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ORIGINAL ARTICLE

Potentiation of the bactericidal activity of *Harungana madagascariensis* Lam. ex Poir. (*Hypericaceae*) leaf extract against oral bacteria using poly (D, L-lactide-co-glycolide) nanoparticles: *in vitro* study

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Abstract

Harungana madagascariensis Lam. ex Poir. (*Hypericaceae*) is known to have biological properties with mainly antibacterial, antifungal, and antiviral effects. The objective of this study was to investigate the *in vitro* bactericidal activity of the ethyl acetate *H. madagascariensis* leaf extract (*HLE*) on the main oral bacterial strains largely implicated in dental caries and gingivitis infections, and the possibility of potentialization of *HLE* antibacterial effects using the poly (D,L-lactide-co-glycolide) nanoparticles (PLG-NP). The microdilution technique and the interfacial polymer deposition following the solvent diffusion method were used to investigate the *in vitro* bactericidal activity of ethyl acetate *HLE* and to prepare nanoparticles, respectively. *HLE* showed significant bactericidal effects against the bacterial strains tested, with minimal bactericidal concentration (MBC) to 5×10^2 mg/l or less, except for *Lactobacillus casei* with 7.5×10^2 mg/l. With the *HLE* incorporated into PLG nanoparticles (*HLE*-PLG-NP), we observed diminution of the bactericidal concentration compared to *HLE*, the upper MBC being of 1.875×10^2 mg/l. Incorporation of the *HLE* into a colloidal carrier optimized its antibacterial performance.

Key Words: *Bactericidal activity, Harungana madagascariensis leaf extract, minimum bactericidal concentration (MBC), nanoparticles, oral bacteria, poly (d,l-lactide-co-glycolide), potentiation*

Introduction

With the rise in resistance of bacteria to antibiotics, there is considerable interest in the development of other classes of antimicrobials for the control of infection. Plant materials are known to offer excellent possibilities for the discovery of anti-infectious agents [1], and in many countries plant products have been successfully incorporated into dentifrice or mouthwash [2].

Harungana madagascariensis Lam. ex Poir. *(Hypericaceae)* is a popular drug native to Africa and Madagascar [3]. Different parts of the plant (leaves, stem bark, roots) are known to possess biological properties, mainly antibacterial, antifungal, and antiviral [4–7]. Phytochemical studies of this plant have shown that it contains components known for their antimicrobial properties; these include anthracenic derivatives, flavonoids, alkaloids, saponins, glycosides, and tannins [5,8–10].

Dental caries is an infection due to oral bacteria and is one of the most prevalent diseases in man [11]. Different types of Gram-positive bacteria, all of which possess the ability to produce organic acids from sugars and to survive under acidic conditions, are closely linked with the development of dental caries [11,12]. Additionally, several types of *Streptococcus mutans*, the primary cariogenic agents in man, have the ability to adhere to tooth surfaces through the synthesis of extracellular polysaccharides from sucrose [12–14]. They subsequently metabolize fermentable sugars in the plaque to organic acids, resulting in the demineralization of tooth enamel. Oral bacteria such as *Streptococcus sanguinis*, *Actinomyces*, and *Lactobacillus* species are

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also associated with root-surface caries formation [12,15]. Thus, the strategy for the prevention of dental caries includes elimination of the cariogenic bacteria from the oral cavity, prevention of the organic acid production, and inhibition of the enzymes responsible for the synthesis of extracellular polysaccharides [16]. Synthetic compounds such as chlorhexidine have been widely used for this purpose [17]. However, unexpected side effects, including brown staining of teeth, predisposition to calculus formation, enhanced bacterial colonization, desquamation, and soreness of the oral mucosa, due to such substances have also been reported [18–20].

In recent years, plant extracts with antibacterial properties have been investigated as alternatives to synthetic compounds [21–25]. In addition, the use of inorganic and organic nanoparticles (NP) as drug delivery devices has been extensively reviewed [26]. It has been reported that the physico-chemical interactions between plant extracts and some macromolecules are capable of forming nanoparticles [27,28]. Poly (D, L-lactide-co-glycolide) (PLG) is a biocompatible and biodegradable polyester biopolymer that is usually used for manufacturing nanoparticles [29].

