International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 6 Nov-Dec 2023, pp: 2048-2061 www.ijprajournal.com ISSN: 2249-7781

Bruce in a Induces Apoptosis by Stabilizing Intracellular Gen P53 and COX2 Protein Expression on Cancer HelaCell Line

Sonlimar Mangunsong
Poltekkes Kemenkes Palembang

Submitted: 10-15-2023 Accepted: 20-12-2023

ABSTRACT
Cancer is is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells. Brucein A from isolated B. javanica has been previously shown to have a proapoptotic effect on cancer cells line through a p53-dependent pathway. One of its intracellular targets, cancer protects against apoptosis under genotoxic stress and interacts with p53 and COX2 Protein. In this current study, we explored the mechanism of the proapoptotic effect of GEN by examining the gen p53 and COX2 protein. We initially showed that the p53 protein level was elevated in GEN-treated human cervical cancer HeLa cells. By examining both protein synthesis and degradation, we found that GEN enhances p53 intracellular stability by interfering with the hole genome, which provided a plausible explanation for how GEN initiates apoptosis. Furthermore, we found that the interaction between COX2 and p53 is important for the degradation of p53 and is dependent on the redox domain mutan HeLa Cells line. Our data brucein has confirm by FTIR, H-C NMR LCMS. Also suggest that brucein recovery degradation of mutan type p53 . Based on this evidence, we hereby report mechanism of p53 repair and reduced COX2 expression through dependent pathway.

Keywords: Brucein A, gen p53, COX2, Brucea javanica, PCR

I. INTRODUCTION

Cancer is is a group of diseases characterized by the uncontrolled proliferation of cells that have evaded central endogenous control mechanisms (Chen and Hunter 2005). Cancers are classified based on their organ or tissue of origin, but increasingly, categorization is also influenced by the molecular characteristics of the respective cancer cells (Stout et al., 2017) . Cancer cervix one of the type cancer leading caused mortality in the world for woman affect by HPV. Human papillomavirus (HPV) infection contributes to virtually all cases of cervical cancer, the fourth most common cancer affecting women worldwide. The oncogenicity of HPV is mainly attributable to the E6 and E7 oncoproteins (de Martel et al., 2017). Cervical cancer, also known as cervical cancer, is a type of cancer primarily caused by the human papillomavirus (HPV) oncogenic, affecting the cervix (Szymonowicz and Chen, 2020). The global annual death toll from cervical cancer is estimated to exceed 300,000, with a significant number of victims being young mothers. In Indonesia, cervical cancer ranks as the leading cancer (Ferlay et al., 2019; Sung et al., 2020) . Given this alarming scenario, there is a pressing need to explore ways to reduce its incidence. Treatment options range from local destruction, such as cauterization, to more extensive measures like hysterectomy. For invasive cases, common treatments involve surgery, radiation, chemotherapy, or a combination of these (Deo et al., 2020) . The goal of chemotherapy is to have high selectivity, targeting cancer cells while sparing normal tissue. Understanding the anticancer mechanisms in genes associated with cervical cancer can contribute to enhancing this selectivity. Rapid technological advances in recent years has contributed, it is now possible to analyze the molecular makeup of different cancer types in detail within short time periods. The accumulating knowledge about the development and progression of cancer can be leveraged to develop more precise diagnostics and more effective and/or less toxic cancer therapies. In the long run, the goal is to offer every cancer patient a therapeutic regimen tailored to their individual disease and situation in the most optimal way. The main cause of cervical cancer is the family member of Papovirida, namely Human Papillomavirus (HPV), with a diameter of 55 µm. This virus is transmitted sexually. HPV has a naked icosahedral capsid with 72 capsomers and contains double-stranded circular DNA (Nishimura, et al., 2006). Cervical cancer cells infected with HPV are known to express 2 oncogenes, namely E6 and E7, which affect E2F5 in the cell cycle, especially the protein synthesis phase. The E6 protein binds to the

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 6 Nov-Dec 2023, pp: 2048-2061 www.ijprajournal.com ISSN: 2249-7781

tumor suppressor protein p53, accelerating the degradation of p53 (Stein et al, 2019), while the E7 protein can bind to the active hypophosphorylated form of pRb. The inhibition of p53 by E6 and E7 affects cell regulation. Another gene influenced by HPV is COX-2 (Hashemi et al., 2019), where there is overexpression of this gene in HeLa cells (Araldi et al., 2018).

Herbal medicine has been a source of medicine to cure ailment. Once of these Brucea javanica. Its commonly known as Java Brucea or Javanese Brucea, is a plant species belonging to the family Simaroubaceae. It is native to Southeast Asia, including countries like Indonesia, Malaysia, and Thailand. The plant contains various bioactive compounds, including quassinoids, alkaloids, and flavonoids (Li et al., 2021). Research has indicated that certain compounds isolated from Brucea javanica may exhibit antimalarial, anticancer, and anti-inflammatory properties (Zhang et al., 2022) It contains bruceantin, brucein chemical groups that already isolate from the seeds has anticancer activity invitro cell lines. But the growing and species seems to be consideration to have isolated. This study was to isolated brucein from B javanica seeds and anticancer assay via the expression of gen p53 and COX 2 immunohistochemistry.

II. METHODS

Tools and Material

A variety of tools, including glassware, clamps, a drying cabinet, a blender, an analytical balance, and specialized equipment such as a Biorad Minioption RT-PCR, were utilized in this research to build a comprehensive foundation for scientific exploration.

The materials used in this research include Brucein A, HeLa cancer cells from the Parasitology Laboratory of the Faculty of Medicine at Universitas Gadjah Mada, and a range of high-quality pro-analysis chemicals such as α-naphthol, ammonium hydroxide, acetic anhydride, glacial acetic acid, HNO_3, H_2SO_4, benzene, chloroform, methanol, n-hexane, anhydrous sodium sulfate, petroleum ether, amyl alcohol, toluene, chloral hydrate, distilled water, Roswell Park Memorial Institute (RPMI) growth medium (Gibco), sodium bicarbonate ($NaHCO_3$) (Merck), [4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid] (HEPES) (Sigma Aldrich), HCl (Merck), Trypsin EDTA, phosphate buffer saline (PBS) (Sigma Aldrich), propidium iodide, Annexin V, dimethyl sulfoxide (DMSO) (Sigma Aldrich), [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] (MTT) (Sigma Aldrich), Sodium Dodecyl Sulphate (SDS) (Sigma Aldrich), hydrogen peroxide blocking, prediluted blocking serum, streptavidin-enzyme horse radish peroxidase, DAB, Mayer-Hematoxylin solution, xylene, and p53 gene.

Media

The media employed in this research include Roswell Park Memorial Institute (RPMI) medium and RPMI culture medium. Composition: RPMI with L-glutamine without $NaHCO_3$, net 10.4 g; [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (HEPES) 2 g; ($NaHCO_3$) 2 g; sufficient 1 N HCl solution; sufficient 1 N NaOH solution; sterilized distilled water up to 1 L.

In the formulation process, 2 grams each of $NaHCO_3$ and HEPES were weighed and combined with 1 packet of RPMI in a 1 L beaker. Subsequently, 800 mL of sterile distilled water was added to the beaker, and the mixture was dissolved and homogenized using a magnetic stirrer. The pH of the resulting mixture was adjusted to the range of 7.2–7.4 using 1 N HCl solution for basic solutions or 1 N NaOH solution for acidic solutions. After adjusting the pH, sterile water was added to achieve a total volume of 1 L. The sterilization process involved using a vacuum filter in a laminar airflow (LAF) hood, with a sterile filter apparatus attached to a labeled 1 L bottle containing the media details. The finalized media were then stored at a temperature of 2–8°C following the protocol outlined by Sambrook et al. (1989).

