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HPTLC-MS: a tool for the efficient dereplication of pentacyclic triterpenic acids from *Platostoma rotundifolium* (Briq.) A. J. Paton (umusékerasúka)

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Abstract

Dereplication is a process that consists of identifying within complex mixtures (crude extracts or first separation fractions) all the bioactive or undesirable molecules (or families of molecules) already known thanks to their physico-chemical properties. Dereplication strategies are very diverse depending on the starting material (plants, animals, microbes, insects or marine organisms), the analytical techniques chosen (chromatographic and/or spectroscopic), the type of compounds sought and the final goal pursued. As previous researches have shown that aerial parts of *P. rotundifolium* (Asteraceae) contained pentacyclic triterpene acids (PTAs) including ursolic, corosolic, tormetic, hyptadenic and jeremic acids, this work aims to make dereplication of these compounds in ethyl acetate extract of the plant without having to isolate them. For this, high performance thin layer chromatography (HPTLC) was coupled with mass spectrometry (MS) to be used in this dereplication process. As results, we found that R_f values of five PTAs contained in the plant extract corresponded exactly to those of the five standards used. These R_f values were (in decreasing order) 0.40; 0.33; 0.16; 0.10 and 0.06 corresponding to ursolic, hyptadienic, jeremic, corosolic and tormentic acids respectively. In addition , the HPTLC–MS full scan mass spectra (of standards and corresponding compounds from the plant extract) showed the same quasi-molecular ion pics $[M-H]^-$ at m/z 455.4, 469.5, 487.4/487.5, 471.4 and 487.4, corresponding to the loss of one proton of ursolic, hyptadienic, jeremic, corosolic and tormentic acids respectively. In view of the interesting results and despite the fact that this technique is little used in the field of triterpenoids, we can affirm that HPTLC-MS is a powerful method in the analysis of natural products such as PTAs contained in the aerial parts of *P. rotundifolium* (Briq.) A. J. Paton.

Keywords: HPTLC-MS, dereplication, pentacyclic triterpenic acids, bioautography, *Platostoma rotundifolium.*

Résumé

La déréplication est un processus qui consiste à identifier au sein de mélanges complexes (extraits bruts ou premières fractions de séparation) toutes les molécules (ou familles de molécules) bioactives ou indésirables déjà connues grâce à leurs propriétés physico-chimiques. Les stratégies de déréplication sont très diversifiées en fonction du matériau de départ (plantes, animaux, microbes, insectes ou organismes marins), les techniques analytiques choisis (chromatographiques et/ou spectroscopiques), le type de composés recherchés et le but final poursuivi. Comme des recherches antérieures ont montré que les parties aériennes de *Platostoma rotundifolium* (Asteraceae) contenaient des acides triterpéniques pentacycliques (ATPs) dont les acides ursolique, corosolique, tormétique, hyptadénique et jérémique, ce travail vise à faire la déréplication de ces composés dans l'extrait acétate d'éthyle de la plante sans devoir les isoler. Pour cela, la chromatographie sur couche mince à haute performance (CCMHP) a été couplée à la spectrométrie de masse (SM) pour être utilisée dans ce processus de déréplication. Comme résultats, nous avons trouvé que les valeurs de R_f des cinq ATPs contenus dans l'extrait de plante correspondaient exactement à celles des cinq standards utilisés. Ces valeurs de R_f étaient (par ordre décroissant) 0,40 ; 0,33 ; 0,16 ; 0,10 et 0,06 correspondant respectivement aux acides ursolique, hyptadiénique, jérémique, corosolique et tormentique. De plus, les spectres de masse à balayage complet (CCMHP-SM) des standards et des composés correspondants de l'extrait de plante ont montré les mêmes pics d'ions quasi-moléculaires $[M-H]^-$ à m/z 455,4 ; 469,5 ; 487,4/487,5 ; 471,4 et 487,4, correspondant à la perte d'un proton des acides ursolique, hyptadiénique, jérémique, corosolique et tourmentique respectivement. Au vu des résultats intéressants et malgré le fait que cette technique soit peu utilisée dans le domaine des triterpénoïdes, nous pouvons affirmer que la CCMHP-SM est une méthode performante dans l'analyse de produits naturels tels que les ATPs contenus dans les parties aériennes de *P. rotundifolium* (Briq.) A. J. Paton

Mots clés: CCMHP-SM, déréplication, acides triterpeniques pentacycliques, bioautographie, *Platostoma rotundifolium.*

1. Introduction

1. 1. Problematic of dereplication and its possible tools

Dereplication is a process that consists in identifying in complex mixtures (raw extracts or first separation fractions) all bioactive or undesirable (already known) molecules (or families of molecules) due to their physicochemical properties. This term was first used in 1978 for the detection and assay of antitumor antibiotics (Hanka *et al*., 1978) and became much more widespread in the 1990s in order to accelerate the process of discovering natural products (Gaudencio and Pereira, 2015). Indeed, since that time, the dereplication has facilitated the research work in several fields such as discovery of new natural compounds having a certain biological activity, identification of known compounds in natural samples from various sources (plants, animals, microbes, venoms and marine), biological analysis to facilitate bioguided fractionation, chemical fingerprinting, quality control of herbal products (used in herbal medicine), chemotaxonomy, metabolomics and biosynthesis of secondary metabolites (Hubert *et al*., 2015; Smyth *et al.*, 2012; Gu *et al*., 2006; Nielsen *et al*., 2011; Ito and Masubuchi, 2014). In addition, it has saved resources (such as solvents) and time that were once wasted during the various stages of extractions and fractionations, as well as during the isolation, purification and spectroscopic identification of compounds already known in literature (VanMiddlesworth and Cannell, 1998; Gaudencio and Pereira, 2015; Mammo and Endale, 2015).

