Quantitative Determination of Jatrophone in "Cachaça" Prepared with *Jatropha elliptica*

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A method for sample preparation and analysis by high performance liquid chromatography with UV detection (HPLC-UV) was developed for analysis of jatrophone in "cachaça" prepared with *Jatropha elliptica*, administered orally, employed in Brazil for the treatment of venomous snake bites. The linearity, accuracy, precision of the procedure was evaluated. Analytical curve for jatrophone was linear in the range of 16.24—81.20 μg ml^{-1}. The recovery of the jatrophone in the samples analyzed was 98.99—99.89%. The percent coefficient of variation for the quantitative analysis of the "cachaça" in the analyses was under 2%.

Key words liquid chromatography; cachaça; *Jatropha*; jatrophone

The genus *Jatropha* (Euphorbiaceae) are used in medicinal plant therapy against skin diseases.[1] *Jatropha elliptica* according to popular wisdom can be used in the form of a tincture prepared by maceration of subterranean stems in a sugar-cane derived spirit "cachaça", administered orally, for the treatment of venomous snake bites. Other uses include as an anti-inflammatory, and an anticholestheremic. Studies phytochemical in ethanolic extract of the subterranean stems showed identification of the diterpene jatrophone (Fig. 1) and also others substances.[2] Other studied in dichloromethanic extract was determined δ-selinene.[3]

Jatrophone is considered cytotoxic and tumor-inhibitory agent.[2,4,5] This substance has been the object of several scientific investigations, and, in Brazil, results have included reports on molluscidal activity,[6] relaxant action in rat portal veins,[7] inhibition of insulin,[8] and inhibition of plateled rich plasma aggregation.[9]

Most of the *Jatropha elliptica* in "cachaça" used in Brazil have no information on their exact composition in relation as substances presents or on the recommended dosages, imposing serious risks to public health.

HPLC equipment is widely disseminated in Brazil. We have developed an analytical procedure suitable for sample preparation and HPLC-UV analysis of *jatrophone* contents in "cachaça".

The HPLC technique has been shown to be a very efficient system separating complex mixtures plants. HPLC methods have been reported for the determination of substances in callus cultures, vitro culture, serum, dermis, plants, citrus, essential oils and phytomedicines, but only the most recent published methods report assay validation.[10—21] There are no descriptions in the literature about sample preparation and determination quantitative of jatrophone present in "cachaça" prepared with *Jatropha elliptica* employed in Brazil.

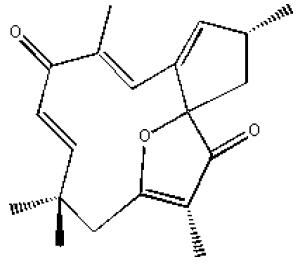

Fig. 1. Chemical Structure of the Jatrophone

The sensitivity, linearity, accuracy, precision were evaluated. The efficiency of the analytical procedure was assessed through calculation of recovery values.

Experimental

Plant Material The samples were collected from the "cerrado" of the Mato Grosso do Sul State, Brazil. A voucher specimen was deposited in the herbarium CCBS/UFMS, in Campo Grande/MS, Brazil.

Chemicals Spectroscopy-grade acetonitrile, hexane, acetone, chloroform and methanol were purchased from Tedia Company/U.S.A. Water was purified using a Milli-Q system (Millipore). Jatrophone were isolated and purified by chromatography and recrystallization in Chemical Laboratories of the Mato Grosso do Sul Federal University. The identity of the standard was confirmed by MS, IR, and ^{1}H- and ^{13}C-NMR and their purity evaluated with HPLC-UV was 97.7% (Fig. 2). The "cachaça" (a commercial product containing distillates of sugar-cane, sugar and water) had and alcohol content of 38%). Stock mixtures of standard were made from the individual solutions in water–acetonitrile (13 : 7) and used as external standards.

TLC Condition The chloroform fractions and the jatrophone standard were performed on 10×10 cm TLC Si F_{254} plates (Merck, Darmstadt, Germany). The solvent system used as eluent was hexane–acetone (4 : 1). The spots were detected under UV light at 254 nm and additionally were sprayed with methanol 10% H_2SO_4.

