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Research Article

# Preliminary findings of the effect of infusion variables on marker contents and antioxidant activity of *Thunbergia laurifolia* tea

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Caffeic acid; Multivariate optimization; Response surface methodology; Rosmarinic acid; *Thunbergia laurifolia*



## ABSTRACT

*Thunbergia laurifolia* (TL) Linn., a Thai medicinal herb, has been used as a herbal tea for detoxification of poisons and antipyretic purpose. Its antioxidant activity and related biological effects have also been reported. The active markers of the TL tea were caffeic acid (CA) and rosmarinic acid (RA). Although this tea has been used for decades, the information relating to its optimal infusion condition was limited. Therefore, this study was aimed to preliminary investigate the effect of infusion variables which were infusion time, water temperature and volume, on the CA and RA contents transferred into the tea solutions. To evaluate the efficiency of extraction methods, the marker contents obtained by infusion and aqueous extraction process were compared. Furthermore, the infusion variables were optimized to obtain maximum antioxidant activity by using response surface methodology (RSM). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity was used as a model response. The results demonstrated that the contents of CA and RA in the tea solutions varied considerably according to the infusion conditions, which may be critical for the therapeutic efficacy of the tea. More importantly, after infusion, the major portion of active markers remained in the tea powder implying low efficiency of the infusion method. By using RSM, it was successfully found that the maximum DPPH activity was obtained when infusing the TL tea in 200 mL of boiling water for 15 min. The results of this study can help further understanding of the maximal health benefits to be attained through consumption of TL tea.

## 1. INTRODUCTION

The leaves, root and bark of *Thunbergia laurifolia* (TL) Linn. or "Rang Cheud", a Thai medicinal herb, has been traditionally used for decades as an antipyretic[1] and detoxifying agent of poisoning from insecticide[1-4], strychnine[5], lead[6, 7] and ethanol[8, 9]. The aqueous leaves extracts have also been reported to possess antioxidant activity[10-13], which may be related to several pharmacological activities of the tea including neuroprotective[6, 7], hepatoprotective[9, 10] and anti-inflammatory properties[10, 14]. The previous studies showed that the aqueous extract of dried leaves exhibited the highest phenolic content and antioxidant activity comparing to ethanol and acetone extracts[10]. The major chemical components of the aqueous extracts were phenolic compounds such as caffeic acid (CA) and rosmarinic acid (RA), which were

also reported to possess antioxidant activity and several pharmacological effects[13-15]. Furthermore, these compounds could be used as markers for quality control of TL raw materials and its products.

Currently, this medicinal herb is available as a capsule, tablet and herbal tea in Thailand. According to the National List of Essential Medicines of Thailand, the products have been categorized as antipyretic and detoxifying herbal medicine[1]. These medicine products have been manufactured and dispensed at some hospitals such as Wang-Nam-Yen hospital and Chao Phya Abhaibhubejhr hospital, etc. At Wang-Nam-Yen hospital, the tea product has been clinically used, especially for detoxification of insecticides and herbicides in farmers, and for the treatment of smoking cessation. However, the infusion conditions suggested by pharmacists were varied as follows; 120-250 mL of warm or boiling water and infusion time of 5-15 min, under non-stirring condition. While the infusion conditions recommended in the National List of Essential Medicines of Thailand were 120-200 mL of hot water, and there was no suggested infusion time.

Several previous studies demonstrated that infusion variables such as infusion time, water volume and temperature are critical infusion parameters for extraction of active ingredients and also the stability of active compounds[16-26]. Although this tea has been used for decades, the information relating to its optimal infusion condition was limited. Therefore, this study was aimed to perform a preliminary investigation of the effect of infusion variables on CA and RA contents. Furthermore, in order to evaluate the efficiency of the extraction methods, the marker contents extracted by infusion were compared to those obtained by the aqueous extraction process. More importantly, since the antioxidant activity and related pharmacological effects of TL teas and its aqueous extracts have been continuously reported[8-11, 14]. The infusion condition that maximized the antioxidant activity of the TL tea was investigated by using a multivariate experimental design. In the present study, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical activity was used as a model response.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and reagents

