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BIOLOGICAL SCREENING ON THE EXTRACT OF *Derris elliptica* (TUBA)

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ABSTRACT

Bio-pesticide has an increasing importance in both commercial agriculture and small plot subsistence farming. One of the sources for bio-pesticide is tuba plant, known as *Derris elliptica*. Rotenone ($C_{23}H_{33}O_6$) is one of the bio-active compounds from *Derris elliptica* known to be harmless to plants, highly toxic to many insects and relatively innocuous to mammals. From the preliminary results, the optimization study is employed for a Central Composite Design (CCD) using the Design Expert software version 6.0.4 (Stat-Ease. Inc., 2002) is to validate the effects of processing parameters of two selected response variables which are the yield of rotenoids resin and rotenone content respectively. The processing parameter studied is types of solvent (acetone & ethanol), solvent-to-solid ratio (10.0 ml/g & 2.0 ml/g) and raw material particles size (0.5 mm & 5.0 mm). In addition, the biological activity is evaluated for all treatment to obtain the Lethal Concentration (LC_{50}) using brine shrimp, *Artemia salina*. Various concentrations of rotenone/Concentrated Liquid Crude Extract (CLCE) were exposed in 2 replicates of each concentration for 6, 12 and 24 hours observation respectively. Subsequently, the mortality of the *Artemia salina* will be evaluated using the probit analysis based on the dose-response curves. The biological activity result shown that the rotenoids resin was considered very active significantly in terms of its toxicity whereby all treatment produced $LC_{50} \leq 100$ ppm after 6, 12 and 24 hours respectively.

Keywords: Bio-pesticide; *Derris elliptica*; rotenone; CLCE; rotenoids resin; *Artemia salina*; bioassay.

INTRODUCTION

Rotenone and its derivatives commonly referred to, as 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 to other plant. It needs at least 75 % moisture and a temperature of 25 ^{0}C to 30 ^{0}C to live. 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 (deguelin and tephrosin) have been shown to be toxic to insects, however they are less active than rotenone (Waterman, 1980). Commercially important plants like *Derris elliptica* and *D. malaccensis* contains 4.0 % (w/w) to 5.0 % (w/w) rotenone (Parmar & Walia, 2001). For several centuries, these plants have been used to prepare hunting and fishing poisons. More recently, rotenone has come of interest because of its selectivity and low environmental hazard.

Rotenone is highly toxic to insects but relatively non-toxic to plants and mammals. This moderate polar molecule is toxic towards cold-blooded animals and when exposed to sunlight, it is easily biodegrades to form dihydrorotenone and water. 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 (Parmar & Walia, 2001).

Bioassays offer a special advantage in the standardization and quality control of heterogeneous botanical products. Products can be "heterogeneous" due to the presence of mixtures of bio-active compounds either from the same or from purposefully mixed botanical sources. A physical analytical method, such as chromatography is inadequate for this purpose as they are usually insensitive to the chemical complexities found in crude botanicals extract. Most often the desired biological response is due to a mixture of bio-active components and the relative proportions of single bio-active compounds can vary from batch to batch while the bioactivity still remains within tolerable limits. Thus, physical or chemical analysis of a single component in such mixtures is not completely satisfactory. Unfortunately, the goal of many phytochemists has been simply to isolate, characterize and publish botanically derived chemical substances without regard to bioactivities. To achieve applied meaning and significance, today's work in natural product chemistry must incorporate bioassay. Extracts must be screened for biological activity, the 'active' extracts selected, fractionations directed with bioassay and then exploited. The pre-screening bioassays that useful are Brine Shrimp Lethality: A rapid general bioassay for bio-active compounds.

METHODOLOGY

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

Raw material - For the preliminary phase, the procured tuba roots are sifted and separated into 2 main particles size, smooth (2.0 mm to 0.5 mm ID) and rough (5.0 mm to 2.0 mm ID) in diameter and for the optimization phase, the procured tuba roots are sifted and separated accordingly to the particles size generated from the software. The amount of 30.0 g of small pieces of dried roots is used for each treatment in the preliminary, optimization and verification phase. The samples are dried separately in a forced air oven 1375 FX (Sheldon Manufacturing, Inc.) at 30 ^{0}C for 3 hours.

