Investigational
Medicinal Chemistry & Pharmacology

Research Article Open Access

Antibacterial potential and mechanism of action of botanicals and phytochemicals from *Stachytarpheta cayennensis* (Verbenaceae) against Gram-negative multidrug-resistant phenotypes expressing efflux pumps

Laetitia M. Youmbi[1,3], Brice C.K. Atontsa[2], Simplice B. Tankeo[1], Brice E.N. Wamba[1], Paul Nayim[1], Blaise K. Nganou[2], Gabin T.M. Bitchagno[2], Ingrid K. Simo[2], James D.S. Mpetga[2], Veronique B. Penlap[3] and Victor Kuete[3]

Abstract

Background: The effectiveness of antibiotics in the fight against bacterial infections motivated their massive use which resulted in the appearance of resistant bacteria. This phenomenon is nowadays in constant evolution and represents a major problem of public health. In this respect, the search for new molecules for fighting against bacterial resistance is essential. This work was committed to studying the *in vitro* antibacterial activity of the ethanol crude extract, fractions and compounds of a medicinal plant of the Cameroonian pharmacopoeia, *Stachytarpheta cayennensis* (Verbenaceae), against some multidrug-resistant (MDR) Gram-negative bacterial strains including *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter aerogenes* and *Providencia stuartii*,.

Methods: The antibacterial activity of the crude extract and fractions (botanicals) of this plant and their constituents (phytochemicals) in the presence and absence of an efflux pump inhibitor phenylalanine-arginine β-naphthylamide (PAβN) was evaluated using the serial micro-dilution method. The determination of the effect of the most active sample on the bacterial H^+-ATPase proton pumps was carried out by a standard method using a pH-meter and the study of the effect of that same sample on the cell growth kinetic was done using a spectrophotometric method.

Results: The phytochemical composition of the crude extract (SC), evaluated using standard qualitative methods, showed a rather selective distribution of secondary metabolites (presence of polyphenols, tannins, steroids, triterpenes, anthocyanins and absence of flavonoids, anthraquinones, alkaloids, saponins). The crude extract (SC) moderately inhibited the growth of 80% of studied bacteria with minimal inhibitory concentrations (MICs) of 256 and 512 µg/mL. Fractions SCc and SCd showed antibacterial activity against 90% and 70% of strains respectively with MICs ranging from 64 to 512 µg/mL, with moderate activity of the crude extract (SC) than the derived fractions. Fraction SCc showed the highest activity spectrum by inhibiting the growth of 90% (9/10) of the studied bacteria, with the least MIC of 64 µg/mL on *Escherichia coli* ATCC8739. In the presence of PAβN, the activity of the extract and derived fractions has been increased to at least 70% with respect of the strains and isolates used. Fraction SCc caused a prolongation of the lag phase and a general decrease of the growth of *E. coli* ATCC8739 bacteria through the blockage of the H^+-ATPase proton pumps of that bacteria, resulting to a decrease of the acidity of the medium.

Conclusion: The overall results obtained in the present work provide important data which constitute a trail to explore regarding the likely use of *Stachytarpheta cayennensis* in antibiotherapy.

Keywords: Gram-negative bacteria; multidrug resistance; efflux pumps; *Stachytarpheta cayennensis*.

*Correspondence: Tel: (237)677355927; P.O. Box 67 Dschang, Cameroon; E-mail: kuetevictor@yahoo.fr (Prof. Victor Kuete)
[1]Department of Chemistry, University of Dschang, P.O. Box 67, Dschang,Cameroon; [2]Department of Chemistry, University of Dschang, P.O. Box 67, Dschang, Cameroon; [3]Department of Biochemistry, University of Yaounde 1, Cameroun P.O. Box 812, Yaounde, Cameroon.

Citation on this article: Youmbi LM, Atontsa BCK, Tankeo SB, Wamba BEN, Nayim P, Nganou BK, Bitchagno GTM, Simo IK, Mpetga JDS, Penlap VB and Kuete V. Antibacterial potential and mechanism of action of botanicals and phytochemicals from Stachytarpheta cayennensis (Verbenaceae) against Gram-negative multidrug-resistant phenotypes expressing efflux pumps. Investigational Medicinal Chemistry and Pharmacology (2020) 3(1):35; Doi: https://dx.doi.org/10.31183/imcp.2020.00035

Background

Infectious diseases constitute nowadays a very important public health problem. They are responsible for 17 million deaths a year worldwide which is about 30% of the overall mortality in the world, especially in developing countries such as South Asia and sub-Saharan Africa. Among the 2.7 million neonatal deaths recorded each year, 560 000 of them are caused by bacterial infection [1]. The discovery of antibiotics constituted an extraordinary medical progress and considerably decreased the mortality rate linked to infectious diseases caused by bacteria. However, these bacteria with time have rapidly developed resistance toward these antibiotics because of their adaptation to the hostile conditions including inappropriate use of antibiotics [2]. Bacteria resistance is a functional or structural property, innate or acquired, which confers to bacteria the capacity to grow in the presence of high concentrations of antibiotics [3]. Furthermore, multidrug resistance (MDR) occur when bacteria can resist against at least three different pharmacological classes of antibiotics. The emergence of MDR bacterial strains appears as the major cause of treatment failures [4]. Among the known mechanisms of resistances, active efflux *via* resistance-nodulation-cell division (RND) pumps is one of the most occurring systems in Gram-negative bacterial strains [5]. This phenomenon allowed most of the commonly used antibiotics less active against these bacteria. Therefore, the search for new molecules able to most efficiently fight against this resistance has become an absolute necessity. Medicinal and edible plants which contain an important number of bioactive substances, particularly those from Cameroon, have proven their ability to inhibit the growth of most MDR Gram-negative bacteria strains [6, 7, 8, 9, 10]. Most of these plants among which *Stachytarpheta cayennensis* (verbenaceae), a Cameroonian pharmacopeia plant, are traditionally used to treat many infectious diseases caused by resistant microorganisms [11]. In the present study, the activity of this plant and its constituents on some MDR bacteria phenotypes was investigated, as well as the role of bacterial efflux pumps in the resistance to the tested plant samples.

