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Full Length Research Paper

Immunomodulatory activities of methanol extract of the whole aerial part of *Phyllanthus niruri* L.

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In this study, the effect of methanol extract of whole aerial parts of *Phyllanthus niruri* on some specific and non-specific immune response was investigated. The effects of *P. niruri* on *in vivo* leucocyte mobilization, delayed type hypersensitivity (DTHR) response, and humoral antibody (HA) response were determined in rats. Acute toxicity profile of *P. niruri* was evaluated in mice. The Agar induced *in vivo* leucocytes mobilization into the rats peritoneal fluid was ($P < 0.05$) increased by *P. niruri* N (200 and 400 mg/kg) in a dose related manner. The total leucocytes count was higher in the extract treated group than the control group. Polymorphonuclear neutrophils (PMNS) were more mobilized than the lymphocytes. *P. niruri* at 100, 200 and 400 mg/kg body weight produced significant ($P < 0.05$) inhibition of DTH response in rat by 30.55, 66.67 and 44.44%, respectively with 200 mg/kg being most significant. The primary and secondary sheep red blood cell antibody titres were significantly elevated when compared with the control group. *P. niruri* administered orally showed no death or signs of acute intoxication at doses up to 5000 mg/kg after 24 h of observation. The result of this study showed that the methanol extract of *P. niruri* whole plant possess immunomodulatory activities and warrant further investigation to determine the specific constituent(s) of the plant responsible for this property.

Key words: *Phyllanthus niruri*, leucocyte mobilization, delayed type hypersensitivity (DTHR), humoral antibody titre, immunomodulation.

INTRODUCTION

The immune system is a collection of cells and proteins that works to protect the body from harmful non-specific agents such as pathogenic bacteria, viruses and cancers.

It utilizes the non-specific (innate) and the specific (adaptive) systems to eliminate these threats. Emerging infectious diseases, bioterrorism, modern day stress are

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some of the factors that have made the use of biological response modifiers such as immunomodulators and adaptogens appealing. Recently, there has been a surge in the number of products claimed to boost the immune system, especially herbs. These developments have invariably stimulated a lot of scientific investigations into the veracity of these claims and on the potential benefits of these products to patients. The two arms of the immune response mechanisms can be downregulated or upregulated by immunomodulators. Substances that stimulate the immune system are indicated in the treatment of cancer, immunodeficiency diseases, for generalized immunosuppression following drug treatment, for combination therapy with antibiotics, and as adjuvants for improving vaccines immunogenicity (Nworu et al., 2007, 2010). It has also been suggested that immunomodulatory regimes offer an attractive approach as an adjunct in the control of microbial diseases and in the management of antibiotic resistance (Masihi, 2001). On the other hand, immunosuppressive agents are indicated for conditions associated with hyperimmune responsiveness such as transplanted organ rejection and autoimmune disorders.

Several studies have shown that herbal extracts and supplements possess immunomodulatory properties that could be beneficial if harnessed. One medicinal plant that has been promoted as an immune-boosting agent is *Phyllanthus niruri* Linn. (Family: Euphorbiaceae). *P. niruri* is well-known, versatile herb used medicinally in Africa, Asia, and South America for a variety of ailments (Bagalkotkar et al., 2006). Extracts of this herb have been proven to have therapeutic effects in many animal and clinical studies (Thyagarajan et al., 1988; Calixto et al., 1998; Liu et al., 2001; Xin-Hua et al., 2001; Nworu et al., 2010a, b). Some of the most prominent therapeutic properties include antihepatotoxic (Prakash et al., 1995), antidiabetic (Calixto et al., 1998), anti-HIV (Ogata et al., 1992; Quian-Cutron, 1996), anti-cancer (Rajesh Kumar et al., 2000) and antihepatitis B. (Venkateswaran et al., 1987; Thyagarajan et al., 1992; Shead et al., 1992). It is believed that most of the beneficial effects attributed to the herb are related to its immunomodulatory properties.

P. niruri is a small, erect, annual herb that grows 30 to 50 cm in height (Wikipedia). It is indigenous to the rainforests of the Amazon and other tropical areas throughout the world, including the Bahamas, Southern India and China. *P. niruri* is prevalent in the Amazon and other wet rainforests, growing and spreading freely. *P. niruri* is known in many languages, as Stonebreaker (English), Chanca Piedra (Spanish), and Quebra Pedra (Portuguese). It is a widespread tropical plant commonly found in coastal areas. Many active constituents, responsible for these pharmacological activities of *P. niruri*, have also been identified. Some of the isolated bioactive constituents have been found only in the *Phyllanthus* genus. Generally, biologically active lignans, glycosides,

flavonoids, alkaloids, ellagitannins, and phenyl propanoids have been identified in the leaf, stem, and root of the plant (Colombo et al., 2009; Rain Tree Data Base, 2009). In South Eastern Nigeria, *P. niruri* is popularly called “Enyikwonwa” and features in herbal recipes used by herbalist to treat a variety of infections, including claims of effectiveness in the management of HIV/AIDS and hepatitis. Although, it is believed that most of these beneficial effects of *P. niruri* could be related to the immunomodulatory activities, there is scarcity of data on the effects of *P. niruri* on the innate, humoral and cellular immune responses. This motivated the present study in which the effects of the methanol extract of the whole aerial part of *P. niruri* on some specific and non specific immune responses were investigated in murine model.

MATERIALS AND METHODS

Animals

Swiss albino mice (17 to 19 g) and adult rats of Wisterstrain (150 to 220 g) of both sexes obtained from the animal facility of the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka were used in the study. The animals were fed with standard livestock pellets and allowed unrestricted access to drinking water. The rodents were housed under room temperature of $25\pm 2^{\circ}\text{C}$ and 12 h light/dark cycle.

