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Article

In vivo antimalarial activity of self-nanoemulsifying drug delivery systems containing ethanolic extract of *Morinda lucida* in combination with other Congolese plants extracts

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Morinda lucida leaves are largely used by Congolese traditional healers for the treatment of uncomplicated malaria. The antimalarial activity of their ethanolic extract has been confirmed both *in vitro* and *in vivo*. However, the development of relevant formulations for potential clinical application is hampered since the active ingredients contained in this extract exhibit poor water solubility and low oral bioavailability. Hence, this work aims not only to develop self-nanoemulsifying drug delivery systems (SNEDDSs) for oral delivery of the ethanolic extract of *Morinda lucida* (ML) but also to evaluate its oral antimalarial activity alone and in combination with other Congolese ethanolic plant extracts (*Alstonia congensis*, *Garcinia kola*, *Lantana camara*, *Morinda morindoides* or *Newbouldia laevis*). Based on the solubility of these different extracts in various excipients, SNEDDS preconcentrates were prepared, and 200 mg/g of each plant extract were suspended in these formulations. The 4-day suppressive Peter's test revealed a significant parasite growth inhibiting effect for all the extract-based SNEDDS (from 55.0 to 82.4 %) at 200 mg/kg. These activities were higher than those of their corresponding ethanolic suspensions given orally at the same dose ($p<0.05$). The combination therapy of ML-SNEDDS with other extract-based SNEDDS exhibited remarkable chemosuppression, ranging from 74.3 % to 95.8 % (for 100 + 100 mg/kg) and 86.7 % to 95.5 % (for 200 + 200 mg/kg/day). In regard to these findings, SNEDDS suspension may constitute a promising approach for oral delivery of ML alone or in combination with other antimalarial plants.

Keywords: *Morinda lucida*. Congolese plants. SNEDDS suspension. Combination therapy. Antimalarial activity.

INTRODUCTION

Affecting 12 million patients and causing nearly 100,000 deaths per year, malaria is the most prevalent parasitic disease and the foremost cause of morbidity and mortality in the Democratic Republic of the Congo (WHO, 2019; PNLP, 2011). Nowadays, the treatment of choice for malaria is based on the association of an artemisinin-type compound with another drug (WHO, 2010). However, the use of traditional medicinal plants remains entrenched in the healing practices of the Congolese population. The use of plant-based products represents a progressive drift in the primary healthcare system of the Democratic Republic of the Congo (DR Congo), in line with the objectives of the "Traditional Medicine Strategy" proposed by the World Health Organization (Memvanga *et al.,* 2015; WHO, 2013).

The potential of many Congolese medicinal plants to yield new antimalarial drugs has been confirmed both *in vitro* and *in vivo* (Memvanga *et al.,* 2015). Among these plants, one can cite *Morinda lucida* Benth (Rubiaceae)

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(Makinde, Obih, 1985; Oko *et al.,* 2012; Unekwuojo, Omale, Aminu*,* 2011; Umar *et al.,* 2013; Memvanga *et al.,* 2015; Adebayo, Adewole, Krettli, 2017). The leaves of *Morinda lucida* are frequently used in the Congolese traditional medicine for the treatment of malaria and febrile diseases. For malaria treatment, *Morinda lucida* is used either alone or in combination with other plants, including *Alstonia congensis*, *Garcinia kola, Lantana camara, Morinda morindoides* or *Newbouldia laevis* (Kambu, 1990).

According to the literature, the management of malaria by these plant extracts is due to the presence of flavonoids (quercetin), triterpenoid acids (ursolic and oleanolic acids), anthraquinones, tannins and/or alkaloids (Sittie *et al.,* 1999; Koumaglo *et al.,* 1992; Cimanga *et al.,* 2006). In fact, quercetin, ursolic acid and oleanolic acid have been reported to exhibit pronounced or good *in vitro* antiplasmodial activity (with IC_{50} values of 3.2 µg/ml, 3.1 µg/ml and 15.2 µg/ml, respectively) (Cimanga *et al.,* 2006; Memvanga *et al.,* 2015). In addition, some of these secondary metabolites were reported to exhibit much higher antimalarial activity in *Plasmodium berghei*-infected mice (Adaramoye *et al.,* 2014; Memvanga *et al.,* 2015). Nevertheless, the limited water solubility and low oral bioavailability of these phytoconstituents remain a deep concern for further pharmaceutical developments and biomedical applications (Mukubwa *et al.,* 2020). Thus, the need for improving the aqueous solubility (and dissolution rate) of these bioactive compounds is highly desired to favor formulation development and increase product bioavailability, which may result in enhanced efficacy (Mukubwa *et al.,* 2020).

