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Research Paper

Characterization of *Pterocarpus erinaceus* kino extract and its gamma-secretase inhibitory properties

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ABSTRACT

Ethnopharmacological relevance: The aqueous decoction of *Pterocarpus erinaceus* has been traditionally used in Benin against memory troubles.
Aim of the study: New strategies are needed against Alzheimer's disease (AD), for, to date, AD treatment is symptomatic and consists in drugs treating the cognitive decline. An interesting target is the β-amyloid peptide (Aβ), whose accumulation and progressive deposition into amyloid plaques are key events in AD aetiology.

Identifying new and more selective γ-secretase inhibitors or modulators (none of the existing has proven so far to be selective or fully efficient) appears in this respect of particular interest. We studied the activity and mechanisms of action of *Pterocarpus erinaceus* kino aqueous extract, after the removal of catechic tannins (KAST).
Methods and results: We tested KAST at non-toxic concentrations on cells expressing the human Amyloid Precursor Protein (APP695), as well as on primary neurons. *Pterocarpus erinaceus* extract was found to inhibit Aβ release in both models. We further showed that KAST inhibited γ-secretase activity in cell-free and *in vitro* assays, strongly suggesting that KAST is a natural γ-secretase inhibitor. Importantly, this extract did not inhibit the cleavage of Notch, another γ-secretase substrate responsible for major detrimental side effects observed with γ-secretase inhibitors. Epicatechin was further identified in KAST by HPLC-MS.
Conclusion: Pterocarpus erinaceus kino extract appears therefore as a new γ-secretase inhibitor selective towards APP processing.

1. Introduction

One of Alzheimer's dementia (AD) major characteristics is the accumulation, in the brain, of β-amyloid peptide (Aβ). This peptide triggers neuronal damage in several ways, e.g. by aggregating into senile plaques causing oxidative stress, synapse disorders and neuritic distrophy, by forming toxic soluble oligomers and/or by intraneuronal accumulation leading to neuronal apoptosis (Kienlen-Campard et al., 2002; Walsh and Selkoe, 2004; Lesné et al., 2006).

Aβ is released by the sequential cleavage of its precursor (APP) by two secretases, namely the β- and γ-secretases (Selkoe and Wolfe, 2007). The cleavage of APP ectodomain by β-secretase generates the soluble β APP (sβAPP) and a membrane-anchored C-terminal fragment β (βCTF or C99), which is further processed by γ-secretase to release Aβ. APP ectodomain can alternatively be cleaved by α-secretase within the Aβ sequence to generate soluble α APP (sαAPP) and the C-terminal fragment α (αCTF or C83), which is also a substrate of the γ-secretase. In both pathways,

Abbreviations: Aβ, β-amyloid peptide; AD, Alzheimer's disease; APP, Amyloid Precursor Protein; BACE, β-site APP cleaving enzyme; BSA, bovine serum albumin; *βIIITub*, Beta III Tubulin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHO, Chinese hamster ovary; CTF, C-terminal fragment; DAPI, 4′,6-Diamidino-2-phenylindole; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester; ECL, Enzymatic Chemi-Luminescence; ECLIA, Electro-Chemiluminescence Immuno-Assay; EDTA, ethylene-diamine-tetra-acetic acid; EGTA, ethylene-glycol-tetraacetic acid; ESI, electro-spray ionisation; FBS, fœtal bovine serum; GSK-3, glycogen-synthase-kinase 3; KAST, kino aqueous extract after tannin removal (*kino aqueux sans tanins*); MAP2, microtubule-associated protein 2; MEF, mouse embryonic fibroblast; MOPS, 3-(*N*-morpholino)-propane-sulfonic acid; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide *or* thiazol blue tetrazolium bromide; PBS, phosphate buffered saline; PDA, photodiode array; PS, presenilins

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γ-cleavage of α- and β-CTFs releases the soluble APP Intracellular Domain (AICD), which acts as nuclear signalling peptide involved in the transcriptional regulation of APP target genes (Huysseune et al., 2009; Pardossi-Piquard and Checler, 2012).

Given AD prevalence and the lack of effective long-term therapies, there is a persistent need to discover active compounds targeting APP processing that can be developed into clinically approved disease-modifying drugs. The vegetal kingdom is a highly abundant resource for the discovery of new active compounds and may have advantages in relation to efficiency versus adverse effect profile. Efficient natural molecules have been isolated, acting on several targets involved in AD pathogenesis, and some of them have already shown promising activity in clinical trials (Williams et al., 2011; Howes and Houghton, 2012). Moreover, plant extracts as such have also been found efficient, either in clinical or in preclinical tests, on several pathological features related to AD (Lai et al., 2006; Williams et al., 2008; Howes and Houghton, 2012; Rendeiro et al., 2013, 2012).

We previously reported a screening of crude extracts of plants used by traditional practitioners for learning and memory problems, potentially targeting APP catabolism and in particular Aβ production. The aqueous extract of the stem-bark of *Pterocarpus erinaceus* Poir. (*Fabaceae* family) lowered Aβ production *in vitro* (Hage et al., 2010). More recently, we showed that this *Pterocarpus erinaceus* extract reduces Aβ production by inhibiting γ-secretase activity, without inhibiting the γ-secretase-dependent cleavage of Notch (Hage et al., 2014).

Here we report the results obtained by analysing new extracts of *Pterocarpus erinaceus*, prepared from its kino, also called *true kino* or *Gambian kino* (Kerharo and Adam, 1974, p. 471). Kinos are red or reddish tannin-rich exudates produced, either spontaneously or after incision, by several vegetal genera (mainly tropical trees), including *Pterocarpus*, but also *Butea*, *Coccoloba*, *Eucalyptus*, *Myristica* and *Rhizophora* (Locher and Currie, 2010); kinos are usually hydrosoluble, which distinguishes them from other red exudates, called *dragon's bloods*, which are insoluble in water (Gupta et al., 2008).

Studying kino activity was first intended as an alternative to the stem–bark: kino is more easily and safely obtained from *Pterocarpus erinaceus*, avoiding overexploitation of the tree. We also noticed (Hage et al., 2010) that the activity of the stem–bark extract was decreased when tannins were removed. Given their high molecular weight (and here their high concentration), tannins can be more difficult to monitor in terms of solubility and resorption. We wanted therefore to find extracts effective even after tannin removal.

In our experiments, *Pterocarpus erinaceus* kino aqueous extract, even after tannin removal, decreased Aβ production not only in CHO cells expressing neuronal APP, but in primary neurons as well; it also inhibited γ-secretase and did not interfere with Notch cleavage. Together, these results indicate that *Pterocarpus erinaceus* kino aqueous extract acts as an APP-specific γ-secretase modulator of high therapeutic interest. The activity of the polar extracts can support the traditional use of the water decoction against memory defects in Benin in rural medicine (Adjanohoun et al., 1989, p. 688).

