ASSESSMENT OF THE POTENTIAL ANTITUMOR EFFECTS OF GLINUS OPPOSITIFOLIUS (L.) AUG. DC. EXTRACTS

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Abstract

The present study aimed to investigate the antitumor activity of two extracts obtained from Glinus oppositifolius aerial parts in MCF-7, A549, PC3 and CACO2 cancer cell lines using the cell viability, cell cycle and DCFH-DA assays. FT-IR analysis revealed triterpenes and carbohydrates in both extracts and sterols in the ethanolic one. The results from the viability assay showed that the aqueous extract had a promising cytotoxic effect on CACO2 cells (IC50 = 97.69 ± 2.03 µg/mL). Induction of apoptosis in CACO2 cells was supported by an increase in the cell population in the sub-G1 phase (55.7 ± 4.03% vs. 8.35 ± 3.85% in control). The DCFH-DA assay revealed that another possible mechanism by which the aqueous extract reduced CACO2 cell viability was an increase in the intracellular oxidative stress. Further in vitro studies are necessary to elucidate other mechanisms underlying the antitumor activity and thus to find new possible therapeutic applications of this plant.

Keywords: Glinus oppositifolius, MTT assay, cell cycle, DCFH-DA assay

Introduction

Glinus oppositifolius (L.) AUG DC. sin. Molugo oppositifolia (Molluginaceae) is used in the traditional medicine in Mali in the treatment of several diseases that are related to the immune response: intestinal pain and parasitosis, diarrhea, joint pain, inflammation, fever, skin diseases, wounds, malaria, urinary infections or liver dysfunctions [16, 17]. For abdominal pain and jaundice, the powder, obtained from the dried aerial parts of the plant, is added to food. An aqueous or oily macerate of the plant material is used to treat open wounds and the juice obtained by cold pressing is used to treat some skin disorders [15]. The aerial parts of Glinus oppositifolius were reported to contain triterpene saponins and glycosides of vitexin [30]. Two pectic polysaccharides were isolated from the hot aqueous extract (50°C) of the aerial parts and their structures have been partially characterized [16]. Previous studies demonstrated that these polysaccharides possess immunomodulating properties consisting in dose-dependent complement fixation activity and induction of chemotaxis of macrophages, T lymphocytes and NK cells [15, 17]. Kandar et al. evaluated the antitumor activity of the methanolic extract from the aerial parts of the plant. Three doses of 100, 200 and 400 mg/kg b.w. were administered for 10 days to Ehrlich ascites carcinoma bearing Swiss albino mice. The tumour volume and the percentage of viable cells in the ascitic fluid were significantly reduced in the mice treated with the methanolic extract in comparison with those treated with the standard anticancer drug, 5-fluorouracil, administered in the same conditions at a dose of 2 mg/kg b.w. [21].

The present study investigated the antitumor activity of the aqueous and ethanolic extracts obtained from Glinus oppositifolius aerial parts in order to find new possible therapeutic applications of this plant.
Materials and Methods

Plant material
Aerial parts of *Glinus oppositifolius* were supplied by the Department of Traditional Medicine, Bamako, Mali. The identity of the plant material was confirmed by Prof. Dr. Drissa Diallo, Director of the Department of Traditional Medicine in Bamako, Mali.

Preparation of extracts
The aqueous (GA) and ethanolic (GE) extracts were obtained as follows: dried and powdered aerial parts (100 g) were extracted with water (2 × 2000 mL, each time for 4 h) under reflux in a 50°C water bath. Another part of the row material (100 g) was extracted with ethanol at room temperature (2 × 2000 mL, each time for 72 h). Both extracts were concentrated to dryness and stored at -20°C until use.

Total saponin content
Total saponin content was estimated using the method of Li et al. [25]. The results were expressed as mg betulinic acid equivalents/g extract. The measurements were performed using an ABLE-JASCO V550 UV-VIS spectrophotometer.

FT-IR Analysis
FT-IR spectra were recorded on an ABB MB3000 FT-IR spectrometer and were acquired over a range of 4000 - 650 cm⁻¹ with 16 scans at a spectrum resolution of 4 cm⁻¹ [14, 33, 35].

Cell Culture
MCF-7 (human breast adenocarcinoma), A549 (human alveolar adenocarcinoma), PC3 (human prostate adenocarcinoma), CACO2 (human colorectal adenocarcinoma) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The four cell lines were routinely cultured in specific media in a humidified incubator with 5% CO₂ at 37°C [13, 23].

Cell viability assay
Cell viability was assessed using the MTT (methyl thiazolyl tetrazolium) colorimetric assay [5, 18, 26]. Briefly, the cells were plated (3 × 10⁵ cells/well) in 96-well tissue culture plates and treated with the extracts (100 µg/mL) for 72 h. The absorbance, measured at 570 nm using a microplate reader (Wallac Victor3 1420-012), was proportional with the number of viable cells per well. IC₅₀ value for the most active extract was further determined.

Cell cycle analysis
Cells were plated (1 × 10⁶ cells/well) in 100 mm plates and treated with the extracts (100 µg/mL) for 72 h. Following incubation, samples were prepared as previously described using propidium iodide (PI) staining [5, 28] and analysed for DNA content using a flow cytometer (Guava EasyCyte™ flow cytometer).