Culture Medium

The culture medium consists of 10% Fetal Bovine Serum (FBS) or Fetal Calf Serum (FCS), 2% Penicillin Streptomycin (Penstrep), 0.5% Amphotericin B (Fungizone), and RPMI media to make up to 100 mL. To create the medium, 10 mL of FBS or FCS, 2 mL of Penstrep as an antibiotic agent, and 0.5 mL of Fungizone as an antifungal agent are combined in a laminar airflow (LAF) hood. The mixture is then adjusted to a total volume of 100 mL with RPMI media. The final mixture is labeled with clear identification on the RPMI culture media bottle, including the media name, production date, expiration date, and creator's name. Following the protocol outlined by Sambrook et al. (1989), the completed culture medium is stored at a temperature of 2–8°C.

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 6 Nov-Dec 2023, pp: 2048-2061 www.ijprajournal.com ISSN: 2249-7781

Cell Growth

The equipment is prepared, and materials are conditioned at room temperature. Subsequently, 10 mL of RPMI media is extracted into a 15 mL conical tube, and an ampoule containing HeLa cancer cells is retrieved from the -80°C freezer or liquid nitrogen tank and thawed at room temperature. The cell suspension from the ampoule is then carefully added drop by drop into the prepared RPMI media, followed by centrifugation at 600 rpm for 5 minutes. The supernatant is discarded, and 4 mL of RPMI culture media is added, followed by thorough resuspension for homogeneity. Portions of 2 mL from the mixture are transferred into new culture flasks, and 5 mL of RPMI culture media is added to each flask before homogenization. The cell conditions are observed using an inverted microscope, ensuring homogeneous distribution across the flask surface without clustering in specific areas. The culture flasks are labeled with cell name, production date, and creator's name, then stored in a 5% CO2 incubator at 37°C (Doyle et al., 2000).

Sub Culture Cell Cancer

All equipment and materials are prepared and conditioned at room temperature. Subculture is performed in a laminar airflow hood. The subculture process for HeLa cancer cells involves taking 500 µL of cell harvest, placing it into a culture flask, adding 6 mL of RPMI culture media, and homogenizing the mixture. The cells are then incubated in a 5% CO2 incubator at 37°C, and their condition is observed the next day (Doyle et al., 2000)

Harvesting Cell Cancer

Equipment is prepared, and materials are conditioned at room temperature, and the cell condition is observed. Harvesting is performed when the cells reach 80% confluence, all work done in a laminar airflow hood. The culture media is removed from the flask using a micropipette or Pasteur pipette, cells are washed twice with 10 mL of phosphate buffer saline (PBS), and 400 µL of 0.25% Trypsin EDTA is evenly added. The flask is then incubated in a 5% CO2 incubator at 37°C for 5 minutes, followed by the addition of 4 mL of RPMI culture media to deactivate Trypsin EDTA. Cell resuspension is performed using a micropipette to ensure single-cell detachment (no clustering), and cell conditions are observed under an inverted microscope. If any cell clusters are observed, immediate resuspension is conducted. Cells are transferred to a conical tube (Doyle et al., 2000)

Preparation sample tested brucein and doxorubicine

Five milligrams of brucein A and doxorubicine were weighed in separated microtubes. They were then dissolved in 100 µL of DMSO solvent, vigorously vortexed to ensure complete solubility, and subsequently supplemented with RPMI culture media. Dilutions were then made to obtain test solutions with concentrations of 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, and 6.25 µg/mL. All sample dilution processes were carried out using culture media, specifically RPMI culture media for the HeLa cell test solution modified method (CCRC, 2010).

Cell Seeding and Viability

Coverslips were placed in each well, and 200 µL of cells were evenly transferred onto the coverslip, followed by a 30-minute incubation in the incubator. Add the test substance (MK) to each well up to 1500 µL. Then, create serial dilutions based on each 1/5 IC50 sample. For each well containing cells, slowly remove the medium, wash the cells once with 500 µL PBS, discard PBS, add the sample, and incubate in the incubator for 15-18 hours. Observe cell conditions after incubation. Fix the cells with 500 µL 70% ethanol (ensuring coverslips are soaked in ethanol). Discard ethanol from the wells, wash with 599 µL PBS three times, each for 5 minutes. Take the coverslip, add 10 µL of a mixture of ethidium bromide and acridine orange reagents onto the coverslip, gently shake to spread it evenly. Examine under a confocal microscope at 40x magnification .

RNA Extraction

HeLa cells (4 x 10^3 cells/well) were seeded into six wells and incubated for 24 hours. Subsequently, the cells were exposed to the test solution and further incubated for another 24 hours. After discarding the media and adding fresh media, the floating and adherent cells were collected by treating them with 0.025% trypsin. The cells were then transferred to conical tubes, washed twice with 1 mL PBS, and centrifuged at 2500 rpm for 5 minutes. The upper layer was discarded, and the pellet was collected, resuspended in PBS, and centrifuged at 3000 rpm for 3 minutes. Discard the supernatant and add 1 mL PBS. Remove the culture

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 6 Nov-Dec 2023, pp: 2048-2061 www.ijprajournal.com ISSN: 2249-7781

medium, wash the cells with PBS, and add trypsin at a concentration of 0.10-0.25%. After the cells detached, add medium and transfer to a 15 mL conical tube, continuing with cell suspension in the culture

Cell Lysis

Add 400 µL RB buffer and 4 µL β-mercaptoethanol (or 8 µL of prepared 2M Dithiothreitol in RNase-free water), then resuspend the mixture. Homogenize the mixture and incubate at room temperature for 5 minutes. Add 500 µL of pre-prepared 70% ethanol (free from Dnase and RNase). Stir vigorously until the mixture is homogeneous, then pipette the pellet. Place an RB column in a 2 mL tube and transfer 500 µL of the mixture onto the RB column. Centrifuge at a force of 14-16,000 x g for 1 minute, then discard the filtrate. Transfer the remaining mixture to the same RB column, centrifuge at a force of 14-26,000 x g for 1 minute. Discard the filtrate and place the RB column in a new 2 mL tube

Washing Stage

Add 400 µL of buffer W1 to the RB column, centrifuge at a force of 14-16,000 x g for 30 seconds. Discard the filtrate, then place the RB column back into the 2 mL tube. Add 600 µL of wash buffer (ensure ethanol has been added) to the RB column. Centrifuge at a force of 14-16,000 x g for 30 seconds, then discard the filtrate. Place the dried RB column into a new 1.5 mL microcentrifuge tube. Add 50 µL RNase-free water to the center of the matrix in the column. Allow it to stand for 1 minute to ensure the RNase-free water has been absorbed. Then, centrifuge at a force of 14-16,000 x g for 1 minute to elute the purified RNA

Extracting RNA

Prepare the DNAse 1 reaction in a 1.5 mL microcentrifuge tube that is RNase-free, combine the DNA sample (X µL), DNAse buffer solution (Y µL), and DNAse 1 (Z µL) in accordance with the specified volumes outlined in Table 3.1. Mix the components thoroughly through vortexing or gentle agitation to achieve a homogeneous solution. Following the mixing, incubate the reaction at the designated temperature and duration as per the experimental protocol. This ensures the effective action of DNAse 1 in degrading DNA. It is essential to use RNase-free reagents to prevent RNA contamination during the reaction. The total reaction volume should be adjusted to 50 µL. Refer to the specific experimental guidelines or product instructions for precise details on incubation conditions and volumes