2. Material and methods

2.1. Chemicals and reagents

Analytical-grade and UPLC-grade solvents, as well as reagents and other chemicals were obtained as previously described (Hage et al., 2014), with the following exceptions. Camptothecin and PMS were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), MTS and reagents for luciferase assay (Dual-Glo Luciferase Assay kit) were purchased from Promega (Madison, WI), and DAPT, used throughout this study as a reference γ-secretase inhibitor, from Calbiochem (Camarillo, CA).

2.2. Plant material and extract preparation

The stem–bark and the kino of wild *Pterocarpus erinaceus* Poir. (called *African rosewood* in English, *vêne* in local French) were collected and identified in Benin in December 2008, by Prof. Pierre Agbani and Prof. Fernand Gbaguidi (Abomey-Calavi University, Benin). A voucher specimen was deposited at the *Herbier National du Bénin* of Abomey-Calavy University (voucher number AA 6355/HNB) and authenticated by Prof. Akpovi Akoegninou, director of the national Herbarium. The plant name was last checked on the website *www.theplantlist.org*, in October 2014. As spontaneous exudate was not found, kino was obtained by practising an incision into the stem–bark in the morning and collecting the exudate 8 h later.

Full aqueous stem–bark extract preparation was previously described (Hage et al., 2010). Similarly, 8 g dry kino were powdered in a mortar, and directly submitted to successive Soxhlet extractions, with 250 ml of *n*-hexane, dichloromethane, ethyl-acetate, methanol, water (the yields obtained are given in Table 1). Organic solvants were eliminated by evaporation under vacuum; water was eliminated by lyophilization. To obtain the aqueous tannin-free fraction (KAST), tannins were removed from 2.6 g kino crude aqueous extract on polyamide column, as described by Houghton and Raman (1998, p. 49); the yield was 25.7% of the raw extract. Both crude and tannin-free aqueous extracts were compared through TLC for tannin removal verification, using *sec*-BuOH–water–acetic acid (14:5:5) as eluent and chlorhydric vanillin as derivatization reagent, as well as by HPLC-MS.

For the biological assays, unless otherwise mentioned, extracts were dissolved at 10 mg/ml in DMSO for organic extracts, in ethanol for MeOH extracts, in water–ethanol 1:1 for aqueous extracts; kino hexane and EtOAc extracts were dissolved in DMSO–EtOH (1:1).

2.3. HPLC-PDA-ESI-HRMS for extract fingerprint

HPLC analysis was performed on an Accela HPLC system hyphenated to a PDA and an LTQ-Orbitrap XL mass spectrometry system, as already described (Dias et al., 2012), with the following modifications. HPLC separation was performed on a reverse-phase C18 column (Lichrospher-Licrocart RP18e, 250 mm length, 4.6 mm width, 5 μm of particle diameter) equipped with a C18 pre-column (5 mm length), both from Merck Millipore (Darmstadt, Germany). Analysis was performed in conditions and settings previously described (Hage et al., 2014).

To assign compounds to the peaks of the HPLC-MS chromatogram, we used standards of epicatechin, catechin, procyanidins B1 and B2, comparing retention times, *m/z* values and ionic fragment patterns. Other oligomers were found by selective ion monitoring (SIM) at 576–580, and 864–867, and 1140–1170 uma, for (epi) catechin dimers, trimers, tetramers, respectively, based on the method described by Fraser et al. (2012).

2.4. Cell lines for activity screening tests

CHO cells stably expressing human APP695 were cultivated and treated as previously described (Hage et al., 2010).

2.5. Neuronal cultures

Primary cultures of cortical neurons were prepared from P0–P1 newborn wild type CD1 mice. Cortices were dissected in Neurobasal medium supplemented with 2% v/v B-27 medium, with 0.5 mM L-glutamine and with penicillin–streptomycin (50 μg/ml of each) and immediately digested with a trypsin solution (220 unit/mg) containing DNAse (1 mg/ml) at 37 °C for 3 min. After removal of the

Table 1
Yields, cytotoxicity of plant extracts and CTF/APP ratios in treated CHO-APP cells.

Extract	Yield[a] (%)	Cytotoxicity on CHO cells[b]		Tested conc. (μg/ml)[c]	CTF/APP ratio[d]	
		IC_{50} (μg/ml), averages	*Confidence intervals*			
Kino Hexane	0.08	25.2	24.1; 26.3	6.25	0.9	± 0.2
Kino CH_2Cl_2	0.04	23.2	21.6; 24.8	6.25	0.9	± 0.1
Kino EtOAc	0.17	> 200	/	50	0.9	± 0.1
Kino MeOH	11.3	105.9	81.4; 138.0	50	7.5	± 0.7**
Kino aqueous	74.9	83.6	80.2; 87.1	50	1.4	± 0.3*
				25	1.4	± 0.1*
				12.5	1.5	± 0.1*
Kino aqueous, *tannins removed* (KAST)	25.7	> 200	/	100	5.2	± 0.7**
				50	2.8	± 0.3**
				25	1.4	± 0.2*
				12.5	1.2	± 0.1
Stem–bark full aqueous (crude extract)	22.0	> 200	/	200	7.4	± 3.2**
				100	5.2	± 2.8**
				50	2.2	± 0.2**
Stem–bark full aqueous, *tannins removed*	75.7	> 200	/	200	4.1	± 1.0**
				100	3.1	± 1.2**
				50	2.0	± 0.6**

[a] Yields of crude extracts are calculated according to the weight of plant powder; yields of fractions are calculated according to the weight of the corresponding extract from which they were prepared.

[b] For the MTT assay, the following concentrations were tried: 3.125, 6.25, 12.5, 25, 50, 100, 150 and 200 μg/ml; $n=5$ at least for each concentration. Camptothecin was used as a reference cytotoxic compound with an IC_{50} of 0.07 μg/ml as (confidence intervals 0.04 and 0.10 μg/ml).

[c] Tested concentrations provide at least 80 % of viability after 72 h treatment.

[d] CHO-APP cells were treated with plant extracts as well as with DAPT 250 nM; cell lysates were used for Western-blotting. CTF/APP ratios (± SD) significantly superior to control cells (1 ± 0.2) are indicated by

** ($p < 0.01$ with Student *t*-test) or by

* ($p < 0.05$); $n=3$ at least; DAPT treatment (250 nM) gave a CTF/APP ratio of 8.3 ± 3.9**.

trypsine/DNAse solution, cortices were further dissociated in Neurobasal medium supplemented with DNAse (0.5 mg/ml). Cells were plated in culture dishes (250×10^3 cells/cm^2) pre-treated with 10 μg/ml poly-L-lysine in PBS. Prior to treatment, cells were cultured for 7 days *in vitro* in Neurobasal medium supplemented with 2% v/v B-27 medium and 0.5 mM L-glutamine and were maintained at 37 °C in a 5% CO_2 atmosphere. Under these conditions, neuronal cultures contain more than 90 % neuronal cells, which display high differentiation and survival rate (Brewer, 1995). Primary neurons were treated by kino extract or DAPT at indicated concentrations. Neuronal morphology has been observed by a standard CKX41 inverted microscope (Olympus Europe, Hamburg). Culture media were harvested 16 h after treatments; primary neurons were scraped in Optimem medium and recovered by quick centrifugation ($28{,}000 \times g$).

