VIETNAM NATIONAL UNIVERSITY-HOCHIMINH CITY
INTERNATIONAL UNIVERSITY (IU-VNU)

STUDY ON TEA PROCESS CONDITIONS FOR TOTAL PHENOLIC CONTENT EXTRACTION OF MULBERRY LEAVES (*MORUS ALBA* L.)

A thesis submitted to
The School of Biotechnology, International University
In partial fulfillment of the requirements for the degree of
B.Eng in Food Technology

Student's name: Luyen Ngoc Do Quyen -ID: BTFTIU14129

Supervisor: Assoc. Prof. Le Hong Phu

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ABBREVIATIONS

ISO	International Organization for Standardization
TCVN	Vietnam Standard
AACC	American Association for Clinical Chemistry
AC	Antioxidant capacity
SSC	Soluble solids content
TPC	Total phenolic content
MC	Moisture content
RT	Room temperature
DPPH	2, 2-diphenyl-1-pycrylhydrazyl
GAE	Gallic acid equivalent

STUDY ON TEA PROCESS CONDITIONS FOR TOTAL PHENOLIC CONTENT EXTRACTION OF MULBERRY LEAF (*MORUS ALBA* L.)

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ABSTRACT

A trend of using leafy herbal tea has been spreading over recent years. In addition, the consumption of mulberry leaves (*Morus alba* L.*)* is on an increase as it is a good source of antioxidants due to its relatively high content of that compound. Therefore, the objective of this study was to propose a tea process made from mulberry leaves based on total phenolic content. Methods used in this research were Folin-Ciocalteu reagent for the determination of total phenolic content and DPPH assay for the determination of antioxidant capacity. Withering duration (0,3,6,9,12,15,18,21 and 24h), temperature and duration of incubation (25, 30, 35 and 40^{o}C within 0, 3, 6, 9, 12, 15, 18, 21 and 24h) as well as drying temperature (60, 90, 120 and 150oC) were examined to select the optimum conditions for reserving total phenolic content (TPC). As the result, the highest yield of total phenolic content of mulberry tea was 25.22 ± 0.35 mg Gallic acid equivalent/ g dry weight, which had been achieved by 12h of withering, 1h of fermentation at 25^{o}C and drying at 150^{o}C. Antioxidant capacity of final product (49.84± 0.38 %) was recorded along with moisture content, ash content and sensory evaluation. This study contributed to both food industry and science as it proposed a complete process of mulberry leaf tea for the current needs.

Keywords: antioxidant capacity, mulberry leaves, tea, total phenolic content.

1. INTRODUCTION

Tea is one of the most widely consumed beverages worldwide, second only to water (Muktar & Ahmad, 2000). After plucking, tea leaves are treated by series of processes called withering (removal of moisture by air flow), curling, which from then, tea leaves are subjected to either drying or further fermentation process. During this fermentation stage, the leaves change the grassy smell gets transformed to floral smell, then, finally, drying is applied to complete the whole tea process (Bhattacharyya, et al, 2007).

Herbs are mainly consumed in the form of tea, an infusion of dried herbs in warm or hot water, brewed from the leaves, flowers, seeds, fruits, and roots of plant species (Aoshima, Hirata, & Ayabe, 2007). Leafy herbal teas (LHT) are widely known to contain a variety of active phytochemicals with biological properties that promote human health and help reduce the risk of chronic diseases such as allergies, insomnia, headaches, anxiety, intestinal disorders, depression, and high blood pressure (Craig, 1999). Major studies have reported that LHT extracts exert beneficial effects on lifestyle-related diseases due to their anti-carcinogenic, anti-atherogenic, chemo-preventive, antioxidant, and antimicrobial activities (Si, et al, 2006). Due to diversified forms of tea products, the vision of science and technology explore deeper the chemical and metabolic processes, which still needs to trigger. At moment, there is huge interest in determining the total phenolic contents and antioxidant capacities of diets. Many herbs and tea have been used to make infusions, and the term "rich in antioxidants" describes such infusions. However, scientific evidence needs to be updated (Sinija, et al, 2007).

In this research, mulberry leaves are used as an input of tea process to examine its total phenolic content during the tea processing. Mulberry belongs to the *Morus* genus of the *Moracea* family. It is recognized as serrate shapes of leaves, which distributed alternatively on a plant body Mulberries are cultivated and grow in the wild across warm, temperate regions in Southern Europe, the Middle East, Northern Africa, the Indian subcontinent, East Asia and the Americas. It is estimated that there are around 10 to16 species of genus *Morus* found in Asia, Africa and North America (Pérez-Gregorio, et al, 2011). Mulberry has been cultivated traditionally in most Asian counties including China, Korea, Japan and Vietnam to use leaves to feed silkworms (Bombyx *mori* L.) or as Chinese herbal tea according to folklore. Mulberry leaf has been used traditionally medical treatment for anti-diabetes, anti-

hyperlipidemics, reducing high blood pressure, high cholesterol and neutral fat (Assy, et al, 2000; Steinberg, et al, 1989). Previous literature confirmed that mulberry leaf has anticancer effects and inhibits hyperglycaemia (Zhou, et al, 2001). These leaves have been being consumed in Korea and Japan for diabetes patients (Katsube, et al, 2006) and are also used as a nutritional addition in noodles, cakes and tea.

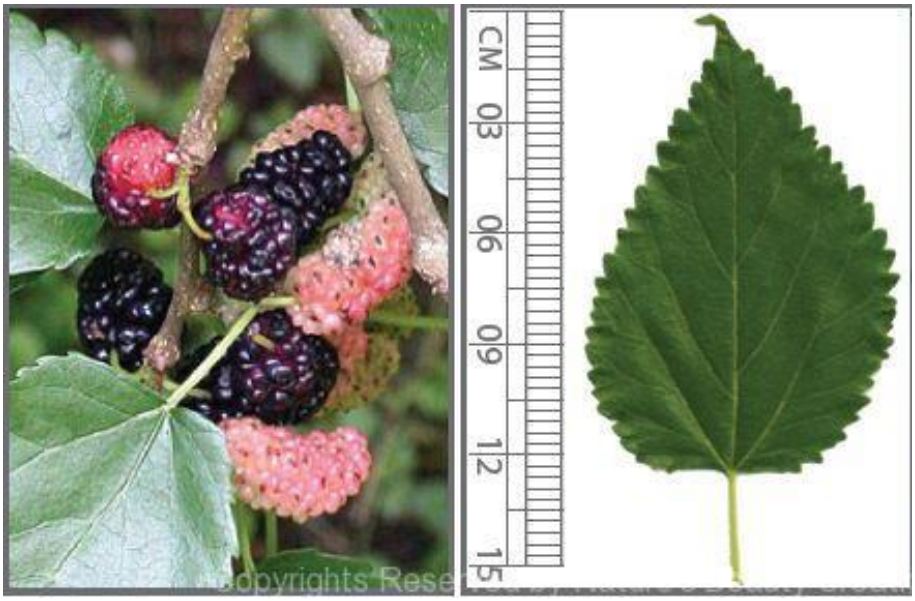

Figure1.1: Mulberry leaf (Morus alba* L.*)

Supplies for the European market mainly come from cultivated sources. The country with the largest area of mulberry is China with approximately 626,000 ha. Several other countries, such as Thailand and Brazil (35,000 ha), still have some mulberry production but on a much smaller scale (Sánchez, M. D., 2002). In Vietnam, survey results (conducted by the Center of Silkworm Research) show that the total area of planting mulberry (*Morus* spp.) trees had been 8,185 ha by the end of 2015. Nowadays, mulberry is cultivated mostly in Central Highlands in which this plant accounted for 49.69% cultivated area in a whole country. Moreover, the productivity of leaves is estimated nearly 20 tons per ha annually. One kilogram of leaves may range from 8,000 to 20,000VND, which is an affordable material for a large scale production.

