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Apport de la Chromatographie de Partage Centrifuge à l'étude phytochimique de 3 plantes utilisées en médecine traditionnelle soudanaise.

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DEDICACES

Au nom de Dieu, le clément; le Miséricordieux.
Nous dédions ce travail à:

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Résumé

Ce travail de thèse est une contribution à l'étude phytochimique de trois plantes utilisées en médecine traditionnelle au Soudan : *Aristolochia bracteolata* Lam. (plante entière), *Ziziphus spina-christi* (L.) Desf (feuilles) et *Hydnora abyssinica* A. Br. (rhizomes). La spécificité de ce programme de recherche est d'avoir mis l'accent sur l'utilisation de la Chromatographie de Partage Centrifuge (CPC) pour le fractionnement de ces trois plantes. Ceci a permis de mettre au point trois méthodologies différentes de purification par CPC, applicables au fractionnement des acides aristolochiques, des flavonoïdes ou de proanthocyanidols (PAC).

Dans ce contexte, la première partie de ce manuscrit est consacrée aux notions générales portant sur la CPC. Les différents modes de développement sont ainsi rappelés.

La deuxième partie porte sur l'étude d'*Aristolochia bracteolata*, utilisée traditionnellement au Soudan pour ses propriétés analgésiques et antipyrétiques notamment. La présence d'acides aristolochiques dans cette plante lui confère pourtant une toxicité notoire principalement au niveau rénal. Du fait de leur toxicité particulière, les acides aristolochiques sont devenus des outils pharmacologiques majeurs pour le développement de modèles d'atteinte rénale, pourtant peu sont commercialisés sous forme pure. Ce travail a permis de mettre au point une méthode innovante pour l'isolement et la purification de ces acides aristolochiques, par CPC en mode d'échange d'ions forts (SIX-CPC). Les acides aristolochiques I, II et IIIa ont ainsi pu être isolés d'un extrait brut d'*Aristolochia bracteolata* en une étape avec un très haut niveau de pureté et en quantités importantes. L'acide aristolochique IIIa n'avait jamais été décrit dans cette plante auparavant. Ces résultats ont fait l'objet d'une publication en 2015 dans *Separation and Purification Technology*.

Dans la troisième partie de cette thèse, la CPC a été appliquée à l'isolement de flavonosides présents dans *Z. spina-christi*. Cette plante est utilisée par les tradipraticiens pour la cicatrisation des blessures et soigner les cas de diarrhées et de malaria, ainsi que pour ses propriétés anti-spasmodiques. L'objectif dans cette partie a été de proposer une stratégie de fractionnement standardisée des flavonosides, adaptée aux non spécialistes de la CPC. Nous appuyant sur l'expérience du laboratoire dans l'extraction par CPC des flavonosides du *Ginkgo biloba*, nous proposons une méthodologie de purification utilisant les systèmes de solvants biphasiques EtOAc/*n*-BuOH/MeOH/H_2O et EtOAc/*n*-BuOH/H_2O à différents ratios en fonction de la polarité des flavonosides. La méthodologie développée a permis une déconvolution rapide des flavonosides majeurs présents dans l'extrait butanolique, en une à deux étapes de CPC.

Dans la dernière partie, l'étude phytochimique de *Hydnora abyssinica* a mis en évidence la présence de PACs, polymères de haut poids moléculaire de flavan-3-ols. La forte teneur en tanins justifie l'utilisation en médecine traditionnelle de cette plante holoparasite des *Acacias* comme anti-diarrhéique dans le continent africain et la péninsule arabique. La méthodologie de fractionnement CPC, précédée d'un pré-fractionnement sur résine LH-20, a permis l'isolement pour la première fois dans cette plante de la katsumadine et du rhodioloside.

Mots clés: *Aristolochia bracteolata*, *Ziziphus spina-christi*, *Hydnora abyssinica*, Chromatographie de partage centrifuge, acides aristolochiques, flavonosides, proanthocyanidols.

Summary

This work was a contribution to the phytochemical study of three Sudanese medicinal plants: *Aristolochia bracteolata* Lam. (Whole plant), *Ziziphus spina-christi* (L.) Desf (Leaves) and *Hydnora abyssinica* A. Br. (Rhizomes). The specificity of this research program was to emphasize the application of Centrifugal Partition Chromatography (CPC) for the fractionation of these plants. Three specific CPC methodologies were developed for the purification of either aristolochic acids, flavonoids or proanthocyanidins (PACs). In this context, the first part of this manuscript was devoted to the presentation of the CPC methodology. The different modes of development are also recalled.

The second part concerned with the study of *Aristolochia bracteolata*, used traditionally in Sudan particularly for its analgesic and antipyretic properties. The presence of aristolochic acids in this plant nevertheless gives it a known toxicity mainly at the renal level. Due to their particular toxicity, aristolochic acids have become major pharmacological tools for the development of models of kidney damage, yet few of them are marketed in pure form.

This work led to the development of an innovative method for the isolation and purification of these aristolochic acids by CPC in Strong Ion eXchange mode (SIX-CPC). The aristolochic acids I, II and IIIa were isolated from crude extract of *Aristolochia bracteolata* in one step with very high level of purity and in large quantities. Aristolochic acid IIIa had never been described in this plant before. These results were published in 2015 in *Separation and Purification Technology*.

In the third part of this thesis, the CPC was applied to the isolation of flavonosides present in *Z. spina-christi*. This plant is used by traditional herbalists for wound healing, diarrhea and malaria; also it's used as antispasmodic. The objective in this part was to propose strategy for standard fractionation of flavonosides, adapted to the non-specialists in CPC. Based on our laboratory experience in CPC extraction of *Ginkgo biloba* flavonosides, we were proposed purification methodology using EtOAc/ *n*-BuOH/MeOH/H_2O and EtOAc/*n*-BuOH/H_2O biphasic solvent systems with different ratios depending on the polarity of the flavonosides. The developed methodology was allowed rapid deconvolution of the major flavonosides present in the butanol extract, in one to two steps of CPC.

In the last part, the phytochemical study of *Hydnora abyssinica* revealed the presence of PACs, polymers of high molecular weight of flavan-3-ols. The high content of tannin justifies the traditional medicinal use of this holoparasite *Acacia* plant as anti-diarrheal in the African continent and the Arabian Peninsula. The CPC fractionation methodology, preceded by LH-20 resin pre-fractionation, allowed the isolation of katsumadine and rhodioloside.

Keywords: *Aristolochia bracteolata*, *Ziziphus spina-christi*, *Hydnora abyssinica*, Centrifugal Partition Chromatography, aristolochic acids, flavonosides, proanthocyanidins.

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List of abbreviations

A	
AA	Aristolochic acid
AAN	Aristolochic acid nephropathy
ACN	Acetonitrile
AHPA	American Herbal Product Association
Al336	Aliquat 336®
Approx.	Approximative
B	
BuOH	Butanol
C	
CC	Column chromatography
CCC	Countercurrent chromatography
CLA	Conjugated Linoleic acid
CNS	Central Nervous system
CPC	Centrifugal Partition Chromatography
CPE	Centriugal Partition Extractor
CYP	Cytochrom P
D	
DAD	Diode array detector
DMAC	4-Dimethylaminocinnamaldehyde
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
E	
EBuWat	Ethylacetate/*n*-Butanol/water
EtOAc	Ethylacetate
F	
FDA	Food and Drug Administration
H	
HEMWat	Heptane Ethylacetate Methanol Water
HPLC	High Performance Liquid Chromatography
HR-ESI-MS	High resolution-electrospray ionisation-mass spectroscopy
I	
IC_{50}	Half maximal inhibitory 50
IEX CPC	Ion exchange centrifugal partition chromatography
L	
LC MS	Liquid chromatography-mass spectroscopy
M	
MDM	Multi Dual Mode
MDR	Multidrug resistant
MeOH	Methanol

MIC	Minimum inhibitory concentration
MS	Mass spectroscopy
MtBE	Methyl-*tert*-butylether
N	
NMR	Nuclear Magnetic Resonance
O	
OK	Oppossum kidney cell line
OTC	Over the counter
P	
PAC	Proanthocyanidol
pHZR	pH zone refining
PTLC	Preparative Thin Laye Chromatography
R	
Rf	Retardation factor
rpm	Rotation per minute
S	
SFDA	State Food and Drug Administration of China
SIX CPC	Strong ion exchange centrifugal partition chromatography
T	
TFA	Trifluoroacetic acid
TGA	Therapeutic Good Administration of Australia
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
U	
UV	Ultraviolet

Objectifs de la thèse

- Application de la CPC à l'étude phytochimique de plantes issues de la Pharmacopée Soudanaise ;

- Développement de différents modes d'élution en CPC appliquées à l'étude phytochimiques (échange d'ions, élution co-courant) ;

- Appronfondissement des connaissances phytochimiques d'*Hydnora abyssinica,* de *Ziziphus spina christi* et d'*Aristolochia bracteolata* ;

- Transfert technologique de la CPC vers l'Université La Gezira.

INTRODUCTION

For thousands of years, natural products have played a very important role in health care and prevention of diseases. The ancient civilizations provide written evidence for the use of natural sources for curing various diseases. Plants continue to be used world-wide for the treatment of disease and novel drug entities continue to be developed through research into their constituents. In the developed countries, high-throughput screening tests are used for bioassay-guided fractionation leading to the isolation of active principles that may be developed into clinical agents either as the natural product or a semi-synthetic or a synthesised analogue with enhanced clinical action or reduced adverse side effects (Phillipson, 2001).

During the past decade, traditional systems of medicine have become a topic of global importance. Current estimates suggest that, in many developing countries, a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs. Although modern medicine may be available in these countries, herbal medicines (phytomedicines) have often maintained popularity for historical and cultural reasons. Concurrently, many people in developed countries have begun to turn to alternative or complementary therapies, including medicinal herbs. However, few plant species that provide medicinal herbs have been scientifically evaluated for their safety, efficacy, ingredients and the preparations containing them. Moreover, in most countries the herbal medicines market is poorly regulated, and herbal products are often neither registered nor controlled (WHO Monograph, 1999).

Sudanese folk medicine represents a unique blend of indigenous cultures with Islamic, Arabic and African traditions. Moreover, Sudan encompasses different terrains and climatic zones, ranging from desert and semi-desert in the north to equatorial with a short rainy season (semi-arid and semi-humid) in the centre to equatorial with a long rainy season (arid-humid and equatorial-humid) in the south. This variation contributes to the immense diversity of vegetation in the region with a lot of species, families and genera. Medicinal and aromatic plants and their derivatives represent an integral part of life in Sudan. Indigenous traditional remedies are the only form of therapy available to the majority of poor people. It has been estimated that only 11% of the population has gained formal health care. Therefore, research on the desired pharmacological effects and possible unwanted side effects or toxicity is required to improve efficacy and safety of Sudanese herbal medicine (Khalid *et al.*, 2012).

Therefore, three species were selected from Sudanese traditional medicinal plants for investigation of CPC as useful tool for rapid phytochemical screening or isolation of

metabolites of interest. These plants include *Aristolochia bracteolata* Lam. (Whole plant), *Ziziphus spina-christi* (L.) Desf. (Leaves) and *Hydnora abyssinica* A. Br. (Rhizomes).

The first part of this manuscript presents the Centrifugal Partition Chromatography (CPC) methodology, which supplies chromatographic techniques for fractionation and purification of natural products. The fractionation and purification procedures developed during this thesis work and described herein were mainly performed using CPC methodology.

The second part of the manuscript deals with the phytochemistry study of *A. bracteolata* Lam. In Sudan, *A. bracteolata* is traditionally used as analgesic, antiscorpion or antisnake bites drugs. It is also used in the treatment of tumors, malaria and for fevers (El-Tahir *et al.*, 1999; De Groot *et al.*; 2006; Abdelgadir *et al.*, 2011).

The fractionation of *Ziziphus spina-christi* is presented in the third part. *Z. spina-christi* leaves are used topically for wound healing. Fruits are traditionally eaten to treat diarrhoea and malaria, and are also used as antispasmodic. The powder of the twigs is used externally to treat rheumatism and scorpion sting (El-Kamali and El-Khalifa, 1999).

Finally, the fourth part presents the preliminary results of the phytochemistry analysis of *Hydnora abyssinica*. It is traditionally used as astringent in dysentery and also for stomach troubles (El-Kamali and El-Khalifa, 1999) and is also used in treatment of cholera and swelling tonsillitis (Yagi *et al.*, 2012).

I. CENTRIFUGAL PARTITION CHROMATOGRAPHY (CPC)

I.1.DEFINITION:

Support-free liquid–liquid separation techniques are promising technologies in pharmaceutical and cosmetic industries for the purification of high added-value bioactive compounds of natural origin (Sutherland *et al.,* 2008). Desirable characteristics of liquid−liquid separation techniques are (a) the easy scale-up to high-capacity (preparative) scale, (b) the array of solvent choices, which allows for considerable flexibility, (c) the capability of separating target analytes from ubiquitous matrix materials, and (d) frequently observed maximum sample recovery (Friesen *et al.,* 2015).

Centrifugal partition chromatography (CPC) is one of the technical solutions to the challenge of maintaining a liquid phase stationary while another one is pumped through it. It is based on partition cells radially engraved in a disk connected to each other by capillary ducts (Maciuk, *et al.,* 2004). These technologies offer clear advantages in terms of column capacity, target compound recovery, selectivity, resolution and process duration compared to more conventional purification techniques using for instance silica gel or RP-18 chromatographic solid supports (Sutherland *et al.,* 2008).

I.2. COLUMN DESIGN AND PUMPING MODE:

A CPC column consists of stacked stainless steel disks separated by polymer plates, which are placed in a centrifuge. In the disks, cells are engraved, connected by narrow ducts. The stationary phase stays in the cells due to the centrifugal force and the special column geometry.

Commercially available columns can consist of 800 or more cells–duct combinations (Figure 1).

A) B)

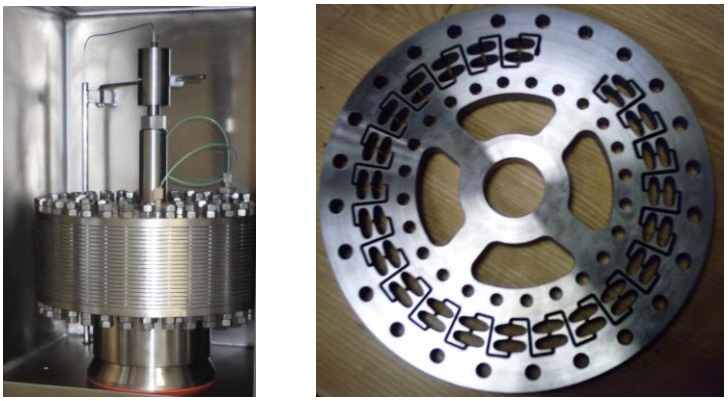


Figure 1. A) CPC rotor used in the laboratory; B) a CPC disk engraved with symmetrical Twin-cells.

The total volume of a CPC column can range from 25 mL for a semi-analytical column to 30 L for a preparative column. Different cells and channel geometries are possible (J. van Buel *et al.*, 1997).

A redesigned CPC column with larger cells and channels has been named Centrifugal Partition Extraction (CPE). The advantages of employing larger cells in CPC are (1) increased flow rates may be achieved with reasonable stationary-phase retention and (2) in most cases higher sample loading, as a percentage of total column volume, may be achieved (Friesen *et al.*, 2015).

In all cases, the mobile phase flows through the stationary phase in the form of droplets, jets or breaking jets depending on the operating conditions.

If the dense phase is used as the mobile phase (Figure 2, on the right), the mobile phase moves in agreement with centrifugal forces field (*i.e.* up to down). This is called the descending mode.

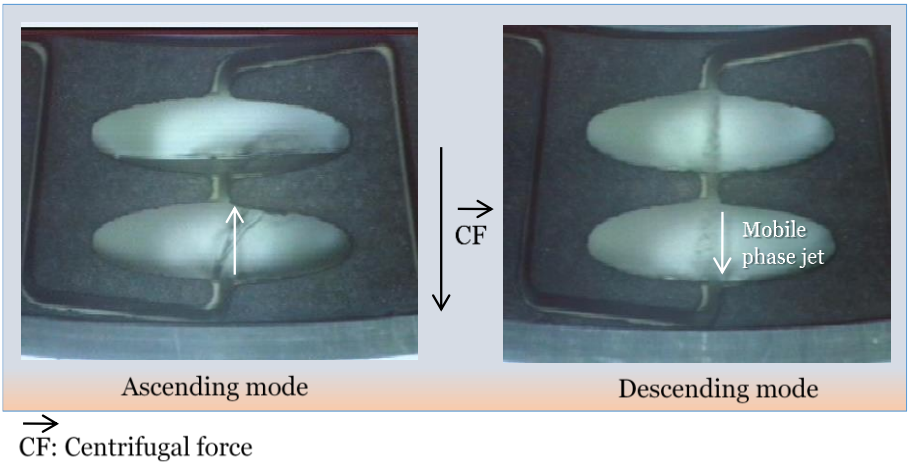


Figure 2. Illustration of pumping modes used in CPC.

If the light phase is used as the mobile phase, the mobile phase should enter the cell at the other side (*i.e.* down to up) and moves against centrifugal forces field. This is called the ascending mode.

One of the most striking aspects of this concept is the ability the user has to customize the mobile and stationary phases to obtain a wide range of chromatographic behaviors. Consequently, most of the development modes used in liquid chromatography can be applied to CPC.

I.3. NATURE OF STATIONARY AND MOBILE PHASE:

The liquid phases used in CPC are built by mixtures of two or three (or more) immiscible solvents and/or solutions to form a two or three phases system. These phases of the same solvent system are in thermodynamic equilibrium with each other. The simplest biphasic system is made by mixing two immiscible solvents, like heptane/methanol system.

The most abundant solvent systems are composed of three solvents. Phase composition of ternary mixtures is described by a ternary phase diagram drawn as an equilateral triangle (Figure 3).

The three apexes correspond to the three pure solvents. The three sides of the triangle represent the three possible binary mixtures. A point in the inner region characterizes the global composition of the mixture. Most often, volume percentages are used, but mass percentages or molar fractions can be used, giving different diagrams for the same ternary system.

In this triangle, the binodal isotherm delimits the two-phase area of the system situated under or inside of the curve.

In the equilateral triangle, binodal curves are drawn to indicate the border between monophasic and biphasic regions. The binodal curve gives the composition of all possible liquid phases that can be used in CPC experiments with this particular ternary liquid system. A specific point of this curve, when it exists, is called the plait point. Close to this point the compositions of the two phases are nearly identical: the partition coefficient of all solutes tends toward unity. In CPC, the liquid system composition must be sufficiently far away from the plait point to avoid coalescence of the phases or mingling in the apparatus. Each point in the biphasic region of the diagram belongs to a so-called "tie-line" that touches the binodal line at points giving the composition of the upper and lower phase.

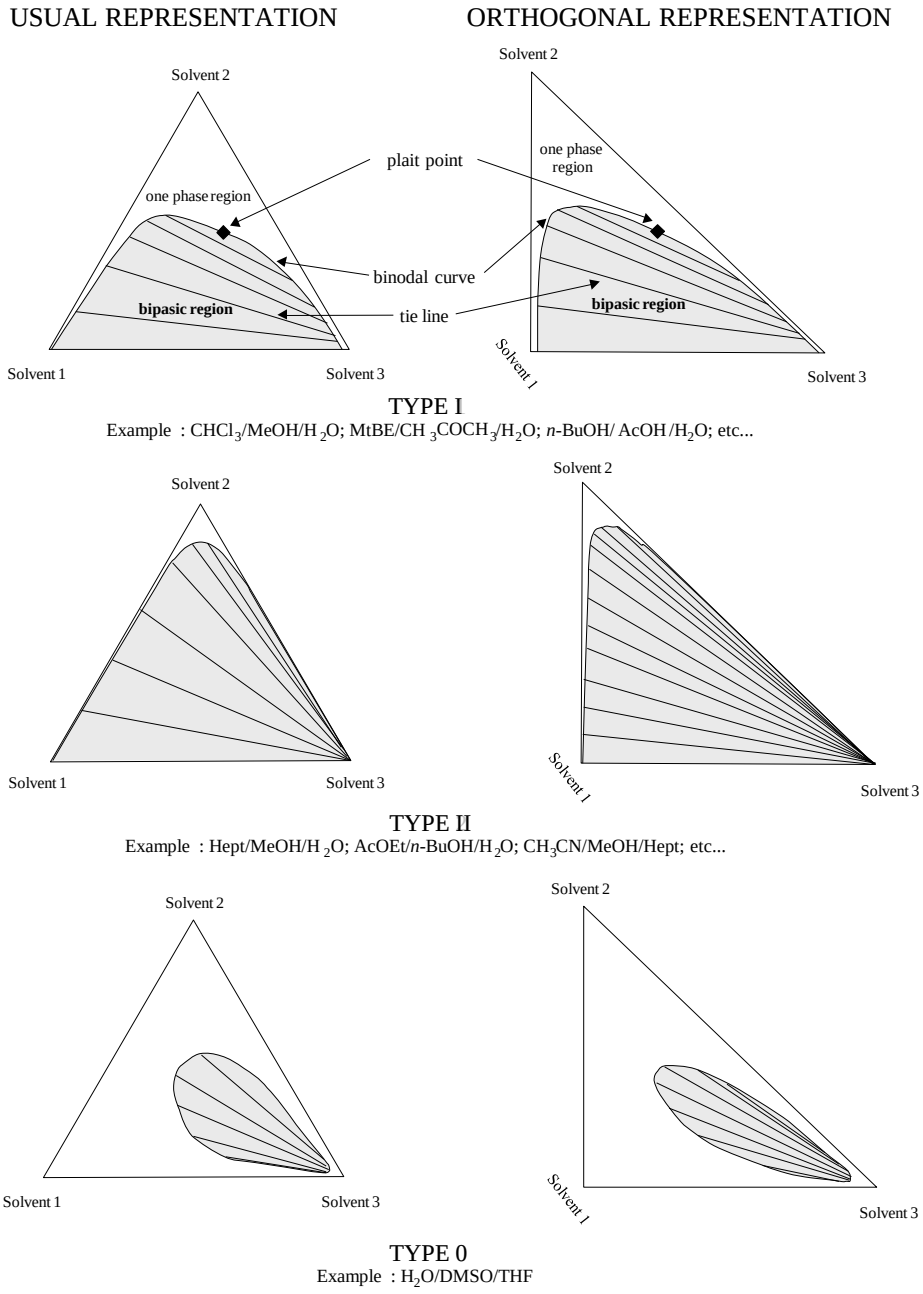


Figure 3. Description of equilateral and orthogonal representations of the three types of ternary diagrams (Renault et al., 2002).

The DMSO-THF-water system is, at the moment, the only Type 0 ternary biphasic system (Figure 3) described in CPC. In this unique system, all binary subsystems, DMSO-THF, DMSO-water, and THF-water, are monophasic. However, for certain compositions, two phases are formed, of which the lower one is rich in water, and the upper one is rich in THF and DMSO.

Solvents are most often chosen so that two are immiscible (Solvents 1 and 3), and a third (Solvent 2) is miscible with mostly one (Type II systems) or both (Type I systems) of the other solvents (Figure 3).

I.4. CHROMATOGRAPHIC PARAMETERS:

CPC has a specific terminology with specific physicochemical parameters, such as the stationary phase retention. However some conventional chromatographic parameters (selectivity, resolution) are also considered in this process.

I.4.1. Partition coefficient:

The partition coefficient (*K*) is the ratio of solute distributed between the mutually equilibrated two solvent phases. Usually it is expressed by the amount of solute in the stationary phase divided by that of the mobile phase as in conventional liquid chromatography (Ito, 2005).

$$K = \frac{C_{stat}}{C_{mob}} \qquad \text{(Equation 1)}$$

With *K*: Partition coefficient.

C_{stat}: The solute concentration in the stationary phase.

C_{mob}: The solute concentration in the mobile phase.

During CPC process, solutes with smaller *K* values are eluted closer to the solvent front with lower resolution while a larger *K* values tends to give better resolution but broader, more dilute peaks due to a longer elution time (Ito, 2005).

In most of the cases, the screening of solvent systems took place without guessing which phase would be stationary or mobile. So in practice, partition coefficient was calculated using following formulae (Equation 2).

$$K = \frac{C_{upper}}{C_{lower}} \qquad \text{(Equation 2)}$$

With *K*: Partition coefficient.

C_{upper}: The solute concentration in the upper phase.

C_{lower}: The solute concentration in the lower phase.

This is definition that we will refer to for the rest of the thesis.

I.4.2. Retention volume (V_r):

Retention volume (V_r) is an important parameter for the liquid-liquid chromatographic separation techniques. It is similar to the retention time in liquid-solid chromatography (Pauli, *et al.;* 2008).

$$V_r = V_{mob} + K\,(V_{stat}) \text{ (Descending mode)} \qquad \text{(Equation 3)}$$
$$V_r = V_{mob} + 1/K\,(V_{stat}) \text{ (Ascending mode)}$$

With V_r = Retention volume

V_{mob}= Volume of mobile phase

V_{stat}= Volume of stationary phase

Volume of stationary phase (V_{stat}) will be calculated in following equation:

$$V_{stat} = V_{col} - V_{mob} \qquad \text{(Equation 4)}$$

With V_{col} = Total volume of the column

I.4.3. Stationary phase retention ratio (S_f):

Stationary phase retention is representing the volume of stationary phase remaining in the column once steady state was reached. It is dependent on the instrument, its parameters, and the solvent system being used. Traditionally, S_f is measured using a pre-run equilibration process. However, it can also be calculated experimentally through several other methods, before, during and after run (Pauli, *et al.;* 2008).

$$S_f = \frac{V_{stat}}{V_{col}} \times 100 \qquad \text{(Equation 5)}$$

With S_f= Stationary phase retention

V_{mob}= Volume of mobile phase

V_{stat}= Volume of stationary phase

S_f has a major impact on process resolution: the higher was S_f, the better was the resolution of the separation. Usual S_f observed during CPC experiments were between 45 and 80 %. A S_f higher than 55 % was generally required to achieve a proper separation in elution mode.

I.5. DIFFERENT DEVELOPMENT MODES:

In liquid/liquid support free chromatography, like CPC, different separation mode are available. The two main modes described are the elution mode and the displacement mode.

I.5.1. Elution mode:

In elution mode, the stationary phase is held in place while the mobile phase is pumped through it. Analytes are eluted as Gaussian peaks, as usually observed in elution chromatography (Figure 4).

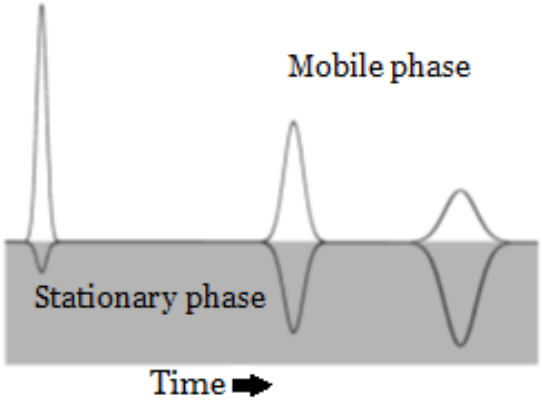


Figure 4. Modeling of an elution chromatogram in elution mode in CPC.

Different elution modes have been developed and applied to deal with actual complex samples in CPC methodology:

I.5.1.1. Isocratic elution:

The isocratic mode was often used in CPC because it was the easiest to perform. In this mode, the composition of the mobile phase remains unchanged throughout the experiment. The isocratic mode is particularly efficient and is recommended for extracts containing a mixture of homologues and/or structural analogues. It is a simple and effective method of isolating and purifying few major compounds from complex mixtures. It may fail when dealing with samples covering a large range of polarity (*K* values) (Renault *et al.*, 2002; Huang *et al.*, 2016).

I.5.1.2. Gradient elution:

An elution mode suitable to perform separations of components with large differences in partition coefficients (*K* values) is the gradient elution mode. During a separation with the gradient elution mode, the composition of the mobile phase is changed, while the composition of the stationary phase is kept (almost) constant (Van Buel *et al.*, 1997). Some studies suggested to employ phase diagrams to build gradients and predict stability of the stationary phase and

even calculate a composition of initial and final phases for gradient elution (Foucault and Nakanishi, 1989; 1990).

Gradient elution is increasingly used in CPC, and two kinds of gradient are described: stepwise and linear gradient elution mode.

In stepwise elution mode, the elution condition is changed stepwise at one or several occasions. It can be treated as a composition of multiple steps isocratic elution and several solutes may be eluted in each step. Stepwise elution mode is frequently used to separate complex samples in CPC. It generally includes the stepwise changes in mobile phase composition, flow rate, pH value or salting-out concentration (Huang *et al.,* 2016).

In linear gradient elution mode, the elution condition is changed continuously toward condition more favourable for separation. It was usually achieved through altering mobile phase composition (Ignatova *et al.,* 2011) and/or pH value (Liu *et al.,* 2004).

Stepwise mode is more common than linear mode; this might be because the gradient elution in liquid-liquid chromatography is quite different from liquid-solid chromatography and stepwise mode is relatively easy to implement concentration (Huang *et al.,* 2016).

The major difficulty of gradient elution in CPC is that any change of operating condition, particularly the change of mobile phase composition, may induce the change of composition of stationary phase and the decrease of stationary phase retention, which affect the chromatographic process (Costa *et al.,* 2013).

Not all ternary biphasic systems are suitable for gradient elution, especially linear gradient. To prevent instability of the stationary phase during a gradient run, the change in stationary phase composition should be small (20% v/v) (Van Buel *et al., 1997).*

To decrease the loss of stationary phase during a three stepwise gradient elution, a descending stepwise flow rate gradient was introduced by Du *et al.* in 2004. In this experiment, the lower phase of solvent system composed of *n*-hexane/*n*-butanol/0.05M NaOH (5:1:6, v/v/v) was used as the stationary phase and the upper phase was used as the initial mobile phase. To decrease the loss of stationary phase, the flow rates were significantly reduced from the initial 5.0 mL/min to 3.0 mL/min (step 1), 2.0 mL/min (step 2) and 1.5 mL/min (step 3). In the mean time, the *n*-hexane/*n*-butanol ratio was stepwise modified in the mobile phase from initial 5:1 to 1: 1 (step 1), 1: 2 (step 2) and 1: 4 (step 3). The retention of stationary phase during the gradient steps decreased to 67% (step 1), to 65% (step 2), and to 64% (step 3). Therefore the decrease of the flow rate while changing the mobile phase minimized the adverse effects on

stationary phase retention and provide satisfaying separation for the target compounds in a single-step CPC separation (Huang *et al.*, 2016).

Beside those classical modes, some modes were developed taking advantages of the liquid nature of the stationary phase: dual mode and multi-dual mode, recycling CPC, elution-extrusion process and co-current.

I.5.1.3. Dual-mode elution and multiple dual-mode elution:

In these processes, both ascending and descending mode were sequentially used to elute the solutes in the same separation. This elution mode is called dual-mode elution, which can be easily achieved by using a switching valve (Figure 5). Initially, classical elution is performed; then the stationary phase is changed to the mobile phase while simultaneously switching the circulation direction at a certain time during the separation process. The dual-mode elution can quickly elute the solutes with high *K* values that have strong affinity for the original stationary phase, which largely reduces the separation time and improves the separation efficiency.

Moreover both phases of the biphasic solvent system can be used as the mobile phase, thus reducing the solvent waste, and without causing sample loss (Huang *et al*., 2016).

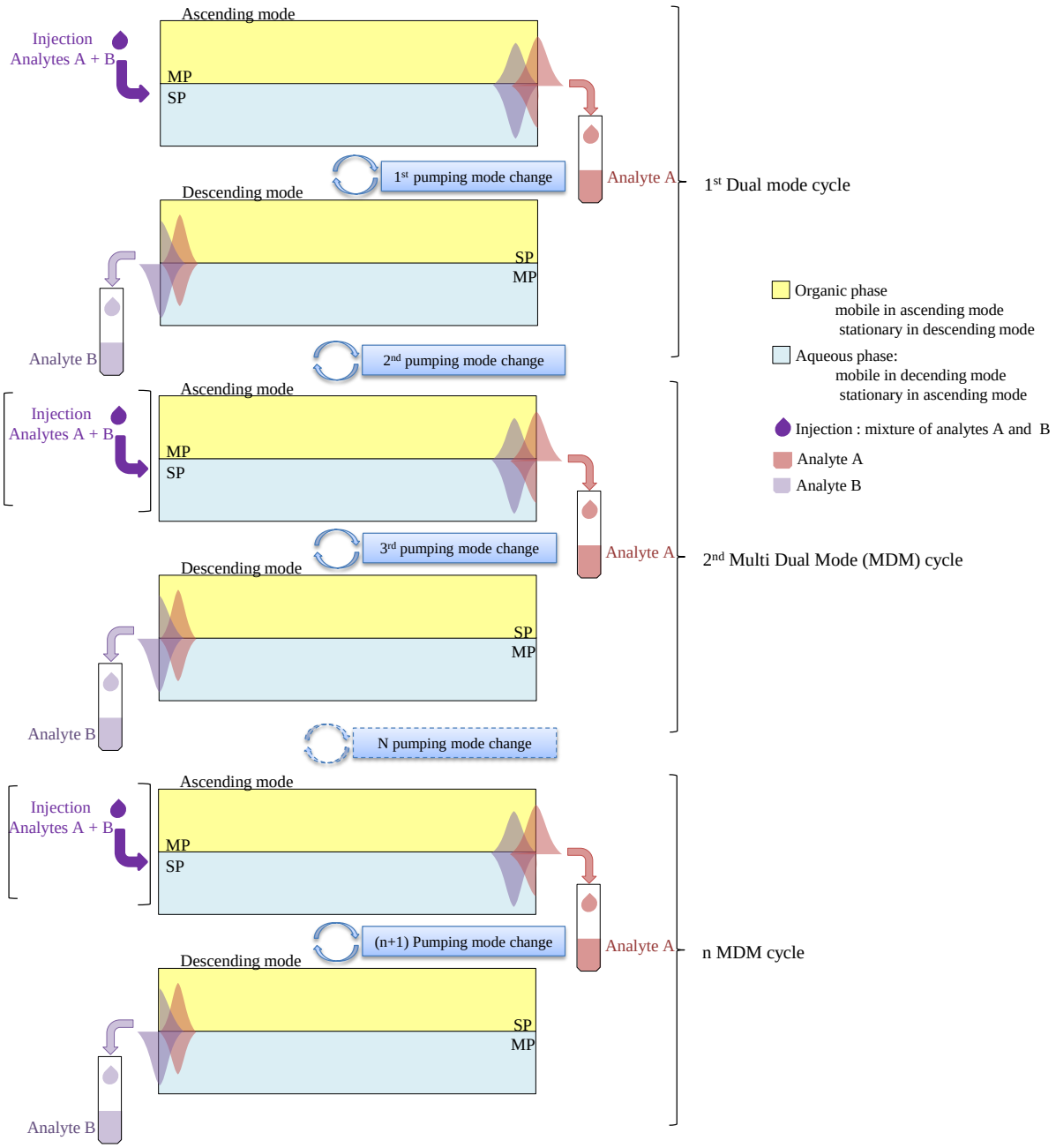
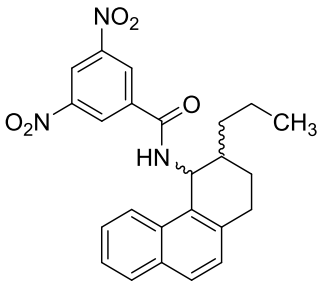
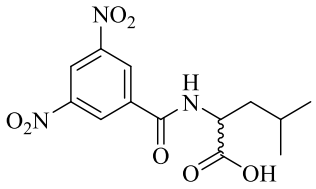


Figure 5. Representation for multiple dual-mode elution in CPC.

Multiple dual-mode (MDM) elution is an extension of dual-mode elution involving a series of consecutive dual-mode steps and used for solutes with close *K* values such as enantiomers (Figure 6) (Rubio *et al*., 2009). The solutes in the column are eluted back and forth until the two peaks are separated by switching between normal and reverse-mode elution operation in a series and finally, the solutes are eluted out from the same end of the column (Figure 5) (Huang *et al*., 2016).



(±)-*N*-(3,4-cis-3-decyl-1,2,3,4-tetrahydro phenanthren-4-yl)-3,5-dinitrobenzamide



N-(3,5-dinitrobenzoyl)-(±)-leucine

Figure 6. Chemical structures of enantiomers purified by multiple dual-mode.

Duration of each elution step is the main parameter to optimize in this case (Figure 7).

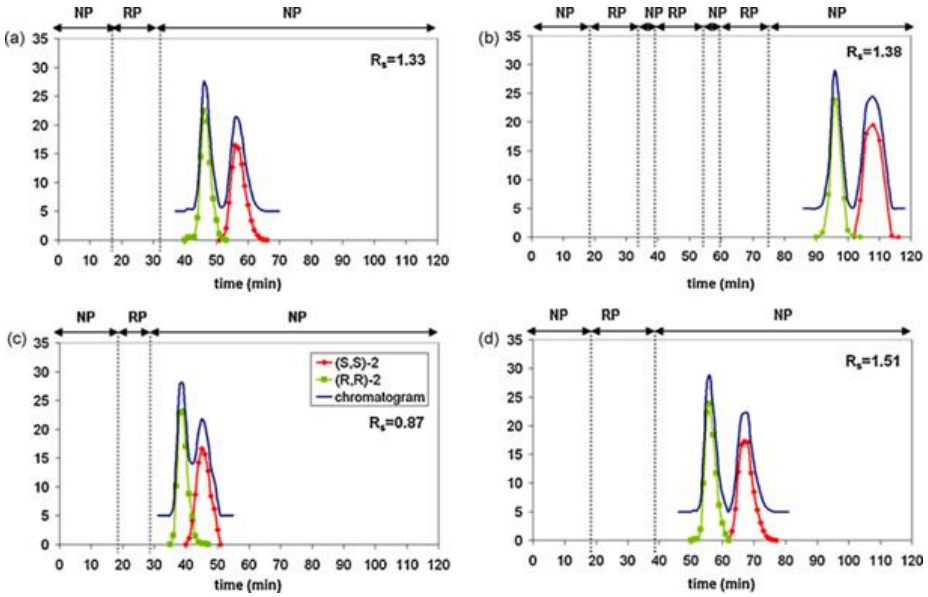


Figure 7. Elution profiles obtained from multiple dual-mode (MDM), the separation of (±)-N-(3,4-cis-3-decyl-1,2,3,4-tetrahydrophenanthren-4-yl)-3,5-dinitrobenzamide. (a) One cycle; RP (reverse phase), NP (normal phase), 15 min; (b) three cycles; RP, 15 min; time between cycles, 5min; (c) one cycle; RP, 10 min; and (d) one cycle, RP, 20 min (Rubio et al., 2009).

As we can notice on Figure 7, comparing a, c and d profiles, duration of elution step in ascending (NP) or descending (RP) mode have a great impact on the resolution between the two peaks. Increasing the elution step from 10 to 20 min, resolution factor increase from 0.87 to 1.51.

I.5.1.4. Recycling elution:

The recycling elution mode can be easily achieved by connecting the outlet of the detector to the inlet of mobile phase pump via a tube and a valve (Figure 8). The separation process is carried out in two system positions, R and S. At the beginning, the system is set at position R

until equilibrium is established. Then sample is dissolved in known volume of mobile phase and the solution is injected through the injection valve. After a dead volume of mobile phase is eluted, the system is set at position S and the mobile phase flowing out of the detector is pumped into the coil again. After the resolution of compounds is good enough, the system must be returned to position R, then the target peak fraction is collected (Yang *et al*., 2012).

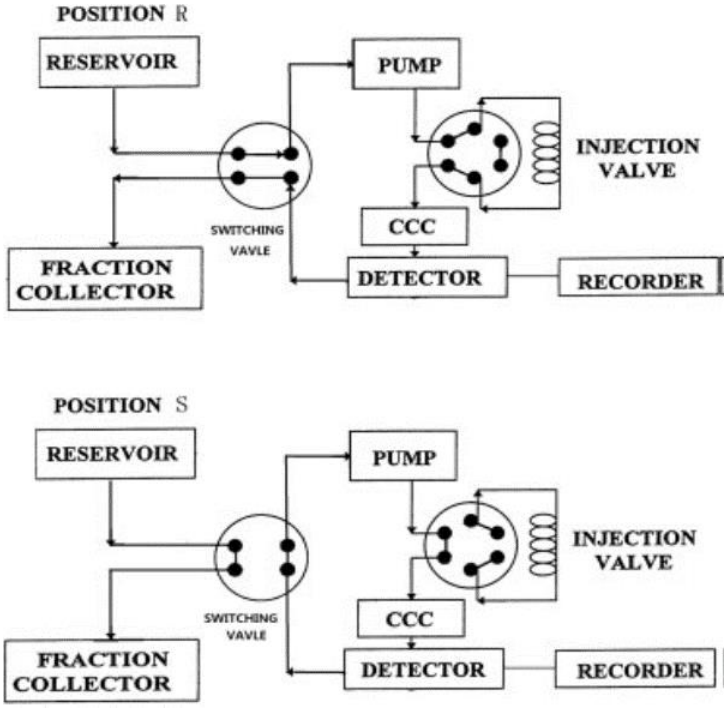


Figure 8. Schematic diagram of CPC set-up configuration during the recycling CPC separation with one set of CPC system, a sixport injection valve and a four-port switching valve (Yang et al., 2012).

This elution mode was used to separate some natural compounds such as epimers (Han *et al*., 2006), which exhibit a very low resolution factor (Figure 9). This methodology dramatically extended the separation time, although the same amount of solvent is consumed, and was not suitable for the simultaneous separation of complex compounds like peptides and polyphenols (Han *et al*., 2006).

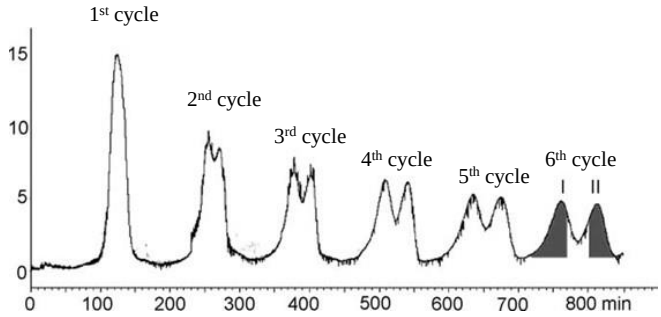


Figure 9. Chromatogram for preparative separation of gambogic acids by recycling CPC, peak I: gambogic acid; peak II: epigambogic acid (Han et al., 2006).

I.5.1.5. Elution-extrusion:

The extrusion-elution mode was developed for complex samples that contain solutes with large range of *K* values. Compared with other elution modes in CPC, it can extensively extend the hydrophobicity window and enhance the separation ability of a single biphasic liquid system. It involves two processes: traditional elution and a stationary phase extrusion procedure. Thus, solutes that are significantly retained on the column can be rapidly eluted, and both solvent consumption and separation time can be considerably reduced in order to save significant amounts of solvents and time (Berthod, 2006).

Generally, elution–extrusion includes three steps: classical elution, sweeping elution and extrusion (Figure 10). The first step is a traditional CPC elution method. After eluting a certain volume of the mobile phase, the elution liquid is changed from the mobile phase to the stationary phase and the elution is continued at the same rotation speed but with higher flow rate, usually between 20 to 30 mL/min. This represents the sweeping elution step, and the mobile phase in the column will be replaced by the stationary phase. Solutes with lower *K* values will be eluted with the mobile phase. In the third step, the stationary phase is continuously pumped into the column and solutes with high *K* values will be pushed out followed by the stationary phase in the column according to the order of their *K* values.

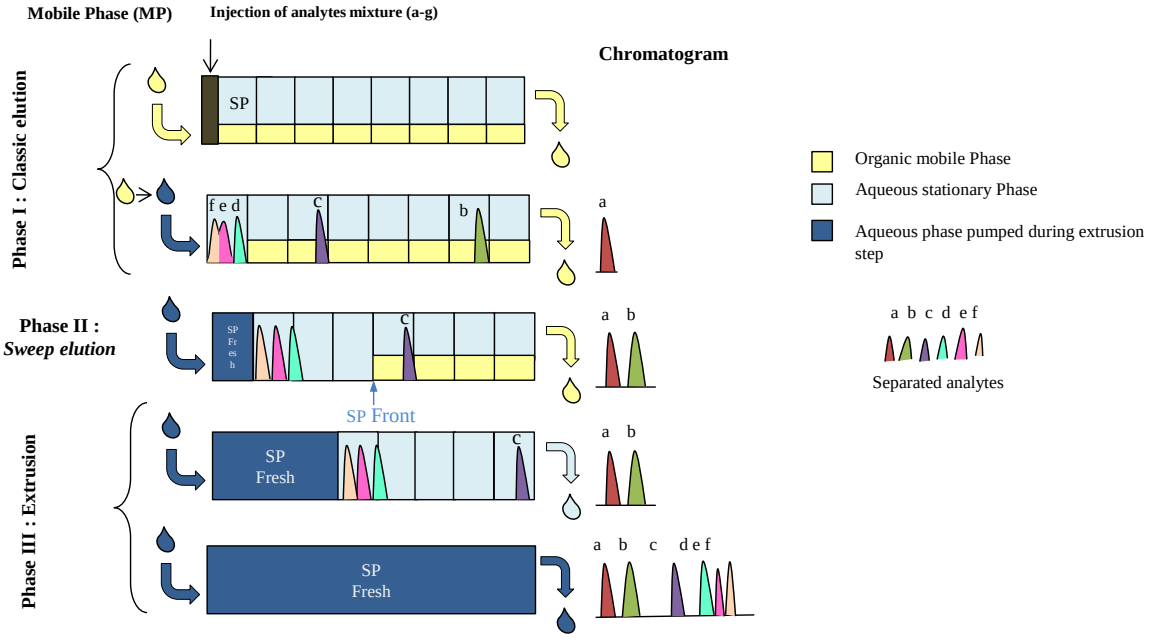


Figure 10. Representation of elution-extrusion process in CPC (Berthod et al., 2007).

After this step, the column is filled again by the stationary phase, which is then prepared for equilibration to begin the next separation process. The major advantage of elution–extrusion process is that it draws on the features of the liquid stationary phase in CPC. It can rapidly elute

all solutes in the column without any irreversible adsorption, most of which are separated with acceptable peak resolution. Moreover, compounds with an extended polarity range and/or in complex biological samples such as in metabolomics can be separated, with the ability to target specific analytes based on their *K* ranges or values (Berthod *et al.,* 2007). Elution–extrusion method drawbacks include that it is discontinuous (Berthod, 2006).

I.5.1.6. Co-current elution:

Co-current elution involves the movement of both the mobile phase and stationary phase in the column in the same direction (Figure 11).

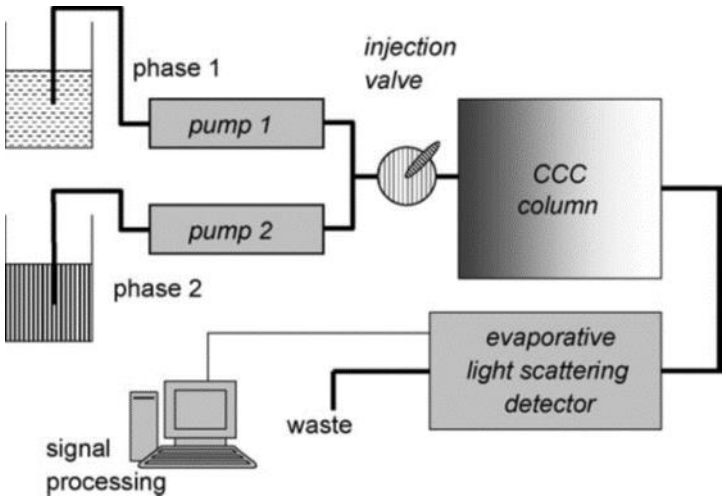


Figure 11. The co-current CPC set-up. Two pumps are needed, one for each phase(Berthod and Hassoun, 2006).

However, the stationary phase moves more slowly than the mobile phase, two pumps must be used, or a gradient pump at least, one for the stationary phase and other for the mobile phase (Berthod *et al.,* 1992). Co-current elution can significantly decrease the retention time and elution volumes of the solutes with high *K* values. It also saves solvents and time, producing well resolved peaks. Unfortunately, serious noise in the detection was usually observed, due to the two immiscible liquid phases simultaneously flowing through the detector (Berthod and Hassoun, 2006).

Elution mode allows recovering most of the compounds and could be applied to all samples but for some metabolites, highly polar, ionized or ionizable, another mode is possible: displacement mode.

I.5.2. Displacement mode:

Displacement CPC was first performed through acid–base equilibrium and referred to as "pH zone refining CPC'' (pHZR). As in conventional displacement chromatography, the feed components emerge from the CPC column as adjacent ''square wave zones'' of homogeneous material. There are two different processes of displacement mode in CPC: pHZR and ion exchange.

I.5.2.1. pH-zone-refining elution (pHZR):

This mode was primarily used for the separation and purification of compounds whose distribution and electric charge depends on the pH value. pHZR CPC was developed based on the peak-sharpening phenomenon caused by the change in pH observed by Weisz *et al.,* in 1994. It is generally employed as a large-scale preparative technique for separating ionizable analytes.

Compounds are eluted as highly concentrated rectangular peaks according to their pK_a values and hydrophobicity. This elution mode was developed for its notable advantages over conventional CPC, and it is now widely used to separate ionizable compounds (Huang *et al*., 2016).

General mechanism depend on three main actors: analytes, retainer and displacer. Analytes refer to ionizable compounds targeted for separation. The retainer is solubilized in stationary phase. It retains the analytes in the stationary phase by maintaining the pH curve as a low flat zone until the elution of the analytes. Displacer or eluter is solubilized in mobile phase and modifies the pH in the column by gradual neutralization of the retainer. This phenomenom is responsible of the sharp retainer border in the column (Ito, 2013).

Mechanism of pHZR could be divided into two types, depending on whether the aqueous or organic phase is mobile.

Normal displacement mode occurs when stationary phase is aqueous and mobile phase is organic. In this case retainer is solubilized in the aqueous phase and displacer in the organic phase. The mechanism in normal elution mode can be demonstrated by the model experiment below (Figure 12), describing the separation of organic acids.

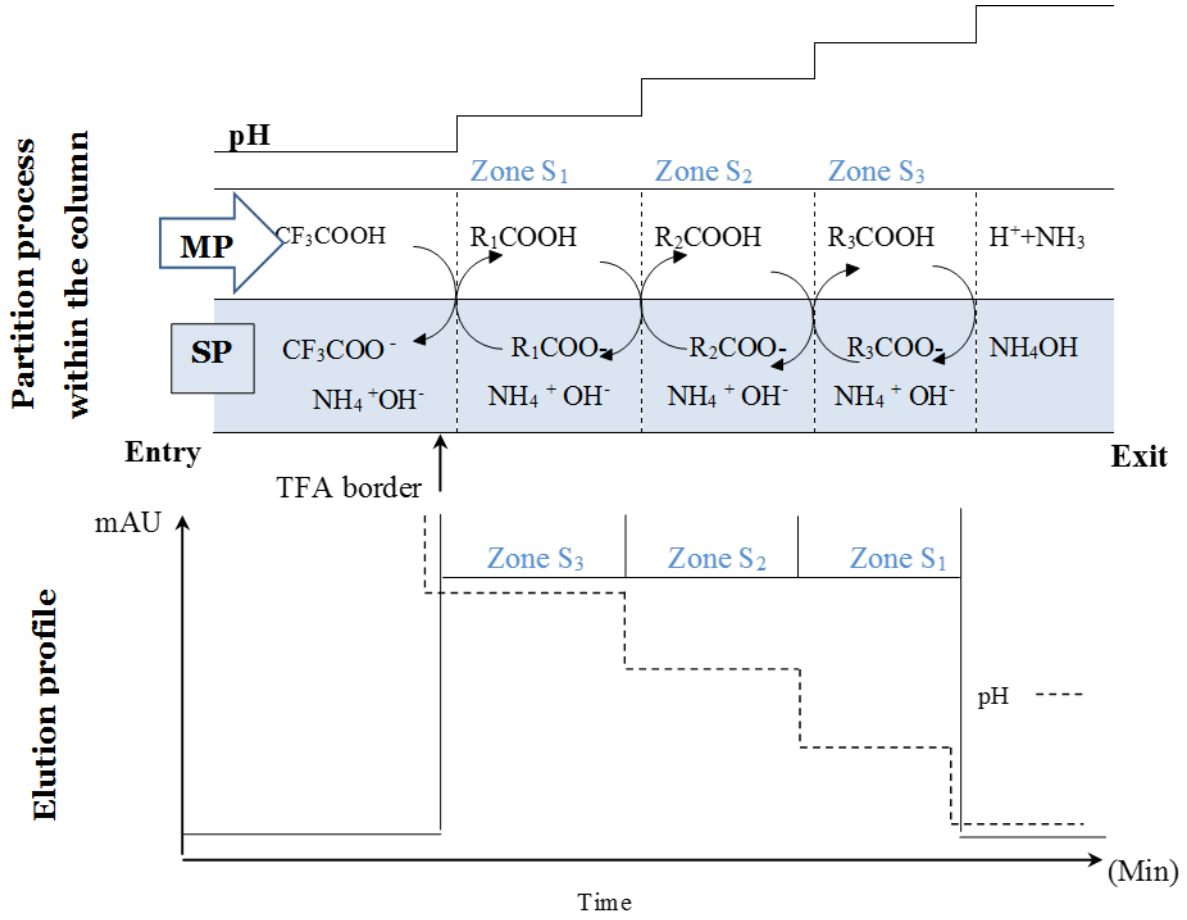


Figure 12. Model experiment for normal displacement pH-zone-refining CPC for separation of three carboxylic acids (S_1, S_2, S_3). TFA<pK_a1<pK_a2<pK_a3<NH_4OH; SP stationary phase, MP Mobile phase (Ito and Ma, 1996).

The column is first filled with the stationary phase containing the retainer, here a hydrophilic base, ammonia (NH_4OH). Sample solution containing three acidic analytes or solutes (S_1, S_2 and $S_{3)}$ is then injected. The mobile phase, containing the displacer, is pumped into the column. The displacer, lipophilic acid trifluoroacetic acid (TFA) form sharp trailing border which moves through the column at an elution rate lower than the mobile phase flow rate. The three analytes S_1, S_2 and S_3 form solute zones in front of the sharp TFA border according to their pK_a values and hydrophobicity. In this case, S_1 with the lowest pK_a is found immediately after the TFA border followed by S_2 and then S_3. Proton transfer (curved arrows) takes place at each zone boundary according to the difference of pH between the two zones. This causes the exchange of the solute between the two phases. As TFA moves through the column, it gradually displaces S_1 from the preceding S_1 zone. Each solute zone advances similarly by displacing the preceding zone at its front border. After equilibrium is established, each solute zone moves at the same

rate as the TFA border while maintaining its width and pH. Charged impurities present in each zone are eliminated according to their pK_a and hydrophobicity and accumulate at the zone boundaries. Consequently, the three analytes are eluted as a train of rectangular peaks associated with sharp impurity peaks at their zone boundaries, called isotachic train.

The mechanism of reverse displacement (Figure 13) in which the aqueous phase is used as the mobile phase, is totally reverse. The retainer is now the organic acid (TFA) and the displacer the hydrophilic base (NH_4OH). Three analytes S_1, S_2 and S_3, competitively form solute zones behind the sharp TFA border according to their pK_a values and hydrophobicity. Among these, S_3 with the highest pK_a and hydrophobicity is located immediately behind the NH_4OH border, while S_1 with the lowest pK_a and hydrophobicity is located at the end of the solute zones where it forms a sharp trailing border. The order of the zone arrangement being reversed from that observed in the normal displacement mode.

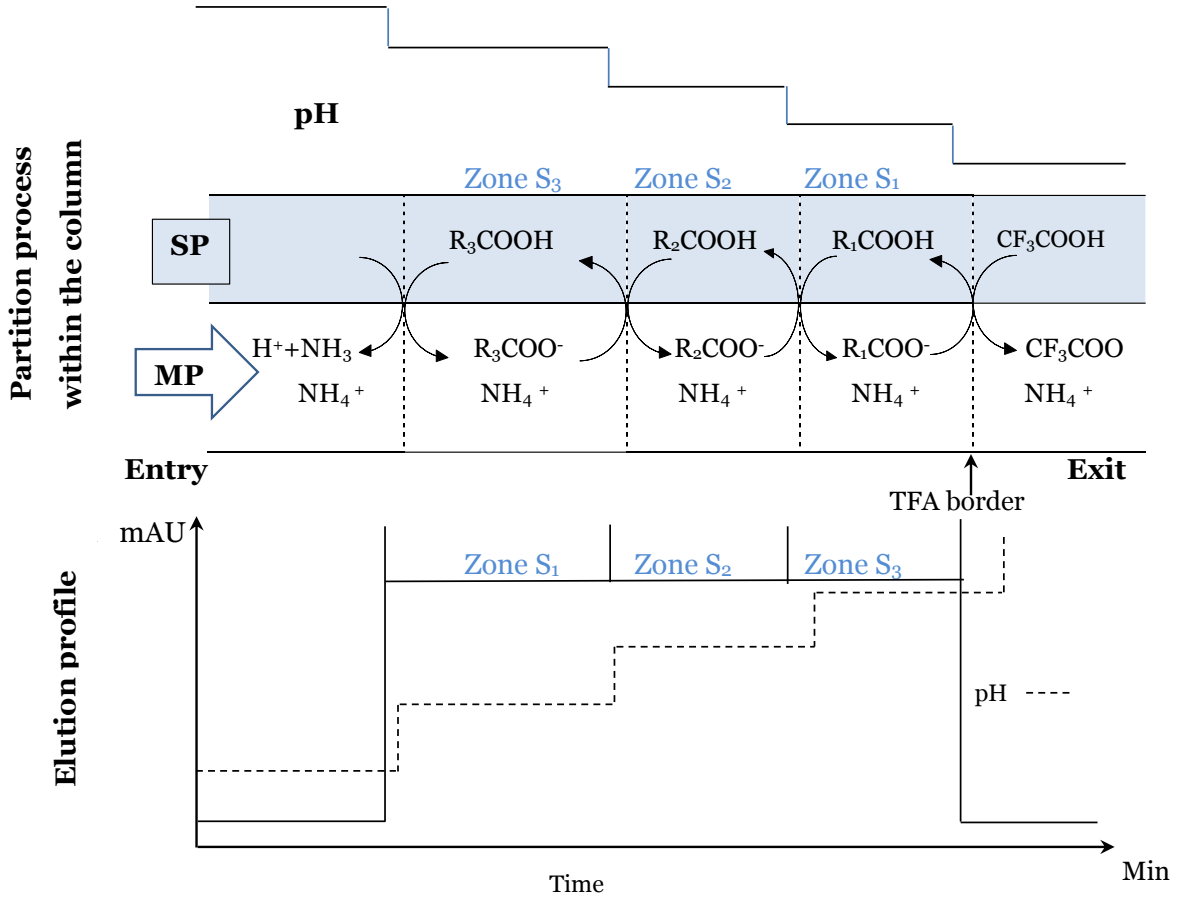


Figure 13. Model experiment for reverse displacement pH-zone-refining CPC for separation of three carboxylic acids (S_1, S_2, S_3): TFA<pK_a1<pK_a2<pK_a3<NH_4OH; SP stationary phase, MP mobile phase (Ito and Ma, 1996).

Proton transfer also takes place at each zone boundary governed by the difference of pH between these zones. The displacement of the solute from the mobile phase to the stationary phase at the zone front is compensated by its return at the back of each zone, while ammonium ion in the aqueous phase serves as counterion for all species. In this reverse displacement mode, the eluting orders of both analytes and pH-zones are completely opposite to those observed in the normal displacement mode.

This mode was used for purification of organic acids, conjugated linoleic acid isomers (CLA) (Figure 14), including *trans*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA and *cis*-9,*trans*-11 CLA (Song *et al.*, 2014). Alkaloids were also successfully purified by pHZR mode like catharanthine, vindoline and vindolinine from the aerial parts of *Catharanthus roseus* (Renault *et al.*, 1999); 10-methoxytetrahydroalstonine and isoreserpiline from the leaves of *Rauwolfia tetraphylla* (Figure 14) (Maurya *et al.*, 2013). Protected peptides and amino acids were purified by pHZR mode (Ito and Ma, 1994; Amarouche *et al.*, 2014).

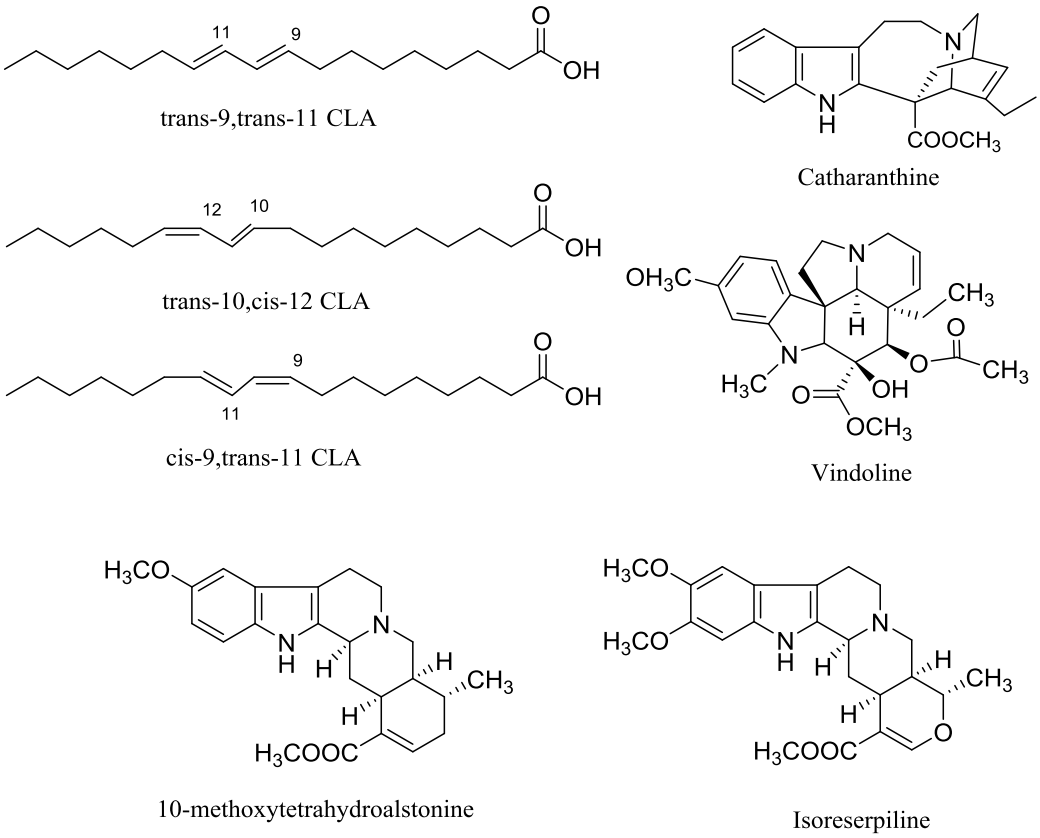


Figure 14. Examples for organic acids and alkaloids purified by pHZR mode.

As conclusion, pHZR mode is powerful tool for separation of some analytes but is not feasible for ionized metabolites with constant charge not depending on pH. For this kind of metabolites, ion-exchange mode could be applied.

I.5.2.2. Ion-Exchange (IEX):

In pHZR, acids and bases are used as retainers and displacers to separate solutes that display a dramatic difference in their relative solubilities in upper and lower phases between their neutral and ionized forms. The same principle is applied in ion-exchange CPC, although it is not strictly pH that is being modulated.

Ion-exchange CPC (IEX-CPC) was introduced by Chevolot *et al.* in 1998. The first applications described were the purification of polysulfated polysaccharides (fucans and heparins) and naphthalen sulfonic acids isomers using the lipophilic secondary amine Amberlite LA2® (*N*-lauryl-*N*-trialkylmethylamine) as a weak anion-exchanger (Chevolot *et al.*, 2000; Intes *et al.*, 2001).

Ion eXchange CPC is based on ion-pair extraction principle, resumed in Figure 15. Ionic analytes are captured into the organic stationary phase by forming stable ion-pairs with the exchanger. The displacer-free mobile phase is not able to elute the extracted analytes.

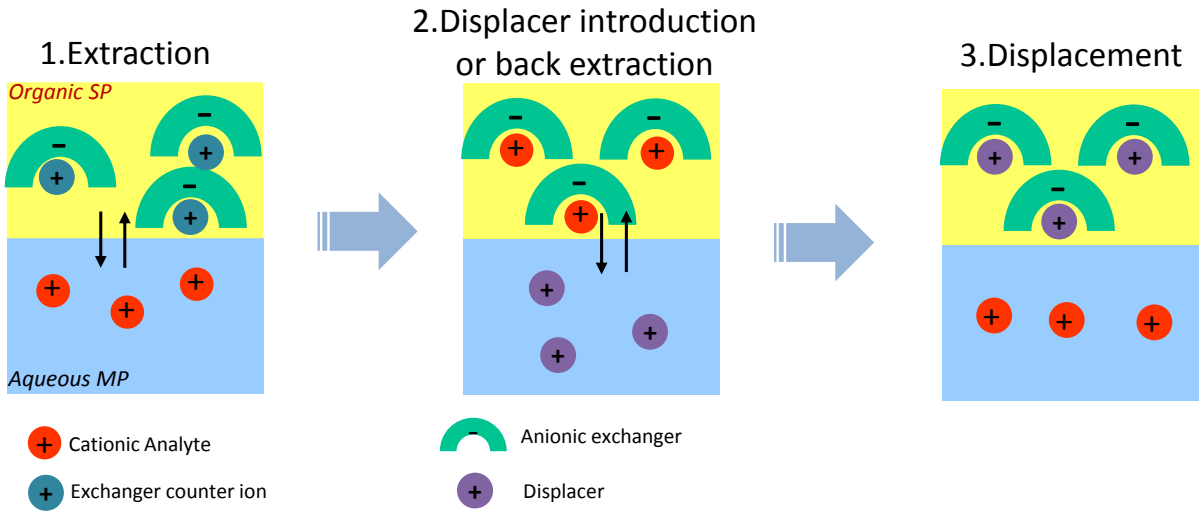


Figure 15. Ion pair extraction principle.

When adding the displacer, analytes return to the mobile phase, which is called the back extraction or displacement step.

IEX-CPC could be considered as a sequential repetitions of ion pair extractions. The separation mechanisms have been well described and modeled by Maciuk *et al.* (2004) using separation of phenolic acid regioisomers as methodological support. All analytes moved along

the column with the same velocity depending only on displacer concentration. This typical column organization is called an isotachic train, like in pHZR.

The CPC column is first filled with the stationary phase containing the exchanger. The mobile phase without displacer is pumped through the column. At the same time, sample is loaded in the column before reaching the equilibrium. The isotachic train is then pre-organized at the head of the column during equilibration step: analyte with the highest affinity for exchanger is extracted first followed by the other analytes in descending affinity order (Step 1, Figure 16).

