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Antibacterial activities of natural lichen compounds against *Streptococcus gordonii* and *Porphyromonas gingivalis*

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ABSTRACT

The oral bacteria not only infect the mouth and reside there, but also travel through the blood and reach distant body organs. If left untreated, the dental biofilm that can cause destructive inflammation in the oral cavity may result in serious medical complications. In dental biofilm, *Streptococcus gordonii*, a primary oral colonizer, constitutes the platform on which late pathogenic colonizers like *Porphyromonas gingivalis*, the causative agent of periodontal diseases, will bind. The aim of this study was to determine the antibacterial activity of eleven natural lichen compounds belonging to different chemical families and spanning from linear into cyclic and aromatic structures to uncover new antibiotics which can fight against the oral bacteria. The compounds were screened by broth microdilution assay. Three compounds were shown to have promising antibacterial activities where the depsidone core with certain functional groups constituted the best compound, psoromic acid, with the lowest MICs = 11.72 and 5.86 μg/mL against *S. gordonii* and *P. gingivalis*, respectively. The compounds screened had promising antibacterial activity which might be attributed to some important functional groups as discussed in our study. The best compounds did not induce the death of gingival epithelial carcinoma cells (Ca9-22). These results introduce new compounds having potent antibacterial activities against oral pathogens causing serious medical complications.

1. Introduction

The early treatment of infections with antibiotics reduces morbidity; however, the erroneous or unsuitable antibiotic prescription reaches 20–50% in hospitals. This misuse and over use of antibiotics is one of the primary reasons behind the bacterial resistance developing globally [1]. The world is registering a substantial increase of the bacterial resistance against the discovered drugs where this resistance has almost touched all the human pathogens. Facing this fact, organizations like World Health Organization have alerted of being very close to the post-antibiotic age where the antibiotic treatments will be dramatically ineffectual against the infectious pathogens. This coincides with the concept of a position paper published by the Infectious diseases Society of America in 2009. It has reported the critical and expeditious need for developing new antibacterial agents to face this serious health crisis [2].

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Facing this public health concern, more effective antimicrobial candidates compared to the current antibiotics were studied. The new drugs, which are of natural origin, are capable to surpass the bacterial resistance mechanisms and the most important is that they can affect the bacteria inside their biofilms [3]. Among the natural sources is the association of fungus and alga and/or cyanobacterium forming a symbiotic organism named lichen which can produce > 1000 distinct secondary metabolites. They include depsones, depsidones, depsides, dibenzofurans, phenolic compounds, lactones, quinones and derivatives of pulvinic acid possessing antitumor, antiviral and antimicrobial activities. They were shown to be effective against sensitive and several multi-drug resistant bacterial strains [4,5]. Some lichen compounds have been already reported acting as antibacterial agents against various bacteria such as evernic acid [6], hybocarpone [7], lichesterinic acid [8], norlichexanthone, hypoprotocetraric [9] and protocetraric acids [10], physodic acid [11,12], secalonic acids [13], vulpinic acid [14,15], or usnic acid [16], the latter being the more studied. More recently, the antibacterial activity against the oral pathogens *Streptococcus mutans* and *Porphyromonas gingivalis* of various diphenylethers and lobaric acid, a depsidone, isolated from *Stereocaulon paschale* has been described [17].

The cost of dental care is the fourth highest one of all diseases and consuming between 5 and 10% of all health care resources. Among the oral complications defined clinically, periodontal diseases stand prominently due to their prevalence, notable effects on individuals and society as well as the required high cost to treat [18]. They can be identified as an infectious inflammation of the teeth-supporting tissues caused by the oral pathogens residing in dental biofilms. A streptococcal layer will form above the salivary pellicle and constitutes a recruitment site on which late pathogenic colonizers can bind. The latter include the etiological agent of this disease, *Porphyromonas gingivalis*. The inflammation commences mildly and can worsen if infections were left untreated destroying the tissues with time and leading to teeth loss [19].

Being the primary colonizer of the oral cavity, an agent of septic arthritis as well as a colonizer of damaged heart valves representing the major causative agent of subacute bacterial endocarditis, *S. gordonii* stands conspicuously as a dangerous bacterium inducing serious medical complications. Alongside, *P. gingivalis*, a maestro in the host's immune system evasion, has been shown to register a lot of capabilities from secreting gingipains which renders its resistance to complement destruction, into its adherence to erythrocytes serving as a safe transport mechanism without being detected by the circulating phagocytes. In addition, this smart bacterium can modify the structure of lipid A in LPS as an escaping mechanism in gingival tissues leading to the pathogenesis of periodontal diseases [20].

