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INNOVATION OF MICROEMULSION SYSTEMS REVERSE MICELLE MENIRAN EXTRACT (*PHYLLANTHUS NIRURI*) AS A CERVICAL ANTICANCER IN THE DEVELOPMENT OF HERBAL MEDICINE

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Prolonged chemotherapy increases side effects that are treated with herbs, such as meniran. Cancer cells have high lipophilicity and Log P corilagin meniran has low bioavailability. The encapsulation of the active ingredient in the reverse micelle system can increase the value of the log P and work selectively. This research aims to determine the effectiveness of the Microemulsion System Reverse Micelle Meniran (MSRMM), a cervical anticancer, in developing herbal medicine. The formulations determine the span of 80 levels that form optimally based on their characterization and effectiveness. The methods included MSRMM making, characterization, and in vitro tests. The results showed that MSRMM F2 (0.50 % span 80) meets optimal requirements, especially droplet size parameters of 2226.4 nm, PDI of 0.2129, and zeta potential of -26.4 mV. MSRMM F2 cervical anticancer IC_{50} was 171.190 µg/mL and P = 0.047 (P<0.05) compared to meniran extract. In conclusion, MSRMM F2 is the selected formula and has potential as a cervical anticancer herbal drug.

***Keywords**: Cervical anticancer; meniran; microemulsion; reverse micelle*

INTRODUCTION

Cancer is a disease caused by abnormal cell growth in body tissue. There were 19.3 million new cases of cancer recorded for a total of 10 million deaths, which is estimated to increase by 47.15% or 28.4 million cases by 2040. The second highest incidence of cancer in Indonesia is cervical cancer with a total of 36,633 patients or 9.2% [1]. Cervical cancer treatment generally uses the chemotherapy agents paclitaxel, doxorubicin, and cisplatin[2]. However, this treatment is expensive and causes side effects such as nausea, vomiting, nephrotoxicity, and hepatotoxicity [3]. Much research has stated that natural ingredients can be chemotherapy agents to reduce side effects in cancer treatment[4]. One of the natural ingredients developed as an anticancer for the cervix is meniran [5].

Meniran (*Phyllanthus niruri*) is a herbal plant that contains corilagin of 1157.87 µg/g [6]. Based on research by Gupta et al, 2019 [2], corilagin can inhibit the proliferation and induction of apoptosis in cancer cells by modulating the NF-κB, Notch-mTOR, and TGF-β signaling pathways. *In silico* research states that the corilagin contained in meniran has a higher docking score than the cancer drug paclitaxel, so it has the potential to be developed as a cervical anticancer agent[5]. Apart from that, the 70% ethanol extract of meniran herb is classified as a strong antioxidant with an IC_{50} of around 17.55 ppm, making it one of the initial parameters that show the potential of a plant as an anticancer [6].

Cancer cells generally have high lipoprotein levels, so preparations with the same lipophilicity are needed to penetrate well[7]. However, corilagin has low permeability

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with a partition coefficient (log P) value of around - 0.29. As a result, the bioavailability of corilagin in the body is low[2]. One way to increase the bioavailability of corilagin so that its effectiveness as a cervical anticancer is optimal is by formulating a reverse micelle system microemulsion preparation.

Efforts to encapsulate drug molecules in a reverse micelle system can increase the log P value. This is proven by the EGCG log P value ranging from 1.0 to 3.1 by forming a reverse micelle system[7]. In addition, the reverse micelle system can increase therapeutic effectiveness by selectively aggregating drugs and reducing systemic toxicity[8]. Therefore, innovative research on reverse micelle system microemulsion preparations of meniran (*Phyllanthus niruri*) extract as a cervical anticancer is proposed in developing herbal medicines through a series of tests. The process of making reverse micelle system microemulsion requires the oil phase and emulgator. The olive oil is selected as an oil phase because it is a preferred oil worldwide for medicine applications and aids in the stabilization of the oil-water interface on the reverse micelle structure [9]. Besides, span 80 was chosen as an emulgator because it can reduce the surface tension between the water phase and the oil phase. In addition, the water-in-oil (w/o) emulsion using span 80 showed good stability [10]. Based on the research of Gao, 2023 [11] the best amount of span is 1%. However, excessive amounts of span can result in too large micelle sizes. In addition, another journal states that the amount of span 0.5% forms a stable microemulsion system. Thus, in this study, we used span percentages of 0.25%, 0.5%, and 1%. This research aims to determine the optimal formula for the Microemulsion System Reverse Micelle Meniran (MSRMM) preparation based on the characterization results and prove that the selected MSRMM formulation has cervical anticancer activity based on the test results *in vitro*.

