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PENTAS LONGIFLORA OLIV. (RUBIACEAE), A PLANT USED IN THE TREATMENT OF PITYRIASIS VERSICOLOR IN RWANDA: CHEMICAL COMPOSITION AND STANDARDIZATION OF LEAVES AND ROOTS

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ABSTRACT

In Rwanda, the roots of Pentas longiflora Oliv. (Rubiaceae) have been used for a long time to treat Pityriasis versicolor. However, many people reported the use of leaves instead of roots. This research was conducted to compare the phytochemical composition and establish chromatographic methods for the standardization of roots and leaves extracts of P. longiflora. During this process, three new pentalongin glycosides (pentalonginoside A, pentalonginoside B, and pentalonginoside C) and two known glycosides of the same type (harounoside and clarinoside), as well as rutin, luteolin-7-rutinoside were isolated from methanol extract of leaves. In addition, pentalongin and psychorubrin, previously isolated from ethylacetate roots extract, were also identified in Pentas longiflora ethylacetate leaves extract. The presence of the antifungal

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compound pentalongin in leaves may explain the traditional use of leaves in the treatment of *Pytiriasis versicolor*.

Furthermore, harounoside, psychorubrin, and pentalongin were selected as markers for HPLC fingerprints of MeOH extract. The accuracy and risk profile demonstrated the reliability of the validated method. In general, considerable variations of concentration in plant metabolites, including pentalongin, were observed between samples from different sites. The content in pentalongin (expressed as juglone) in collected samples ranged between 1.7 and 70.0 mg/100 g. The highest concentration (70.0 ± 17 mg/100 g) was registered in the cultivated samples from Mukoni.

This important variation of pentalongin concentrations according to sampling sites, shows that in order to guarantee equivalent efficacy, finished products with P. longiflora should be standardized based on their pentalongin content.

1. Introduction

Caused by different *Mallassezia* species [1], *Pityriasis versicolor* (PV) is one of the most common disorders of pigmentation in the world. PV is also known as *tiniea versicolar* and, less commonly, as *dermato mycosis furfurraceus*, *achromia parasitica*, and *tinea flava* [2].

PV is estimated to affect as many as 60% of individuals in tropical and humid areas [3]. Some researchers associate its occurrence with occupational and socioeconomic conditions [4]. In contrast, others state that there is no significant relationship between the prevalence and age, gender, profession, family history, and personal hygiene [5].

Most of *Malassezia* species causing PV are killed by imidazole derivatives such as miconazole, econazole, fenticonazole, bifonazole, ketoconazole, fluconazole, and others, because of their ability to penetrate the deep layers of the skin at therapeutic doses [6]. However, the benefits of these drugs are overshadowed by their side effects, such as nausea, headache, and vomiting. At the same time, less common adverse reactions include abdominal discomfort, transient rash, urticaria, diarrhea, and photosensitivity [7]. Apart from the modern medicines, there are phytomedicines that have shown an interesting healing level, such as the India Dill seed (*Anethum graveolens*) ointment, 5% *Artemisia sieberi* essential oil lotion, a polyherbal product containing Ceylon leadwort (*Plumbugo zeylanicum*), black mustard (*Brassica nigra*), sneezewort (*Dregea volubis*), India madder (*Rubia cordifolia*), Radish *(Raphanus sativus*), and vinegar [3], an antimycotic ointment produced using the alcoholic root extract of *Pentas longiflora Oliv.* (Rubiaceae) (*P. Longiflora*) [8], etc.

P. longiflora is an erect stemmed woody herb up to 3 m high from oriental inter-tropical Africa [9], which is reputed to possess several medicinal properties. In Kenya, where it is known as "Nekilango" or "Segimbe," the roots are used as a cure for tapeworm, itchy rashes, and pimples [10]. A decoction of the roots alone [11,12] or mixed with milk [13] is taken as a cure for malaria but

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it causes acute diarrhea and acts as a purgative and antiseptic agent [14]. In addition, the fruits and bark are used to reduce the fever associated with malaria, especially in young children [15] and for back pains [16]. In Uganda, *P. longiflora* is ranked as the most effective antifungal medicinal plant species [17]. In Burundi, both leaves and roots are used in the treatment of microbial diseases, skin mycosis, fever, ringworm, purulent rashes, and cholera [18]. It was the most cited mono-herbal recipe [19]. In Tanzania, a decoction of the roots is mixed with milk and taken as a cure for malaria. In Rwanda, the vernacular name of *P. longiflora* is Isagara, and a study involving several plants used as anti-fungals in traditional medicines was conducted and the higher level of activity was found for the roots of the plant. The same study led to the isolation of the active principle, a naphthoquinone, pentalongin (1). During that study, mollugin, 3-hydroxymollugin, 3-methoxymollugin, scopoletin, methyl-2,3-epoxy-3-prenyl-1,4- naphthoquinone-2-carboxylate, methyl-3-prenyl-1,4-naphthoquinone- 2-carboxylate, *cis*3,4- dihydroxy-3,4-dihydromollugin, trans-3,4- dihydroxy-3,4-dihydromollugin and (3α,3'α,4β,4'β)-3,3'-dimethoxy-cis - [4,4'-bis(3,4,5,10-tetrahydro-1 *H*-naphtho[2,3-c]pyran)]-5, 5',10,10'- tetraone [13, 20] and Munjistin ethyl ester and its three derivatives [21] were also isolated (Fig. S 1; Supplementary Material).

Based on the anti-PV, an ointment was manufactured from the alcoholic extract of the roots and tested clinically on 80 people suffering from PV. All the people treated with the anti-mycotic ointment were healed without any undesirable effects being observed [22].