According to Europe Centre for the Promotion of Imports from developing countries, mulberries are considered as functional foods on the European market. There is an increase for demand of mulberry fruits. Recently updated researches on obesity in the field of food science have indicated on the search for functional food ingredients or herbal extracts that can suppress the

accumulation of body fat (Carling, 2004). Plus, leaves and their extracts have potential in the market for food supplements, because of they are well-known for high level of antioxidant properties and nutritional composition. Weight management is becoming legitimately important as it leads to be healthier and prevent illnesses. Since the World Health Organization estimates that 30–70% of the European population is overweight and 10–30% is obese, this opens up opportunities for weight management products, such as mulberry leaves. Based on their potential to balance blood sugar levels, they are marketed as weight management products. Industry sources also indicated that prices for mulberry leaf extracts are up to around 20 Euro (nearly 550,000 Vietnam Dong) per kg.

Figure1.2: A typical Mulberry leaf tea product on Vietnam market

Plant secondary metabolites such as polyphenols, play an important role in the defense against free radicals. Phenolic substances are a category of phytonutrients that exhibit strong antioxidant properties (Ho, 1992). They can be classified into simple phenols, phenolic acids, hydroxycinnamic acid derivatives and flavonoids. The ability of some of the phenolic substances to act as potent antioxidant components has been reported (Velioglu, et al, 1998; Kahkonen et al, 1999). With regards to scientific trends, many researches have shown the potential of antioxidant, antiviral, anti-inflammatory, hypolipidemic, anti-hyperglycemic, neuro-protective (Pan, et al, 2008), anti-HIV, anti-hypotensive and cytotoxic activities of different species of *Morus* (Du, et al, 2003). Mulberry leaves have significantly noticed since it demonstrated strong

antioxidant properties in rice bran oil and inhibition of oxidative deterioration of oil was observed to be more efficient comparing to synthetic antioxidants (Roy, et al, 2010). Some reports have contributed these most important features related to the many phytochemical constituents that are present in mulberry leaves (Lin, et al, 2009).

Several studies also demonstrated mulberry leaf has potential antioxidant activity (Kim, et al, 1999; Arabshahi-Delouee & Urooj, 2007; Katsube et al., 2006). Polyphenols, found widely in many plants, maintain effective functions in lowering lipid and antioxidant effect. Thus, the single-ring type of polyphenol compounds (Gallic Acid, GA) and multi-ring type of polyphenol compounds (rutin) can be used to determine the standard content of total polyphenol. Previous studies have shown that mulberry leaf extract is rich in polyphenols and can effectively inhibit vascular smooth muscle cells proliferation and migration (Chan et al., 2010; Yang et al., 2011).It contains several functional components, including flavonoids, which are known to be powerful polyphenols and anti-oxidants (Oliaro-Bosso, et al, 2009; Chang, et al, 2013)

Furthermore, the presence of rutin, quercetin, isoquercetin and other flavonoids in fresh mulberry leaves has been detected in recent years, (Zhishen, Mengcheng, Jianming, 1999). Levels of total phenolic content in mulberry leaves from different mulberry based on cultivars ranged from approximately 1 to 1.8 g% per dry weight (Lee, et al, 2012). Three flavonol compounds (rutin, isoquercitrin and astragalin) are identified as main phenolic constituents. Among the three flavonol compounds, isoquercitrin showed the highest content (5.68 mg/g) followed by rutin (3.1 mg per g) and astragalin (2.4 mg per g) (Kim, D. S., et al, 2014). Nutrition constitutions of mulberry leaves have been reported scientifically. Proteins, carbohydrates, calcium, iron, ascorbic acid, β-carotene, vitamin B1, folic acid and vitamin D are contained in mulberry leaves (Bose, 1989). Mulberry leaves are rich in protein and amino acid. The silkworm eats exceptionally mulberry leaves to make its cocoon, releasing silk. It is known that there is high correlation between leaf protein and production efficiency of cocoon shell (Benavides, J. E., 2002).While using as animal and insect feed, mulberry leaves have been shown to contain medicinal properties such as diuretic (causing an increase in the flow of urine), hypoglycemic (lowering the blood sugars) and hypotensive activities (Kelkar, et al, 1996).

Even mulberry leaves are a rich source of nutrition and antioxidant content, little is known about processing with multiple by-products of this plant.

Typical derived mulberry leaf products currently available on Vietnamese market include dried leaves, tea and powder made by Lam Giang Silk Co.,Ltd and Vietnam Center of Silk worm Research. In addition, its quality in terms of antioxidant characteristics and physical parameters of processed mulberry leaves remains unknown. As a result, there is a need to discover these characteristics of mulberry leaves in processing.

With the aim to increase the commercial value of mulberry leaves, the project: "Study on tea process condition for total phenolic extraction of mulberry leaf (*Morus alba* L.)" was proposed to research on a laboratory scale to complete a mulberry leaf tea process with a highest yield of total phenolic content as well as a fundamental foundation in the utilization of raw mulberry leaves into convenient beverage products for future market.

2 MATERIALS AND METHODS

The experimental studies were conducted in laboratories (LA.101, LA.601 and LA.602) of the Department of Food Technology, International University – Vietnam National University in Ho Chi Minh City.

2.1 Materials and equipment

Chemicals used in analysis included: 1, 1-diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu's phenol reagent bought from Meckmilipore Company; Distilled water and Gallic acid taken from laboratories; Sodium Carbonate was purchased from local Chemical for laboratory agents, Hoa Nam Company, district 10, Vietnam.

Fresh mulberry leaves (Morus *alba* L.) were harvested directly from a farm located in Lam Ha, Lam Dong province, Vietnam and transported to Ho Chi Minh city within a day. At the time of picking, all leaves were reached 6 months olds.

2.2 Experimental design

2.2.1. Sample preparation

Fresh mulberry leaves were collected, cleaned with tap water to eliminate residues of dust, sand, soil and other field damaged portion and other undesirable materials before use. Next, the cleaned mulberry leaves were lay on a tray with a thickness layer of 0.5cm^2 and withered at room temperature for a certain time needed for experimental analysis (section 2.2.3). A constant fan air

flow (900rpm, model Sharp PJT16GY) was applied while withering. The withered leaves were then curled by ands and went through fermentation process at RT (25^{o}C) for 2h. Drying stage is finally conducted at 120^{o}C, 20min. Samples were then stored in a desiccator for water extraction before going through further analysis.

To produce mulberry leaf tea, fresh leaves were processed under the fixed conditions including rolling, fermenting, and drying before reaching the final outcome. The tea sample completed was kept in a well-noted plastic bag and stored in the desiccator until being used for extraction.

2.2.2. Extraction

The water extraction, for measuring TPC and SSC, the final dried sample was weighed for 2g in a beaker, then 100 ml boiling water was poured directly to the beaker (sample: water ratio is 1:50) for 6 min (TCVN 5086). Moisture content was determined to calculate the dry weight of extracted sample. Wasted leaf tea was eliminated by the filtration. The extract obtained was aim for measuring TPC and AC.

2.2.3. Effect of withering duration on TPC of mulberry leaf tea

This experiment was conducted following Jabeen, et al, 2015 with a slight modification in withering time. In this experiment, , the treatments comprised of different withering duration (0, 3, 6, 9, 12, 15, 18, 21 and 24h) at a clocked room, at ambient temperature where a constant fan airflow was applied. After each certain timing, 200 g of each withered leaves were followed the procedure mentioned in section 2.2.1 to produce mulberry leaf tea. The sample was extracted, which was followed by the procedure mentioned in the section 2.2.2. TPC and AC were determined. The best result in withering time was chosen according to the yield of TPC.

2.2.4. Effect of incubation temperature on TPC of mulberry leaf tea

100 g of withered leaves obtaining the highest TPC in section 2.2.3 were rolled by hands at a fixed withering time and followed continually by the procedure mentioned in section 2.2.1. The controlled sample was dried immediately after rolling and no incubation (fermentation) was applied. Temperature that was conducted for incubation was respectively at room temperature (25), 30, 35 and 40^{o}C. Temperature, which was equal or higher

than 30°C, was monitored by an incubator. The process of tea making was conducted repeatedly, which was followed by the procedure mentioned in section 2.2.1 to produce mulberry leaf tea. TPC and AC were determined after the extraction noted in section 2.2.2. Data was collected and the highest result in TPC followed by incubation temperature was chosen for further experiments. This experiment were followed by Takeo, T., 1984 and Obanda, et al, 2001 with a modification.

