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HPTLC fingerprinting of *Croton gratissimus* leaf extract with Preparative HPLC-MS-isolated marker compounds



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ABSTRACT

The leaves, root and bark of the aromatic African indigenous plant, *Croton gratissimus* Burch. (Euphorbiaceae), are widely used in traditional medicine to treat coughs, chest complaints, rheumatism, abdominal disorders and fever among others. In Afrikaans it is referred to as “Koorsbessie” which alludes to its traditional use as a pyrogenic. The chemical composition of plants is very complex and analysis and quality control can be very challenging due to natural variability. In addition, very few reference standards from plants, especially from Africa, are commercially available. Due to its visual nature and the holistic fingerprint produced, high performance thin layer chromatography (HPTLC) is often recommended for the quality control of plant material. The aim of this study was to develop an HPTLC fingerprint and isolate marker compounds for inclusion on HPTLC plates to enable quality control. *Croton gratissimus* leaf samples were collected from various parts of South Africa and extraction was optimised. HPTLC fingerprints were developed and optimised and images were captured before and after derivatisation under UV (254 nm, and 366 nm) and white light. Preparative high performance liquid chromatography hyphenated to mass spectrometry (HPLC-MS) was used to isolate marker compounds. Method development and optimisation determined the following: most efficient extraction solvent = methanol:water (8:2 v/v); mobile phase = ethyl acetate:acetic acid:formic acid:water (100:11:11:27 v/v/v/v); and derivatising agent = natural product reagent. UPLC-MS analysis and 1D NMR spectroscopy were used to characterise and identify compound 1 as isoorientin and compound 2 as kaempferol-3-β-D-(6''-O-trans-p-coumaroyl) glucopyranoside, which correlated well with published spectral data. The final HPTLC fingerprint with biomarkers included showed good separation for profiling purposes and well-defined bands. The biomarkers at retention factor (Rf) 0.30 and Rf 0.69 for isoorientin and kaempferol-3-β-D-(6''-O-trans-p-coumaroyl) glucopyranoside, respectively, were present in all samples but varied quantitatively. The HPTLC method developed provided a good fingerprint for species authentication. Preparative HPLC-MS played a major role in successfully isolating marker compounds to be used for the quality control of *C. gratissimus*.

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1. Introduction

Croton is derived from the Greek word *Kroton* meaning “tick” while *gratissimus* is a Latin word meaning “most pleasant” or pleasing; an appropriate name for this tree which is very aromatic, especially when the leaves are crushed. Small cream to golden-yellow flowers is produced in autumn, which yields small fruit capsules. The tree occurs naturally over a large area (North-West Province, Gauteng, Mpumalanga, northern KwaZulu-Natal, Northern Cape, Northern Province) of South Africa (van Wyk et al., 1997; Plantzafrica, 2017). *Croton gratissimus* Burch. (Euphorbiaceae) is commonly used as a traditional medicine. Leaf

infusions are used to treat coughs and reduce fever (Hutchings et al., 1996; van Wyk et al., 1997). The roots are used as purgatives and enemas (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996; van Wyk et al., 1997). Root decoctions have been used to treat chest complaints, coughs, fever and sexually transmitted diseases such as syphilis (Von Koenen, 2001). The bark is most frequently used to treat bleeding gums, abdominal disorders, skin inflammation, ear ache and chest complaints (Hutchings et al., 1996; Von Koenen, 2001). The combinations of roots and bark of *C. gratissimus* in the treatment of respiratory disorders have also been reported when co-administered with other species (van Vuuren and Viljoen, 2008). The major classes of phytochemicals isolated from *C. gratissimus* include terpenoids, alkaloids and flavonoids (Salatino et al., 2007).

Herbal medicines always present a challenge to quality control due to its innate complexity and variability. In some cases identification

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and quality control are performed with few or no characteristic marker compounds; hence, analytical tools which reflect the entire chemical composition are preferable. Chromatographic fingerprinting of herbal medicines has become one of the most commanding approaches to quality control and is accepted by various regulatory authorities and organisations. High performance thin layer chromatography (HPTLC) is such a tool that offers fingerprinting for the identification and characterisation of herbal medicines and products. This established tool offers several advantages; it presents results as colourful images, documents multiple profiles together on one image and it can be fully automated. This technique has gained popularity among various analytical fingerprinting techniques for the quality control of traditional medicines (Fan et al., 2006; Ankli et al., 2008).

