© 2020 Journal of Pharmacy & Pharmacognosy Research, 8 (3), 177-190, 2020
ISSN 0719-4250
http://jppres.com/jppres

Original Article | Artículo Original

Self-nanoemulsifying drug delivery systems (SNEDDS) for oral delivery of *Garcinia kola* seeds ethanolic extract: formulation and *in vivo* antimalarial activity

[Sistemas de administración de fármacos auto-nanoemulsificantes (SNEDDS) para administración oral de extracto etanólico de semillas de *Garcinia kola*: formulación y actividad antipalúdica *in vivo*]

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Abstract

Context: *Garcinia kola* seeds are widely used in Congolese traditional medicine to treat uncomplicated malaria. While the ethanolic extract of these seeds (GK) is reputed for oral antimalarial activity, some of its constituents have shown poor water solubility, which might compromise further phytopharmaceutical developments.

Aims: To develop a self-nanoemulsifying drug delivery system (SNEDDS) for oral delivery of GK, since SNEDDS are promising vehicles for enhancing drug product solubility.

Methods: GK was loaded into liquid SNEDDS (solution and suspension) and solid SNEDDS (S-SNEDDS), and the resultant formulations were characterized using dynamic light scattering and electron microscopy. The antimalarial activity of SNEDDS (200 mg/kg × 4 days, oral) was evaluated in *Plasmodium berghei*-infected mice using a conventional four-day suppressive test.

Results: The characterization of SNEDDS formulations revealed the presence of nanosized structures of spherical morphology and negative surface charge. Data from *in vivo* study showed reduced parasite growth by 77.9, 73.8 and 74.2% for GK-SNEDDS solution, GK-SNEDDS suspension and GK-S-SNEDDS, respectively. The activity of GK-SNEDDS was found to be greater than that of a licensed GK-based syrup (N'sansiphos®) used at the same dose ($p<0.05$).

Conclusions: These findings demonstrate the potential of SNEDDS formulations as a promising alternative for enhancing the antimalarial efficacy of GK following oral administration. The SNEDDS technology holds the promise of improving the oral delivery of herbal-based products for malaria therapy.

Keywords: antimalarial activity; ethanolic extract; *Garcinia kola*; liquid SNEDDS; solid SNEDDS.

Resumen

Contexto: Las semillas de *Garcinia kola* se usan ampliamente en la medicina tradicional congoleña para tratar la malaria no complicada. Si bien el extracto etanólico de estas semillas (GK) tiene fama de actividad antipalúdica oral, algunos de sus componentes han demostrado una baja solubilidad en agua, lo que podría comprometer el desarrollo de fitofármacos.

Objetivos: Desarrollar un sistema de suministro de fármacos auto-nanoemulsionantes (SNEDDS) para el suministro oral de GK, como vehículos para mejorar la solubilidad del producto farmacológico.

Métodos: Se cargó GK en SNEDDS líquido (solución y suspensión) y SNEDDS sólido (S-SNEDDS). Las formulaciones resultantes se caracterizaron usando dispersión dinámica de luz y microscopía electrónica. La actividad antipalúdica de SNEDDS (200 mg/kg × 4 días, oral) se evaluó en ratones infectados con *Plasmodium berghei* utilizando una prueba de supresión convencional de cuatro días.

Resultados: La caracterización de las formulaciones de SNEDDS reveló la presencia de estructuras nanométricas de morfología esférica y carga superficial negativa. Los datos del estudio *in vivo* redujeron el crecimiento del parásito en 77,9; 73,8 y 74,2% para la solución GK-SNEDDS, la suspensión GK-SNEDDS y GK-S-SNEDDS, respectivamente. Se descubrió que la actividad de GK-SNEDDS es mayor que la de un jarabe a base de GK con licencia (N'sansiphos®) utilizado en la misma dosis ($p<0,05$).

Conclusiones: Estos hallazgos demuestran el potencial de las formulaciones de SNEDDS como una alternativa para mejorar la eficacia antipalúdica de GK después de la administración oral. La tecnología SNEDDS promete mejorar la administración oral de productos a base de hierbas para la terapia de la malaria.

Palabras Clave: actividad antipalúdica; extracto etanólico; *Garcinia kola*; SNEDDS líquido; SNEDDS sólidos.

ARTICLE INFO
Received: June 2, 2019.
Received in revised form: November 15, 2019.
Accepted: November 18, 2019.
Available Online: December 11, 2019.
Declaration of interests: The authors declare no conflict of interest.
Funding: This work was partially supported by the National Research Foundation of South Africa and the Sandisa Imbewu Pro-gramme at Rhodes University.

INTRODUCTION

Despite the drop observed in the worldwide incidence, from 237 million cases recorded in 2010 to 216 million cases in 2016, malaria remains the most devastating parasitic disease in Africa, where its burden accounts for 90% of the global 216 million cases and 91 % of the 445 000 deaths (World Health Organization, 2017). This arose mainly from the sub-Saharan African countries, where malaria is a true public health concern. Among many other countries, Democratic Republic of the Congo (DRC) bears the brunt of malaria burden, with nearly 12 million cases and 100,000 deaths per annum. These statistics reflect the urgent need for improving antimalarial therapy, which is currently based on artemisinin-type compounds (PNLP et al., 2017; World Health Organization, 2017). In addition to the fact that research has testified the therapeutic potential of medicinal plants, the use of plants in traditional medicine remains very common in the healing practices of the Congolese population. Nowadays, plant-based products have gained tremendous consideration as potential alternatives for strengthening the primary healthcare system of the DRC, which aligns well with the objectives of the "Traditional Medicine Strategy" proposed by the World Health Organization (Qi and Kelley, 2014; Memvanga et al., 2015).