2.2.5. Effect of incubation duration on TPC of mulberry leaf tea

From the results of previous experiments, the mulberry leaves were withered at a certain time. After rolling, the treatments comprised of different incubation time (0, 0.5, 1, 1.5 and 2h) at a fixed withering time and temperature that was obtained from section 2.2.3 and 2.2.4. The process was conducted repeatedly, which was followed by the procedure mentioned in section 2.2.1 complete the tea process. The sample was extracted to further analyze TPC and AC. The best result in fermentation time was chosen based on the highest TPC obtained. This experiment was modified slightly and based on Muthumani, T., & Kumar, R. S., 2007.

2.2.6. Effect of drying temperature on TPC of final tea product

The tea process was conducted repeatedly after fixing those prior steps in section 2.2.3, 2.2.4 and 2.2.5. The treatments comprised of different drying temperature (60, 90, 120 and 150°C) for 20 min**.** The sample was extracted to further analyze TP, AC as well as MC. This experiment was based on Pothinuch, P., & Tongchitpakdee, S. 2011, for fixing the drying time and Panchariya, et al, 2002, with an extension of temperature range.

2.3 Analytical methods

2.3.1. Determination of total phenolic content (TPC)

Total phenolic content was measured by using Folin-Ciocalteu method reported by Singleton et al., (1999) with some changes reported by Ratiya et al., (2014) using garlic acid as a standard. All test tubes were covered tightly with a layer of aluminum paper. Firstly, aliquots (1ml) of each extract (previously diluted 10-fold with distilled water) were added into test tubes followed by 1 ml of Folin-Ciocalteu reagent (diluted 10 times) and 4 ml of sodium carbonate (7.5g/100ml). Vortex was applied well to neutralize. The volume was filled up to 10 ml with distilled water. Then, the mixture was blended secondly by the vortex

mixer and incubated in the water bath for 45 min at 45°C for blue color development. The absorbance was measured at 765 nm by spectrophotometer. All samples were analyzed in triplicate. TPC was expressed as Gallic acid equivalent (GAE) in mg per gram of dry weight (mg GAE/g DW), according to the equation below:

$$P=\frac{C\times k\times V}{m}$$

Where P is the total content of phenolic content, mg GAE/g DW; C is the concentration of gallic acid equivalents calculated by the substitution of the sample absorbance into the calibration curve, mg/ml; V is the volume of the solvent used to extract the dried sample, ml; k is dilution factor; and m is dry weight of sample to be extracted, g.

2.3.2. Determination of antioxidant capacity by DPPH assay

Antioxidant activity was estimated using the DPPH (1, 1-diphenyl-2-picryl hydrazine) assay described previously by (Hung and Morita, 2008) with a slight odification, aliquots (0.1ml) of each extract were mixed with 3.9 ml of 0.15 mM DPPH in water. The absorbance at 517 nm was measured after 30 min of incubation at room temperature. The remaining DPPH free radical was etermined by absorbance measurement against water blanks. The percentage scavenging effect was calculated from the reduction of absorbance against control (DPPH radical solution in water without sample) using the following equation:

$$\text{Scavenging activity (\%)} = \frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}} \times 100XV$$

2.3.3. Moisture content

5g of tea sample were placed in a forced draft oven at 130°C until the total weigh was unchanged. Treatment samples before and after drying were weighed separately to calculate moisture content of sample. Determination of moisture content was based on AACC International 2010.

$$MC\ (\%) = \frac{mo-m1}{mo}$$

Where: m_o is the weight of leaves before drying

m_1 is the weight of leaves after drying

2.3.4. Measurement of soluble solids content (SSC)

The soluble solids content of tea was measured by a refractometer. Put 2-3 droplets of tea extract in to the machine and close the lid; press start button and wait until the temperature shrink down to 25°C to get the result. Rinse the machine with distilled water and tissues between each measurement. The results was expressed under ° Brix.

2.3.5. Measurement of ash content

The method is described based on AACC International 2010. Ash content is determined by furnace incineration, by firstly labeling and weighing the crucibles, the weighing out three grams of each of the sample into a porcelain crucible of known mass. Placing the crucible into muffle furnace at 550-600°C until the white matters could be seen. Kept the crucible in desiccator for the temperature to cool down and weight it. The ash content (%) was calculated by the formula:

$$\%\text{Ash (wet basis)} = \frac{m2 - m1}{mo} \times 100\%$$

Where: m_2 is the weight of crucible containing ash

m_1 is the weight of crucible

m_0 is the weight of initial sample

2.3.6. Sensory evaluation of final product

After fixing constant conditions of the processing for each stage, a complete mulberry leaf tea product was produced. The final product based on physicochemical parameters including soluble solids content, moisture content, ash were examined. Then sensory test was conducted by scoring method "5 points - 6 scales" based on TCVN 3218-2012 standard for 4 attributes of tea including appearance, color, taste and flavor. 60 trained panelists were invited to laboratory A1.601 for testing.

2.4 Statistical analysis

All measurements are conducted in triplicates and the statistical analysis (ANOVA) was performed by using SPSS version 22 and Excel 2013, on the level of significance $p < 0.05$.

3 RESULTS AND DISCUSSION

3.1 Effect of withering duration on TPC of mulberry leaf tea

Withering, which is also called partial desiccation, is the first significant stage for improving the quality of final tea. Immediately after plucking, the fresh leaf starts to lose water. As withering progresses, the stomata of the lower leaf surface begin to close (Orchard, 199 1; Kramer &Kozlowski, 1979). The picked leaves are usually spread in a series of either enclosed or open trough under forced air circulation (Baruah, 2003). For sensory evaluation, there is no clear trend of preference depending on chemical wither time. Thus, significant deterioration in tea processing at any wither timings has not been recorded (Owuor and Orchard, 1992). For the production of flavorful black tea, traditionally, chemical withering is known to be essential. Similar observations have also been observed for high quality clonal teas in Kenya (Owuor et al., 1987, 1989). In this study withering time was limited to 24h since it had been demonstrated that withering beyond 20 h impaired black tea quality (Owuor et al, 1990). Steps for conducting withering were mentioned previously in section 2.2.3.

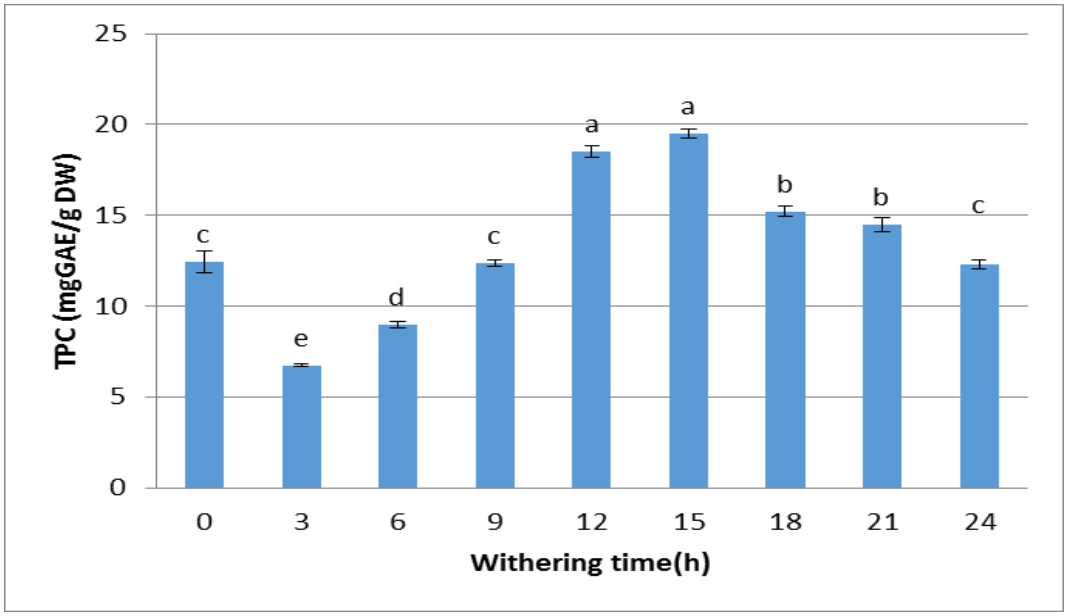

Figure 3.1: Effect of withering duration on TPC of mulberry leaf tea

Note: *All data was presented as means ± standard deviation. Means followed by different lower case letters were significantly different (p<0.05). The error bars are presented for standard deviation.*

