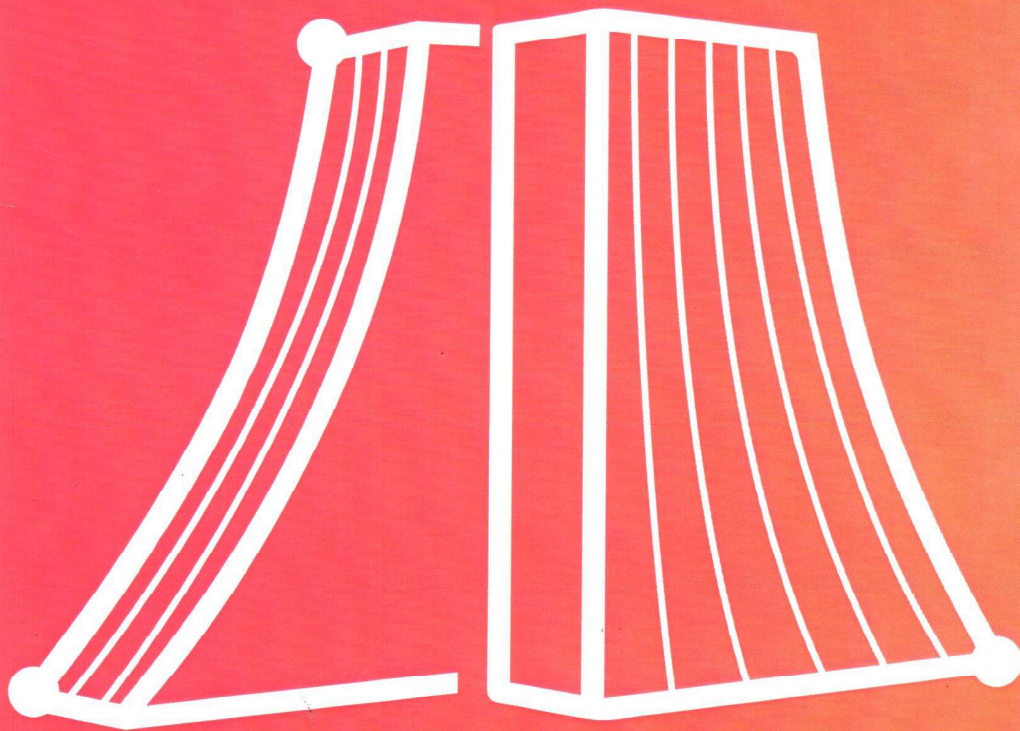


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PRESSURIZED LIQUID EXTRACTION OF PHENOLIC AND FLAVONOID COMPOUNDS FROM NEEM LEAVES

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Azadirachta indica A. Juss (neem) is a medicinal plant with established pharmaceutical applications over time, due to the flavonoid and phenolic compounds presented. Its bioactive compounds can be obtained by distinct extraction methods as maceration, soxhlet, hydrodistillation and pressurized liquid extraction (PLE). PLE is an interesting method to obtain phytochemicals from plants, due to the possibility of using polar and non-polar solvents under high-pressure to enhance the extraction efficiency. This technique can also be used to the exhaustive extraction of analytes in one or more clean-up steps. In this work, a sequential of solvents was used in a PLE process to obtain the flavonoid rutin and phenolic compounds from neem leaves. For this purpose, extractions were carried out at 25 °C, 100 bar, and using 1 mL/min of solvent flow rate. The n-hexane was used in the first step, followed by ethyl acetate, and a mixture of ethanol/water (80/20, v/v) in the final step. One-step extraction using only ethanol/water mixture (80%, v/v) was also performed using the experimental conditions previously mentioned. Total phenolics and total flavonoids were quantitatively determined by spectrophotometry and rutin flavone content was analyzed by chromatographic methods. The solvent ethanol/water promoted the highest global extraction yield in the sequential and one-step extractions. The ethyl acetate fraction resulted in the richest fraction regarding phenolic and flavonoid compounds. Compared to the one-step PLE, the sequential PLE process promoted an enhancement around 50% in the capacity to extract total flavonoid compounds. Rutin flavonoid was found in all extracts investigated. From the results, it can be concluded that the fractionation of the neem leaves using distinct solvents, produced extract fractions with varied chemical profile.