Design of experiments (DOE) - The design of experiments used in the study is a Central Composite Design; CCD with 2nd Order Model - (2^3) with 30 runs including 3 centre point, 2 replicates and 1 alpha point (α). Table 1 shows the CCD specification for the optimisation phase experiment.

Centre Point (CP)	Alpha Point (α)	Replicate
3	1	2

Table 1: Specification of Central Composite Design (CCD)

TOTAL OF EXPERIMENT = $[2^n + 3$ CP (Centre Point)$] \times 2$ Replicates + $(2^n \times (\alpha))$
$= (2^3 \times 2$ Replicates$) + (3$ CP $\times 2$ Replicates $+ [(2^3 \times 1(\alpha)]$
$\therefore = 16 + 6 + 8 = 30$ experiments.

Methods of experimental design or design of experiment (DOE) are used to evaluate the effects of several different factors on a response variable. Statistical tool of analysis of variance (ANOVA) is used to analyze the data from the experiments and to make decisions about whether a given factor has a significant impact on the response variable. Type of experiment often used in experimental design is called factorial experiments where treatments (factors) consist of a combination of levels. The main and interactions effects of the factors towards the response variables can be calculated. Results can be presented in graphical tools such as response surface (Devore & Farnum, 1999). The result of 30 experiments should be subjected to the analysis of mathematical model that have been constructed in order to determine the accuracy of the response variables. All the data from the Design of Experiments (DOE) will be suited to the 2nd order model with the factors of k (Cornell, 1990).

Extraction of rotenoids resin - Normal soaking extraction method (maceration) is used in extracting rotenoids resin. For the preliminary phase, 30.0 g of small pieces tuba roots is added to 300 ml solvent (ethanol, acetone or chloroform); solvent-to-solid ratio: 10.0 ml/g, in an 500 ml beaker. Extraction process is carried out in ambient temperature (26 ^{0}C to 30 ^{0}C) by placing the extraction vessel (500 ml beaker) in a dark place for 24 hours. For the optimization phase, the extraction duration is set to be 10 - 12 hours in order to obtain the exhaustive extraction content (the exhaustive extraction time is obtained from the preliminary studies). The raw material particles size, types of solvent and solvent-to-solid ratio differ according to the phases of the experiments involved. As for the case of optimization phase, the types of solvent, raw material particles size and solvent-to-solid ratio are based on the experimental matrix generated from the software. The kinetic extraction curves of each experiment are constructed by checking the extracts content for 2 hours interval using the High Performance Liquid Chromatography (HPLC) until the exhaustive extraction of rotenone content has been occurred (preliminary studies). The exhaustive extraction time may be vary for each processing parameters generated by the software. The extracts are then filtered using the Whatman filter paper no. 4 with the aid of Altech filter with GAST Laboratory Diaphragm Vacuum Pump and Compressor at 0.3 mbar. The liquid crude extracts (LCE) are subjected to rotenone content determination before concentration process and introduced into a round-bottom flask with reflux condenser in a rotary evaporator Laborata 4001 (Heidolph) with vacuum pump at 0.3 mbar to remove approximately 90.0 % of the solvent to obtain the concentrated liquid crude extract (CLCE). This concentrated liquid crude extract (CLCE) is subjected again to the rotenone content determination prior to brine shrimp lethality study using HPLC and introduced again into a round-bottom flask with reflux condenser to remove 100 % of solvent and weighed the rotenoids resin to determine yield of extract. The rotenoids resin obtained is stored in a cool room (10 ^{0}C).

