RESEARCH ARTICLE

Editorial Process: Submission:11/22/2021 Acceptance:04/09/2022

Polyphenol Content, Antioxidant, Cytotoxic, and Genotoxic Activities of *Bombax ceiba* Flowers in Liver Cancer Cells Huh7

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Abstract

Objective: *Bombax ceiba* (red Silk cotton tree) has great ethnopharmacological significance due to its widespread use to treat various diseases such as dysentery, inflammation, and tuberculosis. Despite decades of research, the studies on the *in vitro* anticancer/genotoxic activity of *B. ceiba* flower remains restricted. Thus, the present research explored the effect of ethanol extract from *B. ceiba* flowers on three human cancer cells, including lung A549 and liver HepG2 and Huh7 cell lines. **Methods:** Cytotoxic and genotoxic activity of *B. ceiba* extract was examined by MTT and comet assay, respectively. Further, *B. ceiba* extract was analysed to determine total polyphenol content and DPPH antiradical scavenging activity. **Results:** ethanol extract from *B. ceiba* flowers had a high polyphenols content with very potent antioxidant activity. Further, *B. ceiba* extract displayed moderate cytotoxicity against Huh7 cells and no cytotoxicity against HepG2 and A549 cells. The comet assay findings showed that Huh7 cells treated with four concentrations of *B. ceiba* extract (¼ IC_{50}, ½ IC_{50}, IC_{50}, and double IC_{50}) increased the comet tail formation within 48 h in a concentration-dependent manner. **Conclusion:** ethanol extract from *B. ceiba* flowers exhibited its cytotoxicity through induction of DNA fragmentation in Huh7 cells.

Keywords: Antioxidant activity- comet assay- liver and lung cancer cells- MTT assay- total polyphenol content

Asian Pac J Cancer Prev, **23 (4)**, 1345-1350

Introduction

Hepatocellular carcinoma (HCC) represents 75% of all primary liver cancer and is one of the primary causes of aggressive malignancy-related death worldwide. The incidence of HCC is associated with several factors: hepatitis virus infection, alcohol abuse, smoking tobacco, toxic chemicals and fungal toxins, non-alcoholic fatty liver disorder, autoimmune hepatitis, dietary iron overload, metabolic syndrome, and mutational signatures. Chemotherapy is very costly, and its efficacy has limited due to its hazardous side effects, such as bone marrow depression, hair loss, postembolization syndrome, and liver and renal failure (Balogh et al., 2016; Liu and Wu 2010; Llovet et al., 2021; Rashed et al., 2020). Therefore, exploring natural products with minimal side effects on the normal cells is a promising approach for drug discovery and development of anticancer drugs.

Bombax ceiba Linnaeus (syn. *Bombax malabaricum* DC) belongs to the family Bombacacae and is called the silk-cotton tree. It has been extensively grown in the tropical and sub-tropical areas in Asia, Africa, and Austria. *B. ceiba* was brought into Egypt several decades ago as a shade tree and an ornamental plant (Chaudhary and Tawar, 2019; Shahat et al., 2003; Harborne, 1994). We selected this plant based on its economic, ecological, and medicinal values. Economically, the fruit of *B. ceiba* is a good source of silk cotton which used for several purposes: (1) insulation material for refrigeration devices and soundproofing walls, (2) padding surgical dressings, (3) stuffing mattresses, quilts, and cushions because it is vermin-proof (Jain and Verma, 2012). Ecologically, *B. ceiba* is considered a pioneer tree in the reforestation system for its capacity to survive in low-rainfall and well-drained environments (Zhou et al., 2015). This plant is suitable for urban greening systems because of its effectiveness in counteracting the detrimental effects of air pollution (Changlian et al., 2002).

In traditional medicine, all parts of *B. ceiba* have been widely used in the treatment of various diseases: dysentery, gonorrhoea, diarrhoea, menorrhagia, skin problems, calculus affections, chronic inflammation, catarrhal inflammation, chronic cystitis, pulmonary tuberculosis, kidney and bladder ulceration, and enteritis (Chaudhary and Khadabadi, 2012; Jain et al., 2009). Pharmacologically, *B. ceiba* possesses several biological activities such as antioxidant, anticancer, anti-acne, antipyretic, antimicrobial, antibacterial, antiobesity, hypotensive, hypoglycaemic, hepatoprotective, and cardioprotective activities (Chaudhary and Tawar, 2019).

