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Breast Cancer Prevention by Dietary Polyphenols: Microemulsion Formulation and In Vitro Studies

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Abstract: Concerns surrounding breast cancer have been increasing, as it leads to the current global cancer incidence and causes a high mortality rate in women. This study investigated the physiological effects of common dietary polyphenols that might prevent breast cancer progression. Quercetin, kaempferol, and rosmarinic acid were selected to explore their potential bioactivities. Each polyphenol was formulated into a microemulsion to improve its bioactivity and bioavailability. In vitro antioxidant and cytotoxicity activities of the selected polyphenols and their microemulsion forms were further investigated. The optimized microemulsion carrier with 1% oleic acid, 3% ethanol, 10% polysorbate 20, and 86% ultrapure water achieved more than 90% polyphenol encapsulation efficiency. The microemulsion was stable for more than 30 days when encapsulating polyphenol in the fluctuating temperature treatment. In vitro studies suggested that rosmarinic acid-loaded microemulsion had the best antioxidant activity compared with other polyphenol-loaded microemulsions (PL-MEs). Blank microemulsion and all PL-MEs significantly inhibited the proliferation of both hormone-dependent (T47D) and hormone-independent (MDA-MB-231) breast cancer cells. More studies are warranted to confirm the contribution of the microemulsion carrier components to the polyphenols' improved antioxidant activity and high toxicity of PL-MEs on breast cancer cells.

Keywords: flavonoid; phenolic acid; microemulsion; encapsulation; antioxidant; breast carcinoma

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1. Introduction

Breast cancer has become the most prevalent cancer in the world and is the main cause of cancer mortality among women, causing 685,000 deaths in 2020 [1,2]. It is predicted that the breast cancer incidence will increase by 31% over the next twenty years [2]. Despite these concerning facts, common treatments for breast cancer such as chemotherapy, surgery, hormone treatment, and radiotherapy have adverse effects that decrease patients' quality of life during and after the treatment [3,4]. Therefore, prevention strategies are an ideal strategy rather than treatment to tackle increasing breast cancer burden [5]. Diet-related intervention is one of the most cost-effective strategies in the primary prevention effort of breast cancer [6]. A dietary modification trial by the Women's Health Initiative (WHI) showed that in addition to reduced fat intake, increasing vegetable, fruit, and grain consumption in the long-term contributed to a 5% reduction in breast cancer risk [7].

From 2000 to 2020, 42% of clinical trials were on polyphenol-rich foods, and 58% were focused on pure compounds or extracts [8]. These data indicate a continuous interest in

the benefits of food bioactives, especially their implications for human health. Regarding breast cancer, a study showed that consuming an acute dose of polyphenol-rich cranberry beverages with low calories improved antioxidant status, and after 8 weeks of daily intake, it regulated glucose levels better, downregulated inflammation, and increased high-density lipoprotein (HDL) cholesterol [9]. Among dietary polyphenols, flavonoids and phenolic acid have been known as effective antioxidant and anti-inflammatory agents, two bioactivities that interfere with breast cancer progression [10]. Quercetin (Que) (Figure 1) is the most abundant flavonoid in vegetables and fruits [11]. Previous studies reported the cytotoxicity of Que in killing breast cancer cell lines, i.e., luminal A MCF-7 and triple-negative breast cancer (TNBC) MDA-MB-231 [12]. Kaempferol (Kae) (Figure 1), another flavonoid that differs from Que only by one hydroxyl group substituted on a phenyl ring, is also a dominant compound in green leafy vegetables [13]. Kae exhibits high radical scavenging activities and inhibits nitric oxide (NO) release [14]. Other studies also showed that Kae inhibited the proliferation of MCF-7 and MDA-MB-231 [15,16]. Rosmarinic acid (RA) (Figure 1), a phenolic acid, has two catechol moieties in its structure. It is commonly found in various herbs and fruits and has comparable intrinsic antioxidant activity to Que [17]. Moreover, RA induces cell cycle arrest in TNBC cells [18].

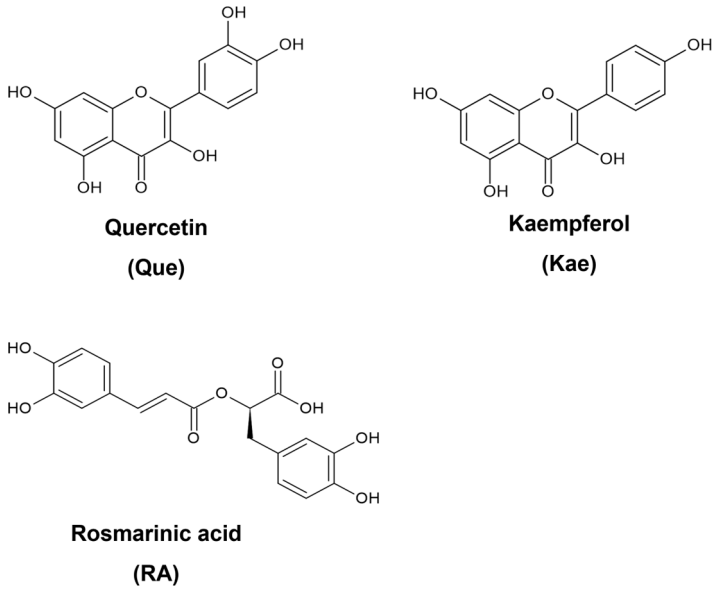

Figure 1. Structures of selected dietary polyphenols.