Reference standards of CA, RA, ascorbic acid and analytical grade of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (MO, USA). High-performance liquid chromatography (HPLC) grade of acetonitrile and methanol were purchased from Honeywell (NJ, USA) and Fisher chemical (Loughborough, UK), respectively. Analytical grade of ortho-phosphoric acid was purchased from Ajax Finechem (MA, USA). The deionized water was obtained from Thai Nakorn Patana (Nonthaburi, Thailand). The commercial sachets containing 3 g of finely ground dried leaves of TL were purchased from Wang-Nam-Yen hospital, Sa Kaeo province, Thailand; (Lot no. 034/009/59).

### 2.2. Weight variation and particle size assessments

A total of 20 sachets were randomly selected for the determination of weight variation. Each sachet was individually weighed, then the tea powder content was removed and the emptied sachet was accurately weighed. The net weight of powder content was calculated by subtracting the weight of the emptied sachet from the weight of intact sachet. The percentage deviation from the average net weight (%WV) of each sachet was determined according to the formula: $\%WV = [(W_{ind}-W_{av})/W_{av}] \times 100\%$, where $W_{ind}$ and $W_{av}$ are individual net weight and average net weight, respectively. After the experiment, all of the tea powders were mixed, and then used for particle size assessment (Mastersizer 2000, Malvern, UK) and for the preparation of TL aqueous extracts.

### 2.3. Investigation of the effect of infusion variables on CA and RA contents

From the previous studies, the infusion time, water temperature and volume were reported to affect the extracted amount of active contents and their stability in tea solutions[18-26]. Therefore, these parameters were chosen to investigate their effect on CA and RA content extracted into the TL tea solutions. In this study, one batch of the tea was used in order to avoid variations in the composition of the plants. The weight of sachets used in this study was within 5% of the mean weight corresponded to 2.75-3.03 g of dried leaves. The studied infusion variable ranges were as follows; infusion time of 3-20 min, water volume of 100-300 mL and water temperature of 60-100ºC. These variable ranges covered the recommended and actual-use conditions employed by consumers. When each parameter was studied, the others were kept constant as follows; infusion time of 10 min,

water temperature of 80ºC and volume of 200 mL. In this study, the boiling water temperature was approximately considered as 100ºC. When the temperature reached the boiling point, the water was further heated for 5 min before using in the experiments. All of the experiments were performed at a controlled room temperature of 25±2ºC.

For the preparation of tea solution, a sachet was weighed and placed in a glass bottle (350 mL). Then, hot water was added, with the sachet staying totally immersed in the water, and the infusion remained at rest. During the experiment, leak sachets were removed and excluded from analysis. After the infusion period, the sachet was immediately removed. The tea solution was filtered through filter paper (Whatman no.1) and evaporated to dryness according to the procedure described by Ruangpayungsak N[15]. The tea solution was poured into an evaporating dish and then evaporated on a water bath (70±2ºC) placed in a laminar air flow cabinet. When a small volume of concentrated solution was obtained (approximately 10 mL), the extract was transferred into a known weight of a glass bottle (20 mL) and was further evaporated on a water bath until obtaining a constant weight (total weight). The total evaporation time for all tea solutions was less than 8 hours. Each extract was kept at -80ºC until analysis. The net weight of the tea extract was calculated by subtracting the weight of the empty bottle from the total weight.

### 2.4. Optimization of infusion condition

A three-level Box-Behnken design (BBD) with 15 trials including three replicates at the center point was used in this study to assess the infusion condition that maximized the antioxidant activity of the TL tea. The DPPH activity was used as a model response. The studied variable ranges were as follows; infusion time of 5-15 min, water volume of 150-250 mL and water temperature of 80-100ºC. The studied ranges were based on the results of the above infusion variable experiments and also covered the actual-used infusion conditions. The detailed levels are shown in Table 1. Each trial was performed in triplicate. The experimental procedures, including the weight of sachet, were under the same condition as previously described in the section 2.3. Then, the TL tea extracts were analyzed for their DPPH activity.