Antigen

The antigen used in the work is fresh sheep red blood cell (SRBC) obtained from the Animal Farm of the Faculty of Veterinary Medicine, University of Nigeria Nsukka. The sheep red blood cells (SRBCs) was washed three times in a large volume of pyrogen-free sterile normal saline by repeated centrifugation at 2500 rev/s for 10 min on each occasion. The washed SRBC was adjusted to a concentration of approximately 1×10^9 cells/ml and used for both immunization and challenge.

Preparation and extraction of plant

The whole aerial parts of *P. niruri* were collected from the wild in May, 2008 at Imilike, a local community in Nsukka District, Enugu State, Nigeria. The plant was identified and authenticated by Mr. Alfred O. Ozioko, a plant taxonomist of the International Centre for Ethnomedicine and Drug Development (InterCEDD), Nsukka, Enugu State, Nigeria. The whole plant was air dried, and then pulverized. The powdered whole plant (1 kg) was extracted with 3 L of methanol in a soxhlet extractor for 24 h and thereafter concentrated in a rotary evaporator to obtain the solid extract of *P. niruri* (PN; 162.89 g; 16.29% w/w). *P. niruri* was subjected to preliminary phytochemical studies according to the procedures outlined by Harbourne (1984) and Trease and Evans (1996).

Acute toxicity studies

Acute toxicity of *P. niruri* (LD_{50}) was performed in mice using the procedures of Lorke (1983). Briefly, the tests involved two phases. The first phase involved the determination of the toxic range. The

mice were placed in three groups ($n=3$) and *P. niruri* (10, 100, and 1,000 mg/kg) was administered orally. The treated mice were observed for 24 h for any deaths. The death pattern in the first phase determined the doses used for the second phase according to the Lorke (1983) estimation. In the second phase, four different doses of *P. niruri* were administered (*per os*) as predetermined in the earlier phase of the study. The animals were observed for lethality and signs of acute intoxication for the next 24 h. The LD₅₀ was calculated as the geometric mean of the highest non-lethal dose and the least toxic dose.

***In vivo* leucocytes mobilization rate**

The method of Ribeiro et al. (1991) was utilized in the *in vivo* leucocyte mobilization study. One hour after oral administration of the *P. niruri* (100, 200, and 400 mg/kg), each rat in the groups ($n=5$) received intraperitoneal injections of 0.5 ml of 3% (w/v) agar suspension in normal saline. Four hours later the rats were sacrificed and the peritoneum washed with 5 ml of a 5% solution of EDTA in phosphate buffered saline (PBS). The peritoneal fluid was recovered and total and differential leucocytes counts (TLC and DLC) performed on the perfusates.

Delayed type hypersensitivity response (DTHR)

Delayed type hypersensitivity was induced in rats using SRBC as antigen. Animals were sensitized by subcutaneous injection of 0.2 ml of 1×10^8 cells/ml SRBC (day 0) in the plantar region of the right hind foot paw and challenged on day 5 by subcutaneous injection of the same amount of antigen into the left hind paw. *P. niruri* (100, 200 and 400 mg/kg) were administered 3 days prior to sensitization and continued till the challenge (Shinde et al., 1999; Naved et al., 2005). The oedema produced by antigenic challenge in the left hind paw was taken as the difference in the paw thickness before and 24 h after the challenge. The paw thickness was measured by volume displacement.

Humoral antibody (HA) response

Rats were immunized by an intraperitoneal injection (i.p.) of 0.2 ml of 1×10^9 SRBC/ml (day 0) and challenged by similar i.p. injection of the same amount on day 5. Primary anti body titer was determined on day 5 (before the challenge) and secondary titre on day 10 (Sharma et al., 1996) by the haemagglutination technique (Nelson and Mildenhall, 1967). *P. niruri* (100, 200 and 400 mg/kg) were administered 3 days prior to immunization and continued daily for 5 days after challenge. Blood samples were obtained by retro-orbital puncture in test tubes and allowed to clot. For each sample, a 25 μ l serum was obtained after centrifugation and serially diluted two-fold in 96 U-bottom microtitre plates using pyrogen free sterile normal saline. The last well on each row contained sterile normal saline as control. The diluted sera were challenged with 25 μ l of 1% (v/v) SRBC in the plates and then incubated at 37°C for 1 h. The highest dilution giving rise to visible haemagglutination was taken as antibody titre. Antibody titre values were expressed in graded manner, the minimum dilution (1/2) being ranked as 1 (calculated as $-\log_2$ of the dilution factor). The mean ranks of different treatment groups were compared for statistical significance.

Statistical analysis

Results of the experiments were analysed using one way analysis of variance (ANOVA, Fischer LSD post hoc test) and expressed as mean \pm standard error of mean. Differences between means of

treated and control groups were considered significance at $P<0.05$.

RESULTS

Yield and phytochemical analysis

The methanol extraction of 1 kg of *P. niruri* yielded 162.89 g of the drug residue (PN) which represent 16.29% w/w. Phytochemical tests on PN indicated the presence of alkaloids, carbohydrates, resins, tannins, glycosides, reducing sugar, flavonoids, acidic compounds and saponins.

Acute toxicity studies on *P. niruri*

P. niruri administered orally to groups of mice did not cause death or signs of acute intoxication even at doses up to 5000 mg/kg after 24 h of observation.

Effect of *P. niruri* on *in vivo* leucocytes mobilization

Oral administration of *P. niruri* (200 and 400 mg/kg) caused a significant ($P<0.05$) and dose-related increase in *in vivo* leucocyte mobilization in mice by 24.6 and 27%, respectively. The increase was not significant in 100 mg/kg group. Groups of mice treated with *P. niruri* also showed significantly ($P<0.05$) higher number of mobilized neutrophils than the control group (Table 1).

Effect of *P. niruri* on delayed type hypersensitivity response (DTHR) in rats

Short term oral administration of *P. niruri* (100, 200 and 400 mg/kg) caused a significant inhibition of DTHR induced by SRBC in rats up to 30.55, 66.67, and 44.44%, respectively. The highest level of inhibition was observed at 200 mg/kg ($P<0.05$) (Table 2).