2.6. Cytotoxicity assays

Cytotoxicity was measured in CHO cells 72 h after treatment by MTT assay (Hage et al., 2010). For primary neurons, cell viability was assessed with a MTS assay after a 32-h treatment, according to manufacturer's instruction (Promega, Madison, WI). Plates were measured at 490 nm using a microplate spectrophotometer Victor *X*3 Multilabel Plate Reader (PerkinElmer, Waltham, MA).

2.7. Western-blotting

Western-blotting was performed on 4–12% Nu-PAGE bis–tris gels, as previously described (Hage et al., 2014). Briefly, membranes were incubated overnight (4 °C) with the primary antibody (1/5000 for the anti-APP C-terminal antibody, 1/1000 for the anti-*myc* antibody), then washed and incubated 1 h with the secondary antibody (1/15,000 horseradish-peroxidase-conjugated anti-rabbit donkey IgG) and revealed by ECL. Signals were quantified using the Quantity One® software coupled to the Gel Doc 2000 device (Bio-Rad, Hercules, CA).

2.8. Aβ quantification

Aβ38, Aβ40 and Aβ42 were quantified in culture media by multiplex Electro-Chemiluminescence Immuno-Assay (ECLIA), carried out using 6E10 (human Aβ triplex kit) or 4G8 (rodent Aβ triplex kit) antibodies on a MSD SECTOR™ Imager 2400 (Meso Scale Discovery, Gaithersburg, MD, USA), following manufacturer's instructions. Preliminary experiments and plate design in triplex assays ensured that there were no significant cross-reactions between Aβ38, Aβ40 and Aβ42 quantifications.

2.9. Cell-free APP cleavage assay

Measurements of γ-secretase activity in cell-free assays were adapted from previous procedures (Hage et al., 2014). All steps were carried out at 4 °C unless otherwise indicated. Confluent CHO-APP cells were washed with PBS buffer, scraped in 0.5 ml hypotonic MOPS buffer (1 mM MOPS, 1 mM KCl, pH 7), incubated 10 min on ice and homogenised in a tight-fitting homogenising syringe. Homogenates were cleared by centrifugation at $1000 \times g$ for 15 min to remove cell debris and nuclei. The post-nuclear supernatants were centrifuged (40 min at $16{,}000 \times g$), and the pellets containing crude membranes were recovered in an assay buffer (150 mM sodium citrate, pH 6.4), with either DAPT or KAST at indicated concentrations, and incubated at 37 °C for 2 h. Membrane fractions were put on ice at the end of the incubation, and sonicated in Laemmli buffer for Western-blotting.

2.10. In vitro fluorogenic γ-secretase assays

γ-Secretase assays with small fluorogenic peptides were adapted from Wang et al. (2009) as already published (Hage et al., 2014). Two fluoroprobes were used: Nma-G-G-V-V-I-A-T-V-K(Dnp)R-R-R-NH_2 (Merck KGaA, ref. VWR 565764-1) mimics the γ-cleavage site of APP, whereas Nma-G-C-G-V-L-L-K(Dnp)R-R-NH_2 (Sigma-Aldrich, ref. N4912) mimics the GVL sequence of the S3 Notch γ-cleavage site. In preliminary experiments, optimal incubation time was found to be 5 h, for protein membrane concentrations above 150 μg/ml.

2.11. Reporter gene assay (dual luciferase assay)

CHO cells were seeded in 12-well plates and co-transfected 24 h after, with the *HES1*-luciferase reporter gene (Hébert et al., 2006) and the pRL-TK Renilla luciferase control vector (Promega, Madison, WI) at 4:1 ratio, to correct for transfection efficiency. One day after transfection, cells were treated during 16 h prior to measurement of luciferase activities following manufacturer's instructions (Dual Glo® luciferase assay, Promega).

MEF cells were handled as previously described (Hage et al., 2010), seeded in 24 well plates and co-transfected 24 h after with the pCS2 Notch1 ΔEMV-6MT plasmid (Kopan et al., 1996) and the two reporter luciferase constructs indicated above. Cells were treated during 16 h one day after transfection, washed with PBS and recovered either for luciferase activity or Western-blotting.

2.12. Quantification of tannins

Tannin quantification was performed following the method of the European Pharmacopœia (2012). Briefly, 15 mg of extract were heated half an hour in 40 ml water, brought to 50 ml and filtrated; 5 ml were diluted 10 times and 2 ml of the dilution were mixed with 1 ml phosphor-molybdo-tungstic reagent (equivalent to tungstene 62.57 g/l), 10 ml water and 13 ml Na_2CO_3 290 g/l. Blanks were done with the same volume of extract dilution, shaken with hide powder to retain the tannins, filtrated and mixed with the reagent. Half an hour later, absorbances were read at 760 nm. Control was done with pyrogallol 2 μg/ml and results were expressed in equivalents of pyrogallol.

2.13. Statistical analysis

The number of samples (n) for each experimental condition is indicated in tables and figure legends. Statistical analyses were done by unpaired t-test or ANOVA followed by Tukey's multiple comparison *post hoc* test (Prism 4.0 and 6.0, GraphPad Software Inc., San Diego, CA).

3. Results and discussion

Up to now, the chemical drugs that are approved for AD are either acetylcholinesterase inhibitors or modulators of the glutamatergic transmission; these treatments are symptomatic and unfortunately do not block the progression of the disease (Anand et al., 2014). It is therefore of prime importance to discover new molecules or approaches with disease-modifying properties.

Recently, several studies have reported a potential therapeutic interest for plant extracts in the treatment of AD (Howes and Houghton, 2012); as an example here, the *Ginkgo biloba* extract (EGb761) is formally approved and registered in Germany and in Belgium as a drug against AD.

Although many phytochemicals have been identified as inhibitors of BACE1 activity (Williams et al., 2011), only a few indirect inhibitors of γ-secretase have been isolated from natural sources so far, *e.g.* beauveriolide III acting through a decrease of acylated cholesterol (Witter et al., 2009), or luteolin through GSK-3 inhibition (Rezai-Zadeh et al., 2009). Very recently, one other vegetal inhibitor of the γ-secretase has been identified in Chinese hop flower extracts (*Humulus lupulus* L.), reducing amyloid charge in mice expressing the Indiana APP mutation (V717F); it was identified as garcinielliptone HC (Sasaoka et al., 2014), a molecule first discovered in the heartwood of *Garcinia subelliptica* Merr. (Lu et al., 2008).

In our study, we report a new plant extract with γ-secretase modulating properties and presenting an interesting therapeutic potential. *Pterocarpus erinaceus* is a papilionoid tree growing in West Africa, its kino was locally used for centuries as traditional medicine, before being used also in European and North American countries, mainly to treat diarrhoea, dysentery and wound ulcers. Despite this, the other pharmacological activities of the tree, and of the kino in particular, have not been sufficiently explored, although they have been traditionally used for numerous indications in Africa (Arbonnier, 2009, p. 324), including cognitive problems.

