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Anticancer effect of aromatic isoniazid derivatives in human gastric adenocarcinoma cells

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ABSTRACT

Current treatments for stomach cancer are often effective in curing cancer. However, these treatments can also have significant side effects, and they may not be effective in all cases. Hence synthetic compounds exhibit promise as potential agents for cancer treatment. In a previous study, we identified (*E*)-N′- (2,3,4-trihydroxybenzylidene) isonicotinohydrazide (ITHB4) as a novel antimycobacterial derivative of isoniazid with cytotoxic effects on the MCF-7 breast cancer cell line. This led us to investigate the potential anti-cancer properties of ITHB4 against adenocarcinoma gastric (AGS) cell line. The cytotoxic effect of ITHB4 has been determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and further confirmed for anticancer properties by means of apoptosis, reactive oxygen species (ROS), nuclear fragmentation, lactate dehydrogenase (LDH), caspases, cytokines and morphological including phenotypic changes of cells assay. The ITHB4 demonstrated a lower IC_{50} in inhibiting growth of AGS cells at 24 h compared to 48 and 72 h. ITHB4 has also shown no toxicity human immune cells. Treatment of ITHB4 against AGS for 24 h eventually lead to formation of early apoptotic AGS cells, reduced mitochondrial membrane potential, nuclear condensation, and nuclear fragmentation lastly increased in ROS levels together with the release of LDH, and secretion of caspases. The altered cytokine profile in ITHB4 treated AGS hints at the possibility that ITHB4 may possess anti-tumor and anti-inflammatory properties. Our results in this study demonstrate that ITHB4 has almost similar chemotherapeutic properties against gastric adenocarcinoma cells compared to breast cancer cell. This is suggesting that the anticancer capabilities of this compound should be *in vivo* and clinically assessed.

1. Introduction

Stomach cancer is the fourth-leading cause of cancer-related death worldwide and ranks fifth in terms of frequency (Ilic and Ilic, 2022). Reports from the World Health Organization (WHO) has stated that there was >800,000 deaths from stomach cancer worldwide in 2020, out of an expected 1.1 million new cases every year (Lin et al., 2021). Stomach cancer continues to pose a significant global health burden, particularly in regions such as Eastern Asia including countries such as China, Japan, and Korea, where it remains a major cause of cancer mortality (Pourhoseingholi et al., 2015). Dietary practices, *Helicobacter pylori* infection, and genetic predisposition are a few of the factors that contribute to the increased incidence rates in these communities (Rueda-Robles et al., 2021).

Although treatments such as surgery, chemotherapy, radiation therapy, immunotherapy and targeted therapy are often used but their efficacy may be constrained in some circumstances (Debela et al., 2021). To enhance outcomes for patients, new therapeutic agents are required.

Abbreviations: ROS, Reactive oxygen species; ITHB4, *(E)-N′*-(2,3,4-trihydroxybenzylidene)isonicotinohydrazide; PBMC, Peripheral blood mononuclear cell; IC_{50}, The half maximal inhibitory concentration; IL, Interleukin; AGS, Adenocarcinoma gastric; LDH, Lactate dehydrogenase.

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Consequently, natural and synthetic compounds have shown promise as potential anticancer agents (Lee et al., 2020). For an instance, the HMGB1/VEGF-D signaling pathway responsible for development and metastasis of tumors was blocked by naturally occurring curcumin, leading to reduced proliferation of gastric cancer cells (Da et al., 2019). Similarly, synthetic compound such as docetaxel, oxaliplatin, and capecitabine (DOC) has been used as a standard treatment for metastatic gastric cancer (Di Lauro et al., 2014). It is a well-tolerated regimen with good efficacy. On the other hand, capecitabine alone is an antimetabolite that interferes with the growth of cancer cells by inhibiting their deoxyribonucleic acid (DNA) synthesis. It is typically prescribed for advanced or metastatic gastric cancer or in cases where surgery is not possible. Meanwhile, trastuzumab (monoclonal antibody), another approved inhibitory target therapy which has demonstrated efficacy in the treatment of HER2-positive advanced gastric cancer. Utilization of herceptin together with other chemotherapy (cisplatin and fluoropyrimidine) has significantly improved the overall survival HER2-positive gastric cancer patients. Another targeted therapy used in the treatment of advanced gastric cancer is ramu-cirumab. It is a monoclonal antibody that specifically targets and inhibits the vascular endothelial growth factor receptor (VEGFR), which plays a role in angiogenesis (formation of new blood vessels) (Di Lauro et al., 2014). The choice between natural and synthetic compounds as potential anticancer agents however depends on various factors, including the specific cancer type, desired mechanisms of action, safety profiles, and availability of resources for drug development (Newman and Cragg, 2020).

In our earlier studies, we have proven the primary use of synthetic isoniazid derivative (*E*)-N′-(2,3,4-trihydroxybenzylidene)isonicotinohydrazide (ITHB4) which primarily involves inhibiting mycolic acid synthesis, an essential component of the mycobacterial cell wall. Recently, we have also demonstrated the potential anti-proliferative effects of our synthetic isoniazid derivative (E)-N′-(2,3,4-trihydroxybenzylidene)isonicotinohydrazide (ITHB4) against breast (MCF-7) (Barathan et al., 2021) and colon (HCT-116) cancer cell lines (unpublished data) by suppressing the growth of tumor cells by targeting specific cellular pathways involved in cancer progression, however, the specific structural features of isoniazid that contribute to its potential anti-proliferative effects in cancer cells are not well characterized till now. To the best of our knowledge, to date, there have been limited reports concerning the effects of isoniazid derivative on human breast and colon. Therefore, further studies are required in order to evaluate the biological function and roles of isoniazid derivative in other cancer cell lines.

This has further prompted us to test the possible anticancer effect of ITHB4 against adenocarcinoma gastric (AGS) cell line. The anticancer mechanisms of ITHB4 will be studied using various approaches that widely used in toxicology studies.

2. Materials and methods

2.1. Synthesis and purification of the test compounds

The ITHB4 was prepared according to the procedure reported in our previous work (Barathan et al., 2021). The structures of the isoniazid analogues and the reaction pathway are given in Fig. 1 accordingly. The isoniazid derivative was prepared following the procedure by Lourenço et al., 2008 (Naveenkumar et al., 2010). The title compound was prepared by the reaction between 2,3,4-trihydroxybenzaldehyde (1.0 eq) with isoniazid (1.0 eq) in ethanol/water. After stirring for 1–3 h at room temperature, the resulting mixture was concentrated under reduced pressure. The residue, purified by washing with cold ethanol and ethyl ether, afforded the pure derivative. The brown-colored single crystals suitable for X-ray analysis were obtained by recrystallization with ethanol.

