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Effect of reverse osmosis concentration coupled with drying processes on polyphenols and antioxidant activity obtained from *Tectona grandis* leaf aqueous extracts



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ABSTRACT

Tectona grandis leaf extracts obtained at pilot scale processes (ultrasound-assisted extraction, cross-flow microfiltration, reverse osmosis concentration) contains phenolic compounds that exhibit antioxidant properties. The final reverse osmosis concentrate extract presented higher content of polyphenol ($21,080 \pm 117 \mu\text{mol g}^{-1}$ GAE) and antioxidant capacity ($8490 \pm 29 \mu\text{mol g}^{-1}$ TE) comparatively to crude extract ($1300 \pm 12 \mu\text{mol g}^{-1}$ GAE for polyphenol and $430 \pm 2 \mu\text{mol g}^{-1}$ TE for antioxidant activity) or cross flow microfiltration extract ($1170 \pm 10 \mu\text{mol g}^{-1}$ GAE for polyphenol and $400 \pm 10 \mu\text{mol g}^{-1}$ TE for antioxidant). The concentration factors of polyphenol and antioxidant capacity were 18 and 21, respectively. High-performance liquid chromatography (HPLC) coupled to electrospray ionization mass spectrometry (ESI-MS) detection negative ion mode has been used to identify and characterized polyphenols in the concentrate extract of *T. grandis* leaves. Seven phenolic acids and flavonoids were characterized. Verbascoside (phenolic acid) was described as the most abundant phenolic compounds in this concentrate extract. Two drying technologies (freeze-drying and spray-drying) were used to obtained stable powder from concentrate extract. The effect of these drying technologies on phenolic compounds and antioxidant activity were studied. Freeze-drying presented a good recovery of phenolic compounds and antioxidant capacity. This drying technology could be used for preservation of *T. grandis* extract.

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1. Introduction

Tectona grandis L. (Verbenaceae), commonly named teak is used in folk medicine for a wide variety of

remedies (Aradhana et al., 2010; Tra Bi et al., 2008). Several pharmacological activities have been attributed to *T. grandis* extracts, mainly antidiabetic activity (Ghaisas et al., 2010; Pooja and Samanta, 2011) and antioxidant activity (Naira and Karvekar, 2011; Rao et al., 2011). Previous studies revealed the presence of phenolic compounds on the extracts of *T. grandis* leaves such flavonoids and phenolic acids (Naira and Karvekar, 2010; Shukla et al., 2010).

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Polyphenols are of a great interest due to their beneficial effect for human health: prevention and treatment of certain cancers, inflammatory diseases, cardiovascular and neurodegenerative diseases (Pandey and Rizvi, 2009). They have often been identified as active principles of numerous folk herbal medicines (Apak et al., 2007).

Local populations of Côte d'Ivoire has been extracted these bioactive compounds from plant materials by decoction or infusion with water as solvent. Many studies have showed that polyphenol contents in aqueous extracts were unstable during storage (Chedea et al., 2011; Malick and Bradford, 2008). In this study, a pilot plant scale processing of dried plant leaves was set to produce stabilized extracts in a powder form to increase the shelf-life of the ready to use medicinal product. The process includes several steps such as ultrasound-assisted water-maceration of dried leaves, membrane filtration and concentration of the extract and stabilization of the concentrate extract by spray-drying or freeze-drying. The effect of stabilization processes on polyphenol contents and antioxidant capacity was studied. Polyphenol contents were also identified and characterized by HPLC coupled to UV–vis diode array detection and mass spectrometry with electrospray ionization (LC/DAD/ESI-MS²).

2. Material and methods

2.1. Plant material

Leaves of *T. grandis* were collected from teak plantations in the centre of Côte d'Ivoire around Yamoussoukro area. After harvesting, the leaves were brought to LAPISEN laboratory (Yamoussoukro, Côte d'Ivoire) for drying at an average temperature of 30 °C during day time, and kept away from direct sun exposure under an open-sided shed. The dried leaves were packed in plastic bags and shipped to CIRAD laboratory (Montpellier, France), where they were stored at 4 °C until processed and analyzed.

