Garcinoic acid: from *Garcinia kola* seeds extraction to an innovative delivery system for nose-to-brain delivery

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Summary

Non-communicable and rare brain diseases' treatments are a crucial challenge worldwide and nose-to-brain delivery route is a promising strategy to address this challenge and to improve drug delivery to central nervous system (CNS). Garcinoic acid (GA) is known for its anti-inflammatory and antioxidant properties [1,2]. In this study, an improved method for extracting GA from *Garcinia kola* seeds was developed using a Naviglio extractor and MeOH as the solvent. This approach enabled GA isolation with a yield of 1.18–1.39% wt (*w*/*w*, based on dried seeds), achieving ≥ 94% purity on a multi-gram scale while maintaining compound stability. *In vitro* studies confirmed the absence of GA toxicity on human nasal (RPMI 2650) and microglial (HMC3) cells. GA was able to reduce intracellular ROS, H_2O_2 release and proinflammatory cytokines on HMC3 cells stimulated with LPS. At the same time, Pregnane X Receptor (PXR) and its downstream genes expression up-regulation was observed. Nanostructured Lipid Carrier (NLCs) encapsulating GA were produced and optimized by Design of Experiment (DoE) evidencing promising particle size properties, i.e., mean diameter < 300 nm and PDI < 0.2. Furthermore, the addition of pegylated phospholipid to NLCs lipid matrix was crucial in improving nanoparticle stability over time and in promoting particle size reduction, as evidenced from the mean diameter < 200 nm.

Key Message

GA extraction procedure appears highly promising for its eco-sustainability, while the microfluidic development of GA-loaded NLCs for nose-to-brain delivery represents an innovative neurotherapeutic approach. NLCs platform improves GA, a PXR modulator [1,2], delivery through olfactory nerve pathway, enhancing the CNS disorders' treatments.

Introduction

Natural products' traditional extraction processes result highly expensive and not-green organic solvents usage such as chloroform is evidenced by literature. Low-cost extraction procedures through more eco-sustainable solvents are raising [3]. GA, contained in *Garcinia kola* seeds, is a vitamin E analogue and a natural multitarget modulator known to activate the PXR [2] and exert redox "scavenger", anti-inflammatory activities and neuroprotective effects in both non-communicable and rare brain diseases. Conventional NLCs production methods often lack precise control of particle dimensions. In contrast, microfluidic technology enables the on-demand production of NLCs with narrow size distribution while minimizing batch-to-batch and operator-related variability, which are crucial for industrial translation and scalability [4]. Furthermore, NLCs system is a promising strategy to vehiculate high lipophilic molecules such as GA (XLogP3=7.3), improving the delivery to CNS through olfactory nerve pathway, bypassing the Blood Brain Barrier (BBB).

Materials: Ultrapure water was obtained from Synergy® UV Water Purification System (Millipore Sigma, USA). Absolute ethanol ≥99.8% (GC), polysorbate 80 (Tween® 80), MeOH (ACS reagent), $CHCl_3$ (ACS reagent), silica (60A 40-63 μm) and dimethyl sulfone (DMS, standard for quantitative NMR, TraceCERT®) were purchased from Merck (Darmstadt, Germany). Cetyl palmitate (CP), propylene glycol monocaprylate (Capryol 90) were donated by Gattefossé (France). Sodium chloride and citric acid were purchased from Carlo Erba (Italy). Phosphatidylethanolamine (PE) 18:0/18:0 - PEG 2000 was obtained from LIPOID (Germany). *Garcinia kola* seeds were purchased from University of Ibadan, Nigeria.

