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Effect of Moringa Leaves Water Extract Combined with Collagen and Vitamin C on Malondialdehyde Levels in Rats Exposed to Oxidative Stress

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Abstract

The increasing prevalence of free radical-related diseases has led to a greater reliance on herbal medicines, such as *Moringa oleifera* (ML), as antioxidants. However, limited evidence exists regarding the combined effect of herbal and conventional antioxidants on malondialdehyde (MDA) levels. This research assessed simplisia characterization, phytochemical screening, antioxidant activity of ML water extract (ML-WE), and serum MDA levels in animal models using ELISA. Forty-two Wistar rats were randomly divided into seven groups: a negative control, a positive control, and five treatment groups. All treatment groups were exposed to lead for seven days, followed by supplementation with either ML-WE (200 mg/kg BW), vitamin C (36 mg/kg BW), collagen (27 mg/kg BW), or their combinations. ML-WE showed strong antioxidant activity (IC_{50}: 81.761 ppm). MDA levels differed significantly among groups ($p < 0.001$). Combining ML-WE with collagen reduced MDA levels more than ML-WE alone (14.65 ± 0.66 vs 21.32 ± 0.95 nmol/mL, $p < 0.05$) but was less effective than collagen monotherapy. Similarly, ML-WE combined with vitamin C showed no statistically significant improvement. These results indicate that while ML-WE has strong antioxidant properties, no synergistic effects were observed in combination therapies.

Keywords: Moringa leaves, MDA, collagen, vitamin C, antioxidant.

1. Introduction

An imbalance between pro-oxidants and antioxidants can impair the elimination of free radicals, disrupting cell signaling and redox control, which leads to molecular damage and oxidative stress [1]. Many diseases have been associated with oxidative stress, such as diabetes mellitus (DM), metabolic syndrome, atherosclerosis and cardiovascular diseases, skin diseases, cancer, and neurodegenerative diseases [2-4]. These diseases are primarily caused by the damaging effects of free radicals on proteins, lipids, and amino acids within cells [5]. The International Diabetes Federation (IDF) estimates that there will be 783 million cases of diabetes worldwide by 2045, and according to GLOBOCAN, there will be 35 million new cancer cases by 2050, with 18.5 million cancer-related deaths [6,7].

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Oxidative stress induces lipid peroxidation within the body, resulting in malondialdehyde (MDA) as one of its final products. Elevated MDA levels indicate the occurrence of oxidation processes in cell membranes [8]. Vitamin C and collagen have been widely used as conventional antioxidants. Vitamin C is commonly used as a standard in antioxidant activity assays because it is a natural, affordable, non-toxic, and readily available compound [9]. The recommended daily intake of vitamin C is approximately 90 mg [10]. Additionally, collagen has been investigated in previous studies for its potential effect in reducing MDA levels [11]. The collagen dosage for Wistar strain white rats is 27 mg/kg body weight (kg/BW) [12].

The use of herbal antioxidants has been widely adopted by society, including the use of *M. oleifera* leaves. *M. oleifera* leaves exhibit numerous pharmacological activities, such as anticancer, antiallergic, antibacterial, antioxidant, anti-inflammatory, immunomodulatory, antidiabetic, antifungal, hepatoprotective, and antispasmodic properties. The abundance of *M. oleifera* leaves in tropical and subtropical regions, combined with their wide range of pharmacological effects, has made them a popular choice for herbal medicine [13].

M. oleifera leaves are used as antioxidants because they contain compounds such as ascorbic acid, flavonoids, phenolics, and carotenoids [14]. According to previous research, the dosage of *M. oleifera* water extract administered to Wistar rats was 200 mg/KgBW [15]. Previous studies on experimental animals have demonstrated that a combination of antioxidants is more effective in reducing oxidative stress compared to antioxidant monotherapy [16]. However, there is still limited research on the combination of herbal and conventional antioxidants. This study aims to evaluate the effect of combining moringa leaf water extract with collagen and vitamin C in reducing malondialdehyde (MDA) levels in oxidative stress-induced experimental animals.

2. Materials and Methods

2.1 Plant Identification

The *M. oleifera* leaves were obtained from Medan City, North Sumatra Province. To ensure accurate identification of the *M. oleifera* plant, a whole plant specimen, including roots, stems, and leaves, was sent to Herbarium Medanense, Faculty of Mathematics and Natural Sciences of Universitas Sumatera Utara for identification. The plant identification number was 1697/MEDA/2024.