Methods

Plant sample and extraction

The tested plant, *Stachytarpheta cayennensis* (verbenaceae), was collected in Bazou locality (5° 09' 00'' North and 10° 31' 00'' East), Nde division, West Region (Cameroon) and was identified at the National Herbarium (Yaounde, Cameroon) where a voucher specimen was deposited under the following reference number 11726/SFR/CAM/NHC.

The air dried and powdered sample (2.5 kg) from this plant was extracted with 10 L of ethanol (EtOH) 98% for 72 h at room temperature. After filtration with Whatman filter paper n° 1, the filtrate was then concentrated under reduced pressure to give residue which constituted the crude extract (140.0 g) denoted SC. It was then stored at 4 °C for further use. The extraction yield in percentage was 5.6% and was obtained by calculating the crude extract weight/powder weight.

Crude extract fractionation and purification

A mass (130.0 g) of the crude extract (SC) was submitted to a silica gel column chromatography (0.200-0.500 mm) and eluted with an increasing polarity of hexane-AcOEt and AcOEt-MeOH mixture (100:0, 90:10, 80:20, 70:30, 60:40 and 50:50). A total of 95 fractions for a total volume of 300 mL were collected, evaporated under reduced pressure and then grouped on the basis of their analytical TLC profile to afford four major fractions namely SCa [(18.0 g) : 1-15], SCb [(24.6 g) : 16-43], SCc [(40.0 g) : 44-75] and SCd [(47.4 g) : 76-95]. Different solvent proportions and fractions grouping are presented in Table S1 (see supplementary files SF1). Fraction SCc which was the most active fraction, was separated by column chromatography over silica gel (0.063-0.200 mm) using gradients of n-hexane-EtOAc and EtOAc-MeOH (100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35 and 60:40) to afford five compounds, a mixture of β-sitosterol and stigmasterol (**1** and **2** respectively, 21.0 mg) at n-hexane-EtOAc 90:10, a β-sitosterol 3-*O*-*β*-D-glucopyranoside (**3**, 16.5 mg) at EtOAc 100:0, and the two triterpenoids, ursolic acid (**4**, 10.0 mg) and oleanolic acid (**5**, 15.0 mg) at n-hexane-EtOAc (85:15). Notice that all ^{1}H and ^{13}C NMR spectra and major chemical shifts of these compounds are shown in the supplementary files (SF 2). The structures of these compounds are shown in Figure 1.

Phytochemical assay

Preliminary phytochemical assay on tested crude extract was investigated to detect the presence of the major secondary metabolite classes, namely alkaloids, flavonoids, phenols, saponins, tannins, anthocyanins, anthraquinones, sterol and triterpenes, using common phytochemical methods as described by [12, 13].

Antibacterial assay

Chemicals

The pure antibiotic chloramphenicol (CHL) ≥ 98% was used as reference. *p*-Iodonitrotetrazolium chloride (INT) 0,2 % and Phenylalanine-Arginine *β*-naphthylamide (PAβN) ≥ 97 % were used as microbial growth indicator and efflux pumps inhibitor (EPI) respectively. All these chemicals were provided from Sigma-Aldrich, St. Quentin Fallavier, France. Dimethylsulfoxide (DMSO) 2.5% at the final concentration was used to dissolve tested samples.

Culture media and microbial strains

Two culture media were used. The solid medium, Mueller Hinton Agar (MHA) for the activation of the bacterial strains and the liquid medium, Mueller Hinton Broth (MHB) for antimicrobial assays. Ten microorganisms, sensitive and multidrug resistant Gram-negative bacterial strains expressing efflux pumps, including *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter aerogenes* and *Providencia stuartii*, provided by American Type Culture Collection, were used. Their main characteristics are summarized in Table 1.

They were maintained on agar slant at 4°C and cultured on fresh appropriate agar plates 24 h prior to any antimicrobial test.

Bacterial susceptibility determination

The respective minimal inhibitory concentrations (MICs) of samples on the studied bacteria were determined using rapid INT colorimetric assay [18, 19]. Briefly, the test samples were first dissolved in DMSO/MHB mixture. The solution obtained was then added to MHB and serially diluted two-fold (in a 96-well microplate). One hundred microlitres (100 µL) of inoculum (1.5×10^6 CFU/mL) prepared in MHB was then added. The plates were covered with a sterile plate sealer, then agitated to mix the contents of the wells using a shaker and incubated at 37°C for 18 h. The final concentration of DMSO was lower than 2.5% and does not affect the microbial growth. Wells containing MHB, 100 µL of inoculum, and DMSO at a final concentration of 2.5% served as a negative control. Chloramphenicol was used as reference antibiotic. The MICs of samples were determined after 18 h of incubation at 37°C, following addition of (40 µL) of 0.2 mg/mL INT and incubation at 37°C for 30 minutes [20]. Viable bacteria reduced the colourless dye to pink. MIC was defined as the lowest sample concentration that prevented this change and exhibited complete inhibition of microbial growth. For the minimal bactericidal concentrations (MBCs) determination, a volume of 150 µL of MHB has been introduced in a new 96-well microplate, following addition of 50 µL of the previous well microplate contents where no microbial growth was observed and which did not received an INT (during the reading of MICs). After 48 h incubation, at 37°C, the MBC of each sample was determined and defined by adding 40 µL of 0.2 mg/mL INT as previously described. It should be noted that samples were tested alone and then, in the presence of PAβN, an efflux pumps inhibitor, at 30 mg/L final concentration. In this last case, the activity improvement factors (AIFs) were determined to qualify the potentiation level of sample activity by this inhibitor, using the $MIC_{sample\ alone}/MIC_{sample\text{-}PA\beta N\ combination}$ ratio. All assays were performed in triplicate and repeated thrice.

Antimicrobial mechanisms of action

Effect of fraction SCc on bacterial growth kinetic

Bacterial growth kinetic study of fraction SCc which showed the best antibacterial activity was done by spectrophotometric method at a wavelength of 600 nm [21]. Bacterium used for this study was a reference strain *Escherichia coli* ATCC8739 and the sample was tested at the concentrations of MIC/2, MIC and 2MIC. Firstly, 500 µL of bacterial suspension ($1.5x10^8$ UFC/mL) from preculture were added to 450 mL MHB (1/100 v/v dilution) and incubated at 37°C for 18 h under magnetic agitation and in the presence of tested sample at different concentrations. Reference antibiotic, chloramphenicol, was used as positive control whereas, inoculum ($1.5x10^8$ UFC/mL)/DMSO (2.5% v/v) mixture constituted the negative control. At 0, 0.5, 1 and 2 h followed by regular interval times of 2 h from 2 to 18 h, aliquots of 1 mL from the preparation were deducted and introduced in a spectrophotometer tab and then, the optical density was read at 600 nm wavelength. From the obtained results, bacterial growth curves [OD = f (times)] were labelled using Microsoft Excel software (Figure 2).