As one of the emerging formulation strategies, self-nanoemulsifying drug delivery systems (SNEDDS) have shown great promise for improved solubility and delivery of poorly water soluble phytoconstituents (e.g. curcumin) (Memvanga, Coco, Préat*,* 2013) and herbal extracts such as *Garcinia kola* and *Ginkgo biloba* (Mukubwa *et al.,* 2020; Tang *et al.,* 2008a) and *Diospyros kaki* extracts (Li *et al.,* 2011). In fact, SNEDDS preconcentrates are isotropic mixtures of oils (i.e. pure triglyceride oils, mixed glycerides, etc.), water-soluble surfactants and hydrophilic co-emulsifiers or co-solvents that form oil-in-water nanoemulsions on mild agitation in the presence of water (Müllertz *et al.,* 2010; Li *et al.,* 2011; Memvanga, Préat, 2012). Recent technological advances have led to the development of several SNEDDS formulations like solutions, suspensions, supersaturables, solids, etc. (Tang *et al.,* 2008b).

In comparison with other lipid-based formulations, SNEDDSs 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.,* 2008b; Li *et al.,* 2011; Memvanga, Préat, 2012; Memvanga, Coco, Préat*,* 2013). This technology is promising for addressing the critical issue of commercial availability and affordability of nanomedicine in developing countries.

The rationale behind the use of self-emulsifying lipid-based formulations as drug carriers is also to utilize the inherent therapeutic potential of fatty acids (oleic acid, linoleic acid, etc.) present in the formulation or released during the *in vivo* lipolysis. Indeed, oleic and linoleic acids are of great benefit in the treatment of blood-stages of *Plasmodium falciparum* infections (Krugliak *et al.,* 1995; Kumaratilake *et al.,* 1992). They have also the ability to augment neutrophil killing of *Plasmodium falciparum* asexual blood forms. Moreover, these fatty acids may also stimulate a protective immune response by the activation of Th2 type CD4 + T cells to increase the clearing of parasitemia (Kumaratilake *et al.,* 1997; Carrillo, Cavia, Alonso-Torre*,* 2012). Fatty acids such as oleic acid have also been reported to inhibit the endothelial expression of the vascular cell adhesion molecule 1 (VCAM-1), E-selectin and the intercellular adhesion molecule 1 (ICAM-1) in several endothelial cells (Carrillo, Cavia, Alonso-Torre*,* 2012), thereby reducing cytoadherence, clumping and sequestration of parasitized red blood cells. In addition, it was reported that many fatty acids have the potential to inhibit the type II fatty acid synthesis pathway (FAS II) of the parasite *Plasmodium falciparum* and have been suggested as a likely strategy to combat the liver-stage of the parasite (Carballeira, 2008; Tarun, Vaughan, Kappe*,* 2009).

Therefore, the present study deals with the development of SNEDDS suspension containing *Morinda lucida* (ML) ethanolic extract and the evaluation of its *in vivo* antimalarial activity in *Plasmodium berghei*-

infected mice, and this, alone and in combination with the ethanolic extracts of *Alstonia congensis* (AC), *Garcinia kola* (GK), *Lantana camara* (LC), *Morinda morindoides* (MM) and *Newbouldia laevis* (NL).

MATERIAL AND METHODS

Material

Plant materials

The leaves of AC, LC, ML and MM were collected in Ndjili Township (Province of Kinshasa, DR Congo) in January 2015, whereas the seeds of *Garcinia kola* harvested in the Province of Kongo Central in May 2015 were purchased from local vendors in Lemba Township (Province of Kinshasa). NL's leaves were collected in Nsele Township (Province of Kinshasa) in October 2017. All these plants were identified by Mr. Nlandu and Mr. Mambwana of the *Institut National d'Etudes et de Recherches en Agronomie* (INERA) of the University of Kinshasa. Voucher specimens were deposited in the herbarium of this institute with a voucher number for each species (see Table I). The plant materials were air-dried over two weeks at room temperature and then reduced to powder.

TABLE I - List of selected plants, with their traditional uses and voucher numbers

Botanical and family names	Vernacular name	Traditional uses	Voucher number
Alstonia congensis Engl. (Apocynaceae)	Okulu	Leaves and bark are used in the treatment of fever, malaria, worms, diarrhea, stomach cramps, spleen problems, hernia, etc.	NP200324
Garcinia kola Heckel (Clusiaceae)	Ngadiadia	Seed is used to treat malaria, fever, worms, colic, headache, dysentery, diarrhea and hypertension. It also used as aphrodisiac and stimulator of digestion.	P120897N1
Lantana camara L. (Vebenaceae)	Maka wabo	Leave decoctions are used against malaria, fever, asthma, cough, colds, pharyngitis, constipation and stomach pain.	NP2003258
Morinda lucida Benth. (Rubiaceae)	Endombe	The leaves have been reported to possess antimalarial, laxative, antibacterial, trypanocidal, aortic vasorelaxant, anticancer, genotoxic, hepatoprotective, antispermatogenic, hypoglycemia and antidiabetic properties.	P1700897NL
Morinda morindoides (Baker) Milne-Redhead (Rubiaceae)	Nkongo bololo	Leaves are used to treat malaria, fever, amebiasis, intestinal worms, diarrhea, hemorrhoids, gonorrhea, rheumatism, etc.	P020897NL
Newbouldia laevis (Bignoniaceae)	Mupesi mpesi	Leaves are used to treat malaria, fever, coughs, tooth ache, stomach ache, constipation, diarrhea, breast cancer, septic wounds, eye problems, etc.	P023895NL

