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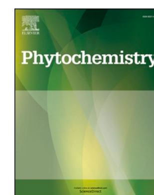

Towards the development of analytical monograph specifications for the quality assessment of the medicinal plant *Phyllanthus urinaria*

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A R T I C L E I N F O

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Phyllanthus urinaria, phyllanthaceae
Quality control
Specifications
Analytical method validation
Contaminants
Metabolomics
Design of experiment

A B S T R A C T

Many people in developing countries rely on herbal remedies for their primary healthcare needs. The challenge however is that several of these products lack proper documentation of quality and safety. To ensure consistent quality, validated methods are needed to establish and control quality attributes associated with identity, purity, and levels of bioactive constituents of the respective herbal materials. The present study focused on *Phyllanthus urinaria* (PU), a widely used medicinal plant in Ghana and West Africa that lacks the necessary quality control standards. The study aimed to develop an HPTLC identification method, which together with UPLC-ESI-Q-TOF-MS/MS analysis established the identity of PU samples and differentiated PU from other closely related *Phyllanthus* species. Quantitative UPLC and HPTLC methods were developed to assess the contents of selected active markers in the PU samples, which invariably led to the proposal of acceptance criteria for the active markers. Prior to the content analyses, the sample extraction procedure was optimized through the use of Design of Experiment method. The effects of harvest time and geographic origin on the content of active compounds were demonstrated in the investigations. PU samples were also found to be contaminated with higher levels of pesticides like chlorpyrifos and folpet. Essentially, this study provides analytical protocols, insights into the quality status of PU samples in Ghana, and analytical specifications contained in a drafted monograph for future consideration in regional and subregional African pharmacopoeias.

1. Introduction

Over the years, concerns and calls for the need to monitor the quality status of herbal medicines (HM) have increasingly been made by different categories of stakeholders in the healthcare industry and value chain (Ghosh, 2018; Govindaraghavan and Sucher, 2015). Although the available data show an encouraging proportion of the public, especially in the developing world, patronising herbal medicines for their basic healthcare needs (Yeboah et al., 2020), the absence and/or limitations in the establishment of the quality and safety statuses of most of these medicines have served as barriers to the general adoption by all and full integration of herbal medicine use and practice into the formal healthcare systems in several countries (Zhang et al., 2012).

It must be emphasised however, that the role of herbal medicines in achieving Universal Health Coverage (UHC) is critical. As the focus of the World Health Organisation (WHO) shifts towards primary

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healthcare in the quest to realise UHC by the year 2025 (World Health Organization WHO, 2020a), the wide availability, easy accessibility as well as the relative affordability of herbal medicines as compared to orthodox medicines (Yeboah et al., 2020) become the more important deciding factors in healthcare delivery in resource-challenged settings. It is for these and many more reasons (World Health Organization WHO, 2013) that the quality and safety of HM has become a great concern in the area of pharmaceutical and medical development. The incidences of plant misidentification, poor quality and the presence of harmful contaminants such as heavy metals and pesticides therefore remain as threats in achieving UHC with HM.

In the current study, we consider an important medicinal plant, *Phyllanthus urinaria* L. (Phyllanthaceae) (PU) (Fig. S1), which is widely used in Ghana and other parts of Africa, Asia and America, as a model herbal remedy, to show how analytical specifications and state-of-the art analytical protocols can be developed by a systematic work-flow to perform quality assessment of HM (Xu et al., 2007). PU is a tropical or perennial herb used traditionally in the management of worm infestations (Agyare et al., 2014), as well as in the treatment of enteritis, hepatitis, jaundice, renal diseases, malaria, diarrhoea, hypertension, among others (Geethangili and Ding, 2018). Ethnopharmacological investigations have demonstrated the following biological effects: anti-inflammatory, antioxidant, antibacterial, antiviral, antihelminthic, anticancer, and antidiabetic effects (Geethangili and Ding, 2018). PU is reported to contain several classes of secondary metabolites including hydrolysable tannins, flavonoids, lignans, coumarins, triterpenoids and phenolic natural products – structural features of typical marker compounds see Supplementary Data, Fig. S2 (Chang et al., 2003; Hu et al., 2014; Jikai et al., 2002; Spiegler et al., 2015; Xu et al., 2007). Some of these secondary products are responsible for the observed pharmacological effects of the plant. For example, the hepatoprotective effects of the plant have been attributed to the presence of phyllanthin (Chirdchupunseree and Pramyothin, 2010), hypophyllanthin (Chirdchupunseree and Pramyothin, 2010), corilagin (Liu et al., 2017), and geraniin (Londhe et al., 2012). Similarly, phyllanthin from the plant has been shown to possess antiamnestic, antiaging, antiapoptotic, and antibacterial effects (Geethangili and Ding, 2018). Norsecurinine has been shown to possess antifungal effects (Sahni et al., 2005). A number of the pharmacological effects of the natural products present in PU have been detailed in the works of Mao et al. (2016), Geethangili & Ding (Geethangili and Ding, 2018), Seyed (2019) among others.

Due to the enumerated therapeutic effects of the plant, *P. urinaria* together with related species in the *Phyllanthus* genus have become mainstay components in several commercial herbal formulations in Ghana (Komlaga et al., 2015; Osei-Djarbeng et al., 2015). Locally, PU is known as '*Bɔwomaguwakyi*', which literally means 'carry your progeny behind you' (Agyare et al., 2014). It is a name that is loosely used also for related species like *P. fraternus* G.L.Webster, *P. niruri* L., *P. amarus* Schumach. & Thonn., and at times for *P. muellerianus* (Kuntze) Exell. Compounded by the similarities in their morphological features, these plants are likely to be mistaken for the other. Thus, in the formulation of commercial herbal products that contain any of the above-mentioned species, there stands a risk of collecting the wrong species when the harvesting of the plant is based only on the knowledge of the local name. Therefore, the need to develop analytical specifications to control the identity of the plant species is obvious. Additionally, some studies have demonstrated concerns with the quality of medicinal plant materials originating from different geographical locations and collections at different times and seasons of the year (Orman et al., 2023). These variations tend to affect the phytochemical content of the plants, especially that of the bioactive compounds and may invariably affect their therapeutic potential. As a result, there is also the need to establish validated analytical standards to ensure batch-to-batch consistency of levels of phytochemical constituents, among others.

The present study was therefore carried out to develop new analytical protocols for the quality assessment of PU plant material, and also to develop analytical specifications based on the results of a representative batch analysis of many PU samples. Additionally, an analytical monograph capturing specifications from the present study is proposed for possible consideration into relevant regional pharmacopoeia such as the Ghana, West Africa, and African herbal pharmacopoeias. The outcomes of our analytical investigations on PU are hereby presented and discussed.

2. Results and discussion

2.1. Phytochemical characterization of P. urinaria by LC-MS/MS

The phytochemical characterization of the acetone: water (7:3) extract of PU by UPLC/+ESI-QqTOF-MS (Fig. 1) resulted in the tentative identification of hydrolysable tannins, flavonoids, lignans, phenolics, and terpenoids. In summary, 56 compounds were tentatively identified by comparing their spectral data with literature and the online database, Reaxys (https://www.reaxys.com/). Most of these compounds identified had previously been reported in the plant species and other *Phyllanthus* species (Geethangili and Ding, 2018; Mao et al., 2016), showing a high degree of similarity in the phytochemistry of the species in the genus. Table 1 describes the compounds identified in PU extract.

2.2. Phytochemical differences between P. urinaria and closely related Phyllanthus species

Building on the knowledge from the phytochemical characterization of the PU extract, further investigations into the similarities and differences in the phytochemistry of eight different *Phyllanthus* species including PU was carried out through metabolomic analysis. Other species investigated were also from the region of West Africa, namely *P. amarus, P. fraternus, P. niruri, P. muellerianus*, and three unknown species were labelled as Unknown A, B, and C. They were collectively labelled as non-PU samples for the purpose of the analysis. In addition to examining the similarities and differences between PU and non-PU samples, feature selection was also carried out to identify molecular features that could be used to distinguish one group from the other (Fig. 2).

The volcano plot obtained from the *t*-test statistics showed some significant differences in terms of the phytochemical composition of PU samples when compared to that of non-PU samples (Fig. 2A). The key information in the plot is defined as molecular features with high fold changes [$\log_2(FC)$] and significant *p*-values, and these are summarized in the plot in red (for features in PU) and blue (for features in non-PU). For instance, PU samples were characterized by the presence of significant intensities of the molecular features *m/z*: 703.3431 (cleistanthoside A, $[M+H]^+$), *m/z*: 407.1992 (urinatetralin, $[M+Na]^+$), *m/z*: 203.2416 (norsecurinine, $[M+H]^+$), and *m/z*: 302.1972 (ellagic acid, $[M+H]^+$). On the other hand, non-PU samples were seen to possess molecular features like *m/z*: 685.5153 (phainanoid G, $[M+H]^+$), *m/z*: 729.5425 (phyllaemblicin H5, $[M+H]^+$), *m/z*: 303.1350 (quercetin, $[M+H]^+$), *m/z*: 328.3184 (phyllangin, $[M + NH_4]^+$), *m/z*: 941.0945 (mallotusinin, $[M+Na]^+$) among others, which were present in significantly higher amounts when compared to the PU samples. Additionally, a PLS-DA classification model with an accuracy of 99% ($R^2 = 0.9639$ and $Q^2 = 0.7942$) was able to discriminate PU samples from non-PU samples based on their LC-MS spectral data (Fig. 2B). The model also showed the molecular features with very high variable importance in projection (VIP) scores summarized in Fig. 2C. Some of the molecular features predicted from the VIP scores plot were also identified from the volcano plot as characteristic of either PU or non-PU samples, for example, *m/z*: 703.3431 (cleistanthoside A, $[M+H]^+$), *m/z*: 407.1992 (urinatetralin, $[M+Na]^+$) and *m/z*: 203.2416 (norsecurinine, $[M+H]^+$) in PU, and *m/z*: 685.5153 (phainanoid G, $[M+H]^+$) in non-PU samples. Other characteristic features also predicted to contribute to the distinct classification of the two set of samples were mostly lignans and included the likes of

