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Identification and Isolation of Constituents Contained in Venoms and Plant Extracts

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Abstract

The present paper is part of publication of the proceedings of the international symposium "Tropical Islands and Biodiversity". The aim is to place emphasis on natural products from plant and animal biodiversity. It also emphazises methods and techniques widely commonly used to identify and isolate components from natural complex mixtures, through two studies. First study is about natural products of animal origin. It presents steps leading to isolation of two high-valued native toxins: Fasciculin-II and Cardiotoxin isolated from *Dendrosaspis angusticeps* and *Naja pallida* venoms, respectively. Second study describes chemical composition of wild growing corsican understudied plant species, with potential biological activities. Phytochemical constituents from *Ilex aquifolium* Linne leaves and berries extracts have been first determined. Among identified compounds, ursolic and oleanolic acids displaying therapeutic interests in various domains have also been quantified by ^{1}H-NMR in a crude leaves extract using a reliable method developed and validated. Then, phytochemical constituents from *Calicotome villosa* (Poiret) Link flowers and roots extracts have been determined. Finally, plant extracts and identified secondary metabolites were evaluated for their antimicrobial activities.

Keywords

Ilex aquifolium Linne, *Calicotome villosa* (Poiret) Link, ^{13}C-NMR Identification, ^{1}H-NMR Quantitation, *Dendroaspis angusticeps*, Fasciculin II, *Naja pallida*, Cardiotoxin

1. Introduction

Natural substances such as venoms or plant extracts, are complex mixtures with several components from various chemical classes [1]-[4]. Natural products from

these complex mixtures have to be identified, quantified and sometimes isolated in order to display their biological properties or enhance their economic value [4]-[6]. Furthermore, these analyses or purification steps have to be optimized procedures for the purposes of facing up challenges such as using fewer organic solvents, reducing analysis or purification times, improving yields, and for a routine use or for adapting to more advanced techniques [7]-[9].

The present paper, focuses on natural products contained in animal venoms and plant extracts through two studies. First study, describes steps leading to isolation of two native peptides, well known for their biological properties. It presents how Fasciculin II, a potent anticholinesterase toxin has been isolated from *Dendrosaspis angusticeps* venom and Cardiotoxin, a potent cytolytic polypeptide that has been isolated from *Naja pallida* venom.

The second is a chemical composition study of corsican understudied plant species extracts. Secondary metabolites contained in *Ilex aquifolium* leaves and berries extracts and also in *Calicotome villosa* flowers and roots extracts have been determined. Then, antibacterial activities of crude plant extracts and chromatography fractions have also been displayed. Both studies were achieved with techniques commonly used to identify and isolate components from natural complex mixtures.

2. Materials and Methods

2.1. Venoms Preparation

Dendroaspis angusticeps and *Naja pallida* lyophilized powder venoms have been obtained from Latoxan Laboratory. Samples were prepared by dissolving *D. angusticeps* venom in an ammonium acetate buffer and *N. pallida* venom in distilled water.

2.2. Plant Material and Solvent Extractions

Leaves and berries from *I. aquifolium* have been harvested in Northern Corsica. Leaves have been crushed and successively extracted with hexane and dichloromethane (Soxhlet apparatus). Berries have also been crushed and successively extracted with hexane, dichloromethane and finally with a dichloromethane/ethyl acetate (50/50, v/v) mixture.

C. villosa flowers have been harvested in Southern Corsica, crushed and successively extracted with hexane, dichloromethane and ethyl acetate. *C. villosa* roots were collected in the same place then extracted with methanol.

2.3. Liquid Chromatography

D. angusticeps whole venom was submitted to a FPLC (Fast Protein Liquid Chromatography) molecular exclusion chromatographic column (AKTA Start Protein Purification System Lab, GE Healthcare). Only fractions containing low molecular weight proteins (≤10,000 Da), have been pooled and fractionated further by ion-exchange chromatography on cation-exchanger resin [10].

Fas-II and CTX purification steps were performed in HPLC system (1260 Infinity LC System, Agilent Technologies) equipped with a reverse-phase preparative column.

Plant extracts were submitted to successive column chromatography over silica gel according to [11].

2.4. Mass Spectrometry

LC/MS experiments were carried out using model 2010 (Shimadzu) apparatus. MALDI-TOF mass spectrometry analyses were performed for identifying the molecular mass of intact main proteins from cation-exchange chromatography, using an AXIMA Performance MALDI-TOF/TOF mass spectrometer (Shimadzu). MS/MS analyses were obtained with Bruker Daltonics Maxis Plus.

