In vitro antioxidant activity of meniran (*Phyllantus urinaria*) functional drink in human low density lipoprotein (LDL)

U Fitrotin¹, N Hilmiati¹, Mardiana¹, Y Triguna¹, A Surahman² and A Hipi¹

¹Assessment Institute for Agricultural Technology West Nusa Tenggara, Mataram, Indonesia
²Indonesian Agency for Agricultural Research and Development, Jakarta, Indonesia

Email: ulyaclariyf@gmail.com

**Abstract.** Preparation process for meniran (*Phyllantus urinaria*) functional drink (MFD) influences its antioxidant activity. This research aims to understand the phenolic content, DPPH Radical Scavenging Activity (RSA), and LDL oxidation of MFD through various preparation processes. Those preparation processes included soaking fresh meniran (SFM), boiling fresh meniran for 5 minutes (BFM5'), boiling fresh meniran for 10 minutes (BFM10'), and soaking dried meniran (DM). The phenolic content was determined with Folin–Ciocalteu, antioxidant activity was assessed using DPPH and TBARS assay with LDL as the oxidation substrate. An antioxidant references in this research used ascorbic acid. The phenolic content in methods of SFM, BFM5', BFM10' and DM were 122±0.022, 182±0.043, 192 ±0.03, and 117 ±0.019 mg GAE/g of meniran respectively. Meanwhile, the DPPH RSA of SFM, BFM5’, BFM10’ and DM accounted for 82.18±0.35, 86.19±0.53, 86.75±0.64 and 69.96% respectively. As comparison, the DPPH RSA of ascorbic acid 50 ppm is 75.65±0.82%. At the same time the optimum inhibition of TBARS formation from BFM5’ and BFM10’ methods were 45.83 % and 48.66%, with MDA concentration in human LDL accounted for 38.30±2.39 and 36.30±1.82 nmol MDA/mg protein, respectively. As comparison, MDA concentration in human LDL added with ascorbic acid 25 ppm accounted for 41.35±2.41 nmol MDA/mg protein. In contrast, the control human LDL was 70.70±2.35 nmol MDA/mg protein. This study concludes that the BFM5’ and BFM10’ methods showed the highest antioxidant properties compared to other methods. All methods showed that MFD extract in concentration more than 25 ppm increased the concentration of MDA in human LDL. Therefore, to produce meniran functional drink in optimum antioxidant properties is best by using BFM5’ and BFM10’ preparation methods in meniran concentration of not more than 25 ppm.

1. Introduction

Natural antioxidants are still one of the safe options for health maintenance and disease therapy. Consumption of natural antioxidants in the right dose poses no side effects when used in a long time [1]. Traditional Chinese medicine using medicinal plants as main ingredients has been used as one of therapy for people with Covid 19 in China where the suitable medicine is not found yet for this particular disease [2]. This shows that traditional medicine is still seen as one of the safest alternative medicines with minimal negative side effects and has great impact for recovery owing to the bio-active contents. At the same time, requirements for functional food have encouraged the development of natural products from medicinal plants and other plant extracts that consist of bioactive compounds [3]. Herbal drink or known as *jamu* is one of functional drinks that has been long known in Indonesia. Up until now, the process for...
jama production emphasises on its sensory quality and often overlooks its functional nature. Jamu preparation process methods vary depending on local conditions and culture such as biopharmaceutical dipping, boiling at various times and drying of the medicinal plant materials [4]. Ingredients and preparation processes are among determinant factors that influence the antioxidant properties of the functional drink.

Preparation processes influence chemical compounds of medicinal plants that have an impact on its bioactivity. For example, steaming ginseng root can decrease its ginsenosides polarity [5]. This treatment will increase its role against cancer [6];[7]. Meanwhile, fresh ginger, dried ginger and steamed ginger have different chemical composition and anti-cancer effects [8];[9]. Another example is fermentation treatment for sesame milk can improve its ability to inhibit MDA development at human blood LDL by two folds as compared to those without fermentation treatment [10].

