



Quality Control and Anti-Inflammatory Activity of the Stem Bark of *Chlorophora regia* A. Chev. (Moraceae)

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Abstract

This study sought to develop a validated reverse-phase high-performance liquid chromatography method for the quality control of the stem bark ingredients and its finished products and investigate the synergistic anti-inflammatory activity of the phytochemical constituents of *C. regia* stem bark. Fractionation and isolation of biomarkers were carried out by column chromatography on silica gel and monitored by thin-layer chromatography. The isolated biomarkers were characterized based on their melting points and extensive analysis of their spectroscopic data (IR, 1D and 2D NMR). The chromatographic separation was investigated and developed for the analysis of the biomarkers using μ BondapakTM C18 (3.9×300 mm, 5 μ m) as stationary phase. The mobile phase composition of 0.1 % trifluoroacetic acid as solvent A, and methanol as solvent B with gradient elution was finally selected. The carrageenan-induced edema in a 7-day-old chick model was used to assess the anti-inflammatory activity of the stem bark extract and compared to diclofenac sodium as a reference drug. Two compounds were successfully isolated and identified, as Regiafuran A (**1**) and 3,5,7,4'-Tetrahydroxy-2'-methoxyflavonol (**2**). The compounds were employed as biomarkers in the RP-HPLC method development. The developed method was validated and was successfully used to quantify the amount of **1** and **2** in the stem bark to be $0.224\% \pm 0.056\%_{w/w}$ and $0.354\% \pm 0.041\%_{w/w}$ respectively. The crude extract showed considerable anti-inflammatory activity compared to the reference drug, diclofenac. The method demonstrated acceptable levels of accuracy, precision, specificity, and robustness hence can be successfully adopted for routine quality control and standardization of the stem bark of *C. regia*. The stem bark of the plant exhibited significant anti-inflammatory activity.

Keywords: *Chlorophora regia*, Quality control, RP-HPLC, Anti-inflammatory, Regiafuran A

1 Introduction

Most people in sub-saharan Africa use traditional medicine for their basic health needs [1,2]. About 70 to 95% of the world's population presently rely on plants as their primary source of treatment as estimated by the World Health Organization. Before the evolution of present-day medicine and its pharmacopeia of 'western' medicines, plants remained the main source of remedy for diseases [3].

The quality of plant material could be assessed by determining the physical and chemical parameters [4]. Herbal medicines are subjected to strict manufacturing standards in certain developed countries like Germany for quality, efficacy, and safety as other orthodox medicines [5]. This is, however, not evident in most developing countries. In Ghana, herbal medicinal products are frequently sold as over-the-counter (OTC) medicines without any knowledge of the number of components consumed [6]. There is thus the need for the development of standard analytical methods to provide useful data on the constituents of herbal medicinal products and their respective amounts for quality control.

Chlorophora regia A. Chev. (Moraceae), also known as *Odum-nua*, is a large dioecious tree frequently used in Ghana for its medicinal properties. It grows to a height of 35–45 m. The trunk is usually straight, often with no branch for as high as 20 m, 2 m in diameter, with no buttresses but may have very large spurs extending into long surface roots. The stem bark extract is employed traditionally in the treatment of several conditions including rheumatism, lumbago, cough, asthma, syphilis, snake and insect bites, dysmenorrhea, and also for the treatment of burns and wounds [7]. These traditional medicinal uses of the stem bark suggest that it may possess anti-inflammatory potential. However, isolates from the stem bark tested so far did not show any significant anti-inflammatory activity [8]. Based on this observation, and traditional uses, the total crude stem bark extract was

investigated for its possible anti-inflammatory activity using the 7-day old chick model.

Herein, we report the isolation of a benzofuran derivative, Regiafuran A (**1**) and a flavonoid, 3,5,7,4'-Tetrahydroxy-2'-methoxyflavonol (**2**) employed as biomarkers for developing a validated reverse phase HPLC method for the quality control of the stem bark of *C. regia* and its finished herbal products. The isolated compounds were identified by detailed spectroscopic techniques including IR, NMR (1D and 2D), and comparing to established data in literature (Figure 1).

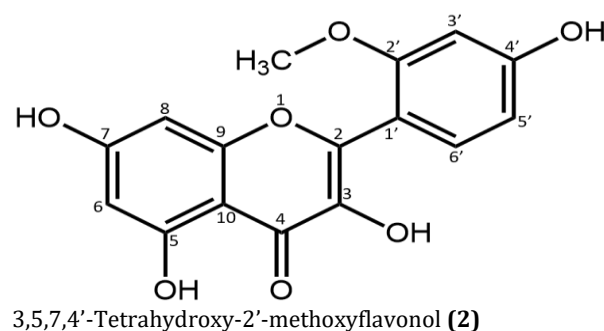
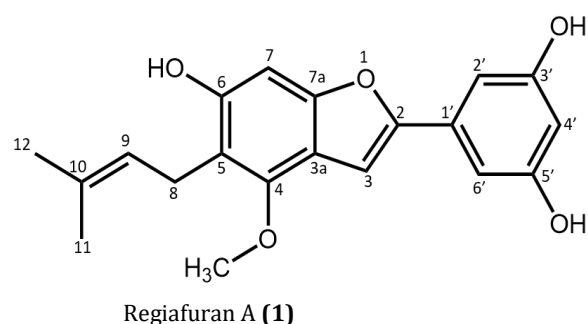


Figure 1. Chemical structures of compounds 1 and 2

2 Experimental Section

2.1 Chemicals and reagents

For extraction and column chromatography, cyclohexane, methanol and chloroform (Fisher Scientific, UK), ethylacetate (Chemiphase, UK) and dichloromethane (Sigma

Aldrich, USA) were used. HPLC grade methanol (Sigma Aldrich, USA) was employed for the RP-HPLC method development. The chemicals and reagents used for the anti-inflammatory activity test included tragacanth (Sigma Aldrich, USA), carrageenan sodium salt (Sigma Aldrich, USA), normal saline (Intravenous Infusions, Ghana) and diclofenac sodium injection (Troge Medical GmbH, Germany). Pre-coated TLC plates (Merck, Germany) for TLC monitoring of isolation. Silica gel (70-230 mesh) (Fisher Scientific, UK) was used for the column chromatography.

