

Oxidative properties of Moringa oleifera kernel oil from different extraction methods during storage

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1 (Title)

2 **Oxidative properties of *Moringa oleifera* kernel oil from different extraction methods**
3 **during storage**

4
5 (Running title)

6 **Oxidative properties of *Moringa oleifera* kernel oil during storage**

7
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20
21 **Abstract**

22
23 **BACKGROUND**

24 *Moringa oleifera* (MO) kernel oil is categorized as high-oleic oil which resembles olive oil.

25 However, different from olive, MO trees are largely available in most subtropical and tropical

26 countries. Therefore, in these countries, the benefits of oleic acid can be obtained at a cheaper
27 price through consumption of MO kernel oil. This study reports on the effect of different
28 extraction methods on oxidative properties of MO kernel oil during storage for 140 days at 13
29 °C, 25 °C, and 37 °C.

30

31 RESULTS

32 All aqueous enzymatic extraction (AEE)-based methods generally resulted in oil with better
33 oxidative properties and higher tocopherol retention than the use of solvent. Prior to AEE,
34 boiling pre-treatment deactivated the hydrolytic enzymes and preserved the oil quality. In
35 contrast, high pressure processing (HPP) pre-treatment accelerated hydrolytic reaction and
36 resulted in higher free fatty acids after 140 days at all temperatures. No significant changes
37 were detected in the oils' iodine values and fatty acid compositions. The tocopherol contents
38 decreased significantly at both 13 °C and 25 °C after 60 days in the oil from SE method, and
39 after 120 days in oils from AEE-based methods.

40

41 CONCLUSION

42 These findings are significant in highlighting the extraction method resulting in crude MO
43 kernel oil with greatest oxidative stability in the storage conditions tested. Subsequently, the
44 suitable storage condition of the oil prior to refining can be determined. Further studies are
45 recommended in determining the suitable refining processes and parameters for the MO
46 kernel oil prior to application in variety food products.

47

48 **Keywords** horseradish, drumstick, murungai, seed, lipid, fat

49

50 Introduction

51

52 *Moringa oleifera* (MO), also known as horse-radish, kelor, or drumstick tree, are widely
53 distributed in Pakistan, India, Nigeria, Philippines, Kenya, Caribbean Island, Cambodia,
54 Malaysia, and Bangladesh. One of the important parts of the tree is its fruit pods which turn
55 into brown color upon maturation and contain mature brown MO seeds. The kernels inside
56 the mature seeds contain the edible MO oil, also known as Ben or Behen oil ^[1-9]. This oil is
57 applicable in perfume industry, hair-care products, in medicinal practices, and act as a good
58 lubricant for fine machineries ^[7]. As an edible oil, MO oil is generally applied as culinary and
59 salad oil in Haiti and other countries ^[10, 11]. Tsaknis and Lalas (2002) ^[12] and Abdulkarim et
60 al. (2007) ^[13] concluded the suitability of MO oil for frying purpose. The fatty acid
61 composition of MO oil resembles that of olive oil, with high oleic acid content in addition to
62 the significant tocopherols content ^[7, 12, 14]. These properties contributed to the oil's oxidative
63 stability ^[6], and consumption of oleic acids was always related to reduced-risk of developing
64 coronary heart disease ^[13, 15].

65 A number of techniques are available for oil extraction from MO kernels, which
66 include solvent extraction (SE) and aqueous enzymatic extraction (AEE) methods, among
67 others. The AEE of oil from oil-bearing materials was reviewed by Mat Yusoff et al. (2015)
68 ^[16] and Rosenthal et al. (1996) ^[17], while studies specifically on AEE of MO oil have been
69 reported by Mat Yusoff et al. (2016) ^[18], Latif et al. (2011) ^[19], and Abdulkarim et al. (2005,
70 2006) ^[4, 20]. According to these studies, MO kernel cells contain approximately 35% (w/w)
71 protein and 40% (w/w) fat content, and the protein is the major component in the MO cell
72 wall surrounding the oil. These findings proved the need to add a proteolytic enzyme to
73 hydrolyze the protein component for higher oil release. At the end of an AEE process, two
74 types of oils are produced – free oil which can be recovered, and emulsified oil in a cream
75 emulsion which is formed due to the use of water as an extraction medium in the presence of

76 MO kernel protein. The earlier studies only succeeded in extracting up to 70% (w/w) total
77 MO oil – measured as the mass of oil extracted using enzymes against the total oil extracted
78 using solvent.

79 Similar issues of lower extracted oil in an AEE process as compared to the use of
80 solvent were also reported in most studies involving other types of oils as reviewed by Mat
81 Yusoff et al. (2015) ^[16]. Many studies attempted to overcome this problem by conducting a
82 pre-treatment in order to assist cells rupture for higher oil release in the following AEE
83 process. One of the pre-treatments conducted was the use of high-pressure processing (HPP)
84 on soybean seeds which resulted in 3.20% (w/w) and 1.30% (w/w) higher free oil recovery at
85 200 MPa and 500 MPa, respectively. In the case of MO kernels, Mat Yusoff et al. (2017) ^[21]
86 reported that the HPP pre-treatment successfully modified the MO protein structure into a
87 form of less emulsifying ability, thus smaller amount of oil got emulsified which resulted in
88 thinner cream emulsion layer and 4.19% (w/w) higher free oil recovery as compared to the
89 use of AEE alone ^[21]. Additionally, the study also reported 4.98% (w/w) increase in free oil
90 recovery when the AEE was pre-treated with boiling (100 °C, 5 min) – this boiling pre-
91 treatment was conducted in earlier studies ^[4, 18, 19, 20] prior to an AEE of MO oil to inactivate
92 the natural hydrolytic enzymes in the seed kernels ^[22].

