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Anti-Quorum Sensing, Antibacterial, Antioxidant Activity and Acute Oral Toxicity of *Acacia hockii* De Wild. (Fabaceae)

Mounirou Tchatchedre[1], Abdou Madjid O Amoussa[1], Martin Kiendrébéogo[2] and Latifou Lagnika[1]*

[1]*Laboratoire de Biochimie and Substances Naturelles Bioactives, Unité de Biochimie et Biologie Moléculaire, Université d'Abomey-Calavi, Cotonou, Bénin*

[2]*Laboratoire de Biochimie et Chimie Appliquées, Université de Ouagadougou, Burkina Faso*

***Corresponding Author:** Latifou Lagnika, Professor, Head of Laboratoire de Biochimie and Substances Naturelles Bioactives, Unité de Biochimie et Biologie Moléculaire, Université d'Abomey-Calavi, Cotonou, Bénin.

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Abstract

Multi-drug resistance in microorganisms is a serious problem of public health at international level. Many researchers have suggested alternatives to antibiotics with minimal or no major side effects. The World Health Organization also has confirmed the importance of the use of medicinal plants in the management of primary health care. Thus, this study aimed to evaluate the anti-quorum sensing, antimicrobial, antioxidant activities and to characterize the phytoconstituents of leafy stems of *Acacia hockii*, a plant used in traditional medicine in Bénin. The ethanolic and aqueous extracts were obtained by maceration and decoction respectively. The fractions were prepared by liquid/liquid partition. Quorum-sensing activity was evaluated by inhibitions of pyocyanin and violacein production using *Chromobacterium violaceum* CV026 and *Pseudomonas aeruginosa* PAO1. Antioxidant potentials of extracts and fractions were evaluated using by 2,2-diphenyl-1-picryl-hydrazyl, Ferric reducing and hydrogen peroxide scavenging methods.

The minimum inhibitory concentration ranged from 0.625 mg/ml to 5 mg/ml. At a sub-inhibitory concentration of 100 µg/mL, ethyl acetate fraction significantly inhibited violacein and pyocyanin production respectively by 69.35% and 45.55% compared to control, without any effect on bacterial kinetic growth. The total phenolics, flavonoids and condensed tannin of extracts ranged from 30.62 to 24.85 mg GAE/100 mg, 32.52 to 26.04 mg QE/100 mg and 17.9 to 13.26 mg CE/100 mg. Ethanolic extract was highest content of total phenolics and condensed tannin while the high rate of total flavonoids was content in aqueus extract. The ethyl acetate fraction showed the highest antioxidant property in comparison to the other extracts and fractions. Ethyl acetate fraction expressed more antibacterial and antioxidant activities. HPLC analysis of ethyl acetate fraction revealed the presence of five phenolic: acid gallic, chlorogenic, tannic, ferrulic, ellargic acid and two flavonoids: hyperoside and Rutin. In the acute oral toxicity of aqueous extract at 2000 mg/kg body weight, no deaths nor biochemical and hematological alterations were observed. The results suggested that *acacia hockii* is not toxic and contains antibacterial compunds justifying its traditional use in the treatment of infectious diseases.

Keywords: *Acacia hockii; Antioxidant; Antibacterial; Quorum Sensing, Toxicity*

Abbreviations

ROS: Reactive Oxygen Species; HNB: Herbier National de l'Université d'Abomey-Calavi (National Herbarium of University of Abomey-Calavi); GAE: Gallic Acid Equivalent; EQ : Quercetin Equivalent; DPPH: 2,2-Diphenyl-1-Picryl-Hydrazyl; FRAP: Ferric Reducing Antioxidant Power; AAE : Equivalent Ascorbic Acid; MIC: Minimum Inhibitory Concentration; TLC: Thin Layer Chromatography; LB: Luria-Bertani; QS: Quorum-Sensing; DMSO: Dimethyl Sulfoxide; HHL: *N*-Hexanoyl-L-Homoserine Lactone; Ht: Haematological Parameters Like Hematocrit; Rbc: Red Blood Cells; Hc: Hemoglobin Concentration; MCHC: Mean Corpuscular Hemoglobin Concentration; MCV: Mean Corpuscular

Volume; MCH: Mean Corpuscular Hemoglobin Levels; Wbc: White Blood Cells; B: Basophils; L: Lymphocytes; M: Monocytes; GLU: Glucose; CREA: Creatinine; URE: Urea; ALT: Alanine Aminotransferase; AST: Aspartate Transaminase; SD: Standard Deviation