Keywords: phenolic compounds, rutin, neem leaves, pressurized liquid extraction, sequential process.

Introduction

Natural products have exhibited potential health benefits over time (Moghadamtousi et al., 2013, 2015). The neem (*Azadirachta indica* A. Juss) medicinal plant has been used for the treatment of several diseases, including diabetes, hypertension, atherosclerosis, infectious illnesses and cancer (Gupta et al., 2017). The medicinal benefits of neem are found due to its phytochemical constituents as alkaloids, terpenoids and phenolic compounds (Hossain et al., 2013). The phenolic compounds include phenolic acids, coumarins, tannins, and flavonoids (Khoddami et al., 2013).

Among these bioactive compounds, the flavonoids are efficient therapeutic molecules with several pharmacological properties recognized for the scientific community. The rutin is a flavonoid which exhibits good pharmacological effects for the maintenance of central nervous, cardiovascular, gastrointestinal, respiratory, excretory, and reproductive systems. Moreover, the rutin shows biological activity for diseases prevention and treatment in various parts of body such as skin, hair, bones, eye, and others (Ganeshpurkar and Saluja, 2017).

To obtain improved extraction yields of phenolic compounds, many solvents such as water, acetone, ethyl acetate, ethanol, methanol, n-hexane, or its mixtures has been used (Khoddami et al., 2013). These solvents with distinct polarity have been applied on distinct extraction methods such as soxhlet, maceration, ultrasonic, microwave, and pressurized liquid (Khoddami et al., 2013; Dahmoune et al., 2014). The use of one-step process with an only solvent results in the inefficient extraction of the metabolites from biomass, due to the high number of compounds with variable physical properties and polarities found in natural source. Thereby, the use of distinct solvents in multiple steps is required for the efficient extraction of bioactive compounds (Dai et al., 2013). Pressurized liquid extraction (PLE) has been used to the exhaustive extraction of analytes in one or more steps (Subedi et al., 2015), using a variety of polar and non-polar solvents under high pressure, that improves the efficiency of the extraction process (Ong and Len, 2003; Eng et al., 2007).

The extraction of plant leaves at high-pressure using solvent in sequence has demonstrated an elevated ability to obtain phenolic compounds (Garmus et al., 2014, 2015). The aim of this study was the development of a methodology using pressurized liquid extraction with distinct solvents to obtain phenolic compounds and flavonoids from neem leaves.

Material and methods

Sample preparation

Neem (*Azadirachta indica* A. Juss) leaves were collected at the Brazilian Agricultural Research Center - Embrapa Coastal Tablelands, in Aracaju, Sergipe, Brazil. All leaves were dried at 45 °C for 36 h in an oven with air circulation. After that, the leaves were milled, and its granulometry classified in the range from 8 to 16 mesh (Tyler sieve series, Bertel, Brazil). Samples were stored under refrigeration and protected from light until the extractions.

Experimental unit and extraction procedure

All extractions were performed in an experimental unit composed by a stainless steel jacketed extraction vessel of 100 mL of internal volume. The extractor was connected to two high-pressure pumps: one HPLC pump (Series III, Lab Alliance, USA) for liquid displacement and a Syringe pump (Teledyne Isco 260D, USA) for compressed gasses displacement. The temperature of the extraction was controlled by a recirculating bath (Q-214 M2, QUIMIS, Brazil). Thermocouples (J-type, Salcas, Brazil), pressure transducer (5436 Wurenlos, Huba Control, Switzerland) and universal indicators (N1500, Novus, Brazil) completed the experimental unit (Figure 1). Details of the experimental unit and procedure can be found in Jesus et al. (2013). Approximately 20 g of the neem leaves were loaded into the extractor cell that was connected to the experimental unit. The extractor was fed with the solvent. The temperature and pressure stabilized at the experimental condition (100 bar, and 25 or 50 °C). Then the solvent was continuously pumped to the system at a constant flow rate (1 and 2 mL/min). Samples were collected periodically after the regulating pressure valve. In the sequential PLE experiments, the solvents were used in an increase polarity sequence: hexane, ethyl acetate, and ethanol/water mixture. In each change of solvent, a flush with carbon

dioxide was performed to remove the first solvent and begin the extraction with the subsequent one.