During the collection of samples in different regions of Rwanda in order to standardize the roots of *P. longiflora*, numerous traditional healers and the local community reported the use of *P. longiflora* leaves to treat PV instead of the roots. This raised a scientific question: do leaves and roots contain similar compounds? This research was conducted to compare the phytochemical composition of leaves and roots extract in order to ascertain the use of the leaves for the treatment of PV and to establish chromatographic methods for the standardization of both roots and leaves of *P. longiflora*. This would help ensure the reproducibility in quality between anti-PV ointment batches.

2. Experimental

2.1. PLANT MATERIAL

The samples of *P. longiflora* Oliv. (Rubiaceae) were collected in Rwanda. The collected samples included the domesticated samples from Mukoni sampling site (2°37'12.65''E, 29°44'27.08'' ; elevation 1685 m) and samples from the wild environment at Rubavu sampling site (1°13'18.47"S, 29°22'44.65''E, elevation: 2277 m), Rusizi sampling site (2°28'02.62"S, 28°56.00.25''E; elevation: 1671 m), Saruheshyi sampling site (2°07'44.23"S, 29°42'56.65''E; elevation: 1892 m), Musanze-Gataraga sampling site (1°31'25.76"S, 29°34'04.17''E; elevation: 2095 m), and Musanze city sampling site (1°30'49.97"S, 29°39'15.82''E; elevation: 1778 m). Specimen for the collected samples were deposited in the National Herbarium of Rwanda (NHR) and given the voucher number

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KAGISHA V. 002, KAGISHA V. 006, KAGISHA V. 007, KAGISHA V. 008, KAGISHA V. 009, KAGISHA V. 010, for Mukoni, Rubavu, Rusizi, Saruheshyi, Musanze-Gataraga, and Musanze city sampling site, respectively. For Mukoni, Rubavu, and Rusizi, samples were collected three times at different periods. For the remaining sites, samples were collected once due to the limited number of plants.

2.2. GENERAL EXPERIMENTAL PROCEDURES

The dried plant material was finely ground using Retsch SM 100 and Retsch ZM 200 grinding machine. HPLC method was developed using an HPLC Agilent 1100 series system. Zorbax eclipse XDB Column (250 mm x 4.6 mm; 5 µm particle size, Agilent) was used as stationary phase. Component of the samples were resolved using the gradient system: mobile phase A: 0.1% of formic acid (pH = 2.5); B: methanol (MeOH); gradient: 0 min: 20% B, 30 min: 33% B, 70 min: 70% B, 72 min: 20% B. Then, re-equilibration was done for 10 min. The injection volume was 10 µL, and separation was carried out at 30 °C with a flow rate of 1 mL/ min. Detection was done at 254 nm, while the injected samples were prepared by extracting 1 g of the fine powder (roots and leaves) with 20 mL of MeOH under sonication for 30 min. Due to the degradation of naphtoquinone in MeOH, samples were finally prepared them in acetonitrile (CH_3N) during validation.

Masses of compounds were identified using the above method on an LC hyphenated with a LTQ-OrbitrapXL(Thermofisher scientific, Belgium) with electrospray ionization(ESI)from the UCL MASSMET platform.

The ESI parameters were set as follows: capillary temperature of 250°C; capillary voltage of 25 V; source voltage of 4.25 kV; tube lens voltage of 110 V. The resolution of the Orbitrap mass analyzer was 30,000. Nitrogenwas used as the sheath gas and helium as auxiliary gas with flow rates of 20 and10 arbitrary units respectively. The experimental data were recorded in full scan mode.

Compounds 1 and 4 were isolated with open column chromatography packed with silica gel. Other compounds (5-9) were isolated with Varian ProStar preparative HPLC (Prep-HPLC) equipped with a Büchi fraction collector C-660 unit and a column of 27 cm length, 1.25 cm ID, packed with Lichroprep stationary phase of 15-25 µm particle sizes, Merck, Hohenbrum, Germany, and samples were eluted with the following system: mobile phase A: Water; B: MeOH; gradient: 0 min: 5% B, 10 min: 20% B, 66 min: 33%, 160 min: 70% B. The injection volume was 10 mL, and separation was carried out at room temperature with a flow rate of 10 mL/min. Detection was conducted at 254 nm and 360 nm.

NMR spectra for purified compounds were recorded in MeOH-d_4 on a Bruker AVANCE NEO 500 MHz spectrometer equipped with a cryoprobe. Chemical shifts were reported in δ values (ppm) relative to internal standard TMS.

UV spectra for new molecules were obtained in CH3OH using a V- 2910 UV spectrophotometer from Hitachi. IR spectra for new molecules were recorded in acetone with FT-IR Frontier Perkin Elmer spectrophotometer equipped with an ATR module.

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Furthermore, the reference of juglone used during validation was purchased from Sigma Aldrich (Syeinheim, Germany). All solvents used were purchased from VWR (Leuven, Belgium) and were HPLC grade (for all HPLC experiments) or analytical grade (for TLC and open column experiments).

TLC experiments were conducted on silica gel $60F_{254}$ TLC plates from Merck, Darmstadt, Germany. The plates were developed with a mobile phase made of n-hexane: ethylacetate (EtOAc) (3:1, v/v) and ethyl acetate: anhydrous formic acid: water: glacial acetic acid (25:2.75:6.50:2.75 v/v/v/v)).

2.3. IDENTIFICATION OF COMPOUNDS WITH HPLC-UV AND LC-MSMS

Based on previous publications about the chemical composition of roots of *P. longiflora*, some commercially available standards, such as scopoletin, tectoquinone, and mollugin were used to screen their presence in the extracts roots and leaves P. longiflora using the existing HPLC-UV methods [23, 24] for their respective analysis. In addition, the HPLC method resolving major compounds of MeOH and EtOAc extract (as described above) was developed for further visualizing the resemblances and differences between different extracts. The extracts were also analyzed with LC-ESI-MSMS in both negative and positive mode, applying the chromatographic conditions of the developed method for compound identification and selectivity evaluation.

