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Bio-Active Constituents of Rotenoids Resin Extracted from *Derris elliptica* Roots: Comparison between Local Plant Extract and SAPHYR (France) Cube Resin

[1]Saiful Irwan Zubairi, [2]Mohamad Roji Sarmidi and [2]Ramlan Abdul Aziz

[1]School of Chemical Sciences and Food Technology, Faculty of Science and Technology, The National University of Malaysia, 43600 UKM Bangi, Malaysia
[2]Institute of Bioproduct Development, Universiti Teknologi Malaysia, 81310 UTM Skudai, Malaysia

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ABSTRACT

It is well known now that some plant species represent an efficient factory of chemicals, which are manufactured and used as bio-weapons against pest attacks. Extensive work has been done since the last few decades on these potentially useful compounds. During the last few decades a growing interest was directed towards a safer agricultural production such as free residual toxicity hazards to human beings and to the environment. For instance, plant-based extracts biocides possess a greater advantage compared with the chemical ones. Their efficacies are also acceptable. In this work, the aim is to standardize and determine the bio-active constituents extracted from local plant of Derris species (*Derris elliptica*) by using an internal standard method of reversed-phase high performance liquid chromatography (RP-HPLC) system. The raw plants were collected from Kota Johor Lama, Malaysia and sorted to collect the roots and stems. Rotenoids resin from the roots and stems were extracted by using a normal soaking extraction (NSE) method at 30 ± 2 °C. A solvent of acetone with an optimum solvent-to-solid ratio of 10 ml/g was utilized in the extraction process. The extraction was carried out for 24 hrs and the extract was filtered to remove any fine debris prior to the RP-HPLC analysis. The commercially available rotenoids resin cube of SAPHYR (France) was analyzed to compare and verify the bio-active constituents available with the extract of local plant species. The RP-HPLC analysis results showed the elicited distinguishable patterns of the bio-active constituents between the extract of *Derris elliptica* and commercial grade of rotenoids cube resin. This extract has a great potential to be used as insecticidal products. However, the rotenone content is still low as compared to the commercial grade rotenoids resin. For that reason, a plant tissue culture is needed to produce a hybrid species between *Derris elliptica* and Amazonia species so that high yield of rotenone could be attained.

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INTRODUCTION

Derris elliptica or 'Tuba' as it is known locally is an insecticidal plant in Malaysia that has been used for the purpose of bio-pesticide production. 'Tuba' plant is a kind of woody creeper plant and climber. It needs at least 75% soil moisture content and the surround temperature should be in between 25 to 30 °C to obtain high content of the rotenone during its development. A calm area with low acidity soil content will enhance the production of rotenone [1]. 'Tuba' is a member of the *Leguminosae* and *Fabaceae* family which comprises 200 genera and 68 species including 21 species of *Tephrosia*, 12 of *Derris*, 12 of *Lonchocarpus*, 10 of *Millettia* and several of *Mundula* [2]. Three species are found in Malaysia, which are *Derris elliptica*, *Derris malaccensis* and *Derris uliginosa*. *Derris* is a climbing plant in Southeast Asia and its roots contain rotenone, a strong insecticide [3]. *Derris elliptica* and *Derris malaccensis* contain approximately 4 to 5% (w/w) rotenone while *Lonchocarpus utilis* and *Lonchocarpus urucu* contain 8 to 10% (w/w) rotenone in dry roots [4]. Rotenone has been found to be used in many applications besides as insecticide. In addition to its effectiveness for both piercing-sucking insects, such as aphids and red bugs and chewing insects, especially caterpillars upon plants, it makes excellent dusts for external parasites of animals such as fleas and lice. The side effect of rotenone to aquatic animals is minimal [5]. The toxic principles all deteriorate rapidly into dihydrorotenone (non-toxic substance) and water when exposed to sunlight and air; spray and dusts usually lose their effectiveness within a week after application

Corresponding Author: Saiful Irwan Zubairi, School of Chemical Sciences & Food Technology, Faculty of Science & Technology, The National University of Malaysia, 43600 UKM Bangi, Malaysia.
Phone: +603-89215989; E-mail: saiful-z@ukm.edu.my

[6]. The outstanding advantages of this group of poisons are that they are harmless to plants (phyto-toxic), relatively non-toxic to man and act as both contact and stomach poisons to insects [2]. Even though it has several outstanding properties, there is one problem has not yet been resolved up to now. A variety of its active ingredients with respect to different kinds of species have been a major problem ever since. In this paper our goal is to examine the rotenone content in the extract of local *Derris* species and to compare it with the commercially available rotenone resin cube extracted from the Amazonian *Derris* species. The aim of this work is to also to produce a standard bio-active constituent profile of rotenone extract from *Derris* plant species available in Malaysia.