Chemicals

Cremophor EL (polyoxyl 35 castor oil) was kindly donated by BASF (Burgbernheim, Germany). Capryol 90 (propylene glycol monocaprylate), Labrafac WL1349 (caprylic/capric acid triglycerides), Labrafil M1944CS (oleoyl polyoxyl glycerides), Labrafil M2125CS (linoleoyl polyoxyl glycerides), Labrasol (caprylocaproyl polyoxyl glycerides), Lauroglycol 90 (propylene glycol monolaurate), Maisine 35-1 (glyceryl monolinoleate) and

Transcutol HP (diethylene glycol monoethyl ether) were kindly provided by Gattefossé (Saint-Priest, France). Aluminum chloride, sodium nitrite, quercetin, gallic acid, caffeic acid, chlorogenic acid, hyperoside, rutin, isoquercitrin, Triton X-100, diphenylboric acid-2-aminoethyl ester and Folin-Ciocalteu reagent were from Sigma-Aldrich (Diegem, Belgium). Sodium carbonate, sodium hydroxide and polyethylene glycol 4000 (PEG 4000) were sourced from Fagron (Waregem, Belgium). Absolute ethanol (99.2 %), ethyl acetate, formic acid, acetic acid and methanol were purchased from Merck (Darmstadt, Germany). Sodium chloride 0.9 %, Tween 80, oleic acid and ethyloleate were gifted by Arauphar (Kinshasa, DR Congo). Quinine was from Pharmakina (Bukavu, DR Congo). Olive oil and groundnut oil were 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).

Animals

NMRI mice (23-27 g, eight weeks of age) obtained from the *Institut National des Recherches Biomédicales* (INRB, Kinshasa, DR Congo) were used in this study. The animals were maintained under conditions of optimum temperature (21 ± 2°C), light (12 h light/dark cycle) and relative humidity (70–80%) with food and water provided *ad libitum*.

Inoculums

The inoculums were the chloroquine-sensitive *Plasmodium berghei* ANKA strain obtained from the Antwerp Tropical Medicine Institute (Belgium) (Memvanga, Préat, 2012).

Methods

Preparation of the crude extracts

Two hundreds grams of each dried and powdered plant material were macerated at room temperature in 1000 ml of ethanol (3 × 24 h), with occasional shaking. The three macerates were pooled, filtered and evaporated to dryness under reduced pressure at 40°C with a rotary evaporator. The obtained extracts were weighed and their yields calculated (Table II). Thereafter, they were stored in amber bottles protected from light until next experiments.

TABLE II - Total phenolic and total flavonoid contents

Plant extracts	Total phenolics (mg gallic acid equivalent/g)*	Total flavonoids (mg quercetin equivalent/g)*	Extraction yield (%)
AC	240.2 ± 12.5	157.7 ± 9.9	11.1 %
GK	113.5 ± 2.3	51.3 ± 3.8	5.3 %
LC	185.9 ± 5.1	102.6 ± 4.2	6.1 %
ML	81.0 ± 5.6	20.3 ± 1.8	12.6 %
MM	83.5 ± 3.4	47.2 ± 6.0	5.8 %
NL	95.2 ± 7.2	38.3 ± 4.6	10.4 %

**Results are expressed as mean ± standard deviation (n=3)*

Thin-layer chromatographic profiles of plant extracts

To achieve chromatographic profiling, the extracts (100 mg/ml) were prepared by maceration in methanol 80 % for 48 hours under constant mechanical agitation. Following filtration, the macerates were concentrated and dried to constant weight in oven at 50°C. The work solutions of each extract consisted of methanolic

solutions of 10 mg/ml, while the standards consisted of methanolic solutions (1 mg/ml) of caffeic acid, chlorogenic acid, quercetin, hyperoside, rutin and isoquercitrin. Aliquots of each work solutions (10 µl) and standards (2 µl) were then spotted on pre-coated silica gel 60 F_{254} plates (10 × 5 cm, on sheet of glass; Merck). The mobile phase employed consisted of ethyl acetate: formic acid: acetic acid: water (100:11:11:27; v/v) (Braz *et al*., 2012). After the development of chromatograms, the plates were dried and spots visualized sequentially in day light and under UV lamp (254 and 366 nm) before and after revelation by Neu's reagent (1% methanolic diphenylboric acid-2-aminoethyl ester and 5% ethanolic polyethylene glycol 400 (10:8, v/v)) (Braz *et al*., 2012). The chromatographic profiles of extracts and standards were comparatively evaluated based on the retention factor (Rf), which was calculated as follow:

