REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 24, No. 1 (2023)
http://www.veterinaria.org
Article Received: 20 December 2022; Revised: 26 February 2023; Accepted: 18 March 2023

The Usage of *Biancaea Sappan* and *Crocus Sativus* as Natural Dyes for the Liver Fluke, *Fasciola Gigantica*

Andini Nurlaelasari[1], A'isyah Retno Wulandari[1], Dimas Ariyanto[1], Penny Humaidah Hamid[1]*

[1] Department of Animal Science, Sebelas Maret University, Surakarta 57126, Indonesia

Abstract

The herbs in Indonesia are rich in substances with medicinal effect as well as natural compounds for many purposes. The herbs are easily cultivated in the supporting climate and thus, guaranteeing it's availability and sustainability. The aim of this study was to utilize *Biancaea sappan* and *Crocus sativus* as natural dye for *Fasciola gigantica* compared to commonly used dye, the acetocarmine. *F. gigantica* itself was frequently found as the liver fluke of cattle in Indonesia with significant economic losses. *B. sappan* and *C. sativus* were mashed and soaked in 100% glacial acetic acid overnight. The extracts were utilized to stain adult *F. gigantica* isolated from infected cattle in Ampel abattoir, Boyolali, Central Java, Indonesia. Acetocarmine 5.5% was a control during staining process. The results showed that structures and internal organs were pigmented in comparable degree with the control. Both of the *B. sappan* and *C. sativus* were successfully stained *F. gigantica* structures such as oral sucker, ventral sucker, uterine, eggs inside the uterine and testes as compared to acetocarmine. The pigmentation result showed that the *C. sativus* stained spina better than acetocarmine. The methods are eco-friendly and safe since the herbs do not have carcinogenic effect.

Keywords: Biancaea sappan; Crocus sativus; Fasciola gigantica.

Introduction

The identification of worms is mainly based on morphology (Haridwal *et al.*, 2021). The species identification helps and gives a rationale on the disease controls. Trematode identifications commonly use various chemical dye such as hematoxylin-eosin, romanowsky, lactophenol cotton blue, lugol's iodine, and malachite green (Zeibig, 2014). The reduced use of natural dyes is due to the presence of more practical synthetic dyes (Bhuyan and Saikia, 2005). However, the use of synthetic dyes may have harmful effects on parasitologist or technician who work on animal health (Akinloye *et al.*, 2010). These problems lead the search for more natural dyes, especially the herbs which are easy to use, more effective, biodegradable, and do not cause health problems for humans and the environment contaminations of discharge. Hence, coloring with the use of natural ingredients tends to increase. Some dyes from extracted plants have been utilized, i.e., the ethanolic extract of *Hibiscus rosa-sinensis*, *Beta vulgaris*, *Curcuma*, *llium cepa*, *Juglans regia*, and *Rubia tinctorum* (Daryani *et al.*, 2011; Kumar *et al.*, 2015; Marhaba and Haniloo, 2018).

Biancaea sappan generally grows to a height of 500-1000 m above the sea level, such as in mountainous areas with rocky topography and moderate temperature. This plant is found in South Asia, Southeast Pacific (Dapson and Bain, 2015; Nirmal *et al.*, 2015). The plant can reach 5-10 m high at maximum. The stems are woody, round, and brownish-green (Hariana, 2006). In Indonesia, *B. sappan* is known locally as "Sappan" or "Secang" (Kurniati *et al.*, 2019). *B. sappan* is utilized as natural dye since contains brazilin, a red pecursor of tropical

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 24, No. 1 (2023)
http://www.veterinaria.org
Article Received: 20 December 2022; Revised: 26 February 2023; Accepted: 18 March 2023

hardwoods (Dapson and Bain, 2015; Tamburini *et al.*, 2019). *Crocus sativus* is known as of Indonesia local saffron. This flowering plant belongs to the family of Iridaceae (Khazdair *et al.*, 2015). The most widespread distribution of *C. sativus* is in Asian coutries, South Europe, and North Africa (Heidari *et al.*, 2022; Vahedi *et al.*, 2018). *C. sativus* is often utilized as drugs since reportedly containing many chemical compounds i.e. safranal, crocin, crocetin, and picrocrocin (Bolhassani, 2018). These herbs can also be used as natural dyes with color between yellow and reddish (Ahmadieh *et al.*, 2018).