3.1. Preparation of extracts, chemical fingerprint and cytotoxicity assay

The kino from *Pterocarpus erinaceus* was extracted as described in Materials and Methods. Yields are given in Table 1. For the crude extracts, Soxhlet percolation was used, instead of maceration, to remain closer to the traditional medicine, where the aqueous decoction is used (Adjanohoun et al., 1989, p. 688). As African kino is rich in catechic tannins, a part of the aqueous extract (2.6 g) was purified to obtain the aqueous tannin-free fraction (hereafter referred to as KAST). The yield of this fraction was 24.7% of the crude extract.

Tannin removal was verified by chromatography. First, a TLC was performed, using chlorhydric vanillin as revelation reagent to hue catechic tannins and related polyphenols in pink. Results indicated the absence of bands related to polymeric catechic compounds in KAST (Suppl. Fig. 1).

We also compared the composition of both extracts by an HPLC-PDA-MS analysis. The fingerprint chromatograms are shown in Fig. 1A and B. The peak at 27.15 min corresponds to epicatechin ($[M+H]^+ = 291.0967$ uma) present in both extracts, whereas (epi) catechic oligomers (among them procyanidin B2), also detected in the crude extract, are reduced or absent in KAST (Fig. 1B). However, a dimer ($[M+H]^+ = 575.43127$ uma) appears in KAST (peak at 54.17 minutes), probably formed during the purification process, as it was absent from the crude extract and not likely to derive from the scission or rearrangement of existing oligomers, which were all retained on the polyamide column used for tannin removal. It is possible that during the drying step a part of the epicatechin isomerised to catechin, which also appeared in KAST (Fig. 1B), or oxidised to other species, allowing thereafter the formation of a new oligomer absent in the native state of the kino. We also observed other peaks not related to (epi)catechin derivatives but whose structure could not be determined. They do not correspond to any other compound previously identified in *Pterocarpus erinaceus*, nor present in our data bank.

Extracts were tested on CHO cells during 72 h for cell viability, to exclude that our further results on Aβ production were due to potential cytotoxicity. Results are given in Table 1; apolar extracts were toxic, while tannin removal significantly lowered the toxicity of the aqueous extract.

3.2. Effects on APP processing and Aβ production in CHO cells

In order to study its effects on APP processing, we treated CHO cells expressing human APP695 during 16 h with each extract and

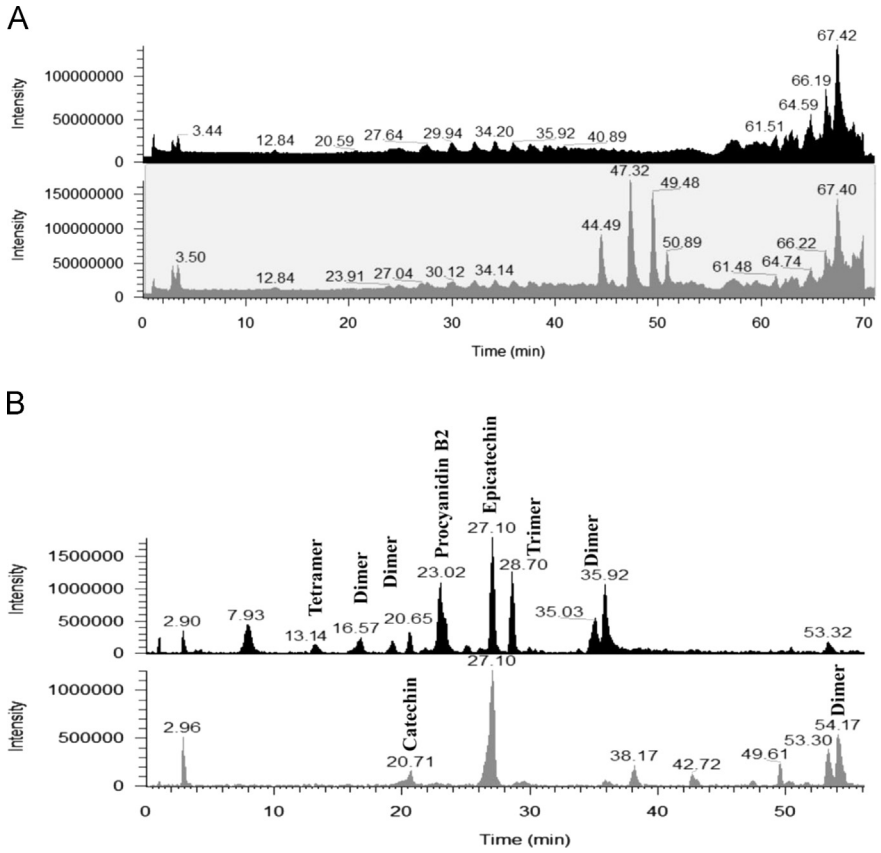

Fig. 1. Comparison of Kino crude aqueous extract and KAST through chromatography. We compared both extracts through column chromatography to verify the removal of tannins. (A and B) HPLC-ESI-HR-MS chromatographic profiles (positive ion mode) of both extracts are given (in black for the crude extract, in grey for KAST). (A) Total ion current. (B) Selected ions (289–292, and 576–580, and 864–867, and 1140–1170 uma) corresponding to epicatechin and (epi)catechin dimers, trimers, tetramers, respectively. The peaks without indications are adducts or fragments of other products, having a MW in the above mentioned ranges. Oligomers of the crude extract are almost absent from KAST, but a new dimer appear at 54.17 min.

the medium was then collected for Aβ quantification by ECLIA (Fig. 2B). Western-blots of cell-lysates were performed to analyse the amounts of full-length APP and CTFs (Table 1 and Fig. 2A).

As already described, *Pterocarpus erinaceus* stem–bark full aqueous extract increased CTF/APP ratio (7.4 ± 3.2 at 200 μg/ml, and 5.2 ± 2.8 at 100 μg/ml), indicating an accumulation of CTFα and CTFβ, and inhibited Aβ production. The pattern was similar to that of DAPT 250 nM (8.3 ± 3.9 CTF/APP ratio), a reference γ-secretase inhibitor (Dovey et al., 2001).

Among kino crude extracts, only MeOH and aqueous extracts significantly increased CTF/APP ratio (Table 1). We observed a significant decrease in Aβ38, Aβ40 and Aβ42 for kino crude MeOH extract at 50 μg/ml and in Aβ40 and Aβ42 for kino aqueous extract (Fig. 2B). On the contrary to what occurred with stem–bark aqueous extract on CTF/APP ratio (Hage et al., 2010) and in dose-dependent Aβ inhibition assays (Suppl. Fig. 2), kino aqueous extract was more active after tannin removal (KAST) on CTF/APP ratio (which raised from 1.4 ± 0.3 to 2.8 ± 0.3 at 50 μg/ml after tannin removal, p-value < 0.01) and on Aβ38, Aβ40 and Aβ42 production.

