Investigation and Comparison of Physicochemical Characteristics of Non-aged and 4-month Aged Mulberry Wine Prepared from Three Different Wine Making Techniques

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Abstract: Winemaking techniques and the aging process of wine are important to the quality of wine. This study was investigated and compared the effect of different winemaking practices on physicochemical characteristics among non-aged and 4-month aged of *Morus alba Linn* (*cv.* Chiang Mai 60) wines. Hand press juice (HPJ), hand press juice with pulp (HPP) and cold press juice (CPJ) were prepared from fresh mulberry fruit which were fermented using *Saccharomyces cerevisiae* (Premier rouge) and then bottled and aged for 4 months in the cold room (12±2˚C). The pH, total titratable acidity (TTA), alcohol content, reducing sugar, color measurements, total phenolic content (TPC), total flavonoid content (TFC), antioxidant capacity and total anthocyanin content (TAC) were observed. The results showed significant differences on the chemical parameters (TTA, alcohol content, reducing sugar, TPC, TFC and TAC) among three treatments of mulberry wines without aging. Among all wine making techniques used, HPP and CJP method produced wine that have similar level of TFC, and color intensity which was higher than the wine prepared by HPJ method. The HPJ method resulted in the wine that have significantly lower level of TPC, TAC, and TFC than the other two techniques. In addition, after aged the mulberry wines for 4 months, the wine made with different technique had significantly lower level of TPC, antioxidant capacity, TAC, TFC and color hue than the non-aged mulberry wine made from the same technique.

1 INTRODUCTION

Mulberry has unique flavour, texture, and color. (Vijayan et al*.,* 2011) considered that mulberry fruits have well balanced sweetness and tartness. It is highly perishable therefore it is used in manufacturing different types of food for example sauce, fruit tea, ice-creams, supplements, syrup, vinegar and also alcoholic beverages (Juan et al*.*, 2012). Mulberry is a potential plant due to the presence of bioactive ingredients (Ercisli and Orhan, 2007). Moreover, Yang and Tsai (1994) found nutritional components such as sugars, organic acids, free amino acids, vitamins and micronutrients in mulberry. Thus, it has significant importance inhuman health. Moreover, Nomura et al. (1976) found that the parts of mulberry such as twigs, leaves, fruits and root bark contain phenolic compounds which can be used to treat various diseases. The mulberry contains functional components such as phenolic, flavonoids and ascorbic acid (Bae and Suh, 2007) which add both interest and value to it. It also contains anthocyanin. According to Kim and Lee (2020) major anthocyanin component found in mulberry are cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside. Due to the presence of the ascorbic acid, flavonoids and anthocyanin, mulberry possess antioxidant characteristics. Besides, anti-microbial, anti-inflammatory and anti-cancer (Butt et al*.*, 2008) and antiradical (Suh et al*.*, 2004) properties are also found in mulberry fruit extract. In addition, Mattivi et al. (2006) and Mcdougall et al. (2005) reviewed anti-atherosclerotic, anti-carcinogenic, and anti-inflammatory properties in mulberry. These significant therapeutic qualities in mulberry may be one of the reasons for its consumption in various possible forms of food and beverages.

The raw materials, environment, temperature, yeasts, different processing techniques, fermentation and aging have significant impact on the physical, chemical and organoleptic properties in wine. Iland et

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al. (2000) considered pre- fermentation, fermentation and post fermentation as vital processes in wine making. For example, Wang et al. (2020) experiment on apple juice extracted by three different mechanical juice extraction methods such as squeezed juice, spiral juicer and broken juice have greater impact on the quality of apple juice. The quality of juice depends upon parameters such as pressing time, flow rate, pressure and heat. Moreover, the processes such as crushing, pressing and filtration can affect the yield, texture and quality of juice. The proper mashing and filtration results in less viscous juice. One of the challenging part in making mulberry wine is high viscosity of juice. The fruits contain fibre of 11.75 ± 1 g/100 g fresh weight (Imran et al., 2010) making juice thicker and syrupy resulting in prolonged filtration time. The quality, time and cost due to extraction may add or reduce the value to winemaking. Chen et al. (2015) suggested that there are lesser information on influence on the quality of fruit and vegetable juice due to processing technologies. Thus, there may be a debate on the quality of fruit juice extracted from different methods. Perez-Cacho and Rouseff (2008) stated that juice processing methods can affect the juice flavour. According to Nadulski et al. (2017) depending on raw material and operation type, different juice extraction techniques are applied. Hydraulic, basket, belt and screw press are popular in food industry for pressing (Jaeger et al., 2012). Cautela et al. (2010) stated that the juice processing method can affect the phytochemical content. However, less information about various wine making techniques and their comparative study in terms of mulberry wine can be found till date