The title isoniazid derivative comprises of ITHB4 molecule and two water molecules of crystallization. The Schiff base molecule exists in an

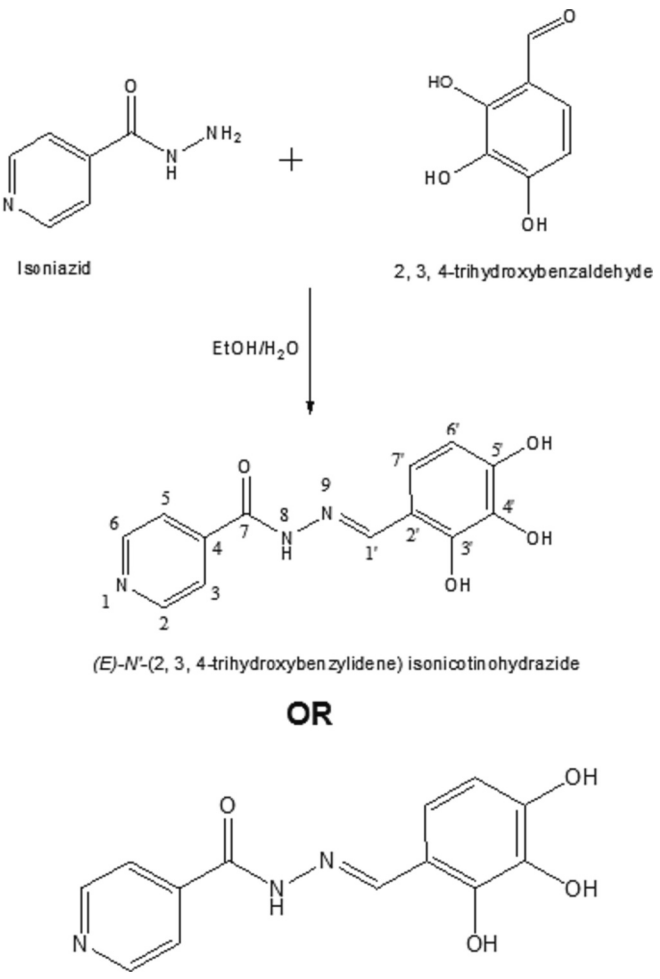

Fig. 1. The structures and synthetic route used for the preparation of *(E)-N′*-(2, 3, 4-trihydroxybenzylidene) isonicotinohydrazide (ITHB4).

E configuration with respect to the acyclic C7═N3 bond [C7═N3 = 1.2921 (Barathan et al., 2021) Å; torsion angle N2—N3—C7—C8 = 178.85 (Da et al., 2019)°]. An intramolecular O2—H1O2···N3 hydrogen bond generates a six-membered ring, producing an *S (Lee et al., 2020)* ring motifs (Sivajeyanthi et al., 2018). The pyridine ring with atom sequence C1/C2/N1/C3/C4/C5 is essentially planar, with a maximum deviation of 0.0119 (Di Lauro et al., 2014) Å at atom C5. There is a slight inclination between the pyridine and benzene rings, as indicated by the dihedral angle formed of 7.30 (Rueda-Robles et al., 2021)°. All bond lengths and angles are consistent to those observed in closely related isoniazid structures (Naveenkumar et al., 2010).

In the crystal packing, water molecules play an extensive part in forming the hydrogen-bonded structure. Neighboring molecules are linked into two-dimensional arrays parallel to the (101) plane by intermolecular O3—H1O3···N1, O4—H1O4···O1W, N2—H1N2···O2W, O1W—H1W1···O1, O2W—H2W2···O4 and C7—H7A···O2W hydrogen bonds. These arrays are further interconnected by intermolecular O1W—H2W1···O2W, O2W—H1W2···O2 and C4—H4A···O1 hydrogen bonds into a three-dimensional extended structure. Weak intermolecular π–π aromatic stacking interactions involving the pyridine and benzene rings [*Cg*1···*Cg*2 = 3.5627 (Debela et al., 2021) Å, symmetry code: -x + 2, −y + 1, −z + 2] stabilizing the crystal structure (Table 1).

ITHB4 spectral data: ^{1}HNMR (DMSO-d_6,400MHz) *δ*: 12.18 [s,1H, H-8(NH)], 11.29[s,1H, H-3(OH)], 9.58[s,1H, H-4(OH)], 8.80–8.78 [dd, 2H, H2 and H6 of pyridine ring; *J* = 5.92 Hz, 2.2 Hz], 8.56[s,1H, H-5(OH)], 8.49(s, 1H, H-1′),7.84–7.82 [dd, 2H, H3 and H5 of pyridine ring; *J* = 5.88 Hz, 2.2 Hz], 6.85–6.82 [d, 1H, H-6(Ar—H); *J* = 11.4 Hz], 6.42–6.39

Table 1
The following table summarizes the key structural features of ITHB4.

Feature	Value
Molecular formula	C13H12N2O4
Molecular weight	246.24 g/mol
Structure	Schiff base
Configuration	E
Hydrogen bonds	Intramolecular and intermolecular
π–π stacking	Weak

[d, 1H, H-7(Ar—H); J = 11.28 Hz]. ^{13}CNMR (DMSO-d_6, 75 MHz) δ; 162.50(C-7), 152.36(C-3′ and C-5′), 151.14(C2 and C6), 149.97(C-4′), 148.58(C-1′), 141.05(C-4), 133.70(C-7′), 122.23 (C3 and C5), 111.81 (C-2′), 108.76(C-6′). MS-ESI m/z-[M + Na]$^+$ 296.2. Anal. Calcd for $C_{13}H_{11}N_3O_4$ (273.07) C, 57.14; H, 4.06; N, 15.38; found C, 57.11; H, 4.09; N, 15.36.

2.2. Chemicals and reagents

All chemicals and solvents used were of the highest analytic grade available. Cell culture supplies and media, fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids, penicillin/streptomycin and DMSO were obtained from Sigma Aldrich (Malaysia).

2.3. Cell culture

Human adenocarcinoma gastric (AGS) cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). MCF10A cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% of fetal bovine serum (FBS) (Sigma), 2 mM L-glutamine, 100 units/ML of penicillin and 100 μg/mL of streptomycin. The cells were propagated in a 37°C humidified incubator with 5% CO2.

2.4. Peripheral blood mononuclear cells (PBMCs) isolation and maintenance

Isolation of PBMCs from buffy coats was carried out as previously described (Barathan et al., 2017). Briefly, PBMCs were separated by density-gradient centrifugation over Ficoll-PaqueTM (Amersham Pharmacia, Piscataway, NJ, USA) from 10 ml of whole blood per healthy control in BD Vacutainer heparin lithium tubes (BD Biosciences, Franklin Lakes, NJ, USA). The cells were cryopreserved in liquid nitrogen after being resuspended in a freezing media (10% DMSO in 90% foetal bovine serum (FBS); Gibco, Carlsbad, CA, USA). This portion of the study was completed after the Medical Ethics Committee (MEC) of the University of Malaya Medical Centre approved the protocols (ref. no. 938.42) for ethical considerations. Prior to research enrolment, all subjects provided written consent.

2.5. Primary cell culture

A RPMI1640 medium supplemented with HEPES buffer (25 mM), L-glutamine (2 mM), penicillin (100U/ml), streptomycin (100 Lg/ml), sodium pyruvate (1 mM), and gentamicin (5 Lg/mL) (all purchased from Life Technologies, Victoria, Australia) was used to cultivate PBMCs. This medium also contained 10% of FBS (Gibco). To raise the cell growth, IL-2 was given to the PBMC culture. These cells were multiplied for 24 h in a 37 °C humid incubator with 5% CO2.

2.6. In vitro cytotoxicity assay of ITHB4 on AGS cells

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the half maximum inhibitory concentration (IC50) of ITHB4 on AGS cells. A 96-well plate with the cells was plated in triplicates at a density of 2 × 10^5 cells per mL in 100 μL of culture media. Prior to treatments, working stocks from the ITHB4 were made in a complete culture medium by dissolving it in DMSO at a concentration of 1 mg/mL. The following working stock concentrations were used to prepare them: 200, 175, 150, 125, 100, 75, 50, and 25 μg/mL. The cells in the 96-well plates received all successive dilutions and one set AGS cells was left untreated which will serve as the experiment's negative control. The vitality of the cells was tested by adding 20 μL of MTT, 5 mg/mL, to the cells in the dark after the treatment point 24 h of incubation. The cells were further incubated for 4 h followed by the removal of all the medium, 100 μL of DMSO was given to the cells in order to dissolve the formazan crystals. A microplate reader was then used to read the absorbance at a wavelength of 570 nm. The IC50 was used to calculate ITHB4's capacity to impede cell growth.

