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Original Research Article

Evaluation of the Antibacterial Activity and Cytotoxicity of Extracts of Endophytic Bacteria Isolated from *Solanum torvum* Sw (Solanaceae)

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Abstract

The study aimed at evaluating the antibacterial and cytotoxic potential of endophytic bacteria extracts isolated from *Solanum torvum* Sw (Solanaceae), a plant traditionally used in the treatment of many pathologies such as diarrheal diseases. Isolation of endophytic bacteria was done on supplemented and non-supplemented nutrient media. The extracts of these endophytes were obtained after fermentation in Muller Hinton Broth (MHB). An antibacterial screening of the extracts was carried out on 04 bacterial strains (*Staphylococcus aureus*, *Shigella flexneri*, *Escherichia coli* and *Salmonella typhimurium*). The strains having demonstrated activity were retained for the determination of the minimum inhibitory concentrations (MIC). The cytotoxicity tests of the extracts were carried out on healthy macrophage cells (Raw 264.7) and healthy kidney cells (Vero). A total of 41 isolates of endophytic bacteria were isolated from *S. torvum*. The antibacterial screening made it possible to retain 08 extracts which demonstrated antibacterial activity on the *S. aureus* and *S. flexneri* strains with MICs ranging from 100 to 200 µg/ml. All extracts exhibited weak cytotoxic activity except for one extract. Extracts of endophytic bacteria isolated from *S. torvum* showed antibacterial activity and low cytotoxicity on the pathogenic strains tested. This study shows that the endophytic bacteria of *S. torvum* are a source of antibacterial compounds to be explored to enrich the therapeutic arsenal against bacterial infections.

Keywords: *Solanum torvum*, endophytic bacteria, antibacterial activity, cytotoxicity.

INTRODUCTION

Infectious diseases are pathologies caused by various pathogenic microorganisms which still constitute a major public health problem today. These pathogens can infect different body systems. Infectious diseases of the digestive tract, also known as enteric diseases, caused nearly 582 million digestive tract cases in 2015, including 351 000 related deaths [1]. The treatment of these pathologies is based on the use of antibiotics. The massive use of the latter, both in urban and hospital environments, has generated a phenomenon of bacterial resistance to these antibiotics. This resistance observed today limits the possibilities of treatment and thus leads to the search for new therapeutic possibilities [2]. The massive use of synthetic drugs and antibiotics is prompting researchers to turn to new natural sources of compounds providing new effective and low-toxic drugs. A new hope for treatment lies in the exploitation of underexploited or even unexploited biological sources in order to produce new effective and safer active compounds. Despite the fact that most bioactive compounds are discovered in plants, endophytic microorganisms are also an important source with more than 200 000 biologically active compounds identified [3]. In recent years, endophytic microorganisms associated with plants have been shown to offer products with high therapeutic potential. Endophytes are defined as any microorganism living in internal plant organs at some point in their life or throughout their life in symbiosis with the host plant [4, 5]. Their main interest lies in the possibility they offer as potential sources of new bioactive compounds

[5]. Molecules produced by endophytes can have antibacterial, antifungal, antioxidant, antiretroviral, anticancer and enzyme inhibitor properties [6]. This observation has stimulated the enthusiasm around endophytic microorganisms and a variety of studies have been carried out on the biological activities of the secondary metabolites produced by them. Within the limits of our research, no study to date has been conducted on the endophytes of *Solanum torvum*, a plant traditionally used for the treatment of pathologies of bacterial origin and indirectly on the potential biological activities of their secondary metabolites. In order to contribute to the search for new bioactive, non-toxic and biodiversity-preserving antibacterial compounds, this study focused on evaluating the antibacterial activity and cytotoxicity of extracts of endophytic bacteria isolated from *Solanum torvum*.

MATERIAL AND METHODS

Plant material

The whole plant of *Solanum torvum* as well as the flowers and the fruits were harvested in the fresh state within the campus of the Faculty of Medicine and Pharmaceutical Sciences of the University of Douala. Harvesting targeted plants showing no visible pathological signs. The sample was placed in a sterile plastic bag to be transported to the laboratory and kept at 4°C for 24 h. A sample was identified at the National Herbarium of Yaounde in comparison with the reference number HNC21103.