Table1: Preparing Reaction DNAse Solution

RNA dalam RNAse free of water	1-40 µL
DNAse I	0,5 µL/µg RNA
Buffer reaaction Dnase I	5 µL
RNAse free of water	Up to volume = 50 µL
Total Volume	50 µL

Carefully pipette the DNAse I reaction solution into the mixture without vortexing, then incubate the microcentrifuge tube at 37ºC for 15-30 minutes. Stop the reaction by adding 1 µL of 20 mM EGTA (pH = 8.0), followed by another incubation of the microcentrifuge tube at 65ºC for 10 minutes. Subsequently, purify the RNA sample again by adding 250 µL of RB buffer to the DNAse I reaction mixture. Mix the contents thoroughly by vortexing. Transfer the entire sample mixture to a new RB column, centrifuge at 14-16,000 x g for 1 minute, and discard the filtrate. This process aids in further purification of the RNA sample, ensuring the removal of any remaining contaminants.

cDNA Synthesis

A total of 3,000 µL of total RNA was used, and PCR water was added to achieve a total volume of 12 µL. To each tube containing RNA, 8 µL of a mixed solution (5x RT-buffer 4 µL, random primer 1 µL, dNTP 2 µL, and Rever Tra-Ace 1 µL) was added, followed by resuspension for PCR amplification. The PCR conditions were set at 30ºC for 10 minutes, 42ºC for 60 minutes, and 99ºC for 5 minutes. The PCR products were then stored at -20ºC in the laboratory. This process involves reverse transcription, converting RNA into complementary DNA (cDNA), which can be further utilized for various molecular biology applications.

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 6 Nov-Dec 2023, pp: 2048-2061 www.ijprajournal.com ISSN: 2249-7781

COX 2 Expression Assay

For the microplate with 6 wells, coverslips were first placed inside. Cells, at a density of 3×10^4 per well, were distributed into the 6-well microplate, which had coverslips placed at the bottom, and incubated for cell adaptation. The cells were then treated with EnHDM at a concentration of 1/5 IC50 (with 5Doxorubicin used as a positive control) and incubated for 24 hours. At the end of the incubation period, the cells were washed with PBS, treated with cold methanol, and further incubated in a freezer at -4°C for 10 minutes. Subsequently, methanol was discarded, and coverslips containing cells were placed in a clean dish. The cells, fixed with methanol, were then washed three times with distilled water and incubated with a hydrogen peroxide blocking solution for 10 minutes at room temperature. This process sets the stage for the analysis of COX-2 expression in the treated cells.

After washing with PBS, the cells were incubated at room temperature and then treated with a prediluted blocking serum for 10 minutes. Following this, the blocking serum was discarded. Antibodies were added to the cells and incubated for 1 hour at room temperature. Subsequently, coverslips were washed with PBS and treated with a secondary antibody (biotinylated universal secondary antibody), followed by a 10-minute incubation. Cells were washed again with PBS and treated with streptavidin-enzyme horse radish peroxidase for 10 minutes. After washing, DAB was added, and the cells were incubated for 5 minutes until a brown color developed. Cells were washed with PBS and distilled water, followed by Mayer-Hematoxylin solution for a 5-minute incubation. After another wash with distilled water until clear, cells were treated with 70% ethanol for 2 minutes, cleaned, dipped in xylene, and air-dried. Once dry, coverslips were placed on glass slides, mounted, and covered with a glass cover slip. Observation was carried out using a microscope equipped with optiLab (Cho, et al., 2009).

P53 Gene Expression Analysis In Vitro Via RT PCR

The examination of p53 gene expression involved extracting 1 µL of cDNA with a concentration of 5 µg/ml (ranging from 0.5 to 6.5 µg/ml) (Wilkerson, 2003). PCR Master Mix was then added to reach a total volume of 25 µL (GoTaxGreen 12.5 µL; forward primer 1 µL; reverse primer 1 µL; DNase/RNase-free water 9.5 µL). Subsequently, RT-PCR was performed for 35-40 cycles under the following conditions: 94°C for 30 seconds (denaturation), 55-60°C for 30 seconds (annealing), and 72°C for 45 seconds (elongation/extension). The final extension phase was set at 72°C for 5 minutes, followed by storage at -20°C. The PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. The resulting bands were documented using the Gel Dox XR image scanner from Bio-Rad. This RT-PCR analysis aimed to assess the expression levels of the p53 gene in the in vitro experimental setting

Tabel 2. Sequences Primer Forward dan Primer Reverse

Gen		Primer sequence (5'-3')	Productlength (bp)
p53	F	5'-ATT CAG CTC CTC CTC CAT GAA GAA TCG GCG-3'	390
	R	5'-GCT TTG CTG CTG AGG CCA CCA GTA TCC ACT-3'	
β-actin	F	5'-GCT CCT CCT AAG CGC GAG T-3 '	100
	R	5'TCA TAC TCG CTG TCC TGC AT TTG -3 '	

III. RESULT AND DISCUSSION

We have already isolated brucein A using the established procedure. (Sonlimar et al. 2011). It was confirm brucein by LC-MS, FTIR, and HNMR -CNMR (Figure 1).

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 6 Nov-Dec 2023, pp: 2048-2061 www.ijprajournal.com ISSN: 2249-7781

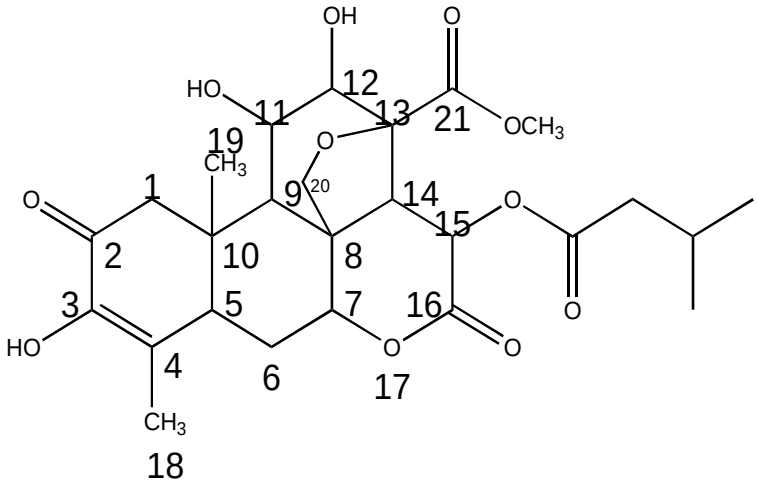

Figure 1. Brucein A($C_{26}H_{32}O_{11}$)

In the HRESI-MS examination, the value of .. originates from .. . However, in the MS examination connected with mass spectrophotometry (LC-MS), a compound brucine is observed with some other impurities, so the purity is not yet 100% (Figure 2 and 4). The Rt (retention time) value has two peaks, one at 1.2 minutes and the base peak at 1.9 minutes. Although there are two peaks, the peak with Rt 1.2 is much smaller compared to the peak at Rt 1.9. This could be due to the influence of solvent impurities or other substances. The obtained retention time values indicate the products of the tested sample. They can also be compared with chromatographic spectra of the control base material. Meanwhile, the area of the HPLC chromatographic peak obtained indicates the amount of the tested substance contained in the analyzed sample (Beccaria and Cabooter , 2020)