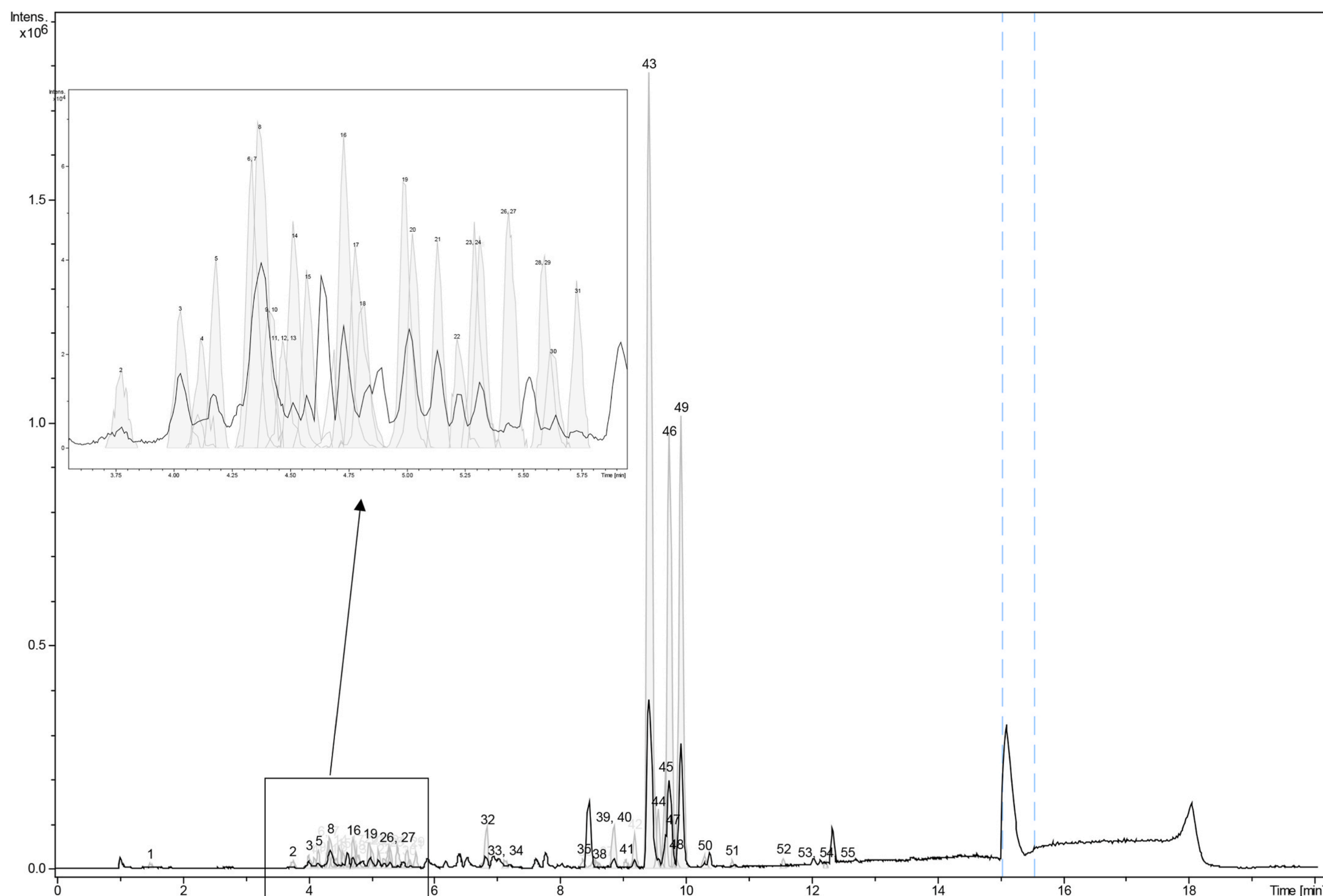

Fig. 1. UPLC-ESI-QTOF-MS analysis of acetone: water extract (7:3) from the aerial parts of *Phyllanthus urinaria*. Interpretation of compounds 1 to 55 see Table 1.

m/z: 383.1853 ((iso)lariciresinol, $[M+Na]^+$) and its glycoside *m/z*: 515.3261 (isolariciresinol-9′-O-β-D-xylopyranoside, $[M+Na]^+$), *m/z*: 401.1642 (urinaligran, $[M+H]^+$), *m/z*: 355.1561 (1-O-galloyl-β-glucopyranose, $[M+Na]^+$), *m/z*: 417.2248 (phyltetralin, $[M+H]^+$).

Based on these observations, it is proposed that the different *Phyllanthus* species (as considered in the study) possessed similar hydrolysable tannins and flavonoid compositions. The lignans present however tend to differ to a certain extent and contribute to the distinction of mostly PU from non-PU samples. For this reason, it could also be argued that biological activities of the plant which are a result of the hydrolysable tannins and flavonoids may be similar across the different species, but those activities as accounted for by the presence of lignans could vary across the different species. That notwithstanding, further studies could be performed to confirm this.

2.3. Validated HPTLC method

The HPTLC method was developed with mobile phase systems optimized from the knowledge of Snyder's classification of solvents (Snyder, 1974) and their corresponding polarity indices. The two HPTLC protocols involved one system developed from a mobile phase containing ethylacetate, water and formic acid (75:15:10, v/v/v). This system was adopted for both qualitative (fingerprint analysis) and quantitative purposes where the assay of marker compounds (including rutin, isoquercitrin, and gallic acid) were carried out (structural formulas of marker compounds are shown in Fig. S2, Supplementary Data File). The second system involved the use of toluene: ethylacetate: formic acid (69:30:1, v/v/v) as mobile phase for the assay of phyllanthin. The HPTLC profiles as developed after derivatization of the plates with NP-PEG and detection at $\lambda = 366$ nm and at $\lambda = 254$ nm for plates from the first and second sets respectively, provided a good characteristic profile of the plant showing part of its phenolic composition as well as providing a basis for its representative quality assessment. Fig. 3 depicts the HPTLC profiles developed along with some highlighted analytical investigations carried out during the validation of the method. The HPTLC method was subsequently validated in accordance with ICH Q2 (R1) guidelines (ICH, 2005) and recommendations from literature (Reich et al., 2008; Renger et al., 2011). Table 2 summarizes the results from the validation carried out.

The method was found to be specific for both qualitative and quantitative purposes. The presence of the marker compounds in the plant extract was confirmed from simultaneous development of the profiles with the reference markers (Table 2). Additionally, for the qualitative use of the method, profiles of different *Phyllanthus* species, *including P. urinaria, P. amarus, P. fraternus, P. muellerianus, P. niruri* and three unknown species, were simultaneously developed and also evaluated. Both visual inspection and chemometric-assisted analysis of the grayscale video-densitometric like profile showed the presence of some differences in the fingerprints for the different species (Fig. 3E). Within the PCA (Fig. 3F), most of the profile for PU were clustered together, and the cluster was quite far off the samples of other *Phyllanthus* species. Thus, the profiles as developed and analysed chemometrically establish intra-species similarities and inter-species differences which confirms the specificity and selectivity of the method for chemical authentication purposes. For the assay of marker compounds, the relationship between concentration and peak areas from densitometric scans were found to be linear for gallic acid ($r^2 = 0.9992$) and non-linear for rutin (adj. $r^2 = 0.9940$), isoquercitrin (adj. $r^2 = 0.9976$), and phyllanthin (adj. $r^2 =$

Table 1
Compounds identified in the aerial parts of *Phyllanthus urinaria* from UPLC-ESI-QTOF-MS/MS analysis.

