

RESEARCH ARTICLE

# Assessment of the effects of *Annona muricata* leaf aqueous extract *in vitro*

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## ABSTRACT

**Background:** The extracts of *Annona muricata*, also known as graviola, form part of traditional medicine. **Objectives:** Verification of their effects and elucidation of their mechanisms is beneficial in terms of the utility of these extracts in treatment or prevention. **Methods:** We monitored the effectiveness of an extract of dried graviola leaves available for direct consumption on cancer cell lines and isolated rat liver mitochondria. **Results:** The Jurkat cell line exhibited the highest sensitivity, with the metabolic activity of cells reaching less than 10% at the highest tested concentration. The effect was dose-dependent. The viability of Jurkat cells was affected after more than the 72 hours. At the same time, there was also a reduction of mitochondrial membrane potential. At 24 to 48 hours incubation, no increase was found in the activity of caspase-3 but increased levels were found for the non-phosphorylated form of Bcl-2. Higher concentrations of graviola affected the mitochondrial redox state by dramatically reducing the levels of reduced glutathione. This was achieved through reduction in the activity of glutathione-S-transferase. **Conclusion:** Our findings indicate that the studied preparation of graviola displayed cytotoxic effects on some cells, depending on the dose and length of action.

**Keywords:** *Annona muricata*; Antioxidant enzyme; Apoptosis; Cell lines; Glutathione.

## INTRODUCTION

The utilisation of herbal extracts as alternative treatments has become more globally widespread in recent years. While plants have been used for centuries to treat diseases, current scientific studies have shown that many of the herbs or essential oils derived from them can actually be used in the prevention and treatment of many symptoms due to their medicinal properties (Tognolini et al., 2006).

*Annona muricata* L. (graviola, GR) is a tree in the family *Annonaceae*, order *Magnoliales*. It comes from the Caribbean in Central America and forms part of traditional medicine in many countries. The main components obtained from leaves, bark and twigs are acetogenins, derivatives of fatty acids with a carbon number of 32-34 in combination with 2-propanol. They also contain quinolines and isoquinolines, and a large amount of B, C and E vitamins in addition to phosphorus. Additionally, the fruit is high in linoleic acid and other unsaturated fatty acids. This specific group of annonaceous compounds,

acetogenins are responsible for anti-cancer activities and also their effectiveness against multidrug-resistant tumor cell lines, described in more details in Prasad et al. (2019). However, as more potent inhibitors of complex I (NADH-ubiquinone reductase) in mammalian, cause insufficient reoxidation of NADH, and consequent reduction in the concentration of NAD below the levels required by citric acid cycle enzymes. The cessation of the cycle together with the inhibition of the respiratory chain causes insufficient production of ATP. These properties, together with the abnormal accumulation of tau proteins, are also responsible for the manifestation of a levodopa-unresponsive form of atypical parkinsonism (Lannuzel et al., 2007).

Graviola extracts have a spectrum of other interesting effects such as anti-viral, -bacterial, -fungal -nociceptive, -inflammatory, -pyretic, -malarial, -hyperglycaemic, -oxidant, -ulcerogenic; insecticidal, hepatoprotective and wound healing (Cesar et al., 2021; Gavamukulya et al., 2017; Saleem et al., 2017; Son et al., 2021).

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In aqueous leaf extracts mostly saponins, tannins, glycosides, alkaloids, flavonoids, anthraquinones, coumarines, and sterols were detected (Ejembí et al., 2021; Fareed et al., 2011; Nga et al., 2018). A wide range of effects of aqueous extracts from cytotoxic to cytoprotective have been detailed (Florence et al., 2014; Balderrama-Carmona et al., 2020; Ogundare et al., 2020). For example, the antiulcerogenic, scarring effect of graviola is dose-dependent and is attributed to its antioxidant properties, mainly mediated by tannins, flavonoids and triterpenes (Baskar et al., 2007; Hamid et al., 2012).

