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Original Article

Quality evaluation using TLC methods with reference to brazilin content of *Caesalpinia sappan* heartwoods in Thailand

Suwipa Intakhiao[1], Chanida Palanuvej[1*], and Nijsiri Ruangrungsi[1, 2]

[1] *Public Health Sciences Program, College of Public Health Sciences, Chulalongkorn University, Pathum Wan, Bangkok, 10330 Thailand*

[2] *Department of Pharmacognosy and Pharmaceutical Botany, College of Pharmacy, Rangsit University, Mueang, Pathum Thani, 12000 Thailand*

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Abstract

Caesalpinia sappan L. is a medicinal plant generally used to promote blood circulation and as an anti-thirst remedy. Fifteen samples were collected from different locations in Thailand. The aim was to identify parameters including the anatomical and histological characters as well as the thin layer chromatography (TLC) fingerprint. The quality parameter results of total ash, acid insoluble ash, ethanol and water soluble extractive values, loss on drying, and water content were 0.87±0.14, 0.44±0.14, 2.94±0.57, 3.77±0.64, 8.50±0.37, and 8.52±0.43% by weight, respectively. TLC-densitometry and TLC-image analysis were performed to detect brazilin using silica gel 60 GF_{254} as the stationary phase and chloroform, ethyl acetate, and formic acid (10:8:2) as the mobile phase. The results of brazilin from the TLC-densitometry and TLC-image analysis were 1.259±0.285 g/100 g and 1.256±0.266 g/100 g of dried heartwood, respectively. The result showed that both techniques were not statistically significantly different.

Keywords: *Caesalpinia sappan* heartwood, brazilin, pharmacognostic specification, TLC-image analysis, TLC-densitometry

1. Introduction

Caesalpinia sappan L. is the well-known plant belonging to the Caesalpiniaceae family. It is generally named sappanwood or brazilwood in English and the local name in Thailand is Fang. This plant is widely distributed in India, Brazil, Burma, South Korea, Philippines, Ceylon, and in all parts of Thailand. Its ecology is in scrub jungle and limestone hills (Smitinand, 1984). The heartwood of this plant is a herbal ingredient in Ayurveda preparations (Kennedy, Srinivasappa, & Farooqi, 2004), traditional Chinese medicine (Efferth *et al*., 2008), and traditional Thai medicine (Det-anand, 1975). Sappanwood is used in Thailand to promote blood circulation and as anti-thirst and anti-hypertension agents. *C. sappan* heartwood has shown pharmacological properties such as anti-microbial, anti-diabetic (Mohan, Anand, & Doss, 2011), anti-bacterial (Xu & Lee, 2004), anti-anemic (Badami, Moorkoth, & Surensh, 2004), anti-inflammatory (Washiyama, Sasaki, Hosokawa, & Nagumo, 2009), and anti-oxidant activities (Nirmal & Panicha yupakaranant, 2015; Sarumathy, Rajan, Vijay, & Dharani, 2011). The wood is very hard and has a deep red color. In addition, the red color from the heartwood is used for various purposes including cosmetics, fabrics, beverages, and foods. Sappanwood contains many phenolic compounds such as flavonoids, flavones, chalcones, and homoisoflavonoids (Cuong *et al*., 2012; Gilbody, Perkin, & Yates, 1902;). Brazilin belongs to the isoflavonoid group and is the major compound from this plant (Puchtler, Meloan, & Waldrop, 1986; Rondao *et al*., 2013;) (Figure 1).

Thin-layer chromatography (TLC) is a well-known and uncomplicated technique used to separate many compounds at the same time. TLC-densitometry is an analytical method to assess different absorbances or fluorescence signals between compound spots and background (Waksmundzka- Hajnos, 2008). TLC-image analysis is

*Corresponding author
Email address: chanida.p@chula.ac.th

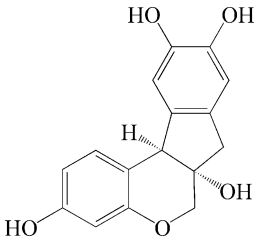


Figure 1. Brazilin.

processed using a digital camera to capture the image of a TLC chromatogram. The image pixels are then calculated by ImageJ free software and interpreted as peak areas (Ferreira & Rasband, 2012). The quantification of brazilin in sappan heartwood uses high performance liquid chromatography (HPLC) (Chen, Bi, & Tu, 2010; Settharaksa, Monton, Pathompak, & Madaka, 2017; Xia, Li, Li, Zhang, & Kang, 2017). This study aimed to investigate the amount of brazilin in *C. sappan* heartwood for a comparative study between TLC-densitometry and TLC-image analysis and to establish the identity and quality parameters of the *C. sappan* heartwood crude drug in Thailand.

