



Title	Formulation and evaluation of a novel megalomeric microemulsion from tamarind seed xyloglucan-megalosaccharides for improved high-dose quercetin delivery
Author(s)	Lang, Weeranuch; Mondol, Debashish; Trakooncharoenvit, Aphichat et al.
Citation	Food Hydrocolloids, 137, 108430 https://doi.org/10.1016/j.foodhyd.2022.108430
Issue Date	2023-04
Doc URL	https://hdl.handle.net/2115/91441
Rights	© 2022. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/
Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	FoodHydrocolloids108430.pdf



1 **Formulation and evaluation of a novel megalomeric microemulsion from tamarind seed**
2 **xyloglucan-megalosaccharides for improved high-dose quercetin delivery**

3
4 Weeranuch Lang^{1,*}, Debashish Mondol², Aphichat Trakooncharoenvit³, Takayoshi Tagami¹,
5 Masayuki Okuyama¹, Tohru Hira³, Nobuo Sakairi⁴, Atsuo Kimura^{1,*}

6
7 ¹Laboratory of Molecular Enzymology, Research Faculty of Agriculture, Hokkaido University,
8 Sapporo 060-8589, Japan.

9 ²Department of Pharmacy, Faculty of Biological Science and Technology, Jashore University of
10 Science and Technology, Jashore 7408, Bangladesh.

11 ³Laboratory of Nutritional Biochemistry, Research Faculty of Agriculture, Hokkaido University,
12 Sapporo 060-8589, Japan.

13 ⁴Division of Environmental Materials Science, Faculty of Environmental Earth Science,
14 Hokkaido University, Sapporo 060-0810, Japan.

15
16 *Corresponding authors.

17 E-mail addresses: weranuch@abs.agr.hokudai.ac.jp, weranuch24@hotmail.com (W. Lang);
18 kimura@abs.agr.hokudai.ac.jp (A. Kimura).

19
20 **Abstract**

21 Megalomeric microemulsion is a new term referring to lipid-based formulation using
22 amphiphilic megalosaccharide as a coexcipient. Quercetin is a dose-dependent bioactive
23 compound and has promising therapeutic potential, but its low water solubility and permeability
24 restrict its treatment efficacy. We aimed to formulate high-dose quercetin loaded into colloidal
25 micelles by the self-micro emulsifying system (SMES) in combination with Tween 80,
26 isopropyl myristate, and xyloglucan megalosaccharide (X-MS). X-MS is a moderate-size
27 heterologous saccharide obtained from enzymatic cleavage of tamarind seed xyloglucan. X-MSs
28 with an average degree of polymerization of 16 and 56 were investigated to bearing their
29 surface hydrophobic interaction with a fluorescence probe 6-(*p*-toluidino)-2-naphthalene-6-
30 sulfonate yielded the binding constant values of 127 and 180 M⁻¹, respectively and X-MS itself
31 displayed a slight effect on quercetin binding. However, the implementation of X-MSs toward
32 SMES was highly compatible because X-MS molecules were confined in micellular solutions.
33 Consequently, X-MSs improved the quercetin loading from 1–2 to 12.5–17.7 mg/mL based on
34 the composition ratio, X-MS chain lengths, and X-MS concentrations (0.15–3.0%, w/v) and

35 stabilized the quercetin-loaded oil-in-water SMES. The optical appearances were transparent
36 yellow containing uniformly fine droplets with diameters of 11–12 nm. In vitro radical
37 scavenging activity tests with 2,2-diphenyl-1-picrylhydrazyl showed that the megalomeric
38 microemulsions improved the half-maximal inhibitory concentration ($IC_{50} = 22\text{--}24 \mu\text{g/mL}$)
39 over that of the X-MS-free microemulsion. This study provided a new approach of liquid
40 supplementation from commercially unavailable-size xyloglucan to be a promising added-value
41 agent for oral uptake of quercetin.

42

43 **Keywords:** tamarind seed xyloglucan, megalosaccharide, antioxidative flavonoid, cosurfactant,
44 microemulsion formulation, self-emulsifying drug delivery system

45

46 **1. Introduction**

47 Quercetin is one of the most common plant flavonoids. It exhibits numerous biological and
48 pharmacological effects, such as antioxidant, anticarcinogenic, and antimicrobial activities
49 (Andrés et al., 2013; Dajas, 2012; Johari, Kianmehr, Mustafa, Abubakar, & Zandi, 2012).
50 However, the level of quercetin in blood plasma is less than 1% of the uptake level, primarily
51 due to practical water insolubility ($0.17\text{--}7.7 \mu\text{g/mL}$) and low permeability (Gao et al., 2009).
52 Lipid-based formulations, predominantly the self-emulsifying drug delivery system (SEDDS),
53 are the most promising strategy for drugs with poor bioavailability, as they retain the drug in a
54 predissolved state in the gastrointestinal tract (Wu et al., 2015). SEDDSs emulsify
55 spontaneously to produce fine oil-in-water (O/W) emulsions when introduced into an aqueous
56 phase and the small size of the formed droplet provides a large interfacial surface area for drug
57 absorption. The SEDDS containing an O/W emulsion with a droplet diameter less than 50 nm
58 entrapping the lipophilic drug molecules absolutely with 100% efficacy is known as the self-
59 micro emulsifying system (SMES) (Phan, Le-Vinh, Efiana, & Bernkop-Schnürch, 2019). The
60 SMES is an isotropic dispersion of oil, emulsifier, coemulsifier (organic solvent or other
61 compatible amphiphilic components), and a drug substance. Quercetin-loaded contents in
62 SEDDSs have been documented in the range of 0.067 to 2 mg/mL, while particle sizes within
63 the range of 20–290 nm and some additional treatments, such as heating, ultrasonic exposure,
64 homogenization, and solvent evaporation, may be applied (Liu, Huang, Jhang, Liu, & Wu,
65 2015; Rogerio et al., 2010; Sermkaew & Plyduang, 2020; Son et al., 2019). For the preparation
66 of a stable SMES formulation, the selection of an appropriate emulsifier and coemulsifier is
67 considered critical since it is a mixture of two immiscible liquids containing nanosized droplets
68 to entrap drug substances (Bai & McClements, 2016). However, to carry a high drug dose, the

69 required emulsifier/coemulsifier contents must be as high as possible, and those high
70 formulations may lead to toxicity aspects of the corresponding delivery systems (Premathilaka,
71 Rashidinejad, Golding, & Singh, 2022).

72 Among the numerous notable polymeric emulsifiers/coemulsifiers, polysaccharides
73 have recently become the most preferable since they are highly stable, safe, nontoxic,
74 biodegradable, and naturally available. However, their solution properties of high molecular
75 weight and viscosity are less proficient, as they are not compatible with SMES. Megalomic
76 microemulsion is a new term designated first time in the present study for lipid formulation-
77 based amphiphilic megalosaccharide (MS) excipients. The terminology of MS, defined by the
78 size composed of 10–100 units of monosaccharide bound together by glycosidic bonds (Thoma
79 & French, 1960), represents the nonsweet middle-size saccharides between oligosaccharides and
80 polysaccharides. Previously, we established a preparation method of homologous glucose-type
81 MS (G-MS) by enzymatic transglucosylation, and G-MS exhibited functional effects in
82 increasing the solubility of quercetin and ethyl red dye and provided a barrier function against
83 paracellular transporters (Shinoki et al., 2013; Lang et al., 2014, 2022a, b; Hara, Kume, Iizuka,
84 Fujimoto, & Kimura, 2017). In the present study, we focused on the preparation of heterologous
85 MSs by the considerably simpler enzymatic hydrolysis of tamarind (*Tamarindus indica* L.) seed
86 xyloglucan. Xyloglucan has versatile industrial and pharmaceutical applications as a neutral
87 thickener, stabilizer, and gelling agent in which the purification method of the seed gum has a
88 great influence on those properties (Crispín-Isidro et al., 2019). The main structure of
89 xyloglucan is a cellulose-like β -(1→4)-linked glucan backbone and branch chains substituted
90 with galactose, xylose, and arabinose (Majee, Avlani, & Biswas, 2016). Accordingly,
91 xyloglucan MS (X-MS) is proposed to aid as a compatible coemulsifier to stabilize quercetin in
92 dissolved form to reach the required therapeutic plasma concentration as well as decrease the
93 toxic quantities conferred by the emulsifier and organic solvents. X-MS was hypothesized to
94 participate in the micelles by taking the place of their branched molecules between the
95 interfacial layer of the head and tail groups of the emulsifier (e.g., Tween 80). The specific
96 hydrophobic force may strongly confine hydrophobic guest molecules into the hydrophobic core
97 and eventually stabilize their conformation.

98 In our study, X-MSs were prepared by cellulase cleavage into two sizes and
99 characterized for their hydrophobic properties. The interaction of X-MSs in terms of
100 synergistically cooperating with Tween 80 micelles was investigated by fluorescence
101 spectroscopy. Polyethylene glycols (PEGs) with several chain lengths are screened as the
102 coadjutant replacing common traditional solvents, such as dimethyl sulfoxide and methanol.

103 The quercetin formulation was adjusted by constructing a ternary phase diagram. Two
104 formulations were applied to compare a load of quercetin with the addition of X-MSs and
105 several saccharides. In vitro antioxidant assays with two free radical compounds, 2,2-di(4-tert-
106 octylphenyl)-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic
107 acid (ABTS), were further examined.

108

109 **2. Materials and Methods**

110 **2.1. Materials.** High-purity-grade tamarind seed xyloglucan and quercetin hydrate were
111 purchased from Tokyo Chemical Industry (Tokyo, Japan). PEG 200, 300, 400, and
112 cyclodextrins (CDs) were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka,
113 Japan). PEG 550 and all higher-molecular-weight PEGs and ABTS diammonium salt were
114 obtained from Fluka (Steinheim, Germany). 6-(*p*-Toluidino)-2-naphthalenesulfonic acid sodium
115 salt (TNS) and DPPH were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dextran T
116 40 was available from Amersham Biosciences (Uppsala, Sweden), and dextran T 3.5 and T 6
117 were ordered from Pharmacosmos (Holbaek, Denmark). Glycerol was purchased from Hampton
118 Research (Aliso Viejo, CA, USA). Maltohexaose was donated from Nihon Shokuhin Kako
119 (Tokyo, Japan). Cellulase Y-C was purchased from Seishin Pharmaceutical (Tokyo, Japan).
120 Xyloglucan octaose (XOS 9) was purified in our laboratory to a purity $\geq 98\%$. Tween 80,
121 isopropyl myristate (IPM), gum arabic, soluble starch, and other required chemicals were
122 purchased from Nacalai Tesque (Kyoto, Japan). The water used was generated with an ion-
123 exchanger ($> 18.2 \text{ M}\Omega$, Direct-Q[®] 3UV, Millipores SAS, Molsheim, France).

