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A PRELIMINARY STUDY ON PHYSICO-CHEMICAL EVALUATION OF AGAR (*AQUILARIA MALACCENSIS)* SEED OIL FROM THREE DIFFERENT LOCATIONS

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ABSTRACT

Aquilaria malaccensis Lam. syn. A. agallocha Roxb (Agar) is one of the spe,cies of the genus Aquitania, belonging to the family Thymelaeaceae producing highly priceless agar wood. As a result of a defence mechanism to fend off pathogens, Aquitania species develop agar wood or resin which can be used for incense, perfumery, and traditional medicines. Many studies have reported reduction in natural populations of Aquileia malaccensis due to the overexploitation of the species which become more threatened and enlisted into CITES Appendix-II. In India, natural habitats of Aquilegias malaccensis are found in North-Eastern States. Due to the commercial value of agar wood, the species is widely grown in Karnataka, Kerala and Tamil Nadu. The formation of agarwood depends on a natural infection of fungus in the wood and no proven artificial methodology available in India. The trees in the established plantations in South India started producing fruits and seeds were available in plenty and could be collected in huge quantities. There were no reports on its traditional use of seed oil of agarwood. On an average 3 5 year-old plant can produce around 1.5 - 3.0 kg of fruits and each fruit is having 1- 2 seeds, predominantly two seeds. The seed index analysis experiment shows that on an average around 10000 numbers of seeds in 1kg with each seed weight of around 0.09g and with an average oil yield of 20-35%. In this study, a preliminary evaluation of physic-chemical properties of seed oil and its variations among three different locations of India is presented.

KEYWORDS: *Aquilegias Malaccensis, Fruit and Seed Index, Physico-Chemical Characterization, Seed Oil*

INTRODUCTION

Aquilaria malaccensis Lam. is usually known as Aloewood, Eaglewood or Agarwood and it belongs to the family Thymelaeaceae. It is found primarily in Bangladesh, Bhutan, India, Indonesia, Iran, Laos, Malaysia, Myanmar, the Philippines and Thailand (Benedict, 2009). Agarwood plant is traded internationally for the agarwood, which is produced by the tree when the trunk and roots of trees infected with *Phaeoacremonium parasiticum* (*Phialophora parasitica*), a dematiaceous (dark-walled) fungus. In reaction to the infection of fungus, the tree produces a resin with a high volatile organic compound to suppress the fungal growth that results in resinous heartwood known as agarwood (Ng *et al*., 1997). Agarwood oil is one of the highly-priced natural products in the world. The oil obtained from the resinous heartwood agarwood is a reddish-brown viscous liquid with sweet aromatic fragrance (Benedict, 2009).

The rate of oil is determined by its grade. The grade and quality of agarwood oil are determined by different factors like country of origin; fragrance strength and longevity; wood density; product purity; resin content; colour and size of the form traded (Barden *et al*., 2000). This oil is used for religious, aromatic, and traditional medicinal preparation. Agarwood oil and powder is mainly used for Chinese medicine and pharmaceutical industries (Hashim and Phirdaous, 2014). The presence of terpenes in the agarwood gives the fragrance and makes it one of the highly demanded ingredients in fine perfumery. Sesquiterpenes present in the resin have plenty of pharmacological actions which include anti-inflammatory and anti-allergy properties (Benedict, 2009). Agarwood oil also proved anticancer activity towards MCF-7 breast cancer cells (Hashim and Phirdaous, 2014). In Malaysia, the fragmented resin is also used to flavour curries and in Taiwan, it is used for the particular aroma to local wines (Singh *et al*., 2010).