Fig-1: A *Solanum torvum* plant

Isolation and purification of endophytic fungal strains

The various organs (leaves, flowers, fruits, twigs, stems and roots) of *Ludwigia octovalvis* were separated and washed under tap water to remove dust and debris. In a sterile hood, the plant organs were cut into fragments of approximately 1 cm x 1 cm using a sterile blade. The explants are disinfected in 70° ethanol for 1 min, followed by immersion in a 1% sodium hypochlorite (NaOCl) solution. The fragments thus sterilized are rinsed with sterile distilled water for 1 min, then dried on sterilized filter paper [7]. In order to validate the effectiveness of surface sterilization, waters from the last rinses were inoculated onto the "nutrient Agar" medium and stored for a period of one week following the protocol described by Schulz *et al*. [8]. Surface sterilization was validated because no bacterial growth was observed. The sterilized fragments of the different organs of the plant were then dried on the blotting paper previously sterilized in the autoclave near the flame. The dried fragments were cultured in Petri dishes on Nutrient Agar with the addition of Ketoconazole to inhibit the growth of fungal endophytes. Dishes were sealed and incubated at room temperature and observed daily for 7 days. The first bacteria to appear in the medium after 24 hours were subcultured on Nutrient Agar without the addition of antifungal.

The strains purification was done by successive subcultures of the bacteria isolated on Nutrient Agar until obtaining pure strains characterized by the uniform morphotypes in Petri dishes according to the method of Rakotoniriana *et al*. [9]. All microbiological manipulations are carried out under aseptic conditions under a fume hood using a Bensen nozzle.

Mass culture and extraction of metabolites from endophytic bacteria

The mass culture of the bacteria was made by the dilution technique described by Eevers *et al*. with some modifications [10]. 1g of sterile plant material was ground with 0.09% NaCl for the preparation of solutions with a concentration of the order of 10-1; 10-2; 10-3. The ground materials were inoculated in Petri dishes on Nutrient Agar supplemented with Ketoconazole (NA+Ketoconazole) and then autoclaved. The boxes were then sealed and incubated at room temperature for 7 days. The first bacteria that appeared in the dishes were subcultured on NA + Ketoconazole. The purification of the strains was done by successive subculturing of the bacteria isolated on NA until obtaining pure strains characterized by the uniform morphotypes in the Petri dish. In preparation for fermentation, these pure strains were subcultured in petri dishes containing MHA (Muller Hinton Agar).

The inoculum of each bacterium was prepared by dilution of a bacterial load: 2 ml of a 0.5 Mc Farland MHB. Each inoculum was then distributed in fermentation flasks (supplemented and non-supplemented medium) and incubated for 1, 2, 3 and 4 weeks at room temperature. They were fermented with daily stirring; the extracts were then prepared according to the protocol of Mohanta *et al*. with some modifications [11]. Methanol (v/v) was added to each flask, after homogenization followed by incubation at room temperature without shaking for 72 hours. The macerate was partitioned in dichloromethane (v/v) then

filtered and the organic phase was collected using a separatory funnel. The organic phase was then added to the pellet from the previous filtration then macerated and incubated for 72 hours. The whole was again subjected to filtration and the filtrate obtained was dried under ventilation until the dry extracts were obtained which constituted the crude extracts of the endophytic bacteria of *Solanum torvum* [11].

Evaluation of the antibacterial activity of extracts of endophytic bacteria of *Solanum torvum*

The following bacterial strains were provided for the Antimicrobial Agents and Biocontrol Unit of the Laboratory of Phytobiochemistry and the Study of Medicinal Plants of the Department of Biochemistry of the University of Yaoundé I: *Salmonella typhimurium* ATCC 13555, *Escherichia coli* ATCC 25922, *Shigella flexneri* ATCC 518, *Staphylococcus aureus* ATCC 43300.