2.2. Chemicals

All reagents were of analytical grade. Sodium hydroxide (NaOH), sodium carbonate salt (Na₂CO₃), monohydrated citric acid, dihydrated monosodium phosphate (NaH₂PO₄, 2H₂O), disodium hydrogen phosphate (Na₂HPO₄), Folin-Ciocalteu's phenol reagent were purchased from Carlo Erba (Spain).

Gallic acid, quercetin, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-1-carboxylic acid), fluorescein, AAPH (2,2'-azobis (2-methylpropanimidamide) dihydrochloride), luteolin, caffeic and chlorogenic acids were purchased from Sigma-Aldrich (Germany).

2.3. Pilot plant extraction and extract concentration

Ultrasound-assisted extraction of dried leaves (1.65 kg) was performed with 100 L of acidified tap water (H₂O, 0.01 N citric acid) during 40 min using ultrasonic (US) pilot plant unit equipped with anchor-shape and slow-motion stirrer (40 kHz US frequency, 200–500 W US variable power, REUS, Contes, France). The water extract obtained

was filtered using a nylon cloth to give a crude extract (CE), which was then clarified by Cross-flow microfiltration (CFM) to give a CFM permeate volume (V_{CFM}) of about 92 L, which was then concentrate by reverse osmosis (RO) at a constant trans-membrane pressure of 40 bar. The final volume of the RO concentrate extract obtained (V_{RO}) was generally 3 L, which was close to that of the dead volume of the RO pilot plant unit. The performance of this RO concentration step was characterized by calculating concentration factor:

$$CF = \frac{TSP_{RO}}{TSP_{CFM}}$$

where CF, concentration factor; TSP_{RO}, total soluble polyphenol content in the RO concentrate and TSP_{CFM}, total soluble polyphenol content in the TSP_{CFM} permeates.

2.4. Freeze drying process

Reverse osmosis (RO) concentrate extract were frozen at –30 °C in cold room and dried using freeze dryer (type cryonext, France) for 48 h. Sample temperature was set at –80 °C and the pressure was set less than 0.2 bar.

2.5. Spray drying process

RO concentrate extracts were dried using spray dryer (Minispray Dryer, B-290 Minispray Dryer) with 120 °C of an inlet air temperature and 60 °C of an outlet air temperature.

2.6. Total polyphenol content

Total polyphenol content was determined by colorimetry, using the Folin-Ciocalteu (F-C) method (Singleton and Rossi, 1965; Wood et al., 2002). To 30 μL sample extract, 2.5 mL of diluted Folin-Ciocalteu's phenol reagent (1/10) were added. After 2 min of incubation in the dark at room temperature, 2 mL of aqueous sodium carbonate (75 g L⁻¹) were added. After slight stirring, the mixture was put in a water bath at 50 °C for 15 min then cooled down. The absorbance was measured at λ = 760 nm using a UV–vis spectrophotometer (Jenway 6705, Barloworld Scientific SAS, France). Total polyphenol content was expressed as μmol GAE (Gallic Acid Equivalent) per gram of dried leaves water-extracted. Samples were analyzed in triplicate.

2.7. Antioxidant capacity

Antioxidant capacity was carried by oxygen radical absorbance capacity (ORAC) assay. The ORAC method used was described by Ou et al. (2001). The automated ORAC assay was carried out on a VICTOR™ X3 Multilabel Plate Reader (Perkin–Elmer, USA) with fluorescence filters for an excitation wavelength at 485 nm and an emission wavelength at 535 nm (Zulueta et al., 2009). The reaction was performed at 37 °C as the reaction was started by thermal decomposition of AAPH in 75 mmol L⁻¹ phosphate buffer (pH 7.4). A stock solution of fluorescein (FL) was prepared by weighing 22 mg of FL, dissolving it in 100 mL of phosphate buffer (PBS) (75 mmol L⁻¹, pH 7.4), and then storing it in complete darkness under refrigeration conditions. The

working solution (78 nmol L^{-1}) was prepared daily by dilution of 0.334 mL of the stock solution in 25 mL of phosphate buffer. The AAPH radical (221 mmol L^{-1}) was prepared daily by taking 0.6 g of AAPH and making it up to 10 mL with PBS. 100 μL of FL and 100 μL of diluted sample, PBS or standard (Trolox 5–50 $\mu\text{mol L}^{-1}$) were placed in each well of a 96 well-plate and pre-incubated during 15 min. After, 50 μL of AAPH were added into the wells. The fluorescence was measured every minute during 60 min with emission and excitation wavelength of 485 and 535 nm, respectively, which was maintained at 37 °C. The ORAC values were calculated as area under the curve (AUC) and were expressed as $\mu\text{mol TE}$ (Trolox Equivalent) per gram of dried leaves water-extracted. Samples were analyzed in triplicate.

