JURNAL ILMU KEFARMASIAN INDONESIA, October 2023, page. 284 - 292 Vol. 21, No. 2
ISSN 1693-1831

Cosmos caudatus Kunth. Leaf Extract Herbal Nanosuspension Formulations, Characterization, and Cytotoxicity Approach Against MCF-7 Breast Cancer Cells

(Formulasi Nanosuspensi Herbal Ekstrak Daun *Cosmos caudatus* Kunth., Karakterisasi, dan Pendekatan Sitotoksisitas Terhadap Sel Kanker Payudara MCF-7)

SAFIRA NAFISA[1,2*], SITI UMRAH NOOR[1,2], AZKANNUFUUS[1], YUSLIA NOVIANI[1,2]

[1]Departement of Pharmaceutical Technology, Faculty of Pharmacy, Universitas Pancasila, South Jakarta, Jakarta 12640, Indonesia

[2]Center for The Study of Natural Product for Degenerative Diseases, Faculty of Pharmacy, Universitas Pancasila, South Jakarta, Jakarta, 12640, Indonesia

Submitted 20 July 2023, Accepted 25 October 2023

Abstract: Kenikir leaves (*Cosmos caudatus* Kunth.) contain quercetin, which has anticancer properties. To provide more effective complementary therapy for breast cancer, nanotechnology was applied to develop preparations containing kenikir leaf extract. This research aimed to formulate a nanosuspension containing kenikir leaf extract with cytotoxic activity against MCF-7 breast cancer cells. Nanosuspension of kenikir leaf extract was prepared using the ionic gelation method with 3%, 4%, and 5% PVP K-30 stabilizer. The nanosuspension formula with the highest entrapment efficiency was further characterised, including particle size, polydispersity index (PDI), zeta potential, pH, and particle morphology. Cytotoxic activity was tested against MCF-7 cells by the MTT assay. The results showed that the formula with 5% PVP has the highest entrapment efficiency value of 85.04±0.08%, a particle size of 221.9 nm, a PDI of 0.211, a zeta potential of -21.7 mV, a pH of 4.08±0.0, and a spherical morphology. The kenikir leaf extract at a concentration of 1 mg/mL inhibited the proliferation of MCF-7 cells by 23.4% ($p<0.05$), whereas the nanosuspension at 10 µg/mL inhibited proliferation by 23.7%. It can be concluded that kenikir leaf extract can be formulated into a nanosuspension that meets the physical criteria and has cytotoxic activity against MCF-7 cells.

Keywords: Cell toxicity, flavonoid, ionic gelation, nanotechnology, PVP K-30.

Abstrak: Daun kenikir (*Cosmos caudatus* Kunth.) mengandung flavonoid kuersetin yang berkhasiat sebagai antikanker. Nanoteknologi diterapkan dalam pengembangan sediaan dari ekstrak daun kenikir sebagai terapi pendamping kanker payudara yang lebih efektif. Penelitian ini bertujuan untuk memformulasi ekstrak daun kenikir menjadi nanosuspensi yang memiliki aktivitas sitotoksik terhadap sel kanker payudara MCF-7. Nanosuspensi ekstrak daun kenikir dibuat dengan menggunakan metode gelasi ionik dengan variasi penstabil PVP K-30 3%, 4% dan 5%. Formula nanosuspensi dengan efisiensi penjerapan tertinggi dikarakterisasi lebih lanjut meliputi ukuran partikel, indeks polidispersitas (PDI), zeta potensial, pH, morfologi partikel dan diuji aktivitas sitotoksik terhadap sel MCF-7 dengan metode MTT assay. Hasil analisis menunjukkan formula dengan penambahan PVP 5% memiliki nilai efisiensi penjerapan tertinggi sebesar 85,04±0,08%; ukuran partikel 221,9 nm; PDI 0,211; zeta potensial -21,7 mV; pH 4,08±0,01 dengan morfologi sferis. Hasil aktivitas sitotoksik tehadap sel MCF-7 ditunjukkan dengan persentasi penghambatan proliferasi yaitu pada ekstrak daun kenikir konsentrasi 1000 µg/mL sebesar 23,368% dan pada nanosuspensi ekstrak daun kenikir konsentrasi 10 µg/mL sebesar 23,686%. Dapat disimpulkan bahwa ekstrak daun kenikir dapat diformulasikan menjadi nanosuspensi yang memenuhi kriteria fisik dan memiliki aktivitas sitotoksik tehadap sel MCF-7.

Kata kunci: Gelasi ionik, kuersetin, nanoteknologi, PVP K-30, toksisitas sel.

*Corresponding author
e-mail: safira.nafisa@univpancasila.ac.id

INTRODUCTION

KENIKIR, scientifically known as *Cosmos caudatus* Kunth, is a plant species that possesses quercetin components. These chemicals are classified as flavonoids and are recognised for their antioxidant properties. The capacity of antioxidants to eliminate free radicals is associated with decreased degenerative ailments, including cancer. This result was achieved by inhibiting the growth of various cell lines associated with human breast cancer[1,2]. According to earlier research findings, the methanol extract derived from kenikir leaves has a notably strong antioxidant effect, as evidenced by the IC_{50} values obtained from macerated extracts (12.5±0.3 µg/mL), percolation extracts (16.8±0.2 µg/mL), and soxhletation (18.8±0.2 µg/mL)[3].

