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In vitro screening on β-amyloid peptide production of plants used in traditional medicine for cognitive disorders

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Trichilia emetica

ABSTRACT

Aim of the study: The aim of the study was to investigate the activity on β-amyloid peptide production of crude extracts of 9 plant species traditionally used in Benin or in Madagascar for the treatment of cognitive disorders, in order to select candidates for Alzheimer's disease treatment.
Materials and methods: For each species, hexane, dichloromethane, ethyl-acetate and water extracts were tested, at non-toxic concentrations, on CHO cells overexpressing the human neuronal β-amyloid peptide precursor (APP695) to measure variations of APP processing (by Western-blotting) and, for the most active, of Aβ-amyloid production (by ECLIA).
Results: We observed, at non-toxic concentrations, a significant increase in CTF/APP ratio with *Oldenlandia affinis* cyclotide-enriched fraction, *Prosopis africana* EtOAc extract, *Pterocarpus erinaceus* aqueous extract and *Trichilia emetica* hexane extract. We also showed that the *Pterocarpus erinaceus* extract significantly decreased Aβ production, displaying effects similar to those of DAPT (γ-secretase inhibitor) on APP processing, but may act on another inhibition site.
Conclusion: These active extracts are worth further studies to isolate the compounds responsible for the observed activities, to analyze their mode of action and determine their clinical potentials.

1. Introduction

Alzheimer's disease is the most widespread cause of age-linked dementia. This incurable dementia is histologically diagnosed by the presence, especially in hippocampus and cortex, of both intraneuronal hyperphosphorylated tau protein tangles and extraneuronal senile plaques; these plaques are mainly constituted by aggregated β-amyloid peptide (Aβ) (Annaert and de Strooper, 2002; Kienlen-Campard and Octave, 2002).

This peptide, in addition to forming plaques, which cause oxidative stress in the neighbouring neurons and synapse disorders, causes a neurotoxic effect by its intraneuronal accumulation and by forming soluble oligomers (Suh and Checler, 2002).

The metabolic pathways involved in Aβ production have been thoroughly studied in the last two decades (Suh and Checler, 2002). Briefly, the β-amyloid precursor protein (APP) is a type I transmembrane protein processed by two catabolic pathways: in the non-amyloidogenic pathway, APP is cleaved by an α-secretase activity generating a soluble α-APP and an α C-terminal fragment (α-CTF) anchored in the membrane; in the amyloidogenic pathway, APP is cleaved by the APP-cleaving enzyme (BACE) generating a soluble β-APP and a transmembrane β-C-terminal fragment (β-CTF); both α- and β-CTFs are substrates of a γ-secretase activity which releases P3 and Aβ, respectively, with the concomitant production of APP intracellular domain (AICD).

Several studies with promising results have been made on plant species traditionally used against memory disorders, especially in Indian and Chinese traditional medicine (*e.g.*, Howes and Houghton, 2003); the most known and used being the *Ginkgo*

Abbreviations: ATCC, American type culture collection; BSA, Bovine serum albumine; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester; DMSO, dimethylsulfoxide; FBS, foetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide *or* thiazol blue tetrazolium bromide; PAF, platelet-activating factor; PS, presenilin; TPA, Trypopylamine.

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Table 1
Studied plant species.

Botanical name	Part studied[a]	Family	Place and date of collection	Voucher number
Centella asiatica (L.) Urban	LF	Apiaceae	Mangoro, sept. 2007	2007sept. Mangoro
Cissampelos owariensis P. Beauvais ex D.C.	AP	Menispermaceae	Azowtisse, oct. 2007	AA 6348/HNB
Heteranthera callifolia Rchb. ex Kunth	WP	Pontederiaceae	Dangbo, oct. 2007	AA 6349/HNB
Oldenlandia affinis (R. et S.) D.C.	AP/WP	Rubiaceae	Abomey-Calavi, oct. 2007	AA 6350/HNB
Parkia biglobosa (Jacq.) R.Br. ex G. Don f.	TW	Fabaceae	Benin, oct. 2007	AA 6351/HNB
Prosopis africana (Guill. & Perr.) Taub	HW	Fabaceae	Benin, oct. 2007	AA 6353/HNB
Pterocarpus erinaceus Poiret	SB	Fabaceae	Benin, oct. 2007	AA 6355/HNB
Rauwolfia vomitoria Afzelius	LF	Apocynaceae	Azowtisse, 16 oct. 2007	AA 6356/HNB
Trichilia emetica Vahl. suberosa J.J.F.E. De Wilde	LF	Meliaceae	Benin, oct. 2007	AA 6357/HNB