No	t_R [min]	Observed *m/z* [Adduct(s)]	Monoisotopic mass	MS/MS Fragments	Accuracy (mDa)	Molecular Formular	Compound name	Ref.
1	1.508	236.0983 [M+H]	235.0913	100.0775; 122.0559; 197.1255	4	$C_{13}H_{17}NO_3$	4-Methoxydihydronorsecurinine	Hassarajani and Mulchandani (1990)
2	3.774	485.0945 [M+H]	484.0875	315.0735; 153.0264	5	$C_{20}H_{20}O_{14}$	Di-*O*-galloyl-β-glucopyranose isomer	Huang et al. (1998)
3	4.029	293.0311 [M+H]	292.0241	191.0380; 107.0455	2	$C_{13}H_8O_8$	Brevifolin carboxylic acid	Huang et al. (2009)
4	4.122	668.1137 [M + NH4]	650.0799	481.0628; 209.0829	13	$C_{27}H_{22}O_{19}$	Furosin	(Agyare et al., 2011; Xu et al., 2007)
5	4.182	975.0810 [M+Na]	952.0918	783.0729; 303.0162; 277.0385; 337.0189	10	$C_{41}H_{28}O_{27}$	Phyllanthusiin A	Yoshida et al. (1992)
6	4.336	465.0689 [M+H]	464.0619	277.0304; 303.0204; 259.0242	5	$C_{21}H_{20}O_{12}$	Myricitrin	Tram et al. (2017)
7	4.336	652.1187 [M + NH4]	634.0849	277.0358; 303.0182; 127.0349	3	$C_{27}H_{22}O_{18}$	Corilagin	(Agyare et al., 2011; Xu et al., 2007)
8	4.369	971.1248 [M+H]	970.1203	783.0747; 463.0456; 303.0149	5	$C_{41}H_{30}O_{28}$	Repandusinic acid A	Xu et al. (2007)
9	4.419	411.1676 [M+Na]	388.1784		4	$C_{19}H_{32}O_8$	Dendranthemoside B	Van Thanh et al. (2014)
10	4.419	970.1206 [M + NH4]	952.0868	783.0749; 463.0453; 303.0135	10	$C_{41}H_{28}O_{27}$	Geraniin	(Agyare et al., 2011; Xu et al., 2007)
11	4.470	388.2541 [M + NH4]	370.2203	355.0702	4	$C_{16}H_{18}O_{10}$	4-Acetyl-bergenin	Wu et al. (2018)
12	4.470	422.2379 [M + NH4]	404.1986	393.2541	9	$C_{16}H_{20}O_{12}$	Mucic acid 1-ethyl 6-methyl ester 2-*O*-gallate	Zhang et al. (2017)
13	4.470	975.0802 [M+Na]	952.0910	783.0710	19	$C_{41}H_{28}O_{27}$	Geraniinic acid B	(Foo, 1995; Yoshida et al., 1992)
14	4.520	387.2046 [M+H]	386.1976	95.0851; 149.0996	4	$C_{22}H_{26}O_6$	Cubebin dimethyl ether	Conrado et al. (2020)
15	4.575	944.1405 [M + NH4]	926.1067	757.0946; 437.0786; 277.0351	5	$C_{40}H_{30}O_{26}$	Phyllanthusiin C	Huang et al. (2009)
16	4.728	581.1550 [M+H]	580.148	303.0519	3	$C_{30}H_{28}O_{12}$	Naringenin-7-*O*-(6″-O-trans-*p*-coumaroyl)-glucoside	Zhang et al. (2002)
17	4.780	972.1337 [M + NH4]	954.0999	785.0854; 277.0374; 429.0633; 153.0211	10	$C_{41}H_{30}O_{27}$	Chebulagic acid	Luo et al. (2012)
18	4.810	249.1115 [M+Na]	226.1223	246.8356; 105.0761	4	$C_{13}H_{22}O_3$	Boscialin	Busch et al. (1998)
19	4.989	611.1656 [M+H]	610.1586	303.0515	6	$C_{27}H_{30}O_{16}$	Rutin	Van Thanh et al. (2014)
20	5.022	303.0188 [M+H]	302.0118		4	$C_{14}H_6O_8$	Ellagic acid	Huang et al. (2009)
21	5.130	465.1061 [M+H]	464.0991	85.0301; 303.0516; 153.0197	5	$C_{21}H_{20}O_{12}$	Isoquercetin	Yao and Zuo (1993)
22	5.214	197.1177 [M+H]	196.1107	100.1177	4	$C_{12}H_8O_6$	Brevifolin	Wu et al. (2012)
23	5.285	119.0853 [M+H]	118.0783		4	$C_4H_6O_4$	Succinic acid	Wei et al. (2005)
24	5.285	524.2501 [M + NH4]	506.2163	303.0505; 204.0992; 153.0135	10	$C_{23}H_{22}O_{13}$	Tri-*O*-methyl ellagic acid 4-*O*-β-glucopyranoside isomer	Tuchinda et al. (2008)
25	5.318	435.0959 [M+H]	434.0889	303.0517	4	$C_{21}H_{22}O_{10}$	Naringenin-7-*O*-glucoside	Zhang et al. (2002)
26	5.434	449.1109 [M+H]	448.1039	303.0512	4	$C_{21}H_{20}O_{11}$	Kaempferol-3-glucoside	Van Thanh et al. (2014)
27	5.434	540.2493 [M + NH4]	522.2155	345.1695; 331.1584	10	$C_{26}H_{34}O_{11}$	Isolarisiresinol 9′-*O*-β-glucopyranoside	Cai et al. (2009)
28	5.581	393.1930 [M+Na]	370.2038	355.1742	4	$C_{21}H_{22}O_6$	Dextrobursehernin	Chang et al. (2003)
29	5.581	800.3019 [M + NH4]	782.2681	371.1193, 251.0582; 311.0850; 517.1747; 637.3690	8	$C_{32}H_{30}O_{23}$	Emblicannin A	Usharani et al. (2013)
30	5.625	461.1436 [M+Na]	438.1544	464.2577; 253.1086; 331.1042	4	$C_{30}H_{62}O$	Triacontanol	Satyan et al. (1995)
31	5.731	261.1591 [M+Na]	238.1699	159.0948; 118.0688	4	$C_{15}H_{26}O_2$	Cloven-2β,9α-diol	Hu et al. (2014)
32	6.851	926.3330 [M + NH4]	908.2992	111.0445; 171.0651; 231.0861; 371.1129	5	$C_{40}H_{28}O_{25}$	Acalyphidin M1	Matou (2019)
33	7.138	313.2070 [M+Na]	290.2430		8	$C_{15}H_{14}O_6$	(Epi)catechin	(Ishimaru et al., 1992; Zhang et al., 2001)
34	7.138	365.0327 [M+H]	364.0257	271.0609; 240.0432; 133.0658	4	$C_{16}H_{12}O_{10}$	Phyllangin	Wei et al. (2004)
35	8.379	401.1687 [M+H]	400.1617	151.0729	4	$C_{23}H_{28}O_6$	(Iso)lintetralin	(Chang et al., 2003; Huang et al., 1992)
36	8.577	333.2046 [M+H]	332.1976		8	$C_{13}H_{16}O_{10}$	β-glucogallin	Subeki et al. (2005)
37	8.577	329.1928 [M+H]	328.1858	247.1323	8	$C_{14}H_{16}O_9$	Bergenin	Tanaka and Matsunaga (1988)
38	8.637	455.2032 [M+Na]	432.2140	332.1731; 231.1030	4	$C_{24}H_{32}O_7$	Niranthin	(Chang et al., 2003; Van Thanh et al., 2014)

(*continued on next page*)

Table 1 (*continued*)

No	t_R [min]	Observed *m/z* [Adduct(s)]	Monoisotopic mass	MS/MS Fragments	Accuracy (mDa)	Molecular Formular	Compound name	Ref.
39	8.863	355.1168 [M+H]	354.1098	135.0441; 231.0875	2	$C_{20}H_{18}O_6$	Hinokinin	Huang et al. (1992)
40	8.863	415.2115 [M+H]	414.2045		4	$C_{29}H_{50}O$	Amarosterol B	Ahmad and Alam (2003)
41	9.062	437.1590 [M+H]	414.1698		4	$C_{29}H_{50}O$	β-sitosterol	Hu et al. (2014)
42	9.197	439.2022 [M+Na]	416.2130	355.1415; 242.8953; 130.0669	4	$C_{24}H_{32}O_6$	Phyltetralin	Fang et al. (2008)
43	9.429	441.2251 [M+Na] 56 anf 57 have been eliminated	418.2359	151.0728; 411.1771; 355.1912	2	$C_{24}H_{34}O_6$	Phyllanthin	Wei et al. (2004)
44	9.572	529.2114 [M+Na]	506.2222	238.1266; 117.0690; 379.2043	3	$C_{26}H_{34}O_{10}$	Phyllanembloid B	Lv et al. (2015)
45	9.679	453.1893 [M+Na]	430.2001	261.1125; 115.0529	2	$C_{24}H_{30}O_7$	Hypophyllanthin	Chang et al. (2003)
46	9.748	201.0908 [M+H]	200.0838		2	$C_{12}H_{24}O_2$	Lauric acid	Islam et al. (2022)
47	9.810	423.1803 [M+Na]	400.1911	295.1796; 100.1105	4	$C_{22}H_{24}O_7$	Urinaligran	Chang et al. (2003)
48	9.849	293.2107 [M+H]	292.2037	277.2166	8	$C_{13}H_8O_8$	Phyllanthusiin E	Wu et al. (2012)
49	9.931	425.1952 [M+Na]	402.2060	135.0412	2	$C_{23}H_{30}O_6$	5-Demethoxyniranthin	Chang et al. (2003)
50	10.306	407.1474 [M+Na]	384.1582	231.1016	4	$C_{22}H_{24}O_6$	Urinatetralin	Chang et al. (2003)
51	10.743	297.2480 [M+H]	296.2362	183.1379; 100.1120	4	$C_{20}H_{24}O_2$	Phyllane B	Duong et al. (2017)
52	11.554	323.1969 [M+Na]	300.2077	279.2323	4	$C_{20}H_{28}O_2$	Spruceanol	Hu et al. (2014)
53	12.228	353.2673 [M+Na]	330.2781	195.1227	4	$C_{15}H_{22}O_8$	3,4-dimethoxy benzyl alcohol-7-O-β-glucopyranoside	Yu et al. (2016)
54	12.228	452.3978 [M + NH_4]	434.3640	313.2724	9	$C_{22}H_{26}O_9$	Phyllaemblic acid methyl ester	Liu et al. (2009)
55	12.228	496.4214 [M + NH_4]	478.3876		10	$C_{26}H_{38}O_8$	19-Hydroxyspruceanol-19-O-β-D-glucopyranoside	Lan et al. (2010)