2.5. Nuclear Magnetic Resonance Spectroscopy

All plant extracts and chromatography fractions from this extracts have been dissolved in suitable solvents. After, all 1D and 2D NMR spectra were recorded on a Bruker AVANCE 400 spectrometer. The study was realized using the computerized NMR method developed over the past thirty years by the University of Corsica "Chimie et Biomasse" group, UMR CNRS "Sciences Pour l'environnement". This method allows individual components identification in natural mixture (essential oil, vegetable oil, bio-oil, and solvent extract) starting from its ^{13}C-NMR spectrum by comparing chemical shift values in the mixture spectrum with those of reference spectra compiled in two libraries devoted to each phytochemical classe [12]. The first spectral data library was constructed with spectra recorded in the laboratory while the second one was built with spectral data picked up in recent literature. Each compound was identified by taking into account three parameters that were directly available from the computer program: 1) the number of observed signals with respect to what was expected, 2) the difference between the chemical shift of each signal in the mixture and in the reference ($\Delta\delta$), and 3) the number of overlapping signals of carbons belonging to two components that fortuitously possess the same chemical shifts [12] [13]. If necessary, the occurrence of "false positive" could be avoided by taking into account the relative intensities of the non-overlapped signals of protonated carbons of a given component. A component is considered identified if 50% of its signals are observed and they belong to that compound.

2.6. Antibacterial Activity Assays

Antibacterial activities have been displayed against six bacterial strains obtained from the Pasteur Institute collection: three Gram-negative bacteria (*Escherichia coli* CIP 54.8 T, *Enterobacter aerogenes* CIP 60.86 T and *Pseudomonas aeruginosa* CIP 103467), three Gram-positive bacteria (*Staphylococcus aureus* CIP 53.156, *Staphylococcus epidermidis* CIP 53.124 and *Bacillus cereus* CIP 78.3). Chloramphenicol have been selected as reference antibiotic. Antibacterial activities have

been determined by measuring the Minimum Inhibitory Concentrations (MICs) values using microdilution method in liquid medium. Bacteria in the liquid medium Müeller-Hinton Broth (MHB) was used as the positive control of bacterial growth.

3. Results and Discussion

3.1. Toxins Isolation and Characterisation

Animals venoms provide peptides with many potential biological activities [2]. However, large quantities of these molecules are difficult to obtain, since they often are a minor venom component [14] [15]. This first study, presents purification and analytical steps leading to Fasiculin II (Fas-II) and Cardiotoxin (CTX) purification from *Dendroaspis angusticeps* and *Naja pallida* venoms, respectively.

3.1.1. Fasciculin II Isolation

D. angusticeps snake has a lethal venom. The main envenomation symptoms reported are respiratory paralysis, longlasting cardiovascular abnormalities and myocardial necrosis. Fas-II is one of the most potent anticholinesterase polypeptide (61 aa residues) in *D. angusticeps* venom, making poisoning by green mamba bites extremely severe [16]-[18].

As part of this study, native Fas-II peptide was purified from *D. angusticeps* snake venom. First, raw venom was fractionated by gel filtration then, fractions containing molecular weight proteins lower than 10.000 Dalton (Da) were fractionated further by ion-exchange chromatography (cation-exchanger). MALDI-TOF (Matrix-Assisted Laser-Desorption/Ionization Time-Of-Flight) mass spectrometry analyses were performed on each fractions obtained by ion-exchange chromatography, in order to highlight which one contained Fas-II: $[M + H]^+$ experimental mass = 6745.078 ± 1.5 Da *vs.* theoretical mass = 6745.412 Da (calculated from Fasc-II aa residues sequency given in literature [19] [20]). Fractions with Fas-II was then purified using reverse-phase preparative HPLC (High Performance Liquid Chromatography). After sequencing by LC-MS (Liquid Chromatography-Mass Spectrometry), molecular mass was confirmed and the fraction predominant component was identified as Fas-II [10].

3.1.2. Cardiotoxin Isolation

In the balance of this study, CTX (60 aa polypeptide) was also purified from *N. pallida* snake venom. CTXs are widely described in literature for their ability to induce systolic cardiac arrest in rodent models, to cause necroses and for their potent cytolytic activity [21] [22]. This membrane-disruptive effect probably gives *N. pallida* venom it antibacterial activity [23].

