Chemical Properties, Total Phenolic Content, Total Anthocyanin, and Antioxidant Activity of *Muntingia calabura* Fermented Vinegar Macerate with *Cannabis sativa* and *Mitragyna speciosa*

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Abstract

Muntingia calabura is a fruit found mainly in northeast of Thailand. The fruit is sweet and red when ripe. Processing into vinegar will encourage more utilization of the fruit. This objective of the study is vinegar production from *Muntingia calabura* wines, chemical properties total phenolic content, total anthocyanin, and antioxidant activity of fermented vinegar from *Muntingia calabura* that is macerated with 2 types of herbs: *Cannabis sativa* and *Mitragyna speciosa*. The first step made *Muntingia calabura* wine by using *Saccharomyces cerevisiae* (0.75% v/v of *Muntingia calabura* juice content) to produce alcohol. The second step made vinegar by inoculating *Acetobacter pasteurianus* 10 %(v/v) into *Muntingia calabura* wines which were adjusted alcohol content to 5.38 %(v/v), fermented at room temperature for 15 days. The results show that on the 5th day of alcoholic fermentation, the alcohol content was 6.45 %(v/v) and vinegar had acetic acid at 4.00 %(v/v). After that bring the herb (*Cannabis sativa* and *Mitragyna speciosa*) macerated in *Muntingia calabura* vinegar for 3 days. The highest antioxidant activity of *Muntingia calabura* vinegar macerated with Cannabis sativa was found to be 24.75 mg/mL. The highest total phenolic content and total anthocyanin of *Muntingia calabura* vinegar macerated with *Mitragyna speciosa* were 4.32 g/L and

50.94 mg/L, respectively. From this research, *Muntingia calabura* could be processed to make new, healthy products and further lead to income generation for people.

Keywords: *Mitragyna speciosa*, *Cannabis sativa*, *Muntingia calabura*, Fermented vinegar, Antioxidant activity

Introduction

The scientific name of the Jamaican cherry is *Muntingia calabura* L. family Elaeocarpaceae. It is the only plant species in the genus Muntingia. It is a perennial native to southern Mexico, Central America, the Caribbean, west of South America Bolivia and Argentina. It is imported into Southeast Asia and became a native plant. Found distributed throughout Thailand. *Muntingia calabura* is little fruit (average weight of 1.60 g) and red-colored at a mature stage and presents a sweet flavor, which fascinates birds. These ripe Jamaican cherries are very sweet due to the high content of soluble solids (10.24°Brix) and little total titratable acidity (0.11 g citric acid per 100 g fruit, pH 5.64). It contains high antioxidant properties suppressing human LDL (Low-Density Lipoprotein) and high content of soluble phenolic compounds, (Pereira et al, 2018). Hemp (*Cannabis sativa* L.) is a herbaceous annual dioecious plant notable for its particular spiky leaves from the Cannabaceae family. *Cannabis sativa* L. is a complex plant with more than 480 compounds that can be separated into diverse phytochemical classes, and the most studied class is the cannabinoids such as cannabidiol (CBD), cannabichromene (CBC), Delta-9 tetrahydrocannabinol (THC), and cannabigerol (CBG) (Drint'c et al, 2018). The ethanolic extract was remarked of the hemp in terms of terpenes (6.00 mg/mL), The monoterpenes were 48.76% of total terpenes (β-pinene, limonene, linalool, camphor, borneol, fenchol) while sesquiterpenes (cis-nerolidol, β-caryophyllene, α- eudesmol, bisabolol, α-humulene, and cedrol) and diterpenes (Phytol) were 47.65% and 3.59% respectively, The most plenty compound was β-pinene (28.17% of the total; 1.69 ± 0.45 mg/mL), followed by cedrol (14.5% of the total; 0.87 ± 0.07 mg/mL) and β-caryophyllene (12% of the total; 0.72 ± 0.06 mg/mL), phenolic compounds (1.80 mg gallic acid equivalents (GAE)/mL), cannabidiol (4.99% w/w),

antiradical (0.39 mg/mL) and metal ion-chelating activities (50% effective concentration (EC_{50}) of 2.47 mg/mL) (Cantele et al, 2020). *Mitragyna speciosa* is widely known as kratom in Thailand. *M. speciosa* leaf is revealed to possess opioid-like sedative narcotic effects. The bioactive of *Mitragyna speciosa* such alkaloids, indole, triterpenoids, flavonoids, saponins, and glycoside, this plant has been presented with numerous biological properties such antibacterial, antioxidant, anti-inflammatory, antiproliferative, and antinociceptive (Goh et al, 2021). Vinegar is rich in nutrients including amino acids, vitamins, sugar, organic acids, and polyphenols, they comprise bioactive and exhibit anti-obesity, antioxidant, antimicrobial activities, and antidiabetic. (Ozdemir et al., 2022)