Introduction

Infectious diseases are nowadays a major public health concern in the world and especially in developing countries. They are responsible for more than 17 million deaths per year worldwide, developing world are the most affected with an estimated prevalence of 90% 9 [1,2]. This prevalence is still very high in Africa because of low standard living of populations, lack of hygiene, non-control access to antibiotics and inadequate practice of prescribing antibiotics [3]. This increase in resistance has become alarming with the emergence of multidrug resistance associated with the production of several virulence factors [4]. The expression of these virulence factors is controlled by a system of social life called quorum sensing (QS). This system enables bacteria to detect their population density through the production of small diffusible molecules called autoinducers and to coordinate gene expression accordingly [5]. Currently, research on anti-QS strategies is directed towards the identification of molecules interfering with bacterial QS. Among these molecules, there are compounds identified from chemical synthesis programs [6,7] and natural sources [8]. In addition, Reactive oxygen species (ROS) are highly reactive molecules produced during normal cellular metabolism but also as a result of environmental factors. Even if they have physiological role as promoters of natural defenses, they are responsible for the alteration of macromolecules such as lipid, nucleic acids and proteins, thus altering their functions. Previous studies have demonstrated that oxidative stress plays a central role in a common pathophysiology of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, also in cancer where they promote many aspects of tumor development and progression [9,10]. The treatment of infectious diseases and diseases caused by ROS is not easy because of the difficulties associated with the management of these diseases. Natural antioxidants such as polyphenols, thiols, ascorbic acid, β-carotene and tocopherol especially from plants has been suggested as potential molecules capable of resisting oxidative damages to human cells [11]. In Africa and especially in Benin, peoples use medicinal plants for the management of diseases. Thus, several studies have demonstrated the effectiveness of these medicinal plants. However, the effectiveness of many species remains to be demonstrated. Belonging to the Fabaceae family, *Acacia hockii* is a species used in traditional medicine in Bénin. The leaves, root and stem are used in traditioanl medicine for the treatment of illnesses such as bacterial infection, malaria, abscess, gastritises diseases and hookworm problems [12]. To our knowledge, no chemical or biological data has been found in the literature. Therefore, the present study, aimed to investigate the antibacterial, anti-quorum sensing and antioxidative effects of extracts and fractions from the leafy stems of *Acacia hockii* used in Benin traditional medicine.

Materials and Methods

Plant material

The leafy stems of *Acacia hockii* were collected in April 2015 from Natitingou, department of Atacora. Specimen was deposited in the National Herbarium of University of Abomey-Calavi in Bénin under the identification code AA6624/HNB. The samples were dried in laboratory conditions (22 ± 3°C) and then reduced to powder using an electric mill (Longyue LY-989).

Preparation of plant extracts and fractions

Acacia hockii powder (200g) was extracted with 1L of distilled water using a heating mantle with temperature controller (Electromantle MA Solid State Stirrer, 60°C). After 30 minutes of extraction, the resulting mixture was filtered through Whatman paper. The residue obtained was again extracted two times. The resulting filtrate was concentrated using a rotary evaporator and the extract was stored at 4°C. For solvent extraction, the air-dried powder of *Acacia hockii* (200g) was extracted with ethanol (800 ml) by mechanical stirring for 24 hours at laboratory temperature (22 ± 3°C). The mixture obtained after maceration was filtered through Whatman paper (Qualitative Circles 150 mm Cat No. 1001 150). The extraction was repeated twice (02) during 1 hour. The filtrates were concentrated under reduced pressure using a rotary evaporator (Buchi Rotavapor R II). Liquid-liquid extraction is a method to separate compounds based on their relative solubility in two different immiscible liquids. The ethanolic extract was subjected to using partition method. Ethanolic extract

from *A. hockii* was subjected to liquid-liquid fractionation by dissolving 75g of extract in 700 ml of ethanol/water (20:80, v/v). The mixture was partitioned using hexane, dichloromethane and ethyl acetate. The biological activity of crude extracts and obtained fractions was evaluated.

Total phenolic content

Folin-Ciocalteu method was used for measuring the phenolic content as described previously with slight modification [13]. Briefly, 1 mL of extract at 100 µg/mL was added to 1 mL of Folin-Ciocalteu reagent (1:10) diluted with distilled water. After 5 min, 800 µl of saturated sodium carbonate solution (75 g/L) was added. After 2h of incubation at room temperature, the absorbance was measured at 765 nm. Gallic acid was used as a reference and the results were expressed as milligram gallic acid equivalent (GAE) per 100 µg dry plant material.

Total flavonoid content

Aluminium trichloride method was used to determined the flavonoids content [14]. Quercetin was used as reference compound. A volume of 1 mL of extract (100 µg/mL) is added to 1 mL of aluminium trichloride (10%). After 10 minutes of incubation absorbance was measured at 415 nm. The total flavonoids content was expressed as mg quercetin equivalent (EQ) per 100 µg dry weight of plant material.

Total condensed tannin contents

The condensed tannin contents were determined according to Sun., *et al.* method [15]. Catechin was used as a reference compound. A volume of 400 µL of extract at 100 µg/mL were added to 3 mL of a solution of vanillin (4% in methanol) and 1.5 mL of concentrated hydrochloric acid. After 15 minutes of incubation, the absorbance was read at 500 nm. The condensed tannin was expressed as mg Catechin equivalent per 100 µg of dry plant material.

Identification and quantification of phenolic compounds by HPLC-DAD

Phenolic compounds and flavonoids in active extract or fraction were analysed using U-HPLC-DAD liquid chromatograph system equipped with a degasser, binary gradient pump, a UV multiwavelength detector (DAD - 3000 RS and MWD- 3000 RS) and a C_{18} reversed phase column (150 × 4.6 mm, 5 µm Hypersil BDS). The mobile phases consisted of water (A) with 0.1% formic acid and acetonitrile (B) with 0.1% formic acid. The elution gradient (0 - 20 minutes, 20 - 50% B; 20 - 25 minutes 50 - 70% B; 25 - 30 minutes, 70 - 80% B; 30-35 minutes, 80 - 20% B; 35-40 minutes, 20% B). The flow rate was 1 ml/min and injection volume 20 µl. Data analysis was performed using Chromleon v.6.80 Software (Dionex, Thermo Fisher Scientific). Phenolic compounds and flavonoids were identified according to their retention times, UV-Vis spectra and comparison with standard compounds.

Antioxidant activity

The antioxidant activity of hexane, dichloromethane, ethyl acetate and aqueous fractions was evaluated using three assays based on different mechanisms, namely the DPPH radical scavenging, Ferric Reducing Antioxidant Power and H_2O_2 radicals scavenging methods.