Effect of *P. niruri* on primary and secondary antibody production

Haemagglutination titre (HA) showed that oral administration of *P. niruri* in rats produced a dose-dependent increase in primary and secondary antibody production. Administration of *P. niruri* (100, 200, and 400 mg/kg) increased the mean primary anti-SRBC antibody from 3.5 ± 0.29 in the untreated control group of rats to 4.0 ± 0.32 , 4.4 ± 0.24 and 4.6 ± 0.24 , representing a significant percentage increase of 14.29, 25.71 and 31.43%, respectively. Similarly, the mean secondary anti-SRBC antibody was increased from 4.5 ± 0.29 in negative control to 5.4 ± 0.51 , 5.2 ± 0.20 and 7.6 ± 0.40 in groups treated with *P. niruri* (100, 200, and 400 mg/kg), also representing a

Table 1. The effect of PN on *in vivo* leucocyte mobilization in rats.

Treatment	Dose (mg/kg)	TLC (cells mm ⁻³)	Increase in leucocytes mobilization (%)	DLC (%)	
				Neutrophils	Lymphocyte
PN	100	652±108	-	49.6±5.0	50.4±5.0
	200	1228±260	24.7*	55.6±6.9*	44.4±6.9
	400	1241±413	26.0*	54.8±8.9*	45.2±8.9
Negative control	Normalsaline	985±180	-	42.0±3.9	58.0±3.9

P < 0.05, n = 5, TLC: Total leucocytes count, DLC: differential leucocytes count, PN: methanol extract of *Phyllanthus niruri*, ±: standard error of mean (SEM).

Table 2. Effects of *Phyllanthus niruri* on delayed type hypersensitivity response (DTHR) in rats

Treatment	Dose (mg/kg)	DTHR Oedema (cm ³)	Inhibition (%)
PN	100	0.5±0.09	30.55*
	200	0.2±0.10	66.67*
	400	0.4±0.09	44.44*
Levamisole	25	0.24±0.08	66.67
Negative control	Normal saline	0.72±0.10	-

P < 0.05, n=5, PN: Methanol extract of *Phyllanthus niruri*, ±: standard error of mean (SEM).

increase of 20.0, 15.56 and 68.89%, respectively (Figure 1).

DISCUSSION

Strengthening the immune system is a veritable approach that has gained prominence to counter the threats posed by immune destructive diseases like the acquired immune deficiency syndrome (AIDS), emerging virulent and highly pathogenic viruses, and cancers. The innate immunity (which is present at birth and responsible for the provision of first barrier against microorganisms) and the adaptive immunity (which is acquired later in life and acts as second barrier against infection) are the two aspects of immune protection. These two aspects of immune protection could be modified by substances to either suppress or enhance their ability to resist invasion by pathogens (William, 2001).

In this study, the immunomodulatory activities of the whole methanol extract of *P. niruri* on some specific and non-specific immune responses using rodent models was evaluated. Results of the study showed that the administration of the whole methanol extract of *P. niruri* caused a significant (P < 0.05) increase in the mobilization of leucocytes into the peritoneum of treated mice in response to injection of agar suspension. The neutrophils count was also correspondingly higher in groups treated with *P. niruri*. Neutrophils are important phagocytic cells

involved in innate surveillance and protection against a broad spectrum of pathogens and invaders. They play the main role as effective or killer cells for many types of antigenic challenges especially for infections (Basaran et al., 1997). The primary function of neutrophils in host resistance is their chemotactic migration towards the challenge and the intracellular killing of microorganisms by the formation of oxygen radicals (Badway and Karnovski, 1980).

The results of this study also showed that the delayed type hypersensitivity reaction evoked by SRBCs in mice was inhibited in groups that were treated with the *P. niruri* extract. Macrophages, memory T cells, CD4 and CD8 T cells have been shown to be required for the manifestation of DTHR (Allen, 2013; Sachdeva et al., 2014). It occurs within 24 to 72 h and it has been postulated that Th1 cell is the inducer of DTHR since it secretes interferon gamma (IFN γ), a potent stimulator of macrophages. In DTH reactions, T cells are first recruited into tissues and then activated by antigen presenting cells to produce cytokine that mediate local inflammation (Kalish and Askenase, 1999). It is a major mechanism of defense against various intracellular pathogens, including mycobacteria, fungi and certain parasites, and it occurs in transplant rejection and tumor immunity. This result shows that *P. niruri* can modulate cell mediated adaptive immune response in rodents as shown by the inhibition of DTHR.