In the present study, the *in vitro* bactericidal activity of the *H. madagascariensis* ethyl acetate leaf extract has been compared to those of the ethyl acetate extract loaded PLG nanoparticles, against cariogenic bacteria and those implicated in gingivitis infections. Since *streptococci* species are involved in caries formation, we have chosen them as the main test microorganisms. *Actinomyces* sp., *Fusobacterium*, *lactobacilli*, *Prevotella*, and *Propionibacterium* species were also selected because of their relation to root caries.

Material and methods

Plant extraction

H. madagascariensis Lam. ex Poir *(Hypericaceae)* leaves were collected from Lome in Togo, between February and March 2002. Voucher specimens were preserved at the herbarium of the Botany Department of the Sciences Faculty at the University of Lome under the following codes: FWTA 1:290; AKPAGANA: 386; GUELLY: 128; BRUNEL: 5393. The plant materials were dried at room temperature and then pulverized. Pulverized leaves (200 g) were extracted using a Soxhlet apparatus (A1137706; Fischer Scientific Labosi, Elancourt, France) by a successive exhaustion method with petroleum ether, dichloromethane, and ethyl acetate, as shown in Figure 1. Each extract was concentrated under reduced pressure in a rotary evaporator and the dried extract preserved at $+4^{\circ}$C.

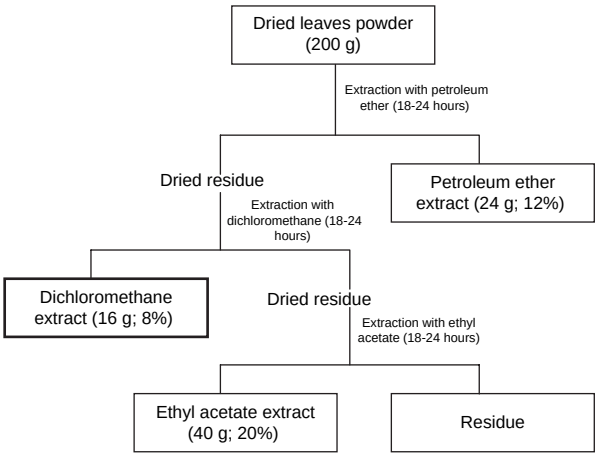

Figure 1. The procedure used to obtain the ethyl acetate leaf extracts of *Harungana madagascariensis* (Soxhlet extractor).

Materials

The ethyl acetate *H. madagascariensis* Lam. ex Poir *(Hypericaceae)* leaf extract (*HLE*) was prepared as described above. Poly (D, L-lactide-co-glycolide: 85/15%) (PLG, MW = 50,000–75,000), acetone, ethanol (EtOH) (99%), petroleum ether, dichloromethane, ethyl acetate were provided by Prolabo (Lyon, France). Soybean phospholipids (Emulmetik® 900) were obtained from Lucas Meyer (Saint Maur des Fossés, France). Polyoxyethylene 20 sorbitane monooleate (Tween® 80) was received as a gift from ICI Surfactant (Sceaux, France). 1.3% Nutrient Broth medium (liquid medium) was purchased from AES Laboratory (Combourg, France), Columbia Blood agar base, Tryptic Soy Agar, and Lactobacillus MRS agar medium were purchased from Becton Dickinson (Le Pont de Claix, France). The bacterial strains studied (Table III) were provided by the Institut Pasteur collection (CIP), the Zahnärzliches Institut of Zürich collection (OMZ), and the Bacteriology Laboratory of University of Franche–Comté (L).

Qualitative phytochemical analysis

Ethyl acetate leaf extract was analysed by thin layer chromatography (TLC) on Merck silica gel 60 F_{254} with chloroform/n-propanol/formic acid/water (15:10:3:1) as eluent. Plates were sprayed using standard phytochemical methods [30,31]. The spots were examined under UV and developed by the following reagents: Draggendorf, H_2SO_4 in ethanol, 5% alcoholic KOH, 1% alcoholic $AlCl_3$, 5% alcoholic $FeCl_3$, natural products/polyethylene glycol (NP/PEG), 0.1% paranitrosodimethylalanin in pyridine.

Preparation of PLG nanoparticles

HLE loaded and unloaded PLG NPs were prepared by interfacial polymer deposition following the

solvent diffusion method [32]. Briefly, 200 mg of PLG and 100 mg of Emulmetik® 900 (lecithin) were dissolved in 25 ml of acetone supplemented with 25 mg of *HLE* at 40°C. The resulting organic mixture was then poured, under magnetic stirring, into 50 ml of 0.25% (m/v) aqueous Tween® 80 solution. The PLG-NP formed spontaneously as the two solutions mixed. Finally, the PLG-NP suspension was concentrated by evaporation under reduced pressure to a final volume of 10 ml. Unloaded PLG-NPs were prepared without *HLE* as described previously.