2.2 Plant collection and authentication

Fresh *C. regia* stem bark (Figure 2) was sourced from Kwahu-Asakraka, Eastern region of Ghana. The collected stem bark was identified and authenticated with a voucher number of KNUST/HM/CR1/2014/R002.



Figure 2. The fresh stem bark of *Chlorophora regia*

2.3 Sample preparation, extraction, and isolation

The stem bark was cut into pieces, air-dried and milled into a coarse powder using a hammer mill. 2 kg of the powdered dried plant sample of *C. regia* was extracted using 7.5 L of methanol-chloroform (4:1) mixture by cold maceration for 5 days. The obtained extract was concentrated using a rotary evaporator to a brownish residue (120 g). Cyclohexane, dichloromethane, and methanol were sequentially used to partition the residue. The methanol fraction (100 g) was subjected to

column chromatography on silica gel using gradient elution with cyclohexane, ethyl acetate, and methanol to obtain 23 major sub-fractions (CR1-CR23) following TLC monitoring.

Based on the TLC results, further fractionation was carried out on sub-fraction CR6 resulting in five sub-fractions (CR6a-e) out of which sub-fraction CR6d gave **1** after repeated washing in ethylacetate. Sub-fraction CR7 yielded **2** after repeated washing in methanol and recrystallization in ethyl acetate.

2.4 Anti-inflammatory Activity Testing

2.4.1 Experimental Animals

One hundred day-old chicks (*Gallus gallus*) were obtained from Akate Farms in Kumasi, Ghana, and kept in a large chicken coop at the animal house, Department of Pharmacology, KNUST. Standard poultry feed and hygienic water were provided *ad libitum*. Coop maintenance was done daily and the chicks were checked for weight and good health daily. All experimental procedures and techniques used were done following the National Institute of Health guidelines for care and use of laboratory animals [9]. All experimental procedures were also approved by the Ethics committee, College of Health Sciences, KNUST (CoHS/EC/2018/186).

2.4.2 Carrageenan-induced footpad edema in chicks

The carrageenan-induced edema in the 7-day-old chick model [10,11] was used to assess the possible anti-inflammatory activity of the stem bark extract of *C. regia* (CRE) and compared to diclofenac sodium as a reference drug. Carrageenan (10 μ L of a 2% solution in saline) was injected subplantar into the right footpads of the chicks. Foot volume was measured before the injection and at hourly intervals for 5 hours after injection by water displacement.

Fifty-six 7-day-old chicks were randomly put into seven groups (n=8). Two hours after the carrageenan challenge, chicks were selected randomly to carry out one of the study groups: Vehicle control; CRE (30, 100 and 300 mg kg⁻¹, *p.o.*), and diclofenac sodium (10, 30 and 100 mg kg⁻¹, *i.p.*). The extract was prepared in 2 % tragacanth mucilage.

2.5 Identification and Characterization of Isolated Compounds

Melting point determination was carried out to confirm the purity of the isolated compounds. The identity and chemical structure of the compounds isolated were investigated using spectroscopic techniques including infrared spectroscopy, 1D, and 2D NMR.

2.5.1 Infrared Spectroscopy

The Attenuated Total Reflectance Fourier Transform Infrared (ATR FTIR) spectrophotometer was employed in this analysis. IR spectra over the wavenumber range of 4000 cm⁻¹ to 400 cm⁻¹ were obtained.

2.5.2 Nuclear Magnetic Resonance Spectroscopy

The Bruker Biospin NMR spectrometer was used to obtain the 1D and 2D NMR spectra. 20 mg of **1** and 10 mg of **2** was dissolved in deuterated methanol and dimethyl sulfoxide (DMSO) respectively and their various NMR spectra obtained at a frequency of 500 MHz and a temperature of 298.0 K.

2.6 RP-HPLC Method Development

Suitable conditions for the chromatographic separation were investigated and developed for the analysis of **1** and **2** using µBondapak™ C18 (3.9×300 mm, 5 µm) (Waters, USA). The mobile phase composition of 0.1 % trifluoroacetic acid (TFA) as solvent A, and methanol as solvent B with gradient elution was finally selected after trying several solvent compositions. The gradient elution was conducted with the following conditions; at time 0 min, the mobile phase was 20% B, from time 0 to 1 min was 20% B, at time 16 min, B was 100%, at time 16.1 min, B was 20% and time 21.1 min was 20% B. The detection wavelength was selected as 250 nm since both compounds exhibited very high absorption at that wavelength. The flow rate for the analysis was 1.0 mL/min with a runtime of 21.1 mins. A temperature of 25 °C was maintained throughout the experiment with 20 µL injection volume. The retention time of each compound was recorded. Data were acquired using the Chromera software (Table 2).

2.6.1 RP-HPLC Method Validation

The RP-HPLC method developed was validated using both **1** and **2** according to ICH guidelines for linearity, robustness, accuracy, specificity, intra-day and inter-day precision, detection limit, quantitation limit, range and linearity [12].

2.6.2 Linearity

Five different concentrations of each biomarker were prepared from the stock solutions (200 µg/mL for **1** and 400 µg/mL for **2**). Concentrations of 32 µg/mL, 16 µg/mL, 8 µg/mL, 4 µg/mL and 2 µg/mL were used for **1** and concentrations of 160 µg/mL, 80 µg/mL, 40 µg/mL, 20 µg/mL and 10 µg/mL were used for **2**.

Using suitable chromatographic conditions, 20 µL of each concentration prepared was injected in triplicates and the peak area of each chromatogram recorded. A plot of average peak area against the concentrations was obtained for each biomarker using Microsoft Excel, 2016. The correlation coefficient and equation of the line for each biomarker were obtained from the plot. These were used to ascertain the linearity of the analytical method developed.

2.6.3 Limit of Detection and Limit of Quantitation

The limit of detection and limit of quantitation were calculated from the data obtained from the calibration plot for both biomarkers using the equations 1.

$$LOD = 3.3 \times \frac{\sigma}{S}$$

$$LOQ = 10 \times \frac{\sigma}{S} \quad (\text{equation 1})$$

Where σ is the standard error of the intercept and S is the slope of the line obtained for linearity.