In this study, administration of the whole extracts of *P.*

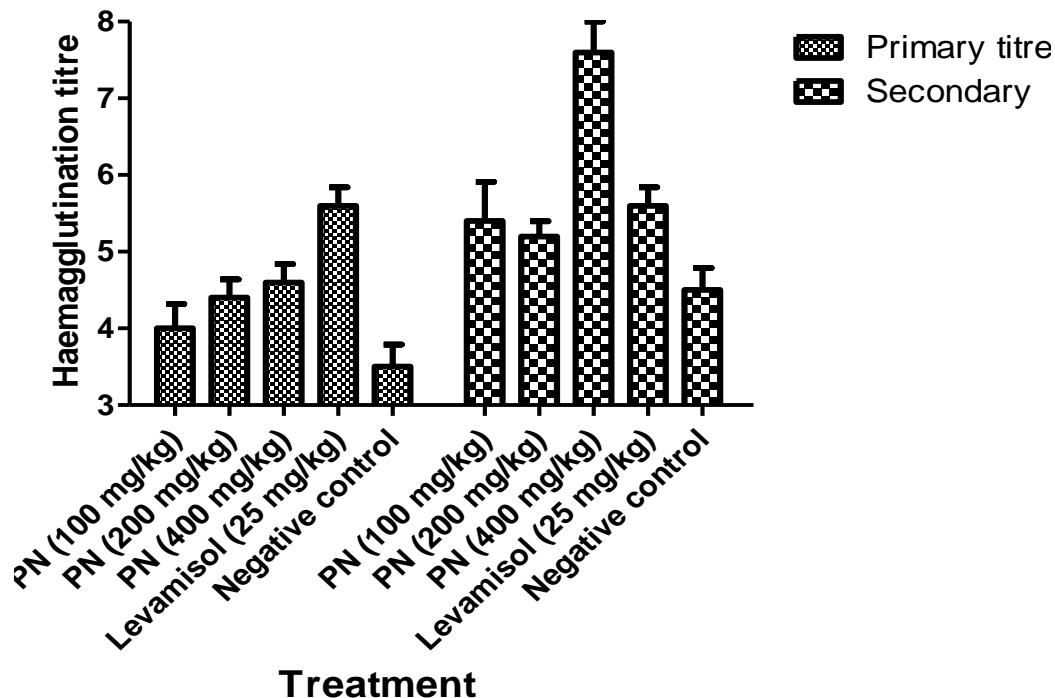


Figure 1. The effects of *Phyllanthus niruri* on primary and secondary antibody responses in rats. $P < 0.05$, $n = 5$, PN = methanol extract of *Phyllanthus niruri*, haemagglutination titre values are calculated as \log_2 of the highest dilution showing visible agglutination and expressed as mean \pm standard error of mean (SEM) of 5 animals in each group.
Note: PN: *Phyllanthus niruri*.

niruri caused a significant ($P < 0.05$) elevation of the primary and secondary humoral immune response (antibody) to sheep red cell antigen. The immunoregulatory properties of antibody have been recognized since the earliest passive immunization experiments, and the potential to modulate an immune response by deliberate immunization with antigen bound by antibody has been demonstrated in numerous instances over the decades (Brady et al., 2000; Alber et al., 2001; Antoniou and Watts, 2002; Rafiq et al., 2002). The ability of *P. niruri* to influence humoral response will confer protection to animals or man. This is possible through the utilization of any or combination of the various functions of antibodies synthesized which include agglutination of particulate matter, including bacteria and viruses, opsonization, neutralization of toxins released by bacteria, immobilization of bacteria, activation of complement, mucosal protection, expulsion as a consequence of mast cell degranulation, precipitation of soluble antigen by immune complex formation and antibody dependent cell mediated cytotoxicity. The secondary response are usually far more rapid, high in magnitude, long lived, as witnessed in the results, because during the primary response, some B-lymphocytes in addition to those differentiating into antibody secreting plasma cells, become memory cells which are long lived.

Conclusion

The results of this preliminary investigation have shown that the whole plant methanol extract of *P. niruri* possess immunomodulatory activities and modulates both the innate and adaptive immune components of the immune system. This study postulates that this immunomodulatory activity may be related to the wide ethnomedicinal use of the plant. The specific phytochemical constituent(s) responsible for these immunomodulatory activities was not ascertained in this study and will be the focus of our future investigation.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Acute toxicity studies, antioxidant and *in vitro* antibacterial activities of extract from the barks of *Ricinodendron heudelottii* (Euphorbiaceae)

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Many bacteria are involved in infectious diseases. Most of these bacteria become resistant to the most commonly used synthetic drugs. In Cameroon, natural substance seem to be an alternative to this problem. Thus the aim of this research was to investigate the acute toxicity, antioxidant activities and the *in vitro* antibacterial of the methanol extract of *Ricinodendron heudelottii* (Euphorbiaceae) against twelve pathogenic bacteria involved in infectious diseases. The major bioactive components were also screened. The antibacterial activity of the extract was investigated against 12 strains including 10 Gram- and 2 Gram+ bacteria by disc diffusion method and micro dilution method, followed by another agar disc diffusion for the determination of inhibition diameters, the minimum inhibitory concentration (MICs) and the minimum bactericidal concentration (MBC), respectively. 2,2-Diphényl-1-Picrylhydrazyl (DPPH) assay was used to evaluate antiradical activity. The acute toxicity study was performed according to World Health Organization (WHO) protocol. The results of the antibacterial assays indicated that the crude extract was active on 8 of 12 strains tested, with MIC ranging from 188 to 750 µg/ml and MBC from 375 to 1500 µg/ml for the extract from barks of *R. heudelottii*. Overall, the results of this study indicated that the crude extract represented a potential source of antibacterial and antiradical compounds as shown in previous studies and justified their traditional use in the treatment of bacterial infections and other diseases in Cameroon.

Key words: *Ricinodendron heudelottii*, antibacterial activities, bacteria, toxicity.

INTRODUCTION

Ricinodendron heudelottii is a perennial native tree in the tropic and subtropic areas, reaching 40 m in height and 1.2 m of diameter, belonging to the Euphorbiaceae family

and widely distributed in the Centre, South and West regions of Cameroon (Noumi and Yomi, 2001). The barks are brown or grey color and smooth. *R. heudelottii*

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morphology varies from one region to another by the color of the bark, the foliage and the fruits produced ; some species like those found in South region of Cameroun have deciduous leaves and big fruits, with 2 or 3 lobes in yellow green color and black color in maturity and this is different from those in other regions of Cameroon (Noumi and Yomi, 2001). The bark extract of this plant is used against cough, as poison antidote and for the treatment of intestinal diseases. Barks is also used to treat yellow fever, malaria, headache, stomach pains and some times it can help pregnant women (Momeni et al., 2005). Infusion of barks of *R. heudelotii* has been shown to possess diuretic and aphrodisiac effects. The leaves are used to treat dysentery and the fruits can be used as spices (Momeni et al., 2005). Plants possess secondary metabolites different from primary metabolites such as proteins, lipid and carbohydrate. Secondary metabolites are classified into many groups: phenolic compounds, terpenes, essential oils and nitrogenous compounds as alkaloids. Each group contains a diversity of components with biological activities (Li et al., 2007). The stem bark of *R. heudelotii* contains dinorditerpenoids for example, heudelotenol, heudelotinone as well as E-ferulic acid octacosylate and some natural chemopreventive agents (Suh et al., 1995).