93 Fotouo et al. (2016) ^[23] demonstrated the effect of storage of MO seeds on the
94 quantity and quality of the MO oil for its potential in biodiesel production. However, the
95 effect of storage of MO oil at different temperatures on its quality attributes had never been
96 reported, which is of great importance in determining the shelf life of the oil. Furthermore, to
97 the best of our knowledge, the study on HPP pre-treatment prior to AEE of MO oil was
98 conducted for the first time ^[21], and no study has reported on the quality attributes of the MO
99 oil extracted from this process.

100 The main objective of this study was to further explore the differences, particularly in
101 oxidative properties of MO oil from different extraction methods - the solvent extraction
102 (SE), aqueous enzymatic extraction (AEE), AEE with boiling pre-treatment (B-AEE), and
103 AEE with high-pressure processing pre-treatment (HPP-AEE). The MO oils were stored for
104 140 days at different temperatures of 13 °C, 25 °C, and 37 °C, and changes in the oxidative
105 properties were evaluated during the storage period in terms of their peroxide value (PV), *p*-
106 Anisidine value (*p*-AV), total oxidation (TOTOX) value, free fatty acids (FFA), iodine value
107 (IV), fatty acid composition (FAC), and tocopherol content.

108

109 **Materials and Methods**

110

111 **Materials**

112

113 Mature MO seeds (PKM1 hybrid) were purchased from Genius Nature Herbs Pvt. Ltd.,
114 Coimbatore, India. All solvents and enzymes used in this study were purchased from Sigma-
115 Aldrich Company Ltd., Dorset, UK.

116

117 **Statistical Analysis**

118

119 All statistical analyses in this study were done by using Minitab® 14.12.0 Statistical
120 Software. A one-way analysis of variance (ANOVA) with Tukey's multiple comparison test
121 (confidence level 95.0%) was applied for the determination of significant differences between
122 more than two samples (each sample with replicates data). A 2-Sample t-test was used to
123 determine significant differences between two samples (replicates data for each sample),

124 while a 1-Sample t-test was used when a sample (with replicates data) was statistically
125 compared with another sample which has one datum only.

126

127 **Preparation of *Moringa oleifera* Kernels for Oil Extraction**

128

129 The MO kernels were randomly collected and conditioned at 50 °C for 8 hr followed by
130 grinding (De'Longhi KG49 Electric Coffee Grinder, Hampshire, UK) and sieving using a
131 vibratory sieve shaker (Fritsch, Analysette 3E) to $\leq 710 \mu\text{m}$ particle size. According to Mat
132 Yusoff et al. (2016) ^[18], the use of ground-sieved MO kernels at this particle size resulted in
133 highest amount of MO oil (410.3 g kg^{-1}) as extracted using hexane. All oil extraction methods
134 conducted in this study were based on studies done by Mat Yusoff et al. (2016, 2017) ^[18, 21]
135 and were performed on the ground-sieved MO kernels.

136

137 **Solvent Extraction (SE) Method**

138

139 Soxhlet method was used to extract the MO oil with the use of hexane for 6 hr extraction
140 time. A total of six refluxes were used each time. The hexane was evaporated from the
141 extracted oil in a round bottom flask of pre-determined weight by using a rotary evaporator
142 ($60 \text{ }^\circ\text{C}$, 10 min), followed by heating in an oven ($100 \text{ }^\circ\text{C}$, 15 min). The difference between the
143 initial (empty) and final (containing the extracted oil) weight of the round bottom flask used
144 was measured as the oil yield in the meal by normalizing this against the weight of the
145 kernels taken initially.

146

147 **Aqueous Enzymatic Extraction (AEE) Method**

148

149 A mixture of ground-sieved MO kernels and distilled water at 1:4 (w/w) ratio was prepared
150 and adjusted to pH 6.0. A mixture of 2% (g enzyme / g kernel) of protease (Neutrase 0.8L,
151 optimum pH 6.8) and cellulase (Celluclast 1.5L, optimum pH 4.8) enzymes at 3:1 (w/w) ratio
152 was added into the mixture, followed by incubation at 50 °C for 12.5 hr at 300 stroke/min
153 shaking speed. The incubated mixture was centrifuged at 4000 rev/min for 20 min which
154 induced separation into four distinct layers of free oil at the top, followed by the cream
155 emulsion layer, the aqueous phase, and the meal at the bottom. Recovery of the free oil is
156 explained in section 'Recovery of free oil'.

157

158 **Aqueous Enzymatic Extraction with Boiling Pre-treatment (B-AEE)**

159

160 Similar AEE as in the previous section was conducted, with addition of boiling pre-treatment
161 in a water bath (100 °C, 5 min), followed by cooling to room temperature prior to adjusting
162 the mixture's pH, Recovery of the free oil is explained in section 'Recovery of free oil'.

163

164 **Aqueous Enzymatic Extraction with High Pressure Processing Pre-treatment (HPP- 165 AEE)**

166

167 The ground-sieved MO kernels were mixed with distilled water at 1:1 (w/w) ratio and
168 vacuum sealed in polyethylene bags. According to Mat Yusoff et al. (2017) ^[21], some
169 preliminary tests were carried out to determine this solid-to-liquid ratio. The use of smaller
170 amount of water caused formation of a very thick paste which adhered to the polyester bag,
171 thus wasted some of the sample. In another way, addition of higher water content resulted in
172 a very dilute mixture which thus allowed only small amount of ground-sieved MO kernel to
173 be processed at one time.