In India, *A. malaccensis* grows mostly in the foothills of the North-eastern region (Assam, Meghalaya, Nagaland, Mizoram, Manipur, Arunachal Pradesh, and Tripura) and West Bengal up to an altitude of 1000 m. In Assam and Meghalaya, it occurs sporadically in the district of Sibsagar, Sadiya, Now gong, Darrang, Goalpara, Garo Hills and Cachar Hills (Atal and Kapoor, 1982). The high international demand and shortage of *A. malaccensis* due to overexploitation made the species more threatened and entered into CITES Appendix-II (CITES, 1994). *Adularia* sp. has adapted to live in various habitats, including those that are rocky, sandy or calcareous, well-drained slopes and ridges and land near swamps (Chakra arty *et al*., 1994). The number of trees in the natural habitats is reduced and the species is widely grown in the home gardens of North-East India to meet the demand. The formation of agarwood in the tree depends on natural infection and only a few trees produce agarwood. Considering the economic value of the agarwood, the species is introduced in the Western Ghats regions of Karnataka and Kerala and some places of Tamil Nadu. More than 5 lakh plants have been planted in Karnataka alone and most of them produce fruits. As the formation of agarwood depends on a fungal infection, it will take years to provide income to the growers through agarwood. The tree produces fruits at the age of 3 years. The fruit is a capsule (Schmidt *et al*., 2004) and having 1- 2 seeds, but predominantly two-seeded. The seeds are recalcitrant, which cannot be stored for a longer time and the seeds fall from the fruits once it is matured. Identifying the utilization value of the seed is important to provide additional income to the farmers. In recent times, it was found out that the oil extracted from agarwood leaves contains bioactive compounds which are phytol, squalene, n-hexadecanoic acid and octadecatrienoic acid. Therefore, oil extracted from agarwood leaves has the potential to be applied in food, pharmaceutical, nutraceutical and cosmetics industries (Lee *et al*., 2017).

The agar wood tree is also used as herbal medicine. It has been used for traditional medicine on account of its effectiveness as a sedative, in detoxifying the body and in maintaining stomach health. Agarwood is used for treating sore throats with its anaesthetic qualities, extreme fatigue with a prescription that includes bear bile, cattle gallstone, ginseng and camphor (Compton and Ishihara, 2004), small pox and for various abdominal complaints through complex ointment. It is also used as a prescription for dropsy, as a carminative, a stimulant, a tonic especially during pregnancy, after child birth and for diseases of the female genital organs and for palpitations of the heart (Chakrabarty *et al*., 1994). Recently, the *Aquilaria malaccensis* has been reported as a potential plant species to uptake and translocate heavy metals found in sewage sludge (Rajoo *et al*., 2013) which indicate its importance in different line of applications.

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trees produces agar wood. Considering the economic value of the agar wood, the species is introduced in the Western Ghats regions of Karnataka and Kerala and in some places of Tamil Nadu. More than 5 lakh plants have been planted in Karnataka alone and most of them produce fruits. As the formation of agar wood depends on fungal infection, it will take years to provide income to the growers through agar wood. The tree produces fruits at the age of 3 years. The fruit is capsule (Schmidt and Nguyen, 2004) and having 1- 2 seeds, but predominantly two seeded. The seeds are recalcitrant, which cannot be stored for longer time and the seeds falls from the fruits once it is matured.

However, there have been no much reports on its traditional use of seed oil of agar. On an average 3-5 year-old plant can produce around 1.5 - 3.0 kg of fruits and each fruit is having 1 - 2 seeds, predominantly two seeds. The seed index analysis experiment shows that on an average around 10000 numbers of seeds in 1kg and each seed weight around 0.09g and the average yield of oil is around 20-35%. As there is not much study on the seed oil of agarwood, a preliminary evaluation of physicochemical properties of seed oil collected from three different locations of India is depicted in this study.

MATERIALS AND METHODS

Source of Seed Materials

Samples of *A. malaccensis* fruits were collected from three different locations. To compare the seed oil characteristics, the seeds were collected first from the native growing area i.e., Jorhat (Assam), as this area is well known for agarwood trade. The seeds were also collected from the plantation in introduced locations i.e. from Ponnempet (Karnataka) and Karumanthurai (Tamil Nadu) of which the trees were more than 7 years old. The fruits were freshly collected in 2019 and received within 3 days from the day of collection for the study purpose.

Seed Index Evaluation

Three sets of good healthy fruits and seeds of one hundred each were selected for their seed index evaluation of three replications. The weight and size of each fruit and seeds were measured. Descriptive statistical analysis using Sigma stat (Version 3.5) for 100 fruit and seed weight and its fruit/seed weight ratio were used to evaluate and understand the seed yield and other seed parameters.