Chemical markers are vital in the current practice of quality control. The lack of chemical markers and many technical challenges in its isolation severely hampers the quality control of herbal medicines. Hyphenated techniques, originating from the coupling between HPLC and MS have become requisite tools for the isolation of chemical markers (Li et al., 2008). Preparative HPLC has been used for high-throughput purification of the target compounds from herbal medicines. The aim of this study was to develop an HPTLC fingerprint that can be used for identification purposes and to isolate marker compounds for inclusion on HPTLC plates to enable effective quality control.

2. Material and methods

2.1. Plant material and extraction

The aerial parts (leaves) of *C. gratissimus* ($n = 70$) were collected from Limpopo, Mpumalanga and Pretoria, identified, and voucher specimens were deposited in the Department of Pharmaceutical Sciences, Faculty of Science, Tshwane University of Technology, Pretoria, South Africa. The plant material was dried and the leaves were pulverised into fine powder using a ball mill (Retsch GmbH, Haan, Germany). Seven representative samples (TUT0000721–TUT0000727) were selected for HPTLC analysis and 0.5 g of each sample was sonicated for 10 min with 10 mL of the following solvents: heptane, toluene, dichloromethane, chloroform, acetone, ethanol, methanol, water, ethanol:water (7:3 v/v), methanol:water (8:2 v/v), methanol:acetic acid (9:1 v/v), water:acetic acid (9:1 v/v), methanol:ammonia 25% (8:2 v/v), water:ammonia 25% (8:2 v/v) to determine the best extraction solvent for HPTLC analysis. For Preparative HPLC-MS, 250 g were weighed and extracted three times to maximise the yield with 1.0 L of methanol:water (80:20 v/v). The extract was filtered using Whatman (No.1) filter paper after each interval. Filtrates were collected in conical flasks and evaporated *in vacuo* using a rotavapor (Büchi Rotavapor R-215) at 40 °C to remove residual solvent and lyophilised using a freeze dryer to remove water residue. The yield of hydro alcoholic extract was 8.13% as brown mass.

2.2. Isolation of marker compounds using Preparative HPLC-MS

Isolation of marker compounds was performed on a Waters chromatographic system with Waters PDA (2998) and MS detector (Waters, Milford, MA, USA). The extract (100 mg) was diluted to 2.5 mL with methanol, and the solution was filtered through a 0.22 µm membrane (Millipore). To achieve chromatograms with better resolution in a short analysis time, the chromatographic conditions were optimised. Separation was achieved on an XBridge Preparative C₁₈ column (19 × 250 mm, i.d., 5 µm particle size, Waters) maintained at 40 °C. The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) at a flow rate of 20 mL/min; gradient elution was applied as follows: Initial ratio 80% A: 20% B, keeping for 1 min, changed to 60% A: 40% B in 2 min, changed to 47% A: 53% B in 9 min, to 5% A: 95% B in 0.5 min, maintaining for 0.5 min and back to initial ratio in 0.5 min. The total run time was 14 min. The injection volume was 500 µL. Data

were collected using MassLynx 4.1™ (Waters, USA) software. The Preparative HPLC system was interfaced with a QDa mass spectrometer. Negative ion mode was selected. The probe temperature was set at 600 °C. The source temperature was 120 °C. The capillary and cone voltages were set to 800 and 10 V, respectively. Data were collected between 100 and 650 *m/z*. The eluents were fractionated into 250 drops/tube (about 2.5 mL) using a fraction collector. The target compounds were collected in various fractions, subsequently combined, and concentrated to give residues, which were analysed by UPLC-MS.

2.3. UPLC-MS

The residues were analysed with a Waters Acquity Ultra Performance Liquid Chromatographic system with PDA detector (Waters, Milford, MA, USA). Separation was achieved on an Acquity UPLC BEH C₁₈ column (150 mm × 2.1 mm, i.d., 1.7 µm particle size, Waters) maintained at 40 °C. Some preliminary analyses were performed prior to setting the chromatographic conditions to obtain chromatograms with better resolution and a short analysis time. The mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.3 mL/min. The gradient elution was executed as follows: initial ratio was 90% A: 10% B, changed to 50% A: 50% B in 3 min, to 10% A: 90% B in 9 min, keeping for 2 min and back to initial ratio in 1 min, equilibrate the system for 3 min. The total run time was 20 min and the injection volume was 1.0 µL (full-loop injection). The positive and negative ion modes were examined and the negative ion mode provided results with more information and higher sensitivity. Thus, the mass spectrometer was operated in negative ion electrospray mode and nitrogen (N₂) was used as the desolvation gas. Data were acquired between 100 and 1500 *m/z*. The following parameters were then set: capillary voltages were 2500 V; sampling cone voltages were 45 V; extraction cone voltage was 4 V; source temperature was 100 °C; desolvation temperature was 350 °C; desolvation gas flow was 500 L/h. MassLynx 4.1™ (Waters, USA) software was used to process and obtain all the chromatographic data.