174 The mixtures at 1:1 (w/w) ratio were treated with high pressure (Stansted Fluid
175 Powder Ltd., Stansted, UK) at 50 MPa and 60 °C for 35 min, followed by addition of distilled
176 water up to 4:1 water/kernel (w/w) ratio for the subsequent AEE as in the earlier section.
177 Recovery of the free oil is explained in section ‘Recovery of free oil’.

178

179 **Recovery of Free Oil**

180

181 The centrifuged mixtures obtained in all the AEE-based methods explained earlier were kept
182 at -20 °C for 24 h. The solidified oil was transferred to a crucible of pre-determined weight
183 and heated in an oven at 100 °C for 15 min to ensure complete removal of any aqueous phase
184 that may present in the recovered oil. The crucible containing the oil was cooled to room
185 temperature in a desiccant containing silica gel for approximately 10 min before being
186 weighed. The free oil yield and recovery were calculated as follows:

187

$$188 \text{ Oil yield (\%)} = \frac{[\text{Mass of crucible containing the oil (g)} - \text{Mass of crucible (g)}] \times 100}{\text{Mass of kernels initially taken (g)}}$$

189

$$190 \text{ Free oil recovery (\%)} = \frac{\text{Mass of oil extracted from a given mass of kernel (g)} \times 100}{\text{Mass of oil contained in the kernels initially taken (g)}}$$

191

192 **Storage of *Moringa oleifera* Oil**

193

194 MO oil samples extracted using the SE method and all AEE-based methods explained in
195 previous sections were filled in transparent glass bottles with screw-caps, up to the bottle's
196 neck in order to minimize the headspace. The bottles were wrapped in aluminium foil and
197 stored in dark to avoid light exposure. The storage temperatures used in this study were in

198 reference to Pristouri et al. (2010) [24]. At 13 °C, the MO oil was stored to simulate the
199 temperature of the cellar commercially used for storing olive oil. Storage at 25 °C and 37 °C
200 were selected for simulating room temperature and elevated ambient temperature normally
201 occurred during the summer, respectively. All oil samples from different extraction methods
202 were stored in these temperatures for 140 days, and the analysis of their PV, *p*-AV, TOTOX,
203 FFA, and tocopherol content were performed on day 0, day 60, day 120, and day 140.
204 Additionally, differences in the IV and FAC between the extracted oils before (i.e. day 0) and
205 after the whole storage period (i.e. day 140) were also examined. Determination of these
206 oxidative properties was performed on each oil sample in triplicate.

207

208 **Determination of Peroxide Value (PV)**

209

210 Peroxide value (PV) of the oil samples was determined in reference to AOCS Official
211 Method Cd 8-53 (2000) [25] and MPOB Test Method p2.3 (2004) [26] with modification. A
212 mixture of 5.0 g oil sample and 20 ml glacial acetic acid/chloroform (1.5:1 v/v) was prepared
213 and swirled until completely dissolved. Excess of saturated potassium iodide solution was
214 added to the mixture, followed by swirling for 1 min. The mixture was combined with 30 ml
215 distilled water and few drops of starch indicator, before being titrated with 0.01 N sodium
216 thiosulphate until the blue-gray color disappeared. The above steps were repeated without
217 adding the oil sample for blank purpose. The following formula was used to calculate the PV
218 of the oil sample:

219

$$220 \text{ Peroxide value } \left(\text{mEq } \frac{\text{O}_2}{\text{kg}} \right) = \frac{(V_b - V_s) \times 0.01 \times 1000}{W}$$

221

222 V_b = Titre for blank (ml)

223 V_s = Titre for sample (ml)

224 W = Weight of sample (g)

225 0.01 = Normality of titrant (N)

226 1000 = Unit conversion (g/kg)

227

228 **Determination of *p*-Anisidine Value (*p*-AV)**

229

230 *p*-Anisidine value (*p*-AV) of oil samples was slightly modified according to AOCS Official

231 Method Cd18-90 (2000) [25]. An oil sample of 0.5 g was weighed into a 25 ml volumetric

232 flask and topped up with isooctane. The absorbance of the oil-isooctane solution (A_1) was

233 determined at 350 nm against isooctane (blank 1) (Cecil CE 1021 UV/Visible

234 Spectrophotometer 1000 series). 5 ml of the oil-isooctane solution was transferred into a 10

235 ml glass bottle (with screw cap), added with 1 ml of anisidine reagent (0.25% w/v anisidine

236 reagent in glacial acetic acid), shook vigorously, and kept in dark for 10 min. Similarly, 5 ml

237 of isooctane in a glass bottle was also added with 1 ml anisidine reagent, shook vigorously,

238 and kept in dark for 10 min (blank 2). The absorbance of the oil-isooctane containing

239 anidisine reagent (A_2) was determined at 350 nm against blank 2. The *p*-AV was calculated

240 as follow:

241

$$242 \quad p - \text{Anisidine value} = \frac{25 \times (1.2A_2 - A_1)}{W}$$

243

244 A_1 = Absorbance of the oil-isooctane solution

245 A_2 = Absorbance of the oil-isooctane containing anidisine reagent

246 25 = Volume of which the oil sample is dissolved with isooctane (ml)

