**Ficus septica**, an ecosystem keystone species induced ROS-mediated cytotoxicity in HepG2 hepatocarcinoma cells

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**Abstract.** *Ficus septica* grows all around Indonesia, as one of the key species in various ecosystem types. This plant is a food resource and habitat for some animals. This study aimed to examine cytotoxic activity of *F. septica* extract in HepG2 cells. The leaves powder was macerated using ethanol 96%. Cytotoxic activity was evaluated by MTT assay. The determination of cell cycle profile and reactive oxygen species (ROS) were done by flow cytometry. The extract inhibited the growth of HepG2 and Vero cells with an IC\textsubscript{50} of 50.9 and 286.2 µg/mL, while doxorubicin 0.8 and 12.8 µg/mL. The selectivity index of the extract and doxorubicin was 5.6 and 16 respectively. The extract triggered cell cycle arrest in HepG2 at the G0/G1 phase, whereas doxorubicin in the S phase. The extract and doxorubicin significantly increased intracellular ROS in HepG2, but not in normal Vero cells. In conclusion, our findings suggest that *F. septica* induced cytotoxicity in HepG2 cells is mediated by excessive ROS generation leading to oxidative stress.

**1. Introduction**

*Ficus* is a genus of plants belonging to the Moraceae family commonly found in various types of ecosystems. *Ficus* is usually named as the fig tree, a keystone species serving as a food source in all seasons for some animals. The presence and role of keystone species in an ecosystem are so important that they have a disproportionate impact on other organisms in the system [1]. Fig trees are reported to be pioneer tree species that develop quickly and can be found on every island in Indonesia. *Ficus* can be employed as protection plants for other primary forest types to help damaged regions [2]. *Ficus septica* Burm.f. is a kind of fig tree, served as a species indicator in the Watuji region of Kebumen, Indonesia. Its existence is critical for detecting environmental changes in pine forests around the water sources [3].

*F. septica* has been used in traditional medicine for a long time. The leaves are used to treat colds, coughs, fevers, and diarrhoea. The leaves are also used as a rheumatism and headache treatment [4]. The ability of *F. septica* leaves to suppress breast cancer cell lines has been demonstrated in several research [5–7]. Considering an estimated incidence of >1 million cases by 2025, liver cancer remains a global health concern. Hepatocellular carcinoma (HCC) is the most common type of liver cancer, accounting for 90% of incidences. Hepatitis B and C virus infection are the most common causes of HCC, while non-alcoholic steatohepatitis linked with metabolic syndrome or diabetes mellitus is becoming more common [8]. One of the most prevalent cytotoxic medications used in HCC is
doxorubicin. Despite the fact that doxorubicin has a wide range of anti-tumor action, systemic dosing can often result in fatal cardiotoxicity and other negative side effects [9]. Therefore, alternative sources of the discovery for novel anticancer agents are still urgently required.

The potential effect of *F. septica* for HCC treatment has not been explored widely. Therefore, this study was purposed to investigate the cytotoxic effect of *F. septica* leaves towards HepG2 and Vero cell lines. HepG2 was differentiated from hepatocellular carcinoma cells, derived from a 15-year-old Caucasian male liver biopsy. HepG2 was a highly proliferative cell grown in large-scale culture systems, and widely used in pharmaco-toxicological research [10]. Vero cells were developed in the 1960s from the kidney of an African green monkey (*Cercopithecus aethiops*). The cells have been extensively applied in virology research, intracellular bacteria, parasites, and the impact of drugs, poisons, and other compounds on mammalian cells [11]. In this study, we evaluated the cytotoxicity of *F. septica* leaves extract and doxorubicin using MTT assay. Then we investigated the cytotoxic mechanism through cell cycle profile and ROS intracellular using flow cytometry.

2. Materials and methods
The study was conducted in the Laboratory of Molecular Biology, Medicinal Plant and Traditional Medicine Research and Development Centre, Tawangmangu, Indonesia.

