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Optimization of Ultrasound-Assisted Extraction of Phenolic Antioxidants from *Tectona grandis* Leaves, Using Experimental Design

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Authors' contributions

This work was carried out in collaboration between all authors. Author ENK designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Authors IC, ABBK, PRL, AAA, ENA and YAB managed the manuscript and managed literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study aims to apply central composite design to optimize ultrasound-assisted extraction conditions in order to maximize simultaneously total polyphenols and antioxidant activity from aqueous leaves extract of *Tectona grandis*.
Study Design: Young teak leaves were collected in June 2011 from teak plantations in the center

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of Côte d'Ivoire. After harvesting, the dried leaves were packed and shipped to CIRAD laboratory (Montpellier, France), where they were stored until processed and analyzed.
Place and Duration of Study: This study was carried out during season 2011-2012 in the CIRAD laboratory (Montpellier, France).
Methodology: Central composite design was used to simultaneous maximize ultrasound-assisted extraction of total polyphenols and antioxidant activity from *Tectona grandis* leaves. The design independent variables selected for this study were vegetal to liquid ratio (X_1, leaves: citric acid; w:v), extraction time (X_2, min) and solvent concentration (X_3, citric acid concentration).
Results: Optimal condition obtained includes 10^{-2} N citric acid concentration, 16.25 g/L vegetal to citric acid ratio and 37.5 min for extraction. Under the above-mentioned condition, the experimental content of total polyphenols and antioxidant activity from aqueous leaves extract of *T. grandis* were 1,310 µmol.g^{-1} GAE and 431 µmol.g^{-1} TE, respectively. These results were well matched with their predicted values which are 1,300 µmol.g^{-1} GAE and 429 µmol.g^{-1} TE for polyphenols and antioxidant activity, respectively.
Conclusion: Ultrasound-assisted extraction was successfully optimized using central composite design to obtain an aqueous leaves extract of *Tectona grandis*, with optimized polyphenol content and antioxidant activity. Results indicated that this extraction method is a promising technique for extraction of phenolic antioxidants from *T. grandis*, as compared as infusion and decoction, and aqueous extracts of *T. grandis* leaves could be explored as a potential antioxidant agent for use in medicine against cardiovascular and cancer diseases.

Keywords: Tectona grandis; ultrasound-assisted extraction; optimization; central composite design; polyphenols; antioxidant activity.

1. INTRODUCTION

Tectona grandis Linn (common name: teak; Familly: Verbenaceae), is grown in Côte d'Ivoire for its wood which have high commercial value. Different parts of this plant are also used by rural communities to prepare aqueous extracts used as traditional medicinal beverage [1,2]. Furthermore, several pharmacological activities have been attributed to *T. grandis* extracts, mainly antidiabetic activity [3,4] and antioxidant activity [5-8]. Antioxidants present in extracts of *T. grandis* could play an important role in the defense of human body against cardiovascular, aging and cancer diseases [9]. Previous studies showed that antioxidant activity exercise by plant extracts was due to their bioactive constituents such as polyphenols [10-12]. Polyphenols are also reported to have other pharmacologic properties such as anti-ulcer, anti-carcinogenic, anti-mutagenic activities, antibacterial, antiviral, anti-inflammatory, estrogenic, and anti-estrogenic properties [13,14]. To extract these compounds, Ivoirians used decoction and infusion. These extraction methods are time-consuming and thermally unsafe and the analysis of numerous constituents in plant material is limited by the extraction step [15]. For this reason, modern extraction techniques were described as alternative techniques to accelerate the extraction process. These modern techniques include: Supercritical fluid extraction, pressurized liquid extraction, microwave-assisted extraction and ultrasound-assisted extraction [16]. The applications of these technologies, particularly ultrasound-assisted extraction offers many advantages, including temperature reduction and extraction time, which are very useful for the extraction of thermo labile and unstable compounds [17,18].

