IOSR Journal Of Pharmacy And Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 17, Issue 4 Ser. II (Jul. –Aug. 2022), PP 20-31
www.Iosrjournals.Org

Neuroprotective Activity of *Limbarda crithmoïdes* L. Phenolic Fractions Obtained by Centrifugal Partition Chromatography

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Abstract:
Background*: Alzheimer's disease is a progressive neurodegenerative disease characterized by abundant protein deposits in neurons that trigger their degeneration. Recently, a large number of phenolic compounds are suspected to have neuroprotective effects. Some reports indicate that these metabolites can prevent the development of Alzheimer's disease, not only by inhibiting ROS, but also by directly inhibiting the formation of fibrillar amyloid peptide deposits in the brain. The present study consists of an exploration of the in Vitro anti-amyloidogenic activity of L. crithmoïds phenolic fractions obtained by centrifugal partition chromatography (CPC) of a crude extract after a solvent fractionation.*
Materials and Methods*: L. crithmoïds was harvested during the flowering stage. Plant material was air dried then subjected to a differential extraction using different polarity solvents. The ethyl acetate crude extract was injected in a CPC apparatus to separate its phenolic compounds. Samples of collected tubes were analyzed by Thin Layer Chromatography (TLC) and HPLC. Those containing the same compounds were gathered resulting in 24 different fractions that were tested for their neuroprotective abilities using the Thioflavin T fluorescence test.*
Results*: CPC procedure permitted an efficient separation of L. crithmoïds phenolics in 24 different fractions: 18 fractions belonging to the ascending mode and 6 ones to the descending mode. Yields of the 24 fractions are variable and depend on phenolic polarities and their affinity to the solvents used in the chromatographic separation. Besides, the results of the anti-amyloidogenic activity testify to the great variability between the fractions expressing very different percentages of inhibition with a maximum of activity exhibited by the fraction 15 capable of inhibiting 39% of the aggregation of the β-peptides amyloids. These results support the interest of using CPC as a technique for separating bioactive molecules.*
Conclusion: *L. crithmoïds flowers are rich in phenolic compounds exhibiting important neuroprotective activities via the inhibition of the accumulation of the β-amyloid peptides forming the senile plaques. Elucidation of the real biological potential of these natural products implies going through a series of separation and purification procedures that enhance the targeted biological effect. CPC is one of the most effective tools in this field.*
Key Word*: Limbarda crithmoïdes; Phenolic compounds; CPC separation; Alzheimer's disease; anti-amyloidogenic activity.*

Date of Submission: 01-08-2022 Date of Acceptance: 13-08-2022

I. Introduction

The plant kingdom constitutes an inexhaustible mine of bioactive molecules. In particular, phenolic compounds have shown excellent multi-use biological properties ranging from food preservation to protecting agents from serious pathologies, such as atherosclerosis, degenerative brain diseases and cancer[1]. To benefit from the effects of these biomolecules, a sequence of steps of extraction, separation/purification and identification must be followed to achieve the characterization of the phenolic compounds of plants. In this context, the use of efficient and adequate techniques to accomplish these steps is a mandatory condition for the valorization of polyphenols[2].

Alzheimer's disease is a progressive neurodegenerative disease characterized by abundant protein deposits in neurons that trigger neuronal degeneration. These deposits result in the intracellular accumulation of the phosphorylated form of tau protein (Pτ) and the extracellular accumulation of *β*-amyloid peptides leading to the formation of neurofibrillary tangles and amyloid plaques, respectively. This promotes pro-inflammatory responses and activates the neurotoxic pathway, leading to brain cell dysfunction and death[3]. Despite recent advances in symptomatic treatment, an effective therapeutic approach that directly interferes with the neurodegenerative process in Alzheimer's disease, in particular the accumulation of *β*-amyloid peptides, is still lacking. Recently, a large number of phenolic compounds are suspected to have neuroprotective effects i*n Vitro* as well as *in Vivo*. Indeed, some studies suggest that polyphenols exert their protective effect via their ability to inhibit reactive oxygen species (ROS). In addition, other reports indicate that these metabolites can prevent the development of Alzheimer's disease, not only by inhibiting ROS, but also by directly inhibiting the formation of fibrillar amyloids peptide deposits in the brain[4,5].

The objectives set for this work are therefore the separation of the phenolic compounds from the extract of the flowers of *L. crithmoïds* by centrifugal partition chromatography, one of the techniques most used in these applications. The second step will consist in carrying out a bio-guided screening, using anti-amyloidogenic activity, of the most interesting fractions.

II. Material And Methods

Plant sampling

Flowers of *L. crithmoïdes* were sampled by the end of September 2021 from Kairouan region (N35°48′53; E10°09′07) in Tunisia, belonging to the Upper-Arid bioclimatic stage (mean annual rainfall >300 mm)[6]. The harvested plants were identified at the Biotechnology Center at the Technopark of Borj-Cédria, and a voucher specimen [PLM78] was deposited at the Herbarium of the Laboratory.

Extraction of phenolic compounds

Aerial part powder was subjected to a differential extraction using diverse polarity solvents (petroleum ether, ethyl acetate, acetone mixed with water) as described by Jallali et al.[7]. A solid/liquid extraction of 100 g of sample powder by 60% aqueous acetone (v/v) (2 x 200 mL) was carried out. Mixture was kept in frequent agitation at ambient temperature for two hours then filtered through a Whatman N°4 filter paper. After filtration, the aqueous acetone extracts were combined and concentrated at 35 °C under reduced pressure. The residual aqueous phase (100 mL) was extracted by petroleum ether (3 x 100 mL) for two hours to eliminate pigments and waxes then subjected to a liquid/liquid extraction by ethyl acetate (4 x 100mL). Mixture was transferred in a funnel and left to stand until two phases were observed. Aqueous phase was discarded and the organic phase was collected and evaporated under vacuum at 35 °C to dryness then dissolved in water to be freeze-dried. The ethyl acetate phase yielded 10.55 g (1.06% of the primary used plant material). Dry residue was stored in the darkness at 4 °C until analysis.

