Characterization and toxicity of citral incorporated with nanostructured lipid carrier

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Nanoparticle as a cancer drug delivery vehicle is rapidly under investigation due to its promising applicability as a novel drug delivery system for anticancer agents. This study describes the development, characterization and toxicity studies of a nanostructured lipid carrier (NLC) system for citral. Citral was loaded into the NLC using high pressure homogenization methods. The characterizations of NLC-citral were then determined through various methods. Based on Transmission Electron Microscope (TEM) analysis, NLC-Citral showed a spherical shape with an average diameter size of 54.12 ± 0.30 nm and a polydipersity index of 0.224 ± 0.005. The zeta potential of NLC-Citral was -12.73 ± 0.34 mV with an entrapment efficiency of 98.9 ± 0.124%, and drug loading of 9.84 ± 0.041%. Safety profile of the formulation was examined via *in vitro* and *in vivo* routes to study its effects toward normal cells. NLC-Citral exhibited no toxic effects towards the proliferation of mice splenocytes. Moreover, no mortality and toxic signs were observed in the treated groups after 28 days of treatment. There were also no significant alterations in serum biochemical analysis for all treatments. Increase in immunomodulatory effects of treated NLC-Citral and Citral groups was verified from the increase in CD4/CD3 and CD8/CD3 T cell population in both NLC-citral and citral treated splenocytes. This study suggests that NLC is a promising drug delivery system for citral as it has the potential in sustaining drug release without inducing any toxicity.

1 **Characterization and Toxicity of Citral Incorporated With Nanostructured Lipid Carrier**

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35 **Abstract**

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37 applicability as a novel drug delivery system for anticancer agents. This study describes the development,

38 characterization and toxicity studies of a nanostructured lipid carrier (NLC) system for citral. Citral was

39 loaded into the NLC using high pressure homogenization methods. The characterizations of NLC-citral

40 were then determined through various methods. Based on Transmission Electron Microscope (TEM)

41 analysis, NLC-Citral showed a spherical shape with an average diameter size of 54.12 ± 0.30 nm and a

42 polydipersity index of 0.224 ± 0.005. The zeta potential of NLC-Citral was -12.73 ± 0.34 mV with an

43 entrapment efficiency of $98.9 \pm 0.124\%$, and drug loading of $9.84 \pm 0.041\%$.NLC-Citralexhibited no toxic

44 effects towards the proliferation of mice splenocytes. Moreover, no mortality and toxic signs were

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46 serum biochemical analysis for all treatments. Increase in immunomodulatory effects of treated NLC-

47 Citral and Citral groups was verified from the increase in CD4/CD3 and CD8/CD3 T cell population in

48 both NLC-citral and citral treated splenocytes. This study suggests that NLC is a promising drug delivery

49 system for citral as it has the potential in sustaining drug release without inducing any toxicity.

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56   **Introduction**

57   Exploring natural plant products as an avenue to discover novel chemical entities is one of the

58   fastest growing areas of pharmaceutical research, considering that bioactive agents are pivotal as

59   potential cures for various diseases. There have been more than one thousand plants identified to

60   possess significant anticancer properties (Cragg & Newman, 2005). Citral, with the molecular

61   formula $C_{10}H_{16}O$ is an aldehyde that is widely used in perfumes for its citrus scent. Citral has

62   been claimed to possess anti-inflammatory effects, as it inhibited NO production and suppresses

63   the activation of NF-kappa B in RAW264.7 cells (Jeong *et al.*, 2008). Treatment with citral also

64   decreased cell proliferation and altered the mitochondrial membrane potential resulting in

65   apoptotic induction of HeLa and ME-180 cervical cancer cell lines (Ghosh, 2013). Together,

66   these versatile characteristics of citral have attracted researchers to further exploit its increased

67   efficiency. However, despite its multi-beneficial effects citral remains an unstable molecule in

68   acidic environments and non-soluble in water (Weerawatanakorn *et al.,* 2015). Therefore, the

69   incorporation of citral into colloidal dispersions such as nano-emulsions in particular, could be

70   the best way to stabilize the poorly-soluble compound.

71

72   To increase the body fluid saturation solubility of poorly soluble compounds, a new delivery

73   system needs to be developed. The interest of using nanoparticles as a drug delivery system is

74   aimed to improve the bioavailability of drugs, increase the release time in the human body,

75   targeting the drug to its site of action to introduce its physiochemical stability of the drug

76   (Mudshinge, Deore, Patil, & Bhalgat, 2011). Different types of nanocarriers, including lipid

77   nanoparticles have been developed over time for this purpose. Lipid nanoparticles have many

78   desirable features which includes low toxicity, a biodegradable matrix, high capacity to

79   incorporate lipophilic and hydrophilic drugs, and controlled release properties of incorporated

80   drugs (Kasongo*et al.,* 2011). The nanostructured lipid carrier (NLC), a second generation of Solid

81   Lipid nanoparticle (SLN)confers all these properties and holds great promise to become an

82   excellent drug carrier system (Pardeshi *et al.,* 2012). NLC has greater flexibility for drug loading

83   and exhibits a reduced drug expulsion of the formulation over time that contributes towards long

84   term stability and better modulation of drug release (Almeida*et al*., 2014) as compared to SLN.

85   NLCs are considered as one of the most versatile generation of lipid nanoparticle as oral drug

86   delivery systems to treat cancer. Studies has reported that small particles of NLCs possessing a

87   particle size less than 200 nm have reduced uptake by the Kupffer cells in the liver and

88   prolonged circulation time in blood, allowing better uptake by tumors (Montenegro *et al*., 2011).

89   Additionally, NLC appears to be an attractive approach for the delivery of lipophilic cancer

90   drugs such as Tamoxifen and 5-Fluorouracil (Andalib *et al.,* 2012; How *et al*., 2013). With NLC,

91   the drug is expected to solubilize into the hydrophobicphase incorporated into the core of the

92   solid lipid. This could enhance the loading capacity, entrapment efficiency and also control the

93   drug release from the NLC in time. Thus far, there exist no reports which disclose the use of

94   nanostructured lipid carrier as an efficient carrier system to increase the solubility of citral and

95   the physicochemical properties.