Bioactivities of dietary polyphenols have an array of roles in breast cancer prevention. However, low bioavailability of dietary polyphenols in the human body (which is primarily composed of water) hinders their ability to provide health benefits to cancer patients. Nanotechnology has evolved over the decades to make various tiny carrier designs available for precisely carrying lipophilic compounds with low bioavailability, like polyphenols. Microemulsion is one of them, and this drug carrier is less than 100 nanometers in size [19]. Microemulsions have an oil core and surfactant layer that dissolve the lipophilic polyphenols and increase the polyphenol solubility in aqueous environment, respectively [20]. Encapsulation of polyphenols in microemulsion results in more precise compound delivery to cancer cells due to their nanometric size. The nanometric size allows better penetration to the targeted cells and improves the biological distribution of the compound [21]. Precise delivery prevents the polyphenols from damaging normal cells, which then lowers risk of side effects and may improve patients' recovery after treatment [22,23].

In this study, considering their abundance in edible plants that showed their potential in cancer prevention, we chose Que, Kae, and RA to be formulated into microemulsions to study how the carrier system may improve cancer prevention-related bioactivities (i.e., antioxidant, cytotoxicity) of polyphenols. Microemulsion is a suitable drug carrier for

non-invasive drug administration, such as topical and transdermal routes [24]. The selected polyphenols and formulated PL-MEs were tested on two notable breast cancer cell lines: T47D, which represents Luminal A, the most common type of breast cancer, and MDA-MB-231, a cell model for triple-negative breast cancer, which has the worst prognosis among other types of breast cancer.

2. Results

2.1. Polyphenol-Loaded Microemulsions Are Homogenous and Stable under Varied Temperatures

Spontaneous emulsification employs organic phase motion; a phase consists of carrier oil, surfactant, and solvent in an aqueous phase. Although the formulations' physicochemical characteristics did not change drastically throughout the accelerated stability test, the effect of temperature change was observed in blank microemulsion (BM) and PL-ME formulations (Table 1). The size of all tested microemulsion systems stayed in the nanometric scale throughout the stability tests; BM and PL-MEs were less than 100 nm in size. BM and PL-ME formulations had decreased transmittance while their droplet size and polydispersity index (PDI) increased. Nonetheless, the final PDI values were still recorded at less than 0.5, meaning that the BM and PL-ME formulations were considered homogenous emulsions [25]. BM showed the highest size change (from 10.487 to 92.317 nm) after the stability test. Negative charge was detected in both BM and PL-MEs after the zeta potential measurement.

Table 1. Particle analysis of PL-MEs before and after subsequent stability tests. All parameters measured in triplicate.

Compound [1]	Before Storage (Day 0)				After Storage, before Stability Test (Day 30, 4 °C)				After Stability Test (Centrifugation, Heating-Cooling, Freeze-Thawing)			
	T (%) [2]	Size (nm) [3]	PDI [4]	Zeta (mV) [5]	T (%)	Size (nm)	PDI	Zeta (mV)	T (%)	Size (nm)	PDI	Zeta (mV)
QM	100.630 ± 0.004	10.443 ± 0.090	0.105 ± 0.011	−3.083 ± −0.300	96.952 ± 0.004	13.333 ± 0.545	0.246 ± 0.012	−2.837 ± −0.102	96.680 ± 0.017	14.870 ± 0.140	0.442 ± 0.003	−2.857 ± −0.163
KM	100.862 ± 0.006	10.223 ± 0.050	0.100 ± 0.004	−2.647 ± −0.333	100.64 ± 0.008	10.853 ± 0.091	0.179 ± 0.009	−1.620 ± −0.239	99.484 ± 0	11.727 ± 0.361	0.289 ± 0.009	−1.573 ± −0.248
RM	101.220 ± 0.003	10.057 ± 0.025	0.101 ± 0.004	−2.987 ± −0.272	99.553 ± 0.005	15.247 ± 0.648	0.271 ± 0.011	−3.010 ± −0.135	97.325 ± 0.003	21.477 ± 3.202	0.428 ± 0.052	−3.377 ± −0.161
BM	101.420 ± 0.005	10.487 ± 0.040	0.119 ± 0.011	−2.617 ± −0.601	100.504 ± 0.008	10.647 ± 0.110	0.154 ± 0.008	−2.657 ± −0.248	99.559 ± 0.003	92.317 ± 37.413	0.244 ± 0.024	−2.263 ± −0.309

[1] Abbreviation: quercetin-loaded microemulsion (QM), kaempferol-loaded microemulsion (KM), rosmarinic-loaded microemulsion (RM), and blank microemulsion (BM). [2] T: percent of transmittance. [3] Size: particle size. [4] PDI: polydispersity index. [5] Zeta: zeta potential value.

The TEM visualization showed that both BM and PL-MEs had non-spherical droplet morphology (Figure 2). All formulations had some aggregations, except for quercetin-loaded microemulsion (QM), which possessed big ellipsoid droplets (Figure 2B). In terms of pH stability, each formulation gradually decreased from week zero to week three, but no drastic change was observed (Figure S1).