Dereplication strategies are very diversified according to natural source (plants, animals, microbes, insects or marine organisms), chosen analytical techniques, type of sought compounds and final goal pursued. To this could be added taxonomic dereplication based on gene sequence analysis of microbial strains (Hubert *et al*., 2015).

Several tools can thus be used and most often require a combination of different methods of separation and spectroscopic analysis (in particular to increase the sensitivity), as well as a careful search in appropriate databases. For that, chromatographic and spectroscopic techniques have been used separately for a long time during identification of compounds from natural sources. For example, high-performance liquid chromatography (HPLC) is used to compare the retention times of compounds contained in a mixture and those of compounds of an existing database; thin layer chromatography (TLC) or high-performance thin layer chromatography (HPTLC) to facilitate the comparison of the retardation factors (R_f) of these products; mass spectrometry to detect and compare the molecular weights of compounds (Horai *et al*., 2010), while nuclear magnetic resonance (NMR) gives information on the peaks of carbon 13 (for comparison in NMR shift database for example).

However, in order to avoid several manipulations, it is currently possible to couple separation methods to certain detection methods. This permit to directly analyze compounds that are previously separated, and this reduces the analysis time and the risk of errors formerly observed when carrying out several manipulations separately. The Table 1 gives an overview of some coupling methods that are currently used in dereplication process. Databases are also diversified and are currently capable of providing two or more characteristics of organic compounds at a time. This is the case of DELEP-NP database designed to facilitate the dereplication of known natural compounds using MS and NMR data (Zani *et al.*, 2017).

1.2. TLC-MS and its use in dereplication

Thin layer chromatography (TLC) is one of the planar chromatographic techniques used in qualitative and quantitative analysis. It is commonly performed on an analytical scale to separate the components of a mixture or to follow evolution of a reaction, and at the preparative scale during purification of compounds (Hao *et al*., 2015). In TLC, the mobile phase is a liquid (solvent), while the stationary phase is a flat thin layer of absorbent material (such as silica gel, aluminum oxide or cellulose) applied on an inert support (glass, aluminum or polyester); hence the term "thin layer chromatography" (Reich and Schibli, 2007).

This technique can be easily performed manually in a simple and inexpensive manner, and has other numerous advantages such as multiple detection, flexibility and cost-effectiveness, speed of separation, good sensitivity, visual and fast results, parallel analysis of samples, etc. (Hao *et al*., 2015, Patel *et al.*, 2011, Reich and Schibli, 2007). However, TLC has certain limitations (such as a large number of variable parameters, an off-line and open system, etc.); and as expectations increase with the quality and value of an analysis, appropriate tools have been developed and are currently available for all stages of TLC. It is in this context that a more advanced form of TLC has been developed and is called "high performance thin layer chromatography" (HPTLC). Indeed, HPTLC uses chromatographic layers with maximum separation efficiency, as well as state-of-the-art instruments at all stages (of the separation process) such as application of precise amounts of samples, development of reproducible chromatograms and standardized, and software-controlled evaluation (Poole, 2013, Reich & Schibli, 2007). In addition, TLC has been revolutionized and transformed into HPTLC which is considered as "a modern analytical

Table 1. Selected hyphenated methods currently used in dereplication process

Analytical tools names	Specificity	References
Chiral liquid chromatography-circular dichroism and NMR (LC-CD-NMR)	The method is required in chiral compounds analysis (such as identifying enantiomers in crude mixtures of compounds)	Mammo & Endale, 2015
Liquid chromatography-ultraviolet/ visible spectroscopy-mass spectrometry & nuclear magnetic resonance (LC-UV-MS-NMR)	This technique has been considered as the most robust and popular hyphenated method employed for marine natural product dereplication	Pérez-Victoria *et al*., 2016
High-performance liquid chromatography-mass spectrometry (HPLC–MS)	The combination of HPLC with MS is widely used in chemical analysis for its high sensitivity and selectivity on the one hand, and because the individual capabilities of each technique are enhanced synergistically on the other hand	Ardrey, 2003
High-performance thin layer chromatography-mass spectrometry (HPTLC–MS)	The technique's merits include its low cost, simplicity, high sensitivity and rapid analysis, as well as molecular weight information (useful for structural characterization)	Hao *et al*., 2015 ; Gupta & Gupta, 2011
High-performance thin layer chromatography-mass spectrometry coupled with nuclear magnetic resonance (HPTLC-MS-NMR)	HPTLC-MS-NMR is considered as one of the fast and high-confident methods for dereplication of natural products	Adhami *et al*., 2012
High-performance thin layer chromatography-fourier transform infrared spectroscopy (HPTLC–FTIR)	The technique is a non-destructive method that makes it possible to analyze a sample without derivatization and discrimination between closely related compounds	Cimpoiu, 2011
Gas chromatography–mass spectrometry (GC-MS)	GC-MS is known for identifying and/or quantifying precisely numerous substances present in very small quantities, even in traces	Vazquez-Roig & Pico, 2012
Liquid chromatography-nuclear magnetic resonance (LC-NMR)	Useful for completing LC-MS data	Bobzin *et al*., 2000
Liquid chromatography coupled to ultraviolet/ visible spectroscopy and a photodiode array detector (LC-UV-DAD)	We use this method for the detection of compounds with characteristic chromophores	Pérez-Victoria *et al*., 2016
Liquid chromatography coupled to a photodiode array detector and a HRMS (LC-DAD-MS)	It has shown that LC-DAD-MS is a powerful approach for the rapid identification and quantification of natural products in plant extracts	He, 2000;
Liquid chromatography-fourier transform infrared spectroscopy (LC-FTIR)	LC-FTIR is used in both on-line (using the flow cell) and in off-line (using an interface) conditions. However, it has been shown that off-line approach exhibits an advantage because there is no interference from the mobile phase	Li *et al*., 2017
Liquid chromatography-nuclear magnetic resonance-mass spectrometry (LC-NMR-MS)	The technology can be used in both on-flow and static approaches, and takes advantage of the rapid and sensitive screening capabilities of MS. This permit to consider this hyphenated strategy as one of the most powerful analytical tools in natural products analysis.	Mammo & Endale, 2015
Ultrahigh high performance liquid chromatography coupled with time-of-flight mass spectrometry (UHPLC-TOFMS)	The technique gives detailed information on the chemotaxonomic relationship between species of the same genus, as well as a comparative analysis of the major constituents contained in these respective species	Mammo & Endale, 2015

