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Original Article

FORMULATION DEVELOPMENT AND *IN VITRO* PENETRATION TEST OF ETHOSOME OF *CHROMOLAENA ODORATA* LEAVES EXTRACT

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ABSTRACT

Objective: This study aimed to develop and assess ethosome preparation using extracts derived from the leaves of *Chromolaena odorata*.

Methods: The study started by obtaining *Chromolaena odorata* leaf extracts. Furthermore, Ethosome formulations were produced using a thermal technique. Ethosome variants were created, each with distinct compositions: Formulation F1, containing 10 ml of ethanol without the extract; Formulation F2, consisting of 0.5 grams of the extract mixed with 10 ml of ethanol; Formulation F3, combining 1 gram of the extract with 20 ml of ethanol; and Formulation F4, incorporating 1.5 grams of the extract with 30 ml of ethanol. The ethosomal systems were thoroughly characterized using various analytical techniques, such as organoleptic analysis, quantification of particle dimensions, zeta potential evaluation, pH metric analysis, transmission electron microscopy (TEM) imaging, and *in vitro* permeability assessment using the Franz Diffusion Cell apparatus.

Results: The findings indicated that the optimized F4 formulation showed 161.2±32.0 nm particle size measurement and a+34.33±0.58 mV zeta potential. All formula possess a pH range of 4.5-6.5, within which the skin can acclimate to preparations. It is evident from all formulations that the pH decreased after the addition of the extract at an acidic pH of 4.11. Following the 12-week storage period, the pH of all treatments exhibited a modest reduction; however, it remained within the acceptable range for skin pH. Furthermore, the F4 formula also had a higher level of penetration activity.

Conclusion: The optimized ethosomal formulations of *Chromolaena odorata* have promising applications in enhancing the permeability and efficacy of plant-derived therapeutic agents.

Keywords: *Chromolaena odorata*, Ethosome, Evaluation, Penetration

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INTRODUCTION

Chromolaena odorata L. is an herbaceous plant belonging to the Asteraceae family. This plant is found in many tropical and subtropical areas and has attracted considerable interest in traditional medicine owing to its healing power [1]. Traditionally, it has been employed in the treatment of wounds and skin infections and as an anti-inflammatory drug [2]. The foliage of *Chromolaena odorata* is well-known for its abundant phytochemical content, including flavonoids, terpenoids, and alkaloids, which are thought to enhance its medicinal effectiveness [3]. According to Nurwahidah's research in 2021, quercetin was the predominant component found in the leaves of *C. odorata* L. after conducting isolation experiments. Quercetin and its glycosides account for approximately 60-75% of the total flavonoids [4]. Quercetin is a chemical within the flavonoid group that can generate complex compounds when reacting with $AlCl_3$.

Numerous studies have established that quercetin is malodorous and unstable, which contributes to its low bioavailability. Quercetin, a compound with limited absorption windows based on its physicochemical properties, is virtually insoluble in water [5]. Problems may arise during the delivery and penetration of the active compound through the epidermis if formulated as a topical preparation.

One approach to address the challenges associated with quercetin compounds is to use an ethosome formula [6]. The primary constituents of ethosome formula are water, phospholipids, alcohol at a relatively high concentration (20-45%), and flexible elastic vesicles that progress inward [7]. The ethosome active compound delivery system shows the capacity to traverse the skin owing to its exceptional deformability. Ethosome possess physicochemical properties that render them suitable for transporting active compounds to the epidermis at greater depth and quantity than alternative drug delivery agents [8]. Furthermore, ethosome can transport medications that are hydrophilic, lipophilic, or amphiphilic [9].

There are several methods for ethosome formulation, such as cold, hot, and classical mechanical dispersion methods [10]. In this study, the hot method was used, in which the lipids were dispersed in water at 40 °C to form a colloidal phase. Furthermore, ethanol and propylene glycol were separately mixed at the same temperature. Plant extracts were added to a solvent with a suitable solubility. An organic phase (ethanol and propylene glycol) was added to the aqueous phase. Particle size reduction was performed using sonication or extrusion. The heat approach was used for the development of these ethosomes because to its potential to influence the formation of the lipid bilayer while ensuring the stability of the active component (quercetin). This study investigates the specific capabilities of *Chromolaena odorata* in the development of ethosomal carriers. This innovative method aimed to improve the skin penetration of substances. This investigation addresses a notable deficiency in current research, as previous studies have barely investigated the utilization of *Chromolaena odorata* in this context. The ethosomal method has demonstrated potential in enhancing the bioavailability of phytoconstituents through the skin barrier, which is a common obstacle faced by traditional topical formulations. This research not only fills the gap by presenting empirical proof of the efficacy of *Chromolaena odorata* ethosomes, but also establishes a new direction for future studies to examine other underexplored natural chemicals for transdermal use. Based on existing literature, researchers are interested in formulating ethosome preparations from the ethanol extracts of *Chromolaena odorata* leaves.