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As far as we know, research in the anticancer/genotoxic effect of *B. ceiba* flower remains limited. Therefore, the present study aimed to evaluate two central objectives: (1) To determine the total polyphenol content and DPPH radical scavenging activity of ethanol extract from *B. ceiba* flower; (2) To examine the cytotoxic and genotoxic effects of *B. ceiba* extract against lung and liver cancer cells using MTT assay and comet assay, respectively.

Materials and Methods

Chemicals

The chemicals were purchased from the suppliers as follows: 2, 2-diphenyl-2-picrylhydrazyl (DPPH) and Folin-Ciocalteu reagent (FCR) from Sigma Aldrich Co, St Louis, United State; L-ascorbic acid 99.5% from Fluka Chemie, Messerschmittstr, Neu-Ulm, Germany; Chlorogenic acid from Alfa Aesar, Karlsruhe, Germany; RPMI-1640 medium, Dulbecco's modified eagle's medium (DMEM), foetal bovine serum (FBS), and trypsin-EDTA from GIBCO™, Grand Island, New York, USA; Low melting agarose (LMA), Normal standard agarose, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl)-tetrazolium bromide (MTT) from Bio Basic Inc, Canada. All other chemicals used throughout the work were of the highest analytical grade.

Plant materials and extraction process

Flowers of *B. ceiba* were collected from the Al-Orman Botanical Garden (Giza, Egypt) in May 2019. The plant specimen was deposited in the herbarium of the National Research Centre (Dokki, Cairo, Egypt) under voucher number 3241. The dried powder flowers (1 kg) was extracted using aqueous ethanol (80% EtOH), and the precipitate extract was composed, following evaporation of EtOH using a rotary evaporator. Then, the precipitate extract was filtrated and dried in a vacuum to give a brownish residue. The chemical constituents of *B. ceiba* flower extract have been previously isolated and identified as follows: (A) nine flavonoids include (1) apigenin, (2) vitexin, (3) isovitexin, (4) vicinine-2, (5) quercetin, (6) quercetin 3-O-β-glucoside, (7) quercetin 3-O-β -arabinoside, (8) isorhamnetin 3-O-β -glucuronide, and (9) rutin; and (B) three xanthones include (1) 4\-O-p-hydroxybenzoyl mangiferin, (2) mangiferin, and (3) isomangiferin (El-Toumy et al.,2013).

Folin–Ciocalteu assay

This assay quantifies the total polyphenol content (TPC) in the plant-derived extracts using FCR, as previously described in detail (Diab, 2016). Briefly, an aliquot of different concentrations of the diluted extract in DMSO (25 µL) was mixed with FCR (1:10 diluted distilled water, 125 µL) and of Na_2CO_3 (10% w/v, 125 µL) in a well of 96 well flat-bottomed microplates. The mixture reaction was incubated at room temperature with intermittent shaking for 10 min. Then, an aliquot of the reaction mixture (200 µL/well) was transferred into a 96-well microplate and allowed to stand for 5 min. The absorbance of the mixture was measured using a microplate reader at 600 nm versus blank. The blank sample was prepared by replacing FCR with distilled water (125 µL). Chlorogenic acid (CA) was used as a standard for constructing a calibration curve which was prepared under the same conditions in the range from 0.0625 to 2 mg/mL. The amount of TPC was expressed as mg CA equivalent per mg of the sample (mg CAE/mg sample) through the calibration curve of CA.

DPPH assay

The ability of the *B. ceiba* to scavenge free radical was measured using DPPH radical assay as previously described in detail (Diab, 2016). Briefly, an aliquot (10 µL) of different concentrations from the diluted extract in DMSO (2.5-1000 µg/mL) was mixed with 70% ethanol (90 µL), 0.1M sodium acetate (100 µL, pH 5.5), and 50 µL of 0.5mM DPPH solution in 100% EtOH. The mixture reaction was shaken thoroughly and kept in the dark at room temperature for 30 min. Then, the mixture (200 µL/well) was transferred into a 96-well microplate, and the optical density (OD) of the mixture was determined using a microplate reader at 517 nm against the blank. The blank sample was prepared by substitution of DPPH with 50 µL of 100% ethanol. The inhibition of the DPPH radical scavenging was calculated as follows:

DPPH radical scavenging activity (%) = 1 – [(OD of sample - OD of blank) /(OD of control - OD of blank)]× 100

The antioxidant activity was expressed as EC_{50} (µg/mL), which is the effective concentration of the plant sample required to scavenge 50% of DPPH. The EC_{50} value was calculated from the graph plotting inhibition percentage against the concentrations of the plant sample. L-ascorbic acid is used as a standard antioxidant reference to convert the DPPH scavenging capacity of the plant sample to ascorbic equivalent antioxidant capacity (AEAC). Ascorbic acid was prepared by the same procedures described above in the concentration ranging from 2.5 to 1000 µg/mL.