Not only dental extraction, periodontal surgery or tooth scaling, but even tooth brushing and flossing can disrupt the barrier between the oral bacterial biofilm and the blood circulation which can vehicle these bacteria so far to reach distant body organs. Recently, periodontal disease has been shown to be related with the cause of Alzheimer's disease [20]. Moreover, periodontal diseases seriousness extends to many dangerous systemic complications like type 2 diabetes and oral and pancreatic cancers [18].

Against this public oral health burden, we have evaluated the antibacterial activity of eleven natural lichen compounds (Fig. 1) against *S. gordonii* (Gram-positive) and *P. gingivalis* (Gram-negative). We have selected a panel of lichen compounds belonging to different classes of structures and spanning from linear into cyclic and aromatic features. Some of them possess close structures to those of already known antibacterial lichen compounds e.g. roccellic acid, an opening form of the butyrolactone lichesterinic acid [8], the four depsidones close to protocetraric [10] and/or physodic [12] or lobaric acids [17], and two depsides close to evernic acid [6]. To our knowledge, this study presents for the first time the activities of these lichen compounds against the targeted bacterial strains. Nevertheless, vulpinic acid [14] and hypoprotocetraric acid [9] have been evaluated against other bacteria and are tested herein as controls. Our promising results introduce new antibiotics that might be able to prevent and treat the periodontal diseases.

2. Materials and methods

2.1. Chemical compounds

Methyl-beta-orcinolcarboxylate (**M**) and Psoromic acid (**P**) were purchased from (Sigma Aldrich, France) and (Extrasynthèse, France). The other compounds were obtained from UMR CNRS ISCR 6226, CORINT, France, and their spectroscopic data were reported in literature [21,22]. Conhypoprotocetraric acid (**C**) was isolated from *Ramalina siliquosa* var. x, demethylbarbatic acid (**D**) from var. *druidarum* and hypoprotocetraric acid (**H**) from var. *zopfii* [21], variolaric acid (**Var**) from *Ochrolechia parella* [23] and vulpinic acid (**Vul**) from *Letharia vulpina* [24]. While (+)-Erythrin (**E**), lepraric acid (**L**) and (+)-acetylportentol(**A**) were isolated from *Roccella fuciformis*, (+)-roccellic acid (**R**) was extracted from *Roccella phycopsis* [22]. Their lichen sources and the solvents used to prepare the initial concentrations are listed in Table 1. Their Log *P* values have been calculated using ALOGPS 2.1 software giving a prediction of their lipophilicity and their aqueous solubility. They are indicated in Table 2. All the tested compounds (Fig. 1) were checked for their > 95% purity by HPLC (data not shown).

2.2. Bacterial strains

Streptococcus gordonii DL1 and *Porphyromonas gingivalis* ATCC 33277 were grown anaerobically (N_2-H_2-CO_2 [80:10:10]) at 37°Caccording to Sweidan et al. [8]. Brain-heart infusion (BHI) medium (DIFCO, France) and blood Columbia agar plates (BioMerieux, France) supplemented with hemin (5 μg/mL) and menadione (1 μg/mL) (Sigma Aldrich, France) were prepared as advised by the manufacturer and utilized for bacterial growth.

2.3. Cell lines

Since the products were tested against the oral bacteria, Ca9-22, gingival epithelial carcinoma cell lines (Health Science Research Resources Bank, Osaka, Japan) were chosen to test the cytotoxicity of these lichen compounds. Temperature of 37 °C, 5% CO_2 atmosphere, and Dulbecco's Modified Eagle Medium or DMEM (Lonza, France) were used for the growth of the gingival cells. The medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS, Lonza, France) and antibiotics (penicillin 100 mg/mL and streptomycin 50 mg/mL) (Sigma Aldrich).