MATERIALS AND METHODS

The materials used in this study were *Chromolaena odorata* leaves with an authentication number by Herbarium Medanense University of Sumatera Utara: 143/MEDA/2022, soya lecithin, cholesterol, distilled water, propylene glycol, ammonium chloride, chloroform, HCl 2N, Meyer's reagent, Bouchardat's reagent, Dragendorf's reagent, Pb $(CH_3COO)_2$ 0.4, H_2SO_4(p), isopropanol, molish reagent, amyl alcohol, HCl(p), $FeCl_3$, CH_3COOH 5% and $AlCl_3$.

Extraction *Chromolaena odorata* leaves ethanol extract.

Fresh *Chromolaena odorata* leaves were washed, separated from the twigs, and dried in a drying cabinet at 30-40 °C for 5 d or until the leaves were sufficiently dry. The dried leaves were ground into powder and sieved through a 40-mesh sieve. 2000 g of *Chromolaena odorata* leaves powder (2000 g) was extracted with 96% ethanol (20,000 ml). It was soaked for the first 6 h with occasional stirring and then allowed to stand for 18 h. The macerate was separated by filtration. The extraction process was repeated twice with the same type of solvent; the total volume of the solvent was half of the volume in the first extraction. All macerate was collected and evaporated using a rotary evaporator until a thick extract was obtained [11]. Phytochemical content was determined using a standard procedure [12].

Ethosome preparation

The ethosome formula design consisted of several components, and the formula variations are shown in table 1.

Table 1: Ethosome formula variation

Composition	Formula I	Formula II	Formula III	Formula IV
Extract	-	0.5g	1g	1.5g
Lecithin	3g	3g	3g	3g
Cholesterol	3g	3g	3g	3g
Ethanol	10 ml	10 ml	20 ml	30 ml
Propylene glycol	1 ml	1 ml	1 ml	1 ml
Distilled water ad	100 ml	100 ml	100 ml	100 ml

The ethosome formula was developed using the heat method. At 40 °C, lecithin was dissolved in water to form a colloidal solution (Mixture 1). The *Chromolaena odorata* extract was dissolved in ethanol and propylene glycol at 40 °C (mixture 2). Mixtures 1 and 2 at 40 °C using a 700-rpm magnetic stirrer. Following refrigeration at room temperature, the suspension was chilled [13].

Evaluation of organoleptic examination

Organoleptic observations included the color and smell of ethosome. Observations were made visually [14].

Evaluation of particle size and zeta potential observations

Particle size and zeta potential measurements were performed using a particle size analyzer, and the sample was placed in a cuvette after 100x dilution. The stability of ethosome preparations was evaluated organoleptically in the form of color changes and the formation of particulates that were dispersed into the carrier liquid [15].

Evaluation of ethosome morphology

Ethosome morphology was examined using Transmission Electron Microscopy (TEM) [16].

Evaluation of pH

A pH test was conducted to determine the pH of the ethosome preparations. The pH was determined at a temperature of 25 °C±2 °C using a pH meter. To determine the pH, the pH meter electrode must be cleaned with distilled water and subsequently dried with tissue. Subsequently, the pH meter was calibrated using a buffer solution with pH 6.0. Subsequently, the electrodes were rinsed with distilled water and dried. The pH meter displays a constant number, which is then recorded in the pH observation table. The replication process was conducted three separate times [17].

***In vitro* penetration test**

The study used male rabbit skin with a weight range of 1.5-2 kg. A modified vertical Franz diffusion cell was used to conduct the penetration tests. The released substance was quantified using a wavelength of 370 nm, and the quantity of the penetrating agent was calculated [18].