Effect of fraction SCc on bacterial H^+-ATPase- dependent proton pumps

The ability of tested sample to inhibit bacterial H^+-ATPase-dependent proton pumps was evaluated following the acidification of the bacterial external environment, through the pH measurement as described by [22]. So, a fresh bacterial colony was dissolved in 20 mL of MHB culture medium and incubated at 37°C under magnetic agitation for 18 h. Aliquots of 1 mL from this bacterial preculture were deducted and added to MHB to afford 100 mL final volume (1/100 v/v dilution) and then re-incubated at 37°C for 18 h under magnetic agitation. One hundred milliliter (100 mL) from this bacterial culture were centrifuged at 4000 rpm for 30 min at 4°C. Collected sediments were washed with sterile distilled water then with KCl 50 mM and were dissolved in 50 mL KCl 50 mM. Obtained bacterial suspension was conserved at 4°C for 18 h (for glucose starvation), after which the pH was adjusted to 8.0 by adding HCl or NaOH solution. Then, 0.5 mL of tested sample was added to 4 mL of this bacterial culture and the mixture was incubated at 37°C for 10 min, after which, 0.5 mL of glucose 20% was added (to activate the environment acidification). Inoculum ($1.5x10^8$ UFC/mL)/DMSO (2.5% v/v) mixture constituted the negative control. The pH values of tested samples at different concentrations were read at room temperature (25°C) each 10 min for 1 h, using a pH-meter. The curves [pH = f (times)] were labelled using Microsoft Excel software (Figure 3).

Results

Antibacterial activity of crude extract, fractions and compounds

Antibacterial susceptibility of tested samples was done by determining the MICs and MBCs of each sample on studied bacteria (Table 2). Bactericidal or bacteriostatic effects of each sample on a bacterial strain were shown by calculating the MBC/MIC ratio. These results show that crude extract SC, fraction SCc and fraction SCd exhibited an antibacterial activity respectively on 80% (8/10), 90% (9/10) and 70% (7/10) of studied strains with MICs ranging from 64-512 µg/mL. Fraction SCc was the most active tested sample with a high inhibitory potential on *Escherichia coli* ATCC8739 (MIC=64 µg/mL) and moderate activities on other bacteria. Whereas, fractions SCa and SCb were the less active samples. They showed weak antibacterial activities (MIC=512 µg/mL) each against 20% (9/10) of strains. However, only fraction SCc showed a bactericidal effect (MBC/MIC≤4) on more than one bacterial strain (*E. coli* ATCC8739, *K. pneumoniae* ATCC11296 and *P. aeruginosa* PA124). Reference antibiotic, chloramphenicol, was active against 90% (9/10) of bacteria with MICs ranging from 4-64 µg/mL and it showed a bactericidal effect almost on three bacteria (*E. coli* 102, *K. pneumoniae* KP55 and *E. aerogenes* ATCC13048). Its activity is compared to that of fraction SCc which was purified to afford some compounds whose antibacterial activities are shown in Table 3. From this Table 3, it was transpired that compound **4** (ursolic acid), the most active compound, exhibited an antibacterial activity against 80% (8/10) of strains, but with a weak antibacterial potential. Its bactericidal effect was shown only on the two *K. pneumoniae* strains (ATCC11296 and KP55).

Compounds **3** (*β*-sitosterol 3-*O*-*β*-D-glucopyranoside) and **5** (oleanolic acid) weakly inhibited the growth of 30% (3/10), meanwhile the mixed compound (**1** : β-sitosterol: and **2** : stigmasterol) did not showed any antibacterial activity against studied strains. For overall results, *P. aeruginosa* PA124 and *E. aerogenes* EA27 strains were more resistant and *E. coli* ATCC8739 strain was most sensitive towards tested samples.

Minimal inhibitory concentrations of samples in association with PAβN

Fractions SCc and SCd which showed the best antibacterial activity and plant crude extract (SC) which also exhibited the growth inhibition of most studied bacterial strains were associated to an efflux pumps inhibitor PAβN which can be able to restore their activity. The overall results are shown in Table 4. Indeed, once associated with PAβN, the activity of all tested samples increased on almost 70% of studied bacteria with the activity improvement factors (AIFs symbolized by R in the table) ranging from >2 to ≥128 for crude extract SC and fractions, and from ≥1 to ≥8 for reference antibiotic, chloramphenicol. This efflux pumps inhibitor mostly potentiated the activity of fraction SCc (90% (9/10)) than the two other samples (70% (7/10). Notice that the activity of almost all tested samples was not improved against *Pseudomonas aeruginosa* PA124. This shows the higher level of this bacterial strain in multidrug resistance and in efflux pumps expression.

Phytochemical composition of crude extract

Phytochemical analysis of *Stachytarpheta cayennensis* crude extract showed the presence of some secondary metabolite classes including polyphenols, tannins, steroids, triterpenes and anthocyanin. Nevertheless, flavonoids, anthraquinones, alkaloids and saponins were absent (Table 5).

Antibacterial mechanisms of action

Effect of fraction SCc on bacterial growth kinetic

Curves presented in Figure 2 show bacterial growth in absence and presence of fraction SCc at different concentrations (MIC/2, MIC and 2MIC) and chloramphenicol (at MIC) during time. Studied bacterial strain was *Escherichia coli* ATCC8739. Growth kinetic was followed during 18 h and the optical density values were measured each 2 h at 600 nm wavelength. Figure 2 shows three stages of bacterial growth for fraction SCc and negative controls (lag, exponential and stationary stages). Lag stage is prolonged till 4 h in presence of fraction SCc at all concentrations and is prolonged till 18 h with reference antibiotic chloramphenicol (CHL). Furthermore, the number of living bacteria in the presence of tested samples considerably decreased compared to negative controls (inoculum and DMSO 2.5%).

