INVESTIGATION OF PHYTOCONSTITUENTS AND SOME BIOACTIVITIES OF LEAVES AND BARKS OF *Aquilaria agallocha* ROXB. (THIT-MHWAE)

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Abstract

The research deals with phytochemical and medico-chemical investigation of *Aquilaria agallocha* Roxb. (Thit-mhwae) (*A. agallocha*) leaves and barks. The samples were collected from Katawe village, Thayatchaung Township, Tanintharyi Region. Antimicrobial activity of three different extracts (PE, EtOAc, EtOH) of *A. Agallocha* leaves and barks were determined by agar well diffusion method. Test microorganisms were *B. subtilis, S. aureus, P. aeruginosa, B. pumilus, C. Albicans* and *E. coli.* EtOAc extracts of leaves and barks of *A. Agallocha* showed the higher antimicrobial activity than other extracts. The cytotoxicity of Thit-mhwae leaves and barks of EtOH and water extract were tested by brine shrimp cytotoxicity bioassay. According to results, the ethanol extract of leaves showed strong cytotoxic effect at LD_{50} = 23.56 μg/mL whereas other extracts (ethanol bark, watery leaves and bark) showed cytotoxic to brine shrimp at LD_{50} = 33.34, 58.50, 60.70 μg/mL respectively. Therefore, the EtOH extract of leaves was the most potent than other extracts because of the lowest LD_{50} value and strong cytotoxic. But all of these samples were lower activity than standards $K_2Cr_2O_7$ (LD_{50} = 4.38 μg/mL) and greater activity than caffeine (LD_{50} = 1000 μg/mL) in cytotoxicity.

Keywords : *Aquilaria agallocha* Roxb., cytotoxicity, EtOAc extracts, EtOH extract

Introduction

During the past decade, traditional system of medicine has become a topic of global interest (WHO, 1998). It is estimated that 80 % of world's population utilize traditional medicines for the treatment of diseases. Herbal

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medicine based on the use of roots, leaves, barks, seeds and flowers of the plants. Depending on the localities or geographic regions with different temperatures, climates, rainfall, altitude and radiations characteristics, the herbal plants may have different chemical composition and different efficacy of biological activities.

Myanmar is rich in varieties of medicinal as well as aromatic plants due to presence of different climate zone in the country. In Myanmar, most of the people depend on traditional medicinal plants and herbal medicines rather than modern medicines for the treatment of various disorders. The study of traditional medicinal plants and their therapeutics play a very important role in health care system of Myanmar because 80% of its population is in the rural area and have been using traditional medicine for centuries.

In this paper Agar wood (Figure 1), a plant drug of controversial identity is taken for investigation. A wood known as Eagle wood (Trade name), Agaru (Hindi), Agil, Akil (Tamil) is credited with several pharmacological properties as per the literature claims; it is also a highly priced incense wood of much popular antiquity.

This plant have been rich sources of medicine because they produce a host of bioactive molecules, most of which probably evolved as chemical defenses against infection. Bioassays are adaptable for screening and testing plant extracts. The aim of the present work is to investigate the isolation of bioactive compounds from leaves and bark of agar wood and also studied some biological activities such as antimicrobial activity, antioxidant activity, cytotoxicity and antitumour activity. Farther work on isolation and characterization of bioactive compound will be published in the future.

Selected Myanmar Medicinal Plant*Aquilaria agallocha* Roxb.
Botanical aspect

Botanical Name	-	*Aquilaria agallocha*
Family	-	Thymelaeaceae
Genus	-	*Aquilaria*
Species	-	*agallocha*
Myanmar Name	-	Thit-mhwae
Common Name	-	Agar wood, eagle wood, Gaharu
Plant Part Used	-	Leaves and barks

(a) Plant

(b) Leaves

(c) Flowers

(d) Fruits

Figure 1. Photographs of *Aquilaria agallocha*. (Thit- mhwae)

Materials And Methods

Collection and Preparation of Samples

The selected medicinal plant used in this study was *Aquilaria agallocha* Roxb. (Thit- Mhwae, TM). The leaves and bark of Thit-mhwae were collected from Katawe village, Thayetchaung Township, Tanintharyi Region on February to May 2013. After collection, the scientific name of *Aquilaria agallocha* was identified by Botanist at Department of Botany, Dawei University. The collected fresh samples were washed with water and dried in an oven at 50 °C. The air dried samples were cut into small pieces and made powder by using grinding machine. And then, the leaves and barks powdered samples were separately stored in the air-tight container to prevent moisture and other contamination. The dried powdered samples were tested for biological activities and isolation of organic compounds.

