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Research Article

Ethanolic Extract of *Garcinia kola* Stem Bark Inhibits LDL-Uptake and LPS-Induced Anxa-1 and ICAM-1 Expression in Human Umbilical Vein Endothelial Cells (HUVECS)

Hadidjatou Daïrou[1,2], Giovana Aparecida Gonçalves Vidotti[3], Lucas Possebon[3], Sara de Souza Costa[3], Melina Mizusaki Iyomasa-Pilon[3], Lucas Azevedo[3], Armelle Deutou Tchamgoué[1], Ana Paula Girol[3], Télesphore Benoît Nguelefack[2], Gabriel Agbor Agbor [1]*

[1] *Center for Research on Medicinal Plants and Traditionnal Medicine, Institute of Medical Research and Medicinal Plants Studies, Yaoundé-Cameroon*

[2] *Department of Animal Biology, Faculty of Sciences, University of Dschang, Cameroon*

[3] *Centro Universitario Padre Albino, Catanduva-Sao Paulo Brazil*

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*Address for Correspondence:

Gabriel Agbor Agbor, Center for Research on Medicinal Plants and Traditionnal Medicine, Institute of Medical Research and Medicinal Plants Studies, Yaoundé-Cameroon

Abstract

The endothelial cells' dysfunction linked to the development of atherosclerosis plays an important role in the regulation of inflammatory responses. Previous studies demonstrated the antiatherogenic effects of *Garcinia kola* seed extracts based on their lipid lowering effects. Our recent studies showed the in vitro antioxidant and anti-inflammatory activities of the ethanolic extract of *Garcinia kola* stem barks (EE). For more insight on the antiatherogenic effect of EE, we investigated its activity on some key points of the atherosclerosis process. The cytotoxicity of EE as well as its effects on LDL-uptake, LPS-induced InterCellular Adhesion Molecule (ICAM-1) and Annexin-1 (Anxa-1) expression and LPS-induced DNA damage were evaluated in Human Umbilical Vein Endothelial Cells (HUVECs). EE significantly ($p<0.0001$) increased the cell viability in both naive and LPS-treated HUVECs. At the concentrations of 50 and 100 µg/ml, EE significantly reduced LDL-uptake by endothelial cells stimulated with LPS. The immunohistochemistry results showed a significant ($p<0.01$) decrease in the ICAM-1 expression at the EE concentration of 250 µg/ml. EE also showed a significant ($p<0.0001$) concentration-dependent reduction of Annexin-1 expression in LPS-treated HUVECs. Besides, EE exhibited significant inhibition on LPS-induced genotoxicity marked by a decrease in tail DNA expression ($p<0.0001$) and tail movement expression ($p<0.001$) for the concentrations of 50 and 100 µg/ml. These findings showed that EE may mitigate the atherogenic process by reducing LDL-uptake and the expression of adhesion molecules. Thus, the EE of *Garcinia kola* turns out to be a potential candidate for the treatment of atherosclerosis with a lower risk of toxicity.

Keywords: *Garcinia kola*, LDL-uptake, LPS-induced inflammation, comet assay.

1- INTRODUCTION

Cardiovascular diseases (CVDs) constitute the first cause of global death. According to the statistics of WHO, 31% of all global deaths in 2016 were from CVDs (WHO, 2017) [1]. According to the Cameroonian Society of Cardiology, cardiovascular diseases are on the rise in Cameroon and already affect nearly 35% of the adult population, which represent a high economic cost for society (Mohamed, 2016) [2]. The development of cardiovascular diseases is deeply linked to the arterial atheromatous process called atherosclerosis. It results from the pooling of a multitude of factors including hypercholesterolemia, and particularly the increase in LDL cholesterol and the maintenance of the inflammatory process by macrophages and lymphocytes which infiltrate atherosclerotic lesions. Hypercholesterolemia, considered as the main trigger of atherosclerosis, results in changes of the arterial endothelial permeability that allow the migration of lipids into the arterial wall, leading to the adherence of monocytes to endothelial cells via the expression of adhesion molecules, such as vascular adhesion molecule-1 (VCAM-1), InterCellular adhesion molecule (ICAM-1) and selectins (Bergheanu *et al.*, 2017) [3]. Atherosclerosis is a chronic inflammatory disease characterized by intense immunological activity which is defined by the formation of fibrofatty lesions in the artery wall that causes much morbidity and mortality worldwide (Libby *et al.*, 2019) [4]. The formation of atherosclerotic plaque, characterized by the lipid accumulation, local inflammation, smooth muscle cell proliferation, cell apoptosis, necrosis, and fibrosis, is a major causative factor of arterial stenosis, and involves a chronic inflammatory response initiated by endothelial damage and inflammatory cell activation (Meng-Yu *et al.*, 2017) [5]. Numerous studies have shown that physiological molecules

such as Annexin A1 (Anxa-1) by instructing endothelial cells and leukocytes, prevents an excessive adhesion of leukocytes on the arterial wall (Shen *et al*., 2020 ; De Jong *et al*., 2017a) [6,7]. Being a multifactorial affection, the existing drugs aim more countering development factors of atherosclerosis and limiting the damage, which does not ensure really a total cure. Statins are very efficient drugs that showed in general consistent clinical event reductions with a good safety profile. However, side effects of importance may occur making these medicines sometimes inappropriate for some individual patients (Bergheanu *et al.,* 2017) [3]. In spite of the important progress realized in the field of medicine, 85% of cardiovascular deaths are due to heart attack and stroke, a large number of deaths than any other disease (WHO, 2017) [1]. A survey conducted by the Centers for Disease Control and Prevention in 2012 revealed that approximately 18% of the U.S. population uses natural products for treatment or prevention of disease but the use of plant-based medicines is even more prevalent in developing countries, where they constitute the primary health care modality for most of the population due to their economic situation. Due to the beneficial "synergistic" interactions of natural product mixtures, proponents of their medicinal use often claim that they are more effective than purified compounds (Lindsay and Nadja, 2019) [8]. Various plants or parts of plants have been studied to assess their antioxidant, anti-inflammatory anti-diabetes capacities and numerous clinical studies have highlighted the essential role of certain molecules from plant in the care and treatment of patients with cardiovascular diseases.