2.4. NMR analysis

Structure elucidation of isolated compounds was carried out using UHPLC-MS and ¹H and ¹³C NMR spectroscopy. NMR spectra were acquired on a Bruker Fourier 600 MHz Avance II spectrometer. Chemical shifts are reported in ppm, referenced to residual solvent resonances (MeOD δ_H 3.31, δ_C 49.0 ppm).

2.5. High performance thin layer chromatography (HPTLC)

The CAMAG semi-automated HPTLC system including the automatic TLC Sampler 4, automatic developing chamber ADC2, chromatogram immersion device, TLC plate heater III, and Reprostar 3 documentation device, all controlled with wincats™ 1.4.1 planar chromatography software was used for HPTLC analysis. The dried extracts and isolated marker compounds were re-dissolved in methanol to a final concentration of 10 mg/mL and 1 mg/mL, respectively, and 2 µL of each sample was spray-applied with a 25 µL Hamilton microsyringe as 8 mm bands, 5 mm from the lower edge of 20 × 10 cm silica gel plates (Silica gel 60 F254, Merck, Germany). The best mobile phase was determined by investigating numerous systems of different polarities. Ascending development was carried out in a 20 × 10 × 4 cm glass twin-trough chamber (CAMAG) up to 75 mm, using the optimised mobile phase ethyl acetate:acetic acid:formic acid:water (100:11:11:27 v/v/v/v), after tank saturation for 20 min with 25 mL of the mobile phase at room temperature (25 ± 2 °C) and relative humidity (49 ± 5%). After development and drying for 5 min, the results were viewed and documented under UV light at 254 nm and 366 nm, derivatised with natural product reagent and viewed under white light.

3. Results and discussion

3.1. Isolation and identification of marker compounds

The target compounds were collected in various fractions, combined and concentrated to give residues, and the structures were elucidated using UPLC-MS, ^1H NMR and ^{13}C NMR spectroscopy and comparison with literature. According to UPLC-MS analysis, compound 1 ($m/z = 447.0947$, $[\text{M}-\text{H}]^-$) and compound 2 ($m/z = 593.1311$ $[\text{M}-\text{H}]^-$) were calculated as $\text{C}_{21}\text{H}_{20}\text{O}_{11}$ and $\text{C}_{30}\text{H}_{26}\text{O}_{13}$, respectively (Fig. 1). The ^1H NMR and ^{13}C NMR data (Table 1) are in agreement with published spectral data for isoorientin (Peng et al., 2005) and kaempferol-3- β -D-(6''-O-trans-p-coumaroyl) glucopyranoside (Tsukamoto et al., 2004; Aderogba et al., 2011). The yield of isoorientin (97.7% purity) and kaempferol-3- β -D-(6''-O-trans-p-coumaroyl) glucopyranoside (99.8% purity) was 12.3 and 15.9 mg, respectively. Flavonoids are ubiquitous in plants and they have received much attention due to their wide range of biological activities. Isoorientin is a flavonoid-C-glycoside that has been isolated from many plant species as well as major cereal crops including maize, wheat, rice and barley. The antiviral activity of isoorientin isolated from the leaves of *Lophatherum gracile* Brongn. (Bamboo leaf) against respiratory syncytial virus (RSV) was determined. Isoorientin exhibited significant anti-RSV activity ($\text{IC}_{50} = 5.7 \pm 1.2 \mu\text{g}/\text{mL}$) compared to the positive control (ribavirin) with an IC_{50} of $3.0 \pm 0.4 \mu\text{g}/\text{mL}$ (Wang et al., 2012). Isoorientin isolated from *Cecropia pachystachya* Trécul (Pumpwood) leaves exhibited moderate quorum sensing inhibition activity using *C. violaceum* and *E. coli* as biosensor models (Brango-Vanegas et al., 2014). Isoorientin isolated from the aerial parts of *Gentiana tenella* Rottb. (Dane's dwarf-gentian) and *G. azurea* Bunge exhibited anti-inflammatory activity through inhibition of the synthesis of thromboxane B_2 of 54.33%, 56.34% and 66.42% at 25, 50 and 100 $\mu\text{g}/\text{mL}$ without significant effect on leukotriene B_4 synthesis (Odontuya et al., 2005; Xiao et al., 2016). Isoorientin could also be beneficial to diabetes patients, as it has been shown to inhibit α -glucosidase. Isoorientin showed 38.22% enzyme inhibition at 25 μM compared to the positive control acarbose with 63.90% inhibition at 250 $\mu\text{g}/\text{mL}$ (Li et al., 2009). The group of Yuan et al. extensively investigated the anticancer activity and mechanisms of isoorientin. They found that it induces apoptosis in HepG2 cells with no toxicity to normal liver cells, through various pathways (Yuan et al., 2012, 2013, 2014). More recently, they investigated