B. sappan and *C. sativus* can be found abundantly in Indonesia. The herbs are well-known for multipurpose pigmentations such as eco-print dyes on textile, herbal medicine, disclosing and therapeutic agents in Indonesia (Kurniati *et al.*, 2019; Sutjaritjai *et al.*, 2022; Utami *et al.*, 2022). Their availability and advantages may serve also as an alternate natural dyes for the parasitic helminths. This study aimed to utilize *B. sappan* and *C. sativus* as natural dye for *F. gigantica* compared to commonly used colouring substance, the acetocarmine.

Materials and Methods

Fasciola gigantica adult stage

Adult worms of *F. gigantica* were collected from the Ampel Abbatoir, Indonesia. The adult worms were isolated in liver parenchyma from naturally-infected cattle. The worms were transported altogether with the liver tissue on ice to Integrated Laboratory Unit, Sebelas Maret University, Indonesia. All of the fresh worms were then gently fixed with double object glass in 10% formalin to keep the intact morphology.

Figure 1. The *B. sappan* wood (A) and *C. sativus* flower (B) in dry forms

Natural dyes preparation

B. sappan (Fig. 1.A) was obtained from Magelang, Java Island, Indonesia with ordinate between 110°09' east longitude, 7°24’ south latitude, and an altitude of 1,109 meters above the sea level. *C. sativus* (Fig. 1.B) was from Balikpapan, Borneo Island, Indonesia with ordinate between 116°48' east longitude, 1°16’ south latitude, and an altitude of 78 meters above the sea level. The dry herbs were mashed by using a homogenizer, 35,000 rpm for 10 minutes. *B. sappan* and *C. sativus* were extracted using 100% glacial acetic acid (Merck, Germany). The herbs were soaked in the acid for 24 hours at room temperature. The natural

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 24, No. 1 (2023)
http://www.veterinaria.org
Article Received: 20 December 2022; Revised: 26 February 2023; Accepted: 18 March 2023

colour came out densely during the extraction process. Extract purifications were performed by filtering the liquid with a Whatman filter paper (Cytiva, US). The dyes were stored in room temperature and or immediately utilized for staining the fixed worms.

Staining processing for adult *F. gigantica*

Fixation was performed by pressing the worm between two object glasses and tied with rubber. The fixed worms were then immersed in formalin 10% for 24 hours or longer (Kumar *et al.*, 2015). After fixation was completed, the worms were washed with tap water and removed from the object glass. Worm specimens were grouped in different staining processes, i.e. the extract *B. sappan*, *C. sativus*, and control, acetocarmine (Himedia, Indonesia).

Each group of treatments were incubated in dyes for 24 hours. Thereafter, the speciments were rinsed using tap water and followed by 70% ethanol. The worms were then immersed in glacial acetic acid for 5 minutes. To remove excess colour without loss of pigmentations, the specimens were dehydrated in ethanol with gradual concentrations, i.e.: 70%, 85%, 95%, and 100% for 5 minutes each. The specimens were then soaked in xylene until they were clear. The stained worms were then permanently fixed by using Canada balsam and then morphologically identified under light microscopy (Olympus, Japan). The internal organ structures were analysed based on the anatomy of *F. gigantica* (Hanna, 2015).

Image acquisition

The figures in detail were acquired by a microscope camera, Optilab (Miconos, Indonesia). The images processing of scale bars was performed by Image Raster 3.0. The contrast ratio on the image acquisitions were set in standard baseline for all treatments.

Results and Discussions

Acetocarmine and the ethanolic extract of *B. sappan* produce red-color dyes, while *C. sativus* produces a yellow dye. The results of staining on worm preparations with acetocarmine showed a brownish red color, *B. sappan* was yellowish brown, and *C. sativus* was yellow. The anterior parts of *F. gigantica* such as the ventral sucker and oral sucker were stained clearly (Fig. 2). All the worms were stained successfully by using acetocarmine. The acetocarmine was able to color surface of the worms, penetrate to internal organs and the color persisted after subsequent washing procedures. The oral suckers were observed clearly after staining procedures with acetocarmine (Fig. 2.A), *B. sappan* (Fig. 2.B), *C. sativus* (Fig. 2.C) and showed as a whole bowl-shaped. The stainings of the pharynx were only in acetocarmine and *B. sappan* (Table 1, Fig. 2.A, 2.B). The dyes utilized were also able to stain ventral sucker (Fig 2.D, 2.E, 2.F) which is located posterior to the intestinal bifurcation. However, *C. sativus* coloring to ventral sucker is less intense compared to acetocarmine and *B. sappan*.