In one of the recent study done by Chang (2020) on cold pressed mulberry wine, the temperature for fermentation of wine has significant impact on physiochemical properties. The same study showed that the alcohol production is faster in room temperature (30±2°C) whereas the higher amount of phenolic (2319.44±2.94 mg/L cyanidin-3-glucoside) was obtained in lower temperature (12±2˚C). Therefore, temperature can also play an important role affecting the phenolics. The cold pressed mulberry wine can have high phenolic contents.

Aging of wine can be another factor that has great impact on its chemical composition. Balga et al. (2015) specified that the phenolic compounds undergo various chemical reactions during aging. The alteration in phenolic affects the quality of wine. Somers (1971) expressed that the phenolic play an important role in the taste and color of wine during maturation, aging and storage. Maturation and aging of wine can change the taste, aroma, texture and flavour of a wine due to the chemical reactions among sugar, phenolic and acids. The wine become smoother creating the complex aroma and taste.

The objective of this study is to analyse and compare the non-aged and 4-month aged mulberry wines which were prepared from three different wine making techniques.

2 MATERIALS AND METHODS

2.1 Wine Preparation

The fresh and ripe mulberry (*Morus alba* Linn.*cv*. Chiang Mai 60) fruits were sorted and directly proceed for hand pressed and cold pressed juice. The juice was prepared using three different processing techniques namely manual mashed mulberry juice without pulp (HPJ), manual mashed mulberry Juice with pulp (HPP) and cold-pressed mulberry juice without pulp (CPJ). HPJ and HPP were hand mashed. The difference between the two treatments was filtration of pulp in HPJ whereas pulp was included in HPP. However, CPJ was prepared using cold press machine (Slow juicer Tefal model ZC150838). Each treatment was then added with distilled water in the ratio 4:1 (v/v) (juice: water). The pH was adjusted to 3.7 using acid blend (citric: malic = 1:1.5 w/w). The ˚Brix was adjusted to 22˚ with granulated cane sugar. Then the pectinase enzyme (L.D. Carlson, OH, USA) was added (0.67 g/L), followed by addition of potassium meta-bisulphite (KMS) (150mg/L). After 10-12 hours, *Saccharomyces cerevisiae* (Premier Rouge UCD#904) was added ($7.5x10^6$ cell/ml) along with 2 g/L diammonium phosphate (DAP). After complete fermentation (6 days), all treatments of wines were filtered, bottled and aged for 4 months at 12±2 ˚C. All treatments were sampled after completed fermentation and 4-month aged then centrifuged (3000 rpm for 2 min) and transferred supernatant into new tubes and subsequently frozen at -20˚C until analysis.

2.2 Chemical Analysis

The chemical compositions of each sample were analyzed in triplicate. The pH was analyzed using the pH meter (pH 211 Microprocessor pH meter, China). The alcohol content was measured using Ebulliometer (160000-complete traditional Ebulliometer, Laboratories Dujardin Salleron^{TM}, France). The official method of analysis (AOAC 926.12, 1990) was used to measure total titratable acidity (TTA) and expressed percentage of citric acid. The 3, 5-dinitrosalicylic acid (DNS) assay (Miller, 1959) was followed to measure the amount of reducing sugar in all wine samples which was expressed in g/L.

2.3 Total Phenolic Content (TPC)

TPC was quantified using Folin-Ciocalteu micro method (Waterhouse, 2002). Wine sample (0.02 mL) was added to 1.58 mL distilled water. To it, 0.1 mL F-C reagent was added. After 5 min, 0.3 mL 20% sodium carbonate (Na_2CO_3) was added, mixed well then incubated for 1.5 hours at 30±2 ºC. The absorbance was read at 765 nm in triplicate. TPC was calculated from the calibration curve and expressed as mg/L of gallic acid equivalent.