Table 1

^1H and ^{13}C NMR chemical shift data for compounds isoorientin and kaempferol-3- β -D-(6''-O-trans-p-coumaroyl) glucopyranoside.

Isoorientin			Kaempferol-3- β -D-(6''-O-trans-p-coumaroyl) glucopyranoside		
Positions	δ H (J in Hz)	C	Positions	δ H (J in Hz)	C
2	–	166.5	2	–	161.2
3	6.56 s	104.1	3	–	135.2
4	–	184.2	4	–	180.9
5	–	162.2	5	–	163.4
6	–	109.3	6	6.13 s	100.0
7	–	164.9	7	–	166.1
8	6.50 s	95.2	8	6.31 s	94.8
9	–	158.9	9	–	158.5
10	–	105.3	10	–	104.0
1'	–	123.8	1'	–	122.7
2'	7.37 s	114.2	2'	7.98 (2H) d (8.7)	132.2
3'	–	151.2	3'	6.81 (2H) d (8.7)	116.1
4'	–	147.2	4'	–	161.5
5'	6.90 d (8.1)	116.9	1''	5.23 d (7.1)	103.9
6'	7.38 d (8.1)	120.5	2''	3.44 m	78.0
1''	4.90 d (9.9)	75.3	3''	3.44 m	75.7
2''	4.16 m	72.7	4''	3.32 m	71.7
3''	3.47 m	71.9	5''	3.44 m	75.8
4''	3.42 m	80.4	6''	4.18 dd (11.9, 6.7)	64.3
5''	3.42 m	82.9	6''	4.28 dd (11.9, 1.6)	–
6''	3.87 m	62.9	1'''	–	168.9
6''	3.73 dd (12.4, 5.4)	–	2'''	6.06 d (15.9)	114.7
			3'''	7.39 d (15.9)	146.5
			4'''	–	127.2
			5'''	7.30 (2H) d (8.4)	131.2
			6'''	6.79 (2H) d (8.4)	116.8
			7'''	–	161.9

NMR spectra were acquired on a Bruker Fourier 600 MHz Avance II spectrometer.

the hepatoprotective effect of isoorientin. It was determined that it prevents hyperlipidaemia and acts as hepatoprotectant through regulation of lipid metabolism, antioxidant activity and regulation of the secretion of inflammatory cytokines (Yuan et al., 2016). The glycoside isolated in this study, kaempferol-3- β -D-(6''-O-trans-p-coumaroyl) glucopyranoside (syn. tiliroside), has also been shown to have an effect on the liver. After isolation from *Fragaria ananassa* Duchesne (strawberry), this compound was shown to inhibit the activity of the drug