2. Materials and Methods

2.1 Plant materials

Heartwoods of *C. sappan* were collected from 15 different places in Thailand (Bangkok 1, Bangkok 2, Kamphaeng Phet, Nakhon Si Thammarat, Nakhon Sawan, Nakhon Ratchasima, Phichit, Chiang Rai, Nakhon Pathom, Phuket, Chanthaburi, Phetchabun, Chiang Mai, Ubon Ratchathani, and NongKhai). The sappan heartwoods were authenticated by Assoc. Prof. Dr. Nijsiri Ruangrungsi and voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand.

2.2 Identity and quality characteristics

The identity and quality parameters including macroscopic-microscopic characteristics, total ash, acid insoluble ash, loss on drying, water content, and extractive matter parameters of *C. sappan* heartwood were examined in accordance with WHO guideline (World Health Organization [WHO], 2011). For the TLC fingerprinting, 1 g of the powdered sample was macerated with 20 mL of methanol for 30 min at room temperature with shaking. The sample was filtered and evaporated to dryness. The residue was dissolved in 0.5 mL of methanol. A volume of 3 µL was applied onto the TLC silica gel 60 GF_{254} plate (Merck). The mobile phase was chloroform and methanol (9:1). The TLC plate was observed under UV light at 254 and 365 nm and dipped with p-anisaldehyde/sulfuric acid.

2.3 Ethanolic extract

C. sappan heartwoods were ground to powders and 5.0 g of the powder was successively extracted with 95% ethanol using the Soxhlet apparatus. The extracts were filtered and evaporated *in vacuo* until dryness.

2.4 Brazilin standard solution

A standard of brazilin was purchased from Tauto Biotech, Shanghai, China. The stock solution was prepared in 95% ethanol and diluted to gain a series of 0.05, 0.075, 0.10, 0.125, 0.15 mg/mL. The standard solutions were stored at 4 °C in the dark.

2.5 Thin-layer chromatography-densitometry

Three microliters of the extract in 95% ethanol (1 mg/mL) and brazilin standard solutions were spotted on the TLC plate (silica gel 60 GF_{254}). The plate was developed in a TLC chamber (10x20 cm) with the mobile phase of chloroform, ethyl acetate, and formic acid (10:8:2). The TLC plate was scanned under a wavelength of 525 nm by a TLC Scanner3 (CAMAG, Switzerland) using winCATS software. Brazilin was quantitated by peak area measurements. The test was done in triplicate.

2.6 Thin-layer chromatography-image analysis

The developed TLC plate was photographed under UV light at 254 nm and saved as tiff files. Quenching bands were changed to chromatographic peak areas by ImageJ software and brazilin contents were calculated. This test was done in triplicate.

2.7 Method validation

The method validation consisting of specificity, calibration range, accuracy, repeatability, intermediate precision, limit of detection, limit of quantitation, and robustness were performed according to ICH guideline (ICH harmonized tripartite guideline, 2005).

2.8 Data analysis

Brazilin contents between TLC-densitometry and TLC-image analysis were compared by paired t-test statistics.

3. Results

3.1 Macroscopic characteristics

C. sappan is a 6–9 m high tree with a 15–25 cm diameter trunk. The branches are rufous-pubescent armed with a few small prickles. The leaves are 20–38 cm long, pinnate 8–12 pairs, 10–15 cm long, and subsessile with small prickles at the base. The leaflets have 10–18 pairs that are 1.3–2.0 × 1 cm, subsessile, close, oblong, and rounded at the apex that are attached at the lowest corner, very inequilateral, glabrous above, more or less puberulous beneath. The flowers in panicles are terminal and in the axils of the upper leaves and 30–40 cm long. The pedicels are 1.3–1.5 cm long with lanceolate bracts, 8 mm long, and caduceus. The calyx is 11 mm long, leathery, glabrous, and the corolla is 2 cm across. The petals are orbicular, subequal, and yellow but at the base of the upper petals there is a red spot. The ovary is grey and velvety. The pods are 7.5–10 × 3.8–5 cm which are woody, obliquely oblong, subcompressed, polished, and indehiscent

with a hard recurved short beak at the upper angle of the obtuse apex (Kirtikar, Basu, & An, 1975) (Figure 2). The heartwood of the *C. sappan* is hard and rough with an orange-red color (Figure 3).