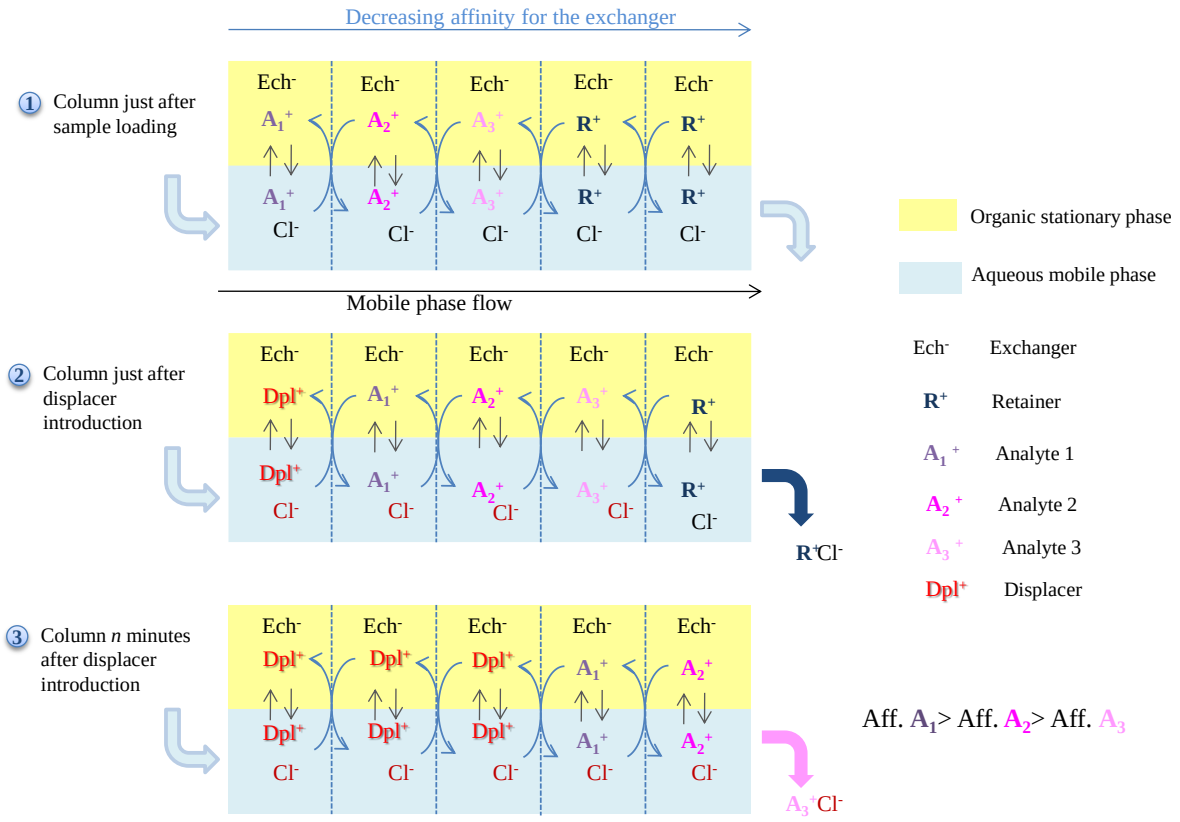


Figure 16. General separation mechanism of IEX-CPC.

Displacer free mobile phase is pumped until all undesired metabolites are eluted from the column, this step is called the washing step.

Then displacer is introduced in the column, and the ion exchange process takes place (Step 2 and 3, Figure 16). Exchanger, with the highest affinity for the exchanger, displaced the analyte 1 from its ion pair. Analyte 1 is then back extracted and moves along the CPC column and arrived in a zone containing exchanger/analyte 2 ion pairs. The analyte 2, with lower affinity for the exchanger, is then displaced by the analyte 1, back extracted in the mobile phase and moves further in the column. Analyte 2 displaces then analyte 3, exhibiting lower affinity, and

so on. IEX-CPC provides so high resolution purification based on the competition between analytes to form ion-pairs with the exchanger.

Ion exchangers are lipophilic anionic or cationic coumpounds like di(2-ethylhexyl)phosphoric acid (DEHPA), benzalkonium chloride or trioctylmethylammonium chloride (Aliquat 336®) (Figure 17).

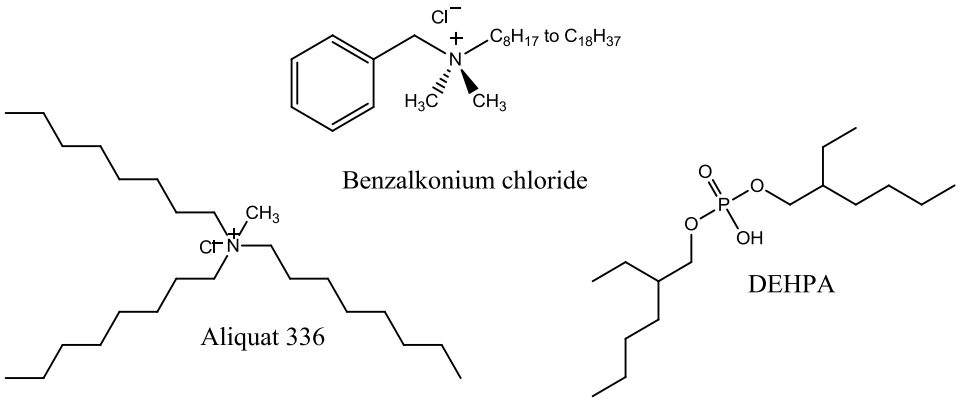


Figure 17. Structures of some exchangers used in CPC.

Displacers could be either acids or bases that modify ionization state of exchanger (Weak Ion eXchange) like HCl or counter ion with high affinity for the displacer like $CaCl_2$, HCl, or KI (Strong Ion eXchange). Exchanger is always solubilized in the organic stationary phase and the displacer in the aqueous mobile phase. This process is then carried out only in a descending mode.

Different IEX CPC processes are described depending on displacement mechanism used. In Weak Ion eXchange, the exchanger is ionizable, like Amberlite LA2® (*N*-lauryl-*N*-trialkylmethylamine) or DEHPA. The displacer is then a base or an acid which neutralizes the exchanger, which can no longer retain the ionic analytes.

In Strong Ion eXchange (SIX)-CPC, the exchanger is permanently ionic and keeps its positive (cationic exchanger) or negative (anionic exchanger) charge at any pH value (Toribio *et al.*, 2007). This mode is the most popular.

IEX-CPC was an efficient method for the purification of anionic or cationic compounds metabolites such as hydroxycinnamic acids (Maciuk *et al.*, 2004), glucosinolates (Toribio *et al.*, 2007; Toribio *et al.*, 2012) (Figure 18) or peptides (Boudesocque *et al.*, 2012).

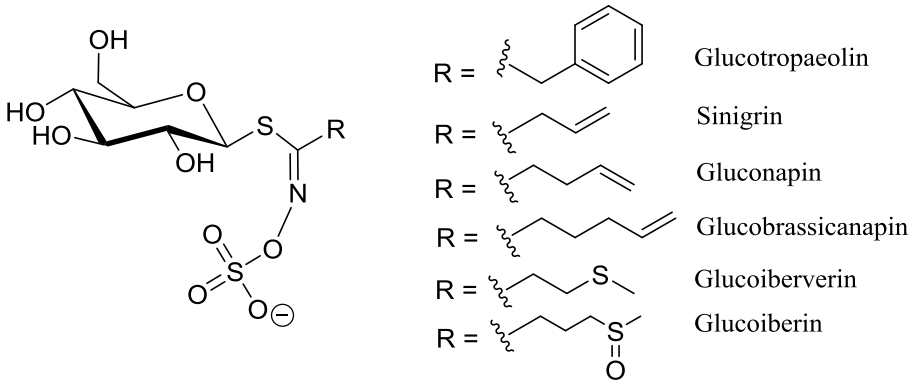


Figure 18. Glucosinolates purified by SIX-CPC.

Unlike pH-zone refining, IEX-CPC does not involve any change of solute ionization state and can be applied to the purification of ionized molecules. IEX-CPC provides wide variety of processes suitable in phytochemistry that open new perspectives for preparative separations of bioactive natural products with large amount of fractions in short time.

II. *ARISTOLOCHIA BRACTEOLATA* LAM.

In this second part, the general characteristics of *Aristolochia* genus and of *Aristolochia bracteolata*. species will be successively described, in terms of geographical localization, botanical identification, phytochemistry and traditional uses.

Then, the Aristolochic acids will be presented: their general structure, their pharmacokinetic properties (Absorption, Distribution, Metabloism and Excretion (ADME)), their toxicity and a review of the literature concerning the protocol of extraction and purification of the aristolochic acids.

The phytochemical investigation and the new CPC protocol developed in this thesis work for aristolochic acid purification will be reported.

II.1. CHARACTERISTICS OF *ARISTOLOCHIA* GENUS:

II.1.1. Classification:

Kingdom: *Plantae*, Plants
Subkingdom: *Tracheobionta* - Vascular plants
Superdivision: *Spermatophyta* - Seed plants
Division: *Magnoliophyta* - Flowering plants
Class: *Magnoliopsida* - Dicotyledons
Subclass: *Magnoliids*
Order: Piperales
Family: Aristolochiaceae - Birthwort family
Genus: *Aristolochia* L. - dutchman's pip (U.S. Department of Agriculture, 2015)

II.1.2. Geographic localization:

Aristolochia is a plant genus with more than 500 species belonging to the family Aristolochiaceae. They are often found in traditional Chinese medicines, whose medicinal parts have distinct Chinese names (WHO, 1997). *Aristolochia* are widely distributed in tropical, subtropical and temperate regions of the world (Figure 19). They are known to occur in Asia, Africa, North and South America, Europe and Australia (Kuo *et al.,* 2012).

The most widely distributed native species include *Aristolochia indica* L., *Aristolochia debilis* Sieb. et Zucc., *Aristolochia contorta* Bunge, *Aristolochia manshuriensis* Kom., *Aristolochia fangchi* Y.C. Wu, *Aristolochia serpentaria* L. (Virginia snakeroot), *Aristolochia*

tomentosa Sims (wooly Dutchman's pipe), *Aristolochia macrophylla* Lam. (Pipevine), and *Aristolochia clematitis* L. (birthwort). In addition, some non-native species such as *A. elegans* is grown as ornamentals (Cullina, 2002; NTP, 2008).

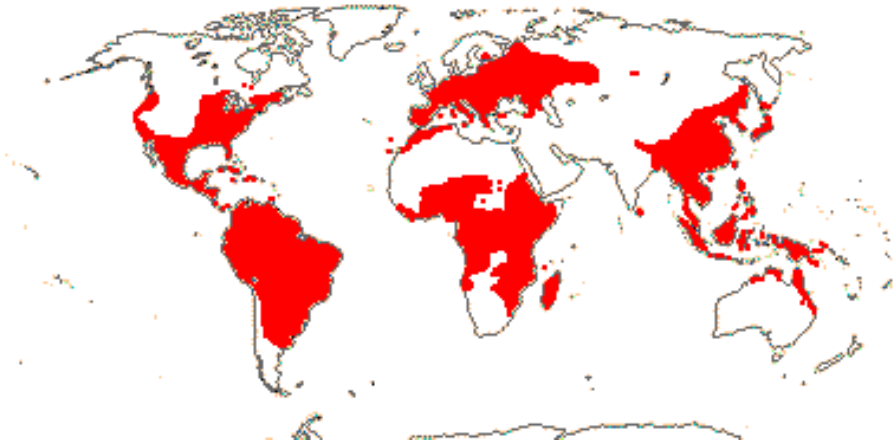


Figure 19. Geographical distribution of Aristolochia genus.

The distribution of the main *Aristolochia* species in Africa is presented in Figure 20 (De Groot *et al.*, 2006). Five main species were reported: *A. baetica* in Morocco, *A. sempervirens* in the north of Algeria, *A. heppii* in Zambia, Zimbabwe and Malawi, *A. rigida* mostly found in Somalia, *A. bracteolata* (Figure 37) widely distributed in East Africa (Soudan, Kenya, Djibouti).

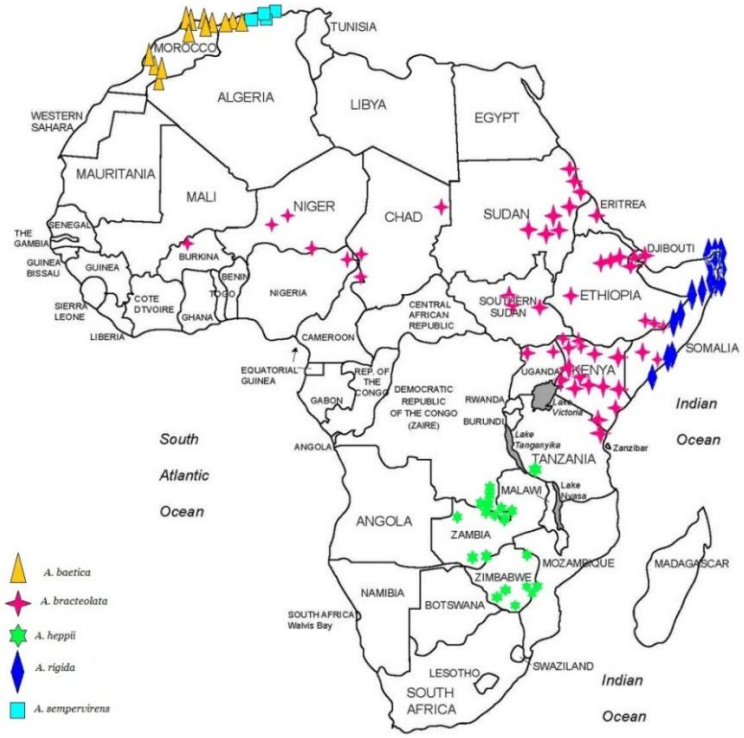
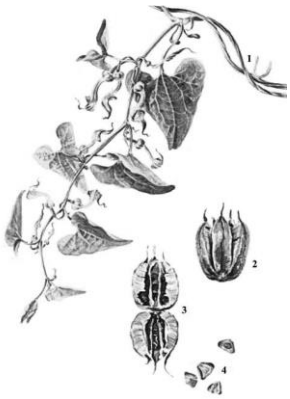


Figure 20. Distribution of some Aristolochia species in Africa (De Groot et al., 2006).

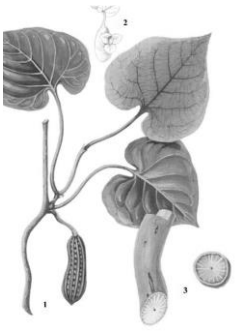
II.1.3. Botanical identification:

Aristolochia is a genus of evergreen and deciduous woody vines and herbaceous perennials. The stem is erect or somewhat twisting (Figure 21). The simple leaves are alternate and often cordate, membranous and growing on leaf stalks. There are no stipules. The flowers grow in the leaf axils. They are inflated and globose at the base, continuing as a long perianth tube, ending in a tongue-shaped, brightly colored lobe. There is no corolla. The calyx is one to three whorled, and three to six toothed. The sepals are united (gamosepalous). There are six to 40 stamens in one whorl, united with the style forming a gynostemium. The ovary is inferior and four to six locular. These flowers have a specialized pollination mechanism. The plants are aromatic and their strong scent attracts insects. The inner part of the perianth tube is covered with hairs, acting as a fly-trap. These hairs wither to release the fly, covered with pollen. The fruit is a dehiscent capsule with many endospermic seeds (Fernald, 1950; Gleason, 1963; Kelly and Favio, 2003; Adams *et al.*, 2005).

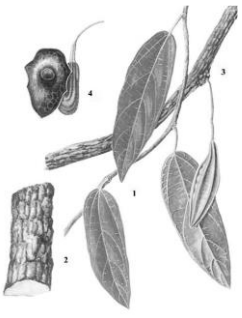


Aristolochia contorta:
1. flower twig; 2. fruit; 3, 4. seeds

Aristolochia debilis:
1. flower twig; 2. root; 3. fruit



Aristolochia manshuriensis:
1. fruit twig; 2. flower; 3. transverse section



Aristolochia fangchi:
1. twig leaf; 2. longitudinal section;
3. fruit twig; 4. flower

Figure 21. Aristolochia species (Qian, 1996).

II.1.4. Traditional and medicanal uses:

Based on the assessment of 566 reference sources, 685 individual sets of data were recorded. Seven species: *A. indica* L. (Asia), *A. serpentaria* L. (North America), *A. debilis* Siebold & Zucc. (China), *A. tagala* Cham. (India), *A. trilobata* L. (Central/South America, Caribbean), *A. clematitis* L. (Europe) and *A. bracteolata* Lam. (Africa) – are reported widely as being used medicinally (Table 1) (Heinrich *et al.*, 2009).

Table 1. The seven most frequently cited species of the genus Aristolochia based on a global assessment of the genus medical use (Heinrich et al., 2009):

Species	**Geographical localization**	**Number of records**
***Aristolochia indica* L.**	Indian subcontinent	105
***Aristolochia bracteolata* Lam.**	Africa	64
***Aristolochia serpentaria* L.**	North America	54
***Aristolochia debilis* Siebold et Zucc.**	China	38
***Aristolochia tagala* Cham.**	India	34
***Aristolochia trilobata* L.**	Central America, Caribbean	34
***Aristolochia clematitis* L.**	Europe	34

Various *Aristolochia* species have been used in herbal medicines since Antiquity in obstetrics and for the treatment of snakebite (Meenatchisundaram, 2009), festering wounds and tumors. They remain in use, particularly in Chinese herbal medicine (Balachandran *et al.*, 2005; NTP, 2008) (Table 2).

Table 2. Some Aristolochia species, their chemical constituents and traditional uses (IARC, 2002; NTP, 2008):

Aristolochia species	**Major chemical constituents**	**Traditional uses**
***Aristolochia contorta* Bunge (China)**	Aristolochic acids and their analogues (Aporphinoid alkaloids).	Dried ripe fruits: a remedy for haemorrhoids, coughs and asthma. Dried stems or leaves: treatment of epigastric pain, arthralgia and edema.
***Aristolochia debilis* Siebold et Zucc. (China)**	Aristolochic acids and their analogues.	Dried roots: treatment of dizziness, headache, abdominal pain, carbuncles, and snake and insect bites.
***Aristolochia manshuriensis* Kom. (China)**	Aristolochic acids and their analogues, tannin and β-sitosterol.	Dried stems: anti-inflammatory and diuretic for acute infections of the urinary system, and as emmenagogue and galactagogue for amenorrhoea and scanty lactation.
***Aristolochia fangchi* Y.C. Wu ex L.D. Chow & S.M. Hwang (China)**	Aristolochic acids and their analogues, *para*-coumaric acid, syringic acid, palmitic acid, allantoin and *β*-sitosterol.	In complex prescriptions as a diuretic for oedema and for antipyretic and analgesic remedies.
***Aristolochia clematitis* L. (Europe)**	Aristolochic acids I and II, as well as the allied aporphinoids alkaloids.	Different parts: stimulation of the immune system and in the treatment of allergically caused gastrointestinal and gall-bladder colic, gynaecological disorders and climacteric symptom; healing of wounds and ulcers in European Folk Medicine.
***Aristolochia indica* L. (India)**	Aristolochic acids and their analogues, monoterpenes, sesquiterpenes and *β*-sitosterol-*β*-D-glucoside	Roots: emmenagogue and abortifacient in Indian folk medicine

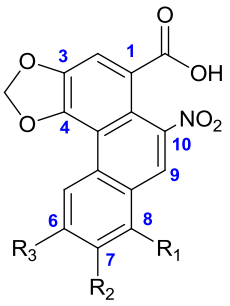
In African traditional medicine, the most abundant *Aristolochia* species used is *A. bracteolata* (Heinrich *et al.*, 2009).

II.1.5. Secondary metabolites isolated from Aristolochia genus and their biological activities:

The major secondary metabolites isolated from *Aristolochia* species were classified according to their chemical structures. They include aristolochic acids and esters, aristolactams and amides, aporphines, protoberberines, isoquinolines, flavonoids, lignans, biphenyl ethers, coumarins, tetralones, terpenoids and steroids (Kuo *et al.*, 2012).

II.1.5.1. Aristolochic acids and esters:

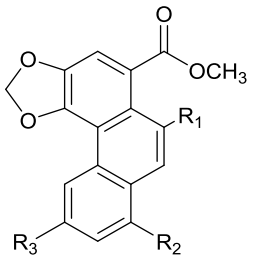
The naturally occurring aristolochic acids possess a 3,4-methylenedioxy-10-nitrophenanthrenic-1-acid skeleton. They are typical constituents of the *Aristolochia* genus and are claimed to be responsible for the various biological activities. Figure 22 lists aristolochic acids that have been described from *Aristolochia*. Aristolochic acid I is the most abundant aristolochic acid found in almost all *Aristolochia* species studied, with few exceptions (Chung *et al.*, 2011).

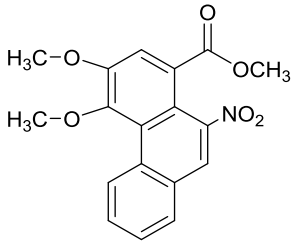


	R_1	R_2	R_3
Aristolochic acid I (A)	OCH_3	H	H
Aristolochic acid Ia	OH	H	H
Aristolochic acid II (B)	H	H	H
Aristolochic acid IIIa (C)	H	H	OH
Aristolochic acid IVa (D)	OCH_3	H	OH
7-Hydroxyaristolochic acid I	OCH_3	OH	H
Aristolochic acid VIIIa	H	OCH_3	OH
Aristoloside	H	Glc	OCH_3
Aristolochic acid F	H	OH	H
Aristolochic acid G	OH	H	OH

Figure 22. Aristolochic acids reported from the Aristolochia species.

In addition, seven methyl esters of aristolochic acid were reported from the *Aristolochia* species and among these only ariskanin A do not possess the 3,4-methylenedioxy substitution pattern. Only few cases of aristolochic acid esters, including aristolic acid methyl ester and 6-methoxyaristolic acid methyl ester, do not possess the nitro group at the C-10 position (Figure 23). The majority of these denitro-aristolochic acids were reported from *A. manshuriensis* (Chung *et al.*, 2011).





Ariskanin A

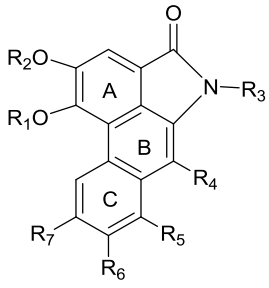
Aristolochic acid esters	R_1	R_2	R_3
Aristolochic acid I (A) methyl ester	NO_2	OCH_3	H
Aristolochic acid Ia methyl ester	NO_2	OH	H
Aristolochic acid IV methyl ester	NO_2	OCH_3	OCH_3
Aristolochic acid IVa (D) methyl ester	NO_2	OCH_3	OH
Aristolic acid methyl ester	H	OCH_3	OCH_3
8-Methoxyaristolic acid methyl ester	H	OCH_3	H

Figure 23. Aristolochic acids esters and ariskanin A structures.

Aristolochic acid I from *A. bracteolata* exhibited antibacterial activity against *Moraxella catarrhalis* with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of 25 and 50 µg/mL respectively (Mohamed *et al.*, 2014).

II.1.5.2. Aristolactams and Amides:

Aristolactams are regarded as biogenetic intermediates in the biosynthetic pathway of aristolochic acids. Twelve aristolactams have been reported from *Aristolochia* species (Figure 24) and among them six compounds present the 3,4-methylenedioxy substitution groups. Aristolactam I, aristolactam AII, and aristolactam Ia *N-β*-D-glucoside were the most frequently encountered aristolactams in the *Aristolochia* species.



Aristolactams	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Aristolactam I	$-CH_2-$		H	H	OCH_3	H	H
Aristolactam II	$-CH_2-$		H	H	H	H	H
Aristolactam AII	CH_3	H	H	H	H	H	H
Isoaristolactam AII	H	CH_3	H	H	H	H	H
Aristolactam IIa	$-CH_2-$		H	H	H	H	OH
Aristolactam AIIa	CH_3	H	H	H	H	H	OH
Cepharanone B or Aristolactam B II	CH_3	CH_3	H	H	H	H	H
Aristolactam E	H	CH_3	H	H	OH	OCH_3	H
Aristolactam AIIIa-6-*O*-β-D-glucoside	CH_3	H	H	H	H	H	OGlc
Aristolactam C *N*-β-D-glucoside	$-CH_2-$		Glc	OCH_3	OCH_3	H	OH
Aristolactam Ia *N*-β-D-glucoside	$-CH_2-$		Glc	H	OH	H	H
Aristolactam Ia 8-β-D-glucoside	$-CH_2-$		H	H	Glc	H	H
Aristolactam 9-*O*-β-D-glucopyranosyl-(1"$_2$)-β-D-glucoside	$-CH_2-$		H	OGlc-Glc	OCH_3	H	H

Figure 24. Aristolactams isolated from the Aristolochia species.

Aristolactam AII found in several species of *Aristolochia* is a simple aristolactam without any substitutions on rings B and C. The 9-oxygenated aristolactams are rare

in *Aristolochia* with only two compounds being reported: Aristolactam C *N*-*β*-D-glucoside and Aristolactam 9-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucoside (Kuo *et al.,* 2012).

Aristolamide and aristolamide II were isolated from *A. manshuriensis* (Chung *et al.*, 2011). They contain an amide group at C-1 in phenanthrene skeleton and represented a new class of amide reported from *Aristolochia* species (Figure 25).

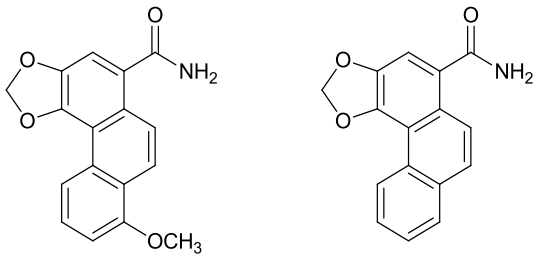


Figure 25. Amides isolated from A. manshuriensis.

Aristolamide II showed a selective inhibitory effect on elastase release by human neutrophils in response to fMLP with an IC_{50} value of 4.11 μg/mL. Aristolactam IIIa exhibited significant inhibitory effects on superoxide anion generation and elastase release with IC_{50} values of 0.12 and 0.20 μg/mL, respectively. Aristolochic acid-IVa also displayed anti-inflammatory effects on superoxide anion generation and elastase release with IC_{50} values of 5.78 and 8.49 μg/mL, respectively (Chung *et al*., 2011).

Considering anti-infectious potential, Aristolactam I exhibited bacteriocid activity against *Mycobacterium tuberculosis* strains with MIC values between 12.5-25 μg/mL (Navarro-García *et al.,* 2011).

II.1.5.3. Aporphines:

Several aporphine alkaloids have been characterized from *Aristolochia* species (Figure 26). Aporphines with *N*-formyl substitution, 6α,7-dihydro-*N*-formylnornantenine and *N*-formylnornantenine, were reported from *A. brevipes* (Navarro-García *et al.,* 2011). The polar quaternary aporphine magnoflorine was found in *A. elegans* and *A. gigantea*.

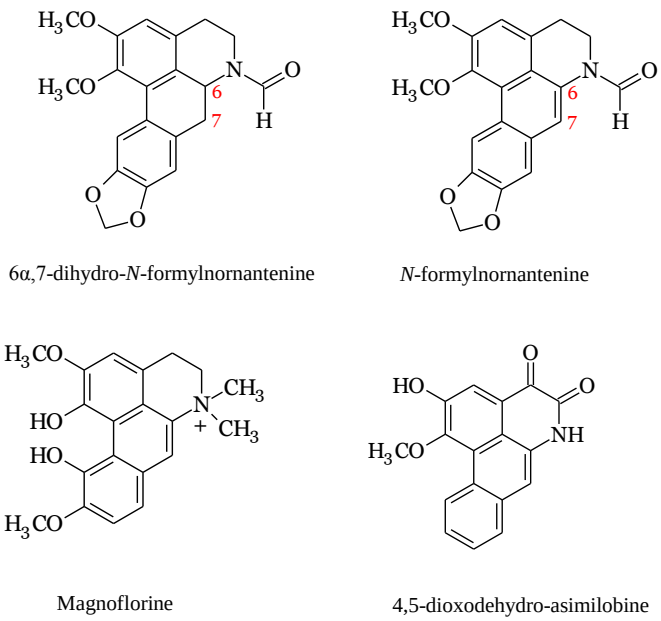


Figure 26. Aporphines isolated from the Aristolochia species.

The 4,5-dioxoaporphine is a small group of aporphine alkaloids found mostly among the *Aristolochiaceae* family and usually considered as possible precursors of aristolactams and aristolochic acids in plants. 4,5-Dioxodehydro-asimilobine was reported from *A. elegans* (Shi *et al.*, 2004).

Dimeric aporphines, as lagesianines B, C and D, were only reported from the leaves of *A. lagesiana* (Ferreira *et al.*, 2010) (Figure 27). Monomers are linked through the substituent on nitrogen, oxygenated functions, or substituent on the phenanthrene ring.

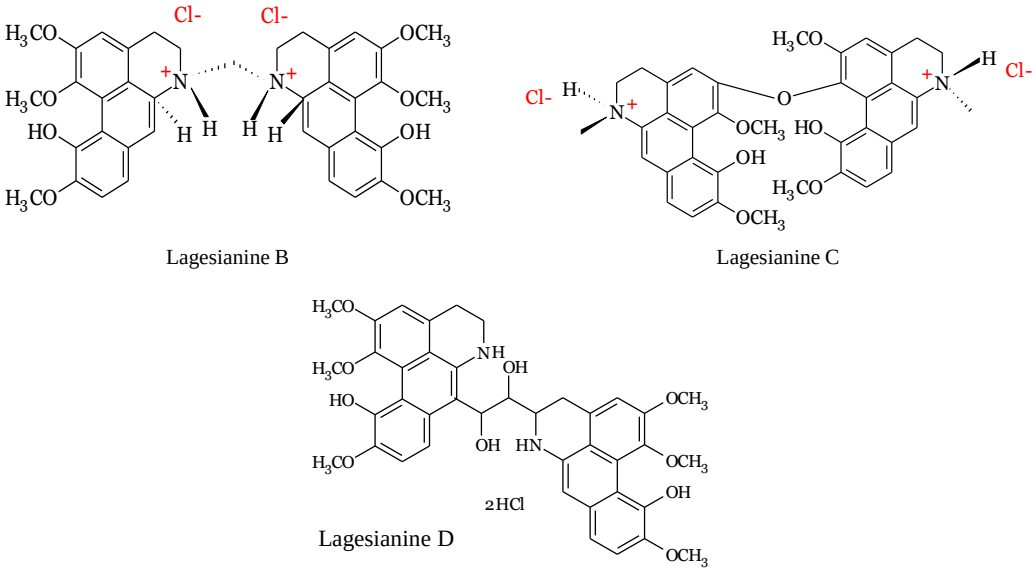


Figure 27. Dimeric aporphines isolated from A. lagesiana (Ferreira et al., 2010).

II.1.5.4. Protoberberines:

The occurrence of protoberberine alkaloids was rare in *Aristolochia*, and they were only reported from *A. constricta* (Capasso *et al.*, 2006). Benzyltetrahydroprotoberberine type alkaloid (Figure 28) in which a benzyl group is introduced at C-8 of berberine, result in an unusual carbon skeleton (Kuo *et al.*, 2012).

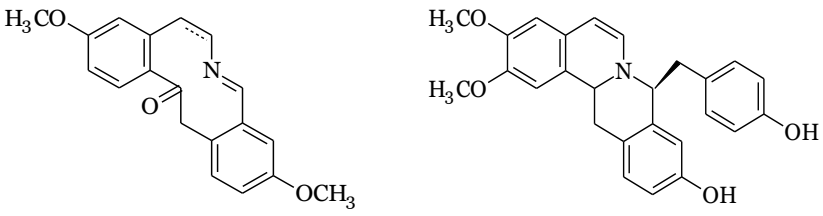
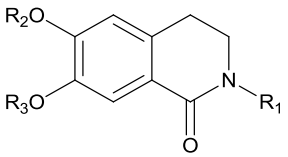


Figure 28. Protoberberines alkaloids isolated from A. constricta.

II.1.5.5. Isoquinoline:

The presence of isoquinoline alkaloids (Figure 29) in the genus *Aristolochia* is limited to *A. elegans*. All the alkaloids reported possess the tetrahydroisoquinolone skeleton. Isoquinoline alkaloids were usually considered as biogenetic intermediates in the catabolic process of bisbenzyl-tetrahydroisoquinoline alkaloids (Shi *et al.*, 2004).

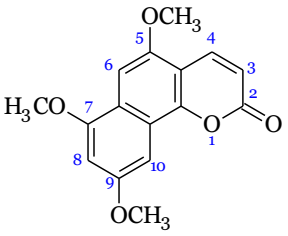


	R_1	R_2	R_3
Pericampylinone A	H	H	H
Corydaldine	H	H	CH_3
Thalifoline `	CH_3	H	CH_3
Northalifoline	H	CH_3	H
N-methylcorydaldine	CH_3	CH_3	CH_3

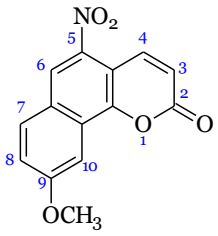
Figure 29. Isoquinolines isolated from A. elegans.

II.1.5.6. Coumarins:

Two coumarins, 7,9-dimethoxytariacuripyrone and 9-methoxytariacuripyrone (Figure 30), were characterized from the roots of *A. brevipes* (Navarro-García *et al.*, 2011).



7,9-dimethoxytariacuripyrone



9-Methoxytariacuripyrone

Figure 30. Coumarins isolated from A. brevipes.

These coumarins were studied for their antimycobacterial effects. The 7,9-dimethoxytariacuripyrone and 9-methoxytariacuripyrone showed antituberculous activity against all of the tested strains: sensitive, monoresistant, clinically isolated and MDR strain. Resulting MIC values were found between 25 and 50 µg/mL, but with the exception of *M. tuberculosis* H37RvIr laboratory strain, for which MIC value of 12.5 µg/mL was obtained with the 7,9-dimethoxytariacuripyrone (Navarro-García *et al.*, 2011).

II.1.5.7. Flavonoids:

Biflavones, chalcone-flavones, and a tetraflavonoid with a new carbon skeleton (Ridiculuflavonylchalcone C) were characterized from *A. ridicula* (Figure 31) (Machado and Lopes, 2005; Machado and Lopes, 2008).

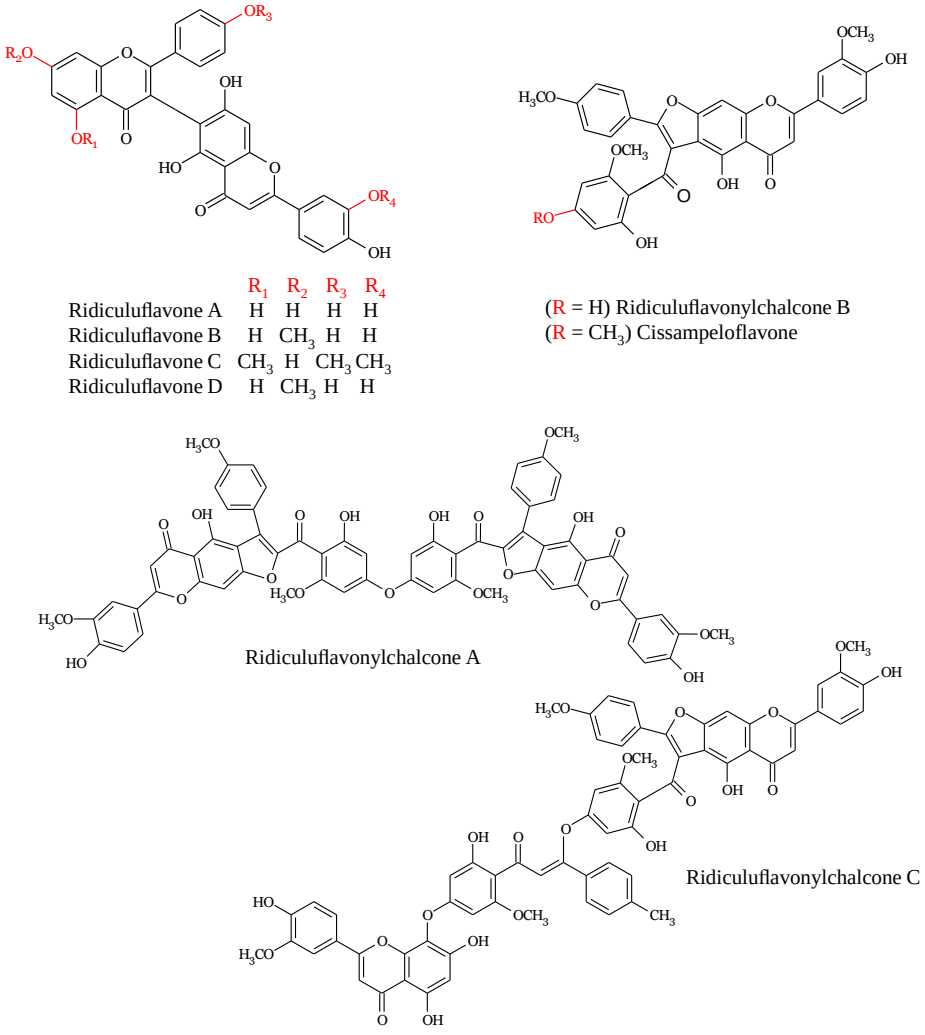


Figure 31. Flavonoids reported from A. ridicula.

II.1.5.8. Lignans:

Some 2,5-Diaryl-3,4-dimethyl-tetrahydrofuranoids were characterized from the roots of *A. arcuata* (Figure 32). They included talaumidin and its analogues, veraguensin, galgravin, aristolignin, nectandrin A, nectandrin B and isonectandrin B (Zhai *et al.*, 2004; Zhai *et al.*, 2005). Licarin A, licarin B, eupomatenoid-1 and eupomatenoid-7 (Figure 32), some benzofuran type lignans, were reported from *A. taliscana* (León-Díaz *et al.*, 2010; Jiménez-Arellanes *et al.*, 2012).

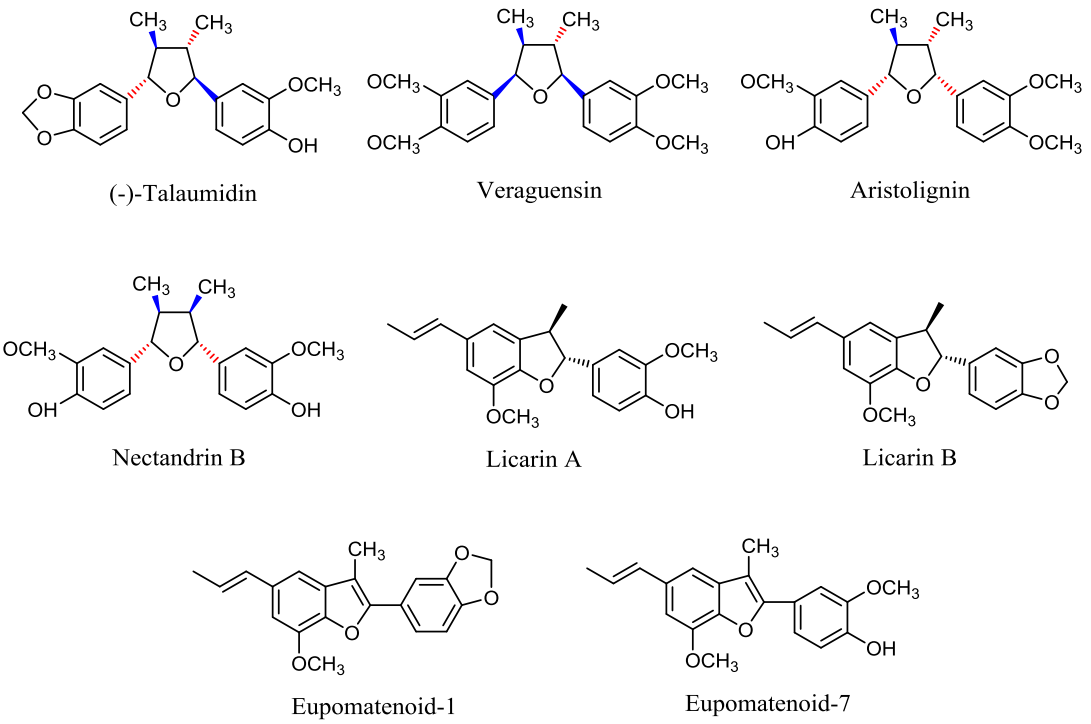


Figure 32. Lignans isolated from A. arcuata and A. taliscana.

The lignans purified from *A. arcuata*, talaumidin (Zhai *et al.*, 2004), galgravin, aristolignin, nectandrin A, isonectandrin B, and nectandrin B all exhibited neuroprotective bioactivity in primary cultured rat neurons with doses 1–20 μM (Zhai *et al.*, 2005).

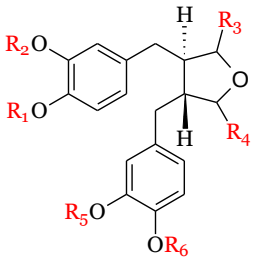
Three lignans, licarin A, licarin B, and eupomatenoid-7 reported from *A. taliscana* showed antimycobacterial activity. Licarin A was the most active compound, with MIC of 3.12-12.5 μg/mL against the following *Mycobacterium tuberculosis* strains: laboratory strain H37Rv, four mono-resistant H37Rv variants and 12 clinical Mutidrug-resistant (MDR) isolates, as well as against five non-tuberculous mycobacteria (NTM) strains.

Licarin B was moderately active against H37Rv and mono-resistant variants *M. tuberculosis* strains (MIC 25-50 µg/mL), but was highly active against the majority of MDR *M. tuberculosis* clinical isolate strains (MIC 12.5-50 µg/mL).

Eupomatenoid-7 was the most active against H37Rv strains (MIC = 25 µg/mL), the four mono-resistant variants of H37Rv and three of the MDR clinical isolates tested (MIC 12.5-25 µg/mL) (León-Díaz *et al.*, 2010).

Also lignan, eupomatenoid-1 from *A. elegans* exhibited antiparasite activity against *Entamoeba histolytica* and *Giardia lamblia* with $IC_{50} < 0.624$ µg/mL (Jiménez-Arellanes *et al.*, 2012).

3,4-Dibenzyldihydrofuran type lignans (Figure 33), (-)-cubebin, (-)-hinokinin, (-)-kusunokinin, (-)-pluviatolide and (-)-haplomyrfolol were isolated from *A. constricta* (Zhang *et al.*, 2008).



	R_1	R_2	R_3	R_4	R_5	R_6
(−)-cubebin	$-CH_2-$		◂OH	H	$-CH_2-$	
(−)-hinokinin	$-CH_2-$		=O	H	$-CH_2-$	
(−)-kusunokinin	$-CH_2-$		=O	H	CH_3	CH_3
(−)-pluviatolide	H	CH_3	=O	H	$-CH_2-$	
(−)-haplomyrfolol	H	CH_3	H	◂OH	$-CH_2-$	

Figure 33. Lignans isolated from A. constricta.

These lignans showed antispasmodic activity on electrically induced (ECI) and acetylcholine-induced (AChI) smooth muscle contraction in isolated guinea-pig ileum with doses 10, 30 and 100 µM (Zhang *et al.*, 2008).

II.1.5.9. Tetralones:

Four tetralones, aristelegones A, B, C and D, were characterized in the stems and roots of *A. elegans* (Wu *et al.*, 2002; Shi *et al.*, 2004). Aristelegone A and (+)-4,7-dimethyl-6-methoxy-1-tetralone were reported from the stems of *A. constricta* (Zhang *et al.*, 2008). Most of the identified tetralones possess a keto substituent at C-1, except for aristelegone D that presents 1,2-diol functionality (Figure 34).

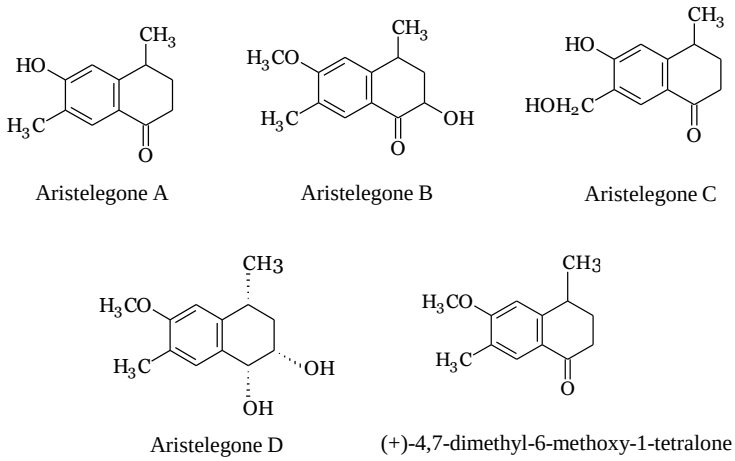
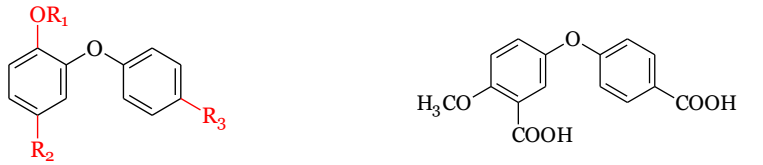


Figure 34. Tetralones isolated from various Aristolochia species.

II.1.5.10. Biphenyl ethers:

Biphenyl ethers were reported from *A. elegans* and were considered as one of the end products in the catabolic process of bisbenzylisoquinoline alkaloids (Shi *et al.*, 2004) (Figure 35).



	R_1	R_2	R_3
Aristogin A	CH_3	CHO	CO_2CH_3
Aristogin B	CH_3	CO_2CH_3	CHO
Aristogin C	CH_3	CO_2CH_3	CO_2CH_3
Aristogin D	H	CO_2CH_3	CO_2CH_3
Aristogin E	CH_3	CO_2CH_3	CH_2OH
Aristogin F	CH_3	CO_2H	CH_2OH

4-methoxy-3,4'-oxydibenzoic acid

Figure 35. Biphenyl ethers isolated from A. elegans.

II.1.5.11. Terpenoids:

Many terpenoids were isolated from *Aristolochia* species, which include monoterpenes, sesquiterpenoids and diterpenoids (Figure 36). The monoterpene 3-hydroxy-α-terpineol was identified from the roots of *A. brevipes* (Navarro-García, *et al.*, 2011). The sesquiterpenoid cadalene was isolated from *A. constricta* (Zhang *et al.*, 2008); aristololide (Shi *et al.*, 2004) and E-nerolidol from stems of *A. gigantea* (Holzbach and Lopes, 2010). Three types of C_{20} carbon skeletons constitute the largest group of diterpenoid metabolites in *Aristolochia* genus, which include 26 kauranes, 29 clerodanes and 9 labdanes diterpene derivatives (Pacheco *et al.*, 2009).

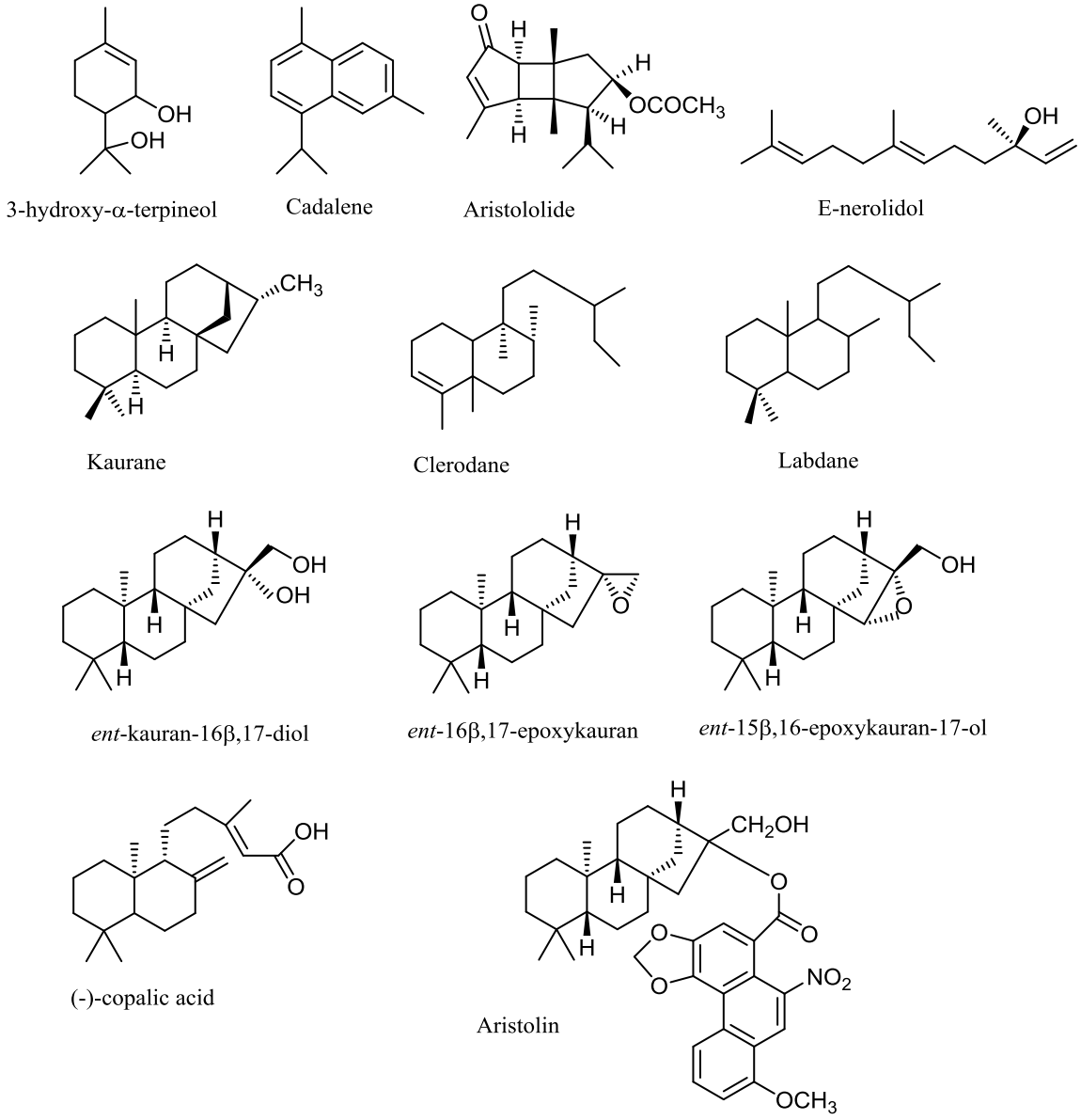


Figure 36. Terpenoids isolated from various Aristolochia species.

Labdane type (-)-copalic acid was isolated from *A. cymbifera* (Sartorelli *et al.,* 2010).

It was found that *Aristolochia* species constitute rich sources of *ent*-kaurane diterpenoids. The *ent*-kaurane diterpenoids, *ent*-kauran-16β,17-diol, *ent*-16β,17-epoxykauran and *ent*-15β,16-epoxykauran-17-ol were reported from *A. constricta, A. elegans* and *A. pubescens*. One *ent*-kaurane diterpenoid ester, aristolin was isolated from *A. elegans* (Shi *et al.,* 2004; Kuo *et al.,* 2012).

(-)-kusunokinin and (-)-copalic acid demonstrated anti-parasitic activity. They were active against trypomastigotes of *Trypanosoma cruzi* with IC_{50} values of 39 µM and 51 µM, respectively (Sartorelli *et al.,* 2010).

In this work, our study focused on *A. bracteolata*, the only *Aristolochia* described in Sudanese traditional medicine.

II.2. *ARISTOLOCHIA BRACTEOLATA:*

II.2.1. Geographic localization:

A. bracteolata Lam. is an indigenous species in Sudan. Several synonyms are known as *A. bracteata* Retz.; *A. sempervirens* Forssk.; *A. mauritania* Pers.; *A. kotschyi* Hochst. ex A. Rich.; *A. abyssinica* Klotzsch and *A. maurorum Klotzsch*.

Its vernacular native names are "Umm gala'gel; Abu Gelagil; Erg el Agrab; Wâlgya G'd; Gegbay; Mtangwa Mitu; Mkulie; Lessurulai and Larndenjal".

It is widespread in lowland plains and water catchment areas (El Gahazali *et al.,* 1994; De Groot *et al*., 2006). It was found in Burkina Faso, Niger, Nigeria, Chad, Ethiopia, Eritrea, Djibouti, Somalia, Uganda, Kenya, and Tanzania (Figure 20). Also it was reported on the Arabian Peninsula, Pakistan, India, and Sri Lanka (Shirwaikar *et al.;* 2003; De Groot *et al*.; 2006).

II.2.2. Botanical description:

A. bracteolata (Figure 37) is perennial herb, suberect to erect, with 10-60 cm tall. Stems are terete to angular and woody at the base. Internodes appear with long 0.7-5 cm. It ascends from a slender rootstock, numerously branched at the top.

Leaves are glaucous, ovate-cordate, 3-10.5 cm long and 2.5-7.5 cm wide. The apex is obtuse to acute, may be rounded and rarely retuse. The base is cordate with rounded auricles. Lamina is five-nerved, prominent beneath, minutely pubescent on both surfaces and less often glabrous. Margin is slightly crenate. Petiole appear with 0.3-6.5 cm long, glabrous.

Bracts are ovate-cordate, 0.3-1 cm in diameter, minutely pubescent and sessile. Its inflorescences are solitary, axillary and subtended by a leafy bract. Flowers are solitary, rarely two, axillary and found in nearly all axils. Perianth is green and glabrous on the outside, 1.9-4.3 cm tall and covered with reddish- brown to dark brown hairs on the inside. Limb is reddish-brown to dark purple. Peduncle is hairy to glabrous at the base with 0.21 cm long. Pedicel is glabrous with 0.3–2 cm long.

Ovary is oblong, hexagonal, glabrous and with 0.5 cm long. Utricle is stipitate, globose, ± 0.1 cm long and 0.4– 0.6 cm in diameter. Tube is funnel shaped, straight or slightly curved,

with 0.5–3 cm long and with 0.2–0.4 cm wide. Gynostemium is found with six anthers and six stigmatic lobes.

Fruit has capsules shape, ovate, glabrous, glaucous, hexagonal, brown in colour, opens at the base with six valves, with 1.5-3 cm long and mostly with 1.3-1.5 cm wide and splitting longitudinally.

Seeds are numerous, triangular- cordate, with 0.4-0.7 cm long, 0.4-0.6 cm wide, ± 0.1 cm thick, not winged, dark brown to black, flat and irregularly verrucate on both sides. Raphe has wedge shaped, with 0.5-0.7 cm long, 0.3-0.5 cm wide, winged, dark brown to black, shiny and rising above the top of the seed (El Gahazali *et al.*, 1994; De Groot *et al*., 2006).

Figure 37. Aristolochia bracteolata herb grown in Central Sudan (Agab, 1998).

II.2.3. Biological activities and traditional medicinal uses of A. bracteolata:

A. bracteolata is used in traditional medicine for its antibacterial, antifungal, antiprotozoal and antioxidant activities.

Leaves' methanol extract exhibited antibacterial activity against *Bacillus subtilis* (Kavitha and Nirmaladevi, 2007), *Staphylococcus aureus* and *Salmonella typhi* (Jebakumar *et al.*, 2011).

The whole plant methanol extract was found active against *Moraxella catarrhalis* (Mohamed, *et al.,* 2014).

Antifungal activity was observed with leaves aqueous extract against *Aspergillus terreus*, with leaves methanol extract against *Rhizopus stolonifer* (Kavitha and Nirmaladevi, 2009) and with whole plant extract against *Aspergillus mucor* (Jebakumar *et al.,* 2011).

Also, whole aerial parts chloroform extract demonstrated antiprotozoal activity against *Trypanosoma evansi* (Samia *et al.,* 2006).

Leaves ethanol extract possess a significant antioxidant and wound healing activity (Shirwaikar *et al.,* 2003).

A. bracteolata preparations have been used in traditional herbal medicine as antirheumatics, diuretics, in the treatment of edema, in wound healing, in obstetrics (to facilitate childbirth), and for other conditions such as hemorrhoids, cough, and asthma (NTP, 2008).

II.2.4. Secondary metabolites isolated from A. bracteolata:

Few papers reported phytochemical investigation of *A. bracteolata*. The preliminary phytochemical tests showed the presence of phenols, terpenoids, tannins, flavonoids, glycosides, carbohydrates in methanol extract (Das *et al.,* 2016) and essential oils (Gbadamosi and Egunyomi, 2012). The most abundant compounds isolated from *A. bracteolata* are Aristolochic acids I and II (Abdelgadir *et al*., 2011).

A. bracteolata was found to be richer in AA I and II than other *Aristolochia* species; we decided to focused on this class of metabolites for our investigation.

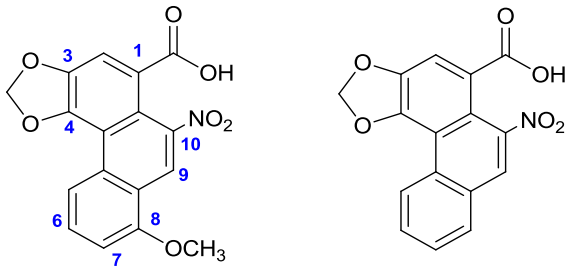
II.3. ARISTOLOCHIC ACIDS:

As mentioned before, aristolochic acids (AAs) are substituted 10-nitro-1-phenantropic acids. Aristolochic acids are highly toxic and carcinogenic agents (IARC, 2002; Jou *et al.,* 2004; Hsieh *et al.,* 2006; Yuan *et al.,* 2007). National Institute of Environmental Health Sciences (NIEHS) and the International Agency for Research on Cancer (IARC) reported that the naturally occurring aristolochic acids are compounds known to be human carcinogens.

The term “aristolochic acids” generally refers to an extract of *Aristolochia* species containing a mixture of aristolochic acid I (AA I) and its demethoxylated derivative, aristolochic acid II (AA II). In fact, the only AA commercially available are pure AA I and a mixture of AA I and II, which are then the most studied AAs. Since they are also the most abundant AA in *A. bracteolata* we focused mainly on these two AAs.

II.3.1. Structures, nomenclature and physicochemical properties:

The chemical abstracts index name of aristolochic acid I (Figure 38) is 8-methoxy-6-nitrophenanthro[3, 4-*d*]-1,3-dioxole-5-carboxylic acid. Its IUPAC name is 8-methoxy-6-nitronaphtho[2,1-*g*][1,3]benzodioxole-5-carboxylic acid. Its synonyms and trade names are aristinic acid, aristolochia yellow, aristolochic acid A, aristolochin, birthwort, aristolochine, Descresept, isoaristolochic acid, 8-methoxy-3,4-methylenedioxy-10-nitrophenanthrene-1-carboxylic acid, 3,4-methylenedioxy-8-methoxy-10-nitro-1-phenanthrenecarboxylic acid, Tardolyt and TR 1736 (IARC, 2002; NTP, 2008).



Aristolochic acid I
$C_{17}H_{11}NO_7$

Aristolochic acid II
$C_{16}H_9NO_6$

Figure 38. Chemical structure of aristolochic acid I and aristolochic acid II.

Aristolochic acid I is a yellow crystalline solid with molecular weight of 341.3 (Table 3).

Table 3. Physical and chemical properties of aristolochic acid I (NTP, 2008):

Property	**Information**
Molecular weight	341.3
Melting point (°C)	281–286
Solubility:	
-Water	Slightly soluble
-Acetic acid, acetone, aniline, alkalies, chloroform, diethyl ether and ethanol	Soluble
-Benzene and carbon disulfide	Practically insoluble

The chemical abstracts index name aristolochic acid II (Figure 38) is 6-nitrophenanthro[3,4-*d*]-1,3-dioxole-5-carboxylic acid. Its IUPAC name is 6-nitronaphtho[2,1-*g*][1,3]benzodioxole-5-carboxylic acid. Its synonyms are aristolochic acid B; 3,4-methylenedioxy-10-nitrophenanthrene-1-carboxylic acid; 3,4-methylenedioxy-10-nitro-1-phenanthroic acid and 3,4-methylenedioxy-10-nitro-1-phenanthrenecarboxylic acid (IARC, 2002; NTP, 2008).

Aristolochic acids I and II are the most common marker compounds used to evaluate the presence of the aristolochic acids family in plant samples (NTP, 2008).

II.3.2. Biosynthesis of Aristolochic acids and their analogs:

The concomitance of aristolochic acids, aristolactams and dioxoaporphines led to the assumption of a close biogenetic relationship between these three classes. A study was carried out for aristolochic acid I biosynthesis in *A. sipho* using tyrosine, dopa, dopamine and noradrenaline as specific precursors. It was found that the nitro group of aristolochic acid is derived from the amino group of tyrosine with (*R*)-orientaline or related benzylisoquinoline as precursors (Comer *et al.*, 1968).

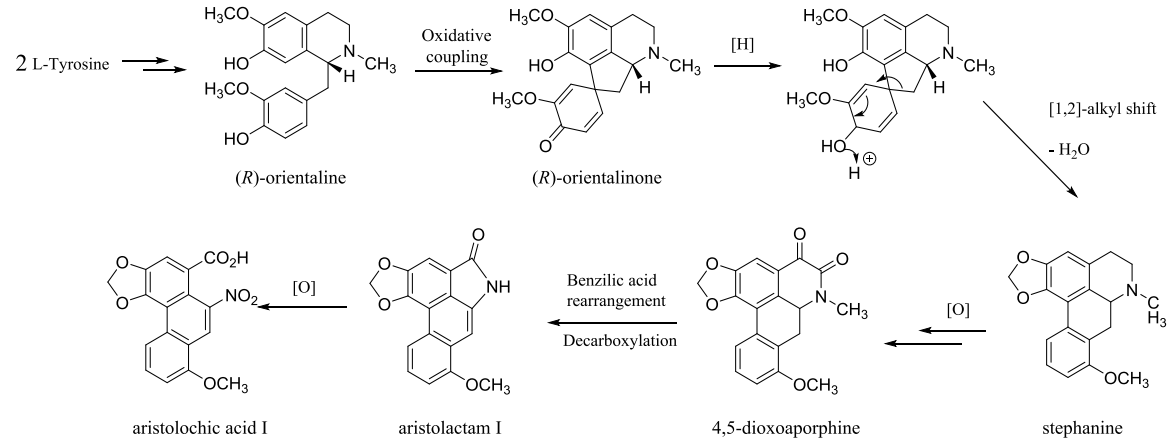


Figure 39. Biosynthesis of aristolochic acids and aristolactams.

In recent review, biosynthesis of aristolochic acid I started from the transformation of two molecules of the amino acid L-tyrosine into (*R*)-orientaline (Figure 39). A de-aromatizing spirocyclization of (*R*)-orientaline leads to (*R*)-orientalinone, which subjected to reduction of the ketone, gives the secondary alcohol. Then this latter undergoes a [1,2]-alkyl migration associated with loss of water. Re-aromatization and dioxolane formation gives stephanine. It is hypothesized that direct oxidation and *N*-methyl cleavage of stephanine forms 4,5-dioxoaporphine, which subjected to benzilic acid rearrangement and decarboxylation provides aristolactam I. Finally, oxidation of the amide leads to formation of aristolochic acid I (Speck and Magauer, 2013). The dioxoaporphines are thought to function as intermediates in the biosynthesis of aristolactams, which are precursors of aristolochic acids (IARC, 2002; Kumar *et al.*, 2003).

II.3.3. Aristolochic acids toxicity:

Aristolochic acids are absorbed from the gastrointestinal tract and distributed unchanged and/or in metabolized form throughout the body (IARC, 2012). They are evidenced by observation of specific DNA adducts in kidney, urinary tract, liver, lung, brain, stomach, and other tissues of humans and experimental animals (Schulz *et al.,* 1971; Schmeiser *et al.,* 1988; Stiborová *et al.,* 1999; Arlt *et al.,* 2004; Shibutani *et al.,* 2007). These adducts are specific markers of exposure to aristolochic acids I and II (Bieler *et al.* 1997).

Kidney is the primary target organ for aristolochic acid toxicity in both animals and humans (Mengs and Stotzem, 1993). Ingestion of herbal remedies containing aristolochic acids is associated with the development of a syndrome designated as aristolochic acid nephropathy (AAN), which is characterized by chronic renal failure, tubulointerstitial fibrosis, anemia and urothelial cancer (Vanherweghem *et al.,* 1993; Cosyns *et al.,* 2001; Tanaka *et al.,* 2001; Lee *et al.,* 2004; Shibutani *et al.,* 2007; Yuan *et al.,* 2007; Lai *et al.,* 2010). AAs are associated with elevation in serum creatinine, significant anemia, and histopathologic changes demonstrating a hypocellular interstitial infiltrate with severe fibrosis. Progression towards end-stage renal disease (ESRD) is rapid, with most patients having chronic kidney disease for less than 2 years. In addition, AAN is associated with a 40–45 % prevalence of urothelial carcinomas (Luciano and Perazella, 2015).

The doses of aristolochic acids required to induce severe tubular necrosis in rats and mice were found between 56 to 203 mg/kg orally and between 38 to 83 mg IV, of body weight. Also the dose 10 mg/kg of body weight needed to induce interstitial fibrosis in rabbits (Cosyns, 2003; Mengs, 1987). Most animal studies used purified aristolochic acids rather than the crude extracts or relatively unprocessed botanical material (NTP, 2008).

Aristolochic acids and their aristolactam derivatives are cytotoxic to oppossum kidney cell line (OK) growing in culture, including rat and human kidney cells and macrophages. The degree of toxicity varies according to cell type and chemical structure of the individual aristolochic acids or aristolactams (NTP, 2008; Tran and Hsiao, 2008).

Urothelial lesions associated with aristolochic acids nephropathy (AAN) are common when rats were fed with 0.1 mg, 1.0 mg, and 10 mg AA/kg body weight/ day for 3 months demonstrated a high incidence of tumors as 25%, 85%, and 100%, respectively. The organs found to have a high association with AA-induced tumor formation included the forestomach (77%), kidney (28%), and urinary tract (17%) as reported by Tran and Hsiao (2008).

The metabolic products of aristolochic acids have been found to be mutagenic and carcinogenic. The aristolactam-nitrenium ion intermediates from the nitro reduction process (Figure 40) are thought to be responsible for the carcinogenicity of AAs and formation of AA-derived DNA adducts (IARC, 2002; Chan *et al.,* 2007; Shibutani *et al.,* 2007; Tran and Hsiao, 2008). Also, AAVIIa and AAIa have shown cytotoxic effects, but to a lesser extent (Pena *et al.,* 1996).