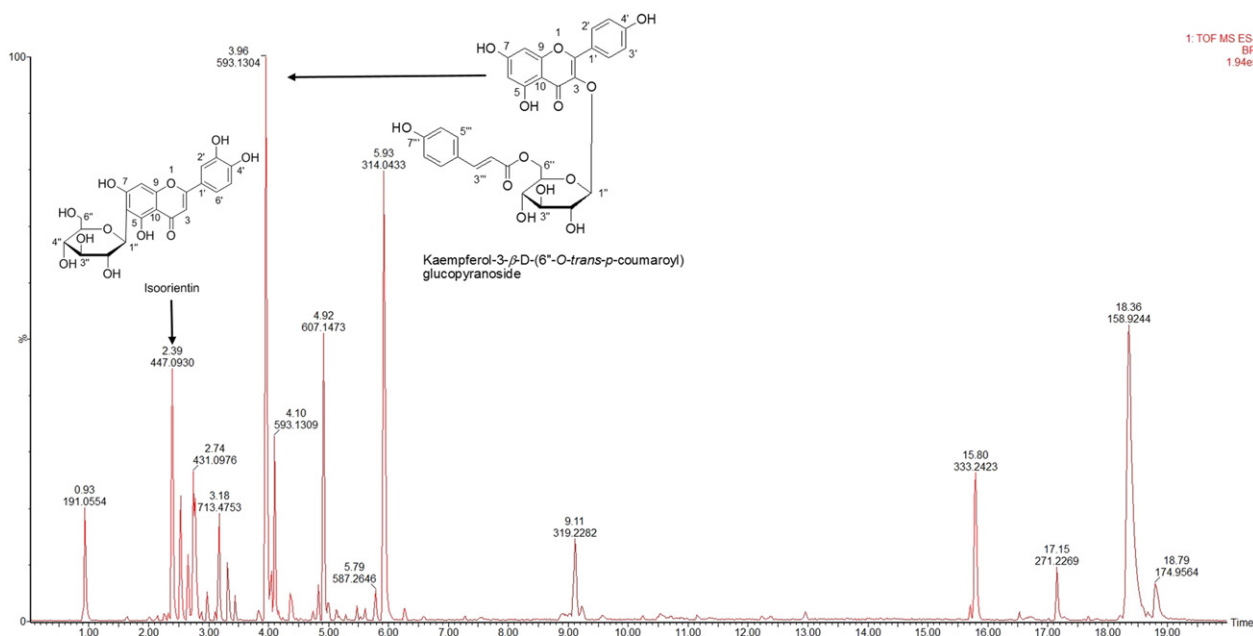


Fig. 1. LC-MS chromatogram of the *C. gratissimus* hydroalcoholic leaf extract indicating the isolated compounds isoorientin and kaempferol-3- β -D-(6''-O-trans-p-coumaroyl) glucopyranoside.