MATERIALS AND METHODS

Plant collection:

Derris elliptica roots were collected in the state of Johor; Kota Johor Lama, Malaysia.

Raw material preparation:

An important aspect of the phytochemical processing is the pre-processing of the herbal material prior to extraction. The treatment of the herbal material affects the viability of the phytochemical as well as the extraction yield. The freshly procured *Derris* roots had to immediately undergo the cleaning process to remove dirt and soil. The roots were kept and dried in an oven overnight at 30 ± 2 ^{o}C and sorted to collect only the roots and stems. The roots and stems were cut into small pieces using knife mill prior to grinding.

Rotenoids cube resin:

The commercial grade of dried rotenoids cube resin was obtained from SAPHYR (France) with the purity of 50% (w/w). The sample was believed to be extracted from the Amazonian native species of *Lonchocarpus nicou* and *Lonchocarpus urucu* dried roots. The cube resin was later dissolved in acetonitrile to a concentration of 0.22 mg/ml prior to the analysis. The molecular structure of essential bio-active constituents available in the extract and SAPHYR (France) cube resin are shown in Figure 1.

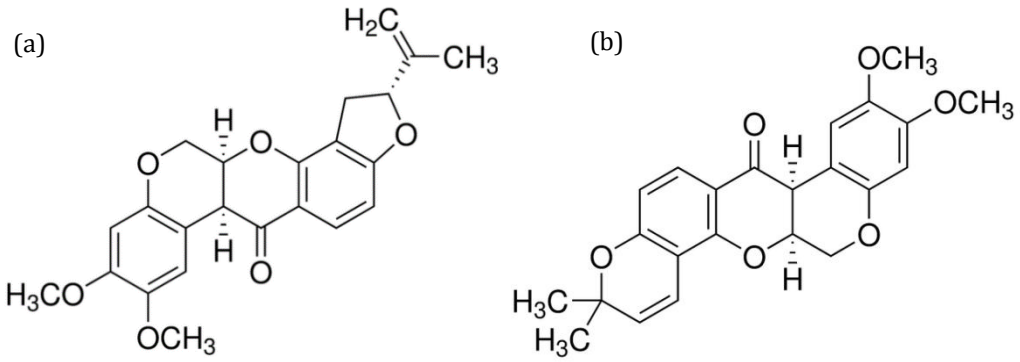


Fig. 1: Molecular structure of (a) rotenone and (b) deguelin that contains in the liquid crude extract and SAPHYR (France) cube resin [4,7,8].

Extraction process:

The extraction was carried out by soaking 30 g of dried roots and stems in 300 ml of solvent; acetone, 95% (v/v) for 24 hrs at 30 ± 2 ^{o}C. The liquid crude extract (LCE) was then filtered using Whatman filter paper No. 4 with the aid of Altech filter GAST laboratory diaphragm vacuum pump at 300 mbar.

Analysis of liquid crude extract and rotenoidscube resin:

The liquid crude extract and SAPHYR (France) rotenoids cube resin were subjected to a quantitative analysis using reverse-phase high performance liquid chromatography (RP-HPLC) to determine the yield of rotenone and other toxic constituents. The ultra violet (UV) photodiode array (PDA) detection was used at a wavelength of 294 nm. The analysis of the extract solutions was carried out using an internal standard method (curcumin, analytical grade, 97% (w/w) - SIGMA-Aldrich™as an internal standard solution) [9]. The operational parameters are shown in Table 1. A C-18 Waters™ Corp. liquid chromatography stainless steel column with particle size of 10 μm (3.9 mm internal diameter× 150 mm length), analytical grade of rotenone standard with known purity (PESTANAL®, analytical grade, 96.2% (w/w); SIGMA-Aldrich™), analytical grade of acetonitrile; 99.9% (v/v) and deionized water (DOW) were utilized in the analysis. The mobile phase system

was prepared by diluting 3000 ml of acetonitrile into 2000 ml of deionized water (60:40) and filtered through a cellulose nitrate membrane filter (0.45 µm pore size filter) to remove impurities and fine dirt [7].