N. pallida raw venom LC-MS analysis allowed to determine the peak of interest. Indeed, m/z (mass-to-charge ratio) values confirmed CTX molecular mass: experimental mass = 6827 Da *vs.* molecular mass from literature = 6827.41 Da [24]. Then, CTX was purified using preparative HPLC.

3.2. Phytochemical Study of Corsican Plant Extracts

This second study describes chemical composition of wild growing corsican understudied plant species mainly using ^{13}C-NMR (Carbon-13 Nuclear Magnetic Resonance). It was realized using the computerized NMR method developed over the past thirty years by the University of Corsica "Chimie et Biomasse" group, UMR CNRS "Sciences Pour l'environnement". In this study, antibacterial activities of crude plant extracts and chromatography fractions have also been determined.

3.2.1. Phytochemical Constituents in Corsican *Ilex aquifolium* Linne Leaves and Berries Extracts

Ilex aquifolium Linne (common Holly; Aquifoliaceae), is a shrub or a small tree with persistent foliage and glowing dark green leaves. Flowers that are small and white or pink and odoriferous appear between April and July. Fruits are red berries that grow during the August-December period and well known for their toxicity [25]. *I. aquifolium* is a decorative tree for Christmas festivities. Leaves have been used in folk medicine for several purposes, such as in intermittent fevers and rheumatisms, for their antipyretic properties, and as astringent, diuretic, and expectorant agents [26]. Various parts of this tree are included in traditional medicinal preparations to treat liver, stomach and intestinal cancers, dropsy, gout, jaundice, malaria, warts, swelling and tumours [27]. This study, describes components contained in the leaves and berries of *I. aquifolium*. Some of this components are known to show biological activities such as anti-inflammatory, antioxidative and antibacterial properties [28]-[31].

In the first place, *I. aquifolium* leaves have been extracted with hexane and crude extract was then submitted to successive fractionations by column chromatography. After ^{13}C-NMR analysis of hexane extract and all chromatography fractions, ten triterpens were identified with spectral data recorded in laboratory or with spectral data picked up in literature (α-amyrin, β-amyrin, β-sitosterol, pseudotaraxasterol, uvaol, erythrodiol, betulin, lupeol, ursolaldehyde, oleanaldehyde). Using the same procedure, *I. aquifolium* leaves previously extracted by hexane have been extracted a second time by dichloromethane. Dichloromethane leaves crude extracts and all chromatography fractions were analyzed by ^{13}C-NMR to allow the identification of two additional triterpens, oleanolic acid and ursolic acid [11].

Ursolic acid is well known to possess a wide range of biological functions, such as antimicrobial, anti-inflammatory and anti-wrinkle activities [32] [33]. Oleanolic acid antioxidative and antifungal activities has been previously reported [34] [35]. So, with the aim to achieve the quantitation of both acids displaying therapeutic interests useful in various domains in dichloromethane leaves crude extract, a reliable method was developed and validated (accuracy, precision linearity of measurements, limit of detection and limit of quantitation), using ^{1}H-NMR (proton Nuclear Magnetic Resonance) [36]. Ursolic and oleanolic acids accounted for 55.3% and 20.8% of the dichloromethane extract, respectively.

In the second place, we determined chemical compositions of *I. aquifolium* berries extracts. Using the same procedure, berries have been extracted with hexane, followed by dichloromethane then, ^{13}C-NMR (2D "bidimensional" NMR sometimes analysis of dichloromethane crude extract and all chromatography fractions allowed identification of nine triterpens previously identified in *I. aquifolium* leaves extracts (*α*-amyrin, *β*-amyrin, oleanolic acid, ursolic acid, lupeol, uvaol, ursolaldehyde, oleanaldehyde, pseudotaraxasterol). We also identified two phenolic derivatives (p-hydroxybenzaldehyde, (p-hydroxyphenyl)acetonitrile) and two lactones (menisdaurilide, aquilegiolide), using spectral data recorded in laboratory or with spectral data picked up in recent literature.