Exploring the properties of natural substances is an inexhaustible well. However, it is often not necessary to choose anything more than the available offer of guaranteed natural “everything”, from which it is difficult for an ordinary person to find reliably verified information about the effects of the dried leaves of the *Annona muricata*. There is an abundance of controversial information, mostly from online sources, about its guaranteed beneficial antioxidant effects on the organism, which is immediately supported by its powerful action against cancer with an effect several times higher than commonly used chemotherapeutics. With such marked adverse effects, it is apparent that the antioxidant, chemopreventive or chemotherapeutic effects will be highly dose dependent. The aim of this work was to verify the cytotoxic effects of freely available dried graviola leaves. Completely new sight is the evaluation of the content of a significant reductant, glutathione and glutathione-using enzyme in an isolated system, mitochondria, which itself is not capable of its synthesis. The results are thus a direct account of the antioxidant or pro-oxidant effect of the extract.

## MATERIALS AND METHODS

For analysis, plant material was chosen in a form freely available to consumers on the market – dried crushed leaves of *Annona Muricata* L. The plant material was provided for the purpose of experiments by Dr. Rafael Alvis Pizzaro from Peru through collaboration with Huminnet Ltd. later EKS-Granite Kft., Hungary and Humac-group, www.humac.bio). For the analysis itself, an aqueous extract was taken from the leaves ( $1 \text{ mg}\cdot\text{ml}^{-1}$ ) for 24 hours, within concentrations  $1000 - 15.6 \text{ }\mu\text{g}\cdot\text{ml}^{-1}$ . The study addressed part of the grant task approved by the Ethics committee of the Faculty of Medicine, Pavol Jozef Šafárik University in Košice no. 1A/2016.

### Cell cultures

The human cancer cell lines MDA-MB-231 (human mammary gland adenocarcinoma), A2780 (human ovarian carcinoma), Jurkat (human leukaemic T cell lymphoma),

U87-MG (human glioblastoma astrocytoma p53wt) and NIH 3T3 (murine fibroblasts) (all ATCC) were cultured in RPMI 1640 medium (Biosera, Kansas City, MO, USA). The MCF-7 (human Caucasian breast adenocarcinoma) and A-2780 (human ovarian carcinoma) cell lines were maintained in a growth medium consisting of high glucose Dulbecco's Modified Eagle Medium with sodium pyruvate (Biosera). Growth medium was supplemented with a 10% foetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1X HyClone™ Antibiotic/Antimycotic Solution (GE Healthcare, Little Chalfont, UK). Cells were cultivated in an atmosphere containing 5% CO<sub>2</sub> in humidified air at 37°C. Cell viability, estimated by trypan exclusion, was greater than 95% before each experiment.

### MTT assay

To analyse cell proliferation and viability, a colorimetric assay was used with MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Aldrich, St. Louis, MO, USA). Cancer and non-cancer cells were seeded at a density of  $5 \times 10^3$  cells/well in 96-well polystyrene microplates. Twenty-four hours after cell seeding, different concentrations ( $1000 - 31.2 \text{ }\mu\text{g}\cdot\text{ml}^{-1}$ ) of the graviola extract were added. After 72 hours of incubation, 10  $\mu\text{l}$  of MTT were added to each well. After 4 hours, the addition of 100  $\mu\text{l}$  of 10% sodium dodecyl sulphate to each well and incubation for another 12-24 h allowed the formed formazan to dissolve. Finally, the metabolic activity of the cell was evaluated by measuring absorbance at a wavelength of 540 nm using the automated Cytation™ 3 Cell Imaging Multi-Mode Reader (Biotek, Winooski, VT, USA). Absorbance of control wells was taken as 100%, and the results were expressed as a percentage of untreated control. IC50 values were calculated from MTT analyses.

### Flow cytometry analysis (FCM)

Based on the best MTT results, only Jurkat cells were used in the following analyses. Jurkat cells ( $1 \times 10^6$ ) were seeded for FCM analysis in Petri dishes and treated with graviola extract at  $500 \text{ }\mu\text{g}\cdot\text{ml}^{-1}$  for 24, 48 or 72 hrs. Floating and adherent cells were harvested, washed in PBS, divided and stained prior to analysis. Fluorescence was detected after 15-30 min incubation at room temperature in the dark using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). A minimum of  $1 \times 10^4$  cells were analysed per analysis. All experiments were performed in triplicate.