3.3. KAST displays γ-secretase inhibitor-like effects on Aβ production and APP processing

Accumulation of CTFs was observed upon treatment by DAPT and by *Pterocarpus erinaceus* extracts (Fig. 2A). This accumulation of CTFs is considered as the hallmark of a decreased γ-cleavage (Hage et al., 2014). We observed by ECLIA a significant reduction in Aβ38, Aβ40 and Aβ42 after treatment by KAST at 50 and 100 μg/ml, with a more potent effect than the one observed with *Pterocarpus erinaceus* stem–bark aqueous extract (Fig. 2B). This indicated that, like the stem-bark aqueous extract, KAST was likely to decrease Aβ production by inhibiting the γ-cleavage of APP. However, as we already noticed (Hage et al., 2014), DAPT at 250 nM inhibited more efficiently Aβ38 and Aβ40 production than *Pterocarpus erinaceus* extracts at the highest non-toxic concentrations. Interestingly the inhibitory effects of KAST and DAPT on Aβ42 release were similar in these conditions (Fig. 2B).

3.4. Role of polyphenols and epicatechin

As the kino and the stem-bark of *Pterocarpus erinaceus* are very rich in proanthocyanidins (Kerharo and Adam, 1974, p. 471), it was important to evaluate their influence in the activity of the extracts. Using the phosphor-molybdo-tungstic reagent, we measured tannin concentrations in the most active extracts, kino extracts being richer in tannins than stem–bark extracts (Table 2). The activity of the kino aqueous extract, which had the highest tannin level, was increased after tannin removal (Table 1 and Fig. 2), which supports the hypothesis that the inhibitory activity is not unspecifically linked to the polyphenols present in *Pterocarpus erinaceus* extracts.

We identified epicatechin as the catechic monomer in *Pterocarpus erinaceus* kino and stem–bark; in accordance with previous observations on other kino producing species of the genus *Pterocarpus*, e.g. *Pterocarpus soyauxii* Taub. and *Pterocarpus marsupium* Roxb. (which gives the Malabar kino), as well as on species that do not produce any kino, e.g. *Pterocarpus santalinus* L. f. (Kameswara Rao et al., 2001; Jain et al., 2010; Tchamadeu et al., 2011). As epicatechin and a dimer were observed in KAST, epicatechin (PubChem CID 72276) 6.25 μg/ml and procyanidin B1 (PubChem CID 11250133) 12 μg/ml were also tested

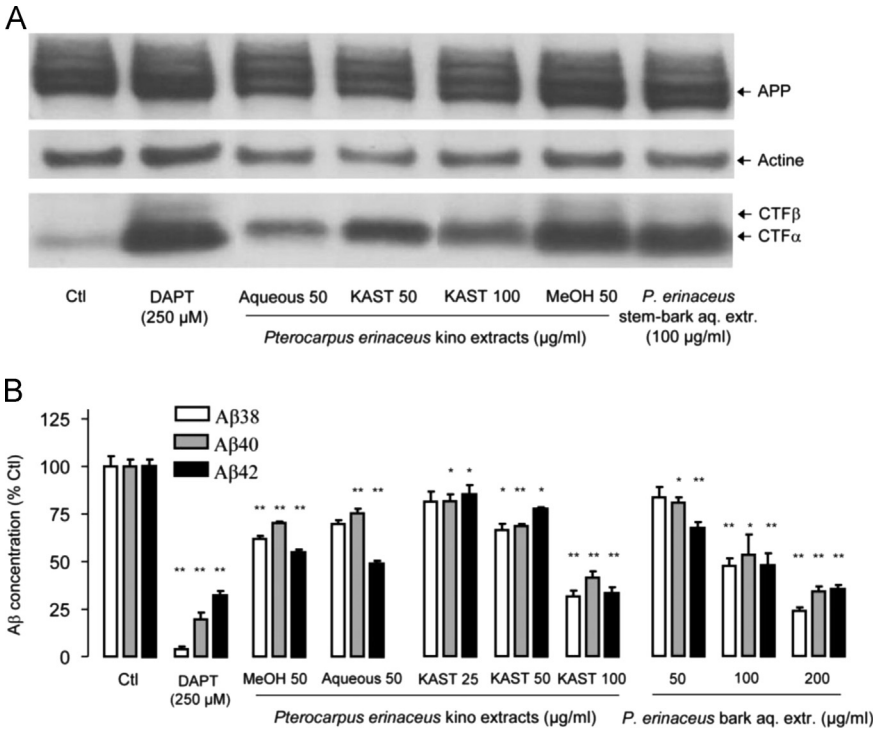

Fig. 2. Effects of *Pterocarpus erinaceus* extracts on Aβ production in CHO cells expressing APP695. CHO-APP cells were treated for 16 h with *Pterocarpus erinaceus* extracts as indicated or with 250 nM DAPT, a functional γ-secretase inhibitor. (A) Western-blot of CHO-APP cell lysates, revealed with the APP C-ter antibody. The expected positions of full-length APP (APP), CTFα and CTFβ are indicated by arrows. (B) Measurement of Aβ concentration in extracellular media from these CHO-APP cells. Human Aβ isoforms (38, 40, 42) were quantified in the extracellular medium by ECLIA method (6E10 antibody). Values in non-transfected cells were below threshold (~ 5 pg/ml) for the three Aβ species (*not shown*). Results (mean ± SEM) are given as percentage of Aβ measured in the medium of non-treated (Ctl) cells; $^{*}p < 0.05$; $^{**}p < 0.01$ ($n=6$).

Table 2
Tannin levels of plant extracts.

Pterocarpus part	Extract[a]	Tannin levels (expressed as % of pyrogallol equivalents)	
		Mean	*SD*
Stem–bark	Full aqueous	14.18	± 2.23
Stem–bark	MeOH	28.57	± 3.35
Stem–bark	Aqueous (post MeOH)	3.89	± 1.54
Kino	MeOH	31.55	± 3.17
Kino	Aqueous (post MeOH)	49.76	± 3.74

[a] Aqueous post MeOH extract corresponds to the aqueous extract prepared from the bark or from the kino after MeOH extraction, whereas the stem–bark full aqueous extract is an aqueous extract prepared without any previous MeOH extraction. For tannin measurements, $n=3$.

in CHO-APP cells. They significantly increased the CTF/APP ratio (1.3 ± 0.2 and 1.5 ± 0.2, respectively, p-value < 0.05), but had no inihibitory effect on Aβ production (Suppl. Fig. 2). Epicatechin seems therefore probably not to be the molecule responsible for the activity measured in the extracts. However, this does not exclude possible beneficial effects of epicatechin on APP amyloidogenic processing, since *in vivo* metabolites can be produced, the active forms of epicatechin still being undefined (Fraga and Oteiza, 2011).