Experimental methods: *Garcinia kola* seeds were finely ground, and extraction was performed on either freeze-dried or fresh (wet) material using a Soxhlet or a Naviglio extractor. MeOH or MeOH/$CHCl_3$ (1:1, v/v) were used as the extraction solvent. For the Naviglio extraction, the ground *Garcinia kola* seeds were pre-treated by sonication (u.s.) for 30 minutes, followed by dynamic extraction using 20 or 40 cycles with 6.67, 13, 20, or 40 volumes (V) of MeOH *per* gram of dry material. Volatile solvents were removed under reduced pressure, and the quantification of GA in the crude extract was performed by quantitative ^{1}H-NMR spectroscopy using dimethyl sulfone as the internal standard. The crude extract was then purified by column chromatography (stationary phase: silica 60A 40-63 μm; Eluent: $CHCl_3$/MeOH from 99.5:0.5 to 98:2, v/v) and the purity of GA was determined by NMR. *In vitro* studies were performed on human nasal epithelial cells (RPMI 2650 CCL-30 from ATCC; passages 10-20) and human microglial cells (HMC3 CRL-3304 from ATCC; passages 25-30) to evaluate the cytotoxicity of GA (MTT assay). Treatment times ranged from 6 to 72 hours and GA was tested at concentrations from 0.1 to 100 μM. Subsequently, HMC3 cells were pre-treated with lipopolysaccharide (LPS, 0.5 μg/mL)

for 24 hours, followed by treatment with GA (25 µM) for an additional 24 hours. Intracellular ROS levels were measured using 2,7-diclorofluorescein diacetate (DCFH-DA) probe while H_2O_2 levels in the cell supernatant were determined by a colorimetric assay kit (Elabscience, Houston, TX, USA). An ELISA assay was performed to quantify the pro-inflammatory cytokines, IL-6 (IL-6 high sensitivity ELISA kit, ENZ-KIT178, Enzo Life Sciences) and IL-1β (RAB0273, Human IL-1 β ELISA Kit, Sigma-Aldrich) following LPS pre-treatment. In addition, a nose-to-brain *in vitro* model, shown in **Figure 1**, was set up, pre-treated with LPS (0.5 µg/mL) for 6 hours and then treated with GA (25 µM) for 24 hours. ROS and H_2O_2 production were assessed using the aforementioned assays. PXR and its downstream genes expression (CYP3A4, MRP1 and MDR1) were evaluated using immunoblot.

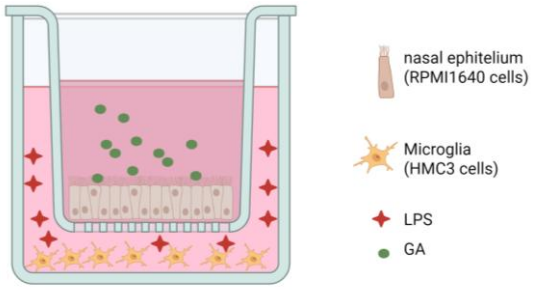

Figure 1 - Nose-to-brain *in vitro* model, constituted by a RPMI 2650 cells layer and an underlying HMC3 cells layer, pre-treated with LPS (0.5 µg/mL) for 6 hours and then treated with GA (25 µM) for 24 hours.

GA-loaded NLCs were prepared by nanoprecipitation technique using a staggered herringbone micromixer at a temperature of 47.5 °C. Lipid mixture (CP:Capryol 90 90:10, % w/w) was solubilized in ethanol, while citrate buffer (0.0025 M, pH 3) containing Tween® 80 was used as anti-solvent. Box-Behnken DoE was set up and 0.4-2 (Lipid mixture, mg/mL), 0.3-5 (Tween 80, mg/mL), 3-10 (Flow Rate Ratio) factors' ranges were used. Total Flow Rate (TFR) was kept constant at 3000 µL/min. GA was added at 5 % w/w (with respect to lipid mixture). Mean diameter (nm), polydispersity index (PDI), Zeta potential (mV) and Encapsulation Efficiency (%) were selected as outcomes. Particle size analysis was performed using *Zeta Potential/Particle Sizer NICOMP 380 ZLS* instrument (Nicomp, USA). A Sephadex LH-20, as stationary phase, and citrate buffer (0.0025 M, pH 3), as mobile phase, were used to promote separation between free drug and GA-loaded NLCs. Recovered NLCs were frozen at - 80 °C and freeze-dried for 48 hours using a TOPT-10A Freeze-Dryer. GA extraction from GA-loaded NLCs was performed using 2 mL ethanol at 45 °C. High Performance Liquid Chromatography (HPLC) analysis was executed using Kinetex Core-Shell RP18 (250 mm × 4,6 mm, 5 µm, 110 Å) as stationary phase and acetonitrile/water (60/40 v/v, 1.0 mL/min) as mobile phase. RF-10A fluorescence detector, using 295 nm and 330 nm as excitation and emission wavelengths, respectively, was employed for GA detection. The analysis of variance (ANOVA) of data was conducted using *Design-Expert* software. In parallel, using parameters of Run 1 (**Table 1)**, PE 18:0/18:0 - PEG 2000 was added at 0.12 (Batch 1) and 0.36 (Batch 2) mg/mL to the lipid mixture.

