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Optimization of NepetaCataria Essential Oil Extraction Yield by Ultrasonic-Soxhlet Extraction Method Using Response Surface Methodology

R M S Marmy[1], N H Norulakmal[1] and G Faridah[1]

1Process Department, Universiti Kuala Lumpur (UniKLMicet), TabohNaning, 78000 Alor Gajah, Melaka, Malaysia.

Abstract. Essential Oil (EO) of *nepetacataria L.* has an attractive benefit due to its potential as alternatives compound of controlling mosquito vectors especially Aedesaegypti. This paper reports on application of response surface methodology (RSM) using Box Behnken design (BBD) to optimize the extraction of EO from *nepetacataria L*. The parameters studied are time of extraction (120-360 min), temperature of sonication (30-50°C) and ratio of sample to solvent (w/v) (30-60 gram/150ml). The extraction was conducted by using soxhlet method assisted by ultrasound using ethanol (polar solvent). Statistical analysis and analysis of variance indicated that the time of extraction and ratio of sample to solvent are significant factors ($p<0.05$) affecting the yield while the temperature of sonication was insignificant ($p>0.05$). For the modelled extraction, the minimum critical values for ethanol solvent was 269.48 minute, 40.18 °C and 33.22 gram/150 ml ethanol. Recovery of oil when 1 gram of raw material was used per 150 ml of solvent was 2.32%. By using High Performance Liquid Chromatography with Ultraviolet detector (HPLC-UV), nepetalactone was detected at time of 12.694 min and 13.941 min using absorption peak 228nm.

1. Introduction

Nowadays, the usage of herbs in daily life become widely spread and common. Herbs been used as medicines to treat illness. Besides, it has been used in a wide range of applications such as dietary supplement, remedies and nutrition. Generally people made herbs beneficial in many manners according to the specified functions. Blumenthal et al. (2006) [1] stated that there are positive growths in total sales of herbal dietary supplements within 10 years from 1996 to 2005. In this study, *nepetacataria L* leaves is choose as the plant material to extract the essential oil. Essential oil (EO) has high potential to be used as medicine to human for many diseases. EO are chemically complex and contain many different substances and compounds. Different types of plants has different chemical characteristic which made essential oil is a reliable source of alternative medical treatment.The plant is choose due to its potential as alternatives compound of controlling mosquito vectors especially Aedesaegypti. The aim of this study is to determine the optimization conditions for the extraction of essential oil from nepeta leaves using ultrasonic assisted soxhlet extraction method.

Recently, dengue had becomes one of the major problems to our country, Malaysia and also few other countries in Asia. According to World Health Organization, WHO (2015) about 500,000 people been hospitalized each year due to severe dengue. Unfortunately, a large number of those affected are among children and this problem associated with more serious infections which have more prevalent as 2.5% of those affected die. Dengue is a systemic viral infection transmitted between humans and Aedes mosquitoes. Prevention of this disease almost entirely dependent on various methods of vector control. Control of vectors by insecticides remains the most important method of reducing disease transmission and protection from mosquito bites [2]. Alternative compounds of controlling mosquito vectors are needed which lies in botanical compound that commonly used and known as insect repellent. The usage of current synthetic insecticides for the control of the vector of mosquito has disturbed the natural biological control system and undesirable effects on non-target organisms, fostered environmental and human health concerns [3]. N,N diethyl-z-toluamide (DEET) are the most conventional repellent used which is now still effective. However there is arising concern on the usage

of DEET which results in unpleasant odour, can damage plastics and synthetic rubber and has high skin penetration characteristics [4]. Due to the problem for the usage of DEET, new improvement on the repellents and strategies to control mosquito attack is needed. The best alternative is to use bioactive compound which extracted from plant because they gives no harmful effect. Thus, much interest has focused on plant extracts, or plant essential oils, as potential mosquito repellent agents.