2.7. In vitro cytotoxicity assay of the ITHB4 on PBMCs culture

To assess the potential cytotoxic effects of ITHB4 on immune cells, testing ITHB4 on PBMCs was used as another valuable approach with help of MTT assay since toxicity of ITHB4 on normal gastric cells was not available in the study. PBMCs were seeded at a density of 1 × 10^5 cells per well in a 96-well plate to investigate the IC50 toxicity of ITHB4 on human immune cells. The cells were then incubated for overnight. The IC50 ITHB4 were added to the culture the following day. Prior to ending the experiment by adding DMSO, MTT was administered to the cells for 4 h while the cells were kept in the dark. Using a microplate reader, the absorbance was measured at a wavelength of 570 nm.

2.8. Morphological changes and phenotypic assay

To determine mitochondrial membrane potential, the tetramethylrhodamine ethyl ester (TMRE) dye (maximal fluorescence wavelength: 595 nm) in the Multi-Parameter Apoptosis Assay Kit (Cayman Chemical, MI, USA) was used. Meanwhile, FITC-conjugated Annexin V was used as a probe for phosphatidylserine (PS) on the outer membrane of apoptotic cells, and Hoechst Dye to demonstrate nuclear morphology of the cells. Briefly, Annexin V, Hoechst Dye and TMRE staining was performed as follows. AGS cell was grown in 24 well plate with 2× 10^5 cells per mL in 500 μL for overnight in incubator at 37 °C followed by ITHB4 treatment. Cells were <80% confluent at the time of staining. The RPMI was removed from each well without disturbing cell layer. Subsequently, 250 μL of the staining solution was added to each dish. The cells were incubated at room temperature in the dark for 15 min. The staining solution was carefully removed, and 500 μL of PBS (pH 7.4) was added. The cells was visualized in fluorescence microscope (Carl Zeiss Microscopy GmbH, Gottingen, Germany) using the filter sets. To test Annexin V FITC staining, Annexin V FITC reagent was diluted in 1:100 in 1× Binding Buffer. The PBS was aspirated from the wells and about 250 μl of the diluted Annexin V FITC was added. The cells was then incubated in the dark for 15 min. The staining solution was carefully removed, and 500 μL of PBS (pH 7.4) was added. The cells was visualized in fluorescence microscope using the filter sets.

2.9. Apoptosis assay

Determination of apoptotic cells in the AGS cell culture after the treatment of IC_{50} of ITHB4 was performed using BD Pharmingen FITC Annexin V Apoptosis Detection Kit I (BD Biosciences). The treated and untreated cells were harvested and pelleted by centrifuging at 600 ×g for 5 min at 4°C. The intact cells (FITC−/PI−), early apoptotic cells (FITC+/PI−) and dead cells (FITC+/PI+) were acquired on a BD FACSCanto II (BD Biosciences, San Jose, CA, USA) flow cytometry using a BD Pharmingen FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer's instructions. The samples were analyzed using the FACSDiva software (BD Biosciences).

2.10. Reactive oxygen species (ROS) analysis

Determination of ROS levels of IC_{50} of ITHB4 on AGS cells culture was performed according to the manufacturer's instructions (Mitosciences, DCFDA Cellular ROS Detection Assay Kit; Mitoscience/Abcam, Cambridge, UK. The kit makes use of the fluorogenic dye 2′,7′-dichlorofluorescein diacetate (DCFDA), a cell permeant reagent that evaluates intracellular ROS activity for hydroxyl, peroxyl, and any other ROS. DCFDA is diffused into the cell, whereupon cellular esterases deacetylate it to a non-fluorescent substance, which intracellular ROS then oxidise into DCF. Due to its high fluorescence, DCF can be found *via* fluorescence spectroscopy at 495 and 529 nm (for excitation and emission, respectively). The AGS cells were grown in 100 μL of culture media in the dark in a 96-well plate overnight. The cells were later exposed to the IC50 of ITHB4 on the day of the experiment, and they were then incubated for 24 h. The cells were then exposed to 25 μM DCFH-DA the following day for 45 min in the dark. Following the removal of DCFH-DA, cells were washed with a 1 × buffer solution. Thermo-Scientific's Varioskan Flash micro-plate reader was used to measure and record the fluorescence coming from the cells of each well at 485 nm (excitation) and 535 nm (emission).

2.11. DNA fragmentation assay

The DNA fragmentation experiment, in which AGS cells were treated appropriately with IC50 of ITHB4 for 24 h, was carried out to investigate the nuclear DNA degradation effect of IC50 of ITHB4 on the cancer cells. The cells were then removed and centrifuged at 4 °C for 5 min at 1800 rpm. Following the manufacturer's instructions, the DNA was extracted from the resultant pellets using a commercial QIAamp DNA mini kit (Qiagen, Valencia, CA, USA). Each sample's DNA content was calculated, and 10 μg of the DNA were electrophoresed on an agarose gel at a 1.2% (*w/v*) concentration. Later, the gel was imaged using a Gel Doc XR gel documentation system (Bio-Rad, Hemel) and visualized using a UV light source (Bio-Rad, Hemel Hempstead, UK).

2.12. Lactate dehydrogenase (LDH) assay

The impact of ITHB4 on the release of lactate dehydrogenase (LDH) from AGS cells was assessed using a lactate dehydrogenase activity assay kit from BioVision, CA, USA. To summarize the procedure, the cells were exposed to the IC50 concentrations of ITHB4 for 24 h, after which they were collected and rinsed with cold saline buffer. The cells were then homogenized in a cold assay buffer, and the resulting supernatant was preserved for subsequent analysis. Prior to analyzing the samples, a standard curve for nicotinamide adenine dinucleotide (NADH) was established. The reaction mixture, consisting of LDH assay buffer and LDH substrate mix, was added to wells containing standards, samples, and positive control samples. After thorough mixing, the optical density (OD) at 450 nm was promptly measured using a microplate reader from Thermo Fisher Scientific, Waltham, MA, USA. The OD measurements were conducted in a kinetic mode, with readings taken every 2 to 3 min for a duration of at least 30 to 60 min at 37°C while protecting the samples from light. Furthermore, untreated cells were also evaluated to verify the actual efficacy of ITHB4.

2.13. Caspases assay

The activity of caspases 3, 8, and 9 was evaluated using caspase kits sourced from Elabscience Biotechnology Co. Ltd., based in TX, USA. These kits were identified by their respective catalog numbers: *E*-CK-A311, E-CK-A312, and E-CK-A313. To conduct the assay, AGS cells were treated with the appropriate IC50 concentration of ITHB4 for a duration of 24 h. Subsequently, the cells were lysed with lysis buffer while maintained on ice, and they were then combined with colorimetric substrates specific to the individual caspases. The resulting samples were incubated at 37 °C for a period of 4 h, after which the optical density (OD) value was determined using a microplate reader. Caspase activity was reported relative to the control or untreated samples.

2.14. Flow cytometry analysis

The supernatant obtained from cells treated with ITHB4 and untreated cells was collected and subsequently filtered through a 0.22-μm steriflip filter manufactured by Millipore, Billerica, MA, USA. To assess the cytokines released from each sample, a BD Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine kit from BD Biosciences was employed, following the provided manufacturer's guidelines. This assay was conducted in triplicate, with mock untreated cells serving as the control. The kit enabled the detection of various cytokines, including IL-2, IFN-γ, TNF-α, IL-6, IL-10, IL-4, and IL-17 A. Data from the samples were acquired using a FACSCanto II flow cytometer from BD Biosciences and subsequently analyzed utilizing the FCAP array software, also provided by BD Biosciences.