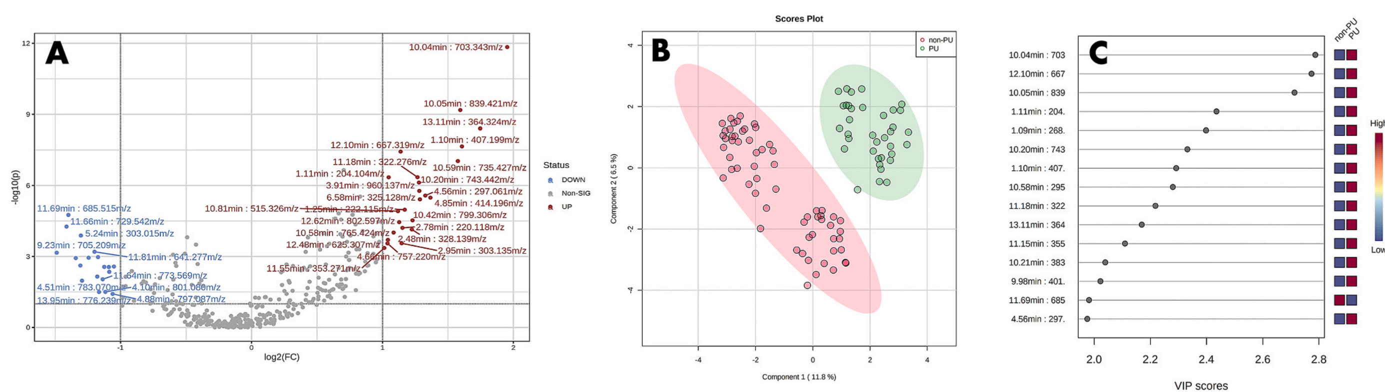

Fig. 2. Results from metabolomic analysis (LC-MS) from eight different *Phyllanthus* species. [**A**] Volcano plot of molecular features present in PU as compared to non-PU. Buckets with a high fold change >2 and *p*-value ≤0.05 in PU and non-PU samples are shown in red and blue colours respectively; [**B**] PLS-DA score plot showing the discrimination of PU samples from the non-PU samples. Classification model achieved with 3 components and had an accuracy of 99% with $R^2 = 0.9639$ and $Q^2 = 0.7942$. [**C**] VIP scores obtained from PLS-DA showing the most important features that contribute to the classification of PU samples different from the non-PU samples.

0.9980). The respective quantitative models described for the relationship between the concentration and response and their consequent use in content assays are shown in Table 2. With recoveries ranging between 98.5 and 101.7%, the use of these quantitative models was also shown to be accurate from the recovery studies. Additionally, the R_Fs and the peak area estimates were demonstrated to be precise (RSD <2%) from both repeatability and intermediate precision tests. The method was then shown to be robust when deliberate changes to the ambient temperature (at 25 &.30 °C), developing chamber (10 × 10 & 20 × 10 cm), application instrument (instruments 1 & 2) and saturation times (20, 25 & 30 min) were made. Finally, the fingerprint and the content of the marker compounds were established to be stable over 48 h for the analytes' solutions, over 60 min for the underivatized and derivatized spots on the plate pre-and post-profile development. The method could then be said to be fit for purpose for both identification and content assessment of PU.

2.4. Validated UPLC method

Existing HPLC methods for the quality assessment of PU and other *Phyllanthus* species mostly target the estimation of selected lignans (Murugaiyah and Chan, 2007; Shanker et al., 2011). However, due to the presence of other classes of bioactive compounds in the herbal material, developing a more specific UPLC method focused on the selection of representative analytical targets for these groups is needed. Thus, geraniin, which is reportedly the most abundant bioactive hydrolysable tannin in the *Phyllanthus* genus (Agyare et al., 2011), rutin and isoquercitrin, which are also bioactive flavonoids and phyllanthin, another prominent bioactive lignan, were selected (Fig. S2). A wavelength of λ = 270 nm was observed to be optimum in detecting the peaks of tannins, flavonoids and lignans in the chromatogram, thus providing a representative fingerprint of the plant's major phytochemistry. Fig. 4 shows a typical UPLC chromatogram developed for the quality assessment of PU.

This UPLC method was similarly validated following the recommendations in the ICH Q2(R1) guidelines (ICH, 2005) (Table 3). The method was initially investigated for specificity, where the presence of

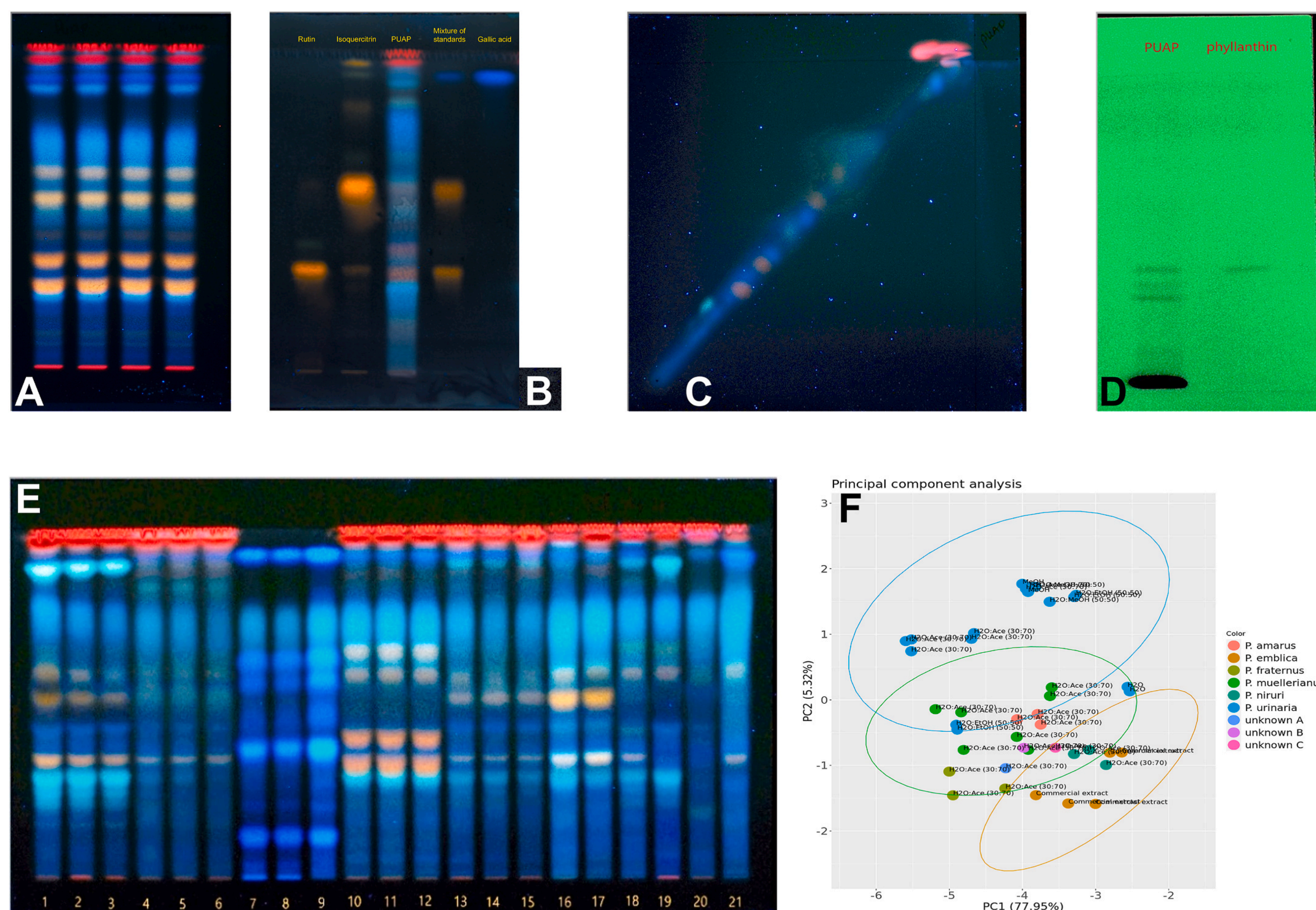

Fig. 3. Typical thin layer chromatograms of *Phyllanthus urinaria* aerial parts (herein referred to as PUAP) under different modes of detection with other *Phyllanthus* species with their fingerprint analysis. **A**: Replicate HPTLC profiles of PUAP extracts showing repeatability of the fingerprint. **B**: TLC identification of analytical marker compounds in PUAP extract by use of reference standards, rutin, isoquercitrin, and gallic acid. **C**: 2D development of PUAP profile using similar conditions in both cases. This forms part of validation investigations. **D**: Simultaneous development of HPTLC profiles of PUAP and the reference compound, phyllanthin using the mobile system (toluene: ethylacetate: formic acid (69:30:1, v/v/v). **E**: Representative profiles of different *Phyllanthus* species (1–3: *P. fraternus;* 4–6: *P. niruri*; 7–9: *P. emblica;* 10–12: *P. urinaria;* 13–15: *P. amarus;* 16–18: *P. muellerianus;* and 19–21: unknown *Phyllanthus* sp. A, B, and C) used in the test for specificity. **F:** Score plot of fingerprints of different *Phyllanthus* species performed by rTLC web application (Fichou et al., 2016) using data from grayscale video-densitometric like channel. Observed differences in the fingerprints for different species, especially for *P. urinaria.* Differences seen in their respective scores.

the marker compounds was confirmed in the PU extract from LC-MS/MS analysis. Also, the retention times, UV spectra and *m/z* values for the respective peaks in the chromatograms from the UPLC and MS analysis were found to be consistent with analytical data of the corresponding reference standards. When the UV and mass spectra were again assessed for peak purity analysis at the respective retention times, it was found that the spectra were comparable at the beginning, middle, and end of the peaks. Linearity ($r^2 > 0.99$) was established within the concentration ranges for the marker compounds (Table 3) and their respective peak areas, thus enabling the quantitative assay of the compounds, a process which was also shown to be accurate, with a recovery ranging between 100.2 and 101.3%. In the test for precision (repeatability and intermediate precision), the RSD for the peak areas ranged between 0.17 and 0.61%. This showed that the peak area calculations were precise and reliable for quantitative purposes. With deliberate changes to the respective column temperature and the flow rate of the method, the RSDs were <2.0%, demonstrating a robust method with respect to variable conditions. Finally, PU test solution used for analysis was shown to be stable >48 h, as the change in response over the period did not change significantly. The outcomes from the validation of the UPLC method are summarized in Table 3.