The cancer repression process has been facilitated by quercetin's antioxidant and pro-oxidant properties, which induce oxidative stress. This effect is comparable to established anticancer medications like doxorubicin[2]. Quercetin is implicated in inhibiting tumour growth due to its pro-oxidant properties. In contrast, quercetin has been observed to promote the initiation of apoptosis and induce cell cycle arrest. The primary objective of cancer therapy is to induce apoptosis, and the inherent capacity of quercetin to induce apoptosis positions it as a potential candidate for cancer investigations[4]. Nevertheless, a notable constraint lies in the inadequate absorption of flavonoids within the gastrointestinal tract. The utilisation of quercetin in therapeutic and pharmacological domains is impeded by its limited water solubility, chemical instability, and brief biological half-life[5,6]. Therefore, the application of nanoformulations is strongly advocated to enhance the bioavailability of flavonoids.

In this study, a nanosuspension of kenikir leaf extract was produced using the ionic gelation technique. This method involves electrostatic interactions between the amine groups (NH_3^+) in chitosan and the phosphate ions (HPO_4^-) acting as polyanions derived from NaTPP. These interactions lead to the formation of a three-dimensional intramolecular structure. The process also incorporates using different quantities of PVP-K30 (Polyvinylpyrrolidone K-30) as a physical stabilizer[7,8]. The cytotoxic activity of the nanosuspension was evaluated to determine its ability to impede the proliferation of MCF-7 (Michigan Cancer Foundation-7) cell cultures using the 3-(4,-5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay technique. The objective of this study was to develop a nanosuspension formulation comprising kenikir leaf extract with the intention of evaluating its cytotoxic effects on MCF-7 breast cancer cells.

MATERIALS AND METHODS

MATERIALS. Plant Material. Kenikir leaf (*Cosmos Caundatus* Kunth.) (BALITTRO, Bogor, Indonesia) Kenikir leaves were determined at the Laboratory of the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, under the reference number 639/UN2.F3.11/PDP.02.00/2022.

Chemicals. Chitosan (Sarchem Labs, New Jersey, USA), sodium tripolyphosphate (Arrow Fine Chemicals, Gujarat, India), PVP K-30 (DC Fine Chemicals, Barcelona, Spain), dimethyl sulfoxide (Merck, Darmstadt, Jerman), propylene glycol (PG) (Dow Chemical Pacific, Singapura), ethanol 96%, quercetin (Sigma, Missouri, USA), phosphate buffer saline (PBS) (Sigma, St Louis, MI, USA), RPMI 1640 medium (Gibco, Paisley, Scotland), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) (Sigma, St Louis, MI, USA).

Equipments. Ultrasonic (GT Sonic-P3, Guangdong, China), rotary vacuum evaporator (Laborota 4011-Digital, Heidolph, Germany), magnetic stirrer (Barnstead/Thermolyne, Dubuque, USA), centrifugation (PLC-03, Hi-care International, Taiwan), Karl Fischer (870 KF Titrino plus, Metrohm, Herisau, Switzerland), spectrophotometer UV-VIS (Shimadzu, UV-1900, Kyoto, Japan), zeta sizer (Malvern, Herrenberg, Germany), transmission electron microscopy (TEM HT7700 Hitachi, Tokyo, Japan), 96-well plate, laminar airflow (Airstream, Esco Micro Ltd, Singapore, Singapore), incubator CO_2 (ICO50, Memmert, Schwabach, Germany), microscope inverted (Zeiss Axiovert 40 CFL, Jena, Germany), ELISA reader (Biotek Instruments, Vermont, USA).

METHODS. Kenikir Leaf Extraction. The Ultrasound-Assisted Extraction (UAE) method using 96% ethanol was used to produce kenikir leaf extract[5]. The kenikir leaf powder is weighed at 100 grammes, followed by adding 1000 mL of solvent (1:10). It is then extracted at 40°C for 30 minutes using an ultrasonic bath with a frequency of 50 kHz. The filtrate was collected and concentrated using a rotary vacuum evaporator (pressure 175 mbar, temperature 40°C, speed 70 rpm) to obtain a crude extract of kenikir leaves. The concentrated extract was weighed, and the yield value was calculated. The characteristics of kenikir leaf extract include

organoleptic, water content, and pH. The organoleptic examination was done by visual observation based on shape, colour, and smell compared to the Indonesian Herbal Pharmacopoeia[10]. The water content of the kenikir leaf extract was determined using the Karl-Fischer titration method. In determining the pH of 1 g of crude extract of kenikir leaves dissolved in 100 mL of distilled water, the pH of the extract was measured using a pH meter. Phytochemical screening of kenikir leaf crude extract was carried out using the Farnsworth method[11].

