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Title	Formulation and evaluation of a novel megalomeric microemulsion from tamarind seed xyloglucan-megalosaccharides for improved high-dose quercetin delivery
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1	Formulation and evaluation of a novel megalomeric microemulsion from tamarind seed
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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 <sup>-1</sup> , 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 (IC50 =  $22-24 \mu g/mL$ ) 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,

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 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 (0.17–7.7 µg/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

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. Megalomeric 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 polysaccharides. Previously, we established a preparation method of homologous glucose-type 80 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  $\beta$ -(1 $\rightarrow$ 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

- 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

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

**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

obtained from Fluka (Steinheim, Germany). 6-(*p*-Toluidino)-2-naphthalenesulfonic acid sodium

salt (TNS) and DPPH were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dextran T

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  $\ge$  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 M $\Omega$ , Direct-Q<sup>®</sup> 3UV, Millipores SAS, Molsheim, France).

124

**2.2. Preparation of X-MSs and their isolation.** A slurry of tamarind seed xyloglucan (2 g)

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

incubated for 2 h. The sample was heated at 121 °C for 15 min to inactivate the enzyme. After

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

dialyzed with a Spectra/Por<sup>®</sup>6 membrane with a molecular weight cutoff of 2 kD against water

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  $\times$ g, 30 min) and dried to collect the sediment at a  $\leq$  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 contents, namely, (70-80)% and (80-95)%. The last fraction was determined as X-MS DP 16. 141 All fractions were dried using a lyophilizer (Eyela FDU-1200, Tokyo, Japan). The four obtained 142 samples were profiled by Dionex HPAEC-PAD ( $4 \times 250$  mm CarboPac<sup>TM</sup> PA1 column, Dionex, 143 144 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 not overlap with each other and were therefore considered for use in the entire study. XOS 9 147 148 was purified from completed cellulase Y-C digests by preparative HPLC on an Imtakt Unison 149 US-Amino column ( $20 \times 250$  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 sodium nitrate solution (0.1 M) at 0.5 mL/min, and the signals were detected by a refractive 155 156 index detector (RI 2013 Plus, Tokyo, Japan). The molecular mass markers used were Shodex<sup>®</sup> 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-18/[(%hexose × 162/100) + (%pentose × 132/100)], whereas MW was the molecular weight 166 obtained in Section 2.3. The molecular weight of a hexose residue in the X-MS chain 167 168 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

**2.5.** Phase solubility assay of quercetin. X-MSs (100  $\mu$ L) in series concentrations (0, 2, 4, 6, 8, and 10 mg/mL) were mixed well with excess quercetin (1 mg) at 25 °C for 6 h in triplicate. The 172 173 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 spectrophotometer (U2900, Hitachi, Tokyo, Japan). The concentration of quercetin in the 175 176 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.

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2.7. Hydrophobic interaction of TNS with X-MSs, TNS with X-MS DP 16 and Tween 80, 184 TNS with Tween 80 and PEG 550. The complexes of 0.5  $\mu$ M TNS with X-MS DP 16 (0, 0.5, 185 186 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 °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 of X-MS DP 16 (5 and 10 mg/mL) and PEG 550 (10 and 30 mg/mL). The determination of the 197 198 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 °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  $\mu$ L (having a final volume of 320  $\mu$ L). 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  $\mu$ L). 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  $\mu$ L to 20, 30, 40, and 50  $\mu$ L; IPM from 10  $\mu$ L to 10, 20, and 30 215  $\mu$ L; and Tween 80 from 70  $\mu$ L to 70, 80, and 90  $\mu$ L. 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  $\mu$ L (having 224 a final volume of 400  $\mu$ L), 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- $\mu$ L 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  $\mu$ g/mL). Sodium 240 phosphate buffer (pH 6.0 and 7.4; 40 mM, 80  $\mu$ L) and the samples (20  $\mu$ L) were added to the 241 96-well plate, and the stock solution of DPPH prepared in methanol (0.2 mM, 100  $\mu$ L) 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 maintained at 25 °C for 16 h in the dark to oxidize ABTS to ABTS<sup>++</sup> radicals. The ABTS 248 249 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  $\mu$ L) to samples 252  $(10 \,\mu\text{L})$  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<sub>3</sub>. 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