2.4. ISOLATION AND STRUCTURE ELUCIDATION OF MAJOR COMPOUNDS FROM LEAVES AND ROOTS OF P. LONGIFLORA

2.4.1. ISOLATION OF COMPOUND (1) AND (4)

EtOAc extract was prepared from a fine powder of *P. longiflora* roots (250 g). The extract was filtered, and the solvent was removed under reduced pressure using a rotary evaporator at 40 °C to obtain 29 g of crude extract.

The dry residues (1.5 g) were separated with an open column packed with silica gel (15 g) and eluted under step gradient with n-hexane and EtOAc, from 100% n-hexane to 30% n-hexane in EtOAc. Collected fractions were analyzed by TLC (silica gel 60F254 TLC plates from Merck, Darmstadt, Germany) and developed with a mobile phase of n-hexane: EtOAc (3:1, v/v) and HPLC-UV method using the conditions previously described. 23 mg of compound (1) was obtained from fractions 44-57 eluted with 0-2% EtOAc in n-hexane, and 7 mg of compound (4) was obtained from fractions 165-169 eluted with 40% EtOAc in n-hexane.

2.4.2. ISOLATION OF COMPOUNDS 4-9

To isolate compounds (4-9), 300 g of a fine powder from leaves of *P. longiflora* was extracted three times with 400 mL EtOAc. After filtration, the solvent was evaporated with a rotary evaporator at low temperature (around 40°C) under reduced pressure. The traces of solvent in the extracts were removed by keeping them in a vacuum oven set at room temperature for 24 h to obtain 46 g of crude extract. 300 mg of EtOAc crude extract was extract with 12 mL of deionized water under sonication. The resulting solution was filtered with a 0.45 ^m filter and injected in Prep-HPLC.

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Collected fractions were analyzed with the developed HPLC-UV method for purity verification (Fig. S 48-S52, Supplementary Material), and fractions containing similar compounds were gathered and evaporated. Compound 5 (8 mg), compound 6 (3.8 mg) compound 7 (5.3 mg), compound 8 (4.5 mg), and compound 9 (3.1 mg) were obtained from fractions 11-21, 40-44, 48-51, and 51-61 respectively.

2.5. VALIDATION OF ANALYTICAL METHODS

The first step was to identify a calibration standard for validation of the HPLC analytical method since the therapeutic molecule ((pentalongin (1)) does not have a standard on the market. The approach consisted of finding out a compound eluted by the developed method, detected at the optimum wavelength of the therapeutic compound, without interfering with any peak in the chromatogram. In addition, the compound should be of low cost and chemically related to pentalongin (1).

During validation, five concentration levels: 14, 28, 70, 140, 210 µg/mL of working solutions, were used to make a calibration curve, and each level was analyzed in triplicate for each of the three series.

Validation standards of the same concentration in juglone were prepared as calibration standards within the matrix by spiking the stock sample solution prepared by sonicating 1 g of powdered roots of *P. longiflora* sample in 10 mL of CH_3CN for 30 min. Total error (systematic + random error) was the main decision criterion during validation [25]. The accuracy was evaluated by generating the accuracy profile with the acceptance limits set at ±10% and the minimum probability to obtain future results within these limits at p = 95%. In addition, the trueness, linearity, range, and precision (repeatability and intermediate precision) were evaluated. Data were analyzed with Enoval V3.0 software (PharmaLex, Mont-St-Guibert, Belgium). Finally, the validated method was applied to the collected samples. The content of pentalongin (1) in collected samples was reported as the percentage of pentalongin (1) in the sample expressed as juglone.

3. Results and discussion

3.1. IDENTIFICATION OF COMPOUNDS WITH HPLC-UV AND LC-ESI-MSMS

The analysis of the reference standards of scopoletin, tectoquinone, and mollugin in the same HPLC experiments with MeOH and EtOAc extracts did not confirm their existence in the plant as they were not detected in both roots and leaves samples. This led us to develop an HPLC method resolving the major compounds (Fig. 1) and allowing their identification with LC-ESI-MSMS.

The analysis of MeOH extracts (leaves and roots) by LC-ESI-MSMS in the negative mode led to the ionization of one compound (compound (5)) from roots extract and seven compounds in leaves extract. None of the compounds were ionized in the positive mode for all extracts. Two of the

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detected masses, i.e., $[M-H]^-$ = 609.14542 (Fig. S 44; Supplementary Material) and $[M-H]^-$ = 593.15066 (Fig. S 45; Supplementary Material), were consistent with the formulae $C_{27}H_{29}O_{16}$ and $C_{21}H_{19}O_{11}$ corresponding to rutin (2) and luteolin-7-rutinoside (3), respectively. The presence of rutine in the leaves was also confirmed by using TLC (Fig. S 55; Supplementary Material) and HPLC experiments through the comparison of their bands, peaks, and UV spectra with standard reference. Flavoinoid, the phytochemical class of rutin (2) and luteolin-7- rutinoside (3), was not yet screened in studied pentas species. Other masses were not consistent with any of the compounds previously isolated from *P. longiflora*.

3.2. ELUCIDATION OF CHEMICAL STRUCTURES OF ISOLATED COMPOUNDS

By comparison of their NMR spectroscopic data (Fig. S 55-S 58; Supplementary Material) to those previously reported in the literature for *P. longiflora* [13], compounds (1) and (4) were elucidated as pentalongin (1) and psychorubrin (1), respectively.

The comparison of experimental spectral data (NMR, MS, UV) (Fig. S 2-S 15; Supplementary Material) of the isolated compounds with data from the literature allowed the identification of two known pentalongin glycosides, harounoside (5) (Harouna et al., 1995) and clarinoside (6) (Audoin et al., 2018), previously isolated from *Mitracarpus scaber* and reported in *P. longiflora* in this work for the first time.

The structures of the new compounds (7-9) were elucidated by the analysis of their 1D and 2D NMR, IR and UV spectra, and MS data.