2.6.4 Intra-day Precision (Repeatability)

20 µL each of 20 µg/mL solution of **1** and 40 µg/mL of **2** was injected six times within 24 h. The peak areas obtained from the chromatograms were recorded and the percentage relative standard deviation (% RSD) calculated.

2.6.5 Inter-day Precision (Intermediate Precision)

20 μ L each of 20 μ g/mL solution of **1** and 40 μ g/mL solution of **2** was injected thrice on three successive days. The peak areas obtained from the chromatograms were recorded and the percentage relative standard deviation (% RSD) calculated for each day. The relative standard deviation in percentage for the three days was also calculated for each biomarker.

2.6.6 Robustness

a. Effect of Wavelength Variation

A 45 μ g/mL solution of **1** and 40 μ g/mL solution of **2** were prepared. 20 μ L each of the two concentrations prepared was injected in triplicates. This determination was done for three different wavelengths of 248 nm, 250 nm, and 252 nm with all other parameters unchanged. The triplicate injections were done at each wavelength used.

b. Effect of Flow Rate Variation

A 16 μ g/mL solution of **1** and 40 μ g/mL solution of **2** were prepared. 20 μ L each of the two concentrations prepared was injected in triplicates. This determination was done for three different flow rates of 0.8 mL/min, 1.0 mL/min, and 1.2 mL/min with all other parameters unchanged. The triplicate injections were done at each flow rate used.

2.6.7 Accuracy

a. Crude Sample

4 mg of the crude solid mass was dissolved in 30 mL of methanol by sonication and quantitatively transferred into a 50 mL volumetric flask and adjusted to volume. Accuracy was estimated by spiking the crude sample at three different concentration levels corresponding to 80%, 100% and 120% of target concentrations of 5.2, 6.5 and 7.8 μ g/mL for **1** and 24.0, 30.0, 36.0 μ g/mL for **2** (**S18**). 20 μ L of the spiked crude extract solution was injected in triplicates and the mean peak area calculated.

b. Quantification of the biomarkers in the stem bark

About 500 g of the powdered stem bark was macerated in 1.5 L of methanol-chloroform (4:1) mixture for 3 days. Complete extraction was ensured using TLC to monitor the extraction process with the reference

compounds **1** and **2**. The various extracts obtained were combined and concentrated using the rotary evaporator to dryness (30 g).

1 mg of the crude solid mass was dissolved in 10 mL of methanol and quantitatively transferred into a 20 mL volumetric flask and adjusted to volume. 20 μ L of the crude extract solution was injected in duplicates and the mean peak area was calculated (Table 3).

2.7 Data Analysis

Statistical analyses were carried out with GraphPad Prism v. 6.01 (GraphPad Software, San Diego, USA). Time-course curves were subjected to the ordinary two-way repeated-measures analysis of variance (RM-ANOVA) with treatment (between-subjects) and time (within-subjects) as factors and the mean of the treatment effects at each time point were compared by Dunnett's post hoc test. The area under the curves (AUCs) was computed to establish the overall treatment effect and total anti-inflammatory score. The difference in total anti-inflammatory score was determined using one-way ANOVA with Turkey's post hoc test using treatment data as the between-subject factor for data that were distributed normally. Differences were considered statistically significant at P value < 0.05.

3 Results and Discussions

3.1 Anti-inflammatory Activity of the crude extract from the stem bark

Carrageenan-induced acute inflammation is one of the most appropriate tests employed in screening anti-inflammatory agents and it remains an acceptable preliminary screening test for possible anti-inflammatory activity [13]. The time course of edema development in carrageenan-induced footpad edema model in chicks is generally represented by a two-phased curve; the first phase occurring within the first two hours of carrageenan injection and is partly due to the trauma of injection and also due to histamine and serotonin release. The second phase occurs due to the release of inflammatory mediators like prostaglandins which may be attributed to cyclooxygenase induction in tissues and usually occurs at the end of the first phase and remain through three

to five hours [14]. 10 μL of 2 % carrageenan suspension was injected into subplantar of chicks to induce acute local edema which peaked around 2 h (Fig. 3A, 3C). Administration of CRE (30, 100 and 300 mg kg^{-1} , *p.o.*) significantly decreased the foot volume of the chicks from the second hour until the sixth-hour time point (Fig. 3A). The total edema, calculated as the area under the time course curve showed a significant reduction in paw

edema after CRE ($F_{3, 112}=110.6$, $P<0.0001$) and diclofenac ($F_{3, 112}=58.92$, $P<0.0001$) treatments (Fig. 3B, D), CRE 300 mg kg^{-1} and diclofenac 100 mg kg^{-1} gave the maximum inhibition of total edema by 39.55 ± 3.70 % and 37.08 ± 4.80 % respectively. This implies a possible inhibitory effect of the stem bark extract on the synthesis and or release of the inflammatory mediators involved in the early and late phases of carrageenan injection.