Determination of the amount of HLE incorporated into PLG-NP

The amount of *HLE* associated with the PLG-NP (*HLE*-PLG-NP) or encapsulation efficiency was determined as follows: 10 ml of *HLE*-PLG-NP just after preparation was centrifuged at 4000 rpm for 10 min by centrifuge Sigma 2-16 (Bioblock Scientific, Illkirch, France). The precipitate containing the non-encapsulated *HLE* was dried and weighed after separation from the supernatant containing the *HLE* associated PLG-NP. Encapsulation efficiency (EE) *HLE*-PLG-NP was calculated as follows:

$$EE_{HLE\text{-PLG-NP}}(\%) = \frac{Q_{\text{Initial } HLE} - Q_{\text{non-encapsulated } HLE}}{Q_{\text{Initial } HLE}} \times 100$$

PLG-NP size and zeta potential measurements

The average diameter and polydispersity index (PI) and the zeta potential of PLG-NP were determined by a Zetamaster (Malvern Instruments, Orsay, France) using photon correlation spectroscopy (PCS; dispersant refractive index = 1.33; detector angle = 90°; wavelength = 670 nm). The surface charge was estimated by electrophoretic measurements (sample dielectric constant = 79; cell field = 28 V/cm). All measurements were done at 20.2 ± 0.9°.

Statistical analysis

The results and data obtained in the physico-chemical characterization were evaluated using the one-way analysis of variance (ANOVA) test between unloaded PLG-NP and *HLE*-PLG-NP, followed by Student's *t*-test. Significant levels were at $p < 0.05$ (95% confidence limits).

Bacterial inoculum preparation

A 24-h culture of each bacterial strain was suspended in 5 ml of 0.85% sterile saline solution with a Vortex apparatus. The concentration was adjusted at 10^7 CFU/ml using 0.5 McFarland standard solution.

Bactericidal assays

The *in vitro* bactericidal activity of *HLE* and *HLE*-PLG-NP was tested by modifying the broth micro-dilution method as described in NCCLS document M27-T [33]. The *HLE* was dissolved in 99% v/v ethanolic solution (stock solution); 1 ml of the stock solution of *HLE* and 1 ml of the *HLE*-PLG-NP suspension were diluted in nutrient broth (NB) in order to obtain serial dilutions to achieve a concentration range from 0.05×10^2 to 10×10^2 mg/l for *HLE* solution, and 0.05×10^2 to 5×10^2 mg/l for *HLE*-PLG-NP suspension. About 100 µl of the bacterial suspension was placed in the wells of a microtiter plate and subsequently 100 µl of the serial dilutions was added. Each lane of the microtiter plate contained 100 µl bacterial suspension and 100 µl plant extract dilution, or 100 µl *HLE* loaded PLG-NP dilution, or *HLE* unloaded PLG-NP (control). The ethanolic control was also utilized. The plates were incubated at 37°C, under CO_2, for 24 h. After 24 h, 100 µl of each lane of the microtiter plate was inoculated on Petri dishes containing Columbia blood agar (*Streptococcus* and *Actinomyces* species) or MRS agar medium (*Lactobacillus* species) or Tryptic Soy agar (*Fusobacterium*, *Prevotella*, and *Propionibacterium* species), using a STEERS multi-ensemencor apparatus. The Petri dishes were incubated at 37°C, under CO_2, for 48 h. The lowest concentration showing no growth was identified as the minimal bactericidal concentration (MBC). In those experimental conditions, the bacterial growth for the ethanolic and unloaded PLG-NP controls was observed. The bactericidal tests were repeated three times.

Results

Phytochemical analysis

The qualitative phytochemical investigation of ethyl acetate leaf extract revealed the presence of saponins, tannins, alkaloids, anthracenic derivatives, and flavonoids (Table I).

