OPTIMIZATION OF SUPERCRITICAL CO_2 EXTRACTION OF ESSENTIAL OIL FROM NEEM LEAVES USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT: *Neem tree or Azadirachta Indica has been proven to give a lot of benefits in medicinal point of view. Each parts of the tree have its own biological activity which could aid in Figurehting and curing diseases. These biological activities were found to come from the sesquiterpenes compounds that exist in the leaf. The main biological activities of neem leaf are anti-inflammatory and anti-allergy properties. Supercritical carbon dioxide extraction was applied for the extraction of essential oil since it gives higher oil yield and does not require extra unit to separate the solvent used. The optimization of supercritical carbon dioxide extraction is studied and the optimized parameters were 20.25MPa pressure and 319.33K using 0.23mm sample determined through RSM. GCMS analysis is carried out on the essential oil of neem leaves and 43 compounds were identified. 1.06wt % of sesquiterpenes compound of γ-elemene and α-farenesene is found. The major compounds in the extract composed mainly carboxylic acids which is 49.11wt %. 17.02wt % of hexadecanoic acid, 31.63wt % of phytol and 24.2wt % of 9, 12, 15-octadecatrienoic acid, (Z, Z, Z) - were also found in the extract. In addition, supercritical carbon dioxide extraction of essential oil from neem leaves has been proven to give higher oil yield (0.6wt %) compared to hydro distillation technique which can only extracted about 0.1wt % of the essential oil. Supercritical CO2 extraction uses environmental friendly solvent such as CO2 and is recyclable.*

Keywords: Neem leaves, super critical carbon dioxide extraction

1. INTRODUCTION

Neem tree or the scientific name *Azadiractha Indica* is part of the *Meliaceae* family which grows fast and can reach up to 50-65 feet. This tree can grow even in drought area, with sub-arid to sub-humid conditions. Neem plant has been very popular in traditional ayurvedic medicine in India [1, 2]. All part of the tree from seeds, flowers, barks, twigs, leaves and also its root have different medicinal uses to human being [3]. Neem tree exhibits biological activities such as anti-ulcer, anti-bacterial, anti-malarial, anti-inflammatory, hypoglycemic, hepatoprotective, spermicidal, chemopreventive and chemotherapeutic properties [4]. Neem seed contains the most oil content among the parts of neem tree. It contains about 45% oil composing of loeic acid, palmatic acid, seric acid, linoleic acid and arachidic acid [5, 6]. There are also some bitter components in neem seed oil which are nimbin, nimbinin, nimbidin and nimbidiol [5]. Neem seed oil has biological activities of anti-malarial, anthelmintic, anti-inflammatory, antipyretic, antimicrobial, antifungal and antiviral properties [5].

Apart from neem seed, neem flower also shows medicinal effect. Neem flower contains sesquiterpenes, aromatics, fatty acids, fatty acid esters, steroids and hydrocarbon and is used traditionally in bile suspension, removal of phlegm and also intestinal worms [2, 7]. Studies have also been done on neem leaf. It has been found to exhibit anti-inflammatory, anti-bacterial, immuno- modulatory, anti-hyperglycaemic, anti-fungal, anti-ulcer, anti-malarial, anti-oxidant, anti-mutagenic, anti-viral, and anti-carcinogenic properties (8). Neem seed and neem flower is seasonal. It can only be obtained around the month of May each year, while neem leaves is abundance and could be obtained for the whole year round. Thus, neem leaves will be studied.

Dastan *et al.* [7] and El-Hawary *et al*. [9] used hydro distillation techniques and found that neem leaves contain a high amount of sesquiterpenes compounds. There is also other technique in extracting neem leaves essential oil such as solvent extraction technique. Akpuaka *et al*. [10] uses solvent extraction technique with n-hexane as solvent and found 45 different constituents in the extract. Some of the components are γ-elemene. phytol, octadecanoic acid, n-hexadecanoic acid, eicosane, 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester and many more. Akpuaka et al. [10] concluded that the neem leaves extract has biological activities against microorganisms as good as conventional drugs while Hossain *et al*. [3] found that using chloroform as solvent shows a good anti-oxidant activity. The compounds found in the extract are (2E)-3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol, methyl 14-methylpentadecanoate, lineoleoyl chloride, phytol, methyl isoheptadecanoate and nanocosane.