Currently, *Cannabis sativa* and *Mitragyna speciosa* are the plants that people are attracted to. Because the whole plant 2 species used to be classified as narcotics. Therefore, this research is interested in using these two plants in the production of *Muntingia Calabura* vinegar.

The overall objective of this research was to develop fermented vinegar from *Muntingia calabura* fruits. This research aimed 1) to produce fermented vinegar from *Muntingia calabura* fruits, 2) to study the chemical properties, total phenolic content, total anthocyanin content, and antioxidant activity of fermented vinegar which macerated with *Cannabis sativa* and *Mitragyna speciosa*.

Methods

Chemicals and reagents

All the reagents and solvents utilized during the experiment were analytical grades and buy from several suppliers. The gallic acid standard was provided by Fluka (Buchs, Switzerland). Folin-ciocalteau reagent was brought from Merck (Darmstadt, Germany). 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) was bought from Sigma–Aldrich (Steinheim, Germany). Potassium hydrogen phthalate from Panreac (Barcelona, Spain) and sodium carbonate from Univar (Downers Grove, IL, USA).

Raw materials

Muntingia calabura (Ripe fruits turn red stage), *Cannabis sativa* (dried leaves), and *Mitragyna speciosa* (dried leaves) were harvested in May 2021 at Mahasarakham province.

Muntingia calabura vinegar production

The vinegar fermentation process started with *Muntingia calabura* fruits were crushed and blended with water at a ratio of 1:1 (w/w) to prepare the juice. After total soluble solid content adjustment up to 21 °Brix by cane sugar.

The *Muntingia calabura* juice was pasteurized at 65 °C for 30 min. Alcoholic fermentation proceeded for 5 days at room temperature. The *Muntingia calabura* juice at 2 L was inoculated with *Saccharomyces cerevisiae* (LALVIN RC212) at a ratio of 0.75% (v/v). Preparation of yeast inoculum was applied by blending 5 g of yeast powder with 60 mL of warm water. At the finish of the fermentation process, the wine was pasteurized at 65 °C for 30 min. The vinegar production begins from the alcohol content of the wine was regulated to 5.4 % (v/v) and inoculated with *Acetobacter pasteurianus* TISTR 521 which was grown in 10% (v/v) glucose yeast broth. The fermentation was processed for 15 d at 30°C on a shaker (150 rpm) in a glass flask containing 135 mL of the *Muntingia calabura* wine. After fermenting vinegar for 15 days, the fermentation process was finished by pasteurization vinegar at 65°C for 30 min. After that, the *Cannabis sativa* (dried leaves) and *Mitragyna speciosa* (dried leaves) were soaked in *Muntingia calabura* vinegar. The ratio of 25 g of *Cannabis sativa* (dried leaves) and *Mitragyna speciosa* (dried leaves) per 125 mL of vinegar, the leaves were soaked for 3 days. (Cejudo-Bastante et al., 2013)

Chemical analysis

The *Muntingia calabura* wine and vinegar samples were filtered through a 0.45 µm filter prepared to be injected into the HPLC system. The analysis was operated on a Shimadzu HPLC-RID system (Shimadzu, Japan) consisting of a RID-10A refractive index detector and Shimadzu LC-20AD pumps. The analytical column was Aminex HPX-87H column (300 mm × 7.8 mm i.d., 9 µm, Bio-Raand Laboratories, Inc., USA) coupled to a cationic exchange precolumn (Bio-Rad Laboratories, Inc., USA). H_2SO_4 (5 mM) was applied as the mobile phase. The column temperature was operated at 45 °C. (Aguiar et al., 2005). A series of standard solutions (ranging from 0-16 % of fructose (w/v), glucose (w/v), alcohol (v/v) and acetic acid (v/v)) were provided. A standard curve with R2 greater than 0.99 was plotted, and then the concentration of the alcohol, sugar and acetic acid in the samples was quantified accordingly.

