Optimization formulation of chamomile-based functional beverage as hypnotic agent using response surface method

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KEYWORDS

Apigenin
EGCG
Functional beverages
Insomnia

ABSTRACT

Insomnia problem can be treated by consuming herbs containing apigenin and EGCG. Chamomile and green tea water extract have been found to contain apigenin and EGCG. Optimization of the formulation of those herbs is necessary to obtain the highest hypnotic activity. The purpose of this study was to optimize formulation of chamomile, green tea, and cinnamon to obtain functional beverage containing the highest phenolic and flavonoid content, but the lowest caffeine content. The best formula of the herbs was then tested in caffeine-induced mice to see the hypnotic effect. The research design in this experiment was Response Surface Model (RSM) using Central Composite Design (CCD) method. The in vivo test used Post Test Only Control Group Design. Consisting five groups of 30 male mice divided to 6 mice for each group. The optimum formula for functional beverages suggested by RSM was chamomile: green tea: cinnamon of 70.32: 30.35 : 4.99. The verification results showed that the actual response of this optimum formula had a total phenol of 23.76 mgGAE/g dry herbs, total flavonoids of 126.43 mgQE/g dry herbs, caffeine content of 29.87 mg caffeine/g dry herbs, apigenin levels of 0.07±0.068 µg/g dry herbs, and EGCG of 6.43±0.218 µg/g dry herbs. Functional beverages showed hypnotic activity in group of mice dosed 26 mL/kg bw, which significantly reduced motor activity to 4.83±0.72 sec. As well as significantly reducing the proinflammatory cytokines TNF-α and IL-6 to 3.17±0.53% and 5.08±0.35% (α=0.05), respectively.

Introduction

Insomnia is one of the world’s health problems with prevalence up to 40%. Specifically, 10-15% of the world population suffers from chronic insomnia and 25-27% suffer from acute insomnia (Roth et al., 2007; Sadehniiat-Haghighi et al., 2014). Insomnia is a person’s condition with less quantity and quality of sleep (Roth and Roehrs, 2003). In the long term, insomnia has a negative impact on physical (Morgan et al., 2012), physiology such as an increase in the body mass index (BMI) (Banks, 2007), as well as psychologies such as depression, anxiety, and major depressive disorder (MDD) (Manber et al., 2008). In addition, people who sleep less than 5 hours a night have lower life expectancies than people who sleep 7-8 hours (Morgan et al., 2012).

As many as 25-30% of patients diagnosed with insomnia are patients with primary insomnia (Kumar and Bhat, 2012). Primary insomnia is both acute or chronic insomnia that affects healthy individuals due to hyperarousal or excessive alert activity in central serotonergic systems (Roth et al., 2007). Hyperarousal is generally triggered by various factors, one of them is caffeine consumption before bedtime (Stein and Stein, 2008; Lara, 2010; Paterson et al., 2007). This hyperarousal condition can be inhibited by inhibitory neurotransmitters type γ-amino butyric acid (GABA) in central nervous system (CNS) (Pham et al., 2009; Hanrahan et al., 2011, 2015). GABA activity can be increased by consuming sedative-hypnotic drugs. However, the use of sedative-hypnotic drugs for four weeks causes various side effects such as imbalance of cognitive response, memory, and daily activities (Dhawan, 2003). In long term, it can also lead to drug tolerance (Hood et al., 2014), decreased cognitive function (Baldessarini et al., 2006), and drugs dependence (Ashton, 2005).
Consuming herbs containing hypnotic compound instead of drugs were suggested for people to reduce insomnia (Morin et al., 2003; Pearson, 2006; Bystritsky et al., 2012). The results prove that functional food can reduce risk factors of insomnia (Johnston, 2005; Stojanovic et al., 2017). A functional beverage with hypnotic effects and has potential for further development is functional beverages based on chamomile, green tea, and cinnamon.