2.1. Plant material
The fresh *F. septica* leaves were collected from Tawangmangu, Central Java, Indonesia. The leaves were dried 40°C in an oven, and grinded into coarse powder. The powder was then macerated in 96% ethanol for 3×24 hours. The macerate was filtered and evaporated in 40°C. The dried extract was dissolved in DMSO (Sigma) and diluted in fresh culture medium.

2.2. Cell culture
The HepG2 and Vero cell lines were obtained from Cancer Chemoprevention Research Center Faculty of Pharmacy, Universitas Gadjah Mada. The cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM, Sigma) supplemented with 10% FBS (Sigma), and incubated in 5% CO2 37°C.

2.3. Cytotoxic assay
HepG2 and Vero cells were planted in 96-well plates at a density of 10⁴ cells per well and incubated for 24 hours. The extract or doxorubicin was delivered in the cells at series concentration for 24 hours. The cellular morphology alterations were documented at 20× magnification using inverted microscope (Olympus, CKX41). The growth media was discarded and washed with phosphate buffer saline (PBS, Sigma). Each well attained 0.5 mg/ml MTT in the media and was incubated for 3 to 4 hours. Sodium dodecyl sulfate (SDS, Sigma) 10% in HCl 0.01 N was used to terminate the MTT reaction, kept in the darkroom overnight. The absorbance was measured using ELISA reader at 595 nm (Biorad). The absorbance values were expressed as a percentage of untreated cells’ viability.

2.4. Cell cycle distribution
In 6 well plates, approximately 5×10⁵ HepG2 cells/well were grown and incubated for 24 hours. The cells are then treated with the extract or doxorubicin for 24 hours. The cells were trypsinsized, rinsed in PBS, and centrifuged for 5 minutes at 500 rpm. Cells were fixed in 70% cold ethanol for 30 minutes, washed in PBS, and centrifuged at 500 rpm for 5 minutes. The cells were then resuspended in a solution containing 40 g/mL propidium iodide (Sigma), 20 g/mL RNAse (Roche), and 0.1 percent TritonX-114 (Sigma). The combination was incubated in the dark for 15 minutes, and analyzed in BD Accuri C6 flow cytometer.

2.5. ROS intracellular
The intracellular ROS level was evaluated by dichlorodihydrofluorescein diacetate (DCFDA) fluorescence dye with the established method earlier [12]. HepG2 or Vero cells (5×10⁴ cells/cell well)
were cultured in 24 well plates for 24 hours at 37°C, 5% CO₂. Cells were harvested with 30 µL of trypsin-EDTA for 3–5 minutes, followed by a 50 µL supplemented buffer (FBS 10% in PBS). The cells were collected in dark microtubes and received a 25 µM DCF-DA (Sigma) solution, incubated for 30 minutes at 37°C, 5% CO₂. The cells then treated with the extract or doxorubicin for 120 minutes. The ROS intracellular was measured with BD Accuri C6 flow cytometer.

2.6. Statistical analysis
The IC₅₀ was determined using linear regression of concentration and cell viability. The selectivity index (SI) was calculated by the ratio of the IC₅₀ of normal cells (Vero) to cancerous cells (HepG2). The data obtained from the flow cytometer was analyzed using BD Accuri C6 software.

3. Results and discussion
The phenotypic characteristics of HepG2 and Vero cells were observed using the inverted light microscope. The decrease of cell density and cell morphology alterations were observed in cells treated for 24 hours, as shown in Figure 1. Both doxorubicin or F. septica ethanolic leaves extract inhibited cell expansion and elongation compared to untreated cells. In this study, vero cells required higher concentration of doxorubicin or extracts than HepG2 to provide the transformation. Both the cells showed different morphological features such as shrinkage and rounded, resulting in a disconnection from culture dishes. Cell detachment indicates a typical hallmark of apoptosis in a tissue culture [13]. Apoptosis causes cell shrinkage, expands from pyknosis to karyorrhexis, and further separates the cells from their surrounding tissues [14].