A few numbers of botanical extract were tested as alternatives mosquito repellents such as eucalyptus (*Eucalyptus citriodora Hook*), citronella grass (*CymbogonnardusRendle*), thyme (*Thymus vulgaris L.*), clove (*Syzygiumaromaticum L.*) and catnip (*NepetaCataria L.*) [5]. In this study, *nepetacataria L.* commonly known as catnip or catmint is used as herbal plant where historically the oil of *nepetacataria L.* has been used in herbal medicine to treat fever, head and tooth aches, colds, colic and spasms in human [6]. Research of *nepetacataria L.* plants had been widely studied by researcher such as a stable effective fly repellent studied by Zhu, et al. 2012 [7] and the usage of essential oil of *nepetacataria L.* as a natural preservative agent in food products [8]. There are continued interests in studies on behavioural response of NepetaCataria L. towards few species of mosquitoes including AedesAegypti.

Essential oils were obtained from parts of the plant by many different ways mainly by extraction method. Ultrasonic assisted solvent extraction (UAE)is being introduced in the extraction industry. Ultrasound is sound waves that comprise frequency between 18 kHzto 100 kHz. The broad range can be divided into high intensity or called aspower ultrasound (20 – 100 kHz) and diagnostic ultrasound (1-10 MHz)[9]. Ultrasonic waves can easily penetrate opaquematerials, where as many other types of radiation such as visible lightcannot. Since ultrasonic wave sources are inexpensive, sensitive andreliable, this provides a highly desirable way to probe and image the interiorof opaque objects [10]. Ultrasound also achievesgreater penetration of a solvent into a plant tissue and improves the masstransfer. A considerable amount of literature has beenpublished on the success of ultrasound [11]; [12]; [13].Recent developments in the field of extraction have opened up somegreat expectations with a promising result of UAE. UAE utilises acousticcavitation to cause molecular movement of solvent and sample. This methodoffer many advantages such as improved efficiency, reduced extraction time, low solvent consumption, and high level of automation as compared toconventional extraction techniques [14]. Currently, thetypically used ultrasonic systems are either bath and probe type sonicator.Both system are based on electromagnetic transducer. Figure 1 shows theschematic diagram of ultrasonic bath [9].

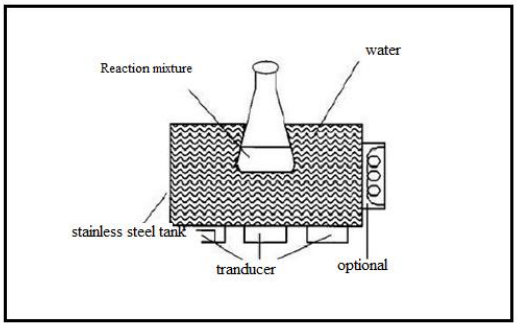

Figure 1. Schematic diagram of ultrasonic bath.

2. Materials and method

The extraction procedure was divided into few subsection where starting with preparation of raw material which is important in order to suit the material structure with the extraction equipment. Preparation of solvent where the two solvent was measured earlier for every run to ease the experiment and avoid any incident to occur if the solvent was being measured right when the solvent is needed. Design of experiment was performed before the start-up of experiment to determine the number of runs and also the matrix of experiment. Prior to extraction of oil, pre-treatment was conducted using Ultrasonic Bath and followed by Soxhlet extractor. Themixture of solvent and oil was separated using rotary evaporator. Selected oil sample was brought to analysis in order to detect the desired constituentwhich was Nepetalactone.

2.1 Selection of design parameters

Thisexperimental design can be generated by Design of experiment method (DOE). It facilitates an arrangement and implementationmethod of the experiment in order to investigate the parameter affecting theexperiment. Once arrangement of method is done, the data obtained arefurther analysed by using statisticasoftware version 10.0 . In this paper, the objectives function forthe optimization of factor affecting the yield of extraction which is to obtainmaximum yield of essential oil. Therefore, three main parameters which aretemperature of the sonication, time of soxhlet extraction and ratio of solventto sample is chosen.