124

125 **2.2. Preparation of X-MSs and their isolation.** A slurry of tamarind seed xyloglucan (2 g)
126 with 1%, w/v was dissolved in 200 ml of sodium phosphate buffer (50 mM, pH 6.0), heated by
127 microwave radiation, and vigorously stirred until boiling 2–3 times to ensure a homogeneous
128 phase. Digestion with cellulase Y-C was further carried out at 37 °C using 9.5 enzyme units and
129 incubated for 2 h. The sample was heated at 121 °C for 15 min to inactivate the enzyme. After
130 cooling, the sample was centrifuged at $11,300 \times g$ for 30 min and then filtered through a 0.2- μm
131 mixed cellulose ester membrane to remove insoluble pellets. Thereafter, the sample was
132 dialyzed with a Spectra/Por[®] 6 membrane with a molecular weight cutoff of 2 kD against water
133 to discard glucose and oligosaccharide, with an average degree of polymerization (DP) less than
134 12, and subsequently desalted by a manually packed ion-exchange column (amberlite MB4,
135 Organo, Tokyo, Japan). Next, 60% by volume of precooled methanol was added with
136 continuous mixing for 1 h and left on ice water for an additional 2 h. The sediment was isolated

137 by centrifugation (13,000 ×g, 30 min) and dried to collect the sediment at a ≤ 60% fraction
138 (later defined as X-MS DP 56). The supernatant was evaporated to decrease the volume, and
139 precooled methanol at 70% of the final volume was then added and mixed to collect the
140 sediment of the (60–70)% fraction. This step was repeated twice but with different methanol
141 contents, namely, (70–80)% and (80–95)%. The last fraction was determined as X-MS DP 16.
142 All fractions were dried using a lyophilizer (Eyela FDU-1200, Tokyo, Japan). The four obtained
143 samples were profiled by Dionex HPAEC-PAD (4 × 250 mm CarboPac™ PA1 column, Dionex,
144 Sunnyvale, CA, USA) (eluent: 16 mM sodium hydroxide for 20 min and further increased to
145 200 mM with a sodium acetate gradient solution of 0–100 mM for 40 min and from 100–250
146 mM for 20 min). Accordingly, the profiled peaks of only two samples (X-MS DP 16 and 56) did
147 not overlap with each other and were therefore considered for use in the entire study. XOS 9
148 was purified from completed cellulase Y-C digests by preparative HPLC on an Imtakt Unison
149 US-Amino column (20 × 250 mm). Eluent: 70%, v/v acetonitrile in water at a flow rate of 4
150 mL/min. Upon evaporation of the solvent, the XOS 9 powder was obtained by lyophilization.
151 The chromatogram was reanalyzed by HPAEC-PAD.

152

153 **2.3. Molecular weight and DP determination.** The molecular weight of X-MSs was
154 determined using a gel filtration HPLC (SB-803 HQ Shodex OHpak, 8.0 × 300 mm) eluted with
155 sodium nitrate solution (0.1 M) at 0.5 mL/min, and the signals were detected by a refractive
156 index detector (RI 2013 Plus, Tokyo, Japan). The molecular mass markers used were Shodex®
157 standard P-82 pullulans (Showa Denko K.K., Kanagawa, Japan).

158

159 **2.4. Monosaccharide composition.** X-MSs (0.5 mg) were hydrolyzed at 120 °C in 0.1 mL of 2
160 M trifluoroacetic acid for 2 h in a glass vial. The acid was vaporized by nitrogen flushing. The
161 sample was dissolved in water and analyzed by HPAEC-PAD under isocratic conditions of 16
162 mM sodium hydroxide to analyze the monosaccharide composition with myo-inositol internal
163 standard. Normalized molar percentages relative to known sugar standards from each peak were
164 obtained. DP was calculated by summation of the molar percentages of hexoses (glucose and
165 galactose) and pentoses (arabinose and xylose), and the equation was expressed as $(MW -$
166 $18)/[(\% \text{hexose} \times 162/100) + (\% \text{pentose} \times 132/100)]$, whereas MW was the molecular weight
167 obtained in Section 2.3. The molecular weight of a hexose residue in the X-MS chain
168 is assumed to be 162 g/mol (180–18 for loss of water), and pentose is 132 g/mol obtained from
169 150–18.

170

171 **2.5. Phase solubility assay of quercetin.** X-MSs (100 μ L) in series concentrations (0, 2, 4, 6, 8,
172 and 10 mg/mL) were mixed well with excess quercetin (1 mg) at 25 $^{\circ}$ C for 6 h in triplicate. The
173 samples were centrifuged three times at 12,000 \times g for 10 min. The supernatant portion was
174 diluted in dimethyl sulfoxide, and the absorbance was measured at 381 nm by a UV-visible
175 spectrophotometer (U2900, Hitachi, Tokyo, Japan). The concentration of quercetin in the
176 supernatant was calculated using the calibration plot. The apparent equilibrium constant (K_S)
177 was determined by following previous methods (Lang et al., 2014, 2022b).

178

179 **2.6. Screening of the excipients.** The maximum solubility of quercetin in several pure
180 components of PEGs (200–550 Dalton), Tween 80, glycerol, IPM, and the combination with
181 water of PEGs (1,000–8,000 Dalton) and X-MS DP 56 was evaluated according to the previous
182 methods described in Section 2.5.

183

184 **2.7. Hydrophobic interaction of TNS with X-MSs, TNS with X-MS DP 16 and Tween 80,**
185 **TNS with Tween 80 and PEG 550.** The complexes of 0.5 μ M TNS with X-MS DP 16 (0, 0.5,
186 1, 3, 5, 8, 10, 12, 14, 16, 18, and 20 mg/mL) and X-MS DP 56 (0, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7,
187 8, 9, and 10 mg/mL) in water were prepared by mixing them for 30 min in the dark at 25 $^{\circ}$ C and
188 then transferred into 96-well black plates. The emission spectra were recorded by a fluorescence
189 microplate reader (Tecan, Infinite M200-HAFH, Tokyo, Japan) at an excitation wavelength of
190 360 nm, and the fluorescence intensity was read at 430 nm for all experiments. The stability
191 constant (K_c) was calculated according to the methods of Buranaboripan, Lang, Motomura, &
192 Sakairi (2014) and Lang et al. (2022b). Tween 80 stock was prepared to 0.2 mg/mL and then
193 diluted to the range of 0.001–0.1 mg/mL (including 0.001, 0.002, 0.003, 0.004, 0.005, 0.006,
194 0.007, 0.008, 0.009, 0.010, 0.015, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, and 0.1 mg/mL)
195 in 96-well black plates. Fluorescence spectra for the complex formation with TNS were
196 recorded with those Tween 80 ranges and those ranges with the addition of two concentrations
197 of X-MS DP 16 (5 and 10 mg/mL) and PEG 550 (10 and 30 mg/mL). The determination of the
198 critical micelle concentration (CMC) value was carried out by plotting the log Tween 80
199 concentrations against the fluorescence emission intensity. Two straight lines were drawn
200 through these points, and their intersection was taken as the CMC.

201

202 **2.8. Preparation of P0 and P14 microemulsions.** Quercetin was dissolved in a series of
203 concentrations in pure PEG 550 at 50, 100, 150, and 200 mg/mL. The last concentration was
204 used routinely as the stock solution. Tween 80 was warmed at 60 $^{\circ}$ C in a heat box only when

205 needed to transfer to the tubes, whereas all samples were mixed at 25 °C. P0 was formulated by
206 mixing PEG 550, Tween 80, IPM, an aqueous solution of 5% X-MS DP56 in water, and water
207 in a volume fraction of 20:70:10:60:160 µL (having a final volume of 320 µL). For the
208 comparison of saccharide excipients, the following maltohexaose, CDs, XOS 9, X-MS DP 16,
209 soluble starch, gum arabic, and dextran T 40 were prepared in 5%, w/v solution and introduced
210 to the P0 formula by replacing the portion of X-MS DP56 (60 µL). The samples were mixed
211 vigorously and incubated at 25 °C in the dark overnight before the appearance was visualized.
212 Centrifugation was performed to remove unloaded quercetin. The ternary phase diagram was
213 constructed based on the P0 formulation without the addition of quercetin and X-MS. The PEG
214 550 volume increased from 20 µL to 20, 30, 40, and 50 µL; IPM from 10 µL to 10, 20, and 30
215 µL; and Tween 80 from 70 µL to 70, 80, and 90 µL. Subsequently, a one-equal volume of the
216 sample by water was added to the medium, yielding 36 formulation trials. A visual assessment
217 of transparency was performed to define the microemulsion zone in the ternary diagram. The
218 type of microemulsion in P0 was identified when water-in-oil (W/O) medium was first prepared
219 from IPM, PEG 550, and Tween 80 (1:2:7 mL to total 10 mL in a glass tube placed in a 25 °C
220 water bath), and the fixed amount of pure water (1 mL each) was then titrated. Conductivity
221 measurements were performed using a TOA EC meter CM-14p model (TOA Electronics,
222 Japan).

223 P14 tests were composed of the same above components of 40:90:10:60:200 µL (having
224 a final volume of 400 µL), whereas 5%, w/v aqueous solution of X-MS DP 16, 56, dextran T
225 3.5, and T 6 were placed in the 60-µL portion. The template microemulsion was prepared
226 without the addition of quercetin but maintained in volume with PEG 550. The droplet sizes
227 were characterized after aqueous dilution 50 times via dynamic light scattering in triplicate by a
228 Zetasizer µV series from Malvern Instruments (Worcestershire, UK).

229

230 **2.9. TEM analysis.** Quercetin-loaded microemulsion (P0 formula) with X-MS DP 56 was
231 diluted with water 100 times to determine the particle size and shape by a JEM-2100
232 transmission electron microscopy (TEM, JEOL, Tokyo, Japan). One drop of the formulation
233 was placed on a formvar-coated copper grid. A drop of a 2% aqueous solution of uranyl acetate
234 was subsequently placed onto the grid for negative staining to improve the contrast and allowed
235 30–60 s until drying. An image was obtained using 300,000× magnification.