Determination of Total Flavonoid Content. The total flavonoid content (TFC) was investigated using the aluminium chloride colorimetry method[12]. In brief, the extracted sample was diluted with methanol up to 100 µg/mL. The calibration curve was prepared by diluting quercetin as the reference standard for flavonoid in methanol (0–100 µg/mL). The diluted extract, or quercetin (2.0 mL), was mixed with 0.1 mL of a 10% (w/v) aluminium chloride solution and 0.1 mL of a 0.1 mM potassium acetate solution. The mixture was kept at room temperature for 30 minutes. Then the maximum absorbance of the mixture was measured at 434.2 nm using a UV-VIS spectrophotometer. TFC was expressed as the percentage of total flavonoids in the sample with the following formula[13]:

Description :

$$F = \frac{c\ x\ V\ x\ f\ x\ 10^{-6}}{m}\ x\ 100\%$$

F = the number of flavonoids $AlCl_3$ method (%)
c = quercetin equivalent (µg/ml)
V = total volume of extract
f = dilution factor
m = sample weight (g)

Extract Solubility Test. The solubility test of the extract in various solvents was determined based on the quercetin content, which could dissolve in aquadest, 96% ethanol, propylene glycol, and dimethyl sulfoxide (DMSO). The crude extract weighed 100 mg and was then dissolved in 100 mL of pure water, 96% ethanol, propylene glycol, and DMSO. Each 1 mL was pipetted, 0.2 mL of 10% $AICI_3$ was added, 0.2 mL of CH_3COONa 1 M was added, 3 mL of 96% ethanol was added, and 5.6 mL of distilled water was added and then homogenized. After that, it was analysed with a UV-Vis spectrophotometer at λ 438 nm. Absorption was used to calculate the solubility of quercetin (mg/mL) in a solution of kenikir leaf extract using the linear regression equation of the quercetin standard calibration curve that had been obtained[14].

Nanosuspension Preparation of Kenikir Leaf Extract. Nanosuspension of kenikir leaf extract was prepared using the ionic gelation method with a variable concentration of PVP K-30 as a physical stabilizer[15,16]. PVP K-30 was not added to FI, while FII, FIII, and FIV were added to the PVP K-30 solution with concentrations of 3%, 4%, and 5% (v/v), respectively. 0.2 g of chitosan was dissolved in 100 mL of 1% acetic acid using a magnetic stirrer at 1200 rpm for 24 hours. As much as 0.1 g of sodium tripolyphosphate was dissolved in 100 mL of distilled water. PVP K-30 was weighed at 0.3 g (FII), 0.4 g (FIII), and 0.5 g (FIV), respectively, and then dissolved in 10 mL of distilled water. 0.10 g of crude extract of kenikir leaves was dissolved in 25 mL of DMSO, 10 mL of propylene glycol, and 15 mL of 96% ethanol solvent. A 0.2% chitosan solution was added to the crude extract solution of kenikir leaves that had been prepared; the mixture was stirred with a magnetic stirrer, each stirring at 500 rpm for 10 minutes. 3.33 mL of PVP K-30 stabiliser solution of 3% (FII), 4% (FIII), and 5% (FIV) was dropped little by little into the mixture and kept stirring with a magnetic stirrer for 30 minutes. Each was added 0.1% sodium tripolyphosphate with a ratio of chitosan and sodium tripolyphosphate of 5:1 at a speed of 1 drop per 3 seconds through a burette and with a magnetic stirrer at 500 rpm for 1 hour to form a homogeneous nanosuspension. The formula with the highest entrapment efficiency was chosen as the optimum formula. The entrapment efficiency was determined using the indirect method[17,18]. A total of 4x10 mL of nanosuspension was centrifuged at 14,000 rpm for 30 minutes, and the residue was separated from the supernatant. The supernatant was homogenised using a vortex for 1 minute, and then its absorbance was measured using a spectrophotometer at λ 438 nm. Absorption is used to calculate the concentration of quercetin that is not adsorbed using the linear regression equation of the standard quercetin calibration curve, and then the active substance supernatant content was converted into the percentage of entrapment efficiency (EE) with the formula:

$$\%EE = \frac{(Ct - Cb)}{Ct}\ x\ 100\%$$

Description :
EE = entrapment efficiency
Ct = quercetin concentration in nanosuspension
Cb = concentration of free quercetin

Optimum Formula Nanosuspension Characterization[19]. The optimum formula's characterization includes morphology, particle

size, polydispersity index, zeta potential, pH, and cytotoxic activity. Nanosuspension morphology using a transmission electron microscope (TEM). Characterization of particle size, polydispersity index, and zeta potential using a zeta sizer, The pH of the nanosuspension was measured using a pH meter.

MCF-7 Breast Cancer Cell Culture[20,21]. MCF-7 human breast cancer cells were grown at 37°C in a humidified incubator with 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and a 1% penicillin-streptomycin mixture. The cells were routinely subcultured every 3 days with 70–80% confluency and harvested using 0.25% trypsin.

Cytotoxic Activity of MCF-7 Breast Cancer Cells with the MTT Assay Method[20,21]. The cytotoxic test used 96-well plates as the test medium. As much as 100 µL of cell suspension in serum RPMI medium was added to each well, then incubated in a 5% CO_2 incubator at 37°C for 24 hours to obtain good growth. After 24 hours, the cells will adhere to the bottom of the microplate. The medium is removed, and into each well was added 100 µL of the test solution (kenikir leaf extract and kenikir leaf nanosuspension) with various concentration series, namely 31.25; 62.5; 125; 250; 500; and 1000 µg/mL in RPMI medium and Doxorubicin control solution (positive control) in RPMI medium with a concentration of 0.375; 0.75; 1.5; 3; 6; and 12 µg/mL and control cells in 100 µL medium (as a blank). They were then incubated at 37°C in a 5% CO_2 incubator for 24 hours. Cells were observed under a microscope after 24 hours of incubation. In the LAF, each medium in the well is removed. Then 100 µL of PBS was added, shaken, and discarded. A total of 100 µL of MTT reagent in RPMI was serum and added to each well, then incubated in a 5% CO_2 incubator at 37 °C for 4 hours, removed from the incubator, and observed the purple formazan crystals formed with a microscope. Then, a 100 µL SDS 10% stopper was added to each well, wrapped in 96 well plates with aluminium foil, and put into the shaker incubator at room temperature overnight. Then each well was read for absorption using an ELISA reader at λ 570 nm[22]. Test data is analysed by calculating the following equation:

$$\text{Percentage of life cell} = \frac{(\text{treated absorbance} - \text{media control absorbance})}{(\text{cell control absorbance} - \text{media control absorbance})} x\ 100\%$$

Percentage of inhibition of proliferation = 100% - percentage of live cells

The IC_{50} value is the antilog of the x-axis on the linear regression graph of the log concentration of the formula (x-axis) and the percent inhibition of cell proliferation (y-axis).

Statistical Analysis. Analysis of variance (ANOVA) was used to analyse significant factors and their interactions using SPSS 26.0. A p-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Kenikir Leaf Extraction. Kenikir leaves were determined at the Laboratory of the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, under the reference number 639/UN2.F3.11/PDP.02.00/2022. The chosen extraction method is ultrasound-assisted extraction (UAE) because of its numerous advantages, including reduced solvent consumption, enhanced yield of active compounds, improved safety, and an expedited extraction procedure. The application of ultrasonic extraction resulted in physical disruptions to the cellular structures, including cell walls and membranes, leading to a reduction in particle size[23]. The UAE method was more efficient than the maceration process in extracting polyphenols, as evidenced by its higher yield and superior antioxidant characteristics[24]. Using 96% ethanol as an extraction solvent demonstrates selectivity in the extraction process, specifically targeting phenolic molecules like flavonoids and other polar chemicals. According to the findings of this study, the crude extract yield was determined to be 13.45%. The current findings demonstrate an improvement compared to the results reported in the prior study[25]. It has been demonstrated that the UAE method increases the yield of extracted components and has more advantages (less time, less solvent, and reduced operational costs) than the maceration method.

The study's findings indicate that the crude extract derived from kenikir leaves exhibited a thick consistency, a dark brown colour, and a characteristic odour associated with kenikir leaves. The extract was found to have a water content of 15.47±1.22% and a pH value of 4.70±0.03. Water in crude extracts can influence microbial development because it is an ideal substrate for microbial proliferation. The water content findings follow the stipulations outlined in the Indonesian Herbal Pharmacopoeia, which specifies a maximum threshold of 18.7%. The acidity of the extract derived from kenikir leaves can be attributed to flavonoids, namely kaempferol and quercetin, which possess acidic characteristics throughout a pH range of 1.8-6.0[26]. The phytochemical screening showed that crude extracts of kenikir leaves contain alkaloids, flavonoids, polyphenols, tannins, glycosides, triterpenoids, steroids, and saponins. Flavonoids have been shown to exert a vast array

of anticancer effects, including the modulation of reactive oxygen species (ROS)-scavenging enzyme activities, participation in cell cycle arrest, induction of apoptosis and autophagy, and suppression of cancer cell proliferation and invasiveness.

Total Flavonoid Content. The research indicated that the overall flavonoid concentration was 5.66±0.01%. The findings of this study satisfied the criteria outlined in the Indonesian Herbal Pharmacopoeia for the overall flavonoid concentration of the crude extract derived from kenikir leaves, which mandates a minimum threshold of 5.20%. There exists a correlation between total flavonoid concentration and antioxidant activity. A positive correlation exists between a material's overall flavonoid content and its antioxidant capacity. The antioxidant properties exhibited by flavonoids can be attributed to the existence of phenolic hydroxyl groups, which have the ability to counteract the harmful effects of free radicals by donating hydrogen atoms. The determination of total flavonoid concentration was employed as a benchmark for quercetin due to its classification as a flavonol subgroup of flavonoids, characterised by a keto group at C-4 and a hydroxy group at either C-3 or C-5 atoms. These structural features make quercetin closely related to flavones and flavonols[27].

Extract Solubility in Various Solvents. Flavonoid extraction and solubilization commonly include the utilisation of organic solvents. The behaviour of the flavonoid in aqueous dimethylsulfoxide mixtures was investigated by Jabbari et al. Through a mixed approach involving experimental techniques and computational molecular dynamics (MD) simulations. The previous study investigated the preferential solvation of flavonoid molecules by DMSO over water, resulting in an enhanced solubility of flavonoids[28]. Two primary factors influence flavonoid solubilization and solute-solvent interactions, namely: (i) the impact of hydrogen bonding and (ii) the phenomenon of preferential solvation in mixed fluids, specifically referring to nanoscopic local heterogeneity[29].