2.2 Simplisia Preparation

The first step in simplisia preparation involved a wet sorting process to remove any adhering dirt from *M. oleifera* leaves. The leaves were washed with running water, sliced into thin pieces, and dried in a drying cabinet until brittle when crushed. A total of 5 kg of fresh moringa leaves yielded 900 g of simplisia. The dried simplisia was then blended into a fine powder, weighed, packed into sealed plastic containers, and stored at room temperature [17].

2.3 Simplisia characterization

The characterization of the simplisia involved determining the water content, water-soluble extract content, ethanol-soluble extract content, total ash content, and acid-insoluble ash content.

2.4 Preparation of moringa leaf water extract

The simplisia of *M. oleifera* leaves was soaked in water. The maceration technique was used to extract *M. oleifera* leaves. One part of simplisia was mixed with ten parts of distilled water and left to absorb for 6 hours with occasional stirring. The mixture was then left to stand for an additional 18 hours. Afterwards, the mixture was filtered using a filtration

process. A second extraction was performed using the same solvent but with half the volume used in the first extraction. The combined filtrates were evaporated using a rotary evaporator until a concentrated extract was obtained. The yield of the extract was measured, and evaporation continued until the extract reached a thick consistency. Finally, the extract was freeze-dried to remove any remaining solvent. Before use, the extract was dissolved in distilled water containing Tween 85 [18].

2.5 Phytochemical screening

Phytochemical screening was performed on both the *M. oleifera* simplisia and water extract to identify the presence of alkaloids, flavonoids, glycosides, tannins, saponins, and triterpenes/steroids.

2.6 Moringa leaf antioxidant activity test

In this study, we used the 1-diphenyl-2-picrylhydrazyl (DPPH) test. 10 mg of DPPH powder was dissolved in methanol to a volume of 50 mL, resulting in a DPPH solution with a concentration of 200 ppm. 1 mL of the standard DPPH solution was pipetted, and methanol was added in a volumetric flask until a solution with a concentration of 40 ppm was obtained. The wavelength was measured using a UV-vis spectrophotometer, resulting in a wavelength of 515 nm. 10 mg of concentrated extract was dissolved in methanol to a volume of 10 mL, resulting in a solution with a concentration of 1000 ppm. 0.125 mL, 0.25 mL, 0.375 mL, 0.5 mL, and 0.625 mL were taken from the 1000 ppm extract solution. Then, 1 mL of DPPH solution (200 ppm concentration) was added to each concentration, and methanol was added up to the mark (5 mL volumetric flask), resulting in concentrations of 25, 50, 75, 100, and 125 ppm. Incubated for 30 minutes, the absorbance was measured using a UV-Vis spectrophotometer at a maximum wavelength of 515 nm. The following process is the calculation of inhibition activity and the calculation of the IC_{50} value [19].

2.7 Animal acclimatization

A total of 42 rats were divided into seven groups: a negative control, a positive control, and five treatment groups. Wistar rats were acclimatized for 7 days in single cages within a laboratory setting and provided with standard feed and water *ad libitum*. The room temperature was 22°C, with a light cycle of 12 hours bright/12 hours dark.

2.8 Induction of oxidative stress using lead (Pb)

Following acclimatization, the positive control group (PC) and all five treatment groups received lead (Pb) orally at a dose of 700 mg/kg BW daily for 7 days to induce free radical generation and oxidative stress [20]. The negative control (NC) group was not exposed to Pb.

2.9 Administration of moringa leaf water extract, collagen, and vitamin C

From days 8 to 21 (14 days), the intervention was administered as follows:

- Group WE-ML received *M. oleifera* leaf water extract at a dose of 200 mg/kgBW.
- Group WE-ML + collagen (WE-ML+Coll) received a combination of *M. oleifera* leaf water extract 200 mg/kgBW and collagen tablets 27 mg/kgBW.
- Group WE-ML + Vit. C (WE-ML+ C) received a combination of *M. oleifera* leaf water extract 200 mg/kg BW and vitamin C tablets 36 mg/kg BW.
- Group collagen (Coll) received collagen tablets 27 mg/kgBW.
- Group vitamin C (Vit.C) received vitamin C tablets 36 mg/kgBW.

2.10 Termination of test animals

At the end of the 14-day intervention, the oxidative stress model rats were anaesthetized with a ketamine injection of 80 mg/kgBW [21]. Blood samples (3 mL per rat) were collected

via cardiac puncture. The blood was centrifuged at 3000 rpm for 10 minutes (Eppendorf Centrifuge 5403). Plasma samples were stored at -20°C until analysis. Serum MDA concentrations were measured using an ELISA kit (BTLAB®, China).