This study aims to apply response surface methodology with central composite design to optimize ultrasound-assisted extraction conditions such as vegetal to liquid ratio, extraction time and solvent concentration, in order to maximize simultaneously total polyphenol contents and antioxidant activity from *T. grandis* leaves. This experimental design provides high prediction (linear, quadratic, and interaction effects) of a response surface over the entire design space as compared as other experimental design [19].

2. MATERIALS AND METHODS

2.1 Plant Material

Entire young teak leaves (purple leaves) were collected from teak plantations (age ≤ 5 years) in the center of Côte d'Ivoire around Yamoussoukro area; then cut in strips of 10 cm of wide. After harvesting, the leaves were brought to LAPISEN laboratory (Yamoussoukro, Côte d'Ivoire) for drying at an average temperature of 30ºC during daytime, and kept away from direct sun exposure

under an open-sided shed. Dried leaves were packed in plastic bags and shipped to CIRAD laboratory (Montpellier, France), where they were stored at 4ºC until processed and analyzed.

2.2 Chemicals

All reagents were of analytical grade. Sodium carbonate salt (Na_2CO_3), monohydrated citric acid, dihydrated monosodium phosphate (NaH_2PO_4, $2H_2O$), disodium hydrogen phosphate (Na_2HPO_4), Folin-Ciocalteu's reagent were purchased from Carlo Erba (Spain). Galic acid, trolox (6-hydroxy-2,5,7,8 tetramethylchroman-1-carboxylic acid), fluorescein, AAPH (2,2'-azobis (2-methylpropanimidamide) dihydrochloride) were purchased from Sigma-Aldrich (Germany).

2.3 Ultrasound-assisted Extraction Procedure

Ultrasound-assisted extraction (UAE) was carried out according to Adjé et al. [20], with a PEX 3 Sonifier ultrasounds system (R.E.U.S., Contes, France) composed of an stainless steel jug having 23 cm × 13.7 cm internal dimensions with a maximal capacity of 3 L, and a transducer, in the base of jug, operating at a frequency of 25 kHz with maximum input power of 150 W. Double layered mantle allowed us to control the temperature of the medium by cooling/heating systems. Generator output power was 150 W while the power dissipated in the medium was about 60 W per kilogram, as measured by calorimetry.

UAE was performed when applying ultrasound to plant material (5 to 20 g) put into water or acidified water (V = 1 L) during 15 to 45 min.

2.4 Total Polyphenol Contents

Total polyphenol content was determined by colorimetry, using Folin-Ciocalteu's (F-C) method [21,22]. To 30 µL sample extract, 2.5 mL of diluted Folin-Ciocalteu's reagent (1/10) were added. After 2 min incubation in dark at room temperature, 2 mL of aqueous sodium carbonate (75 $g.L^{-1}$) were added. After slight stirring, mixture was put in a water bath at 50ºC for 15 min then cooled down. The absorbance was measured at λ = 760 nm using a UV-visible 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.

2.5 Antioxidant Capacity

Antioxidant capacity was carried by Oxygen radical absorbance capacity (ORAC) assay, as described byOu et al. [23]. The automated ORAC assay was carried out on a VICTOR™ *X*3 Multilabel Plate Reader (Perkin–Elmer, USA) with fluorescence filters for an excitation wavelength at 485 nm and an emission wavelength at 535 nm [24]. To start reaction, 100 µl of fluorescein (78 $nmol.L^{-1}$) and 100 µL of diluted sample, phosphate buffer (pH 7.4) or standard (Trolox 5-50 $\mu mol.L^{-1}$) were placed in each well of a 96 well-plate and pre-incubated during 15 min. After, 50 µL of AAPH (221 $mmol.L^{-1}$) were added into the wells. Fluorescence was measured every minute during 60 min with emission and excitation wavelength set at 485 and 535 nm, respectively, at 37ºC. 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.

2.6 Central Composite Experimental Design

A five level, three variable central composite designs was applied to determine the best combination of extraction variables for the extraction of phenolic compounds and antioxidants from *T. grandis* leaves. Central composite design comprised 20 experimental runs with eight factorial points, six axial points (two axial points on each design variable axis at a distance of 1.68 from the design center) and six replicates at the center point. Design independent variables selected for this study were the vegetal to liquid ratio (X_1, leaves: citric acid; w:v), extraction time (X_2, min) and solvent concentration (X_3, citric acid concentration) (Table 1).

