AENSI Journals
Advances in Environmental Biology
ISSN:1995-0756 EISSN: 1998-1066
Journal home page: http://www.aensiweb.com/aeb.html

A Preliminary Study of Rotenone Exhaustive Extraction Kinetic from *Derris elliptica* Dried Roots Using Normal Soaking Extraction (NSE) Method

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ARTICLE INFO

Article history:
Received 14 January 2014
Received in revised form 19 April 2014
Accepted 23 April 2014
Available online 5 May 2014

Keywords:
Derris elliptica, Rotenone, Normal soaking extraction, Exhaustive extraction, Liquid crude extract

ABSTRACT

Currently bio-pesticide is relatively harmless to humans and the environment and thus desirable for the use in the controlling of insect vectors. The importance of bio-pesticide in both scale commercial agriculture and small plot, subsistence farming has been on the rise. One of the sources of bio-pesticide is 'tuba' plant, also known as *Derris elliptica*. *Derris elliptica* contains bio-active compound known as rotenone ($C_{23}H_{33}O_6$) which is harmless to plants, highly toxic to many insects and relatively innocuous to mammals. The study carried out was to investigate the appropriate independent and dependent variables of the exhaustive extraction process by evaluating the kinetic equilibrium phase of the normal soaking extraction (NSE) method. The rotenone from the roots and stems was extracted by using the normal soaking extraction (NSE) at an ambient temperature of 28 to 30 ºC with 95% (v/v) of acetone as a solvent. The solvent-to-solid ratio of the extraction was 10 ml/g. The extraction was carried out for 1440 mins and the fractions of the liquid crude extract were collected for each interval time (30 mins/ml/fraction) and were further cleaned up to remove any fine debris of roots and stems prior to the determination of rotenone content (mg) and its concentration (mg/ml) by using the reverse-phase high performance liquid chromatography (RP-HPLC). Based on the results obtained, it was found that the best extraction time was between 800 mins to 840 mins with the yield of rotenone and rotenone concentration of 830 mg to 890 mg and 2800 ppm to 2950 ppm respectively. Thus, the identified exhaustive extraction parameters can be utilized to optimize the yield of rotenone and its concentration via statistical analysis.

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To Cite This Article: Saiful Irwan Zubairi, Mohamad Roji Sarmidi and Ramlan Abdul Aziz. A Preliminary Study of Rotenone Exhaustive Extraction Kinetic from *Derris elliptica* Dried Roots Using Normal Soaking Extraction (NSE) Method. ***Adv. Environ. Biol., 8(4), 910-915, 2014***

INTRODUCTION

Rotenone and its derivatives that commonly refer to rotenoids are well known for their insecticidal properties. They occur naturally as constituents of the roots, stems, and leaves of many leguminous species of the genera *Derris, Lonchocarpus*, *Tephrosia* and *Amorpha*. The 'Tuba' plant is a woody plant which grows along the ground, crawling and climbing onto other plants. It needs at least 75% moisture and a temperature of 25 to 30 ^{o}C to live [1]. 'Tuba' is known by its botanical name as *Derris elliptica*. Rotenone is the bio-active compound extracted from *Derris elliptica* and other important constituents of *Derris* root (e.g., deguelin and tephrosin) that have been shown to be toxic to insects, although they are less active than rotenone [2]. Commercially important plants like *Derris elliptica* and *Derris malaccensis* contain 4% (w/w) to 5% (w/w) rotenone collected from the Amazonian forest [3]. However, *Derris elliptica* roots collected from the state of Johor, Malaysia contained less than 1.2% (w/w) [4]. Despite of its variety in rotenone content and other bio-active constituents [5,6], for several centuries, these plants have been used to prepare for hunting and fishing poisons. More recently, rotenone has come of interest because of its selectivity, low environmental hazard and relatively non-toxicity to plants and mammals. This moderate polar molecule is toxic towards cold blooded animals and when exposed to sunlight, it easily biodegrades to form *dihydrorotenone* and water (H_2O) [7]. In this preliminary study, we aim to identify the appropriate independent and dependent variables of the exhaustive normal soaking extraction (NSE) process based on the previous exploratory experiment.

