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Determination of the Rutin, Quercetin and Kaempferol Contents in a Ghanaian Polyherbal Formulation (EAF-2011) and Its Raw Materials Using a Simultaneous RP-HPLC Method

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ABSTRACT

Quantitative chemical fingerprinting of herbal medicines provides an avenue for addressing the challenge with their quality. In this report, chemical markers that could be labelled as active markers in the polyherbal product and its component raw materials were assayed using a simultaneous RP-HPLC method. These markers were rutin (RU), quercetin (QE) and kaempferol (KA) which are well known for their antimicrobial activities. The quantitative assay of the markers using a validated HPLC method showed the herbal product (EAF-2011) contained 8.6810% (w/w) of rutin, 0.2670% (w/w) of quercetin and 0.0610% (w/w) of kaempferol. At least two (2) of these flavonoids were detected in each of the component raw plant materials: *Psidium guajava* [(RU-8.5860% (w/w); QE-0.5430% (w/w); KA - 0.1003% (w/w)], *Alchornea cordifolia* [RU-2.5540% (w/w); QE-0.0536% (w/w)], *Zanthoxylum zanthoxyloides* [RU-5.8060 % (w/w); KA-0.0174% (w/w)], *Tridax procumbens* [RU-2.4390% (w/w); QE-0.1040% (w/w); KA-0.0510% (w/w)] and *Eugenia caryophyllata* [QE-0.4080% (w/w); KA-0.0771% (w/w)]. The results obtained indicate that the HPLC method, selected chemical markers and chromatograms produced are ideal for the quality control of the product and its component raw materials.

Keywords: Herbal medicines, Polyherbal product, Quality Control, RP-HPLC, Standardisation.

INTRODUCTION

Herbal medicines play a significant role in primary healthcare delivery that cannot be ignored. In developing countries like Ghana, patronage of these products either as first line treatment or as a complement to conventional treatment is widespread; a situation that emphasises the need for the delivery of quality products to clients. [1]

The quality control of herbal medicines and their products have always posed a challenge for regulators for want of standards to judge quality by and for users because of potential adulteration.

Reports are available for numerous toxic and adverse reactions associated with herbal products that may be the result of poor quality. These untoward effects may be the result of poor agricultural practices, adulteration, misidentification and poor manufacturing procedures. [2-3] Aside the associated toxicity, poor quality products lead to decreased clinical efficacy and increased cost of treatment for patients. Different methods have thus been suggested to address these issues with each of these methods providing varying levels of guarantee

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about quality hence the suggestion to apply multiple methods during the quality assessment of herbal medicine. These methods may involve both chemical and biological assays. [4]

This study follows up on a previous quality evaluation of a Ghanaian polyherbal product *EAF-2011* from the Centre for Plant Medicine Research (CPMR), Mampong-Akwapem. [5] Part of the initial study involved a qualitative assay of the chemical constituents of the raw materials and the finished product using basic phytochemical screening and thin layer chromatography. However, as an improvement on the monograph developed for the product reported in the previous article [5], a quantitative chemical assay of three flavonoid compounds was undertaken for the product and its raw materials using a RP-HPLC method.

MATERIALS AND METHODS

Plant Collection, Extraction and Preparation

Herbal materials used comprised: the dried leaves of *Alchornea cordifolia* (Schum. & Thonn.) Muell.Arg. (Euphorbiaceae) and *Psidium guajava* (Linn) (Myrtaceae), the dried whole plant of *Tridax procumbens* (Linn) (Asteraceae), the dried stem bark of *Zanthoxylum zanthoxyloides* (Lam) (Rutaceae) and the dried flower buds of *Eugenia caryophyllata* (Thumb) (Myrtaceae). Plant samples after authentication by a botanist were air-dried in a cool dry place under shade for 2 weeks at temperatures between 23°C and 27°C. The materials were then pulverized using a hammer mill through 2 mm screen to obtain a fine powdered material. Extract from the plant materials were obtained by macerating 1 kg of the powdered plant material in 5.0 L of 70% (v/v) ethanol for 3 days and then filtered. The ethanol was recovered using the Rotary evaporator (Buchi™ R210). The fluid extract of the plant materials was then lyophilised and stored in a desiccator prior to use.