2.5. *Optimization of sample extraction conditions*

To ensure the adequate recovery of analytes during the sample extraction for quantitative assessment of PU, the extraction conditions for the herbal material were investigated and optimized according to the BBD model. The procedure, as described previously (Orman et al., 2023) involved the investigation of the effects of parameters such as extraction time (A), sample-to-solvent ratio (B), number of extractions (C) and method of extraction (D) on quality attributes including extraction yield, and concentrations of rutin (% wt/wt), isoquercitrin (% wt/wt), geraniin (% wt/wt) and phyllanthin (% wt/wt) (Table S2). Statistical evaluation of the effects with the input variables with ANOVA as well as modelling with multiple linear regression, resulted in a combination of linear and second-order polynomial models (Eqns (2)–(6)) which adequately estimates the contribution of the input variables on each of the outcomes. The results from the ANOVA are also shown in Table 4. With the models having $p < 0.05$ and large *F*-values (range: 5.71–7.87), the predictive models were considered to be too significant to have resulted from noise signals (Orman et al., 2023).

$$Yield\ (\%w/w) = 9.60 + 0.44A - 0.63B + 3.16C + 0.23D_1 - 0.70D_2 \quad (2)$$

$$Sqrt(Rutin)\ (\%w/w) = 0.16 + 0.009A + 0.007B + 0.03C - 0.02D_1 + 0.01D_2 + 0.0007AB - 0.01AC - 0.003AD_1 + 0.008AD_2 + 0.02BC - 0.02BD_1 + 0.02BD_2 - 0.006CD_1 + 0.004CD_2 - 0.01A^2 + 0.0006B^2 - 0.03C^2 \quad (3)$$

$$ISQ\ (\%w/w) = 0.04 + 0.002A - 0.003B + 0.01C - 0.002D_1 + 0.002D_2 + 0.0008AB - 0.003AC - 0.001AD_1 + 0.003AD_2 + 0.009BC - 0.008BD_1 + 0.008BD_2 - 0.003CD_1 + 0.001CD_2 - 0.002A^2 + 0.001B^2 - 0.008C^2 \quad (4)$$

$$Geraniin\ (\%w/w) = -0.86 + 0.08A + 0.09B + 0.20C - 0.24D_1 + 0.14D_2 \quad (5)$$

$$\log_{10} Phyllanthin(\%w/w) = -1.50 + 0.08A + 0.05B + 0.22C - 0.30D_1 + 0.13D_2 \quad (6)$$

Where the coefficient estimates of the experimental factors A, B, and C represent the expected changes in responses per unit changes in factor value when all remaining factors are held constant. In the same stead, D_1 and D_2 indicate mechanical shaking and cold maceration methods respectively, when sonication was held constant.

The extraction parameters, C and D were considered to be the keys as their effects were significant on almost all the quality attributes or outcomes. In effect, the extraction yield, and the concentrations of almost all the marker compounds were much impacted by the number of times the extraction was carried out as well as the method used: As the number of extractions increase, the concentrations and yield also increase and vice versa. Also, when emphasis was placed on the concentrations of geraniin and phyllanthin (considered to be representative markers for two important classes of compounds in the plants (hydrolysable tannins and lignans), using mechanical shaking approach (D_1) produced better outcomes than cold maceration (D_2) and sonication (reference method). The model graphs describing the relationship

Table 2
Summary of validation data for HPTLC analysis of *Phyllanthus urinaria* aerial parts for both qualitative and quantitative purposes.

	Rutin	Isoquercitrin	Gallic acid	Phyllanthin[α]
Identification				
Retardation factor (% RSD)	0.35 (2.09)	0.64 (0.98)	0.96 (0.92)	0.44 (1.50)
System suitability				
Relative retardation (R_{ref}) (% RSD)*	1.00 (0.00)	1.83 (1.73)	2.74 (1.99)	1.26 (2.68)
Specificity	Marker spots confirmed in PU profile. PU profile is different from other *Phyllanthus* species			
Accuracy				
Percentage Recovery (mean ± SD)[a]	98.5 ± 3	101.7 ± 4	100.1 ± 4	99.8 ± 4
Linearity				
Range (ng/band)	100–5000	100–5000	500–5000	10–500
Regression equation	$y = 419.2 + 5.152x - 0.0005248x^2$	$y = 636.7 + 8.149x - 0.0007675x^2$	$y = 0.3944x - 6.096$	$y = 174.2 + 11.02x - 0.002977x^2$
(Adjusted) Correlation coefficient (R^2)	0.9940	0.9976	0.9992	0.9980
Precision				
Instrumental precision (% RSD for R_F, Peak Area)	1.50, 1.70	0.65, 1.90	0.43, 1.03	0.92, 1.94
Repeatability 1[b] (% RSD)	1.55	1.55	1.18	1.19
Repeatability 2[c] (% RSD)	0.90	1.64	1.11	1.47
Intermediate precision 1[d] (% RSD)	1.95	0.65	1.31	1.68
Intermediate precision 2[e] (% RSD)	1.45	1.62	1.31	1.68
Robustness				
Δ saturation time (% RSD for R_F, Peak Area)	1.82, 1.61	1.14, 1.33	0.70, 1.44	1.77, 1.85
Δ development temperature (% RSD for R_F, Peak Area)	1.65, 1.60	0.90, 1.34	0.30, 1.22	1.37, 1.14
Δ developing chamber (% RSD for R_F, Peak Area)	1.64, 1.80	0.90, 0.42	0.60, 1.34	1.31, 1.56
Δ application instrument (% RSD for R_F, Peak Area)	1.63, 1.23	0.91, 1.07	0.60, 1.34	0.65, 1.86
Δ mode of application (% RSD for R_F, Peak Area)	1.55, 1.12	1.33, 2.37	0.30, 2.60	1.29, 1.30
Stability				
Analyte solution (%RSD)	1.56	2.96	2.91	2.76
During development	Marker compounds are stable throughout 2D development. No artefacts detected.			
Analytes on plate	No difference in densitometric profiles within 2 h of sample stay on plate before development			
Derivatized analytes	No difference in densitometric profiles of spots within 1 h after derivatization.			

SD – Standard deviation; RSD – Relative standard deviation; α Mobile system for developed for the assessment of this marker (toluene: ethylacetate: formic acid (69:30:1, v/v/v)) was different from the other three. *Relative retardation (R_{ref}) was calculated as a ratio of R_F of any marker compound to R_F of isoorientin (adopted as an internal reference marker) in every developed fingerprint.

[a] Average of triplicate determinations from three different concentrations (50, 100, 150% of the respective working concentrations) (n = 9) on the same plate.

[b] Repeatability for qualitative purpose, average of R_F determinations from three different plates on the same days and 4 tracks per plate (n = 12).

[c] Repeatability for quantitative purpose, average of triplicate determinations of three concentration levels on same plate at the same day (n = 9).

[d] Intermediate precision of the fingerprint, average of four developments on a plate for three days (n = 12).

[e] Intermediate precision for quantitative purpose, average of triplicate developments of marker compounds on the same plate in three different days.

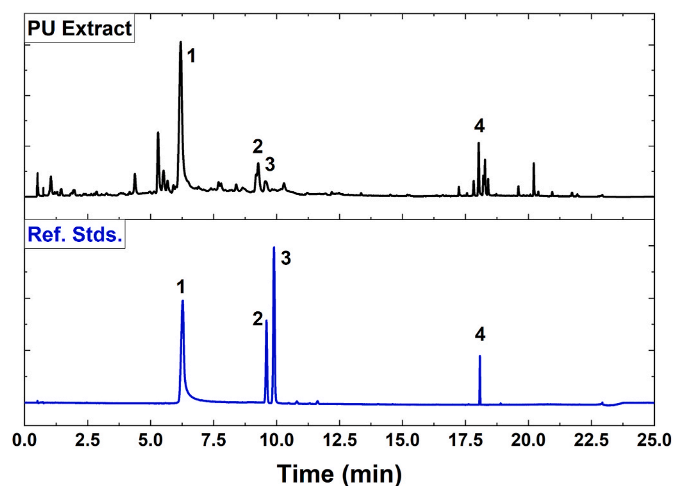

Fig. 4. UPLC chromatograms of acetone: water (7 : 3 v/v) extract of *Phyllanthus urinaria* aerial parts (PU) (top) and the analytical markers considered for assay (bottom). Detection at λ = 270 nm [**1**] Geraniin (t_R = 6.28 min; m/z = 975.0 $[M+Na]^+$); [**2**] rutin (t_R = 9.61 min; m/z = 633.1 $[M+Na]^+$); [**3**] isoquercitrin (t_R = 9.91 min; m/z = 487.0 $[M+Na]^+$); [**4**] phyllanthin (t_R = 18.08 min; m/z = 441.1 $[M+Na]^+$).

between the extraction conditions and the outcomes are shown in the Supplementary Data (Fig. S3). These observations were confirmed by the predictive models, where the coefficients for the C and D variables were comparatively bigger than the coefficients for the other variables (with the exception of the constants). Between the two variables, the coefficients for C were comparatively bigger than that of D, and this showed that the effect of changing the number of extractions was carried out is a bit greater than the effect of changing the method of extraction. Additionally, the results of the experiments indicated that extraction time and ratio of sample-to-solvent were not very critical. As per these observations, it may be proposed that instead of extracting a sample for a longer time (for example, 1–5 h), it may be preferable to rather extract the sample multiple times within the same duration. This assertion could also hold for the preparation of extracts for herbal formulations.

