



Received on 04 March, 2016; received in revised form, 08 April, 2016; accepted, 31 May, 2016; published 01 August, 2016

ISOLATION AND DEVELOPMENT OF AN HPLC METHOD FOR THE QUANTIFICATION OF A BIOMARKER IN THE ROOTS OF *PAULLINIA PINNATA*

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Keywords:

Paullinia pinnata, Biomarker, Validation, Isolation, HPLC

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ABSTRACT: An RP-HPLC method to be used to quantitate the amount of a biomarker in the roots of *Paullinia pinnata* was developed. The root bark was milled and extracted and the dry powder obtained taken through column chromatography (CC). The biomarker was then isolated. The melting point of the biomarker was determined and found to be 117-119 °C. A RP-HPLC method was successfully developed and used to quantify the biomarker in the chloroform extract. The mobile phase used was Methanol:Water (9:1) with an injection volume of 100µl and a range of 1.000. The method employs a Phenomenex Kromasil C-8, 5µ, 250mm × 4.6mm, 100Å reverse phase column at a flow rate of 1.3ml/min, and a wavelength of 210nm. The method gave a retention time of 2.9687 ± 0.2657 (n=15). The percentage content of the biomarker in the root of *Paullinia pinnata* was determined to be 0.052009±0.004321% w/w (n=12). The HPLC method was validated for linearity, repeatability, intermediate precision, and robustness. The limits of detection and quantitation were also determined.

INTRODUCTION: Traditional plant medicine continues to play a major role in the Ghanaian healthcare system. The WHO has stated that about 70% to 80% of people in developing countries have resorted to the use of complementary or alternative medicine (CAM) at one point in time or another¹. The use of CAM in these countries is largely traditional, having been passed on from generation to generation. These traditions however rely heavily on empirical evidence and anecdotal accounts of the benefits of the use of the various healing materials and techniques.

It has therefore been a focus of the Faculty of Pharmacy & Pharmaceutical Sciences, KNUST to conduct scientific experiments to substantiate the claims of efficacy and determine through isolation the compounds responsible for the activities of the plants²⁻⁶.

Paullinia pinnata is a plant that has been used locally for the management of wounds in Ghana. The scientific basis of the use of the plant for wound healing has been investigated and confirmed⁷. It is known locally as Toa-ntini. There are various reports of its use in erectile dysfunction, coughs and pulmonary diseases, and as an antioxidant⁸.

In recent times scientific interest in plants employed in wound healing has gained some ground. This is in view of recent developments with regard to *in vitro* bioassays⁹. Various parts of

<p>QUICK RESPONSE CODE</p> 	<p>DOI: 10.13040/IJPSR.0975-8232.7(8).3446-52</p>
<p>Article can be accessed online on: www.ijpsr.com</p>	
<p>DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.7(8).3446-52</p>	

Paullinia pinnata including more recently the roots have been investigated for their chemical composition. Two novel compounds namely 6 β -(3'-methoxy-4'-hydroxybenzoyl)-lup-20(29)-ene-one and 6 β -(3'-methoxy-4'-hydroxybenzoyl)-lup-20(29)-ene-ol, together with three known compounds, friedelin, β -sitosterol and β -sitosterol-3-D-glucoside have been isolated from the root of the plant¹⁰. The two novel compounds displayed significant fibroblast stimulatory activity¹⁰.

Most medicines from plant sources are used in huge doses (measured in cups and number of tablespoons) and may result in fatal consequences for patients if the plants contain toxic compounds. It has therefore become necessary to include the quantification of the various active principles found in the medicinal plants in the research effort in traditional medicine. Knowledge of the quantity of an active antimicrobial principle in a unit amount of a whole extract of its plant taken for an infection will help to estimate the dose and duration against the background of information regarding the minimum inhibitory concentration, among others, of that active principle.

In view of the above, a biomarker was isolated from the roots and quantified using a RP-HPLC method developed.

MATERIALS AND METHODS:

Plant material:

The *Paullinia pinnata* material was obtained in April 2012 from of the department of Pharmacognosy, KNUST and authenticated at the department.