Chamomile (Chamomilla recutita L.) was known to be binds to the allosteric side and forms a complex with GABA_A receptors (Johnston, 2005). Chamomile-based beverage has been shown to overcome sleep disorders and induce deep sleep within 10 minutes after consumption (Sharafzadeh and Alizadeh, 2011). Green tea (Camellia sinensis) shows hypnotic activity (Adachi et al., 2006) because epigallocatechingallate (EGCG) is able to binds to the allosteric side of the GABA_A receptor (Aoyama and Nakaki, 2013) in GABA_A receptors (Campbell et al., 2004). While the cinnamon bark (Cinnamomum burmanii) contains oleoresin which gives sweet flavor of functional beverages (Guimaraes et al., 2013; Marwa et al., 2017). Caffeine is not expected in functional beverages, so it needs to be minimized by formulating chamomile in higher amounts (above 50%) and formulate green tea in lower amounts (below 50%). This is because chamomile is a caffeine-free herbal tea (Raini, 2011; Guimaraes et al., 2013). Those herbs need to be formulated to obtain a higher synergistic effect between apigenin and EGCG, so the hypnotic activity of functional beverages is higher than using only one type of herb (Johnston, 2005).

Stevia is chosen as a sweetener because it has a sweetness level up to 200-300 times compare to sugarcane, resistant to high temperatures up to 200°C (392° Fahrenheit), does not provide a bitter aftertaste, and does not affect blood sugar levels, making it safe for diabetics (Saniah and Hasimah, 2008; Wuryantoro and Susanto, 2014). Optimization of functional beverage formulations was carried out using RSM method. This method was chosen because it can evaluate the relationship between the independent variable and the dependent variable (Saniah and Hasimah, 2008). The purpose of this study was to analyze the effect of the optimum formula on total phenol, total flavonoids, caffeine content, apigenin levels and EGCG levels on functional beverages, and analyze the effect of optimum functional beverage formula on the treatment of motor activity and proinflammatory cytokines in male mice.

**Research Methods**

**Material**

Dried chamomile flowers were obtained from the Surabaya Distro Herbal, Surabaya, Indonesia. Dried green tea leaf and cinnamon were obtained from Anugrah Alam Herbs, Yogyakarta, Indonesia. Those herbs mixtures were powdered using a dry blender then sieved to pass 60 mesh.

**Analysis of Herbs**

Water content was analyzed using the oven drying method AOAC (AOAC: Official Methods of Analysis, 1995), total phenol used method of Folin-Ciocalteau (Folin and Ciocalteu, 1944), total flavonoid used method of aluminum chloride (AlCl_3) (Atanassova et al., 2011), and caffeine content used UV-Vis Spectrophotometer method (Arwangga, 2016). For those analyses, each herb was extracted using water (infusion) at 90°C for 5 minutes.

**Formulation Optimization**

Optimization process divided into 2 stages, namely the preliminary research stage and the main research stage. Preliminary research is an experiment to obtain a lower limit and the upper limit of each herb to obtain for main study by formulating chamomile above 50% and green tea below 50% in the mixture. The research design used in this experiment was the Surface Response Method (RSM) Central Composite Design (CCD) using Design Expert 7.0 software method with three factors (ratio of chamomile flowers, green tea, and cinnamon) to three responses (total phenol, total flavonoids, and caffeine content). The optimal formula obtained is then verified to obtain % error (Atanassova et al., 2011). % error is a comparison of the suggested predictive value compared to the real test value. If the value of % error is less than 5% it can be ignored and the optimal formula suggested can be considered as the best formula (Silveira et al., 2015). Furthermore, the optimal formula was added 5% of stevia, then levels of apigenin and EGCG was analyzed using the LC-MS/MS method.

**Extraction of Herbs**

The extraction of herbs mixture was carried out by the infusion method using water at 90 °C for 5 min (Harbourne et al., 2009). The ratio of herbs to water was 1:40 (b/v). The proportion of each herbs used was based on the results of the RSM formulation optimization.
Animal Experimental Procedure
The design experiment for animal study was True Experimental Design: Post Test Only Control Group Design with control group and an experimental group. Measurements of were only made once, after the treatment was given to the experimental group.

Thirty healthy male mice (*Mus musculus*) weighting 25±2 g, aged 6-8 weeks old were divided into 5 groups. All groups were as follows: normal control group (K1) received water without caffeine injection; positive control group (K2) was intraperitoneally injected with caffeine 10 mL/kg bw. Group K3 and K4 were mice injected with caffeine 10 mg/kg bw and orally gavaged with 13 and 26 mL/kg bw, respectively. Group K5 were mice injected with caffeine of 10 mg/kg bw and orally gavaged with commercial sedative-hypnotic herbal tea. A summary of the procedure of experimental animals can be seen in Figure 1. During adaptation for 7 days, mice accessed feed and drinking freely (12 hours light and 12 hours dark). Mice underwent a 15-minute rotarod test (Deacon, 2013). After the rotarod test, the mice were dislocated and then spleens were removed. The spleen was harvested and to test the proinflammatory cytokines of TNF-α and IL-6 using a flowcytometer (Dwijayanti and Rifa'i, 2014).