technique with excellent potential for automation, optimization, hyphenation, and multidimensional applications" (Srivastava, 2011). Finally, the technique offers new features that no other method of chromatographic analysis has yet developed. These include the simultaneous analysis of samples and standards under the same experimental conditions, the use of different development methods (ascending, descending, two-dimensional, circular, etc.), as well as the use of UV, Fourier transform infra-red, or Raman spectroscopy that has increased the specificity and the power detection of HPTLC (Dhaneshwar, 2015). HPTLC can be used in many fields (Table 2) including phytochemistry (Galal *et al*., 2015, Ciesla and Waksmundzka-Hajnos, 2011, Ram *et al*., 2011, Waksmundzka-Hajnos *et al*., 2008) and analysis of medicinal plants (Chavan *et al*., 2011, Reich and Schibli, 2007, Waksmundzka-Hajnos et al., 2015), analysis of food and other food products (Kruger and Morlock, 2015, Vovk and Glavnik, 2015), industry (Galal et al., 2015; Coman and Copaciu, 2015), pharmaceutical field (Dhaneshwar, 2015), clinical or biomedical fields (Mohammad and Moheman, 2011), environment (Schwack, 2015), forensic chemistry and cosmetology (Attimarad *et al*., 2011), etc.

For these different applications to be effective, HPTLC is often combined with mass spectrometry (MS) to be used in dereplication; and the result is called HPTLC-MS. In this case, thin-layer chromatography allows the separation of different constituents of the sample to be analyzed (qualitative and quantitative analysis), while mass spectrometry will be used as a means of detection and analysis of separated substances (qualitative analysis). However, HPTLC-MS coupling can also replace densitometry to quantify substances already identified (Waksmundzka-Hajnos *et al*., 2008). Moreover, since the purpose of dereplication is the rapid characterization of known compounds, obtaining the maximum amount of information on the molecule (in order to be able to identify it) is crucial. Thus, thin layer chromatography-mass spectroscopy coupling has its place. For example, by applying standards on a chromatographic plate (TLC or HPTLC plate), information on the R_f value of the molecule to be identified will already be obtained. Indeed, depending on the type of developers chosen, it is possible to know the group of molecules that are facing (e.g, Dragendorf reagent for the detection of alkaloids, or 2-(diphenylboryloxy) -ethylamine and polyethylene glycol (PEG) for identification of polyphenols such as

flavonoids). Then, the mass spectrometry gives additional information concerning in particular the molecular mass and the fragmentation of the molecule. These fragmentation patterns can help to identify clusters present in the molecule and sometimes help to determine the molecule using existing databases (Mandal *et al.*, 2015).

Table 2. Possible applications fields of HPTLC

Applications fields	Possible analytical tasks	Examples of typical research works	References
Herbal medicines and botanical dietary supplements	Identification of natural materials (fingerprint to authenticate herbal phytochemicals), control of purity and stability, detection of adulterants, and quantification of marker compounds	Identity testing of herbal medicinal extracts by comparing conventional TLC and HPTLC	Müller *et al*., 2017 ; Reich & Schibli, 2007
Cosmetics	Identification of raw material, analysis of preservatives or colorants, and screening for illegal substances	Detection of UV filters in cosmetic products	Düsterloh & Do, 2016
Environ-mental applications	Analysis of pesticides as contaminants and residues; control of water, soil and air (*e.g.* identification of polycyclic aromatic hydrocarbons), and quatification of other environmental pollutants such as heavy metals	Determination of the quality of the drinking water	Weiss *et al*., 2014
Food safety	Food quality control (i.e analysis and quantification of food impurities such as contaminants, additives, pesticides) and stability testing to ensure product expiration date	Quality control for pigment formulations	Stiefel et al., 2016
Synthesis in organic and medicinal chemistry	Visual evaluation of chromatograms during synthesis/purification of a chemical compound (rapid assessment of the progress of synthesis/purification, or by-products identification)	Synthesis of triazole analogues of antillatoxin	Goto *et al.*, 2011
Industrial applications	In-process control, quantification of active principles, analysis of impurities in final products, and cleaning validation	Separation and quantification of monoacylglycerides (impurities) in biodiesel	Membrado *et al*., 2015
Pharmaceutical applications	Drug formulation analysis, quality control, identity and purity checks, determination of Content Uniformity, and stability testing	Quantitation of desvenlafaxine in pharmaceutical dosage forms	Pawar *et al*., 2012
Clinical applications	Separation and dosage of various types of analytes (lipids or drugs in serum, carbohydrates in glycosides, bile acids in feces, amines or porphyrins in urine, and vitamins in plasma), metabolism studies, drug testing, or doping control	Analysis of cannabinoids in urine samples of *Cannabis* abusers	Sharma *et al*., 2010
Forensics	Analysis of illicit drugs, detection of falsified documents, investigation of poisoning cases, and analysis of the quality of a finished product	Process monitoring and quality control during synthesis of ergoline psychedelics	Grill & Broszat, 2015
Pharmacolo-gical applications	Discovery of new drugs by isolation from natural material	Bioassy-isolation of plant antibiotics	Moric & Ott, 2014