After, berries previously extracted by dichloromethane have been extracted a third time with a dichloromethane/ethyl acetate (50/50, v/v) mixture. Crude extract and chromatography fractions ^{13}C-NMR spectra revealed presence of four triterpens (*α*-amyrin, *β*-amyrin, oleanolic acid, ursolic acid), three additional phenolic derivatives (vanillic acid, p-hydroxybenzoic acid, 2-hydroxyphenylacetic acid), six monosaccharides (*α* and *β*-D-glucopyranose, *α* and *β*-D-fructopyranose, *α* and *β*-D-fructofuranose) and four lactones. Among these lactones, two were identified in previous berries extract (menisdaurilide, aquilegiolide, dasycarponilide and 7-epi-griffonilide) [11]. These four latest compounds are for the first time described in *I. aquifolium* extracts.

3.2.2. Phytochemical Constituents in Corsican *Calicotome villosa* (Poiret) Link Flowers and Roots Extracts

Calicotome villosa (Poiret) Link (Fabaceae) is a shrub that can reach 2 m in high, with grey-tomentose stems and sharp terminations, villous pods, trifoliate and oval leaves, and yellow and grouped flowers during the spring season [37]. It is very common in the Mediterranean area and particularly in Corsica Island, where it grows near the sea while, the subspecies *C. villosa* subsp. *intermedia* is distributed especially in the North of Africa and Spain [38]. *C. villosa* was used for the treatment of foruncle, cutaneous abscess and chilblain [39]. In Palestine, infusion prepared from flowers is used for the treatment of cardiovascular system and nervous system problems and blood dilution [40]. Roots of this plant are used as corn plasters [41]. Many studies describe aerial parts extracts chemical composition of *C. villosa* [42]-[44], known as well for biological properties including antibacterial, antipyretic or anti-rheumatism activities [45]-[47]. In contrast, few studies describe roots extracts chemical composition and biological properties [41].

In this study, *C. villosa* flowers were first extracted with hexane then by dichloromethane. Dichloromethane extract was partitioned by successive column chromatography. After, crude extract and chromatography fractions ^{13}C-NMR analysis, a flavone (chrysin) and two glycosylated chrysin derivatives (chrysine 7-((4''-*O*-acetyl)-*O*-*β*-D-glucopyranoside); chrysine 7-((6''-*O*-acetyl)-*O*-*β*-D-glucopyranoside)) [11].

Secondly, flowers previously extracted by dichloromethane have been extracted

a third time by ethyl acetate. Crude extracts and chromatography fractions ^{13}C-NMR (2D NMR sometimes) spectra revealed presence of five monosaccharides (*α* and *β*-D-glucopyranose, *β*-D-fructopyranose, *α* and *β*-D-fructofuranose), four phenolic derivatives (caffeic acid; trans-p-coumaric acid; cis-p-coumaric acid; cis-2,4,5-trihydroxycinnamic acid), six flavonoids (apigenine; luteoline; chrysine 7-*O*-*β*-D-glucopyranoside; chrysine 7-((4''-*O*-acetyl)-*O*-*β*-D-glucopyranoside); chrysine 7-((6''-*O*-malonyl)-*O*-*β*-D-glucopyranoside); chrysine 7-((4''-*O*-acetyl)-(6''-*O*-malonyl)-*O*-*β*-D-glucopyranoside)) and a polyol (pinitol). Cis-configuration phenolic derivatives have spare occurrence as natural products and are for the first time described in *C. villosa* extracts.

The following section of this study describe chemical composition of a methanol roots extract of *C. villosa*, which has never been submitted to any chemical composition study. Repetitive column chromatography was carried out on this extract and some of its fractions. Crude extract and all chromatography fractions have been submitted to ^{13}C-NMR (2D NMR sometimes) analysis and many compounds have been identified using spectral data recorded in laboratory or with spectral data picked up in recent literature, including two sterols (*β*-sitosterol; stigmasterol); a stilbene (trans-resveratrol); seven flavonoids (calycosin; daidzein; genistein; liquiritigenin; isoprunetin; bidwillon B; formononetin) and five pterocarpans (maackiain, homoedudiol, isoneorautenol, 11b-hydroxy-11b,1-dihydromaackiain; 3,4-dihydroxy-8,9-methylenedioxypterocarpan). Among identified compounds, two new pterocarpans (4,9-dihydroxy-3-methoxy-2 dimethylallylpterocarpan and 4,9-dihydroxy-3',3'-dimethyl-2,3-pyranopterocarpan) and a new dihydrobenzofuran derivative (2-(1-methylethenyl)-5-hydroxy-6-carbomethoxy-2,3-dihydro-benzofuran) were also identified using a combination of 1D (unidimensional), 2D NMR and MS/MS (tandem mass spectrometry) [48].