[a] Plant part used: AP, aerial parts; LF, leaves; SB, stem bark; SHW, stem and bough heart-wood; TW, twigs; WP, whole plant.

biloba L. (Ginkgoaceae) extract EGb761, which has shown cognitive improvement in animals and humans, likely because of a synergy between various compounds: its flavonoids are antioxidant and vasodilatatory, and rescue neurons from toxicity induced by Aβ or H_2O_2, ginkgolide A inhibits Aβ oligomerization, ginkgolide B inhibits PAF and thus interferes with inflammatory processes. The extract has also been shown to increase choline uptake and synaptic plasticity in hippocampus (Bastianetto et al., 2000; De Feudis and Drieu, 2000; Yao et al., 2001; Howes and Houghton, 2003; Wu et al., 2006).

We selected a series of plants traditionally used for memory problems by traditional practitioners in Benin or Madagascar, and which had never been studied for Alzheimer's disease (except *Centella asiatica*). As popular medicine does not know Alzheimer's disease as such, we chose plants according to Alzheimer-related symptoms: some were indicated for «memory loss», others for «madness», and others for «learning retardation» (Adjanohoun et al., 1989). Nine plants were finally selected. From each species we prepared successive extracts in Soxhlet apparatus by hexane, dichloromethane, ethyl-acetate and water. Crude extracts were evaluated for their action upon APP catabolism and in particular Aβ production.

2. Materials and methods

2.1. Chemicals, reagents and materials

Analytical grade solvents were obtained: ethyl-acetate and *n*-hexane from Sigma–Aldrich Chemie GmbH (Steinheim, Germany); dichloromethane from Fluka (Buchs, Switzerland); ultra-pure water in MilliPore (Bedford, USA) columns, and ethanol and methanol by redistillation.

Cellulose extraction thimbles (MN645) and polyamide SC6 (MN815620) were purchased from Macherey-Nagel GmbH & Co. KG (Düren, Germany); DMSO and MTT from Sigma–Aldrich; Ham-F12 medium, fungizone (amphotericin B) and Nu-Page 4–12% bis-tris gels from Invitrogen (Paisley, UK, and Carlsbad, CA); penicillin-streptomycin solution and G418 from BioWhittaker (Lonza, Walkersville, USA). DAPT was a generous gift from Luc Mercken, Aventis, Paris. *Ginkgo biloba* standardized extract (EGb 761) was a kind gift of Dr. Willmar Schwabe GmbH & Co. KG (Kalsruhe, Germany).

The human specific anti-APP WO-2 antibody was obtained from The Genetics Company (Schlieren, Switzerland). The anti-APP C-terminal antibody was kindly provided by N. Sergeant (INSERM U422, Lille, France). Secondary antibodies were from Amersham Bioscience (Uppsala, Sweden). ECL revelation was performed with the *Western Lightning Chemiluminescence Reagent Plus* kit of Perkin Elmer Inc. (Waltham, Massachusetts, USA).

2.2. Plant materials

Beninese plants were chosen in the ethnopharmacological exploratory book of Adjanohoun et al. (1989), with indications as memory loss, learning retardation, and madness as described in the introduction. They were collected and identified by Dr. Pierre Agbani, vouchers were deposited at the Herbier National of Abomey-Calavy University (Bénin).

Centella asiatica leaves were chosen due to their current popular use in India (already mentioned for memory in Ayurvedic medicine) and Madagascar against Alzheimer's disease and as memory improver (Howes and Houghton, 2003; Zheng and Qin, 2007), and were collected for us in Mangoro (East of Madagascar) in sept. 2007, and voucher specimens were deposited at the *Institut Malgache des Recherches Appliquées* (Antananarivo, Madagascar).

Plant names, families, organs, origins, collecting dates and voucher specimen numbers are given in Table 1.

2.3. Preparation of extracts

Plant materials were dried at room temperature, and then pulverised using a blendor and sieved (365 μm, but 710 μm for *Parkia biglobosa* twigs because of their hardness). However, *Pterocarpus erinaceus* bark and *Prosopis africana* heart-wood were cut with a rabbet hand-plane before powdering. 9–12 g powder of each plant was extracted (Soxhlet apparatus) successively by 250 ml of *n*-hexane, dichloromethane (or chloroform), ethyl-acetate, milli-Q water.

For *Centella asiatica*, a supplementary extraction was made according to the European Pharmacopoeia (2008): 8 h of Soxhlet extraction by methanol.

For *Oldenlandia affinis*, we also made a cyclotide-enriched extract: after maceration of 12 g plant powder 16 h in 280 ml of water:MeOH (1:1) under agitation and elimination of MeOH by vacuum, the aqueous solution was washed by 3 times 250 ml of CH_2Cl_2; the aqueous fraction contains cyclotides, as mentioned by Herrmann et al. (2008), while the CH_2Cl_2 fraction was also concentrated and tested.