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MATERIALS AND METHODS

Plant collection:

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

Raw material:

The collected raw materials were immediately sent to undergo cleaning process to remove dirt and soil. They were kept and dried into an oven for overnight at room temperature (26 to 30 ^{o}C). The cleaned raw materials were 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.

Extraction apparatus and procedure:

The extraction process was carried out by soaking 50 g of dried roots and stems in 500 ml of acetone 95% (v/v) with solvent-to-solid ratio of 10 ml/g for 1440 mins at room temperature (28 to 30 ^{o}C). The fractions of the rotenone liquid crude extract were collected intervally (30 mins/ml/fraction). The sample was further cleaned up to remove any fine debris of roots and stems through organic sample clarification kit (Waters™ Assoc.) containing 0.45 μm/0.5 μm PTFE filter directly into 5 ml of dark vial prior to the determination of rotenone extraction yield, mass content and concentration by using the reverse-phase high performance liquid chromatography (RP-HPLC). Table 2 shows the processing parameters that were involved in the rotenone exhaustive extraction process.

Table 1: Exploratory processing parameters.

Factor names	Factor levels
[a]Types of solvent	Chloroform, ethanol and acetone
[b]Solvent-to-solid ratio	10 ml/g, 5.5 ml/g and 3.3 ml/g
[c]Raw material particles size	Fine and coarse particles size (mm in diameter)
Extraction duration	0 to 1440 mins (2 hrs interval observation)

[a]Purity of the solvents were 95% (v/v).
[b]The solvent-to-solid ratio of 3.3 ml/g and 10 ml/g were selected to evaluate the significant effect on the response variables as compared to the ratio of 5.5 ml/g used by Grinda and Gueyne (1986) [1].
[c]Source: Pagan and Hageman (1949) [8]: (a) Fine; 0.5 to 2 mm in diameter (b) Coarse; 2 to 5 mm in diameter.

Table 2: Processing parameters involved in rotenone exhaustive extraction process.

Variables of extraction process:
Independent variables
☑ Extraction time: 0 to 1440 mins (24 hrs)
Dependent/response variables
☑ Yield of rotenone, mg
☑ Rotenone concentration, mg/ml
Control variables
☑ Ambient temperature (26 ± 2 ^{o}C)
☑ Solvent-to-solid ratio (10 ml/g)
☑ Weight of dried roots (50 g)
☑ Types of solvent (Industrial grade acetone 95% (v/v))
☑ Fine particles size (0.5 to 2 mm in diameter)

Analysis of the rotenone liquid crude extract:

The liquid crude extract solutions were subjected to quantitative analysis using reverse-phase high performance liquid chromatography (RP-HPLC) through UV (Photodiode Array - PDA) detection at 294 nm to determine the rotenone content. The analysis of extract solutions was carried out by using the external standard method (Rotenone PESTANAL®, analytical grade, 96.2% - Sigma-Aldrich™). The flow rate of the reverse phase HPLC was 0.7 ml/min for Genesis™ (C_{18}) stainless steel column with particle size of 4 μm - (3.9 mm I.D. × 120 mm length). For the preparation of rotenone standard solution, about 20 mg of rotenone standard powder (Rotenone PESTANAL®, analytical grade, 96.2% - Sigma-Aldrich™) was weighed and put into 125-ml Erlenmeyer flask and dissolved with 50 ml of acetonitrile in a Gyratory shaker for 10 mins. After shaking, the standard solution was filtered through a 15-cm Whatman filter paper (number 2) directly into 50 ml beakers. About 10 ml of the filtrate was re-filtered through an organic sample clarification kit (Waters™ Assoc.) containing 0.45 μm/0.5μm filters. The solvent system (mobile phase) and amplitude unit full scale (AUFS) were acetonitrile-distilled water (60:40) and 2 respectively [9,10]. The details of the analysis parameter and dependent variable template calculation using the RP-HPLC external standard method are shown in Table 3 and Table 4 respectively.

