P. brazzeana*, *D. gossweileri* and *A. sativum* were capable to resorb the inflammatory processes with a significant level of protection against the mechanisms of protein denaturation and inhibition of lytic enzymes by these bioactive compounds24-26.

In this study, the results demonstrated that the plant extracts inhibited all the resistant strains with the concentrations ranging from 3.125-2500 μg/mL. The *A. sativum* and *P. brazzeana* extracts exhibited better activity than *D. gossweileri* stem barks extract. The activity of hydroethanolic extracts of *A. sativum* have showed more effects against the extensively drug resistant isolate and might be due to the activities of allin and ajene24. For *P. brazzeana* and *D. gossweileri* the extract, many suggested that glycosylates found these plants are the main compounds with antimicrobial and inflammatory and anticancer activities. It the difference of action mechanisms of plants22,23. These plant mechanisms have been reported to have multi-factorial mechanisms due to various phytochemical components that confer their effects at simultaneously25.

These results are in accordance with one of the previous studies which showed that garlic vegetable oil and hydroethanolic extracts Inhibited resistant isolate and, H37Ra and Rifampicin resistant strains of *M. tuberculosis* at concentrations of 0.98, 100 and 200 μg/mL respectively27,28. Another study demonstrated the activity of *P. brazzeana* hydroethanolic extract against H37Ra and clinical isolate of *M. tuberculosis* at the both concentration of 125 μg/mL29. The anti-mycobacterial activity of the tested *D. gossweileri* stem barks extract has not been reported before and therefore our results can be evaluated as the first report about the anti-mycobacterial properties. Compare to the activity of hydroethanolic extracts, the essential oils of these plants exhibited higher activity with MIC ranging 76.17 to 312.50 μg/mL. This difference can be due to the lipophilic character of mycobacterial cell wall. In fact, the mycotic acids contained in cell wall of M. tuberculosis have a high affinity with lipophilic substance like essential oils. This facilitates their uptake in cells and their interaction with cell targets.

The possible explanations for this difference in results among the studies might be due to various species of plant families that differ in concentration of bioactive constituents. This difference in biological activity of ethanolic extracts also be influenced by the vegetative cycle of the plants, the place and the harvest period, the edaphic conditions such as the climate, the type of soil and that are responsible for the variability of their chemical composition2-30. The present study was assumed that the hydroethanolic extracts retain their inhibitory activities due to their various bioactive constituents, against which the gene resistance development might be difficult23.

5. CONCLUSION

Phytochemical studies showed the presence of phenols, polyphenols, flavonoids, alkaloids, catechic tannins, triterpens, steroids, anthocyanins and leucoanthocyanins in hydroethanolic extracts of *D. gossweileri*, *P. brazzeana* and *A. sativum*. Protein denaturation and proteinase inhibitory action assay showed that hydroethanolic extracts of *D. gossweileri*, *P. brazzeana* and *A. sativum* have good anti-inflammatory activity and according to the Microplate Alamar Blue assay a moderate antinocobacterial activity. These results suggest that the hydroethanolic extracts of these plants could be a potential source of natural anti-inflammatory and antinocobacterial compounds. This finding also indicates that at the concentration of 2500 μg/mL each of these hydroethanolic extract could present anti-inflammatory and anti-mycobacterial potential in animal models infected by the multidrug resistant strain of *M. tuberculosis*.

Abbreviations


Competing interests: The authors declare that they have no competing interests.

Author’s contribution

EDFM carried out the research work, wrote the first draft of the manuscript and revised the manuscript. PHDB assisted in the research work, carried out the determination of phytochemical analysis of plant extracts, assisted in the anti-inflammatory and anti-mycobacterial assays, revised the manuscript and submitted the manuscript. CVK assisted in the anti-mycobacterial assay and revised the manuscript. CFMB assisted in the anti-mycobacterial assay and revised the manuscript. NMA identified the project, guided the research and revised the manuscript. All authors have read and approved the final version of the manuscript.

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Consent for publication: Not applicable in this section.
Availability of data and materials: The datasets supporting the conclusions of this article are presented in this main paper. Plant materials used in this study have been identified at the Cameroon National Herbarium where voucher specimens are deposited.

REFERENCES