As presented in the review by Memvanga et al. (2015), most of the Congolese plants commonly used in traditional medicine have proven remarkable antimalarial activities in standardized pharmacological studies under *in vitro* and *in vivo* conditions. Among these plants, *Garcinia kola* Heckel (*Clusiaceae*), also known as bitter kola or false kola, has been reported to be a promising medicinal species for extensive drug developments. Chewing *Garcinia kola* seeds produces both chemopreventive and curative effects against malaria (Tona et al., 1999). In addition, the aqueous macerate of seeds is used for the treatments of bronchitis, mouth infections, colics, colds, laryngitis, sore throat problems, post-partum hemorrhage, urinary tract infections and liver diseases. The seeds are also well known for antiemetic, hepatoprotective, anti-oxidative, aphrodisiac and tonic properties (Iwu, 1985; Farombi et al., 2000; Farombi and Owoeye, 2011; Galam et al., 2013).

Noteworthy, the biological activities of *Garcinia kola* seeds (ethanolic extract) have been associated with their phytochemical composition, which embraces several secondary metabolites. The major of these include the mixture of *Garcinia kola* biflavonoids (GB), namely kolaviron that contains GB-1, GB-1a, GB-2 and kolaflavanone (Kabangu et al., 1987; Olaleye et al., 2000; Farombi et al., 2013; Konziase, 2015; Tshibangu et al., 2016). Despite their poor water solubility, these compounds have demonstrated a wide array of activities, which underlines the importance of *Garcinia kola* in traditional management of infectious diseases such as malaria (Farombi, 2003; Memvanga et al., 2015). In particular, GB-1, GB-1a and GB-2 have been found to exhibit antiplasmodial activity with IC_{50} values of about 0.1 – 10 µM (Antia et al. 2010; Konziase 2015). Moreover, kolaviron and its components were reported to exhibit high antimalarial activity in *Plasmodium berghei*-infected mice (Oluwatosin et al., 2014). Nevertheless, the limited solubility of kolaviron remains a deep concern for further pharmaceutical developments and biomedical applications. Therefore, the need for improving the aqueous solubility of these phytochemicals is highly desired to favor formulation development and increase their bioavailability, which may result in enhanced efficacy (Ajazuddin and Saraf, 2010).

As one of the emerging formulation strategies, self-nanoemulsifying drug delivery systems (SNEDDS) have shown great promise for improved solubility and delivery of plant bioactive and extracts. SNEDDS are isotropic mixtures of oils (i.e., pure triglyceride oils and/or mixed glycerides), water-soluble surfactants and hydrophilic co-solvents or co-emulsifiers that correspond to preconcentrated colloidal solutions. After oral administration, SNEDDS get dispersed in the gut and generate an oil-in-water nanoemulsion upon mild agitation in the gastrointestinal tract (Müllertz et al., 2010; Li et al., 2011; Memvanga and Préat,

2012). The bioavailability of lipophilic drugs can be improved by SNEDDS through several mechanisms, including augmentation of gastrointestinal solubility and stability, prolongation of residence time, induction of biliary secretions and intestinal membrane permeability, reduction of metabolism and efflux pump activity, enhancement of chylomicron level and lymphatic transport, etc. (Porter et al., 2007; O'Driscoll and Griffin, 2008; Li et al., 2011). Many studies have demonstrated the potential of SNEDDS as a promising approach for enhancing oral delivery of poorly water soluble drugs (e.g. *β*-arteether) (Memvanga and Préat, 2012) and phytochemicals (e.g. curcumin) (Memvanga et al., 2013a), as well as herbal extracts such as *Ginkgo biloba* (Tang et al., 2008) and *Diospyros kaki* extracts (Li et al., 2011). Additionally, SNEDDS have shown some clinical success that is reflected by the existence of a couple of marketed formulations, including Sandimmune Neoral®, Norvir® and Fortovase® that contain cyclosporin, ritonavir and saquinavir, respectively (Müllertz et al., 2010; Li et al., 2011).

In comparison with other lipid-based systems, SNEDDS offer the advantage of ease production, which is achieved by means of a simple and cost-effective mixing procedure with no need for heat, apart from the melting of some oils (Tang et al., 2008; Li et al., 2011; Memvanga and Préat, 2012; Memvanga et al., 2013a). This technology is promising for addressing the critical issue of commercial availability and affordability of nanomedicine in developing countries (Memvanga and Préat, 2012; Memvanga et al., 2013a).

The present study aimed at encapsulating the ethanolic extract of *Garcinia kola* seeds (GK) in various SNEDDS formulations for improved oral delivery. Three different formulations, namely GK-SNEDDS solution, GK-SNEDDS suspension and solid GK-SNEDDS; were developed, characterized and evaluated for antimalarial activity in *Plasmodium berghei*-infected mice.

MATERIAL AND METHODS

Plant material

The seeds of *Garcinia kola* harvested in Kongo Central (DR Congo) were purchased from local vendors in Ngaba Township (Kinshasa, DR Congo), in December 2016. The identity of plant materials (vernacular name in Kinshasa: "Ngadiadia") was confirmed by Mrs Nlandu and Mambwana at the *Institut National d'Etudes et de Recherches en Agronomie* (*INERA*) of the University of Kinshasa. A voucher specimen was deposited in the herbarium of this institute, with the voucher number P120897N1. The seeds were air-dried over one week at room temperature in the dark, and then ground to yield a fine powder.

