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Solubility Study and Partition Coefficients of 30% Ethanolic Extract Derived from *Phyllanthus urinaria* and *Phyllanthus niruri between* Newly Introduced Palm Kernel Oil Esters and Aqueous Vehicles.

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Abstract

The purpose of this research was to study of physicochemical properties of the extracts and to develop and validate HPLC method in order to study their solubility in organic and aqueous vehicles and partition coefficients. The physicochemical characteristics such as moisture content, bulk density and were measured using various techniques. HPLC procedure for quantification of ellagic acid in *the extracts* has been modified and validated using binary gradient mode. A 0.2% formic acid and methanol was used to elute ellagic acid reference standard and the extracts on C 18 Hypersil Gold column stationary phase. The HPLC method was validated with respect to, linearity, system suitability, LOD, LOQ, accuracy and precision. The bulk density and moisture content results show of the extracts were varied, while the pH of the two extracts was almost similar. Palm kernel oil esters was solubilised extracts better and Phosphate buffer at pH 7.4 and pH 6.6 were better aqueous vehicle for *Phyllanthus urinaria* and *Phyllanthus niruri* respectively and the extracts were partitioning better between phosphate buffer at pH 7.4 and palm kernel oil esters. The HPLC method was simple, rapid, accurate and safe for the column and successfully applied to pre-formulation study of the extracts as skin cosmetics and palm kernel oil esters / phosphate buffer at pH 7.4 were chosen as oil and aqueous phase to formulate the extracts as skin cosmetics

Keywords: Solubility, Partition coefficients, Ellagic acid, Phyllanthus, Palm kernel oil esters

Introduction

Phyllanthus urinaria (P. urinaria) and Phyllanthus niruri (*P. niruri*) belong to the genus *Phyllanthus*, family *L* (Euphorbiaceae). The genus is found all over the world in to tropical and subtropical countries [1]. The genus Phylanthus is huge and 550 to 750 species have been identified [2, 3]. *P. niruri and P. urinaria* have been used as a herbal medicine to treat broad range of diseases in tropical and sub-tropical countries [2]. The pharmacological effects of *P. niruri and P. urinaria* are attributed to various polyphenolic compounds present in these plants [4, 5]. The polyphenolic compounds identified in the extracts of *P. niruri* and *P. urinaria* were gallic acid, ellagic acid corilagin and geraniin [6-9]. The polyphenolic compounds are antioxidant compounds that are able to scavenging the free radicals and reactive oxygen species [10, 11]. The extracts become attractive for the pharmaceuticals and cosmeceuticals industries because of the presence of these polyphenolic compounds as natural source of antioxidants. Ellagic acid (EA) is one of the major polyphenolic compound identified in the 30% ethanolic extracts derived for local *P. urinaria* and *P. niruri*. EA Chemically is reported to possess low water solubility[12]. EA has lower water solubility compared with the other commercially available compound identified in *P. urinaria* and *P. niruri*. In addition EA is thermodynamically stable molecule with four fused rings representing the lipophilic part and four phenol and two lactones groups which act as hydrogen bond donor and acceptor, respectively [13] Figure 1. Accordingly, EA was selected as biochemical marker to standardize the extracts to reproducible level that naturally found in the extracts. This level will be used to study the physicochemical properties of the extracts such as solubility and partition coefficients of the extracts in oil and aqueous phase. The process of standardization of extract is important since it will facilitate consistency in quality of finished products in terms of quantity and potency. It can also be used to measure possible loss or degradation during manufacturing or shelf-life of the finished product. Our aim of this part of study is to develop and validate simple high performance liquid chromatography (HPLC) analytical method to study the solubility of the extracts in various oil and aqueous phases and to evaluate the partition coefficients of the extracts between the oil that has better solubility to the extracts and various aqueous solvent systems.

Material and Methods

Materials

Ellagic acid (95%) was purchased from Sigma–Aldrich, St. Louis, MO., USA. Methanol was purchased from J. T. Baker (Philipsburg, USA), Formic acid 98-100% assay from Merck, Germany. 99.7% ethanol and acetic acid were purchased from Brightchem Sdn Bhd, Malaysia. Sodium hydroxide potassium dihydrogen phosphate, sodium benzoate and sodium acetate was purchased from R & M Marketing, Essex, UK. *P. niruri, P. urinaria extracts* were obtained as a gift from Nova Laboratories Sepang, Malaysia. Palm kernel oil esters (PKOEs), palm oil esters (POEs) were obtained as a gift from the Chemistry Department, Faculty of Science Universiti Putra Malaysia (UPM), Kuala Lumpur, Malaysia, palm kernel oil BR (PKO), and crude palm oil BR were generous gift from BG Oil Chem, Malaysia and RBD palm olein was a gift from Golden Jomalina Food Industries, Selangor Malaysia.

Determination of Moisture Content

0.3 g of the sample was transferred into each of several Petri dishes and then dried in an oven at 105 C until a constant weight was obtained. The moisture content was then determined as the ratio of weight of moisture loss to weight of sample and was expressed as a percentage[14].

Determination of Bulk and tapped density

The extract was slowly pouring the into a 5 mL graduated glass cylinder to the level of 3mL. The mass of empty and filled measuring cylinder and the volume occupied by sample were recorded. The tapped density (Dt) was determined by tapping 100 times on a flat wooden bench. The bulk (Db) and tapped density was determined by triplicate analysis. The bulk density was calculated by dividing the weight of extract material by the volume occupied by the sample. The Hausners indexc (H) and compressibility index (C%) were calculated using equation 1 and 2 respectively [14].

$$H = \frac{Dt}{Db} \dots\dots\dots\dots (1)$$

$$C\% = \frac{Dt - Db}{Dt} \dots\dots\dots\dots (2)$$

Determination of pH

The pH values of 0.1mg/mL aqueous extract were measured using a microprocessor pH meter (pH211, Hanna Instruments Inc, Woonsocket, RI) at 26 C ± 2 C.