MATERIALS AND METHODS

Chemicals and reagents

Meniran herbs were obtained from Tuban, Indonesia. Distilled water, Ethanol 96%, Span 80, and Olive oil from Merck, Indonesia. HeLa cancer cells were kindly donated from the Faculty of Pharmacy Universitas Airlangga, Indonesia. Roswell Park Memorial Institute (RPMI), Penicillin- streptomycin, Amphotericin B, Phosphate buffered saline (PBS), Dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), Doxorubicin, Sodium dodecyl sulfate (SDS), Trypsin EDTA, Trypan blue from Sigma-Aldrich, St. Louis, MO, USA). All other chemicals and reagents were of the pharmaceutical grade.

Meniran herb extraction

Fresh meniran herbs are sorted and washed with running water until clean, drained, and air-dried without direct exposure to sunlight. The crude extract is then ground with a grinding tool. The method used for extraction is maceration. 250 g of crude extract powder were soaked in 2500 mL of 70% ethanol, left for 24 h, filtered, and the filtrate collected in an Erlenmeyer flask. This activity was repeated three times, and then the filtrate was concentrated using a vacuum rotary evaporator at a temperature of 50°C at a speed of 50 rpm and dried in the oven at a temperature of 50°C overnight. After that, further drying was carried out using a vacuum desiccator until meniran extract powder was obtained.

Formulation of MSRMM

The MSRMM manufacturing stage was carried out by dissolving 40 mg of meniran extract using 12 mL of citrate buffer pH 5.0 and stirring at a speed of 1500 rpm at a constant temperature of 45°C until homogeneous. In another beaker, add olive oil, then add span 80 according to the formulation (F1 = 0.25%, F2 = 0.5%, and F3 = 1%). Stir at a speed of 1500 rpm at a constant temperature of 45°C until homogeneous. Next, the meniran extract solution is added to the mixture of olive oil and span 80 and stirred at a speed of 1500 rpm at a constant temperature of 45°C until clear. Then it was homogenized with an ultraturrax speed of 10,000 rpm for 5 min and ultrasonicated for 60 min.

Visualization test

Visualization test by observing the physical appearance of the MSRMM preparation, including shape, color, clarity, and

phase separation of the preparation using the five senses [12].

Viscosity test

Microemulsion viscosity testing was carried out using a Brookfield viscometer. The MSRMM preparation is put into a container, then a certain spindle is installed and dipped into the container. The viscometer tool is then run and the viscosity value of the preparation stated on it is displayed. The viscosity value that meets the requirements is less than 200 cPs[12].

Stability test

The freeze-thaw method is carried out by storing the microemulsion at 4°C for 24 h, then the microemulsion is transferred to 40°C for 24 h (1 cycle). Testing was carried out in six cycles [12]. The heating method is carried out using an oven with temperatures of 60°C, 70°C, 80°C, 90°C, and 100°C. The samples were kept for 5 h and after the completion of the test, physical characteristics were observed, including organoleptic observations [5]. Zeta potential method using the tool Zetasizer. The optimum zeta potential value of the microemulsion formula ranges between ±30 mV [12].

Microemulsion-type test

The reagents used are Sudan III, which is hydrophobic, and methylene blue, which is hydrophilic. The w/o microemulsion should show mixing at Sudan III for the outer phase and methylene blue for the inner phase [13].

Particle size and homogeneity test

The examination of the size and size distribution of microemulsion droplets was carried out using a particle size analyzer. The microemulsion sample was shaken to homogenize the gtemperature of 25°C. The data observed are the average droplets. diameter and polydispersity index (PDI). PDI parameters describe variations within the sample [13].

pH test

The pH meter used must be calibrated first with a buffer solution of pH 4 before measuring the pH of the microemulsion. This is because the MSRMM preparation is targeted to meet the pH specifications for oral preparations that are absorbed in duodenum (pH 5-6) [14].

Cytotoxicity test

Cytotoxicity testing of cervical cancer cells (HeLa) using MTT reagent. The cells are transferred into a well plate and incubated in a 5% CO_2 incubator for 24 h. Cell cultures are observed with an inverted microscope. The test materials in this research were divided into meniran extract samples, selected MSRMM formulations, MSRMM base, and a reference (doxorubicin) in nine different concentration series with graded dilutions. Each concentration of test material is fed into a well with three replications and re-incubated in a 5% CO_2 incubator. Then, 5 ppm MTT reagent was added. When formazan crystals have been formed, 100 µL of SDS stopper solution is added. Next, the 96-well plate is wrapped in aluminum foil and incubated in a dark place at room temperature overnight. The absorbance of each well is observed with an ELISA microplate reader [15].