The comparison of the spectroscopic data (IR, UV, NMR, MS) of those compounds indicated important similarities between them.

In fact, compounds (7-9) displayed the same UV spectrum in MeOH (UV (MeOH) λ_{max} 316, 303, 234; λ_{min} 310, 286, 225 nm), indicating that they have the same chromophore group. In addition, they displayed almost similar IR spectra (Fig. S 14, Fig. S 33, Fig. S 43; Supplementary Material): IR_{vmax} 3368, 2977, 2905, 1688, 1638, 1349, 1067 cm 1. A broadband at 3368 cm^{-1} slightly overlapping with a band at 2905 cm^{-1} (due to sp^3 CH bond) indicated the presence of O-H of alcohol.

Moreover, the spectrum of compound (9) showed an additional band at 1704 cm^{-1} indicating the presence of the carbonyl group.

Furthermore, NMR data (Table 1 and Fig. S 16-S 21, Fig. S 25-S 30, Fig. S 35-S 40; Supplementary Material) easily confirmed the presence of two sugar moieties for the three of them and the aglycone part presenting identical signals. In fact, for all of them, the ^1H NMR spectrum showed signals of a naphto-pyran ring with an AA'BB' system at δ 7.4 (H-7, m), 8.4 (H-6, m), 7.4 (H-8, m), 8.4 (H-9, m), ppm characteristic for an orthodisubstituted aromatic ring, and a doublet of cis olefinic protons at δ 6.7 (d, J = 5.8 Hz, H-3) and 6.6 (d, J = 5.9 Hz, H-4) and two doublets in an AB system at δ5.3 (d, J = 13.7 Hz, H-1a) and 5.4 (d, J = 13.8 Hz, H- 1b) on the pyran ring.

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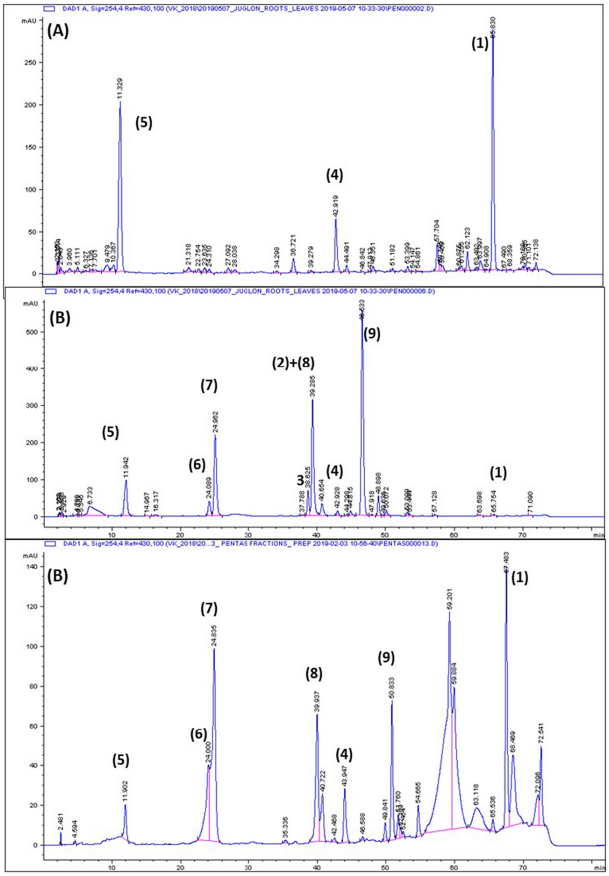
Figure 1. *HPLC profile of MeOH extract of roots (A) and MeOH extract of leaves (B) and EtOAc extract of leaves (C), collected at 254 nm: pentalongin (1), routine (2) luteolin-7-rutinoside (3), psychorubrin (4), harounoside (5), clarinoside (6), pentalonginoside B (7), pentalonginoside A (8), pentalonginoside C (9).*



Two anomeric protons at δ 4.7/ 4.8 ppm (J = 5 Hz) suggested the presence of two sugar moieties. Further analysis of ^{1}H and ^{13}C NMR data suggested the occurrence of one hexose and one desoxyhexose for compound (7) and of two desoxyhexoses for compound (8) and (9) (Fig. 2), with the latter one bearing an acetyl moiety bonded to one of the two desoxyhexoses. Desoxyhexoses were clearly identified by their methyl signals around one ppm connected by COSY and HMBC correlations with the sugar signals.

For compound (7), the sugar moieties were identified as β -D-glucose and β-D-quinovose (6-desoxyglucose), based on their NMR chemical shifts and coupling constants, and comparison with clarinoside (6) and harounoside (5) (Audoin et al., 2018), particularly the chemical shifts of their anomeric protons around 4.7 ppm with a coupling constant of about 5 Hz. The glucopyranosyl was attached to C-10 due to the HMBC correlation between H-1'' and C-10. Accordingly, the quinovose moiety was attached at C-5. This was further confirmed by the long-range correlation of H-1‘ with C-5 (Table 1). After putting everything together, compound (7) was found to be the analog of clarinoside (6) with inversion of the glucose and quinovose moieties and was named pentalonginoside B (Fig. 2). The elucidated structure was consistent with the MS spectrum (Fig. S 22-S23; Supplementary Material) obtained during MS experience with LC-ESI-MSMS. The base peak of 7 at m/z 567.17138 corresponds to C26H31O14 (calc. 567.17146), which is equivalent to [M +

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HCOOH-H]$^-$; the molecular ion peak [MH]$^-$ ($C_{25}H_{29}O_{12}$) and its dimer ion [2 M-H]$^-$ ($C_{50}H_{59}O_{24}$) stand at m/z values of 521.11608 (calc. 521.16597) and 1043.33918 (calc. 1043.33976), respectively. Therefore, molecular ion mass suggested the formula $C_{25}H_{30}O_{12}$ with 11 double bond equivalents (DBE). The peak at m/z of 359.11308 corresponds to the loss of glucose moiety by [M-H]$^-$ resulting from cleavage of O—C bond leaving oxygen to the naphtoquinone ring (aglycone part) and a peak at m/z 212.04762 resulting from the loss of a deoxyhexose and glucose moieties following the same cleavage as above.