Table 1: Operational parameters of an isocratic solvent system RP-HPLC.

Column temperature	Ambient (30 ± 2 ^{o}C)
Flow rate	0.7 ml/min
Wavelength (λ)	294 nm
Injection volume	5 µl
Retention times (t): a) Rotenone b) Internal standard	 3.55 min - 3.60 min 2.87 min - 3.00 min

Preparations of standard solutions:

To prepare an internal standard solution, about 0.035 ± 0.01 g of curcumin standard was dissolved in acetonitrile, making up to a volume of 50 ml of glass-stoppered conical flask. As for rotenone standard solution, about 0.0137 ± 0.01 g of rotenone standard was dissolved in acetonitrile, making up to a volume of 50 ml of glass-stoppered conical flask. Rotenone calibration solution: 10 ml of internal standard solution was added into 10 ml of rotenone standard solution using pipette. The mixture was homogenized using a laboratory shaker to produce a well dissolved solution.

Sample solution preparation and analysis:

To prepare a sample stock solution, 2 ml of the liquid crude extract (LCE) containing unknown rotenone concentration was transferred into a 50 ml glass-stoppered conical flask. By using the same pipette used for the calibration solution, 2 ml of the internal standard solution was mixed and diluted to 100 ml in other volumetric flask. Meanwhile, to start up the analysis, the prepared mobile phase which is a mixture of acetonitrile and water (60:40) was allowed to go through into the column overnight until the system is equilibrated (flat baseline). 5 µl of the calibration solution and sample solution was injected into the system. Repetitive injections of both solutions were carried out to achieve stable responses that agree with 1% of the rotenone peak area (or height) to the internal standard peak area (or height) ratio. Additionally, the peak area (or height) ratio for the sample solution must not differ by more than 10% from the peak area (or height) ratio for the calibration solution. Equation 1 shows the formula of calculating the content (mg) and concentration (mg/ml) of rotenone via internal standard method.

$$A_x/[_x] = F\ (A_{IS}/[_{IS}]) \quad (1)$$

Where:
A_x= Peak area of analyte *x*; $[_x]$ = Concentration of analyte *x*; F = Response factor; A_{IS} = Peak area of internal standard and $[_{IS}]$ = Concentration of internal standard.

Statistical analysis:

Data is presented as mean ± standard deviation (sd) of mean. Statistical comparisons were performed using Students *t*-test (PASW version 17.0 IBM Co.). A $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Figure 2, 3 and 4 show chromatogram of SAPHYR (France) rotenoids cube resin and sample solution using an internal standard (IS) solution of curcumin. The area under the curve of each peak (chromatogram) is proportional to the concentration of each standard solution used and injected into the column. However, if the external standard is implemented, the sensitivity of the RP-HPLC detector in identifying the specific bio-active constituents (rotenone) could generally be compromised with different responses to each standard component used. For that reason, an internal standard method was carried out to determine the concentration of rotenone in the liquid crude extract of *Derris elliptica* and to compare the bio-active constituent profile with the commercially available rotenoids cube resin manufactured by SAPHYR (France). However, the SAPHYR's rotenoids cube resin had to be analyzed to verify the availability of rotenone and other constituents as a certificate of analysis (COA) of the given product was not included and disclosed.