Statistical analysis

Data obtained from the characterization of the three MSRMM preparations were analyzed descriptively, qualitatively, and quantitatively. Stages of determining IC_{50} were carried out by probit analysis of sample concentration series data on the percentage of inhibition in HeLa cervical cancer cells with Statistical Package for the Social Sciences (SPSS) version 26. $P<0.05$ was considered to indicate a statistically significant result [15].

RESULT AND DISCUSSION

Extraction

The determination test proved that the plant used was the green meniran herb with the species name *Phyllanthus niruri* L. from the Phyllanthaceae tribe. The extraction process using 250 g of crude extract in powder form produced 21.91 g, so a yield of around 8.72% w/w was obtained.

Visualization test

The first characterization of the MSRMM formulation was done by a visualization test. The results are shown in **Table 1**. which indicates that F2 and F3 meet the requirements,

namely yellow and clear, which suggests that the microemulsion is homogeneous [9]. Microemulsions appear as clear solutions because of the small particle size. This is also caused by the low surface tension due to the addition of surfactants, resulting in the formation of water droplets in the oil [16].

Microemulsion type test

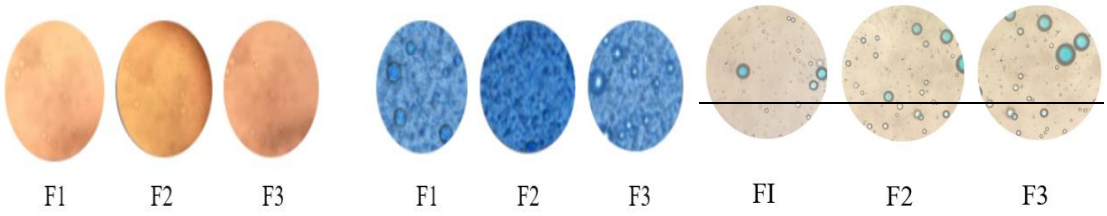
According to the results of the microemulsion type test, yellow droplets from all formulations dissolve when they react with Sudan III (left). Still, they do not dissolve when they react with methylene blue (middle). The mixing of Sudan III and methylene blue with MSRMM forms a blue droplet in the middle with a yellow outer layer (right) (**Fig.1**). The formation of a reverse micelle microemulsion system, or water droplets in oil, is demonstrated by the hydrophobic nature of the Sudan III reagent and the hydrophilic nature of methylene blue [13].

pH test

The pH test findings of all formulations prepared by MSRMM (**Table 2**) meet the standards of oral preparations that are absorbed in duodenum (pH 5-6) [14]. A pH that matches the target environment allows for optimal solubility of the active ingredient, thereby increasing absorption. In addition, it can prevent irritation or tissue damage around the target site of the duodenum [17].

Viscosity test

All of the MSRMM formulation's viscosity tests (**Table 3**) meet the microemulsion viscosity range of <200 cPs [12]. The viscosity value increases with the percentage of span 80 surfactants used. This is because span 80 is a non-ionic surfactant that readily absorbs water during production, increasing its viscosity and expanding its molecular size [14].



(a) (b) (c)

Fig.1: MSRMM microemulsion type test result formed yellow droplets that (a) dissolve in Sudan III (b) do not dissolve in methylene blue and (c) mixing of Sudan III and methylene blue with MSRMM forms a blue droplet in the middle with a yellow outer layer

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Table 1: Visualization testing results of MSRMM.

	F1	F2	F3
Visualization	Yellow, unclear	Yellow, clear	Yellow, clear

Table 2: Results of pH testing of MSRMM.

	F1	F2	F3
pH	5,07 ± 0,008	5,13 ± 0,005	5,15 ± 0,009

Table 3: Viscosity testing results of MSRMM preparations.