The analysis of variance (ANOVA) carried out to determine individual linear, quadratic and interaction regression coefficient using Design Expert trial version 10.0 (State Ease, Inc.). The fitness of the polynomial equation to the DPPH response was estimated using coefficient of determination ($R^2$). The terms of polynomial equation were considered significantly different at *p*-value less than 0.05 ($p<0.05$). The trial version of Design Expert 10.0 software was also used to generate 3D response surface graphs.

**Table 1.** DPPH responses of TL tea extracts prepared according to Box-Behnken design trials

| Experiment | Decodified variables | | | DPPH[b] |
|---|---|---|---|---|
| | Time (min) | Temperature (ºC)[a] | Volume (mL) | |
| 1 | 5 | 80 | 200 | 0.0180±0.0025 |
| 2 | 15 | 80 | 200 | 0.0228±0.0011 |
| 3 | 5 | 100 | 200 | 0.0628±0.0029 |
| 4 | 15 | 100 | 200 | 0.0763±0.0015 |
| 5 | 5 | 90 | 150 | 0.0189±0.0016 |
| 6 | 15 | 90 | 150 | 0.0457±0.0026 |
| 7 | 5 | 90 | 250 | 0.0257±0.0008 |
| 8 | 15 | 90 | 250 | 0.0408±0.0028 |
| 9 | 10 | 80 | 150 | 0.0316±0.0009 |
| 10 | 10 | 100 | 150 | 0.0479±0.0021 |
| 11 | 10 | 80 | 250 | 0.0296±0.0009 |
| 12 | 10 | 100 | 250 | 0.0668±0.0010 |
| 13 | 10 | 90 | 200 | 0.0351±0.0025 |
| 14 | 10 | 90 | 200 | 0.0336±0.0001 |
| 15 | 10 | 90 | 200 | 0.0343±0.0012 |

[a] Boiling water temperature was considered approximately as 100°C.
[b] DPPH = 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability (mg of ascorbic acid equivalent per 1 g of dry extract weight), the data were expressed as mean±S.D (*n*=3).

### 2.5. Analysis of CA and RA contents by HPLC

The tea extract was thawed at room temperature and reconstituted with an appropriate amount of deionized water (2 mg of dried extract/mL). The sample was sonicated until no particles were visually observed (less than 10 min). The extract solution was then filtered using a PTFE syringe filter (13 mm diameter, 0.22 µm pore size, Membrane solution, TX, USA). The filtrate was analyzed for CA and RA contents by using HPLC method[15].

An HPLC system (Waters corporation, MA, USA) equipped with a binary pump (model 1525), autosampler (model 717), dual wavelength UV detector (model 2487), column compartment and 5µm Xterra® $C_{18}$ reversed-phase column (150 × 3.9 mm, Waters, USA) was used for analysis of CA and RA contents. The column temperature was controlled at 30±2°C and the detection wavelength was 330 nm. The peaks of CA and RA were identified by HPLC chromatography with authentic standards. The mobile phase consisted of 0.05% ortho-phosphoric acid in water (A) and acetonitrile (B). A gradient elution was performed as follows: 0.0-3.0 min (10-15% B), 3.1-15.0 min (15-20% B), 15.1-19.0 min (20-25% B), 19.1-21.0 min (25% B), 21.1-25.0 min (25-30% B), 25.1-30.0 min (30-70% B), 30.1-35.0 min (70-10% B) and 35.1-40.0 min (10% B). The flow rate was maintained at 1mL/min with a run time of 40 min, and the injection volume was 20 µL. The integrations were performed using Empower 2 software (Waters, MA, USA). The standard stock solutions of CA and RA were prepared in methanol at a concentration of 1 mg/mL and stored at -20ºC for a maximum of 1 month. The calibration curves (peak area vs. concentration) for individual compounds were constructed in the range of 1-40 µg/mL. Concentrations of investigated compounds were determined, based on the chromatographic data of the standards.