Chemicals

Absolute ethanol (99.2%) was purchased from Merck (Darmstadt, Germany). Triton X-100 was sourced from Sigma-Aldrich (Diegem, Belgium). Polyethylene glycol 400 (Macrogol 400) and propylene glycol were from Certa (Braine-l'Alleud, Belgium). Phosphate buffer saline (PBS) was sourced from Invitrogen Gibco (Merelbeke, Belgium). Maisine 35-1 (glyceryl monolinoleate), Labrafil M1944CS (oleoyl polyoxyl glycerides), Labrafil M2125CS (linoleoyl polyoxyl glycerides), Lauroglycol 90 (propylene glycol monolaurate), Capryol 90 (propylene glycol monocaprylate), Labrasol (caprylocaproyl polyoxyl glycerides) and Transcutol HP (diethylene glycol monoethyl ether) were kindly provided by Gattefossé (Saint-Priest, France). Cremophor EL (polyoxyl 35 castor oil) was kindly donated by BASF (Burgbernheim, Germany). Sodium carboxymethylcellulose, xanthan gum, Aerosil 200, hydroxypropyl-*β*-cyclodextrin, magnesium stearate, sucrose and glycerin were gifted by Kim Pharma and Arauphar (Kinshasa, DR Congo). Quinine was from Pharmakina (Bukavu, DR Congo) whereas N'sansiphos syrup®, an improved traditional med-

icine (ITM) containing seed extract of *Garcinia kola* (12 mg/mL), was obtained from the *Centre de Recherche et de Production des Médicaments Traditionnels Améliorés* (*CRMTA*, Kinshasa, DR Congo). Olive oil was purchased from Shayna (Kinshasa, DR Congo). Ultrapure water was prepared by means of a Milli-Q Plus 185 water purification system (Millipore, Billerica, MA, USA).

Preparation of ethanolic extract

Dry powder of plant material (200 g) was macerated in ethanol (3 × 1000 mL) at room temperature over 24 h, with occasional shaking. The three macerates were pooled and filtered to yield an ethanolic solution that was evaporated to dryness under reduced pressure at 40°C using a rotary evaporator. The resultant solid extract was weighed, and the yield of extraction was estimated to be 7.8%. Using the HPLC-UV method developed by Tshibangu et al. (2016), the content of GB-1, the major compound contained in GK powder, was evaluated and found to be 0.25 – 0.28%.

Preparation of GK self-emulsifying formulations

GK solubility testing

Various vehicles, including oils, surfactants, co-solvents or formulations, were used to evaluate the GK solubility. Briefly, 200 mg of GK was placed in a transparent glass vial and a particular vehicle was added portion wise at 25°C under magnetic stirring at 400 rpm (Memvanga and Préat, 2012). Following each addition, the reaction medium was allowed to stir over 120 min and the extent of solubilization was assessed visually. The process continued until complete solubilization of GK, which was confirmed by the absence of solid particles under an optical microscope (Bressler, Brignoles, France). All the measurements were performed in triplicate.

Preparation of liquid SNEDDS

Liquid SNEDDS (solution and suspension) were prepared by mixing melted Maisine 35-1 (3 g) with olive oil (3 g) and Cremophor EL (3 g) at 25°C under stirring at 400 rpm for 10 min. The obtained mixture was poured into either 1.5 g of ethanol (or Transcutol HP) containing either 0.82 g of GK/g (for SNEDDS solution) or 1.4 g of GK/g (for SNEDDS suspension) and stirred at 400 rpm for 2 h at 25°C to ensure good homogenization.

Preparation of solid SNEDDS

The solid SNEDDS formulation (S-SNEDDS) was prepared by solidification of the SNEDDS suspension. To an aliquot of SNEDDS suspension (3 g), different adsorbent materials (including Aerosil 200, magnesium stearate, carboxymethylcellulose, polyvinylpyrrolidone and 2-hydroxypropyl-β-cyclodextrin) were gradually added and constantly mixed by means of a spatula until the formation of a dry, homogenous and free-flowing powder was achieved. The experiments were conducted in triplicate.

Preparation of the aqueous and ethanolic suspensions

The aqueous suspension of GK (denoted GK-SUS-1) was prepared by dispersing 10 mL of the ethanolic dispersion of GK (50%, w/v) in 90 mL of an aqueous vehicle composed of carboxymethylcellulose (0.25%, w/v), xanthan gum (0.3%, w/v), Aerosil 200 (0.15%, w/v), glycerin (7.5%, w/v), sucrose (30%, w/w) and water. Apart from GK-SUS-1, an ethanolic suspension of GK (GK-SUS-2) was prepared by dispersing 1 g of GK in 10 mL of ethanol under gentle shaking at 25°C for 10 min.

Dispersion of GK lipid-based self-emulsifying systems in aqueous media

Assessment of self-emulsification efficiency

The *in vitro* performance of the self-emulsifying formulations in gastric medium was estimated by the visual grading test previously reported (Khoo et al. 1998). Briefly, 50 mL of HCl 0.1 N was added to 100 mg of SNEDDS (or 200 mg of S-SNEDDS) contained in a volumetric flask. The mixture was firstly stirred at 100 rpm for 10 min at 37°C, then allowed to stand at room temperature for 30 min. The following grading system was used: (A) denotes a clear micro- or nano-emulsion formed within 1 min; (B) denotes a rapidly formed (<1 min) and slightly cloudy emulsion; and (C) de-

notes a bright white emulsion obtained within 2 min. The experiments were conducted in triplicate.

Determination of particle size and Zeta potential

The size and Zeta potential of droplets were determined by dynamic light scattering (DLS) using Zetasizer Nano ZEN 3600 (Malvern Instruments, UK). Briefly, 100 mg of SNEDDS (or 200 mg of S-SNEDDS) were dispersed in 50 – 100 mL of either water or HCl 0.1 N under stirring at 100 rpm for 10 min at room temperature. The resultant dispersion was allowed to settle for 30 min, and an aliquot was withdrawn for particle size analysis or Zeta potential determination. Triplicate measurements were performed at room temperature under the scattering angle of 173°.

Microscopic observation

The samples from DLS experiments were subject to transmission electron microscopy (TEM) for visualization of SNEDDS droplets. Each aliquot was placed on copper grids drop wise using a Pasteur pipette. The grids were then allowed to dry over 48 h at room temperature prior to microscopic observations. TEM experiments were conducted on a Carl Zeiss Libra–120 kV TEM instrument (Oberkochen, Germany). Furthermore, scanning electron microscopy (SEM) was used to investigate the surface morphology of the prepared S-SNEDDS. An aliquot of the sample was dusted on a graphite plate and sputter coated with gold under vacuum for 20 minutes. SEM experiments were carried out on a Tescan Vega® Scanning Electron Microscope (Cambridge, UK).