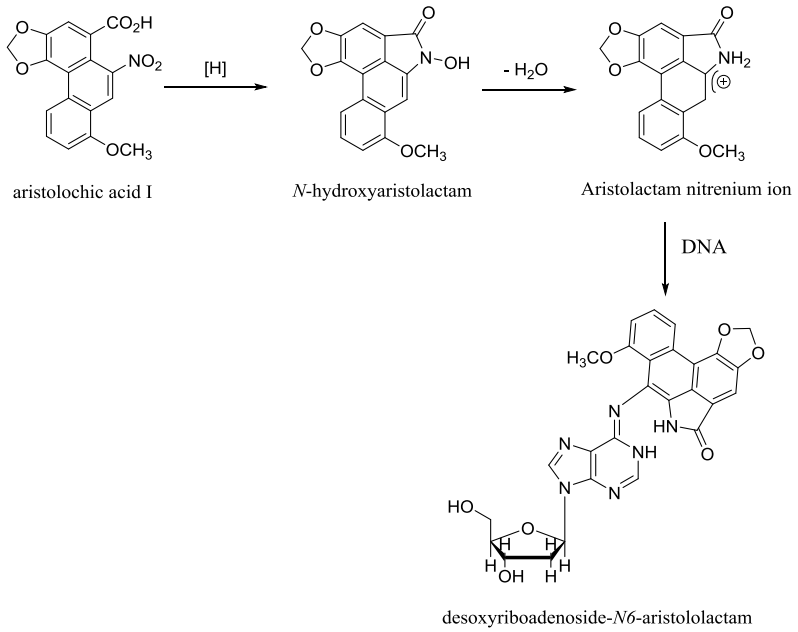


Figure 40. Pathway for the in vivo metabolic activation of aristolochic acids and formation of AA-derived DNA adduct.

Aristolochic acids, when activated by nitroreduction, are consistently active in genotoxicity tests *in vivo* and *in vitro* (Arlt *et al.,* 2002a; IARC, 2002). The major activation pathway involves reduction of the nitro group which is catalysed by several human cytosolic and microsomal enzymes such as hepatic and renal cytosolic NAD(P)H:quinone oxidoreductase (NQO1), hepatic microsomal cytochrome P450 (CYP) 1A2 and renal microsomal NADPH:CYP reductase-NQO1 (Stiborova *et al.,* 2008). In rodent tumours, the major DNA adduct formed by aristolochic acid (7-[deoxyadenosin-N^6-yl]aristolactam I) has been associated with the activation of H-*ras* proto- oncogenes through a specific CAA→CTA transversion mutation in codon 6 (Chen *et al.,* 2006; Shibutani *et al.,* 2007; IARC, 2012). There is significant relationship between cytotoxicity of AAs and functional groups in their structure. Balachandran *et al.* (2005) demonstrated the importance of functional groups in terms of their potency to exert toxic properties. The "nitro" group (-NO_2) and the "methoxyl" (-OCH_3) group were found to be critical in toxicity, whereas addition of hydroxyl group decreased the cytotoxicity. It

appeared that AA I was the most toxic followed by AA II, AA VIIIa, and AA Ia in decreasing levels of toxicity (Balachandran *et al.,* 2005; Tran and Hsiao, 2008).

II.3.4. Human exposure to aristolochic acids:

The risk of human exposure to aristolochic acids remains a global problem (NTP, 2008). *Aristolochia* and related plants have been used since ancient times in traditional herbal and contemporary medicines (Arlt *et al.,* 2002b; Jimenez-Ferrer *et al.,* 2005).

Individuals may potentially be exposed to aristolochic acids by ingesting plants and botanical products made from plants that contain these compounds or by ingesting herbal products contaminated with aristolochic acids (FDA, 2001a; IARC, 2002). In addition to that, exposure to aristolochic acids could result from contamination of wheat flour by seeds of *A. clematitis* growing in wheat fields in the Balkan states (Grollman *et al.,* 2007; De Jonge and Vanrenterghem, 2008).

Exposure to aristolochic acids has also been reported for other countries in which traditional herbal medicine didn't involve *Aristolochia* species, like the United States. Two cases of renal failure in USA have been linked to ingestion of herbal products containing aristolochic acids (Meyer *et al.,* 2000; Grollman *et al.,* 2007).

Herbal preparations containing aristolochic acids have also been used inadvertently as part of a weight-loss regimen (NTP, 2008). Progressive renal interstitial fibrosis frequently associated with urothelial malignancies, was initially reported in a Belgian cohort of more than 100 patients after the intake of slimming regimen based on a Chinese herb *Stephania tetrandra* containing in fact *A. fangchi* in the powdered extracts delivered in Belgium and in France (Vanherweghem, 1998 ; Debelle *et al. ,* 2008). In 1994 in France, two cases of end-stage renal failure (ESRF) related to Chinese herbs were reported similar to those incriminated in the case series reported in Belgium in 1992-93. Those cases led to an epidemiologic investigation of the risk linked to these plants in France. Studies carried out by the Pharmacy Inspection showed that powders labelled *S. tetrandra,* but consisting instead of *A. fangchi* and containing aristolochic acid, were sold in France between 1989 and 1994 (Stengel and Jones, 1998).

One case was reported in Spain after chronic intake of a tea made with a mixture of herbs containing *A. pistolochia*, an herb that was grown in the Catalonia region (Peña *et al.,* 1996).

Many other examples can be given concerning intoxication with AA: two cases in the United Kingdom after treatment of eczema with Mu Tong containing aristolochic acid (Lord *et al.,* 1999), 12 cases in Taiwan related to the use of various unidentified herbal medications for different purposes (Yang *et al.,* 2000), 12 cases in Japan (Tanaka *et al.,* 2001). A similar case

was reported in Germany after intake of Chinese herbal remedy (Akebia® 14, distributed as herbal food combination in both Europe and the United States) (Krumme *et al.*, 2001).

More than 100 suppliers of botanical products that potentially contain aristolochic acids have been identified in recent years (NTP, 2008). In 2001, the FDA issued warnings to consumers, health care professionals, and industry associations concerning herbal products containing aristolochic acids (FDA, 2001a; FDA, 2001b; FDA, 2001c; FDA, 2001d). Other countries, including the United Kingdom, France, Germany, Canada, and Australia, have banned these herbs (Kessler, 2000).

Nevertheless, botanical products potentially containing aristolochic acids are still available legally in other countries and can be bought *via* the Internet (Gold and Slone, 2003; NTP, 2008). The American Herbal Pharmacopoeia®, in collaboration with the American Herbal Products Association (AHPA), the Therapeutic Goods Administration of Australia (TGA), the State Food and Drug Administration of China (SFDA), has completed a monograph on selected plants that contain or may be adulterated with plants that contain aristolochic acids (American Herbal Pharmacopoeia, Aristolochic Acid Evaluation Monograph, 2010).

Aristolochia containing products still reach individuals readily and easily. In particular, it is recognized that language barriers prevent both consumers and distributers from effectively verifying the ingredients listed on many Chinese herbal products. This is particularly challenging when the herbs are sent as gifts from one country to another. Furthermore, the lack of authorities control enables individuals to purchase herbal products *via* the internet (e.g. for slim, beauty) with no valid prescriptions or expert advice from experienced herbalists. It is also difficult to control the legitimacy of merchants as well as the quality of the products. The lack of government regulation on herbal supplements opens the market to a wide overthecounter (OTC) array of natural herbal products. Finally, the crude preparation of herbal medicine, either in raw materials or in powder form, leave many opportunities for error and substitutions. The accidental dispensing of aristolochic acids containing herbs is further magnified because the compound is present in many botanical species.

Due to the serious identified toxic effects of aristolochic acids (AAs) and analogues, the present study was therefore carried out to isolate, characterize and analyze the content of aristolochic acids in *A. bracteolata* indigenous to Sudan.

II.3.5. Previous approach for extraction, fractionation and purification of aristolochic acids:

In the literature, some previous works reported isolation protocols of AA from diverse *Aristolochia spp.*

II.3.5.1. Column, Thin Layer and Preparative Thin Layer Chromatography:

Aristolochic acids from *Aristolochia spp.* were highlighted by different chromatographic methods including Column Chromatography (CC), Thin Layer Chromatography (TLC), Preparative Thin Layer Chromatography (PTLC) (Jou *et al.,* 2004; Ohno *et al.,* 2006).

TLC and PTLC chromatograms clearly demonstrated the presence of AA in *A. bracteolata*. Results also indicated that the obtained AAs produced varying chromatographic responses with different chromatographic solvent systems and/or locating agents (Table 4). Aristolochic acids generally acquired black color on fluorescent silica-gel in UV-light at 365 nm while they appear as yellow spots in visible light. Rf values ranged 0.43–0.46 (chloroform/methanol (6:1, v/v); silica-gel) and 0.83 (chloroform/methanol/water (6:4:1, v/v/v); cellulose) (Abdelgadir *et al*., 2011).

Table 4. Observed chromatographic responses of Aristolochic acids on TLC chromatograms:

Type of adsorbent	**Solvent system**	**Rf**	**Appearance of spots/bands**		
			Visible light	UV at 365 nm	0.5% DPA/ 60% H_2SO_4
Silica-gel 60	$CHCl_3$/MeOH (6:1, v/v)	0.43	Yellow	Black spot	Pale grey spot
Silica-gel G	$CHCl_3$/MeOH, (6:1, v/v)	0.46	Yellow	Faint brown spot	Pale grey spot
Cellulose	$CHCl_3$/MeOH/ Water (6:4:1, v/v)	0.83	Yellow	Faint brown spot	Pale grey spot

** DPA (Diphenylamine)*

II.3.5.2. High Performance Liquid Chromatography (HPLC):

Identification of AAs by HPLC was carried out by using mixtures of methanol and water (Sun *et al.,* 2001; Xie *et al.,* 2003; Jiang *et al.,* 2004; Guan *et al.,* 2005) or of acetonitrile and water as mobile phase (Sun *et al.,* 2001; Lu 2002; Geng and Li, 2005; Kotani *et al.,* 2014).

The presence of AA I and II in *A. bracteolata* was confirmed and quantified by HPLC using a mixture of 1% acetic acid and methanol (40:60, v/v) as mobile phase (Figure 41). Aristolochic acid II and Aristolochic acid I were detected as major peaks at a retention time of about 18.4 min and 27.9 min respectively and exactly corresponding to data profile exhibited by the

Aristolochic acid reference sample. HPLC data also showed that AA-II was represented in a higher calculated amount of 49.03g/kg compared to AA-I (12.98g/kg) in *A. bracteolata* whole plant. It is important to note that other *Aristolochia* species contain far lower amounts of AAs such as *A. debilis* that contains 0.18g/kg of AA-II and 1.01g/kg of AA-I; *A. manshuriensi* 1.0g/kg of AA-II and 8.82g/kg of AA-I; *A. fangchi* 0.22g/kg of AA-II and 2.22g/kg of AA-I (Abdelgadir *et al*., 2011).

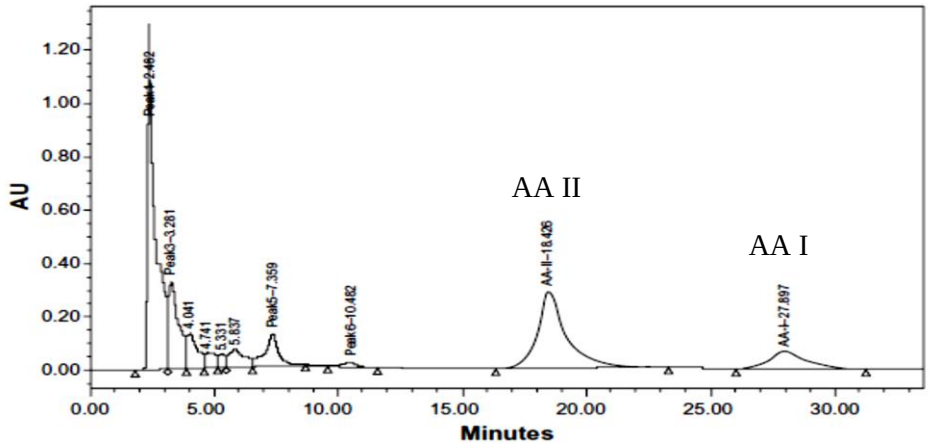


Figure 41. HPLC chromatogram of methanol extract sample of A. bracteolata. Condition: column, C_{18} (Varian® microsorb-MV 300-8 C18 250 × 4.6 mm), mobile phase, 1% acetic acid/methanol (40:60), temperature 20–25 °C, flow rate 1.0 ml/min, UV detection 250 nm (Abdelgadir et al., 2011).

AAs are now used to develop new nephrotoxicity models in zebrafish (Ding and Chen, 2012), in mice (Huang *et al.*, 2014) or to cause acute kidney injury (Roy *et al.*, 2014) in order to elucidate AA-specific renal toxicity mechanisms. So the need of pure AAs compounds is growing.

Isolation of those polar metabolites that exhibit closely-related structures by classical chromatography methods is usually time consuming and highly challenging, involving multistep process. Sequential solid/liquid extraction followed by two chromatographic steps of flash chromatography on silica gel, or on LH-20 resin and then semi-preparative HPLC or preparative TLC were usually reported to obtain pure AAs.

To isolate milligrams of different AAs, support-free liquid-liquid chromatography techniques such as CPC could be an innovative option.

II.4. PHYTOCHEMICAL STUDY OF *ARISTOLOCHIA BRACTEOLATA*:

Sudanese *A. bracteolata* was found to contain high amounts of aristolochic acids. CPC was then explored as an alternative tool for large scale isolation of AAs from *A. bracteolata* extracts.

First trials for purification of AAs were carried out in elution mode, since no reference in the literature reported isolation of aristolochic acids by countercurrent chromatography.

Whole dried *A. bracteolata* aerian parts were ground and then extracted with methanol to yield crude methanolic extract used for this work.

II.4.1. Preparation of crude extract:

Aristolochia bracteolata whole plant was collected during August 2013 from the Southern Gezira in Sudan and taxonomically identified in the Department of Pharmacognosy of the University of Gezira.

The powdered *A. bracteolata* whole plant was extracted by methanol in a Soxhlet apparatus and the solvent was then evaporated to dryness.

II.4.2. Elution approach:

CPC in elution mode was explored using two different types of solvent system: triphasic solvent system, and biphasic solvent system.

II.4.2.1. Triphasic solvent system:

Few studies have demonstrated that the use of a three-phase solvent system in CPC enables the separation of compounds exhibiting a large polarity range in one experiment. For example, CPE in the elution mode using the three-phase solvent system *n*-heptane/Methyl-tert-butylether (MtBE)/Acetonitrile (ACN)/H_2O (1:1:1:1, v/v/v/v) led to a rapid and effective fractionation of the bark crude extracts of *Anogeissus leiocarpus* Guill. & Perr. (Combretaceae) (Hamzaoui *et al.*, 2013).

In this work, CPC experiment for A. bracteolata whole plant crude methanol extract (2 g) was performed using triphasic solvent system n-heptane/MtBE/ACN/H2O (1:1:1:1, v/v/v/v). All fractions were analysed by TLC then similar fractions were combined together leading to six fractions (F1-F6) with moderate fractionation pattern. Aristolochic acids mixture was found in F1 (215 mg) (Figure 42). The main part of the mass (1.3 g) was found in the last hydrophilic fraction F6.

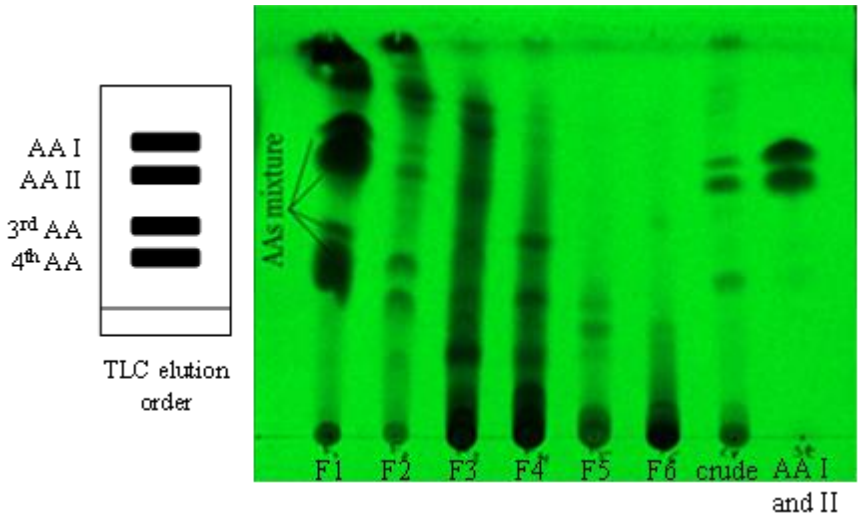


Figure 42. TLC chromatogram for CPC three-phase fractions at UV 254nm, Mobile phase CH_2Cl_2-MeOH (85:15, v/v).

Interestingly, all AAs were grouped in the first fraction, eluted with the most apolar mobile phase. After tin (II) chloride spray reagent, four major AAs were found: AA I and II according to Rf vs reference material, and two other AAs with Rf of 0.51 and 0.62 that needed to be identified. In the literature, only two major AAs (I and II) were reported, other AAs and AA derivatives were described as traces.

CPC experiment using triphasic solvent system was a rapid method to quickly access to aristolochic acids enriched fraction from *A. bracteolata* crude extract, but with a moderately low purity. Classical biphasic solvent systems were then explored to evaluate their selectivity compared to the triphasic system.

II.4.2.2. Biphasic solvent system:

Since AAs were eluted with apolar mobile phase in the previous experiment, apolar and medium polarity biphasic solvent systems were first screened by TLC. The main goal was to obtain distribution constant *K* values between 0.5 and 2 for AAs, and dramatically different for

polar and non polar impurities. *K* difference between the four AAs was also a selection criteria. Results of preliminary screening are presented in Table 5, *K* evaluation for the AA of Rf 0.62 was not included since it was almost not detected during solvent system screening (too low amount).

Table 5. Solvent system screening for elution CPC:

	Solvent system	**K of AA I**		**K of AA II**		**K of 4th AA**		**Conclusion**
		Up.	Low.	Up.	Low.	Up.	Low.	
A	*n*-heptane/MeOH/ACN (4:3:3)	-	+++	-	+++	-	+++	Not good
B	*n*-heptane/MeOH/ACN (4:5:1)	-	+++	-	+++	-	+++	Not good
C	*n*-heptane/MtBE/ MeOH/water (1:1:1:1)	-	+++	-	+++	+	+++	Not good
D	*n*-heptane/MtBE/ACN/MeOH/water (1:1:1:1:1)	-	+++	-	+++	-	+++	Not good
E	EtOAc/MeOH/water (4:2:4)	+++	-	+++	-	+++	+/-	Not good
F	EtOAc/MeOH/water (5:2:3)	+++	+	+++	+	+++	-	Not good
G	MtBE/EtOAc/MeOH/Water (1:3:2:4)	++++	-/+	++++	-	++++	-	Not good
H	MtBE/acetone/MeOH/water (2:1:1:2)	+++	+++	++++	++	++++	+++	Good
I	MtBE/acetone/MeOH/water (4:1:1:2)	++++	+	++++	+	+++	+	Fair

We first investigated apolar systems (A to D, Table 5). In these systems, AAs were partitioned only in the methanolic phase, which was not suitable for CPC. Heptane and MtBE seemed to have a "repulsive" effect toward AAs.

Systems with increased polarity were then tested (E and F, Table 5). The AAs were then partitioned only in the upper organic phase, rich in EtOAc. AAs exhibited high affinity for EtOAc phases.

To combine both "repulsive" effect of MtBE and "attractive" effect of EtOAc, a solvent system combining both solvent in the organic phase was explored (G). In fact, the attractive effect of EtOAc was largely dominating and then AAs were mainly partitioned in the organic phase.

EtOAc was then replaced by acetone, close in polarity but with more balanced partition between organic and aqueous phase (Systems H and I). In these systems, AAs were partitioned between the two phases for the first time. In system H MtBE/acetone/MeOH/water (2:1:1:2, v/v/v/v), resulting *K* values for all AAs were included between 0.5 and 2. This system was then tested in CPC, in descending mode (organic stationary phase).

CPC experiment was performed on crude methanol extract (2 g) in descending mode at 1800 rpm, with Sf of 55 % (Cf chapter I.4.3.). At the end of the experiment, fractions were analyzed by TLC. Similar fractions were combined together into seven fractions (F_1 to F_7) (Figure 43). The four aristolochic acids were isolated in fraction F_4 (157.3 mg).

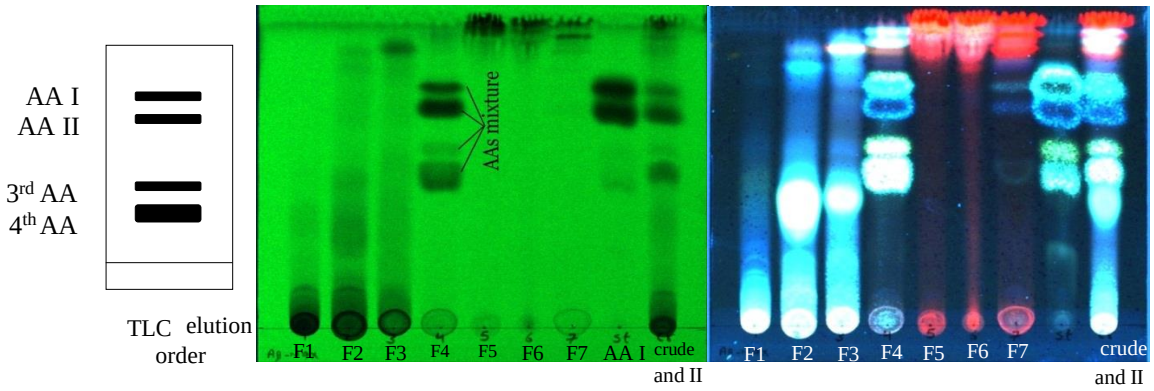


Figure 43. TLC chromatogram for CPC two-phase fractions at UV 254nm and UV 366nm with tin (II) chloride spray reagent, mobile phase CH_2Cl_2/MeOH (85:15, v/v), Réf: AA I and AA II mixture.

The AAs enriched fraction obtained with the biphasic solvent system contained less impurities than the enriched fraction resulting of triphasic CPC experiment (Figure 44). This could be easily explained by the fact that the retention volume of AAs in the biphasic system was higher than in the triphasic system experiment, allowing better purification.

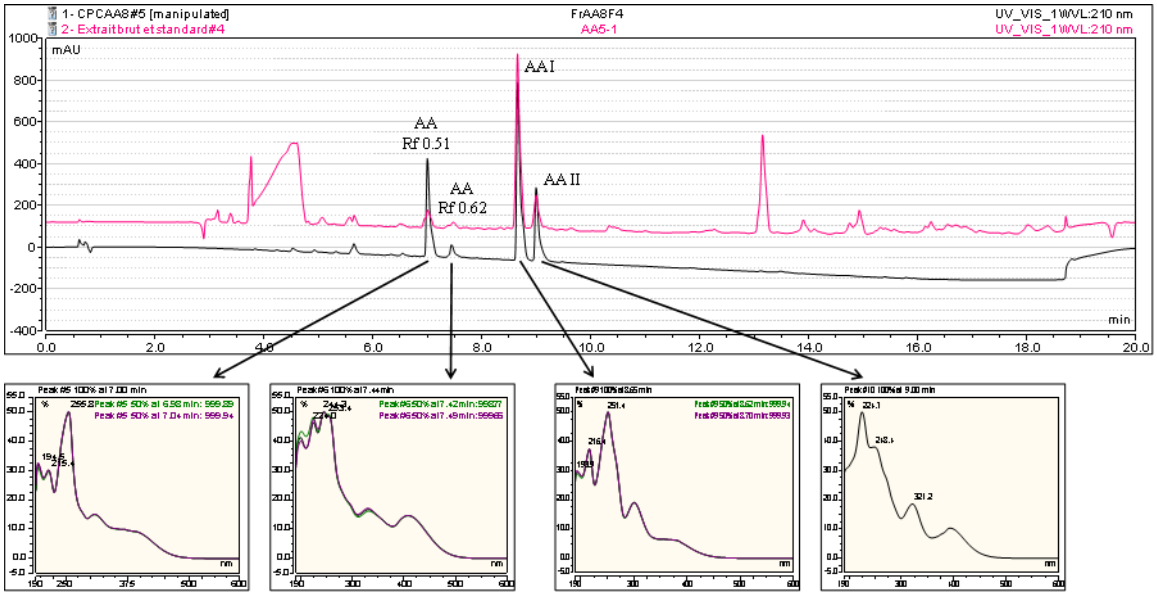


Figure 44. HPLC chromatogram at 210 nm of AAs enriched fractions F1 from triphasic CPC run (in pink) and F4 of biphasic CPC run (in black). UV spectra of each AA were shown at the bottom. Accucore aQ column, the mobile phases were solvent A 0.1% TFA in water, solvent B acetonitrile. The gradient

was set as follow: initial acetonitrile content 0%, raised to 100% in 15.25 min and maintained for 2 min; flow rate 1 mL.min^{-1}, oven temperature 40°C.

Elution CPC allowed the quick access to pre-purified AAs fractions. The fraction F4 was then subjected to further purification step to isolate pure AAs.

F4 was first subjected to flash chromatography with CH_2Cl_2/MeOH gradient mobile phase. At the end of elution process, fractions were analyzed by TLC and similar fractions were combined together into six fractions (Figure 45), F4-1 (4.6 mg), F4-2 (9.4 mg), F4-3 (4.4 mg), F4-4 (5.3 mg), F4-5 (13.7 mg) and F4-6 (24.3 mg).

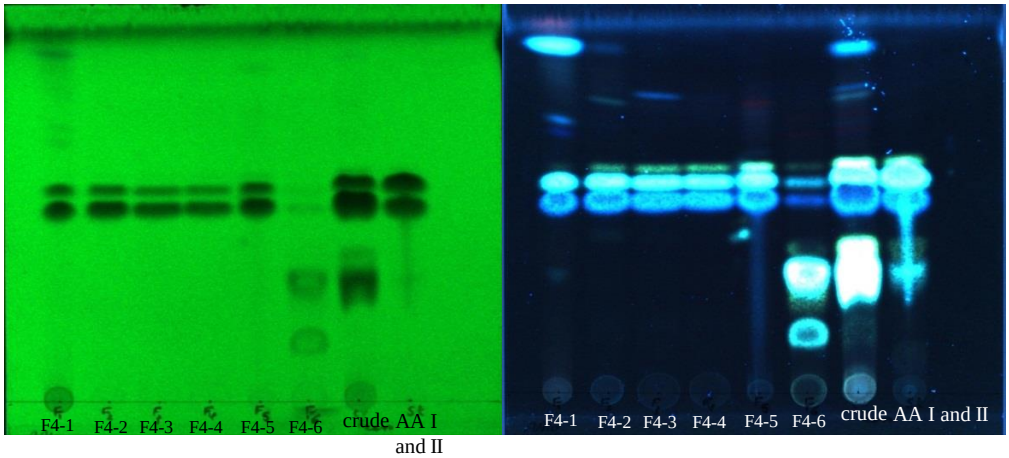


Figure 45. TLC chromatogram for flash chromatography fractions (F4-1-F4-6) at UV 254 nm and UV 366nm after tin (II) chloride spray reagent, mobile phase CH_2Cl_2/MeOH (85:15, v/v).

Fractions F4-1 to F 4-5 contained AA I and AA II, whereas AA of Rf 0.51 and 0.62 were grouped in fraction F4-6.

Fractions F4-1 to F4-5 were combined and subjected to PTLC using lower phase of $CHCl_3$/MeOH/water (13:7:2, v/v/v) as mobile phase. This experiment led to the isolation of 5.5 mg of AA I and 12.3 mg of AA II (Figure 46 on the left). Fraction F4-6 was also subjected to PTLC yielding 7.8 mg of AA of Rf 0.51 and 10.9 mg of AA of Rf 0.62 with moderately high purity (around 92%) (Figure 46 on the right).

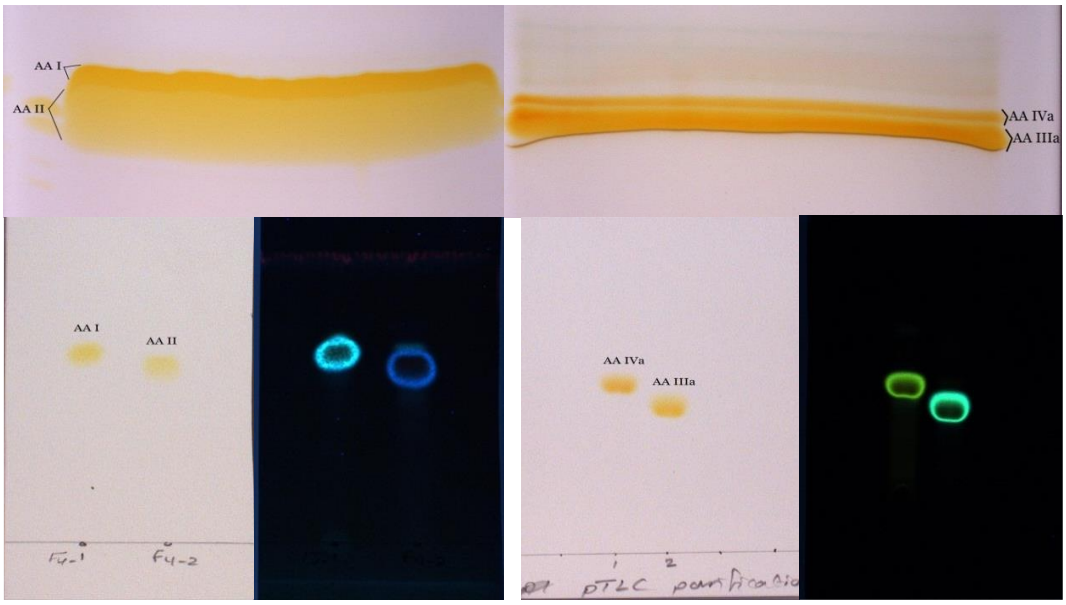
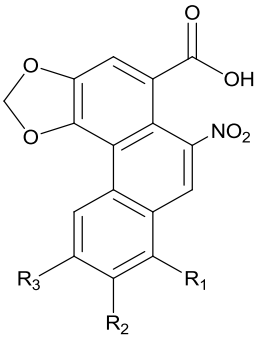


Figure 46. PTLC chromatogram for purification of fractions F4-1 to 5 and F4-6 at daylight on top, and TLC chromatogram of purified fractions at daylight and UV 366nm after tin (II) chloride derivatization; mobile phase $CHCl_3$/MeOH/water (13:7:2, v/v/v).

NMR analysis was performed on purified AAs, confirming the identification of AA I and II. AA of Rf 0.51 was identified as AA IIIa and AA of Rf 0.62 as AA IVa (Figure 47) (Appendix 1-6) by comparison with the literature NMR data (Leu *et al.*, 1998; Nascimento and Lopes, 2003; Zhang and Jiang, 2006; Cai and Cai, 2010; Pistelli *et al.*, 1993).



	R_1	R_2	R_3
Aristolochic acid I	OCH_3	H	H
Aristolochic acid II	H	H	H
Aristolochic acid IIIa	H	H	OH
Aristolochic acid IVa	OCH_3	H	OH

Figure 47. Structures of AAs isolated from A. bracteolata.

In this work, we identified AA IIIa and IVa in *A. bracteolata* for the first time.

As conclusion CPC in elution mode is a fast procedure to access to aristolochic acids enriched fraction, using triphasic or biphasic systems. Nevertheless, the recovery of pure AAs involved a tedious procedure including further purification by two steps: flash chromatography followed by PTLC.

As elution process was not discriminant enough to separate AAs exhibiting closely-related structures differing only by one or two substituents, isolation of those ionizable compounds was then tested using the Ion exchange approach.

II.4.3. Ion exchange approach:

II.4.3.1. Operating conditions selection:

In neutral solutions (pH = 7), AAs are present as anions ($pK_a \approx 3$), and then could interact with a cationic exchanger to form ion pairs. This property was exploited to perform IEX-CPC.

Several solvent systems were tested in order to obtain an AA-distribution, without exchanger, mainly in favor of aqueous phase (Table 6).

Table 6. Two-phase solvent systems screened for AA isolation by IEX CPC and solubility of the A. bracteolata crude extract in those systems:

Code	Solvent system (pH 7)	Solubility
A	$CHCl_3$/*n*-BuOH/H_2O (4.5:1:4.5, v/v/v)	Good
B	AcOEt/*n*-BuOH/H_2O (3:2:5, v/v/v)	Good
C	*n*-Heptane/MtBE/ACN/H_2O (1:1:1:1, v/v/v/v)	Poor
D	*n*-Heptane/MtBE/H_2O (1:1:1:1, v/v/v/v)	Poor
E	MtBE/Acetone/MeOH/H_2O (3:1:1:3, v/v/v/v)	Good
F	MtBE/Acetone/MeOH/H_2O (4:1:1:4, v/v/v/v)	Good
G	MtBE/Acetone/MeOH/H_2O (4:0.5:0.5:5, v/v/v/v)	Good
H	MtBE/MeOH/H_2O (2:1:2, v/v/v)	Poor
I	MtBE/MeOH/H_2O (3:1:3, v/v/v)	Poor
J	MtBE/MeOH/H_2O (4:1:4, v/v/v)	Poor
K	MtBE/MeOH/H_2O (4:0.5:5, v/v/v)	Poor

To develop an IEX-CPC solvent system, the target molecules, here AAs, had to be distributed only in the aqueous phase in exchanger-free solvent system. Also the solubility of the extract in the biphasic solvent system was a critical parameter to evaluate, since IEX-CPC was designed for large scale experiments (several grams up to 30 g in a 200 mL CPC column).

Classical SIX-CPC solvent systems were screened as $CHCl_3$/*n*-BuOH/water pH 7 (A) (Maciuk *et al.*, 2005), AcOEt/*n*-BuOH/water pH 7 (B) (Toribio *et al.*, 2012) or MtBE/MeOH/water pH 7 (H to K) at several ratios. Unfavorable distribution was obtained with systems A and B, with a distribution of AAs between the two phases. With systems C, D and

H to K, the distribution of AAs was suitable, but a poor solubility of the extract was observed (Figure 48).

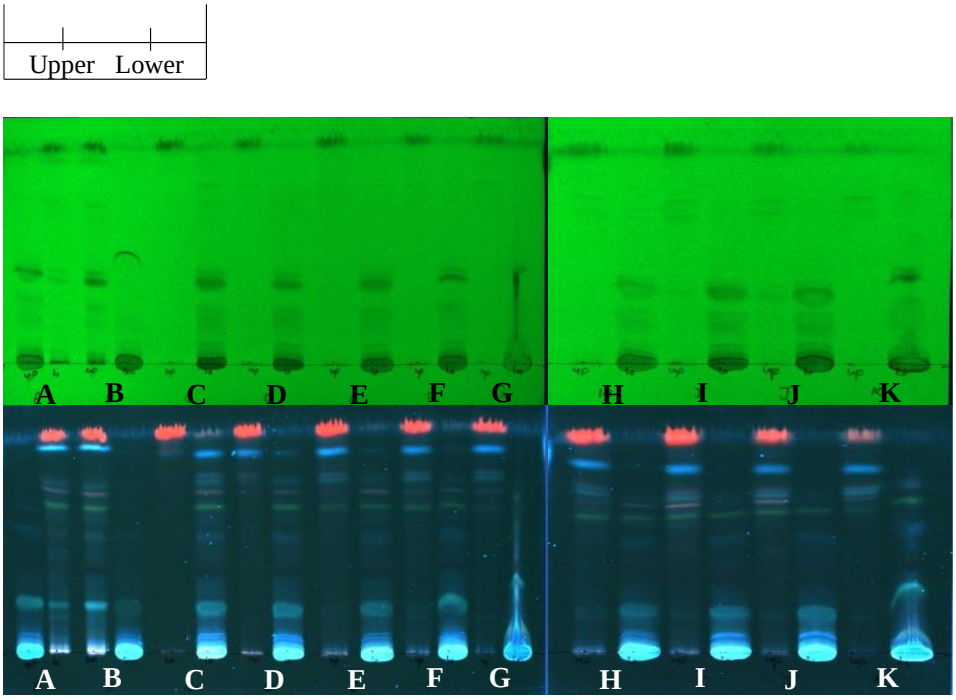


Figure 48. TLC chromatogram for two-phase systems (A-K) were tested for aristolochic acids distribution, up: upper phase, lo: lower phase.

Solvent system MtBE/acetone/MeOH/water allowed a good solubilization of the extract during elution study, we decided to try this solvent system but with decreasing volume of bridge solvent acetone and methanol, to reach favorable distribution for IEX (system H to J).

Finally, the solvent system E: MtBE/acetone/MeOH/water pH 7 (3:1:1:3, v/v) was selected allowing both the best solubilization of the crude extract and a favorable distribution of AAs.

Two cationic exchangers were screened, Aliquat 336® and benzalkonium chloride, which were already used to purify glucosinolates (Toribio *et al.*, 2012) and rosmarinic acid (Maciuk *et al.*, 2005) respectively. As shown in

Figure 49, Al336 was found to be the most suitable to extract AAs from the aqueous phase, while benzalkonium only partially extracted AAs.

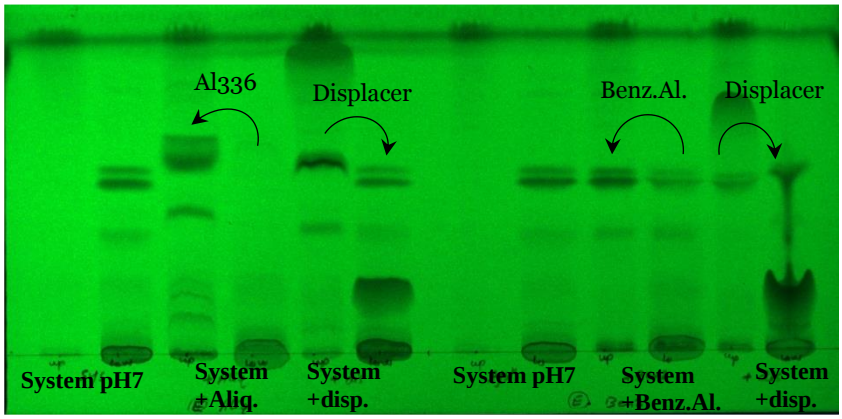


Figure 49. TLC chromatogram for testing Al336 and benzalkonium.

It is well known now that ion exchangers could disturb biphasic solvent system stability, especially Al336 (Toribio *et al.*, 2007). To investigate the Al336 influence on the MtBE/acetone/MeOH/water pH 7(3:1:1:3, v/v) solvent system, pseudo-ternary diagram of organic phase/aqueous phase/Al336 was built (Figure 50).

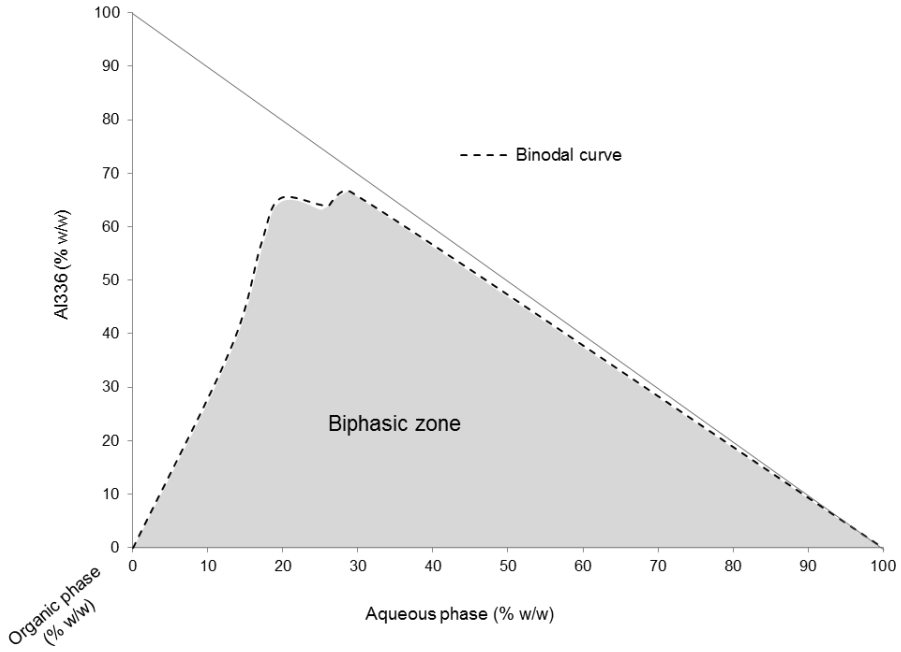


Figure 50. Pseudo-ternary diagram of the system ionic exchanger—aqueous phase–organic phase (in %, w/w) for the initial biphasic system MtBE/acetone/MeOH/water pH 7 (3:1:1:3, v/v).

As shown in Figure 50, Al336 has a minor disturbing effect on biphasic system MtBE/acetone/MeOH/water (3:1:1:3, v/v), allowing addition in large amounts of the exchanger (up to 50 % (w/w)), which is favorable to pilot scale. At this point solvent system MtBE/acetone/MeOH/water pH 7 (3:1:1:3, v/v) and cationic exchanger Al336 were selected. The last actor to cast was the displacer. In SIX-CPC, the displacer is a lipophilic ion, a lipophilic anion here. Sodium iodide, classicaly used as displacer with Al336, was successfully tested as displacer for AAs and then selected (

Figure 49).

II.4.3.2. SIX-CPC Optimization:

SIX-CPC optimization includes the determination of two ratios: the exchanger (Al336)/displacer (NaI) ratio and the exchanger (Al336)/analytes (AAs) ratio.

II.4.3.3. Optimization of Al336/NaI ratio:

As starting point, the Al336/AAs ratio was set at 2, since an excess of exchanger was usually needed to achieve a total extraction during equilibration/washing step. The Al336/NaI ratio was then optimized (see Runs 1- 5, Table 7).

The AAs content in crude extract was estimated, as (~3% = 0.9 mmol) according to the mean yield of AAs mixture (30 mg) eluted in (1 g) crude extract in CPC elution mode.

Table 7. Experimental conditions for Al336/NaI ratio optimization:

CPC run	**1**	**2**	**3**	**4**	**5**	**6**
Crude extract sample	1 g	1 g	1 g	1 g	1 g	1 g
Estimated content of AAs	≈ 3 % (w/w)					
Biphasic solvent system	MtBE/acetone/MeOH/water (3:1:1:3, v/v)					
Pumping mode	Descending					
Al_{336} concentration in organic phase	1.25 mM					
Washing step duration	65 min	65 min	65 min	65 min	65 min	60 min
NaI in aqueous phase	1.25 mM	0.625 mM	0.31 mM	0.125 mM	0.06 mM	0.02 mM
Estimated Al_{336}/AA ratio	2					
Al_{336}/NaI ratio	1	2	4	10	20	62.5
Stationary phase retention	70-73 %					
Back pressure	20-24 bars					
Collection time	1 min (2 mL)					

The Al_{336}/NaI ratio was first set at 1 (Run 1). Resulting CPC chromatogram and TLC fractogram are presented in Figure 51.

A mixing zone between all four AAs was observed (13.5 mg), followed by a mixing zone between AA I and AA II (51 mg). AA IIIa was isolated in small amount (2.2 mg). AAs were eluted rapidly (29 min) as a shock layers series, showing an excess of displacer.

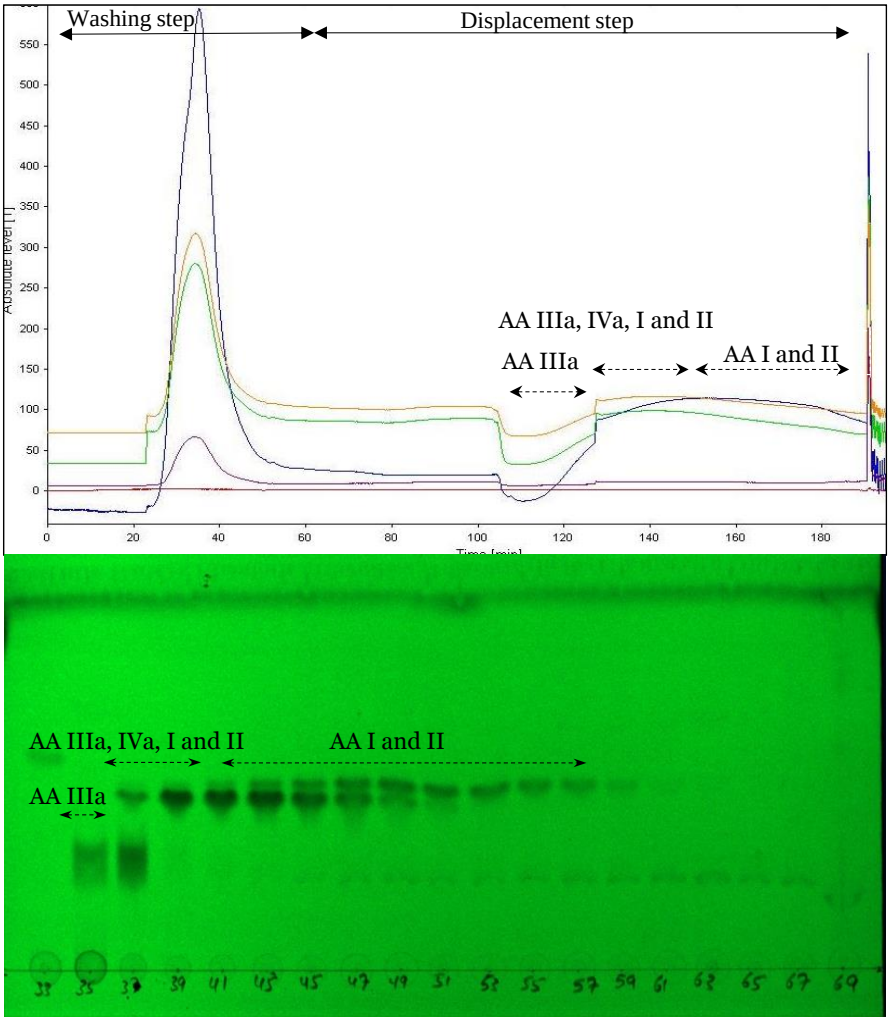


Figure 51. CPC chromatogram and TLC fractogram for IEX-CPC run 1.

In Run 2, the Al336/NaI ratio was then increased to 2. Resulting CPC chromatogram and TLC fractogram are presented in Figure 52.

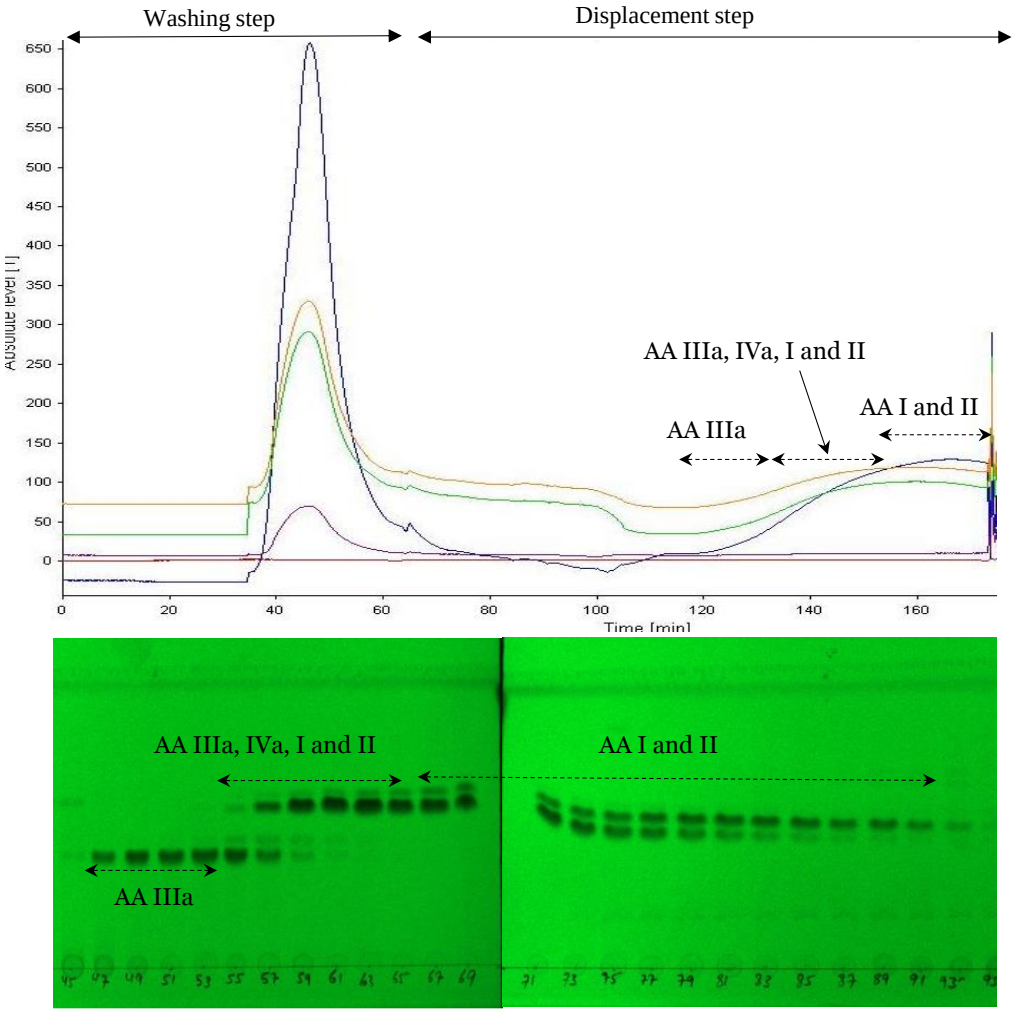


Figure 52. CPC chromatogram and TLC fractogram for IEX-CPC run 2.

The Al336/NaI ratio tested in this run led to isolation of a small amount (3.7 mg) of AA IIIa with high purity (96%), other AAs were eluted in mixture. The TLC-fractogram (Figure 52) of the eluent showed that AAs were eluted again as a series of shock layer, highlighting still an excess of displacer. It was important to note that elution duration was doubled reaching 50 min.

In the run 3, the Al336/NaI ratio was set at 4. Resulting CPC chromatogram and TLC fractogram are shown in Figure 53.

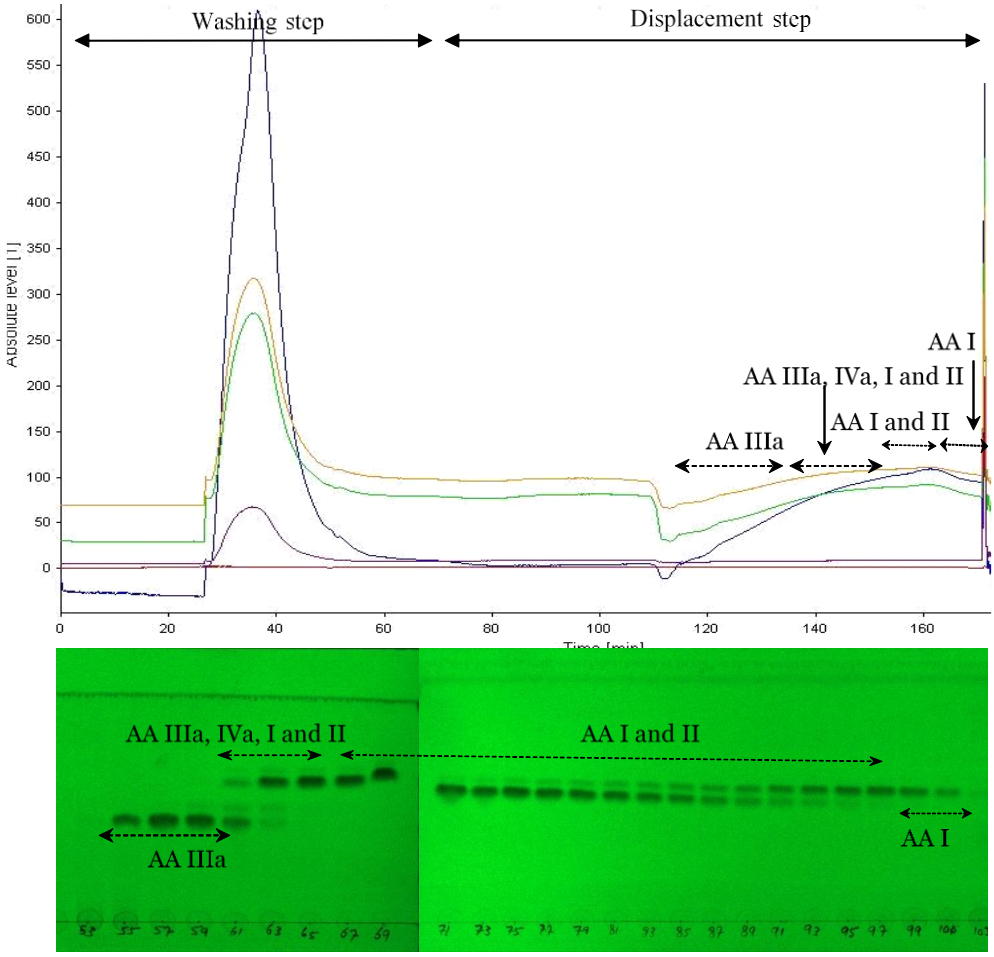


Figure 53. CPC chromatogram and TLC fractogram for IEX-CPC run 3.

The Al336/NaI ratio tested in this run led to similar separation performances than run 2, considering the mixing zone of the 4 AAs. Elution duration was extended to 60 min and a small amount of AA I (2.5 mg) was isolated for the first time. Anyway the displacer amount had still to be decreased.

The Al336/ NaI ratio was then set at 10 (Run 4). TLC fractogram is shown in Figure 54.

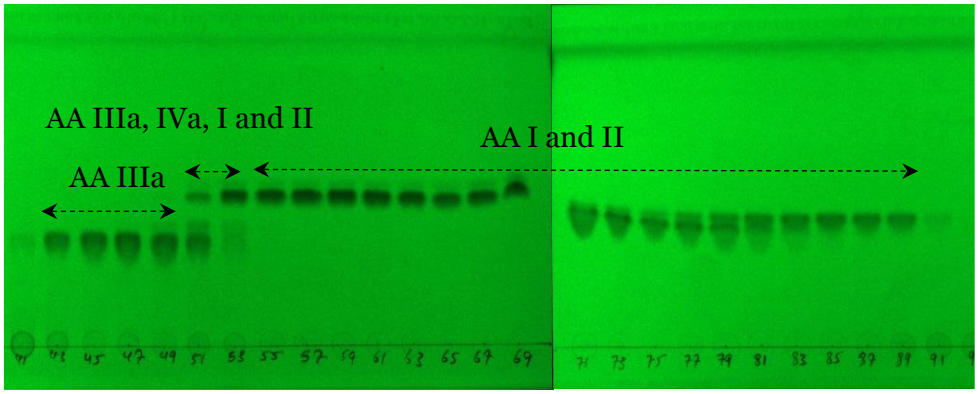


Figure 54. TLC fractogram for IEX-CPC run 4.

Using this ratio, AA IIIa and AA I were obtained in quite larger amount (4.8 and 3.6 mg respectively). Moreover, the mixing zones between the four AAs was reduced (5.3 mg) compared to run 4 (14.2 mg).

Again the Al336/NaI ratio was increased ten fold compared to initial conditions to be set at 20 (Run 5). Resulting CPC chromatogram and TLC fractogram were shown in Figure 55.

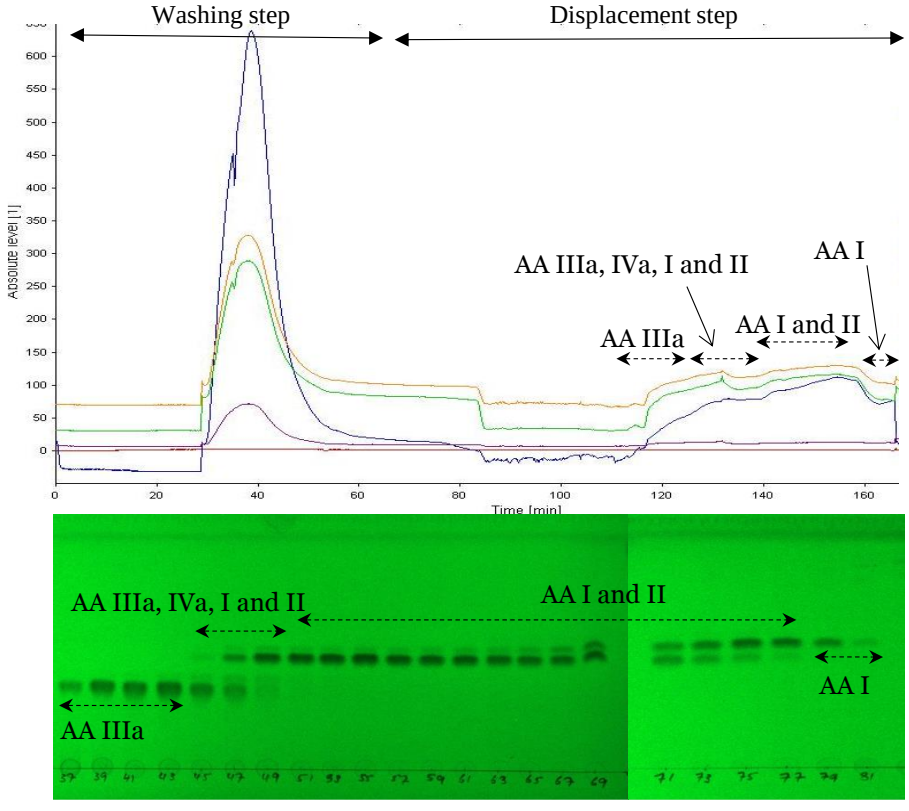


Figure 55. CPC chromatogram and TLC fractogram for IEX-CPC run 5.

Again, the AA IIIa (4.9 mg) and AA I (8.5 mg) were isolated with purity superior to 95 %, in small amounts. Elution duration was stable around 55 min. The mixing zone between all four

AAs was reduced (2.3 mg), but high mixing zones between AA IIIa and AA IVa, and between AA I and II still remain.

Displacer concentration was decreased again, reaching an Al336/NaI ratio of 62.5 (Run 6) without any improvement of AAs separation. The same mixing zone were observed (Figure 56).

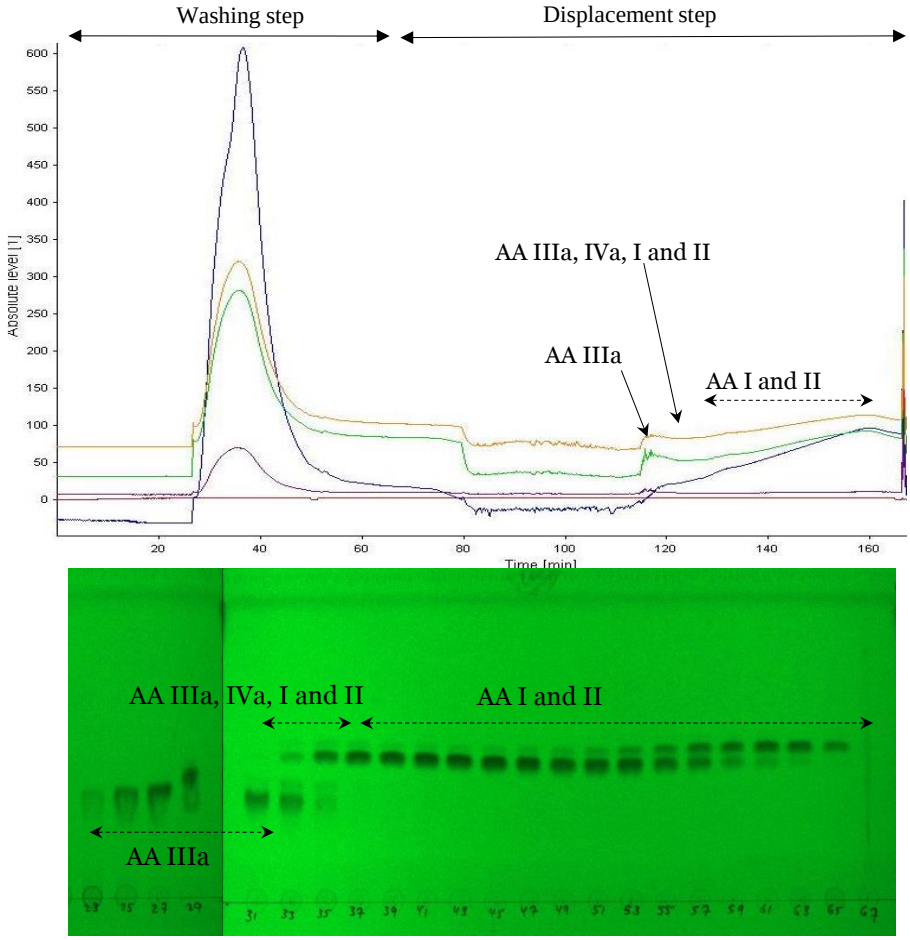


Figure 56. CPC chromatogram and TLC fractogram for IEX-CPC run 6.

Therefore, the displacer does not seem to be responsible for the poor efficiency of the purification process. Thus, the ratio was kept at 20. One hypothesis is that the exchanger concentration is not sufficient to achieve an optimal organization of the isotachic train during the washing step. The lack of exchanger could not allow proper analytes extraction along the whole column. The AAs would then emerge from the column following both displacement and elution mechanisms explaining the important mixing zones. By increasing the Aliquat 336® concentration, the number of theoretical plates would increase as well as the resolution.

II.4.3.4. Optimization of Al336/AA ratio:

Two runs (7-8) were carried out for optimization of Al336/AA as shown below in Table 8.

Table 8. Experimental conditions of IEX-CPC runs for optimization of Al336/AAs ratio:

CPC run	**7**	**8**	**9**
Crude extract sample	1 g	1 g	5 g
Estimated content of AAs	≈ 3 % (w/w)		
Biphasic solvent system	MtBE/acetone/MeOH/water pH 7 (3:1:1:3, v/v)		
Pumping mode	Descending		
Al336 concentration in organic phase	2.4 mM	3.6 mM	18 mM
Washing step duration	60 min	50 min	50 min
NaI in aqueous phase	0.12 mM	0.18 mM	0.9 mM
Estimated Al336/AA ratio	4	6	6
Al336/NaI ratio	20		
Stationary phase retention	70-73 %		
Back pressure	20-24 bars		
Collection time	1 min (2 mL)		

Increasing the Aliquat 336®/AAs ratio to 4 (Run 7), the purification of AA IIIa (4.2 mg) and AA I (11.1 mg) was slightly improved (Figure 57).

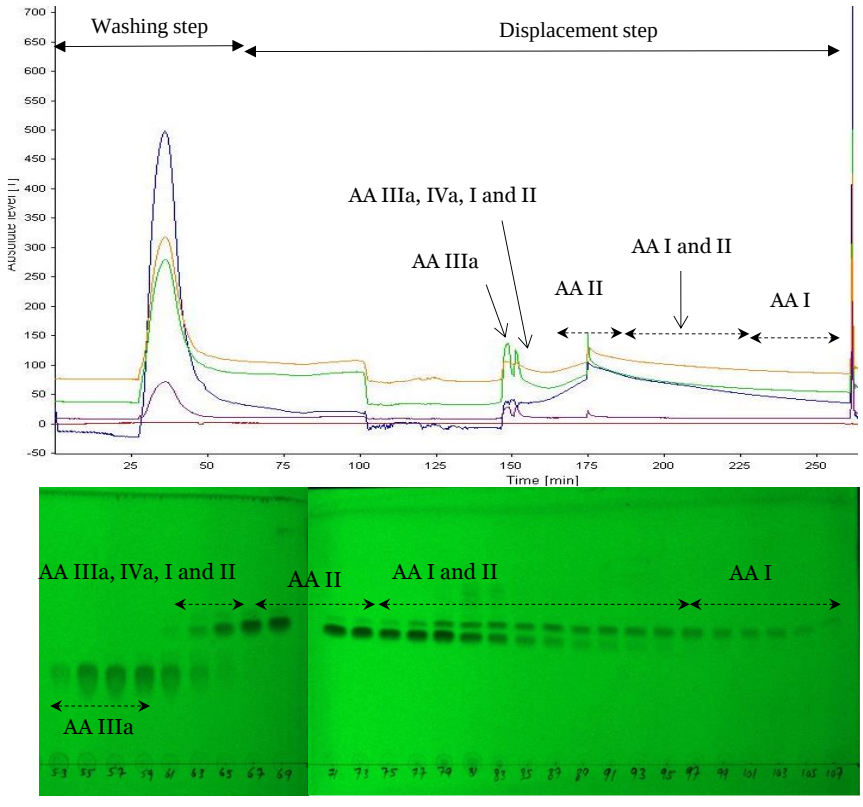


Figure 57. CPC chromatogram and TLC fractogram for CPC run 7.

A latency period between AAs and other lipophilic metabolites elution was visible, but large mixing zones were still noticed–especially between AAs II, IIIa and IVa (4.5 mg).
The Al336 concentration was thus increased again (Run 8) to reach an Al336/AAs ratio of 6 (Figure 58).

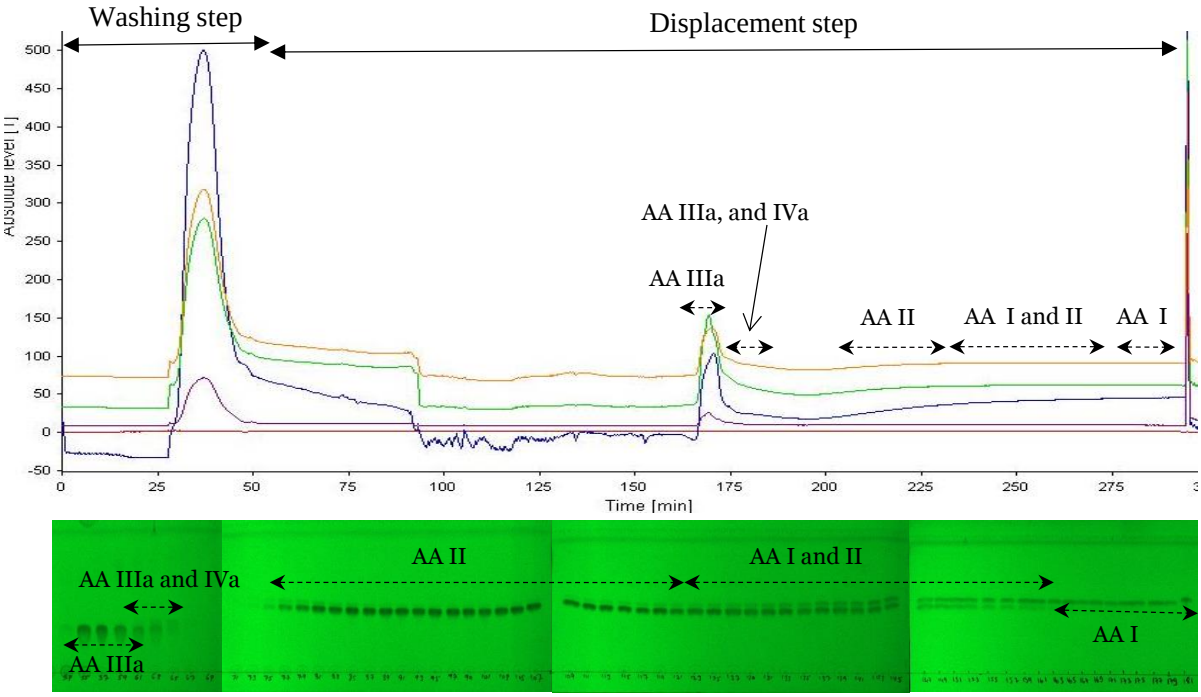


Figure 58. CPC chromatogram and TLC fractogram for CPC run 8.