2.2 Box–Behnken experimental design

Experimental design is widely used for controlling the effects of parameters inmany processes. Its usage decreases number of experiments, using timeand material resources. Furthermore, the analysis performed on the results iseasily realized and experimental errors are minimized.In this study, the Box–Behnken experimental design was chosen for findingout the relationship between the yield of extraction and the three variables.Box–Behnken design is rotatable second-order designs based on three-levelincomplete factorial designs. The special arrangement of the Box–Behnkendesign levels allows the number of design points to increase at the same rateas the number of polynomial coefficients.

2.3 Preparation of Raw Material & Drying Process

Sample was prepared by firstly being washed, driedand grinded. The leaves of raw material which is *nepetacataria L*. was obtained on 2nd July 2017 atthe road sideSimpangEmpat, Alorgajah, Melaka. Firstly, after collected theplant material, whole part of plantwere washed to avoid high contamination. The plants were being cut intosmall pieces by using cutter and divided the parts of plant before being keptinside beaker according to the parts. The plant was then left inside oven at 55°C about 6-12 hours to remove moisture content. Then, the dried plants weregrinded by using conventional blender to obtainsample with smaller particles in order to increase surface area and allowhigher yield of extraction. After grinded, the sample was sieved by using sieve to obtain uniform size of plant particles. The sample was the placedinside small beaker to be weighed according to the ratio. Sample wasweighed by using analytical balance stage by stage to obtain better andaccurate reading for weighing. Weigh of the dry sample were recorded forfurther data processing.

2.4 Extraction method

The optimization of the parameter affecting extraction yield will bestudied by using Statistica 10.0 and meanwhile the method that will be used is Box Behnken design (BBD). The extraction method will be done by using Soxhlet ExtractionMethod assisted by Ultrasound Solvent Extraction. The parameter to bestudied is only the extraction time, temperature of extraction and ratio ofsolvent to sample. The

solvent will be ethanol. The extraction will be done within 120 minutes,240 minutes and 360 minutes. For the ratio of solvent to sample, 10 gram, 20gram and 30 gram of sample will be used and amount of solvent is fixed to150 ml.

2.5 Analysis

According to Zhang (2009) [15] essential oil yield can be expressed in terms ofthe volume of the oil collected in gram per gram of dry plant material. The essential oil obtained will be analysed by using highperformance liquid chromatography (HPLC) to detect the presence ofNepetalactone.HPLC separation was performed on a YMCPackPro C18 column (150 × 4.6 mm i.d.; 3 μm) with a gradient mobile phasesystem of water containing 0.1% (v/v) formic acid(mobile phase A) and acetonitrile (mobile phase B). The wavelength of UV detection was 228 nm and the flow rate was 1 mL/min.

3. Results and discussion

Box Behnken Method was used as the type of response surfacemethodology to develop polynomial regression equation in order to analyse the correlation between the variables chosen to the yield of extraction. The final empirical formula models for the yield of extraction for Ethanol (Y1) are represented by Eq. 1 whereas Eq. 2 shows the general model ofexperiment before the coefficient of each variable been determined. Thecoefficient of the variables were tabulated and the value was been substituted into Eq.2 in order to obtain the predicted second orderpolynomial model.

$$Y1 = B0 + B1X1 + B2X2 + B3X3 + B12X1X2 + B13X1X3 + B23X2X3 + B11X12 + B22X22 + B33X32 \quad (1)$$

$$Y1 = 156.6942 - 0.7892\ X1 - 4.2160\ X2 - 2.1406\ X3 + 0.0019X12 + 0.0346\ X22 + 0.0783\ X32 + 0.0169X1X2 + 0.0002\ X1X3 + 0.0002\ X2X3 \quad (2)$$

Where, Y is the predicted response, B0 is model constant; X1, X2, and X3 areindependent variables; B1, B2, and B3 are linear coefficients; B12, B13, and B23are cross product coefficients and B11, B22, and B33 are the quadraticcoefficients.Positive signs in all equation indicate synergistic effects. In term of this research variable, increase in time of extraction, temperature of sonicationand ratio of sample to solvent will increase the yield of extraction.Conversely, negative signs in all the equation indicate antagonistic effectswhere yield of extraction will increase only if values of time of extraction,temperature of sonication and ratio of sample to solvent decrease.