Table 1. Independent variables and their coded and actual values used for optimization

Independent variable	Symbol	Coded level				
		-1.68	**-1**	**0**	**1**	**1.68**
Vegetal: liquid ratio (g/L)	X_1	5	8	12.5	18	20
Extraction time (min)	X_2	15	21	30	39	45
Citricacidconc. (10^{-3} N)	X_3	0	3.5	5	6.5	10

Two experimental responses were studied (total polyphenol contents (Y_1) andantioxidant capacity (Y_2)). Citric acid volume used was set to 1 L, during all experiments.

2.7 Statistical analysis

Multiple linear regression analysis was performed using the *Statistica 8* software (Stat Soft, Inc., USA). Experimental data were fitted to the following second-order polynomial model and regression coefficients were obtained.

$$Y_n = b_o + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \quad (1)$$

Where $Y_n$is the experimental response; X_1, X_2 and X_3 correspond to the independent variables namely vegetal to liquid ratio, extraction time and citric acid concentration, respectively. The b_n values represent corresponding regression coefficients.

According to the experimental data, the fitting model represented by equation (1) was constructed and the statistical significance of the model terms was examined by regression analysis and analysis of variance (ANOVA).

3. RESULTS AND DISCUSSION

3.1 Experimental Responses Obtained using Central Composite Design

In order to optimize simultaneously of total polyphenol contents and antioxidant activity from *Tectona grandis* leaves under sonication, a central composite design was developed as represented in Table 2. This table presents also experimental values of total polyphenol contents and antioxidant activity from aqueous leaves extract of *T. grandis* at various conditions.

Good correlation (R^2= 0.86) are observed between phenolic compounds and antioxidants extracted from aqueous leaves extracts of *T. grandis* (Fig. 1).

Similar correlation between polyphenol with antioxidant activity was observed by Cortés-Rojas et al.[25] and Lizcano et al. [26]. Therefore, aqueous leaf extract of *T. grandis* could be beneficial to human body. Indeed, natural antioxidants play an important role in the defense of human body against cancer and cardiovascular diseases [9].

Table 2. Response surface central composite design and experimental results

Test set	Independent variables			Experimental responses	
	X_1 (g/L)	X_2 (min)	X_3 (10^{-3} N)	Y1 (µmol.g^{-1} GAE)	Y2 (µmol.g^{-1} TE)
1	-1(8)	-1 (21)	-1 (3.5)	421.45±10	163.27±1
2	1(18)	-1 (21)	-1 (3.5)	693.59±12	251.54±2
3	-1(8)	1 (39)	-1 (3.5)	935.28±7	316.15±5
4	1(18)	1 (39)	-1 (3.5)	1,145.68±15	323.12±3
5	-1(8)	-1 (21)	1 (6.5)	869.4±22	310.52±2
6	1(18)	-1 (21)	1 (6.5)	1,170.85±13	405.03±7
7	-1(8)	1 (39)	1 (6.5)	1,086.31±20	357.61±2
8	1(18)	1 (39)	1 (6.5)	1,276.92±18	391.49±2
9	-1.68 (5)	0 (30)	0 (5)	781.09±15	299.47±5
10	1.68 (20)	0 (30)	0 (5)	1,119.74±12	338.47±5
11	0 (12.5)	-1.68 (15)	0 (5)	592.55±10	157.01±2
12	0 (12.5)	1.68 (45)	0 (5)	1,212.88±13	366.14±3
13	0 (12.5)	0 (30)	-1.68 (0)	843.99±20	313.73±2
14	0 (12.5)	0 (30)	1.68 (10)	1,291.71±18	428.94±1
15	0 (12.5)	0 (30)	0 (5)	1,117.19±15	330.91±3
16	0 (12.5)	0 (30)	0 (5)	1,185.7±23	342.86±5
17	0 (12.5)	0 (30)	0 (5)	1,148.81±25	381.12±7
18	0 (12.5)	0 (30)	0 (5)	1,192.73±10	366.51±3
19	0 (12.5)	0 (30)	0 (5)	1,106.65±12	346.41±2
20	0 (12.5)	0 (30)	0 (5)	1,125.97±22	356.22±5