All PL-ME formulations reached an encapsulation efficiency higher than 90% (Table 2). Among these three PL-MEs, rosmarinic acid-loaded microemulsion (RM) showed the lowest encapsulation efficiency compared with QM and kaempferol-loaded microemulsion (KM).

Table 2. Percent of encapsulation efficiency of each polyphenol-loaded microemulsions.

Compound [1]	Encapsulation Efficiency (Mean ± SD, %)
QM	98.134 ± 0.216 [b]
KM	98.334 ± 0.191 [b]
RM	92.428 ± 0.316 [a]

[1] Abbreviation: quercetin-loaded microemulsion (QM), kaempferol-loaded microemulsion (KM), and rosmarinic-loaded microemulsion (RM). Different letters ([a,b]) denote significant differences between the mean ($p < 0.05$).

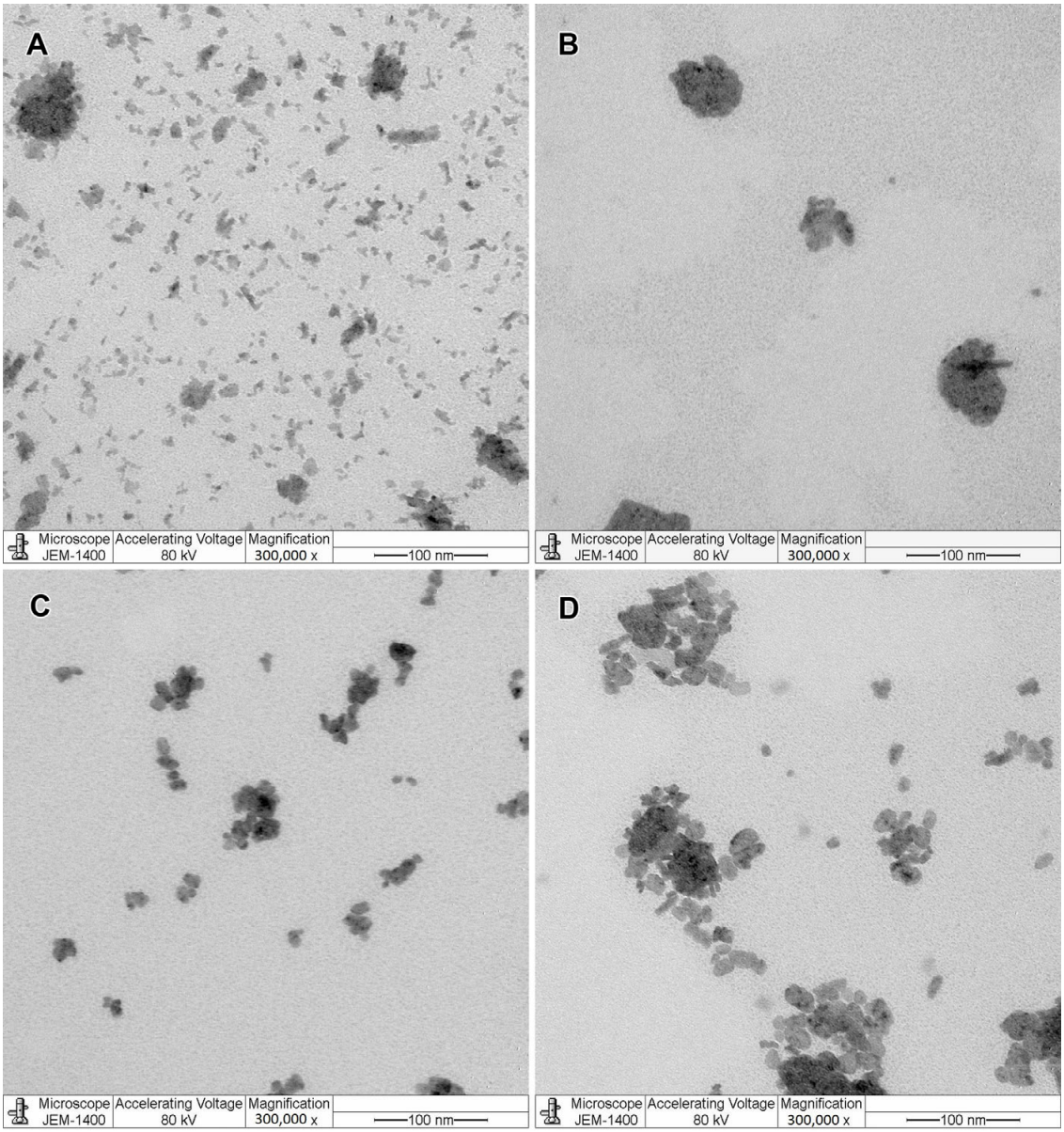

Figure 2. Visualization of blank microemulsion (BM; (**A**)), quercetin-loaded microemulsion (QM; (**B**)), kaempferol-loaded microemulsion (KM; (**C**)), and rosmarinic acid-loaded microemulsion (RM; (**D**)) under a transmission electron microscope (300,000× magnification, negative staining).