The quality of the model developed was evaluatedbased on the correlation coefficient, R^2. The closer the R^2 value to unity themore accurate the response could be predicted by the model. In thisexperiment, the R^2 value for yield of extraction for ethanol was 0.99219. Thisindicated that 99.219%of the total variation in yield of extraction wasattributed to the experimental variables studied. The R^2 value of 0.99219 wasconsidered as relatively high which reflected that predicted values for yield ofextraction would be more accurate to its actual value. The model developedwas successful in capturing the correlation between the variables to theresponses. The plot of experimental value of yield of extraction (%) vs thepredicted values obtained from the model indicated a good fit as presented in Figure 2.

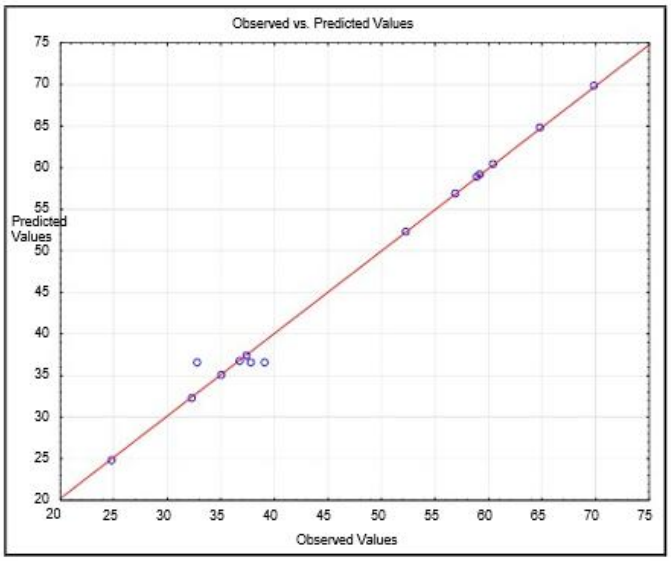

Figure 2 Predicted vs. Actual Extraction Yield (%) for ethanol

3.1 Effect of Parameters and the Interaction Between Parameters on Yield of Extraction for Ethanol

In order to determine the necessity of optimization of the parameter,hypothesis testing was conducted. Statistical analysis heavily relies on test of the hypothesis. It plays animportant role in decision making or analysis. In statistics, hypothesis undertest is referred to as hypothesis null, and associated with alternativehypothesis which is defined to be opposite of the null hypothesis.In this project, the null hypothesis was stated that the yield ofextraction does not affected by the changes of the value of the variablechosen, while the alternative hypothesis was stated that the yield ofextraction was affected by the changes of the value of the variable chosen.For the validation of which hypothesis to be rejected or accepted, anappropriate test statistic which is F distribution test was computed from theexperimental data obtained. Analysis of variance (ANOVA) was performed toobtain the Fishers variance ratio (F-value). The Fishers' variance ratio F-value is the ratio of the mean square owing to regression, to the meansquare owing to error. It is the measure of variation in the data about themean. The mean squares were obtained by dividing the sum of the squares of each of the variation sources, the model and the error variance, by therespective degrees of freedom. The results of the ANOVA are given in Table 1 below.

Table 1 Analysis of Variance (ANOVA) on Yield of Extraction

Sources	Sum of Squares(SS)	Degree of Freedom (df)	Mean Squares (MS)	F-value	R_2
Regression (SSR)	2809.656	12	234.138	21.163	0.99219
Error	22.127	2	11.0635	-	-
Total (SST)	2831.783	14	-	-	-

From Table 1 above, the model F value was 21.163 which fall on the criticalregion of the distribution as the F value from F distribution table was 19.4.Hence in this case, dthe null hypothesis was rejected while alternativehypothesis was accepted. On top of this, the optimization of the parameterwas highly suggested to support the alternative hypothesis which stated thatthe yield of extraction was affected by the changes of the value of thevariable chosen.A better view of the interactions can be seen using Pareto chart as shown in Figure 3 which was used to graphically summarize the importanceof each parameter with respect to yield of extraction.