2.8. HPLC–ESI–SM analyses

An ion trap mass spectrometer (Bruker Daltonics Amazon, Bremen, Germany) was connected via an electrospray ionization (ESI) interface for high performance liquid chromatography–tandem mass spectrometry (HPLC–SM²) to UPLC–DAD (Waters Acquity, Milford, MA) equipped with a RP18 column (Acquity BEH column, 10 mm \times 1 mm, 1.7 μm particle size, Waters, Milford, MA) placed in a controlled temperature oven set at 35 °C. The injection volume was 0.5 μL . The mobile phase was a binary solvent system of A (water:formic acid, 99:1, v/v) and B (methanol:formic acid, 99:1, v/v). The multi-linear gradient profile was: 2% B from start to 1 min, 2–30% B, from 1 to 10 min, 30% B from 10 to 12 min, 30–75% B from 12 to 25 min, 75–90% B from 25 to 30 min, and 90% B from 30 to 35 min. The elution flow rate was set at 0.08 mL min^{-1} . The mass spectrometer operated in negative ion mode (capillary voltage: 2.5 kV; end plate off set: –500 V; temperature: 200 °C; nebulizer gas: 10 psi and dry gas: 5 L min^{-1} ; collision energy for fragmentation in MS/MS set at 1). Polyphenols were detected at 280 nm. UV–vis spectra were recorded from 210 nm to 600 nm. The data analysis software was used for data acquisition and processing.

2.9. Statistical analysis

Results were expressed as mean \pm standard deviation of three replicate. Data were evaluated by one-way analysis of variance (ANOVA) using *Statistica 7.1* (StatSoft, Inc., USA) software. Newman-keuls test was performed to determine significant differences at $p < 0.05$.

3. Results and discussion

3.1. Extraction and concentration process

The ultrasound-assisted extract of *T. grandis* leaves obtained in pilot scale was clarified by cross flow micro-filtration (CFM) and then concentrated by reverse osmosis (RO). Table 1 presents total polyphenol and antioxidant content from the three co-products (crude extract–CFM extract–concentrate RO retentate). The amounts of polyphenols and antioxidants were higher in RO concentrate extract than crude extract or CFM extract. The

Table 1

Total polyphenol contents and antioxidant activity of *T. grandis* L. leaf extract.

Process co-products	Total polyphenols ($\mu\text{mol g}^{-1}$ GAE)	Antioxidant capacity ($\mu\text{mol g}^{-1}$ TE)
Crude extract	1300 \pm 12 ^a	430 \pm 2 ^a
CFM permeate	1170 \pm 10 ^a	400 \pm 10 ^a
RO concentrate	21,080 \pm 117 ^b	8490 \pm 29 ^b
CF	18	21

GAE, gallic acid equivalent; TE, trolox equivalent; CFM, cross flow membrane; RO, reverse osmosis; CF, concentration factor. For each column, letters equals indicate that the means difference is not significant at $p < 0.05$.

concentration factors (CF) of total polyphenol and antioxidant capacity were 18 and 21; respectively. The amount of polyphenols and antioxidant capacity obtained in CFM extract was lower than those obtained in crude extract. Statistical analysis did not show significant differences at $p < 0.05$ between the considered values. These results indicate that concentration process did not degrade polyphenols and antioxidant activity from *T. grandis* leaves, as found by Adjé et al. (2012).

3.2. Identification of phenolic compounds from concentrate RO extract

HPLC–DAD profile of polyphenols from RO concentrate extract was shown in Fig. 1. Phenolic acids and flavonoids were identified.