HPLC Conditions The analyses were performed on a Shimadzu liquid chromatograph with a ternary solvent delivery system—Model LC-6AD, combined with UV–Vis detector—Model SPD-6AV (Shimadzu Co., Kyoto, Japan), and a Rheodyne injection valve filted with a 100-μl sample loop. A microcomputer equipped with Microquimica-MQI18PCA software was used for recording chromatograms and measuring peak areas. HPLC separation of the jatrophone was performed using a Shimadzu octadecyl Shim-pack CLC-ODS (4.6 mm i.d.×25 cm long and 5 μm particle diameter) reversed-phase column with a small pre-column (4.6 mm i.d.×2.5 cm long) containing the same packing, used to protect the analytical column. Before use, the solvents were filtered through a 0.45-μm HV filter (Millipore), then degassed for 20 min in an ultrasonic bath. Elution was performed with water–acetronitrile (60 : 40) by 5 min and gradient in the 20% water in 25 min, return to the initial condition in 30 min. Flow-rate of 1.0 ml/min. Aliquots of 15 μl were injected with a 25 μl Hamilton syringe. After determination the column were clean by 15 min with the same system solvent and flow-rate. Detection of the peaks was recorded at 280 nm. All chromatographic analyses were performed at 25 °C.

Sample Preparation (Optimization) Fragments of the subterranean stem (10 g) *Jatropha elliptica* were maintained in an infusion in 50 ml of the "cachaça" at environmental temperature. After 72 h, a sample of 5 ml was

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removed, and from the dry residue (0,6 g), three quantities, weighing 67 mg each, were submitted to different separation conditions in Sulpelclean™ LC-18 (1 g, 55—105 μm, 6 ml) purchased from SUPELCO Park/U.S.A. In condition **1**, the sample was dissolved in 1 ml of the water–acetonitrile (1 : 1), and added to the column, wich was eluted with water–acetonitrile (1 : 1, 3×1 ml), acetonitrile (4×1 ml) and chloroform (1×4 ml). In condition **2**, the sample was dissolved in 1 ml of the water–acetonitrile (4 : 1) and added to the column, wich was eluted with water–acetonitrile (4 : 1, 3×1 ml), acetonitrile (4×1 ml) and chloroform (1×4 ml). In condition **3**, the sample was dissolved in 1 ml of the water–acetonitrile (13 : 7) and added to the column, wich was eluted with water–acetonitrile (13 : 7, 3×1 ml), acetonitrile (4×1 ml) and chloroform (1×4 ml). Fractions (water: acetonitrile and acetonitrile) were diluted in water–acetonitrile (13 : 7) in a 5 ml volumetric flask, filtered through a 0.45 μm Millex filter and analyzed by HPLC. The fraction chloroform were diluted in chloroform in a 1 ml and analyzed by TLC.

Sample Preparation (Optimal Condition) Fragments of the subterranean stem (10 g) *Jatropha elliptica* were maintained in an infusion in 50 ml of the "cachaça" at environmental temperature. After 48, 72, 96, 168 h, the samples of 1 ml were removed and were submitted the separation in Sulpelclean™ LC-18 (1 g, 55—105 μm, 6 ml) purchased from SUPELCO Park/U.S.A., which were previously conditioned with 5 ml of the water–acetonitrile (13 : 7) and sequentially eluted with water–acetonitrile (13 : 7, 3×1 ml), acetonitrile (4×1 ml) and chloroform (1×4 ml). Fractions were diluted in water–acetonitrile (13 : 7) in a 5 ml volumetric flask, filtered through a 0.45 μm Millex filter and analyzed by HPLC. The fraction chloroform were diluted in chloroform in a 1 ml and analyzed by TLC.

Determination of the Detection and Quantification Limits The detection limits were determined by injecting (n=5) solutions of jatrophone of known concentration (15 μl each), and decreasing the concentrations of the samples until detecting a peak with three times the signal/noise ratio. The corresponding concentration was considered as being the minimal detectable concentration. The quantification limits were performed by the same methodology was considered the chromatography peak having ten times the signal/noise ratio.

Extraction Recovery The extraction efficiency (recovery) was determined in *Jatropha elliptica* with jatrophone in content of the 10 mg of jatrophone by 10 g of the *Jatropha elliptica* (n=5). The spiked samples were submitted to the same procedure as described in sample preparation (Optimal Condition).

Analytical Curve Estimation of the content of jatrophone in the samples was performed by external calibration. The compound in the study were dissolved separately in spectroscopy-grade water–acetronitrile (13 : 7) in order to obtain stock solutions, which were appropriately diluted to concentrations ranging from 16.24—81.20 μg/ml. Aliquots of 5 dilutions for the standard were analysed by HPLC, with each determination being carried out five times. For the standard was obtained the corresponding chromatogram and constructed a graphical plotting the means of areas against the concentration for jatrophone. Linear least squares regression of the peak areas as a function of the concentrations was performed to determine correlation coefficients. Specimens with an analyte concentration exceeding the analytical curve were reassayed upon appropriate dilution of the samples.

