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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 40ºC within 2 hours) 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 18.50 ± 0.33 Gallic acid equivalent/ g dry weight, which had been achieved by 12h of withering, and fermenting at room temperature.

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

Introduction

Tea is one of the most widely consumed beverages worldwide, second only to water [1]. Many herbs and tea have been used to make infusions, and the term "rich in antioxidants" describes such infusions. 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 found in Asian regions. It is recognized as serrate shapes of leaves, which distributed alternatively on a plant body. With regards to scientific trends, many researches have shown the potential of antioxidant, antiviral, anti-inflammatory, hypolipidemic, anti-hyperglycemic, neuro-protective [2], anti-HIV, anti-hypotensive and cytotoxic activities of different species of *Morus* [3]. Some reports have contributed these most important features related to the many phytochemical constituents that are present in mulberry leaves [4]. Several studies also demonstrated mulberry leaf has potential antioxidant activity [5, 6, 7]. Polyphenols, found widely in many plants, maintain effective functions in lowering lipid and antioxidant effect. Previous studies have shown that mulberry leaf extract is rich in polyphenols and can effectively inhibit vascular smooth muscle cells proliferation and migration [8, 9].

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 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.

Materials and methods

Plant material

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 old.

General experimental procedures

Overall, to produce mulberry leaf tea, fresh leaves were processed under 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.

For the first experiment, 500g fresh mulberry leaves were 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 0, 3, 6, 9, 12, 15, 18, 21 and 24h. 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 (25ºC) for 2h. Drying stage is finally conducted at 120ºC, 20min [10]. Samples were then stored in a desiccator for water extraction before going through further analysis to choose the sample which would have the highest TPC. This experiment was conducted following Jabeen, et al, 2015 with a slight modification in withering time. Extraction was conducted for TPC and AC analysis. For the second experiment, 500g fresh clean mulberry leaves were prepared. Withering duration with the highest TPC was applied as a fix step in tea processing. A constant fan air flow (900 rpm, model Sharp PJT16GY) was applied while withering. The withered leaves were then curled by ands and went through fermentation process at varied temperature (25, 30, 35 and 40ºC) for 2h. Drying

stage is finally conducted at 120ºC, 20min [10]. Data was collected and the highest result in TPC followed by incubation temperature was chosen for further experiments. This experiment was followed by [11, 12] with a modification. Extraction was then conducted for TPC and AC analysis.

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.

Determination of Total phenolic content (TPC)

Total phenolic content was measured by using Folin-Ciocalteu method reported by [13] with some changes reported by [14] 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 45ºC 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 Gallic acid equivalent/g Dry weight).

Determination of Antioxidant Capacity (AC)

Antioxidant activity was estimated using the DPPH (1, 1-diphenyl-2-picryl hydrazine) assay described previously by [15] with a slight modification, 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 determined 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:

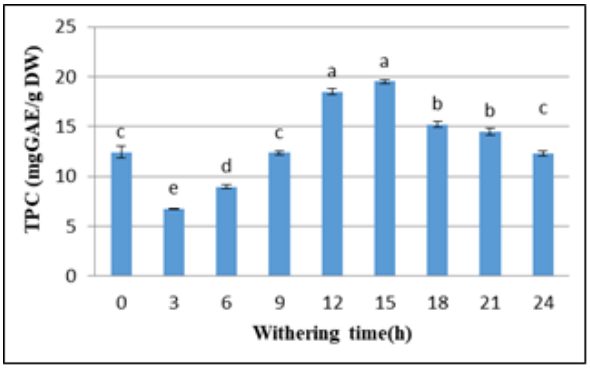
Scavenging activity (%) =

$$\frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}} . 100. volume \quad (1)$$