Organic solvants were eliminated by evaporation under vacuum; water was eliminated by lyophilization. The yields are given in Table 2. For the following tests, unless otherwise mentioned, extracts were dissolved at 20 mg/ml in DMSO for organic extracts, in water: ethanol 2:1 for aqueous extracts; the exceptions are *Centella asiatica* MeOH, and *Pterocarpus erinaceus* hexane dissolved in DMSO: EtOH: water 3:1:2, and *Heteranthera callifolia* CH_2Cl_2 dissolved in DMSO: EtOH: water 3:2:1.

2.4. Cytotoxicity tests

We used CHO-K1 cells (from ATCC, line CCL-61, batch 4765275), cultivated in Ham-F12 medium supplemented with FBS (10%),

Table 2
Yields, cytotoxicity results, chosen concentrations and CTF/APP ratios of all selected plant extracts and reference compounds.

Plant species or substance	Extract	Yield[a] (%)	Cytotoxicity on CHO cells		Chosen conc.[b] (μg/ml)	CTF/APP ratio[c]	
			IC_{50} (μg/ml), averages	Confidence intervals			
Centella asiatica	Hexane	1.53	70.2	64.9; 75.9	25	1.0	±0.1
	CH_2Cl_2	0.84	76.0	71.1; 81.2	25	0.7	±0.2
	EtOAc	2.01	156.3	152.3; 160.4	100	0.9	±0.1
	Water	23.97	>200	/	200	0.7	±0.1
	MeOH (Ph. Eur.)	25.01	>200	/	50	1.1	±0.4
Cissampelos owariensis	Hexane	2.93	122.4	115.0; 130.2	50	0.0	±0.001
	CH_2Cl_2	1.18	20.5	19.7; 21.4	6.25	0,5	±0.1
	EtOAc	1.38	>200	/	100	0.2	±0.03
	Water	6.85	>200	/	200	0.9	±0.1
Heteranthera callifolia	Hexane	0.56	31.6	30. 8; 32.5	6.25	0.6	±0.1
	CH_2Cl_2	0.44	157.9	140.4; 177.6	100	1.1	±0.3
	EtOAc	0.46	199.1	170.4; 232.6	25	0.4	±0.03
	Water	9.39	>200	/	200	0.6	±0.1
Oldenlandia affinis	Hexane	1.95	51.6	50.1; 53.5	12.5	0.5	±0.1
	CH_2Cl_2	1.66	10.4	10.1; 10.6	6.25	0.4	±0.07
	EtOAc	0.71	>200	/	100	0.7	±0.1
	Water	4.25	80.9	79.6; 82.1	50	1.1	±0.2
	Cyclotide-enriched fraction	15.12	72.2	67.8; 77.0	50	2.1	±0.7**
	CH_2Cl_2 fraction	0.65	35.1	33.6; 36.6	25	0.7	±0.07
Parkia biglobosa	Hexane	5.21	33.2	32.3; 34.1	12.5	1.1	±0.3
	CH_2Cl_2	0.64	36.3	35.2; 37.5	12.5	1.0	±0.8
	EtOAc	2.98	>200	/	200	1.3	±0.3
	Water	3.79	>200	/	200	0.6	±0.1
Prosopis africana	Hexane	0.27	73.2	68.1; 78.7	25	0.8	±0.1
	CH_2Cl_2	0.06	124.8	111.4; 139.9	25	1.0	±0.2
	EtOAc	1.06	187.4	177.1; 198.3	100	2.2	±0.6 **
	Water	2.70	>200	/	200	0.6	±0.4
Pterocarpus erinaceus	Hexane	0.48	18.5	17.3; 19.7	6.25	1.2	±0.4
	CH_2Cl_2	0.18	12.5	11.1; 14.2	6.25	0.7	±0.07
	EtOAc	0.26	99.8	90.8; 109.7	12.5	0.5	±0.1
	Water	22.00	262.1	244.5; 281.0	200	7.4	±3.2 **
					100	5.2	±2.8 **
	Water tannins removed	75.73	>200	/	100	2.1	±0.5 **
Rauwolfia vomitoria	Hexane	4.60	>200	/	50	0.2	±0.01
	CH_2Cl_2	1.40	28.0	27.8; 28.3	12.5	1.0	±0.1
	EtOAc	1.98	132.7	128.9; 136.5	25	0.20	±0.04
	Water	6.17	>200	/	200	0.6	±0.2
Trichilia emetica suberosa	Hexane	6.22	>200	/	100	1.5	±0.4 *
	$CHCl_3$	1.37	124.8	120.8; 129.0	25	0.7	±0.08
	EtOAc	0.73	204.2	193.1; 216.0	50	1.1	±0.1
	Water	12.14	>200	/	200	1.2	±0.4
Non-treated CHO-APP cells						1	±0.2
CHO-APP cells without G418						0.8	±0.1
Cycloheximide			0.41	0.35; 0.48	/	/	
EGb 761 (Ginkgo)			>200	/	200	1.0	±0.1
DAPT			n.d.	n.d.	0.108 (i.e. 250 nM)	8.3	±3.9**