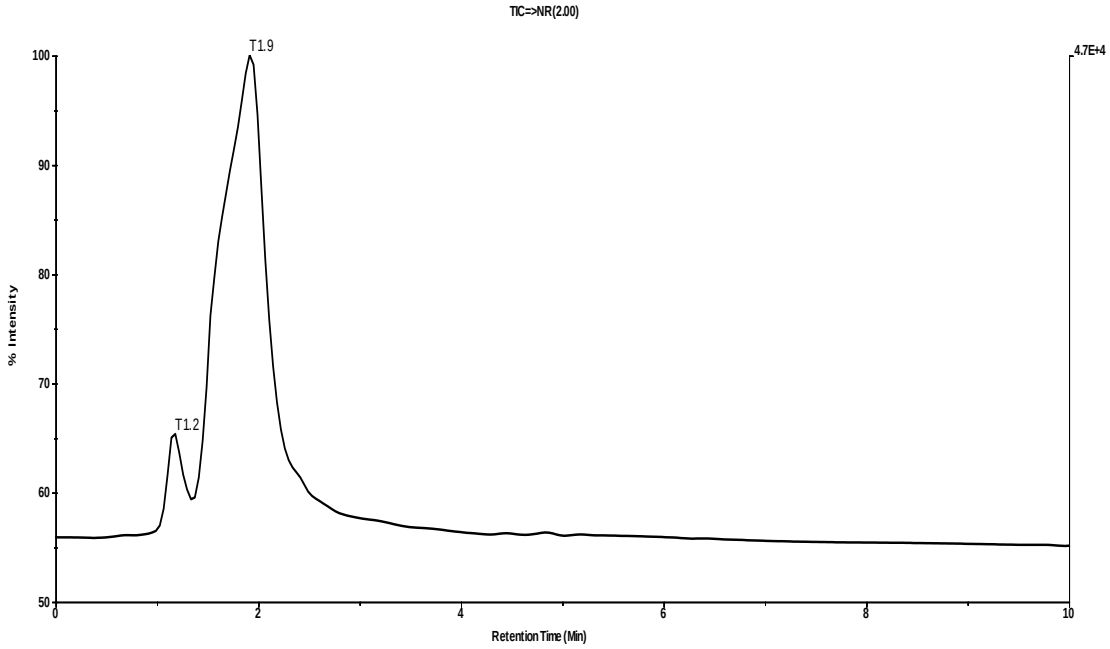

Figure 2 Profile chromatography of brusein with LC-MS on Retention Time (RT) 1,2 and RT 1,9 minutes.

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 6 Nov-Dec 2023, pp: 2048-2061 www.ijprajournal.com ISSN: 2249-7781

LC-MS assays are widely used in various scientific disciplines, including pharmaceuticals, environmental analysis, clinical research, and metabolomics. They offer high sensitivity, selectivity, and the ability to analyze a wide range of compounds in complex mixtures (Seger and Salzman, 2019) .The ionized molecules of bruceine are accelerated into the mass spectrometer, where they are separated based on their mass-to-charge ratio (m/z) of brucein figure 4.

FT-IR is a versatile and widely used analytical technique due to its ability to provide detailed information about the molecular composition of a wide range of materials. It is a valuable tool in both qualitative and quantitative analysis. FT-IR spectra are used to identify functional groups within molecules. Each functional group of brucein absorbs infrared light at characteristic wavelengths, leading to distinctive peaks in the spectrum. Peaks in the fingerprint region (500-1500 cm^{-1}) provide information about the overall molecular structure, while peaks in the functional group region (1500-4000 cm^{-1}) reveal details about specific functional groups.

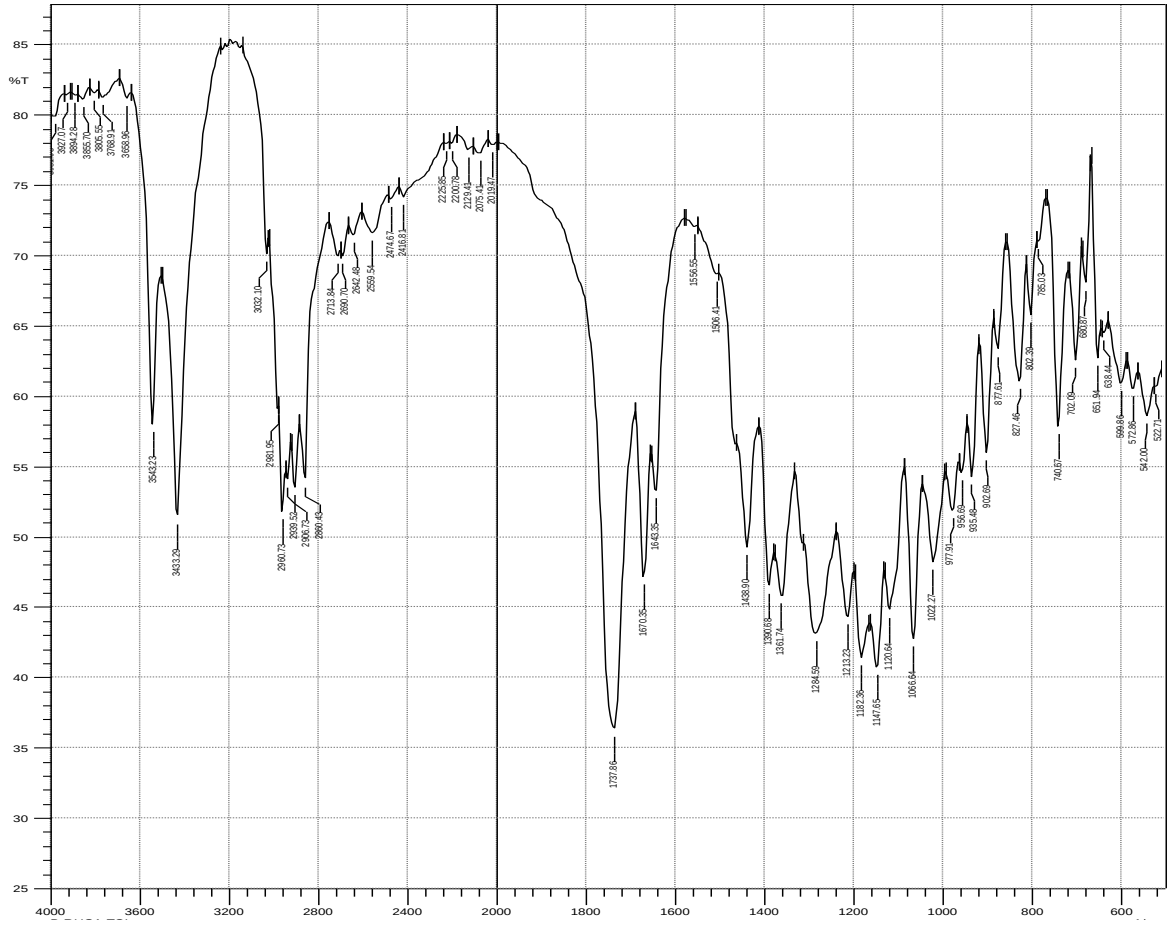

Figure 3 . Spectral of bruceine (KBr) with FT-IR spectroscopy

Based on the infrared spectroscopy image (figure 3), various important peaks provide initial information about several functional groups, namely at 3388.17 cm-1, indicating the presence of hydroxyl groups; at 2360.42 cm-1 for -C=C-; at 1638.36 cm-1, which is close to C=O; at 1402.92 cm-1 for the -C-O group; and at 557.58 cm-1, representing the CH3 group.