For the purpose of this study, the optimization led to the adoption of the following extraction conditions as predicted from the design space modelled with a desirability of 0.637: PU sample extracted in a 2% sample-to-solvent ratio each for 30 min and repeated three times using mechanical shaking method. The predictive ability of the design space was tested, and the responses were found to be within ±5% deviation (Fig. S4).

2.6. QAMS approach to quality assessment of Phyllanthus urinaria samples

In resource-challenged settings, the lack of reference standards for markers described for a developed method could serve as a barrier to its adoption for quality control purposes (Yan et al., 2015). In such instances, the QAMS approach has been proposed, in which case one of the markers is used as an internal reference marker with its content determined by external calibration method (ECM), and the quantitative assays of other marker compounds (irrespective of their number) carried out using their corresponding RCFs as determined with reference to the internal reference from a method development process (Yan et al., 2015). The quantitation of geraniin, isoquercitrin, and phyllanthin were similarly carried out by determining their RCFs using rutin as the internal reference standard. The estimation of the RCFs was then subjected to validation to assess precision, accuracy, and robustness of the QAMS procedure. The results of these investigations are summarized in Table 5.

The validation outcomes show that the approach could provide a reliable alternative to the use of reference standards for all the four markers for PU. Therefore, by determining the content of rutin from the use of its reference compound in an ECM approach, the contents of the other markers could be determined by using their respective RCFs and the peak areas as determined from the chromatogram of the sample.

2.7. Effects of spatio-temporal variations on quality of Phyllanthus urinaria aerial parts and specification setting

The effects of spatio-temporal variations on the content of the marker compounds in PU were evaluated using a batch of 36 samples collected from five different regions in Ghana (Ashanti, Central, Eastern, Volta and Western) which were distributed over 3 agroecological zones (coastal savannah, deciduous and evergreen). The samples were also

Table 3
Summary of validation results for the UPLC method for identity testing and assay of *Phyllanthus urinaria* aerial parts extract.

Parameter	Geraniin	Rutin	Isoquercitrin	Phyllanthin
Identification				
Retention time (% RSD)	6.28 (0.15)	9.61 (0.06)	9.91 (0.11)	18.08 (0.01)
System suitability				
Relative retention time (RRT)[a] (% RSD)	0.65 (0.13)	1.00 (0.00)	1.03 (0.09)	1.88 (0.06)
Specificity	Marker peaks confirmed in PU from PDA and QDa analysis of chromatograms.			
Accuracy				
Percentage Recovery (mean ± SD)[b]	100.4 ± 1.2	101.3 ± 1.0	100.5 ± 1.5	100.2 ± 1.7
Linearity				
Range (μg/mL)	10–100	5–30	20–50	5–20
Regression equation	y = 7588x – 14690	y = 7355x + 858.4	y = 9009x – 14040	y = 2555x – 93.67
Correlation coefficient (R^2)	0.9960	0.9999	0.9994	0.9946
Precision				
Repeatability (% RSD) (n = 6)	0.61	0.47	0.52	0.36
Intermediate Precision (% RSD)[c]	0.30	0.46	0.17	0.27
Robustness				
Δ column temperature (% RSD)	1.86	0.60	0.52	0.85
Δ flow rate (% RSD)	1.59	0.36	0.31	0.76
Stability of analyte solution (% RSD)	1.24	0.69	0.66	1.49

SD – Standard deviation; RSD – Relative standard deviation.

[a] Relative retention time (RRT) was calculated as a ratio of retention time of any other marker compound to that of geraniin (adopted as an internal reference marker).

[b] Average of triplicate determinations from three different concentrations (that is, 50%, 100% and 150% of working concentrations) (n = 9).

[c] Average of triplicate determinations in three different days.

Table 4
Summary of ANOVA results from Box-Behnken experimental model for optimization of sample extraction conditions.

Parameter	Yield		Conc. of rutin(% wt/wt)		Conc. of ISQ(% wt/wt)		Conc. of GER(% wt/wt)		Conc. of PHY(% wt/wt)	
	F-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value
Model	7.65	**< 0.0001**	5.71	<0.0001	7.3	<0.0001	7.01	<0.0001	7.87	<0.0001
A-Extraction time	0.67	0.4168	3.12	0.0885	1.39	0.2494	1.94	0.1714	1.95	0.1709
B-Extraction ratio	1.36	0.2512	1.82	0.1889	2.93	0.0987	2.39	0.1306	0.5674	0.4558
C–No. of extractions	34.58	**< 0.0001**	41.91	**< 0.0001**	62.26	**< 0.0001**	12.7	**0.001**	13.44	**0.0007**
D-Extraction type	0.8331	0.4423	5.03	**0.0139**	1.14	0.3355	9	**0.0006**	11.7	**0.0001**
AB			0.0011	0.917	0.1533	0.6985				
AC			1.76	0.1952	2.5	0.1257				
AD			0.6088	0.5513	0.7586	0.478				
BC			11.65	**0.002**	16.03	**0.0004**				
BD			6.07	**0.0067**	9.14	**0.0009**				
CD			0.3663	0.6967	0.7613	0.4768				
A^2			1.79	0.1924	0.4676	0.4999				
B^2			0.007	0.9342	0.3406	0.5643				
C^2			11.31	**0.0023**	14.26	**0.0008**				

p-values in bold formatting were considered significant.

collected over the two major seasons (rainy and dry) in the country.

Among the markers assessed, geraniin recorded the highest concentration in most of the samples while the least was isoquercitrin. The contents of the markers as determined from the samples collected ranged as follows: geraniin = 0.42–3.22 % wt/wt; rutin = 0.12–0.50 % wt/wt; isoquercitrin = 0.02–0.10% wt/wt; and phyllanthin = 0.07–1.05 % wt/wt (Fig. 5A and B). The predominance of geraniin in PU (as seen in the chromatogram – Fig. 4), and confirmed with its high content is consistent with the results from the study on *P. muellerianus* by Agyare et al., where together with furosin, they were shown to possess significant wound healing effects (Agyare et al., 2011).

When the contents of the marker compounds were further investigated, some variations were seen, and this could partly be attributed to spatio-temporal variations. For instance, samples originating from the evergreen zone contained significantly lower rutin content than those from coastal savannah and deciduous zones ($p = 0.0274$) (Fig. 5C), for which the rutin content was comparable. In terms of the regional distribution, it was also evident that the contents of geraniin ($p = 0.0299$) and rutin ($p = 0.0157$) varied greatly from one region to the other (Fig. 5D). It was further observed that the contents of all four markers were greatly affected by the month of sample collection ($p < 0.05$) (Fig. 5E). Samples generally collected in October recorded the least of the contents for each marker (Fig. S5). Additionally, the content of phyllanthin was observed to be higher in samples collected later in the year than those collected earlier. The final factor to be considered was the potential effect of seasonal variations (dry and rainy seasons). The data indicated that the contents of three of the markers, including geraniin, rutin and isoquercitrin across the two seasons were comparable ($p > 0.05$) (Fig. 5F). For phyllanthin however, the content was significantly higher in the dry season than in the rainy season ($p = 0.0004$). The differences in the contents of the marker compounds in PU may likely affect the therapeutic potential of the same plant from different places. This therefore calls for stringent quality control measures to ensure the quality of starting materials (in this case, PU) used in the formulation of herbal medicinal products as for example, used in Ayuverdic medicine.

For that reason, there is a need to adhere to established acceptance criteria for the control of these markers. As part of the study, we considered setting specifications for these markers by first considering the distribution of the individual contents in the samples. Irrespective of the spatio-temporal effects on the contents, normality tests (including Shapiro-Wilk, Kolmogorov-Smirnov and Anderson-Darling tests) showed that the data were significantly drawn from normally distributed populations. Thus, assuming a maximum of 10% substandard level for each marker content, the acceptance limits for the marker compounds were established as follows: geraniin ≥0.50% (wt/wt); rutin ≥0.14% (wt/wt); isoquercitrin ≥0.03% (wt/wt); and phyllanthin ≥0.10% (wt/wt), related to the dried mass of the plant material, respectively. Considering for example the use of the plant as an anthelmintic, the acceptance limit estimated for geraniin far exceeds the concentration required to exhibit inhibitory effects against *C. elegans* in a mortality assay (Jato et al., 2021). Thus, it could be assumed that the estimated limits may be useful to control the quality of the plant for medicinal use. Applying these limits to the PU plant materials collected, one sample (ID: PU009) could be said to be of a poor quality because the concentrations of all four markers were lower than limits proposed. Few of them had the concentrations of one or two of the markers below the estimated limits. Generally, the compliance rate ranged between 89 and 94%.