2.2. Microemulsion Carrier Improves Antioxidant Activities of Dietary Polyphenols

In comparison with pure polyphenols, Kae was the one that had the lowest activity (Table 3). The activity of RA was similar to Que with no significant difference between their IC_{50} value. This trend was also observed in QM and RM. Kae and KM exhibited the lowest scavenging activity. Overall, the optimized microemulsion carrier did improve the bioactivity of all polyphenols in scavenging *DPPH*. For NO scavenging activity, the results showed that microemulsion carrier helped selected dietary polyphenols (except for Que) to achieve better NO scavenging activity, a trend that was also observed in the *DPPH* experiment. NO scavenging activity of RM was not significantly different to the positive control, gallic acid.

Table 3. Antioxidant activity of all PL-MEs compared with polyphenols against DPPH and NO radicals.

Compound	IC_{50} (µg/mL) for *DPPH* [4]	IC_{50} (µg/mL) for NO [4]
Que	46.491 ± 1.647 [b]	72.087 ± 2.149 [b]
QM	31.725 ± 0.810 [a]	89.503 ± 9.754 [c]
Kae	81.367 ± 2.116 [d]	176.067 ± 8.277 [d]
KM	56.645 ± 1.562 [c]	101.070 ± 10.622 [c]
RA	46.538 ± 1.647 [b]	76.607 ± 5.422 [b]
RM	33.237 ± 0.276 [a]	55.517 ± 2.505 [a]
Que [1]	46.491 ± 1.647 [b]	-
Gallic acid [2]	-	55.260 ± 1.790 [a]
BM [3]	>400	>400

[1] Positive control for *DPPH* radical scavenging activity. [2] Positive control for NO inhibitory activity. [3] Blank microemulsion (BM) as a negative control. [4] Different letters ([a–d]) denote significant difference of the mean ($p < 0.005$).

2.3. Blank Microemulsion and PL-MEs Are More Toxic Than Pure Polyphenols on Breast Cancer Cells

Cytotoxicity tests were conducted to confirm the anti-proliferative potential of PL-MEs on breast cancer cells compared with pure polyphenol compounds. The polyphenols (Que, Kae, and RA) had no cytotoxic effects against T47D cells. After being loaded into microemulsion carrier to make PL-MEs, the PL-MEs exhibited their cytotoxicity. All pure polyphenols and PL-MEs, except Kae, were cytotoxic against MDA-MB-231 (Table 4).

Table 4. Cytotoxicity of polyphenols and all PL-MEs on hormone-dependent breast cancer cell line (T47D) and hormone-independent breast cancer cell line (MDA-MB-231).

Compounds	IC_{50} (µg/mL) for T47D Cells	IC_{50} (µg/mL) for MDA-MB-231 Cells
Que	>50	26.880 ± 0.596 [b]
Kae	>50	>50
RA	>50	26.060 ± 0.137 [b]
QM	1.321 ± 0.018 [b]	1.347 ± 0.006 [a]
KM	1.420 ± 0.067 [b]	1.397 ± 0.012 [a]
RM	1.307 ± 0.210 [b]	1.993 ± 1.005 [a]
Doxorubicin [1]	0.365 ± 0.004 [a]	1.213 ± 0.156 [a]
BM [2]	1.443 ± 0.033 [b]	1.403 ± 0.451 [a]

[1] Positive control. [2] Blank microemulsion (BM) used as a negative control. Different letters ([a,b]) denote significant difference of the mean ($p < 0.005$).

3. Discussion

Storing microemulsions in places with fluctuating temperatures can deteriorate the emulsion system. Oil and non-ionic surfactants have different tendencies in increasing temperature; oil becomes more soluble, but non-ionic surfactants become less soluble. This may lead to phase separation [26,27]. Ostwald ripening, flocculation, and/or coalescence may increase droplet size during storage, which then decreases the transmittance (clarity) of the formula, making it more turbid [19]. Increasing size of microemulsion droplet and PDI value have been reported in a curcumin-loaded microemulsion formula [28]. The notable change in BM size after the stability test could be attributed to the absence of polyphenols in BM [29]. Further study is required to confirm how loaded polyphenol may affect the size of a microemulsion droplet.

Zeta potential is an electrical charge difference between the charge possessed by fluid around nanometric particles and the charge in a layer made of ions surrounding the nanometric particle [30]. Zeta potential value predicts whether a nanometric particle does not undergo significant flocculation or aggregation in a long period of storage [31,32].

Polysorbate 20 is a non-ionic surfactant; it does not give any charge to microemulsions. The minus value in both BM and PL-MEs was possibly due to ionic impurities or fatty acids released upon formulation process or storage [33]. This is why the detected zeta potential values in all formulations were near zero, as the charge came from the byproduct of the side chemical reaction, not from the main emulsification process. Microemulsion with non-ionic surfactant is stabilized more via steric repulsion rather than electrostatic interaction, an interaction that stabilizes microemulsion with ionic surfactant. Steric repulsion occurs when two microemulsion droplets are in close proximity [34]. If the steric repulsion in a microemulsion system fails to overcompensate the Van der Waals interaction among the droplets, droplet aggregation will occur [35]. Aggregation of microemulsion droplets can lower measured transmittance. Given that transmittance in both BM and PL-MEs were maintained above 95% after storage and subsequent stability tests, this indicates the microemulsion carrier provided enough steric repulsion to prevent aggregation.