Based on HRESI-MS we found molecule m/z 522 confirm as Brucein A (Figure 4.) In positive ion mode mass spectrometry, a positively ionized molecule (M+) can interact with various ions to form adducts. The most common example is the attachment of a proton (H+) or sodium ion (Na+) to the molecule. For example, if the Brusein molecule is ionized to (M+), it can form the adduct (M+H)+ by capturing a proton to have m/z 522.

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 6 Nov-Dec 2023, pp: 2048-2061 www.ijprajournal.com ISSN: 2249-7781

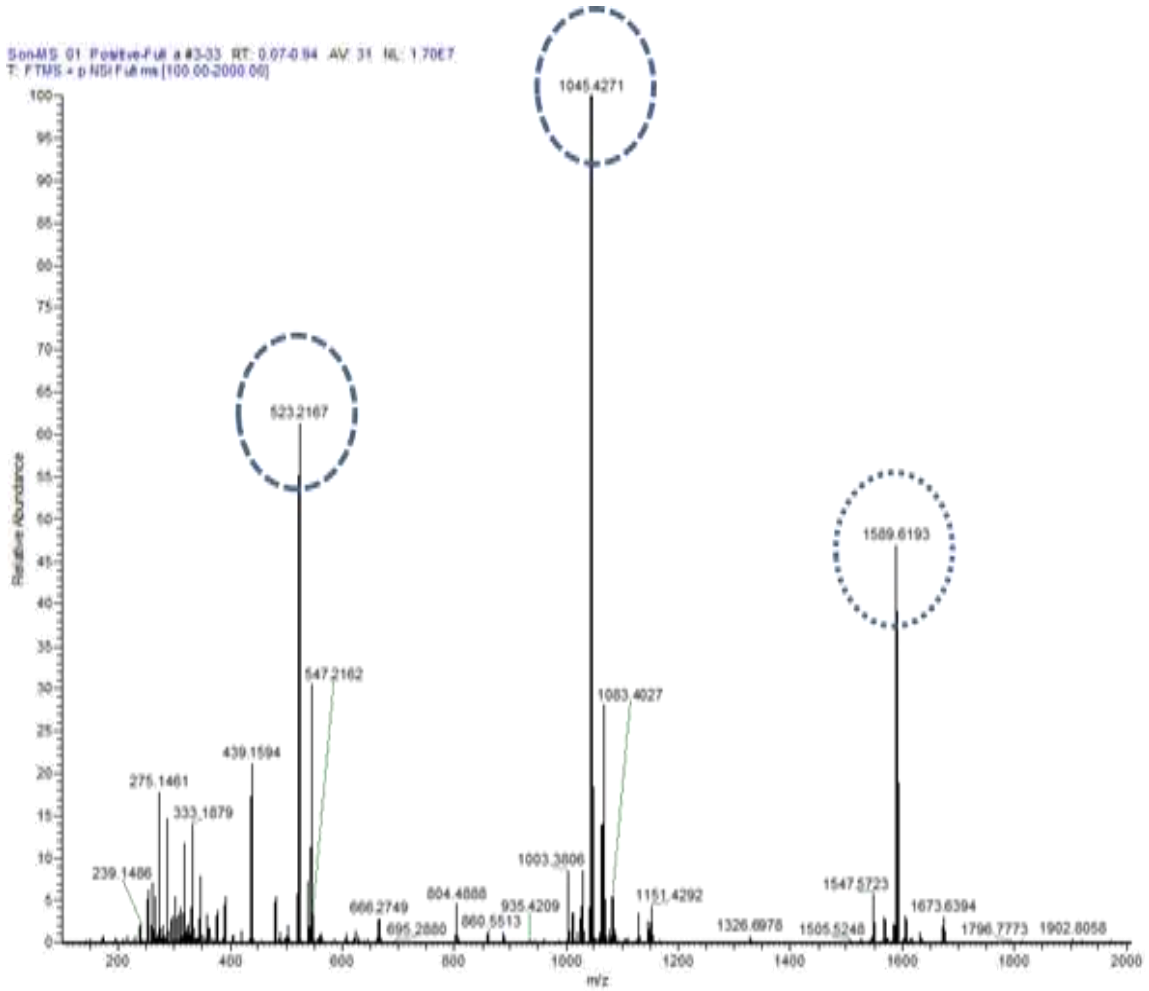

Figure 4: . HRESI-MS brusein molecules m/z 522, 1054 and 1085.

Based on H -NMR and C-NMR tested we have spectral of brucein with protons 32 and Carbons 26 showed in Figure 5 and 6.

Proton NMR is a spectroscopic technique that exploits the magnetic properties of hydrogen nuclei (protons) to elucidate the structure of organic compounds. In a proton NMR spectrum, the x-axis typically represents the chemical shift (δ), measured in parts per million (ppm), and the y-axis represents the signal intensity The chemical shift is influenced by the local electronic environment of the protons, allowing chemists to deduce information about the chemical environment of hydrogen atoms in a molecule. Peaks in the spectrum correspond to different types of protons in the sample, and their integration (area under the peaks) provides information about the relative abundance of each type of proton. Proton NMR is valuable for determining the connectivity of atoms in a molecule and identifying functional groups.

Carbon-13 NMR is spectroscopic technique that utilizes the magnetic properties of carbon-13 nuclei to provide information about the carbon atoms in a molecule In a carbon NMR spectrum, the x-axis represents the chemical shift (δ) of carbon atoms, and the y-axis represents the signal intensity. Unlike proton NMR, carbon NMR spectra are less complex, as they typically show fewer peaks. Carbon NMR of brucein is useful for determining the number and types of carbon atoms in a molecule. It is particularly valuable for characterizing the carbon framework of organic compounds and providing insights into the carbon connectivity. Protons and Carbons position ia not fully explanation in this paper refer to Suwattanasophon et al (2023).

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 6 Nov-Dec 2023, pp: 2048-2061 www.ijprajournal.com ISSN: 2249-7781