Several researches demonstrated that many strains of Gram-positive and Gram-negative bacteria currently developed outstanding drug resistance, making the search of new, safe, non toxic and effective antibacterial agents to become strictly a necessity. Many antibacterial agents are available in nature for the treatment of systemic infections. Plants therefore constitute good source of active agents for this purpose and many plant extracts have been reported to possess various antimicrobial activities (Nawel et al., 2005). Although *R. heudelotii* is widely used for the mentioned biological activities, no toxicological study of the plant has been reported previously as well as its *in vitro* antibacterial and antioxidant activities in Cameroon. As a contribution to the search of non toxic, novel antibacterial principle from medicinal plants of Cameroon, results of *in vitro* antibacterial and antioxidant investigations is being reported here.

METHODOLOGY

Chemicals

Gentamicin usually used was acquired from a local pharmacy. Pure methanol, chloroform, nutrient agar and nutrient broth were purchased from Merck Company and other chemicals used were from Sigma Company.

Plant

The barks of *R. heudelotii* were collected in Minlamizibi, a village in the South region of Cameroon in January, 2009. Botanical identification was made in the Cameroon National Herbarium and

the voucher specimen was deposited under the number 16610/SFR/CAM. Preparation of extract the collected plant was dried at room temperature ($30 \pm 3^\circ\text{C}$), pulverized and sieved. For the extraction, the powders of 500 g each were separately macerated in pure methanol (MeOH) solvents for 72 h. The solution was filtered using Whatman filter paper N°1 and concentrated in an air circulating oven at 54°C until total dryness.

Antibacterial assay

Microorganisms

Twelve species of strains isolated from patients at "Centre Pasteur of Cameroon", a reference center for diagnostic and identified by lab technicians of Centre Pasteur were used for the evaluation of antibacterial activity. These micro-organisms were Gram-negative (*Escherichia coli*, *Salmonella typhi*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Morganella morganii*, *Citrobacter freundii* and *Proteus vulgaris*) and positive gram (*Staphylococcus aureus* and *Streptococcus faecalis*).

Inocula preparation

An inoculum for each micro-organism was prepared from broth cultures containing approximately 5.10^5 to 9.10^6 colony forming units per milliliter (CFU/ml). Each diluted (1:50) inoculum was applied as a lawn with a micropipette calibrated to deliver 50 μl containing around 9.10^6 CFU. The discs impregnated with extract and pure methanol only to show if there is any activity noticed using pure methanol solvent, were evaporated for 24 h at the sterile condition. The inoculated plates were incubated at 37°C for 24 h. Each assay in this experiment was repeated 3 times.

Determination of the diameters of inhibition zone

The extracts was tested *in vitro* for antibacterial activity by the standard disc diffusion method against the micro-organisms at a concentration of 80 mg/ml with pure methanol. Gentamycin used as standard antibiotic (positive control) was tested at a concentration of 1 $\mu\text{g}/\mu\text{l}$. The diameters of inhibition zones produced by these extracts and discs impregnated with pure methanol were then compared to standard antibiotic (gentamycin).

MIC determination

For MICs determination, only the most sensitive microorganisms were tested with the extract. Serial dilutions were from 93.75 to 3000 $\mu\text{g}/\text{ml}$ of extract in the nutrient broth medium. 100 μl of the suspension of each pathogenic bacterium (10^6 cells/ml) were added and incubated at 37°C for 24 h. Standard antibiotic (positive control) was tested in the concentration between 2.5 to 80 $\mu\text{g}/\text{ml}$. The lowest concentration which did not show any macroscopic growth of tested microorganism was identified as the MIC.

Minimum bactericidal concentration (MBC) determination

For MBC determination, 100 μl of each tube following the MIC tube was subcultured in the nutrient broth medium at 37°C for 24 h. The absence of any macroscopic growth of the nearest MIC tube was identified as the MBC (Mims et al., 1993).

Antioxidant activity

Evaluation of the curd extract of *R. heudelotti* is based on the reduction of 2,2-Diphényl-1-Picrylhydrazyl (DPPH) by antioxidant component (Brand-Williams et al., 1995). 100 mM of concentrated methanolic solution of DPPH is prepared and 2 ml shared in tubes where 10 µl of ascorbic acid added. The content was mixed on a plate shaker at 300 rpm for 30 min and incubated at 25°C. Antioxidant activity was determined by absorbance at 517 nm using spectrophotometer UV-120-01 at different concentrations 1, 2, 3, 4, 5 and 6 mg/ml. Each test was repeated 3 times.

Toxicity study

Experimental animals

Albino Wistar rats (100 to 200 g) of about two and half months were obtained from the animal laboratory of the Biochemistry Department of the Yaounde I University, Cameroon. All the rats were kept under environmental conditions ($27 \pm 2^\circ\text{C}$) and they had free access to water and food. These rats were deprived of food but not water (16 to 18 h) prior to the administration of the extract. The principles of laboratory animal care were followed while the Department's ethical committee approved the use of the animals and the study design.