96   One of the major concerns with a nanoparticle system is its inherent toxicity. The safety profiles

97   of citral has been studied on F344/N rat and B6C3F1 mice for two years and been concluded as a

98   non-carcinogenic compound (Ress *et al.*, 2003). The potential success of a nanoparticle system

99   relies on many factors, with one of it the minimum toxicity effects exhibited by the carrier itself.

100 Studies has reported that NLC induces no toxicity and no mortality in an acute toxicity study

101   using BALB/c mice (Rahman *et al*., 2014). Besides the extensive applicability of lipid-based

102   nanoparticle, the toxicity of this system has not been adequately studied thus far. In this present

103   study, combination of hydrogenated palm oil as a solid lipid whilst olive oil a liquid lipid was

104   chosen because this combination was tested to be the least cytotoxic on normal BALB/C mice

105   and 3T3 cells (How, Rasedee, & Abbasalipourkabir, 2013).

106   As a potential drug carrier, it is essential to examine the safety and toxicity of citral incorporated

107   into the NLC system to ensure its suitability for the future therapeutic usage of the formulation.

108   For these reasons, the aim of this present work was to determine the physicochemical properties

109   of NLC-citral and to assess the toxicological effects in BALB/c mice treated with NLC-citral.

110

111   **Materials and Method**

112

113   The materials used to synthesize the Nanostructured Lipid Carrier in this project were received

114   from the Institute of Bioscience, UPM without any alteration in the processing method.

115   Hydrogenated palm oil (HPO) was a gift from Institute of Bioscience (Selangor, Malaysia),

116   Lipoid S-100 (Merck Millipore, Germany), and Olive oil (Basso, Italy). Thimerosal, D-Sorbitol,

117   Tween-80, citral 95%, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum

118   (FBS), Thiazolyl Blue Tetrazolium Bromide were all purchased from Sigma-Aldrich (MO,

119   USA). All of the reagents used were of analytical grade except for olive oil.

120

121 **Preparation of nanostructured lipid carriers and loading of Citral**

122

123 The NLCs were prepared using a high-pressure homogenization technique. The synthesis method

124 of NLC loaded with citral was similar to a previous study with slight modifications (Rahman et

125 al., 2013a). The lipid phase was composed of hydrogenated palm oil (HPO), Lipoid S-100 and

126 Olive oil at a ratio of 7:3:3 that were mixed in a beaker and heated at 70°C.Five hundred mg of

127 citral was added into the lipid fraction with constant stirring (1000 rpm) for 5 minutes. Complete

128 yellowish in color mixture of drug was ensured before proceeding with the next step. For

129 aqueous phase, D-Sorbitol (4.75 % [w/v]), Thimerosal (0.005% [w/v]), and 1% Tween-80 were

130 dissolved in double-distilled water at 70°C and was then added into the lipid phase under

131 constant stirring   for 5 minutes. Further mixing was done by using a high speed stirring method

132 with Ultra-Turrax® (IKA/Staufen, Germany) at 13,000 rpm for 10 minutes. The pre-emulsion

133 mixture was then pressurized using a high pressure homogenizer (Avestin, Canada) at 1000 bar

134 for 15 cycles at 70°C. Finally the hot and clear nanoemulsion was sealed and allowed to cool

135 down at room temperature (27°C) for 24 hours to recrystallize and form the NLCs. The blank

136 NLC was formulated exactly with the same method but without the addition of citral.

137

138 **Zeta potential (ZP)**

139 The magnitude charges between the particles in the NLC were determined by using Zetasizer

140 Nano ZS (Malver, Germany). NLC was diluted 1:1 ratio with double distilled water

141 appropriately prior to measurements to get optimum of 50-200 kilo counts per second

142 (Thatipamula *et al.,* 2011).

Particle size

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144

145 The average particle size diameter and polydispersity index (PDI) of the NLC were analyzed by
146 using dynamic light scattering (DLS) integrated in a Zetasizer Nano ZS (Malvern Instrument,
147 Germany). The NLC solution was diluted at 1:1 ratio with double distilled water preceding the
148 measurement. Five independent measurements were obtained at 25°C with particle size and PDI
149 (size distribution) were calculated.

150

Transmission electron microscopy (TEM)

151

152

153 The NLC-Citral sample was dropped slowly on the surface of a copper grid coated with carbon
154 and then let to air drying for about 5 minutes. Negative staining was performed by using 1%
155 phosphotungstic acid then allowed for air drying for another 3 minutes. The sample was then
156 visualized with transmission electron microscopy (Hitachi H-7100, Japan).

157

Entrapment efficiency (EE) and drug loading (DL) capacity

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159

160 The amount of free drug in the sample was measured in order to determine the entrapment
161 efficiency and the drug loading capacity of the sample by using ultrafiltration method. This was
162 performed using Centisart filter tubes (Sartorious, Germany) with molecular weight cut off
163 300kDa. Five mL of NLC-citral was placed in the upper chamber of the tubes and then was

164 centrifuged using Eppendorf Centrifuge (Hamburg, Germany) at 15000 rpm for 15 minutes. The

165 foundation behind this technique is that citral filtered out to the bottom chamber is unbound to

166 the NLC while the citral remained in the top chamber were still bound as NLC-citral.

167 Subsequently, citral in the bottom chamber was quantified using UV-Vis spectrophotometer at

168 280 nm (Beckton Coulter, USA). The EE and DL percentage of the sample were calculated

169 based on the method previously reported (Rahman *et al.*, 2013).