Effect of fraction SCc on bacterial H^+-ATPase- dependent proton pumps

Figure 3 shows pH evolution of cell culture by incubation time in presence of tested sample at MIC concentration. *Escherichia coli* ATCC8739 was used as bacterial strain and DMSO 2.5% as negative control. Obtained curves showed very low decrease pH values after 60 minutes in presence of fraction SCc (from 8 - 7.6), meanwhile pH values considerably decreased in presence of DMSO (from 8 - 6.2). Notice that a basic pH condition is unfavorable for the studied bacterial growth.

Discussion

Bacterial susceptibility and plant metabolites

The antimicrobial potential of medicinal plant is linked to the presence of one or more metabolite classes or bioactive components in this plant. Phytochemical study of crude extract (SC) in this work revealed the presence of polyphenols, tannins, steroids, triterpenes and anthocyanins and the absence of flavonoids, anthraquinones, alkaloids and saponins. Previous phytochemical studies on *Stachytarpheta cayennensis* extract also showed the absence of flavonoids and the presence of steroids, phenols, saponins and tannins [23]. The antibacterial activity of a plant extract or derived fraction is weak if its MIC>625 µg/mL, moderate if 100<MIC≤625 µg/mL or strong if MIC≤100 µg/mL. For antibiotics and isolated compounds, sample with MIC≤10 µg/mL, 10<MIC≤100 µg/mL or MIC>100 µg/mL is considered to have strong, moderate or weak activity respectively [24]. Based on this scale, it is well notice that tested crude extract SC and fractions exhibited a moderate antibacterial potential against studied strains. Whereas, isolated compounds weakly inhibited the growth of these bactreia. It is known that plant bioactive components can have antagonistic effects against each other, and this considerably decreases the antimicrobial potential of a plant extract. This can explain the fact that tested crude extract was less active than their derived fractions SCc and SCd whose MICs values were from 64 to 256 µg/mL. Some investigations also showed weak or no antimicrobial activities of ethanolic crude extract of *S. cayennensis* root against some Gram negative and positive bacteria [23]. Moreover, previous studies have reported significant antibacterial activities of fractions from *S. cayennensis* against Gram negative strains such as *Pseudomonas aeruginosa, Klebsiella pneumoniae* [11; 25; 26]. Compound **4**, Ursolic acid was shown to exhibit weak antimicrobial activity with MICs ≥ 512 µg/mL against some bacteria among which *P. aeruginosa* and *E. coli* strains [27]. This corroborates the results obtained in the present work. Furthermore, some works showed the weak inhibitory potency of compound **5**, oleanolic acid, against many bacteria including *P. aeruginosa*, *K. pneumoniae* and *E. coli* [28] as shown in the present study. It was reported that compound **3**, β-sitosterol 3-*O*-*β*-D-glucopyranoside, was not active against many Gram-negative bacterial strains including those studied in this work [6]. Tested samples mostly presented bacteriostatic effects (MIB/MIC≤4) indicating that they are only able to inhibit the growth of studied bacteria.

In association with PaβN (Table 4), the activity of tested samples significantly increased indicating that bioactive components contained in crude extract and fractions (SCc and SCd) were expelled from the cytoplasm,

thus reducing their intracellular concentrations and making them less active. This justifies the role of PAβN which inhibits or blocks efflux pumps maintaining high intracellular concentrations of antibacterial substances [29, 30] which can easily act upon their cell targets and inhibit microbial growth. This efflux pumps inhibitor had proved his capacity to improve the activity of many plant constituents [31, 32]. Unchanged activities of some samples in presence of this inhibitor indicate that efflux pumps are not the only mechanism of resistance mediated by studied bacteria.

Antibacterial mode of action

Fraction SCc which was the most active sample was tested to evaluate its influence on growth kinetic of *Escherichia coli* ATCC8739. During lag stage which makes less than 2 h in the normal conditions, bacteria accommodate themselves in their new environment and synthesize enzymes which metabolize nutrient substrates to be used for their growth and multiplication. Compared to negative control, fraction SCc strongly inhibited this bacteria growth by destroying these enzymes, some proteins and transport systems which are essential for bacterial life [33]. This justifies the very low number of multiplied bacteria during the exponential growth stage which persist 4 h, since there was very low quantity of metabolized substrates used by bacteria. In the stationary stage, the quantity of metabolized substrates considerably decreased causing death of half-bacteria and later on the majority of them, due to the accumulation of toxic wastes in the medium [33].

The role of fraction SCc in inhibiting H^+-ATPase-dependent proton pumps of plasma membrane was also investigated. Bacterial proton pumps regulate cytoplasm pH and provide energy in the form of ATP essential for the bacterial growth and survival. Inhibition of these pumps causes H^+ protons decrease of the extracellular environment which becomes less acidic [34]. The increase of environmental pH in presence of an antibacterial substance could result to inhibitory effect of H^+-ATPase-dependent proton pumps by this substance. This could compromise the survival of bacteria since the quantity of energy produced will be very low for their metabolism, growth and multiplication. It is reported that minimum pH supporting bacterial proliferation for an *Escherichia coli* strain is 4.4 [35]. Furthermore, this bacterium is supplied by a multiple complex of pH dependent on many strategies of acid tolerance allowing it to survive in stomach acidic pH conditions [36, 35]. The bacterial cytoplasmic pH which is near of the neutrality is regulated by various cations transport systems. In *E. coli*, cytoplasmic pH is regulated by expulsion of H^+ protons out of the cell via the respiratory chain and potassium ions entrance in the cytoplasm with acidic pH [34, 37]. Moreover, tested fraction SCc compared to negative control (Figure 2), induced the inhibition of H^+-ATPase-dependent proton pumps in *Escherichia coli* ATCC8739, suggesting that they constitute one of the antibacterial action targets of fraction SCc.