[a] Yields are calculated according to the weight of plant powder, excepted for the *Pterocarpus erinaceus* tannin-free aqueous fraction, for which it was calculated according to the weight of crude aqueous extract.
[b] Chosen concentrations provide at least 80% of viability after 72 h treatment.
[c] CTF/APP ratios significantly superior to control cells are indicated by **($p < 0.01$ with Student's *t*-test) or by *($p < 0.05$) ($n = 3$).

Penicillin-Streptomycin (ana 100 UI/ml) and Amphotericin B (2.6 μg/ml). Cells were treated and MTT colorometric assay was performed as previously described by Block et al. (2002). In brief, cells were counted and seeded in 96-well plates (10,000 cells per well in 100 μl of medium); one day after, medium was removed and replaced by 200 μl fresh medium containing the extracts at various concentrations. Each plate contained control wells (non-treated cells); cycloheximide treatments were used as reference cytotoxic conditions and toxicity of the solvents was assayed at their maximal tested concentrations.

72 h after treatment, medium was removed and replaced by 100 μl of a 0.03% MTT solution in culture medium without FBS for 45 min at room temperature; then, MTT solution was removed and replaced by 100 μl DMSO. Absorbance was read at 570 and 620 nm, using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) with the Soft Max Pro program.

Data were analyzed on Graph Pad Prism 4.0, by non-linear regression to determine the IC_{50}. Non-toxic concentrations were determined individually as maintaining at least 80% of viability.

2.5. Cell line for activity screening tests

We used CHO cells stably expressing human APP695 protein (Essalmani et al., 1996). These CHO-APP cells were cultivated as described by Octave et al. (2000), in Ham-F12 medium supplemented with FBS (10%), Penicillin-Streptomycin (ana 100 UI/ml) and with G418 as selection agent (250 μg/ml).

Cells were seeded in 6-well plates (300,000 cells/well). One day after, we treated them by replacing their medium with new medium containing our extract solutions at the maximal non-toxic concentration, or DAPT at 250 nM (0.108 μg/ml), or EGb761 at 200μg/ml. Sixteen hours after treatment, extracel-

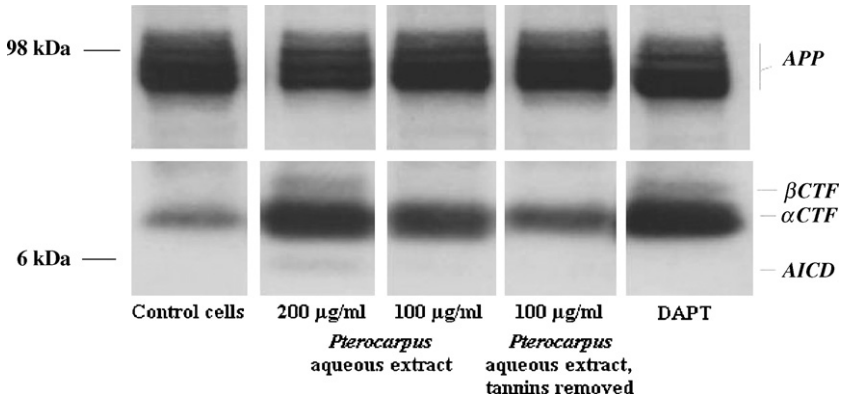

Fig. 1. Western-blot bands of CHO cells expressing APP, treated with DAPT or *Pterocarpus erinaceus* aqueous extract. Cell-lysates of CHO cells expressing human APP695 were analyzed by Western-blotting with the C-ter antibody, directed against the 17 C-terminal residues of APP. Cells were treated for 16 h with *Pterocarpus erinaceus* extracts at indicated concentrations or with 250 nM DAPT, a functional γ-secretase inhibitor. The expected positions of full-length APP (APP), α- and β-cleaved C-terminal fragments (α-CTF and β-CTF) and soluble APP Intracellular C-terminal Domain (AICD) are indicated.

lular media were pipeted and frozen for Aβ measurements, whereas cells were scraped in PBS and recovered by centrifugation (28,000 × *g*).