Antioxidant activity

The Antioxidant activities of the sample were analyzed by DPPH radical assay (Brand-Williams et al,1995) in which 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radical was used as a stable radical. In brief, 5 mL of 0.1 mM DPPH radical solution prepared in ethanol was added to 5 mL of each sample, and the solution was settled for 20 min at room temperature in the dark. After the reaction, absorbance was measured at 517 nm using a Shimadzu UV-1700 spectrophotometer (Shimadzu, Japan), and the DPPH radical scavenging activities were indicated as mg ascorbic acid equivalents in 1 mL of sample (mg/mL).

Total phenolic content analysis

The Folin-Ciocalteu method was utilized for the analysis of the total phenolic contents of the *Muntingia calabura* vinegar (Singleton et al., 1999). In Brief, 1 mL of sample was diluted with 9.5 mL of distilled water and was then blended with 0.5 mL of Folin-Ciocalteu reagent and 2 mL of 10% Na_2CO_3 solution. After 30-min reaction at room temperature, absorbance was measured at 765 nm using a Shimadzu UV-1700 spectrophotometer (Shimadzu, Japan). Results were shown as mg gallic acid equivalents in 1 L of the sample (mg GAE/L).

Total Anthocyanin contents

The analysis of total anthocyanin content (TA) was determined following the pH-differential method described in Phaya. M (2013), The pH values of the diluted samples were 1.0 (0.2 M, 2.98 g KCl in 200 mL distilled water) and 4.5 (0.1 M, 4.08 g $C_8H_5KO_4$ in 200 mL distilled water). The absorbance was measured at both the lambda max of the sample and 700 nm against a distilled water blank. The TA was shown as Cyaniding-3-glucoside equivalents in mg/L.

Statistical analysis

The data were carried out three times. The results were shown as the mean ± standard deviation (SD). The data were determined by one-way analysis of variance (ANOVA) with Duncan multiple range tests (DMRT) to measure the significance between samples. In all data, $p < 0.05$ was considered significant.

Results and Discussion

Chemical properties of the *Muntingia calabura* wines and vinegars

The *Muntingia calabura* wines produced via a 5-day alcoholic fermentation process using *Saccharomyces cerevisiae* as an inoculant were analyzed for their chemical compositions, and the results are presented in Figure 1. It was observed that the *Muntingia calabura* wine contained the highest alcohol content of 6.45 ± 0.01 %(v/v), which was similar to that (5 %(v/v)) detected in *Muntingia calabura* wines which were produced period time 9 days at a yeast ratio of 0.4%. (Minh et al., 2019). As given in Figure 1, glucose and fructose were completely depleted in wine samples on 3rd day of the fermentation. The *Muntingia calabura* fruits also contains other carbohydrates as a component such as sucrose, 1- kestose, maltopentaose, maltohexaose and maltoheptaose that it's could to use as substrates in alcohol fermentation. (Pereira et al., 2018). Usually, the fermentation time depends on the fruit used and its sugar content. It could also have resulted in the concentration of microorganisms, the sugar content, or the fermentation temperature. Similarly, alcoholic fermentation can be processed by spontaneous fermentation or using a starter culture, which also affects the period of the process and the properties of the wine product. (Luzón-Quintana et al., 2021). Originally, the strain RC212 was selected by the Burgundy Wine Board (BIVB) to extract and protect the polyphenols of Pinot Noir. In the information supplied by the manufacturer, it was claimed that wines fermented by RC212 had a good structure with fruity and spicy characteristics (Lallemand Inc., Montreal, QC, Canada) (Gustafsson et al., 2016). The *Muntingia calabura* vinegar produced via a 15-day acetous fermentation process using *Acetobacter pasteurianus* TISTR 521 as an inoculant was analyzed for its chemical compositions, and the results are presented in Figure 2. The vinegar showed a significant decrease in alcohol content as it was converted to acetic acid by acetic acid bacteria. The acetic acid bacteria are mesophilic obligate aerobes that can oxidize sugars, sugar alcohols, and ethanol to allow the production of acetic acid. (Ho et al., 2017). However, the alcohols were not completely depleted, in which at the end of acetous fermentation (0.75 %v/v). At the end of an acetous fermentation process, acetic acid content was 4.00 %(v/v), which was similar to that (4.62 %(v/v)) detected in rosehip

vinegar which was produced for a period time 60 days. (Ozdemir et al., 2022) which is close to the acetic acid content determined in Mulberry vinegar. (Boonsupa, 2019)