Under these conditions, we were delighted to find excellent separation between AAs IIIa and IVa, and AAs I and II, which was achieved for the first time. Moreover, AAs IIIa (7.2 mg), II (7 mg) and I (11.5 mg) were mainly isolated with purity superior to 95 %. AA IVa was obtained as a mixture with AA IIIa, probably due to its small amount insufficient to form a large isotachic zone. These results illustrated the high resolution of SIX-CPC allowing the one step separation of AAs I, II and IIIa that differ from each other by only one methoxyl group.

II.4.3.5. SIX-CPC Scale Up:

A scale up was then performed with a 5 g sample injection (Run 9, Table 8). As usually observed in displacement CPC, separation was improved leading to isolation of AA IIIa (35.1 mg, purity 98.7 %), AA II (77.2 mg, purity 96.7 %) and AA I (45.5 mg, purity 95.0 %) in one step. AA IVa was still obtained in mixture with AA IIIa (See Figure 59).

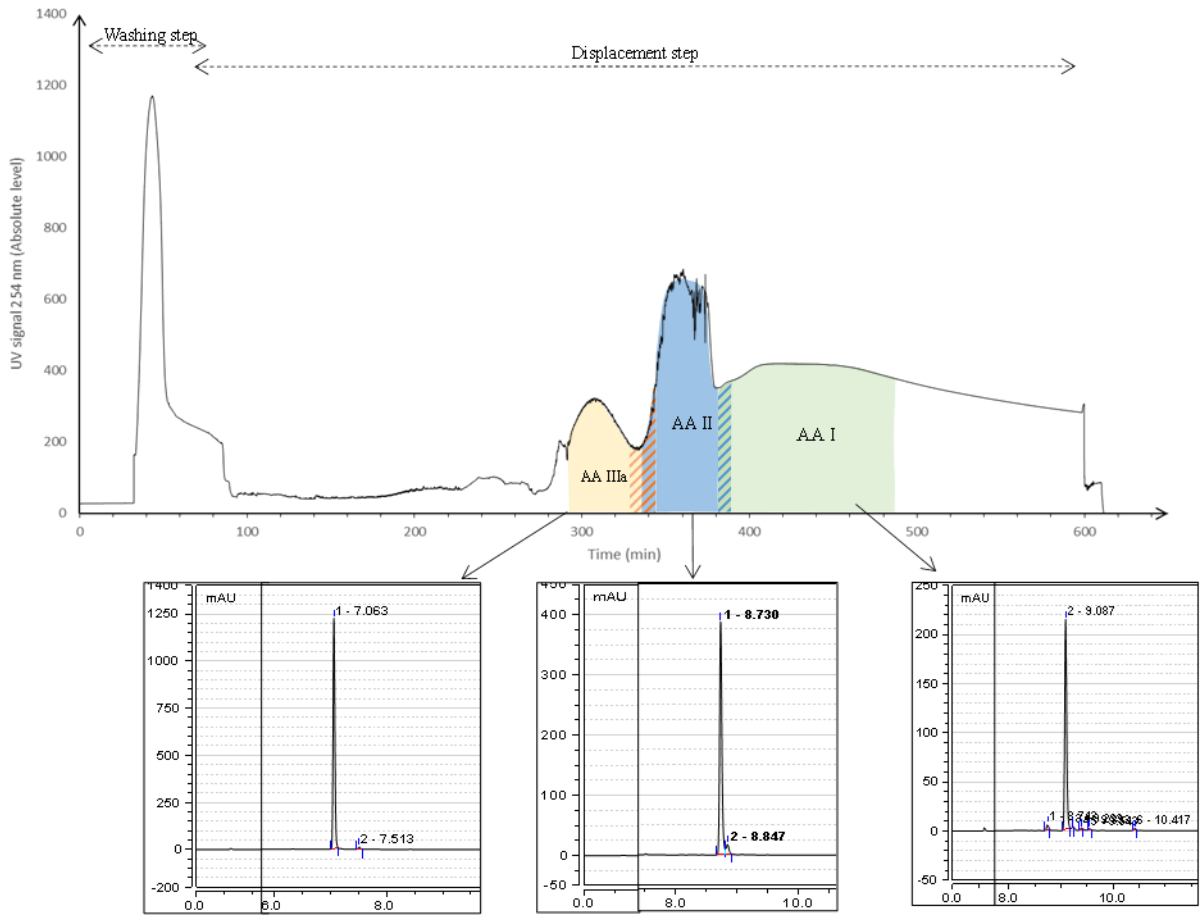


Figure 59. SIX-CPC chromatogram of the purification of AAs (experimental conditions see Run 13, Table 5), and HPLC-DAD analysis of pure AAs.

Moreover, mixing zones were largely reduced compared to pure compounds elution zones. The productivity of the process was acceptable: 4.2 $mg.h^{-1}$ (AA IIIa), 9.3 $mg.h^{-1}$ (AA II) and 5.5 $mg.h^{-1}$ (AA I). In fact the use of a liquid ion exchanger, with tensioactive properties, limit the flow rate of mobile phase to low values, usually 2 or 4 $mL.min^{-1}$. Centrifugal Partition Extraction (CPE) is a possible alternative to improve the productivity of the purification process. CPE, with a smaller number of larger cells, allowed the use of high flow rates. Ion pairing extraction was already described for glucosinolates isolation (Hamzaoui *et al.*, 2012) with a flow rate of 30 $mL.min^{-1}$ and a loading capacity also improved compared to IEX-CPC. Applying CPE and using our optimized ion exchange conditions, the purification duration would be less than 40 minutes with productivity around 50 $mg.h^{-1}$ for AA IIIa and AA I and of 100 $mg.h^{-1}$ for AA II, considering a 5 g sample injection.

II.4.4. Conclusion:

This work led to different interesting results that were published in Separation and Purification Technology in 2015:

- First, AA IIIa and AA IVa were described for the first time in *A. bracteolata*;
- CPC in elution mode was found to be a good process to quickly access to chemically simplified AAs enriched fractions, in one step from crude extract using MtBE/Acetone/MeOH/water (2:1:1:2, v/v/v/v) system;
- SIX-CPC was shown to be an efficient tool for one step isolation of AAs, with high resolution. AA I, AA II and AA IIIa were isolated from crude extracts in one step at a high level of purity in large amounts. This process allows access to new AA standards as AAs II and IIIa for further extensive pharmacological studies.

III. *ZIZIPHUS SPINA-CHRISTI* (L) DESF.

During my PhD, a part of my research program was devoted to the phytochemistry study of *Ziziphus spina-christi*. Using Centrifugal Partition Chromatography methodology, I developed an isolation procedure leading to the purification of different flavonoids and chalcone.

As introduction of this third part, I will rapidly described the general characteristics of *Zizipus* genus and *Ziziphus spina-christi* species successively, in terms of geographical localization, botanical identification, phytochemistry and traditional uses.

III.1. GENERAL CHARACTERISTICS OF *ZIZIPHUS* GENUS:

III.1.1. Classification:

Kingdom:	*Plantae,* Plants
Subkingdom:	*Tracheobionta* - Vascular plants
Superdivision:	*Spermatophyta* - Seed plants
Division:	*Magnoliophyta* - Flowering plants
Class:	*Magnoliopsida* - Dicotyledons
Subclass:	*Rosidae*
Order:	*Rhamnales*
Family:	*Rhamnaceae* – Buckthorn family
Genus:	*Ziziphus* (U.S. Department of Agriculture, 2016)

III.1.2. Geographic localization:

The genus *Ziziphus* belongs to the family *Rhamnaceae* which consists of about 170 species of deciduous or evergreen trees and shrubs distributed throughout the tropical and subtropical regions of the world (Johnston, 1963; Liu and Cheng, 1995). Its geographic localization includes mainly northern Africa, Middle East, Asia, Russia, southern Europe and southwestern USA (Figure 60 and Figure 61). This genus is indigenous to China with a history of over 4000 years (Hernández *et al*., 2015).

Figure 60. Geographical distribution of Ziziphus genus, family Rhamnaceae.

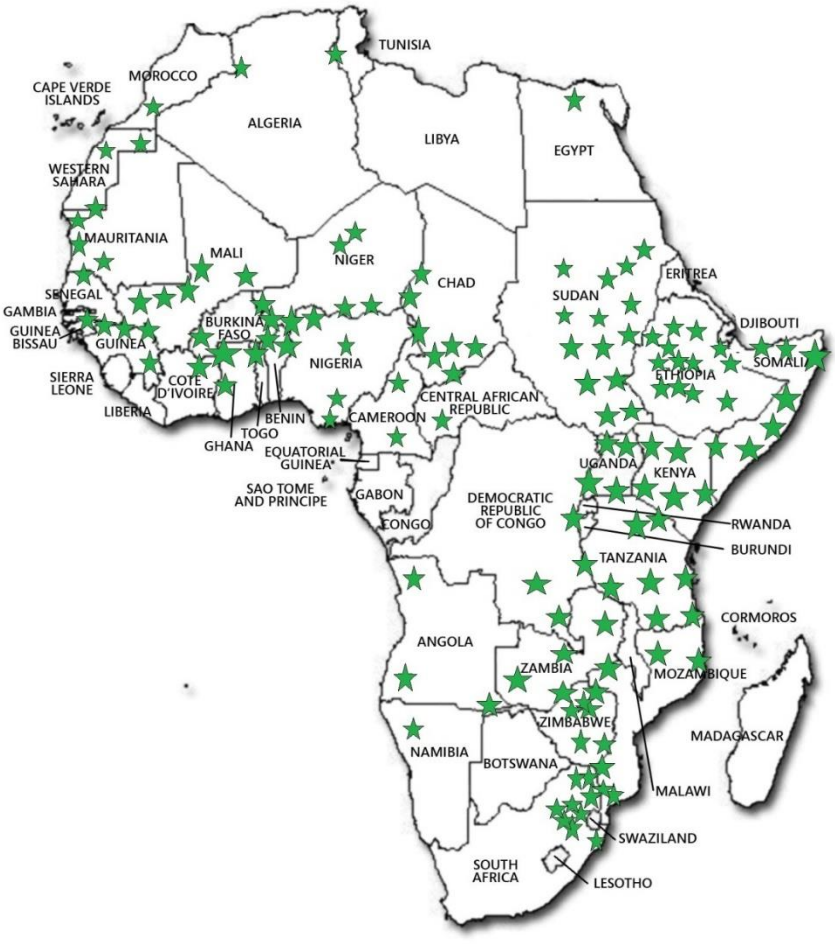
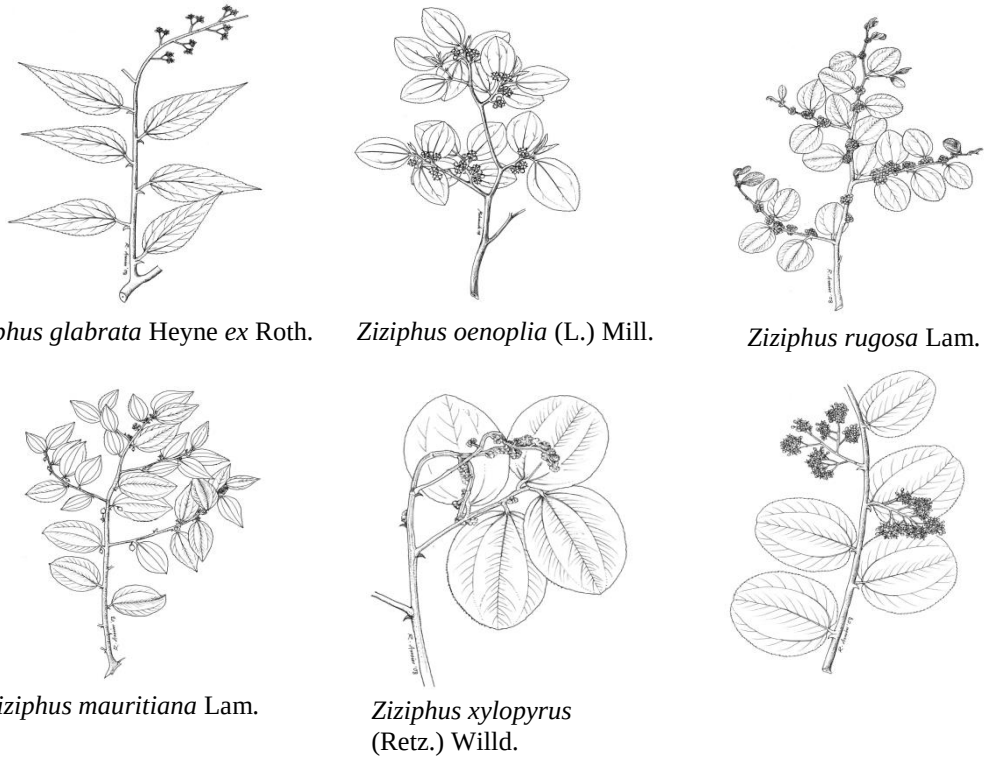


Figure 61. Distribution of some Ziziphus species in Africa (African plant database, 2016).

III.1.3. Botanical identification:

Ziziphus (Figure 62) is an evergreen or deciduous shrub or tree, erect or straggling, often climbing and spinose. Leaves are alternate, petiolate, entire or crenate, coriaceous. Leaves are nerved of 3-5 at the base. Stipules are usually 1 or rarely 2 or curved spines or absent. Inflorescence is axillary or terminal cymes or thyrses. Flowers are small, pentamerous, bisexual, pedicellate and yellow-green. Calyx is tube shallow. Sepals are ovate-triangular or triangular, keeled within. Petals are cucullate, deflexed or incurved, rarely absent. Stamens are 5, included or excluded, inserted below the disc. Disc is shallow or flat, 5-10-lobed. Ovary is globose, 2-4-loculed, sunk in the disk and adnate to its base; styles 2-4 are usually free or partially united. Stigma is papillose. Fruit is a globose or oblong drupe, base with persistent calyx tube, apex mucronulate; putaman woody, 1-3-celled. Seed are 1-3, plano-convex, testa thin, smooth shining; cotyledons thick; radicle short (Ara *et al.*, 2008).



Ziziphus glabrata Heyne *ex* Roth.

Ziziphus oenoplia (L.) Mill.

Ziziphus rugosa Lam.

Ziziphus mauritiana Lam.

Ziziphus xylopyrus (Retz.) Willd.

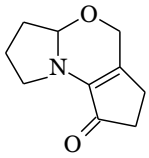
Figure 62. Ziziphus species (Ara et al., 2008).

III.1.4. Secondary metabolites isolated from Ziziphus genus:

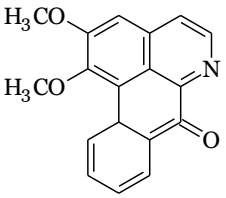
The major secondary metabolites isolated from *Ziziphus* species and presented herein, are classified according to their chemical structures: alkaloids, flavonoids, lignans, saponins, phenolic compounds and triterpenoids.

III.1.4.1. Alkaloids:

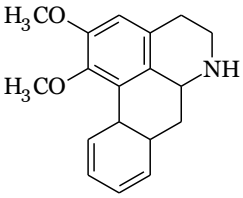
Many alkaloids of different structures were isolaled from *Ziziphus* genus (Figure 62). Daechualkaloid-A, a pyrrolidine alkaloid, and the isoquinoline daechualkaloid-C and E were isolated from the fruits of *Z. Jujuba* (Han *et al.,* 1987; Han and Park, 1987).



Daechualkaloid-A



Daechualkaloid-C
(Lysicamine)



Daechualkaloid-E
(Nurnuciferine)

Figure 62. Alkaloids isolated from Ziziphus species.

Cyclopeptides alkaloids (Figure 63) are characteristic components of *Ziziphus* plants and are heteromonocyclopeptides with a 13-, 14- or 15-membered ring. Amongst the 100 cyclopeptides isolated from *Ziziphus* species (Tripathi, 2001), we can cited: frangufoline from the fruits of *Z. jujuba* (Devi *et al., 1987),* amphibine-H from the stem bark of *Z. xylopyrus* and the root bark of *Z. nummularia* (Tschesche *et al.,* 1975; Devi *et al.,* 1987), lotusine-A and -G from the root bark of *Z. lotus* (Ghedira *et al.,* 1993; Le Crouéoura *et al.,* 2002), jubanine-C from the stem bark of *Z. jujuba* (Tripathi, 2001), mauritine A from the root of *Z. apetala* (Han *et al.,* 2011), mauritine M from the root of *Z. Mauritiana* (Panseeta *et al.,* 2011), oxyphylline B and nummularine-R from the stem and roots of *Z. oxyphylla* (Kaleem *et al.,* 2012).

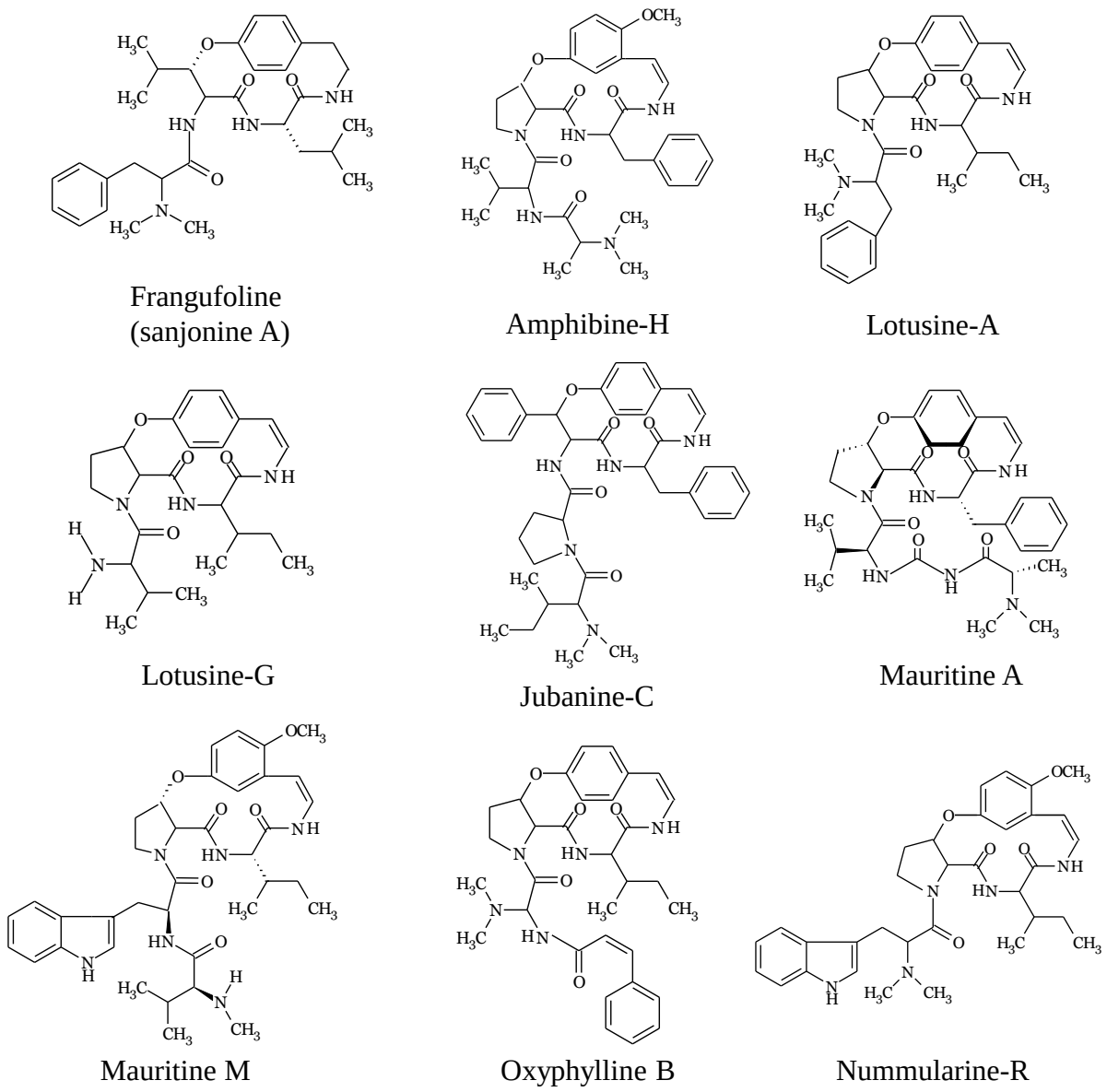


Figure 63. Cyclopeptides alkaloids isolated from Ziziphus species

III.1.4.2. Flavonoids:

From the several flavonoids isolated from *Ziziphus* genus (Figure 64), spinosin was isolated from the seeds of *Z. vulgaris* var. *spinosus* (Woo *et al.*, 1979). Swertish, isovitexin and puerarin were isolated from the seeds of *Z. jujuba Mill* var. *spinosa* (Cheng *et al.*, 2000). Two 8-formyl chalcones, 8-formyl-3',4'-dihydroxy-6,7-dimethoxyflavone and 8-formyl-4'-hydroxy-3',6,7-trimethoxyflavone, were isolated from the fruits of *Z. jujuba* (Li *et al.*, 2013). Quercetin-3-*O*-*β*-glucoside (isoquercitrin) and kaempferol-3-*O*-β-galactoside (trifolin) were isolated from *Z. oxyphylla* (Ahmad *et al.*, 2016).

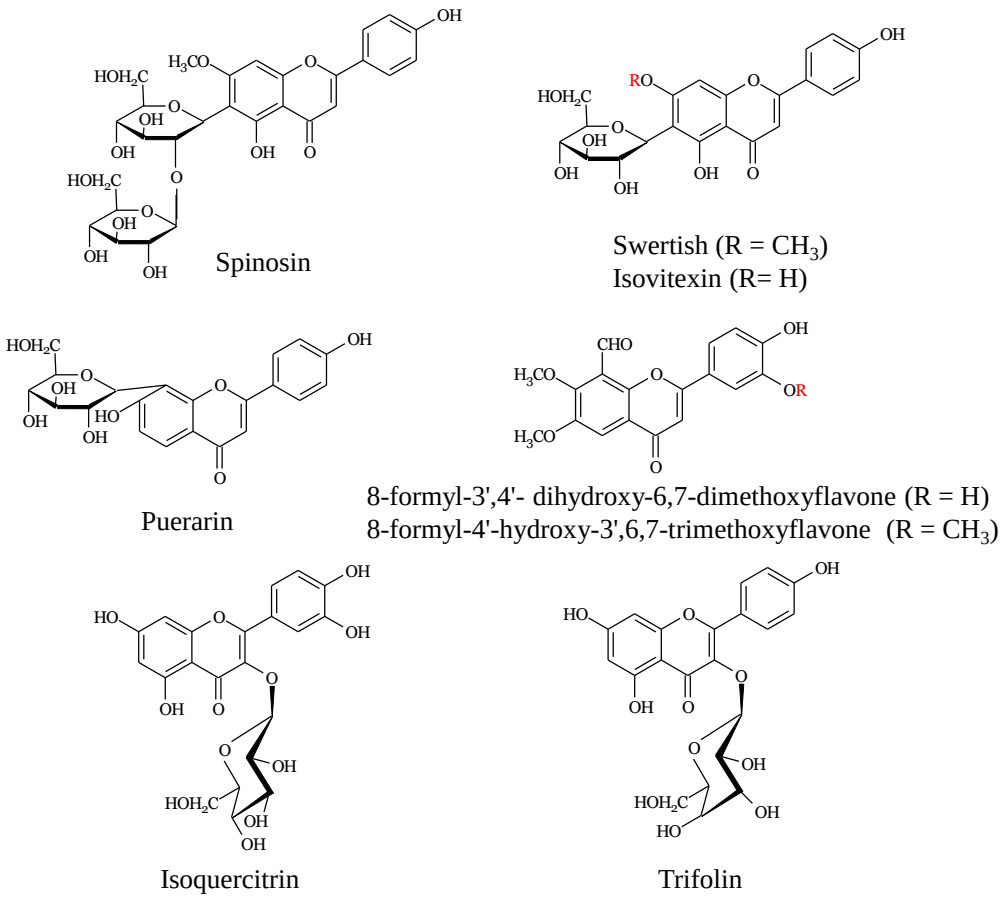
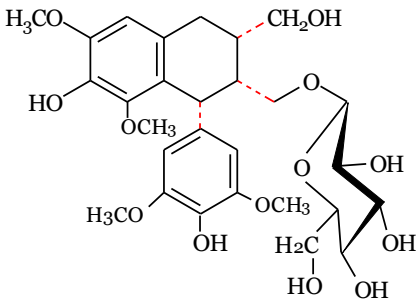


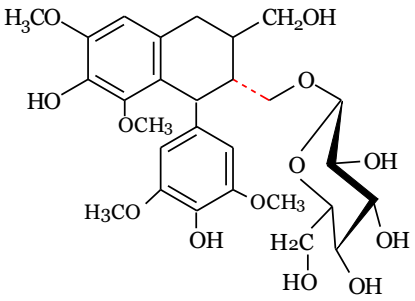
Figure 64. Flavonoids isolated from Ziziphus species

III.1.4.3. Lignans:

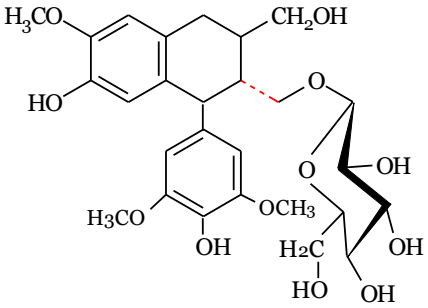
Lignans of aryltetralin type were isolated from the roots of *Z. jujuba* (Figure 65). They include different derivatives of lyoniresinol and isolariciresinol as glucosides and free genins (Meng *et al.*, 2013).



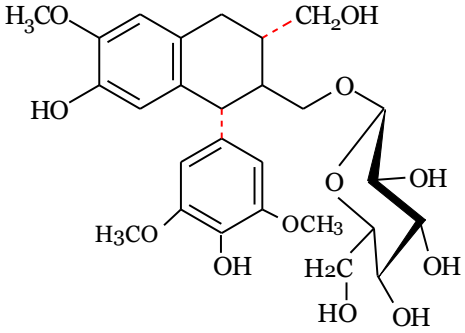
(6*S*,7*R*,8*R*)-7α-[(*β*-glucopyranosyl)oxy] lyoniresinol



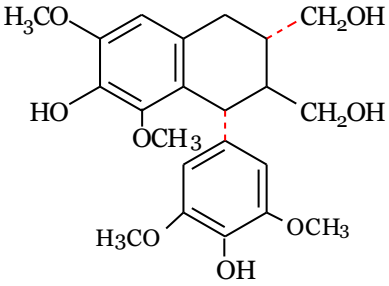
(6*R*,7*R*,8*S*)-7*a*-[(*β*-glucopyranosyl)oxy] lyoniresinol



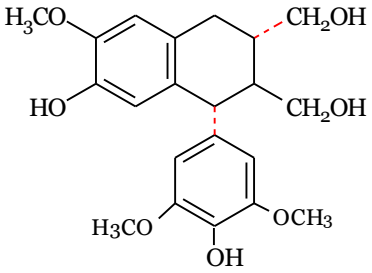
(+)-5'-methoxyisolariciresinol -3*α*-O-*β*-D-glucopyranoside



(-)-5'-methoxyisolariciresinol -3*α*-O-*β*-D-glucopyranoside



(-)-lyoniresinol

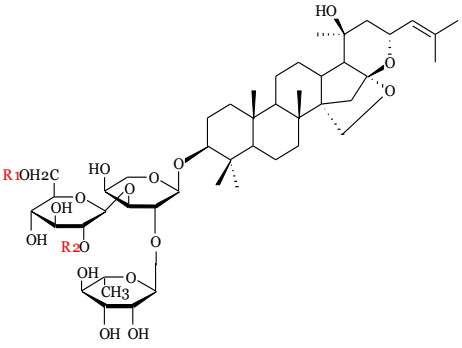


(-)-5'-methoxyisolariciresinol

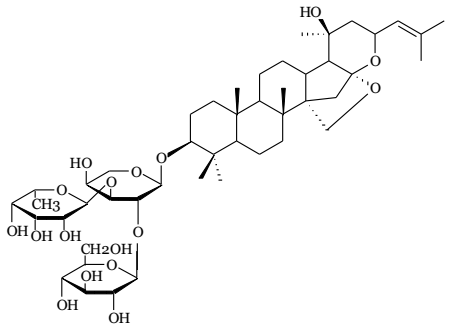
Figure 65. Lignans isolated from Ziziphus species.

III.1.4.4. Saponins:

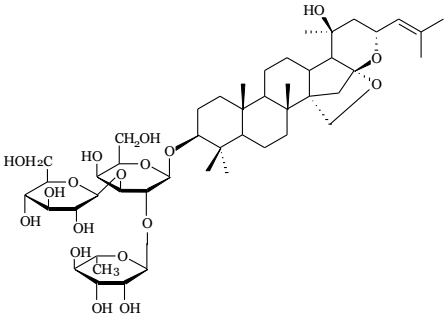
Several dammarane type saponins were characterized from *Ziziphus* species (Figure 66): Jujubosides A and B from *Z. jujuba* (Otsuka *et al.*, 1978), zizynummin from leaves of *Z. nummularia* (Sharma and Kumar, 1983), jujubosides B and C, lotosides I and II, and their free genin (lotogenin) from roots bark of *Z. lotus* (Renault *et al.*, 1997; Maciuk *et al.*, 2004).



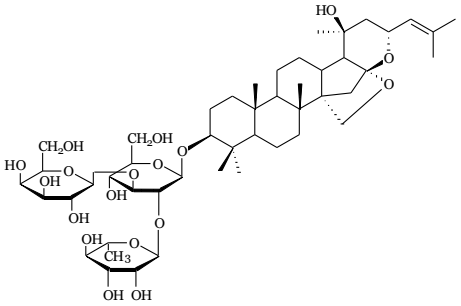
Jujuboside A (R_1, R_2= β-D-glu)
Jujuboside B (R_1= H, R_2= β-D-glu)
Jujuboside C (R_1= β-D-glu, R_2= H)



Zizynummin



Lotoside I

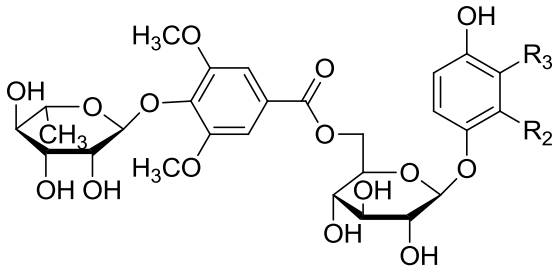


Lotoside II

Figure 66. Saponins isolated from Ziziphus species.

III.1.4.5. Phenolic compounds:

Two related phenolic glycosides were isolated from the roots of *Z. jujuba* in 2013 (Figure 67) (Meng *et al.*, 2013).



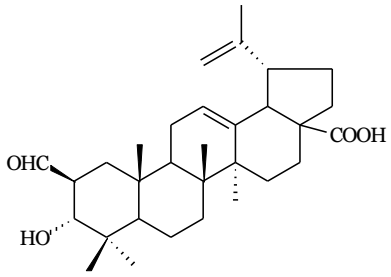
R_2 = OCH_3, R_3 = H: 1-O-(2-methoxyhydroquinone)-6-O-[4-O-(6-deoxy-α-L-mannopyranosyl)syringoyl]-β-D-glucopyranoside

R_2 = H, R_3 = OCH_3: 1-O-(3-methoxyhydroquinone)-6-O-[4-O-(6-deoxy-α-L-mannopyranosyl)syringoyl]-β-D-glucopyranoside

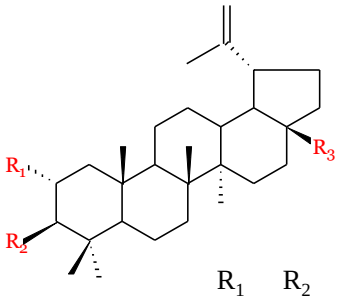
Figure 67. Phenolic compounds isolated from Ziziphus species

III.1.4.6. Triterpenoids:

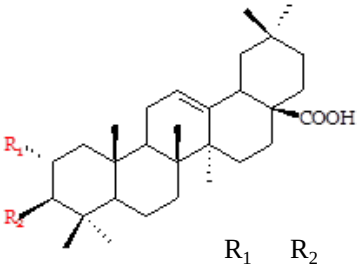
Ziziphus species are known to contain triterpenoids metabolites, especially different types of pentacyclic triterpenoids of lupane structure (Figure 68). Zizyberanalic acid, containing a formyl group in position 2, was isolated from the bark and roots of *Z. jujuba* (Kundu *et al.*, 1989). From the fruits of *Z. jujuba* were isolated triterpenes from different types: (1) lupane type including betulonic acid, betulin, and betulinic acid; (2) oleanane type including oleanonic acid, oleanolic acid and maslinic acid; (3) ursane type including ursonic acid, pomonic acid, and corosolic acid; (4) ceanothane type including ceanothic acid and epiceanothic acid; (5) seco-ursane type including cecropiacic acid (Guo *et al.*, 2011; Fujiwara *et al.*, 2011).



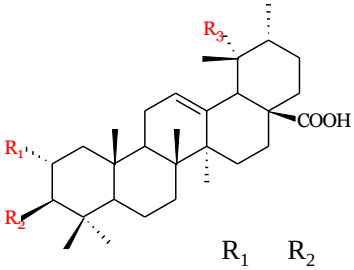
Zizyberanalic acid



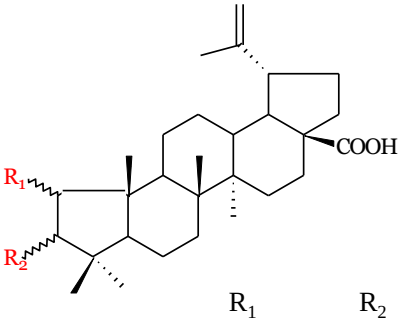
	R_1	R_2	R_3
Betulonic acid	H	O	COOH
Betulin	H	OH	CH_2OH
Betulinic acid	H	OH	COOH



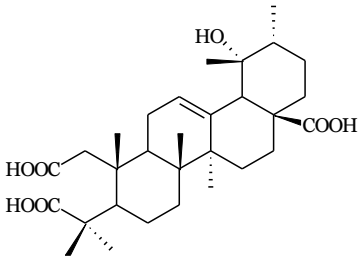
	R_1	R_2
Oleanonic acid	H	O
Oleanolic acid	H	OH
Maslinic acid	OH	OH



	R_1	R_2	R_3
Ursonic acid	H	O	H
Pomonic acid	H	O	OH
Corosolic acid	OH	OH	H



	R_1	R_2
Ceanothic acid	αCOOH	βOH
Epiceanothic acid	βCOOH	βOH



Cecropiacic acid

Figure 68. Triterpenoids isolated from Ziziphus species.

III.1.5. Traditional and medicinal uses:

Ziziphus is known in the system of ancient Chinese medicine since a long time. This genus is traditionnaly used for treatment of heart disorders, abdominal cold, heat and pain in limbs, liver disorder, sweating and anxiety (Singh and Arya, 2011). This genus is also used for treatment of fatigue and insomnia (Hsu and Wang, 1983).

Ziziphus Combination is prescribed in sedative formulas by prominent physicians in China:

Ziziphus jujuba var. Spinosa (suanzaoren)	18 g
Anemarrhena asphodeloides Bunge (zhimu)	10 g
Hoelen, *Poria cocos* (fuling)	10 g
Cnidium, *Ligusticum chuanxiong* (chuanxiong)	5 g
Licorice, *Glycyrrhiza glabra* (gancao)	3 g

This formula is mentioned in books dealing with traditional formulas, as particularly effective (Dharmananda, 2001). The most abundant species used medicinally are: *Z. jujuba* Mill., *Z. nummularia* (Burm.f.) Wight & Arn., *Z. lotus* (L) Lam. and *Z. oxyphylla* Edgew (Table 9).

Table 9. Some Ziziphus species, their chemical constituents and traditional uses:

***Ziziphus* species**	**Major chemical constituents**	**Traditional uses**
***Ziziphus jujuba* Mill. (china)**	Various constituents, including triterpenic acids, cyclopeptide alkaloids, flavonoids, cerebrosides, aminoacids, phenolic acids, mineral constituents and polysaccharides.	Dried seeds are traditionally used as sedative medicine to treat anxiety, nervousness and sleep-related problems for more than one thousand years (Gao *et al.*, 2013). Fruits made into paste, puree, syrup and confection are consumed for digestion improvement and general health maintenance. Also dried fruits are used for treatment of hemorrhage and diarrhea (Wu *et al.*, 2014).
***Ziziphus nummularia* (Burm.f.) Wight & Arn. (India)**	Cyclopeptide alkaloids, saponins, triterpenoids and glycosides.	Leaves and fruits are used in traditional medicine as analgesic, anti-inflammatory, antitussive, anthelmintic. Leaves and root bark of the plant are used as the remedy of inflammation (Ray *et al.*, 2015).
***Ziziphus lotus* (L) Lam. (Nourth Africa and Europe)**	Cyclopeptide alkaloids.	A mixture of dried ground leaves and fruits is applied topically in the treatment of boils, while the root bark is used as antidiabetic (Ghedira *et al.*, 1993; Le Crouéoura *et al.*, 2002).
***Ziziphus oxyphylla* Edgew. (South Asia)**	Cyclopeptide alkaloids.	Fruits are used for treatment of inflammatory, painful conditions especially of rheumatic origin, as an antipyretic, and for the treatment of microbial infections, allergy and diabetes (Kaleem *et al.*, 2013).

III.1.6. Biological activities of isolated molecules:

Different bioactive compounds were identified from the *Ziziphus* species. These compounds were studied for their antioxidant activity, central nervous system (CNS) effects, cardiovascular effects and anti-infectious activity.

III.1.6.1. Antioxidant activity:

Flavonoids are a class of secondary plant phenolic compounds with significant antioxidant activity. Two flavonoids from the fruits of *Z. jujuba*, 8-formyl-3',4'-dihydroxy-6,7-dimethoxyflavone and 8-formyl-4'-hydroxy-3',6,7-trimethoxyflavone (Figure 64) presented an antioxidant activity using 2',7'-dichlorofluorescein diacetate oxidative product (Li *et al.,* 2013). Quercetin-3-*O*-*β*-glucoside (isoquercitrin) and kaempferol-3-*O*-*β*-galactoside (trifolin) from *Z. oxyphylla* exhibited antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) oxidative agent (Ahmad *et al*., 2016).

III.1.6.2. CNS effects:

Isoquinoline alkaloids isolated from *Z. Jujuba,* daechualkaloid C and E, (Figure 62) demonstrated sedative properties in mice after intraperitoneal dose of 3 mg/kg (Han and Park, 1987). Spinosin and swertish (Figure 64), two flavonoids purified from *Z. jujuba*, also presented significant sedative activity after oral dose of 4 x 10^{-5} mol/kg (Cheng *et al.,* 2000). Cyclopeptide alkaloid, mauritine A (Figure 63) showed inhibition of 11*β*-hydroxysteroid dehydrogenase (11*β*-HSD) *in vitro*, an enzyme implicated in the depression, with IC_{50} values of 52.0 µg/ mL in human and 31.2 µg/ mL in mouse (Han *et al.,* 2011). Also cyclopeptide alkaloids purified from stem and roots of *Z. oxyphylla*, oxyphylline B and nummularine-R (Figure 63), exhibited marked antinociceptive activity in mice after intraperitoneal dose of 2.5 and 5 mg/kg respectively (Kaleem *et al.,* 2012).

III.1.6.3. Cardiovascular effects:

Triterpenoids isolated from *Z. Jujuba,* oleanonic acid, pomolic acid, and pomonic acid (Figure 68), may be able to prevent atherosclerosis by inhibiting A: cholesterol acyltransferase (ACAT) *in vitro* (Fujiwara *et al.,* 2011). Saponin purified from *Z. Jujuba,* jujuboside B (Figure 66) exhibited *in vivo* antiplatelet aggregation by inhibition of collagen-, thrombin-, and arachidonic acid-induced platelet aggregation with IC_{50} values of 96.3, 210.6 and 99.5 µg/ml, respectively (Seo *et al.,* 2013).

III.1.6.4. Anti-infectious activity:

The cyclopeptide alkaloids purified from *Z. oenoplia*, Ziziphine N and Q exhibited significant antiplasmodial activity against *Plasmodium falciparum* with IC_{50} values of 3.92 and 3.5 µg/mL respectively (Suksamrarn *et al.*, 2005). Triterpenoids isolated from *Z. cambodiana*, alphitolic acid and zizyberanalic acid (Figure 67) presented antimycobacterial activity against *Mycobacterium tuberculosis* with MIC values of 12.5 and 50 µg/ml, respectively (Suksamrarn *et al.*, 2006). The cyclopeptide alkaloids purified from *Z. mauritiana*, mauritine M (Figure 63) and nummularine H also exhibited antiplasmodial activity against *P. falciparum* with IC_{50} values of 3.7 and 4.2 µM respectively and antimycobacterial activity against *M. tuberculosis* with MIC of 72.8 and 4.5 µM respectively (Panseeta *et al.*, 2011).

III.2. ZIZIPHUS SPINA-CHRISTI SPECIES:

III.2.1. Geographical localization:

Ziziphus spina-christi (L) Desf. is a tropical evergreen wild tree of Sudanese origin commonly known as Christ's thorn (Figure). It is native to semi-arid tropical regions of sub-Saharan Africa and subtropical areas of the Near and Middle East. Its vernacular native names are dum, nabq, sadr, sidr and tsal.

Figure 70. Ziziphus spina-christi near El Obeid, Central Sudan (Saied et al., 2008).

It is usually confined to low elevations below 500 m a.s.l. (Dafni *et al.*, 2005; Wiehle *et al.*, 2014). It strongly resists to heat and drought. It grows in desert areas with an annual rainfall of 50–300 mm and on a wide range of soil types, but prefers light silty ones (Saied *et al.*, 2008).

III.2.2. Botanical description:

Z. spina-christi (Figure 69) is a spiny shrub or small tree. It normally grows into a tree form, but it often acquires a bush form due to intensive grazing during the later part of the dry seasons and heavy destructive cutting for fencing material and fuel. It develops very deep taproot and has an extraordinary regenerative power. It is evergreen but can drop some of its leaves during very dry seasons. Its size is 5–10 m and its trunk diameter is up to 45 cm. The bark is whitish brown or pale grey and is deeply fissured. The crown is rounded or umbelliform with dense branches that spread widely and have a tendency to weep at the ends. Leaves are simple (Figure 69), alternate, narrowly ovate lanceolate and are varying from 1 to 9 cm in length and 1–3.5 cm in width. Leaves also are glabrous above, minutely and densely pubescent beneath, have three basal, conspicuous veins running up to the apex and around 0.5–1 cm long petioles. Spines are light brown in colour and paired, with one of each pair being up to 8 mm long, straight and directed forward while the other is shorter and slightly curved. The flowers are found in dense clusters in the axils of the leaves and having a sweet scent. They are small, greenish yellow, sub-sessile with five sepals of 2 mm long and five petals of 1.5 mm long. Stamens are five inserted at the base of flat lobed disc and their position is opposite to the petals. The ovary is 2-locular; styles short and divided above into two lobes. Fruits are a globose drupe about 1–1.5 cm in diameters, red-brown, with a hard stones surrounded by a sweet edible pulp (Saied *et al*., 2008).

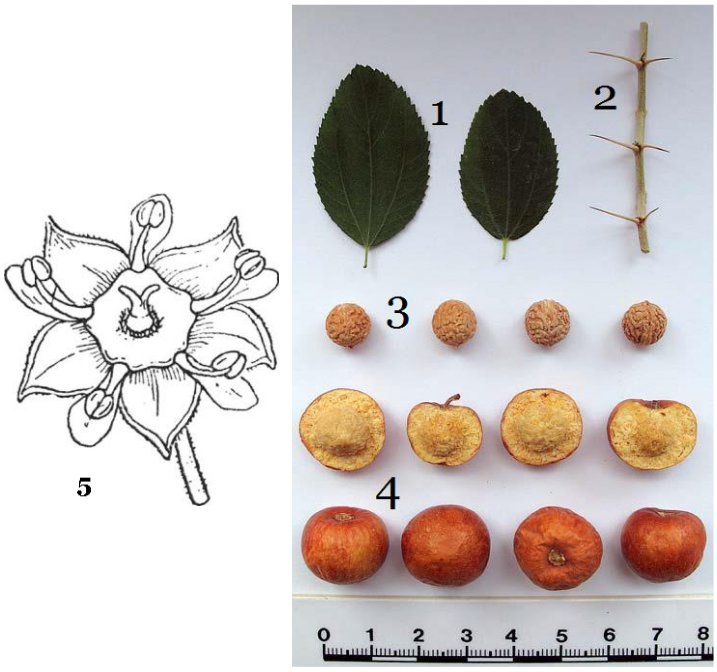


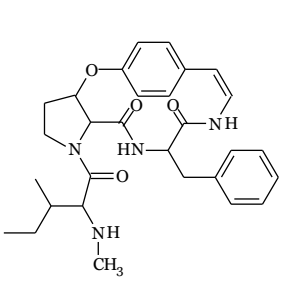
Figure 69. Leaves 1, spines 2, seeds 3, fruits 4, single flower 5 of Z. spina-christi (scale in cm) (Saied et al., 2008).

III.2.3. Secondary metabolites isolated from Ziziphus spina-christi:

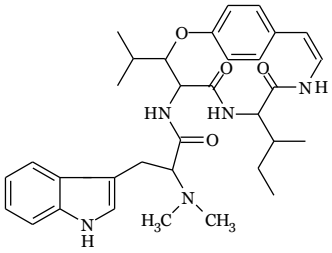
The major secondary metabolites isolated from *Z. spina-christi* species were classified according to their chemical structures: alkaloids, flavonoids, saponins and triterpenoids.

III.2.3.1. Alkaloids:

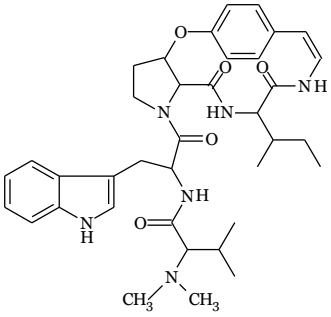
Cyclopeptide alkaloids with a 14-membered ring were isolated from the bark extract (Figure 63 and Figure 70): mauritine-A and -C, amphibine-A and -E (Tschesche *et al.*, 1974), franganine, sativanine-A (Shah *et al.*, 1986), amphibine-H and spinanine-A (Abdel-Galil and El-Jissry, 1991).



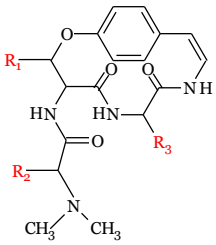
Mauritine-C

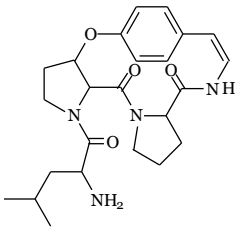


Amphibine-A



Amphibine-E





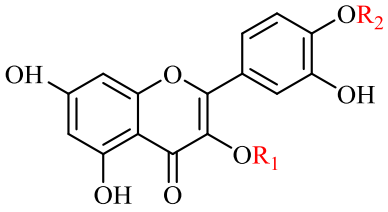
Spinanine-A

	R_1	R_2	R_3
Franganine	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$	$CH_2CH(CH_3)_2$
Sativanine-A	C_6H_6	$CH(CH_3)CH_2CH_3$	$CH(CH_3)_2$

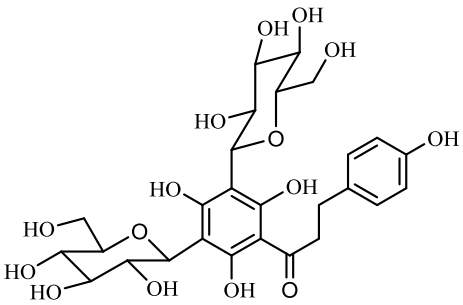
Figure 70. Alkaloids isolated from Z. spina-christi.

III.2.3.2. Flavonoids:

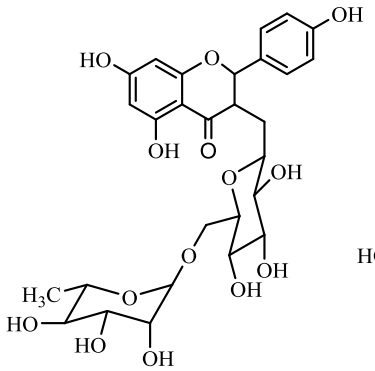
Flavonoids isolated from leaves ethanolic extract are presented in Figure 71: five glycosides of quercetin, one glycoside of kaempferol, two dimers of quercetin (zizyflavosides), one flavanol (epiafzelechin) and a bis-C-*β*-D-glucoside of phloretin (Nawwar *et al.*, 1984; Mostafa *et al.*, 2010).



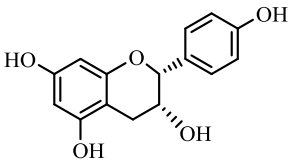
Quercetin-3-O-*β*-D-galactopyranoside (Hyperin)	R_1= *β*-D-Gal	R_2= H
Quercetin-3-O-*α*-L-rhamnoside (Quercitrin)	R_1= *α*-L-Rha	R_2= H
Quercetin-3-O-rutinoside (Rutin)	R_1= *α*-L-Rha-(1→6)-*β*-D-Glu	R_2= H
Quercetin-3-O-[*β*-D-xylosyl-(1→2)-*α*-L-rhamnoside]-4'-O-*α*-L-rhamnoside	R_1= *β*-D-Xyl-(1→2)-*α*-L-Rha	R_2= *a*-L-Rha
Quercetin-3-O-[*β*-D-xylosyl-(1→2)-*α*-L-rhamnoside]	R_1= *β*-D-Xyl-(1→2)-*α*-L-Rha	R_2= H



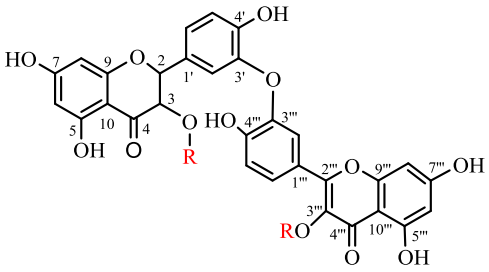
3',5',di-*C*-*β*-glucosylphloretin



Kaempferol-*O*-rutinoside



Epiafzelechin

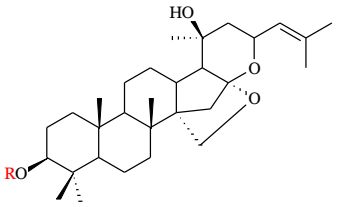


Zizyflavoside A	R= *α*-L-Rha-(1→6)-*β*-D-Glu
Zizyflavoside B	R= *β*-D-Glu

Figure 71. Flavonoids isolated from Z. spina-christi.

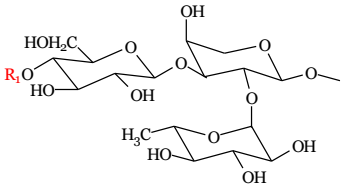
III.2.3.3. Saponins:

Four saponin glycosides (Figure 72), christinin A, B, C and D were isolated from the leaves butanolic extract of *Z. spina-christi* (Mahran *et al.*, 1996).



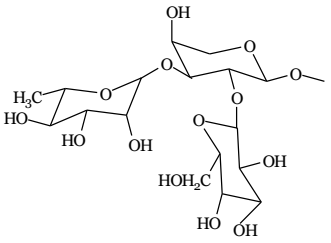
Christinin A (R_1=H)
Christinin B (R_1=HO_3S)

R =



Christinin C

R =



Christinin D

R =

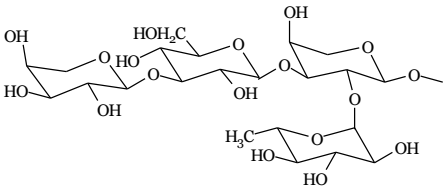


Figure 72. Saponins isolated from Z. spina-christi.

III.2.3.4. Terpenoids:

A pentacyclic triterpenoid, betulinic acid was purified from the root bark chloroform extract (Figure 67) (Adzu *et al.*, 2011).

III.2.4. Biological activities and traditional medicinal use:

Z. spina-christi has long been used in Folklore medicine for treatment of several diseases, such as digestive disorders, weakness, liver compliants, obesity, urinary troubles, skin infections, loss of appetite, fever, pharyngitis, bronchitis, anemia, diarrhea and insomnia (Asgarpanah and Haghighat, 2012).

In Sudan, *Z. spina-christi* fruits are eaten to treat diarrhea, malaria and as an antispasmodic. The powder of the twigs is used externally to treat rheumatism and scorpion sting (El-Kamali and El-Khalifa, 1999). The poultice of the powdered leaves is used to heal swellings while boiled leaves are applied to various surface wounds. Macerated roots and boiled leaves are used as an antipurgative (El Ghazali *et al.*, 1994; El Ghazali *et al.*, 1997). The decocted extract of the bark is used to treat intestinal spasms. In Egypt, a beverage made from fruits is considered to be a sedative. In Palestine, leaves and young branches are used as an anti-inflammatory for eye wash, treat toothache and stomachache and as an antirheumatic. In the United Arab Emirates, leaves are boiled in water and used as a shampoo or mixed with lemon and applied to the face and hair to soften or to soothe it. In Oman, the boiled leaves are given to women in prolonged labour or with a retained placenta for its ocytocic properties (Saied *et al.*, 2008).

In this PhD research program, we decided to focus on the flavonoids composition study of *Ziziphus spina-christi* using CPC methodology. In the following chapter, we thus present a state of the art in this domain.

III.3. ISOLATION AND PURIFICATION OF FLAVONOIDS BY CPC:

Flavonoids form a large class of important naturally occurring bioactive compounds. Their isolation and purification from natural sources can sometimes be difficult and time consuming when traditional chromatographic techniques are used, like silica gel, polyamide or Sephadex LH-20 column chromatography. Support-free liquid–liquid chromatography techniques, like CPC, could be an excellent alternative.

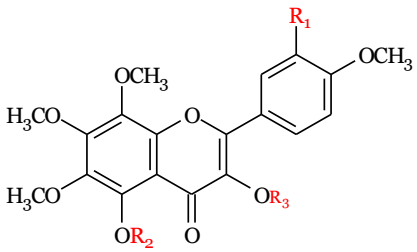
Over 300 different flavonoids were isolated from more than 100 plant species, using support free liquid/liquid chromatography with almost 40 different solvent systems. Different mode of operation columns were used including gradient elution, enhancement of flow rate, pHzone refining, two-step CCC and dual-mode (Costa and Leitão, 2010).

Depending on the nature of the sample, the solvent systems varied (Costa and Leitão, 2010).

III.3.1. Isolation of free flavonoids:

Isolation of free flavonoids was frequently carried out with the solvent system *n*-hexane/EtOAc/MeOH/H_2O (HEMWat), because of the versatility of this quaternary solvent system and of its range of polarities (Friesen and Pauli, 2007).

Different ratios of HEMWat (1:0.8:1:0.8; 1:0.8:0.8:0.8; and 1:0.8:1:1, v/v/v/v) were used for purification of polymethoxylated flavones **1a-d**, from *Citrus reticulata* (Figure 73) (Wang, *et al.;* 2005).



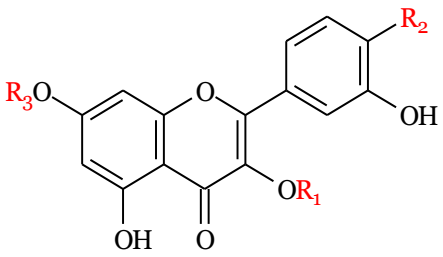
	R_1	R_2	R_3
1a	OCH_3	OCH_3	H
1b	OCH_3	OCH_3	OCH_3
1c	H	OCH_3	H
1d	OCH_3	OH	H

Figure 73. Polymethoxylated flavones purified from Citrus reticulata by CCC.

HEMWat composition modifications by replacing MeOH by ethanol (EtOH), propanol (PrOH), or BuOH; and by replacing EtOAc with $CHCl_3$ or CH_2Cl_2 can cause variations in the properties of each phase to different extents (Costa and Leitão, 2010).

III.3.2. Isolation of glycosylated flavonoids:

The presence of a sugar moiety on the flavonoid structure enhances its polarity. It is necessary to use more polar solvent systems, as the solubility of high-polarity compounds in solvent systems containing *n*-hexane is low. Therefore, the addition of *n*-BuOH to HEMWat system was necessary to isolate glycosylated flavonols from *Ginkgo biloba* (Figure 76) (Zhang *et al.*, 2007). Kaempferol-3-O-rhamnoside-7-O-(6-feruloylgluco(1→3)rhamnoside), quercetin-3-O-rhamnoside-7-O-(6-feruloylgluco(1→3)rhamnoside) and isorhamnetin-3-O-glucosyl(1→2)rhamnoside were eluted in a one-step run using Hex/EtOAc/*n*-BuOH/MeOH/0.5% acetic acid (1:0.5:3.5:1:4, v/v/v/v/v) in ascending mode.



	R_1	R_2	R_3
Kaempferol-3-O-rhamnoside-7-O-(6-feruloylgluco(1→3)rhamnoside)	Rha	H	6-feruloylglc(1→3)rha
Quercetin-3-O-rhamnoside-7-O-(6-feruloylgluco(1→3)rhamnoside)	Rha	OH	6-feruloylglc(1→3)rha
Isorhamnetin-3-O-glucosyl (1→2)rhamnoside	Glu(1→2)rha	OCH_3	H

Figure 74. Glycosylated flavonols isolated from Ginkgo biloba by CCC.

Nevertheless this modified HEMWat system was not the most common found in the literature.

Ternary solvent system based on apolar solvent/alcohol/water mixtures have been extensively used for separations of glycosylated flavanones, flavones, isoflavones and flavonols from plant extracts (Friesen and Pauli, 2007).

The solvent system EtOAc/*n*-BuOH/H_2O (EBuWat) (2:1:3, v/v) was used in descending mode to isolate isoetin-7-O-*β*-D-glucopyranosyl-2'-O-*α*-L-arabinopyranoside and isoetin-7-O-*β*-D-glucopyranosyl-2'-O-*α*-D-glucopyranoside were isolated from *Taraxacum mongolicum* (Shi *et al.*, 2008). This system was also an interesting solvent system for performing gradient elutions in the ascending mode since raising the amount of *n*-BuOH in the solvent system would cause strong variations in the organic mobile phase but would have a minor impact on the aqueous stationary phase (Costa and Leitão, 2010).

The solvent system EtOAc/EtOH/H_2O (5:1:5, v/v/v; descending mode) was successfully used for isolation of quercetin-3-O-L-rhamnoside from *Acer truncatum* Bunge (Figure 77) (Ma *et al.*, 2005).

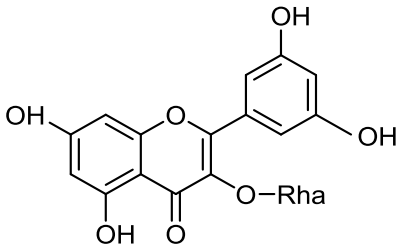
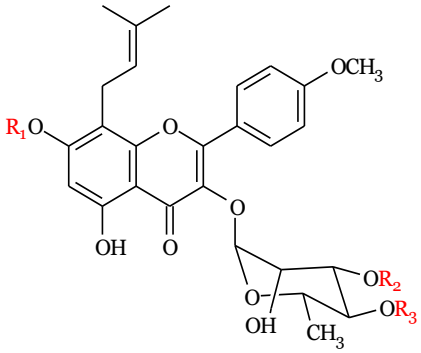


Figure 75. Quercetin-3-O-L-rhamnoside from Acer truncatum Bunge by CCC.

Also, the solvent system composed of $CHCl_3$/MeOH/H_2O was used for the separation of high polarity compounds as an alternative to the EBuWat system (Du *et al.*, 2004). Purification of flavonoids, epimedokoreanoside I and icariin (Figure 78), from the *Epimedium koreamum* was successfully established by using $CHCl_3$/MeOH/H_2O (4:3.5:2, v/v/v/v) (Liu *et al.*, 2005).



	R_1	R_2	R_3
Epimedokoreanoside I	Glu	β-(6-Ac)Glu	Ac
Icariin	Glu	H	H

Figure 76. Flavonoids from Epimedium koreamum by CCC.

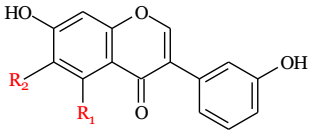
MtBE/ACN/water was an interesting alternative to alcoholic bridge solvent for the isolation of glycosylated flavonoids of moderate to high polarity. The isolation of monoglucosylated/acetylated isoflavones, acetyldaidzin and acetylgenistin, from soybean flour was achieved using this solvent system in two different ratios (2:2:3 and 6:3:8, v/v/v), after a previous clean up step using resin (Degenhardt and Winterhalter, 2001).

III.3.3. Isolation of mixtures of free and glycosylated flavonoids:

In most of the samples, a mixture of free and glycosylated flavonoids occurred leading to a broadened polarity range of target metabolites. Isocratic separations were performed using HEMWat, EtOAc/*n*-BuOH/H_2O, or $CHCl_3$/MeOH/H_2O and the search for the solvent system should begin with the less polar one, moving to the next range of polarity as needed (Costa and Leitão, 2010).

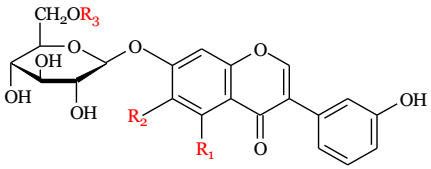
A mixture of isoflavones including the aglycones daidzein, genistein, and glycitein, together with their glycosylated derivatives (daidzin, genistin, glycitin, acetyldaidzin and acetylgenistin) (Figure 77) were separated from a crude soybean extract using chlorinated solvent systems $CHCl_3$/MeOH/H_2O (4:3:2, v/v/v) and $CHCl_3$/MeOH/*n*-BuOH/H_2O (4:3:0.5:2, v/v/v/v) (Yang, *et al.;* 2001).

Aglycones:



	R_1	R_2
Daidzein	H	H
Genistein	OH	H
Glycitein	H	OCH_3

Glucosides:



	R_1	R_2	R_3
Daidzin	H	H	H
Genistin	OH	H	H
Glycitin	H	OCH_3	H
Acetyldaidzin	H	H	$COCH_3$
Acetylgenistin	OH	H	$COCH_3$

Figure 77. Mixture of isoflavones isolated from soybean by CCC.

The first solvent system $CHCl_3$/MeOH/H_2O (4:3:2, v/v/v) was used in descending mode (aqueous stationary phase), to separate less polar isoflavones (daidzein, glycitein), and acetyl glucosides (acetyldaidzin and acetylgenistin) with purity ranging from 98 to 99 %, in one step (Figure). Other compounds stayed in the stationary phase according to their high *K* values.

By adding a small volume of *n*-BuOH in the first solvent system, the retention volume of all compounds was decreased allowing the elution of polar glycosylated compounds. Genistin, glycitin and daidzin were then isolated in descending mode with high purity even if genistin and glycitin, were only partially resolved. The less polar isoflavone and acetylglucoside were eluted faster and so were obtained as a mixture.

To completely achieve the purification of genistin and glycitin, a third solvent system based on MtBE/THF/*n*-BuOH/water 0.5%TFA (2:2:0.15:4, v/v/v/v) was necessary. The CCC was used in descending mode (organic stationary phase). The elution order was totally changed: glycitin was eluted first, followed by daidzin and finally two hours later genistin.

Those results illustrated tha the purification of all flavonoids of wide polarity range was not practical using a unique solvent system in isocratic mode.

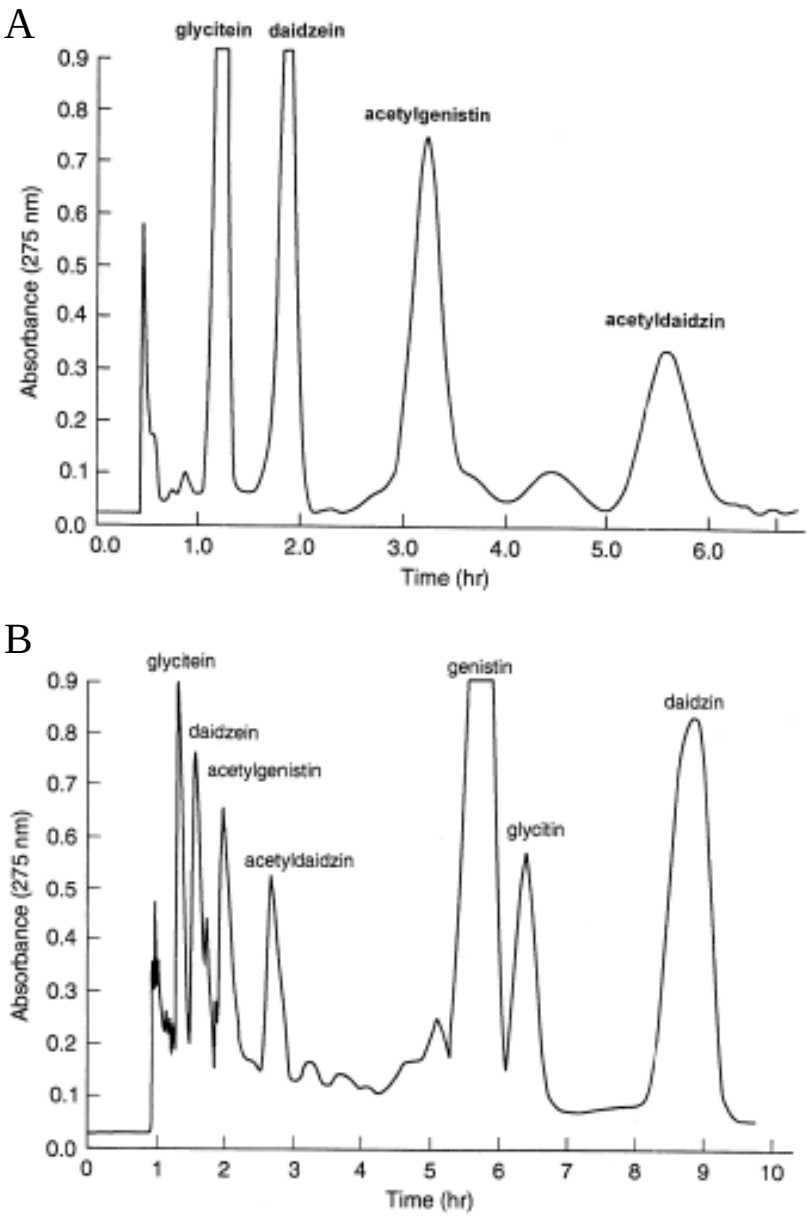


Figure 80. CCC chromatogram of crude soybean extract purification using $CHCl_3$/MeOH/H_2O (4:3:2, v/v/v) solvent system in descending mode, 150 mg of crude extract (A), and using $CHCl_3$/MeOH/n-BuOH/H_2O (4:3:0.5:2, v/v/v/v) in descending mode, 60 mg of crude extract (B) (Zhang et al., 2010).