2.4. Broth microdilution

According to Clinical and Laboratory Standards Institute (CLSI) [25], the compounds were 1:2 serially diluted in BHI in a sterile 96-well plate (untreated, flat bottom, with lid, Evergreen Scientific) starting from their initial concentrations (Table 1). Each well was then inoculated with 3×10^7 CFU/mL of *S. gordonii* and incubated for 24 h or *P. gingivalis* and incubated for 48 h. The solvents used to prepare the compounds were also tested on the bacteria. After that, the minimal inhibitory concentration (MIC), defined as the minimal concentration able to inhibit the visible bacterial growth, was determined as the clear well having the smallest concentration. All the clear wells were then plated on blood Columbia agar and incubated for 24 h as needed by *S. gordonii* or for 5 days as required by *P. gingivalis*. Finally, the minimal bactericidal concentration (MBC), corresponding to the lowest compound concentration killing the bacteria in the well, is determined from the Petri-plate showing no colonies and inoculated from the well with

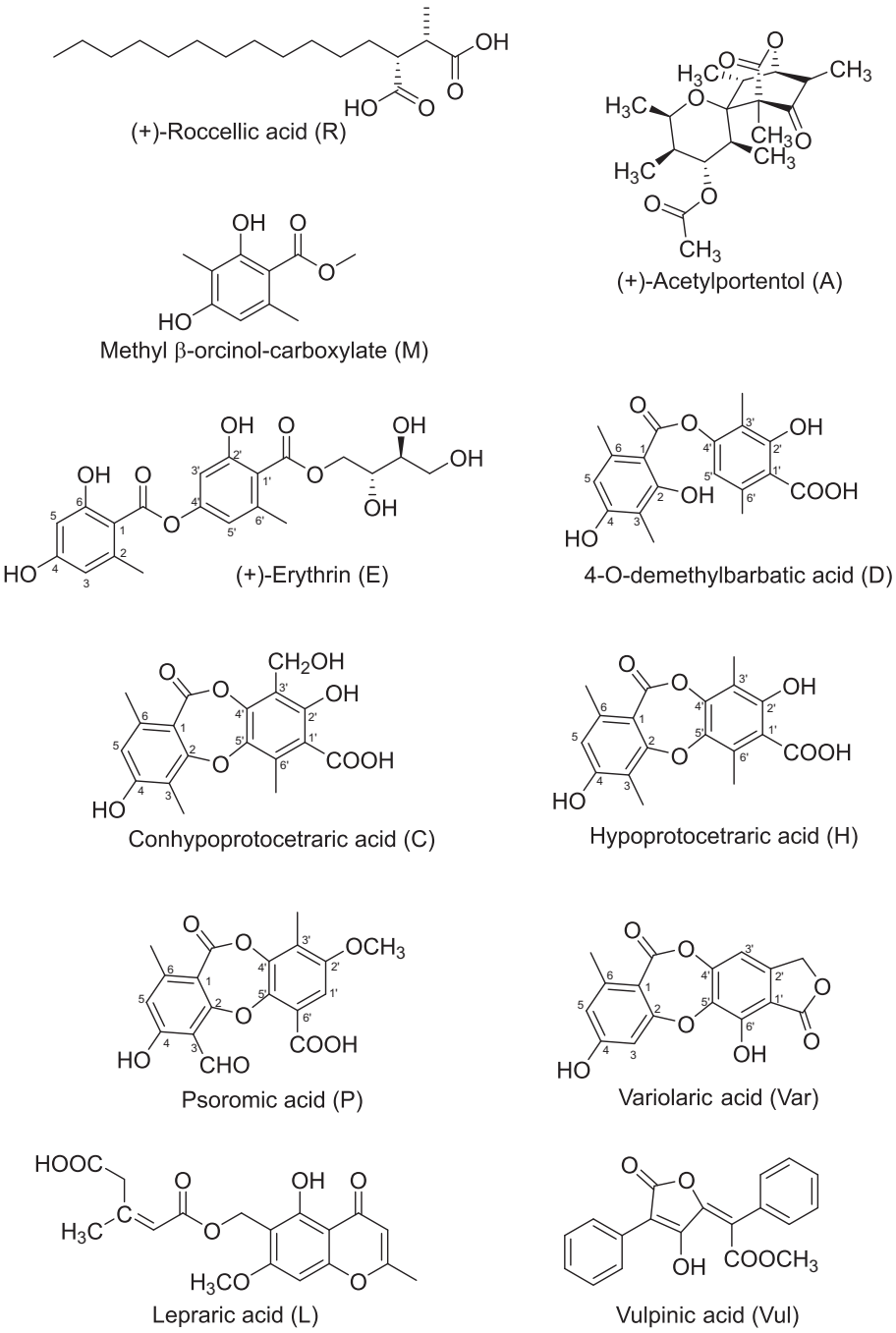

Fig. 1. Chemical structures of the tested lichen compounds listed according to their structural similarities.

