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Nanosuspension Increases the Solubility of Flavonoid Content of *Moringa oleifera* Extract and Antioxidant Activity

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Abstract: The development of natural antioxidants has become increasingly important due to the issues associated with synthetic alternatives. *Moringa oleifera*, rich in flavonoids, shows great promise for powerful antioxidants. This study aims to increase the solubility of flavonoid content and antioxidant activity of an extract from *Moringa oleifera* leaves (e-Mol) by converting it into a nanosuspension. The nanosuspension extract from *Moringa oleifera* leaves (nse-Mol) was prepared using Tween 80, HPMC, and a combination of both as stabilizing agents. The formulations were characterized by measuring particle size, zeta potential, and polydispersity index to assess stability and uniformity. The flavonoid content was determined using a colorimetric method, expressed as quercetin equivalents. Furthermore, antioxidant activity (IC_{50}) was assessed by measuring free radical inhibition using the *2,2-diphenyl-1-picrylhydrazyl* (DPPH) assay. The results indicate that the characterization of nse-Mol with Tween 80 meets the standards for nanomolecular stability, thereby enhancing its antioxidant activity. The flavonoid content of nse-Mol (539.31±8.88 mg QE/g) was higher than that of e-Mol (384.61±57.22 mg QE/g), and the IC_{50} value of nse-Mol (35.67±4.18 μg/mL) was lower than that of e-Mol (99.72±0.40 μg/mL). This suggests that converting *Moringa oleifera* extract into a nanosuspension can effectively increase both flavonoid solubility and antioxidant activity.

Keywords: HPMC; IC_{50}; *Moringa oleifera*; nanosuspension; tween 80

1. INTRODUCTION

Moringa oleifera grows widely across South Asia and globally. It is a plant native to the foothills of the Himalayas in India, flourishing in both tropical and subtropical climates. It has been cultivated and spread to various countries, and it is grown in Africa, Asia, Latin America, the Caribbean, and the Pacific Islands [1]. *Moringa oleifera* is often referred to as "the tree of life" in many parts of the world. Almost all parts of the plant are usable, especially the leaves, which are highly nutritious and rich in amino acids, vitamins, minerals, and natural antioxidants. [2].

Moringa oleifera leaves are highly valued for their numerous active compounds, especially their antioxidant properties. Antioxidants in medicinal plants play a vital biological role [3]. Several degenerative diseases are associated with oxidative stress caused by excess free radicals. Maintaining a balance between free radicals and antioxidants is essential because they play vital physiological roles in metabolic processes. Furthermore, in recent decades, demographic changes resulting from improved well-being have led to an increase in the population over 50 years of age, which is expected to bring an increasingly relevant rise in chronic diseases such as diabetes, cardiovascular disease, and obesity, as inflammatory processes and imbalances in cellular redox status characterize these pathologies. For this reason, scientists and industry are focusing on new strategies to combat the emergence of these diseases by increasing antioxidant intake [4].

Bioactive molecules known for their antioxidant properties in *Moringa oleifera* include flavonoids and other bioactive compounds [5]. However, many active compounds, such as flavonoids from herbal extracts, are large and hydrophilic, hindering their passage through lipid membranes and resulting in low absorption and bioavailability. Additionally, flavonoids often lead to their complexation or precipitation, which inhibits absorption. The breakdown of flavonoids by gut microbiota further reduces their stability and bioavailability [6]. Other strategies to overcome solubility issues and the advantages of nanosuspensions compared to other formulations. These formulations can reduce dosages and minimize side effects [7]. Nanotechnology is employed to create formulations with particles on a nanoscale [8].

A specific application of this technology is the production of nanosuspensions from *Moringa oleifera* to enhance activity and solubility. Nanosuspensions can enhance the solubility of active compounds in the body, thereby optimizing therapeutic effects while reducing the risk of side effects. Increased drug levels are crucial for achieving desired pharmacological effects in certain blood conditions. In such cases, nanoparticles serve as an efficient drug delivery system, providing therapeutic effects at lower doses [9].