170

171
$$EE(\%) = \frac{(Total\ amount\ of\ citral) - (Free\ amount\ of\ citral)}{(Total\ amount\ of\ citral)} x\ 100$$

172
$$DL(\%) = \frac{(Total\ amount\ of\ citral\ encapsulated\ into\ NLC)}{(Total\ amount\ of\ lipid\ used\ in\ NLC - citral\ formulation)} x\ 100$$

173

174 ***In vitro* drug release study**

175

176 The *in vitro* drug release study of NLC-citral was accomplished using a Franz diffusion cell

177 system (PermeGear, USA) with a receptor volume of 20.1 cm^3 and effective diffusion area of

178 4.9 cm^2. This study is essential to determine the suitability of the drug in the system and to check

179 the quality of the formulation. There were donor and receptor compartments in the system with

180 donor cell having the surface area of 20.1 cm^2. The release of the NLC-citral was studied for 48

181 hours. Firstly, the synthetic cellulose acetate membrane (Advantec, Japan) with pore size of 200

182 nm was soaked in the receiving media for 2 hours. Next, the membrane was inserted onto the

183 system and 500 μL of NLC-citral suspension were dispensed in the donor compartment (Salerno

184   *et al.,*2010). The receptor compartment was filled with the receiving medium (PBS + 2% SDS) at

185   pH 7.4. Throughout the study, the temperature of the system was maintained at 37°C under

186   constant stirring. At certain time intervals, 500 µL of sample was withdrawn from the medium

187   in receptor compartment by using a 3 mm^3 syringe needle and then refilled back by using the

188   fresh receiving medium. The samples were then analyzed by UV-Vis spectrophotometer at 280

189   nm (Beckman Coulter, USA) to determine the amount of citral released from the NLC. The

190   concentration of drug release was calculated based on the standard curve for pure citral ranging

191   from 100 µg/mL to 10 µg/mL. The experiment was conducted in triplicate and the drug release

192   data were evaluated by zero-order, first-order, and Higuchi equations to determine the release

193   kinetics (Rahman *et al.*, 2013).

194

195   ***In vitro* splenocyte viability (MTT)**

196

197   In brief, upon mice sacrifice, the spleen collected was washed with 5 mL of PBS. It was then

198   lysed with 2 mL lysis buffer (155 $mMNH_4Cl$, 0.1 M Na_2EDTA, 12 mM KH_2PO_4 at pH 7.4) for

199   10 minutes to remove the red blood cells. Next, the splenocytes were seeded at a density of

200   2×10^6 cells/mL in a 96-well plate. Cells were treated with seven different concentrations: NLC-

201   Blank, NLC-citral and citral starting from 0.8 µg/mLto 30 µg/mL for 24 hours of incubation in a

202   37°C incubator with 5% CO_2 (Memmert, USA). Then, 20 µL of MTT3-[4,5-dimethylthiazol-2-

203   yl]-2,5 diphenyltetrazolium bromide (5 mg/mL) (Merck, USA) solution was added into the cells

204   and incubated for another 4 hours in the incubator. The plate was spun down at 1500 x g for 5

205   minutes. Next, the supernatant was removed and 100 µL of dimethyl sulfoxide (DMSO) (Fisher

206   Scientific, USA) was added in to the plate and incubated for another 15 minutes at room

207   temperature. The plate was read with microplate reader at 570nm. *In vitro* splenocytes viability

208   assay was done according to the previous study (Abu *et al.,* 2015). The percentage of cells

209   viability was calculated based on the formula below:

210

211   $$\text{Viability (\%)} = \frac{\text{OD of sample}}{\text{OD of control}} x\ 100$$

212

213   **Animal**

214

215   Male BALB/c male mice aged seven weeks (20-22 g) were purchased from the Animal House of

216   Faculty of Veterinary, Universiti Putra Malaysia (UPM, Malaysia). This study was approved by

217   the Institutional Animal Care and Use Committee, Universiti Putra Malaysia (R098/2014). The

218   mice were randomly selected and grouped into 3 groups with five mice per group; NLC-Blank,

219   NLC-citral and citral. Fifteen mice were acclimatized in the laboratory environment for 7 days.

220   The mice were accommodated in polypropylene plastic cage with pellet and water provided *ad*

221   *libitum* during the period of study. The treatment of 50 mg/kg/day of NLC-citral , citral and

222   NLC-Blank were administered orally for 28 days following the method described earlier

223   (Mohamad *et al.,* 2015). The animals were observed for any toxic signs such as loss of weight,

224   shedding of fur or behavioral abnormalities after 28 days of study.

225

226 **Serum Biochemistry**

227

228 Blood samples were collected in a plain tube from the sacrificed mice by cardiac puncture. The

229 serum was obtained by centrifugation at 3000 rpm for 15 minutes. The concentration of aspartate

230 aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and

231 creatinine level in mice serum were analyzed accordingly using Hitachi automatic analyzer

232 (Hitachi-902, LTD, JAPAN).

233

234 *In vivo* **Immunophenotyping of Splenocytes**

235

236 Mice spleen obtained from the sacrificed mice were washed with Phosphate Buffered Saline

237 (PBS) and meshed through a 40 μm sterile filter with plunger. Next, the suspension was washed

238 with PBS and centrifuged at 2000 x g for 15 minutes to get the cells pellet. Then, the red blood

239 cell was lysed with 2 mL of lysis buffer (155 mMNH_4Cl, 0.1 M Na_2EDTA, 12 mM KH_2PO_4 at

240 pH 7.4) and incubated for 10 minutes in 4°C.After that, the suspension was again spun at 2000 x

241 g for 5 minutes. Cell was resuspended with PBS for counting purposes. About $1x10^6$ cells/mL

242 was stained with antibodies (CD3, CD4, CD8) (Abcam, USA) for 2 hours with constant shaking.

243 Then, the cells were fixed in 600 μL of 4% paraformaldehyde (Sigma, USA) before being

244 subjected to flow cytometry analysis (FACS Calibur, BD, USA).

245

246

247   *In vivo* **Nitric Oxide detection of the spleen**

248

249   Nitric Oxide detection from the spleen was established with the Griess Reagent Kit (Sigma,

250   USA). The experiment was conducted according to the protocol provided with the kit. The

251   supernatant from the splenocytes was mixed with Griess Reagent and incubated for 30 minutes

252   before being measured at 548 nm using microplate reader (Beckman Coulter, USA).