1.3. *Platostoma rotundifolium* (Briq.) A. J. Paton and its active compounds

1.3.1. Botanical description of the species

P. rotundifolium is a species belonging to the plant kingdom (Plantae), phylum of vascular plants (Tracheophyta), subphylum of angiosperms (flowering plants), class of true dicotyledonous (Eudicotyledonous), subclass of *Lamiidae*, *Lamiales* order, *Lamiaceae* family, *Ocimeae* tribe, *Ociminae* subtribe, and *Platostoma* genus (APG IV, 2016; Paton *et al.*, 2004).

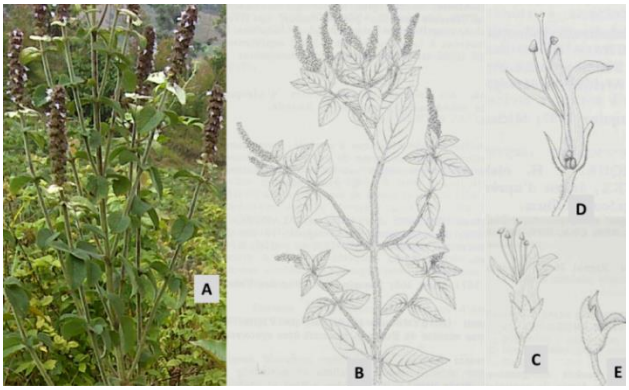

Figure 1. Morphology of *P. rotundifolium* (Briq.) A. J. Paton. : (A) - Photo taken in Burundi (Nyabiraba commune, Bujumbura Rural Province, 1730 m altitude, S 03. 45325 °, E 029. 47607 °); (B-E) – Drawings of Troupin (1985) corresponding to the top of flowering stem (B), flower (C), longitudinal section of the flower (D) and calyx (E).

It is a perennial to small bush that can reach 1.50 m high. Its leaves are little petiolate, sub-coriaceous, oblong, obtuse, sub-cordate at base, crenate, green and pubescent above, gray and more densely pubescent below. It has a sub-woody and densely pubescent-ferruginous stem. The plant has dense, cylindrical inflorescences with broad oval bracts, having the appearance of leaves, white, creamy white or pinkish white. It also has a tubular calyx with four teeth and a corbula slightly bilabiate and whose size is twice as long as that of the calyx (Troupin, 1985). Figure 1 details morphology of some aerial parts of the plant.

1.3.2. Synonyms of the species

In Burundi, this plant is known by the vernacular name "umusekerasuka" (in Kirundi). Its synonyms are:

- Basionym : *Geniosporum rotundifolium* Briq.
- Synomym homotypic: *Geniosporum rotundifolium* Briq. (1894)
- Synonyms heterotypic: *Plectranthus etiolatus* Chiov. (1937) ; *Ocimum konianense* A. Chev. (1911), *Ocimum paludosum* (Baker) Roberty, *Geniosporum paludosum* Baker (1900), *Geniosporum angolense* Briq. (1894), *Geniosporum affine* Gürke (1894), Geniosporum angolense Briq. (1894); *Geniosporum scabridum* Briq. (1894) (African Plant Database, http://www.ville-ge.ch/musinfo/bd/cjb/africa/)

1.3.3. Habitat and geographical distribution

The plant generally grows in open and frequented areas, on slope, in forest edges, in recruits (Troupin, 1985), or in high altitude wet meadows (Ngassapa *et al*., 2016). It is found mainly in the countries of intertropical Africa. In Africa, the plant species is known in Burundi, Cameroon, Congo, Ethiopia, Guinea, Kenya, Malawi, Rwanda, Tanzania, Ouganda and Zambia (Baerts and Lehmann, 1989; Global Biodiversity Information Facility, https://www.gbif.org/occurrence/search?q=Platostoma%20rotundifolium).

1.3.4. Medical-traditional uses, chemistry and biological properties

P. rotundifolium is widely used in African traditional medicine (Figure 2). Indeed, in Burundi, its aerial parts are administered (alone or in combination with leaves of other plants) for a number of medical uses including skin diseases, cough, diarrhea, varicella, otitis and yaws (Ngezahayo, 2015). In Uganda, the plant is used primarily against fungal and bacterial infections (Kamatenesi-Mugisha *et al*., 2008).

Although the plant is not yet highly studied, a number of bioactive products have already been isolated mainly from its aerial parts (Table 3), thus partly justifying its medico-traditional uses. These are mainly essential oils (such as α-terpineol, germacrene D, β-caryophyllene, β-gurjunene et spathulenol) that are active against microbes (Ngassapa *et al.*, 2016, Tchoumbougnang *et al*., 2013), diterpenes (e.g, cassipourol) having anti-virulence properties (Rasamiravaka & Ngezahayo *et al*., 2017), as well as triterpenes (including pentacyclic triterpene acids such as ursolic, corosolic and tormetic acids) which have shown antibacterial activities on susceptible and resistant strains (Ngezahayo *et al*., 2016a).