### Cell cycle analysis

Jurkat cells ( $1 \times 10^6$ ) were harvested at 24, 48 and 72 hours after graviola extract treatment ( $500 \text{ }\mu\text{g}\cdot\text{ml}^{-1}$ ). Complete cell population was washed in phosphate-buffered saline (PBS), fixed in cold 70% ethanol and kept at +4 °C overnight. Before analysis, fixed cells were washed in PBS and stained

in PBS solution (500  $\mu\text{l}$ ) containing 0.2 % Triton X-100, 0.5 mg/ml ribonuclease A and 0.025 mg/ml propidium iodide (all Sigma Aldrich). Samples were incubated for 30-60 minutes at room temperature in the dark. The DNA content of the stained cells, representative of each phase of cell cycle, were analysed using a flow cytometer BD FACSCalibur (BD Biosciences).

#### **Annexin V/PI staining**

For detection of apoptosis, Jurkat cells ( $1 \times 10^6$ ) were harvested 24, 48 and 72 hours after treatment with 500  $\mu\text{g}\cdot\text{ml}^{-1}$  graviola extract treatment. Complete cell population was washed in PBS and stained using Annexin-V-FITC (BD Biosciences) for 15 minutes at room temperature in the dark followed by incubation with propidium iodide (PI; Sigma) and analysed by flow cytometer (BD FACSCalibur).

#### **Detection of mitochondrial membrane potential (MMP)**

Disruption of MMP after graviola extract treatment (500  $\mu\text{g}\cdot\text{ml}^{-1}$ ) was analysed via FCM after staining with 0.1  $\mu\text{M}$  TMRE (Molecular Probes, Eugene, OR, USA). After 30 min of incubation at room temperature in the dark, the stained cells were then washed twice with PBS, resuspended and analysed ( $1 \times 10^4$  cells per sample). Fluorescence was detected with a 585/42 (FL-2) optical filter by flow cytometer (BD FACSCalibur).

#### **Caspase-3 activation analysis**

Changes in caspase 3 activation were analysed with FCM using Active Caspase-3 PE Rabbit mAb (PE Conjugate) (Cell Signaling Technology, Danvers, MA, USA). The cells were harvested 24, 48 and 72 hours after graviola extract treatment (500  $\mu\text{g}\cdot\text{ml}^{-1}$ ). Cells were stained with phycoerythrin (PE) conjugated antibody and incubated for 30 min at room temperature in the dark. The cells were then washed twice with PBS, resuspended in 500  $\mu\text{M}$  to the total volume, and analysed ( $1 \times 10^4$  cell per sample). Fluorescence was detected with a 585/42 (FL-2) optical filter by flow cytometer (BD FACSCalibur).

#### **Measurement of mitochondrial protein dynamics (efflux, activation/deactivation)**

Jurkat cells were harvested 24, 48 and 72 hours after graviola extract treatment (500  $\mu\text{g}\cdot\text{ml}^{-1}$ ), washed with PBS and then stained for 30 minutes with Rabbit anti-Bcl-2 polyclonal Ab FITC conjugate 1:200 (Bioss, Woburn, MA, USA), Phospho-Bcl-2 rabbit mAb Alexa Fluor 488 conjugate 1:200 (Cell Signaling Technology, Danvers, MA, USA), or Mouse anti-AIF polyclonal Ab FITC conjugate 1:200 (Bioss). Additionally, fluorescence was measured using BD FACSCalibur (FL1). Results were quantified as a ratio of the fluorescence median intensity in experimental groups compared to a particular control.

#### **Analysis of antioxidant activity on rat liver mitochondria**

Biological material for analysis were taken from the control group of the 1<sup>st</sup> stage of the procedure approved by ŠVPS SR Ro-2575/14-221 on male Sprague-Dawley laboratory rats from the own breeding of the Laboratory of Research Biomodels, LF UPJŠ. The animals were 6 weeks old and weighed 250 g. Liver mitochondria were isolated as described by Fernández-Vizzara *et al.* (2010) using a homogenization buffer (0.32 mm sucrose, 1mm EDTA, 10 mM Tris-HCl, 0.1% albumin; pH 7.4) at 4 °C. Activity of Glutathione-S-Transferase (GST, EC 2.5.1.18) were measured by means of the Glutathione-S-Transferase Assay Kit (Sigma-Aldrich, Germany). Reduced glutathione (GSH) content was measured by the method described by Floreani *et al.* (1997). Assays were performed on an M 501 single beam UV/VIS spectrophotometer (Spectronic Camspec Ltd., Leeds, United Kingdom). All parameters were measured at least in triplicate and calculated per milligram or gram of protein determined using the bicinchoninic acid assay.