2.11 Measurement of MDA levels using ELISA method

The MDA levels were determined according to the Rat MDA ELISA Kit manual procedure. The stored plasma samples were collected and treated with EDTA-Na_2 as an anticoagulant. The plasma was centrifuged for 15 minutes at 1000 x g at 2–8°C. The resulting supernatant was collected, and 50 µL of biotinylated detection Ab working solution was added to each well. The plate was incubated for 45 minutes at 37°C, aspirated, and washed five times. Subsequently, 90 µL of substrate reagent was added and incubated for 15 minutes at 37°C. Finally, 50 µL of stop solution was added, and absorbance was measured at a wavelength of 450 nm.

2.12 Statistical analysis and ethical clearance

The data were analysed statistically using SPSS ver 26.0. We used the Shapiro-Wilk and Levene's test to assess normality and homogeneity of group distributions. The Kruskal-Wallis test was used to detect overall differences among the groups, and the Mann-Whitney U test was used to identify specific group differences. Ethical approval for this study was obtained from the Research Ethics Committee of Universitas Sumatera Utara No. 254/ UN5.2.1.1.54/SPB/2024.

3. Results and Discussion

3.1 Characteriztion of M. oleifera leaf simplisia

The characterization results of *M. oleifera* leaf simplisia met the Indonesian Herbal Pharmacopoeia requirements: water content (<10%), water-soluble extract (>4.9%), and ethanol-soluble extract (>5.0%). The water content of simplisia is regulated to be below 10% because higher water content can promote the growth of bacteria or fungi, which may degrade the metabolites in the simplisia [22]. The characterization of the *M. oleifera* leaf simplisia is shown in Table 1.

Table 1: Characterization of *M. oleifera* leaf simplisia

Parameters	Content (%)
Water content	8,64
Water-soluble extractive content	9,35
Ethanol-soluble extractive content	14,71
Total ash content	8,18
Acid-insoluble ash content	1,22

3.2 Phytochemical screening of M. oleifera leaf simplisia, and water extract

In the phytochemical screening of *M. oleifera* leaf simplisia, secondary metabolites, such as alkaloids, flavonoids, glycosides, saponins, tannins, and triterpenes/steroids were identified. This finding aligns with previous research [23]. The phytochemical screening of the *M. oleifera* leaf water extract revealed the presence of alkaloids, flavonoids, glycosides, saponins, and tannins, in accordance with previous research [23,24]. However, triterpenes/steroids were not detected in the water extract because triterpenes are lipophilic compounds that are insoluble in water due to their non-polar nature. Water, being a polar solvent, is not effective for extracting triterpenes. Conversely, triterpenes are more readily extracted using non-polar solvents such as methanol, ethanol, 1-butanol, ethyl acetate, diethyl

ether, or acetone [25]. The results of the phytochemical screening of the *M. oleifera* leaf are shown in Table 2 and Figure 1.

Table 2: Phytochemical screening of *M. oleifera* leaf

Secondary Metabolite	Reagent	Result	
		Simplisia	Water extract
Alkaloid	Dragendroff	+	+
	Bouchardat	+	+
	Meyer	+	+
Flavonoid	Mg powder + Amyl Alkohol + HCl_p	+	+
Glycoside	Molish+H_2SO_4	+	+
Saponin	Hot water/shaken	+	+
Tannin	$FeCl_3$	+	+
Triterpene/Steroid	Lieberman-Bourchat	+	-