Among all the references dealing with CCC or CPC purification of flavonoids, each team used a solvent system made to measure, highlighting the high versatility of CPC process. This main advantage was also one of the main limit for non-specialist users, leading to a lack of standardization of CPC purification procedure.

Nevertheless, considering flavonoids, three main solvent systems were described: HEMWat, $CHCl_3$/MeOH/water or EtOAc/Alcohol *(MeOH, n-BuOH, EtOH)*/water.

III.4. PHYTOCHEMICAL STUDY OF *ZIZIPHUS SPINA-CHRISTI*:

n-Hexane and chloroform were highly toxic solvents and should be avoided in new research of solvent system. Using *Z. spina-christi* as a flavonoids rich sample, the purpose of this work was to explore the feasibility of purification of flavonoids from a crude extract using only two solvent systems based on EtOAc/Alcohol(s)/water, to propose a "standardized" solvents range.

III.4.1. Operating conditions selection:

III.4.1.1. Solvent sytems:

In order to select the two biphasic solvent systems we would use for this study, we worked in the so called "good solvent" approach. In this approach, a good solvent of the targeted analytes, here the flavonoids, was first selected as bridge solvent, allowing then good solubility of the sample and partition between organic and aqueous phases. Then to build up the biphasic system, an apolar solvent, miscible with the good solvent, and water were added.

In order to fractionate intensively in one run, the solvent system selected should provide a good retention factor for free and glygosylated flavonoids.

Alcohols were known as good solvents of the flavonoids, especially methanol and *n*-BuOH. The ternary systems EtOAc/*n*-BuOH/water and EtOAc/MeOH/water were studied. Looking at the ternary diagrams of these two solvent systems (Figure 78), we could first notice that the good solvent exhibited dramatically opposite behavior.

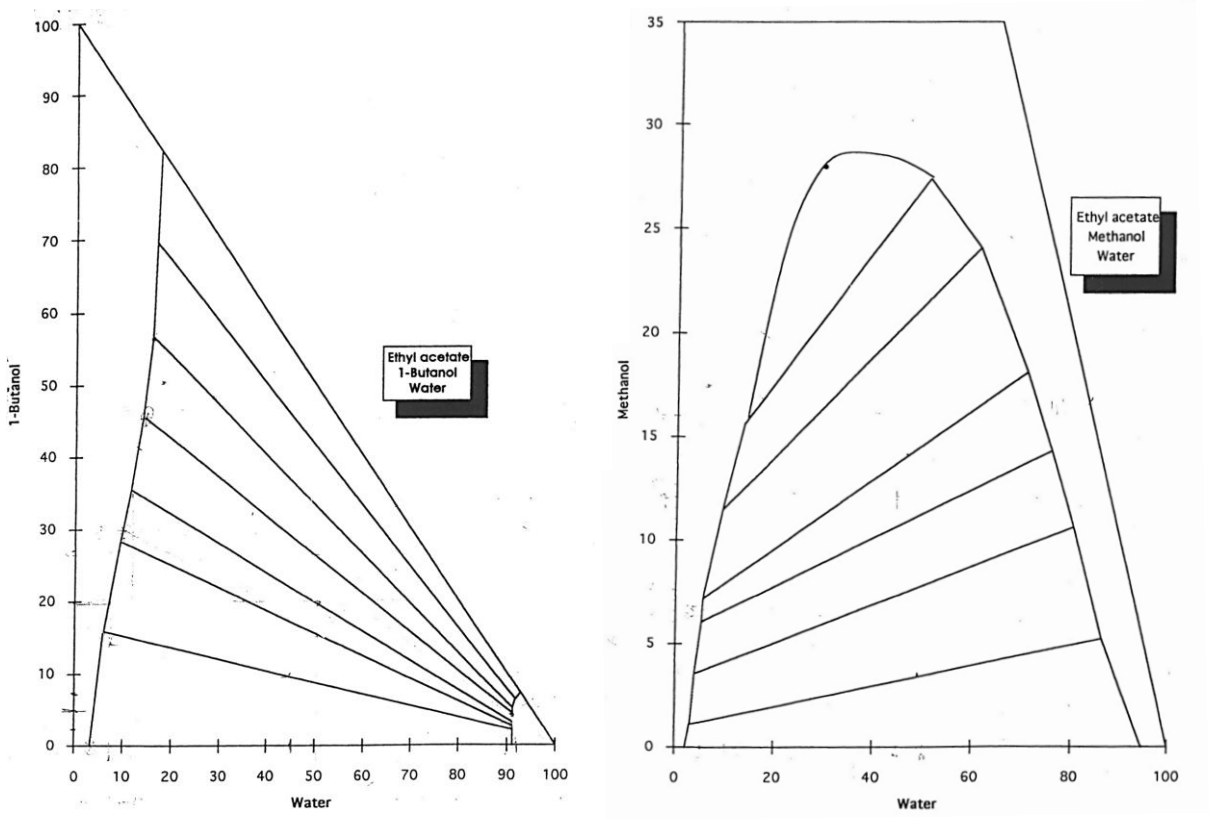


Figure 78. Ternary diagram of EtOAc/n-BuOH/water system (left) and EtOAc/MeOH/water (right).

n-BuOH was mainly partioned in the EtOAc rich phase, whereas MeOH was mainly partitioned in the aqueous phase.

Former data of the laboratory on *Gingko biloba* flavonoids partition demonstrated that flavonoids in these two biphasic solvent systems were partitionned mainly in the alcohol rich phase, which was not suitable for the first fractionation step.

We decided then to mix these two solvent systems to build a quaternary system EtOAc/*n*-BuOH/MeOH/Water, taking advantage of both good solvents:

- an extended solubility of the samples;
- a balanced partition between the two phases, since *n*-BuOH extracted the flavonoids in the organic phase, whereas MeOH extracted them in the aqueous one.

The ternary system EtOAc/*n*-BuOH/water was also chosen for the further fractionation step as it allows a good retention of compounds in simplified mixture and could also be used in gradient mode.

III.4.1.2. Elution mode:

We decided to work in elution mode, since pHZR could lead to a hydrolysis of sugars moieties in acidic conditions, and IEX is not suitable.

Flavonoids, particularly the glycosylated one, were usually responsible for a high level of stationary phase's bleeding. They carried off a small amount of stationary phase together with mobile phase during elution process, leading to an important decrease of Sf during the experiment. Moreover, pulsations were also observed during CPC runs with those metabolites, leading sometimes to a dramatic destabilization of the column and a loss of resolution. In order to overcome these issues, a systematic co-current rate of 10 % could be used. Co-current elution was demonstrated to be a simple option to stabilize pulsations and to overcome stationary phase loss during CPC separation of tensioactive analytes (Amarouche *et al.*, 2013).

In conclusion of this work, two solvent systems were selected, EtOAc/*n*-BuOH/MeOH/water and EtOAc/*n*-BuOH/water, with a co-current rate of 10 %.

III.4.2. Flavonoids enriched extract preparation:

Z. spina-christi leaves methanol extract (80 g) was solubilized in water and extracted successively using petroleum ether, ethyl acetate and *n*-butanol. Flavonoids were mainly present in EtOAc (24.4 g) *n*-BuOH (7.9 g) extracts.

HPLC chromatograms of both extracts are presented in Figure 79. In both extracts, flavonoids were found in high amounts, with retention time between 4 and 7 min. Five major compounds exhibiting a typical flavonoid UV spectra, were highlighted in these chromatograms. We could also notice that the butanol extract contained higher concentrations of flavonoids and less amount of non-flavonoids metabolites compare with ethylacetate extract; we then decided to focus on this extract for our study.

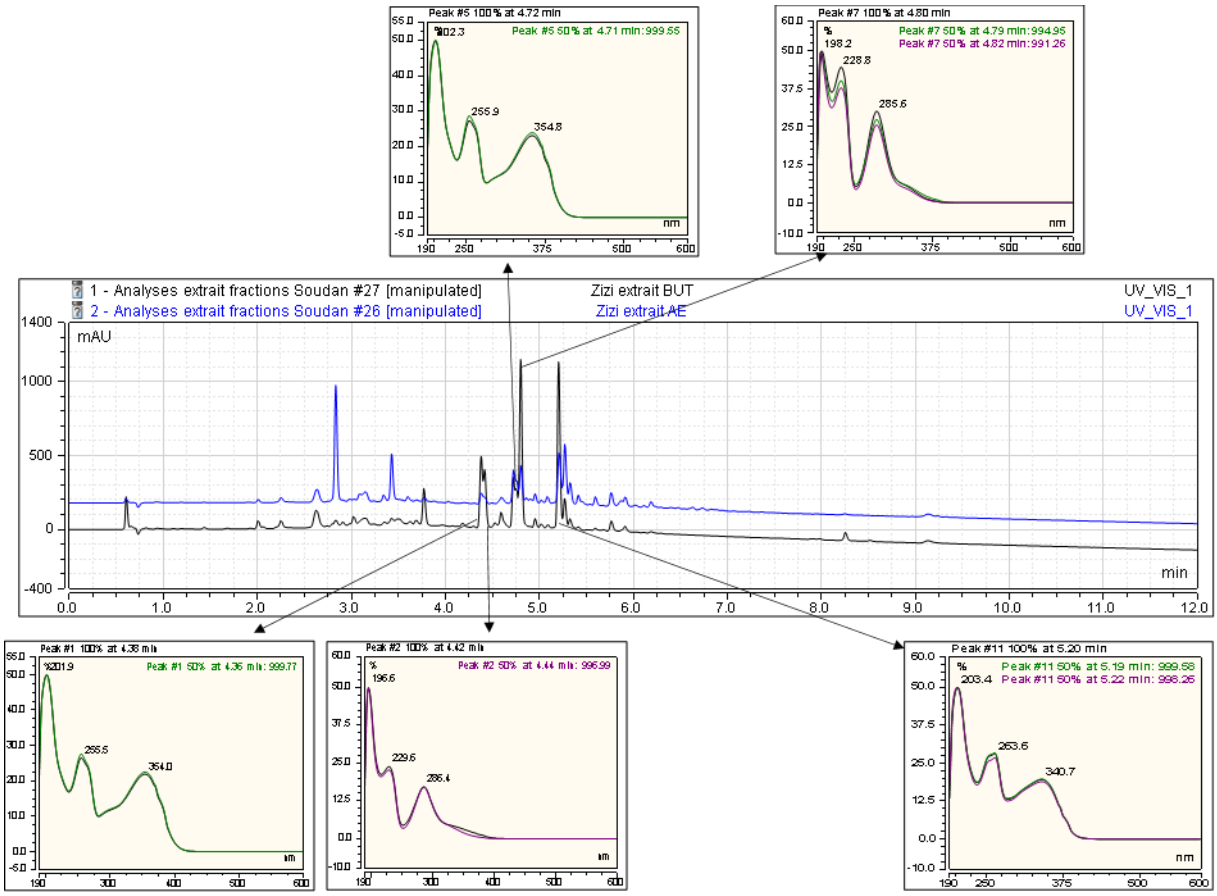


Figure 79. HPLC chromatogram of EtOAc (in blue) and n-BuOH (in black) extracts at 210 nm; UV-spectra of major metabolites.

III.4.3. CPC optimization for Butanol fraction:

The first step was the screening of the two selected solvent systems: EtOAc/*n*-BuOH/MeOH/water and EtOAc/*n*-BuOH/water in different ratios.

III.4.3.1. Solvent system screening:

Four different systems based on EtOAc/*n*-BuOH/MeOH/water were investigated containing different *n*-BuOH/MeOH ratios: equal, excess of *n*-BuOH or excess of MeOH (Systems A to D, Table 10).

Beside them, four different EtOAc/*n*-BuOH/water system were also investigated (systems E to H, Table 10).

On TLC plate, four major flavonoids were visible and used to evaluate the *K* of each compound in the solvent system. Compounds were numbered from Z_1 to Z_4 according to their retardation factor (Rf): Z_1 0.6, Z_2 0.53, Z_3 0.37 and Z_4 0.3 (Figure 80).

Table 10. Solvent systems screened with crude extract and resulting K of major flavonoids:

Code	Solvent system	Z_1 *K*		Z_2 *K*		Z_3 *K*		Z_4 *K*	
	EtOAc/*n*-BuOH/MetOH/H_2O	Up	Low	Up	Low	Up	Low	Up	Low
A	5:2:1:7, v/v/v/v	++++	+	+++	++	++	+++	++	++++
B	5:1:1:7, v/v/v/v	++++	+	+++	++	++	++++	+/-	++++
C	5:1:3:7, v/v/v/v	+++	++	+++	+++	++	++++	+	++++
D	5:1:2:7, v/v/v/v	+++	++	+++	+++	++	+++	+	++++
	EtOAc/*n*-BuOH/H_2O								
E	4:3:5, v/v/v	++++	+	++++	+	+++	++++	+++	++++
F	4:2:5, v/v/v	++++	+	++++	+	+++	+++	++	+++
G	4:1:5, v/v/v	++++	+	++++	+	+++	+++	+	++++
H	4:1:4, v/v/v	++++	+	++++	++	++	+++	+	++++

Again, the goal was to obtain *K* constant for the major products in the so-called *sweet spot* (between 0.5 and 5). Moreover to reach enough selectivity, *K* differences should be noticed between all products, if possible.

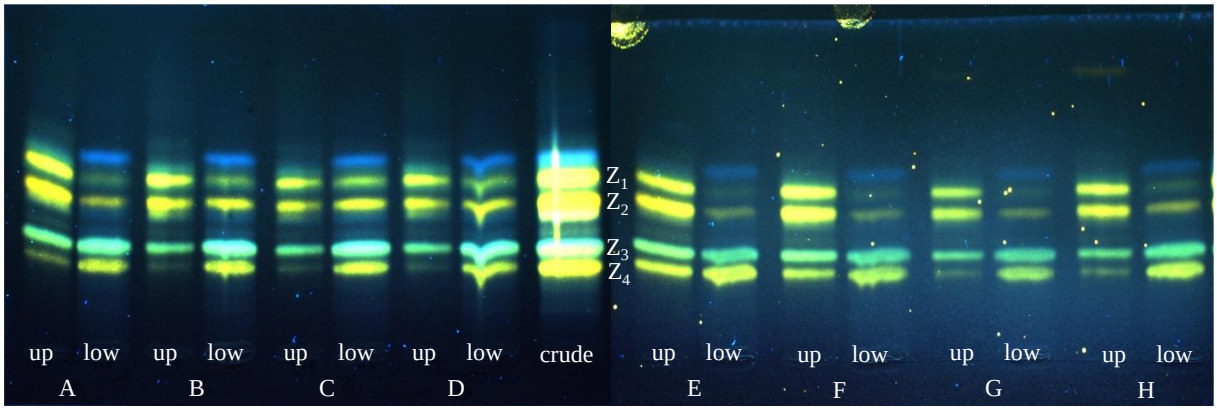


Figure 80. TLC chromatogram of the solvent systems screening for crude BuOH extract at 365 nm after Neu reagent derivatization; mobile phase: EtOAc/Acetic acid/Formic acid/water (100:11:11:26, v/v/v/v), up: upper organic phase, low: lower aqueous phase.

Considering EtOAc/*n*-BuOH/MeOH/water systems (A to D), the extract was perfectly soluble in all systems. In system A containing an excess of *n*-BuOH, the most apolar flavonoids Z_1 and Z_2 were mainly partitioned in the upper phase (approximative *K* of 3 and 2 respectively), the compound Z_3 was almost equally distributed (approx. *K* of 1.5) and product Z_4 was mainly partitioned in the lower phase (approx. *K* of 0.5). This system could be a good option in descending mode (organic stationary phase) for the primary fractionation as all *K* were in the sweet spot.

Decreasing the *n*-BuOH rate (system B), the compounds Z_1 and Z_2 were less concentrated in the upper phase with approximative *K* of 2 and 1 respectively. Also compounds Z_3 and Z_4 were mainly in the aqueous phase (approx. *K* of 0.3 and 0.2). This system would not allow a good retention of compounds Z_1 and Z_2 in ascending or of Z_3 and Z_4 in descending mode.

In systems C and D, containing an excess of MeOH, the compounds behavior was similar: Z_1 was mainly present in upper phase (approx. *K* of 2), Z_2 was equally distributed (approx. *K* of 1), Z_3 was almost equally distributed (approx. *K* of 0.7) and Z_4 was mainly present in aqueous phase (approx. *K* of 0.3). The system D allowed moreover a quicker phase separation compared to system C. The system D could be also a good system for preliminary fractionation in descending mode, since higher *K* differences were noticed either between Z_1 and Z_2 or Z_3 and Z_4.

Considering EtOAc/*n*-BuOH/water systems (E to H), compounds Z_1 and Z_2 were mostly partitionned in the upper phase with approx. *K* around 5 in all of them, leading to unfavorable retention (too long or too short depending on the pumping mode). Compounds Z_3 and Z_4 were well partitioned in system E and F, with *K* around 1, but with no noticeable difference between the two compounds. In system G and H, Z_3 exhibited approx. *K* of 0.5 and Z_4 of 0.3. Those systems would not be suitable for the preliminary fractionation but could be an option for further purification of most polar compounds.

Thus as first fractionation, butanolic extract was fractionated using the two systems EtOAc/*n*-BuOH/MeOH/water A (5:2:1:7, v/v/v/v) and D (5:1:2:7, v/v/v/v), and the fractionation performances were compared.

*III.4.3.2. Preliminary fractionation using EtOAc/*n*-BuOH/MeOH/water:*

The experimental data were summarized in Table 11.

In both cases, descending mode was selected in order to obtain higher retention volume of compounds Z_1 and Z_2. Also a co-current rate of 10 % was used, since butanol rich solvent system were known to be more viscous and thus sensitive to pulsations.

Previous work on *G. biloba* in the laboratory (data not shown) and previous data on a cyclopeptide (Amarouche *et al.*, 2013) showed that the injection protocol was really important to limit the pulsation phenomena and the loss of stationary phase. Optimal protocol was determined as following: an injection after reaching the equilibrium state, in a mixture of the two phases, at low flow rate (2 $mL.min^{-1}$) and lower rotation speed (600 rpm). This protocol was applied systematically.

Table 11. CPC experimental conditions of preliminary fractionation:

Experiment	**ZCPC 1**	**ZCPC 2**
Solvent system	EtOAc/*n*-BuOH/MeOH/water	
Solvent ratio	**D:** 5:1:2:7,v/v/v/v	**A:** 5:2:1:7, v/v/v/v
Mode	Descending, 10 % co-current	
Rotation speed	1800 rpm	
Sample loading	3.00 g	3.77 g
Flow rate	8 mL.min^{-1}	8 mL.min^{-1}
Sf	74 %	71 %
Back pressure	25 bars	29 bars
Fraction collect	Every 30 seconds (8 seconds during extrusion)	

To compare CPC fractionation performances, recap TLC plates of each ZCPC run were shown in Figure 81.

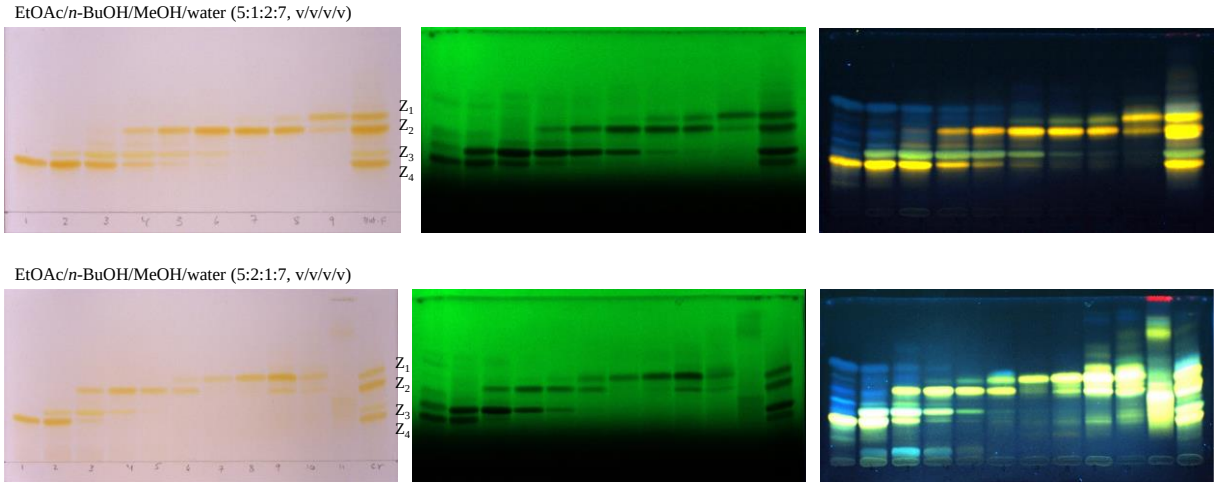


Figure 81. Recap TLC plates of ZCPC 1(Top) and ZCPC 2 (Bottom) at day light, 254 nm and 366 nm after Neu reagent derivatization; Mobile phase EtOAc/Acetic acid/Formic acid/water (100:11:11:26, v/v/v/v).

From ZCPC 1, 9 fractions were obtained (code ZCPC 1-1 to 9). The first flavonoids merged from the column 17 min after the equilibrium, indicating a good retention volume.

Compounds Z_4 was enriched in fraction ZCPC 1-1 (117 mg). According to HPLC analysis, the major peak with a retention time of 4.37 min corresponding to Z_4 in Figure 82, was found to be a mixture of partially co-eluted compounds corresponding to both peaks of Rt 4.36 and 4.44 in (crude extract).

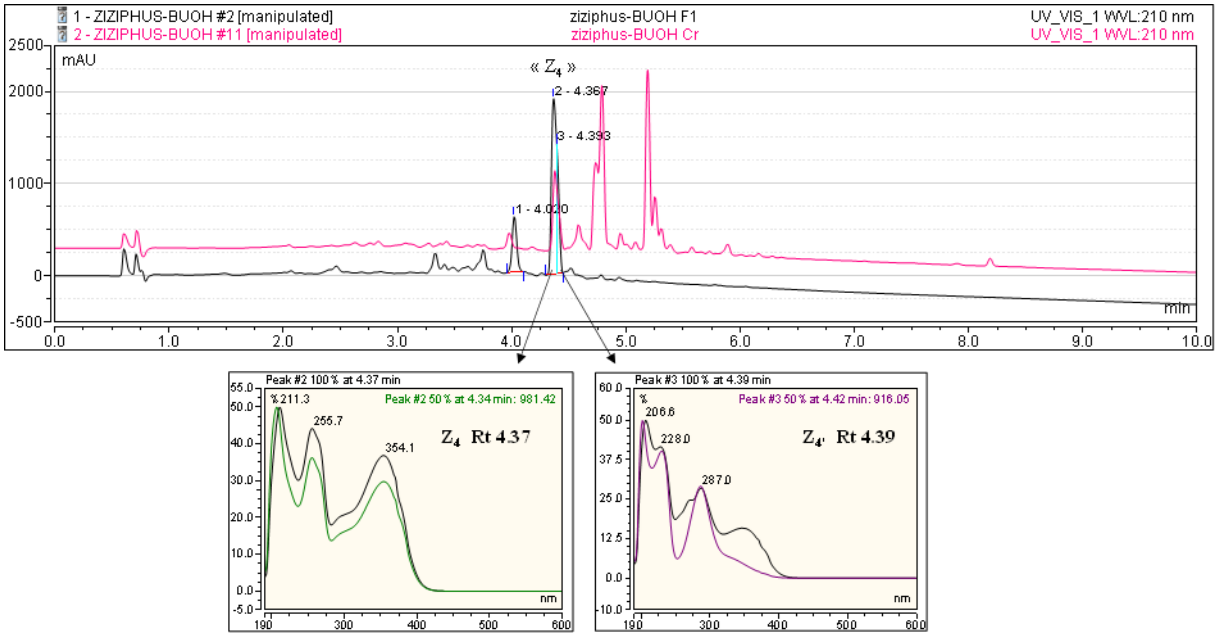


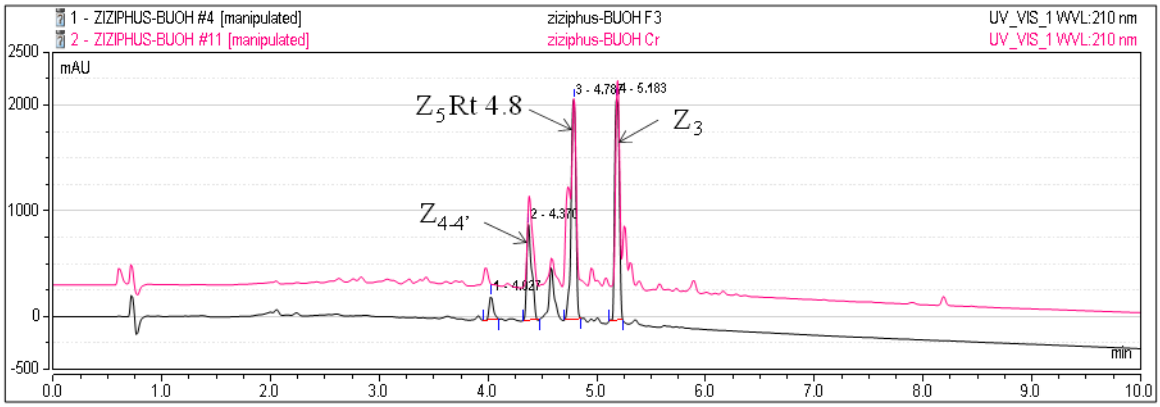
Figure 82. HPLC chromatogram of fraction ZCPC 1-1 (in black) compare to butanolic crude extract (in pink) at 210 nm.

Nevertheless, Z_4 and $Z_{4'}$ were separated from other compounds and obtained in high amount (more than 100 mg), which was promising. But further purification would be tricky, considering the co-elution of the two compounds on TLC and HPLC.

Fractions ZCPC 1-2 (259 mg) and ZCPC 1-3 (247 mg) exhibited almost similar TLC and HPLC profile (Figure 83 and Figure 86) containing mainly $Z_{4\text{-}4'}$, Z_3 and an other flavonoid (Z_5) of Rf 0.42 and Rt 4.78, corresponding to the peak of Rt 4.8 in Figure 83 (peak 7 of the crude extract). Those two fractions were pooled and would be submitted to further purification.

In fractions ZCPC 1-4 to ZCPC 1-6 (196, 88 and 139 mg, respectively), compounds $Z_{4\text{-}4'}$, Z_3, Z_5, and Z_2 were found in mixture. It was also important to note that in HPLC analyses, product Z_2 and Z_5 were co-eluted (Rt of 4.75 and 4.78 respectively), leading to possible confusion (Figure 83). Fortunately, Z_2 and Z_5 exhibited different UV-spectra and could be then distinguished using 3D data of HPLC record (Figure 84).

A



B

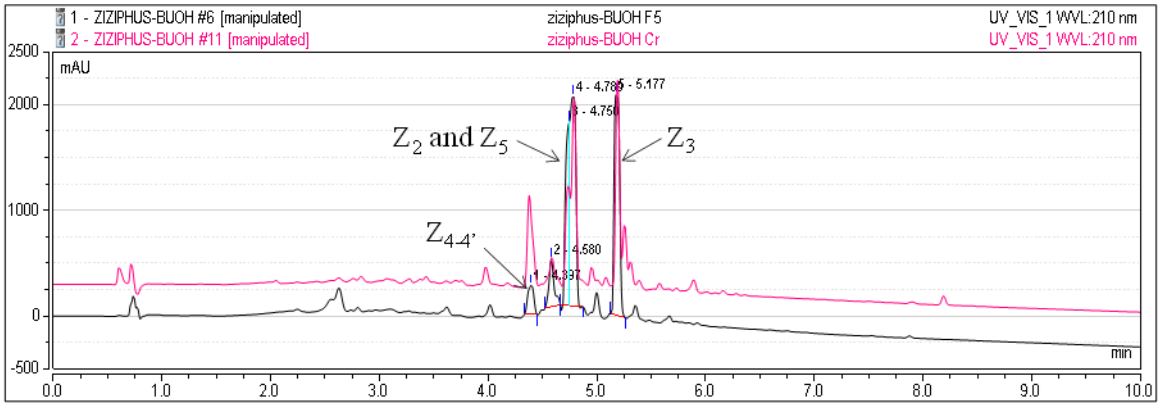


Figure 83. HPLC chromatogram of fraction ZCPC 1-3 (in black) compare to butanolic crude extract (in pink) at 210 nm (A); of fraction ZCPC 1-6 (in black) compare to butanolic crude extract (in pink) at 210 nm (B) .

Fractions ZCPC 1-7 and 8 (37 and 16 mg) contained a mixture of Z_2 and Z_1 in which Z_2 was the major compound contrary to fraction ZCPC 1-9 (488 mg) containing a mixture of those 2 metabolites but with Z_1 as major peak.

The CPC run was stopped after 120 min of experiment, which was quite long according to elution standard. But during washing process, we noticed the elution of yellow colored fractions, highly retained. Thus an extrusion step would be added in further CPC runs.

This first CPC fractionation allowed us to identify HPLC peaks in the crude butanolic extract, using the same numbering as TLC (Figure 84).

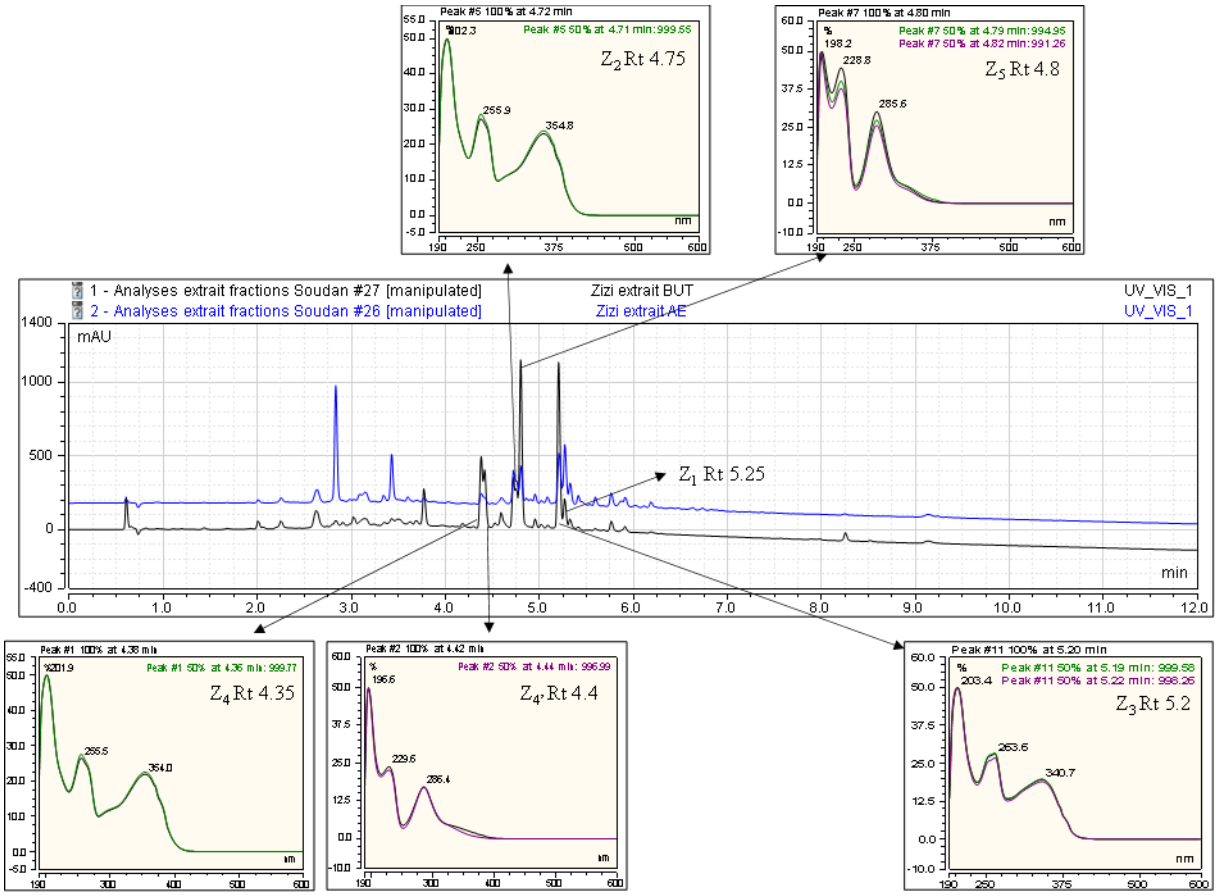


Figure 84. HPLC chromatogram of crude BuOH extract in black at 210 nm, with labeled peaks and corresponding UV spectra.

To conclude on ZCPC 1, compounds $Z_{4\text{-}4'}$ and Z_1 were obtained in enriched fractions but others were obtained in mixture containing 3 or 4 major compounds, which was nevertheless a good deconvolution from a complex crude extract. To reach high enough purity for structure elucidation, all fractions required further purification steps.

Considering ZCPC 2, 11 fractions (ZCPC 2-1 to 11) were obtained, the three last fractions resulting of the extrusion step. A higher retention volume was obtained for the most apolar flavonoid Z_1 leading to a better separation of this compound from Z_2, compared to ZCPC 1. But for compounds Z_3, $Z_{4\text{-}4'}$ and Z_2, retention volumes were slightly decreased leading to extended mixing zones.

As in the first run, fraction ZCPC 2-1 (181 mg) was an enriched fraction of Z_4 and $Z_{4'}$.

Z_1 was obtained with purity around 80 % in fractions ZCPC 2-7 (28 mg) and ZCPC 2-8 (42 mg). Nevertheless, the main part of Z_1 was eluted during the extrusion step in fraction ZCPC

2-9, co-eluted with another compound exhibiting a retardation factor (Rf) closed to Z_2 on TLC plate.

Fractions ZCPC 2-3 and 4 (316 and 281 mg) containing a mixture of Z_5 and Z_3 together with a small amount of Z_2 (Figure 81, at 254 nm), would be interesting to pool to access to Z_3 or Z_5.

Ending extrusion fractions (ZCPC 2-10 and 11, 347 and 501 mg respectively) were composed of minor flavonoids of medium polarity, almost not detected in HPLC and TLC of crude extract. This highlighted the high power of concentration of CPC.

To conclude on ZCPC 2, a good primary fractionation was obtained, improved for apolar compounds compared to ZCPC 1. Enriched fractions should nevertheless be subjected to further purification and structure elucidation.

The EtOAc/*n*-BuOH/MeOH/water system was a good option for preliminary fractionation of flavonoids leading to chemically simplified fractions for major compounds in 2 hours of experiment, from 3 grams of crude extract. Using conventional column chromatography would have been much time consuming.

The further purification of ZCPC 1 and ZCPC 2 fractions was then investigated using an additional CPC step.

III.4.3.3. Further purification using CPC:

III.4.3.3.1. Purification of less polar flavonoid Z_1:

III.4.3.3.1.1. CPC purification of ZCPC 1-9: ZCPC 3:

Solvent system screening:

The ZCPC 1-9 fraction (480 mg) contained mainly Z_1 compound (33 %) in mixture with many minor flavonoids. Considering preliminary solvent screening results (Table 10; Figure 80), and better retention volume of Z_1 during ZCPC 2, the solvent system EtOAc/*n*-BuOH/MeOH/H_2O A (5:2:1:7, v/v/v/v) was selected.

CPC purification:

Experimental data were resumed in Table 12.

Table 12. Experimental data for purification of "apolar" and medium polarity flavonoids by CPC:

Experiment	**ZCPC 3**	**ZCPC 4**
Sample	ZCPC 1-9 (480 mg)	ZCPC 2-3+4
Solvent system	**A:** EtOAc/*n*-BuOH/MeOH/water (5:2:1:7, v/v)	**G:** EtOAc/*n*-BuOH/water (4:1:5, v/v)
Mode	Descending, 10 % co-current	
Rotation speed	1800 rpm	
Flow rate	8 mL.min^{-1}	
Sf	73 %	74 %
Back pressure	26 bars	38 bars
Fraction collect	Every 30 seconds	

The descending mode was used in order to ensure high retention volume of Z_1, increasing the selectivity of the separation.

As expected Z_1 product was eluted 40 min after the equilibrium state for a total CPC run duration of 120 min. Compound Z_1 was isolated with high purity around 97 % from *Z. spina-christi*. HPLC chromatogram is shown in Figure 85, Z_1 peak was a little bit tailing but the UV spectra of the peak and the NMR spectra were clean.

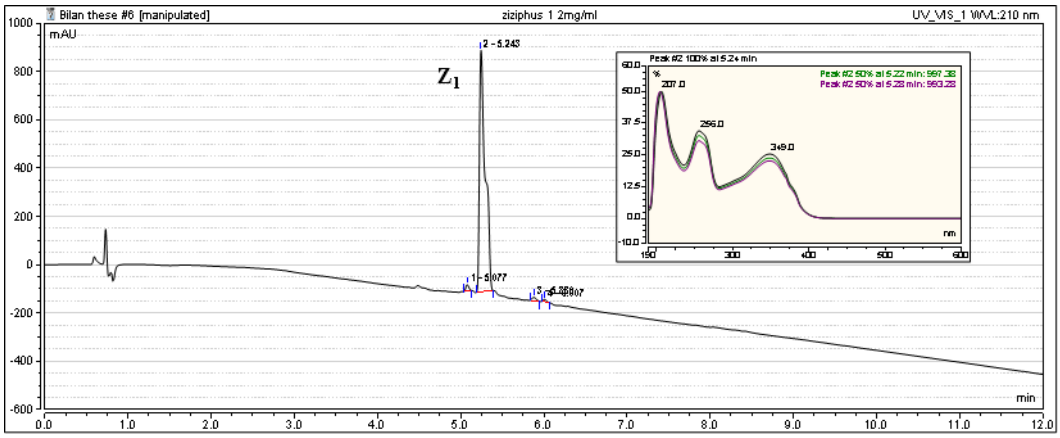


Figure 85. HPLC chromatogram of fraction ZCPC 3-1 at 210 nm; UV spectra of Z_1 peak.

Z_1 (53 mg) was identified by ^{1}H NMR as a glycoside of quercetin (Appendix 7-8). After acid hydrolysis of Z_1, the sugars were isolated and identified by co-TLC with authentic markers. Based on the results of the acid hydrolysis of Z_1, the magnitudes of their $J_{1,2}$ coupling constants and the analysis of 1D- and 2D-NMR data, the sugar units were elucidated as α-L-rhamnopyranose (δH-1 at 5.35 ppm and δC-1 at 101.8 ppm) and β-D-xylopyranose (δH-1 at 4.26 ppm and δC-1 at 106.3 ppm). From the HMBC, the anomeric proton signal of H-1 of

xylofuranose coupled to C-2 of rhamnopyranose was found. The position of the sugar linkage was confirmed by HMBC correlation between anomeric proton signal of rhamnopyranose and C-3 of quercetin moiety.

Moreover, Z_1 displayed a molecular ion peak $[M+H]^+$ at m/z 581.14986 in the positive HR-ESI-MS, indicating the molecular formula $C_{26}H_{29}O_{15}$.

Finaly, by comparison of the 1D- and 2D-NMR data with the literature, Z_1 was identified as quercetin-3-O-(2"-O-*β*-D-xylopyranosyl-*α*-L-rhamnopyranoside) (Nielsen *et al.*, 2005; Ren *et al.*, 2013).

Using this solvent system, purification of apolar Z_1 was easy and fast. It was reasonable to think that Z_1 could have been isolated directly in the first run (ZCPC 2) if the extrusion step would have been started later.

III.4.3.3.2. CPC purification of intermediate polarity flavonoid Z_2:

To access to Z_2, fraction ZCPC 2-3 was pooled with ZCPC 2-4 to obtain a 500 mg sample.

III.4.3.3.2.1. CPC purification of fraction ZCPC 2-3+4: ZCPC 4:

Solvent system selection:

Considering preliminary solvent screening results (Table 10; Figure 80), the solvent system EtOAc/*n*-BuOH/H_2O G (4:1:5, v/v/v) was selected. Indeed, the two main compounds in this fractions presented an important difference of *K* with $K \approx 5$ for Z_2 and $K \approx 0.5$ for Z_3. We could then expect a good resolution. As the main goal of this run was to isolate the compound Z_2, the descending mode was selected to ensure high retention of Z_2 and fast elution of polar flavonoids.

CPC experiment:

Experimental data were resumed in Table 12 and CPC chromatogram together with recap TLC plates were shown in Figure 86.

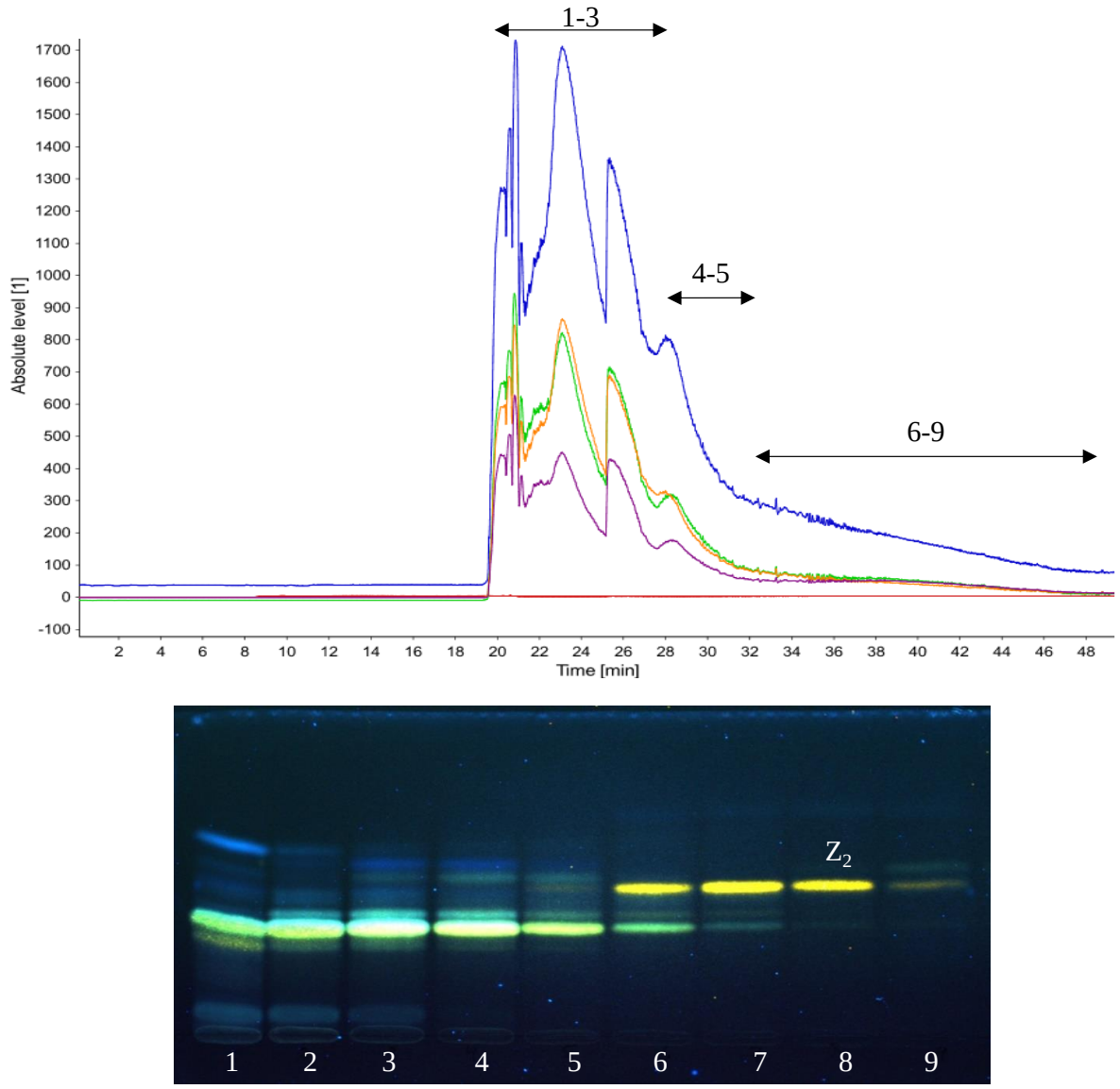


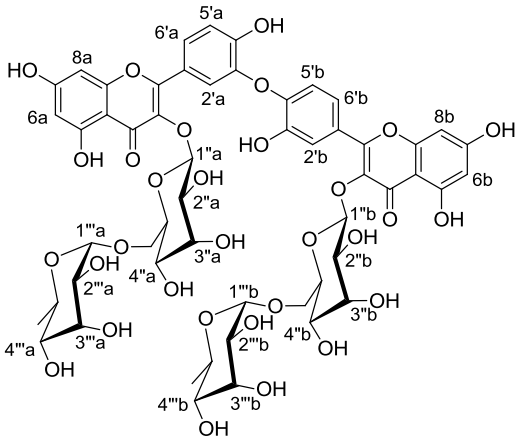
Figure 86. CPC chromatogram (on top) at 210 nm (blue), 254 nm (green), 280 nm (orange) and 365 nm (purple) and recap TLC plate (at bottom) of ZCPC 4 at 366 nm after Neu reagent derivatization; mobile phase EtOAc/acetic acid/formic acid/water (100:11:11:26, v/v).

As expected, polar compounds were eluted just after reaching the equilibrium state in a small volume of mobile phase (70 mL, during 10 min) and then compound Z_2 get out of the column almost pure just after them. Z_2 (46 mg) was then isolated with high purity (up to 96 %) and subjected to NMR and MS. From the 1D- and 2D-NMR data (Appendix 9-16), Z_2 appeared as a mixture of two quercetin squeletons based on the aromatic proton signals. Moreover, Z_2 displayed a molecular ion peak $[M+2H]^{2+}$ at m/z 630.1338 in the positive HR-ESI-MS, with an isotopic repartition indicating a doubly charged ion corresponding to a compound with a masse of 1259.2572.

According to the flavonoids composition of *Ziziphus spina-christi* in the literature, we then deduced that Z_2 is a dimer of rutoside, closely related to zizyflavoside A (Mostafa *et al.*, 2010). Comparing ^{13}C NMR data of zizyflavoside A with our compound Z_2 (Table 13), we noticed a very high matching between them (differences less than 0.5 ppm).

Table 13. ^{13}C NMR Spectroscopic Data (CD_3OD) at 300 MHz for Z_2 and 500 MHz for Zizyflavoside A (Mostafa *et al.*, 2010):

N°	Zizyflavoside A, δC (ppm), mult		Z_2 δC (ppm), mult	
2a,2b	159.36,C	159.03	159.29,C	158.89
3a,3b	135.89,C	135.64	135.85,C	135.62
4a,4b	179.52,C	179.46	179.38,C	179.38
5a,5b	163.02,C	162.97	162.92,C	162.87
6a,6b	99.97,CH	99.96	99.94,CH	99.94
7a,7b	166.12,C	166.03	166.04,C	165.96
8a,8b	94.87,CH	94.84	94.82,CH	94.82
9a,9b	158.55,C	158.50	158.44,C	158.38
10a,10b	105.67,C	105.61	105.58,C	105.51
1'a,1'b	123.56,C	123.18	123.07,C	122.74
2'a,2'b	117.96,CH	117.71	117.96,CH	117.68
3'a,3'b	145.86,C	145.77	145.78,C	145.68
4'a,4'b	150.00,C	149.81	149.96,C	149.77
5'a,5'b	116.14,CH	116.08	116.09,CH	116.03
6'a,6'b	123.07,CH	122.87	123.56,CH	123.00
1a",1b"	104.71,CH	105.96	104.75,CH	105.97
2a",2b"	75.12,CH	73.96	75.05,CH	73.84
3a",3b"	78.23,CH	77.27	78.14,CH	77.15
4a",4b"	72.07,CH	71.90	72.06,CH	72.00
5a",5b"	75.75,CH	75.38	75.70,CH	75.22
6a",6b"	68.58,CH	67.43	68.52,CH	67.28
1a'",1b'"	102.43,CH	101.96	102.38,CH	101.85
2a"',2b"'	72.32,CH	72.28	72.25,CH	72.21
3a"',3b"'	72.12,CH	71.44	72.00,CH	71.34
4a'",4b"'	73.90,CH	73.15	73.90,CH	73.12
5a"',5a"'	69.72,CH	70.22	69.67,CH	70.15
6a"',6b"'	17.95,CH_3	17.87	17.96,CH_3	17.87



Zizyflavoside A

Nevertheless, Zizyflavoside A presented a pseudo-molecular ion peaks at 1203.3038 $[M+2H]^{2+}$ in the positive HR mass spectra. Moreover, we could observed on the ^{13}C NMR spectra two signals corresponding to a carbonyl group (δ CO at 170.2 ppm) and a methoxy group (δCH_3O at 52.6 ppm), with a clear correlation between these two signals in the HBMC. These data suggested the presence of a methyl formiate in Z_2. No coupling between the signal of the carbonyl group at δ 170.2 ppm and the other signals of the molecule could be observed on the HMBC, preventing us to localize this group on the structure of the dimer. The structure elucidation is still under progress.

The last fractions containing flavonoids of highest polarity were then subjected to further purification.

III.4.3.3.3. CPC purification of the flavonoids with the lowest Rf, $Z_{4\text{-}4}$ to Z_3:

Two main fractions were fractionated containing mainly Z_4, $Z_{4'}$, Z_5 and Z_3 compounds: ZCPC 1-2+3 and ZCPC 2-2. These two fractions exhibited very similar composition and could be then fractionated using the same conditions by CPC.

III.4.3.3.3.1. Solvent system selection:

Considering preliminary solvent screening results (Table 10; Figure 80), the solvent systems EtOAc/*n*-BuOH/Water G (4:1:5, v/v/v) and H (4:1:4, v/v/v) were the only systems exhibiting a good partition of products and a significant difference of *K* between Z_3 (0.5) and $Z_{4\text{-}4'}$ (0.25). The system H (4:1:4, v/v/v) settled a little more quickly with the product, it was then selected to perform CPC separation of the fraction ZCPC 1-2+3, as first trial.

III.4.3.3.3.2. CPC fractionation of ZCPC 1-2+3: ZCPC 5:

The solvent system EtOAc/*n*-BuOH/Water H (4:1:4, v/v/v) was then used to perform CPC purification in ascending mode, using co-current. The ascending mode was chosen to ensure higher retention volume of the compounds, since they were partitioned in favor of the aqueous stationary phase. Experimental data were resumed in Table 14 and CPC chromatogram together with recap TLC plate are presented in Figure 87.

Table 14. CPC experimental data for polar flavonoids purification:

Experiment	**ZCPC 5**	**ZCPC 6**
Sample	ZCPC 1-2+3 (500 mg)	ZCPC 2-2 (480 mg)
Solvent system	**H:** EtOAc/*n*-BuOH/water (4:1:4,v/v)	EtOAc/*n*-BuOH/water (6:1:4,v/v)
Mode	Ascending, 10 % co-current	
Rotation speed	1800 rpm	
Flow rate	8 mL.min^{-1}	
Sf	75 %	74 %
Back pressure	37 bars	36 bars
Fraction collect	Every 30 seconds	

The system demonstrated a good stability, with high stationary phase retention and back pressure. Products started to be eluted 40 min after reaching the equilibrium state, highlighting a good retention volume as expected.

On CPC chromatogram, compounds Z_3 and $Z_{4\text{-}4'}$ appeared as two separated peaks. This was confirmed by TLC analyses (Figure 87).

Compound Z_3 was isolated with quite high purity in fraction ZCPC 5-2 (40 mg, 85%). Z_3 was identified by ^{1}H NMR as a bis-glycoside of quercetin (Appendix 17-18). After acid hydrolysis of Z_3, the sugars were isolated and identified by co-TLC with authentic markers. Based on this TLC comparison, the magnitudes of their $J_{1,2}$ coupling constants and the analysis of 1D- and 2D-NMR data, the sugar units were elucidated as previously for Z_1, as α-L-rhamnopyranose (δH-1 at 5.38 ppm and δC-1 at 102.0 ppm) and β-D-xylopyranose (δH-1 at 4.31 ppm and δC-1 at 106.3 ppm); a supplementary unit of α-L-rhamnopyranose is present on the NMR spectra (δH-1 at 5.53 ppm and δC-1 at 99.5 ppm). As previously observed with Z_1, the anomeric proton signal of H-1 of xylopyranose coupled to C-2 of rhamnopyranose was found on HMBC.

The positions of the sugar linkages were confirmed by HMBC correlation between anomeric proton signal of rhamnopyranose and C-3 signal of quercetin moiety, and between the anomeric proton signal of the second rhamnopyranose and C-4' or C-3' signal of quercetine.

Moreover, Z_3 displayed a molecular ion peak $[M+H]^+$ at m/z 727.20748 in the positive HR-ESI-MS, indicating the molecular formula $C_{32}H_{39}O_{19}$.

Comparing NMR data obtained for Z_3 with those described in the literature (Nawwar *et al.*, 1984; Shabat *et al.*, 2001), Z_3 was identified as quercetin-3-O-(2"-O-β-D-xylopyranosyl-α-L-rhamnopyranoside)-4'-O-α-L-rhamnoside.

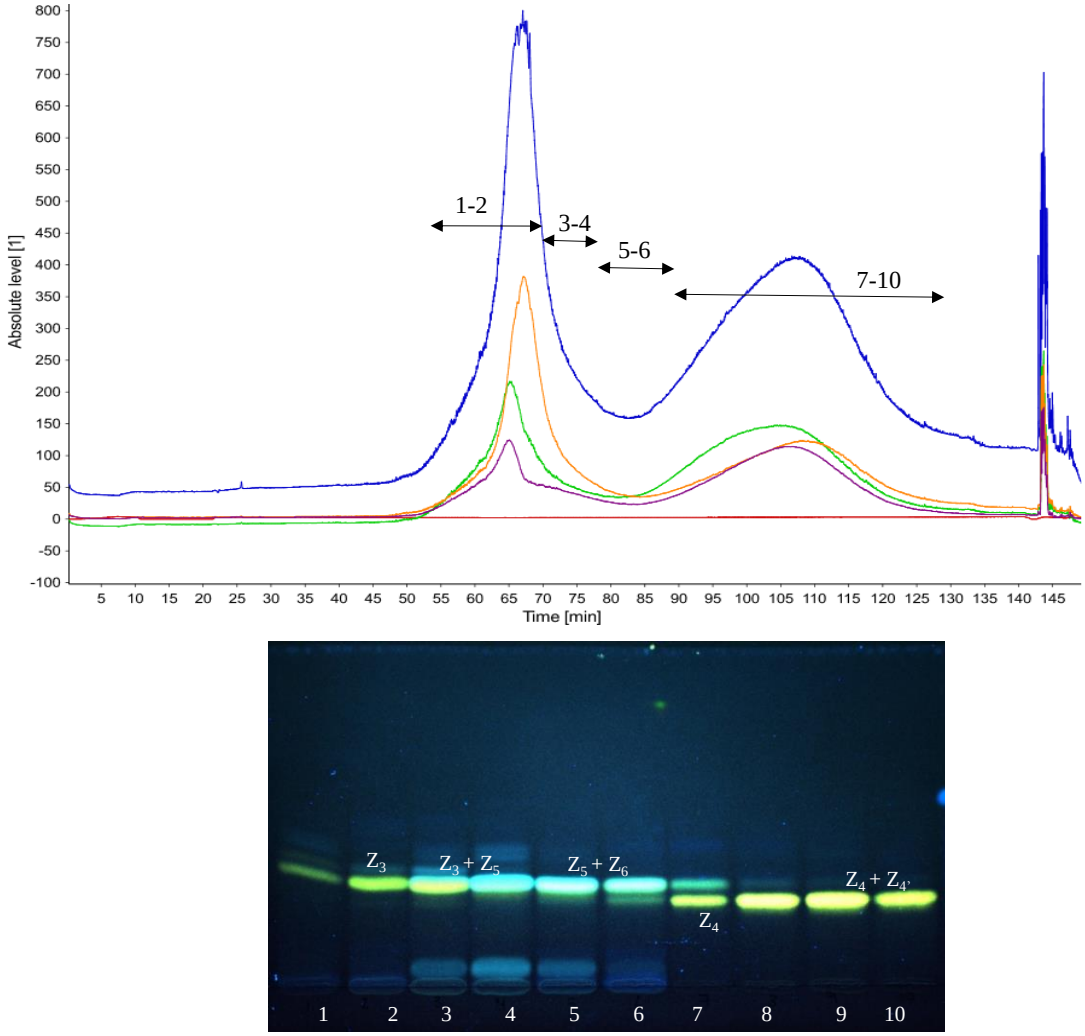


Figure 87. CPC chromatogram (on top) at 210 nm (blue), 254 nm (green), 280 nm (orange) and 365 nm (purple) and recap TLC plate (at bottom) of ZCPC 5 at 366 nm after Neu reagent derivatization; mobile phase EtOAc/acetic acid/formic acid/water (100:11:11:26, v/v).

In fractions ZCPC 5-3 and 5-4 (40 and 47 mg), Z_3 was found in mixture with compound Z_5 according to HPLC analysis. Z_5 was then identified as a fluorescent green spot with Rf closed to Z_3 on TLC plate (Figure 87).

Also in fractions ZCPC 5-5 and 5-6 (63 and 10 mg), compound Z_5 was found in mixture with a new minor compound Z_6, visible on HPLC chromatogram with Rt of 4.6 min (Figure 88).

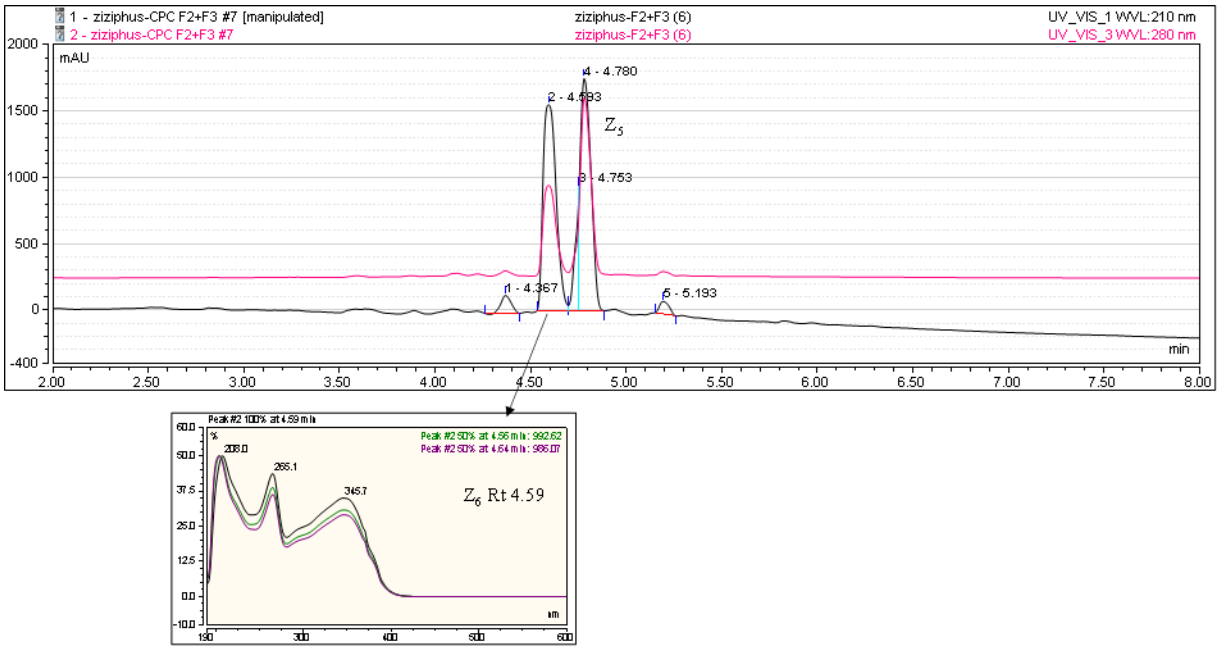


Figure 88. HPLC chromatogram of fraction ZCPC 5-6 at 210 nm (black) and 280 nm (pink) on top; UV spectra of Z_6 peak (at the bottom).

This compound exhibited a UV spectra typical of flavonoid compound. Z_5 and Z_6 were not detected in crude extract but were concentrated after 2 CPC runs.

In fraction ZCPC 5-7 (27 mg), compound Z_4 was obtained with low purity (around 50 %) in mixture with Z_5 and Z_6. But interestingly, Z_4 was not co eluted with $Z_{4'}$ solely in this fraction (Figure 89), highlighting an important selectivity of the system.

Fractions ZCPC 5-8 to 10 (16, 20 and 37 mg) contained co-eluted Z_4 and $Z_{4'}$ product with purity higher than 85 %.

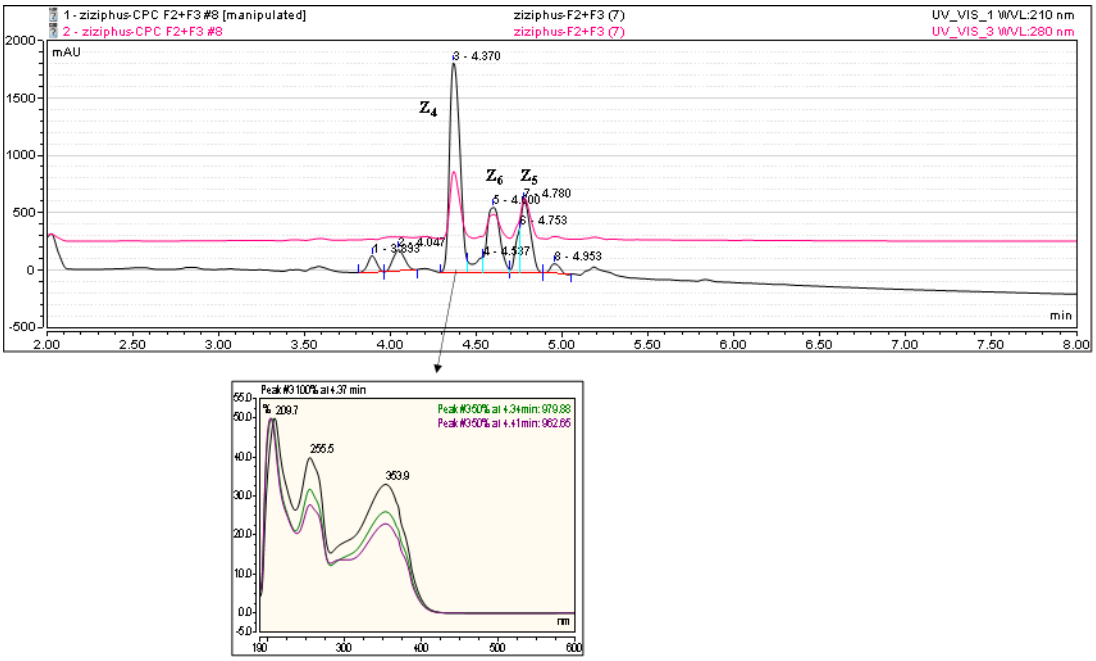


Figure 89. HPLC chromatogram of fraction ZCPC 5-7 at 210 nm (black) and 280 nm (pink) on top; UV spectra of Z_4 peak (at the bottom).

In order to extend the selectivity in ascending mode, additional solvent systems were screened in flask test, with lower butanol content. The solvent system EtOAc/*n*-BuOH/water (6:1:4, v/v/v) allowed a higher partitioning of the flavonoids in aqueous phase, leading to a possible increase of retention volume of the compounds. This system was used to fractionate then the fraction ZCPC 2-2.

III.4.3.3.3.3. CPC purification of fraction ZCPC 2-2: ZCPC 6:

The solvent system EtOAc/*n*-BuOH/ H_2O (6:1:4, v/v/v) was used for CPC purification of ZCPC 2-2 in ascending mode (Experimental data see Table 14). CPC chromatogram and recap TLC plate are presented in Figure 90.

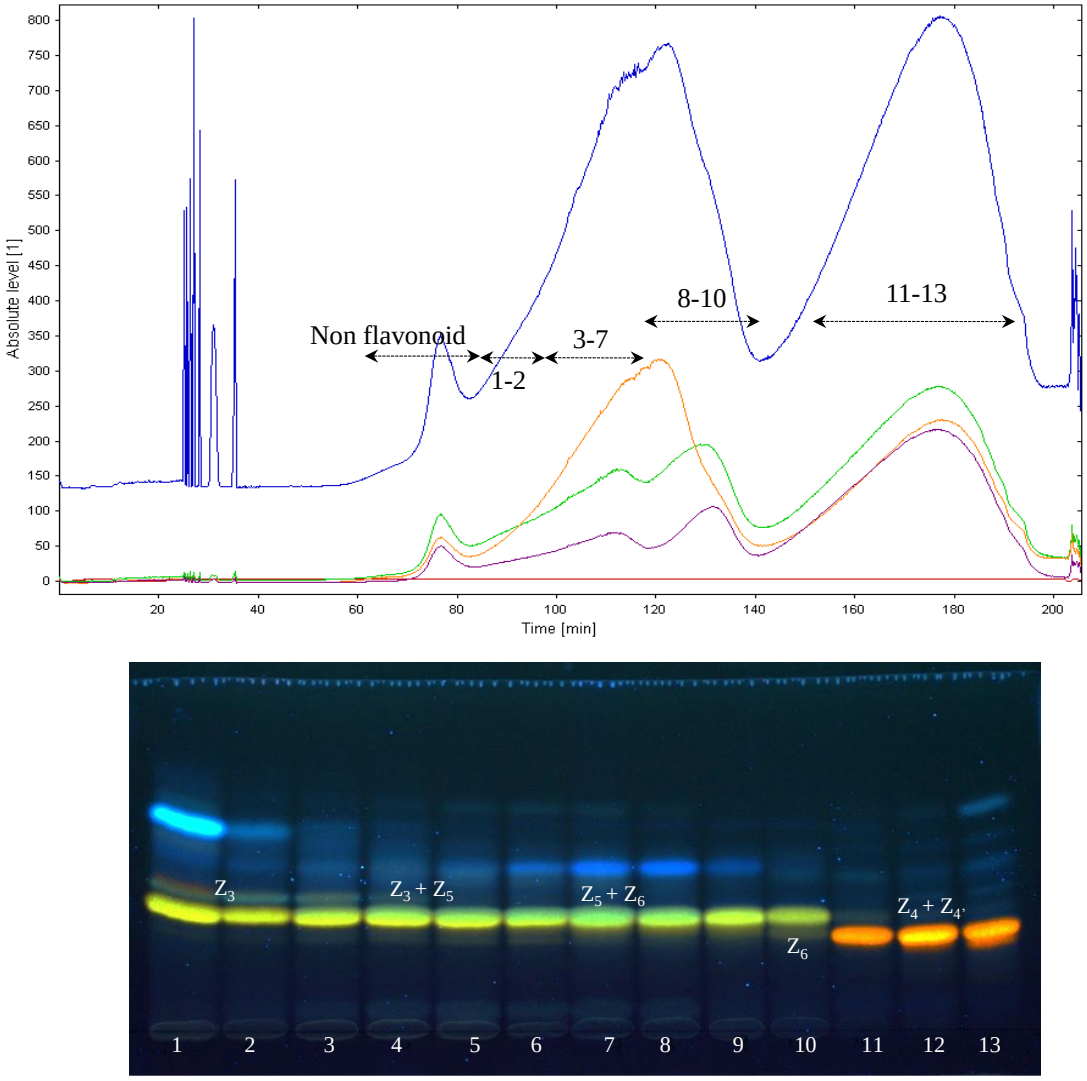


Figure 90. CPC chromatogram (on top) at 210 nm (blue), 254 nm (green), 280 nm (orange) and 365 nm (purple) and recap TLC plate (at bottom) at 365 nm after Neu reagent derivatization, of ZCPC 6.