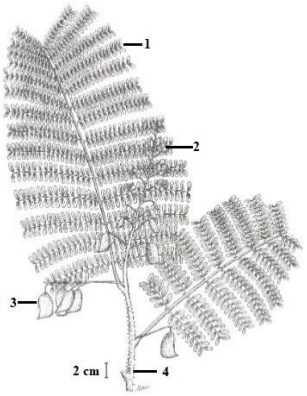


Figure 2. *Caesalpinia sappan* L. 1. Pinnate leaf, 2. Flower, 3. Pod, 4. Branch.

3.2 Microscopic characteristics

The histological characteristics in powdered form showed fragments of wood, fragments of xylem, fragments of bordered pitted vessel, and calcium oxalate. The anatomical characteristics of *C. sappan* heartwood including the transverse section, tangential longitudinal section, and radial longitudinal section presented the wood of parenchyma, vessel, and wood fiber structures (Figure 3).

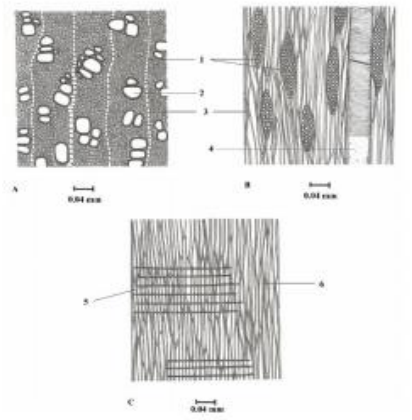
3.3 Physico-chemical parameters

The values of the physico-chemical parameters of *C. sappan* heartwood that included acid-insoluble ash, total ash, ethanol-soluble extractive, water-soluble extractive, loss on drying, and water content are shown in Table 1. The thin layer chromatographic fingerprint is shown in Figure 4.

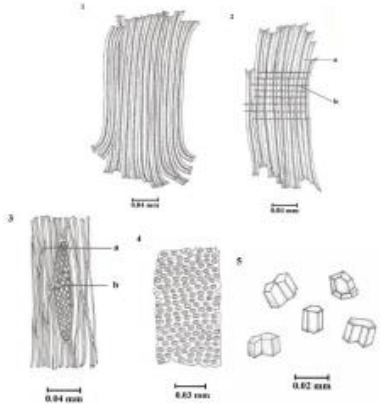
Table 1. Quality parameters of *Caesalpinia sappan* heartwood.

Content (% by weight)	Mean±SD	Min –Max
Acid-insoluble ash	0.44±0.14	0.22–0.71
Total ash	0.87±0.14	0.66–1.12
Ethanol-soluble extractive	2.94±0.57	2.00–4.00
Water-soluble extractive	3.77±0.64	2.70–4.99
Loss on drying	8.50±0.37	7.98–9.58
Water content	8.52±0.43	7.80–9.20

Caesalpinia sappan L. dried heartwoods



A. Transverse section of *C. sappan* heartwood, B. Tangential longitudinal section, C. Radial longitudinal section, 1. Wood parenchyma, 2. Vessel, 3. Wood fiber, 4. Vessel, 5. Wood parenchyma, 6. Wood fiber



Histological characters of *C. sappan* heartwood powder 1. Fragment of wood 2. Fragment of xylem ray in radial longitudinal view (2a. Wood fiber, 2b. Wood parenchyma) 3. Fragment of xylem ray in tangential longitudinal view (3a. Wood fiber, 3b. Wood parenchyma) 4. Fragment of bordered pitted vessel 5. Prism crystals of calcium oxalate

Figure 3. Pharmacognostic specification of *Caesalpinia sappan* heartwood: macroscopic and microscopic characteristics.

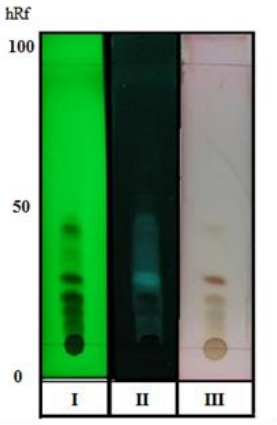


Figure 4. Thin-layer fingerprint of methanolic extract of the wood of *Caesalpinia sappan* L.: Stationary phase, Silica gel 60 GF_{254}; Mobile phase, Chloroform:Methanol (9:1); Detection, I = Detection under UV light 254 nm, II = Detection under UV light 365 nm, III = Detection with p-anisaldehyde/sulfuric acid.