![Figure 1](image1.png)

**Figure 1.** Representative morphological changes of Vero and HepG2 cells treated with the extract and doxorubicin for 24 h. (a) untreated vero cells; (b) and (c) vero cells treated with the extract 300 µg/ml and doxorubicin 5 µg/ml; (d) untreated HepG2 cells; (e) and (f) HepG2 cells treated with the extract 50 µg/ml and doxorubicin 1 µg/ml. The images were captured with inverted microscopy magnification of 200x.

Apoptosis is a regular, sequential order of cell death that maintains a homeostatic balance between cell growth and death [14]. Apoptosis occurs when a cell decides to suicide. This usually happens for better restoration of the whole organism, primarily to eliminate cell damage and carcinogenesis [15]. Apoptosis, or programmed cell death, is closely linked to physiological and pathological processes, including immunological and neurodegenerative diseases, inflammation and cancer [16]. The capacity of cancer cells to escape apoptosis is crucial for their progression. Therefore, targeting the regulation of apoptosis evasion is becoming a promising strategy to develop novel strategies for cancer therapy [17].
In this study the identification of apoptotic cells was not carried out. However, apoptotic induction was associated with reductions in cell viability.

The MTT assay was applied to determine cell viability. The results showed in Figure 2, the extract suppressed HepG2 and vero cells viability with the IC\textsubscript{50} 50.9±1.2 and 286.2±38.2 µg/ml respectively; and selectivity index (SI) value of 5.6. Doxorubicin revealed more potent activity against HepG2 and vero cells with the IC\textsubscript{50} 0.8±0.03 and 12.8±0.8 µg/ml respectively, and SI of 16. According to Mbaveng et al. [18], the SI value of doxorubicin for HepG2 cells was 11.59 as compared to normal AML12 hepatocytes. Several references mention that the SI value greater than 3 were indicated to have a high selectivity towards cancer cells [19]. The extract demonstrated high SI, but it’s lower than doxorubicin. Despite the fact that doxorubicin has a relatively high SI, indeed it shows numerous clinical adverse effects. Therefore, further research into the toxicity of \textit{F. septica} leaves extract is required.

**Figure 2.** Cytotoxic potential of \textit{F. septica} leaves extract (a) and doxorubicin (b) against HepG2 and Vero cell lines. The cells were treated with increasing concentrations for 24 hours. Cell viability was measured by MTT assay. Each diagram represents the mean ± SD of three experiments.

Apoptosis, or programmed cell death, is mediated by many of the same molecular components involved in cell-cycle regulation. The mutations that disrupt these pathways may impair cell-cycle control and lead to genomic instability and cancer. It is based on deciphering the biochemical and genetic mechanisms that preserve the integrity of cell-cycle progression and cell division. Considering the enormous complexity, many components of the cell-cycle system are showing clinical utility as promising chemotherapeutic strategies [20]. However, cancer cells can activate DNA repair pathways to resist the cytotoxic effect of anticancer treatments [21].

Following the cytotoxic effects, the cancer cells could initiate cell cycle arrest to provide DNA repairment. In this study, we determined the impact of the extract to cell cycle progression using flow cytometry by propidium iodide staining. The number of cells arrested at the G0/G1 growth phase was observed significantly after being treated with \textit{F. septica} leaves extract 10 and 50 µg/mL. Although the increase in the percentage of cells in G0/G1 is not large (47.7 to 56%), the results are statistically significant (p < 0.05) and consistent. As shown in Figure 3, the extract caused G0/G1 cell-cycle arrest, accompanied with a proportional decrease in the S phase. The extract of \textit{F. septica} leaves was also reported to interrupt the G0/G1 phase in highly metastatic breast cancer cells 4T1 [5]. The cells treated with doxorubicin caused cell arrest at the S phase, and proportional reduction in G0/G1 and G2/M phase. According to Levi et al. [22] CiPp canin mammary primary tumors cells treated with doxorubicin showed a progressive increase in the S (DNA synthesis) phase, related with a progressive decrease at G2/M (mitosis) phase.