Rotenone calibration solution:

The concentration of rotenone standard comprising theoretical amount of tephrosin and deguelin was prepared as follows: 0.0137 g/50 ml × 96.2% (rotenone purity) = 0.26 mg/ml rotenone, purity tephrosin ≅ 1.5% (w/w): (0.015 × 0.0137 g)/50 ml = 0.004 mg/ml tephrosin and purity deguelin; ≅ 0.5% (w/w) = (0.0005 × 0.0137 g)/50 ml = 0.00014 mg/ml (deguelin). The assumed approximate purity of tephrosin and deguelin was

based on the data acquisition of the rotenone standard (Fig. 2). Meanwhile, the prepared internal standard concentration of curcumin can be calculated based on the observed prominent peak chromatogram (Fig. 2): 0.035 g/50 ml × 97% (purity) = 0.68 mg/ml curcumin. As the respective bio-active constituent concentration was determined, the response factors (F) were calculated with respect to its respective area under the curve (Fig. 2). Equation 1 was used to calculate the F value and is presented in Table 2. All respective peaks show different profiling responses (e.g., area under the curve and retention time) and can be distinguished accordingly (p<0.05). Rotenone produced the highest peak area under the curve as compared to tephrosin (p<0.05). However, deguelin was undetected due to its minute amount in the standard solution and low sensitivity of the detector used.

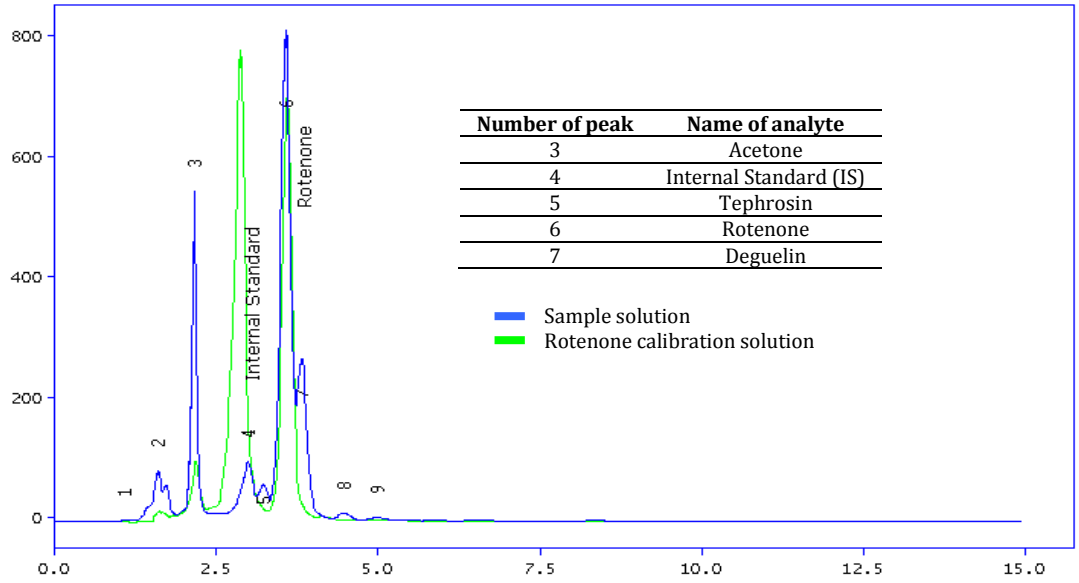


Fig. 2: Chromatogram of sample solution [rotenone crude extract (x) + internal and standard (IS)] calibration solution [rotenone standard solution + internal standard solution (IS)].

Table 2: Bio-active constituent profiles of analyzed standard solutions.

[#]Calibration solution	Concentration (mg/ml)	Peak area (mV*s)	Response factor (F)
Rotenone standard	0.26	8445.57 ± 230[Ψ]	2.08 ± 0.52
Tephrosin	0.004	1111.83 ± 120	17.86 ± 1.22
Deguelin	0.00014	Undetectable	Not determined
Internal standard (IS)	0.68	10583.18 ± 173	Not applicable

[#]Results shown are means (±sd) of 3 injection/sample (n = 3). ([Ψ]) p<0.05 compared to the other compounds.

Table 3: Bio-active constituent profiles of analyzed sample solutions.

[#]Sample solution	[*]Concentration (mg/ml)	Peak area (mV*s)
Rotenone (x)	[a]1.95 ± 0.52[Ψ]	9174.95 ± 112[Ψ]
Tephrosin (y)	0.03 ± 0.01	622.95 ± 233
Internal standard (IS)	[b]0.014	1553.47 ± 120

[a]Rotenone concentration (based on Equation 1): 9174.95/[$_x$] = 2.08 (1553.47/0.0136); [$_x$] = 0.039 mg/ml. Thus, the corrected rotenone concentration using dilution factor: 0.039 mg/ml × (100 ml/2 ml) = 1.95 ml. [b]Concentration of (IS) in the unknown solution is therefore: C (IS) = 1.36 mg/100 ml = 0.0136 mg/ml. [#]Results shown are means (±sd) of 3 injection/sample (n = 3). ([Ψ]) p<0.05 compared to the other compounds.