Extraction of Agar Seed Oil

The classical Soxhlet extraction method provides the fundamental basis for a modern-day solvent extraction system. Normally, crushed seeds are placed inside a thimble made from thick filter paper, which is loaded into the main chamber of the Soxhlet extractor. 100gm of grounded seeds were loaded into the main chamber of the Soxhlet extractor and placed onto a flask containing the extraction solvent petroleum ether (boiling point of 60-80ºC) and also equipped with a condenser. The solvent is heated to reflux for six hours. The solvent vapour moves up a distillation arm and floods into the chamber housing the thimble of the sample. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing the solid material to complete the extraction process. The oils were obtained after the solvent was removed under reduced temperature and pressure and refluxing at 70ºC to remove excess solvent used in the oil. Extracted seed oil was stored in freezer at −4 ºC for subsequent physicochemical analysis.

Determination of Percentage (%) Yield

The percentage (%) yield of oil extracted from seeds which are collected from three different locations was calculated using the equation

- The percentage yield of oil = [Weight of oil /Weight of sample (g)] x 100

PHYSICS-CHEMICAL ANALYSIS

Specific Gravity

Estimation of specific gravity of oil is very important quality criterion for its assessment of purity. The specific gravity of a substance is a comparison of its density to that of water. The Specific Gravity (SG) - is a dimensionless unit defined as the ratio of density of the material to the density of water at a specified temperature.

The specific gravity of the oil sample is measured by a 10 ml. specific gravity bottle. Then the specific gravity bottle was washed and filled with distilled water and weight noted. The Specific gravity was calculated using the following formula

- Specific gravity = [(W2– W1)/ (W3− W1)] x Density of water
- W1: weight of empty specific gravity bottle
- W2: weight of empty specific gravity bottle filled with the oil sample
- W3: weight of empty specific gravity bottle filled with the distilled water

Refractive Index

To determine the refractive index of the *A. malaccensis* seed oil, accurate to one unit in the fourth decimal place, ABBE's Refractometer with a monochromatic light source is used. This method applies to refractive indices in the range between 1.33 and 1.60. Refractive Index is the ratio of the velocity of light (of specific wavelength) in air to the velocity in the substance of sample. Refractive Index may also be defined as the Sine of the angle of incidence divided by the Sine of the angle of refraction, as light passes from air into the sample. Refractive Index is a fundamental property used in conjunction with other properties to characterize hydrocarbons and their mixtures.

Iodine Value

0.25g of the *A. malaccensis* seed oil sample was weighed into a conical flask. 10ml of chloroform was added after which 25ml of Wiji's solution was added and covered tightly using glass stopper measured with potassium iodide and kept in the dark for 30 minutes. 15ml of potassium iodide was added followed by 35ml of distilled water, at that point, the colour changed from brown to wine red. 5ml of 1% starch indicator was added which turned the solution blue-black. The whole solution was titrated with 0.1N sodium thiosulphate till immediately the endpoint is achieved. The solution turns blue-black to colourless. The blank was carried out without oil.

- Iodine value = [12.69 x (B – S) x 0.1/weight of sample used (g)]
- B: ml of sodium thiosulphate required by Blank.
- S: ml of sodium thiosulphate required by oil

Acid Value

1.0gm *A. malaccensis* of seed oil was weighed accurately into a flask to which 10ml of absolute alcohol was added and surface heated for about two minutes. Three drops of phenolphthalein indicator were added and titrated against 0.1N KOH with continuous agitation until the first appearance of the pale pink colour. This was considered as the endpoint and the acid value was calculated using the following formula.

- Acid value= [Titration value x 56.11 x 0.1/ Weight of oil (g)]
- Acid value is the measure of hydrolytic rancidity. In general, it gives an indication about edibility of the lipid.
- Edible oil contain >1%
- Pharmaceutical oil must not have any acidity.