Fractionation of *L. crithmoïdes* flower extract by CPC

Separation procedure was carried out using an FCPC200® apparatus provided by Kromaton Technologies (Angers, France) that is equipped with a rotor made of 20 circular partition disks enclosing 1320 partition cells (0.130 mL per cell); with a total column capacity of 204 mL. The dead volume is about 75 mL. Rotation speed is adjustable in a range of 0-2000 rpm. The resulting centrifugal force field in the partition cell is nearly 1200 ms^{-2} at 1100 rpm and 4200 ms^{-2} at 2000 rpm. The CPC is equipped with a Gilson 321-H1 2-way binary high-pressure gradient pump and a high pressure injection valve (3725(i)038 Rheodyne) equipped with a 10 mL sample loop. The effluent was monitored with an ICS UV-Lambda 1010 detector equipped with a preparative flow cell. Fractions were collected by a Gilson FC 204 fraction collector. The experiments were conducted at room temperature. The quaternary biphasic solvent systems were prepared at room temperature by n-heptane(n-Hept), ethyl acetate (EtOAc), methanol (MeOH), and water in the convenient proportions for systems J (2:5:2:5; v/v/v/v) of Arizona- Margraff systems[8]. The resulting two phases were separated just before use. As a first step, aqueous phase (methanol/water) was used as a stationary phase to fill up the rotor in the ascending mode without rotating. After that, 1 g of dry matter was dissolved in 10 mL of the organic/aqueous phase mixture (1:1) then injected in the apparatus. The pumping of the organic mobile phase into the column was then allowed. The flow-rate was 10 mL min^{-1}, and rotation speed was fixed at 300 rpm. Once the column was filled with the mobile phase, the rotation speed was increased from 300 to 1200 rpm and the flow-rate was fixed at 9 mL min^{-1}. The pressure was maintained at 23 bars along the experience. Fractions of 9 mL were collected every minute. The content of the outgoing organic phase was monitored by online UV absorbance measurement at k = 280 nm.

Thin layer chromatography (TLC) analysis

One of the chromatographic tools used in the analysis of *L. crithmoïdes* flower fractions is TLC. Ten mL of each fraction were deposed on pre-coated silica gel 60 F254 plates (Merck). Plates were then partially immersed in a migration solution consisted of $CHCl_3$–methanol–acetic acid (80/20/3) and left for 15 min to be developed. Revelation of the separated bands was achieved by anisaldehyde sulphuric reagent (5 mL *p*-anisaldehyde, 90 mL ethanol and 5 mL sulphuric acid) permitting their detection at 254 and 366 nm.

HPLC analysis

HPLC analysis was performed on Agilent apparatus equipped with an autosampler model 1100, a Prostar Pump model 1100, diode array detector model 1100, A C18 column (Prontosil, 250 mm x 4.0 mm, 5 µm, Bischoff) was used for analysis. The mobile phase was composed of two solvents: A, 0.025% TFA in H_2O and B, acetonitrile (MeCN). The sample was dissolved in MeOH/H_2O (1:1) and filtered through a 0.45 µm Millipore filter. The elution program at 1 mL/min was as follows: 10% B (0–5 min), 10–100% B (5–55 min), 100–10% B (55–60 min), 10% B (60–65 min), 10 mL of each sample was directly injected and chromatograms were monitored at 280 nm.

Thioflavin T fluorescence spectroscopy

The inhibitory activity of *L. crithmoïdes* fractions on Aβ fibril formation was quantified with a ThT assay using a modification of the method described previously by Naiki et al.[9]. Before experiments, $A\beta_{25–35}$ were sonicated for 10 min to disrupt all aggregates. The peptide stock solution in water (1 mM) was diluted to a concentration of 100 µM into 10 µM ThT, 2 mM phosphate buffer (pH 7.2) and 10 µL MeOH with or without 10 µM curcumin or different fraction extracts (10 mg/L). A blank containing ThT, phosphate buffer, MeOH and H_2O in the same conditions of all samples was sub-tracted. The absence of fluorescence of fraction and curcumin alone was also verified. ThT fluorescence intensity (excitation and emission wavelength of 450 nm and 485 nm, respectively) was assessed under time-resolved fluorescence mode (90 min) at 25°C on a BMG-Labtech spectrofluorimeter. The activity was estimated using the following formula:

Anti-amyloidogenic activity (%) = [(A0− A1)/A0] × 100

where A0 is the absorbance of the control and A1 is the absorbance of the sample.

Statistical analysis

Means were statistically compared using the STATISTICA program. A one-way analysis of variance (ANOVA) and Duncan's multiple range tests were carried out to perceive any significant differences between parameters at $P< 0.05$.

III. Result

Pre-purification of *L. crithmoïdes* flower extract

Chromatographic separation of *L. crithmoïds* flower phenolics by CPC apparatus yielded 131 fractions. Tubes collected were analyzed by TLC to compare their composition. Those containing the same compounds (spots) were combined and so their number was reduced to 24 fractions: 18 fractions from the ascending mode and 6 fractions from the descending one. Obtained fractions were evaporated to dryness then analyzed by TLC (Figure no 1) and HPLC (Figure no 2) to verify compound segregation. Gathered tubes forming the fractions and their corresponding yields are listed in Table no 1.