DPPH radical-scavenging activity

The antioxidant assay of extracts and fractions was evaluated as described previously [16]. A range of eight concentrations of each fraction obtained by two-fold dilution (30 to 0.23 µg/ml) was tested in this assay. The reaction mixture consists of 0.75 ml of fraction and 1.5 ml of the 0.1 mM solution of DPPH in methanol. The blank sample consists of 0.75 ml of methanol and 1.5 mL of DPPH (0,1mM). Ascorbic acid was used as positive control. All tests were performed in triplicate. The mixture was incubated in the dark at laboratory temperature. After 20 minutes of incubation, absorbencies were read at 517 nm with a spectrophotometer (VWR UV- 1600PC). The inhibition percentage (IP) of DPPH radical was determined according to the formula below:

IP (%) = [(Ab – As)/ Ab] x 100

As is the sample absorbance, Ab is the blank absorbance. The IC_{50} were calculated using the equation of curve IP = f([C]).

Ferric Reducing Antioxidant Power (FRAP) method

Reducing ability was performed using the method described previously [17]. Briefly, 2 ml of each sample at 100 µg/ml were mixed with 2 ml of phosphate buffer (0.2 M, pH 6.6) and 2 ml of potassium ferricyanide (10 mg/ml). The mixture was incubated at 50°C for 20 minutes followed by addition of 2 ml of trichloroacetic acid (100 mg/l). The mixture was centrifuged at 3000 rpm for 10 minutes to collect the upper layer. 2 ml of this layer were mixed with 2 ml of distilled water and 0.4 ml of 0.1% (w/v) fresh ferric chloride. After 10 minutes, the absorbances were read at 700 nm. Ascorbic acid was used for calibration curve. Each assay was performed in triplicate. The Fe^{3+} reducing activity was expressed in µMol Equivalent Ascorbic Acid (AAE) per 100 µg of fraction.

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging ability was determined according to the method described by Mohan., *et al* [18]. 100 µg/ml of extract were dissolved in phosphate buffer (0.1nM, pH 7.4) and mixed with 600 µl of hydrogen peroxide solution. The absorbance values of reaction mixture was read at 230 nm after 10 minutes. Gallic acid was used as reference compound. The hydrogen peroxide scavenging ability was calculated as below:

IP (%) = [(Ac – As)/ Ac] x 100

Ac: control absorbance; As: extract absorbance.

Antibacterial activity

Tested microorganisms

Each fractions from ethanolic extract of *Acacia hockii* were tested against a panel of microorganism, including Gram positive: *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* CIP8039, *Staphylococcus aureus methicillin resistant* (SARM) and Gram negative bacteria: *Escherichia coli* CIP 53126, *Pseudomonas aeruginosa* CIP 82118). The microorganisms were maintained on agar media at 4°C and subcultured for 24h before use.

Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of fractions were determined according to the microdilution method using p-iodonitrotetrazolium [19]. Briefly, 100 µl bacterial broth were incubated with 100 µl of fraction for 18h. A range of eight concentrations of each fraction obtained by two-fold dilution was tested. The final concentrations ranging from 5 to 0.078 mg/ml. After 18h incubation, 40 µl of p-iodonitrotetrazolium (INT) at 2% in methanol were added to each well and plate was incubated. After one hour of incubation, wells stained in red or pink indicated bacterial growth. The MIC, which is the lowest extract concentration that inhibited bacterial growth, was then determined.

Bioautography assay

The bioautography test of hexane, dichloromethane and ethyl acetate fractions was performed on six selected bacterial according to a modified version of the method of Srinivas., *et al* [20]. 10 µL (20 mg/ml) of each fraction were loaded onto TLC plates (Pre-coated TLC-sheets ALUGRAM® silica gel 60) and eluted using two different mobile systems, dichloromethane/Methanol, 99.8:0.2 (v/v) and Toluene/ethyl acetate/formic acid/water, 5:100:10:10 (v/v). The developed plates were dried over night to remove traces of solvent and plates were visualized under UV (wavelength 365 and 254 nm). Each plate was sprayed with bacterial culture (10^6 CFU/ml) of *S. aureus*,

Methicillin-resistant, *S. Aureus*, *S. epidermidis*, *E. faecalis*, *P. aeruginosa* and *E. coli*. Each plate was incubated at 37 °C for 24h. The inhibition zones were visualized by spraying the plates with p-iodonitrotetrazolium 2%. Bioautography allowed to select the most active fraction for the evaluation of anti-quorum sensing activity.

Anti-quorum sensing activity

Bacterial and culture conditions

C. violaceum CV026 and *P. aeruginosa* PAO1 were provided from the Laboratoire de Biotechnologie Vegetale (Université Libre de Bruxelles, Gosselies, Belgium). They were grown in LB broth at 37°C, agitation 175 rpm for PAO1 and 30°C, agitation 175 rpm for CV026.

Determination of MIC and MBC

Before evaluated anti quorum-sensing effect, the minimum inhibitory concentration of fractions against PAO1 and CV026 were determined [21,22]. These assay allowed to select the concentration which does not affect the bacterial growth and which will be used for the evaluation of anti-QS activity.

