OPTIMISING AZADIRACHTIN YIELD FROM NEEM TREE SEEDS AND LEAVES USING A BINARY SOLVENT SYSTEM FOR POTENTIAL PEST CONTROL APPLICATION

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ABSTRACT

The neem tree (*Azadirachta indica*) is indigenous to India but is increasingly found in tropical and subtropical regions of Africa, America and Australia. Extracts from the seeds are said to be the most important source of azadirachtin, a limonoid and botanical insecticide. However, neem leafs were also found to contain azadirachtin. Traditionally azadirachtin has been obtained by mechanical extraction and spraying of the oil onto pest-infected plants. Modern extraction methods include either defatting the neem seed with hexane followed by azadirachtin extraction from defatted seeds using a polar solvent, or extraction using supercritical CO_2. In order to reduce the number of process steps involved and maximize the yield of azadirachtin from *A. indica* we combined defatting and azadirachtin extraction in one step using both pulverised neem seeds as well as leaves as source. Extraction was carried out with a Soxhlet device using hexane and ethanol as co-solvents at volumetric ratios of 100:0, 60:40, 50:50, 40:60 and 0:100, while extraction time was varied between 3 and 6 hours. The highest neem oil yield and azadirachtin concentration was obtained using a 50:50 solvent mixture for both neem leaves (44.7 wt%, 1611 ppm) and seeds (53.5 wt%, 1954 ppm), respectively at 6 hours extraction time. Our findings show that single-step azadirachtin extraction using a binary solvent system is not only possible but also helps to save time, solvent and arguably cost. Using both neem seeds and leaves as source of azadirachtin for pest control applications helps to increase the profitability of the plantation and mill without the need to increase plantation size. Further work is needed to study the effectiveness of crude and refined extract as insecticide.

Keywords: Azadirachtin, limonoids, neem tree, extraction, binary solvent system, botanical insecticide.

INTRODUCTION

The neem tree (*Azadirachta indica*) is a tropical plant originating in South Asia but increasingly encountered in Africa, America and Australia. It belongs to the *Meliceae* family and rapidly grows in tropic and semi-tropic climate with an extended dry season [1]. All parts of the neem plant such as leaves, seeds, bark, flowers, fruit and root are very useful in medical treatment and industry products. The ripe neem fruits are oval shaped, have light - coloured seeds inside the fruit [2] and contain the greatest oil content [3] (Hussain et al., 2008).

Neem oil has been used for birth control and to treat diseases such as diabetes, cancer, heart failure and AIDS [4,5. Neem oil is also applied in agriculture as botanical pesticide, insecticide and fungicide[5,6], while it has its importance in cosmetic products such as shower gel, acne care and shampoo [3]. The oil can be used to extend the leather goods [7].

Neem seed oil mainly yields quercetin and nimbosterol as well as a number of other limonoids such as azadirachtin, nimbin and its derivatives [8,9]. Among the various limonoid components, azadirachtin is the most important biologically active component which has many anti-infective, antibacterial, antifungal and antimicrobial properties [10,11. It is found in bark, leaves, fruits, and especially in the seeds. Three derivates of azadirachtin are currently known of which azadirachtin A is the most abundant (80 %) and biologically active compound used as commercial botanic insecticide [12]. While azadirachtin impairs the insect growth regulatory system in all species tested, the effectiveness varies depending on insect order and species [13. Since azadirachtin does not kill adult insects, neem-based insecticides tend to be used with other strategies or beneficial pests. Neem was found to be the best alternative compared to those organic synthetic insecticides which are more dangerous, leave toxic residues in food, harm beneficial pests and not readily biodegradable.