Hemolysis test

A preliminary biocompatibility assessment of both the lipid-based formulations and GK was performed on erythrocytes using the hemolysis test as previously reported (Memvanga et al., 2013a). Briefly, the blood from two healthy volunteers was centrifuged (2000 ×g, 10 min) and the supernatant (plasma) was discarded. The erythrocytes pellet was washed three times and diluted with isotonic phosphate buffered saline (PBS) to achieve a hematocrit level of 8%. The obtained erythrocyte suspension was then incubated (30 min, 37°C) with 1% (v/v) of formulations (0–20 mg/mL in PBS) or the dissolved extract (0-10 mg/mL in ethanol). Triton X-100 (1%, w/v) and ethanol were used as positive and negative control, respectively. PBS was used as standard. After centrifugation (2000 ×g, 5 min, 37°C), the absorbance of the hemoglobin released in the supernatants was measured by spectrophotometric analysis at 540 nm. The percentage of hemolysis was determined using the following equation [1]:

$$\text{Hemolysis (\%)} = \frac{\text{ast} - \text{anc}}{\text{apc} - \text{anc}} \times 100 \quad [1]$$

where ast = absorbance of sample-test, apc = absorbance of positive control, anc = absorbance of negative control. The hemolytic activity of each sample was tested in triplicate.

***In vivo* antimalarial activity evaluation**

To assess the potential *in vivo* antimalarial activity of GK loaded in SNEDDS or in S-SNEDDS, the classical 4-day suppressive test was used as previously described (Memvanga and Préat, 2012; Memvanga et al., 2013a). NMRI mice (23-27 g, eight weeks of age) from the *Institut National des Recherches Biomédicales* (INRB, Kinshasa, DR Congo) were used. All animal experiments were performed according to the National Institutes of Health guidelines for the care and use of laboratory animals (NIH Publications No 85-23, 1985, revised 1996). These experimental protocols were approved by and performed in accordance with the institutional animal care and ethical committee (University of Kinshasa, DR Congo; Approval No 2018/UNIKIN/SS/062). The animals (17 groups of 5 mice) were maintained under optimum conditions of temperature (21 ± 2°C), light (12 h light/dark cycle) and relative humidity (70 – 80%) with food and water provided *ad libitum*.

Briefly, on day 0 the test and control mice were inoculated intraperitoneally with 300 µL of physiological saline containing approximately 1×10^7 *Plasmodium berghei* ANKA parasitized erythrocytes. Two hours after inoculation, test mice were given 0.1 mL of GK-SNEDDS solution, GK-SNEDDS suspension and GK-S-SNEDDS (0, 100

and 200 mg/kg) for four consecutive days. Before each administration to mice, 1 g of GK-SNEDDS (solution and suspension) or 2 g of GK-S-SNEDDS were mixed with water to produce dispersions containing 25 or 50 mg/mL of GK. In the positive- and negative-control groups, mice received 0.1 mL of GK-SUS-1, GK-SUS-2, N'sansiphos (0 and 200 mg/kg) and quinine (0 and 20 mg/kg). The last two drugs were administered after diluting with appropriate volumes of water. The final group of mice was infected but not treated.

On day 4, a thin film was made from the tail-blood sample for each mouse and stained with giemsa. The level of parasitemia was then determined following the method described in the literature (Memvanga and Préat, 2012; Memvanga et al., 2013a). The antimalarial activity was estimated as equation [2] (Memvanga et al., 2015):

$$\text{Antimalarial activity (\%)} = \frac{A - B}{A} \times 100 \quad [2]$$

where A and B represent the mean parasitemia in the negative-control group and in the test groups, respectively.

Statistical analyses

The statistical differences in droplet size and antimalarial activity between GK-SNEDDS, GK-S-SNEDDS and controls were compared by one-way ANOVA using Tukey's post-hoc test (with a significance level of $p<0.05$).

RESULTS AND DISCUSSION

Solubility of GK in various excipients

Most of the lipid and surfactants used to formulate SNEDDS are approved by the Food and Drug Administration and/or the European Medicines Agency. However, in the frame of lipid-based formulation development, a thorough solubility profiling is recommended for selection of appropriate excipients among many others (Memvanga and Préat, 2012). In the case of GK-SNEDDS formulation, this was achieved by evaluating the solubility of GK in a wide range of vehicle candidates including oils, surfactants, co-solvents and formulations.

Data from the solubility study indicated that GK is freely soluble (110–170 mg/g) in Maisine 35-1, olive oil, groundnut oil, sesame oil, Labrafil M2125CS and Labrafil M1944CS; but soluble (40–100 mg/g) in Lauroglycol 90, Capryol 90, Labrasol and Cremophor EL. Among the tested co-solvents, ethanol yielded the highest solubility for GK (~95 mg/g) followed by Transcutol HP (~63 mg/g), PEG (~45 mg/g), and propylene glycol (~40 mg/g). The aqueous solubility of GK was found to be 18 mg/g, which confirmed that this extract was poorly water-soluble.

Among the tested lipid excipients, Maisine 35-1 and olive oil were selected as the oil phase for SNEDDS formulation due to their solubilizing effects towards GK. The selected vehicles are reputed excipients for antimalarial applications. In fact, upon *in vivo* lipolysis, these vehicles release oleic and linolenic acid that show inherent antimalarial activity in addition to improving the bioavailability and lymphatic transport of lipophilic drugs (Seeballuck et al., 2004). Moreover, Cremophor EL was also considered for SNEDDS formulation due to its self-emulsifying efficiency and its ability to inhibit P-glycoprotein activity (Seeballuck et al., 2004; Memvanga et al., 2013a). Regarding the co-solvents, both ethanol and Transcutol HP (an absorption enhancer) were used.