RESULTS AND DISCUSSION

Obtained extract

Chromolaena odorata leaves separated from the dirt were washed thoroughly with a wet weight of 26 kg and then dried. After grinding, the powder weighed 10 kg. The yield of *Chromolaena odorata* leaves ethanol extract obtained from the maceration process is shown in table 2.

Phytochemical content in *Chromolaena odorata* leaves

The identification of the chemical compound content in *Chromolaena odorata* leaves aims to prove the presence or absence of compounds such as alkaloids, flavonoids, tannins, steroids, and saponins. The identification results obtained from the ethanol extract of *Chromolaena odorata* leaves contained alkaloids, flavonoids, tannins, saponins, glycosides and steroids/triterpenoids. These results are consistent with the research of Andika in 2020, which stated that the ethanol extract of *Chromolaena odorata* leaves contains secondary metabolites, such as alkaloids, saponins, flavonoids, phenols, and tannins [18].

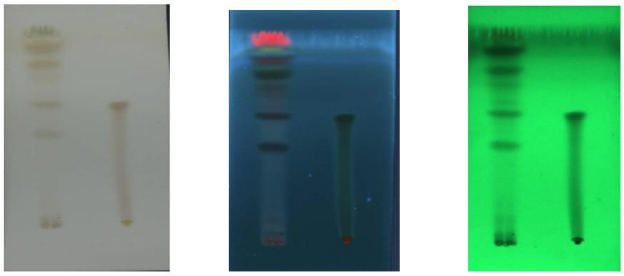

Fig. 1: Visual identification results, UV-366 and UV-254 quercetin compounds

Table 2: Percentage of yield of *Chromolaena odorata* leaves extract

Powder weight (g)	Extract weight (g)	Yield (%)
2000	295.90	14.795

Identification results of quercetin compounds using preparative TLC

Chromolaena odorata is a tropical plant known for its high quercetin content, which is a potent flavonoid. The leaves of this plant are particularly rich in this compound, which is renowned for its antioxidant properties; in this study, the identification of quercetin compounds with preparative TLC can be seen visually, using UV-366 and UV 254. The identification results are shown in fig. 1.

Furthermore, the identification results of the *Chromolaena odorata* leaves extract with quercetin as a comparison can be seen by identifying the R_f value attached to the TLC plate. The results of the Rf values are shown in table 3.

Table 3: Rf value of *Chromolaena odorata* leaves extract

Visualization				UV 366				UV 254			
Sample		Comparison		Sample		Comparison		Sample		Comparison	
Color	R_f	Color	R_f	Color	R_f	Color	R_f	Color	R_f	Color	R_f
Orange	046	Orange	0.6	Orange	0.46	Orange	0.6	Black	0.46	Black	0.6
Orange	0.6			Orange	0.6			Black	0.6		
Yellow	0.73			Indigo	0.73			Black	0.65		
Orange	0.8			Orange	0.8			Black	0.8		
Green	0.86			Yellow	0.86			Black	0.86		
Orange	0.89			Red	0.89			Black	0.89		
				Blue	0.90						
				Yellow	0.92						

Preparative TLC was performed using a silica gel 60GF254 plate as the stationary phase and a combination of chloroform: acetone: formic acid (7:3:0.5) as the mobile phase. Spots visible visually on the extract of *Chromolaena odorata* leaves have an R_f value of 0.6 (orange in color) which has the same R_f value as the Quercetin Standard (orange in color). Spots seen using UV-366 on *Chromolaena odorata* leaves extract had an R_f value of 0.6 (orange in color), the same R_f value as the comparator (quercetin compound). In addition, spots seen using UV-254 on *Chromolaena odorata* leaves extract have an R_f value of 0.6 (black in color), the same R_f value as quercetin.

The preparative thin-layer chromatography isolation process is based on the difference in absorption and partitioning power as well as the solubility of the components; the chemical will move following the polarity of the eluent because the absorption adsorbent to chemical components are not the same, then the components move at different speeds, which causes separation [19]. Before being spotted on the Preparative TLC plate, the sample was dissolved in a small amount of solvent. A volatile solvent is a good solvent. If the solvent used is not volatile, band widening occurs. The sample concentration should also only be 5-10%. The sample to be spotted must be in the form of a band that is as narrow as possible because whether the separation is good also depends on the width of the band [20].