According to the data presented in Figure 1, it can be observed that the solubility of the crude extract derived from kenikir leaves in various solvents is as follows: 67.66 mg/mL in DMSO, 58.71 mg/mL in propylene glycol, 60.35 mg/mL in 96% ethanol, and 19.85 mg/mL in water. Various types of solvents influence the solubility of the resultant extract. DMSO has greater solubility than alternative solvents. This study employed a combination of polar solvents, namely DMSO, PPG, and 96% ethanol, to enhance the solubility of extracts and improve the efficiency of cosolvency procedures[30].

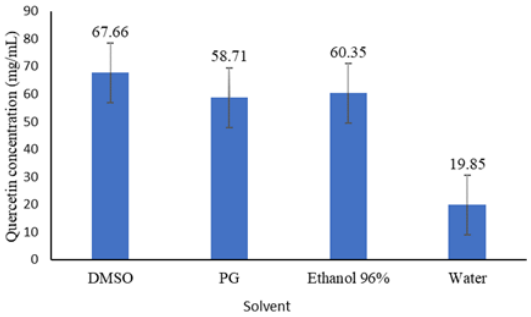

Figure 1. Quercetin level in extracts in various solvents.

Formulation of Kenikir Leaf Extract Nanosuspension. The nanosuspension was prepared using the ionic gelation process, which involves the spontaneous electrostatic contact between 0.2% chitosan and 0.1% sodium tripolyphosphate (Na-TPP). The ionic gelation approach described in this study entails the utilisation of electrostatic interactions between the amine groups of chitosan, which carry a positive charge, and the polyanions of NaTPP, which carry a negative charge[31]. The mechanical strength of nanoparticles can be enhanced through the cross-linking procedure, whereas using Na-TPP as a chitosan ion pair has been found to provide nanoparticles with improved stability[32]. PVP K-30 is used as a polymer stabiliser due to its enhanced efficacy in inhibiting particle aggregation and impeding crystal formation[33].

The nanosuspension obtained exhibits a yellowish-brown hue and possesses a distinctive olfactory profile. Figure 2 illustrates the trapping efficiency of the nanosuspension. The measurement of entrapment efficiency is a crucial parameter in drug delivery systems as it provides insight into the quantity of drug that has been effectively adsorbed within the nanoparticle system. Efficiency values of adsorption exceeding 50% can be deemed satisfactory[34]. The research findings indicate that the three nanosuspension formulations of kenikir leaf extract with PVP K-30 exhibited a greater entrapment efficiency value compared to formula I. Specifically, the entrapment efficiency values ranged from 73.82% to 85.04%, with the greatest average percent adsorption value observed in formula IV at 85.04±0.08%. Formula IV exhibits a notable adsorption percentage, suggesting the presence of ionic interactions among the active ingredient, stabiliser, and cross-linking agent. This phenomenon leads to an augmentation in the number of molecules and extracts adsorbed within the nanosuspension. The employed stabiliser serves the purpose of enveloping the nanoparticles that have been produced, thereby impeding any potential

disruption to the structure of the nanoparticles. This disruption, if left unchecked, could result in the leaking of flavonoids[17]. Statistical analysis ($p<0.05$) showed that changes in the amount of PVP K-30 had a big effect on how well the nanoparticles of kenikir leaf extract were able to trap particles. The Formula IV test findings indicate that it was considered optimal due to its maximum entrapment efficiency value. Further investigation will be conducted to evaluate other characteristics of the nanosuspension.

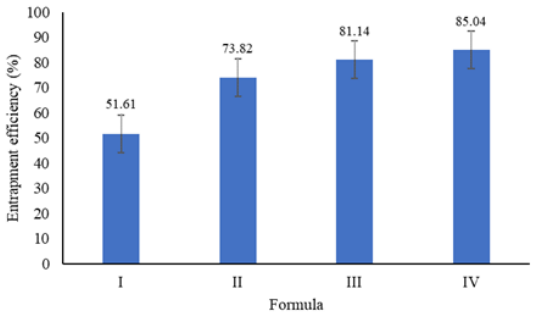

Figure 2. The entrapment efficiency value of kenikir leaf extract nanosuspension (PVP K-30 FI 0%, FII 3%, FIII 4%, FIV 5%).

Optimum Formula Characterization Result. In order to discern the disparities in the characterization of the nanosuspension formulation with and without PVP K-30, an analysis was conducted on many aspects of characterization, such as particle shape, particle size, polydispersity index, and zeta potential, in formulas I and IV. The nanosuspension morphology analysis revealed that the particles obtained were spherical, as depicted in Figure 3. Spherical geometries and smooth textures exhibit enhanced stability and reduced propensity for aggregation owing to the limited frictional forces experienced between constituent particles. The inclusion of PVP in the K-30 formulation reduces particle size due to the stabilising properties exhibited by PVP. In conclusion, the presence of a hydrophobic carbon chain serves as a steric barrier, effectively safeguarding the nanoparticles and impeding inter-particle interactions, thus leading to an augmentation in particle dimensions[34,35]. This finding is consistent with the test outcomes, wherein the diameter of the FI nanoparticles measured 478.2 nm, whereas that of the F IV nanoparticles was 221.9 nm. The nanosuspension crude extract of kenikir leaves formula I, without PVP, exhibited a larger particle size than formula IV, which contained 5% PVP K-30. A polydispersity index value near zero signifies a uniform particle size distribution. In contrast, a value over 0.5 suggests a heterogeneous particle size distribution, perhaps leading to sedimentation due to the presence of aggregated nanoparticles[36]. The obtained values for the FI polydispersity index were 0.336, while the F IV value was measured to be 0.211. The obtained findings suggest that the particles exhibit a high level of uniformity, and including PVP reduces the polydispersity index value.