## **RESULTS**

In our experiments, the most significant effect of graviola was observed in the MTT test on Jurkat cells (Table 1), where the metabolic activity of the cells at the highest tested concentration reached less than 10% of the metabolic activity of the control and 25% of control at the second highest concentration. Incubation of the A-2780 cell line did not significantly reduce the metabolic activities of the cells at any of the tested concentrations of graviola. At the lowest concentration (31.25  $\mu\text{g}\cdot\text{ml}^{-1}$ ) and the highest (1000  $\mu\text{g}\cdot\text{ml}^{-1}$ ), the decrease was only between 80% and 60%. The cell line p53wt (U-87 MG) responded to graviola supplementation similarly to A2780 (Table 1). The addition of graviola had the lowest effect on the metabolic activities of MCF-7 and MDA-MB 231. By comparison, the 3T3 cell line also exhibited changes in metabolic activity at the highest test concentration (1000  $\mu\text{g}\cdot\text{ml}^{-1}$ ), with no changes in the cell metabolic activity at lower concentrations.

As the Jurkat cell line showed the highest sensitivity, the following methodologies were only performed on this cell line. The concentration of graviola used to incubate the cells was  $c_2 = 500 \mu\text{g}\cdot\text{ml}^{-1}$  and measurements were made at 24, 48 and 72 hours in all subsequent experiments. By microscopic monitoring of cell viability with trypan blue, it was observed that the effect of graviola at the tested concentration after 24 and 48 h incubation was almost the same and the most pronounced effect did not appear until 72 h incubation (Table 2), where 35% of cells were observed to have impaired cell membrane permeability, generally considered dead.

**Table 1: Assessment of cell viability and metabolic activity after 72 h incubation by MTT test and IC50 values ( $\mu\text{mol.l}^{-1}$ )**

	c1 1000 $\mu\text{g.ml}^{-1}$	c2 500 $\mu\text{g.ml}^{-1}$	c3 250 $\mu\text{g.ml}^{-1}$	c4 125 $\mu\text{g.ml}^{-1}$	c5 62.5 $\mu\text{g.ml}^{-1}$	c6 31.2 $\mu\text{g.ml}^{-1}$	IC50
3T3	84	87	92	98	100	100	> 1000
Jurkat	7.9	25.1	59.4	72.1	71.3	84.7	320.7
A-2780	22.6	68.7	70.9	71	72	74.2	702.8
U-87 MG	64.6	71.7	72.9	78.1	81.9	82.3	> 1000
MDA-MB 231	78	82	85	87	90	95	> 1000
MCF-7	60.3	90.8	93	96	98	100	> 1000

Analysis of the effect of graviola on the Jurkat cell cycle showed the accumulation of cells in the G2/M phase and a decrease in the G1 phase of the cell cycle after just 24 h incubation. With increasing incubation time, the number of cells in the G2/M phase increased. After 72 h incubation, a very small increase in the number of cells were observed in the sub-G0/G1 population, considered to be cells in apoptosis (Fig. 1). Regarding the detection of phosphatidylserine externalization, a more significant increase in apoptosis was observed in Jurkat cells after 72 h incubation with graviola (Fig. 2a and b). These results also correlate with the trypan blue viability test, where a significant effect was also noted after 72 h incubation.

Flow cytometry analysis showed an increase in the number of cells with decreased MMP in Jurkat cells with increasing incubation time with graviola (Fig. 3a). In our experiments, there was a significant decrease in caspase-3 activity after 24 and 48 h incubation with graviola at a concentration of 500  $\mu\text{g.ml}^{-1}$  (Fig. 3b). After 24 and 48 h incubation, analysis of protein dynamics (Fig. 3c) showed an increase in the level of the unphosphorylated form of Bcl-2 able to form complexes with other proteins, such as proapoptotic Bax, thus preventing its proapoptotic effect.