3.4 Brazilin analysis by quantitative TLC

The average yield of the ethanolic extract of *C. sappan* heartwood was 15.52±2.13 g/100 g of dried crude drug. The quantitative analysis of brazilin was examined by TLC-densitometry and TLC-image analysis. The TLC chromatograms of the brazilin standard solutions and *C. sappan* heartwood extracts visualized under UV 254 nm are shown in Figure 5. The TLC-densitogram scanned at λ_{max} of 525 nm is shown in Figure 6. The calibration curves of brazilin for the TLC-densitometry and TLC-image analysis are presented in Figure 7. The average contents of brazilin from the TLC-densitometry and TLC-image analysis were 1.259±0.285 and 1.256±0.266 g/100 g of crude drug, respectively. Both techniques were not statistically significantly different (P>0.05) using paired t-test.

3.5 Method validity

The absorbance spectra of brazilin scanned in the range of 200–700 nm demonstrated a λ_{max} of 525 nm (Figure 8). The specificity of the developed TLC for the brazilin analysis was represented as identical absorbance spectra

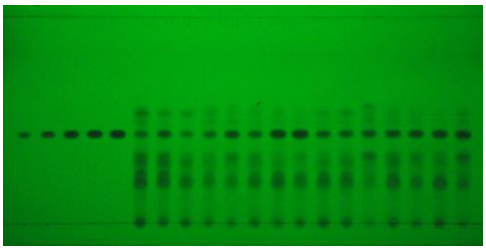


Figure 5. The TLC plate under UV 254 nm; standard brazilin (tracks 1–5), and *Caesalpinia sappan* heartwood extracts from 15 different sources.

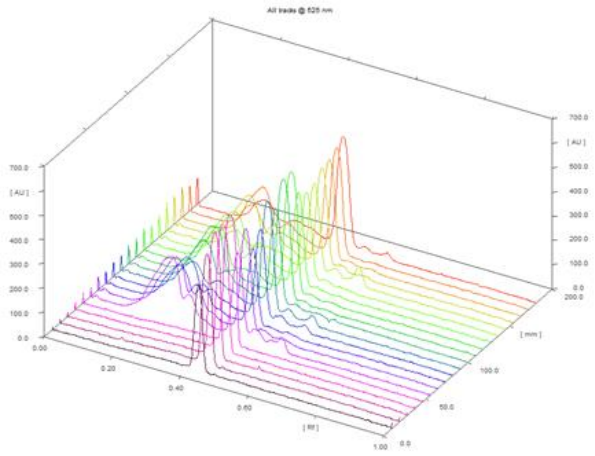
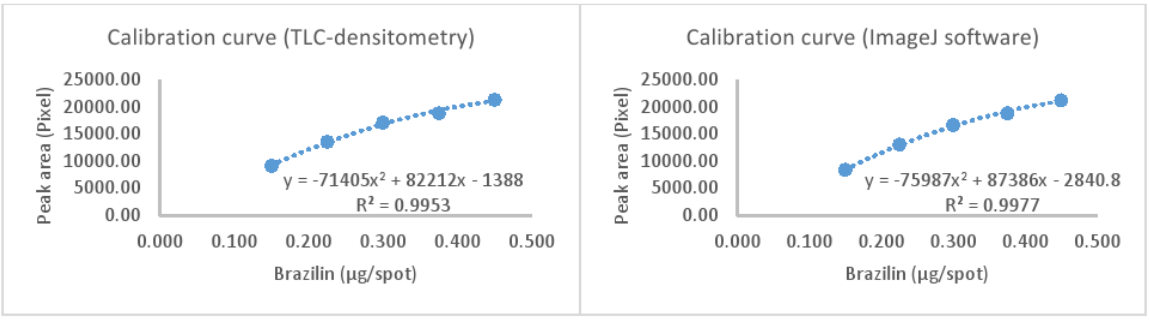