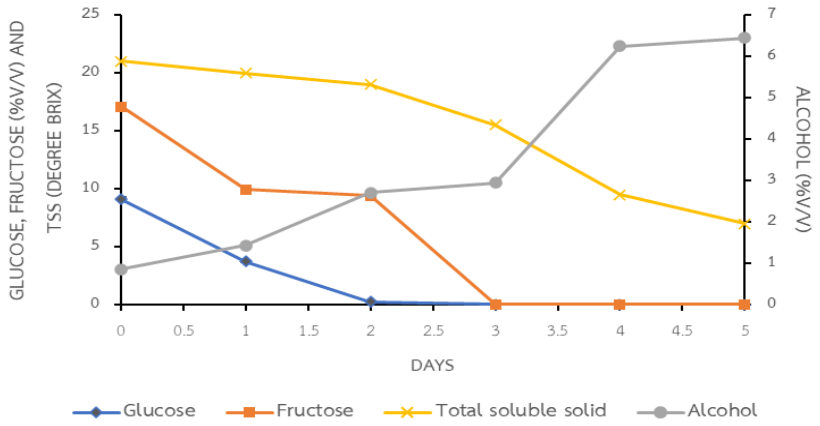

Figure 1 Chemical properties of *Muntingia calabura* wine during a 5-day fermentation process

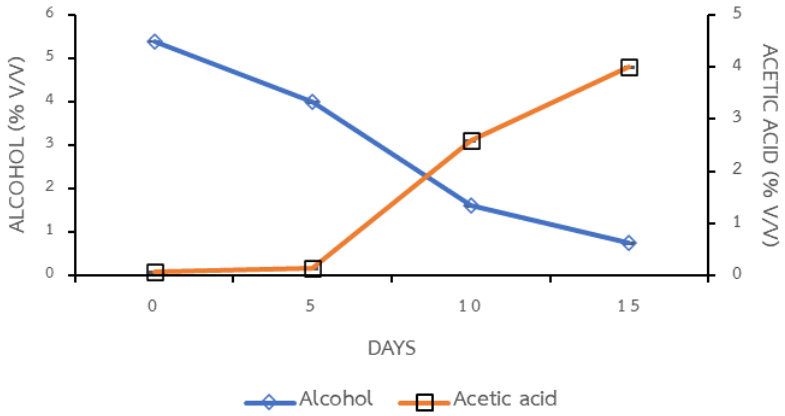

Figure 2 Chemical properties of *Muntingia calabura* vinegar during a 15-day fermentation process

Total phenolic contents, antioxidant activities and Total anthocyanin

The bioactive properties of vinegar could be varied in a wide range depending on the type of raw material. The differences in the antioxidant activities between wine and wine vinegar were attributed to their different phenolic contents and compositions