The bead milling method was employed, which was capable of producing fine particles with high efficiency down to the nanoscale [8]. This study selected a nonionic surfactant, Tween 80, HPMC, and their combination, due to its superior ability to reduce interfacial tension between particles and the dispersion medium [10]. The formulations will be characterized by analyzing particle size, zeta potential, and polydispersity index to evaluate their stability and uniformity. Transmission electron microscopy will be used to confirm the formation of nanoparticles and their stability. Characterization that meets the requirements of a stable nanosuspension will be continued with content and flavonoid tests, as well as antioxidant tests [11,12].

This study investigates a nonionic surfactant that serves as a stabilizing agent for a nanosuspension extracted from *Moringa oleifera* leaves (nse-Mol). The goal is to enhance the stability of the nanosuspension, thereby increasing the flavonoid content and antioxidant activity of the extract from *Moringa oleifera* leaves (e-Mol), which has been modified to create nse-Mol. The flavonoid content of both e-Mol and nse-Mol was determined by a colorimetric method using a UV-Vis spectrophotometer and expressed as quercetin equivalents. The antioxidant activity, measured as IC_{50}, was assessed by monitoring free radical inhibition using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

2. MATERIALS AND METHODS

2.1. Reagents

The ingredients include aquadest, 96% ethanol (Emsure®), Tween 80 (Merck®), HPMC (Merck®), sodium carbonate (Na_2CO_3) (Emsure®), aluminum chloride ($AlCl_3$) (Sigma®), methanol of analytical grade (Merck®), quercetin (Sigma®), and DPPH (2,2-Diphenyl-1-Picrylhydrazyl) (Sigma®).

2.2. Procedures

2.2.1. Collection of the plant material

Moringa oleifera leaves were collected from Ekas Village, Keruak Sub-District, East Lombok District, NTB, Indonesia (8°56'50'' S and 116º35'10'' E). The plant was identified and attested to by a trained Taxonomist. The *Moringa oleifera* plant was assigned a voucher number, the Laboratory of Biology, Universitas Ahmad Dahlan (411/Lab.Bio/B/VI/2025). *Moringa oleifera* was harvested from the leaves that grow after the 3rd branch, air-dried at room temperature until dry, and ground.

2.2.2. Preparation of an extract from *Moringa oleifera* leaves (e-Mol)

The extraction method was adapted from Khalid et al (2023) with adjustments. Dried leaves (300 g) were added to 95% ethanol (1500 mL) and macerated for 48 hours at room temperature. The process was repeated twice, and a colorless supernatant liquid was obtained. Then, the extract solution was filtered through a muslin cloth, rotary-evaporated (Heidolph) at 60°C until it thickened,

and then dried in a water bath (Mammert). The percentage yield of the extract from *Moringa oleifera* leaves (e-Mol) was calculated [13].

2.2.3. Preparation of a nanosuspension extract from *Moringa oleifera* leaves (nse-Mol)

The nanosuspension was prepared using a bead-milling method adapted from Abraham et al. (2020). The e-Mol macrosuspension was made into three formulas, namely Formula 1 (F1) using 10% tween 80 (w/v) as a surfactant, Formula 2 (F2) using 10% HPMC (w/v) in water as a surfactant, and Formula 3 (F3) using a mixture of 5% tween 80 (w/v) and 5% HPMC (w/v). Each macrosuspension of e-Mol was milled with zirconium oxide (Fritsch®) while employing a triangular magnetic stir bar. Before adding the zirconia beads, the mixture was subjected to Ultraturrax for 30 seconds at 1200 rpm. For each macrosuspension, 11.63 mL of the mixture was combined with 7.75 mL of zirconia beads, totaling 50 g, in a 50 mL Erlenmeyer flask equipped with a triangular magnetic stir bar. The mixture was stirred at 700 rpm for approximately 24 hours at 28-30°C [14]. The process was illustrated in Figure 1.