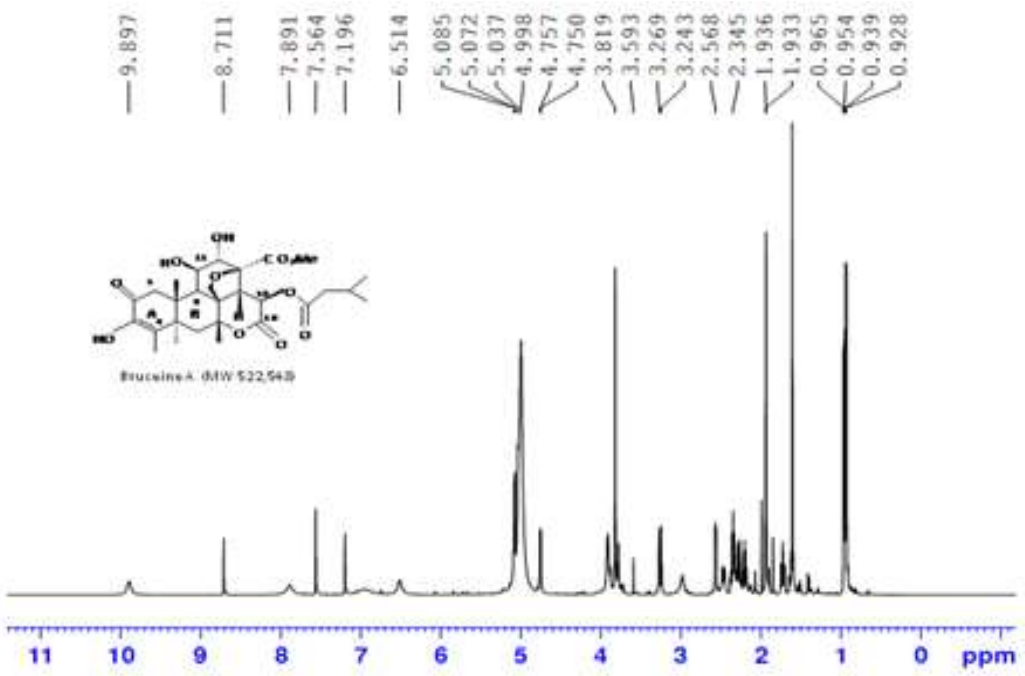

Figure 5 : Spectra H-NMR (32 Proton) of brusein A in piridine

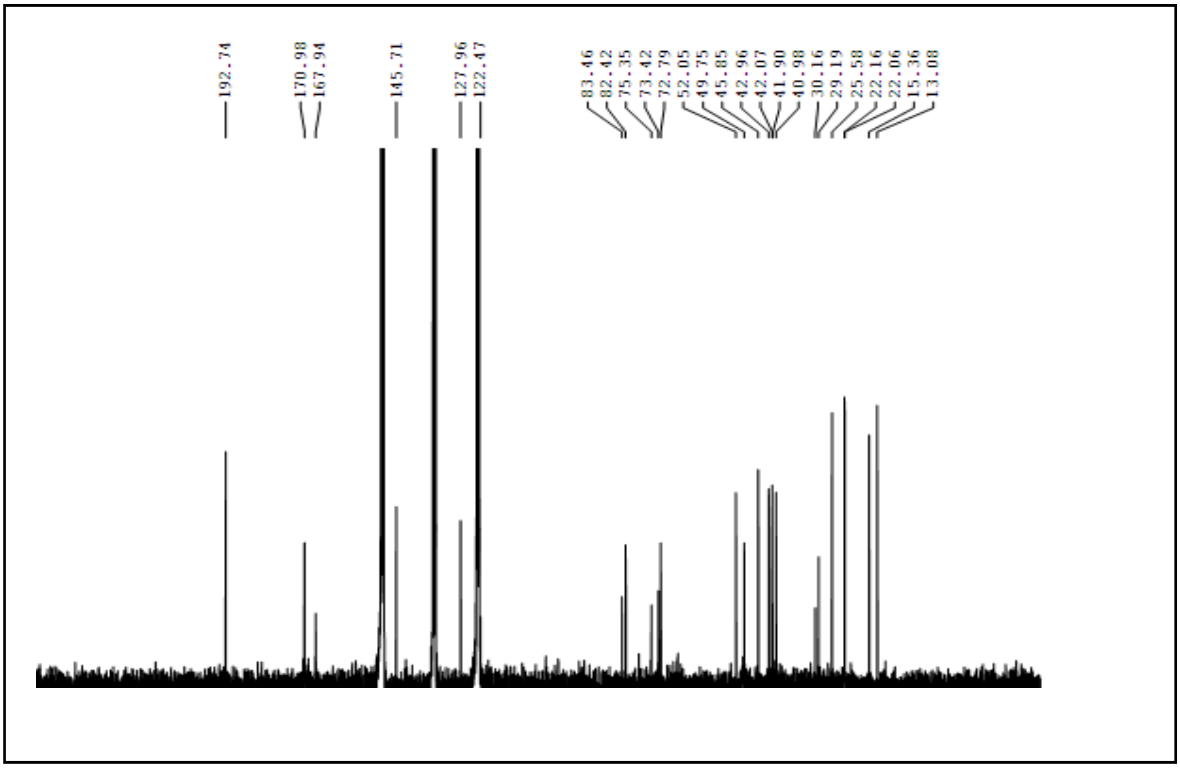

Figure 6 :Spectral C-NMR (26 Carbons) of brusein in piridin

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 6 Nov-Dec 2023, pp: 2048-2061 www.ijprajournal.com ISSN: 2249-7781

Cytotoxicity implies the ability of a substance to cause damage to cells or induce cell death. This can occur through various mechanisms, such as interference with cellular structures, disruption of cell membranes, induction of apoptosis (programmed cell death), or inhibition of essential cellular processes. Based on Cytotoxicity assay, we have IC50 of brucein was 46µg/mL compare to doxorubin 57 µg/mL to two cell tested HeLa Cells and normal cell Vero (Table 4). Sample tested brucein is not fully toxic with in brucein-cell vero if we compare to doxorubicin with in cell vero. This showed that one the advantage of brucein compare to doxorubicin due to its toxicity.

Tabel 3. IC50 sel Vero and HeLa Cell line

Sample Tested	**IC_{50} (µg/mL)**	
	(Cell Vero)	(Cell HeLa)
Bruein	567±35	46±10
Doxorubicin	45± 8	57±5

In the field of pharmacology, cytotoxicity testing is a crucial step in drug development (Li et al., 2021). Before a new drug is tested in animals or humans, its effects on cells are evaluated to identify potential toxicities. Substances with high cytotoxicity may not be suitable for further development. Cytotoxicity refers to the degree to which a substance is toxic or damaging to cells. Cytotoxicity is often assessed to understand the potential harmful effects of a substance on living cells, particularly in the context of drug development, chemical exposure, or research experiments. The cytotoxic effects of a substance often depend on the concentration or dose. Brucein a substance that is harmless or even beneficial at low concentrations its become cytotoxic at higher levels In the context of cancer treatment, certain chemotherapy drugs are intentionally cytotoxic. They target rapidly dividing cells, including cancer cells, to inhibit their growth or induce cell death. However, these drugs can also affect normal, healthy cells and cause side effects, doxorubicin in with vero cell in this studied (Bhatla et al., 2021)

Based on Immunocyto chemistry with antibody COX2 cell tested showed with COX 2 protein expression is much more in cell treated than no treated . It mean that cancer cell is over expression with COX 2 Protein within the consequences with the over expression of COX 2, but can reduced or recover by treated with brucein in Figure 7. Control (a) and (b) brusein tested dose IC50 showed COX2 immunohistochemistry stain with in dark brown.

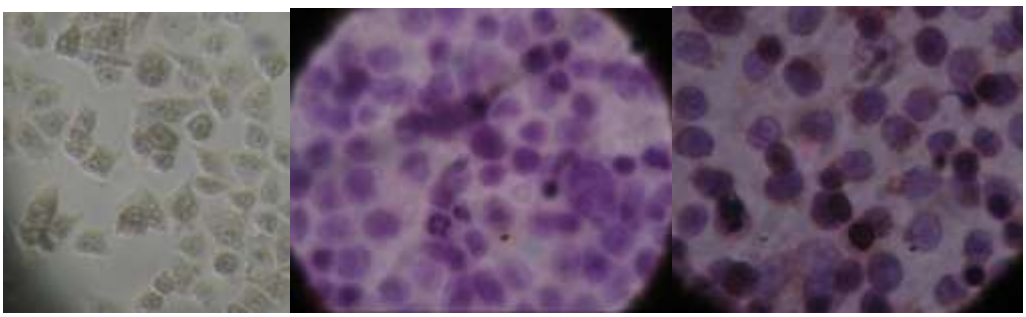

Figure 7. Cell HeLa Tested with COX 2 Protein Expression : Control Cell (a) and (b) Cells with COX2 (c) Brusein tested dose IC50 showed COX2 immunohistochemistry stain with in dark brown.