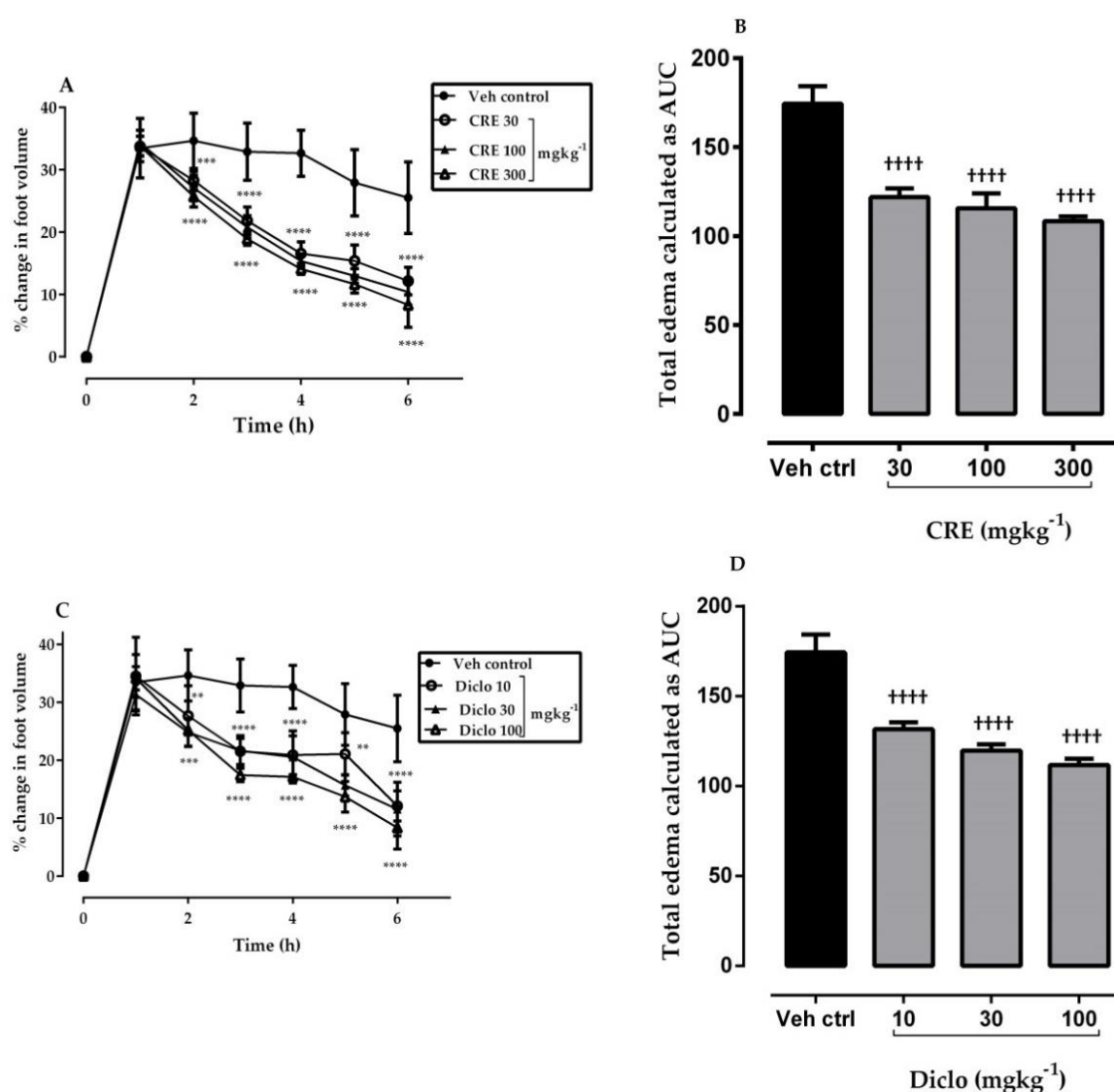


Figure 3. Effect of CRE (30-300 mg kg^{-1} , *p.o.*) and diclofenac (10-100 mg kg^{-1} , *i.p.*) on time-course curves (A and C respectively) and the total edema response (B and D respectively) in the carrageenan-induced foot edema in chicks. Values are mean \pm S.E.M (n=8). The symbols * and † indicate significance levels compared to respective controls: **** $P<0.0001$ and ** $P<0.01$ (two – way ANOVA followed by Dunnett's *post hoc*). †††† $P<0.0001$ (one-way ANOVA followed by Dunnett's *post hoc*)

3.2 Crude Extraction and Isolation of Compounds

The extraction technique used gave a yield of 6%^{w/w} which is comparable to 7%^{w/w} obtained by Kyekyeku *et al* [8]. Phytochemical screening of the stem bark led to the isolation of six compounds of which two were characterized and their structure elucidated due to paucity of the other four isolates. The two compounds were characterized based on their melting points and detailed spectroscopic analysis.

3.3 Melting Point and Spectroscopic Analysis

Melting point determination as a tool to confirm the identity and or purity of compounds was conducted on the two isolated compounds (**1** and **2**). The sharp melting point ranges obtained as shown in the physicochemical constants below confirm the purity of the compounds.

Infrared spectroscopy as a tool to investigate the functional groups present in various compounds was performed to aid in the structure elucidation of the two compounds isolated.

The IR spectrum of **1**(**S4**) shows a broad band at 3297.9 cm⁻¹ which indicates the presence of a hydrogen-bonded O-H stretch. This is confirmed by the O-H bending peak at 673.4 cm⁻¹. A band around 2930.9 cm⁻¹ is indicative of a C-H stretch of sp³ hybridized carbon (CH₃, CH₂, or CH). The weak absorption peak at 1625.3 cm⁻¹ infers the presence of an alkenyl C=C stretch. The medium absorption peaks at 1601.2 cm⁻¹ and 1575.9 cm⁻¹ are also indicative of an aromatic C=C bending which corresponds to the four aromatic C-H bending peaks between 680 cm⁻¹ and 860 cm⁻¹.

The IR spectrum of **2** (**S5**) shows a band at 3316.5 cm⁻¹ which is indicative of the presence of a hydrogen-bonded O-H stretch. A band around 2995.4 cm⁻¹ is indicative of a C-H stretch of sp³ hybridized carbon (CH₃, CH₂, or CH). The medium absorption peak at 1663.1 cm⁻¹ infers the presence of an alkenyl C=C stretch. The three medium absorption peaks at 1615.8 cm⁻¹ and 1574.8 cm⁻¹ and 1506.6 cm⁻¹ are also indicative of an aromatic C=C bending.

The UV-Vis spectrum was scanned for the two compounds (**1** and **2**) which were to be

used in the RP-HPLC method development. The spectrum for **1** showed two maximum wavelengths of absorption at 250 nm and 350 nm. **2** however gave three maximum wavelengths of absorption at 203.2 nm, 251.0 nm, and 348.3 nm (**S2-S3**). Since the UV cutoff for methanol is 205 nm, the first wavelength of absorption was ignored. The wavelengths obtained can be seen to be very close with a very slight deviation. This is suggestive that the two compounds have some similarities in their structure. From literature, it is known that flavonols and polyphenols have wavelengths around the obtained values. This is therefore suggestive that both compounds may belong to the polyphenolic class of compounds. The stem bark of *C. regia* has been confirmed to contain a wide range of polyphenolic compounds as well as flavonols [8].