However, the presence of epicatechin in our active *Pterocarpus erinaceus* extracts is of interest, since this molecule not only crosses the blood–brain barrier *in vivo* (Wu et al., 2012), but has also been shown to exert a wide panel of biological activities: it inhibits Aβ aggregation *in vitro* (Ono and Yamada, 2006); it is an inhibitor of AChE *in vitro*, as reported by a study of the components of areca-nut (fruit of *Areca catechu* L.) and of cutch (extract of the stem-wood of *Acacia catechu* (L.f.) Willd.) (Mukherjee and Houghton, 2006); it enhances spatial memory and increases angiogenesis in mouse hippocampus *in vivo* (van Praag et al., 2007); given *per os* to rats prior to injection of Aβ in the hippocampus, epicatechin increased cellular glutathion in astrocytes, improved memory performances and decreased ROS and lipidic peroxidation in hippocampus (Cuevas et al., 2009); it was also reported to reduce glycæmia of alloxan-induced diabetic rats (Vats et al., 2002; Jain et al., 2010), and it has antiphlogistic and cardioprotective effects *in vivo* (Barnett and Marco, 2011), as well as antihypertensive effects in man (Fraga and Oteiza, 2011). These properties, which are not addressed in our present study, can all be, to some extent, beneficial against AD. They suggest that pure epicatechin, or epicatechin-rich extracts, would have polyvalent benefits when used *in vivo*. We should also mention here that (epi)catechic derivatives, in *Ginkgo biloba* L. as well as cocoa (*Theobroma cacao* L.), have been reported to inhibit Aβ aggregation, another mechanism of Aβ pathological effects in AD (Wang et al., 2014; Xie et al., 2014).

3.5. KAST decreases Aβ in primary neuronal cultures

Although CHO cells are a reliable and efficient tool to study APP processing, we further addressed the effects of KAST on neurons, the most relevant cellular model. Primary cortical neuron cultures were treated with KAST first to test its potential neurotoxicity. Survival assays showed that neuronal survival was not affected by KAST treatment (Fig. 3A), even at 125 μg/ml. APP processing and Aβ production were tested on primary cultures of cortical neurons treated with KAST (50 and 100 μg/ml) or with DAPT. No morphological signs of toxicity were observed after 16 h of treatment (Fig. 3B). CTFs accumulation was measured in cell lysates by Western-blotting (Fig. 3C), and the medium was collected for Aβ quantification by ECLIA (Fig. 3D).

Accumulation of CTFs was observed upon treatment by KAST and by DAPT (Fig. 3C), suggesting a decreased γ-cleavage as in CHO cells. Aβ40 and Aβ42 levels were significantly decreased after treatment by KAST at 100 μg/ml and by DAPT 5 μM. The inhibitory effects of KAST extract on Aβ production measured in primary

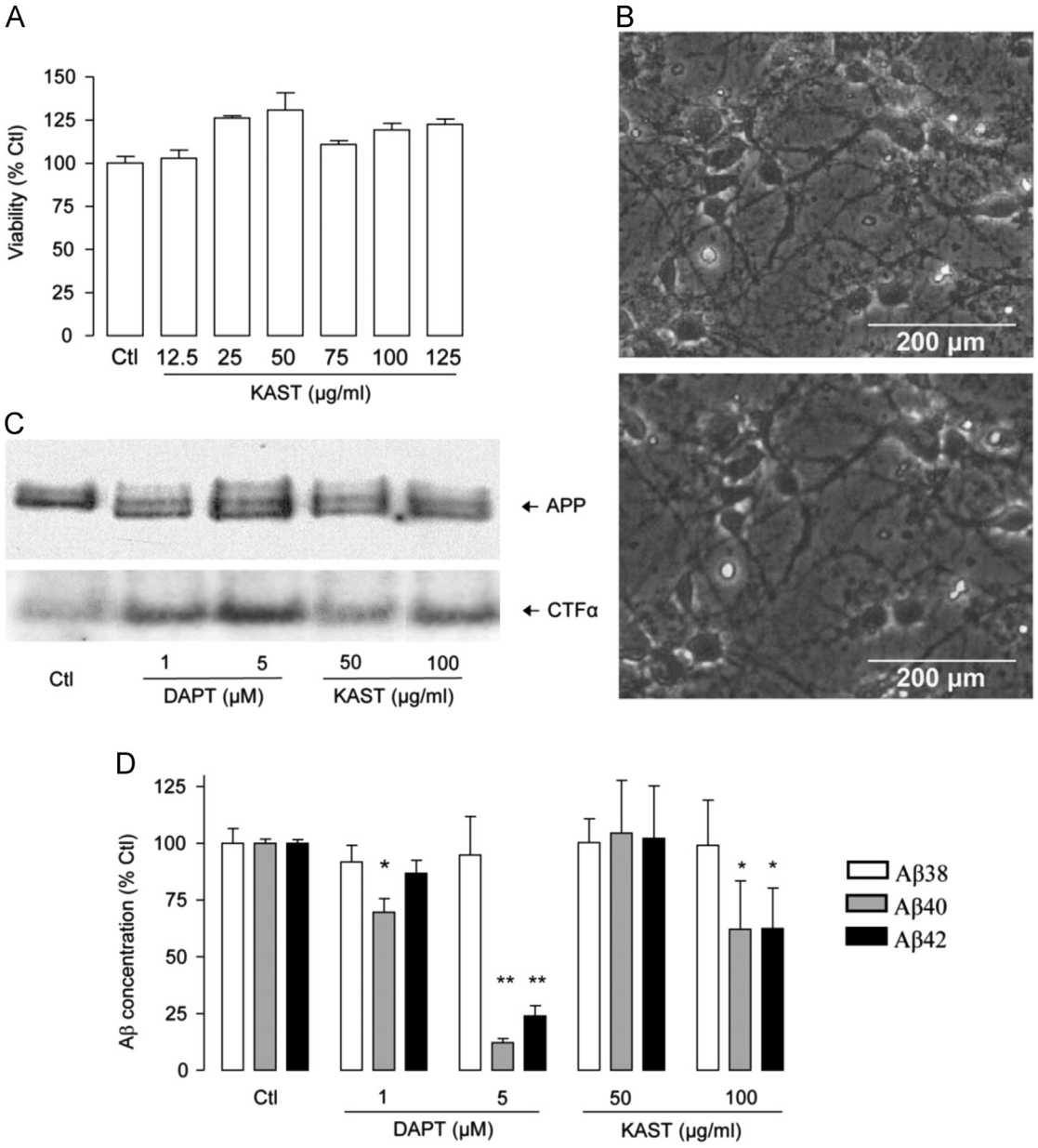

Fig. 3. Effect of *Pterocarpus erinaceus* extract KAST on primary cortical neurons. (A) An MTS assay was performed to measure survival of cortical neurons treated (32 h) at several concentrations of KAST. Results (mean ± SEM) are given as % of the viability (100 ± 3.8) measured in non-treated (Ctl) neurons ($n=4$). No significant toxicity was measured under these conditions. (B–D) Primary neurons were treated for 16 h with KAST at 50 and 100 µg/ml or with 1 and 5 µM DAPT. (B) Neurons photomicrographs before (above) and after treatment (below), show no morphological signs of cell death (original magnification 20×, scale 200 µm). (C) Neuron lysates were analysed by Western-blotting revealed with the C-ter antibody. The expected positions of full-length APP and CTFα are indicated by arrows. (D) Aβ concentration was measured in extracellular media from primary neurons. Rodent Aβ isoforms (38, 40, 42) were quantified by multiplex ECLIA (4G8 antibody). Results (mean ± SEM) are given as percentage of Aβ measured in the medium of non-treated (Ctl) cells; $^{*}p<0.05$; $^{**}p<0.01$ ($n=3$).

neurons (Fig. 3D) were less significant than in CHO-APP cells (Fig. 2A), but the activity of DAPT was also weaker in neurons (Fig. 3D), decreasing Aβ40 only at 1 µM or more, whereas almost complete inhibition was observed at 250 nM in CHO-APP cells (Fig. 2B). It is particularly interesting to note that KAST inhibits Aβ42 and Aβ40 in a similar manner, whereas DAPT is more efficient towards Aβ40. This could be highly beneficial, since Aβ42 is the isoform reported to be the most toxic when it accumulates in the brain or inside the neurons (Wirths et al., 2001; Kienlen-Campard et al., 2002).

