Research Article

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FAST DEVELOPMENT OF RP-HPLC METHOD FOR ESTIMATION OF RUTIN FROM AQUEOUS FRACTION OF NEW POLYHERBAL FORMULATION CONTAINING *CLINACANTHUS NUTANS* AND *ELEPHANTOPUS SCABER*

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Received on: 20/06/16 Revised on: 14/08/16 Accepted on: 12/09/16

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DOI: 10.7897/2277-4343.075199

ABSTRACT

Herbal medicinal products contain a blend of phytochemicals; each of these contains many chemical compounds that may give the anticipated activity. Therefore, it is very vital to analyse and evaluate the quality of various active constituents and markers. Objective of the study was to develop and validate stability indicating RP-HPLC gradient method for simultaneous estimation of rutin as biomarker from an Aqueous fraction of the new polyherbal formulation of *Clinacanthus nutans* and *Elephantopus scaber*. A Shimadzu HPLC was utilized to perform the analysis which was equipped with anautosampler, column oven, and UV/VIS detector. An HPLC column used was Merck Licrochart Purospher Start RP 18 column (250mm, 4.6 mm i.d, 5µm pore size).The temperature was maintained at 40.0 ºC throughout the study. The mobile phase was a binary mixture of methanol–water, 1:1 (v/v), adjusted to pH 3.0 with glacial acetic acid. The flow rate was 1 mL min^{-1}. Absorbance was monitored at λ = 360 nm. Average Retention time, variance and standard deviation of rutin from aqueous fraction of the polyherbal formulation at different concentration was found 4.1327, 0.00157 and 0.0396 respectively. Coefficient correlation of standard and sample was found linear R^2=0.9991 and R^2= 0.998 respectively. The P value of standard and sample was found significant (P <0.0001). The proposed analytical method was found to be fast and stable. It is recommended as a part of more reliable qualitative and quantitative analysis of new herbal formulation in the treatment of the wound.

Keywords: *Clinacanthus nutans*; *Elephantopus scaber*; Polyherbal formulation; Herb-Herb combination; Flavonoids; Wound healing; Rutin; HPLC

INTRODUCTION

Many important and undiscovered medicinal plants may possess the key for the treatments of different kind of diseases. Tropical forest is blessed with the huge gift of many significant medicinal plants from nature[1]. There are many medicinal plants that have been used for thousands of years. These plants can now be found in herbal products and as part of the traditional Malaysian healthcare system because of their therapeutic efficacy[2]. World Health Organization estimated that 80% of the word's inhabitants still rely mainly on traditional medicines for their health care[3]. The selection of plants is based on its commercial potential.

Elephantopus scaber and *Clinacanthus nutans* is a well-known medicinal plant in Malaysia. Both plants are well known in terms of their commercial potential and many types of research are currently being carried out in terms of its medicinal importance [4]. Polyherbal formulation is useful in the treatment of superficial wounds [5]. Our new formulation contains an active aqueous fraction of both herbal medicinal plants. Standardization of herbal formulation is essential in order to assess the quality of drugs [6,7]. Finding new method for identification of biomarkers from the polyherbal formulation is the target of our study to enhance the quality of the new product.

MATERIALS AND METHODS

Plant material

The leaves of *Clinacanthus nutans and Elephantopus scaber* were collected from Institute Of Sustainable Agrotechnology, Sg. Chuchuh, Universiti Malaysia Perlis (UniMAP) and washed using clean water. After that, the leaves were dried in a dryer at the temperature of 35-40ºC for two days. Once dried, the leaves were ground into a fine powder by using a mechanical grinder.

Preparation of Plant Extract

Soxhlet extraction was used in this experiment to extract the herbs. For Soxhlet extraction, a powder sample is weighted approximately. A powdered mixture containing equal proportions of two herbs (5 g each) was extracted with 100 ml of aqueous ethanol 50% for 12-hour extraction. The extract solution was then evaporated by using a rotary evaporator to remove the solvent in the extract solution and dried in an oven at 35-40 ºC for 12 hours.The extract was fractionated using different solvents viz. hexane, chloroform, ethyl acetate, n-butanol, and water. The supernatant was filtered using Whatman No. 1 sheet, pooled and concentrated using vacuum rotary evaporator. The concentrated solutions were then dried in an oven at 35 ºC to get the dry form of respective fractions. Both of medicinal herb is used as a decoction to heal the internal wound and apply externally to heal the wound. This is the reason for

selection of an aqueous fraction of the polyherbal formulation. Further studies are going on to evaluate its in-vitro and in-vivo wound healing activities.

Chemicals

Methanol (HPLC grade; Fischer scientific, USA), ethanol and glacial acetic acid (analytical reagent grade; HmbG, Germany) and Merck (Darmstadt, Germany). The sample was filtered through a 0.45µm nylon membrane filter into an HPLC vial prior to HPLC analysis. Solvent mixtures were filtered through a 0.45µm nylon membrane filter and degassed before use. Rutin as a standard compound was acquired from Sigma-Aldrich (USA).