Cell lines and treatment

Lung adenocarcinoma (A549) and two hepatocarcinoma (Huh7 and HepG2) were obtained from the Medical School of Hannover (Hannover, Germany), Laboratory of Virology and Infectious Disease, The Rockefeller University (New York, USA), and American type culture collection (ATCC; Manassas, United States), respectively. Lung and liver cancer cells were cultured in DMEM and RPMI-16 medium, respectively. The cells were maintained in an appropriate medium, supplemented with 10% FBS, penicillin (100 IU/ mL), and streptomycin (100 µg/mL), in a 5% CO2 incubator at 37ºC with 95% relative humidity. The Cells were passaged every 3–4 days to maintain exponential growth. The plant sample was dissolved in DMSO (<1%) as 1,000 mg/mL stock solution and were further diluted in culture medium before use to achieve final concentrations ranging from 10-1,000 µg/mL.

Determination of cell proliferation by MTT assay

MTT assay determines cytotoxicity or cell viability

through the determination the activity of mitochondrial enzymes inside the viable cells. This assay depends on the reduction of MTT, which is a yellow water-soluble tetrazolium dye, by the mitochondrial dehydrogenases to purple-coloured formazan crystals (Alli et al. 2015). Briefly, the cell lines were harvested in the log phase using trypsinization (0.05% trypsin, 0.02% EDTA, in phosphate buffer solution PBS). The cells (5×10^3 cells/well) were seeded into a 96-well plate in volumes of 100 µL/well and then kept overnight for attachment. Following 24 h of incubation, the complete medium was aspirated, and the cells were exposed to 200 µL /well of freshly prepared medium containing five concentrations of plant extract (10, 30, 100, 300 and 1,000 µg/mL) for 48 h. Four hours before the end of the incubation period, 20 µL aliquots of MTT solution (2.5 mg/mL in PBS) was added to each well and re-incubated for 4 h at 37ºC. Next, the supernatant culture medium containing MTT was dumped off, and DMSO (200 µL) was added to each well and left for 20 min to dissolve the formazan crystals. Then, the 96-well plates were shaken for 5 minutes to ensure a homogeneous dye in the solution. Finally, the optical density (OD) of each well was recorded by a microplate reader at a wavelength of 570 nm. The percentage of cell viability was calculated as follows:

Cell viability (%) = [(OD of treated cells – OD of blank)/ (OD of control – OD of blank

Cytotoxic activity was expressed as IC_{50} (µg/mL), which is inhibitory concentration of the test sample that caused 50% inhibition of cell proliferation. The values of IC_{50} was calculated by plotting cell viability (%) versus sample concentration. According to the IC_{50} value, exploring molecular cytotoxicity was performed on the most sensitive cancer cell line to the *B. ceiba* using comet assay.

Comet assay

The alkaline comet assay was performed according to the procedures described previously by Diab et al. (2018). Briefly, Huh7 cells were seeded at a 6-well microplate (0.5×10^6 cells/well) for 24 h to allow attachment of the cells to the bottom of the wells. Then, the cells were treated with four concentrations of plant extract (¼ IC_{50}, ½ IC_{50}, IC_{50}, and double IC_{50}) for 48 h. Untreated cells and cells treated with DMSO were used as negative and vehicle controls, respectively. The cells were harvested by trypsinization and washed with phosphate buffer solution (PBS, pH 7.2). The cell pellets were suspended in PBS (100 µL) and mixed with LMA (0.8%, 1,000 µL). The mixture was layered into fully a forested slide precoated with 1.0 % normal agarose and covered with a large coverslip. The coverslips were gently removed after solidification of the agarose in a refrigerator at 4°C for 5 min. The slides were kept in ice-cold, freshly lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris HCl, pH 10, 1% Triton X-100, and 10% DMSO) at 4ºC overnight in the dark. The slides were washed with distilled H_2O to remove salt and detergent from the microgels. The slides were kept in a horizontal electrophoresis chamber and covered with chilled, freshly prepared alkaline buffer (300 mM NaOH, 1mM Na_2EDTA, pH>13.0) for 20 min to allow DNA to unwind. Then, electrophoresis was conducted in the dark at 25 V and 300 mA for 30 min. Finally, the slides were washed with 0.4 M Tris-HCl (pH 7.5) three times for 5 min each, fixed with absolute ethanol for 5 min, and air-dried. The slides were stained with ethidium bromide (100 µL, 20µg/mL), and covered with coverslips. The slides were examined at 400× magnification using Zeiss fluorescent microscope equipped with Axiocam 105 colour digital camera and a green excitation filter (510-560 nm) and a barrier filter (590 nm). The experiment was replicated three times for each concentration. Six hundred cells were analysed per concentration (200 cells for each experiment) using automatic comet score™ software (TriTek Corp, version 2.0.0.0, Sumerduck, VA 22742, United State). DNA damage are expressed as the percentage of DNA in the comet tail (% tail DNA), tail moment (TM), and Olive tail moment (OTM).