253

254   **Statistical Analysis**

255   The obtained data statistically analyzed. All of the results were expressed as the means and standard

256   deviations. The statistical analysis was performed using the GraphPad Prism (Version 6.0). Statistical

257   significance ($p < 0.05$) was assayed by one-way ANOVA analysis.

258

259   **Results and discussion**

260   **Physicochemical Characterisation of NLC-Citral formulation**

261   **Particle size and Polydispersity Index**

262   NLC-Citral was successfully synthesized using the high pressure homogenization method. The

263   summary of physicochemical characteristics of NLC-citral formulation is as shown in Table 1.

264   Particle size is the most fundamental parameter in a nanoparticle study. The particle size and

265   polydispersity index is highly important in determining the physical stability of colloidal

266   dispersion system of the nanoparticle (Lee *et al.,* 2007). The mean particle size and

267   polydispersity index of NLC-citral were obtained as 54.12 ± 0.30 nm and 0.224 ± 0.005,

268   respectively.

269   Particle size of the nanoparticle was present in the average diameter of the nanoparticle. It is

270   crucial to confirm that the size of the nanoparticle formulated is within nano-sized range

271   (Mukherjee *et al.,* 2008). As depicted in Figure 1, NLC-citral was observed to confer regular

272   shapes and are relatively uniform suggesting good particle dispersity. There was a minor

273   difference in size distribution data obtained from TEM and zeta sizer. This small disparity in

274   measurement was probably due to differences in the techniques and method of data analysis

275   between both instruments. The measurement of nanoparticles is mostly influenced by the

276   conditions, method of analyzing data and also depends on the operation of instrument in the

277   determination (How *et al.,* 2013). From this result, it was concluded that this formulation has

278   fallen within a nano-sized range. The size of the particle plays a key role towards their adhesion

279   to and interaction with biological cells (Foster *et al.,*2001). Being a small nanoparticle, NLC-

280   Citral holds a potential in lowering the risk of non-specific liver uptake (Seki *et al.,* 2004) as well

281   as increasing the actual distribution and bioavailability of the drug(Fang *et al.*, 2006). The

282   incorporation of a water insoluble anticancer compound (Amoitone B) with NLC was reported to

283   enhance its bioavailability (Luan *et al.,* 2013). Moreover, small nanoparticle with a diameter size

284   of 50-60 nm resulted in high tumor uptake as well as sustained release of the drug in cancer

285   biology studies (Mitra *et al.,* 2001; Sharma *et al.*, 1996).

286

287

288   **Table 1: The summary of characterizations for NLC-Citral**

Element	Value
Zeta potential	-12.59± 0.52mV
Zeta sizer	54.12 ± 1.10 nm
Polydispersity index	0.224 ± 0.005
Entrapment Efficiency	98.9 ± 0.124 %
Drug Loading Capacity	9.84 ± 0.041 %

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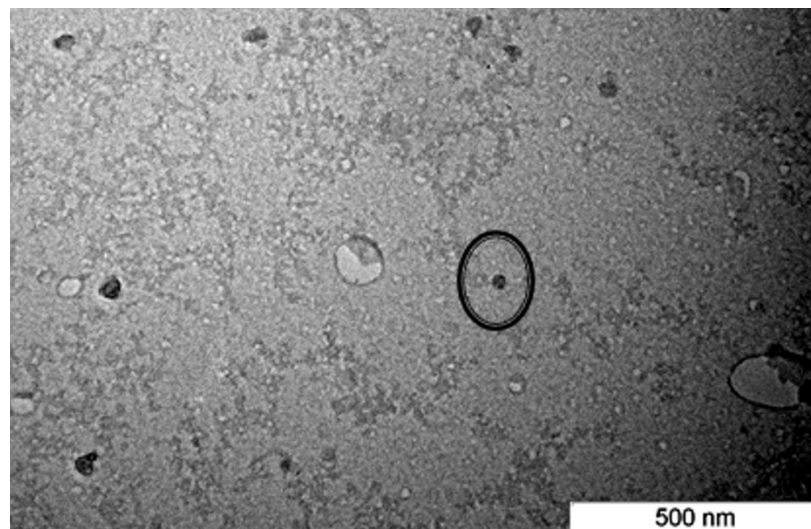
290

291   **Figure 1: Transmission electron microscope (TEM) image of the NLC-Citral prepared by**
292   **negative staining at 80,000X magnification. The circle represents the nanoparticle which**
293   **can be seen in regular circle shape.**

294

295   **Zeta Potential**

296   Determination of zeta potential for NLC-citral was done to evaluate the overall stability of the

297   formulation. The average zeta potential for NLC-citral was -12.73 ± 0.34 mV (Table 1). Stability

298   test for a nano-suspension is very crucial in any nanoparticle studies. Zeta potential is one of the

299   parameters that govern the stability of a nanoparticle formulation. As a rule of thumb, zeta

300   potential ranging from -5mV to +5mV indicates low stability and higher possibility for

301   aggregation (Honary & Zahir, 2013). Generally, a zeta potential value must be higher than

302   +30mV or lower than -30mV (Rahman *et al*., 2013a). Surfactants were used as a steric stabilizer

303   during the nanoparticle formulation to control particle size and the stability of dispersion.

304   Surfactants have a wide range of uses in pharmaceutical preparations. In this formulation, Tween

305   80 was chosen to provide a good steric stabilization. Previous finding has proved that Tween 80

306   can compensate the stability of NLC and SLN dispersions by providing a steric stability in

307   Domeperidone loaded with NLC and SLN (Thatipamula *et al.,* 2011).Conversely, studies have

308   concluded that, nanosuspension with slightly low zeta potential is stable enough with the

309   additional usage of higher molecular weight of stabilizer which will act by steric stabilization in

310   the particle (Honary & Zahir, 2013; Lee *et al.,* 2007).Hence, the stabilizer used in the

311   formulation of nanoparticle effected the average size and charges of the NLC (Abdelwahab *et*

312   *al.*, 2013).