The menira (Phyllanthus niruri) plant has anti-tumor effect [11], antibacterial, antioxidant, and anti-HIV-1 reverse transcriptase activities [12]. Meniran extract contains active compounds which include: phenol, saponins, tannins and flavonoids (based on phytochemical screening) [13], and also contains rutin, gallic acid, and quercetin [14]. In this research, the antioxidant activity of P. niruri was assessed using DPPH RSA method and MDA oxidation inhibition at human Low density lipoprotein (LDL). Esthether cholesterol is the highest component in LDL; hence LDL is prone to oxidation that can lead to many diseases. One LDL molecule consists of a number of lipid acid, 50% of them are Polysaturated Fatty Acids (PUFA) which is very vulnerable to oxidation. LDL oxidation is one of the causes of cardiovascular disease.

Some research shows that bio active compounds assessed using the DPPH RSA method are not able to show the system inside the body. Other research shows that bioactive compounds evaluated using the DPPH RSA method have low antioxidant activities, yet after being evaluated using biological systems, they show high antioxidant activities [15]. Therefore, the antioxidant activities of meniran functional drink are assessed using DPPH RSA method and MDA oxidation inhibition at human Low Density Lipoprotein (LDL). At the same time, the information about the preparation process of meniran functional drink to produce rich antioxidant functional drink is still limited. Therefore, this research aims to understand the phenolic content, DPPH RSA, and LDL oxidation of meniran functional drink through various preparation processes.

2. Methodology
2.1. Materials
All plant samples, including roots, stems and leaves, were collected from the demonstration plot of the West Nusa Tenggara Assessment Institute of Agricultural Technology, Indonesia. Chemical materials and other materials were procured from Merck, Darmstad, Germany (Methanol, Folin–Ciocalteu’s reagent, CuSO4, thiobarbituric acid (TBA), and trichloroacetic acid (TCA), and 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethylene diamine tetra acetic acid (EDTA), and Sigma Chemical Co., St. Louis, MO (ascorbic acid and LDL (low density lipoprotein) human plasma L8292-IVL).

2.2. Preparation of meniran functional drink extract
The composition of this functional drink is only meniran plant. The method of preparation processes adopted local community culture in Indonesia. The Four methods of preparation process were: (1) Soaking Fresh Meniran (SFM) = 10 grams of fresh meniran plant was cut into small pieces, then soaked in 100 ml of distilled water for 48 hours, (2) Boiling Fresh Meniran for 5 minutes (BFM 5’) = 10 grams of fresh meniran plant was cut into small pieces, then put in boiling water for 5 minutes over low heat, (3) Boiling Fresh Meniran for 10 minutes (BFM 10’) = 10 grams of fresh meniran plant was cut into small pieces, then put in boiling water for 10 minutes over low heat, (4) Dried Meniran (DM) = 10 grams of fresh meniran plant was cut into small pieces, then dried in the oven at 60 ℃ for 3 hours, and soaked in 100 mL of distilled water for 48 hours. Crude extracts were made based on the [13] method with modification.
The solution gained from all treatment variations was filtered using Whatman paper No. 42. Furthermore, one millilitre of each solution was taken and placed in Erlenmeyer flasks glass, added by 10 mL of 80% methanol. The solution was shaken (by SIBATA, SU-2TH) at a speed of 120 rpm for 1 hour at room temperature, and then macerated at 4°C for 24 hours. The solution then was filtered again with Whatman paper no. 42 to get supernatant 1 and stored at -4°C. Bottom layer of supernatant 1 called natant then added with 10 mL of 80% methanol and given the exact same treatment as before to get supernatant 2. Supernatant 1 and supernatant 2 were mixed together and stored at -4°C until analysis of phenolic content, DPPH RSA and oxidation inhibition in human blood LDL were carried out.

2.3. Determination of total phenolic content (TPC)
Folin-Ciocalteu reagent was used to determine the total phenolic content (TPC) of meniran functional drink extract [16]. 1 mL of Folin-Ciocalteu reagent was added into 2 mL of meniran functional drink extract, vortexed and left for 1 minute. Next, it was added by 4 mL of 4% sodium carbonate (Na2CO3) solution and left in a dark room for 2 hours at room temperature. A Shimadzu UV-1601 spectrophotometer at λ 760 nm (Shimadzu, Kyoto, Japan) was used to measure the absorbance of complex blue colour. A standard solution used gallic acid and methanol where results were stated in mg gallic acid equivalents (GAE)/g of meniran. Three replications were taken for each process in which data are presented in mean and ± SD values.