236

237 **2.10. Antioxidant assays.** Four quercetin-loaded sample solutions, including two megalomeric
238 microemulsion, X-MS DP 16 and 56, the template microemulsion without X-MS, and methanol,

239 were prepared with appropriate quercetin ranges (0, 10, 20, 40, 60, 80, and 100 µg/mL). Sodium
240 phosphate buffer (pH 6.0 and 7.4; 40 mM, 80 µL) and the samples (20 µL) were added to the
241 96-well plate, and the stock solution of DPPH prepared in methanol (0.2 mM, 100 µL) was
242 transferred to the plate by a multichannel pipette and mixed by tips one time. The samples were
243 incubated at 25 °C in the dark for 30 min. The absorbance was recorded at 517 nm by the Tecan
244 microplate reader. The ABTS assay was performed according to the method guideline of Xiao,
245 Xu, Lu, & Liu (2020) with some modification. Briefly, stock solutions of ABTS (7 mM) and
246 potassium persulfate (2.45 mM) were prepared separately in acetic acid buffer (50 mM, pH 4.0).
247 The ABTS reaction solution was prepared by mixing each stock volume of 5 mL and
248 maintained at 25 °C for 16 h in the dark to oxidize ABTS to ABTS^{•+} radicals. The ABTS
249 solution of 2.8 mL was diluted to 65 mL of 50% methanol in 50 mM sodium acetate buffer (50
250 mM, pH 4.0) to obtain the ABTS working solution and maintained at 25 °C for 30 min in the
251 dark. The ABTS assay was performed by adding ABTS working solution (190 µL) to samples
252 (10 µL) in 96-well plates and incubating at 25 °C in the dark for 30 min. The absorbance was
253 recorded at 734 nm. The measurements were performed in triplicate. Radical scavenging (%)
254 was calculated according to the equation: $A_3 - (A_1 - A_2) / A_3 \times 100$, where the absorbance of free
255 radicals plus the sample solution was recorded as A_1 , the absorbance of methanol plus the
256 sample solution was recorded as A_2 (blank), and the absorbance of free radicals plus methanol
257 solution was recorded as A_3 . The regression equation was estimated from plots of %Scavenging
258 (Y-axis) against quercetin concentration (X-axis), and IC₅₀ was calculated from $(50 - Y -$
259 $intercept) / slope$.

260

261 **2.11. Statistical analysis.** Differences among treatment groups were analyzed with the
262 Tukey–Kramer and Student’s *t* tests after one-way ANOVA. A P value of less than 0.05 was
263 considered significant.

264

265 **3. Results and Discussion**

266 **3.1. Isolation of active X-MSs by enzymatic hydrolysis.** Based on the structural configuration,
267 Janado & Yano (1985) suggested that two aldopentoses, D-xylose and L-arabinose,
268 predominantly possess superior hydrophobicity among other monosaccharides, such as D-
269 galactose and D-glucose, whereas the cellulose skeleton can laterally stack via hydrophobic
270 interactions and form a sheet-like structure (Miyamoto et al., 2009). Accordingly, tamarind seed
271 xyloglucan underlying these patterns was predicted to have the potential to enhance the water
272 solubility of water-insoluble substances. However, polymeric xyloglucan forms molecular

273 aggregates even in very dilute solutions, and the solution after heating is highly viscous. In this
274 study, we established a method to partially cleave and purify xyloglucan into two
275 nonoverlapping MS sizes to evaluate the role of chain length degree in the stabilized SMES.
276 *Trichoderma viride* cellulase (EC 3.2.1.4) acting as endo- β -(1 \rightarrow 4)-glucanohydrolase
277 hydrolyzed xyloglucan at a 2-h incubation to a mixture of four repeating units of xyloglucan
278 oligosaccharides (XOSs: XXXG, XLXG, XXLG, and XLLG with a retention time of 49–55
279 min) and a large number of MS (68–83 min), as depicted in the HPAEC-PAD chromatogram
280 (Fig. 1A, top). We proposed an enzyme mechanism involving the cleavage of random internal
281 β -(1 \rightarrow 4)-glucan at the unsubstituted parts and further access to more highly substituted regions.
282 Therefore, the incubation time was a critical criterion to determine the chain length of X-MS.
283 For instance, the incubation time at 40 min, 1 h, and 2 h enabled X-MS DPs of 122, 73, and 28,
284 respectively. After further prolonged incubation, XOSs eventually became prominent. Isolation
285 of those resulting mixtures of X-MS fragments to DP 16 and 56 by a series of consecutive steps
286 of methanol concentrations was successful as envisaged with nonoverlap peaks by HPAEC-
287 PAD (Fig. 1A, bottom). The composition analysis later revealed four types of sugar elements,
288 arabinose, galactose, glucose, and xylose, from native xyloglucan (255,113 Dalton) with
289 molarity percentages (mol%) of 4.7, 22.6, 44.3, and 28.5, respectively. X-MS DP 16 has the
290 shortest size, with a molecular weight of 2,488 Dalton and mol% values of 1.7, 21.1, 44.5, and
291 32.7. X-MS DP 56 is longer, with 8,493 Dalton and mol% values of 3.4, 23.0, 42.9, and 30.6,
292 respectively. Gel filtration HPLC analysis indicated that X-MS DP 16 and 56 have a better peak
293 profile than parental xyloglucan (Fig. 1B). The sugar compositions of X-MS were similar to
294 those of the native xyloglucan but only lower for arabinose content. The arabinose content in X-
295 MS DP 16 may be negotiable as well as lacking in those repeating units of XOSs. The arabinose
296 substituent plays a structural role in a highly viscous solution. Consequently, both X-MSs
297 exhibited improved solubility in water. Preparation of the X-MS DP 16 solution did not require
298 a rise in temperature for complete dissolution. The DP 56 solution required slight heating;
299 however, it did not form an aggregate in water afterward. The fine components in X-MS
300 molecules were identified by long incubation with the fungal cellulase. The results in HPAEC-
301 PAD indicated that X-MS DP 16 and 56 were enriched in small building blocks of the XXXG,
302 XLXG, XXLG, and XLLG collection with some slight free glucose (data not shown). However,
303 some other unknown shorter XOS fragments and larger resistance molecules were also detected.
304 The model structures of X-MSs are proposed in Fig. 1C. X-MS DP 16 may contain two
305 repeating XOSs, whereas DP 56 has six repeating XOSs with more galactose and arabinose
306 contents. This emphasized the existence of amphiphilicity. The hydrophobic property of X-MS

307 is derived from a large number of xylosyl side chains and additional arabinose molecules
308 substituted in the cellulose backbone, whereas galactose residues facilitate aqueous solubility.
309 Vincken, Keizer, Beldman, & Voragen (1995) demonstrated that the minimum length of at least
310 four repeating units of xyloglucan fragment was required to enable in vitro adsorption of
311 xyloglucan molecules to cellulose microfibrils, whereas XOS with one or two repeating units
312 did not adsorb. Kabel, van den Borne, Vincken, Voragen, & Schols (2007) removed arabinosyl
313 and *O*-acetyl substituents on xylan and found that the adsorption of xylan to cellulose was
314 increased. Hence, in addition to size, the side chain configuration also contributes to the
315 hydrophobic property of X-MS.

316

317 **3.2. Interaction of X-MS and PEG 550 in Tween 80 micelles.** X-MS is an amphiphilic
318 saccharide that possesses both hydrophilic and hydrophobic regions, but the functional groups
319 of the hydrophilic head groups and hydrophobic tail groups were not as well defined as those of
320 Tween 80 molecules. The interaction between Tween 80 with X-MS DP 16 and Tween 80 with
321 PEG 550 was analyzed by fluorescence spectroscopy with a TNS probe. TNS is practically
322 nonfluorescent in an aqueous solution but undergoes a large fluorescence enhancement in
323 appropriate hydrophobic environments or some regions of low polarity (Beyer, Craig, &
324 Gibbons, 1973). Binding interactions of X-MSs with TNS were first determined. The
325 fluorescence emission spectra of TNS at increasing concentrations of X-MS DP 16 and 56 were
326 recorded, and both emission maxima were accordingly detected at 430 nm (Fig. 2A and 2B).
327 The K_c was determined by the double-reciprocal plots (Fig. 2A and 2B; inset) according to the
328 Benesi-Hilderbrand equation ($R^2 > 0.99$), which yielded K_c values of 127 and 180 M^{-1} for X-MS
329 DP 16 and DP 56, respectively. Tween 80 does not have a specific fluorescence spectrum, and
330 the intensity of emission fluorescence is weak. However, the long alkyl chain of Tween 80 in
331 water can self-assemble and form a hydrophobic microenvironment at a CMC of 0.015 mg/mL
332 ($\log \text{CMC} = -1.824$). Two concentrations of X-MS DP 16 at 5 and 10 mg/mL were added to
333 TNS solutions with a series concentration of Tween 80 from 0.001–0.1 mg/mL. It was found
334 that the addition of X-MS DP 16 did not alter the CMC value (Fig. 2C), but the TNS intensity
335 was enhanced slightly at low concentrations of Tween 80. At the Tween 80 monolayer ($<$
336 CMC), X-MS exhibits a strong interaction with TNS based on the increasing X-MS
337 concentrations. Above the CMC, the intensity of TNS increased greatly with increasing
338 concentrations of Tween 80. X-MS binds with Tween 80 molecules at the inner micelles as the
339 intensity increases rapidly. However, increasing the X-MS concentrations had a small effect on
340 the hydrophobic polarity, as the intensity does not increase in proportion to the X-MS

341 concentrations. Notably, the size of the Tween 80 micelles was assumed to not continue to
342 increase, which may restrict the encapsulation of the extra X-MS density. Our results
343 demonstrated that the X-MS branch molecules incorporate to form a hydrophilic outer shell and
344 hydrophobic inner core of Tween 80 micelles, the so-called cosurfactant, and further increase
345 the core stability.

346 PEG 550 was the most prominent solubilizer for quercetin dissolution before SMES
347 was formulated. Thus, we identified the stimulatory role of PEG 550 in the PEG-associated
348 micelles. PEG is hydrophilic and distributes at the surface layer of the micelle corona structure
349 (Agrawal, Tatode, Rarokar, & Umekar, 2020). It may locally induce polarity in Tween 80,
350 which makes the environment slightly polar inside the micelles, and consequently, the
351 hydrophobic environment becomes less hydrophobic. As shown in Fig. 2D, the TNS intensity in
352 the solutions containing PEG 550 and Tween 80 remained constant and was the same as that of
353 the solution containing only Tween 80 when the Tween 80 molecule had not yet formed
354 micelles (< CMC). The quenching of TNS fluorescence was observed when PEG 550
355 participated in the micellization process (> CMC), as the intensity is always less than that of the
356 solution containing only Tween 80. The results implied that PEG 550 does not impact the
357 micellar stability, as the low molecular weight PEG were not hydrophobic enough to bind
358 with a surfactant molecule (Dhara & Shah, 2001). The role of PEG molecular weight on the
359 solubility enhancement of quercetin was subsequently considered in the next section.