Table 1
List of the natural lichen compounds with their lichen species source, along with the solvents used to prepare the solutions and their initial concentrations.

Lichen compounds and their derivations	Lichen species	Solvents used (%)	Initial concentration prepared (μg/mL)
(+)-**A**cetylportentol (**A**)	*Roccella fuciformis* [22]	Methanol (100)	2000
Conhypoprotocetraric acid (**C**)	*Ramalina siliquosa* var. *x* [21]	Methanol (100)	1400
Demethylbarbatic acid (**D**)	*Ramalina siliquosa* var. *druidarum* [21]	Acetone (100)	1400
(+)-**E**rythrin (**E**)	*Roccella fuciformis*, *Roccella phycopsis* [22]	DMSO/methanol (50/50)	3000
Hypoprotocetraric acid (**H**)	*Ramalina siliquosa* var. *zopfii* [21]	DMSO/methanol (50/50)	1000
Lepraric acid (**L**)	*Roccella fuciformis* [22]	DMSO/methanol (50/50)	2500
Methyl-beta-orcinolcarboxylate (**M**)[a]	Various lichens [24]	Acetone/methanol (50/50)	3000
Psoromic acid (**P**)[a]	*Squamarina cartilaginea* [24]	DMSO/methanol (50/50)	3000
(+)-**R**occellic acid (**R**)	*Roccella phycopsis* [22]	Methanol (100)	3000
Variolaric acid (**Var**)	*Ochrolechia parella* [23]	DMSO/methanol (50/50)	3000
Vulpinic acid (**Vul**)	*Letharia vulpine* [24]	Chloroform/methanol (50/50)	3000

[a] Purchased.

Table 2
The antibacterial activity of the natural lichen compounds against *S. gordonii* and *P. gingivalis* and their calculated Log P.

Compound	MIC (μg/mL)		MBC (μg/mL)		Log P[a]
	S. gordonii	*P. gingivalis*	*S. gordonii*	*P. gingivalis*	
A	> 2000	1000	> 2000	2000	2.18 (± 0.66)
C	700	175	> 1400	700	2.19 (± 0.37)
D	21.8	10.94	700	175	3.55 (± 0.67)
E	750	375	3000	1500	1.43 (± 0.44)
H	250	62.5	1000	500	3.49 (± 0.47)
L	> 2500	625	> 2500	2500	1.95 (± 0.40)
M	375	93.75	750	375	2.07 (± 0.23)
P	11.72	5.86	3000	11.72	2.68 (± 0.47)
R	46.9	46.9	750	375	5.28 (± 0.64)
Var	375	375	1500	3000	2.18 (± 0.33)
Vul	187.5	375	1500	375	2.96 (± 0.72)
Doxycycline	0.51	0.13	32.8	75	− 0.88 (± 0.39)

[a] Calculated by ALOGPS 2.1.

the lowest compound concentration. The experiments were repeated three times in monoplicates. Vulpinic acid and hypoprotocetraric acid, as already tested compounds, were used as controls and the reference antibiotic was doxycyline.

2.5. Cytotoxicity

After seeding 55,000 Ca9-22 cells/well in a 96-well plate (Flat bottom, with lid, sterile, Thermofisher, Korea), they were incubated for 24 h at 37 °C under 5% CO_2 environment. Then, the wells contents were removed and replaced with the lichen natural compounds diluted at their higher MICs (Table 2) in the DMEM medium to be incubated for another 24 h. Negative controls containing only compounds with medium or medium alone were also run, and the positive control was represented by Triton 1%.

In the following day, MTT assay was done as previously mentioned by [26] to evaluate the cytotoxicity of the compounds. In nutshell, MTT product of concentration 5 mg/mL was added to the wells (10 μL MTT/100 μL of medium) and the plate is incubated for 4 h under the same conditions as before. Then, acid-isopropanol solution (0.04 N HCL diluted in isopropanol) was used to dissolve the formazan crystals by adding 100 μL in each well which will be mixed thoroughly. After a few minutes at the room temperature, the O.D was measured at 595 nm. The reference wavelength used was 655 nm. The measurements were presented in percentage and the value of the untreated cells was considered as 100%. The experiment was done two times in triplicate.