Data analysis

The experiments were replicated three times with quadruplicate wells in each concentration. Data were analysed using the Statistical Package Social Science (SPSS Inc software, version 20, Armonk, New York: IBM Corp). The data were checked for normality and the homogeneity of the variance using the Kolmogorov-Smirnov's test and Levene's test, respectively. The differences between mean values in all treatments were analysed using one-way analysis of variance (ANOVA) followed by the Tukey's HSD post-hoc test for multiple comparisons. A value of probability ($p < 0.05$ was considered to be statistically significant.

Results

Determination of TPC of B. ceiba extract

The determination of TPC is mainly dependent on the chemical reducing capacity of polyphenols relative to standard equivalents, such as chlorogenic acid and gallic acid, rather than the absolute amount of polyphenol compounds. The estimation of the TPC based on chlorogenic acid equivalent was not performed so far in ethanol extract from *B. ceiba* flowers. As depicted in Table 1, the TPC value of ethanol extract from *B. ceiba* flowers was 60.41 µg CAE/mg extract. Previous studies have evaluated TPC from the leaves and fruits of *B. ceiba* based on gallic acid (GA) and tannic acid (TA) equivalents (Chikatipalli, 2021, Wanjari et al., 2016). For example, Yu et al. (2011) evaluated TPC based on gallic acid equivalent (GAE) on three extracts from *B. malabaricum* flower. They found that 80% acetone possess the highest value of TPC (130.38 mg GAE/g), followed by 50% ethanol