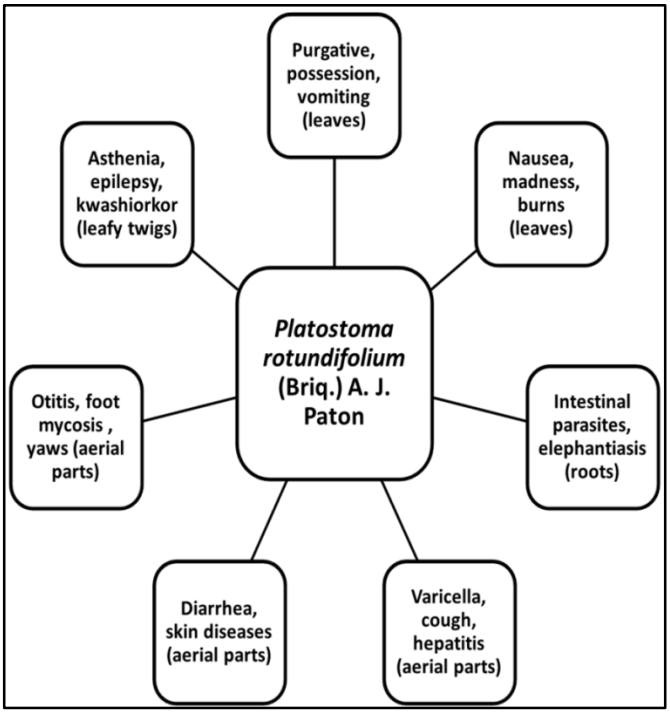

Figure 2. Uses of *P. rotundifolium* in African traditional medicine. Some data in this figure have been provided by Burundian traditional healers (Ngezahayo *et al*., 2015), while others have been adapted from Nieuwinger (2000). The parts plant used are indicated in parentheses for each disease.

2. Objectives of the study

Previous researches have shown that aerial parts of *P. rotundifolium* contained pentacyclic triterpene acids (PTAs) including ursolic, corosolic, tormetic, hyptadenic and jeremic acids (Ngezahayo *et al*., 2016a). The present work aims to make dereplication of these compounds in ethyl acetate extract of the plant. This amounts to identifying these compounds in the extract (without having to isolate them), and HPTLC-MS will be used. In fact, each of the five molecules already known in the plant will be used as a standard in order to compare their retardation factors (R_f) (on HPTLC plates) with those of the molecules present in the plant extract. In addition, terpenes-specific reagents will be used to spray and locate the molecules on HPTLC plates. Finally, mass spectrometry will confirm the presence of these different acids through the m/z ratios, which will also give information on the molecular weights of these compounds.

Table 3. Chemistry and biological properties of *P. rotundifolium*

Classes of compounds	Examples of compounds	Biological activities	References
Monoter-penes	α-terpineol	Moderate antibacterial activity against *Staphylococcus sp.* and other Gram-negative bacteria	Ngassapa *et al.*, 2016
Diterpenes	Cassipourol	Anti-quorum sensing and anti-biofilm activity against *P. aeruginosa* PAO1	Rasamiravaka & Ngezahayo *et al.*, 2017
Sesquiterpenes	Germacrene D, β-caryophyllene et β-gurjunene	Antifungal activity against *Fusarium moniliforme* and R*hizopus stolonifer*	Tchoumbougnang *et al.*, 2013
	Spathulenol, germacrene D	Moderate antibacterial activity against Gram-positive *Staphylococcus sp.* and other Gram-negative bacteria	Ngassapa *et al.*, 2016
Triterpenes	Ursolic acid	Antibacterial activity against Gram-positive *S. aureus* (sensitive and methicillin-resistant) and Gram-negative *E. coli*	Ngezahayo *et al.*, 2016a
	Corosolic acid	Antibacterial activity against Gram-positive *S. aureus* (sensitive and methicillin-resistant) and Gram-negative *E. coli*	Ngezahayo *et al.*, 2016a
	Jeremic acid	No antibacterial activity against Gram-positive *S. aureus* (sensitive and methicillin-resistant) and Gram-negative *E. coli*	Ngezahayo *et al.*, 2016a
	Tormentic acid	Antibacterial activity against Gram-positive *S. aureus* (sensitive and methicillin-resistant) and Gram-negative *E. coli*	Ngezahayo *et al.*, 2016a
	Hyptadienic acid	No antibacterial activity against Gram-positive *S. aureus* (sensitive and methicillin-resistant) and Gram-negative *E. coli*	Ngezahayo *et al.*, 2016a
	α-amyrin	Inhibition properties in biofilm formation by *P. aeruginosa* PAO1	Rasamiravaka & Ngezahayo *et al.*, 2017
	Squalene	No anti-virulence activity against *P. aeruginosa* PAO1	Ngezahayo *et al.*, 2016b
Steroids	β-sitosterol	Anti-quorum sensing and anti-biofilm activity against *P. aeruginosa* PAO1	Rasamiravaka & Ngezahayo *et al.*, 2017

3. Material and methods

3.1. Material and chemicals

The HPTLC Silica gel 60 F254 (MS-grade 20 x 10 cm) plates were obtained from Merck Millipore (Darmstadt, Germany). The TLC-MS interface, automatic TLC sampler 4 (ATS 4), developing chambers, UV lamps, and TLC plate heater were from CAMAG (Muttenz, Switzerland). Electrospray ionization (ESI) experiments were performed on an Advion Expression CMS mass spectrometer. Methanol (HPLC-grade, 99.9 %) and chloroform (analytical grade, 99.9 %) was purchased from Chem-Lab (Redu, Belgium). Ursolic acid (used as standard) was also analytical grade (≥ 98 %, HPLC) and purchased from Sigma Aldrich, while others PTAs used as standards (corosolic, hyptadienic, jeremic and tormentic acids) were isolated in our laboratory during our previous works.