$$Rf = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent front}}$$

Quantitative phytochemical testing

Total phenolic contents of AC, GK, LC, ML, MM and NL were determined by using the Folin-Ciocalteu method (Farahpour *et al.,* 2017). In short, 0.5 ml of each extract was mixed with 3 ml of Folin-Ciocalteu reagent (10 %). After 5 min of incubation, an aqueous solution of sodium carbonate (4 ml, 7.5 %) was added to the mixture. The resultant mixture was then kept in the dark at 30°C for 20 min, after which the absorbance was measured on a spectrophotometer at 765 nm. The total phenolic contents were estimated from a calibration curve using gallic acid (in methanol) as a standard. The phenolic contents were expressed as milligrams of gallic acid equivalents per g of dried extract. All the experiments were performed in triplicate.

Total flavonoid contents of AC, GK, LC, ML, MM and NL were determined by the aluminum chloride method, as described by Ravishankar *et al.* (2018) with some modifications. Briefly, in a 10 ml test tube, 0.3 ml of each methanolic extract was mixed with 3.4 ml of methanol 30 % and 0.15 ml of sodium nitrite 0.5 M under stirring. After incubation period of 5 min, 0.15 ml of aluminum chloride 0.3 M were added to the different mixtures that were further allowed to stand 10 min at 30°C. Then, 1 ml of sodium hydroxide 1M and 2.5 ml of distilled water were added to each of the resultant mixtures. The obtained solutions were vortex-mixed and their absorbances measured at 510 nm using spectrophotometer. Total flavonoids were estimated from calibration curves using quercetin (in methanol) as a standard. The results were expressed as milligrams of quercetin equivalent per gram of dried extract. All the experiments were done in triplicate.

Preparation of the extract-based SNEDDS suspensions

The solubility of AC, GK, LC, ML, MM and NL in various excipients was estimated by dissolving increasing quantities of each extract in 3 g of each excipient at room temperature (20 ± 2°C). After 2 h of stirring, the solubilization of AC, GK, LC, ML, MM and NL was visually verified. The absence of undissolved extracts was confirmed using a microscope (Mukubwa *et al.,* 2020; Memvanga, Préat, 2012). All the trials were conducted in triplicate.

Thereafter, the lipid-based formulations were prepared. Firstly, a mixture of surfactants, co-surfactants (Labrasol and/or Cremophor EL) and oil phase (olive oil, Maisine 35-1 and/or Labrafac WL1349) was stirred at room temperature. After 10 min of mixing, the co-solvent (ethanol or Transcutol HP) was slowly added under stirring. Table III summarizes the composition of the three SNEDDS formulations.

To prepare the different SNEDDS suspensions, the mixture of oils, surfactants and co-surfactants, as per the quantities indicated in Table III, were poured into either 1.5 g of ethanol (or Transcutol HP) suspension (1.4 g of each extract/g) (for Formulation F2 and F3) or 3.6 g Transcutol suspension (0.583 g of each extract/g) (for Formulation F1) and stirred at 400 rpm for 2 h at 25 °C for homogenization (Mukubwa *et al.,* 2020).

TABLE III - Composition of the selected self-emulsifying lipid-based formulations

Formulation	Composition
F1	Labrafac WL1349 (0.95 g) Cremophor EL (3.75 g) Labrasol (1.25 g) Maisine 35-1 (0.95 g) Transcutol HP (3.6 g) Extract (2.1 g)*
F2	Olive oil (3.0 g) Maisine 35-1 (3.0 g) Cremophor EL (3.0 g) Transcutol HP (1.5 g) Extract (2.1 g)*
F3	Olive oil (3.0 g) Maisine 35-1 (3.0 g) Cremophor EL (3.0 g) Ethanol (1.5 g) Extract (2.1 g)*

*Each formulation contained 200 mg of extract per gram of unloaded-formulations (or vehicles)

Preparation of the ethanolic suspensions

The ethanolic suspensions (SUS) containing different extracts were prepared by dispersing 500 mg of each extract in 5 ml of ethanol under gentle agitation at 400 rpm for 10 min at 25°C.

Hemolysis test

The hemolysis test was performed as previously described (Mukubwa *et al.,* 2020; Memvanga, Coco, Préat, 2013). Briefly, 20 ml of human blood from two healthy volunteers was centrifuged (2000 × g, 10 min) and the plasma discarded. Subsequently, the red blood cells were washed three times and diluted with sodium chloride 0.9 % to obtain hematocrit level of 8%. The resulting erythrocyte suspension (9.9 ml) was then incubated with 0.1 ml of the formulations (0–20 mg/ml in PBS) or the dissolved extracts (0–10 mg/ml in ethanol). Triton X-100 (1%, w/v) and ethanol were used as positive and negative controls, respectively. The isotonic solution of sodium chloride 0.9 % was used as standard. The hemoglobin released in the supernatants after centrifugation (2000 × g, 5 min, 37 °C) was quantified by spectrophotometric analysis at 540 nm. The percentage of hemolysis was determined using the following formula:

$$\% \text{ Hemolysis} = \frac{\text{ast - anc}}{\text{apc - anc}} \text{ x } 100$$

where ast = absorbance of the sample-tests, apc = absorbance of the positive control, anc = absorbance of the negative control.