Proton and Carbon-13 NMR spectra were obtained for the two compounds to elucidate their structures. Data obtained from the NMR spectra for **1** and **2** revealed peaks with chemical shifts that are similar to that already published for *Regiafuran A* and *3,5,7,4'-Tetrahydroxy-2'-methoxyflavonol* respectively (**S6-S11**) [8, 15]. Table 1 shows the NMR data for the two compounds.

The two compounds were used as biomarkers in RP-HPLC method development and validation (Figure 4, 5, 6).

3.4 Physicochemical constants of **1** and **2**

3.4.1 Regiafuran A (**1**)

Yellow amorphous solid. Melting point: 111°C – 113°C. λ_{max} 250 nm. Significant IR peak values: 3297.9 cm⁻¹, 2930.9 cm⁻¹, 1625.3 cm⁻¹, 1601.2 cm⁻¹, 1575.9 cm⁻¹, 673.4 cm⁻¹, 680 cm⁻¹ and 860 cm⁻¹. ¹H NMR (500 MHz in CD₃OD) and ¹³C NMR (125 MHz in CD₃OD) data are as shown in Table 1.

3.4.2 3,5,7,4'-Tetrahydroxy-2'-methoxyflavonol (**2**)

Amorphous yellow solid. Melting point: >300°C. λ_{max} 251 nm. Significant IR peak values: 3316.5 cm⁻¹, 2995.4 cm⁻¹, 1663.1 cm⁻¹, 1615.8 cm⁻¹, 1574.8 cm⁻¹ and 1506.6 cm⁻¹. ¹H NMR (500 MHz in DMSO) and ¹³C NMR (125 MHz in DMSO) data are shown in Table 1.

Table 1. ¹H and ¹³C NMR Data of 1 in CD₃OD and 2 in DMSO; δ in ppm and *J* in Hz

Regiafuran A (1)		3,5,7,4'-Tetrahydroxy-2'-methoxyflavonol (2)		
Position	$\delta_{\text{H}}^{\text{a}}$ multi. (<i>J</i> in Hz)	$\delta_{\text{C}}^{\text{b}}$	$\delta_{\text{H}}^{\text{a}}$ multi. (<i>J</i> in Hz)	$\delta_{\text{C}}^{\text{b}}$
2		157.4		148.4
3	6.76, s	102.3		136.7
3a		126.0		
4		145.3		176.2
5		119.7		160.9
6		149.0	6.18, d, (1.9)	98.1
7	6.76, s	104.6		163.8
7a		148.1		
8	3.52, d, (7.5)	24.4	6.28, d, (1.9)	93.4
9	5.28, t, (7.5)	123.4		156.8
10		132.9		103.5
11	1.60, s	25.9		
12	1.79, s,	18.1		
1'		133.8		110.4
2'	6.70, d, (2.0)	104.1		158.6
3'		159.9	6.52, s	99.4
4'	6.17, t, (2.0)	103.7		160.9
5'		159.9	6.47, d, (8.3)	107.1
6'	6.70, d, (2.0)	104.1	7.25, d, (8.3)	131.9
4-OCH ₃	3.71, s	61.6		
2'-OCH ₃			3.73, s	55.6
3-OH			12.56, s	
5-OH			3.35, s	
7-OH			3.35, s	
4'-OH			3.17, s	

^a Recorded at 500 MHz; ^b Recorded at 125 MHz

^a Recorded at 500 MHz; ^b Recorded at 125 MHz

Table 2. A summary of chromatographic conditions

Separation Variable	Selected Conditions
Column	μ Bondapak™ C18 reverse column
Dimension	3.9 × 300 mm
Stationary phase	Octadecylsilane (C18)
Particle size	5 μ m
Mobile phase composition	0.1% trifluoroacetic acid and Methanol
Separation technique	Gradient
Flow rate	1 mL/min
Injection volume	20 μ l
Detection	PDA detector at 250 nm
Temperature	25 °C

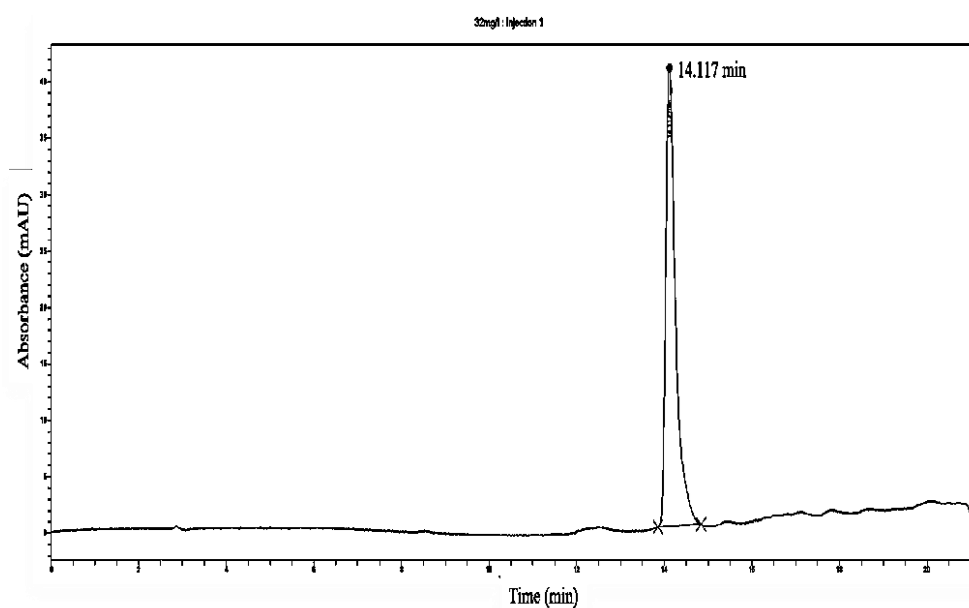


Figure 4. HPLC chromatogram of isolated regiafuran A (1)