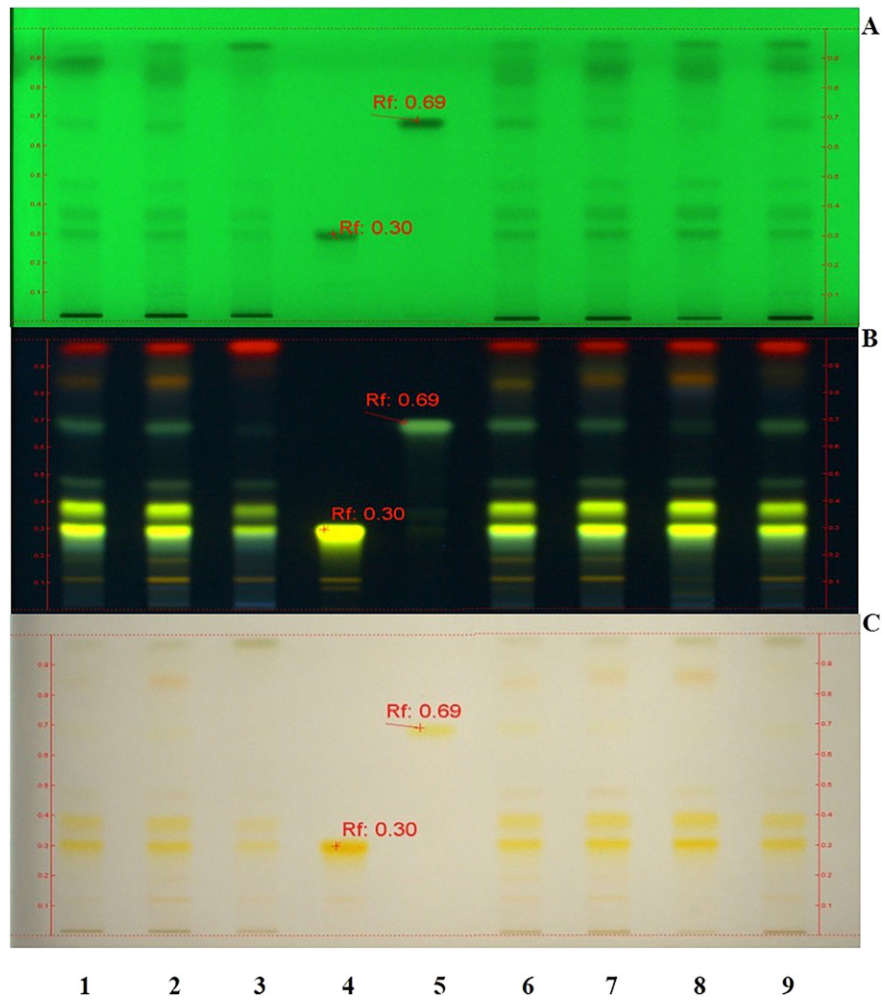


Fig. 2. HPTLC fingerprint of *C. gratissimus* leaf extract (A) under UV 254 nm before derivatisation (B), under 366 nm after derivatisation and (C), under white light after derivatisation. The selected leaf extracts are spotted in Tracks 1–3 and 6–9 and the isolated compounds isoorientin (Track 4; Rf 0.30) and kaempferol-3-β-D-(6''-O-trans-p-coumaroyl) glucopyranoside (Track 5; Rf 0.69) are indicated.

metabolising enzyme CYP3A4 in the cytochrome P450 system (Tsukamoto et al., 2004). Tiliroside isolated from *Agrimonia pilosa* Ledeb. (hairy agrimony) significantly inhibited acetylcholinesterase with an IC₅₀ of 23.5 μM compared to an IC₅₀ value of 37.8 μM for the positive control, dehydroevodiamine. Aderogba et al. isolated antioxidant constituents from *Croton zambesicus* Müll.Arg (Lavender croton; syn. *Croton gratissimus*) leaf extracts. They suggested that the compounds isolated, including kaempferol-3-β-D-(6''-O-trans-p-coumaroyl) glucopyranoside, could provide a rationale for the ethnomedicinal use of this plant in the management of oxidative-stress-related diseases such as diabetes and hypertension. *In vitro* qualitative tests which indicated antioxidant activity were followed up with quantitative tests which determined that kaempferol-3-β-D-(6''-O-trans-p-coumaroyl) glucopyranoside, also isolated in this study, possessed weak antioxidant activity compared to quercetin and rutin. The cytotoxicity for this compound against Vero cells was low (Aderogba et al., 2011).

3.2. HPTLC analysis

Fingerprinting is a useful tool for the quality control of herbal products (Xie et al., 2006) and HPTLC fingerprinting provides an objective source to compare and identify substances (Reich and Schibli, 2006). Fig. 2 shows the HPTLC fingerprint of *C. gratissimus* leaf extract (A) under UV 254 nm before derivatisation (B), under 366 nm after derivatisation and (C), under white light after derivatisation. Track 1 represents the first leaf extract (Nelspruit), Track 2 (Location 1,

Limpopo), Track 3 (Location 2, Limpopo), Track 4 (Compound 1: isoorientin), Track 5 (Compound 2: kaempferol-3-β-D-(6''-O-trans-p-coumaroyl) glucopyranoside), Track 6 (Location 1, Limpopo), Track 7 (Location 2, Limpopo), Track 8 (SANBI, Pretoria), and Track 9 (Location 2, Limpopo). The HPTLC plates show a consistent profile for the majority of samples. The best fingerprint for *C. gratissimus* is represented by Fig. 2B when viewed under 366 nm. Table 2 shows the fingerprint that can be used for comparison based on Rf values and band colour for this plate.

Table 2
HPTLC fingerprint under UV light at 366 nm with band Rf values and colour.

Band	Retention factor (Rf)	Colour
1	0.12	Yellow-orange
2	0.19	Yellow-orange
3	0.30 ^a	Yellow
4	0.36	Yellow
5	0.47	Green
6	0.69 ^b	Green
7	0.85	Orange

^a Isoorientin.

^b Kaempferol-3-β-D-(6''-O-trans-p-coumaroyl) glucopyranoside.

4. Conclusion

The HPTLC method developed provided a good fingerprint for species authentication by direct band comparison and can clearly confirm the presence of isoorientin and kaempferol-3- β -D-(6''-O-trans-p-coumaroyl) glucopyranoside with the extraction protocol and mobile phase developed. A fast and efficient Preparative HPLC-MS method was developed for the first time for the simultaneous and rapid separation and isolation of marker compounds *C. gratissimus*.

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