Figure 3.1 showed the changes in TPC of mulberry leaves due to withering time, expressed in mg GAE/ g DW. There was a variation of TPC during withering, which indicated that wither time affected the TPC significantly. In the first period, after 3h of withering, there was a significant decline in TPC, from 12.43± 0.59^{c} to 6.74 ± 0.06^{e}. The amount of TPC started to increase gradually after 6h withering (8.96±0.17^{e}). TPC reached the highest at 12 and 15h of withering (18.5±0.30^{a} and 19.5±0.23 ^{a}, respectively). Since then, TPC started to decline. Between 18 and 21h of withering, there were no significant difference in the change of TPC (15.2±0.3^{b} and 14.5±0.4^{b}, respectively), which was then followed by a sharp decrease to 12.3 mg GAE/g DW for further 3h of withering, at 24h. With regards to AC, expressed in percentage, showed a similarity to the trend. Figure 3.2 indicated a steadily decrease in AC within the first 3h period (from 36.24±0.22^{e} to 14.5±0.54^{g}), then it jumped until reaching its peak at 12 and 15h (78.6±0.8^{a} and 78.5±0.9 ^{a}, respectively) and from this, it started to decline gradually afterwards.

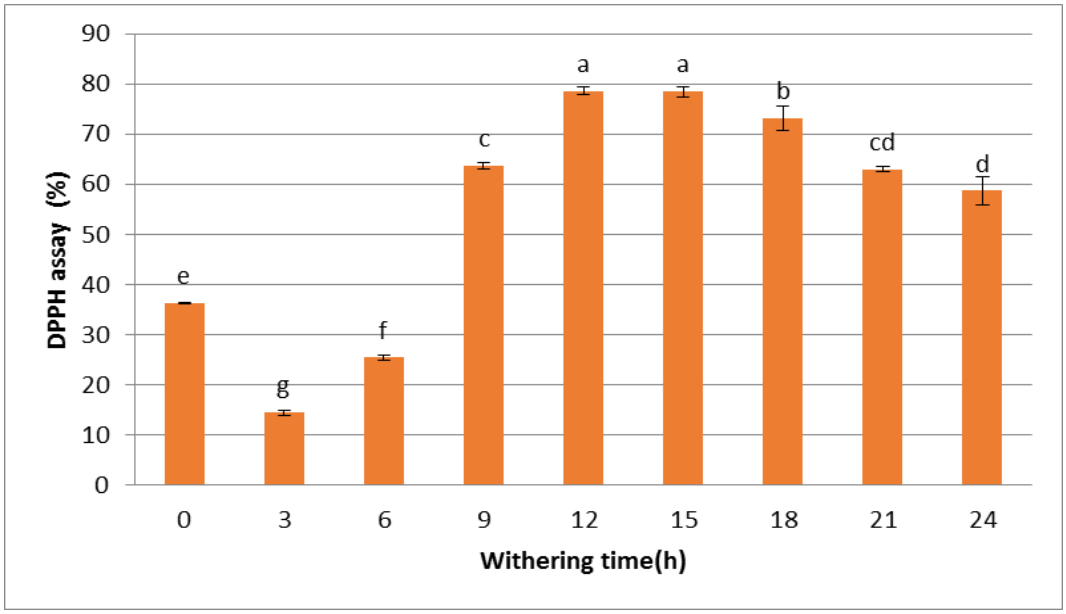

Figure 3.2: Effect of withering duration on AC of mulberry leaf tea

Note: *All data was presented as means ± standard deviation. Means followed by different lower case letters were significantly different (p<0.05). The error bars are presented for standard deviation.*

To explain for the increase of TPC of mulberry leaves in the period of 15h of withering, firstly, polyphenol oxidase (PPO) activities must be examined. It was reported that there is a rise in PPO activity in the mature stage of mulberry leaves, which is relevant to complex modification observed in antioxidant enzyme (Goud, et al, 2012).There are correlations between the presence of high levels of phenolic compounds and PPO or low levels of phenolic and no PPO in some tissue types (Kojima and Conn 1982; Vaughn et al. 1981). Although the developmental role of enzyme to proteins and secondary metabolites has not yet been verified, it is clear that they vary with cultivars and fluctuate with the environment and also with cultural practice. Under the controlled conditions of withering, PPO, peroxidase and protease exhibited maximum activities within 12-16 h (Baruah, 2003), which could explain why TPC of withered mulberry leaves can reach the highest amount after 12 and 15h. In some cases, increased enzyme activity may contribute to appearance of new multiple forms (Saluja and Sachar, 1982).

Besides, it also observed that there was a decline in TPC as well as AC starting from 18h of withering. It could be explained that the lowering of PPO activity was also found to depend on the degree of wither. Hardness of wither due to longer time, followed by a large reduction of moisture content, could inhibit the activity of PPO (Ullah, et al, 1984). Similarly, it was also noted that for traditional tea leaves *(Camelia sinensis),* excessive withering may concentrate the catechins (a type of phenols in tea) due to the water loss to levels that inhibit PPO activity (Robertson, 1992). Moreover, the appearance of death plant cell has been reported after 6h withering (Roberts & Sanderson, 1966). The longer withering is, the more death cells appear, which may lead to the decrease in TPC as well as AC in mulberry withered leaves at 18, 21 and 24h.

Final results of experiment 1 implied that the most appropriate withering duration should be either 12 or 15h as the highest yield of TPC were recorded, however, 12h was selected to further experiment to save time.

3.2 Effect of incubation temperature on TPC of mulberry leaf tea

Incubation or fermentation was applied in rolled leaves. Fermentation is an indispensable step in tea processing. Leaves must be rolled or crushed to initiate the fermentation stage. The main objective of maceration and rolling is to break the cells of the withered tea leaves, which exposes the cell sap (fluid

inside vacuole). The process results in a chemical reaction between the chemical constituents and enzymes in the presence of atmospheric oxygen (Pou, K. J., 2016). In this experiment, after going through withering and rolling process, mulberry leaves continued to be fermented at different temperature (RT, 30, 35 and 40°C). Steps for conducting fermentation were mentioned previously in section 2.2.4.

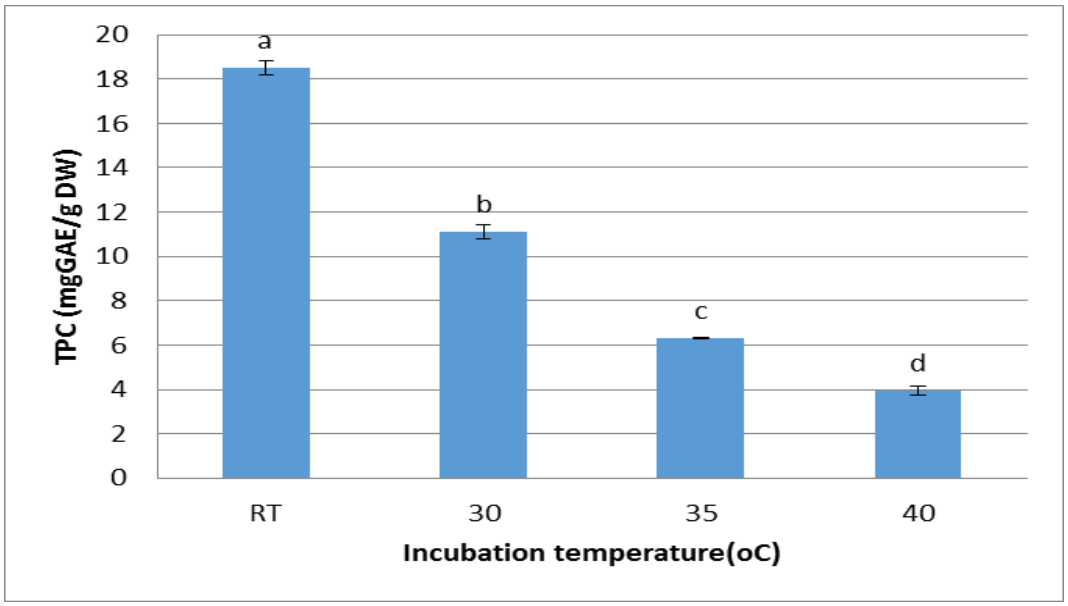

Figure 3.3: Effect of incubation temperature on TPC of mulberry leaf tea

Note: *All data was presented as means ± standard deviation. Means followed by different lower case letters were significantly different ($p<0.05$). The error bars are presented for standard deviation.*

Figure 3.3 showed the relationship of TPC and temperature used for incubation/ fermentation of mulberry tea leaves. There was a statistically difference of TPC and AC at different temperature. Overall, the higher temperature applied during the fermentation process, the more amount of TPC degenerated. Figure 3.3 and 3.4 described a downtrend of TPC and AC where warmer temperature applied. As temperature increased, TPC was decreased respectively. For every increase by 5°C, TPC reduced nearly a half. The figure for TPC at RT was significantly higher than in other temperature (18.50 ± 0.33^{a} mg GAE/ g DW), which is also the highest one, while the lowest TPC was measured at 40°C (3.93 ± 0.20^{d}). Furthermore, in figure 3.4, AC was also depressed due to the increase of temperature during fermentation (from 64.54 ± 3.97^{a} % for RT to 8.09 ± 0.28^{d} for 40°C).