Development of HPLC Method

The HPLC analysis was performed using LC 20 AD Class LC-solution software, connected to SPD-20A UV/VIS detector, binary pump and temperature controlled column oven, (Shimadzu, Japan). Thermo Hypersil Gold TM (260×4.6 mm i.d., 5 μm) reversed phase column was used for all analysis. The column oven temperature was set at 40 C and the external reference standard (EA) and the extracts were eluted with a binary gradient mode at a UV wavelength of 270 nm and 20 μL volume of injection. Methanol (solvent A) and formic acid 0.2% (solvent B) were used as mobile phase at flow rate of 1mL/min and an LC time programme of the modules of the binary gradient of pump (B) was set to 100% at time 0min, 95% at 2 min, 70% at 5min, 66% at 8min, 55% at 11-14 min and the module was return to 100% at 17min and maintained 100% until 20min as described in our previous report with slight modification [15] .

External reference standard solution preparation

Stock solution of external reference standard (EA) was prepared by accurately weighed 2.5 mg and transferred it into 100mL volumetric flax. The reference standard was dissolved in 70mL of 99.7% ethanol and the volume was completed to 100 mL with distilled water. The stock solution was vortexed for 2-3min, ultra-sonicated for 10 min. Six concentrations in the range of 0.5 -16 µg /mL from EA stock solution were prepared. Each concentration was filtered through PTFE membrane syringe filter, 0.45 µm phenomenex, USA before injection. Each concentration was injected six times. Mean standard curve of EA was constructed by plotting the mean area under peaks obtained versus the respective concentrations μg/mL.

Sample solution preparation

The extracts solutions were prepared by dissolving 2.5 mg of each extract in 25 mL of 70:30 ethanol/water. The solutions were vortexed for 2-3 min, ultra-sonicated at room temperature for 10 min and filtered through PTFE membrane syringe filter, 0.45 µm phenomenex, USA before injection.

Method validation

The method validation was performed based on International Conference on Harmonization 2005 (ICH) criteria with respect to, linearity, system suitability, LOD, LOQ, recovery and accuracy and precision [16].

Linearity

The linearity was performed by the external standard method. The method linearity was statistically confirmed by least squares linear regression of six concentrations of EA reference standard in the range of 0.25 μg/mL -8 μg/mL. Each concentration in the range was injected six times and the mean calibration curve was constructed. The slope, intercept, correlation coefficients of the mean calibration curves were used to evaluate the linearity. Moreover, a student's t-test was used to compare the retention time and area under peak of each concentrations of the calibration curve (n=6).

System suitability

The system suitability was performed in order to verify its capability of producing data of acceptable quality. The validation criteria used were number of theoretical plates, tailing factors; resolution, separation, capacity factor, and repeatability (n=6). The value of these parameters were determined and compared with the recommended specifications limits set for the method by ICH, 2005[16].

Precision

The precision of the method was evaluated by analysis of three quality control (QC) samples at two levels, repeatability and intermediate level. Repeatability was evaluated by the intra-day analysis and intermediate level of precision was evaluated by and inter-day analysis for three days. Intra- and inter-day precision was carried multiple runs of EA external reference standard solutions (n=6). The percent of relative standard deviation (R.S.D%.) of the responses was calculated and compared with the specification set by ICH,2005 [16]. Six runs were performed for each concentration each day and were repeated for three consecutive days.

Recovery

Accuracy of the method was evaluated for both *intra-day and inter-day* variations using the six times analysis of the quality control samples (QC). The lower quality control LQC was of 2μg/mL, middle quality control, MQC, 5μg/mL and higher quality control, HQC 7.5μg/mL. Five aliquots of HPLC sample solutions of 3mL each of *P. niruri and P. urinaria* at concentration of 0.1mg/mL were spiked separately with 2 mL of blank solvent of 70:30 ethanol/water and freshly prepared EA standard solution at the QC concentrations levels (2μg /mL, 5μg/mL and 7.5μg/mL) and the fifth aliquot was left as pure extract solution (0.1mg/mL). The resulting solutions mixtures were analyzed and the percentage recoveries *RC* (%) were calculated using the expression (3).

Solubility study

The saturated solubility of the two extracts in various organic and aqueous systems was determined using shake-flask method[17]. EA was used as a marker to determine the solubility of the extracts in the organic and aqueous systems. Excess amount of each extract was placed separately into 25 mL stopper volumetric flasks containing 2 mL of the screened vehicles The mixture in the flasks were kept at ambient temperature (26±2.0 C) on a shaking Device M00 / M01, SV 14-22 / SV 29-45 (MEMMERT GmbH + Co. KG Aubere Rittersbacher StraBe 38 D-91128, Germany) at shaking speed of 90 strokes per minute for 72 h to attain equilibrium. The equilibrated samples were then removed from the shaker and centrifuged at 3,000 rpm for 15 min. 0.1 mL of the supernatant was taken and placed in 10 mL volumetric flask and was dissolved with 7 mL ethanol and completed to 10 with methanol/water. The solutions were vortexed for 2-3 min and filtered before injection. The concentration of the biomarker EA in the extract dissolved in oils or aqueous systems was determined using the above validated HPLC assay.

Apparent partition coefficients of the extracts

The apparent partition coefficient of the extracts between PKOEs and aqueous vehicles were studied using the method described elsewhere with slight modification [18, 19]. A 10 mg amount of each extracts was added to 10 mL of each aqueous system into 20 mL beaker covered with parafilm. The resulting solution was stirred with a magnetic bar at 500 rpm for 3h to reach equilibrium and subsequently centrifuged for 4 minutes at 4000 rpm. The supernatant was then filtered using PTFE membrane syringe filter, 0.45 μm phenomenex, USA. The filtrates concentrations (C1) of each extract were determined using the validated HPLC method. Aliquot (7 mL) of the aqueous phase with known concentration of each extract was added separately to a beaker containing 9 gm of PKOEs. The mixture was agitated by magnetic stirrer at 500 rpm for 3h to reach equilibrium and then was transferred to a separating funnel and allowed to stand for 30 min to separate. The concentration of the extracts in each aqueous phase was determined by the validated HLB method mentioned above and the result concentration was recorded as (CA2). The concentration of the extract in PKOEs was obtained by subtracting the concentration CA2 from CA1, (CA1-CA2). The partition coefficient (P) was calculated using the expression P =CO/CA= (CO = concentration of the extract in the oil (PKOEs), CA is the concentration of the extract in the aqueous phase) at equilibrium which is equal to (C A 1-CA2)/CA2.