The hemolytic activity of each sample was tested three times.

In vivo antimalarial activity

To assess the potential *in vivo* antimalarial activity of SNEDDS suspension of ML alone and in combination with AC, GK, LC, MM and NL, the classical 4-day suppressive test was used as previously described (Peeters, 1965; Memvanga, Coco, Préat, 2013; Mukubwa *et al.,* 2020). Shortly, on day 0, the animals (5 mice per group) were inoculated intraperitoneally with 300 µl of physiological saline containing approximately 1×10^7 *Plasmodium berghei* parasitized erythrocytes. Two hours after inoculation, test mice were orally given 0.1 ml of ML-SNEDDS suspension, AC-SNEDDS suspension, GK-SNEDDS suspension, LC-SNEDDS suspension, MM-SNEDDS suspension, NL-SNEDDS suspension (0 and 200 mg/kg/day), ML-SUS, AC-SUS, GK-SUS, LC-SUS, MM-SUS and NL-SUS (0 and 200 mg/kg/day) for 4 consecutive days. Thereafter, the combinations of ML-SNEDDS suspension (0.1 ml) with self-emulsifying lipid-based formulations of AC, GK, LC, MM and NL (0.1 ml) were also administered by oral gavage in mice (0, 100 and 200 mg/kg/day of each formulation, 4 days). Prior to administration to mice, 1.2 g of each extract-based SNEDDS suspension (or 1 g of blank SNEDDS) was gently dispersed in approximately 3 ml of water, whereas the ethanolic suspension was added over an equal volume of water and then homogenized.

In the positive control groups, the mice received 0.1 ml of an aqueous solution of quinine (25 mg/kg/

day, oral). The final group of mice was infected but not treated. On day 4, a thin film was made from a tail-blood sample from each mouse and stained with giemsa. The level of parasitemia was then determined by counting, in random fields of a light microscope (oil immersion, 1000 × magnification), the number of parasitized erythrocytes per 1000 erythrocytes (Memvanga, Coco, Préat, 2013; Mukubwa *et al.,* 2020). Additionally, the antimalarial activity was calculated as: [(A-B)/A] × 100, where A is the mean parasitemia in the untreated group and B the mean parasitemia in the test groups.

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).

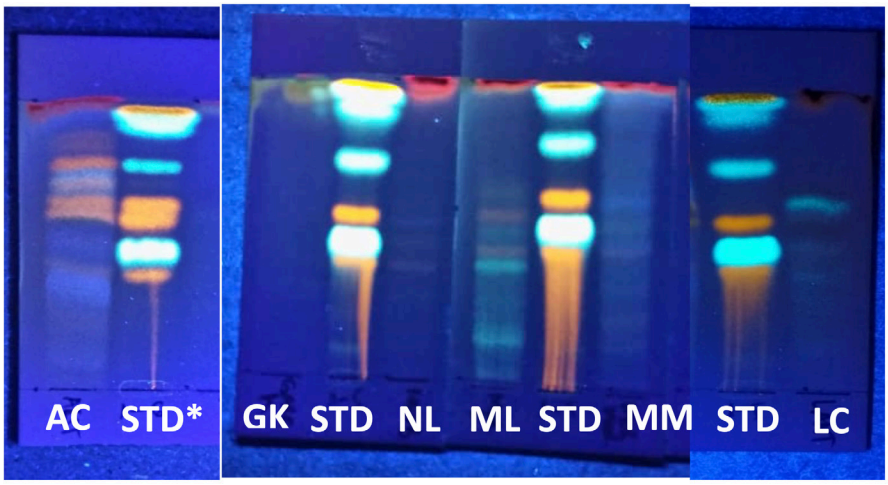
Statistical analyses

Significant differences between the antimalarial activity of SNEDDS, SUS and controls were compared by one-way ANOVA with Tukey's post-hoc test (with a level of significance of $p < 0.05$).

RESULTS AND DISCUSSION

Thin-layer chromatographic profiles of plant extracts

Thin-layer chromatography (TLC) was performed for fingerprint profiling of each plant extract. However, only polyphenols (flavonoids and phenolic acids) were investigated as they are known to be one of the major metabolites responsible for the antioxidant and antimalarial activities of the selected plants (Memvanga *et al.,* 2015). The antioxidant activity may contribute to the malaria therapy when reactive oxygen species (ROS) are overproduced by activated neutrophils in the human host. Indeed, overproduction of ROS can overwhelm the antioxidant defense system and lead to some immune pathologies as well as complications of malaria, though optimal ROS production is essential for intraerythrocytic killing of parasites (Percário *et al.,* 2012). TLC profiles of different extracts observed under UV lamp at 366 nm are shown in Figure 1. In addition, Table IV shows the Rf values of the standards used in the development of TLC, as well as the colors for the respective spots. These data confirm the presence of phenolic and flavonoid compounds (such as quercetin) that possess both antimalarial and antioxidant activities. Additionally, the appearance of compounds in different bands can be useful for further identification and authentication of plant drugs as well as product standardization at the later stage of formulation development. Indeed, in several pharmacopoeias, TLC has been reported as being a reliable method for the analysis of medicinal plants and herbal drugs.