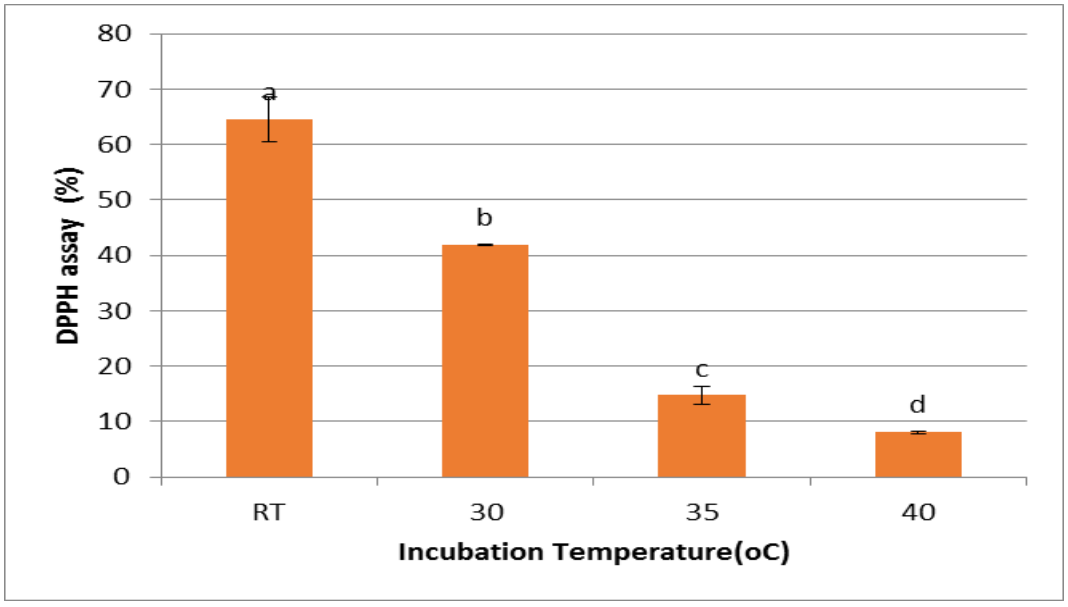

Figure 3.4: Effect of incubation temperature on AC of mulberry leaf tea

Note: *All data was presented as means ± standard deviation. Means followed by different lower case letters were significantly different ($p<0.05$). The error bars are presented for standard deviation.*

The results in this investigation showed temperature is an important factor during the fermentation of mulberry leaf tea. It was reported that along with fermentation process in *Camelia sinensis* leaves, as fermentation proceeded, the levels of total theaflavins (a form of tea polyphenols) declined much more for the higher temperature than the lower temperature (Robertson, 1983). Moreover, the decline of TPC could be explained due to its closed relationship with enzyme activity. The higher the temperature was, the larger degradation of enzyme activity was (Takeo, 1966). Three-dimension structures of proteins forming for catalytic enzymes are subjected to degenerate due to higher temperature. It is believed that, as rising temperature accumulated the fermentation reactions, leading to faster depletion of TPC and AC.

Therefore, there was no need for increasing temperature during fermentation of mulberry leaf tea to maintain the high yield of TPC. RT was chosen for fermentation stage as it maintained the highest TPC as well as AC.

3.3 Effect of incubation durations on TPC of mulberry leaf tea

During incubation or fermentation, time is a necessary factor that allows enough oxidative changes of phenolic contents, which leads to the full development of flavor while eliminating grassy smells of fresh leaves. Color is

also formed during fermentation time. In addition, different clones of tea have a difference in optimum fermentation time (Thanaraj, et al, 1990). Chemical structures in details of polymeric products are unknown, but they contribute to the taste of final tea product. Time of fermentation in traditional black tea varies between 45min to 3h, depending on the nature of the leaf, rolling technique and ambient temperature (Chakraverty, et al, 2003). In this experiment, rolled leaves were placed at RT for a variation of time (0, 0.5, 1, 1.5 and 2h) to obtain the changes of TPC and AC during fermentation. Steps for conducting fermentation durations were mentioned previously in section 2.2.5.

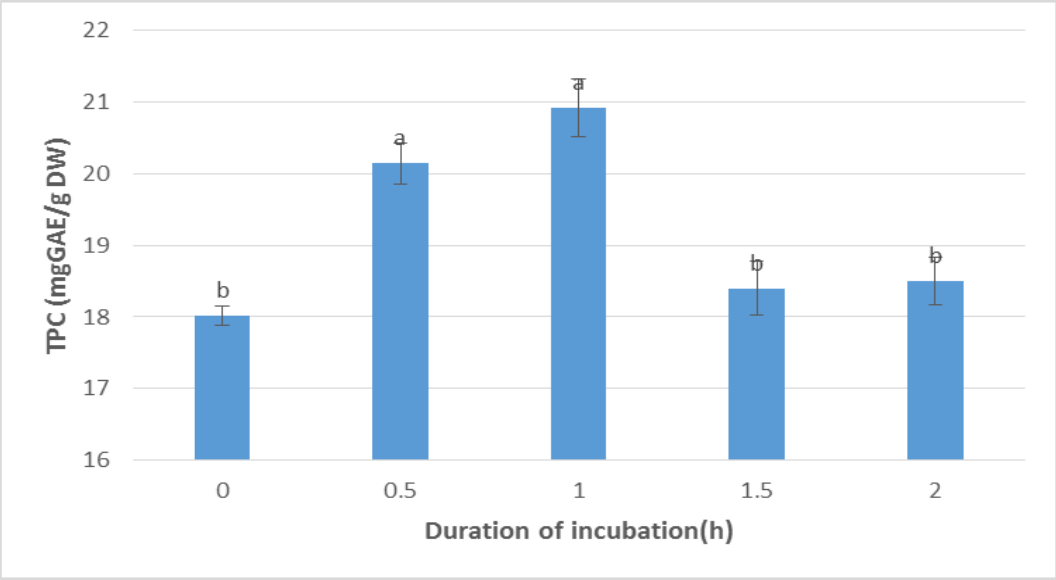

Figure 3.5: Effect of incubation duration on TPC of mulberry leaf tea

Note: *All data was presented as means ± standard deviation. Means followed by different lower case letters were significantly different ($p<0.05$). The error bars are presented for standard deviation.*

Figure 3.5 illustrated the fluctuation in TPC following an extended time. This trend started with rolled leaves without fermentation (0h), which showed the lowest amount of TPC (18.02 ± 0.14^{b}). Following this, after 0.5 and 1h of fermentation, TPC had risen considerably to achieve the highest amount (20.15 ± 0.28^{a} mg GAE/ g DW for 0.5h; 20.92 ± 0.40^{a} for 1h before starting to decline at 1.5h. The result was in agreement with the research paper conducted by Hlahla, L. N. (2010), fermentation of bush tea leaves for 1h had peak TPC as compared to 0h and 2h. This result suggested that desirable TPC could be produced at fermentation time between 0.5 and 1h. As increasing the time, TPC started to drop steadily after 1.5h. This results matched with the findings by Goldstein and Swain (1963), which reported that rising fermentation time

dramatically reduced polyphenol concentration in honey bush tea. As for AC, the longer the fermentation process was, the more AC exhibited. Figure 3.6 showed that there was no significant difference from 1 to 2h of fermentation. The last three patterns of AC were all the highest (61.29± 0.67[a] % for 1h, 63.00± 0.71[a] for 1.5h and 64.54± 3.97[a] for 2h). It could be explained presumably that longer fermentation resulted in increasing overall oxidation processes in mulberry leaves.

