STANDARDIZATION OF THE BIO-ACTIVE COMPOUNDS (ROTENOIDS) FROM THE EXTRACT OF LOCAL PLANT SPECIES (*Derris elliptica*) USING THE INTERNAL STANDARD METHOD OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

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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 during the last few decades on these potentially useful compounds. During the last few decades a growing interest has been paid for safe agricultural production i.e free residual toxicity hazards to human beings and to the environment. Plant extracts-based biocides possess a great advantage compared with the chemical ones. Their efficacies are also acceptable. Research carried out was to standardize and determine the bio-active compounds from the extract of local plant species (*Derris elliptica*) using the internal standard method of the isocratic High Performance Liquid Chromatography (HPLC) analysis system. The raw plants were collected from Kota Johor Lama, Johor and sorted to collect the root and stem. Only the root and stem were utilized as a raw material of the extraction process. The root and stem were extracted using the Normal Soaking Extraction (NSE) process at 28 ^{0}C to 30 ^{0}C with 95.0 % (v/v) of Acetone as a solvent and the solvent-to-solid ratio of the extraction is (10.0 ml/g). The extraction was carried out for 24 hours and further cleaned up to remove fine debris of root and stem prior to determination of the rotenone and its derivatives content. The rotenone cube resin of SAPHYR S.A.R.L (France) was used to verify the appearances of the compounds in the extract. The employed method of analysis shows significant appearances of the bio-active compounds in the extract compared with the commercial grade of rotenone cube resin.

Keywords: *Derris elliptica*; rotenone; rotenone cube resin; internal standard; HPLC

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 75.0 % soil moisture content and the surround temperature should be 25 ^{0}C to 30 ^{0}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 (SAPHYR, 1986). Tuba is a member of the *Leguminosae, 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* (John, 1944). Three species are found in Malaysia, which are *Derris elliptica*, *Derris malaccensis* and *Derris uliginosa* (Gaby Stroll, 1986). *Derris* is a climbing plant of Southeast Asia and its roots contain rotenone, a strong insecticide (Hutchison Encyclopaedia, 2000). *Derris elliptica* and *Derris malaccensis* contains 4.0 % (w/w) to 5.0 % (w/w) rotenone while *Lonchocarpus utilis* and *Lonchocarpus urucu* contain 8.0 % (w/w) to 10.0 % (w/w) percent rotenone in dry roots (Kole *et al.*, 1992). Many uses have been found for these insecticides. In addition to their effectiveness for both piercing-sucking insects, such as aphids and red bugs and chewing insects, especially caterpillars upon plants, they make excellent dusts for external parasites of animals such as fleas and lice. 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 (Schnick, 1974). 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 (John, 1944).

METHODOLOGY

Plant collection - *Derris elliptica* is collected in the state of Johor; Kota Johor Lama, Malaysia.

Raw material - 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 procured Tuba roots were immediately undergoes cleaning process to remove dirt and soil. The procured tuba roots were kept and dried into oven for overnight at room temperature (28 ^{0}C to 30 ^{0}C) and sorted to collect the root and stem. Only root and stem were utilized. The root and stem were cut into small pieces prior to grinding.

Rotenone cube resin - Rotenone cube resin was obtained from the SAPHYR S.A.R.L (France) contains 5.0 % (w/w) rotenone in dried roots of *Lonchocarpus nicou* and *L. urucu* with 50.0 % of pure rotenone in cube resin. Rotenone cube resin was dissolved in acetonitrile with a concentration of 0.22 mg/ml and used to compare the significant appearances between the commercial grade of rotenone cube resin and the extract of local plant species

Extraction apparatus and procedure - The extraction was carried out by soaking 30.0 g of dried root and stem in 300 ml solvent; acetone, 95.0 % (v/v) for 24 hours at room temperature (28 ^{0}C to 30 ^{0}C). The crude extract was then filtered using the Whatman filter paper no. 4 with the aid of Altech filter with GAST laboratory diaphragm vacuum pump and compressor at 300 mbar.

Analysis of crude extract and rotenone cube resin - The extract solution and SAPHYR S.A.R.L (France) rotenone cube resin were subjected to quantitative analysis using reverse-phase High Performance Liquid Chromatography (HPLC) to determine the yield of rotenone and other toxic constituents. UV (Photodiode Array - PDA) detection at 294 nm was implemented and the analysis of extract solutions were carried out using the internal standard method (curcumin, analytical grade, 97.0 % (w/w) - SIGMA-Aldrich^{TM} as an internal standard solution) (Ralph & Ronald, 1976).

Apparatus & Reagents - Waters Corp. (C18) liquid chromatography stainless steel column with particles size of 10.0 µm (3.9 mm I.D × 150 mm length), analytical grade of rotenone standard with known purity (PESTANAL®, analytical grade, 96.2 % (w/w) - SIGMA-Aldrich^{TM}), analytical grade of acetonitrile; 99.9 % (v/v) and deionized water (DOW). Mobile phase (isocratic solvent system) - A volume of 3000 ml of acetonitrile was diluted with 2000 ml of deionized water with a ratio of 60:40 and filtered through cellulose nitrate membrane filter (0.45 µm pore size filter) to remove impurities and fine dirt (Rodney & Alan, 1976)

Operating conditions for High Performance Liquid Chromatography - The conditions given below are typical values and may have to be adjusted to obtain optimum results from the given apparatus (AOAC, 2000).