Inhibition effect of QS-regulated violacein production in C. violaceum CV026

The inhibition of violacein production in *C. violaceum* CV026 by ethanolic extract and ethyl acetate fraction was evaluated according to the method described previously [23]. Violacein production was induced in *C. violaceum* CV026 by adding exogenous N-hexanoyl-l-homoserine lactone (HHL; Sigma-Aldrich Chemie GmbH, Darmstadt, Germany). Extract and fraction at 10 mg/mL were prepared in dimethyl sulfoxide (DMSO). For the test, 100 µL of *C. violaceum* CV026 culture (10^7 CFU/ml) were added to 1860 µL LB broth supplemented with 20 µL HHL (10 mM in DMSO) and 20 µL extract or fraction. The final concentration of extract and fraction was 100 µg/mL and DMSO was used as control. Each assay was performed in triplicate. All tubes were incubated at 30°C, under agitation (175 rpm) for 48h. To evaluate the effect of the extract and fraction on bacterial growth, OD600 nm of the culture was determined every 6 hours for 48 hours. To test the effect of extract and fraction on violacein production, 1 mL of bacterial culture was centrifuged at 7000 rpm for 10 minutes. 1 mL of DMSO was added to the obtained pellet and each solution was vortexed to dissolve violacein. The solution was centrifuged again at 7000 rpm for 10 minutes. Violacein was quantified spectrophotometrically in supernatant at 575 nm using plate reader (Epoch Biotek)

Inhibition effect on growth and pyocyanin production in *P. aeruginosa* PAO1

Inhibition of pyocyanin production was assessed according to Vandeputte., *et al* [23]. *P. aeruginosa* PAO1 was grown in LB broth at 37°C, 175 rpm agitation for 18h. The obtained culture was washed twice with fresh LB medium by centrifugation (7000 rpm for 15 mn). To evaluate the effect of extract and fraction on pyocyanin production, 100 µL of PAO1 (OD600nm between 0.02 and 0.03) was added to 1880 µL LB medium supplemented with 20 µL sample at 10 mg/mL in DMSO. All assay were performed in triplicate and all tubes were incubated at 37°C stirring for 18 h. The tubes contents were sampled each 3 h periodic intervals to assess bacterial growth and pyocyanin production. The pyocyanin was extracted from the supernatant obtained by centrifugation (8,000 rpm, 10 minutes) of the *P. aeruginosa* culture. 2 mL of chloroform was added to 4 mL of supernatant and the solution was mixed vigorously. The chloroform layer containing pyocyanin was extracted again with 1 mL of HCl (0.2 M). The pyocyanin was quantified spectrophotometrically at 380 nm using a plate reader (Epoch Biotek).

Oral acute toxicity testing

The oral acute toxicity of the aqueus extract of *A. hockii* was evaluated on rats using the procedures described by Organization for Economic Co-operation and Development 423 guidelines [24]. A total of six females animals were divided into two groups with three animals each and kept in different cages for easy observation during experiment. Distilled water (10 ml/kg body weight) was given to control group (group I). The animals of group II were given with a single dose of 2000 mg/kg body weight of aqueus extract of *A. hockii*

dissolved in distilled water. Gavage dosing was performed using a curved, ball-tipped intubation needle affixed to a 5 ml syringe. Following administration of extract, rats were closely monitored for 30 min and 2, 4, 8 and 24h. Mortality, food and water consumption and general acute toxicity or clinical symptoms were recorded. Body weight was recorded on day 0, 7 and 14. At the end of the experiment, all the rats were anaesthetized using Chloroform. The blood sample is taken from retro-orbital sinus of the eye using a capillary hematocrit. Haematological parameters like hematocrit (Ht), red blood cells (Rbc), hemoglobin concentration (Hc), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular hemoglobin levels (MCH), white blood cells (Wbc), basophils (B), lymphocytes (L), monocytes (M) were analyzed using an automatic hematological analyzer (Sysmex, XP-300, Japan). Biochemical parameters such as Glucose (GLU), Creatinine (CREA), urea (URE), alanine aminotransferase (ALT), aspartate transaminase (AST) were determined using an autoanalyzer (Erba Chem 7, Germany).

Statistical analysis

Data were analysed using Microsoft Excel and all results are expressed as mean ± standard deviation (SD) with n = 3. Further, the statistical difference between samples were determined via Student Test with significance level at 5% ($p < 0.05$).

Results and Discussion

Total phenolic, flavonoid and condensed tannin contents

Preliminary phytochemical screening of leafy stems of *Acacia hockii* revealed the presence Alkaloid, Coumarin, Flavonoid, Pigment, Saponin, Lignan and anthracene derivatives in ethanolic and aqueous extracts except naphthoquinones [25]. To our knowledge, no study has been conducted on the determination of total phenolic, flavonoid and condensed tannin contents on leafy stems extracts of *A. hockii*. However, interesting results in phenolic compounds of related species have been reported [26,27]. The total phenolics, flavonoids and condensed tannins ranged respectively from 30.62 to 24.85 mg GAE/100 mg, 32.52 to 26.04 mg QE/100 mg and 17.9 to 13.26 mg CE/100 mg. The highest contents of total phenolics (30.62 mg GAE/100 mg) and condensed tannin (17.9 mg EC/100 mg) were detected in ethanolic extract while the highest contents of total flavonoids (32.52 mg EQ/100 mg) were detected in aqueous extract (Table 1). These results indicated the influence of the extraction solvent on the total content of phenolics, flavonoids and condensed tannins in extract. It is reported that phenolic compounds and flavonoids are natural products which possess various biological properties related to antioxidant mechanisms [28]. Phenolic compound have the function to scavenge the free radicals in human body and to help maintain healthy body by scavenging or removing the reactive oxygen species (ROS) [29]. Based on the interesting results obtained, the ethanolic extract was subjected to a liquid partititon as described in methodology. The biological properties of extracts and fractions were then evaluated.