In order to contribute to the strengthening of the program of prevention and fight against the increase of cardiovascular diseases within the Cameroonian population in particular and worldwide in general, we focused our attention on herbal medicine and mainly on *Garcinia kola*, a plant that has a wide range of medicinal uses as reported in the literature for the treatment of gastric and liver disorders, headaches, laryngitis, cough, bronchitis, malaria, gonorrhea and various types of inflammation (Anna *et al*., 2019) [9]. Early studies have shown that *Garcinia kola* throughout its diversity of secondary metabolites polyphenols, flavonoids, alkaloids, tannins, possesses several pharmacological activities such as metabolic, antioxidant and anti-inflammatory (Ahmed *et al*., 2020; Daïrou *et al*., 2021) [10,11]. We studied the pharmacological activity of *Garcinia kola* ethanolic extract against atherosclerosis by evaluating its inhibitory activity on LDL-uptake by endothelial cells which is the intermediate step of LDL trancytosis, LPS-induced ICAM-1 and Anxa-1 expression and LPS-induced DNA damage in Human Umbilical Vein Endothelial Cells (HUVECs).

2- MATERIALS AND METHODS

2-1- Reagents and working solutions

Chemicals and materials were purchased from, Sigma-Alrich Chemical CO, Thermo-fisher, Life Technologies, Invitrogen, abcam, Gibco, Merck, Fisher Scientific and Lonza.

Garcinia kola ethanolic extract and lipopolysaccharide (LPS) working solutions were prepared in culture medium (Endothelial Cell Basal Medium-2) and filtered before use using a sterile Millex Syringe filters (33 mm, MERCK) to avoid any contamination.

2-2- Plant material and extraction

The leaves, fruits and stem barks samples of *Garcinia kola* were harvested in the area of Mdom which belongs to the Log Mbaha village in the Littoral Region of Cameroon in November 2018. Samples were then identified at the National Herbarium of Cameroon (HNC) under number 66984/HNC by the botanist Mr TADJOUTEU. Samples were subsequently kept under the code number 713. After identification, 2 kilograms of fresh stem barks of *Garcinia kola* were cleaned, cut into small pieces, dried for one week at room temperature (25°C) then crushed into powder using an electric blender (Moulinex, LM2211BM-350 W). The powder obtained was sieved using a sieve of 35 cm of diameter with a mesh of 14 µm. At the end of sieving, 1200 grams of powder were obtained and then subjected to extraction.

The ethanolic extract of *Garcinia kola* stem barks, were obtained after maceration of two hundred grams (200 g) of powder in 1L of ethanol (96%, MERCK 100971) for 72 hours at room temperature (Sukhdev *et al*., 2008) [12]. The macerate obtained was filtered using a Whatman filter paper grade 1 (90 mm, Sigma-Aldrich Z240079) and concentrated on a rotary evaporator (Büchi R110) at 78 °C. After ethanol evaporation, 8.9 g of ethanolic extract were obtained with an extraction yield of 2.225%.

2-3- Cell culture and treatment

HUVECs cell line was obtained from the Laboratory of Genetics and Molecular Cardiology LIM 13- InCOR / HCFMUSP of São Paulo (São Paulo / SP CEP 05403-000, BRAZIL) and cultured following the protocol of Baudin *et al.* in 2007 [13]. The culture medium (Endothelial Cell Basal Medium-2 (EMB-2) 500 ml) was supplemented with 10 ml of FBS, 0.2 ml of hydrocortisone, 2 ml of hFGF-B, 0.5ml of VEGF, 0.5ml of R3-IGF-1, 0.5ml of Ascorbic acid, 0.5ml of hEGF, 0.5ml of GA-1000 and 0.5ml of Heparin. HUVECs were seeded at a density of $1x10^4$ cells/well in cell culture flasks (Corning 3290, Fisher Scientific) and incubated at 37°C under 5% of CO_2. Cells were then sub cultured every three days until confluence was reached after ten days. Cells from 3rd passage to 6th passage were used for the experiments.

2-4- LPS-induced cytotoxicity assessment

To be sure that the evaluated parameters will not be biased by any toxic effect of the plant extract on cells, we tested the cytotoxicity activity of the concentrations of the plant extract to be used. In a 24-well plate, HUVECs were plated at a density of $4.7x10^4$ cells/well and then left to incubate for 24 h at 37°C under 5% of CO_2 (Chundong *et al.,* 2019) [14]. Once the supernatant was removed, cells were washed with PBS. In LPS test wells, was added 250 µl of the filtered extract at the concentrations of 50, 100 and 250 µg/ml and after 1h of incubation, 250 µl of filtered LPS 10 µg/ml (L3129, Sigma Life Science) was added. In extract test wells, was added filtered extract (250 µl) at the concentrations of 50, 100 and 250 µg/ml and 250 µl of culture medium. The naive control wells received only 500 µl of culture medium while in LPS control wells was added 250 µl of LPS (10 µg/ml) plus 250 µl of culture medium. The plate was then incubated for 24 hours at 37°C under 5% of CO_2. Each treatment was performed in triplicate and the experiment was repeated three times.

Trypan blue exclusion test of cell viability was assessed using the protocol described by Strober in 2015 [15]. After the incubation period of 24h, the supernatant was removed, cells were washed with PBS (pH 7.2, Gibco 20012-043) then, 1 ml/well of Trypsin-EDTA (0.25%, Gibco 25200-056) was added to allow cell detachment. After five minutes of incubation at 37°C under 5% of CO_2, 10 µl of cells were removed, mixed with 10 µl of Trypan Blue (0.4%, Sigma-Aldrich T8154). The cell count was carried out using a Neubauer improved counting slide (depth: 0.100 mm, surface: 0.0025 mm^2, Sigma-Aldrich BR717805). Living cells were caracterized by clear cytoplasm while death cells by blue cytoplasm. The inclusion criteria of living cells for cell counting were the location of cells within the quadrants, and

the exclusion criteria was their location on lines surrounding the quadrants.