313

314   Entrapment Efficiency and Drug Loading Capacity

315   The entrapment efficiency study is required to determine the high performance from the

316   formulation. In this formulation of NLC, Citral conveyed 98.9 ± 0.124 % of entrapment

317   efficiency in the system with 9.84 ± 0.041 % of drug loading capacity in the lipids concentration.

318   Incorporation of citral has led to a high percentage of entrapment efficiency (EE), probably

319   because of its lipophilicity and low water solubility characters. High EE is required for good

320 encapsulation parameters in any drug nano-carrier system including NLC. Furthermore, the

321 amount of drug entrapped in the nano-carrier also determines the performance of drug delivery

322 system since it influences the rate of the drug release from the system (Tiwari *et al.*, 2012). The

323 addition of drugs in the NLC system minimizes the interfacial retention in between lipid matrix

324 and liquid phase whereby reduces the free energy within phase boundary of lipid and the drug

325 incorporated (How *et al.,* 2013). Ideally a high loading capacity is aimed because it would

326 improve the entrapment efficiency of the drug eventually (Patel *et al.,* 2012). Drug loading is

327 defined as the process of incorporation of the drug into the carrier system; meanwhile entrapment

328 efficiency portrays the effectiveness of the incorporated drug into the carrier. The solubility of

329 the drug in lipid used assures the maximum drug loading in the NLC system (Rahman et al.,

330 2013a).Hence, high loading capacity of the citral in NLC system examined was due to the good

331 solubility of citral in the lipid phase of the NLC. The formation of unordered lipid crystal in the

332 NLC has increased the loading capacity. Imperfect crystal formation in the lipid nanoparticle can

333 be achieved through the mixing of solid lipid with liquid lipid which would lead to a higher drug

334 loading of NLC (Müller, Radtke, & Wissing, 2002).

335

Drug Release Study

337 Furthermore, the drug release profile of NLC-citral was also determined in this study. The

338 cumulative percentage of citral from NLC-citral over 48 hours is as shown in Figure 2. *In vitro*

339 drug release study was performed using the Franz diffusion cell system in order to determine the

340 release profile of the citral from NLC-citral for 48 hours. NLC system has shown a constant

341 release rate of citral release with a total of 96.3±2.1% after 48 hours of study.

342   It has become an essential strategy that drug delivery system should possess a better drug release

343   capacity to improve its effectiveness and reduce side effects of the drug caused by a rapid dosage

344   consumption by the patients in a therapeutic routine(How *et al.,* 2013).It was observed that a

345   slow release profile of citral from NLC was apparent, compared to the administration of citral

346   alone (Figure 2). This study also showed that the drug release kinetics of citral from NLC

347   followed a zero-order kinetic model, with an $r^2 = 0.976$.Relevant to the objective of the study,

348   NLC proved to be a suitable carrier of citral as it provides a slow release of it in time dependent

349   manner. A biphasic drug release pattern was observed in which a drug burst release was

350   observed at the initial stage and followed by sustained release at a constant rate later on. This

351   phenomenon might be explained due to the lipid imperfect crystalline structured of the NLC to

352   allow high drug loaded in the system (Müller *et al.,* 2002). Subsequently, this also affects the

353   release of the drug as it becomes loose and causing high rate of the drug released (Uprit *et*

354   *al.,*2013). In addition, as the size of the nanoparticle is getting smaller, the specific surface area

355   of nanoparticle is increased and thus the drug release became faster (Moghimi, *et al.,* 2005).

356   Therefore, the robust release rate in the initial stage of this in-vitro drug release study was

357   resulted from these two consequences. Consequently, citral in NLC-citral showed slower release

358   profile. Previous study has confirmed that the slow release of the drug was dependent on the

359   entrapment efficiency and homogenous entrapment of the drug throughout the system (Moghimi

360   *et al.,* 2005).

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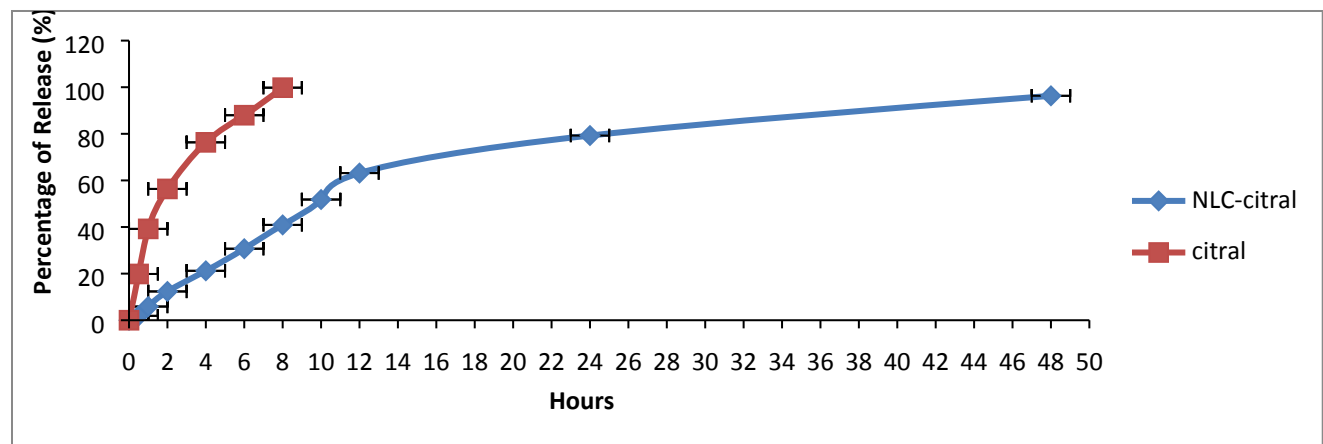

362   **Figure 2: The *in-vitro* drug release study of NLC-citral and citral (control). Each point**
363   **represents mean and standard deviation (n=3).**

364

365   ***In vitro* splenocytes viability (MTT)**

366   To examine the effect of splenocytes proliferation after being treated with NLC-Citral

367   formulation, the MTT assay was conducted. The *in vitro* proliferation response of splenocytes

368   treated with NLC-citral and citral was investigated and showed in Figure 3. There was no

369   significant decline in the viability of splenocytes detected from treatment with NLC-citral (99.8

370   ± 1.7 %) at the highest concentration given to the mice (30μg/ml) after 24 hours post-treatment

371   as compared to NLC-Blank (99.5 ± 0.98 %) which suggests that the formulation did not induce

372   toxicity towards the proliferation of mice splenocytes after being treated (Chen *et al.,* 2009).