After the preparative TLC plate was eluted, the band was scraped from the plate. Furthermore, the compound must be extracted from the adsorbent using a suitable solvent (5 ml per 1 g of adsorbent). Endeavored using the most nonpolar solvents. It should be noted that the more extended the contact between the compound and the adsorbent, the more likely the compound will experience decomposition. The extract was filtered using a funnel with a glass of masonry or a membrane. Preparative thin-layer chromatography has the lowest cost and uses the most basic equipment. Although it can separate ingredients in gram quantities, most are used only in milligram amounts [21].

Organoleptic observation results

The ethosome obtained were in the form of a brownish-yellow solution with no odor. Its composition comprises *Chromolaena odorata* leaves as the active ingredient, cholesterol as a stabilizer to minimize ethosomeal leakage, and lecithin as an emollient and emulsifying agent. Ethanol acts as a facilitator that can enhance the capacity of the drug to permeate, render the vesicles flexible, and expand the structure of the epidermal layer. In addition, they possess antibacterial properties. Distilled water acts as a solvent, whereas propylene glycol acts as a penetration enhancer or wetting agent [22].

Particle and zeta potential measurement

Ethosome particles in the ethanol extract of *Chromolaena odorata* leaves were measured using a Particle Size Analyzer (PSA). Measurements were made on four formulations: F1 (without extract), F2 (extract with 10 ml ethanol), F3 (extract with 20 ml ethanol), and F4 (extract with 30 ml ethanol). Each formula contained ethanol extracts from *Chromolaena odorata* leaves, soya lecithin, propylene glycol, ethanol, and water. The results of the particle size measurements are shown in table 4.

Table 4: Particle size and zeta potential results of ethosome

Formula	Particle size (nm±SD)*	Potential zeta (mV±SD)*
I	273.87±13.78	+27.0±1.0
II	256.93±22.30	+28.0±1.0
III	240.57±29.52	+32.67±0.58
IV	161.2±32.0	+34.33±0.58

*The data present in mean±SD, n= 3. I**=** Without extract with 10 ml ethanol, II= *Chromolaena odorata* leaves ethanol extract 0.5 g and 10 ml ethanol, III= *Chromolaena odorata* leaves ethanol extract 1 g and 20 ml ethanol, IV= *Chromolaena odorata* leaves ethanol extract 1.5 g and 30 ml ethanol

Formula IV had the lowest particle size, measuring 161.2±32.0 nm. Formula I has the largest particle size, which measures 273.87±13.78 nm. Particle size measurements were conducted to ascertain the dimensions of the vesicles and evaluate their capacity to permeate the skin. If the size of a vesicle or ethosome vesicle falls in the range of 10-1000 nm, it satisfies the criteria to be classified as a nanovesicle. The size of the vesicles generated is contingent upon the variability of the constituent components, manufacturing process, and utilization of instruments such as sonicators [23].

In addition, Formula IV had a greater quantity of ethanol (precisely 30 ml) than the other formulations. This affected the magnitude of the final particle size. As the ethanol content was increased, the size of the vesicles decreased. The one-way ANOVA yielded a particle size significance value of 0.003 ($p<0.05$) based on statistical data. This indicates substantial disparities in each formula, depending on the particle size. Furthermore, two distinct groups were identified using Tukey's HSD test. The initial group achieved a significance value of 1.000, indicating that the results were not statistically significant

(p>0.05). This demonstrates that formula IV exhibits a singularity with respect to particle size. The second group achieved a significance score of 0.427, indicating that the probability of obtaining these results by chance alone was greater than 0.005. This indicates that there is no substantial disparity in the particle size among formulas I, II, and III.

Zeta potential is a measure of the stability of ethosome during storage. Vesicles fulfilled the criteria if the zeta potential value exceeded+25 mV or falls below-25 mV. According to Fitrya *et al.* (2021), the degree of vesicle aggregation is inversely proportional to the magnitude of its positive or negative charges [24]. The zeta potential values measured in this investigation ranged from±27.0±1.0 mV to±34.33±0.58 mV. Based on the statistical data, a One-way ANOVA yielded a zeta potential significance value of 0.000 (p<0.005). This demonstrates notable disparities among the formulas with regard to zeta potential. Continuing with the Tukey's HSD test, two groups were identified. The first group had a significance level of 0.480 (p>0.05). Formulas I and II exhibited no substantial disparity in determining zeta potential. The second group achieved a significance score of 0.134, indicating that the probability of obtaining these results by chance was greater than 0.05. These findings indicate no substantial difference between formulae III and IV in their ability to determine zeta potential.