(Ozturk et al., 2015). Several researchers revealed bioactive compounds such as polyphenol-rich foods in the diet may cause protective effects against chronic diseases, such as cancer, and cardiovascular diseases which could be involved in their high antioxidant capacities, including the regulation of cell signalling pathways that were responsible for regulating a variety of enzymes involved in antioxidant defence system (Bakir et al., 2017). The maceration of herps (*Cannabis sativa* and *Mitragyna speciosa*) in vinegar, the herbs contained bioactive compounds that had a positive effect on health. Bioactive properties, namely total phenolic and total anthocyanin (TPC and TA) and DPPH radical scavenging activities of the wine and vinegar samples are shown in Table 1. The results showed that the *Muntingia calabura* wine exhibited the highest antioxidant activity of 80.58 ± 0.10 mg/mL which was greater than that produced from rosehip wine (13.11 mg/mL) (Ozdemir et al., 2022) and Indian pomegranate wine (32.58±0.68 mg/mL)(Boonsupa et al., 2021). The vinegar-soaked with *Cannabis sativa* was observed to show the highest antioxidant activity in all vinegar (24.75 ± 0.12 mg/mL) which was much less than that Chinese plum vinegar (25.83 mg/mL) (Boonsupa & Kerdchan, 2021) and Chinese pomegranate vinegar (28.67 ± 2.15 mg/mL) (Boonsupa et al., 2021). Mnekin and Ripoll (2021) studied the biochemical of *Cannabis sativa* that had the antioxidant activity substances such as delta-9 tetrahydrocannabinol, cannabidiol, cannabigerol, cannabinol, β-caryophyllene and β-myrcene. The total phenolic contents detected in the wine exhibited the highest total phenolic contents of 4.77 g/L which was much greater than that of Japanese cheery wine (1.68 g/L) (Ananda et al., 2012) and mulberry wine (0.45 g/L) (Boonsupa, 2019). The vinegar-soaked with Mitragyna *speciosa* was observed to show the highest total phenolic content in all vinegar (4.32 ± 0.08 g/L) which was much greater than that of citrus vinegar (1.38 ± 0.09 mg/mL) (Chen et al., 2017) and cranberry vinegar (250.02 ± 24.19 mg/L) (Boonsupa, 2019). The total anthocyanin contents detected in the wine exhibited the highest total anthocyanin content (53.19 ± 0.61 mg/L which was much greater than that of cheery wine (27.9 ± 1.3 mg/L) (Sun et al., 2011). The vinegar-soaked with *Mitragyna speciosa* was observed to show a higher total anthocyanin content than the vinegar-soaked with *Cannabis sativa* (50.94 ± 0.09 mg/L) which was much greater than that of strawberry vinegar (10.6 ± 0.9 mg/kg) (Ubeda et al., 2012). Vijayanand and Thomas (2018) studied

the phytochemical in *Muntingia calabura* fruits, the result revealed the presence of six different phytochemicals which include terpenoids, flavonoids, saponins, tannins, reducing sugars, phenols, and carbohydrates. Ahmed et al. (2018) studied the phytochemical in *Cannabis sativa* leaves which were found in alkaloids, glycosides, terpenoids, flavonoids, flavones, steroids, tannins, phenols, and saponins. León et al. (2009) studied the phytochemical characterization of the leaves of *Mitragyna speciosa*, the results showed that *Mitragyna speciosa* leaves had alkaloids, flavonoid epicatechin, a saponin daucosterol, the triterpenoid saponins quinovic acid 3-O-β-D-quinovopyranoside, quinovic acid 3-O- β-D-glucopyranoside, and glycoside derivatives. From the alcoholic and acetous fermentation, all measured parameters decreased during the double fermentation process. Normally, acetous fermentation was related to a higher decrease in antioxidant activity than alcoholic fermentation. Ubeda et al (2012), reported decreasing content of total phenolic, total anthocyanin, and antioxidant activity in strawberry vinegar.

Table 1 The bioactive compound contents and the antioxidant activity of the *Muntingia calabura* wine and vinegar macerate with *Cannabis sativa* and *Mitragyna speciosa*.

Sample	TPC; Total phenolic compound content (g/L)	TA ; Total anthocyanin content (mg/L)	DPPH-radical scavenging activity (mg/mL)
wine	4.77 ± 0.00[a]	53.19 ± 0.61[a]	80.58 ± 0.10[a]
vinegar	4.03 ± 0.17[b]	51.35 ± 0.28[b]	22.58 ± 0.08[c]
vinegar + *Cannabis sativa*	3.55 ± 0.15[c]	49.66 ± 0.27[c]	24.75 ± 0.12[b]
vinegar + *Mitragyna speciosa*	4.32 ± 0.08[b]	50.94 ± 0.09[b]	22.13 ± 0.06[d]

Values with various letters in the same column are significantly different according to Duncan's multiple range test ($p < 0.05$)

Conclusion and recommendations

This study was conducted to examine the levels of acetic acid, total phenolics, total anthocyanin, and antioxidant activity of the *Muntingia calabura* fermented vinegar macerated with *Cannabis sativa* and *Mitragyna speciosa*. The results showed that the vinegar-soaked with *Mitragyna speciosa* had higher total phenolics and total anthocyanin than the vinegar-soaked with *Cannabis sativa*. On the other hand, the vinegar-soaked with *Cannabis sativa* had higher antioxidant activity than the vinegar-soaked with *Mitragyna speciosa*. In this experiment, soaking vinegar with *Cannabis sativa* leaves increased the antioxidant activity. This will help develop new products that are beneficial to health in the future.

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