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Research Article

COMPARATIVE PHYSICOCHEMICAL, PHYTOCHEMICAL AND HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY EVALUATION OF HEART WOOD AND SMALL BRANCHES OF *AQUILARIA AGALLOCHA* ROXB.

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ABSTRACT

Aquilaria agallocha Roxb. commonly called as Agaru is a medicinal plant. Heartwood of this tree is widely used in Ayurveda for various diseases. Removal of heart wood from trunk of this tree may make this plant weak and susceptible to damage due to which availability of this plant may be difficult in near future. Present study outlines the concept of plant part substitution. Heart wood and small branches of *A. agallocha* are compared on the basis of physicochemical analysis, phytochemical analysis, total phenolic contents, total flavonoid contents and high performance thin layer chromatography (HPTLC) to evaluate the possibilities of using small branches instead of heart wood. Physicochemical parameters of heartwood and small branches and phytochemical analysis of *n*-hexane, ethyl acetate and ethanol extract of both heart wood and small branches were carried out using standard methods. Total phenolics and total flavonoids were estimated spectrophotometrically using Folin-ciocalteu assay and aluminum chloride assay methods, respectively. CAMAG HPTLC system equipped with semi-automatic applicator was used for HPTLC of *n*-hexane, ethyl acetate and ethanol extracts of stem bark and small braches using suitable mobile phases. Results of phytochemical analysis and HPTLC of *n*-hexane, ethyl acetate and ethanol extracts showed many similarities which suggest that small branches may have nearly similar active potency like heart wood and may be used as a substitute of heart wood after comparison and confirmation of same for pharmacological activities.

KEYWORDS: *Aquilaria agallocha*, HPTLC profile, Physicochemical analysis, Phytochemical analysis.

INTRODUCTION

Medicinal plants are extensively utilized throughout the world in providing health care for both humans and animals not only in diseased condition but also as potential material for maintaining proper health. Hence the numbers of plant species are individually under attack to accomplish the global demand. *A. agallocha* (Family: Thymeleceae) commonly called as *Agaru* is a precious medicinal plant widely used in Ayurveda. As per Ayurvedic literature, heart wood of this plant is used *Aksiroga, Svasa, Karna roga, Kustha* and *visa*[1]. It is also reported for analgesic, anti-inflammatory[2], anxiolytic, anticonvulsant[3], antioxidant[4] and antibacterial[5] activities. Several chemical constituents like aquillochin[6], 7,8-dimethoxy-2-[2-(3′-acetoxyphenyl)ethyl]chromones, 6-methoxy-2-(2-phenylethyl)chromones, 6,7-dimethoxy-2-(2-phenylethyl)chromones, abietane ester[7], gmelofuran, agarol, 5-iso-propyl-7-methyl-4,5,5a,6,7,8-hexahydro-3H-naphtho-[1,8-bc]-furan-8α-hydroxy-3-one[8], α-agarofuran, 10-epi-γ-eudesmol, oxoagarospirol sesquiterpenes[9], α-agarofuran, agarofuran, dihydroagarofuran[10], norketoagarofuran, 4-hydroxydihydroagarofuran, 3, 4-dihydroxydihydroagarofuran[11], α-agarofuran, β-agarofuran, norketoagarofuran, (-)-10-epi- γ eudesmol, agrospiral, jinkohol, jinkoh-cremol, kusunol, dihydrokaranone, jinkohol II, oxo- agrospiral[12], apigenin-4', 7-dimethyl ether[13] etc. have identified and isolated from this plant.

Removal of heart wood from trunk of this tree may affect the survival of this plant due to which availability of this plant may be difficult in near future for use in Indian system of medicine. To safeguard the survival of this plant and to ensure the availability of heartwood as raw material to manufacturers and dealers of Ayurveda, Siddha and Unani drugs, there is strong need to explore the possibility of substitution of heartwood of the trunk with suitable alternate. An approach which would satisfy the necessities of sustainable harvesting, yet simultaneously provide for health care needs, would be the substitution of heart wood with aerial part of the same plant. Present study is an attempt to evaluate the possibilities of using small branches in place of heart wood. Standard physicochemical parameters of small branches of *A. agallocha* have not been prepared yet. So work is also carried out to establish preliminary physicochemical standards of small branches.