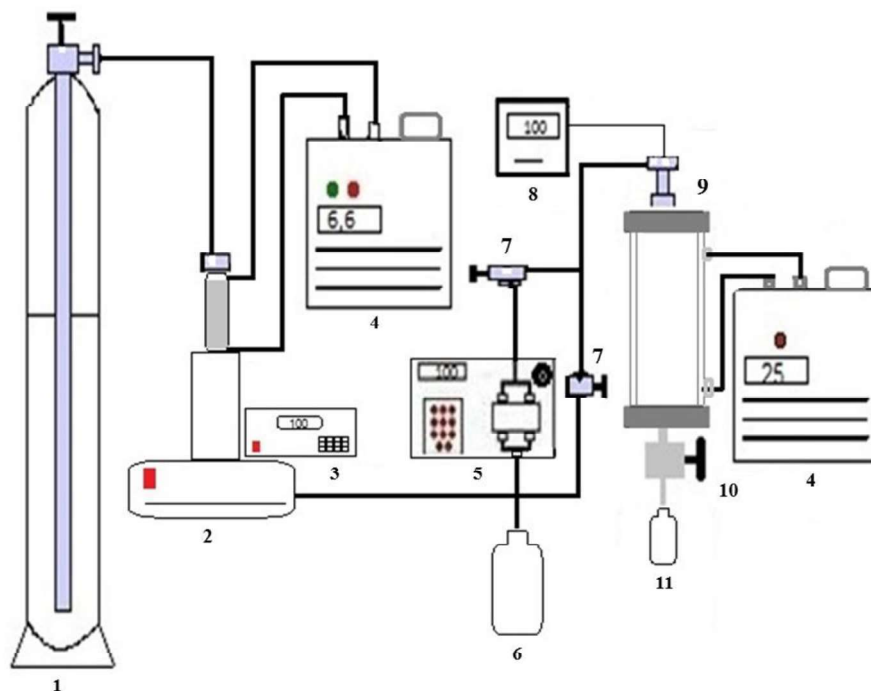


Figure.1. Schematic diagram for the pressurized liquid extraction of neem leaves: (1) CO₂ cylinder, (2) syringe pump, (3) pump control, (4) thermostatic baths, (5) HPLC pump, (6) liquid reservoir, (7) valves, (8) pressure monitoring, (9) extractor cell, (10) needle valves, (11) sample collector.

Determination of total phenolic

The total phenolic was determined colorimetrically using the Folin-Ciocalteu's reagent. Briefly, 500 mL of the neem extract solution (400 µg/mL) was mixed with 2.5 mL of Folin-Ciocalteu's reagent (1:10) and 2 mL of a sodium carbonate solution (7.5% m/v). The mixture was incubated at 45 °C, for 15 min and kept 30 min at room temperature. The phenolic content in the extracts was determined in a microplate reader (Synergy HT, BioTek, USA) at 765 nm. A calibration curve was performed with gallic acid (10-100 mg/L; R²= 0.998), and was used to quantify the phenolics in the extracts. The results obtained were expressed as mg of gallic acid/L of neem extract (Costa et al., 2016).

Determination of Flavonoids contents

Total flavonoids were quantified by a colorimetric method using aluminum trichloride. Briefly, 1 mL of a neem extracts solution (400 µg/mL) or water (blank) was mixed with 300 µL of 5% NaNO₂ and left to stand for 5 min at room temperature in the dark. Then 300 µL of 10% AlCl₃ were added, left to stand for 1 min, and finally, 2 mL of sodium hydroxide (1 mol/L) and 2.4 mL of distilled water were added. The absorbance at 510 nm was determined in the obtained mixture. A calibration curve was prepared with epicatechin (50-450 mg/L; R²= 0.999) and the obtained values reported as mg of epicatechin equivalents (ECE)/L of the extract (Costa et al., 2016).