2.15. Statistical analysis

Data were presented as the mean ± standard deviation (SD) and *n* refers to the number of independent experiments. Levels of significance for comparisons between two or more independent samples were determined using a two-tailed unpaired Student's *t*-test. Differences were considered significant at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Groups were compared by one-way or two-way analysis of variance with Bonferroni's *post hoc* test applied to explore significance.

3. Results

3.1. ITHB4 decreases survival of AGS cells

The study conducted an MTT assay to evaluate the cytotoxic effects of ITHB4 on AGS gastric cancer cells. The results indicate that ITHB4 has the ability to reduce the proliferation of this cancer cells. This is a significant finding as it suggests that ITHB4 may have anti-cancer properties (Fig. 2). An interesting observation is the impact of the treatment duration on ITHB4's effectiveness. A 24-h treatment with ITHB4 resulted in a much lower IC50 compared to longer durations (48 and 72 h). This indicates that a shorter exposure to ITHB4 is more efficient in inhibiting cancer cell growth indicating that ITHB4 is effective at 129 μg/mL for 24 h. This is a positive outcome as it suggests that the agent has high potency against cancer cells and may require lower doses in

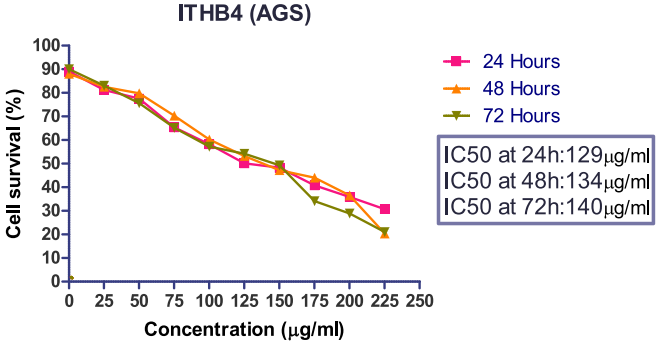

Fig. 2. Effect of ITHB4 on survival of AGS cell line. The viability of AGS cells upon exposure to ITHB4 has been performed *via* MTT assay. ITHB4 was found to gradually inhibit the growth of AGS cells at all treatment points however lower inhibitory concentration of ITHB4 was needed to inhibit 50% growth of AGS cells at 24 h compared to 48 and 72 h. Data are mean ± S.E.M. of $n = 3$ independent experiments carried out in triplicates. Statistically significant differences are labeled as $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with cells using Student's paired *t*-test.

potential clinical applications, potentially reducing the risk of side effects or toxicity associated with higher drug concentrations. Lower IC50 values are desirable in the development of anticancer treatments, as they indicate the potential for more effective and efficient therapies. Hence, this IC50 (129 μg/mL) was used for the other downstream experiments. The study also found that at the highest tested concentration of ITHB4 (225 μg/mL), a 48-h treatment led to the most significant reduction in cancer cells. While this suggests a concentration-dependent effect, it's worth noting that even at this high concentration, not all cancer cells were completely eliminated. This highlights the challenge of achieving complete cancer cell eradication. An encouraging aspect of the study is that the IC50 of ITHB4 did not exhibit cytotoxicity against peripheral blood mononuclear cells (PBMCs). This suggests that ITHB4 may have some selectivity in targeting cancer cells without harming normal immune cells. Selectivity is a crucial factor in cancer therapy to minimize side effects. While these findings are promising, further research is necessary. In particular, *in vivo* studies are needed to confirm the compound's effectiveness and safety. Additionally, researchers should investigate the molecular mechanisms by which ITHB4 exerts its cytotoxic effects on cancer cells.

3.2. ITHB4 alerts morphological and phenotypic characteristics of AGS cells

The data presented in Fig. 4 provides valuable insights into the effects of ITHB4 (129 μg/mL) on AGS cancer cells. Annexin V fluorescence intensity shows that untreated AGS cells emitted low, fluorescence intensity, indicating that the cells were alive (Fig. 3.1 A). Meanwhile, cell morphology showed large and abundant nuclei since high blue fluorescence intensity was observed (Fig. 3.1B), in addition high number of mitochondria was observed in untreated AGS cell (Fig. 3.1C). In treated AGS cell, Annexin V results confirm that the observed cancer cell killing effect elicited by ITHB4 that is caused by apoptosis (Fig. 3.2D). The low blue fluorescence intensity showed the condensed nuclei in treated AGS cell line (Fig. 3.2E). The mitochondria of treated AGS cells was observed to lose their membrane potential due to apoptosis or metabolic stress, the fluorescent dye diffuses throughout the cell, and the fluorescence intensity decreases markedly was observed in treated AGS cells (Fig. 3.2F). In the untreated AGS cells, low Annexin V fluorescence intensity indicates that these cells are viable and not undergoing apoptosis. This fluorescence marker is commonly used to detect early apoptotic events. In contrast, the treated AGS cells display increased Annexin V fluorescence, indicating that ITHB4 treatment leads to apoptosis in these cells. Apoptosis is a programmed cell death process that often occurs in response to cellular stress. The cell morphology data complements the Annexin V results. Untreated AGS cells exhibit large nuclei and a high blue fluorescence intensity, suggesting healthy and proliferating cells. In contrast, treated AGS cells display condensed nuclei and reduced blue fluorescence intensity. These changes are consistent with the characteristic morphological alterations seen in apoptotic cells. Mitochondria are crucial for cell survival, and changes in their function can be indicative of cellular stress or apoptosis. The high

