PURIFICATION AND IDENTIFICATION OF ROTENONE FROM *Derris elliptica* BY USING THE VACUUM LIQUID CHROMATOGRAPHY-THIN LAYER CHROMATOGRAPHY (VLC-TLC) METHOD

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ABSTRACT

History has recorded the use of rotenone as bio-pesticide by plant and vegetables growers worldwide. The re-introduction of this bio-active compound as an environmental friendly bio-pesticide in organic fruits and vegetables plantations is now an emergent trend. Our laboratory is focused on the development of bio-pesticide using rotenone extracted from local *Derris elliptica*. Liquid chromatographic method with high vacuum pressure was developed for the analysis of rotenone in the crude extract of *Derris elliptica.* The treated root and stem were cut into small pieces prior to extraction process using the Normal Soaking Extraction (NSE) method. The crude extract was concentrated further by using rotary evaporator at 40 ^{0}C under reduced pressure of 800 mbar. A high-pressure vacuum liquid chromatographic was then carried out for the purification of rotenone using silica gel (230 mesh to 400 mesh) with variety of eluents polarity (hexane, chloroform, acetone and ultra pure water respectively). All eluents that have been collected after the purification process were subjected to the semi-automatic TLC (CAMAG Linomat 5) for the identification of rotenone and other constituents by using an isocratic elution with a mixture of petroleum ether and ethyl acetate with a ratio of 4:2. The markers for each bio-active constituent were visualized and recorded using UV lamp (wavelength of 254 nm and 365 nm) and the R_f values for desirable constituent (rotenone) was calculated respectively. The employed method shows significant improvement in rotenone separation and purification compared to the normal TLC method.

Keywords: *Derris elliptica*; rotenone; environmental friendly bio-pesticide; TLC; VLC.

INTRODUCTION

Malaysia is embarking on developing its agro-business and to provide food security for the country. The pest control in the country at present relies mainly on toxic chemical pesticides. The conventional pesticides are often toxic to mammals, non-target pests and persist in the environment as recalcitrant. Therefore, the search for bio-active substance which have satisfactory properties that is effective on the target pest is economic viable and biodegradable is great importance. For example, rotenone from a plant belonging to the *Derris elliptica* family known locally as 'Tuba Kapur' has been proven as potent as many conventional synthetic pesticides. Rotenone can be extracted from many tropical *Leguminosae* such as *Derris spp., Lonchocarpus spp.* and *Tephrosia spp. Derris elliptica* is a widely available local plant and contains 4.0 % (w/w) to 5.0 % (w/w) of the active ingredient, rotenone. Rotenone is extremely active as contact and stomach poisons against many crop pests such as Mexican bean beetle, apple and pea aphids, corn borer and household pests. Besides having low mammalian toxicity, they are reasonably safe to honeybees (Opender, 2001).

METHODOLOGY

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

Raw material - The collected raw materials immediately undergo cleaning process to remove dirt and soil. They were kept and dried into oven for overnight at room temperature (26 ^{0}C to 30 ^{0}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 was carried out by soaking 50.0 g of dried root and stem in 500 ml solution of methanol 95.0 % (v/v) for 24 hours at room temperature (26 ^{0}C to 30 ^{0}C). The Liquid Crude Extract (LCE) was filtered through 15.0 cm Whatman No. 4 filter paper directly into 500 ml; PYREX® glassware after 24 hours of the extraction process. The Liquid Crude Extract (LCE) was concentrated further by using rotary evaporator; Laborata 4001 (Heidolph) at 40 ^{0}C, purified by using Vacuum Liquid Chromatography (VLC) under reduced pressure of 800 mbar respectively and analyzed qualitatively by using semi-automatic TLC (CAMAG Linomat 5).

Purification of Concentrated Liquid Crude Extract (CLCE) - The VLC system (4 VLC system were prepared for each eluents) consisted with silica gel powder (230 mesh to 400 mesh) with silica gel-to-CLCE ratio of 40.0 g/g, composition of different eluents as shown in Table 1.0 and vacuum pump with vacuum pressure of 800 mbar. Silica gel solution (silica gel powder + H_2O) was poured into the unit until it dried off as the vacuum pump suck up the air to facilitate the flow of H_2O in the reservoir and eventually dried up silica gel as solid structure in order to obtain a sufficient absorption and separation during the purification process occurred. The Concentrated Liquid Crude Extract (CLCE) was poured onto the surface of the silica gel until 1/3 of the solution absorbed into the dried silica gel. The 15.0 cm of Whatman filter paper No. 4 was putted onto the surface of dried silica gel to stabilize the dried silica gel while pouring the eluents. The amount of 300 ml of eluents were poured onto the dried silica gel until it penetrated and absorbed all over the silica gel. During the flow of eluents occurred thoroughly through dried silica gel, the constituents in the CLCE already been absorbed in the silica gel where the desired constituents were eluted from the CLCE into the eluents accordingly to its similarity of eluents polarity. The eluted or purified solution was subjected to convective concentration process in the fume cupboard and analyzed qualitatively using semi-automatic TLC (CAMAG Linomat 5).