Cell viability percentage was assessed using the Cell Cytotoxicity assay kit ab112118 (colorimetric, abcam) according to the manufacturer's instructions. After cell count, 100 µl of each previous treatment were seeded in a 96-well plate. The positive control wells consisted of cells seeded with LPS, the negative control wells consisted of cells seeded with Phosphate Buffer Saline (pH 7.2, Gibco 20012-043), the conductive control wells consisted of cells seeded in culture medium, the control wells without cells consisted only of culture medium without cells while the test wells consisted of cells seeded with extract (50, 100 and 250 µg/ml). Subsequently, 20 µl of staining reagent were added to each well and after shaking for 30s using a digital microplate shaker (ThermoFisher Scientific 88882006), the plate was incubated in dark at 37 °C under 5% of CO_2 for 24 h. The optical density was recorded at the wavelengths of 570 and 605 nm. The percentage of cell viability was calculated according to the formula below, the experiment was repeated three times:

Cell viability (%) = 100 × [((Rsample-R0))/((Rctrl-R0))]

Rsample absorbance ratio OD570/OD605 of test wells

R0 absorbance ratio OD570/OD605 of control wells without cells

Rctrl absorbance ratio OD570/OD605 conductive control wells

The genotoxicity induced by LPS was measured using comet assay (Møller *et al.,* 2020) [16]. HUVECs were plated at a density of $4.7x10^4$ cells/well and then left to incubate for 24 h at 37°C under 5% of CO_2. At the end of the incubation, the supernatant was descarded, cells were washed with PBS. Then, 250 µl of filtered extract of *Garcinia kola* (50, 100 and 250 µg/ml) were added to LPS test wells. After 1h, 250 µl of LPS 10 µg/ml (Sigma Life Science L3129) were added to the wells. In extract test wells were added 250 µl of filtered extract (50, 100 and 250 µg/ml) and 250 µl of culture medium. The naive control wells received no substance in addition to culture medium (500 µl). In LPS control wells were added 250 µl of LPS (10 µg/ml) and 250 µl of culture medium. The plate was then incubated for 24 hours at 37°C under 5% of CO_2. The final volume of each well was 500 µl. The experiment was performed in triplicate.

At the end of the incubation period, the supernatant was removed, cells were gently washed with PBS (pH 7.2, Gibco 20012-043). 1 ml of Trypsin-EDTA (0.25%, Gibco 25200-056) was added for five minutes at 37°C under 5% of CO_2. Aliquots of 10 µl of each treatment were prepared into micro tubes and placed into water bath (TSGP02S, Fisher Scientific) at 37°C for 5 minutes. After that, 120 µl of heated agarose were added into tubes and once the mixture was homogenized, 3 drops were placed on slides previously coated with normal agarose and slides were covered with coverslips. After 30 minutes of incubation at 4°C, the coverslips were removed and slides were inserted into a dark-rimmed glass vat containing 300 ml of ice-cold lysis solution (NaCl : 2.5M; EDTA : 100mM; Tris : 10mM; Lauril : 8g; NaOH : 10g; pH : 9.8). Slides were left to incubate for 1h at 4°C in fridge, then removed and inserted into an electrophoresis cube. 300 ml of ice cold electrophoresis buffer (NaOH: 300 mM; EDTA: 1mM) were then added, and after 30 minutes of immersion, the migration started at 25 V and 300 mA and lasted 20 minutes. Slides were then rinsed with 300 ml of neutralization buffer (Trizma base: 0.4 M, pH 7.5; Sigma-Aldrich T6066) for 5 minutes three times. After drying slides were placed in a glass vat containing 300 ml of 100% ethanol for 10 minutes. Afterward, the slides were removed from ethanol and allowed to dry in a cooled room. 100 µl of staining solution (Acridine Orange, Sigma-Aldrich A6014) were added on slides and the slides were again covered with coverslips for 5 minutes. The analysis was performed under fluorescence microscope with a 10x objective lens using Comet Score 2.0 software.

2-5- LDL-uptake inhibition

The LDL-uptake assay was performed using LDL-uptake assay kit (ab133127, abcam) according to the manufacturer's instruction. In a 24-well plate, HUVECs were disseminated at a density of $3×10^4$ cells/well and the plate was incubated for 24 hours at 37 °C under 5% of CO_2. In LPS test wells were added 250 µl of extract (50, 100 and 250 µg/ml) and after an incubation time of 1h, 250 µl of LPS (10 µg/ml) were added. In extract test wells were added 250 µl of extract (50, 100 and 250 µg/ml) plus 250 µl of culture medium. LPS control wells received 250 µl of LPS (10 µg/ml) and 250 µl of culture medium, while naive control wells received 500 µl of culture medium. The plate was then incubated for additional 24 hours under the same conditions. The supernatant was subsequently removed and replaced with LDL-DyLight 550 solution (abcam), the plate was incubated under the same conditions for 4 hours and the medium was removed and replaced with PBS (pH 7.2).

For the immunofluorescence staining, PBS was removed and cells were briefly washed with Tris-Buffered-Saline (TBS : pH 7.4, Sigma-Aldrich 93318), fixed for 10 minutes with 100 µl/well of Cell-Based Assay Fixative Solution (abcam), then washed three times for 5 minutes with Tris-Buffered Saline Triton (TBST, 0.1% of Triton X-100 to the final volume of TBS). Cells were first incubated for 30 minutes with 100 µl/well of blocking solution (abcam) and then for 1h with 100 µl/well of primary antibodies against rabbit LDL receptor (abcam). Cells were washed three times with TBST for 5 minutes and incubated in dark for 1h with 100 µl/well of rabbit-448 conjugated secondary antibodies (abcam). They were then washed with TBST three times for 5 minutes each and the coloration was evaluated using a fluorescence microscope equipped with an excitation and emission filter of 485 and 535 nm.