Saponification Value

2g of the *A. malaccensis* seed oil was weighed into a conical flask. 25mL of 0.5N alcoholic solution of potassium hydroxide was added and the solution was boiled for 1hour. The solution was allowed to cool and 3 drops of phenolphthalein were added. The solution was titrated with 0.5N HCl. It was observed that the dark yellow solution turns to colourless. This was considered to be the endpoint. Blank was carried out as well by without adding the oil sample. Saponification value was calculated using the following formula

- Saponification value = [56.1(B-S) x N of HCL/ Weight of oil (g)]
- B: ml of HCl required by Blank
- S: ml of HCl required by oil

The saponification value is the number of milligrams (mg) of potassium hydroxide required to neutralize the free acids and to saponify the esters in 1 g of the substance. The saponification number is a measure of the average molecular weight of the triacylglycerols in a sample. Saponification is the process of breaking down a neutral fat into glycerol and fatty acids by treatment with alkali. The smaller the saponification number the larger the average molecular weight of the triacylglycerols present i.e. Saponification value is inversely proportional to the mean molecular weight of fatty acids (or chain length).

Ester Value

The ester value is the number of milligrams (mg) of potassium hydroxide required to saponify the esters in 1.0 g of the substance

- Ester value = [Saponification value - Acid value]

RESULTS AND DISCUSSIONS

This research was initiated to study the fruit and seed index and evaluate the physicochemical property of oil from *Aquilaria malaccensis* Lam. Seeds collected from three locations of India viz. Jorhat (Assam), Ponnempet (Karnataka) and Karumanthurai (Tamil Nadu) have been evaluated.

Fruit and Seed Index Evaluation

In this study, the weight of 100 *Aquilaria malaccensis* fruits, seeds and number of seeds per 100 fruits were studied for three different locations and given in table -1. The results indicate that among the three different locations the weight of fruits is highest in Ponnempet (214.84g), seed weight is highest in Jorhat (10.08 g) and the number of seeds per 100 fruits is almost similar amongst the fruit and seeds evaluated from three different locations.

Table 1: Estimation of Fruit and Seed in Different Locations

Description	Weight (gram)		
	Jorhat (Assam)	Ponnempet (Karnataka)	Karumanthurai (Tamil Nadu)
Weight of 100 fruits	187.06 (1.31)	214.84 (1.49)	200.36 (0.68)
Weight of 100 seeds	10.18 (0.06)	9.87 (0.03)	9.33 (0.10)
No. of seeds per 100 fruits	184.33 (1.52)	186 (1.0)	184.33 (0.58)
Note: standard deviation is given in the parenthesi			

Set of 100 fruits and seeds from each location were recorded for individual weight and its fruit and seed weight ratio. The analysis of variance between the characters is depicted in table - 2. The seed weight and fruit weight were significant to each other and the fruit weight and fruit/seed weight ratio were significant between them but seed weight and fruit/seed weight ratio was not significant in all the three locations.

Table 2: Analysis of Variance Between the Locations

Parameters	Jorhat	Ponnempet	Karumanthurai
Seed Weight(g)	0.101^{a} (0.016)	0.098^{a} (0.037)	0.927^{a} (0.052)
Fruit Weight(g)	1.872^{ab} (0.277)	2.132^{ab} (0.305)	1.998^{ab} (0.417)
F/S wt. ratio	0.101^{b} (0.029)	0.088^{b} (0.033)	0.089^{b} (0.055)
Note: Significant at $P \leq 0.05$ and standard deviation is given in the parenthesis			

Fruit and Seed Weight Estimation

The two important factors contributing to crop yield are the production of total plant biomass (biological yield) and the Harvest Index (HI) or proportion of biological yield that is converted into reproductive biomass. Because changes in biological yield are largely brought about by changes in cultural techniques, genetic increases in yield among the major crop species have largely been accomplished by increasing the harvest index (Donald and Hamblin, 1976). The information on seeds per plant and seed weight exerts a maximum positive indirect effect on biological yield per plant and harvest index (Heitholt *et al*., 1986). It is well established that seed yield is the final product and many traits contribute to its performance (Kumar *et al*., 2019).