Extracts	Total phenolics (mg GAE/100 mg)	Total flavonoids (mg QE/100 mg)	Condensed Tannins (mg CE/ 100 mg)
EtOH extract	30.62 **± 1.52**	26.04 **± 0.78**	17.9 **± 0.22**
H_2O extract	24.85 **± 1.02**	32.52 **± 1.25**	13.26 **± 0.1**2

Table 1*: Colorimetric quantification of phenolics, flavonoids and condensed tannin in Acacia hockii.*

EtOH: Ethanol; H_2O: Aqueous (Distilled Water); GAE: Gallic Acid Equivalent; QE: Quercetin Equivalent; CE: Catechin equivalent.

Values are mean ± SE (n = 3).

Antioxidant activity

Hydrogen transfer methods measure the overall ability of an antioxidant to suppress free radicals by giving a hydrogen atom while electron transfer methods measure the ability of the antioxidant to transfer an electron that will reduce any type of compounds, including metals, carbonyl groups and radicals. Taking into account the complexity of the oxidation process, a single method alone does not reflect

the antioxidant profile of a sample. Therefore, DPPH radical scavenging, Ferric Reducing Antioxidant Power (FRAP) and H_2O_2 radicals scavenging methods were used to evaluate antioxidant activity of extracts and fractions.

DPPH radical scavenging activity indicates the hydrogen donating ability of secondary metabolites in extract or fraction. The measurement of the absorbance at 517 nm is an indicator of the antioxidant potential of secondary metabolites in extract [30]. Lowest inhibitory concentration 50 (IC_{50}) indicates interesting reducing power of the sample. DPPH scavenging activity of extracts and fraction were dose dependent (Figure 1). The ability of extracts and fractions to reduce the radical DPPH is in the following order: ethyl acetate fraction (IC_{50}: 2.04 µg/ml) > ethanolic extract (IC_{50}: 4.46 µg/ml) > aqueous extract (IC_{50}: 4.79 µg/ml) > aqueous fraction (IC_{50}: 5.06 µg/ml) > dichloromethane fraction (IC_{50}: 10.92 µg/ml) > hexane fraction (IC_{50}: 16.36 µg/ml). The DPPH radical reducing power of ethyl acetate fraction (IC_{50}: 2.04 µg/ml) is higher than that of ascorbic acid (IC_{50}: 3.82 µg/ml) used as a control (Table 2).

Sample	IC_{50} (µg/mL)
Ascorbic acid	3.83
EtOH extract	4,46
AcOEt fraction	2.04
H_2O extract	4,79
Aq fraction	5.06
DcM fraction	10.92
Hex fraction	16.36

Table 2: *Inhibitory concentration 50 (IC_{50}) of extracts and fractions against DPPH radical. EtOH: Ethanol; AcOEt: Ethyl Acetate; DcM: Dichloromethane; Hex: Hexane; Aq: Aqueous.*

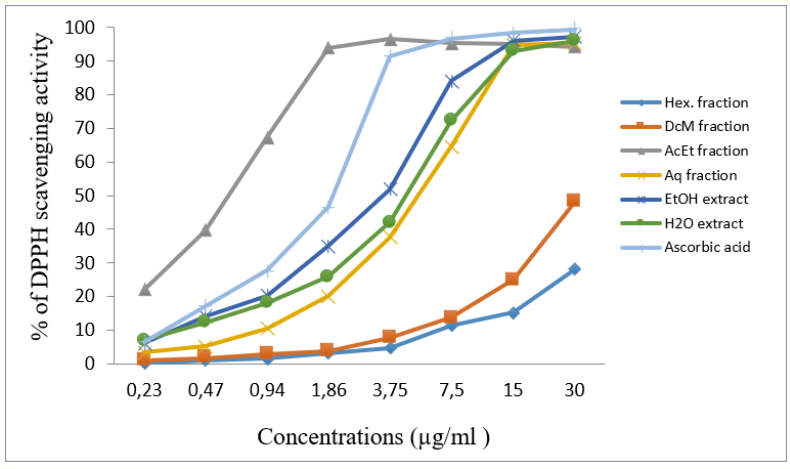

Figure 1: *DPPH radical scavenging activity of extracts and fractions from Acacia hockii. EtOH: Ethanol; AcOEt: Ethyl Acetate; DcM: Dichloromethane; Hex: Hexane; Aq: Aqueous.*

In the Ferric Reducing Antioxidant Power, antioxidant acts as a reductant that can reduce the Fe^{3+}/ferricyanide complex to ferrous form (Fe^{2+}). Thus, ferrous ions (Fe^{2+}) can be measured by the formation of Pearl's Prussian blue at 700 nm [31]. Higher absorbance at 700 nm corresponds to a significant reducing power. In this study, the Ferric Reducing Antioxidant Power values range from 647.41 to 3363.26

µmol AAE g-1 (Figure 2). Ethyl acetate fraction was the most active with FRAP value of 3363.26 µmol AAE g^{-1} followed by ethanolic extract (2836.55 µmol AAE g^{-1}) and dichloromethane (2792.66 µmol AAE g^{-1}). These results indicated significant electron donating capacity of the fraction and extract.