**STD = standards*

FIGURE 1 – Illustrative fingerprint profiling of the investigated plant extracts: Showing the TLC plates (pre-coated silica gel 60 F_{254} plates) and chromatograms (mobile phase composed of ethyl acetate: formic acid: acetic acid: water – 100:11:11:27; v/v) for different extracts and standards after revelation with Neu's reagent (Natural products-polyethylene glycol (NP/PEG) reagent) and visualization at 366 nm.

TABLE IV - Characteristics of chromatograms profiles of the analyzed extracts (Rf values of the standards used in TLC and their respective colors)

Extracts	Rf of found spots	Fluorescence	Corresponding standards
AC	0.11	Blue	-
	0.34	Blue	-
	0.43	Orange	Rutin
	0.61	Orange	Hyperoside
	0.66	Orange	Isoquercitrin
	0.71	Blue	-
	0.74	Orange	-
	0.81	Orange	-
	0.86	Orange	-
	0.90	Orange	-
GK	0.43	Orange	Rutin
	0.96	Yellow-brown	-
LC	0.40	Aquamarine	-

TABLE IV - Characteristics of chromatograms profiles of the analyzed extracts (Rf values of the standards used in TLC and their respective colors)

Extracts	Rf of found spots	Fluorescence	Corresponding standards
	0.52	Aquamarine	Chlorogenic acid
	0.64	Aquamarine	-
	0.97	Orange	Quercetin
ML	0.03	Aquamarine	-
	0.14	Aquamarine	-
	0.37	Aquamarine	-
	0.43	Orange	Rutin
	0.52	Aquamarine	Chlorogenic acid
	0.55	Orange	-
	0.61	Aquamarine	-
MM	0.03	Aquamarine	-
	0.14	Aquamarine	-
	0.25	Aquamarine	-

TABLE IV - Characteristics of chromatograms profiles of the analyzed extracts (Rf values of the standards used in TLC and their respective colors)

Extracts	Rf of found spots	Fluorescence	Corresponding standards
	0.33	Aquamarine	-
	0.37	Aquamarine	-
	0.43	Orange	Rutin
	0.52	Aquamarine	Chlorogenic acid
	0.61	Aquamarine	-
	0.97	Aquamarine	Caffeic acid
NL	0.43	Orange	Rutin
	0.52	Aquamarine	Chlorogenic acid
	0.54	Aquamarine	-

Total phenolic and flavonoid contents

The quantitative determination of phytochemicals (total phenolic and flavonoid contents) of the investigated extracts was performed by means of spectrophotometric methods. All the extracts contained both phenolic and flavonoid compounds. The results from phenolic and flavonoid content determination in plant extracts are summarized in Table II and appear to be in agreement with data from TLC analysis. For future experimental reference, the flavonoids and phenolics contents in each SNEDDS formulation can be estimated by considering the results presented in Table II divided by 5, based on the amount extract (200 mg) dispersed in SNEDDS preconcentrate (1 g or 1000 mg).

Preparation of SNEDDS suspensions

To select the excipients for formulation of self-emulsifying systems, the solubility of each extract was determined in different vehicles. All the extracts exhibited poor solubility (< 20 mg/g) in ethyl oleate, oleic acid, olive oil, groundnut oil, Maisine 35-1, Lauroglycol 90 and Tween 80. This solubility was ranged from 25 to 33 mg/g for Capryol 90, Labrasol, Labrafac WL1349, Labrafil M2125CS, Labrafil M1944CS and Cremophor EL. The tested co-solvents (Transcutol HP and ethanol) yielded the highest solubility for all the extracts (between 60 and 105 mg/g). Maisine 35-1 and olive oil were chosen because, after *in vivo* lipolysis, they may likely release oleic acid and/or linoleic acid, which are reputed for intrinsic antimalarial activity (leading to potential synergy with the active ingredients).