The zeta potential analysis was conducted to characterize the nanoparticle surface's charge properties, specifically in relation to the electrostatic interactions of the nanoparticles. The propensity for aggregation, repulsion, and long-term stability is contingent upon electrostatic interactions. In order to achieve nanosuspensions with favourable stability, sterically stabilised nanosuspensions must possess a zeta potential of at least ±20 mV[37]. The obtained zeta potential value for formula IV is -21.7 mV, as the research findings indicate. The negative charge can be attributed to the negatively charged Na-TPP crosslinker and the PVP stabilizer. The polymer surrounding the particles comprises carbonyl groups, specifically PVP. Due to its electron-donating nature, PVP acquires a negative charge, which also influences the nanoparticles' charge to become negative[8]. The pH analysis revealed that the pH of the FIV nanosuspension was measured to be 4.08±0.01. The pH of the nanosuspension exhibited a lower acidic level than the extract, which can be attributed to the incorporation of glacial acetic acid into the nanosuspension to augment chitosan's solubility. Acids can induce protonation reactions of the amine groups present in chitosan, forming NH_3^+ groups. These NH_3^+ groups can subsequently undergo interactions with NaTPP[31].

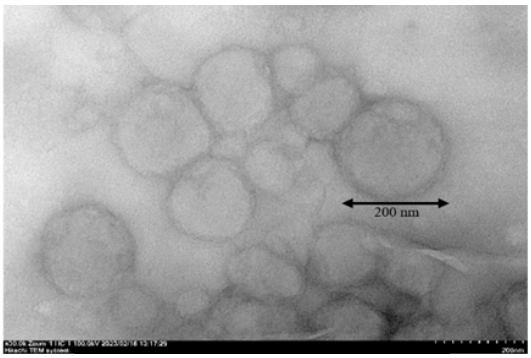

Figure 3. Nanosuspension morphology using TEM, magnification 30,000 times.

Results of the Cytotoxic Activity of MCF-7 Breast Cancer Cells with the MTT Assay Method. Cytotoxicity assessment was conducted via the MTT assay, employing factors related to the enzymatic activity of succinate dehydrogenase in converting MTT substrates into formazan. In order to assess

the potential inhibitory effects of sample treatment on cell growth, a cytotoxic test was conducted on MCF-7 cells. The primary objective of this test was to determine the concentration of the test sample that could effectively inhibit MCF-7 cell growth by 50%, known as the IC_{50} value. This information is crucial for evaluating the suitability of the test sample as a potential cancer drug[38]. In this study, FIV nanosuspension was utilised to assess its cytotoxic potential, and a comparative analysis was conducted with the cytotoxicity results obtained from kenikir leaf extract. The cytotoxic activity of the substance can be assessed by analysing changes in cellular morphology and estimating the degree of inhibition in cell proliferation.

The cellular morphology of MCF-7 cells was examined under a microscope, revealing evidence of either cell division or cell death (Figure 4). The alterations above in cellular structure signify a deleterious impact resulting from the inhibitory mechanism of the test substances on the growth of MCF-7 cells. At a dose of 12 µg/mL, the positive control of doxorubicin had an inhibition of proliferation rate of 75.717%. Additionally, the kenikir leaf extract showed a 23.368% reduction in cell proliferation at a concentration of 1000 µg/mL, and the nanosuspension of kenikir leaf extract showed a 23.686% reduction in cell proliferation at a concentration of 10 µg/mL. The findings suggest that the nanosuspension formulation of kenikir leaf extract did not substantially impact the cytotoxic activity of kenikir leaf extract.

Consequently, it may have the potential to be employed as an adjunctive treatment alongside anticancer drugs like doxorubicin. The findings of this study are consistent with other studies that have shown a synergistic impact on MCF-7 breast cancer cells when combined with kenikir-doxorubicin leaf extract. The synergistic effect of the combined administration of kenikir leaf extract and doxorubicin may be attributed to the distinct mechanisms of action exhibited by these two drugs, which collectively contribute to the reduction of MCF-7 breast cancer cell proliferation. The cellular mechanism by which doxorubicin exerts its effects involves inhibiting the G2/M phase of the cell cycle. The extract derived from Kenikir leaves is known to contain flavonoid chemicals, which have been found to exhibit inhibitory effects on the G1/S phase transition[39,40].

Figure 4. Changes in MCF-7 cell morphology before and after sampling. A) control cells; b) kenikir leaf extract concentration of 1000 µg/mL; c) nanosuspension of kenikir leaf extract at a concentration of 10 µg/mL.

CONCLUSION

The 96% ethanol extract of kenikir (*Cosmos caudatus* Kunth.) leaves can be formulated into chitosan-NaTPP nanosuspension with PVP K-30 stabilizer to meet the physical requirements. Kenikir leaf extract and nanosuspension of kenikir leaf extract had cytotoxic activity against MCF-7 breast cancer cells, as indicated by the percentage inhibition of proliferation, which was 23.368% at 1000 g/mL for kenikir leaf extract and 23.686% at 10 g/mL for nanosuspension of kenikir leaf extract.