3.2 Fitting the Response Surface Models

By referring to Table 3, the coefficient of determination (R^2) value for regression model of polyphenols and antioxidant capacity were 0.99 and 0.93, respectively; which were closed to 1. These suggest that the predicted second order polynomial models defined well the real behavior of the system [27]. Their non-significant lack of fit also indicated that these models were good fit [28]. The lack-of-fit measured the failure of the model to represent data in the experimental domain at points which are not included in the regression [29].

3.3 Effect of Process Variables on Polyphenol Extraction

The polyphenols from *T. grandis* aqueous leaves extracts obtained by UAE based on central composite design are shown in Table 2. Multiple regression analysis was performed on the experimental data and the coefficients of the model are evaluated for significance. Extraction time and solvent concentration effects are highly significant (*P* = .001). Similar results were obtained when extracting phenolic compounds from grape [30]. Also, vegetal ratio played an important role in phenolic compounds extraction from plant material. Coefficients values for polyphenols as presented in Table 3are used for a final predictive equation neglecting the non-significant terms as given below:

$$Y_1 = 1147 + 113\,X_1 + 171\,X_2 + 143\,X_3 - 72\,X_1^2 - 89\,X_2^2 - 30\,X_3^2 - 80X_2X_3 \quad (2)$$

All linear terms (X_1, X_2, and X_3), quadratic term (X_1^2, X_2^2 and X_3^2) and interaction between X_2 and X_3 were significant. Those significant terms had a remarkable impact on polyphenols extraction from *T. grandis* leaves, whereas the non-significant terms had a negligible influence. The negative interaction between X_2 and X_3 indicate that interaction decrease phenolic compounds extracted when increasing both variables during their extraction.

In order to assess the effects of the extraction conditions on phenolic compounds extraction from *T. grandis* leaves, response surfaces and contour plots are constructed in accordance with equation (2). Fig. 2A shows the effects of vegetal to liquid ratio and extraction time on total phenolic contents from *T. grandis* leaves. It shows that lower level of both variables gives low amounts of polyphenols. However, total phenolic Content increases with vegetal to liquid ratio increases at a fixed extraction time, up to a certain limit. Beyond this limit, total polyphenol contents slightly decreases, which indicates that it greater content could be achieved if moderate vegetal to liquid ratio and extraction time were selected. This is due to significant negative quadratic effect of these variables (vegetal to liquid ratio and extraction time) on polyphenol extraction.