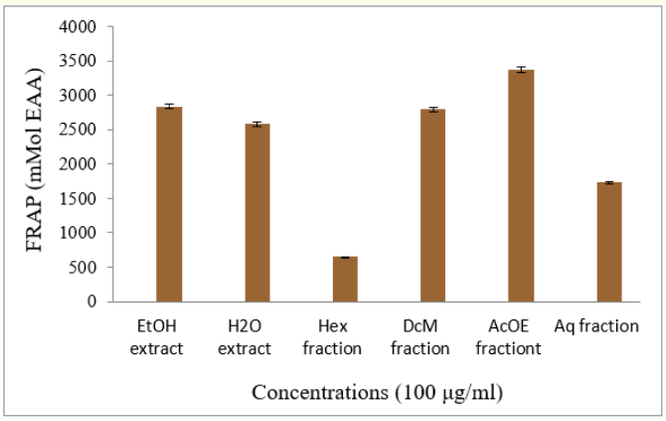

Figure 2: *Ferric Reducing Antioxidant Power of extracts and fractions from Acacia hockii. EtOH: Ethanol; AcOEt: Ethyl Acetate; DcM: Dichloromethane; Hex: Hexane; Aq: Aqueous.*

Hydrogen peroxide (H_2O_2) scavenging is weak oxidizing agent that inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly; once inside the cell, it can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radicals and this may be the origin of many of its oxide effects [32]. In this study, the results show that the scavenging percentage of hydrogen peroxide decreases in the following order: ethanolic extract (97.65%) > ethyl acetate fraction (94.02%) > aqueous fraction (59.46%) > aqueous extract (30.1%) > dichloromethane (17.94%) > hexane (9.3%) (Figure 3). The positive control, gallic acid, demonstrated inhibitory power 93.97% comparable to ethanolic extract and ethyl acetate fraction. These results showed that there is a correlation between the antioxidant activities and the extraction solvents. The high phenolic compounds of ethyl acetate fraction and ethanolic extract could justify their marked antioxidant activity. It is reported that phenolic compounds and flavonoids are natural products which have been shown to possess various biological properties related to antioxidant mechanisms [28]. *Acacia hockii* extracts revealed the presence of flavonoids and phenolics which are major group of compounds that act as antioxidants or free radical scavengers and inhibitors [33,34].

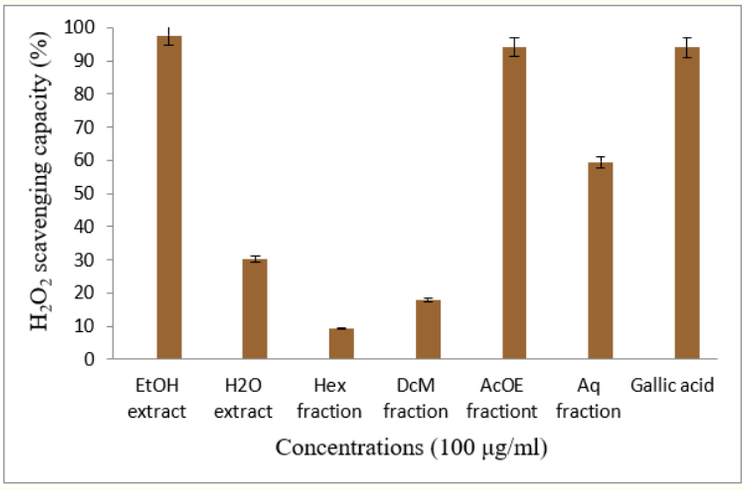

Figure 3: *Hydrogen peroxide (H_2O_2) scavenging of fractions and extracts A. hockii. EtOH: Ethanol; AcOEt: Ethyl Acetate; DcM: Dichloromethane; Hex: Hexane; Aq: Aqueous.*

Identification of phenolic compounds and flavonoids

Analysis of ethyl acetate fraction which was distinguished by it highest biological activity revealed the presence of gallic, chlorogenic, and tannic acids and hyperoside and rutin (Table 3). Other non-identified phenolic acid and flavonoids were also identified.

Identified compounds	Retention time (min)	Relative area (%)
Gallic acid	2.71	45.60
Chlorogenic acid	7,13	1.11
Tannic acid	10.22	0.59
Ferrulic acid	12.42	0.38
Hyperoside	16.38	7.61
Rutin	17.78	4.88
Ellargic acid	18.36	0.11

Table 3: *Phenolics and flavonoids identified in ethyl acetate fraction of A. hockii.*

Antibacterial activity

Minimum inhibitory concentration activity (MIC)

Previous MIC evaluation of ethanolic and aqueous extracts from *Acacia hockii* revealed that ethanolic extract was more effective with MIC values varied from 0.625 to 1.25 mg/mL [25]. In the current study, ethanolic extract was subjected to a partition using hexane, dichloromethane and ethyl acetate and the MIC of obtained fractions were evaluated. The results indicated that fractions were able to prevent microorganism growth. The sensitivity of bacteria to different fractions represented by the MIC values varies from 0.625 to > 5 mg/ml (Table 4). Ethyl acetate fraction was the most effective fraction with a MIC values from 0.625 to 1.25 against all tested bacteria, followed by Dichloromethane fraction (MIC: 0.625 to 2.5). The hexane fraction showed no activity on the bacterial strains used. (MIC: > 5). Previous studies have reported that unlike hexane and dichloromethane fractions, the ethyl acetate fraction was the most active in inhibiting bacterial growth [35]. This activity could be justified by the presence in this fraction of phenolic compounds (gallic acid, tannic, chlorogenic, ferrulic and ellargic) and flavonoids (hyperoside and rutin) which are known for their antibacterial and antioxidant potential [36,37].

	Minimum Inhibitory Concentration (mg/ml)					
	Gram (+) bacteria				Gram (-) bacteria	
Bacteria / Samples	*S. au*	*S.a.m.r*	*S. ep*	*E. fae*	*P.ae*	*E. co*
Hex fraction	> 5	> 5	> 5	> 5	> 5	> 5
DcM fraction	0.625	0.625	0.625	1.25	2.5	1.25
AcOEt fraction	1.25	1.25	1.25	0.625	1.25	1.25
Aq fraction	2.5	2.5	2.5	5	5	2.5
Gentamicin	< 0,0039	< 0,0039	< 0,0039	< 0,0039	< 0,0039	0,0156

Table 4*: Minimum Inhibitory Concentration (mg/ml) of fractions from A. hockii*

S. au: Staphylococcus aureus; S.a.m.r: Staphylococcus aureus methicillin-resistant; S. ep: Staphylococcus epidermidis; E. fae: Enterococcus faecalis; P.ae: Pseudomonas aeruginosa; E. co : Escherichia coli.