As expected, retention volume of the flavonoids was increased and even almost doubled. Looking at CPC chromatogram, it seemed that a better separation occurred between products Z_3 and $Z_{5\text{-}6}$, since three major peaks were visible at 280 and 365 nm.

As during ZCPC 5, Z_3 was obtained with good purity in fraction ZCPC 6-1 (30 mg). Products Z_5 and Z_6 were eluted during a longer period in this run, almost during 50 min. Z_5 was only obtained in mixture with Z_3 and Z_6, with a maximum purity of 45 % (fraction ZCPC 6-3 to 9).

Z_6 was obtained with a purity of 70 % in fraction ZCPC 6-8 to10 (78 mg) and then subjected to further purification.

Z_4 and $Z_{4'}$ were obtained only in mixture in fractions CPCZ 6-11 to 13 (70, 85 and 33 mg).

To conclude on those CPC systems, both allowed the clear separation of the major compounds Z_3 and $Z_{4\text{-}4'}$ from each other. The system EtOAc/*n*-BuOH/water (6:1:4, v/v/v), with increased retention time of flavonoids, was a better option to purify major and minor compounds.

In order to access to Z_5 and Z_6 compounds, further purification was performed on ZCPC 6-3 to 6 and ZCPC 6-8 to 10. Using CPC, it was possible to work on small sample (around 100 mg) without losing product, since no irreversible absorption could happen, contrary to classic silica gel chromatography.

III.4.3.3.3.4. CPC purification of ZCPC 6-3 to 6 and ZCPC 6-8 to 10: ZCPC 7 and 8:

Solvent system selection:

As EtOAc/*n*-BuOH/water system did not give satisfying separation of Z_3/Z_5 and Z_5/Z_6 in previous CPC runs, the system EtOAc/*n*-BuOH/MeOH/water was investigated in order to achieve those separations. Various ratios were screened and the systems EtOAc/*n*-BuOH/MeOH/water (7:1:1:3, v/v/v/v) and (7:1:1:4, v/v/v/v) were selected for separation of Z_3/Z_5 and Z_5/Z_6 respectively. In these systems, the target compounds were partitioned in favor of the aqueous phase, leading to high retention time in ascending mode. The introduction of methanol in the system would hopefully introduce higher selectivity in the system.

CPC experiment:

Experimental data are resumed in Table 15.

Table 15. CPC experimental conditions of ZCPC 7 and 8:

Experiment	**ZCPC 7**	**ZCPC 8**
Sample	ZCPC 6-3to6 (100 mg)	ZCPC 6-8to10 (80 mg)
Solvent system	EtOAc/*n*-BuOH/MeOH/water (7:1:1:3, v/v/v/v)	EtOAc/*n*-BuOH/MeOH/water (7:1:1:4, v/v/v/v)
Mode	Ascending, 10 % co-current	
Rotation speed	1800 rpm	
Flow rate	8 mL.min^{-1}	
Sf	74 %	75 %
Back pressure	29 bars	27 bars
Fraction collect	Every 30 seconds	

CPC purification of ZCPC 6-3 to 6 (~ 100 mg) was performed using the solvent system EtOAc/*n*-BuOH/MetOH/H_2O (7:1:1:3, v/v/v/v). CPC chromatogram, TLC fractogram and HPLC chromatogram were shown Figure 91.

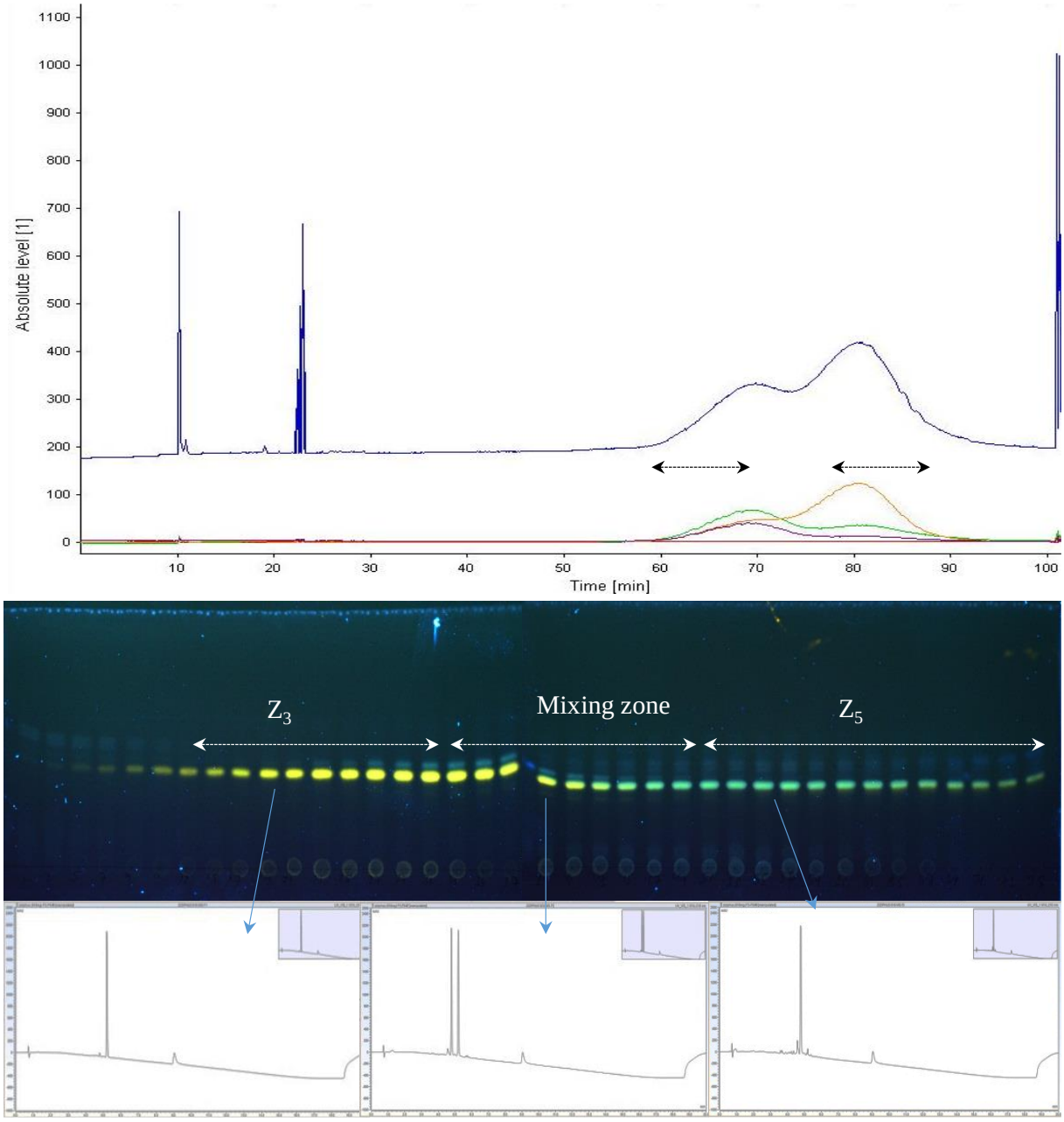


Figure 91. CPC chromatogram (on top) at 210 nm (blue), 254 nm (green), 280 nm (orange) and 365 nm (purple); recap TLC plate (in the middle) at 365 nm after Neu reagent derivatization of ZCPC 7, and HPLC chromatogram of resulting fractions.

As we could notice on CPC chromatogram, Z_3 and Z_5 were well retained and separated using those conditions. Z_3 (22 mg) was obtained with purity of around 95 % and Z_5 (59 mg) was isolated with purity of around 90 %.

Z_5 displayed a molecular ion peak $[M+H]^{2+}$ at m/z 599.19666 in the positive HR-ESI-MS, indicating the molecular formula $C_{27}H_{35}O_{15}$. Z_5 was then submitted to NMR and MS analysis and was identified as phloretin-3',5'-diglycoside (Appendix 21-23) (Sato *et al.*, 2006).

ZCPC 8 was performed on ZCPC 6-8 to10 (80 mg) and led to 3 main fractions. CPC chromatogram and TLC fractogram are presented in Figure 92.

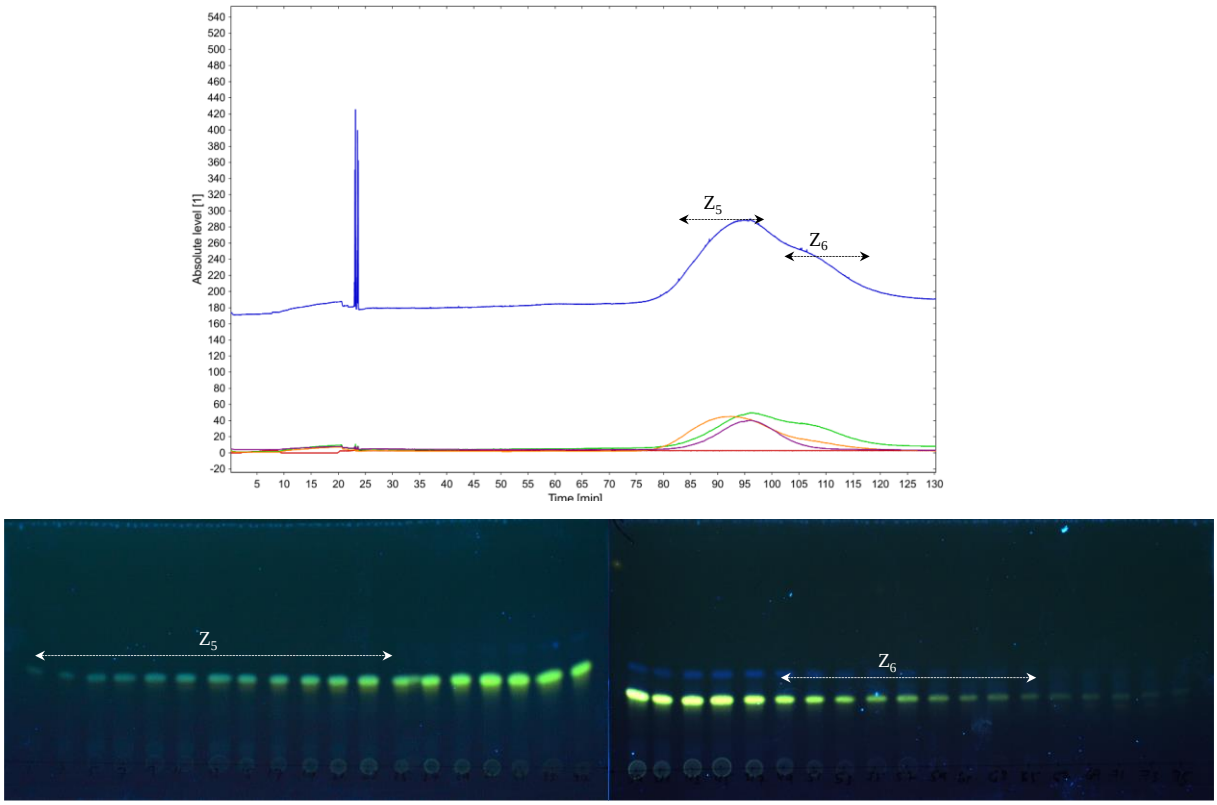


Figure 92. CPC chromatogram (on top) at 210 nm (blue), 254 nm (green), 280 nm (orange) and 365 nm (purple); recap TLC plate (on bottom) at 365 nm after Neu reagent derivatization of ZCPC 8.

Product Z_5 was eluted 60 min after reaching the equilibrium and 10 mg were obtained with quite satisfaying purity of 88 %. Product Z_6 was then eluted first in mixture with Z_5 and alone at the end of the peak (10 mg), with purity reaching 90 %. Z_6 was then subjected to NMR and unfortunately the purity of the fraction wasn't high enough to allow a formal identification of the compound Z_6. Our data allowed us to think that Z_6 is a flavonoside carrying three sugars but the identity of the genin and the sugars were questionable. The structural elucidation of Z_6 was still in progress.

CPC was not very successful in this case, since most of the products were eluted as a mixture.

Butanolic extract

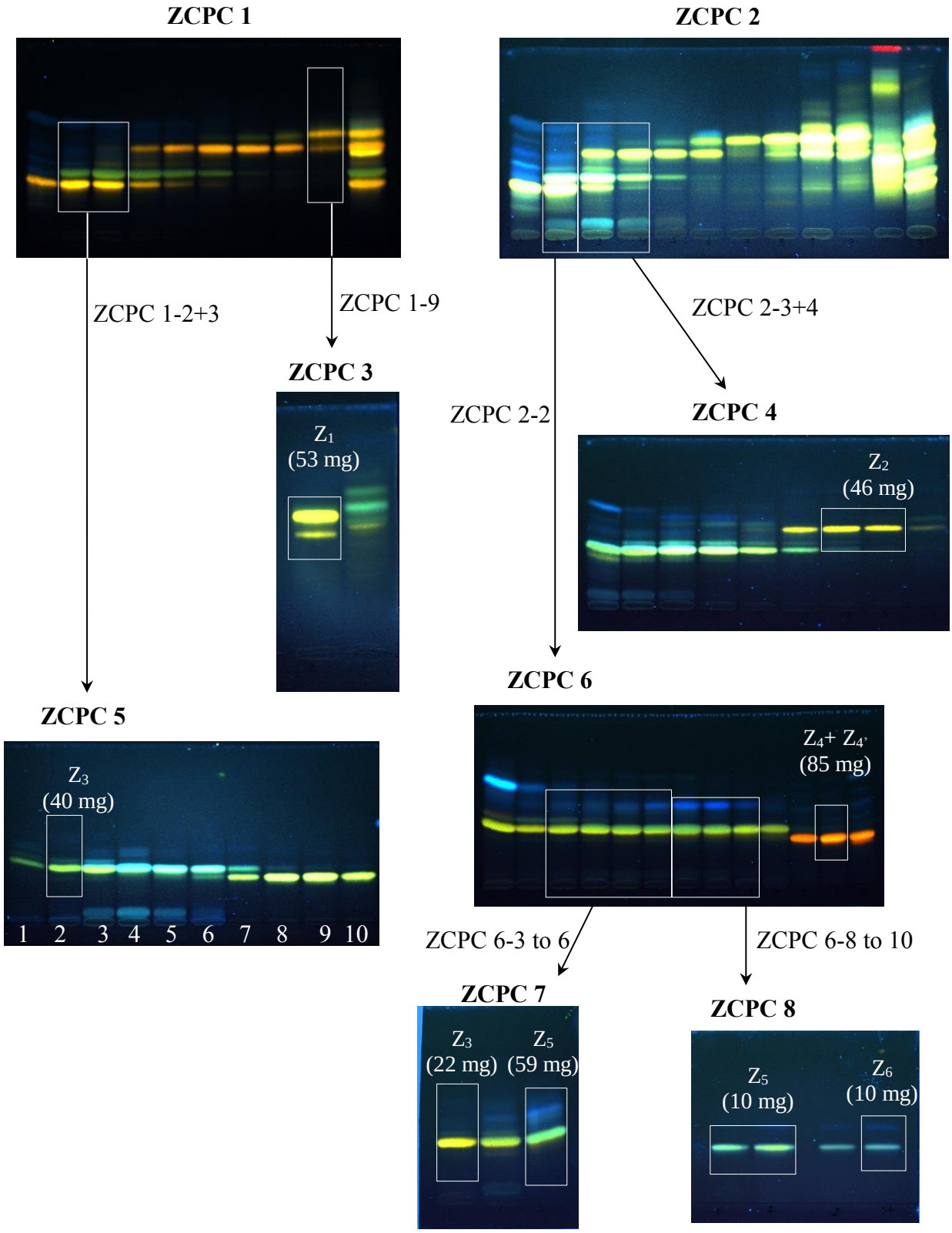
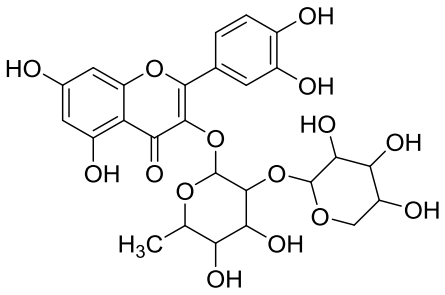


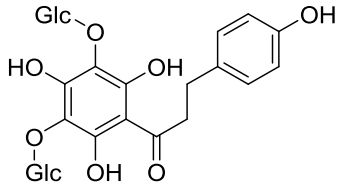
Figure 93. Recapitulative scheme of isolation of falvonoids from Z. spina-christi.

III.4.3.4. Summary of CPC isolated flavonoids from Z. spina-christi:

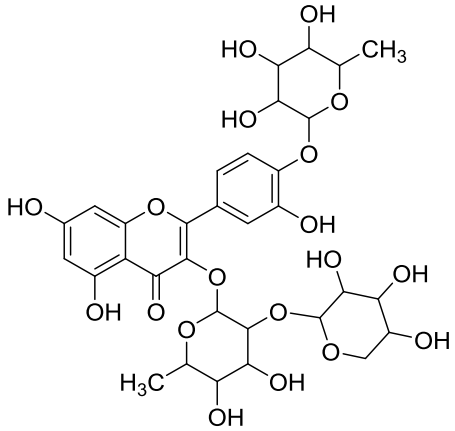
Four major flavonoids were then isolated from *Z. spina-christi* leaves using some CPC experiment (Figure 94). NMR and MS data are presented in Appendix.



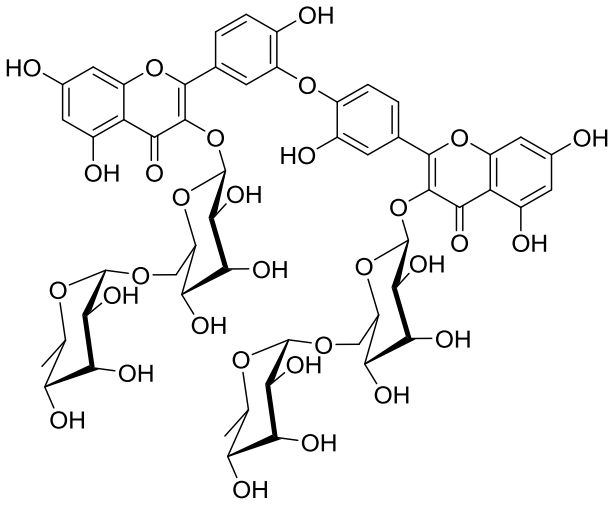
Quercetin-3-O-[β-D-xylosyl-(1→2)-α-L-rhamnoside] Z_1



Phloretin-3',5'-diglucoside Z_5



Quercetin-3-O-[β-D-xylosyl-(1→2)-α-L-rhamnoside]-4'-O-α-L-rhamnoside Z_3



Zizyflavoside A as analogue of Z_2

Figure 94. Structures of flavonoids isolated from Z. spina-christi leaves.

As conclusion, *Z. spina-christi* leaves extract has been representing an example of plant rich in flavonoid glycosides. The separation of these flavonoid glycosides from crude plant extracts has always been a challenge with traditional phytochemical purification techniques, especially those involving solid-support chromatography.

CPC optimization for isolation of flavonoid glycosides was sucessfully performed by using only two solvent systems EtOAc/*n*-BuOH/MeOH/H_2O and EtOAc/*n*-BuOH/H_2O with different ratios either in normal or reverse elution mode. This methodology could be a starting point of a general approach for flavonoids extract rapid purification but should be first exemplify in other plants.

CPC offered many advantages to flavonoids studies. These advantages included: versatility; convenience and speed; good resolution; economy; predictability and reproducibility; and total sample recovery without chemical modifications or loss of biological activity in bioassay-guided fractionations.

If CPC was a powerful tool to quickly fractionate and enriched fractions with major or minor compounds. This approach would be more pertinent for metabolomics-like works. In fact, enriched fractions containing 40 to 70 % of the flavonoids were obtained in one or two CPC run, which would be convenient to run LC-MS analysis but not systematically to perform NMR identification.

IV. *Hydnora abyssinica* A. Br.

In this last part, the general characteristics of *Hydnora* genus and *Hydnora abyssinica* species will be described in terms of geographical localization, botanical identification, phytochemistry and traditional uses. Contrary to the previous plants studied, *Hydnora* genus contains few species, so the description of the genus and the *H. abyssinica* species will be done jointly. Then, the isolation and purification processes of metabolites using CPC will be described.

IV.1. GENERAL CHARACTERISTICS OF *HYDNORA* GENUS AND *HYDNORA ABYSSINICA*:

IV.1.1. Classification:

Kingdom: Plantae, Plants
Subkingdom: Tracheophyta
Superdivision: Spermatophyta - Seed plants
Division: Magnoliophyta - Flowering plants
Class: Magnoliopsida - Dicotyledons
Order: Piperales
Family: Hydnoraceae
Genus: Hydnora

IV.1.2. Geographic localization:

The family of *Hydnoraceae* contains only two holoparasitic genera: *Hydnora*, distributed in Africa and *Prosopanche*, distributed in South America (Figure 95). The genus *Hydnora* is estimated to consist of 5–12 species in the world. It is a genus of subterranean holoparasitic plants found in arid and semiarid regions of Africa with a very large range of distribution, but its pattern of distribution is determined by the amount of rainfall (Nyafuono *et al.*, 2000).

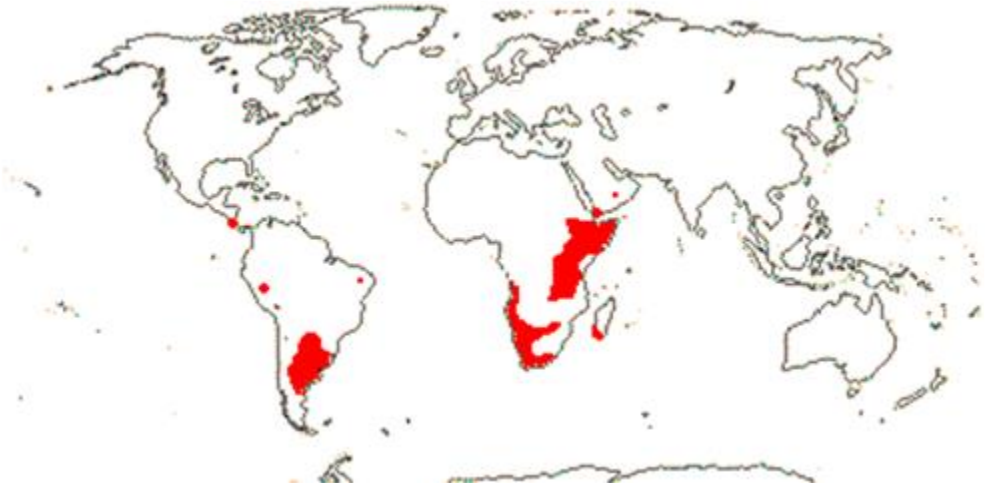


Figure 95. Geographical distribution of Hydnoraceae in the world.

Figure 96. Distribution of Hydnora in Africa (African plant database, 2016).

Among *Hydnora* species, the hypogeous herb *Hydnora abyssinica* A. Br., more frequently referred as its synonym *H. johannis* Becc. or *H. solmsiana* Dinter (The Plant List, 2016), was the most widespread species. It is distributed from Southern Africa, accross to Eastern Africa to the Arabian Peninsula (Al-Fatimi *et al.*, 2016). Its vernacular native name is "Tartous" in Sudan.

IV.1.3. Botanical identification:

Hydnora genus is subterranean root holoparasites, fleshy and coriaceous plants (Figure 97). Its often massive root systems spread laterally from the host. The plant body is a root-like rhizome with extremely reduced vegetative features, a dark brown periderm and fleshy red/pink interior. These rhizomes are bearing series of vermiform outgrowths (haustorial roots) that connect the parasite to its host. Leafy structures are missing. Flower buds are developed underground and emerging at anthesis. The flowers are actinomorphic and are usually bisexual with cylindrical flower tube, often swollen at the base, apically with 2-5 valvate and fleshy lobes.

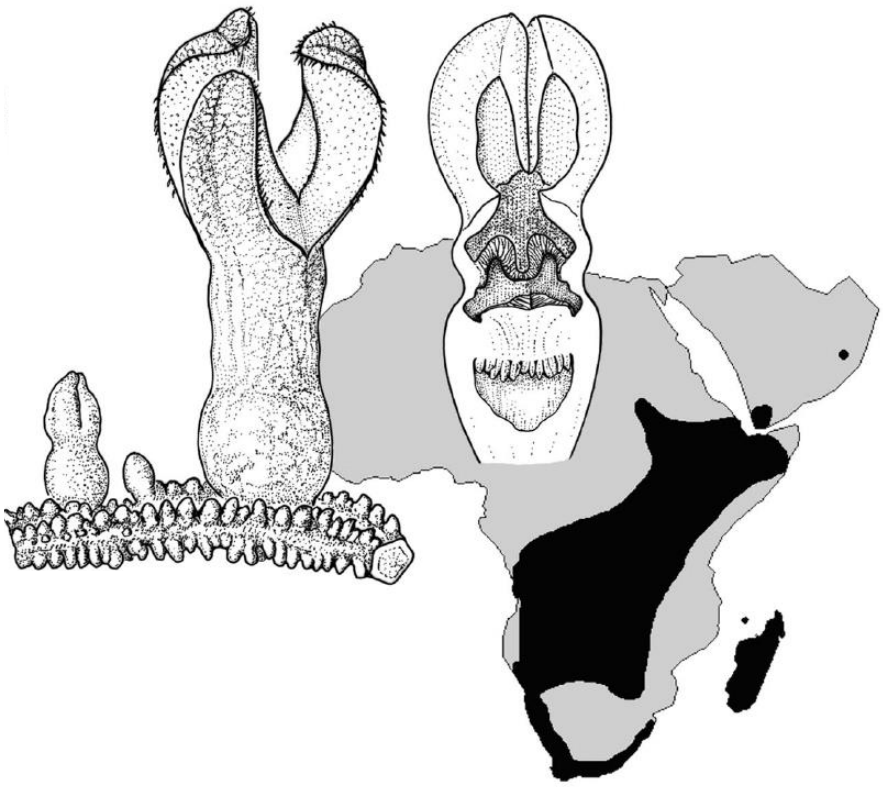


Figure 97. General morphology of Hydnora africana, with flower longitudinal section. Distribution of H. africana in black on the map (Nickrent et al., 2002).

Androecium is synandrial, rarely with filaments and with numerous pollen sacs. Staminal structures are formed of a wavy ring with distinct lobes opposite to the perigon lobes. Ovary is inferior, usually subterraneous, with a flat, sessile stigma, 3-5 carpellate, unilocular and with numerous placenta. Ovules are numerous, orthotropous, tenuinucellate and unitegmic. Fruit is an underground berry, with a thick and leathery wall. Seeds are numerous, globular or ovate, very small, with a hard, thick testa, embedded in a fleshy or gelatinous and edible pulp which is rich in starch. Embryo is small, surrounded by endosperm and perisperm (Meijer, 1993; Williams *et al*., 2011).

H. abyssinica flowers grow on the roots of *Acacia* species. Flowers formed a distally 4-lobes, fleshy, outside orange, inside reddish to orange of up to 12 cm length and 3-4 cm diameter (Figure 98).

Figure 98. H. abyssinica, A: rhizomes, B: flowers, C: fruits.

IV.1.4. Traditional medicinal uses:

Most of the publications dealing with *Hydnora* genus, focused on botany and phylogeny. Few works reported medicinal uses for the whole *Hydnora* genus.

H. africana roots were used as antidysenteric in Eastern Cape region of South Africa (Wintola and Afolayan, 2015).

More data reported the medicinal use of *H. abyssinica*. In Ethiopia, the flowers are used against haemorrhage, diarrhea and wound or mouth infection (Al-Fatimi *et al.*, 2016). In Oman, the fruits are used as food and tanning agent. In Kenya, decoction of roots is used by traditional healers to cure throat pain, dysentery and stomach pain (Onyancha *et al.*, 2015). In Sudan, roots are mostly used, as in other countries, against diarrhea, dysentery or cholera but also to cure swelling tonsillitis (El-Kamali and El-Khalifa, 1999; Yagi *et al.*, 2012).

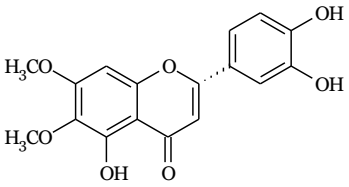
IV.1.5. Secondary metabolites from Hydnora genus:

Few secondary metabolites were reported from *Hydnora* genus.

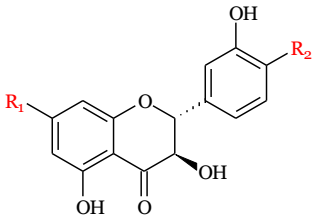
Wintola and Afolayan (2015) reported preliminary screening of *H. africana* roots extracts. They found mainly flavonoids, tannins especially proanthocyanidols, and other phenolic compounds.

Some preliminary screening tests were also performed on roots of *H. abyssinica*. The results were not totally homogeneous: tannins, phenolics, flavonoids were reported in all cases and alkaloids were reported in some work (Onyancha *et al.*, 2015) but not recovered in other publication (Osman *et al.*, 2015).

Few secondary metabolites were purified from *H. abyssinica* (Figure 99). Six compounds were isolated from *H. abyssinica* roots ethylacetate extract, as cirsiliol (3′,4′,5-trihydroxy-6,7-dimethoxyflavone), *trans*-3′,5-dihydroxy-4′,7-dimethoxydihydroflavonol, catechin, vanillin (4-hydroxy-3-methoxybenzaldehyde), protocatechuic acid (3,4-dihydroxybenzoic acid) and oleic acid (Yagi *et al.*, 2012). Also, catechin, tyrosol and ethyl 3,4-dihydroxybenzoate were isolated from *H. abyssinica* ethanolic extract (Koko *et al.*, 2015).

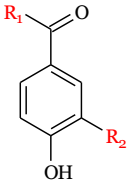


Cirsiliol



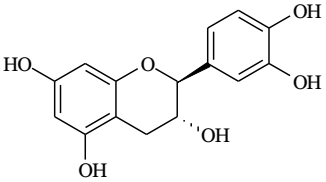
Trans-3′,5-dihydroxy-4′,7-dimethoxydihydroflavonol

(R_1, R_2 = OCH_3)

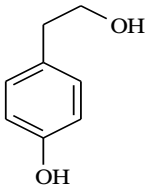


Vanillin (R_1 = H, R_2 = OCH_3)

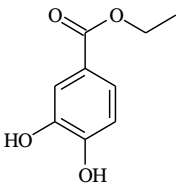
Protocatechuic acid (R_1, R_2 = OH)



Catechin



Tyrosol



Ethyl 3,4-dihydroxybenzoate

Figure 99. Secondary metabolites purified from H. abyssinica

As only few compounds were reported from *H. abyssinica*, we were then interested in the preliminary phytochemical study of *H. abyssinica* for potentialy isolation and identification of new secondary metabolites.

IV.2. PHYTOCHEMICAL STUDY OF *HYDNORA ABYSSINICA*:

To start the study, a preliminary screening of metabolites was performed on our *H. abyssinica* sample, as some discrepancies were noticed in the literature in the results of the different screenings, notably concerning the alkaloid presence.

IV.2.1. First preliminary screening:

The preliminary phytochemical screening of *H. abyssinica* rhizomes methanol extract gave positive test reactions for tannins and flavonoids, while negative test reactions for saponins, alkaloids, sterols, quinones and cardenolides were obtained.

Tannins appeared as the most abundant metabolites in the rhizomes extract of *H. abyssinica*, which was in accordance with the literature. Using DMAC reagent, we could also confirm that those tannins were mostly catechic tannins, i.e. proanthocyadinins (PAC).

IV.2.2. Extracts preparation:

H. abyssinica methanol extract (40 g) was resuspended in water and then extracted sucessively using ethylacetate (9.25 g) and butanol (19.1 g). TLC chromatogram of these two extracts together with aqueous residue (7.5 g) is presented in Figure 100.

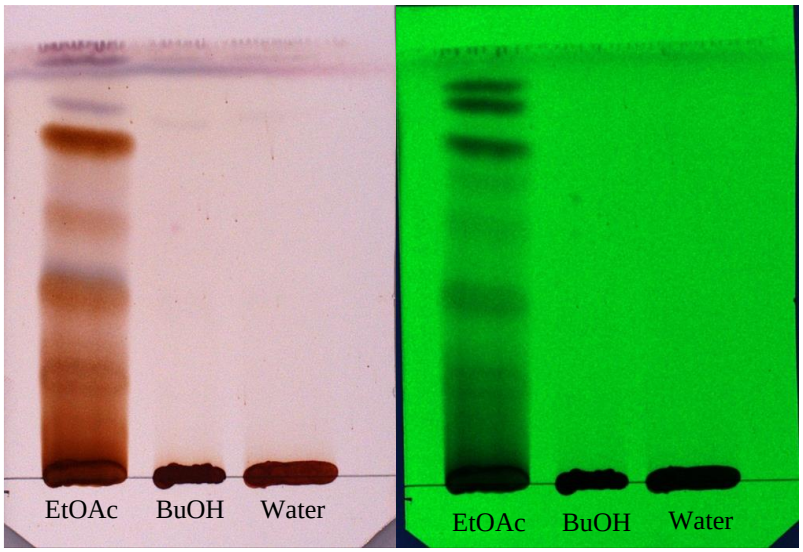


Figure 100. TLC chromatogram of EtOAc, butanol and water fraction of H. abyssinica at daylight (on the left) after sulfuric anisaldehyde derivatization and at 254 nm (on the right), mobile phase: $CHCl_3$/EtOAc/formic acid (6:10:2, v/v/v).

Water residue and butanol extract were mostly composed of high molecular weight tannins. We focused then on ethylacetate extract that exhibited more metabolites diversity.

IV.2.3. EtOAc extract purification using CPC:

IV.2.3.1. Solvent system screening:

Considering that tannins, especially PACs, were the main metabolites in our *H. abyssinica* extract (Figure 100), we focused on solvent systems that we previously developed for cranberry PAC fractionation (data not shown).

Those polar systems contained acetone or acetonitrile as good solvents.

Different solvent systems were tested for CPC purification of EtOAc extract (Table 16 and Figure 101).

Table 16. Solvent systems screened for purification of EtOAc extract:

Code	Solvent	Apolar		Medium		Polar	
		Up	Low	Up	Low	Up	Low
	EtOAc/acetone/water						
A	4:2:4, v/v/v	+++	++	+	+++	+	++++
B	3:3:3.5, v/v/v	+++	++	++	+++	+	++++
C	3:3:3, v/v/v	+++	++	+++	+++	+	++++
	EtOAc/ACN/water						
D	3:2:3, v/v/v	+++	+	+++	+	+	++++
E	2.5:2:3, v/v/v	+++	+	+++	+++	++	++++
F	2:2:3, v/v/v	+++	+	++	++	+	++++
	EtOAc/ACN/acetone/water						
G	1.5:1.5:1.5:6, v/v/v/v	++	++	++++	+	++	++
H	1:0.5:0.5:5,v/v/v/v	+++	++	+++	++	++	++

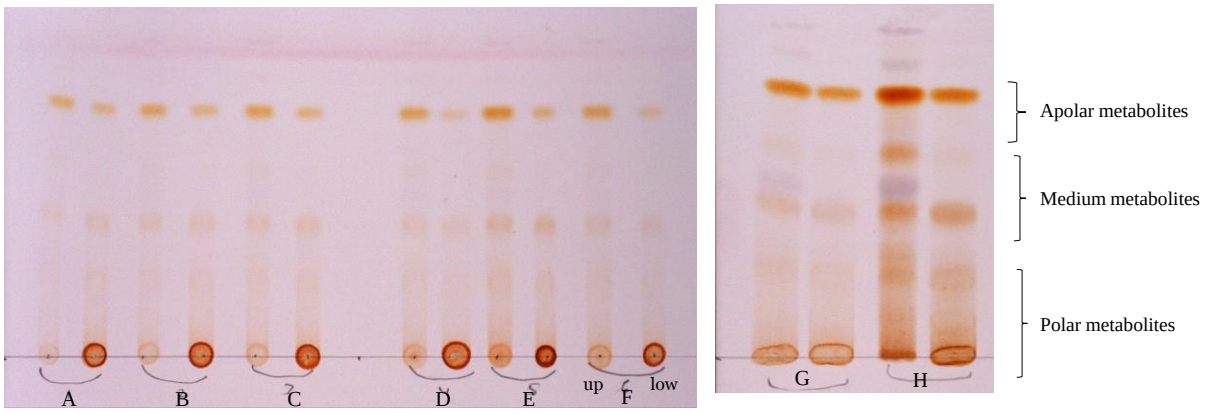


Figure 101. TLC plates obtained for solvent system screening at daylight after sulfuric anisaldehyde derivatization, mobile phase: $CHCl_3$/EtOAc/formic acid (6:10:2, v/v/v).

All systems allowed a good solubilization of the sample. As acetone and acetonitrile were found to be good solvents of PACs in previous work on cranberry, ternary or quaternary solvent systems, were built up around these two solvents.

In all systems, apolar and medium analytes were almost equally partitioned between upper and lower phase. Polar metabolites were partitioned mainly in aqueous lower phase in all systems except for system H, in which they were equally partitioned.

The decantation speed in samples was then used as major selection criteria. In this case, two systems were selected EtOAc/ACN/water F (2:2:3, v/v/v) and EtOAc/ACN/Acetone/water H (1:0.5:0.5:5, v/v). The triphasic solvent system *n*-heptane/MtBE/ACN/water (1:1:1:1, v/v/v/v) would also be screened to quickly remove tannins from other metabolites present in EtOAc extract.

Those systems were then tested on CPC.

IV.2.3.2. CPC fractionation of EtOAc extract:

All CPC experimental data were resumed in Table 17.

Table 17. Experimental data of CPC run HCPC1 to HCPC 4:

Experiment	**HCPC 1**	**HCPC 2**	**HCPC 3**	**HCPC 4**
Sample	1 g	3 g	1 g	1 g
Solvent system	EtOAc/ACN/water 2:2:3, v/v		EtOAc/ACN/acetone/water 1:0.5:0.5:5, v/v	*n*-heptane/MtBE/ACN/water 1:1:1:1, v/v
Mode	Descending		Descending	Ascending
Rotation speed	1800 rpm		1900 rpm	1000 rpm
Flow rate	8 mL.min^{-1}			
Sf	68%	69%	72%	73%
Back pressure	35 bars	36 bars	37 bars	30 bars
Fraction collect	Every 30 s			

Tannins were known to disturd solvent system during sample loading, which was the reason why the sample loading protocol used in all CPC runs was adapted to minimize disturbation of the column. Sample was introduced at low flow rate and rotation speed: 2 mL.min^{-1} at 600 rpm.

IV.2.3.2.1. HCPC 1 and HCPC 2:

Solvent system EtOAc/ACN/water (2:2:3, v/v/v) was used for CPC fractionation of EtOAc extract in a descending mode, in order to elute high molecular weight tannins first. The recap TLC plate of HCPC 1 is presented in Figure 102.

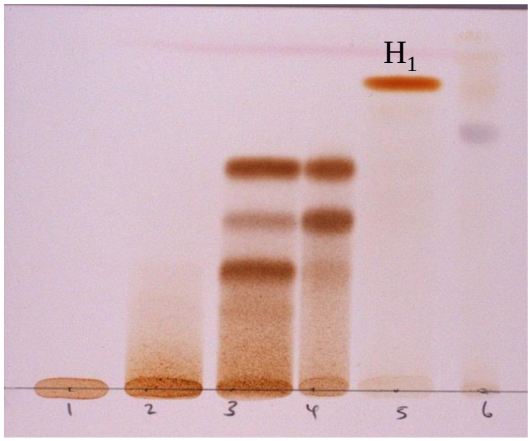


Figure 102. Recap TLC plate of HCPC 1 at daylight after sulfuric anisaldehyde derivatization; mobile phase CH_2Cl_2/EtOAc/Formic acid (6:10:2, v/v/v).

The main part of the sample was eluted in fractions HCPC 1-1 and 2 (577 and 97 mg), containing polar tannins. Medium analytes were eluted in mixture in fractions HCPC 1-3 and 4 (26 and 40 mg). Interestingly in fraction HCPC 1-5 (25mg), an apolar metabolite was isolated with good purity (almost 90 %). This metabolite was labeled as H_1 and subjected to HPLC, NMR and MS analysis. It will be described later in this manuscript.

The fractionation profile was suitable and a scale up was performed with a 3 g sample loading in order to obtain more mass of fractions containing medium analytes.

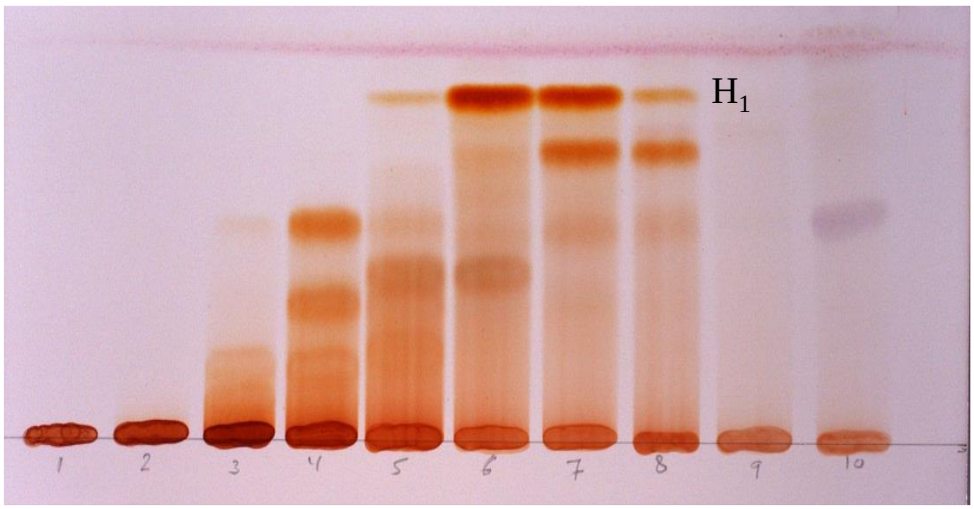


Figure 103. Recap TLC plate of HCPC 2 at daylight after sulfuric anisaldehyde derivatization; mobile phase CH_2Cl_2/EtOAc/Formic acid (6:10:2, v/v/v).

Looking at the recap TLC plates (Figure 103), we could notice that more analytes were visible on the plate than in the first run. Polar tannins were found mostly in fractions HCPC 2-1 and 2 (1.19 and 1.32 g) which represented the major part of the sample.

Medium metabolites were more fractionated and were found in fractions HCPC 2-3 to 5 (91, 261 and 38 mg) in increased quantities.

However for compound H_1, the elution profile was damaged and it was eluted in mixture with other medium or apolar metabolites.

In fraction HCPC 2-7 and 8 (37 and 20 mg) a second apolar metabolite was noticed.

In conclusion, using this biphasic solvent system EtOAc/ACN/water (2:2:3, v/v/v), apolar compounds were well separated from the other, but most of the medium compounds were eluted together in a short time period. In order to improve the separation of those compounds we explored the system EtOAc/ACN/acetone/water H (1:0.5:0.5:5, v/v/v/v).

IV.2.3.2.2. HCPC 3:

Since medium compounds exhibited more balanced *K* in the system EtOAc/ACN/acetone/water H (1:0.5:0.5:5, v/v/v/v), an extended retention volume and a better resolution could be expected for those compounds during this CPC run.

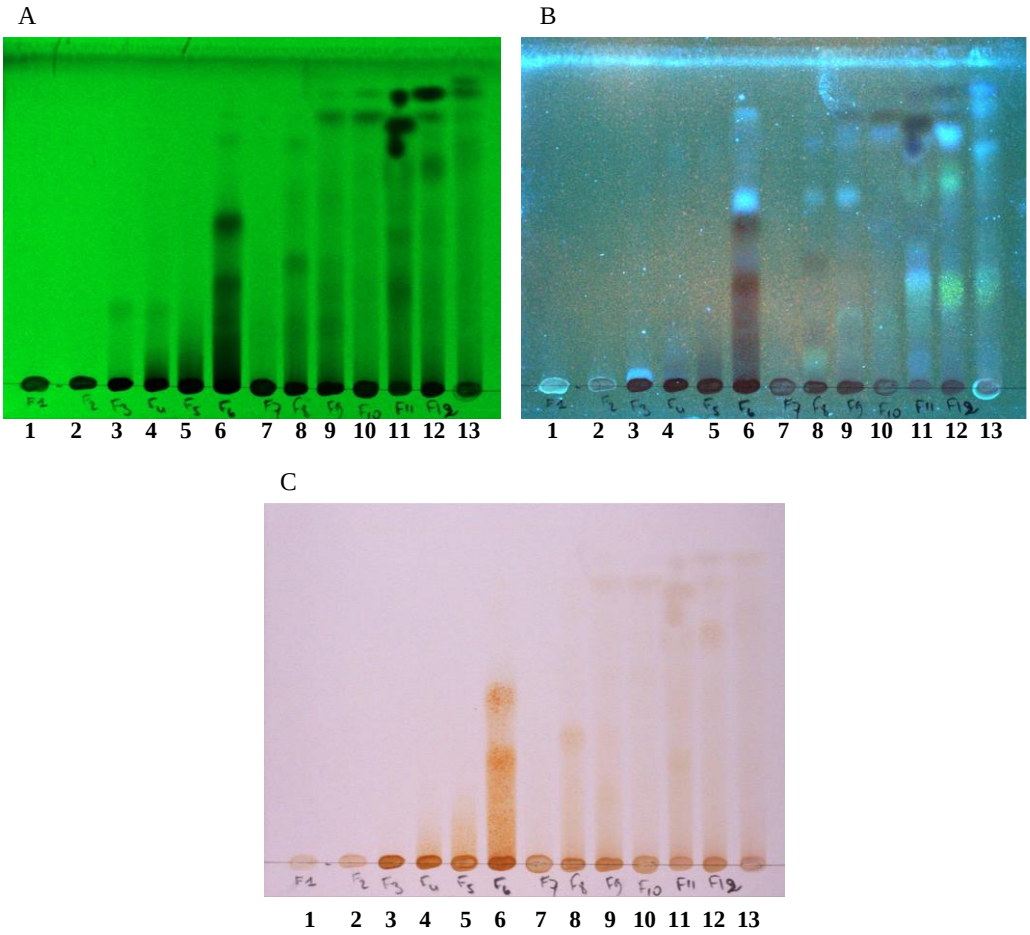


Figure 104. Recap TLC plate of HCPC 3 at UV 254 nm (A), at 365 nm and at daylight after sulfuric anisaldehyde derivatization (B and C). Mobile phase CH_2Cl_2/EtOAc/formic acid (6:10:2, v/v/v).

As we could notice on Figure 104, a better fractionation was obtained but only for non tannic compounds (fractions HCPC 3-8 to 13). For catechic compounds revealed by sulfuric anisaldehyde, they were all eluted in fraction HCPC 3-6 (257 mg), together with some higher oligomers.

Fractionation profile was then less interesting for our purpose. Oligomeric and polymeric tannins were also difficult to totally remove from medium compounds, so we decided to explore the triphasic solvent system *n*-heptane/MtBE/ACN/water (1:1:1:1, v/v/v/v).

IV.2.3.2.3. HCPC 4:

As we already mentioned, the triphasic solvent system was designed to fractionate crude bark extract of trees. The CPC runs were done in ascending mode to block the tannins in the aqueous stationary phase during all the process. Two elution steps with upper heptane/MtBE rich phase then medium MtBE/ACN rich phase as mobile phase were performed and the resulting recap TLC plate is presented in Figure 105.

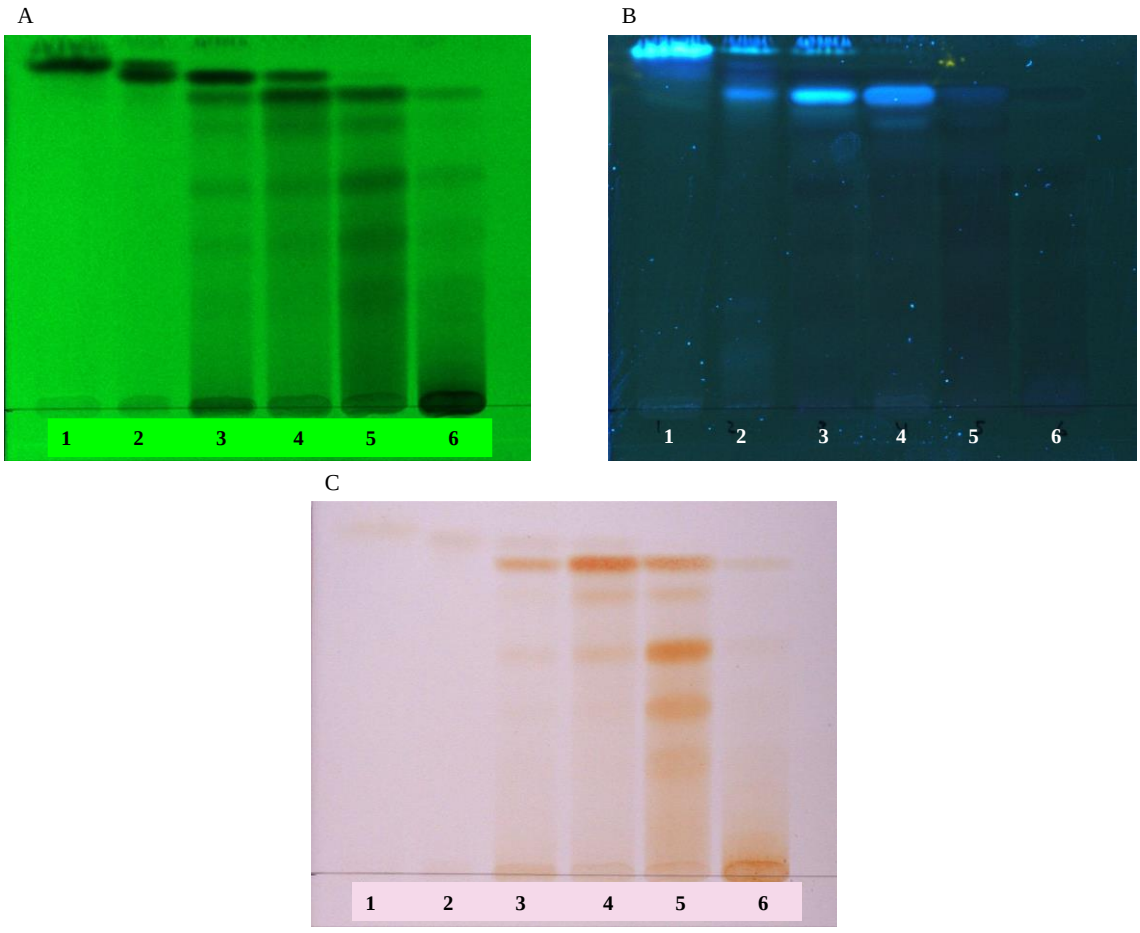


Figure 105. Recap TLC plate of HCPC 4 at UV 254 nm (A), at 365 nm and at daylight after sulfuric anisaldehyde derivatization (B and C). Mobile phase CH_2Cl_2/EtOAc/formic acid (6:10:2, v/v/v).

If tannins were eluted only after extrusion of the stationary phase in fraction HCPC 4-6, the resolution of other compounds was not really satisfying. From all these solvent systems evaluation, the best fractionation profile was obtained with the first system tested EtOAc/ACN/water F (2:2:3, v/v/v).

Further CPC purification was carried out on HCPC 2-4, using solvent system EtOAc/ACN/water (1.5:1.5:4, v/v/v) in descending mode and on HCPC 2-7 using solvent system *n*-heptane/MTBE/ACN/water (1:1:1:1, v/v/v/v). But still, major compounds were eluted

as mixture. In the literature, CPC fractionation on tannin rich extract or PACs rich extract was performed after a first purification step on LH-20 resin (Girardot *et al.;* 2014). We decided then to investigate this protocol for our *H. abyssinica* extract.

IV.2.3.3. LH-20 column chromatography approach:

Sephadex LH 20 resin was largely used to perform a first purification step of tannins rich extracts, especially of proanthocyanidins rich extracts. LH-20 resin allows fractionation according to both polarity and molecular weight of the compounds. Many gradients were described in the literature for cranberry (Macedo *et al.*, 2014), soja (Ito *et al.*, 2013) or blueberry (Gu *et al.*, 2012). In all cases, gradients were step gradient of methanol/water, or acetone/water at different ratios.

As we assumed that most of the tannins and compounds were derived from PAC, we decided to follow Ito *et al.* (2013) protocol. This gradient previously allowed separating phenolic acids from monomer of flavan-3-ol, and oligomer from polymer of PACs of cranberry extract in the laboratory (data to be published in 2017).

IV.2.3.3.1. LH-20 run 1:

Sephadex LH-20 column chromatography was carried out for fractionation of 5g of EtOAc fraction using the following gradient:

- Methanol/water (20:80, v/v);
- Methanol/water (60:40, v/v);
- Methanol;
- Acetone/water (70:30, v/v).

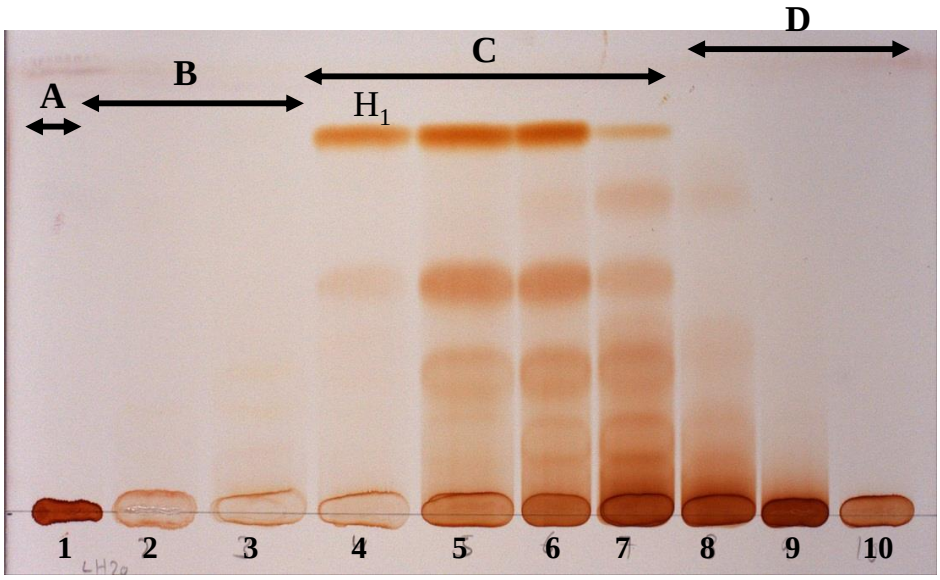


Figure 106. Recap TLC plates of resulting fractions of LH-20 on 5 g of EtOAc extract, at daylight after sulfuric anisaldehyde derivatization. Elution step (above): A MeOH/water (20:80, v/v), B MeOH/water (60:40, v/v), C MeOH, D acetone/water (70:30, v/v). Mobile phase CH_2Cl_2/EtOAc/formic acid (6:10:2, v/v/v).

Highly polar compounds representing half of the extract (F1 2.68 g) were eluted at the beginning. This might be the result of the column overloading. Increasing methanol rate (fractions 2 and 3), few polar compounds (133 and 41 mg respectively) were eluted. Raising to 100% of methanol, most of the medium compounds were eluted but mainly in mixture. In fact looking at recap TLC plate (Figure 106), fractions 5 to 7 (244, 191 and 466 mg) exhibited highly similar profile, which was confirmed by HPLC analysis. The shift to acetone/water allowed then the oligomer and polymer of tannins to be eluted of the column with the main part in fraction 9 (1.1 g).

Fractionation profile was then similar to those obtained using CPC: medium compounds obtained in mixture and "pollution" by polymers. The gradient was then modified to slow down the elution of oligomers and polymers.

IV.2.3.3.1. LH-20 run 2:

Previous gradient was then modified to smooth the transition between 100% MeOH step and acetone/water (70:30, v/v) step. Additional steps of MeOH/water and MeOH/acetone mixture were then added, to design the following gradient:

- Methanol/water (20:80, v/v);
- Methanol/water (60:40, v/v);

- Methanol/water (80:20, v/v);
- Methanol;
- Methanol/acetone (80:20, v/v);
- Methanol/acetone (50:50, v/v);
- Methanol/acetone (30:70, v/v);
- Acetone/water (70:30,v/v).

In order to avoid overloading of the column, the sample mass was decreased to 2.5g (Figure 107).

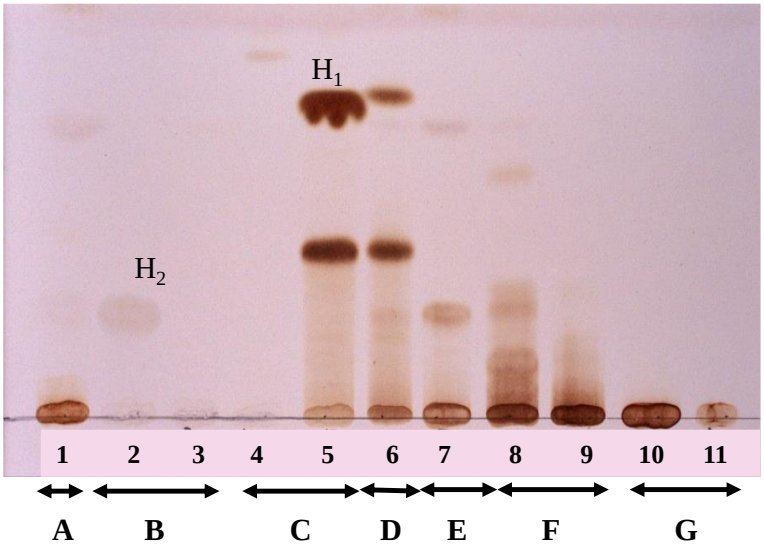


Figure 107. Recap TLC plates of resulting fractions of LH-20 on 2.5 g of EtOAc extract, at daylight after sulfuric anisaldehyde derivatization. Elution step (below): A MeOH/water (20:80,v/v), B MeOH/water (60:40, v/v), C MeOH/water (80:20, v/v), D MeOH, E MeOH/acetone (80:20, v/v), F MeOH/acetone (50:50, v/v), G MeOH/acetone (30:70, v/v). Mobile phase CH_2Cl_2/EtOAc/formic acid (6:10:2, v/v/v).

Using the extended gradient, a better fractionation was obtained. Fractions 5 and 6 (560 and 199 mg) (Figure 107) were chemically simplified, containing mainly two analytes: one apolar and one of medium polarity. The simple profile of these fractions led us to perform a classical silica column chromatography. The apolar compound H_1 (128 mg) was isolated from 200 mg of fraction 5, using CH_2Cl_2/EtOAc/formic acid (65:35:1, v/v/v) as mobile phase.

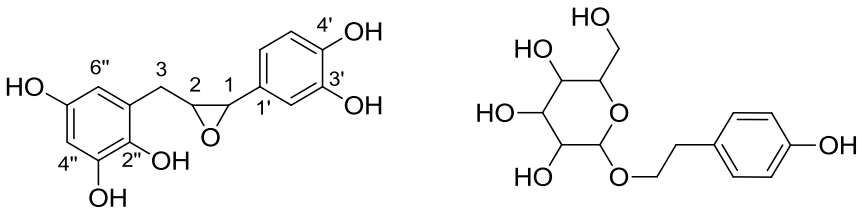
Compound H_1 exhibited 15 carbon signals and 9 protons signals, consistent with the MS peak found at 291.08621 m/z (M+H), leading to the molecular formula $C_{15}H_{14}O_6$.

Analysing NMR data (see Table 18, Appendix 24-28), the molecule contained 7 quaternary carbons all in aromatics and linked to an oxygen for 5 of them (δ ranging from 144.8 to 156.4 ppm). Seven CH and one CH_2 were also highlighted. Among the 15 carbon signals, carbons 1, 2 and 3 were located in the aliphatics zone (δ ranging from 27.1 to 81.4 ppm). Those preliminary data were compatible with two C6 aromatic rings linked by a C3 aliphatic chain.

Table 18. NMR data of compound H_1:

Number	^{1}H δ (ppm) *J* (Hz)	^{13}C δ (ppm)	COSY	HMBC (C→H)
1	4.53 (d, *J*= 7.5)	81.4 (CH)	2	3, 2', 5', 6'
2	3.94 (td, J_1= 7.8; J_2= 5.4)	67.4 (CH)	1, 3	1, 3
3	2.83 (dd, J_1= 16.2; J_2= 5.4) 2.48 (dd, J_1= 16.2; J_2= 8.1)	27.1 (CH_2)	2	1
1'	/	130.8 (Cq)		1, 5'
2'	6.80 (d, *J*= 1.8)	113.8 (CH)		1, 6'
3' (4')	/	144.8 (Cq)		2', 5', 6'
4' (3')	/	144.9 (Cq)		2', 5', 6'
5'	6.72 (d, *J*= 8.1)	114.7 (CH)		1
6'	6.67 (dd, J_1= 8.1, J_2= 1.8)	118.6 (CH)		1, 2'
1"	/	99.4 (Cq)		2, 3, 6", 4"
2" (3")	/	156.2 (Cq)		1, 3, 6", 4"
3" (2")	/	156.4 (Cq)		1, 3, 6", 4"
4"	5.81 (d, *J*= 2.4)	94.0 (CH)		6"
5"	/	155.5 (Cq)		1, 4"
6"	5.88 (d, *J*= 2.4)	94.9 (CH)		4"

On aliphatic moiety, according to chemical shift in proton and carbon spectra, both C_1 and C_2 were linked to an oxygenated group. Looking at molecular formula, only one oxygen was still available, an epoxyde group was then proposed. According to COSY spectra, C_3 was linked to C_2. Looking at the literature, compound H_1 was found to be katsumadin, isolated from *Alpinia katsumadai* (Zingiberaceae) (Huang *et al.*, 2007) (Figure 108). The katsumadin was never described before in the *Hydnora* genus.



Katsumadin **H_1** Rhodioloside **H_2**

Figure 108. Structure of compounds H_1 and H_2.

In fraction 2 (65 mg), a compound H_2 was eluted with a good purity and was then subjected to NMR analysis. By comparison with the literature (Wang *et al.*, 2014), H_2 was identified as 2-(4-hydroxyphenyl)ethyl-*β*-D-glucopyranoside, also known as rhodioloside or salidroside (Figure 108). Rhodioloside was isolated first from *Rhodiola rosea* (Crassulaceae) (Tolonen *et al.*, 2003), but was not described in *H. abyssinica*.

Since at first, the purpose of the LH 20 pre-purification step was to simplify the sample before CPC fractionation, a CPC experiment was performed on pooled fraction 5 to 7 of the first LH 20 gradient.

IV.2.3.3.2. HCPC 5: fractionation after LH 20:

The HCPC 5 was performed in ascending mode at 1600 rpm, using the biphasic solvent system EtOAc/acetone/water A (4:2:4, v/v/v). The ascending mode was selected to retain residual PACs in the stationary phase to avoid possible “pollution” of apolar and medium compounds.

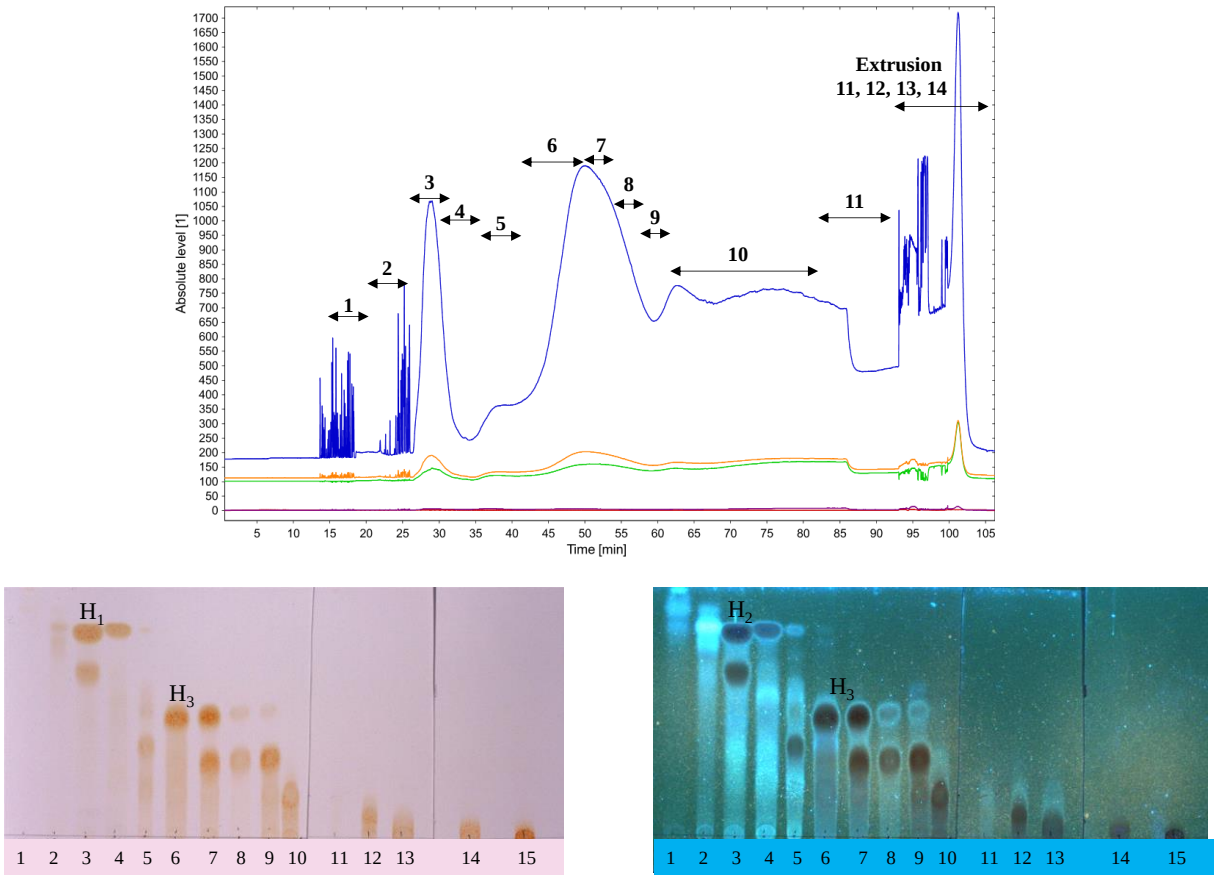


Figure 109. CPC chromatogram (on top) at 210 nm (blue), 254 nm (green), 280 nm (orange) and 365 nm (purple) and recap TLC plate (at bottom) of HCPC 5 at daylight (left) and 366 nm (right) after sulfuric anisaldehyde reagent derivatization; mobile phase CH_2Cl_2/EtOAc/formic acid (6:10:2, v/v).