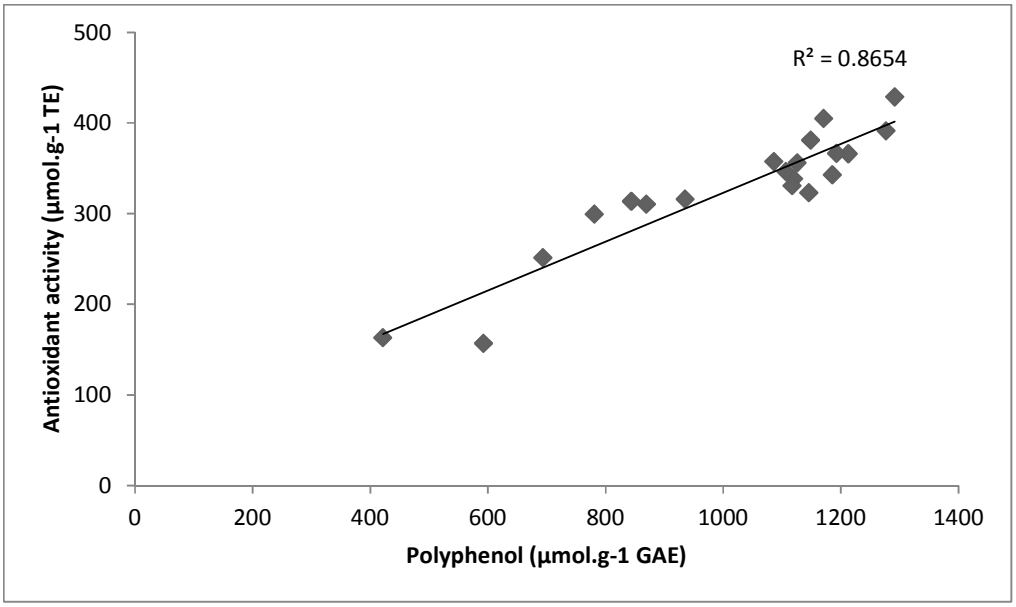


Fig. 1. Correlation between antioxidant capacity and polyphenol contents from *T. grandis* leaves

Table3. Regression coefficients of predicted quadratic polynomial models for polyphenols and antioxidant capacity

Coefficients	Coefficient estimated	
	Polyphenols	**Antioxidant activity**
b_0	1,146.60***	354.01***
Linear		
b_1	113.06***	21.18**
b_2	170.76***	44.64***
b_3	143.54***	44.24***
Quadratic		
b_{11}	-72.00***	-12.43*
b_{22}	-88.86***	-32.72***
b_{33}	-30.49*	6.08ns
Cross products		
b_{12}	-21.57ns	-17.74*
b_{13}	1.19ns	4.14ns
b_{23}	-80.37*	-23.86*
R^2	0.99	0.93
Lack of fit (*P* -value)	0.60ns	0.12ns

*Significant at p= .05; ** Significant at p=.01; *** Significant at p=.001; ns: no significant; R^2: Regression coefficient; p: probability

In fact, a prolonged extraction time would allow all the plant cells to be completely cracked by acoustic cavitations, thus the extraction yield would increase within a certain time. On the other hand, completely ruptured plant cells would also allow various compounds such as insoluble and cytosolic solvent. In addition, target constituents might also be re-absorbed on smashed plants particles, thus affecting recovered compounds [18].

Interaction between vegetal to liquid ratio and citric acid concentration has positive effect on polyphenols extraction (Fig. 2B). Indeed, when increasing vegetal to liquid ratio and citric acid concentration, in experimental design, polyphenol contents increases.

Fig. 2C shows extraction time and citric acid concentration effects on total polyphenols extraction from *T. grandis* leaves at a constant vegetal to liquid ratio (16.25 g/L). Increase of both independent variables enhances polyphenols extraction. Polyphenol contents are particularly increased with prolonged extraction time at certain value. When total polyphenol extracted is maximal, extraction time extension would cause their slight degradation.

3.4 Effect of Process Variables on Antioxidant Extraction

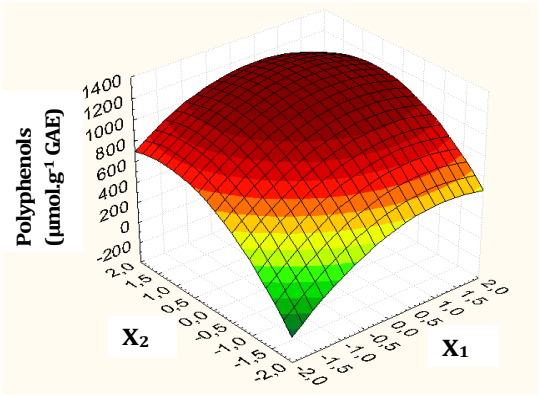
The results show that antioxidant capacity from *Tectona grandis* aqueous leaf extracts, under different combinations, range from 163.27 to 428.94 µmol TE/g of leaves. By applying multiple regression analysis, relationship between tested independent variables and antioxidant extraction are explained in equation (3):

$$Y_3 = 354 + 21\,X_1 + 45\,X_2 + 44\,X_3 - 12\,X_1^2 - 33\,X_2^2 - 18X_1X_2 - 24X_2X_3 \quad (3)$$