2.8. Purity assessment of the Phyllanthus urinaria samples

By way of testing for the purity of the plant materials, a pooled sample from the 36 samples was screened for the presence of pesticides residues. It has become necessary to consider the pesticides residues

Table 5
Summary of results of the QAMS approach for *Phyllanthus urinaria* aerial parts.

	Geraniin	Rutin	Isoquercitrin	Phyllanthin
RCF	0.9489	–	1.1205	0.3359
Precision				
Same day (% RSD)	0.34	–	0.39	0.29
Different days (% RSD)	1.02	–	0.17	0.26
Robustness				
Δ column temperature (% RSD)	0.46	–	0.50	0.29
Δ flow rate (% RSD)	2.03	–	0.41	0.83
Accuracy (%), % RSD	100.1, 0.19	–	93.4, 3.58	95.7, 0.04

QAMS: Quantitative assessment of multicomponents by single markers; RCF: Relative correction factor; RSD: Relative standard deviation; RCF: relative correction factors calculated relative to rutin; RSD: relative standard deviation; ECM: external calibration method.

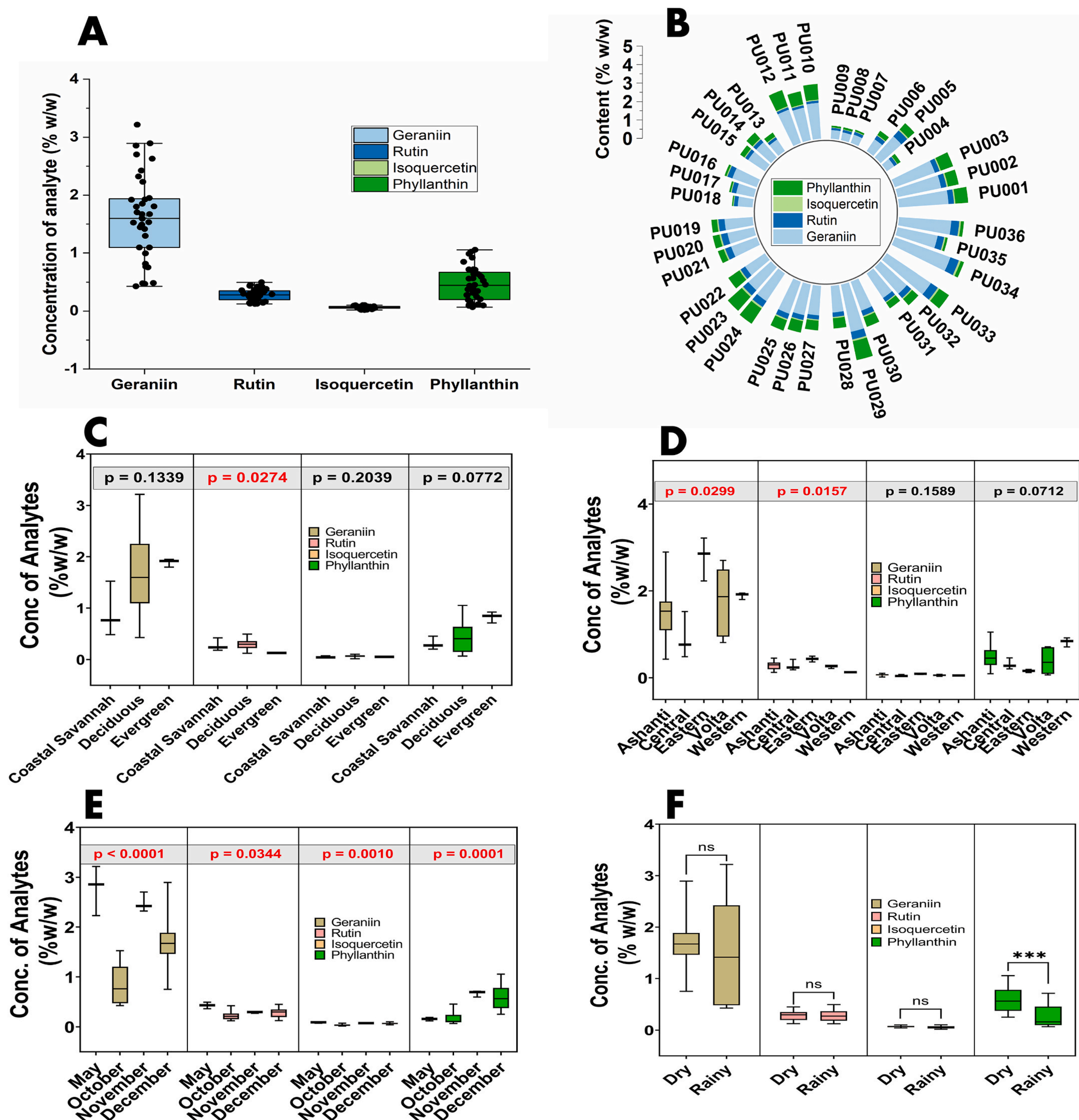

Fig. 5. Batch analysis of 36 samples of *Phyllanthus urinaria* aerial part materials from different places and times of collection in Ghana. **A:** Box plot of the marker contents. Data represented as mean with interquartile range. **B:** Circular stacked plot of the contents of the marker compounds in each of the 36 PU samples. Each content is a representation of the mean from triplicate determinations. **C:** One-way ANOVA to evaluate the effect of agroecological zones on contents of markers. **D:** One-way ANOVA to evaluate the effect of regional origin of sample on contents of markers; **E:** One-way ANOVA to evaluate the effect of different months of collection; **F:** One-way ANOVA to evaluate the effect of different climatic season of sampling on contents of markers.

screening because of recent reports of the detection of several pesticides in food and plant-based medicinal products beyond their maximum residue limits (MRLs) (Agbeve et al., 2013, 2014; Donkor et al., 2016; Opuni et al., 2021). The public therefore gets exposed to these harmful and potentially harmful chemicals at levels that could be of concern because of the current inadequate regulatory framework regarding the rational use of pesticides. For safety reasons, it is crucial that the plant material adheres to the restrictions on particularly hazardous pesticides while maintaining appropriate levels of other less toxic pesticides.

Analysis of the pooled PU sample revealed the presence of chlorpyrifos (0.100 ± 0.000 mg/kg), and folpet (sum of folpet and phthalimide, expressed as folpet, 0.221 ± 0.001 mg/kg) which may be exceeding the MRLs of non-dried, fresh herbs (European Parliament, 2005). The rest of the 349-pesticides panel were either absent or below

their detection limits. Chlorpyrifos, a moderately toxic pesticide, for which the use has been linked to neurodevelopmental problems, a high risk of developing cancer, and even mortality (Ubaid ur Rahman et al., 2021), was present in a considerable residual level that is about 10 times the MRL of 0.01 mg/kg. This situation is deemed very disturbing considering the fact that PU is widely used for different medicinal purposes in Ghana. On the other hand, folpet is considered to be less harmful (World Health Organization WHO, 2020b). Taking a cue from the results of the pesticides analysis, the regulatory structure currently in place to regulate their use needs to be thoroughly reviewed.

3. Conclusions

The current study on the analytical investigations of *Phyllanthus urinaria* aerial parts provides validated analytical methods for quality control, with specifications and a draft monograph. The draft monograph has been written according to the format of the European Pharmacopoeia and is displayed as ***Monograph 1 in the Additional Data File*** for future consideration and discussion by regulatory officials and industry participants in the herbal medicine sector.

4. Experimental

4.1. Solvents, reagents, and reference standards

All solvents and reagents used were of analytical quality and were obtained from VWR International (Darmstadt). The purified water was made in-house by Millipore simplicity 185 system (Merck, Darmstadt). 2-Aminoethyl-diphenylborinate (natural product reagent, NP) and polyethylene glycol 400 (PEG) were from Merck (Darmstadt). The reference standards including rutin (purity >95%), isoquercitrin (purity >95%), gallic acid (purity >95%), and phyllanthin (purity >95%), were obtained from Sigma–Aldrich (Deisenhofen, Germany) and Phytolab (Vestensbergsgreuth). Geraniin (purity >98%) was purchased from Atkin Chemicals, Inc., China.

4.2. Plant material

The leaves of PU and other related species, including *P. amarus, P. fraternus, P. nuriri, P. muellerianus* were collected from different parts of Ghana between March 2020 and December 2021. Commercial extracts of *P. emblica* were donated by an anonymized herbal manufacturer in Germany. The collection sites of the samples are indicated in the Supplementary Data (Table S1). The plants were identified and authenticated by botanists, Dr. George Henry Sam (Department of Herbal Medicine, KNUST, Kumasi, Ghana) and Mr. Tonny Asafo Agyei (Center for Plant Medicine Research, Mampong, Ghana). Voucher specimens are deposited in the herbarium of the University of Münster, Institute of Pharmaceutical Biology and Phytochemistry, Germany. The respective voucher identification numbers (#IPBP728 to 812) are displayed in Table S1 of the Supplementary Data File.