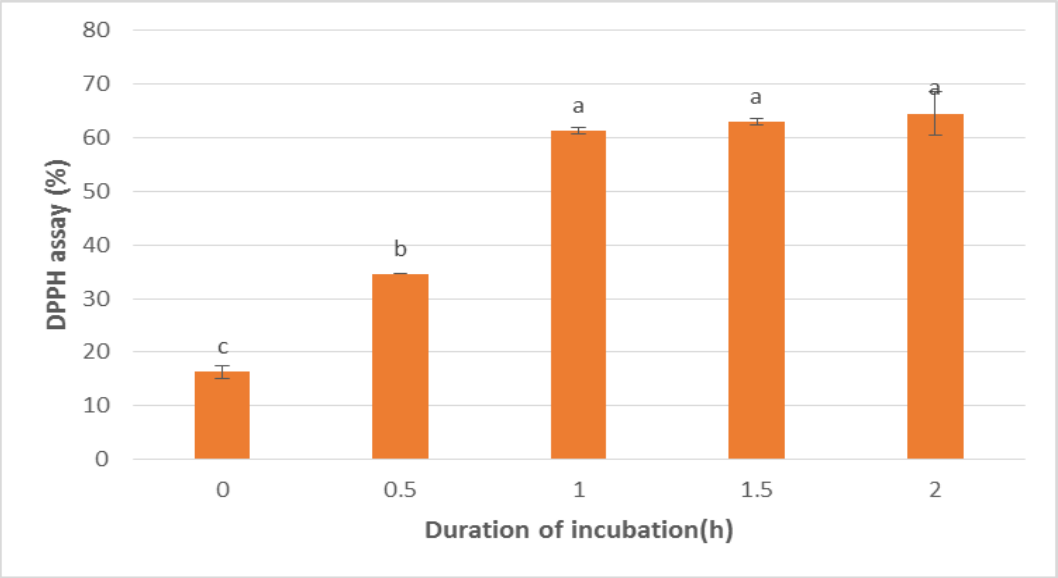

Figure 3.6: Effect of incubation duration on AC of mulberry leaf tea

Note: *All data was presented as means ± standard deviation. Means followed by different lower case letters were significantly different ($p<0.05$). The error bars are presented for standard deviation.*

There was no information that implied about the relationship of incubation or fermentation time and chemical changes in details, which referred to mulberry leaf tea. However, tea quality such as taste and astringent had been enhanced in mulberry tea as it showed a positive increase in TPC when fermented between 0.5-1h. Furthermore, there was a steady drop in TPC at 1.5h and 2h. This could be explained by the degradation of PPO activity due to longer fermentation process (Thanaraj, et al, 1990). It stated that the decline of TPC after 1.5 and 2h of fermentation may relate to activities of both PPO and peroxidase enzymes, which declined as a function of fermentation time (Cloughley, J. B., 1980). However, it seemed that the decline of PPO as well as TPC may be inversely related to AC during fermentation. As it can be seen from figure 3.6, the longer fermentation was, the stronger AC exhibit.

After examining the results from the third experiment, 1h of fermentation was selected as a fixed step in fermentation duration to ensure the full oxidation of mulberry leaf tea as well as the highest amount of TPC.

3.4 Effect of drying temperature on TPC of final tea product

The final step in tea processing is drying. Drying is an important part of tea processing. It is a necessary process to reduce moisture, stop fermentation and increase shelf-life storage (Chong, et al, 2012). This experiment was carried out to identify appropriate temperature required to produce the desired changes without deterioration due to excess heat. Temperatures in the range of 60-150^{o}C were used. The chemical changes undergoing drying process (driving force of heat) may be detected by human senses, plus, they have not been identified or verified scientifically (Temple, et al, 2001). Steps for conducting the drying stage were mentioned previously in section 2.2.6.

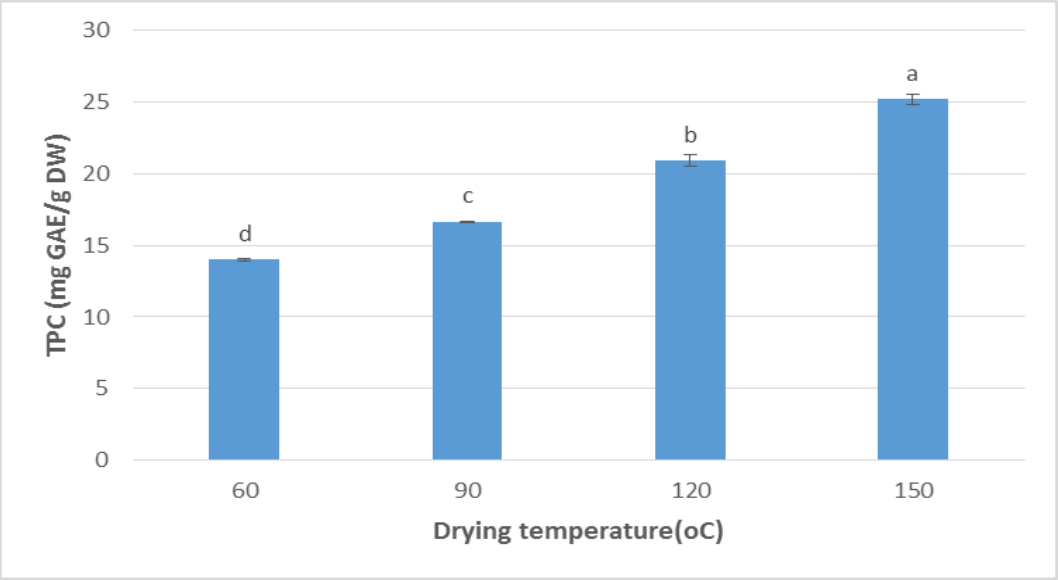

Figure 3.7: Effect of drying temperature on TPC of mulberry leaf tea

Note: *All data was presented as means ± standard deviation. Means followed by different lower case letters were significantly different ($p<0.05$). The error bars are presented for standard deviation.*

Figure 3.7 and 3.8 showed the changes in TPC and AC at different points of drying temperature. As it can be seen in figure 3.7, higher drying temperature resulted in a decrease in TPC of mulberry leaf tea. TPC was found to vary from 25.22 ± 0.35^{a} at 150^{o}C to 14.02 ± 0.10^{d} at 60^{o}C mg GAE/ g DW. There is a significant difference in TPC at 90^{o}C and 120^{o}C (16.67 ± 0.04^{c} versus 20.93± 0.40^{b} mg GAE/ g DW). However, AC was inversely proportional to temperature.

At 60°C, AC accounted for 82 ± 0.14[a] %, which is significantly higher than AC obtained at 90°C, 120°C (61.65± 0.20[b] and 61.29± 0.67[b], respectively). For AC at 150°C, AC remained at a lower rate due to high heat applied (49.84± 0.38[c]).