Screening of Antimicrobial Activity of Leaves and Barks of *Aquilaria agallocha Roxb.*(Thit-mhwae) by Agar Well Diffusion Method

The screening of antimicrobial activity of various crude extracts such as PE, EtOAc and 70 % EtOH extracts of leave and bark of *Aquilaria agallocha* Roxb. (Thit-mhwae) was carried out by agar well diffusion method at Fermentation Laboratory, Pharmaceutial Research Department, Ministry of Industry, Yangon, Myanmar. Six microorganisms namely *Bacillus subtilis*(JAP-022/215), *Staphylococcus aureus* (ATCC-12277*), Pseudomonas aeruginosa* (IFO-3080), *Bacillus pumilus* (IFO-12102), *Candida albicans* (IFO- 1060) and *Escherichia coli* (ACCT-25922) were used for this test.

Meat extract (0.5 g), peptone (0.5 g) and sodium chloride (0.25 g) were mixed with distilled water and the solution made up to 100 mL with distilled water. The pH of this solution was adjusted at 7.2 with 0.1 M sodium hydroxide solution and 1.5 g of agar was then added. The nutrient agar medium was put into sterilized conical flask and plugged with cotton wool and then autoclaved at 121 ºC for 15 min. After cooled down to 40 ºC, one drop of

suspended strain was inoculated to the nutrient agar medium near the burner. About 20 mL of medium was poured into the sterilized Petri dish and left for 10-15 min in order to set the agar. After that the agar wells were made with a 10 mm sterilized cork borer and the wells filled with 0.1 mL of extracts to be tested. Then, the plates were incubated at 27 ºC for 24 h. After incubation, the diameters of inhibition zones including 10 mm wells were measured.

Screening of Anti-Tumor Activity

Anti-tumor activity of ethanol and ethyl acetate extracts and some isolated compounds was examined by Potato disc Assay (PDA) method (Ferrigni *et al.,* 1982) at Fermentation Laboratory, Pharmaceutical Research Department, Ministry of Industry, Yangon, Myanmar.

Potato Crown Gall Test or Potato Assay

Fresh, disease-free potatoes were purchased from a local market. Tubers of moderate size were surface sterilized by immersion in 0.1 % sodium hypochlorite for 20 min. Ends were removed and the potatoes were soaked an additional10 min. A core of the tissue was extracted from each end and discarded. The remainder of the cylinder was cut into 0.5 cm thick discs with a surface sterilized scalpel. The discs were then transferred to agar plates (1.5 g of agar dissolved in 100 mL DDW, autoclaved for 20 min at 121 ˚C , 20 mL poured into each Petridish). Each plate contained four potato discs and 4 plates were used for each sample dilution.

Samples (2.0, 0.1 and 0.5 mg) were respectively dissolved in DMSO (2 mL) and filtered through Millipore filters (0.22 μm) into sterile tube. This solution (0.5 mL) was added to sterile DW (1.5 mL), and broth culture of *A. tumefaciens* in PBS (2 mL) was added. Controls were made in this way; DMSO (0.5 mL) and sterile DW (1.5 mL) were added to the tube containing 2 mL of broth culture of *A. tumefaciens*. Using a sterile disposable pipette, 1 drops (0.05 mL) from these tubes was used to inoculate each potato disc, spreading it over the disc surface. After inoculation, Petri dishes were sealed

by par film and incubated at 27-30 ˚C for3 weeks. Tumors were observed on potato discs after 21 days under stereo-microscope followed by staining with Lugol's iodine (10 % KI and 5 % I_2) after 30 min and compared with control. The antitumor activity was examined by observation of tumor produced or not.

Screening of Antioxidant Activity of Crude Extracts and Isolated Compound from *Aquilaria agallocha* Roxb. (Thit-mhwae) Leaves and Barks

DPPH (2, 2-diphenyl-1-picry-hydrazyl) radical scavenging assay was chosen the antioxidant activity of plant materials. This assay has been widely used to evaluate the free radical scavenging effectiveness of various flavonoids and polyphenols in food system.