360

361 **3.3. Screening of excipients for SMES formulations.** Quercetin is practically insoluble in
362 water (6 µg/mL). Hydrophobic interactions between quercetin and X-MS were responsible for
363 the solubility improvement of quercetin, increasing its apparent water solubility by 4.8- and 5.8-
364 fold with 10% (w/v) X-MS DP 16 and 56 solutions, respectively. The K_S calculated from the
365 phase solubility diagram was found to be consistent with X-MS chain length, as the K_S
366 increased from 114 to 677 M^{-1} for X-MS DP 16 and 56, respectively. To date, dimethyl
367 sulfoxide and alcohol are the most common organic solvents for quercetin dissolution. They are
368 practically used in in vitro cellular tests with the maximum solubility for quercetin at 30 and 2
369 mg/mL, respectively (“Product Information,” 2020). However, quercetin immediately
370 precipitated after aqueous dilution, and dimethyl sulfoxide is not permissible in pharmaceutical
371 formulations. In this study, preformulation studies were carried out to guide the appropriate
372 selection of SMES ingredients. PEG is a synthetic polyether with a commercially broad
373 molecular weight ranging from 200 to 4,000,000 Dalton (Biondi, Motta, & Mosesso, 2002;
374 Dhara & Shah, 2001; Sun, Zhang, & Chu, 2008). PEGs are widely used as a vehicle or base in

375 foods, cosmetics, and pharmaceuticals as they are FDA-approved (Sermkaew & Plyduang,
376 2020). The lower the PEG molecular weight is, the higher the hydrophilic properties, as the
377 ratio of terminal hydroxyl end and polyethylene groups increases. In our study, an excess
378 amount of quercetin powder was mixed vigorously with short-chain PEGs of varying molecular
379 weights (200, 300, 400, 550 Dalton), glycerol, Tween 80, and IPM oil. The aqueous phase was
380 subsequently collected to evaluate their maximum quercetin solubility. It was found that those
381 values of quercetin are the best among the short molecular weight series of PEG (200–550
382 Dalton) between 196.62 ± 0.55 and 204.57 ± 1.37 mg/mL and much higher compared with
383 glycerol (2.60 ± 0.12 mg/mL) and Tween 80 (6.30 ± 0.14 mg/mL) (Table 1), as well as the
384 value found in the literature (14.12 ± 2.18 μ g/mL for PEG 400) (Sermkaew & Plyduang, 2020).
385 Although PEG 400 is the most commonly used coemulsifier in drug formulation (Baksi et al.,
386 2018; Gao et al., 2009; Sermkaew & Plyduang, 2020), PEG 550 was selected for use entirely
387 because it was the best quercetin solubilizer compared with other solvents, including the
388 combination with water, Tween 80, and X-MS DP 56. Polymeric PEGs with longer sized
389 chains, 1000, 4000, 6000, and 8000 Dalton, and X-MS were only tested after being dissolved in
390 water, as they are solid in form. Notably, the combination with water decreased the degree of
391 quercetin solubilization. For instance, 20% PEG 550 in water could dissolve quercetin at only
392 0.16 ± 0.01 mg/mL, a 1,275-fold decrease compared with pure PEG 550 (203.80 ± 1.37
393 mg/mL). Tween 80 is a preferred nonionic emulsifier that has low toxicity. The addition of 10%
394 (w/v) X-MS in water and 10% (v/v) Tween 80 in water was found to increase the quercetin
395 solubility from 0.16 ± 0.01 to 1.38 ± 0.25 mg/mL and 2.51 ± 0.03 mg/mL, respectively. This
396 result emphasized the combined role of Tween 80 and X-MS in the water phase, which could
397 have a synergistic effect with PEG 550 for quercetin solubility. Emulsifiers generally stabilize
398 W/O microemulsions with hydrophilic-lipophilic balance (HLB) values between 4 and 6,
399 whereas for O/W microemulsions, the value is between 8 and 18 (Pichot, Spyropoulos, &
400 Norton, 2010; Syed & Peh, 2014). For effective emulsification in an SMES, specific oils require
401 emulsifiers having a specific HLB (polar or nonpolar characteristic). The HLB values of
402 emulsifiers are mostly preferred to be approximately similar to those of the respective oils to
403 achieve maximum stabilization. The HLB value of Tween 80 is 15; therefore, a high required
404 HLB (RHLB) oil phase, such as IPM (with RHLB 11.5), may be more feasible to formulate
405 O/W SMES. IPM is a low-viscosity oil synthesized from isopropyl alcohol and myristic acid, a
406 naturally occurring fatty acid. We evaluated the maximum solubility of quercetin in pure IPM
407 with the same procedure, which was 0.28 ± 0.01 mg/mL. Accordingly, formulations with Tween
408 80, PEG 550, X-MS, and IPM were evaluated for quercetin-loaded SMES.

409

410 **3.4. Evaluation of the P0 formula for quercetin loading and its microstructure.** In our
411 preliminary trial, one formulation was well established with PEG 550, Tween 80, IPM, an
412 aqueous solution of 5% X-MS DP 56 in water, and water in the volume fraction of
413 20:70:10:60:160 μL (having a final volume of 320 μL), namely, P0. The final mass percentages
414 of PEG 550, Tween 80, IPM, and water in P0 were 6.25, 21.9, 3.12, and 68.8, respectively, with
415 the addition of 0.94% X-MS DP 56 component. Medium before and after the addition of 160 μL
416 of water was categorized as bicontinuous microemulsion (37.5% water; total volume 160 μL)
417 and O/W (68.8% water; total volume 320 μL) medium, respectively, as discussed later. The
418 loading capacity of quercetin in P0 was compared using two methods: (i) from dissolved
419 quercetin form and (ii) from quercetin powder. According to (i), quercetin was initially
420 dissolved in PEG 550 in series concentrations of 50, 100, 150, and 200 mg/mL. A fixed portion
421 (20 μL) of those four series would mix with the medium in a total volume of 320 μL , yielding
422 ideal quercetin loads of 3.13, 6.25, 9.38, and 12.5 mg/mL, respectively. The experiments were
423 performed with a fixed 20- μL quercetin concentration series mixed well with bicontinuous
424 medium containing Tween 80, IPM, and water (70, 10, and 60 μL , respectively), and one equal
425 volume of water (160 μL) was then added as a continuous phase. It was found that the four
426 series suspensions are all clear both in the bicontinuous phase and after the addition of water
427 (160 μL). After standing overnight, the suspension with portions of 50 and 100 mg/mL
428 quercetin in PEG 550 was still clear yellow, but the latter 150- and 200-mg/mL samples became
429 turbid. This result indicates that in the last two samples, quercetin was overloaded and led to
430 precipitation. After centrifugation, the quercetin concentration in the supernatant was 6.8 ± 0.5
431 mg/mL, meaning that P0 could be sufficient to have that load as a maximum. Next, X-MS DP
432 56 (5%, w/v solution) was applied to P0, replacing the water portion (60 μL), and followed the
433 identical protocol. Four portions of the 50–200-mg/mL quercetin series, Tween 80, and IPM
434 were all turbid after mixing with X-MS DP 56 in bicontinuous medium. However, after the bulk
435 water (160 μL) was added, all samples became clear yellow in color and remained stable for
436 over a month. This result indicated that the addition of X-MS DP 56 could stabilize a full load
437 of quercetin at 12.5 mg/mL without precipitation. (ii) In contrast, the direct addition of quercetin
438 powder into the fixed quantity of P0 medium maintained quercetin at only 2.0 ± 0.2 mg/mL,
439 emphasizing the solubilizing role of quercetin with PEG 550 as the main strategy to overcome
440 the poor solubility of quercetin. We strongly recommend not to alternate the methods of (ii) and
441 (i).

442 Among lipid formulations, SMES requires a highly specific balance formulation. The
443 ternary phase diagram was initially constructed based on the P0 formulation. PEG 550 volume
444 was the most critical factor, as it determines the concentration of quercetin loading in each
445 formulation. Among the 36 formulations located in the pentagon area in Fig. 3A, only the green
446 area (18 samples) was found to be a microemulsion. This means that the composition with low
447 IPM, low PEG 550, and a sufficiently large amount of Tween 80 was the desired ratio to obtain
448 the microemulsion template. Next, we replaced the compositions in the green area with the
449 portions of 200 mg/mL quercetin in PEG 550 and 5% X-MS DP 56 solution instead of pure
450 PEG 550 and water to test the full quercetin load, which is proposed to range from 15.79 to
451 23.53 mg/mL. However, such samples were precipitated after standing overnight. This indicates
452 that a high concentration of quercetin could influence the emulsification region. Among those
453 tested formulations, P14 marked inside of the green area with the full load of 20 mg/mL
454 quercetin was used to test the improved loading in Section 3.5.

455 Conductivity measurements were carried out to determine the makeup of the water
456 phase of the P0-based formulation: W/O emulsions are nonconductive, whereas O/W emulsions
457 are conductive. At the first state, the incomplete P0 was W/O medium, as the initial
458 conductivity was very low (0.031 mS/m). Then, after titration with water to 37.5%, the
459 conductivity was 13.67 mS/m, which was consequently classified as a bicontinuous medium.
460 The conductivity values were plotted against the water content in micelles, as shown in Fig. 3B,
461 demonstrating three different structural types of W/O, bicontinuous structures (B.C.) and O/W.
462 The conductivity of the sample rapidly increased to a maximum of 41.1 mS/m after 71.4–72.2%
463 water was introduced and then started to decrease. The result indicates that the P0 medium with
464 68.8% was an O/W microemulsion (carrying a conductivity of 40.5 mS/m) as water became a
465 continuous phase and the conductivity almost reached the maximum value zone. The latter
466 decrease was O/W with excess water.

467

468 **3.5. Loading capacity of quercetin in P0 and P14 containing various saccharides.** Most
469 saccharides are considered to be hydrophilic molecules since a large number of hydroxyl groups
470 are present. Alternatively, the hydrophobic properties derived from the nonpolar methine
471 patches in monosaccharides are possibly stronger if the stereochemical constraints on the chain
472 transform into a better conformation. In this study, we carefully isolated X-MS as an
473 amphiphilic substance with a strong hydrophobic characteristic. The influence of different types
474 of saccharides on the loading capacity of quercetin in P0 was evaluated as a comparison. The
475 solution stocks of 5% (w/v) of four oligosaccharides, including maltohexaose, CDs (α -, β -, and

476 γ -CDs), XOS 9, X-MSs (DP 16 and 56) and three polysaccharides, including soluble starch,
477 gum arabic, and dextran T 40, were introduced to P0 by replacing the portion of 60 μ L of water
478 (used as a control). The SMES medium was evaluated by visual assessment and centrifuged to
479 detect quercetin in the supernatant, and the results are shown in Table 2. The PEG 550 portion
480 determined the load of quercetin, as quercetin was initially dissolved in PEG 550 at a fixed
481 concentration (200 mg/mL). If no precipitation was found, a full load of quercetin in SMES was
482 suggested to be 12.5 mg/mL. Here, we first evaluated the soluble capacity of that PEG 550
483 portion only in a continuous phase. The portion of quercetin in PEG 550 was poured into water
484 instead of P0 medium. The precipitation of quercetin immediately occurred. Only 0.11 ± 0.15
485 mg/mL quercetin in the supernatant could be detected. Next, the portion was poured into P0
486 using water (control) instead of saccharides, following the steps of Tween 80, IPM, and water.
487 In this case, 6.8 ± 0.50 mg/ml quercetin was detected, meaning that in the colloidal solution,
488 quercetin could be more appreciably encapsulated. Between oligosaccharides and
489 polysaccharides, XOS 9 and soluble starch were the most effective saccharides, in which a small
490 quercetin precipitate was found, whereas quercetin in the other samples was highly cloudy and
491 precipitated. The megalomeric microemulsion with X-MSs displayed a better load among all
492 samples. SMES containing X-MS DP 56 had a clear appearance with a full load of quercetin,
493 whereas X-MS DP 16 had very slight precipitates.