### 2.6. Aqueous extract preparation

To evaluate the extraction efficiency, the active marker contents obtained by infusion were compared to those obtained by the aqueous extraction process. The aqueous extracts of TL were prepared according to the procedure described by Ruangpayungsak N[15]. An accurate weight of TL tea powder (2.5 g) was added into a glass tube and 25 mL of deionized water was added (1:10 w/v). Then, the tube was placed in an aluminum container (500 mL) containing 200 mL of water. The container was placed on a heating plate set to 90ºC for 2 hours. After the extraction period, the mixture was centrifuged at 5,000 rpm for 15 min and filtered through Whatman filter No. 1. The extraction process was repeated for three times. Then, the filtrates were combined in a 100-mL volumetric flask and adjusted to the volume with deionized water, and was then evaporated to dryness on water bath at 70ºC. The extracts were kept at -20ºC until analysis. The experiment was performed in triplicate.

### 2.7. DPPH free radical scavenging activity

Each tea extract was thawed unassisted at room temperature and reconstituted with an appropriate amount of deionized water. Then, the sample was sonicated until no particles were visually observed (less than 10 min) and was filtered using a PTFE syringe filter (13 mm diameter, 0.22 µm pore size). The filtrate was then analyzed for antioxidant activity.

The free radical scavenging activity of tea extracts and standard ascorbic acid solution in deionized water was investigated based on their ability to react with the DPPH reagent[15]. A working solution DPPH solution was prepared in methanol at a concentration of 208 µM. The TL extract was reconstituted with deionized water and mixed properly with DPPH solution in the ratio of 1:1. The absorbance of each reaction solution was determined at 515 nm using a UV-VIS spectrophotometer (UV-2600, Shimadzu, Japan). Each sample was investigated in duplicate. The antioxidant activity was obtained from the calculation of ascorbic acid equivalent from a standard curve of ascorbic acid solution (1-12 µg/mL) determined by the same DPPH assay procedure. The antioxidant activity was expressed as ascorbic acid equivalent per 1 g of dried extract weight (mg AEAC/g dw).

## 3. RESULTS AND DISCUSSION

### 3.1. Weight variation and particle size

The average weight of sachets and filled content was 2.99±0.20 and 2.89 ± 0.20 g (Mean±S.D.), with the coefficient of variation of 6.53 and 6.75%, respectively. The mean particle size value (D50) measured by laser diffraction was 123.33 µm with span value of 6.80 µm. For all infusion experiments, the weight of each commercial sachet used was within 5% of the

mean intact sachet weight corresponded to 2.75-3.03 g of dried leaves.

### 3.2. The effect of infusion variables on CA and RA content

Prior to the establishment of a BBD model, a series of preliminary experiments were performed to investigate the effect of each infusion parameter on the contents of CA and RA in tea solutions. From the experiments, it was found that the maximum CA and RA contents were obtained when infusing the tea in 80ºC of 300 mL water for 10 min, and 200 mL of boiling water for 10 min, respectively. The maximum levels of CA and RA were 0.030±0.001 and 0.497±0.008 mg per 1 g of tea, respectively. The relationship between infusion parameters, including infusion time (3-20 min), water temperature (60-100ºC), and water volume (100-300 mL), and the contents of CA and RA per 1 g of tea were demonstrated in Table 2. The results demonstrated that with increasing infusion time, the extracted amounts of CA increased. In the case of RA, increasing this variable favored RA extraction up to a maximum level at 10 min, after which there was a decrease in RA content. Since the long infusion time may result in the degradation of RA in the tea solution. It was also observed that the use of high water temperature, especially boiling water temperature, favored both extractions of CA and RA components. Since, raising the temperature increases the solubility and the diffusion coefficient of phenolic compounds, thereby providing higher dissolution rates[27]. For water volume, the increase in this parameter also enhanced the CA and RA contents which reached a plateau at 250 and 150 mL, respectively. As a result, the infusion condition for the maximum antioxidant activity was further optimized in these following infusion variable ranges; infusion time of 5-15 min, water volume of 150-250 mL and water temperature of 80-100ºC.