The effect on DPPH radical was determined using the method by Marinova and Batchvarov (2011). The control solution was prepared by mixing 1.5 mL of 60 M DPPH solution and 1.5 mL of EtOH using shaker. The test sample solution was also prepared by mixing thoroughly 1.5 mL of 60 μM DPPH solution and 1.5 mL of each sample solution. The mixture solutions were allowed to stand at room temperature for 30 min. Then, the absorbance of these solutions was measured at 517 nm by using UV-7504 spectrophotometer. Absorbance measurements were done in triplicate for each concentration and then mean values so obtained were used to calculate percent inhibition of oxidation and the capability to scavenge the DPPH radical was calculated using the following equation:

$$\% \text{ oxidative inhibition} = \frac{A_{Control} - (A_{Sample} - A_{Blank})}{A_{Control}} \times 100\ \%$$

where, $A_{Control}$ = absorbance of control solution

A_{Sample} = absorbance of sample solution

A_{Blank} = absorbance of the blank (EtOH solution)

Investigation of Cytotoxicity by Brine Shrimp Bioassay

Cytotoxicity of leaves and barks of *Aquilaria agallocha Roxb.*(Thit-mhwae) were investigated by brine shrimp bioassay according to the procedure described by Dockery and Tomkins (2000).Test solution (1 mL) was mixed with 9 mL of artificial sea water and placed in the chamber of ice cup. Alive brine shrimp (10 napulli) was taken with Pasteur pipette and placed into each chamber which was kept at room temperature for about 24 h. After 24 h incubation, the number of survival brine shrimp was counted and 50 % lethality dose (LD_{50}) was calculated (Dockery and Tomkins, 2000). The control solution was prepared as the above procedure by using distilled water instead of sample solution.

Results And Discussion

Antimicrobial Activity of Crude Extracts by Agar Well Diffusion Method

Screening of antimicrobial activity of various crude extracts such as PE, EtOAc and 70 % EtOH extracts of TM leaves and barks were done by employing agar well diffusion method (Table 1 and 2). In this study, the samples were tested on six pathogenic microorganisms such as *Bacillus subtilis, Staphylococus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans* and *Escherichia coli* .

From these results, it was found that PE extract of TM leaves and barks did not exhibit any antimicrobial activity against all tested microorganisms whereas EtOAc and EtOH extracts from TM leave and bark exhibited inhibition zone diameters ranged between in 12 mm ~ 50 mm respectively against all tested microorganisms. The TM leaves and barks of EtOH extract showed less activity and EtOAc extract was observed to be most effective in antimicrobial activity.

Therefore all the crude extracts of two samples, except PE extract of TM exhibited antimicrobial activity against all microorganisms tested. Among the crude extract, EtOAc extract of two samples showed the most pronounced antimicrobial activity against all microorganisms tested. Thus, TM leaves and barks, might be effective in the formulation of medicine for the treatment of

diseases infected by the microorganisms, such as diarrhea, skin disease, aphrodisiac and vomiting.

Table 1. Inhibition Zone Diameters of Crude Extracts of *Aquilaria agallocha* Roxb. (Thit-mhwae) Leaves against Test Microorganisms

Sample	**Extracts**	**Inhibition zone diameters (mm) of various crude extracts against different microorganisms**					
		B.subtilis	***S.aureus***	***P.aeruginosa***	***B.pumilus***	***C.albicans***	***E.coli***
Leave	Pet-ether	-	-	-	-	-	-
	EtOAc	29 (+++)	42 (+++)	32 (+++)	45 (+++)	33 (+++)	34 (+++)
	EtOH	12 (+)	12 (+)	14 (+)	13 (+)	12 (+)	-

Agar well-10mm

10 mm ~ 14 mm (+)

15 mm ~ 19 mm (++)

20 mm above (+++)

Table 2. Inhibition Zone Diameters of Crude Extracts of *Aquilaria agallocha* Roxb. (Thit-mhwae) Bark against Test Microorganisms

Sample	Extracts	Inhibition zone diameters (mm) of various crude extracts against different microorganisms					
		B.subtilis	***S.aureus***	***P.aeruginosa***	***B.pumilus***	***C.albicans***	***E.coli***
Bark	Pet-ether	-	-	-	-	-	-
	EtOAc	32 (+++)	49 (+++)	30 (+++)	40 (+++)	35 (+++)	42 (+++)
	EtOH	16 (+)	12 (+)	15 (+)	16 (+)	12 (+)	12 (+)

Agar well-10 mm

10 mm ~ 14 mm (+)

15 mm ~ 19 mm (+) (+)

20 mm above (+++)

Antitumor Activity of *Aquilaria agallocha* Roxb. (Thit-mhwae) Leaves, Bark and isolated compounds

Screening of antitumor activity of crude extracts such as ethanol and watery extracts from *Aquilacia agallocha Roxb.*was done by agar well diffusion method (Ghanney and Rhouma, 2015). In this investigation, the extracts and some isolated compounds were tested against *Agrobacterium tumefaciens* (Table 3).