2-6- Inhibitory activity of *Garcinia kola* ethanolic extract on LPS-induced ICAM-1 and Anxa-1 expression in Human Umbilical Vein Endothelial Cells (HUVECs)

The method used was previously described by Guo *et al.* in 2016 [17] with some modifications. In a 12-well plate, HUVECs were plated at a density of $4.7×10^4$ cells/well and then left to incubate for 24 h at 37 °C under 5% of CO_2. In LPS test wells was added the filtered extract (250 µl) at the concentrations of 50, 100 and 250 µg/ml and after 1h of incubation, 250 µl of LPS (10 µg/ml) were added. In LPS control wells were added 250 µl of LPS (10 µg/ml) and 250 µl of culture medium. The naive control wells received 500 µl of culture medium (no extract neither LPS). The plate was then incubated for 24 hours at 37°C under 5% of CO_2. The expression of the anti-inflammatory protein Anxa-1 and adhesion molecule ICAM-1 was evaluated by immunohistochemistry and subsequently by optical densitometry of immunostaining (Girol *et al*., 2013) [18]. For this, HUVECs were cultured on slides, fixed with 4% of para formaldehyde (Fisher Scientific, Biotium 22023) for 24 h, washed first with Phosphate Buffered Saline (PBS) then with 0.4% of Tween 20 (Sigma-Aldrich P6585), blocked with 1% of diluted Bovine Serum Albumin (Sigma-Aldrich A9418) and incubated with anti ICAM-1 (1:100) (Sigma, A004877) and anti Anxa-1 (1:1000) (Thermo fisher, 71-3400) polyclonal rabbit antibodies diluted in BSA (1%). After washing, cells were incubated with biotinylated secondary antibodies (Life Technologies, 959943B) and then in diaminobenzidine (DAB)

substrate (Invitrogen, 750118) for development. Cells were then counterstained with hematoxylin (Sigma-Aldrich H9627). The slides were mounted with a solution containing glycerol (Sigma-Aldrich G5516) and PBS (1:1). Cells were then analyzed under a Leica microscope (DM500). For the densitometric analysis, 5 distinct points of the cytoplasm of 100 different cells per slide were evaluated to obtain an average related to the intensity of immunostaining. Values were obtained in arbitrary units from 0 to 255 using Leica Image Analysis software DM2500.

2-7- STATISTICAL ANALYSIS

Data were analyzed using Leica Image Analysis for immunohistochemistry and Comet Score 2.0 for comet test. Data are expressed as mean ± SD of the mean from at least three repetitions. The statistical analysis of data between extract-treated groups and controls (naive and LPS) was performed by analysis of variance (2 ways ANOVA) followed by Tukey's multiple comparisons test using GraphPad Prism software 8.0.2. Differences between treatments were considered significant with $p<0.05$.

3- RESULTS

3-1- Effect of *Garcinia kola* ethanolic extract on cell viability

To evaluate the toxicity of the EE, we tested its effect on the HUVECs growth. The results in Fig. 1 (A and B), show a concentration-dependent increase in endothelial cell growth. Indeed, the different concentrations of the EE significantly ($p<0.0001$) boosted the growth of endothelial cells compared to the naive control (untreated cells).

To test whether EE could prevent cell death, the plant extract was essayed on HUVECs treated with LPS. As shown in Fig. 1 (A, C and D), LPS significantly ($p<0.01$) decreased the cell viability as compared to the naive control and EE significantly reversed the toxic effect of LPS. The effect of EE was inversely proportional to its concentrations with respective p value of ($p<0.0001$), ($p <0.001$) and ($p<0.01$) for the concentrations 50 µg/ml, 100 µg/ml and 250 µg/ml. It is worth noticing that even in presence of LPS, EE at the concentrations of 50 and 100 µg/ml still increased the HUVECs' growth as compared to the naive control.