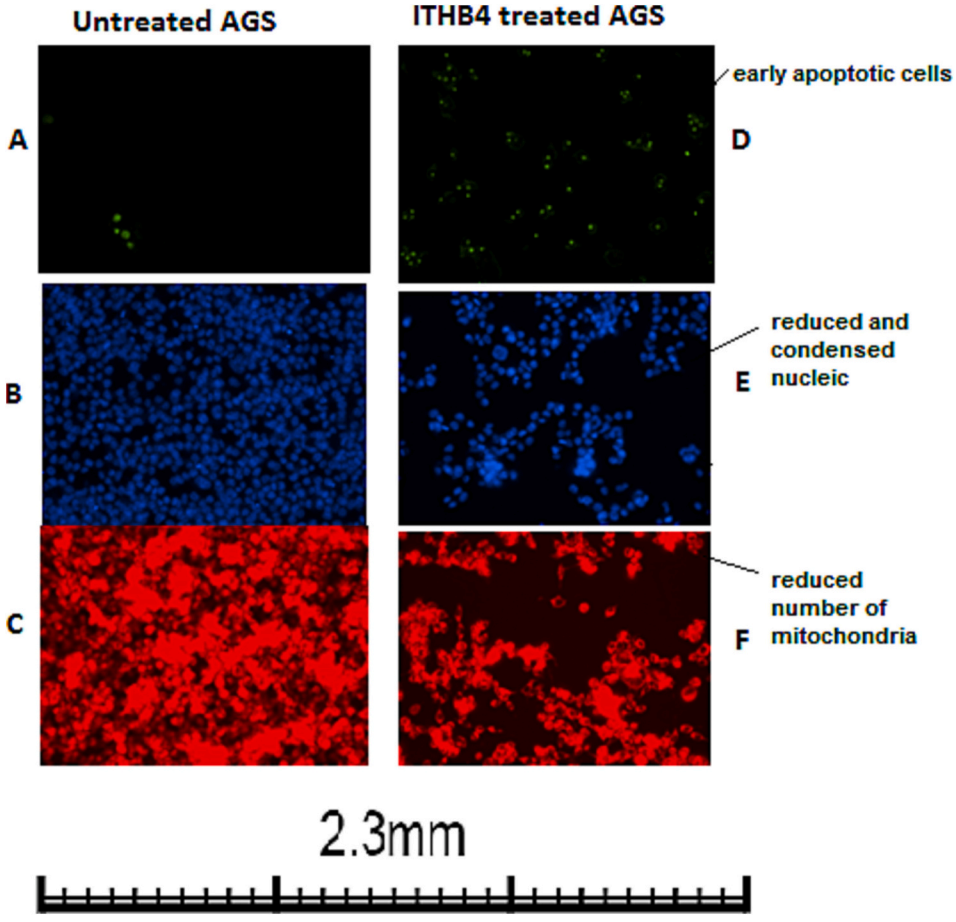

Fig. 3. Effect of ITHB4 on phenotypic features of AGS. The phenotypic and morphological changes of AGS cells upon exposure to ITHB4 has been performed *via* Multi Parameter Apoptosis Kit from Cayman. Fig. 3.1 A shows less number of apoptotic cells, in untreated AGS cell culture, Fig. 3.1B shows healthy cells with no nuclear shrinkage, Fig. 3.1C indicates abundant of healthy mitochondria in untreated AGS cell line, Fig. 3.2D shows increase in number of early apoptotic cells in ITHB4 treated AGS cell culture, Fig. 3.2E displays cell nuclear shrinkage after the treatment of ITHB4 and Fig. 3.2F shows reduced MMP in ITHB4 treated AGS cell culture. Similar cellular morphology was observed in three independent experiments (magnification× 100 and scale bar with 2.3 mm).

number of mitochondria observed in untreated AGS cells suggests robust metabolic activity. However, in treated AGS cells, there is a significant loss of mitochondrial membrane potential, which can be an early sign of apoptosis. The diffusion of a fluorescent dye throughout the cell and the decrease in fluorescence intensity in treated AGS cells indicate mitochondrial dysfunction.

3.3. ITHB4 induces formation of apoptotic cells in AGS cells

This experiment utilized BD Pharmingen FITC Annexin V with 7-AAD to investigate the role of early apoptosis in the anticancer effects of ITHB4 (129 μg/mL) on AGS cell culture. In the untreated AGS cells (Fig. 4.1), a low percentage of early apoptotic cells was observed, indicating that the cells were mostly alive. However, after 24 h of treatment with the IC50 concentration of ITHB4 (Fig. 4.2), there was a significant increase in early apoptosis, as indicated by the presence of BrdU FITC-positive cells. This suggests that ITHB4 effectively induces early apoptosis in AGS cells, a promising outcome for its potential as an anticancer agent. Additionally, natural cell death was observed in both subsets of cells (P1 labeled cells), further supporting the idea that ITHB4 has a cytotoxic effect on AGS cells. These findings provide valuable insights into the mechanism of action of ITHB4 and its potential as a therapeutic agent for gastric adenocarcinoma.

3.4. ITHB4 increases the cellular secretion of ROS in AGS cells

This experiment utilized Mitosciences, DCFDA Cellular ROS Detection Assay to investigate the role of ROS in the anticancer effects of ITHB4 (129 μg/mL) on AGS cell culture. The excessive production of cellular ROS can lead to damage in various cellular components, including proteins, nucleic acids, lipids, membranes, and organelles (Fig. 5). This damage can trigger cell death processes, particularly apoptosis. In the case of ITHB4, its cytotoxic effect may be mediated by the activation of mitochondria-initiated processes. This is suggested by the substantial increase in ROS production observed in cells treated with

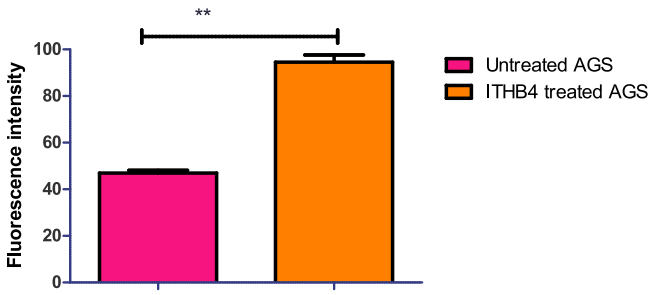

Fig. 5. Effect of ITHB4 on ROS secretion. ROS assay executed by spectrometry-based experiment revealed excessive levels of reactive oxygen species were produced after the treatment of hyperforin, and taxol compared to untreated MDA-MB-231 cell culture. Data are mean ± S.E.M. of $n = 3$ independent experiments carried out in triplicates. Statistically significant differences are labeled as $^{*}P < 0.05$ and $^{**}P < 0.01$, $^{***}P < 0.001$compared with cells using Student's paired *t*-test.

an IC50 concentration of ITHB4, especially when compared to an IC50 of zerumbone. The increase in ROS production is a key indicator of cellular stress and damage, which can ultimately lead to cell death. These findings highlight the potential mechanisms through which ITHB4 exerts its cytotoxic effects on cancer cells.

3.5. ITHB4 activates cell death through DNA fragmentation in AGS cells

This experiment utilized agarose gel electrophoresis to further investigate the anticancer effects of ITHB4 (129 μg/mL) on AGS cell culture. The presence of ladder-like DNA fragments on the agarose gel in (Fig. 6) suggests that the cytotoxic effects of ITHB4 are indeed driven by apoptosis, a regulated form of cell death. During apoptosis, cells undergo a series of orchestrated events, including DNA fragmentation, which ultimately leads to the controlled destruction of the cell. In this context, ITHB4 appears to trigger these apoptotic pathways in AGS cells.