3.2.1. Phenolic acids identification

The molecular structures of phenolic acids in *T. grandis* leaves extract, were confirmed on the basis of their LC–MS fragmentation MS, MS² and MS³ and on the shape of their UV–vis spectra as shown in Table 2, and were compared with previous published studies. Seven phenolic acids were identified in aqueous extract of *T. grandis* leaves.

Compound 1 gave $[\text{M} - \text{H}]^-$ at m/z 153 with UV–vis λ_{max} at 260 and 294 nm spectra are typical for protocatechuic acid. Compounds 2 and 7 had $[\text{M} - \text{H}]^-$ at m/z = 353. The MS² fragmentation patterns produced ions at m/z 191, 179, 135, corresponding to 3-*O*-caffeoylquinic acid (Compound 2). The fragmentation at m/z 191 ($[\text{M} - \text{H} - 162]$), 179 ($[\text{M} - \text{H} - 174]$), 173 ($[\text{M} - \text{H} - 180]$) and 135 ($[\text{M} - \text{H} - 218]$) were characteristics of 4-*O*-caffeoylquinic acid (Stalmach et al., 2009). The product ions m/z 191 for quinic acid and 179 for caffeic acid revealed the constituent of 3-CQA prior to condensation. Loss of a caffeoyl moiety yielded the other dominant fragment ion m/z 173. The structure of 4-CQA (Compound 7) was confirmed by co-elution with the standard. Compound 3 was identified as 2-*O*-caffeoylhydroxycitric acid with m/z^- at 369 and MS² at 207 after loss of 162 amu (caffeoyl moiety). A similar fragmentation was reported by Parveen et al. (2008). Compound 4 with $[\text{M} - \text{H}]^-$ at m/z 487 and a fragment at 179 (caffeic acid) obtained after loss 308 amu. In MS² fragmentation, it observed that compound 3 lost neutral mass ion at m/z 44 corresponding to loss of CO₂. So, compound should be identified as caffeoyl acid derivative. Compound 6, which UV–vis λ_{max} at 325 nm spectra and m/z^- at 179 are

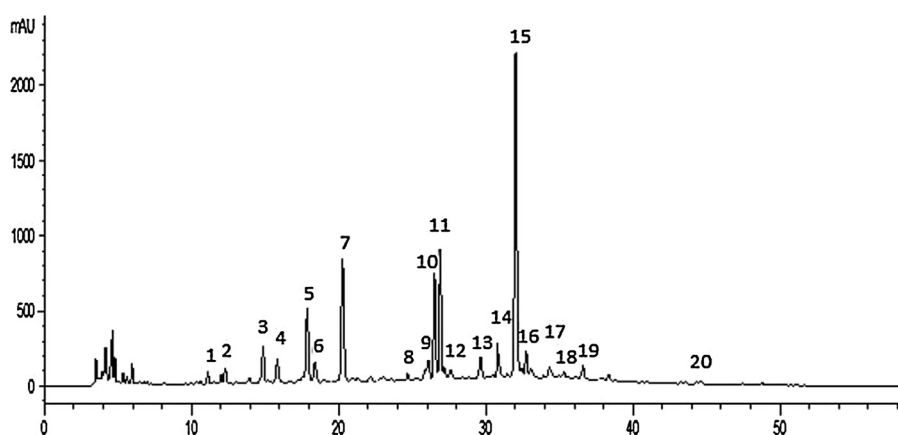


Fig. 1. HPLC chromatogram obtained at 280 nm for aqueous extract of *T. grandis* leaves.

Table 2

LC–MS data obtained from the analysis of *T. grandis* leaves extract.