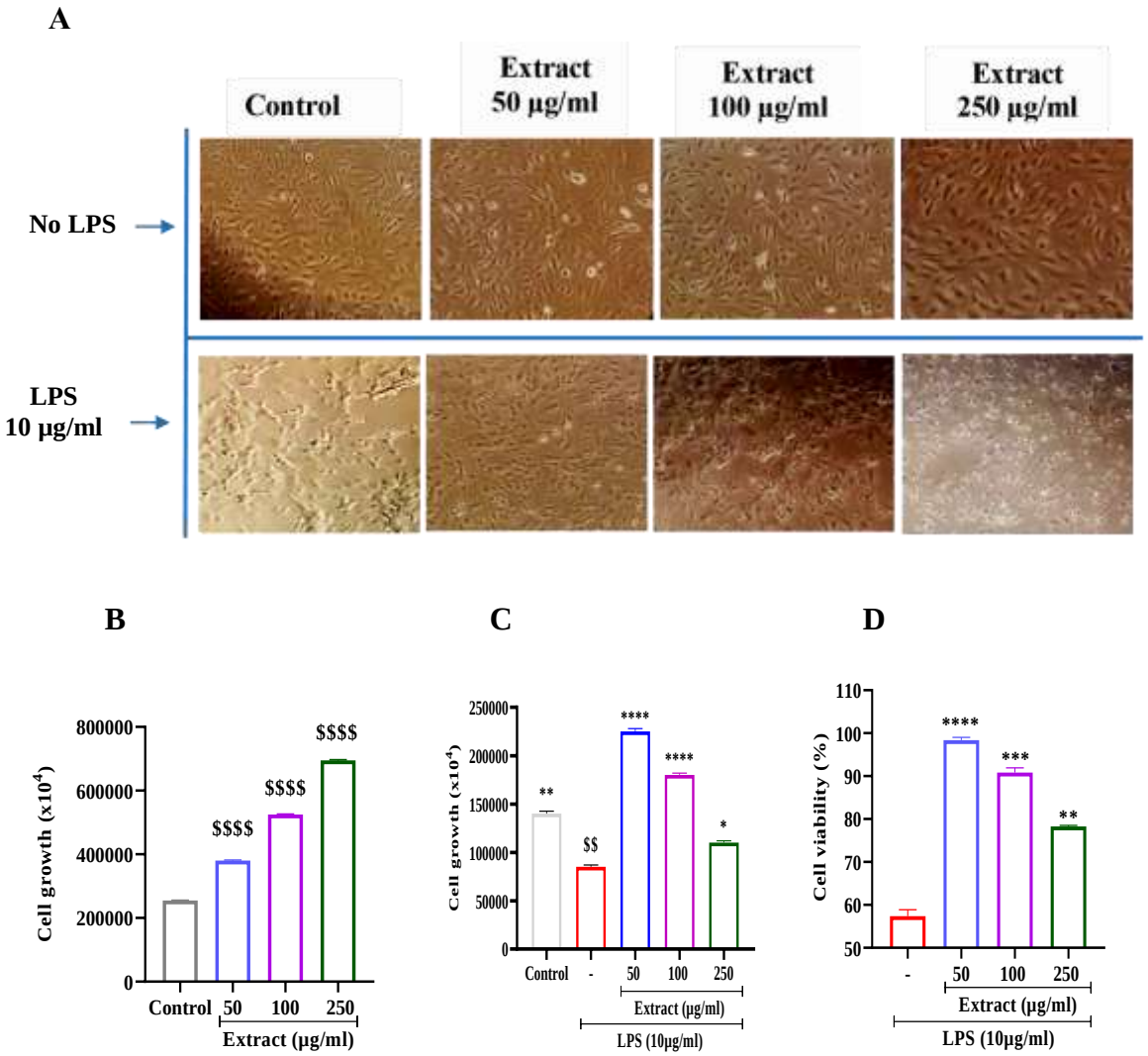


Figure 1: The ethanolic extract of the stem bark of *G. kola* increased the Human Umbilical Vein Endothelial Cells growth and reversed the toxic effect of LPS. Data are presented as mean + standard deviation. The experiment was repeated 3 times. $^{\$\$}p<0.01$ $^{\$\$\$\$}p<0.0001$ significant difference as compared to the naive control. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ significant difference as compared to the LPS-treated control.

3-2- Effect of *Garcinia kola* ethanolic extract on LPS-induced genotoxicity

Ethanolic extract decreased LPS-induced genotoxicity in HUVECs.

As depicted in Fig. 2, EE showed no endothelial cell DNA toxicity. In the expression of tail DNA and tail movement, no significant differences were observed between the naive control and the different concentrations of the ethanolic extract.

The stimulation of HUVECs with LPS, induced DNA damage compared to the naive control (untreated cells). The treatment of cells with the concentrations of 50 and 100 µg/ml of *G. kola* ethanolic extract significantly decreased the LPS-induced DNA damage in comparison to LPS-treated control as shown in Fig. 2 (A and C); while the concentration of 250 µg/ml of extract was unable to significantly protect against LPS-induced DNA damage.

In genotoxicity expression, several elements provide information on DNA damage including tail DNA and tail movement. Tail DNA represents the percentage of DNA found at the tail (area under the light blue line curve in Fig. 2 (B)) and tail movement represents the measure of distance between peaks head and tail (yellow line in Fig. 2 (B)).

EE significantly ($p<0.0001$) reduced the tail DNA Fig. 2 (C), with a percentage of 1.1185±2.698% and 1.66196±2.804% respectively for the concentrations of 50 and 100 µg/ml of extract in comparison to LPS-treated control which showed a percentage of 15.8433±14.313%. No significant difference was observed at the concentration of 250 µg/ml of extract compared to the LPS-treated control. In another side, the extract at its different concentrations significantly ($p<0.001$) reduced tail movement Fig. 2 (D) with an average of 1.64935±3.643%, 1.13508±1.498%, and 4.00321±11.368% respectively for concentrations 50, 100 and 250 µg/ml compared to the LPS-treated control which showed an average of 17.446±22.565%. Tail DNA times tail movement (tail DNA x tail movement), is a parameter that combines information from both tail DNA and tail movement. The concentrations of 50 and 100 µg/ml of EE significantly ($p<0.001$) reduced tail DNA times tail movement Fig. 2 (E), with respectively 11.0007±40.463% and 4.77053±10.481% compared to the LPS-treated control which showed an average of 451.185±670.452%.