247 1.2 = The correction factor for the dilution of the test solution with 1 ml of the anisidine

248 reagent or glacial acetic acid

249 W = Weight of sample taken (g)

250

251 **Determination of Total Oxidation (TOTOX) Value**

252

253 Total oxidation (TOTOX) value of the oil samples was determined according to AOCS

254 Official Method Cg 3-91 (2000) [25]. This value takes into account both the PV and *p*-AV of

255 the oil sample and calculated according to the following formula: TOTOX value = 2PV + *p*-

256 AV

257

258 **Determination of Free Fatty Acids (FFA)**

259

260 Free fatty acids (FFA) of the oil samples was calculated from its acid value (AV) which was

261 determined in accord to AOCS Official Method Cd 3d-63 (2000) [25]. An oil sample of 0.5 g

262 was added to 50 ml of a mixture of diethyl ether and ethanol (95% v/v) in a 250 ml conical

263 flask. Phenolphthalein was added as an indicator, followed by titration on the whole mixture

264 with 0.1 N potassium hydroxide (KOH) solution. The whole steps were repeated without

265 adding the oil sample for blank purpose. The AV of the oil was calculated as follows:

266

$$267 \text{ Acid value (KOH g}^{-1}\text{)} = \frac{(V_b - V_s) \times 5.61}{W}$$

268

269 V_b = Titre for blank (ml)

270 V_s = Titre for sample (ml)

271 W = Weight of sample (g)

272 5.61 = Mass (mg) of KOH in 1 ml of 0.1 N solution

273 Free fatty acids, FFA (%) = Acid value/1.99; where 1.99 is the conversion factor for oleic
274 acid

275

276 **Determination of Iodine Value (IV)**

277

278 Iodine value (IV) of the oil samples was determined according to MPOB Test Method p3:2
279 (2004) ^[26] and AOCS Official Method Cd 1d-92 (2000) ^[25] with slight modification. Earlier
280 studies revealed IV of MO oil which ranged from 60-70 g I₂ / 100 g ^[7, 14, 19]. Thus, an oil
281 sample of 0.2 g was used. The oil was weighed into a conical flask and added with 20 ml
282 chloroform and 25 ml Wijs reagent. A stopper was placed followed by vigorous shaking, and
283 the mixture was kept in dark for 30 min. Following this step was addition of 20 ml KI
284 solution (15% w/v KI in distilled water) and 100 ml distilled water. The mixture was titrated
285 under vigorous shaking with 0.1 M sodium thiosulphate until the yellow colour due to iodine
286 has almost disappeared. Few drops of starch were added afterwards, and the titration was
287 continued until the blue colour just disappeared after very vigorous shaking. The whole steps
288 were repeated without adding the oil sample for blank purpose. The IV was calculated based
289 on the following formula:

290

$$291 \text{ Iodine value } \left(\text{g } \frac{\text{I}_2}{100 \text{ g}} \right) = \frac{0.1269 \times 0.1 \times (V_b - V_s) \times 100}{W}$$

292

293 V_b = Titre for blank (ml)

294 V_s = Titre for sample (ml)

295 W = Weight of sample (g)

296 0.1 = Normality of titrant (N)

297 0.1269 = Mass of iodine in 1 ml of 1 M solution

298

299 **Determination of Fatty Acid Composition (FAC)**

300

301 Fatty acid composition (FAC) of MO oil was determined according to Ezech et al. (2016) ^[27],
302 Agilent Technologies, and *TraceCERT*® (Supelco®) with slight modification. Gas
303 Chromatography (GC, Agilent HP 6890) fitted with flame ionization detector (FID) was used
304 for the analysis, with fused silica capillary column Varian CP-Sil 88 (60 m x 0.25 mm x 0.20
305 µm) and helium as a carrier gas (flowrate 1.0 ml/min). The oil sample was first converted
306 into fatty acids methyl esters (FAME) by dissolving 100 mg oil into 10 ml hexane and added
307 with 100 µl of 2N KOH in methanol (i.e. 11.2 g KOH in 100 ml methanol). The mixture was
308 vortexed for 30 s, centrifuged, and the clear supernatant at the upper layer was transferred
309 into an autosampler vial. The injector and detection temperatures were 250 °C and 260 °C,
310 respectively, while the oven temperature was 230 °C (hold 30 min). The volume of sample
311 injected was 1 µl with split ratio of 100:1. The standard reference used was the Supelco 37
312 Component FAME Mix (1x1ml at varied concentrations in dichloromethane). Identification
313 of the fatty acids was done by comparing retention times with those of standards.

314

315 **Determination of Tocopherol**

316

317 Following the method used by Ezech et al. (2016) ^[27] and Costa et al. (2010) ^[28] with slight
318 modification, the total tocopherols of the oil samples in this study was determined by HPLC-
319 UV system (Agilent 1200, Manchester, UK). The column used was a Nucleosil C-18-100
320 reverse phase column (25 cm x 4.6 mm i.d.) with a particle size of 5 µm (Macherey-Nagel,
321 Duren, Germany), while the mobile phase was a mixture of methanol:tetrahydrofuran:water
322 (67:27:6 v/v/v) at flowrate of 0.8 ml min⁻¹. An oil sample of 0.1 ml was diluted with 1 ml of

323 a mixture of isopropanol:chloroform (75:25 v/v). The mixture was homogenized and 10 μ l
324 was injected into the HPLC system at 25 °C and detection wavelength of 292 nm. The types
325 of tocopherols reported are the α - and γ -tocopherols, and their standard solutions were
326 prepared by dissolving in methanol at 0.02-1.0% (v/v) concentrations. Standard calibration
327 curve was obtained for each type of tocopherol, and identification of the tocopherols in the oil
328 samples was done by comparing their retention times with that of the standard solutions.