Table 1. *NMR data for compounds 7, 8 and 9.*

	Pentalonginoside C (9)					Pentalonginoside A (8)				Pentalonginoside B (7)				
N°	Atom type	δ_h (mult., J (Hz)	δ_c (ppm)	COSY	HMBC C→H	δ_H (mult., J(Hz	δ_c (ppm)	COSY	HMBC C→H	Atom type	δ_H (mult., J (Hz	δ_c (ppm)	COSY	HMBC C→H
1a	CH2	5.3, d (13.7),	65.2	-	3, 10A, 10	5.3, d (13.8)	65.2	-	3,4A 10, 10A	CH_2	5.3, d(13.9)	65.4	-	3, 4A, 10, 10A
1b	CH_2	5.3, d (13.8),	65.2		3, 10A, 10,	5.3, d (13.8)	65.2	-	3, 10, 10A	CH_2	5.4, d(13.9)	65.4	-	3, 4A, 10, 10A
3	CH	6.7, d(5.8)	147.7	-	1b, 4,4A	6.7, d(5.9)	147.8	-	1b, 4,4A	CH	6.7, d(5.8)	147.7	-	1b, 4, 4A
4	CH	6.6, d(5.9)	102.1	-	3, 5, 10A	6.6, d(5.9)	102	-	3, 5, 10A	CH	6.6, d(5.9)	102.1	-	3, 5, 10A
4A	C	-	121.6	-	-	-	121.6	-	-	C	-	121.6	-	-
5	C	-	143.3	-	-	-	143.2	-	-	C	-	143.3	-	-
5A	C		131.1	-			131.1			C	-	131.1	-	
6	CH	8.4, m	124.8	7	5, 8, 9A	8.4, m	124.7	7	5, 9A	CH	8.4, m	124.8	7	5, 9A
7	CH	7.4, m	126.9	6	5A, 6, 9	7.4, m	126.8	6	5A, 9	CH	7.4, m	126.8	6	5A, 9
8	CH	7.4, m	126.4	9	6, 9A, 9	7.4, m	126.2	9	6, 9A	CH	7.4, m	126.3	9	6, 9A
9	CH	8.4, m	123.6	8	5A, 10	8.4, m	123.7	8	5A, 10	CH	8.4, m	123.6	8	5A, 10
9A	C	-	129.1		6,8	-	129.1	-	6, 8	C	-	129.0	-	6, 8
10	C	-	144.7	-	1'', 1a, 1b, 9	-	144.9	-	1'', 1a, 1b, 9	C	-	145.0		1'', 1a, 1b, 9
10A	C	-	122.6	-		-	122.6	-	4	C	-	123.6	-	1a, 1b, 4
11	C	-	172.7	-	-	-	-	-	-	-	-	-	-	-
12	C	2.2, s	21.1	-	11	-	-	-	-	-	-	-	-	-
1'	CH	4.7, d(7.8),	106.6	2'	5	4.7, d(7.8)	106.7	2'	5	CH	4.7, d(7.9)	106.7	2'	5, 5'
2'	CH	3.6, dd	76.1	1', 3'	1', 3'	3.6, m	76	1'	4', 1'	CH	3.6, m	75.7	1'	1', 3'
3'	CH	3.4,m	77.8	2', 4'	4'	3.4, m	77.7	4'	2'	CH	3.4, m	78	4'	2'
4'	CH	3.1, m	77.1	3'	3',5'	3.1, m	77	3'	5'	CH	3.1, d(2.8)	77.1	3'	3', 5', 6'
5'	CH	3.1, m	73.5	6'	6'	3.1, m	73.5	6'	6'	CH	3.1, d(2.8)	73.4	6'	6'
6'	CH_3	1.2, d(5.6)	18	5'	4', 5'	1.2, d(5.5)	18.0	5'	4', 5'	CH_3	1.2, ov	18.0	5'	1', 2', 4', 5'
1"	CH	4.8, d(7.8),	105.9	2"	10	4.7, d(7.8)	106.2	2"	10	CH	4.7, d(7.9)	106.4	2"	5'', 10
2"	CH	3.8, dd	74.2	1'', 3"	1'', 3"	3.6, m	76.1	1"	4'', 1"	CH	3.6, m	76.1	1'', 3"	1'', 3"
3"	CH	5.0, m	78.7	2'', 4"	4'', 11	3.4, m	77.8	4"	2"	CH	3.1, d(6.0)	77.8	2"	2"
4"	CH	3.2, m	75.1	3"	3'', 5''	3.1, m	77.1	3"	5"	CH	3.4, m	71.5	3"	4''6"
5"	CH	3.2, m	73.3	6"	6"	3.1, m	73.5	6"	6"	CH	3.4, m	78	6"	6''
6"	CH_3	1.2, d(5.5)	18.0	5"	5"	1.2, d(5.5)	18.1	5"	4'',5''	CHa CHb	3.7, m 3.7, dd (11.8,	62.7 62.7	6"b 6"a	4"

Compound (8) was also an analog of clarinoside (6) with two quinovose moieties. This was confirmed by the presence of two methyl doublets at δ1.15 (J = 5.5 Hz) and 1.21 (J = 5.5 Hz) correlating in the HMBC spectrum with C-4'' and C-5" and C-4' and C-5' (Table 1), and by the comparison of the spectral data of compound (8) (Fig. S 35-S42; Supplementary Material) with clarinoside (6) spectral data.