Results and Discussion

Physicochemical properties

The results of the physicochemical properties of the extracts were shown in Table 1 and it color in Figure 2. The pH of the extracts was slightly acidic and matches the pH of skin (4.0-6.0) depending on the skin area and the age of the individual [20]. Usually highly acidic cosmetic cause's skin irritation and high alkaline product make the skin susceptible to bacterial infection. Therefore, the pH of the extracts facilitates its application in skin cosmetics.

High moisture contents leads to proliferation of microorganism and spoil of the raw material and affecting the shelf life of the finished product [14]. The moisture content has economic and technical impact. It affect production processes such as drying, packaging and storage[14]. In this study, it was notice that the moisture content of *Phyllanthus urinaria* was very low which mean mostly facilitate formulations stability as well as the quality of the raw material during storage and stability of the finished product compared to *Phyllanthus niruri.*

The bulk and tapped densities shows how the particles of the materials are arranged which affects the compaction factor of the material, flowability and a compressibility [14]. It was cleared from Table 1 that *Phyllanthus urinaria* has good flowability and compressibility compared with *Phyllanthus niruri*. Generally *Phyllanthus urinaria* has better physicochemical properties compared with *Phyllanthus niruri.*

Calibration curve of EA

EA chromatographic peaks of the extracts samples were identified at a retention time of 16.708 min as shown in Figure 3 and Figure 4 compared with the EA external reference standard retention time observed at retention time of 16.851 min (Figure 5). The mean curve equation obtained from the plotted graph of concentrations of ellagic against mean area of the six run (n=6) of each concentration Figure 5, was used as marker to evaluate the solubility of the extracts in various solvents.

Method validation

The method validation was performed based on International Conference on Harmonization 2005 (ICH) criteria with respect to, system suitability, linearity, LOD, LOQ, recovery and accuracy and precision. EA was chosen as external reference since structurally has better oil solubilization and commercially available. This method uses relatively low volumes of formic acid (0.2%) compared with the previous methods which uses 0.4% - 1% of various acids [21-26]. The less volume of acid used will be safe for the column and the instrument on log term of us

Linearity

The results of plotted six calibration curves verify the linearity of the analytical method as shown in Table 2. Six standard calibration curves were plotted using six responses solutions of EA in the range of 0.25 µg/mL and 8 µg/mL (n=6). Each concentration was analyzed six times. The calibration curve was plotted using the main area of six run of each concentrations. The regression coefficient (R^2), intercept (Y) and the slope (S) were used to evaluate the linearity of the method. The RSD% value of the linearity shown in Table 2 were with the limit of requirements set by ICH,2005 [16].

Limits of detection (LOD) and quantification (LOQ)

The limits of detection (LOD) and quantification (LOQ) for the external standard EA results were calculated using equation 3 A and 4 respectively. Where SD is the standard deviation of six responses of lower concentration of the mean calibration curve and *S* is the slope of the calibration curve. From the mean equation of the calibration curve Y= 123801 + 138364 * X, R^2= 0.999184, the slope was 138364 and the standard deviation of six responses of 0.5µg/mL EA external standard *STD*= 2326.36. The LOD and LOQ of the external standard EA is 0.055µg/mL and 0.168µg/mL respectively.

$$\text{LOD} = \frac{3.3SD}{S} \dots\dots\dots\dots..(3)$$

$$\text{LOQ} = \frac{10SD}{S} \dots\dots\dots\dots..(4)$$

System suitability

The system suitability validation criteria used were number of theoretical plates, tailing factors; resolution, separation, capacity factor, and repeatability based on *RSD* retention time and area under the curve for six repetitions of external reference standards (EA), *P. niruri* and *P. urinaria* samples. In this study the concentrations of the external reference standard chosen to be as closed as much to the concentration estimated in the sample so as to give similar response and the sample was intermittent by the reference standard during the analysis. Results of system suitability studied included area under peak (AUP), retention time (Rt), tailing factor (TF), resolution (Rs), separation factor (S), number of theoretical plates (TP) and capacity factor ($K^/$) for the extracts and the external reference standard EA (Table 3). The results of these parameters (Table 3) were found within the recommended specifications limits set for the method validation by ICH, 2005 [16]. The results give indication of column efficacy and the system suitability of the method for the intended application of uses.

Precision (repeatability)

The results of three concentrations at low medium and high level quality control points show that measurements obtained by the method were statistically significant (Table 4). The *intra-day* precision for three days was ranged 0.26 to 1.7 *RSD* and inter-day precision results was ranged *RSD* (%) 1.5 to 3.8. The values of *RSD*% obtained for both *intra-day* and *inter-day* analyses results were within required limit of ICH 2005 and indicate high degree of closeness of measurements.

Accuracy (recovery)

The closeness of the HPLC analysis results of both extracts samples solutions obtained by the method were shown in Table 5 and Figure 6 and Figure 7. The result illustrates analysis of five samples of each extract six times. The analysis of freshly prepared pure extract of *P. Niruri* and *P. Urinaria* (PUE), extract spiked with blanked solvent (SB), extract spiked with lower, middle and high quality control sample (LQC, MQC and HQC) concentrations. The resulting solutions mixtures were analyzed and the percentage recoveries *RC* (%) using equation (5), where RC is the recovery (%), *SK* is the spiked sample solution response at various *QC* levels, *SB* spiked sample with blank solvent response and *PR* is the response of the pure extract sample. The percents recovery obtained were within the limit of the requirement of ICH, 2005.

$$\left[\boldsymbol{RC}\,(\%) = \boldsymbol{SK} - \boldsymbol{SB} + \frac{\boldsymbol{PR}}{\boldsymbol{SK}}\right] \ldots\ldots\ldots\ldots\ldots\ldots (\mathbf{5})$$