329

330 **Results and Discussion**

331

332 **Effect of storage condition on peroxide value, *p*-Anisidine value, and total oxidation** 333 **value of *Moringa oleifera* kernel oil**

334

335 In the following discussion, the following terms will be used: SE-oil, AEE-oil, B-AEE-oil,
336 and HPP-AEE-oil which indicate the MO kernel oil extracted using SE, AEE, B-AEE, and
337 HPP-AEE methods, respectively.

338 Oxidation of lipids takes place by a free radical chain mechanism which can be
339 explained in terms of initiation, propagation, and termination processes. These processes
340 generally comprised of complex sequences and overlapping reactions. Figure 1 revealed
341 changes in PV in the MO oil samples during storage at different temperatures of (a) 13 °C,
342 (b) 25 °C, and (c) 37 °C. PV indicates formation of peroxides and hydroperoxides resulted
343 from propagation reaction. This reaction describes the first oxidation stage involving
344 formation of hydroperoxides as fundamental primary products. These compounds do not have
345 significant effect on the oil's flavor deterioration. Propagation is also the most widely
346 oxidation reaction that takes place in an oil and fat ^[29-33]. According to Figure 1(a), on day 0,
347 there was no significant difference ($p > 0.05$) between PV of MO kernel oil extracted using

348 different methods (approximately 0.49 mEq O₂/kg). However, after 140 days storage at 13
349 °C, PV of the SE-oil, AEE-oil, and HPP-AEE-oil increased significantly ($p < 0.05$) (0.75-
350 0.98 mEq O₂/kg). These findings indicated that at 13 °C, oxidation reaction started to take
351 place in the oil samples after 60 days of storage in SE-oil and AEE-oil. The PV started to
352 increase later after 120 days in HPP-AEE oil, while the PV of B-AEE oil remained
353 unchanged. At 25 °C, Figure 1(b) shows significant increase ($p < 0.05$) in PV of SE-oil from
354 day 0 (0.49 mEq O₂/kg) to day-120 (2.19 mEq O₂/kg) as compared to other oil samples. At
355 37 °C (Figure 1(c)), faster increase in PV was observed in the SE-oil. On day-60, the PV of
356 SE-oil at 25 °C was 0.99 mEq O₂/kg, and the PV was significantly higher ($p < 0.05$) at 37 °C;
357 1.97 mEq O₂/kg. Furthermore, on day-120, the PV of SE-oil at 25 °C was 2.19 mEq O₂/kg,
358 and the PV was significantly higher ($p < 0.05$) at 37 °C; 2.96 mEq O₂/kg. These findings
359 revealed the significant effect of higher storage temperature in increasing the oxidation rate
360 of SE-oil as compared to other oil samples.

361 After 120 days at both 25 °C and 37 °C, the PV of SE-oil started to decrease which
362 indicated the start of initiation reaction. In this reaction, decomposition of hydroperoxides
363 into free radicals took place which is an endothermic reaction promoted by the higher
364 temperatures of 25 °C and 37 °C. Moreover, this thermal oxidation of unsaturated lipids is
365 normally an autocatalytic reaction and is metal-catalyzed. The SE-oil was a crude oil which
366 most likely contained trace metals. These trace metals are very difficult to be eliminated, thus
367 they may act as potent catalysts which catalyzed the initiation reaction ^[33]. Figure 1 (a-c) also
368 shows that the B-AEE-oil exhibited higher oxidative stability upon 140 storage days at 13 °C,
369 since its PV did not increase at this temperature as compared to other oil samples. On the
370 other hand, the maximum PV of HPP-AEE-oil was 0.75 mEq O₂/kg under all storage
371 condition.

372 Earlier studies also reported higher PV of MO oil extracted using solvents (0.94-1.83
373 mEq O₂/kg) as compared to cold-pressed oil (0.11-0.36 mEq O₂/kg) [7, 12]. According to
374 O'Brien (2009) [29], the quality of SE-oil may be lower than that of pressed oil due to
375 simultaneous extraction of non-triglycerides and other undesirable minor components in the
376 former case. Therefore, in this study, the SE-oil exhibited higher PV as compared to other
377 extraction methods. The non-triglycerides and other minor components include fatty acids,
378 phosphatides, sterols, tocopherols, hydrocarbons, colorants, pigments, vitamins, sterol
379 glucosides, protein fragments, glycolipids, traces of pesticides, trace metals, resinous, and
380 mucilagenous materials [29, 34]. A product with PV of 1-5 mEq O₂/kg is categorized as
381 exhibiting low oxidation rate, followed by PV of 5-10 mEq O₂/kg as moderate oxidation rate,
382 while a product with PV of higher than 10 mEq O₂/kg is considered as having high oxidation
383 rate [30, 31]. Moreover, according to Codex (1999) [35], the maximum PV for refined oil is 10
384 mEq O₂/kg, while for cold pressed and virgin oils, the maximum PV is 15 mEq O₂/kg.
385 Despite the high PV of SE-oil, all values in all storage conditions were less than 3 mEq
386 O₂/kg. Thus, in terms of PV, the MO kernel oil samples from SE and enzymatic extraction
387 methods are categorized as oil samples with low oxidation rate within the storage conditions
388 used.