373   According to Figure 3, the percentage of splenocytes viability did not change in the treatment of

374   NLC-citral at any concentration given. However, in the citral treated group there was a decrease

375   in splenocytes viability from 99.7% to 95.2% at the concentration of 15μg/mL and 30μg/mL

376   respectively.

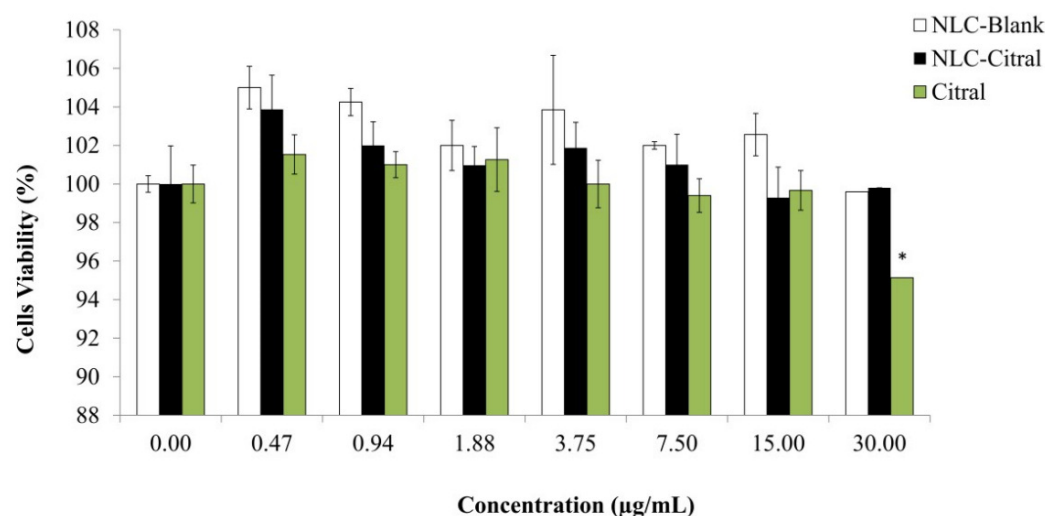
377 It was demonstrated that the NLC-Citral with several concentrations did not alter the effects of

378 splenocytes proliferation treated. As a whole, NLC-Citral has the beneficial effect on immune-

379 stimulation as the splenocytes proliferation was more noticeably better for NLC-citral treated

380 than citral treated group. Immune cells such as T and B cells play an important role in the host

381 defense system. Splenocytes proliferation indicates the ability to increase numbers of immune

382 markers which could stimulate the host immunity (Rescigno *et al.,* 2007).

383

384

385 **Figure 3: The percentage of splenocytes viability after being treated with various**
386 **concentrations of NLC-citral and pure citral ranging from 0.47μg/mL to 30μg/mL for 24**
387 **hours. Each point is represent the mean ± standard deviation (n=3). Significance was set at**
388 **p<0.05 comparing between groups with (**) to NLC-Blank and (*) to citral.**

389 *In vivo* **Toxicity Study**

390 All mice treated with NLC-citral and citral survived till the end of the 28 days of treatment.

391 According to Table 2, there were no mortality, body weight changes, and conspicuous toxic

392 signs developed in treated mice throughout the study time. For biochemical analysis, there was a

393 non-significant increase in the serum ALT level of NLC-citral treated group as compared to

394   NLC-Blank. Meanwhile, the citral treated group showed a decrease in ALT value. Besides,

395   creatinine level of the NLC-citral and citral treated groups has decreased than NLC-Blank.

396   This observation indicates that there was no overt toxicity occurred in NLC-citral and citral

397   treated mice. Even though slight changes were observed in the level of markers from the

398   biochemistry results, there was no significant modification detected. Hepatic damage is a serious

399   issue that should be taken into consideration in the new formulation of compound or drugs.

400   Additional AST enzyme will be released into the bloodstream when liver is damaged that

401   indicates a hepatic injury in the host (Sharma & Shukla, 2011). ALT level is a main indicator in

402   the detection of liver damage by drug or hepatotoxins in the bodies (Shirodkar *et al.*, 2015).

403   Eventually, the increase is AST level caused an elevation in the level of ALT (Giannini *et*

404   *al.,*2005). Moreover, the abnormal levels of ALP, AST and ALT in the serum most often indicate

405   a problem with the liver, gall bladder and heart (Shimizu, 2008). In addition, creatinine level was

406   assessed to view the renal function, an elevation of the creatinine level in the blood signifies

407   impaired kidney function or kidney disease (Shlipak *et al.*, 2013). There were no significant

408   alterations in the levels of kidney and renal biochemical detector molecules in the NLC-Citral

409   and Citral treated groups of mice. Pharmacology study has claimed that Citral is not toxic for

410   human consumption as indicated by no changes and abnormalities in serum glucose, urea,

411   proteins and creatinine level in the urine analysis (Carlini *et al.,* 1986).