Solubility of the extracts in organic solvents

It was known that the solubilization of lipophilic moieties increases with the increase of the chain length of the oily phase [27]. In this study the drug studied is natural plant extracts composes contain polyphenolic compounds. The polyphenolic compounds are known that they have amphiphilic properties [28]. The solubility of both Phyllanthus extracts in palm oil fractions was low (Table 6). It was cleared that the solubility of the extracts in PKOEs were slightly higher compared with the other palm oil fractions. PKOEs is newly synthesized by transesterification method using immobilized lipase *Rhizomucor miehei* (lipozyme RM IM) as catalyst in order to improve vesicular delivery for active ingredient of pharmaceuticals and cosmetics[29]. The newly introduced PKOEs is rich in oleyl laurate ester (54.1%), oleyl myristate ester (13.9%), oleyl oleate (6.4%) and oleyl palmitate 6.2% [29]. The fatty acids esters have several better characteristics compared with non-esterifies fatty acids in term of good novelty, better storage and thermal stability, fat soluble property, less greasiness and excellent wetting behavior at the interface[29-31]. PKOEs is uniquely quite similar to the famous and expensive jojoba oil which has much application in pharmaceuticals and cosmetics. Hence PKOEs can penetrate the skin very well. Moreover PKOEs is very cheap and can reduce the cost of cosmetics. Therefore, PKOEs is the potential oil that can be used as oil phase for delivery of Phyllanthus extracts as skin cosmetic.

It is also shown that the solubility of *P. niruri* in ethanol: water systems were similar. While the solubility of *P. Urinaria* in the ethanol: water systems were slightly different. This could be due to the high content of EA in *P. Urinaria* which has low solubility in water. *P. urinaria* was more soluble in ethanol: methanol system compared with *P. niruri*. This indicates that *P. urinaria* was slightly lipophilic and this due to the high content of EA compared with *P. niruri*. This indicates that *P. urinaria* can be potentially be formulated as skin antiaging due to high content of EA which mainly lipophilic in nature due to the four fused rings Figure 1. The solubility results show that the 30:70 ethanol: water is the most cost effective solvent of choice for extracting the plant materials due to less content of ethanol. While the reverse system 70:30 ethanol: water might be the most effective solvent as shown by high solubility to both extracts compared with their solubility in 30:70 ethanol: water.

Solubility study of extracts in the aqueous solvent

The solubility of the extracts in aqueous solvents is important physicochemical property since it affects the bioavailability of the extract active ingredient, the rate of release and consequently, the therapeutic efficacy. The aqueous solubility information is valuable in developing the formulation because the extracts contained polyphenolic compounds which greatly affected by the pH of the media. The results of extracts solubility in various aqueous vehicles were viewed in Table 7. It was cleared that the extracts were slightly have high solubility in aqueous systems. *P. niruri* has slightly high solubility in water compared with *P. urinaria*. This might be due to more content of ellagic acid in *P. urinaria* as shown in the quantification of EA and the solubility of the extracts in oils. The results of solubility of the extracts in aqueous phases show that *P. niruri* was slightly less acidic compared with *P. urinaria,* since it was highly dissolved in phosphate buffer at pH 6.6. While *P. urinaria* was dissolved better in phosphate buffer at pH 7.4. This result was consistent with the pH measurement of *P urinaria* and *P. niruri* extracts solutions in distilled water which was read 4.75 ± 0.01and 4.91 ± 0.03 respectively. It was also cleared that *P. niruri* solubility in water was slightly higher compared with the solubility of *P. urinaria* which mean more lipophilic compared with *P. niruri.* The highest solubility of *P. niruri* was observed in phosphate buffer system at pH 6.6. While *Phyllanthus urinaria* was highly dissolved in phosphate buffer system at pH 7.4. Therefore, phosphate buffer system at pH6.6 and 7.4 were the suitable solvent of for *P. niruri* and *P. urinaria* respectively. Hence, they will the better solvent to be used to dissolve the extract for HPLC. The extracts were subjected for further study to sect suitable aqueous phase that give high partition coefficients of the extract from PKOEs.

Apparent partition coefficients for the extracts

According to the Nernst law the partition coefficient refers to the concentration ratio of unionized, monomer form of the drug in the immiscible phases [32]. The concentration of the extract in PKOEs was obtained by subtracting the concentration of extract in the aqueous phase after partitioning in the oil (CA2) from CA1 which the initial concentration of the extract in the aqueous phase, (CA1-CA2). The partition coefficient (P) was calculated using the equation (6) where CO = concentration of the extract in the oil

(PKOEs), CW is the concentration of the extract in water at equilibrium which is equal to (CA1-CA2)/CA2. *P. niruri* has low partition coefficient in PKOEs/water system (Table 8) which means that the extract can be entrapped in the water phase. Thus using PKOEs/water system is suitable to prepare sun-blocking agent because large amount of the extract will be entrapped in the water phase and will be on the surface of the skin. In case of *P. urinaria* the most suitable vehicle to prepare sun-blocking is phosphate buffer Ph 6.6 since it has obtained the lowest partition coefficient. The most suitable vehicle to prepare skin antiaging formulation is the phosphate buffer system at pH $^{7.4}$ with PKOEs for both extracts. In this buffer system Phyllanthus urinaria extract has high solubility (2.004±0.058mg/mL) as well as has good partitions coefficient (1.39). While *Phyllanthus niruri* has slightly lower solubility in pH 7.4 (0.827±0.005mg/mL) but has higher partition coefficient (3.05). Therefore PKOEs/phosphate buffer system at pH7.4 is the most suitable system to deliver both Phyllanthus extracts as skin cosmetics preparations. It was noticed that increases the solubility of *P. niruri in* the aqueous decreased its partition coefficient in PKOEs and that aqueous system. This could be due to high intermolecular attraction between the extracts and high solubilization capacity of the vehicle. *P. urinaria* shows the similar character except with the buffer system at pH 7.4. *P. urinaria* shows high solubility in pH 7.4 as well as higher partition coefficient.

$$P = \frac{CO}{CA} \ldots\ldots\ldots\ldots\ldots\ldots\ldots.(\mathbf{6})$$

Conclusions

The result revealed to that the HPLC method applied under the chromatographic condition of binary gradient mode was successfully identified and quantified EA in *P. niruri* and *P. urinaria* extracts. The system was suitable and the method was sensitive and selective for determination of EA composes in the extracts and reference standard solutions. The solubility and partition coefficients of the extracts were greatly affected by the pH of the solvent system and the pPhosphate buffer system at pH6.6 and 7.4 were the suitable solvent for analytical purposes of *P. niruri* and *P. urinaria* for. The results also show that Phosphate buffer systems at pH 7.4 and PKOEs were better aqueous and oil phases to formulate the extracts as skin cosmetics preparations.