Protein analysis by Western-blotting (10% SDS-PAGE) was realized as previously described by Huysseyne et al. (2007). In brief, cells were lysed by 4 s sonication in Laemmli lysis buffer and were loaded (5 μg of protein) onto 10–20% bis-tris gels, and blotted onto nitrocellulose membranes; membranes were then saturated (5% skimmed milk in 0.05% Tween 20/PBS) for 30 min, washed, incubated overnight at 4 °C with the primary antibody (diluted in 0.05% Tween 20/PBS) at the following concentrations: 0.5 μg/ml for the human APP-specific WO-2 antibody and 1:5000 for the anti-APP C-terminal antibody. Membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1/10,000 of the anti-mouse sheep IgG when primary antibody was WO-2, 1/15,000 of the anti-rabbit donkey IgG when primary antibody was anti-C-terminal). After washing, the immunoreactive bands were visualized by chemiluminescence (ECL revelation). Signals were quantified using the Quantity One software coupled to the Gel Doc 2000 device (Bio-Rad, Hercules, CA).

2.6. Aβ measurements

In extracellular media of treated or non-treated cells, we measured the concentration of three species of β-amyloid peptide: Aβ38, Aβ40, Aβ42, by Electro-Chemiluminescence Immuno-Assay (ECLIA). Its principle was described by Best et al. (2005), and the assays were performed according to the manufacturer's instructions. Briefly, samples were analyzed using Meso Scale Discovery (MSD) SECTOR™ Imager 2400 (Meso Scale Discovery, Gaithersburg, MD, USA), with the Human Aβ triplex kit (also from MSD); carbone 96-well plates contained in each well four capture spots, one of which is blocked with BSA (as standard curve control), and the three others coated with isoform specific anti-Aβ antibodies specific for Aβ38, Aβ40, Aβ42, respectively. 100 μl of blocking buffer solution were added to all wells to avoid non-specific binding. The plates were then sealed, wrapped in tin foil, and incubated at room temperature on a plate shaker (600 rpm) for 1 h. At the end of the incubation, the wells were washed three times with washing buffer, and 25 μl of the standards (Aβ38, Aβ40, Aβ42) and samples were then added to the wells, followed by an Aβ-detecting antibody at 1 μg/ml (MSD) labelled with a Ruthenium (II) trisbipyridine N-hydroxysuccinimide ester; this detection antibody is either 4G8 (which recognizes the epitope Aβ18-22 of the human and rodent peptide) or 6E10 (recognizing specifically amino-acids 3–8 of human Aβ). Plates were then aspirated and washed 3 times; MSD read buffer (containing TPA) was added to wells before reading on the Sector Imager; a small electric current through the microelectrodes present in each well produces a redox reaction of the Ru^{2+} cation, with emission of a measured 620 nm red light ray. Using dose–response curves, we calculated for each sample the concentration of each Aβ isoform, the blank being *cell-less* culture medium.

2.7. Elimination of tannins

Pterocarpus erinaceus aqueous extract was impoverished in tannins by retention of these compounds on polyamide column, as described by Houghton and Raman (1998).

2.8. Statistical analysis

The number of samples (*n*) under each experimental condition is indicated in the legends of tables or figures. When two experimental conditions were compared, statistical analysis was performed via unpaired *t*-test. Otherwise, statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Dunnett's multiple-comparisons post-test (Prism 4.0, GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Cytotoxicity tests

The 39 extracts prepared as described in Section 2 (yields are given in Table 2) were tested on CHO cells for their potential cytotoxicity, to exclude that our further results on Aβ production were due to toxicity. We tested each extract during 72 h at least at six different concentrations in at least 12 wells; we determinated for each extract the IC_{50}, the concentration at which 50% of mortality is observed, and the to-be-used concentration as the highest concentration tested with more than 80% viability. Results are given in Table 2.

The results obtained with the reference cytotoxic compound (cycloheximide) and the standardized *Ginkgo biloba* extract (EGb 761) are also given in Table 2.