494 To increase the load of quercetin in tests, the portion of quercetin in PEG 550 (fixed at
495 200 mg/mL) must be increased correspondingly. Accordingly, the composition in P14 was
496 composed of PEG 550, Tween 80, IPM, an aqueous solution of two types of MS in water
497 (dextran T and X-MS with 1, 5, 10, 15, and 20%), and water at the ratio of 40:90:10:60:200 μ L
498 (having a final volume of 400 μ L). The final mass percentages of PEG 550, Tween 80, IPM,
499 and water in P14 were 10.0, 22.5, 2.50, and 65.0, respectively, with additional MSs ranging
500 from 0.15–3.0%. The full load of quercetin was 20.0 mg/mL. Fig. 4A presents an image of the
501 samples in which the dissolved quercetin was added into a P14 template containing two sizes of
502 X-MS and dextran T MS. Dextran T MS is a commercially available hydrophilic linear-
503 isomaltomegalosaccharide with a main α -(1 \rightarrow 6)-linked D-glucopyranose chain. Here, we used
504 dextran T MS with two molecular weights 3,500 Dalton for T 3.5 and 6,000 Dalton for T6. X-
505 MS DP 16 and 56 with molecular weights of 2,488 and 8,493 Daltons, respectively, were again
506 used. In the control set (without the addition of MSs), P14 had a drug load of 7.1 ± 0.6 mg/mL,
507 with no significant ($p > 0.05$) relationships with those of dextran T 3.5 and T 6 (Fig. 4B). Fully
508 coarse precipitates were observed at all sample concentrations. In contrast, it was likely that a
509 growing mass of X-MS DP 16 until 2.25% was required to achieve a high load of quercetin at

510 17.09 ± 0.83 mg/mL, and further loading was then not possibly improved even by the mass
511 increase. In contrast, the high load of quercetin immediately reached 17.70 ± 0.40 mg/mL even
512 with only a tiny amount of X-MS DP 56 (0.15%). Slightly increasing X-MS DP 56 to 0.75%
513 eventually yielded the maximum quercetin solubility at 18.30 ± 1.42 mg/mL. Further increase
514 was not necessary for the load; however, in turn, X-MS DP 56 tended to aggregate instead as
515 fine precipitates were observed. This may be explained by the shorter chain length of X-MS DP
516 16 that could participate in the micelles with the higher masses and concentrations required for
517 quercetin loading. However, the large molecule of X-MS DP 56 was relatively full and required
518 only in low amounts, and only a small concentration was sufficient for the load. The influence
519 of X-MS chain length and concentration on the particle sizes of the megalomeric microemulsion
520 is presented in Fig. 5A. The largest microemulsion template consisted of quercetin and X-MS
521 (12.6 ± 0.3 nm), with a moderate size distribution (polydispersity index (PDI) = 0.20 ± 0.03).
522 With a larger X-MS size (at 3.0% final concentration), the diameters were 12.7 ± 0.2 and 12.1 ±
523 0.0 nm for X-MS DP 16 and 56, respectively. With quercetin loading, both megalomeric
524 microemulsion sizes were further reduced. However, increasing the concentrations of X-MS DP
525 56 was not likely to change the sizes, as it was in the average droplet sizes of 11.5 nm (with PDI
526 of 0.04–0.09). With X-MS DP 16 and quercetin loading, the size slightly increased from 11.5–
527 11.8 nm (with PDI of 0.03–0.10). The micelle was likely not able to expand, in agreement with
528 the results discussed earlier. The TEM micrograph (Fig. 5B) confirmed the globular diameter
529 without aggregation. However, the cylindrical shape of the micelles also appeared consistent
530 with a previous study (Aizawa, 2009). The attempts of improving loads of quercetin in lipid-
531 based carriers have been seriously investigated, as well as of decreasing the particle size to
532 allow better absorption. Hädrich et al. (2016) developed the best SMES with 20-nm droplets
533 using a hot solvent diffusion technique with less emulsifier (1.5% PEG 660-stearate) but still
534 incorporated only 1.5 mg/mL quercetin. Gao et al. (2009) were able to incorporate a higher
535 quercetin content of 4.138 mg/mL in a droplet size of 38.9 nm, but the formulation consisted of
536 a relatively high Tween 80 concentration (48%, w/w). Our megalomeric microemulsions have
537 more advantages, as they incorporate a larger amount of quercetin (12.5–18.3 mg/mL) with
538 smaller droplets, whereas lesser emulsifier/coemulsifier amounts are used. P0 formulated with
539 6.25% PEG 550, 21.9% Tween 80, 3.12% IPM, 68.8% water, 0.94% X-MS DP 56 and a
540 particle size of 11.6 ± 0.2 nm can sustain the load of 12.5 mg/mL quercetin without
541 precipitation over a month. P14 with 10.0% PEG 550, 22.5% Tween 80, 2.50% IPM, 65.0%
542 water, and 0.15% X-MS DP 56 and a particle size of 11.4 ± 0.1 nm was sufficient to hold the

543 higher load of 17.70 ± 0.40 mg/mL with a small precipitate in three days. Formulations and
544 their loading capacity of megalomeric microemulsions are summarized in Scheme 1.

545

546 **3.6. Antioxidation effect.** The DPPH and ABTS assays are commonly applied to estimate the
547 antioxidant activities of plant extracts. It was also very efficacious to identify the state of
548 oxidation under different pH conditions and mimic a physiological fluid. ABTS is found to be
549 stable from pH 3.0 to 6.5. Then, we performed only the tests of ABTS at pH 4.0 and DPPH at
550 6.0 and 7.4 (Table 3), where the original pH values of the quercetin-loaded microemulsion with
551 X-MSs were 5.8. Quercetin H-donor ability was not notably different among the quercetins in
552 methanol and all megalomeric microemulsions based on ABTS assays. However, the
553 antioxidant activity was found to be much improved by the microemulsion containing X-MS DP
554 16 and 56 for DPPH assays, with values of 22.2 ± 1.6 and 24.1 ± 1.2 $\mu\text{g/mL}$, respectively.
555 Although the formulation containing X-MSs could be able to ensure that the antioxidant activity
556 against DPPH and ABTS was maintained compared to the behavior in methanolic solution, the
557 presence of X-MS in the microemulsion has more potent antioxidant properties for DPPH at
558 both pH values but not for ABTS. Since the interactions among different antioxidant
559 components can be synergistic especially between quercetin and X-MS in the oil phase nearly
560 the surfactant film, the antioxidant mechanism and activity in the presence of X-MSs toward the
561 variation of free radicals will be great insight in the following work.

562

563 **4. Conclusion.**

564 The lack of fundamental knowledge derived from unavailable MS-size preparation on
565 the influence of SMES properties seriously hinders development in the field of food
566 supplements, pharmaceutical applications, and even carbohydrate research. X-MS is a branch
567 saccharide amphiphile. The hydrophobic region occurs naturally from methine (CH) groups of
568 the sugar monomers, especially xylose and arabinose residues. X-MS probably has a weaker
569 hydrophobic characteristic, and X-MS itself displayed a slight effect on quercetin binding.
570 However, the implementation of X-MSs toward SMES was highly compatible, as X-MS
571 molecules were confined in micellar solutions depending on the sizes. Additionally, we found
572 that PEG 550 displays excellent efficiency in dissolving quercetin, especially in determining the
573 load of quercetin in the formulations. We carefully chose X-MS to prepare megalomeric
574 microemulsions with two formulations. P0 could sustain the load of 12.5 mg/mL quercetin
575 without precipitation over a month. P14 was sufficient to hold the higher load of 17.70 ± 0.40

576 mg/mL with a small precipitate for three days. This is the first study that explained how
577 effective X-MSs were able to stabilize quercetin in the microemulsion.

578

579 **CRedit authorship contribution statement**

580 Weeranuch Lang: Conceptualization, Investigation, Formal analysis, Writing - original
581 draft, review & editing. Debashish Mondol: Writing - original draft, Investigation,
582 Methodology. Aphichat Trakooncharoenvit: Methodology, Formal analysis. Takayoshi Tagami:
583 Resources, Supervision. Masayuki Okuyama: Data curation, Resources. Tohru Hira:
584 Supervision, Resources. Nobuo Sakairi: Conceptualization, Supervision, Writing - review &
585 editing. Atsuo Kimura: Supervision, Funding acquisition, Project administration.

586

587 **Declaration of competing interest.** The authors have no conflicts of interest to declare.

588

589 **Acknowledgments**

590 We thank the staff of the Instrumental Analysis Division of the Creative Research
591 Institution at Hokkaido University for performing mass spectrometry analysis and Mr. Toshiaki
592 Itou at the facility area of Emission Electron Microscope, Research Faculty of Agriculture,
593 Hokkaido University, for performing the TEM analysis. We thank Elsevier's language editing
594 services for final English proofreads.

595

596 **References**

- 597 Agrawal, R. D., Tatode, A. A., Rarokar, N. R., & Umekar, M. J. (2020). Polymeric micelle as a
598 nanocarrier for delivery of therapeutic agents: A comprehensive review. *Journal of Drug*
599 *Delivery and Therapeutics*, *10*, 191–195. <https://doi.org/10.22270/jddt.v10i1-s.3850>.
- 600 Aizawa, H. (2009). Morphology of polysorbate 80 (Tween 80) micelles in aqueous 1,4-dioxane
601 solutions. *Journal of Applied Crystallography*, *42*, 592–596.
602 <https://doi.org/10.1107/S002188980902295X>.
- 603 Andrés, S., Tejido, M. L., Bodas, R., Morán, L., Prieto, N., Blanco, C., & Giráldez, F. J. (2013).
604 Quercetin dietary supplementation of fattening lambs at 0.2% rate reduces discolouration
605 and microbial growth in meat during refrigerated storage. *Meat Science*, *93*, 207–212.
606 <https://doi.org/10.1016/j.meatsci.2012.08.023>.
- 607 Bai, L., & McClements, D. J. (2016). Formation and stabilization of nanoemulsions using
608 biosurfactants: Rhamnolipids. *Journal of Colloid and Interface Science*, *479*, 71–79.
609 <https://doi.org/10.1016/j.jcis.2016.06.047>.