Cell cycle arrest and DNA repair are the results of DNA damage response (DDR) activation. The DDR is not a single system, but rather a network of biochemical pathways and responses that detect
DNA damage and decide the cell's fate. These include repair throughout different phases of proliferation, delay cell cycle, and cell cycle arrest to allow for more comprehensive DNA repair [23]. If the level of DNA damage exceeds the cell's improvement capacity, cell death is stimulated. As a result, DDR promotes genomically steady cells to survive while preventing the proliferation of unsteady ones. DNA damage is caused by various internal and extrinsic factors including reactive oxygen species (ROS) and environmental mutagens [24].

Figure 3. Flow cytometric performance of HepG2 cells. Cells were treated with doxorubicin 1 μg/mL and *F. septica* leaves extract (10 and 50 μg/mL) for 24 h, compared to untreated cells. The fluorescence of propidium iodide staining was measured to analyze DNA content in the cells. a) Representative flow cytometry graph for each group. b) The histograms of cell percentage in various phases. Data represented mean ± SD of triplicate.

ROS are produced in a strictly regulated manner in normal cells. ROS production is related to the regulation of cell division, immunological modulation, autophagy, inflammation, and stress-related response signaling activities. Uncontrolled production of ROS can induce oxidative stress, leading to the emergence of various diseases [25]. ROS shows a dual role in cancer. The first role is ROS facilitates cancer cell proliferation, survival, and adaptation to hypoxia by promoting protumorigenic signaling. The second role is ROS can activate antitumorigenic signaling and cause cancer cell death due to oxidative stress [26]. Therefore, increasing oxidative stress in cancer cells has been considered as a promising therapeutic approach for cancer therapy [27].

We have determined the quantitative ROS production in HepG2 and Vero cells by flow cytometry with DCFDA staining. As shown in Figure 4., *F. septica* leaves extract 50, 100, and 300 μg/mL increased ROS intracellular in HepG2 cells by 1.6, 1.8 and 2 fold, respectively. It was interesting that the extract treatment considerably lowered intracellular ROS levels in Vero cells, by up to ten fold. This finding supports the selectivity of the extract toward hepatocarcinoma cells. Doxorubicin 5 and 10 μg/mL elevated ROS in HepG2 cells by 1.7 and 3.3 fold respectively, while having slight impact on Vero cells.

Doxorubicin works by increasing ROS production leading to the activation of tumor suppressor p53, resulting in cancer cell death. Emerging regulated cell death mechanisms, such as ferroptosis, necroptosis, and pyroptosis, have been implicated in doxorubicin-induced cardiotoxicity in recent years. Understanding the processes by which doxorubicin causes cardiomyocyte destruction could lead to the development of new chemotherapeutic agents [28]. By exploring the function of elevated ROS generation in cancer, we may be able to specifically target cancer cells while causing minimal damage to normal cells [29].
According to our findings, *F. septica* leaves are potentially developed as a chemotherapeutic agent. Its ability to selectively reduce ROS in normal cells also represents a great potential for further development as a natural exogenous antioxidant. Epidemiological studies have indicated the correlation of natural source antioxidants consumption and a lowered risk of chronic diseases such as cancer, cardiovascular disease, and inflammatory disorders [30].

4. Conclusions

Our findings revealed that ethanolic extract of *F. septica* leaves induced cytotoxicity in HepG2 cells are associated with excessive ROS production leading to oxidative stress, and cell cycle arrest. The extract shows a high selectivity index to HepG2 hepatocarcinoma cells. Further research is required to isolate active compounds and conceive specific mechanisms involved in *F. septica*-induced cell death.

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