3.2. Test on APP cleavage

Each extract was then tested at the highest not toxic concentration on CHO-APP cells during 16 h; the medium was then collected

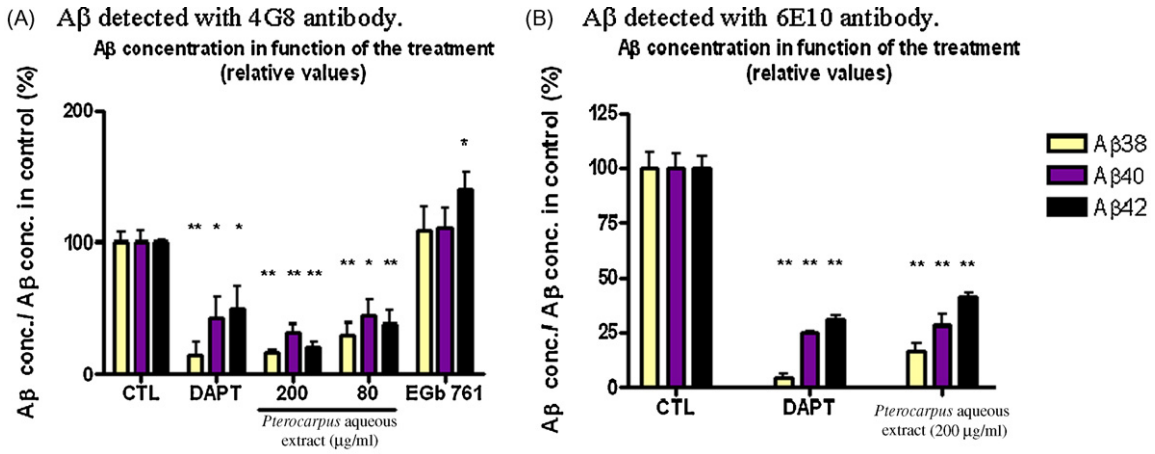

Fig. 2. Measure of Aβ concentration in extracellular medium. (A) Aβ detected with 4G8 antibody. (B) Aβ detected with 6E10 antibody. CHO-APP cells were treated 16 h with DAPT (250 nM), *Pterocarpus erinaceus* aqueous extract (200 or 80 μg/ml) or *Ginkgo biloba* standardized extract (EGb761, 200 μg/ml); Aβ species were quantified in the extracellular medium using ECLIA method, and results were compared to media of non-treated cells (CTL). Each result was compared to control by a Student *t*-test; $^{*}p < 0.05$; $^{**}p < 0.01$ ($n = 3$).

for Aβ quantification. Western-blots of cell-lysates were performed to analyze the amounts of cellular full-length APP (around 98 kDa) and of its C-terminal stubs (α-CTFs and β-CTFs, between 6 and 10 kDa). An increase of the CTF/APP ratio is generally considered as the hallmark of a decreased γ-cleavage of the CTFs, and thus an indicator of decreased γ-secretase activity in the cells (Kienlen-Campard et al., 2008).

Among all the tested extracts, *Pterocarpus erinaceus* aqueous extract gives the highest normalized CTF/APP ratio (5.2 ± 2.8 at 100 μg/ml); some other extracts as *Prosopis africana* EtOAc extract, *Trichilia emetica* hexanic extract and the cyclotide-enriched fraction of *Oldenlandia affinis*, give significantly high CTF/APP ratios, too.

Ginkgo biloba extract does not provide modifications *vs.* the control pattern. A very significant increase in CTF/APP ratio (see Fig. 1) was also observed as expected with DAPT (8.3 ± 3.9), a well-known γ-secretase inhibitor (Dovey et al., 2001).

3.3. Measure of produced Aβ for Pterocarpus erinaceus

Using ECLIA, we measured Aβ concentration in the extracellular medium of cells treated with *Pterocarpus erinaceus* aqueous extract, EGb761 or DAPT. We showed (Fig. 2) a significant decrease of Aβ38, Aβ40 and Aβ42, either using 4G8 or 6E10 antibodies, for *Pterocarpus erinaceus* extracts at 200 μg/ml. The decrease was also visible at 80 μg/ml, with values similar to those of DAPT at 250 nM. EGb761 did not decrease Aβ production, and even increased Aβ42 concentration.

4. Discussion

The cytotoxicity test on CHO cells showed that most hydrophobic extracts have some cytotoxicity, and that some of them, being clearly cytotoxic ($IC_{50} \leq 20$ μg/ml), may have some interest for the isolation of anticancer compounds or some risk of toxicity, especially *Cissampelos owariensis* CH_2Cl_2 extract, *Pterocarpus erinaceus* hexane and CH_2Cl_2 extracts. It is not the case for most water and EtOAc extracts, which may be considered as not toxic.

4.1. Centella asiatica

The Indian Pennywort (*Centella asiatica* (L.) Urban) is a small herbaceous plant of Indian origin, already known in Ayurvedic medicine, and widely used, especially in Asia and Madagascar, as vulnerary, vasoprotector, anti-ulcerous and pro-cognitive, effects now mainly attributed to its triterpenes (Zheng and Qin, 2007). Recent studies of leaf extracts have shown not only *in vivo* spatial learning enhancement and memory retention, but also interesting tracks to an explanation of the traditional use as memory enhancer: extract given to 7-day old rat pups during 6 weeks increased the length and arborization of the dendrites of hippocampal (CA3 regions) neurons implicated in memorization (Mohandas Rao et al., 2006), and extract given *per os* to PS-APP mice (5 mg/kg/day during 8 months, beginning 2 months before the onset of amyloid plaques) diminishes Aβ in the hippocampus and fibrillar amyloid plaques in the cortex (Dhanasekaran et al., 2009).