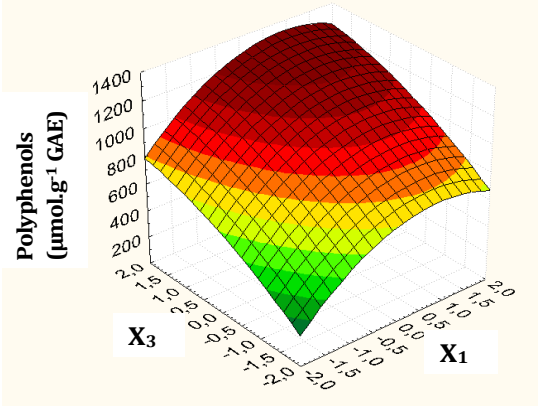
All linear terms (X_1, X_2, and X_3), quadratic term (X_1^2 and X_2^2) and interactions (between X_2 and X_3; between X_2 and X_3) are significant. Those significant terms had a remarkable impact on antioxidant extraction from *T. grandis* leaves. Extraction time and solvent concentration effects are highly significant (P = .001), on antioxidant extraction. Similar results were obtained when extracting total polyphenol from *T. grandis* leaves (Table 2). The surface plot in Fig. 3A shows vegetal to liquid ratio and extraction time effects on antioxidants extraction. Increasing of vegetal to liquid ratio doesn't significantly affect antioxidant extraction. But, extraction time are highly influenced antioxidant contents (P = .001).

Fig. 3B denotes citric acid concentration and vegetal to liquid ratio effects, on antioxidant extraction. When increasing citric acid concentration, antioxidant contents also increase.

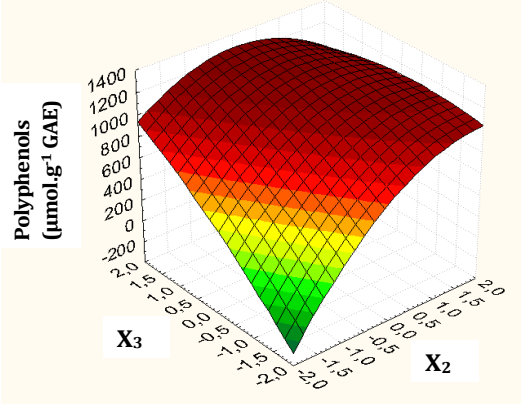
Fig. 3C shows citric acid concentration and extraction time effects on antioxidant from T. grandis leaves. Increase of both variables (acid concentration and extraction time on antioxidant) increases antioxidant extraction. Interaction between these variables is not significant (Table 2).



(A) : an effect of vegetal to liquid ratio and extraction time at citric acid concentration of 5.10-3N



(B) :a function of vegetal to liquid ratio and citric acid concentration at extraction time of 30 min



(C) : a function of extraction time and citric acid concentration at vegetal to liquid ratio of 12.5 g.L-1

Fig. 2. Response surface of phenolic contents of *Tectona grandis* leaves extract

3.5 Optimization and Experimental Validation

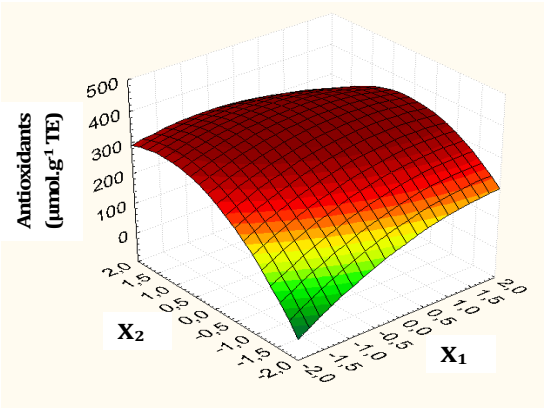
Optimal extraction condition with a citric acid concentration of 10^{-2} N, vegetal: citric acid ratio of 16.25 g/L and extraction time of 37.5 min are predicted using *Statistica 8.0 software* desirability function, to simultaneously maximize polyphenols and antioxidant activity (Table 4).