Reagents and Chemicals

Solvents used: methanol (MEOH), acetonitrile (ACN), acetic acid (CH₃COOH) and tetrahydrofuran (THF) were of HPLC grade (Sigma Aldrich). Deionised water was prepared by a Milli-Q Water purification system (Millipore, MA, USA). The standard compounds rutin, quercetin and kaempferol were also purchased from Sigma-Aldrich, USA.

HPLC Instrumentation and Conditions

The chromatographic system comprised an Agilent Chemstation HPLC system consisting of 1260 quaternary HPLC pump, ASL Prep autosampler, degasser and a multiple wavelength detector. The column used was a Superclo C₁₈ reversed phase column (5µm pore size, Ø 4.6 mm × 250 mm), purchased from Sigma Aldrich, USA.

Preparation of Herbal Extracts

Each of the lyophilised plant materials was reconstituted in methanol to achieve a concentration of 100 mg/ml and then sonicated for 20 minutes. The ointment base for the finished herbal product was

separated from the incorporated herbal extract by macerating 5 g of the product in 50 mls of 0.5N KOH for 24 hours refluxing for 1 hour and then filtering. The filtrate was extracted with methanol (1:2) (v/v) in a separating funnel. All the herbal extracts were filtered through 0.45µm PTFE membrane syringe filters (Thermo Fischer Scientific, USA) prior to injection; each injection was done in triplicate.

Preparation of Standard Solutions

Standard stock solutions of the three flavonoid compounds: rutin, quercetin and kaempferol, were prepared by dissolving them in methanol to give concentrations in the range of 1 mg/ml – 3.9 × 10⁻⁵ mg/ml. All injections were done triplicates.

Table 1: Validation data from the calibration curves of the standard flavonoid compounds

Flavonoid Compound	Regression Equation	Correlation Coefficient (r ²)	Linearity Range (mg/ml)
Rutin	y=719.424 x +12.61	0.9995	0.0313-1.0
Quercetin	y= 813.008 x -24.12	0.9996	0.0625-1.0
Kaempferol	y=4241.882 x -31.91	0.9997	0.0156-1.0

RESULTS

Development of Chromatographic Conditions

Mobile phase for the detection of the three compounds was developed by varying five (5) solvents: tetrahydrofuran (THF), methanol (MEOH), acetonitrile (ACN), H₂O and acetic acid (CH₃COOH) in different ratios. The solvent system of MEOH, ACN and 1% (v/v) CH₃COOH (40:15:45) was settled upon based on the separation and retention time (resolution), height of the peak and the area produced. Detection wavelength for the samples was selected after analysing fingerprints produced from the multiple wavelength detector (250-368 nm). The wavelength of 345 nm was selected as the most suitable based on the fingerprints produced. Flow rate and injection volume were set at 1.0 ml/min and 20µl respectively. Column temperature was also kept at an ambient temperature of 26°C.

Validation of Chromatographic Method

The developed chromatographic method was validated for linearity and range, precision, recovery/accuracy, limits of quantitation, limits of detection, and system suitability according to guidelines by International Conference on Harmonisation (ICH). [6]

A two-fold serial dilution was prepared from the stock solution (1 mg/ml) of the standard compounds to obtain ten (10) concentrations which were then used for the linearity studies. Injections were done in triplicates with the concentrations in the range of 1 mg/ml - 3.9×10⁻⁵ mg/ml. Analysis of the peak area (y-axis) versus concentration (x-axis) was done (Table 1). Correlation coefficient (r²) for all samples tested were ≥0.998, indicating a strong linear relationship between the peaks area and concentration. Retention times (R_T) for rutin, quercetin and kaempferol were 3.549 (± 0.030) mins, 4.999 (± 0.004) mins and 6.561 (± 0.030) mins respectively.