2.4. Determination of DPPH radical scavenging activity.
The value of DPPH RSA Meniran Functional Drink (MFD) extract from each preparation method was determined by the Wang method [17]. 1 mL of MFD extract was added by 3 mL of 0.375 mM DPPH, then incubated for 30 min in the dark at room temperature. The absorbance was measured at λ 522 nm with a Shimadzu UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The value of DPPH RSA is expressed in % DPPH RSA with the following calculation formula:

\[
\text{% DPPH RSA} = \left( \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \right) \times 100\%
\]

Control solution contained 3 mL of DPPH solution with a concentration of 0.375 mM and 1 mL of 80% methanol. A positive control used ascorbic acid. Result data are presented in mean ± SD value from 3 replications.

2.5. Inhibition of LDL oxidation
2.5.1. Preparation of LDL suspension.
A solution of 5 ml of Phosphate Buffered Saline (pH 7.4) was added into Low Density Lipoprotein (Sigma) in a small vial, then shaken by centrifuge at 4000 rpm for 15 minutes. This solution was functioned as a stock solution and stored at a temperature -4°C. The solution should be shaken before analysis by centrifuge at 4000 rpm for 15 minutes. Then the solution was diluted with a solvent suspension of PBS (pH 7.4) as required prior to further testing.

2.5.2. Determination of in vitro inhibition of LDL oxidation.
In vitro inhibition of LDL oxidation was determined by the Warnakulesuriya method [18]. LDL suspension from human plasma (IVL L8292 Sigma-Aldrich) was added into MFD extract in various concentrations (ppm) before the oxidation process was done. Oxidation stress on LDL plasma was carried out by adding CuSO4 as an initiator. Ethylene diamine tetra acetic acid (EDTA) was used to stop the oxidation reaction. Level of LDL oxidation was assessed by the formation of TBARS (Thiobarbituric Acid Reactive Substances). This analysis assessed MDA as a secondary product of lipid peroxidation [19].
TBARS formation was assessed at λ 532 nm and the results were presented in nmol MDA equivalents/g LDL protein. A solution of 1,1,3,3-tetramethoxypropane (TMP) was used as a data standard. The data were presented as mean ± SD of 3 replications.

2.6. **LDL-TBARS (LDL-thiobarbituric acid reactive substances) assay.**

LDL oxidation was described by measuring the formation of TBARS using modification of Xu method [19]. 320 µL LDL suspension (containing 100 g protein/ml) in PBS (pH 7.4) was added into MFD crude extract with various concentrations (25, 50, and 75 ppm phenolic equivalents of extract). Then, it was incubated with 40 µL Cu²⁺ and added 100 µL of crude extract. Controls were made without the addition of crude extract. Oxidation was carried out by centrifuging a small test tube solution at 4000 rpm for 15 minutes. After the incubation process was complete, oxidation reaction process was finished by adding 20 µL of 1 mM EDTA, 2 millilitres of 15% (w/v) trichloroacetic acid (TCA) and 0.67% (w/v) thiobarbituric acid (TBA) in 0.1 N HCl. Then the solution was heated at 95°C for 1 hour and cooled with ice cubes. The solution was centrifuged again at 4000 rpm for 15 minutes to remove the precipitate and then the absorbance of supernatant was measured at λ 532 nm.

2.7. **Statistical Analysis**

All treatments were repeated three times. Data is presented as mean and ± standard deviation. Differences between groups were statistically analyzed using one-way analysis of variance (one way ANOVA) with SPSS Statistics version 17.0 software in the probability values of (P<0.05).

3. **Result and Discussion**

3.1. **The effect of preparation process at phenolic content of meniran functional drink**

Herbal drink has long been used in Indonesia. The preparation process varies depending on local community culture. Preparation process of meniran functional drink has an influence on its antioxidant properties. The effect of the preparation process at phenolic content of meniran functional drink is presented in Figure 1.

![Figure 1](image.png)

**Figure 1.** The effect of preparation process on phenolic content of meniran functional drink. Values with different letters show significant differences (P<0.05).