Under the above-mentioned conditions, the experimental results are very close to predicted one. This implies that there are a high fit degree between observed values in experiment and predicted ones from the regression model [27].

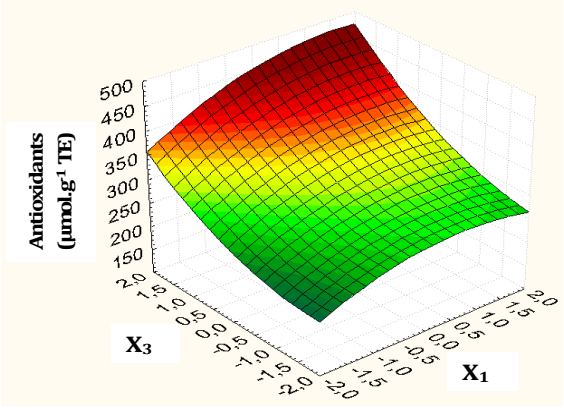
Table 4. Predicted and experimental values of responses under optimal condition and classic extraction

Compounds	UAE optimum condition		Classic extraction	
	Observed value	Predicted value	Infusion	Decoction
Polyphenols (µmol.g^{-1} GAE)	1,310 ± 10^{a}	1,300^{a}	1,117 ± 25^{b}	1,033 ± 13^{c}
Antioxidant activity (µmol.g^{-1} TE)	431 ± 5^{a}	429^{a}	nd	nd

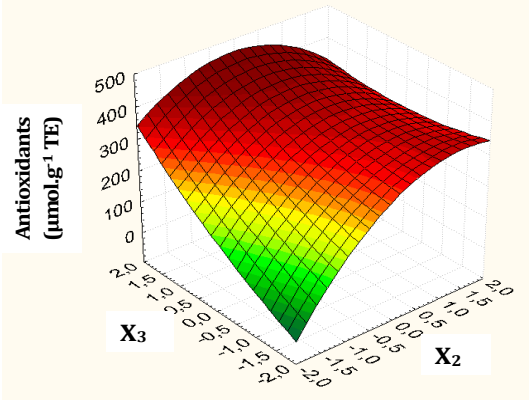
NB: in every line, the averages not followed by the same letter are significantly different at p=.05



(A): an effect of vegetal to liquid ratio and extraction time at citric acid concentration of 5.10-3N



(B): a function of vegetal to liquid ratio and citric acid concentration at extraction time of 30 min



(C): a function of extraction time and citric acid concentration at vegetal to liquid ratio of 12.5 g.L-1

Fig. 3. Response surface of antioxidant contents of *Tectona grandis* leaves extract

The total polyphenol (1,310±10 µmol.g^{-1} GAE) from aqueous leaves ultrasound-assisted optimized extract of *T. grandis* are higher than those of homemade extracts obtained with extraction time varying between 3-24h. Similar result was obtained by Koffiet et al. [31], when extracting phenolic compounds from leaves of *Justicia secunda*. So, ultrasound-assisted extraction gives a shorter extraction time and higher amount of phenolic compounds as compared to classic extraction by infusion and decoction.

4. CONCLUSION

In this study, the optimum condition of ultrasound-assisted extraction (UAE) of polyphenols and antioxidant activity from *T. grandis* was investigated. The optimal condition of UAE was achieved based on response surface methodology with Central Composite design. The optimal extraction condition with a citric acid concentration of 10^{-2} N, vegetal: citric acid ratio of 16.25 g/L and extraction time of 37.5 min was predicted using the desirability function. Under this optimal extraction conditions, polyphenol content and antioxidant activity were 1,310 µmol.g^{-1} EAG and 431 µmol.g^{-1} TE, respectively, which were well closed with the predicted value (1,300 µmol.g^{-1} EAG and 429 µmol.g^{-1} TE, respectively). Results indicated that this extraction method is a promising technique for extraction of phenolic antioxidants from *T. grandis*, as compared as infusion and decoction, and aqueous extracts of *T. grandis* leaves could be explored as a potential antioxidant agent for use in medicine against cardiovascular and cancer diseases.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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