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**

**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
- 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- $\beta$ -(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  $\beta$ -(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 molarity percentages (mol%) of 4.7, 22.6, 44.3, and 28.5, respectively. X-MS DP 16 has the 289 290 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 xyloglucan molecules to cellulose microfibrils, whereas XOS with one or two repeating units 311 312 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 Benesi-Hilderbrand equation ( $R^2 > 0.99$ ), which yielded  $K_c$  values of 127 and 180 M<sup>-1</sup> for X-MS 328 329 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 CMC = -1.824). Two concentrations of X-MS DP 16 at 5 and 10 mg/mL were added to TNS solutions with a series concentration of Tween 80 from 0.001-0.1 mg/mL. It was found 333 334 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

demonstrated that the X-MS branch molecules incorporate to form a hydrophilic outer shell and

hydrophobic inner core of Tween 80 micelles, the so-called cosurfactant, and further increase

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 micellular 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  $K_{\rm S}$  calculated from the phase solubility diagram was found to be consistent with X-MS chain length, as the  $K_{\rm S}$ 365 increased from 114 to 677 M<sup>-1</sup> for X-MS DP 16 and 56, respectively. To date, dimethyl 366 367 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  $196.62 \pm 0.55$  and  $204.57 \pm 1.37$  mg/mL and much higher compared with 383 glycerol (2.60  $\pm$  0.12 mg/mL) and Tween 80 (6.30  $\pm$  0.14 mg/mL) (Table 1), as well as the 384 value found in the literature  $(14.12 \pm 2.18 \,\mu\text{g/mL}$  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 quercetin solubilization. For instance, 20% PEG 550 in water could dissolve quercetin at only 391 392  $0.16 \pm 0.01$  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  $\pm$  0.01 to 1.38  $\pm$  0.25 mg/mL and 2.51  $\pm$  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 emulsifiers having a specific HLB (polar or nonpolar characteristic). The HLB values of 401 402 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 HLB (RHLB) oil phase, such as IPM (with RHLB 11.5), may be more feasible to formulate 404 405 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  $0.28 \pm 0.01$  mg/mL. Accordingly, formulations with Tween 408 80, PEG 550, X-MS, and IPM were evaluated for quercetin-loaded SMES.

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 $\mu L$ (having a final volume of 320 $\mu L$ ), 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 $\mu L$
416	of water was categorized as bicontinuous microemulsion (37.5% water; total volume 160 $\mu$ L)
417	and O/W (68.8% water; total volume 320 $\mu L)$ 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 $\mu$ L) of those four series would mix with the medium in a total volume of 320 $\mu$ L, 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- $\mu$ L quercetin concentration series mixed well with bicontinuous
424	medium containing Tween 80, IPM, and water (70, 10, and 60 $\mu$ L, respectively), and one equal
425	volume of water (160 $\mu L)$ 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 $\mu$ L). 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 $6.8 \pm 0.5$
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 $\mu L$ ), 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 $\mu$ L) 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 $2.0 \pm 0.2$ 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 phase of the P0-based formulation: W/O emulsions are nonconductive, whereas O/W emulsions 456 457 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 68.8% was an O/W microemulsion (carrying a conductivity of 40.5 mS/m) as water became a 464 465 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
- solution stocks of 5% (w/v) of four oligosaccharides, including maltohexaose, CDs ( $\alpha$ -,  $\beta$ -, and