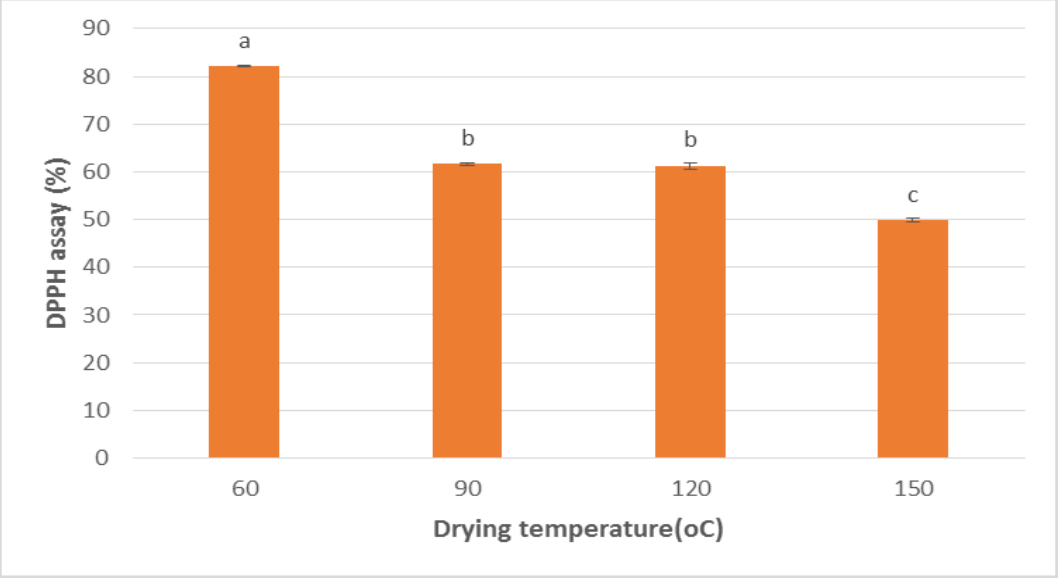

Figure 3.8: Effect of drying temperature on AC of mulberry leaf tea

Note: *All data was presented as means ± standard deviation. Means followed by different lower case letters were significantly different ($p<0.05$). The error bars are presented for standard deviation.*

The rise in temperature may lead to the major loss of moisture content (MC), which condensed TPC in mulberry leaf samples. As the results obtained from figure 3.7, TPC was at a higher rate due to the increasing of drying temperature. There is lack of scientific evidence to imply how the rise of temperature in drying process affect the biochemical transformation. It could be doubtful that Folin-Ciocalteu reagent reacts not only with phenols but also with a variety of other types of nitrogen compounds that may had been generated during drying (Ikawa, et al, 2003). One of the heterocylic compounds generated by the Maillard reaction during tea manufacturing process is pyrrole. This substance could be formed in larger amount at higher drying temperature. Pyrrole is a reducing substance showing its reaction with Folin-Ciocalteu reagent (Ho et al. 2015; Ikawa et al. 2003). In drying process, the changes from yellow to darker colors were caused by the oxidation of phenolic substances. As for the decline of AC taken from figure 3.8, it could be explained that the drying treatment associated to thermal degradation of phytochemicals and antioxidant enzyme activity (Chan, et al, 2013).

Even biochemical changes in during drying process was little known, drying is aimed mostly for controlling MC, though its biochemical changes is little updated. In addition, the requirements of TCVN 7975:2008 for moisture content is below 10%, which could be achieved due to drying stage. As the result, the highest amount of TPC was chosen at 150^{o}C of drying due to the highest TPC obtained (25.22 ± 0.35^{a} mg GAE/ g DW).

3.5 Final quality evaluation

3.5.1. Chemical parameters

Table 3.1: Chemical parameters of final dried product

No.	Parameters	Result	TCVN standards
1	MC (%)	2.15	<10% (TCVN 7975:2008)
2	Ash (%)	3.24	<8% (TCVN 7975:2008)
3	SSC (^{o} Brix)	0.68	-
4	TPC (mg GAE/g DW)	25.22	-
5	AC (%)	49.84	-

3.5.2. Sensory evaluation

Table 3.2: Result of sensory test based on TCVN 3218-2012

Panelists	Attributes			
	Appearance	Color	Flavor	Taste
Mean	4.07	4.28	4.33	4.02
Important factor (IF)	1.0	0.60	1.20	1.20
Mean*IF	4.07	2.57	5.20	4.82
Total	**16.66**			

Table 3.3: Product quality score based on TCVN 3218-2012

No.	Score	Quality
1	18.6 - 20	Good
2	15.2 - 18.5	Fairly good
3	11.2 - 15.1	Moderate
4	7.2 – 11.1	Bad
5	4.0 - 7.1	Very bad
6	0 - 3.9	Fail

The quality score of mulberry leaf product was 16.66, which is in the range between 15.2 and 18.5, which was on the "fairly good" scale according to TCVN 3218-2012.

4 CONCLUSION AND RECOMMENDATIONS

In conclusion, an array of test was conducted to determine the effect of different withering duration, different temperature and time of incubation, and different drying temperature. The results showed the best condition for processing mulberry leaf tea were demonstrated, which had been achieved by 12h of withering, 1h of fermentation at 25^{o}C and drying at 150^{o}C. In one cup of tea (2 g of dried tea per 100 ml of boiled water), SSC (0.68 ^{o} Brix); TPC (25.22 mg GAE/g DW) and AC (49.84%).

After conducting experiment on laboratory scale, there are 3 issues that are implied for further discussion. Firstly, more studies must be conducted on larger and industrial scale. Secondly, more experiments on diversifying the product should be performed by mixing with other ingredients to improve customer acceptability. Thirdly, screening deeply on biochemical changes of mulberry leaves during tea manufacturing.

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APPENDICES

Appendix A: Images taken during the thesis process

Figure A1: Fresh mulberry leaves and withered leaves

Figure A2: leaves after rolling

Figure A3: Final product

Figure A4: A cup of mulberry leaf tea

Appendix B: Data from the experiments

Table B1: TPC changes at different withering times

	Withering hour	N	Subset for alpha = 0.05				
			1	2	3	4	5
Tukey HSD[a]	3h	3	4.8443				
	6h	3		8.9650			
	21h	3			12.2983		
	9h	3			12.3577		
	0h	3			12.4307		
	24h	3				14.4963	
	18h	3				15.2240	
	12h	3					18.4963
	15h	3					19.5047
	Sig.		1.000	1.000	1.000	.900	.634
Tukey B[a]	3h	3	4.8443				
	6h	3		8.9650			
	21h	3			12.2983		
	9h	3			12.3577		
	0h	3			12.4307		
	24h	3				14.4963	
	18h	3				15.2240	
	12h	3					18.4963
	15h	3					19.5047
Duncan[a]	3h	3	4.8443				
	6h	3		8.9650			
	21h	3			12.2983		
	9h	3			12.3577		
	0h	3			12.4307		
	24h	3				14.4963	
	18h	3				15.2240	
	12h	3					18.4963
	15h	3					19.5047
	Sig.		1.000	1.000	.818	.192	.076

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table B2: AC changes at different withering times

			Subset for alpha = 0.05				
	Withering time	N	1	2	3	4	5
Tukey HSD[a]	3h	3	14.4627				
	6h	3	25.4257	25.4257			
	0h	3		36.2490			
	24h	3			58.6893		
	9h	3			60.6927		
	21h	3			62.9857	62.9857	
	18h	3			65.4980	65.4980	
	12h	3				78.6333	
	15h	3				80.8143	
	Sig.		.475	.491	.908	.051	
Tukey B[a]	3h	3	14.4627				
	6h	3	25.4257	25.4257			
	0h	3		36.2490			
	24h	3			58.6893		
	9h	3			60.6927		
	21h	3			62.9857		
	18h	3			65.4980	65.4980	
	12h	3				78.6333	
	15h	3				80.8143	
Duncan[a]	3h	3	14.4627				
	6h	3		25.4257			
	0h	3			36.2490		
	24h	3				58.6893	
	9h	3				60.6927	
	21h	3				62.9857	
	18h	3				65.4980	
	12h	3					78.6333
	15h	3					80.8143
	Sig.		1.000	1.000	1.000	.237	.674

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table B3: TPC changes at different fermentation temperature

	Fermentation temperature	N	Subset for alpha = 0.05			
			1	2	3	4
Tukey HSD[a]	40	3	3.9353			
	35	3		6.2987		
	30	3			11.1083	
	RT	3				18.4963
	Sig.		1.000	1.000	1.000	1.000
Tukey B[a]	40	3	3.9353			
	35	3		6.2987		
	30	3			11.1083	
	RT	3				18.4963
Duncan[a]	40	3	3.9353			
	35	3		6.2987		
	30	3			11.1083	
	RT	3				18.4963
	Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table B4: AC changes at different fermentation temperature

	Fermentation temperature	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	40	3	8.0947		
	35	3	14.7420		
	30	3		39.5380	
	RT	3			60.6927
	Sig.		.330	1.000	1.000
Tukey B[a]	40	3	8.0947		
	35	3	14.7420		
	30	3		39.5380	
	RT	3			60.6927
Duncan[a]	40	3	8.0947		
	35	3	14.7420		
	30	3		39.5380	
	RT	3			60.6927
	Sig.		.106	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table B5: TPC changes at different fermentation duration