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Thus, compound (8) was also determined as a new naphto-pyran to which the trivial name pentalonginoside A (8) was given. Like compound (7), compound (8) had formic acid as adduct, [M + HCOOH-H,]$^-$ (C26H31O13), that gave rise to a peak at m/z 551.17645 (calc. 551.17654), while the parent ion, [M-H]$^-$ ($C_{25}H_{29}O_{11}$) stands at m/z 505.1715 (calc. 505.17105). Therefore, the molecular formula for compound (8) is C25H30O11 and presents with 11 DBE. The loss of deoxyhexose moiety through O—C cleavage that leaves oxygen to the aglycone part explains the presence of an ion peak at m/z 359.11313 (Fig. S 41-S 42, Supplementary Material).

For compound (9), the ^{1}H, ^{13}C, and HMBC NMR spectra revealed resonances and ^{2}JCH and ^{3}JCH correlation consistent with those of an acetate moiety (δ_C 172.7 and 21.1 for C-11 and C-12 and δ_H 2.2 (singlet, 3H) for CH_3-12) attached to C-3'' of one quinovose bonded to C-10 of the aglycone (Table 1). Thus, compound (9) was also determined as a new naphto-pyran to which the trivial name pentalonginoside C (9) (Fig. 2) was given. For this compound (9), the base peak was observed at m/z 593.18716 (calc. 593.1871) and corresponded to compound (9) with an adduct of formic acid, [M + HCOOH—H]$^-$ (C28H33O14) while the molecular ion peak, [M—H] $^-$($C_{27}H_{31}O_{12}$) and its dimer, [2 M—H]$^-$ (C54H61O24) were observed at m/z 547.18206 (calc. 547.18162) and 1095.37030 (calc. 1095. 36,504), respectively. Therefore, the molecular formula was found to be $C_{27}H_{30}O_{12}$ and suggested 12 DBE. The fragment at m/z 212.04762 corresponded to the loss of two substituents of naphtoquinone ring (the aglycone part) following the above mechanism, while the fragment at *m/z* 359.11315 and 401.12357 resulted from the loss of acetylated and non-acetylated deoxyhexose moieties at the same position as above by the parent ion (Fig. S 31-S 32, Supplementary Material).

3.3. CHROMATOGRAPHIC COMPARISON OF LEAVES AND ROOTS EXTRACTS

According to Fig. 1, harounoside (5) and psychorubrin (4) are the only common major compounds in MeOH extract of roots and MeOH extract of leaves. Psychorubrin (4) was isolated for the first time from *Psychotria rubra* [26] and is active against Gram-positive bacteria, with greater activity against the methicillin-resistant species (MRSA), *Staphylococcus aureus* 33,591 and 33,592 and *Staphylococcus pyogenes* 10,096 [27], antitumor, antibiotic and antileishmanial properties [28]. Harounoside (5) was isolated from *Mitracarpus scaber*, an annual plant used in African traditional medicine endowed with antifungal, antimicrobial, and anti-inflammatory properties [29]. Later on, clarinoside (6) was also isolated by Laboratoires Clarins, France, from the same plant, and biological tests confirmed the anti-inflammatory activity of both harounoside (5) and clarinoside (6) [30].

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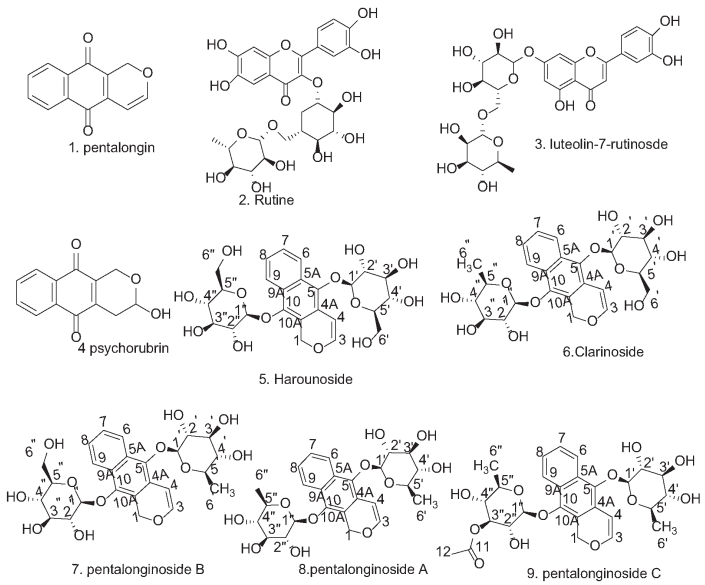
Figure 2. *Chemical structure of identified and isolated compounds from extracts of leaves of P. longiflora.*



In addition to those compounds, MeOH extracts and EtOAc of leaves of *P. longiflora* were found to be rich in other three pentalongin glycosides isolated from EtOAc extract of the same type: pentalonginoside A (8), pentalonginoside B (7), and pentalonginoside C (9), which are either in traces or entirely absent in roots. In addition, the MeOH extract contains rutin, luteolin-7-rutinoside, and phenolic acid (Fig. S54; Supplementary Material and Fig. 1).

Moreover, the analysis of EtOAc leaves extract dissolved in MeOH revealed the presence of other compounds in the leaves that were not detected in MeOH extract, including pentalongin (1). The presence of pentalongin (1) in EtOAC extract may explain why leaves have been reported by many Rwandans to possess the activity against PV [26]. The predominance of pentalongin derivatives (4-9) in the leaves raised another hypothesis about their possible contribution to the activity.

Furthermore, following the WHO guidelines for selecting marker substances of herbs [31]; the therapeutic molecule (pentalongin (1)), psychorubrin (4), and harounoside (5), were selected as makers for MeOH roots extract. Those compounds are not found in any other *Pentas* species, therefore, could be relied on to identify the plant.

3.4. VALIDATION OF ANALYTICAL METHODS

The aim was to validate the analytical method able to quantify pentalongin (1) from roots extracts. During the preliminary works, the reported degradation of naphtoquinones in alcoholic solvent was observed. Pentalongin (1) underwent degradation to give many peaks [9]. However, pentalongin (1) was found to be very stable in CH_3N at room temperature during a stability study that covered a period of three days.