This proves that neem leaves also show biological activities that are beneficial to humans. There are various techniques such as hydro distillation and solvent extraction technique studied by past research to extract the essential oil. There is also a new technique which could yield more oil and does not require any additional unit for separation of solvent [11]. The method of extraction is by using supercritical fluid as solvent. It is called supercritical fluid extraction. The solvent that has been widely used is carbon dioxide [12]. Carbon dioxide critical condition of 30.9°C and pressure 73.8bar is the best solvent because it is cheap, inert, nontoxic, non-flammable, high diffusivity, easily removed from product and also odourless [11,1]. Therefore, the objective of this study is to extract essential oil of neem leaves using supercritical carbon dioxide extraction, Sc-CO_2 and determine its optimized parameters such as pressure, temperature, particle size.

2. METHODOLOGY

2.1 FEEDSTOCK PREPARATION

Neem leaves were collected from Titi Gantung, Bota Agriculture Complex (Kompleks Pertanian Titi Gantung, Bota) and was ensured that it was healthy and in good condition. The leaves will then be packed in a polyethylene bag and kept in the refrigerator at 4°C and pour into extraction. It will then wash with running water in order to remove the dirt and soil and wash with distilled water. Then, last stage the leaves were dried at room temperature for a week under shade.

2.2 EXPERIMENTAL DESIGN

Response surface methodology, RSM was applied to evaluate the effects of the parameters on the essential oil yield. Central composite design, CCD is used to obtain the optimized parameters of Sc-CO_2 extraction of essential oil from neem leaves. The parameters studied are pressure (A), temperature (B) and particle size (C). The experimental is designed and analyzed using Design Expert software version 8.0 which aids in building and evaluating the models and plotting a three-dimensional response surface.

From the modeling using RSM, 15 runs have been arranged. Table 1 show the coded and uncoded parameters used in the RSM.

Table 1: **Coded and uncoded parameters**

Independent variable	Coded levels				
	-1.4	-1	0	1	1.4
A (MPa)	6.69	10.00	18.00	26.00	29.31
B(K)	302.82	308.00	320.50	333.0	333.18
C(mm)	0.06	0.16	0.41	0.65	0.75

2.3 EXPERIMENTAL PROCEDURE

5g of sample was prepared in the pressure vessel and attached to the input and output lines in the external of the supercritical carbon dioxide extraction oven. The flow rate of CO_2 is set at 5ml/min. The extraction was carried out for 30 minutes and the oil was collected at the collection tube. Dichloromethane, universal solvent, was used to dissolve the oil. The extracted oil was kept in the refrigerator at 4°C.

2.4 GCMS ANALYSIS

GCMS analysis was done on the extracted essential oil at optimum parameter. Analysis was carried out using GC Agilent 6890 with MS, Agilent 5973 and HP-5MS tube (30m x 0.25mm x 0.25µm) to analyze the essential oil. The injector (split/splitless injector) and detector temperature are 225°C and 300°C respectively. The carrier gas helium was used with a flow rate of 1ml/min. Oven temperature is set from 80°C to 260°C with a ramping rate of 8°C/min and holding time of 10mins. The components extracts were compared based on NIST library.

3. RESULTS AND DISCUSSION

Neem leaves essential oil extract is yellow in colour and sticks around the collection tube. Dichloromethane, a universal solvent was used to dissolve the oil extracted. Table 2 shows the oil yield of the experimental runs. It was found out that the extracted essential oil yield is about 0.2wt% to only 0.6wt%. Dastan *et al*. [7] and El-Hawary et al. (9) extracted only 0.1wt% and 0.08wt% of neem leaves extracts using hydro distillation technique respectively. This proves that Sc-CO_2 technique extract more essential oil compared to hydro distillation techniques. However, in overall neem leaves are seen to give lower oil yield compared to neem seed oil yield which is 12% to 41wt % [14]. Sc-CO_2 technique is also time saving as a higher oil yield could be obtain in only 30 minutes compared to hydro distillation technique that consumes 3 hours operation with a lesser oil yield [7, 9]. Therefore, Sc-CO_2 technique is a better oil extraction technique as it could obtain higher oil yield and uses shorter time.