HPLC-ESI/MS analysis

The analysis by HPLC-ESI/MS were performed according to Mahapatra et al. (2012), with the change in mobile phase. The extracts were analyzed using a Finnigan LCQ DECA XP MAX (Finnigan Corp, USA) mass detector equipped with electrospray ionization source (ESI) and ion trap quadrupole. The rutin identification was performed in a LICHROART® RP-18 column (150 mm x 4.6 mm, 5 μ m) (Merck Millipore, Germany) with a mobile phase of acetonitrile/water (60:40 v/v) at a flow rate of 0.50 mL min⁻¹ and 40 min of run time. The extract volume injected was of 25 μ L. The mass spectrometry analysis was performed under positive electrospray ionization (ESI+). The mass spectra were obtained in the scan range of 250-1200 m/z, controlled by Xcalibur software version 2.2.

Statistical analysis

All the results obtained were presented as mean \pm standard deviation values. Statistical analysis of the extracted material was conducted with GraphPad Prism® version 5.0 (GraphPad software) using one-way ANOVA, followed by Tukey's test at 95% of confidence level.

Results and discussion

The PLE global extraction yields of the neem leaves are presented in Figure 2. In this figure are presented the yields obtained using the sequential extractions with the distinct solvents: n-hexane (SH), ethyl acetate (SEA), 80/20 (v/v) ethanol/water mixture (SE), along with the results obtained with the one-step pressurized liquid extraction with ethanol/water (80/20, v/v) solvent (O-SE).

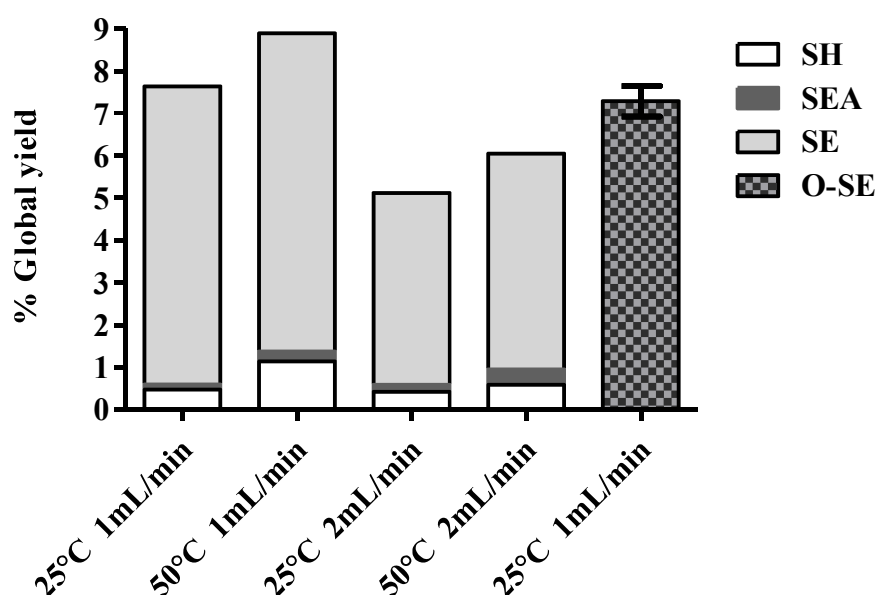


Figure 2. Global yield (%) of the sequential process of pressurized liquid extraction using n-hexane (SH), ethyl acetate (SEA), and 80% ethanol/water (SE). One-step pressurized liquid extraction with 80% ethanol/water (O-SE). Data reported as mean \pm standard deviation.

The results showed that the increase in the solvent flow rate from 1 mL.min⁻¹ during 100 min to 2 mL.min⁻¹ for 50 min, leads to a significant decrease ($p < 0.05$) in the global extraction yield. Moreover, increasing the temperature from 25 °C to 50 °C produced an enhancement of about 1% in the global extraction yield. The effect of solvent flow rate is much more pronounced than the temperature ones in the experimental range investigated (Figure 2). The amount of total phenolic compounds extracted in each sequential fraction compared to the one step ethanol extraction is presented in Figure 3. The results for total amount of flavonoids is presented in Figure 4.