Acute toxicity

The bioassay was conducted according to the World Health Organisation guideline for the evaluation of safety and efficacy of herbal medicines (WHO, 2002). For the study, albino Wistar rats were divided into six groups of five animals each. The methanol extract of *R. heudelotii* was suspended in the distilled water. This extract was employed because of its moderate or high antibacterial activity against all the bacteria considered. This solution was administrated *per os* to rats groups in a single oral dose of 2, 4, 6, 8, and 10 g/kg body weight (bw), respectively by intra gastric gavages using a feeding needle. The control group received an equal volume of distilled water as vehicle. Observations of toxic symptoms were made and recorded systematically at 1, 2, 4, 6 and 24 h after administration of the extract. The number of rats that survived were noted after 24 h and then maintained for the further 8 days with daily observations. This visual observation included skin changes, mobility and aggressiveness, sensitivity to sound and pain, as well as respiratory movements. The toxic effects of the extract were assessed on the basis of mortality which was expressed as LD₅₀ (Schorderet, 1992). During the experiment, the animals were weighed, food and water intake were monitored. At the end of the experiment, all surviving animals fasted overnight and were sacrificed by decapitation. The organs such as liver, lungs, heart and kidneys were excised and weighed. The pathological observations of these tissues were performed on gross. The blood samples were also collected freshly in the dry non-heparinised centrifuge tubes. This blood was allowed to coagulate before being centrifuged to separate the serum. This serum was assayed for biochemical parameters. The liver was excised, rinsed in ice-normal solution followed by cold 0.1 M Tris-HCl (pH 7.5), blotted, dried and weighed. The 20% (w/v) liver homogenates was prepared in the 0.1 M Tris-HCl buffer and the supernatant was used for biochemical analysis. Lung, kidney and heart were removed, washed in 0.9% NaCl weighed and examined as previously mentioned. Determination of biochemical parameters blood collected into non heparinised tubes was centrifuged at 3,000 rpm for 10 min to separate the serum. This serum was used to evaluate the liver enzyme function through some biochemical parameters such as aspartate aminotransferase (AST), alanine aminotransferase (Reitman and Frankel, 1957), alkaline phosphatase

(Oliver et al., 1954), creatinine (Bartels et al., 1972), urea and total protein (Allan et al., 1949), total bilirubine and direct bilirubin (George, 1959).

Phytochemical screening

Qualitative phytochemical tests of *R. heudelotti* methanolic extract were carried out according to Odebiyi and Sofowora (1978) methods to identify some components as alkaloids, saponins, tannins, flavonoids, polyphenols, anthraquinones.

Test for alkaloids: 0.5 g of the sample was stirred with 5 ml of 1% aqueous HCl on a steam bath and then filtered. 1 ml of the filtrate was treated with a few drops of Mayer's reagent and a second 1 ml portion was treated similarly with Dragendroff reagent. Turbidity or precipitation with either of these reagents was taken as evidence for the presence of alkaloids in the extract.

Test for saponins: The ability of saponins to produce frothing in aqueous solution and to haemolyse red blood cells was used for the screening test. 0.5 g of plant extract was shaken with water in a test tube. Frothing which persisted on warming was taken as evidence for the presence of saponins.

Test for tannins: 0.5 g of dried extract was stirred with 5.0 ml of distilled water. This was filtered and ferric chloric reagent was added to the filtrate. A blue-black precipitate was taken as evidence for the presence of tannins.

Test for phenol and polyphenols: 0.5 g of plant extract was heated for 30 min in a water bath. 3 ml of 5% FeCl₂ was added to the mixture then followed by the addition of 1 ml of 1.00% potassium ferrocyanide. The mixture was filtered and green (phenol) and blue (polyphenol) colours were observed.

Test for anthraquinones: 0.5 g of plant extract was shaken with 5 ml of benzene, filtered and 2 ml of 10% ammonia solution was added to the filtrate. The mixture was shaken and the presence of a pink or violet colour in the ammoniacal (lower) phase indicated the presence of free hydroxy anthraquinones.

Test for flavonoids: 0.5 g of plant extract was dissolved in 5 ml of NaOH at 1 N. The change of the yellow colour obtained after adding HCl 1 N indicated the presence of flavonoïds.

Statistical analysis

The values were expressed as mean \pm standard deviation (SD). Each value was a mean of five or six tests. The one-way analysis of variance (ANOVA) was used to determine the significant differences between parameters and the student-Newman Keurls test served to compare these differences at $p < 0.05$ using statistical package used was SPSS 10.1.

RESULTS

Antibacterial activity

The results of the *in vitro* antibacterial activity of methanol extracts determined by diameters of inhibition zones are presented in Table 1. These results indicated that the diameters of inhibition zones varied from 12 to 19.67 mm

Table 1. Antibacterial activities of *R. heudelotii* from the disc diffusion method.

Extract	Bacterial strains											
	<i>S.aureus</i>	<i>Kb. pneumo</i>	<i>S. feacalis</i>	<i>S. typhi</i>	<i>Kb. oxy</i>	<i>S. flexneri</i>	<i>E. coli</i>	<i>P. vulgaris</i>	<i>P. aeruginosa</i>	<i>M. morgani</i>	<i>C. freundi</i>	<i>E. cloacae</i>
Gentamicin	34.33±1.53	26±2.65	31±2	27.33±6.51	32±1	27.33±1.53	26±1	26±3.61	34.67±1.53	30.33±1.53	30.67±2.52	28±1.73
<i>R. heudelotii</i>	12±0	15.33±0.58	12.67±0.58	-	-	-	12.33±0.58	-	19.67±1.59	14.67±0.58	16±1	12.33±1.15

S.aureus =*Staphylococcus aureus*; *Kb. pneumo* =*Klebsiella pneumonia*; *S. feacalis* =*Streptococcus feacalis*; *S. typhi* =*Salmonella typhi*; *Kb. oxy* =*Klebsiella oxytoca*; *S. flexneri* = *Shigella flexneri*; *E. coli* =*Escherichia coli*; *P. vulgaris* =*Proteus vulgaris*; *P. aeruginosa* =*Pseudomonas aeruginosa*; *M. morgani* = *Morganella morgani*; *C. freundi* =*Citrobacter freundi*; *E. cloacae* = *Enterobacter Cloacae*.

Table 2. MIC, MBC values ($\mu\text{g/ml}$) of *R. heudelotii* extract in the macro dilution assay comparable to gentamicin.