As it can be seen, the most important part of *L. crithmoïds* phenolics was eluted in the ascendant mode indicating that its composition is dominated by low to medium weight phenolic compounds, probably belonging to phenolic acids and flavonoids. Polar ones are rather eluted in the descending mode and may be constituted by glycosilated phenols or polymerized tannins. Fraction yields are so fluctuant reflecting a very important qualitative and quantitative variability of this composition as confirmed by the chromatographic results (Figure no 1 and 2). The most important yield was recorded in fraction 11 but when verifying the TLC and HPLC results, we can deduce that fractions 10 to 14 are dominated by one compound. In fact, a previous study reported that we purified this compound and its structure was characterized by RMN to be identified as 1,5-di-*O*-caffeoyl quinic acid, a chlorogenic derivative largely reputed in the *Asteraceae* family; as it was identified to be the major phenolic compound of *L. crithmoïds* flowers[7]. This fact justifies the important yields of fractions 10 to 14, especially fraction 11.

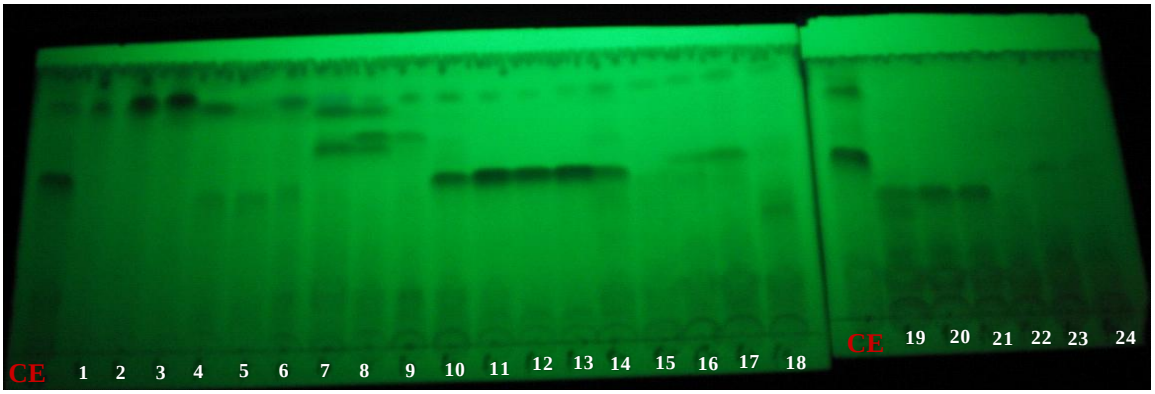
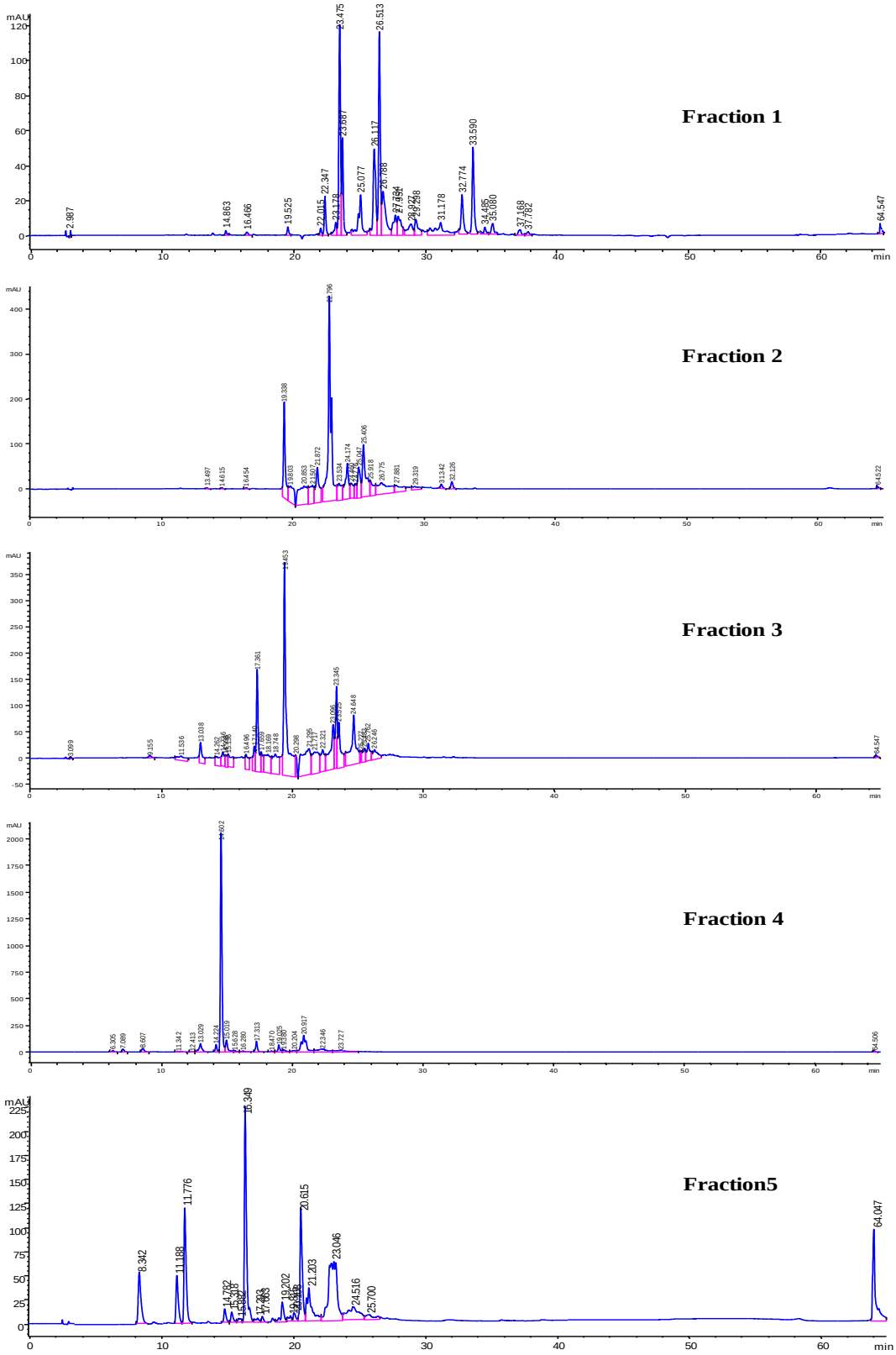
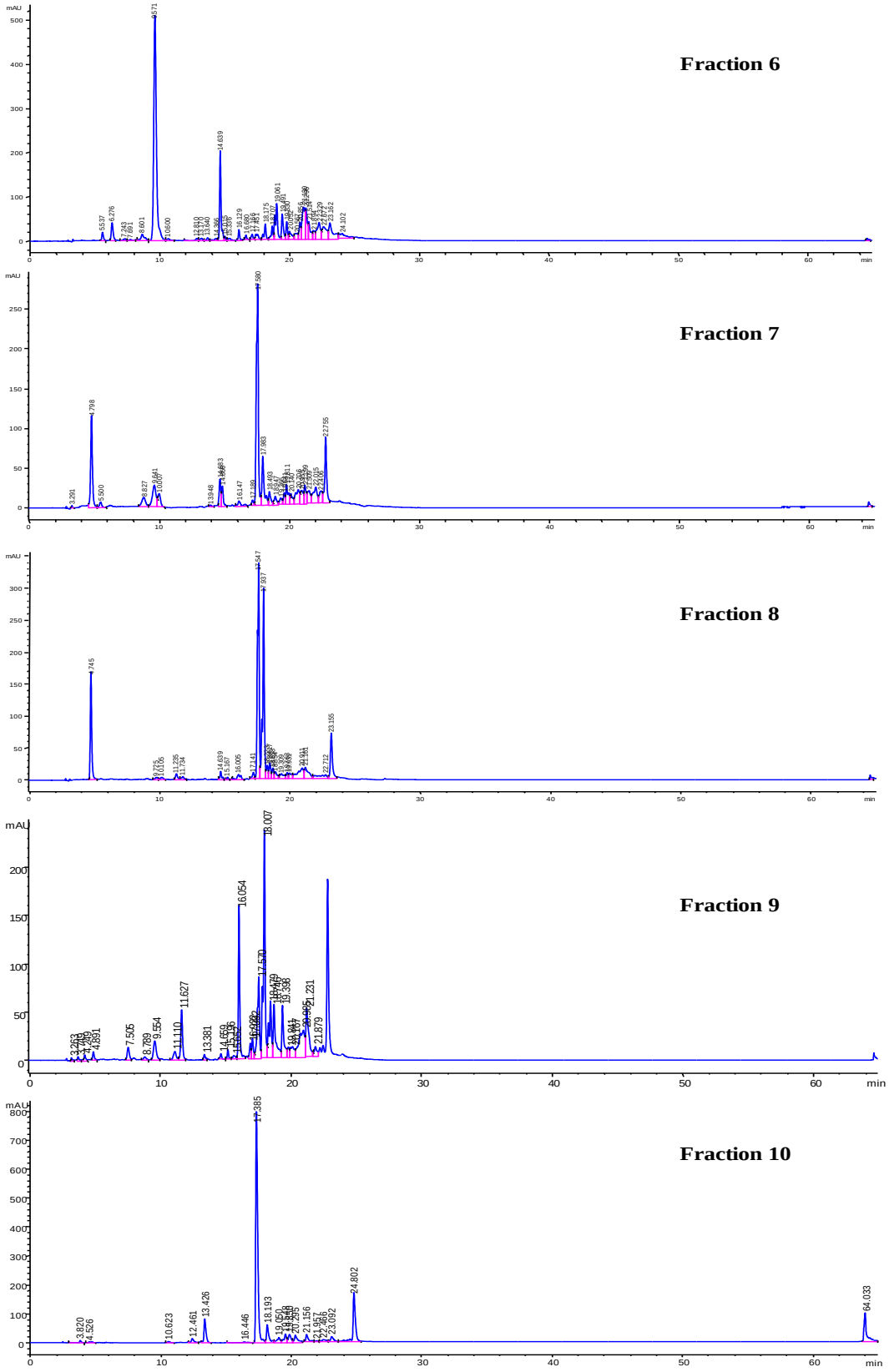