MATERIAL AND METHODS

Plant material

The heart wood and small branches of *A. Agallocha* were collected from Ayurvedic Regional Research Institute, Itanagar, Arunachal Pradesh, India, identified and authenticated by Mr. N.K. Pandey, Research officer (Botany), NRIASHRD, Gwalior.

Instrumentation

CAMAG HPTLC system (Muttenz, Switzerland) equipped with semi automatic TLC applicator Linomat IV, twin trough plate development chamber, Win CATS software version 1.4.2 and Hamilton (Reno, Nevada, USA) Syringe (100 µl).

Material and reagents

All chemicals, reagents and solvents used during the experiments were of analytical grade and HPTLC plates were purchased from E. Merck Pvt. Ltd. (Mumbai, India).

Physicochemical parameters

Heart wood and small branches were studied for various physicochemical standards like foreign matter, loss on drying at 105°C, total ash, acid-insoluble ash, alcohol soluble extractive, water soluble extractive and pH of 10% solution using standard methods[14, 15].

Preliminary phytochemical screening

n-Hexane, ethyl acetate and ethanol extract of both heart wood and small branches were screened for the presence of phenols, tannins, carbohydrates, saponins, alkaloids, proteins, flavonoids, steroids, furanoids, coumarin, quinone and terpenoids by the standard methods of Harbone[16] and Kokate [17].

Estimation of total phenolic and flavonoid content

Five grams of each of the shade-dried plant material was pulverized into coarse powder and subjected to ethanolic extraction using soxhlet apparatus. The extracts were concentrated to dryness. The dried residues were then dissolved in 100 ml of 95% ethanol. The extracts were used for total phenolic and flavonoid assay.

The total phenolics content was determined by using the Folin-Ciocalteu assay[18]. An aliquot (1 ml) of extracts or standard solution of tannic acid (20, 40, 60, 80 and 100 µg/ml) was added to a 25 ml volumetric flask, containing 9 ml of distilled water. A reagent blank was prepared using distilled water. One millilitre of Folin-Ciocalteu phenol reagent was added to the mixture and shaken. After 5 min, 10 ml of 7% Na_2CO_3 solution was added to the mixture. The volume was then made up to the mark. After incubation for 90 min at room temperature, the absorbance against the reagent blank was determined at 550 nm with an UV/Vis spectrophotometer. Total phenolics content was expressed as µg tannic acid equivalents (TAE).

Total flavonoid content was measured by the aluminum chloride colorimetric assay[19]. An aliquot (1 ml) of extracts or standard solutions of quercetin (20, 40, 60, 80 and 100 µg/ml) was added to a 10 ml volumetric flask containing 4 ml of distilled water. To the flask, 0.30 ml of 5% $NaNO_2$ was added and after 5 min, 0.3 ml of 10% $AlCl_3$ was added. After 5 min, 2 ml of 1M NaOH was added and the volume was made up to 10 ml with distilled water. The solution was mixed and absorbance was measured against the blank at 510 nm. The total flavonoid content was expressed as µg quercetin equivalents (QE).

HPTLC profiles

HPTLC studies were carried out following the method of Sethi[20], Stahl[21] and Wagner et al[22]. The heart wood and small branches were powdered coarsely. Ten gram powdered samples of each of heart wood and small branches were accurately weighed and exhaustively extracted by *n*-hexane, ethyl acetate and ethanol (each 100 ml) separately using soxhlet apparatus. The extracts were filtered and concentrated under reduced pressure and made up to10 ml in standard flasks separately.

The mobile phase used for developing the *n*-hexane and ethyl acetate extracts of heart wood and small branches was toluene: ethyl acetate: formic acid (7: 3: 0.1 v/v) and for ethanol extract of heart wood and small branches was toluene: ethyl acetate (7: 3 v/v).

The samples were spotted in the form of bands of width 10 mm with a 100 µl Hamilton syringe on aluminum TLC plates pre-coated with Silica gel 60 F_{254} of 0.2 mm thickness with the help of TLC semi-automatic applicator Linomat IV attached to CAMAG HPTLC system, which was programmed through Win CATS software version 1.4.2. 10 µl of each extracts of heart wood and small branches were applied in two tracks as 10 mm bands at a spraying rate of 10 seconds/µl. Track 1 was heart wood and track 2 was small branches for each of the extracts applied.