RESULTS AND DISCUSSION

Figure 1 and Figure 2 show the kinetic equilibrium of rotenone exhaustive extraction process with respect to yield of rotenone (mg) and its concentration (mg/ml). Table 1 and Table 2 show the exploratory processing parameters and processing parameters involved in the rotenone exhaustive extraction process respectively. All results were analyzed using the external standard method of reversed-phase high performance liquid chromatography (RP-HPLC). The example calculation of an external standard method is shown in Table 4. As time of extraction went by, the yield of rotenone (mg) and rotenone concentration (mg/ml) increased rapidly for the first 30 mins, and gradually increased until it reached to the maximum point at 840 mins. At that point, the extraction process started to decrease as it reached to 1440 mins. Meanwhile, it was observed that around 51.25% to 52.44% of the extraction process had been achieved within 30 mins and 90% prior to the exhaustive point within 620 mins to 650 mins (Table 5).

Table 3: Parameters of RP-HPLC [11].

Parameters	Setting
Column temperature (^oC)	Ambient
Flow rate of separation	0.4 ml/min
UV wavelength (λ)	294 nm
Injection volume	5 µl
Amplitude Unit Full Scale (AUFS)	2

Table 4: Calculation of dependent variables using RP-HPLC external standard method.

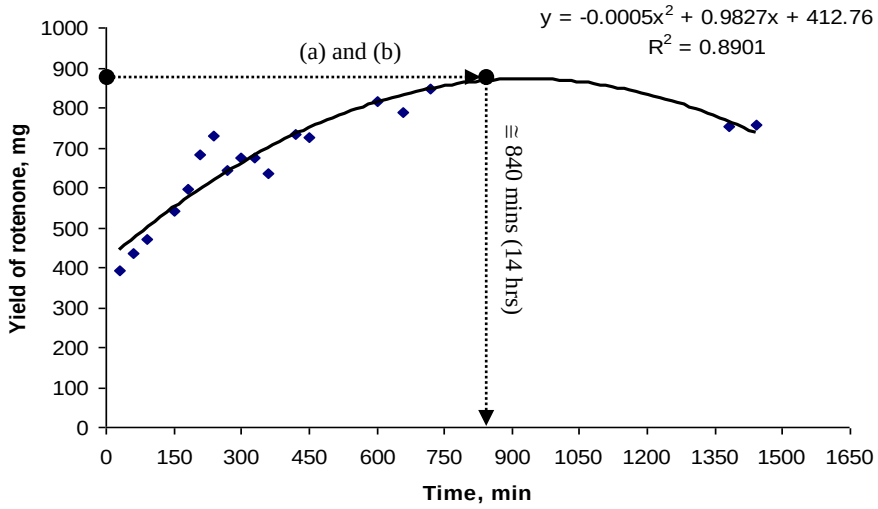
Rotenone standard [Sigma-Aldrich™; purity of 95 - 98% (w/w)]: Rotenone standard concentration (C_{std}) = X (mg/ml) Peak area (A) = Y (mV.s) Sensitivity Factor (SF) = (A)/(C_{std}) = J (mV.s. × ml/mg)
Sample concentration (Liquid crude extract (LCE) or concentrated liquid crude extract (CLCE)): Sample peak area (A_{sample}) = Y (mV.s) Sample concentration (C_{sample}) = (A_{sample})/SF = Q mg/ml.
If, the sample involves dilution: (Dilution factor; DF = flask volume/pipette volume).
Actual concentration (rotenone) = Q mg/ml × DF = G (mg/ml) (10 mg/ml = 1%) - To get 1%: 10/1000 (g/ml) × 100% = 1%
Yield of rotenone = C_{sample} (mg/ml) × volume (ml) of LCE or CLCE = (G) mg/ml × (ml) = K (mg)
Yield of rotenone in dried roots, % (w/w) = Yield of rotenone (mg)/weight of raw material × 100%

Table 5: Data of the dependent and independent variables in the kinetic of rotenone extraction process.