Table 1.0: Composition (%) of different eluents.

No.	Eluents/solvent mixtures	Composition (%)
1.	[a]Hexane + chloroform	1) 100-0 2) 90-10 3) 80-20 4) 70-30 5) 60-40 6) 50-50 7) 40-60 8) 30-70 9) 20-80 10) 10-90 11) 0-100
2.	[a]Chloroform + acetone	12) 100-0 13) 90-10 14) 80-20 15) 70-30 16) 60-40 17) 50-50 18) 40-60 19) 30-70 20) 20-80 21) 10-90 22) 0-100
3.	[a]Acetone + ultra pure water	23) 100-0 24) 90-10 25) 80-20 26) 70-30 27) 60-40 28) 50-50 29) 40-60 30) 30-70 31) 20-80 32) 10-90 33) 0-100
4.	[b]Ultra pure water	34) 100

[a]Purity of the solvents were 95.0 % (v/v).
[b]E-pure, Barnstead: 0.49MΩ-cm.

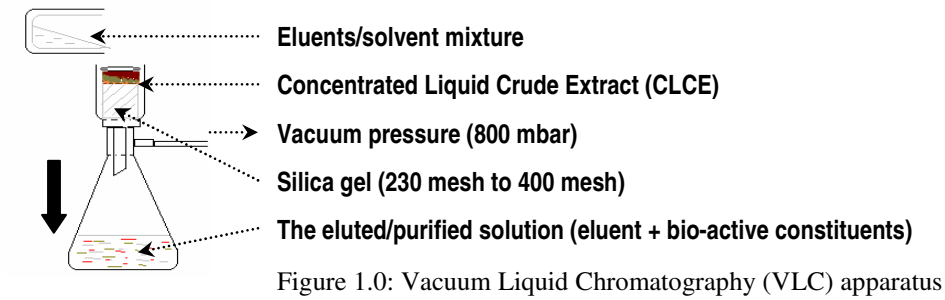


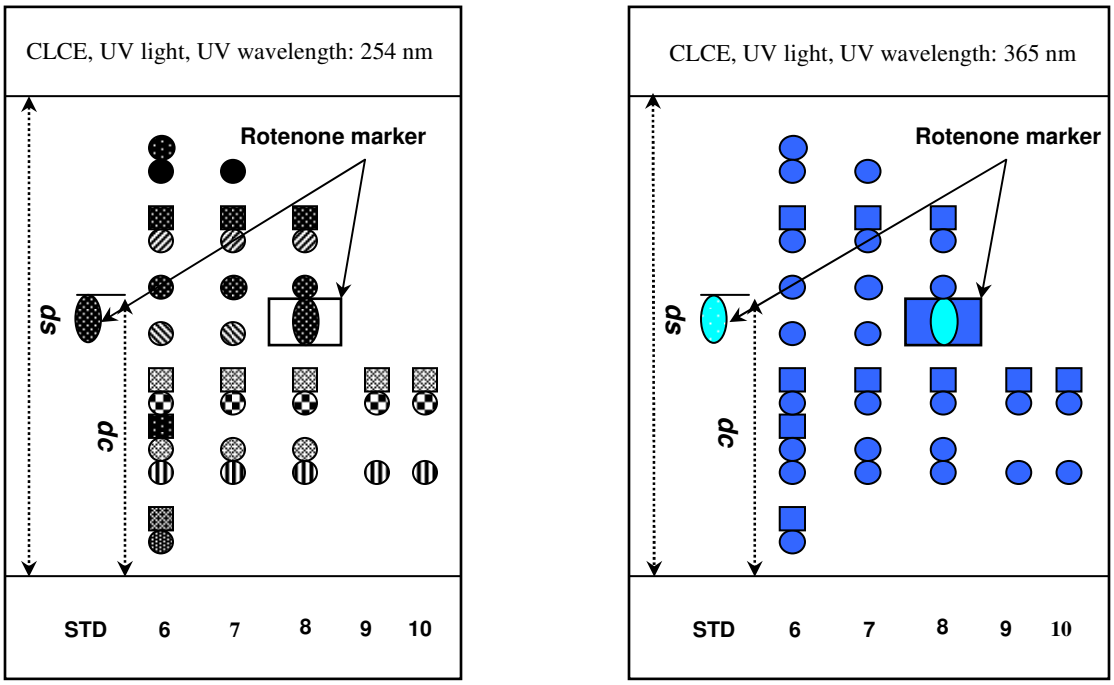
Figure 1.0: Vacuum Liquid Chromatography (VLC) apparatus

Analysis of purified solution - The purified solutions were subjected to the qualitative analysis by using semi-automatic TLC (CAMAG Linomat 5). The operating conditions for CAMAG Linomat 5 were listed as follows: Plate size (TLC aluminium sheets; Silica gel; 60 F_{254}; 10 cm × 10 cm), band of spot: 4.0 mm, *x* and *y*-axis: 11.0 mm and 10.0 mm respectively, track distance: 10.0 mm and spray volume dosage: 4.0 µl. The plates that have been sprayed were subjected into a development chamber using isocratic elution with a mixture of petroleum

eter and ethyl acetate with a ratio of 4:2. The markers for each bio-active constituent found were visualized and recorded using UV lamp (wavelength of 254 nm and 365 nm) and the retardation factor, R_f value of desirable constituent (rotenone) was calculated as shown in Table 1.1.