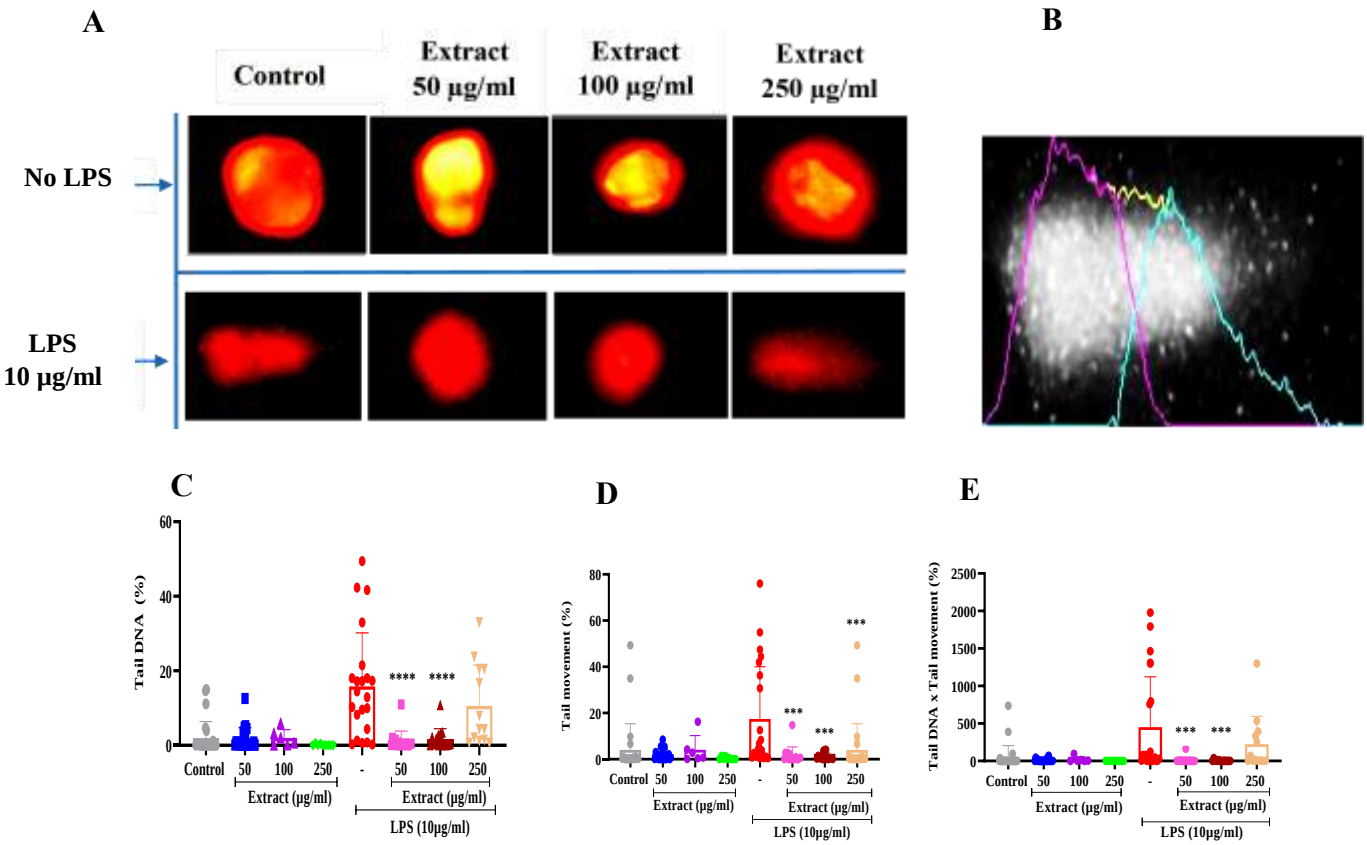


Figure 2: The ethanolic extract of the stem bark of *G. kola* protects against LPS-induced DNA damage in Human Umbilical Vein Endothelial Cells. Fluorescence measure in head and tail using acridine orange as the DNA binding dye. Tail DNA represents the area under the curve of the blue light line in Fig. 2 (B). Tail movement measure the distance between head and tail peaks (yellow line in Fig. 2 (B)). Tail DNA x Tail movement is an computed parameter that combine information from both tail DNA and tail movement. Illustration of the Comet assay's performed measures. Bar graphs are presented as mean + standard deviation. The experiment was repeated 3 times. ***p<0.001, ****p<0.0001 significant difference as compared to the LPS-treated control.

3-3- Effect of the *Garcinia kola* ethanolic extract on LDL-uptake

G. kola ethanolic extract reduced LDL-uptake in LPS-treated HUVECs. As shown in Fig. 3, for cells not stimulated by LPS, the different concentrations of the ethanolic extract of *G. kola* considerably reduced the LDL uptake and the number of cells having absorbed the LDL compared to the number of cells having expressed the LDL receptors compared to the naive control.

For endothelial cells stimulated with LPS and treated with the ethanolic extract of *G. kola*, the extract at concentrations 50 and 100 µg/ml induced a reduction of LDL uptake and the number of cells having absorbed LDL compared to the number of cells having expressed the LDL receptors, when compared to the LPS-treated control. On the other hand, at the concentration of 250 µg/ml of the ethanolic extract, all the cells that expressed the LDL receptors uptake the LDL (Fig. 3).

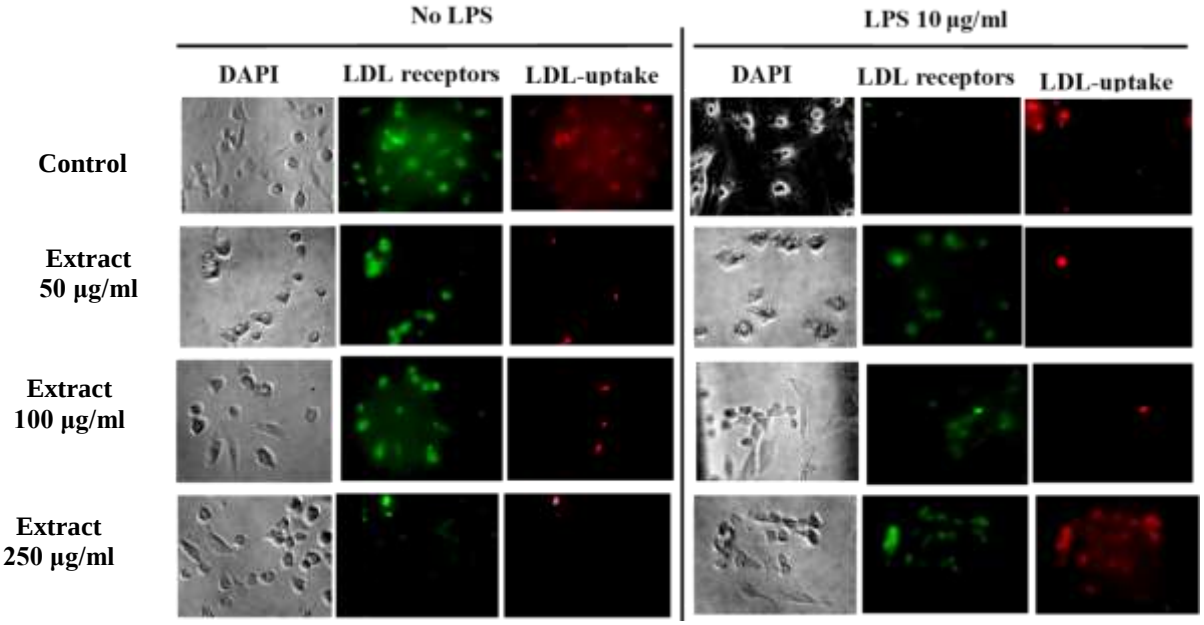
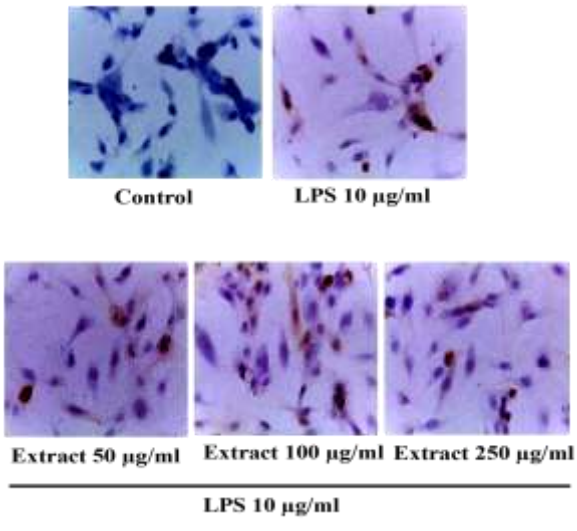