According to the result, the ethanol extracts of TM bark do not show strong antitumor activity against *A. tumefaciens* at the (concentration of 0.1 - 0.3 g/mL). But, watery extracts of TM leave showed strong antitumor activity against *A. tumefaciens* at the (concentration 0.1 - 0.3 g/mL).The watery extract of leave exhibited the antitumor activity significantly at a concentration at (0.1 - 0.3) g/mL. So, the MIC value is 0.1 g/mL.

Table 3. Tumor Inhibition of the Crude Extracts and Isolated Compounds of *Aquilaria agallocha* Roxb. (Thit-mhwae)

Test Sample		**Concentration (g** /mL**)**	**Tumor**
	Control	++	-
***Aquilaria agallocha* Leave**	EtOH extract	0.1	-
		0.2	-
		0.3	-
***Aquilaria agallocha* Bark**	EtOH extract	0.1	+
		0.2	+
		0.3	+
***Aquilaria agallocha* Leave**	H_2O extract	0.1	-
		0.2	-
		0.3	-
***Aquilaria agallocha* Bark**	H_2O extract	0.1	+
		0.2	+
		0.3	+

(+) = tumor appeared
(-) = no tumor appeared
ND = not detected

Antioxidant Activity of Crude Extracts and Isolated Compound of Thit-mhwae

The antioxidant activity of 70 % EtOH and water extracts of two samples, one isolated compound (*p*-hydroxy benzoic acid) and standard vitamin C was studied by DPPH (2, 2 - diphenyl - 1 - picryl - hydrazyl) free radical scavenging assay method. This method is the most widely reported method for screening of antioxidant activity on many plant drugs. It is based on the reduction of color of free radical DPPH in ethanolic solution by different concentrations (6.25, 12.5, 25, 50, 100, 200 μg/mL) of each crude extract, one isolated compound and standard vitamin C in ethanol solvent were used. The absorbance of each solution (control, sample) was measured at λ_{max}517 nm using UV-visible spectrophotometer.

It was found that as the concentrations increased, the absorbance values decreased. The radical scavenging activity of crude extracts were expressed in terms of % inhibition and IC_{50} (50 % inhibition concentration) values and calculated by linear regressive excel program. The results were summarized in Table 4. In the antioxidant activity screening, IC_{50} values of 16.53 μg/mL for H_2O extract and 144.7 μg/mL for EtOH extract in leaves and 90.76 μg/mL for H_2O extract and 57.86 μg/mL for EtOH extract in bark samples were found isolated parahydroxy benzoic acid showed antioxidant activity at (IC_{50} = 59.77 μg/mL). Among the tested crude extracts and isolated compound, watery extract of TM leaves and p-hydroxy benzoic acid compound were found to be more effective than that of ethanol crude extract of leaves and H_2O barks of in free radical scavenging activity. According to this observation water extract of leaves (16.53 μg/mL) was higher activity than others but lower than standard vitamin C (IC_{50} = 7.99 μg/mL).

Table 4. % Free Radical Scavenging Activity of Crude Extracts and Isolated Compounds from *Aquilaria agallocha* Roxb. (Thit-mhwae) Leaves and Bark

Sample	% Inhibition (mean ± SD) in different concentrations (μg/mL)						
	6.25	**12.5**	**25**	**50**	**100**	**200**	**IC_{50} (μg/mL)**
Thit-mhwae leave (H_2O)	13.51 ± 1.96	44.97 ± 1.96	60.57 ± 0.75	71.47 ± 0.74	73.07 ± 1.20	76.81 ± 0.45	16.53
Thit-mhwae bark (H_2O)	10.96 ± 1.09	22.61 ± 1.56	26.57 ± 1.86	35.69 ± 1.01	53.33 ± 0.24	60.78 ± 1.15	90.76
Thit-mhwae leave (EtOH)	10.21 ± 1.49	11.10 ± 1.48	11.84 ± 1.72	22.54 ± 1.60	37.66 ± 1.49	65.27 ± 1.73	144.7
Thit-mhwae bark (EtOH)	18.04 ± 1.73	19.05 ± 1.50	32.16 ± 1.50	48.35 ± 1.20	58.11 ± 1.01	73.96 ± 1.49	57.86