Table 2: Oil Yield of the Experimental Runs

Experimental run	A Pressure (MPa)	B Temperature (K)	C Particle size (mm)	Oil Yield (wt %)
1	10.00	308.00	0.16	0.2
2	18.00	320.50	0.75	0.2
3	10.00	333.00	0.65	0.2
4	26.00	308.00	0.65	0.2
5	18.00	320.50	0.41	0.4
6	18.00	320.50	0.41	0.6
7	18.00	320.50	0.41	0.6
8	26.00	333.00	0.16	0.4
9	18.00	338.18	0.41	0.4
10	18.00	320.50	0.41	0.5
11	18.00	320.50	0.41	0.6
12	29.31	320.50	0.41	0.2
13	18.00	320.50	0.06	0.6
14	6.69	320.50	0.41	0.2
15	18.00	302.82	0.41	0.4

Table 3: **ANOVA analysis neem leaves extraction**

Source	Sum of square	df	Mean square	F-value	p-value (prob>F)
Model	0.3714	9	0.0413	6.3308	0.0280
A-Pressure	0.0000	1	0.0000	0.0000	1.0000
B-Temperature	0.0000	1	0.0000	0.0000	1.0000
C-Particle Size	0.0800	1	0.0800	12.2727	0.0172
AB	0.0167	1	0.0167	2.5643	0.1702
AC	0.0050	1	0.0050	0.7670	0.4212
BC	0.0050	1	0.0050	0.7670	0.4212
A^2	0.2096	1	0.2096	32.1469	0.0024
B^2	0.0324	1	0.0324	4.9716	0.0762
C^2	0.0324	1	0.0324	4.9716	0.0762
Residual	0.0326	5	0.0065		
Lack of fit	0.0006	1	0.0006	0.0741	0.7990
Pure error	0.0320	4	0.0800		
Cor. total	0.4040	14			

*Std. Dev. 0.08074 **R-squared** **0.919325**

3.1 ANOVA ANALYSIS

From the ANOVA analysis as in Table 3, the p-value of the analysis is 0.0280 which is <0.05 and this shows that the model is significant with a confidence level of 95%. Significance of the model is represented by p-value while the factor that influences the study is represented by F-value [15]. The significance of the analysis parameters on the essential oil yield was in the order of particle size > pressure > temperature. The R^2 value obtained from ANOVA is 0.92. From the data of essential oil yield using the parameters A, B and C, a second-degree polynomial is suggested to fit the most as shown in Eq. (1) below;

$$\text{Oil yield} = 0.537037 - 0.14142C - 0.09142AB - 0.05AC - 0.05BC - 0.016481A^2 - 0.06481B^2 - 0.1681C^2 \quad (1)$$

where A, B and C are the pressure, temperature and particle size. The sign in front of the terms gives different meaning. Synergistic effect is signified by positive sign and negative sign in front of the terms signified the antagonistic effect [16].

3.2 RSM 3D PLOT

The most influential parameter on the yield of essential oil is particle size. This could also be seen in the perturbation plot in figure (1). The middle point shown is the centre point and the curve which varies the most from the centre point is the most influential parameter which is particle size of the feedstock. The second most influencing parameter is pressure and the least influencing parameter is temperature. Figure (2) shows the difference between actual values from experiment and predicted values calculated by the model. It can be seen from the plot that the experimental value does not deviate much from the linear line. The relationship between pressure and temperature is less obvious. When both temperature and pressure are either very low or high, the oil yield decreases as in figure (3). Nevertheless, at low pressure, the oil yield is seen to be decreasing with an increment in temperature [17]. In the extraction of neem leaves essential oil, at low pressure the oil yield decreases with a decrease in temperature. The maximum yield as can be obtained is at 20MPa and temperature of 319K. For the effect of pressure, when the pressure increases, the oil yield increases. Increase in pressure will increase the density of solvent and thus increases the solubility of solute in solvent [13, 18]. However, equilibrium is achieved until the oil yield started to decrease. This shows that there is an optimum condition for pressure. Similar trend be observed with respect to temperature. When the temperature increases the oil yield will also increase until a point when it will started to decreases when the temperature keep on increasing. A further increased with temperature due to decreasing the density of the solvent and thus reduces the solubility of solute [13]. Particles size influences the oil yield largely. From figure (4) and figure (5) smaller particle size showed a higher oil yield. Generally smaller particle size will result much in extraction the oil yield [13]. Large surface areas are available for extraction when the size of particle is smaller. This exposes the feedstock more to the solvent for extraction to occur. Besides, grinding feedstocks to smaller particle size disrupted the cell wall of the sample and reduce the intra particle diffusion resistance with shorter path for diffusion of solute [19]. However, the oil yield will decrease when the particle size is smaller.