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 24, No. 1 (2023)
http://www.veterinaria.org
Article Received: 20 December 2022; Revised: 26 February 2023; Accepted: 18 March 2023

Table 1. Staining evaluation of herbs utilized to internal morphology of a Trematode, *F. gigantica*

Morphology	***Acetocarmine***	***Biancaea sappan***	***Crocus sativus***
Oral Sucker	4+	4+	4+
Ventral sucker	4+	4+	4+
Uterus	4+	2+	3+
Testis and Vitelaria	4+	2+	4+
Egg	4+	3+	4+
Spina	2+	2+	4+

1+ negative, 2+ weak, 3+ moderate, 4+ strong

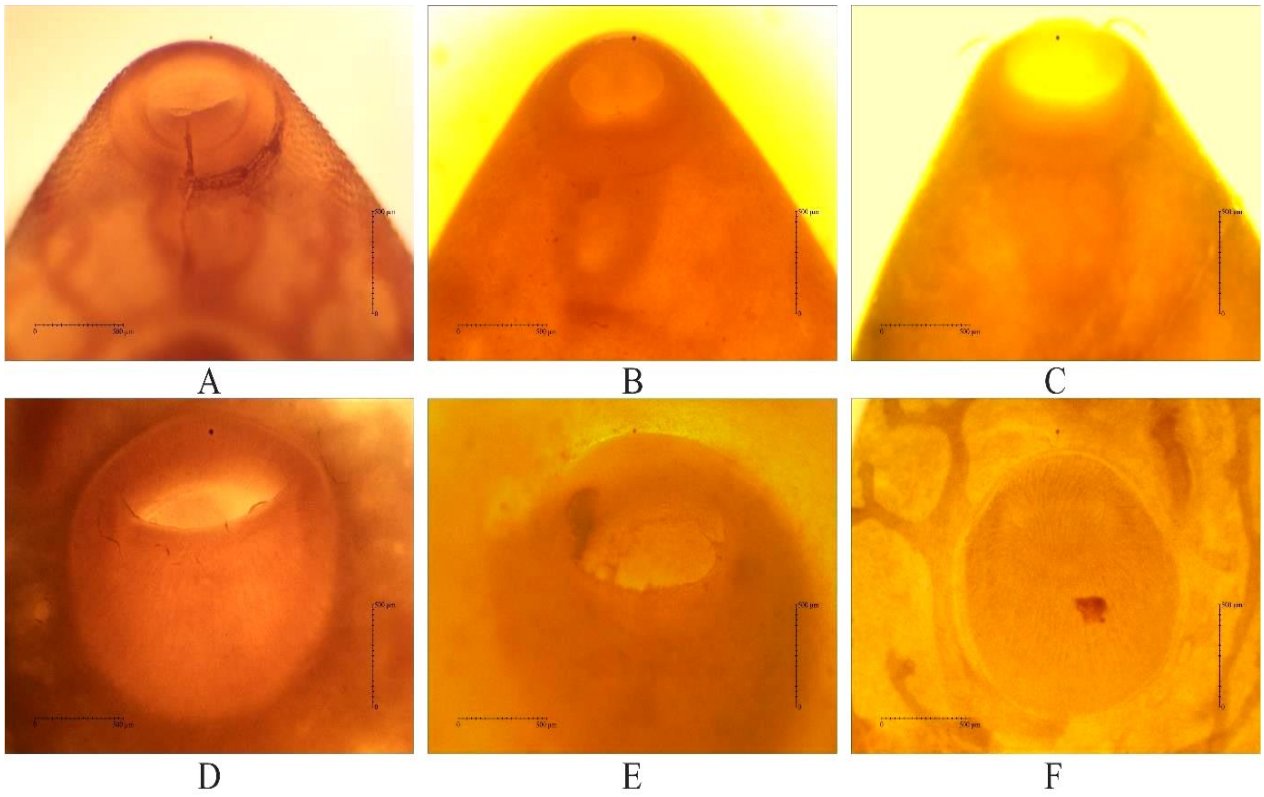

Figure 2. Staining of anterior part of the adult *F. gigantica*. Oral sucker was stained with acetocarmine (A), *B. sappan* (B) and *C. sativus* (C). The ventral sucker was stained with acetocarmine (D), *B. sappan* (E) and *C. sativus* (F). Scale bars are 500 µm.