476  $\gamma$ -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  $\mu$ L of water 478 (used as a control). The SMES medium was evaluated by visual assessment and centrifuged to detect quercetin in the supernatant, and the results are shown in Table 2. The PEG 550 portion 479 480 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  $0.11 \pm 0.15$ 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,  $6.8 \pm 0.50$  mg/ml quercetin was detected, meaning that in the colloidal solution, 488 quercetin could be more appreciably encapsulated. Between oligosaccharides and polysaccharides, XOS 9 and soluble starch were the most effective saccharides, in which a small 489 490 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  $40:90:10:60:200 \mu$ L 498 (having a final volume of 400  $\mu$ 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  $\alpha$ -(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-MS DP 16 and 56 with molecular weights of 2,488 and 8,493 Daltons, respectively, were again 505 506 used. In the control set (without the addition of MSs), P14 had a drug load of  $7.1 \pm 0.6$  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  $17.09 \pm 0.83$  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  $17.70 \pm 0.40$  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  $18.30 \pm 1.42$  mg/mL. Further increase was not necessary for the load; however, in turn, X-MS DP 56 tended to aggregate instead as 514 515 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  $(12.6 \pm 0.3 \text{ nm})$ , with a moderate size distribution (polydispersity index (PDI) =  $0.20 \pm 0.03$ ). 522 With a larger X-MS size (at 3.0% final concentration), the diameters were  $12.7 \pm 0.2$  and  $12.1 \pm$ 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 the results discussed earlier. The TEM micrograph (Fig. 5B) confirmed the globular diameter 528 529 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 a relatively high Tween 80 concentration (48%, w/w). Our megalomeric microemulsions have 536 537 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 6.25% PEG 550, 21.9% Tween 80, 3.12% IPM, 68.8% water, 0.94% X-MS DP 56 and a 539 540 particle size of  $11.6 \pm 0.2$  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% water, and 0.15% X-MS DP 56 and a particle size of  $11.4 \pm 0.1$  nm was sufficient to hold the 542

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

545

3.6. Antioxidation effect. The DPPH and ABTS assays are commonly applied to estimate the 546 547 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  $22.2 \pm 1.6$  and  $24.1 \pm 1.2 \mu g/mL$ , 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 presence of X-MS in the microemulsion has more potent antioxidant properties for DPPH at 557 558 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 hydrophobic characteristic, and X-MS itself displayed a slight effect on quercetin binding. 569 570 However, the implementation of X-MSs toward SMES was highly compatible, as X-MS 571 molecules were confined in micellular 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  $17.70 \pm 0.40$ 

576	mg/mL with a small	precipitate for three day	s. This is the first stud	v that explained how
	0			

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 &
- editing. Atsuo Kimura: Supervision, Funding acquisition, Project administration.
- 586

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588

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### 727 Figure captions

- 728 Fig. 1. Size characterization of X-MSs prepared by cellulase hydrolysis of tamarind seed
- xyloglucan. HPAEC-PAD analyses of (A, top) the major saccharides from the original cellulase
- reaction and (A, bottom) X-MS DP 16 and 56 obtained by methanol purification, and (B) gel
- 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 unambiquous nomenclature system as follows:
- unsubstituted D-Glcp is designated G,  $\alpha$ -D-Xylp-(1 $\rightarrow$ 6)- $\beta$ -D-Glcp residue is designated X,  $\beta$ -D-
- 735 Galp- $(1\rightarrow 2)-\alpha$ -D-Xylp- $(1\rightarrow 6)-\beta$ -D-Glcp is designated L, and  $\alpha$ -L-Arap- $(1\rightarrow 2)-\alpha$ -D-Xylp-
- 736  $(1\rightarrow 6)$ - $\beta$ -D-Glc*p* is designated **A**.
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- **Fig. 2.** Fluorescence spectra of TNS (0.5  $\mu$ M) in the presence of (A) X-MS DP 16 (0–20
- mg/mL) and (B) DP 56 (0–10 mg/mL). Inset: the Benesi-Hildebrand double-reciprocal plots for
- the determination of  $K_c$  values of the corresponding X-MSs. Fluorescence intensity at a series
- 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.
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745	<b>Fig. 3.</b> (A) Ternary phase diagram (by mass fraction) for the mixture of PEG 550. Tween 80.
746	and IPM without guercetin 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).
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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.
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764	Scheme 1. Formulations of megalomeric microemulsions containing quercetin.
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853 Fig. 4. Effect of MS types.