389 With reference to Figure 1(c) at 37 °C, the PV of SE oil started to decrease after 120
390 days which indicated decomposition of primary oxidation products into secondary oxidation
391 products as explained earlier, besides indicating the faster oxidation reaction in the SE-oil as
392 compared to other oil samples. This phenomenon is reinforced by the sudden increase in the
393 *p*-AV of SE oil up to 10.72±1.41. The *p*-AV represents the formation of secondary oxidation
394 products in the form of 2-alkenals and 2,4-alkadienals. In this same storage condition, the *p*-
395 AV of other oil samples remained as low as 0.70-1.44. Latif et al. (2011) [19] reported
396 approximately similar *p*-AV of MO kernel oil extracted using solvent and enzymes which

397 ranged from 1.60-1.92. An oil is considered as having good quality if its *p*-AV is less than
398 10.0 according to Rossell (1989) ^[36], or less than 2.0 according to Subramaniam et al. (2000)
399 ^[37]. Therefore, to conclude, SE-oil is considered unacceptable after 120 days of storage at 37
400 °C due to the high *p*-AV, and TOTOX value of 16.64.

401

402 **Effect of storage condition on free fatty acids of *Moringa oleifera* kernel oil**

403

404 FFA is responsible for the off-flavor and off-odor in fats and oils products ^[29, 38]. Prolong
405 storage time causes decomposition and oxidation of secondary oxidation products into FFA
406 as tertiary oxidation product ^[31]. In crude vegetable oils, improper-stored or field-damaged
407 seeds contribute to abnormally high FFA level. Lipases and other enzymes in seeds and fruits
408 are activated in the presence of moisture which initiates a hydrolysis reaction, causing
409 formation of FFA ^[29].

410 Figure 2 shows the FFA (as oleic acid) of oil samples stored at different temperatures
411 of (a) 13 °C, (b) 25 °C, and (c) 37 °C. On day 0, the SE-oil exhibited significantly higher (*p* <
412 0.05) FFA (2.02±0.14%) than the B-AEE-oil (1.09±0.32%). Abdulkarim et al. (2005) ^[4] also
413 reported higher FFA in SE-oil (2.48%) as compared to B-AEE-oil (1.13%). The higher FFA
414 in SE-oil may be due to simultaneous extraction of other non-triglycerides and minor
415 components by the solvent which also contributed to higher PV as explained earlier.

416 Additionally, on day 0, the B-AEE-oil exhibited nearly 4 times lower FFA
417 (1.09±0.32%) than the AEE-oil (3.85±0.26%). This finding proved the importance of boiling
418 pre-treatment on the ground MO kernels to inactivate hydrolytic enzymes prior to oil
419 extraction. Along the storage period at all temperatures, the FFA in B-AEE-oil remained at
420 low level of 0.96-1.71%, while the FFA in AEE-oil decreased to 1.34-2.08%. These values
421 were significantly lower (*p* < 0.05) as compared to the FFA in HPP-AEE-oil which

422 significantly increased ($p < 0.05$) from day 0 to day 140 at all temperatures of 13 °C
423 (6.66±0.19%), 25 °C (7.19±0.32%), and 37 °C (5.96±0.12%). These significantly higher FFA
424 in HPP-AEE-oil samples were most likely due to possible presence of minute moisture
425 content in the oil.

426 According to O'Brien (2009) ^[29], presence of moisture in combination with high
427 pressure may results in acceleration of hydrolytic reaction, therefore resulted in higher FFA
428 as compared to other enzymatic extraction methods in this study. Increase in FFA was also
429 observed in the SE-oil along the storage period, yet was still lower (2.47-3.62%) than that of
430 HPP-AEE-oil samples.

431 Codex (1999) ^[35] indicates maximum FFA level in oil in terms of its AV which is 0.6
432 mg KOH / g for refined oils, 4.0 mg KOH / g for cold pressed and virgin oils, and 10.0 mg
433 KOH / g for virgin palm oils. In this study, highest FFA was observed in HPP-AEE-oil on
434 day 140: 13.26 mg KOH / g (13 °C), 14.30±0.64 mg KOH / g (25 °C), and 11.86 mg KOH / g
435 (37 °C). The B-AEE-oil exhibited lowest AV of below 4.0 mg KOH / g throughout the
436 storage conditions (1.34-3.40 mg KOH / g). These findings further highlighted the significant
437 effect of boiling pre-treatment in inactivating the hydrolytic enzymes, prevents enzymatic
438 hydrolysis, and thus preserving the oil's oxidative stability.

439

440 **Effect of storage condition on iodine value and fatty acid composition of *Moringa***

441 ***oleifera* oil**

442

443 There was no significant difference ($p > 0.05$) in IV of all oil samples from all extraction
444 methods on day 0 (58-65 g I₂ / 100 g) and after 140 days (54-60 g I₂ / 100 g) at both 25 °C
445 and 37 °C. Abdulkarim et al. (2005) ^[4] also reported similar IV of SE-oil (65.4 g I₂ / 100 g)
446 and B-AEE-oil (66.1 g I₂ / 100 g). Additionally, there was no difference in IV between SE-oil

447 (66.6-66.8 g I₂ / 100 g) and cold-pressed oil (66.8 g I₂ / 100 g) in a study done by Tsaknis et
448 al. (1999)^[7]. These findings indicated that the MO oil did not undergo severe changes in
449 degree of unsaturation within the storage conditions used, despite the production of oxidation
450 products in certain oil samples as explained earlier.