2.4 Total Flavonoids Content (TFC)

TFC was determined using aluminium chloride colorimetric method (Ivanova et al., 2010, Zhishen et al., 1999) in triplicate. Wine sample (1 mL) was mixed with distilled water (4 mL) in a tube. Then 0.3 mL of 5% sodium nitrite ($NaNO_2$) was added and rested for 5 min at room temperature. Then 0.3 mL of 10% aluminium chloride ($AlCl_3$) was added and reacted for 5 min. Then 2 mL of 1M sodium hydroxide (NaOH) was added and the total was made up 10 mL with distilled water. The solution was mixed well, and the absorbance was read at 510 nm in triplicate. TFC was calculated from the calibration curve and expressed as mg/L of Rutin equivalent.

2.5 Total Anthocyanin Content (TAC)

TAC was determined in triplicate by using the pH differential method described by AOAC Official method 2002.02. TAC was determined as mg/L of cyanidin-3-glucoside equivalent according to the following equation (1):

$$TAC = (A \times MW \times DF \times 10^3) / (\varepsilon \times L) \quad (1)$$

Where A is absorbance [(A520nm – A700nm) pH 1.0 – (A520nm – A700nm) pH 4.5]; MW is the molecular weight of cyanidin-3-glucoside (449.2 g/mol); DF is the dilution factor (100); L is the path length in cm (1); and ε is the molar extinction coefficient of cyanidin-3-glucoside (26900 L/mol cm).

2.6 Antioxidant Content

Antioxidant was determined by using DPPH radical scavenging method described by Šimić et al. (2017). The DPPH radical scavenging activity is calculated in terms of percentage inhibition of DPPH activity according to the following equation (2);

$$\% \text{ inhibition} = [(AC - AA) / AC] \times 100 \quad (2)$$

Here AA and AC are the absorbance values of the samples and the control, respectively. The percentage inhibition of DPPH activity of Trolox solution was also tested. The calibration curve was constructed with the Trolox solution concentrations versus the percent inhibition of DPPH activity. The antioxidant capacity of the samples were quantified and expressed as Trolox equivalents mg/L from the calibration curve.

2.7 Color Measurement

The absorbance of the wine samples at 420, 520, and 620 nm was measured directly by use of a spectrophotometer with an optical path length of 10 mm. The color intensity was calculated as the sum of the absorbance at 420, 520, and 620 nm ($A_{420} + A_{520} + A_{620}$). Hue was obtained as the ratio of absorbance measured at 420 and 520 nm (A_{420}/A_{520}).

2.8 Statistical Analysis

All the physicochemical parameters of non-aged and 4-month aged wines were carried out in triplicates. All the data were analysed using Statistical Analysis System (SAS) program version 9.4 (SAS Institute, Cary, NC and USA). The statistical difference between the means was evaluated using least significant difference (LSD) test. T-test was used to compare the difference in the mean between each parameter of non-aged and 4-month aged wines.

3 RESULTS AND DISCUSSION

3.1 Chemical Compositions

The chemical composition of mulberry wines prepared by three different wine making techniques (HPJ, HPP, and CPJ) was determined before and after 4 months aging period (Figure 1). The HPJ has given significantly higher alcohol content in all three treatments. HPJ and CPJ contained only juice making them lesser viscous than HPP. In this study, the alcohol content in HPJ with lesser viscous fluid is higher. The amount of sugar was well utilized by *Saccharomyces cerevisiae* in HPJ and CPJ resulting in higher alcohol content in them. According to Abdullah et al*.* (2007), the use of pectinase aid in the hydrolysis of soluble polysaccharides (high viscosity) to soluble sugars and short chain (low viscosity).