ACKNOWLEDGMENTS

The authors would like to thank the Faculty of Pharmacy, Universitas Pancasila, Indonesia, for allowing this study to be conducted in their laboratory.

FUNDING

This research did not receive any specific grants from public, commercial, or non-profit funding agencies.

REFERENCES

1. Adawiyah R, Setiawan A, Nita S. Effect of active fraction of kenikir (*Cosmos caudatus* Kunth) leaf extract on cytotoxic test, apoptosis and antiproliferation of T47D breast cancer cells in vitro. Biomedical Journal of Indonesia. 2017;3(3):138-44.
2. Ezzati M, Yousefi B, Velaei K, Safa A. A review on anticancer properties of querce tin in breast cancer. Life sciences. 2020;248:117463.
3. Widiyantoro A, Harlia H. Antioxidant activity of leaves extract of kenikir (*Cosmos caudatus* Kunth) with various extraction methods. Indones J Pure Appl Chem. 2021;3(1):9-20.
4. Kasiri N, Rahmati M, Ahmadi L, Eskandari N, Motedayyen H. Therapeutic potential of quercetin on human breast cancer in different dimensions. Inflammopharmacology. 2020;28:39-62.
5. Andriani M, Permana IDGM, Widarta IR. The effect of time and temperature extraction on antioxidant activity of starfruit wuluh leaf (*Averrhoa bilimbi* L.) using ultrasonic assisted extraction (UAE) method. J. Ilmu dan Teknol Pangan. 2019;8(3):330–40.
6. Khan H, Ullah H, Martorell M, Valdes SE, Belwal T, Tejada S, Sureda A, Kamal MA. Flavonoids nanoparticles in cancer: Treatment, prevention and clinical prospects. Seminars in Cancer Biology. 2021;69:200-11.
7. Mudgil M, Gupta N, Nagpal M, Pawar P. Nanotechnology: a new approach for ocular drug delivery system. Int J Pharm Pharm Sci. 2012;4(2):105-2.
8. Koczkur KM, Mourdikoudis S, Polavarapu L, Skrabalak SE. Polyvinylpyrrolidone (PVP) in nanoparticle synthesis. Dalton transactions. 2015;44(41):17883-905.
9. Pamungkas AR, Indrayudha P. Cytotoxic activity of ethanol, ethanol-water, ethyl acetate, and *n*-hexane extracts of *Momordica charantia* against mcf-7 cells in-vitro. Pharmacon: Jurnal Farmasi Indonesia. 2019;16(2):73-82.
10. Kemenkes RI. Farmakope Herbal Indonesia Edisi II. Jakarta: Kementrian Kesehatan RI. 2017.
11. Arba M, Susilawati Y, Mustarichie R. Hair growth promoting activity of langir bark (*Albizia saponaria* Lour.) ethanol extract: In-vivoASSAY. Rasayan Journal of Chemistry. 2022;15(3):2065-71.
12. Do QD, Angkawijaya AE, Tran-Nguyen PL, Huynh LH, Soetaredjo FE, Ismadji S, Ju YH. Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. Journal of Food and Drug Analysis. 2014;22(3):296-302.
13. Azizah DN, Kumolowati E, Faramayuda F. Determination of flavonoid content $AlCl_3$ method in methanol extract of cocoa fruit (*Theobroma cacao* l.). Kartika Jurnal Ilmiah Farmasi. 2014;2(2):45-9.
14. Gayo Z, Lucida H, Zaini E. Solid dispersion of quercetin-PVP K-30 and its effects on the antioxidant activity. Jurnal Ilmiah Farmasi. 2020;16(2):144-54.
15. Thandapani G, Prasad S, Sudha PN, Sukumaran A. Size optimization and in vitro biocompatibility studies of chitosan nanoparticles. International Journal Of Biological Macromolecules. 2017;104:1794-806.
16. Sivasankarapillai VS, Das SS, Sabir F, Sundaramahalingam MA, Colmenares JC, Prasannakumar S, Rajan M, Rahdar A, Kyzas GZ. Progress in natural polymer engineered biomaterials for transdermal drug delivery systems. Materials Today Chemistry. 2021;19:100382.
17. Moon H, Lertpatipanpong P, Hong Y, Kim CT, Baek SJ. Nano-encapsulated quercetin by soluble soybean polysaccharide/chitosan enhances anticancer, anti-inflammation, and antioxidant activities. Journal of Functional Foods. 2021;87:104756.
18. Suciati T, Nafisa S, Nareswari TL, Juniatik M, Julianti E, Wibowo MS, Yudhistira T, Ihsanawati I, Triyani Y, Khairurrijal K. ArtinM. Grafted phospholipid nanoparticles for enhancing antibiotic cellular uptake against intracellular infection. International Journal of Nanomedicine. 2020;8829-43.
19. Arifin MF, Noviani Y, Nafisa S, Sheilabel A. Preparation, characterization, and optimization of ionic gelated nanoparticles dried extract of temulawak rhizome (*Curcuma xanthorrhiza* R.) using a factorial design 2^2. Jurnal Ilmu Kefarmasian Indonesia. 2022;20(2):272-80.
20. Wang X, Luo N, Xu Z, Zheng X, Huang B, Pan X. The estrogenic proliferative effects of two alkylphenols and a preliminary mechanism exploration in MCF-7 breast cancer cells. Environmental Toxicology. 2020;35(5):628-38.
21. de Oliveira Gonçalves K, Vieira DP, Levy D, Bydlowski SP, Courrol LC. Uptake of silver, gold, and hybrids silver-iron, gold-iron, and silver-gold aminolevulinic acid nanoparticles by MCF-7 breast cancer cells. Photodiagnosis and Photodynamic Therapy. 2020;32:102080.
22. El-Atawy MA, Alsubaie MS, Alazmi ML, Hamed EA, Hanna DH, Ahmed HA, Omar AZ. Synthesis, characterization, and anticancer activity of new N, N′-Diarylthiourea derivative against breast cancer cells. Molecules. 2023;28(17):6420.
23. Utami NF, Sutanto S, Nurdayanty SM, Suhendar U. The effect of various methods of extraction on flavonoid levels flower leaf extract (*Plenctranthus scutellariodes*). FITOFARMAKA: Jurnal Ilmiah Farmasi. 2020;10(1):76-83.