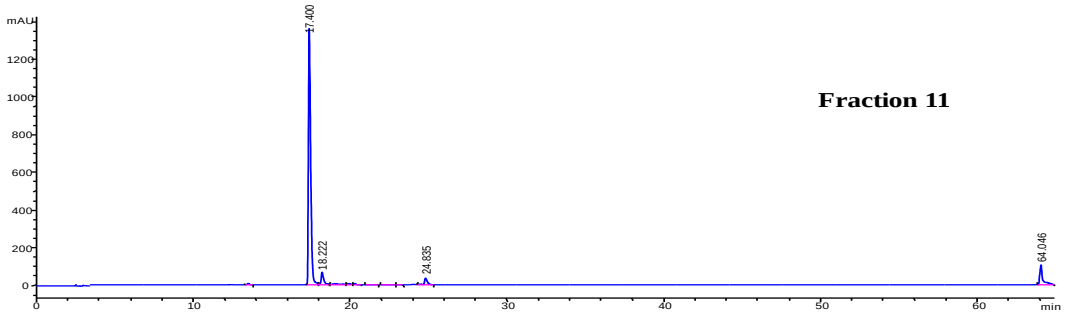
Figure no 1: Thin Layer Chromatography (TLC) of *L. crithmoïdes* crude extract (CE) and derived fractions obtained by Centrifugal Partition Chromatography (CPC).