Column temperature	Ambient
Flow rate	0.7 ml/min
Wavelength (λ)	294 nm
Injection volume	5 µl
Retention times:	
a) Rotenone	3.55 min - 3.60 min
b) Internal standard	2.87 min - 3.00 min

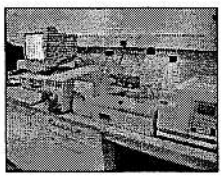


Figure 1.0: HPLC equipment – An isocratic solvent system

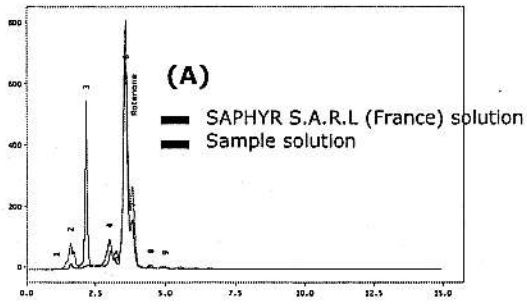
a) Internal standard solution - Weigh about 0.035 g of curcumin standard into 50 ml glass-stoppered conical flask and top up with Acetonitrile until it reaches up to 50 ml of solution volume.
b) Rotenone standard solution - Weigh about 0.0137 g of rotenone standard into 50 ml glass-stoppered conical flask and top up with Acetonitrile until it reaches up to 50 ml of solution volume.
c) Rotenone calibration solution - Add by pipette 10.0 ml of internal standard solution with 10.0 ml of rotenone standard solution and mix to dissolve.

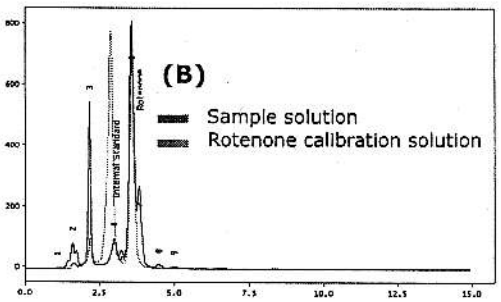
Sample solution preparation and analysis:
Measure 2.0 ml of the crude extract containing unknown rotenone concentration into a 50.0 ml glass-stoppered conical flask. Add using the same pipette as that used for the calibration solution, 2.0 mL of the internal standard solution and then the mixture is diluted to 100 ml in volumetric flask.

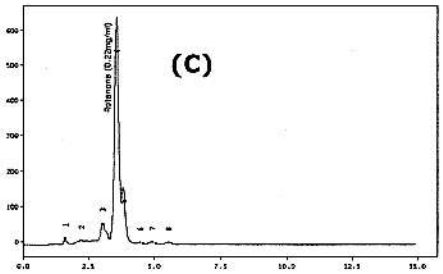
Pump the mobile phase, a mixture of acetonitrile and water (60:40), through column overnight until the system is equilibrated (flat baseline). Inject 5.0 µl of the calibration solution. Make repetitive injections of calibration solution until the response is stable and the ratios of the rotenone peak area (or height) to the internal standard peak area (or height) for successive injections agree to within 1.0 %.

Inject 5.0 µl of sample solution. The peak area (or height) ratio for the sample solution must not differ by more than 10.0 % from the peak area (or height) ratio for the calibration solution.

RESULT & DISCUSSION







(A) Comparison between SAPHYR S.A.R.L (France) rotenone cube resin and sample solution

(B) Chromatogram of sample solution [rotenone crude extract (X) + internal and standard (IS)] calibration solution [rotenone standard solution + internal standard solution (IS)]

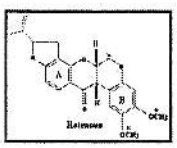
(C) Chromatogram of SAPHYR (FR) rotenone cube resin (0.22 mg/ml cube resin concentration)

(A) & (B)

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

(C)

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



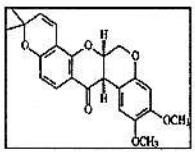


Figure 2: Molecular structure of rotenone & deguelin that contains in the extract and SAPHYR S.A.R.L (France) cube resin.

1.0 Calculation

In the chromatogram, the area under each peak is proportional to the species injected into the column. However, the detector generally has a different response to each component.