EtOH: Ethanol; AcOEt: Ethyl Acetate; DcM: Dichloromethane; Hex: Hexane; Aq: Aqueous.

Bioautography and identification of antimicrobial compounds

Bioautography aimed to identify the active compounds in the fractions. Bioautography was performed on dichloromethane and ethyl acetate fractions, which were active against tested bacterial. Inhibition zones of antimicrobial compounds were observed as white spots on a purple red background. These white areas indicate the presence of antimicrobial compounds which inhibit the growth of microorganisms [38]. The bioautography of dichloromethane and ethyl acetate revealed inhibition zones for ethyl acetate fraction against both Gram positive and Gram negative bacteria. Secondary metabolites of dichloromethane fraction almost did not inhibit the growth of bacteria. The absence of activity could be due to very little amount of the active compounds [38]. It is also possible that the synergy between molecules plays a major role in the activity of the actives fractions which showed interesting activity during the MIC evaluation while the separated molecules of the same fraction on the bioautography plate lose their activity.

Anti qorum-sensing activity

Inhibition effect of QS-regulated violacein production in *C. violaceum* CV026

Quantitative analysis of violacein production was carried out using the *C. violaceum* CV026 reporter strain. This strain is able to produce violacein when the natural inducer HHL is supplemented to the growth medium [39]. In this study, ethanolic extract and ethyl acetate fraction were added to HHL-induced *C. violaceum* CV026 cultures. At a concentration of 100 µg/mL, the ethyl acetate fraction and the ethanolic extract did not inhibit the growth of *C. violaceum* CV026. At the same concentration, the ethanolic extract and the ethyl acetate fraction significantly inhibited by 69.35% and 50% the production of violacein after 48 hours, with no effect on bacterial kinetic growth (Figure 4). Numerous studies showed the anti-QS potential of the compounds such as polyphenols and flavonoids [23,40]. The presence of phenolic and flavonoid compounds identified in the ethyl acetate fraction of *A. hockii* would justify its potential to reduce violacein production. Violacein was not produced in *C. violaceum* CV026 cultures non induced by HHL indicating that ethanolic extract and ethyl acetate fraction do not contain HHL compounds but contain QS inhibitory compounds.

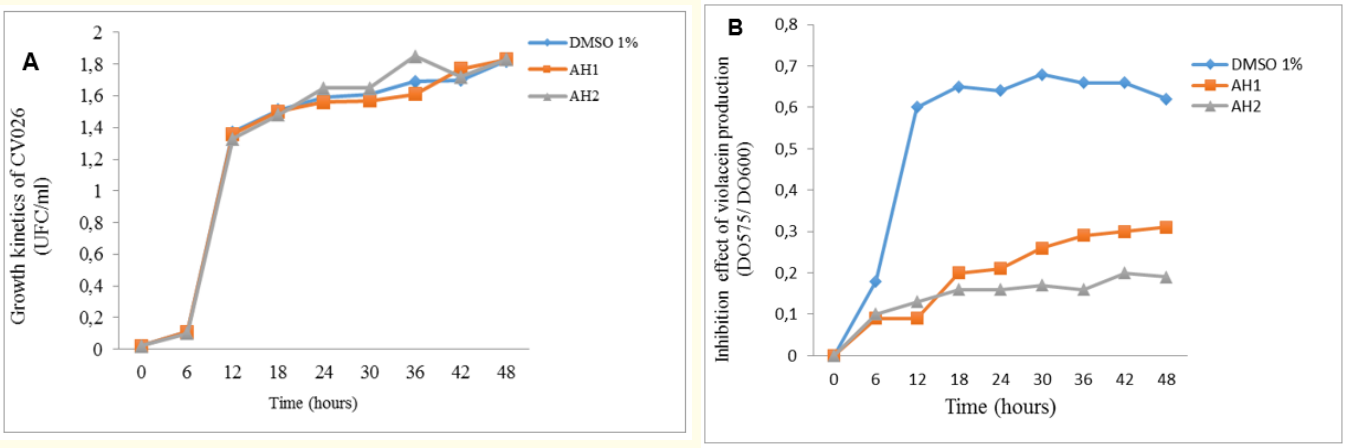

Figure 4: *Growth kinetics (A) and violacein production (B) in C. violaceum CVO26.*
AH1: Ethanolic Extract; AH2: Ethyl Acetate Fraction. Dimethyl sulfoxide (DMSO 1%) was used as negative control.

Inhibition effect on pyocyanin production in *P. aeruginosa* PAO1

Ethanolic extract and ethyl acetate fraction were also tested on *P. aeruginosa* PAO1, where the extracellular virulence factor pyocyanin can be easily detected [41]. Evaluation of their ability to interfere with the production of pyocyanin showed that the acetate fraction reduced the production by 45.55% and ethanolic extract 22.22% (Figure 5). Reduction of pyocyanin production observed within our study might be due to presence of polyphenols and flavonoids, previously quantified. Polyphenols and flavonoids are known for their anti-QS potentiality. These compounds have been reported to inhibit QS-regulated virulence factors expression in *P. aeruginosa* and thought to possibly interfere with the perception of the native AHL by LasR and RhlR [42]. Pyocyanin is one of the virulence factors produced by *P. aeruginosa*. It is involved in the degradation of the host cell but also in the production of reactive oxygen species by modifying the oxidation-reduction cycle involved in cellular respiration and thus increasing the oxidative stress of the host cells [43,44]. Polyphenols and flavonoids, known for their antioxidant activity and identified in ethyl acetate fraction of *A. hockii*, could contribute to the reduction of the oxidative stress caused by pyocyanin and thus reduce inflammatory intensity.