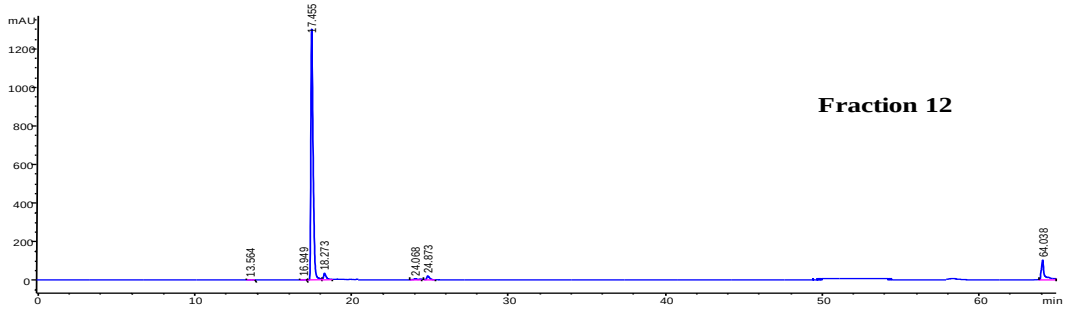
Table no 1: *L. crithmoïdes* fraction collections and their corresponding yields. Means (three replicates) followed by the same letter in the same column are not significantly different at $P < 0.05$.

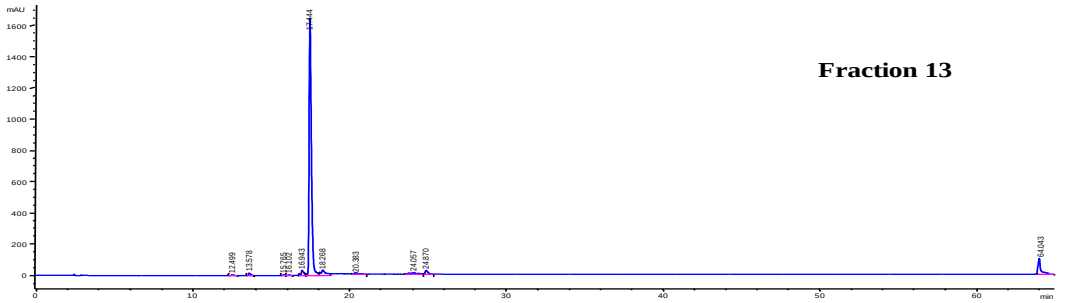
	Fraction N°	Fraction yield (mg)
Ascending mode	F1 (8)	28.1 gh
	F2 (9)	40.1 d
	F3 (10-12)	62.6 c
	F4 (13-17)	74.2 b
	F5 (18)	4.6 q
	F6 (19-22)	17.6 k
	F7 (23-25)	7.6 o
	F8 (26-32)	26.1 h
	F9 (33-34)	5.5 p
	F10 (35-39)	32.5 f
	F11 (40-45)	81.5 a
	F12 (46-47)	30.9 g
	F13 (48-50)	38.3 e
	F14 (51-58)	29.6 g
	F15 (59-70)	19.4 j
	F16 (71-83)	9.5 mn
	F17 (84-98)	8.9 n
	F18 (99-125)	11.5 l
Descending mode	F19 (126)	8.4 n
	F20 (127)	8.5 n
	F21 (128)	10.1 m
	F22 (129)	9.8 m
	F23 (130)	20.6 j
	F24 (131)	24.4 i