General experimental conditions:

Thin layer chromatography (TLC) was carried out with pre-coated analytical TLC plates using solvent systems involving varying proportions of petroleum ether 40–60°C (PE) and ethyl acetate (EtOAc) and acidic anisaldehyde and/or sulphuric acid/methanol solution spray for visualisation. ¹H NMR spectra were determined in deuterated chloroform (CDCl₃) on a Mercury-300BB Varian Spectrometer using tetramethylsilane as internal standard. The UV spectrum was obtained on T90+ UV/VIS Spectrometer (PG Instruments Ltd.) with UV Win 5.2.0 software.

Preparation of extract:

The coarsely powdered plant material (5.4kg) was extracted with about 15L of chloroform (CHCl₃) using a soxhlet extractor for 48 hours till the plant material was well exhausted. The extract obtained was concentrated using a Buchi rotavapor under reduced pressure to a syrupy mass. The syrupy mass was then transferred into a crucible and air dried to obtain a brown powder of weight 41.67g which was transferred into a glass bottle and kept in a desiccator for later use.

30g of the brown powder (chloroform extract) was dissolved in just enough chloroform and transferred into a separating funnel containing water/petroleum ether 1:1 (400ml). The petroleum ether layer was collected and further aliquots of petroleum ether added and collected until the green colour of the layer was discharged. The suspension was then extracted with 5×200ml chloroform. The petroleum ether and chloroform fractions thus obtained were then concentrated and air-dried. (PE – 15g, Chloroform – 8g)

Isolation of biomarker:

The biomarker was isolated from the chloroform fraction (7.0g), which has been determined to be the active fraction¹⁰, by column chromatography using silica gel (70–230 mesh from BDH). Gradient elution was carried out using petroleum ether containing increasing amounts of ethyl acetate. Fractions were collected and bulked into eighteen sub fractions C₁ to C₁₈ based on their profiles on TLC plates and air dried. The components of the dried sub fractions were re-dissolved using petroleum ether, ethyl acetate, chloroform and methanol.

The content of fraction C₃ was dissolved with chloroform. Insoluble orange to yellow crystals were observed at the base of the container. These crystals were filtered out. Methanol was then added to the filtrate after which white amorphous crystals precipitated out and was filtered. The crystals were washed repeatedly with methanol and air dried to afford the biomarker.

High performance liquid chromatography (HPLC): Five mg of the isolate was dissolved in 1ml of ethyl acetate, transferred into a 50ml

volumetric flask and topped up to the 50ml mark using methanol/water (90:10) to give a stock solution of concentration 0.01% (w/v). Solutions A - E of concentrations of 0.001%, 0.0006%, 0.00036%, 0.000216%, and 0.0001296% respectively were prepared from the stock. Solution A was scanned between a wavelength range of 200nm and 400nm with the T90+ UV-VIS Spectrometer (PG Instruments) using 1ml ethyl acetate in 25ml methanol as the blank and the wavelength of absorption determined. The solutions were then injected into the HPLC and their peak areas noted and recorded.

Milled *P. pinnata* roots (361.67g), was exhaustively extracted with 2.5L of chloroform. The extract was then concentrated to a volume of 500ml. 10ml of the resulting solution was pipetted into a 100ml volumetric flask and made up to volume using methanol. The resulting solution was labeled E_A. 10ml of E_A was then pipetted and transferred into a 100ml volumetric flask and also made to volume using methanol and labeled E_B.

Method validation:

The method developed was validated according to the ICH protocol for linearity, repeatability, intermediate precision, robustness and limits of detection and quantification¹¹.

RESULTS:

Identification of biomarker:

The biomarker was isolated as a white amorphous powder with melting point 117-119 °C.

TABLE 1: EXTRACTION AND FRACTIONATION

Weight of ground root material	Weight of extract obtained	Weight of Pet ether fraction	Weight of chloroform fraction
5.4kg	41.67g	15g	7.8g

TABLE 2: SOLUBILITY PROFILE

Solvent	Solubility
Petroleum ether	-
Chloroform	+
Ethyl Acetate	+
Methanol	-

HPLC analysis:

Conditions for chromatographic analysis:

- Column: Kromasil C-8, 5 μ , 250mm \times 4.6mm, 100 \AA by Phenomenex

- Flow rate: 1.3ml/min
- Wavelength of detection: 210nm
- Mobile phase: Methanol (90%) and Water (10%)
- Injection volume: 100 μ l
- Range: 1.000

Retention time:

Retention time (mean \pm SD) = 2.9687 \pm 0.2657 ; n=15

Method validation:

Calibration curve:

TABLE 3: VALUES FOR CALIBRATION CURVE PLOT

Solution	Concentration	Peak area
A	0.00%	8.12
B	0.00%	4.77
C	0.00%	2.95
D	0.00%	1.55
E	0.00%	1.08

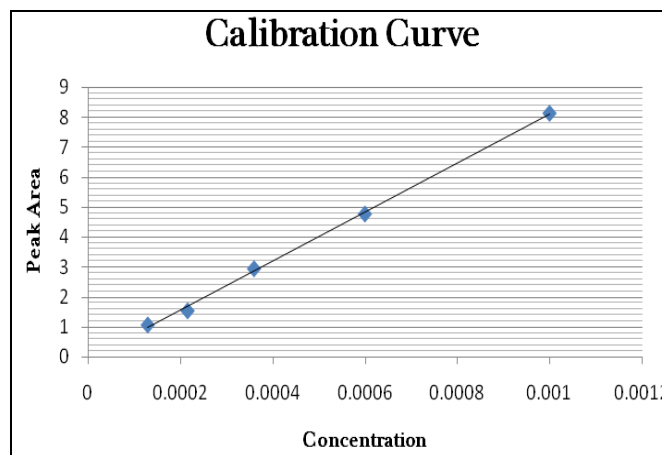


FIG. 1: CALIBRATION CURVE

Slope of the curve = 8168.9, Coefficient of regression = 0.9988, Range = 1.296 μ g/ml - 11 μ g/ml

LOD and LOQ:

The formulae for LOD and LOQ are = 0.11496692 and $S = 8168.87432$ as calculated using Microsoft Excel Therefore, the limit of detection (LOD) = 0.0000464435 = 0.464435 μ g/ml and the limit of quantification (LOQ) = 0.000140738 = 1.40738 μ g/ml.

Repeatability:

For repeatability, six determinations were made for solution E of concentration 0.0001296% (w/v). The Relative Standard Deviation (RSD) was then calculated to determine if the method developed has intra-day precision.

For actual concentration, put peak area value into equation of the line of the calibration curve, then solve for actual concentration, X which becomes equal to. The percentage purity is calculated as.

Therefore for determination 1, actual concentration = 0.000141% and the percentage purity = 108.8893

Sample calculation:**TABLE 4: REPEATABILITY OF THE METHOD FOR THE ISOLATED BIOMARKER**

Determination	Peak Area	Actual Concentration	% Purity
1	1.08	0.000141%	108.8893
2	1.05	0.000137%	106.0557
3	1.03	0.000135%	104.1665
4	1.05	0.000137%	106.0557
5	1.07	0.00014%	107.9448
6	1.08	0.000141%	108.8893
	Mean	Standard Deviation	RSD (%)
Using Peak Area	1.06	0.02	1.886792
Using % Purity	107.0002	1.889128	1.765537

Intermediate precision:

The intermediate precision of the method developed was also determined. Solution E was run on three different days.

TABLE 5: INTERMEDIATE PRECISION USING PEAK AREAS

Day	Mean Area (n=6)	SD	RSD (%)
1	1.06	0.02	1.88679
2	1.02333	0.01211	1.18345
3	0.97	0.01789	1.84418

TABLE 6: INTERMEDIATE PRECISION USING % PURITY

Day	Mean % Purity (n=6)	SD	RSD (%)
1	107.0002	1.889128	1.765537
2	103.5368	1.143924	1.104847
3	98.499141	1.6896876	1.715434

Robustness:**TABLE 7: ROBUSTNESS USING WAVELENGTH FOR BIOMARKER CONCENTRATION 0.000236% (w/v)**

Wavelength (nm)	Effect on peak	SD	RSD (%)
210	Symmetrical peak with no significant tailing	0.028577	1.894633
220	Symmetrical peak with no significant tailing	0.012111	1.942877

Percentage content of biomarker:**Sample calculation:**

Equation of the line is $y = 8168.9x - 0.0728$

Therefore for peak area 25.62, the concentration of dilute solution of extract = 0.003145% (w/v).

The dilution factor is 10 so the concentration of the stock solution = $10 \times 0.003145\%$ (w/v) = 0.03145% (w/v).

The concentration of the stock solution is in grams per 100ml of solution but the volume of the stock used is 500ml. The amount of the biomarker in the stock solution is therefore 5 times the concentration and is given by $5 \times$ concentration of stock solution.