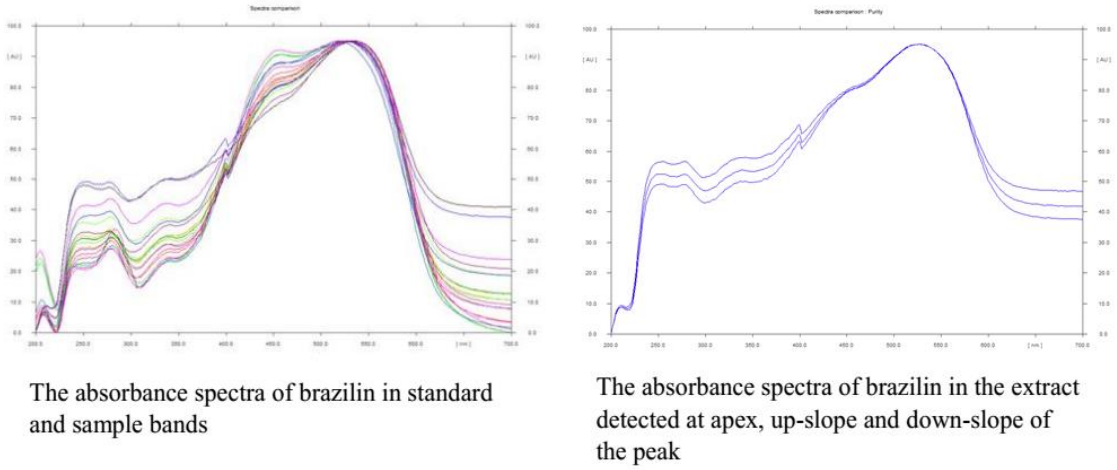
Figure 6. TLC densitogram of brazilin standard solutions and brazilin in *Caesalpinia sappan* heartwood samples.

among standard and sample bands as well as identical absorbance spectra among apex up-slope and down-slope of peak. The accuracy was represented by 98% of recovery of three concentrations of brazilin spiked into the sample. The repeatability and intermediate precision performed on the sample with four different concentrations of brazilin in the same day and in three different days were less than 10% residual standard deviation (RSD). The limit of detection and limit of quantitation calculated by the RSD of the regression line and the slope of calibration curve were less than 0.1 µg/spot. The robustness was confirmed using mobile phase parameter. The method validity is shown in Table 2.



Calibration curve of brazilin by TLC densitometry Calibration curve of brazilin by TLC image analysis

Figure 7. Calibration curve of brazilin by TLC densitometry and TLC image analysis.



The absorbance spectra of brazilin in standard and sample bands

The absorbance spectra of brazilin in the extract detected at apex, up-slope and down-slope of the peak

Figure 8. Absorbance spectra of brazilin.

Table 2. Method validity of TLC-densitometry and TLC-image analysis of brazilin in *Caesalpinia sappan* heartwood.

Parameter	TLC-densitometry	TLC-image analysis
Accuracy (% recovery)	98.10±4.98	97.86±5.90
Precision: Repeatability (%RSD)	3.17±0.94	4.87±2.37
Precision: Intermediate precision (%RSD)	5.44±2.28	9.39±3.97
Limit of detection (µg/ spot)	0.02	0.01
Limit of quantitation (µg/ spot)	0.06	0.04
Robustness (%RSD)	2.12	3.96

Abbreviations: TLC, thin layer chromatography; RSD, residual standard deviation.

4. Discussion

Macroscopic and microscopic characteristics are the first steps for plant sample identification. The main structures in the powder of the heartwood are xylem and pitted vessel (Alex, 2003). This research demonstrated the microscopic characteristics of *C. sappan* heartwood in both sections and powders which presented fragments of wood, fragments of xylem (wood fiber and wood parenchyma), fragments of bordered pitted vessel, and prism crystals of calcium oxalate. The physico-chemical parameters including total ash, acid insoluble ash, ethanol and water soluble extractive values, loss on drying, and water content were specified to assess the quality of *C. sappan* heartwood in Thailand. The content of ethanol and water soluble extractives should not be less than 2.94 and 3.77% by weight. On the other hand, the content of total ash and acid-insoluble ash, loss on drying, and water content should not be more than 0.87, 0.44, 8.50, and 8.52% by weight, respectively. Badami, Rai, Moorkoth, Rajan, and Suresh (2003) reported that the total ash, acid insoluble ash, water soluble ash, and sulphated ash values of *C. sappan* heartwood in India were 1.22, 0.13, 0.38, and 1.14%. The ethanol and water soluble extractive values were 4.80 and 2.69%. Chen, Bi, and Tu (2010) established the quality standard of Sappan Lignum in China and the ethanol soluble extractives of 18 samples varied from 6.4 to 11.3%. The values might be different from other countries depending on intrinsic and extrinsic factors (e.g., atmospheric conditions and the physical features of the land).