The other infusion parameter that has been reported to affect the amount of active contents transferred into the tea solution was stirring effect. The stirring process was reported to increase the rate of extraction and also enhance the extraction yields[21]. As this effect produces a decrease in the thickness of the diffusion films resulted into higher active contents in tea infusion[28]. However, the TL tea product used in this study was designed to infuse under non-stirring condition. Most of the sachet seals were leaked because of stirring action and cloudy tea solutions were obtained. Therefore, a new design of sachet is needed to infuse the tea under stirring condition.

### 3.3. Comparison of marker contents obtained from infusion and aqueous extraction

From the experiment, it was found that the average amount of CA and RA obtained by aqueous extraction process was 0.398±0.026 and 5.636±0.419 mg per 1 g of TL tea powder (Mean±S.D.), respectively. This means that the maximum amount of CA and RA obtained from the infusion variable studies was 7.46 and 8.81%

**Table 2.** Effect of infusions variable on marker contents

| Infusion time (min) | Temperature (°C) | Volume (mL) | Content (mg/g tea)[a] | |
|---|---|---|---|---|
| | | | Caffeic acid | Rosmarinic acid |
| 3 | 80 | 200 | 0.0047±0.0003 | 0.0467±0.0029 |
| 5 | 80 | 200 | 0.0062±0.0006 | 0.1171±0.0054 |
| 10 | 80 | 200 | 0.0166±0.0006 | 0.1755±0.0064 |
| 15 | 80 | 200 | 0.0184±0.0002 | 0.1482±0.0025 |
| 20 | 80 | 200 | 0.0244±0.0006 | 0.1287±0.0070 |
| 10 | 60 | 200 | 0.0111±0.0007 | 0.0455±0.0041 |
| 10 | 70 | 200 | 0.0122±0.0004 | 0.0625±0.0038 |
| 10 | 80 | 200 | 0.0166±0.0006 | 0.1755±0.0064 |
| 10 | 90 | 200 | 0.0205±0.0009 | 0.3430±0.0036 |
| 10 | 100 | 200 | 0.0280±0.0012 | 0.4967±0.0079 |
| 10 | 80 | 100 | 0.0073±0.0008 | 0.0981±0.0079 |
| 10 | 80 | 150 | 0.0114±0.0006 | 0.1883±0.0064 |
| 10 | 80 | 200 | 0.0166±0.0006 | 0.1755±0.0064 |
| 10 | 80 | 250 | 0.0280±0.0014 | 0.1750±0.0069 |
| 10 | 80 | 300 | 0.0297±0.0008 | 0.1594±0.0087 |

[a] Data were expressed as mean±S.D (*n*=3).

**Table 3.** Significant Regression Coefficients for Coded Variables of Quadratic Models

| Parameters | | Coefficient | Standard error | *p* value[a] |
|---|---|---|---|---|
| DPPH | | | | |
| Intercept | | 0.0337 | 2.66E-03 | < 0.0001 |
| time | $x_1$ | 0.0074 | 2.49E-03 | 0.0126 |
| temperature | $x_2$ | 0.0189 | 2.49E-03 | < 0.0001 |
| Temperature x Temperature | $x_2^2$ | 0.0108 | 3.64E-03 | 0.0129 |

[a] Significant at the 95% confidence level

of those contained in the aqueous extracts, respectively. Although the optimal infusion condition that maximized both CA and RA contents have not yet been investigated, the preliminary results could be used to imply that after infusion, the major portion of active markers remained in the tea powder. Therefore, the infusion process could be considered as poor extraction efficiency method.

In addition to infusion conditions, the marker contents in the tea solutions could be affected by the variation of plant material which leads to variation in therapeutic efficacy[22, 23, 29]. Therefore, the use of standardized extract would be benefits in terms of efficacy and quality consistency. As a result, the extraction condition for preparing TL extract should be optimized to obtain optimal amounts of marker contents.