The effect of selected concentrations of graviola extract was monitored on reduced glutathione levels (GSH) and glutathione-S-transferase (GST) activity on liver mitochondria. As mitochondria from healthy cells were involved, the concentrations tested were lower than those found to be effective on cancer cell line. GSH levels varied depending on the graviola extract concentration used (Table 3). Low concentrations (15.6 and 31.2  $\mu\text{g.ml}^{-1}$ ) led to a significant increase in GSH levels (Table 3), while higher concentrations led to a statistically significant decrease. The concentration of 62.5  $\mu\text{g.ml}^{-1}$  had no effect on GSH levels in the liver mitochondria. The addition of different concentrations of graviola to isolated liver mitochondria suggested a concentration-dependent effect (Table 3), where higher concentrations reduced GST activity (without statistical significance).

## DISCUSSION

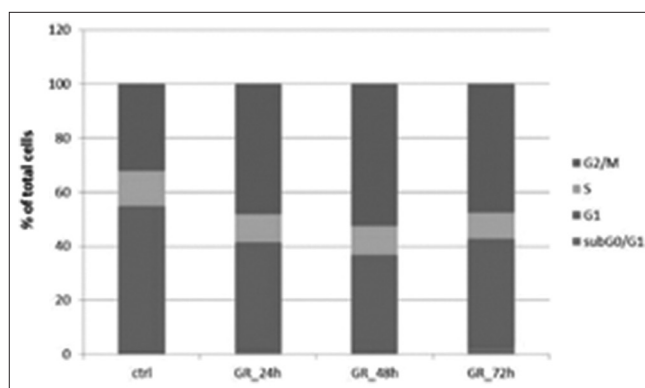
Exploitation of the effects of plants is probably as old as humanity itself. It has been found that many plant

**Table 2: Viability of Jurkat Cells after 24, 48 and 72 h incubation with graviola (GR) at concentration c2 = 500  $\mu\text{g.ml}^{-1}$** 

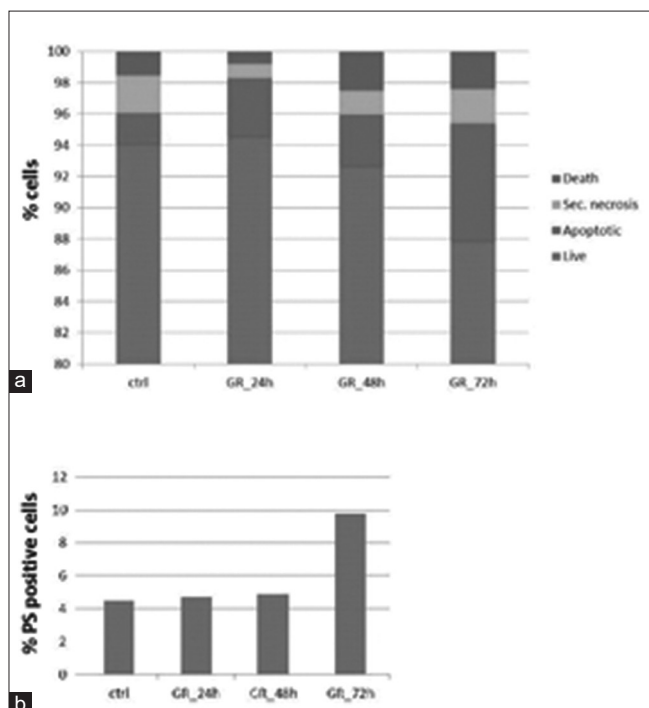
	% living cells	% dead cells
Ctrl	98	2
GR 24 h	87	13
GR 48 h	83	17
GR 72 h	65	35

**Table 3: Levels of reduced glutathione (GSH) in isolated liver mitochondria and glutathione-S-transferase activity in comparison to control after treatment with different concentrations of graviola**