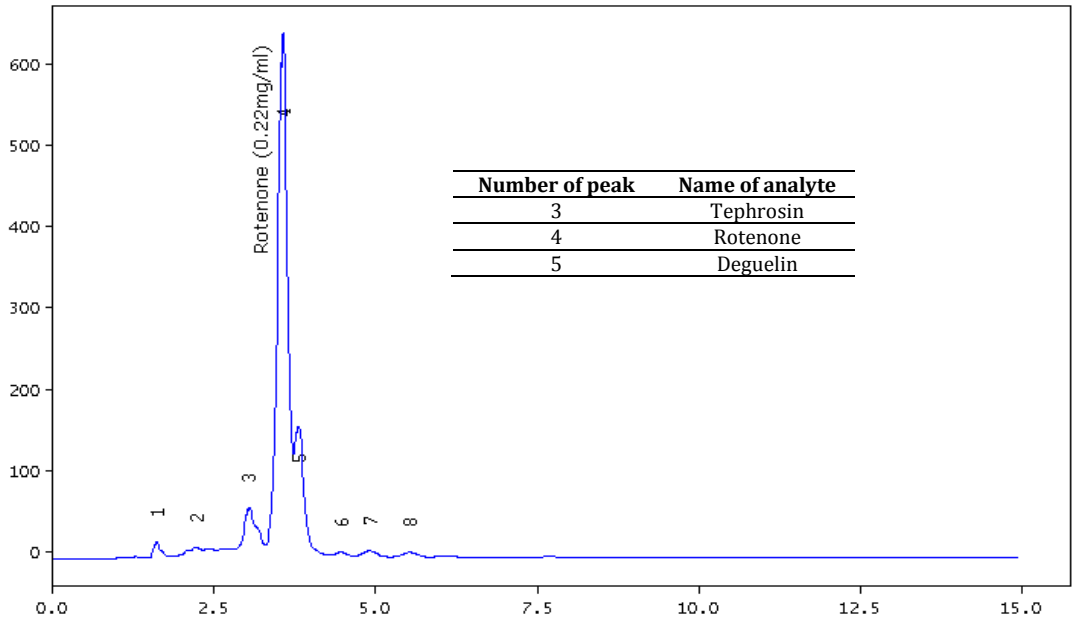
Table 4: Yield of bio-active constituents available inside *Derris* liquid crude extract.

Sample solution	[a]Yield (mg)	[b]% Yield (w/w)
Rotenone	140.4 ± 0.02	1.40 ± 0.02
Tephrosin	1.44 ± 0.01	0.014 ± 0.01
[c]Rotenoids resin	2,070 ± 0.05	20.70 ± 0.04
[d]Other constituents	7,780 ± 0.03	77.84 ± 0.05

[a]Yield of rotenone (mg) = C (sample) × volume of liquid crude extract (ml). [b]Yield of rotenone in dried roots (%) = Yield of rotenone (mg)/weight of dried roots × 100%. Density of LCE: 0.98 ± 0.02 g/ml. Acetone density: 0.791 g/ml. Density of rotenoids resin: 0.82 - 0.791 = 0.03 g/ml. Total volume of LCE: 70.56 ± 0.5 ml. [c]Theoretical rotenoids resin available inside the LCE = 0.03 × 70.56 ml = 2.07 g. [d]10 g of dried roots consist of dried bark, stem, lipid and wax (exclude active ingredients and resin).