1.1 Rotenone calibration solution

a) Concentration of rotenone standard = 0.0137 g/50 ml × 96.2 % (w/w); purity = 0.26 mg/ml
Concentration of tephrosin; purity ≅ 1.5 % (w/w) = (0.015 × 0.0137 g)/50 ml = 0.004 mg/ml
Concentration of deguelin; purity ≅ 0.5 % (w/w) = (0.0005 × 0.0137 g)/50 ml = 0.00014 mg/ml
(Purity of tephrosin & deguelin are approximately based on the data acquisition rotenone standard)

b) Concentration of internal standard = 0.035 g/50 ml × 97.0 % (w/w); purity = 0.68 mg/ml

Calibration solution	Concentration (mg/ml)	Peak area (mV*s)
Rotenone standard	0.26	8445.57
Tephrosin	0.004	1111.83
Deguelin	0.00014	Undetectable
Internal standard (IS)	0.68	10583.18

A_x = Peak area of analyte X
$[x]$ = Concentration of analyte X
F = Response factor
A_{IS} = Peak area of internal standard
$[IS]$ = Concentration of internal standard

$A_x/[x] = F\ (A_{IS}/[IS])$ ---------- (a)

8445.57/0.26 = F (10583.18/0.68)
F (rotenone) = 32482.96/15563.5 = **2.08**

1111.83/0.004 = F (10583.18/0.68)
F (tephrosin) = 277957.5/15563.5 = **17.86**

1.2 Sample solution

Sample solution	Concentration (mg/ml)	Peak area (mV*s)
Rotenone (x)	?	9174.95
Tephrosin (y)	?	622.95
Internal standard (IS)	0.014	1553.47

To determine an unknown concentration of the analyte (X), 2.0 ml of 0.68 mg/ml internal standard is added to 2.0 ml of unknown solution then the mixture is diluted to 100 ml in volumetric flask. Analysis of the mixture resulted in peak areas of A_x = 9174.95 and A_{IS} = 1553.47.

The 2.0 ml of 0.68 mg/ml of internal standard represents 1.36 mg (0.68 mg/ml × 2.0 ml = 1.36 mg) of IS added to 2.0 ml of unknown solution then the mixture is diluted to 100 ml in volumetric flask for a total volume of 100 ml. So, the concentration of (IS) in the unknown solution is therefore:

C (IS) = 1.36 mg/100 ml = **0.0136 mg/ml**

$A_x/[x] = F\ (A_{IS}/[IS])$ ---------- (a)

To determine the rotenone concentration:

9174.95/$[x]$ = 2.08 (1553.47/0.0136)
9174.95/$[x]$ = 237589.53
$[x]$ = 9174.95/237589.53
$[x]$ = 0.039 mg/ml

A_x = Peak area of analyte X
$[x]$ = Concentration of analyte X
F = Response factor
A_{IS} = Peak area of internal standard
$[IS]$ = Concentration of internal standard

Remember that this concentration represents of rotenone after some dilution from 2.0 ml to 100 ml when the mixture with internal standard (IS) is prepared in the volumetric flask. Therefore, the concentration of rotenone and its derivatives in the original 2.0 ml sample are:

∴ **$[x]$ ORIGINAL** = 0.039 mg/ml × (100/2) = **1.95 mg/ml**
$[y]$ ORIGINAL = 0.0003 mg/ml × (100/2) = **0.02 mg/ml**

Yield of rotenone = C (sample) × volume of crude extract (ml) = 1.95 mg/ml × 72.0 ml = 140.4 mg
Yield of tephrosin = C (sample) × volume of crude extract (ml) = 0.02 mg/ml × 72.0 ml = 1.44 mg

Yield of rotenone in dried roots = Yield of rotenone (mg) /weight of raw material × 100 %
= 140.4 mg /10.0 g × 100 % ∴ = **1.40 % (w/w)**
Yield of tephrosin in dried roots = Yield of tephrosin (mg) /weight of raw material × 100 %
= 1.44 mg /10.0 g × 100 % ∴ = **0.014 % (w/w)**
Impurities = 100 % - 0.014 % - 1.40 % = ∴ 98.6 % (w/w).
IMPURITIES INCLUDE: 2 other toxic constituents, wax, lipid, solvent residue, resinous substances and fine dust.

CONCLUSION

a) The HPLC chromatogram (A) indicated that the rotenone extracted from local species are similar to the standard analytical grade purchased from SIGMA-Aldrich™ and identical to the commercial grade of rotenone given by the SAPHYR S.A.R.L (France).
b) Based on the chromatogram (B), beside rotenone (6), the liquid crude extract consisted with other insecticidal compounds which were tephrosin (5) & deguelin (7). These compounds are essential for the insecticidal action against the Lepidopteron insect pest of cabbage (*Spodoptera litura*).
c) Since 2002, Chemical Engineering Pilot Plant (CEPP) has been claimed and confirmed for the amount of rotenone % (w/w) in dried root of local plant species which is *Derris elliptica* and *Derris malaccensis* using simpler and environmental-friendly procedures than the SAPHYR S.A.R.L (France).
d) The analysis method that has been used in the research is based on the AOAC official method, 2000 and analysis parameters are adjusted accordingly in order to achieve the best accuracy of rotenone determination.
e) The internal standard method is the most reliable and accurate analysis method compared with the external standard method. This method has all the advantages of the external standard method but in addition, it compensates for variations in injection volume and also for small changes in detector sensitivity or chromatographic changes that might occur. 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 taken further consideration in order to obtain the optimum result of separation.

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