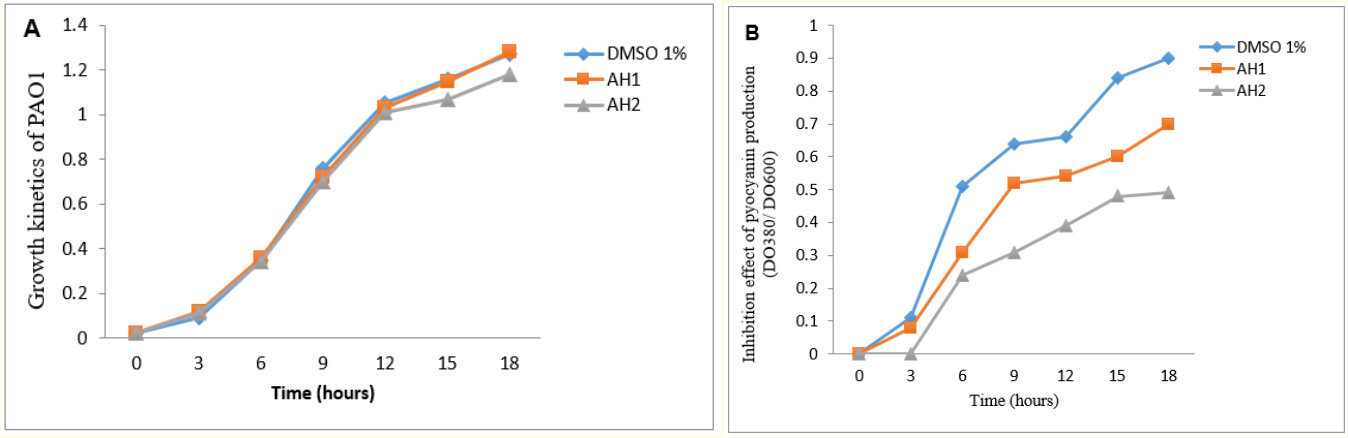

Figure 5: *Growth kinetics (A) and pyocyanin production (B) by P. aeruginosa PAO1.*
AH1: Ethanolic Extract; AH2: Ethyl Acetate Fraction. Dimethyl sulfoxide (DMSO 1%) was used as negative control.

Acute oral toxicity of aqueous extract of *Acacia hockii*

Acute oral toxicity results revealed that rats did not showed any visual symptoms of toxicity or mortality during the observation period. Haematological parameters including Hematocrit (HCT), red blood cells (RBC), hemoglobin (HGB), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular hemoglobin levels (MCH), Red blood cells (RBC), white blood cells (WBC), lymphocytes (LYM) and Red Cell Distribution Width (RDW) did not show any noticeable adverse effects (Table 5). Otherwise, the biochemical analysis including Glucose (GLU), Creatinine (CREAT), Urea (URE), alanine aminotransferase (ALT) and aspartate transaminase (AST) revealed a significant increase in urea and decrease in creatinine (Table 6). It is well know that increase in the levels of urea and creatinine, provides a positive indication towards adverse effects in kidney functions. Therefore, it is likely that diet, degree of hydration and protein metabolism may be responsible for increasing the level of urea in the blood [45,46]. Levels of creatinine are often linked with muscle mass or the amount of muscle in the body, which can decrease with age or illness. Low levels may indicate that muscles are less strong or are deteriorating, for example with a disease such as muscular dystrophy.

Biochemical parameters	Control	Experimental
GLU (g/L)	1.427 ± 0.03	1.193 ± 0.31
CREAT (mg/L)	5.627 ± 4.44	3.193 ± 0.11
UREE (g/L)	0.337 ± 0.25	0.603 ± 0.10
ALT (IU/L)	91.273 ± 2.01	64.683 ± 3.75
AST (IU/L)	160.867 ± 4.92	153.47 ± 3.76

Table 6: *Effect of aqueous extract of A. hockii on biochemical parameters in Wistar rats. GLU: Glucose; CREAT: Creatinine; Urea (UREE); ALT: Alanine Aminotransferase; AST: Aspartate Transaminase. Values are mean ± SEM (n = 3 per group), differences were considered significant when p-values were less than 0.05 (p < 0.05).*

The plasma concentration of creatinine depends almost exclusively on glomerular filtration. Creatinine is therefore a reliable part of kidney failure as elevation of serum urea levels. However, creatinine does increase significantly in plasma only when the glomerular filtration rate is reduced by about half [47]. Accordingly, the significant decrease in creatinine in rat plasma could be due to good perfomance of the glomeruli.

Conclusion

The present study revealed that aqueous and ethanolic extracts of *A. hockii* stems leafy possess antioxidant and antimicrobial properties, which could be explored in the treatment of bacterial diseases and in control of oxidative stress. Our study also demonstrated the anti-QS activity of the ethyl acetate fraction of *A. hockii*. Based on bibliographic reports, polyphenols and flavonoids might be responsible for the anti-QS property demonstrated. By reducing the pyocyanin production and related oxidative stress within infected tissues in a QS manner, *A. hockii* benefits to the healing process of resistant bacteria. Acute toxicity test results revealed that rats did not showed any visual symptoms of toxicity or mortality in animals during the observation period. We are planning to continue experiments to separate the ethyl acetate fraction obtained and further identify the active molecules, responsible for activities.

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