Development of the plate up to a migration distance of 80 mm was performed at 27 ± 2°C with mobile phase for each extracts in a CAMAG HPTLC chamber previously saturated for 30 min. After development the plate was dried at 60°C in an oven for 5 min and visualized under wavelength 254 nm and 366 nm for ultra violet detection. The developed plate was then dipped in anisaldehyde sulphuric acid reagent for derivatization and dried at 105°C in hot air oven till the colour of the band appears and visualized under white light. Images were captured by keeping the plates in photodocumentation chamber and R_f values were recorded by Win CATS software.

RESULTS AND DISCUSSION

Physicochemical parameters like foreign matter, loss on drying at 105º C, ash values, acid insoluble ash, extractive values and pH are given in Table 1. These data can be used for identification of the drug. Both the parts of *A. agallocha* were found to possess little moisture and hence can be stored at room temperature without fear of spoilage. Approximately same value of alcohol soluble extractives for both heart wood and small branches indicates the presence of approximately same amount of polar extractable compounds in heart wood and small branches.

Table 1: Physicochemical Parameters of Heart Wood and Small Branches of *A. agallocha.*

S. No.	Parameters	Results	
		Heart wood	Small branches
1.	Foreign matter (% w/w)	Nil	Nil
2.	Loss on drying (% w/w)	6.074	6.78
3.	Total ash (% w/w)	3.13	3.94
4.	Acid insoluble ash (% w/w)	0.62	0.53
5.	Alcohol soluble extractive value (% w/w)	1.70	1.88
6.	Water soluble extractive value (% w/w)	3.0	7.55
7.	pH of 10 % aqueous solution	6.53	5.75

Phytochemical analysis of different extracts of heart wood and small branches are shown in Table 2. Results reveal the presence of similar phytochemicals in heart wood and small branches except for quinone and furanoids which were found present only in ethanolic extract of heartwood.

Table 2: Phytochemical Analysis of Extracts of Heart Wood and Small Branches of *A. agallocha.*

Phytochemicals	Heart wood			Small branches		
	n-Hexane	Ethyl acetate	Ethanol	*n*-Hexane	Ethyl acetate	Ethanol
Phenols	-ve	-ve	+ve	-ve	-ve	+ve
Tannins	-ve	-ve	+ve	-ve	-ve	+ve
Alkaloids	-ve	-ve	+ve	-ve	-ve	+ve
Carbohydrates	-ve	+ve	+ve	-ve	+ve	+ve
Saponins	-ve	-ve	-ve	-ve	-ve	-ve
Proteins	+ve	+ve	+ve	+ve	+ve	+ve
Steroids	-ve	+ve	-ve	-ve	+ve	-ve
Flavonoids	-ve	-ve	+ve	-ve	-ve	+ve
Coumarin	-ve	-ve	+ve	-ve	-ve	+ve
Quinone	-ve	-ve	+ve	-ve	-ve	-ve
Furanoids	-ve	-ve	+ve	-ve	-ve	-ve
Terpenoids	-ve	-ve	+ve	-ve	-ve	+ve

Total amount of phenolics and flavonoids content of ethanolic extract of heart wood and small branches of *A. agallocha* are summarized in Table 3. Results indicate that in comparison to small branches, heart wood had the high total phenolic and flavonoid content.

Table 3: Total Phenolic and Total Flavonoid Content of Ethanolic Extracts of Heart Wood and Small Branches of *A. agallocha.*

S. No.	Plant parts	Total phenolics µg of TAE/10 g dry weight*	Total flavonoids µg of QE/10 g dry weight*
1.	Heart wood	233.0 ± 0.260	272.0 ± 0.133
2.	Small branches	19.7 ± 0.001	224.0 ± 1.105

*Values are expressed as Mean ± SD

Comparative HPTLC profile of *n*-hexane, ethyl acetate and ethanol extracts of heart wood and small branches of *A. agallocha* were recorded to reveal the chemical pattern of each extract. The HPTLC profile of *n*-hexane extract of both heart wood and small branches (Figure 1 and Table 4) showed one and two bands, respectively out of which only one band at R_f 0.68 (black) found similar when visualized under UV at 254 nm. At UV 366 nm, heart wood and small branches showed five and four bands, respectively out of which one band at R_f 0.62 (florescent blue) found similar. Visualization under white light after derivatization with anisaldehyde sulphuric acid reagent both heart wood and small branches showed two bands with similar R_f. This indicates the presence of many similar compounds in hexane extract of heart wood and small branches.