913 Table 1. Phase solubility of quercetin in fixed-dose excipients with or without the addition of

Excipient	Quercetin solubility (mg/mL)			
	no water	with water <sup>a</sup>	with X-MS and	with Tween 80
			water <sup>b</sup>	and water <sup>c</sup>
PEG 200	$204.57 \pm 1.37$	$0.08 \pm 0.01$	$0.82 \pm 0.02$	$2.17 \pm 0.10$
PEG 300	$201.86 \pm 1.37$	$0.10\pm0.00$	$0.74 \pm 0.04$	$2.36 \pm 0.12$
PEG 400	$196.62 \pm 0.55$	$0.12\pm0.00$	$1.09 \pm 0.03$	$2.48 \pm 0.01$
PEG 550	$203.80 \pm 1.37$	$0.16 \pm 0.01$	1.38 ±0.25	$2.51\pm0.03$
PEG 1000	N.D	$0.05\pm0.00$	$0.31 \pm 0.01$	$1.33 \pm 0.04$
PEG 4000	N.D	$0.08 \pm 0.00$	$0.26 \pm 0.01$	$1.14 \pm 0.00$
PEG 6000	N.D.	$0.07 \pm 0.00$	$0.21 \pm 0.00$	$1.18 \pm 0.10$
PEG 8000	N.D.	$0.07\pm0.01$	$0.20\pm0.00$	$0.94 \pm 0.01$
Glycerol	$2.60 \pm 0.12$	$0.01 \pm 0.00$	$0.15 \pm 0.00$	$1.81 \pm 0.41$
Tween 80	$6.30 \pm 0.14$	$1.63 \pm 0.04$	$1.83 \pm 0.13$	

914 X-MS DP 56 or Tween 80.

915 N.D. = not determined because the original forms are semisolid or solid. <sup>a</sup> The combination

916 contains 20% PEG in water.  $^{\rm b}$  The combination contains 10%, (w/v) X-MS and 20% PEG and

917 replenished with water to be 100% (v/v). <sup>c</sup> The combination contains 10% (v/v) Tween 80 and

918 20% PEG or 10% (v/v) glycerol.

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	Saccharides	Quercetin solubility	Precipitant found
		(mg/mL)	
	_a	0.11 ± 0.15	+++
	_b	$6.80 \pm 0.50$	+++
	Maltoheptaose	$8.37 \pm 0.15$	++
	α-CD	$8.32 \pm 1.43$	+++
	β-CD	$6.02\pm0.70$	+++
	γ-CD	$7.39 \pm 0.68$	+++
	XOS 9	$11.57 \pm 0.16$	+
	X-MS DP 16	$12.04 \pm 0.07$	-/+
	X-MX DP 56	$12.50 \pm 0.20$	-
	Soluble starch	$11.53 \pm 0.05$	+
	Gum arabic	$10.57 \pm 0.66$	+
	Dextran T 40	$8.97 \pm 0.49$	++
935	<sup>a</sup> = Dissolved qu	ercetin with PEG 550 and	then loaded into water, $b = Dissolved$ quercetin with
936	PEG 550 and the	en loaded into P0 using wa	ater instead of saccharides = no precipitate, +, ++,
937	+++ = small, mo	oderate, and complete prec	ipitate, respectively.
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**Table 2.** Maximum quercetin load in the P0 microemulsion containing saccharides.

	DPPH		ABTS
Quercetin in	at pH 6.0	at pH 7.4	at pH 4.0
Methanol	$38.0 \pm 10$	$176.9 \pm 10.0$	$48.3 \pm 2.5$
Microemulsion without X-MS	$89.7\pm0.3$	$125.1 \pm 9.8$	$49.8 \pm 2.8$
Microemulsion with X-MS DP 16	$22.2 \pm 1.6$	$111.2 \pm 9.8$	$48.8 \pm 8.0$
Microemulsion with X-MS DP 56	$24.1 \pm 1.2$	122.7 ± 9.6	$48.7 \pm 11.0$

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