First of all, the stability of the column was extended compare to previous CPC experiments, the flow rate was stable without any pulsations. The back pressure was 29 bars, and the CPC chromatogram showed clearly different peaks (Figure 109 on top).

Fractionation profile was satisfaying compare to previous CPC experiments. We can notice on Figure 109 that more metabolites on low and medium polarity were visible on the TLC plates, highlighting again the high concentration power of CPC. Also, apolar and medium metabolites were well separated from each other this time.

In fraction 4, katsumadin H_1 (8 mg) was obtained almost pure. In fraction 6, a compound H_3 (37 mg) was obtained in enriched fraction but with a purity not satisfaying for NMR analysis.

As conclusion the phytochemical investigation of *H. abyssinica* led to the isolation of two compounds that were never described in this plant. CPC fractionation of tannins and PACs

mixture was a tricky challenge. A pre-purification step on LH-20 resin allowed a better fractionation but the separation process still need to be improved. Further structural elucidation and separation would be necessary to complete this study.

V. General Conclusion

This project aimed to put a valuation on the phytochemical study of three Sudanese medicinal plants.

The first part of the thesis project is an overview mainly focused on the CPC definition, column design and pumping mode, nature of stationary and mobile phase, CPC chromatographic parameters and elution modes with advanced approaches in application and theory.

The second part was focused on the development of an innovative and new methodology for isolation and purification of aristolochic acids from *Aristolochia bracteolata* using strong ion exchange CPC (SIX-CPC). AA I, AA II and AA IIIa were isolated from crude extracts in one step at a high level of purity in large amounts. In a 200 mL CPC column, the productivity of the process was calculated considering each AAs: 4.2 $mg.h^{-1}$ (AA IIIa), 9.3 $mg.h^{-1}$ (AA II) and 5.5 $mg.h^{-1}$ (AA I). This process allows access of new AA standards as AAs II and IIIa for further extensive pharmacological studies.

In the third part CPC was optimized for isolation of flavonoid glycosides from *Z. spina-christi* which represents an example of plant rich in flavonoid glycosides. CPC optimization was successfully performed for the isolation of three known flavonoid glycosides by using two phase solvent systems EtOAc/*n*-BuOH/MeOH/H_2O and EtOAc/*n*-BuOH/H_2O with different ratios either in normal or reverse elution mode.

The fourth part deals with the isolation and purification processes for two known simple phenolic compounds from *Hydnora abyssinica* using LH-20 resin and silica gel column chromatography. These compounds were never described in this plant.

VI. General Experimental Part

VI.1. PLANT MATERIAL:

Aristolochia bracteolata whole plant parts were collected during August 2013 from the Southern Gezira, Sudan and taxonomically identified in the Department of Pharmacognosy, University of Gezira. A voucher specimen was deposited (A.b. 01-8-013) in the faculty of Pharmacy, University of Gezira.

Ziziphus spina-christi leaves were collected during September 2013 from Ganib area, Sudan and taxonomically identified in the Department of Pharmacognosy, University of Gezira. A voucher specimen was deposited (Z.s. 02-9-013) in the faculty of Pharmacy, University of Gezira.

Hydnora abyssinica rhizomes were collected during September 2013 from Abu Haraz, Sudan and taxonomically identified in the Department Pharmacognosy, University of Gezira. A voucher specimen deposited (H.a. 03-9-013) in the faculty of Pharmacy, University of Gezira.

VI.2. PREPARATION OF CRUDE EXTRACT:

VI.2.1. Aristolochia bracteolata:

Dried whole plant was milled into coarse powder using electrical blender. Powdered material of *A. bracteolata* (289 g) were extracted by 1.25 L of Methanol in a Soxhlet apparatus during 20 hours. Methanol was evaporated under reduced pressure and the extract was then freeze-dried (yielding 71.21 g of freeze dried crude extract).

VI.2.2. Ziziphus spina-christi:

Dried leaves were milled into coarse powder using electrical blender. Powdered material of *Z. spina*-christi (500 g) were extracted by 2.6 L of Methanol in a Soxhlet apparatus during 20 hours. Methanol was evaporated under reduced pressure and the extract was then freeze-dried (yield 92 g of freeze dried crude extract).

80 g of crude methanol extract was resuspended in 300 mL of 50% ethanol and extracted with petroleum ether (0.9 L), ethylacetate (0.9 L), *n*-butanol (0.5 L) to yield 9.3, 24.4, 7.9 g of corresponding extracts and 38.4 g of aqueous residue.

VI.2.3. Hydnora abyssinica:

Dried rhizomes were milled into coarse powder using electrical blender. Powdered material of *H. abyssinica* (492 g) were extracted by 1.2 L of Methanol in a Soxhlet apparatus during 20 hours. Methanol was evaporated under reduced pressure and the extract was then freeze-dried (yield 95 g of freeze dried crude extract).

88 g of crude methanol extract was resuspended in 300 mL of 50% ethanol and extracted with ethylacetate (0.9 L) and *n*-butanol (0.5 L) to yield 12.9 and 32.6 g of corresponding extracts and 42.4 g of aqueous residue.

VI.3. PRELIMINARY PHYTOCHEMICAL SCREENING:

VI.3.1. Preparation of extract:

The three extracts were prepared from the coarse powdered plant materials:

a) 1 g of the powder was mixed with 5% HCl and then filtered: acidic extract;

b) 2 g of powder was placed in a 100 ml beaker. 40 ml of boiled distilled water was then added to the powder and filtered after one hour of infusion: aqueous extract;

c) In well closed bottle, 1 g of the powder was macerated with 10 ml of diethyl ether for 3 hours and then filtered: ether extract;

d) In well closed bottle, 1 g of the powder was macerated with 10 ml of 50% ethanol for 3 hours and then filtered: ethanolic aqueous extract.

VI.3.2. Tests methodology:

The tests were repeated three times.

VI.3.2.1. Alkaloids:

The acidic extract was divided in three test tubes. Then into two tubes, few drops of Dragendorff and Mayer reagent were added. The third tube was kept as control. The formation of a precipitate which was not solubilized by addition of ethanol indicated the presence of alkaloids.

There was no formation of a precipitate with those reagents in the three plant samples.

VI.3.2.2. Sterols:

Liebermann-Burchard reaction: A few drops of ether extract were evaporated in a two watch glasses. Two drops of acetic anhydride were added to the residue of one watch glass. A drop of concentrated sulfuric acid was then added to each watch glass. If purple-green colour appeared after few minutes in watch glass treated with acetic anhydride, it indicates the presence of of sterols. The appearance of color in the watch glass treated only with sulfuric acid reveals the presence of other metabolites (red coloration in the presence of terpene derivatives and blue coloration in the presence of carotenoid derivatives).

There was formation of yellow colour in watch glass treated with sulfuric acid with *Aristolochia bracteolata*, while negative reaction was obtained with the two other plant samples.

VI.3.2.3. Saponins:

15 ml of infusion extract are placed in test tube with 16 mm diameter and 160 mm height. After stirring vigorously for about 10 seconds, the formed foam height was evaluated in cm. This height was measured again after standing for 10 min. If the foaming was important and persists after the 10 min rest, it reveals presence of saponins.

After stirring, a height one centimeter of foam was formed that disappeared after 10 min rest, thus the three plant samples did not contain saponins.

VI.3.2.4. Tannins:

Two milliliters of infusion extract were placed into two test tubes. Few drops of a 1% ferric chloride were added to the first tube solution and the second tube was supplemented with saline gelatin (1% aqueous solution of gelatin and 10% sodium chloride). In the presence of tannins, a precipitate occured in both tubes.

After the addition of ferric chloride, a green-brown precipitate appeared in the three plant samples, demonstrating the presence of tannins.With the gelatin solution salt, there was no reaction.

VI.3.2.5. Quinones:

Borntrager's test: two milliliters of diethyl ether extract were poured into test tube and few drops of an aqueous ammonia solution were added. After stirring, the appearance of a violet red color indicates the presence of free quinones.

The color of the aqueous ammonia layer remained yellowish with the three plant samples.

VI.3.2.6. Cardenolides:

A few milliliters of ethanolic maceration were poured into two glass watchs, then few drops of Kedde's reagent (3,5-dinitrobenzoic acid in potassium hydroxide) were added. The formation of a purple violet colour indicates the presence of cardenolides.

There was no reaction with the three plant samples.

VI.3.2.7. Flavonoids:

A few milliliters of ethanolic maceration were poured into test tubes. Few drops of concentrated HCl and 0.5 g magnesium turnings were added. Pink colour was observed for presence of flavonoids. Minor change in colour appeared for the three plant samples.

Few drops of ethanolic extracts were also placed on strips of Whatmann filter paper. Each strip was dipped in a 2% aluminum chloride solution, intense fluorescence under UV at 365 nm develops in the presence of flavonoids: yellow fluorescence with flavones and flavanones, green with xanthones and orange with chalcones.

A second strips was dipped in a 3% potassium hydroxide solution, yellow-orange color develops in the presence of flavonoids. All three plant samples gave positive reaction for flavonoids.

VI.4. CENTRIFUGAL PARTITION CHROMATOGRAPHY:

***VI.4.1. Description*:**

Centrifugal partition chromatography (CPC) separations were performed on a FCPC® Preparative 200 Kromaton Technologies apparatus (RousseletRobatel, Annonay, France) using a rotor made of 20 circular partition disks (840 partition twin-cells; total column capacity: 205 mL, dead volume: 32.3 mL). Rotation speed could be adjusted from 200 to 3000 rpm, thus producing a centrifugal force field in the partition cells of about 120 × g at 1000 rpm and 480 × g at 2000 rpm. The solvents were pumped by a preparative Ecom Beta 50 Gradient pump binary low-pressure gradient pump (Praha, Czech Republic). The samples were introduced into the column through a PEEK dual mode preparative scale sample injector 3725i (Rheodyne, Rohnert Park, CA, USA) equipped with a 20 mL sample loop. Effluent content was monitored by an Ecom Flash 06 DAD 600 detector equipped with a preparative flow cell (45 µL internal

volume, optical path 0.3 mm). Fractions were collected by an Advantec Super Fraction collector (Otowa, Japan) (Figure 4).

VI.4.2. Solvent system screening test:

A small amount of each two-phase system to be tested was prepared by mixing appropriate quantities of each solvent in small test tube. After solubilization of the sample and phase separation, an equivalent volume of each phase was spotted in thin layer chromatography (TLC). Then, TLC was developed by using different solvent systems and visualization was performed by different spray reagents. The intensity of spots obtained in each phase was evaluated under white light and UV 366 nm, by densitometry.

VI.4.3. CPC in elution mode:

VI.4.3.1. General procedure:

Before each experiment, the column was washed by MeOH/water (50:50, v/v) in the ascending mode at 20 mL/min with a 600 rpm rotation speed.

VI.4.3.2. CPC using triphasic solvent system:

Three-phase solvent system was prepared by mixing 300 ml of *n*-heptane, methyl-tert-butyl ether (MTBE), acetonitrile and water. The solvent mixture was shaked well in a separatory funnel. After separation of the *n*-heptane-rich upper phase, one equivalent volume of MTBE (300 mL) was added to the mixture of middle and lower phases in order to slightly reduce the polarity of the remaining two-phase solvent system. After separation, the final middle and lower phases were separated. Column was filled with lower phase at the rotation speed of 600 rpm in ascending mode. The rotation speed was then increased to 1300 rpm with pumping of upper phase at flow rate 8 ml/min.

The sample was prepared by dissolving 2 g of crude extract in a mixture of lower phase/middle phase/upper phase/MTBE (15.6:3:1:1 v/v). Effluent content was monitored at 210, 254, 280 and 365 nm.

VI.4.3.3. CPC using biphasic solvent system:

Two-phase solvent system was prepared by mixing solvents in appropriate amounts. The solvent mixture was thoroughly equilibrated in a separatory funnel. After separation, the two phases were separated. The sample was prepared by dissolving crude extract in a mixture of lower phase/upper phase (50:50 v/v).

Then, the column was filled with stationary phase at the rotation speed of 600 rpm in appropriate pumping mode, i.e.descending mode for organic stationary phase or ascending for aqueous stationary phase. The rotation speed was then increased to reach optimal rotation speed (between 1500 to 2000 rpm) with pumping of mobile phase at flow rate of 6-8 ml/min.

VI.4.4. Ion exchange CPC:

VI.4.4.1. CPC general procedures:

The particular experimental conditions are contained in tables 5, 7 and 8. Before each experiment, the column was washed by MeOH/water (50:50, v/v) in the ascending mode at 20 mL/min with a 600 rpm rotation speed. Two column volumes (410 mL) of the organic stationary phase (SP) were then pumped in the descending mode at the same flow rate and rotation speed. The sample was injected through the sample loop at 2 mL/min at 1200 rpm. Displacer-free mobile phase (MP) (40 mL on average) was pumped at 2 mL/min in order to allow column equilibration. Finally, the aqueous mobile phase that contained the displacer was pumped at 2 mL/min, and the fractions were collected every minute. Effluent content was monitored at λ = 254 nm. Stationary phase retention was about 73% on average. The pressure was approximately 20 bars. The experiments were conducted at room temperature (22±2 ◦C).

VI.4.4.2. Preparation of the biphasic solvent system for CPC separations:

The biphasic system (2 L) was prepared by mixing methyl-*tert*-butyl ether (MtBE), acetone, methanol and water known proportions in a separatory funnel. The solvents were vigorously shaken and then allowed to settle until the two phases became limpid. The pH of the aqueous phase was adjusted to 7 by adding the appropriate amount of NaOH 0.1 M. After phases separation, Aliquat 336 (Al336) was added to the organic stationary phase. The mobile phase was prepared by adding known amount of displacer NaI.

VI.4.4.3. Pseudoternary diagram construction:

A solubility isotherm (or a binodal curve at 22 ± 2◦C) of the exchanger Al336 in the MtBE, acetone, MeOH and water pH 7 (3:1:1:3, v/v) system was generated. Pre-defined ratios (w/w) of stationary phase to mobile phase were successively added to 500 mg of Al336 until the appearance of the conjugated phase. The coordinates of these points were plotted to design the ternary diagram, presented here in its orthogonal form.

VI.4.4.4. Preparation of sample solutions:

The crude *A. bracteolata* extract was dissolved in 18 mL of NaI-free aqueous phase. This aqueous solution was then resaturated by 2 mL of Al336-free organic phase.

VI.5. FLASH CHROMATOGRAPHY:

Flash chromatography was performed on Flashsmart one chain (AIT, Houilles, France) using silica gel column Interchim PF-30-SiHP or silica-gel precoated column (Chromabond® flash, RS4SiOH, 15-40 µm, 4 g, MACHEREY-NAGEL).

VI.6. FRACTIONS ANALYSIS:

***VI.6.1. High Performance Liquid Chromatography (HPLC)*:**

HPLC analysis were performed on a Dionex UHPLC U3000RS system equipped with a LPG-3400RS quaternary pump, a RSLC WPS-300T RS automated injector, a TCC-300SD column oven and a UHPLC+ DAD-3000 diode array detector (ThermoFisher SA, Voisins le Bretonneux, France). The system was fitted with an AccucoreaQ (15 cm x 3 mm i.d., 2.6 µm particle size) column, itself protected by an AccucoreaQ defender guard 13 x 3.0 mm cartridge (ThermoFisher SA, Voisins le Bretonneux, France).

VI.6.1.1. Aristolochia bracteolata:

The mobile phases were solvent A 0.1% TFA in water, solvent B acetonitrile. The gradient was set as follows: initial acetonitrile content was 0%, it was raised to 100% in 15.25 min and maintained for 2 min; the flow rate was 1 $mL.min^{-1}$, the oven temperature was set at 40°C and the chromatogram was monitored at 210, 254, 280 and 365 nm and using 3D data.

VI.6.1.2. Ziziphus spina-christi:

The mobile phases were solvent A 0.1% TFA in water, solvent B acetonitrile. The gradient was set as follow: initial acetonitrile content was 0%, it was raised to 100% in 15.25 min and maintained for 2 min. Oven temperature was set at 40 °C, UV detection was monitored at λ 210, 254, 280 and 366 nm.

VI.6.1.3. Hydnora abyssinica:

The mobile phases were solvent A 0.1% TFA in water, solvent B acetonitrile. The gradient was set as follow: initial acetonitrile content was 0%, it was raised to 60% in 7.6 min, then raised to 100 % in 0.31 min and maintained for 1 min. Oven temperature was set at 40 °C, UV detection was monitored at λ 210, 254, 280 and 520 nm.

VI.6.2. Thin Layer Chromatography (TLC):

TLC analyses of fractions were performed on Merck 60 F_{254} silica gel plates.

VI.6.2.1. Aristolochia bracteolata:

The mobile phase was CH_2Cl_2/MeOH (85:15, v/v), then observed at UV 254 nm and 365 nm after tin (II) chloride spray reagent derivatization.

Tin chloride reagent was prepared by solubilizing 1g of tin (II) chloride in 1.5 ml HCl 36% and 8 ml water.

Preparative thin layer chromatography (PTLC) was performed using PLC silica-gel 60 F_{254} glass plates, 0.5 mm with concentrating zone 20 x 4 cm. The plates were developed by $CHCl_3$/MeOH/Water (13:7:2) lower phase and $CHCl_3$/MeOH/Water/Formic acid (13:7:2:0.1) lower phase.

VI.6.2.2. Ziziphus spina-christi:

The mobile phase was EtOAc/formic acid/acetic acid/water (100:11:11:26, v/v/v/v), then observed at UV 254 nm and 366 nm after derivatization with diphenylboric acid-2-aminoethylester reagent (Neu reagent).

VI.6.2.3. Hydnora abyssinica:

The mobile phase was CH_2Cl_2/EtOAc/formic acid (6:10:2, v/v/v), then observed at UV 254 nm and 366 nm after derivatization using anisaldehyde sulfuric acid reagent.

VI.6.3. Nuclear Magnetic Resonance (NMR) and mass spectroscopy (MS):

VI.6.3.1. General procedure and materials:

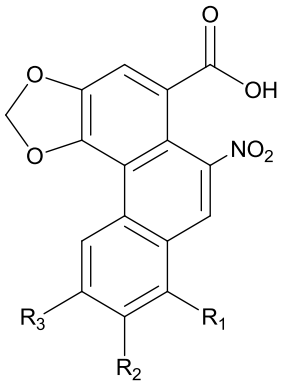
^1H, ^{13}C and 2D-NMR experiments were performed at 300 MHz (^1H) and 75 MHz (^{13}C) on a Brucker-Avance 300 MHz spectrometer (Bruker Biospin, Wissenbourg, France).

HRMS spectra were recorded on an ExactivePlus Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), equipped with a heated electrospray probe (H-ESI II). The instrument was operated in the positive ionization mode using the MS full-scan mode over a mass range of 100–1000 Da. The system was controlled by Xcalibur 2.2 (Thermo Fisher Scientific). ESI and MS parameters were set as follows: spray voltage −4.0 kV, sheath gas and auxiliary nitrogen pressures 40 and 5 arbitrary units, respectively, capillary and heater temperatures 260 and 350 °C, respectively, tube lens voltage 100 V. The automatic gain control (AGC) target value was set at 1 × 106 charges and maximum injection time was set to 200 ms.

VI.6.3.2. Aristolochic acids:

NMR data of aristolochic acids isolated from *A. bracteolata* were summarized below (Table 19 and Table 20).

AA IIIa was subjected to HR-MS leading to a molecular peak M-H of 326.031 m/z in negative mode, corresponding to a molecular formula of $C_{16}H_8NO_7$, confirming the aristolochic acid structure. Other AAs were identified only by NMR.



	R1	R2	R3
Aristolochic acid I	OCH3	H	H
Aristolochic acid II	H	H	H
Aristolochic acid IIIa	H	H	OH
Aristolochic acid IVa	OCH3	H	OH

Figure 110. AA isolated from A. bracteolata.

Table 19. ^{1}H-NMR Data for aristolochic acids I, II, IIIa and IVa isolated from A. bracteolata (δ ppm) (300 MHz in CD_3OD):

N°	AA I	AA II	AA IIIa	AA IVa
2	7.68 *d* (0.9)	7.67 *d* (1.5)	7.63	7.62
5	8.74 *dt* (8.7, 0.9)	9.18 *dd* (8.7, 0.9)	8.56 *d* (2.4)	8.15 *dd* (2.1, 0.6)
6	7.71 *t* (8.1)	7.78 *td* (8.2, 1.5)	-	-
7	7.23 *dd* (8.1, 0.9)	7.70 *td* (7.5, 0.9)	7.20 *dd* (8.7, 2.4)	6.73 *d* (2.1)
8	-	8.04 *dd* (7.8, 1.5)	7.88 *d* (8.4)	-
9	8.58 *d* (0.9)	8.25 *s*	8.18 *s*	8.50 *d* (0.9)
CH_2	6.35 *s*	6.37 *s*	6.33 *s*	6.31
OCH_3	4.08 *s*	-	-	4.03

Table 20. ^{13}C-NMR Data for aristolochic acids I, II, and IIIa isolated from A. bracteolata (δ ppm) (75 MHz in CD_3OD):

N°	AA I	AA II	AA IIIa
1	133.0	133.2	132.8
2	111.2	111.0	110.9
3	146.0	146.1	145.6
4	144.1	144.0	144.1
4a	117.8	117.7	
4b	130.6	129.5	131.1
5	118.9	126.8	111.3
6	129.9	129.1	159.2
7	107.3	127.6	117.8
8	156.5	129.3	131.1
8a	119.8	129.3	122.3
9	117.9	123.8	124.4
10	147.4	148.0	145.6
10a	116.8	116.5	117.1
11	173.6	173.7	174.0
12	102.0	102.1	101.9
OCH_3	55.1		

VI.6.4. Acidic hydrolysis of heterosidic compounds:

VI.6.4.1. General procedures:

25 mg of isolated compound were mixed with 5 mL of trifluoroacetic acid 2N, and then heated to reflux during 4 hours.

The resulting media was extracted with 5 mL of EtOAc (3 fold). The aqueous residue was neutralized at pH 6 using KOH (5 mM), then concentrated under vaccum and lyophylised.

Hydrolysat were resuspended in MeOH or water as convenient and subjected to TLC. Mobile phase used was methylacetoacetate/isopropanol/acetone/water (20:10:7:6, v/v/v/v), plates were observed at daylight after derivatization using sulfuric acid in methanol.

VI.6.4.2. Ziziphus spina-christi:

Compounds Z1 and Z3 were hydrolyzated following the general procedure and then analysed toward different monosaccharide standards. Resulting TLC plates are presented in Figure 111.

Compounds Z_1 and Z_3 exhibited a xylose and a rhamnose substituent.

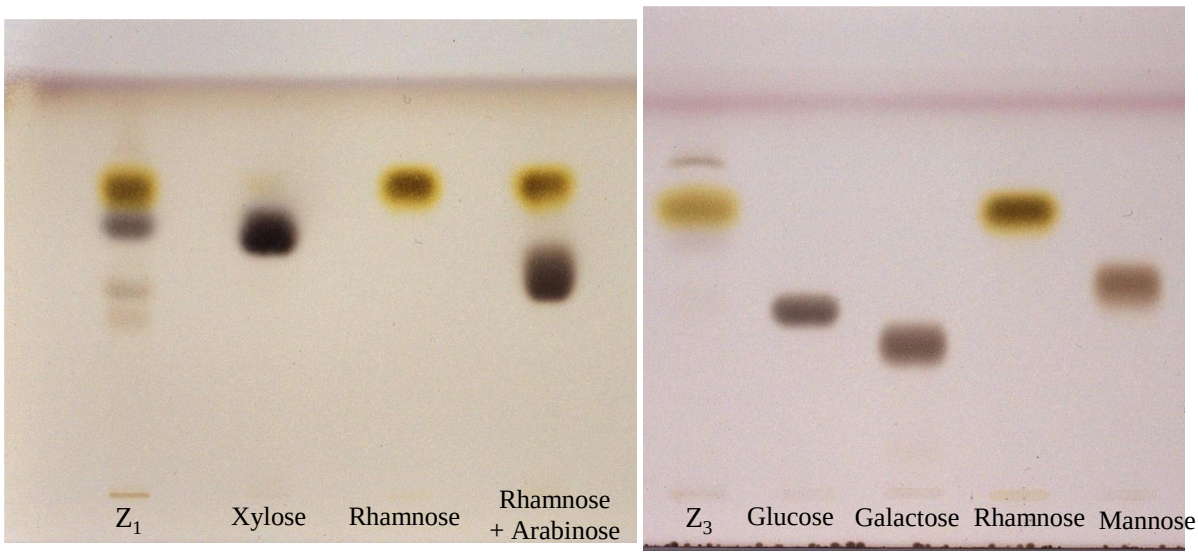


Figure 111. TLC plates of acidic hydrolysis of Z_1 and Z_3.

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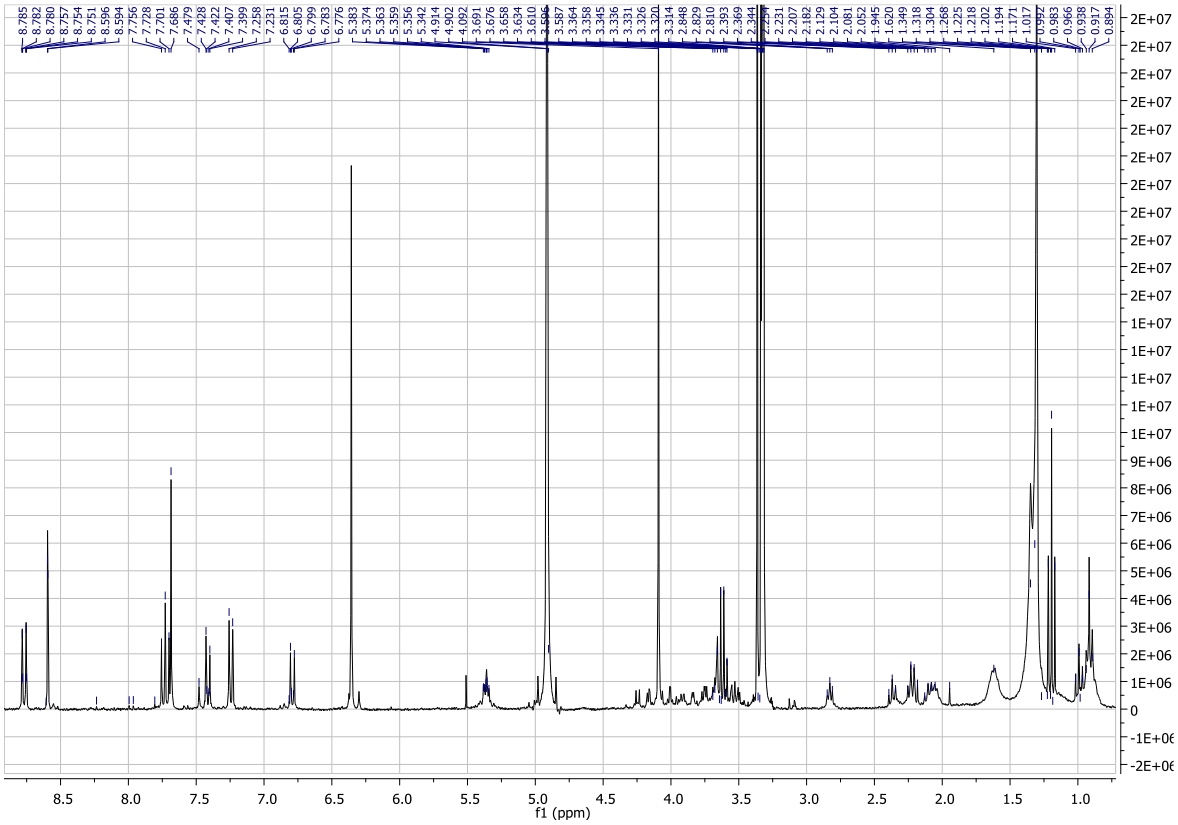
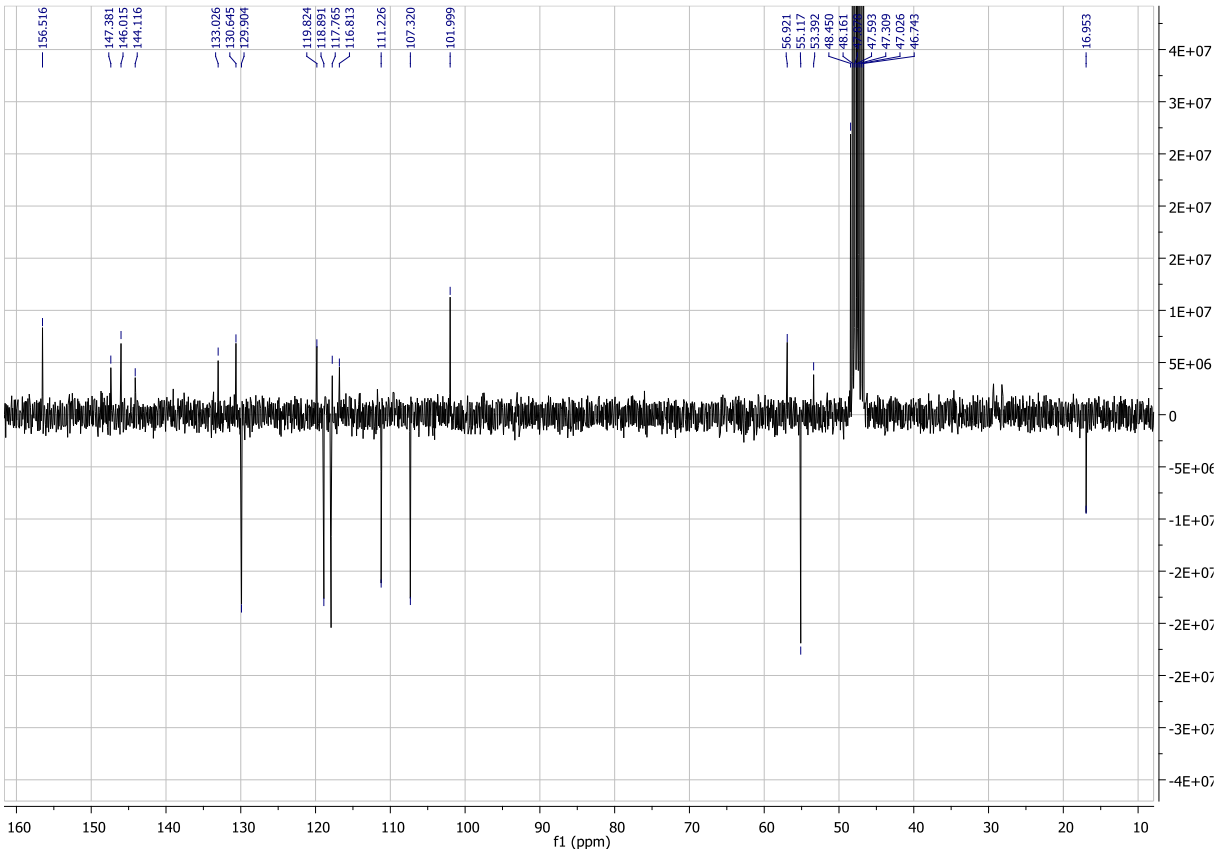
Appendix

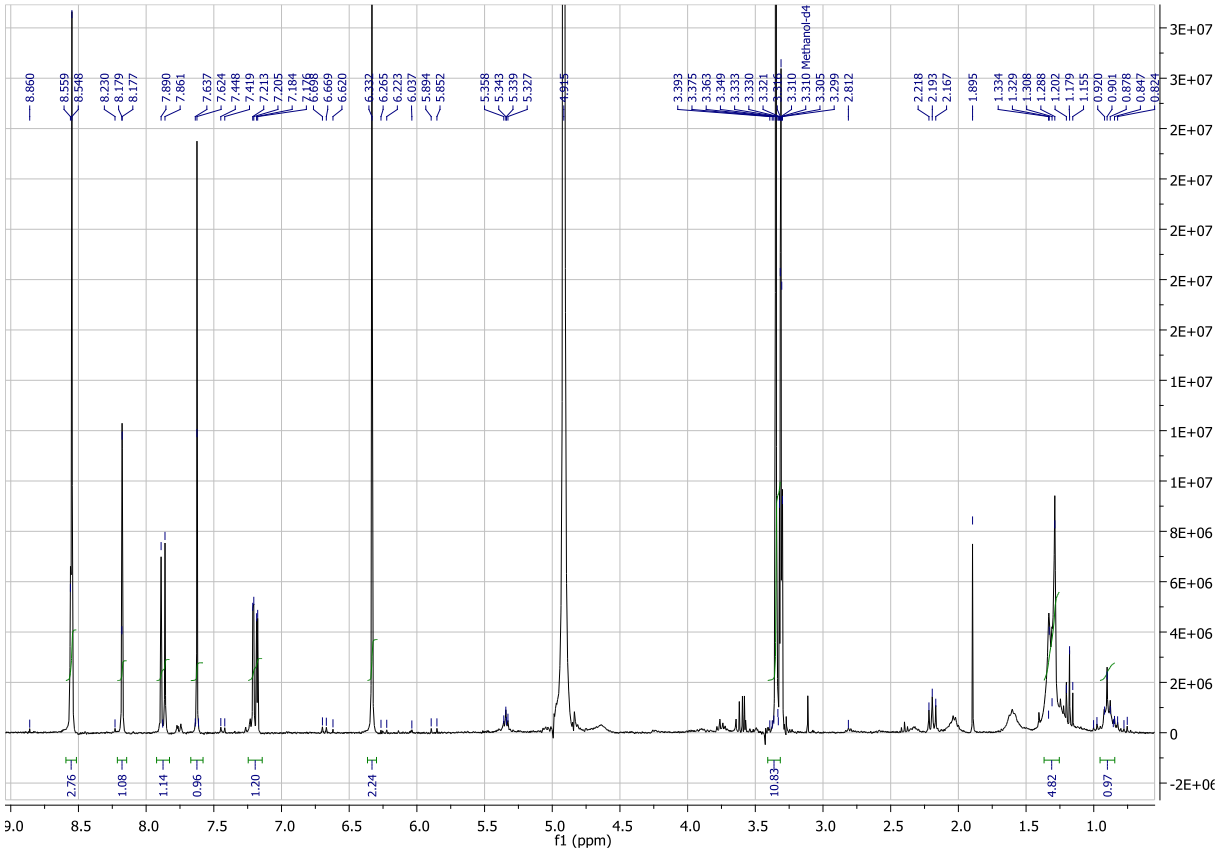
Appendix 1 : ^{1}H NMR spectra of AA I 300 MHz, in MeOD



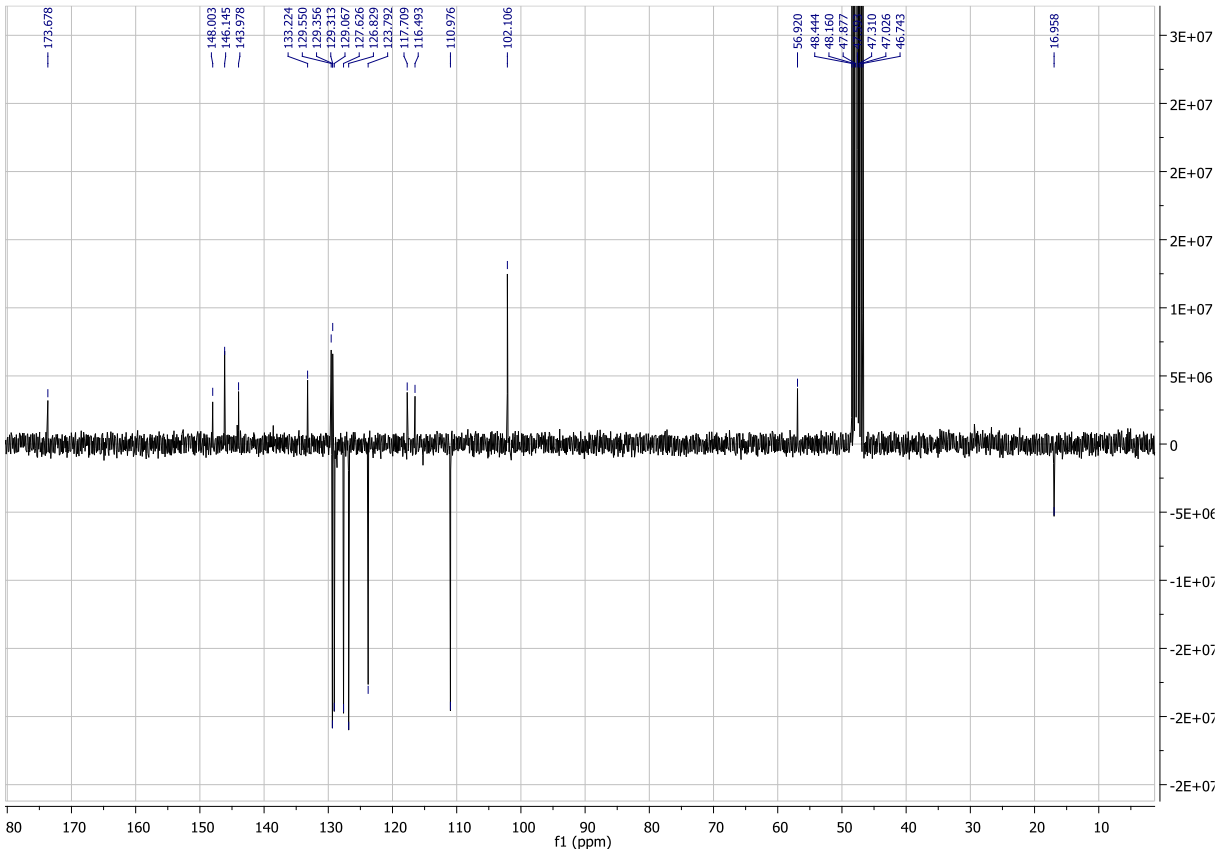
Appendix 2 : ^{13}C NMR spectra of AA II, 75 MHz in MeOD



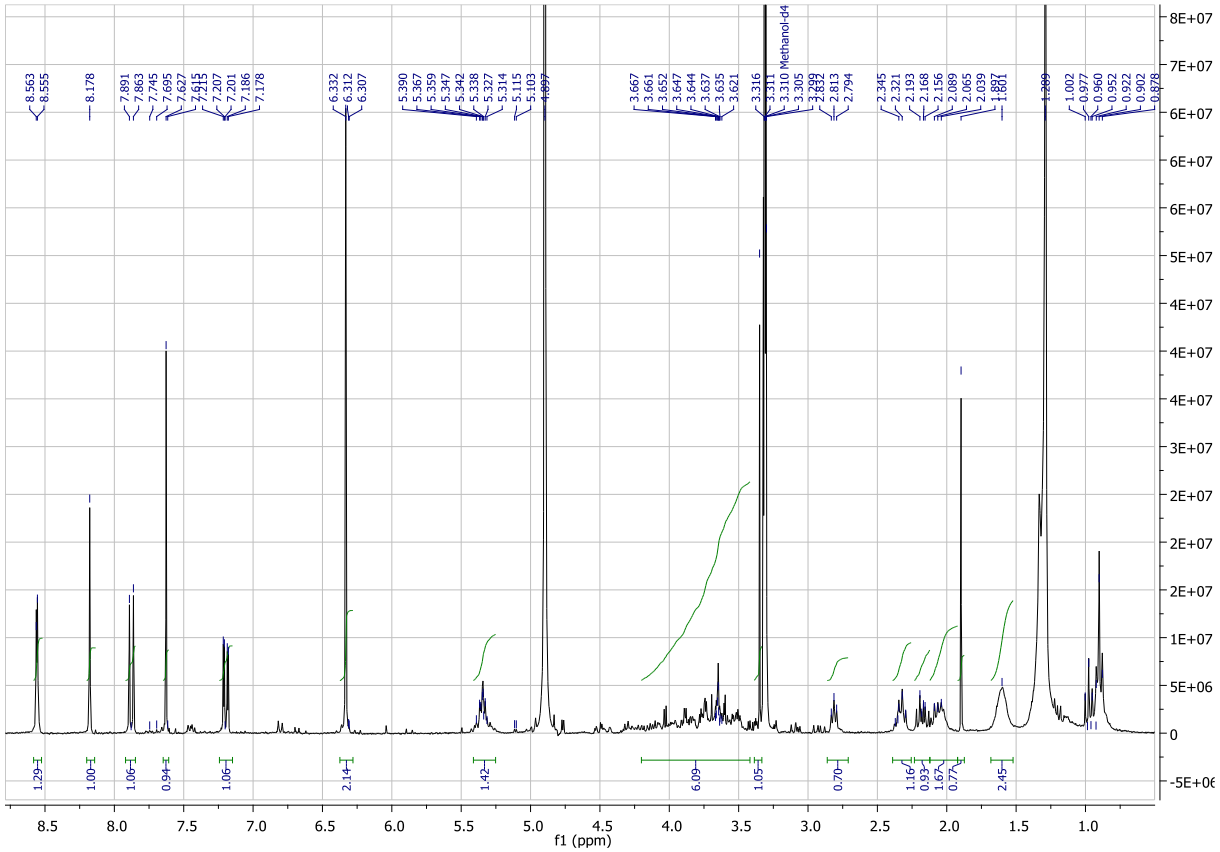
Appendix 3 : ^{1}H NMR of AA II (MeOD, 300 MHz)



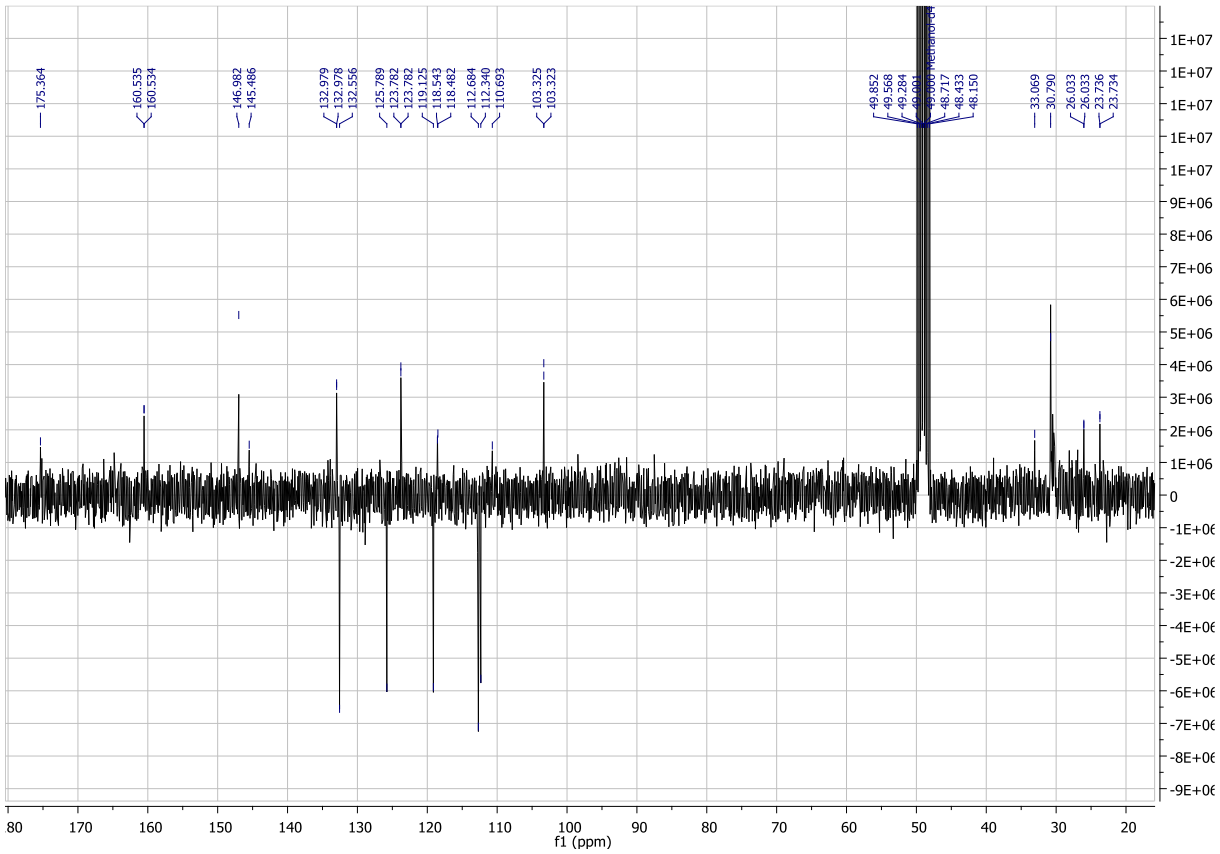
Appendix 4 : ^{13}C jmod NMR of AA II (75 MHz, MeOD)



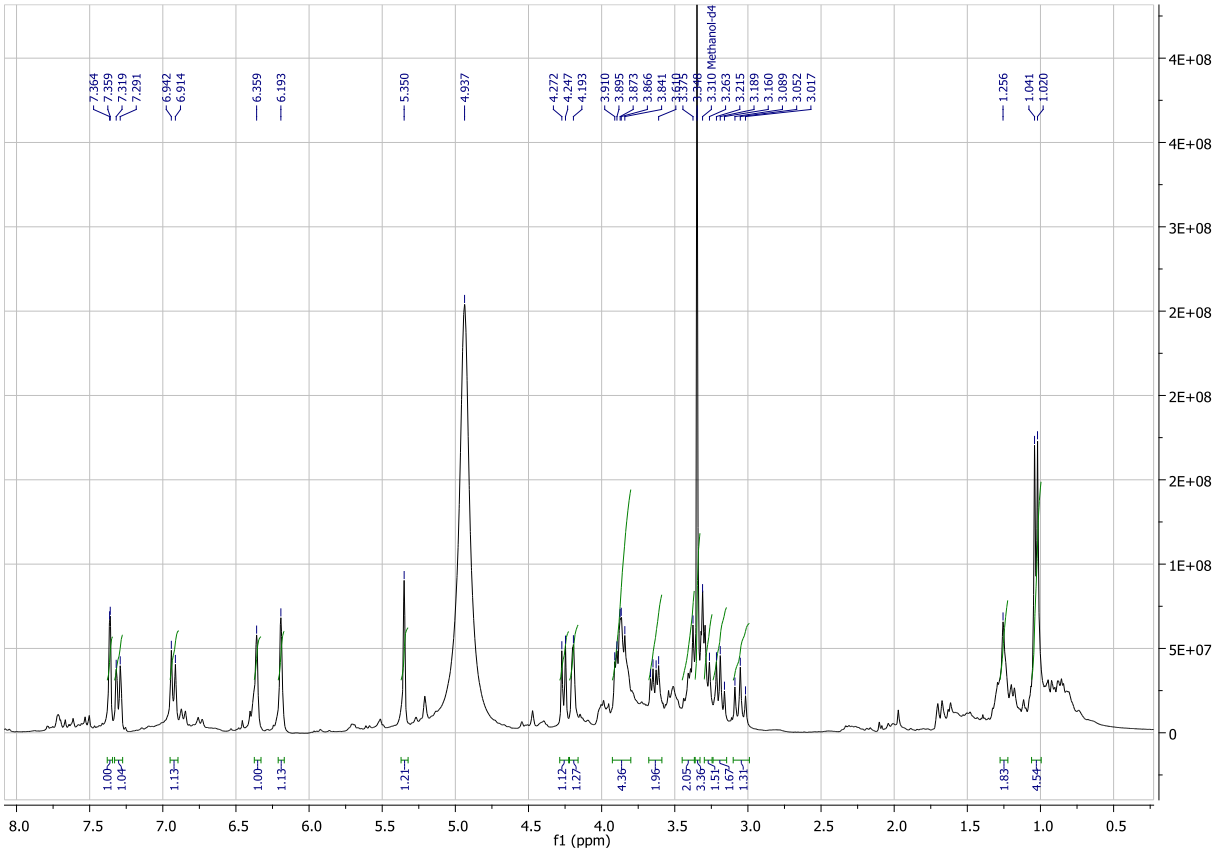
Appendix 5 : ^{1}H NMR of AA IIIa (MeOD, 300 MHz)



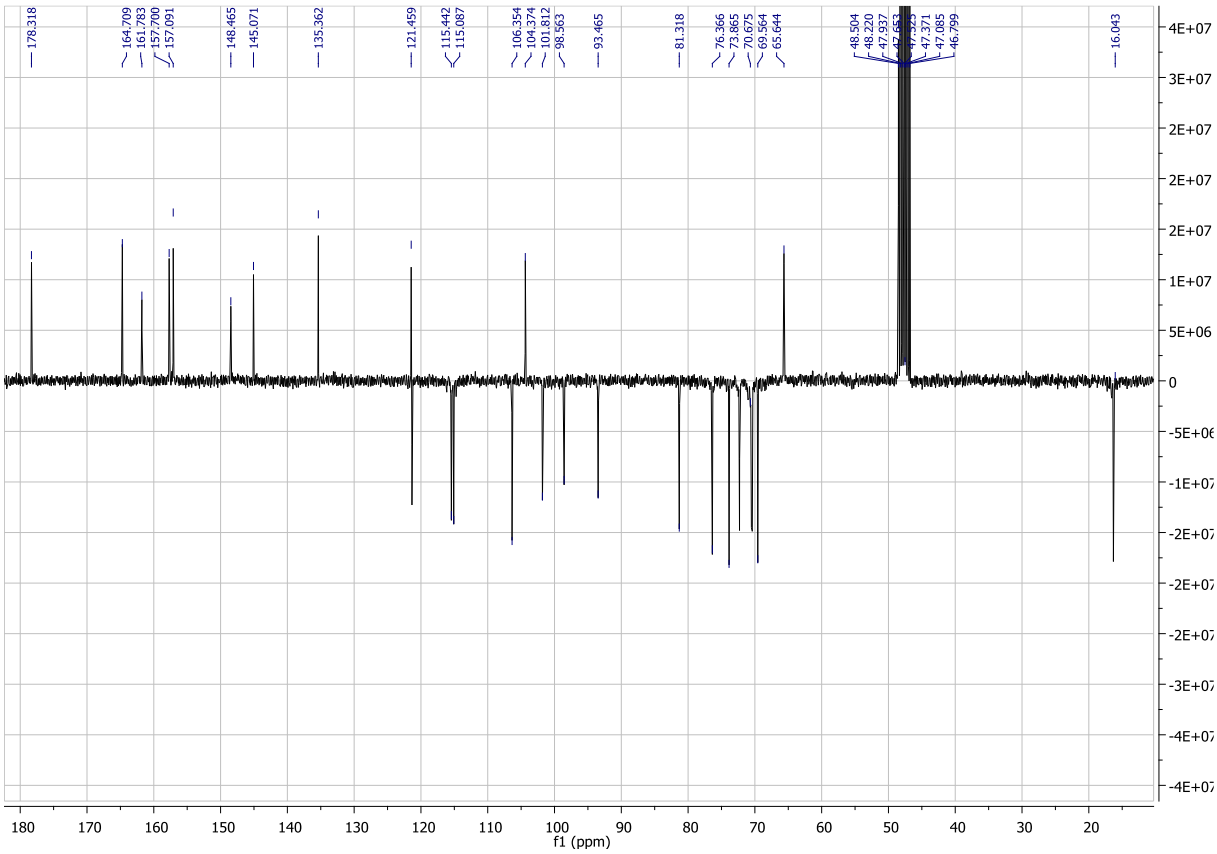
Appendix 6 : ^{13}C jmod NMR of AA IIIa (75 MHz, MeOD)



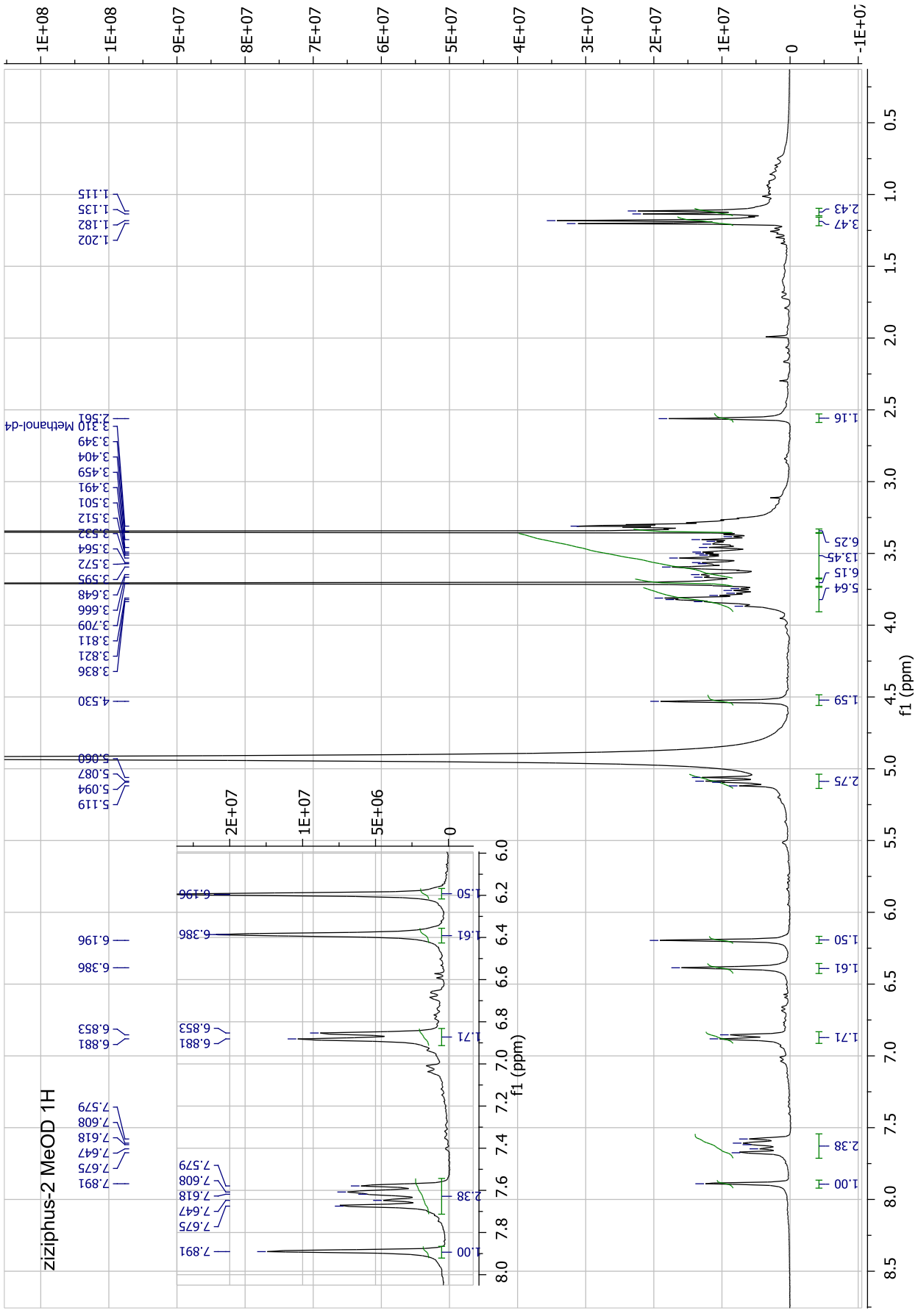
Appendix 7 : ^{1}H NMR of Z_1 (MeOD, 300 MHz)



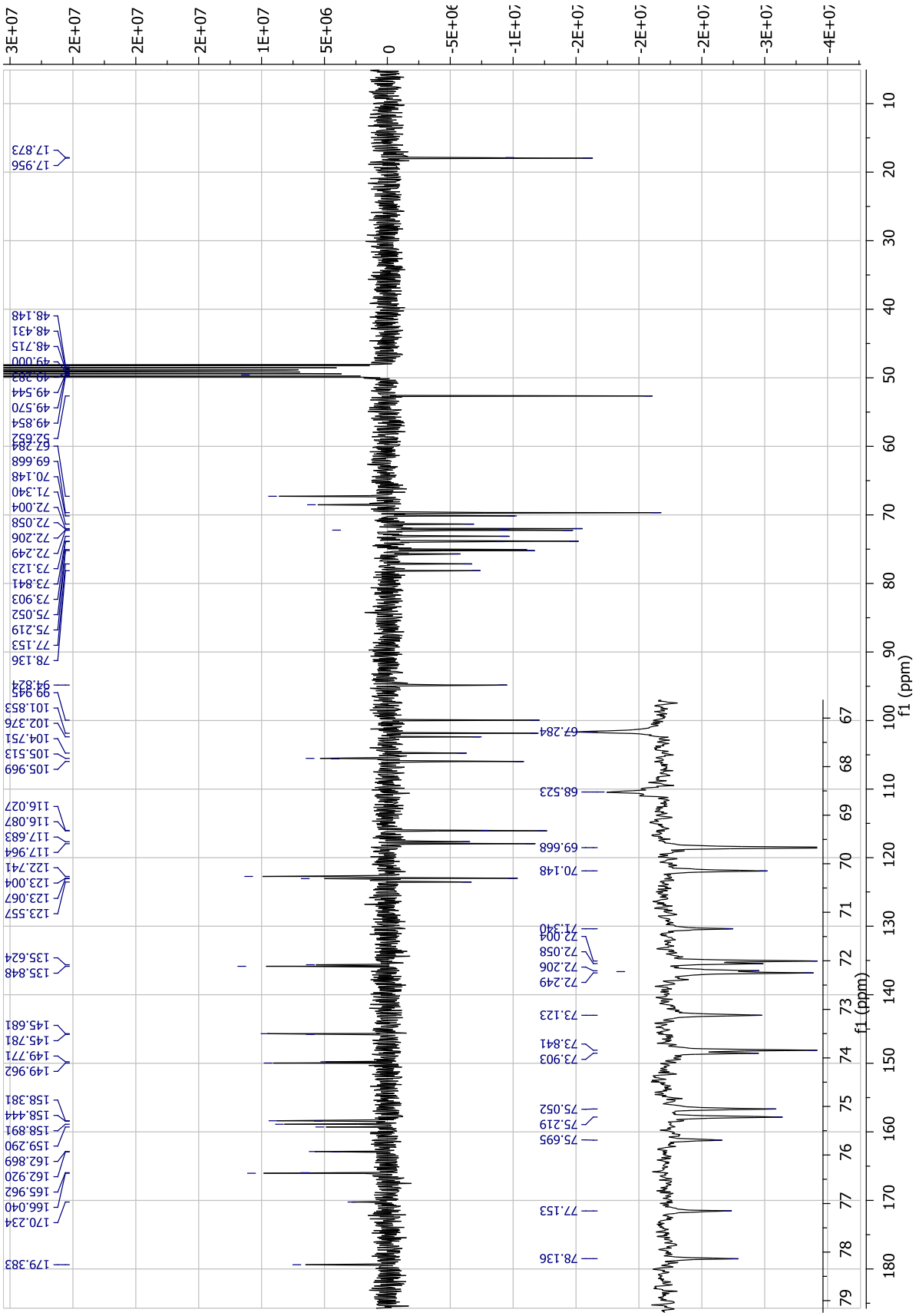
Appendix 8: ^{13}C jmod NMR of Z_1 (75 MHz, MeOD)



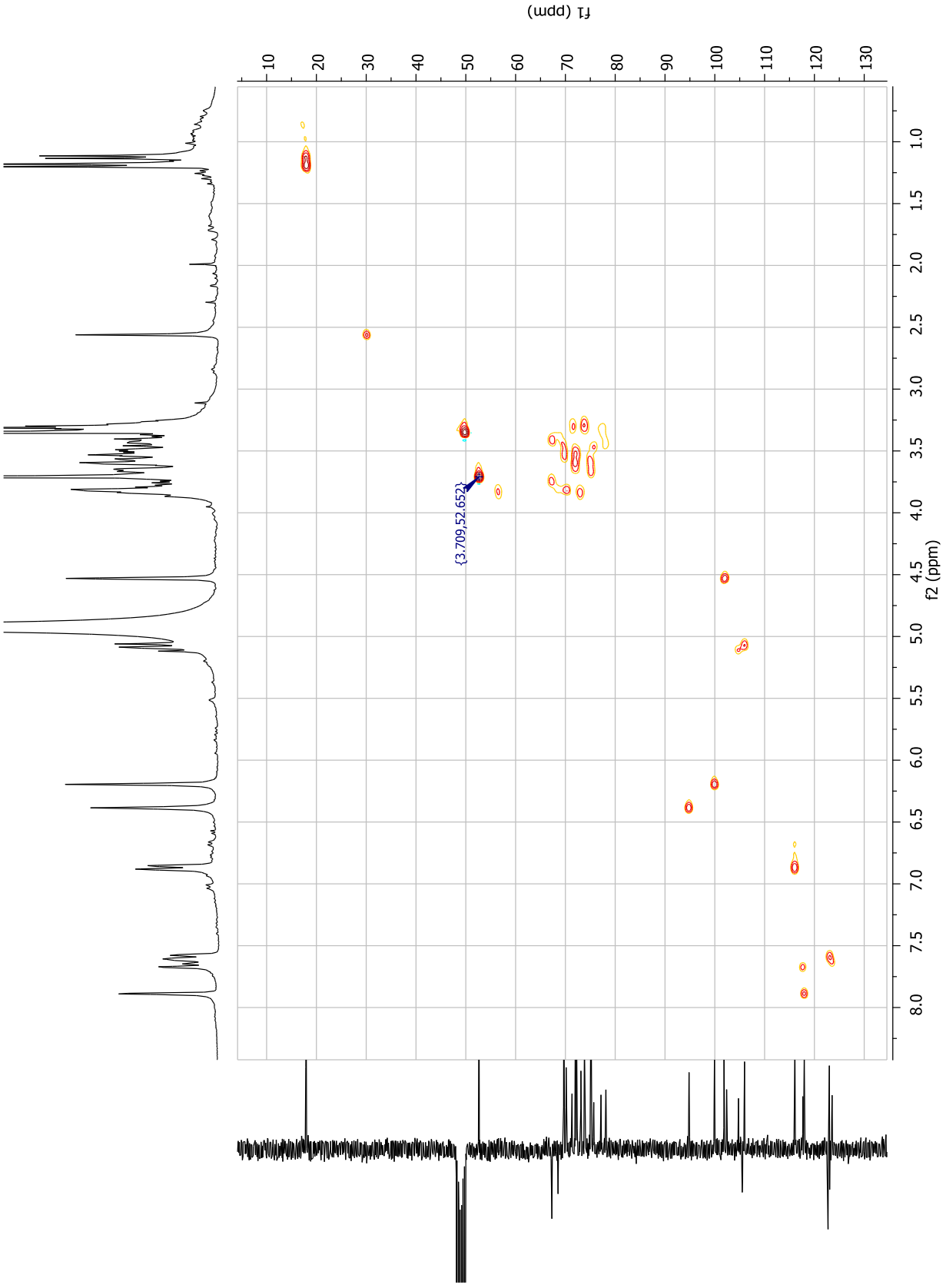
Appendix 9 : ^{1}H NMR of Z_2 (MeOD, 300 MHz)



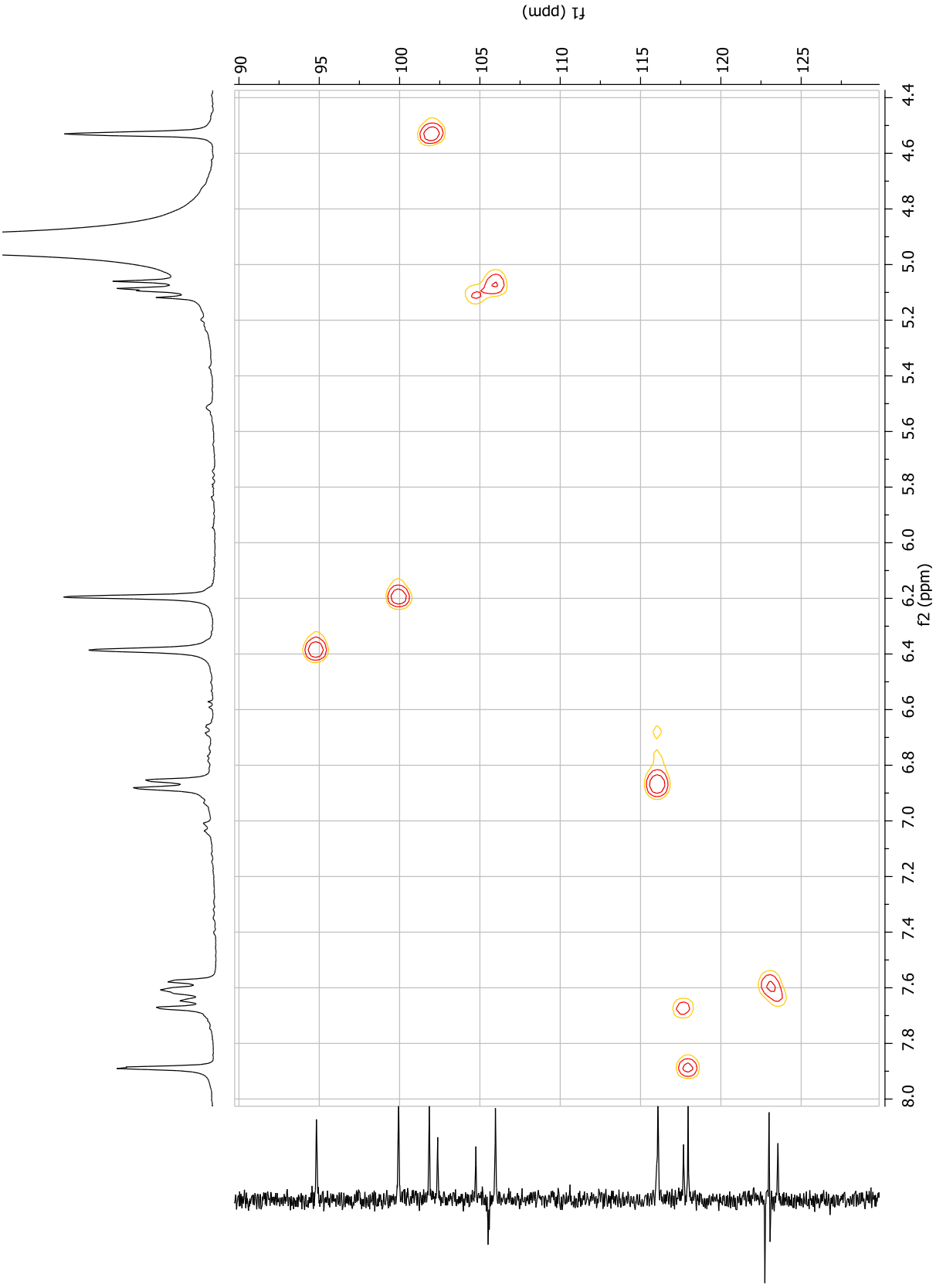
Appendix 10 : ^{13}C jmod NMR of Z_2 (75 MHz, MeOD)