Group/tested concentration ( $\mu\text{g.ml}^{-1}$ )	GST ( $\mu\text{kat.mg prot}^{-1}$ )	GSH (nmol.mg prot <sup>-1</sup> )
Ctrl	1.298 $\pm$ 0.049	1.786 $\pm$ 0.379***
15.6	1.446 $\pm$ 0.051	5.241 $\pm$ 0.487***
31.2	1.352 $\pm$ 0.063	10.227 $\pm$ 0.501
62.5	0.998 $\pm$ 0.088	2.086 $\pm$ 0.511
125	0.856 $\pm$ 0.154	0.658 $\pm$ 0.258*
250	0.954 $\pm$ 0.016	0.635 $\pm$ 0.208*

**Fig 1.** Analysis of the cell cycle of Jurkat cells after 24, 48 and 72 h incubation with graviola (GR) at a concentration of c2 = 500  $\mu\text{g.ml}^{-1}$ 

species used for this purpose contain therapeutically active substances which can either be extracted or used directly. The important role of herbal extracts in the development of new drugs is further emphasized by the fact that natural products were involved in the development of approximately 60% of all therapeutic agents between 1981 and 2012 (Newman and Cragg, 2012). In using active compounds from plants, several dosage forms are suitable, including dried drugs or aqueous and alcoholic extracts. Even today, the search for and elucidation of plant

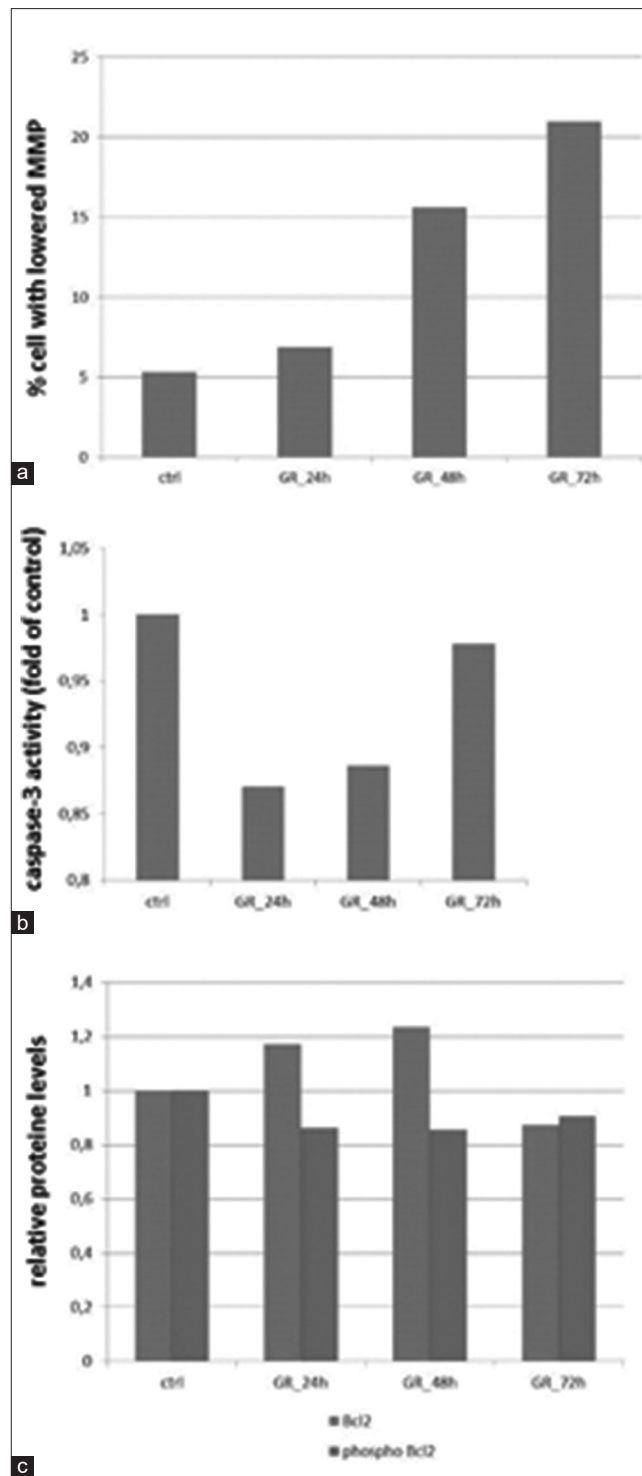


**Fig 2.** Detection of phosphatidylserine (PS) externalization (a) and cells with phosphatidylserine externalization (b) after 24, 48 and 72 h incubation with graviola (GR) at a concentration of  $c_2 = 500 \mu\text{g.ml}^{-1}$