Table 1. TPC and Antioxidant Activity in Ethanol Extract from *B. ceiba* Flower

Variable	*B. ceiba* extract
TPC	60.41 µg CAE/mg extract
EC_{50}	8.01µg/mL

EC_{50} for ascorbic acid was 3.67µg /mL

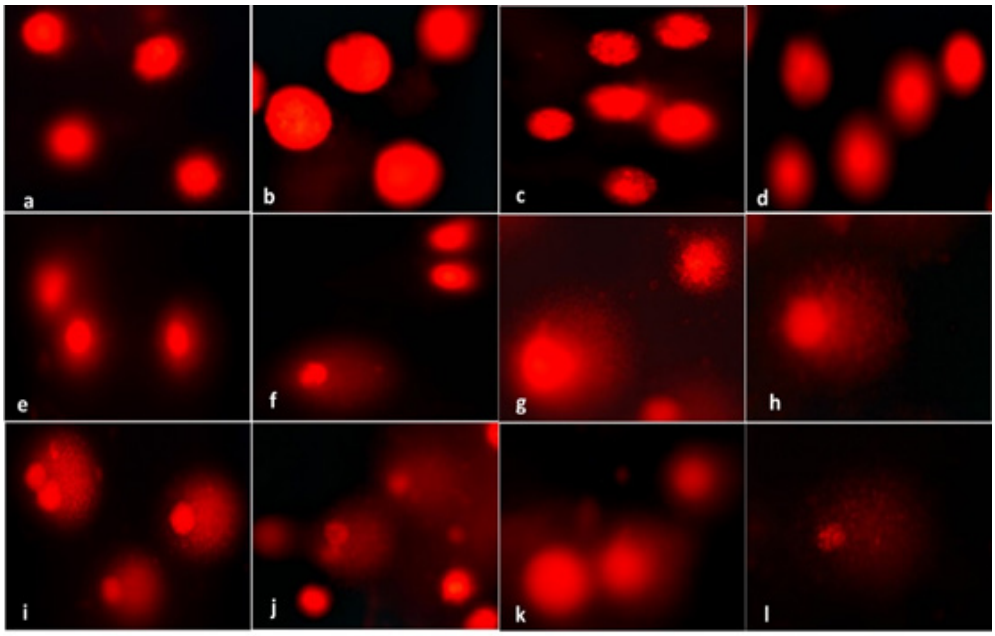

Figure 1. Fluorescent Photomicrographs of Different Patterns of Comet Tail Formation in Huh7 Cells Treated with Various Concentrations of *B. ceiba* Extract Showing: (a), untreated cells; (b), DMSO-treated cells; (c &d), cells treated with ¼ IC_{50} value; (e &f), cells treated with ½ IC_{50} value; (g, h & i), cells treated with IC_{50} value; (j, k & l), cell treated with double IC_{50} value (original magnification 400×)

(106.67 mg GAE/g) and water (57.09 mg GAE/g).

Determination of antiradical activity of B. ceiba extract

Screening the antioxidant activity of the plant extracts plays a vital role in understanding the protection mechanism by which the plants block free radical-induced different diseases, including cancer. DPPH antiradical assay depends on the scavenging of DPPH, a stable nitrogen-centred free radical which reduced by hydrogen-donating antioxidants. This reduction reaction produces the decolourization of DPPH alcohol solution from violet to yellow colour (Mishra et al., 2012).

As depicted in Table 1, *B. ceiba* extract and ascorbic acid significantly decreased the purple colour of the DPPH solution in a concentration-dependent manner. The EC_{50} values of antiradical activity of *B. ceiba* extract and ascorbic acid were 8.09 µg/mL and 3.67 µg /mL, respectively. As mentioned by Fidrianny et al. (2018), the antioxidant activity of a crude extract, based on the EC_{50} value, was classified into four groups: very potent (≤ 50 µg/mL), potent (50–100 µg/mL), medium (101–150 µg/mL), and weak (>150 µg/mL). According to this category, *B. ceiba* extract was very potent antiradical activity as indicated by low EC_{50} value (8.09 µg/mL). Our findings match those mentioned in previous reports displaying that methanol extract from *B. ceiba* flowers possessed potent antiradical scavenging activity (El-Toumy et al., 2013; Vieira et al., 2009).

Table 2. Cytotoxic Aactivity (IC_{50}) of *B. ceiba* Extract against Human Cancer Lines at 48 h Following Treatment

Cell lines	IC_{50} (µg/mL)
A549	962.95
Huh7	198.11
HepG2	540.63

Cancer cell lines (5×10^3 cells/well), grown in a 96-well plate, were treated with five concentrations of *B. ceiba* extract for 48 h. MTT dye (20 µL/ well) was added 4-h before the end of the incubation period.

Effect of B. ceiba extract on the cell viability

Table 2 displays the cytotoxic activity of *B. ceiba* extract against three human cancer cell lines during 48 h of incubation using MTT assay. The cytotoxic activity of the plant extracts has been categorized into four classes as follows: highly active with IC_{50} ≤ 20µg/mL; moderately active with IC_{50} >21–200 µg/mL, weakly active with IC_{50} > 201–500 µg/mL, no cytotoxicity with IC_{50} >501 µg/mL (Goldin et al., 1981; Srisawat et al., 2013). Based on this category, *B. ceiba* extract exerted a moderate cytotoxic activity against Huh7 cells (IC_{50}= 198.11 µg/mL) and no cytotoxicity against HepG2 and A549 cells with IC_{50} > 500 µg/mL). These results reflect the variation in the susceptibility of the cell lines to *B. ceiba* extract attributed to the differences in the origin, morphology and genomes of the cell lines. Similar findings showed that petroleum and diethyl ether extracts from *B. ceiba*

Table 3. Genotoxic Activity after 48 h Treatment of Huh7 Cells with *B. ceiba* Extract in Alkaline Comet Assay