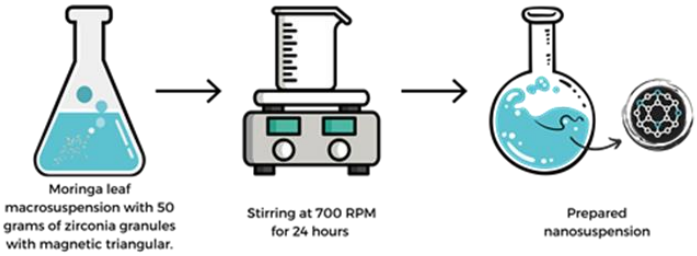


Figure 1. The nanosuspension was prepared using bead milling. The e-Mol macrosuspension was mixed with each surfactant (10% Tween 80 (w/v), 10% HPMC (w/v), 5% Tween 80 (w/v), and 5% HPMC (w/v)). Each macrosuspension was milled using zirconium oxide (Fritsch) with a triangular magnetic stir bar. The mixture was stirred at 700 rpm for 24 hours at 28-30°C.

After 24 hours, the zirconia beads were removed, and the nanosuspension was characterized by a particle size analyzer Zetasizer Nano ZS (Malvern).

2.2.4. Characterization Particle Size, Polydispersity Index, and Zeta Potential of nse-Mol

The sample was taken from 50 microliters of the nanosuspension preparation and added to 1450 microliters of conductivity water, resulting in a sample-to-medium ratio of 1:29. The conductivity water was prepared by diluting 100 mL of Aquadest with distilled water and storing it in the refrigerator until the temperature reached 20°C. Next, a small amount of 0.9% NaCl was added using a dropper, while monitoring the temperature and conductivity until it reached 50 µS/cm at 20°C, as measured with a conductivity/TDS meter. The particle size measurements were then conducted using a Particle Size Analyzer (PSA)[14].

2.2.5. Flavonoid content of e-Mol and nse-Mol test

The flavonoid content in both the e-Mol and nse-Mol samples was analyzed using UV-Vis spectrophotometry. For each sample, 0.5 mL was combined with 1.5 mL of ethanol, 0.1 mL of 1 M sodium acetate, 0.1 mL of a 10% (w/v) $AlCl_3$ solution in methanol, and 2.8 mL of distilled water. This mixture was then incubated for 30 minutes. The absorbance was measured at 433 nm. The standard curve used for comparison was based on quercetin [15]. The experiment was replicated three times.

2.2.6. Antioxidant Activity of e-Mol and nse-Mol test

The antioxidant activity of e-Mol and nse-Mol was tested by *2,2-diphenyl-1-picrylhydrazyl* (DPPH) to inhibit free radicals. A 0.30 mM DPPH solution was prepared in methanol of analytical grade in a dark room. The samples were dissolved in alcohol of analytical grade at concentrations of 100, 50, 25, 12.5, and 6.25 (µg/mL). The 0.5 mL samples of each concentration were added to 0.5 mL of DPPH solution, homogenized, and incubated for 30 minutes in a dark, closed environment. Ascorbic acid was used as a positive control. The absorbance of the sample was measured using a

UV-Vis spectrophotometer (Shimadzu UV-1800) at 507 nm. The results show the inhibition of DPPH free radicals (%).

$$\% \text{ Free radical scavenging} = \frac{\text{Abs control DPPH} - \text{abs sample}}{\text{Abs control DPPH}} \text{x } 100\%$$

The experiment was replicated three times. The concentration of the test compound was then analyzed by linear regression to determine the radical-scavenging concentration that yielded 50% inhibition (IC_{50}). The antioxidant test using the DPPH method is presented as an IC_{50} value.

2.3. Data analysis

The distinction of e-Mol and nse-Mol was analyzed using SPSS Statistics 26 at the 95% confidence level. Data were tested for normality and homogeneity using the Shapiro-Wilk and Levene's tests. Normal ($p>0.05$) or homogeneous ($p>0.05$) data were then subjected to ANOVA. Post hoc (Tukey) testing was used to determine the significance of the data.