Before validation, a calibration standard was identified. In this regard, four standards of naphto- and anthraquinones were tested. Arbutoside and aloin interfered with the peaks from the roots

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sample. In addition, the degradation of aloin in MeOH reported in other studies was also observed [32]. Sennoside presented an important tailing peak. Only juglone gave a symmetrical peak standing at the position in the chromatogram without any other peak for all collected samples. In addition, juglone and pentalongin (1) absorb at the same maximum wavelength and share the same phytochemical class. Therefore, juglone was selected for validation.

The selectivity of the method was verified by collecting and comparing the UV spectra at the beginning, apex, and at the end of both pentalongin (1) and juglone chromatographic peaks, and none of them revealed any interfering compound.

During the data analysis, different regression models were tested, including the linear (simple and weighted), the quadratic (simple and weighted), and the transformed (roots square and logarithmic), to find out the best-fitted model that can ensure the reliability of results within the selected range of analysis. Only the linear regression through 0 and the highest concentration level (level 5) presented the highest accuracy indexes of 0.8639 and 0.8529 within and out of the matrix, respectively, and all over the dosing range. This can be observed by the relative P-expectation tolerance limits which were included within the acceptance limits, as shown in Fig. 3. The closeness between the accuracy indexes indicates that results obtained in and out of the matrix are almost the same [33].

Thus, the trueness of the method, which expresses the closeness of agreement between a conventionally accepted value or reference value and a mean experimental [34], was evaluated. The absolute bias (in μg/ mL) and relative bias (%) presented (Table 2) for each concentration level of the validation standards shows the consistency of this parameter between different repetitions.

In addition, the assessment of precision, which is a validation parameter describing the closeness of agreement among measurements from multiple sampling of a homogeneous sample under the recommended conditions [35], provided good results for both repeatability and intermediate precision as their relative standard deviation (RSD) values were less than 2.2%. This shows that the random errors were negligible during the validation process [36]. Therefore, the method can be reproduced routinely.

Furthermore, the accuracy evaluation using the accuracy profile (Fig. 3) showed that the total errors (systematic error and random error) were very low as the β-expectation tolerance limits were within acceptable limits set at ±10%. This guarantees that each further measurement of unknown samples is included within the tolerance limits at the 5.0% level. Thus, the method presents the guarantee for accurate results between 14.13 μg/mL and 212 μg/mL. In addition, the risk profile was far below the acceptance limits, which shows that the probability of generating results that are out of the acceptance limit is very low [37].

The application of the validated method to collected samples showed an important variation in metabolite contents (Fig. 4). Pentalongin (1), the active molecule, was among the major varying compounds; it was the minor compound in the Musanze city sample while in other samples, it was the major compound. The highest concentrations in pentalongin (1) (70.0 ± 17.0 mg/100 g) were recorded in cultivated samples from the Mukoni sampling site followed by samples from the

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Rubavu sampling site (31.8 ± 12.2 mg/100 g) and Rusizi sampling site (9.3 ± 3.8 mg/100 g). The lowest concentrations, 1.7 ± 0.9 mg/100 g and 2.8 ± 0.7 mg/100 g, were recorded from the samples collected at Saruheshyi and Musanze city sampling sites, respectively. For the Musanze city sampling site, pentalongin (1) was below the limit of quantification of the method. ANOVA test showed that there was a significant difference in the mean concentrations between Mukoni, Rubavu, and Rusizi sampling sites ($p < 0.05$). The results of the multiple comparisons showed that the concentrations in pentalongin (1) in domesticated plants were very high compared to those in samples from the other sites.

Table 2. *Method validation parameters for the quantification of pentalongin.*

Response function	y = 31.47x (with p = 3 and n = 3)	
Linear regression	Through 0 and level 5	
Trueness	Absolute bias (µg/mL)	Relative bias (%)
Level 1	- 0.17	- 1.18
Level 2	0.23	0.80
Level 3	0.087	0.12
Level 4	1.44	1.02
Level 5	1.66	0.79
Precision	**Repeatability (% RSD)**	**Intermediate precision (%RSD)**
Level 1	1.93	1.93
Level 2	1.41	2.17
Level 3	0.95	1.44
Level 4	0.83	0.83
Level 5	0.74	1.00
Accuracy	Relative β-expectation lower and upper tolerance limits (%)within and out of matrix respectively	
Level 1	[- 5.89, 3.53] [- 6.154, 2.25]	
Level 2	[- 6.14, 7.74] [- 8.055, 8.09]	
Level 3	[- 4.56, 4.80] [- 3.40, 2.31]	
Level 4	[- 1.02, 3.06] [- 2.86, 3.33]	
Level 5	[- 2.18, 3.75] [- 1.55, 1.55]	
LOD = 4.28 µg/mL and LOQ = 14.13 µg/mL		
Linearity	Y = - 0.24 + 1.01 X	
Concentration range	14.13 µg/mL to 212.00 µg/mL (that is the ULOQ)	

p = number of series; n = number of repetitions.
Level 1 = 14.0 µµg/mL, Level 2 = 28 µg/mL, Level 3 = 70 µg/mL, Level 4 = 140 µg/mL, Level 5 = 210 µg/mL; LOD: limit of detection; LLOQ: lower limit of quantification; ULOQ: upper limit of quantification.

4. Conclusions

This study led to the isolation and identification of three new pentalongin glycosides and of other known compounds from EtOAc extract of leaves of *P. longiflora*. The presence of a low quantity of pentalongin (1) and its derivatives in leaves of P. longiflora may explain the traditional use of the leaves in the treatment of PV. In addition, an HPLC-UV method for the quality control of roots of *P. longiflora* was developed and validated. It allows the quantitation of pentalongin (1) expressed in juglone from 14.0 to 212 µg/mL using a linear model. Cultivated plants are richer in pentalongin (1) than wild plants sampled in different areas. This important variation of pentalongin (1) concentrations according to sampling sites shows that, to guarantee equivalent efficacy, finished products with P. longiflora should be standardized based on their pentalongin (1) content.