Soxhlet, invented in 1879 by Franz von Soxhlet [14, is a continuous solid-liquid extraction process commonly used to extract oil from seeds and other plant components. Solvent selection is important to provide a good yield from the extraction. The solvent typically used for oil extraction is n-hexane due to its high stability, low greasy effect residual, boiling point, apolarity, low corrosiveness and high oil yield. Oil extraction depends on moisture content, solvent type, extraction time, size of particle and solvent:solid ratio. The Soxhlet extraction technique has disadvantages which is the analytes are exposes to the high temperature due to the long heating time and lead to the thermal degradation of the compounds. The samples that are recovered are diluted in the solvent oil mixture and must be in concentrated by the evaporation process which can result in the loss of solvent and other volatiles [15. In the case of azadirachtin extraction, a combination of n-hexane extraction to remove the oil followed by ethanol extraction of azadirachtin from the defatted neem seed cake has been reported [16].

The aim of this paper is to compare neem oil yield extracted from neem leaves and neem seeds through Soxhlet extraction with various extraction time to determine extraction efficiency of solvents as single solvent and binary solvent to enhance high azadirachtin recovery. The extracted neem oils that contained high azadirachtin concentration has potential to be powdered through aqueous extraction so it is best to be used in pest control as it can be mixed with soil or tinted on surface of plant or capable to use as household scent where its pleasant odour prevent insecticide problem at home.

METHODOLOGY

Preparation of neem leaves sample

The fresh neem leaves were soaked with the tap water for 24 hours at room temperature [17] in order to remove impurities such dust and any unwanted metals. After 24 hours, the soaked neem leaves were then rinsed to separate excess water at the plant material. Next, the cleaned neem leaves were dried in drying oven at 50˚C in 48 hours to reduce the moisture content and calculated by using Equation 1 shown below. According to Drabu et al. [18], the desired moisture content for extraction process should be below of 10% prior to the process. Referring to [19]Carvalho et al. (2012), the dried neem leaves must be finely chopped and grinded in blender with repeated grinding until fine to form a powder to increase the surface area and speed up the reaction during extraction process [20]. Finally, 425 and 710 µm size of Endecotts sieves were used in order to sieve the grinded powder to have particle size as shown in Table 1 because different particle size range gives yields the highest quality of oil [21. This step performed as to get the evenly or uniform size powder and stored in the air tight container to prevent oxidation and contamination in neem leaves powder.

Preparation of neem seed sample

The neem seeds were cleaned to remove sticks, unwanted leaves, bad seeds, sand and dirt to ensure oil produced not contaminated and high quality. Next, the cleaned neem fruits were dried in drying oven at temperature of 55°C for 72 hours to remove the moisture content where the fruits were weighted before it was placed in the oven and thereafter weight was re-taken at interval of 24 hour until constant weight obtained. The percentage of moisture content of neem fruits was calculated by using Equation 1 [22. Then, dried neem fruits were decorticated that involved the removal of pulp and hull to release seed that embedded inside the neem fruit. Firstly, depulped process involved separation of pulp and seeds from dried neem fruits and followed by dehulled process that involved removal of hull that present at the outer surface layer of dried neem seeds surface by peelling off using hand. Dehulled process ensured high extraction efficiency as seed outer surface layer coats contained very little or no oil delayed oil extraction process [23]. After that, the dehulled neem seeds was roasted for about 5 minutes using cooking pan under low flame to open neem seeds pores at surface layer to enhance better and faster oil extraction. Later, the roasted neem seeds were crushed in a blender [22] and sieved using 425 and 710 µm size Endecotts sieves to have particle size as shown in Table 1. Neem powder was then stored in the air tight containers and was placed in a refrigerator at temperature of 20°C for long storage period [10].

$$\text{Moisture Content } (\%) = \frac{(\text{Weight before drying (g)} - \text{Weight after drying (g)})}{\text{Weight after drying (g)}} \times 100 \quad (1)$$

Preparation of solvent

Ethanol and n-hexane were two solvents used for the extraction process. Hexane and ethanol has boiling point of 68.8˚C and 78.8ºC was used as single and binary solvent where both hexane and ethanol solvents were mixed together at several ratios to produce solvent mixtures as shown in Table 1 in Soxhlet extraction process.