3. RESULTS AND DISCUSSION

3.1. Characterization of particle size, polydispersity index, and zeta potential of nse-Mol

The preparation of nse-Mol involved dispersing the e-Mol in a surfactant solution. The surfactants were intended to aid in dispersing the extract in the solvent, resulting in a more homogeneous mixture. Tween 80 acts as a stabilizer, helping prevent nanoparticle agglomeration by reducing surface tension and forming a protective layer around the particles. Additionally, HPMC increased viscosity and effectively dispersed the particles [16].

The nse-Mol was produced by milling the solution of e-Mol and surfactant in zirconia beads for 24 hours using a magnetic stirrer at 700 rpm. The nanosuspension consisted of colloidal particles less than 1 μm in the dispersing medium. The nse-Mol process uses bead milling, which breaks down e-Mol using the milling medium, resulting in smaller particle sizes. This method was simple and capable of producing small particle sizes with the aid of surfactants. The particle size distribution, commonly referred to as the polydispersity index, indicates that the smaller the value, the more uniform the particle sizes are, with a range of 0 to 1. A value greater than 1 depends on the particle size distribution. If the number approaches 1, it indicates a greater variation in particle sizes within the nanosuspension preparation [14]. Furthermore, zeta potential is measured to assess the strength of repulsive forces between adjacent particles in the dispersion medium. In non-ionic surfactants, the expected zeta potential value is greater than +20 mV or less than -20 mV. The particle size, polydispersity index, and zeta potential of nse-Mol were shown in Table 1.

Table 1. The particle size, polydispersity index, and zeta potential of nse-Mol using a variation of surfactant

Surfactant	Particle size (nm)	polydispersity index (nm)	zeta potential (mV)
Tween 80	29.27 ± 2.69	0.36 ± 0.01	-21.49 ± 0.82
HPMC	385.40 ± 9.43	0.88 ± 0.01	-10.86 ± 0.58
Combination of Tween 80 and HPMC	173.17 ± 3.56	0.39 ± 0.01	-11.16 ± 0.46

n = 3; Data represented as Mean ± S.D.
nse-Mol : nanosuspension of extract from *Moringa oleifera* leaves
HPMC : Hydroxypropyl methylcellulose
nm : nano meter
mV : mVolt

The particle size of nse-Mol formed ranged from 29.27 ± 2.69 to 385.40 ± 9.43 nm, with the smallest size observed in the formulation containing 10% Tween 80. Statistically, surfactant variation significantly affects particle size ($p < 0.05$). The smallest particle size is achieved with the surfactant Tween 80, which reduces the interfacial tension between the particles and the suspension's dispersing medium, a property commonly used in nanosuspensions. In contrast, HPMC was more often used as a viscosity stabilizer. The combination of Tween 80 and HPMC can yield particle sizes that vary with the concentration and ratio of the two surfactants, due to their differing properties.

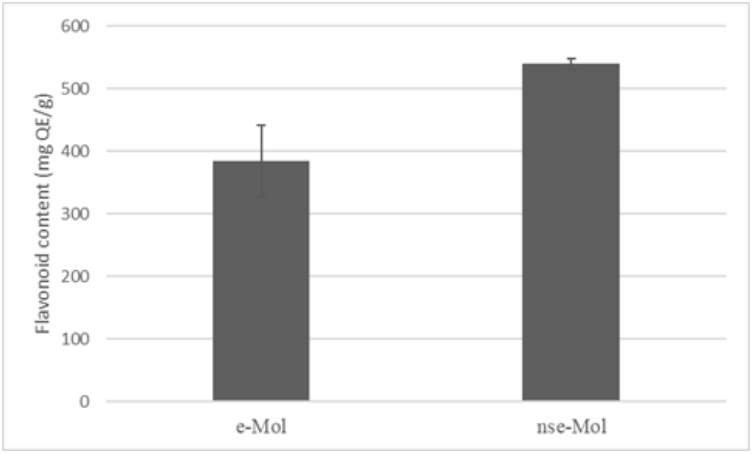
The polydispersity index indicates the degree of heterogeneity in particle size or molecular weight within a sample. A particle size distribution below 0.5 indicates that the preparation has uniform, homogeneous particle sizes. In contrast, a particle size distribution value above 0.5 shows heterogeneous or broad particle sizes in the preparation due to differences in surfactant concentration. Statistically, variations in surfactant (Table 1) significantly affect the particle size distribution (p <0.05). In conditions where the material exhibits hydrophobic properties, it tends to remain on the water surface, making effective milling difficult and resulting in larger particle sizes and uneven distribution. The ability of surfactants to enhance the wetting of hydrophobic samples enables them to be evenly distributed within the dispersing medium, enabling effective milling.