Table 1 - Box-Behnken DoE parameters used and outcomes (mean diameter, PDI and Zeta potential) analyzed.

Run	Lipid mixture (mg/mL)	Tween 80 (mg/mL)	FRR	Mean diameter (nm)	PDI	Zeta potential (mV)
1	1.2	0.3	10	241.2	0.127	-0.53
2	1.2	0.3	3	429.3	0.426	-1.15
3	0.4	0.3	6.5	286.6	0.158	-1.29
4	1.2	2.65	6.5	277.2	0.092	-0.52
5	0.4	2.65	3	397.8	0.155	-1.34
6	1.2	2.65	6.5	267.2	0.105	-0.72
7	1.2	2.65	6.5	274.7	0.127	0.13
8	0.4	5	6.5	333.3	0.13	-0.11
9	2	0.3	6.5	322.7	0.359	-0.17
10	2	2.65	10	256.1	0.202	-0.36
11	2	5	6.5	276.6	0.192	-1.14
12	1.2	5	10	247.1	0.125	1.55
13	1.2	5	3	438.5	0.222	-0.28
14	1.2	2.65	6.5	313	0.144	-1.82
15	2	2.65	3	491.2	0.384	0.41
16	0.4	2.65	10	170	0.54	0.82

Results: Extraction processes, performed by *Soxhlet* apparatus, led to very poor results, i.e., low Purity (%) and low Isolated yield (% wt). On the other hand, GA extraction with Naviglio apparatus was performed selecting MeOH as the solvent of choice thanks to its lower toxicity compared to $CHCl_3$. Better outcomes were obtained in terms of both Purity (%), Isolated yield (% wt) and minimized solvent volume for extraction 1, which process was performed on freeze-dried seeds, as observed in **Table 3**.

Table 3 - Conditions explored for GA extraction using Naviglio apparatus. In [c] extraction, conditions were applied on freeze-dried seeds, in [d] extraction, conditions were applied on wet seeds. Purity (%) [a] was analysed by qNMR, Isolated yield (% wt) [b] was calculated with respect to dry seeds.

Extraction	Conditions	Purity (%) [a]	Isolated yield (% wt) [b]
1	6.67 V MeOH, 40 cycles [c]	94.8-97.8	1.18-1.39
2	13 V MeOH, 40 cycles [d]	91.6	1.37
3	20 V MeOH, 40 cycles [d]	92.8	1.34
4	40 V MeOH, 20 cycles [d]	96.4	1.03

GA exhibited low toxicity, up to 100 µM concentration, at all time points analysis (6, 24, 48, 72 h) in both HMC3 and RPMI 2650 cells. In microglial cells inflamed with LPS (0.5 µg/mL), GA evidenced redox "scavenger" and anti-inflammatory activity. Specifically, treatment with GA (25 µM) resulted in ROS and H_2O_2 production decrease (**Figure 2A-B**), along with reduced expression of the pro-inflammatory cytokines IL-6 and IL-1β (**Figure 2C-D**).