Lethal Concentration (LC_{50}) of the biological activity analysis - Bio-active compounds are almost always toxic in high doses (Jerry & Lingling, 1998). Thus, *in vivo* lethality in a simple zoological organism can be used as a convenient monitor for screening and fractionation in discovery and monitoring of bio-active natural products. The eggs of brine shrimp, *Artemia salinas* are readily available in pet shops at low cost and remain viable for years in dry state. Upon being placed in seawater, the eggs hatch within 48 hours to provide large numbers of larvae (nauplii) for experimental use. The sea salt can be obtained also from the fish store. The small tank (hatching chamber) is used to grow shrimp with dividing dam, cover and lamp to attract shrimp eggs. Allow 3 days for the shrimp to hatch and mature as nauplii. The concentrated liquid crude extract (CLCE) concentration is the initial concentration and dilution will be prepared accordingly to the amount of test concentration. Prepare vial for testing; for each fraction, test initially at 1000, 500, 100, 50, 10, 1.0 µg/ml; prepare 2 vials at each concentration for a total 10 vials plus 1 control vial; from this solution transfer 100 µl to vials corresponding to 1000, 500, 100, 50, 10, 1.0 µg/ml, respectively. Evaporate solvent under dark place and ambient temperature; volatile solvents will evaporate overnight. Evaporation is needed so that only the rotenoids resin is remained in the vials, not the CLCE. After 4 days (when the shrimp larvae are ready), add about 2.0 ml of seawater to each vial, count 10 shrimp per vial (50 shrimp/dilution) and adjust the volume with the seawater to 4.0 ml/vial. Place the vials, uncovered, under the lamp. Be sure that the lamp does not overheat vials. Time of treatment is 6, 12 and 24 hours and each phase is counted and recorded the number of survivors or dying. Subsequently, the mortality of the *Artemia salina* will be evaluated using the probit analysis (Finney, 1971) based on the dose-response curves to determine the LC_{50} (Lethal Concentration of rotenone for obtaining the 50 % mortality). By plotting probit mortality against log-dose of CLCE (ppm), the approximately linear plot can be obtained and the LC_{50} data can be observed by anti-log the dose (10^x; x - log-dose of CLCE).

RESULTS AND DISCUSSION

Design of Experiment (DOE) of 30 experiments in this screening are evaluated, listed and summarized in the Table 2. They are distributed among 3 sets of experiment on the 2-centre point (CP), 2 sets of experiment on the 8 replicates point and 1 set of experiment on the (α) point. The model for this response variable (bioassay) generated from the Design Expert software version 6.0.4 (Stat-Ease. Inc., 2002) is appeared to be **NOT SIGNIFICANT** in order to obtain the optimum processing parameters. Data interpretation are made accordingly to the protocol established by McLaughlin (1991), all treatment should be observed minimally 24 hours and the extracts with $LC_{50} \leq 100$ ppm were considered active.

i) The effect of raw material particles size and types of solvent on the bio-toxic constituents of *Derris*

Based on the results of the biological assays at the 24 hours of treatment as shown in Table 1.0 and Table 2.0, the diameters of *Derris elliptica* dried root groups 0.5 mm ID to 2.75 mm ID in the acetone extract was the most toxic to produce the LC_{50} < 1.00 ppm overall. Compared with ethanol extract, the toxicity level were also nearly as toxic as the acetone extract to produce LC_{50} < 1.00 ppm from the finest (0.5 mm ID) to the thickest roots (5.00 mm ID) as shown in Figure 2.0: (C). The ability of ethanol (polarity of 5.2) to diffuse into the thickest diameter of *D. elliptica* dried roots was the main reason why the toxicity values (LC_{50}) is significantly more less at 1.00 ppm. The result would be strongly indicated that ethanol can be extracted more other constituents such as deguelin, elliptone and toxicarol compared with acetone (polarity of 5.1) that can be only extracted rotenone more than the other constituents only at the 4.09 mm ID. The decrease in toxicological value for particles size more than 4.09 mm ID is possibly due to less other constituents appeared in the Concentrated Liquid Crude Extract (CLCE). According to Pagan & Hageman (1949), the increase in toxicity due to rotenoids is very noticeable for the roots 2.0 mm to 4.0 mm ID. In addition, the previous research done by Pagan, (1948) indicated that the acetone extracts of *Derris* roots might be fairly accurate measure of the total toxicological value whereby there was a close agreement between the known content of other toxic constituents (rotenoids) and the transmittance values (rotenone). On top of that, the yield of rotenone in dried roots for the group of 0.5 mm ID to 2.75 mm ID in the acetone extract indicated that the capability of acetone to extract more rotenone compared with ethanol in the same group were undeniable in the facts that the toxic constituents particularly rotenone is tend to be more stable in acetone than in the other solvents (Pagan & Loustalot, 1948). The yield results as shown in Figure 1.0: (A) were comparable with the previous statement whereas acetone can extracts more rotenone and ethanol extracts less in rotenone content but possibly more on the other constituents

ii) The effect of solvent-to-solid ratio and types of solvent on the bio-toxic constituents of *Derris*