extract effects gives great importance to the history of the reactions of humans and animals to naturally occurring compounds, which are complex mixtures of naturally occurring compounds rather than pure compounds of synthetic origin. In general, these crude plant extracts are less toxic than their individual components or synthetically prepared compounds as they are a mixture of known and unknown substances, which at the same time reduces the manifestation of side effects (Koul et al., 2005).

To accurately assess the effects of graviola, a herbal preparation of *Annona muricata* was chosen in a form available to the direct consumer, namely crushed dried leaves. The cytotoxic effect of graviola extracts, also of interest in the study, is further underlined by the fact that graviola leaves are used in cancer treatment in areas of South America and tropical African countries, including Nigeria (Adewole et al., 2008; Mishra et al., 2013).

The MTT assay is based on the conversion of MTT<sup>+</sup> (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble formazan precipitate in mitochondria. MTT is reduced by mitochondrial enzymes, so changes in metabolism led to changes in the rate of its reduction. Among the monitored cell lines, the Jurkat cell line showed the highest sensitivity, where the most significant effect of graviola was noticed. The inhibitory effect was dose-dependent, with the metabolic activity of



**Fig 3.** Analysis of changes in mitochondrial membrane potential (MMP) of Jurkat cells (a), changes in caspase activity - 3 Jurkat cells (b) and protein dynamics in mitochondria in Jurkat cells after 24, 48 and 72 h incubation with graviola (GR) at concentration  $c_2 = 500 \mu\text{g.ml}^{-1}$  (c)

cells at the highest tested concentration reaching less than 10% of the metabolic activity of the control and 25% at the second highest tested. The non-tumour cell line 3T3 showed the lowest sensitivity. Graviola stems, bark, and leaves have shown remarkable antiproliferative effects

against cancer cells without affecting healthy cells (George et al., 2012; Jaramillo et al., 2000; Mishra et al., 2013). Due to the highest sensitivity of Jurkat cells, further experiments were only performed on this cell line at the second highest tested concentration ( $500 \mu\text{g}\cdot\text{ml}^{-1}$ ).

Through microscopic monitoring of cell viability with trypan blue, it was observed that the effect of graviola at the tested concentration was almost the same during 24 and 48 h of incubation and the most pronounced effect did not appear until 72 h of incubation, where 35% of cells were observed to have impaired cell membrane permeability, generally considered dead. Despite several studies on the effect on cancer cell lines, there is no comparison of the effect of the same formulation. The method of extract preparation impacts the composition and effects of active substances (Chen et al., 2015). In aqueous graviola extract cytotoxic effects are expressed as a function of compounds such as flavonoids, saponins, tannins (Braguini et al., 2018; Daglia, 2012).

For example, graviola ethyl acetate extract showed significant cytotoxic effects on colon cancer cell lines HCT-116 and HT-29 (Zorofchian Moghadamtousi et al., 2014). Ethanolic extract of graviola leaves induced apoptosis in the myeloid leukaemia cell line K562, but the involvement of signalling pathways and the influence of the cell cycle have not been elucidated (Ezirim et al., 2013). Analysis of the effect of graviola on the Jurkat cell cycle showed an accumulation of cells in the G2/M phase and a decrease in the G1 phase of the cell cycle after only 24 h incubation. As the incubation time increased, the number of cells in the G2/M phase increased, and after 72 h incubation, a very small increase was measured in the number of cells in the sub-G0/G1 population, which are considered to be cells in apoptosis. A more significant increase in apoptotic cells was observed after 72 h incubation of Jurkat cells with graviola. The results also correlate with the trypan blue viability test, where a significant effect was also observed after 72 h incubation. Cancer is generally well known as a cell cycle dysfunction disease. Deregulation of molecular processes of the cell cycle is one of the most important changes during tumour progression (Williams and Stoeber, 2012). The ability to block the cell cycle of tumour cells effectively enhances the antitumor effects of natural substances (Mantena et al., 2006).