3. Results and discussion

We have tested the antibacterial activity of some natural lichen compounds due to the potent antibacterial reputation of lichen compounds as reported by several authors against different bacterial strains of different sensitivity, Gram types, and respiration styles [4]. The set of lichen compounds used here has shown promising antibacterial activities against two bacterial strains differing in their Gram type, *S. gordonii* as a Gram-positive strain and *P. gingivalis* as a Gram-negative counterpart. All of them were found active except **A** and **L** on *S. gordonii*, which registered more resistance, compared to *P. gingivalis*. Doxycycline, the positive control, was the most active except for its bactericidal activity against *P. gingivalis* where **P** was stronger (Table 2).

The activity alternates with the compounds structures reflecting their ability to inhibit and/or kill the bacteria. The structure spanned from linear chains into aromatic and cyclic compounds. Both, their chemical structure and the bacterial type (Gram-positive or Gram-negative) have defined their antibacterial potency.

Concerning *S. gordonii*, the least active compound was **E** with MIC = 750 μg/mL and MBC = 3000 μg/mL. The bacteriostatic activity increased to register MIC = 700 μg/mL for **C**, 375 μg/mL for **M** or **Var**, 250 μg/mL and 187.5 for the controls, **H** and **Vul**, respectively, 46.9 μg/mL for **R**, and 21.8 μg/mL for **D**. Then, it reached the maximum with **P** having MIC = 11.72 μg/mL. On the other side, **A**, **C** and **L** have shown no bactericidal potency. The lowest killing activity was shown for **E** and **P** with 3000 μg/mL as MBC. Then, it increased to display MBC = 1500 μg/mL for **Var** or **Vul**, 1000 μg/mL for **H**, 750 μg/mL for **R** or **M**, and700 μg/mL for **D** as the maximum bactericidal activity. However, doxycycline was the most efficient having MIC = 0.51 μg/mL and MBC = 32.8 μg/mL (Table 2).

Regarding *P. gingivalis*, which was shown to be more sensitive than *S. gordonii*, **A** was the least active compound with MIC = 1000 μg/mL and MBC = 2000 μg/mL. **L** came in the second place with MIC = 625 μg/mL and MBC = 2500 μg/mL. Compounds **E**, and **Var** have shown the same inhibitory activity as **Vul**, with MIC = 375 μg/mL. The inhibitory activity increased to display MICs = 175, 93.75, 62.5, 46.9, and 10.94 μg/mL for **C**, **M**, **H**, **R**, and **D**, respectively. It continued enhancing to reach the best value of MIC = 5.86 μg/mL for **P**, but doxycycline was more active with MIC = 0.13 μg/mL. With respect to the bactericidal activity, this strain needed 3000 μg/mL to be killed by the weakest compound, **Var**. Compound **L** came after with MBC = 2500 μg/mL, then, compound **A** with MBC = 2000 μg/mL and compound **E** with MBC = 1500 μg/mL. The MBC value decreased after that to be 700, 500, 375, 175, and 75 μg/mL for **C**, **H**, **M** or **R** or **Vul**, **D**, and doxycycline, respectively. The MBC has finally reached the maximum with 11.72 μg/mL displayed by the strongest compound, **P**, concluding that thiswas a stronger killer compared to the antibiotic, doxycyline, by about 6 times.

Three compounds were shown to have promising antibacterial activities and can be listed from the least into the most active as **R**, **D** and **P**.

Starting with compound **R**, it showed the same MIC value, 46.9 μg/mL, against both bacterial strains, suggesting that it may have the same bacterial target in the two Gram types. As the butyrolactones, it has the same long chain and the carboxyl group suggested to be involved in the antibacterial activity by Sweidan et al. [8]. This compound appears to be the most lipophilic regarding its Log *P* value.

Compound **D** showed a strong inhibitory activity on both bacteria with the lower MIC against the Gram-negative strain compared to the other bacterium, but a weak killing potential has been realized for **D**.

However, compound **P** had a weak cidal activity against *S. gordonii* with MBC = 3000 μg/mL compared to a strong activity of much lower MBC = 11.72 μg/mL which were enough to kill *P. gingivalis*. Like **D**, **P** had a strong bacteriostatic activity with the lower MIC (5.86 μg/mL) for the Gram-negative strain where the MIC was 11.72 μg/mL against the Gram-positive counterpart. However a previous study has shown no antibacterial activity of **P** against methicillin-resistant Staphylococci