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 6 Nov-Dec 2023, pp: 2048-2061 www.ijprajournal.com ISSN: 2249-7781

Detection of COX-2 protein expression can be detected and quantified using various laboratory techniques, including immunohisto chemistry (IHC), Western blotting, and polymerase chain reaction (PCR) COX-2 is often upregulated in response to inflammation. It is responsible for producing prostaglandins, which contribute to the inflammatory response. Monitoring COX-2 expression can be relevant in understanding the inflammatory processes in various diseases. Elevated COX-2 expression has been observed in various types of cancer. The overexpression of COX-2 is associated with increased cell proliferation, angiogenesis (formation of new blood vessels), and resistance to apoptosis (programmed cell death). Therefore, COX-2 inhibitors have been investigated for their potential anti-cancer properties. The understanding of COX-2 expression has led to the development of drugs that specifically inhibit COX-2, providing targeted anti-inflammatory and analgesic effects (Yuniarti et al., 2018)

Based on this studied using PCR assessment we showed the express gen p53 in Cell HeLa with brucein treatment and also with doxorubicin. It mean that these compound guard the genom p53 recover to monitor the cell cycle goes to apoptosis. p53 is often called the "guardian of the genome" because it monitors the cell cycle and can halt it at various checkpoints to allow for DNA repair or initiate programmed cell death (apoptosis) if the damage is severe and irreparable (Zhang et al., 2020). This studi refer to Zhao (2014) Herb can be a potential sources to cure ailment.

As a tumor suppressed gen the protein produced by the p53 gene acts as a transcription factor, regulating the cell cycle and preventing cells from growing and dividing too rapidly or in an uncontrolled way. It helps maintain the stability of the genome and promotes DNA repair. The status of the p53 gene is often assessed in cancer diagnosis and treatment planning. If p53 is mutated in a cancerous cell, it may influence the choice of treatment options, as cells with non-functional p53 may be more resistant to certain therapies (Yuniarti et al., 2018). In now days due to target therapy its central role in cancer biology, p53 has been a focus of extensive research. Scientists are exploring ways to reactivate or restore the normal function of p53 in cancer cells as a potential therapeutic strategy (Jamieson et al, 2015).

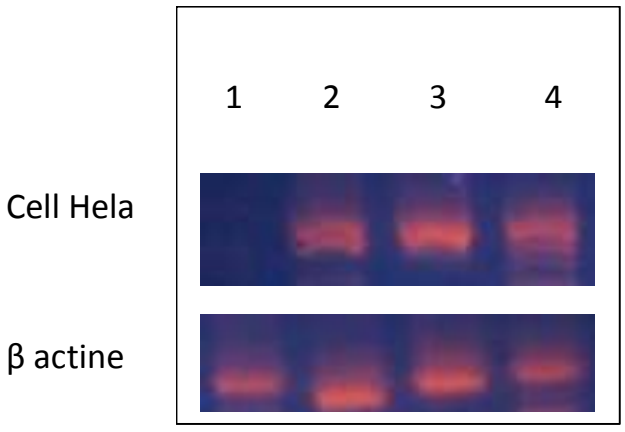

Figure 8 . Gen p53 expression on HeLa Cell treated Cell HeLa; No Expression (1); Treated Brucein 25 µg/mL(2) ; Treated Brucein 12,5 µg/mL (3) ; Doxorubicin 5 µg/mL

IV. CONCLUSION

Brucein has confirm by FTIR, H-C NMR LCMS.It has cytotoxic activity. Also suggest that brucein recovery degradation of mutan type p53 to apoptosis. Based on this evidence, we hereby report mechanism anticancer activity via p53 repair and also can reduced COX2 expression through dependent pathway.

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 6 Nov-Dec 2023, pp: 2048-2061 www.ijprajournal.com ISSN: 2249-7781

REFERENCE

[1]. Araldi RP, Sant'Ana TA, Módolo DG, de Melo TC, Spadacci-Morena DD, de Cassia Stocco R, Cerutti JM, de Souza EB. The human papillomavirus (HPV)-related cancer biology: An overview. Biomed Pharmacother. 2018 Oct;106:1537-1556. doi: 10.1016/j.biopha.2018.06.149. Epub 2018 Jul 26. PMID: 30119229.

[2]. Bagheri E, Hajiaghaalipour F, Nyamathulla S, Salehen N. The apoptotic effects of Brucea javanica fruit extract against HT29 cells associated with p53 upregulation and inhibition of NF-κB translocation. Drug Des Devel Ther. 2018 Mar 29;12:657-671. doi: 10.2147/DDDT.S155115. PMID: 29636600; PMCID: PMC5881282.

[3]. Beccaria M , Cabooter D . Current developments in LC-MS for pharmaceutical analysis. Analyst. 2020 Feb 17;145(4):1129-1157. doi: 10.1039/c9an02145k. PMID: 31971527.

[4]. Bhatla N, Aoki D, Sharma DN, Sankaranarayanan R. Cancer of the cervix uteri: 2021 update. Int J Gynaecol Obstet. 2021 Oct;155 Suppl 1(Suppl 1):28-44. doi: 10.1002/ijgo.13865. PMID: 34669203; PMCID: PMC9298213.

[5]. CCRC (Cancer Chemoprevention Research Centre). (2010). Apoptosis Double Staining. Yogyakarta: Cancer Chemoprevention Research Centre. Halaman 1 – 5.

[6]. Chen YC, Hunter DJ. Molecular epidemiology of cancer. CA Cancer J Clin. 2005 Jan-Feb;55(1):45-54; quiz 57. doi: 10.3322/canjclin.55.1.45. PMID: 15661686.

[7]. de Martel C, Plummer M, Vignat J, Franceschi S. Worldwide burden of cancer attributable to HPV by site, country and HPV type. Int J Cancer. 2017 Aug 15;141(4):664-670. doi: 10.1002/ijc.30716. Epub 2017 Jun 8. PMID: 28369882; PMCID: PMC5520228.

[8]. Deo SVS, Sharma J, Kumar S. GLOBOCAN 2020 Report on Global Cancer Burden: Challenges and Opportunities for Surgical Oncologists. Ann Surg Oncol. 2022 Oct;29(11):6497-6500. doi: 10.1245/s10434-022-12151-6. Epub 2022 Jul 15. PMID: 35838905.

[9]. Doyle, A., Griffiths, J.B., Newell, D.G. (2000). Cell and Tissue Culture: Laboratory Procedures, Edisi Ketiga. New York: John Wiley and Son Incoorporation. Halaman 23 – 24

[10]. Ferlay J, Colombet M, Soerjomataram I, Mathers C, Parkin DM, Piñeros M, Znaor A, Bray F. Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. Int J Cancer. 2019 Apr 15;144(8):1941-1953. doi: 10.1002/ijc.31937. Epub 2018 Dec 6. PMID: 30350310.

[11]. Hashemi Goradel N, Najafi M, Salehi E, Farhood B, Mortezaee K. Cyclooxygenase-2 in cancer: A review. J Cell Physiol. 2019 May;234(5):5683-5699. doi: 10.1002/jcp.27411. Epub 2018 Oct 20. PMID: 30341914.

[12]. Hayes DF, Schott AF. Personalized Medicine: Genomics Trials in Oncology. Trans Am Clin Climatol Assoc. 2015;126:133-43. PMID: 26330667; PMCID: PMC4530676.

[13]. Jamieson NB, Chang DK, Biankin AV. Cancer Genetics and Implications for Clinical Management. Surg Clin North Am. 2015 Oct;95(5):919-34. doi: 10.1016/j.suc.2015.05.003. Epub 2015 Jun 23. PMID: 26315514.