Data analysis
The normality of the data was analyzed by the Kolmogorov-Smirnov test. Then the data was analyzed using one-way ANOVA and post-hoc Fisher Pairwise Comparisons (p <0.05) in Minitab 16 (Minitab Inc.).

Results and Discussion
Analysis of Herbs
The extraction of herbs mixture was carried out by the infusion method using water at 90 °C for 5 min because water infusion at 90 °C was the optimal temperature for the extraction of phenol from the three herbs (Kawiji et al., 2009). The duration of herbs water extraction is based on the research of Cleverdon et al. (2018), that 80-90% of phenol is extracted well in the first 5 minutes. Addition of extraction time 5 minutes later did not increase the concentration of phenol obtained. This is also supported by the study of McAlpine and Ward (2016) that more than 50% of phenol is recovered in the first 5 minutes of steeping and that the addition of steeping time does not significantly increase the amount of phenol that is increased. The difference in total phenol compared with the literature is suspected because other factors that influence other than extraction methods include the differences in plant genetics, cultivation location, harvest time, drying process, extraction process, type of solvent, standard used, and others (Haghi, 2014). Result analysis of each herb shown in Table 1.

Table 1 shows the moisture content of chamomile, green tea, and cinnamon are below 10% which met the water content standard (Thomas, 2003). The analysis of total phenol of chamomile flower infusion is higher than the average of total phenol from McAlpine and Ward (2016) study which brewed chamomile flowers for 5 minutes at 90 °C which is 1.1 ± 0.1 mg GAE/g. When compared with the highest total phenol of chamomile steeping in the McAlpine and Ward (2016) study, which was 3.0 ± 0.2 mg GAE/g, the total phenol infusion of chamomile flowers in this study remained higher. When compared with the highest total phenol of green tea steeping in the McAlpine and Ward (2016) study, which was 27.7±5.5 mg GAE/g, the average total phenol of green tea infusion in this study was appropriate. The total phenol of cinnamon infusion was in accordance with total polyphenols from the results of Vallverdú-Queralt et al. (2014) which was 5.82 ± 0.44 mg GAE/g, but lower than the total phenol average of the results of the study by Muchuweti et al. (2007) which was 13.66 mg GAE/g.
The total flavonoid of infused chamomile in this study was in accordance with the results of the study by Akinseye and Yetunde (2016) which made the infusion of chamomile flowers at 60-80 mg QE/g. However, it was higher than the total mean of flavonoids from Zekovic et al. (2015) which extracted chamomile flowers at a temperature of 80 °C with the value of 8.85 ± 0.1 mg RE/mL. The total flavonoid of green tea infusion in this study was in accordance with the results of the study Akinseye and Yetunde (2016) which was 160-190 mg QE/g. The average of total green tea flavonoids of the study was higher than the total flavonoids average of the results of Bansode (2015) study which was 23.17 mg QE/g-48.81 mg QE/g. But it was lower than the total flavonoids from the study of Nadiah and Uthumporn (2015) which was 252.67 ± 3.79 mg CEG/g. The total flavonoid of cinnamon infusion in this study was higher than the total flavonoids from the study results Pasakawee and Utama-ang (2018) which extracted cinnamon using water at a temperature of 95±2 °C which was 5.22±0.27 mg QE/g.

Green tea infusion contains 30.70±0.75 mg caffeine/g. This is in accordance with the research of Tim Hortons Research and Development (2017) that the caffeine content of green tea infusion varies between 25-60 mg of caffeine/g. Whereas Friedman et al. (2006) showed that the caffeine content of green tea can vary between 1-33 mg/g. According to Tfouni et al. (2018), green tea contains caffeine 18.3-25.3 mg/g.

### Formulation of Functional Beverages

Preliminary research aimed to obtain the upper limit and lower limit that was then inputted to RSM. Functional beverage formulations containing chamomile, green tea, and cinnamon in the preliminary research is presented in Table 2.

