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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 ± 117 μmol g⁻¹ GAE) and antioxidant capacity (8490 ± 29 μmol g⁻¹ TE) comparatively to crude extract (1300 ± 12 μmol g⁻¹ GAE for polyphenol and 430 ± 2 μmol g⁻¹ TE for antioxidant activity) or cross flow microfiltration extract (1170 ± 10 μmol g⁻¹ GAE for polyphenol and 400 ± 10 μmol 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 electro-spray 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 anti-diabetic 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).
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 extracting 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 involves 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 acidic 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_CFМ) 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:

\[ CF = \frac{\text{TSP}_{\text{RO}}}{\text{TSP}_{\text{CF}}}, \]

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 about –30 °C in cold room and dried using freeze dryer (type cryonext, France) for 48 h. Sample temperature was set at about –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 (Galic 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 μmol 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 μmol 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 Amana, Bremen, Germany) was connected via an electrospray ionization (ESI) interface for high performance liquid chromatography–tandem mass spectrometry (HPLC-SM\(^{2}\)) to UPLC-DAD (Waters Acquity, Milford, MA) equipped with a RP18 column (Acquity BEH column, 10 mm × 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 ± 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 microfiltration (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 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, MS\(^{2}\) and MS\(^{3}\) 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.

| Table 1 | Total polyphenol contents and antioxidant activity of \(T.\) grandis L. leaf extract. |
|---------|-----------------------------------------------------------------------------------|
| Process co-products | Total polyphenols (μmol g\(^{-1}\) GAE) | Antioxidant capacity (μmol g\(^{-1}\) TE) |
| Crude extract | 1300 ± 12\(^{a}\) | 430 ± 2\(^{a}\) |
| CFM permeate | 1170 ± 10\(^{b}\) | 400 ± 10\(^{b}\) |
| RO concentrate | 21,080 ± 17\(^{ab}\) | 8490 ± 29\(^{a}\) |

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 \(p < 0.05\).
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 | \(\lambda_{\text{max}}\) | MW | \([\text{M–H}]^-\) | Fragments | Neutral loss | Percentage (%) |
|------|-----------|-------------------------|----|----------------|----------|-------------|---------------|
| 1    | Protocatechuic acid | 296 | 154 | 153 | 191-179-(135) | 179-135 | 0.9 |
| 2    | 3-O-caffeoyl quinic (3-CQA) | 324 | 354 | 353 | 207 | 162 | 1.3 |
| 3    | 2-O-caffeoylhydroxyxeric acid | 326 | 370 | 369 | 207 | 162 | 3.2 |
| 4    | Caffeoyl acid derivative | 326 | 488 | 487 | 179-135 | 308-44 | 2.2 |
| 5    | Caffeic acid | 297 | 180 | 179 | 461-369 | 180-174 | 1.8 |
| 6    | 4-O-caffeoyl quinic (4-CQA) | 326 | 354 | 353 | 173-191-179-135 | 176-174 | 12.2 |
| 7    | Apigenin7-O-diglucuronide | 269 | 624 | 623 | 447-271 | 176 (2x176) | 8.0 |
| 8    | Luteolin 7-O-diglucuronide | 255-349 | 638 | 637 | 351-285-193 | 286 (352-444) | 9.5 |
| 9    | Luteolin 7-O-glucuronide | 289sh-333 | 462 | 461 | 285 | 176 | 2.8 |
| 10   | Verbascoside | 289sh-333 | 624 | 623 | 461-315 | 162-308 | 31 |
| 11   | Luteolin diglucuronide | 269-340 | 637 | 636 | 461-285 | 176±2 | 2.3 |
| 12   | Apigenin glucuronide | 266-336 | 446 | 445 | 269 | 176 | 1.0 |
| 13   | Luteolin glucuronide | 268-340 | 462 | 461 | 285 | 176 | 0.9 |
| 14   | Luteolin | 254-349 | 286 | 285 | 175-239-241 | 0.4 |

Table 3

Effect of drying processes on polyphenol contents and antioxidant activity.

| Co-product | Polyphenol Content (µmol·g⁻¹ GAE) | Recovery (%) | Antioxidant Capacity (µmol·g⁻¹ TE) | Recovery (%) |
|------------|----------------------------------|--------------|-----------------------------------|--------------|
| RO concentrate | 21,080 ± 117a | 100.0 | 8490 ± 29a | 100.0 |
| Powder 1 | 18,170 ± 75b | 86.19 | 6980 ± 12b | 82.21 |
| Powder 2 | 13,960 ± 38c | 66.22 | 5210 ± 5c | 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 SM\(^1\), \(m/z\) at 637 [M–H\(^+\)]\(^-\) with fragments ions at \(m/z\) 461 ([M–H–176\(^-\)]\(^-\) and 285 ([M–H–(2 x 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 Russian salepequisetiformis 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 MS\(^2\) 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-cafeoylhydroxycitric acid, Caffeoyl acid derivative, 4-O-cafeoyl quinic acid, apigenin7-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 ± 38 μmol g\(^-1\) GAE) < powder 1 (18,170 ± 75 μmol g\(^-1\) GAE) < RO concentrate extract (21,080 ± 117 μmol g\(^-1\) GAE). The amount of antioxidant capacity of powder 2 (5210 ± 5 μmol g\(^-1\) TE) was also lower than those of powder 1 (6980 ± 12 μmol g\(^-1\) TE) and RO concentrate extract (8490 ± 29 μmol 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. Phaeochamud et al. (2012) also demonstrated that thermal drying process affected significantly the amount of polyphenolic 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 polyphenolic compounds that exhibit antioxidant properties. Reverse osmosis concentrate extract have higher amount of polyphenolic compounds and antioxidant capacity comparatively to crude extract. In this extract, fourteen polyphenolic compounds as flavonoids and polyphenolic 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-cafeoyl quinic acid, 2-O-cafeoylhydroxyxycitric acid, Caffeoyl acid derivative, 4-O-cafeoyl quinic acid, apigenin7-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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