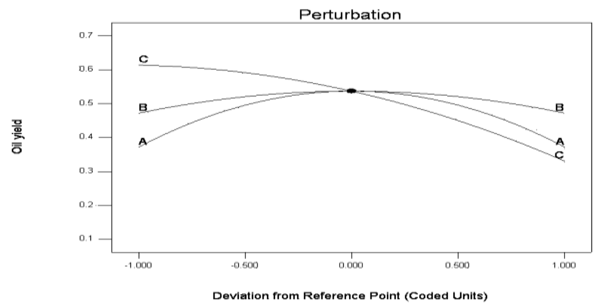

Figure (1) Perturbation plot (A: Pressure, B: Temperature, C: Particle size)

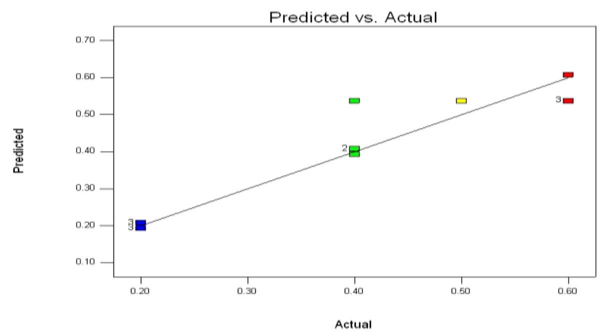

Figure (2) Predicted values versus experimental values

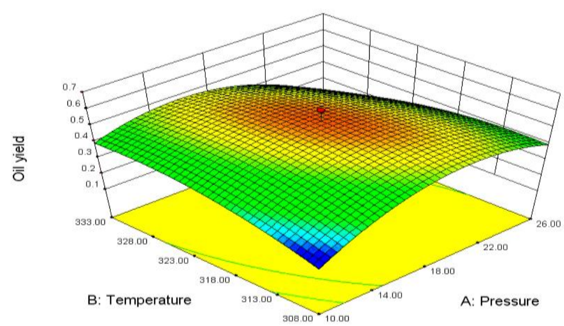

Figure (3) Three dimensional plots on the combined effect of pressure and temperature

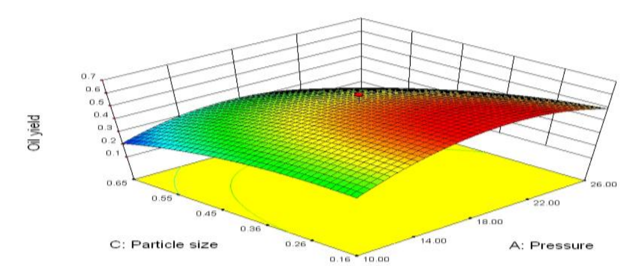

Figure (4) Three dimensional plots on the combined effect of pressure and particle size

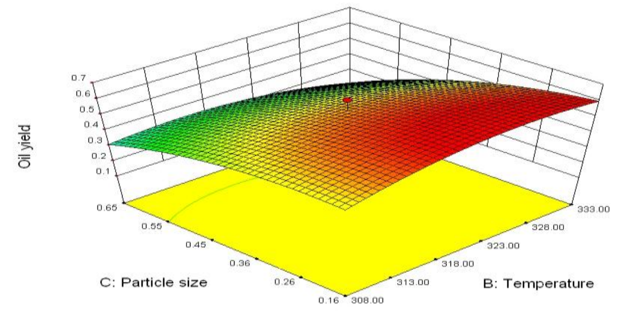

Figure (5) Three dimensional plots on the combined effect of temperature and particle size

3.3 OPTIMIZATION ANALYSIS

The optimum condition of supercritical carbon dioxide extraction of neem leaves is 20.25MPa, 319.33K and 0.23mm particle size. It is reported that with these parameters, the highest oil yield of 0.6% can be obtained with a standard deviation of 0.08 at confidence level of 95%. Two repeated runs (Table 4) were carried out to confirm the result and an average of 0.6013% oil yield was obtained with a standard deviation of 0.08. This proved that the optimum conditions for Sc-CO_2 extraction of neem leaves are at 20.25MPa, 319.33K and 0.23mm particle size.