3.6. KAST directly inhibits γ-secretase activity

The direct inhibition of γ-secretase by KAST was further assessed by another *in vitro* assay on CHO cell membrane preparations using γ-secretase fluorogenic substrates. In this assay, the activities of DAPT 10 µM and of KAST 50 µg/ml can be measured on the γ-secretase cleavage of two fluoroprobes: one mimicking the γ-cleavage site of APP (fluoroprobe APP), the other one mimicking the S3-cleavage site of Notch, the other major substrate of γ-secretase. DAPT and KAST treatments induced an inhibition of APP substrate cleavage, in line with the results discussed above on Aβ production and CTFs accumulation. In addition, KAST was also found to induce accumulation of APP CTFs in cell-free assays, consistent with direct inhibition of γ-secretase activity (Suppl. Fig. 3). Contrary to DAPT, which showed no selectivity between both fluoroprobes, KAST inhibited more specifically APP fluoroprobe cleavage *vs.* Notch fluoroprobe cleavage (Fig. 4A). This selectivity was found to be even more pronounced in KAST than in the stem-bark aqueous extract (Hage et al., 2014).

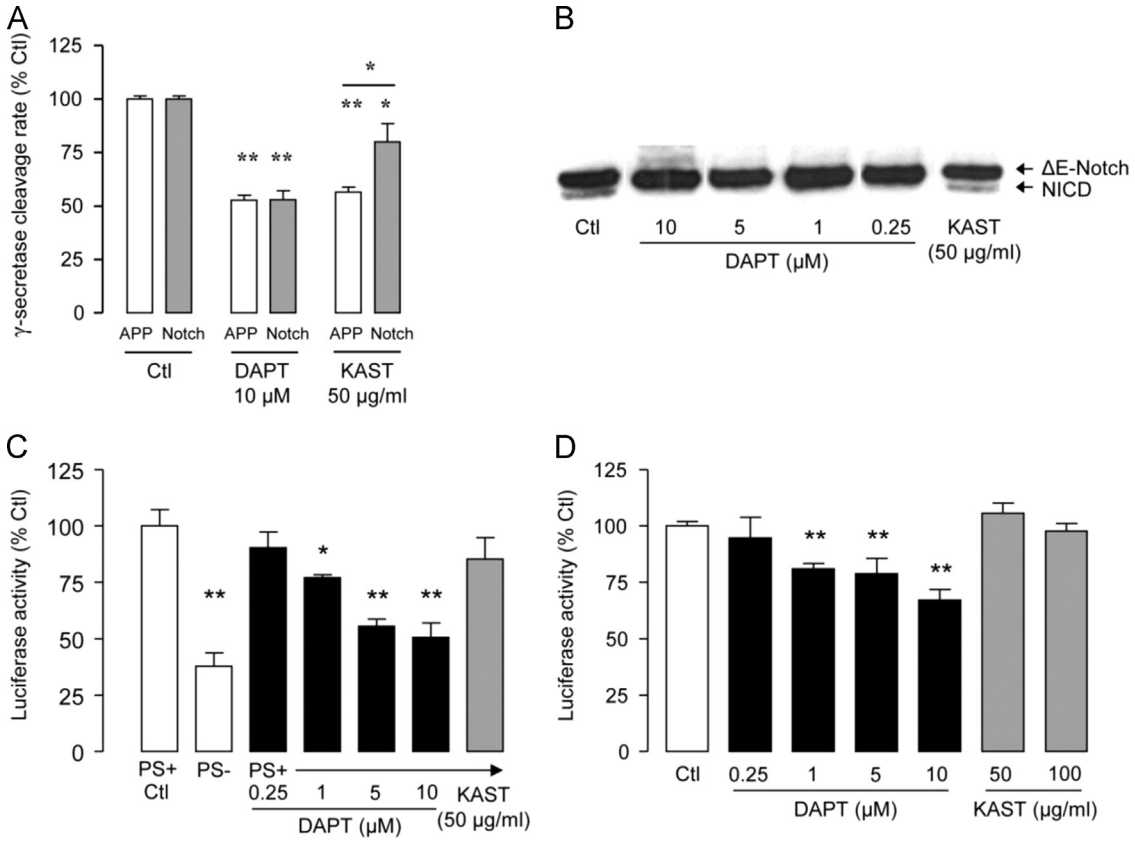

Fig. 4. Effect of KAST on NICD release. Three experiments were carried out to assess the selectivity of the extract for APP *vs.* Notch processing. (A) An *in vitro* γ-secretase assay was performed on membranes isolated from CHO cells and incubated 5 h with DAPT and kino extract, in presence of a fluorogenic peptide mimicking either APP γ-cleavage site (white) or Notch S3-cleavage site (grey), as indicated. Results (means ± SEM) are given as percentage of activity measured in non-treated (Ctl) cells; $^{*}p < 0.05$; $^{**}p < 0.01$ ($n=3$ at least), as compared to control (non-treated). (B and C) MEF cells were co-transfected with a *HES1*-luciferase reporter gene, the pRL-TK *Renilla* construct and *myc*-tagged ΔE-Notch plasmid, and treated with DAPT or with KAST as indicated. NICD release was measured by Western-blotting using an anti-*myc* antibody (B) and by a functional transactivation assay (C). (D) CHO cells were transfected with a *HES1*-luciferase reporter gene and the pRL-TK *Renilla* construct and treated as indicated. Endogenous NICD release was measured by the functional transactivation assay used in MEF cells. Luciferase activities (C and D) were normalised by calculating firefly luciferase/*Renilla* luciferase ratio. Results (means ± SEM) are given as percentage of luciferase activity measured in control (non-treated PS+ MEF or CHO, respectively) cells; $^{*}p < 0.05$; $^{**}p < 0.01$ ($n=6$), as compared to control.