Non-spherical morphology of PL-MEs agrees with the description of oil-in-water microemulsion droplet made by McClements, which is a worm-like or ellipsoid droplet due to low interfacial tension of its surfactant monolayer [19]. BM exhibited a worm-like morphology, while QM, KM, and RM morphologies were more ellipsoid. Big ellipsoid droplets of QM might be formed from fused droplets, which was caused by a 1:10 dilution of the formulations in the sample preparation. Dilution could affect the microemulsion droplet as it changes component concentrations in the microemulsion system. Regarding the impact of different morphologies in BM and PL-MEs, some studies indicated that worm-like or elongated (rather than spherical) emulsion droplets tend to have improved photostability, better skin permeation, and a higher drug loading [36].

Decreasing pH after three weeks is expected, as fatty acid ester bond in polysorbate can be hydrolyzed and release free fatty acids during storage [37]. The acidity of a microemulsion formula should be considered before developing the formula for an intended application. For instance, to develop a microemulsion-based topical or transdermal therapeutic system, the recommended pH is around 4.1 to 5.8 [38,39]. With a pH that matches normal skin condition, the microemulsion formulation will possess lower risk of irritation and discomfort upon application onto the patient's skin [38,40]. BM and PL-MEs had a pH of less than 4, which is too acidic for transdermal delivery. Therefore, to make the formulated PL-MEs suitable for development of non-invasive transdermal or topical breast cancer treatment, the pH might be slightly adjusted to reach pH 4 through dilution with aqueous phase or dispersion in buffer [41].

In the measurement of encapsulation efficiency, the concentration of unencapsulated polyphenol in the water phase reflected the performance of the microemulsion system to encapsulate polyphenol in its core [42]. Components of the microemulsion carrier affect its encapsulation efficiency. The components must protect the polyphenol from hydrolytic, oxidative, and light-induced chemical interactions. They also should be able to prevent the interaction between polyphenol and other elements in the dispersed microemulsion system [22]. RA is slightly soluble in water, and this might be the reason why RA was harder to attract into the oil core inside the microemulsion carrier, considering the dispersion system was hydrophilic and the microemulsion core was hydrophobic [43].

Natural antioxidant compounds may prevent or alleviate breast cancer [44]. A compound with high *DPPH* scavenging activity is better in neutralizing peroxyl radicals, which reduces proinflammatory mediator production and then halts chronic inflammation-mediated carcinogenesis. Nitric oxide (NO) is a water-soluble radical gas commonly found in the human body. Although it has a confusing double-faced role in tumor progression, NO has been reported to be involved in angiogenesis promotion and encouraging the progression of breast carcinoma to the metastasis stage, leading to grave patient prognosis [45]. The role of NO in cancer progression may be linked to its activity as a mediator in inflammation [46]. Hydrophilic surfactant like polysorbate 20 improves water dispersibility of polyphenols in the microemulsion system and protect them from degradation, hence the higher antioxidant activity of PL-MEs than pure polyphenols [47]. Que and RA exhibited

comparable antioxidant activities, whereas Kae had the lowest activity, whether in its pure form or as a microemulsion (KM). Previous study reported similar results; Que is a stronger antioxidant than Kae because it has more hydroxyl groups than Kae, and Que has the same amount of hydroxyl as RA [48]. Nevertheless, the activity of Kae was enhanced after being carried into the microemulsion system. A study on myricetin microemulsion displayed similar results, where the bioactivity of the antioxidant compound was enhanced after microemulsion encapsulation [49]. When incorporated into a carrier system, antioxidants will be protected from oxidation and retain their radical scavenging activity compared with pure compounds without a carrier system [50]. BM exhibited poor antioxidant activities against both *DPPH* and NO radicals. This confirms that improved activity of PL-MEs was caused by the well-protected polyphenol in the optimized microemulsion carrier.

Each cancer cell line has its own unique molecular features that affect its drug response; hence, different cytotoxicity of drugs on different cell lines is expected [51]. In this study, all pure polyphenols showed no cytotoxicity on T47D, while on MDA-MB-231, Kae was the only noncytotoxic tested compound. The structure–activity relationship may also influence the cytotoxicity of the polyphenols. In flavonoids, cytotoxicity comes from an adequate amount of hydroxyl group, the presence of ortho-dihydroxy and 3′-OH in ring B, and the presence of a 2,3-double bond. Kae has the lowest hydroxyl number among the tested polyphenols, and its B ring has no ortho-dihydroxy and 3′-OH. Additionally, Kae has 5-OH, which has been reported before to lower the cytotoxicity of Kae [52].

Although BM did not carry any polyphenol, it showed comparable cytotoxicity to the PL-MEs in both cell lines. An additional study conducted on HaCaT cells also showed higher toxicity of all PL-MEs and BM than pure polyphenols (Figures S2 and S3). It could potentially be caused by components of the microemulsion carrier, i.e., polysorbate, oil, ethanol. Ethanol at concentrations ranging from 0.15% to 1.25% is non-toxic to MDA-MB-231 and MCF-7 cells [53]. Ethanol in PL-MEs was only 0.6% in 50 μg/mL PL-ME, meaning that the concentration was too low to induce a cytotoxicity effect on both cell lines. Polysorbate 20 has been reported to significantly decrease the viability of human colon adenocarcinoma, HT-29, at a concentration of 0.02% [54]. In 50 μg/mL PL-ME, the concentration of polysorbate 20 was 1%, which is higher than the reported safe concentration. This suggests the contribution of polysorbate 20 in the PL-ME toxicity. Several mechanisms allow surfactants to exhibit high toxicity in cells. First, as an emulsifier, a surfactant might break down the cell membranes, or change the position and function of active transporters located in the cell membranes. Second, in the in vitro studies (i.e., MTT assay), surfactants may compete with cells for surface attachment in culture plates, leading to detachment of the cells [54].