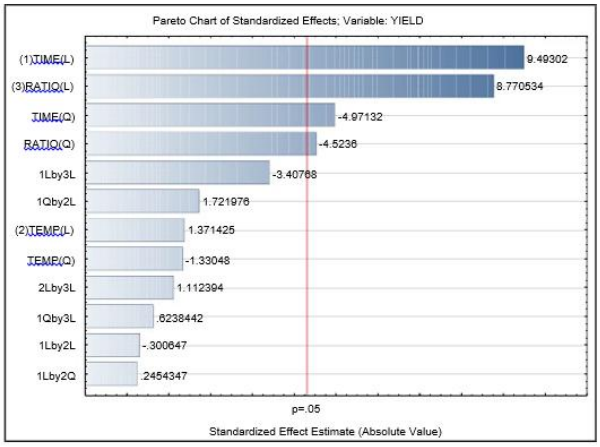

Figure 3 Pareto Chart for the yield of extraction using ethanol as solvent

NOTE :L is linear relation and Q is quadratic relation while the parameter 1, parameter 2 and parameter 3 indicated parameter time of extraction, temperature of sonication, and ratio of sample to solvent

The Pareto chart(Figure 3) above showed all the effect linear, quadratic and also the effect of interactionsbetween parameter. It also displayed a frequency histogram with the lengthof each bar proportional to each of the estimated standardized effect. Thevertical line on the chart functions as indicator whether the effect isstatistically significant within the generated response surface methodology.The bars that extend beyond the lines are statistically significant at 95%confidence level. The sequences of the bar are based on the level ofsignificance of the effect of parameters and the interaction which increasesfrom bottom to top. The least significance bar was at the bottom and thesequence followed by the higher significant parameters.Threedimensional response surface was plotted which can be seen in Figure 4. The use of surface plots was also to explore the effect of changingfactor levels on the response. There are basically three interactions between the parameter to be studied which were the parameter time of extraction vs temperature of sonication, time of extraction vs ratio of sample to solvent, and ratio of sample to solvent vs temperature of sonication. All of those three interactions are not significant based on the p value as stated in the Pareto chart.

Based on the Pareto chart, bar of interactions between parameters did not extend beyond the vertical red line which proven the insignificance of the interactions.