These assays carried out *in vitro* on crude membrane preparations allowed us to show a direct effect of the extract on γ-secretase activity, avoiding the possible interferences due to side effects on cell metabolism or cell trafficking (Hage et al., 2014). As already mentioned, a basal activity is measured upon DAPT treatment (Fig. 4A) which corresponds to the basal activity measured in PS1/PS KO cells and which has to be considered as PS-independent γ-secretase activity (Hage et al., 2014). Although our results strongly supports that KAST directly inhibits γ-secretase activity (Fig. 4A), we cannot firmly rule out at this stage the hypothesis that cellular processes like endocytosis, which are known to be essential in Aβ production (Haass et al., 2012), could also be targeted by KAST and partially responsible for the effects observed in treated cells. Evaluation of *Pterocarpus erinaceus* effects on cell physiology and cell trafficking await further investigations, which are beyond the scope of this study.

3.7. KAST specifically inhibits APP but not Notch γ-cleavage

Another issue that needs to be addressed for γ-secretase inhibitors/modulators, in a therapeutic perspective, is their selectivity towards APP cleavage. The different γ-secretases are intricate and unusual proteolytic complexes, with an increasing number of substrates (Beel and Sanders, 2008; Tolia and De Strooper, 2009). Some of them like the Notch proteins are critically involved in cell functions. Indeed, the PS1 *KO* lethal phenotype is linked to the loss of Notch function during development (De Strooper et al., 1998).

We observed in an *in vitro* assay using fluorogenic substrates (Fig. 4A) that KAST did not inhibit the cleavage of a Notch probe as strongly as it inhibited the cleavage of an APP probe, on the contrary to DAPT. We wanted to confirm this potential selectivity in cell models. This was addressed by a NICD-dependent (Notch Intracellular Domain) transactivation assay. Cells were transfected by a ΔE-Notch construct (analogous to NEXT) allowing to monitor the *myc*-tagged NICD release and to measure NICD activity by the transctivation of a HES1-luciferase reporter gene. Notch γ-cleavage releases NICD, which activates transcription of Notch target genes, among which Hairy Enhancer of Split 1 (HES1); transcriptional activity of a HES1-luciferase reporter gene provides a good functional measurement of NICD production (Hébert et al., 2006). We performed this experiments in MEF cells (co-transfected with *myc*-tagged ΔE-Notch Fig. 4B and C) and in CHO cells (expressing only endogenous Notch, Fig. 4D). Under these conditions, NICD production from ΔE-Notch construct in MEF cells was reduced by DAPT (even at 250 nM) as showed by Western blotting, but remained unaffected by KAST even at 100 μg/ml (Fig. 4B). DAPT treatment (at 1 μM and above) significantly inhibited NICD-dependent transactivation (Fig. 4C); the inhibition was comparable to that observed in MEF cells lacking PS-dependent γ-secretase activity (*shown in* Hage et al., 2014). In strong contrast to DAPT, KAST did not inhibit NICD-dependent transactivation of the HES1 promoter, and did not block the release of NICD from NEXT in MEF cells (Fig. 4B and C). Comparable results were obtained for endogenous NICD release in CHO cells (Fig. 4D).

Taken together, these results strongly indicate that KAST might selectively inhibit the γ-cleavage of APP, without significantly affecting Notch processing, contrary to DAPT. This is a key observation for the potential therapeutic interest of this kino extract. Several hypotheses have been proposed for the selectivity of inhibitors towards a subset of γ-secretase substrates. They are based either on the modulation of substrate properties (Sastre et al., 2001; Kienlen-Campard et al., 2008; Sato et al., 2009) or on the binding to the enzyme complex, changing either its interaction or catalytic properties (Dunn et al., 2010; Thathiah et al., 2013).

3.8. Ethnopharmacological relevance and comparison of stem–bark and kino extracts

The activities that we observed in *Pterocarpus erinaceus* extracts may support the traditional indication of its bark and kino for memory improvement. We observed that polar Soxhlet extracts obtained with MeOH and water were effective and safe on *in vitro* models (whereas the most apolar extracts were toxic). This is in accordance with the use by the local practitioners as a water decoction, to be taken twice a day, simultaneously as a potion and as bath (Adjanohoun et al., 1989, p. 688). Nevertheless, *in vivo* tests should be performed to determine whether such concentrations could be obtained in the brain (Vauzour et al., 2008).

Although kino is abundantly present in a network and as droplets on the internal surface of the bark, we observed several differences between stem–bark and kino: 1) kino EtOAc extract was less cytotoxic on CHO cells ($IC_{50} > 200$ μg/ml) than the bark EtOAc extract ($IC_{50} > 99.8 \pm 9.5$ μg/ml); 2) aqueous extract of kino had a far bigger yield than that of the bark; 3) when tannins were removed from the bark extract, activity was decreased, whereas the contrary was observed with kino; 4) considering the tannin concentrations, we observed that aqueous extract contains much less tannins (stem–bark), or much more tannins (kino) than the corresponding MeOH extracts. These features indicate a chemical difference, resulting either from the presence in the bark of compounds not present in kino (or *vice-versa*), or from a difference in degrees of polymerisation of common compounds, namely (epi) catechic derivatives.

However, kino extract has at least two advantages over bark extract. First, the recent overexploitation of *Pterocarpus erinaceus* on local West-African level, for medicinal purposes but also for woodworking, leads to the gradual disappearance of the tree, except in the protected natural reserves (Adjonou et al., 2010); in this perspective, collecting kino (exuding either spontaneously or after incision) instead of stem–bark would be less harmful to the trees and eventually to the conservation of the species in non-protected areas. Second, as kino extract was more active after tannin removal, the absence of catechic compounds of high MW would make easier the solubilisation, bioavailability and control analysis of the kino extract, when compared to the stem–bark extract.

4. Conclusion

Extracts of *Pterocarpus erinaceus*, used in Beninese traditional medicine to treat cognitive deficits, showed promising effects against amyloidosis, a major feature of Alzheimer's disease. Our results demonstrated that, in cellular models, including primary neurons, *Pterocarpus erinaceus* kino aqueous extract after tannin removal (KAST) strongly inhibits production of Aβ, which is a primary target for AD therapeutics. We found that KAST directly inhibited γ-secretase activity in enzymatic and cell-free assays.

Clinical trials with wide spectrum γ-secretase inhibitors indicated that global inhibition of all γ-secretase activities causes serious adverse effects, because of their inhibition of signalling pathways (e.g. Notch signalling) playing a key role in cell functions (De Strooper and Chávez Gutiérrez, 2015). However, we showed that both bark and kino extracts did not inhibit Notch S3 cleavage in cells, which is the major concern for the therapeutic use of γ-secretase inhibitors. These potentially interesting Notch-sparing inhibitor properties should be further evaluated in accurate animal models to investigate their safety, their specificity towards AD lesions and symptoms and their bioavailability, which is especially critical for potential drugs intended to act in the central nervous system (Imbimbo, 2008). Further research (through bioguided fractionation) should be also carried out to identify the constituents responsible for the *in vitro* observed activities. Up to now, only epicatechin was identified in KAST but was not effective in decreasing Aβ production in our CHO-APP model.

Taken together, our study show the important potential of botanical extracts acting as specific γ-secretase inhibitors to be further evaluated as potential AD treatments.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2015.01.028.

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