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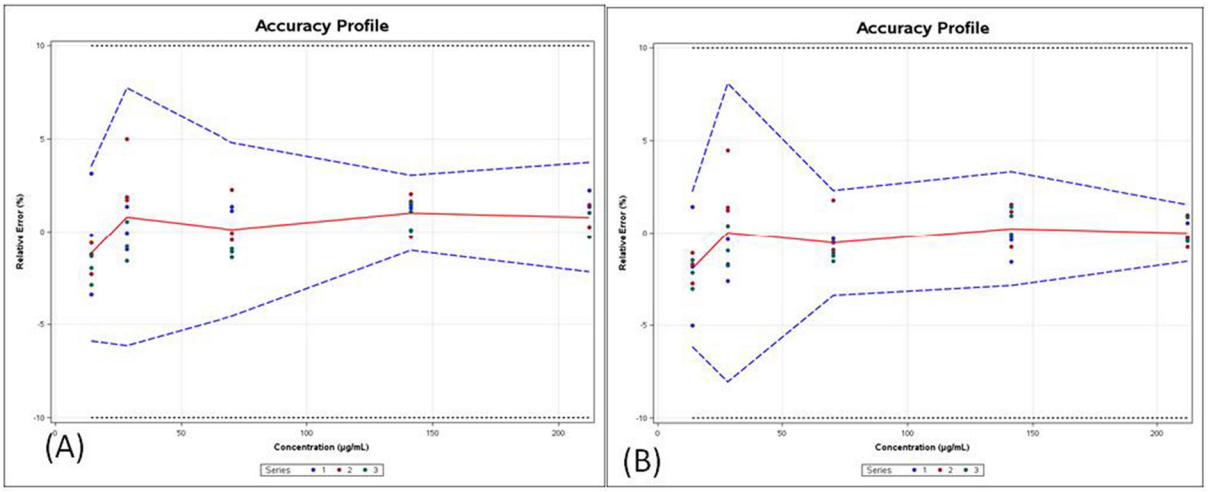
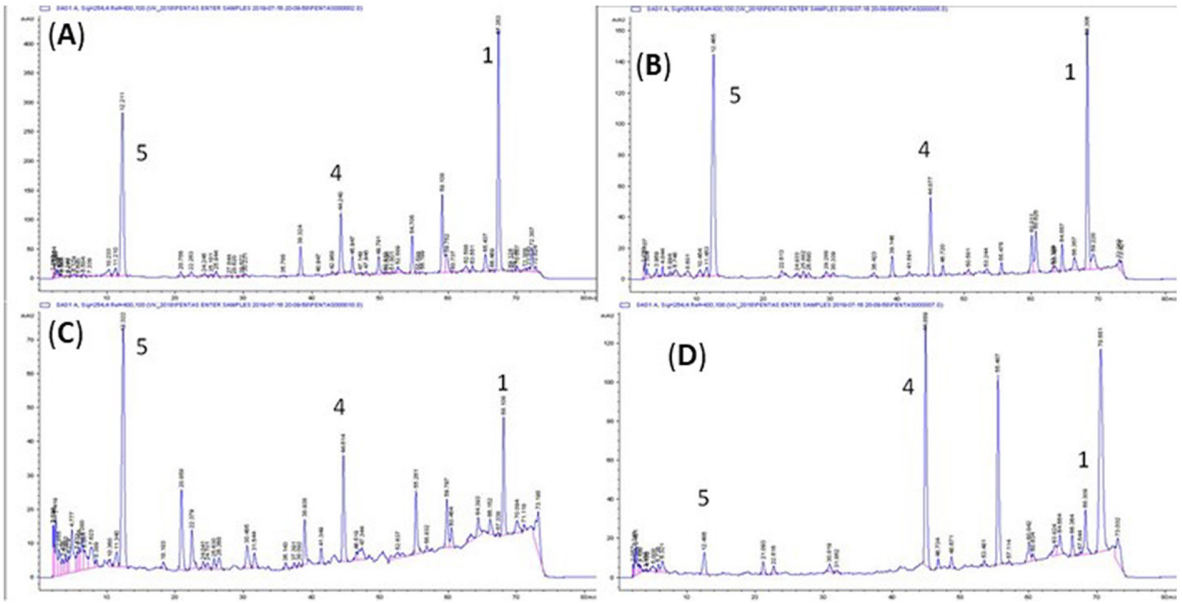
Figure 3. *Accuracy profile obtained by considering linear regression through 0 using the highest level only (5) without (Fig. 3A) and within matrix (Fig.3B).*



Legend: The plain red line is the relative bias, the dashed blue lines are the β-expectation tolerance limits, and the dashed black lines represent the acceptance limits.
The dots represent the relative error of the back-calculated concentrations and are plotted with respect to their targeted concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Figure 4. *HPLC chromatogram of methanol extracts root of P. longiflora collected from: (A) Mukoni (March 2018), (B) Rubavu (December 2019), (C) Rusizi (March 2018), (D) Musanze (March 2018), harounoside (5), psychorubrin (4) and pentalongin (1).*



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Authors' contribution

Conception and Design: MF MR and RMD. Acquisition of Data: VK, NR, AL, JCT, OJ and AN. Analysis and Interpretation of Data: VK, MF, AC, MR and MRB. Drafting the Manuscript: VK, MF and AC. Revising for Intellectual Content: VK, MF, RM, RMD, OJ, AC. Final Approval of the Completed Article: MF, MRD and MR.

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Declaration of Competing Interest

The authors declare that they have no financial or personal relationships that may have inappropriately influenced them in writing this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2021.104974.

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