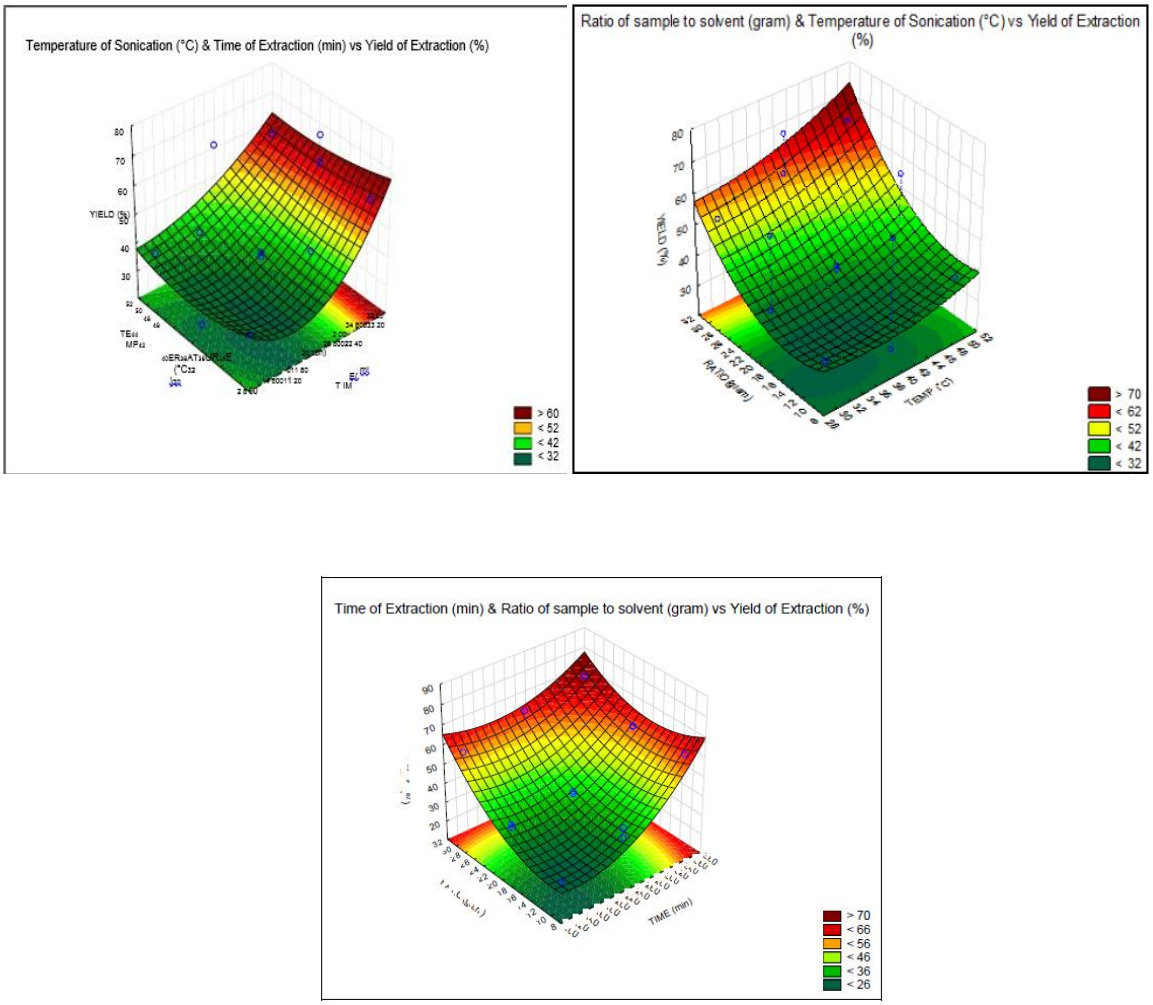

Figure 4 Response Surface Plot of oil yield for ethanol

The effect of single parameters, threedimensional response surface was plotted which can be seen in Figure 4. The use of surface plots was also to explore the effect of changing factor levels on the response. From the 3D fitted surface graph, dark areawith color tone of orange to brown indicates high yield of extraction whilebrighter area with color tone of green to yellow indicates low yield ofextraction. Increment from bright color to dark color shows an increase inyield of extraction due from the effect of all parameters involves.The effect of time of extraction on yield of extraction was illustrated asshown in Figure 4 which reported the interaction between the time ofextraction and temperature of sonication on yield of extraction while the interaction between time of extraction and ratio of sample tosolvent on yield of extraction. As can be seen from both plotted responsesurface, the yield of extraction was high at time of extraction higher than 340minutes. It is apparent from the result which indicate that time of extractiondisplayed a linear effect on the response, where the longer the time ofextraction, the higher the yield of extraction. Therefore, optimum time ofextraction has not

been achieved from the response surface when ethanolwas used as solvent.The minimum critical value obtained for the time of extraction was at157.28 minutes which demonstrated that at the beginning of extraction,before reaching the minute of 157.28, the yield was higher. This had proventhe theoretical background for the extraction, on the basis of Fick's secondlaw of diffusion. The osmotic pressure causes the solvent to penetrate theplant matrix from the beginning of extraction, and later at certain level,concentration of solute in plant matrix and the solvent achieved finalequilibrium causes a slow rate of extraction to occur [16].