Therefore, actual amount of biomarker in the stock solution = 0.03145% (w/v) $\times 5$

The amount of root sample used for the preparation of the 500ml extract is 361.67g.

Therefore, % content of biomarker in the root sample = 0.043482% (w/w)

TABLE 8: % CONTENT OF BIOMARKER FOR 1 IN 100 DILUTION OF STOCK SOLUTION

Peak Area	Concentration of solution (% w/v)	Concentration of stock	Actual Amount of biomarker in stock solution	% w/w of biomarker in roots
25.62	0.003145	0.031451970	0.15725985	0.043482
32.87	0.004033	0.040327094	0.20163547	0.055751
32.41	0.003976	0.039763983	0.19881991	0.054973
30.32	0.003721	0.037205499	0.18602749	0.051436
31.89	0.003913	0.039127422	0.19563711	0.054093
28.76	0.00353	0.035295817	0.17647909	0.048796

TABLE 9: % CONTENT OF BIOMARKER FOR 1 IN 10 DILUTION OF STOCK SOLUTION

Peak Area	Concentration of solution (% w/v)	Concentration of stock	Actual Amount of biomarker in stock solution	% w/w of biomarker in roots
2.88	0.000361	0.036146850	0.18073425	0.049972
2.91	0.000365	0.036514096	0.18257048	0.05048
3.22	0.000403	0.040308977	0.20154488	0.055726
2.67	0.000336	0.033576124	0.16788062	0.046418
3.19	0.000399	0.03994173	0.19970865	0.055218
3.34	0.000418	0.041777963	0.20888981	0.057757

The mean content of biomarker in the roots of *Paullinia pinnata* is therefore 0.052009 \pm 0.004321% w/w (n=12)

DISCUSSION:

Structure of biomarker:

The ¹H NMR spectra of the biomarker reveal a large cluster at chemical shifts between δ 0.8 and δ 2.4 which is in the methylene envelope (chemical shifts between δ 2.6 and δ 0.7). This suggests the likelihood of a steroidal or triterpenoid structure. Also, the signals obtained at δ 4.597, δ 4.586, δ 4.456 and δ 1.563 are similar to the signals obtained by Annan and Houghton at δ 4.67, δ 4.54 and δ 1.68 and suggest the presence of a lup-20(29)-ene system¹⁰.

The chemical shift obtained at δ 5.301 deviates only slightly from that obtained at δ 5.22 by Annan and Houghton¹⁰ which was similar to the chemical shift of carbinol methine proton at H-6 in the 6 β hydroxyl lupane derivatives reported by Dantanaranyaya *et al.*¹². The signal between δ 8 and δ 7 in the spectrum may be resulting from aromatic protons and may signify the presence of a substituted aromatic ring attached to a triterpenoid structure but is also characteristic of chloroform or deuterated chloroform, the solvent used for the analysis.

Using the ¹H NMR spectra, the biomarker is suspected to be a 6 β -(3-methoxy-4-hydroxybenzoyl) ester of lupeone. The identity of the biomarker is not absolutely confirmed due to the lack of ¹³CNMR

HPLC method development and validation:

Following the isolation of the biomarker, an HPLC method was developed and used for the quantification of the amount of the biomarker in the roots of *Paullinia pinnata*. A number of conditions were tried during the method development and the method that yielded the best results with respect to the consistency of the peak shape and symmetry chosen.

The method/conditions chosen eluted the biomarker at a time of 2.9687 \pm 0.2657mins (n=15). This retention time is quite short and can therefore be considered efficient for routine analytical work

Percentage content of biomarker:

The method developed and used for the quantitative analysis showed the root bark to contain 0.052009 \pm 0.004321% w/w (n=12) of the biomarker. This means that every 100g of root material contains about 52mg of the biomarker. This information is useful in determining the

dosage and use of the plant material vis-à-vis information about the minimum concentration at which the fibroblast stimulatory activity or wound healing effect is achieved. Annan & Houghton¹⁰ determined the EC50 of the biomarkers from the roots of *Paullinia pinnata* and noted that above certain concentrations, the biomarkers may have become cytotoxic as the proliferative activity decreased.