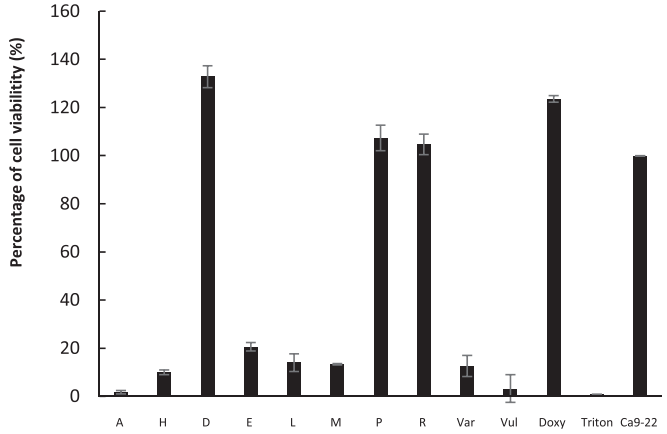

Fig. 2. Evaluation of cytotoxic activities of 11 lichen compounds against gingival epithelial carcinoma cells (Ca9-22) by MTT assay.

[27].

The Log *P* values, the coefficient describing their relative lipophilicity, decrease from **R** to **P** but remain high. This parameter seems not to have influenced their antibacterial activity because doxycycline inhibiting the growth of the two bacteria possesses a very low Log *P* value. We suggest that there is no correlation between lipophilicity and antibacterial activity.

Compound **Vul** was reported to be active against several bacterial strains. Its best MIC was 4 μg/mL against *Propionibacterium acnes* [14]. We have found in this study that it is active against *S. gordonii* and *P. gingivalis* but to a much less efficiency than what Lauterwein et al. have found. On the other hand, while **H** has been described active against various strains [9], we found it weakly active against the two tested oral bacterial strains.

Among the compounds we can find 5 compounds that possess close structure, **C**, **D**, **H**, **P** and **Var** (Fig. 1). Compounds **D** and **P** have displayed stronger results than compounds **C**, **H** and Var. We can conclude that some functional groups could be implicated in the antibacterial activity that will target a certain type. If we compare the structurally closer depsidones **C** and **H** to the depside **D**, we find that one or two structural changes have taken place: substituting CH_2OH at carbon number 3′ in compound **C** instead of CH_3 in compound **D** and the presence of ether linkage in **C** and **H** at C-5′. These changes have weakened the antibacterial activity and showed the importance of the methyl group in C-3′ and of the flexibility around the ester linkage. In comparison with the compound **P** which had a lower MIC than **D**, two CH_3 groups at carbons 3 and 6′ in **D** were replaced with aldehyde and carboxyl groups, respectively. In addition to the ether linkage between C-2 and C-5′, the carboxyl group of **D** at C-1′ was lost in **P** and the hydroxyl group attached to C-2′ was replaced by a methoxy group. The best antibacterial activity of **P** could be attributed to the presence of an aldehyde group at C-3. Moreover **P** showed a better bactericidal activity concerning the Gram-negative bacteria, *P. gingivalis*, in comparison to the Gram-positive counterpart, *S. gordonii*. This result is in accordance with those of protocetraric and lobaric acids which showed a good activity against *Salmonella typhi* [10] and *P. gingivalis* [17], respectively.

Other physicochemical properties than Log P such as pKa could be an important parameter to determine the solubility of these lichen compounds as already mentioned by Honda et al. [28]. All the active compounds possess a carboxylic group indicating that these compounds are mostly ionized at pH 7. Our results are in agreement with those reported previously [28]. Further investigations will be carried out to determine the means they used to penetrate bacterial cells and to precise the mechanism of action of these compounds.

At their higher MICs, the most active compounds (**R**, **D** and **P**) showed no cytotoxicity against gingival epithelial carcinoma cells whereas the weak antibacterial compounds (**A**, **H**, **E**, **L**, **M**, **Var** and **Vul**) exhibited a relative cytotoxicity (Fig. 2). These results augur well for the development of new treatments for periodontal diseases.

4. Conclusion

The natural lichen compounds screened had promising antibacterial activity against the oral bacteria, *S. gordonii* and *P. gingivalis*. Compounds (+)-Roccellic acid (**R**), Demethylbarbatic acid (**D**) and Psoromic acid (**P**) had the highest activity. Chemically, some structural changes among the compounds have shown some important sites that might be involved in the antibacterial activity. However, this activity is not attributed to their Log *P* values. These results introduce new compounds without cell cytotoxicity and with potent antibacterial activities against oral pathogens causing serious medical complications.

Conflict of interests

None to declare.

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