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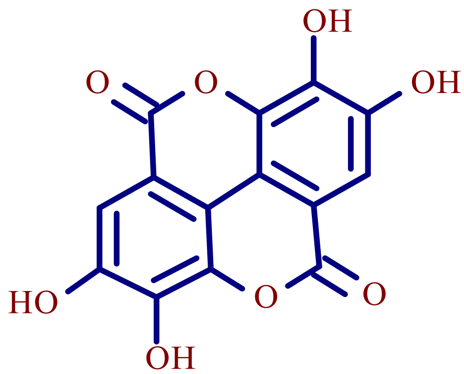

Figure 1. ***Chemical structure of ellagic acid (2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione)***

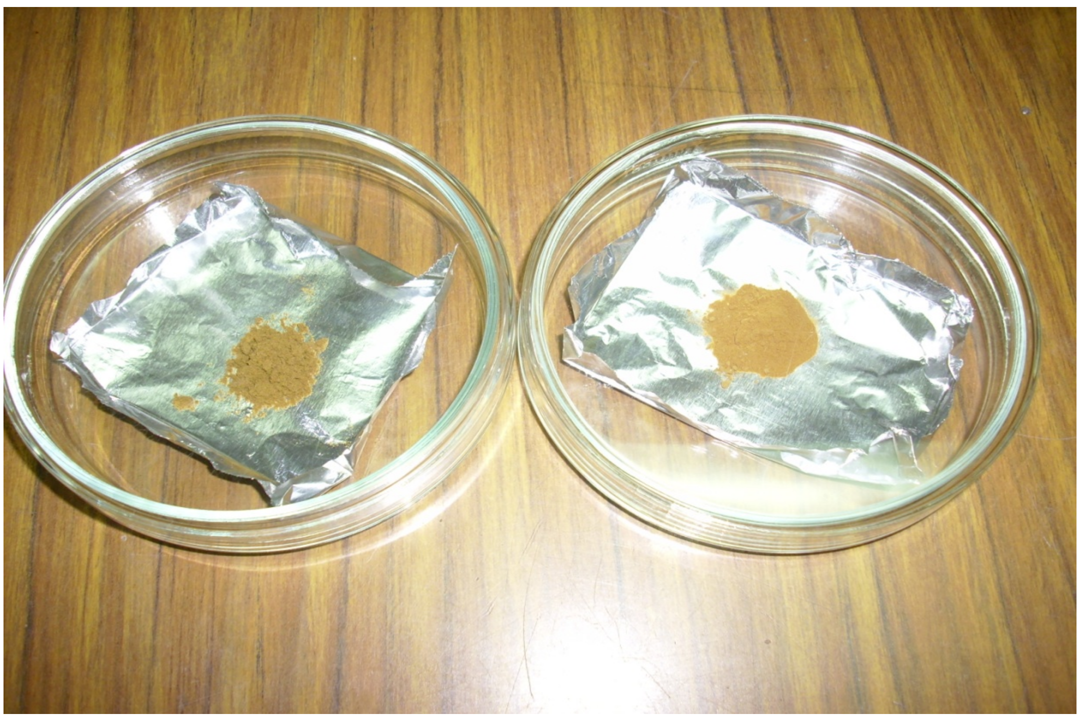

Figure 2. Phyllanthus niruri to the left and *Phyllanthus urinaria* extract powder to right