RESULT AND DISCUSSION

Figure 1.1: The presence of rotenone under UV light of wavelength 254 nm and 365 nm respectively.



$$Rf = \frac{\text{Distance traveled by the compound (dc)}}{\text{Distance traveled by the solvent front (ds)}}$$

CLCE [Methanol extract; 95.0 %]	
Rotenone standard; 96.2 %, R_f	≅0.63
Rotenone (8), R_f	≅0.61

Table 1.1: R_f for eluent no. (8): Hexane + chloroform [30-70]

Analysis of purified rotenone solution - The markers of each eluents composition for hexane + chloroform from no. 6 to 10 using the semi-automatic TLC (CAMAG Linomat 5) is shown in Figure 1.1 and R_f for the rotenone standard (Rotenone PESTANAL®, analytical grade, 96.2 % - SIGMA ALDRICH™) and desirable constituent (rotenone) are listed in Table 1.1. The retardation factor, R_f of rotenone was fairly similar as rotenone standard with approximately 0.61 and 0.63 respectively. Although rotenone standard with purity of 96.2 % can be seen easily on the silica plats, actually there was a small markers encircle the rotenone marker which is represented as other constituents (tephrosin, 6αβ, 12αβ-rotenolone and deguelin). The encircle markers were insignificant for the R_f determination due to it appearances (not as a solid spot) and small amount of constituents comprise in the standard (≅ 3.8 %). From Figure 1.1, the desirable constituents and rotenone standard (STD) markers were highlighted to show that the purification process using hexane + chloroform [30-70] as an eluent were the best composition that procured small amount of impurities marker compared with the other eluents composition. The desirable constituents were eluted and absorbed along with the composition of hexane + chloroform [30-70] eluents gradually disappeared after increasing the concentration chloroform and decreasing the concentration of hexane by 90 % and 10 % respectively. Rotenone and other constituents were started to elute from composition no. 6 to no. 8 and gradually disappeared (exhaustively flush out) or still being absorbed by the remaining constituents in silica gel along with the changes of the composition.

By using a mixture of two different eluents polarity [hexane (0) and chloroform (4.1)], rotenone appeared to be existed in the composition between these values and there was a small amount of unknown markers compared

by using only non-conventional TLC method. Non-conventional TLC method showed 13 to 15 markers compared by using the VLC-TLC method that showed only 8 markers. The other eluents composition as shown in Table 1.0 showed hard visibility for observing the desired markers after the semi-automatic TLC (CAMAG Linomat 5) development process. As for the ultra pure water, this eluent produced a milky solution in which dissolved and flush out almost all CLCE solution absorbed in the silica gel. In fact, the TLC plat revealed that there was no separation occurred (no markers) either during the VLC or TLC development process. This was due to all constituents in the eluent (ultra pure water) were possibly be to polar and the separation on the non-polar material (silica gel) either in the VLC or TLC system would be impossible to solubilize into the eluent (absorption to silica gel > solubility in the eluent) and separate accordingly into their own polarity.

Pure rotenone has following solubility in gram per 100 cubic centimeters of solution at 20 ^{0}C as follows: water, ≅ 0.00002; ethyl alcohol, 0.2; carbon tetrachloride, 0.6; amyl acetate, 1.6; xylene, 3.4; acetone, 6.6; benzene, 8.0; chlorobenzene, 13.5; ethylene dichloride, 33.0; and chloroform, 47.0. The solubility of pure rotenone in chloroform (47 grams per 100 cubic centimeters of solution at 20 ^{0}C) indicates that the elution of rotenone has been suspected in a mixture of hexane + chloroform or chloroform + acetone and the most suitable mixture composition (%) itself were to be unknown until this study has been done extensively. After rigorous study done, it can be concluded that the higher polarity eluents used as an eluent, the less constituents can be eluted or absorbed into the eluents and resulted less impurities in the purified solution. The techniques to procure and achieve as an USP (U.S. Pharmacopeia) standard for rotenone constituent particularly on this *Derris* species should be further investigated.

Sequel of eluents based on the best separation and identification of rotenone constituent

Hexane (0) + Chloroform (4.1) > Chloroform + Acetone (5.1) > Acetone (5.1) + H_2O (9.0) > H_2O (9.0)

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