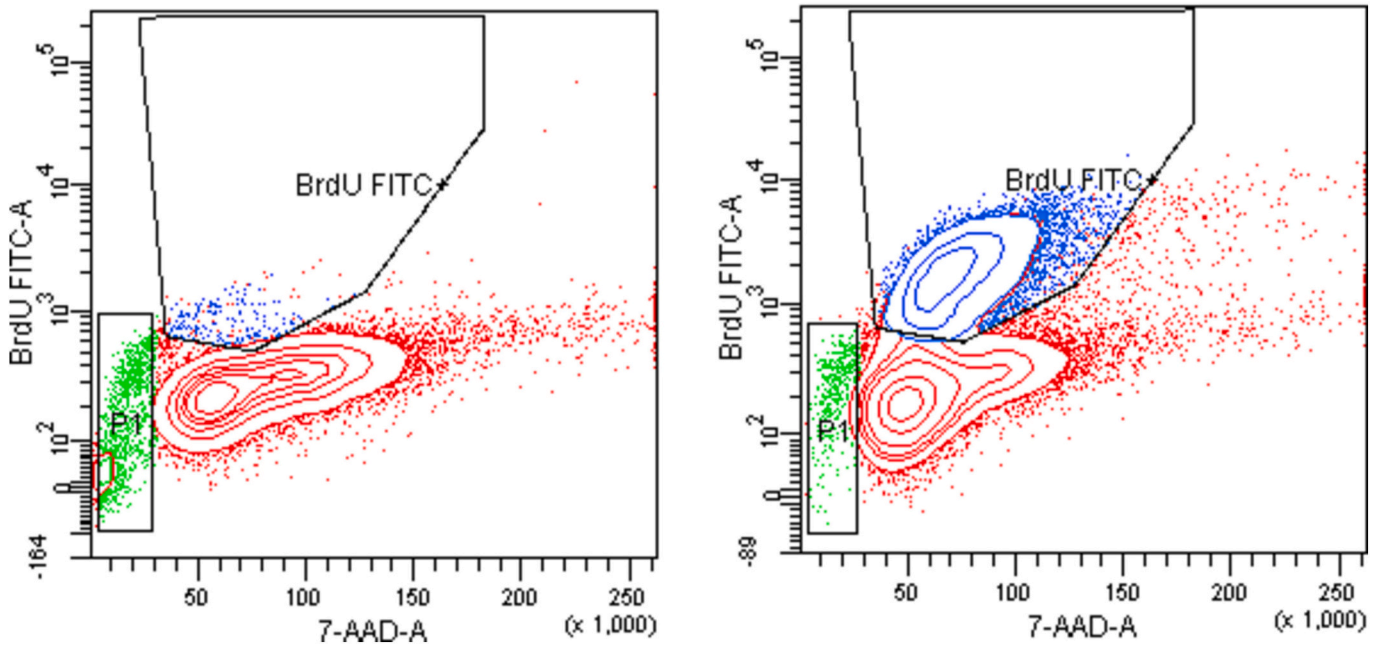

Fig. 4. Effect of ITHB4 on occurrence of early apoptotic cell. Apoptotic cells were identified by employing 7-AAD and BrdU, which demonstrated increase in apoptosis activity after the treatment of ITHB4. Fig. 4.1 shows untreated AGS cell line with a low number of early apoptotic cells in untreated AGS cells (BrdU FITC labeled, blue). Fig. 4.2 shows an increase in apoptosis activity in the AGS cells culture following treatment with ITHB4 ((BrdU FITC labeled, blue) after 24 h. Apoptotic cells are represented by the blue cell population, whereas dividing cells and possibly dead cells are represented by the red cell population. Meanwhile, cell labeled in green are represented the cell undergoing natural cell death with any stimulator. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

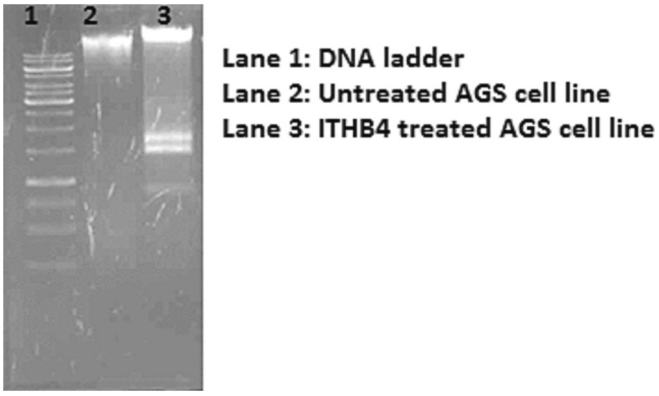

Fig. 6. Effect of ITHB4 on DNA of AGS cell line. DNA fragmentation was observed through the gel electrophoresis. DNA degradation was apparent in ITHB4 treated AGS cells seen in 1.2% agarose gel electrophoresis. Complete and undamaged DNA was seen in untreated AGS cell line compared to treated cells.

3.6. ITHB4 induces cytotoxicity through release of LDH in AGS cells

This experiment utilized a lactate dehydrogenase activity assay kit to further investigate the anticancer effects of ITHB4 (129 μg/mL) on the release of LDH from AGS cell culture. The release of LDH into the extracellular space serves as an indirect marker for cell membrane damage and early cell death. When cell membranes are compromised, LDH leaks out into the surrounding environment. This phenomenon is used as an indicator of cytotoxicity in anticancer research. In this experiment, the findings demonstrated a significant increase in LDH release from AGS cell culture treated with ITHB4 when compared to untreated cells (Fig. 7). This increase in LDH release suggests that ITHB4 has cytotoxic effects on the gastric cancer cells, leading to cell membrane damage and early cell death. These results underscore the potential of ITHB4 as cytotoxic agents in the context of gastric cancer treatment. The detection of LDH release provides valuable insights into the efficacy of these compounds as potential anticancer agents, supporting further investigation and research into their mechanisms of action and clinical applications.

3.7. ITHB4 activates expression of caspases in AGS cells

This experiment utilized a caspase kit *via* spectrophotometrically to further investigate the role of caspases on the anticancer effects of ITHB4 (129 μg/mL) against AGS cell culture. The expression of caspases and the induction of apoptosis play crucial roles in understanding the mechanism of action of potential anticancer agents. Caspases are enzymes involved in apoptosis, acting as key regulators of cell death. In this experiment, the significant activation of caspases 3, 8, and 9 was found in AGS gastric cancer cells treated with ITHB4 compared to the untreated cells (Fig. 8). The findings revealed that ITHB4 treatment led to the activation of caspases 3, 8, and 9 in AGS cells. The detection of caspases can provide deeper insights into the molecular mechanisms through which ITHB4 induces apoptosis.

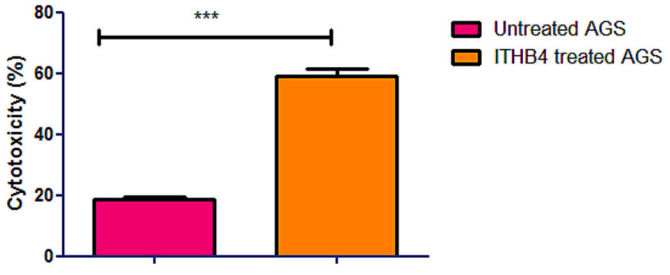

Fig. 7. Effect of ITHB4 on the release of LDH from AGS cell culture. Treated cells were assayed using LDH assay and measured spectrometrically to obtain release of LDH level. Higher cytotoxicity and release of LDH in ITHB4 treated AGS cells was detected. Data are mean ± S.E.M. of $n = 3$ independent experiments carried out in triplicates. Statistically significant differences are labeled as $^{*}P < 0.05$ and $^{**}P < 0.01$, $^{***}P < 0.001$compared with cells using Student's paired *t*-test.

3.8. ITHB4 expresses secretion of cytokines in AGS cells

This experiment utilized flow cytometry approach to measure secretion of cytokines related to the anticancer effects of ITHB4 (129 μg/mL) against AGS cell culture. The expression of proinflammatory cytokines, which are signaling molecules that play a central role in the body's immune response and inflammatory processes. The results showed that ITHB4 treatment of AGS gastric cancer cells had an impact on proinflammatory cytokines, specifically IL-6 compared to untreated AGS cell culture (Fig. 9). The altered expression of proinflammatory cytokines and their potential impact on cancer cells suggest that ITHB4 may have anti-tumor and anti-inflammatory properties.

4. Discussion

There is a need for new anticancer agents because the current treatments are often have many challenges hence leading to treatment which is not effective enough or have too many side effects eventually resulting tumor recurrence and becoming more aggressive (Bayat Mokhtari et al., 2017). In addition, cancer is a complex disease, and there is no one-size-fits-all treatment. The best treatment for each patient will depend on the type of cancer, the stage of the cancer, and the patient's overall health (Krzyszczyk et al., 2018). To overcome this, oncologists may need to increase the dosage of chemotherapeutic drugs, which can increase toxicity and impair the body's immunity (Bracci et al., 2014). Hence, it is hoped that new and more effective anticancer agents will be developed to improve the survival rates for gastric cancer. One of the promising areas of research is utilizing synthetic anticancer agent to kill cancer cells (Bisht and Rayamajhi, 2016). It is made in a laboratory, rather than being found in nature. Synthetic anticancer agents are often more effective and have fewer side effects than natural anticancer agents (Sharifi-Rad et al., 2019).