3.2. Collection and identification of plant material

The plant material (aerial parts) was harvested from Nyabiraba area (1730 m, S03.45325◦, E029.47607◦) in Bujumbura Rural Province (Burundi). The botanical identification was realized by specialists of the herbaria of 'Université du Burundi', and the authentication by the researchers of the Herbarium of the National Botanical Garden of Meise (Belgium) where a voucher specimen has been deposited under the number BR0000013315900.

3.3. Plant extracts preparation

Extraction was described in our previous works (Ngezahayo *et al.*, 20017). Brifly, powdered aerial parts were percolated successively with solvents of increasing polarity (n-hexane, dichloromethane, ethyl acetate, methanol and water); and the extracts obtained were evaporated at 40° C under vacuum.

3.4. Bacterial strains

Four Gram-positive (MRSA ATCC 33591, MRSA C 98506, MRSA C 100459 and MSSA ATCC 6538) and one Gram-negative (*Escherichia coli* ATCC 25922) bacterial strains were used in this work. Both bacterial strains typed "C" were clinical isolates from the 'Centre Hospitalier Universitaire de Charleroi' (Belgium), while the "ATCC" ones were obtained from the American Type Culture Collection.

3.5. HPTLC-bioautography analysis

Previous research had shown that ursolic acid was present in ethyl acetate extract of *P. rotundifolium*, and that it had antibacterial activity on susceptible and resistant strains (Ngezahayo *et al.*, 2016a). To ensure the presence of this acid (as well as other antibacterial compounds) in this new extract, a bio-autographic test was carried out with Gram-positive *S.*

aureus (sensitive and methicillin-resistant) and Gram-negative *E. coli*. Thus, thin-layer chromatographies were first performed in duplicate (one plate for the test itself and the other one for comparison of spots under visible light at 254 nm or ultraviolet at 366 nm). After migration and evaporation of the solvent from the plate, a mixture of Mueller-Hinton broth/Mueller-Hinton agar media (9: 1) was prepared and kept at 50° C as recommended by Okusa *et al.* (2010). A bacterial suspension (0.5 Mc Farland) was then added to this culture medium (1 ml of suspension in 9 ml of medium) and the mixture was carefully (and aseptically) poured onto the plate. After solidification of the medium at room temperature, the plate was incubated for 18-24 hours at 37° C. After incubation, a sterile solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) (0.8 mg/ml) was sprayed onto the plate and this was again incubated at 37° C for 4-6 hours. After this operation, active substances were observed as clear bands (characteristic of bacterial growth inhibition) against a purple or dark background (Figure 3).

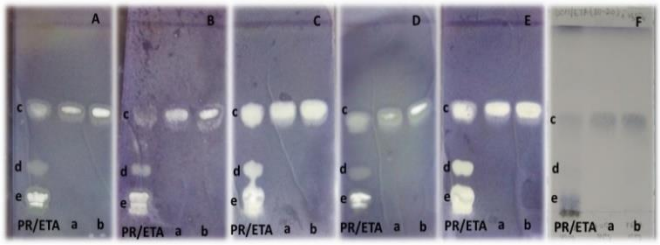

Figure 3. HPTLC-bioautography analysis of dereplicated pentacyclic triterpenic acids from ethyl acetate extract of *P. rotundifolium* (PR/ETA). Standards: commercial ursolic acid (a) and ursolic acid from *P. rotundifolium* (b). Active compounds in the plant extract: ursolic acid (c), corosolic acid (d), tormentic acid (e). Mobile phase: dichloromethane-ethyl acetate (80:20, v/v). Bacterial strains: MSSA ATCC 6538 (A), *E. coli* ATCC 25922 (B), MRSA ATCC 33591 (C), MRSA C98506 (D) and MRSA C100459 (E). Spots visualization: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) (A-E) and vanillin-sulfuric acid reagent (F). Both plates were observed under visible light, and white spots represent inhibition zones of bacterial growth. Standards (10 µg) and plant extract (40 µg) were deposited on each HPTLC plate and at the same relative position.

3.6. HPTLC-MS

Samples (dry ethyl acetate extract and pure compound powders) were solubilized in methanol (1 mg/ml). The plates were prepared (in triplicate) by applying 20 µl of the extract solution and 10 µl of each of the PTAs solutions (standards). Deposits were realized by an automatic TLC sampler 4 (ATS 4) in bands 6 to 12 mm from the left edge and 10 mm from the bottom edge. Then, the plates were developed in a saturated tank, containing a mixture of dichloromethane-methanol (96: 4, v/v) solvents and the development was done up to 8 cm height. After solvents migration, the plates were dried in an open air. The first plate was first analyzed under visible light, UV 254 nm and 366 nm (to visualize all the products available on the plate), then sprayed with a vanillin-sulfuric acid solution. Finally, the plate was placed on a TLC plate heater at 100° C. The other plates were also revealed by vanillin-sulfuric acid, but only the part corresponding to the deposit of the extract. This permitted to visualize all molecules contained in the plant extract, and to locate their positions on the non-pulverized plates. In addition, to be analyzed by the mass spectrometer, the molecules must not be derived. A glass plate was thus placed in front of the part corresponding to the deposits of the products to be analyzed. The plate was then gridded using a pencil to determine the probable position of the spots.