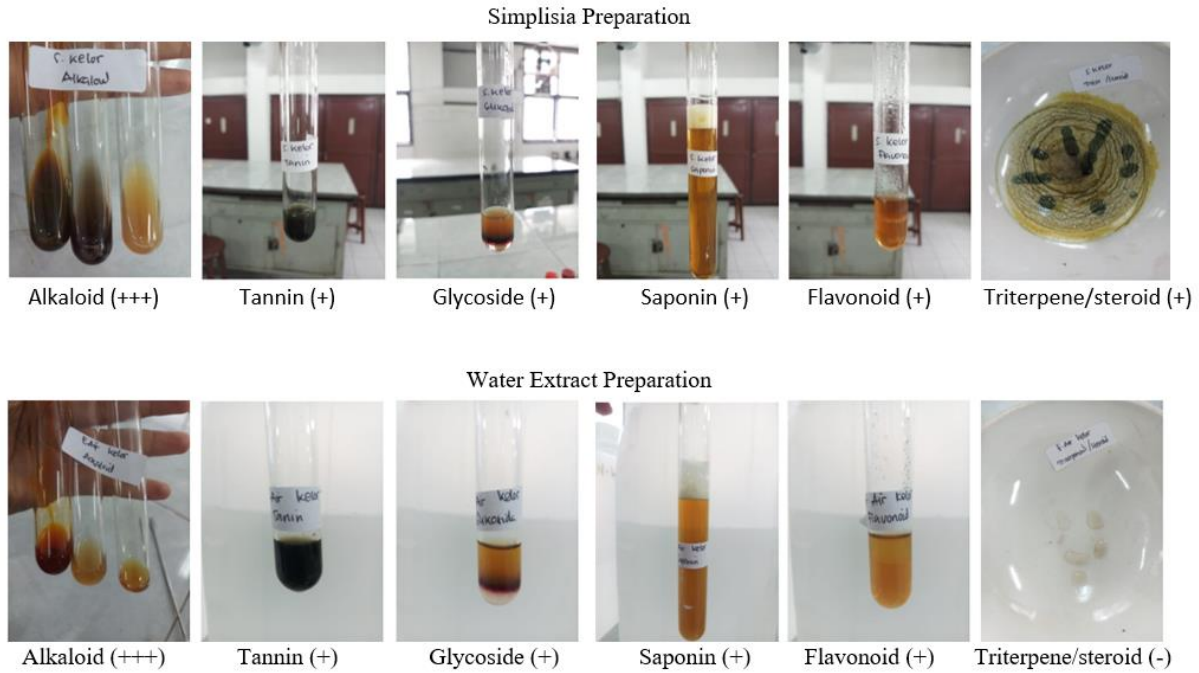

Figure 1: Phytochemical screening of *M. oleifera* simplisia and water extract

3.3 Antioxidant Activity

The antioxidant test of *M. oleifera* leaves using the DPPH method yielded an IC_{50} value of 81.761 ppm, indicating strong antioxidant potential. The IC_{50} range can be classified into strong (50-100 ppm), moderate (100-150 ppm), and weak (>150 ppm) [26]

3.4 The effect of the extract combination with collagen and vitamin C on reducing MDA levels

Malondialdehyde (MDA) is the end product of lipid peroxidation in the body. An increase in MDA levels indicates the occurrence of oxidation processes in cell membranes [27]. ML-WE + collagen group had higher MDA level than the monotherapy collagen group and vitamin C group (14.65 ± 0.66 vs 9.45 ± 0.68 vs 8.28 ± 1.78 nmol/mL), and the differentiation of MDA levels was statistically significant ($p<0.05$). This indicates that the combination group ML-WE + collagen was not superior in reducing MDA levels compared

to the collagen and vitamin C monotherapy groups. However, the ML-WE + collagen group showed better MDA reduction (14.65 ± 0.66 nmol/mL) than the monotherapy group ML-WE (21.32 ± 0.95 nmol/mL). The current results found that the combination of *M. oleifera* water extract and collagen was more effective in reducing MDA levels than the *M. oleifera* water extract monotherapy, but it was not superior to the collagen and vitamin C monotherapy groups. The combination group of ML-WE + vitamin C with MDA level of 20.85 ± 1.12 nmol/mL, showed higher values than the monotherapy collagen group (9.45 ± 0.68 nmol/mL) and vitamin C group (8.28 ± 1.78 nmol/mL), indicating that the combination of *M. oleifera* water extract and vitamin C was not superior in reducing MDA levels compared to collagen and vitamin C monotherapy. The ML-WE + vitamin C group also had a slightly lower MDA level than the monotherapy ML-WE group (21.32 ± 0.95 nmol/mL), although the differentiation of MDA levels was not statistically significant.

In our study, the ML-WE + collagen group showed lower MDA levels than the monotherapy ML-WE group, but higher levels than the collagen group. Similarly, the ML-WE + vitamin C group showed lower MDA levels than the monotherapy ML-WE group, but higher levels than the vitamin C group, indicating no synergistic effect. This is because the MDA levels in the combination groups were not lower than those in the monotherapy groups. The lack of synergistic effects in the combinations of *M. oleifera* water extract with collagen or vitamin C may be attributed to the following possibilities: (I) A potential antagonistic interaction between secondary metabolites in the *M. oleifera* water extract and collagen, which may interfere with the pharmacodynamic activity of collagen in reducing MDA levels. (II)The optimal dosage combination of *M. oleifera* water extract and collagen may not have been achieved to produce an optimal synergistic effect, suggesting that further studies using various dosage ratios are required. (III) A higher number of compounds in the *M. oleifera* water extract may antagonize vitamin C more than collagen, resulting in the combination of *M. oleifera* and collagen showing better MDA reduction than the combination of *M. oleifera* and vitamin C.