Efforts to reduce caffeine levels by regulating green tea formulations in functional beverages (Palma et al., 2006; Everson et al., 2005; Vgontzas et al., 2000; Opp et al., 2005). Therefore, in the preliminary study, chamomile in higher amounts (above 50%) and lower amounts of green tea (below 50%) is formulated. This is because chamomile is a caffeine-free herbal tea (Netto et al., 2009; Olayiwola et al., 2007; Head and Kelly, 2009; Shinomiya et al., 2005). Whereas cinnamon added was 5% as the results of the study by Anggraini et al. (2015) found that the addition of 5% cinnamon shows the highest aroma and taste preference compared to the addition of cinnamon as much as 4%.

### Values in the form of average ± standard deviation from 3 replications. Herbs in powder form for analysis of moisture content. Herbs in liquid form for analysis of TPC, TFC, and CC

### Table 1. Average of result analysis of each herbs

<table>
<thead>
<tr>
<th>Herbs</th>
<th>Moisture Content (%)</th>
<th>Total Phenolic Content (mg GAE/g)</th>
<th>Total Flavonoid Content (mg QE/g)</th>
<th>Caffeine Content (mg caffeine/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chamomile</td>
<td>6.42±0.004</td>
<td>15.83±1.51</td>
<td>91.58±9.01</td>
<td>0.92±0.23</td>
</tr>
<tr>
<td>Green Tea</td>
<td>4.92±0.004</td>
<td>28.53±2.36</td>
<td>194.89±14.60</td>
<td>30.70±0.75</td>
</tr>
<tr>
<td>Cinnamon</td>
<td>7.96±0.001</td>
<td>5.16±1.12</td>
<td>8.30±8.86</td>
<td>0.96±0.32</td>
</tr>
</tbody>
</table>

### Table 2. Formulation of chamomile, green tea, and cinnamon

<table>
<thead>
<tr>
<th>Proportion Formulation</th>
<th>Dependent Variable</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Total phenolic content (mg GAE/g)*</td>
</tr>
<tr>
<td>Chamomile</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>55</td>
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<tr>
<td></td>
<td>60</td>
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*Values in the form of average mg/g infusion ± standard deviation from 3 replications
Based on the results of preliminary research, the best functional beverage formulations were found in the treatment of chamomile : green tea : cinnamon (70:30:5) which had a total phenol of 23.84±0.24 mg GAE/g, total flavonoids of 127.93±0.23 mg QE/g, and caffeine content of 28.36±0.15 mg caffeine/g.

The formulation was chosen because when compared with the treatment of chamomile:green tea:cinnamon (70:25:5) there was an increase in total phenol by 3.35 mg GAE/g and an increase in total flavonoids by 9.68 mg QE/g, but the caffeine content was only increased by 0.86 mg of caffeine/g. This increase in total phenol and flavonoids was much higher than that of in other treatments. This increase in caffeine levels was also much lower than the increase in caffeine levels among other treatments. When green tea was added to the formulation into chamomile:green tea: cinnamon (65:35:5) only increased total phenol by 0.92 mg GAE/g and increases in total flavonoids by 1.55 mg QE/g, but there was a significant increase in caffeine levels by 5.53 mg of caffeine/g. Therefore, the formulation of chamomile:green tea:cinnamon (70:30:5) was used as a center point for formulation optimization in the main research.

**Main Research**

The best formulation in preliminary studies was 70% chamomile, 30% green tea, and 5% cinnamon. For chamomile, the lower limit and the upper are 65% (-5) and 75% (+5), respectively. For green tea, the lower limit and upper limit were 25% (-5) and 35% (+5), respectively. For cinnamon, the lower limit and upper limit were 3% (-2) and 7% (+2), respectively. The variables studied in the optimization of functional beverage formulations using the CCD method can be seen in Table 3.