Formulation of SNEDDS solution and suspension

A series of liquid SNEDDS formulations previously developed using some of the selected vehicles were characterized and established as safe products (Khoo et al., 1998; Memvanga et al., 2013a;b). Two of these formulations were used in the present study with slight modifications, particularly with respect to their quantitative and qualitative composition based on the Lipid Formulation Classification System (Pouton and Porter, 2008). The SNEDDS vehicles used in this study were composed of olive oil (3 g), Maisine 35-1 (3 g) and Cremophor EL (3 g), with either ethanol or Transcutol HP (1.5 g).

The solubility of GK in these SNEDDS preconcentrates was evaluated over 24 h of incubation at room temperature. The results from GK solubility testing indicated that the ethanol-based SNEDDS was better than the Transcutol HP-based SNEDDS, with respective GK solubility of 133 and 124 mg/g. The observed solubility is due to additive or synergistic dissolving effects of the incorporated excipients. In fact, the presence of the drug in the dissolved state circumvents the need for dissolution to ensure drug release. This feature aligns with one of the advantages of self-nanoemulsifying systems (Larsen et al., 2008; Siqueira et al., 2017). Interestingly, the drug loading for the SNEDDS solution went up to 87.5% of the equilibrium solubility (S_{eq}) of GK in each formulation (i.e. ethanol-based and Transcutol HP-based vehicles, which were about 117 and 109 mg/g, respectively).

However, since the daily dose of GK in humans is estimated to be 600 – 1000 mg based on both dose translation from animal to human and previous pharmacological investigations (Tona et al., 1999; Reagan-Shaw et al., 2007; Memvanga et al., 2015), the observed GK solubility in the formulated SNEDDS would lead to an impasse at late phases of clinical development. Therefore, the possibility of developing a suspension of GK in the lipid formulation was investigated. Although not frequently reported, lipid-based suspensions of lipophilic compounds exhibit the same bioavailability patterns as lipid-based solutions. A few studies reported so far include successful delivery of several hydrophobic drugs such as danazol, fenofibrate, cinnarizine, griseofulvin, phenytoin and 17-*β*-acetoxy-2-*a*-chloro-3-(*p*-nitrophenoxy)-imino-5-*a*-androstane (I) in the form of lipid-based suspensions (Larsen et al., 2008; Mu et al., 2013; Thomas et al., 2014; Siqueira et al., 2017).

For the formulation of our SNEDDS suspension, we have considered 150% of drug loading in the ethanol-based and Transcutol HP-based SNEDDS, i.e. a concentration that would be compatible with the required clinical dose (200 – 300 mg three times per day). A trial comparing the dispersibility of GK in the two vehicles revealed that the ethanol-based vehicle would be more suitable for GK-SNEDDS suspension than the Transcutol HP-based vehicle. Thus, in the scope of this study, the ethanol-based vehicle was used for formulation of both SNEDDS solution and suspension.

Formulation of solid SNEDDS

To prevent drug precipitation and particle agglomeration in the self-emulsifying suspension, GK-S-SNEDDS was blended with different adsorbents that can trap its components inside their pores, limiting drug exposure at the surface. Adsorption saturation was deemed to be achieved upon formation of free-flowing dry powders. Aerosil 200 exhibited minimal absorption capacity at the "Adsorbent–SNEDDS" ratio of 8:10 (w/w), while other tested adsorbents showed much higher mass ratios (>12:10) (Fig. 1). Aerosil 200 was therefore used for preparation of GK-based self-emulsifying powder (GK-S-SNEDDS). The blank S-SNEDDS formulation was prepared from the GK-free liquid SNEDDS. Table 1 summarizes the composition of GK-SNEEDS solution, GK-SNEDDS suspension and GK-S-SNEDDS.

Table 1. Detailed composition of the GK-based SNEDDS formulations.

GK-SNEDDS solution (S_{eq} 87.5%, 117 mg/g)	**GK-SNEDDS suspension (S_{eq} 150%, 200 mg/g)**	**GK-S-SNEDDS (100 mg/g)**
Olive oil (3 g)	Olive oil (3 g)	Olive oil (3 g)
Maisine 35-1 (3 g)	Maisine 35-1 (3 g)	Maisine 35-1 (3 g)
Cremophor EL (3 g)	Cremophor EL (3 g)	Cremophor EL (3 g)
Ethanol (1.5 g)	Ethanol (1.5 g)	Ethanol (1.5 g)
-	-	Aerosil 200 (10.5 g)
GK (1.23 g)	GK (2.1 g)	GK (2.1 g)

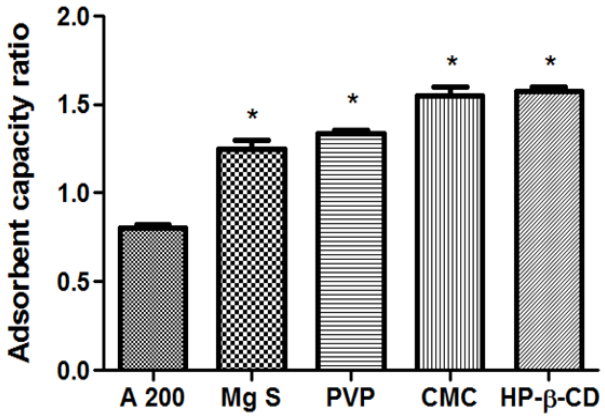

Figure 1. Adsorbent capacity ratio (g of solid carrier/g SNEDDS). (A 200: Aerosil 200; Mg S: magnesium stearate; PVP: polyvinylpyrrolidone; CMC: carboxymethylcellulose and HP-β-CD: 2-hydroxypropyl-*β*-cyclodextrin).

Values are presented as mean ± S.D, *n* = 3. *p<0.05 significantly different from Aerosil 200. One-way ANOVA followed by post-test Tukey.