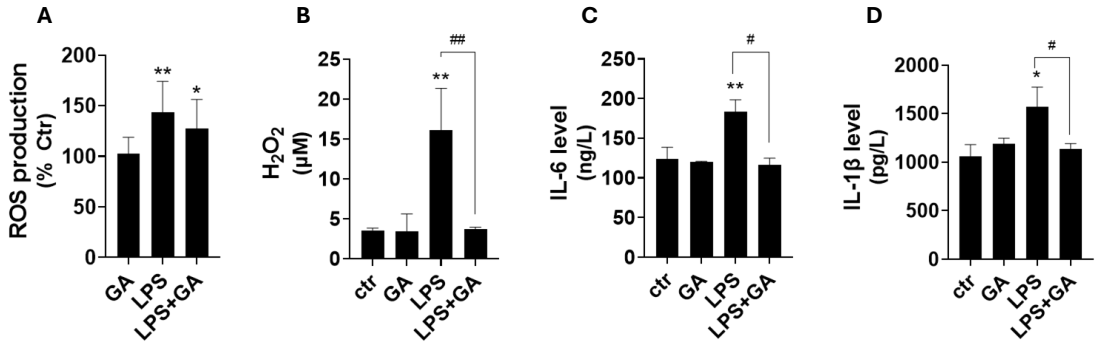

Figure 2A-D - ROS (%Ctr, A) and H_2O_2 (µM, B) production by microglial cells after GA, LPS and LPS+GA treatments is displayed. Cytokines IL-6 (ng/L, C) and IL-1β (pg/L, D) expression by microglial cells after GA, LPS and LPS+GA treatments is shown. ctr=untreated cells. Data are the means ± SDs of triplicate determinations. *p<0.05; **p<0.001; #p<0.05; ##p<0.001.

Using the *in vitro* nose-to-brain model illustrated in **Figure 1**, pre-treated with LPS (0.5 µg/mL) for 6 hours and then treated with GA (25 µM) for 24 hours, a significant reduction in H_2O_2 (µM) levels was observed in both HMC3 and RPMI 2650 cells as well as a decrease in ROS production in the HMC3-RPMI 2650 co-culture, compared to LPS treatment alone. Immunoblotting analysis revealed that, GA treatment (25 µM for 24 hours following a 6-hour LPS pre-treatment) led to increase expression of PXR and its downstream target genes including CYP3A4, MRP1 and MDR1 (**Figure 3A-D**).

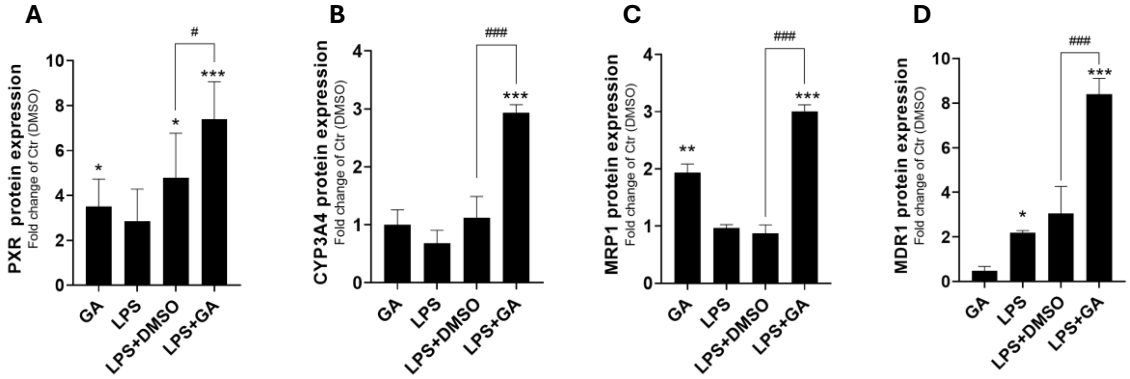

Figure 3A-D - Analysis on nose-to-brain *in vitro* model: PXR (A), CYP3A4 (B), MRP1 (C) and MDR1 (D) protein expression, expressed as fold change of Ctr (DMSO), after GA, LPS, LPS+DSMO and

LPS+GA treatments is shown. ctr=untreated cells. Data are the means ± SDs of triplicate determinations. *p<0.05; **p<0.001; ***p<0.0001; #p<0.05; ##p<0.001; ###p<0.0001.