Soxhlet extraction

A Buchi Extraction System B-811 of Soxhlet extraction was carried out with 4 units in a run. Firstly, Soxhlet extractor was placed in a fume to reduce the fugitive emissions of n-hexane and thus the airborne concentration. Then, the weight of flask and thimble of the extractor were noted respectively.10g of sample was weighted and placed into the thimble then weight of thimble with samples before extraction was noted. The filter paper was folded carefully and then inserted on top of sample inside the thimble to prevent sample escape and penetrate into the extractor throughout extraction period which influenced extraction efficiency and indirectly stimulus oil yield and quality [24]. After that, 150 ml of the pre-heated solvent was poured into each extractor flask which was 4/5 of the volume of the flask with mass to solvent ratio of 10g :150 ml. Then, the automatic Soxhlet apparatus was heated up by using heating mantle at fixed temperature based on types of solvent used by selecting heating mode. The oil was evaporated alongside with solvent and then condensed into the extractor, which poured back into the flask. This process was continued with repeated similar process for respective hours for selective solvent types shown in Table 1. After heating and cooling for required time, the wade thimble contained the sample was brought out. Then, the weight of thimble with samples after extraction was noted [25]. After 30 minutes of cooling down process, the mixture solution inside the flask was removed out from Soxhlet apparatus. The mixture solution was poured into a round bottom flask to distil off solvent at 80°C under vacuum in a rotary evaporator. The distillation process was continued until no solvent was distilled off from the mixture solution [24]. After the solvent was removed from the sample, a small quantity of oil was remained inside the flask and was weighted as it was pure neem oil extracted by using Soxhlet extraction. The neem oil was stored in a glass vial at 20ºC for further analysis [26]. The yield of neem oil was extracted using Soxhlet extraction was calculated by using Equation 2.

$$Extraction\ Yield\ (\%) = \frac{Weight\ before\ extraction\ (g) - Weight\ after\ extraction\ (g)}{Weight\ after\ extraction\ (g)} \times 100\% \quad (2)$$

Table 1 : The parameter used for Soxhlet extraction

Parameters					
n-Hexane to ethanol ratio (ml:ml)	100:0	60:40	50:50	40:60	0:100
Soxhlet temperature (℃)	70	70	70	70	80
Soxhlet Heating Mode	10	10	10	10	16
Time (hours)			3, 4, 5 and 6		
Particle size (mm)			0.425 – 0.700		
Mass to solvent ratio (g/ml)			1:15		

High performance liquid chromatography (hplc)

Perkin-Elmer system comprising of series 200 pump and series 200 UV-VIS detector was set at 217 nm be used. The mobile phase usually consisted of acetonitrile: water mixture of 65:35 (%v/v) with mobile phase temperature of 45˚C using column C18 of 250 mm x 4.6 mm id x 5 μm [27]. A known weight of neem oil was dissolved in methanol (Merck HPLC Grade) at sample to dilution solvent ratio of 1 g:10 ml to ensure the mixture was diluted and sonicated until complete solubilization. Then, the mixture solution was filtered through Millipore 0.45 µm membrane syringe filter before injected into the HPLC. Injection volume was 20 µL and the mobile phase flow rate was 1 mL/min. The retention time for Azadirachtin (AZA-A) was detected around 15.85 min with an isocratic elution time of 20 min [28].

RESULTS AND DISCUSSION

The binary solvents were more environmentally friendly, less toxic and gave highest neem oil yields than single solvent hexane and ethanol to increased extraction efficiency [29]. The ability of the binary solvents to perform better than ethanol and hexane because the binary solvent was able to reduce the flammability challenges usually associated with pure hexane solvent [30]. Hence binary solvent has effect of being able to operate at a temperature higher than the boiling point temperature due to increased rate of diffusion, evaporation and condensation. This invariably causes increased contact time between the binary solvent and the oil bearing material which thus increases solubility and oil yield [31].