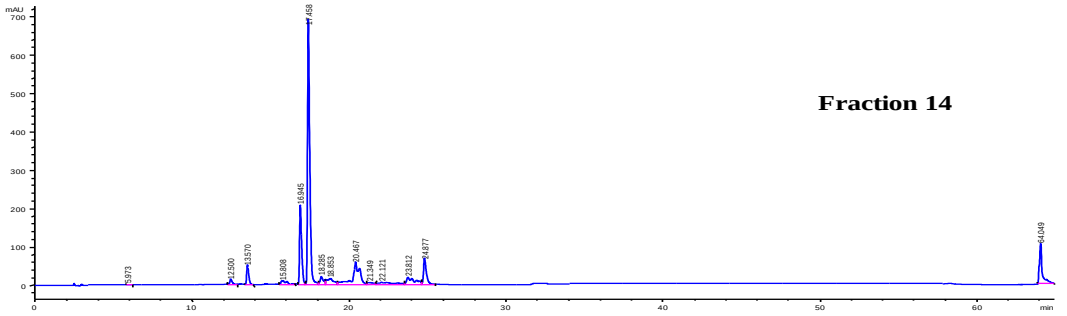

Fraction 1
Fraction 2
Fraction 3
Fraction 4
Fraction5
mAU
min

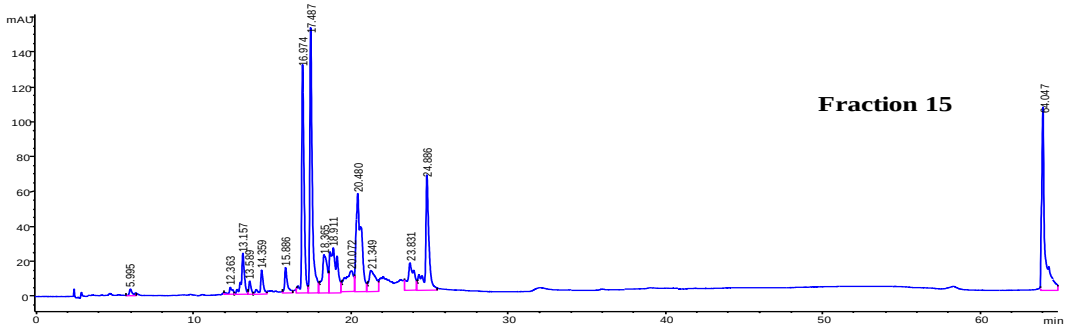

Fraction 6
Fraction 7
Fraction 8
Fraction 9
Fraction 10
mAU
min

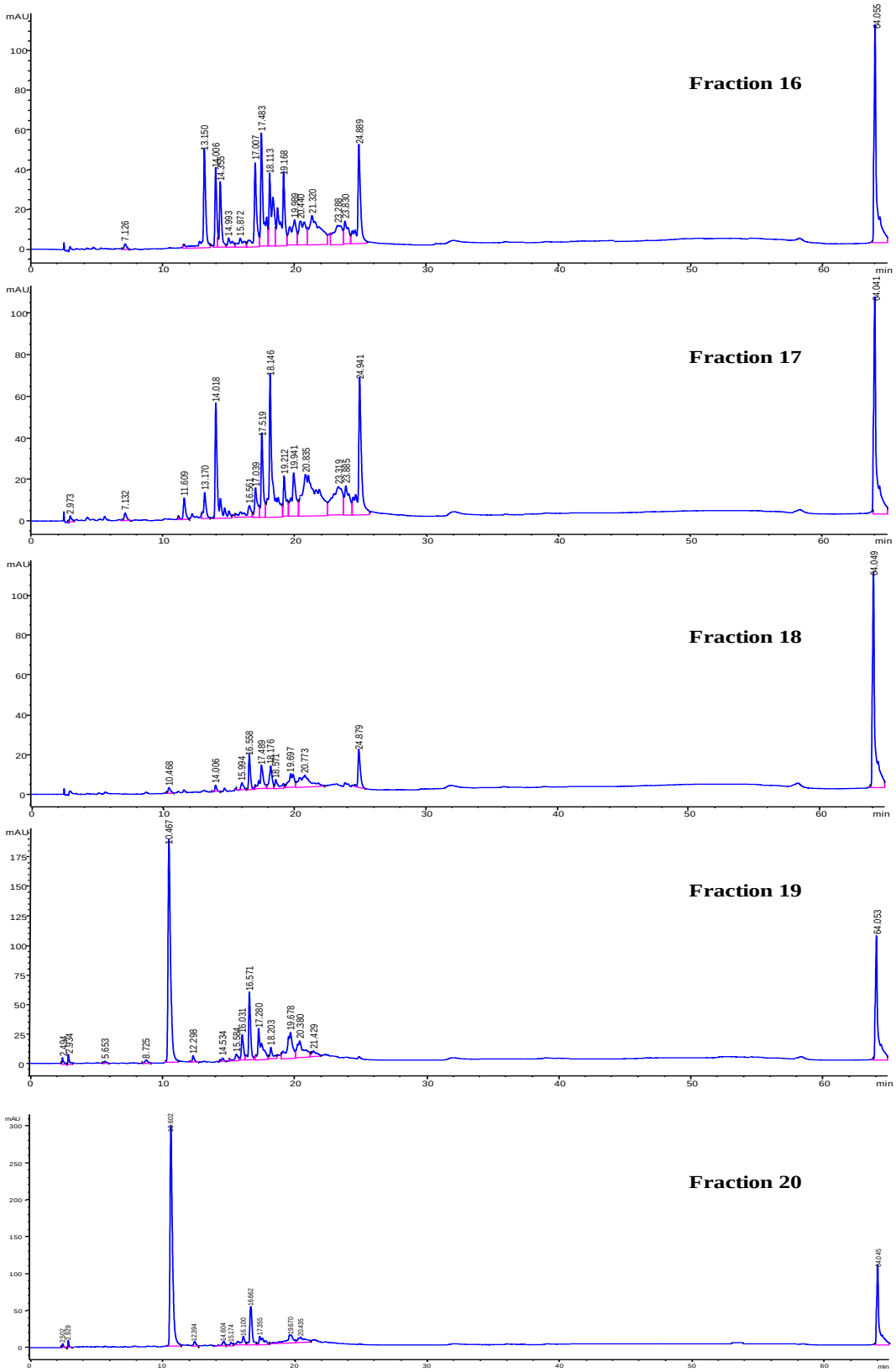

mAU
17.400
18.222
24.835
64.046
min
Fraction 11


mAU
17.455
13.564
16.949
18.273
24.068
24.873
64.038
min
Fraction 12


mAU
17.444
64.043
min
Fraction 13


mAU
17.458
16.945
64.049
min
Fraction 14


mAU
17.487
16.974
20.480
24.886
64.047
min
Fraction 15


Fraction 16
Fraction 17
Fraction 18
Fraction 19
Fraction 20
mAU
min

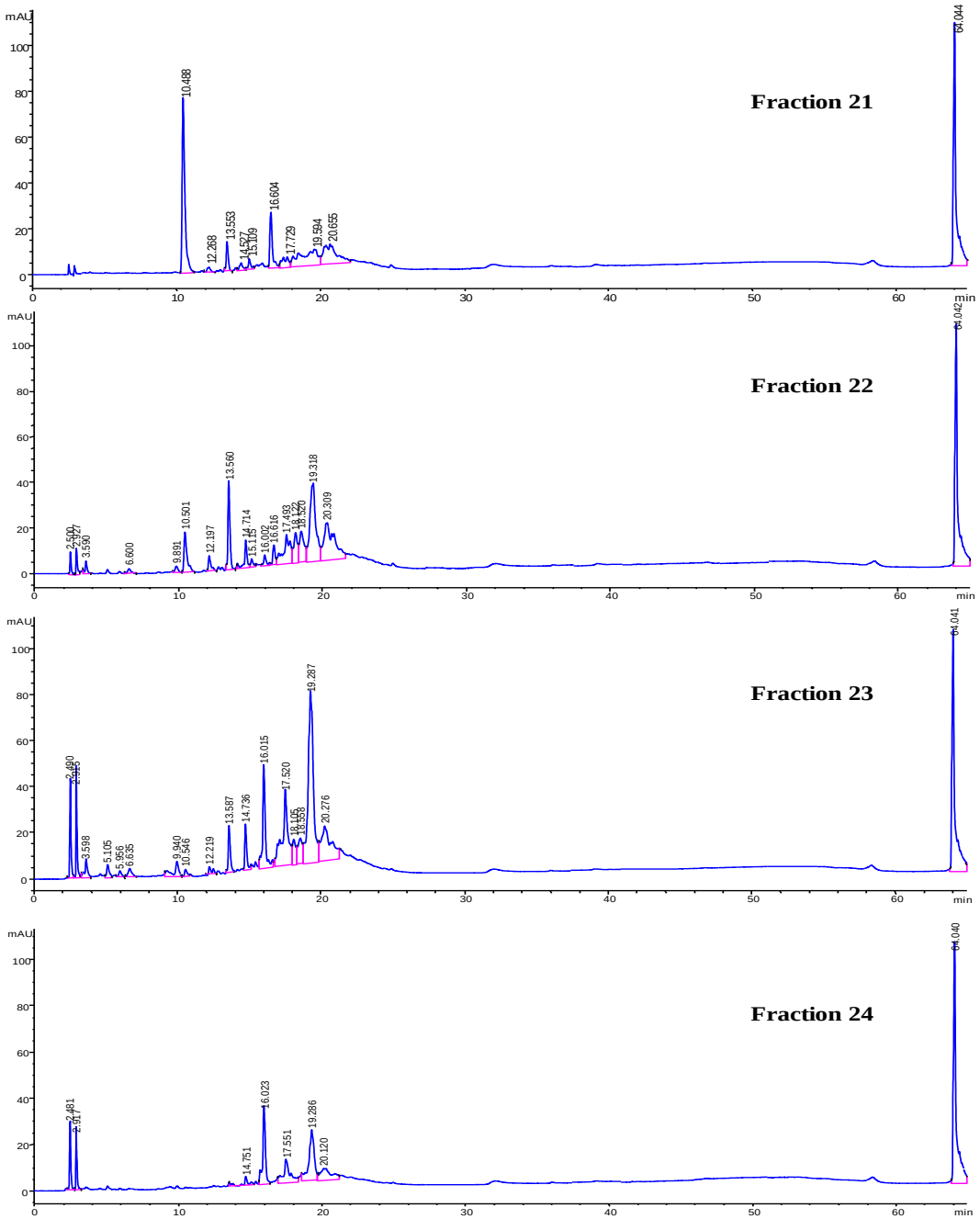


Figure no 2: HPLC chromatograms of the 24 fraction obtained by Centrifugal Partition Chromatography (CPC) from *L. crithmoïdes* flower extract and monitored at 280 nm.

The inhibitory activity of *L. crithmoïdes* fractions on Aβ fibril formation