Instrumentation

A Shimadzu HPLC was utilized to perform the analysis which was equipped with anautosampler, column oven, and UV/VIS detector. An HPLC column used was Merck LicrochartPurospher Start RP 18 column (250mm, 4.6 mm i.d, 5µm pore size).The temperature was maintained at 40.0 ◦C throughout the study.

Chromatography conditions

The mobile phase was a binary mixture of methanol–water, 1:1 (v/v), adjusted to pH 3.0 with glacial acetic acid. The flow rate was 1 mL min^{-1}. Absorbance was monitored at λ = 360 nm.

Preparation of stock solution

A standard stock solution of rutin was prepared by dissolving 10.2 mg of rutin in ethanol, yielding 25 mL of a concentration stock = 0.41 mg mL^{-1}. Series of dilutions were prepared by aliquoting 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0 and 5.0 mL of the standard stock solution and diluted with the mobile phase to yield 10 mL of standard solutions containing 2, 4, 8, 20, 40, 80, 120 and 200µg mL^{-1} of rutin, respectively. The sample was prepared in the same manner as standard.

RESULT

Method development

The main objective of the chromatographic method was to identify rutin as one of bioactive compound inside a new polyherbal formulation. This new polyherbal formulation contains an equal amount of *Clinacanthus nutans* and *Elephantopus scaber* in the treatment of the wound. We successful identify rutin with respect to retention time as compared to standard purchased from sigma-aldrich.

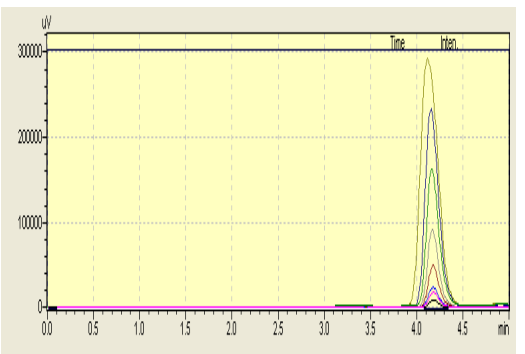

Figure 1: Chromatogram for Standard Rutin at different concentration

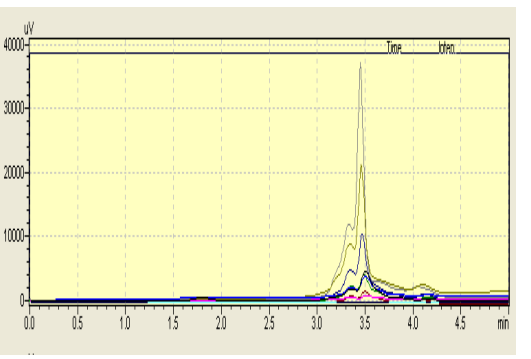

Figure 2: Chromatogram from aqueous fraction of polyherbal formulation at different concentrations

Table 1: Retention time for Standard Rutin at different concentration

Standard concentration (ug/ml)	Retention time (Rt)	Concentration found	Area	%Area
2	4.179	4.08	81217	89.24
4	4.146	7.37	146937	96.59
8	4.174	9.82	195336	96.82
20	4.174	21.44	426475	98.69
40	4.167	49.77	870998	99.26
80	4.161	78.05	1552511	97.80
120	4.151	123.48	2456005	98.08
200	4.115	198.22	3942422	98.34

Table 2: Retention time for aqueous fraction of polyherbal formulation at different concentration

Standard concentration (µg/ml)	Retention time (Rt)	Concentration found	Area	%Area
2	4.160	0.16	3313	3.90
4	4.160	0.06	1329	9.84
8	4.160	0.16	3238	5.75
20	4.158	0.13	2611	8.03
40	4.149	0.37	7537	10.03
80	4.125	0.70	14889	8.94
120	4.099	0.97	19398	7.32
200	4.051	0.56	11139	2.93

Table 3: Average Retention time, variance and standard deviation for aqueous fraction of polyherbal formulation at different concentration

Parameters	Average Retention time	Variance (s^2)	Standard deviation (s)
Standard	4.1583	0.00044	0.0209
Sample	4.1327	0.00157	0.0396

Correlation coefficient

The results of standard and sample were found to be statistically significant. Figure 3 and 4 explain the results in the form of concentration (µg/ml) on x-axis and Area under y-axis