3.2.3. Antibacterial Activities

In order to determine some previously identified compounds antibacterial activities, microbiologic assays were performed, measuring the MICs (Minimum Inhibitory Concentrations) of extracts and chromatography fractions against three Gram-negative bacteria (*Escherichia coli*, *Enterobacter aerogenes* and *Pseudomonas aeruginosa*), three Gram-positive bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis* and *Bacillus cereus*) and using chloramphenicol as reference antibiotic.

In the first instance, *I. aquifolium* leaves extracts and chromatography fractions antibacterial activities have been determined. Only Gram-positive bacteria were sensitive to extracts and chromatography fractions with high content in ursolic acid and oleanolic acid. Both triterpen acids and chloramphenicol displayed similar antibacterial activities (MIC = 4 and 8 mg·L^{-1} *vs.* 2 and 4 mg·L^{-1}). Following this study, a structure-activity relationship has been displayed. Comparing, ursolic acid, oleanolic acid and three structurally related compounds (ursolaldehyde, *α*-amyrin, uvaol) MICs, this study show that ursolic acid and oleanolic acid antibacterial activities are attributable to the carboxylic acid group (C28 carbon). Ursolic

acid having similar MIC with oleanolic acid indicate that methyl groups (C29 and C30 carbons) are not influencing ursolic acid and oleanolic acid antibacterial activities [11].

In the second instance, we determined antibacterial activities of *C. villosa* flowers and roots extracts and also antibacterial activities of chromatography fractions from this extracts. Only methanol roots extract displayed antibacterial activity with MIC lower than 32 $mg{\cdot}L^{-1}$. So, different chromatography fractions and subfractions from this extract were submitted to assays. Bidwillon B displayed the highest antibacterial activity with MICs similar with those of chloramphenicol (MIC = 4 $mg{\cdot}L^{-1}$ *vs.* 2 et 4 $mg{\cdot}L^{-1}$), followed by homoedudiol (MIC = 8, 16 $mg{\cdot}L^{-1}$) and daidzein (MIC = 16 $mg{\cdot}L^{-1}$). New pterocarpans and chloramphenicol also displayed similar antibacterial activities against *S. aureus* and *B. cereus* (MIC = 8 and 16 $mg{\cdot}L^{-1}$ *vs.* 2 and 4 $mg{\cdot}L^{-1}$). Further investigation into specific targets and mode of action of the identified antibacterial compounds will come soon.

4. Conclusions

Natural complex mixtures such as animal venoms or plant extracts require an optimized analysis step in order to identify, quantify, or isolate natural products with potential biological properties. This paper presents two studies. First study highlights purification and analytical steps leading to the isolation of two toxins with strong added value. Native Fasciculin II, a potent anticholinesterase polypeptide (61 aa residues), has been isolated from *Dendroaspis angusticeps* venom using successively gel filtration, cation-exchange chromatography, MALDI-TOF mass spectrometry, preparative HPLC and LC-MS analysis. Native Cardiotoxin has also been isolated from *Naja pallida* venom using preparative HPLC and LC-MS analysis.

Second study describes phytochemical components in Corsican plant extracts. The chemical compositions of extracts from *Ilex aquifolium* leaves and berries have been determined, mainly using ^{13}C-NMR. Several compounds from various phytochemical classes have been identified including triterpens, phenolic derivatives, monosaccharides and lactones described for the first time in *I. aquifolium* extracts. Among identified triterpens, ursolic acid and oleanolic acid, two biologically active compounds, have been quantified in dichloromethane leaves extract with a developed and validated ^{1}H-NMR method. Both triterpen acids accounted for 55.3% and 20.8% of the extract, respectively. Phytochemical constituents in flowers and roots extracts of *Calicotome villosa* have also been determined. Compounds from sterols, stilbenes, flavonoids, monosaccharides, phenolic derivatives, polyols and pterocarpans phytochemical classes, have been identified. Among these compounds, two new pterocarpans and a new dihydrobenzofuran derivative were also identified, using a combination of 1D, 2D NMR and MS/MS.

Identified secondary metabolites were then evaluated for their antimicrobial properties. Ursolic acid, oleanolic acid, bidwillon B, homoedudiol, daidzein and new pterocarpans displayed the highest antibacterial activities with MICs similar

with those of chloramphenicol.

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Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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