- 610 Baksi, R., Singh, D. P., Borse, S. P., Rana, R., Sharma, V., & Nivsarkar, M. (2018). In vitro and
611 in vivo anticancer efficacy potential of Quercetin loaded polymeric nanoparticles.
612 *Biomedicine and Pharmacotherapy*, *106*, 1513–1526.
613 <https://doi.org/10.1016/j.biopha.2018.07.106>.
- 614 Beyer, C. F., Craig, L. C., & Gibbons, W. A. (1973). Structural requirements for binding and
615 fluorescence enhancement of the fluorescent probe TNS with peptides. *Nature New Biology*,
616 *241*, 78–80. <https://doi.org/doi:10.1038/newbio241078a0>.
- 617 Biondi, O., Motta, S., & Mosesso, P. (2002). Low molecular weight polyethylene glycol
618 induces chromosome aberrations in Chinese hamster cells cultured in vitro. *Mutagenesis*,
619 *17*, 261–264. <https://doi.org/10.1093/mutage/17.3.261>.
- 620 Buranaboripan, W., Lang, W., Motomura, E., & Sakairi, N. (2014). Preparation and
621 characterization of polymeric host molecules, β -cyclodextrin linked chitosan derivatives
622 having different linkers. *International Journal of Biological Macromolecules*, *69*, 27–34.
623 <https://doi.org/10.1016/j.ijbiomac.2014.05.016>.
- 624 Crispín-Isidro, G., Hernández-Rodríguez, L., Ramírez-Santiago, C., Sandoval-Castilla, O.,
625 Lobato-Calleros, C., & Vernon-Carter, E. J. (2019). Influence of purification on
626 physicochemical and emulsifying properties of tamarind (*Tamarindus indica* L.) seed
627 gum. *Food Hydrocolloids*, *93*, 402–412. <https://doi.org/10.1016/j.foodhyd.2019.02.046>.
- 628 Dajas, F. (2012). Life or death: Neuroprotective and anticancer effects of quercetin. *Journal of*
629 *Ethnopharmacology*, *143*, 383–396. <https://doi.org/10.1016/j.jep.2012.07.005>.
- 630 Dhara, D., & Shah, D. O. (2001). Effect of poly(ethylene glycol)s on micellar stability of
631 sodium dodecyl sulfate. *Langmuir*, *17*, 7233–7236. <https://doi.org/10.1021/la001030i>.
- 632 Gao, Y., Wang, Y., Ma, Y., Yu, A., Cai, F., Shao, W., & Zhai, G. (2009). Formulation
633 optimization and in situ absorption in rat intestinal tract of quercetin-loaded
634 microemulsion. *Colloids and Surfaces B: Biointerfaces*, *71*, 306–314.
635 <https://doi.org/10.1016/j.colsurfb.2009.03.005>.
- 636 Hädrich, G., Monteiro, S. O., Rodrigues, M. R., de Lima, V. R., Putaux, J. L., Bidone, J.,
637 Teixeira, H. F., Muccillo-Baisch, A. L., & Dora, C. L. (2016). Lipid-based nanocarrier for
638 quercetin delivery: system characterization and molecular interactions studies. *Drug*
639 *Development and Industrial Pharmacy*, *42*, 1165–1173.
640 <https://doi.org/10.3109/03639045.2015.1118491>.
- 641 Hara, H., Kume, S., Iizuka, T., Fujimoto, Y., & Kimura, A. (2017). Enzymatically synthesized
642 megalo-type isomaltosaccharides enhance the barrier function of the tight junction in the
643 intestinal epithelium. *Bioscience, Biotechnology and Biochemistry*, *82*, 629–635.

644 <https://doi.org/10.1080/09168451.2017.1398065>.

645 Janado, M., & Yano, Y. (1985). Hydrophobic nature of saccharides as evidenced by their
646 differential affinity for polystyrene gel in aqueous media. *Journal of Solution Chemistry*,
647 *14*, 891–902. <https://doi.org/10.1007/BF00646298>.

648 Johari, J., Kianmehr, A., Mustafa, M. R., Abubakar, S., & Zandi, K. (2012). Antiviral activity of
649 baicalein and quercetin against the Japanese encephalitis virus. *International Journal of*
650 *Molecular Sciences*, *13*, 16020–16045. <https://doi.org/10.3390/ijms131216785>.

651 Kabel, M. A., van den Borne, H., Vincken, J. P., Voragen, A. G. J., & Schols, H. A. (2007).
652 Structural differences of xylans affect their interaction with cellulose. *Carbohydrate*
653 *Polymers*, *69*, 94–105. <https://doi.org/10.1016/j.carbpol.2006.09.006>.

654 Lang, W., Kumagai, Y., Sadahiro, J., Maneesan, J., Okuyama, M., Mori, H., Sakairi, N., &
655 Kimura, A. (2014). Different molecular complexity of linear-isomaltomegalosaccharides
656 and β -cyclodextrin on enhancing solubility of azo dye ethyl red: Towards dye
657 biodegradation. *Bioresource Technology*, *169*, 518–524.
658 <https://doi.org/10.1016/j.biortech.2014.07.025>.

659 Lang, W., Kumagai, Y., Sadahiro, J., Saburi, W., Sarnthima, R., Tagami, T., Okuyama, M.,
660 Mori, H., Sakairi, N., Kim, D., & Kimura, A. (2022a). A practical approach to producing
661 isomaltomegalosaccharide using dextran dextrinase from *Gluconobater oxydans* ATCC
662 11894. *Applied Microbiology and Biotechnology*, *106*, 689–698.
663 <https://doi.org/10.1007/s00253-021-11753-6>.

664 Lang, W., Kumagai, Y., Habu, S., Sadahiro, J., Tagami, T., Okuyama, M., Kitamura, S.,
665 Sakairi, N., & Kimura, A. (2022b). Physicochemical functionality of chimeric
666 isomaltomegalosaccharides with α -(1 \rightarrow 4)-glucosidic segments of various lengths.
667 *Carbohydrate Polymers*, *291*, 119562. <https://doi.org/10.1016/j.carbpol.2022.119562>.

668 Liu, C. H., Huang, Y. C., Jhang, J. W., Liu, Y. H., & Wu, W. C. (2015). Quercetin delivery to
669 porcine cornea and sclera by solid lipid nanoparticles and nanoemulsion. *RSC Advances*, *5*,
670 100923–100933. <https://doi.org/10.1039/c5ra17423f>.

671 Majee, S. B., Avlani, D., & Biswas, G. R. (2016). Non-starch plant polysaccharides:
672 Physicochemical modifications and pharmaceutical applications. *Journal of Applied*
673 *Pharmaceutical Science*, *6*, 231–241. <https://doi.org/10.7324/JAPS.2016.601033>.

674 Miyamoto, H., Umemura, M., Aoyagi, T., Yamane, C., Ueda, K., & Takahashi, K. (2009).
675 Structural reorganization of molecular sheets derived from cellulose II by molecular
676 dynamics simulations. *Carbohydrate Research*, *344*, 1085–1094.
677 <https://doi.org/10.1016/j.carres.2009.03.014>.

- 678 Phan, T. N. Q., Le-Vinh, B., Efiana, N. A., & Bernkop-Schnürch, A. (2019). Oral self-
679 emulsifying delivery systems for systemic administration of therapeutic proteins: science
680 fiction? *Journal of Drug Targeting*, *27*, 1017–1024.
681 <https://doi.org/10.1080/1061186X.2019.1584200>.
- 682 Pichot, R., Spyropoulos, F., & Norton, I. T. (2010). O/W emulsions stabilised by both low
683 molecular weight surfactants and colloidal particles: The effect of surfactant type and
684 concentration. *Journal of Colloid and Interface Science*, *352*, 128–135.
685 <https://doi.org/10.1016/j.jcis.2010.08.021>.
- 686 Premathilaka, R., Rashidinejad, A., Golding, M., & Singh, J. (2022). Oral delivery of
687 hydrophobic flavonoids and their incorporation into functional foods: Opportunities and
688 challenges. *Food Hydrocolloids*, *128*, 107567.
689 <https://doi.org/10.1016/j.foodhyd.2022.107567>.
- 690 Product Information. (2020). *Transplantation*, *104*, S13–S16.
691 <https://doi.org/10.1097/TP.0000000000003330>.
- 692 Rogerio, A. P., Dora, C. L., Andrade, E. L., Chaves, J. S., Silva, L. F. C., Lemos-Senna, E., &
693 Calixto, J. B. (2010). Anti-inflammatory effect of quercetin-loaded microemulsion in the
694 airways allergic inflammatory model in mice. *Pharmacological Research*, *61*, 288–297.
695 <https://doi.org/10.1016/j.phrs.2009.10.005>.
- 696 Sermkaew, N., & Plyduang, T. (2020). Self-microemulsifying drug delivery systems of
697 *Moringa oleifera* extract for enhanced dissolution of kaempferol and quercetin. *Acta*
698 *Pharmaceutica*, *70*, 77–88. <https://doi.org/10.2478/acph-2020-0012>.
- 699 Shinoki, A., Lang, W., Thawornkuno, C., Kang, H. K., Kumagai, Y., Okuyama, M., Mori, H.,
700 Kimura, A., Ishizuka, S., & Hara, H. (2013). A novel mechanism for the promotion of
701 quercetin glycoside absorption by megalin α -1,6-glucosaccharide in the rat small intestine.
702 *Food Chemistry*, *136*, 293–296. <https://doi.org/10.1016/j.foodchem.2012.08.028>.
- 703 Son, H. Y., Lee, M. S., Chang, E., Kim, S. Y., Kang, B., Ko, H., Kim, I. H., Zhong, Q., Jo, Y.
704 H., Kim, C. T., & Kim, Y. (2019). Formulation and characterization of quercetin-loaded
705 oil in water nanoemulsion and evaluation of hypocholesterolemic activity in rats.
706 *Nutrients*, *11*, 1–19. <https://doi.org/10.3390/nu11020244>.
- 707 Sun, G., Zhang, X. Z., & Chu, C. C. (2008). Effect of the molecular weight of polyethylene
708 glycol (PEG) on the properties of chitosan-PEG-poly(*N*-isopropylacrylamide) hydrogels.
709 *Journal of Materials Science: Materials in Medicine*, *19*, 2865–2872.
710 <https://doi.org/10.1007/s10856-008-3410-9>.
- 711 Syed, H. K., & Peh, K. K. (2014). Identification of phases of various oil, surfactant/co-

712 surfactants and water system by ternary phase diagram. *Acta Poloniae Pharmaceutica -*
713 *Drug Research*, 71, 301–309.