Accuracy and Precision The accuracy of the assayed method was evaluated by performing replicate analyses against an analytical calibration curve and calculating the mean percent differences between the theoretical values and the measured values. The accuracy values variation studies using HPLC in the amount of the 10 mg of jatrophone by 10 g of the *Jatropha elliptica* (n=5). The precision of a method is expressed as the percentage of the coefficient of variation (CV) of replicate measurements. The variability of the method was determined for each sample with addiction of known amount of the 10 mg of jatrophone by 10 g of the *Jatropha elliptica* (n=5).

Stability Study The stability of the working standard solutions was tested at 25 °C (working temperature), 4 °C and −20 °C (storage temperatures). The stability of jatrophone was evaluated during all the storage steps (*i.e.* at room temperature, at 4 °C and at −20 °C). Spiked samples were analyzed, against the analytical calibration curves, immediately after preparation (reference values) and after storage. Stability was defined as being less than 2% loss of the initial drug concentration in the stated time.

Results and Discussion

Preliminary sample preparation and HPLC experiments employing *Jatropha elliptica* in the "cachaça" were performed to establish optimal conditions for analysis of jatrophone.

In this report, a method based on reversed-phase LC separation combined with UV detection was developed for jatrophone assay in "cachaça". HPLC analysis showed baseline separation for the compound of interest, which could be analysed in a satisfactory time interval (jatrophone 16.41±0.07 min) (Fig. 2).

The conditions **1**, **2** and **3** were tested for developed optimal condition. In condition **1**, the HPLC analysis showed the presence of tannins in both the water–acetonitrile (1 : 1) fraction as well as in the acetonitrile fraction with higher concentration in the more polar fraction. Jatrophone was also present in both fractions, having however a larger concentration in the less polar fraction. In condition **2**, jatrophone was absent in the water–acetonitrile (4 : 1) fraction but present in the acetonitrile fraction, with a significant part of the tannins not totally eluted in the more polar fraction. In condition **3**, the HPLC analysis showed almost total elution of the tannins in the water–acetonitrile (13 : 7) fraction, and total concentration of the jatrophone in the acetonitrile fraction (Fig. 3). The TLC analysis demonstrated the absence of jatrophone in the chloroform fractions.

The quantification was performed in optimal condition (Fig. 3).

The identification of jatrophone in the *Jatropha elliptica* was performed by comparison of their retention time with