412

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416

417 **Table 2: The observation of mortality, body weight changes, toxicity signs and serum**

418 **biochemical analysis of NLC-Blank, NLC-Citral and Citral treated groups. Values**

419 **represent means and standard deviation.**

Group	Mortality at 28th days	Body weight Changes	Toxic Signs	ALT (U/L)	ALP (U/L)	AST (U/L)	CREATININ (µmol/L)
NLC-Blank (Control)	NONE	NONE	NONE	75.2 ± 0.37	230 ± 0.65	348.2 ± 1.56	49.0 ± 1.09
NLC-CITRAL	NONE	NONE	NONE	76.1 ± 1.03	225 ± 0.98	351.9 ± 0.98	47.0 ± 0.97
CITRAL	NONE	NONE	NONE	71.5 ± 0.76	232 ± 1.34	359.0 ± 0.56	47.6± 1.0

420

421 ***In vivo* Nitric Oxide detection from the spleen**

422

423 NO level from the NLC-citral and citral treated groups was evaluated to study the anti-

424 inflammatory effects of the formulations. As illustrated in Figure 4, both NLC-citral and citral

425 have significantly reduced the level of NO. The level of nitric oxide in the NLC-citral group was

426 declined from 101± 3.02 µM/mg (NLC-Blank) to 40.91 ± 3.02 µM/mg of protein while in citral

427 the value was dropped to 46.16 ± 4.02 µM/mg of protein.

428 Nitric Oxide (NO) is a signaling molecule that initiates inflammation under normal conditions.

429 High concentrations of NO in the immune system was relatively regulated by cytokine-activated

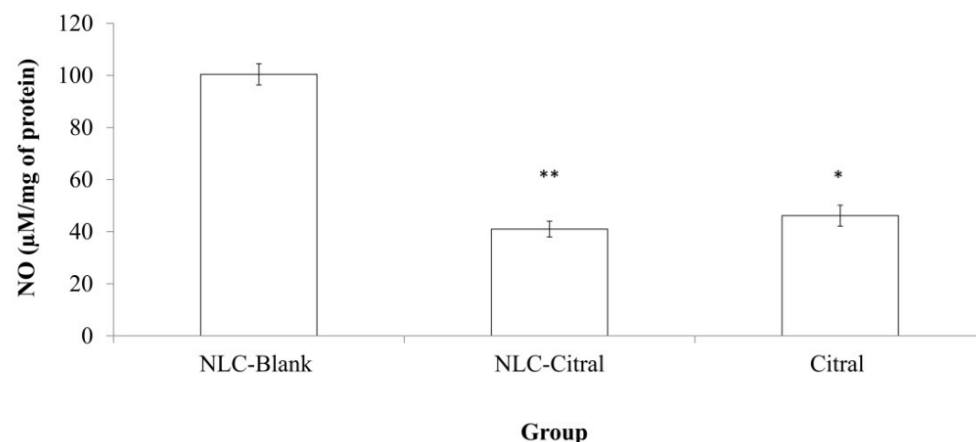
430   macrophage(Sharma *et al.,* 2007). The level of NO in the NLC-citral treated spleen has

431   significantly reduced as compared to the NLC-Blank. Chronic inflammation facilitates cancer-

432   related potentials such as inhibition of cell death programs, induce angiogenesis and assists

433   metastasis (Multhoff, Molls, & Radons, 2011). Hence, the reduction NO level in NLC-citral

434   depicts the anti-inflammatory effect possessed in the formulation.

435

436   **Figure 4: Bar chart analysis of the level of Nitric Oxide (NO) detected in the mice**
437   **splenocytes harvested from the NLC-Blank, NLC-citral and citral treated groups after 28**
438   **days of treatment (50mg/kg). Each value represents the means ± standard deviation.**
439   **Significance was set at p<0.05 comparing between groups with (*) to NLC-Blank and (**)**
440   **to citral.**

441

442   **Splenocytes immunophenotyping**

443   To determine the immune response affected from the NLC-citral and citral treatment as

444   compared to the NLC-Blank, *in vivo* immunophenotyping assay was done. Three different

445   conjugated staining of CD3 (FITC), CD4 (PE) and CD8 (APC) T-cells immune markers were

446   selected to stain the harvested splenocytes from all groups. As shown in Figure 5, the percentage

447   of CD4/CD3 T-cell population has significantly increased in the NLC-citral and citral treated

448   groups as compared to the NLC-Blank. The level of CD4/CD3 T-cells population increased from

449   13.97±0.19% in NLC-Blank to 20.12± 0.40 % in the NLC-citral treated splenocytes and 18.44 ±

450   0.50 % in the citral treated splenocytes (50 mg/kg). Furthermore, the level of CD8/CD3 T-cell

451   population was also increased, similar to the pattern of elevation observed in the CD3/CD4

452   population. It can be observed from Figure 5 that the percentage of CD8/CD3 T-cells population

453   in the NLC-citral and citral treated splenocytes has significantly increased to 9.56±0.32% and

454   8.82±0.16% respectively.

455   NLC-citral has increased the percentage of T-cells population higher than citral for all three T-

456   cells immune markers as compared to the NLC-Blank within the splenocytes population.

457   Previous study reported that citral exhibited potential as an immunomodulatory agent in murine

458   macrophage (Bachiega & Sforcin, 2011). Therefore, the increase of CD4/CD3 and CD8/CD3 in

459   both NLC-citral and citral treated splenocytes conveyed that these treatments have induced the

460   immunomodulatory effect of the mice. Studies on the immune responses have continued to gain

461   significant attraction due to the importance of immune system in combating against common

462   critical diseases such as cancer (Dunkelberger & Song, 2010). T-cell immune response

463   impairments and dysfunction almost appear in many chronic diseases such as cancer and Human

464   immunodeficiency virus (HIV) infection disease (Kinter *et al.,* 2004). CD4/CD3 is the T-helper

465   lymphocyte cells while CD8/CD3 is the cytotoxic T cells (Anderson *et al.,*1988). The depletion

466   of T-helper cells creates an immune hyper activation conditioned which is usually associated

467   with the immune response deficiency (Février *et al.,* 2011). In addition, a cytotoxic T

468   lymphocyte cell CD8/CD3 leads to the death of target cells by apoptosis in host defense against

469   various pathogens (Janeway *et al.,*2001b). Much attention has focused on the role of CD8T-cells

470   in immunotherapy of cancer as it is capable to destroy tumor cells in *in vitro* and *in vivo* studies

471   (Finn, 2003).