The endophytic bacteria extracts diluted in DMSO were tested at 200 µg/ml on the bacterial strains selected by the method of microdilution in liquid medium. In each well, 60 µl of the extract at 1 mg/ml, then 90 µl of MHB and finally 150 µl of the bacterial inoculum at 10^6 CFU/ml were added. The microplate was incubated for 24 h at 37° C, then 15 µl of freshly prepared resazurin (0.15 mg in 100 ml) were added to each well. After 30 min of incubation, the wells having kept the blue color of the resazurin were retained as active, the cupules colored in pink are those where the bacterial growth was effective [12].

Only extracts that showed total inhibition at 200 µg/ml were selected for determination of minimum inhibitory concentrations (MIC) by the dilution method following the protocol described by Ganfon *et al* [13]. In the first horizontal line of a microplate, 200 µl of extract at 400 µg/ml were added, then 100 µl of extract were taken and diluted in the next well on the vertical line previously containing 100 µl of MHB. After mixing, 100 µl were taken and diluted to the content of the following well, and so on for 5 dilutions on the vertical line. A bacterial suspension at 10^6 CFU/ml of 100 µl was added to each well. The concentration of extract in each well is 200, 100, 50, 25 and 12.5 µg/ml and the bacteria concentrated at 5.10^5 CFU/ml. The microplate was then sealed and incubated for 18-24h at 37°C.

The MIC corresponded to the lowest concentration at which the inhibition of bacterial growth is visible to the naked eye (absence of turbidity). In order to facilitate the development, a colored developer (resazurin) was used.

Evaluation of the cytotoxicity of extracts of endophytic bacteria of *Solanum torvum*

The cytotoxicity of extracts of endophytic bacteria on healthy macrophage cells (Raw) and healthy kidney cells (Vero) was evaluated according to the protocol described by Czekanska, the principle being the enzymatic reduction of rezasurine (non-fluorescent blue compound) by resofurine (pink fluorescent compound) by the mitochondria of living cells [14]. A 72h culture was introduced into the wells of a 96-well microplate and incubated for 15-24h. The culture medium and the non-adherent cells were aspirated, then replaced with a new medium containing the extracts to be tested at different concentrations and incubated for 24 hours. After this incubation, resazurin corresponding to 10% of the total volume of each well was introduced and incubated for 4 hours. The plates were then read with a fluorescence spectrophotometer at 590 nm [14].

The percentages of inhibition were determined by the formula:

% Inhibition: [Nc-(extrait-Pc) /Nc] x 100]
Nc: absorbance of the negative control
Pc: absorbance of the positive control

RESULTS AND DISCUSSION

1. Endophytic bacterial strains isolated from *Solanum torvum*

A total of 67 endophytic isolates were isolated from *Solanum torvum* organs after purification and based on morphological characteristics. After several subcultures on supplemented and non-supplemented medium, 41 strains of endophytic bacteria survived, which could be due to residues and specific metabolites of the host plant still present during isolation but absent after several subcultures [15]. All organs of *S. torvum* are colonized by endophytic bacteria, roots and stems had a greater number of strains isolated (Table 1). Previous work has shown that species of the Solanaceae family such as *S. nigrum* and *S. mauritianum* are also colonized by endophytic microorganisms [16, 17].

The supplemented medium (MS) provided more endophytic bacteria (28) than the non-supplemented medium (13). Supplementation of the medium aims to optimize the processes of endophyte isolation and subsequent production of secondary metabolites of interest. The cultures obtained made it possible to observe a greater number of endophytes isolated from the supplemented medium than from the non-supplemented medium. This result is corroborated by those of Eevers *et al.* in 2015 who demonstrated that the addition of plant extracts to culture media would significantly increase the number of cultivable endophyte bacteria [10]. Although the distribution of endophyte bacteria was done in a homogeneous way in the different organs of *S. torvum*, the roots and the fruits showed a predominance of colonization which can be explained for the roots by the fact that in ligneous plants, the Endophytic bacteria are found more in organs with a long lifespan and in permanent contact with a large inoculum, as is the case for roots in constant contact with the rhizosphere [18]. Wilson *et al*.

demonstrated in his work in 2000 that the seeds contained in the fruits were responsible for the transmission of endophytes from one generation to another [19].