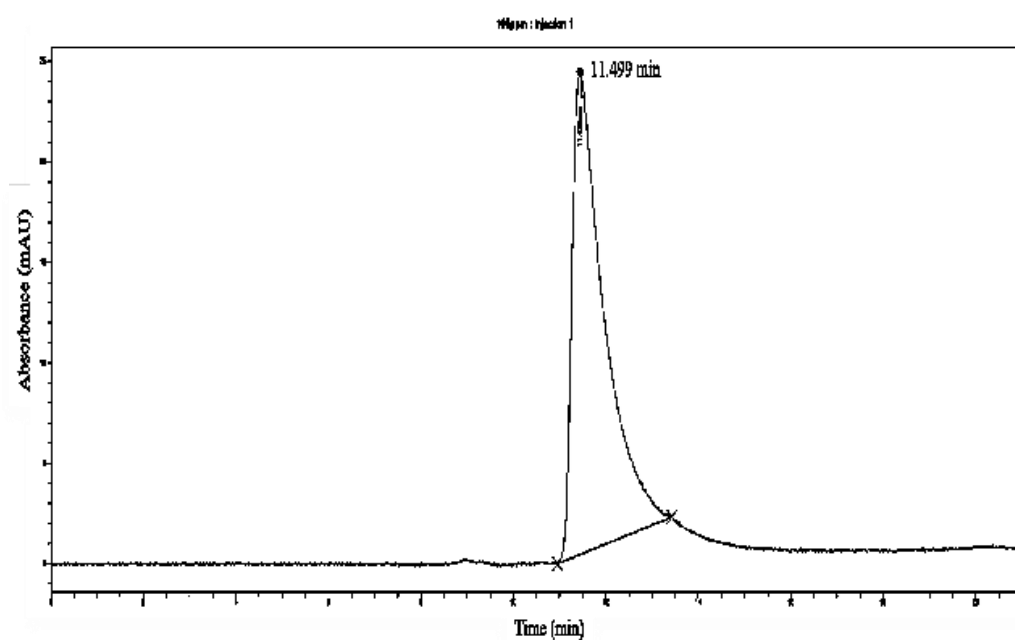


Figure 5. HPLC chromatogram of 160 µg/ml of isolated 3,5,7,4'-Tetrahydroxy-2'-methoxy flavonol (2)

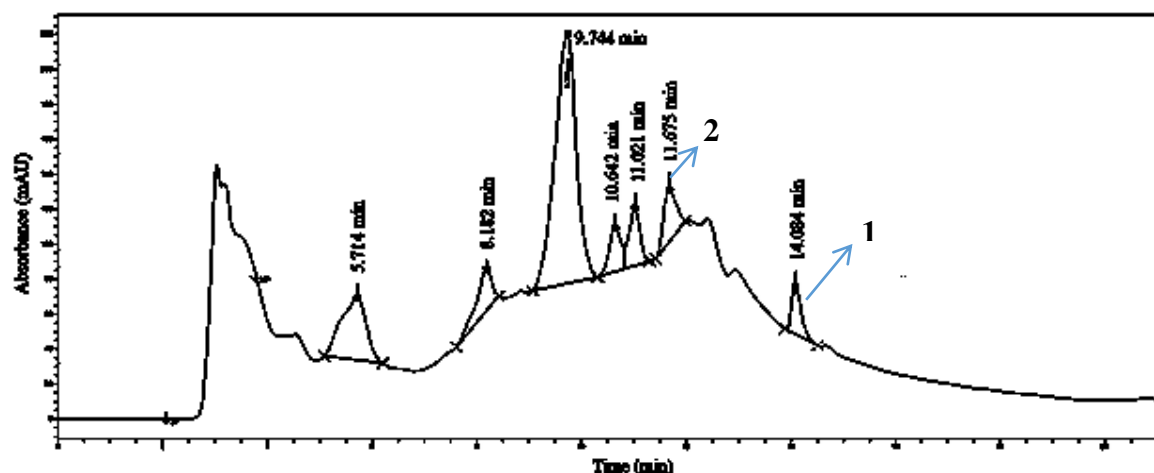


Figure 6. HPLC chromatogram of crude extract of the stem bark of *C. regia*

3.5 RP-HPLC Analytical Method Development

The developed method was finally optimized by using a mobile phase of 0.1% trifluoroacetic acid and Methanol in a gradient mode. The flow rate for the analysis was 1 mL/min at 25 °C with a UV detection at 250 nm (table 2). The total runtime of the analysis was 21.1 mins. The retention time of 1 and 2 were observed to be 14.13 (± 0.015) and 11.50 (± 0.058) respectively, as shown in table 4.

3.6 RP-HPLC Method Validation

3.6.1 Accuracy

The mean recovery was the in range of 99-103%, which indicates acceptable recovery values as shown in Table 4.

3.6.2 Precision

Three replicates injection at working concentration of standard solutions exhibited a percentage relative standard deviation (% RSD) less than 2% for each of the biomarkers for inter-day precision. The intra-day precision study was performed in sextuplicate at one concentration level of each biomarker to afford a percentage relative standard deviation (% RSD) less than 2%. The above results demonstrates clearly that the analytical system is working under the conditions specified as shown in Table 4.

3.6.3 Sensitivity

The sensitivity of the developed method was determined using Limit of detection (LOD) and Limit of quantitation (LOQ). The LOD and LOQ were computed to be based on the standard deviation and slope from the calibration curve as shown in table 4. The method is therefore sensitive to the biomarkers selected.

3.6.4 Robustness

As indicated in table 4, the % RSD obtained for both biomarkers when the flow rates and wavelengths were varied were less than 2%. This shows the developed method is reasonably robust.

3.6.5 Specificity

The developed method was found to be sensitive for both biomarkers as no interference of any components was observed at the retention times of 1 and 2 (Figure 9, 10, 11).

3.6.6 Linearity

The developed method demonstrated linear responses in the concentration range of 2 to 32 $\mu\text{g/mL}$ for 1 and 10 to 160 $\mu\text{g/mL}$ for 2. Five different concentrations of each biomarker were injected in triplicates and the average areas were plotted against the concentrations of each biomarker (Figure 7, 8).

The correlation coefficient greater than 0.99 was observed for each marker which falls within the acceptable criteria for linearity. The developed method is therefore linear.

The results of the other validation parameters are summarized in Table 4.