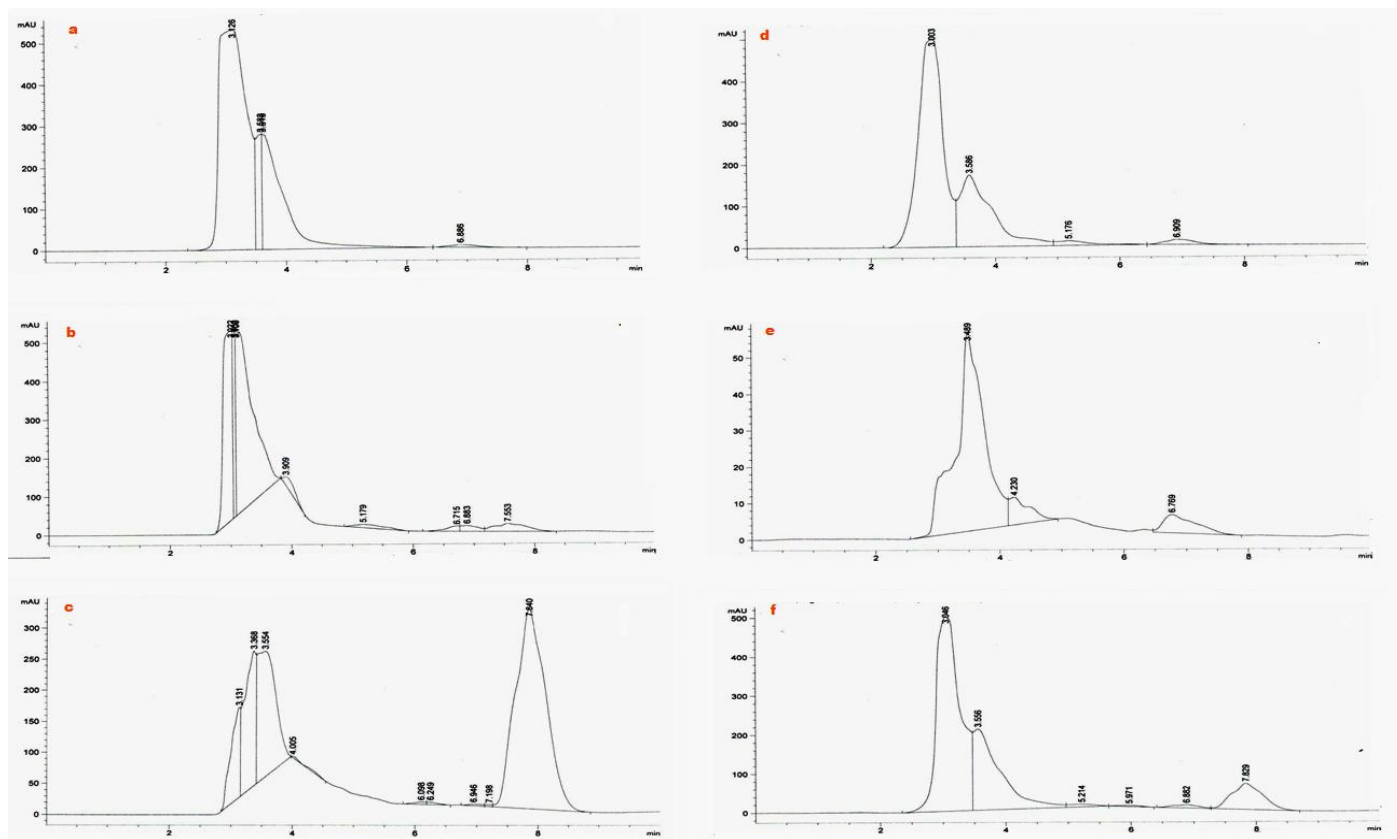


Fig. 1: Chromatographic fingerprints for the component raw materials used and the product (EAF-2011): a- *Alchornea cordifolia*, b-*Eugenia caryophyllata*, c- *Zanthoxylum zanthoxyloides*, d- *Psidium guajava*, e- *Tridax procumbens*, f- Ointment (EAF-2011)

Table 2: Results of inter-day repeatability test presented as % RSD for the HPLC method

Standard	Precision for R _T	Precision for Peak Area
Rutin		
1 mg/ml	0.12	0.05
0.5 mg/ml	0.06	0.11
Quercetin		
1 mg/ml	0.21	0.01
0.5 mg/ml	0.18	0.02
Kaempferol		
1 mg/ml	0.08	0.01
0.5 mg/ml	0.04	0.04

Table 3: System suitability analysis for the HPLC assay for the three flavonoid compounds

Standard	Theoretical Plates (>2000)	Injection Precision for area (% RSD)	Injection Precision for retention time (% RSD)
Rutin	6180.019	0.18	0.17
Quercetin	8641.921	0.07	0.034
Kaempferol	11774.8657	0.04	0.06