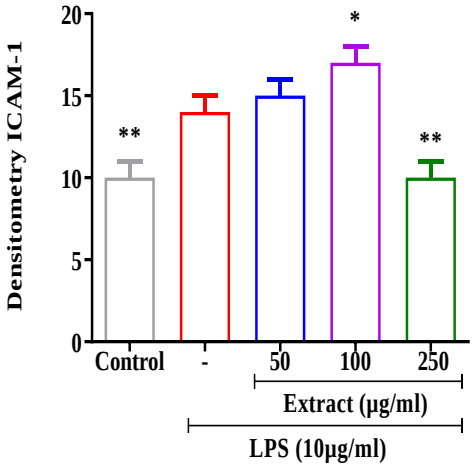
Figure 3: The ethanolic extract of the stem bark of *G. kola* reduced LDL-uptake by the Human Umbilical Vein Endothelial Cells.

3-4- Effect of *Garcinia kola* ethanolic extract on the expression of ICAM-1

LPS stimulation increased the level of ICAM-1. EE showed a dual effect on the ICAM-1 expression in LPS-treated HUVECs (Fig. 4, A and B). Treatment of LPS-stimulated cells with the EE concentrations of 50 and 100 µg/ml led to a concentration dependent increase in ICAM-1 expression. However, a significant increase (p<0.05) by 17±1 as compared to the LPS-treated control was observed only at the concentration of 100 µg/ml. At the concentration of 250 µg/ml of EE, instead, ICAM-1 expression significantly (p<0.01) decreased in comparison to LPS-treated control with a value of 10±1, which is equal to that of the negative control.



A

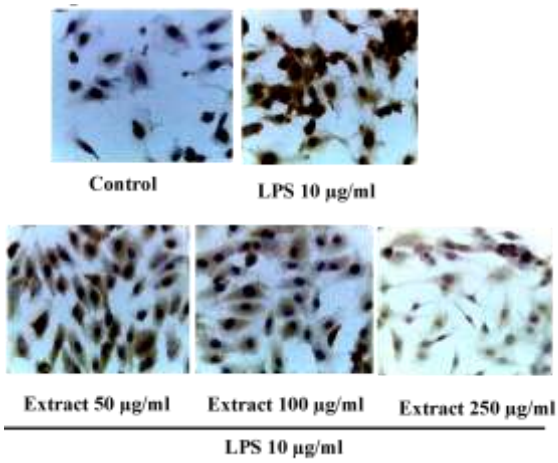


B

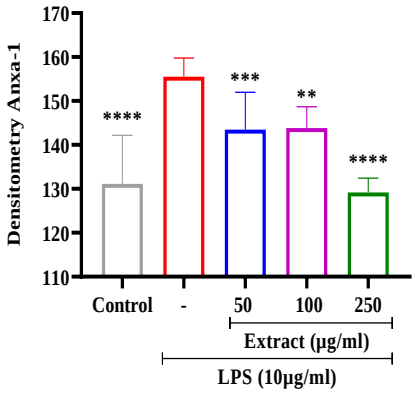
Figure 4: The ethanolic extract of the stem bark of *G. kola* showed a dual effect on the LPS-induced ICAM-1 expression. Bar graphs are presented as mean + standard deviation. The experiment was repeated 3 times. *p<0.05, **p<0.01 significant difference as compared to the LPS-treated control.

3.5. Effect of the *Garcinia kola* ethanolic extract on the expression of Anxa-1

The immunohistochemistry revealed that the HUVECs treatment with LPS significantly (p<0.0001) increased the expression level of Anxa-1. Treatment of LPS-stimulated HUVECs with EE led to a significant drop in the Annexin-1 expression at the different concentrations of 50 µg/ml (p<0.001), 100 µg/ml (p<0.01) and 250 µg/ml (p<0.0001). A highly significant reduction of the expression of Anxa-1 was observed at the concentration of 250 µg/ml of extract with an expression rate of 129.193±14.262 compared to control LPS which showed a value of 155.500±4.263 (Fig. 5, A and B).



A



B

Figure 5: The ethanolic extract of the stem bark of *G. kola* reduced the LPS-induced overexpression of Anxa-1 in HUVECs. Bar graphs are presented as mean + standard deviation. The experiment was repeated 3 times. **p<0.01, ***p<0.001, ****p<0.0001 significant difference as compared to the LPS-treated control.

4- DISCUSSION

Growing evidence suggests that oxidative stress and inflammation induced by lipopolysaccharide stimulation can lead to apoptosis or endothelial cell death. Indeed, in vitro stimulation by LPS, alters the normal functioning of endothelial cells and activate the production of cytokines (TNF-α, IL-1), chemokines and adhesion molecules (VCAM-1, ICAM-1, E-selectin) which facilitate interaction between endothelial cells and leucocytes resulting in leucocyte recruitment into inflamed tissues (Dayang *et al*., 2019) [19]. Molecules able to protect endothelium from dysfunction or injury that can lead to cell death, can reduce the incidence of cardiovascular diseases. The ethanolic extract of *G. kola* in its various concentrations significantly protected endothelial cells from cell death in an inversely proportional way to the increase in concentration with a percentage of cell viability of more than 80%.

This protection of endothelial cells would be correlated with the presence of antioxidant molecules such as polyphenols in the ethanolic extract of *G. kola*. Indeed, many antioxidant molecules were linked to a lower heart disease risk factor. Some analysis found that Vitamin C significantly reduced LDL cholesterol and blood triglycerides level (Ryan Raman, 2020) [20]. Moreover, the protective role of Berberine against arterial plaque formation and its attenuating effect on the inflammatory response in the aortic tissues in atherosclerotic rats with damp-heat syndrome by promoting autophagy has been shown (Ke *et al*., 2020) [21].