The descriptive analysis of the fruit weight, seed weight and fruit/seed weight ratio are given in tables - 3 to 5. The analysis provides information on evaluation and outlines the data arranged in selected fruit and seed characters. The descriptive data analysis information highlights about the variability and uncertainty in the data, it provides a focal tendency of distribution also (Kumar *et al*., 2019). The data provides the arithmetic mean which is an average of all values with standard deviation from the mean value for the group. The average estimated value of fruit weight, seed weight and fruit/seed weight ratio from different locations are given in tables - 3 to 5

Table 3: Descriptive Statistical Analysis for Seed Weight (G)

	Jorhat	Ponnempet	Karumanthurai
Mean	0.1012	0.0984	0.092651786
Standard Error	0.001622319	0.003672777	0.005229248
Median	0.1	0.09	0.085
Mode	0.1	0.085	0.08
Standard Deviation	0.016223191	0.036727773	0.05229248
Sample Variance	0.002631919	0.001348929	0.002734503
Kurtosis	5.167520143	23.39727651	81.30505898
Skewness	-1.579270928	4.696574182	8.572084475
Range	0.1	0.24	0.555
Minimum	0.03	0.055	0.03
Maximum	0.13	0.295	0.585
Sum	10.12	9.84	9.265178571
Count	100	100	100
Largest (1)	0.13	0.295	0.585
Smallest (1)	0.03	0.055	0.03
Confidence Level (95.0%)	0.003219033	0.007287587	0.010375962

Table 4: Descriptive Statistical Analysis for Fruit Weight (G)

	Jorhat	Ponnempet	Karumanthurai
Mean	1.8724	2.1317	1.9978
Standard Error	0.027705566	0.030471399	0.041691164
Median	1.85	2.18	1.935
Mode	1.96	2.44	1.65
Standard Deviation	0.277055659	0.304713991	0.416911637
Sample Variance	0.076759838	0.092850616	0.173815313
Kurtosis	0.172326091	-0.838696846	-0.355435537
Skewness	0.509288169	-0.228764079	0.601299307
Range	1.36	1.17	1.62
Minimum	1.35	1.51	1.26
Maximum	2.71	2.68	2.88
Sum	187.24	213.17	199.78
Count	100	100	100
Largest (1)	2.71	2.68	2.88
Smallest (1)	1.35	1.51	1.26
Confidence Level (95.0%)	0.054973854	0.060461867	0.082724314

Table 5: Descriptive Statistical Analysis for Fruit/ Seed Weight Ratio

	Jorhat	Ponnempet	Karumanthurai
Mean	0.101478023	0.088037548	0.089344925
Standard Error	0.002921797	0.003263635	0.005441734
Median	0.108593109	0.085302174	0.088469728
Mode	0.112244898	0.086065574	0.103030303
Standard Deviation	0.029217965	0.032636353	0.054417343
Sample Variance	0.000853689	0.001065132	0.002961247
Kurtosis	-0.095221856	7.30438494	55.74007186
Skewness	-0.657859645	2.168444501	6.414648805
Range	0.139307346	0.181073193	0.545238095
Minimum	0.021276596	0.044117647	0.011904762
Maximum	0.160583942	0.22519084	0.557142857
Sum	10.14780233	8.803754778	8.934492476

Table 5 Contd.,			
Count	100	100	100
Largest (1)	0.160583942	0.22519084	0.557142857
Smallest (1)	0.021276596	0.044117647	0.011904762
Confidence Level (95.0%)	0.005797478	0.006475761	0.010797582

Physical-Chemical Analysis

The *Aquilaria malaccensis* seed oil is pale yellow and the Appearance is viscous. Table-6 shows the range of values of yield and physical properties of *Aquilaria malaccensis* seed oil with respect to location

Table 6: Physical-Chemical Parameters of Agar Seed Oil from Different Locations

Description	Jorhat	Ponnempet	Karumanthurai
Colour	Pale yellow	Pale yellow	Pale yellow
Appearance	Viscous	Viscous	Viscous
Specific gravity	0.91	0.912	0.908
Yield of oil	24.72	36.41	38.41
Refractive index	1.46570	1.46575	1.46575
Acid value	6.5(0.22)	4.35(0.21)	4.23(0.21)
Saponification value	149.6 (0.75)	154.74(0.80)	146.79(0.80)
Ester value	143.09(0.19)	150.38(0.69)	142.55 (1.02)
Iodine value	79.69 (0.19)	92.24 (0.03)	95.17 (0.05)