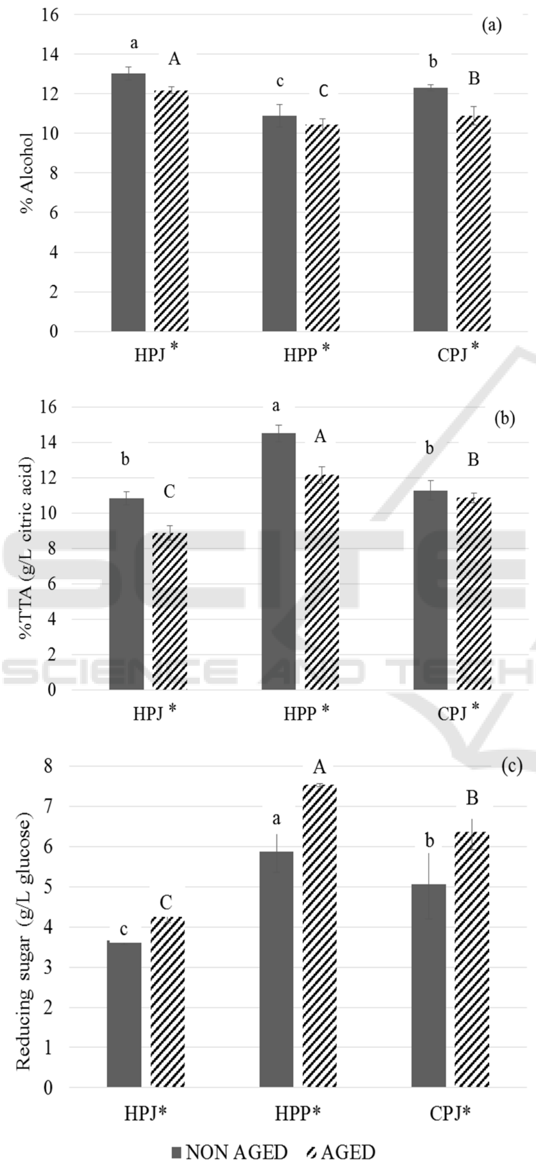

Figure 1: The changes in chemical composition of non-aged and 4 months aged mulberry prepared from three different wine making techniques (HPJ, HPP and CPJ).: (a) alcohol content, (b) acidity (TTA) percentage, and (c) reducing sugar. The data are expressed as mean ±SD (n=3). The different letters indicate significant difference ($p < 0.05$). '*' represents the significant difference.

However, the alcohol percentage significantly decreased [Figure 1(a)] during 4 months aging in all three treatments. During fermentation or aging of wine, the ethyl esters formation such as ethyl esters of lactic, malic and tartaric acid occurs developing odor and taste of wine (Shinohara et al*.*, 1979). These might be the reason for decrease in the percentage of alcohol in all three treatments after 4 months aging.

When the acidity of all three treatments were observed [Figure 1(b)] in 4-month aged mulberry wine, the result showed significantly reduction in the acidity in all treatments. Shinohara et al. (1979) stated that during aging, ethyl esters acid are formed rapidly. The acid in wine reacts with alcohol to form esters. Ancin-Azpiicueta et al. (2008) stated that during aging the ethyl esters of organic acid increased leading to decrease in acidity.

The reducing sugar content [Figure 1 (c)] of the three treatments was compared. HPP had significantly higher reducing sugar. Comparatively, HPJ had the least reducing sugar content (3.66 g/L glucose). This indicated that the most of the sugar was well consumed by yeast and converted to alcohol in HPJ. However, after 4-month of aging, the reducing sugar content was increased in all the treatments. As stated by Butt et al. (2008), mulberry fruit contains a good amount of proteins, carbohydrates, fats, fiber and vitamins. Carbohydrates from mulberry may have been remained in the wine especially in the HPP treatment. Chapman et al. (1991) stated that pectin in fruits can be hydrolyzed into free sugars by a pectinase enzyme. As the pectinase enzymes has been added to the fermentation from the beginning, remaining activity from the pectinase could have slowly released these sugars into wine cause the increase in reducing sugar during 4-months aging.