Moreover, the average zeta potential values range from -10.86 ± 0.58 to -21.49 ± 0.82 mV. According to research by Bhakay *et al*. (2018), a zeta potential value that provides particle stability through steric hindrance by non-ionic surfactants is approximately ±20 mV [17]. The zeta potential value of -20 mV in the formulation with Tween 80 was expected to provide stability in particle size within the nanosuspension. A zeta potential value approaching zero is due to the reduction of repulsive forces between particles, leading to aggregation and decreased stability of the particle dispersion. Statistically, the surfactant and concentration variations have a significant effect (Table 1) on the zeta potential values (p< 0.05).

3.2. The flavonoid content of e-Mol and nse-Mol

The analysis of flavonoid content was conducted using colorimetric or UV-Vis spectrophotometry methods. The determination of total flavonoids in the sample was performed by adding $AlCl_3$ to the sample solution to form a stable acid complex with the ketone group at position C-4 and the hydroxyl groups at positions C-3 or C-5 of flavon and flavonoids. A stable complex with $AlCl_3$ was formed at the optimal duration (operating time) for forming was determined, which was approximately 30 minutes at a wavelength of 425 nm. This time range was then used in the preparation stage of both the quercetin standard solution and the sample [15]. Based on the results, the maximum absorption wavelength of quercetin was found to be 433 nm, which was then used as a standard for testing quercetin compounds (in concentrations of 30, 40, 50, 60, and 70 (μg/mL) and for analyzing samples. The samples were triplicated, and the linear equation obtained was y = 0.0107x - 0.0704, with a correlation coefficient of 0.9982, indicating a strong linear relationship between concentration and absorbance, enabling accurate calculations of flavonoid content.

The flavonoid content in the e-Mol and nse-Mol (Tween-80 as surfactant) was calculated in triplicate using a linear equation relating the concentration and absorbance of quercetin. The results were expressed as milligrams of quercetin equivalent per gram of sample. The flavonoid content of nse-Mol (384.61±57.22 mg QE/g) was significantly higher than that of e-Mol (539.31±8.88 mg QE/g) (Figure 2, p < 0.05). The flavonoid contents of e-Mol and nse-Mol were shown in Figure 2.

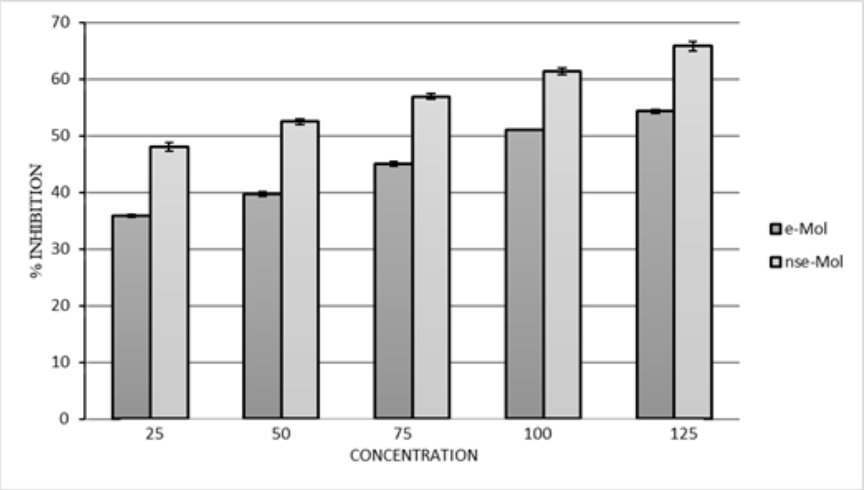


e-Mol: extract from *Moringa oleifera* leaves; nse-Mol: nanosuspension extract from *Moringa oleifera* leaves