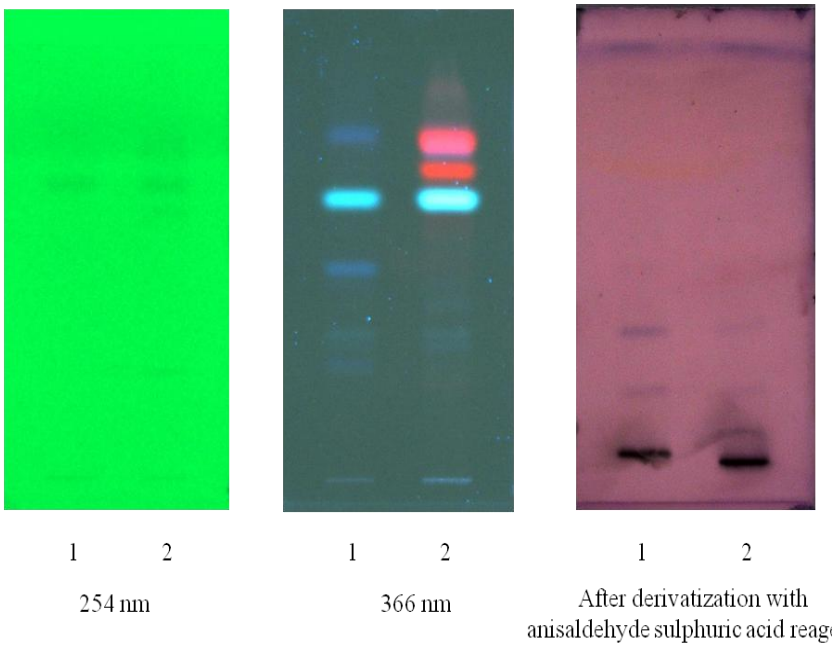

Figure 1: HPTLC Profile of *n*-Hexane Extracts of Heart Wood and Small Branches of *A. agallocha* (Track 1: Heart Wood, Track 2: Small Branches)

Table 4: R_f Value of *n*-Hexane Extract of *A. agallocha*.

S. No.	Wavelength	R_f value	
		Heart wood	Heart wood
1.	254 nm	0.68	0.27, 0.68
2.	366 nm	0.26, 0.33, 0.47, 0.62, 0.77	0.33, 0.62, 0.68, 0.74
3.	Visible light after derivatization	0.26, 0.33	0.26, 0.33

HPTLC profile of ethyl acetate extract of heart wood and small branches (Figure 2 and Table 5) showed two and one bands, respectively at UV at 254 nm out of which one band at R_f 0.25 (black) found similar. At UV 366 heart wood and small branches showed five and six bands, respectively out of which two bands at R_f 0.25 and 0.62 found similar but color of these bands found different in heart wood and small branches. Visualization under white light after derivatization with anisaldehyde sulphuric acid reagent, both heart wood and small branches showed six bands with similar R_f. This indicates the presence of many similar compounds also in ethyl acetate extract of heart wood and small branches.

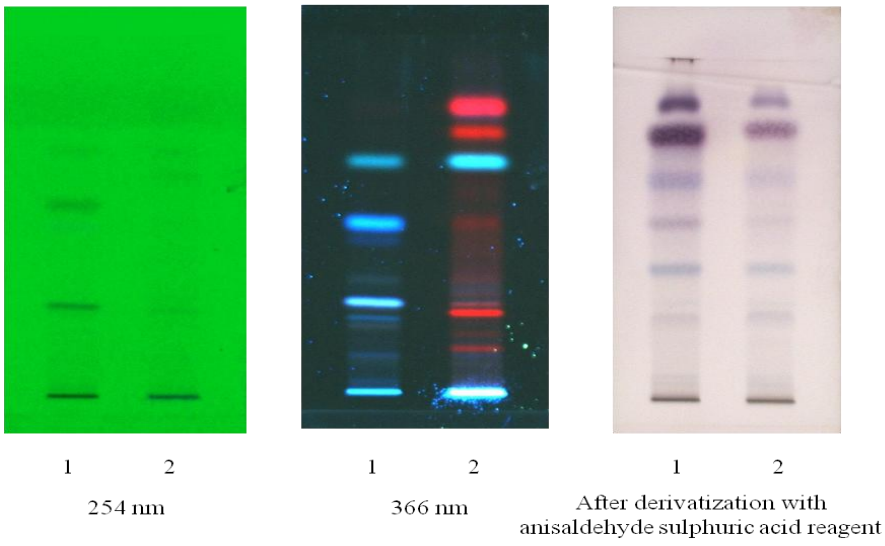

Figure 2: HPTLC Profile of Ethyl Acetate Extracts of Heart Wood and Small Branches of *A. agallocha* (Track 1: Heart Wood, Track 2: Small Branches)

Table 5: R_f Value of Ethyl Acetate Extract of *A. agallocha*.