Extraction time (min)	Volume of LCE (ml)	Yield of rotenone (mg)	Rotenone concentration (mg/ml)	Yield of rotenone in dried roots, % (w/w)
30	470	391.96	0.83	0.78
60	460	435.16	0.95	0.87
90	450	469.85	1.04	0.94
150	430	540.51	1.26	1.08
180	420	594.66	1.42	1.19
210	418	683.43	1.64	1.37
240	406	727.64	1.79	1.46
270	390	642.68	1.65	1.29
300	385	675.64	1.76	1.35
330	375	674.45	1.80	1.35
360	370	635.77	1.72	1.27
420	355	732.80	2.06	1.47
450	345	727.07	2.11	1.45
600	320	816.55	2.55	1.63
660	310	789.88	2.55	1.58
720	300	848.60	2.83	1.70
1380	250	751.45	3.01	1.51
1440	240	756.46	3.15	1.51

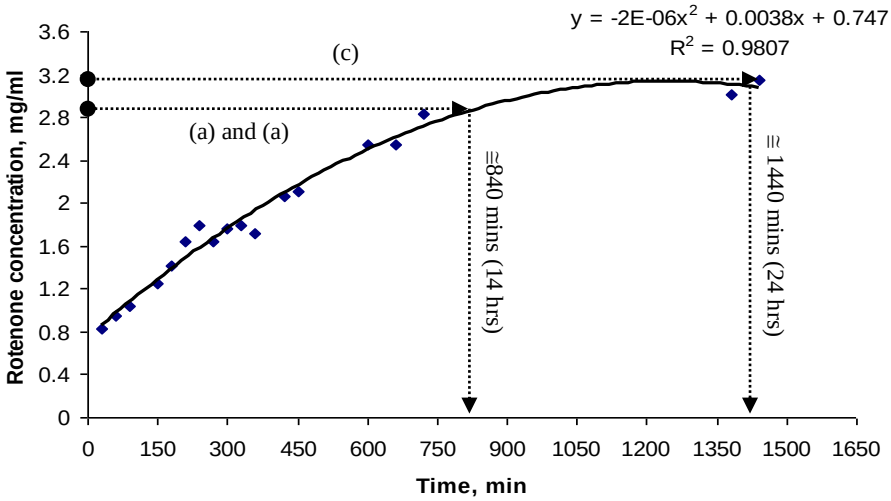
The exhaustive extraction of *Derris elliptica* occurred approximately between 800 mins to 840 mins with the amount of 895 mg rotenone/50 g dried roots. This phenomenon is called the steep rate of extraction. The steep rate of extraction at the beginning was possibly due to the washing [12,13] of solute from the ruptured cells rather than leaching alone, where the phytochemicals were released from within the cells by crushing or grinding was quickly dissolved into the bulk solution. In contrast to Suraphon and Manthana's idea [14], the exhaustive extraction time occurred at 480 mins of ethanolic extraction process at room temperature using the stirring soaking method. The early exhaustive extraction period was due to the usage of stirring which facilitated the internal diffusion of phytochemicals as well as the mass transfer coefficient values.