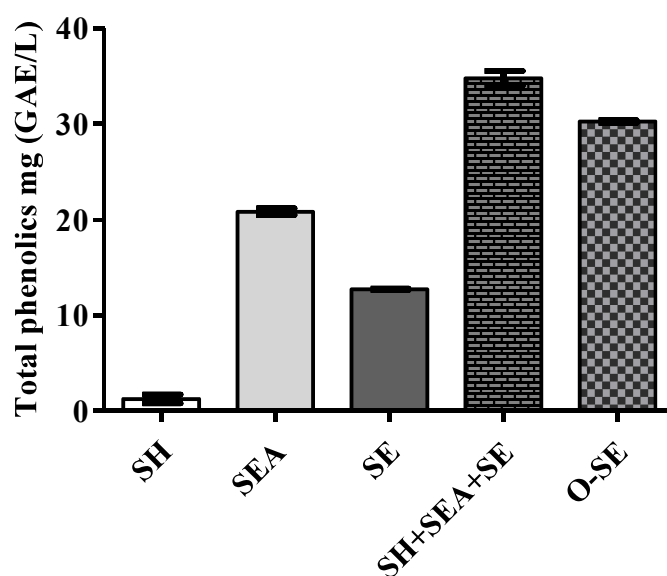


Figure 3. Total phenolic content (mg of gallic acid equivalents (GAE)/L of neem extract) presented in the neem extracts obtained by PLE at 1mL/min, 25 °C and 100 bar with distinct solvents: SH, SEA, SE and O-SE. Data reported as mean \pm standard deviation.

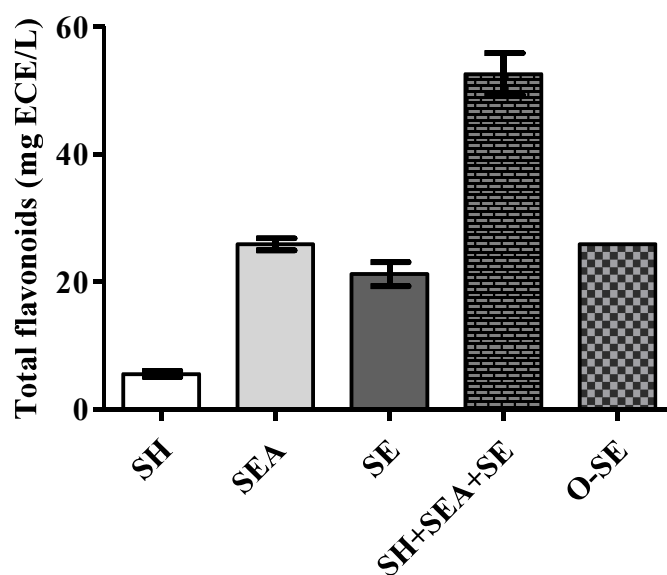


Figure 4. Total flavonoid content (mg of epicatechin equivalents (ECE)/L of neem extract) presented in the neem extracts obtained by PLE at 1mL/min, 25 °C and 100 bar with distinct solvents: SH, SEA, SE and O-SE. Data reported as mean \pm standard deviation.

It can be observed in figure 4 that the SEA, SE, and O-SE extracts showed no significant differences in the total flavonoids content. However, the SH+SEA+SE shows a significantly higher capacity to extract total flavonoids than using just one solvent O-SE. According to Garmus et al., 2014, 2015, the sequential extraction exhibits an efficient method to produce the more concentrated extracts on total phenolic compounds and flavonoids. Moreover, this extraction process is significantly influenced by solvent type, showing different results for the increasing their polarity.

The affinity of the targeted compounds with the solvent used in the extraction is very important to obtain bioactive compounds, such as phenolics (Cowan, 1999; Azmir et al., 2013). The phenolics compounds and flavonoids obtained in this study are more soluble in solvents such as ethyl acetate and 80% ethanol/water compared to the less polar solvent n-hexane (Figures 3 and 4). According to the results, ethyl acetate and 80% ethanol/water seems to be good options to obtain phenolic compounds and flavonoids.