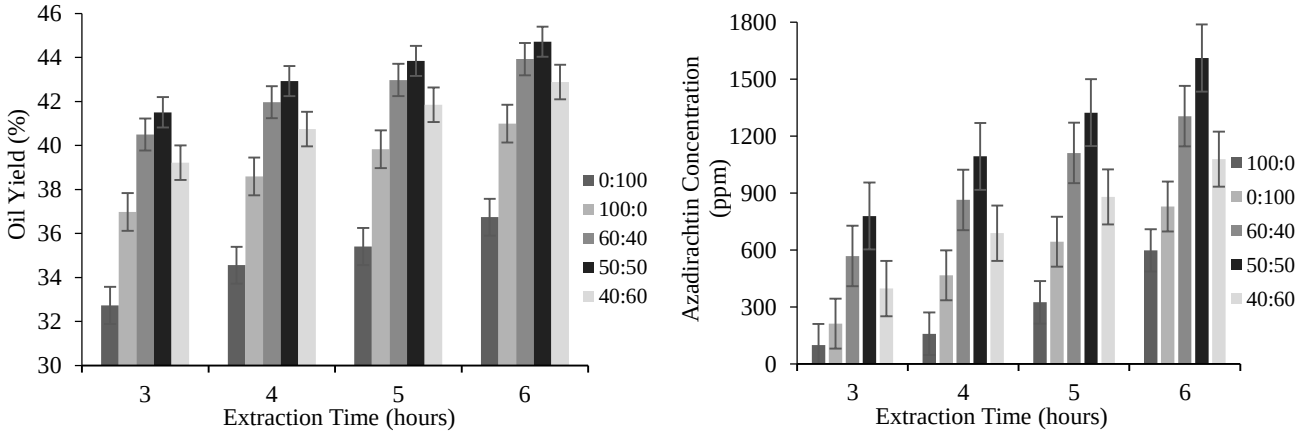

Figure 1 : Yield of and Azadirachtin concentration in oil from neem leafs at various solvent ratios and extraction times.

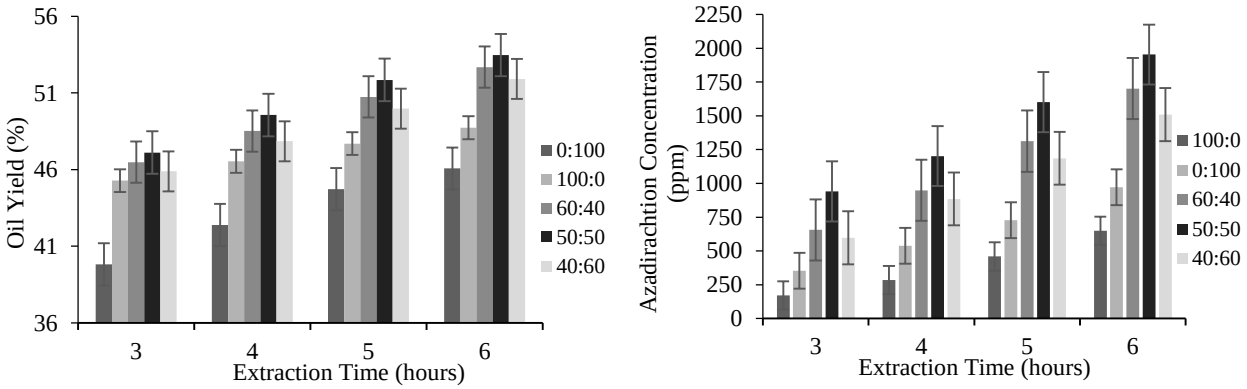

Figure 2 : Yield of and Azadirachtin concentration in oil from neem seeds at various solvent ratios and extraction times