BFM10’ method has higher phenolic content and significantly different statistically than other treatments, followed by BFM5’, SFM and DM ((p≥5%). Long boiling triggers dilution of water soluble phenolic compounds. In general, foods contain flavonoid content that are water soluble conjugated...
(glycosides) and non-soluble forms [20]. Naturally, polyphenol is bound to the sugar component in glycoside form. Some heating treatments such as boiling can cause a hydrolysis process that weakens covalent bond leading to phenolic compound release into the water. Prolonged heating causes a longer hydrolysis process producing more phenolic. This result is in line with a study by [21] that boiling turmeric in 7.5 minute has higher phenolic content compared to those with 2.5 minutes boiling time. The phenolic compound will be oxidized by folin ciocalteu reagent into phenolate ions. These ions will reduce heteropoly acid creating dark blue substance; therefore the absorption value will be greater at $\lambda$ 762 nm. The amount of phenolic content in the meniran functional drink will influence its ability to scavenge DPPH free radicals.

3.2. **The effect of preparation process at DPPH RSA of meniran functional drink**

Preparation process includes soaking, boiling, drying will influence DPPH RSA of meniran functional drink. Figure 2 explained the effect of the preparation process at DPPH RSA of meniran functional drink.

![Figure 2](image-url)

**Figure 2.** The effect of the preparation process at DPPH RSA of *meniran* functional drink. Values with different letters show significant differences ($P<0.05$).

BFM 5’ and BFM 10’ methods have similar percentages of DPPH RSA and greater than other treatments. This is because those two treatments have greater phenolic content than the other treatments. Phenolic compound has antioxidant activity owing to the hydroxyl groups that play as hydrogen atom donors when reacted with radical compounds. The higher the phenolic content, the greater the ability to scavenge DPPH free radicals. Similar observation was also previously reported that phenolic content has positive correlation with DPPH RSA [21];[10];[12]. In this research, ascorbic acid is used as a comparative antioxidant against DPPH RSA ability. The use of ascorbic acid was based on its wide application as a commercial antioxidant and as aqueous. The ascorbic acid concentration with similar DPPH RSA ability to meniran functional drink extract is 50 ppm.

3.3. **Inhibition of LDL oxidation in vitro**

This research reveals that high antioxidant activity is reflected by small MDA production in human LDL. This method considers that the LDL substrate of human blood plasma has been fully oxidized to produce MDA as the final product [22]. Calibrating baseline of this research which used human blood plasma LDL substrate without the addition of Cu$^{2+}$ initiator has zero absorbance value. This indicates that the production of the MDA-TBA complex has not yet been formed, meaning that the baseline of this study starts from zero. Treatment for human blood plasma LDL by additional Cu$^{2+}$ initiator, and
ascorbic acid 25, 50 and 75 ppm was used as comparison. The effect of the preparation process of meniran functional drink extract in inhibition of MDA production in human LDL is presented in Table 1.

**Table 1. In vitro antioxidant activity of meniran functional drink extract in human LDL.**

| Treatments                  | Concentrations of phenolic equivalents of extract (ppm) | Concentration of MDA in human LDL (nmol MDA/mg protein) | Decrease of MDA concentration from control (%) |
|-----------------------------|---------------------------------------------------------|-------------------------------------------------------|-----------------------------------------------|
| LDL + CuSO4 (kontrol)       | 70.70±2.35                                              |                                                       |                                               |
| LDL + Cu^{2+} extract of SFM | 25           | 44.70±1.41 def                                         | 36.77                                         |
|                             | 50           | 47.87±2.27 cde                                         | 32.29                                         |
|                             | 75           | 50.67±3.12 cd                                          | 28.33                                         |
| LDL + Cu^{2+} extract of BF5’ | 25           | 38.30±2.39 fg                                          | 45.83                                         |
|                             | 50           | 47.87±1.25 cde                                         | 32.34                                         |
|                             | 75           | 51.83±1.05 c                                          | 26.69                                         |
| LDL + Cu^{2+} extract of BF10’ | 25          | 36.30±1.82 g                                          | 48.66                                         |
|                             | 50           | 43.54±1.32 defg                                        | 38.41                                         |
|                             | 75           | 61.47±1.93 b                                          | 13.05                                         |
| LDL + Cu^{2+} extract of DM | 25           | 40.80±1.05 efg                                         | 42.29                                         |
|                             | 50           | 48.54±1.79 cd                                          | 31.35                                         |
|                             | 75           | 52.25±2.05 e                                          | 26.09                                         |
| LDL + Cu^{2+} extract of AA | 25           | 41.35±2.41 efg                                         | 41.51                                         |
|                             | 50           | 51.25±1.18 cd                                          | 27.56                                         |
|                             | 75           | 105.15±2.14 a                                         | -48.72                                        |