<table>
<thead>
<tr>
<th>Table 3. Factors and responses used in RSM design</th>
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<tbody>
<tr>
<td><strong>Independent Variable</strong></td>
</tr>
<tr>
<td>Factor</td>
</tr>
<tr>
<td>Chamomile</td>
</tr>
<tr>
<td>Green Tea</td>
</tr>
<tr>
<td>Cinnamon</td>
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<th>Table 4. Factor and response of RSM design</th>
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<tr>
<td><strong>No</strong></td>
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</table>

*Values in the form of average mg/g herb ± standard deviation from 3 replications
Modeling and Response Analysis of Total Phenolic Content

The results of the analysis of the total phenolic content response, indicate that the quadratic model was the suggested model. In the summary statistical model, the quadratic model has the highest adjusted $R^2$ and predicted $R^2$ values compared to the linear and 2FI models, which were 0.8292 and 0.5393, respectively. The quadratic model is known to have a low PRESS (Prediction Error Sum of Squares) value of 75.20. Figure 2 shows the model produced by the total phenol response was a quadratic model. This model has a $R^2$ value of 0.9101 which approaches the value of 1. A close value to 1, shows that the correlation between the value of observation and predictive value is increasingly appropriate (Saniah and Hasimah, 2008).

![Figure 2. Surface response of total phenolic content](image)

Modeling and Response Analysis of Total Flavonoids Content

The results of the analysis of total flavonoid content responses indicate that the quadratic model was the suggested model. In the summary statistical model, the quadratic model has the highest adjusted $R^2$ and predicted $R^2$ values compared to the linear and 2FI models, which were 0.9609 and 0.8744, respectively. The quadratic model is known to have a low PRESS value of 471.59. Figure 3 shows the model produced by the total response of flavonoids is a quadratic model. This model has a $R^2$ value of 0.9794 closed to the value 1.

![Figure 3. Surface response of total flavonoid content](image)

Modeling and Response Analysis of Caffeine Levels

The results of the program analysis on the response of caffeine levels indicate that the quadratic model was the recommended model. In the summary statistical model, the quadratic model has the highest adjusted $R^2$ and predicted $R^2$ values compared to the linear and 2FI models, which were 0.9406 and 0.8001 respectively. The quadratic model is known to have low PRESS value of 248.20. Figure 4 shows the model produced by the response to caffeine content was a quadratic model. This model has a $R^2$ value of 0.9687 which approaches the value 1.

![Figure 4. Surface response of caffeine levels](image)

Formulation Optimization of Total Phenolic Content, Total Flavonoid Content, and Caffeine Levels Response

The recommended optimal formulation (prediction on RSM) is verified and compared with actual testing, can be seen in Table 5.
Table 5. Results of verification of the optimum formula suggested by RSM

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>Response</th>
<th>Response Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>X₁ (%)</td>
<td>X₂ (%)</td>
<td>X₃ (%)</td>
</tr>
<tr>
<td>70.32</td>
<td>30.35</td>
<td>4.99</td>
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</table>

Based on Table 5, the recommended formulation was the proportion of chamomile 70.32%, green tea 30.35%, and cinnamon 4.99%. This formulation produces optimal response predictions, namely the total phenol value of 23.0387 mg GAE/g, total flavonoids of 124.908 mg QE/g, and caffeine content of 20.9932 mg caffeine/g with a fairly good desirability value of 0.8886, as shown in Figure 5.

Quantitative Analysis of Apigenin and EGCG

The chromatogram analysis of the content of apigenin and EGCG in the optimum functional beverage formula can be seen in Figure 6. The results of quantitative analysis of the content of apigenin and EGCG can be seen in Table 6.

Table 6. Results of analysis of the content of apigenin and EGCG

<table>
<thead>
<tr>
<th>Compound</th>
<th>Average Content (µg/g)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apigenin aglycone</td>
<td>0.07±0.068</td>
</tr>
<tr>
<td>EGCG</td>
<td>6.43±0.218</td>
</tr>
</tbody>
</table>

*Values are the mean ± standard deviation of 4 replications

The content of apigenin and EGCG of functional beverage is shown in Table 6. Apigenin results are in accordance with Haghi et al. (2014) who used the maceration extraction method for 24 hours. Their results indicated that chamomile...
flower water extract contained 0.68% hydrolyzed apigenin-7-glucoside and 0.28% apigenin-7-O-glycoside not hydrolyzed. While the amount of apigenin aglycone in the lower amount is 0.38% for hydrolyzed apigenin and 0.04% for apigenin not hydrolyzed. EGCG results are in accordance with the results of Cabrera et al. (2003) who analyzed EGCG using HPLC, which giving the value in the range of 0.4-128.0 µg/mL. The results of EGCG results vary depending on the type and method of processing tea. This is in accordance with the statement of Haghi et al. (2014) that flavonoid levels are influenced by various factors, plant genetics, cultivation location, harvesting time, drying process but the extraction process is the most influential factor (Ramlah, 2017).