Results and discussion

Effect of withering duration on TPC of mulberry leaves

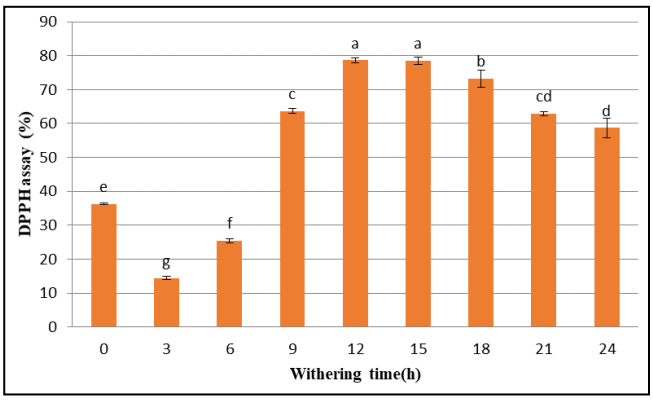
Withering, which is also called partial desiccation, is the first significant stage for improving the quality of final tea. Immediately after plucking, the fresh leaf starts to lose water. As withering progresses, the stomata of the lower leaf surface begin to close [16, 17]. The picked leaves are usually spread in a series of either enclosed or open trough under forced air circulation [18]. 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 [19]. 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 [20]. In this study withering time was limited to 24h since it had been demonstrated that withering beyond 20 h impaired black tea quality.



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.

Fig 1: Effect of withering duration on TPC of mulberry leaf tea

Fig 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.



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.

Fig 2: Effect of withering duration on AC of mulberry leaf tea

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

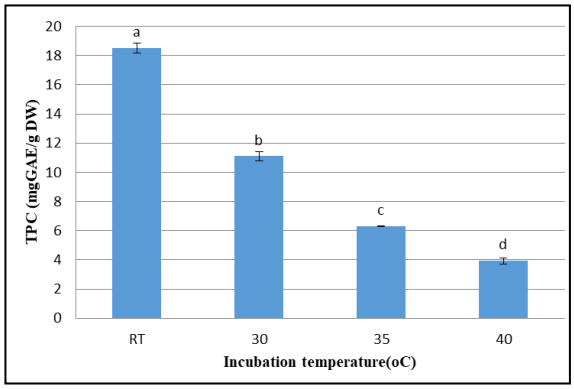
mulberry leaves, which is relevant to complex modification observed in antioxidant enzyme [21]. There are correlations between the presence of high levels of phenolic compounds and PPO or low levels of phenolic and no PPO in some tissue types [22, 23]. Although the developmental role of enzyme to proteins 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 [18], 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 [24].

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 [25]. 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 [26]. Moreover, the appearance of death plant cell has been reported after 6h withering [27]. 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.

Effect of incubation temperature on TPC of mulberry leaves

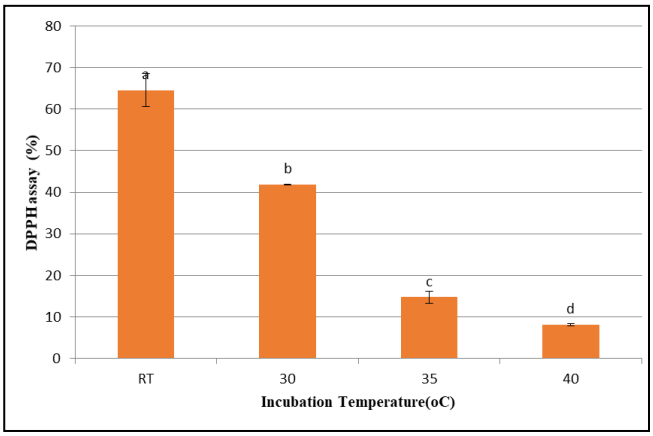
Incubation or fermentation was applied in rolled leaves. Fermentation is an indispensable step in tea processing. Leaves must be rolled or crushed to initiate the fermentation stage. The main objective of maceration and rolling is to break the cells of the withered tea leaves, which exposes the cell sap (fluid inside vacuole). The process results in a chemical reaction between the chemical constituents and enzymes in the presence of atmospheric oxygen [28]. In this experiment, after going through withering and rolling process, mulberry leaves continued to be fermented at different temperature (RT, 30, 35 and 40ºC).



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.

Fig 3: Effect of incubation temperature on TPC of mulberry leaf tea

Fig 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. Fig 3 and Fig 4 described a downtrend of TPC and AC where warmer temperature applied. As temperature increased, TPC was decreased respectively. For every increase by 5ºC, 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 40ºC (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 40ºC).



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.

Fig 4: Effect of incubation temperature on AC of mulberry leaf tea

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 [26]. 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 [11]. 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.

Conclusion

In conclusion, an array of test was conducted to determine the effect of different withering duration and fermentation at different temperatures. The results showed the best condition for processing mulberry leaf tea were demonstrated, which had been achieved by 12h of withering, followed by fermentation at 25ºC. As the result,

the highest yield of total phenolic content of mulberry tea was 18.50 ± 0.33 GAE/ g dry weight after 12h of withering, and fermentation at 25ºC.

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