Table 2. Droplet characteristics of the SNEDDS formulations

Medium	Formulations	Droplet size (nm) / PDI*	Zeta potential (mV)
Water	Blank SNEDDS	167.2 ± 7.8 (0.22)	–12.41 ± 1.64
	GK-SNEDDS solution	88.5 ± 3.8 (0.28)	–18.10 ± 0.76
	GK-SNEDDS suspension	138.4 ± 3.5 (0.50)	–28.12 ± 0.32
	Blank S-SNEDDS	79.9 ± 4.3 (0.35)	–47.40 ± 1.82
	GK-S-SNEDDS	66.6 ± 13.4 (0.26)	–16.00 ± 1.12
HCl 0.1N	Blank SNEDDS	153.4 ± 9.4 (0.18)	+0.47 ± 0.88
	GK-SNEDDS solution	82.7 ± 6.5 (0.25)	–0.36 ± 0.76
	GK-SNEDDS suspension	124.1 ± 21.7 (0.91)	+0.28 ± 0.67
	Blank S-SNEDDS	57.5 ± 8.1 (0.31)	+0.22 ± 1.10
	GK-S-SNEDDS	95.1 ± 31.3 (0.25)	–2.11 ± 0.10

Data represent mean ± SD, *n*=3. *The polydispersity index (PDI) is given in the parentheses.

Dispersibility of GK-based SNEDDS

Self-emulsification grading test

The visual observation represents the primary means to assess the self-emulsification efficiency of lipid-based formulations. The visual grading system described by Khoo et al. (1998) was used in this study. Upon dispersion in aqueous media with gentle shaking, all the liquid SNEDDS formed clear and fine emulsions with emulsification time of <1 min. Similar behavior was also exhibited by solid SNEDDS, which produced ultrafine and transparent dispersion when dispersed in aqueous media under gentle stirring within 1 min. Due to this emulsification profile, all the SNEDDS formulations were graded A.

Droplet size and Zeta potential

Dynamic light scattering (DLS) was used to determine the droplet sizes of all the formulations in order to confirm the self-emulsification efficiency. As presented in Table 2, the unloaded-SNEDDS formulations generated small droplets in the nanoscale range of 153–167 nm (Z-average) with polydispersity index (PDI) <0.25, indicating good uniformity in particle sizes (Cavalcanti et al., 2011). These droplet sizes remained unchanged at various dilution levels, confirming the SNEDDS na-

ture of the formulation. Despite the incorporation of GK (up to 87.5% S_{eq}), SNEDDS solution still exhibited spontaneous dispersibility into nanoemulsion systems, with droplet sizes of 80 – 90 nm and PDI <0.35. When the drug load went up to 150% S_{eq} (for SNEDDS suspension), the nanoemulsion droplet sizes increased two-fold on dispersion in aqueous media. This was associated with remarkable increase in PDI values (0.5 – 0.9), which may be due to the presence of undissolved GK particles.

The droplet size distribution of S-SNEDDS also confirmed the self-emulsification nature of this lipid-based system (Table 2). This shows the ability of the excipients mixture used to preserve its emulsification properties despite the change in the physical state of the formulation. Nevertheless, there was significant difference in droplet sizes between GK-S-SNEDDS and its parent formulation (i.e. GK-SNEDDS suspension) ($p<0.05$). This is most likely due to the presence of Aerosil 200 in S-SNEDDS, which may be located at the interphase between water and oil thereby stabilizing oil/water nanoemulsion and preventing the dispersed phases from coalescing. This is consistent with the observation previously reported (Kang et al., 2012).

Regarding the droplet surface charge, SNEDDS dispersed in Millipore water exhibited completely different Zeta potential compared to those dispersed in HCl (0.1 N), about –12 to –47 mV versus +0.2 to –2.1 mV, respectively (Table 2). This surface charge feature may be due to the presence of fatty acids that may undergo deprotonation in water and protonation upon acidification, leading to negative and neutral Zeta potential, respectively. In practice, the surface charge neutralization can lead to attraction and then flocculation of lipid droplets. However, no remarkable droplet size difference was observed between SNEDDS in the two media. This suggests that the droplets remained individually dispersed in acidic medium due to the presence of a huge water layer on droplet surface arising from particle hydration. This phenomenon is part of the reasons why DLS size measurement requires complementary microscopy analyses, since nanoparticles solvation may lead to false readings of particle sizes (Eaton et al., 2017; Eltobshi et al., 2018).

Microscopic analysis

SEM study was performed for particle shape and morphology analysis of only solid formulations, while TEM experiments involved all the formulated SNEDDS. The microscopic observation of S-SNEDDS under SEM instrument revealed the presence of particle agglomerates endowed with rough surface morphology, irrespective of the presence or absence of GK (Fig. 2). This microstructure corresponds to a bulky matrix within which several channels form in aqueous media (Hu et al., 2012; Dash et al., 2016), facilitating water infiltration and spontaneous dispersion of nanodroplets upon hydration. Further, TEM experiments allowed to confirm the presence of SNEDDS in all the diluted formulations (with or without GK). As illustrated in Fig. 2, TEM analysis unveiled the presence of individually dispersed particles in nanometric range (around 10 nm), confirming the nanoparticulate nature of the prepared formulations. However, the observed particle sizes disagree with the data from DLS experiments, where mean sizes were found to be greater than 50 nm. Although previous studies have reported SNEDDS with similar DLS sizes (e.g. around 100 nm) (Khoo et al., 1998; Kassem et al., 2016; Dou et al., 2018), the difference between TEM and DLS particle sizes may be due to particle solvation that results in large hydrodynamic diameters (Eaton et al., 2017; Eltobshi et al., 2018). Nevertheless, the overall outcomes from microscopic analyses confirm good self-emulsification efficiency of our lipid-based formulations, which would lead to improved bioavailability and enhanced therapeutic efficacy of the encapsulated active ingredients (Tang et al., 2008; Li et al., 2011; Memvanga et al., 2013a).