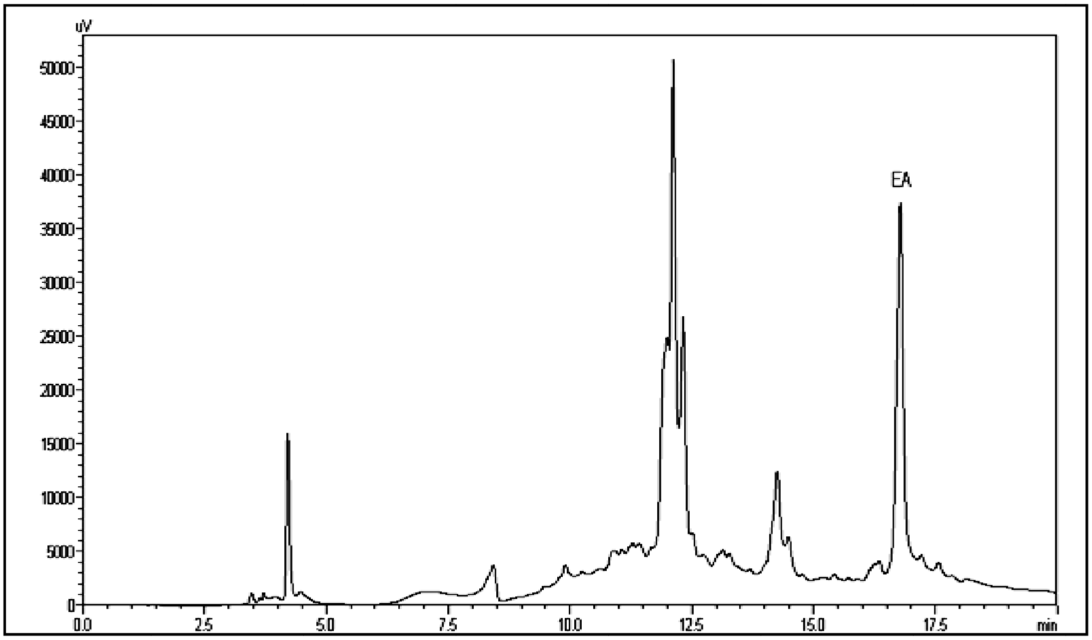

Figure 3. Chromatogram of *P. niruri extract* shows the peak of EA at 16.708 min

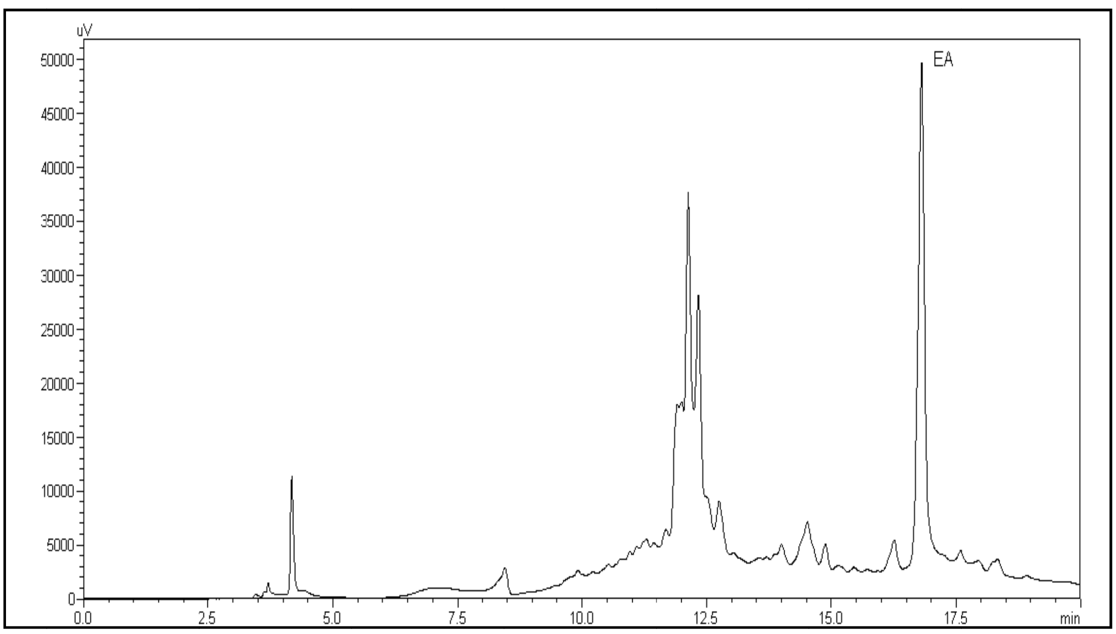

Figure 4. Chromatogram of *P. urinaria* extract shows the peak of EA at 16.708 min

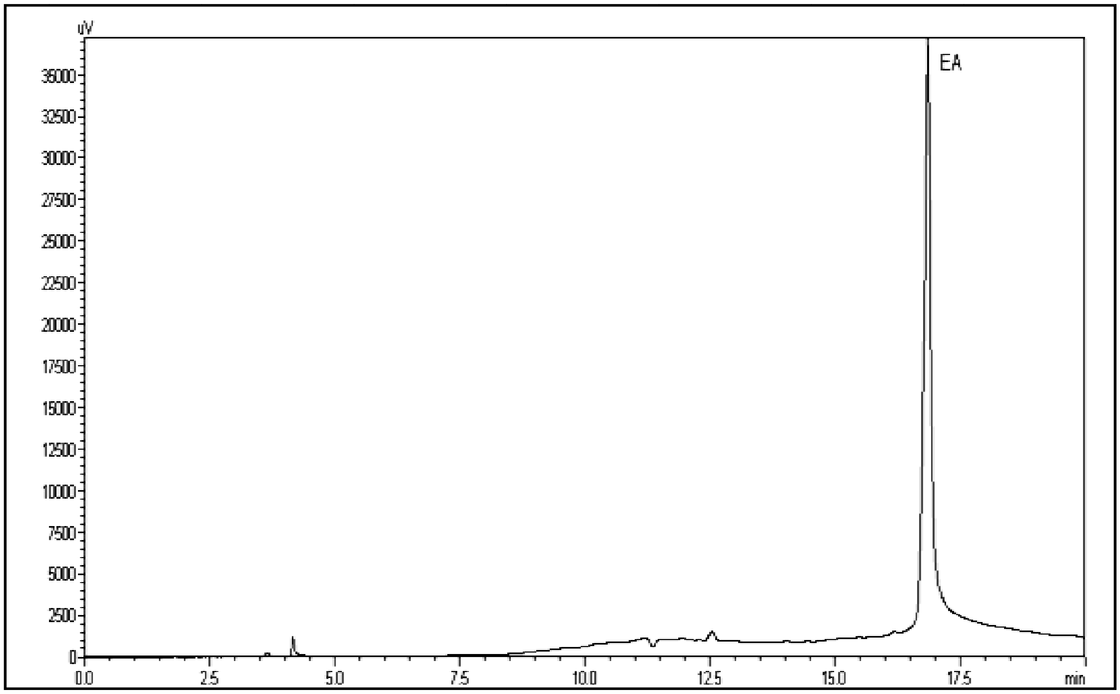

Figure 5. Chromatogram of EA reference standard peak at 16.851 min, with calibration curve equation Y= 123801 + 138364 * X, R^2= 0.999184, the slope was 138364