Cytotoxicity of Ethanol and Water Crude Extracts of *Aquilaria agallocha* Roxb. (Thit-mhwae)

The cytoxicity of *Aquilaria agallocha Roxb.* leave and bark were investigated by brine shrimp cytoxicity bioassay (Dockery and Tomkins, 2000). The tested organisms used were brine shrimp (*Artemia salina*). The cytotoxic effect was expressed at LD_{50} values (50 % lethality dose). The cytotoxicity of ethanol and water crude extracts of leave and bark evaluated in this study are reported in Table 5 . All of four tested extracts were found to possess cytotoxic in the brine shrimp bioassay. As shown in Table 5, the most cytotoxic extract was found to be the ethanol leave extract (LD_{50} = 23.56 μg/mL) whereas other extracts (ethanol barks, watery leaves and barks) were 33.34, 58.50 and 60.70 μg/mL respectively.

These result suggested that the EtOH extract of leave is the most potent, and the other extracts were less cytotoxic to brine shrimp. All of these samples are lower than standards $K_2Cr_2O_7$ (LD_{50} 4.38 μg/mL) and greater than caffeine (LD_{50} 1000 μg/mL) in cytotoxicity.

Table 5. Cytotoxicity of Different Doses of Watery and Ethanol Crude Extracts of *Aquilaria agallocha* Roxb. (Thit-mhwae)

Tested	Sample	Numbers of Death Brine Shrimp (Mean± SEM) in Various Concentration (µg/mL)				
		1	**10**	**100**	**1000**	**LD_{50} (µg/mL)**
1	Watery Extract(L)	1.33 ± 0.58	4.33 ± 0.58	5.67 ± 0.58	8.00 ± 1.00	58.50
2	EtOH Extract(L)	1.67 ± 0.58	4.67 ± 0.58	7.00 ± 0.00	7.67 ± 0.58	**23.56**
3	Watery Extract(B)	1.00 ± 0.00	3.33 ± 0.58	6.33 ± 0.58	7.33 ± 0.58	60.70
4	EtOH Extract(B)	3.33 ± 0.58	4.33 ± 0.58	7.00 ± 1.00	8.33 ± 0.58	33.34
5	$K_2Cr_2O_7$	4.00 ± 1.00	6.67 ± 0.58	8.33 ± 0.58	9.33 ± 0.58	4.38
6	Caffeine	0	2.33 ± 1.20	3.00 ± 0.58	5.00 ± 1.16	1000

Conclusion

Investigation of Myanmar indigenous plant, leaves and barks of *Aquilaria agallocha* Roxb. used to be ingredient for traditional medicine was systematically carried out. Antimicrobial activity of three different extracts (PE, EtOAc, EtOH) of *Aquilaria agallocha* Roxb. leaves and barks were determined by agar well diffusion method. Test microorganisms were *B. subtilis, S. aureus, P. aeruginosa, B. pumilus, C. albicans* and *E. coli.* EtOAc extracts of leaves and barks of *Aquilaria agallocha* Roxb. showed the higher

antimicrobial activity than other extracts. Antitumor activity investigated by potato crown gall (PCG) assay revealed that EtOH and H_2O extracts and isolated compounds hydroquinone (benzene 1,4-diol) and *p*-hydroxy benzoic acid inhibited tumor growth in a concentration dependent manner. Significant tumor inhibition was observed at 0.1 g/mL of the leaves EtOH extracts and 0.01 g/mL bark H_2O for the isolated compounds. The antioxidant activity screening of EtOH and water extracts and isolated compounds were determined by using DPPH assay. According to this observation water extract of leave (16.53 µg/mL) was higher activity than others but lower than standard vitamin C (IC_{50} = 7.99 µg/mL).The cytotoxicity of *Aquilaria agallocha* Roxb. leaves and barks of EtOH and water extract were tested by brine shrimp cytotoxicity bioassay. From the results, the leave EtOH extract was the most potent in other extracts because it possessed the lowest LD_{50} value in cytotoxic to brine shrimp. ID_{50} values of these samples were lower than standards $K_2Cr_2O_7$ (LD_{50} = 4.38 µg/mL) and caffeine (LD_{50} = 1000 µg/mL) in cytotoxicity.

Acknowledgements

The authors would like to express their profound gratitude to the Department of Higher Education (Yangon Office), Ministry of Education, Yangon, Myanmar, for provision of opportunity to do this research and Myanmar Academy of Arts and Science for allowing to present this paper.

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