In vitro hemolytic effects

Since *Plasmodium falciparum* parasites are mainly located in the blood stream, erythrocytes represent the site of great interest for investigating a potential malaria therapy. When induced by the compounds under investigations, the lysis of

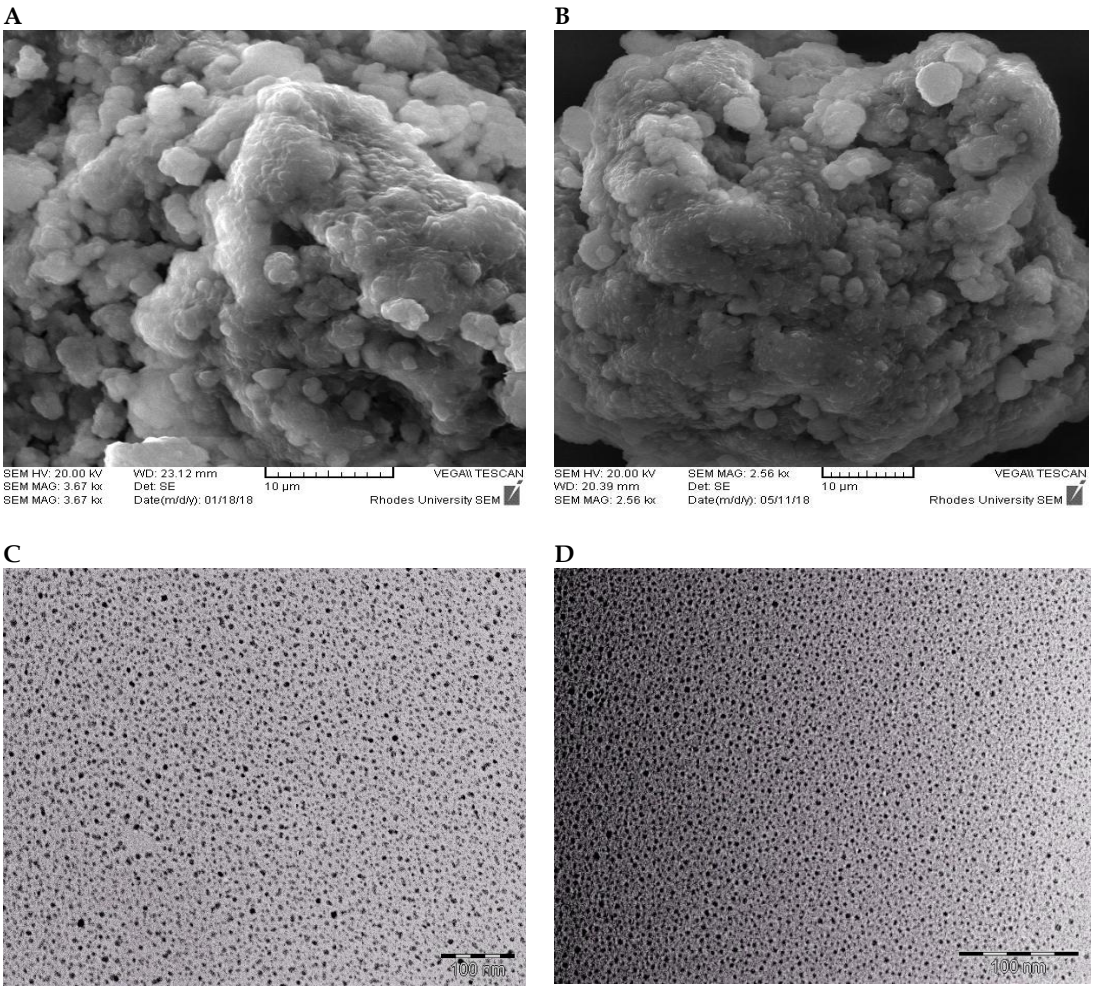

Figure 2. Representative scanning electron microscopy **(A-B)** and transmission electron microscopy **(C-D)** micrographs of the prepared formulations.

(A) Blank-(S-SNEDDS); **(B)** GK-S-SNEDDS; **(C)** Blank-S-SNEDDS; **(D)** GK-S-SNEDDS.

S-SNEDDS: solid self-nanoemulsifying drug delivery system; GK: *Garcinia kola*.

erythrocytes can cause false positives of the antimalarial assays. As the activity was expected to arise from the antiparasitic action of GK constituents in the systemic circulation (and not from any hemolytic actions), the *in vitro* hemolytic study was performed to ensure trustworthy readings of antimalarial effects. Interestingly, both the extract and the formulation showed negligible hemolytic effect (<5%). The hemotoxicity of Cremophor EL, which is also present in injectable Taxol®, might have been reduced due to its low concentration and the presence of other excipients. Nevertheless, this estimated *in vitro* erythrocyte toxicity may be largely minimized *in vivo* due to metabolism and lipid digestion that occurs in the gastrointestinal tract (Memvanga et al., 2013b; 2015).

In vivo antimalarial activity

The antimalarial activity of GK-SNEDDS solution, GK-SNEDDS suspension and GK-S-SNEDDS was assessed in a *Plasmodium berghei* mouse model of malaria. This allowed to determine whether their biological effects could or not reduce parasitemia at early stage of infection. GK-SUS-1,

Table 3. Antimalarial activity in infected mice on day 4 of the treatment.