Pro-oxidant activity (DCFH-DA assay)
The pro-oxidant effect of GA extract in CACO2 cells was evaluated according to the method reported by Girard-Lalancette et al. [12], with some modifications [35]. Cells were plated (4 × 10⁴ cells/well) in 12-well plates and treated with the vehicle control [doubled distilled (DD) water], GA extract (100 µg/mL) for 1, 2, 3 and 24 h or H₂O₂ (100 µM) as a positive control for 30 min. Fluorescence intensity, which was proportional to the amount of reactive oxygen species (ROS) produced by cells, was measured in an automated plate reader (Infinite F200 PRO Tecan) (excitation wavelength 485 nm, emission wavelength 535 nm) and was compared with the positive control (H₂O₂ - treated cells).

Statistical analysis
All experiments were performed in triplicate and the results were expressed as mean ± SEM. Data are representative of three individual experiments. Statistical evaluation was performed using Student’s *t* test; *p* < 0.05 was considered statistically significant.

Results and Discussion

Chemical study of GE and GA extracts
The total saponin content was higher in GE extract (548.23 ± 6.57 mg/g) than in GA extract (47.67 ± 0.55 mg/g). FT-IR analysis showed the presence of triterpenes and carbohydrates (mono-, oligosaccharides in GE extract, polysaccharides in GA extract) in both extracts and sterols in GE extract.
Figure 1A shows the FT-IR spectra of GE extract. Characteristic absorption bands for saponins are present at 3335 cm⁻¹ (O-H group), 2923 cm⁻¹ (methyl group), 1704 cm⁻¹ (C=O group), 1652 cm⁻¹ (C=C group) and 1066 cm⁻¹ (C-O group) [38, 39]. For sterols characteristic absorption bands are present at 3419 - 3334 cm⁻¹ (for O-H group), 2923 cm⁻¹ (bending vibration of methyl/methylene groups), 1635 cm⁻¹ (stretching vibration of C-C group). Characteristic band for C-H group (-CH₃CH₂, n ≥ 4) vibration was identified at 669 cm⁻¹ [40]. GA extract presents characteristic absorption bands for saponins at 3338 - 3284 cm⁻¹, 2941 cm⁻¹, 1595 cm⁻¹ and 1070 - 1039 cm⁻¹ [38, 39]. Absorption bands at approx. 1000 cm⁻¹ (1070 and 1039 cm⁻¹) are characteristic for carbohydrates (Figure 1B) [37].

Spectral data are consistent with previous studies that reported that the aerial parts of *Glinus oppositifolius* contain triterpenes (glinoside A, B and C, spergulin A and B) and carbohydrates (acidic and neutral pectic polysaccharides) [15, 16, 29, 31].

**Cell viability assay**

Malignant diseases are a major public health concern around the world. According to the latest statistics, one in four deaths in USA is caused by cancer. In 2012 in Europe, Ferlay *et al.* estimated 3.45 million of new cancer cases and 1.75 million deaths due to cancer. The most common cancers were breast cancer (464,000 cases), followed by colorectal cancer (447,000 cases), prostate cancer (417,000 cases) and lung cancer (410,000 cases) [9]. Colorectal cancer is the third most common cancer in men and the second in women worldwide [11]. Cytotoxicity is considered to be the potential of a compound to induce cell death. The *in vitro* cytotoxicity assays are widely used as quick predictors of toxicity of new drugs in animals or humans. They also allow the reduction in animal use for toxicity testing [8].

In the present study, we investigated the antitumor effects of *Glinus oppositifolius* extracts on four human cancer cell lines (MCF-7, A549, PC3 and CACO2). The cells were initially exposed to 100 µg/mL of GA or GE extracts for 72 h. The cytotoxic activities of the extracts are presented in Figure 2A. The negative controls (vehicle controls) were similarly processed using DMSO and DD water instead of GE and GA extracts, respectively.

The two extracts had insignificant cytotoxic effects on MCF-7, A549 and PC3 cells. In contrast, GA extract had a remarkable cytotoxic effect on CACO2 cells. Therefore, the effect of GA extract on CACO2 cells was further investigated using different concentrations ranging from 10 to 100 µg/mL. The dose-response graph showed that GA extract had an IC₅₀ of 97.69 ± 2.03 µg/mL (Figure 2B).

A similar study conducted by Boulaba *et al.* showed that the methanolic extract of *Arthrocnemum indicum* aerial parts reduced CACO2 cells proliferation to approximately 55% at 100 µg/mL (72 h treatment) [4]. The cytotoxic effect of GA extract on CACO2 cells was slightly stronger than the one reported for the methanolic extract of *Chrysanthemum coronarium* L. (IC₅₀ = 106.4 µg/mL; 72 h treatment) [1] but comparable to the one reported by Lawson-Evi *et al.* for the hydro-ethanolic extract of *Plumeria alba* L. roots (IC₅₀ = 94.5 µg/mL; 72 h treatment) [24].