The reproductive organs of *F. gigantica* were stained densely, i.e., the uterus, testes, and vitellaria. The acetocarmine, *B. sappan*, and *C. sativus* all stained uterine and therefore, can be well-distinguished. The colours produced were reflected the colour produced from each dye accordingly (Fig. 3.A, 3.B, 3.C). Testes and vitellaria sections from *B. sappan* staining were less pigmented compared to *C. sativus* and acetocarmine (Fig. 3.D, 3.E, 3.F). Furthermore, the eggs pigmentation from acetocarmine, *B. sappan*, and *C. sativus* in this experiment showed a comparable degree of intensities (Fig. 3.G, 3.H, 3.I). By observing the pigmentation, *C. sativus* was best in staining the spina of *F. gigantica* (Fig. 4.A, 4.B, 4.C, Table 1). The dyes color of acetocarmine and *B. sappan* were not well persistent in spina after washing procedures and therefore the pigmentations result in the end were less.

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 24, No. 1 (2023)
http://www.veterinaria.org
Article Received: 20 December 2022; Revised: 26 February 2023; Accepted: 18 March 2023

Natural herbs can be used as appropriate dyes for staining worm parasites as they are easily processed, inexpensive, abundant in nature and more environmentally friendly (Marhaba and Haniloo, 2018). The ethanol extraction from *B. sappan* and *C. sativus* in this report had a potential to be regularly utilized and the results of the stains were comparable to commonly used substance, acetocarmine. Oral sucker staining with *B. sappan* and *C. sativus* were comparable to the Hena and Curcuma previously reported (Daryani *et al.*, 2011). Pharynx staining with *B. sappan* has almost the same quality as alizarin extract dye (Daryani *et al.*, 2011). Whilst, ventral sucker stainings with acetocarmine, *B. sappan*, and C. *sativus* were seemed to be more pigmented when compared to *Rosa hybrida* (Kumar *et al.*, 2015).