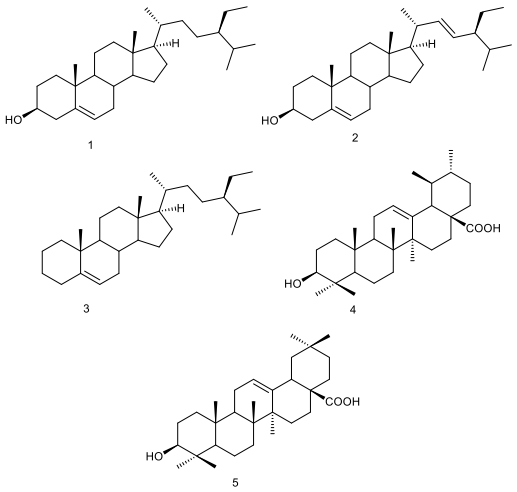

Figure 1. Structures of compounds from *Stachytarpheta cayennensis*

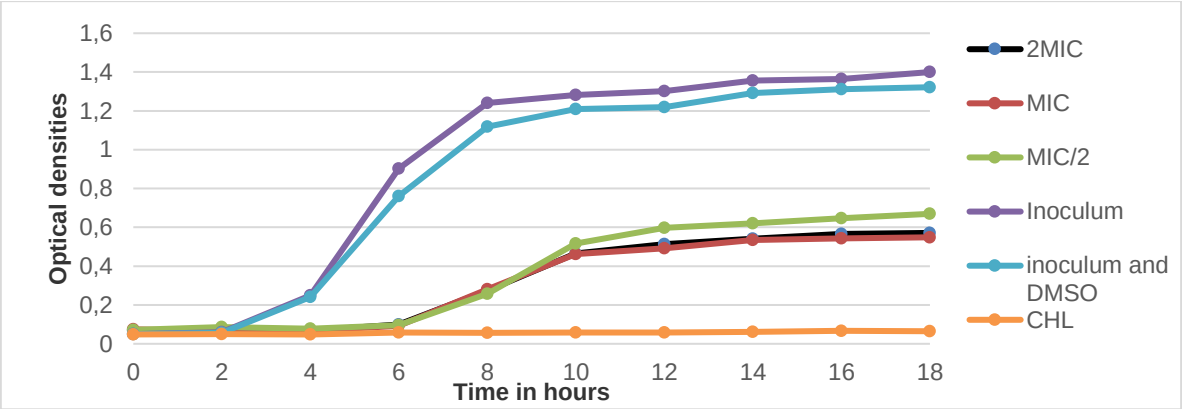

Figure 2. Effect of fraction SCc at different concentrations on growth kinetic of *Escherichia coli* ATCC8739

These results show that DMSO at 2.5% concentration used to dissolve tested samples, did not inhibit the growth of studied bacterial strains and did not influent the activity of these samples

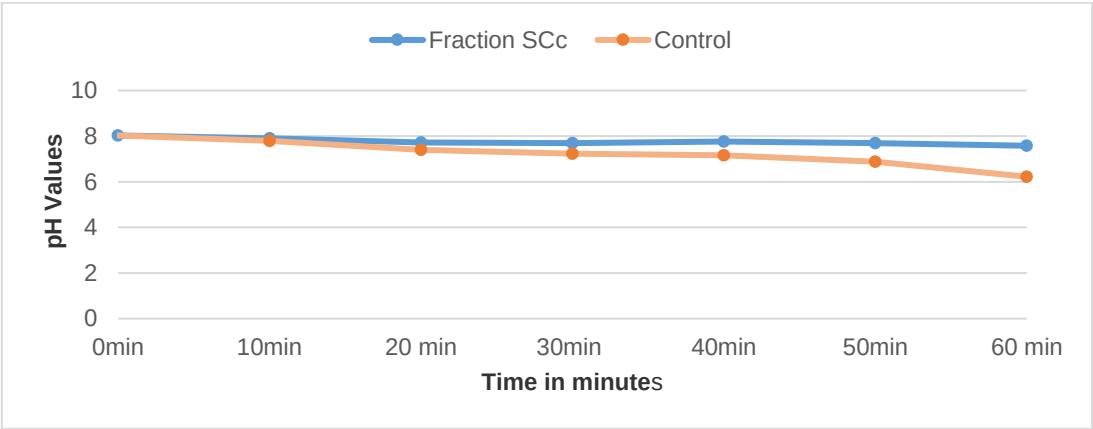

Figure 3. Effect of fraction SCc on *Escherichia coli* ATCC8739 H^+-ATPase- dependent proton pumps

Table 1. Characteristics of the studied bacterial strains

Bacterial species	Types	Characteristics	References
Escherichia coli	ATCC 8739	Reference strain	[14]
	AG102	*AcrAB* mutant AG100, owing acrF gene markedly over expressed; Tet^r	[14]
Klebsiella pneumoniae	ATCC 11296	Reference strain	[15]
	KP55	Clinical MDR isolate Tet^r, Amp^r, Atm^r, Cef^r	[15]
Pseudomonas aeruginosa	PA01	Reference strain	[16]
	PA124	MDR clinical isolate expressing Mex efflux pump	[16]
Enterobacter aerogenes	ATCC13048	Reference strain	[14]
	EA 27	Clinical MDR isolate exhibiting energy-dependent norfloxacin and chloramphenicol efflux with Kan^r, Amp^r, Nal^r, Str^r, Tet^r	[14]
Providencia stuartii	ATCC29916	Reference strain	[17]
	PS 2636	Clinical MDR isolate, *AcrAB-TolC*	[17]

$Ofxa^r$, Kan^r, Tet^r, Erm^r, Amp^r, Nal^r, Str^r, Atm^r, Cef^r, Cip^r, Im/Cs^r, Chl^r, Gen^r, Nis^r, Flx^r : resistant (r) to ofloxacin, kanamycin, tetracycline, erythromycin, ampicillin, nalidixic acid, streptomycin, aztreonam, cefepime, ciprofloxacin, imipenem/Cilastatin sodium, chloramphenicol, gentamycin, nisin and flomoxef respectively; MDR : Multidrug.resistant ;. *AcrAB-TolC, AcrAB* are efflux pumps

Table 2. Minimal inhibitory and bactericidal concentrations of *S. cayennensis* crude extract, its derived fractions and chloramphenicol