451 These outcomes are also reflected by insignificant changes in FAC of the oil samples
452 (Table 1(a-d)) at all storage temperatures. All oil samples consist of up to 76% oleic acid
453 (C18:1) which contributes to the oil's oxidative stability and is related to reduced risk of
454 developing coronary heart disease^[13, 15]. Additionally, the oil samples consist of up to 6.60%
455 behenic fatty acid (C22:0) in all storage conditions, thus suits its other names as Ben or
456 Behen oil as described in the Introduction.

457

458 **Effect of storage condition on α -tocopherol content in *Moringa oleifera* oil**

459

460 Figure 3 shows the α -tocopherol content in oil samples stored at different temperatures of (a)
461 13 °C, (b) 25 °C, and (c) 37 °C. On day 0, highest α -tocopherol content was discovered in B-
462 AEE-oil (31.17±3.52 mg/l) which was insignificantly different ($p > 0.05$) with the AEE-oil
463 (28.04±1.26 mg/l) and HPP-AEE-oil (28.77±1.05 mg/l). As compared to these enzymatic
464 extraction methods, significantly lower ($p < 0.05$) α -tocopherol content was observed in SE-
465 oil (23.33±0.99 mg/l). In a study done by Tsaknis et al. (1999)^[7] using MO seed kernels of
466 Kenya origin, the α -tocopherol content in the oil samples were similar in the case of solvent
467 (98-105 mg/kg) and cold press (101.46 mg/kg) methods. With the use of MO seed kernels of
468 Bangladesh origin, Rahman et al. (2009)^[14] also revealed as high as 121-154 mg/kg α -
469 tocopherol content in the oil extracted using different types of solvents. In another study done
470 by Tsaknis and Lalas (2002)^[12] on seed kernels of India origin, the SE-oil contained higher
471 α -tocopherol (15.38 mg/kg) as compared to cold-pressed oil (5.06 mg/kg). To summarize,

472 regardless of the extraction methods, the α -tocopherol contents reported in this present study
473 on day 0 (23.33-31.17 mg/l) and those reported by Tsaknis and Lalas (2002) ^[12] (5.06-15.38
474 mg/kg) were far too low than that of reported by Tsaknis et al. (1999) ^[7] (98-105 mg/kg) and
475 Rahman et al. (2009) ^[14] (121-154 mg/kg). These findings highlighted variations in the MO
476 seed kernels of different origins which resulted in different oil properties. Besides α -
477 tocopherol, earlier studies reported presence of γ - and δ -tocopherols in MO kernel oils
478 extracted using solvents, enzymes, cold press, and supercritical fluid extraction method ^{[6, 7, 12,}
479 ^{14]}, yet the values varied significantly. In this study, the tocopherols reported are the α - and γ -
480 tocopherols only, due to low amount of δ -tocopherol detected.

481 Presence of higher oxidation products in SE-oil as indicated by increased in its PV, *p*-
482 AV, and TOTOX as compared to enzymatic extraction methods was reflected by significant
483 decrease ($p < 0.05$) in the oil's α -tocopherol content during storage. On day 60, the lowest α -
484 tocopherol content was detected in SE-oil at 37 °C (18.14±1.24 mg/l) as compared to 13 °C
485 (29.51±0.75 mg/l) and 25 °C (25.60±2.24 mg/l). Greatest effect of storage temperature took
486 place on day 120 where the α -tocopherol content in SE-oil decreased with temperature
487 increased from 13 °C (27.81±0.89 mg/l) to 25 °C (7.89±0.14 mg/l). On day 140, the α -
488 tocopherol content in SE-oil was not significantly affected ($p > 0.05$) by the storage
489 temperatures, yet highest α -tocopherol content was detected at 37 °C in AEE-oil (31.22±1.73
490 mg/l), B-AEE-oil (28.79±3.56 mg/l), and HPP-AEE-oil (32.86±0.56 mg/l) as compared to
491 storage at lower temperatures of 13 °C (14-19 mg/l) and 25 °C (14-16 mg/l). The reason
492 behind this finding is not yet been understood.

493

494 **Effect of storage condition on γ -tocopherol content in *Moringa oleifera* oil**

495

496 Figure 4 shows the γ -tocopherol content in oil samples stored at different temperatures: (a) 13
497 °C, (b) 25 °C, and (c) 37 °C. In this study, all MO oil samples exhibited lower γ -tocopherol
498 content as compared to α -tocopherol. On day 0, all extraction methods resulted in oil samples
499 with approximately similar γ -tocopherol content: 14.74±1.29 mg/l (SE), 12.79±1.26 mg/l
500 (AEE), 15.32±1.57 mg/l (B-AEE), and 13.84±0.97 mg/l (HPP-AEE). Differently, Tsaknis et
501 al. (1999) [7] reported higher γ -tocopherol content in cold-pressed oil (39.54 mg/kg) than that
502 of SE-oil (27.90-33.45 mg/kg) with the use of MO seed kernels of Kenya origin. Tsaknis and
503 Lalas (2002) [12] also reported higher γ -tocopherol content in cold pressed-oil (25.40 mg/kg)
504 as compared to SE-oil (4.47-5.52 mg/kg) from MO seed kernels of India origin. In a study
505 done by Rahman et al. (2009) [14] using seed kernels of Bangladesh origin, different types of
506 solvents resulted in oil samples with approximately similar γ -tocopherol content (62.2-77.4
507 mg/kg). To conclude, similar with α -tocopherol, the γ -tocopherol content varied in MO oil
508 samples from seed kernels of different origins and is also dependent on extraction methods
509 used.