S. No.	Wavelength	R_f value	
		Heart wood	Small branches
1.	254 nm	0.25, 0.52	0.25
2.	366 nm	0.21, 0.25, 0.41, 0.47, 0.62	0.23, 0.25, 0.46, 0.62, 0.70, 0.77
3.	Visible light after derivatization	0.26,0.40,0.52,0.64,0.80, 0.88	0.26,0.40,0.52,0.64,0.80,0.88

HPTLC profile of ethanol extract of heart wood and small branches (Figure 3 and Table 6) showed two bands and no band when visualized under UV at 254 nm. At UV 366 heart wood and small branches showed nine and six bands, respectively out of which four bands at R_f 0.26 (florescent blue), 0.63 (florescent blue), 0.72 (red), 0.80 (red) found similar. Visualization under white light after derivatization with anisaldehyde sulphuric acid reagent, both heart wood and small branches showed two bands with similar R_f. This indicates the presence of many similar compounds in ethanol extract of heart wood and small branches also.

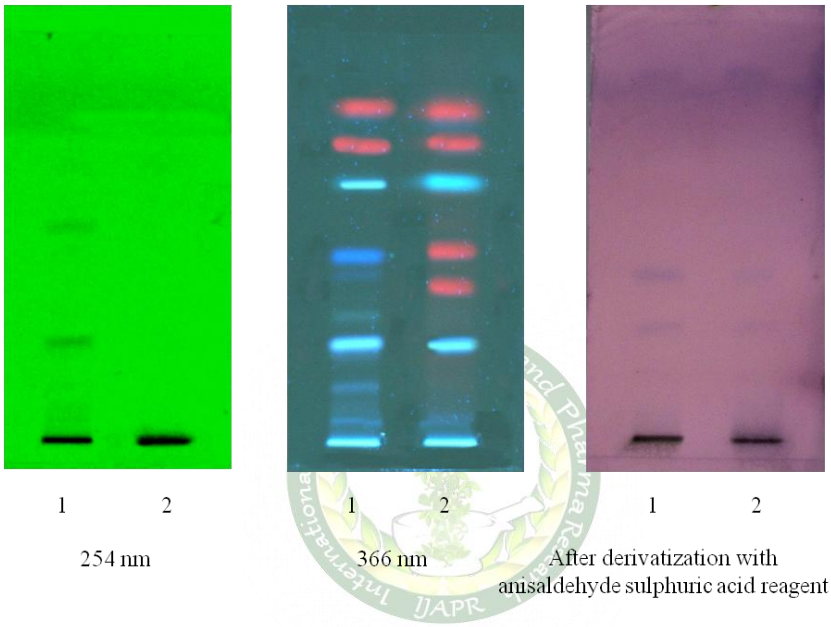

Figure 3: HPTLC Profile of Ethanol Extracts of Heart Wood and Small Branches of *A. agallocha* (Track 1: Heart Wood, Track 2: Small Branches)

Table 6: R_f Value of Ethanol Extract of *A. agallocha*.

S. No.	Wavelength	R_f value	
		Heart wood	Small branches
1.	254 nm	0.26, 0.58	No band
2.	366 nm	0.04, 0.16, 0.26, 0.34, 0.42, 0.47, 0.63, 0.72, 0.80	0.26, 0.39, 0.42, 0.63, 0.72, 0.80
3.	Visible light after derivatization	0.26, 0.29	0.26, 0.29

CONCLUSION

The present study carried out in *A. agallocha* to evaluate the possibilities of using small branches in place of heartwood will help sustainable utilization. The study will also be useful in identification and quality control of drug and can provide standard HPTLC profiles with selected solvent system for proper identification/ authentication of drug. Similarity in HPTLC profiles of *n*-hexane, ethyl acetate and ethanol extracts of heart wood and small branches suggests that small branches may have nearly similar active potency like heart wood and may provide the base for further study to use the small branches as a substitute of heart wood of *A. agallocha*.

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