3.2 TPC

The TPC of all the treatments were analyzed [Figure 2 (a)]. The results showed that there was a significant difference ($p < 0.05$) between all the treatments. Mulberry contains phenolic and polysaccharides (Chen et al*.*, 2015) which might give higher phenolic content in HPP. The wine with filtered juice (HPJ) with absence of whole fruit had significantly lesser phenolic in it. Martinez-Lapuente et al*.* (2017) stated that winemaking step such as filtration causes decrease in wine polysaccharide content which is why HPJ had the lesser phenolic content. However, after 4-months aging, the decreased in TPC in all the treatments was observed. Mulberry is rich in phytochemicals such as phenolic compounds, flavonoids, anthocyanin and ascorbic acid (Bae and

Suh, 2007). According to Ribereau-Gayon et al. (2006), the chemical reactions such as thermal and oxidative degradation of anthocyanin, interaction of tannins with proteins and polysaccharides, polymerization of procyanidin and copigments and formations of anthocyanindins are the reasons for changes in level of phenolic compounds. Whereas Yıldırım et al. (2015) emphasized on the subsequent transformation of wine phenolic in bottled (i.e. anaerobic) condition where slow polymerization and condensation reaction occurs. This process can cause the decrease in TPC. Moreover, the similar reduction of phenolic in mulberry juice was seen in a study done by Zou et al. (2017). However, the reason for the reduction in the same study was the formation of sediments in mulberry juice during storage in which was not a case in this study as the sediments was not observed.

3.3 TFC

The TFC of the treatments were analyzed [Figure 2 (b)]. The HPP resulted in the higher flavonoid content (2785.24 ± 136.53 mg/L rutin) before aging. The fermentation with pulp in HPP may account for the higher flavonoid content due to the increasing time for extraction. The fruit of mulberry is rich in phytochemicals such as phenolic, flavonoids, anthocyanin and ascorbic acid (Bae and Suh, 2007). Thus, the addition of pulp in the juice may promote the higher flavonoids in wine. After 4-month of aging, the reduction in TFC was seen in all the three treatments. This result may show the importance of presence of pulp in the juice for the higher flavonoid content in wine.

The flavonoids and non-flavonoids are polyphenolic contents (Lachman et al., 2009) which get affected by factors such as pH, temperature and aging. The total flavonoid phenols tended to decrease with aging of wine. Furthermore, the flavonol, a major class of flavonoids in wine can react with sugar to form flavonol glycosides (Hertog et al., 1993). During wine aging the formation of flavonol glycosides occurs which undergo self-hydroxylation (Somers, 1971). High antioxidant properties and free radical scavenging characteristics are due to flavonoids (Scherer and Godoy, 2009) thus the degradation in TFC may affect the antioxidant content.