Formulation and characterization of HLE-PLG-NP

Table II gives the physico-chemical characterization of *HLE* incorporated into PLG-NP and unloaded PLG-NP. A significant ($p < 0.005$) increase in *HLE*-PLG-NP size compared with unloaded PLG-NP was observed. Therefore, the high size of *HLE*-PLG-NP was likely due to the conjugated components of *HLE* (i) adsorbed at the surface of the polymer matrix and/or (ii) dispersed in the polymer matrix. *HLE*-PLG-NP did not seem to have any significant ($p > 0.05$) effect on the PI when compared to unloaded PLG-NP, so the particle size distribution could be considered as similar in all formulations. It is worth mentioning that the surface

Table I. Phytochemical investigation of ethyl acetate leaves extract of *Harungana madagascariensis* (*HLE*)

Spray reagents	Observation	Secondary metabolites	Inference
Dragendoff's reagent	Appearance of red-orange-colored zones in the visible one	−Alkaloids	+
5% ethanolic KOH	Appearance of red-colored zones at 254 nm	−Anthraquinones	+
1% $FeCl_3$	Appearance of blue-black-colored zones at 254 nm	−Tannins	+
1% ethanolic $AlCl_3$	Appearance of white-yellow-colored zones at 254 nm	−Flavonoids	+
NP/PEG	Appearance of orange-fluorescence-colored zones at 366 nm	−Flavonols	+
		−Flavones	+
0.1% pyridine para-nitrosodimethylalanin	Appearance of grey-colored zones at 254 nm	−Anthrones	+
Distilled H_2O+extract in stirring	Frothing which lasts about 3 min	−Saponins	+

+Secondary metabolite present.

charge measurements of PLG-NP showed a negative charge and no significant ($p > 0.05$) difference between all formulations. The encapsulation efficiency value indicated that 75% of the initial amount of *HLE* was encapsulated. Therefore, the *HLE*-PLG-NP dilutions contained 25% less *HLE* than equivalent amounts of *HLE* extracts alone under standard assay conditions.

Bactericidal activity

The bactericidal activity results of the *HLE* and the *HLE*-PLG-NP were expressed in minimal bactericidal concentration (MBC). The *HLE*-PLG-NP yielded significant bactericidal activity against the oral bacterial strains, with MBCs varying from 0.375×10^2 to 1.875×10^2 mg/l, whereas the *HLE* solution showed MBCs varying from 2.0×10^2 to 7.5×10^2 mg/l. The *HLE*-PLG-NP enhanced the bactericidal effect against the whole bacterial strain with the upper MBC at 1.875×10^2 mg/l (Table III).

Discussion

The aim of this study was to investigate the bactericidal activity of the *HLE* solution with the same PLG-encapsulated extract. The ethyl acetate extract of *H. madagascariensis* leaf (*HLE*) and the ethyl acetate extract of *H. madagascariensis* leaf incorporated into PLG nanoparticles (*HLE*-PLG-NP) tested in this study presented bactericidal properties against cariogenic bacteria and those implicated in gingivitis infections.

The qualitative phytochemical analysis of *HLE* showed the presence of secondary metabolites (saponins, tannins, alkaloids, anthracenic derivatives, and flavonoids), substances the presence of which had already been confirmed in this plant by Iinuma et al. [9], Olagunju et al. [5], and Capasso et al. [10]. These secondary metabolites are known to have antimicrobial properties, as shown by Scalbert [34] and Favel et al. [35], either singly or in combination, as has been suggested by Leven et al. [36].

The physico-chemical characterization results reflected a significant increase of *HLE*-PLG-NP size compared with unloaded PLG-NP, confirming the association of *HLE* with nanoparticles. This was due to the fact that the PLG-lecithin drug is complexed to the matrix and/or onto the surface of the nanoparticles [37]. So, the complex phenomena increased the particle size significantly without modifying the electric charge surface (zeta potential). *HLE*-PLG-NP did not seem to have any significant effect on the PI compared to unloaded PLG-NP. Indeed, the PI values showed that the particles were similarly dispersed. The particle size distribution was considered as monodisperse ($PI \leq 0.2$) [38]. The significant encapsulation efficiency obtained (75%) substantiates the nanoparticle preparation technique used.

The antimicrobial results obtained in this study showed that the ethyl acetate extract of *H. madagascariensis* (*HLE*) possesses an important bactericidal effect against the Gram-positive and Gram-negative bacteria tested, with MBC around 5.0×10^2 mg/l, except for *Lactobacillus casei* with 7.5×10^2 mg/l.