We observed a significant decrease in caspase-3 activity in our experiments after 24 and 48 h incubation with graviola. Only active caspase is able to cleave the substrate, and in the case of reduced caspase activity, it can be assumed to be in the form of an inactive procaspase. This could indicate that the apoptosis observed in previous experiments is triggered by a different, alternative and non-caspase pathway. The mitochondrial apoptotic pathway is regulated by Bcl-2 family proteins (Cory and Adams,

2002). Pro- and anti-apoptotic proteins of this family adjust the permeability of the mitochondrial membrane. Pro-apoptotic Bax protein mediates the release of cytochrome C through conformational changes and translocation into mitochondria, while anti-apoptotic Bcl-2 protein release suppresses cytochrome C from mitochondria (Liu et al., 2003; Martinou et al., 2011). Our experiments showed the increase in the level in the non-phosphorylated form of Bcl-2 after 24 and 48 h incubation with graviola, which is capable of forming complexes with other proteins, for example with pro-apoptotic Bax and defending its pro-apoptotic effect. For these results, we can assume that the predominantly anti-apoptotic effects of Bcl-2 were active for 24 and 48 hours. Reducing the phosphorylated form of Bcl-2 showed that other mechanisms are likely to be involved in apoptosis.

Mitochondria are often the site of action for toxicants and toxins, directly affecting mitochondrial function, which can be reflected immediately in changes in homeostatic capacity and cell viability (Robertson and Orrenius, 2002). Flow cytometric analysis showed an increase in the number of cells with reduced mitochondrial membrane potential with increasing incubation time of Jurkat cells with graviola. Very low concentrations of graviola led to a significant increase in mitochondrial glutathione levels, but graviola concentrations above  $62.5 \mu\text{g}\cdot\text{ml}^{-1}$  caused a significant decrease. Changes in the levels of reduced glutathione disrupt redox homeostasis of the cell and are a sign of increased oxidation of glutathione or changes in its transport, indicative of the initiation or propagation of the apoptotic cascade (Circu and Aw, 2012). Increased glutathione-S-transferase activity is therefore associated with increased resistance to apoptotic stimuli and is also the cause of cancer cell resistance to active substances (Di Pietro et al., 2010). Our results showed that the concentrations of graviola used ( $31.25 \mu\text{g}\cdot\text{ml}^{-1}$ ) reduced GST activity in mitochondria, although without statistically demonstrable significance in our study.

Despite several studies on the effect on cancer cell lines, there is no comparison of the effect of the same formulation. The method of extract preparation impacts the composition and effects of active substances (Chen et al., 2015).

Cytotoxicity of fruit of *A. muricata* has been previously reported by (Kuete et al., 2016), and expressed as a function of plant compounds such as flavonoids, saponins, tannins (Braguini et al., 2018; Daglia, 2012).

## CONCLUSIONS

Our findings show that extract from the dried leaves of graviola demonstrates cytotoxic effects on some cells,

depending on the dose and duration of action. The Jurkat cell line showed the highest sensitivity in a dose-dependent manner, where the metabolic activity of the cells at the highest tested concentration reached less than 10%. We found that the viability of Jurkat cells was most affected after 72 hours, at which point a growing number of cells were found to be apoptotic by cell cycle analysis. Concurrent with the increasing incubation time, the mitochondrial membrane potential also decreased. At 24-48h incubation, no increase in caspase-3 activity was detected and the analysis of protein dynamics pointed to increased levels of non-phosphorylated Bcl-2, thereby indicating that apoptosis is triggered by an alternative, non-caspase route. Higher concentrations of graviola affected the redox state of mitochondria by a significant reduction in reduced glutathione levels and compromised cells, reducing glutathione-S-transferase activity to slow the metabolization of the active compounds and mediating their effect over a longer period.

## ACKNOWLEDGEMENTS

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