Previously, we demonstrated the cytotoxic effect of ITHB4 against MCF-7 human breast cancer cells whereby ITHB4 was able to kill the cancer cells and activate secretion of several cytokines responsible inflammation which may result in activation of immune response against cancer cells (Barathan et al., 2021). This compounds based on the isoniazid drug, which has been used to treat tuberculosis (Khan et al., 2019), could be effective against breast cancer (Barathan et al.,

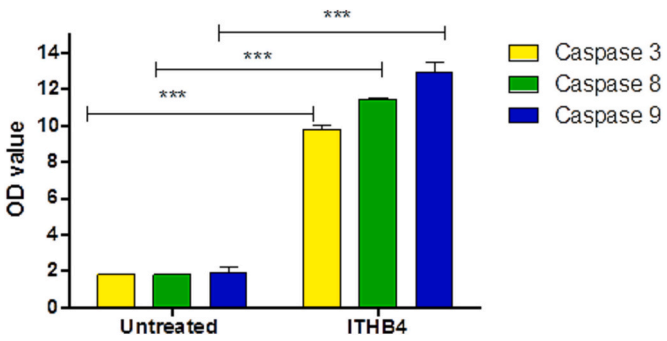

Fig. 8. Effect of ITHB4 on expression of caspases in AGS cell line. Release of caspase 3, 8 and 9 was observed through ELISA. Prominent release of caspase 3, 8 and 9 was apparent in ITHB4 treated AGS cells compared to untreated AGS cell culture. Data are mean ± S.E.M. of $n = 3$ independent experiments carried out in triplicates. Statistically significant differences are labeled as $^{*}P < 0.05$ and $^{**}P < 0.01$, $^{***}P < 0.001$compared with cells using Student's paired *t*-test.

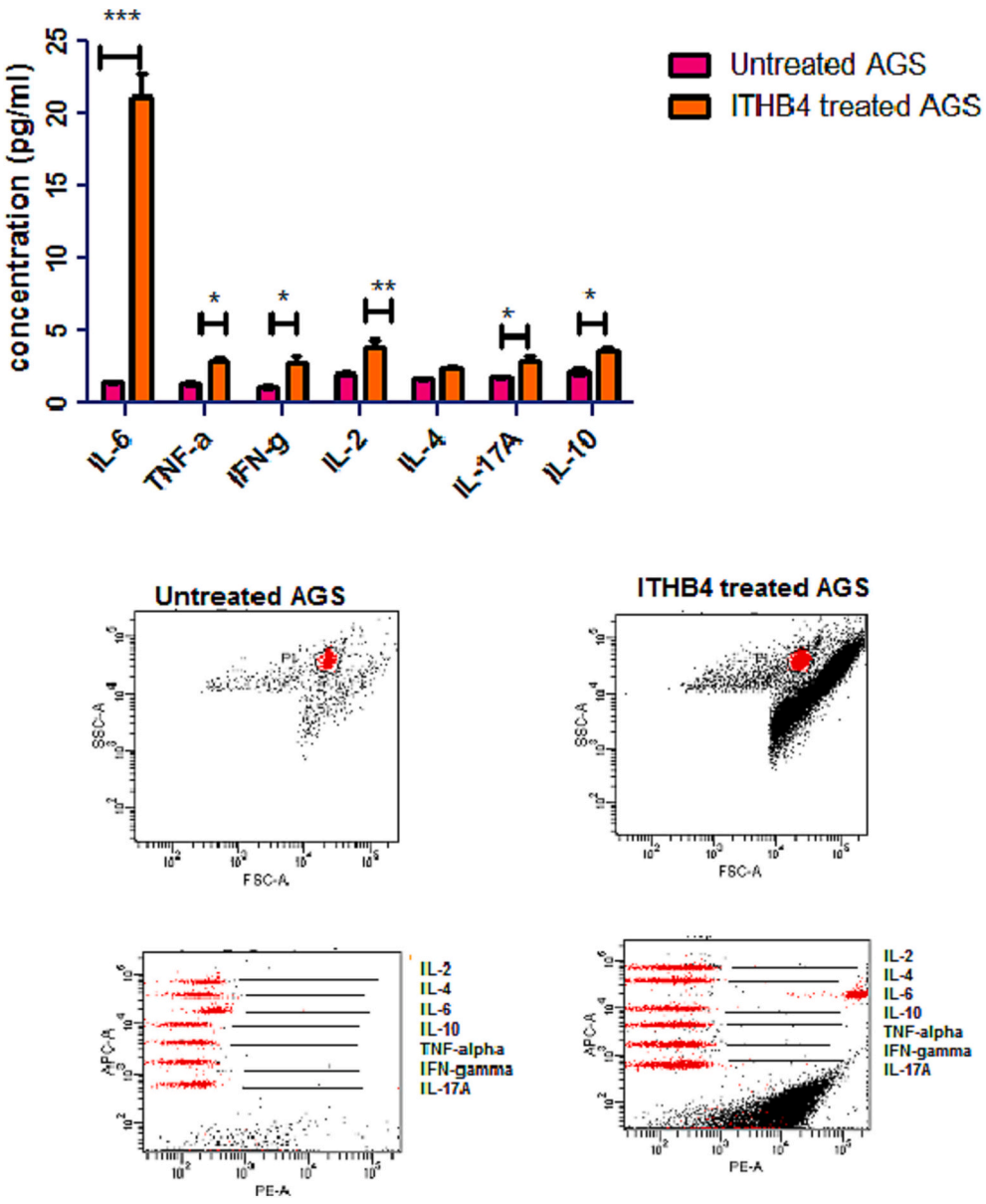

Fig. 9. Expression of pro and anti-inflammatory cytokines levels of AGS cell culture after ITHB4 treatment and untreated cells. Significant expression of IL-6 level was apparent in ITHB4 treated AGS compared to untreated cells. Data are mean ± S.E.M. of n = 3 independent experiments carried out in triplicates. Statistically significant differences are labeled as *P < 0.05 and **P < 0.01, ***P < 0.001compared with cells using Student's paired t-test.

2021). This would be a significant breakthrough in the fight against this deadly disease. Hence, in this new study, we wanted to explore the cancer killing effect of ITHB4 against gastric adenocarcinoma cells, AGS cell line. A study published in the journal "International Journal of Nanomedicine" in 2022 has investigated the effectiveness of a combination therapy involving isoniazid and core-shell magnetic nano-radiotherapy against gastrointestinal tumor cell types (Chen et al., 2022). Our isoniazid, ITHB4 has six-membered ring of benzene with alternate single and double bonds and five-membered pyridine which has four carbon atoms and one nitrogen atom. Both aromatic rings exhibit a high level of electron delocalization and are planar. Because of these characteristics, benzene and pyridine rings are vulnerable to electrophilic attack (Vianello, 2011). This makes them possible targets for anticancer medications that fight cancer by concentrating on enzymes involved in DNA synthesis eventually cause apoptosis (Tylińska et al., 2021). Anthracyclines, including doxorubicin and epirubicin, are a class of anticancer drugs widely used in the treatment of various types of cancer, including gastric cancer (Thorn et al., 2011). These drugs have a complex structure that includes a benzene ring and a sugar moiety, which attack primarily DNA and topoisomerase II of cancer cells.