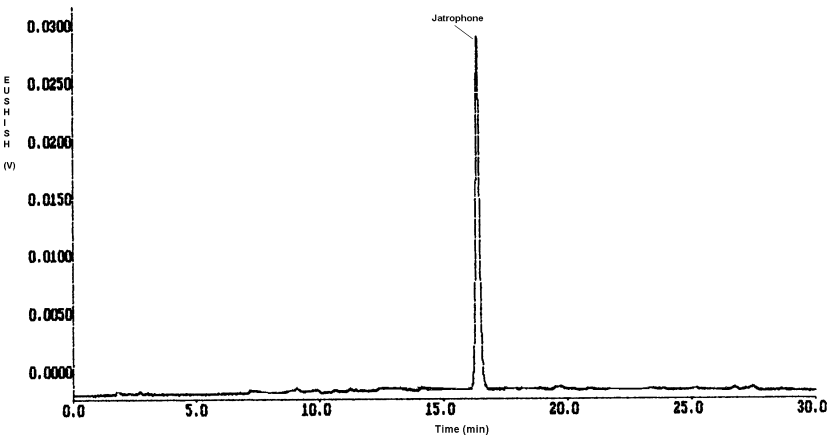

Fig. 2. Chromatogram of Standard (Jatrophone) Analysis by HPLC

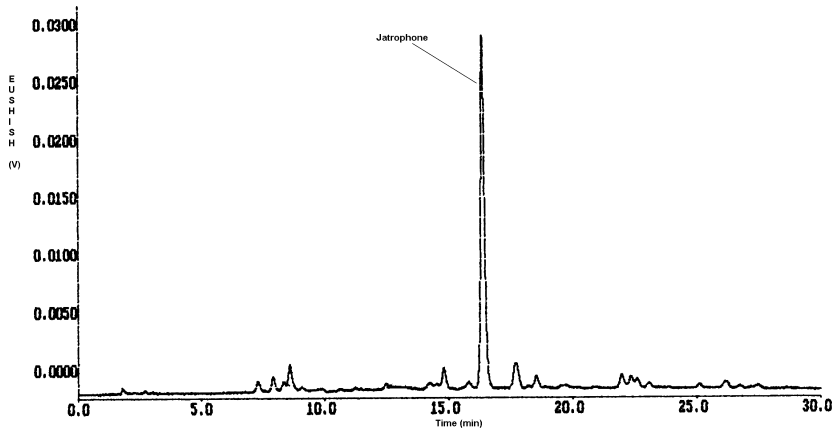

Fig. 3. Chromatogram of a Typical "Cachaça" (48 h) Prepared with *Jatropha elliptica* Analysis (Optimal Condition) by HPLC

Table 1. Precision and Accuracy of HPLC Method for Determination of the Jatrophone in "Cachaça" Prepared with *Jatropha elliptica* Samples ($n=5$)

Times (h)	Amount added (μg)	Jatrophone (μg) (mean±S.D.)			
		Jatrophone (%)	Amount found (μg) (mean±S.D.)	Ac (%)	CV (%)
48	10000	98.99	9899.20±123.35	1.02	1.25
72	10000	99.30	9930.00±100.03	0.70	1.01
96	10000	99.59	9959.10±121.21	0.41	1.22
168	10000	99.89	9989.00±111.77	0.11	1.12

CV, coefficient of variation; Ac, accuracy ; S.D., standard deviations.

those of authentic standard and by standard addiction in the samples in analyzed by HPLC-UV. The intervals, where the compound eluted, were free of interference in all samples tested, employed sample preparation optimized.

No changes of jatrophone were detected in working solutions after 24 h at 25 °C, 2 months at 4 °C and 6 months of storage at −20 °C. Jatrophone one were stable in the samples after 24 h at 25 °C, one month of storage at 4 °C and 6 months of storage at −20 °C. Thus this validated method for the assay of jatrophone may be regarded as indicating stability of the solutions.

The calibration curve was determined by linear regression. The calibration curve was linear in the range of 16.24—81.20 μg/ml, with excellent correlation coefficients (r). The representative linear equation, $y=-0.0712+0.20406x$ ($n=5$). Average standard errors for the peak areas of replicate injections ($n=5$) were smaller than 1% showing good repeatability of the calibration curve. The correlation coefficient 0.99989 showing good calibration curve.

Detection limit was 0.012 μg/ml and quantification limit was 0.040 μg/ml for jatrophone. HPLC procedure using ultraviolet detection presents lower quantification and detection limits.

The results showed good recovery in 4 times (48, 72, 96 168 h) in the "cachaça" prepared with *Jatropha elliptica*. The accuracy values were less than 2% (Table 1). Regarding the precision of the assay and CVs were less than ±2%. The "cachaça" was prepared with *Jatropha elliptica* in accordance with indications popular. Fragments of the subterranean stem *Jatropha elliptica* were maintained in an infusion of the "cachaça" at environmental temperature by years. The "cachaça" is initially consumed after 48 h of the prepared.

Table 2. Contents in "Cachaça" (μg of Jatrophone by ml of "Cachaça") (mean±S.D.) Employing the HPLC Method (Optimal Condition)

Time (h)	Jatrophone
48	132.59±1.02
72	142.63±1.22
96	159.44±2.40
168	160.31±1.93

S.D., standard deviations; S.D. of five determinations.

In this study, amount of the 160 μg were found for jatrophone in the 1 ml of the "cachaça" prepared with *Jatropha elliptica* in an time of the 96 or 168 h and 133 μg/ml in 48 h (Table 2).

Cachaça prepared with *Jatropha elliptica* are very consumed by people in Mato Grosso do Sul, Brazil, and showed the presence of the jatrophone. However, the lack of knowledge of the presence of jatrophone in the cachaça offers risks to the public health, since they are not adversed about the potencialization of the effect toxic with consumption of the several ml. Therefore, it is important know the levels of jatrophone in "cachaça" consumed by humans.

Conclusion

The HPLC analysis of the "cachaça" prepared with *Jatropha elliptica* was developed for the determination of jatrophone, is the first report of the separation and sample preparation of the substance.

Validation experiments showed good precision and accuracy for the method with coefficients of variation and relative errors less than ±2%.

In conclusion, the proposed LC method shows an excellent performance to separate and quantitative the jatrophone in "cachaça" prepared with *Jatropha elliptica*.

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