The average yield of oil from three different locations; Jorhat, Ponnempet and Karumanthurai were estimated on seed weight/weight basis and it is 24.72%, 36.41and 38.41% respectively. The lowest yield 24.72% was observed in Jorhat and the highest yield were observed in Karumanthurai (38.41%). This result showed that variation in oil yield with location might be attributed to the diversity of natural habitats, agro-climatic and ripening stage. The Specific gravity of *Aquilaria malaccensis* seed oil was found to be in the range of 0.90-0.91. The specific gravity of seed oil from Jorhat, Ponnampet and Karumanthurai were almost similar (0.91, 0.912 and 0.90). The refractive index (RI) of oil is related to the degree of saturation and is indicative of oil purity. RI is a very important parameter of oil which increases with the increase in unsaturation and chain length of fatty acids and it is correlated to the molecular weight. The refractive index of the *Aquilaria malaccensis* seed oil was in the range of 1.4657 and there is no significant difference in RI of seed oil among Jorhat (1.46570), Ponnampet (1.46575) and Karumanthurai (1.46575).The results of Specific gravity and Refractive index showed there was no significant difference among the oil from three locations. Thus, to infer that the quality of seed oil obtained from two different locations like Ponnempet in Karnataka and Karumathurai in Tamil Nadu are almost similar when compared to the seed oil obtained from the natural habitat place like Jorhat.

The Saponification values of the *Aquilaria malaccensis* seed oil were ranged from 149 to 154. The maximum value was recorded from the sample collected from Ponnampet (154.74) and the minimum value was recorded from Karumathurai (146.79). The saponification value is an indicator of molecular weight or size as a function of the chain lengths of the constituent fatty acid. The value of around 154 indicates that seed oil of Ponnampet contains more fatty acid of high molecular mass compared to Jorhat and Karumanthurai.

The acid value of the *Aquilaria malaccensis* seed oil from three different locations was ranged from 4 - 6. The acid value of Jorhat is 6.5 and that of Ponnampet and Karumathurai is 4.35 and 4.23 respectively. The result showed that there is similarity in acid value among Ponnampet and Karumathurai but Jorhat is different. The acid value indicates the

freshness of fatty oil and also is indicative of its shelf life properties. Lower the acid value the quality of the oil will be fresher. So, comparing the result, the seed oil from Ponnampet and Karumanthurai are fresher than Assam.

The range of Iodine value is from 79-96 (I/100g). The highest iodine value was recorded from seed collected from Karumanthurai (95.17) and the lowest iodine value was from Jorhat seed oil (79.69). The iodine value of Ponnampet (92.24) and Karumanthurai (95.17) showed no significant variation. But comparing with Jorhat the iodine value of Ponnampet showed more variation. The Iodine Value gives a measure of the average degree of instauration of oils and fats. The higher the iodine value, the greater the number of C=C double bonds and lower the iodine value the lesser the number of unsaturated bonds (Aremu *et al*., 2006).

CONCLUSIONS

The physico-chemical characteristics of oil samples like specific gravity, acid value, saponification value and ester value etc. from three different locations have been studied. As the global demand for novel cosmetic agents is ever increasing, Agarwood seed oil could enter the market as a cosmetic ingredient that could also act as a vehicle for other oil-soluble agents. As there are very few research studies pertaining to Agarwood seed oil, further extensive research work could continue on oxidative stability and pharmacological effects such as anti-inflammatory and cytotoxicity properties that could make the agarwood seed oil as an acceptable pharmaceutical and cosmetic ingredient. The analysis of fruit and seeds weight of *Aquilaria malaccensis* is showing significant variations among fruit and seed weights index. The physicochemical analysis of *Aquilaria malaccensis* seed oil was analysed and compared with three different locations. The findings offer that there is considerable variation in some of the physiochemical parameters of oil in three locations. Thus, this important commercially valuable timber species deserves more attention and detailed research investigations of *Aquilaria malaccensis* seed oil for its properties and value-added products of pharmaceutical importance.

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