24. Saini A, Panesar PS, Bera MB. Comparative study on the extraction and quantification of polyphenols from citrus peels using maceration and ultrasonic technique. Current Research in Nutrition & Food Science. 2019;7(3):678-85.
25. Rukmana RM, Sawal AA, Wibawa DA. Combination of antibacterial activity of ethanol extract of meniran leaves and kenikir leaves against *Shigella dysenteriae*. In1st International Conference for Health Research BRIN ICHR 2022. 2023;7-20.
26. Pakade VE, Lesaoana M, Tavengwa NT. Effect of pH, time and temperature on forced degradation studies of quercetin in presence of polymers. Asian Journal of Chemistry. 2016;28(10):2181-7.
27. Fadillah A, Rahmadani A, Rijai L. Analysis of total flavonoid levels and antioxidant activity test of kelubut leaf extract (*Passiflora foetida* L.). In Proceeding of Mulawarman Pharmaceuticals Conferences. 2017; 5: 21-8.
28. Jabbari M, Khosravi N, Feizabadi M, Ajloo D. Solubility temperature and solvent dependence and preferential solvation of citrus flavonoid naringin in aqueous DMSO mixtures: an experimental and molecular dynamics simulation study. RSC advances. 2017;7(24):14776-89.
29. Tamayo-Ramos JA, Martel S, Barros R, Bol A, Atilhan M, Aparicio S. On the behavior of quercetin+ organic solvent solutions and their role for C60 fullerene solubilization. Journal of Molecular Liquids. 2022;345:117714.
30. Alshehri S, Shakeel F, Alam P, Pena A, Jouyban A, Martinez F. Effect of temperature and polarity on the solubility and preferential solvation of sinapic acid in aqueous mixtures of DMSO and Carbitol. Journal of Molecular Liquids. 2021;340:117268.
31. Marudova M, Zahariev N, Milenkova S, Pilicheva B, Viraneva A, Yovcheva T. Development and in-vitro characterization of benzydamine loaded chitosan nanoparticles. In Macromolecular Symposia. 2021;395(1):2000279.
32. Ranjit K, Baquee AA. Nanoparticle: an overview of preparation, characterization, and application. Int Res J Pharm. 2013;4(4):47-57.
33. Verma S, Kumar S, Gokhale R, Burgess DJ. Physical stability of nanosuspensions: Investigation of the role of stabilizers on ostwald ripening. Int J Pharm. 2011; 406(1- 2):45-52.
34. Goel S, Sachdeva M, Agarwal V. Nanosuspension technology: recent patents on drug delivery and their characterizations. Recent pat drug deliv formul. 2019; 13(2): 91–104.
35. Vecchi CF, Cesar GB, Souza PR, Caetano W, Bruschi ML. Mucoadhesive polymeric films comprising polyvinyl alcohol, polyvinylpyrrolidone, and poloxamer 407 for pharmaceutical applications. Pharmaceutical Development and Technology. 2021;26(2):138-49.
36. Bandulasena MV, Goran TV, Omololu GO, Brahim B. Continuous synthesis of PVP stabilized biocompatible gold nanoparticles with a controlled size using a 3D glass capillary microfluidic device. Chem Eng Sci. 2017; 171: 233–43.
37. Lakshmi P, Kumar GA. Nanosuspension technology: a review. Int J Pharm Pharm Sci. 2010;2(4):35-40.
38. Kusuma AW, Aries NN, Hartanti D. Cytotoxic and antiproliferative effects of quercetin on colon cancer cells WiDr. PHARMACY: Jurnal Farmasi Indonesia. 2010; 7(03):107-22
39. Oueslati MA, Gunenc A, Rigane G, Ghazghazi H, Valencia C, SALEM RB, Boukhchina S, Willmore WG, Hosseinian F. Chemical composition, antioxidant and cytotoxic activities of *Onopordum acanthium* L. crude oil and defatted meal. Rev. Roum. Chim. 2019;64(6):503-10.
40. Fauzia A, Wati UA, Rizkita CG, Qori'ah EW, Arifin I. The effect of marigold leaves and doxorubicin toward cell cycle and apoptosis of T47D cells. Indonesian Journal of Cancer Chemoprevention. 2016;7(3):79-86.