3.7 Determination of Content

The percentage content of both compounds **1** and **2** was calculated using the suitable calibration equation to be $0.224 \pm 0.056\%w/w$ and $0.354 \pm 0.041\%w/w$ respectively as shown in table 3.

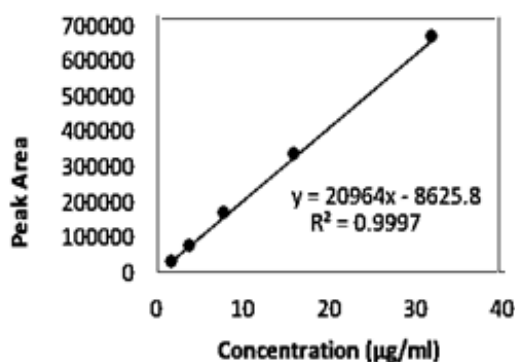


Figure 7. Calibration curve for 1

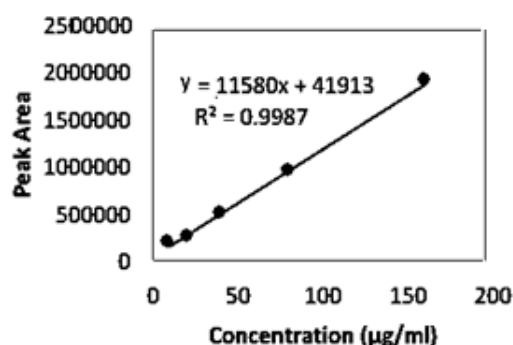


Figure 8. Calibration curve for 2

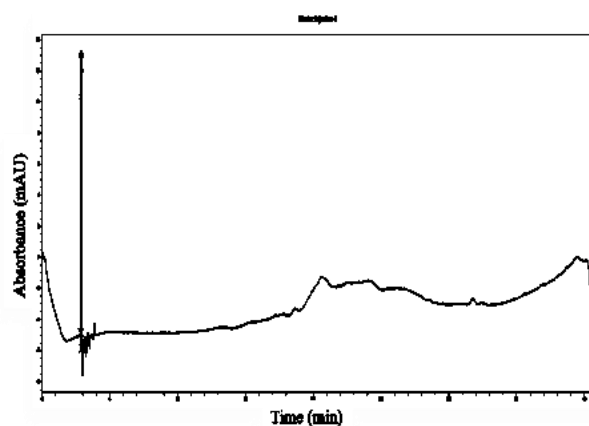


Figure 9. Chromatogram of blank

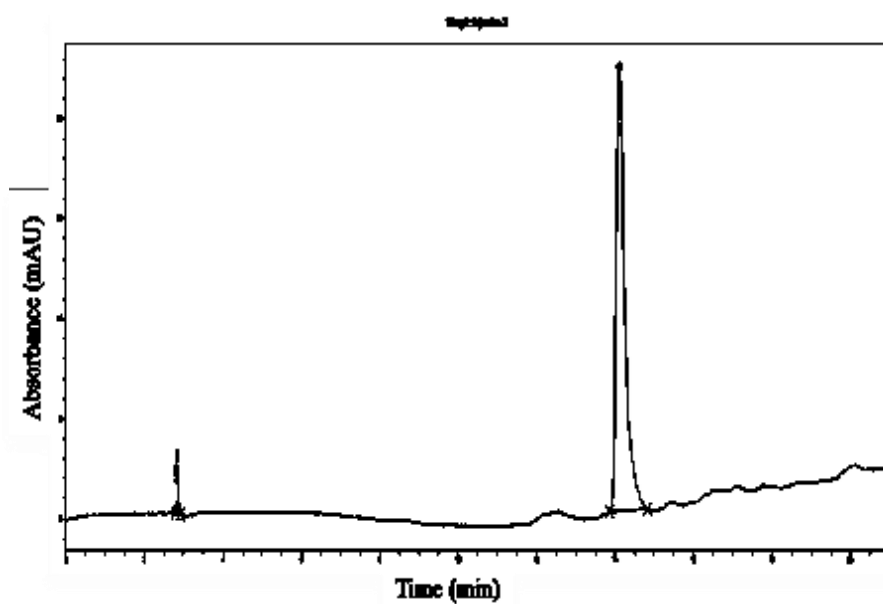


Figure 10. Chromatogram of 1

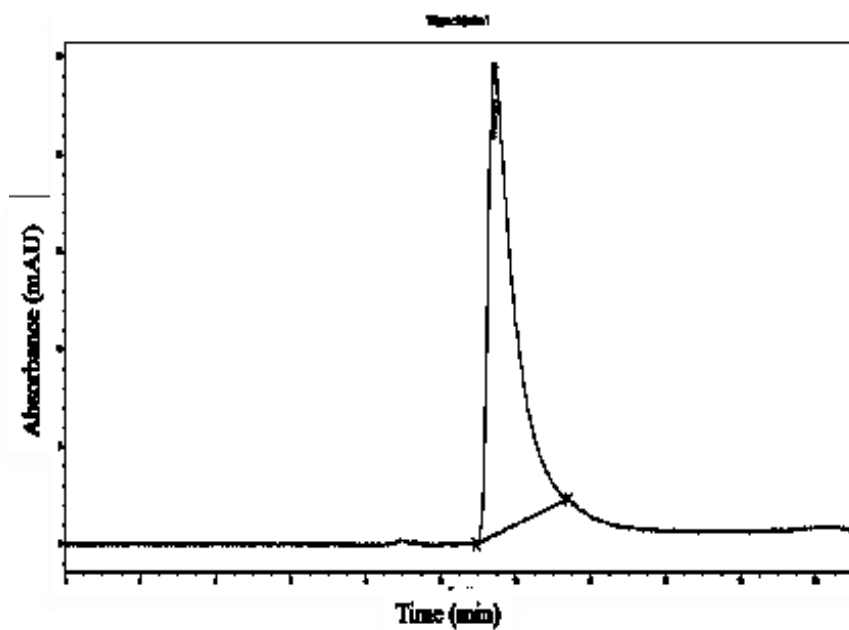


Figure 11. Chromatogram of 2

Table 3. Percentage content of **1** and **2** in the stem bark

Compound	Mean peak area*	Mean Concentration* (µg/ml)	Mean Content in 500 g of the stem bark* (g)	Mean Percentage Content* (%w/w)
1	383407.2	18.70	11.220	0.224 ± 0.056
2	383407.2	29.49	17.694	0.354 ± 0.041