Extract/ gentamicin	Parameter ($\mu\text{g/ml}$)	Bacterial strain											
		SA	KP	AB	ST	KO	SF	EC	PV	PA	MM	CF	EC*
<i>R. heudelotii</i>	MIC	750	375	750	-	-	-	750	-	188	375	375	750
	MBC	1500	750	1500	-	-	-	1500	-	375	750	750	1500
	MBC/MIC	2	2	2	-	-	-	2	-	2	2	2	2
Gentamicin	MIC	10	10	10	20	10	10	10	10	10	5	10	10
	MBC	20	20	20	40	20	20	20	40	20	10	20	20
	MBC/MIC	2	2	2	2	2	2	2	2	2	2	2	2

SA=*Staphylococcus aureus*; KB=*Klebsiella pneumonia*; AB=*Streptococcus feacalis*; ST=*Salmonella typhi*; KO=*Klebsiella oxytoca*; SF= *Shigella flexneri*; EC=*Escherichia coli*; PV=*Proteus vulgaris*; PA=*Pseudomonas aeruginosa*; MM= *Morganella morgani*; CF=*Citrobacter freundi*; EC*= *Enterobacter cloacae*. Minimum inhibitory concentration (MICs); minimum bactericidal concentration (MBC).

and 26 to 34.67 for the extracts and gentamicin, respectively. Gentamicin used as a standard antibiotic at the concentration of 1 $\mu\text{g}/\mu\text{l}$ exhibited higher diameters of inhibition than other extracts. No diameters of inhibition zones was obtained with discs impregnated with pure methanol. Among the twelve isolates, eight bacteria (*S. aureus*, *K. pneumonia*, *S. feacalis*, *E. coli*, *P. aeruginosa*, *M. morgani*, *C. freundi* and *E. cloacae*) were sensitive to the extract. However, *P. aeruginosa* was the most sensitive, with 19.67 mm. *S. typhi*, *K. oxytoca*, *S. flexneri* and *P. vulgaris* were the most resistant bacteria isolate with no diameters

of inhibition zones. The results of the antibacterial activity of the extract determined by minimal inhibitory concentration (MIC) and minimal bactericide concentration (MBC) are represented in Table 2. The MICs ranged between 188 and 750 $\mu\text{g/ml}$ and the MBC ranged between 375 and 1500 $\mu\text{g/ml}$. These results revealed that *P. aeruginosa* was the most sensitive with values of 188 $\mu\text{g/ml}$ (Table 2).

The MIC of the extract of *R. heudelotii* was less active than standard antibiotic (gentamicin) ranged between 5 and 10 $\mu\text{g/ml}$. No result of MIC and MBC was determined for *S. typhi*, *K. oxytoca*,

S. flexneri and *P. vulgaris* isolates.

Antioxidant activity

2,2-Diphényl-1-Picrylhydrazyl (DPPH) radical scavenging assay revealed a high antiradical activity of the extract from barks of *R. heudelotii* with a SC_{50} of 7.21 mg/ml (Table 3).

Acute toxicity

For the acute toxicity studies, no death of rats was

Table 3. Trapping percentage of the extract/ascorbic acid at different concentration.

Extracts	Concentration of extract/ascorbic acid (mg/ml)						Concentration of antioxidant 50 of extract/ascorbic acid	
	1	2	3	4	5	6	SC ₅₀ (mg/ml)	R ²
Ascorbic acid	36.67±0.53	40.91±0.00	50.30±1.39	55.46±0.91	60.30±0.51	63.64±0.91	3.28	0.978
<i>R. heudelotii</i>	20.30±0.53	24.85±0.53	27.88±0.53	35.46±0.91	40.00±0.00	43.64±0.91	7.21	0.988

Table 4. Biomedical parameters values of male rats in acute toxicity of the methanol extract from the barks of *R. heudelotii*.

Parameter tests	Extract dose (g/kg body weight)					
	0	2	4	6	8	10
Weight	124.15±10.15	116.87±20.70	117.9±21.46	102.80±15.65	109.56±18.64	124.36±13.34
Urea (mg/ml)	0.90±0.74	0.94±0.69	1.23±0.67	1.65±1.05	2.04±0.55	2.48±1.27
Creatinin (mg/ml)	1.1±0.08	1.2±0.04	1.6±0.15	2±0.47	2.01±0.13	2.45±0.22
Total protein (mg/ml)	3.77±0.53	3.93±0.51	4.55±0.55	5.5±0.71	6.1±1.19	7.35±2.31
ALP (UI/L)	10.69±0.59	11.95±0.29	13.24±1.2	14.9±0.56	16.2±1.58	18.56±1.98
AST (UI/L)	42±2.62	50.75±4.49	52.50±4.79	61.5±6.78	76±1.84	93.5±13.88
ALT (UI/L)	26±3.18	29.9±0.62	29.15±4.27	36.75±8.82	39.5±4.99	54.1±7.84
Total bilirubin (mg/l)	1.05±0.05	1.07±0.10	1.14±0.04	1.09±0.04	1.09±0.065	1.36±0.09
Conjugated bilirubin (mg/l)	0.32±0.001	0.33±0.07	0.34±0.005	0.31±0.05	0.29±0.01	0.29±0.04

neither recorded in the control nor in the treated groups. The animals exhibited slight changes in general behaviour (slow response to stimuli, stretching and sluggishness) but did not expressed changes in their physiopatological activities. Although there was an increase in the weights of rats and their organs in the treated groups, this addition remained statistically not significant compared with control. The pathological examinations of the tissues on a gross basis indicated no detectable abnormalities at the end of the experiment. Table 4 shows the results of the blood and hepatic parameters. These results indicated that alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) of the treated rats

increased significantly ($p > 0.05$) at the higher concentration of the extract compared to the control. We noticed also an increase of urea, total protein, creatinin (CRT) and bilirubin of the treated rats but the difference is not significant ($p < 0.05$).

Phytochemical screening

The phytochemical screening of the extract of *R. heudelotii* indicated the presence of three main classes of compounds: polyphenols, alkaloids and steroids. The polyphenols group constitutes the principal component of *R. heudelotii* extract with three bioactives subgroups: tannins, flavonoids and saponins. Anthraquinone was absent in

the extract of *R. heudelotii*. Alkaloids and steroids whose antimicrobial activities have been demonstrated were also found in this extract (Table 5)

DISCUSSION

This work permitted the evaluation of some biological properties of *R. heudelotii*; among which the antimicrobial activity directed on some pathogens frequently encountered in infections as well as the toxicological and antioxidant studies of this extract. The diameters of inhibition zones varied from 12 to 19.67 mm and 26 to 34.67 for the extracts and gentamycin, respectively. The

Table 5. Phytochemical screening of crude methanol extract.