714 Thoma, J. A., & French, D. (1960). The starch-iodine-iodide interaction. Part I.
715 spectrophotometric investigations. *Journal of the American Chemical Society*, 82, 4144–
716 4147. <https://doi.org/10.1021/ja01501a004>.

717 Vincken, J. P., De Keizer, A., Beldman, G., & Voragen, A. G. (1995). Fractionation of
718 xyloglucan fragments and their interaction with cellulose. *Plant Physiology*, 108, 1579–
719 1585. <https://doi.org/10.1104/pp.108.4.1579>.

720 Wu, L., Qiao, Y., Wang, L., Guo, J., Wang, G., He, W., Yin, L., & Zhao, J. (2015). A Self-
721 microemulsifying drug delivery system (SMEDDS) for a novel medicative compound
722 against depression: a preparation and bioavailability study in rats. *AAPS PharmSciTech*,
723 16, 1051–1058. <https://doi.org/10.1208/s12249-014-0280-y>.

724 Xiao, F., Xu, T., Lu, B., & Liu, R. (2020). Guidelines for antioxidant assays for food
725 components. *Food Frontiers*, 1, 60–69. <https://doi.org/10.1002/fft2.10>.

726

727 **Figure captions**

728 **Fig. 1.** Size characterization of X-MSs prepared by cellulase hydrolysis of tamarind seed
729 xyloglucan. HPAEC-PAD analyses of (A, top) the major saccharides from the original cellulase
730 reaction and (A, bottom) X-MS DP 16 and 56 obtained by methanol purification, and (B) gel
731 filtration HPLC chromatogram of X-MS DP 16 and 56 and the parental xyloglucan. (C)
732 Proposed model structure of X-MS DP 16 and 56 coexisting with the fine structures of XOSs.
733 Subunit names are presented as a one-letter unambiguous nomenclature system as follows:
734 unsubstituted D-Glcp is designated **G**, α -D-Xylp-(1→6)- β -D-Glcp residue is designated **X**, β -D-
735 Galp-(1→2)- α -D-Xylp-(1→6)- β -D-Glcp is designated **L**, and α -L-Arap-(1→2)- α -D-Xylp-
736 (1→6)- β -D-Glcp is designated **A**.

737

738 **Fig. 2.** Fluorescence spectra of TNS (0.5 μ M) in the presence of (A) X-MS DP 16 (0–20
739 mg/mL) and (B) DP 56 (0–10 mg/mL). Inset: the Benesi-Hildebrand double-reciprocal plots for
740 the determination of K_c values of the corresponding X-MSs. Fluorescence intensity at a series
741 concentration of Tween 80 (0.001–0.1 mg/mL) coexisting with (C) X-MS DP 16 (5 and 10
742 mg/mL) and (D) PEG 550 (10 and 30 mg/mL). Black arrows indicate the direction of the
743 growing concentrations of X-IMSs and PEG. Dashed lines depict the CMC value.

744

745 **Fig. 3.** (A) Ternary phase diagram (by mass fraction) for the mixture of PEG 550, Tween 80,
746 and IPM without quercetin and X-MS. An equal volume of water was added to each fraction.
747 The opened and filled cycles represent the two formulations P0 and P14, respectively, and
748 SMES P0 was categorized as an O/W microemulsion according to Fig. 3B. (B) Variation in
749 microemulsion conductivity at various water contents.

750

751 **Fig. 4.** Effect of MS types and concentrations on the carried dosage of quercetin in the P14
752 microemulsion. (A) Visual inspection of quercetin loaded into a megalomeric microemulsion of
753 X-MS DP 16, 56, T 3.5, and T 6 with various concentrations after standing overnight (up rows)
754 and then centrifugation at 12,000 $\times g$ and 25 °C for 10 min (down rows). The dissolved
755 quercetin in the supernatant of each sample was determined and is presented in (B).

756

757 **Fig. 5.** Droplet analysis of the megalomeric microemulsion. (A) A zetasizer was used to
758 determine the droplet sizes of the microemulsion containing various concentrations of X-MS
759 DP 16 and 56 in the P14 formula. CTRs are the microemulsion template (without quercetin
760 load). The panel compares the sizes of microemulsions containing 3.0% X-MS DP 16 and 56
761 with and without quercetin loading. (B) The particle appearance of the P0 image obtained by
762 TEM.

763

764 **Scheme 1.** Formulations of megalomeric microemulsions containing quercetin.

765

766

767

768

769

770

771

772

773

774

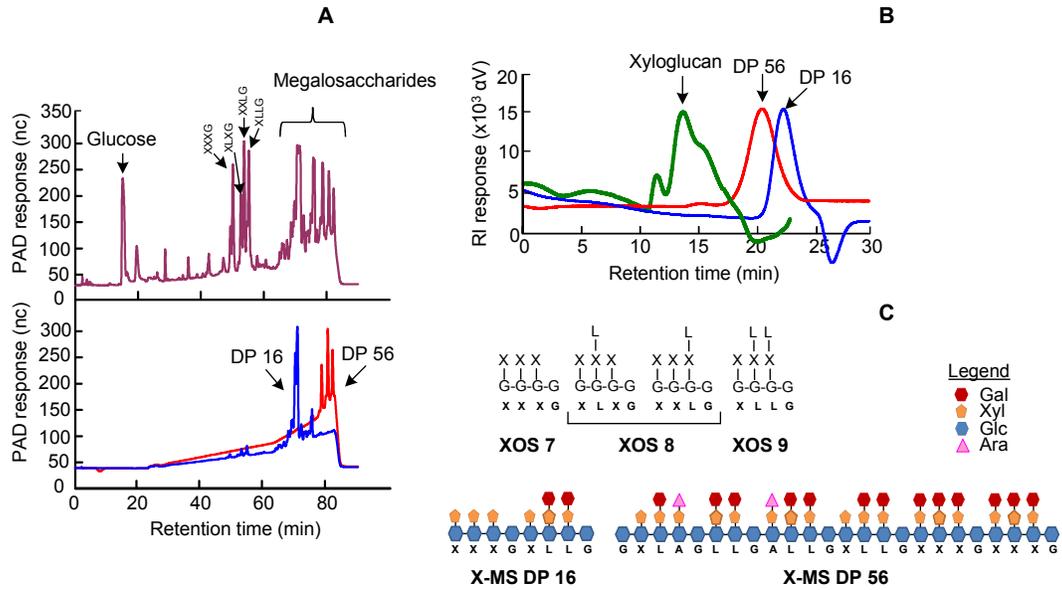
775

776

777

778

779



780

781 **Fig. 1.** Size characterization of X-MSs prepared from cellulase hydrolysis.

782

783

784

785

786

787

788

789

790

791

792

793

794

795

796

797

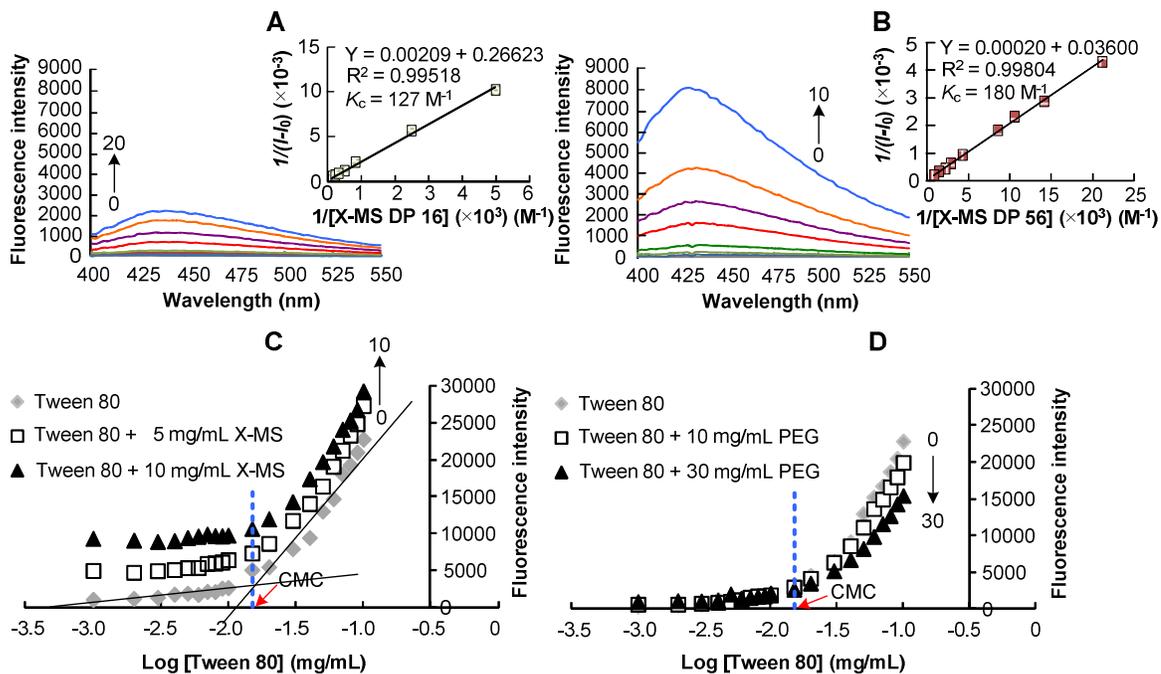
798

799

800

801

802



803

804

805 **Fig. 2.** Fluorescence spectra.

806

807

808

809

810

811

812

813

814

815

816

817

818

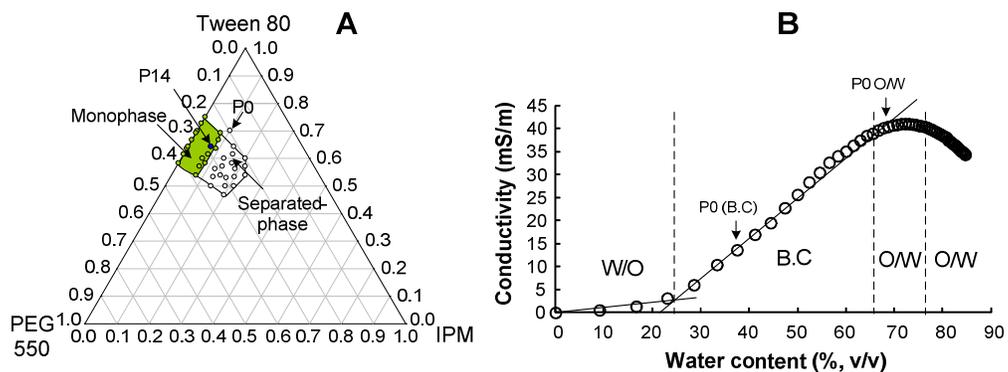
819

820

821

822

823



824

825 **Fig. 3.** (A) Ternary phase diagram.