	F1	F2	F3
Viscosity (cPs)	74,2 ± 0,082	75,8 ± 0,047	81,3 ± 0,094

Droplet size and homogeneity test

Three formulations were found to meet the requirements for microemulsion preparations, with F2 demonstrating the best results (**Table 4**). This can be attributed to the fact that all three formulations have droplet sizes between 0.5 and 10 μm and a PDI value <7, indicating a homogeneous particle size [13]. The droplet size and PDI value decreases with increasing span 80 surfactant concentration, but the opposite occurs with excessive surfactant. The flocculation and deflocculation systems of the preparation have an impact on this result. Deflocculation happens when the formulation's droplets split apart into smaller droplets, whereas flocculation happens when oil droplet size increases, which can facilitate the coalescence of oil droplets [18]. According to **Fig. 2,** tumor cell blood vessels show differences with normal cells, namely the endothelial cell layer is discontinuous, showing cavities between endothelial cells. These cavities range in size from 200 to 10.000 nm [19]. Therefore, MSRMM with sizes between 2.000 to 6.000 nm (**Table 4**) can enter these cavities and increase their bioavailability in tumor cell.

Stability test

The three MSRMM formulations were tested for stability using three methods, that is freeze-thaw method, heating method, and zeta potential method.

Freeze-thaw method

The freeze-thaw method shows no separation between the base and the extract when the MSRMM preparation is stored at a cold temperature (4°C) and then heated at a high temperature (40°C). These results show that F1 separated the base from the extract (**Table 5**), so it is unstable. It also shows that the MSRMM F2 and F3 are not separated, so they are able to remain stable and effective during storage and transportation in extreme conditions [20].

Heating method

The three formulations exhibit stability at high temperatures within the range of 60°C to 100°C, as demonstrated by stability testing using the heating method (**Table 6**). Thus, all three MSRMM formulas are able to maintain the stability and quality of the product under extreme heat conditions, whether during storage, transportation, or use, such as in tropical climates [5].

Zeta potential method

The purpose of the zeta potential method stability testing is to quantify droplet charges that contribute to the inhibition of flocculation and coalescence. **Table 7** indicates that F2's ideal zeta potential is around -30 mV. The potential zeta value is negative because three formula use non-ionic surfactants (span 80) as emulgators to reduce particle mobility and avoid aggregation formation [12].

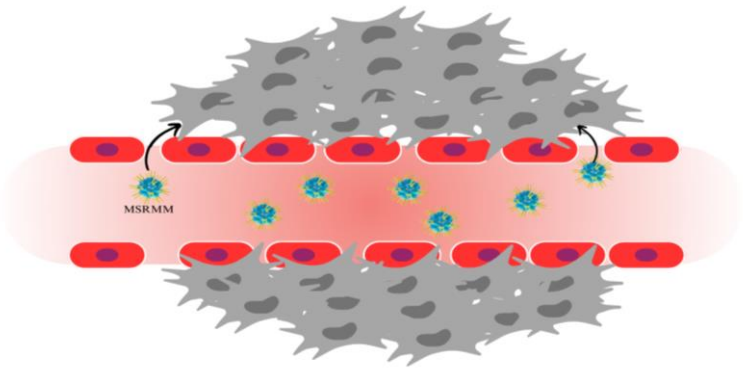


Fig.2: Illustration of the increased bioavailability of MSRMM in cancer cells due to the size of MSRMM that can enter the cavity of the blood vessels of cancer cells.

Table 4: Droplet size and homogeneity testing results of MSRMM.

Sample	Result	
	PDI	Droplet size (nm)
F1	0,5739 ± 0,012	5390,0 ± 0,178
F2	0,2129 ± 0,003	2226,4 ± 0,111
F3	0,3980 ± 0,042	4250,0 ± 0,255

Table 5: Stability testing results of MSRMM using the freeze-thaw method.

	F1	F2	F3
Result	Separated	Not separated	Not separated

Table 6: Stability testing results of MSRMM using heating method.

Sample	60 °C	70 °C	80 °C	90 °C	100 °C
F1	Not separated	Not separated	Not separated	Not separated	Not separated
F2	Not separated	Not separated	Not separated	Not separated	Not separated
F3	Not separated	Not separated	Not separated	Not separated	Not separated

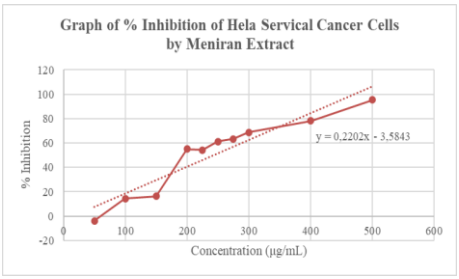
Table 7: Stability testing results of MSRMM using zeta potential.