Chemical group	polyphenols	phenols	alkaloids	favanoids	saponins	tanins	Anthraquinone
Results	+	+	+	+	+	+	-

(diameter of inhibition between 7 and 10 mm), moderate activity (diameter of inhibition between 11 and 16 mm) and good or higher activity (diameter of inhibition between > 16 mm). All the micro-organisms (*S. aureus*, *K. pneumonia*, *S. fecalis*, *E. coli*, *P. aeruginosa*, *M. morgani*, *C. freundii* and *E. cloacae*) tested here were harmful and involved in infectious diseases. The broad antimicrobial activity of the extract in our study can be attributed to the presence of various bio-actives components such as tannins, polyphenols, alkaloids, glycosides, flavonoids, steroids and saponins found in this extract (Dinan et al., 2000). The variation observed from the diameters of inhibition zone of the bacteria tested can be attributed either to the difference of the bioactive molecules present in the extract or to their mechanism of action on Gram-positive and Gram-negative bacteria. The mechanism of action of the glycosides, polyphenols, tannins and alkaloids on Gram-positive and Gram-negative bacteria was demonstrated (Deeni and Sadiq, 2002; Kalemba, 2003). The MICs ranged between 188 and 750 µg/ml and the MBC ranged between 375 and 1500 µg/ml. These results revealed that *P. aeruginosa* was the most sensitive with values of 188 µg/ml. The MIC of the extract of *R. heudelotii* was less active than standard antibiotic (gentamycin) ranged between 5 and 10 µg/ml. The antibacterial property of *P. aeruginosa* is well documented (Kuete et al., 2007; Armelle et al., 2008). The rate MBC/MIC revealed that the extract presents a bactericidal action (MBC/MIC < 4). This action can justify the use of this plant against bacterial infections. No result of MIC and MBC was determined for *S. typhi*, *K. oxytoca*, *S. flexneri* and *P. vulgaris* whose isolates were the most resistant bacteria isolate with no diameters of inhibition zones and this can explain the result obtained.

The evaluation of trapping of DPPH radical revealed a high antiradical activity of the extract from barks of *R. heudelotii* with a SC₅₀ of 7.21 mg/ml. This antioxidant activity observed in our study can be attributed to the presence of various bio-actives components such as tannins, polyphenols, alkaloids, flavonoids and steroids found in this extract (Sokol-Letowska et al., 2007). However this trapping rate remained lower than the one of ascorbic acid (SC₅₀ = 3,28 mg/ml). The work done by Afolabi et al., in 2007 (Afolabi et al., 2007) showed a large correlation between the percentage of phenolic components of the extract and the inhibitory activity of the extract on the DPPH.

The acute toxicity test has been investigated to establish the adverse effects of the administration of the

methanol extract of *R. heudelotii* on some behavioural and biochemical parameters. The results indicated that up to 10 g/kg bw, no abnormal symptoms and no death of the rats was observed. According to the OCED protocol (Organisation of Economic Co-operation and Development, 2001) *R. heudelotii* extract can be classified as non toxic since the limited dose of an acute toxicity is generally considered to be 5.0 g/kg bw (Schorderet, 1992). If no mortality is observed at this level, a higher dosage is generally not necessary (Wallace, 1989). However, the body weight of the rats increased during the experiment. Compared to the control, the weight gained by the treated rats was higher but statistically not significant. This result showed that the extract slightly stimulates the appetite of the rats and probably not irritates directly the gastrointestinal tract.

The significant changes noted in some of the blood chemistry parameters such as ALT, AST, ALP and CRT were an indicator of some toxic effects undoubted. It should be noted that these modifications were observed at high dose (more than 6 g/kg bw). Even though the changes noted were not significant, they were statistically different compared to control. The variation of biochemical parameters indicated the malfunctioning of one or many organs. The increase of the activity of ALP after the administration of the extract may indicate the obstruction of the bile duct. However, this variation can not be attributed only to a dysfunction of the bile duct since many sources of ALP are known (liver cells; osteoblast, intestinal cells and placenta tissue). Although the variation of ALT and AST activities are associated with the hepato-cellular damage, only ALT is specific for the evaluation of liver damage. AST is highly concentrated in cardiac muscle, liver, skeletal muscle and kidneys. The significant increase of ALT and AST activities after treatment of the rats with 8 and 10 g/kg bw of extract implies an injury of the liver as well as the heart or other sources of these enzymes. The creatinine level of treated rats increased compared with the control. This last result confirms that kidneys are slightly affected at the dose of 10 g/kg body weight of the extract. The variation of other parameters such as protein and bilirubin were not statistically significant.

It has been recognized that eventual therapeutic bioactive products from plants may also contain substances which act as poisons in human (Ekramul et al., 2002). Several researches demonstrated that phenolics and polyphenols compound have antimicrobial activities. Other workers have shown that the sites and the number of hydroxyl groups on the phenol are thought to be related

to their relative toxicity to microorganisms, with the evidence that an increase of hydroxylation will result to an increased toxicity (Kareem et al., 2008). The presence of three subclasses of polyphenols (tannins, flavonoids and saponins) in the extract may have significant inhibitory effect on all isolates tested. The antibacterial effects of each of these subclasses of polyphenols on Gram-positive and Gram-negative have been demonstrated as well as the microbicidal effects of alkaloids (Kalemba and Kunicka, 2003). The presence of multiple phytochemical components confers to *R. heudelotii* extracts *in vitro* antibacterial and antioxidant activities.

Conclusion

The methanol extract of *R. heudelotii* demonstrated effective *in vitro* antibacterial and antioxidant activities. However some toxic effects have been discovered after administration of high dose of this extract. Further research needs to be carried out to identify the active molecules and evaluate the *in vivo* antibacterial and antioxidant activities as well as sub-acute or chronic toxicities.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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