Since the first plate was entirely sprayed with vanillin-sulfuric acid, only the other two plates were analyzed. To do this, the gridded plates were passed through a CAMAG TLC-MS interface. For the sequence of analysis, two blanks solutions (methanol) were first eluted, then a purified molecule, followed by the supposed spot of this molecule in the extract. Before moving on to the next molecule, a blank solution was eluted again. The following operating parameters were used: flow rate of 0.2 ml/min, a contact time of 60 seconds and a voltage applied at the output of the capillary of -2500 V. Finally, the purified molecules were injected directly into the system. Two blanks (methanol) were first injected and then alternating between molecule and blank to clean the system. The mass spectra of all compounds and extracts were acquired in the m/z range 300-600, and the molecules were ionized with the electron spray ionization (ESI) source in negative mode.

4. Results and discussion

4.1. HPTLC and chemical detection of pentacyclic triterpenoids acids

With some exceptions (such as glycyrrhizic acid and its derivatives), most of triterpenoids (including PTAs) are not seen on the (HP)TLC plate either in natural light or under UV exposure without any chemical treatment (Oleszek *et al.*, 2008). This would be due to lack of chromophore unit in their chemical structures. During this work, PTAs were detected on the plate under visible light after derivatization with vanillin-sulfuric reagent (Figure 4A), as proposed by Wagner and Bladt (1996). A second plate prepared under the same conditions as the first (but without chemical treatment) was gridded with a pencil lead to determine the probable position of the spots (Figure 4B). This grid system is absolutely mandatory in this case, since the compounds (PTAs) were invisible on plate not treated with vanillin-sulfuric reagent. For this, a comparison of PTAs R_f values (Figure 4, Table 4) on the first plate was carried out with respect to those of the second plate; then another verification was carried out by spraying the second plate with the same reagent (10% vanillin-sulfuric solution in ethanol) as that used for the

reference plate (first plate). This operation was done after sampling and permitted to be reassured that one has well stung all the spots corresponding to the compounds of interest.

Table 4. Retardation factor (R_f) values and negative-mode ESI-MS characterization of HPTLC spots of compounds 1–5 dereplicated from ethyl acetate extract of *P. rotundifolium*. R_f and m/z values of quasi-molecular ion pics are similar for standards and their homologues in the extract.

Dereplicated compounds	R_f values of standards (¤)	Quasi-molecular ion pics of standards (m/z = $[M-H]^-$)	R_f values of from mixture (¤)	Quasi-molecular ion pics of PTAs from the extract (m/z = $[M-H]^-$)
UA (**1**)	0.40	455.4	0.40	455.4
HA (**2**)	0.33	469.5	0.33	469.5
JA (**3**)	0.16	487.4	0.16	487.5
CA (**4**)	0.10	471.4	0.10	471.4
TA (**5**)	0.06	487.4	0.06	487.4

UA: Ursolic acid; HA: Hyptadienic acid; JA: Jeremic acid; CA: Corosolic acid; TA: Tormentic acid. ¤ Mobile phase: chloroform-methanol (96: 4, v/v).

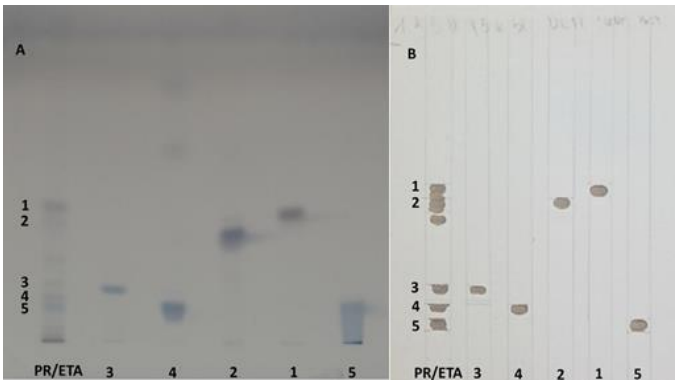

Figure 4. HPTLC analysis of dereplicated pentacyclic triterpenic acids from ethyl acetate extract of *P. rotundifolium* (PR/ETA). Mobile phase: dichloromethane-methanol (96:4, v/v); compounds: ursolic acid (1); hyptadienic acid (2); jeremic acid (3); corosolic acid (4) and tormentic acid (5). The plates were observed under visible light after derivatization with vanillin-sulfuric reagent (A) and after elution of target zones with the TLC-MS interface.

4.2. HPTLC and effect-directed analysis (bio-autography)

Bio-autography test (technique for detecting antimicrobial compounds on a TLC plate) can be realized after migration of an extract/compound solution on the TLC plate (TLC-bio-autography), or directly without prior migration of the extract/compound solution (direct bio-autography). In both cases, it is necessary that the culture medium (to be flowed on the plate) is sufficiently fluid to facilitate the preparation of bacterial suspensions and, consequently, the adhesion of this medium to the plate. In the present study, TLC-bio-autography was performed after migration of the extract and pure compounds (standards) solutions on the TLC plate (Figure 3A-E). A comparison of R_f values of active compounds (white spot on a colored background) on the plate (for TLC-bio-autography) with a second reference plate (realized under the same conditions as the first but observed under visible light at 254 nm or ultraviolet at 366 nm) revealed at least three antibacterial compounds in the plant extract. These compounds were active both on Gram-negative *E. coli* and Gram-positive *Staphylococcus* species (Figure 3 A-E). In addition, one of these compounds was easily identified as ursolic acid by the standard. Finally, using their R_f values on TLC-chromatograms (Figure 4), the other two compounds were identified as corosolic and tormentic acids (Figure 3, see bands "**d**" and "**e**" on the TLC plate A). Moreover, combination of bio-autographic (Figure 3) and MS (Figure 5) data confirmed the identity of these active compounds on TLC plate. Thus, with all this information above, the TLC-MS/bio-autography allows in some cases not to waste time isolating the molecules already known; and in addition, the knowledge of the structural class of compounds is sometimes sufficient information that does not require going further in pure products isolation.