Product designation	Antimalarial activity (%) at different doses of GK		
	0 mg/kg	100 mg/kg	200 mg/kg
GK-SNEDDS solution[1]	9.8 ± 1.8*	59.6 ± 3.3	77.9 ± 2.9*
GK-SNEDDS suspension	9.3 ± 0.8*	60.1 ± 4.2	73.8 ± 3.6*
GK-S-SNEDDS	9.1 ± 0.9*	58.2 ± 2.9	74.2 ± 2.5*
GK-SUS-1	0.2 ± 0.1[ns]	>nd[4]	62.5 ± 2.4[ns]
GK-SUS-2	0.6 ± 0.3[ns]	nd	61.4 ± 3.0[ns]
N'sansiphos[2]	0.8 ± 0.2	nd	64.1 ± 2.8
Quinine[3]	0.8 ± 0.3	nd	86.6 ± 4.1

[1]The GK-free SNEDDS formulations (liquid and solid blank SNEDDS) were considered as test samples at 0 mg/kg; [2]The placebo contained sugar (65%), water (35%), nipagin (0.08%) and nipasol (0.02%); [3]The blank is only composed of water; [4]nd: not determined. Values are presented as mean ± S.E.M., n = 5. *p< 0.05, significantly different from N'sansiphos, the commercialized GK-based syrup; ns: not significantly different from N'sansiphos. One-way ANOVA followed by post-test Tukey.

GK-SUS-2, N'sansiphos syrup and quinine were used as controls. Data from antimalarial assessment are summarized in Table 3.

The unloaded SNEDDS showed antimalarial activity of 9.1–9.8%, while the aqueous placebo vehicles exhibited <0.8% chemosuppression. This observation agrees with the previous studies that reported inherent antimalarial activity of lipid-based vehicles (Kumaratilake et al., 1997; Joshi et al., 2008; Memvanga and Préat, 2012; Memvanga et al., 2013a). With a daily dose of 100 mg/kg consecutively administered over four days, all the GK-loaded SNEDDS formulations caused parasitemia suppression of about 60%. This activity was significantly enhanced (up to about 78%) when a dose regimen of 200 mg/kg/day was used (Table 3). Nevertheless, these results are to be further confronted with data from the pharmacokinetic investigations at later stage of product development. Noteworthy, both GK-SUS-1 and GK-SUS-2 administered at 200 mg/kg/day showed lower antiparasitic activity (61–64%) than the GK self-nanoemulsifying formulations. There were no significant differences in antimalarial activities of GK-loaded SNEDDS formulations (100 mg/kg), N'sansiphos (200 mg/kg), GK-SUS-1 (200 mg/kg) and GK-SUS-2 (200 mg/kg) with p>0.05. Interestingly, the activity of GK-loaded lipid-based systems (200 mg/kg) was significantly higher than that of N'sansiphos (200 mg/kg), which is a Congolese licensed GK-based syrup currently used in malaria treatment (p<0.05).

Although the prepared formulations did not completely delay the course of early infection in mice (same as quinine that showed even higher activity). It appears evident that GK (with or without SNEDDS) exhibits antimalarial activity at the blood stage, which is consistent with previous studies (Kabangu et al., 1987; Farombi, 2003; Antia et al., 2010; Farombi et al., 2013; Oluwatosin et al., 2014; Konziase, 2015; Tshibangu et al., 2016). Nevertheless, we hypothesized that the use of extended dose regimen and prolonged treatment duration would provide better clearance of *Plasmodium* parasites for overcoming recrudescence and resistance development. Apart from the GRAS status of the excipients used (Memvanga and Préat, 2012), the observed antimalarial activity is encouraging for GK-based product development, since the safety profiles of both GK and GB-1 were reported to be in good standing, with no acute toxicity seen below 5000 mg/kg and 200 mg/kg respectively (Iwu, 1985; Farombi et al., 2000).

CONCLUSIONS

The present study presents SNEDDS formulations containing ethanolic extract of *Garcinia kola*'s seeds (GK) for potential oral management of malaria. The encapsulation of GK in the lipid-based

vehicles was successfully achieved with an associated enhanced solubility and good self-emulsification profile. Upon dispersion, the prepared formulations demonstrated attractive self-nanoemulsifying characteristics, including the ability to generate nanosized droplets, which promotes dissolution of GK constituents. Following oral administration, SNEDDS exhibited much greater *in vivo* antiparasitic activity in *Plasmodium berghei*-infected mice than unformulated GK and a branded GK-based antimalarial syrup. The use of SNEDDS technology is therefore promising for improving herbal-based malaria therapy in developing countries. Nevertheless, further investigations are needed for (i) standardization and stability assessment of GK-based formulations through quantitative analysis of the biflavonoids (GB-1, GB-1a, GB-2) to establish quality control tools for potential product development, (ii) evaluation of the influence of lipolysis, and (iii) assessment of oral bioavailability, which is key to the translation of the preclinical performance of plant extracts to potential therapeutic efficacy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This work was partially supported by the National Research Foundation of South Africa and the Sandisa Imbewu Programme at Rhodes University. Grady K. Mukubwa and Christian I. Nkanga are indebted to the NGO *Förderverein Uni Kinshasa e.V.*- BEBUC/*Else-Kroener-Fresenius Stiftung & Holger-Poehlmann* for their advice and moral support. The technical assistance provided by Guy Midingi (from the *Institut National de Recherches Biomédicales*, Kinshasa, DR Congo) is gratefully acknowledged.

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Contribution	Mukubwa GK	Nkanga CI	Buya AB	Mbinze JK	Krause RWM	Memvanga PB
Concepts or ideas						x
Design	x	x	x	x	x	x
Definition of intellectual content	x	x		x	x	x
Literature search	x	x	x		x	x
Experimental studies	x	x	x	x		
Data acquisition	x	x	x	x		
Data analysis	x	x	x	x	x	x
Statistical analysis	x	x		x	x	x
Manuscript preparation			x	x		x
Manuscript editing	x	x			x	x
Manuscript review	x	x	x	x	x	x

Citation Format: Mukubwa GK, Nkanga CI, Buya AB, Mbinze JK, Krause RWM, Memvanga PBSI (2020) Self-nanoemulsifying drug delivery systems (SNEDDS) for oral delivery of *Garcinia kola* seeds ethanolic extract: formulation and *in vivo* antimalarial activity. J Pharm Pharmacogn Res 8(3): 177–190.