### 3.4. Optimization of infusion condition to obtain maximum DPPH response

Table 1 shows the studies variables and DPPH responses of the tea solutions prepared under experimental conditions of BBD. The experimental data were subjected to multivariate analysis, and the generated regression coefficients of the model of DPPH response are shown in Table 3. The regression for the quadratic model was significant at the 95% confidence level, and the residuals were shown to be random. The ANOVA (Table 4) results indicated that the quadratic model showed no lack of fit for the data ($p$>0.05) suggesting that the model fit the data well. The coefficient of determination ($R^2$) from multiple correlation coefficients represented the relationship between predicted and actual values in each quadratic equation. The $R^2$ value of DPPH response was 0.8729, suggesting that there was a high degree of correlation between observed and predicted values. Therefore, the mathematical model was accepted and further used to explain the effects of the variables on the response and can be applied to optimize the condition for preparing TL tea to obtain optimal DPPH activity.

From Table 3, the results suggested that the variables significantly affecting DPPH responses were infusion time and water temperature, and this activity was not significantly affected by the water volume. The quadratic effect of temperature implied that increasing this variable dramatically favored the extraction of compounds possessing antioxidant activity into the tea solution, which led to high DPPH response. Three-dimensional response surface and contour plots are illustrated in Figure 1. The plots show the effect of infusion time and water temperature on the DPPH response, while the water volume was kept constant at 200 mL. As can be seen, the DPPH responses continuously increased as increasing infusion time and water temperature. The maximum DPPH activity of TL tea was obtained when boiling water and long infusion time (15 min) were used for tea infusion. These results agreed with previous studies whereby the antioxidant activity of teas was directly proportional to water temperature and infusion time. Since these factors affected the transfer of chemical contents possessing antioxidant activity of herbal extracts[18, 20, 21, 25].

**Table 4.** Analysis of Variance (ANOVA) of Quadratic Polynomial Models for DPPH Response

| Sources of variations | Sum of square | DF | Mean square | F-value | *p* value[a] |
|---|---|---|---|---|---|
| DPPH ($R^2$ = 0.8729) | | | | | |
| Regression | 3.74E-03 | 3 | 1.25E-03 | 25.18 | < 0.0001 |
| Residual error | 5.44E-04 | 11 | 4.90E-05 | | |
| Lack of fit | 3.37E-04 | 5 | 6.70E-05 | 1.95 | 0.2200 |
| Pure error | 2.07E-04 | 6 | 3.50E-05 | | |
| Total SS | 4.28E-03 | 14 | | | |

DF, degree of freedom; $R^2$, coefficient of determination.[a] Significant at the 95% confidence level