Another possible source of toxicity in BM is oleic acid. A study reported a high tumoricidal effect of oleic acid-modified liposomes on MDA-MB-231 [55]. Moreover, oleic acid enhances growth inhibitory effect of trastuzumab in SKBr-3 and BT-474 cells, which both are breast cancer cell lines [56]. Furthermore, cytotoxicity of microemulsion without drug (blank microemulsion) has also been reported in several studies. In a study on mitomycin-C microemulsion, a blank microemulsion that consisted of polysorbate 80, lecithin, soybean oil, ethanol, and water reduced viability of A549 carcinoma cells by 41% at 300 μM dose. The study further suggested that free polysorbate 80 concentration in the formula might lead to significant toxicity to cells. Therefore, enough excipient (i.e., co-surfactant like lecithin) may be necessary for the formula to bind the polysorbate 80 and reduce its toxicity [57]. Another study reported that drug-free microemulsion made from polysorbate 80, span 20, isopropyl myristate, and ethanol had comparable cytotoxicity with gemcitabine-loaded microemulsion on MCF-7 and HCT116 [58]. Furthermore, the morphology of BM seemed more elongated than that of QM, KM, and RM. This factor could also contribute to the cytotoxicity activity of BM, although to confirm this, an extensive investigation is needed. Considering the overall results and supporting data from the aforementioned studies, the microemulsion formulation in this study might lack a co-surfactant (e.g., lecithin) to mask the toxicity of polysorbate 20. This finding highlights

the role of co-surfactants and other excipients in reducing the toxicity of microemulsion carriers. It also opens a potential anticancer activity of blank microemulsion itself after further optimization, without the need for drug loading.

4. Materials and Methods

4.1. Microemulsion Formulation

The low-energy method was utilized based on the previous study [59]. Que (≥95% purity; Sigma-Aldrich, St. Louis, MO, USA), Kae (>97%; Tokyo Chemical Industry, Tokyo, Japan), RA (≥96% purity; Sigma-Aldrich, USA) with concentration of 500 µg/mL were mixed with various concentrations of carrier oil: oleic acid (Tokyo Chemical Industry, Japan), olive oil (Rumah Atsiri Indonesia, Karanganyar, Indonesia), or MCT oil (Coco' Care, Bangkok, Thailand). To the organic mixture, ethanol (AR grade; RCI Labscan, Bangkok, Thailand) and surfactant (polysorbate 80 or polysorbate 20; Sigma-Aldrich, USA) were added and homogenized using a magnetic stirrer (Corning Hot Plate Stirrer PC-351, Corning, Inc., New York, NY, USA) to make an organic phase (Tables S1–S3). Ultrapure water was added drop-by-drop to the organic phase mixture while stirring at 550 rpm for 30 min at room temperature. The exact concentration of surfactant, carrier oil, and ethanol of optimized microemulsion carrier was obtained after a series of component screening experiments (Tables S1–S3, Figure S4). The optimized microemulsion carrier was produced from 1% (*v*/*v*) oleic acid, 3% (*v*/*v*) ethanol, 10% (*v*/*v*) polysorbate 20, and 86% (*v*/*v*) ultrapure water.

4.2. Microemulsion Characterization

4.2.1. Particle Size

Particle size and polydispersity index (PDI) of BM and polyphenol-loaded microemulsions (PL-MEs), i.e., quercetin-loaded microemulsion (QM), kaempferol-loaded microemulsion (KM), and rosmarinic acid-loaded microemulsion (RM), were measured by the dynamic laser scattering method [60]. Each formula was diluted in ultrapure water (1:2 dilution) for the measurement. The analysis was carried out in triplicate at 25 °C by Zetasizer Nano ZSP (Malvern Instruments Ltd., Malvern, UK).

4.2.2. Zeta Potential

The electric charge on microemulsion droplets was determined [61]. BM and PL-MEs (3 mL, 1:2 dilution in ultrapure water) were analyzed with particle electrophoresis instrument (Zetasizer Nano ZSP, Malvern Instruments Ltd., Malvern, UK). The analysis was carried out thrice at 25 °C.

4.2.3. Transmission Electron Microscopy (TEM) Visualization

Negative staining was used to visualize BM and PL-ME droplets [61]. The microemulsion formulations (7 µL) were ten-fold diluted, then positioned on a copper grid, and washed with ultrapure water. A solution of 2% uranyl acetate (7 µL) was used to stain the droplets. The TEM (JEM-1400; JEOL Ltd., Tokyo, Japan) micrographs were acquired at 200 kV. A grating replica 3 mm grid calibrated the scales.