The data presented in Table 2.0 and Figure 2.0: (D) shows a significant effect of solvent-to-solid ratio on the toxicological values. Acetone extract is dominated the most toxic to produce LC_{50} below the lowest dose of 1.0 ppm. Ethanol extract is also considered toxic by producing the LC_{50} < 1.00 ppm at nearly with all ratios without posses any major fluctuation. Theoretically, the increase of solvent-to-solid ratio with the increases of the yield of rotenone is consistent with mass transfer principles (Frank, Downey & Gupta, 1999). Figure 1.0: (B) shows the comparison yield of rotenone for the acetone extract and ethanol extract whereas the results were comparable with the theoretical statement. This figure indicates that the yield of rotenone increase with the increase of solvent-to-solid ratio for both acetone and ethanol extract respectively. There is a strong correlation between the solvent-to-solid ratio and LC_{50} for the ethanol extract which means that the increase of the ratios, increase the toxicity values significantly but for the Acetone extract, the correlation is designated only for ratio 2.0 ml/g to 3.62 ml/g and 8.38 ml/g to 10.0 ml/g respectively. The main consequence was due to the losses of rotenone during the concentration process. Previous study has shown that rotenone deteriorate rapidly at the first 15 minutes of 50 ^{0}C exposure during the period of concentration process (Saiful & Roji, 2003). Insufficient pressure of vacuum pump would be the reason to lead the degradation of rotenone in the Concentrated Liquid Crude Extract (CLCE). A sufficient vacuum pressure reduced boiling point below acceptable limit, reduced time of consumption subsequently minimizes any thermal degradation in order to retain as much as possible rotenone content in CLCE. The research done by Pagan & Hageman, (1949) demonstrated that the toxicological value (rotenone equivalent) of *Derris* roots is not perfectly correlated with the rotenone content only. The data from these papers indicate that substances other than rotenone also contributed to the toxicity of the roots (rotenoids).

After an extensive study done, there are several conclusions can be drawn:

1) On the basis of these data it felt that rotenone is generally considered the most important toxic constituents of *Derris* root and its concentration is the criterion most often used in evaluating the roots.
2) The biological activity result shown that the rotenoids resin was considered very active significantly in terms of its toxicity whereas all treatment produced $LC_{50} \leq 100$ ppm after 6, 12 and 24 hours respectively. Extracts with $LC_{50} \leq 100$ ppm were considered active (McLaughlin, 1991).

3) The toxicological value of the *Derris* roots was strongly affected by other rotenoids substances (other constituents) and not only the rotenone itself despite of having the most toxic of all constituents.
4) Acetone is the best solvent to obtain the best yield of rotenone and procured the highest LC_{50} (< 1.00 ppm). Ethanol is the best solvent to extract the greatest amount of other toxic constituents but less of major substance (rotenone).
5) The fine roots (0.5 mm ID to 2.75 mm ID) tend to be more superior in rotenone content because of the resin cell tissue that contains the rotenoids (Rotenone and its derivatives) was relatively abundant in roots of small and medium diameters (Waterman, 1980).
6) Increasing the solvent-to-solid ratio increases the yield of toxic constituents. Hence, increases the LC_{50} significantly. The ratio is always subjected to the mass transfer's principles, which mean that there is a particular ratios limitation (optimum point) to be observed in order to procure the yield rotenone and other toxic constituents as well as the toxicological values.
7) Concentrating the phyto-extract material particularly on the rotenone extract should be taken under consideration in terms of its operational specification (temperature, pressure & room light) so that any thermal deterioration could be minimized. Although there is no data and any extensive research have been done yet for the dissipation of the rotenone content, the operating temperature either for the extraction and concentration process should not be surpassing 40 ^{0}C to retain the rotenone content as much as possible or to minimize any thermal degradation to other substances that occurred in the extract for market value added purposes since that this fine chemical substances are so valuable and difficult to obtain in mass production.