Linearity:

The biomarker was used as a secondary standard and the method developed used to determine the amount of the biomarker in the root extract. A calibration curve was therefore drawn to determine the concentration range within which the method gives a good linear relationship between the concentration of the biomarker and the peak area obtained. The results indicate that the method has a coefficient of regression (R^2) of 0.9988. The ICH guidelines (1994) recommend a coefficient of regression value above 0.998 to indicate good linearity. The linearity of the methods holds true for concentrations between 1.296 μ g/ml and 11 μ g/ml.

LOD and LOQ:

The LOD and LOQ represent a measure of the sensitivity of a given method. The lesser the LOD, the more sensitive a method is. The LOD of the method developed was calculated as 0.464435 μ g/ml which means that the method is very sensitive to the biomarker being analyzed. The LOQ, much like the LOD, gives accurate information regarding the quantity of the analyte that can be correctly determined quantitatively. The lower the LOQ the more sensitive therefore the method is. The LOQ was calculated as 1.40738 μ g/ml which is low and signifies that using this method one can accurately determine microgram quantities of the biomarker.

Precision:

Precision may be in the form of the intra-day precision (repeatability) of the method or the inter-day precision (intermediate). The intra-day day precision judges the repeatability (consistency of results) of the analytical method being used using results obtained on the same day of analysis whereas the inter-day precision determines the

consistency of results obtained on different days of analysis. The ICH guidelines (1994) stipulate an RSD of $\leq 2\%$ for both intra- and inter-day precision to make an analytical method repeatable or reproducible.

The RSD of the results obtained for the repeatability study was calculated as 1.886792% and 1.765537% using the peak areas and the % purity respectively.

The intermediate precision of the method was also determined using six replicate determinations of one concentration level on three days. The RSD of the method in this respect was found to be 1.886792%, 1.183446% and 1.84418% for days 1, 2 and 3 respectively using the peak areas in the calculation. All values were below the recommended ≤ 2 mark and suggest good between-day precision of the method developed. The method showed good between-day precision as well when the %purity values were used in the calculation with RSD values of 1.765537%, 1.104847% and 1.715434% representing days 1, 2 and 3 respectively.

Robustness:

Robustness gives a measure of the ability of the method to give accurate results within variations of certain conditions. The method developed was found to be robust with respect to wavelength change. The RSD determined using 210nm and 220nm were found to be 1.894633 and 1.942877 respectively indicating that the method can be used satisfactorily at any of these wavelengths of measurement.

CONCLUSION: A reverse phase HPLC method was developed for compound 1 with the following parameters: Column: Kromasil C-8, 5 μ , 250mm \times 4.6mm, 100 \AA by Phenomenex, Flow rate: 1.3ml/min, Wavelength of detection: 210nm, Mobile phase: Methanol (90%) and Water (10%), Injection volume: 100 μ l and Range: 1.000.

The percentage content of the biomarker in the root bark of *P. pinnata* was determined to be 0.052009 \pm 0.004321% w/w (n=12) using the RP-HPLC method developed.

RECOMMENDATIONS:

Based on the work that has been carried out in this project, I make the following recommendations

- The identity of the compounds isolated in this work should be determined using NMR data (i.e. HSQC, HMBC, ¹³CNMR) that will help to conclusively identify them.
- The seasonal variations in the amount of the isolates in the root should be determined to ascertain the best time to harvest the roots of *P. pinnata* for medicinal purposes.
- Different and possibly easier/faster methods of isolation of the compounds obtained from the root bark should be investigated.
- Stability studies should be done on extracts of *P. pinnata* and on the isolates to determine the effect of temperature and storage conditions on the isolates.
- Where stability studies reveal degradation with respect to temperature or time, suitable methods should be developed for detection of possible degradation products and establish limits of toxicity for those that may be toxic.

ACKNOWLEDGEMENT: The authors are grateful to the technicians of the Department of Pharmaceutical Chemistry, K.N.U.S.T., Kumasi, Ghana for their contribution to this study.

CONFLICT OF INTEREST: The authors declare no conflict of interest.

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How to cite this article:

Cudjoe EK, Addotey JNA, Okine NNA, Adosraku RK, Annan K: Isolation and development of an HPLC method for the quantification of a biomarker in the roots of *Paullinia pinnata*. *Int J Pharm Sci Res* 2016; 7(8): 3446-52. doi: 10.13040/IJPSR.0975-8232.7(8).3446-52.

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