The main constituent of *C. sappan* heartwood is brazilin. Therefore, brazilin quantification is essential for quality control of this crude drug. TLC-densitometry and TLC-image analysis were performed for brazilin quantification. The quantities of brazilin in the *C. sappan* heartwood from the two methods were not statistically significantly different ($P>0.05$). Brazilin in the heartwood by TLC-densitometry compared to TLC-image analysis revealed 1.259±0.285 and 1.256±0.266 g/100 g crude drug, respectively. As a result the TLC-image analysis, which is inexpensive, can be used as an optional technique to examine brazilin compound.

The accuracy was done by recovery of spiking three concentrations of brazilin standard solution in the sample. Recovery values from the TLC-densitometry and TLC-image analysis were within admissible limits (98.10 and 97.86%). An analysis of the precision, that included repeatability and intermediate precision, was tested by determination of four concentrations (each in triplicate) at the same and different days. Variations in intra-day and inter-day precision were not more than 10%. The detection limits from TLC-densitometry and TLC-image analysis were 0.02 and 0.01 µg/spot, respectively. The quantitative limits were 0.06 and 0.04 µg/spot. Robustness was evaluated by an analysis of peak area after deliberate differences of mobile phase ratios of

chloroform:ethyl acetate:formic acid (10.2:8.2:1.8; 10:8:2; and 9.8:7.8:2.2) and it indicated that the %RSD was less than five. The method validation showed that both techniques were reliable, efficient, and proper for the quantitation of brazilin in *C. sappan* heartwood.

5. Conclusions

The pharmacognostic specifications with reference to brazilin content of *C. sappan* heartwood in Thailand were established. Quantitative TLC was performed for brazilin analysis that included TLC-densitometry and TLC-image analysis. ImageJ software was used to process the chromatogram images and found that it can be an alternated method for quantification of brazilin in *C. sappan* heartwood.

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References

Alex L. (2003). *Modern Arboriculture – A System approach to the care of trees and their associates* (3rd ed.). (p. 56-57).

Badami, S., Rai, S.R., Moorkoth, S., Rajan, S., & Suresh, B. (2003) Pharmacognostical Evaluation of *Caesalpinia sappan* heartwood. *Ancient Science of Life Vol: XXIII (2)*, 100-107. Retrieved from https://pdfs.semanticscholar.org/b14b/b199516df4a15df788574c3fad783b26e619.pdf

Badami, S., Moorkoth, S., & Suresh, B. (2004) *Caesalpinia sappan* A medicinal and dye yielding plant. *Natural Product Radiance*, *3*(2), 75-82. Retrieved from https://pdfs.semanticscholar.org/e1dd/71363ea75e3dcda1e2d00b31f38b73996e4f.pdf

Chen, Y., Bi, D., & Tu, P. (2010) Quality standard of Sappan Lignum. *Zhongguo Zhong Yao Za Zhi*, *35*(16), 2068-2071. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/21046730

Cuong, T., Hung, T., Kim, J., Kim, E., Woo, M., Choi, J., Lee, J., & Min, B. (2012) Phenolic Compounds from *Caesalpinia sappan* Heartwood and Their Anti-inflammatory Activity. *Journal of Natural Products*, *75*, 2069-2075. Retrieved from https://cdn-pubs.acs.org/doi/pdf/10.1021/np3003673

Det-anand, K. (1975) Pigments from Sappan wood. *Vanasarn*, *33*(1), 63-65.