An optimal zeta potential value signifies an increased ability of the particles to repel one another, resulting in the formation of a stable dispersion during preparation. Conversely, an unfavorable zeta potential value indicates a decrease in the ability of particles to repel each other, leading to an increased tendency for aggregation and reduced preparation stability [25].

Ethosome morphology

The TEM morphology test utilized a single formula that was optimal in terms of particle size and dispersion. The specified size was included in formulation 4, which contained the highest concentration of extract (1.5 g) and ethanol (30 ml). The observed TEM data encompassed both the pre-and post-purification TEM images. Fig. 3 shows the TEM observations of the ethanol extract of *Chromolaena odorata* leaves.

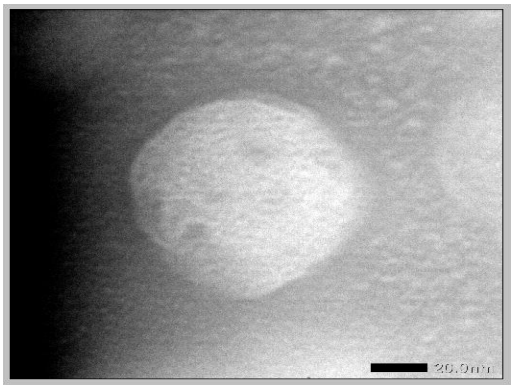

Fig. 3: TEM results from the ethanol extract of *Chromolaena odorata* leaves formulation-4. (400 X magnification)

Fig. 3 shows the findings from observations of a closed spherical object. The findings of this observation align with the discoveries made by Limsuwang in 2012, that the closed spherical shape of the ethosome' morphology [26].

pH measurement

The pH measurements were performed to determine the pH of the preparation. The pH affects the availability of drugs in the molecular form. Drugs in molecular form can easily penetrate. The results of the ethosomal pH measurements were recorded for 12 w. The results are presented in table 5.

Table 5: Results of ethosome pH measurement

Formula	Initial pH	Week to-2	Week to-4	Week to-6	Week to-8	Week to-10	Week to-12
I	6.33±0.06	6.30±0.00	6.30±0.00	6.23±0.06	6.23±0.06	6.20±0.10	6.20±0.17
II	5.21±0.15	5.20±0.00	5.17±0.06	5.13±0.06	5.03±0.01	5.03±0.01	5.03±0.01
III	4.43±0.12	4.43±0.06	4.43±0.06	4.43±0.12	4.27±0.06	4.27±0.06	4.27±0.06
IV	4.23±0.12	4.23±0.12	4.23±0.06	4.13±0.06	4.10±0.10	4.10±0.00	4.10±0.00

*The data present in mean±SD, with n = 3. I**=** Without extract with 10 ml ethanol, II= *Chromolaena odorata* leaves ethanol extract 0.5 g and 10 ml ethanol, III= *Chromolaena odorata* leaves ethanol extract 1 g and 20 ml ethanol, IV= *Chromolaena odorata* leaves ethanol extract 1.5 g and 30 ml ethanol

The pH measurement in this study was performed by correlating its utilization with topical application. The typical pH range for normal skin is 4.5-6.5. The investigation followed the required preparation criteria, which indicated that the skin may tolerate a pH range of 4.5-6.5 [27]. It is evident from all formulations that the pH decreased after the addition of the extract. The extract had an acidic pH of 4.11. Following the 12-week storage period, the pH of all treatments exhibited a modest reduction; however, it remained within the acceptable range for skin pH.

***In vitro* penetration test**

The penetration test aims to determine the total amount of ethosome flavonoids transported through the rabbit skin membrane per unit area and time. Flavonoid compounds released from the base are transported through the skin membrane to the dissolution medium. Release testing was performed using the Franz Diffusion Cells method. Phosphate buffer was chosen as the receptor fluid because it simulates the pH of human biological fluids.