Design of experiment (DOE), CCD with 2nd Order Model - (2^3)	[a]6 hours Ÿ (Mean LC_{50})	[a]12 hours Ÿ (Mean LC_{50})	[a]24 hours Ÿ (Mean LC_{50})	[b]CLCE (mg/ml) Ÿ (Mean)	[d]Rotenone content Ÿ (Mean)
CP POINT (3 sets of experiment)					
2.75 mm ID, 6.00 ml/g & Acetone (13,17&24) - A	5.15	2.62	[c]<1.00	10.47	0.66
2.75 mm ID, 6.00 ml/g & Ethanol (10,18&23) - B	4.83	1.82	[c]<1.00	2.00	0.31
REPLICATES POINT (2 sets of experiment)					
4.09 mm ID, 3.62 ml/g & Ethanol (3&14) - C	7.83	3.00	1.50	1.99	0.20
1.41 mm ID, 3.62 ml/g & Acetone (4&9) - D	5.42	0.69	[c]<1.00	38.44	0.69
4.09 mm ID, 8.38 ml/g & Ethanol (5&8) - E	4.88	0.61	0.52	1.92	0.46
4.09 mm ID, 3.62 ml/g & Acetone (7&16) - F	5.74	1.21	0.84	12.48	0.52
1.41 mm ID, 8.38 ml/g & Ethanol (12&22) - G	9.65	4.51	[c]<1.00	1.69	0.34
1.41 mm ID, 8.38 ml/g & Acetone (19&27) - H	1.27	2.69	[c]<1.00	2.39	0.41
4.09 mm ID, 8.38 ml/g & Acetone (20&25) - I	3.68	2.50	0.75	7.95	0.49
1.41 mm ID, 3.62 ml/g & Ethanol (26&30) - J	2.36	2.12	[c]<1.00	5.03	0.39
α POINT (1 set of experiment)	**Y**	**Y**	**Y**		
2.75 mm ID, 2.00 ml/g & Acetone (6) - K	0.56	[c]<1.00	[c]<1.00	5.12	0.09
5.00 mm ID, 6.00 ml/g & Ethanol (11) - L	5.62	0.77	[c]<1.00	2.63	0.23
0.50 mm ID, 8.38 ml/g & Ethanol (15) - N	0.59	[c]<1.00	[c]<1.00	6.64	0.75
0.50 mm ID, 6.00 ml/g & Acetone (21) - M	5.50	5.50	0.77	36.03	0.69
2.75 mm ID, 2.00 ml/g & Ethanol (28) - O	6.44	4.21	[c]<1.00	1.31	0.05
2.75 mm ID, 10.00 ml/g & Acetone (29) - P	7.43	3.08	0.67	60.20	2.40
5.00 mm ID, 6.00 ml/g & Acetone (1) - Q[e]	10.00	7.16	5.91	11.61	0.39
2.75 mm ID, 10.00 ml/g & Ethanol (2) -R	1.82	[c]<1.00	[c]<1.00	2.86	0.99

[a]Lethal Concentration (LC_{50}) - ppm.
[b]Concentrated Liquid Crude Extract (CLCE) is obtained after the concentration process. CLCE is used as an initial concentration (C_0) for the biological activity corresponding to the testing fractions of 1000, 500, 100, 50, 10, 1.0 ppm respectively.
[c]100 % mortality achieved at the lowest dose (1.0 mg/ml). LC_{50} value is predicted < 1.00 ppm.
[d]Yield of rotenone in dried roots after the concentration process, % (w/w). This response variable is not significant for the identification of optimum parameters model. [Yield of rotenone in dried roots, % (w/w) = mass of rotenone (mg)/weight of dried roots × 100 %]
[e]Only S1 is eligible for the probit analysis on the 24 hours of treatment due to majority of the treatment excluded S1 produced 100 % mortality at the lowest dose (1.0 ppm) and numbers of reasonable point of % mortality for constructing the % mortality versus log dose (ppm) is less than 1 point. LC_{50} data for all treatment excluded S1 are obtained from the interpolation method.

Table 1.0: Biological activity (LC_{50}) of Concentrated Liquid Crude Extract (CLCE) against *Artemia salina.*