Figure 2. The flavonoid content of e-Mol and nse-Mol (10% Tween-80 (w/v) as surfactant) was determined by the colorimetric method using $AlCl_3$ (n=3). The linear equation relating the concentration and absorbance of quercetin was equated as quercetin (mg) equivalent of sample (g).

3.3. The antioxidant activity of e-Mol and nse-Mol

The antioxidant activity was tested using the DPPH based on the principle of transferring a single hydrogen atom to a free radical, thereby reducing DPPH to a non-radical form. This reduction process was indicated by the fading of the purple color in the DPPH solution, as evidenced by a decrease in absorbance at 507 nm measured with a UV-Vis Spectrophotometer. The more DPPH that remains, the lower the percentage of inhibition observed in the sample, indicating a decrease in free radical inhibition. This inhibition percentage shows an inverse relationship with absorbance: as absorbance decreases, the inhibition percentage against DPPH radicals increases. The inhibition percentage graph of e-Mol and nse-Mol is shown in Figure 3.



e-Mol: extract from *Moringa oleifera* leaves concentration (µg/mL)
nse-Mol: nanosuspension extract from *Moringa oleifera* leaves concentration (µg/mL)

Figure 3. The inhibition percentage graph from the radical DPPH assay of the sample e-Mol and nse-Mol (10% Tween-80 (v/v) as surfactant) at 507 nm wavelength.

The antioxidant activity test using the DPPH method was conducted at the IC_{50} level, indicating free radical inhibition capacity at 50% sample concentration. Therefore, a lower IC_{50} value indicates a higher antioxidant capacity. The IC_{50} values of the e-Mol, nse-Mol, and ascorbic acid, used as the standard, are shown in Table 2.

Table 2. The IC_{50} values and antioxidant classification of the e-Mol, nse-Mol, and ascorbic acid using the DPPH method [18]

Samples	IC_{50} values (µg/mL)	Antioxidant classification
e-Mol	99.72 ± 0.40*†	strong
nse-Mol	35.67 ± 4.18#†	very strong
Ascorbic acid	5.28 ± 0.37*#	very strong

n = 3; Data represented as Mean ± S.D.;
*) significant ($p<0.05$) IC_{50}: Inhibitory concentration at 50%
#) significant ($p<0.05$) e-Mol: extract from *Moringa oleifera* leaves;
†) significant ($p<0.05$) nse-Mol: nanosuspension extract from *Moringa oleifera* leaves

It was known that ascorbic acid, a powerful antioxidant ($IC_{50} < 50$ µg/ml), and the antioxidant activity of nse-Mol and ascorbic acid differed significantly ($p < 0.05$). Meanwhile, e-Mol showed a strong antioxidant activity ($50 < IC_{50} < 100$) and differed statistically from nse-Mol and ascorbic acid. There was a significant difference in the antioxidant effect of crude from Moringa oleifera and its nanosuspension form.

3.3. Discussion

The combination of nanotechnology and flavonoid research offers a new method for delivering and stabilizing flavonoids, powerful natural antioxidants found in *Moringa oleifera*. The flavonoids in *Moringa oleifera* leaves have biological and antioxidant properties that aid in scavenging free radicals

[19]. Flavonoids are among the most common and extensively studied natural phenolic compounds. However, flavonoids have low bioavailability. To address these challenges, ongoing research explores novel delivery systems such as nanoencapsulation and structural modifications to improve bioavailability [20]. For instance, the double bonds in flavonoids and flavonols at positions 2, 3, and 4 can easily form planar structures, resulting in tightly packed molecules that impede membrane penetration. Ongoing research on flavonoids and other phenolic compounds aims to better understand their potential as free radical scavengers capable of neutralizing singlet and triplet oxygen. Their capacity to degrade peroxides and repair oxidative damage caused by reactive oxygen species (ROS) underscores the active efforts to unlock their health benefits, inspiring continued investigation. [21].