Since GA was the sole extract showing a significant effect in reducing cell viability, only the effects of this extract (influence on cell cycle, pro-oxidant activity) were further analysed.

**Cell cycle analysis**

Cell death is a central mechanism controlling multicellular organism development. Apoptosis is the most common form of programmed cell death, through which intracellular activation of controlled self-destruction occurs [2]. It is characterized by structural changes, including degradation of nuclear chromatin by fragmentation, decondensation and despiralization. Endonucleases cleave DNA phosphodiester bonds at internucleosomal linker sites [36]. It is also known that apoptosis and cell cycle arrest are closely related processes; cell cycle arrest may lead to cell death.
via apoptosis [2]. As cancer is characterized by uncontrolled cellular proliferation, there is considerable interest in treatment-induced apoptosis [19].

To elucidate whether the reduction in CACO2 cell viability caused by GA extract was due to the inhibition of cell cycle progression, the DNA content of CACO2 cells was determined using flow cytometric analysis after 72 h exposure to GA extract (100 µg/mL). The percentages of cells in the G0/G1, S, G2/M and sub-G1 phases were determined by measuring PI fluorescence upon binding to DNA. As shown in the histograms in Figure 3A, GA extract induced massive apoptosis in CACO2 cells and led to a significant accumulation of cell population in the sub-G1 phase (55.7 ± 4.03% vs. 8.35 ± 3.85% in control; Figure 3B); cells in the sub-G1 phase are apoptotic, sub-diploid cells (DNA content < 2n). This was accompanied by a marked reduction in the percentages of cells in the G0/G1, S and G2/M phases (Figure 3B).

Another recent study concerning the anti-proliferative activity of the water extract from Inula viscosa (L.) Ait. leaves on HCT116 and Colo320 human colorectal cancer cells was conducted by Bar-Shalom et al. The study revealed a significant increase of cell accumulation in sub-G1 phase in both cell lines following 72 h treatment (HCT116 cells: 48.47 ± 5.67% vs. 5.54 ± 1.52% in control; Colo320 cells: 40.18 ± 4.81% vs. 5.68 ± 1.48% in control) [3]. Daddiouaisa et al. performed a study on ionic liquid extract of Graviola (Annona muricata L.) fruits and the results showed that the treatment increased the cells in the G0/G1 phase from 59.07% in control to 72.82% in cells treated with the extract for 72 h, while decreasing the cells in the S and G2M phases compared with the control [7]. Boulaba et al. reported the cell cycle arrest at the G2/M phase in CACO2 cells following 72 h exposure to Arthrocnemum indicum methanolic extract at 100 µg/mL (42.2% vs. 38.95% in control) [4].

To conclude, our data demonstrate that the reduction in CACO2 cell viability by GA extract can be attributed, in part, to induction of apoptosis.

**Pro-oxidant activity (DCFH-DA assay)**

Intracellular ROS production was measured in terms of fluorescence using dichlorodihydrofluorescein diacetate (DCFH-DA). Low concentrations of ROS promote cell growth, medium concentrations induce apoptosis and high concentrations induce cell death through necrosis [20]. Schulze-Osthoff et al. and Lancaster et al. reported the mitochondrial ROS as second messengers in cell death [22, 32]. The inhibition of mitochondrial respiratory chain leads to an increased ROS production, ROS acting as mediators of the cell death signalling pathway. In addition, ROS modulate the activity of proteins that are involved in the regulation of apoptosis such as Bcl-2 [10, 34].

Figure 4 depicts the fluorescence intensities of DCF signals in CACO2 cells exposed for 1, 2, 3 and 24 h to GA extract (100 µg/mL). Pro-oxidant activity of GA extract varied in a time-dependent manner. Thus, CACO2 cells exposed to GA extract displayed an increasing fluorescence intensity of DCF signals, which indicated an increase in the production of ROS in cells from 14.86 ± 1.45% (1 h exposure) to 63.23 ± 3.40% (24 h exposure). Ciani et al. reported that 24 h exposure of A431 human epidermoid carcinoma cells to 3 mg/mL of Uncaria tomentosa (Willd.) DC. bark aqueous extract increased intracellular ROS production to 70% [6]. Piccolella et al. reported that 72 h exposure of SH-SY5Y human neuroblastoma cells to 100 µg/mL of Pistacia lentiscus L. leaves chloroform extract increased intracellular ROS production to 93.4% [27].

Our results also suggest that another possible mechanism by which GA extract reduces CACO2 cell viability is an increase in the intracellular oxidative stress.

![Figure 3.](image_url)
References

Mali) for providing the plant material

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Conclusions

Antitumor screening of *Glinus oppositifolius* extracts revealed that the aqueous extract reduced CACO2 tumour cell viability to 49.36 ± 0.979% at the concentration of 100 µg/mL. One of the mechanisms by which the extract reduced CACO2 cell viability was the induction of apoptosis. In addition, the extract increased the intracellular oxidative stress in CACO2 cells. Further *in vitro* studies are necessary to elucidate other mechanisms by which the aqueous extract reduces tumour cell viability. Sub-fractionation of the extract may reveal the presence of novel compounds with antitumor activity.

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References

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