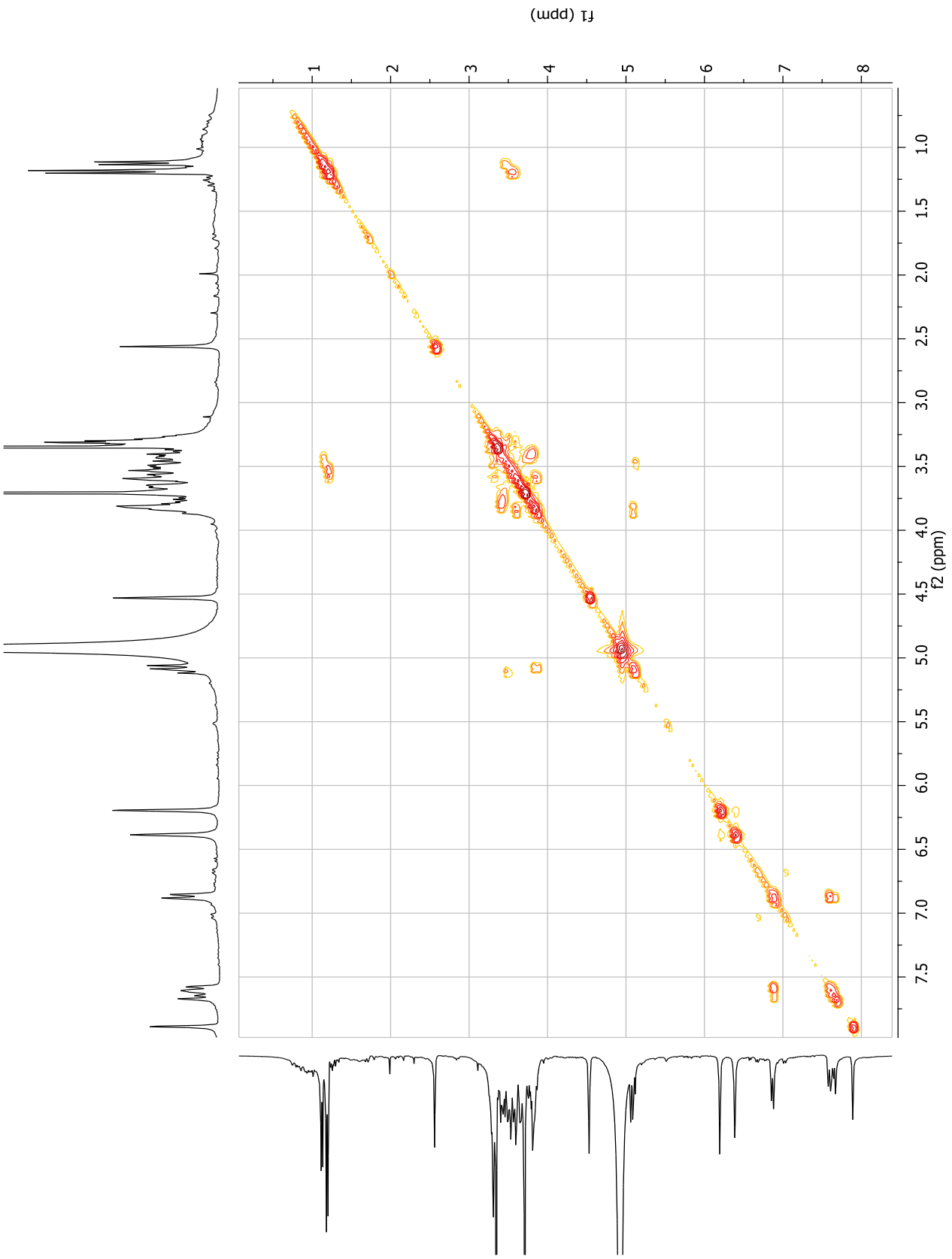
Appendix 11 : HSQC NMR of Z_2 (MeOD)



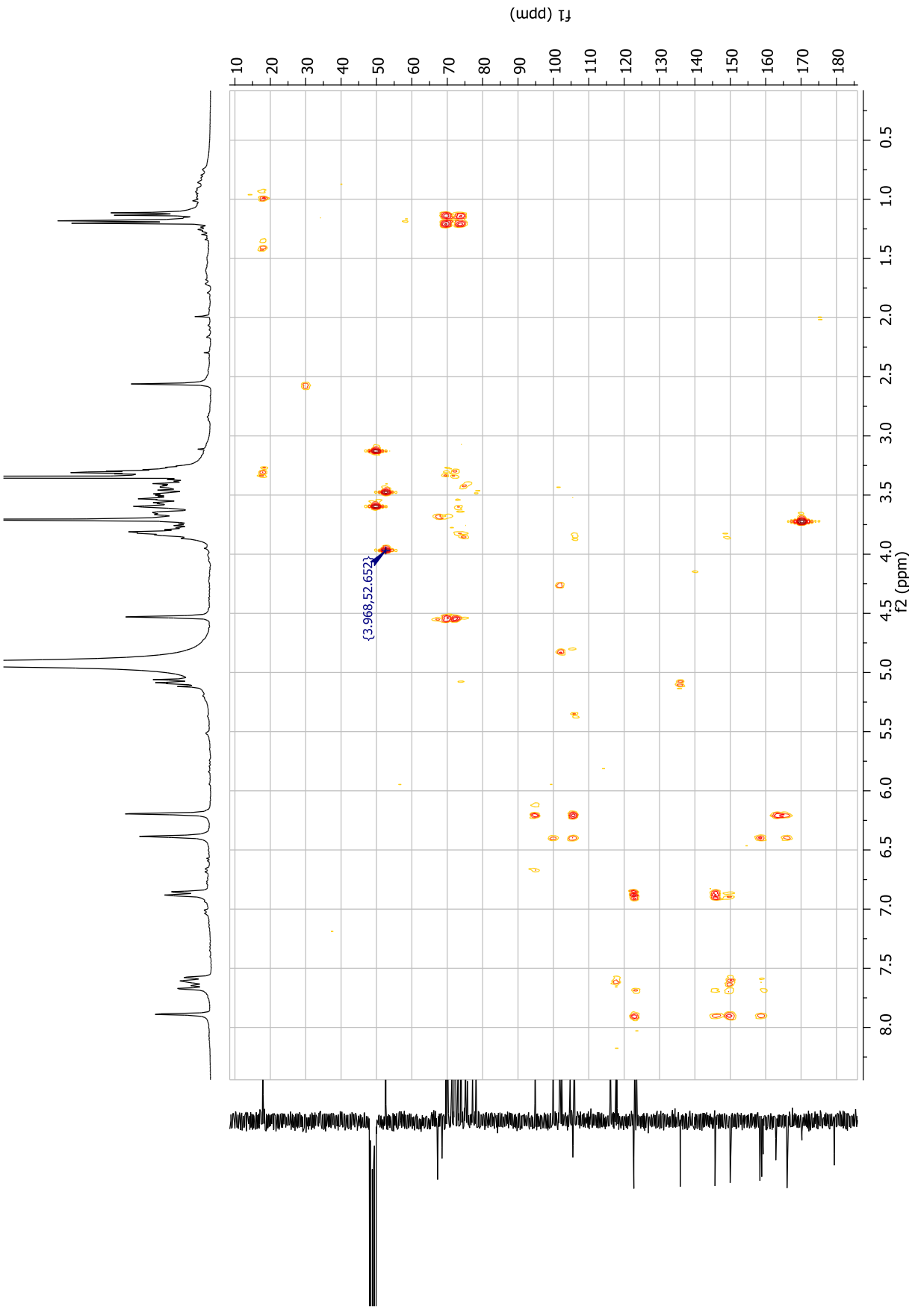
Appendix 12 : HSQC NMR of Z_2 (MeOD) zoom



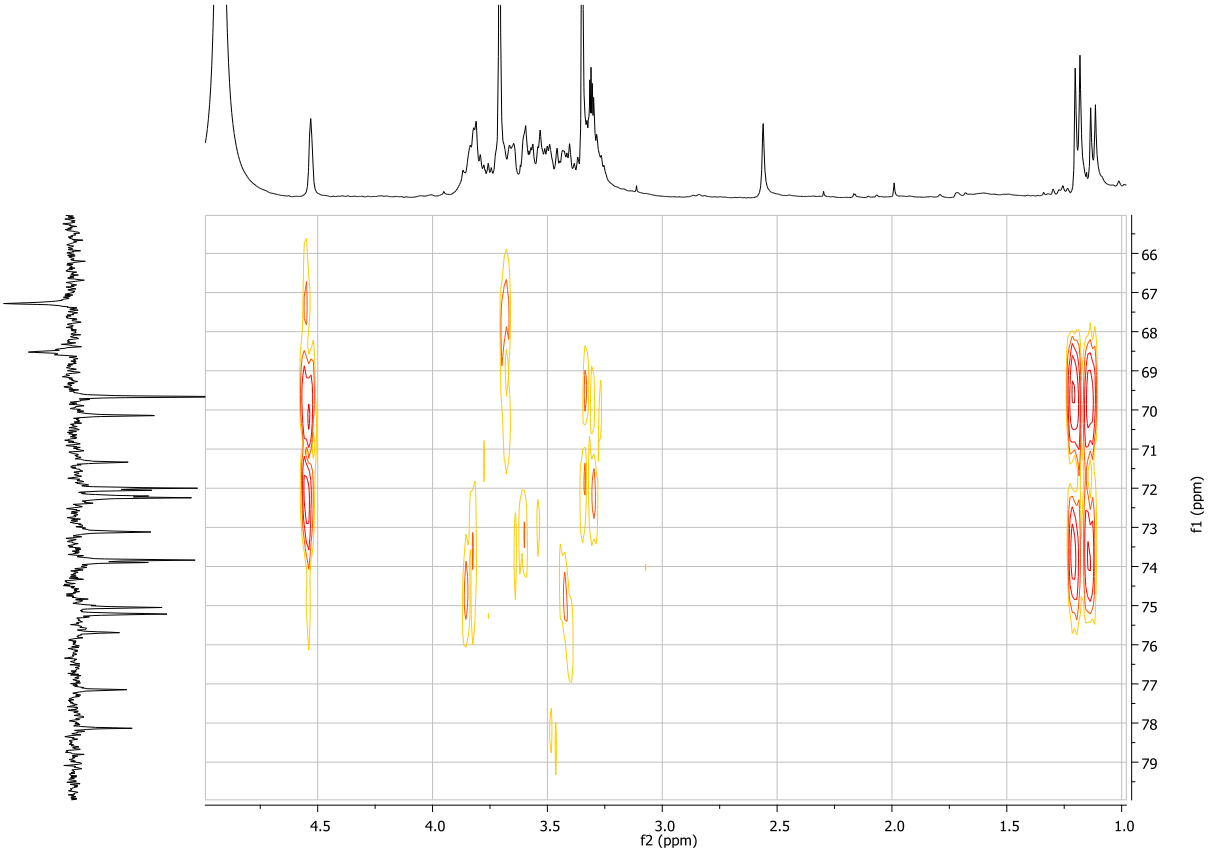
Appendix 13: COSY spectra of Z_2 (MeOD)



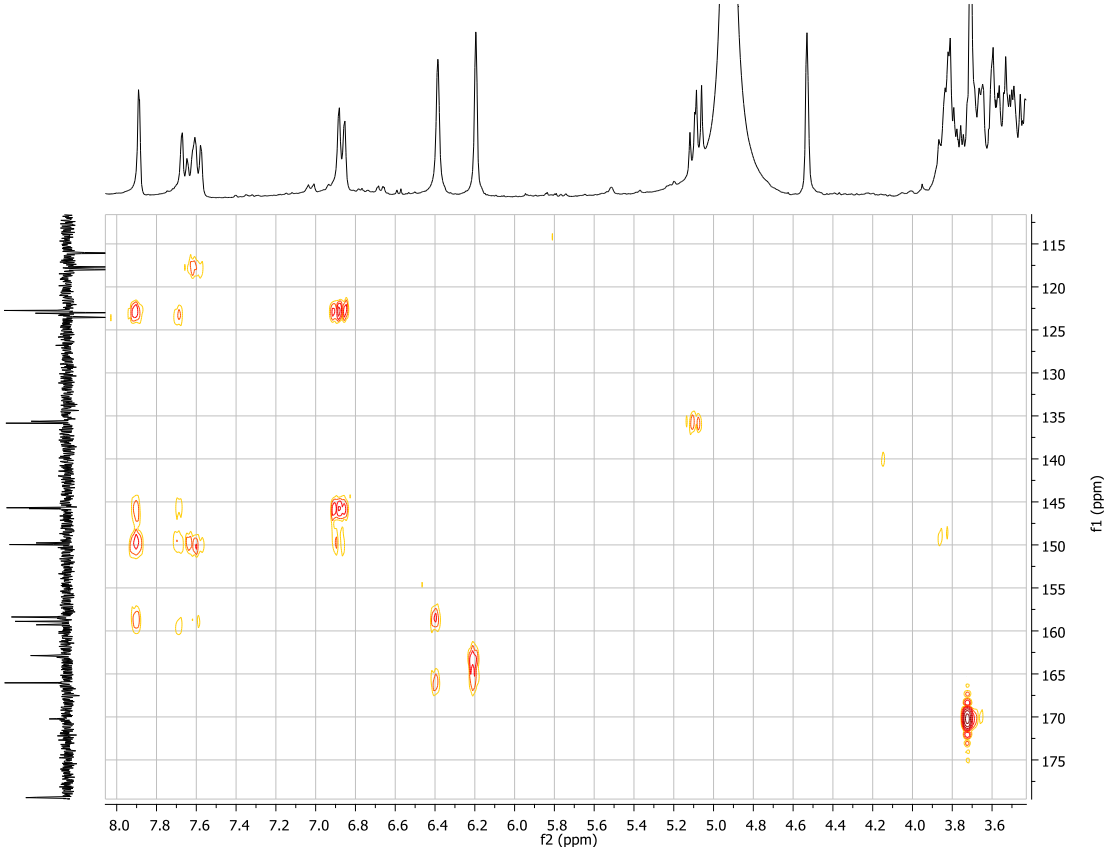
Appendix 14: HMBC spectra of Z_2 (MeOD)



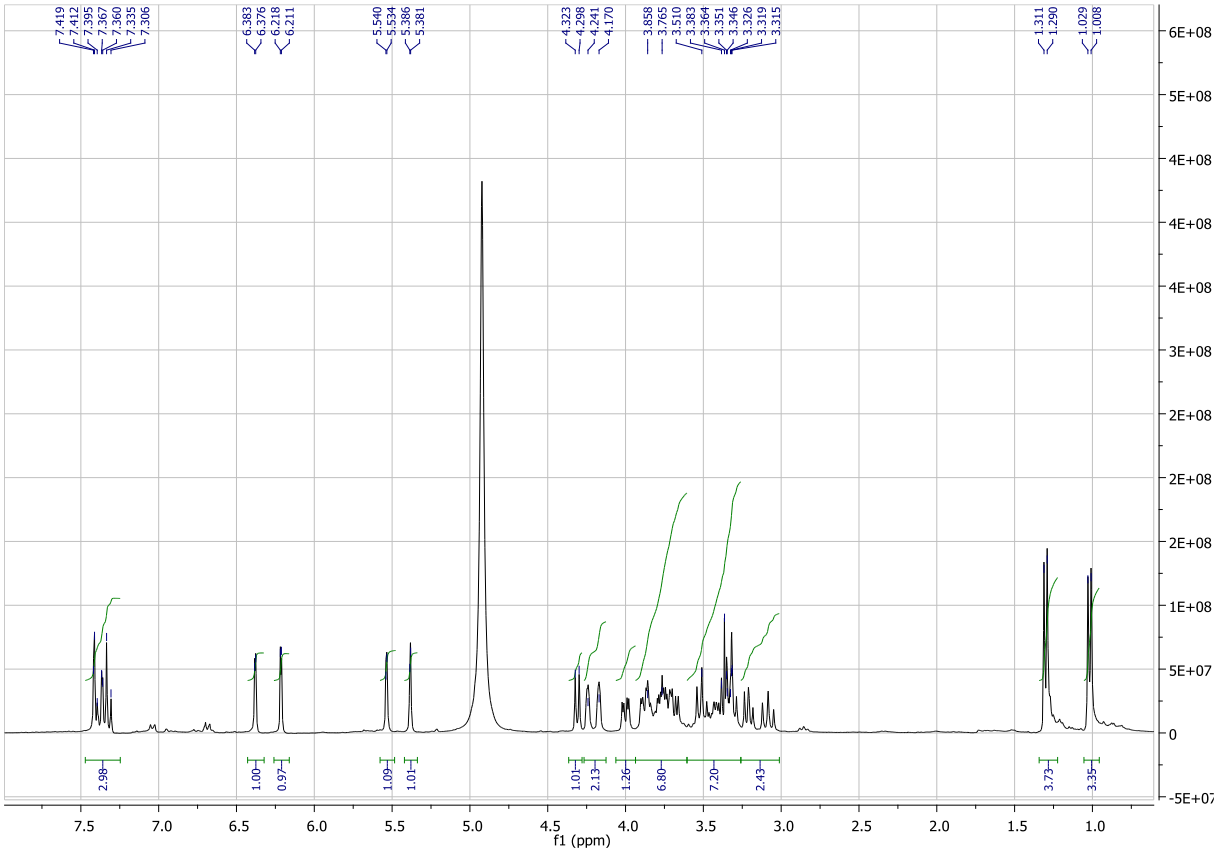
Appendix 15: HMBC spectra of Z_2 (MeOD) zoom 1



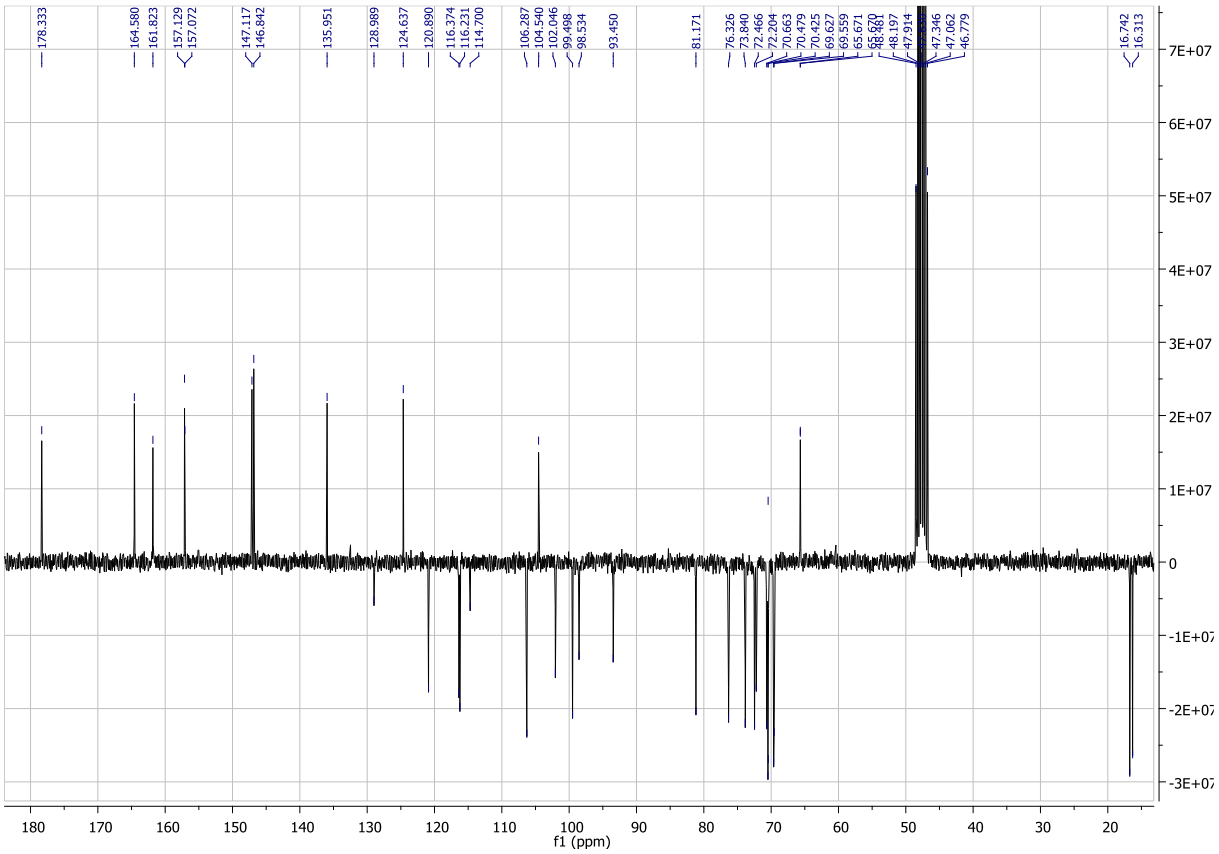
Appendix 16: HMBC spectra of Z_2 (MeOD) zoom 2



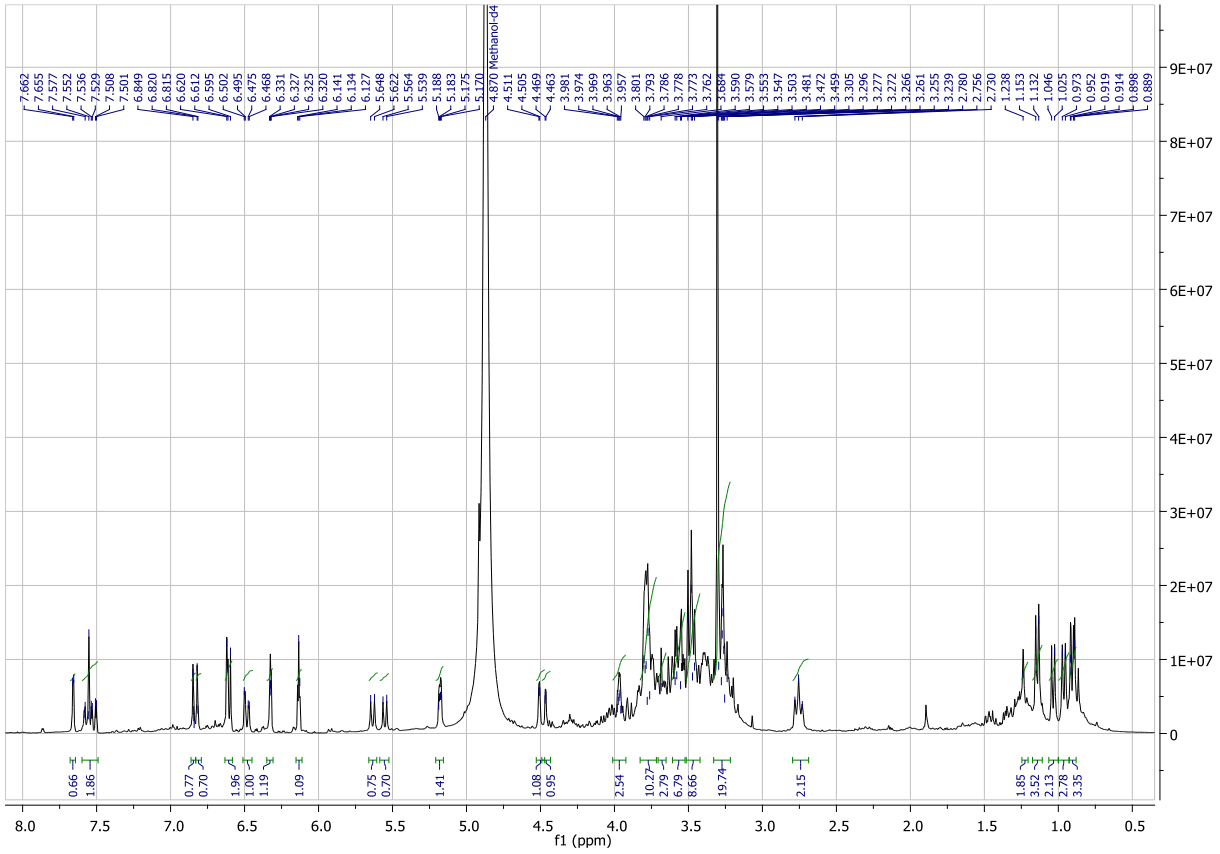
Appendix 17 : ^{1}H NMR of Z_3 (MeOD, 300 MHz)



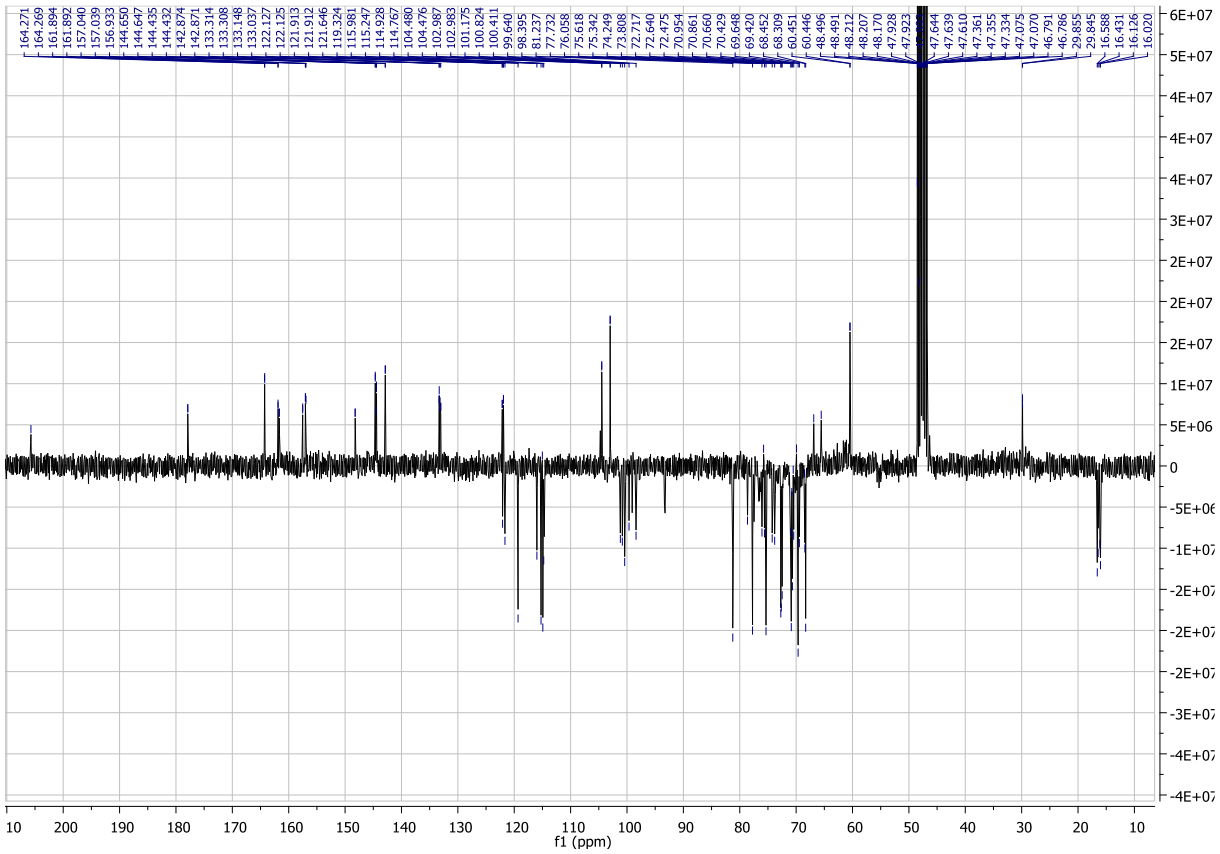
Appendix 18 : ^{13}C jmod NMR of Z_3 (MeOD, 75 MHz)



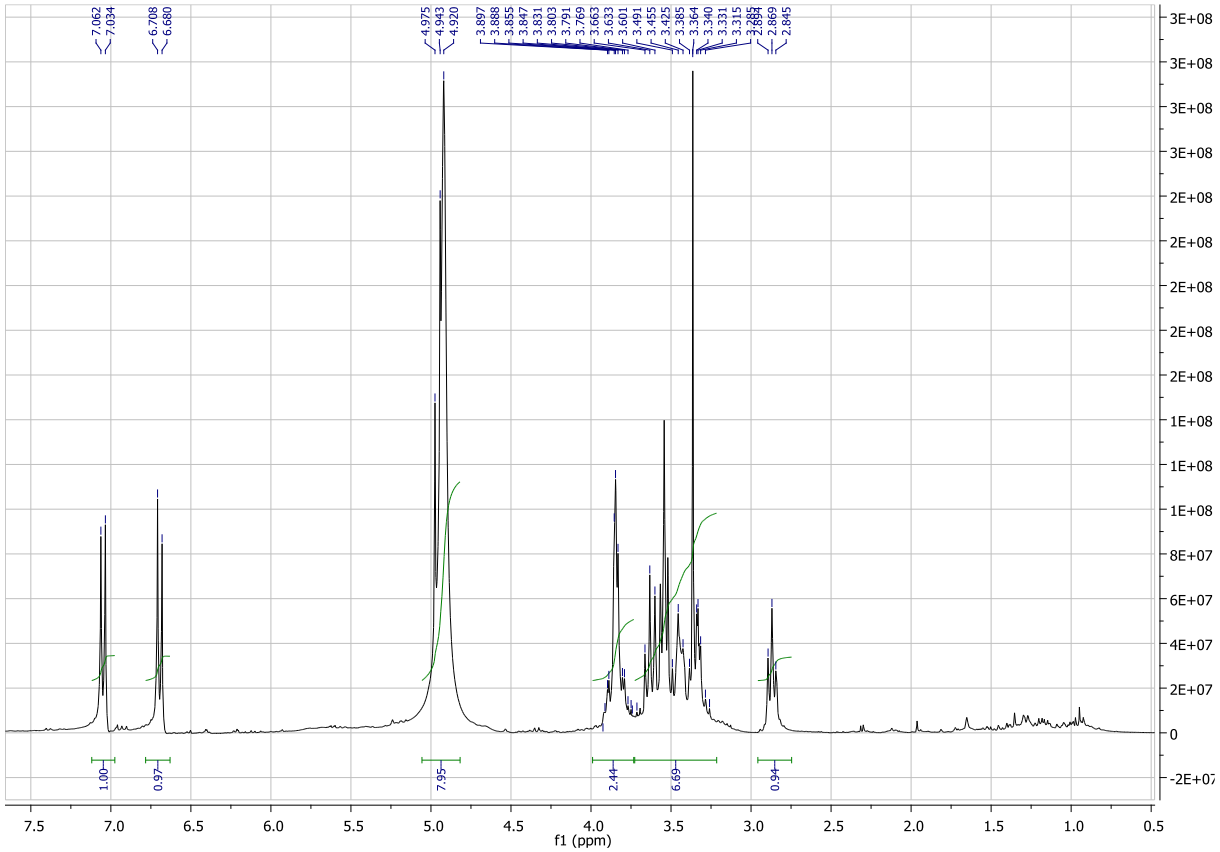
Appendix 19 : ^{1}H NMR of $Z_{4\text{-}4'}$ (MeOD, 300 MHz)



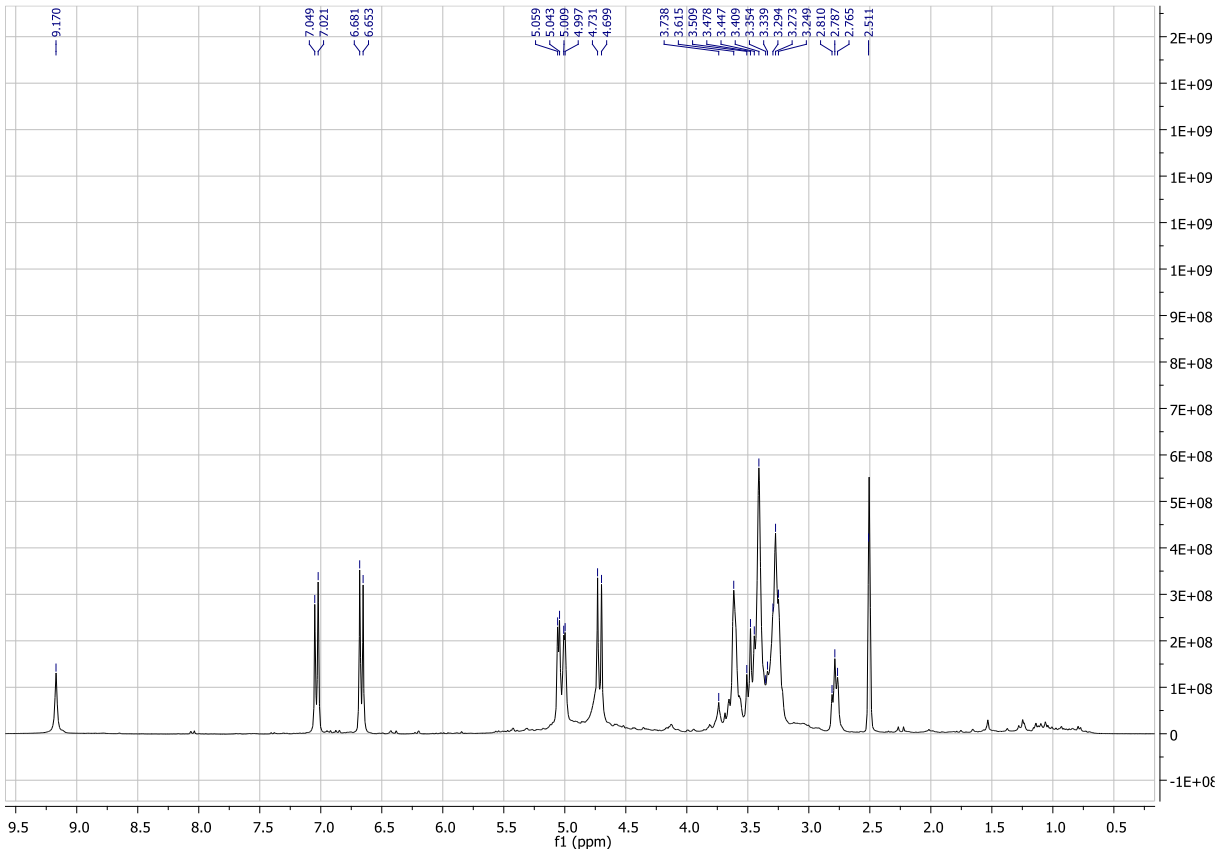
Appendix 20 : ^{13}C jmod NMR of $Z_{4\text{-}4'}$ (MeOD, 75 MHz)



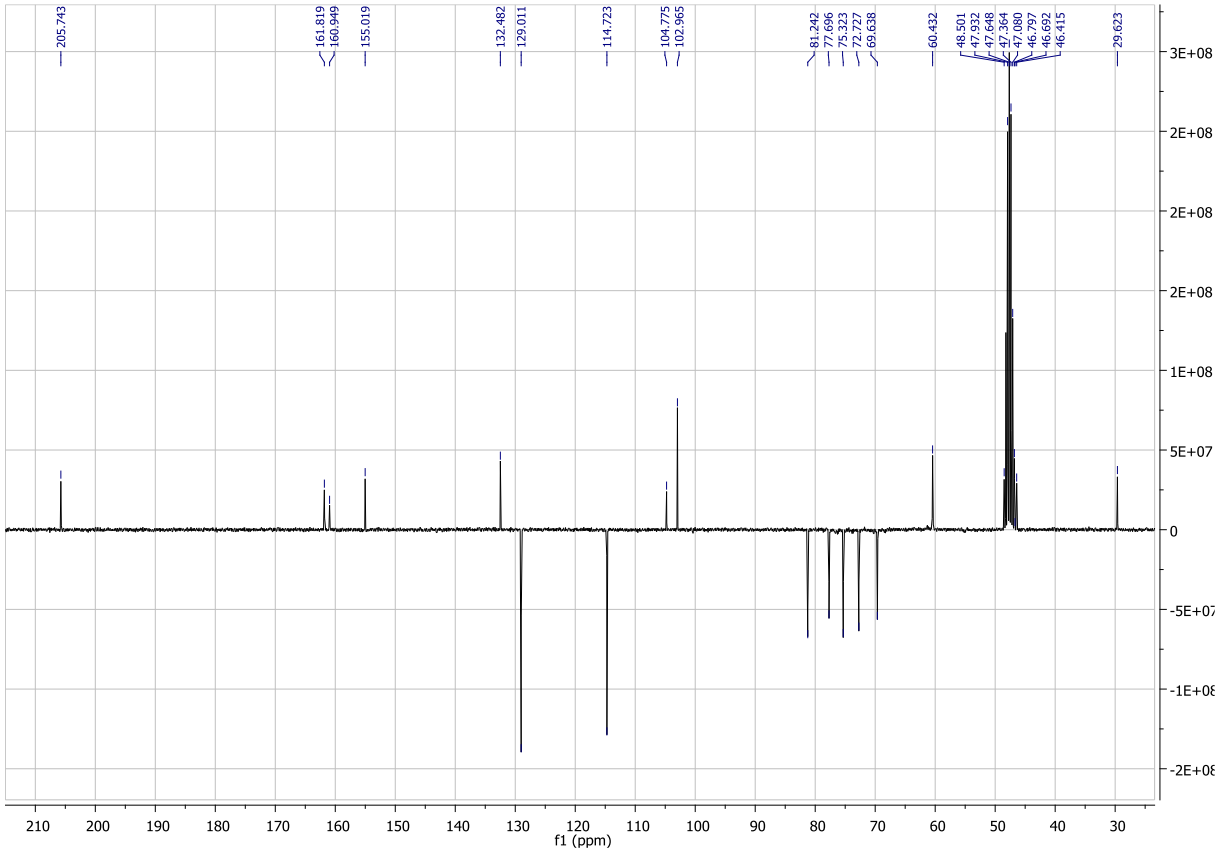
Appendix 21 : ^{1}H NMR of Z_5 (MeOD, 300 MHz)



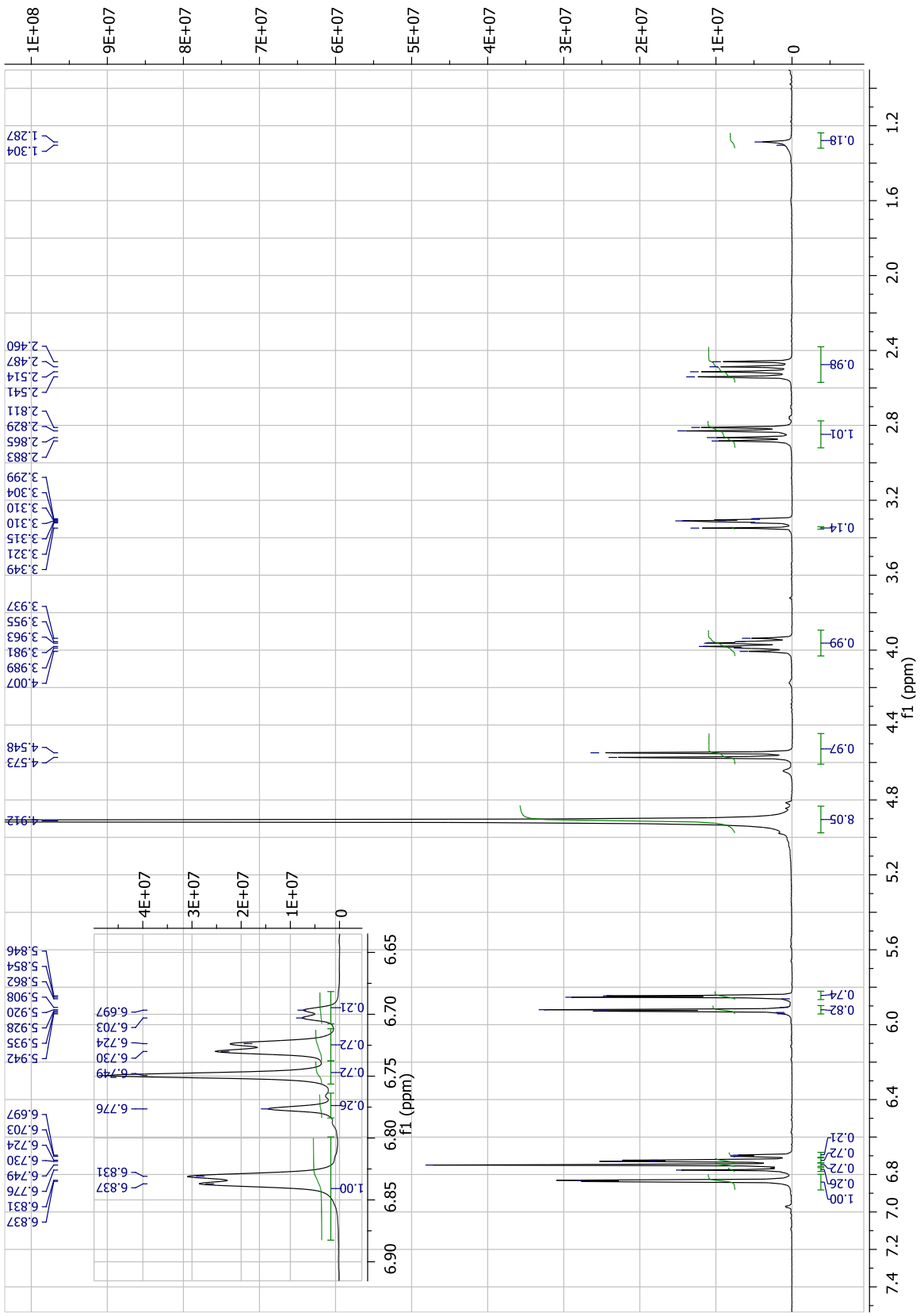
Appendix 22 : ^{1}H NMR of Z_5 (DMSO D_6, 300 MHz)



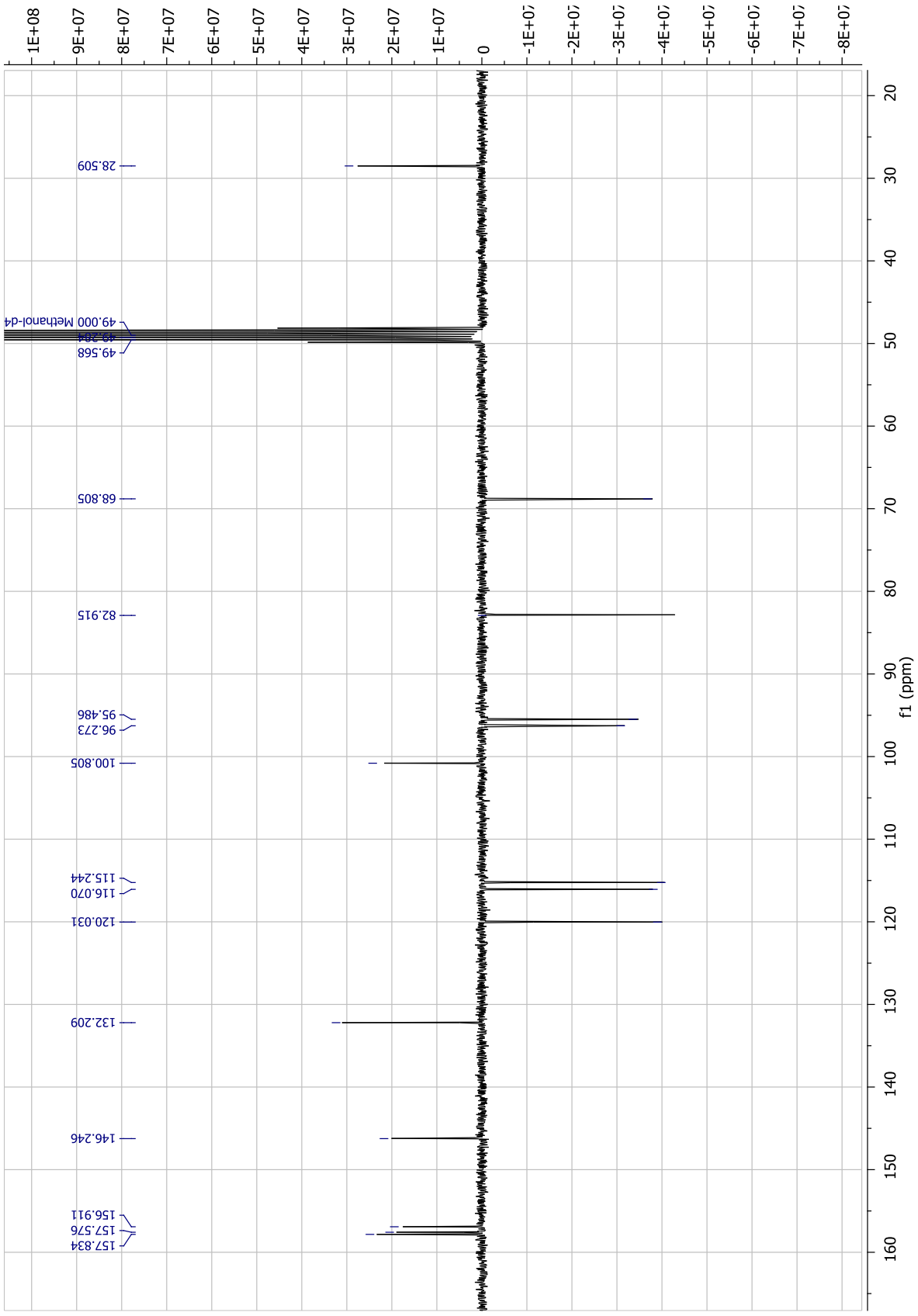
Appendix 23 : ^{13}C jmod NMR of Z_5 (MeOD, 75 MHz)



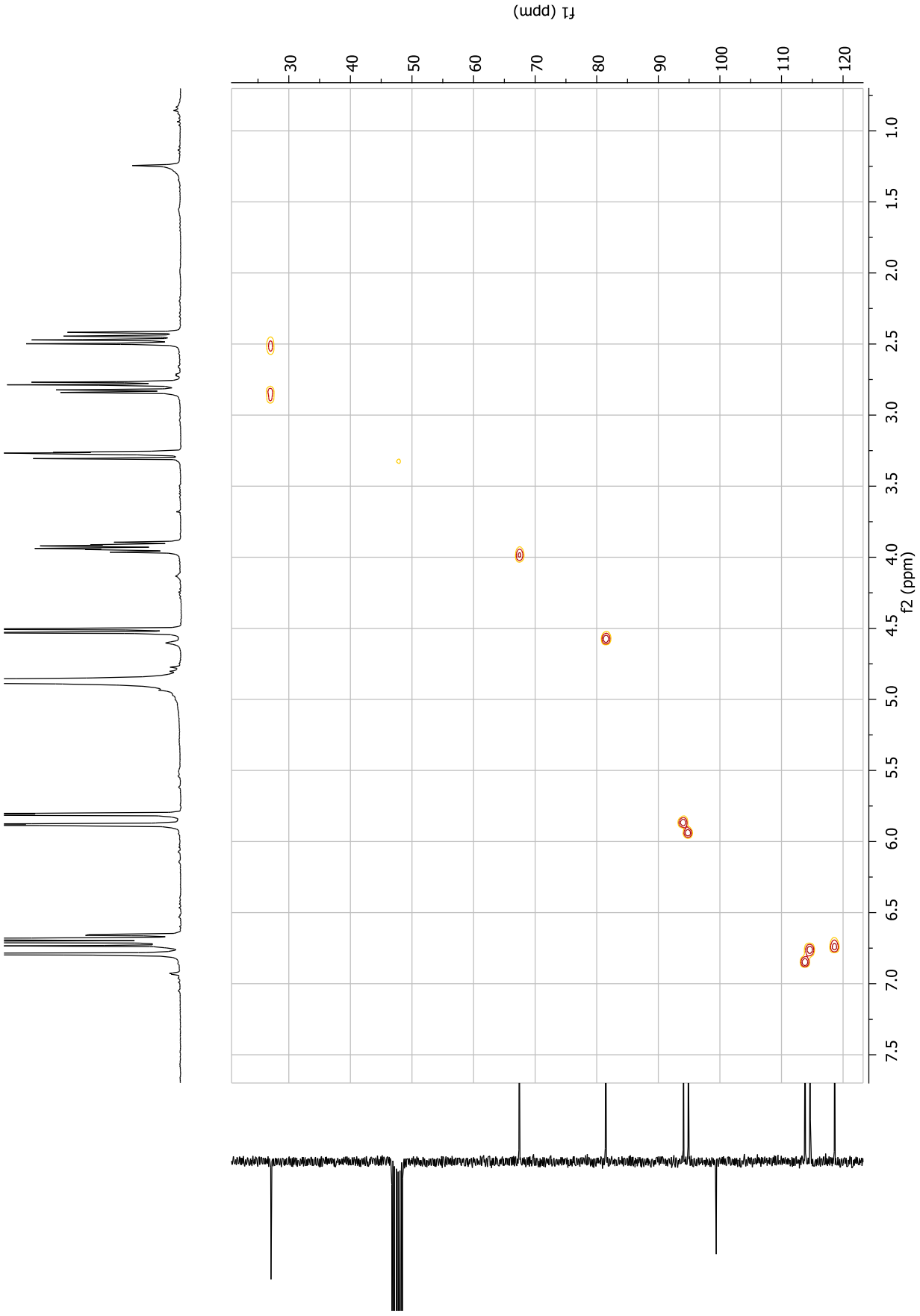
Appendix 24 : ^{1}H NMR of H_1 (MeOD, 300 MHz)



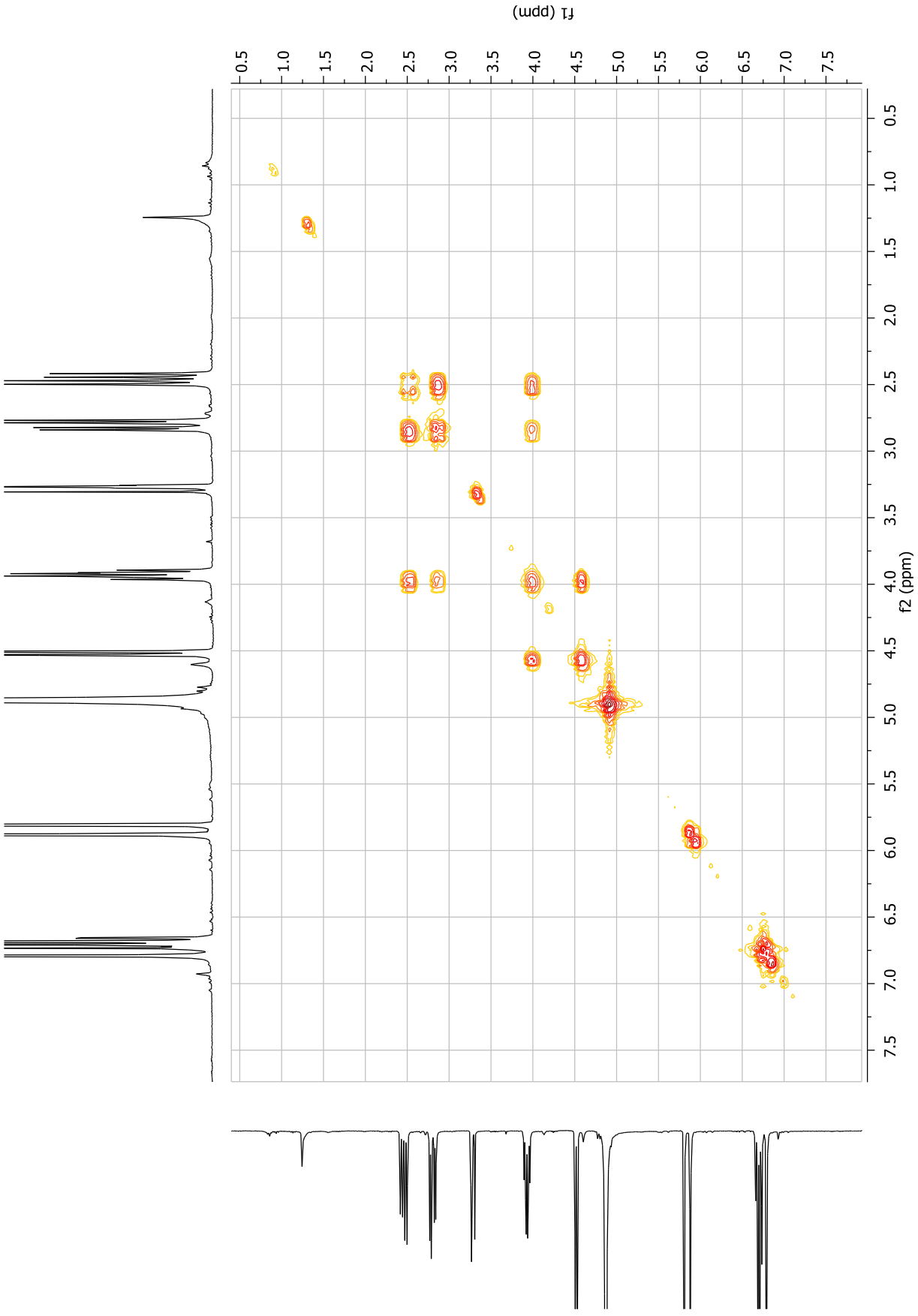
Appendix 25 : ^{13}C jmod NMR of H_1 (MeOD, 75 MHz)



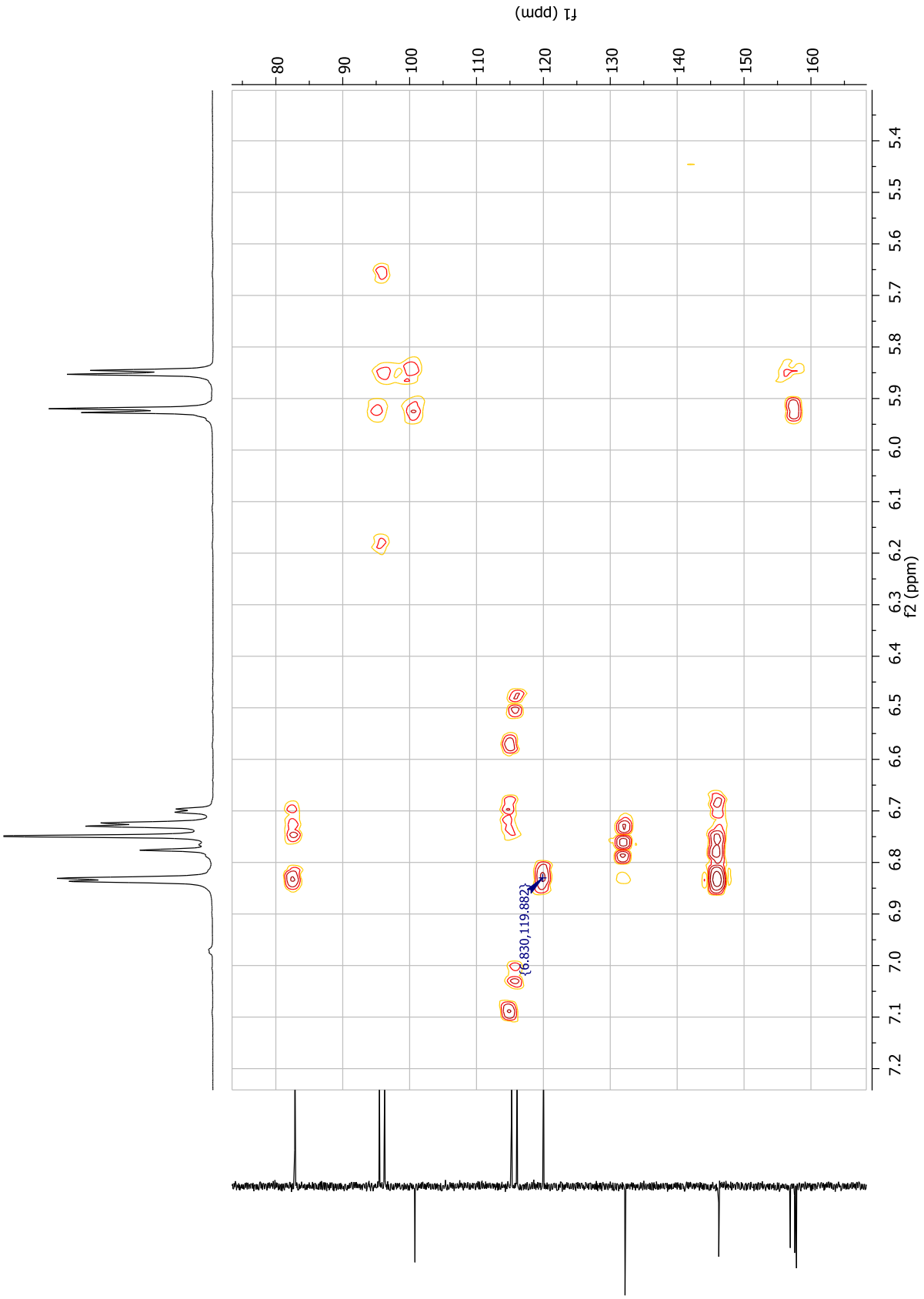
Appendix 26 : HSQC spectra of H_1 (MeOD)



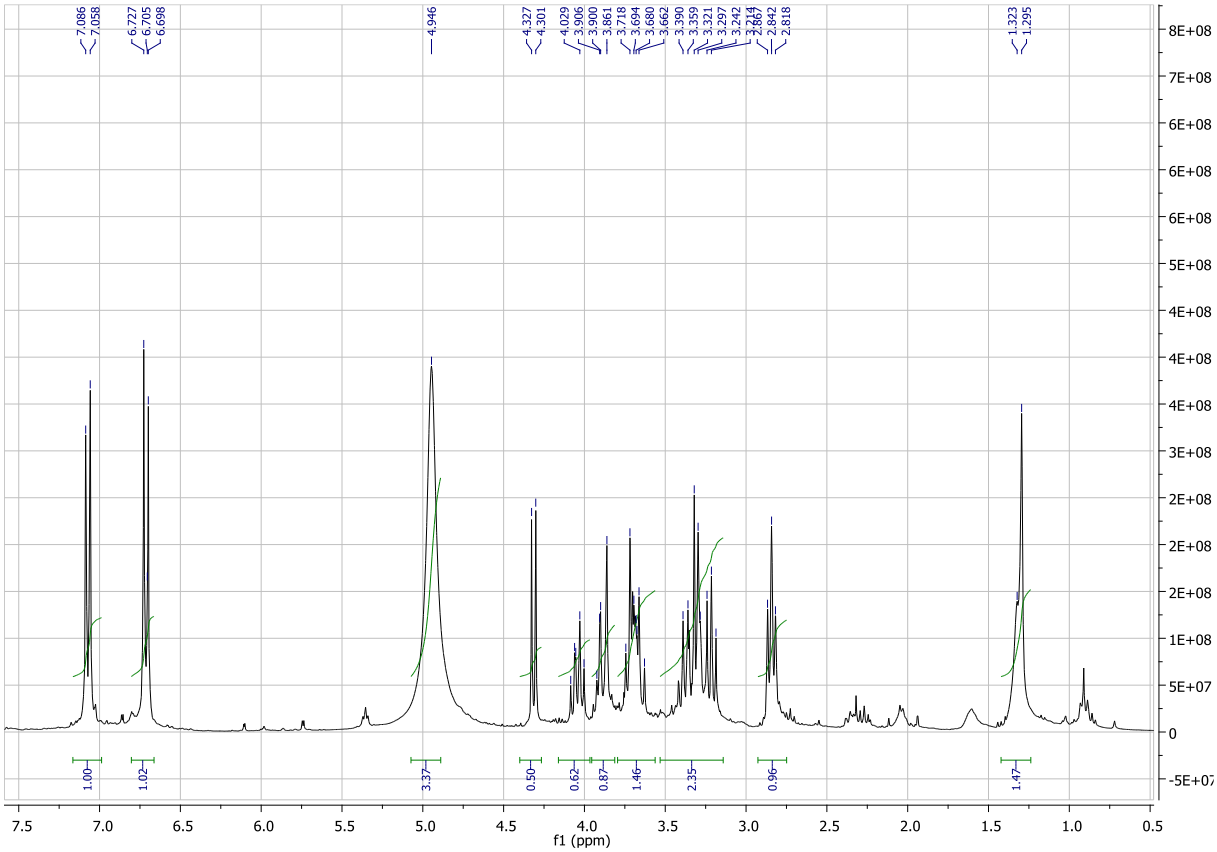
Appendix 27 : COSY spectra of H_1 (MeOD)



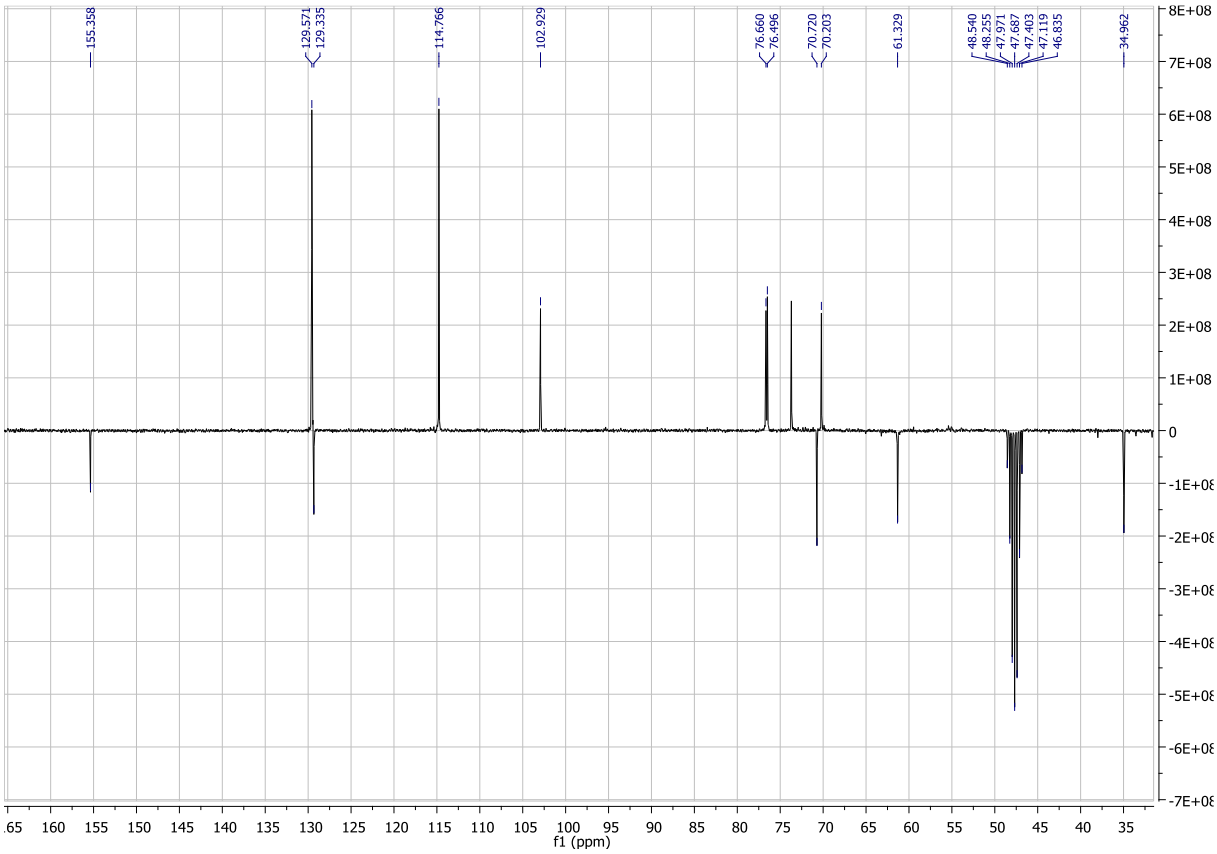
Appendix 28 : HMBC spectra of H_1 (MeOD)



Appendix 29 : ^{1}H NMR of H_2 (MeOD, 300 MHz)



Appendix 30 : ^{13}C NMR of H_2 (MeOD, 75 MHz)



Résumé

Ce travail de thèse est une contribution à l'étude phytochimique par Chromatographie de Partage Centrifuge (CPC), de trois plantes utilisées en médecine traditionnelle au Soudan : *Aristolochia bracteolata* (plante entière), *Ziziphus spina-christi* (feuilles) et *Hydnora abyssinica* (rhizomes). Ce travail a permis de mettre au point trois méthodologies de purification par CPC, applicables au fractionnement des acides aristolochiques, des flavonoïdes ou des proanthocyanidols (PAC). Dans ce contexte, la première partie de ce manuscrit est consacrée aux notions générales portant sur la CPC.

La deuxième partie porte sur l'étude d'*Aristolochia bracteolata*. Cette plante est utilisée en médecine traditionnelle, malgré la présence d'acides aristolochiques qui confèrent une néphrotoxicité élevée. Ce travail a permis de mettre au point une méthode innovante pour l'isolement et la purification, avec un très haut niveau de pureté, des acides aristolochiques I, II et IIIa à partir d'un extrait brut, en une étape par CPC en mode d'échange d'ions forts (SIX-CPC). L'acide aristolochique IIIa n'avait jamais été décrit dans cette plante auparavant. Ces résultats ont fait l'objet d'une publication en 2015 dans *Separation and Purification Technology*.

Dans la troisième partie de cette thèse, la CPC a été appliquée à l'isolement de flavonosides présents dans *Z. spina-christi*. Nous appuyant sur l'expérience du laboratoire dans l'extraction par CPC des flavonosides du *Ginkgo biloba*, nous proposons une méthodologie de purification utilisant les systèmes de solvant biphasiques EtOAc/*n*-BuOH/MeOH/H_2O et EtOAc/*n*-BuOH/H_2O à différents ratios en fonction de la polarité des flavonosides.

Dans la dernière partie, l'étude phytochimique de *Hydnora abyssinica* a mis en évidence la présence de PACs, polymères de hauts poids moléculaires de flavanols. La méthodologie de fractionnement CPC, précédée d'un pré-fractionnement sur résine LH-20, a permis l'isolement pour la première fois dans cette plante de la katsumadine et du rhodioloside.

Mots clés: *Aristolochia bracteolata* Lam, *Ziziphus spina-christi* (L.) Desf, *Hydnora abyssinica* A. Br., Chromatographie de partage centrifuge, acides aristolochiques, flavonosides, proanthocyanidols.

Abstract

This work was a contribution to the phytochemical study of three Sudanese medicinal plants: *Aristolochia bracteolata* (Whole plant), *Ziziphus spina-christi* (Leaves) and *Hydnora abyssinica* (Rhizomes). The specificity of this research program was to emphasize the application of Centrifugal Partition Chromatography (CPC) for the fractionation of these plants. Three specific CPC methodologies were developed for the purification of either aristolochic acids, flavonoids or proanthocyanidins (PACs). In this context, the first part of this manuscript was devoted to the presentation of the CPC methodology.

The second part focused on the fractionation of crude extract of *Aristolochia bracteolata*. This plant is used in traditional medicine, in spite of the presence of aristolochic acids that confer a high nephrotoxicity. In this work was developed an innovating procedure for the isolation and purification in high purity of aristolochic acids I, II and IIIa, in one step from crude extract, using Strong Ions eXchange CPC (SIX-CPC). These results were published in 2015 in *Separation and Purification Technology*.

In the third part, the flavonosides present in *Z. spina-christi* were isolated using CPC, either in normal or reverse elution mode, using two phases solvent systems EtOAc/*n*-BuOH/MeOH/H_2O or EtOAc/*n*-BuOH/H_2O with different ratios.

In the last part, the phytochemical study of *Hydnora abyssinica* led to the fractionation of PACs, polymers of high molecular weight of flavanols. The CPC fractionation methodology, preceded by LH-20 resin pre-fractionation, allowed the isolation of katsumadine and rhodioloside.

Keywords: *Aristolochia bracteolata* Lam, *Ziziphus spina-christi* (L.) Desf, *Hydnora abyssinica* A. Br., Centrifugal Partition Chromatography, aristolochic acids, flavonosides, proanthocyanidin.