*mean of 3 determinations

Table 4. HPLC method development and validation parameters

Parameters				1		2			
Mean Retention Time * (minutes)		14.13± 0.015				11.50± 0.058			
Range (µg/mL)		2 - 32				10 - 160			
Calibration equation		y =20964x - 8625.8				y =11580x + 41913			
Coefficient of Correlation (R)		0.9998				0.9993			
Limit of Detection (µg/mL)		0.57				5.66			
Limit of Quan-titation (µg/mL)		1.73				17.14			
Intra-day Precision	Concentration (µg/mL)	(Mean peak Area ± SD) x 10 ⁶		RSD (%)		Concentration (µg/mL)	(Mean peak Area ± SD) x 10 ⁶		RSD (%)
	22.00	n = 6 0.4419 ± 0.0037		0.83		40.00	n = 6 0.5083 ± 0.0025		0.50
Inter-day Precision	Day	(Mean peak Area ± SD) x 10 ⁶				Day	(Mean peak Area ± SD) x 10 ⁶		
		n = 3					n = 3		
Robustness (Flow rate)	1	0.4405 ± 0.0032		0.73		1	0.5062 ± 0.0014		0.28
	2	0.4433 ± 0.0041		0.93		2	0.5093 ± 0.0017		0.34
	3	0.4469 ± 0.0030		0.68		3	0.5068 ± 0.0063		1.25
Robustness (Flow rate)	Flow rate (ml/min)	Mean peak area*				Flow rate (ml/min)	Mean peak area*		
	0.8	327158.9		0.91		0.8	510756.0		0.91
	1.0 (original condition)	322158.4		1.04		1.0 (original condition)	508909.7		1.04
Robustness (Wavelength)	1.2	318727.8		0.88		1.2	496593.5		1.18
	Wavelength (nm)	Mean peak area*				Wavelength (nm)	Mean peak area*		
	248	951784.12		0.81		248	513879.5		1.21
Accuracy	250 (original condition)	968842.3		1.15		250 (original condition)	508909.7		1.12
	252	984448.24		0.68		252	499784.12		0.98
	Amount of compound added (µg)	Mean amount recovered* (µg)	Mean % Recovery*			Amount of compound added (µg)	Mean amount recovered* (µg)	Mean % Recovery*	
Accuracy	5.20	5.3711742	103.29 ± 0.452	0.438		24.00	23.968825	99.87 ± 0.878	0.879
	6.50	6.5703524	101.08 ± 1.136	1.124		30.00	30.062527	100.21 ± 0.895	0.893
	7.80	8.1703524	104.75 ± 1.122	1.071		36.00	37.174833	103.26 ± 0.414	0.401

*Mean of 3 determinations; Acceptance criteria for mean % recovery (accuracy): 90-110 %; Acceptance criteria for RSD: ≤ 2.0

A novel reverse-phase HPLC method which was proven to be robust, precise, accurate, and specific was developed for the simultaneous quantitation of **1** and **2** present in the stem bark of *C. regia*. Octadecylsilane was used as the reverse stationary phase and a mobile phase composition of methanol and 0.1% trifluoroacetic (TFA) acid in a gradient elution mode at a flow rate of 1.0 mL/min. With a run time of 21.1 mins, the mobile phase was able to elute both compounds with the peak of each compound possessing good shape and symmetry. Methanol was employed as an organic modifier in this mobile phase because it was able to dissolve both compounds to an appreciable degree. TFA has been shown to improve the separation and resolution of peaks of the components in RP-HPLC. It does so by acting as a pH modifier; lowering the mobile phase pH, generally to enhance retention of acidic solutes through avoidance of partial ionization of analytes and suppression of ionization of residual silanols on the stationary phase. TFA also serves as an ion-pairing agent [16]. The wavelength chosen for the analysis of

both compounds was 250 nm because both are detectable to higher degrees at that wavelength and it gave the best sensitivity and results. Coefficient of correlation values of 0.9997 and 0.9987 for compounds **1** and **2** respectively show that the method is very linear within the range of 2 to 32 µg/mL for **1** and 10 to 160 µg/mL for **2**. The results of robustness studies depicted that minor changes in the conditions of the proposed method such as slight changes in flow rate and wavelength did not affect the integrity of the method as the percentage RSD values recorded were all below the ICH guideline standard of 2%. The method was again found to be accurate as the mean recovery values obtained for **1** and **2** were all within 95%-105% with a percentage RSD below 2%. The method showed high precision with percentage RSD values for intra-day precision of 0.83% and 0.50% for **1** and **2** respectively as shown in table 3. All percentage RSD values for inter-day precision studies were below 2% and in accordance with the ICH guidelines [12]. The low LOD and LOQ values recorded in table 3 shows the high level of

sensitivity of the method. The developed and validated RP-HPLC method was employed successfully in the determination of the amount of **1** and **2** in the stem bark to be $0.224 \pm 0.056\%w/w$ and $0.354 \pm 0.041\%w/w$ respectively. The method development, validation and its use in the quantitation of the selected biomarkers are comparable to reported literature in the area [17,18]. The crude extract from the stem bark of the plant also showed a good level of anti-inflammatory activity similar to that of the reference standard, diclofenac as shown in figure 3.

4 Conclusions

The present study has confirmed that the total crude extract of the stem bark of *C. regia* has considerable acute anti-inflammatory activity compared to the reference drug, diclofenac in the 7-day old chick carrageenan. A dual-component RP-HPLC method with UV detection was successfully developed and validated per the ICH guidelines for the identification and quantitation of the characterized compounds in the stem bark of *C. regia* with their percentage contents as $0.224 \pm 0.056\%w/w$ for **1** and $0.354 \pm 0.041\%w/w$ for **2**. The developed method could efficiently be used routinely for the quality control of the extracts of the stem bark of *C. regia*.

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