**Male Mice in Vivo Test**

**Effect of Functional Beverages on Motor Activity**

One of the most common tests for analysis of motor activity is rotarod (Rustay et al., 2003). The results of the analysis of motoric activity of mice using the rotarod test can be seen in Table 7.

In the positive control group, mice can last the longest compared to other groups in the rotarod. This is in accordance with the study of Connole et al. (2004) that the administration of acute caffeine (20 mg/kg) increases mice motor activity evaluated in rotarod acceleration. The results of this study are also supported by López-Cruz et al. (2013) and López-Cruz et al. (2014) which show that low-dose acute caffeine can increase motor activity, alertness, and reduce the effect of coordination. Caffeine is chosen because at doses equivalent to one cup of coffee, it can prolong sleep time and increase locomotor activity (Roth and Roehrs, 2003).

Mice in the group dosage 26 mL/kg bw fell faster than the rotarod compared to the other groups. This shows that flavonoids especially apigenin and EGCG have hypnotic effects. Flavonoids are ligands for GABAₐ receptors in the CNS that show effects such as benzodiazepines (Bhattacharya et al., 2011). These flavonoids exhibit high affinity bonds in the GABAₐ-benzodiazepine binding receptor Roth and Roehrs, 2003; Rustay et al., 2003) so that they have anxiolytic effects, sedation, decreased motor activity (Netto et al., 2009), and hypnotics (Olayiwola et al., 2007).

Based on the results of further tests, functional beverages showed hypnotic activity which could be observed from a decrease in motor activity of mice that was significant at the rotarod. This is supported by research (Head and Kelly, 2009) that 10 out of 12 insomnia patients can sleep 10 minutes after consuming chamomile tea. The results of this study are also reinforced by research by Shinomiya et al. (2005) that in trials in experimental animals, chamomile can reduce sleep time. While 1/2 dose functional beverages and commercial functional beverages did not show significant differences from each other. It is suspected that the two beverages do not contain bioactive components which sufficiently show hypnotic activity.

**Effect of Functional Beverages on Proinflammatory Cytokines on Mice**

Insomnia affects cytokine levels in humans (Chavez-Valdez, 2011; Motivala and Irwin, 2007) and rodents (Motivala and Irwin, 2007; An et al., 2015). The results of the analysis of the proinflammatory cytokines of mice using a flow meter can be seen in Figure 7.

Based on the results of the plot flow meter in Figure 7, spleen mice in the positive control group showed the highest expression of TNF-α, whereas spleen mice in the negative control group showed the lowest expression of TNF-α. Optimized extract at a dose of 26 mL/kg bw can reduce TNF-α expression as indicated by the results of the plot flow meter almost matching the expression of negative control TNF-α. Whereas spleen mice in optimized extract at a dose of 13 mL/kg bw and commercial sedative-hypnotic showed relatively similar TNF-α expression. The results of the analysis of TNF-α expression are relevant with the expression of the results of IL-6 proinflammatory cytokines in Figure 8.

**Table 7. Results of motoric activity analysis of mice using the rotarod test**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Time of Falling Mice (sec)</th>
<th>Rotarod Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control</td>
<td>11.00±1.48c</td>
<td>3</td>
</tr>
<tr>
<td>Negative Control</td>
<td>21.22±1.85a</td>
<td>4</td>
</tr>
<tr>
<td>Sleep Well Beverage</td>
<td>18.17±1.01b</td>
<td>3</td>
</tr>
<tr>
<td>Functional Beverage Dosage 13 ml/kg bw</td>
<td>18.22±1.44b</td>
<td>3</td>
</tr>
<tr>
<td>Functional Beverage Dosage 26 ml/kg bw</td>
<td>4.83±0.72d</td>
<td>1</td>
</tr>
</tbody>
</table>

*Values are in the mean ± standard deviation of 6 replications. Numbers followed by different letter notations show significant differences in Fisher Pairwise Comparisons (α = 0.05)
Figure 7. Results of analysis of proinflammatory cytokines of TNF-α in mice spleen (Area M1: cells expressing TNF-α.) (a) negative controls, (b) positive controls, (c) optimized extract at a dose of 26 mL/kg bw, (d) optimized extract at a dose of 13 mL/kg bw, (e) commercial sedative-hypnotic