Table 4: **Oil Yield (%) with Optimized Parameters**

Run	A Pressure (MPa)	B Temperature (K)	C Particle size (mm)	Oil Yield (wt %)
1	20.25	319.33	0.23	0.70
2	20.25	319.33	0.23	0.54
Average				0.62

3.4 ANALYSIS OF NEEM LEAVES ESSENTIAL OIL

GCMS analysis was carried out on the essential oil obtained using the optimum conditions. Figureure 6 shows the components identified in neem leave essential oil. Neem leaves mainly consist of sesquiterpenes compounds. However, from the GCMS analysis, carboxylic acid is found to be the highest amount of compounds in neem leaves. Two sesuiterpenes compounds found which are γ-elemene (0.72 %) at the retention time of 15.336min and α-farenesene (0.34%) at the retention time of 15.410min. A total of 1.06 wt % of sesquiterpenes is only found in the extracted essential oil.

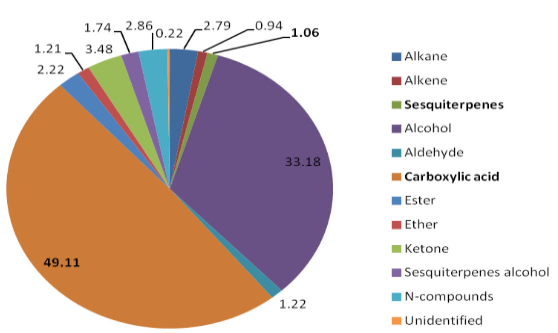

Figure (6) Components identified in neem leaves essential oil

The compounds are n-hxadecamoic acid (17.02%) at 22.673 minute, phytol (31.63 wt %) at 31.63 minute and 9, 12, 15-octadecatrienoic acid, (Z,Z,Z) –(24.2 wt%) at 25.558 minutes. These compounds also show some biological activities which are beneficial to humankind. Studies shows that n-hexadecanoic acid, shows anti-inflammatory activity against phosphalipase [20] whereas, phytol has been widely known to be synthetic form of vitamin E and K1. Phytol is also been studied to show antimycobacterial activity against mycobacterium tuberculosis [21. 9, 12, 15]-octadecatrienoic acid, (Z, Z, Z) - which is a family of linoleic acid group has shows medicinal properties such as anti-thitaminic, anti-inflammatory, anti-eczemic and many more [22]. Therefore, despite having fewer amounts of sesquiterpenes in the essential oil extracted from neem leaves using Sc-CO_2 extraction, carboxylic acid was also present which shows medicinal properties. n-hexadecanoic acid, phytol and 9,12,15- octadecatrienoic acid, (Z,Z,Z)- have the properties which give good effect towards human.

4. CONCLUSION

Using the method of supercritical carbon dioxide extraction of essential oil from neem leaves has been proven to give a higher oil yield of 0.6 wt% compared to hydro distillation technique which extracted only 0.1 wt%. Supercritical CO_2 extraction uses environmental friendly solvent CO_2 to extract its oil and this extraction process is found to be giving better oil yield and time saving. Application of RSM method of analysis allows obtaining the optimum parameters such as pressure, temperature and particle size for Sc-CO_2 extraction of neem leaves which are at 20.25MPa pressure, and 319.33K using 0.23mm sample particle size. GCMS analysis done at the optimum condition of Sc-CO_2 extraction of neem leaves was found to consist 1.06 wt% of sesquiterpenes such as γ-elemene and α-farenesene. Apart from that, 1.74% sesquiterpenes alcohol is also found. The most compounds found in the neem leaves extract is carboxylic acids (49.11 %). The carboxylic acids are n-hexadecanoic acid, phytol, and 9, 12, and 15-octadecatrienoic acid, (Z, Z, Z)-. Therefore the essential oil extract of neem leaves using Sc-CO_2 extraction method is able to extract a valuable compound which have good effects towards human and also found to have reliance against biological activity against microorganisms.

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