Table 4: Flavonoid contents of the component plant materials and the product

Plant Materials	Rutin (w/w)	Quercetin (w/w)	Kaempferol (w/w)
<i>Eugenia caryophyllata</i>	0.00	0.4080 (0.001)	0.0771 (0.00)
<i>Psidium guajava</i>	8.5860 (0.057)	0.5430 (0.00)	0.1003 (0.00)
<i>Alchornea cordifolia</i>	2.5540 (0.001)	0.0536 (0.00)	0.00
<i>Zanthoxylum zanthoxyloides</i>	5.8060 (0.001)	0.00	0.0174 (0.00)
<i>Tridax procumbens</i>	2.4390 (0.01)	0.1044 (0.00)	0.0505 (0.00)
Herbal product (EAF-2011)	8.6810 (0.00)	0.2665 (0.00)	0.0610 (0.00)

Inter-day repeatability performed as a measure of precision of the HPLC method confirmed procedure as being robust to some random laboratory variation such as temperature and humidity. Percentage relative standard deviation (% RSD) for all samples analysed was <2% (Table 2). This was also confirmed by the system suitability analysis which indicated a theoretical plate number >2000 and % RSD for area precision and retention time <1% (Table 3).

Limits of detection and quantitation for rutin were 0.0048 mg/ml and 0.0148 mg/ml respectively, quercetin (0.00304 mg/ml and 0.00921 mg/ml) and kaempferol (0.000142 mg/ml and 0.00043 mg/ml). The accuracy of the method was also demonstrated by the recovery studies after the injection of 1.5 mg/ml of rutin, quercetin and kaempferol individually. The peak area produced after indicated percentage recovery for rutin as 99.13%, quercetin (104%) and kaempferol (99.33%) and the RSD for the entire test samples as <1%.

Determination of the flavonoid content in plant raw materials and EAF-2011

Using the above chromatographic conditions, the content of rutin, quercetin and kaempferol in the five plant materials and the final product (EAF-2011) were determined (Table 4). Concentrations of rutin when present were found to be higher than other constituents present e.g. 8.586 mg/ml in *Psidium guajava*. The ointment also had all the assayed flavonoid compounds present (Figure 1).

DISCUSSION

The use of chemical markers during the standardisation of herbal medicines is a reliable technique for addressing the challenges associated with their quality control. Regulators require that when therapeutic constituents are known, those chemicals are used in the identification and standardisation of the herbal materials and their products. [3,7]

The markers selected for the quality control of the raw materials and the finished herbal product was based on their reported therapeutic activity and presence in the plant materials. [7] The flavonoid compounds were of interest because of their prospects and role in the antimicrobial activity of numerous medicinal plants. [8-9] Rutin has been reported to be active against *Candida albicans* and the gastrointestinal organisms like *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, *Staphylococcus aureus* and the dermatophytes: *Microsporum spp*, *Trichophyton spp* and *Epidermophyton spp*. [10-12] Quercetin and kaempferol have also proven to be efficacious against a similar spectrum of organisms. [13-14] These chemical markers can therefore be considered as ideal for use as active markers for the product and its raw materials. However, their ubiquitous presence in several other plant species means that they cannot be used as characteristic markers to check adulteration of the raw materials hence the importance of the other methods of standardisation previously described by Dickson. [5]

The suggested HPLC method was also ideal per the guidelines stipulated by the International Committee on Harmonisation (ICH). This document specifies that the regression coefficient (r^2) for all analytes should be >0.998 , % RSD for accuracy and precision should also be $<2\%$. System suitability analysis must also have theoretical plates >2000 and % RSD for injection precision (area and retention time) $\leq 1\%$. The HPLC method described is therefore ideal to be used for the quality control of the product based on these standard requirements.

The results of the analysis indicated that *Eugenia caryophyllata*, *Alchornea cordifolia* and *Zanthoxylum zanthoxyloides* did not have detectable quantities of rutin, kaempferol and quercetin respectively. The final product however contained all the tested flavonoids: 8.6810 % (w/w) of rutin, 0.2670 % (w/w) of quercetin and 0.0610 % (w/w) of kaempferol.

In conclusion, the high performance liquid chromatography remains an important tool in the step towards addressing the inconsistency of herbal medicinal products.

The flavonoid contents reported in this study can be included as part of the monograph for the product. The RP-HPLC method is also suitable for the detection and quantification of rutin, quercetin and kaempferol in the product (EAF-2011) and the raw materials used in its production to ensure consistency.

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