In our results however, we obtained no significant effect on CTF/APP ratio, even with the methanolic extract. It should be noticed that the *in vivo* Aβ reduction mentioned by Dhanasekaran et al. (2009), took place after 8 months of treatment, and was not visible after 2 months; such treatment length is impossible in our screening test.

4.2. Cissampelos owariensis and Heteranthera callifolia

Mud-plantain (*Heteranthera callifolia* Rchb. ex Kunth), a small aquatic flower, and the twiner *Cissampelos owariensis* P. Beauvais ex D.C. are used together in decoction in Benin against dementia-type loss of memory. *Cissampelos owariensis* is also used in Benin against circulatory gynecological problems, against asthenia and (in association with *Rauwolfia vomitoria*) against madness (Adjanohoun et al., 1989). In the other African countries where *Cissampelos owariensis* is present, it is used for several purposes: against human and animal diarrhoea, to cure wounds or snake-bits, and, in Tanzania, against amnesia and psychoses (Schmelzer, 2008). No study has been made on these plants.

In our test, we did not observe any effect on APP processing for *Cissampelos owariensis* and *Heteranthera callifolia* extracts.

4.3. Oldenlandia affinis

Oldenlandia affinis (R. & S.) D.C. is used in Benin mixed in honey against learning retardation and in aqueous decoction as anticolic drink (Adjanohoun et al., 1989); it is also used in Centrafrican Republic and Congo D.R. (Zaire) as an ocytocic infusion (Gran et al., 2000). Interestingly, the plant was shown to contain, beside serotonin, several cyclotides, cyclic proteins of ab. 40 amino-acids, with three Cys–Cys bridges responsible for their special, compact structure, and first isolated from *Oldenlandia affinis*; cyclotides are

proteolysis-resistant, can cross the digestive barrier and have an ocytocin-like effect on uterine receptors (Gran et al., 2000). The interaction of cyclotides with β-amyloid peptide (which has the same length as cyclotides) has not been investigated.

In our study, cyclotide-enriched fraction of the MeOH-water maceration (but not the other extracts) raised significantly the CTF/APP ratio (2.1 ± 0.7).

4.4. Parkia biglobosa

Parkia biglobosa (Jacq.) R.Br. ex G. Don f. is an African tree; it is used in Benin against madness (twigs), against hypertension and hemorrhoids (bark), against abcesses (leaves), against snake-bits (also in Nigeria) (Adjanohoun et al., 1989; Asuzu and Harvey, 2003); fruits are used as mosquito repellents in Guinea Bissau (Pålsson and Jaenson, 1999); fruit aqueous extract shows antidiabetic activity (Odetola et al., 2006).

Our results showed that EtOAc twig extract provided a slight but not significant increase in CTF/APP ratio (1.3 ± 0.3).

4.5. Prosopis africana

African Mesquite, *Prosopis africana* (Guill. & Perr.) Taub, is a tree used for different purposes in Benin. Leaved stem decoction is used against epilepsy, bark decoction against dermatose, and heart-wood decoction against learning retardation (Adjanohoun et al., 1989). In other countries, the plant is also largely used against dermatoses and for anemia; leaves, roots and stems have been found to contain alkaloids (Kerharo and Adam, 1966; Khuong-Huu-Qui et al., 1972). This heart-wood, frequently used as *frotte-dents*, is imputrescible, maybe because of the antifungical and anti-insect activity of its extracts; the durability is due to its impenetrability by water: the wood cell lumen is filled by high-rated hydrophobic, non-extractible gums (Gerardin et al., 2004).

In our results, its EtOAc extract raised about twice CTF/APP ratio (2.2 ± 0.6).

4.6. Pterocarpus erinaceus

Barwood or African rosewood, *Pterocarpus erinaceus* Poiret, is a well-known workwood and dyestuff source; in Benin, the stem bark decoction is used against anaemia, dysmenorrhoea, dyssentery and growth or learning retardation. It is also used in Senegal against dyssentery and as breathing improver. The stem bark produces, spontaneously or after incision, a red exsudate, the kino of Gambia, which contains catechic tannins at 60% m/m (Kerharo and Adam, 1966; Adjanohoun et al., 1989).