4.2.4. Physicochemical Stability Test

Optimized PL-MEs and BM were subjected to an accelerated stability test that subsequently started from centrifugation test (3500 rpm for 30 min; Velocity 14R Refrigerated Benchtop Centrifuge, Dynamica Scientific Ltd., Livingston, UK), six cycles of heating–cooling, to three cycles of freeze–thawing [62]. One cycle of heating–cooling needed 4 days to finish; it involved 48 h of microemulsion storage at 40 °C, followed by 48 h of storage at 4 °C. Similar to heating–cooling, one cycle of freeze–thawing also needed 4 days to be performed; it consisted of 48 h of storage at −19 °C, then the thawing process continued at 30 °C for 48 h. Before and after the stability test, particle size, PDI, and zeta potential were measured. Aggregation, turbidity, and transmittance were measured with

ultraviolet–visible (UV-Vis) spectrophotometer (650 nm; Shimadzu UV-1800, Kyoto, Japan) after specific cycles. The pH stability was observed at 37 °C and measured by a pH meter (Apera PH700 Benchtop, Columbus, OH, USA) once a week during a 3-week observation.

4.2.5. Encapsulation Efficiency

Efficiency of the optimized microemulsion carrier to encapsulate selected polyphenols was analyzed [42]. BM and PL-ME (500 µL) were placed in ultrafiltration units with 5 KDa cut-off (Sartorius, Goettingen, Germany) and centrifuged for 30 min (10,000 rpm, 28 °C). The optical density of polyphenol in the filtrate was measured in a 96-well microplate at the maximum wavelength (λ_{max}) of each polyphenol (Que = 374 nm, Kae = 363 nm, and RA = 325 nm; BioTek Synergy H1 plate reader, BioTek Instruments Inc., Winooski, VT, USA). Standard curves used in determination of polyphenol concentration were prepared by making separated two-fold dilution for Que, Kae, and RA in a known range of concentration from 125 to 0.488 µg/mL. The concentrations were measured using the same maximum wavelength of each compound mentioned before. The concentration of free polyphenol in the filtrate was estimated by standard curves. The final encapsulation efficiency was calculated by the following equation:

$$EE\ (\%) = \frac{total\ polyphenol\ mass - free\ polyphenol\ mass}{total\ polyphenol\ mass} \times 100$$

Total polyphenol mass was the initial amount of polyphenol that was added into the microemulsion formula (500 µg/mL), and *free polyphenol mass* was the estimated amount of polyphenol outside the microemulsion carrier.

4.3. In Vitro Antioxidant Activities

4.3.1. Free Radical Scavenging Activity against 2,2-Diphenyl-1-Picrylhydrazyl (*DPPH*)

The free radical scavenging ability of BM and PL-MEs against *DPPH* (Sigma-Aldrich, USA) was determined based on a previous study [63]. Diluted BM and PL-MEs in distilled water were made at concentrations ranging from 3.125 µg/mL to 100 µg/mL. Polyphenols (Que, Kae, and RA) were prepared in DMSO (RCI Labscan, Thailand) to compare the activity. Each solution (20 µL) was then placed into a 96-well plate and measured with a microplate reader (517 nm, Biotek Synergy H1 plate reader). A solution of 120 µM *DPPH* in MeOH (180 µL) was added into the wells, and the mixture was dark-incubated for 30 min (250 rpm, 37 °C). Ultrapure water and DMSO were used as blanks. The solution absorbance was measured at 517 nm, and the radical *scavenging activity* was calculated by the equation below:

$$DPPH\ scavenging\ activity\ (\%) = \frac{A0 - A}{A0} \times 100$$

A0 is expressed as an absorbance of negative control, and *A* is expressed as the absorbance of a tested sample after the reaction.

4.3.2. Free Radical Scavenging Activity against Nitric Oxide (NO)

Griess reaction from previous study was utilized to assess the *scavenging activity* of polyphenols, BM, and PL-MEs against NO [64]. Polyphenols (Que, Kae, and RA) were dissolved in MeOH and diluted in distilled water. BM and PL-MEs were diluted in distilled water. Gallic acid (Sigma-Aldrich, USA) was used as a positive control. All microemulsions and compounds were diluted in a range of concentrations (400, 200, 100, 50, 25, 12.5, and 0 µg/mL). Each solution (10 µL) was placed into a 96-well plate, followed by adding 10 mM SNP (Sigma-Aldrich, USA) solution (90 µL) under polychromatic light condition for 90 min in an open incubator shaker. A solution of 1% sulfanilamide was then added (50 µL) and incubation continued under dark conditions (5 min). The 0.1% NED (Sigma-Aldrich, USA) solution (50 µL) was sequentially added and incubated again (dark condition, 30 min). The

absorbance was immediately measured at 540 nm. The inhibition of *NO* radical formation was calculated by the equation below:

$$NO\ scavenging\ activity\ (\%) = \frac{A0 - A}{A0} \times 100$$

$A0$ is expressed as an absorbance of a negative control, and A is expressed as the absorbance of a tested sample after the reaction.