The membrane is placed between the receptor and donor compartments, where the membrane must contact the receptor fluid so that the preparation applied to the membrane can be released and penetrate the membrane. The receptor compartment functions for homogenization, accelerating the dissolution of the released substance. Stirring was performed using a magnetic stirrer at 150 rpm. During this process, the temperature was maintained using a thermometer at 37 °C which represents the human body temperature. The quercetin compound was released, and the test results were measured by measuring the absorbance at a wavelength of 374 nm. The release profiles of the quercetin ethosome extract compounds per unit area are shown in table 6.

According in table 6, the ethosome containing the most ethanol in F4 (1.5 g ethosome extract with 30 ml ethanol) exhibited a release of flavonoid substances at 480 min, measuring approximately 1099.789±83.409 mcg/cm2. Ethanol serves as a substance that enhances the penetration of other substances. Elevated ethanol concentrations can interfere with control of the lipid bilayer in the skin. Hence, upon incorporation into a bubble, the membrane exerts a propulsive force that enables the bubble to pierce the corneum. The lipid membrane of ethosome is rendered less dense than traditional bubbles, resulting in a softer and more flexible shape [28]. This increased pliability provides greater freedom and stability to the membrane, allowing it to easily penetrate integrated stratum corneum lipids. Elevated ethanol concentrations led to a reduction in particle size, whereas membrane thickness decreased as a result of

interactions with hydrocarbon chains. Ethanol decreased the surface tension by altering the overall charge of the solution, thereby enhancing the stability and reducing the particle size. The particle size was directly proportional to the decrease in ethanol concentration [29]. Finally, ethosome formula F4 was identified as the most optimal formula. A range of studies have explored the potential of ethosomes as a delivery system for quercetin, a natural antioxidant with various health benefits. Another research found that quercetin-loaded ethosomes significantly enhanced skin permeation and bioavailability [30, 31]. Ferrara in 2022 further demonstrated the potential of transethosomes, a type of ethosome, in improving quercetin permeation and stability. These findings suggest that ethosomes, particularly transethosomes, could be a promising vehicle for enhancing the delivery of quercetin [32].

Table 6: Quercetin compound release from ethosome prepartion of *chromolaena odorata* leaves

Time (min)	F2(mcg/cm²)	F3(mcg/cm²)	F4(mcg/cm²)
0'	0.000±0.000	0.000±0.000	0.000±0.000
5'	5051.434±21.146	4816.411±2275.098	4543.077±227.787
10'	5311.599±32.874	3666.596±200.940	5101.165±241.830
15'	5590.643±45.236	4063.726±114.081	5475.066±276.664
30'	5732.468±22.030	4345.536±116.975	5718.653±233.933
60'	5982.502±70.185	4680.757±228.801	5947.507±349.332
90'	6143.666±24.980	5137.082±150.974	6459.087±439.792
120'	6457.245±44.140	5454.023±383.553	6892.849±545.693
150'	6691.624±88.122	5664.779±334.781	7403.048±606.830
180'	7030.529±227.572	5930.469±331.418	7932.588±771.294
210'	7376.802±171.619	6099.001±333.386	8359.442±756.901
240'	7716.167±145.752	6558.100±226.226	8869.181±866.859
270'	7923.838±123.142	7016.253±239.783	9267.486±873.120
300'	8143.482±225.205	7258.922±200.567	9485.288±855.994
360'	8564.811±30.589	7611.641±292.768	9871.161±714.796
390'	9123.820±108.353	7889.764±299.762	10130.679±733.531
420'	9366.487±277.893	8127.826±277.296	10478.9797±675.105
450'	9808.535±96.071	8432.656±268.011	10790.257±769.682
480'	9989.182±114.787	8873.325±335.690	11210.204±815.049

*The data present in mean±SD, with n = 3.

CONCLUSION

The optimal ethosome *Chromolaena odorata* extract was F4, made with 1.5 g of Chromolaena odorata extract in 30 ml ethanol, effectively delivers active compounds to the skin, featuring particle size of 161.2±32.0 nm and zeta potential of+34.33±0.58 mV. All formulas maintained a skin-safe pH of 4.5-6.5, slightly decreasing over 12 w but within tolerance limits. F4 also exhibited enhanced penetration activity.

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AUTHORS CONTRIBUTIONS

Sofia Rahmi took the lead on the formulation work as part of her doctoral research. Julia Reveny, Panal Sitorus, and Anayanti Arianto provided supervision and guidance throughout the study.

CONFLICT OF INTERESTS

Declared none

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