The fractions from the flowers of *L. crithmoïds* have been explored for their anti-amyloidogenic potency. The results illustrated in Figure no 3 testify to the significant variability of this activity across the 24 fractions with percentages of inhibition ranging from 0 to 39%. Fraction 15 expresses the strongest activity, being almost two-thirds that of curcumin used as positive control. Fraction 2 also shows quite significant activity (32% of inhibition), both of which belong to the ascending mode. However, fractions 19 and 20 of the descending mode are endowed with a certain anti-amyloidogenic power since they manage to inhibit the aggregation of *β*-amyloid peptides at 26.5 and 28%, respectively.

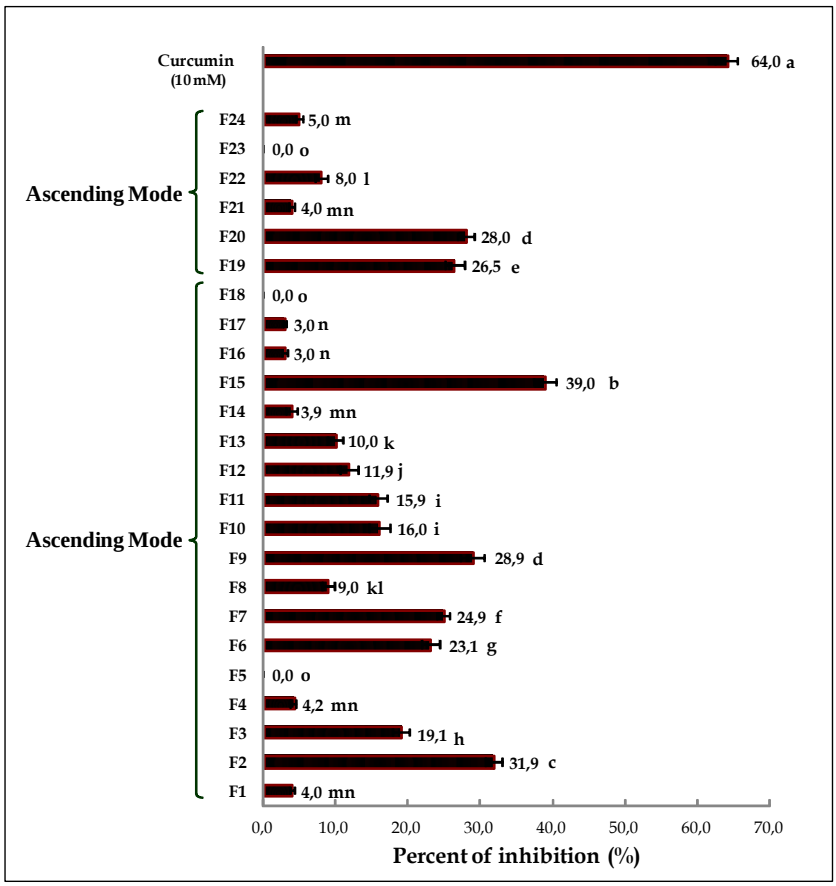


Figure no 3: Anti-amyloidogenic activity of *L. crithmoïdes* fractions (tested at a concentration of 10 mg.L^{-1}) and positive control (Curcumin) expressed as % of inhibition of $A\beta_{25-35}$ aggregation. Means (three replicates) followed by the same letter are not significantly different at $P < 0.05$.