Similar to our previous study (Barathan et al., 2021), we discovered that ITHB4 caused dose-dependent cell death in gastric adenocarcinoma cells. The capacity of ITHB4 to kill AGS cells was dose-dependent at all time points (24, 48 and 72 h). However, ITHB4 showed a lower IC50 at 24 h treatment point compared to others. This is possibly indicating the shorter incubation time of anticancer drug in the cell culture, which reduces the risk of harming normal cells (Eastman, 2017). However, the optimal incubation time for an anticancer drug in cell culture will be determined by a number of factors, including the specific drug, the type of cancer being studied, and the goal of the study (Evans et al., 2019). Additionally, ITHB4 had any cytotoxic effects on human immune cells (PBMC). In light of this, the results of the current investigation may have important ramifications for the addition of synthetic chemicals to conventional anticancer treatment. We have also highlighted a number of crucial aspects of ITHB4 as a prospective gastric cancer anticancer candidate. First off, treatment of IC50 of ITHB4 at 24 h on AGS cells

showed a considerable increase of number of cells that exposed inner phosphatidylserine on the cell surface, which makes the binding of Annexin V to apoptotic cells. This resulted in identifying and measure the extent of apoptosis in the cells (Liu et al., 2009). Occurrence of apoptosis in gastric cancer cell culture, which is a key mechanism underlying the compound's cytotoxicity. In addition, ITHB4 was found to reduce mitochondrial membrane potential of the AGS cells, resulting cell death in cell culture. It can disrupt energy production, impair cellular metabolism, promote the release of pro-apoptotic factors, and increase ROS levels (Mani et al., 2020). These combined effects can induce cell death through apoptotic or other cell death pathways. Occurrence of oxidative stress was also evident in the treated AGS cells, indicating possible occurrence of apoptosis through mitochondrial cell death pathway. Elevated ROS levels can lead to DNA damage, protein oxidation, and lipid peroxidation, triggering apoptotic cell death pathways (Arfin et al., 2021). Excessive ROS can overwhelm the antioxidant defence mechanisms of cancer cells, resulting in apoptosis and the elimination of cancer cells (Perillo et al., 2020). Nuclear condensation along with DNA fragmentation were some of hallmark observed during the treatment of ITHB4 against AGS cell culture. DNA fragmentation is reportedly suggestive of nuclear damage during the late stages of chromatin condensation (Sun et al., 2016). This is possible due to benzene and pyridine ring present in the compound, which cause the DNA to become distorted and can lead to breaks in the DNA strand (De et al., 2022; Fu et al., 2015). In addition, pyridine can also act as a free radical together with ROS. This can damage DNA directly (Wheelock et al., 2008). To further support the statement, the release of LDH from damaged AGS cells due to ITHB4 treatment, is a crucial indicator of cytotoxicity. LDH is an enzyme present in cells, and its release into the extracellular space signifies damage to the cell membrane and, potentially, early cell death. This phenomenon is a valuable marker for assessing the effectiveness of ITHB4 as an anticancer agent, as it indirectly reveals their impact on the permeability and integrity of cancer cell membranes. This also suggest, making ITHB4 as a promising candidate for further exploration as potential anticancer agents. Similarly, release of LDH from AGS cell culture after the treatment of procyanidin B2 was observed and interpreted as marker for cell death (Li et al., 2021).

The ITHB4 treatment also led to the significant activation of caspases 3, 8, and 9 in AGS cells. Caspase 3 is often referred to as an "executioner" caspase, as it plays a central role in the final steps of apoptosis, leading to cell death. Caspases 8 and 9, on the other hand, are known as "initiator" caspases, as they trigger the apoptotic process. Their activation signifies the beginning of apoptosis. The activation of these caspases in response to ITHB4 treatment is a crucial finding. It suggests that ITHB4 has the potential to induce apoptosis in AGS gastric cancer cells through the activation of caspases. A study also mentioned that phenolic constituents of *Xylopia aethiopica* (Dunal) A. Rich might contribute to the cytotoxic effects, particularly *via* caspase-3 activation in AGS cells (Ribeiro et al., 2021) hence this activation is a clear indicator of the molecular mechanisms through which ITHB4 exerts its anticancer effects. Apoptosis is a desirable outcome in cancer therapy, as it leads to the controlled death of cancer cells, preventing uncontrolled growth and proliferation. The treatment of AGS gastric cancer cells with ITHB4 had a discernible impact on the expression of proinflammatory cytokines, with a specific emphasis on IL-6. The secretion of proinflammatory cytokines was altered in the presence of ITHB4, compared to untreated AGS cell cultures. This alteration in cytokine expression is a significant finding and these he changes in proinflammatory cytokines, especially IL-6, indicate that ITHB4 has the potential to influence the immune and inflammatory response within AGS gastric cancer cells (Gornowicz et al., 2023). The altered cytokine profile hints at the possibility that ITHB4 may possess anti-tumor and anti-inflammatory properties. ITHB4 may not only have anticancer effects, as indicated by the activation of caspases in a previous part of the study, but it might also exhibit anti-inflammatory properties. Inflammation is closely linked to cancer progression, and substances that can modulate the immune response and inflammation could have a dual benefit in cancer therapy. This could be a mechanism used by certain immunotherapies or treatments that stimulate the body's immune system to recognize and attack cancer cells. Overall, this is suggesting that ITHB4 potentially utilizes intrinsic mitochondrial cell death pathway to cause apoptosis in AGS cells. Mitochondrial cell death involves the disruption of mitochondrial function, which can lead to the release of pro-apoptotic factors and activation of caspases. This, in turn, triggers a cascade of events that ultimately results in cell death (McIlwain et al., 2013). The study's findings are consistent with the idea that ITHB4 could be initiating these mitochondrial cell death pathways, ultimately leading to cell death in AGS gastric cancer cells.

But it is important to recognize that this study has certain limitations. This study did not compare the anticancer activity of ITHB4 against clinically used anticancer drugs. This information would be helpful in understanding the capacity of this synthetic compound by which ITHB4 inhibits the growth of AGS cells. The study primarily focused on AGS gastric cancer cells, and the results may not be directly generalizable to other cancer cell types or *in vivo* conditions. Different cell lines may respond differently to ITHB4. In addition, it is also valuable to include non-neoplastic or normal gastric cells as counterparts in anticancer research. These normal cells can serve as a baseline for comparison with the neoplastic gastric cells when assessing the effects of the anticancer agent. By doing so, we can differentiate the ITHB4's impact on gastric cancer cells from its effects on healthy cells, helping to gauge its selectivity and potential toxicity. This comparative approach provides a more comprehensive understanding of the agent's specific anticancer properties while considering its potential impact on non-cancerous tissues. The study also did not investigate the impact of ITHB4 on the human immune system in a comprehensive manner. Understanding the immune response is crucial in cancer treatment. In addition, genes associated to cycles, apoptosis, and DNA damage were not examined for gene expression in this study. This suggests that the study's lack of examination of how ITHB4 altered the expression of genes related to DNA damage, apoptosis, and cell cycle progression. The study was conducted *in vitro*, which means that the results may not be applicable to *in vivo* studies. Results observed in a controlled laboratory setting may not completely mimic real-life conditions.

5. Conclusion

This research offers a novel and significant contribution by unveiling the potential of ITHB4, initially known for its antimycobacterial properties, as a promising anticancer agent. As a result, our findings are consistent with our previous study that found ITHB4 to be cytotoxic to MCF-7 cells. However, ITHB4 was found to inhibit growth of AGS at 24 h compared to previous study on MCF-7 cells. ITHB4 causes increased cytotoxicity through release of LDH, reduced mitochondrial membrane potential, increased nuclear condensation, upregulation of ROS levels, increased in early apoptotic cells, activation of DNA fragmentation and caspases (caspases 3, 8, and 9), collectively indicate that ITHB4 may induce apoptosis, a process often linked to mitochondrial cell death. ITHB4 has a multi-pronged impact on cancer cells, potentially inducing cell death through mitochondrial pathways and influencing the immune and inflammatory response through cytokine modulation. The development of ITHB4 as a possible candidate medication for the treatment of gastric cancer cells is well supported by these findings.

Author contributions

MB and JV designed the project. HSNK synthesized ITHB4 and provided reagents, MB performed the experiments. MB analyzed the data and wrote the manuscript. JV provided funding. All authors discussed the results and contributed to the manuscript.

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Declaration of Competing Interest

No potential conflicts of interest were disclosed.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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