Next, three SNEDDS preconcentrates previously characterized in terms of nano-dispersity, droplet size, potential zeta, emulsification time and kinetic stability were prepared for the present study (Mukubwa *et al.,* 2020; Zhu *et al.,* 2009). The solubility of each extract in these lipid-based formulations was evaluated. The obtained results are presented in Table V, and show that no extracts exhibited solubility higher than 150 mg/g. This would make a serious bottleneck to product development at the late phase of clinical development, since the daily dose of all these extracts in humans is estimated to be in the range of 600–1000 mg (Memvanga *et al.,* 2015; Reagan-Shaw, Nihal, Ahmad, 2008; Cimanga *et al.,* 2019). Therefore, the possibility of developing suspensions of ML, AC, GK, LC, MM and NL in SNEDDS formulations was investigated (Table III). Indeed, lipid-based suspensions (e.g. SNEDDS suspension), a relatively unexplored formulation type, perform just as good as lipid-based solutions (e.g. SNEDDS solution), as demonstrated for some lipophilic compounds (i.e. griseofulvin, phenytoin, danazol, etc.) (Larsen *et al.,* 2008; Mu, Holm, Müllertz*,* 2013).

In line with our SNEDDS, the formulation F3 was found to be a suitable vehicle for the preparation of our different SNEDDS suspensions, due to the ability of ethanol to better disperse different extracts compared to Transcutol HP (which is present in Formulations F1 and F2). This stands to the reason since ethanol was used as solvent for the preparation of plant extracts. Interestingly, the unloaded-formulation F3 was found to be nontoxic against Caco-2 intestinal cells at the administered doses (IC_{50} ranged between 5.0 and 7.7 mg/ml) (Memvanga, Préat, 2012).

TABLE V - Solubility of extracts in the different blank-SNEDDS

	Solubility (mg extract/g formulation)					
	AC	GK	LC	ML	MM	NL
F1	< 60	< 120	< 95	< 125	< 110	nd*
F2	< 85	< 120	< 100	< 110	< 125	Nd
F3	< 85	< 135	< 120	< 130	< 140	< 150

** nd = not determined*

Hemolysis

The hemolytic activity was evaluated to determine whether SNEDDSs or extracts can likely cause damage to the erythrocyte membrane, since the latter is the primary target of antimalarial treatment (Memvanga, Coco, Préat, 2013; Memvanga *et al.,* 2015). Noteworthy, all the formulations and extracts showed negligible hemolytic effects (less than 3 %) (data not shown). The hemotoxicity of Cremophor EL, which is widely used in oral and intravenous drug formulations, might have been reduced due to its low concentration (≤ 6 mg per 100 ml of erythrocyte suspension) and its mixture with other excipients generally known to be safe (e.g. olive oil and Maisine 35-1) (Müllertz *et al.*, 2010). Nonetheless, it is important to note that, after oral administration, the estimated *in vitro* erythrocyte toxicity may be considerably reduced due to lipid digestion and metabolism of extracts in the gastrointestinal tract. In fact, when counting parasitemia of the treated mice, no sign of hemolysis or anemia was observed.

In vivo antimalarial activity

The present *in vivo* investigation was carried out in the context of the previously reported antimalarial activity of the plant materials used herein (Memvanga *et al.,* 2015; Cimanga *et al.,* 2019). The antimalarial activity of extract-based SNEDDS suspensions and SUS was determined in mice earlier infected with *Plasmodium berghei*.

Based on the results of the assessment of *in vivo* antimalarial activity (Table VI), it is evident that all the extract-based SNEDDS suspensions possess blood schizontocidal activities. At the dose of 200 mg/kg/day, all the formulations exhibited average % of chemosuppression in the range of 50–60% for GK-SNEDDS suspension, 60–80% for AC-SNEDDS, LC-SNEDDS and ML-SNEDDS suspensions, and 80–90% for MM-SNEDDS and NL-SNEDDS suspensions. Based on the thresholds for *in vivo* activity of antimalarial extracts proposed by Rasoanaivo *et al.* (2004), all these formulations exhibited moderate to good antimalarial activity in treated mice. These parasitemia suppressions may be due, inter alia, to the presence of flavonoids (4 to 32 mg/g of SNEDDS) and phenolic compounds (16 to 48 mg/g of SNEDDS). A clear difference was observed between the chemosuppression of GK-SNEDDS suspension from this study and that of Mukubwa *et al.* (2020). This could be explained by the difference in the geographical location and harvest time of GK materials used in the two studies. The unloaded SNEDDS (for 0.1 ml/day) showed a chemosuppression of 8.6%, which is consistent with the literature regarding the intrinsic antimalarial activity of fatty acids contained in the lipid-based vehicles (i.e. inhibition of the fatty acid synthesis (FAS) II pathway, ability to augment neutrophil killing, stimulation of protective immune response, etc.) (Kumaratilake *et al.,* 1997; Memvanga, Préat, 2012; Memvanga, Coco, Préat, 2013; Mukubwa *et al.,* 2020).