IV. Discussion

For the present work we have chosen to go through a differential extraction using several types of solvents with different polarities and, consequently, with different affinities to extract the maximum of phenolic compounds from the flowers of *L. crithmoïds*. Indeed, the different classes of these molecules express a marked dependence on the solvent used. Several studies have described alcohol/water mixtures as the best solvents for the extraction of the majority of classes of phenolic compounds[10,11]. Thus a liquid solid extraction of the dry matter of the flowers by acetone at 60% was carried out. Subsequently, the extract obtained was "cleaned" with petroleum ether. In fact, previous research has indicated the use of this solvent to rid the crude extract of pigments and lipids[12,13]. The extract thus depigmented and defatted has undergone a liquid/liquid extraction by a solvent of intermediate polarity, which is ethyl acetate, towards which the majority of the polyphenols will migrate. Indeed, several other works have shown that the ethyl acetate fractions of several plants enjoy the most significant antioxidant activities, compared to other fractions, closely correlated with their spectacular richness in phenolic compounds, mainly flavonoids[7,13,14]. Our previous work confirmed the usefulness of this differential separation which made it possible to obtain extracts enriched in polyphenols, especially *L. crithmoïds* flower extract whose total polyphenol contents were about 100.4 mg EAG.g^{-1} DM[7]. The next step consisted in phenolic separation for better evaluation of their bioactivity. In this context, the use of CPC for the purification of natural products has attracted more attention during the last two decades. The CPC process differs from solid-support chromatographic methods, such as HPLC, in several criteria: high selectivity can be achieved by designing the right solvent system, the amount of solvent can be significantly reduced, a single method of separation/purification is suitable for samples ranging from mg to several kg, but it is above all the absence of solid support that gives CPC its effectiveness as a tool for the separation and purification of biological molecules[15,16]. TLC (Figure no 1) and HPLC (data not shown) chromatograms of *L. crithmoïds* flower extract confirm the efficient separation of phenolic compounds by this method. The different 24 fractions thus obtained were subjected to a bio-guided screening of the most active ones, based on the neuroprotective activity. In fact, the aggregation of amyloid peptides forming senile plaques is one of the two types of lesion of the cerebral

cortex highlighted in Alzheimer's disease, the other being neurofibrillary degeneration. One of the approaches to combating this incurable neurodegenerative disease of the brain tissue is the prevention of the formation of these senile plaques, which implies the need to inhibit the aggregation of *β*-amyloid peptides. In fact, pathological and biochemical studies suggest that fibrils are generators of ROS, while soluble monomeric forms of amyloid peptides act as natural antioxidants that prevent neuronal cell death due to oxidative stress[5,17]. Polyphenols are able to inhibit the formation of fibrils in vitro by forming a complex with *β*-amyloid peptides. This mechanism, unrelated to oxidative conditions, is highly relevant for the design of therapeutic inhibitors for the treatment of Alzheimer's disease in the future[5]. In this context, we tried to investigate the ability of polyphenols of *L. crithmoïds* to inhibit the aggregation of *β*-amyloid peptides.

The results displayed showed the variability of the effectiveness of the 24 fractions. Indeed, the percentages of inhibition expressed varied between 0 and 39%, with a maximum of activity exhibited by fraction 15, followed by fraction 2 (32%). This activity is quite appreciable if we compare it to pure products such as curcumin used as a positive control (64%), the stilbenoids *E*-resveratrol (63%) and (+)-ampelopsin A (46%) tested at the concentration of 10 µM[16], or by comparison with soy isoflavones (genistein and equol) having an ability to inhibit the formation of fibrils equal to 36 and 31% respectively (at a concentration of 50 µM)[18]. Apart from fractions 15 and 2, fraction 9 from the ascending mode and fractions 19 and 20 from the descending mode expressed significant activities too. In General, fractions from the first mode of separation are more active than the second one, as shown by the statistical analysis.

Indeed, it should be kept in mind that the percentages of inhibition expressed by the fractions of *L. crithmoïds* do not reflect the actual anti-amyloïdogenic potential of the molecules included. Fraction 15, for example, expressing the most interesting activity, presents an encumbered chromatographic profile (figure no 3). The effectiveness of a compound is then diluted in this complex mixture, as it can be negatively influenced by different modalities of antagonistic interactions with the other compounds of the fraction. It is therefore necessary to consider all these factors to better enhance the anti-amyloidogenic capacities of *L. crithmoïds* which, even with these fluctuant inhibition percentages expressed by its fractions, can constitute, with more thoroughly investigations, an important source of neuroprotective bioactive molecules.

V. Conclusion

The results of this investigation have identified several points of interest concerning the phytochemical and biological characterization of plant phenolic compounds. CPC is a relevant technique for the separation of these metabolites, allowing their concentration in precise fractions according to their structures and their modes of interaction, in close relation to their biological properties. The results testify to the important neuroprotective effects of some obtained fractions of *L. crithmoïdes* and confirm the usefulness of the pre-purification of crude extracts by CPC as well as the need for investigation of the multiple biological effects of the same fraction by several tests, such as antioxidant and anti-inflammatory activities, to better specify the fractions of interest with the aim to purify active phenolic compounds and their formulation by the pharmaceutical industry.

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