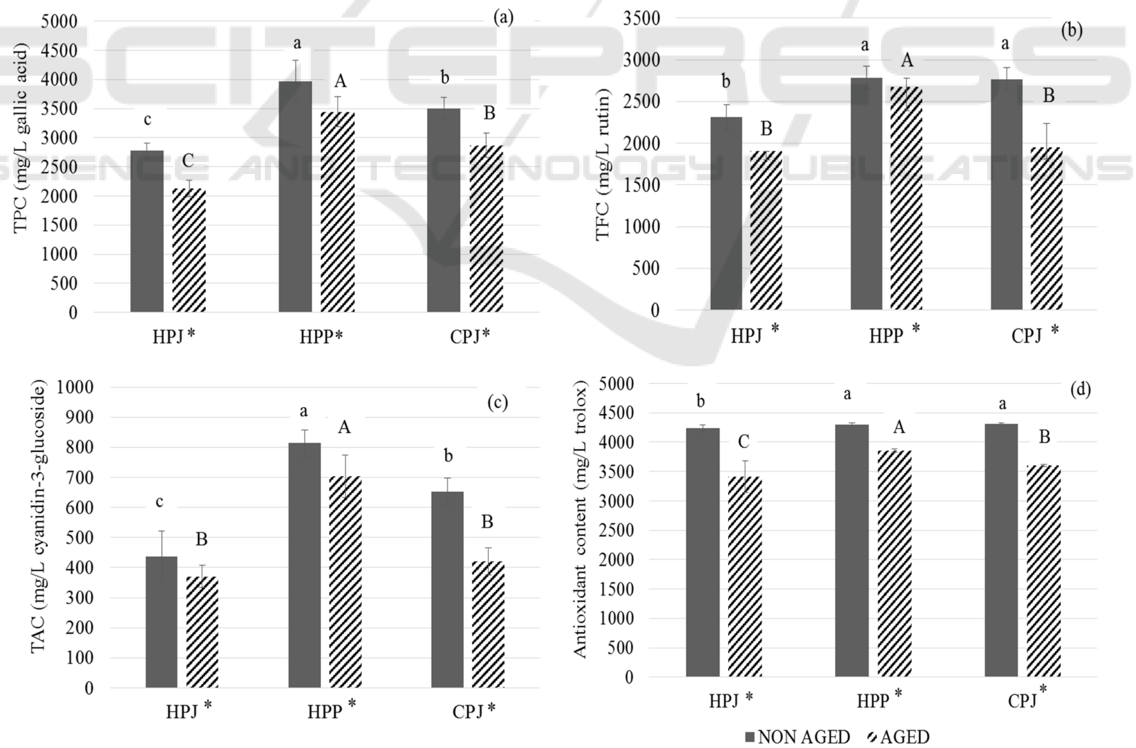

Figure 2: The changes in phenolic of non-aged and 4 month aged mulberry prepared from three different wine making techniques (HPJ, HPP and CPJ).: (a) total phenolic content, (b) total flavonoids content, (c) total anthocyanin content, and (d) antioxidant content. The data are expressed as mean ±SD (n=3). The different letter indicates significant difference ($p < 0.05$). '*' represents the significant difference between non aged and 4 months aged mulberry wines.

3.4 TAC

The comparative study of TAC in all the treatments [Figure 2(c)] showed that HPP had the higher anthocyanin content (813.79±44.73 mg of cyanidin-3-glucoside per L) before aging whereas HPJ had the lower TAC. According to Hunjaroen and Tongchitpakee (2010), the young mulberry has total anthocyanin content of 2.6-6.8 mg of cyanidin-3-glucoside. Chen et al. (2006) states that anthocyanin is an important constituent of mulberry fruit. Thus the presence or absence of pulp in juice affects the TAC in juice and wine. However, slight decrease in the TPC was observed in three treatments after 4-months aging. The TAC of non-aged wine showed significant difference ($p < 0.05$) with that of 4-month aged wines. The reduction may be due to the conversion of anthocyanin into non-pigmented compounds. The anthocyanin undergoes oxidative polymerization resulting in oligomeric and polymeric pigments which convert red wine color to brown (Somers, 1971). Wang et al. (2015) states that the anthocyanin possesses low stability and factors such as pH, temperature and oxygen can affect it. Anthocyanin are more stable at low pH resulting in red pigment (Wahyuningsih et al., 2017). In this study, the reduction in acidity during aging may alter the anthocyanin content as according to (Khoo et al., 2017), the anthocyanin pigments decreases when there is lower acidity. These can be the reason for anthocyanin reduction after 4 months aging.

3.5 Antioxidant Content

The analysis of antioxidant content of all three treatments was done [Figure 2(d)]. The antioxidant content of all three treatments ranged from 4297.54 to 4306.09 mg of gallic acid/L. According to Rice-Evans et al. (1996), polyphenolic components in higher plants give antioxidant activity. The presence of other functional bioactive components such as ascorbic acid (Ercisli and Orhan, 2007) and niacin (Imran et al., 2010) besides anthocyanins, phenolic and flavonoids might be the reason for similar amount of antioxidant content in all the treatments.

However, the decrease in all three treatments was seen after 4 months of aging. The abundant bioactive compounds in mulberry give it great antioxidant properties (Castrejon et al., 2008). The antioxidant capacity is due to phenolics (Wu et al., 2013) thus change in phenolics can change the antioxidant capacity. The decrease in antioxidant content is due to the reduction of phenolic content, the similar result can be previously seen in mulberry juice (Zou et al., 2017).

This lower antioxidant is corelated with the result of TPC, TFC and TAC presented earlier. The wine made with HPP demonstrated the highest amount of TPC, TFC, TAC and also antioxidant content which could be a contribution of longer pulp extraction time during fermentation.