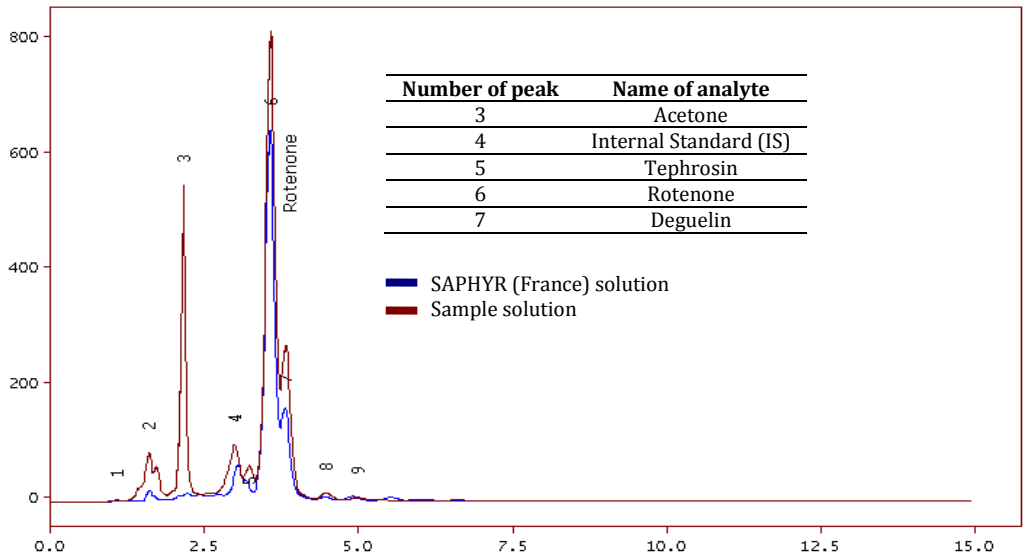
Sample solution:

To determine the unknown concentration of rotenone and tephrosin (analyte *x* and *y*), 2 ml of internal standard solution (0.68 mg/ml) was added to another 2 ml of unknown solution. The mixed solution was diluted to 100 ml in volumetric flask. As predicted, the analysis of the mixture showed in similar retention time ($p>0.05$) with diverse peak area under the curves of rotenone, tephrosin and curcumin ($p<0.05$). The bio-active constituent profiles of the analyzed sample solutions available inside the LCE are shown in Figure 2 and Table 3. The availability of the bio-active constituents especially rotenone was quite consistent with the findings from the previous reports [10, 11]. Finally, the yield of all constituents available inside the sample solution (LCE) was calculated based on the area under the curves obtained (Table 4). The yield of rotenone in dried roots was slightly higher than the previous reported study (1.14% (w/w)) ($p<0.05$) [13]. In addition, the sample solution profile was compared with the commercially available rotenoids cube resin manufactured by SAPHYR (France) (Fig. 3). Comparable peaks retention time was presented in Figure 4. Both solutions presented the same pattern and this result confirmed the existence of rotenone in the liquid crude extract [9,12].



Number of peak	Name of analyte
3	Tephrosin
4	Rotenone
5	Deguelin

Fig. 3: Chromatogram of SAPHYR (France) rotenoids cube resin (C_{cube} = 0.22 mg/ml).



Number of peak	Name of analyte
3	Acetone
4	Internal Standard (IS)
5	Tephrosin
6	Rotenone
7	Deguelin

Fig. 4: Comparison between SAPHYR (France) rotenoids cube resin and sample solution.

Conclusion:

The results showed that rotenone extracted from local plant species was comparable to the standard analytical grade purchased from SIGMA-Aldrich^{TM}. It was also identical to the commercial grade rotenoids cube resin given by SAPHYR (France). Based on the chromatogram in Figure 3, besides rotenone (6), the liquid crude extract consisted of other insecticidal compounds which were tephrosin (5) and deguelin (7). These compounds are essential for the insecticidal action against the Lepidopteron insect pest of cabbage (*Spodoptera litura*). Furthermore, the analysis method used in this work was based on the AOAC official method. However, several parameters were adjusted in order to achieve high accuracy in identifying bio-active constituents. Moreover, the internal standard method used was considered the most reliable and accurate analysis method employed as compared to the external standard method. This method not only has all the advantages of the external standard method but it also compensates for variations in injection volume and for small changes in detector sensitivity or chromatographic changes that might have occurred. Because we do not need to inject exactly the same amount each time, this method generally has better precision than the use of an external standard. On top of that, ultra clean apparatus, equipment and chemical should also be given due consideration in order to obtain the optimum result of separation.

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