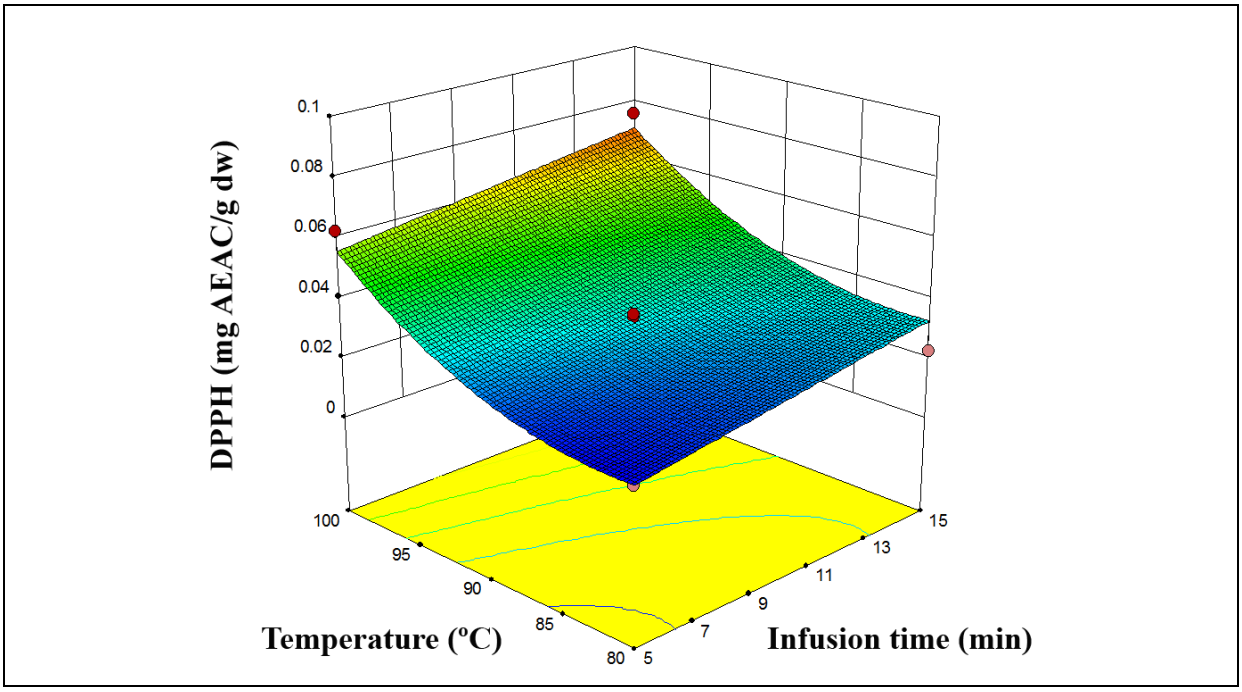


**Figure 1.** Response surface and contour plots of DPPH response (mg AEAC/g dw). Variables: Temperature (ºC) × Infusion time (min), with the volume fixed at 200 mL.

The optimal infusion condition for each herbal tea depends on what active contents or biological effects were desired. For this study, the infusion condition that maximized antioxidant activity of TL tea was investigated by using DPPH activity as a model response. This activity may come from a synergistic effect that occurs between several compounds in the TL tea. Furthermore, since the therapeutic efficacy or biological activities were directly correlated to the amounts of absorbed active ingredients[30]. Therefore, for the optimal use of TL tea, the infusion condition that maximizes active marker contents and the interactions between infusion variable on marker contents will be further investigated in the same studied infusion ranges for DPPH activity. In the case of RA, the preliminary results suggested that the infusion condition should be carefully optimized. Because RA trended to degrade in hot water with increasing infusion time as reported for other teas when high water temperature and/or long infusion time were used for infusion[19, 20]. More importantly, the relationship between the clinical efficacy of TL tea and its oral dose of active markers was still unclear. The optimal infusion condition should be applied in a clinical trial to find a relationship between the oral dose of active markers and their clinical efficacy. Ultimately, the therapeutic oral dose of TL tea will be obtained and could be used as support information to calculate the oral dose of the aqueous extract.

## 4. CONCLUSIONS

In summary, this study demonstrated that the infusion time, water temperature and volume were critical infusion variables affecting the extraction of CA and RA into the TL tea solutions. More importantly, when comparing the marker contents obtained by infusion to those of aqueous extracts, it was found that the major portions of markers remained in the discarded tea powder. Therefore, the infusion could be considered as a low-efficiency extraction method. With the use of RSM, it was successfully found that the significant infusion variables affecting DPPH response were infusion time and water temperature. The maximum DPPH activity was obtained when infusing the TL tea in 200 mL of boiling water for 15 min. The results of this study can help further understanding of the maximal health benefits to be attained through consumption of TL tea. Furthermore, since the therapeutic efficacy was directly correlated to the amounts of absorbed active ingredients[30], the infusion condition that maximized CA and RA contents will be further assessed by using RSM. Then, the optimal infusion will be applied in a clinical trial to find a relationship between active contents and therapeutic efficacy.

## 5. ACKNOWLEDGEMENTS

### Conflict of interest
The authors declare no conflicts of interest.

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### Ethical approval
None to declare

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