Figure 2 shows the profile of rotenone concentration that can be obtained from the normal soaking extraction (NSE) method. The maximum rotenone concentration was approximately 3.15 mg/ml at 1440 mins of extraction. The mass of rotenone in this period was approximately 760 mg rotenone/50 g dried roots which were approximately 15% less than the mass at the maximum or exhaustive time (895 mg/50 g dried roots). These mass losses were possibly due to the over exposure to room lighting during the liquid crude extract sampling, RP-HPLC sample preparation, unstable ambient temperature, inappropriate insulation of extraction vessel and inaccurate RP-HPLC detector. On top of that, the volume of solvent (ml) in liquid crude extracts (LCE) rapidly decreased within 720 mins to 1200 mins during the extraction process due to the rapid solvent evaporation which might perhaps explain why rotenone concentration (mg/ml) had increased greatly. Hence, the solution of liquid crude extract (LCE) became highly concentrated with the insufficient amount of solvent to penetrate deep inside the plant cell walls and extract more rotenone and other important phytochemicals. The most important consideration to construct an appropriate procedure for extracting the bio-active constituents that are susceptible to heat, light and rapid solvent evaporation is by installing or constructing the extraction vessel that monitors systematically all critical processing parameters (e.g., solvent volatility, operating temperature, pressure and time). The configuration of that system will minimize the dissipation of valuable constituents in the extract. Moreover, the ambient temperature and light exposure in the analysis room should be controlled efficiently during sample preparation to minimize any effects to the measurement. Calibration and cleaning all instruments (e.g., PDA or UV detector, columns, column heater, isocratic pump and etc.) periodically could also minimize errors and at the same time give precise, reasonable and acceptable results.



(a) Exhaustive extraction time: ≅ 840 mins (14 hrs)
(b) Exhaustive yield of rotenone in dried roots: ≅ 885.50 mg/50 g

Fig. 1: Kinetic equilibrium of rotenone extraction process: Yield of rotenone in dried roots, mg.



(a) Exhaustive extraction time: ≅ 840 mins (14 hrs)
(b) Exhaustive rotenone concentration: ≅ 2.84 mg/ml
(c) Maximum rotenone concentration: ≅ 3.15 mg/ml

Fig. 2: Kinetic equilibrium of rotenone extraction process: Concentration of rotenone, mg/ml.

Conclusion:

It was observed that within 30 mins of the extraction process, around 51.25% to 52.44% of the extraction process had been accomplished and 90% prior to the exhaustive extraction within 620 mins to 650 mins. The exhaustive extraction of *Derris elliptica* occurred approximately between 800 mins to 840 mins with the amount of 895 mg rotenone/50 g dried roots. Thus, the identified exhaustive extraction parameters can be utilized to further optimize the yield of rotenone and its concentration via statistical analysis (central composite design, CCD). Moreover, the maximum concentration that could be obtained in the normal soaking extraction (NSE) process was 3.15 mg/ml at the 1440 mins of extraction time. However, the yield of rotenone at this period was approximately 760 mg rotenone/50 g dried roots which was approximately 15% fewer than the mass at the maximum or exhaustive time (895 mg rotenone/50 g dried roots). These anomalies were possibly due to the volume of solvent (acetone) in the liquid crude extract that rapidly decreased within 720 mins to 1200 mins of the extraction time. Consequently, the concentration of rotenone (mg/ml) increased tremendously and hence, the extract solution became concentrated with less amount of solvent required to extract more of the phytochemicals from the dried roots. The most important thing that should be taken into consideration is the whole normal soaking extraction (NSE) system should be insulated properly in order to minimize any dissipation of rotenone due to the fluctuation of ambient temperature, pressure and increasing the amount of rotenone by controlling the high volatility of organic solvent (acetone). Rotenone is light and heat sensitive and as such, with prolonged exposure to extreme environments and lack of proper extraction system, a major loss of bio-active compounds and less effectiveness of insecticidal action will be undesirable.

ACKNOWLEDGEMENT

All thanks are due to the Malaysia Ministry of Science, Technology & Environment (MOSTE) for the financial assistance under RM8 IRPA 04-01-06-SF0077 and the Universiti Teknologi Malaysia (UTM) for providing the research facilities throughout the study.

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