826

827

828

829

830

831

832

833

834

835

836

837

838

839

840

841

842

843

844

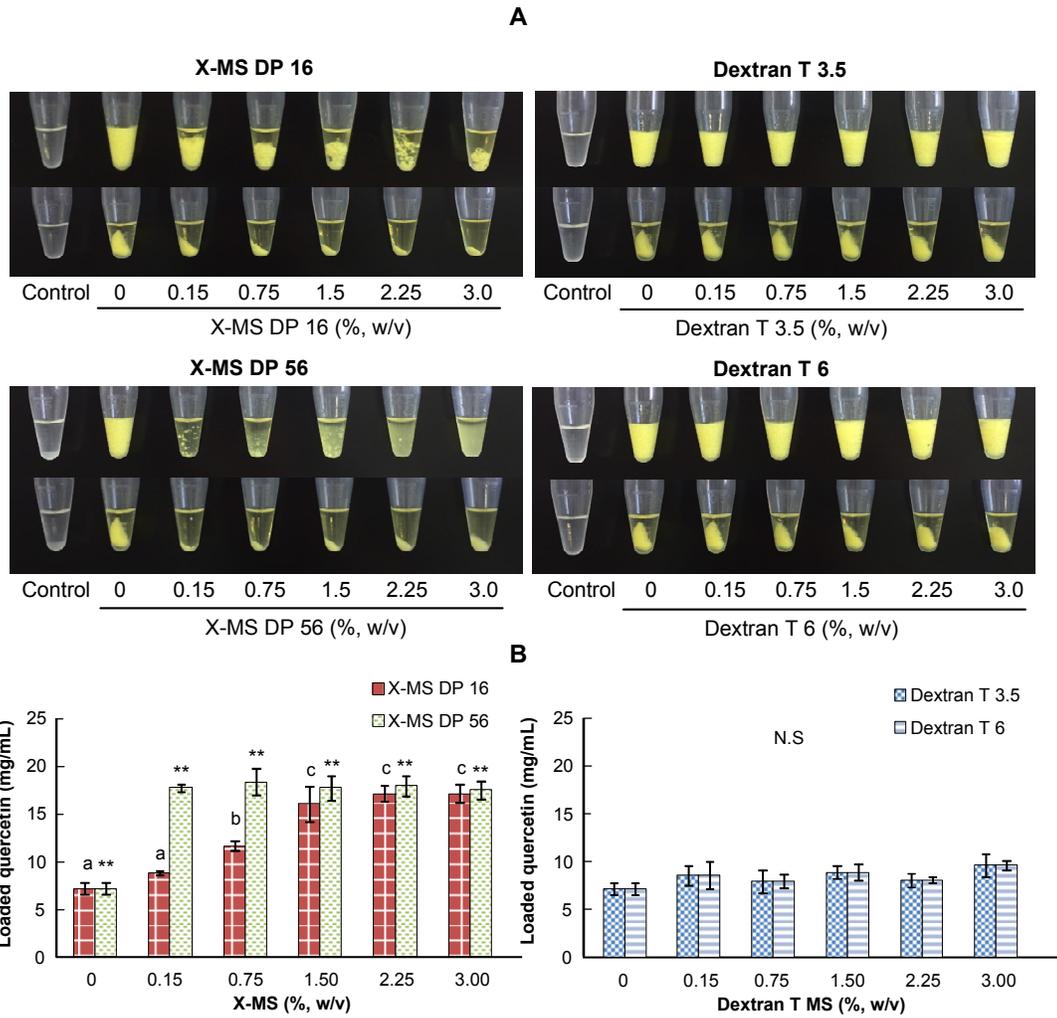
845

846

847

848

849



851
852

853 **Fig. 4.** Effect of MS types.

854

855

856

857

858

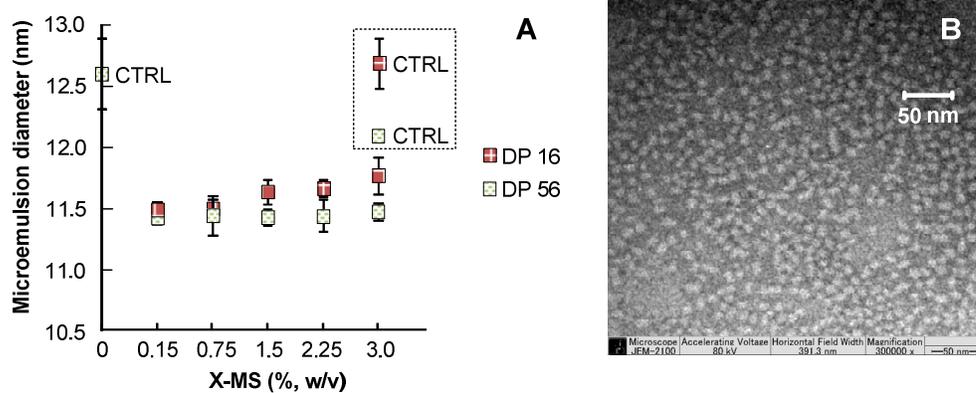
859

860

861

862

863



864

865 **Fig. 5.** Droplet analysis of the megalomeric microemulsion.

866

867

868

869

870

871

872

873

874

875

876

877

878

879

880

881

882

883

884

885

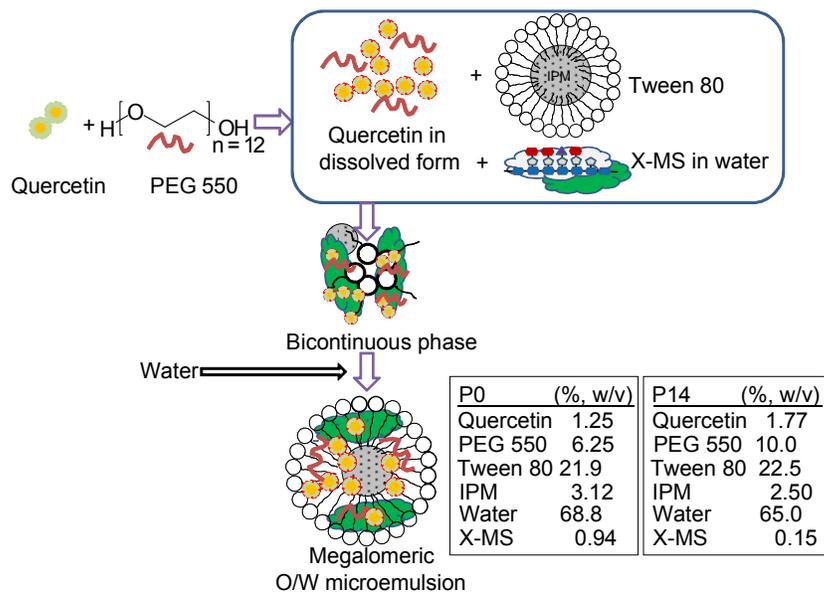
886

887

888

889

890



891

892 **Scheme 1.** Formulations of the megalomeric microemulsion.

893

894

895

896

897

898

899

900

901

902

903

904

905

906

907

908

909

910

911

912

913 **Table 1.** Phase solubility of quercetin in fixed-dose excipients with or without the addition of
 914 X-MS DP 56 or Tween 80.

Excipient	Quercetin solubility (mg/mL)			
	no water	with water ^a	with X-MS and water ^b	with Tween 80 and water ^c
PEG 200	204.57 ± 1.37	0.08 ± 0.01	0.82 ± 0.02	2.17 ± 0.10
PEG 300	201.86 ± 1.37	0.10 ± 0.00	0.74 ± 0.04	2.36 ± 0.12
PEG 400	196.62 ± 0.55	0.12 ± 0.00	1.09 ± 0.03	2.48 ± 0.01
PEG 550	203.80 ± 1.37	0.16 ± 0.01	1.38 ± 0.25	2.51 ± 0.03
PEG 1000	N.D	0.05 ± 0.00	0.31 ± 0.01	1.33 ± 0.04
PEG 4000	N.D	0.08 ± 0.00	0.26 ± 0.01	1.14 ± 0.00
PEG 6000	N.D.	0.07 ± 0.00	0.21 ± 0.00	1.18 ± 0.10
PEG 8000	N.D.	0.07 ± 0.01	0.20 ± 0.00	0.94 ± 0.01
Glycerol	2.60 ± 0.12	0.01 ± 0.00	0.15 ± 0.00	1.81 ± 0.41
Tween 80	6.30 ± 0.14	1.63 ± 0.04	1.83 ± 0.13	

915 N.D. = not determined because the original forms are semisolid or solid. ^a The combination
 916 contains 20% PEG in water. ^b The combination contains 10%, (w/v) X-MS and 20% PEG and
 917 replenished with water to be 100% (v/v). ^c The combination contains 10% (v/v) Tween 80 and
 918 20% PEG or 10% (v/v) glycerol.

919
 920
 921
 922
 923
 924
 925
 926
 927
 928
 929
 930
 931
 932
 933

934 **Table 2.** Maximum quercetin load in the P0 microemulsion containing saccharides.

Saccharides	Quercetin solubility (mg/mL)	Precipitant found
- ^a	0.11 ± 0.15	+++
- ^b	6.80 ± 0.50	+++
Maltoheptaose	8.37 ± 0.15	++
α-CD	8.32 ± 1.43	+++
β-CD	6.02 ± 0.70	+++
γ-CD	7.39 ± 0.68	+++
XOS 9	11.57 ± 0.16	+
X-MS DP 16	12.04 ± 0.07	-/+
X-MX DP 56	12.50 ± 0.20	-
Soluble starch	11.53 ± 0.05	+
Gum arabic	10.57 ± 0.66	+
Dextran T 40	8.97 ± 0.49	++

935 ^a = Dissolved quercetin with PEG 550 and then loaded into water, ^b = Dissolved quercetin with
 936 PEG 550 and then loaded into P0 using water instead of saccharides. - = no precipitate, +, ++,
 937 +++ = small, moderate, and complete precipitate, respectively.

938
 939
 940
 941
 942
 943
 944
 945
 946
 947
 948
 949
 950
 951
 952
 953

954 **Table 3.** IC₅₀ (μg/mL) determination by DPPH and ABTS assays.

Quercetin in	DPPH		ABTS
	at pH 6.0	at pH 7.4	at pH 4.0
Methanol	38.0 ± 10	176.9 ± 10.0	48.3 ± 2.5
Microemulsion without X-MS	89.7 ± 0.3	125.1 ± 9.8	49.8 ± 2.8
Microemulsion with X-MS DP 16	22.2 ± 1.6	111.2 ± 9.8	48.8 ± 8.0
Microemulsion with X-MS DP 56	24.1 ± 1.2	122.7 ± 9.6	48.7 ± 11.0

955