510 On day 140, the γ -tocopherol content in all oil samples was significantly higher ($p <$
511 0.05) at 37 °C (12.48-16.07 mg/l) as compared to lower storage temperatures of 13 °C (7.76-
512 9.61 mg/l) and 25 °C (7.58-8.20 mg/l). The reasons for this sudden increment was not
513 identified. In the SE-oil, this trend was different from that of α -tocopherol which did not
514 change upon different storage temperatures on day 140.

515 At 13 °C, the γ -tocopherol content in all oil samples from all extraction methods
516 decreased significantly ($p < 0.05$) after 120 days. Similarly, at 25 °C, the γ -tocopherol content
517 in oil samples from enzymatic extraction methods decreased after 120 days, while in SE-oil,
518 the γ -tocopherol content started to decrease earlier which was after 60 days. At 37 °C,
519 insignificant ($p > 0.05$) decrease in γ -tocopherol content in SE-oil was observed, which was
520 different from significant decrease ($p < 0.05$) in the case of α -tocopherol in the same storage

521 condition. In overall at 37 °C, the storage time imparted no significant changes in the γ -
522 tocopherol content in oil samples from all extraction methods.

523

524 **Conclusions**

525

526 In most MO oil samples, changes in oxidative properties and tocopherol contents started to
527 take place after 120 days of storage, and the rate of changes increased with increased in
528 temperature. The SE-oil underwent greater oxidative deterioration as compared to other AEE-
529 based oils. The SE-oil was not in good quality after 120 days at 37 °C, while it is still
530 acceptable during storage at 13 °C up to 140 days of storage. The AEE-based oils exhibited
531 approximately similar oxidative properties throughout the whole storage conditions, except in
532 the case of HPP-AEE-oil which exhibited high FFA content after 120 days, even at as low as
533 13 °C storage temperature. This may be due to the high pressure applied which caused
534 acceleration of hydrolytic reaction. On the other hand, the boiling pre-treatment was
535 necessary to inactivate the hydrolytic enzymes in the seed kernels for better oil quality during
536 storage. Thus, to conclude, within the storage conditions tested, B-AEE-oil exhibited greatest
537 oxidative properties, followed by the AEE-oil, HPP-AEE-oil, and the SE-oil. No significant
538 changes occurred in IV of all oil samples, indicating no changes in their degree of
539 unsaturation throughout the storage condition. After 140 days at 37 °C, the concentration of
540 both α - and γ -tocopherols in all oil samples were nearly two times higher than their
541 concentrations at lower temperatures, and the reasons for this finding is not yet discovered.
542 Both the boiling and HPP pre-treatments did not significantly affect the tocopherol contents
543 of the MO oil. Moreover, the AEE-based methods resulted in oils with better oxidative
544 properties as compared to the use of solvent. This advantage assists in minimizing refinery
545 loss and therefore should further be explored.

546

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553

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681
682

683 **Table Caption**

684
685 **Table 1** Fatty acid composition (%) of *Moringa oleifera* oil from (a) solvent (hexane)
686 extraction method (SE), (b) aqueous enzymatic extraction (AEE) method, (c) aqueous
687 enzymatic extraction method with boiling pre-treatment (B-AEE), and (d) aqueous enzymatic

688 extraction method with high pressure processing pre-treatment (HPP-AEE) on day 0 and after
689 140 days of storage at different temperatures. nd, not detected

690

691

692 **Figure Captions**

693

694 **Fig. 1.** Effect of different extraction methods on the peroxide values of *Moringa oleifera*
695 kernel oil stored for 140 days at different temperatures of (a) 13 °C; (b) 25 °C; and (c) 37 °C.
696 SE, solvent extraction; AEE, aqueous enzymatic extraction; B-AEE, aqueous enzymatic
697 extraction with boiling pre-treatment; HPP-AEE, aqueous enzymatic extraction with high
698 pressure processing pre-treatment.

699

700 **Fig. 2.** Effect of different extraction methods on the free fatty acids of *Moringa oleifera*
701 kernel oil stored for 140 days at different temperatures of (a) 13 °C; (b) 25 °C; and (c) 37 °C.
702 SE, solvent extraction; AEE, aqueous enzymatic extraction; B-AEE, aqueous enzymatic
703 extraction with boiling pre-treatment; HPP-AEE, aqueous enzymatic extraction with high
704 pressure processing pre-treatment.

705

706 **Fig. 3.** Effect of different extraction methods on the alpha tocopherol content in *Moringa*
707 *oleifera* kernel oil stored for 140 days at different temperatures of (a) 13 °C; (b) 25 °C; and
708 (c) 37 °C. SE, solvent extraction; AEE, aqueous enzymatic extraction; B-AEE, aqueous
709 enzymatic extraction with boiling pre-treatment; HPP-AEE, aqueous enzymatic extraction
710 with high pressure processing pre-treatment.

711

712 **Fig. 4** Effect of different extraction methods on the gamma tocopherol content in *Moringa*
713 *oleifera* kernel oil stored for 140 days at different temperatures of (a) 13 °C; (b) 25 °C; and
714 (c) 37 °C. SE, solvent extraction; AEE, aqueous enzymatic extraction; B-AEE, aqueous
715 enzymatic extraction with boiling pre-treatment; HPP-AEE, aqueous enzymatic extraction
716 with high pressure processing pre-treatment.

717

718

719