4.3. HPTLC and HPTLC-MS analysis of dereplicated pentacyclic triterpenic acids

As indicated previously, identification of each of the five PTAs was performed by comparing their R_f values with those of standards as indicated on chromatograms (Figure 4). In this figure, we found that ethyl acetate extract from aerial parts of *P. rotundifolium* contained several compounds including the five PTAs studied. Indeed, R_f values of five compounds contained in the plant extract corresponded exactly to those of the five standards used. These R_f values were (in decreasing order) 0.40; 0.33; 0.16; 0.10 and 0.06 corresponding to ursolic, hyptadienic, jeremic, corosolic and tormentic acids respectively (Figure 4, Table 4). Next, we confirmed the above by comparing mass spectra of standards with those of the compounds in the plant extract. Thus, the HPTLC–MS full scan mass spectra (of standards and corresponding compounds from the extract) showed the same quasi-molecular ion pics $[M-H]^-$ at m/z 455.4, 469.5, 487.4/487.5, 471.4 and 487.4 corresponding to the loss of one proton of ursolic, hyptadienic, jeremic, corosolic and tormentic acids respectively (Table 4, Figure 5). These results permitted to conclude that molecular masses of five compounds contained in the plant extract were identical to those of pure molecules (standards) positioned at the same R_f values. Since R_f values were the same and ESI-MS spectra identical, it was possible to say that the molecules having these two identical properties were the same. All five PTAs

were therefore identified without being isolated from the plant extract thanks to HPTLC-MS dereplication strategy.

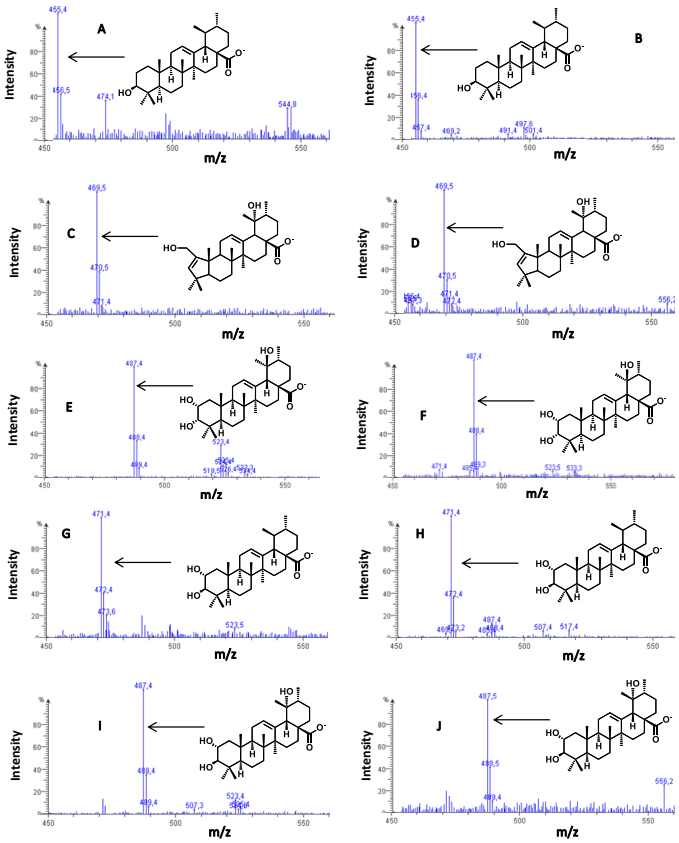

Figure 5. HPTLC-MS mass spectra of pentacyclic triterpenic acids. Five couples of ESI-MS spectra of PTAs are represented: spectra of standards compounds (A, C, E, G, I) are located on the left (of each couple of spectra), while the spectra of compounds (to be identified) in the plant extract and positioned at the same R_f values as standards, are found on the right. HPTLC–MS mass spectra (of standards and corresponding compounds from the extract) showed the same quasi-molecular ion pics $[M-H]^-$ at m/z 455.4, 469.5, 487.4/487.5, 471.4 and 487.4 corresponding to the loss of one proton of ursolic (A-B), hyptadienic (C-D), jeremic (E-F), corosolic (G-H) and tormentic (I-J) acids respectively.

5. Conclusions

The aim of this study was to dereplicate five previously known pentacyclic triterpenoids acids from aerial parts extract of *P. rotundifolium* without isolating them, and using high performance thin layer chromatography coupled to mass spectrometry (HPTLC-MS). Thus, with an HPTLC-MS analysis of spots of the products present on HPTLC plate, and with the use of standards, we were able to conclude very easily the presence of five PTAs used as standards in the plant extract. Added to this is the simplicity and speed of the method compared to the traditional method for isolating and determining the structures of natural products such as PTAs. This coupling is also suitable in effect-directed analysis (bio-autography). Indeed, by combining bio-autography and MS, one has a quick suspicion of active compounds (antibacterial activity in particular), and this avoids wasting time in non-bioactive compounds isolation. In view of the interesting results and despite the fact that this technique is not widely used in the triterpenoids field (Vovk and Albreht, 2015), we can confirm that HPTLC-MS is a powerful method in the analysis of natural products such as PTAs contained in the aerial parts of *P. rotundifolium* (Briq.) A. J. Paton.

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