Based on Figure 1 and Figure 2, as the extraction time increased, solvents; 50:50 gave the maximum oil yield, followed by 60:40, 40:60, 100:0 and finally least by 0:100. These showed that the binary solvents favour higher oil yield compared using single solvents because hexane performed better as extraction solvent for shorter operation period while ethanol was preferred at longer extraction period. Figure 1 and Figure 2 displayed that Azadirachtin concentrations in neem oils using hexane was less than ethanol as single solvents for extraction for both neem leaves and seeds simultaneously with time increment. However, vice versa scenario occurred for the neem oil yields when used single solvents for extraction for both neem leaves and seeds although time increased as hexane performed better than ethanol.

It was observed when extraction time increased from 3 hours to 6 hours, hexane was the best solvent that can extracted highest yield of neem oil as well as sustained in volume compared to ethanol for both neem leaves and seeds respectively when used as single solvent extraction. This was because hexane has high capacity to dissolve non-polar compounds in neem oil than ethanol has high capacity to dissolve polar compounds in neem oil [32].Therefore, hexane was a better solvent for neem oil extraction as hexane rarely extract free fatty acid compounds in neem oil. The loss of ethanol was potential lied due to increase of evaporation as result of high volatility during extraction process. Thus, neem oil yield extracted by ethanol can be seen much lower in quantity compare to hexane throughout the extraction times. The result was supported with previous study by Lafka et al. [33] stated that hexane was the best solvent for the extraction compared to ethanol in research on the extraction and antioxidant activity of phenolic compounds from winery wastes.

The binary solvent extraction of neem oil involved with nonpolar (hexane) and polar (ethanol) solvent that simultaneously removed hydrophilic and hydrophobic extracted Azadhirachtin from the neem leaves and seeds in greater percent [34].Azadirachtin was only extracted from hydrophobic fatty acids and highest oil yields were removed when nonpolar solvent used extraction of neem leaves and seeds. The neem leaves and seeds were then extracted with polar solvent to remove the hydrophilic fatty acids rich with Azadirachtin containing neem fractions meanwhile extracted less oil yields. Therefore, binary solvent able to extract higher neem oil yield plus Azadirachtin concentrations in a single extraction to prevent double extraction to reduce cost and extraction time [3].

The results in Figure 1 and 2 showed that the longer the extraction time, the higher the concentration of Azadirachtin obtained. An increasing in extraction time was reported to improve all overall extraction because of the enhancement of mass transfer and diffusivity rate of solvents into neem matrix boosted up while viscosity of solvent was declined [35]. The time of extraction increased, the solubility of bioactive compounds in solvents also increased. The solvents diffused into neem leaves and seeds as the bioactive compounds from active site within medium dissolved into the solvents [31].The net upshot of this enriched more neem oil was dissolved in solvent per contact time which increased neem oil yield and Azadirachtin concentration for both neem leaves and seeds [36]. Thus, longer extraction time, higher percentage of neem oil yield extracted. However, prolonged extraction process leading to evaporation of solvents and reduced oil yield.

Thus, the binary solvents of 60:40, 50:50 and 40:60 succeeded to extract highest neem oil yields and Azadirachtin concentrations for both neem leaves and seeds. The binary solvent 50:50 extracted highest oil yield and Azadiractin concentration of 44.72% (1611.06 ppm) and 53.47% (1953.62 ppm) followed by binary solvent 60:40 scored second place with 43.93% (1304.52 ppm) and 52.68% (1702.33 ppm) and the binary solvent 40:60 excelled in third place with 42.89% (1078.98 ppm) and 51.91% (1509.04 ppm) for neem leaf and seed oils respectively at 6 hours extraction time. Then, followed by single solvent hexane with 40.99% and 48.73% oil yields with lowest Azadirachtin concentration of 598.13 ppm and 650.06 ppm and finally single solvent ethanol with lowest oil yields of 32.73% and 39.81% with Azadirachtin concentration of 829.27 ppm and 1095.75 ppm at 6 hours extraction time for neem leaf and seed oils respectively.