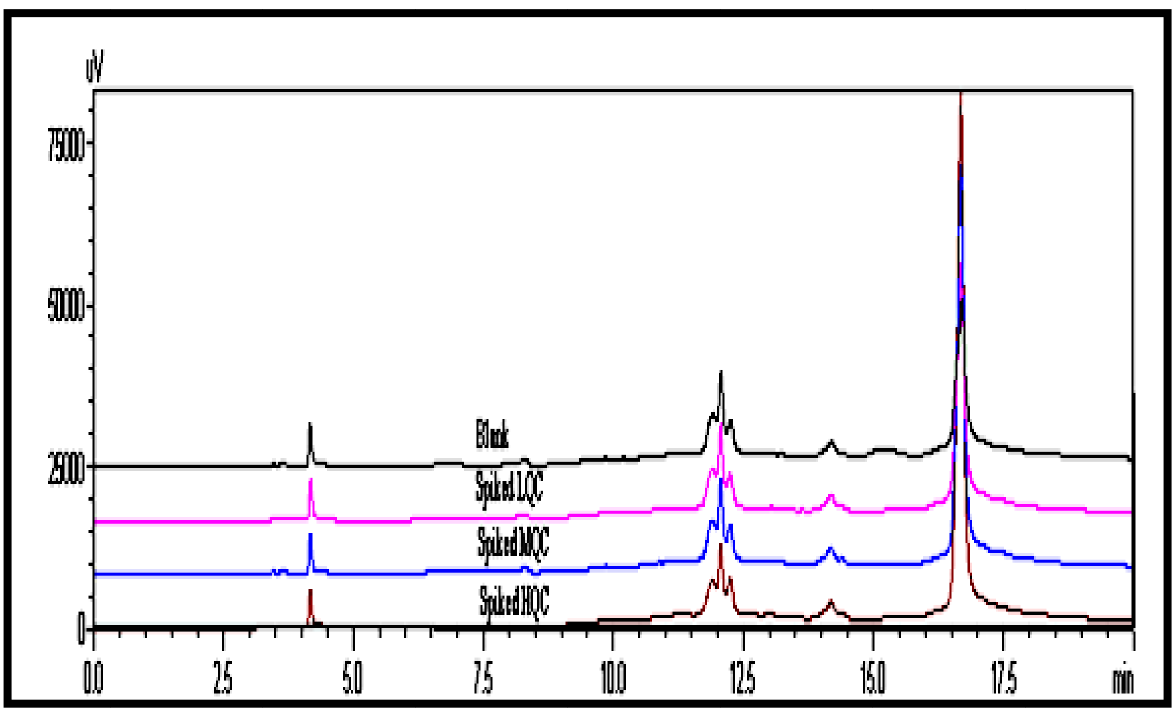

Figure.6. Chromatogram of base-shift overlay peaks of *P.niruri* spiked with blank solvent and various QC levels of EA external reference standard.

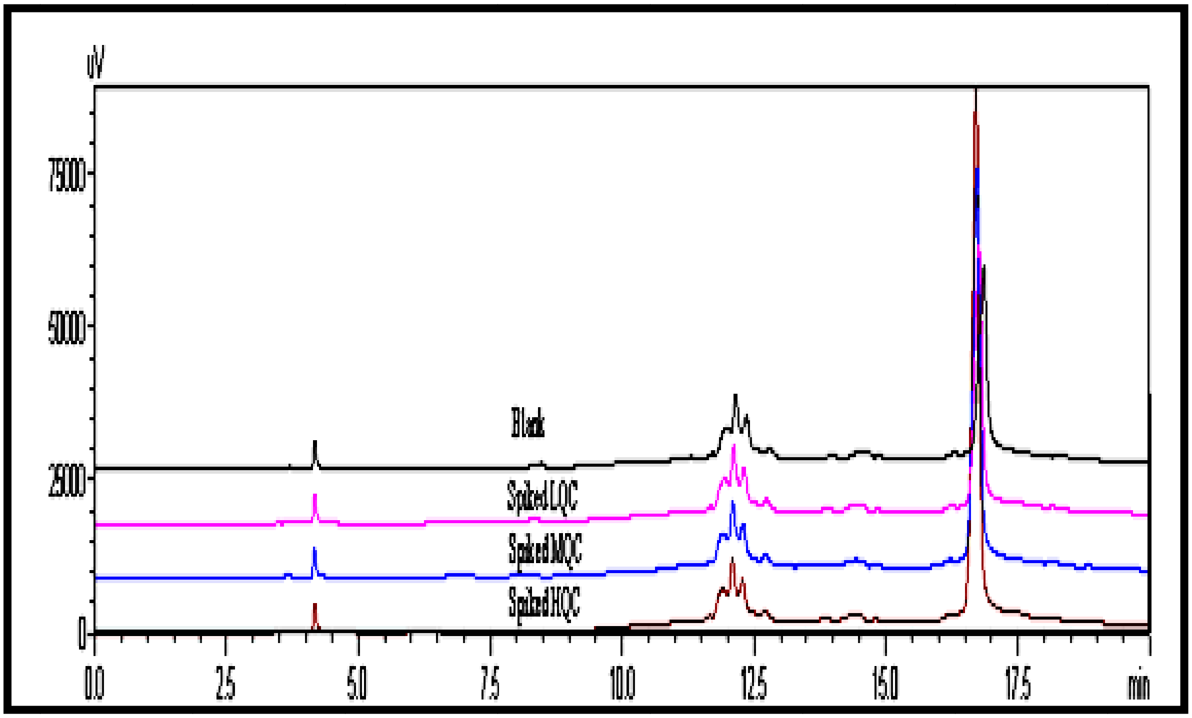

Figure 7. Chromatogram of Base-shift Overlay Peaks of *P. Urinaria S*piked with Blank and Various QC Levels of EA External Reference standard.

Table 1 *Physicochemical Properties of the Extracts*

Parameter	*P. urinaria*	*P. niruri*
Color	Red brown	Dark brown
pH	4.75±0.01	4.91±0.03
Moisture content (%)	7.40	0.036
Bulk density " Pb" (gm/mL^{3})	0.595±0.017	0.146±0.002
Tapped density$_{100}$ «Pt» (Pgm/mL^{3})	0.671±0.000	0.349±0.007
Hausners indexc (H)	1.128±0.023	2.393±0.0345
Compressibility index (C%)	11.30±1.84	58.21±0.60

Table 2 *Parameters of Linearity of the HPLC Method (n=6)*

concentration (µg/mL)	Mean area	*STD*	*RSD* (%)
0.25	169200.6	2326.4	1.38
0.50	206379.1	2255.2	1.09
1.00	246706.5	2174.0	0.88
2.00	392539.0	3400.6	0.87
4.00	670232.9	8514.8	1.27
8.00	1236976.4	3937.1	0.32
Mean R^2	0.999184	.0001	0.10
Mean slope	138364	809.2	0.58
Mean intercept	123801.0	2026.3	1.60

Table 3. System Suitability Regarding the External Reference Standard (EA 2µg/mL) and EA of *P. niruri* and *P. urinaria* samples (n=6)