Neem compounds analyzed by HPLC-MS presented H₂O-adduct resulted in an additional fragment $[M+18]^+$. Other fragments can result from the rupture of ester bonds from $[M+H]^+$ (Schaaf et al., 2000). In Figure 5 is identified a flavonoid compound by mass fragmentation, corresponding to rutin, $[M+H]^+$ at 611 m/z (Savic et al., 2013; Siebert et al., 2017).

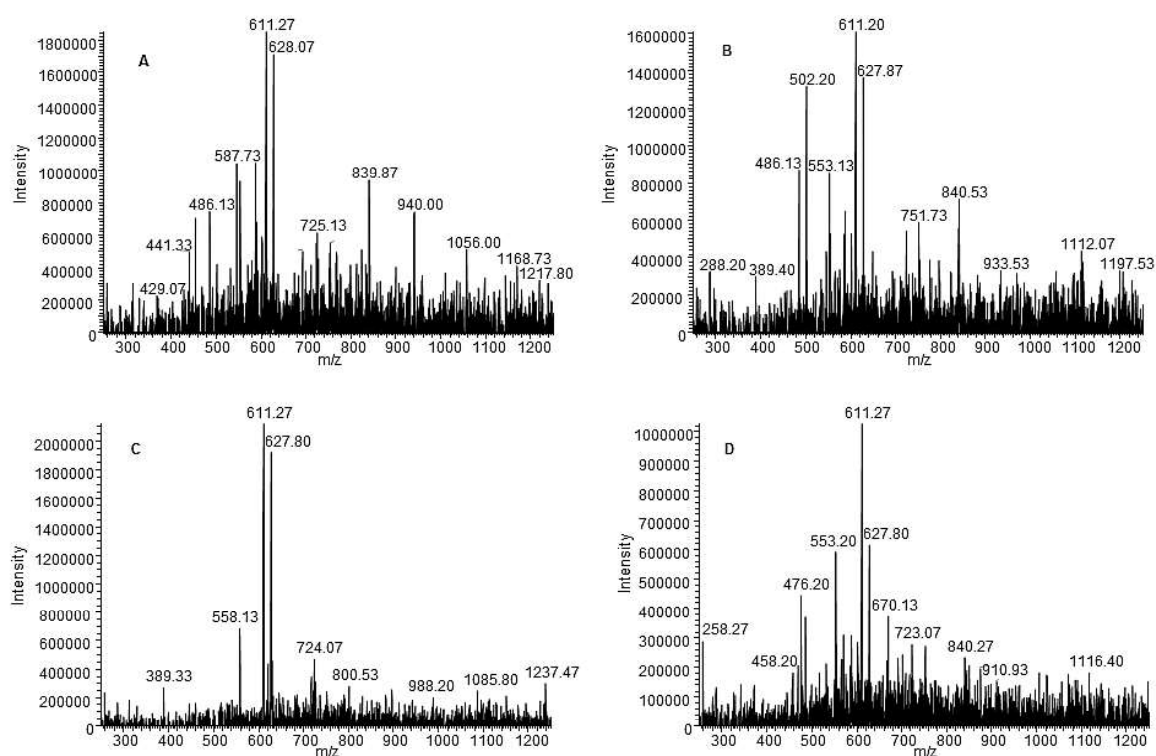


Figure 5. Mass spectra of rutin extracted by pressurized liquid extraction. Capital letters A, B, C, and D correspond to SH, SEA, SE and O-SE, respectively.

In figure 5, we observe the presence of rutin making adducts with H₂O, forming 628 m/z (610+18) in all extracts obtained in the present study. Rutin has been extracted in others studies using n-hexane (Guneser and Yilmaz, 2017), ethyl acetate (Shon et al., 2004; Siebert et al., 2017), and 80% ethanol/water (Liao et al., 2015) as solvents. However, the sequential

process of PLE using n-hexane, ethyl acetate and 80% ethanol has a great potential to obtain the rutin.

Conclusion

In the present study, it was demonstrated that the sequential extraction is an efficient methodology for obtaining phenolic compounds and flavonoids from neem leaves. The use of three extraction solvents with distinct polarities provides cleaner fractions of the extract with distinct phenolic concentrations. The ethyl acetate fraction (SEA) was the richest one in phenolic compounds and flavonoids. These extracts show potential for future application as therapeutic agents.

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