Bacterial strains	Samples and concentrations (µg/mL)																	
	SC			SCa			SCb			SCc			SCd			Chloramphenicol		
	MIC	MBC	R	MIC	MBC	R	MIC	MBC	R	MIC	MBC	R	MIC	MBC	R	MIC	MBC	R
Escherichia coli																		
ATCC8739	256	-	>2	-	nt	nd	512	-	>1	64	128	2	128	256	2	8	256	32
AG102	512	-	>1	512	-	>1	-	nt	nd	128	-	>4	128	-	>4	32	64	2
Klebsiella pneumoniae																		
ATCC11296	256	512	2	-	nt	nd	-	nt	nd	128	256	2	128	-	>4	8	256	32
KP55	512	-	>1	512	512	1	-	nt	nd	256	-	>2	128	-	>4	4	8	2
Enterobacter aerogenes																		
ATCC13048	512	-	>1	-	nt	nd	-	nt	nd	128	-	>4	-	nt	nd	8	32	4
EA27	-	nt	nd	-	nt	nd	-	nt	nd	256	-	>2	-	nt	nd	-	nt	nd
Pseudomonas aeruginosa																		
PA01	512	-	>1	-	nt	nd	512	-	>1	-	nt	nd	256	-	>2	32	-	>8
PA124	-	nt	nd	-	nt	nd	-	nt	nd	128	512	4	-	nt	nd	64	-	>4
Providencia stuartii																		
ATCC29916	512	-	>1	-	nt	nd	-	nt	nd	128	-	>4	128	-	>4	4	32	8
PS2636	-	nt	nd	-	nt	nd	-	nt	nd	128	-	>4	256	-	>2	4	64	16
PSBS (%)	70			20			20			90			70			90		

MIC : minimal inhibitory concentration; MBC : minimal bactericidal concentration; R : MBC / MIC ratio (a sample is considered as bacteriostatic or bactericidal when this ratio is >4 or ≤4 respectively) (-) : MIC or MBC > 512 µg/mL for crude extract and fractions and > 256 µg/mL for chloramphenicol; nt : not tested; nd : not determined (as no MIC and MBC values were not observed till 512 µg/mL); PSBS : percentage of susceptible bacteria to substances SC is *Stachytarpheta cayennensis* crude extract; SCa, SCb, SCc and SCd are different fractions obtained from crude extract SC

Table 3. Minimal inhibitory and bactericidal concentrations of compounds from fraction SCc

Bacterial strains	Compounds									Chloramphenicol		
	3			**4**			**5**					
	MIC	MBC	R	MIC	MBC	R	MIC	MBC	R	MIC	MBC	R
Escherichia coli												
ATCC8739	512	-	>1	512	-	>1	512	-	>1	8	256	32
AG102	-	nt	nd	256	-	>2	-	nt	nd	32	64	2
Klebsiella pneumoniae												
ATCC11296	-	nt	nd	256	512	2	-	nt	nd	8	256	32
KP55	-	nt	nd	256	512	2	-	nt	nd	4	8	2
Enterobacter aerogenes												
ATCC13048	-	nt	nd	256	-	>2	512	-	>1	8	32	4
EA27	512	-	>1	512	-	>1	-	nt	nd	-	nt	nd
Pseudomonas aeruginosa												
PA01	-	nt	nd	512	-	>1	-	nt	nd	32	-	>16
PA124	-	nt	nd	-	nt	nd	-	nt	nd	64	-	>8
Providencia stuartii												
ATCC29916	256	512	2	512	-	>&	512	-	>1	4	32	8
PS2636	-	nt	nd	-	nt	nd	-	nt	nd	4	64	16
PSBS (%)	30			80			30			90		

MIC : minimal inhibitory concentration MBC : minimal bactericidal concentration R : MBC / MIC ratio (a sample is considered as bacteriostatic or bactericidal when this ratio is >4 or ≤4 respectively) (-) : MIC or MBC > 512 µg/mL for compounds and > 256 µg/mL for chloramphenicol nt : not tested nd : not determined (as no MIC and MBC values were not observed till 512 µg/mL) PSBS : percentage of susceptible bacteria to substances Compounds **1** and **2** mixture did not showed any activity against all studied bacterial strains All these compounds were isolated from fraction SCc

Table 4. Minimal inhibitory concentrations of samples in the presence of PAβN

Bacterial strains	Samples (µg/mL)											
	SC			SCc			SCd			Chloramphenicol		
	MIC	+PAβN	R	MIC	+PAβN	R	MIC	+PAβN	R	MI	+PAβN	R
Escherichia coli												
ATCC8739	256	<8	>32	128	8	16	128	<4	≥32	4	<4	≥1
AG102	512	<8	>64	128	<4	≥32	128	128	1	32	<4	≥8
Klebsiella pneumoniae												
ATCC11296	256	64	4	128	-	≤0,2	128	<4	≥32	4	<4	≥1
KP55	512	-	<1	256	16	16	128	<4	≥32	64	8	8
Enterobacter aerogene												
ATCC13048	512	32	16	64	8	8	-	64	>8	8	<4	≥2
EA27	-	64	>8	256	32	8	-	128	>4	-	<4	>64
Pseudomonas aeruginosa												
PA01	512	-	<1	-	<4	≥12	-	<4	>128	16	<4	≥4
PA124	-	-	nd	128	16	8	256	256	1	32	<4	≥8
Providensia stuartii												
ATCC29916	512	128	4	128	<4	≥32	128	<4	≥32	16	8	2
PS2636	-	<8	>64	128	64	2	256	256	1	32	<4	≥8
PIA (%)		70			90			70			100	

MIC : minimal inhibitory concentration; R=AIF : MICsample alone / MICsample$_{+PAβN}$ ratio (this means the factor which determines the improvement of the activity of samples by PAβN; the activity of a sample was considered to be improved when its AIF was > 2); (+ PAβN) : represent MICs values of tested samples obtained in presence of PAβN ; **(-)** : MIC > 512 µg/mL for crude extract and fractions and > 256 for chloramphenicol; PIA : percentage of improved activity; nd : not determined (as no MIC values were not observed till 512 µg/mL) SC is *Stachytarpheta cayennensis* crude extract and SCc and SCd are fractions obtained from crude extract SC

Table 5. Phytochemical composition of *Stachytarpheta cayennensis*

Phytochemicals	Inference
Polyphenols	+
Flavonoids	-
Tanins	+
Anthraquinons	-
Alkaloids	-
Saponins	+
Steroids	+
Triterpens	+
Anthocyanins	+

(+): presence of metabolites; **(-)**: absence of metabolites

Conclusions

The overall results obtained in the present work indicate that the moderate antibacterial activity of *Stachytarpheta cayennensis* constituents can be significantly improved by an efflux pumps inhibitor to overcome multidrug-resistant bacteria overexpressing efflux pumps. This report provides additional information for the traditional use of this plant in the treatment of infections and diseases caused by these bacteria. Most interesting, the mechanisms of action of a constituent from this plant are reported in this work for the first time. In order to have most interesting activities, the effects of these constituent in combination with commonly used antibiotics could be investigated.