[14]. Krieghoff-Henning E, Folkerts J, Penzkofer A, Weg-Remers S. Cancer – an overview. Med Monatsschr Pharm. 2017 Feb;40(2):48-54. English, German. PMID: 29952494.

[15]. Lewandowska AM, Rudzki M, Rudzki S, Lewandowski T, Laskowska B. Environmental risk factors for cancer - review paper. Ann Agric Environ Med. 2019 Mar 22;26(1):1-7. doi: 10.26444/aaem/94299. Epub 2018 Oct 17. PMID: 30922021.

[16]. Li KW, Liang YY, Wang Q, Li Y, Zhou SJ, Wei HC, Zhou CZ, Wan XH. Brucea javanica: A review on anticancer of its pharmacological properties and clinical researches. Phytomedicine. 2021 Jun;86:153560. doi: 10.1016/j.phymed.2021.153560. Epub 2021 Mar 28. PMID: 33858739.

[17]. Seger C, Salzmann L. After another decade: LC-MS/MS became routine in clinical diagnostics. Clin Biochem. 2020 Aug;82:2-11. doi:

10.1016/j.clinbiochem.2020.03.004. Epub 2020 Mar 15. PMID: 32188572.

[18]. Stout NL, Baima J, Swisher AK, Winters-Stone KM, Welsh J. A Systematic Review of Exercise Systematic Reviews in the Cancer Literature (2005-2017). PM R. 2017 Sep;9(9S2):S347-S384. doi: 10.1016/j.pmrj.2017.07.074. PMID: 28942909; PMCID: PMC5679711.

[19]. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021 May;71(3):209-249. doi: 10.3322/caac.21660. Epub 2021 Feb 4. PMID: 33538338.

[20]. Suwattanasophon C, Mistlberger-Reiner A, Alberdi-Cedeño J, Pignitter M, Somoza V, König J, Lamtha T, Wanaragthai P, Kiriwan D, Choowongkomon K. Identification of the Brucea javanica Constituent Brusatol as a EGFR-Tyrosine Kinase Inhibitor in a Cell-Free Assay. ACS Omega. 2023 Jul 28;8(31):28543-28552. doi: 10.1021/acsomega.3c02931. PMID: 37576644; PMCID: PMC10413460.

[21]. Szymonowicz KA, Chen J. Biological and clinical aspects of HPV-related cancers. Cancer Biol Med. 2020 Nov 15;17(4):864-878. doi: 10.20892/j.issn.2095-3941.2020.0370. Epub 2020 Dec 15. PMID: 33299640; PMCID: PMC7721094.

[22]. Wang X, Li Y, Mou Y, Yue Z, Zhang H, Li R, Sun H. Ethanol Extract from Brucea Javanica Seed Inhibits Angiogenesis Mediated by Platelet Derived Growth Factor Receptor-beta. Int J Med Sci. 2018 Oct 20;15(13):1517-1521. doi: 10.7150/ijms.28337. PMID: 30443173; PMCID: PMC6216067.

[23]. Wang X, Li Y, Mou Y, Yue Z, Zhang H, Li R, Sun H. Ethanol Extract from Brucea Javanica Seed Inhibits Angiogenesis Mediated by Platelet Derived Growth Factor Receptor-beta. Int J Med Sci. 2018 Oct 20;15(13):1517-1521. doi: 10.7150/ijms.28337. PMID: 30443173; PMCID: PMC6216067.

[24]. Wang X, Wang H, Cao L, Wu J, Lu T, Li S, Li J. Efficacy and Safety of Brucea javanica Oil Emulsion Injection in the Treatment of Gastric Cancer: A Systematic Review and Meta-Analysis. Front Nutr. 2021 Dec 9;8:784164. doi: 10.3389/fnut.2021.784164. PMID: 34957186; PMCID: PMC8696120.

[25]. Yan Z, Guo GF, Zhang B. Research of Brucea javanica against cancer. Chin J Integr Med. 2017 Feb;23(2):153-160. doi: 10.1007/s11655-016-2501-6. Epub 2016 Apr 4. PMID: 27041332.

[26]. Ye L, Zhao JF, Wang YM, Chen WH, Qian S, Zhou ZG, Xu M. Brucea javanica oil emulsion suppresses tumor growth in human cervical cancer cells through inhibition of the E6 oncogene and induction of apoptosis. Transl Cancer Res. 2020 Feb;9(2):918-929. doi: 10.21037/tcr.2019.12.62. PMID: 35117437; PMCID: PMC8797272.

[27]. Ye QM, Bai LL, Hu SZ, Tian HY, Ruan LJ, Tan YF, Hu LP, Ye WC, Zhang DM, Jiang RW. Isolation, chemotaxonomic significance and cytotoxic effects of quassinoids from Brucea javanica. Fitoterapia. 2015 Sep;105:66-72. doi: 10.1016/j.fitote.2015.06.004. Epub 2015 Jun 9. PMID: 26071073.

[28]. Yu XQ, Shang XY, Huang XX, Yao GD, Song SJ. Brusatol: A potential anti-tumor quassinoid from Brucea javanica. Chin Herb Med. 2020 Aug 19;12(4):359-366. doi: 10.1016/j.chmed.2020.05.007. PMID: 36120179; PMCID: PMC9476775.

[29]. Yuniarti L, Mustofa M, Aryandono T, Haryana SM. Synergistic Action of 1,2-Epoxy-3 (3- (3,4-dimethoxyphenyl)- 4H-1-benzopiyran-4-on) Propane with Doxorubicin and Cisplatin through Increasing of p53, TIMP-3, and MicroRNA-34a in Cervical Cancer Cell Line (HeLa). Asian Pac J Cancer Prev. 2018 Oct 26;19(10):2955-2962. doi: 10.22034/APJCP.2018.19.10.2955. PMID: 30362332; PMCID: PMC6291055.

[30]. Zhan Y, Tan T, Qian K, Yang S, Feng Y, Wen Q. Quassinoids from seeds of Brucea Javanica and their anticomplement activities. Nat Prod Res. 2020 Apr;34(8):1186-1191. doi: 10.1080/14786419.2018.1550764. Epub 2019 Jan 8. PMID: 30618292.

[31]. Zhang C, Liu J, Xu D, Zhang T, Hu W, Feng Z. Gain-of-function mutant p53 in cancer progression and therapy. J Mol

Cell Biol. 2020 Sep 1;12(9):674-687. doi: 10.1093/jmcb/mjaa040. PMID: 32722796; PMCID: PMC7749743.

[32]. Zhang J, Xu HX, Dou YX, Huang QH, Xian YF, Lin ZX. Major Constituents From Brucea javanica and Their Pharmacological Actions. Front Pharmacol. 2022 Mar 18;13:853119. doi: 10.3389/fphar.2022.853119. PMID: 35370639; PMCID: PMC8971814.

[33]. Zhao L, Li C, Zhang Y, Wen Q, Ren D. Phytochemical and biological activities of an anticancer plant medicine: Brucea javanica. Anticancer Agents Med Chem. 2014 Mar;14(3):440-58. doi: 10.2174/18715206113136660336. PMID: 24066797.

[34]. Zhu G, Pan C, Bei JX, Li B, Liang C, Xu Y, Fu X. Mutant p53 in Cancer Progression and Targeted Therapies. Front Oncol. 2020 Nov 6;10:595187. doi: 10.3389/fonc.2020.595187. PMID: 33240819; PMCID: PMC7677253.