The cytotoxic effect of high level of Reactive Oxygen Species (ROS), results in necrotic cell death and cell apoptosis. The oxidative injuries caused by ROS to macromolecules, include DNA damage and DNA strand breaks (Yuan *et al*., 2019) [22]. The comet assay is a widely used test that aims to detect, DNA damage or DNA strand breaks and repair activity (Møller *et al*., 2020) [16]. The size (tail movement) and amount of DNA found inside the comet (tail DNA) give the gravity of the DNA damage. The evaluation of the repair activity of *G. kola* ethanolic extract in LPS-induced genotoxicity in HUVECs, has shown that *G. kola extract* significantly reduced tail DNA and tail movement expression. Many studies showed that bioactive components possess many activities such as antioxidant and DNA damage protection activities (Kaur *et al*., 2018) [23]. Naringenin a citrus bitter component, protected plasmid DNA from UVB-induced DNA damage (Kaur *et al*., 2019) [24]. *G. kola* ethanolic extract could therefore act via its antioxidant molecules by inhibiting free radicals activity on cell DNA.

For many researchers, tail movement does not provide precise information on comet formation, as maximum DNA migration is usually achieved at low doses of exposure to strand breaking agents (at least when analyzed with commonly used comet testing protocols). Therefore, it was recommended to evaluate the tail DNA times tail movement (tail DNA x tail movement) measurement in this scenario. The ethanolic extract of *G. kola* significantly (p<0.001) reduced the tail DNA times tail movement at the concentrations of 50 et 100 µg/ml compared to the LPS-treated control.

Atherosclerosis is a chronic maladaptive inflammatory disease in which arteries harden through build-up of plaques and lose their elasticity (Bartlett *et al*., 2019) [25]. Its starting point has been linked to the increase in plasma cholesterol resulting in changes of the arterial endothelial permeability that allows the migration of LDL-Cholesterol particles into the arterial wall where they are oxidized and become highly chemoattractant. Even if the mechanism of LDL absorption by cardiovascular system cells is still poorly understood, regulating the amount of LDL-cholesterol and its absorption by cells are important therapeutic targets in the treatment of atherosclerosis and associated diseases (Kraehling *et al*., 2016) [26]. The evaluation of *G. kola* ethanolic extract activity on LDL-uptake revealed a significant decrease in LDL uptake by endothelial cells stimulated with LPS and treated with the ethanolic extract at the concentrations of 50 and 100 µg/ml. Some studies using an in vitro permeability system and blocking LDL binding to

LDL-receptors using an anti-LDL receptor antibody, revealed that LDL was responsible for an increase of the permeability of endothelial cells in an LDL-receptor-dependent manner (Magalhaes *et al*., 2016) [27]. The ethanolic extract of *G. kola* would therefore act by inhibiting specific LDL-receptors.

In the development process of atherosclerosis, after the penetration and oxidation of LDL particles into the inner wall of artery, endothelial cells express a network of adhesion molecules (VCAM-1, ICAM-1, selectins) which induce the adhesion of circulating monocytes (Bergheanu *et al*., 2017) [3]. These steps are the most significant in the mechanism for setting up atherosclerosis. The search for substances that can inhibit monocytes adhesion to the endothelial wall by reducing the expression of adhesion molecules, can be an effective way of prevention and/or treatment. The evaluation of *Garcinia kola* ethanolic extract activity on LPS-induced ICAM-1 expression, showed a significant decrease of ICAM-1 expression in LPS-stimulated cells at the concentration of 250 µg/ml of extract. The antiatherogenic effects of a polyphenol, resveratrol, was shown through the inhibition of lipopolysaccharide-stimulated adhesion of THP-1 human monocytes by the decrease of the expression of ICAM-1 (Youngsik *et al*., 2019) [28]. Thus, this inhibitory effect of *G. kola* ethanolic extract, could be related to the presence of polyphenols and flavonoids. Indeed, according to some several epidemiological studies, an inverse association between flavonoids intake and mortality by cardiovascular diseases was found (Santos *et al*., 2018) [29]. In the same way, Ciumarnean *et al*., in 2020 [30] showed that the beneficial cardiovascular effects of flavonoids are due to their ability to produce vasodilatation and regulate the apoptotic processes in the endothelium.

Since inflammation is intimately involved in all stages of atherosclerosis, a different view of that disease as an inflammatory disease has emerged. Thus, targeting the inflammation has become a promising way to improve current approaches.

Annexin-1, that promotes the end of inflammation by engaging various important pro-resolution properties, is expressed by multiple cells including leukocytes, endothelial cells and mast cells. Since its designation as an important regulator in leukocyte recruitment and in anti-inflammatory response by protecting cells from attacks and chronic inflammation (De Jong *et al*., 2017a) [7], Annexin-1 may be a suitable candidate for limiting inflammation during atheromatous plaque formation and related cardiovascular disease.

The expression of Annexin-1 during LPS-induced inflammation was evaluated and results showed that it decreases with increasing concentration of extract. The ethanolic extract lowers inflammation as evidenced by low expression of Anxa-1. Studies have shown that Anxa-1 inhibits leukocyte migration to inflammatory site by binding to and activating the formylpeptide (FPR) receptor on leukocytes and endothelial cells (Parisi *et al*., 2019) [31]. It also prevents inflammatory responses by reducing the expression of nitric oxide synthase (iNOS) involving a protective role of Anxa-1 in atherosclerosis (Shen *et al*., 2020) [6].

5- CONCLUSION

The ethanolic extract of *G. kola* induced an inhibition of LDL uptake and LPS-induced ICAM-1 and Anxa-1 expression. It also prevented LPS-induced apoptosis and DNA damage and showed no cell toxicity. In view of all the results obtained showing the effect of *G. kola* ethanolic extract on various stages of atherosclerosis development, namely the uptake and oxidation of LDL, the release of adhesion molecules necessary for the adhesion of monocytes, combined with its antioxidant power, *Garcinia kola* could be a probable source of metabolites for the development of an effective treatment against atherosclerosis.

Conflict of Interest: The authors declare no conflict of interest

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