	F1	F2	F3
Zeta potential (mV)	-0,2 ± 0,010	-26,4 ± 0,085	-24,7 ± 0,131

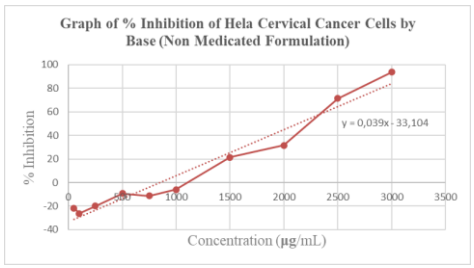
Inhibit concentration 50% (IC_{50})

According to **Fig. 3**, the IC_{50} value of meniran extract that inhibits HeLa cancer cell growth 50% (IC_{50}) is 231.467 µg/mL, whereas the IC_{50} MSRMM value is 171.190 µg/mL, so it is significantly different from the value of P = 0.047 (P<0.05). Both are included in the moderate category of cervical anticancer, while the base doesn't have anticancer effect. Despite being in the same anticancer category, it is more effective because its IC_{50} MSRMM value is lower than that of the extract [15]. Additionally, MSRMM preparations include less meniran extract and yield superior outcomes. Therefore, adding meniran extract to MSRMM's dosage form can improve the active components' absorption toward therapeutic goals such as cervical anticancer.

(a)



(b)



(c)

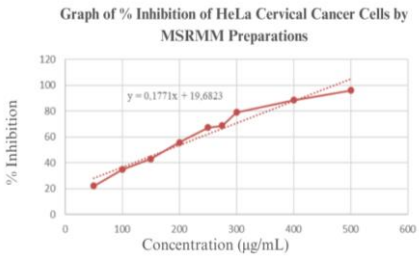


Fig. 3: Graph of % inhibition of HeLa cervical cancer cells by (a) meniran extract (b) base and (c) MSRMM preparations.

Conclusion

Based on the research and analysis that has been carried out, it can be concluded that the innovative use of meniran extract in the MSRMM dosage form can be a potential candidate for cervical anticancer herbal medicine. Formula 2 with a span of 80 of 0.5% showed the best dosage characteristic results based on visualization tests, microemulsion type, pH, viscosity, particle size, homogeneity, and stability (freeze-thaw method, heating, and zeta potential). Test results in vitro cervical anticancer activity with HeLa cells also show that the effectiveness of MSRMM preparations is better than meniran extract alone with IC_{50} values amounting to 171,190 µg/mL.

Acknowledgments

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نشرة العلوم الصيدليـــة
جامعة أسيوط

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ابتكار أنظمة المستحلبات الدقيقة بنظام (مقلوب الميسلات) باستخدام مستخلص مينيران (فيلانثوس نيروري) كمضاد لسرطان عنق الرحم كهدف لتطوير الطب العشبي

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يؤدي العلاج الكيميائي لفترات طويلة إلى زيادة الآثار الجانبية التي ممكن أن يتم علاجها بالأعشاب، مثل المينيران. تمتلك الخلايا السرطانية قدرة عالية على محبة الدهون، كما أن التوافر البيولوجي لـ Log P corilagin meniran منخفض. وإن تغليف المادة الفعالة في نظام الميسيل المقلوب يمكن أن يزيد من قيمة سجل P ويعمل بشكل انتقائي.

ويهدف هذا البحث إلى تعيين فعالية نظام المستحلب الدقيق بنظام الميسيل المقلوب لمستخلص مينيران (MSRMM)، وهو مضاد للسرطان في عنق الرحم، و تطوير الطب العشبي. وتحدد الدراسة تركيز الاسبان ٨٠ فى الصيغ المحضرة و مدى تأثيره على خصائصها وفعاليتها. وتضمنت الدراسة طريقة التحضير (MSRM)، وتوصيفها، وإجراء الاختبارات المعملية عليها. وأظهرت النتائج أن مستخلص MSRMM (F2) (0.50% span 80) يعتبر الصيغة المثلى، وخاصة أن حجم القطرات بلغت ٢٢٢٦,٤ نانومتر، وقيمة PDI البالغ ٠,٢١٢٩، وجهد زيتا البالغ -٢٦,٤ ملي فولت. و بلغ التركيز الموضعي المضاد لسرطان عنق الرحم MSRMM F2 171.190 ميكروغرام/مل، وقيمة P (P = 0.047 <٠,٠٥) مقارنةً بمستخلص المينيران.

و الخلاصة أثبتت النتائج ، أن (MSRMM F2) هي الصيغة المختارة ولها إمكانية استخدامها كدواء عشبي مضاد للسرطان في عنق الرحم.