Peak	Compounds	λ max	MW	$[M-H]^+$	Fragments	Neutral loss	Percentage (%)
1	Protocatechuic acid	296	154	153			0.9
2	3- <i>O</i> -caffeoyl quinic (3-CQA)	324	354	353	191-179-(135)		1.3
3	2- <i>O</i> -caffeoylhydroxycitric acid	326	370	369	207	162	3.2
4	Caffeoyl acid derivative	326	488	487	179-135	308-44	2.2
6	Caffeic acid	297sh-325	180	179			1.8
7	4- <i>O</i> -caffeoyl quinic (4-CQA)	326	354	353	173-191-179-135	180-162-174-218	12.2
10	Apigenin 7- <i>O</i> -diglucuronide	269–330	624	623	447 (271)	176 (2 \times 176)	8.0
11	Luteolin 7- <i>O</i> -diglucuronide	255–349	638	637	351-(285-193)	286-(352-444)	9.5
14	luteolin 7- <i>O</i> -glucuronide	289sh-333	462	461	285	176	2.8
15	Verbascoside	289sh-333	624	623	461-315	162-308	31
16	Luteolin diglucuronide	269–340	637	637	461–285	176*2	2.3
17	Apigenin glucuronide	266–336	446	445	269	176	1.0
19	Luteolin glucuronide	268–340	462	461	285	176	0.9
20	Luteolin	254–349	286	285	175-239-241		0.4

typical for caffeic acid (Fernandes et al., 2011). The nature of this compound was confirmed by co-elution with the standard. Caffeic acid was already reported in *T. grandis* extract (Nayeem and Karvekar, 2010). Compound 15 gave $[M-H]^-$ at 623 and fragments at 461 amu and 315 amu after loss of 162 amu (caffeoyl moiety) and 308 amu (rhamnoside moiety), respectively. Its UV–vis spectrum showed a maximal at $\lambda_{max} = 333$ with a shoulder at 289sh typical for verbascoside (Petreska et al., 2011). Verbascoside was identified as the most abundant compound (30% of total peak area at 280 nm) in extract of *T. grandis* leaves. Previous study showed significant antihyperglycemic activity of verbascoside (Shukla et al., 2010). This property can be beneficial for the treatment of diabetics. Other studies assigned to this compound antioxidant, anti-inflammatory,

photo-protective and anti-gastric ulcer activities (Singh et al., 2010; Vertuani et al., 2011). So, leaves which are the discharges of wood industries could be interested by pharmaceutical or cosmetic industries.

3.2.2. Flavonoid identification

Seven flavonoids were identified on between basis of HPLC coupled to UV–vis diode array detection and mass spectrometry with electrospray ionization (LC/DAD/ESI-MS²).

The molecular ion peak $[M-H]^-$ for the compound 11 was m/z 623 with fragments ions at m/z 447 ($[M-H-176]^-$) and 271 ($[M-H-176-176]^-$), typical fragments of apigenin 7-*O*-diglucuronide (Meng et al., 2006). Compounds 12 and 16 were assigned to luteolin diglucuronide. HPLC-SM

Table 3

Effect of drying processes on polyphenol contents and antioxidant activity.

Co-product	Polyphenol		Antioxidant	
	Content ($\mu\text{mol g}^{-1}$ GAE)	Recovery (%)	Capacity ($\mu\text{mol g}^{-1}$ TE)	Recovery (%)
RO concentrate	21,080 \pm 117 ^a	100.0	8490 \pm 29 ^a	100.0
Powder 1	18,170 \pm 75 ^b	86.19	6980 \pm 12 ^b	82.21
Powder 2	13,960 \pm 38 ^c	66.22	5210 \pm 5 ^c	61.36

RO, reverse osmosis; GAE, gallic acid equivalent; TE, trolox equivalent. For each column, letters equals indicate that the means difference is not significant at $p < 0.05$.

characteristics indicated in SM¹, m/z at 637 [M–H][–] with fragments ions at m/z 461([M–H–176][–] and 285 ([M–H–(2 × 176)][–]), respectively after loss of one glucuronic acid and two glucuronic acids. Compound 14 with [M–H][–] at m/z 461 and a fragment at 285 (luteolin) obtained after loss 176 (glucuronic acid) was assigned to luteolin 7-*O*-glucuronide. A similar fragmentation of the compound was reported by Johnson et al. (2011) in *Russelia equisetiformis* extract. Compound 18 was identified as apigenin glucuronide showed the loss of a glucuronic acid (m/z 176) and produced the predominant fragment at m/z 269 corresponding to deprotonated apigenin. Similar fragmentation of the compound was reported by Zimmermann et al. (2011) when analysing *Salvia officinalis* L. extracts. Compound 19 was luteolin glucuronide with m/z at 461 and MS² ion at 285 (luteolin) due to the loss of 176 amu corresponding to glucuronic acid. The similar fragmentation has previously been by Patora and Klimek (2002) from the leaves of *Melissa officinalis*. Compound 20 had a [M–H][–] ion at m/z 285 and was assigned to luteolin aglycone. The co-elution with a standard confirmed the presence of luteolin.