The colour of neem oil was dark greenish-brown for neem leaf oil meanwhile, golden to dark golden for neem seed oil. The consistency of neem leaf oil was liquid form indicated less viscous due to present of chlorophylls compared to semi-solid form of neem seed oil resulted more viscous due to high Azadirachtin concentration [33].. The colour of neem seed oil extracted using ethanol was darker than neem seed oil extracted using hexane and binary solvents. Therefore, higher the ratio of ethanol in the binary solvents, darker the colour of neem seed oil due to absence of chlorophylls. Besides that, the odour of neem seed oil was stronger (peanur - garlic mixed smell) compared to neem leaf oil (pungent smell) [19] hence the texture of neem seed oil was smooth and neem leaf oil was silky [19].

The results obtained for neem seeds were higher than neem leaves for both percentage of neem oil yield and Azadirachtin concentration in neem oils for all solvent types throughout all extraction time. This was consequences present of chlorophyll in neem leaves that more prone to sunlight as well absence of chlorophyll in neem seeds that was not liable to sunlight effect.

Potential Pest Control Application

Azadirachtin is now known to affect a broad spectrum of over 200 insect species including Aphids, Mealybugs, Caterpillars, Japanese Beetle, Whiteflies, Mites, Root Aphids and Thrips. Azadirachtin's primary mode of action is as an anti-feedant, but it also disrupts normal insect growth, repels larvae and adults, sterilizes adults and deters egg laying. Azadirachtin products are preferred by some due to their compatibility with a wide range of insecticides and biocontrols, such as the beneficial fungus Beauveria bassiana. with no re-entry restrictions where Azadirachtin allows for use in a variety of settings and breaks down rapidly after application, minimizing unwanted effects on the environment [37]. Alternating Azadirachtin applications with other insecticides provides variable control with less risk of insects developing resistance. Diseases caused by various pathogenic

microorganisms such as fungi, bacteria, and viruses not only damage the plant as a whole and result in more than 30-40% crop losses all over the world but also severely affect food quality of the crops [38]. Neem has been in the central to this idea and a number of neem products have safely been used to control a variety of plant pathogens. Azadirachtin extracts of seeds and leaves parts of neem have been used successfully in vitro to inhibit the growth of various plant pathogenic fungi. Neem oil has been reported to be antifungal in vitro and excellent control of many plant diseases and various fungicidal commercial formulations based on the oil have been developed [1]. Neem AZA-A, a product of neem has been found to induce resistance in pea (Pisum sativum) against the fungus in vitro and gave excellent control of pea powdery mildew under field conditions alone and also in combination with plant growth promoting rhizobacteria. Most of the studies on neem research were conducted on insectspests damaging crops and the effect of neem on insects affecting man and animals are relatively. Seed kernel extract was found to be effective against young fourth instar larvae of certain mosquitoes such as Aedes aegypti, A. togi, Culex quinquefasciatus, and Anopheles stephensi [39].

CONCLUSION

Extraction time played an important role in this extraction process, where good duration allowed the extraction process to reach equilibrium which promoted an increased concentration gradient, resulted in increased of diffusion rate that allowed greater extraction of solids by solvent. Therefore, 6 hours extraction time gave highest neem oil yields and Azadirachtin concentration. Thus, longer the extraction time, higher the percentage of oil yield extracted. Furthermore, binary solvents showed potential as best solvent for extraction of neem leaf and seed oils compared to single solvent hexane and ethanol due to binary solvent has higher polarity which increased extraction yield. Therefore, binary solvents of 60:40, 50:50 and 40:60 extracted both hydrophilic and hydrophobic lipid components in neem oils resulted higher diffusion rate that allowed greater extraction of neem oil yields and Azadirachtin concentrations.

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