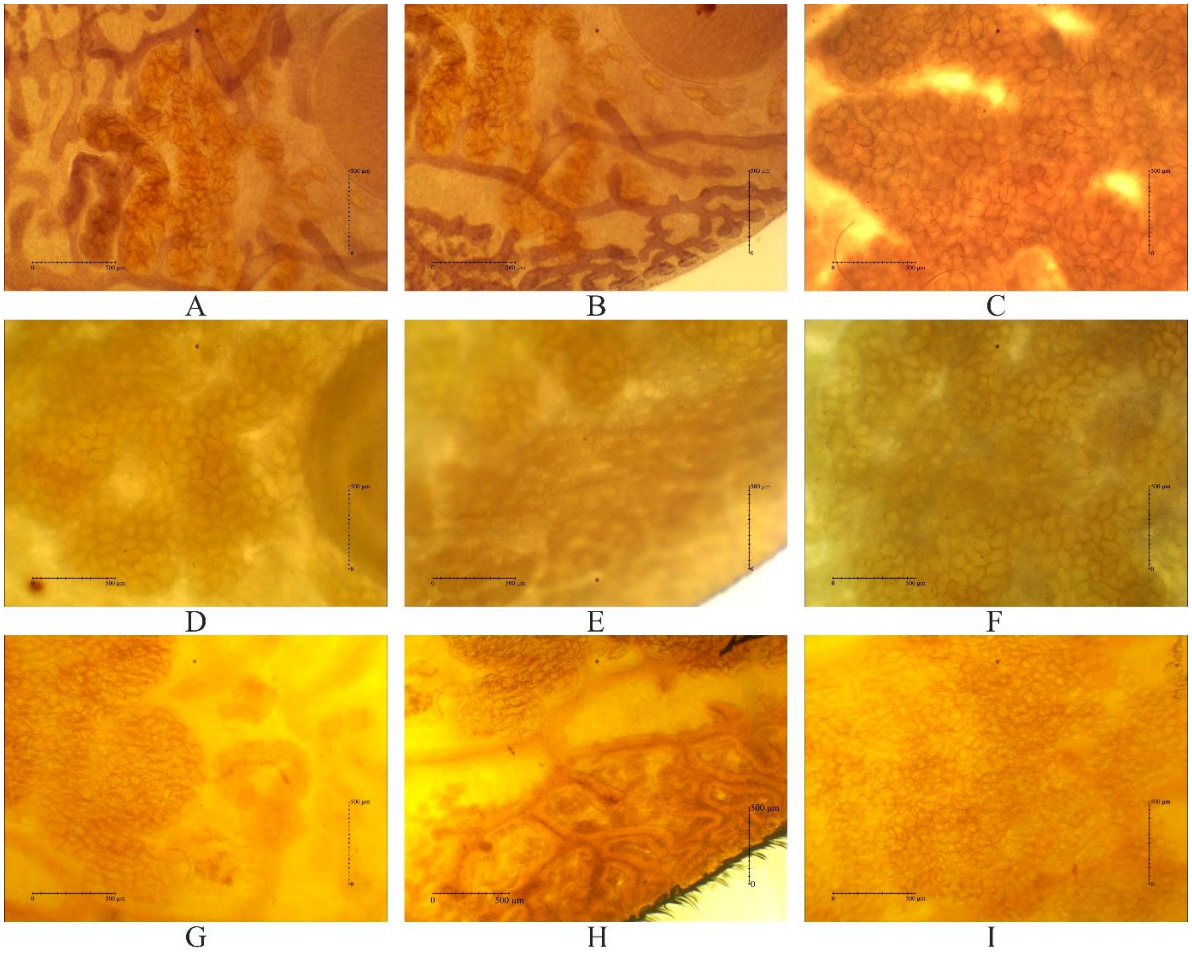

Figure 3. Staining of reproductive organs from the adult *F. gigantica*. Uterus, testes and vitellaria, eggs were stained with acetocarmine: A. uterus, B. testes and vitellaria, C. eggs. *B. sappan* stainings were: D. uterus, E. testes and vitellaria, F. eggs. *C. vernus* stainings were: G. uterus, H. testes and vitellaria, I. eggs.

The dyes colour of acetocarmine and *B. sappan* were not well persistent in spina after washing procedures and therefore the pigmentations result in the end were less. The results are in accordance to previous report that acetocarmine produced less pigmentation to stain the spine of *F. gigantica* (Daryani *et al.*, 2011; Marhaba and Haniloo, 2018). Staining of body spine with *B. sappan* and *C. sativus* has the comparable results as other natural dyes previously reported, such as Alizarin, Hena, and Curcuma (Daryani *et al.*, 2011).

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 24, No. 1 (2023)
http://www.veterinaria.org
Article Received: 20 December 2022; Revised: 26 February 2023; Accepted: 18 March 2023

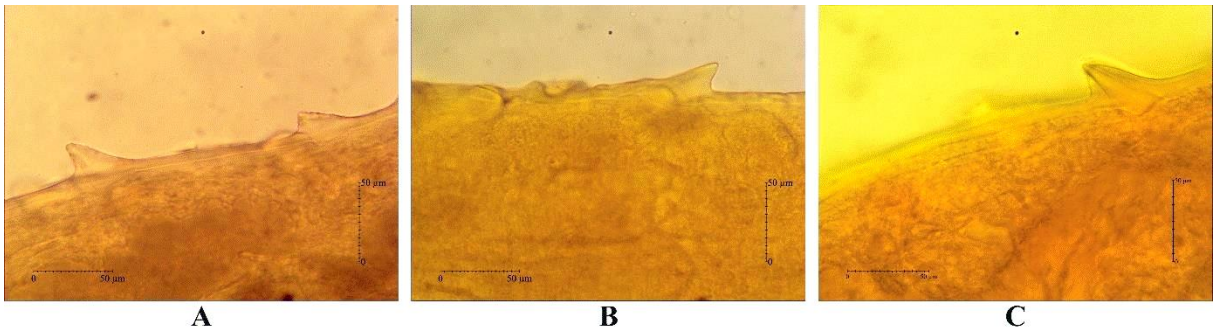

Figure 4. Spina of *F. gigantica* stained with *acetocarmine* (A), *B. sappan* (B) and *C. sativus* (C)

Conclusion

Staining with natural dyes for Trematode, i.e., *F. gigantica* is efficient and environmentally friendly. The color produced from the natural pigment is able to distinguish the internal structures and organs of the worm, such as oral sucker, ventral sucker, uterus, testis, vitellaria, egg, and spina. Both *B. sappan* and *C. sativus* can be used as dyes and serve as an alternate to acetocarmine. Moreover, the *C. sativus* stained spina with higher pigmentation degree in this study.

Acknowledgement

This research can be carried out with the help of related parties. Thank you to RPH Ampel, Boyolali for supporting and assisting in taking samples for research purposes. Thanks to the Planning, Research and Development Agency of Boyolali District, Indonesia, for granting permission to perform this study in the region.