A nanotechnology approach, utilizing the bead milling method, is an alternative to overcome these problems [22]. The selection of a suitable surfactant has a significant influence on the formation of nanosuspensions [23]. The use of Tween 80 as a surfactant produces ideal nanosuspension characteristics, including particle size (nM), polydispersity index (homogeneous), and zeta potential (mV), which were 29.27 ± 2.69 nm, 0.36 ± 0.01, and -21.49 ± 0.82 mV, respectively. Tween 80, also known as Polysorbate 80, is a nonionic surfactant with a Hydrophilic-Lipophilic Balance (HLB) value of 15. Tween 80 consists of 20 polyoxyethylene groups and one oleic acid group. The polyoxyethylene group is hydrophilic, providing steric hindrance to prevent agglomeration. The oleic acid group is hydrophobic and covers the particle surface. The use of Tween 80 can increase the solubility of active substances [24]. Tween 80 has a better ability to reduce the interfacial tension between particles and the dispersing medium. The results of this nanosuspension differ from those obtained with HPMC, which yields less favorable results for nanosuspensions [25,26].

Nanosuspensions are nanoparticles with an average particle diameter of less than 1 μm. Generally, the particle size for nanosuspensions ranges from 1 to 500 nm. The particle size offers advantages over nanosuspensions, specifically increasing the dissolution rate and accelerating drug absorption in the body, as smaller particle sizes result in greater solubility and dissolution [17]. The Polydispersity Index, also known as particle size distribution, is a characteristic of nanoparticle systems that can significantly influence nanoparticle properties. A good particle size distribution is indicated by improved drug distribution during preparation, enabling optimal therapeutic effects. A particle size distribution value of 0.1–0.25 indicates a relatively narrow particle size distribution, while a particle size distribution value greater than 0.5 indicates an extensive particle size distribution [27]. Furthermore, the electrostatic potential of nanoparticles in a dispersion system is called the zeta potential. A good zeta potential indicates that the repulsive forces between particles are more potent than the attractive forces, resulting in stability in the dispersion. A high stability value is greater than +30 mV or less than -30 mV. A ±30 mV value is required for electrostatically stabilized nanosuspensions with ionic surfactants. In contrast, non-ionic surfactants in sterically stabilized suspension systems require a zeta potential greater than +20 mV or less than -20 mV. This ensures the stability of the formed nanosuspensions [28].

The smaller the particle size produced, the higher the detected flavonoid content. The flavonoid content of nse-Mol was higher compared to that of e-Mol, which was associated with optimal stabilization effects at lower concentrations, thereby enhancing solubility and preventing aggregation [29]. Conversely, higher concentrations may increase interactions, thereby reducing the availability of flavonoids. These findings indicate that the formulation in the form of a nanosuspension contributes to the increased flavonoid content in the preparation [30]. Moreover, small particle size increases bioavailability and therapeutic effects, including increased antioxidant effects [31]

Although the antioxidant activity test was performed in vitro, an increase in antioxidant effect was detected. The antioxidant activity of nse-Mol was stronger than that of e-Mol, characterized by a significantly lower IC_{50} value than e-Mol (Table 2). The small particle size enables increased dispersion of particles with antioxidant activity, leading to more effective free radical scavenging. Increased activity was also observed in enzymatic antioxidant tests [32–35]. Furthermore, increased activity was observed in nanotechnology-based preparations. This smaller particle size can improve

the stability of the dosage form, including increasing the active ingredient content and its therapeutic activity [36].

4. CONCLUSION

A nanosuspension of Moringa oleifera extract, made using the Tween 80 surfactant, exhibits good characteristics, which enable it to increase flavonoid content and antioxidant activity.

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