Table 3: Difference in MDA average levels among croups

Groups	MDA (Mean ± SD)	Min-Max	P value
Positive control (PC)[a]	23.77 ± 1.58	20.56 – 24.74	<.001*
Negative control (NC)[b]	8.46 ± 0.09	8.30 – 8.58	0.025[a,c]; 0.004[a,d]; 0.016[a,e]
Moringa leaves water extract 200 mg/kgBW (WE-ML)[c]	21.32 ± 0.95	20.12 – 22.73	0.004[a,f]; 0.004[a,g]; 0.004[c,d]; 0.004[c,f]; 0.004[c,g]; 0.004[d,e];
Moringa leaves water extract 200 mg/kgBW + collagen(WE-ML+ Coll)[d]	14.65 ± 0.66	13.78 – 15.66	0.004[d,f]; 0.004[d,g]; 0.004[e,f]; 0.004[e,g]
Moringa leaves water extract 200 mg/kgBB + Vitamin C (WE-ML+ C)[e]	20.85 ± 1.12	19.61 – 22.47	
Collagen (Coll)[f]	9.45 ± 0.68	8.51 – 10.60	
Vitamin C (Vit.C)[g]	8.28 ± 1.78	4.72 – 9.41	

**Kruskal-Wallis*, [a,c; a,d; a,e; a,f; a,g; c,d; c,f; c,g; d,e; d,f; d,g; e,f; e,g] *Mann-Whitney test*

Significant differences between two groups were found for the following pairs: (PC, WE-ML); (PC, WE-ML+Coll); (PC, WE-ML+C); (PC, Coll); (PC, Vit.C); (WE-ML, WE-ML+Coll); (WE-ML, Coll); (WE-ML, Vit.C); (WE-ML+Coll, WE-ML+C); (WE-ML+Coll, Coll); (WE-ML+Coll, Vit.C); (WE-ML+C, Coll); and (WE-ML+C, Vit.C). The presentation of differences between groups in the graph is shown in Figure 2.

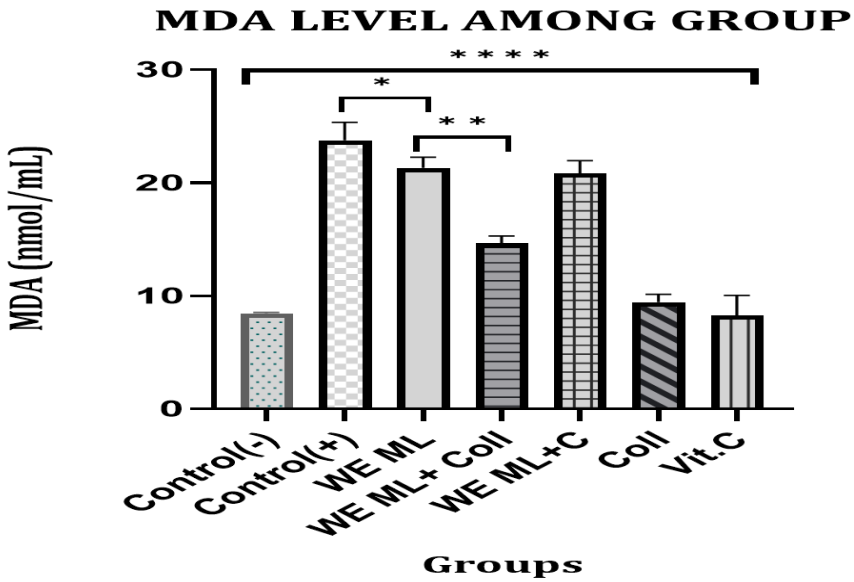

Figure 2. Differences in the MDA level. * p<0.05 vs. control (+);** p<0.01; **** p <0.0001.

Conclusion

Moringa leaves water extract had strong antioxidant potency. The combination of Moringa leaves water extract with collagen and with vitamin C can reduce MDA levels in animal models of oxidative stress, but there is no synergistic effect in the combination form.

Conflict of Interest

We declare there was no conflict of interest in our research.

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