4.4. In Vitro Cytotoxic Activities

4.4.1. Cytotoxicity on Breast Cancer Cells

The hormone-dependent T47D cells (ATCC: HTB-133) were cultured in RPMI-1640 (HyClone Laboratories, Inc., Logan, UT, USA) with L-glutamine (2 mM; Sigma-Aldrich, USA) and supplemented with penicillin-streptomycin (100 U/mL; Sigma-Aldrich, USA), insulin (0.2 U/mL; Sigma-Aldrich, USA), glucose (4.5 g/L; Sigma-Aldrich, USA), and fetal bovine serum (10%; JR Scientific, Inc., USA). The cells were added to each well at a density of 0.5×10^4 to 2×10^4 cells and grown for 24 h (37 °C, 5% CO_2, 95% humidity). DMEM (HyClone Laboratories, USA) was used to culture hormone-independent MDA-MB-231 cells (ATCC: HTB-26), containing 100 U/mL penicillin-streptomycin and 10% fetal bovine serum. The culture conditions were similar to T47D cell culture; each well contained a range of 0.5×10^4 and 2×10^4 cells and the temperature was set at 37 °C, with 5% CO_2, and 95% humidity. Doxorubicin was used as a standard drug. The cytotoxicity of polyphenols, BM, and PL-MEs was assessed by MTT assay. Doxorubicin (Sigma-Aldrich, USA), DMSO, and tested samples (Que, Kae, RA, BM, and PL-MEs) were dissolved in DMEM. The solutions were added to the well filled with T47D or MDA-MB-231. The treatment incubation was allowed for 48 h. The MTT (Sigma-Aldrich, USA) solution (100 µL, in serum-free cell culture medium) was added into the wells, and the incubation continued at 37 °C (95% humidity, 5% CO_2). After that, the solution was replaced with DMSO (100 µL). The absorbance was measured at 550 nm with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The experiments were carried out in triplicate. The cytotoxicity against breast cancer cell lines was reported as an IC_{50} value in µg/mL.

4.4.2. Cytotoxicity on Human Keratinocyte

The human epidermal keratinocytes (HaCaT) cells were cultured in DMEM supplemented with fetal bovine serum (10%), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37 °C with 5% CO_2. The cells were seeded into a 96-well plate (10^4 cells/well) and grown for 16 h. Polyphenols, BM, and PL-MEs were added and incubated with the cells for 24 h. Cisplatin was used as a standard drug. The cell viability was determined by MTT assay. The culture media was discarded and changed with MTT solution (0.5 mg MTT in 1 mL of complete medium). This mixture was incubated at 37 °C for 3 h. Next, the solution was replaced with DMSO (100 µL). The absorbance was measured at 570 nm using a microplate reader (Varioskan™ LUX multimode microplate reader, Thermo Scientific™). The experiments were conducted in triplicate. The percentage of inhibition was determined.

4.5. Statistical Analysis

One-way analysis of variance by Duncan's Multiple Range Test ($p < 0.05$) was accomplished with IBM SPSS Statistics version 29 to determine whether there was a significant difference in the mean between groups.

5. Conclusions

This study addressed the different bioactivities of common dietary polyphenols and how a microemulsion carrier may improve their bioactivities. The optimized microemulsion carrier was made from 1% oleic acid, 3% ethanol, 10% polysorbate 20, and 86% ultrapure water. After loading pure polyphenols into a microemulsion, the size and PDI of all formulations were maintained under 100 nm and 0.5, respectively, even after more than

two months of stability tests. These characteristics confirmed the homogeneity and stability of the PL-ME formulations. QM, KM, and RM had a high polyphenol encapsulation efficiency, which was higher than 90%. All polyphenol-loaded microemulsion formulations exhibited non-spherical droplet morphologies. This type of morphology is commonly observed in oil-in-water microemulsion systems. Among all tested dietary polyphenols and their microemulsion formulations, RM performed comparably well or even better than standard compounds and pure rosmarinic acid as an antioxidant that inhibited *DPPH* and nitric oxide radicals. Incorporation of polyphenols into the optimized microemulsion carrier not only drastically inhibited the proliferation of both hormone-dependent and hormone-independent breast cancer cells, but also the normal dermal cells. Further studies are recommended to assess the potential involvement of polysorbate 20 and oleic acid in the toxicity of the microemulsion carrier system. Nevertheless, the results of this study demonstrate the potential use of common dietary polyphenols (especially RA), and how microemulsion carriers protect the antioxidant activity of oxidation-prone polyphenols, which may be valuable in developing antioxidant supplements for breast cancer prevention. For the future direction of this study, it would be interesting to explore additional modifications of the microemulsion formula, such as the utilization of water-soluble polyphenolate sodium salts, to possibly improve the solubility of lipophilic polyphenols without excessive surfactants [65].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/scipharm92020025/s1, Figure S1: Gradual change of blank microemulsion (BM) and PL-ME formulations (QM, KM, and RM) at different pH values and times; Figure S2: Dietary polyphenols exhibit safer inhibitory activity on normal dermal cells (HaCaT) proliferation than cisplatin; Figure S3: Inhibitory effect of PL-MEs on proliferation of normal dermal cells (HaCaT); Figure S4: Appearance of quercetin (Q)- and rosmarinic acid (R)-loaded microemulsions based on experiment in Table S3; Table S1: First screening of component and optimization of microemulsion carrier without polyphenols; Table S2: Second screening of components and further optimization of FA3 without polyphenols to reduce EtOH concentration; Table S3: Incorporating dietary polyphenols (Que and RA) into selected microemulsion carrier candidates (microemulsion carrier B).

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