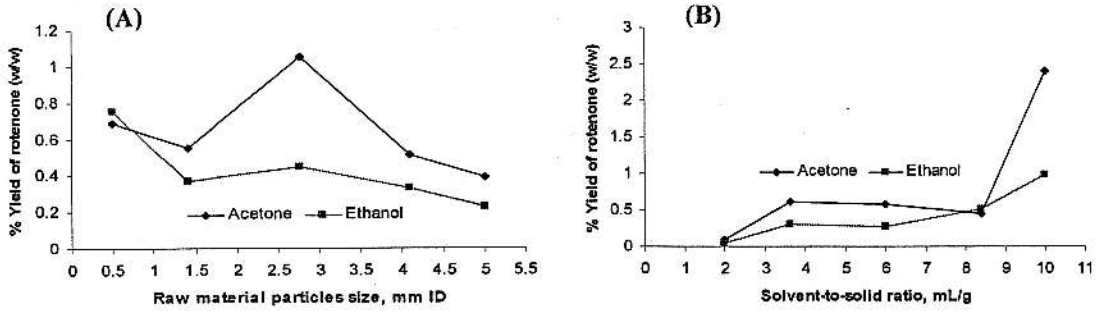

Figure 1.0: Yield of rotenone in dried roots, % (w/w) obtained from the extract of *Derris elliptica*: **(A)** The effect of raw material particles size, mm ID; **(B)** The effect of solvent-to-solid ratio, ml/g.

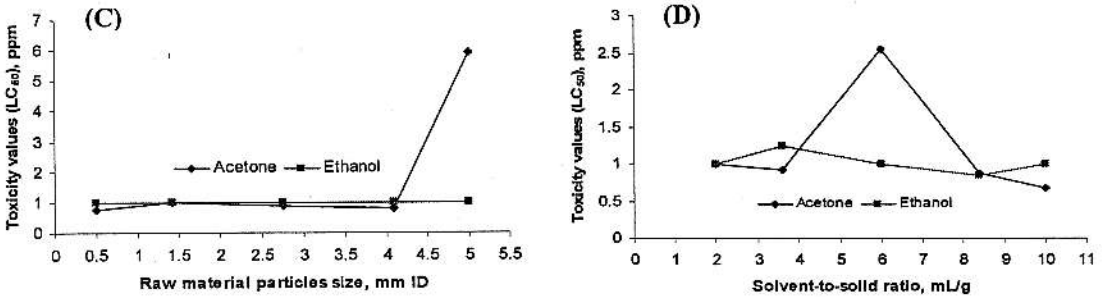

Figure 2.0: Lethal concentration (LC_{50}) of *Artemia salina* when exposed to the extracts of *Derris elliptica*: **(C)** The effect of raw material particles size, mm ID; **(D)** The effect of solvent-to-solid ratio, ml/g.

Raw material particles size	(C) LC_{50}	(C) LC_{50}	[g](A) Yield, % (w/w)	[g](A) Yield, % (w/w)
Types of solvent	Acetone	[f]Ethanol	Acetone	[f]Ethanol
0.5 mm ID	0.77	<1.00	0.69	0.75
1.41 mm ID	<1.00	<1.00	0.55 ± 0.20	0.37 ± 0.04
2.75 mm ID	0.89 ± 0.19	<1.00	1.05 ± 1.20	0.45 ± 0.49
4.09 mm ID	0.80 ± 0.06	1.01 ± 0.69	0.51 ± 0.02	0.33 ± 0.18
5.00 mm ID	5.91	<1.00	0.39	0.23
Solvent-to-solid ratio	**(D) LC_{50}**	**(D) LC_{50}**	**[g](B) Yield, % (w/w)**	**[g](B) Yield, % (w/w)**
Types of solvent	**Acetone**	**[f]Ethanol**	**Acetone**	**[f]Ethanol**
2.00 ml/g	<1.00	<1.00	0.09	0.05
3.62 ml/g	0.92 ± 0.11	1.25 ± 0.35	0.61 ± 0.12	0.3 ± 0.13
6.00 ml/g	2.56 ± 2.90	<1.00	0.58 ± 0.17	0.27 ± 0.06
8.38 ml/g	0.88 ± 0.18	0.84 ± 0.28	0.45 ± 0.06	0.52 ± 0.21
10.00 ml/g	0.67	<1.00	2.4	0.99

[f]Ethanol is added with the H_2O & oxalic acid (OA) - Ratio (9: 1 [Prepare 1.0 mg/ml of (OA) solution from the H_2O ratio]).
[g]Yield of rotenone in dried roots after concentration process, % (w/w) = Yield of rotenone (mg)/weight of dried roots (g) × 100 %.

Table 2.0: Statistically summarized for the biological activity (LC_{50}) of Concentrated Liquid Crude Extract (CLCE) against *Artemia salina* on the 24 hours of treatment established by McLaughlin (1991).

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