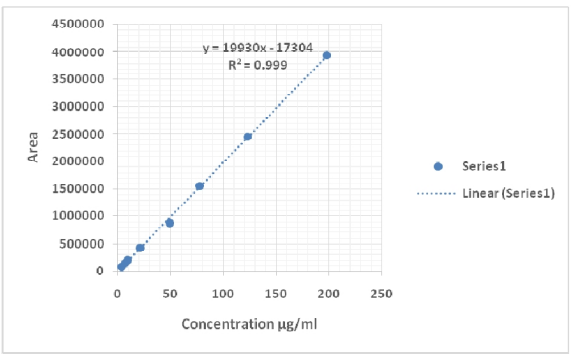

Figure 3: Correlation coefficient of Standard Rutin at different concentration

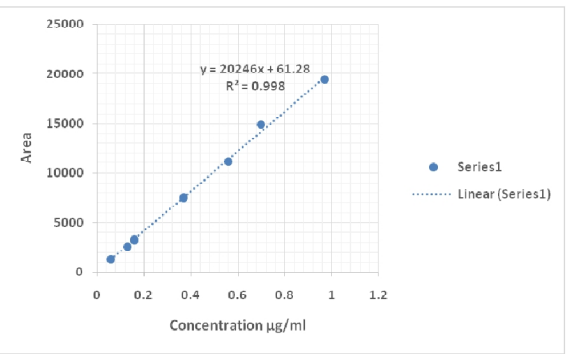

Figure 4: Correlation coefficient of aqueous fraction of polyherbal formulation at different concentration

Linearity

Linearity data were obtained by plotting the area of the standard rutin and sample peak, expressed in area units, against the concentration of rutin expressed as µg ml^{-1}. A linear regression least square analysis was performed in order to determine the slope, intercept, and coefficient of determination. The values of the slope, intercept, and coefficient of determination of the calibration curve for standard and sample are given in figure 3 and 4 respectively. The high value of the coefficient of determination indicated good linearity.

Limit of detection (LOD) and Limit of quantification (LOQ)

LOD and LOQ were determined based on the standard deviation of the response (Y-intercept) and the slope of the calibration curve at low concentration levels according to ICH guidelines. The LOD and LOQ for standard and sample were mentioned in table 4 .

Table 4: Linearity, LOD, LOQ

Parameter	Standard	Sample
LOD	7.50	0.05
LOQ	22.74	0.15
Slope	19930	20246
SEM	45336.61	316.8471
Corr. Coefficient (R^2)	0.9991	0.998
95% Confidence interval	0.9973 to 0.9999	0.9943 to 0.9998
P value	<0.0001	<0.0001

DISCUSSION

An easy, cost-effective, specific and precise method has been developed for the analysis of rutin in polyherbal formulations of *Clinacanthus nutans* and *Elephantopus scaber*.The relative retention times of characteristic peaks in the HPLC fingerprint will helps to establish as an important parameter for identification of rutin from new formulation. This will help to increase the quality of herbal formulations. *Clinacanthus nutans* contains C-glycosidic flavones such as shaftoside, isoorientin, orientin, isovitexin, and vitexin in the leaves of this plant[8]. *Elephantopus scaber* contains flavonoids aglycosides such as luteolin[9].Rutin was not identified as a biomarker in both of medicinal plants. By a combination of both herbs, rutin may arise as new flavonoids. Rutin plays an important role in healing wound and Antioxidant[10].Anti-oxidant plays an important role in preventing oxidizing of molecules [11].The results of standard and sample were found to be statistically significant ($P<0.0001$) with coefficient correlation (R^2) 0.9991 and 0.998 respectively. LOD and LOQ of thesample (rutin) were found only 0.05 and 0.15 respectively.Further fractionation using column chromatography is required to isolate rutin.

CONCLUSION

Rutin which is also known as rutoside, quercetin-3-O-rutinoside and sophorin are well-known flavonoids. We successfully identified rutin from the newly developed formulation in the treatment of the wound. This method will help to identify rutin in the much faster way and improving the quality of herbal formulation for the future.

ACKNOWLEDGEMENTS

Special thanks to the Institute of Sustainable Agrotechnology, Sg. Chuchuh, Universiti Malaysia Perlis (UniMAP) for

providing the samples. This research was supported by the Prototype ResearchGrant Scheme (PRGS; Reference no : PRGS/9013-00016) awarded by the Ministry of Higher Education (MOHE), Malaysia, Universiti Malaysia Perlis (UniMAP), Ministry of Higher Education Malaysia [Malaysian International Scholarship; Reference no. KPT.B.600-18/3 JLD 6 (31)] and Research Collaborative Effort from Malaysian Agricultural Research and Development Institute, Serdang, Malaysia.

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Cite this article as:

Muhammad Shahzad Aslam, Muhammad Syarhabil Ahmad, AwangSoh Mamat, Muhammad Zamharir Ahmad. Fast development of RP-HPLC method for estimation of Rutin from aqueous fraction of new polyherbal formulation containing *Clinacanthus nutans* and *Elephantopus scaber*. Int. J. Res. Ayurveda Pharm. Sep - Oct 2016;7(5):78-81 http://dx.doi.org/10.7897/2277-4343.075199

Source of support: Prototype Research Grant Scheme, Malaysia, Conflict of interest: None Declared