Additional file

Supplementary file.Docx: G1. Data and graphic for the effect of sub-fraction SCc2 on the bacterial growth kinetic, G2. Data and graphic for the effect of sub-fraction SCc2 on bacterial H+-ATPase-depending proton pumps; SF1. Tables showing the fractionation and purification of *Stachytarpheta cayennensis*; SF2. 1H and 13C NMR and major chemical shifts of studied compounds. Available online at: https://www.investchempharma.com/imcp35-supplementary-file/

Abbreviations

SC : *Stachytarpheta cayennensis*
ATCC : American type culture collection
MIC : Minimal inhibitory concentration
MBC : Minimal bactericidal concentration
DMSO: Dimethylsulfoxide
INT : p-Iodonitrotetrazolium chloride
PAβN : Phenylalanine arginine-*β*- naphthylamide
SFR/CAM : Society of forest reserve of Cameroon
NHC : National herbarium of Cameroon

CHL : Chloramphenicol
MHA : Mueller Hinton agar
MHB : Mueller Hinton broth
OD : Optical density
RND : Resistance-nodulation-cell division
EPI : Efflux pumps inhibitor
AIF : Activity improvement factor

Authors' Contribution

VK designed the experiment and supervised the work together with VBP. LMY carried out the antibacterial activities of samples. The antibacterial mechanisms of action of the most active sample were done by BENW and PN. The plant extraction and purification as well as the phytochemical analysis were done by BCKA. The 1H and 13C NMR spectra were analyzed by GTMB, BKN, JDSM and the structures of different compounds were determined by ISK. The chemical part of the manuscript was written by BCKA and GTMB and the biological part by SBT. All authors read and approved the final manuscript.

Acknowledgments

The authors wish to autograph this work in memory for Pierre Tane (Professor) who was the supervisor of the chemical part of this work. We also thank the American Type Culture Collection laboratory which provided the reference bacterial strains and Cameroon National Herbarium (Yaoundé) for plants identification.

Conflict of interest

No conflict of interest

Article history:

Received: February 13, 2020
Received in revised form: March 17, 2020
Accepted: March 25, 2020
Available online; March 25, 2020