References

[1] Ahmadieh, H., Kanavi, M. R., Moradian, S., & Bonyadi, M. H. J. (2018). Saffron for staining the anterior lens capsule. Journal of Cataract Refractive Surgery, 44(12), 1524-1525.

[2] Akinloye, A., Illoh, H., & Olagoke, A. (2010). Screening of some indigenous herbal dyes for use in plant histological staining. Journal of Forestry Research, 21(1), 81-84.

[3] Bhuyan, R., & Saikia, C. (2005). Isolation of colour components from native dye-bearing plants in northeastern India. Bioresource technology, 96(3), 363-372.

[4] Bolhassani, A. (2018). Bioactive components of saffron and their pharmacological properties. Studies in Natural Products Chemistry, 58, 289-311.

[5] Dapson, R., & Bain, C. (2015). Brazilwood, sappanwood, brazilin and the red dye brazilein: from textile dyeing and folk medicine to biological staining and musical instruments. Biotechnic Histochemistry, 90(6), 401-423.

[6] Daryani, A., Sharif, M., & Meigouni, M. (2011). Staining of *Fasciola hepatica* by natural herbal dyes. Comparative clinical pathology, 20(4), 305-308.

[7] Hanna, R. (2015). *Fasciola hepatica*: histology of the reproductive organs and differential effects of triclabendazole on drug-sensitive and drug-resistant fluke isolates and on flukes from selected field cases. Pathogens 4(3), 431-456.

[8] Hariana, A. (2006). Tumbuhan Obat Dan Khasiatnya, Penebar Swadaya, Jakarta. In: Hal.

[9] Haridwal, S., Malatji, M. P., & Mukaratirwa, S. (2021). Morphological and molecular characterization of *Fasciola hepatica* and *Fasciola gigantica* phenotypes from co-endemic

localities in Mpumalanga and KwaZulu-Natal provinces of South Africa. Food Waterborne Parasitology 22, e00114.

[10] Heidari, F., Shekari, F., Andalibi, B., Saba, J., Uberti, D., & Mastinu, A. (2022). Comparative effects of four plant growth regulators on yield and field performance of *Crocus sativus* L. Horticulturae, 8(9), 799.

[11] Khazdair, M. R., Boskabady, M. H., Hosseini, M., Rezaee, R., & Tsatsakis, A. M. (2015). The effects of *Crocus sativus* (saffron) and its constituents on nervous system: A review. Avicenna journal of phytomedicine, 5(5), 376.

[12] Kumar, N., Mehul, J., Das, B., & Solanki, J. (2015). Staining of Platyhelminthes by herbal dyes: An eco-friendly technique for the taxonomist. Veterinary world, 8(11), 1321.

[13] Marhaba, Z., & Haniloo, A. (2018). Staining of parasitic helminths by extracts of *Allium cepa*, *Juglans regia*, and *Rubia tinctorum:* An approach to herbal dyes. Iranian Journal of Parasitology, 13(2), 293.

[14] Nirmal, N. P., Rajput, M. S., Prasad, R. G., & Ahmad, M. (2015). Brazilin from *Caesalpinia sappan* heartwood and its pharmacological activities: A review. Asian Pacific Journal of Tropical Medicine, 8(6), 421-430.

[15] Sutjaritjai, N., Panyadee, P., Phumthum, M., Inta, A., & Balslev, H. (2022). High diversity of medicinal uses of *Thai legumes* (Fabaceae) and their potential in public herbal medicine. Diversity, 14(8), 588.

[16] Tamburini, D., Cartwright, C. R., Pullan, M., & Vickers, H. (2019). An investigation of the dye palette in Chinese silk embroidery from Dunhuang (Tang dynasty). Archaeological Anthropological Sciences, 11(4), 1221-1239.

[17] Utami, N. K., Amperawati, M., & Rizki, M. I. (2022). Quantitative analysis of secondary metabolites and solvent optimization of Secang wood extract (*Caesalpinia sappan* L/*Biancaea sappan*) as disclosing agent. Jurnal Kesehatan Gigi, 9(1), 70-74.

[18] Vahedi, M., Kabiri, M., Salami, S. A., Rezadoost, H., Mirzaie, M., & Kanani, M. R. (2018). Quantitative HPLC-based metabolomics of some Iranian saffron (*Crocus sativus* L.) accessions. Industrial Crops Products, 118, 26-29.

[19] Zeibig, E. (2014). Clinical parasitology: A practical approach: Elsevier Health Sciences.