4.3. Optimization of sample extraction procedure for chromatographic analysis

The procedure used to extract the *Phyllanthus* samples for analytical investigations was optimized using the Box-Behnken Design (BBD) model from Design Expert software (version 11, Stat Ease Inc., 2017) in an experimental design approach reported in literature (Orman et al., 2023). The quality attributes monitored were extraction yield (% wt/wt), and concentrations (% wt/wt) of the marker compounds, geraniin, rutin, isoquercitrin, and phyllanthin. A second-order polynomial function (Eqn. (1)) was used to describe the interaction between the extraction parameters and the quality attributes.

$$Y = \beta_o + \sum \beta_i x_i + \sum \beta_{ii} x_{ii}^2 + \sum \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

Where Y represents the measured response associated with each factor level combination, β_o is a constant, β_i is the slope or linear effect of the input factor x_i, β_{ii} is the quadratic effect of input factor x_i, and β_{ij} is the linear-by-linear interaction effect between the input factor x_i and x_j.

4.4. Extract preparation for subsequent analytical investigations

The plant material was cleaned, air-dried at room temperature for three weeks, and pulverized in a mortar after freezing with liquid nitrogen. An amount of 0.5 g of the pulverized plant material was extracted 3 times, each for 30 min with 25 mL of acetone: water (7:3, v/v) mixture by use of a mechanical shaker (GFL 3018 model, Germany). The suspensions were centrifuged for 10 min at 3000×*g* and the clear supernatant was evaporated and dried *in-vacuo*. The residue was resuspended in a small amount of water (Aqua Millipore) and lyophilized. The lyophilized samples were stored at – 20 °C prior to analysis. For analysis, 10 mg/mL methanol (MeOH) solution of the extract was prepared, centrifuged at 6020×*g* (Mikro 120, Hettich Zentrifugen) for 5 min and 2 mL of the supernatant was transferred into HPLC vials.

4.5. LC-MS/MS characterization of P. urinaria aerial part extract

The phytochemical characterization of the acetone: water extract (7:3) of *P. urinaria* was performed using an LC-MS/MS analytical approach and instrumentation system described previously (Orman et al., 2023). The elution system comprised of a binary solvent composition with solvent A: water with 0.1% formic acid, and solvent B: acetonitrile with 0.1% formic acid. Eluting was carried out at 0.4 mL/min, using the following gradient system: t_{0min}: 13% B, t_{5min}: 36% B, t_{8min}: 100% B, t_{15min}: 100% B, $t_{15.1min}$: 5% B, t_{20min}: 5% B. The injection volume was 2 μL. Metlin (Smith et al., 2005), MassBank (Horai et al., 2010), MMCD (Cui et al., 2008) and KNApSAcK (Afendi et al., 2012) mass spectral databases as well as Reaxys enabled the identification of the eluted compounds. A typical chromatogram is displayed in Fig. 1 and compounds identified are summarized in Table 1.

4.6. Phytochemical differences between P. urinaria and closely related Phyllanthus species

The composition of PU was comparatively studied with that of authenticated closely related *Phyllanthus* species including *P. fraternus*, *P. niruri, P. amarus, P. muellerianus,* and *P emblica* in an untargeted LC-MS metabolomic analysis using an analytical procedure previously described in literature (Orman et al., 2023). After pre-preprocessing the mass spectral data, the resulting bucket table consisted of 378 buckets which was subsequently used as data matrix for the statistical modelling using the MetaboAnalyst 5.0 server (https://www.metaboanalyst.ca). Prior to the modelling, the data were further pre-processed by log transformation and pareto scaling.

4.7. HPTLC analysis of PU extract

The HPTLC procedure used for both qualitative and quantitative analyses of PU was developed with conditions previously described in literature (Orman et al., 2023). Where applicable, the different conditions are highlighted.

Standard solutions: 1 mg/mL stock solution of each of the reference compounds, rutin, isoquercitrin, phyllanthin and gallic acid, was separately prepared in MeOH and stored at - 20 °C for use. The stock solutions were then serially diluted with MeOH to obtain the following working concentrations for validation and further analysis: rutin and isoquercitrin (10–500 μg/mL equivalent to 100–5000 ng/band); phyllanthin (1–50 μg/mL equivalent to 10–500 ng/band); and gallic acid (50–500 μg/mL equivalent to 500–5000 ng/band).

Chromatographic conditions: The analysis was performed by use of a

CAMAG HPTLC system (Muttenz, Switzerland). This set up comprised an Automatic TLC sampler ATS 4, a Twin Trough Chamber with steel lid (20 × 10 cm and 10 × 10 cm), TLC scanner 3 in combination with winCATS software (version: 1.April 4, 6337) and TLC Visualizer 2 controlled by visionCATS software (version: 3.0). Test solutions were applied at 10 μL as 6 mm bands using a 25 μL Hamilton syringe (Bonaduz) on silica gel 60 F_{254} coated HPTLC plates (10 × 10 cm and 20 × 10 cm; Merck, Germany). A saturation time of 25 min at room temperature was ensured. Two different mobile phase systems were used: í. toluene: ethylacetate: formic acid (69:30:1, v/v/v), which has been optimized for the detection and quantitation of phyllanthin; ii. ethylacetate: water: formic acid (75:15:10, v/v/v) has been optimized for the detection and quantitation of rutin, isoquercitrin, and gallic acid. Detection: λ = 254 nm, and 366 nm pre- and post-derivatization with Natural Product Reagent and polyethylene glycol (NP-PEG). Densitometric scanning of the underivatized plate was performed at λ = 254 nm, and derivatized plates at λ = 360 nm in the absorbance mode.

Validation of the HPTLC Method (ICH Q2(R1)): The HPTLC method as developed for both qualitative and quantitative purposes, was validated according to International Council of Harmonization (ICH) Q2(R1) guidelines (ICH, 2005) using procedures previously described in literature (Orman et al., 2023). The following parameters were assessed: specificity, accuracy, linearity, precision, reproducibility, and stability of analytes. The outcomes of the validation are summarized in Table 2.

4.8. UPLC analysis of PU extract

Standard solutions: Stock solutions of the reference compounds (each 1 mg/mL in MeOH) were prepared. The working standard solutions were prepared by diluting the stock standard solutions with MeOH to the following series of concentrations: geraniin (10–100 μg/mL), rutin (5–30 μg/mL), isoquercitrin (20–50 μg/mL) and phyllanthin (5–20 μg/mL).

*Test solutions:*10 mg/mL in MeOH of the lyophilized extracts.

UPLC analysis was performed with an Acquity UPLC™ system (Waters) equipped with PDA eλ detector (λ = 210–400 nm), QDa detector (ESI, positive and negative modes, single quadrupole, 100–1250 Da), RP-18 stationary phase (Acquity UPLC HSS T3 column; 1.8 μm, 2.1 × 100 mm), Autosampler, and binary solvent manager. The mobile phase was made of solvent A (water + 0.1% formic acid) and solvent B (acetonitrile + 0.1% formic acid). The gradient elution system employed was as follows: t_{0min}: 2% B, t_{2min}: 8% B, t_{11min}: 18% B, t_{15min}: 35% B, t_{20min}: 100% B, t_{22min}: 100% B, t_{24min}: 2% B, t_{25min}: 2% B. A flow rate of 0.5 mL/min, injection volume of 2 μL, and column temperature of 40 °C were adopted. Data acquisition and processing were performed using Waters Empower 3 (Waters, Milford, Milwaukee, USA).

Validation of the UPLC Method (ICH Q2(R1)): Similarly, the validation of the UPLC method for PU was carried out in accordance with ICH Q2 (R1) (ICH, 2005) guidelines, using procedures previously described in literature (Orman et al., 2023). The parameters investigated included specificity, accuracy, linearity, precision, reproducibility, and stability of analytes.

4.9. Quantitative assessment of multicomponents by single markers (QAMS) approach to content assay in Phyllanthus urinaria

The QAMS approach to content assay was investigated by determining the relative correction factors (RCFs) of geraniin, isoquercitrin, and phyllanthin from their respective peak areas, in relation to rutin used as an internal reference marker (Zhu et al., 2017). The procedures adopted are previously reported in literature (Orman et al., 2023).

4.10. Pesticides residues analysis

Similarly, a pooled PU sample was screened for the presence of contaminants from a panel of 349 pesticides and related contaminants using LC-MS/MS and GC-MS/MS techniques. Depending on the polarities of the pesticides targeted, two sample preparation approaches were used, including the modified Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method (European Committee for Standardization, 2018) and the Quick Polar Pesticide (QuPPe) method (Anastassiades et al., 2020). The details of the procedures carried out are as described previously in literature (Orman et al., 2023). The list of pesticides screened with their corresponding analytical data are reproduced in Table S3.

4.11. Statistical analysis

To analyse the data, the study used both univariate and multivariate data analysis techniques, determining the mean, standard deviation, and relative standard deviation. Within method validation and batch PU samples analyses, statistical tests such as the *t*-test, and ANOVA with *post-hoc* tests were used to compare analytical results. Multiple linear regression analysis and principal component analysis (PCA) were used to optimize the sample extraction procedure and assess marker content in batch samples, respectively. To authenticate PU samples, the LC-MS metabolomics study in several *Phyllanthus* species employed techniques such as volcano plot analysis, clustered heatmap analysis, and Partial Least-Squares Discriminant Analysis (PLS-DA). Statistical significance was assessed using $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***)

Ethical approval

This article does not contain studies with human participants performed by any of the authors.

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Contributors statement

EO, EOB and AH conceptualized the study. EO, IK carried out the experimental work and evaluated the data; EO wrote the manuscript draft; JJ, VS, EOB, AH revised the MS; VS, EOB, AH, CA applied for the research grants, SOB, SAN commented on the MS. The study was designed by EO, EOB and AH.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2023.113854.

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