	Fermentation duration	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	0h	3	18.0170		
	1.5h	3	18.3970		
	2h	3	18.4963		
	0.5h	3		20.1490	
	1h	3		20.9257	
	Sig.		.586	.185	
Tukey B[a]	0h	3	18.0170		
	1.5h	3	18.3970		
	2h	3	18.4963		
	0.5h	3		20.1490	
	1h	3		20.9257	
Duncan[a]	0h	3	18.0170		
	1.5h	3	18.3970		
	2h	3	18.4963		
	0.5h	3		20.1490	
	1h	3			20.9257
	Sig.		.183	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table B6: AC changes at different fermentation duration

	Fermentation duration	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a,b]	0h	3	16.3240		
	0.5h	2		34.6050	
	1h	2			61.2950
	1.5h	3			63.0043
	2h	2			64.5415
	Sig.		1.000	1.000	.594
Tukey B[a,b]	0h	3	16.3240		
	0.5h	2		34.6050	
	1h	2			61.2950
	1.5h	3			63.0043
	2h	2			64.5415
Duncan[a,b]	0h	3	16.3240		
	0.5h	2		34.6050	
	1h	2			61.2950
	1.5h	3			63.0043
	2h	2			64.5415
	Sig.		1.000	1.000	.193

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.308.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Table B7: TPC changes at different drying temperature

	Drying temperature	N	Subset for alpha = 0.05			
			1	2	3	4
Tukey HSD[a,b]	60	3	13.9513			
	90	3		16.6453		
	120	3			20.9257	
	150	2				24.8760
	Sig.		1.000	1.000	1.000	1.000
Tukey B[a,b]	60	3	13.9513			
	90	3		16.6453		
	120	3			20.9257	
	150	2				24.8760
Duncan[a,b]	60	3	13.9513			
	90	3		16.6453		
	120	3			20.9257	
	150	2				24.8760
	Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.667.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Table B8: AC changes at different drying temperature

	Drying temperature	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a,b]	150	3	49.8483		
	120	2		61.2950	
	90	3		61.5120	
	60	3			82.0617
	Sig.		1.000	.947	1.000
Tukey B[a,b]	150	3	49.8483		
	120	2		61.2950	
	90	3		61.5120	
	60	3			82.0617
Duncan[a,b]	150	3	49.8483		
	120	2		61.2950	
	90	3		61.5120	
	60	3			82.0617
	Sig.		1.000	.607	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.667.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Table B9: Standard for sensory evaluation

Sensory attributes	Score	Description
Color	5	Reddish brown color, very transparent
	4	Reddish brown color, slightly transparent
	3	Light reddish brown or dark yellow, very slightly transparent
	2	Pale or dark yellow color, slight turbidity, little residues at the bottom of the glass
	1	Pale yellow, turbidity, more residues
	0	Strange color, very turbid, too much residue
Appearance	5	Small and even particle size, yellowish brown, color, typical for the product
	4	Relatively even particle size and yellowish brown color
	3	Relatively even particle size, light brown or dark brown
	2	Uneven size and dark brown color
	1	Uneven size and black color of burned sample
	0	Heterogeneous size, clear difference between particle, black color of burned sample
Taste	5	Pleasant and harmonious taste, sweetness aftertaste
	4	Pleasant and harmonious taste, slight sweetness aftertaste
	3	No taste at first, then slight sweetness aftertaste
	2	Slight sour or bitter taste, no aftertaste
	1	Typical sour taste of fresh fruit or bitter taste due to burned tea, no aftertaste
	0	Strange taste, no aftertaste, cannot drink
Flavor	5	Natural and harmonious aroma for liquid, very strong smell for the powder, no strange flavor.
	4	Natural and relatively harmonious aroma for liquid, strong smell for the powder, no strange flavor.
	3	Typical aroma for both liquid and powder, no strange flavor
	2	Breathable fragrance, slight burned smell
	1	Slight burned smell
	0	Strange or burned smell, no breathable aroma.

Table B10: Sensory evaluation score of 60 panelists

Panelists	**Attributes**			
	Appearance	Color	Flavor	Taste
1	5	4	4	4
2	4	4	4	4
3	4	4	4	5
4	4	4	4	4
5	3	4	4	4
6	4	4	5	4
7	5	4	4	3
8	3	4	5	4
9	4	5	5	5
10	4	5	5	4
11	4	4	4	3
12	4	4	3	4
13	4	4	4	3
14	4	5	5	5
15	5	4	5	5
16	4	4	5	4
17	4	4	5	4
18	4	5	4	4
19	4	4	3	4
20	4	5	5	4
21	4	4	4	3
22	4	4	4	4
23	4	4	4	4
24	3	4	4	4
25	4	5	5	5
26	5	4	5	4
27	5	4	5	4
28	4	4	4	4
29	5	5	4	3
30	3	5	4	4
31	5	4	4	4
32	4	4	4	4
33	4	4	4	5
34	4	4	4	4
35	3	4	4	4
36	4	4	5	4
37	5	4	4	3
38	3	4	5	4
39	4	5	5	5
40	4	5	5	4

41	4	4	4	3
42	4	4	3	4
43	4	4	4	3
44	4	5	5	5
45	5	4	5	5
46	4	4	5	4
47	4	4	5	4
48	4	5	4	4
49	4	4	3	4
50	4	5	5	4
51	5	4	5	4
52	4	4	4	4
53	5	5	4	3
54	3	5	4	4
55	5	4	4	4
56	4	4	4	4
57	4	4	4	5
58	3	5	4	5
59	4	4	5	4
60	4	5	5	3

Appendix C: Standard curve

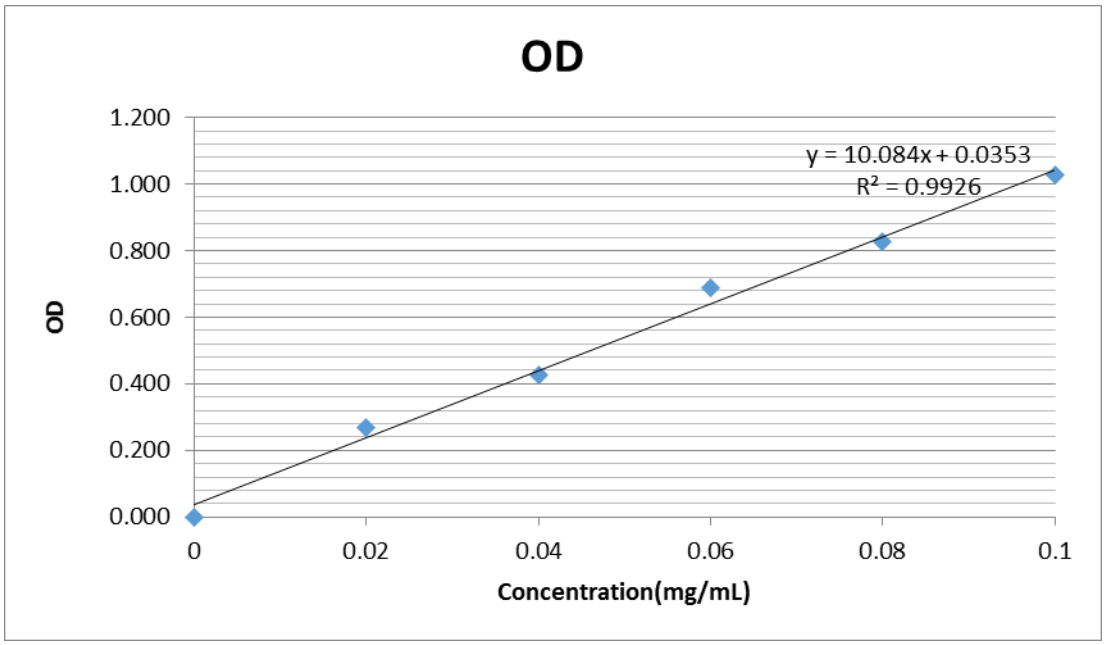

Figure C1: Gallic acid standard curve

SUPERVISOR APPROVAL

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