However, the bactericidal effects obtained with *HLE*-PLG-NP were better than those given by the *HLE* solution. For example, the mutans streptococci group species (*S. mutans, S. mutans* serotypes *c, f, k, S. cricetus* and *S. rattus*), the main species implicated in the formation of dental caries, were destroyed by the *HLE*-PLG-NP formulation at 1.875×10^2 mg/l, just as were the species causing gingivitis (*Actinomyces naeslundii* and *Lactobacillus* sp.), whereas the

Table II. Physico-chemical characterization of *HLE*-PLG-NP

Formulations	Size (nm)	Zeta potential (mV)	Polydispersity index (PI)	Encapsulation efficiency (%)
HLE-PLG-NP	255.0±31.1‡	−31.1±0.4	0.20±0.07	75±5.00
PLG-NP	184.7±23.7	−30.0±1.0	0.19±0.02	–

‡ $p < 0.05$ as compared to *HLE*-PLG-NP and PLG-NP group (Student's *t*-test).All data are the mean±SD ($n = 3$).

Table III. Minimal bactericidal concentration of *Harungana madagascariensis* ethyl acetate leaves extract unloaded (*HLE*) and loaded in PLG-nanoparticles (*HLE*-PLG-NP) on oral bacterial strains

Oral bacterial strains	*HLE*	*HLE-PLG-NP*
	MBC (mg/l)	
Actinomyces naeslundii CIP 103128	2.5×10^2	0.75×10^2
Actinomyces viscosus OMZ 206	2.0×10^2	0.75×10^2
Fusobacterium nucleatum subsp. Nucleatum CIP 101130	4.0×10^2	1.5×10^2
Lactobacillus acidophilus CIP 76.13	1.25×10^2	0.375×10^2
Lactobacillus casei CIP 107868	7.5×10^2	1.875×10^2
Prevotella melaninogenica CIP 105346	2.5×10^2	0.75×10^2
Propionibacterium acnes CIP 53.117	4.0×10^2	1.0×10^2
Streptococcus anginosus (L)	3.5×10^2	1.125×10^2
Streptococcus cricetus OMZ 52	5.0×10^2	1.5×10^2
Streptococcus mitis OMZ 89	2.0×10^2	0.375×10^2
Streptococcus mutans CIP 103220	5.0×10^2	1.5×10^2
Streptococcus mutans serotype c OMZ 64	3.0×10^2	0.75×10^2
Streptococcus mutans serotype f (L)	3.0×10^2	0.5625×10^2
Streptococcus mutans serotype k OMZ 65	5.0×10^2	1.5×10^2
Streptococcus rattus OMZ 51	4.0×10^2	0.75×10^2
Streptococcus salivarius OMZ 36	2.5×10^2	1.5×10^2
Streptococcus sanguinis CIP 103231	5.0×10^2	1.875×10^2

same concentration of *HLE* solution was ineffective against *S. mutans* species. This may be because the incorporation, adsorption, and/or conjugation of the plant extract on/or into the PLG nanoparticles facilitated the slow and continuous diffusion of the extract from the nanoparticles matrix to the bacterial cells and maintained the drug concentration. These interactions are the outcome of the tendency of polymers and surfactants to adsorb at the interface, mostly due to steric and electrostatic mechanisms [37]. The enhanced bactericidal activity of *HLE*-PLG-NP could also be due to the bioadhesive property of the PLG biopolymer that stays on the bacterial cells for a prolonged period, thus prolonging the drug action [39]. Indeed, the ability of the polymer to support bacterial attachment is directly linked to its hydrophobicity and surface properties. PLG nanoparticles are described as hydrophobic matrix on which several substances could be adsorbed or conjugated [40–42]. Therefore, in the present study, the highly hydrophobic character of PLG may have the advantage of maintaining the *HLE* stably on the surface of the bacteria, optimizing its bactericidal effects.

In conclusion, on the one hand the data presented in this study suggest that the ethyl acetate extract of *H. madagascariensis* may be useful in reducing the incidence of dental caries, since it demonstrated significant bactericidal activity against the microorganisms involved in such diseases. On the other hand, the results obtained with the encapsulated *H. madagascariensis* clearly demonstrated the increased antimicrobial effects, so the association of the extract to PLG nanoparticles enhanced its antibacterial activity *in vitro*. Encapsulation is a promising approach for the formulation of biologically effective plant extracts against oral diseases.

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