In our CHO-APP model, *Pterocarpus erinaceus* aqueous extract raised CTF/APP ratio (7.4 ± 3.2 at 200 μg/ml, and 5.2 ± 2.8 at 100 μg/ml). Like DAPT (which gives a 8.3 ± 3.9 CTF/APP ratio), it increases intensity of bands α- and β-CTF on cell-lysates Western-blots; however, unlike DAPT, it does not prevent AICD production (see Fig. 1), although it inhibits Aβ production as strongly as DAPT does at 250 nM (see Fig. 2). These observations joined together could be explained by the inhibition of γ-secretase activity at the γ-site (where Aβ is produced) without inhibition at the ε-site (where AICD is produced). Indeed, recent studies have shown that some compounds can inhibit one cleavage without inhibiting the other, and some mutations of APP have also an unequal effect on the two cleavages (Tolia and De Strooper, 2009). Such an effect on APP has the advantage not to decrease the production of AICD, which is involved in nuclear signaling and participates to APP physiological function by controling gene transcription (Huysseune et al., 2009).

As this aqueous extract is rich in tannins, we also tested it after removal of polyphenols by retention on polyamide; this purified extract did show a reduced but still well significant increase in CTF/APP ratio (2.1 ± 0.5; $p < 0.01$ *vs.* control cells as well as *vs.* crude aqueous extract). It indicates thus that tannins may play a role in the observed activity but are not the only effective compounds.

4.7. Rauwolfia vomitoria

Rauwolfia vomitoria Afzelius is a very well-known plant used in popular medicine in Benin, Ivory Coast and other African countries for several purposes, the main one being always madness (in decoction or brushed in a pasta); the used parts are roots (especially root bark) and, far less, leaves (Kerharo and Adam, 1966; Adjanohoun et al., 1989). It is one of the richest plants in indolic alkaloids (dry roots contain 25% m/m), variously distributed in the organs of the plant, as for example reserpine (hypotensive, bradycardic and sedative) and ajmaline (Kerharo and Adam, 1966). We did not observe any increase in CTF/APP ratio with the leaves.

4.8. Trichilia emetica subsp. suberosa

Trichilia emetica Vahl. subsp. *suberosa* J.J.F.E. De Wilde has received its name from the strong emetic properties of its stem bark (which is also strongly cathartic); leaves are used as anthelminthic, vulnerary, against fever and against trypanosomias and malaria, to relieve rheumatical pain or to treat convulsions or school retardation (Hoet, 2005); the roots contain phenolic acids (Germano et al., 2006), whereas leaves were studied for their polysaccharides (Diallo et al., 2003). In our study, hexanic extract of the leaves showed a significant increase of CTF/APP ratio (1.5 ± 0.4).

5. Conclusion

Our study presents elements indicating that some of these plants, used in Beninese and Malagasy traditional medicine to treat cognitive problems, contain compounds affecting the metabolism of APP, the precursor of Aβ peptide, the accumulation of which is characteristic of Alzheimer's disease. Especially, *Pterocarpus erinaceus* bark aqueous extract could be a promising object of study against Alzheimer's disease, due to the fact that it decreases β-amyloid peptide production; the next step will be the isolation and identification by bioguided fractionation of the constituents responsible for the observed *in vitro* activities.

Other extracts (*Prosopis africana* EtOAc and *Trichilia emetica* hexane, as well as *Oldenlandia affinis* cyclotide-enriched fraction) were also shown to significantly increase the CTF/APP ratio, an indicator of a possible inhibition of γ-secretase.

However, as this study provides only results obtained on cell cultures, we are aware that further studies should also be carried out in animals, to assess the activity but also the bioavailability of the isolated tested compounds or extracts, which is especially critical for an action in the central nervous system.

It must also be noted that the other extracts could be useful against memory problems, and even against Alzheimer's disease progression, without having shown any activity in our study. Their molecules could need a metabolic transformation before being active, or act upon other mechanisms implicated in Alzheimer's disease (for example, against amyloid aggregation, or against Aβ-induced neurotoxicity or oxidative stress). They could also act on dementia-related symptoms by enhancing cholinergic transmission like several Asian or European plants traditionally used for memory loss and now known for highly neuroprotective antioxidants and acetylcholine-esterase inhibitors (Perry et al., 1998; Howes and Houghton, 2003). Alzheimer's disease is also a very slow process, and effects may be only visible with long-term treatments, which are not possible on *in vitro* tests.

Finally, we found the *Pterocarpus erinaceus* aqueous extract to be a potent inhibitor of Aβ release in CHO cells expressing human

APP, and that this effect is still significant after removal of the tannins. This prompts us to further investigate precisely how the active compounds presents in the extract inhibit Aβ production in order to evaluate their therapeutic interest.

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