The change in temperature thus was notsignificant to the yield of extraction. The reason for this insignificance wasbecause the range of temperature or the level of minimum and maximumwas not relevant. Contrary to expectation, the usage of ultrasound expectedto allow changes in extraction condition as mentioned by Romdhane&Gourdon (2002) [17] in their research where decrease in temperature of theextraction process was achieved. Due to this expectation the level of factorswas chosen between 30°C to 50°C only however other factors such as thesize of plant material and polarity of solvent used, possibility of obtainingdifferent results when different type of material had been used is relativelyvery high. The later factor contributed highest influence on the extractionprocess. This evidence is supported by Wang and Weller(2006) [13] who claimedthat co-solvent could give higher yield of essential oil. In addition, the effect oftemperature in the range of 40–66 °C on the yield is negligible, such thatoptimal extraction occurs across the range of temperature from 40 to 66 °C. Therefore, use of ultrasound-assisted extraction is advisable for extraction ofcompounds, which may be altered under Soxhlet operating conditions due tothe high extraction temperature [13].From the graphical representation shown in Figure 4,the plots showed that rising in temperature of sonication does not affect theyield of extraction.The minimum critical temperature obtained from thestatistical analysis was 34.84°C. However the optimum temperature ofsonication need to be determined because too high temperature might givethermal degradation effect throughout the experiment.

Based on graphical representation high yield could be obtain at ratio of range from 30 to 32 gram of sample per150 ml of solvent. This indicated that the yield of extraction increased alongwith the increment of the weight of plant material. A linear effect can beinterpreted hence again, optimum ratio could not be determined at this levelof experiment. In order to obtain higher yield of essential oil, higher amount ofdried plant material is needed however thimble of soxhlet extractor can onlyfit 30 gram of sample. Therefore modification on the parameter ratio can onlybe made if the size of particle was reduced to smaller dimension. At certainextent, the extraction remains unchanged as most of the oil in the plantmaterial had been extracted. According to Sahin and Samli (2012) [18] the yield of extraction decreases with the increase of solventvolume. This is because theconcentration gradient which is driving force is supposed to be higher when alower solid to solvent ratio used, leading to higher diffusion. The optimumratio of solvent to sample need to be determined as in economical point ofview, consuming less solvent or plant material for the extraction is extremelyreasonable and practical.

3.2 Analysis of Nepetalactones

Nepetalactones is the volatile oil produced from this plant. The determinationof this constituent is necessary for this project in order to prove the presenceof nepetalactones. This compound is the desired compound due to its repellence towards for aedes mosquitoes. By using highperformance liquid chromatography (HPLC) it was possible to separate themajor component in the essential oil sample extracted. The method waschosen based on the method used by Wang et al.(2007) [19] whoconducted a comparison of quantification of nepetalactone in NepetaCatariaL. by using HPLC-UV coupled with Ultraviolet and Mass Spectrometric. Based on the analysis, the peakswere observed with maximum ultraviolet (UV) absorptions at 228 nm at flowrate 1mL/min. The detection time at two peak which were12.694 min and 13.941 respectively.

4. Conclusion

Response Surface Methodology (RSM) was successfully been usedin determining the optimum condition for the process of extraction based onthree chosen parameters which were time of extraction(min), temperature ofsonication (°C) and ratio of sample to solvent (gram/150ml). Ethanol andPetroleum Ether were used as solvent and ethanol were proven to befeasible to produce higher yield of essential oil, with several modifications onparameters are needed. Based on regression model, correlation factor (R^2)for ethanol was 0.99219.The optimum condition for ethanol solvent could not be achieved asthe model developed showed a linear effect with minimum critical value. Theminimum critical values for ethanol solvent was 269.48 minute, 40.18 °C and33.22 gram/150 ml. Recovery of oil when 1 gram of raw material was used per 150 ml of solventwas 2.32%. The aim of detection of nepetalactoneto strengthen the evidence of potentiality of this constituent to repel aedesaegypti was proven fruitful with detection of nepetalactone at absorption of228nm at detection time of about 12.694 min and 13.941 min.

5. Acknowledgement

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