References

1. WHO 2017. Déclaration conjointe OMS/UNICEF. Prise en charge d'une infection bactérienne potentiellement grave chez le jeune nourrisson âgé de 0 à 59 jours lorsqu'un transfert vers une structure hospitalière est impossible. Disponible sur http://www.who.int. Consulté le 15/09/18.
2. Lemaoui C, Layaida H, Badi A, Foudi N. 2017. Stratégies actuelles de lutte contre la résistance aux antibiotiques. *Journal Des Anti-Infectieux*, 19(1) :12-19.
3. Muylaert A, Mainil JG. 201). Résistances bactériennes aux antibiotiques: les mécanismes et leur « contagiosité ». *Annales de Medecine vétérinaire*, 156 :109-123.
4. Koyama J. 2006. "Anti-infective quinone derivatives of recent patents," *Recent Patents on Anti-infective Drg Discovery*, 1(1):113–125,
5. Lomovskaya, O, Watkins W. 2001. "Inhibition of efflux pumps as a novel approach to combat drug resistance in bacteria". *Journal of Molecular Microbiology and Biotechnology*, 3(2):225–236.
6. Tankeo SB, Damen F, Awouafack MD, Mpetga J, Tane P, Eloff JN, Kuete, V. 2015. Antibacterial activities of the methanol extracts, fractions and compounds from *Fagara tessmannii*. *Journal of Ethnopharmacology*, 169(2015): 275–279.
7. Dzotam JK, Touani KF, Kuete V. 2016. Antibacterial and antibiotic-modifying activities of three food plants *Xanthosoma mafaffa* Lam., *Moringa oleifera* (L.) *Schott* and *Passiflora edulis* (Sims) against multidrug-resistant (MDR) Gram-negative bacteria. *Biomed central Complementary and Alternative Medicine*, 16(1): 1-9.
8. Nayim P, Mbaveng AT, Wamba BEN, Fankam AG, Dzotam JK, Kuete V. 2018. Antibacterial and antibiotics-potentiating activities of thirteen Cameroonian edible plants against Gram-negative resistant phenotypes. *The Scientific World Journal,* 2018: 1-14. doi: 10.1155/2018/4020294.
9, Nguenang SG, Mbaveng TA, Fankam GA, Manekeng TH, Nayim P, Wamba NEB, Kuete V. 2018. *Tristemma hirtum* and five other Cameroonian edible plants with weak or no antibacterial effects modulate the activities of antibiotics against Gram-Negative Multidrug-Resistant Phenotypes. *The Scientific World Journal,* 2018, 1-12. doi: 10.1155/2018/7651482.
10. Wamba NEB, Nayim P, Mbaveng TA, Voukeng KI, Dzotam KJ, Ngalani OJT, Kuete V. 2018. *Syzigium jambos* displayed antibacterial and antibiotic-modulation activities against resistances phenotypes. *Evidence-Based Complementary and Alternative Medecine*, 2018: 1-12.
11. Okoye TC, Akah PA, Okoli CO, Ezike AC, Mbaoji FN. 2010. Antimicrobial and antispasmodic activity of leaf extract and fractions of *Stachytarpheta cayennensis. Asian Pacific Journal of tropical medicine*, 3(3): 189-192.
12. Harborne JB. 1998. Phytochemical methods: A guide to modern techniques of plant analysis (3e éd.). New york : Chapman and Hall Limited.
13. Evans WC. 2002. Trease and Evans Pharmacognosy. (15th edn), Elsevier (A Division of Reed Elsevier India Pvt. Limited), pp. 585
14. Kuete V, Ngameni B, Tangmouo JG, Bolla JM, Albert-Franco S, Ngadjui BT, Pagès J-M. 2010a. '' Efflux pumps are involved in the defense of Gram negative bacteria against the natural products isobavachalcone and diospyrone*", Antimicrobial Agents and Chemotherapy* 54(5): 1749-1752.
15. Chevalier J, Pages JM, Eyraud A, Mallea M. 2000. Membrane permeability modifications are involved in antibiotic resistance in *Klebsiella pneumoniae. Biochem Biophys. Res. Commun*. 274: 496–499.
16. Lorenzi V, Muselli A, Bernardini AF, Berti L, Pages JM, Amaral L, Bolla JM. 2009. Geraniol restores antibiotic activities against multidrug-resistant isolates from Gram-negative species. *Antimicrob. Agents Chemother*. 53: 2209–2211.
17. Tran Q-T., Mahendra, K.R., Hajjar, A., Ceccarelli, M., Davin-Regli, A., Winterhalter, M., Weingart, H, Pagès J-M. 2010. Implication of porins in ß-lactam resistance of *Providencia stuartii. The Journal of Biological Chemistry*, 285: 32273-32281.
18. Eloff JN. 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Med*, 64: 711-713.
19 Mativandlela SPN, Lall N, Meyer JJM. 2006. "Antibacterial, antifungal and antitubercular activity of (the roots of) *Pelargonium reniforme* (CURT) and *Pelargonium sidoides* (DC) (Geraniaceae) root extracts." *South African Journal of Botany*, 72(2): 232–237.
20 Kuete V, Ngameni B, Fotso SCC, Kengap TR, Tchaleu NB, Meyer JJM *et al.* 2008. Antimicrobial activity of the crude extracts and compounds from *Ficus chlamydocarpa* and *Ficus cordata* (Moraceae). *J Ethnopharmacol*, 120: 17-24.
21. Cox SD, Mann CM, Markham JI, Bell HC, Gustafson JE, Warmington JR, Wyllie SG. 2000. The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *Journal of Applied Microbiology*, 88: 170-175.
22. Manavathu EK, Dimmock JR, Sarvesh CV, Chandrasekar PH. 2001. Inhibition of H^+-ATPase-mediated proton pumping in *Cryptococcus neoformans* by a novel conjugated styryl ketone. *Journal of Antimicrobial Chemotherapy*, 47: 491–494
23. Iwu IC, Onu UL, Ukaopma AA, Oze RN. 2018. Phytochemical, antimicrobial, and Gc/Ms analysis of the root of *Stachytarpheta cayennensis* (L. Vahl) grown in Eastern Nigeria. *International Research of Natural Sciences*, 6(2):1-14.
24 Kuete V. 2010. Potential of Cameroonians plants and derived products against microbial infections : A Review. *Planta Medica*, 76: 1-13.
25. De Souza PA, Silva CG, Machado BRP, De lucas NC, Leitao GG, Eleutherio ECA, Ortiz GMD, Benchetrit LC. 2010. Evaluation of antimicrobial, antioxidant and phototoxic activities of extracts and isolated compounds from *Stachytarpheta cayennensis* (Rich.) Vahl, Verbenaceae. *Brazilian Journal of Pharmacognosy*, 20(6) : 922-928.
26. Sujan GPS, Schruthi SD, Ramachandra YL. 2016. Antibacterial and molecular docking studies of bioactive component from leaves of *Stachytarpheta cayennensis* (Rich) Vahl. *Research Journal of Phytochemistry.* 2-7. doi: 10.3923/rjphyto.2016.
27. Do Nascimento PGG, Lemos TLG, Bizerra AMC, Arriaga ÂMC, Ferreira DA, Santiago GMP, Braz-Filho R, Costa JGM. 2014. Antibacterial and antioxidant activities of ursolic acid and derivatives. *Molecules,* 19: 1317-1327
28. Krystyna I.W, Grudniak AM, Fiecek B, Kraczkiewicz-Dowjat A, Kurek A. 2010. Antibacterial activity of oleanolic and ursolic acids and their derivatives. *Central European Journal of Biology*, 5(5): 543-553
29. Pagès, JM, Amaral L. 2009. Mechanisms of drug efflux and strategies to combat them: Challenging the efflux pump of Gram-negative bacteria. *Biochemistry and Biophysic Acta*, 1794: 826- 833
30. Van Bambeke F, Pagès J-M, Lee VJ. 2010. Inhbitor of bacterial efflux pupms as adjuvants in antibacterial therapy and diagnostic tools for detection of resistance by efflux. *Frontier In Anti-Infective Drug Discovery*, 1: 138-175.
31. Tankeo SB, Lacmata ST, Noumedem JAK, Dzoyem JP, Kuiate JR, Kuete V. 2014. Antibacterial and antibiotic-potentiation activities of some Cameroonian food plants against multidrug resistant Gram-negative bacteria. *Chinese Journal of Integrative Medicine*, 20(7):546-554
32. Badawe G, Fankam AG, Nayim P, Wamba BEN, Mbaveng AT, Kuete V. 2018. Anti-staphylococcal activity and antibiotic-modulating effect of *Olax subscorpioidea*, *Piper guineense, Scorodophloeus zenkeri, Fagara leprieurii*, and *Monodora myristica* against resistant phenotypes. *Investigational Medicinal Chemistry and Pharmacology*, 1(2):17
33. Delhalle L, Daube G, Adolphe Y, Crevecoeur S, Clinquart A. 2012. Les modèles de croissance en microbiologie prévisionnelle pour la maitrise de la sécurité des aliments (synthèse bibliographique). *Biotechnology and Agronomic Society of Environment*, 16(3): 369-381
34. Kobayashi H. 1985. A Proto-translocating of ATPase Regulates PH of The bacterial Cytoplasm. *The Journal of Biological Chemistry*, 260(5): 72-75.
35. Bavishi C, DuPont HL. 2010. Systematic review: the use of proton pumps inhibitors and increased susceptibility to enteric infection. *Alimentary Pharmacology and Therapeutics*, 34: 1269–1281

36. Foster JW. 2004. Escherichia coli acid resistance: tales of an amateur acidophile. *Natural Review of Microbiology*, 2: 898-907
37. Kuete V, Poumale Poumale HM, Guedem AN, Shiono Y, Randrianasolo R, Ngadjui BT. 2010b. Antimycobacterial, antibacterial and antifungal activities of the methanol extract and compounds from *Thecacoris annobonae* (Euphorbiaceae). *South African Journal of Botany*, 76: 536 – 542