Figure 8. Results of analysis of proinflammatory cytokines of IL-6 in mice spleen (Area M1: cells that express IL-6. (a) negative controls, (b) positive controls, (c) optimized extract at a dose of 26 mL/kg bw, (d) optimized extract at a dose of 13 mL/kg bw, (e) commercial sedative-hypnotic
Table 8. Results analysis of proinflammatory cytokines of mice using flowcytometers

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Proinflammatory Cytokines</th>
<th>% total TNF-α*</th>
<th>% total IL-6*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control</td>
<td></td>
<td>1.15±0.22c</td>
<td>2.31±0.74c</td>
</tr>
<tr>
<td>Negative Control</td>
<td></td>
<td>20.35±0.40a</td>
<td>25.03±0.66a</td>
</tr>
<tr>
<td>Sleep Well Beverage</td>
<td></td>
<td>14.22±0.29b</td>
<td>17.15±0.27b</td>
</tr>
<tr>
<td>Functional Beverage Dosage 13 mL/kg bw</td>
<td></td>
<td>12.99±0.34c</td>
<td>16.15±0.27c</td>
</tr>
<tr>
<td>Functional Beverage Dosage 26 mL/kg bw</td>
<td></td>
<td>3.17±0.53d</td>
<td>5.08±0.35d</td>
</tr>
</tbody>
</table>

*Values are mean ± standard deviation of 6 replications. Values followed by different notations show significant differences (α = 0.05)

Based on the results of the flow meter plot in Figure 8, spleen mice in the positive control group showed the highest IL-6 expression, while the spleen mice in the negative control group showed the lowest expression of IL-6. Dosing 1 functional beverage can reduce IL-6 expression as indicated by the results of the plot flow meter almost equal to the expression of negative control IL-6. Whereas, spleen mice in the 1/2 dose functional beverage group and commercial functional beverages showed relatively similar IL-6 expression. The results of the analysis of proinflammatory cytokines TNF-α and IL-6 expressed in spleen mice can be seen in Table 8.

Table 8 shows the increase in both TNF-α and IL-6 was significant in the positive control group due to injection of caffeine. Caffeine causing insomnia (López-Cruz et al., 2013). If insomnia occurs in the first half of sleep (nighttime for humans or morning for mice), cytokine IL-6 increases more significantly than in the latter half of sleep (Vgontzas et al., 2000). Increasing IL-6 also activates which is directly proportional to the increase in body temperature, increased awareness, and higher concentration of cytokines in plasma. This is because an increase of 10-20 μg/mL of caffeine in plasma correlates with an increase in proinflammatory cytokines such as IL-1 (Oppe, 2005; Vgontzas et al., 2000), IL-6 (An et al., 2015; Motivala and Irwin, 2007), and TNF-α (Chavez-Valdez et al., 2011).

Low levels of proinflammatory cytokines in the negative control group is because of the mice were not induced by caffeine, so the levels of proinflammatory cytokines were low as normal mice. This shows that improving inflammatory conditions due to insomnia can reduce proinflammatory cytokine levels, but it cannot reverse cytokine levels to normal conditions. This is supported by various studies that flavonoids can reduce proinflammatory cytokines in the brain (An et al., 2015) and inhibit TNF-α production in microglial cells of mice (Patil et al., 2014), but do not return the amount to normal conditions.

The results of further analysis showed that the treatment of dosage 26 mL/kg bw had the most significant effect in reducing proinflammatory compared dosage 13 mL/kg bw treatment and commercial functional beverage treatment. This shows that dosage 26 mL/kg bw is the appropriate dose of functional beverage to reduce proinflammatory cytokines. This is supported by research results (Head and Kelly, 2009) that 10 out of 12 insomnia patients can sleep 10 minutes after consuming chamomile tea. The results of this study are also reinforced by research by Shinomiya et al. (2005) that in trials in experimental animals, chamomile can reduce sleep onset. Whereas dosage of 13 mL/kg bw and Sleep Well beverage indicating that the contained bioactive compounds have not reached sufficient amounts as hypnotics.

Conflict of interest
The authors declare that there is no conflict of interest in this publication.

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