Sesame milk extract equivalent with total phenolic concentration (ppm).
Mean values within a column followed by the same letters were not significantly different at P<0.05.

Preparation process using BF5’, BF10’ and DM methods at 25 ppm concentration inhibited MDA production at the same level with ascorbic acid at 25 ppm concentration. This method had the highest inhibition percentage shown by low MDA production as compared to other methods.

Concentration of phenolic at 25 ppm has the smallest MDA value and goes up along with increasing concentration. Similar condition was found for increasing ascorbic acid concentration. This study found that concentration of meniran extract exceeding 25 ppm showed increasing MDA content, meaning that the use of meniran at concentrations above 25 ppm was no longer effective because its ability to inhibit MDA formation had decreased. Meniran plant contains (1) lignins, in form of 1,4-diaryltetralin and 1-arlyltetralin as well as neo lignans and several other lignans, (2) coumarins, tannins, and polyphenols that have been isolated from this plant, such as gallic acid, ellagic acid, brevifolin, carboxylic acid, ethyl brevifolin carboxylate, methyl brevifolin carboxylate, geraniin, corilagin, phyllanthusin D, amarin, amariniacid, claeroceparusin, geraniinic acid B, catechin, epicatechin, gallocatechin, epigallocatechin, epicatechino 3-Ocatechin, 3-Ogallate, (3) Flavonoid which is isolated from this meniran plant are quercetin, rutin, astragalin, quercitrin, isoquercitrin, kaempferol-4’, rhamnopyranoside, eridictyol-7-rhamno pyranoside, fisetin-4’-Oglucoside, quercetin-3-O-glucopyranoside, kaempferol-3-O rutinoside [23]. High content of active compounds in meniran plants is able to inhibit the formation of MDA at small concentrations. In addition, meniran also contains vitamin C of 9506.69 mg/100 grams. Vitamin C in meniran is directly proportional to the amount of plant extracts use. High content of Vitamin C used will change the role of Vitamin C into a pro-oxidant. It is also seen in using ascorbic acid as a comparison antioxidant.
Table 1 showed that the use of high ascorbic acid concentration at 75 ppm produced a very high MDA value. It is supposed to have ascorbic acid's role as pro-oxidant at high concentration. This is because vitamin C has the ability to reduce metal ion Fe$^{3+}$ and Cu$^{2+}$. The reduction process forms Fe$^{2+}$ and Cu$^{+}$ and through metal ion reduction by hydrogen peroxide (Fenton reaction) or lipid peroxide (LOOH) will form radical peroxide (.OOH), radical lipid alkoxyl (LO$^*$) [24]. Other studies report that the pro-oxidant effect of vitamin C is shown by increased DNA damage and MDA concentration at ascorbic acid supplementation in healthy people [25],[26]. DNA mutation and lipid peroxide can trigger various diseases such as cardiovascular, stroke, cancer, neuro degenerative, and ageing process [27]. Based on the research result above, it can be seen that BFM5' and BFM10' methods as a suitable process to produce meniran functional drink.

4. Conclusion
BFM10' preparation method has greater phenolic content and is significantly different from other treatments. Preparation process using BFM10' and BFM5’ statistically has similar DPPH RSA value and significantly different from other treatments. The use of BFM5’ and BFM10 methods can deter smallest MDA formation compared to other treatments. This research concludes that to produce meniran functional drink in optimum antioxidant properties is best by using BFM5’ and BFM10’ preparation methods in meniran concentration of not more than 25 ppm.

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