Efferth, T., Kah, S., Paulus, K., Adams, M., Rauh, R., Boechzelt, H., Hao, X., Kaina, B., & Bauer, R. (2008) Phytochemistry and pharmacogenomics of natural products derived from traditional Chinese medicine and Chinese materia medica with activity against tumor cells. *Molecular Cancer Therapeutics*, *7*(1), 152-161. Retrieved from http://mct.aacrjournals.org/content/molcanther/7/1/152.full.pdf

Ferreira, T. & Rasband, W. (2012). *ImageJ User Guide*. IJ 1.46r. Retrieved from http:// https://imagej.nih.gov/ij/docs/guide/user-guide.pdf

Gilbody, A.W., Perkin, W.H., & Yates, J. (1902) Brazilin and Haematoxylin Part I. *Journal of Chemical Society*, *79*, 1396. Retrieved from http://pubs.rsc.org/-/content/articlelanding/1901/ct/ct9017901396/unauth#!divAbstract

ICH Harmonized Tripatide Guideline. (2005). Validation of analytical procedures: Text and methodology, Q2 (R1). *Proceeding of International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use*. Geneva, Switzerland

Kennedy, R., Srinivasappa, N., & Farooqi, A. (2004) Sappan wood - a multipurpose tree. *Indian Journal of Arecanut, Spices and Medicinal Plants*, *6*(3), 93-94.

Kirtikar, K. R., Basu, B. D., & An, I. C. S. (1975). *Indian Medicinal Plants, Vol. II. Reprint edition* (pp. 5-6). Delhi, India: Jayyed Press.

Mohan, G., Anand, S.P., & Doss, A. (2011) Efficacy of aqueous and methanol extracts of *Caesalpinia sappan* L. and *Mimosa pudica* L. for their potential antimicrobial activity. *South as Journal of Biological Sciences*, *1*(2), 48-57. Retrieved from https://pdfs.semanticscholar.org/4713/9ea7e8711503965ed4d5b002f6875578c051.pdf

Nirmal, N. P. & Panichayupakaranant, P. (2015) Antioxidant, antibacterial, and anti-inflammatory activities of standardized brazilin-rich *Caesalpinia sappan* extract. *Pharmaceutical Biology*, *53*(9), 1339-1343. doi: 10.3109/13880209.2014.982295

Puchtler, H., Meloan, S. N., & Waldrop, FS. (1986) Application of current chemical concepts to metal - heamatein and –brazilein stains. *Histochemistry*, *85*, 353-364. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/2430916

Rondao, R., Melo, S. S., Pina, J., Melo, M. J., Vitorino, T., & Parola, A. J. (2013) Brazilwood reds: The photo chemical of Brazilin and Brazilein. *The Journal of Physical Chemistry*, *117*, 10650-10660. Retrieved from https://datapdf.com/brazilwood-reds-the-photochemistry-of-brazilin-and-brazilein.html

Sarumathy, K., Rajan, M. S. D., Vijay, T., & Dharani, A. (2011) In *Vitro* study on antioxidant activity and phytochemical analysis of *Caesalpinia Sappan*. *International Journal of Institutional Pharmacy and Life Sciences*, *1*(1), 31-39. Retrieved from https://pdfs.semanticscholar.org/f546/0c35abd6ad720fbfbf363e6751d33318d57e.pdf

Settharaksa, S., Monton, C., Pathompak, P., & Madaka, F. (2017) HPLC method validation for determination of brazilin in *Caesalpinia sappan* L. heartwood extract. *Thai Journal of Pharmaceutical Sciences (TJPS)*, *41* (Supplement Issue), 141-144. Retrieved from http://am-2017.weebly.com/uploads/1/5/1/8/15182734/fp-36_sukanya_settharaksa__141-144__ok_v1.pdf

Smitinand, T. (1984) *Flora of Thailand Volume Four Part One* (pp. 65).

Waksmundzka- Hajnos, M. (2008) Thin- Layer chromatography. *Journal of Chromatographic Science*, *46*(4), 289-290. doi: 10.1093/chromsci/46.4.289

Washiyama, M., Sasaki, Y., Hosokawa, T., & Nagumo, S. (2009) Anti-inflammatory Constituents of Sappan Lignum. *Biological and Pharmaceutical Bulletin*, *32*(5), 941-944. doi:10.1248/bpb.32.941

World Health Organization. (2011). *Quality control methods for herbal materials* (pp. 1-40). Geneva, Switzerland: Author.

Xia, Z., Li, D., Li, Q., Zhang, Y., & Kang, W. (2017) Simultaneous determination of brazilin and protosappanin B in *Caesalpinia sappan* by ionic-liquid dispersive liquid-phase microextraction method combined with HPLC. *Chemistry Central Journal*, *11:114*. doi:10.1186/s13065-017-0342-9

Xu, H. & Lee, S. (2004) The antibacterial principle of *Caesalpinia sappan*. *Phytoterapy Research*, *18*, 647-651. doi:10.1002/ptr.1524