Parameter	External standard (EA)			*Phyllanthus niruri*			*Phyllanthus urinaria*		
	mean	*SD*	*RSD* (%)	mean	*SD*	*RSD* (%)	mean	*SD*	*RSD* (%)
Area (AUP)	393539.0	3400.6	0.89	316435.1	2973.8	0.94	437912.0	2371.0	0.54
Retention time (Rt)	16.754	0.0359	0.21	16.790	0.02	0.09	16.808	0.04	0.21
Tailing factor (TF)	1.318	0.0201	1.53	1.0603	0.009	0.91	1.1307	0.0072	0.64
Resolution (Rs)	17.233	0.2288	1.33	10.5967	0.111	1.05	6.9087	0.0407	0.59
Separation (S)	1.474	0.0027	0.19	1.233	0.000	0.00	1.206	0.0006	0.05
Theoretical plate (TP)	75866.2	1328.6	1.754	75831.9	356.0	0.47	78281.6	578.5	0.74
Capacity factor ($K^/$)	3.617	0.0096	0.27	3.865	0.0021	0.05	3.804	0.0032	0.08

Table 4 *Intraday and Inter-day Precession (n=6)*

Variables	*Intra-day*			*Inter-day*
	Day 1	Day 2	Day 3	
QCL (2µg/mL) mean	300313.9	290866.3	305910.7	299030.3
QCL STD	5069.5	4456.3	4239.1	7603.9
QCL RSD (%)	1.7	1.5	1.4	2.5
QCM(5µg/mL) mean	836028.3	860715.2	845134.3	847356.5
QCM STD	4514.6	11066.2	10131.1	12468.5
QCM RSD (%)	0.54	1.3	1.2	1.5
QCH (7.5µg/mL) mean	1179430.7	1173911.8	1256509.6	1203284
QCH STD	3118.4	3816.9	9420.7	46177.2
QCH RSD (%)	0.26	0.325	0.75	3.8

Table 5 The Percent Recovery of *P. niruri* and *P. urinaria* Extracts (n=6 for each concentration)

Extract	Aliquots	Mean	*STD*	*RSD* (%)	*RC* (%)
P. niruri	PNE (non spiked).	298454.4	438.0	0.15	-
	SB (spiked with 2mL blank solvent).	283517.6	904.6	0.32	-
	SLQC (spiked with 2 mL of 2µg/mL EA).	538328.5	8962.4	1.7	97.3
	SMQC (spiked with 2mL of 5µg/mL EA).	731032.8	5925.8	0.81	98.0
	SHQC (spiked with 2mL of 7.5µg/mL EA).	901910.6	4617.6	0.51	98.3
P. urinaria	PUE (non spiked)	372892.6	984.7	0.26	-
	SB (spiked with 2mL blank solvent).	353026.7	2569.6	0.73	-
	SLQC (spiked with 2 mL of 2µg/mL EA)	439762.7	3037.7	0.69	95.7
	SMQC (spiked with 2mL of 5µg/mL EA)	621639.3	829.9	0.13	96.9
	SHQC (spiked with 2mL of 7.5µg/mL EA)	811372.7	1334.2	0.16	97.7

Legends: PNE=Phyllanthus niruri extract non spiked, PUE= Phyllanthus urinaria extract non spiked, SB=sample of the extract sample solution spiked with 2mL blanked solvent (matrix), SLQC= extract sample solution spiked with 2 mL of low quality control concentration of EA 2µg/mL, SMQC= extract sample solution spiked with 2 mL of medium quality control concentration of EA 5µg/mL, SHQC= extract sample solution spiked with 2 mL of high quality control concentration of EA 7.5µg/mL.

Table 6 The solubility of the Extracts in Various Palm Oil Fractions and Organic Solvents

Vehicle	Solubility (mg/mL)	
	P. niruri	*P. urinaria*
Palm kernel oil (PKO)	0.550±0.001	0.440±0.020
BR crude palm oil (PO)	0.250±0.013	0.204±0.008
RBD palm olein (POl)	0.642±0.082	0.530±0.082
Palm oil esters (POEs)	0.622±0.011	0.612±0.014
Palm kernel oil esters (PKOEs)	0.676±0.011	0.733±0.003
1:1 mixture of POEs and PKOEs	0.592±0.033	0.717±0.015
Methanol	0.7192±0.005	0.934±0.006
Ethanol (99.7%)	1.149± 0.004	1.054±0.001
Ethanol: Methanol 70:30	1.151±0.011	1.579±0.020
Ethanol: water 30:70	1.428±0.003	1.096±0.005
Ethanol: water 70:30	1.473± 0.007	1.211± 0.002

Table 7Solubility of Extracts in Aqueous Systems

Vehicle	Solubility (mg/mL)	
	P. niruri	*P. urinaria*
Distilled Water	1.537±0.026	1.469±0.009
Buffer system at pH 4.0	0.377±0.005	0.431±0.002
Buffer system at pH 5.0	0.707±0.004	0.505±0.002
Buffer system at pH 5.4	0.606±0.005	0.457±0.002
Buffer system at pH 6.0	1.332±0.023	0.791±0.001
Buffer system pH 6.6	1.994±0.031	1.365±0.009
Buffer system at pH 7.0	1.122±0.021	1.485±0.014
Buffer system at pH 7.4	0.827±0.005	2.004±0.058

Table 8 Apparent Partition and Log P Coefficients for the Extracts in Palm Kernel Oil Esters

Vehicle	*Phyllanthus niruri*		*Phyllanthus urinaria*	
	Partition coefficient (P)	*Log P/ Log D*	*Partition coefficient (P)*	*Log P/ Log D*
Water	0.24	-0.63	0.68	-0.17
P^H 4.0	0.64	-0.19	0.58	-0.24
P^H 5.0	1.83	0.26	0.41	-0.39
P^H 5.4	2.32	0.37	0.85	-.007
P^H 6.0	0.65	-0.18	0.77	-0.11
P^H 6.6	0.51	-0.30	0.25	-0.60
P^H 7.0	2.35	0.37	0.65	-0.19
P^H 7.4	**3.05**	**0.48**	**1.39**	**0.14**

Log P is log partition coefficient when the aqueous phase is water, Log D is the log distribution coefficient at a particular P^H, Log P = 1means 10:1 Organic: Aqueous, Log P = 0 means 1:1 Organic: Aqueous, Log P = -1 means 1:10 Organic: Aqueous