Many studies were investigated polyphenol contents of *T. grandis* leaf extracts. Among of polyphenol identified only chlorogenic acid (Ghareeb et al., 2013), caffeic acid (Naira and Karvekar, 2010; Shalini and Rachana, 2009), verbascoside (Shukla et al., 2010; Singh et al., 2010) and luteolin (Shukla et al., 2010) were identified during previous studies. Others phenolic compounds were reported for the first time (namely protocatechuic acid, 2-*O*-caffeoylhydroxycitric acid, Caffeoyl acid derivative, 4-*O*-caffeoyl quinic acid, apigenin 7-*O*-diglucuronide, luteolin 7-*O*-diglucuronide, luteolin glucuronide, luteolin diglucuronide, apigenin glucuronide, luteolin glucuronide). The composition in polyphenol of our extracts was for greater part different from those of previous studies. Manah et al. (2004) was reported that polyphenol contents of plants were affected by numerous factors. These factors include genetic, ripeness at time harvest, environmental factors (soil type, sun exposure, and rainfall), processing, and storage.

3.3. Effect of drying processes on reverse osmosis concentrate extract

The reverse osmosis concentrate extracts were dried by freeze-drying and spray-drying to obtain powder 1 and 2, respectively. The powders obtained are all brown. As shown in Table 3, the effect of drying processes on polyphenols and antioxidant capacity from reverse osmosis (RO) concentrate extract. The amounts of polyphenols obtained after drying process are lower than those of the concentrate extract: powder 2 ($13,960 \pm 38 \mu\text{mol g}^{-1}$ GAE) < powder 1 ($18,170 \pm 75 \mu\text{mol g}^{-1}$ GAE) < RO concentrate extract ($21,080 \pm 117 \mu\text{mol g}^{-1}$ GAE). The amount of antioxidant capacity of powder 2 ($5210 \pm 5 \mu\text{mol g}^{-1}$ TE) was also lower than those of powder 1 ($6980 \pm 12 \mu\text{mol g}^{-1}$ TE) and RO concentrate extract ($8490 \pm 29 \mu\text{mol g}^{-1}$ GAE). Recovery of polyphenol contents and antioxidant capacity in powders, were generally better than 61%. Freeze-drying

gave better yields (>82%) than did spray-drying (61–67%). Similar result was reported by Da Silva et al. (2011) when drying propolis. Phaechamud et al. (2012) also were demonstrated that thermal drying process affected significantly the amount of phenolic compounds in extract.

This study showed that freeze-drying is a good process to stabilize phenolic antioxidant from *T. grandis* leaves, as indicated by Munin and Edwards-Lévy (2011). They reported that freeze-dried particles were stable over long periods and provided to polyphenols an effective protection against oxidation phenomenon during their storage, whereas antioxidant activity remained identical.

4. Conclusion

T. grandis leaves extracts obtained at pilot scale contain phenolic compounds that exhibit antioxidant properties. Reverse osmosis concentrate extract have higher amount of phenolic compounds and antioxidant capacity comparatively to crude extract. In this extract, fourteen phenolic compounds as flavonoids and phenolic acids were identified and characterized. The most abundant polyphenol in this extract was verbascoside. Others compounds were reported for the first time in *T. grandis* (namely protocatechuic acid, 3-*O*-caffeoyl quinic acid, 2-*O*-caffeoylhydroxycitric acid, Caffeoyl acid derivative, 4-*O*-caffeoyl quinic acid, apigenin 7-*O*-diglucuronide, luteolin 7-*O*-diglucuronide, luteolin glucuronide, luteolin diglucuronide, apigenin glucuronide, luteolin glucuronide). When a concentrate extract was dried by freeze-drying and spray-drying, the freeze-dried extract has been presented a good recovery of polyphenols and antioxidant capacity. The powder form of leaf water-extracts obtained by freeze-drying could be a potential advantage for preservation of its quality during storage and marketing of this traditional medicine at village level in tropical countries.

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