Table-1: Distribution of endophytes by environment and organ

***Solanum torvum* organs**	**Medium with no supplement**	**Supplemented medium**	**Total**
Leaves	1	2	3
Fruits	2	8	10
Flowers	2	3	5
Branches	2	0	2
Roots	4	9	13
Stems	1	4	5
Stem barks	1	2	3
Total	**13**	**28**	**41**

Antibacterial potential of endophytic bacteria isolated from *Solenum torvum*

The antibacterial screening was evaluated at 200 µg/ml of extract of each endophyte bacterium isolated from *Solanum turvum* on 04 bacteria: *Salmonella typhimurium* ATCC 13555, *Escherichia coli* ATCC 25922, *Shigella flexneri* ATCC 518, *Staphylococcus aureus* ATCC 43300. From this screening, 08 extracts of endophytic bacteria were active on 02 bacterial strains: *S. aureus* ATCC 43300 and *S. flexner* ATCC 518. Extracts of endophytic bacteria of strains 2035 and 2058 demonstrated bacterial activity on *S. flexneri* while extracts from strains 2016, 2022, 2035, 2052, 2037, 2038 and 2065 demonstrated activity against *S. aureus* (Table 2).

Microdilution in liquid medium made it possible to determine the minimum inhibitory concentration of the extracts of isolated endophytic bacteria. The extracts of these bacteria selected after the screening presented an MIC equal to 200 µg/mL on *S. aureus* with the exception of the endophyte strain 2058. Only the endophyte strains 2035 and 2058 were active on *S. flexneri* with MICs 200 µg/mL and 100 µg/ml respectively. These results are in agreement with the work of Maroyi *et al.* who proved that bacterial endophytes could be sources of bioactive compounds with antimicrobial properties. In addition, of all these active extracts of endophyte bacteria are mostly isolated from fermentation in an unsupplemented medium. The bacterial endophytes of the plant would produce their own bioactive substances [20].

Table-2: Minimum inhibitory concentration of extracts of endophytic bacteria from *Solanum turvum* organs

Strains	**Medium**	**weeks**	**Organs**	***Staphylococcus aureus***	***Shigella Flexneri***
				MIC (µg/mL)	
2016	MNS	4	Stems	200	-
2022	MNS	2	Stem barks	200	-
2035	MS	3	Roots	200	200
2037	MNS	2	Roots	200	-
2038	MNS	4	Roots	200	-
2052	MS	1	Flowers	200	-
2058	MNS	2	Fruits	-	100
2065	MS	2	Fruits	200	-

Cytotoxic potential of endophytic bacteria isolated from *Solenum torvum*

The extracts that demonstrated antibacterial activity on pathogenic strains were selected so that they could assess their cytotoxicity on healthy macrophage cells (Raw) and healthy kidney cells (Vero). Only the endophytic bacterial strain 2052 exhibited cytotoxicity on renal cells (Table 3). According to the National Cancer Institute (NCI), an extract is cytotoxic when its percentage inhibition of living cells is greater than 30µg/ml [21].

Table-3: Percentage of inhibition of endophytic bacteria extracts of Solanum torvum extracts

Extract code	**Vero cells (%)**	**Raw cells (%)**
2035	26.25	5.14
2016	8.49	7.55
2052	51.02	28.14
2065	12.02	5.21
2037	-31.56	0.8
2038	-32.70	1.74
2022	-82.50	19.89
2058	22.73	-8.23

CONCLUSION

This study was conducted with the aim to assess the antibacterial potential and the cytotoxicity of extracts of endophytic bacteria isolated from the organs of *Solanum torvum*. It appeared that the organs of *S. torvum* are colonized by endophytic bacteria. The evaluation of the antibacterial activity showed that the extracts of the isolated endophytic bacteria have an inhibitory activity on 02 bacterial strains *Staphylococcus aureus* ATCC 43300 and *Shigella flexneri* ATCC 518. All the extracts of the endophytic bacteria showed low cytotoxicity except, of the endophyte strain 2052 which proved to be cytotoxic towards healthy renal cells (vero). Endophytic bacteria isolated from *S. torvum* organs could offer opportunities for discovering new sources of active molecules.

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