TABLE VI - *In vivo* antimalarial activity of extract-based SNEDDS suspension and SUS in monotherapy

Extract/ drug	Formulation/ vehicle	Dose (mg/kg/day)	Parasitemia (%)	Antimalarial activity (%)*
AC	SNEDDS suspension	200	13.8 ± 1.6	68.3
	SUS	200	19.4 ± 2.8	55.6
GK	SNEDDS suspension	200	19.6 ± 2.1	55.0
	SUS	200	27.0 ± 2.4	38.1
LC	SNEDDS suspension	200	16.6 ± 1.5	61.9
	SUS	200	22.8 ± 2.0	47.8
ML	SNEDDS suspension	200	15.8 ± 1.9	63.7
	SUS	200	23.4 ± 3.3	46.3
MM	SNEDDS suspension	200	8.6 ± 1.1	80.2
	SUS	200	15.8 ± 1.2	63.7
NL	SNEDDS suspension	200	7.7 ± 0.6	82.4
	SUS	200	14.7 ± 1.3	66.2
Quinine	Water	25	5.5 ± 0.4	87.3
-	SNEDDS	-	39.8 ± 2.7	8.6
-	SUS	-	43.2 ± 3.4	0.9
-	Water	-	43.3 ± 4.6	0.7
Untreated	-	-	43.6 ± 3.8	-

**The average activity of the different formulations (and vehicles) was determined on day 4 of treatment.*

Significant differences were observed between the parasitemia and the antimalarial activity of extract-based SNEDDS suspensions and those of the extract-based SUSs ($p<0.05$). At the same dose as before (i.e. 200 mg/kg × 4 days), extracts administered in a mixture of ethanol:water (50:50, v/v) reduced parasitaemia by 30–40% (for GK), 40–60% (for AC, LC and ML) and 60-70% (for MM and NL). On the other hand, the blank SUSs exhibited no antimalarial activity (< 1%). Used as controls, quinine and water exhibited antiparasitic activity of 87.3% and of 0.7%, respectively.

In order to overcome the incomplete chemosuppression observed in the monotherapy which is often subject to the rise of *Plasmodium* drug resistance, combination therapies of SNEDDS suspensions were introduced and assessed in *Plasmodium berghei*-infected mice based on the traditional use of ML. The combination of ML-SNEDDS suspension with NL-SNEDDS suspension (oral dose of "100 + 100" mg/kg/day) exhibited a percentage of parasite suppression greater than 95 % (Table VII). However, at the same dose, the SNEDDS suspension-based therapeutic combinations of "ML + AC", "ML + GK", "ML + LC" and "ML + MM" exhibited percentages of parasite suppression between 70 and 80 %. Nevertheless, it is interesting to note that a significant improvement in antimalarial

activity (85–95%) was observed in using a double dose (i.e. "200 + 200" mg/kg per day) of theses combinations. In addition, blank-SNEDDS (for 0.1 + 0.1 ml/day) showed antimalarial activity of 13.2%. These results are appealing but not conclusive of an additive or synergistic effect of MM-SNEDDS suspension in combination with other plant extracts. Therefore, further investigations using isobologram analysis are needed to provide more information and enhance the biological understanding of these emerging formulations.

TABLE VII - *In vivo* antimalarial activity of ML-based SNEDDS suspension in combination therapy at different doses

Non-fixed product combination designation	100 + 100 mg/kg/day		200 + 200 mg/kg/day	
	Parasitemia (%)	Antimalarial activity(%)*	Parasitemia (%)	Antimalarial activity(%)*
ML-SNEDDS suspension + AC-SNEDDS suspension	9.6 ± 1.0	79.0	5.8 ± 0.7	86.7
ML-SNEDDS suspension + GC-SNEDDS suspension	10.3 ± 0.9	76.3	5.4 ± 0.5	87.6
ML-SNEDDS suspension + LC-SNEDDS suspension	10.2 ± 1.2	76.6	2.0 ± 0.4	95.5
ML-SNEDDS suspension + MM-SNEDDS suspension	11.2 ± 0.8	74.3	2.6 ± 0.3	94.0
ML-SNEDDS suspension + NL-SNEDDS suspension	1.8 ± 0.3	95.8	nd***	nd***

**The average activity of the different combinations was determined on day 4 of treatment.*
***The parasitemia and parasitemia suppression (antimalarial activity) in the blank-SNEDDS treated group (0.1 ml + 0.1 ml) were 37.8 ± 3.1 % and 13.2 ± 0.8 %, respectively.*
**** nd = not determined*

CONCLUSION

The present investigation was a preliminary study that aimed to evaluate the ability of SNEDDS to increase the *in vivo* antimalarial activity of the ethanolic extract of ML when administered alone or in combination with other plant extracts (AC, GK, LC, MM and NL), with which it is associated in the Congolese traditional medicine. The results clearly demonstrate for the first time that the antimalarial activity of all the extracts is enhanced by formulating them in SNEDDS suspension, in comparison with their ethanolic suspension. The combination of ML-based SNEDDS suspension with the other plants extracts also exhibited marked antimalarial activity. The combination therapy with SNEDDS containing extracts appears to be therefore a promising approach for the treatment of uncomplicated malaria in developing countries. Nevertheless, further studies are needed to standardize the different plant extracts and assess their oral bioavailability.

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