Box-Behnken DoE's outcomes are displayed in **Table 1**. Run 1 and 12 reveal promising particle size properties, i.e., minimized mean diameter and PDI, which is < 0.2. Results were statistically significant only for the mean diameter, for which a modified linear model was applied, with a p-value <0.0001 (significant) and a Lack of Fit with a p-value of 0.1362 (not significant). R2 was 0.8155 and Adeq Precision was 15.735. The parameter that influenced to a larger extent the mean diameter is FRR. An increase of FRR led to a decrease in the mean diameter. Furthermore, Box-Behnken DoE's central point (Run 4,6,7,14) reveals an Encapsulation Efficiency of 97.3 ± 4.4. Considering Run 1 promising particle sizes, pegylated phospholipid was added to this formulation at different concentrations, 0.12 (Batch 1) and 0.36 (Batch 2) mg/mL. Pegylated phospholipid led to mean diameter decrease, while PDI value was < 0.3 (**Figure 4**).

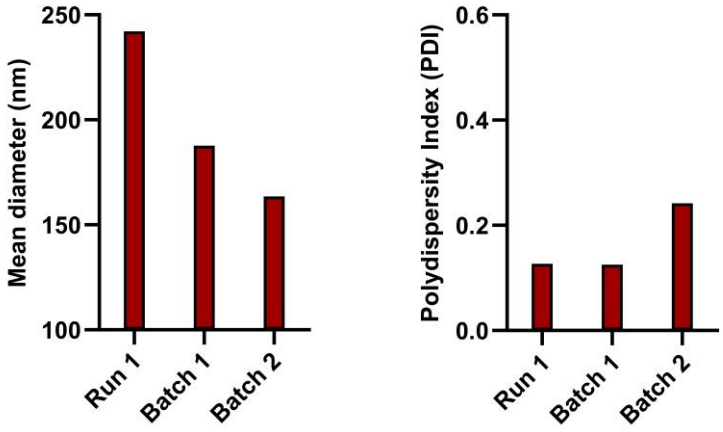

Figure 4 - Particle size properties (mean diameter and PDI) of Batch 1 and 2, compared to Run1. GA was used at 5 % w/w (with respect to the lipid mixture).

Discussion and Conclusions: An efficient and robust protocol for the extraction of GA from *Garcinia kola* seeds was successfully optimized. Under the optimized conditions (freeze-dried seeds, 40-80 g per extraction, 30 min u.s., then extraction with Naviglio using 6.67 V MeOH and 40 cycles), GA was isolated in 1.18-1.39% wt yield and high purity suitable for its formulation and biological appraisals. GA was well tolerated by human nasal and human microglial cells. Moreover, pre-inflamed microglial cells treated with GA evidenced an important reduction of ROS, H_2O_2 and pro-inflammatory cytokines. Using the developed *in vitro* nose-to-brain model, GA was able to reduce inflammation, as evidenced from PXR and PXR downstream genes up-regulation. NLCs, selected as innovative nose-to-brain delivery platform, were able to encapsulate GA. In fact, the selection of anti-solvent at pH 3 promotes improved drug loading inside the lipid matrix. As evidenced from DoE set-up and in progress DoE optimization, GA-loaded NLCs show promising particle size properties for nose-to-brain delivery. In parallel, pegylated phospholipid seems to be involved in nanoparticles particle size reduction, controlling the nanoprecipitation process. These results aim to demonstrate GA anti-inflammatory and antioxidant properties and to elevate it as a promising molecule in non-communicable and rare brain diseases' treatments.

References

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