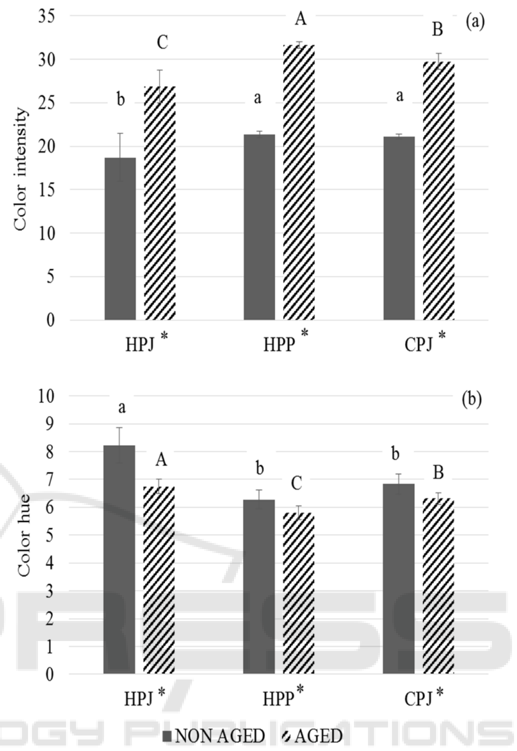

Figure 3: The changes in color measurement of non-aged and 4 month aged mulberry prepared from three different wine making techniques (HPJ, HPP and CPJ): (a) color intensity, and (b) color hue. The data are expressed as mean ±SD (n=3). The different letter indicates significant difference ($p < 0.05$). '*' represents the significant difference between non aged and 4 months aged mulberry.

3.6 Color Measurement

The change in the wine color measurement of three treatments was analyzed (Figure 3). The wine color intensity in HPJ, HPP and CPJ are found to be in range of 18.69 to 21.34 and, the wine color hue of the same treatments ranged from 6.28 to 8.23 before aging. Khoo et al. (2017) suggested that the anthocyanins are responsible for the colors of fruits, flowers and vegetables. Mulberry is dark red fruit. The wine color intensity and color hue measured after 4 months aging was found to be increased [Figure 3(a)] and decreased [Figure 3(b)] respectively. After 4 months aging, the color intensity of HPJ, HPP and CPJ were increased after aging (26.90, 31.64, and

29.75, respectively). This study showed that HPP has the highest color intensity before and after aging. However, the color hue of HPJ, HPP and CPJ were decreased after 4 months aging (6.75, 5.81, and 6.32, respectively). In both wine color intensity and hue, there found to be significant difference ($p < 0.05$) between aged and non-aged mulberry wines. HPP has the highest color intensity whereas HPJ has the highest color hue. Color intensity shows how dark the wine is whereas wine color hue measures the appearance of yellow and red color present in wine.

Anthocyanin is less stable and easily degrade by pH, storage temperature, light, oxygen, concentration, enzyme's presence, proteins and flavonoids (Rein, 2005). The structure of anthocyanin and pH has high effect on pigments of anthocyanin (Torskangerpoll and Andersen, 2005). According to Ummi et al. (2011), experiment on anthocyanin, when the acidity decreases, the color changes to purplish tones causing the decrease in color hue. Boulton (2001) suggested that anthocyanins can react with other molecules and convert into co-pigments and polymerized pigments. In one of the study done by Heras-Roger et al. (2016), the copigments decreases hue but increases color intensity in wine and the copigmentation increases A_{520} more than in A_{420}. This condition resembles with current study in which A_{520} had increased causing decrease in hue.

4 CONCLUSION

The comparison analysis of different treatments of mulberry wine before aging showed that the different wine making techniques affect the physicochemical characteristics of wine. Among all wine making techniques used, HPP and CJP method produced wine that have similar level of TFC, and color intensity which was higher than the wine prepared by HPJ method. The 4 months aging of mulberry wine at 12±2˚C resulted in increasing level of reducing sugar, and color intensity of wine. In contrast, the alcohol content, acidity, phenolic, and color hue was decreased after aged for 4 months. However, sensory analysis of wines will be worthwhile to perform in the future which will help to understand the sensory characteristic along with consumer preference of these wines from different wine making techniques.

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