Treatment	Tail DNA (%)	TM (A.U)	OTM (A.U)
Untreated Control	6.90 ± 0.15^{a}	0.29 ± 0.02^{a}	1.13 ± 0.03^{a}
Vehicle control	7.52 ± 0.15^{a}	0.38 ± 0.04^{ab}	1.31 ± 0.04^{a}
¼ IC_{50}	9.07 ± 0.76^{a}	1.13 ± 0.29^{ab}	1.785 ± 0.21^{ab}
½ IC_{50}	13.18 ± 0.57^{b}	2.94 ± 0.59^{bc}	3.16 ± 0.39^{bc}
IC_{50}	15.14 ± 0.76^{bc}	4.17 ± 0.69^{cd}	3.86 ± 0.48^{cd}
Double IC_{50}	17.25 ± 0.60^{c}	6.57 ± 0.94^{d}	5.32 ± 0.55^{d}

Data expressed as Mean% ± Standard error; A.U, Arbitrary Unit .The values with different superscript letters in each column are significantly different from one another as calculated by ANOVA (Tukey's HSD test, P<0.05).

flowers exhibited moderate cytotoxicity, based on sulforhodamine B (SRB) assay, against different human cancer cells, including lung carcinoma COR-L23 (Tundis et al., 2014). Further, aqueous ethanol extract (50%) from *B. anceps* twigs exhibited a moderate cytotoxic activity (IC_{50} = 212 µg/mL) against HepG2 cells using a neural red assay (Weerapreeyakul et al., 2016). Furthermore, flavonoids identified in *B. ceiba*, including rutin, quercetin, 3-O-β-glucoside, apigenin, and vitexin, exhibited potent cytotoxic activity on liver and lung human cancer cells (Fawzy et al., 2014; Imran et al. 2020; Li et al. ,2014; Ren and Tang., 2011; Wang et al., 2014; Zhaorigetu et al., 2021).

Effect of B. ceiba extract on the induction of DNA strand breaks

To understand whether the inhibition of cell viability was associated with induction of DNA fragmentation in Huh7 cells, we employed the comet assay after 48 h treatment. In this assay, negatively charged DNA breaks migrate through agarose gel under the influence of an electric field toward the direction of the anode, producing different patterns that resemble a comet in shape (Figure 1). The results showed no significant differences between negative and vehicle control cells in all comet tail parameters (Table 3). No significant increases in the values of all comet tail parameters were observed between the cells treated with ¼ the IC_{50} concentration of *B. ceiba* extract and control cells. Treatment with three concentrations of *B. ceiba* extract (½ IC_{50}, IC_{50}, and double IC_{50}) remarkably increased all comet tail parameters in Huh7 cells in a concentration-dependent manner. The percentage of DNA in the comet tail reached its maximum values (17.25% versus 7.52% for vehicle control, i.e. a 2.3-fold increase) after treatment with double IC_{50} value of *B. ceiba* extract. These data support the genotoxicity activity of *B. ceiba* extract and its constituents against cancer cells. These findings match those reported in earlier studies. For example, aqueous ethanol extract from *B. anceps* twig induces DNA fragmentation in human liver cancer HepG2 as indicated by a significant increase in the percentage of apoptotic cells-stained with DAPI (Weerapreeyakul et al., 2016). High concentrations of flavonoids (myricetin, kaempferol, quercetin, luteolin, and apigenin) induce comet tail formation in human leukaemia K562 cells (Das et al., 2017). It appears that chemical constituents of *B. ceiba* flower exert their molecular genotoxicity toward cancer cells through two main mechanisms: (1) Polyphenol compounds bind directly to DNA molecules via intercalation, groove binding, and backbone binding (Das et al., 2017; Kanakis et al., 2009); (2) high levels of polyphenol compounds induced pro-oxidative activities leading to overproduction of peroxidation species. These species interact with macromolecules causing selective cytotoxicity to cancer cells (Arif et al., 2015; Tu et al., 2015).

In conclusion, ethanol extract from *B. ceiba* flower is considered a genotoxic agent toward cancer cells at its cytotoxic concentrations. This extract exerted moderate cytotoxic activity via induction of DNA damage in liver cancer Huh7 cells. However, further studies are needed to expand the knowledge of mechanisms of genotoxicity and anticancer activity of *B. ceiba* extract against cancer cells.

Author Contribution Statement

KAD: Experimental planning and execution, data analysis, and editing the manuscript. RE and NMH: Experimental planning and execution. SAE: Collection the plant and extraction process.

Acknowledgments

This study was carried out with the support of the fluorescence microscopy infrastructure provided by the Microbial Biotechnology Department, National Research Centre.

Funding Statement

The authors received no financial support for the research.

Ethical Issues

There are no ethical issues as the present work was carried out on human cancer cell lines as in vitro model system.

Conflict of interest

The authors declare that they have no conflicts of interests.

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