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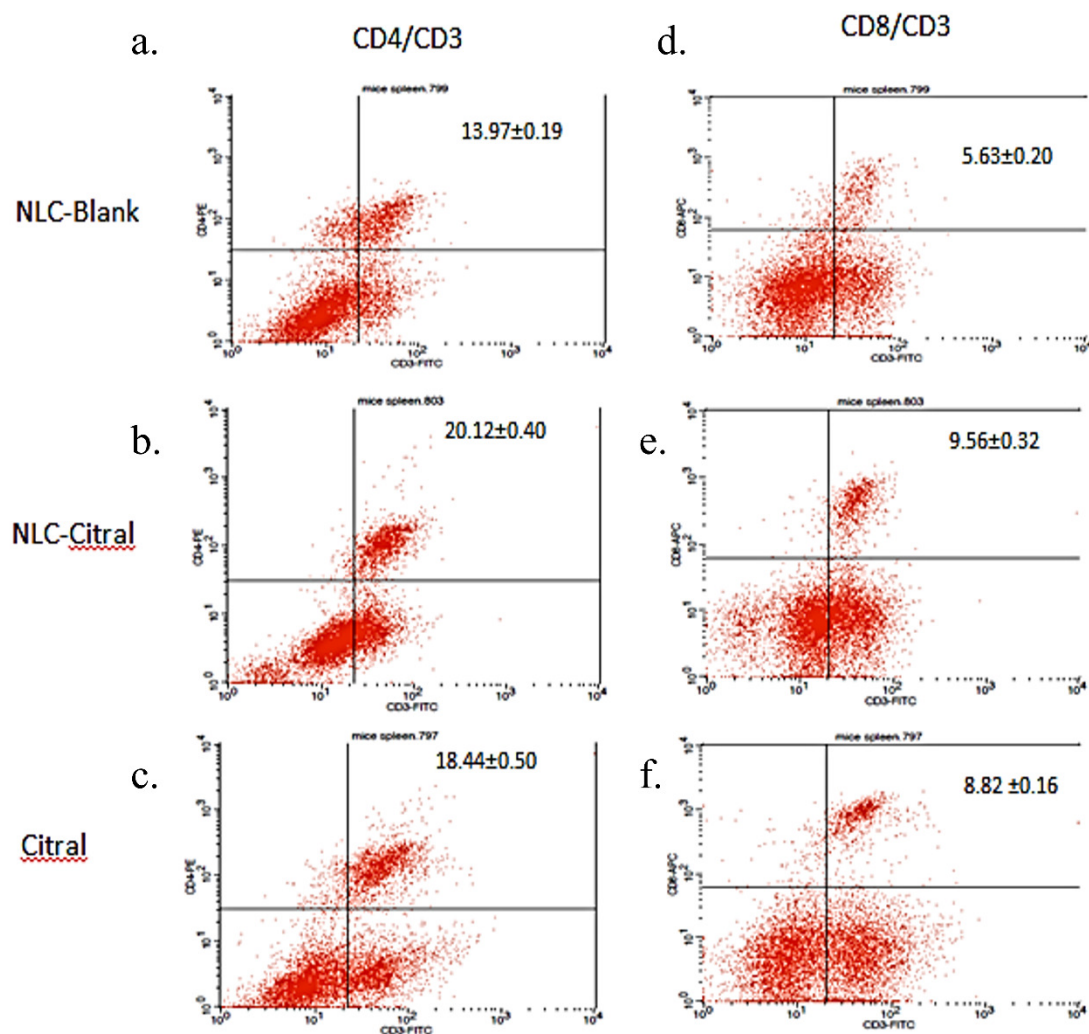
Figure 5: The histogram analysis of (a-c) CD3$^+$CD4$^+$and (d-f) CD3$^+$CD8$^+$ T lymphocytes in the splenocytes harvested after 28 days of treatment with 50 mg/kg of NLC-Blank, NLC-citral and citral. Each value represents mean ± std with n=5 mice per group

476

Conclusion

477

478 In the present study, the formulation of nanostructured lipid carrier encapsulated with citral

479 (NLC-citral) was characterized. The formulation was confirmed to be stable and fell within the

480 nano size range. This was proved based on the results from zeta potential, zeta sizer,

481 polydispersity index and TEM analysis of the nanoparticle. Furthermore, NLC-citral formulation

482 imposes a slow release profile of citral and has enhanced the solubility of the pure citral in the

483 water. Additionally, there was no aberrant toxic sign detected in the blood biochemistry analysis,

484 flow-cytometry immunophenotyping assay, lipid peroxidation (NO) and splenocytes viability

485 between the NLC-Blank (control) and NLC-citral and citral treated mice in the toxicity study

486 conducted. The physicochemical properties have validated that NLC-citral is suitable as a

487 potential delivery system of citral and non-toxic towards healthy cells. Hence, the NLC-citral

488 formulation can be further investigated to confirm its potential as a new delivery system for citral

489 in the treatment of cancer as citral is known to have anti-cancer properties.

490

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650 **List of abbreviation**

ALP   Alkaline phosphatase

ALT   Alanine aminotransferase

APC	Antigen-presenting cells
AST	Aspartate Aminotransferase
ATCC	Animal Tissue Culture Collection
CDK	Cyclin Dependent Kinase
DL	Drug Loading
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EE	Entrapment Efficiency
FACS	Fluorescence-activated cell sorter
HPO	Hydrogenated Palm Oil
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NO	Nitric oxide
PBS	Phosphate buffer saline
PI	Propidium Iodide
TEM	Transmission Electron Microscope
WHO	World Health Organization

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